# Targeting GARP:TGF-β1 complexes on Tregs for the immunotherapy of myeloproliferative neoplasms

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# ABBREVIATIONS

| | |
|---|---|
| **ADCC** | Antibody-dependent cellular cytotoxicity |
| **ADCP** | Antibody-dependent cellular phagocytosis |
| **AML** | Acute myeloid leukemia |
| **BCR** | B cell receptor |
| **BET** | Bromodomain and extraterminal domain |
| **BM** | Bone marrow |
| **CALR** | Calreticulin |
| **CHIP** | Clonal hematopoiesis of indeterminate potential |
| **cIAP** | Cellular inhibitor of apoptosis |
| **CML** | Chronic myeloid leukemia |
| **CTL** | Cytotoxic T lymphocytes |
| **CTLA4** | Cytotoxic T Lymphocyte Antigen 4 |
| **DC** | Dendritic cell |
| **DTR** | Diphtheria toxin receptor |
| **ECM** | Extracellular matrix |
| **ELISPOT** | Enzyme linked immunospot |
| **EPO-R** | Erythropoietin receptor |
| **ER** | Endoplasmic reticulum |
| **ET** | Essential thrombocythemia |
| **GARP** | Glycoprotein A Repetition Predominant |
| **GF** | Graft failure |
| **GvHD** | Graft versus host disease |
| **GvL** | Graft versus leukemia |
| **HLA** | Human leukocyte antigen |
| **HMA** | Hypomethylating agent |
| **HSC** | Hematopoietic stem cell |

| | |
|---|---|
| **HSCT** | Allogeneic stem cell transplantation |
| **ICS** | Intracellular cytokine staining |
| **IFN-α** | Interferon-α |
| **IFN-γ** | Interferon-γ |
| **IL** | Interleukin |
| **ISRE** | Interferon responsive element |
| **ITIM** | Immunoreceptor tyrosine-based inhibitory motif |
| **ITSM** | Immunoreceptor tyrosine-based switch motif |
| **JAK** | Janus kinase |
| **KI** | Knock-in |
| **KO** | Knock-out |
| **LAP** | Latency Associated Peptide |
| **LRRC32** | Leucine Rich Repeats Containing protein 32 |
| **mAb** | Monoclonal antibody |
| **MAPK** | Mitogen-activated protein kinase |
| **MDS** | Myelodysplastic syndrome |
| **MDSC** | Myeloid-derived suppressive cell |
| **MHC** | Major Histocompatibility Complex |
| **MK** | Megakaryocyte |
| **MPN** | Myeloproliferative neoplasm |
| **MSC** | Mesenchymal stem cell |
| **NF-κB** | Nuclear factor κB |
| **NK** | Natural killer |
| **OS** | Overall survival |
| **PBMC** | Peripheral blood mononuclear cell |
| **PD-1** | Programmed cell death protein 1 |
| **PD-L1** | Programmed death ligand 1 |
| **PGF** | Poor graft function |
| **PI3K** | Phosphatidylinositol-3 kinase |

| | |
|---|---|
| **PLT** | Platelet |
| **PMF** | Primary myelofibrosis |
| **PRC2** | Polycomb repressive complex 2 |
| **PV** | Polycythemia Vera |
| **RBC** | Red blood cell |
| **ROS** | Reactive oxygen species |
| **SIRPα** | Signal regulatory protein alpha |
| **STAT** | Signal transducers and activators of transcription |
| **TCR** | T cell receptor |
| **TGF-β1** | Transforming Growth Factor β1 |
| **TGFβRI** | TGF-β1 receptor class I |
| **TGFβRII** | TGF-β1 receptor class II |
| **Th** | Helper T cell |
| **TIL** | Tumor infiltrating lymphocyte |
| **TMB** | Tumor mutational burden |
| **TPO** | Thrombopoietin |
| **Tregs** | Regulatory T cells |
| **TRM** | Treatment related mortality |
| **WBC** | White blood cell |
| **WT** | Wild-type |

# ABSTRACT

Philadelphia-negative myeloproliferative neoplasms (MPNs) regroup hematological malignancies arising from the clonal expansion of a hematopoietic stem cell (HSC) and lead to the accumulation of differentiated myeloid cells. They are mainly composed of polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). Although each MPN is associated to significant morbidity and a risk of evolution to acute myeloid leukemia (AML), PMF bears the worst prognosis and its only potentially curative option remains allogenic stem cell transplantation (HSCT). PMF is characterized by immature megakaryocytes (MKs) proliferation and aberrant production of pro-inflammatory cytokines, leading to progressive bone marrow (BM) fibrosis, extramedullary hematopoiesis and BM failure. Many efforts have been invested into the development of new therapeutic approaches, including immunotherapy, but have so far failed to transform patient care.

TGF-β1 is one of various cytokines incriminated in PMF development. It is not only potently pro-fibrotic but also immunosuppressive. However, which cells produce active TGF-β1 remains to be specified. Indeed, TGF-β1 is produced by nearly all cells in an inactive form, called latent TGF-β1. To exert biological activity, the mature cytokine must be released from latent TGF-β1 via mechanisms that are cell-type specific. Only a few cell types are known to activate latent TGF-β1. Our laboratory previously showed that stimulated regulatory T cells (Tregs) present latent TGF-β1 on their surface in association with the GARP transmembrane protein for activation by integrin αVβ8. Only activated B cells and platelets (PLT) are also known to express GARP:TGF-β1 complexes and activate TGF-β1 in a GARP-dependent manner. Our group developed anti-GARP:TGF-β1 monoclonal antibodies (mAb) that block TGF-β1 activation by GARP-expressing cells. In transplantable mouse tumor models, anti-GARP:TGF-β1 was shown to block TGF-β1 activation and immunosuppression by Tregs, overcoming resistance to immune checkpoint inhibitors and promoting immune-mediated tumor rejections.

Here, we hypothesized that pathogenic active TGF-β1 is produced by GARP-expressing cells during PMF development. We used a mouse model of PMF to test the therapeutic potential of anti-GARP:TGF-β1 mAb. In addition to a moderate reduction of fibrosis, we observed a significant decrease in disease burden upon treatment. We also found that the therapeutic activity of anti-GARP:TGF-β1 required blocking TGF-β1 activation by GARP-expressing Tregs and implied $CD8^+$ T cells. Altogether, our results suggest that selective blockade of TGF-β1 activation by GARP-expressing Tregs with anti-GARP:TGF-β1 mAb exerts immune-mediated therapeutic activity in mouse PMF, and could thus pave the way for novel immunotherapies in patients with MPN.

# INTRODUCTION

# INTRODUCTION

As many other myeloid malignancies, MPNs are considered poorly immunogenic. This often leads to questioning the interest and feasibility of immunotherapies in these diseases. However, our results suggest that blocking TGF-β1 activation and immunosuppression by GARP-expressing Tregs could be beneficial in MPN patients. For this reason, our introduction will discuss the inflammatory context and the tumor-specific T cell responses that were described in MPNs. We will then summarize the immune cell alterations found in MPNs that could explain or contribute to suppression of anti-tumor immune responses. Finally, after describing the standard of care in this context, we will highlight several therapeutic strategies that could improve anti-tumor immunity in PMF. Being written as a basis for a future review article, this introductory part will already include a few of the experimental results that are fully detailed in the research article of the Results section.

# 1. Myeloproliferative neoplasms: classification and clinical considerations

MPNs form a group of hematological malignancies characterized by clonal expansion of a hematopoietic progenitor, leading to the accumulation of differentiated and functional myeloid cells. According to the latest update of WHO classification of tumors of hematopoietic and lymphoid tissues in 2016, MPNs comprise chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia not otherwise specified (CEL-NOS) and unclassifiable MPN (MPN-U). Here, we will discuss the group formed by PV, ET and PMF, the most frequent *BCR::ABL1* negative MPNs. For the rest of the manuscript, “MPNs” will refer collectively to these three entities.

MPNs arise from a single mutated hematopoietic stem cell (HSC). Cytokine-autonomous expansion of this progenitor is associated to a persistent activation of JAK2, arising from few driver mutations which are well described and shared between the three pathological entities. Keeping its self-renewal and differentiation potential, the mutated HSC drives a myeloid biased hematopoiesis, with single or multilineage hyperplasia. In addition to sharing common genetic alterations, the different MPNs also possess common clinical features and are sometimes perceived as a continuum ultimately culminating in the most severe PMF. All are associated to

increased risk of vascular thrombosis and bleeding, but also to progression toward other MPNs and acute myeloid leukemia (AML). Many efforts were made recently, notably with the latest edition of the WHO classification, to improve differential diagnosis between MPN subtypes, based on strict clinical, histological and genetic criteria [1].

MPNs are rare diseases. Incidence rates of PV, ET and PMF have been reported to be 2.8, 1.7 and 1.0 per $10^5$ habitants, respectively [2]. Median age at diagnosis was 62 years in a recent report [3]. PMF patients were generally the oldest.

PV diagnosis is based on increased numbers of circulating red blood cells (hemoglobin superior to 16,5 g/dl for men or 16 g/dl for women), with BM examination revealing a panmyelosis with increased development of the three myeloid lineages. Classical symptoms mainly relate to the elevated red blood cell mass, responsible of hyperviscosity and hypoxia. ET is characterized by increased blood PLT values (>450x$10^9$/L). BM evaluation reveals proliferation of mature megakaryocytes (MKs), without significant increase of the erythroid or granulocytic compartments. General symptoms of PV or ET are usually the mildest among MPNs. Venous and arterial thromboses are the major causes of morbidity in PV and ET. Their treatment therefore aims at preventing such complications without promoting evolution to AML. Risk stratification is based on the age of the patients, cardio-vascular risk factors and previous history of thrombosis. Leukocytosis is an increasingly recognized risk factor, especially in PV. In the case of ET, a specific scoring system regroups risk factors with *JAK2* mutational status (IPSET-thrombosis system). Low risk patients are treated with low-dose aspirin while phlebotomies maintain hematocrit under 45%. Cautions must be taken regarding aspirin in ET patients with pronounced thrombocytosis (>10x$10^9$/L), as an increased bleeding risk can arise from an acquired von Willebrand syndrome. Additional cytoreductive therapy is reserved to high-risk patients. Hydroxyurea or IFN$\alpha$ are recommended options as first line, with IFN$\alpha$ preferred in young patients who will need long-term treatment [4]. Highlighting the biological continuum between MPNs, a low rate (1 - 5%) of transformation of ET into PV has been reported. Moreover, secondary transformation of PV and ET into myelofibrosis will happen in 16% and 13% of the patients, respectively.

PMF is a more heterogenous disorder. The BM shows distinctive accumulation of atypical MKs accompanied by reticulin and/or collagen fibrosis. Clinical

manifestations are variable but are more severe than in PV and ET. Symptoms are due to high systemic inflammation and extramedullary hematopoiesis which leads to a more life-treating condition. Usual manifestations consist of anemia, bulky splenomegaly and debilitating constitutional symptoms (fatigue, night sweats, fever) that can ultimately lead to cachexia [5]. The main causes of death include transformation into AML, cardio-vascular events and infections. Given the dismal prognosis of PMF, treatment aims to cure the disease. Scoring systems have evolved and are essential for treatment decision, particularly with regards to transplantation. While initially solely based on clinical parameters (IPSS, DIPSS, and DIPSS-plus), newer prognosis scores include high molecular risk (HMR) mutations like in Mutation-Enhanced International Prognostic Scoring System 70+ v2.0 (MIPSS70+ v2.0) and Genetically Inspired Prognostic Scoring System (GIPSS) [6, 7]. Unfortunately, although allogenic hematopoietic stem cell transplantation (HSCT) is the only potentially curative treatment for PMF, it is only applicable to a minority of patients because of its high toxicity (up to 50% mortality and morbidity). Accordingly, low-risk patients are treated with a symptom-based approach and disease-modifying strategies are reserved for the highest risk diseases [4]. Of note, poor prognosis factors such as HMR mutations, refractory or transfusion-dependent anemia and increased peripheral blast counts may prompt the decision of HSCT in some intermediate-low risk patients.

Recently, updated diagnosis criteria edited by the WHO define a new category of MPN described as pre-PMF. Diagnosis criteria are close to that of overt PMF, the main difference residing in the lack of fibrosis on histological BM sections. While pre-PMF seems to have a better prognosis than overt PMF, it needs to be discriminated from true ET because of a lower survival and higher risk of evolution to fibrosis and AML [8] [9]. Treatment recommendations are limited given the novelty of the description.

Altogether, survival in PV and ET is close to that in the healthy population, while PMF patients have the poorest prognosis (median overall survival, or OS: 15, 18 and 4,4 years, for PV, ET and PMF, respectively). One of the ultimate threats in MPNs is evolution towards AML, with the highest risk in PMF (40%, 7% and 2% of blast transformation after 15 years of follow-up in PMF, PV and ET, respectively) [10].

# 2. Mutational landscape of MPNs

## 2.1. Main driver mutations

Thrombopoietin receptor (TPO-R or MPL) is essential for HSCs renewal, MK differentiation and PLT production. MPL plays a central role in MPNs, which result from persistent and pathological activation of this signaling pathway. Driver mutations in MPNs thus mainly include mutations in three genes implicated in the pathway, namely *JAK2*, *MPL* and *CALR*.
Activation of the erythropoietin receptor (EPO-R) and granulocyte colony stimulating factor receptor (G-CSFR) also plays complementary roles In *JAK2*-mutated MPNs.

Like other type I cytokine receptors, MPL lacks intrinsic catalytic activity and relies on pre-association to Janus kinase (JAK) family members to mediate intracellular signaling. JAK2 is the main JAK of MPL, EPO-R and G-CSFR signaling. TYK2 is also implicated but seems more dispensable. Ligand binding induces and stabilizes dimerization of two MPL chains which will become oriented in an active conformation. This configuration allows activation of JAK2 via reciprocal trans-phosphorylation. Activated JAKs then phosphorylate tyrosine residues on the cytoplasmic portion of MPL, allowing recruitment of Signal transducers and activators of transcription (STATs). STATs are in turn phosphorylated by activated JAKs and ultimately translocate to the nucleus where they will exert their transcriptional activities. STATs implicated in MPL signaling are STAT3 and STAT5, but also STAT1. Moreover, JAK2 activate other downstream pathways such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K).

Somatic mutations in either *JAK2*, *MPL* or calreticulin (*CALR*) have been shown to induce persistent activation of MPL-JAK2 axis in MPNs, which results in clonal expansion of myeloid progenitor and reproduce disease phenotype in animal models. Noteworthy, all of them need surface expression of MPL to exert their full signaling potential and are mutually exclusive.

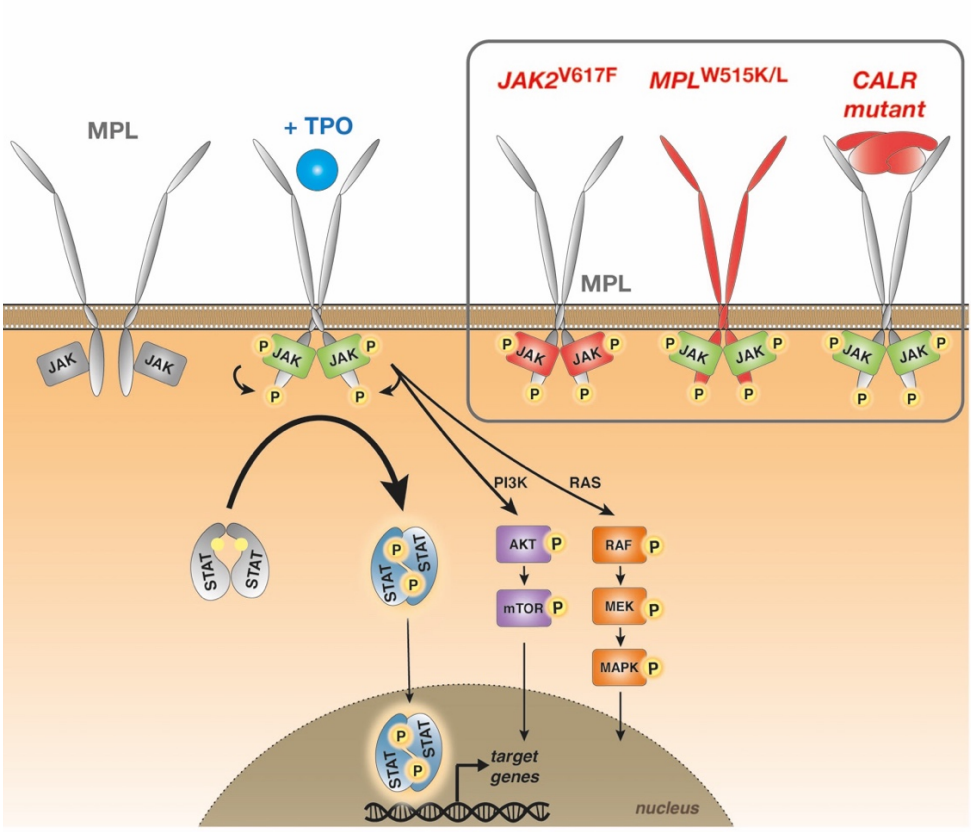


**Figure 1: The MPL signaling pathway**
Thrombopoietin (TPO) binding to MPL induce and stabilize a dimeric active conformation of the receptor. It allows transphosphorylation of JAKs (mainly JAK2), which will phosphorylate tyrosine residues on the receptor, recruit and activate STATs. The transcription factors will then translocate to the nucleus to drive the transcription of their target genes. Apart from STATs, MPL signaling also activates PI3K and MAPK pathways. In MPNs, TPO-independent activation of MPL-JAK2 signaling pathway is mediated by mutated *Jak2, MPL* or *CALR*.

*JAK2* mutations are mostly represented by *JAK2*$^{V671F}$, the first driver mutation identified in MPNs. Its identification in 2005 revolutionized our understanding of these diseases [11]. *JAK2*$^{V671F}$ is also the most frequent mutation in MPNs. It is found in 95% of PV and 50 to 60% of ET or PMF. In *JAK2*$^{V671F}$, a valine encoded by exon 14 is replaced by a phenylalanine, leading to constitutive activation of JAK2 in the absence of cytokine binding to the cognate receptor. The V617F mutation is located in the pseudokinase domain (JH2), which normally prevents spontaneous activity of

the catalytic kinase domain (JH1). It is thought to destabilize the inhibitory JH2-JH1 interface. Mutant JAK2$^{V671F}$ associates to MPL or EPO-R, but not IFNγ or GH receptors [12]. A minority of PV cases harbor other gain-of-function mutations in *JAK2* encoded by exon 12. They are not found in other MPNs and their mechanism is less well understood.

Introduction of the *JAK2*$^{V671F}$ mutation in mice has successfully modeled PV, ET and PMF diseases. Retroviral transduction of a JAK2$^{V671F}$-encoding transgene in BM cells leads to high mutant protein expression. Transplantation of JAK2$^{V671F}$ BM cells into irradiated recipients leads to a PV-like phenotype evolving within several months towards myelofibrosis. In more recent mouse models, the transgene is knocked-in (KI) the endogenous *Jak2* locus. For instance, Cre-lox based recombination systems allow conditional replacement of wild type (WT) exon 14 of *Jak2* by a sequence encoding the V617F mutation. Thus, *Jak2*$^{V671F}$ heterozygosity or homozygosity can be generated in mice and their BM cells, which will express mutant JAK2$^{V671F}$ at controlled and more physiological levels by comparison to the retrovirus-based method. Depending on the level of expression, mice will develop ET or PV, secondary evolving to myelofibrosis after 6 months. Those observations indicate a link between expression levels of mutant JAK2 and the MPN phenotype.

Activating mutations in the *MPL* gene are found in a small proportion of ET and PMF patients (3% and 6%, respectively). The majority of these mutations are located in exon 10, in the codon encoding tryptophan in position 515 (W515). This amino acid is part of the juxtamembrane cytosolic domain of MPL and plays a key role in preventing activation of the MPL in the absence of cytokine binding [13]. Nearly all substitutions of W515 by another amino-acid tilt MPL monomer and induce persistent dimerization in an active orientation. MPL$^{W515K}$ and MPL$^{W515L}$ are the most prevalent substitutions found in MPNs. Using a BM cell retroviral transduction and transplantation strategy similar to the one described for JAK2$^{V671F}$, these MPL mutations were shown to induce an aggressive ET phenotype which rapidly evolves to PMF in mice [14]. Of note, the amino-acid homologous to W515 in human MPL is W508 in murine MPL.

Mutations in *CALR* were identified d more recently in MPNs. *CALR* encodes a chaperone protein located in the endoplasmic reticulum (ER). CALR has multiple functions including quality control of glycoproteins and control of calcium homeostasis. More than 40 *CALR* mutations have been reported in ET and PMF

patients. Interestingly, all mutations induce a +1 frameshift within exon 9. This shift in the reading frame leads to a modified CALR bearing a new methionine-rich C-terminus. This sequence is positively charged and devoid of the “KDEL” ER retention motif present in the C-terminus of wild type CALR. Although the precise mechanism is still subject of investigations, robust evidence indicates that pathogenic CALR mutants have the unique ability to promote cytokine-independent cell growth through specific activation of the MPL signaling pathway. The mechanism implicates the ability of mutant CALR to bind the extracellular domain of immature MPL chains in the ER and promote their transport to the cell surface via the Golgi apparatus, where they are expressed in a dimeric and activated configuration [15]. Cell surface localization of the MPL:mutant CALR complexes was confirmed on primary $CD34^{+}$ cells from MPN patients, and is required for complete activation of the JAK2-STAT3 and STAT5 pathways in mutant cells. Other biological functions were attributed to CALR mutants, including alteration of the calcium efflux from the ER, “rogue cytokine” signaling and inhibition of phagocytosis by a paracrine mechanism.

Overall, *CALR* mutations are found in approximately 25% of ET and PMF cases. In ET, by comparison to $JAK2^{V671F}$, *CALR* mutations are associated to a younger age at diagnosis [16]. Accounting together for 85% of the *CALR* mutations, the two most frequent mutations correspond to a 52-bp deletion, called “type 1 mutation”, and to a 5-bp insertion, called “type 2 mutation”. Whereas type 1 is predominant in PMF, type 2 is more frequent in ET. Survival benefits have also been attributed to *CALR* mutations, especially type 1 mutations in PMF which independently predicts a better outcome in HSCT.

Retrovirus and CRISPR-Cas9 strategies were used to generate *Calr* mutations in mice, which display ET and secondary myelofibrosis [17]. Functional differences between type 1 and type 2 mutations remain intriguing. Indeed, while both are able to confer autonomous growth to several cell lines, the ET phenotype is consistently induced only in mouse models with the type 1 mutants [17, 18].

As a final remark, the mutated C-terminus of *CALR*-exon 9 mutants, being derived from a frame shift shared between many patients, represents an attractive target for tumor antigen- specific immunotherapies.

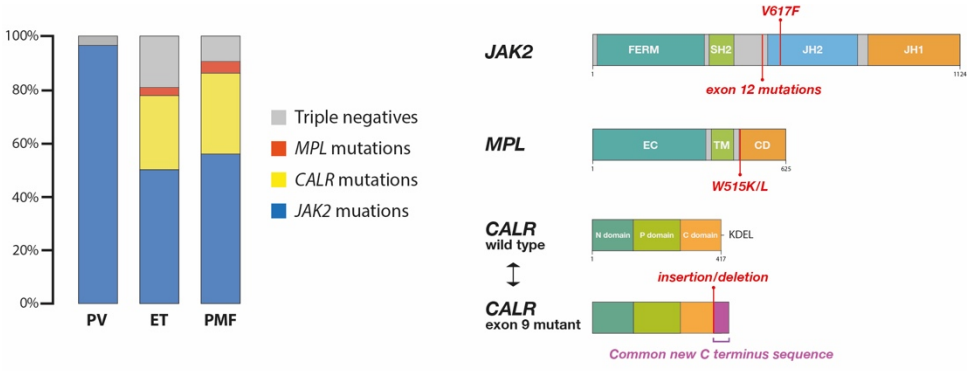


**Figure 2: Distribution of driver mutations across MPNs and structural localization**
(left) Mutations of *JAK2* are the most common in MPNs, found in 96% of PV, 55% of ET and 65% of PMF (blue). *CALR* mutations are found in ET and PMF, with a respective frequency of 20% and 25% (yellow). MPL mutations are the rarest, accounting for 3% of ET and up to 10% of PMF (red). Triple negative category corresponds to unknown or non-canonical mutations (grey). (right) Schematic representation of structural domains of *JAK2*, *MPL* and *CALR*. Numbers represent the amino-acid position. Localizations of classical MPN driver mutations are indicated in red. *JAK2* comprises FERM domain (4.1 Ezrin, Radixin, Moesin), SH2 domain (Src Homology 2), a pseudo-kinase JAK homology-2 domain (JH2) and tyrosine kinase JAK homology-1 domain (JH1). MPL comprises extracellular, transmembrane and cytoplasmic domains (EC, TM and CD respectively). CALR comprises NH2 domain (N-domain), proline-rich domain (P-domain) and COOH domain implicated in ER retention (KDEL motif) but also in calcium buffering (C-domain). Common C-terminus sequence shared by CALR exon 9 mutants is represented in purple.

## 2.2. Other driver mutations common to myeloid malignancies

In addition to driver mutations leading to JAK-STAT pathway activation, mutations of genes implicated in epigenetic regulation (*TET2, DNMT3A, IDH1/2, EZH2, ASLX1*) and RNA splicing (*SF3B1, SRSF2, U2AF1*) are regularly found in MPNs [19, 20]. These mutations are not unique to MPNs but also found in other myeloid malignancies. They are found in 35% to 40% of MPN cases, most often in PMF. Indeed, a majority of PV and ET patients harbor only one mutation of *JAK2*, *CALR* or *MPL*.

Although precise mechanisms behind their contribution to MPN physiology remain unclear, their prognosis significance in PMF is well established. Indeed, the latest prognosis scores recognize several HMR mutations as being the ones located in *ASXL1*, *SRSF2*, *EZH2*, *IDH1*, *IDH2* and *U2AF1*.

Among those genes, the most commonly mutated is *TET2*, with loss-of-function mutation identified in up to 17% of PV and PMF patients. This enzyme is implicated in the conversion of 5-methylcytosine into 5-hydroxymethylcytosine, a process thought to play an important role in promoter hypomethylation and transcriptional activation of target genes promoting HSCs differentiation. Its defect has been shown to promote increased self-renewal with myeloid bias and could then result in a differentiation block.

DNMT3A is less frequently impacted by loss-of-function mutations (around 7% of PV and PMF). This DNA methyltransferase is responsible of *de novo* methylation of CpG dinucleotides. A decrease of its activity could downregulate expression of differentiation factors, also resulting in differentiation blockade and HSCs expansion.

EZH2 is a histone methyltransferase, a catalytic component of the polycomb repressive complex 2 (PRC2) implicated in transcriptional repression. Loss-of function mutations are seen in 3% of PV and 7% of PMF. De-repressed genes could include some oncogenes and favor self-renewal. Animal models with both *JAK2*$^{V671F}$ and *EZH2* deletion presented accelerated PMF phenotype with reduced survival and increased megakaryopoiesis.

Mutations of *ASLX1* are nearly restricted to PMF in which they are quite common (up to 36% according to some series). *ASLX1* have a wider regulatory role than *EZH2* but acts notably by recruiting PRC2. In mouse models, *ASLX1* gene deletion leads to myelodysplasia accompanied by cytopenia and profound defect of HSC-renewal.

Spliceosome gene mutations in *SF3B1, SRSF2* and *U2AF1* are also mainly restricted to PMF (with respective frequency of 7%, 17% and 16%). Associations with anemia or thrombopenia are often reported. Interestingly, splicing alterations could theoretically be good provider of antigens in the perspective of immunotherapy.

Apart from overt malignancies, both *JAK2*$^{V671F}$ and those mutations of *TET2, DNMT3A* or *ASLX1* are genetic abnormalities commonly found in clonal hematopoiesis of indeterminate potential (CHIP), which corresponds to infra-clinic proliferation of a HSC clone. Accordingly, they are supposed to confer a fitness advantage to the HSCs and predispose to the development of overt myeloid malignancies including MPN, myelodysplastic syndromes (MDS) and LMA. While the interplay between those epigenetic alterations and *JAK2*/*CALR*/*MPL* mutations will require further explorations, some of them may be implicated in disease initiation, progression and transformation.

## 2.3. Determinants of the various MPN phenotypes

Mutations in *JAK2, CALR* or *MPL* affect signaling cytokine receptors (mostly MPL, but not only) and all converge to increased JAK2 activity. They are important for the definition of MPNs, but how different mutations in the same pathway can lead to different disease phenotypes remains a fundamental yet unanswered question.

A first element of response lies in the identity of the driver mutation itself. *CALR* and *MPL* mutations are restricted to ET and PMF but not found in PV. This could be because they affect signaling mostly by MPL, but not by other cytokine receptors such as EPO-R. MPL signaling plays a role in megakaryocyte differentiation, thus promoting megakaryopoiesis and disease phenotypes including thrombocytosis. Mutation of $JAK2^{V671F}$, in contrast, can lead to PV, ET or PMF. However, $JAK2^{V671F}$ activity in PV is not restricted to the EPO-R pathway. This has been demonstrated in animal models, in which loss of MPL signaling via $JAK2^{V671F}$ resulted in an attenuated PV phenotype [12, 21]. MPL signaling is thus implicated in all MPNs.

Another factor may pertain to quantitative differences in $JAK2^{V671F}$ allele burden, suggested to account for part of the phenotypic heterogeneity. In patients, homozygous $JAK2^{V671F}$ mutations in BM progenitors are associated with PV, whereas heterozygous $JAK2^{V671F}$ mutations are more frequent in ET [22]. Moreover, the size of the homozygous $JAK2^{V671F}$ clone has been positively linked to the degree of erythrocytosis and the probability of myelofibrosis transformation [23-25]. These observations were also made in $Jak2^{V671F}$ KI mice [26].

Qualitative differences in JAK2 mediated signaling also influence the MPN phenotype. Whereas STAT5 was shown essential to induce PV in mice [27], STAT3 deletion normalized neutrophilia but aggravated thrombocytosis and disease severity in a $Jak2^{V671F}$ mouse model [28]. This observation suggests increased compensatory STAT1 phosphorylation. The prominent role of increased STAT1 in ET by comparison to PV was also suggested by studies on erythroid cells isolated from MPN patients [29]. The balance between the various STATs engaged downstream of JAK2 mediated signaling is thus thought to influence the MPN phenotype.

As mentioned previously, *JAK2*$^{V671F}$ in HSC is not always assorted to overt neoplasm, as it is frequently reported in CHIP. Cordua et al., studying a Danish population, reported a prevalence of *JAK2*$^{V617F}$ mutations in healthy subjects as high as 0,2 % [30]. This means that JAK2 mutations increase the risk but do not always lead to MPN. The divergence between occurrence of *JAK2*$^{V671F}$ in HSC and MPN development was also reported in an animal model using single *Jak2*$^{V617F}$ HSC transplantation [31]. Several intrinsic and extrinsic factors favoring MPN clone dominance were proposed and could also account for the diversity of the phenotypes.

Among cell-intrinsic factors, the most obvious would be co-existence of mutations that would increase the proliferative advantage of a specific cell progenitor. As already mentioned, mutations in epigenetic regulators are common, especially in PMF. The mechanisms are not entirely clear, but other CHIP-associated mutations could further increase the fitness of HSC, especially in aging individuals. Interestingly, mutations of genes involved in DNA methylation, like *TET2*, have been modeled in mouse. Here, Chen et al. shown that *Jak2*$^{V617F}$ KI mice having lost *TET2* expression displayed a more aggressive MPN phenotype, with double mutant *Jak2*$^{V617F}$ *TET2*$^{-/-}$ BM cells having a competitive advantage over *Jak2*$^{V617F}$ *TET2*$^{WT}$ cells [32]. Interestingly, the order of acquisition of those mutations is also significant and influences MPN phenotype [33]. Using patient samples, it was shown that acquisition of *JAK2*$^{V617F}$ before *TET2* mutation ("*JAK2*$^{V617F}$ first") was associated to earlier diagnosis, increased *JAK2*$^{V617F}$ allele burden, increased risk of PV and more thrombosis than patient having acquired *TET2* mutation in the first place. Accordingly, "*TET2* first" patients had milder diseases, with more cases of ET. Moreover, by increasing HSC proliferation, epigenetic alterations could favor disease progression by promoting additional mutations. Thus, beyond proliferative advantage, those mutations can contribute to phenotype heterogenicity.

Several cell-extrinsic factors could also be involved. First, heterogenicity of HSC populations in term of lineage commitment or self-renewal capacity could orient the phenotype of the clone or favor its disease-propagating potential [31, 34]. Secondly, the inflammatory milieu triggered by mutated clones has been showed to promote their proliferation at the expanse of the WT HSCs and could favor certain phenotypes. Here again, inflammatory cytokine production has been demonstrated since the stage of CHIP (also with *TET2* mutants), where it could even promote the atherosclerotic cardiovascular diseases associated to such clonal hematopoiesis

[35]. Moreover, the resultant oxidative stress could further increase the risk of new mutations and subsequent disease progression. At this subject, an increased susceptibility to DNA damages mediated by reactive oxygen species (ROS) has been reported in JAK2$^{V617F}$ HSCs [36]. ROS accumulation seemed to depend on a decreased expression of catalase, an antioxidant enzyme, directly suppressed by AKT and PI3K pathway under the control of JAK2.

# 3. Immunity and inflammation in MPNs

## 3.1. Chronic inflammation in MPNs, particularly in PMF

Chronic inflammation is a mainstay manifestation in MPNs that is well recapitulated by constitutive MPL-JAK-STAT activation in animal models. Although more modest in PV and ET, massive overproduction of a wide range of inflammatory mediators is observed in primary and secondary MF. This inflammatory cytokine environment is highly complex. It implicates various cell populations of the bone marrow niche, of clonal or non-clonal origin, and a lot of elements that are still to be discovered. Accumulating evidence suggests direct and indirect impacts of the remodeled BM environment on the emergence of clonal dominance of the mutated HSC. We will review here some major publications which demonstrate the permissive role of inflammation in progression and putative immune escape of the MPN clone.

### *3.1.1. Elevated production of inflammatory cytokines*

The pathological and persistent activation of the MPL-JAK-STAT pathway is required for cytokine-independent growth of the MPN clone. In addition to STAT phosphorylation and its downstream transcriptional effects, constitutive JAK activation also activates MAPK and PI3K signaling. However, signaling alterations observed in MPNs are not restricted to these signatures of autonomous cell growth. For instance, the role of nuclear factor kB (NF-κB) signaling has been increasingly acknowledged in disease progression, and its activation is thought to be driven by inflammatory cytokines produced by mutated and non-mutated cells. This highlights the supporting role of non-mutated cells in the disease and the major role of inflammatory mediators in MPN development.

An instructive report was published by Tefferi et al. in 2011, describing the cytokine abnormalities in a cohort of 127 PMF patients [37]. By comparison to healthy controls, PMF patients had increased plasma levels of numerous cytokines (IL-1β, IL-2R, IL-6, IL-8, IL-10, IL-12, TNF-α, IFNα, MIP-1α, MIP-1β, HGF and CXCL10) and growth factors (G-CSF and VEGF). In multivariate analysis, some of those cytokines were associated to clinical outcomes: IL-8 was associated to constitutional symptoms, HGF with splenomegaly, IL-2R and IL-8 with anemia (or transfusion-dependency), IL-2R with leukocytosis and CXCL10 with thrombocytopenia. Regarding prognosis, IL-8, IL2-R, IL-12, IL-15 and CXCL10 were corelated to a reduced OS and leukemia-free-survival. Overall, the strongest correlations were found for IL-8 and IL2-R. In PV and ET, cytokine production was also specifically evaluated. While some of the aforementioned cytokines were increased (such as IL2-R, IL-6, IL-8 or TNF-α), their respective levels were generally lower compared to PMF [38]. Interestingly, their level could be positively associated to the risk of fibrotic transformation in PV [39]. Those correlations would highlight a progressively growing inflammatory continuum in PMF.

Using a $MPL^{W515L}$-based murine model of PMF, Kleppe et al. performed an elegant study, putting emphasis on the role played by non-malignant BM cells in the development of the disease and their cytokine releasing profile [40]. As the plasma cytokine profile was similar between mice and humans, they intended to discriminate the respective contributions of the $MPL^{W515L}$ mutant cells and non-malignant cells, using single cell cytokine analysis. Thus, whereas IL-6 were more produced by mutant cells and CXCL9 were more produced by non-mutant cells, both populations secreted increased quantities of various cytokines such as TNF-α and IL-10. Interestingly, pan-hematopoietic deletion of *Stat3* significatively reduced the severity of the phenotype and cytokine production. This could be expected given the role of STAT3 signaling in the autonomous proliferation of $MPL^{W515L}$ cells. However, *Stat3* deletion specifically in mutant cells failed to recapitulate the improvement. This suggests that cytokine signaling in non-mutated cells, mediated by STAT3, also plays a significant role in disease progression.

A third group of studies further explored cytokine production in PMF, with a particular attention to the NF-κB pathway. Working with PBMC from PMF patients, the authors observed increased activation of MAP kinase and NF-κB signaling pathways using mass cytometry and transcriptomic analysis. NF-κB activation seemed to participate

to myeloproliferation, as suggested *in vitro* by reduced colony formation upon NF-κB blockade. As NF-κB activation affected every WBC compartment, it appeared unlikely that a mechanism restricted to mutated cells (i.e., collateral NF-κB activation by constitutively activated JAK2) would be the sole explanation. This suspicion was confirmed in animals with the help of *in vivo* imaging [41]. The authors used a reporter mouse strain expressing firefly luciferase under the control of NF-κB DNA binding sites. Reporter mice were used as BM cell donors in the *MPL*$^{W515L}$ PMF model. When reporter cells were transduced with mutated *MPL* and transplanted into WT recipients, luciferase activity could be detected after transplantation and PMF development. If reporter cells were not transduced but co-transplanted with reporter-negative *MPL*$^{W515L}$ BM cells, bioluminescence imaging showed similar NF-κB activation, indicating that NF-κB is activated in mutated and non-mutated cells. This led to the hypothesis that NF-κB activation contributing to MPN expansion would, at least in part, be activated in a non-cell-autonomous fashion. Cytokine production, namely increased secretion of TNFα, is a plausible candidate. Interestingly, secretion of TNFα could be induced in Ba/F3 cells after *JAK2*$^{V617F}$ transduction, but it could also be stimulated *in vitro* on patient samples by TNFα itself, raising the possibility of a positive feedback loop.
In plasma they could confirm once again the higher TNFα, IL-10 and IL-6 levels seen by others, but they went further with mass cytometry characterization of WBC populations [42]. At the single cell level, they could demonstrate that the monocytes were the WBC population displaying the highest overproduction of cytokines, including TNFα, IL-10 and latent-TGFβ. Noteworthy, neither cytokine secretion nor NF-κB activation was significantly abolished in the patients currently treated with JAK inhibitor ruxolitinib. Indeed, *in vitro* studies confirmed that TNFα, IL-6, IL-8 or IL-10 secretion was incompletely sensitive to JAK1/2 inhibition, but effectively suppressed by pharmacological targeting of NF-κB activation or MAP kinases. It suggests that a JAK2 independent mechanism participate to cytokine production and that a combination of inhibitors may be clinically relevant.

As we have seen, numerous pro-inflammatory cytokines are overproduced in MPNs. Whereas some may be linked to robust clinical endpoints, the specific role of each cytokine remains incompletely understood. This task is complex, given the elaborate crosstalk between various cell types and between WT and mutated cells. Accumulating evidence points toward a global remodeling and dysregulation of the bone marrow regulatory niche. Some cytokines have emerged as having a more

prominent impact than others: some may confer a proliferation advantage to the mutant cell, favoring a phenomenon of clonal dominance, while some may more indirectly reshape the BM environment to favor tumor progression.

#### 3.1.1.1. Cytokines that give a proliferative advantage

TNF-α, IL-6 and IL-1 form a group of inflammatory cytokines (monokines) produced by macrophages, among other cell types. These mediators are physiologically involved since the earliest steps of the inflammatory response and are involved in essential functions in both innate and adaptative immune responses. NF-κB activation is part of their intracellular signaling pathway and is also a common trigger for their secretion. It is thus without surprise that they are in the foreground of PMF, a disease fueled by constitutive NF-κB activation.

TNF-α has complex and cell-type dependent physiological effects. While being a major activator of monocytes or granulocytes, it is also a negative regulator of HSCs expansion and an inducer of apoptosis. Intracellular signaling involves two distinct receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2), being respectively expressed ubiquitously or largely confined to hematopoietic cells. While both TNFRs can activate NF-κB transcriptional factors, only TNFR1 is able to recruit the pro-apoptotic complex, in a facultative second step of its activation. Cell fate is then dependent on complementary factors, like cellular inhibitor of apoptosis (cIAP), which stabilize signaling complex toward inflammation and maintain pro-apoptotic complex inactive.
While TNF-α is implicated in the inflammatory symptoms of PMF patients, it has also been implicated in promoting the survival of the mutated clone over that of WT cells. First clues were reported with CD34+ cells isolated from MPN patients, which showed resistance to suppressive effects of TNF-α in colony formation assays [43]. The research group later reproduced these results upon transduction of *JAK2*$^{V617F}$ mutant in human and murine BM cells. Further validation was obtained in the *Jak2*$^{V617F}$ mouse model, where TNF-α *knock out* (KO) mice presented an attenuated disease. A better understanding of the underlying mechanism was brought by Heaton et al., who described a TNFR2 and *JAK2*$^{V617F}$-dependent downregulation of essential components of the TNF-α-mediated apoptosis machinery (*Xiap* and *Mapk8*). The consequence of this TNFR2 signal reprogramming in *JAK2*$^{V617F}$ cell consisted in increased expression of cIAP, redirecting TNF-α signaling toward

survival and inflammatory response, rather than apoptosis [44]. Mutant cells seem able to generate an autocrine feed-back loop relying on the TNF-α/TNFR2 axis to escape apoptosis. A third animal-based study built on this evidence, suggesting a potential benefit to target a specific TNF-α receptor rather than the cytokine itself, and used distinct antibodies against TNFR1 and TNFR2 in a conditional *Jak2*$^{V617F}$ KI mouse model [45]. They showed only modest but dichotomous function of the two receptors on disease manifestations, with TNFR2 antagonism being responsible of decreased white blood cell and minor inflammatory cytokine decrease. To date, contrary to a large amount of inflammatory disease, TNF inhibitors have not been explored as potential PMF therapeutic agents. A pilot study using etanercept, a fusion protein neutralizing TNF-α, has been reported in 2002 with limited affect restricted to constitutional symptoms improvement [46].

IL-6 is another inflammation monokine produced by macrophages, endothelial cells, fibroblasts or activated T cells. During inflammatory response, it triggers acute phase protein release by hepatocytes, PLT production, $CD4^+$ T cell and B cell differentiation. It was also linked to angiogenesis and bone remodeling in chronic inflammatory diseases. Limited benefits of IL-6 blockade were reported in PMF mouse models. They included isolated decrease of white blood cell in the MPL$^{W515L}$ model or erythropoiesis suppression in a JAK2$^{V617F}$ model [40, 47]. Interestingly, several *in vivo* studies suggested a deleterious role of the cytokine in anti-tumor immunity, notably by promoting differentiation of $CD4^+$ T cell toward Th2 instead of Th1 cells phenotype and by impairing priming ability of dendritic cells in this specific context [48].

Finally, IL-1 was also the subject of dedicated studies in MPNs. As for TNF-α, a study on human CD34+ PBMC suggested a proliferative advantage conferred by IL-1 to the mutant cells in culture [49]. Those cells displayed a higher viability and a better proliferative potential in clonogenic assays than control cord blood cells exposed to IL-1. The role of IL-1 in disease progression was also assessed in a *Jak2*$^{V617F}$ KI mouse model by Rahman et al. [50]. Treatment with a blocking mAb against the IL-1 receptor reduced WBC and PLT counts, and reduced HSCs and myeloid progenitors in the BM. Fibrosis and splenomegaly were also reduced. In addition, competitive transplantation experiments between *Jak2*$^{V617F}$ BM cells with or without IL-1 receptor confirmed an advantage of the *Jak2*$^{V617F}$ cells able to respond to IL-1 signaling.

Overall, preclinical models suggest that inflammatory monokines, particularly TNF-α and IL-1, confer a proliferative advantage to MPN cells. Nevertheless, targeting of a single cytokine has not been associated to dramatic improvements in the clinics. Noteworthy, NF-κB activation, both in mutated and non-mutated cells, is probably a major common denominator for the production of those cytokines and represent a promising target for future treatments. This precise topic will be further discussed.

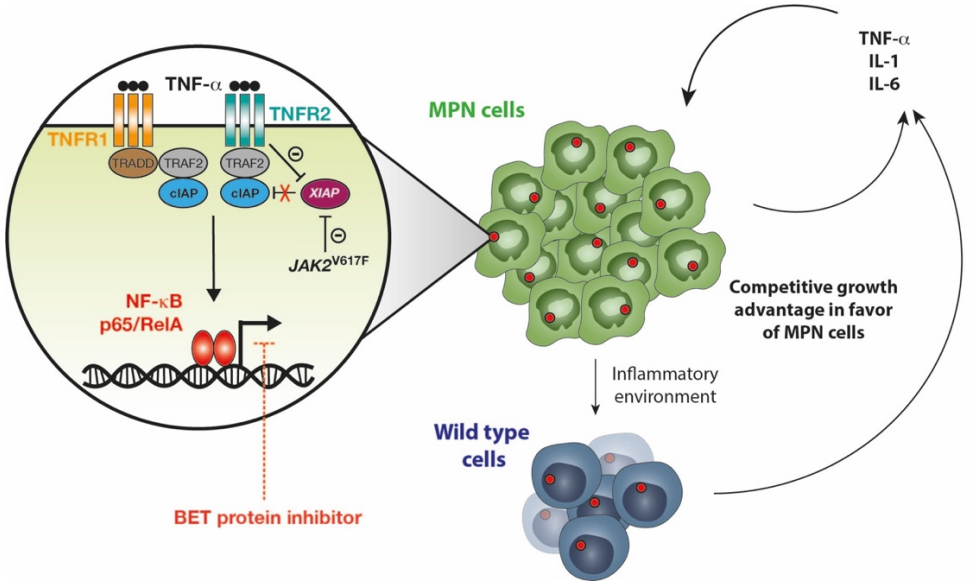


**Figure 3: Dysregulate inflammatory cytokine production confers a competitive growth advantage to the mutated clone**
MPNs and especially PMF are characterized by increased pro-inflammatory cytokine production. Cytokine production of the clone is self-reinforced and triggers further cytokine release by the wild type hematopoiesis. Constitutive NF-κB signaling fueled by monokines (TNF-α, IL-1 and IL-6) play a central role in this process which confers a proliferative advantage to MPN cells over their wild type counterpart. Indeed, TNF-α signaling is notably altered in *JAK2*$^{V617F}$ cells. They avoid TNF-α induced apoptosis via downregulation of Xiap, leading to upregulation of cIAP and redirection of TNF-α signaling toward pro-survival and pro-inflammatory response. Accordingly, inhibition of NF-κB transcriptional program with BET inhibitors is a promising investigational therapy.

### 3.1.1.2. Cytokines that remodel the BM environment

HSCs are capable of self-renewal and differentiation into every cell-populations of the hematopoietic system. In resting condition, HSCs are mainly kept in a quiescent state, corresponding to the $G_0$ phase of the cell cycle, in order to preserve their life-long capacity. Limiting their replication avoids genomic alterations and exhaustion but they must be able to quickly react and increase their output of hematopoietic cells in response to any situation of stress. This precise balance is operated by a complex niche microenvironment comprising highly organized and specialized cells. This regulatory environment is composed of endothelial cells, mesenchymal stromal cells (MSC), MKs, osteoblasts and nerve cells. Many evidences point at a BM niche disrupted at many levels in MPNs, ending by promoting mutant clone over normal hematopoiesis.

The article of Arranz et al. reported an elegant study which brought new insights into another mechanism enforced by IL-1 in MPNs, namely the ability of IL-1 secretion by mutated cells to alter the HSC niche to favor its own development [51]. In physiological condition, nestin$^+$ MSC regulate the dormancy and retention of the HSCs in the BM niche via secretion of CXCL12. The MSC are themselves stimulated by noradrenaline secretion, via their β3 adrenergic receptors, coming from sympathetic nerve fibers. It has been shown in a murine MPN model that secretion of IL-1 by *Jak2*$^{V617F}$ hematopoietic progenitors was neurotoxic, inducing a neuropathy responsible of MSC apoptosis and decreased CXCL12 production. The result was the expansion of mutant HSCs and progression of the disease. Noteworthy, sympathetic regulation of the nestin$^+$ MSC could be rescued by treating the mice with β3-adrenergic agonist, allowing the preservation of CXCL12 content and blocking MPN progression by reducing mutant HSCs number. Those results legitimated a phase 2 clinical trial, investigating sympathomimetic agonist mirabegron in a cohort of 39 JAK2$^{V617F}$ MPN patients [52]. Unfortunately, the study failed to achieve its primary end point which was an allele burden reduction of 50% (one patient showed a 25% decrease). Nevertheless, they observed a modest increase of nestin$^+$ MSC and a slight decrease of reticulin fibrosis, suggesting that this approach can indeed modify human MPN microenvironment (unfortunately without affecting mutant HSC). As mirabegron was safe and well tolerated, it could still have future potential in combination with another clone directed therapy.

Implication of other inflammatory chemokines have been revealed in the remodeling of the bone marrow niche. MSC expressing the transcription factor Gli1 can differentiate into collagen secreting myofibroblasts, which are considered as one of the key drivers of fibrosis in PMF. This observation has been made in murine models, transplanted with BM cells overexpressing TPO or transduced with *Jak2*$^{V617F}$ mutant. Indeed, Gli1+ MSC depletion or pharmacological inhibition of Gli1 is able to abolish the fibrotic phenotype of the transplanted mice [53]. CXCL4 is a chemokine produced by MKs and PLT, implicated in HSC dormancy, but also in various inflammatory diseases. In PMF, the chemokine is overproduced, especially by the pathological MKs, and responsible of the reprogramming of Gli1+ MSC leading to their differentiation into fibrosis-driving myofibroblasts [54]. Moreover, CXCL4 seems to exert even more diverse activities, as CXCL4 KO in animal models is not only responsible of decreased fibrosis but also decreased inflammatory monokines production, NF-κB activation and thrombocytosis. The underlying molecular mechanism will require further characterization.

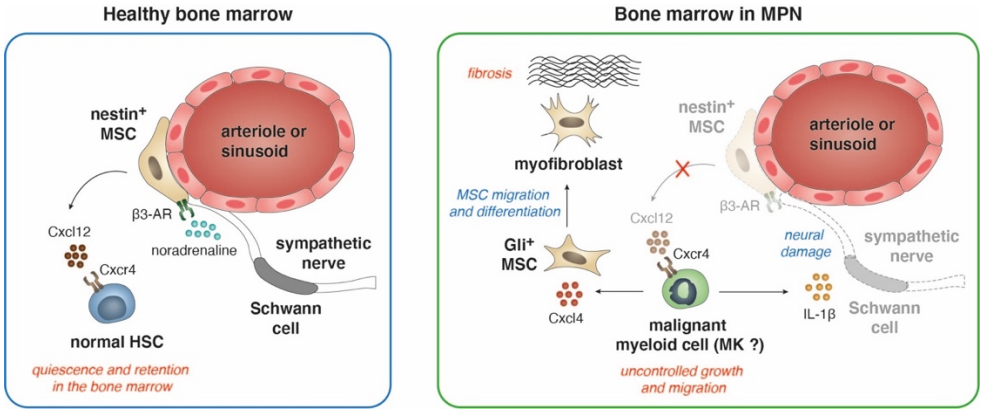


**Figure 4: Inflammation transforms bone marrow niche in MPNs (figure adapted from S. Lecomte thesis)**

(left) In healthy condition, adrenergic stimulation of nestin$^{+}$ MSC (via β3-adrenergic receptor or β3- AR) promote their *Cxcl12* release. CXCL12 binds to CXCR4 on HSC, regulating their quiescence and retention in the BM.

(right) In MPNs, neuropathy induced by IL-1β producing mutant cells is responsible of the death of nestin$^{+}$ MSC. Eventually, the loss of this regulatory cell population results in uncontrolled growth and migration of mutant cells. Moreover, mutant cells also produce CXCL4 that induces migration of Gli$^{+}$ MSC and their differentiation into myofibroblasts, responsible of BM fibrosis.

#### 3.1.1.3. Inhibition of cytokine signaling in clinical trials

Several *in vitro* and *in vivo* results have suggested a potential synergy between JAK inhibitors and NF-κB directed therapy. Indeed, whereas JAK1/2 inhibitor ruxolitinib is a potent anti-inflammatory drug, able to decrease secretion of several cytokines overexpressed in PMF, success have been clinically modest and its inability to decrease disease burden leaves some unmet medical needs [55]. Bromodomain and extraterminal domain (BET) proteins are epigenetic readers, binding acetylated residues on histone tails and promoting gene transcription. Those proteins particularly facilitate NF-κB transcriptional program. Competitive inhibitor JQ1 has been tested in *MPL*$^{W515L}$ and *Jak2*$^{V617F}$ mouse models [41]. Benefits of the treatment included decreased disease burden, decreased inflammation and reduced fibrosis. Whereas JAK inhibitor alone failed to counter MPL$^{W515L}$ cell expansion in mice, combination of JAK and BET inhibitor reduced the number of transformed cells, decreased inflammatory cytokines (notably IL-1 and IL-6) and reversed fibrosis to a greater extent than any of the single agents. Preclinical data were completed with *in vitro* experiments on CD34+ progenitor cells from MPN patients and confirmed the synergy between ruxolitinib and JQ1 on colony formation assays. This rational prompted clinical investigation of pan-BET inhibitor pelabrasib (CPI-0610), alone or in combination with ruxolitib [56]. Results of the phase 2 clinical trial, MANIFEST, were especially encouraging in the combination arm with symptoms improvements, spleen volume reduction and indication of disease modifying activity, such as anemia and fibrosis reductions (at least one grade improvement in a third of the patients). Phase 3 randomized clinical trial MANIFEST-2 is currently ongoing and assesses the benefits of the combination therapy in ruxolitinib-naïve patients.

### *3.1.2. Elevated production of anti-inflammatory cytokines*

Adding to the complexity of the inflammatory landscape in MPNs, all cytokines overexpressed in the disease are not pro-inflammatory. Indeed, IL-10, and more importantly TGF-β1, are rather associated to inhibition of inflammation and immunosuppression.

### 3.1.2.1. TGF-β1 in MPNs

TGF-β1 is pleotropic cytokine that was suspected to be associated with PMF a long time ago. It belongs to the TGF-β superfamily, which comprises 33 structurally related members. Three closely related TGF-β isoforms (TGF-β1, 2 and 3) are expressed in mammals. They share homologous amino-acid sequences and signal through the same TGF-β receptor. Of the three, only TGF-β1 is overexpressed in PMF. Remarkably, almost all cell types in adult mammals produce TGF-β1, but they all produce it in an inactive form, called latent TGF-β1. Latent TGF-β1 is composed of a mature TGF- β1 dimer, which remains non-covalently associated to the Latency Associated Peptide (LAP) dimer, comprised of the N-terminal portion of the pro-TGF-β1 precursor generated by Furin-mediated cleavage. When associated with LAP, mature TGF-β1 cannot interact with the TGF-β receptor. To become biologically active, mature TGF-β1 must be released from LAP, a process called "TGF-β1 activation". Few cell types can accomplish this tightly regulated activation process, which involves cell-type dependent mechanisms. Of the many activation mechanisms described in *in vitro* settings, only those involving thrombospondin 1 and RGD-binding integrins were shown to have *in vivo* relevance.

The three TGF-β isoforms bind the same hetero-tetrameric receptor composed of two high affinity TGF-β receptor class 2 chains (TGF−βRII), and two TGF-β receptor class 1 chains (TGF−βRI). Upon ligand binding, TGF−βRII chains will recruit and phosphorylate the intracytoplasmic domains of the TGF−βRI chains. Downstream signaling involves recruitment and phosphorylation of R-SMADs, namely SMAD2 and SMAD3, that will bind SMAD4. Trimeric complexes migrate to the nucleus to exert transcriptional activity upon binding to SMAD-responsive elements in numerous genes. Regulation of gene expression by SMADs requires the contribution of various other DNA-binding cofactors. The diversity of the latter contributes to the pleiotropic actions of TGF−βs. In addition to this so-called canonical signaling pathway, SMAD-independent signaling can also be mediated by MAPK, PI3 kinase or Rho GTPases pathways.

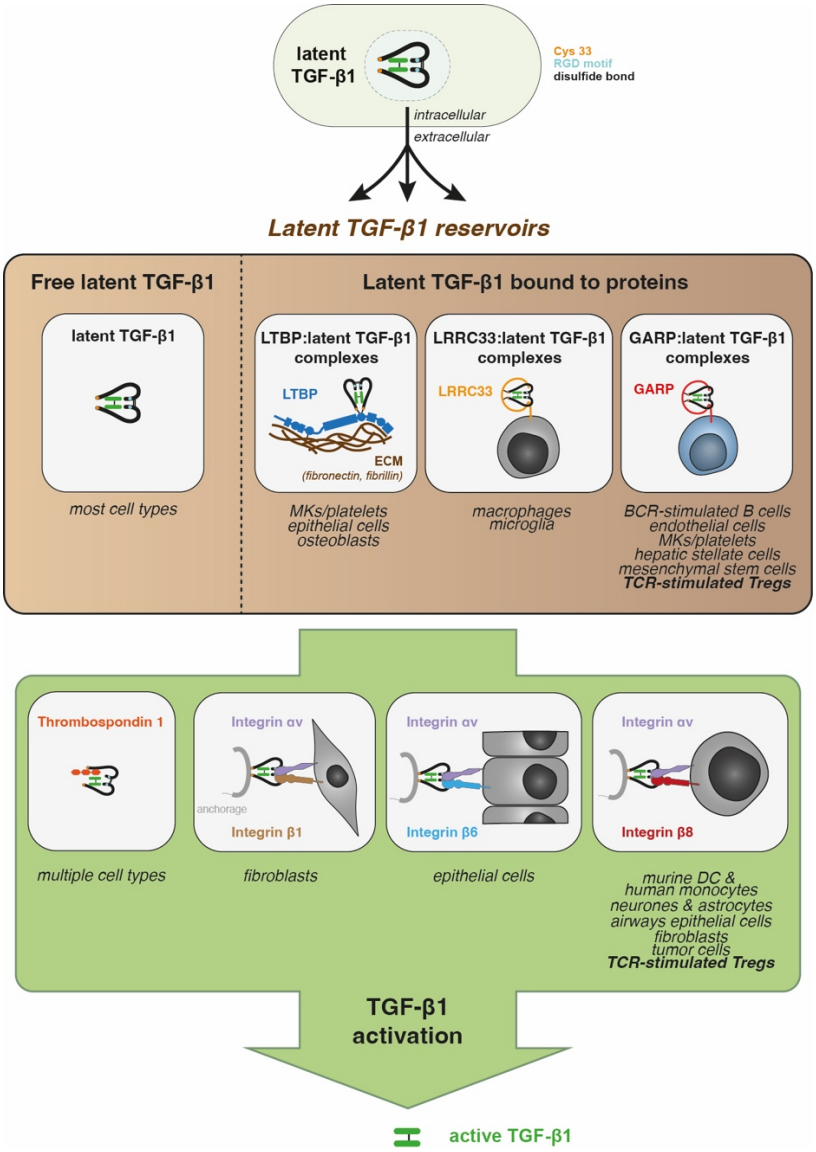


**Figure 5: Latent TGF-β1 reservoirs and activation mechanisms**
TGF-β1 is produced by most cell types as an inactive complex (latent TGF-β1), in which LAP remains non-covalently associated to mature TGF-β1. While most of the cells secrete soluble latent TGF-β1 in the extracellular space, some of them secrete latent TGF-β1 bound to other proteins, via formation of disulfide bonds involving Cys33 in LAP. This pool of latent TGF-β1 is stored in the extracellular matrix (in complex with Latent TGF-β1 Binding Proteins, LTBP) or on the cell surface (in complex with LRRC33 or GARP). To bind its receptor, mature TGF-β1 needs to be released from the LAP (TGF-β1 activation). Only very few cell types activate TGF-β1, via mechanisms that are cell-type specific, consisting in latent TGF-β1 presenting molecules (*i.e.* LTBPs, LRRC33 or GARP) that cooperate with dedicated activation partners. Among these partners, 2 groups have proved *in vivo* relevance: thrombospondin 1 and RGD-binding integrins (like αvβ1, αvβ6 and αvβ8). The best-understood mechanisms of TGF-β1 activation involves αvβ6 and αvβ8, which require latent TGF-β1 anchor to LTBP or GARP, respectively.

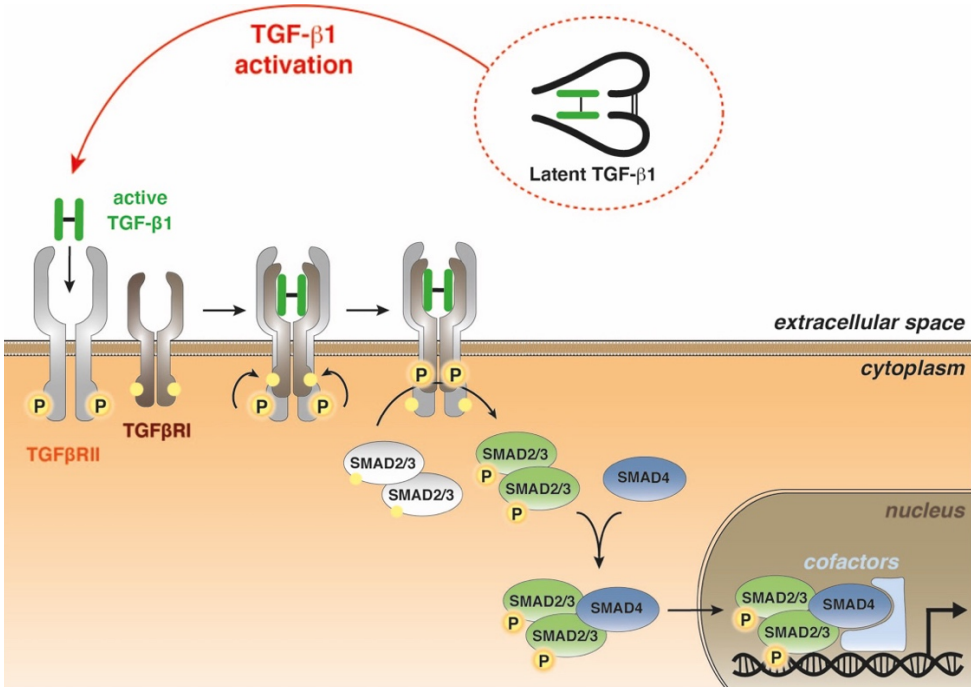


**Figure 6: Canonical TGF-β1 signaling pathway (figure adapted from S. Lecomte thesis)** Following activation from latent-TGF-β1, active TGF-β1 binds to TGFβRII. It triggers the recruitment and phosphorylation of TGFβRI. The activated tetrameric receptor complex phosphorylates SMAD2/3 cytosolic transcription factors (R-SMADs), that will then join SMAD4. The hetero-trimeric complex finally translocates to the nucleus. There, it will induce or repress the transcription of a variety of genes, modulated by its association to a variety of co-factors.

As highlighted by many studies, TGF- β1 concentration in plasma is increased in MPNs, particularly in PMF. Unfortunately, many of the studies in patients fail to make a clear distinction between concentrations of total TGF- β1 (*i.e.* active + latent TGFβ1) and active TGF- β1. This is a limitation as an increased pool of latent TGF- β1 may not necessarily imply increased TGF-β1 activity, as TGF-β1 is a tightly regulated process. Among the few studies that paid attention to this distinction, Campanelli et al. rigorously quantified active TGF-β1 in the plasma of PMF patients, along with PV/ET patients and healthy control [57]. They found increased blood and BM plasma levels of active TGF-β1 in the three types of MPN.

Which cell type is responsible for increased TGF- β1 activity in PMF is an even more difficult question. Results from several studies, using complementary techniques,

suggest that MKs are the main producers of the increased amounts of latent TGF-β1 in PMF. A first study used immunohistochemistry on BM samples from ET, pre-PMF or fibrotic PMF patients. The authors reported increased amounts of latent TGF-β1 in the fibrotic forms of the diseases and identified MKs as producers. Unfortunately, the technique cannot discriminate active from latent TGF-β1 and is hardly quantitative [58]. A second study analyzed the transcriptome MKs isolated by laser microdissection in BM sections from 45 PMF patients. They confirmed increased TGF-β1 mRNA expression in the MKs [59]. One last study reported an increased ability of human MKs to secrete total TGF-β1 *in vitro,* using ELISA [60]. Therefore, considering that active TGF-β1 production is regulated mostly at post-transcriptional and post-translational levels and cannot be measured by immunohistochemistry, there is no study that formally identified the cellular source of increased, active TGF-β1 in PMF. Nevertheless, MKs appear to represent at least a major reservoir of latent TGF-β1.

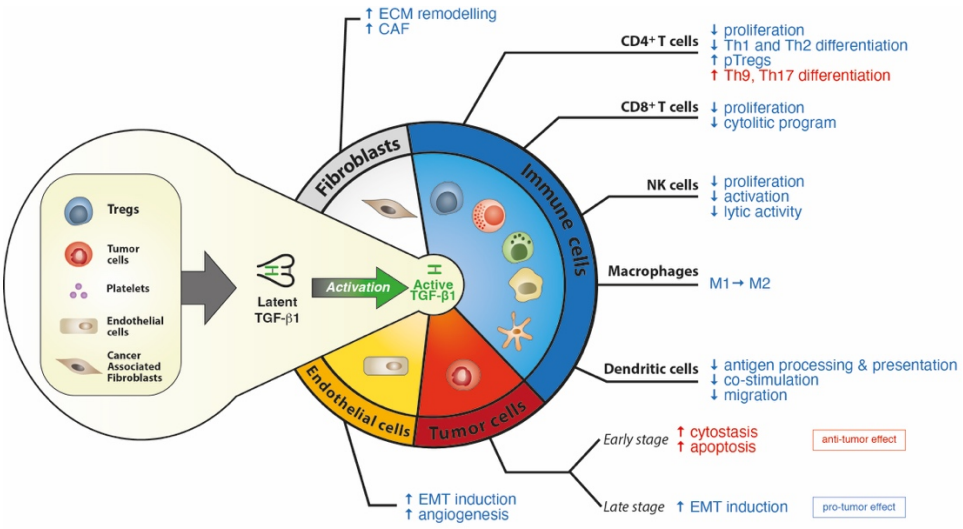


**Figure 7: TGF-β1 sources and activities in cancer (figure adapted from G. de Streel thesis)**
Various cell types produce latent TGF-β1 in cancer, such as Tregs, tumor cells, platelets, endothelial cells and cancer associated fibroblasts (CAF). Noteworthy, only some of them have brought indisputable proofs of their ability to activate the cytokine (Tregs in particular). On tumor cells, active TGF-β1 plays a dual role as it can promote both suppression of pre-malignant lesions and cancer progression with metastasis (epithelial-mesenchymal transition or EMT induction). It is also a potent immunosuppressive cytokine inhibiting proliferation, differentiation and anti-tumor function of numerous immune cells. Finally, it also remodels the tumor microenvironment (TME) through additional effect on fibroblasts and endothelial cells.

Since embryonic development, TGF-β1 is a key mediator of a wide array of biological processes in mammals, including cell proliferation, cell and tissue differentiation, vasculogenesis, wound healing and immune response. During tumorigenesis, the cytokine even plays a dual role. Indeed, although it suppresses pre-malignant lesions, it promotes cancer progression by increasing tumor invasiveness and metastatic activity (EMT induction), while remodeling the microenvironment toward an immunosuppressive tumor-permissive bed.

For the purpose of this review, we will only discus here of its roles in hematopoiesis, tissue repair and immunity, as they are the only ones to have been specifically investigated in MPNs.

#### *3.1.2.1.1. Effects of TGF-β1 on HSC quiescence and differentiation*

TGF-β1 is an essential regulator of cell proliferation and differentiation, including during hematopoiesis. The prominent role of the cytokine in preventing HSC exhaustion by maintaining their quiescence was described in several studies. In a conditional TGF−βRII KO mouse strain (*Mx-1*$^{cre}$ *Tgfbr2*$^{fl/-}$ in a *Rag2*$^{-/-}$ background), Yamazaki et al. showed that suppression of TGF-β signaling in HSC was associated with increased cycling [61]. This was associated with progressive exhaustion, demonstrated by a decreased repopulating capacity in serial transplantation experiments. Non-myelinating Schwann cells were proposed to activate TGF-β1, on the basis of their close contacts with HSC and the reproduction of the TGF−βRII KO phenotype upon microsurgical BM denervation. In addition to nerve cells, another well-known actor of the bone marrow niche was proposed to exert a similar role. Indeed, conditional deletion of MKs by diphtheria toxin in *Pf4*$^{cre}$;iDTR mice was also shown to nearly abrogate R-SMAD phosphorylation and induce HSC cycling [62]. In the two models, the phenotypes were rescued by recombinant TGF-β1 administration. Outside of any pathology, MKs seem then to be once again closely associated to TGF-β1 production. Whether non-myelinating Schwann cells and MKs are both independent activators of TGF-β1 or if a collaboration between the two cell types is required is not known.

Based on its anti-proliferative properties, TGF-β1 would be intuitively considered as a tumor-suppressor in MPNs. However, as in many other neoplasms, growing evidence points at a subversion of the effects of TGF-β1. Acquisition of resistance to the cytostatic properties of TGF-β1 would represent a first step, allowing the MPN clone to gain clonal dominance on non-mutated BM cells, but also to exploit the full spectrum of its biological properties to further remodel the BM niche to its advantage.

Arguments supporting an acquired resistance of PMF progenitors to the cytostatic effects of TGF-β1 were brought by two publications studying PBMCs *in vitro* [63]. Ceglia et al. compared the effect of SB-431542, a TGF−βRI inhibitor, on PBMC cultures from PV patients, PMF patients and healthy subjects. While SB-431542 increased the number of progenitor and precursor cells derived from healthy and PV PBMCs, it had no impact on the proliferation of mononucleated cells from PMF donors. The authors considered this lack of effect as indirect evidence for TGF-β resistance by PMF cells. Another group went a little further. Using colony formation assays with PBMC from *JAK2*$^{V617F}$ PMF patients, they tested the effect of AVID-200, a potent TGF-β receptor trap which is able to consume TGF-β1 and TGF-β3 [64]. Treatment with AVID-200 had no impact on the absolute number of hematopoietic colonies generated by PMF PBMC. However, in 5 out of the 8 PMF samples tested, they observed a decreased number of *JAK2*$^{V617F}$ colonies with a concomitant increased number of WT colonies (1,6-fold). This could again indirectly suggest that in some PMF samples, active TGF-β is produced and inhibits the proliferation of WT but not mutant cells. We performed proliferation assays with murine 32D myeloid cells stably expressing MPL$^{WT}$ or the MPL$^{W508A}$ mutant in the presence of recombinant TGF-β1. We observed that *Mpl*$^{W508A}$ mutant cells are indeed resistant to the cytostatic effects of recombinant TGF-β1 (manuscript in preparation).

The question was also addressed *in vivo*, in a murine MPN model resulting from transplantation of *Jak2*$^{V617F}$ BM cells [65]. In competitive transplantation experiments, the authors compared *Jak2*$^{WT}$ to *Jak2*$^{V617F}$ BM cells and confirmed the proliferative advantage of mutated cells, which represented an increasing fraction of the circulating WBC over time. They next combined expression of the *Jak2*$^{V617F}$ transgene with an inducible deletion of the gene encoding TGF−βRII. In competitive transplantation experiments, the proliferative advantage of *Jak2*$^{V617F}$ over *Jak2*$^{WT}$ BM cells was conserved as long as the *Jak2*$^{WT}$ cells had an intact TGF−βRII expression. In contrast, if TGF−βRII was KO in *Jak2*$^{WT}$ cells, the competitive growth advantage

of *Jak2*$^{V617F}$ cells was lost. As expected, maintenance of TGF−βRII in *Jak2*$^{V617F}$ was not essential for their expansion. TGF-β1 is thus suggested to participate to the acquisition of clonal dominance by *Jak2*$^{V617F}$ cells only if WT cells remain sensitive to its anti-proliferative effect.

The mechanism behind the lack of sensitivity of MPN cells to the cytostatic effects of TGF-β1 remains elusive. Decreased expression of TGF−βRII, due to hypermethylation of the *Tgfbr2* promoter, was described in CD34+ cells from some PMF patients [66]. Decreased expression of SMAD4 was also detected, but more rarely. It should be noted that while the phenomenon is seen in numerous solid tumors, many of them present a functional TGF-β signaling pathway. Cytostatic effects of TGF-β1 could be bypassed by mutations in other tumor-suppressor or tumor promoting pathways.

#### *3.1.2.1.2. Pro-fibrotic effects of TGF-β1*

Following acute injury, the activation of a matrix-preserving fibrogenic program induces rapid reparative response, aiming at the preservation of the basic structure of the organ. Dysregulated, persistent or hyperactive fibrogenic response will lead to fibrosis, characterized by an increased deposition of extra-cellular matrix (ECM) proteins and impaired organ function [67]. TGF-β1 is implicated in tissue repair, but also in fibrosis across a wide variety of diseases and organs. While SMAD-dependent or SMAD-independent signaling pathways are implicated, the process remains incompletely understood and context-dependent. Overall, TGF-β1 effects can be grouped in two categories. On one hand, TGF-β1 will stimulate matrix biosynthesis, by inducing the differentiation of central effectors called myofibroblasts. Depending on the context, the origin of these cells has been attributed to resident fibroblasts, epithelial cells, endothelial cells, MSCs or even monocytes. Myofibroblasts stimulated by TGF-β1 will then produce ECM components such as type I, III and IV collagens. On the other hand, TGF-β1 will inhibit degradation of the ECM proteins, through induction of various protease inhibitors.

Fibrosis is by definition a hallmark of PMF. Most of the elements gathered so far regarding the implication TGF-β1 in this particular process come from MPN animal models.

In a landmark study published twenty years ago, Chagraoui et al. used a $TPO^{high}$ mouse model to reproduce PMF [68]. In this model, irradiated recipient mice were transplanted with BM cells retrovirally transduced to overexpress TPO. Recipients developed an ET-like phenotype evolving within months toward myelofibrosis, with a dense reticulin network observed in BM and spleen. Strikingly, when donor BM cells were from TGF-β1 KO mice (*Tgfb1*$^{-/-}$), MPN still developed but fibrosis was abolished. Those results supported the implication of TGF-β1 produced by the mutant clone in PMF.

Another study relied on $Gata1^{low}$ mice. Due to an hypomorphic mutation of the transcription factor Gata1, these mice show impaired MKs development associated to anemia and thrombocytopenia [69]. At 1 year of age, they develop progressive extramedullary hematopoiesis and fibrosis similar to PMF. Interestingly, $Gata1^{low}$ mice displayed increased plasma level of total and active TGF-β1. Treatment with a TGF−βRI inhibitor (SB-431542) rescued normal hematopoiesis, notably by improving MKs maturation, and decreased both splenomegaly and fibrosis.

Finally, Yue et al. produced convincing data showing reduced fibrosis with galunisertib, another TGF−βRI inhibitor, in $MPL^{W515L}$ and $JAK2^{V617F}$ mouse models [70]. Here, beyond confirming MKs accumulation and increased TGF-β1 production, the authors showed that MSCs were directly stimulated by the cytokine which increased their production of collagen I and III in a SMAD3-dependent manner. As shown by others, MSCs could differentiate into myofibroblasts, one of the fibrosis-driving cell types in PMF. Interestingly, MSCs isolated from *MPL*$^{W515L}$ retained an abnormal phenotype *in vitro*, secreting more collagen than MSC isolated from *MPL*$^{WT}$ mice. Nevertheless, galunisertib was still able to decrease their collagen production *in vitro* and decreased fibrosis *in vivo* in the two MPN models. It should be noted that here, the effect of TGF−βRI blockade did not reduce disease burden.

In PMF patients, TGF-β1 implication in fibrosis has not been established with a comparable level of confidence. Some evidence can be found in transcriptional studies of BM samples. Microarray analysis, based on mononucleated cells isolated

from BM and spleen of PMF patients, revealed an up-regulation of several genes (such as *JUN*, *EVI1* and *STAT1*) that can relate to the non-canonical MAPK-dependent signaling pathway of TGF- β [71]. This proposed hypothesis is however less robust than modifications in the canonical SMAD-dependent signaling pathway. Nonetheless, it would correspond to a TGF-β1 signaling pathway already described in auto-immune models such as those of scleroderma [72]. Interestingly, those observations were confirmed by another group in PMF, but not in ET. This would reinforce the prominent role played TGF- β1 in PMF compared to other MPNs.

#### *3.1.2.1.3. Immunosuppressive effect of TGF-β1*

TGF-β1 exerts critical roles in regulating immunity and inflammation. This was particularly well illustrated by the phenotype of *Tgfb1*$^{-/-}$ mice, which die from multi-organ inflammation and autoimmunity as early as 3-4 weeks after birth [73]. Immunoregulatory properties of TGF-β1 affect both innate and adaptive immune responses at multiple levels.

In innate immune cells, TGF-β1 polarizes macrophages toward a regulatory M2 phenotype, decreases maturation and cytokine production of NK cells, but also decreases the ability of dendritic cells (DC) to prime and activate T-cells [74].
Adaptative immune responses are also dampened, with major immunosuppressive effects of TGF-β1 on T cells. Here again, it was well illustrated by CD4$^{cre}$ x T*gfbr2*$^{fl/fl}$ mice, which have a conditional deletion of TGF−βRII in all T cells [75]. The absence of TGF-β signaling in T cells leads to a similar lethal phenotype as that of *Tgfb1*$^{-/-}$ mice. TGF-β1 is a potent inhibitor of T cell proliferation, notably inhibiting their IL-2 production [76]. Effector functions of cytolytic T lymphocytes (CTL) is also reversibly decreased following downregulated expression of CTL effector programs (*Ifng*, *Gzma*, *Gzmb*, *Prf1* and *Faslg*). TGF-β1 impairs differentiation of naïve CD4$^{+}$ T cells into Th1 and Th2 cells, by downregulating essential transcription factors, T-bet and GATA-3, respectively. Moreover, cytokine production by Th1 (IFNγ) and Th2 cells (IL-4) is also inhibited by TGF-β1 [77, 78].

T lymphocytes are able to recognize and mount specific immune response against tumor cells. In the context of tumor immunology, TGF-β1 was shown to favor tumor development through its inhibitory effect on the T cell compartment. This has notably

been shown with transgenic mice expressing a dominant negative TGF−βRII on T cells ($CD4^{cre}$ x T*gfbr2*$^{dn}$ mice). In these mice, attenuated TGF-β1 signaling in T lymphocytes lead to more potent anti-tumor CTL responses and increased resistance to transplanted tumors [79, 80]. However, if we want to counter this pathway for cancer treatment, complexity arises because of the pleiotropic activities of TGF-β1, which plays dual roles in cancer. On one hand, it exerts tumor-suppression on pre-malignant cells by inducing cytostasis. On the other hand, it exerts pro-tumoral activities through pro-metastatic effects (EMT) and immunosuppression of anti-tumor T cell responses. To selectively target the pool of active TGF-β1 that contributes to cancer progression, an interesting approach would be to identify its origin and precise mechanism of activation. To this aim, our laboratory has recently shown that TGF-β1 activated from GARP:TGF-β1 complexes on Tregs contributed to the suppression of anti-tumor T cell responses in mouse tumor models. Indeed, in CT26 and MC38 transplantable tumors models, treatment with a blocking anti-GARP:TGF-β1 mAb was sufficient to overcome resistance to anti-PD1 based immunotherapy [81]. This approach is interesting because of its selectivity, as a limited number of cell types, including activated Tregs, activated via a GARP-dependent mechanism. Thus, blocking GARP:TGF-β1 complexes may preserve other sources of active, and potentially tumor-suppressive TGF-β1.

Impact of TGF-β1 on T cell responses in MPNs has not yet received any attention. Building on previous results gathered by our laboratory in mouse tumor models, we obtained original results suggesting that TGF-β1 activated from GARP:TGF-β1 complexes on Tregs could also be implicated in the development of mouse PMF. Our results support the use of immunotherapy in MPNs and will be further discussed in the chapter dedicated to Tregs.

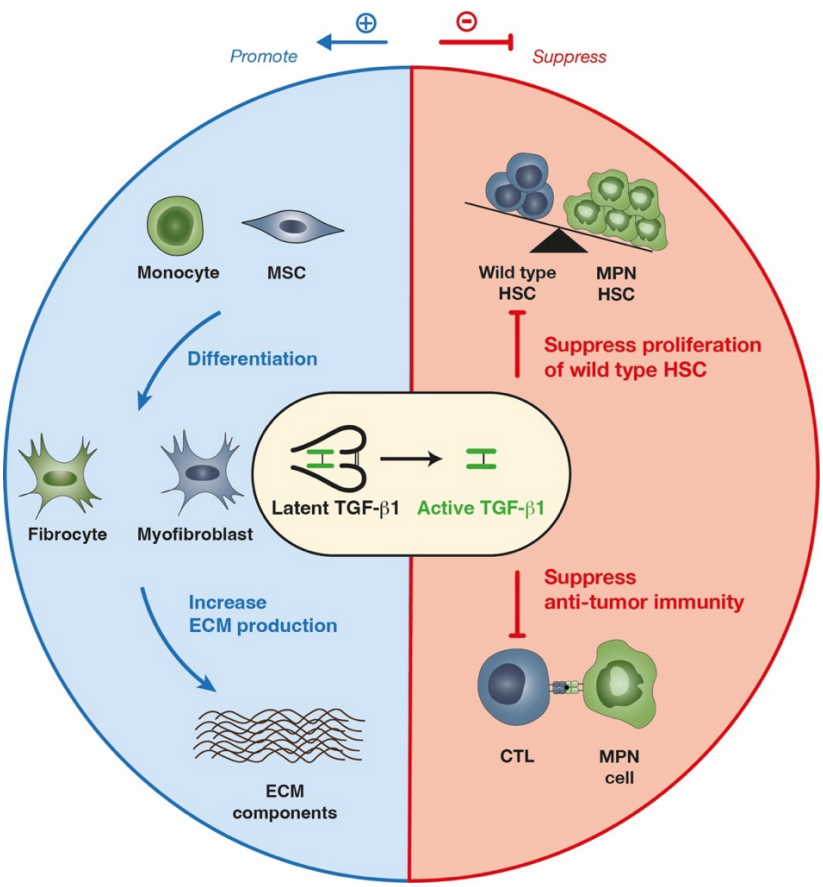


**Figure 8: Subverted functions of TGF-β1 in MPN**
MPNs and particularly PMF are characterized by an increased TGF-β1 activation. Firstly, as mutated hematopoietic progenitors are resistant to its cytostatic effect, it gives them of proliferative advantage by preferentially inhibiting the growth of wild type BM cells. Secondly, through its immunosuppressive properties, it dampens putative anti-MPN T cell responses, allowing tumor immune escape. Finally, it contributes to fibrosis development by promoting the differentiation of MSC (and monocytes) into fibrosis driving cells. Furthermore, it stimulates their production of ECM components, including type I and type III collagen.

#### 3.1.2.2. IL-10 in MPNs

As mentioned previously, IL-10 is overexpressed in PMF, but to our knowledge its contribution has never been comprehensively studied in this particular context. IL-10 is a cytokine exerting essential function in immune homeostasis, targeting both innate and adaptive immune responses to prevent tissue damage due to excessive immune effector activity. It's of particular importance for the resolution phase of inflammation and in the gut to maintain homeostasis to the microbiome. Accordingly, multiple murine *Il10* KO models are affected by inflammatory colitis [82]. IL-10 has been targeted in multiple clinical trials, notably in inflammatory bowel diseases. A vast majority of activated immune cells are able to produce the cytokine. Myeloid cells, such as DCs and macrophages, produce it along with proinflammatory cytokines, secondary to the activation of intracellular pathways including NF-κB. IL-10 acts on these cells to dampen proinflammatory cytokine production and antigen presentation. T cells, including Tregs, are also able to secrete IL-10. This ability of Tregs has been of particular interest because it was also shown to be an immunosuppressive mechanism responsible for the exhaustion of anti-tumor CTLs. IL-10 secretion by intra-tumoral Tregs could favor the escape of tumoral cells from the anti-tumor immunity [83]. Whether increased IL-10 is just a bystander elevated cytokine due to NF-κB activation or is responsible of an immunoediting mechanism by any means is completely unknown but warrants further investigations in PMF.

### 3.2. Anti-tumor T-cell responses in MPNs

It's has been several decades since the discovery of human tumor antigens recognition by T cells, confirming the existence of an adaptive immune response directed against cancer cells. Over the years, many therapeutics improved this anti-tumor response with notably cancer immunotherapy which can be defined as the "deliberate use of the adaptive immune system to reject tumors or to prevent their recurrence". This led to major therapeutic advances for certain families of cancers. The existence of such immune response in MPNs is still a matter of debate, but if so, it could pave the way to new treatment perspectives.

Among T cells, CTL have been recognized as the primary mediator of anti-tumor immunity. In order the generate an effective T cell response capable of killing the

tumor cells, several steps are mandatory. Those have been described as a “cancer-immunity cycle”, supposedly able of self-reinforcement [84]. Tumor neoantigen will first need to be captured by antigen presenting cells like DC. Then DC will need to process the antigen into peptides that will be cross presented to T cells on major histocompatibility complex (MHC) class I molecules. This priming step will activate effector T cells against this specific tumor antigen, if it is indeed recognized as foreign (no prior elimination of this T cell by negative selection in the thymus). Next, activated effector T cells will need to traffic and infiltrate the tumor bed. There, they should be able to specifically recognize cancer cells, upon engagement of their T cell receptor (TCR) with the cognate antigen presented on MHC class I at the surface of the tumor cells. Tumor cell lysis will release more antigens, increasing anti-tumor immune response by repeating the steps of the cycle.

After a general discussion on tumor antigens, we will critically review the arguments that support the occurrence of spontaneous T cell responses against MPN cells.

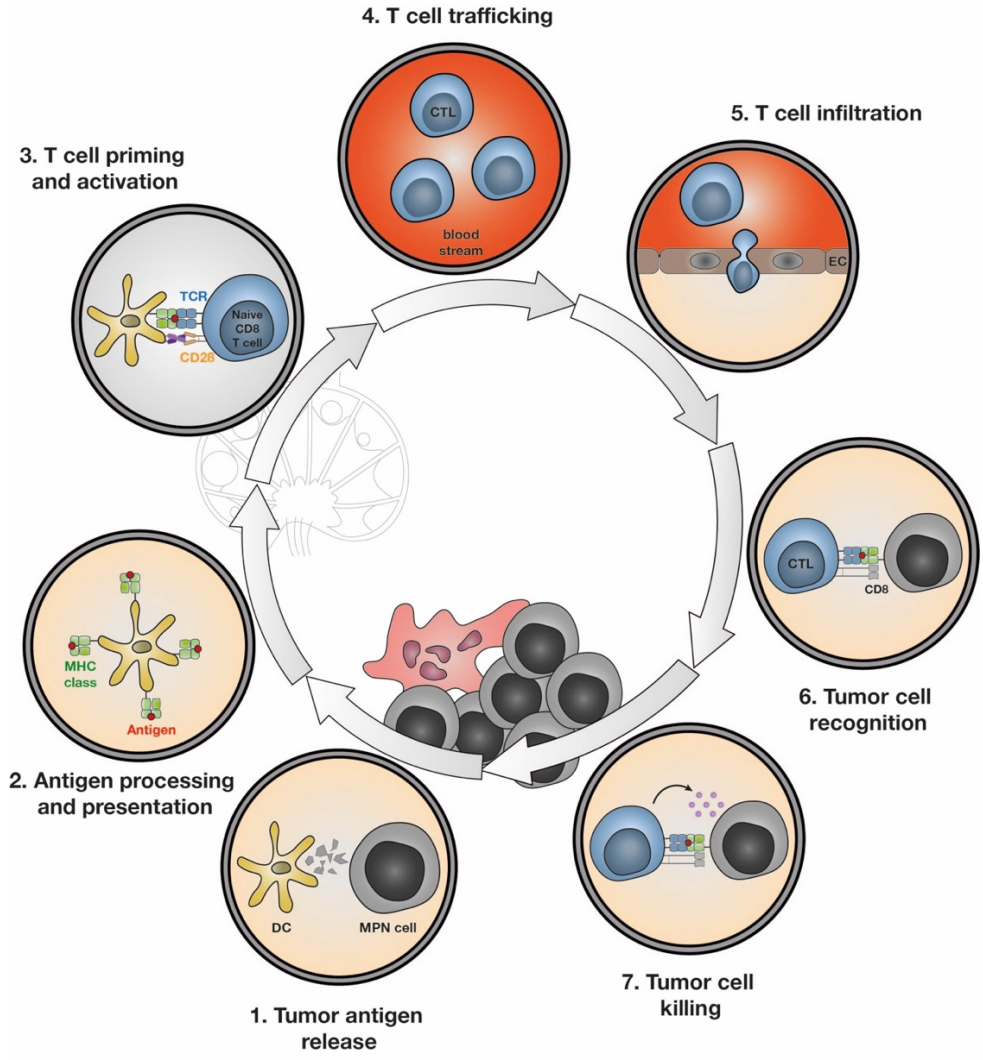


**Figure 9: Cancer-immunity cycle**
Mounting a T cell response leading to the effective killing of tumor cells is a stepwise process. In step 1, the tumor antigen needs to be captured by antigen presenting cells like DC. In step 2, this antigen needs to be processed into smaller peptides and mounted on MHC class I molecules in order to be cross presented to $CD8^+$ T cells. In step 3, antigen presentation leads to T cell priming in lymph nodes. Adequate TCR stimulation by MHC-bound antigen, in combination with co-stimulation signal (CD28-B7 engagement) and cytokine (notably IL-2), will activate naïve $CD8^+$ T cell and lead to its differentiation into effector CTL. In step 4 and 5, CTL will migrate to and infiltrate the tumor bed. There, in step 6, CTLs need to recognize tumor cells via engagement of their TCR with the specific MHC class I bound antigen. Lastly, CTL activation will lead to tumor cell lysis requiring specific effector function deployment (perforin/granzyme, Fas ligand). This process can be perceived as a cycle as tumor cell lysis will further increase the release of tumor antigens and the breadth of the immune response.

### *3.2.1. Tumor antigens in cancer and tumor mutational burden*

Anti-tumor T cell responses presume the expression of tumor antigens. According their tumoral specificity, these antigens can be divided in two categories [85]. Among the antigens of low tumoral specificity, we find differentiation antigens (expressed in the tumor and in its organ of origin) and antigens that are overexpressed in tumors. Their expression is shared between tumor and normal tissues. The second category regroups the antigens of high tumor specificity and includes: viral antigens, neoantigens (that result from a mutation or a rearrangement of a gene coding sequence) and antigens encoded by cancer germline genes (normally expressed only in germline cells, which do not expressed MHC molecules). To mount an adaptive immune response that is truly tumor-specific, this last category is thus a particularly interesting target.

Identification of tumor specific antigens and their epitopes recognized by T cells is a challenging task, requiring dedicated methods. Without looking for exhaustivity, some of them would be relevant to mention.

On one hand, a reverse immunology approach consists at identifying a candidate antigen based on abnormal expression profile or on identification of mutated gene sequence. After that, computer-based algorithm will be used to predict possible epitopes and the HLA molecule they would bind to (usually short 9 amino-acids sequences for MHC class I molecules) [86]. Candidate small peptides that effectively bind HLA molecules will then be validated in *in vitro* T-cell assays. If activating T-cells, they can be used to stimulate the generation of specific T cell populations or clones [87]. Successful generation of an antigen specific CTL clone is although insufficient in itself. Indeed, at this point, we don't know if the antigen will be processed by tumor cells in order to produce the right immunogenic peptide. Tumor-specific and biologically relevant CTL clones must then recognize tumor cells displaying the appropriate HLA molecule and not previously pulsed with candidate peptide. At the end, only less than a 1% of the predicted candidates are true HLA-presented neoepitopes.

On the other hand, we can directly study the HLA-bound peptides of tumor cells (or immunopeptidome). This unbiased method relies on mass spectrometry with the advantage to directly focus on epitopes that are presented *in vivo* [88]. However, the

sensitivity remains a drawback as neoantigen-derived peptides usually account for a small proportion of the immunopeptidome.

Neoantigen have been particularly well studied in recent years and mutated genes proved to greatly contribute to the immunogenicity of human tumors. It has been well acknowledged that the frequency of those mutations greatly varies across cancer types, notably favored by environmental factors (like tobacco smoking or ultraviolet radiations) or tumor intrinsic factors such as defective DNA repair pathways [89]. This can be observed in lung cancers and melanomas, which are usually comprising around 200 non-synonymous mutations per tumor, whereas most of breast or digestive tumors only have around 50 of those. Standardized frequency of non-synonymous nucleoid variants in tumor genome is usually referred as tumor mutational burden (TMB, i.e. number of mutations per megabases). Intuitively, one would expect that higher TMB would translate into higher number of altered amino-acid sequences, thus increasing the chances to obtain peptides presented on HLA molecules to cognate anti-tumor T cells. Accordingly, some results obtained with checkpoint inhibitor therapies initially tended to agree with this hypothesis. In a meta-analysis published in 2017, objective response rate to anti-PD-1 or anti-PD-L1 therapy was positively correlated with TMB across 27 tumor types [90]. Tumors bearing the lowest TMB such uveal or pancreatic cancers had the lowest response rate, whereas high TMB non-small cell lung carcinomas, melanomas and cutaneous squamous cell carcinomas were the best responders. Nevertheless, predicting value of TMB has been contested by several exceptions. As seen in the same meta-analysis, Merckel cell carcinomas, although having a relatively low TMB, had an unexpectedly high response rate to immunotherapy. At the opposite, gliomas in which high TMB had been induced by alkylating chemotherapy conserved a low rate of response. Thus, a higher mutation load in tumor is associated to a higher probability to develop anti-tumor immune response, but TMB alone remains an imperfect biomarker. Many reasons can explain this inability to determine a universal TMB cut-off threshold [91]. One of them is probably that TMB emphasizes the quantity of mutations without considering their quality. Mutations alter protein sequence differently if they are replacing one single nucleotide, inducing a frameshift or even influencing RNA splicing [92]. Single nucleotide replacements tend to dominate TMB but are not necessarily the most immunogenetic mutations. Secondly, another explanation could be found in intratumor heterogeneity, as the immunogenic neoantigen could be distributed non-clonally and only targetable in a fraction of tumor cells. Finally, driver mutations, located in tumor driver genes, would

also be ideal targets for T cell because they are required for the selective growth advantage of the tumor clone (as opposed to passenger mutations).

Overall, an ideal tumor antigen can be described as immunogenic, specific of the tumor, derived from driver genes and homogenously distributed among tumor cells.

### *3.2.2. Putative tumor antigens in MPN patients*

#### 3.2.2.1. Neoantigens

In the field of MPNs and more precisely PMF, tumor associated neoantigens has been by far the most investigated category. As a general finding, TMB of myeloid malignancies has always been depicted as low and conventional immunotherapies have not been as successful as in certain solid tumors. Although we still don't know if MPNs can be eliminated by an anti-tumor immune response, remissions obtained through HSCT can help to maintain a reasonable hope. On the few exomes that have been reported, MPNs makes no exception to low myeloid TMB. In a publication from 2013, a research group performed whole exome sequencing (WES) of 151 MPN patients, including PV, ET and PMF [93]. They found a median of 6,5 mutations per patient in PV or ET and 13,0 mutations per patient in PMF. Those results are coherent with the notion that PMF is more frequently accompanied by mutations, notably in genes coding for epigenetic regulators. Nonsynonymous variants represented the majority of mutations found in the total cohort (1130/1405), the most frequent being driver mutation *JAK2*$^{V617F}$. Further confirmations are given by other data sets, with limited number of sequenced genes [94]. Grinfeld et al., having sequenced only 69 myeloid cancer genes, confirmed low TMB in MPNs and shown that mutation in *JAK2, MPL* or *CALR* was the only mutation in 45% of the patients (but short coverage and less than 10% of PMF patients). Another group sequenced 1,1 Mb of coding sequence per sample, which is supposed to confer a more reasonable estimation of TMB based on WES. It compared mutation frequencies from 167 cancer types and found MPNs, *ex aequo* with MDS, with the lowest TMB of 0,8 [95].

Based on their TMB, MPNs like the other myeloid neoplasms seem unlikely to benefit from immunotherapy. Nonetheless, it has been regularly hypothesized that this low number of mutations may be partially compensated by its particular landscape of

mutated driver genes. If immunogenic, driver mutations in *JAK2*, *CALR* and *MPL* would represent valuable neoantigens. They would represent T cell targets directly implicated in clonal autonomous proliferation, homogeneously distributed within diseases and shared between patients. Although converging reports have suggested that driver mutations, especially *CALR* frameshift mutations, are able to produce immunogenic neoantigens, evidence of a spontaneous MPN-specific T cell responses in patients still need to be strengthened. Most relevant discoveries will be reviewed here.

Beginning with predicted epitopes, an Austrian group reported a comprehensive study of the MPN mutational landscape from 113 patients, half of them with PMF, in order to find putative targets for immunotherapy [96]. Compared to previous reports based on exons sequencing, they looked at the transcriptome of MPN granulocytes using RNA sequencing data. This whole transcriptomic approach allowed them to find a number of gene fusions, nonsynonymous single nucleotide variants and insertions or deletions in 106 patients. Moreover, splicing aberrations could also be interrogated with this technique. In *SF3B-1* mutated PMF patients (K700E and K666N/R/T), distinctive 3' splicing patterns could be found and were frequently associated with a protein altering potential. With a virtual peptide library generated from the total RNAseq dataset, they used a predicting algorithm to determine potential neoantigens that could be bound to MHC class I molecules. Having selected the 4 most frequent alleles of HLA-A, HLA-B and HLA-C in their patient population, the *in-silico* method provided 102 peptides that were predicted to bind one of those MHC class I molecules with a high affinity. Quite expectedly, neoantigen candidates were mainly derived from frameshift mutations. The vast majority of those putative neoantigens were derived from *CALR* mutations and co-occurrence of *SF3B-1* mutations seemed to further increase the number of candidates per patient. Noteworthy, *MPL* mutations (W515K/L/A) were able to generate 17 candidates. In the end, we don't really go further from computerized MHC binding predictions. Only a small fraction of those peptides, if any, could be biologically relevant as processing and immunogenicity have not been questioned here. However, this study is still instructive as it strengthens the immunotherapeutic potential of *CALR*, *MPL* and epigenetic regulator mutations.

With a dedicated focus on *CALR* mutations, two research groups reported the immunogenic potential of the altered CALR proteins. While more than 50 types of mutations have been reported in exon 9 of *CALR* gene, all of them create a +1 shift

in the open reading frame. They lead to a new C-terminal portion with a 36 amino-acids sequence conveniently shared by all the mutant CALR proteins. Both works relied on healthy donor PBMC, pulsed with a collection of overlapping peptides spanning the whole consensus C-terminal region of CALR mutants. T cells activation were usually assessed by their cytokine secretion via ELISPOT and intracellular cytokine staining (ICS).

In the publication of Bozkus et al., PBMC have been pulsed and amplified *in vitro* for 9 to 11 days with pools of overlapping 15 amino acids peptides [97]. Among 16 donors, in ELISPOT, half of them were able to secrete IFN$\gamma$ upon restimulation with the pool of CALR mutant peptides. The specificity of T cell activation was established by comparison with an irrelevant stimulation using myelin oligodendrocyte glycoprotein. Those results were confirmed with ICS, indicating that both $CD4^+$ and $CD8^+$ T cells had been activated by fragments of mutant CALR proteins. As suggested by the mandatory preamplification step with mutant CALR derived peptides, we probably assist here at an *in vitro* priming of naïve T cells. To precise the immunogenicity and HLA presentation of the altered protein, MHC typing was retrospectively done on few selected donors and peptide-binding predictions were made. Short peptides with high affinity binding to MHC class I were then designed. One of them has been successfully validated *in vitro*, activating T cells originally generated with the original long mutant peptides. Accordingly, this minimal epitope of the mutated CALR protein induced T-cell response in the two healthy donors sharing the adequate HLA allele. Interestingly, first clue of an *in vivo* processing of this mutant CALR epitope has been found. Indeed, T-cell expanded with the short peptide were activated by autologous APC pulsed with a long mutant CALR peptide. With similar procedures, a second group came to radically different conclusions [98]. Their publication is based on nearly 30 healthy volunteers and PBMC were pulsed with longer overlapping peptides (29 amino acids), also spanning the entire length of the new C terminus portion of mutated CALR protein. Without clear evidence of previous T cell amplification *in vitro*, 75% of the donors responded to the peptides with T cell activation seen in ELISPOT and ICS. Here, control stimulation was made of scrambled CALR sequence. A major divergence with the previous study is the high frequency of responders, which is furthermore described to rely on $CD4^+$ T cells. Very questionably, their following experiments suggested that T cell responses directed against mutant CALR peptides relied on memory T cell compartment, instead of the naïve one. Indeed, IFN$\gamma$ ELISPOT showed no more signs of T cell activation if pulsed PBMC had been depleted from memory T cells (not $CD62L^+$ and

CD45RA$^+$). With such surprising results, they wanted to suggest that a previous immunity against mutant CALR protein is common amongst healthy individuals. This must be tempered by the selection of patients used for the experiments pointing at memory T cells (only two). Besides, the risk that their observations were attributed to cross reactivity with another commensal antigen is high. Indeed, their antigenic stimulation with long peptides only led to CD4$^+$ T cells responses and lacked controls. With the screening of a collection of overlapping nonamer peptides, they better defined the epitope recognized in mutant CLAR and found a hotspot of 11 amino acids. Unsurprisingly, it does not seem to superimpose with the one described by Bozkus et al. Indeed, T cell responses observed here mainly consist of CD4$^+$ T cells, thus assuming a presentation of the antigen by HLA class II molecules instead of HLA class I molecules.

When it comes to non-*CALR* driver mutations, experimental results on the immunogenic potential of those single amino-acid substitutions are rare. Outside of potential neoepitopes predicted *in silico*, we don't have found any relevant study about *MPL* mutations. For *JAK2*$^{V617F}$, another publication from Holmström and his group reported the successful generation of CD8$^+$ T cells, able to lyse *JAK2*$^{V617F}$ cell lines upon recognition of a specific epitope presented in a HLA class I restricted manner [99]. To this aim, they started by identifying one HLA-A2 restricted epitope in the sequence of *JAK2*$^{V617F}$ (online database). Then, they used PBMC collected from a healthy individual, stimulated *in vitro* with autologous dendric cells pulsed with the aforementioned candidate peptide. The amplified CD8$^+$ T cell population was able to release IFN$\gamma$ and TNF-$\alpha$ upon restimulation with the *JAK2*$^{V617F}$ derived peptide. Cytotoxic ability of *JAK2*$^{V617F}$-specific T cells was confirmed in $Cr^{51}$ release assay, as their ability to recognize spontaneously processed mutant JAK2. This was done by stimulating the T cells with human UKE-1 cell lines, expressing both *JAK2*$^{V617F}$ and HLA-A2. Moreover, specificity of the recognition was validated using THP-1 cells, transfected with an irrelevant gene or a mutant JAK2. No lysis nor cytokine release were observed in the absence of *JAK2*$^{V617F}$ transfection. Those results are interesting, suggesting a feasible priming and generation of *JAK2*$^{V617F}$-specific T cells in human but warrant further explorations.

Overall, we can conclude at this point that *in vitro* generation of T cells directed against *CALR* mutations and maybe *JAK2*$^{V617F}$ seems feasible with healthy donor cells. From this theoretical immunogenicity, a lot of work remains to be done to

assess their true specificity and their spontaneous occurrence *in vivo* remains to be confirmed.

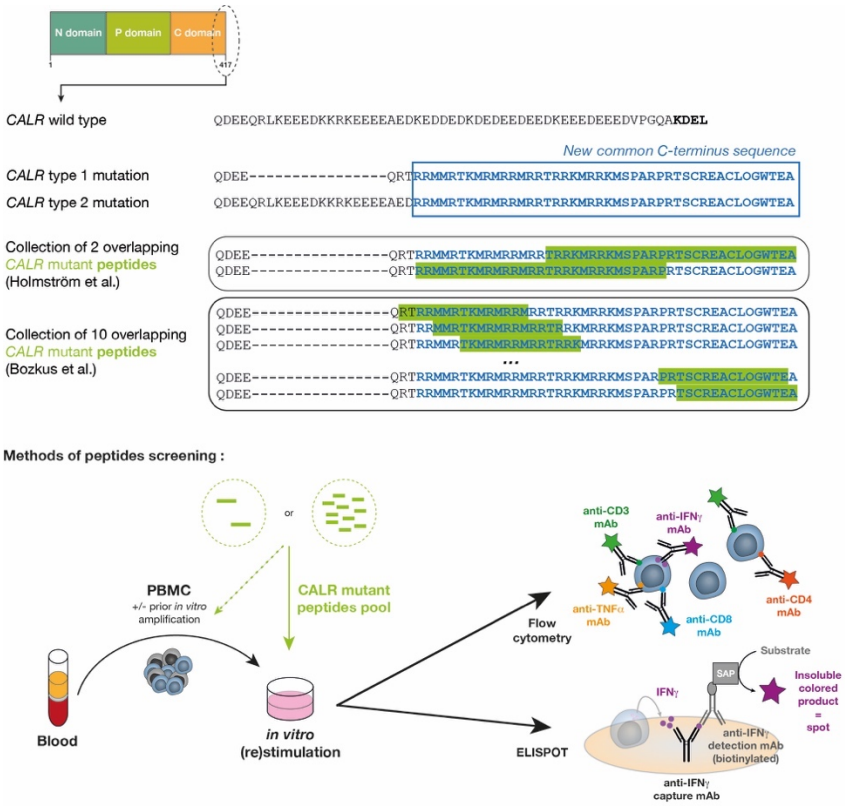


**Figure 10: Isolation of T cells recognizing mutated CALR protein**
All the mutations in exon 9 of *CALR* gene lead to a +1 shift in the open reading frame, which results in the formation of a new C-terminus in CALR protein. All mutant CARL proteins thus share a common 36 amino acids sequence. This is illustrated on the top by the comparison of wild type CARL protein sequence with the ones of type 1 and type 2 CALR mutants (accounting for 85% of total CALR mutations). New common C-terminus is highlighted in blue. Two independent research groups looked for T cell responses directed against CALR mutants in PBMC of healthy donors or MPN patients. Those cells have been pulsed with their respective collection of CALR mutant derived peptides, overlapping the new C-terminus sequence. The sequences of those peptides are reported in green, superimposed on original CALR mutant. For the 10 peptides pool of Bozkus et al., only the first and the last peptides have been illustrated (the others are spanning the remaining mutant sequence, progressively shifting towards C-terminus). For the majority of the experiments, except maybe from the first screenings of Holmström et al., T cells have been first amplified *in vitro*, in culture with CALR mutant derived peptides (expectable *in vitro* priming). Upon stimulation of PBMC with candidate peptides, T cell responses, more specifically IFNγ (+/- TNF-α), is measured by ELISPOT and flow cytometry.

#### 3.2.2.2. Cancer-germline antigens

Other MPN neoantigens have been occasionally evoked amongst cancer-germline genes, without pointing at any convincing target to date. One single study made on PV patients has suggested two candidates: eukaryotic translation initiation factor 2A (eIF-2A) and protamine 2 [100]. They've been designated by serological analysis of expression cDNA libraries (SEREX), which is supposed to detect antigens that elicit high IgG response. The biological relevance of the discovery remains to question, as the (over)expression of those proteins have not been clearly demonstrated in MPNs.

### *3.2.3. Evidence of anti-tumor T-cell responses in MPN patients*

Several publications have reported T cell responses directed against MPN associated antigens in patients. Those responses are not numerous and have only been directed against CALR mutant proteins. The studies have been conducted by the same research groups than the one who characterized mutant CALR-specific T cell responses in healthy donors. As the same techniques were used, similar limitations need to be considered.

The Danish group still used the same 29 amino acid long peptides, covering together the new C terminus portion of the mutant CALR. PBMC from more than 20 MPN patients were assessed directly *ex vivo*. IFN$\gamma$ secretion were detectable by ELISPOT for half of them [101]. Of note, responses in PMF occurred less frequently than in ET patients (36% vs 80% respectively). This frequency is high. Given the responses previously described in healthy volunteers, we would stay careful regarding the specificity the assay. ICS made on the peptide pulsed PBMC allowed them to identify this time $CD4^+$ and $CD8^+$ T cells as being cytokine producing cells. Their next observations were reproduced with T cell enriched culture then with T clone [102]. To that aim, T cells from one patient had been amplified *in vitro* (pulsed with the long CALR mutant peptide against which he originally responded). Again, those selected T cells were $CD4^+$, but suspected to display cytotoxic phenotype ($CD4^+$ CTL). It was suggested by their cytotoxicity in $Cr^{51}$ release assay and surface expression of both CD107 and CRTAM upon restimulation with mutated CALR long peptides. The immunogenic epitope of the mutated CALR protein was also clarified, with the screening of shorter peptides. Finally, they could show that co-culture of autologous

MPN monocytes with their selected T cell clone was also able to activate its IFNγ and TNFα secretion. This is an important observation because it suggests for the first time that the putative anti-tumor T cell clone is able to recognize MPN cells with a spontaneously processed epitope. The experiment was repeated with autologous monocytes transfected with siRNA directed against CALR or with an anti-HLA-DR blocking mAb. Both resulted in antagonizing the activation of the T cell clone, further confirming it recognized a neoepitope from mutant CALR protein that was endogenously processed and presented on MHC class II molecules. We should note that mutant CALR T cell clone has been generated from one patient's PBMC, after prolonged co-culture with autologous DC pulsed with one of the long CALR mutant peptides. The most robust experiments have thus been performed on T cells from one single patient, potentially primed *in vitro*.

The group of Bozkus also described T cell responses with PBMC isolated from MPN patients. Like with healthy volunteers, in order to detect any cytokine secretion in ELISPOT or ICS, the cells had to be expanded *in vitro* with the mutated CALR peptides. It is then clear that responder T cells may have been also primed outside of the patient. In total, 8 of the 18 patients of the cohort had a T cell response after peptide restimulation. Interestingly, immune checkpoints expression was increased on MPN T cells and *in vitro* treatment with anti-PD1 or anti-CTLA-4 mAb moderately increased the proportion of responders. Furthermore, as reported by Holmström, response rate in ELISPOT was lower for PMF patients than for ET patients. Combined together, it has been tempting to suggest that the lack of T cell reactivity observed in patients could be favored by defective immune responses, promoted by immunosuppressive mechanism progressing all along the MPN continuum.

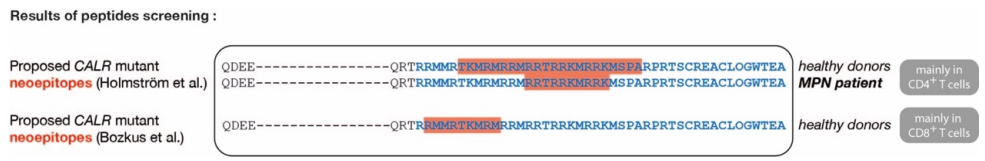


**Figure 11: Candidate neoantigens in mutant *CALR***
Based on similar approaches, two groups have reported T cell responses directed against mutant CALR protein. Having debuted with long peptides, they specified the neoepitopes recognized in their experiments using nonamers peptides derived from mutant sequence. Here, partially overlapping "immunogenic hotspots" of mutant CALR protein according to their work are highlighted in pale red. We should note that Holmström group mainly observed $CD4^+$ T cell responses, whereas Bozkus et al. mainly studied $CD8^+$ T cell responses, limiting direct comparison.

On the other hand, another group tried to identify neoantigens derived from *CALR* exon 9 mutation, while only working with preselected peptides predicted to bind HLA class I [103]. They used MHC class I multimers containing the putative neoantigens in order to sort out MPN-specific CTL among PBMC. This straightforward technique, aiming at directly identify a precise T cell clone *in vivo*, was not used by the previous groups. Unfortunately, the attempt was not successful with MPN patients, but interestingly multimer specific $CD8^+$ T cells could again be isolated from healthy donors. TCR was cloned, but further *in vitro* testing was not conclusive as the subsequent reactivity of the T cell clones against transformed cell lines were low.

Their weaknesses being put aside, some of these results indicate that lymphocytes of a subset of patients with MPN, including PMF, may be able to mount a T cell response against CALR mutant cells (at least after an *in vitro* amplification or priming). As for now, definitive proof of a spontaneous neoantigen presentation and $CD8^+$ T cell response against MPN cells in patients is still lacking. A better comprehension of the altered immune landscape of MPNs may bring some clues, helping to transpose *in vitro* T cells response into clinical practice.

### 3.3. Global immunosuppression in MPN

As we have seen thus far, the MPN clone is responsible of a chronic inflammatory state characterized by a complex interplay of numerous cytokines. Some are produced by the clone itself whereas others are secreted by non-clonal cells from the BM environment. The cytokine crosstalk between those two populations is not fully elucidated but fueled disease evolution and brings some clues for a better treatment of the disease. Although T cell clones directed against MPN neoantigens have been obtained in well-defined *in vitro* protocols, indisputable proof of a spontaneous immune response against MPN cells in patients is still lacking. As described in the “cancer immunity cycle”, mounting a potent anti-tumor CTL response is a complex process with multiple steps and partners. It offers numerous opportunities of dysfunctions that can be mediated by the tumor or its environment, like impaired antigen capture by DC, defective antigen presentation by tumor cells, impaired T cell activation via co-inhibitory receptors or immunosuppressive soluble mediators. Between proinflammatory and immunosuppressive cytokines, various immune cell types display dysfunctional phenotypes in MPNs. To a certain extent, some degree of systemic immunosuppression could be proposed to explain the

apparent inability to generate or maintain an effective anti-tumor immune response in MPNs.

### *3.3.1. Signs of systemic immunosuppression in MPN*

#### 3.3.1.1. Increased susceptibility to infections

A first clue for a significant level of immunosuppression in MPN patients have been suggested by their increased risk of infections. A large study made on Swedish cancer registers showed a hazard ratio (HR) of 2,0 for the occurrence of any infection, reaching even 3,7 for patients with PMF [104]. Bacterial and viral infections were similarly implicated, but one of the highest increased risks was for sepsis (HR of 2,6). Interestingly, the likelihood of infectious complications was independent of the treatment status. Moreover, as the period of data collection ended in 2013, most recent immunosuppressive drugs such as JAK inhibitors only had minor influence in the cohort.

This vulnerability was also noticeable over the course of the recent pandemic. Several retrospective studies have reported a higher risk of COVID-19-related death for MPN patients [105, 106]. Beyond older age, always predictive of an adverse outcome in multivariate analysis, one large European cohort also suggested an even worse prognosis associated to the diagnosis of PMF. Indeed, whereas the percentage of death for PV, ET and pre-PMF patients was 28%, it reached 48% in PMF patients ($p$=0,016). Another particular element from that study regarded the prognosis implications of ruxolitinib treatment. While it could be expected to be deleterious because of its immunosuppressive properties, no significant consequence on survival resisted to multivariate analysis. Interestingly, it was rather ruxolitinib discontinuation that conferred the highest risk of COVID-19-related death. This could reflect a potential benefit from the anti-inflammatory effects of the drug (or highlight the danger of ruxolitinib withdrawal syndrome).

#### 3.3.1.2. Decreased responses to vaccines

Another insight into the immunosuppression of MPN patients can be obtained by looking at their altered response to vaccines. In a short report, an English group

examined the immunization following seasonal influenza A vaccination of 19 patients with ET, PV or PMF [107]. They performed an immunophenotyping of PBMC by single cell analysis with mass spectrometry. As previously described, $CD4^+$ T cells were decreased compared to healthy donors. 3-weeks and 3-months after vaccination, MPN patients were characterized by a reduction in the memory cell clusters, affecting both T and B cells populations. No complementary serological or functional testing has been done. Whereas no clear information is available about an inferior clinical response to influenza vaccine in MPN patients, a delayed response could be at least proposed on this basis.

While MPN patients seem to suffer of higher mortality from COVID-19, their response to vaccination has been reported with contradictory results. Some cohorts of MPN patients, including PMF patients, have shown preserved T cells and B cells responses to a single dose of BNT162b2 mRNA vaccine [108]. At the opposite, other groups have shown lower rates of anti-SARS-CoV-2 IgG response after two doses of Pfizer-BioNTech or Moderna vaccine [109, 110]. Interestingly, seroconversion was less frequent in MPNs compared to control (57,7% vs 97,7%) but especially in PMF compared to ET (60% vs 93,8%). Moreover, poor serological response was not statistically associated to JAK inhibitor treatment (but maybe helped by a lack of statistical power). These are the results of small cohorts, without corresponding clinical data. Nonetheless, they still suggest some degree of immune dysfunction, especially in PMF patients.

#### 3.3.1.3. Increased incidence of solid tumor

The Swedish Cancer Registry has been used to establish an increased risk of secondary malignancies for MPN patients [111]. Whereas evolution to AML is part of the natural history of the disease, non-hematological cancers are supposedly not. Increased risk of solid tumors in MPN patients was seen with a statistically significative hazard ratio (HR) of 1,6. Non-melanoma skin cancers, kidney cancers and brain cancers were the most strongly associated to MPN history. Whether it is favored by the immunosuppressive/cytotoxic treatment previously administered for MPN or whether those diseases shared a common predisposing factor mediated by genetic/environment factors cannot be addressed here. Nonetheless, albeit not bringing solid proof, an immunosuppressive environment favoring tumor development through immune escape can be one of the plausible hypotheses, along

with cytoreductive treatments (hydroxyurea and radioactive phosphorus) or germline predisposition.

### 3.3.2. Altered number or function of innate immune cells

Innate immune cells encompass granulocytes, mast cells, natural killer (NK) cells, macrophages, DC and monocytes. These cells can be implicated in tumor suppression, either by direct killing of tumor cells and/or by recruiting an adaptive immune response.

#### 3.3.2.1. Monocytes and phagocytes

Monocytes are myeloid cells playing major role in tissue homeostasis and immunity. Besides other inflammatory functions, monocytes have the ability to leave the blood stream for the tissues, where they can differentiate into macrophages and DC. Nonetheless, a vast portion of macrophages and DC are issued from dedicated precursors [112]. While they share these abilities, macrophages are the dominant phagocytic population in tumors, whereas DC represent the quintessential antigen presenting cells.

Several studies designated monocytosis, corresponding to monocytes blood count above $10^9$/L, as indicative of adverse prognosis in PV and PMF [113, 114]. It's associated to older age, increased leukocytosis, frequent HRM (*SRSF2* in PV or *ASLX1* in PMF) and increased leukemic transformation. Accordingly, a growing body of evidence incriminated monocytes into various pathological processes of MPNs. As previously mentioned, they have been proposed as major inflammatory cytokine producers and could bear a huge responsibility in the constitutive activation of NF-κB which favors PMF progression. Here we would like to specifically address their impaired phagocytic and T cell priming function but also their implication in BM niche remodeling through differentiation into fibrosis-producing fibrocytes.

##### *3.3.2.1.1. Impaired phagocytosis and priming*

Functional testing has suggested altered monocytes functions in PMBC from PMF patients, as reported in a cohort of 38 untreated individuals comprising a similar

fraction of *JAK2*$^{V617F}$ and *CALR* mutated diseases [115]. *In vitro* differentiation assay revealed impaired monocyte's capacity to differentiate into DC, especially in *CALR* mutated patients. Moreover, across all groups, CD1a and CD80 expression at the surface of DC failed to be upregulated, with further *in vitro* testing confirming an altered function as they failed to stimulate allogenic T cells proliferation and CD25 expression. This observation suggests a potential reduced capacity of T cells priming, but other observations are needed as monocytes-derived DC may not reflect the other DC types (especially regarding T cell activation capability).

Macrophages and DC are professional phagocytes and antigen presenting cells. Tumoricidal role of macrophages is well described in oncology, especially with antibody-dependent cellular phagocytosis (ADCP) following various mAb based treatments [116]. Apart from a direct killing activity, phagocytosis of apoptotic or tumoral cells by macrophages and DC is also required for their antigen presentation capability. This process is mandatory to prime and activate T cells, crosslinking innate and adaptive immunity. To this aim, phagocytes are able to recognize target cells through various surface elements, such as exposed phosphatidylserine on dying cells, but it is further facilitated by the exposition of proteins such CALR [117]. Surface expression of CALR is indeed increased in cells undergoing stress response, which also include cancer cells exposure to numerous chemotherapeutic agents, and acts as a potent pro-phagocytic signal. Anti-phagocytic signals or "phagocytosis checkpoints" balance this recognition pathway in healthy cells. Among them, CD47, a widely expressed transmembrane protein, is able to engage signal regulatory protein alpha (SIRP$\alpha$) on phagocytes and counters pro-phagocytic signals [118]. Upon ligand binding, phosphorylated SIRP$\alpha$ will recruit tyrosine phosphatases that will orchestrate cytoskeleton rearrangement inhibition and prevent phagocytosis. CALR exposition is then balanced in healthy cells by concomitant CD47 expression, whereas in stressed cells, such as apoptotic cells, CD47 is lost or segregated away from CALR.

CD47 acting as a "don't eat me signal" has been shown to be overridden by several tumors. Expression of both CALR and CD47 is associated to worse prognosis in various cancers, including AML [119]. *In vitro* experiments on primary human cancer cells, such as myeloid malignancies, have shown that even if CALR were increased compared to normal tissues, phagocytosis by macrophages was blocked by a parallel increased expression of CD47, maintaining the equilibrium between pro- and anti-phagocytic signals [120]. Anti-phagocytic signal from CD47 could be prevented

by blocking anti-CD47 mAb *in vitro* and in mouse models. Such treatment affected preferentially tumor cells as healthy cells does not expressed higher level of dominant pro-phagocytic CALR. In addition, preclinical work with several transplantable tumor mouse models have shown that the efficacy of anti-CD47 blockade was mediated trough the induction of an anti-tumor T cell response [121, 122]. As a dynamic process, efficacy of CD47-SIRP$\alpha$ disruption can be improved by increasing pro-phagocytic signals on target cells, with cytotoxic agents or by combination with another mAb leading to apoptosis or ADCP.

CD47-SIRP$\alpha$ axis has been widely studied in myeloid malignancies such as AML, with encouraging results of combination therapies in phase 1 clinical trials. Up to now, few interests has been given to MPNs, but some preclinical rational may suggest its relevance and warrant further investigations. On PBMC, similar levels of CALR and CD47 expression have been reported between a group of ET patients and healthy donors [123]. As this expression levels may not seem promising for a therapy against CD47, it doesn't take into account the other types of MPNs, BM progenitors or altered CALR/CD47 ratio due to combined treatment. A short report, relying on a diverse cohort of 30 MPN patients (PV, ET and PMF) drawn somewhat different conclusions, with increased CD47 compared to CALR especially in PV or PMF treated with prior cytoreductive therapies [124]. Unfortunately, those results were obtained by western blotting, done on total or membrane cellular fractions, limiting precise quantitative interpretation. Further studies, using flow cytometry for instance, are required to confirm those observations.

Another way phagocytosis and subsequent T cell priming could be impaired in MPN cells has been specifically proposed for *CALR* mutants. Multiple pathological consequences of the altered CALR protein comes from the loss of its ER retention signal, allowing its escape from this cellular compartment. Secretion of mutated CALR protein is even found in the plasma from murine models and patients. Using type 1 and type 2 *CALR* mutants found in MPNs, a research group has recently suggested that paracrine secretion of those proteins could block the phagocytosis of tumor cell lines by DC, *in vitro* and in tumor mouse models [122]. This effect was particularly marked with tumor cell lines previously primed with cytostatic agents, supposed to induce "immunogenic cell death" by increasing presentation of pro-phagocytic signals. Recombinant protein injection, as well as its endogenous production by transduced BM cells or KI tumor cells, led to similar effects. Interestingly, the altered CALR proteins were able to preclude the protection

normally conferred by tumor vaccination in MCA-205 tumor model and to impede anti-tumor effect of anti-PD1 treatment in two mouse transplantable models (MAC and TC1). However, the effect on anti-tumor T cell response has not been precisely addressed, which is a limitation. How the secreted CALR mutant acts on DC is not known, saturation of CALR binding sites on DC has been proposed. Whether CD-47 is able to reverse this putative altered CALR function is not known either. Even if not directly addressing the question in MPN model, this work is nonetheless still interesting for our field.

#### *3.3.2.1.2. Fibrocytes*

In a different perspective, monocytes have also been directly associated to the fibrotic process. Indeed, a subpopulation of CD14+ monocytes can differentiate into fibrocytes, spindle-shaped fibroblast-like cells, implicated in the fibrosis of various diseases such as pulmonary fibrosis, end-stage liver or kidney diseases and auto-immune disorders [125]. In PMF, two research groups have identified these fibrocytes as yet another fibrosis-driving cells, complementing the myofibroblasts that derive from MSC.

In a first publication, Verstovsek et al. were able to detect high frequencies of clonal collagen-producing fibrocytes in the BM from animal model and patients with PMF [126]. In previous research works, several groups had already reported the ability of serum glycoprotein pentraxine-2 to inhibit differentiation of monocyte into fibrocytes. Moreover, like in other fibrotic diseases, level of pentraxine-2 was lower in plasma samples from PMF compared to healthy individuals. Recombinant pentraxin-2 (PRM-151) has thus been tested *in vitro* on monocytes from PMF patients and successfully blocked their differentiation into fibrocytes. Those results motivated the evaluation of PRM-151 administration in xenograft mouse model, which resulted in increased survival, reduced fibrocyte count and improved BM fibrosis.
In a later publication, a second group supported these observations in another transplantation mouse model [127]. While recipients were WT mice, BM donors were transgenic mice with a double *Jak2*$^{V617F}$ and CD11b-DTR KI. This strategy allowed a conditional DTR-mediated ablation of *Jak2*$^{V617F}$ monocytes and fibrocytes after transplantation. CD11b-replete *Jak2*$^{V617F}$ mice developed a PMF phenotype, with increased BM infiltration by *Jak2*$^{V617F}$ fibrocytes and WT myofibroblasts. By comparison, combined depletion of monocytes and fibrocytes resulted in attenuated

phenotype with reduced number of collagen-producing fibrocytes and decreased fibrosis. Noteworthy, anemia and splenomegaly were also improved, which could be explained by a global reduction of proinflammatory cytokines. This was not previously associated to PRM-151 treatment and could be favored by the low specificity of their fibrocyte depletion methodology which also target cytokine-producing monocytes and granulocytes. However, increased production of total TGF-β1 from *Jak2*$^{V617F}$ fibrocytes was demonstrated *in vitro* and *in vivo*. Unfortunately, more precisions regarding actual TGF-β1 activation were not investigated in their protocol. Overall, fibrocytes would represent a second type of fibrosis-driving cells, deriving from the mutated HSC, by opposition to the myofibroblasts which come from the non-mutated cell population (differentiated from Gli1+ MSC). The question whether fibrocytes only contribute to collagen deposition or significantly participate to PMF inflammatory milieu is unanswered. The question deserves to be addressed, because, even as latent TGF-β1 reservoir, they could be implicated in the activation of a cytokine affecting numerous PMF pathways, including stimulation of two fibrosis-driving cell populations.

Following this work, targeted therapies against fibrocytes have been proposed. On one hand, PRM-151 itself is evaluated in PMF patients, with an ongoing phase 2 trial (NCT01981850), recruiting intermediate to high-risk patients being not eligible for ruxolitinib. Interim results from the first stage seemed to confirm a reduction of fibrosis but more results are needed [128]. One the other hand, a drug-targetable marker has been proposed for fibrocytes. Signaling lymphocytic activation molecule-F7 (SLAMF7) is highly expressed on fibrocytes but also on a specific subpopulation of monocytes which are their precursors. Increased proportion of those SLAMF7$^{high}$ monocytes have been confirmed in PMF patients, in correlation with their respective *JAK2*$^{V617F}$ allele burden [129]. Elotuzumab, an anti-SLAMF7 mAb has thus been tested *in vitro* and in a murine xenograft model of PMF. There, it suppressed fibrocyte differentiation and decreased fibrosis, along with splenomegaly. Elotuzumab, which have shown activity and good tolerability in multiple myeloma, could potentially being repurposed for PMF [130]. Accordingly, a phase 2 clinical trial has been started in 2020 (NCT04517851).

#### 3.3.2.2. NK cells

NK cells form a group of innate immunes cells expressing spontaneous lytic activity. Target cells are recognized in an MHC-independent manner, upon antibody-mediated binding (antibody-dependent cell cytotoxicity or ADCC) or upon an imbalance between activator and inhibitor receptors stimulation. Activated NK cells kill target cells directly by secreting cytotoxic molecules (perforin, granzyme) but also indirectly by secreting cytokines, such as IFN$\gamma$ which favor Th1 adaptive immune response. NK cells play a role in defense against infected cell but also have an increasingly acknowledged function against tumor cells [131]. In CML for instance, another MPN, high proportion of mature NK cells has been repeatedly associated with successful treatment discontinuation [132]. Frequent MHC class I downregulation of tumor cells, allowing their escape from CTL, sensitize them to a NK cells-mediated cytotoxicity. This is explained by the fact that MCH class I is normally binding inhibitory receptor on NK, dampening their activation. MHC class I decrease is then supposed to activate lytic activity of NK cells against tumor cells. Unfortunately, tumor escape mechanisms have been described such as secretion of prostaglandin E2, TGF-$\beta$1 and IL-10 by tumor environment cells like Tregs, MDSC or fibroblasts. Little attention has been dedicated to NK cells in MPNs. While a recent study reported a decreased percentage of those cells in *JAK2*$^{V617F}$ MPN patients, another one found an impaired cytotoxicity on the basis of *in vitro* experiments [133, 134]. Notably, they could be involved in the immunomodulatory mechanisms triggered by IFN$\alpha$ therapy, of whom we will discuss later.

#### 3.3.2.3. MDSC

Myeloid-derived suppressive cells (MDSC) are a group of pathologically activated neutrophils and monocytes characterized by potent immunosuppressive properties. Their ability to inhibit T-cell, B-cell and NK cell-mediated immune response is associated with adverse outcome in cancer patients [135]. In PMF, there is only indirect proofs of their implication. Wang et al. studied PBMC from 55 MPN patients (half with PMF) by flow cytometry. They identified a significantly increased number of MDSC in those patients [136]. MDSC in the study were defined as CD11b+CD14-CD33+, which fulfil some criteria of the actual definition of granulocytic MDSC. It should be noted that precise discrimination of MDSC from their classical counterpart has always been a challenging task, requiring continuous standardization efforts.

Besides their increased number, MPN MDSC also exhibited a superior T-cell antiproliferative capacity compared to those from healthy donors. Looking for an explanation, they noted an increased mRNA expression of arginase-1 in PBMC from MPN patients. They associated this finding to MDSC, as this arginine-depleting enzyme has been one of the first immunosuppressive mechanisms identified in MDSC. In line with those observations, the publication of Bozkus et al. also mentioned the increase of a similar WBC population, but no further testing has been done [97]. Clinical relevance of all those findings requires more data.

### *3.3.3. Altered T cell responses*

#### 3.3.3.1. Reduced T cell numbers in MPNs

$CD8^+$ T cell, as CTL, are probably the most important immune effectors implicated in tumor cells lysis. $CD4^+$ T cells largely remained in the background but have revealed a dual role, as Th1 helper cells supporting anti-tumor specific CTL response or as Treg suppressive CTL by their production of IL-10 and TGF-β1. Several authors have reported reduced $CD3^+$ blood cell counts in PMF patients, generally reported as a percentage of WBC which limits interpretation [97, 137, 138]. The decrease of T cells was generally well balanced between $CD4^+$ and $CD8^+$. Whereas some have described a reduction of Th1 helper cells amongst $CD4^+$ T cells, other have reported a decrease of naïve populations which could be coherent with the chronic inflammatory state.

#### 3.3.3.2. Reduced MHC class I expression in MPNs

Antigen recognition by cytotoxic T cell requires prior peptide binding to MHC class I molecule and presentation of the complex at the cell surface. Intracellular proteins are processed into smaller peptides by the proteasome, then imported to the ER by a transporter called TAP [139]. In ER, they will join the MHC molecule, a heterodimer composed of HLA heavy chain and β2-microglobulin, on which they will bound with the help of the peptide loading complex. Loaded MHC class I complex will finally be exported to the plasma membrane in order to be presented to $CD8^+$ T cells.

Among the mechanisms used by tumor cells to evade immune surveillance, downregulation of MHC class I pathway is common in many tumor types and have been related to shorter survival [140]. Indeed, without antigen presentation, tumor specific CTL are unable to recognize and lyse the tumor cells. Accordingly, this mechanism has also been implicated in resistance to immunotherapy treatments such as check point inhibitors [141]. Suspicions that such mechanism could be implicated in MPN progression has been suggested by two studies looking at the transcriptomic profile of PBMC from MPN patients [142, 143]. Analysis of the microarray data sets showed a significant decrease of the expression of many MHC class I and class II genes but also of genes implicated in peptide processing and loading. However, there is no corresponding data regarding protein expression, antigen presentation capability or clinical outcome. Another limitation is the lack of precisions regarding the MPN driver mutations in their cohort. If MHC class I decrease was confirmed, underlying mechanism remains elusive.

It has to be considered that peptide-empty MHC class I molecules are unstable and need to be associated to chaperone proteins until the complex is finalized. CALR is one of those chaperon proteins. As they must be properly loaded to gain stability and reach cell surface, any defect in the whole pathway (MHC heavy chain, β2-microglobulin, proteasome, chaperons, transporter, peptide editor) may lead to decreased MHC class I expression on cell surface. Outside PMF, it has been shown that such defects can be due to irreversible events, such as inactivating gene mutations, or to potentially reversible events, such as decreased transcriptional factors, epigenetic silencing, histone modifications, suppressive signaling pathways [144, 145].

Regarding the contribution of mutated genes, *CALR* exon 9 mutations could represent a good candidate [146]. *E*xon 9 mutations translate into the loss of CALR ER retention motive (KDEL) and lead to its secretion. One could think that loss of function of this chaperone protein could impaired peptide loading complex and preclude MHC class I surface expression. To explore the hypothesis, Arshad and al. compared MHC class I loading and surface expression between *CALR* KO 293T cells transfected with *CALR* WT or exon 9 mutants. In the mutant 293T, they confirmed impaired incorporation of *CALR* into the peptide loading complex, decreased MHC class I expression at cell surface and suboptimal loading with lower affinity peptides. Those observations are to temper as they have been obtained on cell lines lacking any functional *CALR* gene copy. Indeed, homozygous *CALR*

mutated cells will not reflect the majority of the patients and when *CALR* mutant was co-expressed with WT *CALR*, those abnormalities were less sticking.

Regarding transcriptional regulation, several hypotheses inspired by other neoplasm can be made.
Firstly, IFN signaling, especially type 2 IFN (or IFNγ), is a potent transcriptional activator of the MHC class I antigen processing machinery [147]. IFNγ is mainly produced by activated T cells but also by ILC1 and NK cells. Intracellular signaling of IFNγ is mediated by tyrosine kinases JAK1 and JAK2, leading to the formation of phosphorylated STAT1 homodimers and downstream transcriptional response. Here, transcriptional modifications are mediated by upregulation of IRF1 which later binds MHC class I promoter. Whether IFNγ signaling is downregulated in PMF is not absolutely clear. Plasma concentrations of IFNγ have been reported to be decreased in PMF patients by two different groups [37, 38]. Moreover, other researchers have noted some intriguing transcriptomic alterations regarding this question. By comparing microarray data from CD34+ cells isolated from *JAK2*$^{V617F}$ negative PMF patients or healthy controls, they found a downregulated expression of HLA-A but also of genes implicated in IFNγ signaling pathway (like IRF1 and IFNγ receptor) in PMF cells [143]. Interestingly, they made opposite observations with *JAK2*$^{V617F}$ PMF patients, with increased expression of those genes. This contradiction may be explained by the implication of JAK2 in IFNγ signaling pathway. Thus, if MHC class I and IFNγ downregulation are implicated in immune escape of MPNs, the phenomenon could affect diseases differently depending on their driver-mutations. Whereas IFNγ treatment is poorly tolerated, type 1 IFN have been successfully used in MPNs. We will come back later to this precise topic, but MHC class I upregulation may be part of IFNα immunomodulatory effect. Secondly, overexpressed cytokines like TGF-β1 have been shown to decrease the expression of various genes implicated in the antigen-presentation machinery. Such observations have been made outside of MPNs, in mouse tumor models, and were reversed by anti- TGF-β1 treatment [148]. Thirdly, several altered signaling pathways in tumors have been seen to downregulate MHC class I expression. As such, MAPK pathway has been associated to MHC class I downregulation trough interference with IFN signaling and IRF1 decrease [149]. As being one of the downstream effectors of the constitutive JAK2 activation of MPNs, MAP kinases could maybe play a similar role here. Lastly, epigenetic silencing could be implicated, especially in PMF. Mechanism such as

DNA methylation or histone modifications can be suspected, especially given the frequency of known mutations affecting those processes in the disease.

As we have seen, MHC class I expression is suspected to be decreased in some MPN groups and multiple explanations could be proposed. One of the reasons for this inconsistency could the multiplicity of mechanisms, as only certain genetic alterations or certain combinations of them could lead to impaired MHC expression.

#### 3.3.3.3. PD1/PD-L1 axis in MPNs

T cell activation upon MHC-restricted TCR engagement is controlled by a series of stimulatory and inhibitory receptors-ligands interactions. Nearly 30 years ago, discovery of “immune checkpoints” which refer to T cells co-inhibitory receptors paved the way to some impressive successes in cancer therapy [150]. While various members of this family of immunoreceptors have been described in the last decades, the most clinically successful so far have been cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death- 1 (PD-1). Only the later have been studied in MPNs.

PD-1 (or CD279) expression is induced on activated T cells, following TCR engagement, and interacts with 2 ligands known as PD-L1 and PD-L2. As the other immune checkpoints, PD-1 plays an important role in limiting inflammatory tissue damages and promoting self-tolerance. This has been firstly demonstrated in mouse KO models which developed auto-immune disorders such as lupus-like diseases or auto-immune cardiomyopathies [151]. PD-1 is a transmembrane protein belonging to the immunoglobulin superfamily [152]. Its intracellular portion contains immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) which will be phosphorylated upon ligand binding in order to recruit SHP-2 and SHP-1 tyrosine phosphatases. These enzymes will then attenuate T cell activation by dephosphorylating key signaling molecules from the TCR complex but also from CD28 co-stimulatory receptor [153]. Consequently, PD-1 engagement is able to inhibit T cell proliferation, survival and cytokine release amongst other effector functions. Following persistent antigen stimulation, such as in the well-studied mouse model of chronic LCMV infection, T-cells may harbor a dysfunctional phenotype characterized by the loss of effector functions. So called “exhausted” T cell populations have also been described in cancer and high PD-1

expression is part of their defining characteristics. Cytokines such as IL-10 and TGF-β1 have also been associated to this phenomenon. In mouse tumor models and in cancer patients, tumor reductions have been obtained with mAb disrupting the interaction between PD-1 and its ligand, in parallel of increased effector functions of tumor infiltrating lymphocytes (TIL) [154]. Whether "exhausted" TIL recover their effector functions or whether this relies on newly primed TIL is still a matter of debate.

In MPNs, PD-1 expression on T cells have been established with flow cytometry in several publications. PD-1 expression is globally increased compared to healthy donors but as disease subtypes and driver mutations are not always precise in each study, a detailed interpretation is limited. Only one of them analyzed PV, ET and PMF separately, confirming increased PD-1 expression in $CD8^+$ T cells across the three groups [155]. Those results were confirmed in a second independent report focusing solely on PMF patients [137]. Interestingly, they were also able to associate PD-1 expression with clinical parameters, finding positive correlations with leukocytosis and spleen size but also negative correlation with splenomegaly resolution following treatment with JAK inhibitor ruxolitinib. In terms of prognosis, higher proportion of $PD\text{-}1^+$ cells amongst $CD8^+$ T cells (above 6,12%) was predictive of inferior OS (HR= 2,48). Here again, PD-1 probably reflects disease progression and inflammatory environment. Further validation was provided by a last study which examined PBMC from *CALR* mutated MPNs, adding the notion that other inhibitory receptors such as CTLA-4, TIM3 or LAG3 were concomitantly expressed on $CD3^+$ T cells [97]. In summary, PD-1 is overexpressed in MPNs, especially in PMF in which it correlates with advanced disease, decreased life expectancy and maybe T cell dysfunction.

Amongst PD-1 ligands, PD-L1 has been the most studied. PD-L1 is expressed by various hematopoietic cell types, including macrophages and DC, but is also frequently overexpressed in various tumors. Underlying mechanisms of this increased expression is divided in two categories, representing respectively intrinsic and acquired immune resistance phenomenon. On one hand, it can be overexpressed following the constitutive activation of specific signaling pathways in the tumor, such as STAT3 or AKT. On the other hand, PD-L1 expression could be acquired under the influence of cytokines produced by anti-tumor immune cells, such as IFNγ by activated T cells and NK cells. This dynamic PD-L1 expression may thus partially explain some discrepancies regarding predictive value of PD-L1 expression on anti-PD-1 treatment outcome. Indeed, PD-L1 overexpression on tumor cells has

been shown to reliably predict anti-PD1 treatment response in several studies, but some “PD-L1 low” cancer patients can also benefit from anti-PD1 mAb. This could be linked to the blockade of a *de novo* PD-L1 expression, acquired secondarily to the cytokines produced by newly activated anti-tumor CTL. Another explanation could be that PD-L1 expression on APC or other immune cells from tumor environment is also an important target.

An increased PD-L1 expression has been repeatedly reported in MPNs. A publication by Prestipino et al. provided some clarifications regarding the underlying mechanism, pointing at a direct contribution of *JAK2*$^{V617F}$ mutation [156]. Using murine and human cell lines, they could reproduce PD-L1 overexpression after *JAK2*$^{V617F}$ transfection. They showed that PD-L1 expression was directly dependent of the constitutive JAK-STAT activation as it was abrogated by JAK inhibitors. Moreover, while both STAT3 and STAT5 were implicated in *JAK2*$^{V617F}$ downstream signaling, using gain-of-function or loss-of-function STATs mutants, they were able to show a predominant contribution of JAK2-STAT3 pathway over JAK2-STAT5 pathway. Finally, PD-L1 overexpression was confirmed in *JAK2*$^{V617F}$ KI mouse model and in MPN samples. Here, compared to lymphocytes, PD-L1 expression was the highest in monocytes and MKs, reflecting higher allele burden of *JAK2*$^{V617F}$ in those populations. PD-L1 expression is then proposed to be at least intrinsically increased in *JAK2*$^{V617F}$ mutant cells. Accordingly, another group have reported increased PD-L1 expression on blood myeloid cells from MPN patients, with good correlation with *JAK2*$^{V617F}$ allele burden. Where it comes more contradictory, is whether this expression profile can be generalized to other JAK-STAT pathway driving mutations. In PBMC from mutated *CALR* MPNs, Prestipino found normal expression of PD-L1 while other reported increased expression whatever *JAK2* mutational status [157]. BM samples are maybe more relevant, as they may more faithfully reflect disease stem cells, but they led to similar discordances [158]. Noteworthy, technical concerns, such as sensitivity and standardization issues, may account for such discrepancies and we would give more credit to flow cytometry than to immunohistochemistry staining. Accordingly, CD34+CD45$^{dim}$CD38$^{-}$ HSCs from MPN patients have revealed increased PD-L1 expression in both *JAK2* and *CALR* mutated patients [157]. Without dedicated study, final conclusion is delicate. We could agree that subtle modulation in JAK-STAT pathway activation, following different driver mutations, may account for a differential expression of PD-L1 across MPN patients. Nevertheless, as previously stated, absence of PD-L1 overexpression in itself is not a reliable predictor factor of checkpoint inhibitor primary resistance.

Preclinical rational for therapeutic anti-PD1 blockade in MPNs is limited. A xenotransplantation model has been depicted by Prestipino, in which *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice were injected with PBMC from *JAK2*$^{V617F}$ patients and developed a lethal phenotype with *JAK2*$^{V617F}$ mediated thrombocytosis [156]. Anti-PD-1 or anti-PD-L1 mAb treatment on day 8 was responsible of increased survival, reduced thrombocytosis and profound decrease of BM infiltration by human CD45$^{+}$ cells. Treatment efficacy was seemingly mediated by T cells, as it was abrogated if PBMC were depleted from CD3$^{+}$ cells prior to transplantation. Unfortunately, the lack of a proper control group transplanted with healthy donor cells and the absence of further precision about T cell response require careful interpretation.

Overall, increased PD-1 and PD-L1 are seen in MPNs, especially in *JAK2*$^{V617F}$ and in PMF patients. Constitutive activation of JAK-STAT pathway is directly implicated, as it drives inflammatory cytokine secretion, their signaling pathway and PD-L1 expression. Whether checkpoint inhibitors are responsible of impaired T cell response against MPN cells is not proven.

#### 3.3.3.4. Tregs in MPNs

Among T cells, regulatory T cells (Tregs) are an immunosuppressive subset of CD4$^{+}$ T lymphocytes specialized in the maintenance of immune tolerance in the periphery. They are characterized by their stable expression of transcription factor FOXP3, mandatory for their development. Here, TGF-β1 plays a crucial role in the maintenance of the Tregs but also as one of their key effector cytokines, enable them to suppress other immune cells. Our lab has demonstrated that human Tregs stimulated trough their TCR activated latent-TGF-β1, with a mechanism requiring its presentation by transmembrane protein GARP and integrin αVβ8.

The immunological repercussions of Tregs and TGF-β1 had not been previously studied in MPNs. In a PMF mouse model, our group recently described the role of TGF-β1 activated from GARP-expressing Tregs (manuscript in preparation). We modeled the disease in murine recipients transplanted with retrovirally transduced MPL$^{W508A}$ BM cells. MPL$^{W508A}$ transplanted mice developed an aggressive PMF phenotype with elevated WBC and PLT, mostly derived from MPL$^{W508A}$ HSC. Interestingly, the expression of GARP:TGF-β1 complexes in hematopoiesis sites was increased but still largely restricted to Tregs and MKs. When GARP-dependent

TGF-β1 activation was blocked by an anti-GARP:TGF-β1 mAb treatment, we observed a significant decrease of WBC and PLT. Remarkably, this reduction of disease burden only affected myeloid cells derived from $MPL^{W508A}$ HSC, without impairing wild type cell populations. The selective reduction of disease burden observed in $MPL^{W508A}$ treated mice required blocking TGF-β1 activation from the GARP-expressing Tregs, as mice with conditional GARP KO on Tregs (*Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$) recapitulated the therapeutic effect of the mAb. Comforting Tregs as an essential source of GARP-dependent active TGF-β1, no improvement was seen in mice with a MK-specific GARP KO (*Pf4*$^{cre}$ x *Garp*$^{fl/fl}$). Moreover, therapeutic activity was associated to an increased IFNγ signaling and required $CD8^+$ T cells integrity. Altogether, our results suggest that selective blockade of TGF-β1 activation by GARP-expressing Tregs with anti-GARP:TGF-β1 mAb exerts immune-mediated therapeutic activity in mouse PMF.

Apart from those encouraging preclinical observations, Tregs have drawn little attention in PMF. With inconsistent results, decreased Tregs counts in the blood of PMF patients have been reported, while their function remains poorly evaluated [159-161]. Overall, blood Treg proportion is conserved or maybe decreased in PMF, but not increased compared to healthy donors. To note, in solid malignancies, an adverse prognosis has rather been associated to an increased number of Tregs [162]. A publication mentioned increased soluble IL2 receptor (sIL2R) levels, observed *in vitro* on stimulated PBMC from MPN patients [163]. This observation is of limited relevance, but we know that plasma sIL2R is globally elevated in PMF, where it correlates with anemia, leukocytosis and prognosis. sIL2R receptor comes from the proteolytic cleavage of the ectodomain of the IL-2 receptor alpha chain and results from T cell activation. Conflicting results exist regarding the biological activity of sIL2R itself, but the majority agrees to see it as a marker of ongoing inflammatory process. No new element can thus be found here. Further exploration would be needed, especially on BM sample from PMF patients to quantify Treg infiltration and activity.

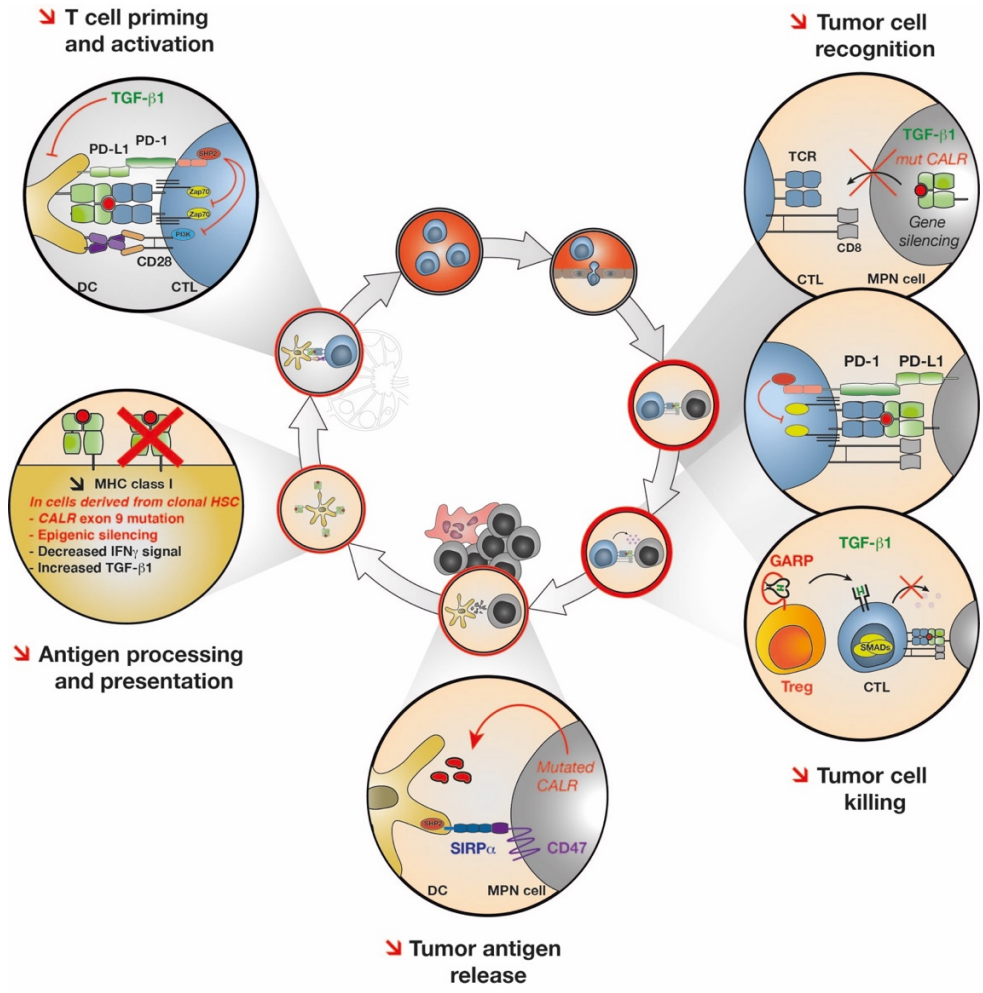


**Figure 12: Altered innate and adaptive immune functions that could impair anti-MPN immune response**

As review with cancer-immunity cycle, development of an effective immune response against MPN cells is a complex stepwise process. Several immune dysfunctions of MPN may allow its immune escape and may deserve specific attention in the perspective of immunotherapy. Antigen capture by APC could be decreased, phagocytosis of MPN cells being prevented by CD47-SIRPα axis or mutant CALR protein secretion. Antigen presentation on MHC class I complexes could be decreased, especially in DC derived from MPN clone, due to CALR mutation, IFNγ downregulation, TGF-β1 or epigenetic silencing. T cell priming and activation could even be further decreased by TGF-β1 or in a PD-L1 dependent manner. Later, MPN cell recognition by CTL and subsequent killing activity could again be countered by decreased MHC class I and/or increased PD-L1 expression by MPN cells. Interestingly, CTL activation and function could also be dampened by TGF-β1 activate by Tregs in a GARP-dependent manner.

# 4. Current and future therapies in development that impact the immune landscape in PMF

As we have seen, some antigens that are specific to MPNs have been described and have been proposed to be immunogenic. Neoantigens and more specifically *CALR* mutants seems to be the most promising candidates. Unfortunately, clinical evidence of an immune response directed against those neoantigens during the disease is still lacking. Accordingly, the perspectives of a successful application of immunotherapy in PMF is still highly debated. Numerous immune cell dysfunctions have been observed in MPNs, especially in PMF. The question whether they contribute to the lack of immune response observed so far in the disease is still opened. Based on this hypothesis, we would like to focus our review on therapeutic approaches harnessing the functions of immune effectors, with the goal to propose strategies that could favor the success of immunotherapies.

## 4.1. Standard of care and risk-adapted therapy

Over the years, various scoring systems have been progressively improved to refine the prognosis prediction of patients with PMF. Originally composed of clinical parameters, the latest scoring systems such as MIPSS70 also takes into account genetic variables (absence of *CALR* type 1 mutation, presence of one or more HMR mutation found in *ASLX1, SRSF2, EZH2, IDH1* or *IDH2*) while GIPSS only consider genetic abnormalities. Beyond bringing expected survival information, those scores are mandatory to guide the treatment decision process. Indeed, whereas JAK inhibitors have shown a limited capacity to improve OS, the only potentially curative modality remains HSCT. Unfortunately, transplantation is associated to a high rate of mortality and morbidity, regardless the intensity of the conditioning. Thus, the decision to perform HSCT must be carefully balanced against the expect survival without transplantation and is typically reserved for high risk fit patients. Other approved treatment modalities aim at symptoms control and are mostly palliative. For non-transplant candidates, because of the lack of disease-modifying agent, participation in a clinical trial is then the more appropriate option [5]. Although it does not directly fit into the scope of our review, we would like to briefly summarize two main standard of care modalities of PMF: HSCT and JAK inhibitors. The goal will be

to insist on chosen topics that might be relevant when considering inflammatory background and immunotherapy perspectives.

### *4.1.1. Allogenic stem cell transplantation*

HSCT consists in the transfer of HSCs of a healthy donor after the administration of a conditioning regimen containing high dose (radio)chemotherapy. Efficacy of HSCT relies on two complementary mechanisms: the anti-tumor potency of the conditioning regimen itself and the graft versus leukemia effect (GvL) mediated by donor-derived immune cells. Indeed, donor cells are supposed to be able to clear the residual hematopoietic cells of the recipient, either upon recognition of foreign histocompatibility or upon recognition of a tumor associated neoantigen. This GvL mechanism has been increasingly acknowledged and highlighted by the successes obtained with reduced intensity conditioning. However, GvL must be cautiously balanced with graft versus host disease (GvHD), targeting the healthy cells of the recipient, which is the major cause of morbidity and non-relapse mortality in transplanted patients.

In one of the largest studies reported by the American Society of Blood and Marrow Transplantation, with a matched related donor, 5-years disease free survival and treatment-related mortality (TRM) were respectively of 33% and 35% [164]. TRM was even higher when a matched unrelated donor was used (50%). A more recent study confirmed those results, with a 5-years survival of around 50%, and tended to confirm the absence of major difference between myeloablative and reduced intensity conditioning [165]. HSCT is then able to cure the disease in some but should be restricted to high risk scored patients in order to avoid unbalanced toxicities [166]. Age and co-morbidities are also carefully considered in the process.

A particular concern in PMF is a higher incidence of complications related to an impaired donor HSC function after transplantation. Amongst these life-threatening pancytopenias, we can discriminate graft failure (GF) and poor graft function (PGF), the presence of a full donor chimerism being conserved in the later [167]. Prognosis in GF is particularly adverse, with a recent EBMT study indicating a 5-years survival rate of only 14%. Whereas other parameters are involved (older age, iron overload, HLA mismatch or prior sensitization, CD34+ source and number), more PMF specific factors have been suggested such as splenomegaly and remodeled bone marrow

niche. Interestingly, a German study compared the risk of delayed engraftment in PMF compared to AML [168]. They could show that neutrophils and platelets engraftment were significantly delayed in PMF, while GF only occurred in PMF patients with an incidence of 6%. The only factor influencing engraftment seemed to be splenomegaly. Circulating donor CD34+ cells were reduced and BM examination revealed a loss of VCAM-1, an adhesion molecule implicated in the homing of HSCs to the BM vascular niche. Thus, they proposed the hypothesis of an early CD34+ pooling in the spleen, that could be the consequence of the altered hematopoietic niche. In line with those findings, several recent studies have suggested a favorable role for splenectomy before transplantation. Whereas splenectomy remains a heavy procedure, a retrospective analysis performed on 530 patients seemed to be reassuring regarding its risks [169]. Moreover, another retrospective study on nearly 1200 transplanted patients suggested a survival benefits for splenectomyzed patients with marked splenomegaly [170]. Such results were encouraging to test JAK inhibitor prior to transplantation, as they are able to reduce spleen volume. Reduction of inflammatory cytokines and constitutional symptoms could also help to reduce complications by improving the performance status and maybe acting on inflammatory BM niche derangement. Discordant results have been obtained thus far, between improved outcomes, increased infections or withdrawal syndrome. While it could still be debated, a recent large study on 551 patients has been reassuring [171]. It did not reveal any increased complications and pointed at a decreased risk of relapse (8,1% vs 19,1%) and an increased 2-year event-free survival (68,9% vs 53,7%) in ruxolitinib treated patients compared to controls, if they had a spleen response at the time of HSCT. The exact impact of ruxolitinib on post-transplant outcomes, including GF and PGF, remains to clarify. Even if more results are still needed, the majority agrees to recommend ruxolitinib in the pre-HSCT setting, especially in patients with high symptom burden and splenomegaly. Furthermore, one could wonder if more potent JAK inhibitors (or BET inhibitors) will not be even more efficient options.

As already mentioned, genetic mutations are increasingly recognized in prognosis systems. Among them, *CALR* type 1 mutation has been associated with improved survival. In the transplantation setting, first observations have been reported several years ago in a retrospective study on 133 PMF patients [172]. Compared to other driver mutations, *CALR* mutated patients showed increased 4-years OS (56% versus 80% respectively) and parallel tendency of more frequent GvHD. Survival was mainly due to better non-relapse mortality, but relapses also tended to

decrease. While it remains a speculation, enhanced immune response within those patients could be implicated, GvL having a supposedly more immunogenic neoantigen to target.

There is no randomized controlled trial to define the best option following PMF relapse after HSCT. Donor lymphocyte infusion (DLI) is a common approach in such setting, followed or not by a second HSCT. DLI aims at reinforcing GvL effect but is limited by the associated risk of GvHD. Moreover, its efficacy is variable depending on the hematological disease considered. Several retrospective reports have suggested reasonable success of DLI in PMF. In a small cohort of 26 relapsed patients, a median administration of 3 DLI led to 39% of complete response [173]. A larger retrospective study reported a median OS of 76 months with such approach [174]. However, numerous confounding factors are present, due to the retrospective design of these studies, and preclude accurate comparison of DLI versus second HSCT. Moreover, these studies have not detailed the gene mutations harbored by the diseases of the responders. This could have been instructive to gather complementary clues of putative tumor-neoantigens.

### *4.1.2. JAK inhibitors*

As previously stated, HSCT is potentially curative but not best suited to elderly, co-morbid or low risk patients because of its toxicity. Pathological and persistent activation of the JAK-STAT pathway is essential for MPNs and *JAK2*$^{V617F}$ has been its first identified driver mutation. Given this importance of JAK2 in MPNs, many efforts were invested into the development of dedicated inhibitors and in 2011, the first targeted therapy was approved by FDA. Ruxolitinib (Jakavi) is an orally bioavailable JAK1 and JAK 2 inhibitor. First phase 1-2 clinical trials suggested responses in terms of symptoms relief (fatigue, weight loss, night sweats), spleen reduction and decreased levels of inflammatory cytokines, notably IL-1, TNF-$\alpha$ and IL-6. It has been followed by phase 3 randomized clinical trials, CONFORT-I (comparing ruxolitinib to placebo) and CONFORT-II (comparing ruxolitinib to best available therapy), which all confirmed those preliminary data in a population of intermediate or high-risk patients ineligible for HSCT [175, 176]. Both trials reported improved MF-related symptoms and decreased splenomegaly. Upon 5-years follow up, patients treated with ruxolitinib also showed a statistically improved median OS (not reach for ruxolitinib treated patients versus 4,1 years in control group according CONFORT-II). However, disease modifying activity is lacking, without meaningful

effect on disease burden, fibrosis or clonal evolution. Furthermore, therapy duration is limited, with half of the patients discontinuing ruxolitinib over 2 years, because of side effect (including anemia and thrombopenia) or disease progression [177].

In order to improve ruxolitinib efficacy, some have proposed to treat the patients earlier, before reaching the highest-risk categories investigated by the CONFORT studies [178]. A secondary analysis of the JUMP study and another retrospective study found out that predictors of ruxolitinib resistance included spleen size (>10cm), delayed initiation of ruxolitinib therapy (more than 2 years after PMF diagnosis) and high-risk score according IPSS [179]. The same studies also showed a negative correlation between baseline symptoms score and response to therapy. Early treatment of less advanced diseases in order to maximize ruxolitinib efficacy and survival benefit seems possible but will require additional studies.

Beside its benefits in the treatment of PMF and other MPNs, inhibition of the JAK-STAT pathway is also associated to immunosuppressive effects. Indeed, the JAK family also play a major role in the signaling cascade of cytokine receptors implicated in hemopoiesis and various immunological processes. Although it has been used with benefits in the treatment of inflammatory diseases, this aspect of ruxolitinib treatment raises some concerns. Given our focus on the immune landscape of PMF and its immunotherapy perspectives, potential impact of ruxolitinib on immunosurveillance and putative tumor-specific T cells is of great interest. Over the years, several groups have reported various damages to immune effector cells following treatment with ruxolitinib. In DC, which are crucial for T cell priming, differentiation, activation and migration is inhibited by ruxolitinib [180]. Similar observations were made on NK cells, decreasing in number because of an impaired maturation and manifesting reduced killing activity *in vitro* [181]. T cells have also been well studied. Several publications corroborated a global T cell count reduction, affecting both $CD4^+$ and $CD8^+$ T cells in ruxolitinib treated patients [159, 182]. This is supported by an impaired *in vitro* differentiation of $CD4^+$ T cells into Th1, Th17 and Tregs. Cytokine release from activated $CD4^+$ and $CD8^+$ T cells, including IFN$\gamma$, was decreased upon *in vitro* activation and *in vivo*. On the other hand, reduced number of Tregs did not always correlate with a decrease of their suppressive function [183].

The most obvious manifestations are the infectious complications, already increased by nature in PMF patients [184, 185]. Looking at large clinical trials, the risk of infection was usually well balanced with the control group. The precise

contribution of ruxolitinib in the middle of other confounding factors such advanced stage diseases complicate the interpretation of many retrospective studies. However, significant increase of Herpes zoster infection has been noted in CONFORT-I trial and many case reports have been published on ruxolitinib-associated opportunistic infections, including tuberculosis, HBV reactivations and *Pneumocystis jirovecii* diseases. Moreover, increased risk of lymphoma following ruxolitinib treatment has been sporadically suggested, like in the small cohort reported by Porpaczy et al. [186]. They described 4 cases of aggressive B cell lymphomas reminiscent of the ones occurring in immunocompromised patients. Reassuring data were later provided by larger retrospective studies [187]. For example, a publication of Pemmaraju analyzed 2583 MPN patients, including 1617 PMF, and did not confirm such elevated risk of secondary lymphoma [188].

Interestingly, part of the immunosuppressive potential of ruxolitinib is directly dependent of its inhibitory activity against JAK1. In contrast, JAK2 inhibition, which is at first glance more relevant for targeting PMF clone, does not seem to drive a potent T cell blockade. Indeed, a well performed study, has compared T cell suppression capacity of several JAK inhibitors targeting JAK1 or JAK2 or both [189]. On PBMC from healthy donors, they showed *in vitro* that specific JAK2 inhibition was not impairing T cell proliferation by comparison to classical JAK1/JAK2 inhibitor. These results were confirmed upon JAK1 or JAK2 gene silencing using shRNA, in proliferation and allogenic lysis assays. T cell activity was also preserved in JAK2 KO cells but not in JAK1 KO cells in a GvHD mouse model. Following those evidences, an inhibitor devoid of activity against JAK1 would be of interest. It would supposedly induce less immunosuppression, therefore leading to less infectious complications but also be a more promising candidate in combination with an immunotherapy. Amongst the JAK inhibitors being in active clinical development, fedratinib is a JAK2 selective inhibitor [190]. It also exhibits interesting off-target inhibition of FLT3 and BRD4 (member of BET protein family). The JAKARTA phase 2 study showed benefits comparable to ruxolitinib with decreased MF related symptoms, decreased splenomegaly and decreased inflammatory cytokines. The drug is well tolerated but special attention has to be dedicated to thiamine monitoring, because of a potential association with Wernicke's encephalopathy. Promising clinical trials are going forward with phase 3 reaching completion. This large clinical study has been focused on a second line positioning, after ruxolitinib failure. Given its potency and its activity putatively sparing the T cells, its positioning and combination potential is likely to evolve.

## 4.2. Targeting monocytes and phagocytes

As we have seen, monocytes and phagocytes could have an important role to play in PMF, as cytokine producing cells, precursors of fibrosis-driving cells and defective antigen presenting cells. In PMF, several drugs are being investigated or could be considered to counter these mechanisms.

### *4.2.1. Anti-SLAMF7 antibodies*

Elotuzumab in a humanized IgG1 mAb directed again SLAMF7, originally developed and approved for treatment of relapsed/refractory multiple myeloma. It's primary mechanism of action relies on cell depletion trough ADCC by NK cells. Recent interest has been shown for elotuzumab in PMF. The main reason is the high expression of SLAMF7 on a particular subset of monocytes, supposed to be the precursor of fibrocytes, one of the fibrosis-driving cells in PMF. Noteworthy, depletion of those cells in animal models was associate with benefits on fibrosis but also on cytopenias. Interestingly, TGF-β1 production was also decreased. NCT04517851, a phase 2 clinical trial, has been started in 2020 and investigates efficacy of elotuzumab monotherapy in PMF patients. First results are still awaited.

### *4.2.2. Anti-CD123 immunotoxin*

CD123, the alpha chain of interleukin 3 receptor, is expressed at low level in various myeloid cells, including their progenitors, but is overexpressed in numerous myeloid malignancies. Targeted therapy against such malignant cells has been developed with tagraxofusp, an immunotoxin consisting in the fusion of IL-3 and truncated diphtheria toxin. The mechanism of action relies on the binding of the fusion protein to CD123 expressing cells, leading to the internalization of diphtheria toxin that will migrate into the nucleus and block proteins synthesis. Tagraxofusp treatment initially proved its efficacy with blastic plasmacytoid dendritic cell neoplasm (BPDCN), for which it has been approved in 2018.
CD123 expression has been reported to be increased in PMF, notably on monocytes and plasmacytoid dendritic cells, promoting the development of a dedicated phase 1/2 clinical trial (NCT02268253). Tagraxofusp is evaluated as monotherapy in PMF patients relapsing after or refractory to JAK inhibitors. To this day, this study is still

actively recruiting, but first efficacy results have been reported [191]. In heavily pre-treated patients with intermediate-2 or high-risk DIPPS plus scores, tagraxofusp was associate to spleen responses (53% of patients) and symptom responses (45% of patients). Most grade 3 treatment related adverse events were thrombocytopenia (8% of patients) and anemia (15% of patients). The best response rates were obtained in patients with monocytosis and splenomegaly, which could help to further select patients. Interestingly, synergy with JAK inhibitors has been shown *in vitro* on PMF primary cells [192]. Further results and combination studies will be needed, especially for PMF patients with accelerated-phase and blast-phase.

### *4.2.3. Promoting phagocytosis with anti-CD47 antibodies*

As previously mentioned, antigen presenting cells may present an altered function in PMF. A paracrine anti-phagocytic property of mutated CALR protein or an overexpression of CD47 have been described and could play a supporting role by blocking phagocytosis of MPN cells. Whether an impaired phagocytosis and subsequent decreased T cell priming is responsible of the paucity of anti-tumor response in PMF is an attractive hypothesis, but it remains speculative.

Overexpression of CD47 has been well demonstrated in a series of myeloid malignancies, prompting clinical trials with magrolimab, a first-in-class humanized anti-CD47 mAb. Although monotherapy proved to be disappointing in AML and MDS, combination with therapies improving pro-phagocytic signals have demonstrated synergistic effects in phase 1b trials. We can cite the combination of magrolimab and azacytidine in AML, leading to an encouraging 71% of objective response rate (complete for 48%) in the highest risk group with *TP53* mutation [193]. Phase 3 clinical trials are ongoing for AML and MDS (ENHANCE trails) to confirm these results. To our knowledge, T cell responses have not been analyzed in these patients whereas therapeutic benefits of CD47 blockade has been attributed to improved T cell priming in several mouse tumor models. Such investigations would be welcomed in AML or MDS, which are not considered as potent immunogenic malignancies, similarly to MPNs.
Given these early results, combined therapy with magrolimab in PMF should deserve attention, especially in patients with mutated *CALR*. Association with cytoreductive (hydroxyurea) or hypomethylating agents (HMA) would be close to what have been done so far in AML/MDS. Other combinations with mAb, like with anti-PD-L1, could

promote ADCP and be really interesting. Apart from reduced disease burden, increased T cell response mediated by improved priming should be researched.

## 4.3. Immunomodulatory treatments

### *4.3.1. Interferon alpha*

IFNα, a member of type I IFN family, is an innate immunity cytokine primary implicated in the defense against viruses and intracellular pathogens [147]. It binds to type I IFN receptor, composed of two units, IFNAR1 and IFNAR2, constitutively associated tyrosine kinases JAK1 and TYK2. Activation of the receptor leads to phosphorylation of those tyrosine kinases, phosphorylating in turn STAT1 and STAT2. Together with IRF9, they form the "ISG factor 3 complex" which will exert its transcriptional activity on IFN-stimulated genes. Beyond apoptosis or cell cycle arrest, the effects of IFNα encompass stimulation of innate immune response (promote antigen presentation and NK cell function) and adaptative immune response.

While anti-tumor properties have been attributed to the cytokine since decades, clinical applications have been historically hampered by its toxicities and need of frequent parenteral injections. Later, improved pharmaceutical formulations, thanks to the addition of a polyethylene glycol tail, led to increased half-life and stability. Improved tolerance to pegylated IFNα helped to renew interest for its use, but it remains a second-line treatment in MPNs. Many reports have proposed a specific activity of IFNα against the stem cell pool of MPN cells, leading to complete responses, even durable molecular remission, in some patients [194]. On the basis of its potential disease-modifying activity, clinical research is still ongoing with the most promising results gathered in PV and ET. In PV patients, comparison between ropeginterferon-α2b and standard therapy (hydroxyurea) in a phase 3 clinical study initially confirmed non-inferiority and good tolerability of IFNα (PROUD-PV and subsequent CONTINUATION-PV study). However, IFNα responses continued to increase overtime [195]. At 36 months, complete hematological responses and *JAK2*$^{V617F}$ allele burden decreases statistically surpassed hydroxyurea. Toxicity was manageable, 8% of the participants having left the study due to adverse events. These results are encouraging, questioning the place of IFNα in first line, but

addressing the question of a true modification of the disease evolution will require longer follow up. Indeed, no differences have been seen on the complications of PV, such as cardiovascular events, and decrease disease burden does not necessary imply disease cure or increased survival.

Successful use of IFNα in PMF has been more modest, with only non-controlled studies and highest efficacy being obtained in the early phase of the disease. Ianotto et al. reported a prospective cohort of 62 patients, with intermediate to high-risk score diseases, treated for a median of 39 months [196]. Here again, *JAK2*$^{V617F}$ allele burden only started to decrease after one year of treatment (from more than 50% in 37% of the patients) but was not associated to increased survival or reduced AML transformation. Although the design of the study was not meant to assess it, OS was suggested to be improved with IFNα treatment. It's not possible to conclude on this point as OS was compared to historical controls and did not take into account further treatments (ruxolitinib, HSCT, …). Drop-out rate was high (72,6%) and half of it was due to intolerance (dose of IFNα not mentioned). Trying to improve efficacy, some attempted to combine low doses of IFNα with JAK inhibitor. COMBI study included 32 PV and 16 PMF patients for a 2 years treatment [197]. Pegasys 45µg/week or PegIntron 35 µg/week was associated to ruxolitinib (5 to 20mg twice a day). In PMF patients, 44% of patients achieved complete or partial response based on ELN 2013 criteria. Drop-out rate was lower than previously reported as 12 of the 16 patients completed the pre-planned treatment schedule. Half of them had a molecular remission with undetectable *JAK2*$^{V617F}$ transcript. The major hematologic side effect was anemia. Even if no information regarding survival is available, this study is interesting as it confirmed feasibility and tolerability of the combination. Another phase I/II clinical trial, called RUXOPEG, is asking similar questions and is currently ongoing [198]. Here, a first part of the study will determine more accurately the most tolerable and effective combination dose. Up to now, no relevant efficacy data have emerged, but safety assessment was reassuring with IFNα up to 135µg/week.

While future results of clinical trials such as RUXOPEG will be interesting, mechanism of action of IFNα in MPNs remains debated. One of the dominant hypotheses would be an eradication of the mutated HSCs pool trough reduced quiescence and secondary exhaustion. In their publication, Mullally et al. showed increased cell cycle activation of long term HCSs from *Jak2*$^{V617F}$ mice in response to IFNα administration [199]. Repopulating capacity was impaired in ulterior

transplantation assay. In a similar way, the group from Skoda described a MK-biased CD41$^{high}$ HSC population, characterized by reduced quiescence and poor long-term capacity [200]. Its proportion was increased by *Jak2*$^{V617F}$ mutation and even further increased following long term IFNα treatment, favoring depletion of multipotent *Jak2*$^{V617F}$ HSC pool and resurgence of WT HSC. Interestingly, reduced quiescence was not counterbalanced by ruxolitib, which acted preferentially on downward erythroid progenitors [201]. Also, *JAK2*$^{V617F}$ mutation seems to sensitize cells to the effect of IFNα, higher dose being needed to achieve similar effects on proliferation of *CALR* mutants *in vitro* [202]. Priming due to preexisting STAT1 phosphorylation in *JAK2*$^{V617F}$ cells had been suggested. This finding has no demonstrated clinical relevance to date.

Beyond HSC exhaustion, immunological properties of IFNα have been proposed to mediate its effects in MPNs, but evidences are weaker. The evoked mechanisms are those expected from IFNα in anti-viral response. Some have been extrapolated from CML, as an increased of NK cells number [203]. In MPNs, increased HLA expression was shown by flow cytometry, especially on DC [204]. The same DC also expressed higher levels of maturation markers and IFNα has the capability to restore their T cell activation potential. Overall, no conclusion can be drawn regarding the true contribution of immune properties of IFNα to its clinical benefit in MPNs. Further studied will be needed in order to refine further combinations.

### *4.3.2. Hypomethylating agents*

Gene mutations coding for epigenetic regulators are regularly found in PMF. Aberrant methylation is a hallmark of cancer and myeloid malignancies are no exception. This abnormal methylation within promoter regions can lead to silencing of critical tumor suppressor genes and may favor tumor progression and escape to treatment. Hypomethylating agents (HMA), such as 5-azacytidine (Vidaza), are cytidine nucleoside analogs that inhibit DNA methyltransferase after their incorporation into DNA during cellular replication. They induce DNA demethylation and reprogram tumor cell, leading to reactivation of aberrantly silenced genes involved in multiple pathways (apoptosis, DNA repair, differentiation, angiogenesis). While they proved to be successful in high risk MDS and AML, used as first line therapy for older patients, their application in PMF has not met the same success [205]. Indeed, phase 2 trial evaluating monotherapy only found limited activity, with

clinical improvement in 24% of patients but no significant response [206]. More encouraging results were collected more recently, in another phase 2 trial combining 5-azacytidine with ruxolitinib [207]. Here, 72% of patients responded with prolonged spleen responses (95% at week 48) and 57% had improvement in fibrosis. However, no further studies seem to be planned.

Mechanism of action of HMA seems to partially rely on immunological effects that have been proposed to stimulate anti-tumor immunity. Those effects, mainly observed *in vitro* or in mouse models, include increased cancer germline genes expression, increased antigen presentation on MHC class I, upregulation of IFN$\gamma$ pathway, suppression of MDSC and improved activation of NK cells [208-210]. Nevertheless, clinical benefits of HMA could be tempered by the concomitant expression of checkpoint inhibitors they induce (PD-1, PD-L1, CTLA-4). This rational prompted the assessment of HMA combination with anti-PD-1 mAb, leading to encouraging responses in phase 2 trial for some AML patients. In PMF also, HMA association could also be evaluated with immunomodulatory or immunotherapeutic approaches.

## 4.4. Immunotherapies

### *4.4.1. Therapeutic cancer vaccines*

Therapeutic cancer vaccines have been poorly explored in MPNs. Here again, the C-terminus portion created by the majority of *CALR* exon 9 mutations is the most promising candidate. This neoantigen is a driver mutation, specific of MPN cells, expressed by nearly all mutant cells and have shown evidences of immunogenicity. A recent report has been made by Grauslund et al., reporting the negative results of a clinical trial using vaccination against mutant CALR in a series of 10 MPN patients with various diagnostics (ET, pre-PMF, PMF). They used a peptide-based vaccine, comprising the 36 amino-acids of the C-terminus portion of the mutated CALR protein. Peptides were emulsified in Montadine$^{TM}$ ISA-51 and injected subcutaneously, for a total of 15 doses over a year. No other adjuvant has been used and other treatment were allowed in parallel, notably IFN$\alpha$ in 8 patients. Unfortunately, apart from a slight temporary PLT decrease, no clinical response has been observed independently of MPN subtype. More detailed analysis revealed *in vitro* T-cell responses against CALR mutant peptide using IFN$\gamma$ ELISPOT and ICS

as previously described. Interestingly, the frequency of positive patients progressively increased over treatment period (from 4/10 to 7/10 at the end of the study). Again, these responses were present in ET patients, but absent in PMF. Cytokine producing cells were $CD4^+$ and/or $CD8^+$ T lymphocytes. Given the technical limitations, we should be careful in the interpretation of this so-called increased immune responses.

This strategy in nonetheless conceptually elegant, but even more limited in PMF patients. Combination therapies could be imagined, as with PD-1 inhibitors or IFN$\alpha$, but the choice of the vector could be improved in the first place [211]. Indeed, while peptide vaccine is a simple approach, it has not been associated to high chances of success [212]. Cell based vectors could be interesting, notably with autologous DC that can be loaded with the desired peptide, bypassing the question of the endogenous antigen processing. An alternative could be a molecule-based vaccine, in which immunogenic peptide would be encoded by mRNA [213].

### *4.4.2. Immune checkpoint inhibitors*

As previously reviewed, both PD-1 and PD-L1 have been reported to be overexpressed in PMF. Some encouraging preclinical rational has also been obtained with anti-PD1 treatment, suggested to restore T cell response in mouse model and in human PBMC. Unfortunately, recent phase 2 clinical trials failed to confirm the efficacy of PD-1 inhibition in PMF patients. As a general remark, these cohorts were characterized by advanced diseases (interemediate-2 or high-risk DIPPS scores), few *CALR* patients, frequent HRM mutations and numerous pretreatments (prior ruxolitinib for the majority of patients). The first study administered nivolumab to 8 patients leading at best to stable diseases in 5 of them after 8 doses [214]. The second study used pembrolizumab on 9 patients, all attaining stable disease at the end of the sixth cycle [215]. Both studies were interrupted beyond this stage given the lack of response.

Interestingly, T cells populations were studied upon pembrolizumab treatment. Flow cytometry showed an increased T cell frequency ($CD4^+$ and $CD8^+$) in 3 patients while TCR sequencing showed significant expansion of some T cell clones. As no further characterization has been done, it is not possible to relate these clones to a specific response against PMF cells. However, one of the few mutated *CALR* patient has

been more precisely studied than the others, in the related publication of Bozkus et al. As previously detailed, they assessed IFNγ production in ELISPOT upon PBMC stimulation with mutated CALR peptides. While no response was observed with PBMC collected before treatment, *in vitro* T cell activation appeared in PBMC collected after first cycle of pembrolizumab. Moreover, some of the amplified T cell clones observed in pembrolizumab treated cohort were also increased in PBMC amplified *in vitro* with mutated CALR peptide and checkpoint inhibitor treatment. Nevertheless, in the absence of precise characterization of these clones, it remains clearly possible that they corresponded to by standers T cells, specific of other unrelated epitopes.

Regarding the failure of those studies, we have also to keep in mind the advanced stages and multiple pretreatments of the patients that potentially limited clinical responses. Indeed, high tumor burden has been seen to compromise anti-PD1 response and long-standing treated diseases have a higher probability to have impaired T cell function [216]. Notably, treatment with ruxolitinib has been shown to impair T cell function for a prolonged time, even months after withdrawal. Of course, this problem is not easy to solve and first line usage of such medications could seem provocative given the few arguments for their activity in PMF. Immune checkpoint inhibitors in an adjuvant setting, after another drug decreasing the tumor burden, could be interesting as long as it does not impair T cell function.

Other immune checkpoint inhibitors could be envisioned. To our knowledge, whereas overexpressed, anti-CTLA4 and anti-PD-L1 mAb have not been tested in PMF. Sabatolimab, targeting TIM-3, has shown preliminary efficacy with HMA in high risk MDS and AML. It is planned to be also tested in PMF in combination with ruxolitinib (NCT04097821).

While it would be reasonable to be pessimistic regarding to chance of success of such approaches, other combination therapies may increase response rate. First, check point inhibitors could be associated with improved efficacy, as previously done in other malignancies. Anti-PD1 therapies were well tolerated in phase 2, but addition of anti-CTLA4 or anti-TIM3 could lead to high toxicity. Secondly, they could be combined with other immunotherapy platforms such as tumor vaccination or Treg-targeted therapies. Finally, combination with immunomodulatory drugs improving antigen presentation or T cell priming would also be logical.

### *4.4.3. Inhibition of TGF-β1*

As previously detailed, active TGF-β1 is proposed to be involved in various pathological processes of PMF, including fibrosis deposition, growth suppression of normal hematopoiesis and inhibition of the immune response directed against PMF cells. However, therapeutic inhibition of active TGF-β1 has always been a challenging task, given the wide range of physiological processes in which the cytokine is implicated and the dual role it plays in neoplasms general. Accordingly, clinical developments have been slow but strategies aiming at targeting only a restricted portion of the total amount of active TGF-β1 is promising. As a general remark, by narrowing the specify of TGF-β blockade, depending on the mechanism of activation or the isoform considered, different biological pathways are expected to be impacted.

To begin with one of the least specific approaches, inhibitors of TGF−βRI kinase activity has been developed but raised major concerns about on-target cardiac toxicities. Preclinical experiments in adult rats have indeed indicated that TGF−βRI was essential for heart valves homeostasis, as the inhibitor was responsible of shear stress-associated inflammatory lesions [217]. Even if this potential side effect has been mitigated, by limiting drug exposition and selecting inhibitors with the lowest toxicity profile, TGF−βRI inhibitors like the most advanced galunisertib have proved limited success to date in oncology. Clinical research is still ongoing, but without actual plans in PMF despite preclinical rational [70].

Most documented strategies concern inhibitors working upstream, by preventing the interaction of active TGF-β with its receptor.

One small phase 1 study has been reported with neutralizing antibodies against the mature form of the cytokine. Fresolimumab is a human IgG4 mAb capable of neutralizing mature TGF-β1, -β2 and -β3. Tolerability had been previously demonstrated in phase 1 clinical trial including 29 patients with advanced melanomas and renal cell carcinomas. At the time, no tumor responses had been observed and treatment had nonetheless been responsible of serious skin rashes, including squamous cell carcinomas that resolved after treatment interruption [218]. In PMF, the study was prematurely interrupted by the pharmaceutical company after having included only 3 patients [219]. Only two patients reached the 12 planned

treatment cycles (one died after cycle 1 from an independent cause). Analysis is thereof impossible on this cohort. To note, a red blood cell count improvement was seen in one the two patients, as he became transfusion-independent after cycle 6. Development of this mAb, with a broad inhibition profile, has stopped.

Soluble TGF-β receptor traps represent an emerging class of drugs, notably in PMF. The underlying principle is to use a soluble molecule, formed by the fusion of the extracellular domain of TGF-β receptor and an antibody Fc fragment, that will consume the circulating active cytokine without mediating any signal. AVID200 is composed of extracellular domains of TGF-βRII fused with the Fc portion of human IgG. It is a potent trap for TGF-β1 and TGF-β3, but not for TGF-β2. Aforementioned preclinical data on PBMC reported its ability to promote proliferation of WT progenitor cells rather than *JAK2*$^{V617F}$ ones. AVID200 was subsequently tested in a phase 1 clinical trial on 21 PMF patients, resistant or ineligible for ruxolitinib [220]. While it failed to induce any response based on IWG-MRT criteria, 17 patients in the study experienced improvement of their PLT counts compared to baseline, including 2 normalizations. Further clinical development will then focus on disease-associated thrombocytopenia. The analyses that have been done so far with AVID200 in PMF, both in preclinical and in clinical setting, have not assessed the consequences of a broad TGF-β1 and TGF-β3 neutralization on immune response.

Finally, anti-GARP:TGF-β1 mAb are an attractive way to directly target a source of TGF-β1 that specifically exerts a deleterious effect on anti-tumor immune response, without affecting TGF-β1 implicated in other biological processes and activated in a GARP-independent manner. This TGF-β1 activated from GARP:TGF-β1 complexes comes from a limited number of cell types including TCR stimulated Tregs, BCR stimulated B cells and activated PLT. While Tregs are the only demonstrated source of TGF-β1 activated from GARP:TGF-β1 complexes involved in anti-tumor immunity, anti-GARP:TGF-β1 mAb activity on B cells and PLT have not raised major preclinical toxicity concerns. Moreover, the mAb acts by functional blocking of TGF-β1 activation by Tregs, rather than by depleting Tregs, probably helping to preserve some part of their regulatory functions. The results gathered in mouse tumor models showed that anti-GARP:TGF-β1 mAb treatment overcame resistance to anti-PD1 blockade [81]. Accordingly, this combination is currently tested in phase 1 clinical trials with cancer patients (NCT03821935). The same combination therapy would be

also relevant in PMF and should deserve attention as shown by our preclinical results in MPL$^{W508A}$ mice.

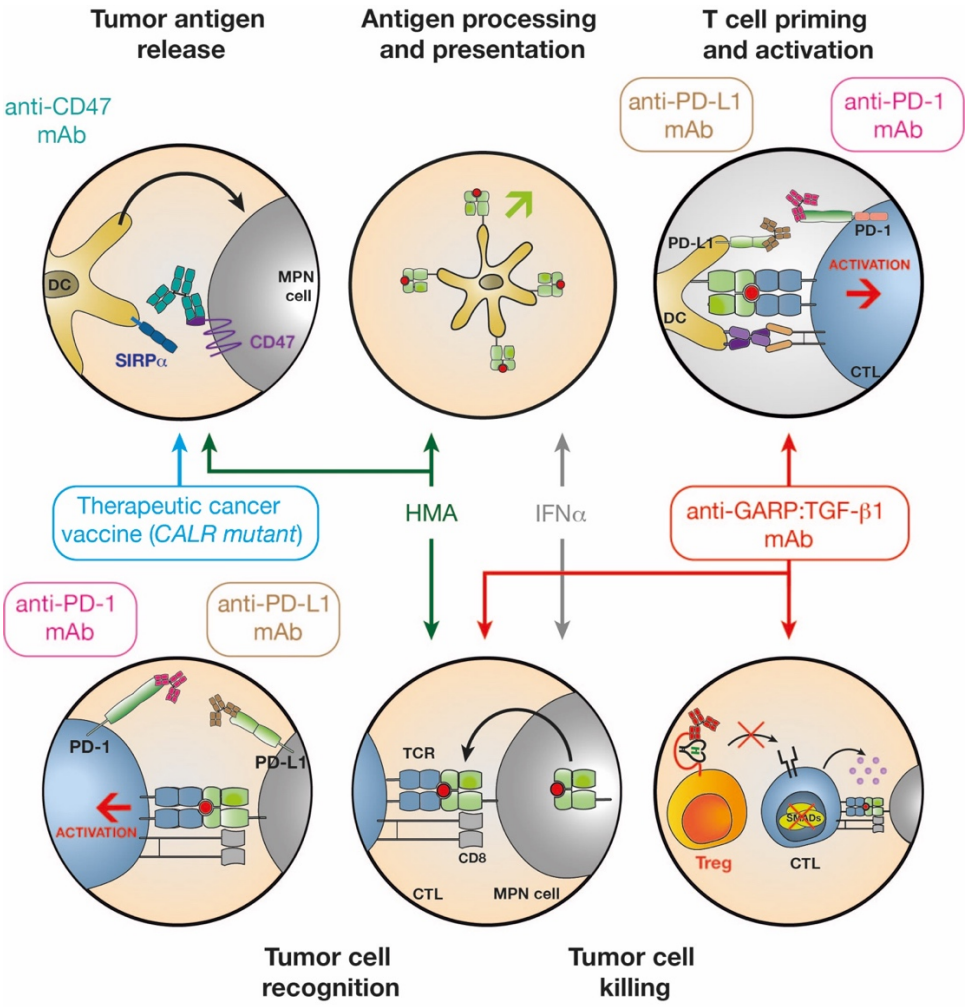


**Figure 13: Potential immunomodulatory and immunotherapeutic agents in PMF**
Visual summary of the proposed drugs that could be further investigated for an immunotherapeutic approach of PMF. Proposed mechanisms of action only focus on altered processes observed or suggested in PMF and are thus non-exhaustive. Immunotherapies have been highlighted by color boxes, to make a clear distinction with immunomodulatory drugs that could be used in combination.

# AIM OF THE STUDY

TGF-β1 is associated to the pathophysiology of MPNs, and particularly PMF. Considered mainly because of its pro-fibrotic properties, TGF-β1 is a pleiotropic cytokine that could contribute more fundamentally to disease progression and deserves more attention. TGF-β1 is produced as an inactive precursor that requires activation by cell type-specific mechanisms. Precise identification of the main cell type(s) producing and activating TGF-β1 in PMF or MPNs more generally would be important to therapeutically target the cytokine more safely and efficiently.

Transmembrane protein GARP is implicated in TGF-β1 activation by Tregs, but also by PLTs which derive from MKs. GARP-expressing cells are interesting candidate producers of pathogenic TGF-β1 in PMF. Our laboratory derived monoclonal antibodies that inhibit TGF-β1 activation by GARP-expressing cells, but not by other cells, and showed that GARP:TGF-β1 blockade inhibits TGF-β1 activation and immunosuppression by human and mouse Tregs *in vitro* and *in vivo*.

The main objective of my work was to investigate the involvement of GARP-dependent TGF- β1 activation in mouse MPN, to assess the therapeutic potential of antibody-mediated GARP:TGF-β1 blockade in this disease. The results of this work, done in collaboration with Sara Lecomte, are presented here in the form of a manuscript that will be submitted for publication shortly.

# AIM OF THE STUDY

# RESULTS

# RESULTS

# Therapeutic activity of GARP:TGF-β1 blockade in murine primary myelofibrosis

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## Abstract

Primary myelofibrosis (PMF) is a myeloproliferative neoplasm characterized by clonal expansion of myeloid cells, notably megakaryocytes (MKs), and aberrant cytokine production leading to bone marrow (BM) fibrosis and insufficiency. Current treatment options are limited. TGF-β1, a pro-fibrotic and immunosuppressive cytokine, is involved in PMF pathogenesis. While all cell types secrete inactive, latent TGF-β1, only a few activate the cytokine *via* cell type-specific mechanisms. The cellular source of the active TGF-β1 implicated in PMF is not known. Transmembrane protein GARP binds and activates latent TGF-β1 on the surface of regulatory T lymphocytes (Tregs) and MKs or platelets. Here, we found increased expression of GARP in BM and spleen of mice undergoing PMF and tested the therapeutic potential of a monoclonal antibody that blocks TGF-β1 activation by GARP-expressing cells. GARP:TGF-β1 blockade reduced not only fibrosis, but also clonal expansion of transformed cells. Using mice carrying a genetic deletion of *Garp* in either Tregs or MKs, we found that the therapeutic effects of GARP:TGF-β1 blockade in PMF imply targeting GARP on Tregs. These therapeutic effects, accompanied by increased IFN-g signals in the spleen, were lost upon CD8 T cell depletion. Our results suggest that selective blockade of TGF-β1 activation by GARP-expressing Tregs increase a CD8 T cell-mediated immune reaction that limits

transformed cell expansion, providing a novel approach that could be tested to treat patients with myeloproliferative neoplasms.

## Introduction

Philadelphia-negative myeloproliferative neoplasms are hematopoietic stem cell disorders characterized by clonal expansion of differentiated myeloid cells. They encompass polycythemia vera, essential thrombocythemia and primary myelofibrosis (PMF). PMF bears the worst prognosis with a median overall survival of 4.4 years (*1*). Characteristic features of PMF include abnormal proliferation of immature megakaryocytes (MKs) and aberrant production of pro-inflammatory cytokines, leading to progressive bone marrow (BM) fibrosis, BM failure and extramedullary hematopoiesis. Transformation into acute myeloid leukemia can also arise and constitutes a major concern. The physiopathology of the disease is not completely understood. Identification of somatic mutations in *JAK2*, *CALR* or *MPL* in nearly 90% of PMF patients underscores the central role of JAK/STAT pathway activation in the disease (*2*). Although JAK inhibitors provide symptomatic relief and a limited survival benefit (*3-6*), allogeneic stem cell transplantation remains the only curative treatment option. However, only the youngest patients are eligible given the high toxicity of this approach (*7*). Novel therapeutic strategies are thus needed.

Several cytokines are suspected to be abnormally produced and contribute to PMF pathogenesis. Among these, TGF-β1 has received much attention owing to its pro-fibrotic properties that contribute to PMF in several mouse models (*8-11*). However, which cell types, if any, produce the pathogenic TGF-β1 in PMF is unknown.

Virtually all cell types produce TGF-β1 in a latent, inactive form. In latent TGF-β1, the mature TGF-β1 dimer remains non-covalently associated with the Latency Associated Peptide (LAP), which forms a ring around mature TGF-β1 and masks sites of interaction with the TGF-β receptor. To exert activity, mature TGF-β1 must be released from LAP, a process referred to as “TGF-β1 activation”. Only a few cell types were shown to activate TGF-β1, *via* molecular mechanisms that are cell type-specific. In stimulated regulatory T lymphocytes (Tregs), transmembrane protein GARP binds LAP through disulfide linkage, allowing surface presentation and

activation of latent TGF-β1 by integrin αVβ8 (*12-17*). GARP:(latent)TGF-β1 complexes are also expressed on the surface of B cells, megakaryocytes and platelets (*18, 19*). Upon stimulation, B cells and platelets release active TGF-β1 from surface GARP:TGF-β1 complexes, but which other protein contributes to TGF-β1 activation by these cells is not fully elucidated (*20*).

We previously derived anti-human or mouse GARP:TGF-β1 monoclonal antibodies (mAbs) that block TGF-β1 activation by GARP-expressing cells *in vitro* and *in vivo* (*15, 17*). Anti-GARP:TGF-β1 clone 58A2 blocked TGF-β1 activation and immunosuppression by mouse Tregs, and induced immune-mediated rejection of mouse tumors when combined with anti-PD-1 (*17*). Antibody-mediated GARP:TGF-β1 blockade is currently tested in patients with locally advanced or metastatic solid tumors (ClinicalTrials.gov: NCT03821935).

Here, we hypothesized that pathogenic TGF-β1 is produced by GARP-expressing cells during PMF development, and we tested whether anti-GARP:TGF-β1 could exert therapeutic activity in a mouse model of the disease.

# Results

## Increased GARP levels in bone marrow and spleen of mice with PMF

TGF-β1 is thought to be pathogenic and contribute to fibrosis in PMF, but which cells produce and activate the cytokine during disease progression is unknown. GARP-expressing cells are interesting candidates as TGF-β1 activation by these cells, but not by others, can be blocked with anti-GARP:TGF-β1 mAbs. We thus examined whether GARP-expressing cells are present or enriched in BM and spleen of mice undergoing PMF.

Most clinical features observed in PMF patients, including leukocytosis, splenomegaly, BM and spleen fibrosis, can be induced in mice by transplanting syngeneic BM cells that have been retrovirally transduced to express a constitutively active mutant thrombopoietin receptor ($MPL^{W508A}$), which is the homolog of the pathogenic human $MPL^{W515A}$ variant. Expression of $MPL^{W508A}$ in BM cells activates the JAK/STAT pathway (*21, 22*). Here, briefly, BM cells were isolated from 5-FU treated C57BL/6 mice, transduced with a retroviral vector encoding $MPL^{W508A}$ and GFP (*$Mpl^{W508A}$-IRES-GFP*), and transplanted into lethally irradiated C57BL/6 recipients (Fig. 1A). Transduction efficacy ranged from 5 to 15% according to GFP expression. Severe leukocytosis developed within 14 to 28 days after transplantation of BM cells transduced with *$Mpl^{W508A}$-IRES-GFP*, but not *$Mpl^{WT(wild\ type)}$-IRES-GFP,* taken here as controls (fig. S1A). We will further refer to these mice as $MPL^{W508A}$ or $MPL^{WT}$ controls, respectively. Leukocytosis reflects the proliferation of transformed $MPL^{W508A}/GFP^{+}$ cells. It is accompanied by the development of spleen and BM fibrosis, measured by histological analyses of organs collected 4 to 5 weeks after transplantation (fig. S1B). Phosphorylated STAT5 (pSTAT5) levels were increased in BM and splenocytes of $MPL^{W508A}$ mice by comparison to $MPL^{WT}$ controls, confirming constitutive activation of the JAK/STAT pathway in this murine model of PMF (Fig. 1B).

GARP protein levels were significantly increased in BM and spleen of $MPL^{W508A}$ mice

by comparison to controls (Fig. 1B). Flow cytometry analyses indicated that Tregs ($FOXP3^+$ cells) and MKs ($CD41^{high}$ cells) represented the vast majority (>95%) of GARP-expressing cells in these organs (fig. S2A) and virtually all Tregs and MKs expressed GARP (fig. S2B). Whereas nearly all $GARP^+$ cells were Tregs in BM and spleen of $MPL^{WT}$ mice, Tregs only represented ±50% of $GARP^+$ cells in $MPL^{W508A}$ mice, with MKs representing the other ±50% (fig. S2A). Altogether, proportions of $GARP^+$ Tregs within live cells were reduced 2- to 3- fold, but proportions of $GARP^+$ MKs increased 20- to 30- fold in $MPL^{W508A}$ mice by comparison to controls (Fig. 1C). Increased proportions of MKs in BM and spleen were expected, as activation of the JAK/STAT pathway in PMF drives differentiation and proliferation of myeloid cells, notably MKs. Increased proportions of MKs, combined with very high expression of GARP per single MK (fig. S2B) most probably accounts for the observed increase in total GARP protein levels in BM and spleens of $MPL^{W508A}$ mice (Fig. 1B). As expected, all MKs were $GFP^+$, indicating that they originate from transformed cells expressing $MPL^{W508A}$, whereas Tregs were $GFP^-$, indicating that they mostly originate from non-transformed lymphoid progenitors (Fig. 1D).

Altogether, results above show that in mice undergoing $MPL^{W508A}$-induced PMF, GARP-expressing cells are present and GARP levels are increased in BM and spleen, as a consequence of increased proportions of tumoral $GARP^+$ MKs.

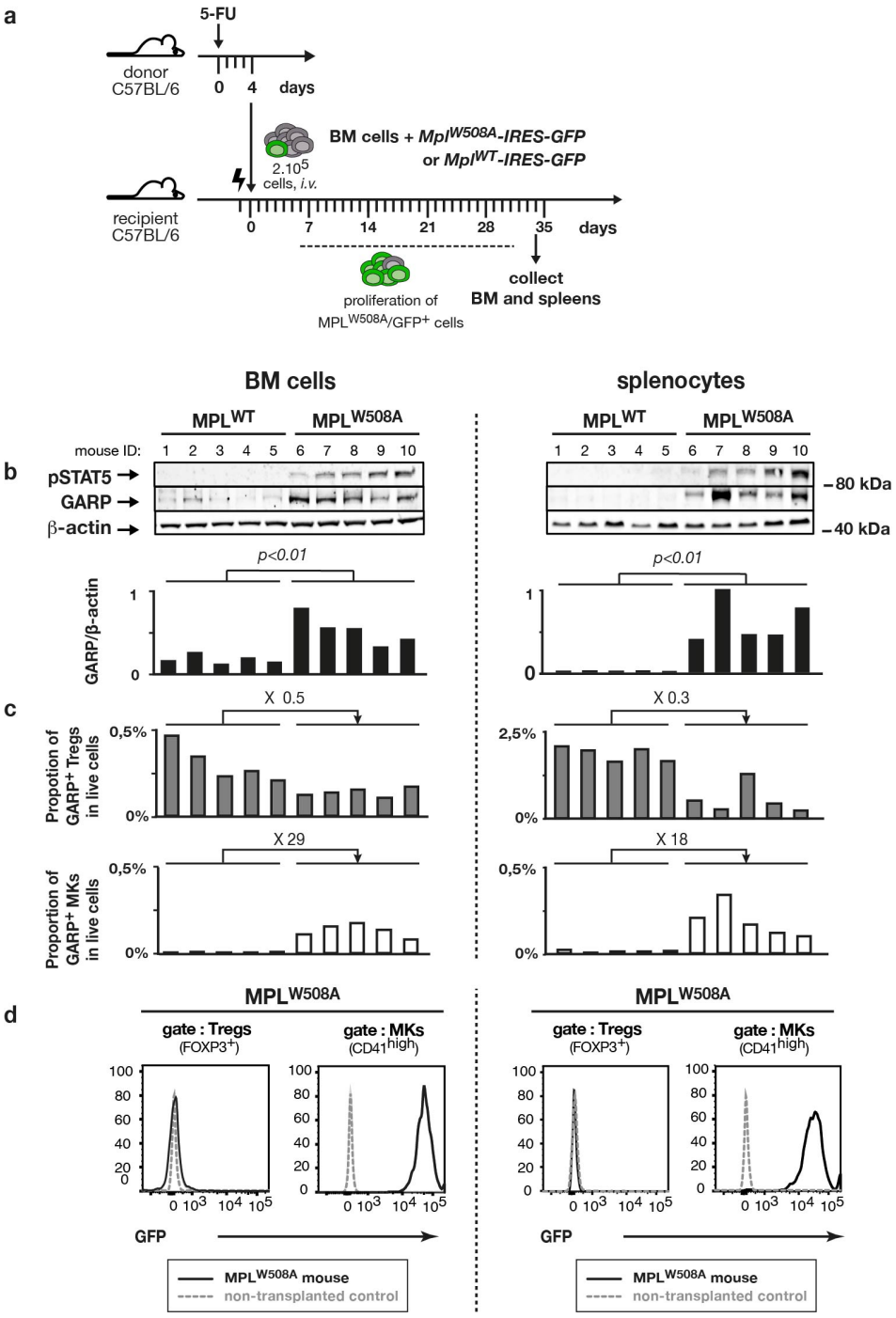
a
5-FU
donor C57BL/6
0 4 days
BM cells + Mpl^W508A-IRES-GFP
or Mpl^WT-IRES-GFP
2.10^5 cells, i.v.
recipient C57BL/6
0 7 14 21 28 35 days
collect BM and spleens
proliferation of MPL^W508A/GFP+ cells
BM cells
splenocytes
b
MPL^WT
MPL^W508A
mouse ID: 1 2 3 4 5 6 7 8 9 10
pSTAT5
GARP
β-actin
80 kDa
40 kDa
p<0.01
GARP/β-actin
c
X 0.5
X 0.3
Propotion of GARP+ Tregs in live cells
0,5%
2,5%
0%
X 29
X 18
Proportion of GARP+ MKs in live cells
d
MPL^W508A
gate : Tregs (FOXP3+)
gate : MKs (CD41high)
GFP
MPL^W508A mouse
non-transplanted control

**Fig. 1. GARP protein is increased in murine PMF and is expressed exclusively on MKs and Tregs.**

(**A**) Schematic representation of the experimental design. BM cells were isolated from 5-FU-treated donor mice, transduced with a retrovirus encoding $MPL^{W508A}$ or $MPL^{WT}$, and transplanted into lethally irradiated recipient mice (n=5 mice/group). BM cells and splenocytes were isolated from $MPL^{W508A}$ or $MPL^{WT}$ control mice 34 days after transplantation. (**B**) Western blot analyses of BM cells and splenocytes with antibodies against GARP, pSTAT5 or β-actin. Bar graphs show quantification of ECL signals. P values were calculated using a two-tailed Student t-test. (**C**) Proportions of $GARP^+$ Tregs and $GARP^+$ MKs in live single cells, as determined by flow cytometry. Fold differences between mean proportions in $MPL^{W508A}$ and $MPL^{WT}$ mice are indicated above bar graphs. (**D**) Histograms showing GFP expression on Tregs and MKs in BM and spleen from one representative $MPL^{W508A}$ mouse. Cells from a non-transplanted C57BL/6 mouse were used as negative controls. Experiment representative of at least 3 other independent experiments.

## A blocking anti-GARP:TGF-β1 mAb reduces tumor burden in mice with PMF

We next explored whether blocking TGF-β1 activation by GARP-expressing cells could exert a therapeutic activity in PMF. We used anti-GARP:TGF-β1 clone 58A2 (mouse IgG2a), which was shown to block TGF-β1 activation by mouse Tregs (*17*). We first verified that 58A2 also blocks TGF-β1 activation by mouse platelets, easier to isolate than MKs. Production of active TGF-β1 in mouse serum during blood coagulation *in vitro* was significantly reduced in the presence of 58A2 **(**fig. S3**)**. Next, we administered 58A2 to $MPL^{W508A}$ and $MPL^{WT}$ mice, via weekly *i.p.* injections starting one day before BM transplantation. By comparison to the mIgG2a isotype control, 58A2 reduced spleen and bone marrow fibrosis measured on day 34 in $MPL^{W508A}$ mice, although this did not reach statistical significance **(**Fig. 2A**)**. Unexpectedly, 58A2 significantly reduced tumor burden in $MPL^{W508A}$ mice: counts of total ($GFP^{+ \text{ and } -}$) platelets and white blood cells (WBC) were markedly reduced (Fig. 2B). This reduction was due to reduced numbers of tumoral $MPL^{W508A}/GFP^{+}$ cells, and notably $GFP^{+}$ neutrophils which accounted for the majority of the $GFP^{+}$ WBC in $MPL^{W508A}$ mice. Counts of non-transduced $GFP^{-}$ WBC, neutrophils and platelets were not reduced. GARP:TGF-β1 blockade by 58A2 did not reduce counts of non-tumoral $MPL^{WT}/GFP^{+ \text{ or } -}$ cells in $MPL^{WT}$ controls, which did not develop pathological leukocytosis or fibrosis (Fig. 2A, and B). In line with this, no reduction in WBC and platelet counts was observed in mice transplanted with non-transduced BM cells and treated with 58A2, by comparison to isotype control (fig. S4).

Altogether, our results suggest that anti-GARP:TGF-β1 reduces tumor burden in murine PMF by selectively killing or limiting the expansion of $MPL^{W508A}$ transformed cells.

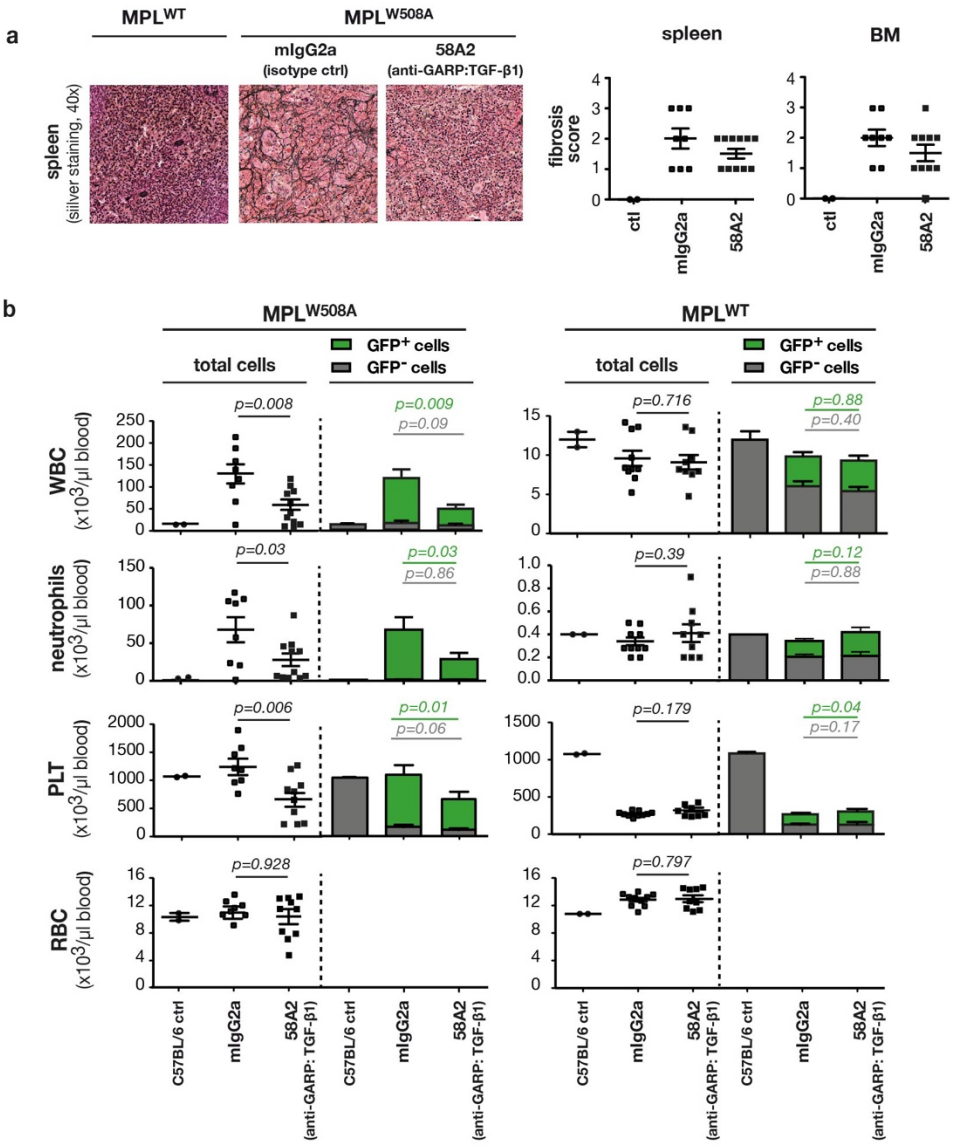
a
MPL^WT
MPL^W508A
mIgG2a
(isotype ctrl)
58A2
(anti-GARP:TGF-β1)
spleen
(silver staining, 40x)
spleen
BM
fibrosis score
ctl
mIgG2a
58A2
b
MPL^W508A
MPL^WT
total cells
GFP+ cells
GFP- cells
WBC (x10^3/µl blood)
neutrophils (x10^3/µl blood)
PLT (x10^3/µl blood)
RBC (x10^3/µl blood)
p=0.008
p=0.009
p=0.09
p=0.716
p=0.88
p=0.40
p=0.03
p=0.03
p=0.86
p=0.39
p=0.12
p=0.88
p=0.006
p=0.01
p=0.06
p=0.179
p=0.04
p=0.17
p=0.928
p=0.797
C57BL/6 ctrl
mIgG2a
58A2 (anti-GARP: TGF-β1)

**Fig. 2. Blocking anti-GARP:TGF-β1 mAbs reduce fibrosis and tumor burden in mouse PMF.** $MPL^{W508A}$ and $MPL^{WT}$ control mice, generated as indicated in Fig. 1, were injected i.p. weekly with the indicated mAbs, starting one day before transplantation of the transduced BM cells (n=8-10 mice per group). Non-transplanted C57BL/6 mice served as additional controls (ctrl). Blood was taken on day 28 and mice were sacrificed on day 34 to collect femurs and spleens. (**A**) Histological assessment of fibrosis in sections of paraffin-embedded spleens and femurs after Silver staining. Representative sections are shown on the left. Fibrosis scores, corresponding to reticulin staining grades expressed on a modified Bauermeister scale, are indicated on the right. Data points: scores in individual mice; horizontal bars: mean + SEM per group. (**B**) Blood cell counts and GFP expression were determined using a hematological analyzer and flow cytometry. Data points on the left : blood cell counts in individual mice; horizontal bars : mean + SEM per group. Stacked bar graphs on the right : mean + SEM of $GFP^+$ and $GFP^-$ cells. Data from 1 of at least 4 independent experiments. P values calculated using a two-tailed Student t-test.

## Reduced tumor burden in PMF does not require effector functions of anti-GARP:TGF-β1

58A2 could reduce tumor burden in PMF by killing tumoral GARP$^+$ cells *via* antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis (ADCP). To test this possibility, we used an Fc-dead version of 58A2 containing D265A/N297A substitutions in the Fc region that preclude binding to mouse FcgRs. We previously showed that Fc-dead 58A2 exerts anti-tumor activity against CT26 and MC38 tumors as efficiently as wild-type 58A2 (*17*). As shown in Fig. 3A**,** tumor burden and fibrosis in MPL$^{W508A}$ mice were reduced to similar levels upon treatment with wild type or Fc-dead 58A2, indicating that the therapeutic effects of anti-GARP:TGF-β1 in PMF did not require antibody binding to FcgRs, and thus did not imply killing of GARP$^+$ tumor cells by ADCC or ADCP.

## Reduced tumor burden may result from reduced cytostasis exerted by TGF-β1 on normal hematopoietic progenitors

Alternatively, 58A2 could reduce the cytostasis exerted by TGF-β1 on non-transformed hematopoietic progenitors, favoring their proliferation and reducing in turn the competitive growth advantage of malignant cells that resist to TGF-β1 cytostasis. Resistance to TGF-β1 cytostasis was suggested to favor transformed cell expansion in mouse and human PMF (*23-25*). We observed that expression of constitutively active MPL$^{W508A}$, but not MPL$^{WT}$, reduced the ability of recombinant TGF-β1 to suppress the proliferation of murine myeloid 32D cells *in vitro* (Fig. 3B). This was true using transduced 32D cells or clones, in the absence or presence of IL-3 or thrombopoietin (TPO), and similar observations were made with murine myeloid Ba/F3 cells transduced to express the constitutively active human MPL$^{W515K}$ mutant (Fig. 3B and fig. S5). Thus, our results show that constitutive activation of the JAK/STAT pathway by mutant MPL in myeloid cells induces resistance to the cytostatic effects of TGF-β1 *in vitro*. It remains to be determined whether this mechanism contributes to the therapeutic effect of GARP:TGF-β1 blockade in mouse or human PMF.

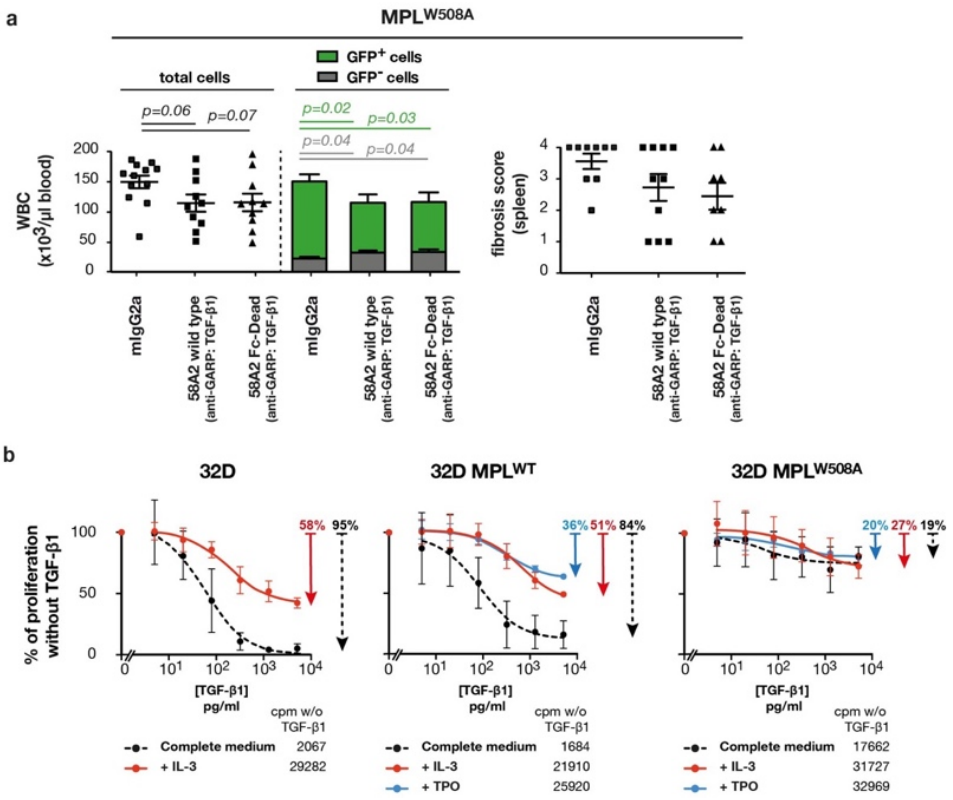


**Fig. 3. Reduced tumor burden and fibrosis in PMF do not require effector functions of anti-GARP:TGF-β1 mAbs.**

(**A**) MPL$^{W508A}$ mice were treated with wild-type or Fc-dead mAbs as indicated Fig. 2 (n=8-10 mice per group). Blood was taken on day 21 and mice were sacrificed on day 32 to collect femurs and spleens. Blood cell counts, GFP expression and spleen fibrosis were measured as indicated in Fig. 2. Data points: values in individual mice; horizontal lines: mean +/- SEM per group; stacked bars : mean + SEM. P values were calculated using a two-tailed Student t-test. (**B**) 32D cells were transduced with *Mpl$^{WT}$-IRES-GFP* or *Mpl$^{W508A}$-IRES-GFP* RV, and GFP$^+$ cells were sorted by flow cytometry. Parental 32D cells, sorted MPL$^{WT}$/GFP$^+$ or MPL$^{W508A}$/GFP$^+$ bulk cell populations or clones (fig. S6) were cultured in complete medium, in the absence or presence of recombinant IL-3, TPO or TGF-β1, as indicated in the graphical legend. Proliferation was measured in $^3$H-thymidine incorporation assays. cpm w/o rTGF-β1: counts per minute in the absence of rTGF-β1. Maximum growth inhibition (%) in the presence of rTGF-β1 is indicated in colour font on the right and was calculated as follows: (cpm w/o rTGF-β1 - cpm at the highest concentration of rTGF-β1) / (cpm w/o rTGF-β1). Data points: mean cpm +/- SD (triplicates). Results representative of 2 independent experiments.

## Targeting GARP:TGF-β1 complexes on Tregs reduces tumor burden in mouse PMF

Tregs and MKs represent the vast majority of GARP-expressing cells in spleen and BM of mice undergoing PMF. To define which GARP-expressing cells need to be targeted by anti-GARP:TGF-β1 to observe therapeutic effects, we induced PMF in mice carrying a Treg- or an MK/platelet-specific deletion of the *Garp* gene (*17*).

In preliminary experiments with UBI-GFP BM donor cells, 100% of MKs and platelets ($CD41^+$) were of donor origin as early as 19 days after transplantation. However, more than 50% Tregs ($CD4^+GARP^+$) were still of recipient origin on day 36, when full reconstitution was achieved (fig. S6A to C). This is due to the known resistance of T cells to irradiation, by comparison to B cells and myeloid cells ((*26*) and fig. S6D). Thus, to ensure that all Tregs (donor and recipient origin) carry a deletion of *Garp* in $MPL^{W508A}$ mice, BM cells from $Foxp3^{Cre}$ x $Garp^{fl/fl}$ mice were transduced with $Mpl^{W508A}$-*IRES*-*GFP* and transplanted into irradiated $Foxp3^{Cre}$ x $Garp^{fl/fl}$ recipients. PMF in these mice was compared to that induced by transplanting transduced BM cells from $Foxp3^{Cre}$ x $Garp^{wt/wt}$ donors into $Foxp3^{Cre}$ x $Garp^{wt/wt}$ recipients. Similar to what was observed by blocking GARP:TGF-β1 with a mAb, fibrosis and most significantly counts of transformed $MPL^{W508A}/GFP^+$ WBC were reduced in the absence of GARP:TGF-β1 on Tregs (Fig. 4A).
Next, we transplanted transduced BM cells from MK/platelet-specific *Garp* KO [Tg($Pf4^{Cre}$) x $Garp^{fl/fl}$] or WT littermates ($Garp^{fl/fl}$) into WT recipients ($Garp^{fl/fl}$). Same WT recipients could be used in the two conditions because all MKs and platelets are from donor origin very early after transplantation. In contrast to observations with Treg-specific *Garp* KOs, tumor burden was not reduced in $MPL^{W508A}$ mice transplanted with MK/platelet-specific *Garp* KO cells, by comparison to WT (Fig. 4B).

Altogether, our results suggest that anti-GARP:TGF-β1 mAb reduce tumor burden in PMF by blocking GARP:TGF-β1 on Tregs, but not on MKs or platelets. This was unexpected. Whilst a role for MKs in PMF pathogenesis is well established (*27, 28*), a role for Tregs has not been reported. A plausible hypothesis to explain our

observations is that Tregs suppress anti-tumor T cell responses in PMF, as they do in many types of human or mouse cancers, and this immunosuppression is relieved at least in part by GARP:TGF-β1 blockade.

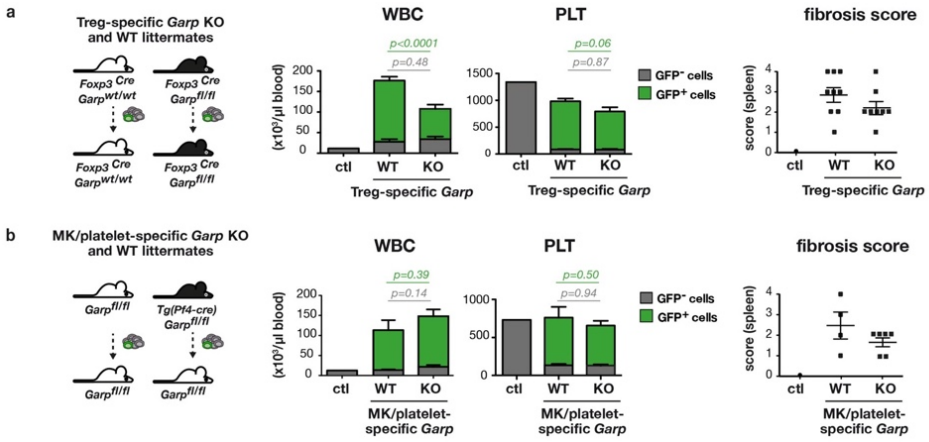


**Fig. 4. Absence of GARP:TGF-β1 complexes on Tregs, but not on MKs, reduces fibrosis and tumor burden in PMF mouse model.**

(**A**) BM cells were isolated from 5-FU-treated *Foxp3*$^{Cre}$ *x Garp*$^{wt/wt}$ mice or *Foxp3*$^{Cre}$ *x Garp*$^{fl/fl}$ mice, transduced with a retrovirus encoding MPL$^{W508A}$, and transplanted into irradiated recipient mice of the same genotype as the donor mice (n=8-9 mice per group). Non-transplanted C57BL/6 mouse were used as controls. Blood was taken on day 28 and mice were sacrificed on day 34 to collect femurs and spleens. Blood cell counts, GFP expression and spleen fibrosis were measured as indicated in Fig. 2. Data points: values in individual mice; horizontal lines: mean +/- SEM per group; stacked bars : mean + SEM. P values were calculated using a two-tailed Student t-test. (**B**) Same as in (**A**), except that BM cells were isolated from *Garp*$^{fl/fl}$ or *Tg(Pf4*$^{Cre}$*) x Garp*$^{fl/fl}$ mice and transplanted into irradiated *Garp*$^{fl/fl}$ recipients (n=4-6 mice per group).

## GARP:TGF-β1 blockade during PMF increases IFNg signals in spleen

We used RNA sequencing (RNAseq) to confirm the effects of 58A2 on tumor burden and fibrosis, and tested the hypothesis that GARP:TGF-β1 blockade could relieve suppression of T cell-mediated anti-tumor immunity by Tregs.

RNAseq of spleens collected from non-treated $MPL^{W508A}$ mice on days 13 and 20 were compared to establish signatures of PMF progression. RNAseq of spleens collected on day 34 from $MPL^{W508A}$ mice responding to 58A2 were compared to non-treated controls to analyse effects of treatment with 58A2 (Fig. 5A). We performed Gene Set Enrichment Analysis (GSEA, (*29*)) of genes ordered according to fold-change expression during PMF progression (day 20 *vs* 13 in controls), or according to fold-change expression in response to treatment (58A2 responders *vs* controls on day 34).

As shown in Fig. 5B (white bars), 5 myeloid cell-specific gene signatures from the PanglaoDB (*30*) and a signature of mouse myelofibrosis (*31*) showed a significant positive enrichment during PMF progression (ES > 0.4 and FDR <5%). The same signatures were negatively enriched in 58A2 responders (ES < -0.4 and FDR < 5%; black bars in Fig. 5B). Thus, GSEA detects progression of tumor burden and fibrosis between day 13 and 20 in non-treated controls, and reduced tumor burden and fibrosis on day 34 in responders *vs* controls.

We next used the 50 hallmark signatures from the MSigDB database (*32*) to search for mechanisms of actions by which 58A2 could exert this anti-tumor activity. Five and six hallmark signatures were positively or negatively enriched in 58A2 responders, respectively. Reverse enrichments were observed during PMF progression in controls (Fig. 5B). Signatures that were negatively enriched in responders comprised hallmarks of platelet components or functions (“coagulation”), fibrotic process (“EMT”), and inflammation (“inflammatory response” and “IL6 JAK STAT3 signaling”), again likely reflecting reductions in tumor burden, fibrosis or other

PMF features such as inflammation in response to treatment (*28, 33*). Less expected were the hallmarks of IFNa and IFNg signaling, which were positively enriched in responders (Fig. 5B). Genes composing the IFNa and IFNg signatures partially overlap, but 43% of the genes in the leading edge of the IFNg signature are not present in the IFNa signature (Fig. 5C). Levels of IFNg- and/or IFNa- induced mRNAs inversely correlated with $MPL^{W508A}/GFP^{+}$ WBC counts, suggesting an association between increased interferon signaling and decreased tumor burden (Fig. 5D).

Finally, negative enrichments for signatures of TGF-β1 signaling in $CD4^{+}$ T cells and fibroblasts (*13, 34*) were also found in responders, even though they did not reach statistical significance (FDR = 7,6% and 10,6 %, respectively) (Fig. 5B).

Altogether, analyses of transcriptional profiles in murine PMF confirmed reduced disease burden in response to GARP:TGF-β1 blockade, but also suggested that therapeutic activity of 58A2 is associated with reduced TGF-β1- and increased interferon- signals. Reduced TGF-β1 activity was expected as 58A2 blocks TGF-β1 activation by GARP-expressing cells such as Tregs. Increased interferon signals may indicate increased T cell activity resulting from reduced Treg immunosuppression.

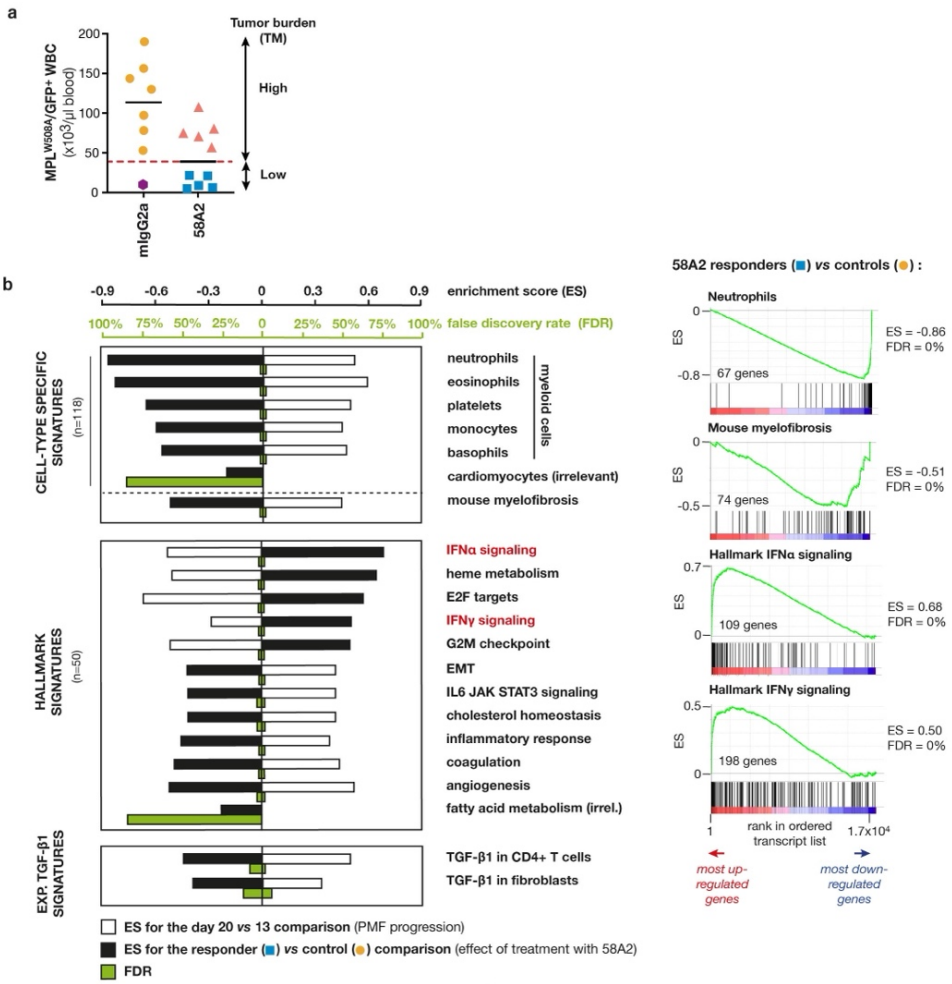
a
Tumor burden (TM)
High
Low
MPL^W508A/GFP+ WBC (x10^3/µl blood)
mIgG2a
58A2
b
enrichment score (ES)
false discovery rate (FDR)
CELL-TYPE SPECIFIC SIGNATURES (n=118)
neutrophils
eosinophils
platelets
monocytes
basophils
myeloid cells
cardiomyocytes (irrelevant)
mouse myelofibrosis
HALLMARK SIGNATURES (n=50)
IFNα signaling
heme metabolism
E2F targets
IFNγ signaling
G2M checkpoint
EMT
IL6 JAK STAT3 signaling
cholesterol homeostasis
inflammatory response
coagulation
angiogenesis
fatty acid metabolism (irrel.)
EXP. TGF-β1 SIGNATURES
TGF-β1 in CD4+ T cells
TGF-β1 in fibroblasts
ES for the day 20 vs 13 comparison (PMF progression)
ES for the responder (■) vs control (●) comparison (effect of treatment with 58A2)
FDR
58A2 responders (■) vs controls (●) :
Neutrophils
ES = -0.86
FDR = 0%
67 genes
Mouse myelofibrosis
ES = -0.51
FDR = 0%
74 genes
Hallmark IFNα signaling
ES = 0.68
FDR = 0%
109 genes
Hallmark IFNγ signaling
ES = 0.50
FDR = 0%
198 genes
rank in ordered transcript list
most up-regulated genes
most down-regulated genes
c
Leading edge of the IFNγ signaling signature :
(68 out of 198 genes in the signature)
overlap with the IFNα signature (57%)
58A2 responders
mIgG2a controls
d
R2 = 0,60
Irf7 (read count)
R2 = 0,55
Nlrc5 (read count)
GFP+ WBC (10^3/µl blood)

**Fig. 5. Anti-GARP:TGF-β1 mAb induce an IFNγ signature in mouse PMF.**

**(A**) $MPL^{W508A}/GFP^{+}$ WBC counts on day 34 in mice from experiment in Fig. 2. Data points: individual mice; horizontal lines: median + SEM per group. $MPL^{W508A}$ mice responding to 58A2 (n=5, blue symbols) are defined as mice with a low tumor burden (cut-off: median $MPL^{W508A}/GFP^{+}$ WBC counts in the 58A2 group). Controls correspond to non-treated (mIgG2a) $MPL^{W508A}$ mice with a high tumor burden (n=7, orange symbols). (**B**) GSEA of RNAseq data from spleens collected from $MPL^{W508A}$ mice on days 13, 20 or 34. White bars: enrichment score (ES) of the indicated signatures in the list of transcripts ordered by mean fold-change between day 20 and 13 in non-treated controls. Black bars: ES in transcripts ordered by mean fold-change between 58A2 responders and non-treated controls. Green bars: False Discovery Rate (FDR) obtained by calculating ES for 1000-gene-set permutations. Right panel shows representative GSEA plots, with black vertical bars indicating position of genes from various gene sets in the ordered transcript list for the responder vs control comparison. (**B**) Heatmap representation of the expression of genes in the leading edge of the IFNγ response signature. Each row represents a gene and each column a mouse. Black boxes on the right indicate genes that are also present in IFNα response signature. (**D**) Correlation between expression of IFN-induced genes by RNAseq analysis and number of $MPL^{W508A}/GFP^{+}$ WBC cells in all mice.

## Therapeutic efficacy of anti-GARP:TGF-β1 mAbs in PMF requires CD8⁺ T cell function

We previously observed in CT26-bearing mice that blocking GARP:TGF-β1 on Tregs increased IFNg production by anti-tumor CD8⁺ T cells (*17*). To test the involvement of CD8⁺ T cells in the therapeutic activity of GARP:TGF-β1 blockade in PMF, we depleted these cells by repeated injections of an anti-CD8α mAb (Fig. 6A). CD8⁺ T cell depletion abolished the reduction of MPL$^{W508A}$/GFP⁺ neutrophil counts obtained with 58A2 (Fig. 6B). It had no effect in mice that did not receive 58A2, which was expected because PMF develops very rapidly in this model, leaving little opportunity for spontaneous anti-tumor CD8 T cells to control the disease. Thus, therapeutic efficacy of 58A2 in murine PMF requires CD8⁺ T cells.

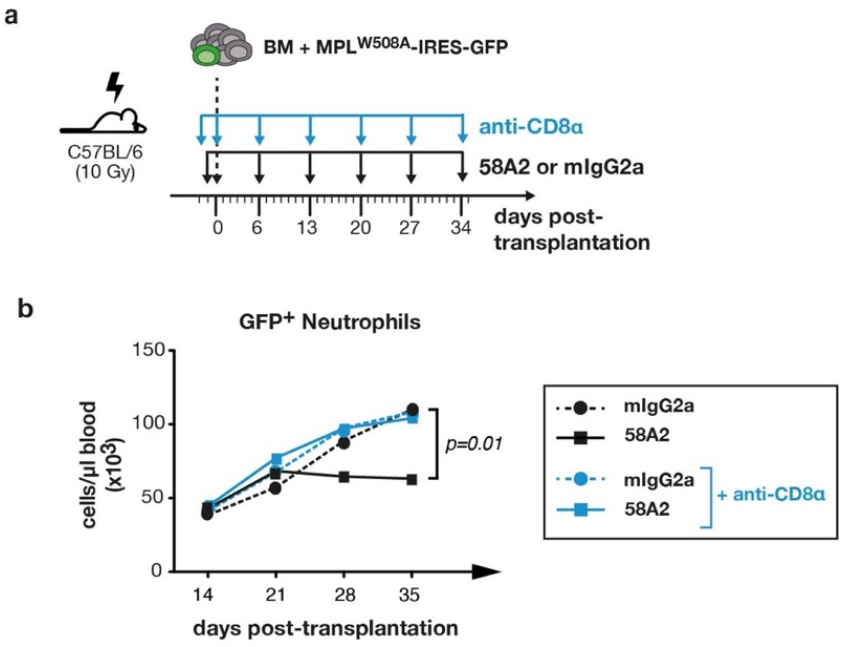


**Fig. 6. Therapeutic efficacy of anti-GARP:TGF-β1 mAb in mouse PMF requires T cell function.**

(**A**) Recipient mice were transplanted with transformed MPL$^{W508A}$/GFP⁺ cells and injected i.p. weekly with 58A2 and/or anti-CD8α mAbs at the time points indicated. Blood samples were collected weekly starting on day 14 for blood cell count and flow cytometry. Mice were sacrificed at

day 35. (**B**) Counts of $GFP^+$ blood neutrophiles during PMF progression. Data points: mean values per group (n=6-8 mice per group). P values were calculated with a repeated measure ANOVA.

## GARP:TGF-β1 blockade reduces tumor burden in another murine model of myeloproliferative neoplasm

We tested whether 58A2 could exert anti-tumor activity in a non-PMF model of myeloproliferative neoplasm. BM cells from mice carrying a heterozygous $JAK2^{V617F}$ knock-in mutation in hematopoietic stem cells (*35*) were mixed at a 1-to-1 ratio with BM cells from UBI-GFP mice and transferred into irradiated UBI-GFP recipients (fig. S7A). Recipients developed a moderate form of myeloproliferative neoplasm that resembles more polycythemia vera than PMF, and includes mild erythrocytosis, thrombocytosis and leukocytosis. 58A2 reduced WBC counts in recipient mice, mostly in $JAK2^{V617F/WT}$ ($GFP^-$) neutrophils (fig. S7B). Counts of other $JAK2^{V617F/WT}$ myeloid cells including monocytes, eosinophils and basophils were also reduced, although this did not reach statistical significance. Counts of $JAK2^{WT/WT}$ myeloid cells and $JAK2^{V617F/WT}$ or $JAK2^{WT/WT}$ lymphocytes were not modified (fig. S7C). These results suggest that GARP:TGF-β1 blockade may also reduce tumor burden in non-PMF myeloproliferative neoplasms.

## Discussion

The effects of GARP:TGF-β1 blockade on tumor burden in murine PMF are the most important results of this report. They were completely unexpected, as our hypothesis was that GARP:TGF-β1 blockade would reduce fibrosis owing to the previously reported pro-fibrotic roles of TGF-β1 in PMF (*8-11*). We repeatedly observed reductions in spleen and bone marrow fibrosis, but it did not reach statistical significance and may be secondary to the significant tumor burden reduction in response to treatment. Thus, although we do not exclude a direct pro-fibrotic role for TGF-β1 in PMF, our data suggest that TGF-β1 exerts deleterious effects by favoring clonal expansion of transformed cells. In homeostatic conditions, TGF-β1 reduces hematopoiesis. It imposes cytostasis on normal BM progenitors, maintaining them in a state of quiescence to prevent exhaustion (*36-39*). Recent studies suggested that in myeloproliferative neoplasms, malignant cells fail to respond to the cytostatic activity of TGF-β1, which could even promote their proliferation, either directly or competitively by decreasing the growth of normal hematopoietic stem cells (*24, 25*). In these studies, the clonal dominance of $JAK2^{V616F}$ cells over WT cells in competitive transplantation assays was abrogated by genetic deletion of the TGF-βRII receptor in WT cells, but not $JAK2^{V616F}$ cells (*25*). Here, we observed that expression of mutant $MPL^{W508A}$ in murine myeloid cells induced resistance to the cytostatic effect of TGF-β1. Thus, the anti-tumor activity of GARP:TGF-β1 blockade in PMF could result from reactivation of normal hematopoiesis and competition with transformed cells. A direct anti-proliferative effect of GARP:TGF-β1 blockade on transformed cells, *via* a mechanism to be determined, cannot be excluded.

A second unexpected result is that tumor burden reduction in response to GARP:TGF-β1 blockade depends on GARP on Tregs but not on tumoral MKs or platelets. Given their known pathogenic role in PMF, MKs were considered as the most likely source of pathogenic TGF-β1 in this disease (*10, 40-42*). Our data however, comparing Treg- and MK/platelet-specific *Garp* KO mice, suggest that

TGF-β1 presented and activated by GARP on the Treg surface plays a more important role than MK/platelet-derived TGF-β1 in PMF. Tregs are potent suppressors of adaptive immunity and are known to exert detrimental immunosuppression in patients with solid cancer (*43, 44*). Immunosuppression by Tregs is mediated at least in part by the ability of Treg-derived TGF-β1 to inhibit the effector functions of CD8 T cells, which include IFNg production and cytolysis. Strikingly here, we observed that the therapeutic activity of anti-GARP:TGF-β1 in PMF was accompanied by increased IFNg signals, and was lost upon CD8 T cell depletion. Thus, in addition to limiting the clonal expansion of transformed cells, blocking TGF-β1 activation could reduce immunosuppression by Tregs, and thus increase the activity of CD8 T cells directed against transformed cells in PMF. Whether T lymphocytes can recognize and kill transformed cells in murine or human PMF has not been established. Improved survival upon PD-1 blockade in murine myeloproliferative neoplasms suggests the existence of an anti-tumor T cell activity (*45*), and clinical trials with antibodies blocking the CTLA-4 or PD-1 inhibitory pathways are underway (ClinicalTrial.gov NCT02871323, NCT03065400, NCT01822509). Recent studies identified T cells against mutated CALR in the blood of patients carrying a *CALR* mutation (*46-48*), and several MHC class I binding peptides were identified in mutated CALR and MPL proteins (*49*). Whether Tregs suppress the activity of the putative anti-tumor T cells is not known. Our results suggest it could be the case, at least in murine PMF.

Therapeutic approaches to block TGF-β signals are tested in early phase clinical trials in patients with myeloproliferative neoplasms. Current approaches either neutralize the activity of all three TGF-β1, -β2 and -β3 isoforms, or block TGF-β signals regardless of the cellular source. Anti-GARP:TGF-β1 mAbs, by selectively blocking TGF-β1 production from GARP-expressing cells such as Tregs, could represent an interesting alternative, less toxic but as efficient as broad blockade of all TGF-β activity.

Altogether, our results suggest that selective blockade of TGF-β1 activation by Tregs with anti-GARP:TGF-β1 mAbs could be a novel immunotherapeutic approach in patients with myeloproliferative neoplasms.

# Methods

## Mice

WT C57BL/6J mice, *Vav*$^{Cre}$ x *JAK2*$^{L2/WT}$ mice, Tg(*Pf4*$^{Cre}$) x *Garp*$^{fl/fl}$ mice and Foxp3$^{Cre}$ x Garp$^{fl/fl}$ mice were bred at the SPF animal facility of the UCLouvain. Tg(*Pf4*$^{Cre}$) x *Garp*$^{fl/fl}$ mice and Foxp3$^{Cre}$ x Garp$^{fl/fl}$ mice (C57BL/6 background) were kindly provided by Wim Maes (IRF Life Sciences, KULeuven). UBC-GFP mice (C57BL/6-Tg(UBC-GFP)30Scha/J) were purchased from Jackson Laboratory. Male mice (7- to 8-week-old) were used as donors and female mice (7- to 8- week-old) were used as recipients. All animal studies were performed in accordance with national and institutional guidelines for animal care, under permit numbers 2017/UCL/MD/19 at the UCLouvain.

## Retrovirus production and titration

For retroviruses production, 293 EBNA cells ($2{,}5x10^6$) were seeded in a 10-cm dish the day before the transfection and then transfected with retroviruses pMegix-MPL$^{W508A}$-IRES-GFP or pMegix-MPL$^{WT}$-IRES-GFP together with packaging plasmids. 48h after transfection, viral supernatant was collected and store at 4°C until the next day. Fresh medium was added on cells for an additional 24h before to be collected. Supernatants collected at both time points were pooled and concentrated using Centricon Plus 70 (Merck Millipore, UFC703008). Concentration factors varied between 60 and 100. Concentrated viruses were stored in liquid nitrogen until use.

For retroviruses titration, 293T cells ($2{,}5x10^5$) were seeded in a 8-well plate. The next day, cells were counted, concentrated viruses were added on cells (dilution 10 by 10) and spin-infection was performed at 300 x *g* for 2 hours at 37°C in presence of Polybrene (8 µg/ml). Cells were then incubated for 48 hours, in presence of the viruses. Retrovirally transduced cells were finally collected, washed and analyzed by flow cytometry for GFP expression. Virus titer was defined as the number of

transducing units per ml of virus, corresponding to the number of GFP-positive cells per ml of virus.

## Mouse experiments

All animal studies were performed in accordance with national and institutional guidelines for animal care, under permit numbers 2017/UCL/MD/019 and 2019/UCL/MD/032 at the UCLouvain.

For the $MPL^{W508A}$ model, femurs and tibiaes were collected from donor mice 4 days after 5-fluorouracil (5FU) injection (3 mg, *i.p.*). 5FU is used to increase the proportion of cycling hematopoietic stem cells (HSCs) in the BM. BM cells were flushed with a 21-gauge needle under sterile conditions, filtered through a 70µm nylon filter and lyzed for red blood cells. Cells were prestimulated for 24 hours in $\alpha$-minimal essential medium (Gibco # 32561-029) containing 5 recombinant cytokines (mu-FLT3-L, mu-SCF, mu-IL3, mu-IL6, muTpo ; 10 ng/ml all). All cytokines were purchased from Miltenyi. Retrovirus encoding either $MPL^{WT}$ or $MPL^{W508A}$ was added on prestimulated cells (ratio 5:1, resp.) and spin-infection was performed at 300 x *g* for 2 hours at 37°C in presence of Polybrene 8 µg/ml. Cells were then incubated for 48 hours, in presence of the virus and of the 5 recombinant cytokines. Retrovirally transduced cells were collected, washed and injected (*r.o.*) into lethally irradiated recipient mice (2x500 cGy) at $2x10^5$cells/mouse. Recipient mice receiving $MPL^{W508A}$-transduced BM cells ($MPL^{W508A}$ mice) developed leukocytosis and fibrosis with 2 to 4 weeks. Recipient mice receiving $MPL^{WT}$-transduced BM cells ($MPL^{WT}$ mice) served as controls.

For transplantation with untransduced BM cells, BM cells were isolated from donor mice without prior 5-FU injection and transplanted directly into lethally irradiated recipient mice (2x500 cGy). $2x10^6$ BM cells from donors were transplanted by retro-orbital injection in each recipient.

For experiments using $Vav^{Cre}$ x $JAK2^{L2/WT}$, a competitive transplantation setting has been used. BM cells from 14-week-old donor male mice ($Vav^{Cre}$ x $JAK2^{L2/WT}$ and

UBC-GFP) have been isolated without prior 5-FU injection. BM cells have then been mixed following a 1:1 ratio and transplanted into lethally irradiated UBC-GFP female recipients ($3x10^6$ BM cells/mice by retro-orbital injection).

As indicated in the figures, mice received weekly intraperitoneal (*i.p.)* injections of isotype control (motavizumab, mIgG2a) or anti-GARP:TGF-β1 (clone 58A2, mIgG2a WT or mIgG2a FcDead) mAbs (400 µg/mouse), starting from the day before the transplantation. For in vivo $CD8^+$ T cells depletion, recipient mice were injected *i.p.* two days before and the day of the transplantation with 500 µg of anti-mouse CD8α mAb (Bio X Cell, clone 2.43). The injections were then repeated weekly (250 µg/mouse) until the end of the experiment.

Peripheral blood cell count and flow cytometry analyses were performed on blood during and at the end of the experiment to monitor the development of the disease. At the end of the experiment, mice were sacrificed to take spleen and femurs for further analyses (histopathology, Western Blot, flow cytometry, RNA sequencing).

## Peripheral blood cell counts

Blood (80µl/sampling) was collected with Minivette® (Sarstedt #17.2113.050) from the end of the tail, then placed into EDTA-coated Microvette® (Sarstedt #20.1341). Peripheral blood cell counts were performed on Melet Schloesing MS9-5 hematological analyzer.

## Histopathology

Femurs and spleens were collected at the end of the experiment, fixed in formaldehyde (4%) and paraffin embedded. Femurs were decalcified during 1 hour (VWR, 09128300) before paraffinization. Sections of paraffin-embedded organs (5µm) were cut with microtome, and reticulin fibers were stained with silver staining kit according to the Gordon & Sweets (Sigma, 1002510001). Fibrosis scores were defined according to modified Bauermeister scale (*50*).

## Flow Cytometry

Blood cells, total splenocytes and bone marrow cells were analyzed by flow cytometry for GFP expression and, in some cases, GARP protein, lymphoid markers and myeloid markers expression. Cells were isolated from mice and prepared as single-cell suspension as the following. Spleens were mashed in 2 mL ID + 5%FBS + AJ, and then filtered through a 70µm nylon filter. Bone marrow cells were flushed with a 21-gauge needle from femurs and tibiae and then also filtered through a 70µm nylon filter. Red blood cell lysis was performed on blood, splenocytes and bone marrow cells. Cells are finally pelleted and resuspended in PBS containing 2 mM EDTA and 1% FCS for immediate staining.

Cells were first incubated with anti-CD16/32 to block FcγRs, then with antibodies against surface markers (CD4, CD8$\alpha$, B220, CD41, Ly-6G, CD11b, GARP ; Biolegend) in the presence of a viability dye (eBioscience). For intra-cellular staining, cells were fixed and permeabilized using FOXP3/ Transcription Factor Staining Buffer set (eBioscience) during 20h at 4°C, then stained with anti-FOXP3 in the presence of anti-CD16/32. Analyses were performed on a FACS LSR Fortessa flow cytometer (BD Biosciences) and data were computed using the FlowJo software (Tree Star).

## Western Blots

BM cells and total splenocytes were isolated from mice at the end of the experiment. Cells were lyzed in 2X Laemmli sample buffer, passed through a 25-gauge needle and then incubated for 5 minutes at 100°C. Samples ($10^5$ equivalents/well) were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4-12% Bis-Tris BOLT gel ; Thermo Fisher Scientific) under reducing conditions. Gels were blotted on nitrocellulose membranes with the iBlot system (Thermo Fisher Scientific). After blocking, membranes were incubated with primary antibodies directed against pSTAT5 (Cell Signaling #9359, 1:1000), GARP (R&D Systems #AF6229 ; 1:500) or $\beta$-actin (Sigma #A5441, 1:10000), then with secondary HRP-coupled antibodies, and revealed finally with an ECL substrate (Thermo Fisher

Scientific). ECL signals were detected with Fusion SOLO 4S device and quantified with Bio1D software.

## Generation of cell lines and cloning

Ba/F3 and 32D cell line was maintained in RPMI medium, supplemented with 10% fetal bovine serum and mu-IL-3. Ba/F3 and 32D cell lines stably expressing $MPL^{WT}$ or mutants were generated by retroviral transduction of the adequate transgene, as described above for murine BM cells (pMegix-IRES-GFP containing construct). After 48h, population of cells expressing green fluorescent protein (GFP) were purified by fluorescence-activated cell sorting (BD FACSAria). Further cloning was performed by single cell sorting in 96-well culture plate. Surface expression of the HA-tagged transduced MPL and GFP positivity were validated for each selected clone by flow cytometry.

## Cell proliferation assay

In $^{3}$H-thymidine incorporation assays, Ba/F3 or 32D cells stably expressing wild type or mutated MPL were washed twice in RPMI. 8000 cells were seeded by triplicate in a 96-well plate in RPMI, supplemented with 10% FBS and the indicated growth factor. IL-3 was used at 125 pg/ml and TPO at 2.5 ng/ml. After 24 h, $^{3}$H-thymidine (0.5 µCi/well, GE Healthcare) was added for 4h. Cells were then harvested, and the incorporation of $^{3}$H-thymidine was quantified using a scintillation counter (Packard TopCount scintillation counter).

## RNAseq analysis on spleen tissue samples

Mouse spleens were collected and stored at -80°C until processing. After spleen disruption using Ultra Turrax (Ika), total RNA was extracted using NucleoSpin RNA kit (Macherey Nagel, 740955.50) and RNA quality was verified with an Agilent Bioanalyzer. Sequencing was outsourced to Macrogen and performed using Illumina SBS technology with the following requirement: paired-end reads and 30M reads/sample. Sequences were aligned to the GRCm38 mouse genome using the

STAR software, and read counts were determined with the HTSeqCount software. Raw read counts were normalized using the DESeq2 package (*51*) and the R software. For RNAseq analyses, 58A2-treated mice were considered as "Responders" if their $MPL^{W508A}/GFP^{+}$ WBC counts was inferior to the median WBC count in the group (median: $3.8x10^4$ /µl). Non-responders had $MPL^{W508A}/GFP^{+}$ WBC counts similar to that of the isotype control group. They were excluded to avoid biased effect sizes and identify transcriptional changes associated with a measurable therapeutic effect. Gene Set Enrichment Analyses (GSEA) were performed on normalized read counts using the GSEA Software (*29*). Molecular Signature Database v7.2 (MSigDB) was used for hallmark signatures (*32*) and Panglao Database for cell-type signatures (*30*). Of the 178 existing cell-type signatures in Panglao database, 60 were excluded by GSEA software due to small gene set size (size < 15). Mouse fibrosis signatures was finally extracted from published results (*31*).

## Serum generation assay

WT C57BL/6J mice were anesthetized and blood was withdrawn by retro-orbital puncture using uncoated capillaries (Hirschmann Laborgeräte, #9000110). Blood was collected in normal Eppendorf tubes and mixed with either anti-GARP:TGF-β1 mAb (clone 58A2) or an isotype control (motavizumab), at a final concentration in the blood of 340µg/ml. Blood was left to coagulate for 1 hour at room temperature, and then centrifuged at 12000 g for 30 minutes at 4°C. Supernatant (serum) was collected and its content in active TGF-β1 was measured by ELISA the same day according to manufacturer instructions (ThermoFisher, 88-8350-88).

## Statistical analyses

All of the statistical analyses were performed using GraphPad Prism software (version 9). Differences between the groups were compared using unpaired two-tailed Student's t test for blood cell counts, and unpaired two-tailed Mann-Whitney test for fibrosis and active TGF-β1 quantification in serum by ELISA. All tests were evaluated at a statistical significance of 0.05.

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## Author contributions

SLe, JD, ND, CP performed animal and molecular and cellular biology experiments. SLe, GDS, NVB, CVanhaver and CVanderaa performed bioinformatics analyses. SLe, JD and SLu conceived the study. SLe, JD, PGC, SNC and SLu analyzed results and wrote the manuscript.

## Conflicts of interest

Patents pertaining to blocking antibodies against human GARP:TGF-β1 have been filed under the Patent Cooperation Treaty (International application Number PCT/IB2019/053753), with Sophie Lucas and Pierre G. Coulie as inventors and UCLouvain as applicant. The anti-mouse GARP:TGF-β1 antibody (clone 58A2) used

herein is a surrogate for these antibodies. All other authors declare no competing interests.

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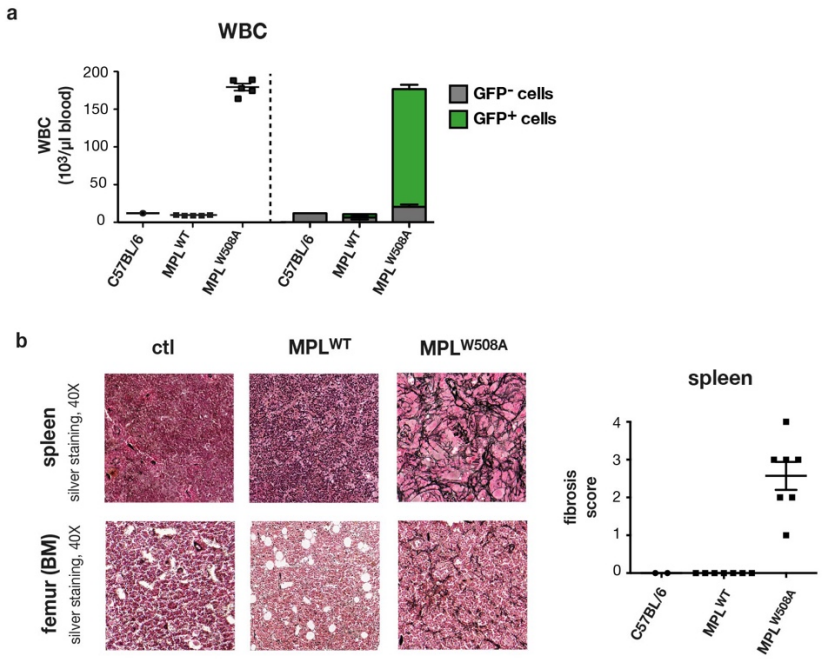
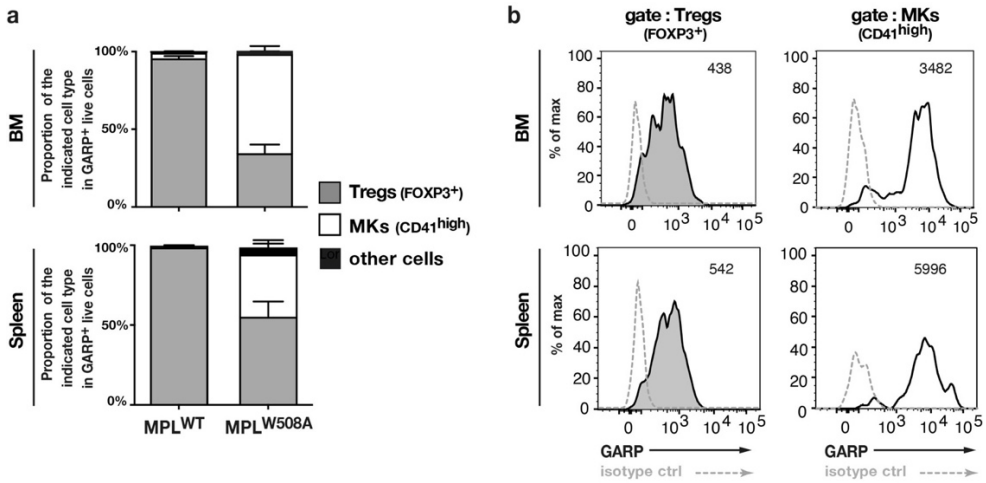
# RESULTS

# Supplementary Figure

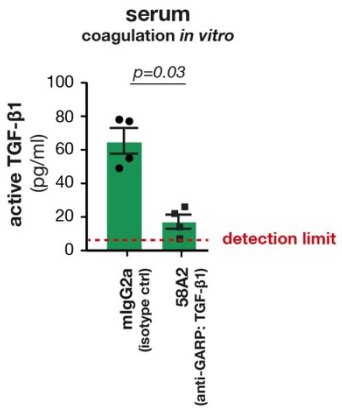



**Supplementary Figure 1. Mice transplanted with BM cells overexpressing MPL$^{W508A}$ develop leukocytosis, BM fibrosis and spleen fibrosis.**

MPL$^{W508A}$ and MPL$^{WT}$ control mice were generated as indicated in Fig. 1. Non-transplanted C57BL/6 mice were used as additional controls. Blood was taken on day 28 after transplantation of transduced BM cells to determine various blood cell counts and analyse GFP expression by flow cytometry. Mice were sacrificed on day 32 to collect femurs and spleens. (**A**) Counts of total, GFP$^+$ and GFP$^-$ blood cells, determined using a hematological analyzer and flow cytometry. Data points on the left : counts in individual mice; horizontal bars : mean + SEM per group. Stacked bar graphs on the right : mean + SEM of GFP$^+$ (green) and GFP$^-$ (grey) cells. (**B**) Histological assessment of fibrosis in sections of paraffin-embedded spleens and femurs after Silver staining. Representative sections are shown on the left. Fibrosis scores, established according to a modified Bauermeister scale, are indicated on the right. Data points: scores in individual mice; horizontal bars: mean + SEM per group.

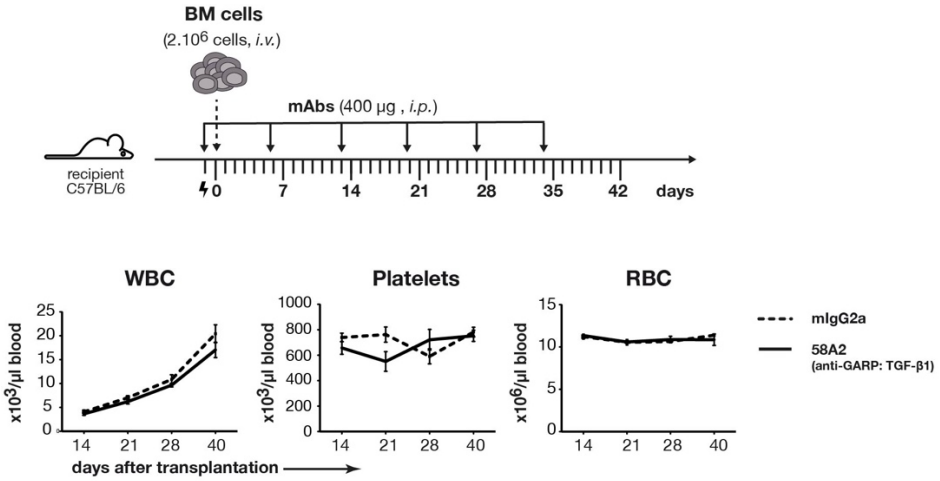



**Supplementary Figure 2. GARP expression in BM cells and splenocytes from MPL$^{W508A}$ mice.** BM cells and splenocytes isolated from MPL$^{WT}$ and MPL$^{W508A}$ mice at the end of the experiment illustrated in Fig. 1 were analyzed by flow cytometry after staining with antibodies against CD4, FOXP3, CD8, B220, CD11b, Ly6G, CD41 and GARP. Analyses were performed on gates containing live single cells, after exclusion of platelets and red blood cells. (**A**) Stacked bar graphs indicate proportions (mean + SEM) of the indicated cell types within GARP-expressing cells. Cells with a non-specific GARP staining (as assessed with an isotype control antibody) were excluded from the analysis. (**B**) Histograms are gated on the indicated cell type (Tregs or MKs). Numbers on top right indicate geometric mean fluorescence intensity after staining with anti-GARP.



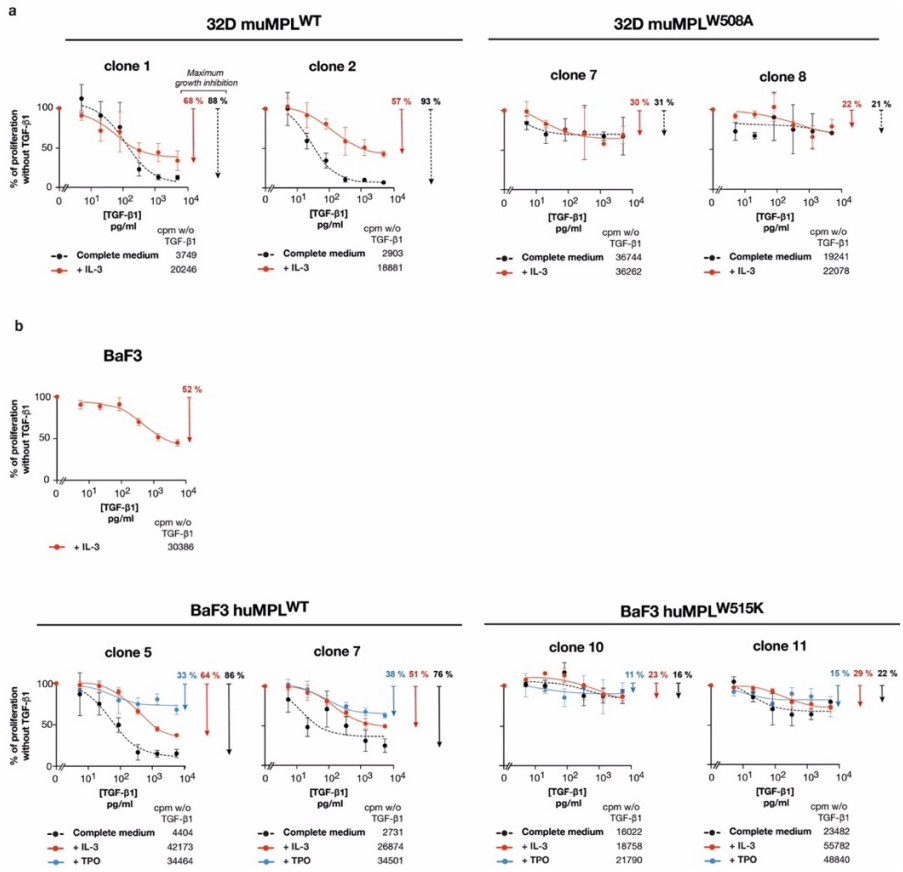

**Supplementary Figure 3. Blocking anti-GARP:TGF-β1 antibody blocks active TGF-β1 production by mouse platelets during blood coagulation *in vitro*.**

Blood taken from C57BL/6 mice was directly mixed with 58A2 or mIgG2a control, incubated at RT for 1 hour to allow coagulation, and centrifugated to collect serum. Active TGF-β1 concentration in serum was measured by ELISA (without acidification). Data points: values in individual C57BL/6 mice (n=4). Bar graphs and horizontal lines: mean + SEM. P value was calculated using a two-tailed Student t-test.



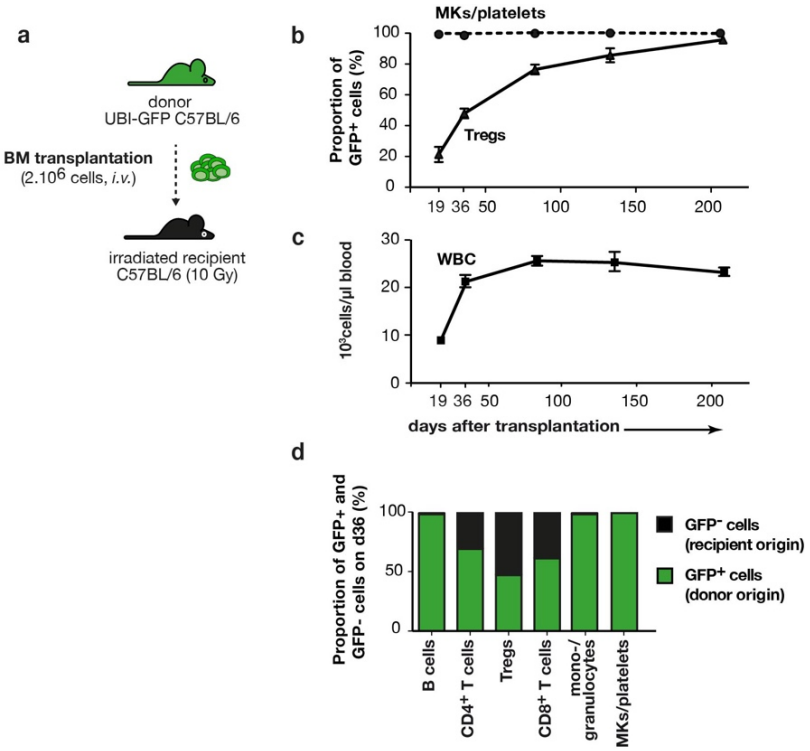

**Supplementary Figure 4. Blocking anti-mGARP: TGF-β1 antibody does not affect reconstitution after bone marrow transplantation.**

BM cells isolated from C57BL/6 mice were transplanted into irradiated C57BL/6 recipients ($2.10^6$ cells, i.v.). Recipient mice were injected i.p. weekly with the indicated mAbs (400 µg/mouse), starting one day before transplantation. Blood samples were collected on days 14, 21, 28 and 40 to count white blood cells, red blood cells and platelets. Graphs indicate means per group + SEM. n=5 mice per group.



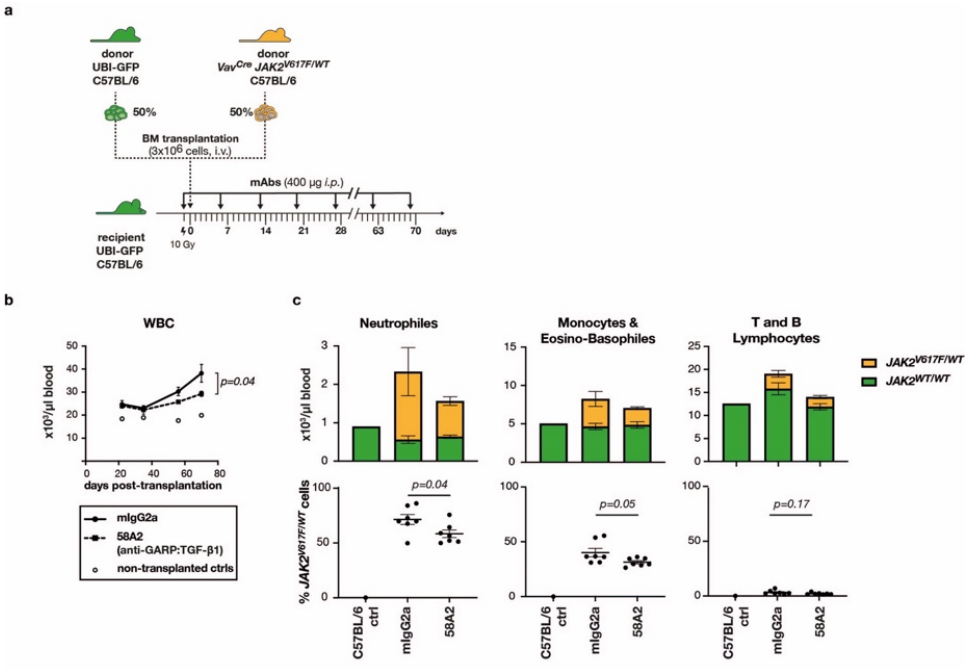

**Supplementary Figure 5. 32D and Ba/F3 clones expressing constitutively mutant MPL are resistant to the cytostatic effect of TGF-β1.**

(**A**) 32D cells were transduced with *Mpl*$^{WT}$*-IRES-GFP* or *Mpl*$^{W508A}$*-IRES-GFP* RVs encoding wild type (WT) or mutated (W508A) murine MPL, respectively. GFP$^+$ cells were sorted and cloned by flow cytometry. Proliferation of clones was measured in $^3$H-thymidine incorporation assays, as indicated in Fig. 3B. (**B**)**.** Murine myeloid Ba/F3 cells were transduced with *MPL*$^{WT}$*-IRES-GFP* or *MPL*$^{W515K}$*-IRES-GFP* RVs, encoding human wild type (WT) or mutated (W515K) human MPL, respectively. GFP$^+$ cells were sorted and cloned by flow cytometry. Proliferation of non-transduced, parental Ba/F3 cells or GFP$^+$ clones was measured in $^3$H-thymidine incorporation assays, as indicated in Fig. 3B and fig. S5A. cpm w/o rTGF-β1: counts per minute in the absence of rTGF-β1. Maximum growth inhibition (%) in the presence of rTGF-β1 is indicated in colour font on the right and was calculated as follows: (cpm w/o rTGF-β1 - cpm at the highest concentration of rTGF-β1) / (cpm w/o rTGF-β1). Data points: mean cpm +/- SD (triplicates).




**Supplementary Figure 6. T cells of recipient origin, including Tregs, persist long-term after irradiation and BM transplantation in recipient mice.**

(**A**) Schematic representation of the experimental design. BM cells were isolated from C57BL/6-Tg(UBC-GFP)30Scha/J donor mice and transplanted into lethally irradiated C 57BL/6 recipient mice ($2.10^6$ cells, i.v.). Blood was taken at days 19, 36, 83, 133 and 208 for blood cell count and flow cytometry analyses. (**B**) Proportions of donor cells within Tregs ($CD4^+GARP^+$) or MKs/platelets ($CD41^+$) according to time (mean + SEM). (**C**) Evolution of WBC counts after transplantation (mean + SEM). (**D**) Proportions of donor cells in B cells ($B220^+$ cells), $CD4^+$ T cells ($CD4^+$ cells), Tregs ($CD4^+Garp^+$ cells), $CD8^+$ T cells ($CD8\alpha^+$ cells), monocytes and granulocytes ($CD11b^+$ cells) and MKs/platelets ($CD41^+$ cells) at day 36 after transplantation, when reconstitution was achieved.




**Supplementary Figure 7. Blocking anti-mGARP: TGF-β1 antibody also reduces disease burden in a model of myeloproliferative neoplasm induced by JAK2$^{V617F}$.**

*Vav$^{Cre}$*x*JAK2$^{L2/WT}$* mice are conditional JAK2$^{V617F}$ knock-in mice, in which constitutive expression of a mutated *JAK2* allele is restricted to HSC and endothelial cells. Mice develop a polycythemia vera-like phenotype, secondary evolving to myelofibrosis within 6 months (*35*).

(**A**) BM cells isolated from *Vav$^{Cre}$* x *JAK2$^{L2/WT}$* and UBI-GFP C57BL/6 mice were mixed at a 1-to-1 ratio and transplanted into irradiated UBI-GFP recipients (3.10$^6$ cells/recipient, i.v.). Recipients are thus reconstituted with GFP$^-$ JAK2$^{V617F/WT}$ and GFP$^+$ JAK2$^{WT/WT}$ hematopoietic cells. Recipients were injected i.p. weekly with the indicated mAbs (400 µg/mouse), starting one day before transplantation (n=7 mice per group). Blood samples were collected on days 21, 35, 56 and 69 for blood cell count and flow cytometry analyses. Results are representative of 1 experiment. (**B**) WBC counts during disease progression. Data points: mean + SEM per group (n= per group). P values were calculated by repeated measure ANOVA. (**C**) Cell counts and flow cytometry analyses on day 69. Stacked bars on top: mean + SEM of GFP$^+$ JAK2$^{WT/WT}$ cells (green) and GFP$^-$ JAK2$^{V617F/WT}$ cells (orange) in neutrophiles, MEB and platelets. Data points on bottom: proportions of GFP$^-$ JAK2$^{V617F/WT}$ cells within neutrophiles, MEB and platelets in individual mice; horizontal lines: mean +/- SEM. P values were calculated using a two-tailed Student t-test.

# DISCUSSION AND PERSPECTIVES

# DISCUSSION AND PERSPECTIVES

# 1. How could TGF-β1 activation blockade from Tregs alter PMF development in *MPL*$^{W508A}$ mouse model?

## 1.1. How is TGF-β1 activated from GARP:TGF-β1 complexes and how does anti-GARP:TGF-β1 mAb blocks activation ?

As previously mentioned, TGF-β1 is produced as inactive latent TGF-β1 by almost every cell of the organism but especially by hematopoietic and immune cells. While latent TGF-β1 can be released as a soluble molecule, it can also be covalently tethered to dedicated proteins via disulfide bonds, located on cysteine 33 of LAP. GARP (or LRRC32 for leucine rich repeat-containing protein 32) is a transmembrane protein covalently binding LAP via its large extracellular domain. Main biological function of GARP is to present latent TGF-β1 at the surface of a limited number of cell-types. In Tregs, the only TGF-β1 activation mechanism to our knowledge requires GARP and integrin αVβ8 [221]. Although the precise molecular mechanism of TGF-β1 activation by integrin αVβ8 remains not fully understood, it has been hypothesized based on the one described for αVβ6, another RGD-binding integrin. The resolution of the 3D structure of latent TGF-β1 bound to αVβ6 showed that a region of the LAP, called “latency lasso”, is covering the sites necessary for the interaction of mature TGF-β1 with its receptor [222, 223]. By modeling the linear traction forces exerted by the αVβ6 trough RDG motive of LAP and the resistance opposed by cysteine 33 anchorage on the other side of the LAP, an elongation of the latency lasso allowing the release of the mature TGF-β1 could be shown. The LAP, acting as a straitjacket for mature TGF-β1, is thus distorted between its covalent tethering on one side and contractile forces coming from cytoskeleton but transmitted through integrin binding on the other side. Could this mechanism be identically applicated to αVβ8 is not absolutely clear, as the cytoplasmic tail of the integrin is different from αVβ6 and does not seem essential for its TGF-β1 activation ability. Nonetheless, activation of TGF-β1 by integrin αVβ8 requires its binding to LAP via RGD motif, latent TGF-β1 disulfide linkage to GARP and anchoring of GARP to the cell surface [221, 224]. Moreover, a recent study has suggested that integrin αVβ8 was able to induce a deformation of the LAP, unmasking mature TGF-β1

interaction site [225]. Altogether, it gives credit to a mechanism relying on tensile forces transmission trough integrin.

With those elements in mind, we can wonder what is the mechanism of action of our anti-GARP:TGF-β1 blocking mAb. Instead of precluding the binding of integrin αVβ8 to GARP:TGF-β1 complex, it rather prevents structural deformation of LAP mediated by the integrin. Our group has recently resolved and published the crystal structure of human GARP:TGF-β1 complex bound to Fab fragment of anti-human GARP:TGF-β1 blocking mAb (clone MHG-8). Interestingly, the mAb recognizes a mixed conformational epitope comprising amino acids from GARP, LAP and mature TGF-β1 [226]. Thus, blocking mAb seems to act like "a glue", locking these three elements together, impeding elongation of LAP and subsequent TGF-β1 activation. At this point, it should be noted that we don't have such precisions concerning the mAb directed against murine GARP:TGF-β1 complexes. Whereas binding specificities for the complexes are similar, epitope mapping revealed that different regions of GARP were bound by the mAb.

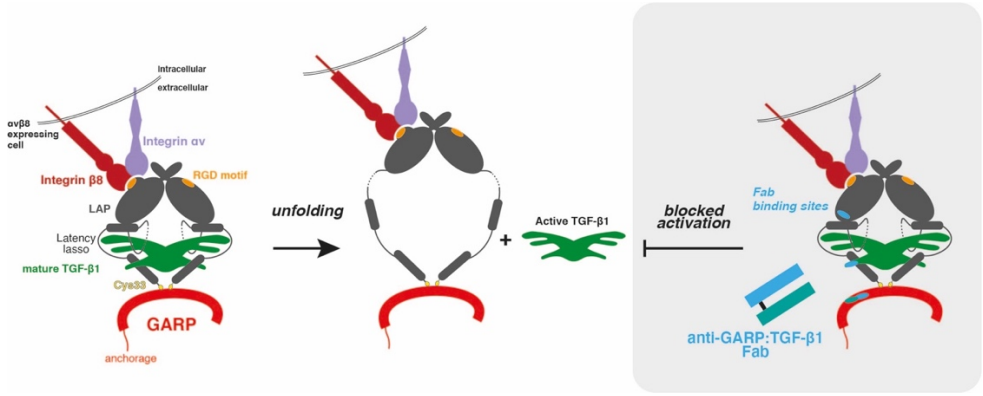



**Figure 14: Proposed activation mechanism of latent TGF-β1 by GARP and integrin αVβ8 (figure adapted from S. Liénart's thesis)**

Hypothesis extrapolated from the mechanism of activation of latent TGF- β1 by integrin αVβ6. On one side, integrin αVβ8 binds to RGD-containing loop of the LAP. On the other side, latent TGF- β1 is covalently linked to GARP, via Cys33 of the LAP, while GARP:TGF-β1 complex is anchored at cell surface. Opposed tensile forces on the LAP are thought to unfold the latency lasso and release mature TGF-β1. In human, this deformation of the LAP is supposedly prevented by anti-GARP:TGF-β1 mAb which recognizes a mixed conformational epitope and unites GARP, LAP and active TGF-β1.

## 1.2. Inhibition of TGF-β1 activation from Tregs GARP:TGF-β1 complexes could deprive MPN cells from a competing growth advantage ?

WT hematopoiesis is expected to be suppressed by the increased level of the active TGF-β1 observed in PMF. As repeatedly suggested in the literature, MPN cells are thought to be resistant to this cytostasis induced by the cytokine [64, 65, 227]. In an environment enriched in active TGF-β1, those mutant cells could thus benefit from a proliferative advantage favoring their clonal dominance. Here, we have confirmed that transduction of *Mpl*$^{W508A}$ in 32D cells confers them a resistance to the anti-proliferative effect of high concentrations of recombinant TGF-β1. By contrast, similar concentrations effectively inhibited proliferation of parental 32D or *Mpl*$^{WT}$ transduced cells. This observation had not been previously reported with *MPL* driver mutation. It only gives additional credit to the hypothesis of reduced competitive growth advantage of *Mpl*$^{W508A}$ cells upon active TGF-β1 reduction by anti-GARP:TGF-β1 mAb. Nevertheless, *in vitro* experiments with myeloid cell line are only a simplified model of MPN. We have not proven that *Mpl*$^{W508A}$ BM cells could benefit from this lower TGF-β1 sensitivity *in vivo*, neither that myelosuppressive TGF-β1 was indeed activated from GARP:TGF-β1 complexes.

In *Mpl*$^{W508A}$ mice treated with anti-GARP:TGF-β1 mAb, GFP$^{+}$ cells are significantly decreased, but the number of GFP$^{-}$ cells remains unchanged. Following the hypothesis of a restored competition, we could intuitively expect an increase of non-transduced cells following active TGF-β1 reduction, which is not the case here. Based on the aggressivity of this short living model, this measure may lack of sensitivity. Furthermore, following anti-GARP:TGF-β1 mAb injections, RNAseq analysis revealed enrichment for hallmark signatures that could correspond to GFP$^{-}$ cells increased proliferation (E2F targets and heme metabolism). However, the relevance of this remains purely speculative, the cells types driving these transcriptional modifications and their precise biological consequences remaining elusive. A dedicated analysis of GFP$^{-}$ cells would have been interesting, with flow cytometry or RNAseq on sorted population.

Interestingly, TGF-β1 is well described as a potent inducer of HSC quiescence, preserving progenitors from exhaustion. In this regard, MKs have been suggested as a source of TGF-β1 [62]. Accordingly, previous experiments from our group have

investigated the role of GARP:TGF-β1 complexes in BM cells quiescence. Using mice carrying a genetic deletion of *Garp* in either Tregs or MKs, the two main GARP expressing BM cell populations, we have not observed any difference of blood cells counts or BM composition in homeostasis conditions. More precisely, HSC numbers and their cycling status were similar between mice with or without GARP expression on their MKs or Tregs. Moreover, reconstitution following chemotherapy was unaltered in conditional *Garp* KO mice. This would suggest that if active TGF-β1 regulates proliferation of BM cells, it would not be activated from GARP:TGF-β1 complexes of MKs or Tregs in healthy mice. Nonetheless, we cannot rule out their contribution in the inflammatory context of MPNs, with potential modification of TGF-β1 activation pathways. Interestingly, while Tregs may not seem proper candidates at first glance, they have already been described as positive regulators of HSC quiescence and promoters of allogenic HSC engraftment. It this case, niche associated Tregs were characterized by high CD150 expression and their properties were dependent of adenosine generation [228].

### 1.3. Inhibition of TGF-β1 activation from Tregs GARP:TGF-β1 complexes could increase production of IFNα ?

Based on GSEA of RNAseq data, splenocytes collected from $MPL^{W508A}$ mice treated with anti-GARP:TGF-β1 mAb display an enriched IFNα gene signature. As mentioned in the first part of the manuscript, IFNα is a key cytokine of the immune response against viruses. Its secretion can be triggered in a large variety of cell types, but particularly in DC, upon activation of pattern recognition receptors by foreign or mislocated endogenous components. One of the best-known examples is its high production by pDC upon stimulation of toll-like receptors by viral DNA or RNA. While limiting the spread of the pathogen by inducing a cell-intrinsic antimicrobial transcriptional program, IFNα has also diverse effects on the innate and adaptative immune response. Indeed, beyond anti-proliferative and pro-apoptotic properties, it also promotes antigen presentation and cross-presentation by DC, increase cytotoxicity of NK cells and CTL, together with stimulation of inflammatory cytokine production by macrophages. In MPNs, clinical efficacy of IFNα have been demonstrated over the years with the belief of a disease-modifying activity, as molecular remissions are seen in a subset of patients. The best documented mechanism of action in those diseases would rely on MPN stem cells

depletion, as IFNα preferentially promote quiescence exit of mutated HSCs leading to their proliferation-associated exhaustion.

The PMF mouse model used in our experiments is obtained by transducing BM cells with *Mpl*$^{W508A}$ using a retroviral vector. Given the role played by IFNα in anti-viral response, this is legitimate to wonder if its enriched signature is not primarily related to a technical artifact of our PMF model. The retrovirus that we used is non-replicative and the infection of BM donor cells has been performed *in vitro*, followed by extensive washing steps. Contamination of transplanted cells by retroviral particles is improbable. Moreover, RNAseq analysis has been performed on splenocytes collected nearly 5 weeks after transplantation. It is then even more unlikely that we could detect at that time an acute response against a virus incapable of replication.

In parallel of IFNα, IFNγ gene signature is also enriched in anti-GARP:TGF-β1 mAb treated mice. This could be explained by the substantial overlap existing between the two gene signatures [229]. Indeed, the two types of IFNs have distinct receptors and functions but share some part of their signaling pathways and target genes. On one hand, receptor of IFNα is composed of two subunits, IFNAR1 and IFNAR2, and mediate its canonical signal trough a transcriptional trimeric complex, STAT1-STAT2-IRF9, which will modulate expression of ISRE-containing genes. One the other hand, IFNγ, a major effector cytokine of activated T cells, binds a receptor composed of IFNGR1 and IFNGR2 subunits. Its intracellular signaling leads to the formation of STAT1 homodimers that will bind to GAS (IFNγ activated sites) elements in the promoters of a different panel of interferon stimulated genes (ISG). Interestingly, IFNAR downstream signaling can be modulated in order to elicit different downstream transcriptional responses, trough STAT1 or STAT3 homodimers, that will also bind to GAS. This shared gene sets account for the impossibility to accurately define the respective contributions of IFNα and IFNγ on the sole basis of GSEA in our mice. A dedicated experimental approach should help us to precise if induction of IFNα or IFNγ or both is required for the therapeutical effect of anti-GARP:TGF-β1 mAb in murine PMF. Using the MPL$^{W508A}$ model, we could assess if the efficacy of our treatment is maintained or abrogated without type 1 or type 2 IFN signaling. Anti-GARP:TGF-β1 mAb could be combined with neutralizing mAb directed against IFNγ, as previously done in tumor models. The

same approach could be used for IFNα, targeting its receptor with an mAb or by using dedicated transgenic *Ifnar1*$^{-/-}$ mice available in our institute.

### 1.4. Inhibition of TGF-β1 activation from Tregs GARP:TGF-β1 complexes could increase T cell responses against transformed cells ?

Our observations collected in MPL$^{W508A}$ mice treated with anti-GARP:TGF-β1 mAb suggest at least a contribution of an immune mediated activity of the treatment. First suspected by an enriched IFNγ gene signature in responder mice, abrogation of the anti-GARP:TGF-β1 mAb response upon CD8$^{+}$ T cells depletion further confirmed the pivotal of role of effector T cells in the efficacy of the treatment. This is coherent with previous results obtained by our group in solid tumors models, in which selective blockade of TGF-β1 activation from Tregs increased function of anti-tumor CTL and overcame checkpoint inhibitor resistance in combination therapy. Like it has been extensively reviewed in the first part of the manuscript, description of an immune response directed against MPN cells has already been done by others but stays controversial. Here, our observations would add new evidence supporting this hypothesis by describing an original immune escape mechanism.

An obvious question is which antigen is recognized by putative anti-MPN T cells. As previously recalled, transplanted MPL$^{W508A}$ cells have been generated by retroviral infection, but here again, response against a viral antigen seems unlikely. Besides *MPL*$^{W508A}$ mice, control *MPL*$^{WT}$ mice have been generated with the same viral vector. Upon anti-GARP:TGF-β1 mAb treatment, the number of GFP$^{+}$ transformed cells is only decreased in MPL$^{W508A}$ mice, while it stays unaltered in MPL$^{WT}$ mice. These two viruses only differing by one nonsynonymous substitution, this would suggest that the epitope recognized by T cells would be part of the altered *Mpl*$^{W508A}$ sequence. Previous publications on MPNs have already pointed at neoantigens as being the most promising T cell target candidates in human, but they are mostly derived from *CALR* exon 9 frameshift mutations. However, non-synonymous single nucleotide variants, like *MPL*$^{W515K}$, have been predicted to generate MHC bound immunogenic peptides, without bringing any proof beyond *in silico* simulation [96]. It would then be valuable to investigate how 9 amino acids peptides derived from murine *Mpl*$^{W508A}$ would be predicted to bind H-2Db or H-2Kb MHC class I alleles of C57BL/6 mice. Apart from this evident putative neoantigen, expression of other class of tumor

antigens could be envisioned from the deregulated transcriptome of transformed cells like cancer-germline antigens or some overexpressed genes which are less tumor-specific.

The most convincing argument for CTL implication in anti-GARP:TGF-β1 mAb response is its abrogation upon $CD8^+$ T cells depletion. Noteworthy other arguments have been gathered in $MPL^{W508A}$ mice and deserve to be noted. First, in several experiments we have seen an increased number of blood $CD4^+$ and $CD8^+$ T cells upon anti-GARP:TGF-β1 mAb treatment. Secondly, after *in vitro* stimulation of splenocytes with PMA-ionomycin, we performed flow cytometry analysis for multiple T cells effector functions. We saw that the proportion of $CD4^+$ T cells expressing both IFNγ and TNFα but also the proportion of $CD8^+$ T cells expressing $IFN\gamma^+$ $TNF\alpha^+$ $CD107a^+$ were higher in the anti-GARP:TGF-β1 mAbs treated mice compared to isotype control. This would at least suggest that the treatment was responsible of an increased differentiation of effector T cells. Lastly, a negative correlation could be made between the number of GFP+ neutrophils and the aforementioned percentage of $IFN\gamma^+$ $TNF\alpha^+$ $CD107a^+$ $CD8^+$ T cells.

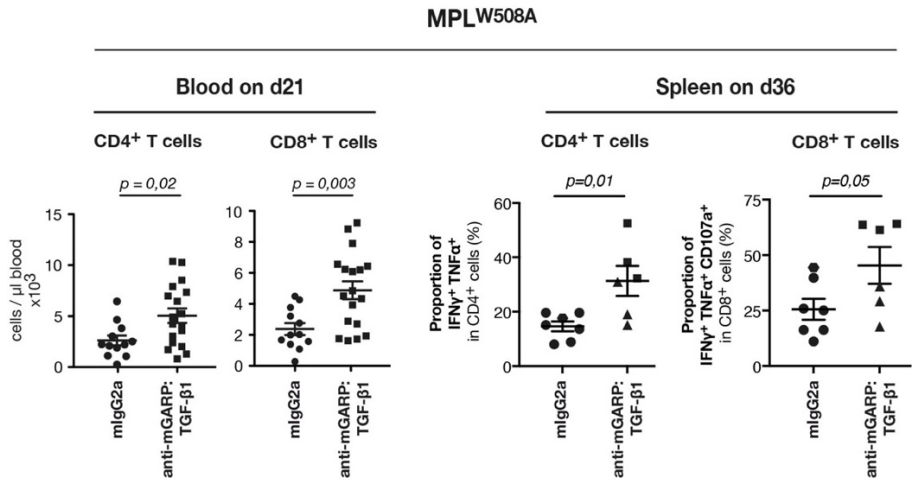



**Figure 15: Anti-GARP:TGF-β1 mAb improves differentiation of effector T cells in *MPL*$^{W508A}$ mice**

*MPL*$^{W508A}$ mice have been injected with anti-GARP:TGF-β1 mAb as previously described. The two figures come from unrelated experiments. (left) Bleeding has been done on day 21. WBC have been counted on a hemocytometer and further analyzed with flow cytometry. (right) Splenocytes has been isolated on day 36 and stimulated 4 hours *in vitro* with PMA and ionomycin before flow cytometry staining.

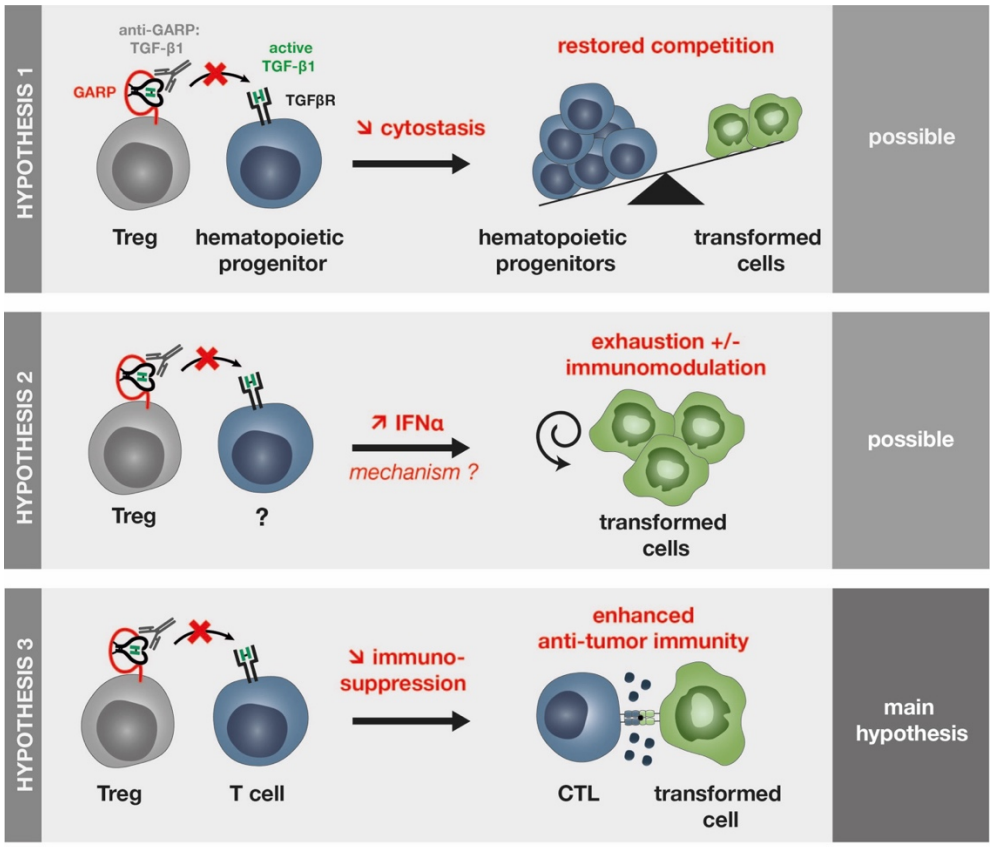



**Figure 16: Summary of putative mechanisms of action of anti-GARP:TGF-β1 mAb in murine PMF (figure adapted from S. Lecomte thesis)**
(1) blocking TGF-β1-induced cytostasis on normal hematopoietic progenitors would increase the proliferation of normal cells *versus* the proliferation of transformed cells, intrinsically resistant to TGF- β1-induced cytostasis ; (2) blocking TGF-β1 signals would increase IFNα signals *via* an unknown mechanism, that would increase cycling of transformed cells and subsequent exhaustion (associated or not to immunomodulatory effect) ; (3) blocking TGF-β1-induced immunosuppression on T cells would increase immune responses against MPN cells. Although additional experiments are needed, the last hypothesis is actually the most supported one.

## 2. Is TGF-β1 activation blockade specific of Tregs in MPL$^{W508A}$ mouse model?

Expression of GARP:TGF-β1 complexes has been detected in a limited number of cell types including Tregs stimulated *via* their TCR, activated PLT and MKs, B cells stimulated *via* their BCR, hepatic stellate cells, MSC and endothelial cells [230]. Among them, a capability to activate TGF-β1 in a GARP-dependent manner has only been demonstrated in activated Tregs, B cells and PLT. For the later, this release of mature TGF-β1 has been shown with mouse samples, during *in vitro* PLT aggregation and blood coagulation in serum generation assays.

Treg-specific deletion of GARP in *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice reproduce the decrease of MPL$^{W508A}$ disease burden obtained in WT C57BL/6 mice injected with anti-GARP:TGF-β1 mAbs. This is an important observation, indicating that Tregs are a predominant target for the therapeutic activity of the blocking mAb. Immunophenotyping *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice have been previously reported and focused on GARP expression of known GARP-dependent TGF-β1 activating cells [81]. By comparison to littermate controls *Foxp3*$^{cre}$ x *Garp*$^{WT/WT}$, GARP expression is completely abrogated on Tregs. Although its expression is globally conserved on activated B cells and PLT, we have observed a non-specific and discrete GARP deletion in a minority of mice. The consequence of this reduced GARP expression in B cells and PLT is not known but is not expected to jeopardize our observations and highlight the interest to cross our results with the ones obtained from other cell-type specific GARP KO mice colonies.

MSC has been described to express GARP and to play an important role in PMF physiopathology. The relation between these two properties have never been studied, but their ability to activate TGF-β1 from GARP:TGF-β1 has been suggested without being formally demonstrated [231]. However, *Foxp3* expression has been reported in MSC and their examination was not included in the original phenotyping of our transgenic mice [232]. Moreover, these cells being presents at a very low percentage amongst BM cells, we could have missed them while looking at GARP expressing cell populations in MPL$^{W508A}$ mice. This is why we sought to complete the analysis of our *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice and evaluated GARP expression on their MSC.

This cell type representing only a small fraction of total BM cells (0,01%), we needed to isolate and amplify the MSC *in vitro* in the first place. We choose a classical method relying on their preferential adherence to plastic, allowing a progressive enrichment of MSC in the cell culture flask along the passages [233]. Direct examination revealed the expected spindle-shaped morphology and using flow cytometry, we confirmed their MSC phenotype as being defined as $CD45^-CD11b^-CD34^-CD29^+CD44^+Sca\text{-}1^+$ expressing cells. By comparing cells isolated from *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice and littermate controls, we have found a low but similar surface expression of GARP between the two genotypes. Further confirmation was obtained by RT-qPCR analysis. Overall, even if we still don't know if MSC are able to activate TGF-β1 in a GARP-dependent manner in our MPL$^{W508A}$ mouse model, we can rule out their contribution to the predominant TGF-β1 activation attributed to Tregs with *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice colony.

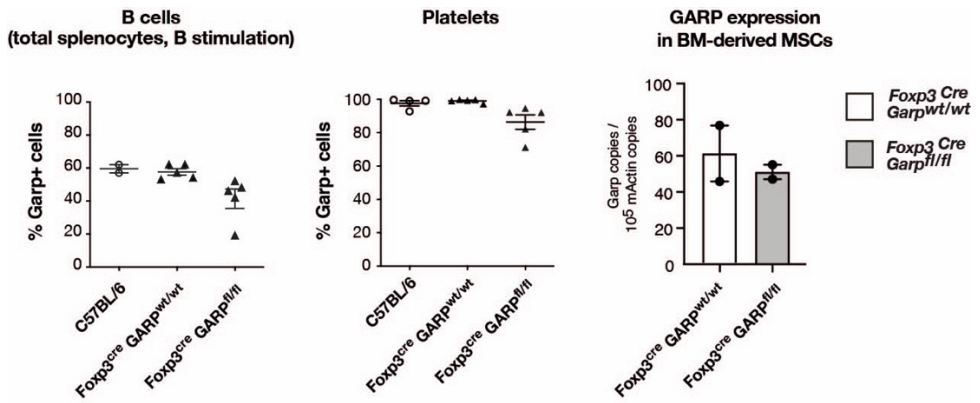



**Figure 17: Specificity of GARP deletion in *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice**
All comparisons have been made between *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice and their *Foxp3*$^{cre}$ x *Garp*$^{WT/WT}$ littermate controls. (left) Flow cytometry analysis on B cells. Isolated splenocytes have been stimulated overnight to induce GARP expression. Percentage of GARP positive events has been determined on $B220^+$ live cells. (center) Flow cytometry analysis on PLT, purified from whole blood. Percentage of GARP positive events has been determined on $CD41^+$ cells. (right) Total BM cells were cultivated for 11 days following recommendations of a protocol used for specific mouse MSC isolation. Cells were then collected, RNA extracted and RT-qPCR analysis for *Garp* has been performed (normalization with mActin).

The results obtained with *Pf4*$^{cre}$ x *Garp*$^{fl/fl}$ mice have been decisive. Our initial hypothesis was incriminating MKs, instead of Tregs, as essential contributors to the activation of TGF-β1 from GARP:TGF-β1 complexes. Indeed, the review of the available literature pointed at a closer relation between MKs and TGF-β1 in the disease. Surprisingly, while deletion of GARP on Tregs recapitulated the observations made with the blocking mAb, there was no disease burden reduction to be seen when GARP expression was specifically lost on MKs and PLT. Thus, targeting MKs or PLT do not seem to be required to observe the benefits of anti-GARP:TGF-β1 mAbs treatment. Combined with the observations made with *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice, it further strengthened to dominant role played by the Tregs in the PMF-promoting GARP-dependent TGF-β1 activation.

Nonetheless, the results collected in *Pf4*$^{cre}$ mice GARP$^{fl/fl}$ mice have been difficult to obtain and can thus be discussed. Here, the efficacy and the specificity of GARP deletion in PLT has not been problematic, with no expression of GARP on desired cells and virtually no aspecific deletion on B cells or Tregs. However, the mice from this transgenic colony, including the phenotypically normal littermate controls, have repeatedly failed to reconstitute their hematopoiesis and develop PMF phenotype following transplantation of *Mpl*$^{W508A}$ BM cells. No clear explanation has been found to justify this lack of reconstitution. Moreover, this seemed to be associated to the retroviral infection step, as unmanipulated BM cells from *Pf4*$^{cre}$ x *Garp*$^{fl/fl}$ or *Pf4*$^{WT}$ x *Garp*$^{fl/fl}$ mice behaved normally in transplantation experiments. We finally circumvented this issue by adapting our protocol with a shorter infection step. The rational of this approach was to maximize the fitness of the BM cells, by limiting hypothetical exhaustion or toxicity during *in vitro* manipulations. This strategy allowed convincing results but remains limited in terms of reproducibility. Given these limitations, complementary experiments have been designed to further precise of the role played by GARP:TGF-β1 expressing MKs (or another unnoticed rare cell type). A first option keeps the same PMF mouse model and would consist to treat *Mpl*$^{W508A}$ *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice with anti-GARP:TGF-β1 mAbs. This method could help us to verify if an additional effect could be obtained beyond deletion of GARP on Tregs. Unfortunately, such experiment has failed to reproduce interpretable data and underlines the technical complexity inherent to this mouse model. As a second option, another mouse model independent of any retroviral infection and transplantation is an attractive alternative. Thus, we sought to generate

myelofibrosis with a pharmacological product, like romiplostim which is a MPL agonist able to reproduce a TPO$^{high}$ mouse model (more details in the next section).

In summary, we can conclude that Tregs are an important source of GARP-dependent TGF-β1 activation in our PMF model, but we can't definitively rule out the contribution of another unrecognized cell type.

## 3. Could we translate therapeutical efficacy of anti-GARP:TGF-β1 mAbs to other MPN models or human disease ?

### 3.1. Advantages and limitations of the MPL$^{W508A}$ mouse model

MPL$^{W508A}$ mouse model is capable of rapidly induce a robust phenotype of PMF in mice. This short period of time is convenient in an experimental setting dependent of mAb injections. Disease modeling being based on retroviral transduction, it has also the advantage of being potentially transposed to a wide variety of transgenic mouse colonies. However, substantial limitations must be considered. Firstly, while resembling to PMF, the disease we are modeling is only a simplified version of its human counterpart. As being *a priori* purely monogenic it doesn't comprise the other mutations frequently found in PMF, affecting notably epigenetic or splicing regulators. This could restrict the behavior of the clone or putative tumor antigens. Secondly, the transduction procedure inserts the *Mpl*$^{W508A}$ transgene into a wide variety of cells in diverse differentiation states which does not recapitulate the true genesis of the disease, arising from one single mutated HSC. This confers an unusual presentation of PMF due to polyclonal proliferation of heterogenous progenitors and dominated by unusual erythrocytosis and leukocytosis. Thirdly, a highly aggressive PMF presentation could be disadvantageous in order to demonstrate the activity of a treatment, especially in immunotherapy. At this regard, the requirement to start anti-GARP:TGF-β1 mAbs injections before transplantation, meaning prior disease development, can be related to this rapid kinetic and is not engaging for translational perspectives. Fourthly, the immunogenicity of the transformed cells could be artificially increased. On one hand, *Mpl*$^{W508A}$ transgene is

randomly inserted in its retroviral vector and expressed independently of *Mpl* transcriptional regulation. This high-level of *Mpl*$^{W508A}$ expression, escaping physiological regulation, may positively affect the immunogenicity of the mutated receptor and would not reflect human disease [234]. On the other hand, it has also been previously demonstrated that GFP, also comprised in the bicistronic vector, can be immunogenic in mice recipients [235]. GFP has been less considered as a putative antigen in the MPL$^{W508A}$ model as it is also expressed in MPL$^{WT}$ mice, which do not present any decreased of GFP$^{+}$ transformed cells upon anti-GARP:TGF-β1 mAbs treatment. We could argue that GFP may be more prone to elicit immune response in MPL$^{W508A}$ than in MPL$^{WT}$ mice. This could be due to better infection efficacy of MPL$^{W508A}$ cells in some experiments, responsible of an increased protein expression, or higher cell turn over in the PMF model compared to its control. Lastly, the establishment of the model requires many complicated steps, offering many opportunities to endanger reproducibility between experiments. Overall, MPL$^{W508A}$ mouse model is a powerful yet delicate tool. It suggested new opportunities for future MPN treatment, including immunotherapy, but they will need to be validated in complementary models.

## 3.2. Other models to address some of the MPL$^{W508A}$ mice shortcomings

### *3.2.1. Jak2$^{V617F}$ knock in model*

MPN mouse models relying on *Jak2*$^{V671F}$ mutation are widely used and could help to confirm the hypothesis made with the MPL$^{W508A}$ model. Indeed, by comparison to the rare *MPL* mutants, this driver mutation is the most commonly found, encompassing both PV, ET and PMF. This is an advantage in order to make more generalizable observations on MPNs. Depending on the *Jak2*$^{V671F}$ transfer method, mouse phenotype is variable but is generally characterized by a slower and less aggressive presentation than the one conferred by *Mpl*$^{W508A}$. Interestingly, by contrast to viral transduction technique, KI approach allows the expression of *Jak2*$^{V671F}$ from the original *Jak2* locus. By replacing the WT exon 14 of endogenous *Jak2* by the one carrying V617F mutation, the transgene can be expressed at near physiological level in the hematopoietic cells. No equivalent system exists for *Mpl*$^{W508A}$ and may bring more relevance. Moreover, artificial tumor antigen like GFP

is absent from *Jak2*$^{V671F}$ KI cells, adding credit to the relevant immunogenicity that we could attribute to them. On the other side, usage of *Jak2*$^{V671F}$ KI mice is restricted to the assessment of anti-GARP:TGF-β1 mAb, as crossing those mice with our cell-type specific GARP KO colonies would be challenging.
In our experimental protocol with *Jak2*$^{V671F}$ KI mice, instead of treating them directly with anti-GARP:TGF-β1 mAb, we choose to use them as donors of BM cells. We've elaborated a competitive transplantation assay in which we could discriminate the engraftment of *Jak2*$^{V671F}$ and *Jak2*$^{WT}$ cells, with or without anti-GARP:TGF-β1 mAb. Proliferative advantage of each cell population could be followed in the recipients and compared between treatment groups. We obtained a PV-like phenotype characterized by a progressive excess of *Jak2*$^{V671F}$ myeloid cells. Proportion of *Jak2*$^{V671F}$ cells was the highest in PLT and neutrophils. Interestingly, these two cells types were the most vulnerable to a decrease following anti-GARP:TGF-β1 mAb. While *Jak2*$^{V671F}$ PLT reduction was not sustained until the end of the follow up, the decrease of *Jak2*$^{V671F}$ neutrophils persisted, without affecting their *Jak2*$^{WT}$ counterpart and tended to reach statistical significance in terms of absolute number. Fibrosis has not been evaluated because of a too short follow up. These results are interesting because they tend to confirm the observations made in MPL$^{W508A}$ mice with a more general and slightly less artificial MPN model in steady-state hematopoiesis. However, we have not precise the mechanism of anti-GARP:TGF-β1 mAb in *Jak2*$^{V671F}$ mice. On the basis of a more indolent evolution and less sticking effect of anti-GARP:TGF-β1 monotherapy, this *Jak2*$^{V671F}$ model would also be a valuable platform to test combination therapies.

### *3.2.2. CARL exon 9 mutations*

As extensively reviewed in the first part of the manuscript, CALR exon 9 mutations are the most promising source of neoantigens in human. Based on the potential immune-mediated effect of anti-GARP:TGF-β1 mAb in the other models, MPN induced in mice with *CALR* type 1 mutation would disserved to be explored. Noteworthy, the predicted immunogenicity of *CALR* frameshift mutants in humans may not be identically transposable in mice. At first glance, a similar transplantation strategy that the one used for *Jak2*$^{V671F}$ could be envisioned.

### 3.2.3. $TPO^{high}$ model

PMF mouse models based on increased MPL stimulation have been the first ones described and preceded *JAK2*$^{V671F}$ discovery. For instance, they have been used to demonstrated to role of TGF-β1 in the fibrosis observed in the disease [68]. While TPO can be overexpressed by gene transfer methods in transplanted BM cells, direct administration of TPO or another MPL agonist can lead to similar outcomes. Romiplostim, a clinically approved TPO mimetic, has previously demonstrated its ability to model PMF in mice and appeared as a suitable option to circumvent the transplantation issues encountered in *Pf4*$^{cre}$ x *Garp*$^{fl/fl}$ mice [236]. On the downsides, romiplostim-induced PMF is devoid of any MPN driver mutations and therefore may be less relevant to study a process suspected to depend on an increased T cell response against MPN cells. Without prejudging of a single mode of action, it could still be interesting as a complementary tool to increase our coverage of GARP-dependent TGF-β1 function in PMF.

Only one set up experiment has been done with *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice and their littermate controls, as we wanted to compare the results to the ones collected in the MPL$^{W508A}$ model. With a three weeks injection schedule, mice developed thrombocytosis and mild myeloid leukocytosis compared to untreated mice. Their bone marrow examination revealed discrete fibrosis and increased p-SMAD2 signal. *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice presented similar grades of fibrosis than *Foxp3*$^{cre}$ x *Garp*$^{WT/WT}$ mice, but our analysis was not sensitive enough given the small size of the groups and the poor induction of fibrosis in the experiment. Interestingly, *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice shown decreased neutrophil counts compared to their WT controls, but it failed to reach statistical significance. This could be another element pointing at the role played by TGF-β1 activated from Tregs GARP:TGF-β1 complexes in the development of the disease. However, before such conclusions, the experiment should be repeated with a prolonged romiplostim treatment in larger groups. A similar design should also be applied to *Pf4*$^{cre}$ x *Garp*$^{fl/fl}$ mice. Unfortunately, such experiments have been kept on hold given their lack of financial sustainability and other priority MPN models.

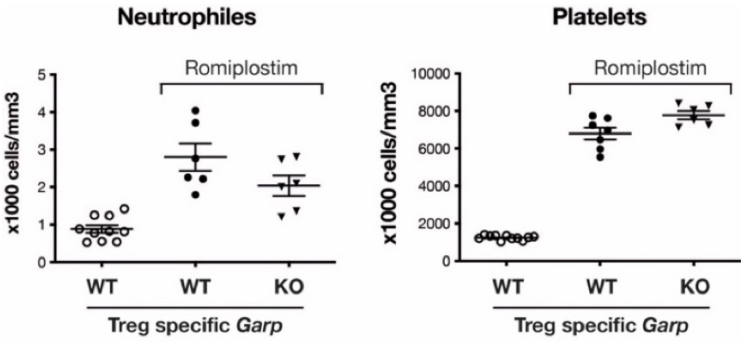



**Figure 18: TPO$^{high}$ myelofibrosis model with *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice**
*Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice (n=6) and wild type littermate controls (n=6) have been injected with romiplostim (1000μg/kg) or NaCl 0,9%. Sub-cutaneous injections have been performed on day 1, 8 and 15. Blood has been collected on day 21. WBC and PLT have been counted and further analyzed with flow cytometry (neutrophils defined as CD11b$^+$ Ly6G$^+$ single cells).

## 3.3. How could we extrapolate the effect of anti-GARP:TGF-β1 mAbs to humans

### *3.3.1. TGF-β1 activation from Tregs: differences between mice and humans*

While human Tregs strictly express GARP:TGF-β1 complexes at their surface following stimulation trough their TCR, those complexes can be detected at low level on mouse resting Tregs [237]. Notwithstanding this discrepancy, both human and mouse Tregs only activate TGF-β1 after TCR-stimulation [238]. However, whereas TGF-β1 activated from human Tregs is solely produced from GARP:TGF-β1 complexes, stimulated mouse Tregs seem to possess a GARP-independent TGF-β1 activation mechanism. This have been suggested by previous work done in our laboratory, with the help of blocking mAb specifically binding human (clone MHG-8) or mouse (clone 58A2) GARP:TGF-β1 complexes. When looking at the pSMAD2 signal from activated Tregs by Western Blot, pSMAD2 is abrogated when human Tregs have been treated with MHG-8 whereas pSMAD2 signal is only reduced of 50% when murine cells have been treated with 58A2 [81, 239]. A similar incomplete abrogation of TGF-β signaling has been seen when comparing activated Tregs

isolated *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice to their littermate controls. Finally, transgenic mice have been engineered with a mutation in their *Garp* gene in order to allow binding of anti-human GARP:TGF-β1 mAb (YSG mice). This modified *Garp* sequence does not impair binding and efficacy of anti-mouse GARP:TGF-β1 mAb. Comparing both blocking mAbs, the anti-human GARP:TGF-β1 mAb failed to demonstrate any superiority on pSMAD2 signal suppression in activated Tregs from YSG mice. Altogether, these observations tend to demonstrate an alternative TGF-β1 activation mechanism in mouse Tregs. As absent in humans, this might suggest a potential increased activity of anti-GARP:TGF-β1 mAb in patients.

### *3.3.2. Are GARP:TGF-β1 complexes expressed in MPN patients?*

As the existence of a GARP-dependent TGF-β1 activation in MPN patients is unknown, confirming the expression of GARP:TGF-β1 complexes on their Tregs or MKs would be a first valuable information. BM samples would be a proper material to look for GARP expression on MKs or for an infiltration by GARP$^+$ Tregs. Moreover, we could look at the TGF-β1 responsive cells and integrate those data to determine their spatial interaction with putative GARP-expressing cells. Immunofluorescence techniques are currently being developed in our group in order to perform such analysis on tumor biopsies. Multicolor staining including GARP, FOXP3 and pSMAD2 are being validated. Unfortunately, our anti-human GARP staining is restrictive and only works with frozen samples, excluding the vast majority of the formaldehyde-fixed BM biopsies stored in biobanks or other collections. A prospective recruitment of dedicated patients is not yet planned.

As a surrogate, we want to collect PBMC from PMF patients and analyze them with flow cytometry. The primary question addressed here is whether those individuals have spontaneously activated Tregs, expressing GARP:TGF-β1 complexes at their surface. Although unlikely, a positive result could be an argument favoring the hypothesis of a spontaneous Treg activation in those patients, putatively suppressing anti-PMF T cell response like in our mouse model. The first samples have been collected and the flow cytometry staining protocol has been validated on healthy donors.

### *3.3.3. Would our experimental readouts be relevant for MPN patients?*

In $MPL^{W508A}$ mouse model, anti-GARP:TGF-β1 mAb treatment have not demonstrated any benefit in terms of survival. However, such interpretation is limited by the preemptive sacrifice of the mice engaged in our experiments. This was generally requested for proper organ collection that we needed for histological, transcriptional and/or flow cytometry analyses.
In clinical trials, surrogate markers are increasingly used to accelerate the availability of promising drugs addressing unmet medical needs. In PMF, surrogate biomarkers of disease modification of been proposed [240]. Among them, reduction of variant allele frequency (VAF) or reduction of bone marrow fibrosis have been frequently used in clinical trials, but definitive proof of their translation into progression or survival improvement is still lacking. The reduction of transformed cells observed in the blood of $MPL^{W508A}$ mice after anti-GARP:TGF-β1 mAb treatment (and to a lesser extent in *Jak2*$^{V671F}$ mice) can be considered as an alternative measurement of VAF. Moreover, our measures indicated a non-significant decrease of spleen fibrosis, that we consider reflecting femur fibrosis. This equivalence has been confirmed in our first experiments and spleen has been later preferred because of an easier processing.

Interestingly, a decrease of neutrophils derived from transformed HSCs have been seen across all our MPN models treated with anti-GARP:TGF-β1 mAb. While not being used as a clinical marker of response, neutrophils have been associated to particular complications of MPNs. Thus, some particular benefits may be suggested for patients. First, neutrophils have been associated to a process called emperipolesis. In PMF, this uncommon biological phenomenon refers at the passage of neutrophils trough MKs, supposed to trigger TGF-β1 release and disease development. The interaction between the two cell types is increased in PMF and mediated trough a higher expression of P-selectin by MKs [241]. Secondly, leukocytosis and especially its granulocytic fraction has been designated as a reliable biomarker to predict the risk of thrombosis in MPN patients. More precisely, activated neutrophils in MPNs have been suggested to accelerate atherosclerosis, have a pro-thrombotic increased adherence to endothelium or PLT and favor thrombosis via formation of neutrophil extracellular traps [242, 243]. Whether a positive effect on cardiovascular complications after anti-GARP:TGF-β1 mAb

treatment is possible, it remains unknown as not explored by our experimental design.

## 4. Advantages and safety of targeting GARP: TGF-β1 complexes with blocking mAb

### 4.1. How does it compare with the other TGF-β1 inhibition strategies?

TGF-β1 is implicated in wide variety of physiological processes and shares its receptor with the other TGF-β isoforms. A dual role in tumor progression has even been attributed to the cytokine as it can promote both suppression of pre-malignant lesions and cancer progression with metastasis. Therapies targeting TGF-β1 have thus been limited by recurrent safety and efficacy issues. However, the complex biology of TGF-β1, produced as inactive cytokine requiring tightly regulated and cell-type specific activation mechanisms, could offer us the possibility to restrict our intervention to the supposedly harmful active TGF-β1.
As mentioned in the first part of the manuscript, the less specific approaches neutralizing the three mature TGF-β isoforms (Frezolizumab) or inhibiting their common receptor have not demonstrated convincing efficacy and were responsible of worrying side effects (squamous cell carcinomas or cardiac toxicity via TGF-β2 inhibition). In PMF, soluble TGF-β receptor traps narrowed the inhibition spectrum to mature TGF-β1 and TGF-β3. This led to good tolerability, but only obtained marginal hematological responses.

More recently, the efficacy of strategies directed against TGF-β1 activation mechanism has been reported in preclinical tumor models. Another research group has derived mAb binding the latency lasso of the LAP, reinforcing the straitjacket it exerts on mature TGF-β1 [244]. This treatment successfully improved immune checkpoint inhibitor efficacy in mouse tumor models. Compared to mature TGF-β1 blockade, a therapy acting upstream by targeting latent-TGF-β1 itself could possess superior therapeutic potential, precisely because it acts prior the activation step. Indeed, mature TGF-β1 could not be widely released in the extracellular space. As we already know, the interaction sites of mature TGF-β1 with its receptors are covered by the latency lasso of the LAP and must be unmasked to allow the

biological effect of the cytokine. Using non cleaved latent-TGF-β1 mutants, Campbell et al. have recently suggested that whereas those sites were freed by structural deformation of the latency lasso during activation, mature TGF-β1 did not require a physical separation from the LAP to bind its receptor [225]. Part of the TGF-β1 activation could then be restricted to close cellular contacts and limit the availability of neutralizable mature TGF-β1. Although it has the advantages of an early grip on the activation process and only target TGF-β1 isoform, anti-LAP mAb could still be responsible of substantial toxicities as it potentially affects all the latent TGF-β1 reservoir. The fear of promoting the growth of pre-malignant lesions is persisting. This drawback could be addressed by anti-GARP:TGF-β1 mAb, because it only preclude activation of TGF-β1 of a limited number of cell types. It would mainly block the immunosuppressive TGF-β1 activated by the Tregs, associated to a clearly deleterious effect on anti-tumoral immune response, while sparing the remaining active TGF-β1 pool implicated in other non-harmful physiological functions.

### 4.2. Potential toxicities of anti-GARP:TGF-β1 treatment

In the perspective of blocking TGF-β1 activation mediated by Tregs in MPN patients, we should try to envisioned which toxicities could arise from anti-GARP:TGF-β1 mAb treatment.

Beyond its immunosuppressive activity on anti-tumor T cells, GARP-dependent TGF-β1 activation of Tregs could be anticipated to maintain immune tolerance and its blockade could raise concerns about the occurrence of autoimmune adverse events. Data observed in our group are reassuring, as *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice do not have overt autoimmune phenotype. At steady state, GARP-expression by Tregs would thus not be required for immune homeostasis. In inflammatory conditions, Tregs contribution may be more important but conflicting results have emerged. In mice with GARP-deficient Tregs, another group have reported the development of a spontaneous lupus-like disease [245]. We have been unable to reproduce these results in one-year old *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice. Nonetheless, our Treg-specific GARP KO mice developed a lupus-like disease under chronic inflammatory conditions induced by a persistent IFNα production (electroporation of IFNα-coding plasmid). Further contradictions were found in inflammatory models. Salem et al. described a reduced capability of GARP-deficient Tregs to suppressed DSS-induced colitis or

colitis induced by WT naïve $CD4^+$ T cells transferred into immunodeficient *Rag2*$^{-/-}$ mice. These observations contrast with the ones obtained by our group. Indeed, we found that Tregs isolated from *Foxp3*$^{cre}$ x *Garp*$^{fl/fl}$ mice or their WT littermates similarly controlled colitis induced by similar T cells transfer. To summarize, GARP expression by Tregs could be implicated in the control of autoimmunity in the specific context of ongoing inflammation. With respect to the inflamed environment of PMF, combination with an anti-inflammatory drug sparing T cell function could be a reasonable approach.

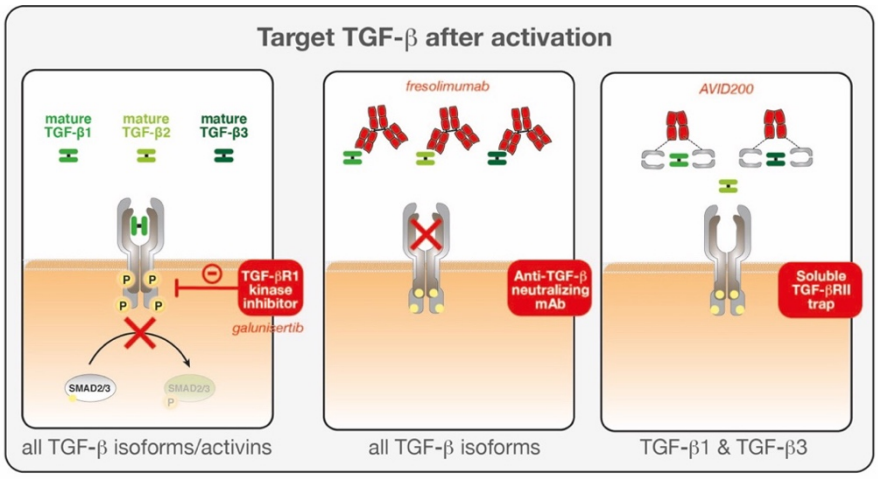
Beyond activated Tregs, TGF-β1 activation from GARP:TGF-β1 complexes has also been shown in activated B cells and PLT *in vitro*. Whereas it has been implicated isotype switch and IgA production of B cells, the biological function of this activation from PLT remains elusive [246].

Apart from autoimmunity, immunosuppressive consequences have been envisioned for anti-GARP:TGF-β1 treatment. As TGF-β1 is known to participate to IgA production and Th17 cells, it could be feared that anti-GARP:TGF-β1 mAb could impair two important effectors implicated in mucosal immunity. This hypothesis has been tested in a murine model of intestinal bacterial infection [247]. Transgenic *il22r*$^{/-}$ mice are at risk to develop severe colitis upon oral gavage with *Citrobacter rodentium*, a natural murine intestinal pathogen that mimic human diseases caused by pathogenic *Escherichia coli*. Treatment with anti-GARP:TGF-β1 mAb did not aggravated the severity of the disease while it preserved the innate and adaptative immune response against the pathogen. Moreover, consequence of GARP:TGF-β1 blockade on IgA production has been further evaluated by comparing immunoglobulin response to ovalbumin (OVA) immunization in Treg-, B cell- or PLT/MK-specific GARP KO mice. No reduction of anti-OVA immunoglobulin production was observed in any of these GARP deficiencies. This would suggest that GARP-dependent TGF-β1 activation is not required for IgA production *in vivo*.

Activation of TGF-β1 dependent on GARP:TGF-β1 complexes has been demonstrated *in vitro*, following PLT aggregation in serum generation assays. Although it has been robustly demonstrated, the physiological relevance of this discovery can be questioned [248]. A first hypothesis has been a role in hemostasis. Dedicated studies using PLT/MK- specific GARP KO mice failed to demonstrate any functional consequence of this deletion on PLT activation, aggregation and thrombus

formation, both *in vitro* and *in vivo* [249]. Another hypothesis would be that GARP:TGF-β1 complexes on MKs could be a source of active TGF-β1 implicated in the regulation of HSC quiescence. As already mentioned, our previous work with *Pf4*$^{cre}$ x *Garp*$^{fl/fl}$ mice suggested that even if MKs activate TGF-β1 to regulate HSCs quiescence, it would be by an alternative mechanism than the one requiring GARP.

Those preclinical observations are globally reassuring regarding the potential toxicities associated to anti-GARP:TGF-β1 therapy. A phase 1 clinical trial is currently ongoing in cancer patients and will collect essential data regarding the safety profile and the risk of autoimmune manifestations due to GARP-dependent TGF-β1 activation blockade.



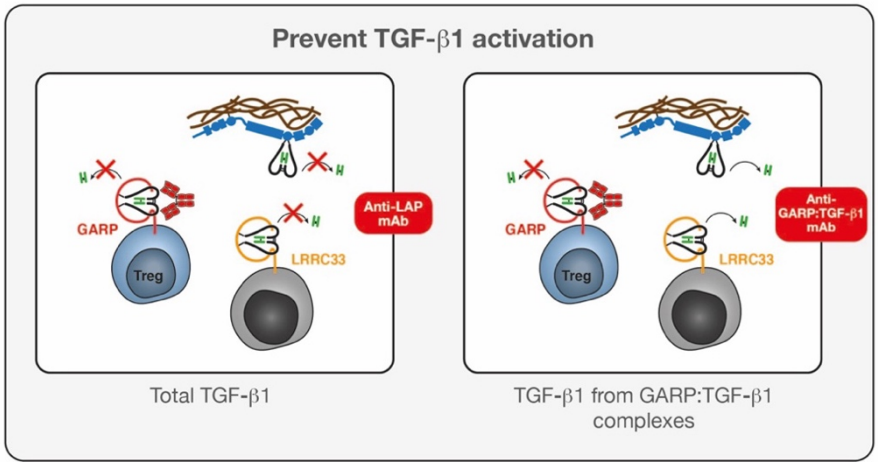



**Figure 19: Comparison of the TGF-β1 blockade strategies (figure adapted from C. Bertrand thesis)**
Here are described several strategies of TGF-β1 blockade, highlighting the specificity of anti-GARP:TGF-β1 mAb. The majority of them have been tested in PMF (in murine model or in patients).
A first group targets the cytokine after its activation, like receptor TGF-β1R1 inhibitors (galunisertib has shown some benefits in murine PMF) or neutralizing mAb (fresolimumab had no therapeutic effect in a small clinical trial). All of these are active against the 3 TGF-β isoforms. Targeting only mature TGF-β1 and -β3, TGF-β1RII trap like AVID200 has shown better tolerability, but its relevance in human disease is still investigated. A second group of molecules aims at blocking TGF-β1 activation, thus preventing the release of the mature cytokine. Anti-LAP mAb blocks all latent-TGF-β1 reservoir (no experience in PMF). Anti-GARP:TGF-β1 mAb allows a precise targeting of TGF-β1 activated trough a GARP-dependent mechanism by a limited number of cell-type, including Tregs.

## 5. Improving efficacy of anti-GARP:TGF-β1 therapy with combination therapies

Our results suggest that blocking TGF-β1 activation from GARP-expressing Tregs could be an interesting approach for the treatment of MPNs and especially PMF. While it provided original results on the role of GARP:TGF-β1 in the disease, the clinical activity obtained in $MPL^{W508A}$ mice remains limited. Moreover, the therapeutic activity of our blocking mAb requires to start the injections prior $Mpl^{W508A}$ cells transfer which is seemingly less relevant. The reason of this requirement remains elusive, high disease burden secondary to the aggressive phenotype of the $MPL^{W508A}$ model could be envisioned. Another explanation could be related to the timing of action of TGF-β1 on putative anti-MPN CTL leading to their exhaustion. An improved efficacy can be sought trough combination therapies.

Our work suggests that TGF-β1 activated from GARP-expressing Tregs could contribute to the establishment of an immunosuppressive environment which is reversed by blocking mAb treatment. Therapeutical benefits of the blockade would indeed rely on an increased T cell response putatively directed against MPN cells. PD1/PD-L1 axis has also been widely described in the suppression of anti-tumor immune responses and suspected to be implicated in PMF progression. Such coexisting immunosuppressive mechanism in the $MPL^{W508A}$ model could be one of the explanations for the anti-GARP:TGF-β1 mAb resistance displayed by some of the “non-responders” mice. No dedicated analysis has been done in $MPL^{W508A}$ mice, but we’ve been able to confirm PD-L1 overexpression in Ba/F3 cells transduced with

*MPL*$^{W515K}$. Comforted by the synergy observed between anti-PD1 mAb and anti-GARP:TGF-β1 in tumor bearing mice, we looked for repeating such promising combination in PMF model. Unfortunately, our tentative with MPL$^{W508A}$ mice have led to mixed results, notably due to recent technical difficulties in the model. As being more reliable and leading to a milder phenotype, our latest transplantation model based on *Jak2*$^{V671F}$ KI mice would be a suitable candidate for further attempts with such mAb combination.

Another strategy could be a combination with immunostimulatory drugs. HMA like 5-azacytidine could be interesting, notably via its potential propriety to favor cancer germline antigens expression. IFNα is another possibility but there is no formal proof that its effects are immune mediated in MPNs. Moreover, preclinical results with Treg-specific GARP deficient mice may have suggested that IFNα could favor autoimmune complications.

Finally, reducing the disease burden prior or during anti-GARP:TGF-β1 mAb treatment could be another alternative. A careful choice of drug should aim at avoiding any detrimental effect on T cell function. Ruxolitinib (which inhibit JAK1) or BET inhibitor like pelabresib (which suppress NF-kB pathway) should be avoided, at least in simultaneous treatments. A same remark could be addressed at PI3K inhibitors like parsaclisib. JAK inhibitors more specific of JAK2 could be a reasonable alternative. Medications targeting apoptotic pathways such as Bcl-2 inhibitors (navitoclax) or MDM2 inhibitors (KRT-232) could be interesting.

# DISCUSSION AND PERSPECTIVES

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