A pro-inflammatory stem cell niche drives myelofibrosis through a targetable galectin-1 axis

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OVERLINE:

One Sentence Summary: Unravelling the cancer-stroma interactome in myelofibrosis reveals key drivers of inflammation and galectin-1 as a clinically-actionable target.

Editor's summary:

Abstract:

Myeloproliferative neoplasms are stem cell-driven cancers associated with a large burden of morbidity and mortality. The majority of patients present with early-stage disease, but a substantial proportion progress to myelofibrosis and/or secondary leukemia, advanced cancers with a poor prognosis and high symptom burden. Currently, it remains difficult to predict progression, and therapies that reliably prevent or reverse fibrosis are lacking. A major bottleneck to the discovery of disease-modifying therapies has been an incomplete understanding of the interplay between perturbed cellular and molecular states. Several cell types have individually been implicated, but a comprehensive analysis of myelofibrotic bone marrow is lacking. We therefore mapped the crosstalk between bone marrow cell types in myelofibrotic bone marrow. We found that inflammation and fibrosis are orchestrated by a 'quartet' of immune and stromal cell lineages – with basophils and mast cells creating a TNF signaling hub, communicating with megakaryocytes, mesenchymal stromal cells and pro-inflammatory fibroblasts. We identified the β-

galactoside binding protein galectin-1 as a striking biomarker of progression to myelofibrosis and poor survival in multiple patient cohorts, and as a promising therapeutic target, with reduced myeloproliferation and fibrosis in vitro and in vivo and improved survival following galectin-1 inhibition. In human bone marrow organoids, TNF increased galectin-1 expression, suggesting a feedback loop wherein the pro-inflammatory MPN clone creates a self-reinforcing niche, fueling progression to advanced disease. This study provides a valuable resource for studying hematopoietic cell-niche interactions, with broad relevance for cancer-associated inflammation and disorders of tissue fibrosis.

INTRODUCTION

In most cancers, one or more genetic perturbations are initiating events that confer a survival advantage to the cell-of-origin and its progeny, but the stromal-immune context in which the emergent clone operates determines its ultimate impact. Myeloproliferative neoplasms (MPNs) are initiated by somatic mutations in hematopoietic stem cells (HSCs) that cause clonal expansion and an over-production of blood cells and their progenitors (*1*). The underlying genetic lesions are well described, with mutations affecting either the gene encoding the Janus kinase signal transducer *JAK2* (JAK2V617F), the chaperone protein calreticulin (CALR) or the thrombopoietin receptor (*MPL*) occurring in almost all patients (*2*). Interactions between the MPN clone and its microenvironment influence the rate and likelihood of progression to advanced disease (*3*-*5*). Although most patients present with slow-growing malignancies that only modestly impact life expectancy, some patients develop a severe form of MPN called myelofibrosis. In these patients, fibrotic bone marrow remodeling and pronounced systemic inflammation cause bone marrow failure, extramedullary hematopoiesis, splenomegaly, severe symptoms and a median survival of around 5 years (*6*).

Myelofibrosis results when cytokines produced by the MPN clone stimulate bone marrow stromal cells to deposit an excess of collagens and other extracellular matrix proteins, consequently destroying

99 the hematopoietic microenvironment. A pivotal role for certain pro-fibrotic and pro-inflammatory growth
100 factors, such as megakaryocyte-derived transforming growth factor β (TGF), is well recognized (*7-9*).
101 However, the complexity of cell lineages that send and receive the signals that fuel bone marrow
102 inflammation and fibrosis has not been fully elucidated. For example, whereas various mesenchymal
103 stromal cell (MSC) subsets have been studied, including Nestin+ (*10*), GLI Family Zinc Finger 1 (Gli1+) (*11*)
104 and Leptin receptor+ MSCs (*12*), little is known about the subtypes and transcriptional states of bone
105 marrow fibroblasts in myelofibrosis, and the specific cellular mediators and receptor-ligand (R-L)
106 interactions that lead to pathological stromal cell activation.

107 Our aim was to build a comprehensive atlas of myelofibrotic bone marrow including
108 hematopoietic stem/progenitor cells (HSPCs), mature hematopoietic cells and their stromal cell
109 neighbors, to identify potentially targetable mediators of inflammation and fibrosis. To achieve this, we
110 first mapped the cellular and molecular cross-talk in myelofibrotic bone marrow at single cell resolution
111 in a mouse model of myelofibrosis, and corroborated findings using bone marrow biopsies and blood
112 samples from patients. Unexpectedly, we found that basophils and mast cells – populations not previously
113 highlighted as important inflammatory drivers in MPNs – are increased in abundance and act as the 'hub'
114 for the enhanced tumor necrosis factor (TNF) signaling (*13*). We also showed that while MSCs divert to
115 produce extracellular matrix (ECM) components and downregulate their production of hematopoietic
116 support factors, a compensatory increase in production of hematopoietic cytokines occurs from basophils,
117 mast cells and a subset of pro-inflammatory bone marrow fibroblasts (iFibs). Therefore, paracrine
118 hematopoietic support within myelofibrotic bone marrow derives from alternative cellular sources to that
119 in healthy marrow.

120 The β-galactoside binding protein galectin-1 emerged as one of only two genes differentially
121 expressed in both the MPN clone and the inflamed stroma in myelofibrosis. We confirmed a clear positive
122 correlation between galectin-1 expression and myeloid cancer progression in three large patient cohorts,

and showed that inhibition of galectin-1 using a neutralizing anti-galectin-1 monoclonal antibody (mAb) ameliorated myeloproliferation and fibrosis in a mouse model and in 3D, multi-lineage human bone marrow organoids. This identifies galectin-1 as a biomarker for progressive disease and therapeutic target for MPNs and potentially other myeloid malignancies and fibrotic disorders.

RESULTS

Generating a high-resolution cellular atlas of myelofibrotic bone marrow

To enable detailed analysis of the cellular landscape of myelofibrotic bone marrow, we utilized a well-characterized murine model in which *Mpl*^{W515L}, the third most common driver mutation occurring in patients with MPN, is introduced into murine HSPCs by retroviral transduction and the cells transplanted into lethally irradiated, wild-type recipients (*14*). Control C57BL/6OlaHsd mice received HSPCs transduced with enhanced green fluorescent protein (EGFP) alone. As previously described, this resulted in a severe and rapidly progressive myeloproliferative disease that was typically lethal within 4 weeks (*14*). Mice receiving MPL^{W515L} bone marrow developed leukocytosis, thrombocytosis, polycythemia, pronounced splenomegaly, a reduction in body weight, bone marrow fibrosis, reduced cellularity and increased, atypical megakaryocytes (Figs. 1, A and B, fig. S1A). Histology of the spleens revealed loss of the normal lymphoid follicle architecture and markedly increased splenic megakaryocytes and other myeloid cells (fig. S1B).

We devised a workflow enabling simultaneous capture of hematopoietic and stromal cells from murine femurs, tibiae and iliac crests, and performed high-throughput, droplet-based, single cell RNA-sequencing, isolating total mononuclear cells (MNCs) and enriching for rarer relevant cell types including lineage negative (Lin-) cKit+ HSPCs and cells expressing the megakaryocyte cell surface marker CD41 (Fig. 1C). Capture of non-hematopoietic stromal cells was achieved by performing collagenase digestion of flushed and crushed bone pieces, beads depletion of CD45+ hematopoietic cells and then fluorescence-activated cell sorting (FACS) to isolate the CD45-, Lin-, Ter119-, CD71mid/- non-hematopoietic cell fraction (Fig. 1C, fig. S1C and data file S1) (*15*).

Following data integration, doublet removal and quality control (fig. S1D), 77,288 cells were analyzed from 23 mice in 3 independent experiments, including 42,319 hematopoietic and 34,969

stromal cells, generating a comprehensive atlas of normal and myelofibrotic bone marrow (Fig. 1, D to G, dataset can be queried in an online data explorer at https://mouse10x.shinyapps.io/myelofibrosis/.) Differentially expressed genes for each cluster were calculated after dimensional reduction and clustering, and cell types identified by their expression of canonical marker genes (Fig. 1, E and G, fig. S1E, data file S2).

We successfully captured the major cellular subsets annotated in recently published atlases of murine bone marrow (*15-18*). Within the bone marrow stroma, this included: MSCs (expressing *Lepr*, *Cxcl12*, *Adipoq*), fibroblasts (*Dcn*, *Pdgfra*, *Pdgfrb*) (*19*), osteolineage cells (OLC, *Bglap*, *Bglap2*, *Alpl*), chondrocytes (*Acan*, *Sox9*), pericytes (*Myh11*, *Rgs5*) (*20*) and neuronal (*Ncam1*) cells, and distinct arteriolar (*Bcam*, *C1qtnf9*) and sinusoidal (*Plvap*, *Lrg1*) endothelial cell subtypes (Fig. 1D and 1E, fig. S1E). Eleven hematopoietic cell types were captured, including hematopoietic stem and multipotent progenitor cells (HSC/MPP, *Cd34*, *Ly6a*, *Cd27*), megakaryocytes (*Pf4*), T (*Lck*) and B (*Cd79a*, *Ebf1*, *Vpreb3*) lymphocytes, eosinophil/basophil/mast cells (*Prss34*, *Fcer1a*), erythroid (*Car2*, *Gata1*), granulocyte-monocyte progenitors and pro-monocytes (*Mpo*, *Elane*), monocytes/macrophages (*Ms4a6c*) and neutrophils (*Camp*, *Retnlg*; Fig. 1, F and G, fig. S1E).

To compare the cell types captured in our study to previously published datasets of normal (*15*) and myelofibrotic (*21*) bone marrow, Symphony analysis (*22*) was performed, using our data as the reference dataset and projecting cells from existing datasets onto to the reference embeddings. This confirmed annotation in our dataset of several major cell types including fibroblasts, chondrocytes, endothelial, osteolineage, mature neutrophils, eosinophils, basophils, and mast cells that were not captured in previous studies of myelofibrotic bone marrow, particularly in the stromal cell compartment (fig. S2, A and B) (*18, 21*). This dataset therefore represents an unbiased cellular and molecular atlas of the bone marrow in myelofibrosis, enabling a more comprehensive analysis of cellular and molecular interactions and perturbations than has been possible to date.

Alterations to the cellular constituents of the bone marrow in myelofibrosis

The relative abundance of cell lineages was altered in myelofibrotic bone marrow. In concordance with the expected disease phenotype, erythroid, neutrophil and megakaryocyte cells were expanded in the hematopoietic compartment of MPL^{W515L} mice (Fig. 2A). A decrease in HSPCs was also observed, with a near absence of B and T lymphocytes (Fig. 2A). Within the stromal compartment, the most striking change in myelofibrosis mice was an expansion of Lepr+ MSCs, with a more modest increase in fibroblasts and decrease in chondrocytes, OLCs and endothelial cells compared to controls (Fig. 2B). We did not find any evidence of monocyte-derived fibrocytes in myelofibrosis bone marrow, with no expression of Slam family member 7 (*Slamf7)* or monocyte-affiliated markers detected in stromal cell subsets (fig. S2C).

Basophils and mast cells were increased in abundance in myelofibrosis mice compared to controls (Fig. 2A). We found that the eosinophil, basophil and mast cell (EBM) population had primarily been captured by the enrichment sort for CD41+ cells (Fig. 2C), a canonical cell surface marker of megakaryocyte cells but also expressed on murine basophils at steady-stage, and upregulated after cytokine activation (*23*).

Altered cellular sources of ECM components

A defining feature of myelofibrosis is the aberrant deposition of ECM in the bone marrow, causing reticulin fibrosis, bone marrow failure and extramedullary hematopoiesis in the spleen. The specific constituents and cellular origin of ECM factors in normal and myelofibrotic bone marrow have not been well described, although ECM components are recognized as important regulators of HSC function (*24*). To determine the cellular sources of ECM proteins in the bone marrow, we utilized an ECM gene list derived from proteomic analysis of normal and malignant tissues (*25*). Higher numbers of ECM genes were expressed by cells from the stroma than the hematopoietic compartment (total ECM genes: n = 233 versus

198 107; collagens: n = 42 versus 17; glycoproteins: n =159 versus 76 and proteoglycans: n = 32 versus 14 for
199 stroma versus hematopoietic respectively). Within the stromal cell subsets, high *per cell* expression of
200 ECM genes was detected in all cell types apart from endothelial cells, neurons and pericytes (Fig. 2D and
201 fig. S2D). Expression of collagen subtypes and glycoproteins was higher in OLCs and chondrocytes than
202 other stromal cell subtypes, whereas fibroblasts and fibro-chondrocytes were the primary cellular source
203 of proteoglycans, and MSCs predominantly expressed glycoproteins (fig. S2D). Expression of ECM
204 components were also detected in the hematopoietic compartment, although in lower abundance than
205 in the stroma (Fig. 2E). Prominent expression of glycoproteins and proteoglycans were detected in EBM
206 cells, as well as a small fraction of monocytes/macrophages and mature neutrophils (fig. S2D).

207 To validate these findings at protein level, we performed low-input mass spectrometry
208 proteomics on hematopoietic cells (CD45+) and the key stromal cell subfractions including $Lepr^{+}$ MSCs,
209 fibroblasts and endothelial cells. 131 ECM proteins were detected in the proteomes, and both the number
210 of ECM proteins detected and their abundance was significantly higher in stromal cell fractions than
211 hematopoietic cells, confirming the stroma as the dominant source of ECM protein in the bone marrow
212 ($P < 0.0001$, Fig. 2F). We confirmed that this is also true in human bone marrow by interrogating a recently
213 published atlas capturing both hematopoietic and stromal cell subtypes (*26*) for expression of ECM
214 proteins (fig. S2E). In myelofibrotic bone marrow, *per cell* expression of ECM genes was increased in MSCs
215 and EBM cells but decreased in fibroblasts (Fig. 2, D and E), suggesting that MSCs and EBM cells are major
216 contributors to the altered deposition of extracellular matrix proteins in myelofibrosis.

217

218 **Altered cellular sources of hematopoietic support factors in myelofibrosis**

Bone marrow Lepr+ MSCs transdifferentiate into myofibroblasts in myelofibrosis in response to platelet derived growth factor receptor (PDGFR) stimulation, downregulating their production of hematopoietic niche support factors in parallel with their increased expression of fibrogenic and osteogenic genes (*12, 21*). We detected clear transcriptional reprogramming of MSCs in myelofibrotic bone marrow, with a pronounced reduction in expression of hematopoietic niche support factors (Fig. 3A, data file S4) in parallel with the increased expression of ECM factors (Fig. 2D). Although the reduction in expression of hematopoietic support factors by MSCs in myelofibrosis has been documented (*12, 21, 27*), prior studies did not examine whether the production of hematopoietic support factors 'shifts' from MSCs to other cellular components of the bone marrow niche. We found that the reduction in gene expression of niche support factors (NSF) from MSCs was compensated by a significant increase in expression of NSF in fibroblasts and also EBM cells in myelofibrosis versus control cells ($P < 0.001$)(Fig. 3, A and B). The *per cell* expression of NSF, in particular *Cxcl12* and *Csf1*, was decreased in myelofibrosis versus control bone marrow MSCs but increased in fibroblasts (fig. S3, A and B).

Myelofibrosis MSCs were transcriptionally distinct, with enrichment of genes and pathways associated with myofibroblast transition including alpha smooth muscle actin 2 (*Acta2)*, KRAS and phosphoinositide-3-kinase (PI3K) signaling, inflammatory response genes and IL2-STAT5 signaling (Fig. 3, C and D). Therefore, myelofibrosis-induced MSC trans-differentiation leads to increased ECM production but reduced hematopoietic support from MSCs. Assuming that protein expression reflects the observed changes in gene expression, this suggests that hematopoiesis is guided by alternative cellular sources in the setting of MPNs, potentially influencing the competitive advantage of the MPN clone over healthy hematopoiesis.

Emergence of a distinct inflammatory fibroblast subset in the myelofibrotic niche

The relative proportion of fibroblast cells overall was only minimally increased in myelofibrotic bone marrow (Fig. 2B). As fibroblasts were the most abundant stromal cell type captured, and as distinct fibroblasts subsets have been reported to be important in other pathologies (*28, 29*), we selected the fibroblasts for further analysis, confirming their expression of the canonical fibroblast markers *Pdgfra/Pdgfrb* and performing unsupervised sub-clustering (fig. S3C).

Five transcriptionally distinct sub-clusters were identified (Fig. 3E, data file S2), of which one cluster (Fib4) uniquely showed striking enrichment for inflammatory pathways (Fig. 3, F and G) and was therefore annotated as representing inflammatory fibroblasts (iFibs). iFibs were enriched for TNF signaling through NFκB, inflammatory response signaling, IL6-JAK-STAT3 signaling and interferon gamma response (Fig. 3G). Pseudotime analysis showed that iFibs arise from a separate trajectory to Fib1 cluster (fig S3D). The relative frequency of iFibs was 2-fold higher in myelofibrosis mice than controls (Fig. 3H), and expression of chemokine genes was strongly enriched in the iFibs with significantly increased *per cell* expression of chemokines in MPL versus control fibroblasts ($P < 0.001$) (Fig. 3I, data file S3), including *Kitl*, *Cxcl12*, *Ccl2*, *Cxcl1* (fig. S3, A and E). The iFib cluster also expresses *Cxcl5*, which has been identified in a recent fibroblast atlas as a marker for perturbation-specific, activated fibroblast states and not detected in steady-state fibroblasts (*19*) (fig. S3F). Collectively these data support that, although overall fibroblast numbers are only slightly altered in myelofibrosis, distinct inflammatory fibroblast subsets producing hematopoietic support factors are markedly expanded in number, thereby contributing to the development of an aberrant hematopoietic niche in myelofibrosis.

Expanded pro-inflammatory basophils, mast cells and megakaryocytes in myelofibrosis

Megakaryocyte proliferation and morphological atypia are hallmark features of overt and pre-fibrotic myelofibrosis (*1*), and we found an expansion of megakaryocytes with angiogenic, proliferative

and inflammatory gene expression programs in the myelofibrosis mice (MK3, 4 and 5, fig. S4, A to E). Although megakaryocytes are well recognized as important drivers of fibrosis (*9, 30*), the pathological contributions of basophil and mast cell subsets in myelofibrosis have not been extensively studied (*31*). Having noted a significant increase in the abundance of EBM cells (Fig. 2A), we extracted cells from the EBM cluster for a more detailed analysis (Fig. 4A). Four distinct subtypes of EBM cells were annotated – EBM progenitors, mast cells, basophils and a small population of mature eosinophils (Fig. 4, A and B, Fig. S4F, data file S2). The relative proportions and transcriptional activity of these cellular subsets were distinct in myelofibrosis bone marrow, with a dramatic expansion of basophils and mast cells, and relatively few eosinophils in myelofibrosis mice compared to controls (Fig. 4C), and significant enrichment of IL2-STAT5, TGFβ, and TNF via NF-κB inflammatory signaling pathways (FDR < 0.25, Fig. 4, D and E).

Basophils and mast cells emerge as the 'hub' of TNF and pro-inflammatory cytokine signaling

To identify how the cellular cross-talk was altered in myelofibrotic bone marrow, we computationally inferred the interacting receptor-ligand (R-L) pairs that might mediate communication between cell types (*32*). The overall number of predicted R-L interactions was 20% higher in MPL^{W515L} than control mice (Fig. 5A), and the aberrant signaling was largely due to increased interactions deriving from basophils, mast cells and megakaryocytes in the hematopoietic compartment and MSCs and inflammatory fibroblasts in the stroma (Fig. 5B), highlighting these 4 cell types as 'orchestrators' of inflammatory signaling in myelofibrotic bone marrow. Basophils and mast cells emerged as the hub of TNF and IL4 signaling in MPL^{W515L} mice, with fibroblasts, inflammatory fibroblasts, MSCs and neutrophils and monocytes/macrophages as their key interacting partners (Fig. 5, C and D, fig. S5, A and B). Intracellular flow cytometry confirmed increased TNF and IL4 protein abundance in MPL^{W515L} basophils compared to controls (Fig. 5E and fig. S5C). This effect was seen both in basophils analysed ex vivo from bone marrow

cells of MPL^{W515L} mice (Fig. 5E) as well as basophils differentiated ex vivo from MPL^{W515L} versus control HSPCs (fig. S5C), indicating that the induction of TNF and IL4 occurred as a direct effect of the MPN driver mutation and was not dependent on niche reprogramming.

Lgals1, the gene encoding the protein galectin-1, a β-galactoside binding protein which interacts with β-1 integrin (*Itgb1*), emerged as a R-L pair with substantially enhanced predicted signaling across the key interacting cell types (Fig. 5F, fig. S5, D and E). When we looked for genes which were differentially expressed in the key interacting cell types in myelofibrosis, only two genes were concordantly dysregulated across cell types – S100 calcium binding protein a6 (*S100a6)* and *Lgals1* (Fig. 5G). A role for *S100a6* and other *S100* family members in inflammation and malignant hematopoiesis has previously been reported (*33-35*), whereas galectin-1 has not been extensively studied in myeloid malignancies. Expression of *Lgals1* was strikingly increased in basophils and mast cells, MSCs and megakaryocytes in MPL^{W515L} mice compared to control mice, with high expression in fibroblasts overall but no significant difference in *per cell* expression (Fig. 5H). *Lgals1* was also highly expressed in monocytes, pro-monocytes and GMPs, but neither the abundance of these cell types nor the *per cell Lgals1* expression was increased in myelofibrosis, indicating that monocytes and their precursors are not a source of excess galectin-1 production in myelofibrosis (fig. S5F). Together, these data suggested that galectin-1 signaling might play a key pathological role in myelofibrosis progression and warranted further exploration.

Galectin-1 inhibition ameliorates myelofibrosis disease phenotype *in vivo*

To test whether galectin-1 signaling contributes to the pathobiology of myelofibrosis in vivo, we tested the impact of a neutralizing anti-galectin-1 monoclonal antibody (Gal-1-mAb3) that binds to a specific sequence in galectin-1 not present in other galectin family proteins (*36*) in the MPL^{W515L} mouse model. Control and MPL^{W515L} mice were treated with either IgG isotype control or Gal-1-mAb3 by

intraperitoneal injection (Fig. 6A). Galectin-1 neutralization led to a reduction in bone marrow fibrosis and cellular architecture in the MPL^{W515L} mice (Fig. 6B) and reduced the myeloproliferative phenotype with significantly reduced thrombocytosis, polycythemia (*$P < 0.05$, **$P < 0.01$) and splenomegaly (*$P < 0.05$, **$P < 0.01$)(Fig. 6, C to E). The reduction in splenomegaly with Gal-1-mAb3 treatment was similar to that with fedratinib, a JAK2 inhibitor in clinical use (Fig. 6F), and no cytopenias were observed following galectin-1 inhibition in the control mice (Fig. 6C), indicating specific inhibition of the MPN clone rather than a non-specific cytoreductive impact. Furthermore, inhibition of galectin-1 led to significantly improved MPN-free survival (*** $P < 0.001$, **** $P < 0.0001$)(fig. S6A).

Galectin-1 is a robust biomarker of fibrosis progression in patients with MPNs

Given the amelioration of disease phenotype in vivo in the mouse model, we next sought to validate galectin-1 in myeloid malignancies in the setting of human disease, using a series of patient cohorts (data file S4). We first tested whether galectin-1 expression correlated with fibrosis progression in patients with myeloproliferative neoplasms, quantifying galectin-1 protein in bone marrow biopsies of 30 patients, including those with myelofibrosis (n = 14), non-fibrotic MPNs (essential thrombocythemia [ET], n = 9 and polycythemia vera [PV], n = 7) and age-matched healthy controls (n = 7, data file S4). Galectin-1 was increased in myelofibrotic bone marrow (Fig. 7A). Objective quantification of staining intensity per high power field view showed a significant increase in galectin-1 with progression to myelofibrosis across patient groups (Fig. 7B, $P < 0.001$ for myelofibrosis versus healthy donors and $P < 0.001$ for myelofibrosis versus ET and PV). Bone marrow fibrosis is often unevenly distributed in the bone marrow space, and this heterogeneity is inadequately captured by the standard categorical fibrosis grading system that is typically employed in clinical assessments (for example WHO grade MF 0 – 3). In order to measure the association between galectin-1 expression and reticulin fibrosis more precisely, we

employed a recently developed machine learning pipeline that enables automated fibrosis quantification by allocating a Continuous Index of Fibrosis (CIF) score for each bone marrow region, creating a heatmap representing the density of fibrosis across the entire marrow specimen (*37*). This showed clear correlation between the intensity of galectin-1 immunostaining and the density of fibrosis within the marrow sections (Fig. 7C), as well as between patient samples (Fig. 7B).

To further validate galectin-1 as a biomarker and to see if it could be utilized as a non-invasive peripheral blood biomarker of fibrosis, we investigated galectin-1 expression in a cohort of 120 patients where platelet transcriptomes were available from patients with myelofibrosis (n=42), ET (n=24), PV (n=33) and healthy controls (n=21, data file S4) (*38*). A progressive and highly significant increase in galectin-1 expression was observed with progression of MPN to fibrosis (monotonic trend from controls to PV/ET to myelofibrosis, $P < 0.0055$, Fig. 7D), with a 3.4-fold increase in myelofibrosis versus controls.

Galectin-1 validates as a targetable mediator of fibrosis in human cellular assays and bone marrow organoids

MPN mouse models are useful surrogates for the human disease, but evidence that a potential target can be functionally validated using human experimental systems is more compelling. We therefore explored whether galectin-1 was mediating a severe disease phenotype using human disease models. We derived bone marrow stromal cells (BMSCs) from marrow aspirates of patients with MPNs (data file S4) and utilized these in a TGFβ-induced fibroblast-to-myofibroblast transition assay (*39*). Treatment of BMSCs with recombinant human TGFβ led to increased collagen 1 deposition and αSMA expression, which was reversible on inhibition of TGFβ signaling with SB431542, an inhibitor of the TGFβ activin receptor-like kinase (ALK) receptors (*40*) (Fig. 7E). OTX008, a small molecule galectin-1 inhibitor previously shown

to inhibit pulmonary fibrosis, inhibited TGFβ-induced fibroblast-to-myofibroblast transition (Fig. 7E, fig. S6, B and C) (*41*).

To confirm a role for galectin-1 as a mediator of TGFβ-induced bone marrow fibrosis in a multi-cellular bone marrow microenvironment, we utilized a three dimensional model that better recapitulates the complexity of human bone marrow. Bone marrow organoids were generated from human induced pluripotent stem cells using an optimized protocol that gives rise to the key stromal and hematopoietic cellular elements of the central marrow space, approximating the transcriptional and architectural features of the native human hematopoietic tissues (*42*). In this model, OTX008 significantly inhibited TGFβ-induced collagen 1 and αSMA expression at both protein and mRNA levels (**P* < 0.05, ***P* < 0.01, *****P* < 0.0001) (Fig. 7F and fig. S6D).

TNF upregulates galectin-1 gene expression

Given the increase in TNF signaling from basophils and mast cells in the MPL^{W515L} mouse model (Figs. 4E and 5C), and as TNF-NF-κβ signaling has previously been shown to regulate *LGALS1* expression by T cells (*43*), we hypothesized that TNF might stimulate galectin-1 production in human bone marrow. We first corroborated that basophils and mast cells were increased in frequency and had an inflammatory phenotype in the setting of myelofibrosis in patients by interrogating a scRNAseq dataset of ~120,000 CD34+ Lin- HSPCs isolated from a cohort of 15 patients with myelofibrosis and 6 age-matched healthy donors (9). A population of EBM progenitors was identified (fig. S6E, data file S3), which were substantially more abundant in patients with myelofibrosis than healthy controls (Fig. 7G). Similar to our findings in the mouse model, these cells showed an enrichment of inflammatory response, IL2-STAT and TNF signaling (Fig. 7H). A significant increase in *LGALS1* (*P* < 0.001) was detected in patients with myelofibrosis due to

either *JAK2V617F* or *mutCALR* (fig. S6F), confirming that basophils and mast cells are likely to play an important role in the pathobiology of myelofibrosis and contribute to TNF pro-inflammatory pathways.

TNF is a potent activator of nuclear factor (NF)-κB (*44*), and NF-κB directly binds to regulatory elements in exon 1 of the *LGALS1* gene, enhancing gene expression (*43*). We therefore tested whether the mechanism of galectin-1 increase in myelofibrosis might occur secondary to TNF stimulation. Indeed, TNF treatment of bone marrow organoids robustly led to a dose-dependent increase in *LGALS1* expression (Fig. 7I), suggesting a model wherein a self-reinforcing, inflammatory MPN niche is created by expanded populations of basophils, mast cells, MSCs and inflammatory fibroblasts with a central role for TGFβ, TNF and galectin-1 signaling (fig. S7A).

High galectin-1 is associated with poor survival in *de novo* acute myeloid leukemia and progression to blast phase MPN

Given the disease modifying activity of the anti-galectin-1 antibody treatment in the MPL^{W515L} mouse model, we hypothesized that high expression of galectin-1 may be detrimental more broadly in myeloid malignancies. We therefore interrogated The Cancer Genome Atlas (TCGA) to test whether expression of galectin-1 correlated with overall survival in 132 patients with acute myeloid leukemia (*45*). There was a clear correlation between *LGALS1* expression and poor survival (Fig. 7J, $P = 0.0005$), with highly significant enrichment of inflammatory signaling pathways in patients with high *LGALS1* levels and poor survival, including inflammatory response, IL6 – JAK – STAT signaling and TNF signaling (Fig. 7K, FDR < 0.001). We also found significantly increased *LGALS1* expression in accelerated/blast phase MPN (AP/BP-MPN), suggesting a role for galectin-1 in leukemic progression of MPN. *LGALS1* expression was significantly increased in RNA-sequencing data from 200-cell 'mini-bulks' of CD34+ HSPCs from patients with AP/BP-MPN (n=10, pre-treatment) versus healthy controls (n=5, Log_2FC 2.09; $P = 0.01$, fig. S7B) (*46*).

Interrogating a scRNAseq dataset capturing both HSPCs and total MNCs from healthy donors (13,713 cells) and patients with AP/BP-MPN (44,107 cells) revealed significantly increased *LGALS1* expression in HSCs, multipotent progenitors and megakaryocyte-erythroid progenitors ($P < 0.0001$) but not granulocyte-monocyte progenitors or mature blood cell lineages (fig. S7C). Collectively, these results highlight galectin-1 as a central pathological mediator in myeloid malignancies, a promising biomarker, and a therapeutic target that may alter the disease course, which is not possible to achieve for the majority of patients using currently available medical therapies.

DISCUSSION

MPNs are inflammatory pathologies that result in a large burden of morbidity and mortality. The majority of patients present with early-stage malignancies, presenting an opportunity for intervention. However, at present, there are no drug therapies that robustly impede or reverse progression to fibrosis, and a more detailed understanding of the genetic and non-genetic drivers of MPN progression is crucial. In this study, we present a comprehensive road-map of the cellular composition of myelofibrotic bone marrow, providing a platform for the discovery and characterization of cellular and molecular targets for therapy. Although prior studies highlighted important aspects of disease pathophysiology (*9, 18, 21*), these datasets have not simultaneously captured hematopoietic and stromal cells, precluding accurate delineation of the multi-lineage interactions that occur between myeloid cells of the MPN clone and components of their niche. The analyses presented here revealed perturbations to cellular frequencies and transcriptional phenotypes that were previously unappreciated, noting that an expansion of basophils, mast cells and a distinct subset of inflammatory fibroblasts collectively underlie pathogenic cellular interactions in myelofibrosis.

In individuals who acquire an MPN cancer driver mutation, the inflammatory microenvironment is an important determinant of clinical phenotype, symptom severity and the risk of disease progression (*47*). The same mutations can present with diverse clinical phenotypes, including in healthy individuals without overt hematologic disease (*48*). Although specific genetic contexts (high molecular risk mutations like concurrent ASXL Transcriptional Regulator 1 (*ASXL1)*, Serine And Arginine Rich Splicing Factor 2 (*SRSF2)* mutations or a high *JAK2V617F* allele burden) increase the likelihood of progression to fibrosis, these are not essential, suggesting a major role for cell-extrinsic signaling in driving disease evolution. Recent studies revealed that MPN driver mutations are typically acquired early in life, often several decades before clinical presentation (*49-51*), yet myelofibrosis usually presents in the later decades of life. One explanation for the long latency observed between mutation acquisition and clinically overt disease is that the composition and function of the bone marrow stroma becomes more permissive for MPN outgrowth with age. A pro-inflammatory, TGFβ-rich stroma (*52*) and reduced MSC-derived hematopoietic support factors (*53*) develop with physiological ageing and induce a myeloid bias even in individuals without an MPN driver mutation. Here, we show that an MPN induces an exacerbation of the inflammatory and myeloid-biased hematopoiesis phenotype that occurs as part of healthy ageing (*54*), encouraging speculation that aging might accelerate the development of the self-reinforcing, malignant niche in myelofibrosis (*55*).

We demonstrated the utility of the dataset in identifying clinically-actionable targets by focusing on galectin-1, a β-galactoside binding protein that has been previously implicated in cancer, tissue fibrosis and immunoregulation (*56, 57*) although its role in myeloid malignancies has not been fully investigated. Exploration of galectin-1 expression in large patient cohorts showed a clear association with fibrosis progression and correlation with survival in patients with myeloid leukemias. A functional role for galectin-1 was confirmed, using 2D and 3D in vitro models of bone marrow fibrosis and also in vivo by demonstrating efficacy of a neutralizing anti-galectin-1 mAb (*36*).

Previous studies have suggested modes of action for galectin-1 that may be relevant in myeloid malignancies. Galectin-1 has been identified as a mediator of TGFβ- and hypoxia-induced lung fibrosis (*41*), and direct anti-proliferative effects have been shown using shRNA knock-down of galectin-1 as well as treatment with OTX008, a small molecule inhibitor that reached phase I clinical trials for patients with advanced solid tumors. Proliferative effects are mediated by ERK1/2 and AKT-dependent survival pathways, and galectin-1 inhibition induces of G2/M cell cycle arrest (*58*). Immunomodulatory activities are well documented for galectin-1, which acts as a suppressor of T cell anti-tumor immunity (*59*), enhances regulatory monocyte/macrophage subsets (*60*), promotes tolerogenic dendritic cells and in certain scenarios has been shown to trigger damage-associated molecular pattern (DAMP) pathway activation (*61*). Galectin-1 is a transcriptional target of NFκB, and its expression and release are enhanced through TNF signaling and NFκB (*62*). We show that a feedback loop exists wherein expanded basophil, mast cell, megakaryocyte and stromal cell subsets induce a self-reinforcing pro-inflammatory niche and galectin-1 expression, fueling inflammation and fibrosis. Targeting galectin-1 using small molecule glycan inhibitors, natural polysaccharides, peptides (OTX008) or anti-galectin-1 monoclonal antibodies may counteract fibrosis and also the immunomodulation that occurs in myeloid neoplasms (*61, 63*).

Our study has several limitations. Additional work is required to determine the mechanisms of action for galectin-1 in myeloid neoplasms. Further validation of the efficacy of galectin-1 targeting in additional disease models and identification of the most clinically-tractable targeting modality are also required. In addition, the mechanistic role for galectin-1 in leukemic progression is unknown.

Collectively, the data presented here confirm a role for galectin-1 as a mediator of pathobiology in myeloid malignancies and worthy of further exploration as a therapeutic target that has the potential to modify the disease course. The road-map of cellular interactions in myelofibrotic bone marrow has broad implications for other hematological malignancies, cancer-associated inflammation and non-malignant fibrotic disorders.

MATERIALS AND METHODS

Study Design

The overall aim of this study was to identify mediators of the pathological crosstalk between hematopoietic and stromal cells in myelofibrotic bone marrow. Power calculations were performed to measure a 50% difference (effect size=2), with at least 2 independent experiments of 5 mice per cohort to ensure reproducibility, based on variability observed in our experience using MPN models and published literature. Mice were randomized for treatment with either IgG isotype control or Gal-1-mAb3 within the control or MPL^{W515L} groups. Mouse and human primary cells were used for in vitro assays, and all in vitro experiments were performed in a minimum of 3 independent experiments each with appropriate controls to ensure reproducibility. Experiments were unblinded. The exact sample size used for each experiment is indicated in the respective figure legend and in the results section.

Animal Studies

All mice were bred and maintained in accordance with UK Home Office regulations, and experiments were conducted in accordance with approvals from the University of Oxford Animal Welfare and Ethical Review Body (project license P22FF90EE8). For transplantation experiments, C57BL/6OlaHsd (CD45.2) mice were used as donors, and CD45.1 B6.SJL-Ptprca Pepcb/BoyJ mice were used as recipient mice and as a source of competitor cells to establish the MPL^{W515L} murine model (*14*).

In vivo treatments

The anti-galectin-1 neutralising antibody (mAb3) was produced as previously reported (*36*). Seven days after transplantation, either an isotype IgG control antibody or mAb3 were administered in MPL^{W515L}

and control mice twice weekly for 14 days by intraperitoneal (i.p.) injections, at alternating locations at 25 mg/kg. For comparison with a standard-of-care agent, we treated a subset of mice with fedratinib (MedChemExpress). In this cohort, seven days after transplantation, PBS or fedratinib (120 mg/kg) (*64*) were administered 5 days per week for 2 weeks by once daily oral gavage treatment.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (9 or later version) or R (version 3.6.1 and 4.0.4) software. Unpaired t test with Welch's correction was used to compare differences in mice spleen weights and blood counts (Fig. 1B and fig. S1A) and in basophils IL4 and TNF expression (Fig. 5E) from control and MPL^{W515L} mice and for differences in mice spleen weights and blood counts in MPL^{W515L} mice treated with isotype IgG control antibody and mAb3 or Fedratinib (Fig. 6C, E and F). Wilcoxon test was used to compare ECM genes (Fig. 2D and E), NSF score (Fig. 3A and B), chemokine score (Fig. 3E), *Acta2* (Fig. 3C) and *Lgals1* (Fig. 5H) expression in relevant cell clusters from control versus MPL^{W515L} mice. The Kruskal-Wallis test was used to compare Gal-1 protein expression in immunohistochemistry of human bone marrow biopsies (Fig. 7A). The Wilcoxon test was used to assess differences in low-input proteomics results (Fig. 2F and fig. S2E) and to analyse the response to treatments in the in vitro fibrosis assay (Fig. 7E and fig. S6B and S6C). A one-way ANOVA with Tukey's post hoc test was used to compare the impact of inhibitors on TGFβ-induced fibrosis in bone marrow organoids (Fig. 7F and 7I). All bar charts show mean ± standard error of the mean. The number of independent experiments, donors and replicates for each experiment are detailed in figure legends. Methods for computational analysis of scRNA seq data, correlating galectin-1 expression with AML survival from TCGA AML dataset and GSEA are described in the Supplementary methods.

Mpl^{W515L} murine model

To produce retroviral supernatant, transient co-transfection of HEK293T cells with pCL_Ampho retroviral packaging vector and the MSCV-*MplW515*-IRES-EGFP were performed using PEI Pro (Polysciences, Inc) according to manufacturer's guidelines. The MSCV-IRES-EGFP empty vector was used as control. The retroviral supernatant was collected 48h or 72h post transfection, filtered and stored at -80°C.

For each experiment, bone marrow cells were harvested from 7-8 weeks old C57BL/6OlaHsd (CD45.2) female mice by isolating and crushing the bones. Bone marrow cKit+ cells were enriched using mouse CD117 microbeads (Miltenyi Biotec), and pre-stimulated in IMDM (Gibco) with 10% FBS (Sigma Aldrich), 100 ng/ml stem cell factor (SCF) (Peprotech), 20 ng/ml Flt-3 ligand (Flt3L) (Peprotech), 20 ng/ml interleukin (IL)-3 (Peprotech) and 20 ng/ml IL-11 (Peprotech) for 24 hours. 12-well tissue culture plates were coated with retronectin (Takarabio) and left overnight at 4°C. After 24 hours, retronectin-coated wells were pre-loaded with GFP only or *MPL^{W515L}*-GFP virus supernatant by centrifugation at 2000rpm for 60 min at 4 °C. Meanwhile, the pre-stimulated cKit+ cells were harvested and resuspended in IMDM with 10% FBS, 200 ng/ml SCF, 40 ng/ml Flt-3 Flt3L, 40 ng/ml IL-3 and 40 ng/ml IL-11. The virus supernatant was removed from the wells, and the pre-stimulated cKit+ cells were added, together with the same volume of GFP or *MPL^{W515L}* virus supernatant into the preloaded plate. After 24 hours of transduction, cells were washed 3 times and intravenously injected along with wild-type CD45.1 B6.SJL-Ptprca Pepcb/BoyJ bone marrow cells into lethally irradiated (2 × 4.5 Gy [450 rad]), CD45.1 B6.SJL-Ptprca Pepcb/BoyJ recipient mice. Animals were humanely killed when they had palpable splenomegaly or were moribund. Peripheral blood was collected from the tail vein or via cardiac puncture into EDTA-coated microvettes (Sarstedt) and diluted five times in PBS prior to analysis on a Pentra ES 60 Cell Counter (HORIBA ABX SAS). Mice were monitored and culled if the body weight loss was more than 15%. For the MPN-free survival curve analysis,

MPN was defined as white blood cell count ≥ $60*10^9$/L, HGB ≤ 10 g/dL or ≥ 15 g/dL, platelet count ≥ $1600*10^9$/L or spleen size > 1g.

Immunohistochemistry

Mouse bones and spleens were fixed using 4% Formaldehyde solution and processed for IHC, or hematoxylin/eosin stain, or reticulin staining. Human bone marrow biopsy samples were fixed in 10% neutral buffered formalin prior to decalcification in 10% EDTA for 48 hrs. Histopathological diagnosis was carried out according to the WHO classification (*65*). Galectin1 staining was performed using 1:400 Anti-Galectin-1 antibody (Abcam). Antigens were visualized using diaminobenzidine (DAB) as chromogen.

Murine stromal cells isolation

Murine bone marrow stromal cells (BMSCs) were isolated as previously described (*15*). In brief, long bones were flushed and the central bone marrow was digested with 2mg/ml Collagenase IV (Thermo Fisher Scientific) at 37°C for 20min. The bones were cut or crushed and digested with 3mg/ml Collagenase I (Thermo Fisher Scientific) at 37°C for 1.5h. Cells were pooled, treated for 10 min with NH4Cl solution (STEMCELL Technologies), washed with PBS and CD45 negative cells were enriched using mouse CD45 microbeads (Miltenyi Biotec) depletion of CD45+ cells.

Fluorescent activated cell sorting (FACS) for single cell RNA sequencing

To capture stromal cells, CD45+ cell bead-depleted cells were incubated with Fc block for 15 minutes at 4°C. Cells were then stained with anti-mouse CD45, anti-mouse hematopoietic lineage marker cocktail (CD11b, CD3e, CD19, B220, Gr1), anti-mouse Ter119 and anti-mouse CD71 for 20 min at 4°C in

PBS 1% FCS 2mM EDTA (Thermo Fisher Scientific), antibody details in Supplemental Table 1. DAPI (Sigma Aldrich) was added prior to analysis and sorting as a cell viability dye. Stromal cells were identified as DAPI-CD45-Lin- Ter119- CD71- and were sorted into 2µL PBS/0.05% BSA (non-acetylated) on an Aria™ Fusion Cell Sorter (BD Biosciences).

To capture hematopoietic cells, bone marrow cells obtained by crushing the femurs, tibias and cristae were suspended in PBS + 1% BSA and incubated with Fc block for 15 minutes at 4°C. Antibody staining was performed using the antibodies listed in Supplemental Table 1 for 20 min at 4°C in PBS + 1% FCS + 2mM EDTA and sorted into 2µL PBS + 0.05% BSA (non-acetylated) on an Aria™ Fusion Cell Sorter (BD Biosciences). The following populations were sorted: (i) Total viable mononuclear cells (DAPI- GFP+); (ii) HSC/MPP or lineage negative, cKit+ cells (DAPI- GFP+ Lin- CD117+ cells) and (iii) CD41+ cells (DAPI- GFP+ CD41+). The CD41+ fraction was sorted using a 130nm nozzle. All other hematopoietic and stromal cell populations were sorted using a 100nm nozzle. Cell number and volume was adjusted prior to loading onto the 10x Chromium Controller for droplet generation.

High throughput single cell transcriptomics sequencing (10x Genomics)

FACS sorted cells from each sample were processed according to the 10x Genomics protocol using the Chromium Single Cell 3' library and Gel Bead Kits v3 (10x Genomics). Briefly, cells and reagents were prepared and loaded onto the chip and into the Chromium Controller for droplet generation. RT was conducted in the droplets and cDNA recovered through demulsification and bead purification. Pre-amplified cDNA was used for library preparation, multiplexed and sequenced aiming to obtain > 50,000 reads per cell.

Single cell transcriptomics analysis

We used CellRanger software version 3.0.1 (10x Genomics) to obtain cell counts using "cellranger count" command to align the reads to the mm10 genome to identify cell barcodes and generate the expression matrix. Single-cell RNA sequencing analysis was performed using SingCellaR software (v1.2.0)(*66*) . Briefly, we analyzed the cells that passed the following QC parameters: min UMI counts > 1,000 and ≤ maximum UMIs; min number of detected genes > 500 and ≤ maximum number of detected genes and genes expressed at least in 10 cells and 10% as the mitochondria cut-off. Then, individual objects were integrated and highly variable genes were identified using the 'get_variable_genes_by_fitting_GLM_model' function, retaining 1536 highly variable genes for stromal populations and 1306 genes for haematopoietic populations respectively for downstream analysis. Stromal and haematopoietic populations were analysed separately to ensure that all the cells were correctly clustered and annotated. Principal component analysis (PCA) was performed using the top 50 PCs and the Harmony method was used ('runHarmony' function in SingCellaR) on the top 30 PCs to integrate the datasets and correct the batch effects for downstream analyses including UMAP analysis, Louvain clustering and cell type annotations. Cell types were annotated by combining three strategies: 1) annotation of the clusters by canonical marker genes; 2) implementation the semi-automatic annotation method in SingCellaR; 3) visualization of multiple lineages genesets on top of the UMAP plot using 'plot_umap_label_by_multiple_gene_sets' function.

Hematopoietic 'contamination' removal in the stromal samples

We aimed to investigate the gene expression profiles of stromal cells in the bone marrow, however, a key challenge was the potential 'contamination' of hematopoietic cells in the stromal samples. To solve this problem, we obtained a list of cell-type-specific marker genes for hematopoietic cells and stromal cells from previous studies (*15-17*) and then performed two rounds of removal of hematopoietic cell clusters.

Doublet removal

We implemented a two-step doublet removal method to ensure data quality. First, we applied the Scrublet algorithm to each individual sample as per the documentation. Secondly, the doublets were projected onto the UMAP plot of integrated objects to visualize the doublets. We then examined the expression of multiple genes lineages using the 'plot_umap_label_by_multiple_gene_sets' function in SingCellaR to confirm their cellular identities. We identified two additional doublet clusters in hematopoietic populations (Fig. S1C). We removed the doublets identified by Scrublet (279 cells in hematopoietic populations and 89 cells in stromal populations) and the doublet clusters (374 cells in hematopoietic populations, 0 cell in stroma population). Following doublet removal, the objects were used for differentially expressed genes analysis by standard SingCellaR workflow.

Symphony analysis

We applied Symphony (*22*) to overlay published healthy and myelofibrotic mouse bone marrow stroma and hematopoietic scRNAseq datasets onto our datasets. We first used 'buildReference' function to build the reference UMAP plots using the control mice only stroma, control + *Mpl*^{W515L} stroma and all hematopoietic cells in our study and colored by the annotated cell types. Then we used 'mapQuery' function to perform dataset projection.

Differential abundance test

We performed differential abundance testing using the MiloR software (v0.1.0) (*67*) between Control and *Mpl*^{W515L} mice for MNCs in the hematopoietic populations and stroma cells respectively. MiloR is an R package designed for differential abundance testing in single-cell transcriptomics analysis. We created a miloR object using the 'Milo' function. 30 dimensions were used to calculated neighborhood

distance. We then used the 'buildNhoodGraph' function to perform differential abundance testing between different clusters. The differential abundance test generated a list of significantly differentially abundant fractions with their respective P-values and fold changes.

Extra cellular matrix (ECM), chemokine and Niche Supporting Factor (NSF) scores

We analyzed the expression of ECM factors from a previously published database (*25*)in each single cell by defining an ECM score using the total expression of expressed ECM genes divided by total expression of all the genes within a cell. The expressed ECM genes were defined as having a minimum count of 50 UMIs across all cells in hematopoietic populations or stromal populations. For the chemokine and NSF gene scores, we curated the genes from published studies (Supplemental Table 4) and analyzed the NSF gene score as described for the ECM score.

Gene set enrichment analysis (GSEA)

We used the 'Run_fGSEA_analysis' function to compare two groups of cells in SingCellaR. Genes were pre-ranked using the function 'identifyGSEAPrerankedGenes'.

Differentiation state analysis

We applied CytoTrace (*68*) on the EBM cluster to investigate the differentiation state. Briefly, we extracted the expression matrix (counts) from the SingcellaR object and then used the function 'CytoTRACE' to calculate the CytoTRACE score for each cell. This score implies the differentiation state for each cell. To visualize the result on a 2D plot, we plotted a 2D plot with UMAP embeddings of the EBM object.

scTour anlaysis

scTour (*69*) is a method for dissecting cellular dynamics. We extracted the expression matrix (counts), metadata and UMAP from the SingCellaR fibroblast object and transformed to AnnData. Then

we counted the number of genes detected in each cell and trained the model using negative binomial distribution as the loss function to get the pseudotime variable for each cell. We further counted the latent representations and transcriptomic vector field and projected it onto a UMAP embedding.

Cell-cell interaction analysis

We applied CellChat (*32*) to analyze the cellular interactions. We first built a customized ligand-receptor database followed by the tutorial and merged the bone marrow stroma and hematopoietic cells into one object and normalized the counts. The objects were then split to 'Control' and 'MPL^{W515L}' groups and the cellular interactions were analyzed separately. To compare the differential interactions between 'Control' and 'MPL^{W515L}' groups, we used the 'mergeCellChat' function to merge the 'Control' and 'MPL^{W515L}' Cellchat objects and compared the interaction by the 'compareInteractions' function. We further applied the 'netVisual_heatmap' function to show the differentially expressed number of interactions of MSC, iFibs, other Fibs, EBM and MK. Selected differentially expressed ligand-receptor pairs were shown in Fig. 5E using the 'netVisual_bubble' function.

Fluorescent activated cell sorting (FACS) for low-input proteomics

BM stromal cells from n = 4 GFP control mice were isolated as reported below and then stained at RT for 30 minutes with the following antibody panel: AF700 anti-CD45, PerCP Cy5.5 anti-TER-119, BV605 anti-Sca-1, PE Cy7 anti-CD31, Biotin anti-LEPR/Streptavidin PE CF594, APC anti-PDGFRA, and PE anti-CD71. DAPI was used as a live-dead marker. Fibroblasts were defined as Ter-119$^-$CD71$^{-/low}$-CD45$^-$PDGFRA$^+$, Endothelial cells (ECs) as Ter-119$^-$CD71$^{-/low}$-CD45$^-$PDGFRA$^-$Sca-1$^+$CD31$^+$ and leptin receptor positive mesenchymal stromal cells (LEPR+ MSC) as Ter-119$^-$CD71$^{-/low}$-CD45$^-$PDGFRA$^-$CD31$^-$LEPR$^+$. For each population, 500 cells per well were sorted into 384-well plates (Eppendorf twin.tec 384 LoBind) containing 1µl of lysis buffer (0.2% DDM, 80mM TEAB). After sorting, the plates were briefly spun, snap-frozen on dry ice, and boiled for 5 minutes at

95°C. Subsequently, the plates were cooled on ice, briefly spun again, and stored at -80°C until further analysis.

Low-input proteomics

Low input proteomic on the different cells populations was performed as previously reported (*72*). Briefly, protein digestion was conducted overnight at 37 °C by adding Trypsin (Promega) at a concentration of 10 ng/uL in 100 mM TEAB (pH 8.5) and subsequently stopped by the addition of 4 % (v/v) trifluoroacetic acid (TFA). Prior to mass spectrometry analysis, digested samples were loaded on Evotip pure (Evosep) columns for online desalting following the manufacturers recommendations. Chromatographic separation of peptides derived was conducted over a 58-minute gradient on an EvosepOne UHPLC system (Evosep) connected to a 15 cm Aurora Elite TS (Ion Opticks) maintained at 50 °C. Following ionization, MS-spectra were collected using a Orbitrap Eclipse Tribrid mass spectrometer equipped with FAIMS Pro interface (Thermo Scientific) and operated in positive mode with a compensation voltage of -45 V. MS1 spectra were collected in the Orbitrap at a resolution 120k and a mass range of 400 to 1000 Th. Automatic gain control (AGC) was set at 300 % and a maximum injection time set to 246 ms. Fragmentation of precursor ions was achieved through higher energy collisional dissociation (HCD) using a normalized collision energy of 33%. Data-independent acquisition was conducted in the Orbitrap at the same resolution utilizing loop control set to 12 spectra per loop and isolation windows of 17 Th over a mass range of 200 to 1200 Th resulting in 36 windows across all looped cycles. For this, AGC was set at 1000 % and the maximum injection time was configured to automatic.

Mass spectrometry data analysis

Obtained .raw files were processed with Spectronaut (v.18) in directDIA mode using standard settings with the following modifications: Quantity MS level was changed to MS1 and Carbamidomethylation of cysteines was removed as fixed modification. Protein quantification matrices were then exported and further downstream analysis. Log normalization was performed to stabilize the variance and reduce skewness throughout the dataset. The data was subsequently scaled to a fixed range (0 – 1) unsing min-max normalization to ensure that all protein expression amounts levels were on a comparable scale. This dual normalization approach facilitated the precise comparison of protein expression across the different samples. A Wilcoxon test was conducted to ascertain the differences in protein abundance between cell types after normalization steps.

Intracellular flow of basophils for IL4 and TNF

For analysis of basophils differentiated *in vitro*, cKit+ murine stem/progenitor cells were transduced with *MPL^{W515L}-GFP* or control-GFP vectors and cultured as previously described (*70*) at a density of 2 x 10^6 cells/ml in IMDM containing 10% FBS, 1% Pen/Strep and 10% conditioned media from BHK/MKL cells (as a source of SCF), 150 uM monothioglycerol (Sigma) and 10 ng/ml m-IL3 (PeproTech). Every 2 to 3 days cells were transferred into new media, and basophils analysed on day 7 of the differentiation (defined as live cells, GFP+, FcER1a$^+$/CD117$^-$). For *ex vivo* analysis of bone marrow basophils, flushed bone marrow cells were gated as 7AADneg, GFP$^+$, lineage (B220, CD3, CD11b, Gr-1, Ter-119) neg, FcER1a$^+$ CD117neg. Expression of IL4 and TNF was analysed by intracellular flow cytometry. In brief, cells were transferred into 96-well plates and incubated with 1 µl/ml BD GolgiPlug for 4 hours at 37° Celsius. Following live dead staining with Zombi Red (BioLegend) and subsequent staining of cell surface antigens (CD117, SiglecF and

715 FcER1a), cells were then fixed and permeabilised using the BD Cytofix/Cytoperm™
716 Fixation/Permeabilization Kit, and IL4 and TNF antibodies were added (Table S1). Samples were
717 acquired using the LSR Fortessa X-20 (BD) and data were analysed with FlowJo.

718 **Banking and processing of human samples**

719 Patients and healthy donors provided written informed consent in accordance with the
720 Declaration of Helsinki for sample collection, tissue banking and use in research under the
721 Informed study (Investigating the genetic and cellular basis of sporadic and Familial Myeloid
722 Disorders; IRAS ID: 199833; REC reference: 16/LO/1376; PI: Prof AJ Mead). Cryopreserved bone
723 marrow mononuclear cells isolated by density gradient centrifugation using Ficoll-Paque
724 Premium (Sigma Aldrich) were cryopreserved in FCS with 10% DMSO (Sigma Aldrich) and thawed
725 and processed by warming briefly at 37°C, gradually diluted into RPMI-1640 (Gibco),
726 supplemented with 10% FCS and 0.1mg/mL DNase I (Sigma), centrifuged at 500G for 5 minutes
727 and washed in FACS buffer (PBS + 2mM EDTA + 5% FCS).

728 Primary human BMSCs were isolated as previously reported (*71*). Briefly, cryopreserved
729 mononuclear cells from bone marrow aspirates were thawed and cultured in αMEM (Gibco)
730 supplemented with 10% FBS for 3-4 days. Subsequently non-adherent cells were removed,
731 whereas stromal cells were selected by their adherence to plasticware.

732 For analysis of galectin-1 mRNA expression in platelets, published platelet RNA sequencing data
733 was analyzed (*38*). Eligibility criteria included age ≥18 years and Stanford MPN clinic diagnosis of essential
734 thrombocythemia, polycythemia vera or myelofibrosis (defined using the consensus criteria at the time
735 of this study). For healthy controls, blood was collected from twenty-one asymptomatic adult donors
736 selected at random from the Stanford Blood Center. All donors provided written consent for genetic

research. For both MPN patients and healthy controls, blood was collected into acid citrate-dextrose (ACD, 3.2%) sterile yellow-top tubes (Becton, Dickinson and Co.) and platelets were isolated by established (*72-75*) purification protocols. Blood was processed within 4 h of collection for all samples. The time from whole blood collection to platelet isolation was similar between healthy donors and MPN patients.

Fibroblast to myofibroblast transition assay using human BMSC

BMSCs were seeded into collagen-treated 348 wells imaging plates (Corning cat # 356667) at 5000 cells/well and cultured for 24h in αMEM (Gibco) supplemented with 0.3% FBS, 200µM Hepes (Gibco), 50µM ß-mercaptoethanol (Gibco) and 30µg/mL ascorbic acid (Sigma Aldrich). Then media was replaced, and cells were cultured for 72h in FMT media in presence or absence of 10ng/ml TGFβ (Biolegend), 4µM OTX008 (MedChemExpress), or 20µM SB431542 (MedChemExpress). At the end of the assay, cells were fixed in cold methanol, blocked with 6% FBS in PBS and then stained for 1h with the primary antibodies for aSMA (Sigma Aldrich, 1:500) and Collagen 1 IgG1 (Sigma Aldrich, 1:4000) for 1h at RT. After that, wells were washed for 3 times with PBS and incubated with the secondary antibody Alexa488 (Thermo Scientific, 1:2000) or Alexa 568 (Thermo Scientific, 1:1000) – at RT for 2h. DRAQ5 was used to stain the nuclei. Images were acquired using the IN Cell Analyzer 6000 (GE Healthcare).

For image analysis, we used a bespoke imaging analysis program to automate calculation of the mean intensity of fluorescence for collagen 1 per well. The program takes paired grey-scale images for nuclei and collagen 1, counts the number of cells (nuclei) using edge detection and calculates the mean intensity for collagen 1 staining per cell. The source code is available at https://zenodo.org/records/13349071.

***Lgals1* expression in TNF-treated human bone marrow organoids**

Human bone marrow organoids were derived from human induced pluripotent stem cells as previously described (*42*). Mature organoids (day 18-24) were treated with TNF for 18-20h with 2ng/ml, 20ng/ml and 50ng/ml TNF (Life). After treatment, organoids were collected for RNA extraction and qRT-PCR.

Fibrosis assay with human bone marrow organoids

Human bone marrow organoids were derived from human induced pluripotent stem cells (hiPSCs) as previously described (*42*). At day 18 of the differentiation protocol, organoids were cultured for 72h with 10ng/ml TGFβ (Biolegend) in presence of 30µg/ml ascorbic acid (Sigma Aldrich), followed by 72h in the presence or absence of 10 ng/ml TGFβ, 4µM or 8µM OTX008 (MedChemExpress), or 20µM SB431542 (MedChemExpress). After treatment, organoids were collected for either fixation and imaging, or digested with 5mg/ml Collagenase II in HBSS (Sigma Aldrich) for 20min at 37°C with gentle agitation to perform RNA extraction and qRT-PCR.

For imaging, organoids were fixed in 4% PFA for 30 minutes with gentle agitation before a series of PBS washes. Washed samples were blocked in 2% goat serum, 1% BSA, Triton X100 (Sigma Aldrich), 250µL Tween-20 (Sigma Aldrich), and 500 µL sodium deoxycholate (w/v) (Sigma Aldrich) in PBS before labelling in blocking buffer with the same antibodies used in the FMT assay. Labelled samples were then embedded in low molecular weight agarose and subject to a serial dehydration and ethyl cinnamate clearance before imaging on a Zeiss LSM 880 AiryScan confocal (*42*). For image analysis, images were processed using ImageJ/Fiji. Z stacks were subject to a maximum intensity projection before denoising and background subtraction (rolling ball). Regions of interest were drawn around organoids, and the fluorescence intensity calculated per organoid.

RNA extraction and qRT-PCR

Total RNA was isolated using the Qiagen Mini RNA isolation kit (Qiagen) and cDNA was prepared using EvoScript Universal cDNA Master (Roche) according to manufacturers' instructions. Quantitative real time PCR (qRT-PCR) was performed on a StepOne plus machine (Applied Biosystem) using the 2-ΔCt analysis method. Details of the TaqMan gene expression assays (Thermofisher Scientific) used are available in Supplemental Table 1.

Continuous Indexing of Fibrosis (CIF) scores assessment

A Learning to Rank (LTR) strategy known as RankNet (*76*)is used to assess the severity of fibrosis within and between myelofibrosis grades (MFs). The RankNet model predicts the order in which features are ranked according to their severity. A Convolutional Neural Network (CNN) is used as a feature extractor for a model to learn to rank because of its high performance in many applications, especially in medical image analysis (*77*). Therefore, the Ranking-CNN model was developed by combining RankNet with a CNN (*37*). The trained model then outputs the score. This was used as a reference for fibrosis severity which is called Continuous Indexing of Fibrosis (CIF) scores (*37*). CIF scores approach 1 when the sample is more fibrotic. To visualize the spectrum of the fibrosis within the sample, a map of fibrosis severity is generated using CIF scores.

Galectin-1 quantification

To quantify galectin-1 expression, we identified pixels with positive staining in the tissue. We applied stain deconvolution (*78*) to computationally separate the galectin-1 stain channel from the DAPI stain channel and employed stain normalization (*79*) to address staining variability across different sample batches. To identify galectin-1-positive pixels, we obtained the galectin1 pixel intensity distribution from all tissue samples and used the Otsu method (*80*) to determine an appropriate cut-off. A heatmap showing

the level and variation of galectin1 expression within the same tissue is generated by calculating the ratio of galectin1-positive pixels in multiple small tissue areas (256-by-256 microns).

Analysis of *LGALS1* in human *de novo* AML and leukemic transformation of MPN

TCGA AML patient survival data and gene expression values were retrieved as previously described (*81*). In total, 132 patients with survival and gene expression data were available and included for survival and gene set enrichment analysis (GSEA) (*82*). To interrogate *LGALS1* expression in patients with blast phase MPN, we explored published RNA-sequencing data of CD34+ HSPCs and total mononuclear cells from patients with accelerated/blast phase MPN (AP/BP-MPN, n = 10) and healthy donors (n = 5) (*46*).

For survival analysis, we stratified the patients into *LGALS1*-high and *LGALS1*-low based on the median gene expression value of *LGALS1* across the cohort. Cox proportional hazards regression model was fitted using the coxph function from the R package survival to estimate the hazard ratio of *LGALS1*-high patients by using *LGALS1*-low patients as the reference group. The Kaplan-Meier (KM) curve was plotted using the ggsurvplot function from the survminer R package.

For GSEA, we first identified differentially expressed genes between the *LGALS1*-high and *LGALS1*-low patient groups. To this end, we included genes that were expressed in at least 10% of patients in either group. A gene was considered to be expressed when its FPKM (Fragments Per Kilobase of transcript per Million mapped reads) value was at least 1 and above. The gene expression values were subsequently offset by +1 and log2-transformed. Then, the expression values of each gene were compared between the two patient groups using t-test and the log2(fold change) was computed for *LGALS1*-high relative to *LGALS1*-low patient group using the mean expression value of each patient group. Next, a score was generated for each gene by computing -log10(P value) and then multiplying by -1 if the log2fc < 0. This score was used to rank the genes from the most-regulated to most-downregulated in *LGALS1*-high relative

826 to *LGALS1*-low patient group, and subsequently used as input for GSEA software. Our ranked gene list was
827 assessed for enrichment of the HALLMARK gene set using RunGSEAPreranked option. Gene sets with false
828 discovery rate (FDR) < 0.25 were considered to be significantly enriched among our ranked gene list.

829

830 **List of Supplementary Materials**

831 Fig. S1 to S6

832 Data files S1 to S6

833 References (65-82)

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Acknowledgments

We thank Professor R. Levine for sharing the MPL^{W515L} plasmid, the patients who kindly consented to research and: P. Ciccone, N. Hayder and S. Reed who helped with sample banking; K. Clark, C. Waugh and P. Sopp in the MRC WIMM Flow Cytometry facility which is supported by the MRC Human Immunology Unit and MRC Molecular Haematology Unit; Dr. N. Ashley in the MRC WIMM Single Cell Facility; V. Millar (Target Discovery Institute, University of Oxford); I. Parisi (Histology Lab, Kennedy Institute); R. Beveridge (MRC WIMM Virus Screening Facility) and all the staff of the Biomedical Science Division, University of Oxford. We thank Professor J. Xu from University of Texas Southwestern Medical Center, for sharing the mouse HSPCs R objects (Liu et al, Nat Comms 2021) and Prof. A. Kusumbe from the MRC WIMM for her support with immunostaining. The computation in this research was supported by MRC WIMM server of University of Oxford and the Medical Science Data Center of Fudan University.

Funding

This work was supported by the Kay Kendall Leukaemia Fund (KKL1057) and Blood Cancer UK (project grants to B.P and A.J.M), the Chinese Academy of Medical Sciences (CAMS) Innovation Fund for Medical Science (CIFMS), China (Grant number: 2018-I2M-2-002 to R.L), CRUK Advanced Clinician Scientist

Fellowship (to B.P, Grant number C67633/A29034), CRUK Senior Cancer Research Fellowship (to A.J.M., Grant number C42639/A26988), Sir Henry Wellcome Fellowship (to A.O.K; 218649/Z/19/Z), the Novo Nordisk Foundation (NNF21OC0071016) and the Danish Cancer Society (R324-A17978) to E.M.S., Agencia Nacional de Promocion Cientifica y Tecnologica (PICT 2020-01552), Ministerio de Ciencia, Tecnología e Innovación (Redes Federales de Alto Impacto) and Fundacion Sales (the three to G.A.R.), and US National Institutes of Health grant 1K08HG010061-01A1 to A.K. The authors would like to acknowledge the National Institute for Health Research (NIHR), Oxford Biomedical Research Centre (BRC); John Fell Fund (131/030 and 101/517), the EPA fund (CF182 and CF170) and by the MRC WIMM Strategic Alliance awards G0902418 and MC_UU_12025, and the contribution of the WIMM Sequencing Facility, supported by the MRC Human Immunology Unit and by the EPA fund (CF268). The views expressed are those of the authors and not necessarily those of the National Health Service (NHS), the NIHR or the Department of Health.

Author contributions

R. Li performed in vitro and in vivo experiments, designed methodology, curated and analysed data, performed data visualisation, contributed to writing the original draft and editing and to funding acquisition. M. Colombo performed in vitro and in vivo experiments, designed methodology, curated and analysed data, performed data visualisation and contributed to writing the original draft and editing the manuscript. G. Wang performed and supervised the computational analysis, developed methodology, contributed to writing and editing the original draft of the manuscript and data curation. A. Rodriguez-Romera contributed to in vivo experiments, C. Benlabiod contributed to in vivo and in vitro experiments, validation of findings, curation and visualisation of data. N.J. Jooss contributed to in vivo and in vitro experiments, validation of findings, curation and visualisation of data. J. O'Sullivan contributed to project conceptualization. C. K Brierley contributed to computational analyses, data curation and analysis and

conceptualization. S.A. Clark performed to flow cytometry analysis and cell sorting and experimental design. J. M. Pérez Sáez provided resources (generating galectin-1 neutralising antibody). P. Aragón Fernández, E.M. Schoof and B. Porse contributed to experimental design, generation and analysis of low-input proteomics data. Y. Meng contributed to data generation for the in vivo experiments. A.O. Khan performed and analysed the human bone marrow organoid experiments and contributed to conceptualization. S. Wen contributed to data analysis. P. Dong, W. Zho and Q. Cheng performed computational analysis. N. Sousos contributed to collation and clinical annotation of human samples. L. Murphy contributed to data acquisition and imaging. M. Clarke collated the clinical information for human samples. A. Olijnik. Z. C. Wong, J. Carrelha, Z. Red, R. Norfo and C. Simoglou Karali contributed to in vitro and in vivo experiments. K. Sirinukunwattana, H. Ryou and D. Royston developed the methodology and performed the bone marrow immunohistochemistry analysis for galectin-1 quantification. S. Thongjuea developed computational methodology. V.A. Rathinam contributed to conceptualisation of in vivo galectin-1 targeting experiments. A. Krishnan generated, analysed and performed visualisation of the platelet transcriptomics data. G. A. Rabinovich contributed to conceptualization, methodology, supervision and project administration as well as provision of resources (galectin-1 neutralising antibody) and editing the manuscript. A. J. Mead and B. Psaila conceived of the project, acquired funding, supervised and administered the project, curated data, designed methodology, provided clinical samples and wrote and edited the manuscript. All authors read and approved the submitted manuscript.

Competing interests: B. Psaila is a co-founder and equity holder in Alethiomics Ltd, a spin out company from the University of Oxford, and has received research funding from Alethiomics, Incyte and Galecto, and honoraria for consulting and/or paid speaking engagements from Incyte, Constellation Therapeutics, Blueprint Medicines, Novartis, GSK and BMS. A. J. Mead is a co-founder and equity holder in Alethiomics Ltd, a spin out company from the University of Oxford, has received research funding from Celgene/BMS,

Novartis, Roche, Alethiomics and Galecto, and honoraria for consulting and/or speaker fees from Novartis, Celgene/BMS, Abbvie, CTI, MD-Education, Sierra Oncology, Medialis, Morphosys, Ionis, Mescape, Karyopharm, Sensyn, Incyte, Galecto, Pfizer, Relay Therapeutics, GSK, Alethiomics & Gilead. A.O. Khan has received honoraria for consultancy from Alethiomics Ltd. B. Porse has received honoraria for consulting from Trailhead Biosystem. J. O'Sullivan has received honoraria for consulting and/or speaking engagements from Constellation Therapeutics, Novartis, Karyopharm and Medscape. Two patents have been filed by A.O. Khan and B. Psaila relating to the human bone marrow organoids platform utilised in this manuscript (GB2202025.9, GB2216647 and 2402478.8). G. Rabinovich and J.M. Pérez Sáez are co-founders of Galtec and co-inventors of the US Patent 10294295B2: 'Methods for modulating angiogenesis of cancers refractory to anti-VEGF treatment'.

Data and materials availability: All data associated with this study are present in the paper or supplementary materials. All raw and processed sequencing data generated in this study have been submitted to the NCBI Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE228995 (data file S5) and processed objects can be visualized at https://mouse10x.shinyapps.io/myelofibrosis/. The corresponding sample information is contained in data file S5. All processed data and code is deposited at Zenodo https://doi.org/10.5281/zenodo.13318764 and https://doi.org/10.5281/zenodo.13341379 . The galectin-1 neutralising antibody was generated by G. Rabinovich and J.M. Pérez Sáez and provided to Oxford under a material transfer agreement. Raw data for experiments with n<20 is provided in data file S6.

Figure Legends

Figure 1. A cellular atlas of myelofibrotic bone marrow. (**A**) H&E (top) and reticulin stained (bottom) femur sections from control and MPL^{W515L} mice. Red arrows highlight megakaryocytes (top) and reticulin fibrosis (bottom), representative images shown. (**B**) Spleen weights (grams, g) with representative images of control (*n* = 24) and MPL^{W515L} (*n*=24) mice. ****$P < 0.0001$ for unpaired t test with Welch's correction. Mean ± SEM. (**C**) Schematic of experimental workflow to capture hematopoietic cells including lineage negative (lin-) cKit+ HPSCs, CD41+ and total mononuclear cells, as well as bone marrow stromal cells from control (*n*=12) and MPL^{W515L} mice (*n*=11) for single cell RNA sequencing. *n*=3 independent experiments. (**D)** Uniform Manifold Approximation and Projection (UMAP) of 34,969 stromal cells from 12 GFP control mice and 11 MPL^{W515L} mice, colored by annotated cell cluster. **(E)** Dot plot showing expression of canonical marker genes used to annotate stromal cells. (**F**) UMAP of 42,319 hematopoietic cells from 12 GFP control mice and 11 MPL^{W515L} mice, colored by annotated cell cluster. (**G**) Dot plot showing expression of canonical marker genes used to annotate hematopoietic cells. Abbreviations: BM, bone marrow; MNC, mononuclear cells; Fibro-chondro, fibroblast-chondrocytes; Chondro, chondrocytes; OLC, osteolineage cells; Fibro-osteo, fibroblast-osteoblasts; Fibro, Fibroblasts; MSC, mesenchymal stromal cells; A-endo, arterial endothelial cells; S-endo, sinusoidal endothelial cells; Neutro, neutrophils; GMP, granulocyte-monocyte progenitors; Pro-mono, monocyte progenitors; Mono/MG, monocyte/macrophages; HSC/MPP, hematopoietic stem and multipotent progenitor cells; MK, megakaryocytes; EBM, eosinophil, basophil, mast cells; DC, dendritic cells; B, B-cell; T, T-cell; Ery, erythrocytes. , neutrophils; GMP, granulocyte-monocyte progenitors; Pro-mono, monocyte progenitors; Mono/MG, monocyte/macrophages; HSC/MPP, hematopoietic stem and multipotent progenitor cells; MK, megakaryocytes; EBM, eosinophil, basophil, mast cells; DC, dendritic cells; B, B-cell; T, T-cell; Ery, erythrocytes.

Figure 2. Alterations to the cellular constituents of myelofibrotic bone marrow and source of extracellular matrix components. (**A)** Differential abundance of mononuclear cell (MNC) subsets and (**B**) stromal cell subsets in control (green) versus MPL^{W515L} mice (purple), shown with relevant UMAPs to indicate relative frequency of each cell type. Each dot in the differential abundance plots represents a KNN cluster of the indicated cell type, clusters marked green and purple indicate those significantly depleted or enriched in MPL^{W515L} mice respectively. Sinusoidal and arterial endothelial cells are merged (endo) for the purpose of differential abundance in panel B. (**C**) Derivation of the total bone marrow hematopoietic cells captured (full dataset) from the three flow cytometric sorting strategies for MNCs, lineage negative cKit+ HSPCs (LK) and CD41+ cells (CD41), indicating that eosinophil, basophil and mast (EBM) cells and megakaryocytes (MK) were primarily captured by the CD41+ cell sort. (**D and E**) UMAPs (left) showing expression of a gene set of extracellular matrix factors (ECM) in (**D**) stromal and (**E**) full hematopoietic cell dataset, with violin plots (right) showing expression in relevant cell clusters from control (green) and MPL^{W515L} mice (purple). Yellow diamond indicates mean value. ***$P < 0.001$ for Wilcoxon test. (**F**) Abundance of ECM proteins detected by low-input mass spectrometry proteomics in hematopoietic cells (CD45+), endothelial cells (EC), leptin receptor + mesenchymal stromal cells (Lepr+) and fibroblasts (fibro). **** $P < 0001$ for adjusted p value comparing each stromal cell subtype to CD45+ hematopoietic cells, n = 4 control mice.

Figure 3. Altered cellular sources of hematopoietic support factors and expansion of inflammatory fibroblasts in myelofibrosis bone marrow. (**A and B**) Uniform manifold Approximation and Projection (UMAP, left) and violin plots (right) showing expression of niche supporting factors (NSFs) in (**A**) stromal and (**B**) hematopoietic cell datasets. Violin plots show expression in mesenchymal stromal cells (MSC), fibroblasts (Fibro) and eosinophil, basophil & mast cells (EBM) from control (green) and MPL^{W515L} mice

(purple). ***$P < 0.001$ for Wilcoxon test. (**C**) MSCs from control (green) and MPL^{W515L} mice (purple) cluster separately, reflecting marked transcriptional reprogramming and myofibroblast trans-differentiation as indicated by increased *Acta2*. (**D**) Significantly enriched HALLMARK gene sets in MSCs from myelofibrosis mice. Selected gene sets shown. (**E**) UMAP showing 5 fibroblast sub-clusters. (**F**) Top 5 differentially expressed genes in each fibroblast subcluster. (**G**) Selected HALLMARK gene sets significantly enriched in Cluster 4 (FDR < 0.25), reflecting inflammatory fibroblast (iFib) phenotype. (**H**) UMAP from panel E highlighting the iFib cluster is shown on the left. The inset shows the UMAP embeddings of fibroblasts colored according to their derivation from MPL^{W515L} (purple) or GFP control (green) mice. Frequency of iFibs in MPL^{W515L} *vs*. control mice is shown in the bar chart on the right. (**I**) UMAP on the left shows the expression of chemokine genes in the fibroblast UMAP from panel E, and the violin plot on the right shows the chemokine score of iFibs in MPL^{W515L} *vs*. control mice. ***$P < 0.001$ for Wilcoxon test.

Figure 4. Expansion of pro-inflammatory basophils and mast cells in myelofibrosis. (A) UMAP showing annotated sub-clusters of cells from the eosinophil, basophil and mast (EBM) cell cluster. (**B**) CytoTRACE differentiation state analysis of EBM cells, with blue indicating primitive state and yellow showing differentiation trajectory. (**C**) UMAP identifying cells originating from MPL^{W515L} (purple) and control (green) mice. (**D**) Significantly (FDR < 0.25) enriched HALLMARK gene sets in basophils and mast cells from MPL^{W515L} versus control mice. (**E**) Expression of TNF-NFκB pathway genes projected onto the EBM cell UMAP.

Figure 5. Basophils and mast cells emerge as the 'hub' of TNF and interleukin 4 pro-inflammatory signaling. (**A**) Number of inferred Ligand (L) – Receptor (R) interactions in control and MPL^{W515L} mice bone marrow. (**B**) Differential number of L-R interactions in MPL^{W515L} versus control bone marrow. Total Number

of enriched L-R interactions is shown as a bar on the x/y axes and relative strength of the interactions (MPL^{W515L} versus control bone marrow) is shown in the heatmap for key stromal and hematopoietic cell populations. (**C**) Circus plot depicting interaction pathway of TNF and (**D**) IL4 uniquely upregulated in MPL^{W515L} mice. The width of the connections reflects the strength of the interactions between two populations. (**E**) Percentage of TNF-positive (left) and IL4-positive (right) basophils in MPL^{W515L} versus control bone marrow (n=4) assessed by intracellular flow cytometry. Data are shown as mean ± SD* $P <$ 0.05. (**F**) The left plot shows selected L-R interactions predicted to be upregulated in MPL^{W515L} mice between EBM cells ('sender' cells – i.e. source of ligands) and selected stromal 'recipient' populations (expressing cognate receptors, iFibs, all other fibroblasts and mesenchymal stromal cells [MSCs]) and the right plot shows L-R interactions between stromal populations ('sender' cells expressing the ligands) and EBM (recipents, expressing receptors). **(G)** Venn diagram showing distinct and overlapping differentially expressed genes in EBM, MSC and MK clusters. (**H**) Violin plots showing expression of *Lgals1* in EBM, MSC, MK and fibroblasts in control and MPL^{W515L} mice. Abbreviations: R – L, receptor-ligand; L – R, ligand-receptor; TNF, tumor necrosis factor alpha; IL, interleukin; EBM, eosinophil, basophil, mast cells; iFibs, inflammatory fibroblasts; MSCs, mesenchymal stromal cells; Fibro, fibroblast; HSC/MPP, hematopoietic stem and multipotent progenitor cells. ***$P < 0.001$; ns – non-significant for Wilcoxon test.

Figure 6. Inhibition of Galectin-1 ameliorates fibrosis and myeloproliferation in vivo (**A**) Schematic of treatment with isotype control (IgG) or anti-galectin-1 (anti-Gal1) mAb3, initiated on day 7 following transplantation of control or MPL^{W515L} BM cells in C57BL/6OlaHsd mice. (**B**) Representative H&E (top) and reticulin staining (bottom) of femur sections from MPL^{W515L} mice treated with IgG control or anti-Gal1 mAb3 at day 24. (**C**) Mean ± SEM platelet counts and hematocrit (HCT) in IgG or anti-Gal1 treated control (n=8 and n=8) and MPL^{W515L} mice (n=13 and n=11). *$P < 0.05$, **$P < 0.01$ for unpaired t test with Welch's

correction. (**D)** Representative images and weights (**E**) of spleens from IgG or anti-Gal1 mAb3 treated control (n=8 and n=8) and MPL^{W515L} mice (n=13 and n=11), at day 24. Data are shown as mean ± SD. (**F**) Mean ± SEM spleen weights of mice treated with PBS control (n=5) or the JAK2 inhibitor fedratinib (n=4). **P* < 0.05, ***P* < 0.01 for unpaired t test with Welch's correction.

Figure 7. Galectin-1 is a robust biomarker for fibrosis and poor outcomes in myeloid malignancies.

(A) Representative immunohistochemistry staining for galectin-1 (Gal-1) of bone marrow biopsy sections from healthy donors (HD) (n=7) and patients with myelofibrosis (MF) (n=14) . **(B)** Gal-1 expression per high power field (HPF) view in bone marrow biopsy sections from HDs (n=7) and patients with essential thrombocythemia (ET, n = 9), polycythemia vera (PV, n=7), and MF (n=14). Median ± 95% CI shown, *****P* < 0.0001 for Kruskal-Wallis test. **(C)** Gal-1 staining intensity correlated with reticulin fibrosis density across bone marrow biopsy sections from HDs and patients with MF. Color scale from blue to red as fibrosis density increases. Representative images shown. **(D)** *LGALS1* expression in platelets from a cohort of 120 HDs and patients with MPNs (HD=21, ET=24, PV=33, MF=42). Median ± 95% CI. (**E**) TGFβ-induced fibroblast to myofibroblast transition assay using human BMSCs treated with TGFβ alone ± OTX001 (galectin-1 inhibitor) or SB431542 (TGFβ inhibitor). Representative images shown for high-throughput, 384-well imaging plate (left). Each treatment was performed in quadruplicate and 4 images acquired per well (n=7 patients). Chart (right) shows MFI per cell for collagen 1 normalized to the no-TGFβ control. Data are mean ± SEM (n=7). **P* < 0.05 for wilcoxon matched pairs signed rank test. (**F**) Impact of OTX008 on TGFβ-induced Collagen 1 and aSMA in human iPSC-derived BM organoids. Representative images (left); Mean ± SEM for protein/mRNA expression quantification of Collagen 1/*COL1A1* (right). n=5-8 organoids from 3 independent experiments. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001 for one-way ANOVA and Tukey's post hoc test. (**G**) Bar chart showing relative proportion of cell subtypes from a previously published dataset of ~120,000 cells from human CD34+ hematopoietic stem/progenitor cells from patients with MF (n=15) and

age-matched controls (HD, n=6). (**H**) Enriched HALLMARK gene sets in EBM progenitor cells from patients with MF versus HD. Abbreviations: UMAP, Uniform manifold Approximation and Projection; EBM, eosinophil (eosino)-basophil (baso)-mast cells; MF, myelofibrosis; HD, healthy donors; NES, normalized enrichment score; FDR, false discovery rate. **(I)** Mean ± SEM *Lgals1* mRNA expression in human bone marrow organoids with/without treatment with TNF at doses shown. n=80 organoids across 2 independent experiments. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001 for one-way ANOVA with Dunnett's T3 post hoc test. (**J**) Kaplan-Meier survival curves showing correlation between high *LGALS1* expression and poor survival in 132 patients with acute myeloid leukemia (AML) in The Cancer Genome Atlas (TCGA) dataset. (**K**) Gene set enrichment analysis show significant enrichment of IL6-JAK-STAT3 signalling, inflammatory response and TNF signalling via NFKB in patients with high *LGALS1* expression in TCGA database (FDR < 0.001). Abbreviations: HR, hazard ratio; NES, normalized enrichment score; FDR, false discovery rate.