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Genetic deletion of P-selectin prevents fibrosis development by inhibiting the neutrophil megakaryocyte emperipolesis in the Gata1^{low} mouse model for myelofibrosis

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ABSTRACT

Myelofibrosis (MF) is a rare chronic hematological disorder, within the family of myeloproliferative neoplasms. The MF patients present clinical abnormalities such as anemia, and thrombosis, as well as alterations in the bone marrow (BM) microenvironment, an increased number of megakaryocytes (MKs), most of which are found in emperipolesis with neutrophils. In MF, the MKs emperipolesis is induced by an altered MK secretome, containing increased levels of pro-inflammatory cytokines, proteins, and growth factors such as interleukin-8 (IL-8) and P-selectin (P-sel). These, allow the altered cell-to-cell interactions and cause the transforming growth factor-β (TGF-β) to be released into the BM microenvironment. This fibrogenic cytokine contributes to BM fibrosis and disease progression. Emperipolesis has already been identified as a pathobiological event that contributes to MF and it is widely recognized in the most advanced stages of the disease. In this study, we evaluated the role of P-sel in BM alterations associated with emperipolesis in the Gata1^{low} mouse model of MF. Our data show that emperipolesis is driven by P-sel. Genetic ablation of P-sel rescued the BM microenvironment, by decreasing fibrosis, suggesting that pharmacological targeting of P-sel could contribute to reduce the BM dysfunction and disease progression.

Key words: P-selectin; emperipolesis; myelofibrosis; megakaryocytes; Gata1^{low}.

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Introduction

In myeloproliferative neoplasm, megakaryocytes (MKs) are hyperplastic and immature cells with altered platelet granules as a consequence of the abnormal expression of platelet factor IV and other key megakaryocytic proteins.[1] In myelofibrosis (MF), MKs exhibit both biochemical and morphological abnormalities, including reduced levels of the transcription factor GATA1[2-4] and of genes related to platelets, abnormal clustering, and an enlarged cytoplasm.[5,6] These features are the main pathological hallmarks of the fibrotic bone marrow (BM) microenvironment.

The pathogenesis of MF is strongly linked to the constitutive activation of the Janus kinase / signal transducer and activator of transcription JAK2/STAT pathway,[7-9] which drives the dysregulation of megakaryopoiesis and the proliferation of atypical MKs.[1,10] Together with the hemostatic function, the MKs are cells that promote fibroblast proliferation and activation by the releasing of profibrotic growth factors and cytokines, including transforming growth factor-β (TGF-β).[11-17] Aberrant TGF-β secretion appears to result from altered communication between MKs and leukocytes mediated by the P-selectin (P-sel).[18]

Under physiological conditions, P-sel (CD62P), mediates a well-defined process, known as emperipolesis, which is characterized by neutrophil passing within the MK cytoplasm, by binding to their counter-receptor P-sel glycoprotein ligand-1 (PSGL-1), on neutrophils.[18-20]

P-sel is an adhesive protein even found on the surfaces of activated platelets and endothelial cells.[21] When secreted, its soluble form P-sel is responsible for pre-inflammatory events, such as thrombosis, and the establishment of an inflammatory environment and related alterations.[22]

In MF, the emperipolesis is markedly increased due to elevated expression of P-sel within megakaryocyte cytoplasmic vacuoles and on the membrane demarcation systems.[23] This increased expression of P-sel facilitates pathological emperipolesis and enhances cell-to-cell interaction. Differently from the physiological emperipolesis, which is a passive and transient event;[24] during the pathological emperipolesis in MF, neutrophils are able to release proteases and reactive oxygen species in the MK cytoplasm, thereby altering the megakaryocytic signaling and trafficking machinery.[25] This abnormal interaction contributes to the reshaping of the MKs secretome. Indeed, the release of neutrophil lytic granules into MKs leads to the destruction of α-granules in their cytoplasm, and the abnormal secretion of growth factors and proinflammatory mediators as platelet derived grow factor (PDGF), vascular endothelial grow factor (VEGF), fibroblast grow factor (FGF), and in particular TGF-β, driving stromal remodeling and BM fibrosis.[10,26]

Evidence suggests that aberrant emperipolesis, sustained by P-sel/PSGL-1 interactions, amplifies the altered secretory profile, and reinforces the pathological feed-forward loop that underlying the fibrotic niche.[27,28]

In this study, we examined the function of P-sel and its direct correlation with emperipolesis in the BM of Gata1^{low} mice, which are a well-established animal model of MF.

Materials and Methods

Mice

Transgenic mice were bred at the animal facility of Istituto Superiore di Sanità (Rome, Italy) as previously described.[3,29] Littermates were genotyped at birth by PCR[3] and those not carrying the expected mutations were used as wild-type (WT) controls. The study analyzed Gata1^{low} mice, mice lacking P-sel (P-sel^{null}),[30] and double Gata1^{low}P-sel^{null} mice. [31]

The study was approved by the Italian Ministry of Health (protocol n. 419/2015-PR and D9997.121) and conducted according to the European Directive 2010/63/EU for the protection of animals used for scientific purposes.

Histological and immunohistochemical analyses

For the BM analysis, femurs were collected from 15-month-old WT, Gata1^{low} mice, P-sel^{null} mice, and Gata1^{low}P-sel^{null} double knockout mice. Femurs samples were fixed in 10% formaldehyde solution with a neutral buffer, treated with decalcifying solution EDTA 10% (pH=7.4). Following decalcification, samples were immersed in a sucrose gradient (15% for 3 h and 30% overnight). After decalcification protocol, samples were processed and embedded in paraffin wax. For each experimental group, femurs samples were cut in 3 µm sections and stained with hematoxylin and eosin (hematoxylin Cat. #01HEMH2500, eosin cat#01EOY101000; Histo-Line Laboratories, Milan, Italy) and Gomori silver impregnation (04-040801; Bio-Optica, Milan, Italy) following the standard procedure.

Reticulin fibers were quantified on five different areas of the femur of at least three mice per group at a magnification 40x, from at least three mice per group using the ImageJ program (version 1.52t) (National Institutes of Health, Bethesda, MD, USA).

For the immunohistochemical staining, samples were antigen retrieved with citrate buffer solution (pH=6) at 98°C for 30 min. Endogenous peroxidases were blocked by immersion in H_2O_2 in methanol (3%, v/v) for 30 min at room temperature. After blocking of non-specific antigenic sites by incubation with goat serum in phosphate buffer solution (PBS) (10%, v/V) for 30' at room temperature, slides were incubated using the following primary antibodies anti-P-sel (CD62P), 1:100 titre (cat#PA5-79973, Thermo Fisher Scientific, Waltham, MA, USA), anti-TGF-β1, 1:100 titre (cat# sc-130348, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti- C-X-C motif ligand 1 (CXCL1), 1:100 titre (PA5-11538, Thermo Fisher Scientific). All antibodies were incubated overnight (ON) at +4°C. After 3 washes in 0.1M PBS, the immunoreactions were detected using the 1-Step POLYMER HISTO-STATTM-Multivalent DAB Staining System (INNOVEX; BD Biosciences, Franklin Lakes, NJ, USA). The slides were then counterstained with hematoxylin (cat# 01HEMH2500). The secondary antibody controls for the immunostaining experiments were performed by omitting the primary antibody. Images were acquired with an optical microscope Olympus BX51 (Tokyo, Japan) equipped with the imaging source Micropublisher 6 camera (Crisel Instruments, Rome, Italy).

Confocal microscopy determinations

Formalin fixed paraffin embedded femur tissues were cut into consecutive 3-µm sections, dewaxed in xylene, rehydrated and treated with citrate buffer solution (pH=6) for antigen retrieval at 90°C for 30 minutes. Sections were then incubated with the appropriate primary antibodies anti-CD42b, 1:150 titre, (Ab183345, rabbit monoclonal, Abcam, Cambridge, UK; and SC-271171, mouse monoclonal, Santa Cruz Biotechnology) and anti-Ly6B, 1:100 titre, (Ab25377, rat monoclonal; Abcam) for 2 h room temperature. After 3 washes in 0.1M PBS, for each case, secondary Alexa Fluor 488 and/or Alexa Fluor 568-conjugated donkey anti-rabbit, anti-rat (Cat. N A21206 and A21434, respectively; Thermo Fisher Scientific), 1:200 titre, were then added for 1 h at room temperature. Control sections were incubated with 0.01M PBS, without primary antibody. Nuclear counterstaining was performed after 3 washes in 0.1M PBS for 10 min, by using Hoechst 33342, trihy-

drochloride, trihydrate (Thermo Fisher Scientific), 1:1000 titre for 5 min at room temperature. The samples then were washed in 0.1M PBS for 3 times, 10 min each, and mounted with Fluor-shield histology mounting medium (Catalog F6182-10MG, Sigma-Aldrich, St. Louis, MO, USA). Fluorescent images were collected using a Nikon (Tokyo, Japan)A1 confocal laser microscope, acquired with imaging software NIS-Elements (Nikon), and processed with Fiji software (US National Institutes of Health). Events were quantified by examining images acquired at 60× magnification from at least ten randomly selected areas per section. The total area analyzed for each sample was 0.448 mm^2.

Statistical analyses

The data were analyzed and presented as mean ±SD values. Comparisons between the groups were performed using one way ANOVA while comparisons between multiple groups were performed using the Tukey's multiple comparisons test. Linear correlations were calculated by the Pearson R test. Differences were considered statistically significant at $p<0.05$. Each dataset was plotted using GraphPad Prism 8.0.2 software (GraphPad Software, San Diego, CA, USA).

Results

P-selectin is expressed at high levels in megakaryocytes that directly interact with neutrophils

As the primary objective of this study is to compare the role of P-sel in MF, we analyzed the P-sel expression in the BM from mice of different strains. All of these were obtained from CD1 mice, which were used here as WT. The other groups are models that host either the Gata1^{low} mutation, the P-sel ablation (P-sel^{null}), or both (Gata1^{low}P-sel^{null}).

The hematoxylin and eosin BM staining of Gata1^{low} mice showed the canonical features of the MF, characterized by an increased number of clustered MKs (Figure 1A). Immunohistochemistry then confirmed significant P-sel expression in the BM cells of the Gata1^{low} mice compared with the respective WT and the P-sel ablated mice (Figure 1 A,B). Interestingly, morphological analyses revealed that most of the recognized MKs, in the Gata1^{low} mice were characterized by the increased P-sel content (Figure 1C). Using optical microscopy, we found that the in

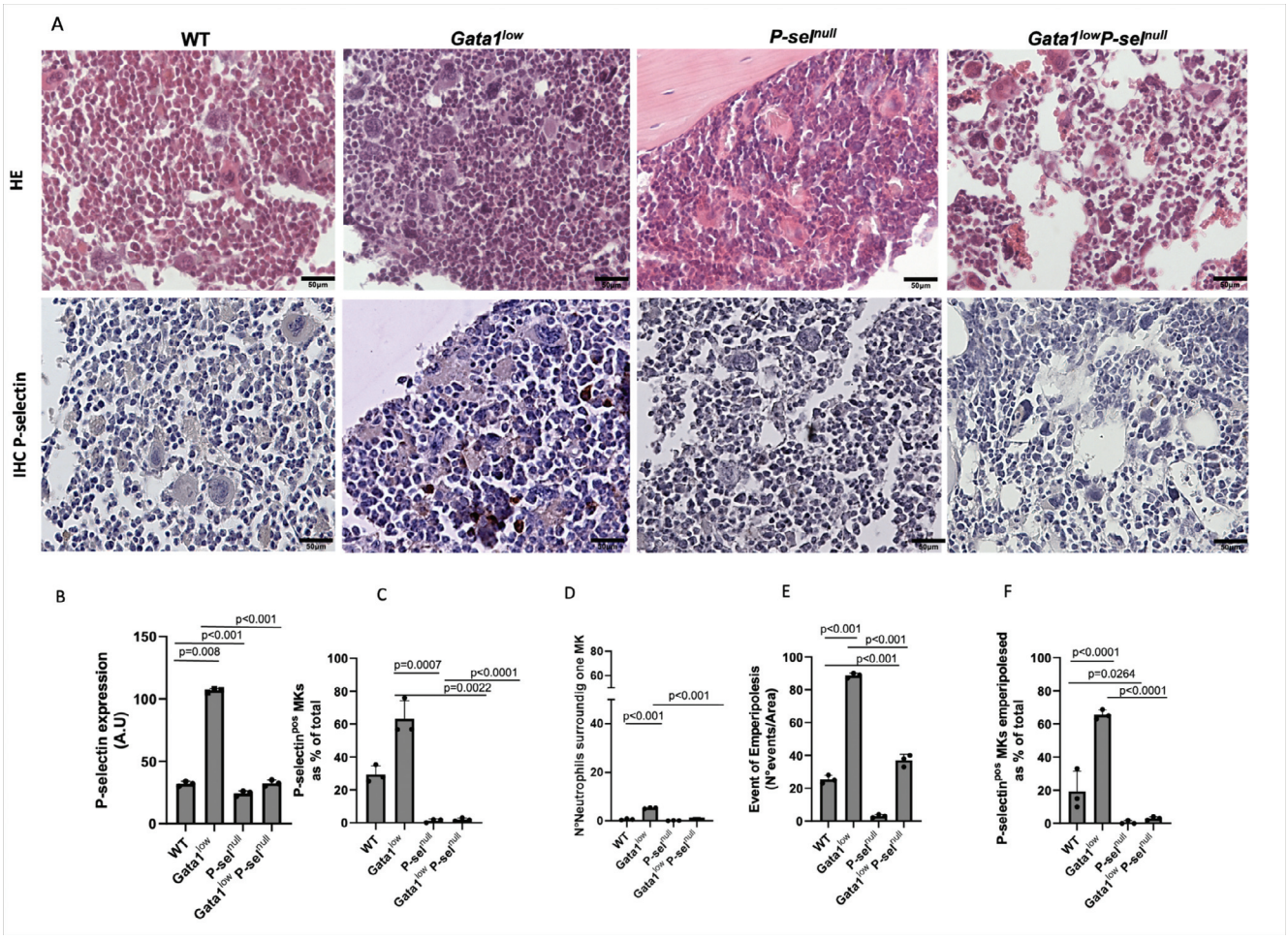

Figure 1. P-sel is expressed at high levels in MF megakaryocytes that directly interact with neutrophils. **A**) Hematoxylin and Eosin (H&E, top panels) and immunohistochemistry for P-sel (bottom panels) were performed on BM samples from WT, Gata1^{low}, P-sel^{null}, and Gata1^{low}P-sel^{null} mice; compared to WT and P-sel-ablated mice, Gata1^{low} mice display increased numbers of clustered MKs and strong P-sel expression; original magnification 40x. **B**) Quantification of P-sel expression. **C**) Percentage of P-sel positive MKs. **D**) Number of neutrophils surrounding one Mk; each dots represent as single mouse. **E**) Frequency of neutrophil emperipolesed MKs and in of P-sel positive Mks (**F**). Data are expressed as a percentage of total. Data were analyzed using one-way ANOVA and significant *p*-values are indicated within the panels.

Gata1^{low} mice, the P-sel positive MKs were also found mostly surrounded by neutrophils (Figure 1D) or in emperipolesis with them (Figure 1E). The data for Gata1^{low}P-sel^{null} mice differs, although the significant impairment of the emperipolesis, a small amount of emperipolesed cells negative for P-sel were observed, indicating significant impairment of the emperipolesis. This suggests that the emperipolesis event is triggered by the combined action of different pro-inflammatory cytokines.

Genetic ablation of P-selectin reduces the events of emperipolesis

Confocal microscopy analyses confirmed a change in the frequency of the neutrophils (Ly6b positive cells) engulfed (emperipolesed) in the CD42b positive MKs (Figure 2A). Morphological determinations revealed a significant difference in the frequency of total MKs and of those observed in emperipolesis (Figure 2 B,C), which were increased significantly in both MF mouse models compared to the respective controls. To note, in the BM from Gata1^{low}P-sel^{null} mice, the emperipolesed MKs were highly half decreased (from 32±4.32 to 11.67±1.25) than in the Gata1^{low} ones (Figure 2D). Given the significant decrease in emperipolesis following P-sel ablation, but not its complete elimination, we sought to verify whether the maintenance of this event is related to the overexpression of other proinflammatory cytokines. CXCL1 is a proinflammatory cytokines, which is highly expressed in the Gata1^{low} mice[13,14] and that is known to be involved in many pathological cell-to-cell interaction. CXCL1 expression was significantly increased in the MF BM mice (Figure 2E), particularly in MKs of both the MF mice (Gata1^{low} and Gata1^{low}P-sel^{null}) (Figure 2F), in which most neutrophil emperipolesis was observed (Figure 2G).

P-selectin sustains TGF-β accumulation and bone marrow fibrosis in the Gata1^{low} mice

Since the neutrophil-MK emperipolesis has been suggested as a cause of the TGF-β accumulation,[32,33] which is implicated in pathologic BM fibrosis, we evaluated TGF-β expression immunohistochemically in the BM of all the transgenic mouse models. TGF-β expression was significantly increased in the microenvironment (Figure 3A) and especially in MKs in Gata1^{low} mice (Figure 3B). Furthermore, morphological analyses confirmed that the majority of emperipolesed MKs were positive for TGF- β, in the BM of Gata1^{low} mutant mice (Figure 3C). Statistical analyses revealed a direct correlation between the levels of CXCL1 with the TGF-β content in Gata1^{low} mice in respect to the relative controls, as well as in the Gata1^{low} P-sel^{null} mice (Figure 3D). Similarly, the positive correlation between TGF- β and P-sel expression, suggests a direct link between the pro-adhesive effect of the P-sel molecule and the fibrotic changes in the BM microenvironment (Figure 3E). Lastly, reticulin fibers analyses (Figure 3F) confirmed the increased fibrosis in the BM of Gata1^{low} mice, compared with control subjects and the MF P-sel ablated mouse model. This confirmed the direct correlation between the TGF-β levels and the degree of BM fibrosis (Figure 3G).

Discussion

MF is characterized by the accumulation of atypical, hypolobulated MKs with reduced DNA content and abnormal clustering.[34-36] These cells contribute directly to disease progression and fibrosis by secreting high levels of cytokines, such as TGF-β, which remodel the BM microenvironment.[37]

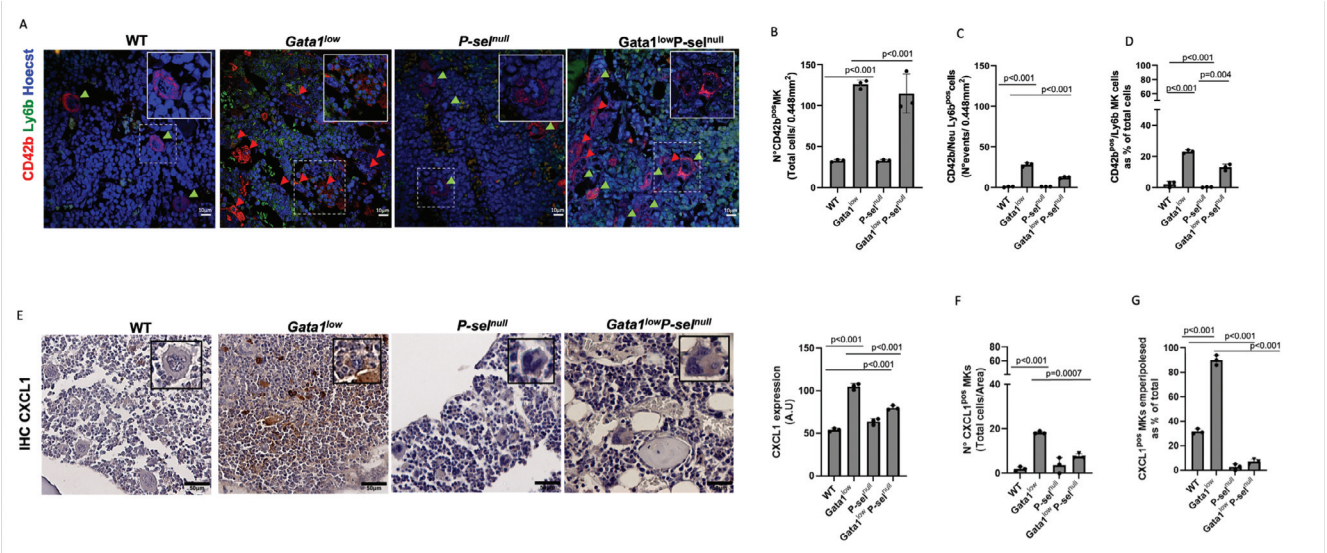

Figure 2. Emperipolesis is significantly reduced in MF mice genetically ablated for P-sel. **A**) Confocal microscopy analysis shows BM sections showing with neutrophils (Ly6b, green) undergoing emperipolesis within CD42b MKs (red); nuclei are stained with DAPI (blue); images highlight an increased number of emperipolesis events in Gata1^{low} and Gata1^{low}P-sel^{null} mice compared to the control groups; original magnification 60x. **B**) Quantification of total MKs, recognized as CD42b positive cells. **C**) Percentage of MKs engaged in emperipolesis (defined by internalized Ly6b positive cells) in the different mouse models. **D**) Percentage of CD42b positive Mks emperipolesed with neutrophils (Ly6b positive cells) in BM section from different groups; a significant reduction (>50%) in emperipolesis frequency is observed in Gata1^{low}P-sel^{null} mice (from 32±4.32 to 11.67±1.25 emperipolesed MKs per field; $p<0.01$). **E**) Overall CXCL1 levels in the BM microenvironment from all the strand investigated. **F**) Total number of CXCL1 positive MKs are presented as mean (±SD), each dot represents a single mouse. **G**) The percentage of total CXCL1 positive MK in emperipolesis with neutrophils (morphologically recognized) is expressed as percentage of total MKs. Data were analyzed using one-way ANOVA and significant *p*-values are indicated within the panels.

Our previous studies, in both MF mouse models and patients, demonstrated a significant increase in morphologically immature MKs with 1-2 nuclear lobes.[38] These cells appear to shape the fibrotic microenvironment, likely as a consequence of pathological interactions with other cells, particularly through neutrophil emperipolesis.[39] Alongside TGF-β, both P-sel and Interleukin 8 (IL-8) or C-X-C motif ligand-8 (CXCL8), are overexpressed in the MF patients BM microenvironment,[31,40-42] where they contribute to disease pathogenesis by sustaining neutrophil chemotaxis and MK-neutrophil interactions. Consistently, we have already shown that both MF patient samples and Gata1^{low} mice display an accumulation of neutrophils surrounding MKs or undergoing emperipolesis.[38]

In this study, we used the Gata1^{low}P-sel^{null} mouse model in which P-sel has been genetically ablated, to specifically investigate the role of P-sel in emperipolesis.

We demonstrated that the majority of P-sel positive MF MKs engage in emperipolesis, whereas this phenomenon is significantly reduced in P-sel-deficient MF mice. This finding highlights the central role of P-sel in facilitating MK–neutrophil interactions. Notably, the reduction in emperipolesis observed in the Gata1^{low}P-sel^{null} mice was associated with lower TGF-β levels and reduced BM fibrosis, suggesting a direct link between pathological emperipolesis, changes in the MK secretome, and microenvironmental remodeling.

P-sel is a pro-adhesive molecule stored in MK/platelet α-granules, where it interacts with the neutrophil receptor PSGL-1.[43,44] Therefore, its role in MF extends beyond emperipolesis, potentially contributing to platelet activation and thrombotic complications[45,46] in myeloproliferative neoplasms.[47] Mechanistically, emperipolesed para-apoptotic MKs release their cytoplasmic content into the BM niche, by contributing to the development of an inflammation-related phenotype, and resulting in a supportive microenvironment for the stromal rearrangement.[11,27,48] The CXCL8 is a potent chemoattractant cytokine that promotes both neutrophil emperipolesis[38] and NETosis,[49-51] thereby amplifying inflammation.

IL-8 expression in the BM microenvironment from Gata1^{low} and Gata1^{low}P-sel^{null} mice was detected in both the MF mouse models, however in Gata1^{low}P-sel^{null} mice the levels of IL-8 in the BM microenvironment were significantly decreased.

Interestingly, the P-sel genetic ablation significantly contributes to the emperipolesis event reduction, and of those emperipolesed MKs, they were positive for IL-8, confirming the residual role of this proinflammatory cytokine in emperipolesis.

Both the significant decrease in emperipolesis as well as the decrease in IL-8 microenvironmental availability in the P-sel silenced mouse model for the MF, suggest that the increased amount of IL-8 in the MF BM microenvironment is a consequence of the emperipolesis induced by P-sel and that this process sustains IL-8 secretion, by fueling this vicious cycle.

We have previously demonstrated that the increased proportion of CXCL1positive MKs in Gata1^{low} mice is associated with an increase in niche MKs, a specialized subset involved in extracellular matrix (ECM) deposition and fibrosis.[52] Consistently, pharmacological inhibition of IL-8 signaling has been shown to reduce

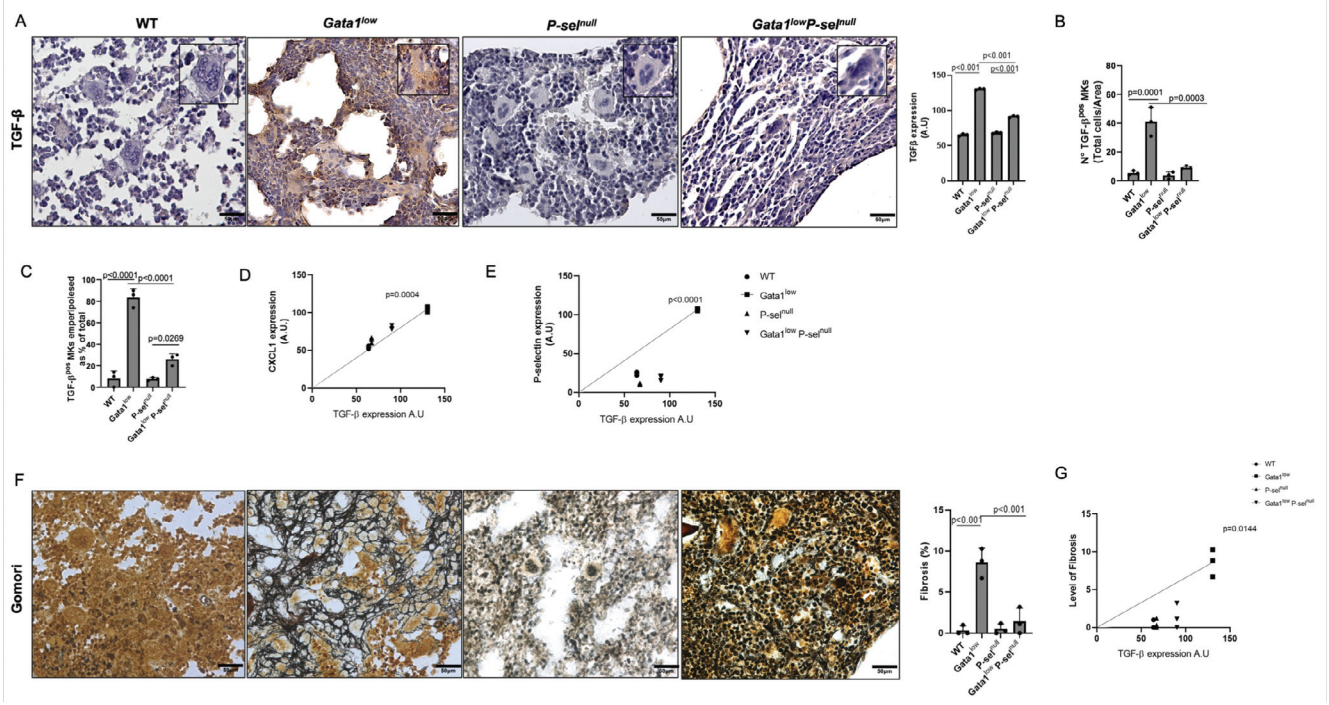

Figure 3. Reduction in emperipolesis in the Gata1^{low}P-sel^{null} mice was associated with lower TGF-β levels and decreased BM fibrosis. **A**) Immunohistochemical staining for TGF-β in BM sections from WT, Gata1^{low}, P-sel^{null}, and Gata1^{low}P-sel^{null} mice with relative quantification; original magnification 40x. **B**) Total number of TGF-β positive Mks, each dots represent a single mouse. **C**) Percentage of TGF-β positive emperipolesed Mks. **D**) Linear regression analyses between levels of TGF-β with either CXCL1 (Pearson R=0.99 p=0.0004). **E**) Linear regression analyses between levels of TGF-β and P-sel in individual mice (Pearson R=0.9994, p<0.0001); each dot represents an individual mouse. **F**) Gomori staining of BM sections from representative WT, P-sel^{null}, Gata1^{low} and Gata1^{low}P-sel^{null} mice. **G**) Linear regression between level of fibrosis and TGF-β direct positive correlation is observed, particularly in Gata1^{low} mice (p=0.0144). **F**) Linear regression between level of fibrosis and TGF-β (Pearson R=0.971, p=0.0144).

BM fibrosis, emperipolesis, and the frequency of niche MKs as well as of TGF-β, [53] providing direct evidence of a causal link between IL-8/TGF-β bioavailability and fibrosis progression.[39,52]

We have also confirmed in this work our previous result, in which both wild-type mice and double Gata1^{low}Psel^{null} mice do not develop BM fibrosis.[54]

Even no data are reported in this paper about the role of Lipocalin 2 (LCN2), this pro inflammatory cytokine is strictly related to the BM alteration in MF.[55] Indeed, the increased plasma level of LCN2 was detected in sera from MF patients.[56,57] Our group have already investigated the role of LCN2 in fibroblast proliferation and ECM neodeposition in the Gata1^{low} P-sel^{null} model.

Cytokines profiling assay revealed that together with the significant decrease of cytokines directly involved in the stromal rearrangement, also LCN2 was found decreased after P-sel ablation. Lipocalin 2 expression in the BM from Gata1^{low} mice was significantly marked in megakaryocytes and in neutrophil cluster, suggesting its key regulatory role in the cellular crosstalk.[13] These findings indicate that P-sel ablation modifies neutrophil activation and LCN2 secretion, thereby attenuating the inflammatory and fibrotic response. In our model, where P-sel ablation reduces emperipolesis and IL-8 production, modulation of LCN2 release may represent an additional mechanism contributing to the reduced fibrotic phenotype. Thus, decreased P-sel-dependent emperipolesis may limit both IL-8 and LCN2 availability, interrupting the self-reinforcing loop that sustains TGF-β activation and fibrosis.

Another important feature of the altered cell crosstalk is related to the recent results of Khatib-Massalha *et al*., that demonstrated the accumulation of senescent, CD24 positive neutrophils in JAK2V617F-driven myeloproliferative neoplasms amplifies inflammation and promotes MF.[58] The defective CD24 neutrophil clearance, promote the interaction with MK by the P-sel binding on the demarcation membranes system. Even the different perspective, this work points to the P-sel contribution to the fibrotic transformation of the BM, by sustaining the central role of both the inflammatory milieu and the pathological emperipolesis, characteristic of MF BM microenvironment.

Here we demonstrate that the ablation of P-sel in Gata1^{low} mice reduces emperipolesis, TGF-β accumulation, and BM fibrosis. The translational relevance of targeting P-sel is reinforced by further antibody-mediated blockade studies in Gata1^{low} mice,[59] that reproduced the protective effects observed with genetic ablation. Notably, anti-P-sel treatment reduced BM fibrosis and demonstrating an additive effect when combined with inhibition of the JAK-STAT pathway.[52] These concordant results strengthen the hypothesis that P-sel is a key mediator of pathological MK–neutrophil interactions in MF.

Together with the evidence that CXCL1/IL-8 signaling contributes to emperipolesis and fibrosis,[38,60] these results suggest that a combined therapeutic strategy targeting both adhesive interactions (P-sel) and chemoattractant signaling (CXCR1/2–IL-8 axis) could be effective in MF. Overall, our data suggest that both P-sel and IL-8 are involved in pathological emperipolesis in MF, albeit through different mechanisms. While IL-8 amplifies neutrophil recruitment and MK activation, P-sel acts as the key adhesion molecule enabling direct MK-neutrophil interaction. Convergence of these pathways results in enhanced TGF-β release and microenvironmental fibrosis, placing P-sel at the core of aberrant cellular interactions in MF. Future studies should assess drug dosing, timing, and combination approaches (*e.g*., with JAK inhibitors or CXCR1/2 antagonists) to better define the translational potential of P-sel inhibition either alone or in combination with new molecules or currently approved therapies.

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