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Preclinical studies on the use of a P-selectin blocking monoclonal antibody to halt progression of myelofibrosis in the *Gata1^{low}* mouse model

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Abstract

The bone marrow (BM) and spleen from myelofibrosis patients, as well as those from the *Gata1*^{low} mouse model of the disease, contain increased numbers of abnormal megakaryocytes. These cells express on their surface high levels of the adhesion receptor P-selectin that, by triggering a pathological neutrophil emperipolesis, lead to increased bioavailability of TGF-β in the

microenvironment and disease progression. *Gata1*^{low} mice develop with age a phenotype similar to that of patients with myelofibrosis, the most severe of the Philadelphia-negative myeloproliferative neoplasms. We previously demonstrated that *Gata1*^{low} mice lacking the *P-selectin* gene do not develop myelofibrosis. In the current study, we test the hypothesis that pharmacological inhibition of P-selectin may normalize the phenotype of *Gata1*^{low} mice which have already developed myelofibrosis. To test this hypothesis, we have investigated the phenotype expressed by aged *Gata1*^{low} mice treated with the anti-mouse monoclonal antibody RB40.34, alone or in combination with Ruxolitinib. The results indicate that RB40.34 in combination with Ruxolitinib normalize the phenotype of *Gata1*^{low} mice with limited toxicity by reducing fibrosis, and TGF-β and CXCL1 (two drivers of fibrosis in this model) content in the BM and spleen and by restoring hematopoiesis in the bone marrow and the architecture of the spleen. In conclusion, we provide pre-clinical evidence that treatment with an antibody against P-selectin in combination with Ruxolitinib may be more effective than Ruxulotinib alone to treat myelofibrosis in patients.

1 Introduction

Myelofibrosis (MF) is the most severe of Philadelphia chromosome negative myeloproliferative neoplasms (MPN). The complex phenotype of the MF patients includes fibrosis and hematopoietic failure in bone marrow (BM), stem/progenitor cell mobilization, development of extramedullary hematopoiesis with splenomegaly and their clinical course is associated with increased risk of thrombosis, bleeding and evolution to acute leukemia (1–4). MF may be driven by gain of function mutations in several genes of the thrombopoietin axes such as *MPL*, the thrombopoietin receptor, *JAK2*, the first element of the MPL signaling, and *calreticulin*, a chaperon protein that when mutated binds MPL on the cell surface, inducing conformational changes which lead to ligand independent constitutive activation of the receptor (5). Regardless of the driver mutation, is has been recognized that MF has a distinctive cellular signature. In fact, both the BM and spleen from these patients contain numerous clusters of morphologically immature megakaryocytes (MK)(6) endowed with great proliferation potential (7,8). In MF, MK are retained immature by a mutation-driven RSP14 ribosomopathy that impairs the translation of the mRNA for GATA1 (9,10), the transcription factor which plays a pivotal role in the progression of MK maturation (11). The causative role of the resulting abnormal MK in the pathogenesis of this disease is strongly supported by experiments in mice indicating that those carrying a hypomorphic mutation which selectively reduces GATA1 in

MK (*Gata1^{low}* mice) develop myelofibrosis with age (12) while transgenic mice expressing *JAK2*V617F, one of the driver mutations of the disease(1–4), only in MK develop myelofibrosis even if their hematopoietic stem cells are normal (13,14). As first hypothesized by Schmitt et al (7), mechanistically, malignant MK are thought to drive MF by engaging in a pathological process of emperipolesis with the neutrophils which increases the bioavailability of transforming growth factor-β (TGF-β), and possibly of other pro-inflammatory cytokines, in the BM of MF patients and animal models (8,15–17) The pathobiological role of TGF-β in the development of myelofibrosis has been further confirmed by the observation that in animal models development of myelofibrosis is prevented by genetic ablation of the *TGF-β* gene (17,18) and reverted by treating myelofibrosis mice with a small TGF-β receptor-1 kinase inhibitors (19) or with the TGF-β trap AVID200 (20). The TGF-β trap AVID200 is currently in clinical phase-1/2 clinical trials for MF who are resistant to therapy with the JAK1/2 inhibitor Ruxolitinib (Rux)(21).

Later studies have indicated that the adhesion receptor P-selectin (P-SEL) may represent an element upstream to TGF-β in the pathobiological pathway leading to MF. In fact, the MK abnormalities observed in this disease include abnormal cytoplasmic trafficking of P-SEL which instead to be partitioned in the granules is displayed on the cell surface(22). The high levels of P-SEL on the cell surface, by interacting with its ligand (P-selectin glycoprotein ligand-1, PSGL-1) expressed by the neutrophils (23,24) has been hypothesis to drive a process of pathological emperipolesis between the neutrophils and the MK which leads to death of the MK by para-apoptosis and release of TGF-β in the microenvironment (25,26). This hypothesis has been mechanistically demonstrated by the observation that the TGF-β bio-availability in the BM of *Gata1*^{low} mice lacking the *P-sel* gene is normal and that these mice do not develop myelofibrosis with age and live, on average, 2 months longer than their *Gata1*^{low} littermates (27). These findings support the hypothesis that in MF, the disease is established and progresses thanks to a pathological P-SEL/TGF-β circuit established by the malignant MK(28). Whether inhibition of P-SEL would also be effective in reverting to normal myelofibrosis once the disease is established has not been demonstrated as yet.

Recently, the P-SEL inhibitor Crizanlizumab (SEG101) has been demonstrated to reduce the frequency of vaso-occlusive crises in patients with Sickle Cell Disease with limited toxicity (29). Based on these observations, in November 2019, the Federal Drug Administration approved the use of Crizanlizumab for the treatment of pain crisis in Sickle Cell Disease. The rationale for the clinical study with Crizanlizumab had been provided by a pre-clinical study that evaluated the effects of the commercially available monoclonal antibody RB40.34 targeting the murine P-SEL as antithrombotic

agent in a mouse model of Sickle Cell Disease (30). Using the fact that Crizanlizumab had been already approved for clinical use and that conditions for effective treatment of mice with RB40.34 had been already described, we test here the hypothesis that pharmacological inhibition of P-SEL with RB40.34, alone or in combination with Rux, is effective in reverting the myelofibrotic phenotype expressed by *Gata1*^{low} mice.

2 Materials and Methods

2.1 Mice. *Gata1*^{low} mice are bred in the animal facility of Istituto Superiore di Sanità as described (31). Littermates are genotyped at birth by PCR and those found not to carry the mutation are used as wild-type (WT) controls. All the experiments, including the size of the experimental groups, are performed according to the protocols D9997.121 approved by the Italian Ministry of Health on September 2nd 2021, and according to the European Directive 86/609/EEC.

2.2 Treatments. A total of 47 *Gata1*^{low} mice were implanted with 14mm micro-chips (one chip/mouse) (AVID, Norco, CA, USA) and divided into two separate experiments (**Figure S1**). In the first experiment, 24 11-months-old mice were randomly divided in four groups (3 males and 3 females each) that were treated as follows: Group 1: Vehicle (2% v/v DMSO by gavage, negative control for group 3 and 4); Group 2: Biotin-conjugated rat anti-mouse CD62P (RB40.34, Cat. n. 553743, BD Pharmigen, San Diego, CA, USA; 30 μg/mouse per day x three days per week by iv, as described (30), until day 45, and then ip); Group 3: Rux (Novartis Pharma AG, Basel, Switzerland; 45mg/Kg twice per day x 5 days a week by gavage as described (32)); Group 4: biotin-labeled RB40.34 and Rux in combination. On Day 5, all the mice were weighed and bled for blood cell counts determinations and detection of RB34.40 on platelets. Mice were sacrificed at day 5 (males) and day 12 (females) and BM and spleen collected for cell signaling and histopathological determinations. In the second experiment, 23 8-months-old *Gata1*^{low} mice were divided in the same groups described above and treated for 7 weeks. In this experiment, we used the purified RB40.34 (Cat. n. 553742, BD Pharmigen). The treatment was interrupted for two weeks during the holyday break (from day 24 to day 43). On day 54, all the mice were weighed, bled for blood counts determination, and sacrificed for histopathology observations of their BM and spleen.

2.3 Blood counts determination. Mice were topically anesthetized with Novesina (Cat. n. s01ha02, Novartis, Basel, CH, one drop/eye) and blood collected from the retro-orbital plexus into heparinized

microcapillary tubes. Blood counts were evaluated on deidentified samples by an accredited commercial laboratory which provide diagnostic services for laboratory animals (Plaisant Laboratory, Rome, Italy).

2.4 Flow cytometry. *Binding of RB40.34 to platelets.* Platelet-enriched plasma was prepared by centrifugation of 200µL of heparinized blood (10,000rpm for 20min with the Eppendorf™ Centrifuge 5425/5425 R, Eppendorf, Milan, Italy) and the binding of biotinylated RB40.34 to platelets measured by flow cytometry following incubation with PE-Cy7 Streptavidin (ca. no. 557598, BD Pharmingen). Platelets were identified based on size (FS: forward scatter) and internal cell complexity (SS: side scatter), as described(22). *MK identification and binding to RB40.34.* BM and spleen cells were resuspended in Ca^{++} Mg^{++}-free PBS containing 0.5% (v/v) fetal bovine serum (FBS, Cat. n. F7524, Sigma-Aldrich) and incubated with PE-CD41, FITC-CD61 and PE-Cy7-streptavidin. Cells were then divided by flow cytometry into four populations corresponding to non-MK ($CD41^{neg}/CD61^{neg}$); immature MK ($CD41^{neg}/CD61^{high}$); mature MK ($CD41^{high}/CD61^{high}$) and very mature MK ($CD41^{high}/CD61^{neg}$), as described(19). The levels of PE-Cy7-streptavidin staining was assessed as a measure of biotinylated-RB40.34 binding to the MK. *Hematopoietic stem/progenitor cell determinations.* Mononuclear BM and spleen suspensions were incubated with a cocktail of antibodies including APC-CD117, APC-Cy7-Sca1, PE-Cy7-CD150, biotin-labeled anti–mouse CD48 and biotin-labeled anti-lineage antibodies. After 30min of incubation on ice, cells were washed and incubated with streptavidin-PE-Cy5 (all from BD Pharmingen). Hematopoietic progenitor cells were defined as lineage negative cells (Lin-). Hematopoietic stem cells were defined as LSK ($Lin-/CD48^{neg}/c\text{-}Kit^{pos}/Sca\text{-}1^{pos}$) while long-term repopulating hematopoietic stem cells were defined as SLAM ($Lin-/CD48^{neg}/c\text{-}Kit^{pos}/Sca\text{-}1^{pos}/CD150^{pos}$) as described(27,33). Nonspecific signals and dead cells were excluded, respectively, by appropriate fluorochrome-conjugated isotype and propidium iodide staining. All the flow cytometry analyses were performed with the Gallios analyzer (Beckman Coulter) and the results analyzed with the Kaluza analysis program, version 2.1 (Beckman Coulter).

2.5 Western blot analysis. BM and spleen from *Gata1*^{low} mice treated for 5 days were dissolved in lysis buffer containing protease and phosphatase inhibitors and stored at -80°C. Protein extracts were separated by electrophoresis under denaturing conditions using 7.5-10% mini-Protean TGX pre-casted gels (Bio-Rad, CA, USA) and transferred to nitrocellulose filters with the Transblot-Turbo system (Bio-Rad, Hercules). Filters were probed with antibodies against proteins of the canonical (SMAD2/3, cat no. 8685, Cell Signaling, Boston, MA, USA), p-SMAD2/3 (cat no. 8828, Cell

Signaling), TGF-βRII (cat no. ab186838, Abcam, Cambridge, UK) and non-canonical (p38, cat no. 9212; p-p38, cat no. 4511; ERK1/2, cat no. 9102; p-ERK1/2, cat no. 9101; all from Cell Signaling) TGF-β signaling and of the JAK/STAT signaling (JAK2 (cat. No 3230, Cell Signaling), STAT5 (cat no. sc-74442, Santa Cruz, Dallas, Texas, USA), pJAK2 (Phospho-Tyr1007/1008 JAK2, cat no. 3771, Cell Signaling) and p-STAT5 (cat no, 9351, Cell Signaling). GAPDH (cat no. G9545, Sigma Aldrich) was used as a loading control. The bands were quantified with the ImageJ 1.52q software (National Institutes of Health, Bethesda, MD, USA) and normalized against GAPDH. Stoichiometry determinations of phospho-proteins levels were obtained by normalizing the content of the phosphoprotein with that of the corresponding total protein.

2.6 Histological analyses. Femurs were fixed in formaldehyde (10% v/v with neutral buffer), treated for 1h with a decalcifying kit (Osteodec; Bio-Optica, Milan, Italy) and included in paraffin. Spleens were fixed in formaldehyde and then included in paraffin (19). Paraffin-embedded tissues were cut into consecutive 3μm sections and stained either with Hematoxylin-Eosin (H&E; cat no. 01HEMH2500 and 01EOY101000, respectively; Histo-Line Laboratories, Pantigliate, MI, Italy), Gomori silver or Reticulin staining and Mallory Trichrome staining (cat no. 04-040801, 04-040802, 04-020802 respectively; Bio-Optica). For immune-microscopy, BM sections were incubated with anti-CXCL1 (cat no. ab86436, Abcam), anti-TGF-β1 (cat no. sc-130348, Santa Cruz Biotechnology) antibodies and reactions detected by avidin-biotin immune-peroxidase staining and 3,3′-diaminobenzidine (0.05% w/v) (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA). Slides were counterstained with Papanicolaou's hematoxylin (Histo-Line Laboratories). Images were acquired with the optical microscope Eclipse E600 (Nikon, Shinjuku, Japan) equipped with the Imaging Source "33" Series USB 3.0 Camera (cat no. DFK 33UX264; Bremen, DE) and the signal quantified acquiring at least 5 different areas/femur/mouse from at least 4 mice per group using the ImageJ program (version 1.52t) (National Institutes of Health), as described(34,35). For Immuno-fluorescence microscopy - determinations, three micron-thick BM sections were dewaxed in xylene and antigens were retrieved by treatment with EDTA buffer (pH=8) for 20' in a pressure cooker (110-120°C, high pressure) and incubated with antibodies against CD42b (cat no. ab183345, Abcam), GATA1 (cat no. sc-265, Santa Cruz), CD3 (cat no. ab16669, Abcam) and CD45R/B220 (cat no. 553085, BD-Pharmingen) over night at 4°C. Primary antibodies were visualized with the secondary antibody goat anti rat Alexa Fluor 488 (cat no. ab150165, Abcam) or goat anti rabbit Alexa Fluor 555 (cat no. ab150078, Abcam). All sections were counterstained with DAPI (cat no. D9542-5MG, Sigma-Aldrich, Darmstadt, DE),

184 mounted with Fluor-shield histology mounting medium (cat. F6182-10MG, Sigma-Aldrich), and
185 examined using a Nikon Eclipse Ni microscope equipped with filters appropriate for the
186 fluorochrome to be analyzed. Images were recorded with a Nikon DS-Qi1Nc digital camera and NIS
187 190 Elements software BR 4.20.01 and quantified with the ImageJ program by counting the number
188 of cells that exceeded the intensity set as threshold, as described(35). Reconstruction of the image of
189 the all femur was obtained by the combining the entire set of stack images (15 images at 20x or 34
190 images at 63x) with the Zen Blue software (Zeiss, Oberkochen, DE). Microvessel density was
191 determined by incubating bone marrow and spleen sections with anti-CD34 (cat no. MAB7100,
192 AbNova, Taiwan, primary) and Alexa Fluor 568-conjugated donkey anti-rat (Invitrogen, Carlsbad,
193 CA, USA, secondary) and with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA).

194 **2.7 Data analysis.** Data were analyzed and plotted using GraphPad Prism 8.0.2 software (GraphPad
195 Software, San Diego, CA, USA) and presented as Mean (±SD) or as box charts, as more appropriate.
196 All the data had a normal distribution, as assessed by Shapiro Wilk T test. Values between two
197 groups were compared by T test while those among multiple groups were compared by Tukey's
198 multiple comparisons test or Anova, as indicated. Kaplan–Meier survival curves were compared by
199 log-rank (Mantel-Cox) test. Differences were considered statistically significant with a $p< 0.05$.

200

201 **3 Results**

202 **3.1 The RB40.34 antibody readily binds to platelets in the blood and reaches the fibrotic BM of**
203 ***Gata1*^{low} mice.** Since the underlying fibrosis in the BM of *Gata1*^{low} mice may restrain the RB40.34
204 antibody to reach the BM, we conducted a feasibility study to determine whether the biotinylated-
205 RB40.34 was detectable in BM sections from mice treated for 5 days. In addition, since we, and
206 others, have demonstrated that platelets from *Gata1*^{low} mice express greater levels of P-SEL on their
207 surface(22,36), we determined whether biotinylated-RB40.34 was detectable on platelets present in
208 the blood 5hr after its administration as control of the persistence of the antibody in the circulation
209 after its injection (**Figure 1**). Biotin is produced in the liver and is present, albeit at low levels, in
210 several cell types(37). Therefore, it is not surprising that the APC-Cy7 Streptavidin signal is detected
211 also on platelets and BM sections from mice in the vehicle and Rux groups which did not receive
212 biotinylated-RB40.34. However, the signals on platelets and BM sections from mice which had
213 received biotinylated-RB40.34 is clearly greater than background levels (**Figure 1**).

3.2 Five days treatment with RB40.34 in combination with Rux reduces TGF-β signaling in bone marrow and JAK2/STAT5 signaling in the spleen from *Gata1^{low}* mice. To investigate the effects of the treatments on the signaling state of the BM and spleen from *Gata1^{low}* mice, western-blot analyses of these organs from untreated *Gata1^{low}* mice and from mice treated for 5 days were performed. These studies used a panel of antibodies which target SMAD2/3, and TGF-βRII (canonical TGF-β signaling); p38, p-p38, ERK1/2 and p-ERK1/2 (non-canonical TGF-β signaling) and JAK2 and STAT5 (JAK/STAT signaling). Untreated WT mice were analyzed in parallel as control (**Figures 2,3** and **S3**). pJAK2 and pSTAT5 were not investigated because the phosphorylation of these two proteins in extracts from primary tissues is very sensitive to degradation upon storage (**Figure S4**).

The BM from untreated *Gata1^{low}* mice expresses levels of TGF-βRII significantly greater than WT mice, which are likely a reflection of the increased number of MK present in this organ. Treatment with RB40.34 or Rux alone had no effect to the levels of SMAD2/3 and TGF-βRII proteins which remain similar to that of untreated *Gata1^{low}* mice. By contrast, treatment with RB40.34 in combination with Rux reduce the content of SMAD2/3 and of TGF-βRII in the BM down to levels expressed by BM from WT mice, suggesting that the treatment reduced the canonical TGF-β signaling in this organ (**Figure 2A,C)**. The levels of total ERK and p38, that are two elements of the MAPK-dependent non canonical TGF-β signaling (38), and of their phosphorylated forms in the BM of untreated *Gata1^{low}* mice are not significantly greater than normal. Although none of the treatments affected the p38 content/activation state, treatment with RB40.34 in combination with Rux reduces to barely detectable levels the activation of p-ERK, an indication that this treatment is reducing the non-canonical TGF-β signaling possibly responsible for fibrosis in the BM (**Figure 2B,C)**. By contrast, the content and activation state of canonical and non-canonical TGF-β signaling in the spleen from untreated *Gata1^{low}* mice are similar to that of spleen from WT mice and are not significantly affected by any of the treatments (**Figure S3**).

JAK2 is not detected in BM from untreated *Gata1^{low}* and WT littermates and, with the exception of two out of three mice in the vehicle and Rux alone groups, remains undetectable in BM from the treated groups as well. The content of STAT5 is instead robust and not statistically different across all groups in BM (**Figure 3A,C**). By contrast, the content of JAK2 and STAT5 in the spleen from untreated *Gata1^{low}* mice is significantly greater than that from WT littermates (**Figure 3B,D**), possibly reflecting the great levels of extramedullary hematopoiesis occurring in the spleen of the

mutant animals(39). The levels of STAT5 in the spleen from *Gata1^{low}* mice remain robust after treatment with either Rux or RB40.34 alone or in combination. By contrast, the levels of JAK2 in the spleen of the mutant mice are drastically reduced upon the combined treatment with Rux and RB40.34, but not with either of the drugs alone. These data suggest that the combination of Rux+RB40.34 is targeting the extramedullary hematopoiesis in spleen.

These data indicate that treatment for only 5 days with Rux+RB03.34 in combination induces detectable biochemical changes in the BM and spleen from *Gata1^{low}* mice. Additional experiments, associated with expression profiling of individual cell populations, are necessary to assess whether these biochemical changes are due to alterations in cell composition and/or in the signaling cascade of individual cell populations in these organs.

3.3 All the treatments are well tolerated with no significant effects on survival and body weight. Encouraged by the results described above, we performed longer treatments (day 12 and day 54) to assess whether these drugs may affect the myelofibrosis phenotype expressed by *Gata1^{low}* mice. To determine safety, all the treated mice were daily monitored by a veterinarian who recorded no significant modifications in physical activity and behavior (no lethargy, no excessive grooming, no change in coat luster) during all the period of observation. None of the treatments affected the weight of the animals which remains similar to that observed before treatment in all the experimental groups (**Figure S5A**). Although few deaths were recorded during treatment (**Table S1**), overall log-rank test of the Kaplan-Meier survival curves of the treated *Gata1*^{low} mice showed no significance difference in death rate among groups (**Figure S5B**).

3.4 RB40.34, alone or in combination with Rux, reduces anisocytosis and lymphocyte counts. Hct levels remained within normal ranges in all experimental groups for all the duration of the treatments (**Table 1**). A significant raise in red blood cell (RBC) distribution width (RDW) at levels that meet the criteria for anysocytosis is observed in some of the mice treated with vehicle and in all of those treated with Rux for 54 days while the RDW in the groups treated with RB40.34 alone or in combination with Rux is within normal ranges (**Figure 4A**).

None of the treatments rescue the platelet deficiency of *Gata1*^{low} mice which remains significantly lower than normal in all the groups (**Table 1**).

The difference in WBC counts between untreated *Gata1^{low}* and WT littermates is not statistically significant (**Table 1**) and none of the drugs investigated induces significant changes in the WBC counts since even the two-fold reductions observed at day 54 in the Rux and RB40.54 plus Rux

groups are not statistically significant by Tukey multiple comparison test with those of untreated mice. However, a comparison of the frequencies of the different WBC subpopulations reveals that RB40.34 in combination with Rux significantly decreases the lymphocytes counts by day 54 (**Figure 4B**).

In conclusion, none of the treatments induced anemia nor rescued thrombocytopenia of *Gata1*^{low} mice. However, treatment for 54 days with RB40.34 in combination with Rux reduced anisocytosis and lymphocyte counts.

3.5 Treatment with RB40.34 in combination with Rux reduces fibrosis and restores hematopoiesis in the bone marrow from *Gata1*^{low} mice. By 8-11 months of age, the femur of *Gata1*^{low} mice is hypocellular and contain great levels of fibrosis (**Figure S6**). None of the treatments alters the BM cellularity (which remains lower than normal) and the level of fibrosis observed by day 5 (data not shown). By day 12, however, although the BM from all the groups remains hypocellular (data not shown), the level of fibrosis in the diaphysis of the femur from mice treated with RB40.34 and Rux in combination is reduced (**Figure S7**), suggesting that this combination is starting to be effective. In agreement with this hypothesis, by day 54, the femur from mice of the RB40.36 plus Rux group appears reddish, a sign of improved erythropoiesis (**Figure 5A**), and contains significantly greater number of cells than that from the vehicle group (**Figure 5B**). This increased cellularity is also evident by hematoxilin/eosin staining of the BM sections (**Figure 5C**). In addition, reticulin staining indicates strong reductions of fibrosis in the BM of the entire femur of mice treated with RB40.34 plus Rux for 54 days (**Figure 5C,D).** By contrast, single treatment with RB40.34 significantly reduces fibrosis, but does not increase BM cellularity while, as previously reported(32), treatment with Rux alone does not increase BM cellularity and does not reduce fibrosis in *Gata1*^{low} mice. The reason while, by contrast with our data, Rux is effective in reducing fibrosis in the *JAK2*^{V617F}-driven mouse model (40) is unclear and deserves to be further investigated.

The abnormal *Gata1*^{low} MK release several bone morphogenic proteins that are responsible for increased bone formation starting at 1-months of age(12,41,42). In spite of the increased collagen deposition, the bone from the *Gata1*^{low} mice remains immature with poor Ca++ deposition and, similarly to what observed in MF patients, the mice develop osteopetrosis(43,44). Differences in the integrity of the femur from mice treated with the different drug combinations (**Figure 5C)** suggest possible differences in the levels of osteopetrosis expressed in the four experimental groups. To test this hypothesis, we analyzed by Mallory trichrome staining femur from untreated WT and *Gata1*^{low}

littermates and from *Gata1^{low}* mice treated with the different drug combination (**Figure S8)**. As expected, the cortical bone of WT mice is characterized by red-mature lamellar bone with limited blue areas of osteoids rich in collagen fibers but poor in Ca++. By contrast, the cortical bone of both the epiphysis and the diaphysis from *Gata1*^{low} mice contains large blue areas with unmineralized osteoids and limited areas of red-mature lamellar bone. The diaphysis of the mutant mice also contains large areas of trabecular unmineralized bone protruding in the medulla. These results are similar to those published in (40, 41). After 54 days of treatment, the histopathology of the femur from the vehicle and RB40.34 group is similar to that of untreated *Gata1^{low}* mice of comparable age. However, the medulla of the femur from the mice treated with Rux, alone and in combination with RB40.34, contains significant less areas of neo-bone formation while the maturation of the cortical bone from the femurs of mice treated with Rux is normal (**Figure S8**).

Another of the features associated with myelofibrosis which is conserved in animal models is increased neo-angiogenesis (12). To assess whether the treatments had reduced the neo-angionesis in the bone marrow and spleen from *Gata1^{low}* mice, confocal microscopy studies with CD34, which in mice recognize endothelial cells, and Hoechst, to identify the nucleated cells, were performed (**Figure 6**). Indeed, by day 54, the vessel density of all the treatment groups was significantly lower than in the vehicle.

The BM hematopoietic failure associated with the myelofibrotic phenotype of *Gata1^{low}* mice includes barely detectable levels of hematopoietic stem/progenitor cells in this organ(27). To confirm that RB40.34 in combination with Rux improves hematopoiesis in BM, the frequency and total numbers of progenitor (Lin-) and short term (LSK) and long (SLAM) term repopulating stem cells in the BM from mice treated for 54 days with the various drug combination was evaluated (**Figures 5E** and **S9**). Indeed, the BM from mice treated with RB40.34 in combination with Rux contains significantly greater frequency of Lin- and LSK and greater total numbers of all three populations than that from the vehicle-treated group.

3.6 Treatment with RB40.34 in combination with Rux reduces fibrosis, extramedullary hematopoiesis and restores the architecture of the spleen from *Gata1^{low}* mice. Given the great relevance of JAK2 signaling in hematopoiesis(45), the observation that treatment with RB40.34 and Rux in combination greatly reduces the JAK2 content in the spleen suggest that this treatment decreases hematopoiesis in this organ. In agreement with this hypothesis, we observe marked reductions of fibrosis in spleen from *Gata1^{low}* mice treated with RB40.34 plus Rux for only 12 days (**Figure S7**). By day 54, a trend toward reduction in spleen size (both as weight, as ratio between

spleen weight and body weight, and as cell numbers) is observed in the group treated with RB40.34 plus Rux (**Figure 7A-C**). Furthermore, a significant reduction in the total number of hematopoietic progenitor cells (Lin- cells) was observed at day 54 in the spleen from this group (**Figures 7D** and **S9),** supporting the hypothesis that RB40.34 and Rux in combination reduces extramedullary hematopoiesis in this organ.

The architecture of the spleen from *Gata1^{low}* mice is greatly altered by the fibrosis and by the underlying extramedullary hematopoiesis (**Figure 8A,B,D**). Significant reductions in fibrosis are observed in mice treated with RB40.34 alone, Rux alone and RB40.34 and Rux in combination, although the greater reductions are observed in mice treated with the combination (**Figure 8C,E)**. As expected(46), CD45R/CD3 staining indicates that the architecture of the spleen from WT mice is characterized by the presence of large aggregates of lymphoid cells and a well-developed white pulp. Red blood cells are embedded in the reticular connective tissue which contains few megakaryocytes and supporting trabeculae. The T (CD3pos, in red) and B (CD45Rpos, in green) lymphocytes are numerous and localized around the central arterioles: T lymphocytes form a sleeve around the central arteriole, the periarteriolar lymphoid sheath, while B cells are mainly localized in the outer region of the white pulp, defined the marginal zone. By contrast, the spleen from *Gata1*^{low} mice contains a hypoplastic white pulp and its periarteriolar lymphoid sheath contains a markedly reduced number of T cells. In addition, the red pulp appears disorganized by the presence of numerous MK and fibrosis (**Figure 8A-C**). Treatment of *Gata1*^{low} mice with RB40.34 and Rux in combination, and to a lesser extent by the two drugs as single agents, restores the normal architecture of the spleen with a great expansion of white pulp and a nearly normal organization of the periarteriolar lymphoid sheath and of the marginal zone (**Figure 8C**).

3.7 RB40.34 in combination with Rux for 54 days improves MK maturation but does not decrease the MK content in the bone marrow and spleen from *Gata1^{low}* mice. The process of terminal megakaryocyte maturation involves a series of precursors that progressively acquire features of mature cells that release platelets(16,47). As these precursors progress along the maturation pathway, they express increased levels of CD41 and CD61 on their surface(47,48). Therefore, flow cytometry analyses for CD41 and CD61 expression divides MK precursors into three classes: immature (CD41negCD61pos), mature (CD41posCD61pos) and very mature (CD41posCD61low) while non-MK are negative for both markers. On the basis of this flow cytometry criteria, we determined whether the treatments rescued the defective MK maturation of *Gata1^{low}* mice (**Figure 9**).

Since the BM of *Gata1^{low}* mice contains great number of MK (12), it is not surprising that CD61pos cells represent amost 30% of the total cells of BM and spleen from mutant mice (**Figure 9**). The total frequency of the CD61pos cells in the BM and spleen from all the experimental groups remains high for all the duration of the treatments. By day 5 and 12, very few of the MK in the BM and spleen from *Gata1^{low}* mice in all the experimental groups had the very mature CD41bposCD61low phenotype. The frequency of very mature CD41bposCD61low MK is low and that of immature CD41bnegCD61pos MK high also in BM and spleen from mice treated with vehicle for 54 days. By contrast, by day 54, the frequency of MK with the very mature and immature phenotypes in the groups treated with RB40.34, Rux or RB40.34 and Rux in combination is, respectively, significantly greater and lower that in the vehicle group. These results suggest that the drugs, although ineffective in reducing the proliferation of the MK, are improving their maturation.

The abnormal maturation of MK from mouse models and MF patients includes localization of P-SEL on the DMS instead than in the α-granules (22,25,26). Since the DMS increases with maturation, the amount of P-SEL exposed to the extracellular space also increases during this process. Therefore, the biotinylated RB40.34 which reach the BM should bind great numbers of *Gata1^{low}* MK and its binding should be greater as these cells mature. To assess whether the improved MK maturation induced by the treatments for 54 days included rescue of the altered cell surface expression of P-SEL, the binding of PE-Cy7-streptavidin to MK from the BM and spleen of mice treated for 5, 12 or 54 days was determined (**Figures 9A-C**). As expected, PE-Cy7-streptavidin binding is barely detected on BM and spleen cells from mice treated with either vehicle or Rux alone which had not received the antibody in any of the time points. The low levels PE-Cy7-streptavidin binding observed in these group probably represent background signals due to endogenously produced biotin and are not informative on the levels of P-SEL expressed by MK. By day 5 and 12, PE-Cy7-streptavidin binding is detected in cells both in the non-MK and in the MK gate. The binding of PE-Cy7-streptavidin to the non-MK cells is possibly related to the presence in this population of endothelial cells, also known to express P-SEL (17). By day 5 and 12, great numbers of MK from the BM and spleen of mice treated with RB40.34 alone or in combination with Rux bind PE-Cy7-streptavidin. As expected, the MFI of the binding increases in cells with a more mature phenotype, a reflection of the greater levels of P-SEL on the cell surface of *Gata1^{low}* MK as they mature. In addition, increases in MFI are also observed among MK of comparable maturation stage analyzed at day 5 and day 12, a possible reflection of increased bioavailability of the antibody in the microenvironment due to the reduction of fibrosis induced by the treatments. By contrast, PE-Cy7-streptavidin is found barely bound to MK

from BM and spleen of mice treated with RB40.34 alone or in combination with Rux by day 54. These last results indicate that the improved MK maturation induced by these two drug combinations may include reduced localization of P-SEL on the DMS. By day 54, PE-Cy7-streptavidin binding is also barely detectable in the non-MK populations from mice treated with RB40.34 alone or in combination with Rux. Since the cells responsible for binding PE-Cy7-streptavidin in the non-MK population are probably endothelial cells and that P-SEL expression in endothelial cells is up-regulated by inflammation(49), these data provide further support for the hypothesis that the treatments are reducing the inflammatory milieu of the BM and spleen from *Gata1^{low}* mice.

3.8 Treatment with Rux increases the frequency of MK expressing detectable levels of GATA1 in the BM from *Gata1^{low}* mice. The abnormal maturation of the MK which is thought to drive myelofibrosis in patients and mouse models is driven by defective content of GATA1(6,9,10), the transcription factor which plays a pivotal role in supporting MK maturation(6,11). As expected, confocal microscopy analyses with antibodies against GATA1 and CD42b (as a marker of MK) indicated that the BM from *Gata1^{low}* mice contains great numbers of MK the nuclei of which are not stained by the GATA1 antibody (**Figure S10**). To generate insights on the possible mechanism(s) that rescues MK maturation in mice treated with RB40.34 and Rux, alone or in combination, we performed confocal microscopy analyses with the same antibodies of BM section from mice treated for 54 days with either vehicle, RB40.34 alone, Rux alone or the two drugs in combination **(Figure 10)**. These analyses confirm the indications provided by flow cytometry (**Figure 9**) that none of the treatments affected the number of MK (as CD42bpos cells) present in the BM, which remains high. As expected, very few of the MK from mice treated with vehicle contain GATA1. By contrast, a significant number of MK in the BM from mice treated with either RB40.34 or Rux alone contain detectable levels of GATA1 in their nuclei (**Figures 10** and **S11**). It is surprising instead that GATA1 is not detected in MK in the BM from mice treated with RB40.34 and Rux in combination.

3.9 Treatment with RB40.34 in combination with Rux reduces the TGF-β and CXCL1 content of the BM of *Gata1^{low}* mice. Increased bioavailability of the pro-inflammatory cytokines TGF-β and CXCL1, the murine equivalent of human IL-8 has been suggested to represent the driver for fibrosis and hematopoietic failure in BM of MF patients and mouse models(4,50,51). In previous studies we demonstrated that the cells responsible for increasing the bioavailability of these two cytokines in the BM (and spleen) from *Gata1*^{low} mice are the abnormal MK(35). To test whether treatment with RB40.34 in combination with Rux decreases the proinflammatory milieu of the BM

433 microenvironment of *Gata1^{low}* mice, we performed histochemical evaluations with antibodies against
434 TGF-β and CXCL1 of BM sections from *Gata1^{low}* mice treated for 54 days with either vehicle,
435 RB4034 alone, Rux alone or the two drugs in combinations (**Figure 11**). As expected, the BM from
436 *Gata1^{low}* mice treated with vehicle contains great levels of TGF-β and CXCL1. The levels of TGF-β
437 are significantly decreased by treatment with both Rux alone or in combination with RB40.34
438 (**Figure 11A, B**). Morphological analyses of the cells which expressed TGF-β indicates that the
439 reductions are mainly due to reduced numbers of MK expressing this factor (**Figure 11C**). CXCL1
440 instead is reduced only by RB40.34 in combination with Rux and the numbers of MK which express
441 this factor remain high in all the groups (**Figure 11A-C**). Since in addition to MK, CXCL1 is
442 expressed by many other cell types, we suggest that the two-drugs in combination reduce the pro-
443 inflammatory milieu of *Gata1^{low}* mice by targeting not only the MK but also additional cells in the
444 microenvironment.

445

446 **4 Discussion**

447 The hypomorphic *Gata1^{low}* mutation deletes only one of the three major hypersensitive sites which
448 regulate the expression of the gene (52). After birth, the hematopoietic cells of these mice activate the
449 expression of the gene from the two regulatory sites not affected by the mutation so that the levels of
450 Gata1 mRNA in the hematopoietic cells are overall normal (39). However, the thrombocytopenia
451 induced by the mutation activates the TPO/Mpl axis(32) which results in a RSP14 ribosomopathy,
452 similar to that observed in MF patients(10), which reduces the efficiency of the translation of *Gata1*
453 mRNA reducing the content of the protein. The mechanism(s) linking the TPO/Mpl axis to the
454 RSP14 ribosomopathy in MF, and in animal models including *Gata1^{low}* mice, is still unknown. It has
455 been suggested that it is represented by aurora kinases since their inhibition increase GATA1 in the
456 MK while reducing fibrosis in animal models and in MF patients(53).

457 *Gata1^{low}* mice, although they do not carry any of the MF driver mutations, are considered a bona fide
458 animal model of MF because their HSC express an activated TPO/MPL axis, which may be drugged
459 by JAK inhibitors, and a RSP14 ribosomopathy, which is responsible for low GATA1 content and
460 altered MK maturation and P-SEL expression. In turn, altered P-SEL expression on the MK is
461 responsible for the pathological cell interaction that increase the bioavailability of pro-inflammatory
462 cytokines and drives fibrosis. Over the years we, and others(54–56), have extensively used *Gata1^{low}*
463 mice as a tool to identify lesions which may be targeted to normalize their myelofibrosis phenotype.

Based on previous observations indicating that deletion of *P-sel* prevents *Gata1^{low}* mice for developing myelofibrosis (27), we establish here whether inhibition of P-SEL, alone or in combination with Rux, may also normalize the phenotype of *Gata1^{low}* mice that have already established myelofibrosis. A summary of the results is presented in **Table S2**.

We first demonstrated that after short term-treatment, RB40.34 is bound to the platelets and the MK from the BM of *Gata1^{low}* mice, suggesting that the drug is retained in the circulation for at least 5h and that, in spite of fibrosis, reaches the BM of the animals. We also found that after 5 days, RB40.34 in combination with Rux normalizes non only the abnormal non-canonical TGF-β signals, which is a signature of a pro-fibrotic microenvironment, but also the abnormal canonical TGF-β signature, which indicates reduced hematopoiesis in the BM. These data suggests that after only 5 days the combination of RB40.34 and Rux is more effective than any of the two drugs alone in suppressing the cells responsible for fibrosis while reactivating hematopoiesis in the BM. The drug combination was also more effective than the two drugs as single agent in reducing JAK2 in the spleen from the mutant mice, suggesting that it is reducing extramedullary hematopoiesis in this organ.

Encouraged by these results, we analyzed the effects of long-term treatments with RB40.34 and Rux, alone or in combination, on the myelofibrotic phenotype expressed by *Gata1^{low}* mice using a vast range of clinically relevant end points. The results indicate that none of the treatments induce anemia nor rescue the thrombocytopenia of *Gata1*^{low} mice. However, treatment for 54 days with RB40.34 in combination with Rux, and to a less extent the two drugs alone, reduced anisocytosis, expression of P-SEL on MK, and probably on endothelial cells, and lymphocyte counts. Since the iron metabolism of old *Gata1^{low}* mice is normal (Stefano Rivella, unpublished observation) and the mean corpuscular volume and Hb content of the RBC remain within normal values in all the treated mice (**Table S3**), it is unlikely that the high RDW detected in *Gata1^{low}* mice is a sign of impaired iron metabolism. Anisocytosis without raises in mean corpuscolar volume is induced by pro-inflammatory cytokines in several benign and malignant disorders, including MF where it has been proposed as a marker that predict inferior survival (57). We hypothesize that the reduction in anisocytosis observed at day 54 in the RB40.34 alone or in combination with Rux groups reflects reductions in the proinflammatory cytokines TGF-β and/or CXCL1 that drives myelofibrosis in this model. Since TGF-β and CXCL1 are well known to affect directly (TGF-β) or indirectly (through neutrophil activation, CXCL1) lymphocyte counts (58–61), this hypothesis is also consistent with the reduced lymphocyte counts observed by day 54 in the mice treated with the two drugs in combination. Reduction in

microenvironment bioavailability of pro-inflammatory cytokines was directly tested by showing that RB40.34 and Rux in combination significantly reduces the TGF-β and CXCL1 content of the BM.

Finally, treatment for 54 days with RB40.34 in combination with Rux also reduced fibrosis in BM and spleen while improving effective hematopoiesis in BM and reducing extramedullary hematopoiesis restoring the architecture of the spleen.

Treatments for 54 days with RB40.34 and Rux, alone or in combination, were ineffective in reducing the proliferation of the MK which may be driven in our model, as well as in the patients, by the activated TPO/Mpl axis. They were, however, all effective in improving the maturation profile of the MK, including reducing the abnormally high level of cell surface expression of P-SEL and TGF-β content. RB40.34 and Rux alone were also effective in increasing the GATA1 content in a proportion of CD42bpos MK. The mechanistic interpretation of these data is complicated by the recent single cell profiling indicating that murine (and human) BM contains four distinctive MK subpopulations, each one exerting a different function (47,62–64). The BM of adult mice and men contains at least three subpopulations: the platelet producing MK, the niche supportive MK, and the immune MK. Only platelet producing MK have the morphology of mature MK. Niche supportive MK and immune MK have instead the morphology of immature MK. In addition, by characterizing the MK subpopulations present in the embryos, Wang et al(63) identified a fourth subpopulation, which they defined niche-poised-MK that has an immature morphology and is characterized by high expression of extracellular matrix genes such as *COL1A1*, *COL3A1*, and *COL6A2* and enrichment of the "response to TGF- β signature", which indicate that this subpopulation is sustained by TGF-β. Of interest for this paper, reduced GATA1 content blocks the maturation of platelet producing MK but not favors the maturation of the other three subpopulations(47,62–64). This new knowledge indicates that the nature of the immature GATA1^{low} MK found in great numbers in MF is presently not known. Preliminary observations indicating that great numbers of the morphologically immature MKs in the BM from *Gata1*^{low} mice, as well as that of MF patients, express collagen(65,66) suggests that at least some of these MK are represented by niche poised MK the maturation of which is reactivated by TGF-β(19). It is, therefore, possible, that low levels of GATA1 drive the disease not only by retaining platelet forming MK immature (leading to the thrombocytopenia), but also by increasing the frequency of other megakaryocyte subtypes. According to this hypothesis, although RB40.34 and Rux in combination did not induce detectable increase in GATA1 content in MK (and did not increased platelet counts), it reduced the frequency of MK containing TGF-β, which may correspond to niche supporting MK. Reduced TGF-β bioavailability may have then limit the number of niche

MK, which are sustained by this growth factors and are responsible for. On the other hand, Rux alone, which induced the greater increase of GATA1 in MK, also reduced the TGF-β containing MK but did not altered fibrosis, suggesting that the number of MK expressing collagen was not significantly affected. These data support the need of further studies, clearly outside the purpose of the current manuscript, to clarify the MK subpopulations which are altered in MF, which of them is responsible for the different traits of the myelofibrosis phenotype and how they are affected by RB40.34 and Rux alone or in combination.

In conclusion, these data provide pre-clinical evidence that treatment with the RB40.34 antibody in combination with Rux is more effective than the use of Rux alone for reverting the myelofibrotic trait in the *Gata1^{low}* mouse model and encourage clinical studies to validate the effects of Crizanlizumab, in combination with Rux, for the treatment of human PMF.

5 Conflict of Interest

PV, FG, FM, MF, AV, GS and MZ declare no conflict. CW, AB and AP are employee of Novartis Pharmaceutical Corporation. ARM received research funds from Novartis Pharmaceutical Corporation.

6 Author Contributions

PV, FG, FM, MF, FA and AV performed experiments and analyzed the data. PV and FG performed statistical analyses. GS reviewed all the histopathogical determinations. ARM and MZ designed the study, interpreted the data and wrote the manuscript. CW, AB and AP revised the data and wrote the manuscript. All the authors read the manuscript and concur with its content.

7 Data Availability Statement

The individual data for each mouse are available on request.

8 Disclosure

The content of the manuscript was presented as a poster at the 62rd ASH Annual Meeting & Exposition (Paola Verachi, Fabrizio Martelli, Maria Zingariello, Shalini Chaturvedi, Celine Wilke, Valerie Campello-Iddison, Anna Rita Migliaccio; Preclinical Rationale for the Use of Crizanlizumab (SEG101) in Myelofibrosis. *Blood* 2020; 136 (Supplement 1): 26–27. doi: https://doi.org/10.1182/blood-2020-133896). The data are unpublished and have not been submitted for publication to any other journal.

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LEGEND to FIGURES

Figure 1. At day 5, RB40.34 is readily detected on the platelets present in the blood and on sections of bone marrow from *Gata1*^{low} mice. (A) Flow cytometry analyses with APC-Cy7-streptavidin of platelets present in the blood from *Gata1*^{low} mice after 5 days of treatment. The mice had been treated 5hr earlier with vehicle, biotinylated-RB40.34, Rux or the two drugs in combination, as indicated. Platelets are recognized based on size (FS: forward scatter) and internal cell complexity (SS: side scatter). Representative FS/SS gating and histograms of the APC-Cy7-streptavidin staining are presented on the left. Mean Fluorescence Intensity (MFI) (±SD) of APC-Cy7-streptavidin staining and values in individual mice (each symbol a mouse) are presented on the right. p values are calculated with Tukey multiple comparison test and significant differences are indicated in the panels. (B) Representative sections of BM from *Gata1*^{low} mice treated with either vehicle (first panel) or biotinylated-RB40.34, Rux and Rux+ biotinylated-RB40.34 incubated with APC-Cy7-streptavidin (top panels). The panel on the bottom shows the computer-generated signal specific for mAb RB40.34 obtained by subtracting the background from vehicle with the ImageJ program. Areas exciding the threshold are artificially labeled in red. Detail of the ImageJ processing of the images are provided in **Figure S2**. Magnification 40x. The intensity of APC-Cy7 staining as percent of areas above the threshold in sections from the BM of multiple mice are presented on the right.

Figure 2. Treatment with RB40.34 in combination with Rux restores the abnormal canonical and non-canonical TGF-β signaling observed in the bone marrow from *Gata1*^{low} mice. (A-C) Western blot analyses for the content of elements downstream to the canonical and non-canonical TGF-β signaling of the BM of untreated wild-type (WT) and *Gata1*^{low} mice and from *Gata1*^{low} treated with vehicle, RB40.34, Rux or the two drugs in combination, as indicated. Blots are presented on the right and quantifications on the left. Quantitative values are presented as Means (± SD) and as individual values for each mouse. Total protein levels are normalized toward the corresponding GPDH levels while, to take into account differences in total protein, the levels of the phosphoproteins are expressed stoichiometrically as a ratio with the total level of the corresponding protein. p values were calculated with Tukey multiple comparison test and statistically significant differences are indicated in the panels.

Figure 3. Treatment with RB40.34 in combination with Rux restores the abnormal JAK2/STAT5 signaling observed in the spleen from *Gata1*^{low} mice. Western blot analyses for JAK2, STAT5 and GAPDH (as loading control) of BM (A,C) and spleen (B,D) from untreated wild-type (WT) and *Gata1*^{low} mice and from *Gata1*^{low} mice treated with vehicle, RB40.34 and Rux, alone or in combination. Blots are presented on the right and quantifications on the left. In C and D, quantitative values are presented as Means (± SD) and as individual values for each mouse. p values are calculated with Tukey multiple comparison test and statistically significant differences are indicated in the panels.

Figure 4. Treatment for 54 days with RB40.34 in combination with Rux reduces the frequency of red blood cell anisocytosis and lymphocyte counts in the blood from *Gata1*^{low} mice. (A) Red blood cell distribution width (RDW, in %) detected in the blood from *Gata1*^{low} mice treated with vehicle, RB40.34, Rux or the two drugs in combination for 54 days, as indicated. Data are presented as Mean (±SD) and as individual values from each mouse. The asterisks indicate the groups containing the deidentified samples flagged for anisocytosis by the accredited laboratory. (B) Lymphocyte counts observed in the blood from *Gata1*^{low} mice treated with vehicle, RB40.34, Rux or the two drugs in combination for 54 days, as indicated. Data are presented as Mean (± SD) and as individual values from each mouse. p values are calculated with Tukey multiple comparison test and statistically significant differences ($p<0.05$) are indicated in the panels.

- **Figure 5. Treatment for 54 days with RB40.34 in combination with Rux increases the cellularity, reduces fibrosis and restores hematopoiesis in the bone marrow from *Gata1*^{low} mice.** A) Photographs of the femur and tibia from representative mice treated for 54 days with either

Vehicle or RB40.34 in combination with Rux, as indicated. B) Number of cells per femur observed at day 54 in *Gata1^{low}* mice treated with either vehicle, RB40.34, Rux or the two drugs in combination. C) Hematoxylin/Eosin (H&E) and Reticulin staining of femurs from representative *Gata1^{low}* mice treated for 54 days with either vehicle, RB40.34, Rux or the two drugs in combination, as indicated. The femurs are presented as stack images (at 4x) and as representative sections at 4 and 20x magnification, as indicated. D) Levels of fibrosis quantified by image analyses of the reticulin staining of BM sections from *Gata1^{low}* mice treated for 54 days as indicated above. E) Frequency and total number of Lin-, LSK and SLAM cells in the femur of *Gata*1^{low} mice treated with the various drug combinations. In B, D and E, results are presented as Mean (±SD) and as values per individual mice (each symbol a mouse) and were analyzed by Tukey's multiple comparisons test. Statistically significant groups are indicated within the panels.

- **Figure 6. Treatment for 54 days with RB40.34 and Rux, alone or in combination, reduces the vessel density in the bone and spleen from *Gata1^{low}* mice.** Confocal microscopy with CD34 and Hoechst (to counterstain the nuclei) of bone marrow (A) and spleen (B) sections from *Gata1^{low}* mice treated for 54 days with either vehicle or with RB40.34 and Rux alone and in combinations. The panels in the first and third lanes are at 20x magnification, and the area depicted in the rectangles are shown at 60x in the corresponding panels in the second and third lanes. At 60x magnification, microvessels (dashed lines) are identified as structures surrounded by CD34pos cells (indicated by arrows) and containing red cells (autofluorescent cells not counterstained by Hoechst, asterisks). Quantitative results are shown on the right as Mean (±SD) and as values per individual mice (each symbol a mouse). Statistical analysis was performed by Tukey's multiple comparisons test and significant p-values are indicated within the panels.

Figure 7. Treatment for 54 days with RB40.34 in combination with Rux decreases hematopoiesis of the spleen from *Gata1^{low}* mice. A) Photographs of representative spleens treated for 54 days with the various drug combinations, as indicated. B,C) Spleen size, as weight and ratio between spleen weight and body weight (B), and total cell numbers (C), of mice treated for 54 days with the various drug combinations. D) Frequency and total number of Lin- and LSK cells in the spleen of *Gata*1^{low} mice treated with the various drug combinations. SLAM cells are not presented because they are almost 100% of the LSK cells detected in the spleen (see **Figure S9)**. In B, C and D, results are presented as Mean (±SD) and as values per individual mice (each symbol a mouse) and

are analyzed by Tukey's multiple comparisons test. Statistically significant groups are indicated within the panels.

Figure 8. Treatment for 54 days with RB40.34 in combination with Rux decreases fibrosis and restores the architecture of the spleen from *Gata1^{low}* mice. A) Hematoxylin/Eosin (H&E) and reticulin staining of spleen from representative 8-11-months old WT and *Gata1^{low}* mice. WT spleens are characterized by the presence of large aggregates of lymphoid cells, well developed white pulp, with the presence of red blood cells embedded in reticular connective tissue containing few megakaryocytes and supporting trabeculae. By contrast, *Gata1*^{low} spleen is characterized by hypoplastic white pulp and red pulp rich of megakaryocytes. Reticulin staining of the consecutive section indicates that fibrosis is localized mostly in the red pulp. Results are representative of those observed in at least three WT and three *Gata1^{low}* littermates, all 11-months old. B) Triple immunofluorescent analyses for CD3 (as a marker of T cells, red), CD45R (B220, as a marker for B cells, green) and DAPI (nuclei) of spleen sections from representative WT and *Gata1^{low}* mice, as indicated. As expected (50), the white pulp of WT spleen contains numerous T and B lymphocytes, organized around central arterioles. T lymphocytes form a sleeve around the central arteriole, the periarteriolar lymphoid sheath while B cells are mainly localized in the outer white pulp region, the marginal zone. In *Gata1*^{low} spleens the white pulp is smaller than that in the WT organ and the periarteriolar lymphoid sheath contain a markedly reduced number of T cells. Magnification 4x, 10x and 20x, as indicated. C) Hematoxylin/Eosin (H&E), reticulin staining and triple staining with CD45R (green), CD3 (red) and DAPI (blue) of sections from the spleen of representative *Gata1^{low}* mice treated for 54 days with the various drug combinations, as indicated. Images are presented at 4x and 20x magnification. D) Levels of fibrosis quantified by image analyses of the reticulin staining of spleen sections from untreated 8-11-months old WT and *Gata1^{low}* littermates, as indicated. E) Levels of fibrosis, quantified by image analyses of the reticulin staining, in spleen sections, from *Gata1^{low}* mice treated for 54 days, as indicated. In D and E) results are presented as Mean (±SD) and as values per individual mice (each symbol a mouse) and were analyzed by t test. Statistically significant groups are indicated within the panels.

Figure 9. Treatment for 54 days with RB40.34 and Rux alone in combination improves the maturation profile of the MK from the bone marrow and spleen of *Gata1^{low}* mice. A) Representative dot-plots and histograms of MK from the bone marrow (left quadrant) and spleen (right quadrant) of one representative mouse from each experimental group treated for 5 days. MKs were labeled with CD41, CD61 and PE-Cy7-streptavidin. The a, b, c and d gates identify non-MK,

immature MK, mature MK and very mature MK, respectively. The levels of APC-Cy7-streptavidin bound to the MKs at their different stage of maturation is presented by histograms. Since P-sel is abnormally expressed at high levels on the surface of *Gata1^{low}* MK, the APC-Cy7-streptaviddin signal identifies the MK expressing P-sel which have bound the biotinylated RB40.34 injected 5 hr earlier in the mice. B,C) Frequency of cells in the non-MK (a) and immature (b), mature (c) and very mature (d) MK gate (% in percent of total cell number) and percentage and MFI of the events positive for PE-Cy7-streptavidin staining in each gate in the BM (A) and spleen (B) from *Gata1^{low}* mice treated for 5, 12 and 54 days with either vehicle or the different drug combinations, as indicated. Values were reported as Means (±SD) of those detected in at least three mice per experimental group. Data are analyzed by Tukey's multiple comparisons and statistically significant differences among groups are indicated within the panels.

Figure 10. Treatment for 54 days with RB40.34 or Rux alone, but not in combination, increases the GATA1 content in the MK from the BM of *Gata1^{low}* mice. A) Merged GATA1 (FITCH-green) and CD42b (TRITCH-red, as a marker of MKs) images of the confocal microscopy analyses with the corresponding antibodies in BM sections from representative *Gata1^{low}* mice treated for 54 days with either vehicle, RB40.34 alone, Rux alone, or the two drugs in combination, as indicated. The corresponding images acquired in the single cannels, in the cannel for DAPI (as indication of the nuclear localization of GATA1) and in the bright field (to exclude autofluorescence) are presented in **Figure S11.** Magnification 40x. B) Frequency of MK (CD42b positive cells) and percentage of MK positive for GATA1 in BM sections from *Gata1^{low}* mice treated for 54 days as indicated. Data are presented as Mean (±SD) and as values in individual mice (each symbol a mouse). Results were analyzed by Tukey's multiple comparisons test and significant differences among groups indicated within the panels.

Figure 11. Treatment for 54 days with RB40.34 and Rux in combination decreases the TGF-β, mainly in the MK, and CXCL1 content of bone marrow from *Gata1^{low}* mice. A) BM sections from representative mice treated for 54 days with either vehicle, RB40.34, Rux or the two drugs in combination immune-stained for TGF-β or CXCL1, as indicated. Representative MK are indicated by arrows. Magnification 40x. B) Quantification by computer assisted imaging of the TGF-β1 and CXCL1 content in the BM from *Gata1^{low}* mice treated for 54 days as indicated. C) Frequency of MK and percentage of MK expressing high levels of TGF-β1 and CXCL1 in BM sections from *Gata1^{low}* mice treated as indicated. MK were identified on the basis of size (10 times greater than that of any

893 other cell type in the section) and the polylobate morphology of their nuclei. In B and C, data are
894 presented as Mean (±SD) and as values per individual mice (each symbol a different mouse) and are
895 analyzed by Tukey's multiple comparisons test. Values statistically different are indicated within the
896 panels.

897 **10.1 Tables**

898 **Table 1.** Hematocrit (Hct, %), platelets (plt) and white blood cells (WBC) counts determinations
899 at day 5, 12 and 54 in *Gata1*^{low} mice treated with vehicle, RB40.34, Rux or with the combination of drugs.
900 Historical values from *Gata1*^{low} untreated mice and WT littermates are reported. $^{(n)}$: number of mice.
901 Values among the treatment groups are not statistically different by Tukey multiple comparison test.
902

Hct (%)	**Controls**	**Day 5**	**Day 12**	**Day 54**
WT	39.65 ± 0.49 $^{(2)}$			
Gata1^{low} untreat	21.10 ± 6.64 $^{(16)}$			
Vehicle		33.03 ± 4.23 $^{(6)}$	44.30 ± 0.10 $^{(3)}$	33.52 ± 3.08 $^{(5)}$
RB40.34		32.15 ± 5.51 $^{(6)}$	43.25 ± 0.21 $^{(2)}$	33.47 ± 0.23 $^{(3)}$
Rux		38.12 ± 1.46 $^{(6)}$	44.43 ± 0.81 $^{(3)}$	29.46 ± 5.00 $^{(5)}$
Rux + RB40.34		38.00 ± 1.58 $^{(6)}$	44.07 ± 0.58 $^{(2)}$	29.19 ± 1.23 $^{(3)}$

903

Plt (10^3/µL)	**Controls**	**Day 5**	**Day 12**	**Day 54**
WT	775 ± 466.69 $^{(2)}$			
Gata1^{low} untreate	115.06 ± 42.10 $^{(16)}$			
Vehicle		138.67 ± 40.23 $^{(6)}$	100.67 ± 17.67 $^{(3)}$	92.40 ± 26.34 $^{(5)}$
RB40.34		111 ± 46.23 $^{(6)}$	79 ± 19.80 $^{(2)}$	65.00 ± 28.48 $^{(3)}$
Rux		137.83 ± 33.81 $^{(6)}$	105.33 ± 26.41 $^{(3)}$	94.60 ± 59.32 $^{(5)}$
Rux + RB40.34		142.60 ± 26.45 $^{(6)}$	123 ± 31.43 $^{(2)}$	126.33 ± 34.59 $^{(3)}$

904

Plt (10^3/µL)	**Controls**	**Day 5**	**Day 12**	**Day 54**

WT	775 ± 466.69 (2)			
Gata1^{low} untreate	115.06 ± 42.10 (16)			
Vehicle		138.67 ± 40.23 (6)	100.67 ± 17.67 (3)	92.40 ± 26.34 (5)
RB40.34		111 ± 46.23 (6)	79 ± 19.80 (2)	65.00 ± 28.48 (3)
Rux		137.83 ± 33.81 (6)	105.33 ± 26.41 (3)	94.60 ± 59.32 (5)
Rux + RB40.34		142.60 ± 26.45 (6)	123 ± 31.43 (2)	126.33 ± 34.59 (3)

905