Hematopoietic expression of a chimeric murine-human CALR oncoprotein allows the assessment of anti-CALR antibody immunotherapies in vivo

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Abstract
Myeloproliferative neoplasms (MPNs) are characterized by a pathologic expansion of myeloid lineages. Mutations in JAK2, CALR and MPL genes are known to be three prominent MPN disease drivers. Mutant CALR (mutCALR) is an oncoprotein that interacts with and activates the thrombopoietin receptor (encoded by the MPL gene). The mutCALR protein acts as a rogue chaperone and is known to stabilize the dimeric form of MPL. Also, mutCALR cannot be recycled in endoplasmic reticulum (ER) as its wild type counterpart because of the absence of the KDEL residues at the C-terminus. Notably, mutCALR physically interacts

1 | INTRODUCTION

The identification of CALR gene mutations has been a breakthrough towards understanding the molecular basis and diagnosis of patients with MPN. Type 1 (del52) and type 2 (ins5) mutations are the most recurrent mutations in MPN patients.1,2 Mutant CALR (mutCALR) proteins induce ligand-independent activation of the thrombopoietin receptor (encoded by the MPL gene).3-7 The mutCALR protein acts as a rogue chaperone and is known to stabilize the dimeric form of MPL. Also, mutCALR cannot be recycled in endoplasmic reticulum (ER) as its wild type counterpart because of the absence of the KDEL residues at the C-terminus. Notably, mutCALR physically interacts...
with the MPL receptor and is presented on the cell surface as a complex with MPL and it was also shown to be secreted into the extracellular milieu.4,9 The mutant C-terminus of the protein serves as a bona fide neo-antigen, offering the possibility of developing immunotherapeutic strategies to target the oncprotein.10

The alternative reading frame used by the human mutCALR oncprotein displays a 60–70% homology to the murine Calr exon 9 alternative reading frame. Thus, potential therapeutic antibodies raised against the mutant C-terminus of human mutCALR may not necessarily recognize the mouse frameshifted sequence. Therefore, we aimed to generate a chimeric murine-human transgenic model that can be used for studying immunotherapeutic interventions targeting human mutCALR. To preserve the epitope composition of the mutant C-terminus, we conditionally knocked-in the human exon 9 carrying the del52 mutation into the endogenous locus of the mouse Calr gene. The resulting mouse-human CALR-del52 chimeric oncogene recapitulated the MPN phenotype in vivo. Here in, we provide a proof of concept that the mutCALR murine transgenic model can be used to assess the in vivo efficacy of immunotherapeutic interventions using antibodies or their derivatives.

2 | MATERIAL AND METHODS

2.1 | Generation of conditional knock-in CALR-del52 transgenic mice

The CALR-del52 transgenic mice were generated by homologous recombination of embryonic stem cells in C57BL/6 mice (Ogene, Perth, Australia). The targeting vector consisted of two loxP sites flanking the murine endogenous exons 8 and 9 with a poly A sequence, a PGK-neomycin selection cassette (flanked by Flp recombinase target FRT sequences) and a cDNA sequence of murine exon 8 ligated with the human exon 9 del52 with the endogenous murine 3' UTR. Embryonic stem cells were transfected with the targeting construct, selected with neomycin and the cells with homologous recombination were identified by Southern blotting. The targeted stem cells were then microinjected into C57BL/6 blastocysts to obtain chimeras that were bred with C57BL/6 mice to generate germline transmitting CALR-del52 conditional knock-in mice. The PGK-neo cassette was deleted using the OzFlp: Ubic-Flpe mice (Figure 1A). The conditional transgenic mice were bred with OZCre mice (PGK-cre in ROSA26') to generate germline heterozygous CALR-del52 mice. The germline knock-in mice did not survive past embryogenesis in the homozygous state. The mice were bred with Vav-iCre transgenic mice11 for hematopoietic specific expression of CALR-del52.

2.2 | Animal experiments

The mice were housed and maintained under standard conditions and all experiments were performed in accordance with Austrian law under the animal experimentation license number BMWFV-66.015/0004-WF/V/3b/2016. The genotyping primers are listed in Table S1. Blood was obtained from Vena facialis and at end point by heart puncture. Blood parameters were measured using the animal blood counter scil Vet abc. The sternum and a part of the spleen were processed for histopathology. Single cell suspensions were made from the rest of the spleen and the bone marrow from the femurs and tibia and analyzed by flow cytometry.

2.3 | Enzyme-linked immunosorbent assay (ELISA) for quantification of secreted mutCALR

To quantify secreted mutCALR (sCALR) in murine and human sera, two antibodies against mutCALR were used – polyclonal antibodies against a 22-mer peptide derived from the mutCALR C-terminus in rabbits (capture antibody) and polyclonal antibodies against the recombinant CALR-ins5 protein in chicken (detection antibody). The rabbit anti-mutCALR specific polyclonal antibody (anti-mutCALRaB - SAT601) was diluted in PBS at a concentration of 2 μg/mL and 50 μL was used to coat each well of a 96-well plate. The assay plates were incubated overnight at 4°C and later washed with wash buffer (0.05% Tween20 in PBS). Plasma samples from vavCre, CALRdel52fl/fl:vavCre (mutCALRht), CALRdel52fl/fl:vavCre (mutCALRht), CALR mutated patients and healthy control were diluted 1:4 in the blocking buffer (2% BSA, 0.05% Tween20 in 1xPBS) and were added to the plates. The samples were incubated for 2 h at 30°C and later washed with wash buffer. Rabbit anti-mutCALR polyclonal antibody was added to all the wells at a concentration of 0.5 μg/mL for 1 h at 30°C. Following washing of the plates, secondary antibody anti-chicken IgY-HRP was used in the assay (1:50 000) for 1 h at room temperature. The plates were then washed and developed with TMB for 5 min at room temperature. The absorbance was read at 450 and 620 nm using Spectra-Max i3 (Molecular Devices). Recombinant CALRdel52 and CALRins5 proteins (MyeloPro) were used as a standard to calculate the concentration of the samples.

2.4 | Anti-mutCALR antibody treatment of mice

The mice (3n) were injected intraperitoneally with 3 mg/kg of mouse IgG2a anti-mutCALR monoclonal antibody twice a day, for 2.5 days. The control group received an equal volume of the vehicle (PBS). The mice (3n) were injected intraperitoneally with 3 mg/kg of mouse IgG2a anti-mutCALR monoclonal antibody twice a day, for 2.5 days. The control group received an equal volume of the vehicle (PBS). The end point analysis was performed as described above.

3 | RESULTS

3.1 | CALR-del52 transgenic mice express the mutant CALR protein and develop myeloproliferative disease

In the presence of Cre recombinase, the endogenous murine exon 9 of Calr is replaced by human del52 mutated exon 9 (Figure 1A). This
FIGURE 1  CALR mutant mice develop myeloproliferative disease. (A) Schematic representation of the transgenic Calr locus. (B) Western blot analysis of wild type and mutant CALR proteins (high and low exposure) from splenocytes of transgenic mice. (C) Peripheral blood counts of platelets, WBC and RBC from transgenic mice over time. (D) Representative images of spleens from transgenic mice at 6 months and 1 year of age. (E) Spleen to body weight ratio. Percentage of different populations in the bone marrow of transgenic mice at 6 months and 1 year of age (F). Megakaryocytes (G), LSK cells (H), LT-HSCs (I), ST-HSCs and MPPs (J). (K) Histopathological analyses of sterna of transgenic mice at 6 months and 1 year of age, by H&E and reticulin staining. Black bars correspond to 50 μm.
results in the generation of a mouse-human chimeric CALR-del52 (murine Calr with human mutant C-terminal end), under the control of the endogenous murine Calr promoter. We generated the conditional CALR-del52 mice by breeding with vavCre transgenic animals to restrict the expression of CALR-del52 to the hematopoietic system. To confirm the expression of the mutant CALR protein, Western blot analysis was performed with the whole cell extracts of splenocytes and probed using antibodies against total CALR and mutant CALR. The expression level of mutant CALR were higher in mutCALR homozygous (mutCALRhom) mice for the CALR-del52 transgene compared to mutCALR heterozygous (mutCALRhet) mice. The expression level of total CALR is significantly lower in the mutCALRhom mice compared to that in the wild type mice due to the increased degradation and secretion of the mutCALR protein (Figure 1B). We followed the peripheral blood values of a cohort of mutCALRhet and mutCALRhom mice and vav-Cre control mice (Figure 1C). Compared to wild type controls, mutCALRhet mice had elevated platelet counts from 6 weeks of age without any significant change in total white blood count (WBC) and red blood count (RBC). However, in the mutCALRhom mice, the platelet counts were significantly higher. In older mice, leukocytosis and erythrocytosis were evident along with thrombocytosis. We analyzed the mice in more detail at 6 months and 1 year of age and made similar observations in the peripheral blood (Figure S1A).

The mutCALRhom mice manifested splenomegaly at 6 months of age with high spleen to body weight ratio (2.25-fold) that increased at 1 year of age (3.3-fold). The mutCALRhet mice developed mild splenomegaly only at 1 year (1.3-fold) (Figure 1D,E). The total number of cells in the bone marrow (BM) was significantly reduced in 6 months (4-fold) and 1 year (6-fold) old mutCALRhom mice (Figure 1C). We saw a significant increase in the percentage of megakaryocytes (Mks) in the bone marrow of mutCALRhet (1.7-fold) and mutCALRhom (6-fold) mice at 6 months. The percentage of Mks increased further in mutCALRhet mice at 1 year (7-fold) (Figure 1F). The percentage of hematopoietic stem cells (HSCs, LSK, lineage− sca1+ kit+) was also significantly increased in the mutCALRhet mice at 6 months (2-fold), which further increased at 1 year (6-fold) of age. However, the mutCALRhet mice showed increased LSK cells only at 1 year (1.7-fold) of age (Figure 1G). In mutCALRhet mice this increase in LSK cells was due to increase in all three sub-populations of the stem cells - long term HSCs (LT-HSCs, Lineage− sca1+ CD117+ c-KIT+ CD150− CD48−), short term HSCs (ST-HSCs, Lineage− sca1+ c-KIT− CD150− CD48−) and multipotent progenitor cells (MPPs, Lineage− sca1+ c-KIT+ CD48+) (Figure 1H–J). Similar to the bone marrow, we observed increased percentage of progenitor populations in the spleen of mutCALRhet mice at both time points and in mutCALRhet mice at 1 year of age (Figure S1D–G).

Histopathological analysis of the bone marrow confirmed a myeloproliferative phenotype with prominent megakaryocytosis and megakaryocytic dyspoiesis in the bone marrow mutCALRhom mice (Figure 1K, Figure S1B). These features were milder in the mutCALRhet mice and in 6 months old mice. The one-year-old mutCALRhom mice had mild to moderate trabecular osteosclerosis and minimal to mild increase in reticulin positive fibers. Overt myelofibrosis was not a prominent feature in these mice (Figure 1K).

In the spleen, the splenic red pulp was significantly expanded by the myeloproliferative process with marked megakaryocytosis and megakaryocytic dyspoiesis in 6 month- and 1 year-old mutCALRhom mice. Lymphoid follicles were significantly attenuated in the spleens of these mice. In the mutCALRhet mice, these features were much less prominent at 1 year of age, and insignificant to marginal at 6 months (Figure S1H).

The conditional CALR-del52 mice were bred with Oz-Cre transgenic animals (which express Cre in germ line cells) to generate mice with germ line expression of CALR-del52 (CALRdel52Δ/Δ). Germline homozygous mice are not present in these litters suggesting embryonic lethality as previously reported. Germline heterozygous mice are viable and develop a phenotype similar to the mutCALRhet mice (Figure S2). Taken together, the CALR-del52 transgenic animals develop a disease reminiscent of human essential thrombocythemia.

### 3.2 CALR mutant stem cells have increased proliferation capacity in a competitive bone marrow transplant

We performed a competitive bone marrow transplant assay by transplanting a mixture of total bone marrow cells from mutCALRhom mice (CD45.2) and vavCre mice (F1:CD45.1/45.2), in a ratio of 10:90; into lethally irradiated wild type recipient mice (CD45.1) (Figure 2A). The recipient mice were bled regularly, and the blood parameters and percentage of mutant cells (by FACS) were determined (gating strategies have been shown in Figure S4). There was a steady increase in the number of platelets and WBC in the recipient mice, while the number of RBC remained unchanged (Figure 2B). The percentage of CALR mutant cells kept steadily increasing with time, in all lineages (Figure S3A). Five months after the transplant, the mice were euthanized, and the hematological organs were analyzed by FACS. A majority of myeloid cells (around 60%) in the blood were derived from the CALR mutant cells (Figure 2C). In the bone marrow, 75% of LSK cells and nearly 100% of LT-HSCs were of the mutant cell origin (Figure 2D).

We then performed a competitive bone marrow transplant wherein sorted LSK cells from mutCALRhet and mutCALRhom mice (CD45.2) and vavCre mice (F1 CD45.1/45.2), were transplanted into lethally irradiated wild type recipient mice (CD45.1); in a ratio of 20:80 (Figure 2E). The mice were euthanized and analyzed after 20 weeks by assessing blood parameters and by flow cytometry. We did not find any significant differences in the percentage of different populations in the blood or bone marrow among the four groups (Figure S3C,D). We also did not see a significant increase in the percentage of mutant cells in the myeloid lineage, in the blood; although the percentage of chimerism was significantly reduced in the T-cells, in mice that received LSK cells from mutCALRhom mice. In the bone marrow, we saw a significant increase in the mutant chimerism in LSK cells only in mice that received mutCALRhom transplants. Within the LSK cells, all the three progenitor populations (LT-HSCs, ST-HSCs and MPPs) showed significant increase of the mutant chimerism.
percentage in mutCALR<sup>hom</sup> transplanted mice (Figure 2G). In conclusion, the stem cells from the CALR-del52 homozygous mice have an increased proliferative advantage compared to those from wildtype and heterozygous mice.

### 3.3 Transcriptome profiling reveals a distinctive gene signature pattern in murine hematopoietic stem cells

To understand the effect of mutCALR, we determined the transcriptome of bone marrow LSK (Lin-Sca+Kit+) cells (Figure 3A). Principal Component Analysis reveals hierarchical clustering of samples based on genotype (Figure 3B). We identified 460 differentially expressed genes between wild type and mutCALR LSK cells (Figure S5B) and the top 25 upregulated and downregulated genes are shown (Figure 3C). Gene set enrichment analysis (GSEA) against hallmarks cohort revealed 12 gene-sets that are significantly enriched, and six gene sets significantly depleted in mutCALR LSKs. We saw upregulation of genes in the IL-6 JAK–STAT signaling pathway, including the bona fide STAT5 targets such as Mycn, Socs1, and Junb (Figures 3D). Components of the cholesterol homeostasis (Clu, Trib3, Plscr1, Aldoc, Jag) were ranked significantly high in the GSEA. Interferon alpha response, TNFA signaling via NFKB, bile acid metabolism, MTORC1 signaling, P53 pathway, interferon gamma response and hypoxia are few other gene-sets that are significantly upregulated in the mutCALR LSK cells. We see a significant downregulation in gene-sets associated with E2F targets, G2M checkpoint, mitotic spindle, epithelial to mesenchymal transition, Kras signaling and coagulation in mutCALR LSK cells (Figure 3E,F). The transcriptome analysis identified...
specific pathways associated with the oncogenic functions of mutCALR and reveals the inflammatory gene expression signature in the bone marrow stem cells.

3.4 Treatment with anti-mutCALR monoclonal antibody reduces platelet and LSK counts

The mutant CALR protein represents a bona fide tumor antigen that is a target for immunotherapeutic strategies. We were able to detect the presence of the mutCALR protein on the surface of the total cells, lineage negative cells, LSK cells and megakaryocytes in the bone marrow of mutCALR<sup>hom</sup> mice, by FACS (Figure 4A). Since, the expression of the protein was higher in the homozygous mice and the homozygous mice best represent the human MPN phenotype, we chose to use these mice for immunotherapy experiments. We treated the mice with a monoclonal antibody targeting the human mutant C-terminus. The mutCALR<sup>hom</sup> mice were injected with anti-mutCALR mAb, intraperitoneally 3 mg/kg, twice a day for a period of 2.5 days (Figure 4B). After treatment, only platelet counts were significantly reduced

FIGURE 3 Transcriptome analysis of stem cells with CALR mutations. (A) Schematic representation of the RNA-seq pipeline (B) Hierarchical clustering of the reads according to genotype of the samples: vavCre vs CALR<sup>del52fl/fl</sup>;vavCre (C) Heatmap annotation of top 25 upregulated and downregulated genes in mutCALR LSK cells (D) FPKM values of STAT5 target genes (E) Volcano plot representing differentially regulated gene-sets (Hallmark GSEA) in mutCALR LSK cells (F) Bar plot representing statistically significant gene-sets upregulated and downregulated in mutCALR LSK cells. (* - show statistical significance, which has been explained in the supplementary methods)
FIGURE 4  Immunodepletion of platelets and stem cells by anti-mutCALR antibody. (A) FACS analysis of different lineages in bone marrow for expression of mutCALR on the cell surface. (B) Schematic representation of anti-mutCALR mAb treatment plan. (C) Peripheral blood parameters of platelets over 2 day time-period. (D) Percentage of different populations in bone marrow and spleen of treatment and control groups. (E) sCALR quantification in 1 year old transgenic mice and (F) patient samples and healthy donors (Kruskal-Wallis test). (G) sCALR quantification in 8-month old mice treated with antibody. (H) ELISA assay to detect presence of mutCALR-Ab immune complexes in plasma of mice treated with anti-mutCALR antibody.
altered in the peripheral blood. Platelet values normalized 6 h post antibody treatment. The platelet counts returned to thrombocytopenic levels in 24 h after treatment in the mutCALR<sup>hom</sup> mice. This fluctuation persisted on day 2. We did not observe any effects of the treatment on blood parameters in wild type mice (Figures 4C, 5A).

At terminal workup, the antibody treated mice had a significant reduction in the percentage of LSKs and MPPs in the spleen of mutCALR<sup>hom</sup> mice. There was no change in the levels of ST-HSCs, and LT-HSCs and megakaryocytes (Figure S6B). The mutCALR LSK cells in the bone-marrow were also significantly reduced in the antibody treated mice, while the other cell populations remained unchanged (Figure 4D). In conclusion, anti-mutCALR antibody treatment of mice resulted in immunodepletion of platelets and hematopoietic stem cells in vivo in mutCALR-dependent manner.

Since shedding and secretion of antigen constitutes a potential antibody sink, we examined the soluble mutCALR (sCALR) levels in the plasma of transgenic mice. The mutCALR<sup>hom</sup> mice had a significantly increased amount of sCALR compared to the wild type and mutCALR<sup>het</sup> mice (Figure 4E, ELISA setup in Figure S6C,D). This is consistent with the levels of sCALR detected in MPN patients with CALR mutations, using the same ELISA assay (Figure 4F). Notably, the mutCALR<sup>hom</sup> mice treated with anti-mutCALR antibody have significantly reduced levels of sCALR in the plasma in comparison to PBS treated mice (Figure 4G). We ruled out any competition between the two antibodies by performing a competitive ELISA (data not shown). Therefore, immunological depletion of the antigen (by formation of antigen–antibody complexes) could be a possible reason for the reduction of ELISA signal. Accordingly, we were able to detect antigen–antibody complexes of the monoclonal antibody bound to sCALR in the plasma of the treated mice (Figures 4H, S6D). These data indicate that sCALR was reduced in the plasma due to immunodepletion.

4 | DISCUSSION

A relevant transgenic mouse model is required for screening and understanding the molecular mechanisms of therapeutic interventions in a pre-clinical setting. We have developed a murine model that expresses the mutCALR oncoprotein with human mutant C-terminal peptide (del52) under the control of the endogenous murine Calr promoter.

The use of vavCre transgenic mice restricted the expression of the mutCALR protein to the hematopoietic tissue. We were able to detect the expression of the mutCALR protein in the splenocytes of transgenic mice. Notably, the mutCALR<sup>hom</sup> mice expressed higher levels of the CALR-del52 protein than the mutCALR<sup>het</sup> mice. Transgenic mice expressing human CALR-del52 under MHC-I promoter<sup>13</sup> or murine mutant CALR protein<sup>12</sup> have a relatively mild increase in platelets. However, transgenic mice expressing mouse-human hybrid CALR mutant proteins generated by "knock-in" strategy similar to ours,<sup>14,15</sup> developed a myeloproliferative disease which was more severe in homozygous mice. Similarly, the phenotype in the mutCALR<sup>hom</sup> mice was characterized by increased platelets and WBC and reduced RBC in the peripheral blood. The mutCALR<sup>hom</sup> mice also developed splenomegaly and increased percentage of megakaryocytes and stem cells in the bone marrow (Figure 1). The LSK cells from mutCALR<sup>hom</sup> mice also showed proliferative advantage in a competitive bone marrow transplantation assay, as previously reported by Benlabeled et al.<sup>15</sup> Using transgenic mice with the expression of Cre in germ line tissue, we generated mice with systemic CALR-del52 expression (Calr<sup>del52/+</sup>). The mice do not survive past embryogenesis when they are homozygous for CALR-del52, similar to the Calr knockout mice and CRISPR/Cas9 knock-in mice.<sup>12,16</sup>

To dissect the signature pattern associated with the mutant CALR, we performed transcriptome profiling of the LSK cells sorted from the mutCALR<sup>hom</sup> mice. The pathways revealed by GSEA, fall into four major categories: oncogenic signaling (K-ras signaling, mTORC1, PS3 and JAK–STAT signaling pathways), cell cycle control (E2F targets, G2M checkpoint), inflammation (TNFα, IFNα and IFNγ signaling) and metabolism (bile acid, cholesterol homeostasis). Constitutive JAK–STAT signaling is one of the hallmarks of classical MPNs. We see an enrichment of genes that are known to be bona fide STAT5 targets. Major signaling networks are also known to feed into the JAK–STAT signaling.<sup>17</sup> The prominent ones that have emerged in the transcriptome data are categorized into oncogenic signaling list. One of the hallmarks of cancer is deregulation of cell cycle. Interestingly, we see that the components of G2M checkpoints and E2F targets are downregulated in mutCALR LSKs. The crucial role of CDK6 in stem cell quiescence, cytokine secretion and cell proliferation has already been demonstrated, in the context of JAKV617F.<sup>18</sup> It would be interesting to investigate the role of cell cycle control associated genes in CALR-positive MPN progression. A chronic state of inflammation is another hallmark of MPN.<sup>19</sup> NFKB is known to be the master regulator in promoting inflammation. We see that the components of TNFα signaling via NFKB, IFNα and IFNγ are upregulated in mutCALR LSKs. We also see that cholesterol homeostasis is significantly upregulated in mutCALR LSKs. As the ER luminal calcium plays an important role in sensing cholesterol,<sup>20</sup> the perturbation of cholesterol homeostasis may be due to the loss of calcium binding capacity in the del52-homozygous cells. Compared to the report of Prins et al.<sup>21</sup> we identified several other pathways in the GSEA analysis. This is likely because we performed transcriptome analysis from 1000 LSK cells rather than single cells. While single cell RNA-seq has the advantages of identifying transcriptional signature of individual cells, information of low-expressed genes can be lost in the process, which can be accessed in conventional transcriptome analysis.

MutCALR associates with the thrombopoietin receptor and is trafficked onto the cell surface as a complex.<sup>2</sup> We were able detect the presence of the mutCALR protein on the surface of the target cells in the bone marrow of mutCALR mice. The cell-surface residing protein can act as a neoantigen and, therefore, be targeted by antibody-based immunotherapy. To test this hypothesis, we administrated a murine anti-mutCALR IgG2a raised against the human oncoprotein to the mutCALR<sup>hom</sup> mice. During antibody treatment, we observed a short-term platelet immunodepletion in the mutCALR<sup>hom</sup>
mice. At the end of treatment, we detected a significant reduction in the percentage of LSK cells in both the spleen and the bone marrow, in the monoclonal antibody treated mutCALR mice. The control vavCre mice were not affected by the antibody treatment, demonstrating the specificity of the antibody against mutCALR. Interestingly, the platelet values were replenished within the 18-h gap between two injections. The amount of antibody used here may not be sufficient to combat the very high rate at which the mutCALR megakaryocytes shed platelets.

Secretion of mutCALR has been shown in cell culture supernatants. However, the biologic effects of the secreted neoantigen has not yet been investigated in mice or patients. To test if the transgenic mutants. However, the biologic effects of the secreted neoantigen has not yet been investigated in mice or patients. To test if the transgenic mice could be used to model the pathophysiologic role of secreted mutCALR, we measured the soluble mutCALR (sCALR) in the transgenic mice and in MPN patients. The plasma levels in patients with CALR mutations and in mutCALR mice were comparable suggesting that the mutCALR mice also recapitulate this aspect of the MPN phenotype. The sCALR in plasma may act as a “sink” for the anti-mutCALR monoclonal antibody and lead to dampening of the antibody therapeutic effects. Although we detected the presence of immunocomplexes of the anti-mutCALR antibody and the sCALR in the plasma of treated mice, this did not prevent the immunodepletion of LSK cells in both spleen and bone marrow (Figure 4). These data suggest that the secreted mutCALR and the abundant platelets in the peripheral blood do not constitute a significant antibody sink. The mechanisms of how the antibody treatment leads to platelet and LSK cell depletion is unclear at the moment and further studies are needed to clarify the mode of action.

In summary, we have generated transgenic mice that recapitulate the MPN phenotype and provide proof of concept that an antibody raised against the mutant C-terminus can be effectively used to target the platelets and mutant LSK cells. The mutCALR mice serve as a modeling platform to study the molecular mechanisms and immuno-therapeutic strategies to target the disease.

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AUTHOR CONTRIBUTIONS

Robert Kralovics and Harini Nivarthi conceived and designed the study, interpreted the data and contributed to the manuscript writing. Harini Nivarthi (mouse model generation, phenotyping and bone marrow transplantation) and Sarada Achyutuni (transcriptome analysis, antibody treatment and ELISA) designed and performed the experiments, analyzed the data and wrote the manuscript. Andrea Majoros, Ruochen Jia, Christina Schueller and Cecilia Varga conducted specific experiments and analyzed the data. Anoop Kavirayani coordinated the histology processing and analyzed and interpreted the histopathology data. Eva Hug designed the ELISA assay. Michael Schuster and Martin Senekowitsch contributed in processing the NGS samples and in analyzing the sequencing files. Oleh Zagrijtschuk, Dimitrios Tsiantoulas, Christoph Bock and Christoph Binder contributed to the intellectual input.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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