# Hematopoiesis after anti-CD117 monoclonal antibody treatment in the settings of wild-type and Fanconi anemia mice

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## Abstract

Anti-CD117 monoclonal antibody (mAb) agents have emerged as exciting alternative conditioning strategies to traditional genotoxic irradiation or chemotherapy for both allogeneic and autologous gene-modified hematopoietic stem cell transplantation. Furthermore, these agents are concurrently being explored in the treatment of mast cell disorders. Despite promising results in animal models and more recently in patients, the short- and long-term effects of these treatments have not been fully explored. We conducted rigorous assessments to evaluate the effects of an antagonistic anti-mCD117 mAb, ACK2, on hematopoiesis in wild-type and Fanconi anemia (FA) mice. Importantly, we found no evidence of short-term DNA damage in either setting following this treatment, suggesting that ACK2 does not induce immediate genotoxicity, providing crucial insights into its safety profile. Surprisingly, FA mice exhibited an increase in colony formation after ACK2 treatment, indicating a potential targeting of hematopoietic stem cells and expansion of hematopoietic progenitor cells. Moreover, the long-term phenotypic and functional changes in hematopoietic stem and progenitor cells did not differ significantly between the ACK2-treated and control groups, in either setting, suggesting that ACK2 does not adversely affect hematopoietic capacity. These findings underscore the safety of these agents when utilized as a short-course treatment in the context of conditioning, as they did not induce significant DNA damage in hematopoietic stem or progenitor cells. However, single-cell RNA sequencing, used to compare gene expression between untreated and treated mice, revealed that the ACK2 mAb, via c-Kit downregulation, effectively modulated the MAPK pathway with Fos downregulation in wild-type and FA mice. Importantly, this modulation was achieved without causing prolonged disruptions. These findings validate the safety of anti-CD117 mAb treatment and also enhance our understanding of its intricate mode of action at the molecular level.

## Introduction

Bone marrow transplantation/hematopoietic stem cell transplantation (BMT/HSCT) is a powerful curative treatment for many different blood and immune diseases, including both non-malignant and malignant diseases, which affect millions of people world-wide. Furthermore, it is the only proven therapy for bone marrow failure syndromes such as Fanconi anemia (FA).[1] While bone marrow failure can arise from a variety of genetic or acquired factors, in both scenarios, hematopoietic stem cells (HSC) and hematopoietic progenitors are lost, leading to a decline of blood and immune cell production which results in anemia, thrombocytopenia, and neutropenia. Only through replacement of HSC from a healthy donor can a definitive cure be achieved. FA, one of the most frequent genetic causes of bone marrow failure, arises from mutations in the Fanconi anemia complementation (FANC) genes, which are required to repair DNA interstrand crosslinks.[2] FA patients have chromosomal fragility leading to hematopoietic failure (aplastic anemia, myelodysplasia, or leukemia), increased susceptibility to systemic cancers, other rare organ dysfunctions including congenital structural anomalies[3,4] and, ultimately, shortened lifespans. Given that more than 90% of FA patients develop hematopoietic failure over their lifetimes, almost all require HSCT.[5]

While HSCT can effectively replace a defective blood and

immune system, current transplantation methods necessitate the use of nonspecific, genotoxic chemotherapy and/or irradiation conditioning.[6] These conventional methods of conditioning cause substantial DNA damage which is typically repaired by DNA repair pathways that necessitate FA proteins. Consequently, the multi-organ damage, mucositis, infertility, and secondary malignancies resulting from classic conditioning are especially profound in FA patients who are unable to repair DNA interstrand crosslinks.[1] This leads to considerable morbidity and mortality in this particularly vulnerable group of patients, and restricts the use of BMT/HSCT primarily to patients with severe hematopoietic failure or malignant transformation.[7,8] To improve outcomes for all individuals requiring HSCT, including those with FA, it is crucial to identify a safe, non-genotoxic conditioning strategy.

Over the past decade, various alternative antibody-based conditioning strategies have been developed by our group and others to improve the safety and efficacy of BMT/HSCT. Based upon experiments showcasing that competing HSC limited engraftment,[8] our group initially pioneered a novel antibody-based conditioning approach by targeting the HSC surface protein, c-Kit (CD117), using antagonistic anti-mouse (m)CD117 monoclonal antibodies (mAb) which we found caused effective depletion of host HSC in certain mouse models, enabling non-toxic BMT/HSCT.[9,10] Given these results, clinical equivalent antagonistic anti-human (h)CD117 mAb have been developed which have shown promise in human xenografts and in a recent pivotal clinical trial in patients with severe combined immunodeficiency (SCID).[11,12] Moreover, these strategies could potentially improve outcomes for patients with all types of genetic and acquired blood and immune diseases and are, therefore, now being tested in a variety of clinical trials. These include as conditioning agents for HSCT for myelodysplastic syndrome/acute myeloid leukemia, hemoglobinopathies, and FA, as well as for treatment of mast cell disorders. However, an important question remaining for the translation of these strategies to patients, especially those with FA who are known to be more fragile, is whether antagonistic anti-CD117 mAb induce cellular stress and DNA damage in HSC in either the short-term or the long-term.

The preliminary safety of antagonistic anti-CD117 mAb has been established in wild-type (WT) mouse models, nonhuman primates, normal human xenografts and short-term in patients with SCID with no evidence of resulting myelodysplastic syndrome or leukemia. However, a more substantive evaluation of cellular and genotoxic stresses in association with antagonistic anti-CD117 mAb treatments is needed to understand their effects, especially on DNA repair-defective genetic backgrounds such as FA. We have addressed this need by assessing changes in transcriptomic profiles of HSC and progenitors by single-cell RNA sequencing analysis as well as genotoxicity and systemic toxicity in response to treatment with ACK2, an anti-mCD117 mAb, in wild-type (WT) C57BL/6N mice and FA complementation group D2 knockout (FANCD2$^{-/-}$) mice – which model the disease and display a hematopoietic phenotype. While prior studies have showcased the utility of antagonistic anti-CD117 mAb in this and other FA mouse models,[13,14] our study underscores the safety of this treatment in FA mice, both in the short- and long-term, with no evidence of DNA damage induction. Moreover, our investigation into potential downstream effects reveals intriguing disruptions of various regulatory pathways, including the MAPK pathway, with downregulation of Fos, opening avenues for further exploration of antagonistic anti-mCD117 mAb as therapeutic agents in the context of bone marrow failure and other conditions. These findings contribute valuable insights that may inform future clinical studies and therapeutic approaches to various hematologic disorders including FA. This work is especially timely as the parallel anti-hCD117 mAb clinical agents are currently being tested in these settings.

# Methods

### Mice

All animal housing and procedures were approved by the institutional ethics Animal Care and Use Committee at Stanford University. FANCD2$^{-/-}$ mice were a generous gift from Dr. Kenneth I. Weinberg (Stanford University) and originated from Dr. Marcus Grompe at Oregon Health and Science University.[15] Mice were backcrossed onto C57BL/6N (CD45.2), originally derived on a 129/SvJ background. Designate WT C57BL/6N mice were purchased from Charles River Laboratories. In all studies, adult female and male animals between 8 to 12 weeks of age were used.

### Antibody treatment

Two hundred micrograms of Benadryl (Sigma-Aldrich, St Louis, MO, USA) was given via intra-peritoneal injection, followed by anti-mCD117 ACK2 mAb (BioXcell) or IgG mAb (Sigma), which were administered intravenously as a one-time dose of 500 μg per animal as previously published.[10] For each experiment, 1 week or 24 weeks after treatment (Untreated, IgG and ACK2), blood was collected, and mice were euthanized to collect whole bone marrow from tibia, femur, pelvis and spine. Cell suspensions from whole bone marrow were divided for each experiment described as follows.

### Colony-formation cell assay

Twelve thousand whole bone marrow cells were plated in triplicate in a methylcellulose-based culture medium, Methocult (M3434, StemCell Technologies, Vancouver, Canada). Colony formation was evaluated after 7 days of culture by StemVision (StemCell Technologies) and recorded accordingly.

### Immunofluorescence of γH2AX

After c-Kit enrichment on whole bone marrow using autoMACS® columns (CD117 MicroBeads, mouse, 130-091-224, Miltenyi Biotec), 250,000 c-Kit-enriched cells were resus-

pended in 100 μL 1x phosphate-buffered saline (PBS) and pipetted directly onto poly-L-lysine coated coverslips. After fixing the cells with 3.7% formaldehyde/PBS, cells were stained. Briefly, after permeabilization and 1 hour of blocking with 2% bovine serum albumin/PBS 0.1% Tween 20, we stained the cells with 1:250 γH2AX antibody (clone JBW301, Millipore) for 1 hour at room temperature. After a washing cycle, the secondary antibody goat anti-Mouse IgG Alexa Fluor 488 was added at 1:1000 for 1 hour at room temperature. After a washing cycle the coverslip was mounted onto DAPI Fluoromount-G on a microscope slide. The slides were scanned with a Keyence microscope and cells were counted using ImageJ software.

### Micronuclei assay

Blood was collected via retro-orbital bleeding with 2x heparinized capillary tubes into EDTA-coated BD Microtainer® tubes. The assay was performed as previously described.[16] Briefly, the heparinized red blood cells were mixed directly over 2 mL cold methanol taken directly from stock at -80°C and stored at -80°C for at least 12 hours. The cells were stained with anti-CD71-FITC (Southern Biotech) and anti-CD61-BV421 (BD Bioscience) in a solution containing 5 mg/mL RNase (Sigma) for 45 minutes at 4°C with gentle agitation. After washing with bicarbonate buffer, cells were resuspended in bicarbonate buffer containing 5 mg/mL propidium iodide and analyzed on a FACSymphony A5 (BD Biosciences, San José, CA, USA).

### Flow cytometry

One million whole bone marrow cells were stained with the antibodies listed in Table 1 for 30 minutes at 4°C in the dark. After washing with PBS 1X, cells were analyzed using a BD FACSymphony A5 (BD Biosciences, San José, CA, USA). FlowJo software (TreeStar, Ashland, OR, USA) was used to analyze the flow cytometry data.

### Single-cell RNA sequencing

Two hundred thousand whole bone marrow cells from each mouse were pooled into groups and sequenced using the 10X Genomics Chromium Next GEM Single Cell 3' Kit v3.1 and Chip G Kit; libraries were developed by the Stanford Functional Genomics Facility. Sequence alignment was performed using the mm10 (GSE63472) and 10X Genomics Cell Ranger V7 bioinformatics suite. Data were analyzed using R (version 4.3.0) and the Seurat package (version 2.3.1).[17] For downstream analysis, the *FindVariableGenes* function was used to identify variable genes and *FindMarkersFunction* was used to identify differentially expressed genes. Moreover, pathway analysis was performed using reactome with *Griss J. ReactomeGSA* (*https://github.com/reactome/ReactomeGSA* [2019]) and gene set enrichment analysis was carried out using the Enrichr function.[18]

### Statistics

Statistical analyses were performed in R version 4.3.0 or Prism9 (GraphPad Software). Unpaired two-tailed *t* tests were used to define statistical significance (**P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001).

## Results

### No short-term DNA damage was detected in wild-type or Fanconi anemia mice after antagonistic anti-mouse CD117 monoclonal antibody treatment

To assess the short-term toxicity of antagonistic anti-mCD117 ACK2 mAb and investigate whether it can induce DNA damage, we conducted three different assays: colony formation counts, γH2AX analysis, and micronuclei assay.

Interestingly, at 7 days after antagonistic anti-mCD117 mAb treatment in WT mice, we observed no significant differences in colony formation compared to that in the control groups (Figure 1A). However, we did observe an increase in the percentage of γH2AX-positive cells following antagonistic anti-mCD117 mAb treatment compared to the untreated and IgG-treated groups (*P*<0.05) (Figure 1B).

To assess genome instability in mouse erythrocytes, we employed the micronuclei assay, which involves the identification of micronuclei in normochromatic erythrocytes. These micronuclei are indicative of damage occurring more than 72 hours earlier and are identified by propidium iodide-positive and CD71-negative staining (*Online Supplementary Figure S1*). Micronuclei assays are commonly used

**Table 1.** Antibodies used in the flow cytometry investigations.

| Antibody | Fluorophore | Supplier | Catalog reference |
|---|---|---|---|
| CD117 | BV785 | Invitrogen | 78-1171-82 |
| Sca1 | BV650 | Invitrogen | 64-5981-82 |
| Lineage | AF700 | Biolegend | 799223 |
| CD150 | PeCy7 | Biolegend | 115914 |
| CD48 | PerCpCy5.5 | Biolegend | 103422 |
| CD135 | PeCy5 | Biolegend | 135312 |
| CD34 | AF647 | BD Biosciences | 560230 |
| IL-7 Ra | BV605 | Biolegend | 135041 |
| Aqua Viability Dye | BV510 | Invitrogen | L34957 |

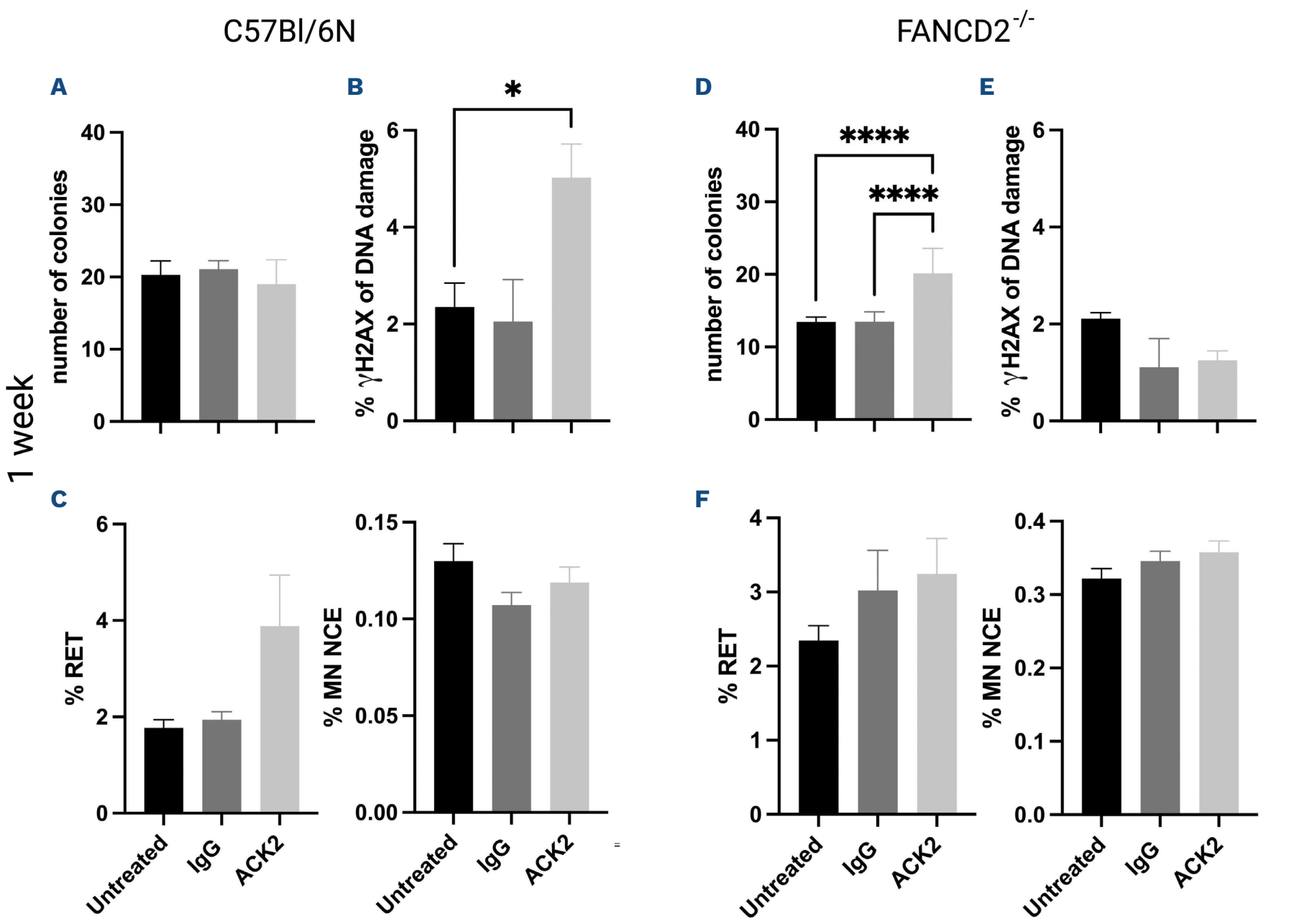


**Figure 1. No acute DNA damage was detected after antagonistic anti-CD117 monoclonal antibody treatment in FANCD2$^{-/-}$ mice.** The short-term toxicity of ACK2, in terms of DNA damage induction, was investigated in wild-type (C57Bl/6N) mice and FANCD2$^{-/-}$ mice. (A) Functional assessment by *in vitro* colony-forming capacity in wild-type mice. (B) Percentage of γH2AX, detected by immunofluorescence, in wild-type mice. (C) Micronuclei assay analysis representing percentage of reticulocytes and micronuclei in wild-type mice. (D) Functional assessment by *in vitro* colony-forming capacity in FANCD2$^{-/-}$ mice. (E) Percentage of γH2AX, detected by immunofluorescence, in FANCD2$^{-/-}$ mice. (F) Micronuclei assay analysis representing percentage of reticulocytes and micronuclei in FANCD2$^{-/-}$ mice. Data shown are mean values and error bars represent the standard error of mean. N=3-5 mice per group. *$P$<0.05; ****$P$<0.0001, using a Mann-Whitney $t$ test. RET: reticulocytes; MN NCE: micronuclei in normochromatic erythrocytes.

in toxicity studies to evaluate the potential carcinogenicity of substances and were used to assess potential risks associated with the antagonistic anti-mCD117 mAb.[19] Additionally, this assay allowed us to determine the regenerative activity of the bone marrow during and after treatment. As previously reported, our results indicated that the percentage of reticulocytes typically falls within the range of 1-3%. However, as expected, after 7 days, the reticulocyte count tended to increase following antagonistic anti-mCD117 mAb treatment, indicative of the bone marrow's ability to respond appropriately following disruption of its function. Moreover, no upregulation of micronuclei in normochromatic erythrocytes was detected following treatment. This reticulocyte response suggests that the bone marrow remains functional and possesses the necessary components to produce red blood cells after this bone marrow insult (Figure 1C).

In contrast, in FANCD2$^{-/-}$ mice, we observed an upregulation in the number of colonies following the antagonistic anti-mCD117 mAb treatment compared to the colony formation in the control groups ($P$<0.001) (Figure 1D). This suggests that the antagonistic anti-mCD117 mAb may preferentially target HSC with less self-renewal capability in this assay or alternatively that a progenitor response was elicited. Importantly, we did not detect any changes in γH2AX levels or the percentage of micronuclei in normochromatic erythrocytes, indicating that the antagonistic anti-mCD117 mAb does not induce DNA damage (Figure 1E, F).

To further explore the effects of the antagonistic anti-mCD117 mAb, we used flow cytometry to assess changes in the number of long-term HSC (LT-HSC: Lin$^-$CD117$^+$Sca1$^+$CD150$^+$CD48$^-$),

short-term HSC (ST-HSC: Lin⁻CD117⁺Sca1⁺CD150⁻CD48⁻), and multipotent progenitors (MPP: Lin⁻CD117⁺Sca1⁺CD150⁻CD48⁺) at the indicated time points after treatment with ACK2 and compared these to the changes after treatment with IgG mAb or no treatment. The flow cytometry gating strategy is shown in *Online Supplementary Figure S2*. Surprisingly, we observed a decrease of the Lin⁻Sca1⁺Kit⁺ (LSK) population after both IgG and antagonistic anti-mCD117 mAb treatments in WT mice compared to the untreated group ($P<0.05$ and $P<0.01$, respectively) (Figure 2A). Additionally, within the LSK population, we detected an increase of ST-HSC after IgG and antagonistic anti-mCD117 mAb treatments compared to the untreated group in WT mice, while no differences were detected in MPP and LT-HSC populations (Figure 2B-D). This upregulation of the ST-HSC population indicates that the hematopoietic system is responding to overcome depletion once anti-mCD117 blockade is triggered. In contrast, focusing on FANCD2$^{-/-}$ mice, we observed no significant differences across the groups and subpopulations (Figure 2E-H). This observation indicates that one-time treatment of with the anti-mCD117 mAb does not alter HSC or progenitor distribution dramatically in this setting.

### No long-term DNA damage was detected in wild-type or Fanconi Anemia mice after antagonistic anti-mouse CD117 monoclonal antibody treatment

We additionally conducted analyses at 24 weeks after treatment with the aim of uncovering potential long-term phenotypic and functional changes in residual/recovered hematopoietic stem and progenitor cells (HSPC) following antagonistic anti-mCD117 mAb treatment. Similar assays to those used 1 week after treatment with ACK2 mAb were employed. We did not detect differences at the 24-week time point in either the WT or FANCD2$^{-/-}$ groups of mice

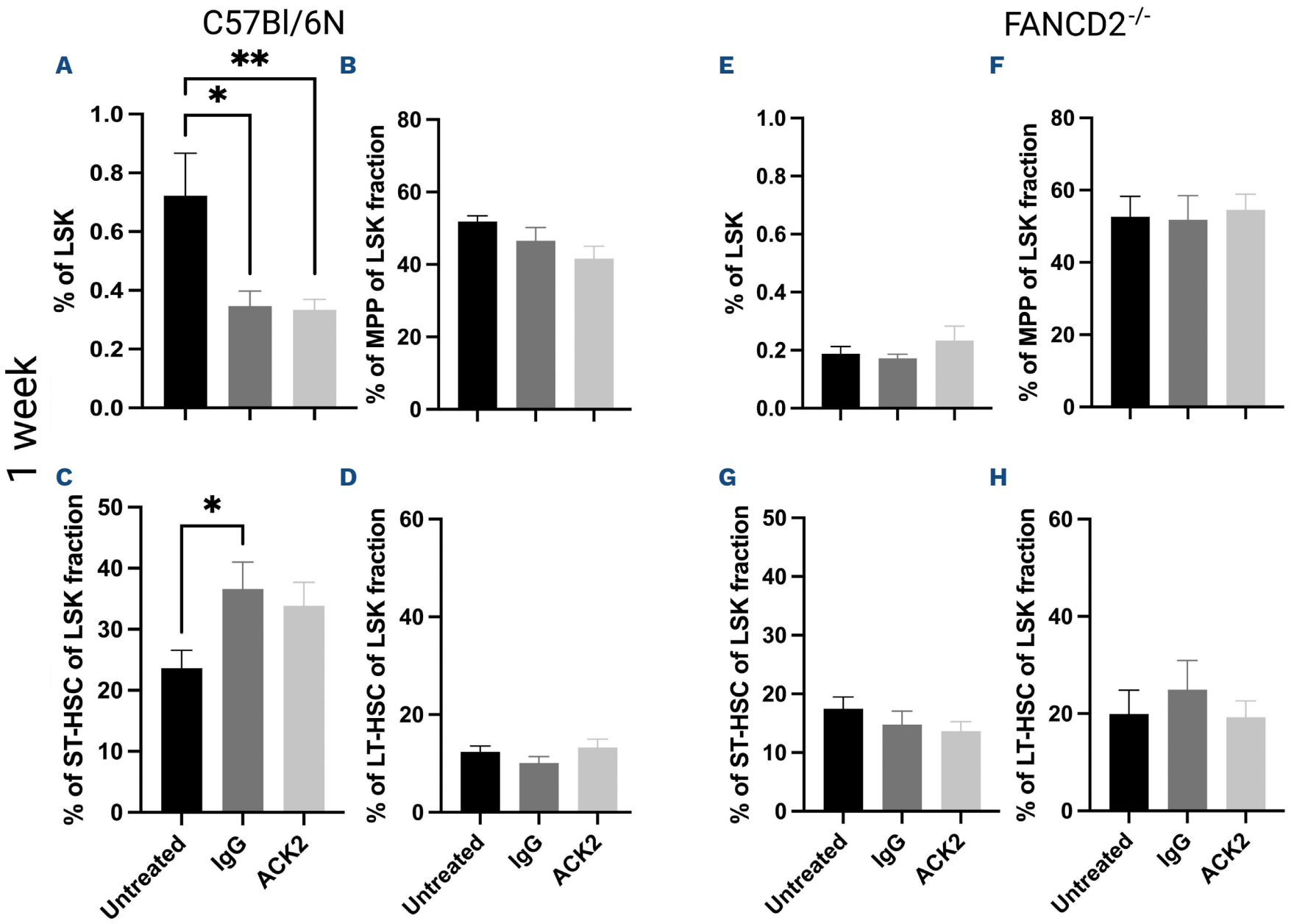


**Figure 2. Impact of IgG or antagonistic anti-CD117 monoclonal antibody on hematopoietic stem and progenitor cells, assessed by flow cytometry 1 week after administration.** (A-D) Wild-type (C57Bl/6N) or (E-H) FANCD2$^{-/-}$ mice were treated with IgG or ACK2 or left untreated. (A, E) Histograms represent the percentage of Lin⁻Sca1⁺Kit⁺ (LSK) cells in the Lin⁻ fraction. (B, F) Histograms represent the percentage of multipotent progenitors in the LSK cell fraction. (C, G) Histograms represent the percentage of short-term hematopoietic stem cells (ST-HSC) in the LSK cell fraction. (D, H) Histograms represent the percentage of long-term hematopoietic stem cells in the LSK cell fraction. Data shown are mean values and error bars are ± standard error of mean. N=5 mice per group: *$P<0.05$, **$P<0.01$, using a Mann-Whitney *t* test. MPP: multipotent progenitors; ST-HSC: short-term hematopoietic stem cells; LT-HSC: long-term hematopoietic stem cells.

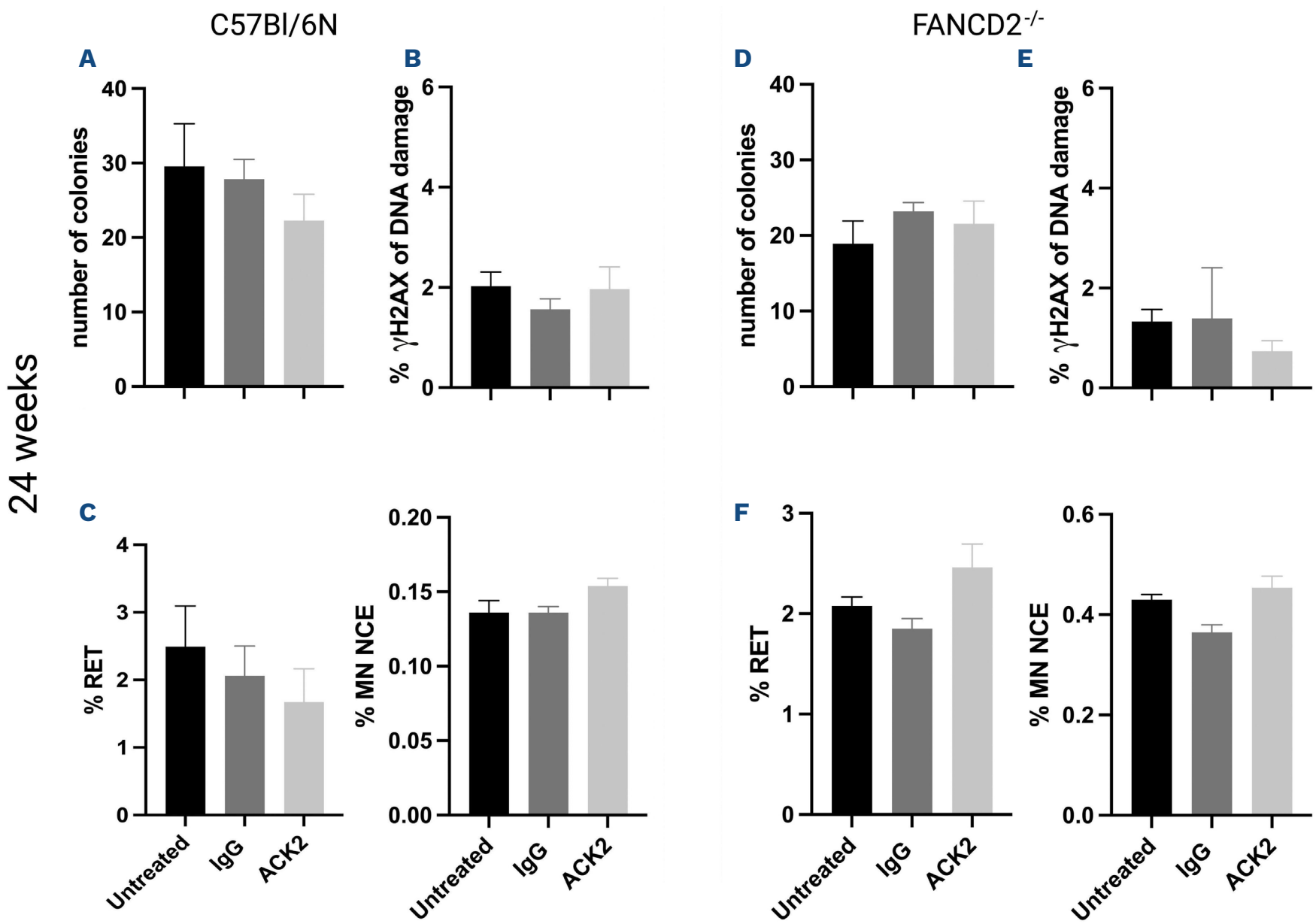


**Figure 3. No long-term DNA damage was detected in FANCD2$^{-/-}$ mice after antagonistic anti-CD117 monoclonal antibody treatment.** The long-term toxicity of ACK2 was investigated in wild-type (C57Bl/6N) mice and FANCD2$^{-/-}$ mice. (A) Functional assessment by *in vitro* colony-forming capacity in wild-type mice. (B) Percentage of γH2AX, detected by immunofluorescence in wild-type mice. (C) Micronuclei assay analysis representing percentage of reticulocytes and micronuclei in wild-type mice. (D) Functional assessment by *in vitro* colony-forming capacity in FANCD2$^{-/-}$ mice. (E) Percentage of γH2AX detected by immunofluorescence in FANCD2$^{-/-}$ mice. (F) Micronuclei assay analysis representing percentage of reticulocytes and micronuclei in FANCD2$^{-/-}$ mice. Data shown are mean values and error bars represent the standard error of mean. N=2-5 mice per group. No statistically significant differences were found using a Mann-Whitney *t* test. RET: reticulocytes; MN NCE: micronuclei in normochromatic erythrocytes.

between animals treated with ACK2 mAb, IgG mAb or left untreated (Figure 3).

Furthermore, the FACS analysis indicated a complete recovery in the percentage of LSK (Lin$^{-}$Sca-1$^{+}$c-Kit$^{+}$) cells in WT mice after the antagonistic anti-mCD117 mAb treatment (Figure 4A). This finding supports the hypothesis that the antagonistic anti-mCD117 mAb does not have any adverse effects on the renewal capability of HSC. Moreover, no significant differences were observed for other subpopulations following antagonistic anti-mCD117 mAb treatment (Figure 4B-D). However, in the case of FANCD2$^{-/-}$ mice, we observed a slight increase of LSK and MPP following the antagonistic anti-mCD117 mAb treatment compared to the untreated group ($P$<0.01 and $P$<0.05) (Figure 4E, F), whereas ST-HSC were decreased ($P$<0.01) (Figure 4G). Interestingly, no significant differences were detected in LT-HSC (Figure 4H).

These findings indicate that long-term treatment with the antagonistic anti-mCD117 mAb may lead to an increase in the number of LSK cells in the bone marrow of FANCD2$^{-/-}$ mice. This suggests that the antagonistic anti-mCD117 mAb may have eliminated the most dysregulated LSK cells, thereby allowing the other LSK pool with a higher renewal capability to compensate for this hematopoietic stress.

## Single-cell RNA-sequencing analysis highlights the involvement of the MAPK pathway with antagonistic anti-mouse CD117 monoclonal antibody blockade

To enhance our understanding of the effects of the antagonistic anti-mCD117 mAb and to ensure that it does not significantly alter long-term gene expression of cells, we performed single-cell RNA-sequencing analysis on the whole bone marrow of untreated and ACK2-treated WT and

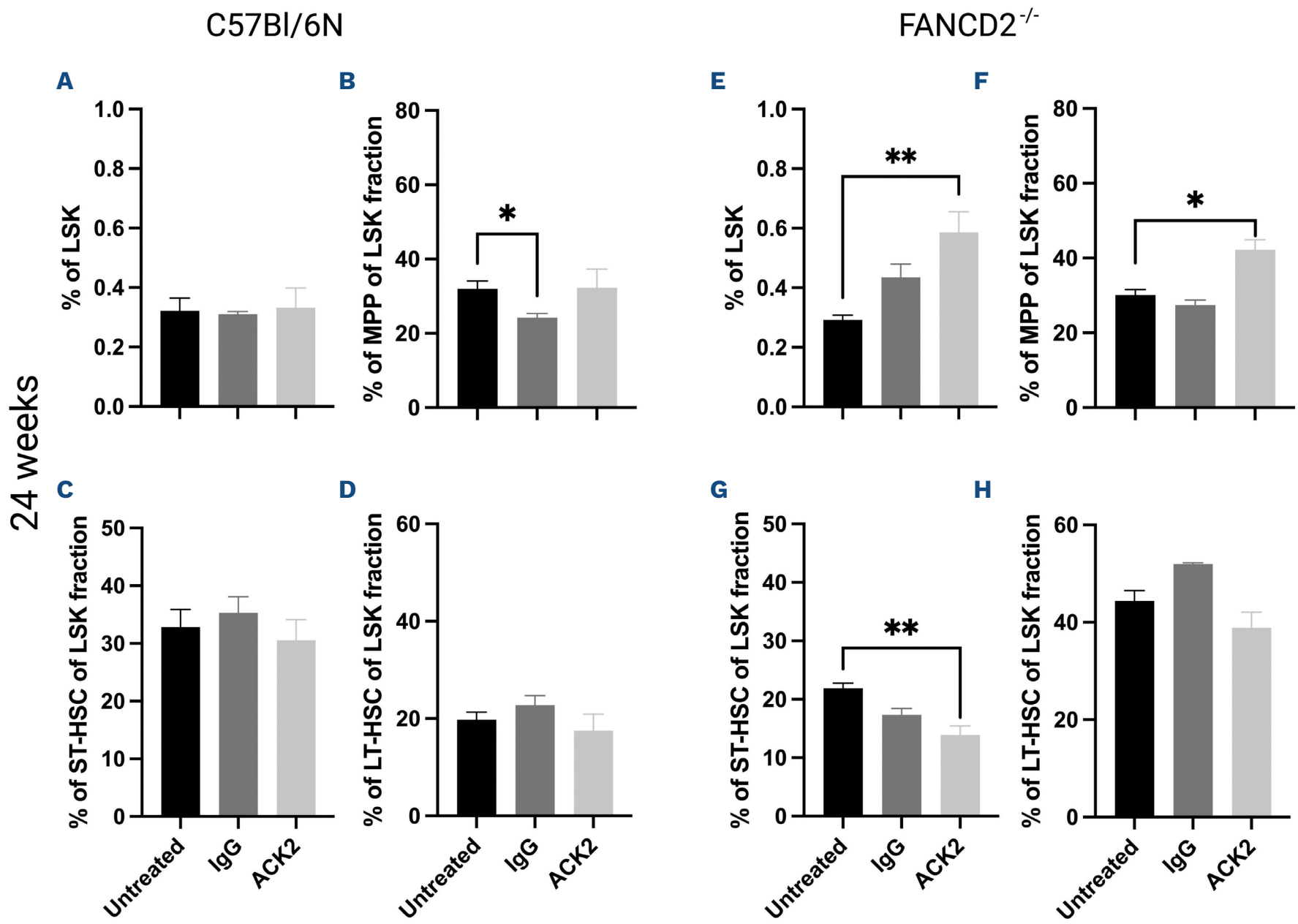


**Figure 4. Impact of IgG and antagonistic anti-CD117 monoclonal antibody on hematopoietic stem and progenitor cells, assessed by flow cytometry at 24 weeks after administration.** (A-D) Wild-type (C57Bl/6N) or (E-H) FANCD2$^{-/-}$ mice were treated with IgG or ACK2 or left untreated. (A, E) Histograms represent the percentage of Lin$^{-}$Sca1$^{+}$Kit$^{+}$ (LSK) cells in the Lin$^{-}$ fraction. (B, F) Histograms represent the percentage of multipotent progenitors in the LSK cell fraction. (C, G) Histograms represent the percentage of short-term hematopoietic stem cells (ST-HSC) in the LSK cell fraction. (D, H) Histograms represent the percentage of long-term hematopoietic stem cells in the LSK cell fraction. Data shown are mean values and error bars are ± standard error of mean. N=2-5 mice per group: *$P$<0.05, **$P$<0.01, using a Mann-Whitney $t$ test. MPP: multipotent progenitors; ST-HSC: short-term hematopoietic stem cells; LT-HSC: long-term hematopoietic stem cells.

FANCD2$^{-/-}$ mice 1 week and 24 weeks after treatment. First, we determined the clustering of 14 cell subsets. We used an automated method with specific markers to identify each cluster.[20] This method allowed us to have the most probable subset for each of the clusters (*Online Supplementary Figure S3*). To probe deeper, for each cluster we identified the ten most expressed genes that allowed us to determine the cell identity according to the literature for each cluster.[21] For the myeloid component, we classified cells into monocytes (mainly expressing Ly6ca and Il1b), neutrophils (mainly expressing Mmp8, Mmp9, and Ly6g), macrophages (mainly expressing Csf1r and Klf4), and myeloid progenitors, divided into four subgroups using non-supervised clustering: monocyte/neutrophil progenitors and 1-3 myeloid progenitors (mainly expressing Cebpe, Elane, Mpo, and Csf1r), and dendritic cells (mainly expressing Siglech and Irf8). In the lymphoid component, focusing on B cells, we identified four different subsets: B-cell progenitors, immature B cells, B cells and plasma cells (mainly expressing Pax5, Ebf1, and Iglc1). We also identified common lymphoid progenitors (CLP, mainly expressing Lck, Tox, and Prkch). The last cluster was related to erythroblasts (Hbb-bs, Hba-a1, and Hba-a2) (*Online Supplementary Figure S4A-C*). We subdivided the data by condition to determine the distribution of each cluster (*Online Supplementary Figure S4D*).

To delve further, we first conducted an analysis of the 100 pathways with the maximum difference in expression across samples using the *ReactomeGSA* package. No drastic changes or pathway activations were found between any of the samples (*Online Supplementary Figure S5*). We conducted a sec-

ond non-supervised analysis using *WikiPathways_2019_Mouse data base* to identify which pathways were most upregulated and downregulated after the antagonistic anti-mCD117 mAb treatment compared to the control for each group. Using this assessment, several pathways were found to be affected. Notably, we found that the MAPK signaling pathway ranked as the second and third most downregulated pathway 1 week after the antagonistic anti-mCD117 mAb treatment in WT mice and FANCD2$^{-/-}$ mice, respectively (*Online Supplementary Figure S6A, B*). To assess whether this downregulation was reversible, we conducted the same analysis at 24 weeks after antagonistic anti-mCD117 mAb treatment. Surprisingly, we found that the MAPK pathway was upregulated in WT mice, supporting the hypothesis that the antagonistic anti-mCD117 mAb treatment does not have a permanent impact on this pathway in hematopoietic cells (*Online Supplementary Figure S6C, D*).

To probe further, we decided to explore and conduct an analysis of differential gene expression between untreated and treated groups, considering different time points and mouse strains for each cluster. For this analysis, we selected all genes that were upregulated or downregulated with $P<0.05$ and an average $\log_2$ fold change (avg_$\log_2$FC) $<\pm0.5$ for each cluster (*Online Supplementary Table S1*). Subsequently, we created Venn diagrams for each cluster to assess whether genes were commonly upregulated or downregulated following antagonistic anti-mCD117 mAb treatment (*Online Supplementary Figures S7-S20*). Interestingly, we observed across clusters that Fos was significantly downregulated 1 week after treatment in both WT and FANCD2$^{-/-}$ mice whereas at 24 weeks after treatment Fos was upregulated in WT mice (*Online Supplementary Figures S7-S20*).

Considering that CD117 (c-Kit) is known to be involved in the activation of various signaling pathways, such as the MAPK pathway, and that Fos is associated with MAPK activation as a transcription factor, we decided to examine the activation of the genes associated with this pathway. We found, in accordance with our previous results, that Fos and Jun are downregulated at 1 week after antagonistic anti-mCD117 treatment in both mice models. Moreover, we assessed other genes associated with PI3K/AKT, JAK2/STAT5, Src or PKC pathways: only Pi3k was differentially expressed between groups. More precisely, it was upregulated at 1 week after antagonistic anti-mCD117 mAb treatment in both murine models (*Online Supplementary Figure S6E*).

These data provide evidence that antagonistic anti-mCD117 ACK2 mAb, by blocking c-Kit, disrupts the MAPK pathway and downregulates the expression of Fos/Jun transcription factors, overcome by activation of the PI3K/AKT pathway (*Online Supplementary Figure S6F*).

# Discussion

The use of a targeted antibody to deplete host HSC before transplantation in patients with hematopoietic diseases such as FA is one of the first promising alternative treatments to chemotherapy or radiotherapy.[10] We elucidated the safety of antagonistic anti-CD117 mAb-mediated BMT/HSCT in both WT and FA mice with the specific goal of generating biological insights clarifying the vulnerability of HSC to antagonistic anti-mCD117 ACK2-mediated stem cell factor-CD117 proliferative signaling blockade and additionally to provide a preclinical toxicity analysis of antagonistic anti-CD117 mAb to assess their safety and aid their clinical development. Given the heightened sensitivity of FA patients to genotoxic and proliferative stressors and the potential for resulting mixed chimerism with this approach, our experiments are of paramount importance to inform potential future clinical studies in this group of patients. The effects of antagonistic anti-mouse CD117 mAb-ACK2 treatment were compared to those of no treatment or mock treatment with IgG mAb in WT or FANCD2$^{-/-}$ mutant mice 1 week and 24 weeks after mAb treatment. We elucidated the short- and long-term safety of the antagonistic anti-mCD117 ACK2 mAb and showed that no drastic cellular stress or DNA damage, which could lead to the induction of myelodysplastic syndrome in the long-term, was generated. This result indicated that the antagonistic anti-mCD117 ACK2 mAb does not produce a selective pressure of clonal proliferation which could predispose to myelodysplastic syndrome.

Our research, along with the work of our colleagues, has demonstrated that a single treatment with the antagonistic anti-mCD117 mAb ACK2 can selectively deplete host HSC *in vivo*, thereby facilitating effective engraftment of donor hematopoietic cells in syngeneic immunodeficient murine models. However, it is important to note that ACK2 mAb alone does not enhance donor engraftment in WT hosts and currently has limited conditioning efficacy in this setting, making it primarily applicable to specific genetic disease contexts, at present. Notably, recent reports have highlighted the effectiveness of antagonistic anti-mCD117 ACK2 mAb as a pre-transplant conditioning agent in FA mouse models in combination with immunosuppression. Specifically, when ACK2 mAb treatment is combined with anti-CD4 mAb (GK1.5), it has enabled remarkable long-term, multi-lineage donor engraftment, reaching up to 63% and 93% in homozygous mutant FANCA$^{-/-}$ and FANCD2$^{-/-}$ recipients, respectively, after BMT.[13]

Recently, similar experiments were conducted in our laboratory to assess the conditioning capacity of antagonistic anti-mCD117 ACK2 mAb in combination with GK1.5 in FANCD2$^{-/-}$ animals.[14] We chose the FANCD2$^{-/-}$ model for these studies because of its well-established severe hematopoietic phenotype, which aligns with prior investigations. Our preliminary observations were that pre-treatment with ACK2 and anti-mCD4 GK1.5 mAb enabled engraftment of WT donor HSC in FANCD2$^{-/-}$ animals. This finding supports the hypothesis that ACK2 mAb may facilitate the depletion of host FANCD2$^{-/-}$ HSC, thereby promoting successful donor

HSCT. Furthermore, our detailed analysis of these studies suggests that the proliferative and hematopoietic reconstitution capacity of WT donor HSC and HSPC can outcompete FANCD2$^{-/-}$ host HSPC over time, which was also accomplished with anti-mCD4 GK1.5 mAb alone. This competition leads to high levels of donor hematopoietic chimerism in both the bone marrow and peripheral blood. In line with successful donor hematopoietic engraftment in this experiment, we observed an increase in progenitor cells with functional DNA interstrand crosslink repair activity following conditioned transplantation, as demonstrated by *in vitro* colony-forming assays in the presence of mitomycin C.

However, our results also indicate that a significant population of FANCD2$^{-/-}$ HSC remains in the host long term, resulting in a state of mixed hematopoietic chimerism. This mixed chimerism raises potential concerns in the context of FA, as residual host HSC, subjected to additional hematopoietic stress through antibody-based HSCT, might be predisposed to malignant transformation. While our studies did not find any evidence of development of myelodysplastic syndrome or leukemia in the antagonistic anti-mCD117 mAb ACK2-conditioned FA animals that underwent BMT, understanding the extent of antagonistic anti-mCD117 mAb ACK2-induced hematopoietic stress and DNA damage remains of critical importance. Importantly, there is a prior report of epithelial tumor development in a FANCD2$^{-/-}$ model between 14 and 19 months of age, but hematologic malignancies have not been observed in this model.[22]

Our single-cell RNA-sequencing data analysis revealed that antagonistic anti-mCD117 ACK2 mAb blockade of the c-Kit/stem cell factor axis inhibited the MAPK pathway and downregulated the transcription factor Fos. These findings are the first to shed light on the potential action of antagonistic anti-mCD117 ACK2 mAb. Previous studies underscored the pivotal role of the MAPK pathway in regulating HSC quiescence.[23-25] Indeed, the MAPK signaling pathway is instrumental in promoting cell proliferation by activating cyclin-CDK complexes, thereby facilitating cell cycle progression and gene transcription. Additionally, this pathway exerts its influence on various transcriptional regulators, further bolstering its involvement in the regulation of cellular proliferation. It is worth noting that several tyrosine kinase inhibitors known for their ability to target c-Kit – such as imatinib, sunitinib, and dasatinib – have been extensively characterized and used in the context of cancer therapy, primarily for their anti-proliferative effects. Our results suggest that the antagonistic anti-mCD117 ACK2 mAb may induce a similar effect by specifically targeting c-Kit on cells that overexpress it. This highlights the potential therapeutic implications of antagonistic anti-CD117 mAb in modulating c-Kit-mediated signaling pathways and cellular proliferation.

Our research signifies a significant advance in the understanding of the safety and efficacy of HSC-targeted antibody-based therapies with a focus on bone marrow failure conditions, offering hope for improved treatments in the future for these patients. The potential benefits and insights gained from this study underscore the importance of continued investigation into the clinical utility of antagonistic anti-CD117 mAb for FA and related disorders. This discovery has prompted efforts to translate these findings into clinical applications to evaluate the potential adoption of this conditioning approach for FA patients. Based upon these reassuring results, we have recently opened a clinical trial to test the use of antagonistic anti-CD117 mAb conditioning in FA patients in combination with transient immunosuppression and TCR$\alpha\beta^+$ T-cell/CD19$^+$ B-cell-depleted grafts, thereby eliminating the use of total body irradiation or busulfan from the conditioning regimens for these high-risk patients (NCT04784052). Antagonistic anti-CD117 mAb treatment is also being utilized in a number of other conditions and insights into its effects on hematopoiesis may be pertinent to other settings as well.

## Disclosures

*ADC discloses financial interests in the following entities working in the rare genetic disease space: Beam Therapeutics, Decibel Therapeutics, Editas Medicines, GV, Magenta Therapeutics, Prime Medicines and Spotlight Therapeutics.*

## Contributions

*MD, YYC, AC, and ADC designed the study. MD, LS, HW, EH, YYC, AC, and MRK performed research and analyzed data. LS and EH managed animal colonies and provided technical assistance. MD, QRB, and ADC interpreted data. MD and ADC wrote the manuscript. ADC provided mentorship to the first author.*

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## Data-sharing statement

*Data that support the findings of this study are available from the corresponding author upon reasonable request.*

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