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TITLE: Biology and Therapeutic Targeting of Malignant Monocytes in Myeloid Blood Cancers

PRINCIPAL INVESTIGATOR: Ann Mullally, MD

CONTRACTING ORGANIZATION: The Brigham and Women's Hospital, Inc., Boston, MA

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project is focused on the FY20 PRCRP topic area of blood cancers. This project addresses several Military Health Focus Areas, including: (i) environmental / exposure risk factors associated with blood cancer development (Aim 1), (ii) gaps in blood cancer prevention, early detection/diagnosis, prognosis and treatment (Aims 1, 2, 3). There is a fundamental gap in understanding the role of clonal monocytes in the pathogenesis and progression of myeloid blood cancers. Continued existence of this gap represents an important problem because despite the poor prognosis of myeloid blood cancers characterized by malignant monocytosis, there are currently no treatment strategies to effectively target monocytes in patients with myeloid malignancies.

The central hypothesis of this project is that clonal monocytes have cell-intrinsic abnormalities that result in the development of aberrant inflammatory responses to common stimuli (e.g. infection), which drives the pathogenesis and progression of myeloid blood cancers. The overall objectives are to determine the molecular consequences of inflammation in clonal monocytes, to understand how this contributes to the pathogenesis and progression of myeloid blood cancers and to use this information to develop novel therapeutic approaches to target malignant monocytes in patients.

15. SUBJECT TERMS
Clonal Hematopoiesis, clonal monocytosis, chronic myelomonocytic leukemia (CMML), myleproliferative neoplasms (MPN).

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The main focus of this project is in understanding the role of clonal monocytes in the pathogenesis and progression of myeloid blood cancers. The ***central hypothesis*** of the project is that clonal monocytes have cell-intrinsic abnormalities that result in the development of aberrant inflammatory responses to common stimuli (e.g. infection), which drives the pathogenesis and progression of myeloid blood cancers. The ***overall objectives*** in this application are to determine the molecular consequences of inflammation in clonal monocytes, to understand how this contributes to the pathogenesis and progression of myeloid blood cancers and to use this information to develop novel therapeutic approaches to target malignant monocytes in patients. The project focuses on specific mutated genes that commonly occur in myeloid blood cancers with monocytosis (i.e. *JAK2*, *ASXL1* and *SRSF2*) and on myeloid blood cancers associated with monocytosis (i.e. CMML, MPN).

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Clonal hematopoiesis, monocytosis, myeloproliferative neoplasms, myeloid blood cancers, pro-inflammatory cytokines, obesity, JAK2, ASXL1

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The major goals (specific aims) of this project are:
Aim 1: To determine the genomic and environmental factors that drive the development of myeloid malignancies in individuals with isolated monocytosis in the general population
Aim 2: To identify differentially activated inflammatory pathways in Asxl1- and Srsf2- mutant monocytes
Aim 3: To determine the impact of therapeutically targeting JAK2-mutant monocytes in vivo

I have listed the major tasks for years 1 and 2 below and commented on progress:
Aim 1: Major Task 1: NGS and analysis of 323 gDNA samples will occur in months 6 – 18. This task was not pursued due to insurmountable regulatory issues (see Section 5 for full explanation).
Major Task 2: Statistical analyses to investigate association between gene mutations & outcomes will occur in months 21 – 33. This task has been completed on UK Biobank data (Figure 1).
Major Task 3: Validation of association findings in independent cohort (e.g. UK-Biobank) will occur in months 24 – 36. We have now completed analysis of 500,000 whole exomes from UK Biobank. Based on our findings we have performed validation studies in *Jak2*-mutant and *Asxl1*-mutant mice (Figures 2, 3, 4).

Aim 2: Major Task 4: RNA-seq and analysis of pro-inflammatory pathways in mice will occur in months 6 – 18. We have completed bulk RNA-sequencing and analysis of Jak2V617F mice and identified several pathways that are differentially activated in the context of obesity (Figure 4).
Major Task 5: Validation experiments in primary human monocytes will occur in months 15 – 21
These studies have not yet been performed as we are have not completed the studies in mice to identify the top candidate pathways for validation in primary human samples.
Major Task 6: BMT studies in mice + lineage tracing will occur in months 18 – 36
These studies have been completed in Jak2V617F mice (Figures 2 and 3).
Aim 3: Major Task 7: scRNA-seq & genotyping + analyses will occur in months 1 – 18.
Completed and results published in Experimental Hematology paper in 2022, reported last year.
Major Task 8: IHC staining on MPN BM samples will occur in months 12 – 18 (partially completed and reported last year)
Major Task 9: Validation flow studies in primary MPN samples will occur in months 18 – 24
Completed and results published in Experimental Hematology paper in 2022, reported last year.
In summary, Aim 1 has been completed using UK Biobank data. Aim 2 has almost been completed with the exception of validation studies in primary human monocytes. Aim 3 is on schedule. Therapeutic targeting studies in mice are planned for the coming year (as proposed for year 2-3).

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major activities:
During this reporting period, we have made progress on all three aims:
Aim 1: We have analyzed data from 500,000 whole exomes from the UK Biobank and identified divergent associations between *JAK2* and *ASXL1* mutations and obesity (Figure 1). We have developed novel mouse models of *Jak2*-mutant clonal hematopoiesis and obesity (Figure 2,3)
Aim 2: We have identified differentially activated pro-inflammatory pathways in Jak2-mutant HSC in the context of obesity. Studies in *Asxl1*-mutant HSC in the context of obesity are ongoing.
Aim 3: We have identified several candidate pro-inflammatory pathways for therapeutic targeting including interleukin 17, interferon gamma, interferon alpha and tumor necrosis factor alpha (Figure 4). We are planning functional studies on a subset of these pathways with the goal of preventing the transition from clonal hematopoiesis to overt MPN (i.e. preventing blood cancer development).

Specific objectives:
Aim 1: To determine the genomic and environmental factors that drive the development of myeloid malignancies in individuals with isolated monocytosis in the general population
Aim 2: To identify differentially activated inflammatory pathways in Asxl1- and Srsf2- mutant monocytes
Aim 3: To determine the impact of therapeutically targeting JAK2-V617F-mutant monocytes in vivo

Major activities:

Significant results or key outcomes, including major findings, developments, or conclusions:
Aim 1: Aim 1 is focused on studying the interaction between clonal genetic mutations found in monocytosis and the environmental influences exerted in the bone marrow microenvironment on these cells. In our application, we proposed to study the impact of lifestyle factors, including body mass index, on clonal monocytes. We began by interrogating the UK Biobank to determine if two mutations commonly found in myeloid malignancies with monocytosis (i.e. *JAK2* and *ASXL1*) are associated with obesity in the context of clonal hematopoiesis (CH). At the time of last year's report, we had analyzed 200,000 exomes from the UK Biobank. Over the past year, we extended our analysis to 500,000 exomes from UK Biobank. When we applied our inclusion and exclusion criteria for the analysis to the cohort, 425,573 exomes met the criteria. Using this dataset we found a negative association (OR = 0.5) between *JAK2*-mutant CH and obesity (BMI>30) and a positive association (OR = 1.4) between *ASXL1*-mutant CH & obesity (BMI>30) (Figure 1A, B, Appendix). At the time of last year's report, we had developed mouse models of obesity and the Jak2V617F mutation. However, we found that Jak2V617F-VavCre expressing mice did not develop obesity. We believe this is due to the fact that VavCre simultaneously induces Cre expression in all hematopoietic cells and is therefore modeling overt *JAK2*-mutant myeloproliferative neoplasm (MPN) and not *JAK2*-mutant CH. To overcome this issue, we developed an entirely novel genetic mouse model of Jak2V617F-CH (Figure 2, Appendix), which we believe accurately re-creates in a mouse the same characteristics of human JAK2V617F-CH. Briefly, we crossed Fdg5 Cre-ER mice with Jak2V617F conditional knockin animals (Figure 2A). In this model Jak2V617F expression is driven by the Fdg5 promoter and since Fdg5 is expressed primarily in long-term (LT) hematopoietic stem cells (HSC) (Figure 2B), this more accurately re-creates the acquisition of the JAK2V617F in LT-HSC as it occurs in humans. Furthermore, the Cre-ER element enables tamoxifen-inducible Cre expression, with higher doses of tamoxifen causing Cre recombination (and hence Jak2V617F expression) in a higher number of LT-HSC (Figure 2C). Having generated the novel mouse model, we next generated cohorts of Fdg5 Cre-ER Jak2V617F-expressing mice fed either a control or an obesity diet as well as Fdg5 Cre-ER Jak2 wildtype-expressing mice fed a control or an obesity diet (Figure 2D). Fdg5 Cre-ER Jak2V617F-expressing mice fed the control diet demonstrated a progressive increase in the % of dsTomato expressing cells in the peripheral blood (Figure 2E) and concordant with this developed an increased hematocrit (HCT) starting at 20 weeks (Figure 2F). Of note, these findings were not observed in Jak2V617F-expressing mice fed an obesity diet. This suggests that obesity inhibits the expansion of Jak2V617F-expressing LT-HSC and aligns with our data from the UK Biobank showing a negative correlation between *JAK2*-mutant CH and obesity (OR = 0.5). In aggregate our findings suggest that obesity may protect against the expansion of *JAK2*-mutant LT-HSC in bone marrow. We are currently investigating the factors mediating this protective effect, with the goal of developing new approaches to prevent the progression of *JAK2*-mutant CH to an overt JAK2-mutant MPN i.e. preventing blood cancer development.
Aim 2: Aim 2 is focused on studying monocytes and inflammatory pathways in the relevant mouse models**.** Given our findings from the UK Biobank (Figure 1) we focused primarily on *Jak2*-mutant and *Asxl1*-mutant mice. In addition to the novel genetic mouse model of Jak2V617F-CH (Figure 2), we also developed a Jak2V617F chimeric bone marrow transplant (BMT) model (Figure 3A, B, C). In this model we found that obese recipient mice transplanted with Jak2V617F and wild-type cells in equal ratios developed more marked monocytosis than control recipient mice (Figure 3D). To further characterize the inflammatory pathways driving this phenotype, we performed bulk RNA-sequencing on Jak2V617F and Jak2 wild-type HSCs isolated from both control and obese mice (i.e. 4 groups total). Using gene set enrichment analysis (GSEA), we found several pro-inflammatory pathways differentially upregulated in Jak2V617F-mutant HSC (as compared to Jak2 wild-type HSC) in mice fed an obesity diet. (Figure 4). Similar studies are ongoing in *Asxl1*-mutant mice.

Significant results or key outcomes, including major findings, developments, or conclusions:

Aim 3: Aim 3 is focused on targeting *JAK2*-mutant monocytes. We have identified several candidate pro-inflammatory pathways for targeting including interleukin 17, interferon gamma, interferon alpha and tumor necrosis factor alpha (TNFA). (Figure 4). Given our findings from the UK Biobank (Figure 1) showing opposite relationships between obesity and *JAK2*-mutant CH (negative association) and *ASXL1*-mutant CH (positive association), we are in the process of performing bulk RNA-sequencing of *Asxl1*-mutant and *Asxl1* wild-type HSC isolated from both control and obese mice (i.e. 4 groups total). We intend to compare the results from the *Asxl1*-mutant studies with our findings from the Jak2V617F studies (Figure 4) with the goal of focusing on pathways that demonstrate divergent directionality with respect to pro-inflammatory pathways in *Jak2*-mutant and *Asxl1*-mutant HSC, to align with our findings of *JAK2*-mutant and *ASXL1*-mutant human clonal hematopoiesis in the UK Biobank dataset (Figure 1).

Other achievements:
Dr. Mullally gave the 2023 Richard T. Silver Visiting Professorship Lecture at Weill Cornell Medical Grand Rounds, New York

Stated goals not met:
None.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."
Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."
Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."
Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Research:
Aim 1: (i) Interrogate additional data from the UK Biobank, in particular data on plasma proteins which is expected to become publically available in the coming year, to further explore the relationship between *JAK2*- and *ASXL1*- mutant clonal hematopoiesis, monocytosis and obesity. (ii) Further explore the impact of obesity on *Jak2*-mutant and *Asxl1*-mutant clonal hematopoiesis using the novel mouse models we have developed.
Aim 2: (i) Analyze bulk RNA-sequencing data of *Asxl1*-mutant HSC in control and obese mice and compare with previously generated data from *Jak2V617F*-mutant HSC (ii) Perform single-cell RNA sequencing of HSC and mesenchymal stromal cells (MSC) from Jak2V617F control and obese mice to identify candidate pro-inflammatory pathways underlying the differential behavior of Jak2V617F-mutant HSC in the context of obesity.
Aim 3: (i) Perform functional studies to validate and therapeutically target pro-inflammatory pathways underlying the differential behavior of Jak2V617F-mutant HSC in the context of obesity and use this knowledge to develop new treatments to prevent progression to *JAK2*-mutant MPN in individuals with JAK2-mutant clonal hematopoiesis.

Laboratory:
I have explanded my laboratory with the addition of two postdoctoral fellows in the past year.

Meetings:
I have been invited to speak at the following events:

(i) Faculty Speaker, Oncode Annual Scientific Conference, Amsterdam, October 2023
(ii) Faculty Speaker, European School of Hematology MPN Meeting, April 2024

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

We have developed a novel genetic mouse model of Jak2V617F clonal hematopoiesis (Figure 2) that more accurately recreates in a mouse what occurs in human *JAK2*-mutant clonal hematopoiesis. Specifically, this model does not require transplantation of Jak2V617F cells and eliminates the need to irradiate recipient mice, thus preserving the normal bone marrow microenvironment. Furthermore, through the use of Fdg5 Cre-ER mice Jak2V617F expression can be induced preferentially in the LT-HSC compartment and the number of LT-HSC in which Jak2V617F expression is induced can be controlled through titrating the tamoxifen dose. This model can be used to study other genetic mutations that cause clonal hematopoiesis.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Problem/delay: In Aim 1, we originally proposed to perform NGS on 323 primary human PB samples to be obtained from our collaborator (Dr. Ellervik) in Denmark. We proposed that the samples be sent to Boston where we would perform NGS and bioinformatic analysis. We encountered a significant problem whereby under new EU and Danish regulations substantial documentation and additional data sharing agreements were required in order to proceed with NGS data generation and analysis. Despite our best efforts we were unable to resolve this issue.
Action/Plans to resolve:Thankfully, we were able to proceed with what we originally had proposed as an alternative approach i.e. analysis of publically accessible whole exome sequencing data via an existing database (i.e. UK Biobank) i.e. ask the same scientific question but use a different dataset. This turned out to be a superior approach as we were able to access data from 500,000 exomes, which is a larger dataset than originally planned.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to report.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to report.

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

  DOD-funded review articles (DOD support is acknowledged in the article):

  1. How, J, Garcia, JS, **Mullally, A**. Biology and therapeutic targeting of molecular mechanisms in MPN. Blood 2023 Apr 20;141(16):1922-1933.

  2. Kramer, F, **Mullally, A**. Antibody targeting of mutant calreticulin in myeloproliferative neoplasms. J Cell Mol Med 2023 Aug 7. Online ahead of print.

Other original publications not directly related to DOD funding:

1. Gigoux, M, Holmstrom, MO, Zappasodi R, Park, JJ, Pourpe, S, Bozkus, CC, Mangarin, LMB, Redmond, D, Verma, S, Schad, S, George, MM, Venkatesh, D, Ghosh, A, Hoyos, D, Molvi, Z, Kamaz, B, Marneth, AE, Duke, W, Leventhal, MJ, Jan, M, Ho, VT, Hobbs, GS, Knudsen, TA, Skov, V, Kjaer, L, Larsen, TS, Hansen, DL, Lindsley, RC, Hasselbalch, H, Grauslund, JH, Lisle, TL, Met, O, Wilkinson, P, Greenbaum, B, Sepulveda, MA, Chan, T, Rampal, R, Andersen, MH, Abdel-Wahab, O, Bhardwaj, N, Wolchok, JD*, **Mullally, A***, Merghoub, T*. *Equal contribution. Calreticulin mutant myeloproliferative neoplasms induce MHC-I skewing, which can be overcome by an optimized peptide cancer vaccine. Sci Transl Med. 2022 Jun 15;14(649)
2. Jutzi, JS, Marneth, AE, Ciboddo, M, Guerra-Moreno, A, Jimenez-Santos, MJ, Kosmidou, A, Dressman, JW, Liang, H, Hamel, RSL, Lozano, PR, Rumi, E, Doench, J, Gotlib, JR, Krishnan, A, Elf, S, Al-Shahrour, F, **Mullally, A**. Whole-genome CRISPR screening identifies N-glycosylation as a genetic and therapeutic vulnerability in CALR-mutant MPN. Blood 2022 Sep 15;140(11):1291-1304. *Featured in Blood Commentary:* Rao, S, Carlson, K. Mutant CALR's "sweet tooth". Blood 2022 Sep 15;140(11):1187-1189.
3. Vining, KH, Marneth, AE, Adu-Berchie, K, Grolman, JM, Tringides, CM, Liu, Y, Wong, WJ, Pozdnyakova, O, Severgnini, M, Stafford, A, Duda, GN, Hodi, FS, **Mullally, A***, Wucherpfenning, KW, Mooney, DJ. Mechanical checkpoint regulates monocyte differentiation in fibrotic niches. Nat Mater. 2022 Aug;21(8):939-950.
4. Jutzi, JS, Marneth, AE, Jimenez-Santos, MJ, Hem, J, Guerra-Moreno, A, Rolles, B, Bhatt, S, Myers, SA, Carr, SA, Hong, Y, Pozdnyakova, O, van Galen, P, Al-Shahrour, F, Nam, AS, **Mullally, A**. CALR-mutated cells are vulnerable to combined inhibition of the proteasome and the endoplasmic reticulum response. Leukemia 2023 Feb;37(2):359-369.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: **Ann Mullally, MD**
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID): 0000-0001-9727-8495
Nearest person month worked: 4.52 CM

Contribution to Project: Oversight of all aspects of the project

Name: **Swarna Bale, PhD**
Project Role: Postdoctoral Research Fellow
Nearest person month worked: 6.74 CM
Contribution to Project: *In vivo* mouse models

Name: **Jessica Hem**
Project Role: Research Technician
Nearest person month worked: 6.2 CM
Contribution to Project: Mouse colony maintenance and mouse experiments

Name: **Adela Herce**
Project Role: Research Technician
Nearest person month worked: 5.95 CM
Contribution to Project: Mouse colony maintenance and mouse experiments

Name: **Chulwoo Kim**
Project Role: Research Technician
Nearest person month worked: 2.70 CM
Contribution to Project: Mouse colony maintenance and mouse experiments

Name: **Benjamin Rolles, MD**
Project Role: Postdoctoral Research Fellow
Nearest person month worked: 2.62 CM
Contribution to Project: UK Biobank studies, *in vivo* mouse models

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

MULLALLY, ANN - OTHER SUPPORT

Ended Projects since award activation

Title: Enhancing the clonal selectivity of existing therapies in myeloproliferative neoplasms
Time Commitment: 4.80 calendar months
Supporting Agency: The Leukemia & Lymphoma Society
Name, Address of Supporting Agency: The Leukemia & Lymphoma Society, 3 International Drive, Suite 200

Rye Brook, New York 10573.
Contracting/Grants Officer: Lee Greenberger
Performance Period: 07/01/2017 - 06/30/2022
Level of Funding:
Goals/List of Specific Aims: The goal of this project is to enhance the clonal selectivity of JAK2 inhibitor in MPN using genome-wide CRISPR Cas9 synthetic lethal approaches.
Role: PD/PI
Overlap: none

Title: Biology and Therapeutic Targeting of mutant CALR in MPN
Time Commitment: 0.60 calendar months
Supporting Agency: Gabrielle's Angels Foundation for Cancer Research
Name, Address of Supporting Agency:142 W. 57th Street, Floor 11, New York, New York 10019
Contracting/Grants Officer: Denise Rich
Performance Period: 06/01/2019 - 05/31/2022
Level of Funding:
Goals/List of Specific Aims: The goal of this project is to advance strategies to therapeutically target mutant CALR in MPN.
Role: PD/PI
Overlap: none

Title: A Spatial and Lineage Atlas of Bone Marrow Hematopoiesis
Time Commitment: 1.20 calendar months
Supporting Agency: The Chan Zuckerberg Initiative
Name, Address of Supporting Agency: The Chan Zuckerberg Initiative, 2440 West El Camino Real, Suite 300 Mountain View, California 94040.
Contracting/Grants Officer: Nina Cardoza
Performance Period: 07/01/2019 - 06/30/2022
Level of Funding:
Goals/List of Specific Aims: The goal of this project is build an atlas of normal human bone marrow hematopoiesis using single-cell transcriptomics and multiplexed spatial imaging
Role: Co-PI
Overlap: none

Title: Targeting mutant calreticulin in myeloproliferative neoplasms
Time Commitment: 1.20 calendar months
Supporting Agency: Janssen Pharmaceutica, Inc.
Name, Address of Supporting Agency: Janssen Pharmaceutica, Inc., 800 Ridgeway Drive, Horsham, Pennsylvania 19044.
Contracting/Grants Officer: Thomas Cavanaugh
Performance Period: 03/28/2019 - 03/27/2021
Level of Funding:
Goals/List of Specific Aims: The goal of this project is to validate mtCALR as a target for antibody approach and generate tool reagents and therapeutics for the mtCALR antibody while studying the effect of anti-mtCALR in *in vitro* and *in vivo* models.
Role: PD/PI
Overlap: none

Title: Pre-clinical Studies of 9-ING-41 in Myelofibrosis
Time Commitment: 0 calendar months
Supporting Agency: Actuate Therapeutics, Inc.
Name, Address of Supporting Agency: Actuate Therapeutics, Inc., 1751 River Run, Suite 400, Fort Worth, TX 76107.
Contracting/Grants Officer: Francis Giles
Performance Period: 02/02/20 - 02/01/22
Level of Funding:
Goals/List of Specific Aims: Study the consequences of treatment with 9-ING-41 in MPN cell lines, MPN mouse models and primary MPN.
Role: PD/PI
Overlap: none

Title: Characterization of JAK2 V617F inhibitors in MPN mouse models
Time Commitment: 2.4 calendar months in year 2
Supporting Agency: Relay Therapeutics Inc.
Name, Address of Supporting Agency: Relay Therapeutics, 399 Binney Street, Cambridge, MA 02139
Contracting/Grants Officer: Sanjiv K. Patel
Performance Period: 09/01/2021 - 08/31/2023
Level of Funding: $253,458
Goals/List of Specific Aims: To test JAK2V617F inhibitors in MPN pre-clinical mouse models
Role: PD/PI
Overlap: none

<u>New Active Projects since award activation (page 1)</u>

Title: Functional and Molecular Dissection of Mutant Calreticulin in Myeloproliferative Neoplasms
Time Commitment: 3 calendar months
Supporting Agency: NIH-National Institutes of Health
Name, Address of Supporting Agency: NHLBI, BG 6705 ROCKLEDGE DR RK1 RM 201-E, 6705 ROCKLEDGE DR, BETHESDA MD 20817
Contracting/Grants Officer: KIMBERLY GREGG Hall
Performance Period: 07/2021 - 06/2026
Level of Funding:
Goals/List of Specific Aims: The goal of this project is to advance the biological understanding and treatment of mutant CALR-driven MPN.
Role: PD/PI
Overlap: none

Title: Early intervention for the prevention of blood cancer development
Time Commitment: 1.2 calendar months
Supporting Agency: Immediate sponsor: Broad Institute, inc.; originating sponsor: Starr Foundation
Name, Address of Supporting Agency: Broad Institute, 415 Main Street, Cambridge, MA 02142
Contracting/Grants Officer: Ashlin Bolton

Performance Period: 01/2022 - 12/2023
Level of Funding:
Goals/List of Specific Aims: To identify genotype-specific therapeutic agents for clonal hematopoiesis in advanced preclinical models
Role: PD/PI
Overlap: none

Title: Elucidating Mechanisms of Therapy-Resistance to Interferon-alfa in Myeloproliferative Neoplasm Stem Cells
Time Commitment: 2.4 calendar months
Supporting Agency: immediate sponsor: Cornell University; originating sponsor: NIH – National Institutes of Health
Performance Period: 09/2022 - 08/2027
Level of Funding:
Goals/List of Specific Aims: To test novel therapeutic approaches to overcome therapy-resistance to interferon in the hematopoietic stem cells (HSC) compartment of MPN.
Role: Co-PD/PI
Overlap: none

Title: Mutant CALR-targeted CAR T cells
Time Commitment: 0.6 calendar months
Supporting Agency: Dana Farber / Harvard Cancer Center
Performance Period: 12/1/2022-11/30/2024
Level of Funding:
Goals/List of Specific Aims: The goal of this project is to develop a CAR T-cell to target mutant calreticulin.
Role: Co-PD/PI
Overlap: none

Title: Targeting integrin-mediated TGFb activation in Myeloproliferative Neoplasms
Time Commitment: 0 calendar months
Supporting Agency: Morphic Therapeutic
Performance Period: 02/10/2023-02/09/2025
Level of Funding:
Goals/List of Specific Aims: To test integrin inhibition as an approach to inhibit TGFb activation in mouse models of MPN
Role: PD/PI
Overlap: none

Title: Predicting progression in myeloproliferative neoplasm patients by reconstructing the history of disease in each patient
Time Commitment: 0.6 calendar months
Supporting Agency: immediate sponsor: Dana Farber Cancer Institute, originating sponsor: Leukemia and Lymphoma Society
Performance Period: 10/01/2023-09/30/2026
Level of Funding:

Goals/List of Specific Aims: Aim 1. Optimize recollective measurements to accurately infer the dynamics of MPN expansion from bulk sequencing of peripheral blood.
Aim 2. Conduct a recollective study of MPN patients and develop an accurate prognostic model for predicting risk of progression.
Role: Co-PD/PI
Overlap: none

Pending Projects since award activation

n/a

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)
- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other*

Nothing to report.

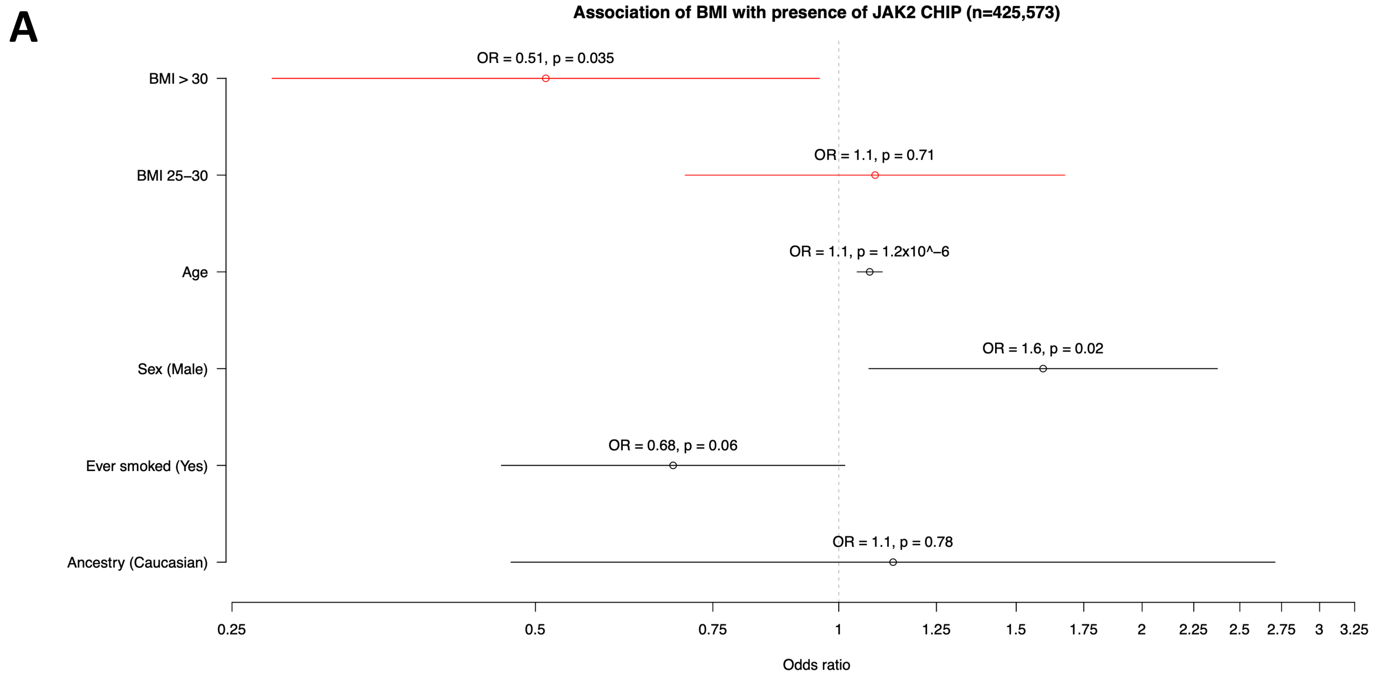
8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* **https://ebrap.org/eBRAP/public/index.htm** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

I have included the following as appendices:
Figures 1 – 4 data referenced in the accomplishments section
Two review articles – published during year 2 of funding; DOD funding is acknowledged.



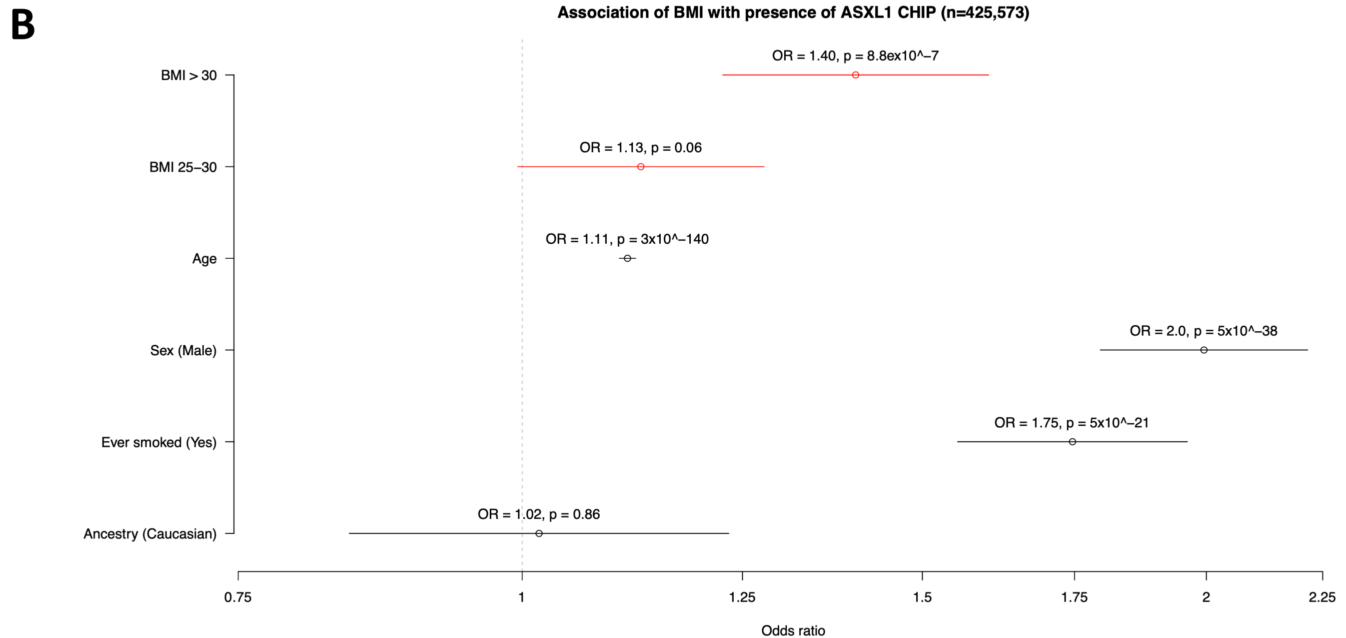


Figure 1: Association between BMI and *JAK2*-mutant or *ASXL1*-mutant clonal hematopoiesis
(A) Analysis of 425, 573 exomes from UK Biobank showing a negative association (OR = 0.51) between *JAK2*-mutant clonal hematopoiesis& obesity (BMI>30) (B) Analysis of 425, 573 exomes from UK Biobank showing a positive association (OR = 1.4) between *ASXL1*-mutant clonal hematopoiesis & obesity (BMI>30)

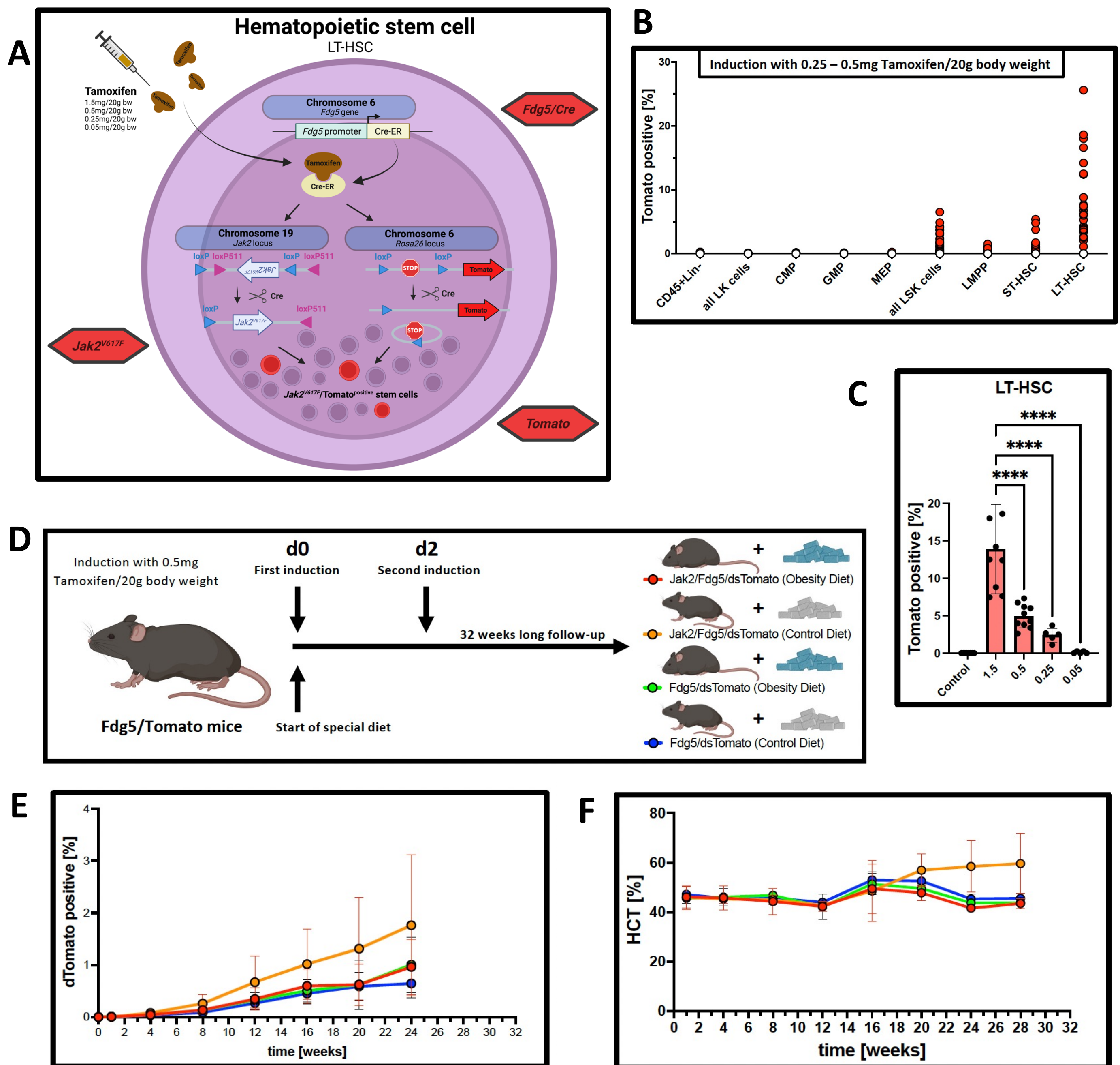
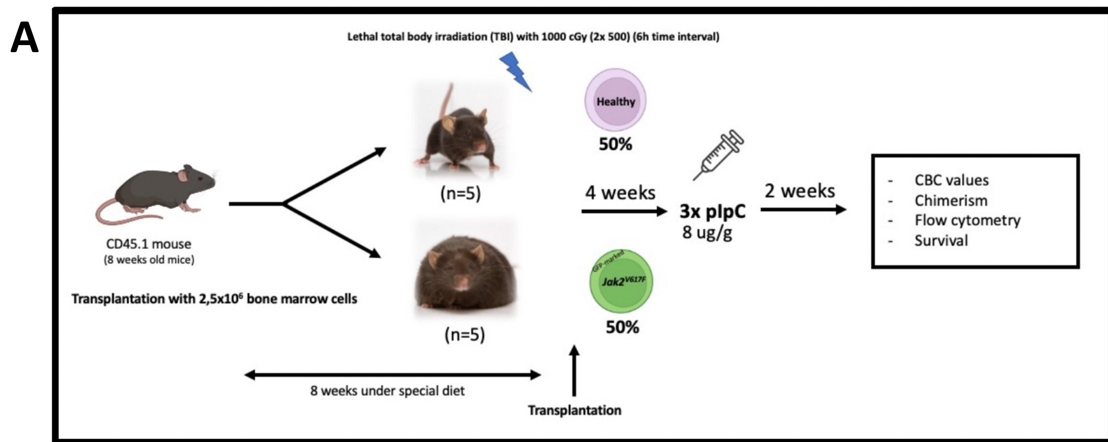
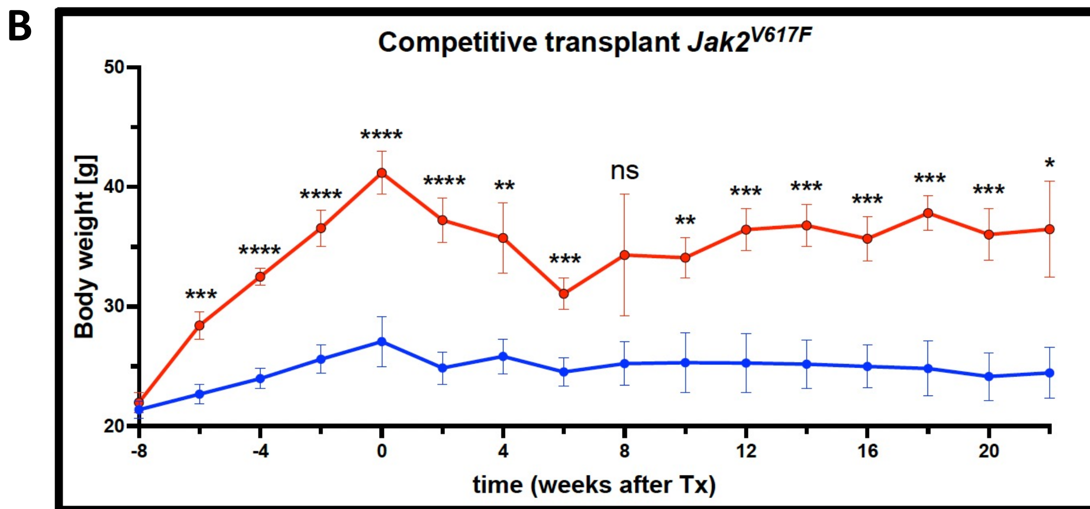
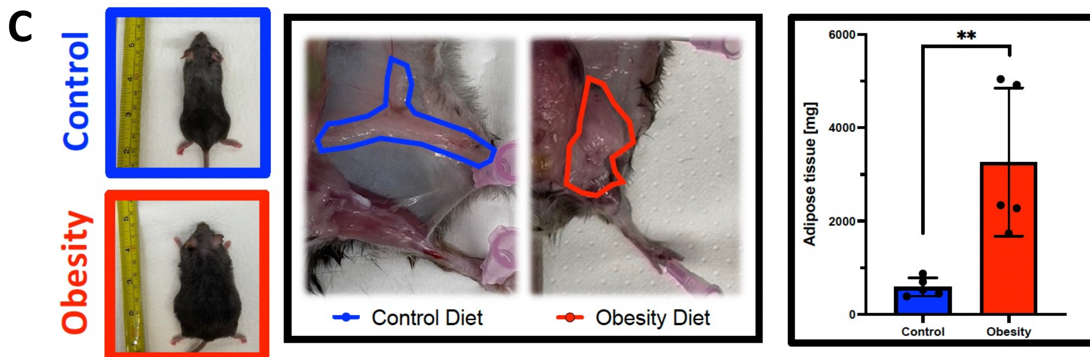


Figure 2: Development of a novel genetic mouse model of Jak2V617F clonal hematopoiesis
(A) Schematic showing the novel tamoxifen-inducible Fdg5 Cre-ER Jak2V617F genetic mouse model (B) Flow cytometry analysis of bone marrow from Fdg5 Cre-ER Jak2V617F mice showing Cre recombination (as indicated by dsTomato positivity) occurs in the hematopoietic stem cell (HSC) compartment only, and is highest in long-term HSCs (C) Flow cytometry analysis of bone marrow from Fdg5 Cre-ER Jak2V617F mice showing that induction with higher concentrations of tamoxifen results in a higher % of dsTomato cells (indicating more Cre recombination & more LT-HSC expressing Jak2V617F) (D) Schematic showing experimental design to induce Jak2V617F expression in LT-HSC in mice fed a control (orange) or obesity (red) diet and in Jak2 wild-type control mice fed a control (blue) or obesity (green) diet (E)) Flow cytometry analysis of peripheral blood showing that Fdg5 Cre-ER Jak2V617F-expressing mice fed a control diet (orange) have the highest % of dsTomato cells (F) Hematocrit (HCT) measurement showing that Fdg5 Cre-ER Jak2V617F-expressing mice fed a control diet (orange) develop increased HCT starting at 20 weeks. Of note Fdg5 Cre-ER Jak2V617F-expressing mice fed a high-fat diet (red) do not develop elevated HCT in same time.







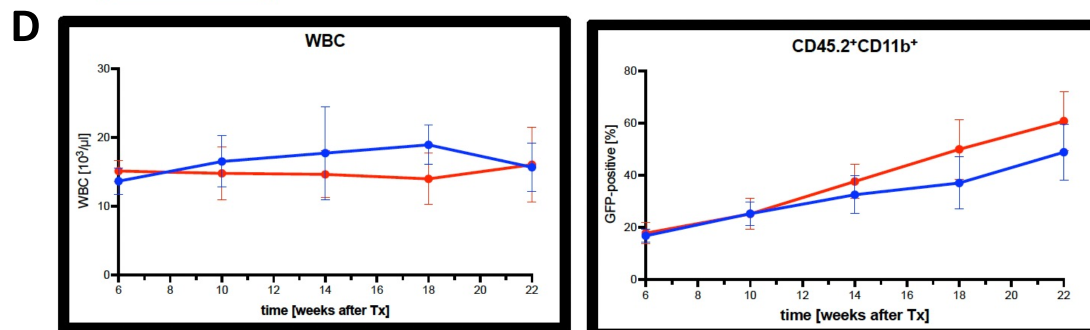


Figure 3: Obesity drives monocytosis in a Jak2V617F bone marrow transplant model
(A) Schematic showing competitive bone marrow transplant (BMT) model where wild-type and Jak2V617F donor bone marrow cells are transplanted in a 1:1 ratio in obese or control recipients. (B) Sequential body weights in obese and control recipient mice out to 22 weeks post transplantation. (C) Characterization of obesity model showing obese phenotype, increased inguinal fat pad & quantitatively more adipose tissue (D) Even in the absence of leukocytosis (left panel), obese recipient mice develop more marked monocytosis as compared with control recipient mice.

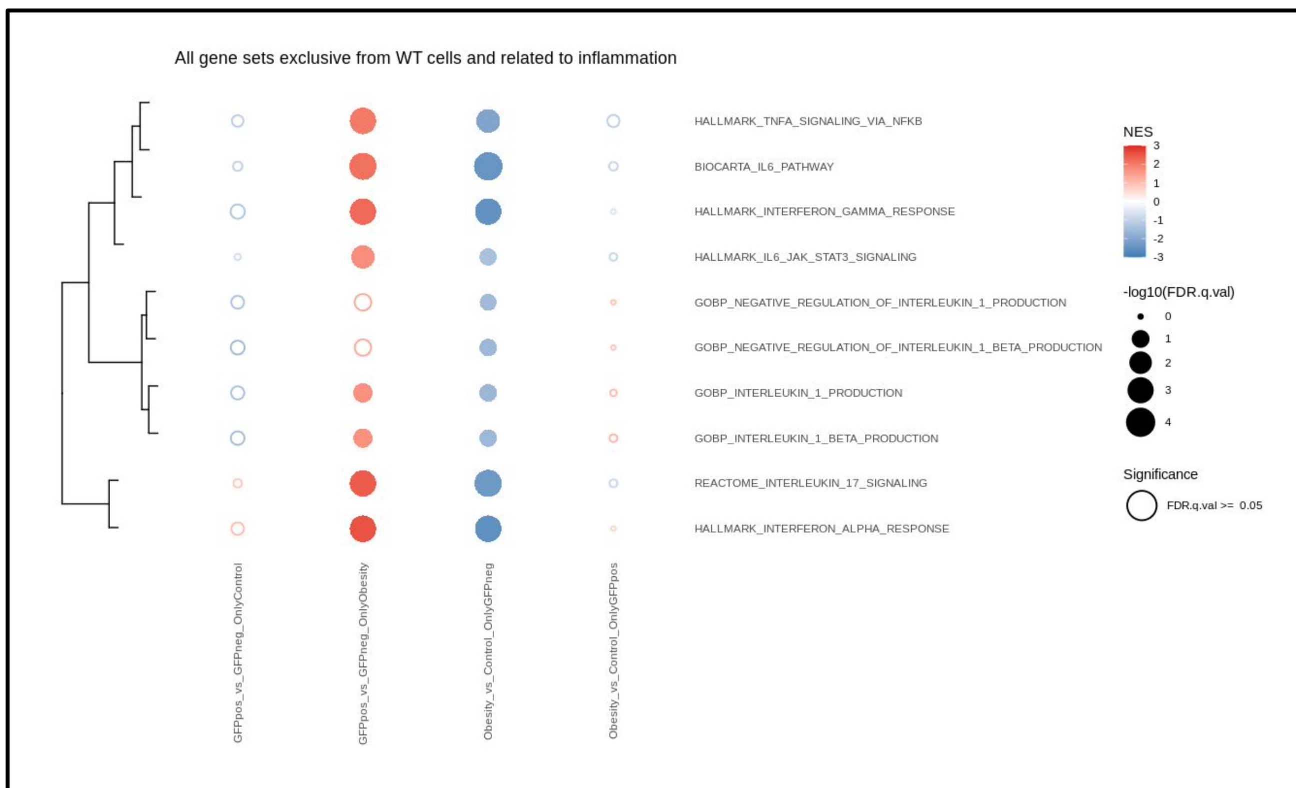
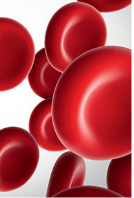


Figure 4: Summary gene set enrichment analysis focused on inflammatory pathways in Jak2V617F mice
Schematic summarizing gene set enrichment analysis (GSEA) comparing Jak2V617F-mutant hematopoietic stem cells (HSC) and Jak2 wild-type HSC isolated from chimeric transplant mice fed either control or obesity diets. GFPpos indicates Jak2V617F-mutant cells, GFPneg indicates Jak2 wild-type cells. Control indicates mice fed a control diet, obesity indicates mice fed an obesity diet. The main finding is that pro-inflammatory pathways are differentially upregulated in Jak2V617F-mutant HSC (as compared to Jak2 wild-type HSC) in mice fed an obesity diet (second column). These findings are not seen in Jak2V617F-mutant HSC in mice fed a control diet (first column). These pathways are not differentially upregulated in Jak2 wild-type HSC in mice fed an obesity diet (as compared to a control diet) (third column) or in Jak2V617F-mutant HSC in mice fed an obesity diet (as compared to a control diet) (fourth column). The specific pathways differentially upregulated in Jak2V617F-mutant HSC (as compared to Jak2 wild-type HSC) in mice fed an obesity diet include tumor necrosis factor alpha (TNFA), interferon gamma, interferon alpha and interleukin 17.



blood® Review Series

CLASSIC MYELOPROLIFERATIVE NEOPLASMS

Biology and therapeutic targeting of molecular mechanisms in MPNs

Joan How,[1,2] Jacqueline S. Garcia,[2] and Ann Mullally[1-3]

[1]Division of Hematology, Department of Medicine, Brigham and Women's Hospital, and [2]Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA; and [3]Cancer Program, Broad Institute, Cambridge, MA

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell disorders characterized by activated Janus kinase (JAK)–signal transducer and activator of transcription signaling. As a result, JAK inhibitors have been the standard therapy for treatment of patients with myelofibrosis (MF). Although currently approved JAK inhibitors successfully ameliorate MPN-related symptoms, they are not known to substantially alter the MF disease course. Similarly, in essential thrombocythemia and polycythemia vera, treatments are primarily aimed at reducing the risk of cardiovascular and thromboembolic complications, with a watchful waiting approach often used in patients who are considered to be at a lower risk for thrombosis. However, better understanding of MPN biology has led to the development of rationally designed therapies, with the goal of not only addressing disease complications but also potentially modifying disease course. We review the most recent data elucidating mechanisms of disease pathogenesis and highlight emerging therapies that target MPN on several biologic levels, including *JAK2*-mutant MPN stem cells, JAK and non-JAK signaling pathways, mutant calreticulin, and the inflammatory bone marrow microenvironment.

Introduction

Philadelphia chromosome–negative myeloproliferative neoplasms (MPNs) including polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF) are chronic blood cancers characterized by an excessive production of mature blood cells of the myeloid lineage. In more than 90% of the cases, MPNs develop because of the acquisition of an MPN phenotypic driver mutation in 1 of 3 genes: *JAK2*, *CALR*, or *MPL*, with the resultant activations of Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signaling.[1,2] Current treatment for PV and ET is largely focused on reducing the risk of thrombosis, with cytoreduction indicated for those patients deemed to be at high risk. Older age (>60 years), prior history of thrombosis, presence of the *JAK2V617F* mutation, and coexisting cardiovascular risk factors are all considered to be factors associated with increased thrombotic risk. In contrast, mutations in *CALR* have been demonstrated to confer lower risk of thrombosis,[3] and patients with type 1 *CALR*-mutated MF also have better leukemia-free and overall survival than those with *JAK2*- or *MPL*-mutated MF.[4] Although ruxolitinib was reported to confer an overall survival advantage in a pooled analysis of the COMFORT trials,[5] current treatments in MF are largely palliative to reduce symptom burden, blood counts, and spleen size, with no therapies known to alter the disease course substantially, except for allogeneic hematopoietic stem cell (HSC) transplantation.

However, greater understanding of the biological mechanisms underlying MPN pathogenesis have led to more rationally designed therapies, including interventions that may result in disease modification.[6] Clinical trials have primarily been focused on MF, where prognosis and complications are often more grave, but novel treatments are also being evaluated in ET and PV. Here, we review the most recent data elucidating mechanisms of disease in MPNs, with a focus on those with therapeutic potential. This review will not cover MPN-related thrombosis or stem cell transplantation, both of which have been reviewed elsewhere.[7,8]

MPNs are HSC disorders

Understanding of the pathophysiology of MPNs was transformed after the discovery in 2005 that mutations in *JAK2*, specifically the *JAK2V617F* mutation, were found in nearly all patients with PV and in ~50% to 60% of patients with ET and MF.[9-12] It was subsequently established that the *JAK2V617F* mutation arises in the long-term HSC compartment, consistent with the fact that it can be detected in both myeloid and lymphoid lineage cells.[13-15] Similarly, *CALR* and *MPL* mutations have also been found to occur in multipotent HSCs.[16,17] Although sequencing studies in humans[1,18] and multiple MPN mouse models[19-21] have shown that an MPN phenotypic driver mutation alone is sufficient to cause a full MPN phenotype, the *JAK2V617F* mutation can be detected in asymptomatic individuals as clonal hematopoiesis, indicative of a preclinical phase of disease.[22] Consistent with this, 2 independent studies have recently shown evidence for a long latency period between *JAK2V617F* mutation acquisition and MPN presentation.[23,24]

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Through whole-genome sequencing of HSCs, Williams et al and Van Egeren et al performed phylogenetic reconstruction of clonal lineage histories in patients with *JAK2*-mutated MPNs.[23,24] Both groups found that the *JAK2* mutation arose decades before disease presentation, and in some cases was acquired in utero, with a mean latency between mutation acquisition and disease presentation of ~30 years. Similar findings were recently reported for *CALR*-mutant MPN, in which monozygotic twins presented at ages 37 and 38 with MF. Using whole-genome sequencing and lineage tracing, the authors identified a common in utero origin for the *CALR*-mutant clone, with twin-to-twin transplacental transmission and subsequent MPN development in adulthood.[25] A recent study using mathematical modeling to infer disease initiation in patients with *CALR* and *JAK2* mutation found that, in general, *CALR* mutations are acquired later in life and have a stronger HSC growth advantage than *JAK2* mutations.[26] This is consistent with a younger age of MPN onset in patients with *CALR* mutation than in those with *JAK2* mutation[27] and the finding that the *CALR*-mutant variant allele fraction (VAF) measured in granulocytes is typically 40% to 50% at the time of ET diagnosis,[28] suggesting that heterozygous *CALR*-mutant HSC quickly become clonally dominant. Following acquisition of an MPN phenotypic driver mutation, the subsequent clonal expansion of the HSC-mutated clone is likely influenced by additional factors, including germ line factors,[29] occurrence of other mutations in a particular order,[30,31] inflammation,[32,33] and the bone marrow (BM) microenvironment.[34,35] These recent findings reframe MPNs as a chronic disease with a long precursor state, in which the disease presentation is a late phase and fit clones have reached a detectable level that results in symptoms. Given the lifelong trajectories of MPNs, one wonders if the current treatment paradigm for ET and PV, in which therapies are targeted at reducing vascular risk and low-risk patients are often observed, should shift to earlier interventions.

Targeting the HSC compartment is an appealing approach, given that it would eradicate disease at its source. This, however, has been challenging given that long-term HSCs are generally resistant to therapeutic targeting, likely because of their quiescent state. It is also difficult to identify therapeutic targets unique to the MPN stem cell that spare healthy HSCs and leave them unaffected. Among current MPN therapies, interferons are notable for their capacity to induce durable molecular responses. In addition, interferons have a long history of clinical efficacy in MPNs, particularly in patients with ET and PV.[36,37] Although the mechanism of action of interferons is multifactorial, molecular responses observed in patients with *JAK2*-mutant MPN treated with pegylated interferons are likely due to the differential effects of interferon on *JAK2*-mutated HSCs over healthy HSCs.

Mechanism of action of interferons and targeting of HSCs

There has been a resurgence of interest in interferons as treatment for MPNs, given clinical data indicating that interferons can reduce the *JAK2V617F* mutant allele burden and, in some cases, even induce complete molecular remission.[38,39] In mice, it has been shown that interferon induces HSC exit from quiescence and differentiation into mature progenitors, resulting in preferential depletion of *JAK2*-mutated HSCs,[40,41] a finding that has also been validated in human MPNs[42,43] (Figure 1A). Using *JAK2V617F* mouse models and patient samples, Rao et al found that prolonged interferon stimulation preferentially induced expansion of a megakaryocyte-biased CD41^{Hi} HSC subset at the expense of more primitive CD41^{Lo} populations.[44] Because CD41^{Hi} subsets have less self-renewing capabilities, this biased expansion could result in the eventual exhaustion of MPN-sustaining HSCs. Treatment with interferons has also been shown to result in accumulation of reactive oxygen species and induction of DNA damage preferentially in *JAK2*-mutant HSCs in mice.[45] Interestingly, molecular responses are distinct in patients with *JAK2*- vs *CALR*-mutated MPNs.[46] Sequential next-generation sequencing of patients treated with pegylated interferons, in the DALIAH trial, showed that patients with a *JAK2* mutation had a significant reduction in VAF at 24 months in response to interferon, whereas patients with a *CALR* mutation did not,[46] consistent with retrospective data and a more recent prospective observational study showing that *CALR*-mutant progenitor cells are less responsive to interferon than *JAK2*-mutant progenitors.[43,47] Overall, hematologic responses with hydroxyurea and pegylated interferon were similar in both the DALIAH and MPN-RC 112 randomized phase 3 trials, which may be partially because of the high discontinuation rates observed in those who experienced pegylated interferon-related toxicity. However, greater decreases in *JAK2V617F* VAF were observed with longer treatment duration of pegylated interferon.[46,48] Notably, treatment-emergent DNA-methyltransferase-3α (*DNMT3A*) mutations were identified in patients treated with interferons in the DALIAH trial, highlighting the importance of assessing molecular response not just in MPN phenotypic driver mutations, but more broadly, using sequential genomic profiling because divergent molecular responses may occur in independent clones or subclones in the same patient.[46]

There have been ongoing efforts to improve existing interferon treatments in MPNs. The development of ropeginterferon, a monopegylated form of interferon with improved tolerability, has demonstrated superior hematologic and molecular responses than the use of hydroxyurea in patients with PV, although superior molecular responses only became apparent at 2 years.[49] The slow rate of molecular responses is partially because of the fact that titration of ropeginterferon to therapeutic dosing takes more than 6 months. However, sustained *JAK2V617F* molecular responses to ropeginterferon have been reported at 5 years.[50] Ropeginterferon is now approved in the United States and Europe for patients with PV in the frontline setting, regardless of risk status.

Identification of the downstream pathways that potentiate or bypass interferon response signaling may also lay the groundwork for combination therapies that improve interferon's efficacy. For instance, promyelocytic protein (PML) is a tumor suppressor that is important in the induction of senescence and programmed cell death. PML is a target of arsenic trioxide (ATO) and is transcriptionally activated by interferons.[51] In experimental studies, ex vivo treatment with ATO potentiated interferon-induced growth suppression in *JAK2V617F* progenitor cells and the combination of ATO with interferon enhanced hematologic and molecular responses in MPN mouse models compared with ATO or interferon alone.[52] Unc-51–like kinase 1 (ULK1) has been identified as a mediator of interferon's effects

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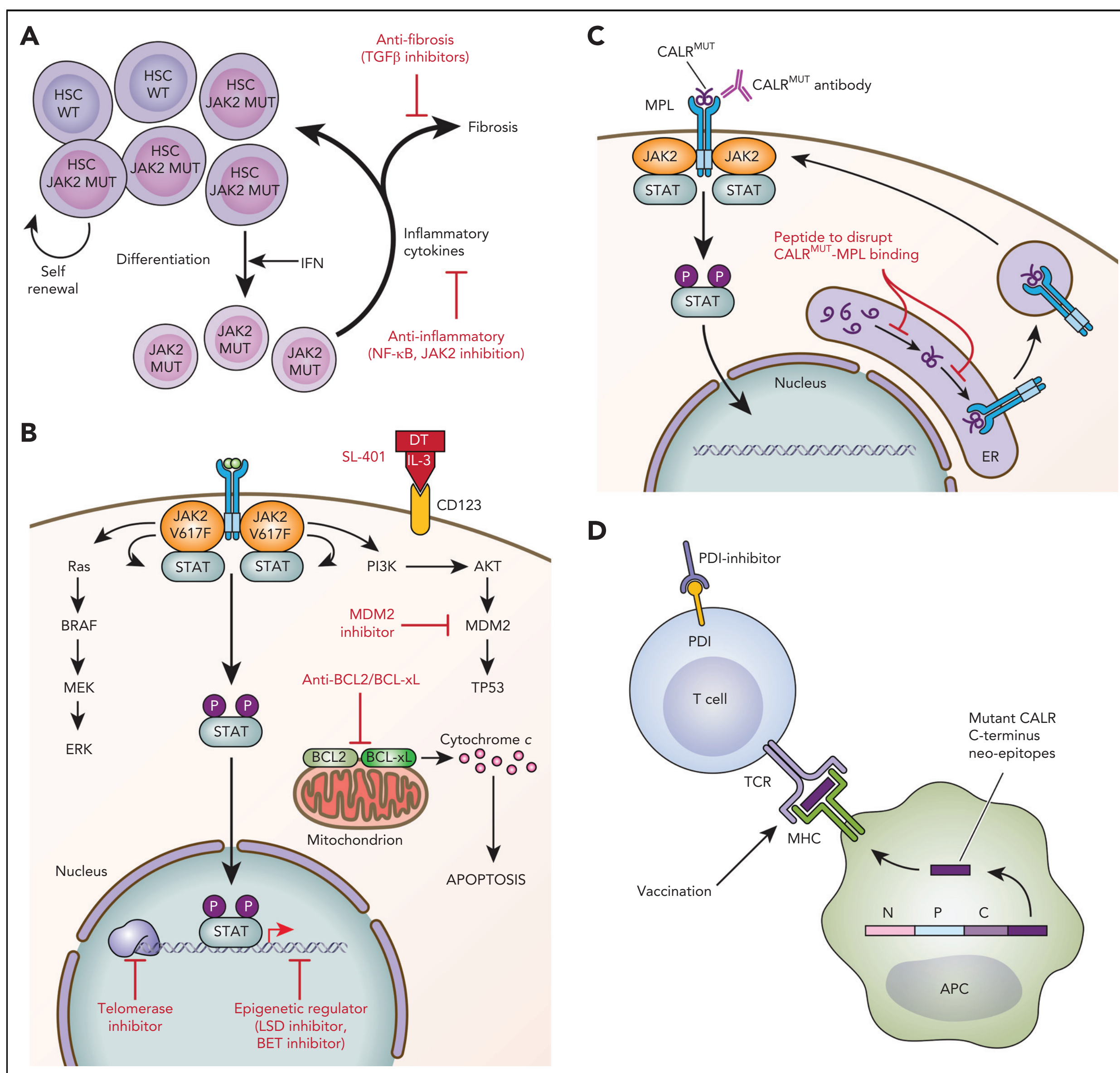

Figure 1. Schematic of novel therapies in development for the treatment of MPNs. (A) Targeting the HSC and microenvironment. JAK2V617F–mutated HSCs show clonal dominance over wild-type HSCs. Interferon preferentially targets *JAK2*-mutated HSCs to induce exit from quiescence and promote terminal myeloid differentiation, resulting in preferential depletion of *JAK2*-mutated HSCs. The expanded myeloid clone also disrupts the BM microenvironment through secretion of inflammatory mediators. Novel therapies targeting inflammation and profibrotic cytokines may delay or prevent the progression of early MPNs to MF. (B) Targeting cell signaling, epigenetics, and apoptosis. MPNs are characterized by activated JAK/STAT signaling. Multiple signaling pathways are activated downstream of mutant JAK2 and represent targets for therapeutic intervention. (C) Novel therapies targeting CALR-mutated MPNs. Novel therapies in preclinical development against CALR-mutated MPNs include antibodies to block mutant CALR on the cell surface and peptides to disrupt intracellular MPL/mutant CALR binding. (D) Immune therapies targeting CALR-mutated MPNs. Vaccination strategies to induce T-cell–directed immune activation against CALR-mutated clones take advantage of the mutant CALR C-terminus neoepitopes generated by CALR mutations. APC, antigen-presenting cell; DT, diphtheria toxin; ER, endoplasmic reticulum; IFN, interferon; MUT, mutated; P, phosphorylated; TCR, T-cell receptor; WT, wild-type. Professional illustration by Patrick Lane, ScEYEnce Studios.

against clonal progenitor cells in MPNs. Interferon treatment activates ULK1-interacting Rho-associated kinases (ROCK1/2) resulting in a negative feedback loop suppressing interferon responses,[53] suggesting that combining ROCK inhibition with interferon may enhance treatment response in MPNs. Interferon activity has also been studied in combination with ruxolitinib, a JAK1/2 inhibitor. Although ruxolitinib antagonizes interferon-induced JAK1 signaling, the favorable activity of both treatments in MPNs can be retained with combination therapy. It has been shown that JAK/STAT pathway activation is enhanced with chronic interferon stimulation in *JAK2V617F* mouse models, and ruxolitinib therapy does not inhibit interferon-induced reactive oxygen species and DNA damage to *JAK2V617F* HSCs.[45] A phase 2 trial of ruxolitinib and pegylated interferon showed improvements in cell counts in 32 patients with PV and 18 with MF, although it was met with significant toxicity, particularly in patients with MF (32% discontinuation rate).[54]

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JAK2 inhibition in MPNs

The *JAK2V617F* mutation lies within the pseudokinase domain of JAK2, which sits adjacent to its C-terminal tyrosine kinase domain. A crystal structure of the JAK2 pseudokinase domain harboring the *JAK2V617F* mutation was solved in 2012, and in 2022 the full-length structure of a homologous JAK mutant (*JAK1V657F*), complexed with a cytokine receptor intracellular domain, was revealed using cryo-electron microscopy.[55,56] This recent study advances the understanding of the basis for JAK dimeric activation, in the context of a V617F homologous mutant. Efforts to develop *JAK2V617F*-selective inhibitors are ongoing, but none have yet advanced to clinical testing.

Current JAK2 inhibitors

Currently approved JAK2 inhibitors are not strongly clonally selective for the *JAK2*-mutant clone and act as competitive inhibitors on the adenosine triphosphate–binding site. Ruxolitinib, a JAK1/2 inhibitor, was first approved in patients with MF and later in those with PV who were refractory or intolerant to hydroxyurea.[57-59] Since ruxolitinib, there have been 2 additional Food and Drug Administration–approved JAK2 inhibitors for the treatment of MF. Fedratinib is a JAK2 and fms-like tyrosine kinase (FLT3) inhibitor that can be used as an alternative first- or second-line agent for MF.[60] Both ruxolitinib and fedratinib are limited by treatment-related adverse effects including cytopenias and are not recommended for use in patients with platelet counts $<50 \times 10^9$/L. Pacritinib, a JAK2, FLT3, and interleukin 1 receptor associated kinase 1 (IRAK1) inhibitor, was the third JAK2 inhibitor approved in MF and its label allows use in patients with baseline platelet counts $<50 \times 10^9$/L, based on the results of the PAC203 dose-finding and phase 3 PERSIST-1 and 2 trials.[61-64] Finally, since IRAK1 is also involved in inflammatory signaling pathways that may promote fibrotic progression,[35] inhibiting IRAK1 in MF may provide additional benefit.

Because one of the on-target toxicities of JAK2 inhibition is anemia, several JAK2 inhibitors worsen anemia, which is a common feature in MF. Anemia in MF is multifactorial and partially due to the inflammatory milieu in MF, and upregulation of hepcidin is associated with worsened anemia of inflammation.[65] Momelotinib is a selective inhibitor of JAK1, JAK2, and activin receptor type 1 (ACVR1), the latter of which is a transforming growth factor β (TGF-β) family member that controls iron storage and upregulates hepcidin. Inhibition of ACVR1 leads to decreased hepcidin production, allowing mobilization of sequestered iron and improved markers of erythropoiesis.[66] In patients with upfront intermediate-2/high-risk MF, momelitinib was associated with reduced transfusion requirements compared with ruxolitinib in the phase 3 SIMPLIFY-1 trial and was noninferior to ruxolitinib for spleen response but not for symptom response.[67] Recently presented data from the phase 3 MOMENTUM study among patients with MF previously treated with ruxolitinib demonstrated the superiority of momelitinib to danazol in symptom improvement and spleen reduction and noninferiority in transfusion independence. Though danazol is a supportive agent recommended by expert consensus guidelines,[68] it might have been a weak comparator arm because danazol is not expected to induce significant spleen or symptom responses in patients with MF.[69]

Novel approaches to targeting JAK2V617F

Although JAK2 inhibitors represent a major step forward in the treatment of MPNs, they do not significantly decrease the *JAK2*- or *CALR*-mutant clonal burden, and with prolonged treatment, clinical resistance inevitably develops.[70,71] Concordant with this, JAK2 inhibitor resistance appears to be mediated by nonmutational mechanisms, suggesting that a strong selective pressure is not exerted on the *JAK2*-mutant clone in patients treated with JAK2 inhibitors (ie, mutations in JAK2 kinase domain are not observed).[72] Advancement in the knowledge of JAK2V617F dimeric activation, as outlined here, continues to drive an interest in developing JAK2V617F-mutant–specific inhibitors.[56] Conditionally inducible JAK2V617F mouse models in which *JAK2V617F* expression can be switched off and the MPN phenotype is reversed support this approach.[73,74] Type II JAK2 inhibitors that bind JAK2 in an inactive conformation have been developed and reported to show enhanced selectivity for mutant JAK2 in preclinical models.[75,76] Targeting ubiquitination and degradation of mutated JAK2 is another novel approach to JAK2 inhibition currently under investigation, including design of proteolysis-targeting chimeras that degrade oncogenic JAKs and inhibition of deubiquitinases that stabilize the *JAK2V617F* protein.[77,78]

Non-JAK2 targets in MPNs

Given the limitations of JAK2 inhibition, particularly in MF, there is an ongoing effort to develop several non-JAK2 inhibitors and regimens in combination with JAK2 inhibitors to increase pathway inhibition or to address MPN clones that are not JAK2 dependent. An in-depth overview of all novel inhibitors being investigated in the treatment of MPNs is outside the scope of this review, however, we highlight some promising therapies here. Table 1 also summarizes drugs with clinical data and known plans for future development.

Targeting signaling pathways

Multiple signaling pathways are activated in MPNs and contribute to disease progression and failure of JAK2 inhibition. These pathways include phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin[93] and extracellular signal-regulated kinases/mitogen-activated protein kinase [94,95] (Figure 1B), providing rationale for combination therapies with ruxolitinib. Positive phase 2 data for the PI3Kδ inhibitor parsaclisib in combination with ruxolitinib in patients with MF with suboptimal responses to ruxolitinib have been reported in abstract form (Table 1)[85] and are the basis for ongoing phase 3 studies in the first-line (#NCT04551066) and second-line (#NCT04551053) settings.

Targeting antiapoptosis pathways

JAK/STAT signaling pathway activation and resistance to JAK inhibitors also provide opportunities for targeting antiapoptotic pathways.[96,97] Antiapoptotic proteins including BCL-2, BCL-xL, and MCL-1 sequester proapoptotic BCL-2 homology 3 (BH3)–only proteins by binding to their BH3 motifs, thereby preventing the initiation of apoptosis.[96] Small molecules targeting these antiapoptotic proteins have been developed to trigger apoptosis by binding these proteins, freeing proapoptotic proteins, and promoting apoptosis.[96] Functional dissection of the critical survival pathways in human and mouse models

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Table 1. Summary of selected drugs approved or in clinical development for MPNs

MF	Mechanism	Trial	Clinical response
Rux[57,58] (approved)	JAK2 inhibitor	3	SVR_{35} = 42% TSS_{50} = 50%
Fedratinib[79] (approved)	JAK2 inhibitor	3 Rux naïve 2 Prior rux	SVR_{35} = 37% TSS_{50} = 40% SVR_{35} = 55% TSS_{50} = 26%
Pacritinib[61] (approved)	JAK2 inhibitor	3 Prior rux allowed Platelets <100 × 10^9/L	SVR_{35} = 29% TSS_{50} = 32%
Momelitinib[67]	JAK2 inhibitor	3 Rux naïve	SVR_{35} = 27% TSS_{50} = 38% Transfusion independence at wk 24 in 67%
AVID200[80]	TGF-β trap	1B (N = 21) Prior rux	SVR_{35} = 19% TSS_{50} = 43% 3/4 patients treated to cycle 12 with clinical improvement
Bomedemstat[81]	LSD1 inhibitor	1/2 (N = 89) Prior rux	SVR_{35} = 37% TSS_{50} = 39% BM fibrosis improvement in 17%
Imetelstat[82]	Telomerase inhibitor	2 (N = 59) Prior rux	SVR_{35} = 10% TSS_{50} = 32% BM fibrosis improvement in 40.5%
Navtemadlin[83]	MDM2 inhibitor	2 (N = 32*) Prior rux	SVR_{35} = 16% TSS_{50} = 30% BM fibrosis improvement in 27%
Navitoclax + rux[84]	BCL2 inhibitor	2 (N = 34)	SVR_{35} = 27% TSS_{50} = 30% Anemia response in 64% BM fibrosis improvement in 33%
Parsaclisib + rux[85]	PI3K inhibitor	2 (N = 51)	SVR_{35} = 27% TSS_{50} = 50%
Pelabresib[86]	BET inhibitor	2 (N = 86)	SVR_{35} = 11% TSS_{50} = 28% TD to TI conversion rate in 16% BM fibrosis improvement in 23.4%
Pelabresib + rux[87]	BET inhibitor	2 (N = 78) Rux naïve 2 (N = 70) Prior rux	SVR_{35} = 67% TSS_{50} = 57% BM fibrosis improvement in 33% SVR_{35} = 21% TSS_{50} = 53% TD to TI conversion rate 36%
Selinexor[88]	Selective inhibitor of nuclear export	2 (N = 10)	SVR_{35} = 30% Reduction in TSS in 8 evaluable patients
Tagraxofusp[89]	CD123 (IL-3Rα) targeted therapy (recombinant human IL-3 fused to diphtheria toxin)	1/2 (N = 39)	SVR_{10} = 47%; SVR_{50} = 29% TSS_{50} = 36%
PV/ET			
Pegylated interferon[46,48]	Interferon	3	CHR: 35% at 1 y; 21% at 2 y MR: 16% at 18 mo

CHR, complete hematologic response; IL-3, interleukin 3; IL-3Rα, interleukin 3 receptor α; MR, molecular response; rux, ruxolitinib; $SVR_{10/35/50}$, spleen volume reduction by 10%/35%/50% (shown are observations at week 24); TD, transfusion dependence; TI, transfusion independence; TSS_{50}, total symptom score improvement by 50%.

*Sample size of cohort receiving dosing selected for phase 3 testing; response rates are reported for this cohort.

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Table 1 (continued)

MF	Mechanism	Trial	Clinical response
Ropeginterferon[49]	Interferon	3	CHR: 43% at 1 y; 48% at 2 y; 54% at 3 and 4 y MR: 34% at 1 and 2 y; 50% at 3 and 4 y
Rusfertide[90,91]	Hepcidin mimetic	2 (N = 63) PV	Mean phlebotomy after enrollment: 0.43 (vs 4.63 prior)
Bomedemstat[92]	LSD1 inhibitor	2 (N = 29) ET	TSS_{50} = 53% 100% with normalization of platelet counts

CHR, complete hematologic response; IL-3, interleukin 3; IL-3Rα, interleukin 3 receptor α; MR, molecular response; rux, ruxolitinib; $SVR_{10/35/50}$, spleen volume reduction by 10%/35%/50% (shown are observations at week 24); TD, transfusion dependence; TI, transfusion independence; TSS_{50}, total symptom score improvement by 50%.

*Sample size of cohort receiving dosing selected for phase 3 testing; response rates are reported for this cohort.

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bearing *JAK2*-mutated leukemias revealed upregulation of prosurvival (antiapoptotic) *Bcl-2* family genes, dependence on BCL-2/BCL-xL, and the ability to overcome JAK2 inhibitor-acquired resistance with combined targeting of JAK2 and BCL-2/BCL-xL[98] (Figure 1B). Gain-of-function studies in *JAK2V617F*-mutated MPNs identified resistance to JAK inhibition upon activation of RAS and its effector pathways, which results in dependence on BCL-xL for survival.[97] Navitoclax (ABT-263) is an orally bioavailable BH3 mimetic that binds with high affinity to prosurvival BCL-xL, BCL-2, and BCL-W. Preclinical data indicate synergistic activity of combination JAK2 inhibition and navitoclax.[99] The REFINE phase 2 trial (n = 34) demonstrated safety and clinical activity with the addition of navitoclax to ongoing ruxolitinib in patients with persistent or progressive MF (Table 1).[84] An exploratory study provided preliminary evidence for BM fibrosis grade reduction in approximately one-third of patients with MF that received combination navitoclax and ruxolitinib, regardless of high-molecular risk mutation status, and reduction of ≥20% in driver VAF, suggesting disease modifying benefits as these treatment-induced biological changes were associated with increased survival.[100] Results from the ongoing phase 3 trials in the upfront (TRANSFORM-1) and relapsed/refractory (TRANSFORM-2) settings are awaited.

Targeting epigenetic regulators: BET and LSD1

Epigenetic dysregulation is a feature of MPNs, and mutations in genes involved in DNA methylation (eg, *TET2*, *DNMT3A*, and *IDH1/2)* and chromatin modification (eg, *ASXL1* and EZH2) are frequently found.[1] In the context of MF, *IDH1/2*, *ASXL1*, and *EZH2* mutations are defined as high-molecular risk mutations and associated with adverse prognosis.[101] Although *ASXL1* and *EZH2* mutations are associated with decreased response to JAK inhibitor therapy,[102,103] there are currently no rationally designed approaches to directly target mutant ASXL1 or EZH2. Except for small molecule inhibitors of mutant IDH1/2, current therapies targeting epigenetic regulators in MPN exert their effects indirectly.

NF-kB signaling is activated in MF and contributes to the proinflammatory state that characterizes MPNs and may drive the development of fibrosis. The bromodomain and extra-terminal domain (BET) family of proteins bind to acetylated histones, facilitating transcription of genes regulated by NF-kB.[104] Preclinical work by Kleppe et al has demonstrated that dual JAK/BET inhibition results in the reduction of inflammatory cytokines and BM fibrosis in MPN mouse models.[104] Data from studies on mice suggest that *EZH2* and *ASXL1* mutations can sensitize MF-initiating cells to BET inhibition.[105,106] Pelabrisib, a first-in-class oral BET inhibitor, is currently being evaluated in the phase 2 MANIFEST study as a single agent and in combination with ruxolitinib, both in the upfront and refractory setting (#NCT04603495) (Table 1 and Figure 1B).[87]

LSD1 is a histone demethylase important for regulating HSC self-renewal and proliferation through the epigenetic regulation of gene transcription. LSD1 activity is essential for enhancer silencing during cell differentiation and in myelopoiesis.[107] LSD1 allows myeloid progenitors to differentiate into mature myeloid lineage cells.[108] In a conditional in vivo knockdown mouse model of LSD1, loss of LSD1 resulted in an extensive expansion of myeloid progenitor cells with a concomitant severe inhibition of terminal granulopoiesis, erythropoiesis, and platelet production, with reversal of the cytopenias upon knockdown termination.[109] Increased LSD1 expression has been reported in MPNs, mainly in megakaryocytes.[110] LSD1 inhibition in MPN mouse models resulted in reduced blood counts and spleen volume, improved BM fibrosis, and increased survival.[111] In human MPNs, results of monotherapy with bomedemstat, an oral LSD1 inhibitor, have been reported in abstract form for both patients with MF and ET in phase 1/2 clinical trials (Table 1), with reduced splenomegaly and symptoms observed in MF and reduction in platelet counts observed in ET (Figure 1B).[81,112] The impact of LSD1 inhibition on myeloid differentiation in human MPN BM has not yet been reported from these clinical trials; this will be an important parameter to follow given that loss of LSD1 in normal murine hematopoiesis results in an expansion of myeloid progenitor cells by blocking myeloid differentiation.[109] Interestingly, LSD1 inhibitors have been reported to induce myeloid differentiation in MLL-translocated acute myeloid leukemia, a mechanism initially thought to result from blocking LSD1 demethylase activity but more recently reported to be a consequence of disrupting the LSD1-GFI1B interaction on chromatin.[113-115]

Other targets: MDM2 pathway, telomerase inhibition, and cell cycle

Other non-JAK inhibitor therapies currently in development for MPNs include the mouse double-minute homolog 2 (MDM2) inhibitor, which exerts its mechanism of action by restoring the

TP53 pathway (Figure 1B).[116] Gastrointestinal toxicities seen with this drug limit its future clinical development for PV and ET, although it is being evaluated in the relapsed or refractory setting in patients with MF with *TP53* wild-type disease after JAK inhibitor failure (#NCT03662126) (Table 1). Increased telomerase activity and shortened telomeres are seen in MPNs, and telomerase inhibition results in the selective reduction of clonal megakaryocyte colony-forming units in MF and ET.[117] A randomized, phase 2 trial of imetelstat, a telomerase inhibitor (Figure 1B), was completed in patients with intermediate-2/high-risk MF who were relapsed or refractory to ruxolitinib and demonstrated clinical improvements, including spleen and symptom responses at the higher dose and reduction of BM fibrosis in 40.5% and driver VAF in 42.1% of the patients (Table 1).[82] At the recommended phase 2 dose, a median overall survival of 29.9 months was observed. A phase 3 trial (#NCT04576156) is currently underway. There are also data indicating that cell cycle genes regulated by activated JAK/STAT signaling promote MPN disease.[118] Preclinical studies evaluating combination CDK4/6 inhibitors with ruxolitinib in mice show improvements in disease phenotypes.[119] The addition of inhibitors of proto-oncogene serine/threonine-protein kinase (PIM1), also a cell cycle regulator, to ruxolitinib and CDK4/6 inhibitors similarly reduces disease features in MPN mouse models.[120]

Targeting hepcidin and altered iron metabolism in PV

A novel approach, rationally designed based on preclinical studies, is the development of hepcidin mimetics in PV, with the goal of constraining mutant *JAK2*-driven erythrocytosis, and thus reducing/eliminating the need for therapeutic phlebotomy. Unlike in MF, in which increased hepcidin levels contribute to an inflammatory anemia, in PV, there is relative suppression of hepcidin without mobilization of hepatic iron stores or increased iron absorption.[121] As a result, patients with PV often show evidence of iron deficiency even before therapeutic phlebotomy is initiated, including lower serum iron, ferritin, and transferrin saturation.[121] Hepcidin mimetics provide a chemical therapeutic phlebotomy, with the goal of restricting iron for accelerated erythropoiesis while reducing systemic iron deficiency. Mini-hepcidin mimetics normalized splenomegaly and hematocrit levels in *JAK2V617F*–mutated mouse models of PV,[122] and in phase 2 trials, the hepcidin mimetic rusfertide (PTG-300) similarly reduced hematocrit levels and therapeutic phlebotomy needs in patients with PV.[90] A phase 3 trial evaluating rusfertide vs placebo in patients with PV requiring frequent phlebotomy is currently underway (#NCT04057040 and #NCT04767802).

CALR-mutated MPNs provide novel therapeutic targets

Approximately 80% of patients with ET and MF without *JAK2* mutations will have mutations in *CALR*.[16,123] *CALR* encodes for calreticulin, an endoplasmic reticulum chaperone protein. MPN-associated *CALR* mutations are insertions and/or deletions in exon 9 that induce a frameshift, resulting in the generation of a novel positively charged C-terminus to the mutant CALR protein. This mutant-specific C-terminus of mutant CALR enables pathogenic binding with MPL, ultimately resulting in ligand-independent MPL/JAK/STAT signaling pathway activation[124-127] (Figure 1C). The specific requirements for the interaction of mutant CALR with MPL have revealed potential opportunities for therapeutic targeting. For instance, mutant CALR forms homomultimers to bind and activate MPL.[128] Zinc is a cofactor necessary for mutant CALR homomultimerization, and ex vivo treatment with zinc chelators disrupted $CALR^{del52}$-MPL signaling complexes in *CALR*-mutated cells, identifying the reduction in intracellular zinc levels as a potential treatment approach in *CALR*-mutated MPNs.[129] N-glycosylation of the extracellular domain of MPL and the lectin-binding sites of mutant CALR are also required for the mutant CALR-MPL interaction.[127,130,131] Whole-genome CRISPR screening recently identified N-glycosylation as a therapeutic vulnerability in mutant CALR-transformed hematopoietic cells, and chemical inhibition of N-glycosylation resulted in an improvement of disease features in *CALR*-mutant mouse models and reduced growth of *CALR*-mutant patient-derived BM in vitro.[132] Other approaches tested in the preclinical setting include (1) delivering a wild-type CALR C-terminal synthetic peptide that is taken up by the cell with the goal of abolishing the intracellular mutant CALR binding interaction with MPL (Figure 1C) and (2) targeting the inositol requiring enzyme 1a (IRE1a)/X-box binding protein 1 (XBP1) axis of the unfolded protein response, since CALR is an endoplasmic reticulum chaperone.[133-135]

CALR-mutated MPNs also provide unique opportunities for immunologic treatment strategies given that the novel mutated C-terminus is tumor specific. Because the mutant CALR-MPL complex traffics to the cell surface, interest in therapeutically targeting mutant CALR using a mutant CALR blocking antibody (Figure 1C) has grown. Three recent studies reported the development and testing of therapeutic monoclonal antibodies targeting the C-terminal mutant CALR sequence in preclinical models,[136-138] with 1 of the studies also testing the antibody (4D7) against primary MPN cells in vitro and demonstrating reduced megakaryocyte proliferation, specifically in *CALR*-mutant (but not *JAK2*-mutant) cells.[136] Similar findings in primary MPN cells were recently reported for a fully human immunoglobulin G1–mutant CALR antibody (INCA033989) as a plenary abstract at the 2022 American Society of Hematology meeting.[139] In addition, using a chimeric mutant CALR BM transplant model, the authors reported that treatment with INCA033989 selectively targeted *CALR*-mutant platelets, megakaryocytes, and long-term HSCs. In aggregate, these preclinical studies indicate that therapeutic mutant CALR antibodies have specificity and efficacy, and further clinical development is awaited.

Using in silico approaches, it has been shown that the mutant-specific C-terminus of mutant CALR is predicted to generate multiple neoepitopes that bind to major histocompatibility class I (MHC-I) with high affinity.[140] This has led to interest in developing T-cell receptor–mediated immune therapy, including vaccination approaches (Figure 1D). However, peptide-stimulated T-cell responses against mutant CALR epitopes are decreased in patients with MPN compared with those in healthy control subjects, suggesting defects in immune response and/or antigen presentation in MPN.[141] Consistent with this, T cells from patients with MPN exhibit an exhausted phenotype with increased expression of immune checkpoint receptors,[142] and programmed cell death protein 1 (PD-1)

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inhibitor monotherapy failed to show clinical responses in a phase 2 single-arm study.[143] Although it has not been directly demonstrated that mutant CALR–derived neoepitopes are presented by MHC-I,[144] indirect evidence suggests that they are. MHC-I alleles predicted to bind to *CALR*-mutated neo-epitopes with high affinity are underrepresented in patients with *CALR*-mutant MPN (as compared with patients with *JAK2*-mutant MPN and population-matched healthy control subjects). This suggests that immune-mediated clearance by individuals expressing a mutant CALR neo-epitope high-affinity binding MHC-I allele occurs such that *CALR*-mutant MPN may not manifest clinically in these individuals.[140] In the first vaccine trial with a mutant CALR peptide, none of the 10 patients had any clinical response and only 2 had a $CD8^+$ T-cell response, consistent with impaired MHC processing and/or presentation of neoepitope antigens.[145] Alternative approaches to enhance the mutant CALR-directed T-cell immune response include the use of a modified vaccine approach, such as heteroclitic peptides, which optimize MHC-I binding affinity,[140] or combination approaches with an MPN-directed neoantigen vaccine plus an immune checkpoint inhibitor, which is the focus of a recently opened phase 1 clinical trial (#NCT05444530).

The inflammatory microenvironment promotes MPN progression

The BM microenvironment and, in particular, the development of fibrosis are key aspects of disease pathophysiology in MF. The cell nonautonomous effects of the MPN clone on the BM niche via the release of inflammatory mediators are central to the pathogenesis of myelofibrotic transformation.[146] Notably, the physical properties of a fibrotic BM may also propagate a proinflammatory environment, which further establishes a self-reinforcing fibrotic niche.[147] Nonclonal mesenchymal stromal cells (MSCs) are thought to be a key MF-promoting cellular population within the BM niche, with recent studies implicating $Gli1^+$ and $Lepr^+$ MSCs as fibrosis-driving cells in the BM.[35,148] Activation of the stroma by clonal MPN cells is likely driven by multiple inflammatory mediators; for example, an increased expression of CXCL4, a chemokine secreted by megakaryocytes, has been linked to progression of BM fibrosis and may be a potential drug target.[33] TGF-β is also critical to MSC differentiation, with the loss of TGF-β signaling abrogating the development of MF in MPL and JAK2 MPN mouse models.[149] Drugs targeting TGF-β, including TGF-β1–trap AVID-200, have therefore come under clinical investigation for the treatment of MF. Recently presented data demonstrated that, in patients with MF previously treated with ruxolitinib, AVID200 reduced TGF-β1 plasma levels and circulating inflammatory cytokines, although no reduction in BM fibrosis grade or cellularity was observed.[80]

Future directions

Although MPN encompasses a heterogenous and biologically complex set of chronic blood cancers, advances in understanding its molecular pathogenesis provide a framework toward developing improved treatments with the potential to target MPN disease–propagating stem cells. MPNs arise in the HSC compartment and have a long preclinical phase. MPN phenotypic driver mutations alone are sufficient to induce overt MPN. Increasing genomic complexity is observed with increased age and longevity of disease. The development of rationally designed therapies focused on targeting the clonal advantage conferred by MPN phenotypic driver mutations is likely to be clinically impactful, particularly if instituted in earlier stages of overt MPN or even in individuals with *JAK2*-mutant clonal hematopoiesis. An increased understanding of which individuals with *JAK2*-mutant clonal hematopoiesis are at the highest risk of developing overt MPN will help in designing clinical trials focused on MPN prevention. Continued efforts to uncover therapeutic vulnerabilities for high-molecular risk mutations associated with adverse prognosis and therapeutic resistance (eg, *ASXL1*, *EZH2*, *SRSF2*, *U2AF1*, and *TP53*) remain a high priority. The development of mutational-agnostic immunological therapies (eg, antibodies) offers the potential to overcome the therapeutic resistance of these mutations to currently available MPN therapies. Research advances enabling increasingly sophisticated studies of primary MPN tissues will continue to reveal new insights into MPN biology and uncover novel targets for therapeutic intervention.

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Footnote

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REFERENCES

1. Grinfeld J, Nangalia J, Baxter EJ, et al. Classification and personalized prognosis in myeloproliferative neoplasms. *N Engl J Med*. 2018;379(15):1416-1430.

2. Rampal R, Al-Shahrour F, Abdel-Wahab O, et al. Integrated genomic analysis illustrates the central role of JAK-STAT pathway activation in myeloproliferative neoplasm pathogenesis. *Blood*. 2014;123(22):e123-133.

3. Rotunno G, Mannarelli C, Guglielmelli P, et al. Impact of calreticulin mutations on clinical and hematological phenotype and outcome in essential thrombocythemia. *Blood*. 2014;123(10):1552-1555.

4. Tefferi A, Lasho TL, Finke CM, et al. CALR vs JAK2 vs MPL-mutated or triple-negative myelofibrosis: clinical, cytogenetic and molecular comparisons. *Leukemia*. 2014;28(7):1472-1477.

5. Verstovsek S, Gotlib J, Mesa RA, et al. Long-term survival in patients treated with ruxolitinib for myelofibrosis: COMFORT-I and -II pooled analyses. *J Hematol Oncol*. 2017;10(1):156.

6. Pemmaraju N, Verstovsek S, Mesa R, et al. Defining disease modification in myelofibrosis in the era of targeted therapy. *Cancer*. 2022;128(13):2420-2432.

7. Ali H, Bacigalupo A. 2021 update on allogeneic hematopoietic stem cell transplant for myelofibrosis: a review of current data and applications on risk stratification and management. *Am J Hematol*. 2021;96(11):1532-1538.

8. Hasselbalch HC, Elvers M, Schafer AI. The pathobiology of thrombosis, microvascular disease, and hemorrhage in the myeloproliferative neoplasms. *Blood*. 2021;137(16):2152-2160.

9. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet*. 2005;365(9464):1054-1061.

10. James C, Ugo V, Le Couédic J-P, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*. 2005;434(7037):1144-1148.

11. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med*. 2005;352(17):1779-1790.

12. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell*. 2005;7(4):387-397.

13. Jamieson CH, Gotlib J, Durocher JA, et al. The JAK2 V617F mutation occurs in hematopoietic stem cells in polycythemia vera and predisposes toward erythroid differentiation. *Proc Natl Acad Sci U S A*. 2006;103(16):6224-6229.

14. Ishii T, Bruno E, Hoffman R, Xu M. Involvement of various hematopoietic-cell lineages by the JAK2V617F mutation in polycythemia vera. *Blood*. 2006;108(9):3128-3134.

15. Delhommeau F, Dupont S, Tonetti C, et al. Evidence that the JAK2 G1849T (V617F) mutation occurs in a lymphomyeloid progenitor in polycythemia vera and idiopathic myelofibrosis. *Blood*. 2007;109(1):71-77.

16. Nangalia J, Massie CE, Baxter EJ, et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med*. 2013;369(25):2391-2405.

17. Chaligné R, James C, Tonetti C, et al. Evidence for MPL W515L/K mutations in hematopoietic stem cells in primitive myelofibrosis. *Blood*. 2007;110(10):3735-3743.

18. Lundberg P, Karow A, Nienhold R, et al. Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. *Blood*. 2014;123(14):2220-2228.

19. Mullally A, Lane SW, Brumme K, Ebert BL. Myeloproliferative neoplasm animal models. *Hematol Oncol Clin North Am*. 2012;26(5):1065-1081.

20. Benlabiod C, Cacemiro MDC, Nédélec A, et al. Calreticulin del52 and ins5 knock-in mice recapitulate different myeloproliferative phenotypes observed in patients with MPN. *Nat Commun*. 2020;11(1):4886.

21. Li J, Prins D, Park HJ, et al. Mutant calreticulin knockin mice develop thrombocytosis and myelofibrosis without a stem cell self-renewal advantage. *Blood*. 2018;131(6):649-661.

22. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371(26):2488-2498.

23. Van Egeren D, Escabi J, Nguyen M, et al. Reconstructing the lineage histories and differentiation trajectories of individual cancer cells in myeloproliferative neoplasms. *Cell Stem Cell*. 2021;28(3):514-523.e519.

24. Williams N, Lee J, Mitchell E, et al. Life histories of myeloproliferative neoplasms inferred from phylogenies. *Nature*. 2022;602(7895):162-168.

25. Sousos N, M NL, Simoglou Karali C, et al. In utero origin of myelofibrosis presenting in adult monozygotic twins. *Nat Med*. 2022;28(6):1207-1211.

26. Hermange G, Rakotonirainy A, Bentriou M, et al. Inferring the initiation and development of myeloproliferative neoplasms. *Proc Natl Acad Sci U S A*. 2022;119(37):e2120374119.

27. Rumi E, Pietra D, Pascutto C, et al. Clinical effect of driver mutations of JAK2, CALR, or MPL in primary myelofibrosis. *Blood*. 2014;124(7):1062-1069.

28. Rumi E, Pietra D, Ferretti V, et al. JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. *Blood*. 2014;123(10):1544-1551.

29. Bao EL, Nandakumar SK, Liao X, et al. Inherited myeloproliferative neoplasm risk affects haematopoietic stem cells. *Nature*. 2020;586(7831):769-775.

30. Rodriguez-Meira A, Buck G, Clark SA, et al. Unravelling intratumoral heterogeneity through high-sensitivity single-cell mutational analysis and parallel RNA sequencing. *Mol Cell*. 2019;73(6):1292-1305.e1298.

31. Ortmann CA, Kent DG, Nangalia J, et al. Effect of mutation order on myeloproliferative neoplasms. *N Engl J Med*. 2015;372(7):601-612.

32. Fleischman AG, Aichberger KJ, Luty SB, et al. TNFα facilitates clonal expansion of JAK2V617F positive cells in myeloproliferative neoplasms. *Blood*. 2011;118(24):6392-6398.

33. Gleitz HFE, Dugourd AJF, Leimkühler NB, et al. Increased CXCL4 expression in hematopoietic cells links inflammation and progression of bone marrow fibrosis in MPN. *Blood*. 2020;136(18):2051-2064.

34. Decker M, Martinez-Morentin L, Wang G, et al. Leptin-receptor-expressing bone marrow stromal cells are myofibroblasts in primary myelofibrosis. *Nat Cell Biol*. 2017;19(6):677-688.

35. Leimkühler NB, Gleitz HFE, Ronghui L, et al. Heterogeneous bone-marrow stromal progenitors drive myelofibrosis via a druggable alarmin axis. *Cell Stem Cell*. 2021;28(4):637-652.e638.

36. Bellucci S, Harousseau JL, Brice P, Tobelem G. Treatment of essential thrombocythaemia by alpha 2a interferon. *Lancet*. 1988;2(8617):960-961.

37. Silver RT. Recombinant interferon-alpha for treatment of polycythaemia vera. *Lancet*. 1988;2(8607):403.

38. Kiladjian JJ, Cassinat B, Chevret S, et al. Pegylated interferon-alfa-2a induces complete hematologic and molecular responses with low toxicity in polycythemia vera. *Blood*. 2008;112(8):3065-3072.

39. Quintás-Cardama A, Kantarjian H, Manshouri T, et al. Pegylated interferon alfa-2a yields high rates of hematologic and molecular response in patients with advanced essential thrombocythemia and polycythemia vera. *J Clin Oncol*. 2009;27(32):5418-5424.

40. Hasan S, Lacout C, Marty C, et al. JAK2V617F expression in mice amplifies early hematopoietic cells and gives them a competitive advantage that is hampered by IFNα. *Blood*. 2013;122(8):1464-1477.

41. Mullally A, Bruedigam C, Poveromo L, et al. Depletion of Jak2V617F myeloproliferative neoplasm-propagating stem cells by interferon-α in a murine model of polycythemia vera. *Blood*. 2013;121(18):3692-3702.

42. King KY, Matatall KA, Shen CC, Goodell MA, Swierczek SI, Prchal JT.

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Comparative long-term effects of interferon α and hydroxyurea on human hematopoietic progenitor cells. *Exp Hematol*. 2015;43(10):912-918.e912.

43. Mosca M, Hermange G, Tisserand A, et al. Inferring the dynamics of mutated hematopoietic stem and progenitor cells induced by IFNα in myeloproliferative neoplasms. *Blood*. 2021;138(22):2231-2243.
44. Rao TN, Hansen N, Stetka J, et al. JAK2-V617F and interferon-α induce megakaryocyte-biased stem cells characterized by decreased long-term functionality. *Blood*. 2021;137(16):2139-2151.
45. Austin RJ, Straube J, Bruedigam C, et al. Distinct effects of ruxolitinib and interferon-alpha on murine JAK2V617F myeloproliferative neoplasm hematopoietic stem cell populations. *Leukemia*. 2020;34(4):1075-1089.
46. Knudsen TA, Skov V, Stevenson K, et al. Genomic profiling of a randomized trial of interferon-α vs hydroxyurea in MPN reveals mutation-specific responses. *Blood Adv*. 2022;6(7):2107-2119.
47. Czech J, Cordua S, Weinbergerova B, et al. JAK2V617F but not CALR mutations confer increased molecular responses to interferon-α via JAK1/STAT1 activation. *Leukemia*. 2019;33(4):995-1010.
48. Mascarenhas J, Kosiorek HE, Prchal JT, et al. A randomized phase 3 trial of interferon-α vs hydroxyurea in polycythemia vera and essential thrombocythemia. *Blood*. 2022;139(19):2931-2941.
49. Gisslinger H, Klade C, Georgiev P, et al. Ropeginterferon alfa-2b versus standard therapy for polycythaemia vera (PROUD-PV and CONTINUATION-PV): a randomised, non-inferiority, phase 3 trial and its extension study. *Lancet Haematol*. 2020;7(3):e196-e208.
50. Kiladjian JJ, Klade C, Georgiev P, et al. Long-term outcomes of polycythemia vera patients treated with ropeginterferon alfa-2b. *Leukemia*. 2022;36(5):1408-1411.
51. Stadler M, Chelbi-Alix MK, Koken MH, et al. Transcriptional induction of the PML growth suppressor gene by interferons is mediated through an ISRE and a GAS element. *Oncogene*. 1995;11(12):2565-2573.
52. Dagher T, Maslah N, Edmond V, et al. JAK2V617F myeloproliferative neoplasm eradication by a novel interferon/arsenic therapy involves PML. *J Exp Med*. 2021;218(2):e20201268.
53. Saleiro D, Wen JQ, Kosciuczuk EM, et al. Discovery of a signaling feedback circuit that defines interferon responses in myeloproliferative neoplasms. *Nat Commun*. 2022;13(1):1750.
54. Sørensen AL, Mikkelsen SU, Knudsen TA, et al. Ruxolitinib and interferon-α2 combination therapy for patients with polycythemia vera or myelofibrosis: a phase II study. *Haematologica*. 2020;105(9):2262-2272.
55. Bandaranayake RM, Ungureanu D, Shan Y, Shaw DE, Silvennoinen O, Hubbard SR. Crystal structures of the JAK2 pseudokinase domain and the pathogenic mutant V617F. *Nat Struct Mol Biol*. 2012;19(8):754-759.
56. Glassman CR, Tsutsumi N, Saxton RA, Lupardus PJ, Jude KM, Garcia KC. Structure of a Janus kinase cytokine receptor complex reveals the basis for dimeric activation. *Science*. 2022;376(6589):163-169.
57. Verstovsek S, Mesa RA, Gotlib J, et al. A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. *N Engl J Med*. 2012;366(9):799-807.
58. Harrison C, Kiladjian JJ, Al-Ali HK, et al. JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis. *N Engl J Med*. 2012;366(9):787-798.
59. Vannucchi AM, Kiladjian JJ, Griesshammer M, et al. Ruxolitinib versus standard therapy for the treatment of polycythemia vera. *N Engl J Med*. 2015;372(5):426-435.
60. Zhou T, Georgeon S, Moser R, Moore DJ, Caflisch A, Hantschel O. Specificity and mechanism-of-action of the JAK2 tyrosine kinase inhibitors ruxolitinib and SAR302503 (TG101348). *Leukemia*. 2014;28(2):404-407.
61. Mascarenhas J, Hoffman R, Talpaz M, et al. Pacritinib vs best available therapy, including ruxolitinib, in patients with myelofibrosis: a randomized clinical trial. *JAMA Oncol*. 2018;4(5):652-659.
62. Singer JW, Al-Fayoumi S, Ma H, Komrokji RS, Mesa R, Verstovsek S. Comprehensive kinase profile of pacritinib, a nonmyelosuppressive Janus kinase 2 inhibitor. *J Exp Pharmacol*. 2016;8:11-19.
63. Gerds AT, Savona MR, Scott BL, et al. Determining the recommended dose of pacritinib: results from the PAC203 dose-finding trial in advanced myelofibrosis. *Blood Adv*. 2020;4(22):5825-5835.
64. Mesa RA, Vannucchi AM, Mead A, et al. Pacritinib versus best available therapy for the treatment of myelofibrosis irrespective of baseline cytopenias (PERSIST-1): an international, randomised, phase 3 trial. *Lancet Haematol*. 2017;4(5):e225-e236.
65. Ganz T. Anemia of inflammation. *N Engl J Med*. 2019;381(12):1148-1157.
66. Oh ST, Talpaz M, Gerds AT, et al. ACVR1/JAK1/JAK2 inhibitor momelotinib reverses transfusion dependency and suppresses hepcidin in myelofibrosis phase 2 trial. *Blood Adv*. 2020;4(18):4282-4291.
67. Mesa RA, Kiladjian J-J, Catalano JV, et al. SIMPLIFY-1: a phase III randomized trial of momelotinib versus ruxolitinib in Janus kinase inhibitor–naïve patients with myelofibrosis. *J Clin Oncol*. 2017;35(34):3844-3850.
68. National Comprehensive Cancer Network, Myeloproliferative Neoplasms Version 2, 2022. Accessed 11 September 2022. https://www.nccn.org/professionals/physician_gls/pdf/mpn.pdf
69. Mesa RA, Gerds AT, Vannucchi A, et al. MOMENTUM: phase 3 randomized study of momelotinib (MMB) versus danazol (DAN) in symptomatic and anemic myelofibrosis (MF) patients previously treated with a JAK inhibitor. *J Clin Oncol*. 2022;40(suppl 16):7002.
70. Pardanani A, Laborde R, Lasho T, et al. Safety and efficacy of CYT387, a JAK1 and JAK2 inhibitor, in myelofibrosis. *Leukemia*. 2013;27(6):1322-1327.
71. Guglielmelli P, Rotunno G, Bogani C, et al. Ruxolitinib is an effective treatment for CALR-positive patients with myelofibrosis. *Br J Haematol*. 2015;173(6):938-940.
72. Koppikar P, Bhagwat N, Kilpivaara O, et al. Heterodimeric JAK-STAT activation as a mechanism of persistence to JAK2 inhibitor therapy. *Nature*. 2012;489(7414):155-159.
73. Dunbar A, Bowman RL, Park Y, et al. $Jak2^{V617F}$ reversible activation shows an essential requirement for $Jak2^{V617F}$ in myeloproliferative neoplasms. *bioRxiv*. Preprint posted online 18 May 2022. https://doi.org/10.1101/2022.05.18.492332
74. Chapeau EA, Mandon E, Gill J, et al. A conditional inducible JAK2V617F transgenic mouse model reveals myeloproliferative disease that is reversible upon switching off transgene expression. *PLoS One*. 2019;14(10):e0221635.
75. Meyer SC, Keller MD, Chiu S, et al. CHZ868, a type II JAK2 inhibitor, reverses type I JAK inhibitor persistence and demonstrates efficacy in myeloproliferative neoplasms. *Cancer Cell*. 2015;28(1):15-28.
76. Rai S, Stetka J, Usart M, et al. The second generation type II JAK2 inhibitor, AJ1-10502, demonstrates enhanced selectivity, improved therapeutic efficacy and reduced mutant cell fraction compared to type I JAK2 inhibitors in models of myeloproliferative neoplasms (MPNs) [abstract]. *Blood*. 2022;140(suppl 1). Abstract 2992.
77. Yang J, Weisberg EL, Liu X, et al. Small molecule inhibition of deubiquitinating enzyme JOSD1 as a novel targeted therapy for leukemias with mutant JAK2. *Leukemia*. 2022;36(1):210-220.
78. Chang Y, Min J, Jarusiewicz JA, et al. Degradation of Janus kinases in CRLF2-rearranged acute lymphoblastic leukemia. *Blood*. 2021;138(23):2313-2326.
79. Pardanani A, Harrison C, Cortes JE, et al. Safety and efficacy of fedratinib in patients with primary or secondary myelofibrosis: a randomized clinical trial. *JAMA Oncol*. 2015;1(5):643-651.
80. Mascarenhas J, Kosiorek HE, Bhave R, et al. Treatment of myelofibrosis patients with the TGF-β 1/3 inhibitor AVID200 (MPN-RC 118)

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induces a profound effect on platelet production. *Blood*. 2021;138:142.

81. Gill H, Yacoub A, Pettit KM, et al. A phase 2 study of the LSD1 inhibitor Img-7289 (bomedemstat) for the treatment of advanced myelofibrosis [abstract]. *Blood*. 2021;138(suppl 1). Abstract 139.

82. Mascarenhas J, Komrokji RS, Palandri F, et al. Randomized, single-blind, multicenter phase II study of two doses of imetelstat in relapsed or refractory myelofibrosis. *J Clin Oncol*. 2021;39(26):2881-2892.

83. Vachani P, Lange A, Delgado RG, et al. Potential disease-modifying activity of navtemadlin (KRT-232), a first-in-class MDM2 inhibitor, correlates with clinical benefits in relapsed/refractory myelofibrosis (MF) [abstract]. *Blood*. 2021;138. Abstract 3581.

84. Harrison CN, Garcia JS, Somervaille TCP, et al. Addition of navitoclax to ongoing ruxolitinib therapy for patients with myelofibrosis with progression or suboptimal response: phase II safety and efficacy. *J Clin Oncol*. 2022;40(15): 1671-1680.

85. Yacoub A, Wang ES, Rampal RK, et al. Abstract CT162: Addition of parsaclisib (INCB050465), a PI3Kδ inhibitor, in patients with suboptimal response to ruxolitinib: A phase 2 study in patients with myelofibrosis. *Clin Trials*. 2021;81(suppl 13):CT162.

86. Kremyanskaya M, Mascarenhas J, Palandri F, et al. Pelabresib (CPI-0610) monotherapy in patients with myelofibrosis-update of clinical and translational data from the ongoing manifest trial [abstract]. *Blood*. 2021; 138(suppl 1). Abstract 141.

87. Mascarenhas J, Kremyanskaya M, Patriarca A, et al. S198: BET inhibitor pelabresib (CPI-0610) combined with ruxolitinib in patients with myelofibrosis—JAK inhibitor-naïve or with suboptimal response to ruxolitinib—preliminary data from the manifest study. *HemaSphere*. 2022;6:99-100.

88. Tantravahi SK, Kim SJ, Sundar D, et al. A phase 2 study to evaluate the efficacy and safety of selinexor in patients with myelofibrosis refractory or intolerant to JAK inhibitors [abstract]. *Blood*. 2021;138(suppl 1). Abstract 143.

89. Yacoub A, Patnaik MM, Ali H, et al. A phase 1/2 study of single agent tagraxofusp, a firstin- class CD123-targeted therapy, in patients with myelofibrosis that is relapsed/refractory following JAK inhibitor therapy [abstract]. *Blood*. 2021;138(suppl 1). Abstract 140.

90. Hoffman R, Ginzburg Y, Kremyanskaya M, et al. Rusfertide (PTG-300) treatment in phlebotomy-dependent polycythemia vera patients. *J Clin Oncol*. 2022;40(suppl 16):7003.

91. Ginzburg Y, Kirubamoorthy K, Salleh S, et al. Rusfertide (PTG-300) induction therapy rapidly achieves hematocrit control in polycythemia vera patients without the need for therapeutic phlebotomy [abstract]. *Blood*. 2021;138(suppl 1). Abstract 90.

92. Palandri F, Ross D, Cochrane T, et al. P1033: a phase 2 study of the LSD1 inhibitor IMG-7289 (bomedemstat) for the treatment of essential thrombocythemia (ET). *HemaSphere*. 2022;6:923-924.

93. Bogani C, Bartalucci N, Martinelli S, et al. mTOR inhibitors alone and in combination with JAK2 inhibitors effectively inhibit cells of myeloproliferative neoplasms. *PLoS One*. 2013;8(1):e54826.

94. Stivala S, Codilupi T, Brkic S, et al. Targeting compensatory MEK/ERK activation increases JAK inhibitor efficacy in myeloproliferative neoplasms. *J Clin Invest*. 2019;129(4):1596-1611.

95. Brkic S, Stivala S, Santopolo A, et al. Dual targeting of JAK2 and ERK interferes with the myeloproliferative neoplasm clone and enhances therapeutic efficacy. *Leukemia*. 2021;35(10):2875-2884.

96. Leverson JD, Phillips DC, Mitten MJ, et al. Exploiting selective BCL-2 family inhibitors to dissect cell survival dependencies and define improved strategies for cancer therapy. *Sci Transl Med*. 2015;7(279): 279ra240.

97. Winter PS, Sarosiek KA, Lin KH, et al. RAS signaling promotes resistance to JAK inhibitors by suppressing BAD-mediated apoptosis. *Sci Signal*. 2014;7(357):ra122.

98. Waibel M, Solomon VS, Knight DA, et al. Combined targeting of JAK2 and Bcl-2/Bcl-xL to cure mutant JAK2-driven malignancies and overcome acquired resistance to JAK2 inhibitors. *Cell Rep*. 2013;5(4):1047-1059.

99. Guo J, Roberts L, Chen Z, Merta PJ, Glaser KB, Shah OJ. JAK2V617F drives Mcl-1 expression and sensitizes hematologic cell lines to dual inhibition of JAK2 and Bcl-xL. *PLoS One*. 2015;10(3):e0114363.

100. Pemmaraju N, Garcia JS, Potluri J, et al. Addition of navitoclax to ongoing ruxolitinib treatment in patients with myelofibrosis (REFINE): a post-hoc analysis of molecular biomarkers in a phase 2 study. *Lancet Haematol*. 2022;9(6):e434-e444.

101. Tefferi A, Guglielmelli P, Lasho TL, et al. MIPSS70+ version 2.0: Mutation and Karyotype-Enhanced International Prognostic Scoring System for primary myelofibrosis. *J Clin Oncol*. 2018;36(17): 1769-1770.

102. Patel KP, Newberry KJ, Luthra R, et al. Correlation of mutation profile and response in patients with myelofibrosis treated with ruxolitinib. *Blood*. 2015;126(6):790-797.

103. Spiegel JY, McNamara C, Kennedy JA, et al. Impact of genomic alterations on outcomes in myelofibrosis patients undergoing JAK1/2 inhibitor therapy. *Blood Adv*. 2017;1(20): 1729-1738.

104. Kleppe M, Koche R, Zou L, et al. Dual targeting of oncogenic activation and inflammatory signaling increases therapeutic efficacy in myeloproliferative neoplasms. *Cancer Cell*. 2018;33(1):29-43.e27.

105. Sashida G, Wang C, Tomioka T, et al. The loss of Ezh2 drives the pathogenesis of myelofibrosis and sensitizes tumor-initiating cells to bromodomain inhibition. *J Exp Med*. 2016;213(8):1459-1477.

106. Yang H, Kurtenbach S, Guo Y, et al. Gain of function of ASXL1 truncating protein in the pathogenesis of myeloid malignancies. *Blood*. 2018;131(3):328-341.

107. Whyte WA, Bilodeau S, Orlando DA, et al. Enhancer decommissioning by LSD1 during embryonic stem cell differentiation. *Nature*. 2012;482(7384):221-225.

108. Lara-Astiaso D, Weiner A, Lorenzo-Vivas E, et al. Immunogenetics. chromatin state dynamics during blood formation. *Science*. 2014;345(6199):943-949.

109. Sprüssel A, Schulte JH, Weber S, et al. Lysine-specific demethylase 1 restricts hematopoietic progenitor proliferation and is essential for terminal differentiation. *Leukemia*. 2012;26(9):2039-2051.

110. Niebel D, Kirfel J, Janzen V, Höller T, Majores M, Gütgemann I. Lysine-specific demethylase 1 (LSD1) in hematopoietic and lymphoid neoplasms. *Blood*. 2014;124(1): 151-152.

111. Jutzi JS, Kleppe M, Dias J, et al. LSD1 inhibition prolongs survival in mouse models of MPN by selectively targeting the disease clone. *Hemasphere*. 2018;2(3):e54.

112. Palandri F, Vianelli N, Ross DM, et al. A phase 2 study of the LSD1 inhibitor Img-7289 (bomedemstat) for the treatment of essential thrombocythemia (ET) [abstract]. *Blood*. 2021;138(suppl 1). Abstract 386.

113. Cusan M, Cai SF, Mohammad HP, et al. LSD1 inhibition exerts its antileukemic effect by recommissioning PU.1- and C/EBPα-dependent enhancers in AML. *Blood*. 2018; 131(15):1730-1742.

114. Maiques-Diaz A, Spencer GJ, Lynch JT, et al. Enhancer activation by pharmacologic displacement of LSD1 from GFI1 induces differentiation in acute myeloid leukemia. *Cell Rep*. 2018;22(13):3641-3659.

115. Vinyard ME, Su C, Siegenfeld AP, et al. CRISPR-suppressor scanning reveals a nonenzymatic role of LSD1 in AML. *Nat Chem Biol*. 2019;15(5):529-539.

116. Vassilev LT, Vu BT, Graves B, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science*. 2004;303(5659):844-848.

117. Iancu-Rubin C, Mosoyan G, Parker CC, Eng K, Hoffman R. Imetelstat (GRN163L), a telomerase inhibitor selectively affects malignant megakaryopoiesis in myeloproliferative neoplasms (MPN) [abstract]. *Blood*. 2014;124(21). Abstract 4582.

118. Uras IZ, Maurer B, Nivarthi H, et al. CDK6 coordinates JAK2 (V617F) mutant MPN via

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NF-κB and apoptotic networks. *Blood*. 2019;133(15):1677-1690.

119. Dutta A, Nath D, Yang Y, Le BT, Mohi G. CDK6 is a therapeutic target in myelofibrosis. *Cancer Res*. 2021;81(16):4332-4345.

120. Rampal RK, Pinzon-Ortiz M, Somasundara AVH, et al. Therapeutic efficacy of combined JAK1/2, Pan-PIM, and CDK4/6 inhibition in myeloproliferative neoplasms. *Clin Cancer Res*. 2021;27(12):3456-3468.

121. Ginzburg YZ, Feola M, Zimran E, Varkonyi J, Ganz T, Hoffman R. Dysregulated iron metabolism in polycythemia vera: etiology and consequences. *Leukemia*. 2018;32(10):2105-2116.

122. Casu C, Oikonomidou PR, Chen H, et al. Minihepcidin peptides as disease modifiers in mice affected by β-thalassemia and polycythemia vera. *Blood*. 2016;128(2):265-276.

123. Klampfl T, Gisslinger H, Harutyunyan AS, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med*. 2013;369(25):2379-2390.

124. How J, Hobbs GS, Mullally A. Mutant calreticulin in myeloproliferative neoplasms. *Blood*. 2019;134(25):2242-2248.

125. Elf S, Abdelfattah NS, Chen E, et al. Mutant calreticulin requires both its mutant C-terminus and the thrombopoietin receptor for oncogenic transformation. *Cancer Discov*. 2016;6(4):368-381.

126. Araki M, Yang Y, Masubuchi N, et al. Activation of the thrombopoietin receptor by mutant calreticulin in CALR-mutant myeloproliferative neoplasms. *Blood*. 2016;127(10):1307-1316.

127. Chachoua I, Pecquet C, El-Khoury M, et al. Thrombopoietin receptor activation by myeloproliferative neoplasm associated calreticulin mutants. *Blood*. 2016;127(10):1325-1335.

128. Araki M, Yang Y, Imai M, et al. Homomultimerization of mutant calreticulin is a prerequisite for MPL binding and activation. *Leukemia*. 2019;33(1):122-131.

129. Rivera JF, Baral AJ, Nadat F, et al. Zinc-dependent multimerization of mutant calreticulin is required for MPL binding and MPN pathogenesis. *Blood Adv*. 2021;5(7):1922-1932.

130. Elf S, Abdelfattah NS, Baral AJ, et al. Defining the requirements for the pathogenic interaction between mutant calreticulin and MPL in MPN. *Blood*. 2018;131(7):782-786.

131. Pecquet C, Chachoua I, Roy A, et al. Calreticulin mutants as oncogenic rogue chaperones for TpoR and traffic-defective pathogenic TpoR mutants. *Blood*. 2019;133(25):2669-2681.

132. Jutzi JS, Marneth AE, Ciboddo M, et al. Whole-genome CRISPR screening identifies N-glycosylation as a genetic and therapeutic vulnerability in CALR-mutant MPN. *Blood*. 2022;140(11):1291-1304.

133. Pronier E, Cifani P, Merlinsky TR, et al. Targeting the CALR interactome in myeloproliferative neoplasms. *JCI Insight*. 2018;3(22):e122703.

134. Ibarra J, Elbanna YA, Kurylowicz K, et al. Type I but not type II calreticulin mutations activate the IRE1α/XBP1 pathway of the unfolded protein response to drive myeloproliferative neoplasms. *Blood Cancer Discov*. 2022;3(4):298-315.

135. Jutzi JS, Marneth AE, Jiménez-Santos MJ, et al. CALR-mutated cells are vulnerable to combined inhibition of the proteasome and the endoplasmic reticulum stress response. *Leukemia*. 2023;37(2):359-369.

136. Tvorogov D, Thompson-Peach CAL, Foßelteder J, et al. Targeting human CALR-mutated MPN progenitors with a neoepitope-directed monoclonal antibody. *EMBO Rep*. 2022;23(4):e52904.

137. Achyutuni S, Nivarthi H, Majoros A, et al. Hematopoietic expression of a chimeric murine-human CALR oncoprotein allows the assessment of anti-CALR antibody immunotherapies in vivo. *Am J Hematol*. 2021;96(6):698-707.

138. Kihara Y, Araki M, Imai M, et al. Therapeutic potential of an antibody targeting the cleaved form of mutant calreticulin in myeloproliferative neoplasms [abstract]. *Blood*. 2020;36(suppl 1). Abstract 716.

139. Reis E, Buonpane R, Celik H, et al. Discovery of INCA033989, a monoclonal antibody that selectively antagonizes mutant calreticulin oncogenic function in myeloproliferative neoplasms (MPNs) [abstract]. *Blood*. 2022;140(suppl 1). Abstract 6.

140. Gigoux M, Holmström MO, Zappasodi R, et al. Calreticulin mutant myeloproliferative neoplasms induce MHC-I skewing, which can be overcome by an optimized peptide cancer vaccine. *Sci Transl Med*. 2022;14(649):eaba4380.

141. Holmström MO, Ahmad SM, Klausen U, et al. High frequencies of circulating memory T cells specific for calreticulin exon 9 mutations in healthy individuals. *Blood Cancer J*. 2019;9(2):8.

142. Cimen Bozkus C, Roudko V, Finnigan JP, et al. Immune checkpoint blockade enhances shared neoantigen-induced T-cell immunity directed against mutated calreticulin in myeloproliferative neoplasms. *Cancer Discov*. 2019;9(9):1192-1207.

143. Hobbs G, Cimen Bozkus C, Moshier E, et al. PD-1 inhibition in advanced myeloproliferative neoplasms. *Blood Adv*. 2021;5(23):5086-5097.

144. Tubb VM, Schrikkema DS, Croft NP, et al. Isolation of T cell receptors targeting recurrent neoantigens in hematological malignancies. *J Immunother Cancer*. 2018;6(1):70.

145. Handlos Grauslund J, Holmström MO, Jørgensen NG, et al. Therapeutic cancer vaccination with a peptide derived from the calreticulin exon 9 mutations induces strong cellular immune responses in patients with CALR-mutant chronic myeloproliferative neoplasms. *Front Oncol*. 2021;11:637420.

146. Fisher DAC, Malkova O, Engle EK, et al. Mass cytometry analysis reveals hyperactive NF Kappa B signaling in myelofibrosis and secondary acute myeloid leukemia. *Leukemia*. 2017;31(9):1962-1974.

147. Vining KH, Marneth AE, Adu-Berchie K, et al. Mechanical checkpoint regulates monocyte differentiation in fibrotic niches. *Nat Mater*. 2022;21(8):939-950.

148. Schneider RK, Mullally A, Dugourd A, et al. Gli1(+) mesenchymal stromal cells are a key driver of bone marrow fibrosis and an important cellular therapeutic target. *Cell Stem Cell*. 2017;20(6):785-800.e788.

149. Yao JC, Oetjen KA, Wang T, et al. TGF-β signaling in myeloproliferative neoplasms contributes to myelofibrosis without disrupting the hematopoietic niche. *J Clin Invest*. 2022;132(11):e154092.

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REVIEW

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Antibody targeting of mutant calreticulin in myeloproliferative neoplasms

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Abstract

Mutations in calreticulin are one of the key disease-initiating mutations in myeloproliferative neoplasms (MPN). In MPN, mutant calreticulin translates with a novel C-terminus that leads to aberrant binding to the extracellular domain of the thrombopoietin receptor, MPL. This cell surface neoantigen has become an attractive target for immunological intervention. Here, we summarize recent advances in the development of mutant calreticulin targeting antibodies as a novel therapeutic approach in MPN.

1 | INTRODUCTION

Myeloproliferative neoplasms (MPN) are malignant haematological disorders that are caused by clonal proliferation of haematopoietic stem cells (HSC) in the bone marrow. There are three classical, BCR-ABL-negative MPN: essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF), which present with distinct clinical features. Whereas ET and PV are characterized by platelet and erythrocyte overproduction, respectively, PMF is marked by aberrant proliferation of cells of the megakaryocytic lineage and progressive bone marrow fibrosis.[1] All three classical, BCR-ABL-negative MPN are initiated by acquired, somatic mutations in HSC. In more than 90% of MPN patients, one of three driver mutations in *JAK2*, *CALR* or the thrombopoietin receptor *MPL* are present.[2–8] Mutations in *CALR* are responsible for 20%–30% of MPN cases and, like the other driver mutations, lead to the constitutive activation of Janus kinase–signal transducer and activator of transcription (JAK–STAT) signalling.[9]

2 | PATHOGENIC MECHANISMS OF MUTANT CALRETICULIN IN MPN

The majority of MPN driver mutations in *CALR* are caused by two mutations in exon 9 of the *CALR* gene, typically occurring in a heterozygous manner: (i) a 52 bp deletion (type I mutation), present in approximately 50% *CALR*-mutant patients, and (ii) a 5 bp insertion (type II mutation), present in approximately 30% *CALR*-mutant patients. The majority of the remaining *CALR* mutations are classified as type I-like or type II-like, with these categories defined on the basis of the deletion of stretches of negatively charged amino acids in the wild-type calreticulin C-terminus.[5,6] All MPN-associated

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CALR mutations lead to a +1 bp frameshift that results in translation of an altered C-terminus of the calreticulin protein. The novel C-terminus is lacking the endoplasmic reticulum (ER) retention signal (KDEL domain) and primarily consists of positively charged amino acids. Since its discovery in 2013, the mechanisms by which mutant calreticulin causes MPN were elucidated by several groups. Mutant calreticulin forms homomultimers via its novel C-terminus and acquires a pathogenic binding interaction with MPL in the ER.[10] Lacking the KDEL domain, the mutant calreticulin-MPL complex is shuttled to the cell surface.[11–13] Mutant calreticulin stabilizes trans-membranous MPL, resulting in ligand-independent activation of MPL and downstream JAK–STAT signalling pathway activation.[11,13–17] Consequently, MPL expression is required for cell transformation by mutant calreticulin.[11,12,16] For MPL binding, mutant calreticulin requires the positive electrostatic charge of the novel C-terminus, as well as the lectin-dependent function.[11,13,14] Recent work has provided detailed insights into the protein conformation of mutant calreticulin and the formation of a tetrameric mutant calreticulin-MPL complex, resulting in MPL dimerization and activation.[18] Enhanced accessibility of the N-terminal N-glycan binding domain of mutant calreticulin facilitates binding to the extracellular domain of an immature, partially glycosylated MPL, while the mutant C-terminus of mutant calreticulin also interacts with MPL via acidic patches (e.g. TFED, PDQEE and WEEP) in the extracellular domain of MPL.[18] Cell intrinsically, mutant calreticulin promotes megakaryocytic differentiation through MPL activation, which is consistent with the fact that *CALR* mutations engender MPN with a megakaryocytic lineage phenotype (i.e. ET and PMF).[19] Several groups have shown that wild-type as well as mutant calreticulin are secreted, and become damage-associated molecular patterns exhibiting immunomodulatory functions.[20,21] Whereas secretion of wild-type calreticulin is mainly considered to be a reaction to ER stress,[22] other studies indicate that in MPN, secretion of mutant calreticulin is facilitated by the lack of the KDEL domain. Interestingly, the majority of soluble mutant calreticulin detectable in the plasma has been found to be secreted from non-MPL-expressing cells in MPN.[23] Circulating mutant calreticulin has been shown to have an immunosuppressive role, for example, by reducing phagocytosis mediated by CD11c$^+$ bone marrow-derived dendritic cells.[24]

3 | CURRENT TREATMENT OPTIONS FOR *CALR*-MUTANT MPN

Treatment of MPN relies on cytoreductive agents, including hydroxyurea, pegylated interferons, as well as the JAK1/2 inhibitor, ruxolitinib. Cytoreductive therapy can reduce blood counts, thrombotic risk, splenomegaly and improve symptoms.[25,26] Despite these benefits, none of the current medical treatments for MPN eliminate the disease-initiating *CALR*-mutant HSC clone. Over time, patients develop resistance to JAK2 inhibition, further limiting the efficacy of ruxolitinib. To date, the only curative treatment for *CALR*-mutant MPN remains allogeneic stem cell transplantation, a procedure associated with substantial morbidity, in addition to a mortality risk. With the presentation of a neoantigen, the development of therapeutic antibodies targeting the novel cell surface mutant calreticulin C-terminus has become a strategy of great interest for the inhibition of pathogenic MPL activation.

4 | TARGETED THERAPY USING MUTANT CALRETICULIN TARGETING MONOCLONAL ANTIBODIES

The development of therapeutic, monoclonal antibodies (mAbs) for cancer therapy has been successful, broadly speaking (e.g. targeting CD20 in non-Hodgkin lymphoma). The progress in unravelling the structural properties and the mechanisms of the pathogenic interaction of mutant calreticulin with MPL provides the understanding needed to translate this knowledge into efficacious treatment. Recent research efforts have therefore centered on immunotherapeutic approaches with the goal of targeting the mutant calreticulin neoepitope while sparing normal haematopoiesis. In 2020, Kihara et al. reported (in abstract form) the generation of the mouse chimeric monoclonal antibody B3, specifically targeting mutant calreticulin.[27] In a *CALR*^{del52} ET mouse model, treatment with B3 reduced platelets in the peripheral blood and numbers of megakaryocytes in the bone marrow of the mice. Soon after, Achyutuni and colleagues generated a murine IgG2a raised against the human calreticulin neoantigen and treated homozygous *CALR*^{del52} transgenic mice.[28] Although treatment with the antibody was only for 2.5 days (5 doses total), the platelet count rapidly dropped before rising again 24 h after completion of treatment.[28] In 2022, Mughal et al. generated and characterized eight peptide antibodies recognizing mutant calreticulin. This study provides important information on essential sites within mutant calreticulin epitopes, however these antibodies have yet to be tested in pre-clinical models.[29] In the same year, Tvorogov et al. reported the development of the monoclonal antibody 4D7, targeting mutant calreticulin on the cell surface.[30] 4D7 was generated using a synthetic peptide corresponding to the novel C-terminus of mutant calreticulin using a hybridoma approach. The antibody is directed against the common C-terminus of both Type I and Type II *CALR* mutations, and it effectively blocked binding of mutant calreticulin to MPL, abrogating aberrant JAK–STAT activation. Tvorogov and colleagues also showed that 4D7 inhibited TPO-independent megakaryocyte differentiation in patient-derived *CALR*-mutant CD34$^+$ cells. Treatment with 4D7 did not show any inhibitory effect on in vitro haematopoiesis in non-mutated cells. The authors further showed that 4D7 has efficacy on ruxolitinib resistant cells in vitro, suggesting that treatment with 4D7 might be a promising therapeutic approach for patients with an acquired ruxolitinib resistance. 4D7 showed beneficial effects on survival in cell line xenograft models, both in calreticulin-mutant as well as ruxolitinib-resistant cells. The antibody has yet to be tested in

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patient-derived xenograft models or in genetic MPN mouse models. Another milestone in the development of mutant calreticulin-targeting antibodies was the generation of the human IgG1 mAb INCA033989, introduced by Reis et al. in a plenary abstract at the 2022 American Society of Haematology (ASH) meeting.[31] Based on the data presented in the abstract (currently unpublished), INCA033989 inhibited mutant calreticulin-induced MPL signalling in murine Ba/F3 cells, but showed no effects on non-mutated cells. Reis et al. also reported enhanced efficacy of INCA033989-ruxolitinib combination in mouse cells in vitro. In patient-derived $CD34^+$ cells, treatment with INCA033989 inhibited JAK–STAT signalling and proliferation of progenitor cells, an effect not observed in non-mutated or *JAK2*-mutant cells. In competitive transplant calreticulin-mutant mouse models, 10 weeks of treatment with INCA033989 prevented thrombocytosis and significantly decreased the numbers of *CALR*-mutant stem and progenitor cells, as well as megakaryocytes in the bone marrow, without affecting cellularity of wild-type mice. Secondary transplantation did not result in development of MPN in mice, suggesting that treatment with INCA033989 successfully targeted the *CALR*-mutant MPN disease-propagating HSC. The expectation is that INCA033989 will enter Phase 1 clinical trials in patients with *CALR*-mutant MPN in 2023 [verbal communication, Dr. Reis, ASH plenary presentation 2022].

5 | FUTURE DIRECTIONS

Research in the last few years has shown immense progress in developing a specific, mutant calreticulin targeting treatment approach in MPN. Of note, other promising immunological approaches, such as mutant calreticulin peptide vaccination and T cell-directed targeting have been investigated and reviewed elsewhere,[32,33] and are therefore not the primary focus of this review. To date, peptide vaccination targeting the mutant calreticulin neoantigen is the immune therapy approach that has advanced the furthest clinically. One Phase 1 vaccine trial (NCT03566446), using a 36 amino acid peptide vaccine spanning the novel mutant calreticulin C terminus, has been completed and the vaccine was found to be safe and tolerable.[34] While 8/10 patients with MPN who received the peptide vaccine showed evidence of T-cell responses, no patient demonstrated a clinical response.[34] There are several potential reasons why a mutant calreticulin-directed peptide vaccine might not induce an immune response in patients, including (i) the patients who received the vaccine may not have expressed human leukocyte antigen (HLA) subtypes that present the mutant calreticulin neo-epitope with high affinity, (ii) defects in major histocompatibility complex (MHC)-mediated presentation of the mutant calreticulin neo-epitope, (iii) inadequate immune stimulation by the adjuvant and (iv) an immunosuppressive microenvironment in the context of MPN-related chronic inflammation. Two other mutant calreticulin vaccine trials (NCT05444530 and NCT05025488) are currently open, with some differences in their approaches compared to the published trial and results from these ongoing studies are eagerly awaited.

Targeting mutant calreticulin with a cell-surface blocking mAb may circumvent some of the challenges of peptide vaccination. Recent advances in the development of mutant calreticulin targeting mAb have taken advantage of the blocking properties of the antibody binding to the cell surface neoantigen, preventing MPL dimerization, activation and thus abrogating aberrant activation of JAK–STAT signalling. INCA033989, which has been tested in pre-clinical models by Reis et al., showed efficacy as an Fc-silent IgG1, and the abrogation of JAK–STAT signalling indicates successful inhibition of MPL activation. Based on its mechanism of action, INCA033989 is expected to inhibit proliferation of *CALR*-mutant cells, however it remains to be determined if treatment with INCA033989 will preferentially target the mutant *CALR* clone in patients to achieve molecular responses and/or remissions. While the lack of the Fc domain reduces toxicity, it also precludes Fc-mediated cell death that could improve therapeutic potency of the antibody. Future efforts could therefore be aimed at optimizing treatment efficacy by Fc engineering to enable Fcγ receptor-mediated antibody-dependent cellular cytotoxicity or phagocytosis. Additional approaches to enhance the efficacy of the mutant calreticulin mAb may include the development of antibody-drug conjugates, and/or integrating the antibody into a chimeric antigen receptor (CAR)-T construct. CAR T therapy, a significant advance in oncology immunotherapy, has been successfully used in ALL and lymphoma.[35,36] A constraint of targeting mutant calreticulin using therapeutic antibodies could be circulating mutant calreticulin, potentially acting as decoy for antibody binding, thus reducing the availability of the mutant calreticulin mAb to bind on the cell surface. Even though it might be advantageous to prevent secreted mutant calreticulin from (i) binding extracellularly to the mutant calreticulin-MPL complex on the cell surface and (ii) mediating its cell-extrinsic effects as an immunosuppressor, the potential therapeutic benefit of blocking secreted mutant calreticulin is currently unclear. Developing a bispecific antibody, targeting both mutant calreticulin and MPL in the complex, could enhance specificity by selectively targeting the protein complex, and preventing secreted mutant calreticulin from functioning as a decoy. A bispecific approach could also be applied to allow concomitant binding of mutant calreticulin expressing MPN cells and T cells. This approach has been successfully used to target CD19 in acute lymphoblastic leukaemia (ALL) through engaging CD3 on T-cells.[37]

6 | CONCLUSION

MPN are a group of chronic blood cancers that have an unmet need for treatment options which eliminate the disease-propagating clone. Recent advances in identifying the mechanisms by which mutant calreticulin causes MPN paved the path for immunological targeting of *CALR*-mutant MPN cells, and specific mutant calreticulin

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targeting mAbs have been developed and found to be efficacious in preclinical mouse models. The safety and efficacy of these novel antibodies have yet to be evaluated in MPN patients.

AUTHOR CONTRIBUTIONS

Frederike Kramer: Conceptualization (lead); writing – original draft (lead). **Ann Mullally:** Funding acquisition (lead); supervision (lead); writing – review and editing (lead).

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DATA AVAILABILITY STATEMENT

Data sharing not applicable - no new data generated, or the article describes entirely theoretical research.

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REFERENCES

1. Campbell PJ, Green AR. The myeloproliferative disorders. *N Engl J Med*. 2006;355(23):2452-2466.
2. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet*. 2005;365(9464):1054-1061.
3. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med*. 2005;352(17):1779-1790.
4. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell*. 2005;7(4):387-397.
5. Nangalia J, Massie CE, Baxter EJ, et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med*. 2013;369(25):2391-2405.
6. Klampfl T, Gisslinger H, Harutyunyan AS, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med*. 2013;369(25):2379-2390.
7. Pikman Y, Lee BH, Mercher T, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Med*. 2006;3(7):e270.
8. James C, Ugo V, Le Couedic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*. 2005;434(7037):1144-1148.
9. Kleppe M, Kwak M, Koppikar P, et al. JAK-STAT pathway activation in malignant and nonmalignant cells contributes to MPN pathogenesis and therapeutic response. *Cancer Discov*. 2015;5(3):316-331.
10. Araki M, Yang Y, Imai M, et al. Homomultimerization of mutant calreticulin is a prerequisite for MPL binding and activation. *Leukemia*. 2019;33(1):122-131.
11. Elf S, Abdelfattah NS, Chen E, et al. Mutant calreticulin requires both its mutant C-terminus and the thrombopoietin receptor for oncogenic transformation. *Cancer Discov*. 2016;6(4):368-381.
12. Araki M, Yang Y, Masubuchi N, et al. Activation of the thrombopoietin receptor by mutant calreticulin in CALR-mutant myeloproliferative neoplasms. *Blood*. 2016;127(10):1307-1316.
13. Chachoua I, Pecquet C, El-Khoury M, et al. Thrombopoietin receptor activation by myeloproliferative neoplasm associated calreticulin mutants. *Blood*. 2016;127(10):1325-1335.
14. Elf S, Abdelfattah NS, Baral AJ, et al. Defining the requirements for the pathogenic interaction between mutant calreticulin and MPL in MPN. *Blood*. 2018;131(7):782-786.
15. Lau WW, Hannah R, Green AR, Gottgens B. The JAK-STAT signaling pathway is differentially activated in CALR-positive compared with JAK2V617F-positive ET patients. *Blood*. 2015;125(10):1679-1681.
16. Marty C, Pecquet C, Nivarthi H, et al. Calreticulin mutants in mice induce an MPL-dependent thrombocytosis with frequent progression to myelofibrosis. *Blood*. 2016;127(10):1317-1324.
17. Nivarthi H, Chen D, Cleary C, et al. Thrombopoietin receptor is required for the oncogenic function of CALR mutants. *Leukemia*. 2016;30(8):1759-1763.
18. Papadopoulos N, Nedelec A, Derenne A, et al. Oncogenic CALR mutant C-terminus mediates dual binding to the thrombopoietin receptor triggering complex dimerization and activation. *Nat Commun*. 2023;14(1):1881.
19. Kollmann K, Warsch W, Gonzalez-Arias C, et al. A novel signalling screen demonstrates that CALR mutations activate essential MAPK signalling and facilitate megakaryocyte differentiation. *Leukemia*. 2017;31(4):934-944.
20. Han L, Schubert C, Kohler J, et al. Calreticulin-mutant proteins induce megakaryocytic signaling to transform hematopoietic cells and undergo accelerated degradation and Golgi-mediated secretion. *J Hematol Oncol*. 2016;9(1):45.
21. Sollazzo D, Forte D, Polverelli N, et al. Circulating calreticulin is increased in myelofibrosis: correlation with Interleukin-6 plasma levels, bone marrow fibrosis, and splenomegaly. *Mediators Inflamm*. 2016;2016:5860657.
22. Wiersma VR, Michalak M, Abdullah TM, Bremer E, Eggleton P. Mechanisms of translocation of ER chaperones to the cell surface and immunomodulatory roles in cancer and autoimmunity. *Front Oncol*. 2015;5:7.
23. Pecquet C, Papadopoulos N, Balligand T, et al. Secreted mutant calreticulins as rogue cytokines in myeloproliferative neoplasms. *Blood*. 2023;141(8):917-929.
24. Liu P, Zhao L, Loos F, et al. Immunosuppression by mutated calreticulin released from malignant cells. *Mol Cell*. 2020;77(4):748-760 e749.
25. Verger E, Cassinat B, Chauveau A, et al. Clinical and molecular response to interferon-alpha therapy in essential thrombocythemia patients with CALR mutations. *Blood*. 2015;126(24):2585-2591.
26. Guglielmelli P, Rotunno G, Bogani C, et al. Ruxolitinib is an effective treatment for CALR-positive patients with myelofibrosis. *Br J Haematol*. 2016;173(6):938-940.
27. Kihara YAM, Imai M, Mori Y, et al. Therapeutic potential of an antibody targeting the cleaved form of mutant calreticulin in myeloproliferative neoplasms. *Blood*. 2020;136(Supplement 1):9-10. doi:10.1182/blood-2020-141159
28. Achyutuni S, Nivarthi H, Majoros A, et al. Hematopoietic expression of a chimeric murine-human CALR oncoprotein allows the assessment of anti-CALR antibody immunotherapies in vivo. *Am J Hematol*. 2021;96(6):698-707.

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29. Mughal FP, Bergmann AC, Huynh HUB, et al. Production and characterization of peptide antibodies to the C-terminal of frameshifted calreticulin associated with myeloproliferative diseases. *Int J Mol Sci*. 2022;23(12):6803.
30. Tvorogov D, Thompson-Peach CAL, Fosselteder J, et al. Targeting human CALR-mutated MPN progenitors with a neoepitope-directed monoclonal antibody. *EMBO Rep*. 2022;23(4):e52904.
31. Reis EBR, Celik H, Marty C, et al. Discovery of INCA033989, a monoclonal antibody that selectively antagonizes mutant calreticulin oncogenic function in myeloproliferative neoplasms. *Blood*. 2022;140(Supplement 1):14-15. doi: 10.1182/blood-2022-159435
32. How J, Garcia JS, Mullally A. Biology and therapeutic targeting of molecular mechanisms in MPNs. *Blood*. 2023;141(16):1922-1933.
33. Holmstrom MO, Hasselbalch HC, Andersen MH. Cancer immune therapy for Philadelphia chromosome-negative chronic myeloproliferative neoplasms. *Cancers (Basel)*. 2020;12(7):1763.
34. Handlos Grauslund J, Holmstrom MO, Jorgensen NG, et al. Therapeutic cancer vaccination with a peptide derived from the calreticulin exon 9 mutations induces strong cellular immune responses in patients with CALR-mutant chronic myeloproliferative neoplasms. *Front Oncol*. 2021;11:637420.
35. Park JH, Riviere I, Gonen M, et al. Long-term follow-up of CD19 CAR therapy in acute lymphoblastic leukemia. *N Engl J Med*. 2018;378(5):449-459.
36. Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *N Engl J Med*. 2017;377(26):2531-2544.
37. Ribera JM. Efficacy and safety of bispecific T-cell engager blinatumomab and the potential to improve leukemia-free survival in B-cell acute lymphoblastic leukemia. *Expert Rev Hematol*. 2017;10(12):1057-1067.

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