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TITLE: Macrophage-Mediated HSC Dysfunction in Bone Marrow Failure

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RECIPIENT: Albany Medical College
Albany, NY 12208

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Albany Medical College
47 New Scotland Avenue,
Albany, NY 12208

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Interferon-gamma (IFNγ) is increased in patients with a type of bone marrow failure (BMF) called aplastic anemia (AA). IFNγ has been linked with hematopoietic dysfunction and exhaustion of hematopoietic stem cells (HSCs). However, it is still unclear how IFNγ drives BMF. HSCs are rare, multipotent cells residing in the BM that give rise to all cells of the blood. The BM microenvironment, or HSC niche, plays a key role in blood cell production by controlling HSC function and maintaining HSCs in a quiescent state, necessary for their self-renewal and life-long blood production. Macrophages (Ms) are myeloid cells that maintain tissue homeostasis throughout the body and also play a key role in initiating innate immunity and countering infection. They respond to, and produce, many factors that direct host immune responses, and their function is highly plastic. In fact, recent evidence suggests that at steady-state M□s are important for maintaining HSC dormancy, yet how inflammation impacts M function and HSC maintenance is not yet clear. Notably, Ms are exquisitely sensitive to IFNγ. We found that Ms are required for the IFNγ-dependent loss of HSCs in a mouse model of AA and depletion of Ms increases HSC self-renewal (McCabe et al., 2015). Thus, in contrast to the assumption that IFNγ acts directly on HSCs to drive their loss, Ms are the key cellular targets of IFNγ. We also identified the chemokine CCL5 as an IFN- dependent chemokine responsible for HSC loss. Moreover, Ms aberrantly express podoplanin (PDPN) during AA and this signaling is also important for mediating HSC loss in AA. The rationale for this proposal is that Ms are essential targets of IFNγ and control numbers of HSCs in vivo, in part via CCL5 and PDPN expression thus, suggesting novel therapeutic targets for treatment of AA.

15. SUBJECT TERMS

Severe aplastic anemia, macrophage, interferon-gamma, hematopoietic stem cell, CCL5, RANTES, podoplanin, bone marrow, anemia, thrombocytopenia

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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TABLE OF CONTENTS

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

Aplastic anemia (AA) is a bone marrow failure syndrome characterized by destruction or exhaustion of hematopoietic stem cells (HSCs) leading to anemia, hemorrhage, or infection and invariably fatal if untreated. Elevated levels of interferon gamma (IFNγ) have long been associated with hematopoietic suppression in aplastic anemia, though it is unclear precisely how IFNγ mediates loss of or exhaustion of HSCs *in vivo*. We identified an essential role for macrophages (MΦs) as key mediators of IFNγ-dependent HSC loss in a mouse model of AA. The overall **goal** of this project is to determine mechanisms whereby macrophages (MΦs) reduce the number and function of HSCs to reveal novel strategies for specific treatments to rescue hematopoiesis in AA. In Aim 1 we propose to pursue a deeper understanding of how expression of the chemokine CCL5 and the molecule podoplanin contribute to disease progression. In Aim 2 we propose to evaluate the therapeutic potential of these targets for amelioration of AA disease using a mouse model.

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

Severe aplastic anemia, macrophage, interferon-gamma, hematopoietic stem cell, CCL5, RANTES, podoplanin, bone marrow, anemia, thrombocytopenia

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.

Aim 1.1: *Evaluate impact of CCL5 and PDPN on BM megakaryocytes*
Aim 1.2: *Evaluate whether blockade of CCL5 and/or PDPN rescues BM stromal niche cells.*
Aim 1.3: *Determine the impact of CCL5 and PDPN neutralization on MΦ function*

Aim 2.1: *Determine whether targeting MΦ self-renewal (using M-CSF-R antagonists) and/or targeting CCL5- and/or PDPN-mediated signaling can reverse disease in AA and increase survival*
Aim 2.2: *Test whether the c-Mpl-independent, iron-chelating ability of Eltrombopag can modulate MΦ number/function, PDPN expression, and rescue mice with AA*

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.

1. **Major Activities during Year 1:** During this funding period the major activities included in vivo experiments in the mouse model of SAA, breeding to generate appropriate models to study the disease, performing gene expression and cell analysis by flow cytometry, and analyzing and preparing data for dissemination. A second major activity was writing and submitting a manuscript on the role of macrophages and podoplanin in driving bone marrow failure.

2. **Specific Objectives for Year 1:**
   a. *Evaluate impact of CCL5 and PDPN on BM megakaryocytes.*
   b. *Evaluate whether blockade of CCL5 and/or PDPN rescues BM stromal niche cell*
   c. *Determine the impact of CCL5 and PDPN neutralization on MΦ function*
   d. *Determine whether targeting MΦ self-renewal (using M-CSF-R antagonists) and/or targeting CCL5- and/or PDPN-mediated signaling can reverse disease in AA and increase survival*

3. **Significant Results.**

We observed that blockade of podoplanin during SAA impacted thrombocytopenia, megakaryocyte number, and macrophage function (**Figure 1**). We observed that podoplanin targeting increased platelet counts (**Fig. 1A**), moderately increased megakaryocyte numbers (**Fig. 1B**), and altered macrophage function as indicated by increased expression of *arginase* (**Fig. 1C**). These date demonstrate that aberrant podoplanin expression on macrophages is a correlate of disease and is also associated with macrophage polarization.

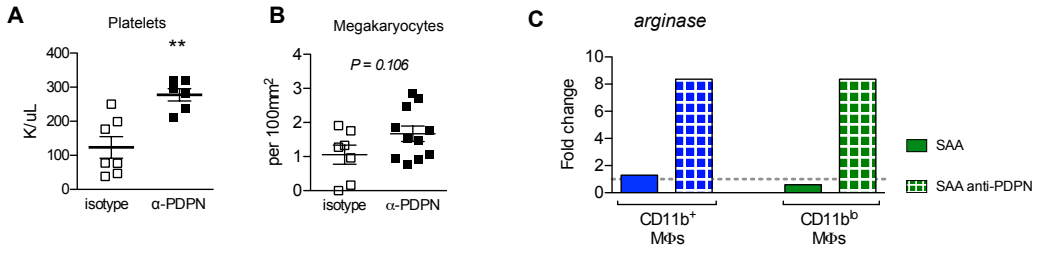

Figure 1. Targeting podoplanin *in vivo* with an antibody (clone 8.1.1) improves thrombocytopenia and alters macrophage function. Mice were treated anti-PDPN (■; α-PDPN) or isotype control (□) on days 4, 7, and 10, and analyzed days 8 and 14 post-splenocyte transfer (p.s.t.) (**A**) Platelets in the blood of isotype control- (□) and α-PDPN (■)-treated mice 14 d.p.s.t. (**B**) GP1bβ^{+} megakaryocytes per 100mm^{2} of BM 14 d.p.s.t. Data represent one experiment repeated at least twice, n=4-18 mice/group. Mean ± SEM is shown. **p<0.01, ***p<0.001. (**C**) Sort purified MΦs were obtained on day 8 post SAA induction from control and anti-PDPN treated mice and evaluated for expression of *iNos* and *arginase1*. Data is normalized to CD11b^{+} MΦs from LC SAA mice. Data come from two independent experiments and two independent sorts, reflecting at least 3 individual mice. (Adapted from our manuscript published in *Haematologica*, September 2018)

Our previous observations that CCL5 or RANTES was elevated during SAA prompted our analysis of the key receptors for this chemokine. We observed a specific increase in CCR5 during SAA, whereas the CCL5 receptor CCR3 was either unchanged or decreased (**Fig. 2A**). These findings were consistent with our observations that CCR5 antagonism with the small molecule inhibitor maraviroc (MVC) was effective at ameliorating disease (**Fig. 2C**). At the same time, targeting CCR5 with MVC during SAA was associated with reduced live or viable macrophages and increased Annexin V-positive, apoptotic macrophages (**Figure 2D**), thus suggesting that CCR5 signaling may be important for maintaining macrophages during disease. Though these trends were not significant, current work is aimed at repeating these studies to determine whether there is a biological impact on macrophage numbers and/or function. Together, these data demonstrate the importance CCR5 signaling in the pathogenesis of SAA (**Figure 2D**).

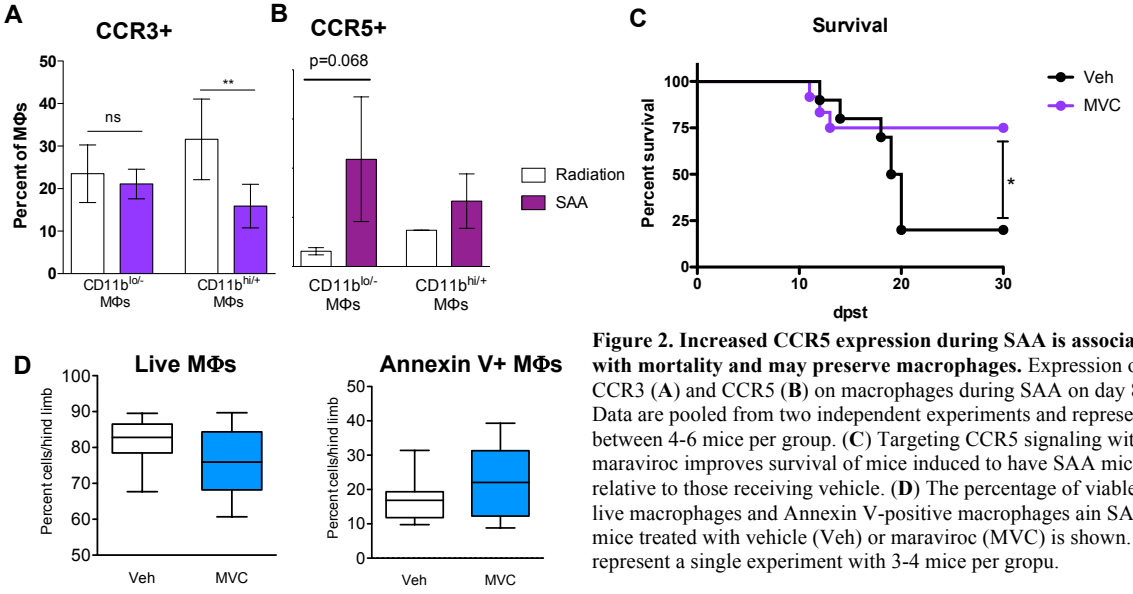

Figure 2. Increased CCR5 expression during SAA is associated with mortality and may preserve macrophages. Expression of CCR3 (**A**) and CCR5 (**B**) on macrophages during SAA on day 8 p.s.t. Data are pooled from two independent experiments and represent between 4-6 mice per group. (**C**) Targeting CCR5 signaling with maraviroc improves survival of mice induced to have SAA mice, relative to those receiving vehicle. (**D**) The percentage of viable or live macrophages and Annexin V-positive macrophages ain SAA mice treated with vehicle (Veh) or maraviroc (MVC) is shown. Data represent a single experiment with 3-4 mice per gropu.

We discovered that administering a neutralizing antibody to CCL5 induced a slight improvement in HSC numbers during SAA (**Figure 3A**). Anti-CCL5 treatment increased CD41^{lo} HSCs, but decreased CD41^{hi}, platelet-biased, HSCs (**Figure 3B**). In contrast, administering MVC, the small molecule antagonist against the main CCL5 receptor (CCR5) increased platelet-biased (CD41^{hi}) HSCs and moderately improved platelet numbers (**Figure 3C-E**), which correlated with improved survival outcomes (*Figure 2C, above*). This is significant because small molecules are translationally-relevant, and our data reveal a specific role for a single receptor (CCR5) in SAA disease pathogenesis.

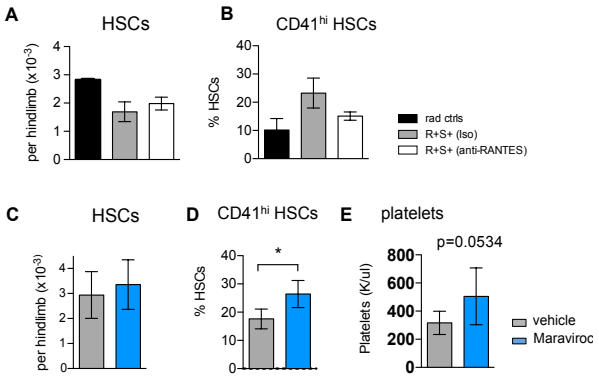

Figure 3. Targeting CCR5, but not CCL5/RANTES, increases platelet-biased HSCs during SAA. Mice were induced to have SAA and treated with either an anti-CCL5 antibody or the CCR5 antagonist maraviroc, and bone marrow analyzed day 8 post induction. (**A**) The total number of HSCs in radiation controls (filled black bar), SAA (gray bar), or SAA mice treated with anti-RANTES on days 3, 5, and 7 post-induction (open bars). (**B**) The percent of CD41^{hi} HSCs in each group is shown. (**C**) Absolute numbers of HSCs in SAA mice treated with vehicle or maraviroc on days 5, 6, and 7 is shown. (**D**) The proportion of CD41^{hi} HSCs in each group is shown. (**E**) Peripheral blood was analyzed by complete blood count using a Heska machine; platelets are shown on day 12 post-induction in vehicle and maraviroc-treated mice. Data are representative of two experiments pooled and reflect 6-8 mice per group.

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.

We first plan to finalize data sets not yet completed during year 1. Specifically, we will complete our anlaysis on the role of targeting CCL5 receptor CCR5 on disease outcomes and macrophage function and polarization, and we will evaluate its impact on megakaryocyte number and platelet production.
Our overall goal for year 2 is to determine effectiveness of Mϕ depletion as a therapeutic treatment in a model of BMF. To do this will determine whether targeting MΦ self-renewal (using M-CSF-R antagonists) and/or targeting CCL5- and./or PDPN-mediated signaling can reverse disease in AA

and increase survival. We will combine CCR5 antagonism with M-CSFR antagonism during acute disease to test whether the combined strategies can reverse disease progression.
We will also test whether the c-Mpl-independent, iron-chelating ability of Eltrombopag, can modulate MΦ number/function, PDPN expression, and rescue mice with AA. Eltrombopag is a primate specific c-Mpl agonist and therefore should not have agonist activity in mice. However, the ability of this drug to chelate iron should still be intact. We propose to determine whether iron chelation can impact the course of disease and impact macrophage numbers and function.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report." Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Our work is the first to definitively describe an essential role for macrophages in the pathogenesis of severe aplastic anemia. There have been clinical reports to suggest a role for the bone marrow microenvironment, or stroma, on bone marrow failure and stem cell loss, but no conclusive or causative role for the bone marrow stroma. Macrophages are one component of the bone marrow microenvironment and we demonstrate that macrophages respond to interferon gamma, and that interferon gamma specifically maintains macrophages during the course of disease. We find that targeting marrow macrophage proliferation and survival is novel therapeutic strategy to alleviate bone marrow failure. Our findings provide a novel paradigm in the pathophysiology of this devastating disease and open numerous new avenues of study. Importantly, our studies also reveal novel potential therapeutic targets for the treatment of patients with severe aplastic anemia.

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.

Our studies define a role for podoplanin expression in macrophages in the pathology of bone marrow failure. We observe that podoplanin ligation by a specific antibody that mimics ligand binding regulates polarization and macrophage function. This is relevant to other tissue systems where macrophages may express podoplanin, and where the ligand may be limiting. Though it is currently unknown how podoplanin expression is linked to macrophage polarization, this information is likely relevant to other cell types and diseases.

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.

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.

We experienced somewhat of a delay in progress due to the departure of Julianne Smith from the lab, due to family relocation out of state. It also took some time to recruit a graduate student Allison Seyfried. Allison joined the lab in December 2017 and Julie Smith left the lab in January 2018, thus they spent approximately 6 weeks working together on the project. Allison is now fully trained on the project, and it is expected that the pace of her progress will continue. In addition, we hired a new postdoc, Jing Jing Li, in February 2018. Dr. Li has imaging experience and is working with Allie to conduct the proposed studies, with a particular focus on imaging megakaryocytes in the bone marrow. Our delays were primarily due to not having sufficient workforce for the studies and now that the personnel is in place, we envision achieving the stated goals and having an accelerated pace of progress.

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.

The salary lines were impacted as there was a delay in filling the student position, requested in the budget. This also impacted a delay in spending as experiments slowed during the time when she and another postdoc joined the lab. It is anticipated that the pace will make up for this lag, in the coming months.

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).

McCabe A, Smith JN, Costello A, Maloney J, Katikaneni D, and **MacNamara KC**. **2018**. Hematopoietic stem cell loss and hematopoietic failure in severe aplastic anemia requires macrophages and aberrant podoplanin expression. *Haematologica* (accepted, *pre-published online*).

Acknowledgement of federal support: *yes.*

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).*

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.*

NewYork Immunology Conference, October 24th, 2018 (Bolton Landing, NY)
Invited Speaker
Title: Macrophages Drive Hematopoietic Stem Cell Loss and Bone Marrow Failure in Severe Aplastic Anemia

Pennsylvania State University, April 18th, 2018 (College Park, PA)
Invited Speaker
Title: Macrophage-Dependent Regulation of Hematopoiesis In Infection and Disease

- **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.

- **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: Katherine C. MacNamara
Project Role: PI
Researcher Identifier:
Nearest Person Month worked: 1.3
Contribution to Project: Dr. MacNamara has provided guidance to all members of the team working on the project. She has performed data analysis and presented findings.
Funding Support: NIH NIGMS, Albany Medical College

Name: Julianne NP Smith
Project Role: postdoctoral researcher
Researcher Identifier:
Nearest Person Month Worked: 4
Contribution to Project: Dr. Smith performed experiments, collected and analyzed data, and worked on preparing a manuscript.

Name: Allison Seyfried
Project Role: Graduate Student
Researcher Identifier:
Nearest Person Month Worked: 4
Contribution to Project: Dr. Smith performed experiments, collected and analyzed data, and worked on preparing a manuscript.

Name: Hui Jin Jo
Project Role: Laboratory Technician
Researcher Identifier:
Nearest Person Month Worked: 12

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. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. 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.

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.*

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://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) 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.

ARTICLE

Hematopoietic stem cell loss and hematopoietic failure in severe aplastic anemia is driven by macrophages and aberrant podoplanin expression

Amanda McCabe,[1*] Julianne N.P. Smith,[1**] Angelica Costello,[1] Jackson Maloney,[1] Divya Katikaneni[1] and Katherine C. MacNamara[1]

[1]Department for Immunology and Microbial Disease, Albany Medical College, NY, USA

*AM and JNPS contributed equally to this work. *Current address: Boston Children's Hospital, Division of Hematology/Oncology, Harvard Medical School, Karp Family Research Lab, Boston, MA, USA. **Current address: Case Western Reserve University, Department of Medicine, Wolstein Research Building, Cleveland, OH, USA*

Haematologica 2018
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ABSTRACT

Severe aplastic anemia (SAA) results from profound hematopoietic stem cell loss. T cells and interferon gamma (IFNγ) have long been associated with SAA, yet the underlying mechanisms driving hematopoietic stem cell loss remain unknown. Using a mouse model of SAA, we demonstrate that IFNγ-dependent hematopoietic stem cell loss required macrophages. IFNγ was necessary for bone marrow macrophage persistence, despite loss of other myeloid cells and hematopoietic stem cells. Depleting macrophages or abrogating IFNγ signaling specifically in macrophages did not impair T-cell activation or IFNγ production in the bone marrow but rescued hematopoietic stem cells and reduced mortality. Thus, macrophages are not required for induction of IFNγ in SAA and rather act as sensors of IFNγ. Macrophage depletion rescued thrombocytopenia, increased bone marrow megakaryocytes, preserved platelet-primed stem cells, and increased the platelet-repopulating capacity of transplanted hematopoietic stem cells. In addition to the hematopoietic effects, SAA induced loss of non-hematopoietic stromal populations, including podoplanin-positive stromal cells. However, a subset of podoplanin-positive macrophages was increased during disease, and blockade of podoplanin in mice was sufficient to rescue disease. Our data further our understanding of disease pathogenesis, demonstrating a novel role for macrophages as sensors of IFNγ, thus illustrating an important role for the microenvironment in the pathogenesis of SAA.

Introduction

Severe aplastic anemia (SAA) is a rare, lethal bone marrow (BM) failure disease that can be inherited or acquired. The most effective treatment for SAA is BM transplantation but disease management also includes immunosuppressive therapy (IST). Not all patients are good transplant candidates, however, and IST responsiveness varies. Therefore more specific treatments are necessary.[1,2]

Chemical-induced toxicity, myeloablation, and lymphocyte infusion-based BM destruction have been used to model SAA in mice and define factors critical for initiating disease.[1,2] SAA can be acquired as a result of radiation, toxic drug exposure, or infection. Acquired forms are often immune-mediated,[3] thus the lymphocyte-infusion model is clinically relevant. Sublethal irradiation and subsequent lymphocyte or bulk splenocyte transfer elicits pancytopenia and death within 2-3 weeks.[4] Importantly, disease progression and IST treatment responses are similar to those in SAA patients.[5] T cells promote hematopoietic stem cell (HSC) loss during SAA through a "bystander effect" involving inflammatory cytokines, including IFNγ.[6-9] IFNγ negatively regulates HSC function, and it was first observed over thirty years ago that SAA patients show elevated IFNγ levels.[10] Despite this knowledge, the

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underlying mechanisms whereby IFNγ drives SAA are still unknown.

Thrombocytopenia causes substantial morbidity and mortality in SAA patients.[11] Megakaryocytes (Mks) not only produce platelets *via* thrombopoiesis, they serve as critical niches for HSCs.[12,13] Thrombopoiesis is regulated by soluble factors, vascular integrity, and extracellular matrix composition, and requires adequate numbers and location of Mks.[14] Platelet-biased HSCs, including HSCs that highly express CD41, have been observed in settings of inflammation and aging, where increased platelet output may be necessary to maintain vascular function.[15,16] Inflammation can impact megakaryopoiesis and thrombopoiesis, though it is unclear whether this process is modulated in SAA.

Bone marrow macrophages (Mϕs) support stromal niche cell function at steady-state,[17-19] however, little is known about the impact of inflammation on BM Mϕs. Herein, we demonstrate that Mϕs are essential for IFNγ-dependent HSC loss in murine SAA. IFNγ signaling in Mϕs was necessary for the selective maintenance of BM resident Mϕs, whereas all other myeloid cells were diminished. Targeting Mϕs during SAA, *via* depletion or blocking their ability to respond to IFNγ, rescued HSCs and markedly improved survival. We demonstrate a key role for BM Mϕs in sensing IFNγ, and our findings suggest that dysfunctional megakaryopoiesis and thrombopoiesis underlie hematopoietic collapse in SAA.

Methods

Mice

C57BL/6 ($H^{b/b}$) and BALB/c ($H^{d/d}$) mice were from Taconic (Albany, NY, USA). C57BL/6-TG(UBC-GFP)30Scha/J mice and ACTB-tdTomato mice were from Jackson Laboratory (Bar Harbor, ME, USA). MIIG (Mϕ-insensitive to IFNγ) mice[20] were a gift from Dr. Michael Jordan. Hybrid B6 F1 (Hb/d) were generated by crossing C57BL/6 with BALB/c mice. To generate MIIG F1 (Hb/d) mice, MIIG (C57BL/6 background) and BALB/c mice were crossed. Hybrid F1 progeny were screened by PCR to identify mice carrying the MIIG transgene, and MIIG-negative mice were included as littermate controls (LC). Mice were bred and housed in the Animal Research Facility at Albany Medical College (AMC) under microisolator conditions. Protocols were approved by the AMC Institutional Animal Care and Use Committee.

SAA induction

Age- (6-8 weeks) and sex-matched B6 F1 mice were sublethally irradiated (300 RADs) using a ^{137}Cs source four hours prior to intraperitoneal (i.p.) transfer of $5x10^7$ C57BL/6 splenocytes.[4] Mice were euthanized by CO_2 inhalation at the indicated day post splenocyte transfer (d.p.s.t.). For survival studies, mice were examined twice daily and humanely euthanized upon 20% loss of initial body weight or if found moribund. Surviving mice were euthanized approximately ten days after the last mouse succumbed to disease.

Cell preparation and flow cytometry

Bone marrow was flushed from femurs and tibias, and spleens were homogenized. After red blood cell (RBC) lysis single-cell suspensions were plated and stained. Surface-stained cells underwent nuclear or cytoplasmic permeabilization (BD Pharmingen) prior to T-bet (4B10) and IFNγ staining, respectively. Data were collected on an LSR II (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

Bone-associated cell analysis

Bones were crushed using a mortar and pestle in HBSS, washed and twice digested in 2 mg/mL collagenase type 2 and 1X trypsin enzyme solution at 37°C (with rocking, 30 min).

Complete blood cell count

Complete blood counts (CBCs) were determined using an automated hematology analyzer (Cell Dyn 3700, Abott Laboratories).

Tissue preparation for protein quantification

Bone marrow cell lysates were homogenized with a pestle in a buffer containing IGEPAL CA-630 and proteinase inhibitors for protein analysis.

Transplantation

150 HSCs (Lin^- $cKit^+$ CD48- $CD150^+$) were sorted from PBS- and clod-lip-treated ACTB-tdTomato F1 mice 8 d.p.s.t. and transplanted separately into lethally irradiated F1 recipients with $2.5x10^5$ protective F1 whole BM cells.

Macrophage depletion and antagonist delivery

250 μL of PBS- or clodronate-liposomes (*ClodronateLiposomes.com*) was administered intravenous (i.v.) at 1 d.p.s.t. (day 7 analysis) or 1 and 7 d.p.s.t. (day 15 analysis). Anti-podoplanin (PDPN) antibody (clone 8.1.1) or Syrian Hamster IgG (both from BioXcell) was administered i.v. 3, 7, and 10 d.p.s.t. at 125 μg/dose.

Platelet analysis

Fluorescently-tagged anti-Gp1bβ antibodies (Emfret Analytics) were administered to mice 5 or 10 d.p.s.t., according to the manufacturer's instructions.

Histology

Sternums were fixed in 10% buffered formalin, decalcified in 14% EDTA, and paraffin-embedded. Megakaryocytes were stained with anti-rat GP1bβ (Emfret) and nuclear fast red (Poly Scientific R&D) counterstaining. Images were taken on an Olympus SC30 light microscope using CellSens software.

Statistical analysis

Analysis was performed using Prism software. Two-tailed Student *t*-test was used to compare between indicated groups, unless otherwise reported.

Results

BM macrophages are maintained during SAA

Hematopoietic stem cell loss and BM destruction are key features of SAA and are associated with cytokine production by T cells.[6-8] It is unclear, however, if inflammation depletes HSCs directly or through the microenvironment. To examine resident Mϕs in SAA, we used an established murine model of BM failure involving histocompatibility mismatched recipients and splenocyte infusion.[4] SAA was induced *via* sublethal irradiation of F1 hybrids (C57BL/6 x BALB/c), followed by adoptive transfer of C57BL/6 splenocytes (*Online Supplementary Figure S1A*). Significant cytopenias were observed in SAA mice at 8 and 15 d.p.s.t. compared to radiation controls (Rad) (*Online Supplementary Figure S1B-D*). Thrombocytopenia was evident 8 d.p.s.t in control and SAA mice, and progressed in SAA mice (*Online Supplementary Figure S1D*). SAA-associated cytopenias coincided with BM hypocellularity (Figure 1A) and HSC loss (*Online Supplementary Figure S1E-G*).

Monocytes (CD11b^{+} Ly6C^{hi}) and Mϕs (defined as CD11b$^{lo/-}$ Mϕs: F4/80^{+} VCAM1^{+} CD169^{+} CD11b$^{lo/-}$ SSC^{lo} and CD11b^{+} Mϕs: F4/80^{+} VCAM1^{+} CD169^{+} CD11b^{+}Ly6C^{int} SSC^{lo}; *Online Supplementary Figure S2*), were increased by frequency 8 d.p.s.t., relative to Rad mice (Figure 1C). At 15 d.p.s.t., CD11b^{+} Mϕs, monocytes, and neutrophils were reduced by frequency whereas CD11b$^{lo/-}$ Mϕ frequencies were significantly increased (Figure 1D). Despite severe BM hypocellularity at 15 d.p.s.t., CD11b$^{lo/-}$ Mϕ numbers remained stable (Figure 1F). Thus, BM CD11b$^{lo/-}$ Mϕs persist despite SAA-associated cytopenias, myeloid cell loss, and HSC loss in SAA. These data suggest that CD11b$^{lo/-}$ Mϕs do not require hematopoietic input during SAA, potentially due to their long lifespan or ability to self-renew.

IFNγ-dependent increase in BM macrophages during SAA drives HSC loss and thrombocytopenia

Interferon-γ mediates SAA pathology[5,9] and maintains BM Mϕs during infection,[21] thus we next addressed

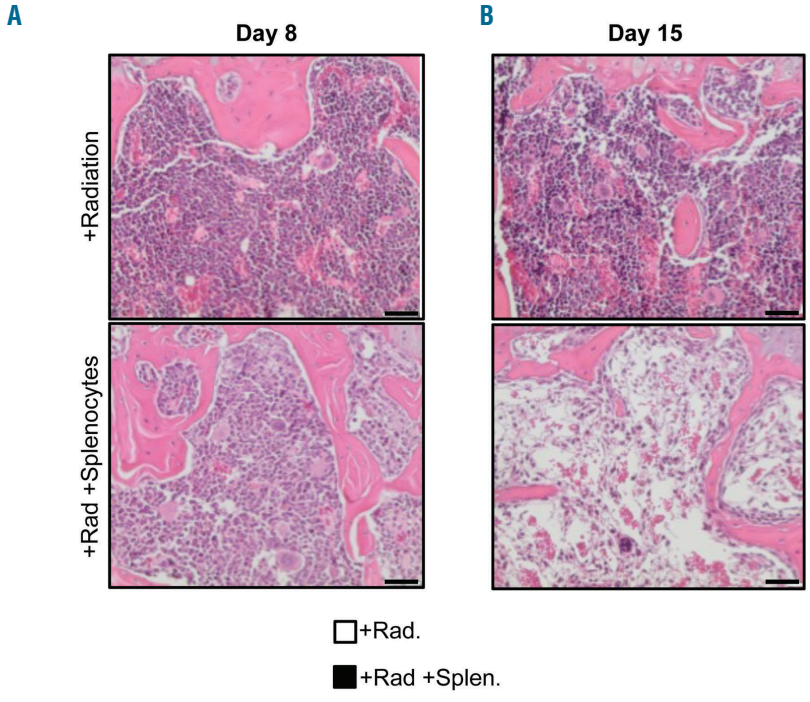

Figure. 1. Bone marrow (BM) macrophages are maintained during aplastic anemia. (A and B) Hematoxylin and eosin-stained BM in radiation control (Rad) (top) and severe aplastic anemia (SAA) (bottom) mice on days 8 and 15. Scale bar=100μm. Frequencies (C and D) and numbers (E and F) of CD11b$^{lo/-}$ Mϕs, CD11b^{+} Mϕs, monocytes, and neutrophils in radiation control (open bars) and SAA (filled bars) mice on days 8 and 15. Data represent one experiment repeated at least twice, n=3-6 mice/group. Mean±Standard Error of Mean is shown. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

whether IFNγ was necessary for preservation of Mϕs during SAA. Using transgenic mice in which Mϕ-lineage cells are insensitive to IFNγ (referred to as MIIG mice) due to a CD68-driven dominant-negative IFNγ receptor,[20] we noted improved cellularity upon induction of SAA (Figure 2A and B). MIIG and littermate control (LC) mice showed no significant difference in response to sublethal radiation; however, $CD11b^{lo/-}$ and $CD11b^{+}$ Mϕs were significantly reduced in MIIG mice relative to LC counterparts 8 d.p.s.t, when HSC loss is first noted (Figure 2C). Thus, we define a novel role for IFNγ in maintaining and increasing BM Mϕs during SAA.

Based on our prior findings in bacterial infection, where Mϕs drive IFNγ-induced HSC depletion,[21] we predicted that the BM HSC pool would be preserved in MIIG mice during SAA. Indeed, HSCs were preserved in MIIG mice, relative to LCs (Figure 2D and E), demonstrating IFNγ-sensing by Mϕ-lineage cells drives HSC loss in SAA. Anemia was slightly, but significantly, ameliorated (Figure 2F and G) whereas thrombocytopenia was strikingly rescued in MIIG relative to LC controls (Figure 2H). In fact, platelet levels were higher in MIIG mice with SAA than in radiation-control mice. The robust platelet rescue suggested that IFNγ-stimulated Mϕs contribute specifically to thrombocytopenia in SAA.

MIIG mice exhibit increased $CD41^{hi}$ HSCs and megakaryocytes during SAA

Inflammation-induced megakaryopoiesis reportedly relies on the emergence of a $CD41^{hi}$ stem-like Mk progenitor cell type (SL-MkP) within the phenotypic HSC gate.[15] SL-MkPs self-renew and rapidly produce Mks and platelets, while $CD41^{lo/int}$ HSCs contain multi-lineage potential.[15,16] CD41 expression increased robustly on HSCs in MIIG relative to LC mice at day 15 p.s.t. (Figure 3A), suggesting that IFNγ-sensing Mϕs limit $CD41^{hi}$ HSC emergence in response to SAA-induced inflammation. MIIG and LC radiation-control mice exhibited similar numbers of $CD41^{lo/int}$ and $CD41^{hi}$ at days 8 and 15 p.s.t. (Figure 3B). In SAA conditions, however, MIIG mice exhibited significantly more $CD41^{hi}$ HSCs (Figure 3C), and increased $CD41^{lo/int}$ on day 15 p.s.t. than LC. Consistent with increased phenotypic SL-MkPs we observed increased BM Mks in MIIG SAA mice, relative to LC (Figure 3D and E). Our data indicate that IFNγ signaling in Mϕs during SAA is associated with rapid loss of both $CD41^{lo/int}$ and $CD41^{hi}$ HSCs, which correlates with Mk depletion and severe thrombocytopenia. Moreover, SAA-induced mortality was significantly reduced in MIIG compared to LC mice (Figure 3F). Thus, Mϕs are key sensors of IFNγ, and our data strongly suggest that Mϕs drive disease and death by reducing platelet-biased $CD41^{hi}$ HSCs.

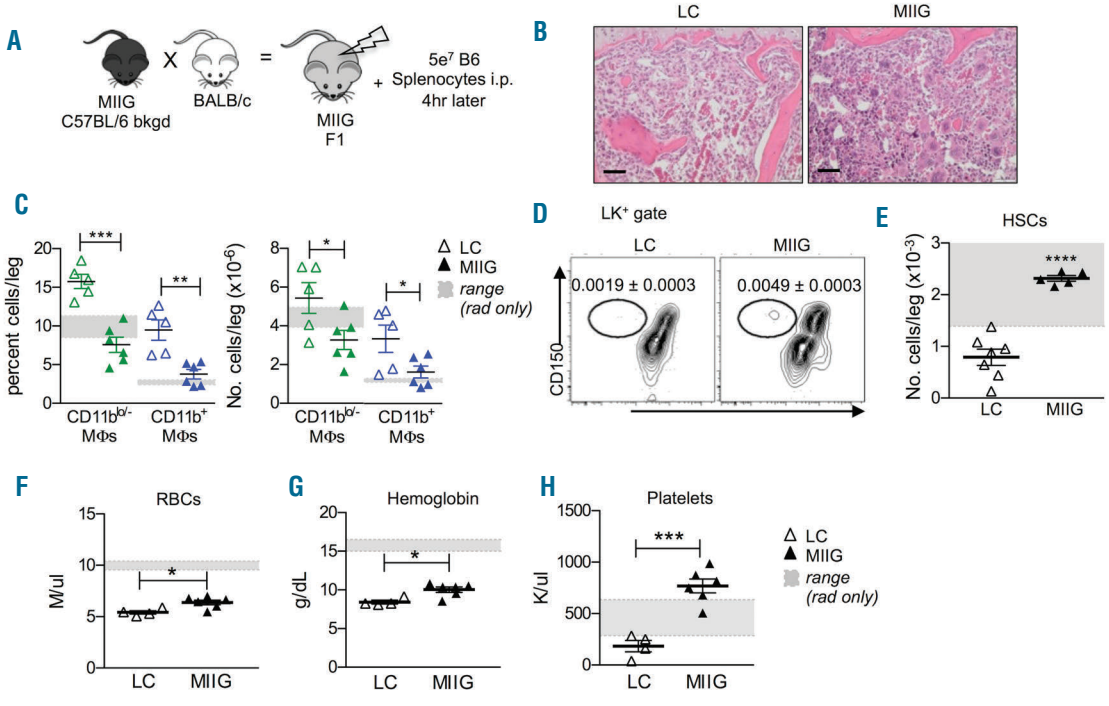

Figure 2. IFNγ sensing by macrophages is required for bone marrow (BM) macrophage maintenance and hematopoietic stem cell (HSC) loss in aplastic anemia. (A) Severe aplastic anemia (SAA) was induced in MIIG and littermate control (LC) F1 hybrids. (B) Hematoxylin and eosin-stained BM of LC and MIIG mice 15 days post induction. Scale bar=50 μm. (C) Frequencies and absolute numbers of $CD11b^{lo/-}$ Mϕs and $CD11b^{+}$ Mϕs in LC (Δ) and MIIG (▲) mice 8 days post induction. Shading represents ranges of each Mϕ population in radiation control LC and MIIG mice. (D) CD150 and CD48 expression on BM Lin- c-Kit^{+} (LK^{+}) cells. Numbers represent mean HSC (LK^{+} $CD150^{+}CD48^{-}$) frequency±Standard Error of Mean (SEM). (E) HSC numbers in MIIG (Δ) and LC (▲) mice 8 days post induction. Shading represents ranges of radiation control LC and MIIG mice. (F) Red blood cells (RBCs), (F) hemoglobin, and (H) platelets in the blood 15 days post induction. Shading represents ranges of radiation control LC and MIIG mice. Data represent one experiment repeated at least two times, n=5-7 mice/group. Mean±SEM is shown. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Clodronate-liposomes specifically deplete macrophages, increase CD41^{hi} HSCs and platelets, and rescue survival during SAA

To test the impact of Mϕ depletion on SAA pathogenesis, we administered clodronate-encapsulated liposomes (clod-lip) to mice one day after SAA induction. BM Mϕs were significantly and specifically reduced 8 d.p.s.t. (Figure 4A). Monocytes and neutrophils are also phagocytic and may be transiently depleted; however, they were quickly replaced and no sustained depletion was observed with clod-lip. Mϕ depletion correlated with improved cellularity at day 15 (Figure 4B and C), increased total HSCs (*Online Supplementary Figure S3A*), and increased CD41^{hi} HSCs at 8 and 15 p.s.t (Figure 4D), thus supporting the idea that Mϕs negatively regulate HSCs during SAA. Macrophage-colony stimulating factor (M-CSF) is critical for tissue Mϕ survival and self-renewal,[22,23] and similar to clod-lip administration, M-CSFR antagonism significantly increased HSCs during SAA (*Online Supplementary Figure S3B*). Similar to MIIG SAA mice, CD41^{hi} HSCs correlated with increased circulating platelets, significantly increased BM Mks, and improved survival (Figure 4E-G). HSCs were increased in both MIIG SAA and Mϕ-depleted SAA mice, while more downstream progenitors, including short-term HSCs and multipotent progenitors (MPPs), were more variable (*Online Supplementary Figure S4A*). Consistent with improved thrombocytopenia in both models, a similar and significant increase in megakaryocyte progenitors was observed in MIIG SAA mice and Mϕ-depleted SAA mice (*Online Supplementary Figure S4B*).

A similar rate of platelet removal from circulation was observed in PBS- and clod-lip-treated SAA mice (Figure 4H and I). Thus, improved platelet counts were not due to loss of consumption by Mϕs. The increase in BM Mks and significantly reduced mortality in Mϕ-depleted mice compared to PBS-lip-treated controls demonstrate Mϕs drive SAA mortality, possibly *via* their ability to restrict phenotypically-defined platelet-biased HSCs. Thus, HSC loss and thrombocytopenia is dependent on Mϕs and Mϕ growth factors in SAA.

T-cell responses are not impaired in clod-lip-treated and MIIG mice

Macrophages may drive HSC loss by enhancing activated T-cell infiltration into the BM; therefore, we tracked donor T cells by inducing SAA with splenocytes from UBC-GFP mice (C57BL/6 background). Expression of the T-helper 1 transcription factor T-bet, which is expressed in T cells of SAA patients and increases IFNγ gene transcription,[7] was not diminished in T cells from Mϕ-depleted mice (*Online Supplementary Figure S5A*). IFNγ protein levels (*Online Supplementary Figure S5B*) and IFNγ-secreting donor T cells (*Online Supplementary Figure S5C-E*) in the BM of SAA mice were also unaffected by Mϕ depletion. T-

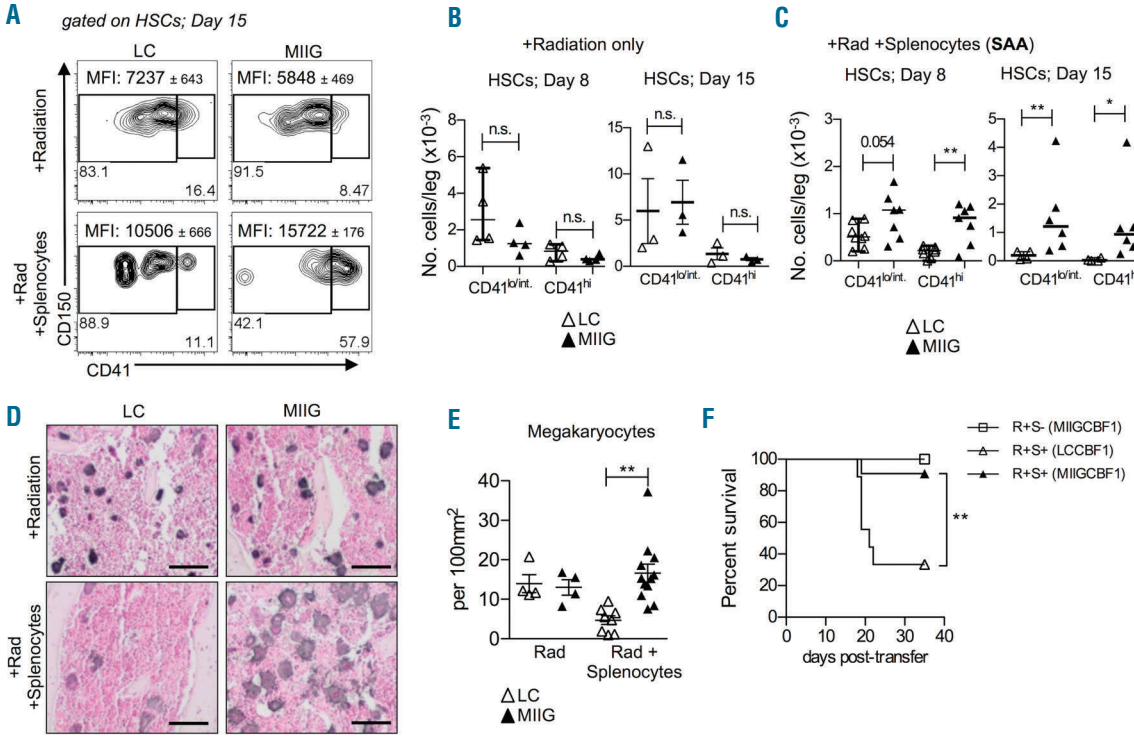

Figure 3. MIIG mice exhibit increased CD41^{hi} hematopoietic stem cells (HSC), increased megakaryocytes in the bone marrow (BM), and reduced mortality during aplastic anemia. (A) CD41 expression on BM HSCs in radiation control (Rad) (top) and severe aplastic anemia (SAA) (bottom) MIIG and LC mice 15 days post-splenocyte transfer (d.p.s.t.). CD41 mean fluorescence intensity (MFI) on HSCs is shown on the plots and gates represent CD41$^{lo/int}$ and CD41^{hi} HSCs. (B and C) CD41$^{lo/int}$ and CD41^{hi} HSC numbers in MIIG (▲) *versus* LC (Δ) mice on days 8 (mean is shown) and 15 (median is shown, and a Mann-Whitney test was used to compare between groups). *P<0.05, **P<0.01. (D) Gp1bβ staining in BM of Rad and SAA MIIG (▲) and LC (r) mice 15 d.p.s.t. Scale bar=100 μm. (E) GP1bβ^{+} megakaryocytes per 100 mm^2 of sternal BM. Mean±Standard Error of Mean (SEM) is shown. **P<0.01. (F) Kaplan-Meier survival curve for radiation control (Rad; LC and MIIG mice; □, n=4) and SAA MIIG (▲; n=9) and LC (Δ; n=11) mice. Log-rank (Mantel-Cox) test was used to compare between groups. *P<0.05.

bet^{+} donor T-cell numbers, IFNγ protein levels, and IFNγ^{+} donor T cells were also comparable between LC and MIIG mice in SAA (*Online Supplementary Figure S5F-J*). Though donor T lymphocytes are necessary for disease initiation, HSC loss during SAA occurred independently of the direct effects of T-cell-derived IFNγ. Rather, HSC loss occurred through Mϕ-dependent sensing of T-cell-derived IFNγ.

Interferon-γ acts on Mϕs to promote inflammation during disease contributing to M1 polarization of Mϕs,[24,25] and inflammation can impact HSC pool size and function.[26,27] However, we observed similar levels of inflammatory factors previously associated with SAA, including TNFα, IL-6, and IL-1β, in the BM of MIIG and LC mice during SAA (*Online Supplementary Figure S6A*). Furthermore, Mϕ depletion did not alter TNFα, IL-6, and IL-1β levels (*Online Supplementary Figure S6B*), demonstrating that HSC loss is not due to induction of a broad inflammatory response during SAA. Despite the similar inflammatory milieu, purified Mϕs from LC and MIIG SAA mice exhibited differential expression of genes associated with M1 and M2 polarization, programs associated with inflammatory and wound healing, respectively.[28] In MIIG-derived CD11b^{+} Mϕs, we observed a reduction in inducible nitric oxide synthesis (*Nos2*) expression, whereas induction of *arginase 1* was seen in CD11b$^{lo/-}$ Mϕs (*Online Supplementary Figure S6C*). Elevated *Nos2* expression and increased nitric oxide concentrations are associated with disease in SAA patients,[29] thus IFNγ signaling in Mϕs may drive pathological M1 polarization in SAA.

Mϕ depletion increases functional platelet-biased HSCs

To examine Mϕ-dependent regulation of HSC function and lineage bias in SAA, we transplanted HSCs sorted from the BM of PBS- or clod-lip-treated SAA mice 8 d.p.s.t. (Figure 5A). To our knowledge, HSC function and lineage bias have not previously been assayed in models of SAA, likely due to the severity of BM hypocellularity. HSCs exposed to Mϕs during SAA showed little repopulating activity, indicating that exposure to secondary stress severely compromised their function, whereas HSCs from clod-lip-treated SAA mice exhibited platelet-, myeloid-, and lymphoid-repopulating capacity (Figure 5B). Thus our data demonstrate that in SAA, Mϕs reduce HSC function and impair platelet output.

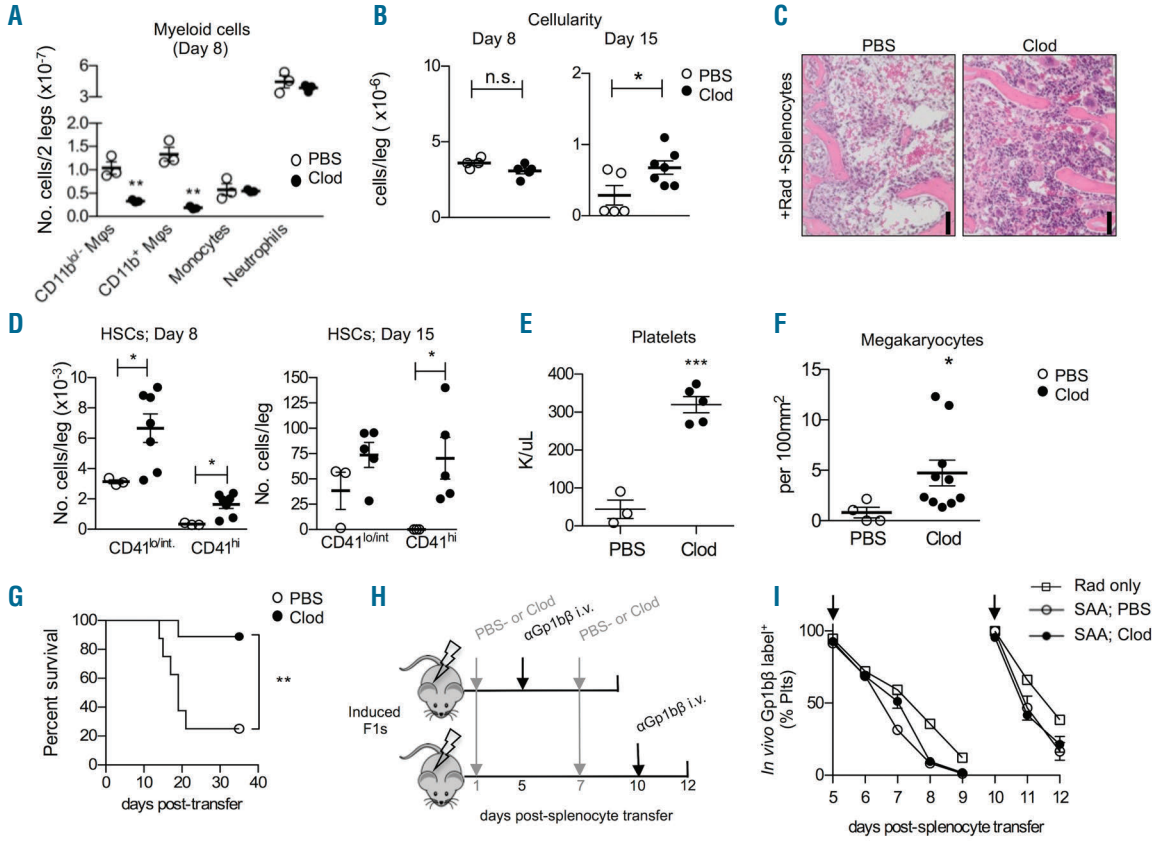

Figure 4. Clodronate-liposomes specifically deplete macrophages and rescue hematopoietic stem cell numbers during aplastic anemia. (A) Myeloid bone marrow (BM) cell numbers in PBS- (○) or clodronate-loaded (Clod; ●) liposome-treated severe aplastic anemia (SAA) mice. (B) BM cellularity in PBS- (○) or clodronate-loaded (Clod; ●) liposome-treated SAA mice on days 8 and 15 post-splenocyte transfer (p.s.t.) is shown. (C) Hematoxylin and eosin-stained BM from PBS- or clod-lip-treated radiation control (top) and SAA (bottom) mice 15 days post splenocyte transfer (d.p.s.t.). Scale bar=50 μm. (D) CD41$^{lo/int}$ and CD41^{hi} HSC numbers 8 and 15 days d.p.s.t. *P<0.05. (E) Platelets in the blood 15 d.p.s.t. ***P<0.001. (F) GP1bβ^{+} megakaryocytes per 100mm^{2} of BM 15 d.p.s.t. Mean±Standard Error of Mean is shown. *P<0.05. (G) Kaplan-Meier survival curve for mice with SAA treated with PBS- (○; n=8) or clod-lip (●; n=9) 1 and 7 d.p.s.t. Log-rank (Mantel-Cox) test was used to compare between groups. **P<0.01. (H) Schematic showing administration of anti-Gp1bβ antibodies to mice 5 or 10 d.p.s.t. (I) Labeled platelets were measured in the blood over time. Two-way ANOVA was used to compare between groups. P<0.0001 and P=0.0007 for SAA-PBS *versus* radiation control (Rad) at days 5-9 p.s.t. and days 10-12 p.s.t., respectively; P<0.0001 for SAA-Clod *versus* Rad at days 5-9 p.s.t. and days 10-12 p.s.t.; P=0.004 and P=0.7 for SAA-PBS *versus* SAA-Clod at days 5-9 p.s.t. and days 10-12 p.s.t., respectively.

Aberrant podoplanin expression during SAA drives HSC loss, thrombocytopenia, and death

Macrophages negatively regulated both HSCs and Mks, and we questioned whether Mϕs also regulate non-hematopoietic BM stromal cells, known to be impaired in SAA patients.[30-32] In contrast to radiation alone, SAA reduced osteoblastic and endothelial cells (*Online Supplementary Figure S7A* and *B*). Because SAA was associated with severe thrombocytopenia we examined expression of podoplanin (PDPN), recently identified in the BM and shown to increase platelet production.[33] We noted a striking loss in PDPN^{+} stromal cells in SAA (*Online Supplementary Figure S7C* and *D*). At the same time, however, we observed induction of PDPN on hematopoietic cells that appeared to be entirely restricted to Mϕs and a majority were CD11b$^{lo/-}$ Mϕs (Figure 6A and B, and *Online Supplementary Figure S8A*). We found no change in PDPN expression among other hematopoietic or non-hematopoietic stromal cells (*data not shown*). In addition, we observed increased podoplanin (gp38) transcripts specifically in the CD11b$^{lo/-}$ Mϕs, relative to CD11b^{+} Mϕs, T cells, and neutrophils in SAA mice (Figure 6C). PDPN^{+} Mϕs were reduced in MIIG mice relative to controls, though PDPN MFI was unchanged (*Online Supplementary Figure S8B* and *C*). This suggests that IFNγ increased numbers of PDPN^{+}BM Mϕs, rather than directly regulating PDPN expression, during SAA.

To determine if aberrant PDPN expression on Mϕs mediated pathology during SAA, we administered an anti-PDPN monoclonal antibody during SAA. PDPN blockade significantly increased CD41$^{lo/int}$ and CD41^{hi} HSCs and resulted in a preservation of BM cellularity compared to isotype control treatment (Figure 7A and B). Administration of an anti-PDPN antibody did not rescue HSCs by reducing or impairing T-cell activation because similar numbers of T-bet^{+} CD4 and CD8 T cells and IFNγ levels were observed in the BM of anti-PDPN and control-treated mice during SAA (*Online Supplementary Figure S9A* and *B*). Consistent with improved HSC numbers PDPN blockade rescued thrombocytopenia, increased BM Mks, and increased survival in SAA (Figure 7C-E). Anti-PDPN antibody conferred significant protection, whereas isotype-control antibody-treated mice had a median survival of only 16.5 days and died between days 12 and 19 induction of SAA.

PDPN-CLEC-2 interaction was reported to induce RANTES,[33] which can support platelet production, thus our finding that PDPN blockade improved thrombocy-

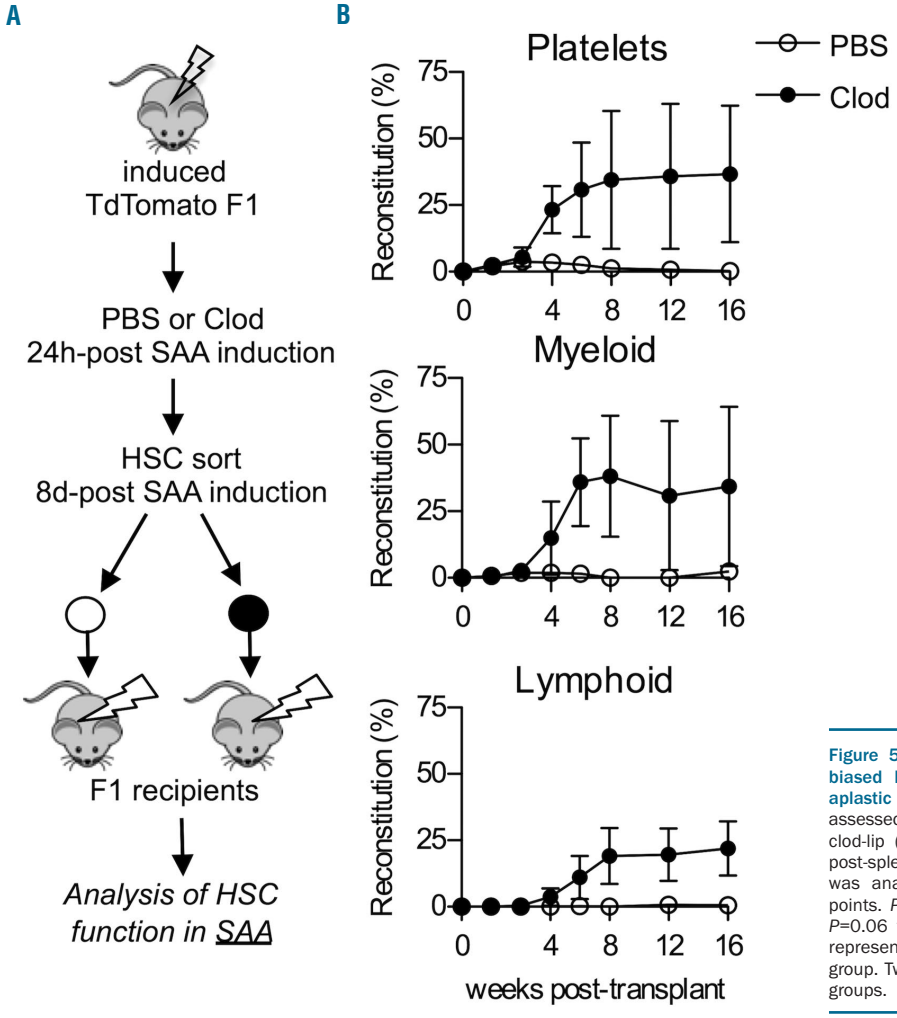

Figure 5. Macrophage depletion increases platelet-biased hematopoietic stem cells (HSC) in severe aplastic anemia (SAA). (A) HSC function in SAA was assessed by transplantation of HSCs from PBS- (™) or clod-lip (Clod;˜)-treated TdTomato+ F1 mice 8 days post-splenocyte transfer (d.p.s.t.). (B) Peripheral blood was analyzed for reconstitution at indicated time points. *P*<0.0001 for platelets, *P*=0.002 for myeloid, *P*=0.06 for lymphoid. Each transplantation data set represents one experiment, n=4-5 recipient mice per group. Two-way ANOVA was used to compare between groups.

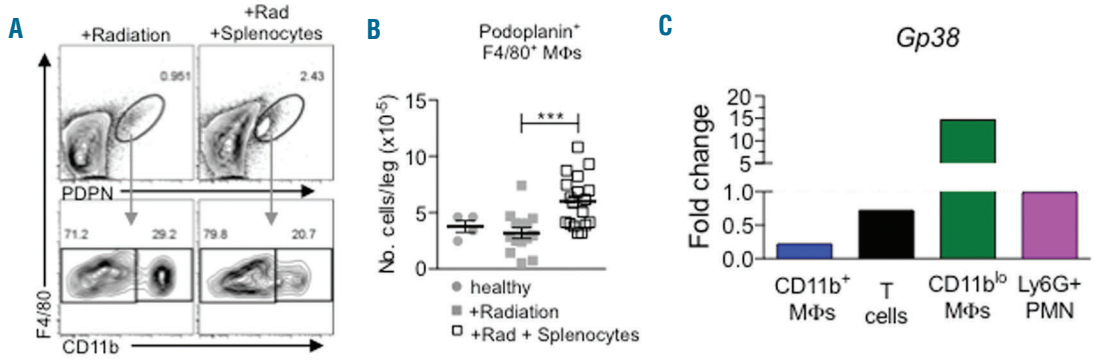

Figure 6. Macrophages exhibit increased expression of podoplanin (PDPN) during severe aplastic anemia (SAA). (A) PDPN expression in bone marrow (BM) cells (top) 8 days post-splenocyte transfer (d.p.s.t.). CD11b expression among PDPN$^+$ F4/80$^+$ cells (bottom). (B) PDPN$^+$ F4/80$^+$ MΦ numbers in healthy (●), radiation control (■), and SAA (+Rad +Splenocytes; □) mice 8 d.p.s.t. (C) Gp38 expression in sort-purified BM populations, relative to *β-actin* and normalized to expression in neutrophils. Data represent data pooled from 3 independent experiments n=5-10 mice/group.

topenia was somewhat surprising. Consistent with a role for PDPN in RANTES production we observed increased RANTES during SAA (*Online Supplementary Figure S9C*), where we also observe increased PDPN$^+$ Mϕs. PDPN blockade did not impact BM RANTES in SAA, likely because the antibody clone (8.1.1) does not interfere with CLEC-2 binding *in vivo* or *in vitro* (*Online Supplementary Figure S9D-F*).[34] Thus, PDPN-dependent HSC loss and hematopoietic failure occurs *via* a RANTES-independent mechanism.

Administration of anti-PDPN antibody induced a specific decrease in CD11b$^{lo/-}$ Mϕs whereas CD11^{b+} Mϕ numbers were not significantly different (Figure 7F). This suggests PDPN signaling may be important for CD11b$^{lo/-}$ Mϕ survival during SAA. It also demonstrates that selective reduction of CD11b$^{lo/-}$ Mϕs is associated with improved survival during SAA. PDPN can bind and activate ezrin, radixin, and moesin family proteins to promote cytoskeletal reorganization and contractility of fibroblastic reticular cells in lymph nodes.[34] Microenvironmental stiffness can reduce physical support for HSCs and Mks,[35] and we observed reduced expression of α-smooth muscle actin (αSMA), a marker of contractile stress fibers,[36] by PDPN$^+$ BM Mϕs at day 8 p.s.t. upon anti-PDPN treatment (*Online Supplementary Figure S10A* and *B*). We also noted a striking increase in expression of *arginase-1*, a marker of M2-polarized Mϕs, in both CD11b$^{lo/-}$ and CD11b$^+$ Mϕs upon anti-PDPN treatment (*Online Supplementary Figure S10C*). Thus, CD11b$^{lo/-}$ Mϕs aberrantly express PDPN in the BM during SAA, correlating with hematopoietic failure. Future studies are warranted to determine the precise impact of PDPN-expressing Mϕs on the microenvironment and whether stiffness and Mϕ-activation state contribute to SAA pathology.

Discussion

Hematopoietic stem cell loss and BM destruction are key features of SAA, and are associated with cytokine production by T cells.[6-8] It is still unclear, however, if inflammation depletes HSCs directly or does so through the microenvironment. Findings from SAA patient BM suggest that stromal support of hematopoietic cells is significantly reduced.[30-32,37,38] In a mouse model of SAA, we observed reduced stromal cells, but, at the same time, BM Mϕs were maintained. CD11b^{lo} Mϕs exhibited a unique survival advantage in SAA that correlated with their expression of PDPN. Consistent with our findings, SAA patient BM exhibited CD169$^+$ Mϕs persistence despite significant reductions in nearly all other hematopoietic cell types.[31] Our findings reveal that, rather than direct IFNγ-mediated HSC depletion, IFNγ signaling in Mϕs promotes HSC loss during SAA. IFNγ and Mϕs limit CD41^{hi} HSCs during disease, thus contributing to severe thrombocytopenia and mortality in SAA (Figure 7G). To the best of our knowledge, this is the first *in vivo* study addressing the mechanistic role of the BM microenvironment in HSC loss and disease progression during SAA.

HSCs reportedly undergo apoptosis during SAA,[39] yet studies in models of infection suggest that excessive differentiation and reduced self-renewal contribute to IFNγ-dependent HSC depletion.[40,41] We previously identified an IFNγ-dependent increase in monopoiesis during *Ehrlichia muris* infection, which occurred at the expense of HSCs.[42-44] Monocytes are increased early in SAA, prior to their ultimate loss, supporting the idea that increased IFNγ-driven HSC differentiation contributes to HSC loss. It is also possible that increased apoptosis in SAA is a product of enhanced differentiating divisions that render HSCs more susceptible to inflammatory stress and/or cell death.

Aberrant immune cell function, specifically T-cell activation and homing to the BM, is associated with SAA.[7] Since IFNγ primes Mϕs for activation,[25] and Mϕs produce cytokines and present antigen to T cells, we predicted that IFNγ signaling in Mϕs increase T-cell activation. SAA progression is mitigated in MIIG mice, however, despite similar numbers of activated and IFNγ-secreting donor T cells in the BM. Cytokines associated with SAA (TNFα, IL-1β, and IFNγ) were also similarly induced. Thus, resident Mϕs do not appear to drive disease through their capacity to present antigen to T cells or general inflammatory disposition. Mϕ polarization can contribute to disease through exaggerated inflammation and wound healing responses.[28] During SAA, differential expression of M1-associated *Nos2* was observed between the MIIG model and anti-PDPN treatment and between CD11b$^+$ and CD11b$^{lo/-}$ Mϕs, indicating functional differences between these two Mϕ

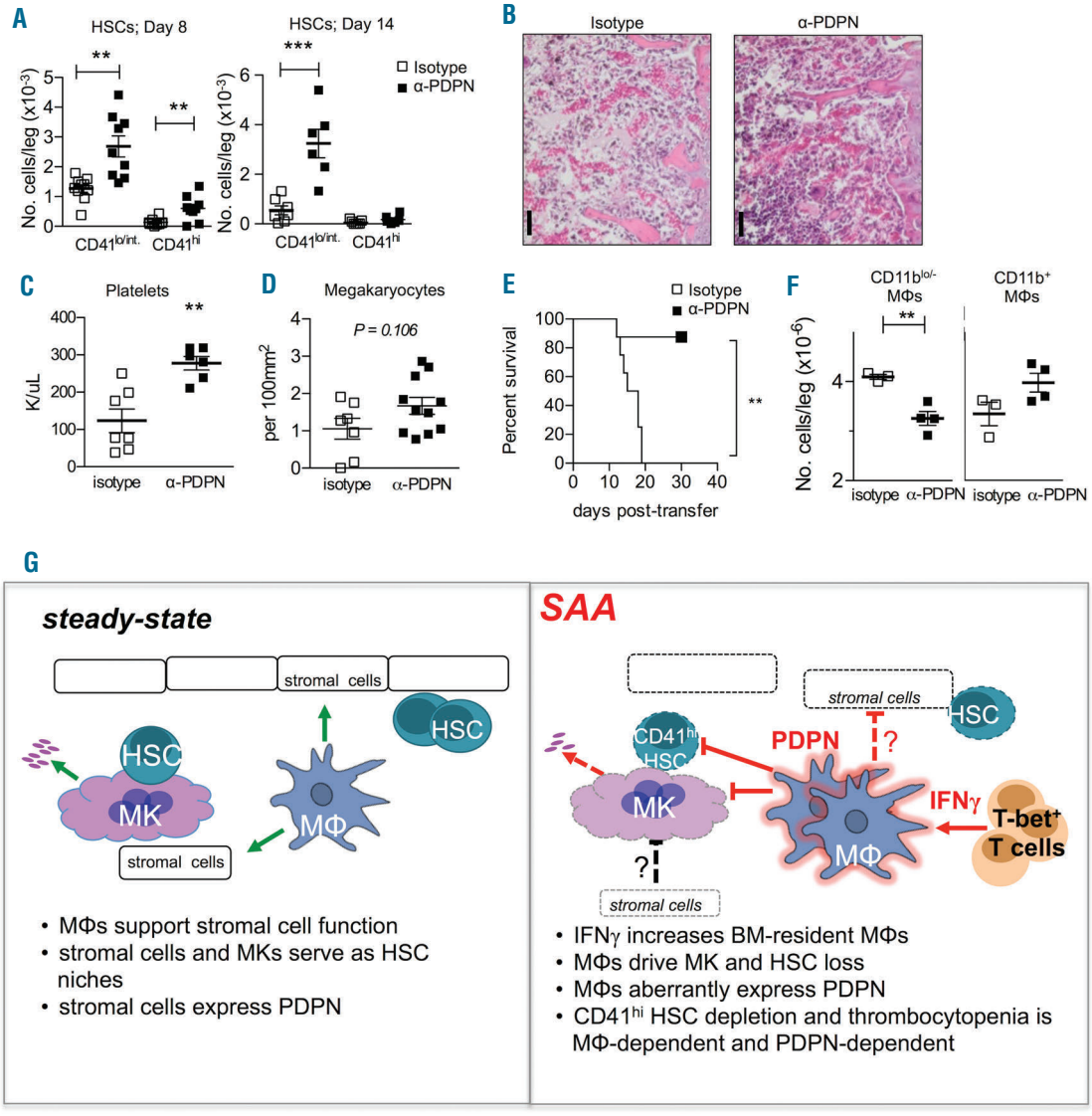

Figure 7. Podoplanin (PDPN) antagonism rescues hematopoietic stem cells (HSC), circulating platelet levels, and mortality. (A) CD41$^{lo/int.}$ and CD41^{hi} HSC numbers in anti-PDPN (■; α-PDPN) or isotype control (□)-treated mice 8 and 14 days post-splenocyte transfer (d.p.s.t.). (B) Hematoxylin and eosin-stained bone marrow (BM) from isotype control- or α-PDPN-treated mice 14 d.p.s.t. Scale bar=50 μm. (C) Platelets in the blood of isotype control- (□) and α-PDPN (■)-treated mice 14 d.p.s.t. (D) GP1bβ^{+} megakaryocytes per 100mm^{2} of BM 14 d.p.s.t. Data represent one experiment repeated at least twice, n=4-18 mice/group. Mean±Standard Error of Mean is shown. $^{**}P<0.01$, $^{***}P<0.001$. (E) Kaplan-Meier survival curve for α-PDPN- (■) or isotype control (□)-treated severe aplastic anemia (SAA) mice. Data represent one experiment, n=8 mice/group. Log-rank (Mantel-Cox) test was used to compare between groups. (F) CD11b$^{lo/-}$ and CD11b^{+} MΦ populations were enumerated 8 d.p.s.t. (G) Schematic summarizing the steady state role(s) for MΦs in the BM (left) and the impact of IFNγ on the BM microenvironment and resulting HSC loss in SAA (right): increased PDPN-expressing MΦs that drive reduced Mks, impaired platelet production, and correlate with reduced stromal.

populations. Of note, we observed increased expression of M2-associated *arginase1* in CD11b$^{lo/-}$ Mφs from MIIG mice and after anti-PDPN treatment. Mechanistically, a role for M1 activation in SAA pathogenesis may differ between the MIIG and anti-PDPN models; however, enhanced M2 activity correlates with protection in SAA.

Macrophages participate in immune-mediated thrombocytopenia (ITP), where increased platelet clearance drives thrombocytopenia and reduced platelet production.[45] Mφ clearance of platelets does not appear to drive thrombocytopenia in SAA, however, as clearance rates were similar in Mφ–depleted and control mice. Our findings are consistent with and add to previous reports of Mφ–dependent impairment of megakaryopoiesis and platelet production at steady state.[46,47] Additionally, macrophage-colony stimulating factor M-CSF, a factor that increases Mφ self-renewal, transiently causes thrombocytopenia.[47] Our study builds upon these findings and identify Mφs as key sensors or target cells of IFNγ.

Acute inflammation increases CD41^{hi} HSCs or SL-MkPs[15] and we observed the emergence of a CD41^{hi} HSC population in SAA that is accompanied by increased Mk numbers and platelet recovery when Mφs are depleted or unresponsive to IFNγ. While CD41^{hi} HSC-derived Mks may support sustained platelet production during SAA, another intriguing possibility is that Mk-lineage cells protect HSCs through a positive feedback loop. Mk-derived factors promote HSC quiescence and protect

HSC from myeloablative injury.[12,13] Therefore, CD41^{hi} HSCs may represent more committed progenitors that augment HSC-protective niches during SAA. It is currently unclear if Mk preservation is necessary for or predictive of HSC rescue. However, our finding that HSC loss precedes Mk loss would argue in favor of a decline in megakaryopoiesis as a result of reduced CD41^{hi} HSCs.

We identify a unique population of PDPN-expressing Mϕs that restrict the HSC compartment and contribute to thrombocytopenia during SAA. PDPN regulates contractility and migration of lymphatic endothelial cells, FRCs, and tumor cells.[48] Thus PDPN signaling in Mϕs may influence hematopoiesis in a similar manner by regulating BM stiffness or migration within BM niches. Because increased or decreased matrix stiffness impairs proplatelet extension by Mks *in vitro*, it is possible that SAA-induced stromal stiffness restricts thrombopoiesis.[45] PDPN expression is low and confined mainly to stromal cells in the BM at steady state and even upon radiation injury. PDPN is specifically increased on Mϕs in the BM during SAA, and anti-PDPN antibody specifically reduced CD11b$^{lo/-}$ Mϕs, but not CD11b^{+} Mϕs, during SAA. Our data demonstrate a direct correlation between CD11b$^{lo/-}$ Mϕs and SAA pathogenesis. Future studies to test the impact of PDPN expression on Mϕ migration and survival during SAA are warranted.

Podoplanin-expressing Mϕs are increased in SAA, consistent with previous reports that PDPN is expressed on inflammatory Mϕs in response to IFNγ during infection.[49] PDPN is a CLEC-2 ligand that triggers downstream signaling in CLEC2-expressing cells. However CLEC-2/PDPN ligation also elicits bidirectional signaling, eliciting RANTES production from PDPN-expressing cells.[33,34] We noted increased RANTES in SAA, relative to radiation controls. Thus, our observation that PDPN blockade did not impact SAA-induced RANTES was somewhat surprising. However, our data and a previous report demonstrate that clone 8.1.1 does not interfere with CLEC-2 binding.[34] Thus, PDPN blockade with clone 8.1.1 during SAA likely does not interfere with CLEC-2-driven RANTES production. Clone 8.1.1 may interfere with CLEC-2 binding,[50] though very high concentrations of antibody were needed, and it is unlikely that this can be achieved *in vivo*. Our observations demonstrate protection independently of CLEC-2; however, future studies are necessary to test the involvement of the CLEC-2-PDPN axis and define the precise action of PDPN in the BM during SAA.

Interferon-γ impacts SAA disease progression, yet neutralization of IFNγ is not currently a treatment option during this disease. Our finding that IFNγ maintains Mϕs provides the rationale for targeting Mϕs during BM failure. We reveal that Mϕs errantly express PDPN during SAA and antagonizing PDPN signaling rescues HSCs and enhances platelet output, thus revealing a novel circuit in the microenvironment during BM failure. Understanding the mechanisms whereby PDPN expression in Mϕs regulates HSC function and platelet production may reveal novel treatment options for SAA.

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