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**TITLE:** The Role of Histone Demethylase Jmjd3 in Immune-Mediated Aplastic Anemia

**PRINCIPAL INVESTIGATOR:** Yi Zhang

**CONTRACTING ORGANIZATION:** Temple University
**Philadelphia, PA 19140**

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**PREPARED FOR:** U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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**6. AUTHOR(S)**
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Temple University of the Commonwealth System
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**14. ABSTRACT**
Acquired aplastic anemia (AA) is a condition of bone marrow failure (BMF) characterized by blood pancytopenia and BM hypoplasia. In most cases, AA is an immune-mediated disorder with destruction of hematopoietic stem and progenitor cells by T cells. There is increasing evidence that CD4+ T effector cells that produce high levels of IFN-γ are associated with AA in patients and experimental AA mice. IFN-γ displays potent effects on suppressing hematopoiesis. Immunosuppressive therapy with antithymocyte globulin in combination with cyclosporin A (CsA) can induce a hematologic response in about two-thirds of AA patients. However, relapse occurs in up to 35% of AA patients when CsA is withdrawn at 6 months. Allogeneic BM transplantation (BMT) has significantly improved the survival of AA. However, graft-versus-host disease (GVHD) remains a major barrier to the success of the procedure. Novel approaches are needed to improve the outcomes of AA treatment.

**15. SUBJECT TERMS-**

None listed

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## 1. INTRODUCTION:

Acquired aplastic anemia (AA) is a condition of bone marrow failure (BMF) characterized by blood pancytopenia and BM hypoplasia. In most cases, AA is an immune-mediated disorder with destruction of hematopoietic stem and progenitor cells by T cells. There is increasing evidence that $CD4^+$ T effector cells that produce high levels of IFN-$\gamma$ are associated with AA in patients and experimental AA mice. IFN-$\gamma$ displays potent effects on suppressing hematopoiesis. Immunosuppressive therapy with antithymocyte globulin in combination with cyclosporin A (CsA) can induce a hematologic response in about two-thirds of AA patients. However, relapse occurs in up to 35% of AA patients when CsA is withdrawn at 6 months. Allogeneic BM transplantation (BMT) has significantly improved the survival of AA. However, graft-versus-host disease (GVHD) remains a major barrier to the success of the procedure. Novel approaches are needed to improve the outcomes of AA treatment.

The long-term goal of our studies is to develop novel approaches to control inflammatory T cells causing BMF and GVHD. The objective of this application is to define the role of Jmjd3, which is a histone demethylase, in regulating inflammatory T cell responses, and identify an optimal approach to reduce BMF and GVHD by inhibiting Jmjd3. The **rationale** of these studies is that if we identify the critical roles of JMJD3 and its regulated mechanisms in BM-destructing T cells, we can further define optimal therapeutic approaches to modulate inflammatory T cell responses for controlling AA. These studies are **highly significant** because they would potentially lead to novel and clinically relevant strategies to improve the outcomes of therapy for AA, and may have broad implications in other T cell-mediated disorders such as autoimmune diseases and chronic infection.

## 2. KEYWORDS:

1.1. Aplastic anemia
1.2. T cells
1.3. Th1 cells
1.4. Th17 cells
1.5. MDSC (Myeloid derived suppressive cells)
1.6. Jmjd3
1.7. Utx
1.8. GSK-J4
1.9. Ezh2
2.9. H3K27me3
2.10. Graft-versus-host disease (GVHD)
2.11. Bone marrow transplantation
2.12. Hematopoietic stem cells (HSCs)
2.13. Myeloid derived suppressive cells (MDSCs)

**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?**

The major goals of the project include:

**1)** Understanding the roles of JMJD3 in the generation and maintenance of BM-destructive T cells;

**2)** Understanding the cellular mechanisms of JMJD3 action in inflammatory T cells is essential to establish how BMF-mediating T cells develop and persist during AA process;

**3)** Identifying the molecular mechanisms by which JMJD3 and its-counteracting enzyme Ezh2 orchestrate transcriptional programs (such as T-bet) for inducing and sustaining T cells mediating BMF.

**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.*

1. **Major activities:** The major activities during the past one year of support focus on optimizing the pharmacological and genetic approaches of inhibiting Jmjd3 for the purpose of reducing AA in mice.

2. **Specific objectives:** The objectives are: 1) identifying the effect of in vivo administration of GSK-J3 on modulating inflammatory T cell-mediated AA in mice; 2) using genetic approach to define the precise role of Jmjd3 in T cell-mediated AA in mice; and 3) establishing a pharmacological inhibition of Jmjd3-based approach to control AA in mice.

3. **Significant results:** Over the past year of support, we have achieved significant progress in understanding the impact of inhibiting Jmjd3 on inflammatory T cell-mediated AA in mice. Furthermore, we have established that combined inhibition of Jmjd3 using its specific inhibitor and administration of myeloid derived suppressive cells (MDSCs). Below describe our experimental findings:

**A. Jmjd3 is rapidly induced in TCR-activated T cells and important for their survival and expansion in cultures.** Jmjd3 is rapidly induced in TCR-activated T cells and important for their survival and expansion in cultures. To examine the role of Jmjdj3 in activated CD8$^+$ T cells, we activated mouse CD8$^+$ T cells by anti-CD3 Ab and cells within ani-CD28 Ab over a period of 24 hours. RNA was extracted from 2 hours of TCR these cells at indicated time points of culture. The expression of Jmjd3 increased by 4 hours and peaked by 6 hours. TCR activation induced Ezh2 in CD8 T cells at a slower rate than Jmjd3, peaked by 24 hours after activation (**Fig.1a**). Notably, TCR ligation did not significantly influence the expression of Utx (**Fig.1a**), which also demethylates H3K27me3. Thus, Jmjd3 is selectively and rapidly induced in T cells early after TCR ligation.

JMJD3 is a histone demthylase that mediates demethylation of H3K27me3 and acts primarily as a gene activator. To determine whether inhibiting JMJD3 may influence the survival and expansion of alloantigen-activated T cells, we cultured

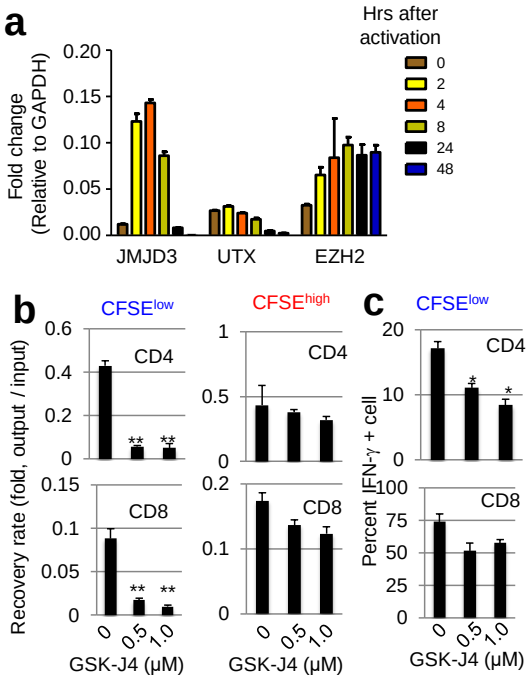


**Fig.1.**

C57BL/6 (B6) T cells In the presence of DCs derived from BALB/C BM cells, with or without addition of the Jmjd3 Inhibitor GSK3-J4. T cells were labeled with CFSE to measure whether inhibiting Jmjd3 may selectively impair the expansion of highly proliferative T cells. We found that addition of GSK-J4 led to 8- and 6-fold reduction of $CFSE^{low}$ CD4 and CD8 T cells, but have minimal effect on the survival of non-dividing T cells (**Fig.1b**). Notably, GSK-J4 treatment caused 2-fold decrease in frequency of IFN-γ of proliferating CD4 T cells, rather than CD8 T cells (**Fig.1c**). Our findings identify for the first time that Jmjd3 promotes the survival and expansion of antigen-driven proliferating T cells. However, although Jmjd3 is important for inducing IFN-γ in proliferating CD4 T cells, it may be dispensable for CD8 T cell production of effector cytokines.

**B. Administration of GSK-J4 only results in limited effect on reducing AA.** We next examined the impact of GSK-J4 on BMF in mice. To identify appropriate doses of GSK-J4 for in vivo modulation of inflammatory T cells -mediating BMF, we transferred 2x10^7 B6 mouse-derived lymph node cells($H2K^b$), which were labeled with CFSE, into non-irradiated BDF1 mice ($H2K^{d/b}$). In this setting, alloantigen-activated T cells undergo proliferation and show dilution of CFSE, whereas alloantigen-nonresponding T cells do not divide without CFSE dilution. GSK-J4 was administered at day 4, day 5 and day 6 after transfer of lymph node cells. These recipients were killed at day 7 to measure proliferation, expansion and IFN-γ production by B6 T cells (**Fig.2**). PBS was administered as control (CTR). We found that in vivo administration of 30 mg / Kg GSK-J4 only moderately decreased the expansion of B6 CD8 T cells but not CD4 T cells in the spleen compared to control treatment (**Fig.2a**). This dose of GSK-J4 treatment resulted in 1.5-fold in frequency of IFN-γ producing T cells in the spleen (**Fig.2b**). These data suggest that GSK-J4 treatment demonstrates moderately effects on reducing expansion and IFN-γ production by alloantigen-activated T cells in vivo. Since by the time of GSK-J4 treatment, B6 T cells have already been activated, it is possible delayed administration od GSK-J4 may have limited effect on inhibiting already activated T cells.

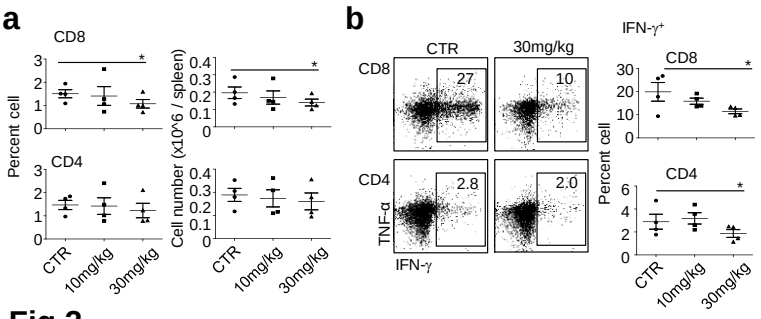


**Fig.2.**

To rule out this possibility and to examine whether GSK-J4 treatment may have differential roles in modulating inflammatory T cell responses in AA mice, we tested whether administration of GSK-J4 to sub-lethally irradiated BDF1 receiving B6 lymph node cells reduced the development of BMF. As expected, transfer of 10x10^6 lymph node

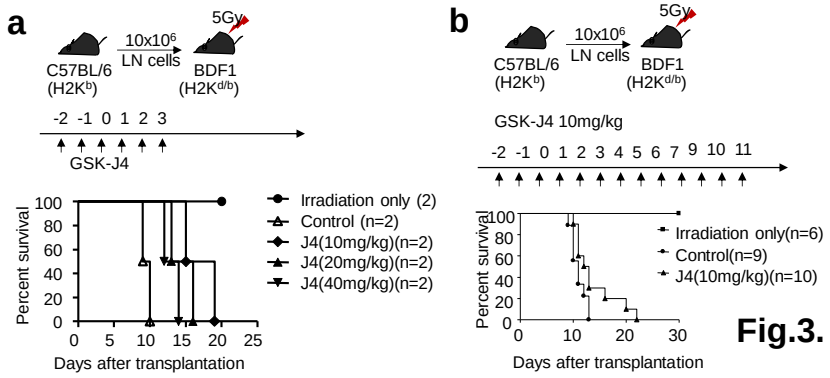


**Fig.3.**

cells induced lethal BFM 10 days after transfer (**Fig.3a**). Administration of GSK-J4 10 mg / Kg slightly prolonged survival time, however, all BDF1 mice died from BMF by day 23 (**Fig.3a,b**). Increasing the dose of in vivo administered GSK-J4 to 40mg / kg did not prolonged the survival, instead, it caused early death compared to 10mg / Kg of GSK-J4 (**Fig.3a**).

Taken together, these results suggest that although Jmjd3 could effectively inhibit the ex vivo proliferation and cytokine production in cultures, in vivo administration of GSK-J4 results in limited effect on inhibiting the inflammatory T cell response that mediates BMF in mice. This raises an important question that Jmjd3 in T cells might not be an important target for reducing BMF in mice. Alternatively, systemic administration of GSK-J4 treatment in vivo could influence non-T cells that could potentially decrease the effect of systemic GSK-J4 on inflammatory T cells.

**C. Jmjd3 in T cells is important for their induction of AA in mice.** To address these two key issues, we examined whether conditional deletion of JMJD3 in T cells may influence their capacity to mediate BMF in mice. We crossed Jmjd3$^{fl/fl}$ B6 mice to Cd4-Cre B6 mice to generate Cd4-Cre/ Jmjd3$^{fl/fl}$ B6 mice, who are termed Jmjd3-cKO mice. We found that transfer of low dose of lymph node cells (2~3x10^6 cells / mouse) from these Cd4-Cre/ Jmjd3$^{fl/fl}$ B6 mice protected 70% of BDF1 mice from lethal BMF (**Fig.4a**). However, BDF1 mice receiving higher dose of Cd4-Cre/ Jmjd3$^{fl/fl}$ B6 lymph node cells (10x10^6 cells / mouse) developed lethal severe BMF, with all dying from the disease (**Fig.4b**). These data indicate that while inhibiting Jmjd3 in T cells can ameliorate the severity of AA in mice, but this effect disappeared when the dose of inflammatory T cells surpass certain threshold, e.g., more than 3x10^6 cells / mouse. Nevertheless, these data suggest that T cell Jmjd3 is an important target for modulating BMF. Thus, it is likely that inhibiting Jmjd3 in T cells may not completely block effector function of inflammatory T cells.

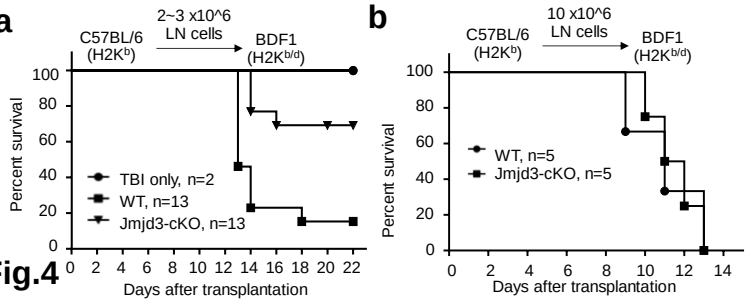


**Fig.4**

**D. Loss of Jmjd3 leads to dramatically impaired expansion of CD4 T cells in in vivo in AA mice**. To understand the mechanisms by which loss of T cell Jmjd3 reduces BMF in mice, we transferred WT and Jmjd3-cKO B6 mouse-derived lymph node cells into sublethally irradiated BDF1 mice (n=3 ~ 5), isolated donor T cells at day 12 after transplantation and measure the expansion and IFN-γ production by these T cells (**Fig.5**). We found that loss of Jmjd3 resulted in significantly decreased frequency and numbers of donor CD4 and CD8 T cells in the spleen of BDF1 mice during AA (**Fig.5a**) Similar effects of Jmjd3 deficiency on decreasing CD4 T cell accumulatio and IFN-γ production also occurred in the BM of these BDF1 mice (**Fig.5c**). Intriguingly, as we observed in prior experiments (**Fig.1b**), we found that Jmjd3 inhibition showed minimal effect on reducing the frequency of IFN-γ-producing CD8 T cells in the spleen and BM (**Fig.5b, c**). These different impacts of Jmjd3 deficiency on inhibiting the expansion and IFN-γ production between CD4 and CD8 T cells may explain why inhibiting Jmjd3 results in moderate effects on reducing AA in these mice educed T cell response in spleen.

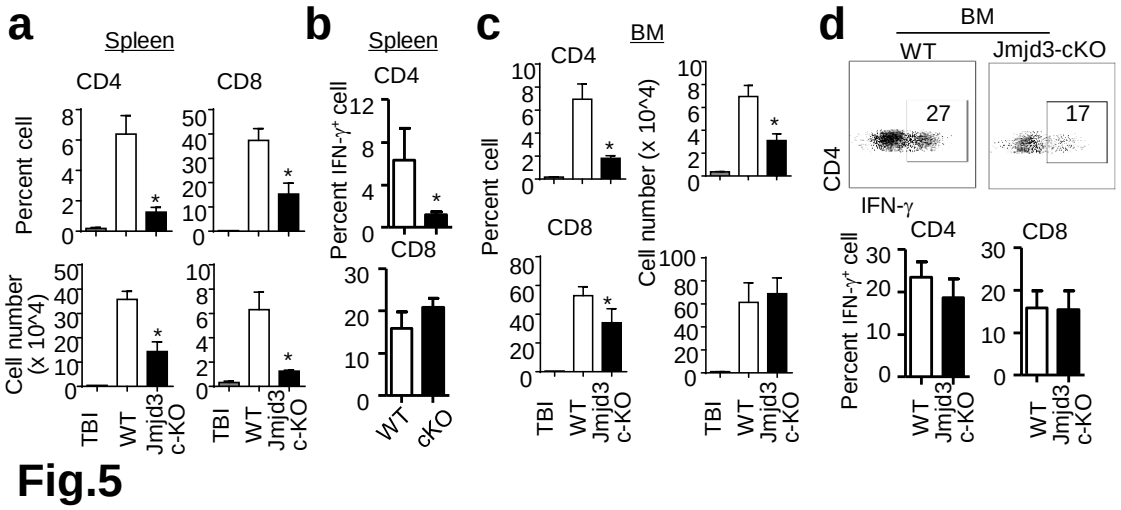


**Fig.5**

**E. Combined administration of GSK-J and MDSCs significantly improves the efficacy on reducing AA in mice**. We wanted to identify an optimal approach to reduce AA that can be potentially translated into patients in future. To this end, we asked whether combined therapy using Jmjd3 inhibitor and MDSCs may enhance the effect of Jmjd3 inhibition on reducing AA. Previously studies have established that MDSCs are potently immune regulators that can suppress T cell immune responses. Using ex vivo cultures, we observed that B6 mouse BM cell-derived MDSCs suppressed the expansion and effector cytokine production (e.g., IFN-γ and TNF-α) (**Fig.6a,b**). Interestingly, ex vivo treatment of MDSCs with GSK-J4 markedly decreased the expression of genes encoding immune suppressive molecules (e.g., Tgfb1, Il10, Ido and Arg1) (**Fig.6c**). This effect of GSK-J4 explains that systemic administration of GSK-J4 results in limited effects on reducing antigen-driven T cell responses compared to GSK-J4 effect on inhibiting in vitro activated T cells (**Fig.1b**).

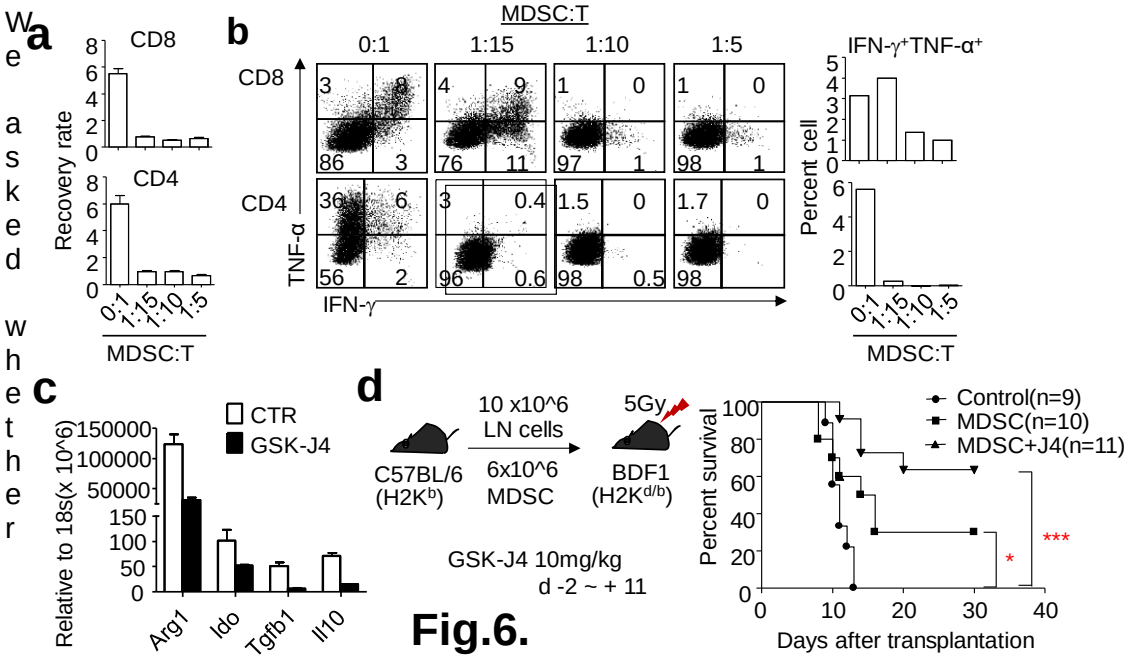


**Fig.6.**

We asked whether MDSCs or the combination of MDSCs an GSK-J4 treatment in vivo may enhance the capacity of GSK-J4 to reduce AA in mice. To test it, we administered MDSCs with or without GSK-J4 to sublethally irradiated BDF1 mice (**Fig.6d**). We found that combined administration of MDSCs + GSK-J4 significantly improved the survival of these BDF1 mice compared to control mice and mice treated with MDSCs along (**Fig.6d**).

In summary, results from the past two years of studies have illuminated the role of inhibiting Jmjd3 in the modulation of AA in mice. Further studies are needed to define the mechanisms by which combined MDSCs and Jmjd3 inhibition leads to augmented effects on reducing AA in mice. In particularly, we want to define whether this combined approach helps reducing the expansion and effector differentiation of both CD4 and CD8 T cells during AA.

**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.*

N/A

**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

*Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.*

1. Understanding the mechanisms by which combined administration of MDSCs and GSK-J4 induces enhanced effects on controlling AA in mice
2. Writing up and publishing the paper

## *4.* IMPACT:

**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).*

Nothing to report

**What was the 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:*

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.*

Our research was significantly slowed down due to: 1) acquiring Jmjd3 conditional knockout mice; 2) unexpected results showing that systemic administration of GSK-J4 results in limited effects on reducing AA. We now understand that GSK-J4 have limited effect on reducing CD8 T cell-mediated inflammatory responses in vivo, and inhibiting Jmjd3 may impair the function of MDSCs, which are important for regulating T cell inflammation; 3) delayed approval of our animal protocol during the time period of applying to no-cost-extension. We have solved these problems and will be able to complete the project as 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.*

No significant changes.

**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**

No significant change.

**Significant changes in use of biohazards and/or select agents**

No significant change.

## 6. PRODUCTS:

- **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).*

| |
|---|
| No 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.*

| |
|---|
| Nothing to report. |

- **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.*

| |
|---|
| N/A. |

- **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.*

N/A.

## 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.)* |

Yi Zhang
Hongxing Sun
Yuanyuan Tian

**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.*

| Nothing to 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:*
*<u>Organization Name:</u>*
*<u>Location of Organization: (if foreign location list country)</u>*
*<u>Partner's contribution to the project</u> (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:** *N/A*

**QUAD CHARTS:** *N/A*

**9. APPENDICES:** *N/A*