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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 However, relapse occurs in up to 35% of AA patients when CsA is withdrawn at 6 months.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.

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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-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 However, relapse occurs in up to 35% of AA patients when CsA is withdrawn at 6 months.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.

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?

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

The major goals 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.

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 three year of support include: **1)** defining the role of Jmjd3 in regulating BM-destructing T cells; **2)** optimizing the pharmacological and genetic approaches of inhibiting Jmjd3 for the purpose of reducing AA in mice; and **3)** establishing the beneficial effects of targeting epigenetic regulators (Ezh2, Jmjd3) and antigen-presenting cells (APCs) on reducing graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT)

2. **Specific objectives:** The objectives will: **1)** identify the critical role of T cell Jmjd3 in the regulation of effector T cell responses that mediate BMF; **2)** establish an optimized pharmacological approach to reduce BMF in mice inhibition of Jmjd3-based approach to control AA in mice; and **3)** develop novel approaches to reduce GVHD for improving the safety and efficacy of allo-HSCT that is a potentially curative therapy for patients with advanced AA.

3. **Significant results:** Over the past three years of support, we have achieved significant progress in understanding the impact of inhibiting Jmjd3 on inflammatory T cell-mediated AA in mice. Building on these findings, we have established that combined Jmjd3 inhibition and administration of myeloid derived suppressive cells (MDSCs) effectively reduces AA in mice (See **Accomplishment A**). In addition, with the great support of this project, we have extended our studies to better understanding of pathophysiology of GVHD and biology of allo-HSCT. Our group has developed new and clinically relevant approaches to reduce GVHD and improve the safety of allo-HSCT (**Accomplishment B**). Below describe our experimental findings:

Accomplishment A: Identifying the impact of inhibiting Jmjd3 on inflammatory T cell-mediated AA in mice.

1). Jmjd3 is rapidly induced in TCR-activated T cells and important for their survival and expansion in cultures. To examine the role of Jmjd3, which demethylates H3K27me3, in T cell responses, we first determined the dynamic expression level of Jmjd3 after T cell activation. Mouse $CD4^+$ T cells and $CD8^+$ T cells were stimulated with antibodies specific for CD3 and CD28. Quantitative reverse transcription PCR (RT-qPCR) analysis showed that expression of Jmjd3 was rapidly increased in both $CD4^+$ T cells and $CD8^+$ T cells 2 hours after activation of TCR and CD28, gradually declined by 24 hours (**Fig.1**). In contrast, TCR activation had no significantly impact on the expression of Utx mRNA (**Fig.1**). As expected, TCR activation induced Ezh2, which catalyzes H3K27me3, in T cells at a slower rate than Jmjd3, peaked by 24 hours after activation and maintained throughout 48 hours (**Fig.1**). Thus, Jmjd3 is selectively and rapidly induced in T cells early after TCR ligation.

2). Jmjd3 in T cells is important for their induction of AA in mice. To understand the precise role of Jmjd3 in T cell-mediated BMF, we crossed C57BL/6 (B6) mice with floxed alleles of Jmjd3 ($Jmjd3^{fl/fl}$) to B6 mice expressing Cre recombinase under control of the CD4 promoter, and generated T cell-specific Jmjd3 conditional knockout B6 mice (named Jmjd3-cKO mice). Lymph node cells were isolated from WT and Jmjd3-cKO B6 mice ($H2K^b$) and transferred into irradiated (6.5 Gy) BDF1 recipients ($H2K^{d/b}$). In this setting, BDF1 mice receiving WT donor LN cells developed BMF, as evidenced by significant weight loss and 90% of them dying from the disease within 12 days after transfer. In contrast, transfer of Jmjd3-cKO LN cells protected 70% of BDF1 recipients from lethal BMF (**Fig.2a, b)**. As compared with control mice receiving total body irradiation, there was no significant reduction of BM cellularity and peripheral blood platelets in these BDF1 mice receiving Jmjd3-cKO LN cells, 12 days after transfer (**Fig.2c, d)**. These data suggest that selective inhibition of Jmjd3 in T cells leads to reduction of BMF in mice.

We next examined the mechanism by which deletion of Jmjd3 in T cells reduced their capacity to mediate BMF in mice. Donor T cells were isolated from the spleen and BM of BDF1 recipient mice 12 days after transfer of WT or Jmjd3-cKO LN cells. Deletion of T cell Jmjd3 resulted in significantly decreased frequency of donor $CD4^+$ and $CD8^+$ T cells in the BM and spleen (**Fig.3a, b**). Similar effects of Jmjd3 deficiency on decreasing T cell

accumulation and IFN-γ production also occurred in the BM of these BDF1 mice (**Fig.3c,d,e**). Collectively, these results suggest that during the AA process, Jmjd3 is involved in regulating the expansion and IFN-γ production of activated T cells. This explains the effect of inhibiting Jmjd3 in T cells on their mediated BMF in mice.

3). Pharmacological inhibition of Jmjd3 using GSK-J4 reduces the expansion and IFN-γ production of TCR-activated T cells. To test the impact of pharmacological Inhibition of JMJD3 on reducing BMF in mice, we examined whether the Jmjd3 inhibitor GSK-J4 may influence the survival and expansion of alloantigen-activated T cells. B6 T cells ($H2K^b$) were cultured in the presence of allogeneic DCs derived from BALB/C BM cells ($H2K^d$), with or without addition of GSK-J4. T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) to monitor their proliferation and examine the impact of Jmjd3 inhibition on alloantigen-responding T cells ($CFSE^{low}$) and non-responding T cells ($CFSE^{high}$). Addition of GSK-J4 from the day of culture led to 8- and 6-fold reduction of $CFSE^{low}$ CD4 and CD8 T cells (**Fig.4a**), but had minimal effect on the survival of non-dividing $CFSE^{high}$ T cells (**Fig.4b**). Inhibition of Jmjd3 significantly decreased the frequency of IFN-γ secreting cells (**Fig.4c**). These data suggest that Jmjd3 promotes the survival and expansion of antigen-driven proliferating T cells and their production of IFN-γ in response alloantigens. However, Jmjd3 is dispensable for alloantigen non-responding T cells.

Given the fact that TCR-ligation induced rapid expression of Jmjd3 as shown in our prior experiments (**Fig.3**), we reasoned that inhibited activation of T cells by adding GSK-J4 from the beginning of culture might account for their subsequent proliferation and effector differentiation. To rule out this possibility, we addedGSK-J4 to the MLR cultures 3 days after DC activation of allogenic T cells. This would allow us to test the impact of Jmjd3 inhibition on prior activated T cells. Delayed addition of GSK-J4 markedly decreased expansion of alloantigen-activated T cells and their production of IFN-γ (**Fig.4e-g**), confirming the role of Jmjd3 in regulating allogeneic T cell responses.

4). Administration of GSK-J4 results in limited effect on reducing AA. We next examined the potential effect of pharmacological inhibition of Jmjd3 using GSK-J4 on T cell response in mouse models of 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.5a**). PBS was administered as control (CTR). Administration of GSK-J4 reduced the percentage of donor CD4 and CD8 T cells in a dose-dependent manner (**Fig.5b**), and significantly inhibited production of IFN-γ in CD8 T cells (**Fig.5c**) in the spleen. These data suggested that in vivo administration of GSK-J4 markedly reduces expansion of alloantigen-activated T cells and their production of and IFN-γ.

To determine the effect of GSK-J4 treatment on BMF in mice, we transferred $1x10^7$ B6 lymph node cells into sub-lethally irradiated BDF1 recipients, followed by treatment with GSK-J4 (10 mg/Kg) from day -2 of transplantation. As expected, transfer of B6 lymph node

cells induced lethal BMF in these mice around 10 days after transfer (**Fig.5d**). Administration of GSK-J4 slightly prolonged survival time, however, all BDF1 mice died from BMF by day 23 (**Fig.5d**). Increasing the dose of in vivo administered GSK-J4 to 40mg/kg caused early death due to drug toxicity (**data not shown**).

Taken together, these results suggest that although inhibiting Jmjd3 markedly inhibits the ex vivo proliferation and cytokine production in cultures, in vivo administration of GSK-J4 results in limited effect on inhibiting BMF in mice. This raises an important question that pharmacological inhibition of Jmjd3 by systemic administration of GSK-J4 in vivo might influence non-T cells, leading to reduced effect of inhibiting Jmjd3 on decreasing inflammatory T cells.

5). Combined administration of GSK-J4 and MDSCs significantly improves the efficacy on reducing AA in mice. Myeloid-derived suppressor cells (MDSCs) are known for their immunosuppressive function[19,20]. MDSCs produce high levels of immunosuppression molecules, such as arginase (Arg)1 and nitric oxide synthase (iNOS), to suppress T cell proliferation and survival[19,21-23]. Thus, MDSCs have been implicated in modulation of autoimmunity, tumor immunity and GVHD. BMF is characterized by blood pancytopenia and BM hypoplasia. We hypothesized that BMF caused the defect in generation of MDSCs, thereby impairing the restoration of immune regulatory mechanisms during GSK-J4 treatment to BMF.

To test this possibility, we generated MDSCs by culturing BM cells in the presence of G-CSF + GM-CSF for five days. IL-13 was added from day 4 to activate suppressive function. $CD11b^{+}Gr1^{+}$ MDSCs were purified from the culture and added to cultures of T cells that were stimulated with anti-CD3Ab + anti-CD28Ab. As expected, we observed that B6 mouse BM cell-derived MDSCs suppressed the expansion and effector cytokine production of both CD4 and CD8 T cells (e.g., IFN-γ and TNF-α) (**Fig.6a, b**).

We finally asked whether combined therapy using GSK-J4 and MDSCs may lead to effective control of AA in mice. Previously studies have established that MDSCs are potently immune regulators that can suppress T cell immune responses. MDSCs were transferred together B6 LN cells into sublethally irradiated BDF1 mice. We found that while BDF1 mice receiving B6 LN cells all died from BMF, addition of MDSCs protected 30% of BDF1 mice from lethal BMF. Combined administration of GSK-J4 and MDSCs significantly reduced the incidence of BMF, leading to 64% them surviving without BFM (**Fig.6c**). Thus, Combined treatment of MDSCs together with Jmjd3 inhibition provide greater protection than either method to reducing AA in mice.

In summary, results from the past three years of studies have illuminated the role of inhibiting Jmjd3 in the modulation of AA in mice. Future studies are needed to define whether this combined approach of transferring MDSCs and inhibiting Jmjd3 helps reducing the expansion and effector differentiation of BM-destructing T cells during AA, and facilitates the restoration of immune regulatory mechanisms. Identifying the underlying mechanisms by which combined treatment of MDSCs and Jmjd3 inhibition leads to augmented effects on reducing AA in mice may lead to new methods to control AA. The

manuscript reporting these findings is in preparation and will be submitted to Journal of Immunology.

Accomplishment B: Developing new and clinically relevant approaches to reduce GVHD after allo-HSCT.

With the support of this DOD-sponsored project, we have expanded our studies to understanding of pathophysiology of GVHD and allo-HSCT. New and clinically relevant approaches have been developed by our group for reducing GVHD after allo-HCT. As a result, improved the safety of allo-HSCT will allow the application of this procedure to treatment of BMF. We have achieved two major accomplishments in understanding of pathophysiology of GVHD and allo-HSCT and development of novel strategies to inhibit GVHD as described below:

1). We have discovered that targeting Ezh2 represents an effective strategy to modulate alloimmunity. Ezh2 is a histone methyltransferase that specifically catalyzes H3K27me3. We have previously discovered the essential roles of Ezh2 in the regulation of alloreactive T cell responses. During the past three years, we discovered: i). pharmacological inhibition of Hsp90 destabilizes Ezh2 protein in alloreactive T cells and reduces GVHD; and ii). Ezh2 controls the generation and maintenance of memory T cells that play important roles in sustaining GVHD and BMF. Thus, these findings provide new molecular targets for investigating the role of T cell immunity in mediating GVHD and BMF. Below brief these findings:

i). Qingrong Huang, Shan He, Yuanyuan Tian, Yuting Gu, Pan Chen, Changhong Li, Jiefang Huang, Yongnian Liu, Min Jin, Shaoyan Hu, Qing Tong, Anqi Ma, Jian Jin, Elizabeth Hexner, Henry Fung, Ran Reshef, **Yi Zhang (Correspondence author)** and Yanyun Zhang. Hsp90 inhibition destabilizes Ezh2 protein in alloreactive T cells and reduces graft-versus-host disease in mice. (Blood 2017, 129(20):2737-2748; **Cover story**).

Modulating T-cell alloreactivity has been a main strategy to reduce GVHD, a life-threatening complication after allo-HSCT. Genetic deletion of T-cell Ezh2, which catalyzes trimethylation of histone H3 at lysine 27 (H3K27me3), inhibits GVHD. Therefore, reducing Ezh2-mediated H3K27me3 is thought to be essential for inhibiting GVHD. We tested this hypothesis in mouse GVHD models. Unexpectedly, administration of the Ezh2 inhibitor GSK126, which specifically decreases H3K27me3 without affecting Ezh2 protein, failed to prevent the disease. In contrast, destabilizing T-cell Ezh2 protein by inhibiting Hsp90 using its specific inhibitor AUY922 reduced GVHD in mice undergoing allo-HSCT. In vivo administration of AUY922 selectively induced apoptosis of activated T cells and decreased the production of effector cells producing interferon γ and tumor necrosis factor α, similar to genetic deletion of Ezh2. Introduction of Ezh2 into alloreactive T cells restored their expansion and production of effector cytokines upon AUY922 treatment, suggesting that impaired T-cell alloreactivity by inhibiting Hsp90 is achieved mainly through depleting Ezh2. Mechanistic analysis revealed that the enzymatic SET domain of Ezh2 directly interacted with Hsp90 to prevent Ezh2 from rapid degradation in activated T cells.

Importantly, pharmacological inhibition of Hsp90 preserved antileukemia activity of donor T cells, leading to improved overall survival of recipient mice after allogeneic HSCT. Our findings identify the Ezh2-Hsp90 interaction as a previously unrecognized mechanism essential for T-cell responses and an effective target for controlling GVHD.

ii). Shan He, Yongnian Liu, Lijun Meng, Hongxing Sun, Janaki Purushe, Pan Chen, Changhong Li, Jozef Madzo, Jean-Pierre Issa, Jonathan Soboloff, Bethany B Moore, Luca Gattinoni and **Yi Zhang**. The Phosphorylation State of Ezh2 Determines its Capacity to Maintain $CD8^+$ Memory T Cells for Antitumor Immunity. Nature Communications, 2017 (DOI:10.1038/s41467-017-02187-8).

Memory T cells sustain effector T-cell production while self-renewing in reaction to persistent antigen; yet, excessive expansion reduces memory potential and impairs antitumor immunity. Epigenetic mechanisms are thought to be important for balancing effector and memory differentiation; however, the epigenetic regulator(s) underpinning this process remains unknown. Herein, we show that the histone methyltransferase Ezh2 controls $CD8^+$ T memory precursor formation and antitumor activity. Ezh2 activates *Id3* while silencing *Id2*, *Prdm1* and *Eomes*, promoting the expansion of memory precursor cells and their differentiation into functional memory cells. Akt activation phosphorylates Ezh2 and decreases its control of these transcriptional programs, causing enhanced effector differentiation at the expense of T memory precursors. Engineering T cells with an Akt-insensitive Ezh2 mutant markedly improves their memory potential and capability of controlling tumor growth compared to transiently inhibiting Akt. These findings establish Akt-mediated phosphorylation of Ezh2 as a critical target to potentiate antitumor immunotherapeutic strategies.

2). We have identified the crucial role of $DLL4^+$ dendritic cells in the regulation of alloimmunity. Notch signaling is critical for GVHD responses. Notch receptors interact with Notch ligands of the δ-like and Jagged families, triggering the release of intracellular Notch that activates Notch target genes. Inhibiting pan-Notch signaling in donor T cells reduced their production of IFN-γ-producing T helper (Th)1 and IL-17-producing Th17 cells. Notch ligand DLL4 mediates a dominant role for activating Notch signaling in alloreactive T cells. We previously discovered that DLL4 identified a population of human and murine $DLL4^+$DCs that had greater ability than DLL4-negative ($DLL4^-$) DCs to induce alloreactive Th1 and Th17 cells. We have identified the crucial roles of both human and murine $DLL4^+$DCs in T cell alloimmunity and are investigating how DCs can be exploited for modulating GVHD and BMF in experimental models.

i). Meng L, Bai ZJ, He S, Mochizuki K, Liu YN, Purushe J, Sun HX, Wang J, Yagita H, Mineishi S, Fung H, Yanik GA, Caricchio R, Fan X, Crisalli LM, Reshef R, Zhang YY, and **Zhang Y.** The Notch ligand DLL4 derived from human dendritic cells is critical for promoting T helper (Th)1 and Th17 cell differentiation. J Immunol. 2016 Feb 1;196(3):1070-80. doi: 10.4049/jimmunol.1501310.

Notch signaling regulates multiple helper $CD4^+$ T cell programs. We have recently demonstrated that DCs expressing the Notch ligand DLL4 are critical for eliciting

alloreactive T cell responses and induction of graft-versus-host disease in mice. However, the human counterpart of murine DLL4$^+$ DCs has yet to be examined. We report the identification of human DLL4$^+$ DCs and their critical role in regulating Th1 and Th17 differentiation. CD1c$^+$ DCs and plasmacytoid DCs (pDCs) from the peripheral blood (PB) of healthy donors did not express DLL4. In contrast, patients undergoing allogeneic hematopoietic stem cell transplantation had a 16-fold more DLL4$^+$CD1c$^+$ DCs than healthy donors. Upon activation of TLR signaling, healthy donor-derived CD1c$^+$ DCs dramatically upregulated DLL4, as did pDCs to a lesser extent. Activated DLL4$^+$ DCs were better able to promote Th1 and Th17 differentiation than unstimulated PB DCs. Blocking DLL4 using a neutralizing Ab decreased Notch signaling in T cells stimulated with DLL4$^+$ DCs, and it reduced the generation of Th1 and Th17 cells. Both NF-κB and STAT3 were crucial for inducing DLL4 in human DCs. Interestingly, STAT3 directly activated DLL4 transcription and inhibiting STAT3 alone was sufficient to reduce DLL4 in activated PB DCs. Thus, DLL4 is a unique functional molecule of human circulating DCs critical for directing Th1 and Th17 differentiation. These findings identify a pathway for therapeutic intervention for inflammatory disorders in humans, such as graft-versus-host disease after allogeneic hematopoietic stem cell transplantation, autoimmunity, and tumor immunity.

ii). Meng L, Hu S, Wang J, He S, **Zhang Y (Correspondence author)**. DLL4+ Dendritic Cells: Key Regulators of Notch Signaling in Effector T Cell Responses. Pharmacol Res. 2016 Sep 14. pii: S1043-6618(16)30873-8.

DCs are critical regulators of adaptive immune responses. DCs can elicit primary T cell responses at low DC:T cell ratios through their expression of high levels of antigen-presenting molecules and costimulatory molecules. DCs are important for induction of functionally diverse T cell subsets such as CD4$^+$ T helper (Th)1 and Th17 cells and effector CD8$^+$ T cells able to reside in epithelial tissues. Recent studies begin illuminating the underlying mechanism by which DCs regulate specialized T cell subsets. DCs are composed of subsets that differ in their phenotype, localization and function. DCs expressing high levels of DLL4 (DLL4$^+$ DCs), which is a member of Notch ligand family, are newly discovered cells that have greater ability than DLL4$^-$ DCs to promote the generation of Th1 and Th17 CD4$^+$ T cells. DLL4 derived from DLL4$^+$ DCs is also important for promoting the differentiation and expansion of effector CD8$^+$ T cells. Experimental studies have demonstrated that selective deletion of DLL4 in DCs causes impaired antitumor immunity. In contrast, blocking DLL4 leads to dramatic reduction of inflammatory T cell responses and their-mediated tissue damage. We will discuss emerging functional specialization within the DLL4$^+$ DC compartment, DLL4$^+$ DC biology and the impact of pharmacological modulation of DLL4 to control inflammatory disorders.

iii). Mochizuki K, Meng L, Mochizuki I, Tong Q, He S, Liu Y, Purushe J, Sun H, Fung H, Zaidi MR, Reshef R, Blazar BR, Yagita H, Mineishi S, and **Zhang Y (Correspondence author)**. Programming of Donor T Cells Using Allogeneic Delta-like ligand 4-positive Dendritic Cells to Reduce GVHD but Retain GVL activity. Blood (DOI 10.1182/blood-2015-05-644476), Blood 2016, 127:3270-328.

Alloreactive T cells play a critical role in eliminating hematopoietic malignant cells but are also the mediators of GVHD, a major complication that subverts the success of allo-HSCT. However, induction of alloreactive T cells does not necessarily lead to GVHD. Here we report the development of a cellular programming approach to render alloreactive T cells incapable of causing severe GVHD in both major histocompatibility complex (MHC)–mismatched and MHC-identical but minor histocompatibility antigen–mismatched mouse models. We established a novel platform that produced δ-like ligand 4–positive dendritic cells (Dll4^{hi}DCs) from murine bone marrow using Flt3 ligand and Toll-like receptor agonists. Upon allogeneic Dll4^{hi}DC stimulation, CD4^{+} naïve T cells underwent effector differentiation and produced high levels of interferon γ (IFN-γ) and interleukin-17 in vitro, depending on Dll4 activation of Notch signaling. Following transfer, allogeneic Dll4^{hi}DC-induced T cells were unable to mediate severe GVHD but preserved antileukemic activity, significantly improving the survival of leukemic mice undergoing allogeneic HSCT. This effect of Dll4^{hi}DC-induced T cells was associated with their impaired expansion in GVHD target tissues. IFN-γ was important for Dll4^{hi}DC programming to reduce GVHD toxicities of alloreactive T cells. Absence of T-cell IFN-γ led to improved survival and expansion of Dll4^{hi}DC-induced CD4^{+} T cells in transplant recipients and caused lethal GVHD. Our findings demonstrate that Dll4^{hi}DC programming can overcome GVHD toxicity of donor T cells and produce leukemia-reactive T cells for effective immunotherapy.

iv). Tian Y, Meng L, **Yi Zhang (Correspondence)**.Epigenetic Regulation of Dendritic Cell Development and Function. The Cancer Journal. 2017 Sep/Oct;23(5):302-307. PMID: 28926431.

The immune system is characterized by the generation of structurally and functionally heterogeneous immune cells that constitute complex innate and adaptive immunity. This heterogeneity of immune cells results from changes in the expression of genes without altering DNA sequence. To achieve this heterogeneity, immune cells orchestrate the expression and functional status of transcription factor (TF) networks, which can be broadly categorized into 3 classes: pioneer TFs that facilitate initial commitment and differentiation of hematopoietic cells, subset-specific TFs that promote the generation of selected cell lineages, and immune-signaling TFs that regulate specialized function in differentiated cells. Epigenetic mechanisms are known to be critical for organizing the TF networks, thereby controlling immune cell lineage-fate decisions, plasticity, and function. The effects of epigenetic regulators can be heritable during cell mitosis, primarily through the modification of DNA and histone methylation patterns at gene loci. By doing so, the immune system is enabled to mount a selective but robust response to stimuli, such as pathogens, tumor cells, autoantigens, or allogeneic antigens in the setting of transplantation, while preserving the immune cell reservoir necessary for protecting the host against numerous other unexpected stimuli and limit detrimental effect of systemic inflammatory reactions.

v). Yu H, Tian Y, Wang Y, Mineishi S, **Zhang Y (Correspondence author)**. Dendritic Cell Regulation of Graft-Vs.-Host Disease: Immunostimulation and Tolerance. Front Immunol. 2019 Feb 1;10:93. doi: 10.3389/fimmu.2019.00093.

GVHD remains a significant cause of morbidity and mortality after allo-HSCT. Significant progresses have been made in defining the dichotomous role of DCs in the development of GVHD. Host-derived DCs are important to elicit allogeneic T cell responses, whereas certain donor-types of DCs derived from newly engrafted hematopoietic stem/progenitor cells (HSPCs) can amply this graft-vs.-host reaction. In contrast, some DCs also play non-redundant roles in mediating immune tolerance. They induce apoptotic deletion of host-reactive donor T cells while promoting expansion and function of regulatory T cells (Treg). Unfortunately, this tolerogenic effect of DCs is impaired during GVHD. Severe GVHD in patients subject to allo-HSCT is associated with significantly decreased number of circulating peripheral blood DCs during engraftment. Existing studies reveal that GVHD causes delayed reconstitution of donor DCs from engrafted HSPCs, impairs the antigen presentation function of newly generated DCs and reduces the capacity of DCs to regulate Treg. The present review will discuss the importance of DCs in alloimmunity and the mechanism underlying DC reconstitution after allo-HSCT.

In summary: In this part of accomplishment B, we list 7 papers (4 research articles and 3 review papers) that are published in peer-reviewed journals. These accomplishments indicate that we have established the beneficial effects of targeting Ezh2 and DLL4+ DCs on modulating GVHD and BMF. These findings open new perspectives to better understand pathophysiology of GVHD and BMF and develop new methods to modulate GVHD and BMF. We are grateful for the support from DOD over these many years for our fruitful research.

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

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.

Our research findings have been published in peered reviewed journals and reported in national and international conferences, such as ASH conference and ASBMT conference.

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

This is the final report. Our future studies are to apply for new research funds to support our continual understanding of pathophysiological role of epigenetic regulators in BMF and GVHD.

4. **IMPACT:** D*escribe 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).

As described above in the section of Accomplishments, results from our studies have the significant impact on the development of new approaches to control BMF and GVHD. **First**, we elucidate the crucial role of and epigenetic regulators, including Ezh2 Jmjd3, in regulating inflammatory T cell immunity and identify the potent effect of combined treatment with transfer of MDSCs and administration of Jmjd3 inhibitor on reducing BMF. These findings provide molecular insights into pathophysiology of BMF and GVHD. They have significant impact on the development of novel approaches that lead to control GVHD and BMF in patients. The journal Blood choses our paper as a cover story (Blood 2017, 129(20):2737-2748; **Cover story**). **Second**, we have established novel culture systems to produce large amounts of dendritic cells and new methods to program allogeneic T cells for inhibition of GVHD. Furthermore, we have identified the importance of $DLL4^{+}$ dendritic cells in the regulation T cell immunity and the underlying mechanism. These accomplishments have significant impact on the disciplines of dendritic cell biology, T cell biology and development of novel and clinically relevant cellular therapy for GVHD and BMF.

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

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

Nothing to report

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

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

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

Nothing to report.

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: *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).*

The bibliography of all publications that acknowledge the support of DOD:

1. Meng L, Bai ZJ, He S, Mochizuki K, Liu YN, Purushe J, Sun HX, Wang J, Yagita H, Mineishi S, Fung H, Yanik GA, Caricchio R, Fan X, Crisalli LM, Reshef R, Zhang YY, and **Zhang Y (Correspondence author)**. The Notch ligand DLL4 derived from human dendritic cells is critical for promoting T helper (Th)1 and Th17 cell differentiation. J Immunol. 2016 Feb 1;196(3):1070-80. doi: 10.4049/jimmunol.1501310.
2. Mochizuki K, Meng L, Mochizuki I, Tong Q, He S, Liu Y, Purushe J, Sun H, Fung H, Zaidi MR, Reshef R, Blazar BR, Yagita H, Mineishi S, and **Zhang Y (Correspondence author)**. Meng L, Hu S, Wang J, He S, **Zhang Y (Correspondence author)**. DLL4+ Dendritic Cells: Key Regulators of Notch Signaling in Effector T Cell Responses. Pharmacol Res. 2016 Sep 14. pii: S1043-6618(16)30873-8.
3. Meng L, Hu S, Wang J, He S, **Zhang Y (Correspondence author)**. DLL4+ Dendritic Cells: Key Regulators of Notch Signaling in Effector T Cell Responses. Pharmacol Res. 2016 Sep 14. pii: S1043-6618(16)30873-8.Programming of Donor T Cells Using Allogeneic Delta-like ligand 4-positive Dendritic Cells to Reduce GVHD but Retain GVL activity. Blood (DOI 10.1182/blood-2015-05-644476), Blood 2016, 127:3270-328.
4. Qingrong Huang, Shan He, Yuanyuan Tian, Yuting Gu, Pan Chen, Changhong Li, Jiefang Huang, Yongnian Liu, Min Jin, Shaoyan Hu, Qing Tong, Anqi Ma, Jian Jin, Elizabeth Hexner, Henry Fung, Ran Reshef, **Yi Zhang (Correspondence author)** and Yanyun Zhang. Hsp90 inhibition destabilizes Ezh2 protein in alloreactive T cells and reduces graft-versus-host disease in mice. (Blood 2017:blood-2016-08-735886; doi: https://doi.org/10.1182/blood-2016-08-735886; **Cover story**).
5. Tian Y, Meng L, **Yi Zhang (Correspondence)**.Epigenetic Regulation of Dendritic Cell Development and Function. The Cancer Journal. 2017 Sep/Oct;23(5):302-307. PMID: 28926431
6. Shan He, Yongnian Liu, Lijun Meng, Hongxing Sun, Janaki Purushe, Pan Chen, Changhong Li, Jozef Madzo, Jean-Pierre Issa, Jonathan Soboloff, Bethany B Moore, Luca Gattinoni and **Yi Zhang (Correspondence author)**. The Phosphorylation State of Ezh2 Determines its Capacity to Maintain $CD8^+$ Memory T Cells for Antitumor Immunity. Nature Communications (DOI:10.1038/s41467-017-02187-8).
7. Yu H, Tian Y, Wang Y, Mineishi S, **Zhang Y (Correspondence author)**. Dendritic Cell Regulation of Graft-Vs.-Host Disease: Immunostimulation and Tolerance. Front Immunol. 2019 Feb 1;10:93. doi: 10.3389/fimmu.2019.00093.

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.*

No 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.)*

The list of personnel (not salaries) receiving pay from the research effort:

Yi Zhang (PI), MD, PhD
Hongxing Sun (Postdoctoral fellow), PhD
Yuanyuan Tian (PhD Candidate)

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.*