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14. ABSTRACT

anemia (AA) is a fatal disorder characterized by immune-mediated destruction of hematopoietic stem and progenitor cells.1 Immunosuppressive therapy and allogeneic BMT have significantly improved the survival of severe AA. However, relapse still occurs in approximately 35% of AA patients when immunosuppressive therapy is withdrawn.2,3 Furthermore, GVHD remains a major barrier to the success of allogeneic BMT.4,5 Memory T cells may present a significant barrier to the success of controlling various inflammatory conditions. Memory T cells, derived from proliferating T cells during primary immune response,6-13 can undergo self-renewal to survive throughout the lifetime of an individual and continually generate differentiated effector T cells.9-11,14-18 Compared to naïve T cells, memory T cells respond more rapidly to T cell receptor (TCR) activation, require lower concentrations of antigen and are less dependent on costimulatory signals such as CD28.6,7,9,11,15,19 This could explain that memory T cells can be refractory to many of the tolerance-inducing strategies and immunosuppressive agents that are effective against naïve T cells.20,21 Notably, BM can function as a secondary lymphoid organ where memory T cells are maintained as "resting" cells undergoing homeostasis and can be reactivated to become effector T cells.22,23 These observations suggest that memory T cells could be responsible for the relapse and treatment refractory of AA.

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INTRODUCTION: .

Acquired aplastic anemia (AA) is a fatal disorder characterized by immune-mediated destruction of hematopoietic stem and progenitor cells.[1] Immunosuppressive therapy and allogeneic BMT have significantly improved the survival of severe AA. However, relapse still occurs in approximately 35% of AA patients when immunosuppressive therapy is withdrawn.[2,3] Furthermore, graft-versus-host disease (GVHD) remains a major barrier to the success of allogeneic BMT.[4,5] Memory T cells may present a significant barrier to the success of controlling various inflammatory conditions. Memory T cells, derived from proliferating T cells during primary immune response,[6-13] can undergo self-renewal to survive throughout the lifetime of an individual and continually generate differentiated effector T cells.[9-11,14-18] Compared to naïve T cells, memory T cells respond more rapidly to T cell receptor (TCR) activation, require lower concentrations of antigen and are less dependent on costimulatory signals such as CD28.[6,7,9,11,15,19] This could explain that memory T cells can be refractory to many of the tolerance-inducing strategies and immunosuppressive agents that are effective against naïve T cells.[20,21] Notably, BM can function as a secondary lymphoid organ where memory T cells are maintained as "resting" cells undergoing homeostasis and can be reactivated to become effector T cells.[22,23] These observations suggest that memory T cells could be responsible for the relapse and treatment refractory of AA. Notch signaling is critical for the development of Th1, Th2 and Th17 $CD4^+$ T cells.[24-27] Recently, Notch was described to control the effector program of $CD8^+$ cytotoxic T cells.[28,29] However, some T cell responses were shown to be unaffected by Notch inhibition.[24,30-32] Thus, Notch can be an important regulator of antigen-driven T cell differentiation and function. The purpose of this proposed project is to define the role of memory T cells in mediating BM failure in mouse models; determine the mechanisms whereby marrow-destructive memory T cells are generated and maintained during immunosuppression; and exploit the beneficial effects of memory T cell inhibition in mouse models of BM failure. Results from these experiments will identify the impact of memory T cells in mediating marrow destruction and the beneficial effects of blocking Notch and Notch ligands in antigen-presenting cells in the treatment of aplastic anemia in mouse models.

2. KEYWORDS:

2.1. Aplastic anemia
2.2. Memory T cells
2.3. Th1 cells
2.4. Th17 cells
2.5. Inflammatory dendritic cells
2.6. Regulatory antigen-presenting cells
2.7. Notch signaling
2.8. Notch ligands
2.9. Delta-like ligand 4 (Dll4)
2.9. Delta-like ligand 1 (Dll1)
2.10. Graft-versus-host disease (GVHD)
2.11. Bone marrow transplantation

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.

3.1. What were the major goals of the project?

The major goals of the project are to identify the impact of inflammatory T cells in immune-mediated marrow destruction and the beneficial effects of blocking Notch signaling on the treatment of aplastic anemia in mouse models. If blocking memory T cells can prevent the relapse of bone marrow failure, the overall outcomes of BMF treatment can be potentially improved using an alternative immunomodulation approaches.

3.2. 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.

Overview of our research accomplishments:

Over the past four years supported by this DOD award, we have achieved two major accomplishments in understanding of mechanisms that regulate the development and maintenance of inflammatory T cells that mediate BMF and GVHD both in murine models and humans. **First,** we have discovered that Dll4 shows greater capacity than other Notch ligands (Dll1, Jagged1 and Jagged2) to induce the generation of T helper (Th)1 and Th17 cells, which are characterized by producing high levels of IFN-γ and IL-17, respectively.[33-36] This leads to the discovery of Dll4-positive ($Dll4^+$) DCs that have greater capacity than Dll4-negative ($Dll4^-$) DCs to induce Th1 and Th17 $CD4^+$ T cells.[33-35] Blocking Dll4 by specific neutralizing antibody significantly decreased the generation of both Th1 and Th17 $CD4^+$ T cells activated by $Dll4^+$ DCs.[33-35] **Second,** we have also discovered that the histone methyltransferase Ezh2, which catalyzes histone H3 lysine 27 trimethylation, plays a central role in regulating GVH reactions. Inhibition of Ezh2 in donor T cells inhibited GVHD and prevented BMF in experimental mouse models.[37,38] Mechanistic studies reveal that Ezh2 was required to prevent proteasome-mediated degradation of T-bet protein in Th1 cells, thereby promoting Th1 differentiation.[37,38]

Our research accomplishments have been well recognized both at the national and international levels. For example, I have been invited to serve as an Ad Hoc reviewer of research grants for NIH. I was also invited to be a moderator of conference, American Society of Hematology. In addition, numerous universities and medical institutions, both within and outside of USA, invited me to present our research progress. Most importantly, this DOD award helped us to gain a new R01 support in 2016. We are grateful for this timing support from Bone Marrow Failure Research Program, DOD. We outlined these major research accomplishments in our final report

as the follows.

3.2.1. Year-1, establishment of three different mouse models of human AA
3.2.2. Year-2, discovery of a previously uncharacterized Dll4$^+$ DCs and their function (***JI 2013***)
3.2.3. Year-3 to Year-4, completion of three major projects, including**:**

A. Discovering the essential role of the histone methyltransferase Ezh2 in Th1 cell-mediated AA in mice (***JI 2014***).[37]
B. Establishment of a cellular programming approach that is able to reduce the toxicity of donor CD4$^+$ naïve T cell (T_N) to mediate GVHD in mice receiving allogeneic BMT (***Blood, 2016***).[33]
C. Discovering the role of human DCs expressing DLL4 and their roles in regulating Th1 and Th17 differentiation (***JI 2016***).[34]

Detailed report of our research accomplishments:

3.2.1. During the **first year** of support, we have established three different mouse models of human AA (**Fig.1**). These experimental animal models to study human AA are based on bone marrow destruction mediated by allogeneic T cells that recognize and react to alloantigens presented by hematopoietic cells.[39-41] Using these animal models, we identified that Notch signaling played important roles in regulating the generation of inflammatory effector T cells mediating BMF in the setting of allogeneic T cell transfer (**Fig.1A**). Donor T cells deprived of Notch signaling fail to mediate BM destruction. Inhibition of Notch signaling in donor T cells reduces allogeneic T cell-mediated GVH reaction in BALB/B mice receiving MHC-identical minor histocompatibility-mismatched B6 T cells (**Fig.1B**). To our surprising, in vivo administration of anti-thymocyte globulin (ATG) failed to affect the development of BMF in the b6 anti-BDF1 mouse model (**Fig.1C**). These results suggest that ATG-based immunosuppression therapy may not prevent the development of BMF in this mouse model. Alternative approaches are needed to reduce alloreactive T cell-mediated BMF.

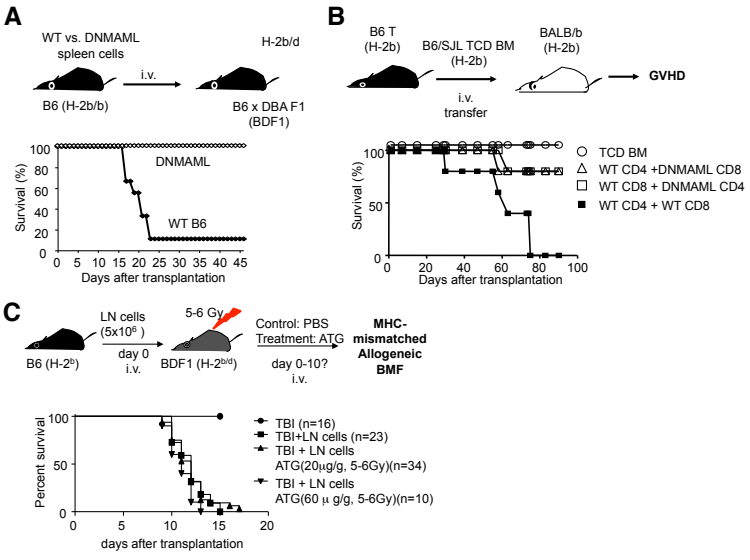


Fig.1. Establishment of three different mouse models of human AA. (A) *Splenocytes and LN cells (5x10^7) from wild-type (WT) or DNMAML B6 mice were transplanted into unirradiated BDF1 recipients. BDF1 recipients (H-2$^{b/d}$) are tolerant to the infused B6 cells (H-2^b), but donor T cells mount a potent alloreactive response against host H-2^d antigens. Improved overall survival of BDF1 recipients receiving DNMAML B6 T cells (n=9 in each group) (p<0.01).* ***(B)*** *Lethally irradiated BALB/b mice were transplanted with TCD BM, with or without WT CD4$^+$ + WT CD8$^+$ T cells, WT CD4$^+$ + DNMAML CD8$^+$ T cells, or DNMAMLCD4$^+$ + WT CD8$^+$ T cells. TCD BM recipients survived, while recipients of WT T cells all succumbed to allogeneic T cell-mediated GVHD. In contrast, recipients of WT CD4$^+$ + DNMAML CD8$^+$ T cells, or DNMAMLCD4$^+$ + WT CD8$^+$ T cells displayed attenuated GVHD (p<0.05).* ***(C)*** *Transfer of lymph node (LN) cells (5x10^6) from normal B6 mice into sublethally total body irradiated (5.0 Gys, TBI) BDF1 recipients (TBI + LN cells). BDF1 mice receiving TBI without given LN cells were used as control (TBI). ATG was administered to these recipients as indicated in (**C**).*

3.2.2. During the **second year** of grant support, we continued investigating the pathophysiological role of Dll4$^+$ i-DCs in regulating inflammatory T cell responses, including the development and maintenance of marrow-destructive effector and memory T cells. Two major objectives include: **1)** assessing the impact of pharmacologic inhibition of Notch in BM failure, and analyzing the effect of immunomodulation agents on memory T cells; and **2)** identification and characterization of different subsets of stimulatory and regulatory APCs, including inflammatory DCs. During this period, we have made a critical discovery that the Dll4 Notch ligand is implicated in the generation of bone marrow-destructive effector T cells, and Dll4-positive inflammatory dendritic cells play important roles in eliciting allogeneic T cell responses in mice. Blocking Dll4 led to the decreased production of effector T cells, reduced GVHD, and significantly improved survival of mice after allogeneic bone marrow transplantation. **These findings are published in JI 2013.[35], which are briefed as the followings:**

A. Host DCs upregulate Notch ligands early during GVHD induction.

To determine the role of Notch ligands in regulating allogeneic T cell responses, we examined the expression of Notch ligands on the surface of APCs after transplantation. B6 TCD-BM plus CD4$^+$ T cells were injected into lethally irradiated BALB/c mice to induce GVHD. As expected, GVHD occurred in these allogeneic recipients, with all of them dying of the disease between days 7 and 35 after transplantation (**Fig. 2A**). Given the importance of host APCs in eliciting GVH reaction,[42-46] we first assessed the expression of Notch ligands on host CD11c$^+$ DCs. On days 1 and 3 after transplantation CD11c$^+$ cells were all of host origin (**Fig.2B**). By 7 days after transplantation, host CD11c$^+$ cells were reduced about 20-fold in the spleen of these allogeneic HSCT mice compared to day 1 (**Fig. 2B**), which coincides with previous studies.[46,47] Notch ligand Dll4, J1 and J2 were dramatically upregulated on the surface of host CD11c$^+$ DCs from the spleen of allo-HSCT recipients by 3 days after transplantation and declined by 7 days (**Fig. 2C,D**). Interestingly, there were only few host CD11c$^+$ DCs expressing low levels of Dll1 (**Fig. 2C,D**), although Dll1 has been implicated in other types of antigen-driven T cell responses.[48,49] These host CD11c$^+$ DCs expressed high levels of MHC class II molecule Ia and costimulatory molecules CD80 and CD86 (**Fig. 2E**), resembling the phenotype of i-DCs.[44,50-52] Donor-derived CD11c$^+$ cells did not occur by 7 days after transplantation (**Fig. 2B**). These donor origin CD11c$^+$ cells expressed low levels of Dll4, J1 and moderate levels of J2 (**Fig. 2F**). These results suggest that host DCs upregulate the expression of Dll4, J1 and J2 during early phase of GVHD induction.

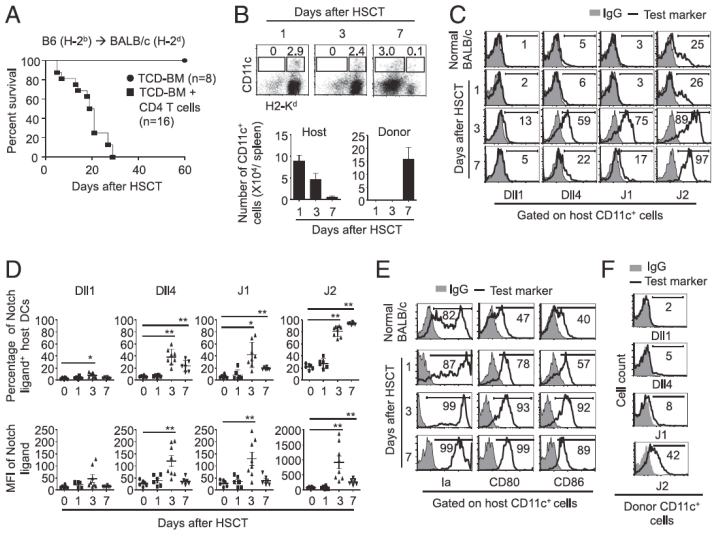


Fig.2. Notch ligands are up-regulated on the surface of CD11c$^+$ DCs in the recipient mice early during GVHD induction. *Lethally irradiated (8Gy) BALB/c mice were injected with B6 TCD-BM (5.0×10^6) mixed with or without CD4 T cells (1.0×10^6). Cells were isolated from the spleens of these recipients at various time points after transplantation. (**A**) Survival of animals was monitored over time. Data shown here are pooled from three independent experiments. (**B**) Dot plots and graphs show the percentage and number of host (H2-Kd$^+$) or donor (H2-Kd$^-$) origin CD11c$^+$ cells (mean ± SD, n=6 to 8 mice per group). (**C**) Histograms show the*

*expression of Notch ligands on the surface of host CD11c$^+$ cells which were recovered from the spleens of normal BALB/c mice and allogeneic HSCT BALB/c mice at the time point as indicated. Representative histograms are shown. (**D**) Graphs show the percentage and mean fluorescent intensity (MFI) of Notch ligand expression on the surface of host CD11c$^+$ cells (mean ± SD, n=6 to 8 mice per group). (**E**) Histograms show the expression of tested markers on the surface of host CD11c$^+$ cells. Representative histograms are shown. (**F**) Histograms show the expression of Notch ligands on the surface of donor CD11c$^+$ cells that were recovered from the spleens of BALB/c recipients 7 days after HSCT. Representative histograms are shown.*: P<0.05, **: p<0.01.*

B. Dll4^{hi} i-DCs have greater ability than Dll4^{lo} i-DCs to promote the development of effector T cells producing IFN-γ and IL-17

To further define the biological properties of these Dll4^{hi} i-DCs and Dll4^{lo} i-DCs, we purified these two DC subsets from GVHD mice based on their characteristic phenotype of CD11c$^+$PDCA-1$^+$B220$^+$ and CD11c$^+$PDCA-1$^-$B220, respectively (**Fig.3A**). This allowed us to precisely evaluate the functional activity of Dll4 in these i-DC subsets without using neutralizing anti-Dll4 Ab during the process. Morphological examination showed that Dll4^{hi} i-DCs appeared large in cell size and contained more vesicles as compared to DCs at steady state conditions (Fig. 4B). Real-time RT-PCR analysis showed that Dll4^{hi} i-DCs expressed higher levels of *Dll4*, *Ifnb* and *Il23* but lower levels *Il12* than Dll4^{lo} i-DCs (**Fig. 3B**). In addition, compared to pDCs, Dll4^{hi} i-DCs produced more *Dll4*, *Ifnb* and *Il23* but less *Il12* and Arginase 1 (*Arg1*) (**Fig. 3B**), further confirming that Dll4^{hi} i-DCs are distinct from Dll4^{lo} i-DCs and those pDCs and cDCs at steady state conditions.

We then used MLR assays to examine the difference in function between Dll4^{hi} i-DCs and Dll4^{lo} i-DCs in vitro. Highly purified Dll4^{hi} i-DCs and Dll4^{lo} i-DCs were added to cultures containing B6 CD4$^+$ T cells, with or without addition of neutralizing anti-Dll4 Ab (**Fig. 3C,D**). Dll4^{hi} i-DCs induced approximately 3-fold and 10-fold more IFN-γ- and IL-17-producing T cells, respectively, compared to Dll4^{lo} i-DCs (**Fig. 3C,D**). Addition of anti-Dll4 Ab abrogated the ability of Dll4^{hi} i-DCs to promote effector differentiation, but had little effect on cytokine production in T cells stimulated by Dll4^{lo} i-DCs (**Fig. 3C,D**). IL-2 was added to the culture to enhance the proliferation and differentiation of alloantigen-activated T cells.[53] However, IL-2 alone did not induce production of high levels of IFN-γ and IL-17 by donor T cells cultured in the absence of allogeneic DCs (**Fig. 3C,D**). Thus, Dll4^{hi} i-DCs may represent a unique i-DC subset and have greater capability than Dll4^{lo} i-DCs to promote the development of Th1 and Th17 cells.

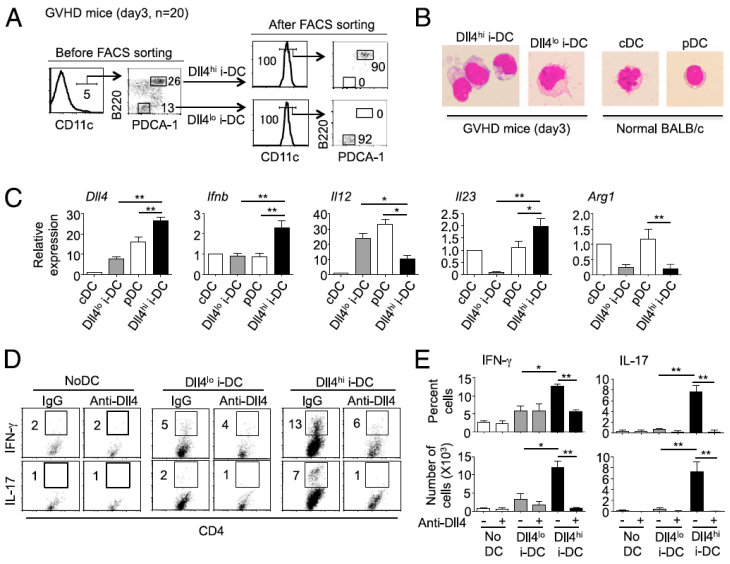


Fig.3. Gene expression and function of host Dll4^{hi} i-DCs. *Lethally irradiated (8Gy) BALB/c mice (n=20) were injected with B6 TCD-BM (5.0×10^6) mixed with CD4 T cells (1.0×10^6). Cells were recovered from the spleens of allogeneic HSCT BALB/c mice 3 days after transplantation. Cells purified from the spleens of normal BALB/c mice were also accessed as controls. **(A)** Dll4^{hi} i-DCs and Dll4^{lo} i-DCs were highly purified by using FACS Sorter. Histogram and dot plots show representative*

results of DC purification from the spleen of 20 mice receiving allogeneic HSCT. Approximately 0.5 to 0.7 X 10^5 cells of each DC subset were acquired. ***(B)*** *Graphs show the relative expression of indicated genes in each DC subset (mean ± SD). Results shown are representative of three independent experiments. (****C, D****) DC subsets purified from the allogeneic recipient mice were cultured ex vivo with donor $CD4^+$ T cells (1.0 X 10^5) derived from normal B6 mice in the presence of IL-2 (DC and $CD4^+$ T cell ratio was 1:10). Neutralizing anti-Dll4 Ab (20 μg/ml) was added into the culture. Four days later, cells were collected to measure their production of IFN-γ and IL-17. Data show mean ± SD of the percentage and number of cytokine producing cells. Results shown are representative of two independent experiments.*: P<0.05, **: p<0.01.*

C. In vivo blocking Dll4 reduces the production of alloreactive effector T cells and GVHD

To test the impact of blocking Dll4 on alloreactive T-cell response in vivo, we transplanted B6 donor $CD4^+$ T cells with TCD-BM into lethally irradiated BALB/c mice. Anti-Dll4 Ab was given to these recipients at day 0, 2 and 4 after transplantation. Donor T cells were recovered 5 days after transplantation from these recipients (**Fig. 4A**). We observed that in vivo blockade of Dll4 resulted in a marked reduction of donor effector T cells producing high levels of IFN-γ and IL-17 in the spleen and intestine (**Fig. 4B,C**). Anti-Dll4 treatment reduced the expression of the Notch target gene Dtx1 in donor T cells (**Fig. 4D**), but had no effect on the recovery of $Dll4^+$ i-DCs in the spleen compared to IgG control (0.98±0.2 $X10^4$ versus 0.83±0.1 $X10^4$, respectively). This is in agreement with previous studies demonstrate that antibody to any single Notch ligand has no impact on depletion of DCs in vivo.[54] These results suggest that Dll4 may play an important role in the regulation of alloreactive effector T cells in GVHD target organs.

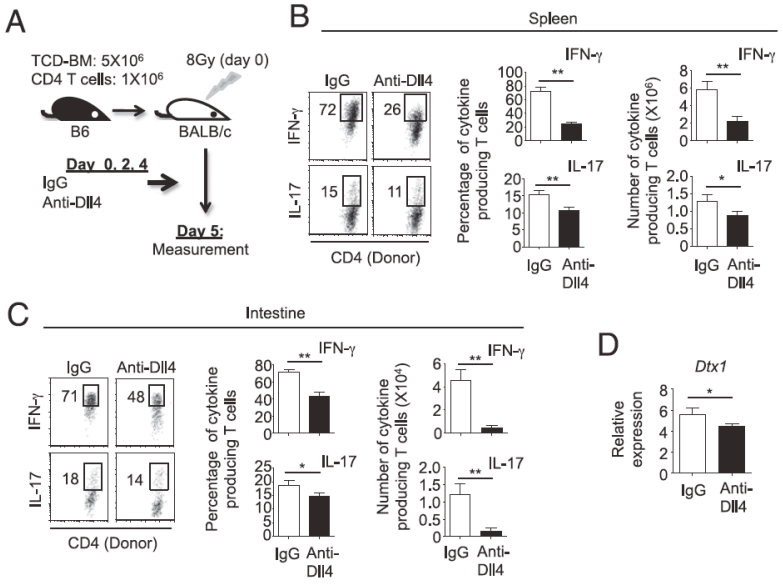
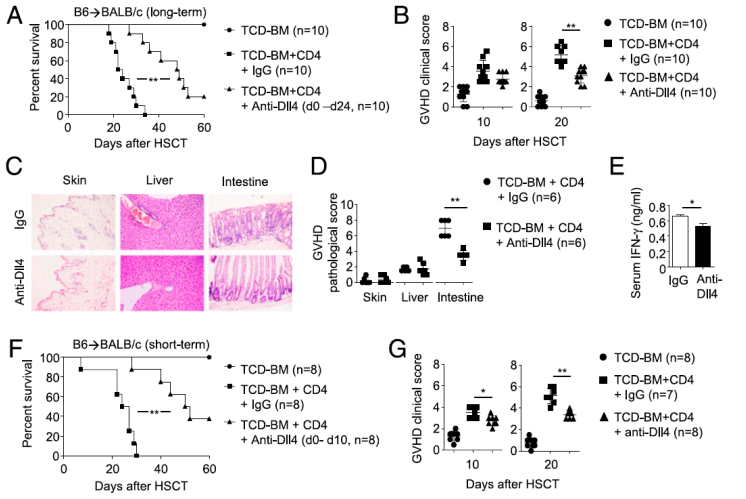


Fig.4. In vivo blocking of Dll4 reduces effecter differentiation of alloreactive $CD4^+$ T cells. *(****A****) Lethally irradiated (8Gy) BALB/c mice were transplanted with B6 TCD-BM (5.0×10^6) with $CD4^+$ T cells (1.0×10^6) , followed treatment with or without anti-Dll4 Ab at day 0, 2 and 4 after transplantation (3 mice per group). Control IgG was given as control. At day 5, cells were recovered from the spleen and intestine lamina propria to assess cytokine production and gene expression. (****B,C****) Dot plots and graphs show the percentage and number of donor $CD4^+$ T cells producing IFN-γ and IL-17 in the spleen and intestine. Data show mean ± SD. (****D****) Graphs show relative expression of Notch target gene Dtx1 in donor $CD4^+$ T cells (mean ± SD). Results shown are representative of two independent experiments. *: P<0.05, **: p<0.01.*

D. In vivo administration of anti-Dll4 reduces GVHD in MHC mismatched recipient mice. We next asked if blockade of Dll4 could prevent production of GVHD. Donor B6 T cells were transplanted with TCD-BM into lethally irradiated BALB/c mice to induce GVHD. Nine doses of anti-Dll4 Ab were administered to these recipients once every three days from day 0 to day 24 after transplantation. In vivo administration of Dll4 Ab significantly attenuated GVHD in mice

receiving high dose of donor $CD4^+$ T cells, with markedly prolonged survival and reduced clinical signs of GVHD (**Fig. 5A,B**). Histology examination showed markedly reduced inflammation in the intestine of these recipients treated by anti-Dll4 Ab (**Fig. 5C,D**). This decrease of donor effector T cells was accompanied with significant reduction of serum IFN-γ at day 7 after HSCT (**Fig. 5E**). Interestingly, administration of 6 doses of anti-Dll4 Ab from day 0 to day 10 after transplantation effectively reduced GVHD in these BALB/c recipients (**Fig. 5F,G**). These data suggest that a short-term blockade of Dll4 during early phase of GVHD is sufficient to reduce the disease, which coincides with the occurrence of $Dll4^{hi}$ i-DCs during this period of GVH reaction.

Fig.5. In vivo administration of anti-Dll4 reduces GVHD in MHC mismatched recipient mice. *Lethally irradiated (8Gy) BALB/c mice were injected with B6 TCD-BM (5.0×10^6) mixed with $CD4^+$ T cells (1.0×10^6). (**A,B**) Nine doses of anti-Dll4 antibody (250 μg/mouse) were given to BALB/c recipients once every three days from day 0 to day 24 after HSCT. BALB/c recipients treated with anti-Hamster IgG were used as controls. Survival and GVHD clinical score of the recipients were monitored over time. (**C**) Representative images show the tissues from one of 6 recipients in each group at day 7 after transplantation. Images were obtained with an OlympusBX41 microscope (10/0.3 NA lens, 200x magnification, digital DP70 camera). (**D**) Pathological scores of GVHD 7 days after HSCT (6 mice per group). (**E**) ELISA assays show the serum IFNγ in allogeneic BALB/c recipients (4 mice for each group) 7 days after HSCT. Data show mean ± SD. (**F,G**) Six doses of anti-Dll4 antibody (250 μg/mouse) were administered once every other day from day 0 to day 10 after HSCT. BALB/c recipients treated with anti-Hamster IgG were used as controls. Survival and GVHD clinical score of the recipients were monitored over time.*: P<0.05, **: p<0.01.*



E. In summary, these studies provide evidence for a function of Dll4 and $Dll4^{hi}$ i-DCs in eliciting allogeneic T-cell responses early during GVHD. Upon preparative conditioning for allogeneic HSCT, Notch ligands Dll4, J1 and J2 were markedly upregulated on the surface of host origin i-DCs early during GVHD induction. Importantly, based on the expression of Dll4, i-DCs could be divided into two subsets: $Dll4^{hi}$ i-DCs and $Dll4^{lo}$ i-DCs. $Dll4^{hi}$ i-DCs and $Dll4^{lo}$ i-DCs are different entities in the context of their surface phenotype, expression of cytokine transcripts and capability to promote the production of alloreactive effector T cells. As compared to $Dll4^{lo}$ i-DCs, $Dll4^{hi}$ i-DCs had greater ability to stimulate the generation of alloreactive effector T cells producing IFN-γ and IL-17. Neutralizing Dll4 using anti-Dll4 Ab could abrogate this effect of $Dll4^{hi}$ i-DCs. Furthermore, in vivo administration of anti-Dll4 Ab caused a marked reduction of alloreactive effector T cells in GVHD target organs, leading to reduction of GVHD and significantly improved survival of mice after allogeneic HSCT. Our findings indicate that $Dll4^{hi}$ i-DCs and Dll4 are important for the generation of alloreactive effector T cells capable of mediating host tissue injury and could be beneficial targets for improving the efficacy of allogeneic HSCT.

3.2.3. During the period of final years, we have accomplished three major projects related to this proposal. These include:

A. Discovering the essential role of the histone methyltransferase Ezh2 in Th1 cell-mediated AA in mice (***JI 2014***).[37]
B. Establishment of a cellular programming approach that is able to reduce the toxicity of donor $CD4^+$ naïve T cell (T_N) to mediate GVHD in mice receiving allogeneic BMT (***Blood, 2016***).[33]
C. Discovering the role of human DCs expressing DLL4 and their roles in regulating Th1 and Th17 differentiation (***JI 2016***).[34]

These findings not only provide cellular and molecular insights into the generation of alloreactive T cells that mediate BMF and GVHD, but also are important for the development of novel strategies to reduce BMF and GVHD by inhibiting inflammatory T cell responses. We summarize these major findings as the follows.

A. Discovering the essential role of the histone methyltransferase Ezh2 in Th1 cell-mediated AA in mice (JI 2014)[37].

To better understand how Notch signaling regulate BM-destructive memory T cells in mediating AA in mice, we investigated the epigenetic mechanism that regulated the expression and function of T-bet, which is a transcription factor key to the generation of Th1 cells. However, transcription factors are difficult drug targets.[55] Thus, identifying the molecular pathway(s) that control T-bet expression in Th1 cells may lead to new strategies to control AA. Ezh2 is a histone methyltransferase that specifically catalyzes trimethylation of histone H3 at lysine 27 (H3K27me3).[56] Several studies point to an important role of Ezh2 and H3K27me3 in multiple lineages of effector T cells.[57-60] Genome-wide mapping analysis revealed that repressive H3K27me3 marked genes associated with differentiation and maintenance of effector and memory T cells.[61,62] Most recently, we have demonstrated new and essential roles of Ezh2 in regulating inflammatory T cell responses in mice after allogeneic BMT.[38] Loss of Ezh2 led to impaired production of alloreactive T cells that induce damage to epithelial organs.[38]

A.1. To elucidate whether Ezh2 mediates pathogenic Th1 responses in AA and the mechanism of Ezh2 action in regulating Th1 cells, we studied the effects of Ezh2 inhibition in $CD4^+$ T cells using a mouse model of human AA. Conditionally deleting Ezh2 in mature T cells dramatically reduced the production of BM-destructive Th1 cells in vivo, decreased BM-infiltrating Th1 cells, and rescued mice from BM failure (**Fig.6, reference-**37).

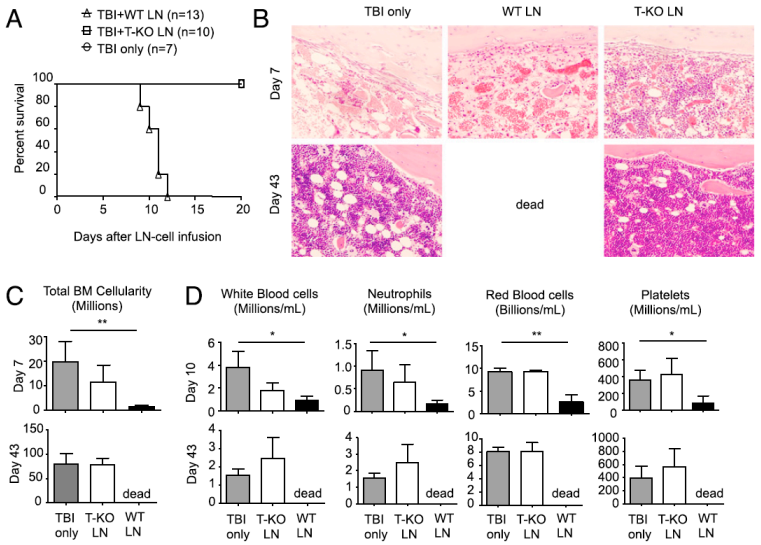
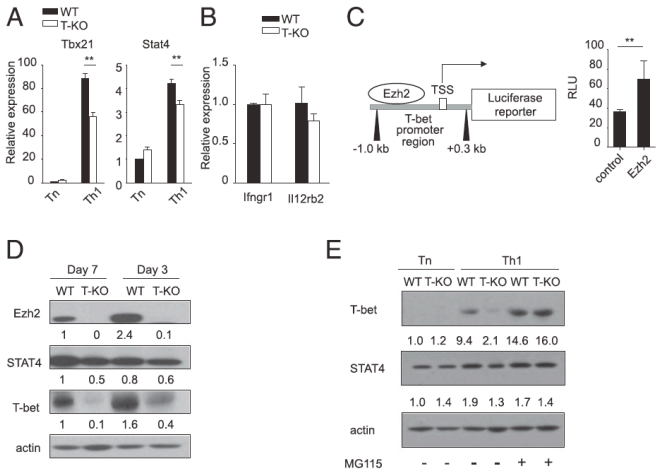


Fig. 6. In the absence of Ezh2, LN cells are defective in mediating AA in mice. *To assess whether conditionally deleting Ezh2 in T cells affected their ability to mediate AA, we transferred donor LN cells derived from WT and T-KO B6 mice into irradiated (6.5Gy) BDF1 recipients. In this setting, transfer of donor LN cells causes severe BM destruction and blood pancytopenia in these haplo-identical recipients, which closely*

reflects the pathogenesis of human AA.[41,63,64] As expected, BDF1 mice receiving WT B6 LN cells developed BM hypoplasia and severe blood pancytopenia after LN cell infusion compared to control total body irradiation (TBI) only mice, with all of them dying from the disease within 12 days after transfer (Fig.1A). Histological examination showed the destruction of BM and lack of hematopoietic cell islands in the BM of these recipients (Fig.1B). In contrast, transfer of T-KO LN cells did not cause severe AA in these BDF1 recipients (Fig.1A, B). As compared to control TBI mice, there was no significant reduction of BM cellularity and peripheral blood WBCs in these BDF1 mice receiving T-KO LN cells (Fig.1C, D). Importantly, all T-KO LN cell recipients survived without clinical signs and histological evidence of AA (Fig.1A, B). Thus, T cells required Ezh2 to mediate AA.

A.2. Ezh2 inhibition resulted in significant decrease in the expression of *Tbx21 and Stat4* (which encode transcription factors T-bet and STAT4, respectively) (**Fig.7**).

Fig.7. Ezh2 regulates T-bet at both the transcriptional level and post-translational level. *To determine how Ezh2 regulated the expression of T-bet and STAT4 in Th1 cells, we examined the expression of T-bet and STAT4 mRNA in WT and T-KO CD4$^+$ T cell 7 days after culture under Th1-skewing conditions. CD4$^+$ Tn were assessed as controls. Seven days after culture under Th1-skewing conditions, T-KO CD4$^+$ T cells expressed approximately 1.8-fold less T-bet mRNA than their WT counterparts (Fig. 7A). STAT4 mRNA was slightly decreased in activated T-KO CD4$^+$ T cells (Fig.7A). In addition, Ezh2 deficiency had no effect on the expression of Th1-related Ifngr1 and Il12rb2 genes (Fig.7B), two critical signaling molecules upstream of T-bet and STAT4, respectively.[65] These data indicate that Ezh2 promotes the expression of T-bet at the transcriptional level.*



To assess whether Ezh2 directly activated Tbx21 transcription, we co-transfected 3T3 cells with pGL3-Tbx21 reporter and MigR1 viral plasmid encoding Ezh2 or empty MigR1 plasmid. These 3T3 cells were harvested 48 hours after transfection and analyzed with the Dual Luciferase system. Overexpression of Ezh2 resulted in moderate induction of Tbx21 reporter activity (Fig.7C). This indicates that Ezh2 can directly activate Tbx21 transcription.

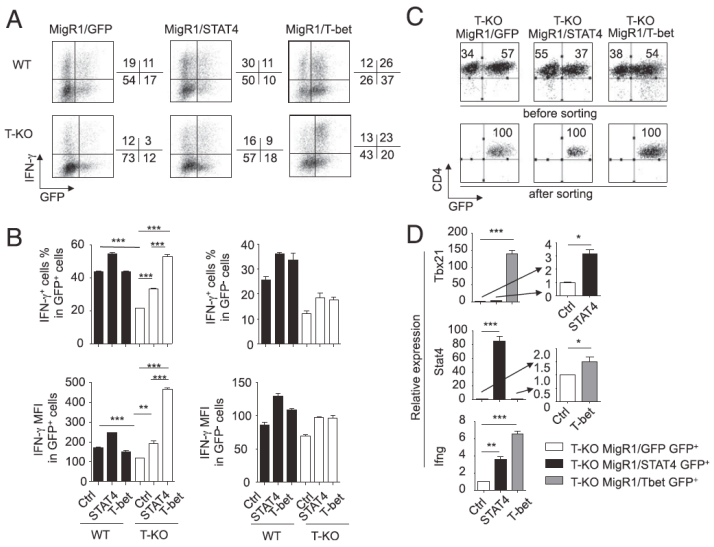
We further verified whether loss of Ezh2 led to reduction of STAT4 and T-bet protein in Th1 cells. As compared to activated WT CD4$^+$ T cells, there was only minimal reduction of STAT4 protein in these activated T-KO CD4$^+$ T cells (Fig.7D). Most notably, T-KO CD4$^+$ T cells showed approximately 4-fold and 10-fold less T-bet protein at day 3 and day 7 after culture, respectively, than their WT counterparts (Fig.7D). This dramatic reduction of T-bet protein in T-KO Th1 cells appeared not to be completely supported by moderate reduction of T-bet mRNA in these cells. We reasoned that loss of Ezh2 might lead to increased degradation of T-bet protein in Th1 cells. To test it, we treated WT and T-KO Th1 cells with the proteasome inhibitor MG115.[66] Addition of the proteasome inhibitor MG-115 restored the expression of T-bet protein, but not STAT4 protein, in Ezh2-deficient Th1 cells (Fig.7E), suggesting that T-bet protein may be more susceptible than STAT4 to proteasome-mediated degradation in Th1 cells lacking Ezh2. Altogether, Ezh2 promotes T-bet expression at both transcriptional and post-translational levels, with the later the most extent. These results identify a novel and important role for Ezh2 to regulate Th1 cells.

A.3. Introduction of T-bet but not STAT4 into Ezh2-deficient T cells fully rescued their differentiation into Th1 cells mediating AA. Ezh2 bound to the *Tbx21* promoter in Th1 cells, and directly activated *Tbx21* transcription. Unexpectedly, Ezh2 was also required to prevent

proteasome-mediated degradation of T-bet protein in Th1 cells.

Fig.8. Introduction of T-bet into T-KO CD4$^+$ T cells fully rescues their differentiation into Th1 cells

To determine whether the down-regulation of T-bet caused the impairment of Th1 cell development, we used MigR1 virus bicistronically encoding T-bet and GFP (named MigR1/T-bet) to infect T-KO CD4$^+$ T cells cultured under Th1-skewing conditions. T-KO CD4$^+$ T cells infected with MigR1 encoding STAT4 and GFP (named MigR1/STAT4) or GFP alone (named MigR1/GFP) were assessed in parallel. Expression of GFP allowed us to track cells expressing T-bet or STAT4. WT CD4$^+$ T cells were also infected with each of these viruses as controls.



We found that T-KO GFP-positive (GFP$^+$) CD4$^+$ T cells that were derived from cultures infected by MigR1/T-bet, which over-expressed T-bet, produced similar percentage of IFN-γ^+ T cells to WT GFP$^+$ CD4$^+$ T cells derived from cultures infected by either MigR1/T-bet or MigR1/GFP (Fig.8A,B). Interestingly, as compared to WT GFP$^+$CD4$^+$ T cells infected with MigR1/GFP or MigR1/STAT4, T-KO GFP$^+$CD4$^+$ T cells expressing STAT4 had significantly lower frequency of IFN-γ^+ T cells (Fig.8A,B). Furthermore, T-KO CD4$^+$ T cells expressing STAT4 contained about 40% less IFN-γ^+ T cells than T-KO CD4$^+$ T cells expressing T-bet (Fig.8A,B). These data suggest that viral expression of T-bet fully rescues the ability of Ezh2-deficient CD4$^+$ T cells to differentiate into Th1 cells, whereas overexpression of STAT4 only partially improves Th1 cell differentiation of activated T-KO T cells.

To validate these observations, we highly purified GFP-positive T-KO T cells from these cultures (Fig.8C) and confirmed the overexpression of T-bet and STAT4 in these T-KO CD4$^+$ T cells, respectively, using real-time RT-PCR (Fig.8D). Furthermore, overexpression of T-bet in T-KO T cells induced significantly more Ifng transcripts than did overexpression of STAT4 (Fig.8D). Phosphorylation of STAT4 is critical for IFN-γ production and Th1 cell differentiation.[67,68] Western blot analysis showed that MigR1/STAT4-infected T-KO T cells expressed 7.5-fold more STAT4 protein and 1.2-fold more phosphorylated STAT4 than their WT counterparts (data not shown). Thus, the reduction of T-bet in activated T-KO CD4$^+$ T cells is a major contributor to their impaired development of Th1 cells.

A.4. In summary, our results identify T-bet as the transcriptional and post-translational Ezh2 target that acts together to generate BM-destructive Th1 cells, and highlight the therapeutic potential of Ezh2 inhibition in reducing AA and other autoimmune diseases. Although these studies are not originally planned in our proposal, findings from those experiments suggest that targeting the epigenetic regulator Ezh2 may be an alternative approach to modulate memory T cells and memory T cell-mediated AA.

B. Establishment of a cellular programming approach that is able to reduce the toxicity of donor CD4$^+$ naïve T cell (T_N) to mediate GVHD in mice receiving allogeneic BMT (*Blood, 2016*).[33]

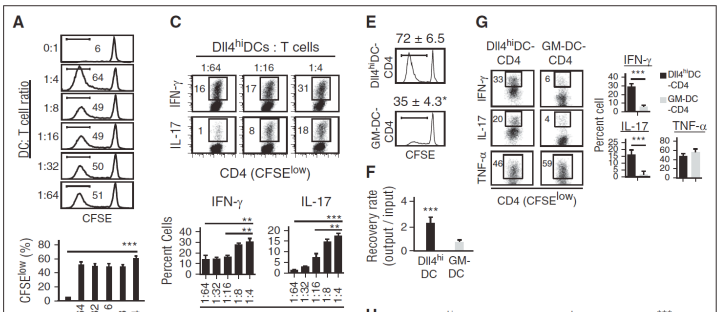
Data from our group and others indicate that induction of alloreactive T cells does not necessarily lead to GVHD. For example, naturally occurred effector memory T cells (nT_{EM}) are unable to mediate GVHD.[69,70] These cells responded to alloantigen and mediated graft-versus-

leukemia (GVL) effect, but showed impaired expansion in local tissues.[69-72] This nT_{EM} pool might have less diverse T cell receptor (TCR) repertoire than the naïve T cell (T_N) pool,[70] however, even host antigen-sensitized T_{EM} showed a reduced ability to trigger GVHD.[73,74] These host-reactive T cells responded to the antigen but died faster than T_N, suggesting cell-intrinsic properties independent of TCR repertoire account for decreased ability of T_{EM} to mediate GVHD.[74] Thus, induction of qualitative changes in donor T cells can reduce their anti-host toxicities.

We previously identified inflammatory DCs that expressed high levels of Dll4 (Dll4^{hi}DCs).[35] They occurred in mice early during GVHD induction and had a greater ability than Dll4-negative DCs to induce IFN-γ and IL-17 in alloantigen-activated T cells.[35] Differentiated effector T cells have reportedly reduced capacity to proliferate and persist in vivo,[75-78] therefore we reasoned that in vitro priming with Dll4^{hi}DCs could allow the induction of alloreactive effector T cells with reduced GVHD toxicity.

B.1. In vitro generated murine Dll4^{hi}DCs induce effector CD4$^+$ T cell differentiation. To test this hypothesis, we first established a novel platform that produced 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 IFN-γ and IL-17 *in vitro,* depending on Dll4 activation of Notch signaling (Fig.9).

Fig.9. Dll4^{hi}DCs induce effector CD4$^+$ T cell differentiation. *To determine if Dll4^{hi}DCs could program T_N for reducing GVHD, we incubated B6 CD4$^+$ T_N with or without escalating numbers of BALB/c Dll4^{hi}DCs for 5 days. These Dll4^{hi}DC-activated CD4$^+$ T cells underwent extensive proliferation, as evidenced by low levels of CFSE (CFSE^{low}) (Fig.9A). As a result, Dll4^{hi}DCs induced expansion of donor CD4$^+$ T cells (Fig.9B), production of high levels of IFN-γ and IL-17 (Fig.9C). These Dll4^{hi}DC-induced T cells killed A20 leukemia cells in vitro (Fig.9D). As compared to Dll4^{hi}DCs, GM-DCs were significantly less potent in promoting proliferation of allogeneic CD4$^+$ T cells and their production of IFN-γ and IL-17 (Fig.2E-G), confirming the functional difference between Dll4^{hi}DCs and GM-DCs.*

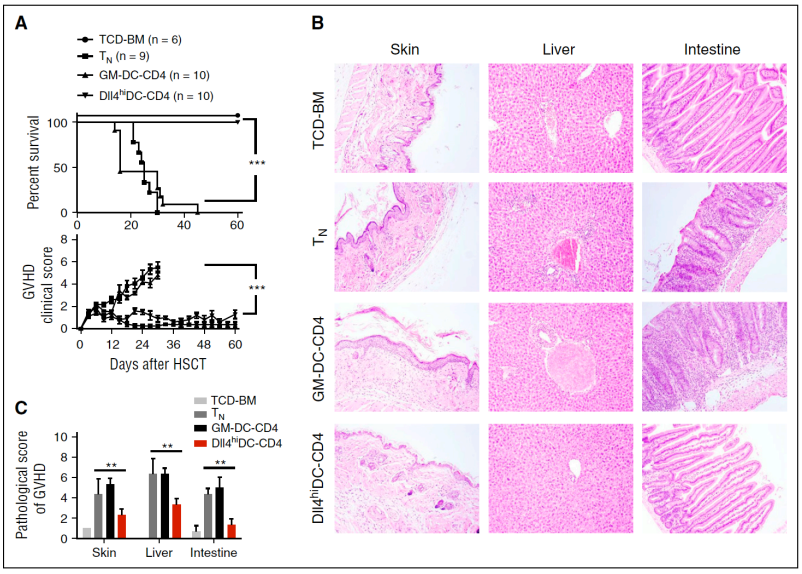


Allogeneic MLR activates polyclonal T cells and is unable to model alloreactivity to a single alloantigen. To test it, we isolated CD4$^+$ T_N specific to OT-II peptide ($OVA_{232-239}$) from TCR transgenic OT-II mice and cultured them with syngeneic Dll4^{hi}DCs pulsed by OT-II peptides. Addition of Dll4^{hi}DCs and OT-II peptides induced production of IFN-γ and IL-17 (Fig.9H). Blocking Dll4 but not Dll1 using neutralizing Abs reduced IFN-γ and IL-17 by CD4$^+$ T cells (Fig.9H). Dll4 blockade also inhibited IFN-γ- and IL-17-production by B6 CD4$^+$ T_N stimulated with BALB/c Dll4^{hi}DCs (Fig.9H). These findings demonstrate that antigenic peptides presented by Dll4^{hi}DCs elicit specific T cell responses and Dll4 is critical for promoting IFN-γ- and IL-17-production.

B.2. Dll4^{hi}DC-induced alloreactive T cells have reduced ability to cause GVHD. Following transfer, allogeneic Dll4^{hi}DC-induced T cells were unable to mediate severe GVHD

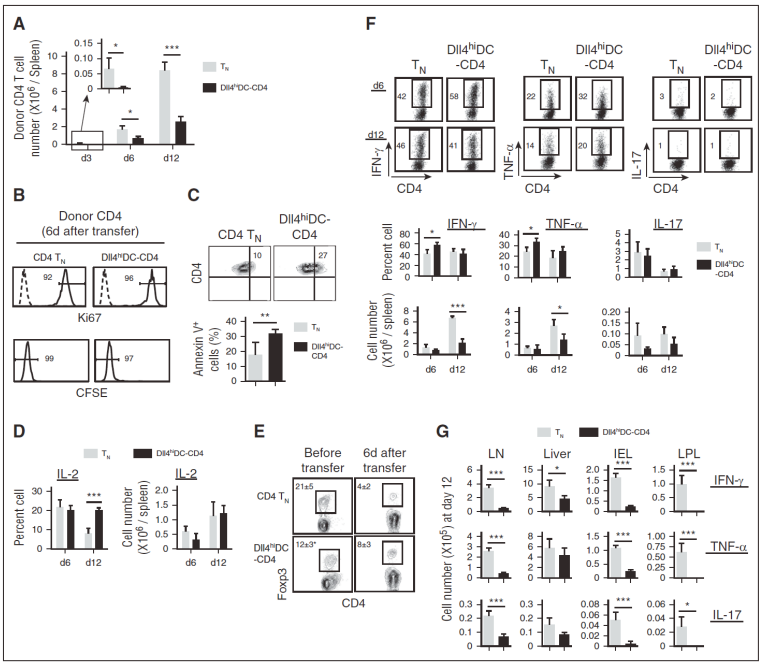
but preserved anti-leukemic activity, significantly improving the survival of leukemic mice undergoing allogeneic HSCT (**Fig.10**).

Fig.10. Dll4^{hi}DC-induced alloreactive T cells have reduced ability to cause GVHD. *To test the ability of allogeneic Dll4^{hi}DC-induced CD4^{+} T cells (Dll4^{hi}DC-CD4) to mediate GVHD, we harvested primed T cells 5 days after stimulation and transferred them with TCD-BM into irradiated BALB/c mice. Transfer of donor CD4^{+} T_N caused lethal GVHD (Fig.10A-C). In contrast, BALB/c mice receiving Dll4^{hi}DC-CD4^{+} T cells developed only minimal GVHD and complete survival (Fig.10A-C). Histological examination demonstrated that Dll4^{hi}DC-CD4^{+} T cells caused less severe tissue inflammation compared to CD4^{+} T_N (Fig.10B,C). Notably, GM-DC-CD4^{+} T cells mediated severe GVHD, with all recipients dying from the disease (Fig.10A-C). Thus, culture with Dll4^{hi}DCs rather than GM-DCs can reduce the GVHD activity of donor CD4^{+} T_N.*



B.3. Dll4^{hi}DC-T cells produced high levels of IFN-γ but have impaired capacity to expand in vivo. This effect of Dll4^{hi}DC-induced T cells was associated with their impaired expansion in GVHD target tissues (**Fig.11**). These data suggest that impaired expansion capability of Dll4^{hi}DC-CD4^{+} T cells in GVHD target organs may account for protection against GVHD.

Fig.11. *We determined the underlying mechanism rendering Dll4^{hi}DC-CD4^{+} T cells ineffective in mediating GVHD. Dll4^{hi}DC-CD4^{+} T cells and CD4^{+} T_N were transferred into irradiated allogeneic BALB/c mice. As compared to T_N recipients, Dll4^{hi}DC-CD4^{+} T cell recipients showed significantly fewer donor T cells in the spleen at day 3, 6 and 12 (Fig.11A). Transferred Dll4^{hi}DC-CD4^{+} T cells had undergone similar proliferation as measured by Ki67 and CFSE dilution 6 days after transplantation (Fig.11B), but had a 1.5-fold higher frequency of apoptotic cells (Fig.11C). Anergic T cells have decreased capacity to produce IL-2 and might contribute to impaired expansion of alloreactive T cells.[79] Dll4^{hi}DC-CD4^{+} T cells produced higher levels of IL-2 than CD4^{+} T_N (Fig.11D). FoxP3^{+} regulatory T cells (Treg) also affect GVH reactions.[4,80] As compared to CD4^{+} T_N, Dll4^{hi}DC-CD4^{+} T cells contained fewer number of Treg prior*



to transfer but showed slightly higher frequency of Treg without statistical significance 6 days after transfer (Fig.11E). These data suggest that impaired expansion of Dll4^{hi}DC-T cells in vivo may be primarily attributable to increased apoptosis.

Interestingly, Dll4^{hi}DC-CD4^{+} T cells produced higher levels of IFN-γ and TNF-α than CD4^{+} T_N cells 6 days after transfer (Fig.11F). Twelve days after transplantation, Dll4^{hi}DC-CD4^{+} T cells and CD4^{+} T_N produced similar levels of IFN-γ and TNF-α (Fig.11F). However, the impaired expansion of transferred Dll4^{hi}DC-CD4^{+} T cells led to an overall reduction of IFN-γ- and TNF-α-producing alloreactive T cells in the spleen, LN, liver and intestine compared to CD4 T_N (Fig.11F,G).

Whether Th17 cells mediate GVHD remains controversial.[81-83] Th17 cells were shown to induce GVHD but donor T cells lacking IL-17 induce worse GVHD when compared with normal T cells.[82] Interestingly, although Dll4^{hi}DC-CD4^{+} T cells produced high levels of IL-17 prior to their transplantation, they did not sustain this capacity following transfer (Fig.11F,G). Th17 cells have a demonstrated plasticity of becoming Th1 cells.[84] Thus, whether the decrease in production of IL-17 seen in Dll4^{hi}DC-CD4^{+} T cells in vivo results from impaired survival and/or IFN-γ-mediated repression of IL-17 during GVH reaction seen by other studies,[85] has to be determined.

B4. In summary, this study presents a novel cellular programming approach that produces alloreactive effector T cells incapable of causing severe GVHD but retaining GVL effects. To facilitate this strategy, we developed a platform that produces Dll4^{hi}DCs from murine BM. Upon in vitro stimulation by allogeneic Dll4^{hi} DCs, donor CD4^{+} T_N became alloreactive effector cells that secreted high levels of IFN-γ and IL-17. Adoptive transfer of these Dll4^{hi} DC-induced T cells eliminated leukemic cells without causing severe GVHD, leading to significantly improved survival of leukemic mice undergoing allogeneic HSCT. This strategy has several potential advantages compared to current and developing methods for the modification of donor grafts to reduce GVHD,[69,70,73,86,87] including no requirement for T cell subset selection, dramatically expanded safety range of infused donor T cell dose. Importantly, our platform does not require transfection with viral vectors, which has limitations of safety and efficiency. Thus, Dll4^{hi} DC programming can overcome GVHD toxicity of donor T cells and produce leukemia-reactive T cells for effective immunotherapy.

C. Discovering the role of human DCs expressing DLL4 and their roles in regulating Th1 and Th17 differentiation (*JI 2016*).[34]

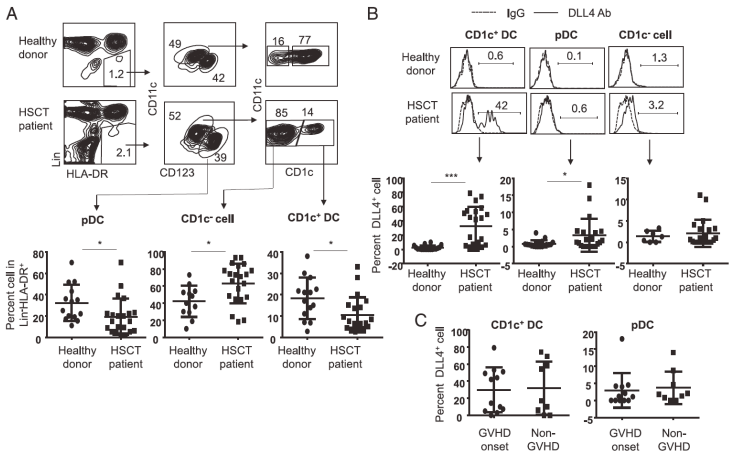
In this study, we report the identification of human DLL4^{+} DCs and their critical role in regulating Th1 and Th17 differentiation. CD4 Th1 cells, which are characterized by production of high levels of IFN-γ, are well defined for AA in patients and experimental AA mice.[1,2,37,41,64,88,89] In human patients with AA, T-bet, which is a transcription factor critical for inducing both Th1 and cytotoxic CD8 effector T cells, is found to be aberrantly overexpressed in T cells and correlates with disease severity.[4] We asked whether the human counterpart of murine DLL4^{+} DCs existed and whether DLL4 derived from human DCs was important for promoting the generation of IFN-γ- and IL-17-producing T cells. Assessing the role of human DC-derived DLL4 in T cell responses will be important for better defining in depth the pathophysiology of AA in human patients in future.

Our understanding of human DCs is derived predominantly from studies of cells isolated from peripheral blood (PB) [90]. Under steady state condition, human PB DCs are defined as cells that lack lineage (Lin) markers (i.e., CD3, CD15, CD19, CD14, CD20 and CD56) and constitutively express HLA-DR (referred as Lin^{-}DR^{+} pan-DCs)[91]. Human PB DCs are broadly categorized into two major subsets: cDCs and pDCs. cDCs are characterized as Lin^{-}HLA-DR^{+}CD11c^{+} cells, whereas pDCs are Lin^{-}HLA-DR^{+}CD11c^{-}CD123^{high}cells[91,92]. In blood, cDCs can be further classified into at least two subsets: CD1c^{+} DCs and CD141^{+} DCs[93]. The former

comprises the predominant cDC subset, whereas the latter is a relatively small population. At least three lines of evidence indicate that these three subsets of DCs may have different functions in mediating T cell immune responses. $CD1c^+$ DCs express toll-like receptor (TLR)4 and TLR7, $CD141^+$ DCs have high expression of TLR3, and pDCs express TLR7 and TLR9 and lack TLR4.[91,94-97] In addition, when activated, $CD1c^+$ DCs produce high levels of IL-12, IL-6, IL-23 and IL-1β, whereas $CD141^+$ DCs secrete IL-12 and IFN-β. In contrast, pDCs produce IFN-α and IFN-β.[97-103] However, whether PB $CD1c^+$ DCs and pDCs produce high levels of DLL4 and whether DLL4 derived from human DCs are critical for regulating Th1 and Th17 differentiation have not been previously defined.

C.1. Circulating blood DCs from HSCT patients upregulate DLL4. $CD1c^+$ DCs and pDCs from the peripheral blood of healthy donors did not express DLL4. In contrast, patients undergoing allogeneic HSCT had a 16-fold more $DLL4^+CD1c^+$ DCs than healthy donors (**Fig.12**).

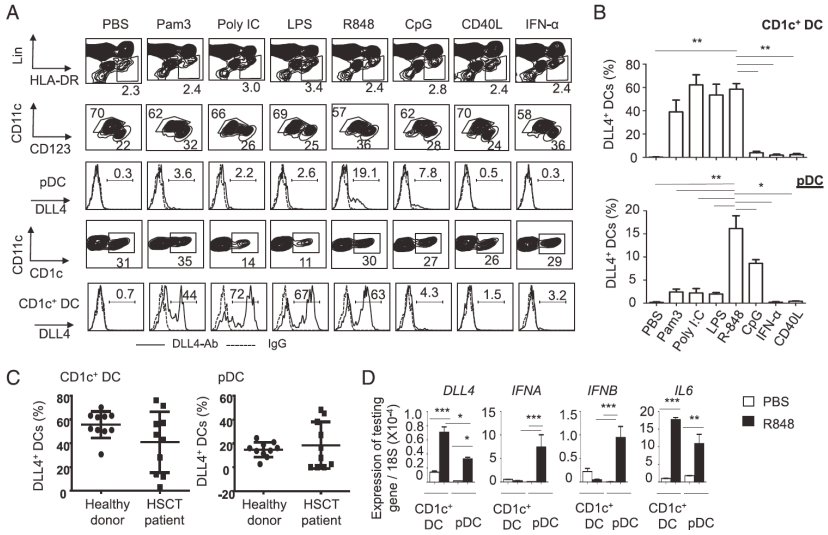
Fig.12. *To identify human $DLL4^+$ DCs and characterize their biological properties, we obtained PB from healthy donors. Flow cytometric analysis revealed that Lin^-DR^+ pan-DCs contained three subsets: pDCs ($CD11c^-CD1c^-CD123^{hi}$), $CD1c^+$ DCs ($CD11c^+CD1c^+CD123^{low}$), and $CD11c^+CD1c^-CD123^{low}$ cells (Fig.12A). We found that only a small fraction of $CD1c^+$ DCs (2.2% ± 0.7%) and pDCs (0.8% ± 0.2%) from PB of healthy donors expressed low levels of DLL4 on their surface (Fig.12B). $CD11c^+CD1c^-CD123^{low}$ cells, which accounted for approximately 35% of Lin^-DR^+ pan-DCs, were also negative for DLL4 (Fig.12B). Thus, under steady-state conditions, most of PB DCs do not express surface DLL4.*



To determine if under inflammatory conditions circulating DCs might upregulate DLL4, we obtained PB from patients undergoing allogeneic HSCT between 21 and 39 days after transplantation when these patients have fully engrafted and were no longer pancytopenic. HSCT recipients had lower proportion of $CD1c^+$ DCs and pDCs than healthy donors (Fig.12A). This is consistent with previous observations of decreased circulating DCs in HSCT patients[104-106]. Interestingly, as compared to healthy donors, HSCT recipients had a 12-fold higher frequency of $DLL4^+CD1c^+$ DCs (Fig.12B). In contrast, both pDCs and $CD11c^+CD1c^-CD123^{low}$ DCs from HSCT patients did not express DLL4 compared to healthy donors (Fig.12B). Our results indicate that upregulation of DLL4 on the surface of DCs is associated with the inflammatory condition that accompanies allogeneic HSCT due to conditioning and alloreactivity.

C.2. Activation of TLR signaling induces DLL4 in human $CD1c^+$ DCs and pDCs. Upon activation of toll-like receptor signaling, healthy donor-derived $CD1c^+$ DCs dramatically upregulated DLL4, as did pDCs to a lesser extent (**Fig.13**). Activated $DLL4^+$DCs were better able to promote Th1 and Th17 differentiation than unstimulated PB DCs. Blocking DLL4 using a neutralizing antibody decreased Notch signaling in T cells stimulated with $DLL4^+$ DCs, and reduced the generation of Th1 and Th17 cells (See Fig.3 to Fig.5, reference- **[34]**)

Fig.13. *Previous studies have demonstrated that activation of TLR signaling is important for inducing Notch ligands in murine antigen-presenting cells[24,26,49,107]. To determine the stimulus capable of inducing high levels of DLL4 in human DCs, we isolated PBMCs from healthy donors and cultured them with a variety of TLR agonists. Pam3 (TLR1/2 stimulus), Poly I:C (TLR3 stimulus), LPS (TLR4 stimulus) and R848 (TLR7/8 stimulus) induced high levels of DLL4 expression on the surface of 50% to 80% of CD1c$^+$ DCs, whereas IFN-α (proinflammatory cytokine) and CD40L (signal from activated T cells) did not (Fig. 13A,B). CpG oligodeoxynucleotides (TLR9 agonists) did not increase DLL4 in CD1c$^+$ DCs (Fig. 13A,B), likely due to their lacking of TLR9[108,109]. pDCs increased DLL4 expression when activated by R848 (16.0% ± 2.7%) and to less extent by CpG oligodeoxynucleotides (8.6% ± 0.8%) (Fig. 13A,B). These results demonstrate that activation of TLR signaling induces high levels of DLL4 in CD1c$^+$ DCs and pDCs, with R848 being the most potent stimulus. R848 also induced DLL4 on the surface of approximately 30% of CD141$^+$ DCs (data not shown). We did not further explore this CD141$^+$ DC subset due to its rarity in the circulation.[110]*



We next focused on assessing biological properties of PB CD1c$^+$ DCs and pDCs that were activated by R848 in subsequent experiments. We observed no significant difference in fraction of DLL4-expressing CD1c$^+$ DCs and pDCs between healthy donors and HSCT patients after R848 stimulation (Fig. 13C). This indicates that CD1c$^+$ DCs and pDCs from healthy donors and HSCT patients have similar capacity to increase DLL4 upon activation of TLR7/8.

To further assess whether these DCs increased DLL4 at the transcriptional level, we isolated PB Lin$^-$ DR$^+$ DCs from healthy donors and incubated them with R848 for 24 hours to induce DLL4. Highly pure populations of CD1c$^+$ DCs and pDCs were then obtained through FACS cell sorting. Real-time RT-PCR validated that activated pDCs expressed significantly higher levels of IFNA and IFNB, which are signature genes for pDCs[94], compared to activated CD1c$^+$ DCs (Fig. 13D). Notably, activated CD1c$^+$ DCs expressed higher levels of DLL4 than pDCs (Fig. 13D), which was consistent with the observation that there was about 4-fold more in frequency of CD1c$^+$ DCs capable of upregulating cell surface DLL4 compared to pDCs (Fig. 13B,C). Taken together, these results show that activation of TLR signaling induces high levels of DLL4 at transcriptional levels in both CD1c$^+$ DCs and pDCs.

C.3. Both NFκB and STAT3 were crucial for inducing DLL4 in human DCs. We further determined he underlying mechanism that regulated DLL4 expression in human DCs. Interestingly, STAT3 directly activated DLL4 transcription and inhibiting STAT3 alone was sufficient to reduce DLL4 in activated PB DCs (**Fig.14** and **Fig.15,** below).

Fig.14. NFκB only is not sufficient to induce DLL4 induction in human DCs. *We determined the molecular mechanism that regulated DLL4 in human DCs. NFκB is a critical pathway downstream of TLR signaling[111]. We found that the NFκB inhibitor PDTC completely blocked DLL4 induction in both DC subsets (Fig. 14A,B), suggesting the important role of NFκB in inducing DC expression of DLL4. Interestingly, stimulation of monocytes with R848 + LPS, which is known to activate NFκB signal in these cells,[112] activated NFκB as evidenced by increased expression of phosphorylated P65 (p-P65) (Fig. 14C), but induced low levels of DLL4 on the surface of monocytes (Fig. 14D). Real-time RT-PCR analysis further revealed that activated monocytes expressed 2 to 5-fold less DLL4 transcripts compared to pDCs and CD1c$^+$DCs (Fig. 14E). Furthermore, DCs derived from monocytes cultured in GM-CSF and IL-4 (referred to as moDCs) had elevated p-P65 following stimulation by R848 + LPS (Fig. 14F) and upregulated the expression of costimulatory molecules (e.g., CD40, CD80, CD83, and CD86) (Fig. 14G), but were DLL4 negative (Fig. 14G). These data suggest that activation of NFκB is important but not sufficient for inducing DLL4 in human DCs. Other uncharacterized factor(s) is required to induce DLL4 in PB DCs.*

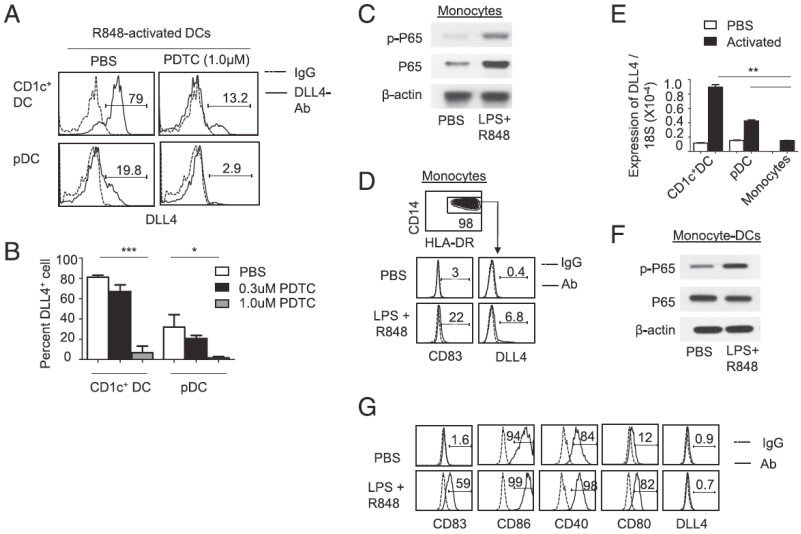
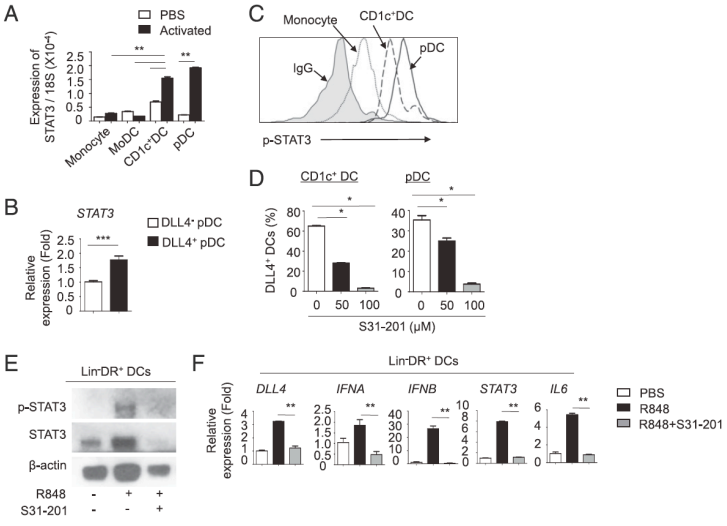


Fig.15. STAT3 is critical for inducing DLL4 in PB CD1c$^+$ DCs and pDCs. *STAT3, which is a transcription factor that regulates genes involved multiple cell processes,[113] is essential for production of Flt3L-dependent DCs rather than monocytes and moDCs.[114] To determine the effect of STAT3 on DC expression of DLL4, we first examined the expression of STAT3 in DCs derived from different sources. We found that R848-activated CD1c$^+$ DCs and pDCs expressed 8- to 10-fold more STAT3 transcript than both monocytes and moDCs that were activated by R848 plus LPS (Fig.15A). As expected, DLL4$^+$ pDCs expressed significantly higher level of STAT3 than DLL4$^-$ pDCs (Fig.15B).*

To further evaluate the effect of STAT3 on DLL4 expression in DCs, we examined the amount of phosphorylated STAT3 (p-STAT3) in both CD1c$^+$DCs and pDCs. Flow cytometric analysis showed that both activated CD1c$^+$DCs and pDCs significantly higher levels of pSTAT3 than monocytes (Fig. 15C). Addition of the STAT3 inhibitor S31-201, which blocks STAT3 phosphorylation and dimerization[115], dramatically reduced DLL4 expression in both CD1c$^+$DCs and pDCs (Fig. 15D). Using Lin$^-$DR$^+$ pan-DCs, we confirmed that S31-201 treatment decreased p-STAT3 in these cells (Fig.15E). This was accompanied with a reduction of DLL4, IFNA and IFNB (Fig. 15F), suggesting that STAT3 inhibition may have a broad effect on DC function. Interestingly, S31-201 also significantly reduced expression of



STAT3 transcripts and protein (Fig. 15E,F). This indicates that STAT3 has self-regulation effect, which is consistent with previous observations[116]. Thus, in addition to NFκB, activation of STAT3 is critical for inducing DLL4 in PB DCs.

C.4. In summary, human DCs are important primary T cell responses, and cytokines produced by DCs are also thought to be essential for promoting Th1 and Th17 differentiation. Both human $CD1c^+$ DCs and pDCs can produce IL-12, promoting the generation of Th1 $CD4^+$ T cells.[110,117] $CD1c^+$ DCs also secreted IL-23 and promoted Th17 cell responses.[98] Our central finding is that both human PB $CD1c^+$ DCs and pDCs require DLL4 to direct Th1 and Th17 differentiation. When activated by TLR ligands, both PB $CD1c^+$ DCs and pDCs upregulated DLL4 and acquired potent capacity to induce production of high levels of IFN-γ and IL-17 by human alloreactive $CD4^+$ T cells. Notably, without ex vivo activation of TLR signaling, patients undergoing allogeneic HSCT had significantly higher frequency of $DLL4^+CD1c^+$ DCs in their PB compared to normal healthy donors. Our findings show that DLL4 derived from human DCs is critical for the priming of human Th1 and Th17 responses and may have a significant role in better understanding of T cell-mediated inflammatory conditions such as chronic infection, autoimmune diseases, tumor rejection and GVHD after allogeneic HSCT.

3.3. 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

3.4. 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

3.5. 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.

Nothing to report

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).

We would like to thank the ***Congressionally Directed Medical Research Programs*** for providing this support to our research. This award from the DOD is critical for us to accomplish the goals designed in this project. Over the past four years of the support, we have published 16 papers and acquired two R01 grants.

Our findings are likely to make an impact on the development of new and clinically relevant strategies that can be used for: **A)** improving the safety and efficacy of allogeneic BMT (which is an effective therapy for BMF); and **B)** reducing the generation and persistence of BM-destructive T cells. Specifically, we have made significant progress in understanding the pathophysiology of AA in mice and developing novel approaches to modulate inflammatory T cell responses-mediated BMF. These include: **1)** identifying a previously unrecognized DLL4-expressing DCs that are important for mediating T cell responses in mice; **2)** establishing a cellular programming approach to reduce T cell toxicity of mediating GVHD, thereby improving the safety and efficacy of allogeneic BMT; **3)** discovering the critical role of human DCs expressing DLL4 in mediating human T cell responses, which will facilitate the translation of our bench studies into human patients in future; and **4)** identifying the essential role of chromatin-modifying enzyme Ezh2 in the production and persistence of BM-destructing T cells. This

discovery opens a new perspective to study how Notch and Notch ligands may interact with epigenetic regulators to control memory T cell development and subsequent production of AA. Most importantly, pharmacological EZH2 inhibitors are in clinical trials for the treatment of cancer. We hope these inhibitors will be used to develop effective approaches to treat AA.

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

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

Nothing to report

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

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:
- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report.

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

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. CHANGES/PROBLEMS: The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously

reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

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

N/A

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.

N/A

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.

N/A

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

N/A

Significant changes in use or care of vertebrate animals

N/A

Significant changes in use of biohazards and/or select agents

N/A

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 followings are our major publications over the period of this grant support:

1. He S, Wang J, Kato K, Varambally S, Xie F, Kuick R, Mineishi S, Liu Y, Nieves E, Mani R, Chinnaiyan AM, Marquez VE and **Zhang Y**, Inhibition of histone methylation arrests ongoing graft-versus-host diseases in mice by selectively inducing apoptosis of alloreactive effector T cells. *Blood*, 2012, 119:1274. PMID:22117046 PMCID:PMC3338164

2. Mochizuki K, Xie F, He S, Tong Q, Liu Y, Guo YJ, Kato K, Yagita H, Mineishi S, and **Zhang Y**. Delta-like Ligand 4 Identifies a Previously Uncharacterized Population of Inflammatory Dendritic Cells That Plays Important Roles in Eliciting Allogeneic T-cell Responses in Mice. *Journal of Immunology* 2013. 190(7):3772-82. doi: 10.4049/jimmunol.1202820.

3. He S, Xie F, Liu Y, Tong Q, Mochizuki K, Lapinski PE, Mani RS, Reddy P, Mochizuki I, Chinnaiyan AM, Mineishi S, King PD, and **Zhang Y**. The histone methyltransferase Ezh2 is a crucial epigenetic regulator of allogeneic T cell responses mediating graft-versus-host disease. *Blood* 2013 Dec 12;122(25):4119-28. PMID:24141370

4. Tong Q, He S, Xie F, Mochizuki K, Liu Y, Mochizuki I, Meng L, Sun H, Zhang YY, Guo Y, Hexner E, and **Zhang Y**. Ezh2 regulates transcriptional and post-translational expression of T-bet and promotes Th1 cell responses mediating aplastic anemia in mice. J Immunol 2014, 192(11) 5012-5022, PMID:24760151, PMCID: PMC4075972

5. 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.

6. **Kazuhiro Mochizuki**, Lijun Meng, Izumi Mochizuki, Qing Tong, Shan He, Yongnian Liu, Janaki Purushe, Hongxing Sun, Henry Fung, M. Raza Zaidi, Ran Reshef, Bruce R Blazar, Hideo Yagita, Shin Mineishi, and **Yi Zhang**. 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).

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

Nothing to report.

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

1. *Kazuhiro Mochizuki, Fang Xie, Shan He, Qing Tong, Yongnian Liu, Yajun Guo, Koji Kato, Hideo Yagita, Shin Mineishi, and Yi Zhang. Notch ligand Dll4 derived from inflammatory plasmacytoid DCs plays important roles in eliciting graft-versus-host disease in mice. The 54th ASH Annual Meeting and Exposition (December 8-11, 2012), in Atlanta, GA. Oral presentation.

2. Invited Speaker, Damon Runyon-Rachleff Innovators Symposium, New York, NY, "Therapeutic targeting of Notch signaling in T cell alloimmunity", Oct. 2011.

3. Invited Speaker, "Notch ligands, inflammatory dendritic cells and alloimmunity", The University of Alabama at Birmingham, Alabama, Jul. 2012.

4. Invited Speaker, "Notch and inflammatory T-cell response", Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. China, Apr. 2012.

5. Invited Speaker, "Dll4+ Dendritic cells and alloimmunity", The Fox Chase Cancer Center, Temple University, PA, Mar 2014

6. Invited Speaker, "Inflammatory Dendritic cells and alloimmunity", CBG&E, Temple University, PA, May 2014

7. Invited Speaker, "Target histone methyltransferase Ezh2 to modulate GVHD", Grand Round, Cancer Institute, Pennsylvania State University, PA, Oct. 2015.

- **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.

Lay Press:

Delta-like Ligand 4 identifies a previously uncharacterized population of inflammatory dendritic cells that plays important roles in eliciting allogeneic T-cell responses in mice. Journal of Immunology, 2013, 190:3772). In this study, we report the discovery of previously uncharacterized inflammatory DCs expressing high levels of Notch ligand Delta-like Ligand 4 (Dll4). These Dll4-positive inflammatory DCs have greater ability than conventional DCs to induce Th1 and Th17 cell responses essential for mediating GVHD. In vivo administration of neutralizing antibody specific to Dll4 leads to reduction of GVHD in mice after allo-BMT. These findings indicate that Dll4 and Dll4-positive DCs play important roles in eliciting GVHD, and represent beneficial targets for modulating allogeneic T cell responses.

The following website shows the highlights:
http://cdmrp.army.mil/bmfrp/research_highlights/13zhang_highlight.shtml;

- **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.

Findings from our studies lead to the application of patent, entitled: "DLL4-expressing cells and vaccine using the same".

- **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.)

Nothing to report.

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

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

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

N/A

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.

We include the following original copies of journal articles:

1. He S, Wang J, Kato K, Varambally S, Xie F, Kuick R, Mineishi S, Liu Y, Nieves E, Mani R, Chinnaiyan AM, Marquez VE and Zhang Y, Inhibition of histone methylation arrests ongoing graft-versus-host diseases in mice by selectively inducing apoptosis of alloreactive effector T cells. *Blood*, 2012, 119:1274. PMID:22117046 PMCID:PMC3338164

2. Mochizuki K, Xie F, He S, Tong Q, Liu Y, Guo YJ, Kato K, Yagita H, Mineishi S, and Zhang Y. Delta-like Ligand 4 Identifies a Previously Uncharacterized Population of Inflammatory Dendritic Cells That Plays Important Roles in Eliciting Allogeneic T-cell Responses in Mice. *Journal of Immunology* 2013. 190(7):3772-82. doi: 10.4049/jimmunol.1202820.

3. He S, Xie F, Liu Y, Tong Q, Mochizuki K, Lapinski PE, Mani RS, Reddy P, Mochizuki I, Chinnaiyan AM, Mineishi S, King PD, and Zhang Y. The histone methyltransferase Ezh2 is a crucial epigenetic regulator of allogeneic T cell responses mediating graft-versus-host disease. *Blood* 2013 Dec 12;122(25):4119-28. PMID:24141370

4. Tong Q, He S, Xie F, Mochizuki K, Liu Y, Mochizuki I, Meng L, Sun H, Zhang YY, Guo Y, Hexner E, and Zhang Y. Ezh2 regulates transcriptional and post-translational expression of T-bet and promotes Th1 cell responses mediating aplastic anemia in mice. J Immunol 2014, 192(11) 5012-5022, PMID:24760151, PMCID: PMC4075972

5. 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.

6. Kazuhiro Mochizuki, Lijun Meng, Izumi Mochizuki, Qing Tong, Shan He, Yongnian Liu, Janaki Purushe, Hongxing Sun, Henry Fung, M. Raza Zaidi, Ran Reshef, Bruce R Blazar, Hideo Yagita, Shin Mineishi, and Yi Zhang. 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).

Reference:

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3. Young, N.S., Bacigalupo, A. & Marsh, J.C. Aplastic anemia: pathophysiology and treatment. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* **16**, S119-125 (2010).
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21. Yang, J.*, et al.* Allograft rejection mediated by memory T cells is resistant to regulation. *Proc Natl Acad Sci U S A* **104**, 19954-19959 (2007).

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