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Myeloid differentiation factor 88 signaling in donor T cells accelerates graft-*versus*-host disease

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ABSTRACT

Myeloid differentiation factor 88 (MyD88) signaling has a crucial role in activation of both innate and adoptive immunity. MyD88 transduces signals *via* Toll-like receptor and interleukin-1 receptor superfamily to the NFκB pathway and inflammasome by forming a molecular complex with interleukin-1 receptor-associated kinase 4. The MyD88/interleukin-1 receptor-associated kinase 4 pathway plays an important role, not only in innate immunity, but also T-cell immunity; however, its role in donor T cells on the pathophysiology of graft-*versus*-host disease (GvHD) remains to be elucidated. We addressed this issue by using MyD88-deficient T cells in a mouse model of allogeneic hematopoietic stem cell transplantation (allo-SCT). While MyD88-deficient and wild-type T cells proliferated equivalently after transplantation, MyD88-deficient T cells demonstrated impaired survival and differentiation toward Th1, Tc1, and Th17, and induced less severe GvHD compared to wild-type T cells. Administration of interleukin-1 receptor-associated kinase 4 inhibitor PF-06650833 significantly ameliorated GvHD after allo-SCT. These results thus demonstrate that donor T-cell MyD88/ interleukin-1 receptor-associated kinase 4 pathway is a novel therapeutic target against GvHD after allo-SCT.

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Introduction

Myeloid differentiation factor 88 (MyD88) is a critical adaptor molecule to transduce the signals through receptors belonging to most of the TLR and IL-1R family (TLR/IL-1R superfamily) to NFκB pathway and inflammasome by recruiting IL-1R-associated kinase 4 (IRAK4).[1] The TLR/MyD88 pathway in myeloid cells plays an essential role in innate immunity by recognizing pathogen-associated molecular patterns (PAMP) released by microbes and damage-associated molecular patterns (DAMP) produced by stressed or dying cells.[2] MyD88 dependent signaling in myeloid cells plays proinflammatory roles by secreting proinflammatory cytokines and enhancing antigen presentation, and also homeostatic roles by maintaining regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC).[3,4] In addition, there is growing evidence to indicate that MyD88 signaling in T cells also plays a critical role in T-cell proliferation, survival, and differentiation upon TCR-stimulation.[5-13]

Graft-*versus*-host disease (GvHD), the major complication of allogeneic hematopoietic stem cell transplantation (allo-SCT), is mediated by donor T cells recognizing host-derived alloantigens expressed on professional or non-professional APC and further accelerated by pre-transplant conditioning-mediated inflammatory milieu and tissue injury.[14,15] In particular, damage of the epithelial and mucous barrier allows translocation of bacteria and its immunostimulatory molecules into systemic circulation, leading to subsequent activation of innate immune responses

through TLR stimulation towards production of inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-1 family cytokines.[16-19]

In this study, we evaluated the role of MyD88 signaling in donor T cells by using a well-established mouse model of allogeneic bone marrow transplantation (allo-BMT), in which lethally irradiated recipient mice were transplanted with *MyD88*$^{-/-}$ T cells and T cell-depleted bone marrow cells (TCD-BM) from *WT* mice. We found that lack of MyD88 signaling in donor T cells directly modulated adaptive T-cell responses and reduced severity of GvHD in association with profoundly impaired donor Th1, Tc1, and Th17 responses. Administration of a pharmacological IRAK4 inhibitor, PF-06650833, significantly ameliorated GvHD. MyD88 in donor T cells was not essential for graft-*versus*-leukemia (GvL) effects, suggesting that MyD88 in T cells is a potential therapeutic target of GvHD, while sparing GvL effects.

Methods

Mice

Female C57BL/6 (B6, H-2^{b}) and B6D2F1 (H-2$^{b/d}$) mice were purchased from Charles River Japan (Yokohama, Japan). *TLR2*$^{-/-}$ and *TLR7*$^{-/-}$ mice with a B6 genetic background were purchased from Oriental Bioservice (Chiba, Japan). B6-*MyD88*$^{-/-}$ mice were produced and maintained as previously described.[20] Age of the mice was 8-10 weeks. All animal experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee (approval n: 12-0106).

Bone marrow transplantation

Mice were transplanted as previously described.[21] In brief, recipient B6D2F1 mice were intravenously (i.v.) injected with 5x10^{6} TCD-BM cells form WT B6 donors plus 1x10^{6} T cells purified from either wild-type (WT) or *MyD88*$^{-/-}$ B6 donors on day 0 following lethal total body irradiation (TBI, 12Gy) delivered in two doses at 3-hour intervals. BALB/c recipients were transplanted with 5x10^{6} TCD-BM cells from WT B6 donors plus 1x10^{6} T cells purified from either WT or *MyD88*$^{-/-}$ B6 donors on day 0 following 6 Gy TBI. Isolation of T cells and TCD were performed using a Pan T cell Isolation kit II and anti-CD90-MicroBeads, respectively, and the autoMACS Pro system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Mice were housed in sterilized microisolator cages and received autoclaved hyperchlorinated drinking water for the first three weeks after BMT, and filtered water thereafter.

Assessment of graft-*versus*-host disease and graft-*versus*-leukemia effect

Clinical GvHD scores were assessed as previously described.[22] GvL was assessed by postmortem examination or on *in vivo* bioluminescent imaging.[23,24] Detailed protocols are described in the *Online Supplementary Methods*.

Quantitative-polymerase chain reaction

RNA extraction and quantitative-polymerase chain reaction (Q-PCR) were performed as described in the *Online Supplementary Methods*. Specific primers and probes used for Q-PCR are listed in *Online Supplementary Table S1*.

Flow cytometric analysis

Flow cytometric analysis was performed as previously described.[21] The cells isolated from the thymus or spleen were incubated with antibodies (Abs) (listed in *Online Supplementary Table S2*) at 4°C for 30 minutes (min). Detailed protocols are described in the *Online Supplementary Methods*.

Cell cultures

All culture media and incubation conditions have been previously described.[21] TCR on purified T cells (5x10^{4} T cells/well) were stimulated with 5x10^{4} /well of Dynabeads Mouse T-Activator CD3/CD28 for T-cell expansion and activation (ThermoFisher Scientific, Waltham, MA, USA) in the presence or absence of TLR ligands at concentrations listed in *Online Supplementary Table S3* or PF-06650833 (20 μM) for up to 96 hours.

T-cell proliferation

To assess T-cell proliferation, purified T cells were labeled using a CellTrace Violet Cell Proliferation Kit (ThermoFisher Scientific) according to the manufacturer's instructions. To measure cellular uptake of BrdU, recipients were intraperitoneally (i.p.) injected with 1 mg of BrdU 2 hours before analyses.

Statistical analysis

Mann-Whitney U tests were used to analyze cell counts, the cytokine data, and the clinical scores. We used the Kaplan-Meier product limit method to obtain the survival probability. and the log-rank test was applied to compare the survival curves. $P<0.05$ was considered statistically significant.

Results

Donor T cells lacking MyD88 pathway induce attenuated graft-*versus*-host disease

We investigated whether ablation of MyD88 signaling in donor cells influenced GvHD in a well-established mouse model of haploidentical BMT. Lethally irradiated B6D2F1 mice were i.v. injected with 5x10^{6} BM plus 5x10^{6} splenocytes from either WT or *MyD88*$^{-/-}$ B6 donors. Frequencies and absolute numbers of CD4^{+} T cells, CD8^{+} T cells, memory T cells, and Foxp3^{+} Tregs in the spleen were equivalent in donor WT and *MyD88*$^{-/-}$ B6 mice (*Online Supplementary Figure S1*). While GvHD was severe in allogeneic controls with 80% mortality by day 50, 67% of recipients of *MyD88*$^{-/-}$ donors survived this period (Figure 1A). Clinical GvHD scores were also significantly lower in recipients of *MyD88*$^{-/-}$ graft compared to those of WT graft (Figure 1B).

Next, we evaluated if effects of MyD88 signaling in donor cells on GvHD could reside in the T-cell compartment of the donor graft. Lethally irradiated B6D2F1 mice were injected with 5x10^{6} TCD-BM from WT B6 mice plus 1x10^{6} T cells from either WT or *MyD88*$^{-/-}$ B6 mice. MyD88 deficiency in donor T cell alone significantly ameliorated mortality and morbidity of GvHD (Figure 1C and D). Histopathological examination of the small intestine and colon performed 6-8 weeks after BMT confirmed attenuated GvHD pathology in recipients of *MyD88*$^{-/-}$ T cells. GvHD pathology in the small intestine, including villous blunting, epithelial apoptosis, and Paneth-cell loss accom-

panied by inflammatory-cell infiltration, was significantly less severe in recipients of *MyD88*$^{-/-}$ T cells (Figure 1E-G), although liver GvHD was the same as in controls. The thymic GvHD characterized by the loss of CD4^{+}CD8^{+} double positive (DP) thymocytes was also less severe in *MyD88*$^{-/-}$ T-cell recipients than in controls (Figure 1H-J). The role of donor T-cell MyD88 signaling in GvHD was not strain dependent, as MyD88 deficiency in donor T cells ameliorates GvHD in the B6 into BALB/c model (Figure 1K).

Absence of MyD88 signaling impairs donor Th1/Tc1/Th17 differentiation

Induction of GvHD is primarily dependent on donor T-cell reactivity to host alloantigens.[14] To evaluate the effects of MyD88 signaling in donor T-cell activation *in vivo*, donor T-cell expansion was evaluated in the spleen early after BMT. Numbers of CD8^{+} T cells were significantly less in *MyD88*$^{-/-}$ T-cell recipients than those in controls seven days after BMT (Figure 2A). Both groups showed complete donor T-cell chimerism, ruling out mixed chimerism as a cause of reduced numbers of donor T cells (Figure 2B). Analysis of cell division using CellTrace Violet-labeled T cells showed equivalent cell division between WT and *MyD88*$^{-/-}$ T cells at both 72 and 96 hours after BMT (Figure 2C and D). Donor T-cell proliferation determined by BrdU uptake on day 6 also showed equivalent T-cell uptake of BrdU in both groups (Figure 2E and F). Proliferative response of WT and *MyD88*$^{-/-}$ T cells to CD3/CD28 stimulation *in vitro* and after allogeneic SCT *in*

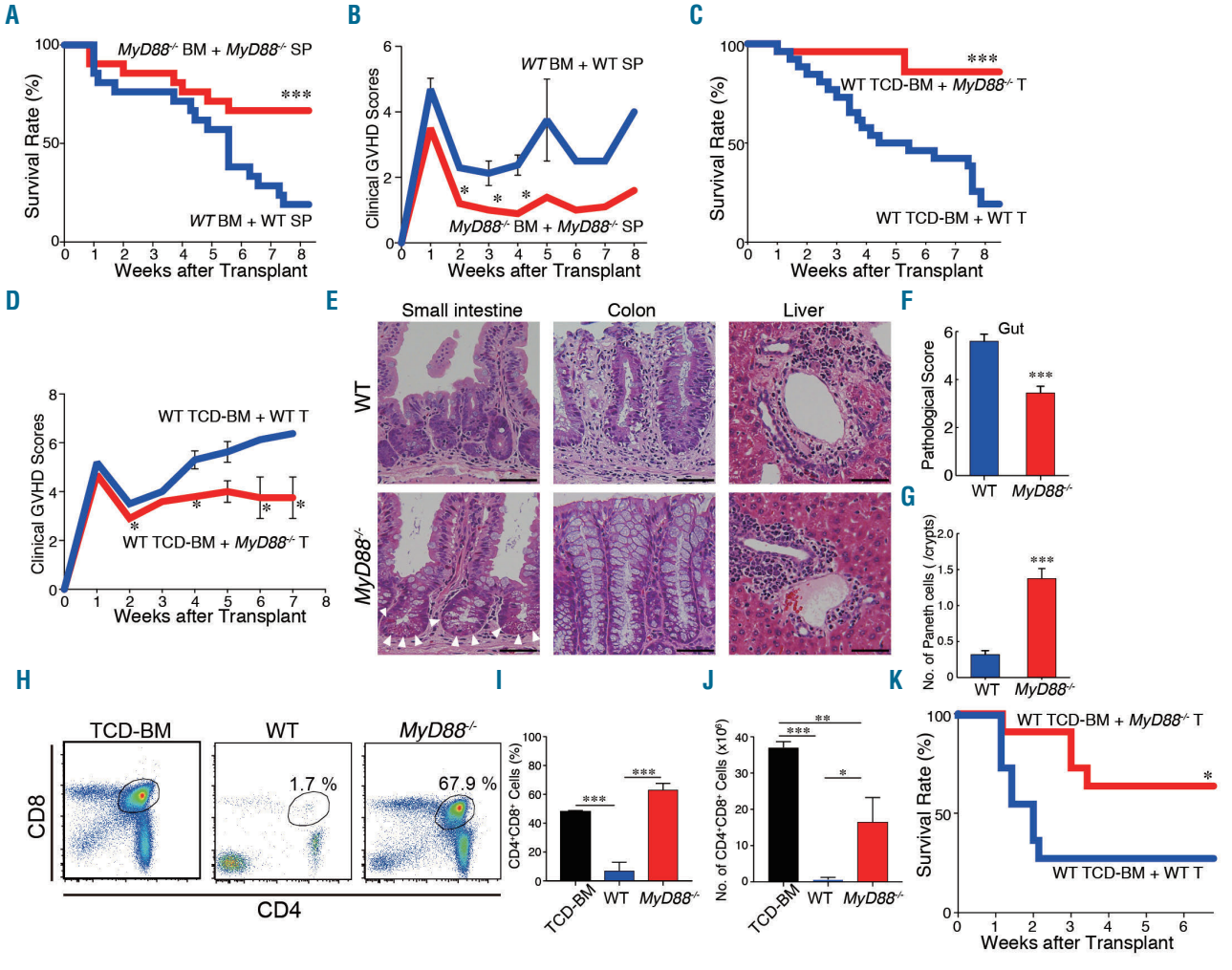

Figure 1. MyD88 signaling in donor T cells exaggerates graft-versus-host disease (GvHD). (A and B) Lethally irradiated B6D2F1 mice were transplanted with 5x10^{6} bone marrow (BM) cells plus 5x10^{6} splenocytes from wild-type (WT) (n=21) or *MyD88*$^{-/-}$ (n=21) B6 donors on day 0. Survival (A) and clinical GvHD scores (B) from four independent experiments are combined. (C-H) Lethally irradiated B6D2F1 mice were transplanted with 5x10^{6} T-cell-depleted bone marrow cells (TCD-BM) cells from WT B6 mice plus 1x10^{6} purified T cells from WT or *MyD88*$^{-/-}$ B6 donors. Survival (C) and clinical GvHD scores (D) from five independent experiments are combined (n=25-26 / group). (E) Representative Hematoxylin & Eosin (H&E) images of the small intestine, colon, and liver harvested 6-8 weeks after BM transplantation (BMT). (F) Pathological GvHD scores of the liver and total pathological scores in the gut which is the sum of the scores of the small intestine and colon. Data from three independent experiments are combined and shown as means ± Standard Error (SE) (n=8-14/group). (G) Numbers of Paneth cells morphologically identified as cells containing eosinophilic granules at crypt base of the small intestines (white arrow heads in Figure 1E) on day +7 after BMT. Data from two similar experiments were combined and shown as means ± SE (n=12 / group). (H-J) CD4^{+}CD8^{+} double positive thymocytes were assessed 6-8 weeks after BMT. Representative dot plots (H), frequencies (I) (means±SE), and absolute numbers (J) (means±SE) of CD4^{+}CD8^{+} thymocytes from one of two similar experiments were shown (n=5/group). (K) BALB/c mice recipients were transplanted with 5x10^{6} TCD-BM cells from WT B6 mice plus 1x10^{6} purified T cells from WT or *MyD88*$^{-/-}$ B6 donors following total body irradiation on day 0 (n=11/group). Data from two similar experiments were combined. Bar, 50 μm. ***P*<0.01; ****P*<0.005.

vivo was also equivalent (Figure S2A and B). T-cell proliferation was much more robust *in vivo* compared to *in vitro*, probably due to the presence of potent inflammatory milieu induced by TBI. On the other hand, annexin V+ apoptotic donor CD8$^+$ T cells were significantly increased in the recipients of *MyD88*$^{-/-}$ T cells on day +7, suggesting that MyD88 is an intrinsic survival factor of donor T cells after allo-BMT (Figure 2G).

To assess the role of MyD88 in donor T-cell differentiation after allo-BMT, cytokine production was evaluated in donor T cells isolated on day +7 after BMT. *MyD88*$^{-/-}$ CD4$^+$ T cells produced significantly less IFN-γ and IL-17, and *MyD88*$^{-/-}$ donor CD8$^+$ T cells produced less INF-γ than their WT counterpart, indicating that MyD88 signaling is critical for Th1, Th17, and Tc1 differentiation (Figure 3A-D). In contrast, IL-4 production from *MyD88*$^{-/-}$ donor CD4$^+$ T cells tend to be greater than that from WT CD4$^+$ T cells (Figure 3E and F). Impaired Th1/Tc1, not Th2, differentiation in *MyD88*$^{-/-}$ T cells was also confirmed *in vitro* after CD3/CD28 stimulation (Figure S2C-F). Furthermore, flow cytometric analysis on day +7 demonstrated that absolute numbers of donor CD4$^+$ Foxp3$^+$ Tregs and pro-

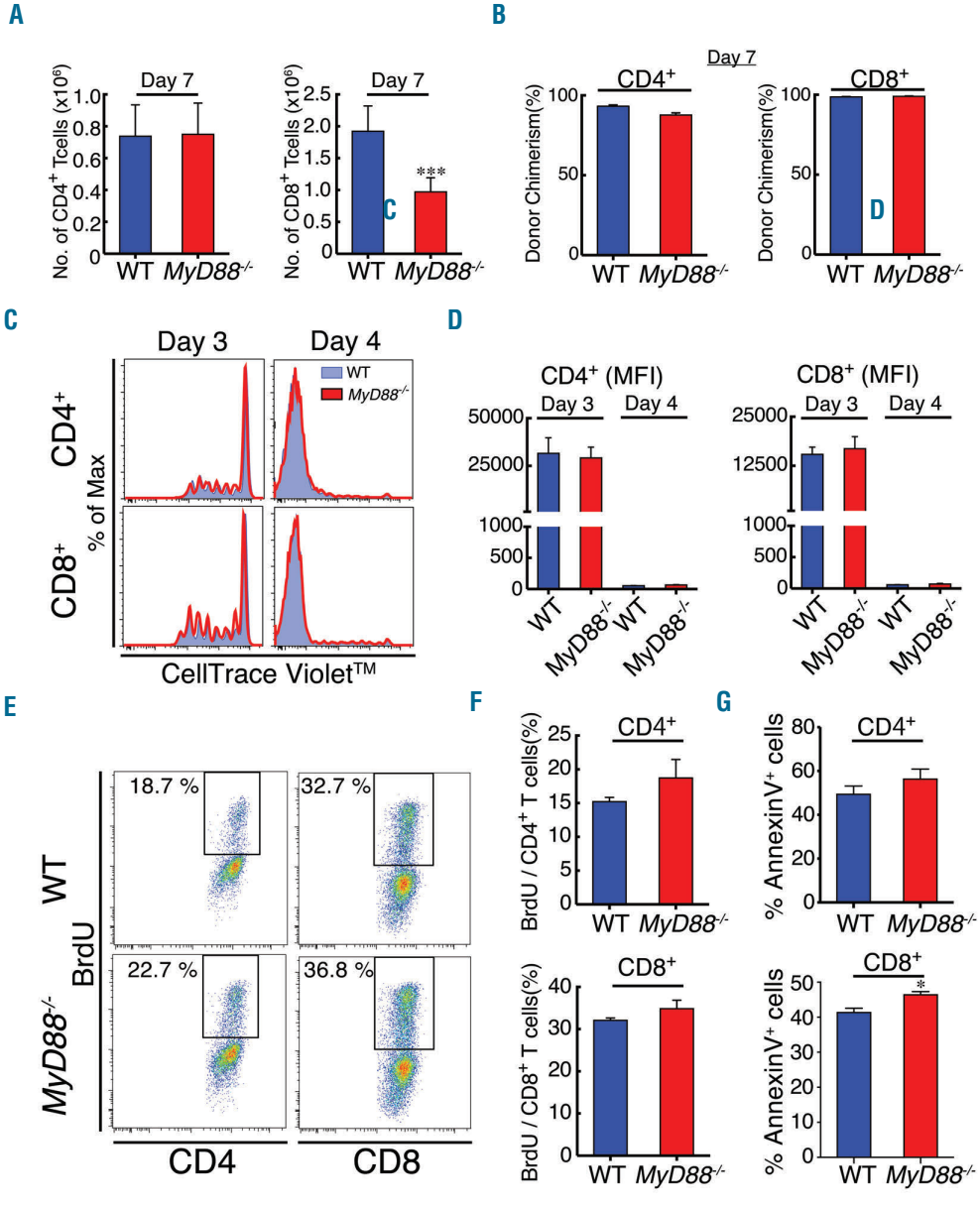

Figure 2. MyD88 is not essential for T-cell proliferation and survival after allogeneic bone marrow transplantation (alloBMT). (A,B,E-H) Mice were transplanted as in Figure 1C. Absolute numbers of donor CD4$^+$ and CD8$^+$T cells (A) and donor chimerism (B) in the spleen on day +7. Data from four independent experiments were combined and shown as means±Standard Error (SE) (n=17-18/group). (C and D) CellTrace Violet-labeled T cells from *WT* or *MyD88*$^{-/-}$ B6 mice were injected and dilution of CellTrace Violet in donor CD4$^+$ and CD8$^+$ T cells in the spleen were assessed on days 3 and 4. Representative histograms (C) and MFI of Cell Trace Violet (D) (means±SE) from two experiments were combined (n=4-6 group). (E and F) Recipients were intraperitoneally injected with 1 mg BrdU on day +6 and BrdU taken up by donor T cells was evaluated two hours later. Representative dot plots (E) and proportions of BrdU positive cells among donor T cells (F) (means±SE). Data from two experiments were combined (n=10/group). (G) Proportion of Annexin V$^+$ cells among donor T cells in the spleen on day +7. Data from a representative experiment of two similar experiments were combined and shown as means±SE (n=9-11 / group). *P<0.05; ***P<0.005.

portions of Foxp3$^+$ cells among donor CD4$^+$ T cells were significantly greater in the spleen of recipients of *MyD88*$^{-/-}$ T cells than those in controls (Figure 3G and H). Taken together, donor T cells require MyD88 signaling to differentiate into Th1, Th17, and Tc1, while it is dispensable for Th2 and Treg differentiation after allo-SCT.

IRAK4 inhibition, but not single TLR deficiency, mitigates lethal graft-*versus*-host disease

MyD88 transduces signals from both TLR and IL-1 family cytokines, such as IL-1, IL-18 and IL-33. Although roles of IL-1, IL-18, and IL-33 in GvHD have been well studied, role of T-cell TLR remained to be clarified. First, we examined expression of MyD88-related TLR and IL-1 family cytokine receptors on CD4$^+$ and CD8$^+$ T cells. Q-PCR demonstrated that resting CD4$^+$ and CD8$^+$ T cells expressed TLR2 and TLR7, but to a lesser extent to the levels in macrophages (Figure 4A). In addition, CD4$^+$ T cells also expressed IL-1R, IL-18R and IL-33R, and CD8$^+$ T cells did IL-18R. Flow cytometric analysis confirmed expression of TLR2 and TLR7 both on CD4$^+$ and CD8$^+$ T cells (Figure 4B). Expression of TLR2 and TLR7 on T cells were functional, such as TLR2 ligand, Pam3CSK4, and TLR7 ligands, ssRNA40 and R848 enhanced T-cell proliferative response to CD3/CD28 stimulation *in vitro*, indicating that TLR2 and TLR7 on T cells have co-stimulatory function in T-cell responses (Figure 4C). In contrast, LPS, flagellin, or CpG-ODN did not enhance T-cell expansion. Next, we evaluated the role of TLR2 and TLR7 in GvHD by transplanting *TLR2*$^{-/-}$ or TLR7$^{-/-}$ T cells plus WT TCD-BM. Mortality and morbidity of GvHD in recipients of *TLR2*$^{-/-}$ or TLR7$^{-/-}$ T cells were identical to those of WT T cells, suggesting that inhibition of single TLR signaling was insufficient to ameliorate GvHD (Figure 4D and E).

Next, we tested if pharmacological inhibition of MyD88 signaling could ameliorate GvHD, using IRAK4 inhibitor PF-06650833 that inhibits signal transduction through MyD88. First, we found that addition of PF-06650833 to culture significantly suppressed T-cell production of IFN-γ after CD3/CD28 stimulation without affecting T-cell proliferative response *in vitro* (Figure 5A-D). PF-06650833 administered daily for three weeks after allogeneic BMT significantly ameliorated morbidity and mortality of GvHD, indicating that MyD88/IRAK4 is a potential pharmacological therapeutic target of GvHD (Figure 5E and F).

MyD88 signaling is dispensable for graft-*versus*-leukemia activity of donor T cells

Considering the significant reduction of GvHD in the absence of MyD88 signaling in donor T cells, it is of interest to evaluate the impact of MyD88 signaling in donor T cells on GvL effect after allogeneic BMT. Lethally irradiat-

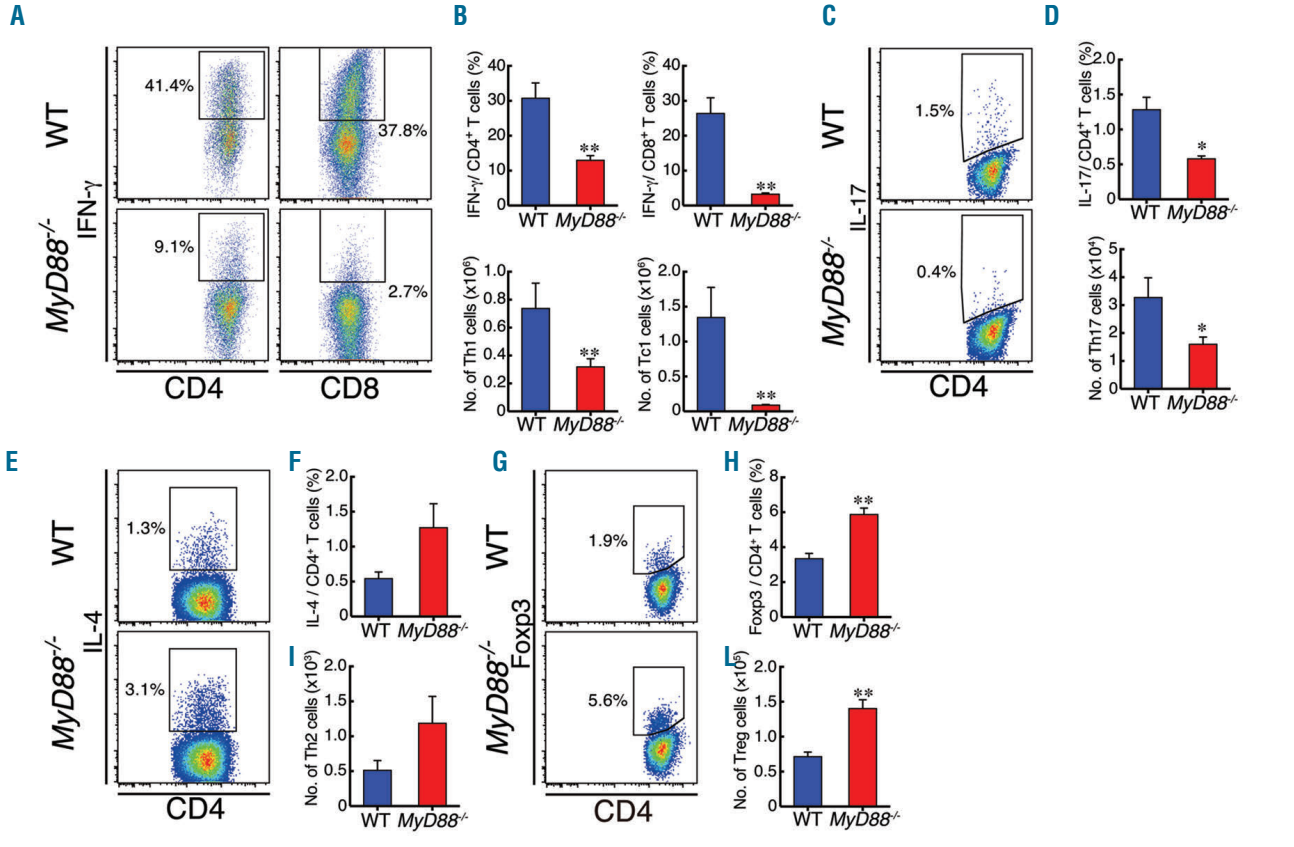

Figure 3. MyD88 deficient donor T cells skew from Th1, Tc1 and Th17 toward T regulatory cell (Treg) differentiation after allogeneic bone marrow transplantation (alloBMT). Mice were transplanted as in Figure 1C. (A-H) Donor T cells harvested from spleens on day +7 were stimulated with PMA/ionomycin and intracellular IFN-γ (A-B), IL-17 (C-D), and IL-4 (E-F) were stained. Representative dot plots (A, C, E), frequencies (B, D, F; top panels) means±Standard Error (SE) and absolute numbers (B, H, E; bottom panels) (means±SE) of cytokine producing donor T cells are shown. Data from two similar experiments were combined (n=10/group). Representative dot plots (G), frequencies (H; top panels) (means±SE), and absolute numbers (H; bottom panels) (means±SE) of donor CD4$^+$Foxp3$^+$ Tregs from a representative experiment of three similar experiments are shown (n=5/group). *P<0.05; **P<0.01.

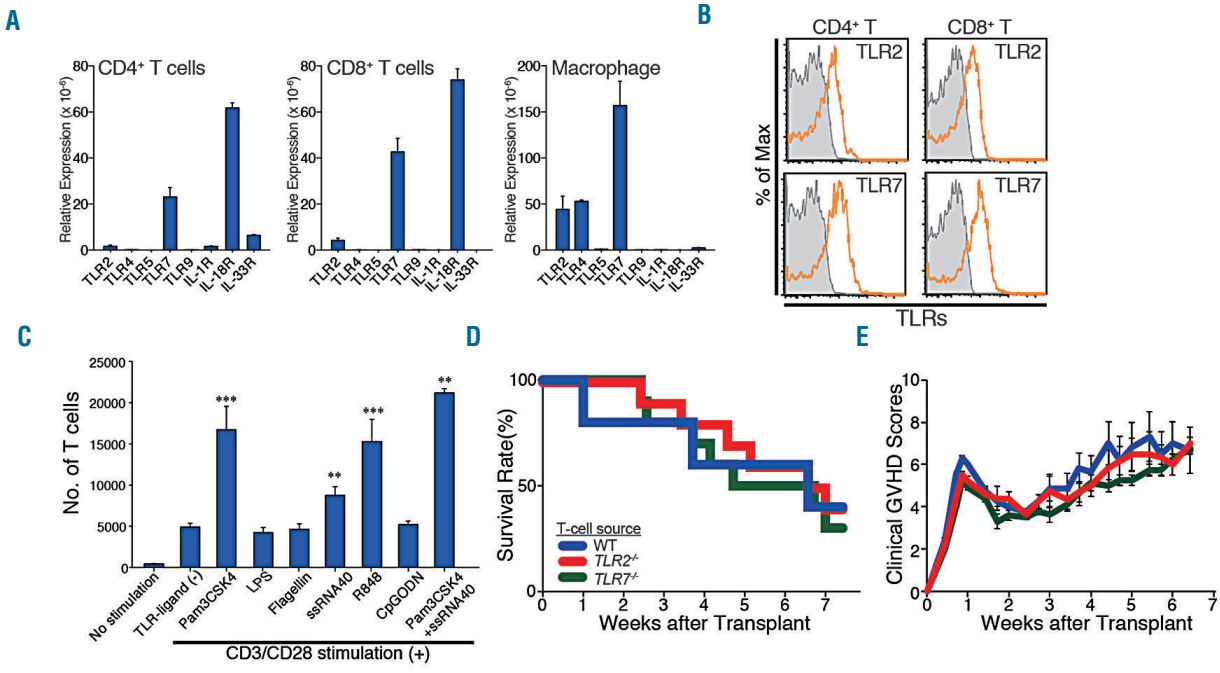

Figure 4. Single TLR blockade is not sufficient to mitigate lethal graft-*versus*-host disease (GvHD). (A) mRNA from CD4+ T cells, CD8+ T cells, and macrophages of naïve *WT* B6 mice was subjected to quantitative polymerase chain reaction of MyD88-related TLRs and cytokine receptors. Data from three similar experiments were combined and shown as means±Standard Error (SE). (B) Flow cytometric analysis of TLR2 (top panels) and TLR7 on T cells (bottom panels) were performed. Representative histograms from one of three similar experiments were shown. (C) 5x10^4 T cells were stimulated with CD3/28 mAb-coated beads in the presence or absence of TLR-ligands for three days (n=12/group). Absolute numbers of T cells after stimulation from four similar experiments were combined and shown as means±SE. (D and E) Lethally irradiated B6D2F1 mice were transplanted with 5x10^6 T cell-depleted bone marrow cells (TCD-BM) cells from *WT* B6 mice plus 1x10^6 purified T cells from *WT*, TLR2$^{-/-}$, or TLR7$^{-/-}$ B6 donors. Survivals (D) and clinical GvHD scores (E) from two independent experiments are combined and shown (n=10/group).

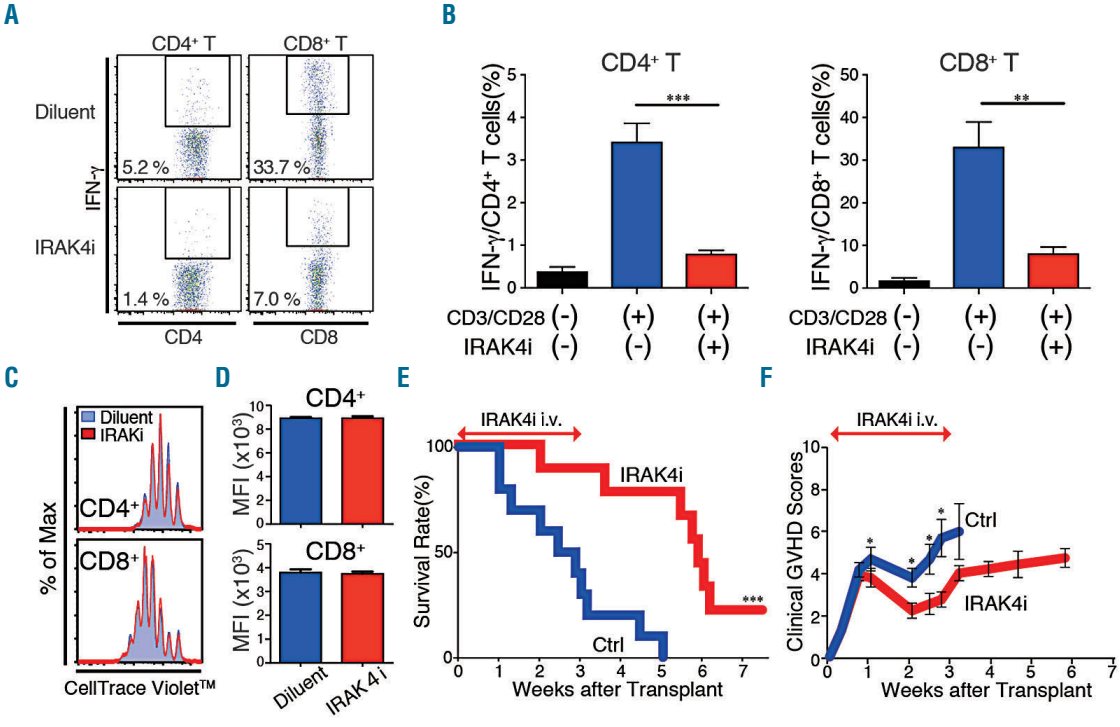

Figure 5. IRAK4 inhibitor PF-06650833 ameliorates graft-*versus*-host disease (GvHD). (A and B) Sorted 5x10^4 T cells from B6 mice were incubated with Dynabeads coated with anti-CD3/CD28 mAbs in the presence or absence of PF-06650833 at a concentration of 20 μM for 96 hours. Representative dot plots (A) and frequency (B), means±Standard Error (SE), of IFN-γ producing T cells from two similar experiments are combined (n=8/group). (C and D) T cells were labeled with CellTrace Violet and stimulated with Dynabeads coated with anti-CD3/CD28 mAbs for 72 hours. Representative histograms (C) and MFI of CellTrace Violet (D) (means±SE) from one of two similar experiments were shown (n=4/group). (E and F) Mice were transplanted as in Figure 1C. A group of mice were intravenously injected with 12.0 mg/kg PF-06650833 daily from day 0 to day 20 after BMT. Survival curves (E) and clinical GvHD scores (F) from two independent experiments are combined (n=10/group). Ctrl: control.

ed B6D2F1 mice were injected with $5x10^6$ TCD-BM from WT B6 plus $1x10^6$ T cells from either WT or *MyD88*$^{-/-}$ B6 mice, with the addition of $1x10^3$ host-type P815 leukemia cells to the donor inoculum. All allogeneic TCD-BM recipients died from leukemia within two weeks after BMT, whereas leukemia mortality was significantly suppressed in the recipients of both WT and *MyD88*$^{-/-}$ T cells. While leukemia mortality was not significantly different between the allogeneic recipients of WT and *MyD88*$^{-/-}$ T cells, overall survival time was significantly prolonged in recipients of *MyD88*$^{-/-}$ T cells compared to controls, suggesting that MyD88 signaling in donor T cells is dispensable for GvL effect and T-cell MyD88 is a therapeutic target of GvHD without GvL reduction (Figure 6A and B). To further assess GvL effects, we have performed *in vivo* bioluminescent imaging (BLI) to track luciferase-transfected P815 (P815-luc) cells after BMT. Survivals were again significantly prolonged in recipients of *MyD88*$^{-/-}$ T cells compared to those of WT T cells (Figure 6C). Whole body BLI clearly demonstrated that growth of P815-luc cells were suppressed both in the recipients of *MyD88*$^{-/-}$ T cells and WT T cells, while P815-luc expanded vigorously in the recipients of TCD-BM alone (Figure 6D). Taken together, we concluded that GvL effects were preserved without donor T-cell MyD88 signaling.

Discussion

The expanding IL-1R/TLR superfamily now consists of more than ten TLR and IL-1R, IL-18R, and IL-33R. TLR are expressed on a wide range of myeloid cells such as macrophages and dendritic cells. However, emerging evidence demonstrates that TLR are also expressed on T cells.[25] Signaling from TLR2, TLR3, TLR5, TLR7/8, and TLR9 has co-stimulatory role in T-cell activation, leading to enhancement of proliferation, survival, and differentiation towards Th1, Th17 and memory CD4$^+$ T cells.[8,25-28] TLR2 is required for expansion and differentiation of virus-specific CD8$^+$ memory T cells.[29,30] TLR4 engagement on CD4$^+$ T cells is required for their effector functions in experimental autoimmune encephalomyelitis.[9] Members of the IL-1R family including IL-1R, IL-18R, and IL-33R are also expressed in T cells and regulate various T-cell functions.[7] IL-1R or IL-18R signaling in T cells is critical for Th1, Th17 and antigen-specific CD8$^+$ T-cell responses.[12,13,31] A previous study demonstrated an impaired proliferation of MyD88-deficient T cells upon antigen stimulation.[12] However, in our study, proliferation of WT T cells and MyD88-deficient T cells were comparable early after allogeneic transplantation or *in vitro* upon CD3/CD28 stimulation, suggesting that the pres-

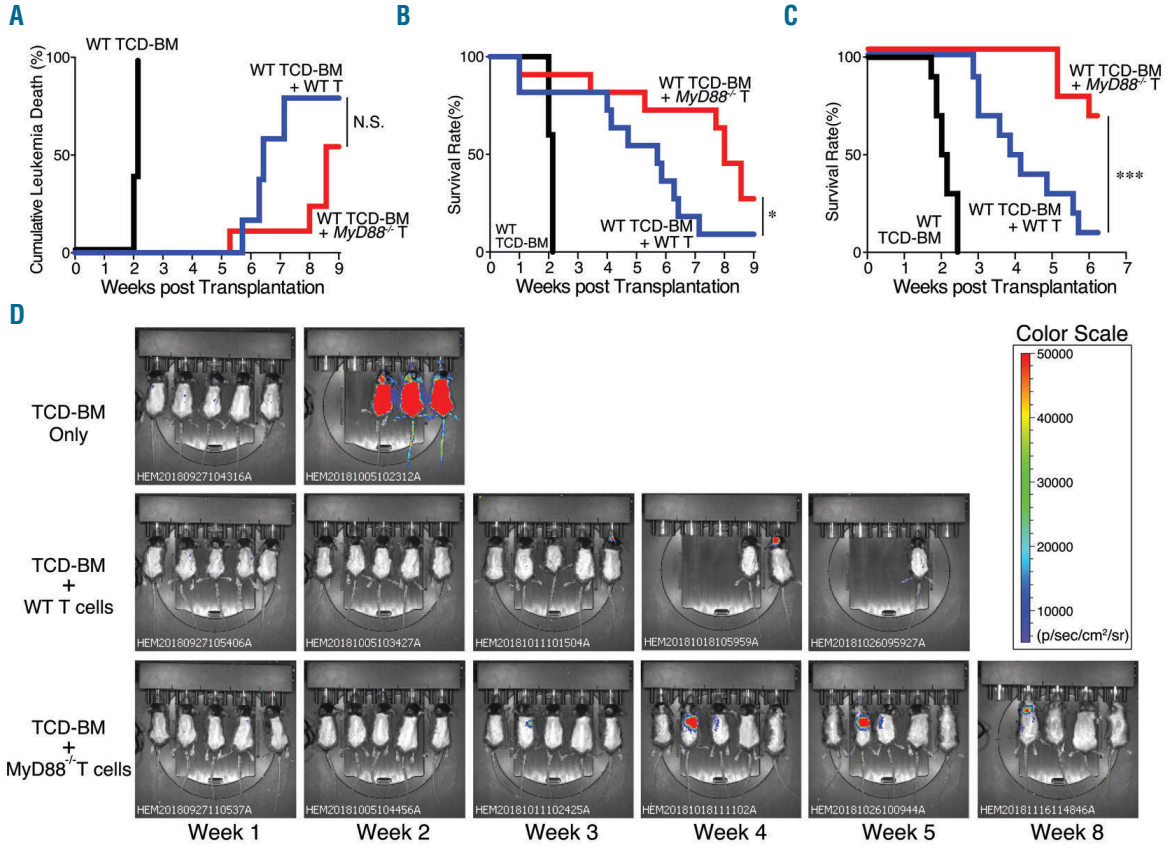

Figure 6. MyD88 signaling in donor T cells is not essential for significant graft-*versus*-leukemia (GvL) effects. (A and B) Lethally irradiated B6D2F1 mice were transplanted with wild-type (WT) T-cell-depleted bone marrow cells (TCD-BM) alone (n=5) or in combination with either WT (n=11) or *MyD88*$^{-/-}$ (n=11) T cells from B6 together with $1x10^3$ P815 leukemia cells (H-2Kd+) on day 0. Cumulative leukemia mortality (A) and overall survival (B) after bone marrow transplantation (BMT) from two experiments are combined. (C and D) Lethally irradiated B6D2F1 mice were transplanted with WT TCD-BM alone (n=10) or in combination with either WT (n=10) or *MyD88*$^{-/-}$ (n=10) T cells from B6 donors together with $5x10^3$ P815-luc cells on day 0. Bioluminescent imaging (BLI) was performed weekly after allogeneic BMT. (C) Overall survival from two independent experiments were combined. (D) The whole body bioluminescent images from 1 of 2 similar experiments are shown.

ence of inflammatory milieu or potent co-stimulation induced robust T-cell proliferation even without T-cell MyD88 signaling. Although we detected only TLR2, TLR7, IL-1R, IL-18R, IL-33R but not TLR4, TLR5, or TLR9 in resting T cells, a diverse combination of expression of the TLR/IL-1R superfamily in T cells including TLR4 and TLR5 has been demonstrated, indicating that the range of TLR/IL-1R expression could be context dependent, such as differentiation and activation status and subtype of T cells, as well as environmental milieu, such as type of inflammation and microbiota.

Although we demonstrated the T-cell co-stimulatory functions of both TLR2 and TLR7 signaling *in vitro*, absence of TLR2 or TLR7 in donor T cells did not change the severity of GvHD. Our results are consistent with previous studies showing that neither TLR2 or TLR7 deficiency in donor cells nor pharmacological inhibition of TLR2 altered morbidity and mortality of GvHD.[32,33] These results suggest that TLR have no role in GvHD. IL-18R and IL-33R signaling in donor T cells has also conflicting roles in GvHD, depending on the experimental models used and the timing of administration of their ligands.[34-37] Given this redundancy and conflicting role of each of IL-1R/TLR superfamily in T cells, we took advantage of *MyD88*$^{-/-}$ T cells that enabled us to block most of the signals from receptors belonging to the TLR/IL-1R superfamily. We found that *MyD88*$^{-/-}$donor T cells showed significantly impaired survival and differentiation toward Th1, Tc1, and Th17 cells, while preserving Foxp3^{+} Treg expansion after allo-SCT, resulting in significant improvement of GvHD. This impairment of *MyD88*$^{-/-}$ T-cell functions after allo-SCT was confirmed in *in vitro* culture. It has been shown that impairment of Th1, Tc1, and Th17 results in mitigated GvHD.[38] A recent study by Lim *et al*. showed that MyD88 deficiency in donor T cells did not ameliorate GvHD, but decreased GvL using a similar model of BMT, even though they also showed impaired Th1 differentiation of *MyD88*$^{-/-}$ T cells after BMT.[39] It should be noted that GvHD of allogeneic controls was less severe with 30% mortality in that study compared to severe GvHD with 80% mortality in our study. Thus, the mild experimental condition in that study may compromise our seeing a reduction in GvHD. GvL activity determined by leukemia mortality and *in vivo* BLI was preserved in the absence of T-cell MyD88, suggesting that T-cell MyD88 signaling plays a more important role on GvHD than on GvL effects in our model. However, its contribution to GvHD and GvL may change in allo-SCT following reduced intensity conditioning, as shown in the Lim *et al*. study.[39]

Pharmacological inhibition of TLR/IL-1R has been studied in murine models and clinical studies. Pharmacological blockade of IL-1/IL-1R or IL-33/IL-33R interaction ameliorates experimental GvHD;[40] however, a randomized trial failed to show any protective effect of the IL-1R antagonist Anakinra against clinical GvHD.[41-43] MyD88 is an adopter molecule to recruit IRAK to most of the receptors belonging to the IL-1R/TLR superfamily to relay signals to downstream proinflammatory pathways.[44,45] Given the redundant roles of a wide range of TLR/IL-1R, inhibition of multiple TLR/IL-1R signals by MyD88 or IRAK4 inhibitors would be more effective than a single pathway blockade. We showed that pharmacological inhibition of MyD88/IRAK4 pathway using IRAK4 inhibitor ameliorated experimental GvHD. IRAK4 also plays a critical role in T-cell activation[46] and IRAK4-deficient T cells showed significantly delayed allogeneic skin graft rejection.[46] The IRAK4 inhibitor could not only affect donor T cells, but also donor accessory cells and recipient cells; however, it has been reported that MyD88 signaling in the donor BM cells or recipient cells did not enhance GvHD, thus it is likely that IRAK4 inhibitor ameliorated GvHD by acting on donor T cells.[4,47] There are several MyD88 or IRAK4 inhibitors that demonstrated anti-inflammatory effects in pre-clinical models. PF-06650833 is one of IRAK4 inhibitors in clinical development, that was previously tested in a phase I trial for patients with systemic lupus erythematosus and phase II clinical study for patients with rheumatoid arthritis is ongoing (*https://clinicaltrials.gov/ct2/show/NCT02996500?term=06650833&rank=3*).[48,49]

In conclusion, our results demonstrate a previously unrevealed role of T-cell MyD88 signaling in the development of GvHD. Because GvL effects were at least preserved in the absence of donor MyD88 signaling, the IRAK-4 inhibitor PF-06650833 is an ideal agent to ameliorate GvHD, likely by inhibiting MyD88/IRAK4 signaling in donor T cells with sparing. Furthermore, the IRAK4 inhibitor is also under development as a therapeutic reagent against B-cell neoplasms with MyD88 mutation, indicating that therapeutic or prophylactic administration of IRAK4 inhibitor after allo-SCT could ameliorate GvHD with enhancing tumor control of B-cell neoplasms.[50] Signaling molecules such as JAK, MEK, AURKA, and many other molecules that integrate the signals from multiple receptors of cytokines or growth factors, are promising therapeutic targets of GvHD.[51,52] Given the critical role of IRAK4 in activation of human T cells, MyD88 and IRAK4 inhibitors that could inhibit signaling from more than ten receptors belonging to the TLR/IL-1R superfamily should be tested in clinical studies to explore their prophylactic and therapeutic potentials against GvHD.[53]

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