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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

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Graft-versus-host disease (GVHD) reflects an exaggerated inflammatory allogeneic T cell response in hosts receiving allogeneic hematopoietic stem cell transplantation (HSCT). Inhibition of pan-Notch receptor signaling in donor T cells causes reduction of GVHD. However, which Notch ligand(s) in what APCs is important for priming graft-versus-host reaction remains unknown. We demonstrate that 6-like ligand-4 (Dll4) and Dll4-positive (Dll4high) inflammatory dendritic cells (i-DCs) play important roles in eliciting allogeneic T cell responses. Host-type Dll4high i-DCs occurred in the spleen and intestine of HSCT mice during GVHD induction phase. These Dll4high i-DCs were CD11c+ B220+PDCA-1+, resembling plasmacytoid dendritic cells (pDCs) of naive mice. However, as compared with unstimulated pDCs, Dll4high i-DCs expressed higher levels of costimulatory molecules, Notch ligands Jagged1 and Jagged2, and CD11b, and produced more IFN-γ and IL-17, and had low levels of Jagged1. In vitro assays showed that Dll4high i-DCs induced significantly more IFN-γ and IL-17–producing effector T cells (3- and 10-fold, respectively) than Dll4low i-DCs. This effect could be blocked by anti-Dll4 Ab. In vivo administration of Dll4 Ab reduced donor-alloreactive effector T cells producing IFN-γ and IL-17 in GVHD target organs, leading to reduction of GVHD and improved survival of mice after allogeneic HSCT. Our findings indicate that Dll4high i-DCs represent a previously uncharacterized i-DC population distinctive from steady state DCs and Dll4low i-DCs. Furthermore, Dll4 and Dll4high i-DCs may be beneficial targets for modulating allogeneic T cell responses, and could facilitate the discovery of human counterparts of mouse Dll4high i-DCs. The Journal of Immunology, 2013, 190: 3772–3782.

Allogeneic hematopoietic stem cell transplantation (HSCT) is potentially a curative therapy for hematopoietic malignancies and nonmalignant disorders (1–8). However, graft-versus-host (GVH) disease (GVHD) remains a major barrier to the success of allogeneic HSCT. GVHD is mediated by donor T cells that recognize and react to histocompatibility differences of the host. These alloreactive T cells and their recruited inflammatory cells attack epithelial organs such as intestine, liver, and skin (2–4). In both human patients and experimental mouse models, severe damage to the gastrointestinal (GI) tract is associated with the high mortality after allogeneic HSCT (2, 3, 9, 10).

The Notch signaling pathway is important for cell-cell communication, which controls multiple cell processes, including proliferation, differentiation, and survival (11, 12). Notch receptors (Notch 1, 2, 3, and 4) interact with Notch ligands of the 6-like (Dll1, Dll3, and Dll4) and Jagged families (J1 and J2) (11–13). Binding of a Notch ligand to its receptor results in the cleavage of the receptor by γ-secretase complex and the subsequent release of intracellular Notch (11–13). The intracellular Notch domain translocates to the nucleus, where it activates the transcription of Notch target genes (11–13). Using a genetic approach, we have recently shown that inhibition of pan-Notch receptor signaling in donor T cells markedly reduced GVHD severity and mortality in mouse models of allogeneic HSCT (14). Donor T cells lacking Notch receptor signaling proliferated and expanded in response to alloantigens in vivo, but showed markedly reduced capability of producing inflammatory cytokines such as IFN-γ, IL-17, and TNF-α (14). These Notch-deprived donor T cells were incapable of mediating GVHD, in particular damage to the GI tract (14). However, pharmacological blockade of Notch receptors may lead to severe toxicity to the host (15). Identifying which Notch ligand is critical for activation of the Notch signaling pathway to elicit allogeneic T cell responses may have significant implications in modulation of GVHD.

Emerging evidence indicates that Notch ligands are important for APCs to direct differentiation of distinct lineages of effector
T cells (16–18). For example, Dll4 is required for the development of CD4+ Th1 cells (19–21) and Th17 cells (21–23), whereas Dll1 regulates CD8+ cytotoxic T cell–mediated antitumor activity (17, 24, 25). In contrast, the Jagged family has been found to mainly influence Th2 cell response (18). Different inflammatory stimuli may stimulate APCs to express different types of Notch ligands (16–18, 26–28). TLR ligands or TLR agonists stimulate APCs expression of both 5-like ligands and Jagged ligands, whereas other inflammatory stimuli such as helminthes, allergens, and toxins may increase expression of Jagged ligands (16–18, 26–28). These observations suggest that the expression of Notch ligands may differentiate the capability of APCs to direct effector differentiation of alloreactive T cells. However, which Notch ligands in what APCs play important roles in the regulation of allogenetic T cell responses and GVHD remains unknown.

In the current study, we identified a previously uncharacterized population of inflammatory dendritic cells (i-DCs) that expressed high levels of Dll4. These Dll4-positive (Dll4\textsuperscript{high}) i-DCs were mainly located in the spleen and GI tract in mice early after allogeneic HSCT, and possessed a greater ability than Dll4-negative (Dll4\textsuperscript{low}) i-DCs to induce the generation of alloreactive effector T cells. This effect of Dll4\textsuperscript{high} i-DCs was abrogated by anti-Dll4 Ab. Importantly, in vivo treatment with Dll4 Ab markedly reduced the generation of alloreactive effector T cells producing IFN-γ and IL-17 in GVHD target organs, leading to inhibition of GVHD and significantly improved survival of mice after allogeneic HSCT. Our findings indicate that Dll4 and Dll4\textsuperscript{high} i-DCs are important for eliciting allogenetic T cell responses and may be beneficial targets for modulating GVHD.

Materials and Methods

**Mice**

C57BL/6 (B6, H-2\textsuperscript{b}), BALB/c (H-2\textsuperscript{k}), and B6 x DBA/2 F\textsubscript{1} (BDF1, H-2\textsuperscript{bd}) mice were purchased from Taconic (Rockville, MD). Experimental protocols were approved by the University of Michigan’s Committee on Use and Care of Animals.

**Abs, flow cytometry analysis, and cell lines**

Neutralizing Abs specific to mouse Dll1, Dll4, J1, and J2 were prepared, as previously described (29–32). All other Abs used for immunofluorescence staining were purchased from eBioscience, BioLegend, or Becton Dickinson Biosciences. Microbead-conjugated Abs and streptavidin were purchased from Miltenyi Biotec (Auburn, CA), and human rIL-2 was from R&D Systems (Minneapolis, MN). Flow cytometry analyses were performed using FACScalibur and Canto cytometer (Becton Dickinson Biosciences), as described (33).

**Cell preparations**

T cell–depleted bone marrow (TCD-BM) was prepared by depleting T cells with microbead-conjugated anti-CD4/CD8 Abs (33). CD4+ and CD8+ lymphocytes were isolated from spleens and lymph nodes using microbead-conjugated Abs (MiniMACS; Miltenyi Biotec). Purity was consistently >92%. The preparation of lumina nonmononuclear cells was performed, as previously described (14).

**Induction of GVHD**

Mice underwent bone marrow transplantation, as described (33). The GVHD score and severity were assessed, as described (34). GVHD severity was also assessed by histopathological analysis (35). For the B6 anti-BALB/c mouse model, BALB/c recipients were irradiated using 800 rad from a \( ^{137} \)Cs source. Donor B6 TCD-BM (5.0 \( \times \) 10\textsuperscript{6}) with B6 CD4+ T cells (1.0 \( \times \) 10\textsuperscript{6}) were transplanted into irradiated BALB/c recipients. In some experiments, we transplanted donor B6 TCD-BM (5.0 \( \times \) 10\textsuperscript{6}) together with B6 CD4+ T cells (1.0 \( \times \) 10\textsuperscript{6}) plus CD8+ T cells (1.0 \( \times \) 10\textsuperscript{5}) to lethally irradiated (1200 rad) BDF1 recipients.

**RT- PCR**

Total RNA was extracted from the sorted donor CD4+ T cell subsets and dendritic cell (DC) subsets using TRizol (Invitrogen Life Technologies). cDNA was quantified through the quantitative real-time PCR technique. Real-time PCR was performed with a SYBR Green PCR mix on a Mastercycler realspace (Eppendorf). Thermocycler conditions included an initial holding at 95˚C for 2 min; this was followed by a three-step PCR program, as follows: 95˚C for 30 s, 55˚C for 30 s, and 72˚C for 40 cycles. Transcript abundance was calculated using the \( \Delta \Delta Ct \) method (normalization with GAPDH). The primer sequences are listed in Table I.

**Mixed leucocyte reaction**

Donor (B6)-derived CD4+ T cells (1–2 \( \times \) 10\textsuperscript{5} cells/well) in 96-well U-bottom plates with complete medium were stimulated with or without DCs (1–4 \( \times \) 10\textsuperscript{6} cells/well) from BALB/c mice. Cells were cultured for 4 or 5 d to assess intracellular cytokines, as described (33, 36–38).

**Statistical analysis**

Survival in different groups was compared using the log-rank test. Comparison of two means was analyzed using the two-tailed unpaired Student t test.

**Results**

**Host DCs upregulate Notch ligands early during GVHD induction**

To determine the role of Notch ligands in regulating allogenetic 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. 1A). Given the importance of host APCs in eliciting GVH reaction (9, 35, 37, 39–44), 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. 1B). By 7 d after transplantation, host CD11c+ cells were reduced ∼20-fold in the spleen of these allogeneic HSCT mice compared with day 1 (Fig. 1B), which coincides with previous studies (37, 45, 46). 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 d after transplantation and declined by 7 d (Fig. 1C, 1D). Interestingly, there were only few host CD11c+ DCs expressing low levels of Dll1 (Fig. 1C, 1D), although Dll1 has been implicated in other types of Ag-driven T cell responses (17, 25). These host CD11c+ DCs expressed high levels of MHC class II molecule Ia and costimulatory molecules CD80 and CD86 (Fig. 1E), resembling the phenotype of i-DCs (47–50). Donor-derived CD11c+ cells did not occur by 7 d after transplantation (Fig. 1B). They expressed low levels of Dll4 and J1, and moderate levels of J2 (Fig. 1F). These results suggest that host DCs upregulate the expression of Dll4, J1, and J2 during early phase of GVHD induction.

**Dll4 derived from host-type DCs promotes production of IFN-γ and TNF-α in alloantigen-activated CD4+ T cells**

We next used in vitro MLR assays to examine whether Notch ligands expressed by DCs were important for effector differentiation of alloantigen-activated T cells. CD11c+ DCs were isolated from BALB/c mice receiving HSCT 3 d after transplantation and cultured ex vivo with normal B6 mouse-derived CD4+ T cells, with or without addition of Ab specific to individual Notch ligand. Blocking Dll1 and Dll4 led to a significant reduction of effector T cells producing IFN-γ and TNF-α compared with control IgG (Fig. 2A). Inhibition of either J1 or J2 had less effect on production of IFN-γ and TNF-α in alloantigen-activated T cells compared with blockade of either Dll1 or Dll4 (Fig. 2A). These data suggest that Dll1 and Dll4 may play important roles in regulating the generation of alloreactive effector T cells.

To ask whether blocking Dll1 and/or Dll4 could reduce production of IFN-γ and TNF-α by CD4+ T cells activated in vivo, we...
transplanted B6 donor T cells with TCD-BM into lethally irradiated BALB/c mice and administered two doses of anti-Dll1 Ab, anti-Dll4 Ab, and anti-Dll1 Ab plus anti-Dll4 Ab at days 0 and 3 after transplantation (Fig. 2B). Donor T cells were recovered at day 5 after transplantation from the spleens of these recipients. In vivo blockade of Dll4 resulted in more profound reduction of donor T cells with TCD-BM + CD4 T cells (1.0 × 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 = 6–8 mice per group). (C) Histograms show the expression of Notch ligands on the surface of host CD11c^+ cells that were recovered from the spleens of normal BALB/c mice and allogeneic HSCT BALB/c mice at the time point, as indicated. Representative histograms from three independent experiments 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 = 6–8 mice per group). (E) Histograms show the expression of tested markers on the surface of host CD11c^+ cells. Representative histograms from three independent experiments 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 d after HSCT. Representative histograms from three independent experiments are shown. *p < 0.05, **p < 0.01.

**FIGURE 1.** Notch ligands are upregulated on the surface of CD11c^+ DCs in the recipient mice early during GVHD induction. Lethally irradiated (8 Gy) 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. (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 = 6–8 mice per group). (C) Histograms show the expression of Notch ligands on the surface of host CD11c^+ cells that were recovered from the spleens of normal BALB/c mice and allogeneic HSCT BALB/c mice at the time point, as indicated. Representative histograms from three independent experiments 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 = 6–8 mice per group). (E) Histograms show the expression of tested markers on the surface of host CD11c^+ cells. Representative histograms from three independent experiments 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 d after HSCT. Representative histograms from three independent experiments are shown. *p < 0.05, **p < 0.01.

DCs expressing high levels of Dll4 represent a unique i-DC subset distinct from Dll4-low i-DCs and steady state DCs

DCs are heterogeneous cell populations (52, 53). In naive mice under steady state conditions, two major types of DCs have been defined based on their surface phenotype, anatomical location, and function, including plasmacytoid DCs (pDCs; CD11c^+PDCA-1^+B220^+) and conventional DCs (cDCs; CD11c^+PDCA-1^−B220^−) (48, 53). Under inflammatory conditions, i-DCs occur and have properties different from steady state DCs, as evidenced by profound phenotypic changes, enhanced Ag-presenting capacity, and altered migration capability (47–50, 54). However, whether distinct i-DC subsets may have differential effects on inducing alloreactive effector T cells was not well defined.

To examine whether Dll4 could be useful for identifying distinct i-DC subsets that have differential capacities to prime allogeneic T cell responses, we isolated cells from the spleens of GVHD BALB/c mice 3 d after transplantation. As shown in Fig. 3A, ~64% of Dll4^high CD11c^+ DCs were pDCDA-1^−B220^+ cells, suggesting that they resemble pDCs (53). When gating on CD11c^+PDCA-1^−B220^+ cells, we noted that ~85% of them expressed high levels of Dll4 (Fig. 3B). However, as compared with steady state pDCs derived from naive mice (Fig. 3B, 3C), GVHD mouse-derived Dll4^high CD11c^+PDCA-1^−B220^+ cells expressed higher levels of Ia, CD40, CD80, and CD86, a typical phenotype of activated DCs (48, 49, 54). Intriguingly, these Dll4^high DCs expressed CD11b (Fig. 3B), which is normally not expressed on the surface of steady state pDCs (Fig. 3C) (53). Furthermore, Dll4^high i-DCs did not express Siglec-H (Fig. 3B) and had higher levels of J1 and J2 compared with pDCs. In addition, Dll4^high i-DCs did not express other lineage markers such as NK1.1, CD19, and surface IgM (data not shown).

In contrast, ~56% of Dll4^low CD11c^+ cells derived from GVHD mice were pDCDA-1^−B220^− cells (Fig. 3A), resembling cDCs from naive mice (47, 48, 50, 53, 55). When gating on these CD11c^+PDCA-1^−B220^− cDC-like cells derived from GVHD mice, we found that whereas they had higher levels of costimulatory molecules (CD40, CD80, and CD86) than steady state cDCs (Fig. 3B, 3C), only <10% of them expressed low levels of Dll4 (Fig. 3B). This suggests that these CD11c^+PDCA-1^−B220^− DCs are activated i-DCs and distinct from steady state cDCs. As

**FIGURE 3.** Representative histograms from three independent experiments are shown. (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 = 6–8 mice per group). (C) Histograms show the expression of Notch ligands on the surface of host CD11c^+ cells (mean ± SD, n = 6–8 mice per group). (D) Histograms show the expression of tested markers on the surface of host CD11c^+ cells. Representative histograms from three independent experiments are shown. (E) 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 d after HSCT. Representative histograms from three independent experiments are shown. *p < 0.05, **p < 0.01.
compared with steady state cDCs, Dll4low i-DCs also upregulated the expression of J2 (Fig. 3D). Collectively, these data indicate that Dll4high i-DCs and Dll4low i-DCs are distinct entities.

**Dll4high i-DCs have greater ability than Dll4low i-DCs to promote the development of effector T cells producing IFN-γ and IL-17**

To further define the biological properties of these Dll4high i-DCs and Dll4low 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. 4A). This allowed us to evaluate the functional activity of Dll4 in these i-DC subsets without using neutralizing anti-Dll4 Ab during the process. Morphological examination demonstrated that Dll4high i-DCs showed irregular shape and contained more cytoplasmic vacuoles compared with pDCs and cDCs (Fig. 4B). Unlike cDCs and Dll4low i-DCs, Dll4high i-DCs did not display long processes on the cell periphery (Fig. 4B). Real-time RT-PCR analysis showed that Dll4high i-DCs expressed higher levels of Dll4, Ifnb, and Il23, but lower levels of Il12 than Dll4low i-DCs (Fig. 4C, Table I). In addition, compared with pDCs, Dll4high i-DCs produced more Dll4, Ifnb, and Il23, but less Il12 and arginase 1 (Arg1) (Fig. 4C, Table I), further confirming that Dll4 high i-DCs and Dll4 low i-DCs are distinct entities.

We then used MLR assays to examine the difference in function between Dll4high i-DCs and Dll4low i-DCs in vitro. Highly purified Dll4high i-DCs and Dll4low i-DCs were added to cultures containing B6 CD4⁺ T cells, respectively, with or without addition of neutralizing anti-Dll4 Ab (Fig. 4D, 4E). Dll4high i-DCs induced ~3- and 10-fold more IFN-γ- and IL-17-producing T cells, respectively, than Dll4low i-DCs (Fig. 4D, 4E). Addition of anti-Dll4 Ab abrogated the ability of Dll4 high i-DCs to promote effector differentiation, but had little effect on cytokine production in T cells stimulated by Dll4low i-DCs (Fig. 4D, 4E). IL-2 was added to the culture to enhance the proliferation and differentiation of alloantigen-activated T cells (36). 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. 4D, 4E). Thus, Dll4high i-DCs may represent a unique i-DC subset and have greater capability than Dll4low i-DCs to promote the development of Th1 and Th17 cells.

**Time kinetics in generation of Dll4high i-DCs and their transcriptional signature**

We next determined the time kinetics in generation of Dll4high i-DCs and Dll4low i-DCs during GVH response by assessing the occurrence of Dll4highCD11c⁺PDCA-1⁺B220⁺ and Dll4lowCD11c⁺PDCA-1⁻B220⁻ cells. As shown in Fig. 5A, host-type Dll4low i-DCs were a dominant DC population in the spleen of these GVHD recipients 1 d after transplantation and markedly declined 3 d after transplantation. This is in agreement with previous observations that host DCs may finally diminish during the GVHD process (37,
In contrast, host origin Dll4 high i-DCs occurred by day 3 after transplantation and declined by day 7 (Fig. 5A). Interestingly, mice receiving TCD-BM plus donor T cells had $\sim$3-fold more number of Dll4 high i-DCs than those mice receiving TCD-BM alone at day 3 after transplantation (Fig. 5B). However, there was no difference in expression of Dll4 on the surface of i-DCs between these two groups (Fig. 5C). These results suggest that infusion of donor T cells is not necessary to the generation of host Dll4-POSITIVE INFLAMMATORY DENDRITIC CELLS by guest on June 4, 2017 http://www.jimmunol.org/ Downloaded from
we highly purified Dll4 high i-DCs from GVHD mice to compare their transcriptional signature with different DC subsets, as described by the Immunological Genome Consortium (56). As their transcriptional signature with different DC subsets, as described by the Immunological Genome Consortium (56). As shown in Fig. 5D, Dll4high i-DCs expressed a transcriptional signature that differed from pDCs, as evidenced by expressing drastically lower levels of Tcf4, Runx2, and Irf8. Id2 and Xcr1, which are two signature genes of CD8+ DCs, were also absent in Dll4high i-DCs. In addition, as compared with CD8+ DCs, Dll4high i-DCs expressed lower levels of Id2 and Flt3 (56). These data suggest that Dll4high i-DCs express a transcriptional signature distinct from each of these tested DC subsets. Further studies are required to determine the developmental origin of Dll4high i-DCs.

**Spleen and GI tract contain more Dll4high i-DCs than other tested tissues**

Data from our studies and others suggest that tissue-resident APCs play important roles in mediating GVHD (10, 38, 41, 57). To understand the potential effect of Dll4high i-DCs on GVH reaction, we examined their tissue distribution during the GVHD process. Mononuclear cells were isolated from various tissues of lethally irradiated BALB/c mice 3 d after receiving B6 TCD-BM and CD4+ T cells. Both the spleen and intestine contained more Dll4high i-DCs than other GVHD target organs such as the mesenteric lymph node, liver, BM, and lung (Fig. 6A). Like spleen Dll4high i-DCs (Fig. 3B), intestinal Dll4high i-DCs expressed high levels of Dll4, Ia, CD80, CD86, and CD11b (Fig. 6B, 6C). We could not recover Peyer’s patch from these recipients due to lethal irradiation-mediated severe lymphopenia 3 d after transplantation.

**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 days 0, 2, and 4 after transplantation. Donor T cells were recovered 5 d after transplantation (Fig. 7A). 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. 7B, 7C). Anti-Dll4 treatment reduced the expression of the Notch target gene Dtx1 in donor T cells (Fig. 7D), but had no effect on the recovery of Dll4high i-DCs in the spleen compared with IgG control (0.98 ± 0.2 × 10^4 versus 0.83 ± 0.1 × 10^4), respectively. This is in agreement with...
previous studies showing that in vivo administration of Ab to any single Notch ligand could not deplete DCs (58). These results suggest that Dll4 may play an important role in the regulation of alloreactive effector T cells in GVHD target organs.

We next asked whether blockade of Dll4 could prevent production of GVHD in BALB/c mice receiving donor B6 CD4 T cells. Nine doses of anti-Dll4 Ab were administered to these recipients once every 3 d 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. 8A, 8B). Histology examination showed markedly reduced inflammation in the intestine of these recipients treated by anti-Dll4 Ab (Fig. 8C, 8D). This decrease of donor effector T cells was accompanied with significant reduction of serum IFN-γ and IL-17. Neutralizing Dll4 abrogated this effect of Dll4 high i-DCs during early phase of GVHD. 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 high i-DCs during this period of GVH reaction.

**DLL4 high i-DCs occur in a second mouse model of GVHD**

To rule out the possibility that Dll4 high i-DCs might be model specific, we finally tested whether they could be detected in a different mouse model. TCD-BM and T cells derived from B6 mice were transplanted into lethally irradiated BDF1 mice (Fig. 9). We found a similar population of host Dll4 high i-DCs in these BDF1 recipients (Fig. 9A, 9B). Notably, short-term treatment with anti-Dll4 Ab reduced GVHD in irradiated BDF1 mice receiving both CD4+ and CD8+ T cells (Fig. 9C). These data indicate that induction of Dll4 high i-DCs can be generalized to other mouse models.

**Discussion**

The regulation of GVHD pathogenesis requires the generation of alloreactive effector T cells capable of mediating host tissue injury (1–3). In the current study, we provide evidence for a function of DLL4 and Dll4 high i-DCs in eliciting allogeic T cell responses. Upon preparative conditioning for allogeneic HSCT, Notch ligands Dll4, J1, and J2 were markedly upregulated on the surface of host origin i-DCs. Importantly, based on the expression of Dll4, i-DCs could be divided into two subsets, as follows: Dll4 high i-DCs and Dll4 low i-DCs. These cells had 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 with Dll4 low i-DCs, Dll4 high i-DCs had greater ability to stimulate the generation of alloreactive effector CD4+ T cells. 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 high i-DCs and Dll4 could be beneficial targets for improving the efficacy of allogeneic HSCT.

i-DCs occur as a result of inflammation and may have altered phenotype and function that are not normally present in steady state DCs (47, 48, 50, 59). This includes the upregulation of Ag-presenting molecules and costimulatory molecules (47, 48, 50, 53). Prior studies suggested that i-DCs can be derived from monocytes transferred into mice with vigorous inflammation, but not in clean and nonirradiated mice (55, 60). Some other studies

FIGURE 8. In vivo administration of anti-Dll4 reduces GVHD in MHC-mismatched recipient mice. Lethally irradiated (8 Gy) BALB/c mice were injected with B6 TCD-BM (5.0 × 10⁶) mixed with CD4⁺ T cells (1.0 × 10⁶), (A and B) Nine doses of anti-Dll4 Ab (250 μg/mouse) were given to BALB/c recipients once every 3 d 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 of H&E staining show the tissues from one of six recipients in each group at day 7 after transplantation. Images were obtained with an OlympusBX41 microscope (10×0.3 NA lens, ×200 original magnification, digital DP70 camera). (D) Pathological scores of GVHD 7 d after HSCT (6 mice per group). (E) ELISAs show the serum IFN-γ in allogeneic BALB/c recipients (4 mice for each group) 7 d after HSCT. Data show mean ± SD. (F and G) Six doses of anti-Dll4 Ab (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.

FIGURE 9. Identification of Dll4 high i-DCs in second mouse model of GVHD. Lethally irradiated (12 Gy) BDF1 mice were injected with B6 TCD-BM (5.0 × 10⁶) mixed with B6 CD4⁺ T cells (1.0 × 10⁶) and CD8⁺ T cells (1.0 × 10⁶). (A and B) Cells were recovered from the spleens of allogeneic HSCT BDF1 recipients 3 d after transplantation. Dot plots and histograms show the expression of Dll4 and other markers on the surface of different DC subsets. Results shown are representative of three independent experiments. (C) Six doses of anti-Dll4 Ab (250 μg/mouse) were administered once every other day from day 0 to day 10 after transplantation. Control recipients were treated with anti-hamster IgG. Survival of animals was monitored over time for BDF1 mice. **p < 0.01.
suggested that upon infection and activation by inflammatory stimuli, BM-derived pDCs differentiated into conventional i-DC–like cells and acquired potent ability to prime naive T cells (49, 54, 59). We found that host origin Dll4high i-DCs occurred in the spleen and GI tract by day 3 after preparative irradiation and HSCT. As compared with pDCs and cDCs, Dll4high i-DCs increased expression of Il6, Cxcl10, and Cxcl16; showed higher levels of Notch ligands Dll4, J1, and J2 on their surface; and produced more abundant IFN-β and IL-23 transcripts. Thus, Dll4high i-DCs may represent a unique and previously uncharacterized inflammatory APC subset.

Delineating the developmental origin of Dll4high i-DCs will be important for better understanding their function. Our studies and others have shown that a large proportion of host-type pDCs and cDCs were depleted within 24 h following HSCT (37, 38, 45, 46). In contrast, host origin Dll4high i-DCs were detected as later as 3 d after transplantation. Thus, it is possible that Dll4high i-DCs could be de novo generated during GVHD induction. To understand the relationship in development between Dll4high i-DCs and other DC subsets, we performed PCR-based gene-profiling analysis. We found that Dll4high i-DCs expressed a transcriptional signature distinct from pDCs, CD8α cDCs, and CD8α cDCs (56). However, because immature DCs may alter their phenotype and gene expression upon inflammatory stimulation (48, 50), we do not rule out the possibility that Dll4high i-DCs could be derived from those immature DCs with altered phenotype during GVHD induction. Further studies are needed to determine whether and how Dll4 may be upregulated in immature DCs and how Dll4high i-DCs are induced in vivo during the GVHD process.

Prior studies have shown that Dll4 may play multiple roles in regulating T cell responses during inflammation. Dll4 may directly regulate the production of effector T cells during infection and autoimmune responses (16, 17). Activation of CD4+ T cells with Dll4 enhanced the generation of Th1 cells in cultures, whereas competitively repressing the binding of endogenous Dll4 to Notch receptors by soluble Dll4 led to a decrease in IFN-γ production by activated CD4+ T cells in vivo (19). Dll4 directed the development of Th1 cell differentiation in the absence of IL-12 (19). Recent studies suggested that Dll4 promoted CD4+ T cells to secrete IL-17 via a mechanism of upregulating Rorc and Il17 transcripts (21–23). In contrast, Dll4 may influence inflammatory T cell response via modulating chemokine receptor expression in activated T cells during experimental autoimmune encephalomyelitis, thereby regulating differentiation of effector T cells (28). Thus, although our in vitro MLR assays clearly showed that blocking Dll4 led to a marked reduction of alloreactive effector T cells producing IFN-γ and IL-17, we do not rule out the possibility that in vivo blocking Dll4 could impair the migration of alloreactive effector T cells and consequently reduced GVH reaction.

Tissue-resident APCs play critical roles in inducing organ-specific GVHD (9, 10, 38, 41, 57). Depletion of APCs in the spleen and liver reduced GVHD in the liver, but had no effect on GVHD in skin (38). Other studies suggest that depletion of Langerhan’s cells in skin caused inhibition of cutaneous GVHD (41). We observed that Dll4high i-DCs represented a major population of CD11c+ cells in the intestine 3 d after allogeneic HSCT. In vivo administration of anti-Dll4 Ab reduced GVH reactions in the GI tract, coincident with our recent observations that donor T cells lacking Notch signaling failed to mediate intestinal GVHD (14). We proposed that Dll4 and intestinal Dll4high i-DCs could play important roles in mediating GVH reaction in these tissues. However, some studies suggested that Dll4 was expressed in inflammatory macrophages and endothelial cells in other models (12, 17, 28, 61, 62). The impact of Dll4 blockade on T cell responses in vivo should consider the potential contribution of Dll4-expressing nonhematopoietic APCs (such as endothelial cells and epithelial cells).

One major arguable point in the field of allogeneic HSCT is whether hematopoietic-derived APCs are essentially required for CD4 T cell–mediated GVHD. Some studies revealed that chimeric mice, in which host hematopoietic APCs lacking MHC class II (therefore incapable of priming donor T cells) developed severe GVHD (9), suggest the importance of nonhematopoietic APCs in GVHD induction. In contrast, many other studies using chimeric mice wherein host nonhematopoietic APCs lack MHC class II revealed that hematopoietic-derived APCs were sufficient to cause GVHD mediated by CD4 T cells (35, 40–42, 63). Although how to reconcile these observations remains controversial, data from previous studies indicate that the function of APCs in regulating allogeneic T cell responses in vitro may closely reflect their ability to mediate GVHD (43, 44, 64–66). For example, deletion of immature pDCs that had suppressive effect on T cells in vitro induced amelioration of GVHD (66). Adoptive transfer of regulatory DCs capable of repressing allogeneic T cell responses in vitro caused GVH inhibition (64, 65). In contrast, adoptive transfer of DCs, which showed potent ability to stimulate allogeneic T cell responses, resulted in lethal GVHD in MHC class II–deficient mice wherein recipient APCs cannot elicit GVHD (43, 44). We found that Dll4high i-DCs had potent effect on stimulating the generation of alloreactive effector T cells producing high levels of effector cytokines (e.g., TNF-α, IFN-γ, and IL-17) essential for mediating GVHD. This effect could be abrogated by neutralizing Dll4. Furthermore, in vivo blockade of Dll4 reduced GVHD, leading to significantly improved survival. These data suggest that Dll4high i-DCs play important roles in eliciting allogeneic T cell responses, although novel approaches are needed to formally assess whether Dll4high i-DCs are essentially required for GVHD induction.

In summary, we have identified previously uncharacterized Dll4high i-DCs that may have important roles in the regulation of GVHD pathogenesis. Most notably, Dll4 derived from these Dll4high i-DCs was required for them to direct effector differentiation of alloantigen-activated donor T cells. Given the central role of DCs in adaptive immunity, our findings may have broad implications in the development of novel strategies to modulate T cell responses for controlling GVHD and other inflammatory disorders such as graft rejection of transplanted solid organs, autoimmune diseases, and other immune cell–mediated blood diseases. Dll4 may facilitate the discovery of human counterparts of mouse Dll4high i-DCs.

Disclosures

The authors have no financial conflicts of interest.

References

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