DNAM-1 regulates Foxp3 expression in regulatory T cells by interfering with TIGIT under inflammatory conditions

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Regulatory T (Treg) cells that express forkhead box P3 (Foxp3) are pivotal for immune tolerance. Although inflammatory mediators cause Foxp3 instability and Treg cell dysfunction, their regulatory mechanisms remain incompletely understood. Here, we show that the transfer of Treg cells deficient in the activating immunoreceptor DNAM-1 ameliorated the development of graft-versus-host disease better than did wild-type Treg cells. We found that DNAM-1 competes with T cell immunoreceptor with Ig and ITIM domains (TIGIT) in binding to their common ligand CD155 and therefore regulates TIGIT signaling to down-regulate Treg cell function without DNAM-1–mediated intracellular signaling. DNAM-1 deficiency augments TIGIT signaling; this subsequently inhibits activation of the protein kinase B–mammalian target of rapamycin complex 1 pathway, resulting in the maintenance of Foxp3 expression and Treg cell function under inflammatory conditions. These findings demonstrate that DNAM-1 regulates Treg cell function via TIGIT signaling and thus, it is a potential molecular target for augmenting Treg function in inflammatory diseases.

regulatory T (Treg) cells | DNAM-1 | TIGIT | Foxp3 | mTORC1

Through their immune-suppressive functions, regulatory T (Treg) cells are crucial for preventing the development of autoimmune diseases (1). Whereas Treg cells inhibit excessive and unnecessary immune reactions during steady-state conditions, their suppressive function in inflamed environments is down-regulated to prevent suppression of the effector immune cell activity necessary for the elimination of pathogens (2); this down-regulation of Treg cell function is achieved by modulating the stability of forkhead box P3 (Foxp3), a key transcription factor in Treg cells (3). Stable Foxp3 expression is essential for Treg cell function because Foxp3 directly regulates the gene expression that organizes the Treg cell signature (4). "Fragile" Treg cells, which have reduced Foxp3 expression, occur in patients with autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus (5, 6). Fragile Treg cells have decreased the ability to suppress pathogenic T cells, which exacerbate autoimmune diseases by producing proinflammatory cytokines (7, 8). Therefore, the molecular mechanisms that cause Treg cell fragility must be elucidated to improve therapeutic strategies for autoimmune diseases.DNAM-1 (also called CD226) is a transmembrane-type glycoprotein receptor that belongs to the immunoglobulin superfamily and is predominantly expressed on T cells and natural killer (NK) cells (9). Upon ligation with its ligands CD155 (poliovirus receptor or nectin-like protein 5) or CD112 (nectin-2 or poliovirus receptor-related 2) (10, 11), DNAM-1 transduces an activating signal for enhancing cell-mediated cytotoxicity and cytokine production through the phosphorylation of a conserved tyrosine in its cytoplasmic domain (12, 13). DNAM-1 shares ligands with T cell immunoreceptor with Ig and ITIM domains (TIGIT), an inhibitory receptor expressed on activated and memory T cells, activated NK cells, and Treg cells (14, 15). In contrast to DNAM-1, the TIGIT-mediated signal inhibits the proliferation, activation, and cytokine production of T and NK cells (16, 17).

Several studies have demonstrated that a deficiency or blockade of DNAM-1 increases the numbers of $Foxp3^+$ cells and enhances Treg cell function in mouse models of acute graft-versus-host disease (GVHD), unilateral ureteral obstruction, and allogeneic skin transplantation (18–20), suggesting that DNAM-1 suppresses, rather than enhances, Treg cell function. In sharp contrast, TIGIT promotes the production of anti-inflammatory cytokines, such as IL-10 and fibrinogen-like protein 2 (Fgl2), by Treg cells, thus suppressing Th1 and Th17 reactions (21) and suggesting that TIGIT is involved in the immune-suppressive function of Treg

Significance

Immune tolerance is essential to prevent autoimmune responses, but it often needs to be limited for proper immune response. Regulatory T (Treg) cells play a crucial role in immune tolerance; however, their immune-suppressive function is restricted under inflammatory conditions. Here, we show that the activated immune receptor DNAM-1 limits Treg cell function regardless of DNAM-1–mediated intracellular signaling. We found that DNAM-1 competes with T cell immunoreceptor with Ig and ITIM domains (TIGIT) in binding to a common ligand. DNAM-1 deficiency enhances TIGIT signaling, thus resulting in the Treg cell function's maintenance under inflammatory conditions. These results suggest that the DNAM-1 and TIGIT balance orchestrate Treg cell function for optimal immune reaction.

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cells. However, the molecular mechanisms by which DNAM-1 and TIGIT modulate Treg cell function are unknown.

Here, we show direct evidence that DNAM-1 competes with TIGIT for ligand binding and regulates TIGIT-mediated signaling for Foxp3 expression via the protein kinase B–mammalian target of rapamycin complex 1 (AKT-mTORC1) pathway.

Results

DNAM-1 Deficiency Maintains Treg Cell Function in an Acute GVHD Model. Although DNAM-1 promotes the differentiation of $CD4^+$ naïve T cells into Th1 cells (12), whether DNAM-1 is also involved in their differentiation into Treg cells was unknown. We, therefore, analyzed spleen cells from $Cd226^{+/+}$ (wild type, WT) and $Cd226^{-/-}$ $Foxp3^{GFP}$ mice and found that the proportions of Treg cells and effector Treg cells and the expression of activation markers on Treg cells were comparable between these mouse lines (*SI Appendix*, Fig. S1 *A–C*). Moreover, WT and $Cd226^{-/-}$ $Foxp3^{GFP+}$ Treg cells similarly suppressed the proliferation of anti-CD3 monoclonal antibodies (mAb)-stimulated conventional T cells (Tconv) in vitro (*SI Appendix*, Fig. S1*D*). Together, these results suggest that DNAM-1 is not involved in the development and function of Treg cells under steady-state conditions.

We next used an acute GVHD model to evaluate the function of DNAM-1 on Treg cells in an inflammatory environment. BALB/c

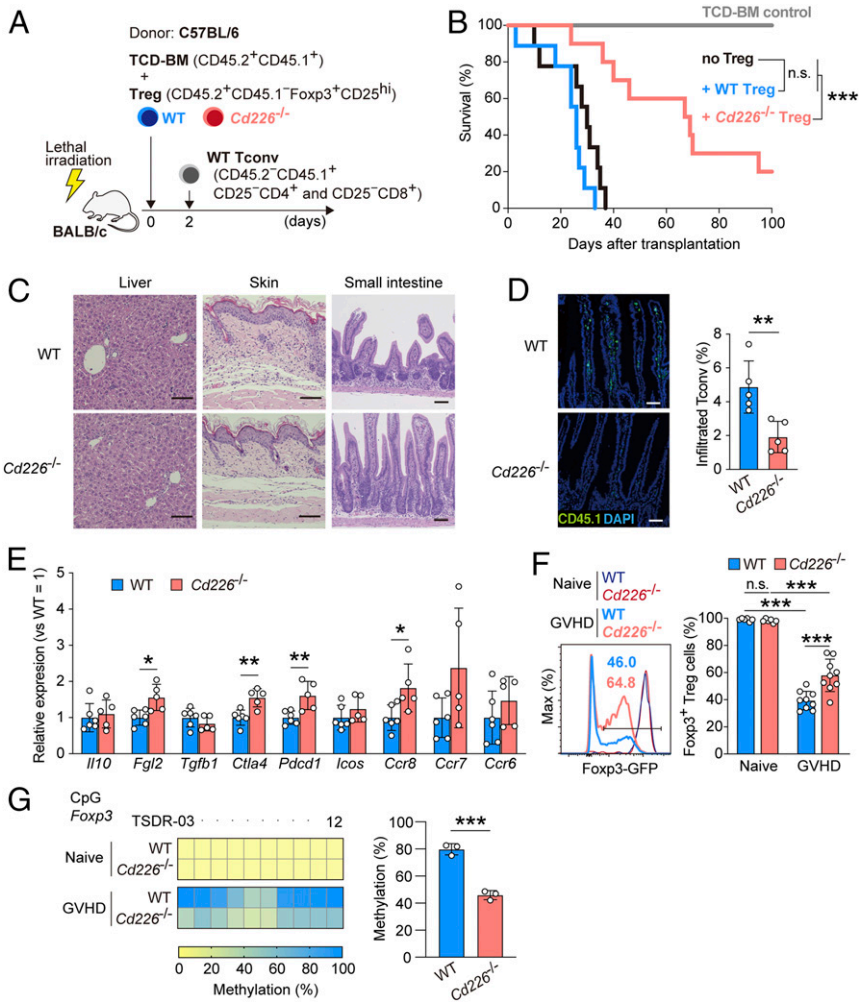

Fig. 1. DNAM-1 (CD226) deficiency maintains Treg cell function in a mouse model of acute GVHD. (*A*) Schematic representation of the experimental approach. Lethally irradiated BALB/c mice received TCD-BM cells from C57BL/6 mice and purified Treg cells from either C57BL/6 $Foxp3^{GFP}$ WT or C57BL/6 $Cd226^{-/-}$ $Foxp3^{GFP}$ ($Cd226^{-/-}$) mice. Recipient mice received $CD25^-$ Tconv cells from C57BL/6 WT mice to induce GVHD 2 d later. TCD-BM cells alone (i.e., without $CD25^-$ Tconv cells from C57BL/6 WT donors) were transferred into irradiated BALB/c recipients to generate non-GVHD controls. (*B*) Survival (log-rank [Mantel–Cox] testing) of mice with GVHD. The data shown are combined from two independent experiments (WT Treg, $n = 9$; $Cd226^{-/-}$ Treg, $n = 10$; no Treg, $n = 9$; control, $n = 3$). (*C*) Representative images of liver, skin, and small intestine from recipient mice on day 14 after the induction of GVHD. (Scale bar, 100 μm.) The data shown are representative of two independent experiments. (*D*, *Left*) Representative images of $CD45.1^+$ Tconv cells (green) and nuclei (blue) in the small intestines of recipient mice on day 8 after GVHD induction, and (*Right*) the proportions of Tconv cells in the tissue sections. (Scale bar, 100 μm.) The data shown are combined from two independent experiments. (*E*) Gene expression according to qRT-PCR analysis of WT and $Cd226^{-/-}$ Treg cells ($H\text{-}2K^{b+}$ $TCR\beta^+$ $CD4^+$ $CD45.2^+$ $Foxp3^{GFP+}$) sorted from the spleens of recipient mice on day 8 after GVHD induction. The data shown are combined from two independent experiments. (*F*, *Left*) Representative flow cytometry analysis of Foxp3 expression in transferred Treg cells ($H\text{-}2K^{b+}$ $TCR\beta^+$ $CD4^+$ $CD45.2^+$) sorted from the spleens of naïve or recipient mice on day 5 after GVHD induction. (*Right*) Quantified data are combined from three independent experiments. (*G*) Transferred Treg cells ($H\text{-}2K^{b+}$ $TCR\beta^+$ $CD4^+$ $CD45.2^+$) were sorted from the spleens of naïve or recipient mice on day 5 after GVHD induction, and (*Left*) the methylation status of the TSDR in genomic DNA was analyzed. Each line represents one CpG motif; the degree of methylation at each CpG motif is color coded. (*Right*) Statistical results. For *E*, *F*, and *G*, circles depict individual biological replicates. Data in graphs are reported as means ± SD. Statistical analysis was performed by using one-way ANOVA (*C* and *F*) and unpaired *t* tests (*G*); $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, n.s., not significant.

mice were lethally irradiated and then transplanted with T cell–depleted bone marrow (TCD-BM) cells together with, or without, WT or *Cd226*$^{-/-}$ Treg cells derived from C57BL/6 mice; 2 d later, the recipient mice received Tconv cells derived from C57BL/6 mice to induce acute GVHD (Fig. 1*A*). We found that all of the recipient mice that had received TCD-BM cells but not Treg cells died within 40 d (Fig. 1*B*). Although Treg cells attenuate the pathogenesis of GVHD by suppressing Tconv cell activation (22), the survival of the recipient mice that we transplanted with WT Treg cells was comparable to that of mice that did not receive any Treg cells. This result might indicate that we transferred too few Treg cells in our GVHD model and that the transplanted Treg cells were functionally down-regulated owing to radiation-induced inflammation (23). Even under these conditions in which WT Treg cells failed to manifest their immune-suppressive function, the transplantation of *Cd226*$^{-/-}$ Treg cells nevertheless prolonged the survival of recipient mice and attenuated gastrointestinal tract injury compared with after the transfer of WT Treg cells (Fig. 1 *B* and *C*). Immunohistochemical analysis showed that the small intestine of recipient mice given *Cd226*$^{-/-}$ Treg cells had fewer infiltrating CD45.1^{+} Tconv cells than that of mice infused with WT Treg cells (Fig. 1*D*). Together, these results suggest that *Cd226*$^{-/-}$ Treg cells are more highly suppressive of Tconv cell activation during GVHD than WT Treg cells.

Indeed, *Cd226*$^{-/-}$ Foxp3^{GFP+} Treg cells isolated from the recipient mice with GVHD showed higher expression of genes, *Fgl2*, *Ctla4*, *Pdcd1*, and *Ccr8*, than did WT Foxp3^{GFP+} Treg cells (Fig. 1*E*), consistent with the features of highly immune-suppressive Treg cells (24). We also observed that the down-regulation of *Foxp3* expression was milder in *Cd226*$^{-/-}$ Treg cells than in WT Treg cells in the inflammatory context of GVHD (Fig. 1*F*). Because the stability of Foxp3 is ensured through strictly maintained hypomethylation at the Treg-specific demethylated region (TSDR), an evolutionarily conserved noncoding element within the *Foxp3* gene locus (25), we analyzed the methylation status at the TSDR in Treg cells isolated from recipient mice with GVHD. Although both WT and *Cd226*$^{-/-}$ Treg cells showed increased methylation at the TSDR after the induction of GVHD, methylation levels were lower in *Cd226*$^{-/-}$ Treg cells than in WT Treg cells (Fig. 1*G*). Together, these results suggest that DNAM-1 deficiency maintains Foxp3 stability and Treg cell function during inflammation.

DNAM-1 Deficiency Reduces mTORC1 Activity and Maintains Foxp3 Expression in an Acute GVHD Model. Various components of tissue environments, including cytokines, inflammatory mediators, and metabolic factors, influence the stability of Foxp3 (26). To elucidate how DNAM-1 regulates Foxp3 stability, we performed a single-cell RNA (scRNA) sequence analysis of donor T cells isolated from the spleens of recipient mice with GVHD by using oligo-conjugated antibodies against the congenic markers CD45.1 and CD45.2 to distinguish Treg cells from Tconv cells (Fig. 1*A*). The proportions of transferred Tconv, Treg, and BM-derived T cells were comparable between mice given WT Treg cells and those that received *Cd226*$^{-/-}$ Treg cells (*SI Appendix*, Fig. S2*A*).

Gene set enrichment analysis (GSEA) showed that the activation status of Tconv cells was higher in mice given WT Treg cells than in those infused with *Cd226*$^{-/-}$ Treg cells (*SI Appendix*, Fig. S2 *B* and *C*), consistent with the severity of GVHD in each group of mice (Fig. 1 *B* and *C*). Donor Treg cells were further categorized into three fractions—*Ikzf2*^{hi} (i.e., effector Treg-like cells), *Ccr9*^{hi} (i.e., nonlymphoid tissues Treg-like cells), and *Mki67*^{hi} (i.e., Treg cells with high proliferative capacity)—according to the predominant gene expression pattern (Fig. 2*A* and *SI Appendix*, Fig. S2*D*). GSEA revealed that, among the three Treg fractions, only the *Mki67*^{hi} fraction showed enrichment of several gene sets in WT Treg cells compared with *Cd226*$^{-/-}$ Treg cells (Fig. 2*B*). In particular, we focused on enriched genes encoding members of the mTORC1 signaling, which is involved in Foxp3 stability and the function of Treg cells (27, 28). Consistent with the GSEA result, *Cd226*$^{-/-}$ Treg cells isolated from the recipient mice with GVHD exhibited a decrease of phosphorylation of ribosomal S6 protein (p-S6), an indicator of mTORC1 activation (Fig. 2*C*). To confirm the involvement of DNAM-1 in the mTORC1 signaling, we evaluated the mTORC1 activity in induced Treg (iTreg) cells, which mimic fragile Treg cells owing to incomplete demethylation of the TSDR (29). Although the p-S6 fraction was comparable between WT and *Cd226*$^{-/-}$ iTreg cells after stimulation with anti-CD3 mAb only, costimulation with the chimeric fusion protein comprising the extracellular portion of mouse CD155 fused to the Fc portion of human IgG1 (CD155-Fc) decreased the p-S6^{+} fraction in *Cd226*$^{-/-}$ iTreg cells more than in WT iTreg cells (*SI Appendix*, Fig. S3*A*). Moreover, the expression of *Myc*, which is up-regulated by mTORC1 activity (28), was higher in WT iTreg cells compared with *Cd226*$^{-/-}$ iTreg cells (*SI Appendix*, Fig. S3*B*). These findings suggest that the interaction of DNAM-1 with CD155 promotes mTORC1 activity.

The signals mediated through the T cell receptor (TCR) and costimulatory molecules activate mTORC1 through AKT phosphorylation (p-AKT) (30). We found that *Cd226*$^{-/-}$ Treg cells isolated from recipient mice with GVHD showed a lower level of p-AKT (threonine 308) compared with WT Treg cells from recipient mice with GVHD (Fig. 2*D*). Consistent with ex vivo Treg cell phenotypes, after being stimulated with anti-CD3 mAb and CD155-Fc, *Cd226*$^{-/-}$ iTreg cells showed reduced p-AKT and increased Foxp3 expression compared with WT iTreg cells (*SI Appendix*, Fig. S3*C* and Fig. 2*E*). We further examined whether the down-regulation of the AKT-mTORC1 activity was involved in higher Foxp3 expression in *Cd226*$^{-/-}$ iTreg cells. When mTORC1 and AKT activity were suppressed by using rapamycin and AKT inhibitor (AKTi), respectively, the Foxp3 expression level was comparable between WT and *Cd226*$^{-/-}$ iTreg cells (Fig. 2*F*). These data indicate that DNAM-1 promotes Foxp3 instability through the mTORC1 pathway in Treg cells.

DNAM-1–Mediated Signaling Does Not Affect Treg Cell Function. We observed that expression of the DNAM-1 ligand CD155 on splenic dendritic cells (DCs) was up-regulated through irradiation in vivo and by stimulation with inflammatory cytokines, including tumor necrosis factor-α (TNFα) and lipopolysaccharide (LPS), in vitro (*SI Appendix*, Fig. S4 *A* and *B*), suggesting that DNAM-1–mediated signaling may be up-regulated because of increased CD155 in the context of inflammation. In addition, a previous study demonstrated that DNAM-1 signaling augments the AKT pathway after the ligation of CD155 (31). Therefore, we hypothesized that the DNAM-1 signaling regulates Foxp3 expression through the AKT-mTORC1 pathway in Treg cells. To test our hypothesis, we stimulated iTreg cells by using anti-CD3 mAb together with, or without, anti–DNAM-1 mAb. TCR/CD3 stimulation alone significantly reduced Foxp3 expression, but costimulation with anti–DNAM-1 mAb did not further decrease Foxp3 levels (Fig. 3 *A* and *B*). Moreover, stimulation with DNAM-1 had no effect on p-S6 and p-AKT (Fig. 3 *C* and *D*). We further examined the effects of DNAM-1–mediated signaling on Treg cells derived from *Cd226*^{Y319F} knock-in mice in which DNAM-1 is not able to bind to Grb2 that is required for the DNAM-1–mediated activating signal in NK cells (31). The p-S6, tp-AKT, and Foxp3 expressions in both WT and *CD226*^{Y319F} iTregs after stimulation with anti-CD3 mAb plus anti–DNAM-1 mAb were comparable to those after stimulation with anti-CD3 mAb plus control Ig (*SI Appendix*, Fig. S4 *C* and *D*). In addition, we performed an in vitro suppression assay of Treg cells for the proliferation of Tconv cells. The suppressive function of iTreg cells was enhanced by DNAM-1 deficiency, while *CD226*^{Y319F} was comparable to WT iTreg cells after stimulation of plate-coated anti-CD3 mAb and CD155-Fc (Fig. 3*E*). These results suggest that DNAM-1–mediated signaling itself, if any also in *CD226*^{Y319F} iTregs, had no effect on the AKT-mTORC1–Foxp3

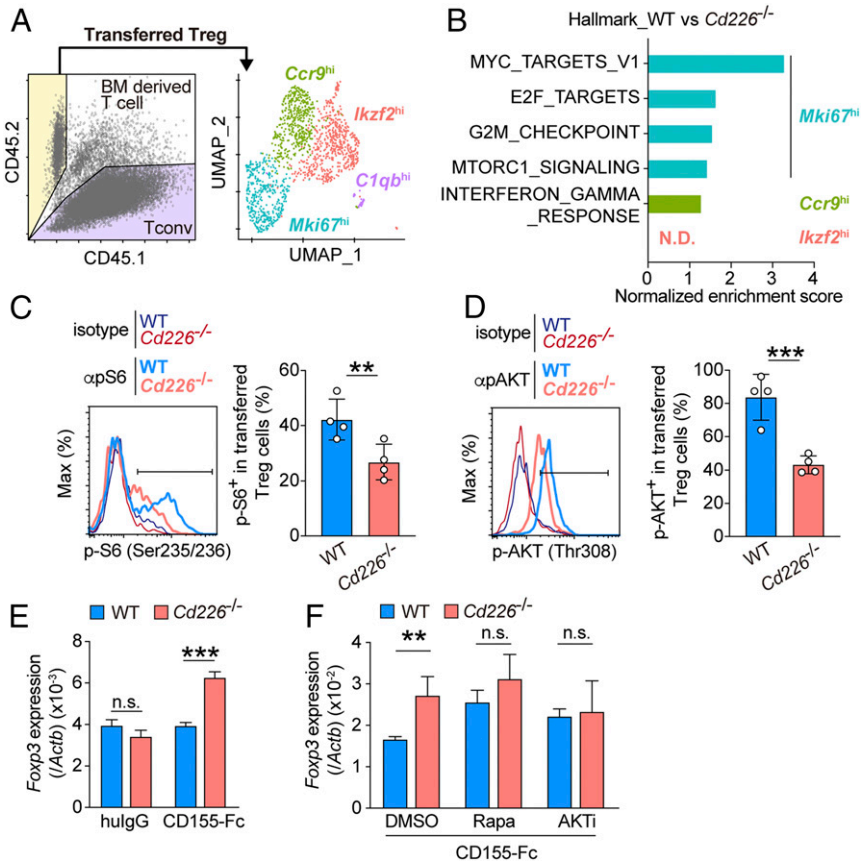

Fig. 2. DNAM-1 deficiency reduces mTORC1 activity and maintains Foxp3 expression in an acute GVHD model. (*A*, *Left*) Donor T cells (H-2K^{b+} TCRβ^{+} CD4^{+}) sorted from the spleens of recipient mice on day 8 after GVHD induction (Fig. 1*A*) as visualized by using CD45.1 and CD45.2 cell-surface expression levels according to Cellular Indexing of Transcriptomes and Epitopes by Sequencing. (*Right*) CD45.1^{-} CD45.2^{+} Treg cells as visualized by using uniform manifold approximation and projection. Sorted cells were used for scRNA sequencing analysis. (*B*) Gene set enrichment analysis showing differentially enriched (false discovery rate <0.25) hallmark gene sets in WT compared with *Cd226*$^{-/-}$ Treg cell populations. (*C* and *D*) Phosphorylation of S6 (*C*) and AKT (*D*) in transferred Treg cells from the spleens of recipient mice on day 5 after GVHD induction. Representative flow cytometry plots (*Left*) and statistical results (*Right*) are shown. Data are representative of two independent experiments. (*E*) *Foxp3* expression in iTreg cells 1 d after stimulation with anti-CD3 mAb plus either CD155-Fc or human IgG1 (huIgG). Data are representative of two independent experiments. (*F*) *Foxp3* expression in iTreg cells 4 d after stimulation with anti-CD3 mAb and CD155-Fc in the presence of the inhibitors indicated. The data shown are combined from two independent experiments. Data in graphs are shown as mean ± SD; (*C* and *D*) unpaired *t* tests, (*E* and *F*) one-way ANOVA, $^{**}P < 0.01$, $^{***}P < 0.001$, n.s., not significant.

pathway and Treg cell function. Consistent with these in vitro results, the transfer of *Cd226*$^{-/-}$ Treg cells again prolonged the survival of recipient mice compared with that of WT Treg cells; however, the transfer of Treg cells derived from *Cd226*^{Y319F} knock-in mice (*SI Appendix*, Fig. S4*E*) achieved recipient survival comparable to that of WT Treg cells (Fig. 3*F*), suggesting that DNAM-1–mediated intracellular signaling is not involved in Treg cell function. To confirm this idea, we generated the transfectants of *Cd226*$^{-/-}$ Treg cells expressing full-length DNAM-1 (DNAM-1^{WT}) or mutated DNAM-1 lacking the cytoplasmic region (DNAM-1$^{\Delta cyt}$) as well as a mock transfectant of *Cd226*$^{-/-}$ Treg cells. DNAM-1^{WT} and DNAM-1$^{\Delta cyt}$ Treg cells showed comparable amounts of DNAM-1 and TIGIT expressions at the cell surface (*SI Appendix*, Fig. S4*F*). These Treg cell transfectants showed higher phosphorylation of S6 and AKT than did the mock transfectant after stimulation with CD155-Fc together with anti-CD3 mAb. Importantly, both transfectants of DNAM-1^{WT} and DNAM-1$^{\Delta cyt}$ Treg cells showed comparable levels of S6 and AKT phosphorylation (*SI Appendix*, Fig. S4*G*). Consistent with these results, Foxp3 expression was down-regulated in both DNAM-1^{WT} or DNAM-1$^{\Delta cyt}$ Treg cells to the similar level after stimulation with anti-CD3 mAb and CD155-Fc (Fig. 3*G*), indicating that the DNAM-1 extracellular region was sufficient to limit the Foxp3 expression in Treg cells. Taken together, these results indicate that DNAM-1 deficiency reduced mTORC1 activity and therefore maintained Foxp3 expression, yet DNAM-1–mediated signaling did not directly affect Foxp3 expression or Treg cell function under inflammatory conditions.

TIGIT Signaling Inhibits the AKT-mTORC1 Pathway to Down-Regulate Foxp3 Expression. Instead of a mechanism through which the DNAM-1–mediated signal directly affects the function of Treg cells, we next examined the possibility that ligand binding to TIGIT or CD96, rather than to DNAM-1, regulates Treg cell function. Consistent with the previous report (18), DNAM-1 and TIGIT expression levels were up-regulated on Foxp3^{GFP+} Treg cells isolated from mice on day 5 after GVHD induction compared with those harvested before (naïve) GVHD induction (*SI Appendix*, Fig. S5*A*). In contrast, CD96 expression was decreased after GVHD induction (*SI Appendix*, Fig. S5*A*). Similarly, Treg cell activation in vitro up-regulated DNAM-1 and TIGIT expression but down-regulated CD96 expression (*SI Appendix*, Fig. S5*B*), thus suggesting that TIGIT, rather than CD96, regulates Treg cell function. Indeed, the stimulation of TIGIT on iTreg cells with plate-coated anti-TIGIT mAb up-regulated *Fgl2* and *Il10* expression (*SI Appendix*, Fig. S5*C*) as previously reported (21, 32). We showed that IL-10 expression, but not Fgl2, was comparable between WT and *CD226*$^{-/-}$ Tregs after GVHD induction

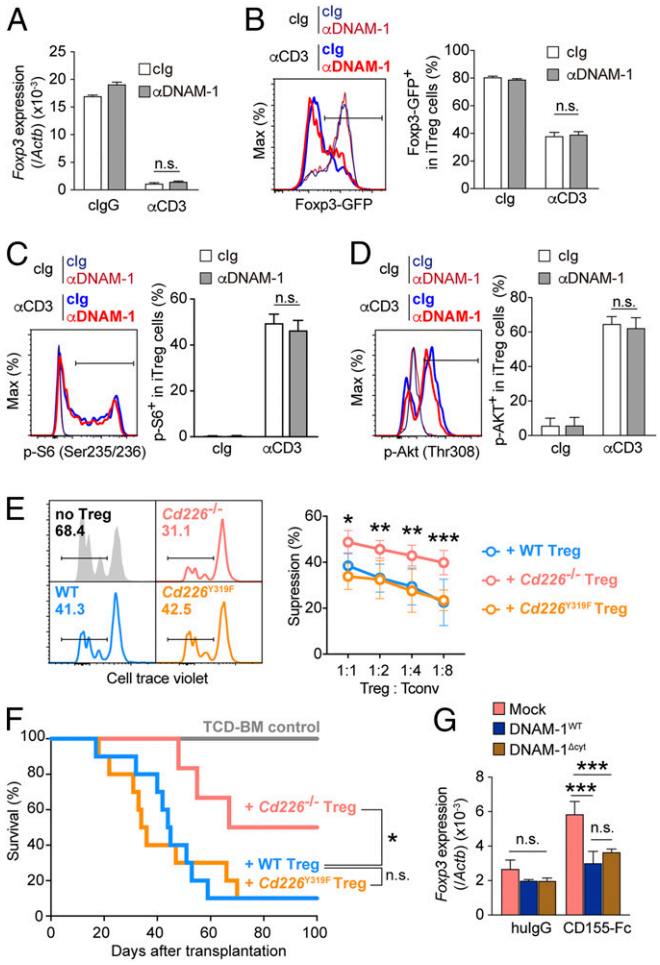

Fig. 3. DNAM-1–mediated signaling does not affect Treg cell function. (*A–D*) WT iTreg cells were stimulated with anti-CD3 mAb or isotype control plus either anti–DNAM-1 mAb or isotype control. (*A* and *B*) Foxp3 expression and phosphorylation of (*C*) S6 and (*D*) AKT on day 3 after stimulation were analyzed through qRT-PCR and flow cytometry, respectively. (*Left*) Representative histograms and (*Right*) statistical results are shown. The data shown are combined from two independent experiments. (*E*) CD45.2$^+$ iTreg cells were cocultured with CD45.1$^+$ CTV-labeled WT Tconv cells with plate-coated anti-CD3 and CD155-Fc. The cell division of responder cells was analyzed 3 d later. Representative histograms (*Left*) and statistical results (*Right*) are shown. Percent suppressions of Tconv cell division in the presence of Treg cells were calculated relative to those in the absence of Treg cells. The data shown are representative of two independent experiments. (*F*) Survival (log-rank [Mantel–Cox] testing) of mice with GVHD (Fig. 1*A*); the data shown are combined from three independent experiments (WT Treg, $n = 10$; *Cd226*$^{-/-}$ Treg, $n = 6$; *Cd226*^{Y319F} Treg, $n = 10$; control, $n = 3$). (*G*) *Cd226*$^{-/-}$ iTreg cells expressing DNAM-1^{WT}, DNAM-1$^{\Delta cyt}$, or mock control vector were stimulated with anti-CD3 mAb plus either CD155-Fc or huIgG. Foxp3 expression on day 1 after stimulation were analyzed through qRT-PCR. The data shown are representative of two independent experiments. Data in graphs are given as mean ± SD; (*A*, *D*, and *G*) one-way ANOVA; (*E*) two-way ANOVA, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, n.s., not significant.

(Fig. 1*E*). This discrepancy may be caused by different time windows for proper analysis of each molecule in inflammatory conditions during GVHD. Nonetheless, how TIGIT regulates Treg cell function and whether TIGIT regulates Foxp3 expression remains unknown. To address these issues, iTreg cells were stimulated by using anti-CD3 mAb alone or with anti-TIGIT mAb. Stimulation with anti-CD3 mAb alone decreased both *Foxp3* transcript and Foxp3–GFP protein levels (Fig. 4 *A* and *B*). However, we found that the addition of anti-TIGIT mAb suppressed the downregulating effect of anti-CD3 mAb on Foxp3 expression (Fig. 4 *A* and *B*). Moreover, anti-TIGIT mAb reduced CD3 mAb-induced p-AKT and p-S6 (Fig. 4 *C* and *D*). These data suggest that the TIGIT-mediated signal suppresses the TCR-AKT-mTORC1 pathway, thus inhibiting the down-regulation of Foxp3 expression in Treg cells.

DNAM-1 Deficiency Enhances TIGIT Signaling in Foxp3$^+$ Treg Cells. DNAM-1 and TIGIT mediate opposing positive and negative signals, respectively, upon binding to the same ligand, CD155 (33, 34). Because the binding affinities of DNAM-1 and TIGIT to CD155 are comparable (K_D values of 5.16 and 1.29 μM, respectively) (17, 35, 36), TIGIT-mediated signaling is likely to be dependent on the level of DNAM-1 expression. To test this scenario, we established nuclear factor of activated T cells (NFAT)–GFP reporter cells (37), which express a chimeric fusion protein comprising the extracellular and transmembrane portions of mouse TIGIT fused to the cytoplasmic portion of the gamma chain of FceRI. The TIGIT reporter cells were then transfected with a DNAM-1$^{\Delta cyt}$. The reporter cells expressing DNAM-1$^{\Delta cyt}$ reduced the NFAT–GFP expression induced by CD155-Fc stimulation compared with that in parental reporter cells, despite similar expression levels of TIGIT (Fig. 5*A* and *SI Appendix*, Fig. S6*A*). Furthermore, treatment with an anti–DNAM-1 neutralizing mAb enhanced NFAT–GFP expression induced by CD155-Fc stimulation in DNAM-1$^{\Delta cyt}$–expressing reporter cells (Fig. 5 *B* and *C*), indicating that DNAM-1 interfered with TIGIT signaling through ligand competition. Conversely, while AKT-mTORC1 activity was decreased in *Cd226*$^{-/-}$ iTreg cells compared with WT iTreg cells after costimulation with anti-CD3 mAb and CD155-Fc, the addition of a neutralizing anti-TIGIT mAb increased AKT-mTORC1 activity of *Cd226*$^{-/-}$ iTreg cells to the comparable level of WT iTreg cells (Fig. 5*D* and *SI Appendix*, Fig. S6*B*). Similarly, the expression of Foxp3 in *Cd226*$^{-/-}$ iTreg was reduced to the comparable level in WT iTreg cells by the addition of a neutralizing anti-TIGIT mAb. (Fig. 5*E*).

We next established *Tigit*$^{-/-}$ and *Cd226*$^{-/-}$ *Tigit*$^{-/-}$ mice and confirmed the proportion of Foxp3$^+$ CD25$^+$ Treg cells were comparable between *Tigit*$^{-/-}$ and *Cd226*$^{-/-}$ *Tigit*$^{-/-}$ mice in the steady state (*SI Appendix*, Fig. S6*C*). Then, we used our GVHD model to

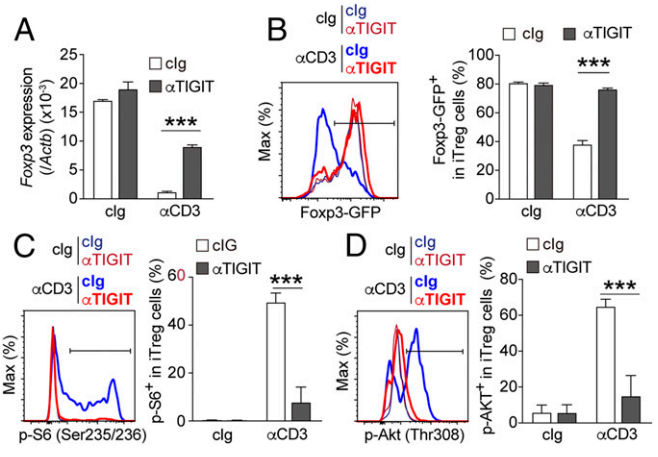

Fig. 4. TIGIT signaling inhibits the AKT-mTORC1 pathway to down-regulate Foxp3 expression. (*A–D*) WT iTreg cells were stimulated by using anti-CD3 mAb or isotype control antibody plus either anti-TIGIT mAb or isotype control antibody. On day 3 after stimulation, (*A* and *B*) *Foxp3* expression and phosphorylation of (*C*) S6 and (*D*) AKT were analyzed by qRT-PCR and flow cytometry, respectively. (*Left*) Representative histograms and (*Right*) statistical results are shown. The data shown are combined from two independent experiments. Data in graphs are given as means ± SD; (*A–D*) one-way ANOVA, $***P < 0.001$.

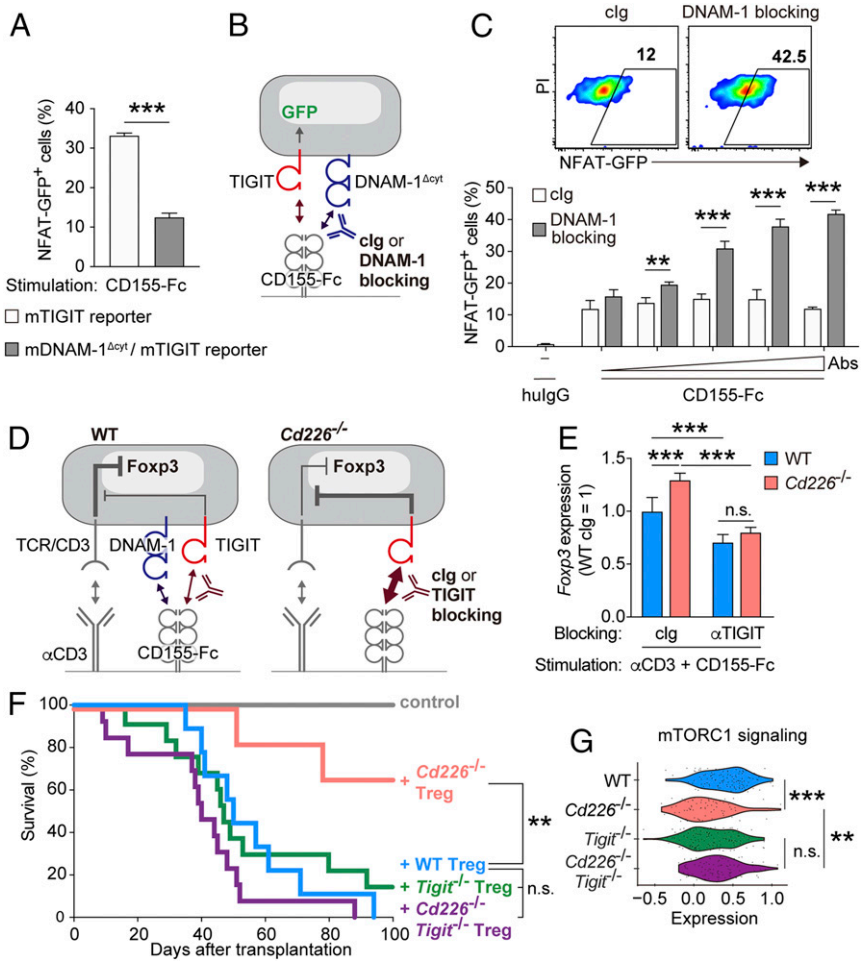

Fig. 5. DNAM-1 deficiency enhances TIGIT signaling in Foxp3$^+$ Treg cells. (*A*) GFP expression in TIGIT reporter cells and in TIGIT reporter cells coexpressing DNAM-1$^{\Delta cyt}$ after stimulation with the plate-bound CD155-Fc protein. Data are representative of two independent experiments. (*B*) Schematic diagram of TIGIT reporter cell analysis. (*C*, *Top*) Representative data of GFP expression in TIGIT reporter cells and TIGIT reporter cells coexpressing DNAM-1$^{\Delta cyt}$ after stimulation with CD155-Fc in the presence of isotype control or anti–DNAM-1 neutralizing mAb. (*Bottom*) Quantified data are representative of two independent experiments. (*D*) Schematic diagram of experimental design for *E*. (*E*) Foxp3 expression in iTreg cells 1 d after stimulation with anti-CD3 mAb and CD155-Fc in the presence of isotype control or an anti-TIGIT neutralizing mAb. The data shown are combined from two independent experiments. (*F*) Survival (log-rank [Mantel–Cox] test) of mice with GVHD (Fig. 1*A*); the data shown are combined from four independent experiments (WT Treg, $n = 9$; *Cd226*$^{-/-}$ Treg, $n = 6$; *Tigit*$^{-/-}$ Treg, $n = 13$; *Cd226*$^{-/-}$*Tigit*$^{-/-}$ Treg, $n = 11$; control, $n = 3$). (*G*) Violin plots showing mean expression (log transformed, normalized, and scaled) of DNAM-1–regulated mTORC1 signaling–related genes in clusters of proliferating Treg cells from WT, *Cd226*$^{-/-}$, *Tigit*$^{-/-}$, and *Cd226*$^{-/-}$ *Tigit*$^{-/-}$ mice (Wilcoxon test with Bonferroni adjustment, $^{**}P < 0.01$; $^{***}P < 0.001$). Data in graphs are shown as means ± SD; (*A*) unpaired *t* test; (*C* and *E*) one-way ANOVA; $^{**}P < 0.01$, $^{***}P < 0.001$, n.s., not significant.

examine whether DNAM-1 regulates Treg cell function in a TIGIT-dependent manner in vivo. Consistent with the results in Fig. 1*B*, mice given *Cd226*$^{-/-}$ Treg cells survived longer after GVHD induction than mice infused with WT Treg cells (Fig. 5*F*). However, mice transplanted with *Cd226*$^{-/-}$ *Tigit*$^{-/-}$ Treg cells died sooner than those given *Cd226*$^{-/-}$ Treg cells (Fig. 5*F*). Conversely, survival after GVHD was comparable between mice given *Cd226*$^{-/-}$ *Tigit*$^{-/-}$ Treg cells and those that received *Tigit*$^{-/-}$ Treg cells (Fig. 5*F*). These results indicate that the prolonged survival of mice transplanted with *Cd226*$^{-/-}$ Treg cells and the function of those cells were dependent on the expression of TIGIT on Treg cells.

To confirm the role of TIGIT in mTORC1 activity, we performed an scRNA sequencing of Treg cells isolated from recipient mice transplanted with *Tigit*$^{-/-}$ or with *Cd226*$^{-/-}$ *Tigit*$^{-/-}$ Treg cells. We observed that the proportions of each Treg cell subset were comparable between the two groups of mice (*SI Appendix*, Fig. S6*D*). mTORC1 activity was lower in *Cd226*$^{-/-}$ Treg cells than in WT Treg cells and *Cd226*$^{-/-}$ *Tigit*$^{-/-}$ Treg cells (Fig. 5*G*), whereas mTORC1 activity was comparable between *Cd226*$^{-/-}$ *Tigit*$^{-/-}$ Treg cells and *Tigit*$^{-/-}$ Treg cells (Fig. 5*G*). Similarly, p-AKT and p-S6 were lower in *Cd226*$^{-/-}$ Treg cells compared to WT Treg cells isolated from recipient mice with GVHD, and *Tigit*$^{-/-}$ or *Cd226*$^{-/-}$ *Tigit*$^{-/-}$ Treg cells showed comparable AKT-mTORC1 activity (*SI Appendix*, Fig. S6*E*). Since the expression of TIGIT was comparable in WT and *Cd226*$^{-/-}$ Treg cells (*SI Appendix*, Fig. S6*F*), these results demonstrate that DNAM-1 deficiency promotes TIGIT–CD155 interaction and TIGIT-mediated signaling, resulting in suppression of the AKT-mTORC1 pathway and leading to the instability of Foxp3 expression and down-regulation of Treg cell function.

DNAM-1 Deficiency Enhances TIGIT Signaling and Foxp3 Expression in a Treg Cell–Intrinsic Manner. Given that inflammatory factors directly affect the stability of Treg cells, we next investigated whether DNAM-1 regulates Foxp3 expression in a cell-intrinsic manner in the GVHD model. We combined CD45.2$^+$ *Cd226*$^{-/-}$, *Tigit*$^{-/-}$, or *Cd226*$^{-/-}$ *Tigit*$^{-/-}$ Treg cells in a 1:1 ratio with CD45.1$^+$ WT Treg cells and then cotransferred this cell mixture; 2 d after cotransfer,

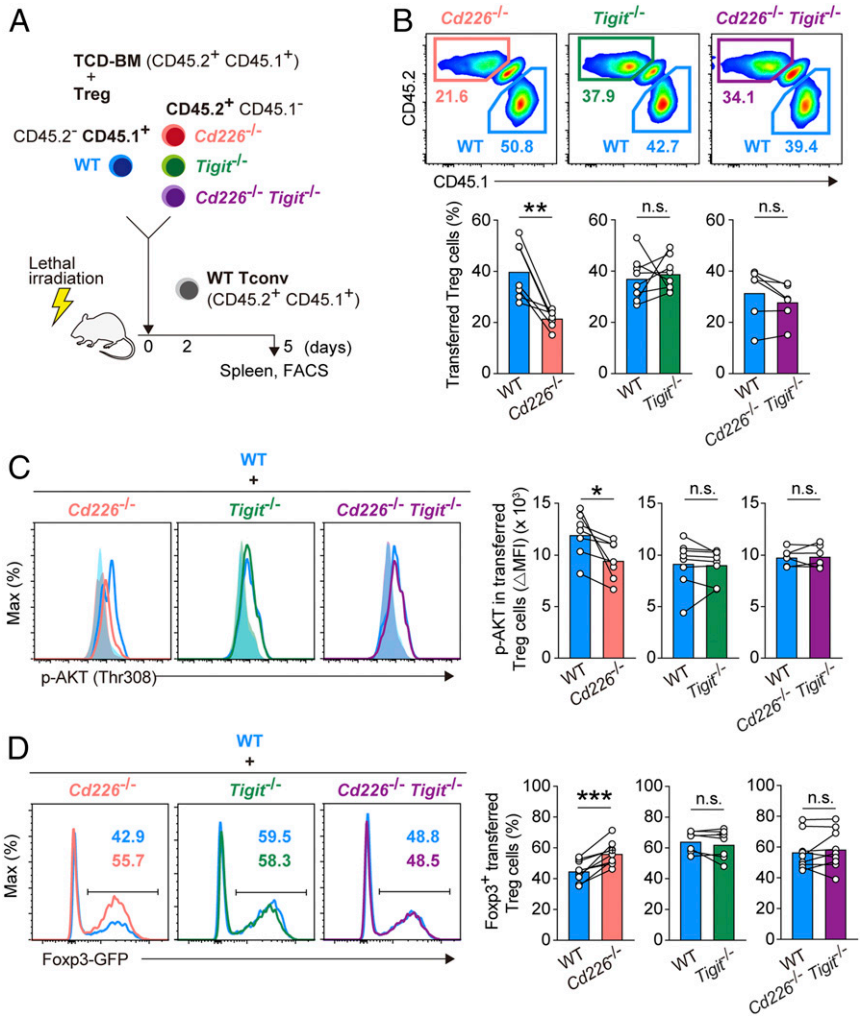

Fig. 6. DNAM-1 deficiency enhances TIGIT signaling and Foxp3 expression in a Treg cell-intrinsic manner. (*A*) Schematic diagram of experimental approach. Lethally irradiated BALB/c WT mice were given CD45.2$^+$ CD45.1$^+$ TCD-BM from C57BL/6 WT mice and a mixture (ratio of 1:1) of CD45.2$^-$ CD45.1$^+$ WT Treg cells and CD45.2$^+$ CD45.1$^-$ Treg cells deficient in *Cd226,* or *Tigit*, or both. Recipient mice were infused with CD45.2$^+$ CD45.1$^+$ Tconv cells from C57BL/6 WT mice 2 d later. (*B*) The proportions of transferred Treg cells in donor T cells (H-2K^{b+} CD4$^+$) from the spleens of recipient mice on day 5 after GVHD induction. (*Top*) Representative flow cytometry plots and (*Bottom*) statistical results are shown. The data shown are combined from two independent experiments. (*C* and *D*) Phosphorylation of (*C*) AKT and (*D*) *Foxp3* expression in transferred Treg cells (H-2K^{b+} CD4$^+$) from the spleens of recipient mice on day 5 after GVHD induction. (*Left*) Representative flow cytometry plots and (*Right*) statistical results are shown. Data are combined from (*C*) two and (*D*) four independent experiments. For *B*, *C*, and *D*, circles depict individual biological replicates. Data in graphs are given as means ± SD; paired *t* test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, n.s., not significant.

mice received CD45.1$^+$ CD45.2$^+$ Tconv cells (Fig. 6*A*). We found that the proportion of *Cd226*$^{-/-}$ Treg cells was smaller than that of WT Treg cells, whereas the proportions of *Tigit*$^{-/-}$ and *Cd226*$^{-/-}$ *Tigit*$^{-/-}$ Treg cells were comparable to that of WT Treg cells (Fig. 6*B*). These results indicated that *Cd226*$^{-/-}$ Treg cells had lower proliferative activity than WT Treg cells in a TIGIT-dependent manner under inflammatory conditions, consistent with the decreased activity of Myc and mTORC1 (Fig. 2*B*). Similarly, *Cd226*$^{-/-}$ Treg cells showed decreased p-AKT and increased Foxp3$^+$ expression compared with the cotransferred WT Treg cells (Fig. 6 *C* and *D*). However, both p-AKT and Foxp3 expression were comparable between *Cd226*$^{-/-}$ *Tigit*$^{-/-}$ and *Tigit*$^{-/-}$ Treg cells (Fig. 6 *C* and *D*). Taken together, these results indicated that DNAM-1 deficiency suppressed AKT phosphorylation and promoted Foxp3 expression through TIGIT in a Treg cell–intrinsic manner under inflammatory conditions.

Treatment with Anti-Human DNAM-1 mAb Attenuates the Development of GVHD in Humanized Mice. To investigate whether DNAM-1 suppresses Treg cell function under inflammatory conditions in humans as well as in mice, we generated human *Pvr*-transgenic NOD/Shi-scid, IL-2Rγnull (hCD155-NOG) mice and established an acute GVHD model by transferring human peripheral blood mononuclear cells (hPBMCs), among which xenoreactive T cells contribute to the development of GVHD. Treatment of hCD155-NOG mice with the F(ab')$_2$ fragments of a neutralizing anti-human DNAM-1 mAb (anti–hDNAM-1) before hPBMC transfer prolonged survival and ameliorated liver injury compared with these indicators in control mice (Fig. 7 *A* and *B*). Consistent with the results in Fig. 6*B*, the number of Treg cells in peripheral blood was decreased in hCD155-NOG mice treated with anti–hDNAM-1 (Fig. 7*C*). Of note, however, treatment with anti–hDNAM-1 increased the proportion of Treg cells among CD4$^+$ T cells (Fig. 7*C*). In addition, we found that Foxp3 expression was higher in Treg cells isolated from recipient hCD155-NOG mice that had been treated with anti–hDNAM-1 compared with the control group (Fig. 7*D*). These results suggest that the DNAM-1 blockade promotes the Foxp3 expression and function of Treg cells under inflammatory conditions in humans as well as in mice. Taken together, these results suggest that DNAM-1 intrinsically limits

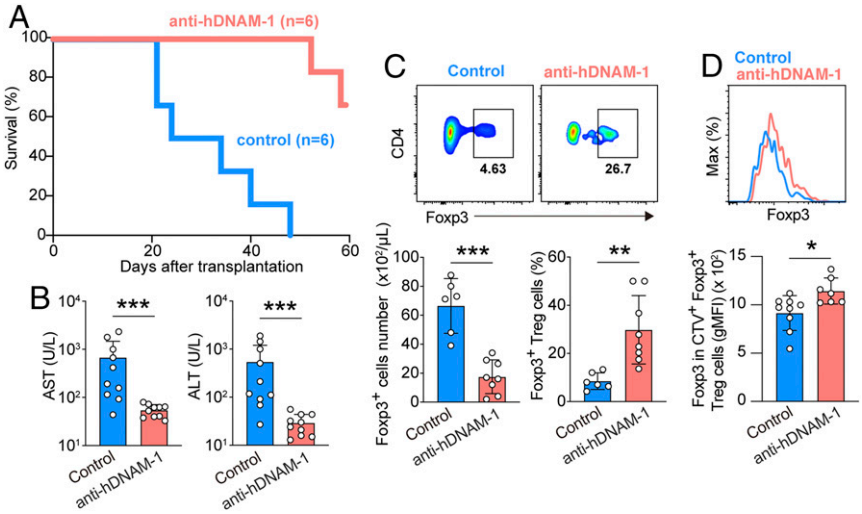

Fig. 7. Treatment with anti–DNAM-1 mAb attenuates the development of GVHD in humanized mice. (*A*) Human peripPBMCs were transferred into lethally irradiated hCD155-NOG mice on day 0. F(ab′)$_2$ of anti-human DNAM-1 (anti–hDNAM-1, $n = 6$) or phosphate-buffered saline (control, $n = 6$) were injected intravenously every 3 d, beginning on day 0. Survival curves were analyzed with log-rank (Mantel–Cox) test. The data shown are combined from two independent experiments. (*B*) Serum asparagine aminotransferase and alanine aminotransferase levels in recipient mice on day 14 after GVHD induction. The data shown are combined from three independent experiments. (*C*) (*Top*) Representative flow cytometry plots of hCD45$^+$ CD3$^+$ CD4$^+$ T cells in the peripheral blood of recipient mice on day 14 after GVHD induction. (*Bottom*) Numbers and proportions of Foxp3$^+$ Treg cells in hCD45$^+$ CD3$^+$ CD4$^+$ T cells. The data shown are combined from two independent experiments. (*D*) Cell Trace Violet–labeled human CD25$^+$ Treg cells combined with human CD25$^-$ PBMC were given to irradiated hCD155-NOG mice that had been injected with anti–hDNAM-1 mAb or isotype control on day 0. *Foxp3* expression in CTV$^+$ Foxp3$^+$ T cells in the spleens of recipient mice was analyzed on day 4 after GVHD induction. The data shown are combined from two independent experiments. For *B*, *C*, and *D*, circles depict individual biological replicates. The data in graphs are shown as means ± SD; unpaired *t* test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

TIGIT signaling by competing with the CD155 binding, resulting in negatively regulated Foxp3 expression and Treg cell function via the uncontrolled AKT-mTORC1 pathway under inflammatory conditions (*SI Appendix*, Fig. S7).

Discussion

Although several studies have demonstrated that the activating and inhibitory receptors DNAM-1 and TIGIT have opposite roles in Treg cell function (18, 32), the molecular mechanism of how DNAM-1 and TIGIT function and signaling interact remains incompletely understood despite the fact that this field is attracting increasing attention. Here, we showed that DNAM-1 competes with TIGIT for binding to the ligand CD155 in an inflammatory environment; DNAM-1 deficiency enhanced TIGIT signaling, which subsequently suppressed the AKT-mTORC1 pathway and led to Foxp3 stability (*SI Appendix*, Fig. S7). Moreover, we have first demonstrated the formal evidence that the roles of DNAM-1 and TIGIT in the regulation of Treg cell function were in a Treg cell–intrinsic manner by using a Treg cell transfer experiment. Although TIGIT on Treg cells has an immune-suppressive role against Th1 and Th17 responses by inducing the production of Fgl2 and IL-10 (21), we showed that, in our GVHD model, mice given *Tigit*$^{-/-}$ Treg cells did not have shorter survival than those infused with WT Treg cells. In addition, WT Treg cells failed to prolong the survival of recipient mice, suggesting that the Treg cell function was suppressed under inflammatory conditions in our GVHD model. The reduced frequency and abnormal function of CD4$^+$ CD25$^+$ Treg cells were also observed in patients with GVHD (38, 39). Despite such conditions, however, DNAM-1 deficiency augmented Treg cell function via increased TIGIT signaling. These results indicate that DNAM-1 regulates Treg cell function under inflammatory conditions.

We further showed that DNAM-1 competes with TIGIT for ligand binding and consequently the regulation of Treg stability and the immune-suppressive function under the inflammatory conditions of our GVHD model. We showed that DNAM-1–mediated signaling was not required for DNAM-1 function in the regulation of Treg cell stability and the immune-suppressive function by using *Cd226*^{Y319F} and DNAM-1$^{\Delta cyt}$ Treg cells. Expression of DNAM-1, as well as of TIGIT, was up-regulated on Treg cells in this context, likely escalating the competition between DNAM-1 and TIGIT for binding to their ligand. Previous reports demonstrated that T cell activation up-regulates DNAM-1 in human CD4$^+$ T cells (40), and DNAM-1 expression on CD8$^+$ T cells is controlled by the strength of TCR signaling, which is carried by transcriptional regulation by Eomes (41). By contrast, Treg cells in tumor tissues show down-regulated expression of DNAM-1, whereas TIGIT is highly expressed on Treg cells (34). In this context, DNAM-1 is unlikely to compete effectively with TIGIT for ligand binding on Treg cells in the tumor microenvironment. Instead, the TIGIT signal may be enhanced, resulting in the down-regulation of the PI3K–AKT pathway and augmentation of Treg cell function.

The PI3K–AKT pathway reportedly activates both FOXO1/FOXO3A and mTORC1 in Treg cells, leading to Foxp3 instability (42). In addition, TIGIT signaling suppresses the AKT-FOXO1 pathway, thereby restoring the immune-suppressive capacities of IFN-γ producing Th1-like Treg cells in the presence of IL-12 (43). However, we showed here that enhanced TIGIT signaling inhibits the AKT-mTORC1 pathway to maintain Foxp3 expression and the immune-suppressive functions of Treg cells in an inflammatory environment, suggesting that the pathway downstream of TIGIT–AKT in Th1-like Treg cells in the presence of IL-12 alone differs from that in fragile Treg cells exposed to multiple inflammatory mediators. Further investigation is required to reveal how factors in the Treg cell environment determine the target molecules downstream of TIGIT–AKT.

Human Treg cells are categorized into four fractions according to their expression patterns of TIGIT and DNAM-1 (44). Compared with the other three populations, CD226$^-$ TIGIT$^+$ Treg cells have higher Foxp3 stability and immune-suppressive capacity. Conversely, CD226$^+$ TIGIT$^+$ Treg cells are associated with low Foxp3 stability and suppressive capacity (44). These findings may be explained by the competition of DNAM-1 with TIGIT for ligand

binding, which regulates TIGIT signaling to influence Foxp3 stability as we demonstrated here. Therefore, DNAM-1 is not only a cell-surface marker for distinguishing Treg cell subsets with diverse functions but also a functional molecule that can be an attractive target for enhancing Treg cell function. Rapamycin—an inhibitor of the mTORC1 complex and an immunosuppressive agent used to prevent rejection of transplanted organs—increases the function of Treg cells (45). However, rapamycin also has an unfavorable effect because mTORC1 is ubiquitously expressed and is a primary regulator of metabolism (46). In contrast, DNAM-1 expression is restricted to immune cells, including Treg cells. Therefore, regulating mTORC1 activity by targeting DNAM-1 likely will be more specific to T cell–mediated immune responses than is rapamycin.

Although most costimulatory molecules such as CD28, ICOS, and OX40 promote the activation and differentiation of both Tconv and Treg cells (47–49), DNAM-1 is a unique receptor that activates Tconv cells but inhibits Treg cell function, suggesting that DNAM-1–targeted therapy regulates both Tconv and Treg cells, but in different directions, and thus contributes to the control of inflammation. Although DNAM-1 deficiency has been reported to decrease pathology associated with experimental autoimmune encephalomyelitis, organ transplantation, and GVHD (20, 50–52), whether these effects are due to the regulation of Treg cells or effector cells is unclear. Further studies are required to better understand the pathophysiological roles of DNAM-1 on Tconv and Treg cells in inflammatory diseases.

Methods

Experimental GVHD Mouse Model. On day 0, recipient BALB/c mice were lethally irradiated (900 cGy), after which they were intravenously infused with TCD-BM cells (5×10^6 cells) from CD45.1$^+$ CD45.2$^+$ C57BL/6 mice with or without Treg cells (2×10^5 cells, Foxp3^{GFP+} CD25$^+$ CD4$^+$; >95% purity) isolated from the spleens of Foxp3^{GFP} CD45.2$^+$ CD45.1$^-$ WT, *Cd226*$^{-/-}$, *Tigit*$^{-/-}$, *Cd226*$^{-/-}$ *Tigit*$^{-/-}$, or *Cd226*^{Y319F} mice on a C57BL/6 background. On day 2, recipient mice were transplanted with CD4$^+$ and CD8$^+$ Tconv cells (5×10^5 cells) isolated from the spleens of CD45.2$^-$ CD45.1$^+$ WT mice on a C57BL/6 background; Tconv cells were purified by negative selection using the biotinylated mouse mAbs (CD25, B220, CD11b, CD11c, Gr-1, and CD49b) and Dynabeads MyOne Streptavidin C1 (Thermo Fisher Scientific). Purities of CD4$^+$ and CD8$^+$ Tconv cells were more than 85% as analyzed by flow cytometry.

For cotransfer experiments, lethally irradiated mice received a mixture of Foxp3^{GFP+} CD45.2$^-$ CD45.1$^+$ WT Treg cells (1×10^5 cells); Foxp3^{GFP+} CD45.2$^+$ CD45.1$^-$ *Cd226*$^{-/-}$, *Tigit*$^{-/-}$, or *Cd226*$^{-/-}$ *Tigit*$^{-/-}$ Treg cells (1×10^5 cells); and CD45.1$^+$ CD45.2$^+$ TCD-BM cells (5×10^6 cells) on a C57BL/6 background. Recipient mice received CD45.1$^+$ CD45.2$^+$ Tconv cells (5×10^5 cells) on a C57BL/6 background 2 d after cotransfer.

Additional methods are available in *SI Appendix, Supplemental Materials and Methods* and Tables S1 and S2.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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