Supporting Information

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SI Materials and Methods

Mice. C57BL/6N (B6) (H-2^b) and B6C3F1 mice (B6 × C3H/NeN F_1 mice: H-$2^{b/k}$) were purchased from Nihon Clea. C3.SW-H2^b-Sn/J (C3) (H-2^b) mice were purchased from the Jackson Laboratory. DNAX accessory molecule-1 (DNAM-1)-heterozygous mice that were generated from BALB/c embryonic stem cells were backcrossed with B6 mice for 10 generations (1), and DNAM-1 WT and DNAM-1–deficient (KO) littermates were obtained by intercrossing with heterozygous B6 mice. All mice were housed and bred under specific-pathogen-free conditions at the Animal Resource Center of the University of Tsukuba. All experiments using mice were approved by the Institutional Review Committee and performed according to the guidelines of the University of Tsukuba.

Graft-Versus-Host-Disease Model. Recipient B6C3F1 mice were sublethally irradiated with 500 cGy by X-ray (Hitachi Medical Corporation). Fifty-million splenocytes from DNAM-1 WT or KO B6 mice were i.v. infused into each recipient B6C3F1 mouse. For prophylaxis, 100 μg TX42 was injected i.p. into the recipient mice 1 d before transplantation (day −1) and then every other day for nine additional doses (total 10 times, 1.0 mg per mouse). For therapy, 300 μg TX42 was injected i.p. beginning on day 14 and then every week for nine additional doses (total 10 times, 3.0 mg per mouse), or 1.0 mg TX42 was injected i.p. once into the recipient mice on day 14.

In some experiments, B6C3F1 recipient mice were transplanted with 3.8×10^7 T cell-depleted splenocytes (TCD-SP) alone or the same number of TCD-SP plus 7.0×10^6 $CD4^+$ T cells (derived from DNAM-1 WT or KO mice) and 5.0×10^6 $CD8^+$ T cells (derived from DNAM-1 WT or KO mice) after sublethal irradiation with 500 cGy by X-ray. TCD-SP were purified from WT B6 mice by negative selection with biotinylated anti-CD4 and CD8 mAbs, followed with Dynabeads MyONE streptavidin (Invitrogen). TCD-SP contained T cells at less than 3%, as determined by flow cytometry. $CD4^+$ and $CD8^+$ T cells were purified from the spleens of DNAM-1 WT or KO B6 mice by negative selection with biotinylated anti-B220, CD11b, CD11c, Gr-1, and DX5 (CD49b) mAbs and either biotinylated CD8 or CD4 mAbs, followed with Dynabeads MyONE streptavidin. Purities of $CD4^+$ and $CD8^+$ T cells were more than 85%, as determined by flow cytometry.

To examine whether DNAM-1 on recipient cells is involved in development of graft-versus-host disease (GVHD), we generated DNAM-1 WT or KO CBF1 mice (H-$2^{b/d}$) by crossing *Cd226*$^{+/-}$ BALB/c mice with *Cd226*$^{+/-}$ B6 mice. These mice received 1×10^7 splenocytes from WT B6 mice after sublethally irradiation (600 cGy).

For a GVHD model after MHC-matched, minor antigen-mismatched bone marrow transplantation (BMT), recipient C3 mice were lethally irradiated with 900 cGy one day before BMT (day −1), and then 5×10^6 bone marrow cells and 4×10^6 spleen T cells from B6 mice were i.v. infused into each recipient mouse on day 0.

Antibodies. The anti–DNAM-1 mAb TX42 (rat IgG2a) and anti-CD155 mAb TX56 (rat IgG2a), neutralizing mAb that is able to block interaction between DNAM-1 and its ligands CD112 or CD155, were generated in our laboratory (1, 2). Intraperitoneal injection with the TX42 mAb did not deplete T cells or NK cells in mice (Fig. S2*A* and ref. 2), and TX42 mAb was detected on $CD8^+$ T cells for more than 10 d after in vivo administration (Fig. S2*B*). Polyclonal rat IgG and anti-H-$2K^k$ mAb was purchased from MP Biomedicals and BioLegend, respectively. All other mAbs used in this study were purchased from BD Biosciences.

ELISA. IFN-γ in the sera was measured by ELISA using an anti-IFN-γ mAb (clone AN-18; BD Biosciences) and biotinylated anti-IFN-γ mAb (clone R4-6A2; BD Biosciences) as capture and detection mAbs, respectively, followed by streptavidin-conjugated horseradish peroxidase (GE Healthcare Biosciences). For evaluation of IFN-γ production from $CD8^+$ T cells in vitro, 10^5 splenic $CD8^+$ T cells from naive DNAM-1 WT or KO B6 mice (resting $CD8^+$ T cells) or recipient mice that received DNAM-1 WT splenocytes (effector $CD8^+$ T cells) were stimulated with plate-bound polyclonal rat IgG or TX42 in addition to plate-bound anti-CD3 mAb for 12 h. For in vitro coculture stimulation, 5×10^4 DNAM-1 WT or KO $CD8^+$ T cells were cocultured with 5×10^5 splenocytes from syngeneic B6 or allogeneic B6C3F1 mice for 3 d.

Assessment of GVHD. Aminotransferase (ALT) and aspartate aminotransferase (AST) values in the sera were measured by using a Fuji Dri-Chem 3500V slide analyzer (Fujifilm). Histopathological diagnosis of the liver and small intestine was performed as described (3, 4). The peripheral blood cells were counted by an automated blood cell counter (Celltaca MEK-6358; Nihon Kohden).

Infiltrating Donor Lymphocytes. The liver and small intestine were isolated from the recipient mice on day 14 after transplantation. Liver was mechanically ground in RPMI-1640 medium with 5% FBS. A cell suspension from the lumen of the small intestine was prepared by using a scraper (BD Biosciences). These cell suspensions were passed through a nylon mesh to remove debris, added with mouse BD Fc Block, and stained with fluorochrome-conjugated anti-CD3, CD4, CD8, CD45.2, NK1.1, or H-$2K^k$ mAbs. Donor-derived lymphocytes were gated by $CD45.2^+$ and H-$2K^{k-}$ populations, and then each lymphocyte subset was defined as follows: $CD4^+$ T cells, $CD3^{high}CD4^+$; $CD8^+$ T cells, $CD3^{high}CD8^+$; NK cells, $CD3^-NK1.1^+$; and NKT cells, $CD3^{low}NK1.1^+$.

Proliferation Assays. For evaluation of the proliferation of effector $CD8^+$ T cells, sublethally (500 cGy) irradiated B6C3F1 mice were i.v. infused with 5×10^7 DNAM-1 WT or KO B6 splenocytes. The donor $CD8^+$ T cells, most of which were $CD44^{high}CD62L^{low}$ effector T cells (Fig. S5), were purified from the spleens of the recipient mice by use of the anti-CD8 (Ly-2) MACS beads (Miltenyi Biotec) at 14 to 35 d after splenocyte infusion and labeled with 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen). CFSE-labeled $CD8^+$ T cells (5×10^4) were stimulated with plate-bound rat IgG or TX42 in addition to plate-bound anti-CD3 mAb for 3 d. For in vitro coculture stimulation, 5×10^4 CFSE-labeled DNAM-1 WT or KO $CD8^+$ T cells were cocultured for 3 d with 5×10^5 splenocytes, 3×10^4 $CD11c^+$ dendritic cells or 4.7×10^5 CD11c-depleted splenocytes from syngeneic B6 or allogeneic B6C3F1 mice. Dendritic cells were purified from the spleen by using the anti-CD11c MACS beads (Miltenyi Biotec). CD11c-depleted splenocytes were purified by negative selection with biotinylated anti-CD11c mAbs (BD Biosciences), followed with Dynabeads MyONE streptavidin (Invitrogen). In some coculture experiments, 5×10^4 CFSE-labeled DNAM-1 WT $CD8^+$ T cells were cocultured for 3 d with 5×10^5 syngeneic B6 or allogeneic B6C3F1 splenocytes that had been treated with control Ig or TX56 after blocking Fc receptors with BD Fc Block. Stimulator cells were used after the treatment with 100 μg/mL mitomycin C

for 30 min. For in vivo proliferation assays, CFSE-labeled donor DNAM-1 WT or KO B6 splenocytes were infused into sublethally irradiated B6C3F1 mice. Splenocytes from the recipients were prepared for flow cytometry on day 3 after the infusion.

Quantitative RT-PCR. *Cd112* and *Cd155* expressions were analyzed by quantitative RT-PCR, as described (1).

Statistical Analyses. To analyze survival, we used Kaplan-Meier estimation with the statistical analysis system-type log-rank test. The correlation between DNAM-1 expression levels and ALT values was evaluated with Spearman's rank-order correlation coefficient. All other statistical analyses were performed with the two-tailed Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

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2. Tahara-Hanaoka S, et al. (2006) Tumor rejection by the poliovirus receptor family ligands of the DNAM-1 (CD226) receptor. *Blood* 107:1491–1496.
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Fig. S1. DNAM-1 expression on donor $CD8^+$ T cells is involved in exacerbation of acute GVHD. (*A–C*) After sublethal irradiation, B6C3F1 mice received splenocytes from DNAM-1 wild-type (WT) (n = 14) or knockout (KO) B6 (n = 16) mice. B6C3F1 mice that received irradiation only are also shown (n = 5). (*A*) The liver and small intestine of mice on day 21 after transplantation were stained with H&E and histologically analyzed. The organs of three mice in each group were analyzed and representative data of a mouse in each is shown. (Scale bars, 100 μm.) (*B*) ALT and AST and (*C*) IFN-γ in the sera of recipient mice was monitored before and after transplantation. Data are representative of two independent experiments. (*D* and *E*) After sublethal irradiation, B6C3F1 recipient mice received T cell-depleted splenocytes plus $CD8^+$ T cells from DNAM-1 WT or KO B6 mice and $CD4^+$ T cells from DNAM-1 WT or KO B6 mice (n = 5 in each group). B6C3F1 mice that received irradiation alone are shown (n = 3). (*D*) ALT and (*E*) IFN-γ in the sera of recipients was measured on day 14 after transplantation. The experiments were performed twice, with the similar results. One experiment is shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$. Error bars show SD.

Fig. S2. Characterization of TX42 mAb in vivo. (*A*) Peripheral blood mononuclear cells (PBMC) from B6C3F1 mice that were i.p. injected with 100 μg of control rat IgG2a (*Upper*) or anti–DNAM-1 mAb, TX42 (rat IgG2a), (*Lower*) were collected 3 d after the injection, and stained with biotin-conjugated TX42 or isotype-matched control antibody followed by FITC-conjugated streptavidin. The PBMC were also stained with FITC-conjugated anti-rat IgG2a or anti-CD8 mAbs. Cells were analyzed by flow cytometry. Although $TX42^+$ population was not detected by in vitro staining with TX42 in PBMC from the mice on day 3 after TX42 injection, the DNAM-1^+ cell population was detected in mice injected with control rat IgG, suggesting that the pure TX42 mAb continued to bind to DNAM-1 on the cell surface in vivo for more than 3 d. The populations of $CD8^+$ T cells were comparable between mice that were injected with control IgG and TX42, indicating that TX42 did not deplete $CD8^+$ T cells. (*B*) PBMC from B6C3F1 mice that were injected i.p. with 100 μg TX42 were collected at the time indicated after the injection and stained with biotin-conjugated TX42, followed by FITC-conjugated streptavidin. $TX42^+$ population still was not detected on day 12 after TX42 injection, but completely recovered on day 20, indicating that TX42 binding to DNAM-1 on the cell surface continues for more than 12 d after TX42 injection. Closed and opened histograms show isotype control and the indicated mAb staining, respectively. Data are representative of three independent experiments with similar results (*A* and *B*). (*C*) After sublethal irradiation, B6C3F1 mice that received DNAM-1 WT splenocytes were i.p. injected with anti–DNAM-1 (TX42) or control antibody every other day from day −1 until day 17 after transplantation. Mean fluorescence intensity (MFI) of DNAM-1 expression on donor $CD4^+$ and $CD8^+$ T cells in the peripheral blood of recipient mice and that on T cells in naive mice were analyzed by flow cytometry. Data are representative of three independent experiments with similar results. *$P < 0.05$. Error bars show SD.

Fig. S3. Anti–DNAM-1 mAb suppressed the development of acute GVHD. After sublethal irradiation, B6C3F1 mice received splenocytes from WT B6 mice. The recipient mice were i.p. injected with anti–DNAM-1 (TX42) ($n = 14$) or control antibodies ($n = 16$) every other day from day −1 until day 17 after transplantation. B6C3F1 mice that received irradiation only are also shown ($n = 10$). (*A*) ALT and AST and (*B*) IFN-γ in the sera of recipient mice were monitored before and after transplantation. Data are pooled from three independent experiments. *$P < 0.05$; ***$P < 0.005$, control Ig vs. TX42. Error bars show SD.

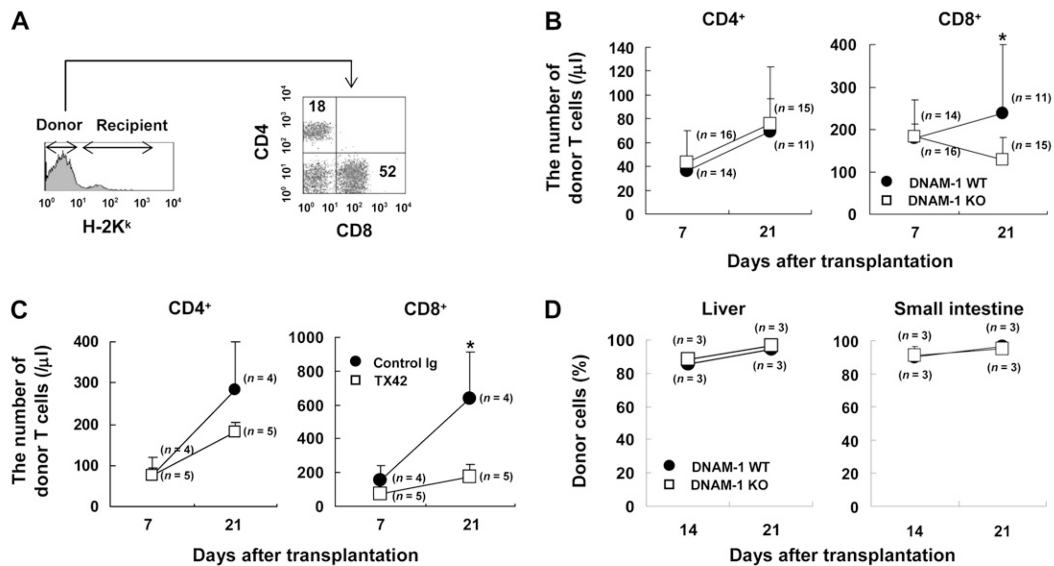

Fig. S4. DNAM-1 is involved in donor $CD8^+$ T-cell proliferation in recipient mice. (*A–D*) After sublethal irradiation, B6C3F1 mice received splenocytes from WT or KO B6 mice. (*C*) Some of the mice that received WT splenocytes were i.p. injected with anti–DNAM-1 (TX42) or control antibodies every other day from day −1 until day 17 after transplantation. (*A*) Peripheral blood cells from the recipient mice were simultaneously stained with FITC-conjugated anti-H-$2K^k$, PE-Cy5-conjugated anti-CD4, and PE-conjugated anti-CD8 mAbs, and analyzed by flow cytometry. Representative profile of flow cytometry data on day 7 after transplantation is shown. Data are representative of more than five independent experiments. (*B* and *C*) The absolute number of donor $CD4^+$ and $CD8^+$ T cells in recipient peripheral blood was calculated as the number of white blood cells multiplied by the percentage of total donor T cells determined by flow cytometric analysis. Data in *B* and *C* are representative of two and three independent experiments, respectively. (*D*) On days 14 and 21 after transplantation, the infiltrating donor cells in the liver and small intestine of recipient mice were separated, stained with PerCP-conjugated anti-CD45.2 and FITC-conjugated anti-H-$2K^k$, and analyzed by flow cytometry. Percentages of donor cells (H-$2K^{k-}$ gated by $CD45.2^+$) are shown. Experiments were performed twice, with similar results. One experiment is shown. *$P < 0.05$, DNAM-1 WT vs. KO mice (*B*) or control Ig vs. TX42 (*C*). Error bars show SD.

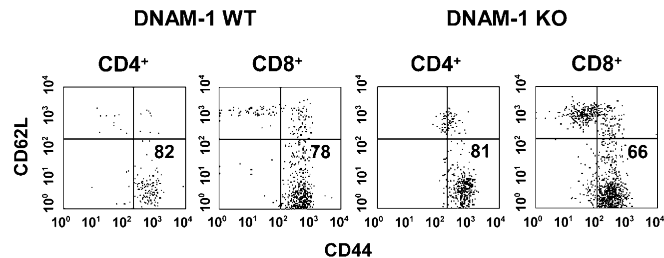

Fig. S5. Differentiation of donor T cells after transplantation. After sublethal irradiation, B6C3F1 mice received splenocytes from DNAM-1 WT or KO B6 mice, as shown in Fig. 1*A*. On day 21 after transplantation, PBMC were collected and stained with FITC-conjugated anti-H-$2K^k$, APC-conjugated anti-CD44, PE-conjugated anti-CD62L, and either PE-Cy5-conjugated anti-CD4 or anti-CD8 mAbs and analyzed by flow cytometry. The profiles on H-$2K^{k-}CD4^+$ or H-$2K^{k-}CD8^+$ gates are shown. Experiments were performed twice independently ($n = 4$ in each experiment), with similar results. Data are representative from four mice of one experiment.

Fig. S6. DNAM-1 mediates a costimulatory signal in $CD8^+$ T cells. (*A* and *B*) CFSE-labeled splenocytes from DNAM-1 WT (n = 7) or KO (n = 7) B6 mice were transplanted into sublethally irradiated B6C3F1 mice (*A*). Recipient B6C3F1 mice were i.p. injected with anti–DNAM-1 (TX42) (n = 3) or control antibodies (n = 3) on day −1 and day 1 after transplantation of CFSE-labeled splenocytes from WT B6 mice (*B*). CFSE-labeled splenocytes from B6 mice were infused into sublethally irradiated syngeneic mice as a control (n = 3). Proliferation of donor $CD8^+$ T cells was analyzed by flow cytometry on day 3 after transplantation. (*C–E*, *H*) Resting $CD8^+$ T cells purified from naïve DNAM-1 WT and KO B6 mice (*C* and *D*), and donor effector $CD8^+$ T cells purified from B6C3F1 mice that received WT B6 splenocytes (*E*) were labeled with CFSE, stimulated or not with plate-coated anti-CD3 mAb plus anti–DNAM-1 mAb (TX42) or control antibodies for 3 d, and analyzed by flow cytometry (*C–E*), and by ELISA for IFN-γ level in the culture supernatants (*H*). Resting $CD8^+$ T cells purified from naïve DNAM-1 WT or KO B6 mice were labeled with CFSE, cocultured with mitomycin C-treated syngeneic (B6) or allogeneic (B6C3F1) splenocytes, dendritic cells ($CD11c^+$), CD11-depleted splenocytes ($CD11c^−$) (*F*), or a neutralizing mAb against CD155 (TX56)- or control antibody-treated splenocytes (*G*) for 3 d, and analyzed by flow cytometry, and by ELISA for IFN-γ level in the culture supernatants (*I*). Data are representative from two or three independent experiments with similar results (*A–I*). *$P < 0.05$. Error bars show SD.

Fig. S7. Expression of the DNAM-1 ligands. Each organ from B6C3F1 mice was subjected to quantitative RT-PCR for *Cd155* and *Cd112.* Data are means of triplicates. The experiments were performed independently twice, with similar results. Error bars show SD.

Fig. S8. Anti–DNAM-1 mAb ameliorated overt acute GVHD. B6C3F1 mice received splenocytes from WT B6 mice after sublethal irradiation. The recipient mice were injected i.p. with anti–DNAM-1 (TX42) (n = 11) or control antibodies (n = 11) every week from day 14 until day 77 after transplantation. B6C3F1 mice that received irradiation only are also shown (n = 8). (*A* and *B*) ALT, AST, and IFN-γ levels in the sera of recipient mice were monitored before and after transplantation. (*C*) MFI of DNAM-1 expression on donor $CD8^+$ T cells in the peripheral blood of the recipient mice was analyzed by flow cytometry. MFI of DNAM-1 expression on $CD8^+$ T cells from naive B6 mice is also shown. (*D*) The correlation between DNAM-1 expression on donor $CD8^+$ T cells on day 21 and ALT values on day 28 in recipient mice injected with control Ig or TX42 was statistically evaluated. (*E*) PBMC from the recipient mice were simultaneously stained with FITC-conjugated anti-H-$2K^k$, PE-Cy5-conjugated anti-CD4, and PE-conjugated anti-CD8 mAbs, and analyzed by flow cytometry. The absolute number of $CD4^+$ and $CD8^+$ T cells was calculated, as described in Fig. S4 *B* and *C*. Data are representative of two independent experiments (*B*–*E*). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$, control Ig vs. TX42. Error bars show SD.