allogeneic organ transplantation.

Genetic modification of DCs with genes encoding immunoregulatory molecules provides a potential approach for Ag-specific regulation of T-cell-mediated immunity by selectively targeting Ag-restricted T cells. The use of these genetically modified DCs was reportedly effective for the prevention of experimental autoimmune and allergic diseases as well as allogeneic organ transplantation in animals, possibly mediated through the downregulation of the activation of Ag-specific T cells[14-17].

Allogeneic bone marrow (BM) transplantation (BMT) is an effective treatment for hematologic malignancies as well as genetic disorders[18-21]. However, acute GVHD, which is caused by alloreactive T cells in donor BM inocula, is a major cause of morbidity and mortality in patients undergoing allogeneic BMT[18-21]. Although the incidence and the severity of acute GVHD can be dramatically improved by T-cell depletion or the combination of immunosuppressive agents, the risk of leukemia relapse may be increased in turn, possibly due to the lack of antileukemia effect of allogeneic T cells infused, the so-called graft-versus-leukemia (GVL) effect[19-21]. Therefore, there is an increasing interest in the development of strategies that suppress acute GVHD but exert a GVL effect.

Here, we report that adenoviral gene transfer of TRAIL involving DCs is effective for the protection of the recipients from the lethality caused by acute GVHD and leukemia relapse mediated through the deletion of pathogenic alloreactive T cells and leukemia cells.

RESULTS

Regulatory function of human DCs genetically modified to express TRAIL

To clarify the regulatory role of TRAIL in T-cell activation, we generated adenovirus vector encoding the human TRAIL gene (hTRAIL-Ad). Introduction of hTRAIL-Ad into 293 cells resulted in specific and functional expression of TRAIL (**Fig. 1a-b**).

To test the potential use of TRAIL for selectively targeting Ag-specific T cells, we examined the conditions for the generation of human DCs genetically engineered to express TRAIL. Although stimulation of immature DCs (iDCs) with interferon (IFN)-γ or lipopolysaccharide (LPS) induced slight expression of TRAIL[22, 23], adenoviral gene transduction of TRAIL into DCs resulted in more potent expression of TRAIL. Of note, introduction of hTRAIL-Ad into iDCs followed by stimulation with LPS resulted in the generation of mature DCs (mDCs) with higher levels of dose-dependent expression of TRAIL compared with adenoviral infection of the hTRAIL gene into iDCs or mDCs (**Fig. 1c-e**).

To determine the functional expression of hTRAIL in DCs genetically modified to express TRAIL (designated as hTRAIL-Ad/DCs), we examined the cytotoxicity of these cells against various cell types. As shown in Figure 2A, adenoviral infection had little or no effect on the expression of MHC and costimulatory molecules (**Fig. 2a**). hTRAIL-Ad/DCs showed more potent killing activity against Jurkat cells known to be hTRAIL-sensitive target cells than mDCs, control Ad-transduced mDCs (control Ad/mDCs) and soluble TRAIL. In addition, their cytotoxicity was blocked by anti-hTRAIL mAb (**Fig. 2b**), indicating that hTRAIL is functionally expressed on genetically modified DCs. In contrast, concanavalin A (Con A)-blasts (**Fig. 2b**) and DCs (data not shown) were relatively resistant to TRAIL-mediated cytotoxicity.

To determine the mechanism responsible for the difference in the sensitivity against TRAIL-mediated cytotoxicity, we examined the expression levels of the receptors for TRAIL in various cell types (**Fig. 2c**). iDCs constitutively expressed DR4, DR5, RcR1 and DcR2 at similar levels, and the expression of these receptors did not changed following maturation. Unlike Jurkat cells, which predominantly expressed DR5, little or

no expression of these receptors was observed on unstimulated $CD4^+$T cells and Con A-blasts. Interestingly, cognate interaction of $CD4^+$T cells with allogeneic DCs induced specific upregulation of DR5 (**Fig. 2c**) whereas the stimulation with mAbs to CD3 and CD28 upregulated its expression to a lesser degree (data not shown). These results indicate that the sensitivity to TRAIL is associated with the expression levels of the receptors for TRAIL.

We also examined the T-cell regulatory function of hTRAIL-Ad/DCs. Soluble TRAIL showed a minimal inhibition of activation of $CD4^+$T cells when the cells were cultured with mAbs to CD3 plus CD28 or allogeneic mDCs (**Fig. 2d**). In contrast, hTRAIL-Ad/DCs, but not mDCs or control-Ad/mDCs, displayed a potent suppressive effect on activation of alloreactive $CD4^+$T cells (**Fig. 2d-e**) although all of these effector cell types showed similar expression of MHC and costimulatory molecules (**Fig. 2a**). In addition, this suppression was abrogated by anti-hTRAIL monoclonal antibody (mAb) and anti-hDR5 mAb (**Fig. 2d**). These results indicate that DCs genetically modified to express TRAIL inhibit the activation of $CD4^+$T cells through the interaction of TRAIL-DR5.

Previous studies have shown that soluble TRAIL induces no apoptosis but inhibits activation of T cells through blockeage of cell cycle progression in humans and animals [6, 7]. To clarify the mechanism underlying T-cell regulatory function of DCs genetically modified to express TRAIL, we characterized the $CD4^+$T cells primed with hTRAIL-Ad/DCs. The proportion of dividing cells was significantly reduced in allogeneic $CD4^+$T cells primed with hTRAIL-Ad/DCs when compared with those primed with mDCs and control-Ad/mDCs (**Fig. 2F**). On the other hand, hTRAIL-Ad/DCs induced more potent apoptosis in allogeneic $CD4^+$T cells than mDCs and control-Ad/mDCs (**Fig. 2G**). Similarly, numerous apoptotic cells were detected in allogeneic $CD4^+$T cells primed with hTRAIL-Ad/DCs, whereas the productive cells (S phase and G2+M phase) were increased in allogeneic $CD4^+$T cells primed with mDCs and control-Ad/mDCs compared with unprimed $CD4^+$T cells (**Fig. 2h**). These results indicate that DCs genetically modified to express TRAIL induce apoptosis rather than

cell cycle arrest.

Expression of TRAIL in adenoviral gene transferred mice

To determine the regulatory role of TRAIL on immunnopathogenesis, we generated adenovirus vector encoding the murine TRAIL gene (mTRAIL-Ad). Infection of 293 cells with mTRAIL-Ad induced cytotoxicity against L929 cells expressing DR5 (**Fig. 3a-b**), indicating that the adenoviral gene transfer of mTRAIL could induce its functional expression of mTRAIL in target cells.

We next examined the effect of adenoviral infection on the numbers of spleen leukocytes and their constitution (**Fig. 3d-e**). A injection of control-Ad into mice increased the number of leukocytes and the rate of T cells compared to normal mice, indicating that these changes involve the immune response against adenoviral infection. On the other hand, the mice injected with mTRAIL-Ad showed a reduced number of leukocytes and constituency of T cells compared to mice injected with control-Ad. In addition, the introduced TRAIL was specifically detected in $F4/80^+$cells (tissue macrophages) and $CD11c^+$cells (resident DCs). These results indicate that adenoviral transduction of the TRAIL gene suppresses the immune response against adenovirus, and transduced TRAIL was mainly expressed in both macrophages and DCs.

Gene transfer of mTRAIL prevents murine acute GVHD

Previous studies have shown the dominant role of the Fas/Fas ligand (FasL) pathway in acute GVHD, whereas TRAIL has a minimal contribution to this pathogenesis[19, 20]. We therefore tested the effect of in vivo gene therapy with adenovirus vector encoding TRAIL on acute GVHD in murine allogeneic BMT (**Fig. 4a**). All recipients of allogeneic BMT died on day 8 following transplantation, and a single injection of control-Ad into mice before allogeneic BMT had no effect on the incidence and the severity of acute GVHD. In contrast, a single injection of mTRAIL-Ad protected mice from acute GVHD-induced lethality in a dose-dependent manner. However, a single injection of mTRAIL-Ad after allogeneic transplantation failed to cause a therapeutic

effect.

To clarify the mechanism underlying the preventive effect of adenoviral transduction of the mTRAIL gene on acute GVHD, we analyzed I-K^{b+}donor-derived T cells in transplanted mice. The number of spleen MNC was lower in the recipients injected with mTRAIL-Ad before transplantation (mTRAIL-Ad-treated recipients) than in untreated recipients and in recipients injected with control-Ad before transplantation (control-Ad -treated recipients) (**Fig. 4b**). In addition, I-$K^{b+}CD3^+$, I-$K^{b+}CD4^+$ and I-$K^{b+}CD8^+$ subpopulations were decreased in mTRAIL-Ad-treated recipients as compared with their counterparts (**Fig. 4c**).

We also examined the allogeneic response of I-$K^{b+}CD4^+$T cells to recipient-type DCs. I-$K^{b+}CD4^+$T cells obtained from mTRAIL-Ad-treated recipients showed a reduced response to DCs (H-2^d) as compared with those obtained from untreated recipients and control-Ad-treated recipients (**Fig. 4d**).

To examine the CTL activity of I-$K^{b+}CD8^+$T cells to recipient tissues (H-2^d), we examined the lytic activity of I-$K^{b+}CD8^+$T cells obtained from the recipients against P815 cells (H-2^d). I-$K^{b+}CD8^+$T cells obtained from mTRAIL-Ad-treated recipients showed a lower lytic activity against recipient-type-targeted cells than those obtained from untreated recipients and control-Ad-treated recipients (**Fig. 4e**).

We further evaluated the levels of proinflammatory cytokines in serum of the recipients of allogeneic transplantation (**Fig. 4f**). The levels of IFN-γ, tumor necrosis factor (TNF)-α and IL-12p40 in serum obtained from mTRAIL-Ad-treated recipients were lower than those in serum from untreated recipients and control-Ad-treated recipients. These results indicate that the protective effect of in vivo gene transfer of TRAIL on acute GVHD-induced lethality involves the suppression of donor-derived T cells.

Cell gene therapy with DCs genetically modified to express mTRAIL against murine acute GVHD

The above studies indicate that DCs genetically modified to express hTRAIL exhibited

a more potent regulatory effect on Ag-specific $CD4^+$T cells than soluble TRAIL through the induction of apoptosis. We therefore tested the in vivo regulatory function of DCs genetically modified to express mTRAIL (mTRAIL-Ad/DCs). Similar to hTRAIL-Ad/mDCs, mTRAIL-Ad/DCs showed the functional expression of mTRAIL (**Fig. 5a-b**). In addition, mTRAIL-Ad/DCs impaired the allogeneic activation of $CD4^+$T cells (**Fig. 5c**), and they induced a higher rate of apoptosis in allogeneic $CD4^+$T cells than their counterparts (data not shown).

We next tested the therapeutic efficacy of cell gene therapy with DCs genetically modified to express mTRAIL against acute GVHD (**Fig. 5d**). A single injection of recipient-type DCs or control-Ad/DCs ($5x10^6$/mouse) 2 days following transplantation enhanced the lethality caused by acute GVHD. In contrast, the therapeutic effect of recipient-type mTRAIL-Ad/DCs on acute GVHD was observed in a dose-dependent fashion ($1x10^6$-$5x10^6$/mouse) when the recipients received a single injection of mTRAIL-Ad/DCs on 2 days after transplantation. In addition, in-vivo blockade experiments showed that treatment with anti-mTRAIL mAb significantly abrogated the therapeutic effect of mTRAIL-Ad/DCs (**Fig. 5e**).

To examine the in vivo regulatory effect of DCs genetically modified to express mTRAIL, the allogeneic responses of donor-derived T cells were analyzed. The recipients treated with recipient-type DCs showed an increased number of leukocytes and proportions of T cells as compared with untreated recipients. In addition, they showed not only enhanced responses of donor-derived T cells against recipient-type cells but also increased production of proinflammatory cytokines (**Fig. 5f-j**). In contrast, the recipients treated with recipient-type mTRAIL-Ad/DCs showed not only a reduced proportion of donor-derived T-cell subsets but also impairment of their responses against recipient-type cells as compared with untreated recipients (**Fig. 5f-j**). Furthermore, the suppressive effect of mTRAIL-Ad/DCs was more potent than the effect of in vivo gene transfer of TRAIL. These results indicate that cell gene therapy with mTRAIL-Ad/DCs is effective for the protection against acute GVHD-induced lethality mediated through the potent suppression of donor-derived alloreactive T cells.

Gene transfer of mTRAIL protects allogeneic transplanted recipients from leukemia relapse

TRAIL reportedly plays a crucial role in the expression of the GVL effect of donor-derived $CD8^+$T cells [20]. In accordance with previous report[20], membrane-bound murine TRAIL expressed on the adenovirally transduced 293 cells showed the cytotoxicity against DR5-expressing P815 leukemic cells (**Figs. 3b, 6a**). To test the potential use of TRAIL for the treatment of leukemia relapse, we examined the effect of in vivo gene therapy with mTRAIL-Ad on tumor burden in leukemia-bearing allogeneic transplanted mice (**Fig. 6b**). In accord with previous reports[20, 21], mice transplanted with allogeneic BM survived over 60 days after transplantation without apparent acute GVHD. All leukemia-bearing mice that received total body irradiation (TBI) died with 13 days with marked hepatosplenomegaly. On the other hand, leukemia-bearing recipients of allogeneic BM cells died within 26 days after transplantation, indicating that allogeneic BM containing $CD8^+$T cells exhibited an insufficient GVL effect. In contrast, treatment with mTRAIL-Ad, but not control-Ad, before allogeneic transplantation prolonged the survival of leukemia-bearing transplanted mice.

We also examined the therapeutic effect of cell gene therapy with DCs genetically modified to express mTRAIL against leukemia relapse. mTRAIL-Ad/DCs showed higher cytotoxicity against P815 cells than 293 cells expressing transduced TRAIL (**Fig. 6a**). A single injection of recipient-type mTRAIL-Ad/DCs, but not DCs or control-Ad/DCs, 2 days after transplantation prolonged the survival of leukemia-bearing transplanted mice (**Fig. 6b**). These results indicate that the adenovirally transduced TRAIL in the transplanted recipients is effective for the suppression of leukemia relapse after hematopoietic cell transplantation.

DISCUSSION

Gene transfer of immunoregulatory molecules provides an attractive immunotherapeutic approach for the treatment of certain diseases. Our findings demonstrare the efficacy of adenoviral-mediated gene transfer of TRAIL for the therapy of lethal acute GVHD and leukemia relapse via elimination of the pathogenic T cells and leukemia cells.

Efficient gene introduction into target cells is crucial for the regulating their function. We showed that the gene transfer of TRAIL into iDCs followed by inflammatory stimulation caused maximal expression of TRAIL. The precise molecular mechanism by which inflammatory stimuli upregulated the transcription of the transduced TRAIL gene in DCs remains unknown, but inflammatory stimuli might activate certain transcriptional factor(s) leading to the transactivation of the transcriptional expression of the transduced TRAIL gene, because inflammatory stimuli could induce the expression of endogenous TRAIL. On the other hand, mDCs show more potent interaction with Ag-specific T cells than iDCs due to their higher expression levels of adhesion/costimulatory molecules. Thus, the generation of mDCs genetically engineered to express TRAIL provides advantages for TRAIL expression as well as Ag-specific interaction with T cells.

The slight suppressive effect of the TRAIL protein on T-cell activation involves the blockage of cell cycle progression rather than the induction of apoptosis (ref. 6, 7) whereas DCs genetically modified to express TRAIL could effectively induce apoptosis in targeted T cells. The difference in the biological function between soluble TRAIL and membrane-bound TRAIL might reflect the strength of the intracellular signaling delivery via the receptors for TRAIL. On the other hand, DCs genetically modified to express TRAIL showed more potent cytotoxicity against Ag-specific T cells than non-specifically activated T cells. Interestingly, Ag-specific T cells primed with DCs specifically upregulated DR5, possibly mediated through the antigenic signaling. Thus, DCs genetically modified to express TRAIL are useful for the Ag-specific regulation of activated T cells.

Gene transfer of TRAIL into normal mice suppressed the adenoviral infection-

associated immune responses. In addition, the transduced TRAIL was specifically detected in tissue macrophages and resident DCs. Previous studies have shown that DCs are more resistant to ligand-induced apoptosis than macrophages [24, 25]. Thus, adenovirus-associated gene product-presenting, transduced TRAIL-expressing resident APCs, especially DCs, are involved in the suppression of adenovirus-restricted T cells in an Ag-specific manner in vivo.

Host resident APCs, especially DCs, reportedly play a crucial role in the initiation and the progression of acute GVHD in allogeneic BMT, and their inactivation lead to the suppression of acute GVHD[18, 21]. We showed that gene transfer of mTRAIL into mice before allogeneic transplantation prevented acute GVHD. The analysis of the protected recipients of allogeneic BMT revealed that they showed a reduction of the number of donor-derived alloreactive peripheral T cells. In addition, donor-derived T cells expressed DR5 as well as TRAIL on the cell surface (data not shown). Therefore, gene-transduced recipient APCs expressing TRAIL act as 'killer APCs' to induce the peripheral elimination of donor-derived alloreactive T cells in a TRAIL/DR5 pathway. On the other hand, donor-derived T cells obtained from the protected recipients also showed the reduced allogeneic responses. Our results imply that the intracellular signaling events involving TRAIL/DR5 induce not only apoptosis but also functional deficiency in the targeted T cells

The introduction of the mTRAIL gene into the recipients of allogeneic transplantation failed to cause a therapeutic effect. We also showed the maximal expression of the transduced TRAIL in DCs was reached 4 days after the infection in vitro. Therefore, the lack of a therapeutic effect of adenoviral transduction of TRAIL might be explained by insufficient expression of the transduced TRAIL in the recipient APCs.

Although the use of DCs genetically modified to express immunoregulatory molecules is thought to be usefulfor the selective regulation of pathogenic T cells, the precise difference in the efficiency between in vivo gene therapy and ex vivo gene therapy with DCs remains unclear. We showed that cell gene therapy with DCs

genetically modified to express TRAIL were more effective for the protection of the transplanted recipient mice from acute GVHD-induced lethality than in vivo gene transfer of TRAIL. In addition, DCs genetically modified to express mTRAIL caused more potent peripheral elimination of donor-derived alloreactive T cells as well as their functional impairment than gene transfer of mTRAIL. The results led us to hypothesize that the selective interaction of alloreactive T cells with the infused DCs genetically modified to express mTRAIL caused further enhancement of the susceptibility of alloreactive T cells to undergoing apoptosis via upregulation of DR5, and that resulting in their potent peripheral elimination in vivo. Thus, this immunoregulation may account for the efficiency of ex vivo gene therapy with genetically modified DCs against acute GVHD in allogeneic BMT.

The strategy to regulate TRAIL-mediated GVL activity is thought to decrease the relapse rates of malignancies after hematopoietic cell transplantation[20]. We showed that the protective effect of cell gene therapy with the transduced TRAIL-expressing DCs against leukemia relapse was more potent than in vivo gene therapy with mTRAIL-Ad, and that this difference may have reflected the ability of the targeted cells (resident recipient cells versus recipient-type DCs) to cause the cytotoxicity against leukemic cells. Thus, our results suggest that gene transfer of mTRAIL in combination with selective depletion of the donor T-cell population in the marrow graft is effective for the suppression of leukemia relapse in the allogeneic BMT.

Collectively, our findings demonstrate that the transduced TRAIL expressed on recipient APCs, especially DCs, is effective for not only the peripheral deletion of Ag-restricted activated T cells in acute GVHD but also the elimination of leukemia cells in leukemia relapse. Further development of TRAIL-mediated regulation of immunity might provide clinical benefits for the treatment of immunopathogenic diseases.

METHODS

Media and Reagents. The medium used throughout was RPMI 1640 (Sigma, St. Louis, MO) or DMEM (Sigma) supplemented with antibiotic-antimycotic (GIBCO BRL, Rockville, MD) and 10% heat inactivated FCS (GIBCO). Granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-2, IL-4, IFN-γ and TRAIL were purchased from Pepro Tech. (London, England).

Cell preparations. Human iDCs were obtained by culturing peripheral blood monocytes with GM-CSF (50 ng/ml) and IL-4 (50 ng/ml) for 7 days[26]. For the preparation of mDCs, cells were subsequently cultured with LPS (1 μg/ml, Sigma, St. Louis, MO) for another 4 days[26]. Murine iDCs were prepared by culturing BM cells obtained from female BALB/c mice (H-2^d; Charles River Laboratories, Raleigh, NC) with murine GM-CSF (20 ng/ml) for 8 days, and mDCs were obtained from culture of iDCs treated with LPS (1 μg/ml) for 4 days[21]. Human T cells were purified from peripheral blood mononuclear cells (PBMC) with a T cell negative isolation kit (Dynal, Oslo, Norway), and $CD4^+$T cells were then negatively selected from T cells with anti-CD8 mAb (BD Bioscience, San Diego, CA) plus goat anti-mouse IgG mAb-conjugated immunomagnetic beads (Dynal)[26]. Murine T cells were negatively selected from spleen MNC obtained from C57/BL6 mice (H-2^b; Charles River Laboratories) with mAbs to Ly-76, B220, Ly-6G and I-A/I-E (all from BD Bioscience) plus sheep anti-rat IgG mAb-conjugated immunomagnetic beads (Dynal) [21]. Subsequently, $CD4^+$T cells were then negatively selected from T cells with anti-CD8 mAb (BD Bioscience) in combination with sheep anti-rat IgG mAb-conjugated immunomagnetic beads [21]. Con A-blasts were obtained from the culture of human or murine T cells treated with Con A (2.5 μg/ml; Sigma) for 3 days.

Production of adenovirus encoding the TRAIL genes. The full-length hTRAIL cDNA (884 bp) was prepared by RT-PCR amplification of total RNA from Con A-blasts with the following oligonucleotide primers: 5'- CAG CAG TCA GAC TCT

GAC AG-3' and 5'- TCT TTC CAG GTC AGT TAG CC-3". The PCR product was subcloned into pCR2.1 vector using TA Cloning Kit (Invitrogen Corp., Carlsbad, CA) and the nucleotide sequence was confirmed by using a 373A automated sequencer (Applied Biosystems , Foster City, CA) and the fluoresceinated dye terminator cycle sequencing method. The full-length mTRAIL cDNA was prepared from mTRAIL/pMKITNeo expression vector[27]. After *Xho*I and *Not*I digestion, the 850-bp of mTRAIL cDNA was obtained, and the nucleotide sequence was confirmed as described above.

A replication-deficient adenovirus vector expressed from the CAG promotor was generated using an Adenovirus Expression Vector Kit (TaKaRa Shuzo, Kyoto, Japan), in which an adenoviral cosmid, pAxCAwt, was included. The cosmid pAxCAwt consisted of E1- and E3-deficient Ad type 5 (Ad5) sequences, and the CAG promoter and rabbit-globin poly A were inserted at the former E1 site in reverse orientation with respect to the Ad5 sequences. Briefly, the entire coding sequence of hTRAIL or mTRAIL was blunted using a DNA Blunting Kit (TaKaRa Shuzo), and was then subcloned into the *Swa I* site of pAxCAwt. The resulting cosmids were named hTRAIL/pAxCAwt or mTRAIL/pAxCAwt, respectively. Transfection of human embryonic kidney 293 cells (RIKEN Cell Bank, Tsukuba, Japan) with these cosmid vectors and Ad backbone sequences (DNA-TPC) that had the E1and E3 genes deleted was performed according to the manufacturer's instructions, to produce replication-incompetent, E1- and E3-deficient Ad expressing hTRAIL or mTRAIL (hTRAIL-Ad or mTRAIL-Ad). The viruses were then prepared by expansion of a single clone generated in 293 cells, which were purified by limiting dilution, and viral particles were isolated and amplified for analysis of hTRAIL or mTRAIL expression by flow cytomerty. Recombinant adenoviruses generated from the homologous recombination of pAxCAwt and DNA-TPC were used as virus controls (control-Ad). Recombinant adenovirus titers were determined by plaque assays on 293 cells. These adenoviruses were suspended in culture medium, adjusted to $2x10^8$ plaque forming units (PFU)/ml and stored at $-80^{\circ}C$ until use.

Adenoviral infection. 293 cells were cultured in culture medium, and permitted to adhere to 24-well plates (Becton Dickinson, Lincoln Park, NJ) for at least 6 h before adding adenoviral vector. Before infection, cells were washed with PBS, and then the vectors were added at multiplicity of infection (MOI) of 10/100 μl/well. After 1 h-incubation at 37 °C, the culture medium (400 μl/well) was added to the cells, and then the cultures were incubated for 24 h at 37 °C. For Ad-mediated gene transfer into human DCs by centrifugal transduction [28], 500 μl of cells (10^6 cells) were mixed with 500 μl of adenoviral vector (MOI of 10 or 50), and 1 ml of the mixture was poured into a polypropylene tube (Becton Dickinson). The tubes were centrifuged at 2000 *g* at 37 °C for 2 h. After the centrifugal transduction, the cell and virus mixture was washed twice in PBS. DCs were resuspended in culture medium under various culture conditions and cultured for the indicated periods in tissue culture dishes (Becton Dickinson). For adenoviral infection of murine DCs, 500 μl of iDCs (10^6 cells) were mixed with 500 μl of adenoviral vector (MOI of 50) in a polypropylene tube, and 1 ml of the mixture was incubated at 37 °C. After a 2-h incubation, culture medium was added to the cells, and then the cultures were incubated with LPS (1 μg/ml) for 4 days in tissue culture dishes.

Flow cytometry. Cells were stained with following mAbs to human and murine markers: CD3, CD4, CD11c, CD40, CD80, CD86, HLA-A/B/C, HLA-DR, B220, I-K^d, I-A/I-E, FITC-conjugated goat anti-mouse IgG Ab (control Ig), FITC-conjugated avidin and isotype-matched control IgG (all from BD Bioscience); CD83 (Coulter Immunology, Hialeah, FL); F4/80 (BMA Biomedicals AG, Rheinstrasse, Switzerland); FITC-conjugated goat anti-rat IgG Ab (Santa Cruz Biotech, Santa Cruz, CA). The purified mAbs to hTRAIL (RIK-2), mTRAIL (N2B2), human DR4 (hDR4; DJR1), human DR5 (hDR5; DJR2), human DcR1 (hDcR1; DJR3), human DcR2 (hDcR2; DJR4) and murine DR5 (mDR5; MD5-1) were prepared as described previously[5, 27, 29, 30]. Fluorescent staining was analyzed with a FACScan flow cytometer and CELLQuest Software (Becton Dickenson, Mountain View, CA), and data are expressed as mean

fluorescence intensity (MFI).

Cytotoxicity assay. Adenoviral gene-transduced cells or untransduced cells were cultured with $Na_2{}^{51}CrO_4$ (NEN™ Life Science Products, Boston, MA)-labeled Jurkat cells, L929 cells , P815 cells (10^4, all from RIKEN Cell Bank) or Con A-blasts (10^4) for 4 h at various effector cell-to-target cell ratios (E/T ratios) in the presence or absence of 10 μg/ml of anti-hTRAIL mAb, anti-mTRAIL mAb or control IgG (control Ig). As a control, soluble hTRAIL was added to the target cells at the indicated concentrations (10-1000 ng/ml). The radioactivity of the supernatants was measured, and the % specific lysis was calculated[21, 26]. The value of spontaneous-release cpm was less than 10 % of the total-release cpm.

Cell proliferation assay. Human $CD4^+$T cells (10^5) were stimulated with or without platebound anti-human CD3 mAb (BD Bioscience) plus soluble anti-human CD28 mAb (BD Bioscience) in the presence or absence of soluble hTRAIL (1000 ng/ml), anti-hTRAIL mAb or control Ig (each 10 μg/ml). For human or murine allogeneic MLR, $CD4^+$T cells (10^5) were cultured in 96-well plates (Becton Dickinson) with various numbers of irradiated (15 Gy from a ^{137}Cs source, MBR-1505R2, Hitachi Medical, Tokyo, Japan) adenoviral gene-transduced or untrnsduced allogeneic DCs in the presence or absence of soluble hTRAIL (1000 ng/ml), anti-hTRAIL mAb (10 μg/ml), anti-mTRAIL mAb (10 μg/ml), anti-hDR5 mAb (10 μg/ml) or control Ig (10 μg/ml). Thymidine incorporation was measured on day 5 after an 18-h pulse with [^{3}H]thymidine[21, 26].

In vitro analysis of T cell responses. For cell division measurement, $CD4^+$T cells were labeled with carboxyfluorescein diacetate-succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) according to the manufacturer's instructions. CFSE-labeled $CD4^+$T cells ($5x10^6$) were primed with irradiated (15 Gy) allogeneic DCs ($5x10^5$) for 3 days, and these T-cell subsets were negatively selected with anti-human CD11c mAb (BD

Bioscience) plus goat anti-mouse IgG mAb-conjugated immunomagnetic beads[26]. These T-cell preparations contained <0.1% $CD11c^+$cells as assessed by FACS analysis. The CFSE-positive cells were then analyzed by flow cytometry. For analysis of apoptosis, apoptotic cell death in Ag-primed $CD4^+$T cells as described above was measured by flow cytometry using an Annexin V-FITC Apoptosis Detection kit (R&D Systems, Minneapolis, MN). For cell cycle analysis, Ag-primed $CD4^+$T cells were incubated with bromodeoxyuridine (BrdU; BD Bioscience) at 10 μM for 1 hr at 37 °C. Staining of incorporated BrdU was performed using reagents from a BrdU Flow Kit (BD Bioscience) according to the manufacturer's instructions. Cells were stained with FITC-conjugated anti-BrdU antibody for 20 min at room temperature. 7-amino-actinomycin D (7-AAD, 20 μg/ml) was added to the cell suspension before flow cytometry analysis. After Ag-priming, $CD4^+$T cells were selected, and analyzed by flow cytometry.

Detection of mTRAIL in gene-transduced mice. Mice (H-2^d; three animals in each group) were intravenously (i.v.) injected with or without control-Ad or mTRAIL-Ad (10^8 pfu/mouse). After 4 days, the spleens were obtained from BALB/c mice, and MNC were analyzed for expression of mTRAIL by flow cytometry with biotinylated anti-mTRAIL mAbs (eBioscience, San Diego, CA).

Models for acute GVHD. Recipient mice (H-2^d; five animals in each group) were i.v. injected with control-Ad or mTRAIL-Ad (10^7-10^8 pfu/mouse). After 4 days, recipients received lethal total body irradiation (TBI) (10 Gy), and allogeneic transplantation was then performed by a single i.v. injection of the recipient-mismatched (H-2^b) BM cells ($1.5x10^7$/mouse) plus spleen MNC ($1.5x10^7$/mouse) through the tail vein[21]. The day of transplantation was designated as day 0. Alternatively, the transplanted recipients were i.v. injected with control-Ad or mTRAIL-Ad (10^8 pfu/mouse) 2 days after transplantation. In another experiment, the transplanted recipients received a single i.v. injection of recipient-type adenoviral gene-transduced or untransduced syngeneic DCs

(10^6-5x10^6/mouse) 2 days after transplantation. For the in-vivo blockade experiments, recipients (H-2^d; five animals in each group) of allogeneic BMT (H-2^b) were intraperitoneally injected with control Ig or anti-mTRAIL mAb (1 mg/mouse) before i.v. injection of recipient-type mTRAIL-Ad/DCs (5x10^6/mouse, H-2^d) 2 days after transplantation. Recipients were monitored once every day from the day of transplantation until they succumbed naturally to GVHD to determine survival time. For analysis of the transplanted mice, recipients were killed 5 days after allogeneic transplantation to obtain the serum and spleen.

Models for leukemia relapse. Recipients (H-2^d, five animals in each group) were inoculated i.v. with P815 cells (2x10^5/0.2 ml) 2 days before TBI (10 Gy) and transplanted i.v. transplantation with allogeneic BM cells (H-2^b;1.5x10^7/mouse)[21]. The transplanted recipients were i.v. injected with control-Ad or mTRAIL-Ad (10^8 pfu/mouse) 4 days before transplantation. In another experiment, the transplanted recipients received a single i.v. injection of recipient-type adenoviral gene-transduced or untransduced syngeneic DCs (5x10^6/mouse) 2 days after transplantation. Recipients were monitored once every day from the day of transplantation until they succumbed to tumor burden to determine survival time.

Statistical analysis. All analyses for statistically significant differences were performed with the Student's paired t test or Mann-Whitney's U test. P values <0.01 were considered significant.

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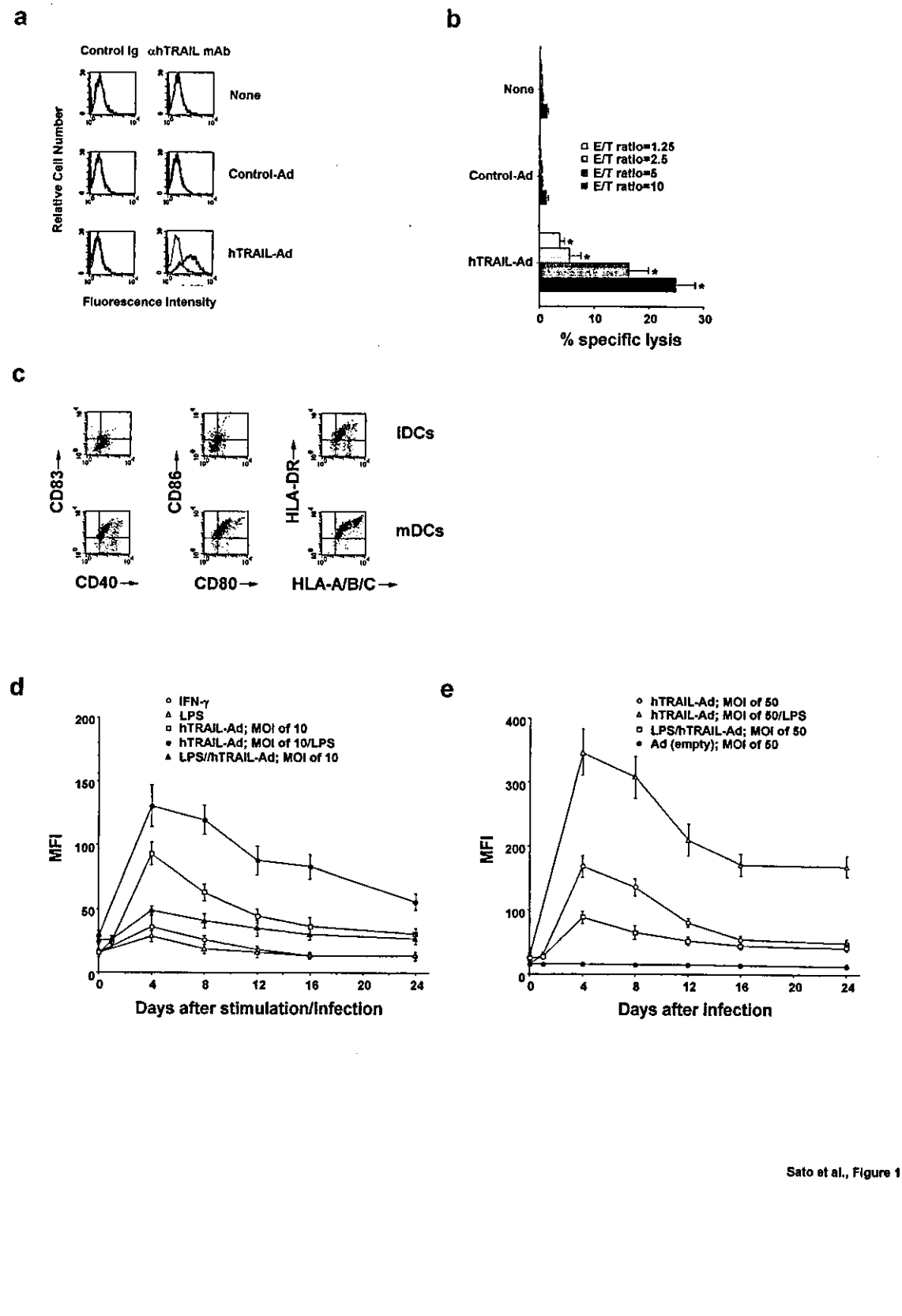

Sato et al., Figure 1