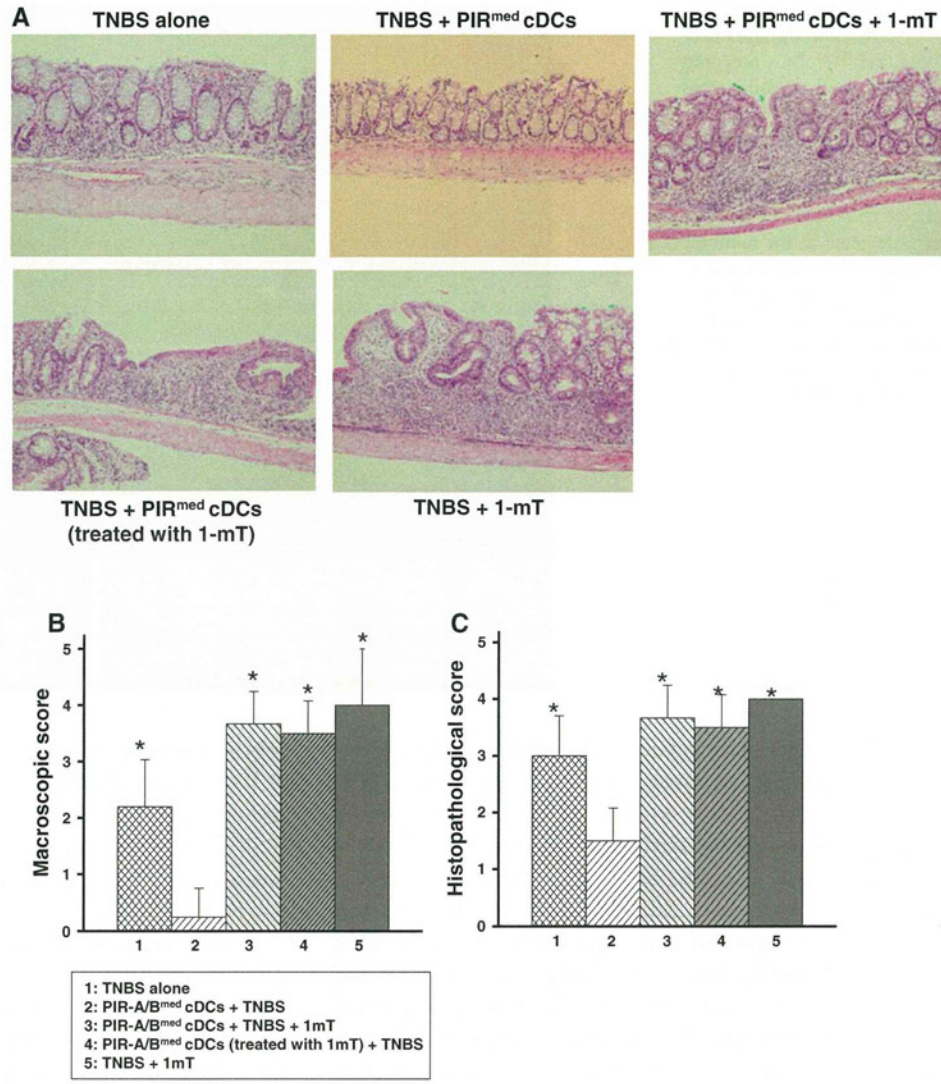

Fig. 8 Effect of inhibition of IDO on the treatment of TNBS-induced colitis by PIR-A/B^{med} cDCs. **a** Representative histological findings in mice with TNBS-induced colitis as assessed by the inoculation of PIR-A/B^{med} cDCs and treatment with 1-mT (TNBS alone, mice that received PIR-A/B^{med} cDCs, mice that received PIR-A/B^{med} cDCs plus 1-mT, mice that received PIR-A/B^{med} cDCs treated with 1-mT, mice treated with 1-mT). Infiltration of inflammatory cells, ulceration, loss of cryptal cells, and thickening of the colon wall were all observed in the mice that received PIR-A/B^{med} cDCs plus 1-mT and in mice that received PIR-A/B^{med} cDCs treated "in vitro" with 1-mT, but not in the mice transferred with PIR-A/B^{med} cDCs alone. **b** Macroscopical scores for the colons from the mice that received PIR-A/B^{med} cDCs plus 1-mT. **c** Histopathological scores for the colons from the mice that received PIR-A/B^{med} cDCs plus 1-mT. Each *column* shows mean ± SD of 4 mice. $^*P < 0.05$ versus group 2

(dendrites) between epithelial cells and into the gut lumen to sample antigens associated with microorganisms above the epithelial layer. These antigen-sampling DCs can activate CD4$^+$ T cells and induce effector T cells producing proinflammatory cytokines, suggesting that antigen-sampling DCs serve as host-defense guardians. In addition to this, DCs (CD103$^+$ DCs) also contribute to the induction of Treg; probably after "conditioning" by epithelial cells, CD103$^+$ DCs present antigens to naïve T cells in the context of RA and TGF-β secretion, and this process might be essential to the induction of Treg [2].

To elucidate the subcellular molecular mechanisms behind the regulatory function of PIR-A/B^{med} cDCs, analyses of some signaling pathways downstream of the PIR-A or -B tail are required. It was recently reported that protein tyrosine phosphatases (SHP-1 and SHP-2) are required for PIR-B-mediated inhibitory signaling [29]. Furthermore, of note is that there is considerable evidence that PIR-B may have the dual function of both inhibitory and activating activities, as suggested by the presence of an additional SH2-binding motif called the immunoreceptor tyrosine-based switch motif (ITSM) "T/SxYxxV/I" (where x represents any amino acid) [14, 30].

In this regard, PIR-B both negatively [14] and positively [31] regulates the eosinophil chemotaxis by recruiting the SHP-1 protein tyrosine phosphatase and by recruiting activating kinases (JAK1, JAK2, Shc, and Crk), respectively. Thus, it is feasible that cDCs bearing both PIR-A and -B have the function of negatively regulating the inflammatory and immune responses elicited by TNBS.

PIRs are now proposed as orthologues of human leukocyte immunoglobulin (Ig)-like receptors [LILRs, also known as Ig-like transcripts (ILTs)], based on their similarities in structure, expression profiles, and genomic localization [32]. LILRs are a family of inhibitory and stimulatory receptors encoded within the leukocyte

receptor complex and are expressed on immune cell types of both myeloid and lymphoid lineage, and may exert an influence on signaling pathways of both innate and adaptive immune systems. It is noted that several members of the LILR family recognize MHC class I, indicating that most cells can recognize LILRs or are recognized by the cells with LILRs. Thus, LILRs with inhibitory and stimulatory activity can also influence the antigen-presenting properties of macrophages and dendritic cells and may thus play a role in T cell tolerance. The wide-ranging effects of LILR signaling on immune cell activity imply that these receptors are likely to play an important role in a range of clinical situations, including autoimmune diseases, infectious diseases, transplantation, and cancer [33, 34]. Actually, it has been reported that triggering of LILRs through their interaction with self proteins can modulate the DC activation status, antigen-presenting functions, and the capacity to elicit T cell responses [34]. Ligation of LILRB4, one of the inhibitory receptors, led to an upregulation of IL-10 secretion by in vitro-cultured macrophages. This inhibitory cytokine may contribute to a feedback loop of LILR-mediated inhibition as it upregulates LILRB1, LILRB2, LILRB3, and LILRB4 on antigen-presenting cells [35–37]. We are now further investigating whether the expression of the LILR family participates in the intestinal inflammation in patients with inflammatory bowel diseases.

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ORIGINAL RESEARCH REPORT

Effects of Intrabone Marrow–Bone Marrow Transplantation Plus Adult Thymus Transplantation on Survival of Mice Bearing Leukemia

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We recently found that allogeneic intrabone marrow–bone marrow transplantation (IBM–BMT) plus adult thymus transplantation (ATT) from the same donor is effective in mice bearing solid tumors. In the current study, we examined the effects of this strategy on the survival of mice with leukemia. One week after intravenous injection of 1×10^6 leukemic cells (EL-4, H-2^b) into 8-week-old B6 (H-2^b) mice, the mice were 8 Gy irradiated and transplanted with 1×10^7 bone marrow cells (BMCs) from 8-week-old BALB/c mice (H-2^d) by IBM–BMT with or without donor lymphocyte infusion (DLI) or ATT. All the mice without treatment died within 70 days after injection of EL-4. About 40% of those treated with IBM–BMT alone died within 100 days due to tumor relapse. In contrast, those treated with IBM–BMT + DLI or ATT showed the longest survival rate without relapse of leukemia. In addition, the former showed less graft versus host disease (GVHD) than the latter. The mice treated with IBM–BMT + ATT also showed an intermediate percentage of effector memory (EM) and central memory (CM) cells between those treated with BMT alone and those treated with IBM–BMT + DLI. The numbers and functions of T cells increased in those treated with IBM–BMT + ATT with interleukin-2 and interferon-γ production. These results suggest that IBM–BMT + ATT is effective in the treatment of leukemia with strong graft versus leukemia without increased risk of GVHD.

Introduction

ALLOGENEIC BONE MARROW transplantation (allo-BMT) has been used for the radical treatment of leukemia. However, allo-BMT has some side effects. Graft versus host disease (GVHD) occurs if anti-host reaction in donor T cells is too strong, whereas relapse occurs if it is too weak [1]. In addition, a failure of bone marrow cell (BMC) engraftment in the early phase of transplantation may induce immunodeficiency, which, in turn, leads to severe infection [2]. Although donor lymphocyte infusion (DLI) is sometimes used to enhance engraftment and/or graft versus leukemia (GVL) activity [3], this is associated with an increased risk of GVHD [4]. Therefore, new cellular-based methods are required.

We recently developed a new BMT method, intrabone marrow–bone marrow transplantation (IBM–BMT), in which BMCs are directly injected into the bone marrow cavity [5]. IBM–BMT results in a reduced incidence of GVHD and greater engraftment of donor cells, including mesenchymal stem cells (MSCs) than the conventional intravenous method [6,7].

We have also developed a BMT method that is combined with thymus transplantation (TT), which includes the transplantation of adult thymus transplantation (ATT), newborn thymus, and fetal thymus. The combination of BMT + TT is effective in restoring donor-derived T cell function even in aged, chimeric-resistant, supralethally irradiated, and low-dose irradiated mice, mice with metabolic diseases, and also in mice injected with a small number of BMCs [8–12]. Further, we demonstrated that IBM–BMT + TT is effective for graft versus tumor (GVT) and long-term survival with a low risk of GVHD [13,14].

In the current study, we examined the BMT + ATT method in mice with leukemia. We also performed BMT alone and BMT + DLI in these mice and compared the survival rate, degree of GVHD, and T-cell functions.

Materials and Methods

Mice

Female 6- to 8-week-old C57BL/6 (B6) (H-2^b) and BALB/c (H-2^d) mice were obtained from Shimizu Laboratory Supplies (Shizuoka, Japan) and maintained until use in our animal facilities under specific pathogen-free conditions. All

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protocols for these animal experiments were performed in accordance with the Guidelines for Animal Experimentation, Kansai Medical University, and received approval from the Committee on Animal Experiments. EL-4 cells (H-2^b) were derived from thymoma in B6 mice. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum with antibiotics. These cells were intravenously transferred to the recipients (B6 mice).

IBM–BMT and ATT

Recipient B6 mice with tumors were irradiated (8 Gy) by using a ^{137}Cs irradiator (Gammacell 40 Exactor; MDS Nordion International, Ottawa, ON, Canada) 7 days after transfer of the EL-4 cells. The next day, BMCs were flushed from the shafts of donor femora and tibiae, and single-cell suspensions were prepared. Next, 1×10^7 BMCs were directly injected into the bone marrow cavity of the recipient's tibia, as previously described for the IBM–BMT method [7]. Briefly, the knee was flexed to 90°, and the proximal side of the tibia was drawn to the anterior. A 26-gauge needle was inserted into the joint surface of the tibia through the patellar tendon and then inserted into the bone cavity. Simultaneously, one quarter of the AT was grafted under the renal capsule of the left kidney in some mice.

Histology

Histological studies were performed in the liver, intestine (for evaluation of GVHD), and engrafted tumors from the recipients 4 weeks after the BMT. The tissues were fixed in 10% formaldehyde and embedded in paraffin. Serial tissue sections (4 μm thick) were prepared and stained by using hematoxylin and eosin. The degree of GVHD was evaluated by using a semiquantitative scoring system for abnormalities known to be associated with GVHD, as previously described [13].

Analysis of surface marker antigens and intracellular FoxP3 and cytokines by flow cytometry

Surface markers on lymphocytes from the spleen were analyzed by 3-color fluorescence staining by using a FACScan system (Becton Dickinson, Franklin Lakes, NJ). Fluorescein isothiocyanate (FITC)-conjugated anti-H-$2K^b$ (Pharmingen, San Diego, CA) mAbs and phycoerythrin (PE)-conjugated anti-H-$2K^d$ mAbs were used to determine chimerism. FITC, PE, or biotin-conjugated CD4, CD8, B220, CD44, or CD62L (Pharmingen) were used to analyze spleen cell subsets. Avidin-Cy5 (Dako, Kyoto, Japan) was used as the third color in the avidin/biotin system. Intracytoplasmic FoxP3 staining was performed by using an eBioscience FITC-anti mouse/rat FoxP3 staining kit in accordance with the manufacturer's instructions (eBioscience, San Diego, CA). Intracellular cytokines [interleukin (IL)-2, IL-4, IL-10, IL-17, interferon (IFN)-γ, and tumor necrosis factor] were detected by using an Intracellular Cytokine Staining Kit in accordance with the manufacturer's instructions (Becton Dickinson).

Mitogen responses

To analyze lymphocyte function, mitogen responses were examined in chimeric mice 2 months after transplantation. For mitogen response, a total of 2×10^5 splenocytes collected from chimeric mice and untreated B6 and BALB/c mice as responders were plated in 96-well flat-bottomed plates (Corning Glass Works, Corning, NY) containing 200 μL of RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 2 μL of glutamine (Wako Pure Chemicals, Tokyo, Japan), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat-inactivated fetal calf serum. For mitogen responses, responder cells were incubated with 2.5 μg/mL of Concanavalin A (ConA) (Calbiochem, San Diego, CA) or 25 μg/mL of lipopolysaccharide (LPS) (Difco Laboratories, Franklin Lakes, NJ) for 48 or 72 h. During the last 18 h of the culture period, 20 mL of 0.5 μCi ^{3}H-thymidine (^{3}H-TdR; New England Nuclear, Cambridge, MA) was introduced. Incorporation of ^{3}H-TdR was measured by using Microbeta TriLux (PerkinElmer, Wellesley, MA). The stimulation index was calculated as the average of ^{3}H-TdR incorporation in triplicate samples of responding cells with mitogen/^{3}H-TdR incorporation of responding cells in medium alone.

Statistical analyses and nonparametric analyses (Mann–Whitney *U*-test and log rank-test) were performed by using StatView software (Abacus Concepts, Berkley, CA). In all analyses, $P<0.05$ was taken to indicate statistical significance.

Results

Survival rate and body weight

First, we examined the effects of BMT alone, BMT + ATT, or BMT + DLI on the survival rate of mice transplanted with EL-4 (Fig. 1A). All the untreated mice transplanted with EL-4 died within 70 days due to tumor growth (Fig. 2B). Those treated with BMT alone showed a survival rate of about 60% 6 months after BMT. The remaining 40% of the mice died due to tumor growth. Interestingly, those treated with BMT + ATT or BMT + DLI showed the longest survival rate. Next, we investigated the weight of these mice. The mice not treated with EL-4 showed a gradual increase in weight (Fig. 1B), which was due to growth of the tumor (Fig. 2B). Those treated with IBM–BMT alone and IBM–BMT + ATT surviving for a long time showed a stable weight, and those treated with IBM–BMT + DLI showed a gradual weight loss.

Chimerism and histology

All mice treated with BMT showed donor-derived chimerism (H-$2K^d$), whereas untreated controls showed host-derived chimerism (H-$2K^b$) (Fig. 2A). The untreated mice showed massive infiltration of tumor cells throughout the whole body, including the liver, lung, mesenterium, muscle, and bone (Fig. 2B). All mice that died treated with BMT alone showed such tumor growth (as just mentioned). In contrast, most of those treated with BMT + ATT or BMT + DLI showed little tumor growth and long-term survival. The engrafted thymus showed a normal structure with cortical and medullar areas under the renal capsule (Fig. 2C). Normal T-cell differentiation was also observed in the thymus. In addition, those treated with BMT + ATT and BMT + DLI showed mild and moderate infiltrations of lymphocytes in the liver and small intestine, and the latter also showed some fibrosis with tissue destruction (Fig. 2D). Since the chimerism was of the donor type, this suggested the occurrence of

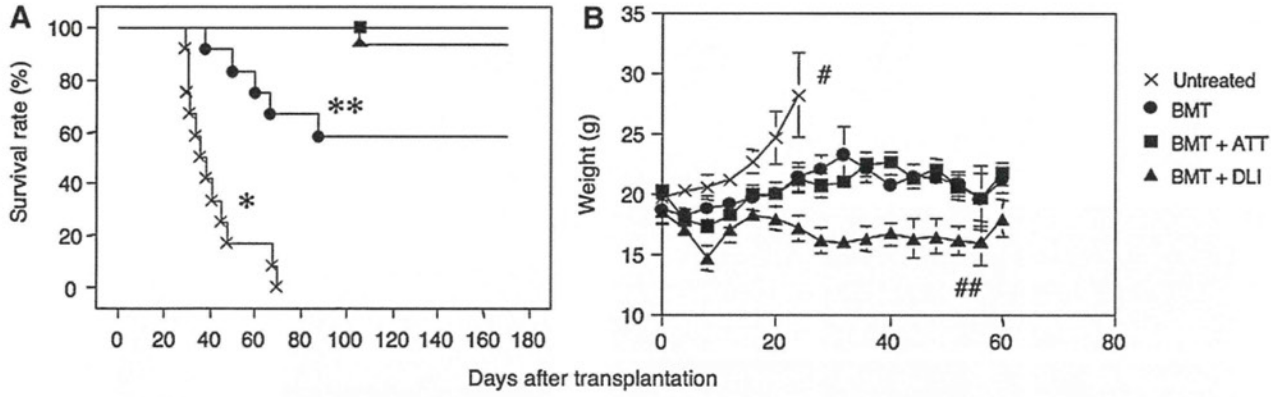

FIG. 1. Survival rate and body weight in mice with leukemia treated with BMT + TT. Survival rate **(A)** and weight **(B)** of mice with advanced tumors are shown. $^{*}P < 0.0001$ compared with those treated with BMT alone, BMT + ATT, or BMT + DLI. $^{**}P < 0.05$ compared with those treated with BMT + ATT or BMT + DLI. $^{\#}P < 0.05$ compared with those treated with BMT alone, BMT + ATT, or BMT + DLI. $^{\#\#}P < 0.05$ compared with those treated with BMT + ATT or BMT + DLI. Data are shown as means ± SE. Untreated controls ($n = 12$), those treated with BMT alone ($n = 12$), BMT + ATT ($n = 12$), or BMT + DLI ($n = 15$). ATT, adult thymus transplantation; BMT, bone marrow transplantation; DLI, donor lymphocyte infusion; TT, thymus transplantation.

GVHD. The GVHD scores are summarized in Fig. 2E. Those treated with BMT alone showed little GVHD, whereas those treated with BMT + ATT showed mild GVHD, and those treated with BMT + DLI showed moderate GVHD.

Lymphocyte subsets

We next analyzed the lymphocyte subsets in the spleen 4 weeks after transplantation. The number of $CD4^+$ T cells was significantly greater in the mice treated with BMT + ATT compared with those treated with BMT alone and those with BMT + DLI, in which the levels were comparable to those in normal BALB/c mice (Fig. 3). This was followed by those treated with BMT alone, followed by those treated with BMT + DLI, which showed the lowest levels. The percentage of $FoxP3^+$ regulatory T cells, which suppress immune responses, including GVH reactions [15,16], among $CD4^+$ T cells was the highest in the mice treated with BMT alone, the percentage being comparable to that in BALB/c mice. This was followed by those treated with BMT + ATT, whereas those treated with BMT + DLI showed the lowest percentage. The results for $CD8^+$ T cells were similar to those for $CD4^+$ T cells, although all values for mice treated with BMT were lower than those of BALB/c mice. The number of $B220^+$ T cells was lowest in those treated with BMT + DLI.

Effector memory, central memory, and naïve T cell subsets

T cells can be functionally divided into $CD62L^-CD44^-$ naïve, $CD62L^+CD44^+$ central memory (CM), and $CD62L^-CD44^+$ effector memory (EM) cells from prestimulation to terminal differentiation [17,18]. Therefore, we examined the proportions of these cells in both $CD4^+$ and $CD8^+$ subsets of T cells in the spleen. The percentage of EM among $CD4^+$ T cells was the highest in the mice treated with BMT + DLI followed by those treated with BMT + ATT (Fig. 4A). The lowest percentage of EM among $CD4^+$ T cells was seen in those treated with BMT alone, being comparable to that in BALB/c mice. Conversely, the percentage of CM was the highest in the mice treated with BMT alone and BALB/c mice, followed by those treated with BMT + ATT, and the lowest percentage of CM was seen in those treated with BMT + DLI. The percentage of naïve T cells was similar to that of CM cells, although the highest percentage was seen only in the BALB mice, and there were no significant differences between those treated with BMT + ATT and those treated with BMT + DLI. The results for $CD8^+$ T cells were similar to those for $CD4^+$ T cells (Fig. 4B).

Mitogen responses and cytokine production

Finally, we examined lymphocyte functions by monitoring mitogen responses (Con A for T cells and LPS for B cells) and cytokine production. The mice treated with BMT + ATT showed significantly increased Con A response compared with those treated with BMT alone, and the level was comparable to that in BALB/c mice (Fig. 5A). Those treated with BMT + DLI showed the lowest response. In contrast, the LPS response was almost the same in those treated with BMT alone, BMT + ATT, and BALB/c mice, and the lowest response was seen in BMT + DLI. With regard to cytokine production, those treated with BMT + ATT showed significant increases in IL-2 production compared with those treated with BMT alone and BMT + DLI, the level being comparable to that in BALB/c mice (Fig. 5B). In contrast, those treated with BMT + DLI showed significantly higher levels of IFN-γ production than those treated with BMT + ATT and BMT alone, although they did not reach the level of BALB/c mice. The production of IFN-γ was also elevated in those treated with BMT + TT, and was higher than in those treated with BMT alone.

Discussion

The current study was performed to examine the effects of BMT + ATT on leukemia in mice. The mice treated with BMT + ATT showed a longer survival than those treated with BMT alone, and milder GVHD than those treated with BMT + DLI. Leukemia showed little growth in BMT + ATT mice comparable to those treated with BMT + DLI. Those treated with BMT + ATT showed higher numbers of both

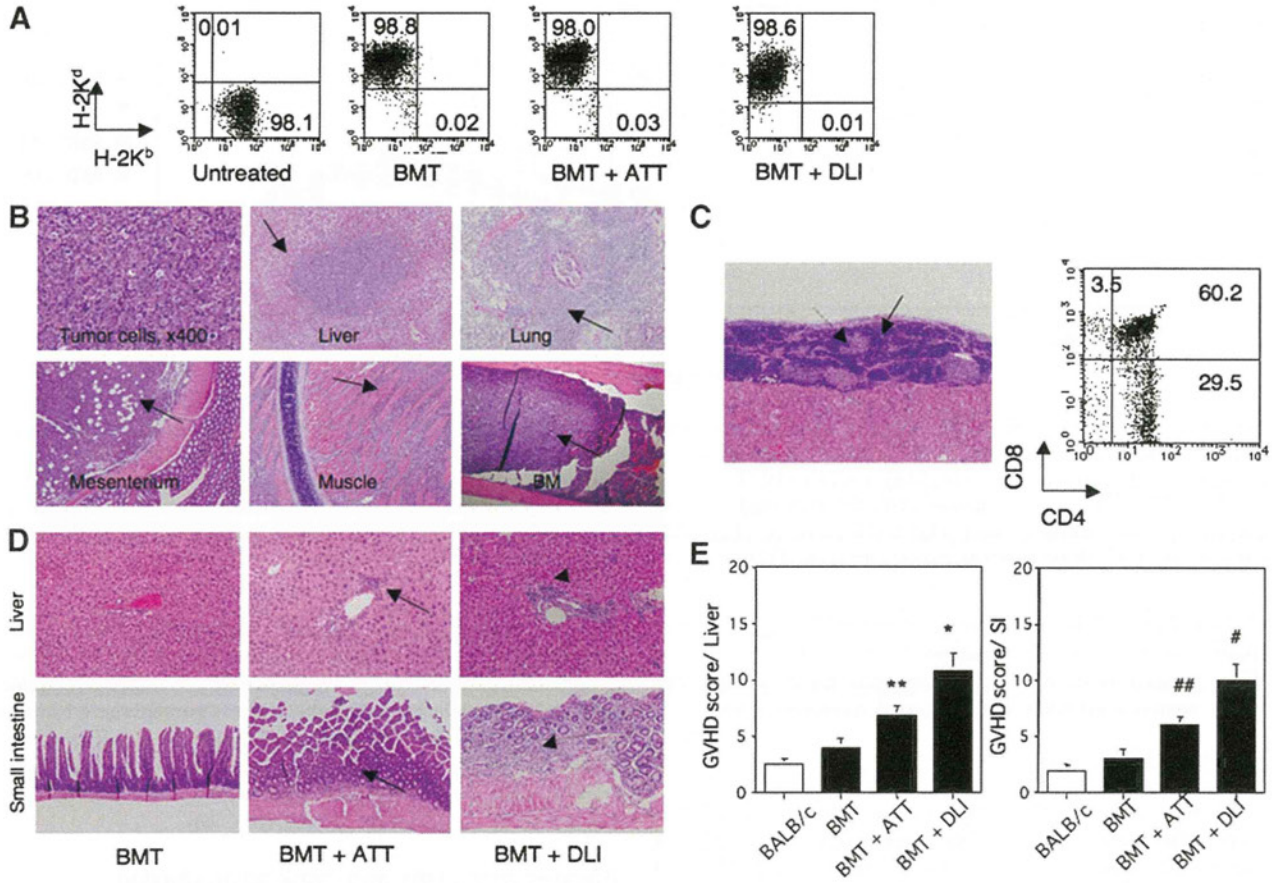

FIG. 2. Chimerism, histology, transplanted thymus, and GVHD in mice with leukemia treated with BMT + TT. Chimerism in experimental mice **(A)**, histology in the liver, lung, mesenterium, muscle, and BM from mice with leukemia **(B)**, histology in transplanted thymus and the FACS profile in **(C)**, histology for GVHD in chimeric mice **(D)** and the GVHD scores in the mice **(E)** are shown. Chimerism was analyzed in the spleens from mice transplanted with leukemia and those treated with BMT alone, BMT + ATT, or BMT + DLI **(A)**. The numbers in the profiles show the percentage. Tumor cells (*upper, left*) infiltrated the liver, lung, mesenterium, muscle, and BM in the mice transplanted with leukemia (*arrows*) **(B)**. Histological findings (*left*) and FACS profile (*right*) of thymocytes in the transplanted thymus from the mice treated with BMT and ATT **(C)**. *Plain arrow*, cortex; *dotted arrow*, medulla. The numbers in the profiles show the percentage. Representative data from 4 experiments are shown. The histology of GVHD is shown in the liver (*upper*) and small intestine (*lower*) **(D)**. Some lymphocytes infiltrated the liver and small intestine in BMT + ATT (*arrows*) and destroyed the tissue in BMT + DLI (*dotted arrow*). GVHD scores are shown in the liver (*left*) and small intestine (*right*) **(E)**. The GVHD score was calculated as described in Materials and Methods. $^{*}P < 0.05$ compared with normal BALB/c mice and mice treated with BMT alone and BMT + ATT. $^{**}P < 0.05$ compared with normal BALB/c mice and the mice treated with BMT alone. $^{\#}P < 0.05$ compared with normal BALB/c mice and mice treated with BMT alone and BMT + ATT. $^{\#\#}P < 0.05$ compared with normal BALB/c mice and mice treated with BMT alone. The mice transplanted with leukemia cells (EL-4) were analyzed 5 weeks after transplantation and those treated with BMT alone, BMT + ATT, or BMT + DLI were analyzed 8 weeks after treatment. Data are shown as means ± SE. Normal BALB/c ($n = 4$), BMT ($n = 4$), BMT + ATT ($n = 4$), BMT + DLI ($n = 4$). GVHD, graft versus host disease.

$CD4^+$ and $CD8^+$ T cell subsets than those treated with BMT alone or with BMT + DLI. Interestingly, the percentages of $FoxP3^+$ regulatory T cells, CM, and EM T cells in those treated with BMT + ATT were intermediate between those treated with BMT alone and those treated with BMT + DLI. T-cell functions with production levels of some cytokines were also elevated in those treated with BMT + ATT. These findings suggest that the BMT + ATT method is more effective in the treatment of leukemia than previous methods.

First, we examined the survival rates in association with GVH and GVL effects. All mice with the development of leukemia died early, whereas those without leukemia showed long-term survival, with or without GVHD, thus indicating that the presence of leukemia is the factor with the greatest influence on mortality. However, we did not examine the further long-term effects of GVHD, and chronic GVHD may also lead to death in the long term [19]. Therefore, these observations suggest that BMT + ATT is superior to BMT alone and BMT + DLI.

We next investigated the mechanism of these effects. The numbers of both T-cell subsets significantly increased in mice treated with BMT + ATT compared with those treated with BMT alone and BMT + DLI. The low numbers of T-cell subsets as well as B cells in those treated with BMT + DLI may have resulted in GVHD [20]. Interestingly, those treated with BMT + ATT showed a lower percentage of regulatory T cells

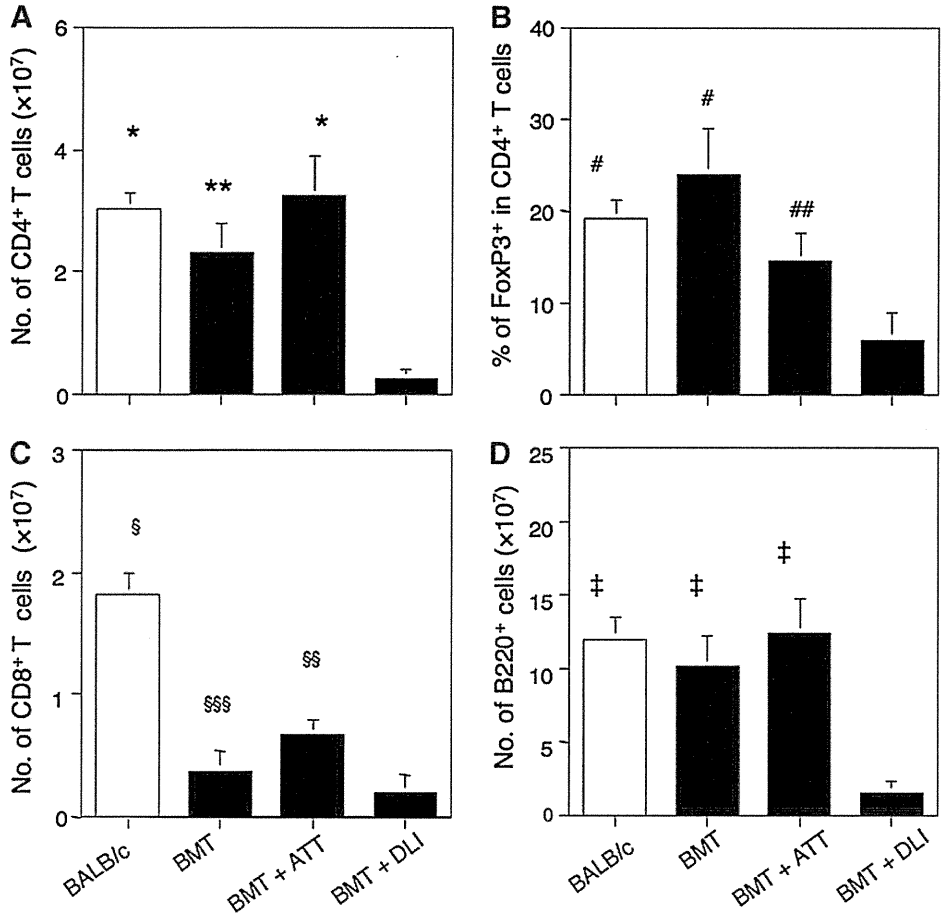

FIG. 3. Numbers of lymphocytes in the spleen from leukemia-bearing mice treated with BMT+ATT. Numbers of $CD4^+$ T cells **(A)**, percentage of $FoxP3^+$ cells in $CD4^+$ T cells **(B)**, numbers of $CD8^+$ T cells **(C)**, and $B220^+$ B cells **(D)** in the spleen were evaluated in normal BALB/c mice, leukemia-bearing mice treated with BMT alone, BMT+ATT, or BMT+DLI. The experiments were performed 5 weeks after BMT. $^*P<0.05$ compared with BMT alone and BMT+DLI. $^{**}P<0.01$ compared with BMT+DLI. $^{\#}P<0.05$ compared with BMT+ATT and BMT+DLI. $^{\#\#}P<0.05$ compared with BMT+DLI. $^{\S}P<0.01$ compared with BMT, BMT+ATT, and BMT+DLI. $^{\S\S}P<0.05$ compared with BMT and BMT+DLI. $^{\S\S\S}P<0.05$ compared with BMT+DLI. $^{\ddagger}P<0.01$ compared with BMT+DLI. Data are shown as means±SD. Normal BALB/c ($n=5$), BMT ($n=5$), BMT+ATT ($n=5$), and BMT+DLI ($n=5$).

than those treated with BMT alone, whereas they showed a higher percentage of regulatory T cells than BMT+DLI. Since regulatory T cells suppressed GVHD, preserving GVT and GVL [21,22] the intermediate percentage of these cells also played an important role in the mechanism underlying our results. The elevation of regulatory T-cell numbers compared with BMT alone suggested that some regulatory T cells in BMT+ATT may be produced from the TT [13].

The percentages of EM T cells were highest in the mice treated with BMT+DLI, followed by those treated with BMT+ATT, and lowest in those treated with BMT alone. In contrast, CM and naïve T cells were lowest in the BMT+DLI group, followed by the BMT+ATT group, and highest in those treated with BMT alone. Since freshly isolated CM and naïve T cells in DLI induce GVHD and GVL [23,24], the observation of the lowest numbers of these cells in BMT+DLI suggested that these cells in DLI had differentiated into EM T cells with activation and/or were consumed [25].

The mice treated with BMT+ATT showed sufficient mitogen responses to both T and B cells, whereas those treated with BMT alone showed low T-cell response, and those treated with BMT+DLI showed low responses to both T and B cells. Such immunodeficiency in BMT+DLI may be induced by GVHD [20]. These findings indicated that BMT+TT is the best method compared with BMT alone or BMT+DLI. Although the mechanism underlying the long-term survival in the BMT+DLI group is unclear, the SPF condition may have prevented severe infection. Alternatively, the IBM–BMT method itself may suppress lethal GVHD by DLI [6].

Analysis of cytokines indicated significantly elevated IL-2 production in the mice treated with BMT+ATT compared with those treated with BMT alone or with BMT+DLI, whereas IFN-γ production was significantly higher in those treated with BMT+DLI. Although several cytokines play a role in GVHD, IL-2 is effective in inducing strong GVL while avoiding GVHD [26]. In addition, the highest degree of elevation of IFN-γ production may contribute to GVHD with lymphoid hypoplasia (immunodeficiency) in BMT+DLI [27]. Therefore, the cytokine patterns may also be at least partly associated with the pathogenesis.

We thus found that donor-derived T cells play an important role in the treatment of leukemia. However, further gene analyses are needed for a more comprehensive understanding. For example, cytokine profiles at the transcriptional level should be analyzed by using DNA microarry in T cells [28]. In addition, differences in T-cell clones may become evident between GVL and GVH, or between host and donor-thymus derived by next-generation sequencing and/or other methods [29]. These findings would help detail the mechanism of BMT+TT.

We examined means of treating several intractable diseases and/or serious complications by BMT+TT [8–14]. In most studies, BMT+TT showed better results than BMT alone or BMT+DLI. The results of the current study were also compatible with these previous findings. The thymus is

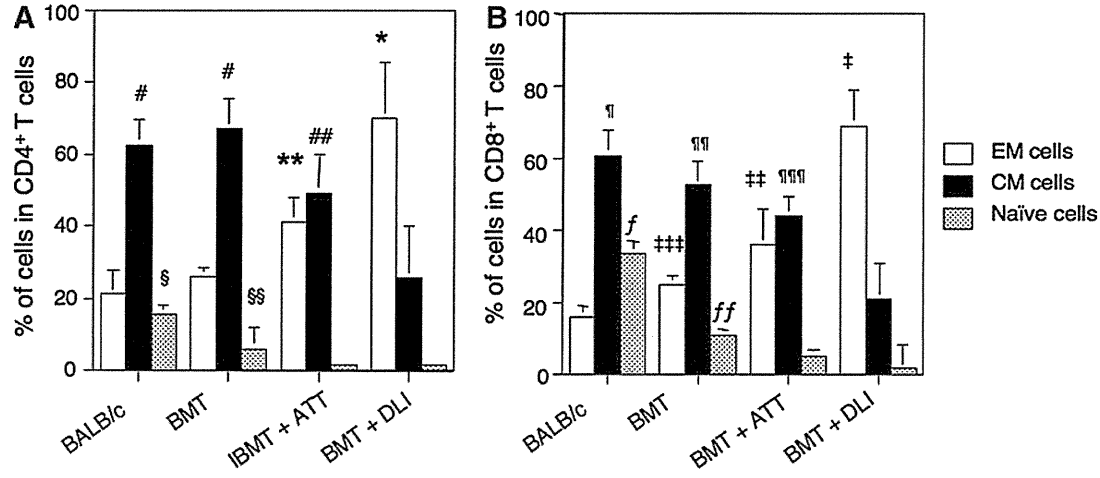

FIG. 4. Proportions of EM, CM, and naïve T cells from leukemia-bearing mice treated with BMT + ATT. Percentages of $CD62L^-CD44^+$ EM, $CD62L^+CD44^+$ CM, and $CD62L^-CD44^-$ naïve cells in $CD4^+$ **(A)** and $CD8^+$ **(B)** T cell subsets were analyzed in the spleens from normal BALB/c mice and leukemia-bearing mice treated with BMT alone, BMT + ATT, and BMT + DLI. Analyses were performed at the same time as for the experiment in Fig. 3. $^*P < 0.01$ compared with BALB/c, BMT, and BMT + ATT. $^{**}P < 0.01$ compared with BALB/c and BMT. $^{\#}P < 0.05$ compared with BMT + ATT and BMT + DLI. $^{\#\#}P < 0.01$ compared with BMT + DLI. $^{\S}P < 0.01$ compared with BMT, BMT + ATT, and BMT + DLI. $^{\S\S}P < 0.01$ compared with BMT + ATT and BMT + DLI. $^{\ddagger}P < 0.01$ compared with BALB/c, BMT, and BMT + ATT. $^{\ddagger\ddagger}P < 0.01$ compared with BALB/c and BMT. $^{\ddagger\ddagger\ddagger}P < 0.01$ compared with BALB/c. $^{\P}P < 0.05$ compared with BMT, BMT + ATT, and BMT + DLI. $^{\P\P}P < 0.05$ compared with BMT + ATT and BMT + DLI. $^{\P\P\P}P < 0.01$ compared with BMT + DLI. $^{f}P < 0.01$ compared with BMT, BMT + ATT, and BMT + DLI. $^{ff}P < 0.05$ compared with BMT + ATT and BMT + DLI. Data are shown as means ± SD. Normal BALB/c ($n = 5$), BMT ($n = 5$), BMT + ATT ($n = 5$), and BMT + DLI ($n = 5$). CM, central memory; EM, effector memory.

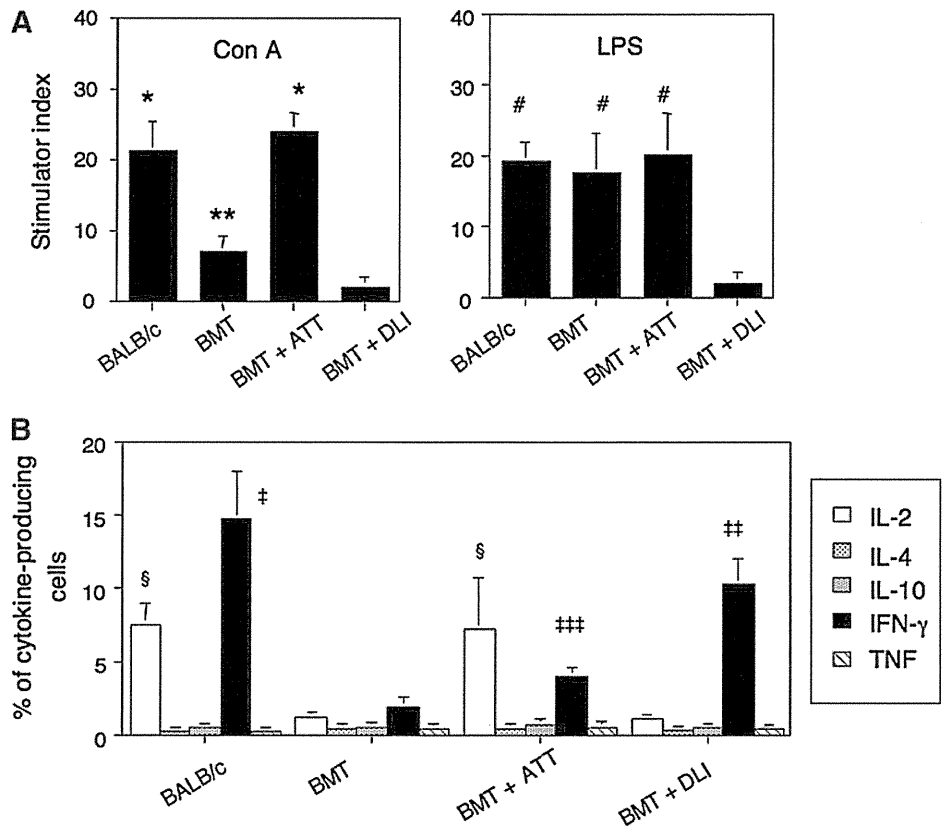

FIG. 5. Mitogen responses and percentages of cytokine-producing cells in the spleens from leukemia-bearing mice treated with BMT and ATT Mitogen responses: Con A and LPS **(A)** and percentages of cytokine-producing cells **(B)** in the spleen were evaluated in the spleens from normal BALB/c mice and leukemia-bearing mice treated with BMT alone, BMT + ATT, and BMT + DLI. Analyses were performed at the same time as for the experiment in Fig. 3. $^*P < 0.05$ compared with BMT and BMT + DLI. $^{**}P < 0.05$ compared with BMT + DLI. $^{\#}P < 0.05$ compared with BMT + DLI. $^{\S}P < 0.05$ compared with BMT and BMT + DLI. $^{\ddagger}P < 0.05$ compared with BMT, BMT + ATT, and BMT + DLI. $^{\ddagger\ddagger}P < 0.05$ compared with BMT and BMT + ATT. $^{\ddagger\ddagger\ddagger}P < 0.05$ compared with BMT. Data are shown as means ± SD. Normal BALB/c ($n = 4$), BMT ($n = 4$), BMT + ATT ($n = 4$), and BMT + DLI ($n = 4$). LPS, lipopolysaccharide.

an organ involved in the maintenance of homeostasis itself and regulates the production of not only T cells but also several cytokines and hormones in a feedback mechanism [30]. Thus, TT is different from DLI, which supplies mature lymphocytes in one direction. Hence, TT is a type of functional organ transplantation, and may represent an approach that significantly regulates the immune function of T cells in vivo for the benefit of the host.

Overall, we found that allogeneic IBM–BMT + ATT induces strong GVL effects with mild GVHD. Although it may be both ethically and technically difficult to obtain adequate thymus tissue in clinical cases (including the question of donor age), grafts could be obtained from patients with congenital heart diseases or from aborted fetuses, as previously utilized [31,32]. We have recently found that even if the thymus donor is different from the BMC donor, then the effect is comparable to that seen with transplantation from the same donor using triple chimeric mice [33]. In addition, a method of regenerating the thymus has also been developed [34–37]. Therefore, IBM–BMT + ATT could become a viable strategy for the treatment of malignant hematological tumors in humans.

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Author Disclosure Statement

The authors disclose no commercial association that might create a conflict in connection with the submitted article. No competing financial interests exist.

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Glypican-3 could be an effective target for immunotherapy combined with chemotherapy against ovarian clear cell carcinoma

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Glypican-3 (GPC3) is useful not only as a novel tumor marker, but also as an oncofetal antigen for immunotherapy. We recently established HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clones from hepatocellular carcinoma patients after $GPC3_{144-152}$ peptide vaccination. The present study was designed to evaluate the tumor reactivity of a HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone against ovarian clear cell carcinoma (CCC) cell lines. The $GPC3_{144-152}$ peptide-specific CTL clone could recognize HLA-A2-positive and GPC3-positive ovarian CCC cell lines on interferon (IFN)-γ enzyme-linked immunospot assay and showed cytotoxicity against KOC-7c cells. The CTL clone recognized naturally processed GPC3-derived peptide on ovarian CCC cells in a HLA class I-restricted manner. Moreover, we confirmed that the level of GPC3 expression was responsible for CTL recognition and that subtoxic-dose chemotherapy made tumor cells more susceptible to the cytotoxic effect of CTL. Thus, it might be possible to treat ovarian CCC patients by combining chemotherapy with immunotherapy. Our data suggest that GPC3 could be an effective target for immunotherapy against ovarian CCC. (*Cancer Sci* 2011; 102: 1622–1629)

Epithelial ovarian carcinoma (EOC) is the leading cause of death from gynecological malignancy. Cytoreductive surgery and systemic combination chemotherapy with a platinum drug and a taxane represent the standard of care for EOC patients. Ovarian clear cell carcinoma (CCC) is the second most frequent subtype of EOC in Japan, although CCC represents 8–10% of all EOC in the United States.[1,2] Compared with other EOC subtypes, ovarian CCC is associated with a poorer prognosis and increased chemoresistance.[1,3] More efficient conventional therapies and novel strategies for effectively treating ovarian CCC are required.

Glypican-3 (GPC3) is a member of the glypican family of heparan sulfate proteoglycans that are attached to the cell surface via the glycosylphosphatidylinositol (GPI) anchor.[4] It is known as an oncofetal antigen specifically overexpressed in hepatocellular carcinoma (HCC).[5] Previous studies have shown that GPC3 was also overexpressed in other malignant tumors, such as melanoma, Wilms' tumor, hepatoblastoma, yolk sac tumor, ovarian CCC and lung squamous cell carcinoma.[6–10]

We previously identified the HLA-A24-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptides, both of which can induce GPC3-reactive cytotoxic T cells (CTL).[11] Recently, HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clones were established from HCC patients after $GPC3_{144-152}$ peptide vaccination in our laboratory.[12] Although CTL reactivity against HCC cell lines was analyzed using these CTL clones, other GPC3-positive tumor cell lines have not been studied. Therefore, we examined the reactivity of a HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone against ovarian CCC cell lines, and whether subtoxic-dose chemotherapy sensitizes ovarian CCC cells to lysis of $GPC3_{144-152}$ peptide-specific CTL.

Materials and Methods

$GPC3_{144-152}$ peptide-specific CTL clone and cell lines. We established the HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone from the PBMC of HCC patients vaccinated with $GPC3_{144-152}$ (FVGEFFTDV) peptide by single-cell sorting using CD107a antibody. The established CTL clone was tested for avidity by using $GPC3_{144-152}$ peptide-pulsed T2 targets with a range of peptide concentrations, starting at 10^{-6} M and decreasing by log steps to 10^{-14} M. The peptide concentration at which the curve crossed 50% cytotoxicity was defined as the avidity of the CTL clone and was rounded to the nearest log. This CTL clone had high avidity CTL (10^{-11} M) and could recognize HCC cell lines expressing GPC3 in a HLA-class-I-restricted manner.[12] Two human ovarian CCC cell lines, KOC-7c (HLA-A*0201/A*3101) and TOV-21G (HLA-A*1101/A*2601), and two human HCC cell lines, HepG2 (HLA-A*0201/A*2402) and SK-Hep-1 (HLA-A*0201/A*2402), were used in the present study. They were conserved in our laboratory. TOV-21G.A2 acquires expression of HLA-A2 following transfection with an HLA-A2 expression plasmid.[13] TOV-21G.A24 was similarly transfected with an HLA-A24 expression plasmid. SK-Hep-1.hG acquires expression of human GPC3 following transfection with a human GPC3 expression plasmid. SK-Hep-1.vec cell line transfected with an empty vector was used as a control. To study the effect of silencing GPC3, KOC-7c GPC3-shRNA and Neg-shRNA (control shRNA) were established by short hairpin RNA knockdown technology as described previously.[14] These cells were maintained in RPMI 1640 or DMEM medium (Sigma, St Louis, MO, USA) supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere containing 5% CO_2.

RNA preparation and quantitative real-time PCR (qRT-PCR). Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. GPC3 gene expression levels were analyzed by qRT-PCR assays using the following primers generated according to the indicated reference sequences: sense, 5′-GAGCCAGTGGTCAGTCAAAT-3′ and antisense, 5′-CTTCATCATCACCGCAGTC-3′. Amplification reactions were carried out in 96-well plates in 25 μL reaction volume using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). All reactions were

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performed in technical triplicate using an ABI 7500 Fast Real-Time PCR System. Relative expression of the GPC3 gene to the endogenous control gene, β-actin, was calculated using the comparative C_T method. β-actin qRT-PCR primer sequences were: sense, 5′-TCCATCATGAAGTGTGACGT-3′ and antisense, 5′-GAGCAATGATCTTGATCTTCAT-3′.

Flow cytometry analysis and cell sorting. Flow cytometry (FCM) was performed to quantify the expression of GPC3 and Fas on the cell surface using the following antibodies: primary anti-GPC3 (clone 1G12; BioMosaics, Burlington, VT, USA); Alexa Fluor 488 conjugated second Ab (Invitrogen); phycoerythrin (PE)-conjugated anti-Fas (clone DX2; BioLegend, San Diego, CA, USA); FITC-conjugated anti-HLA-A2 (clone BB7.2; MBL, Nagoya, Japan); and FITC-conjugated mouse IgG2b isotype control (clone 3D12; MBL).

The FCM data was acquired using the FACSCanto II system (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). Mean fluorescence intensity (MFI) of GPC3 staining was calculated as follows: MFI ratio = MFI with the anti-GPC3 Ab/MFI with the secondary Ab. MFI of HLA-A2 staining was similarly calculated (MFI ratio = MFI with the anti-HLA-A2 Ab/MFI with isotype control Ab).

Cell sorting was performed using the FACSAria II cell sorter (BD Biosciences) to isolate $GPC3^+$ and $GPC3^-$ cells from KOC-7c cells. We purified KOC-7c GPC3 high or low cells with the top or bottom 10% of GPC3 expression, respectively.

Response of $GPC3_{144\text{-}152}$ peptide-specific CTL clone against cancer cell lines. $GPC3_{144\text{-}152}$ peptide-specific CTL clone cells were co-cultured with each cancer cell line as target cells at the indicated effector/target (E/T) ratio and cytotoxicity assay or IFN-γ enzyme-linked immunospot (ELISPOT) assay was performed. Blocking of HLA class I was done as follows. Before coculturing the CTL clone with a cancer cell line in an assay, the target cancer cells were incubated for 1 h with anti-HLA class I mAb (clone W6/32; BioLegend), or isotype control IgG2a mAb, and then the effects of Ab on CTL clone activity was examined.

IFN-γ ELISPOT analysis. ELISPOT assay for detecting antigen-specific IFN-γ-producing T cells was performed using the ELISPOT kit (BD Biosciences). The spots were automatically counted and analyzed with the Eliphoto system (Minerva Tech, Tokyo, Japan).

Cytotoxicity assay. The cytotoxic capacity was analyzed with the Terascan VPC system (Minerva Tech). The CTL clone was used for effector cells. Target cells were labeled in calcein-AM solution for 30 min at 37°C. The labeled cells were then co-cultured with effector cells for 4–6 h. Fluorescence intensity was measured before and after the 4–6 h culture, and specific cytotoxic activity was calculated as previously described.[12]

Cold inhibition assay. Calcein AM-labeled target cells were cultured with effector cells in a 96-well plate with cold target cells. T2 target cells, which were prepulsed with either $HIV_{19\text{–}27}$ peptide or $GPC3_{144\text{-}152}$ peptide, were used as cold target cells.

CD107a degranulation assay. $GPC3_{144\text{-}152}$ peptide-specific CTL clone cells were incubated with cancer cell lines at a 2:1 ratio for 4 h at 37°C. APC-conjugated CD107a-specific mAb (clone H4A3; BD Biosciences) were present during the incubation period; after incubation, cells were stained with additional PE-conjugated anti-CD8 mAb (clone HIT8a; BioLegend) and analyzed by FCM.

Growth inhibition assay. Growth inhibition was evaluated by a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) colorimetric assay using a Cell Counting Kit (Dojindo, Kumamoto, Japan). Cells (5×10^3) were seeded into 96-well plates in 100 μL of culture medium for 24 h prior to drug exposure, and then treated with various concentrations of paclitaxel (PTX) or cisplatin (CDDP) for 18 or 48 h. Cell viability was determined colorimetrically by optical density at 450 nm wavelength using a microplate reader (Bio-Rad, Hercules, CA, USA). The percentage of cell survival for each drug concentration was calculated as: (absorbance of test wells/absorbance of control wells) × 100.

Apoptosis analysis. The Annexin V-FITC Apoptosis Detection Kit (BioVision, Mountain View, CA, USA) was used to determine apoptosis after treatment with PTX or CDDP. After treatment with the chemodrug, floating and adhering cells were collected via trypsinization and centrifuged. The supernatant was removed and resuspended in 500 μL of binding buffer to which 5 μL of Annexin-V-FITC and propidium iodido (PI) was added. The cells were incubated at room temperature for 5 min in the dark and assessed by FCM.

Statistical analysis. Univariate regression analysis was used to evaluate the correlation between GPC3 expression and GPC3-specific CTL recognition. Mann–Whitney *U*-test and Kruskal–Wallis test followed by Scheffe's *post hoc* test were used to detect differences between groups. For all statistical tests, differences were considered significant at $P < 0.05$.

Results

HLA-A2-restricted $GPC3_{144\text{-}152}$ peptide-specific CTL clone recognizes ovarian CCC cell lines. To ascertain whether the HLA-A2-restricted $GPC3_{144\text{-}152}$ peptide-specific CTL clone recognizes ovarian CCC cell lines expressing HLA-A2 and GPC3, we first evaluated the expression of GPC3 on cancer cell lines. We used KOC-7c and HLA-A0201 gene stable transfectant TOV-21G.A2 and two human HCC cell lines for the target cells. As positive controls, we used two HCC cell lines. SK-Hep-1.hG cells were an established stable GPC3-expressing cell line. As we performed qRT-PCR and FCM of GPC3 in these cell lines, GPC3 expression in ovarian CCC cell lines was less than that in HCC cell lines. Representative data of relative mRNA expression (ratio to KOC-7c) and MFI ratio are shown (Fig. 1A). The CTL response generally correlates with the numbers and density of MHC/antigen peptide complex on the target cells. Accordingly, we also evaluated HLA-A2 expression on the cell surface in cancer cell lines with FCM analysis (Fig. 1B). IFN-γ production of the CTL clone was detected against two ovarian CCC cell lines (Fig. 1C). In Figure 1C, we used TOV-21G.A24 as a negative control. Furthermore, we determined whether efficient $GPC3_{144\text{-}152}$ peptide-specific CTL clone recognition was correlated with GPC3 expression levels. We found that CTL clone recognition was correlated with the relative GPC3 mRNA expression and GPC3 MFI ratio in the cell lines ($r^2 = 0.995$ and 0.935, respectively) (Fig. 1D,E). In addition, we also analyzed whether CTL reactivity is correlated with not only GPC3 expression but also the expression of HLA-A2. The correlation between HLA-A2 expression levels on FCM analysis and CTL clone recognition (IFN-γ production or CD107a degranulation) was insufficient in the cell lines (data not shown). Although HLA-A2 expression on the cell surface in TOV-21G.A2 was moderately low, that in three other cell lines was sufficient on FCM analysis. TOV-21G.A2 cells have low expression of not only HLA-A2 but also GPC3. Therefore the GPC3 expression level is more important than the HLA-A2 expression level on $GPC3_{144\text{-}152}$ peptide-specific CTL clone reactivity.

$GPC3_{144\text{-}152}$ peptide-specific CTL clone lyses ovarian CCC cell lines. We detected GPC3-specific CTL responses by a CD107a degranulation assay. GPC3-specific CTL responses against TOV-21G.A2 and KOC-7c cells exhibited 2.79% and 5.42% CD107a staining, respectively, approximately 1.8- and 3.4-fold increases compared with the SK-Hep-1.vec as a negative control (Fig. 2A). CD107a degranulation was also correlated with the

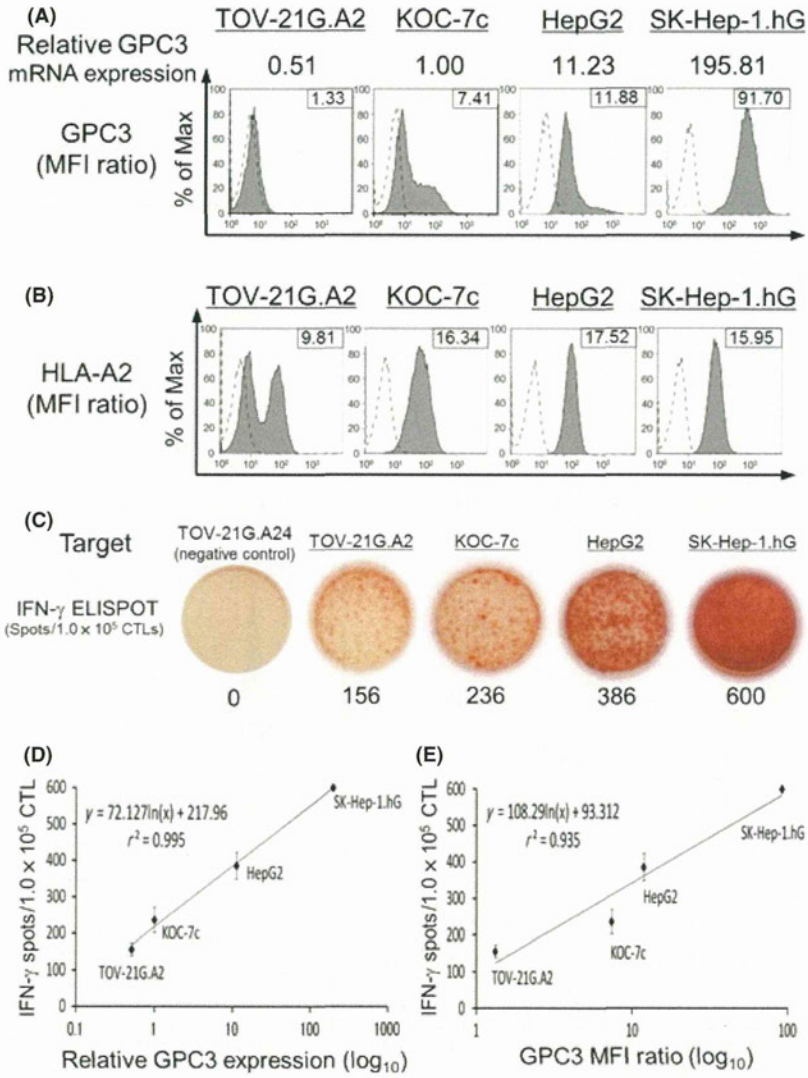

Fig. 1. HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone recognizes ovarian clear cell carcinoma (CCC) cell lines. (A) Expression of GPC3 on cancer cell lines. We used two human ovarian CCC cell lines (TOV-21G.A2 and KOC-7c) and two human HCC cell lines. We performed qRT-PCR and flow cytometry analysis (dashed line, secondary Ab stained control; gray-filled area, GPC3 staining). Numbers in the histograms correspond to the ratio of mean fluorescence intensity (MFI) of GPC3 staining, calculated as: MFI ratio = (MFI with the anti-GPC3 Ab)/(MFI with the secondary Ab). Representative data of relative GPC3 mRNA expression (ratio to KOC-7c) and GPC3 MFI ratio are shown. GPC3 expression in ovarian CCC cell lines was less than in HCC cell lines. (B) Expression of HLA-A2 on cancer cell lines. Numbers in histograms correspond to the ratio of MFI of HLA-A2 staining, calculated as: MFI ratio = (MFI with the anti-HLA-A2 Ab)/(MFI with isotype control Ab). (C) Representative results of IFN-γ ELISPOT analysis are shown. Effector/target ratio = 2. TOV-21G.A24 cells were used as a negative control. (D) IFN-γ production of a $GPC3_{144-152}$ peptide-specific CTL clone was correlated with relative GPC3 mRNA expression (r^2 = 0.995). (E) Similarly, $GPC3_{144-152}$ peptide-specific CTL clone recognition was correlated with the GPC3 MFI ratio (r^2 = 0.935).

relative GPC3 mRNA expression and GPC3 MFI ratio in the cell lines (r^2 = 0.978 and 0.865, respectively) (Fig. 2B). The $GPC3_{144-152}$ peptide-specific CTL clone was further tested for its capacity to kill ovarian CCC cell lines, by a calcein-AM-based cytotoxicity assay. SK-Hep-1.vec cells were used for a negative control. The CTL clone displayed mild, but clear, specific cytotoxicity against KOC-7c cells (Fig. 2C). However, GPC3-specific cytotoxicity was insufficient against TOV-21G.A2 cells compared with TOV-21G.A24 cells (data not shown). In both ovarian CCC cell lines, Fas expression on the cell surface was sufficiently similarly to that of the HCC cell lines on FCM analysis (Fig. 2D).

HLA class I specificity was confirmed by the blockade of reactivity against ovarian CCC cell line KOC-7c. HLA class I-restricted activity was demonstrated by blocking of IFN-γ release and lysis of the $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c after pretreatment with a HLA class I-specific mAb (W6/32) or mouse IgG2a isotype control, respectively, for 1 h. This reactivity could be inhibited by anti-HLA class I mAb but not by isotype control (Fig. 3). These results clearly indicate that the CTL clone recognized KOC-7c in a HLA class I-restricted manner.

Effect of GPC3 silencing using shRNA on the response of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c cells. To verify the GPC3 antigen-specific response of the CTL clone against ovarian CCC cell lines, we examined GPC3 knockdown on the GPC3-positive cell line KOC-7c. KOC-7c GPC3-shRNA was established using shRNA knockdown technology. The GPC3 expression of KOC-7c was obviously decreased by GPC3 shRNA on qRT-PCR. We examined the IFN-γ production and

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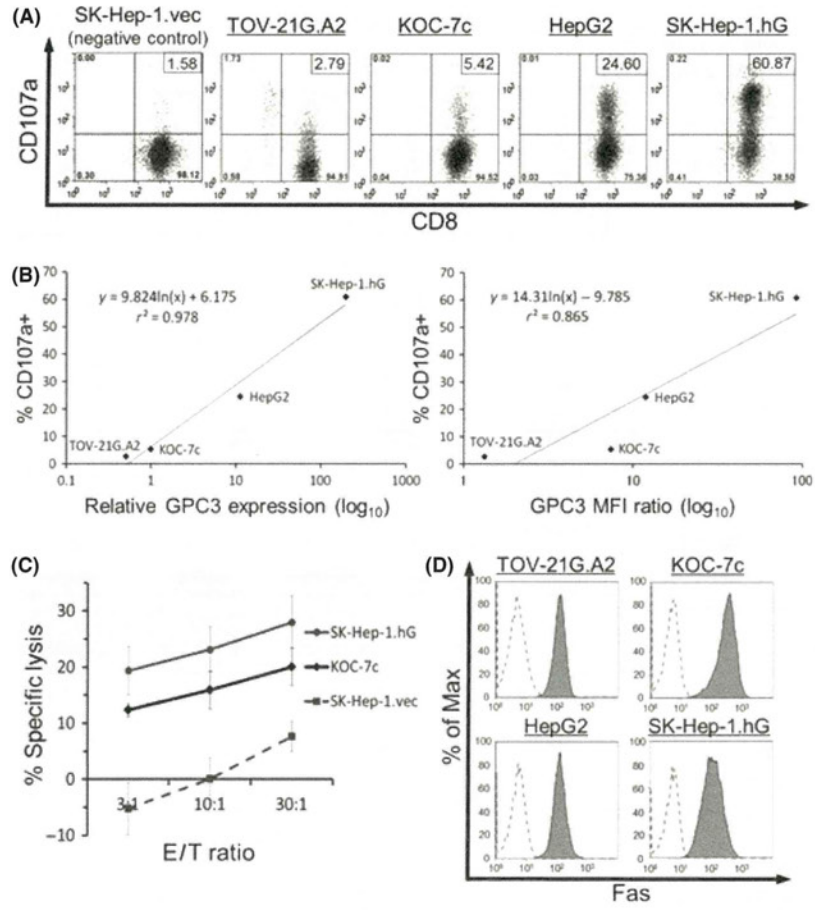

Fig. 2. $GPC3_{144-152}$ peptide-specific CTL clone lyses ovarian clear cell carcinoma (CCC) cell lines. (A) CD107a degranulation assay. Representative data are shown. GPC3-specific CTL responses against TOV-21G.A2 and KOC-7c cells exhibited 2.79% and 5.42% CD107a staining, respectively. (B) CD107a degranulation was correlated with relative GPC3 mRNA expression and GPC3 mean fluorescence intensity (MFI) ratio in cell lines (r^2 = 0.978 and 0.865, respectively). (C) Cytotoxicity (4 h) assay was performed at three effector/target ratios. We used SK-Hep-1.hG as a positive control. SK-Hep-1.vec cells were used as a negative control. The CTL clone showed specific cytotoxicity against KOC-7c cells. Data represent the mean ± SD. (D) Flow cytometry analysis of Fas expression on cancer cell lines. In all cell lines, Fas expression was sufficient (dashed line, unlabelled control; gray-filled area, PE-Fas staining).

lysis of the CTL clone against KOC-7c GPC3-shRNA and KOC-7c GPC3 Neg-shRNA cells. IFN-γ production was significantly decreased by GPC3 shRNA ($P = 0.004$) (Fig. 4A). GPC3-specific cytotoxicity was reduced against KOC-7c GPC3-shRNA cells compared with KOC-7c Neg-shRNA cells (Fig. 4B). These results indicate that HLA-A2-restricted $GPC3_{144-152}$ peptide could be processed naturally by ovarian CCC cells, and the peptides in the context of HLA-A2 could be expressed on the surface of ovarian CCC cells.

Level of GPC3 expression on the cell surface is related to $GPC3_{144-152}$ peptide-specific CTL clone recognition. To confirm that the level of GPC3 expression on the cell surface is responsible for CTL recognition, KOC-7c GPC3 high and low cells were sorted by FACSAria II (Fig. 5A). As shown in Figure 5B, KOC-7c GPC3 high cells expressed higher mRNA of GPC3 than GPC3 low cells. Figure 5C shows the IFN-γ release of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c wild type, GPC3 high and GPC3 low cells. There were significant differences in IFN-γ production between the three populations ($P < 0.001$). GPC3-specific cytotoxicity was increased against KOC-7c GPC3 high cells compared with GPC3 low cells in a cytotoxicity assay without cold target cells. In a cold target inhibition assay, cytotoxicity against KOC-7c GPC3 high cells was suppressed by the addition of $GPC3_{144-152}$ peptide-pulsed T2 cells but not by the addition of HIV_{19-27} peptide-pulsed T2 cells, even though cytotoxicity against KOC-7c GPC3 low cells was not changed by T2 pulsed with either $GPC3_{144-152}$ or HIV_{19-27} peptide (Fig. 5D).

Chemotherapy sensitizes KOC-7c cells to the cytotoxic effect of $GPC3_{144-152}$ peptide-specific CTL clone. Taxane plus platinum combination chemotherapy is generally considered to be the ''gold standard'' regimen for treatment of EOC. As PTX and CDDP have different mechanisms of action, we chose these two agents to investigate whether they sensitize ovarian CCC cells to GPC3-specific lysis. To evaluate the subtoxic dose of each drug, we assessed growth inhibition and apoptosis assays by FCM using Annexin V and PI staining. Growth-inhibitory effects were observed for treatment with either PTX or CDDP alone in a time- and dose-dependent manner. We calculated the 25% inhibitory concentration (IC25) of each drug as the minimum cytotoxic condition and regarded lower values as the subtoxic dose. The IC25 values of PTX and CDDP for 18 h were 22.8 ng/mL and 6.2 μg/mL, respectively (Fig. 6A). Exposure of CTL clone or KOC-7c cells to PTX (10 ng/mL) or CDDP (1 μg/mL) for 18 h had no significant cytotoxic effect, as determined by apoptosis assay. In other words, cell viability in untreated and PTX- and CDDP-treated groups of CTL clone or KOC-7c cells exceeded 95% in all cases (Fig. 6B). These

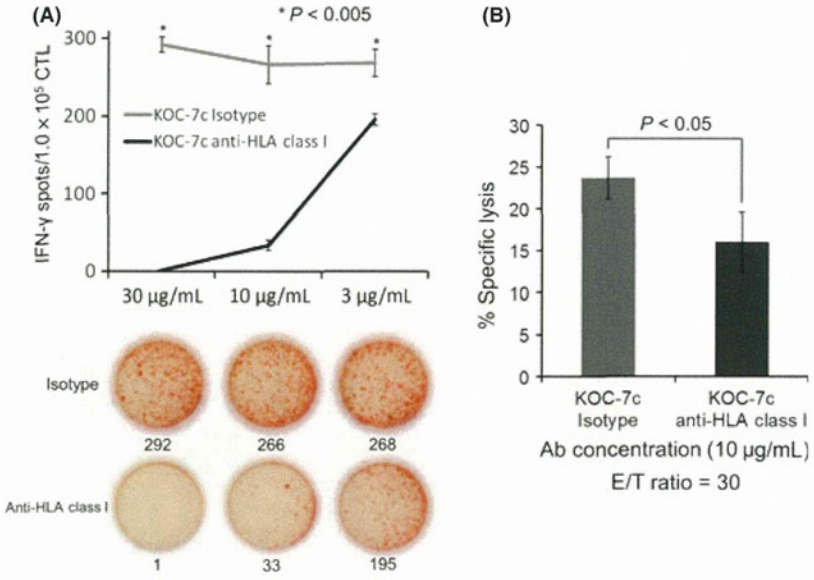

Fig. 3. Analysis of HLA class I restriction. (A) Inhibition of IFN-γ production by anti-HLA class I mAb. Effector/target ratio = 2. Data represent the mean ± SD of six wells. IFN-γ production of the CTL clone was markedly inhibited by anti-HLA class I mAb compared with that by isotype control in a concentration-dependent manner (*$P < 0.005$). (B) Inhibition of cytotoxicity by anti-HLA class I mAb. Effector/target (E/T) ratio = 30. Ab concentration = 10 μg/mL. Data represent the mean ± SD from the 4 h cytotoxicity assay. Cytotoxicity could be inhibited by anti-HLA class I mAb but not by isotype control ($P < 0.05$).

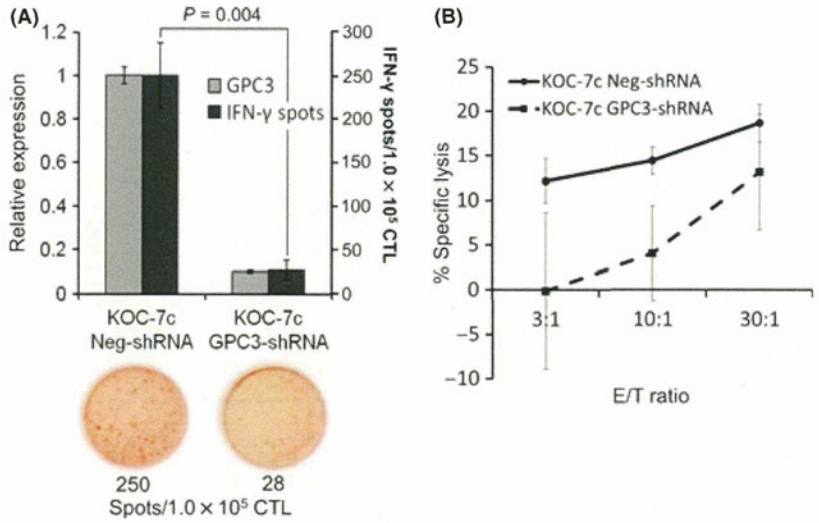

Fig. 4. Effect of GPC3 silencing using shRNA on the response of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c cells. (A) GPC3 expression of KOC-7c was obviously decreased by GPC3 shRNA on qRT-PCR. IFN-γ production was significantly decreased by GPC3 shRNA ($P = 0.004$). Data represent the mean ± SD. Effector/target (E/T) ratio = 2. (B) KOC-7c GPC3-shRNA cells were less cytolytic than KOC-7c Neg-shRNA cells. Data represent the mean ± SD from the 4 h cytotoxicity assay.

conditions excluded direct cytoxic effects of the compounds and effects as a subtoxic dose. In contrast, PTX (10 ng/mL) or CDDP (1 μg/mL) for 48 h showed mild cytotoxicity (basal levels of apoptosis >5%), and PTX (1 μg/mL) or CDDP (10 μg/mL) for 18 h induced substantial cell death (data not shown). KOC-7c cells were exposed to the subtoxic dose of each drug for 18 h and then examined by cytotoxicity assay. Pretreatment of KOC-7c cells with PTX (10 ng/mL) or CDDP (1 μg/mL) significantly increased CTL-mediated cytotoxicity of target cells (Fig. 6C). In all experiments, the level of spontaneous calcein release of target cells treated with chemotherapeutic agents was similar to that of untreated cells.

Discussion

Ovarian CCC has a poor prognosis due to low sensitivity to conventional chemotherapy.[1,3] To improve the prognosis, strategies are needed to efficiently kill all cancer cells by surgery and chemotherapy, as well as to stimulate the immune response to keep residual tumor cells in check. Thus, effective novel treatment strategies combined with surgery and chemotherapy are needed for treating ovarian CCC. Cancer vaccines are an attractive approach because of their low toxicity.

In previous studies, GPC3 was overexpressed in several malignant tumors, including ovarian CCC.[6–10] GPC3 is useful

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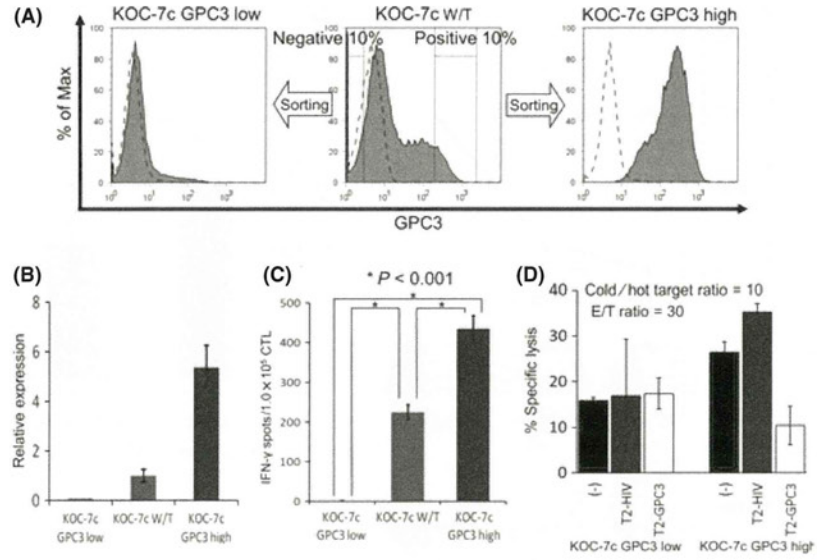

Fig. 5. The level of GPC3 expression on the cell surface is responsible for CTL recognition. (A) KOC-7c GPC3 high and GPC3 low cells were sorted as described in the Materials and Methods. (B) Relative GPC3 mRNA expression (ratio to KOC-7c wild type) is shown. Data represent the mean ± SD. (C) IFN-γ production of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c wild type, GPC3 high and GPC3 low cells. There were significant differences between the three populations (*$P < 0.001$). Mean ± SD of six wells is shown. (D) Cold target inhibition assay of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c GPC3 high and GPC3 low cells. Effector/target (E/T) ratio = 30. T2 was prepulsed with either HIV_{19-27} peptide or $GPC3_{144-152}$ peptide and then used as cold target cells. Cold/hot target ratio = 10. Cytotoxicity of the CTL clone against KOC-7c GPC3 high cells was inhibited by the addition of $GPC3_{144-152}$ peptide-pulsed T2 cells but not by the addition of HIV_{19-27} peptide-pulsed T2 cells. In contrast, cytotoxicity against the KOC-7c GPC3 low cells was not suppressed by T2 pulsed with either $GPC3_{144-152}$ or HIV_{19-27} peptide. Data represent the mean ± SD from the 4 h cytotoxicity assay.

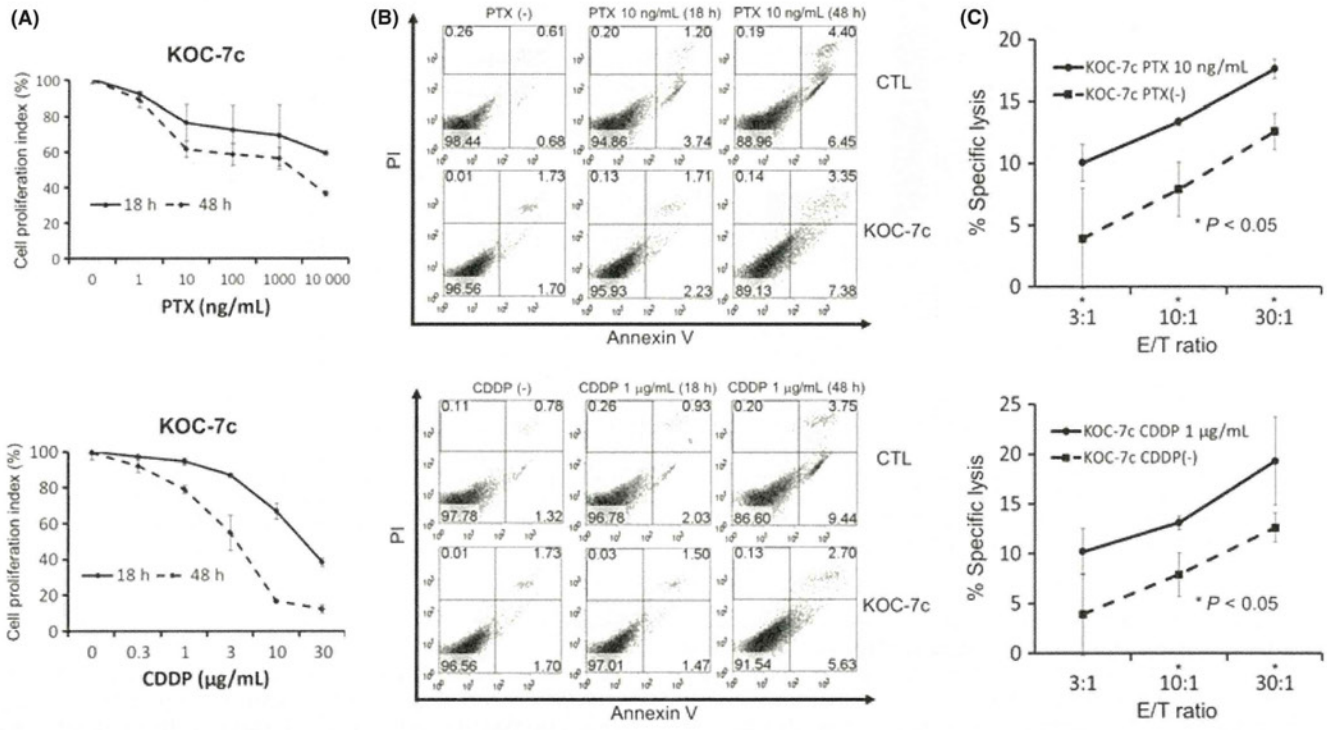

Fig. 6. Subtoxic-dose chemotherapy sensitizes KOC-7c cells to the cytotoxic effect of the $GPC3_{144-152}$ peptide-specific CTL clone. We used two agents (paclitaxel [PTX] and cisplatin [CDDP]) to investigate whether they sensitize ovarian clear cell carcinoma (CCC) cells to GPC3-specific lysis. (A) Growth-inhibitory effects were observed for treatment with each drug alone in a time- and dose-dependent manner. Data represent the mean ± SD. (B) Apoptosis analysis by flow cytometry analysis. Representative data are shown. The numbers in each quadrant represent the percentage of cells in the quadrant. Exposure of CTL clone or KOC-7c cells to PTX (10 ng/mL) or CDDP (1 μg/mL) for 18 h had no significant cytotoxic effect. By contrast, PTX (10 ng/mL) or CDDP (1 μg/mL) for 48 h showed mild cytotoxicity. (C) KOC-7c cells were pretreated with the subtoxic dose of each drug for 18 h and then a cytotoxicity assay (4 h) was performed. Pretreatment of KOC-7c cells with PTX (10 ng/mL) or CDDP (1 μg/mL) significantly increased CTL-mediated cytotoxicity of target cells (*$P < 0.05$). Data represent the mean ± SD.

as a novel biomarker and oncofetal antigen for immunotherapy.[15–22] However, association of ovarian CCC with CTL recognition has not been performed, hindering the selection of appropriate candidates for GPC3-specific immunotherapy. We recently established HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clones.[12] In the present study, we analyzed the IFN-γ production and cytotoxicity of an established CTL clone against ovarian CCC cell lines expressing HLA-A0201 and GPC3. The $GPC3_{144-152}$ peptide-specific CTL clone could recognize HLA-A2-positive and GPC3-positive ovarian CCC cell lines, suggesting that ovarian CCC present endogenously processed $GPC3_{144-152}$ peptide. Even though the CTL clones recognized two ovarian CCC cell lines on the IFN-γ ELISPOT assay, they showed inefficient lysis against TOV-21G.A2 cells. This was not due to a low expression level of HLA-A2 molecules on the cell surface, because the tumor cells were lysed after being pulsed with the antigenic peptide (data not shown). We also confirmed that the level of antigen expression is important in GPC3-specific CTL recognition of malignant cells. Therefore, low-level expression of GPC3 on tumor cells might be insufficient for triggering CTL-mediated killing.

Recent clinical studies have reported high rates of objective clinical response when cancer vaccines are combined with chemotherapy in patients with various cancers.[23–27] To evaluate the feasibility of chemoimmunotherapy for ovarian CCC, we investigated the cytotoxic effect of subtoxic-dose PTX or CDDP combined with $GPC3_{144-152}$ peptide-specific CTL clone in the human ovarian CCC cell line KOC-7c. We found that chemotherapy made ovarian CCC cells more susceptible to the cytotoxic effect of the $GPC3_{144-152}$ peptide-specific CTL clone. Chemotherapeutic drugs generally suppress the immune function, and each drug has a different level of immune suppression. Therefore, combination therapy requires an optimal dose that does not suppress peptide-induced immune activation. Importantly, the synergistic cytotoxic effect remained when both CTL and tumor cells were pretreated with PTX or CDDP under identical conditions (data not shown). However, high-dose chemotherapy has been shown to be toxic and the synergistic effect increased slightly more compared with the subtoxic dose, therefore limiting its potential therapeutic usefulness *in vitro*. The mechanism of improvement in immunotherapy with chemotherapy remains unclear, but the two possible types of mechanism are: systemic factors and local tumor microenvironment factors. For example, possible systemic effects include the elimination of cells with immunosuppressive activity such as regulatory T cells[28] and myeloid-derived suppressor cells,[29] or improved cross-presentation of tumor antigens. Examples of possible local effects include the disruption of tumor stroma that results in improved penetration of CTL into the tumor site, increased permeability of tumor cells to CTL-derived granzymes via upregulation of mannose-6-phosphate (M6P) receptors on the surface of tumor cells,[30] increased expression of tumor-associated antigens by tumor cells or upregulation of Fas (and other death receptors) on tumor cells, or FasL on CTL, etc.[31,32] We performed experiments to address the change in permeability for GrzB and the expression of M6P receptors in KOC-7c cells pretreated with PTX or CDDP. However, both drugs had no significant effect on the expression of M6P receptors. Moreover, we could not confirm the mechanism through an increase in permeability to GrzB in CCC cell line KOC-7c cells. Paclitaxel is known to upregulate the expression of Fas on the surface of tumor cells, resulting in an increase in Fas–FasL interaction.[33] However, Fas expression was sufficient in ovarian CCC cell lines without chemotherapy, and both drugs had no significant effect on Fas expression. The threshold for Fas-induced apoptosis in ovarian CCC is high and/or Fas signaling in CCC is altered through unknown mechanisms. In addition, both drugs had no significant effect on GPC3 expression under subtoxic-dose conditions (data not shown).

In conclusion, the present study suggests that GPC3 could become an effective target for HLA-A2-restricted peptide vaccine therapy against ovarian CCC. Moreover, our data suggest the possibility of treating ovarian CCC patients by combining standard chemotherapy with relatively non-toxic and highly specific immunotherapy. We will clarify the mechanisms of this phenomenon in our next study.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest.

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ELSEVIER

Intracellular Interferon-γ Staining Analysis of Donor-Specific T-Cell Responses in Liver Transplant Recipients

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ABSTRACT

Objective. Biomarkers that accurately reflect, detect, and/or predict detrimental immune responses to grafts are important in organ transplantation. We established a new detection method for alloreactive T cells on the basis of intracellular staining for interferon (IFN)-γ, using CD40-activated B cells as stimulators, and assessed temporal changes in alloreactive T-cell frequencies in patients who received liver transplantation.

Methods. Peripheral blood mononuclear cells and CD40-activated B cells were used as responder and stimulator cells, respectively. The responder cells were cultured with the stimulator cells for 7 days, restimulated for 5 hours, and flow cytometrically tested by intracellular staining for IFN-γ.

Results. The relative postoperative-preoperative ratio of donor-specific $CD8^+$ T cells in the nonrejection group was significantly lower than that in the rejection group and found to be <1 in most individuals of the group throughout the postoperative periods, indicating an induction of donor-specific suppression of the $CD8^+$ T-cell responses. In contrast, such differences were not found in the donor-specific $CD4^+$ T cells. These results suggest that the relative postoperative–preoperative ratio of the donor-specific $CD8^+$ T cells is a good indicator of graft rejection.

Conclusion. We established a new flow cytometric method for the detection of alloreactive T cells by intracellular staining for IFN-γ, using CD40-activated B cells as stimulator cells. Using this system, we found that the relative postoperative–preoperative ratio of the donor-specific $CD8^+$ T cells is a possible evaluative indicator of the risk for graft rejection.

MOST HEPATIC allograft recipients have remained under long-term maintenance of immunosuppression, and the evaluation and the adjustment of the immunosuppressant levels are largely determined on the basis of empirical data.[1–3] In this regard, the development and validation of noninvasive biomarkers are important to determine the timing of withdrawal of immunosuppressive regimens, and several candidates for tolerance assays for this purpose have been reported.[3–5] Of these, the profiling of alloreactive T cells (cytotoxicity, surface phenotype, cytokine production, etc) reflects the potential direct donor-specific immune responses in the host.

Recent advances in immunology revealed that various types of T cells with distinct functions could be distinguished by the expression profiles of their cytokines, surface markers, and transcription factors.[6–9] T-cell alloreactivity has been measured in several ways, but flow cytometric analysis seems to bring us the most informative results because it can analyze these factors simultaneously.

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