Allogeneic MHC Gene Transfer Enhances Antitumor Activity of Allogeneic Hematopoietic Stem Cell Transplantation without Exacerbating Graft-versus-Host Disease

Masaki Ohashi,1,4 Akihiko Kobayashi,2 Hidehiko Hara,2 Yoshiaki Miura,1 Kimiko Yoshida,1 Miwa Kushida,2 Yoshinori Ikaraishi,3 Masaki Mandai,5 Masaki Kitajima,4 Teruhiko Yoshida,1 and Kazunori Aoki2

Abstract
Enhancement of the specific antitumor activity of allogeneic hematopoietic stem cell transplantation (alloHSCT) against solid cancers is a major issue in the clinical oncology. In this study, we examined whether intratumoral allogeneic MHC (alloMHC) gene transfer can enhance the recognition of tumor-associated antigens by donor T cells and augment the antitumor activity of alloHSCT. In minor histocompatibility antigen–mismatched alloHSCT (DBA/2→BALB/c: H-2d) recipients, alloMHC gene (H-2Kb) was transduced directly into a s.c. tumor of CT26 colon cancer cells. Because CT26 cells have an aggressive tumorigenicity in syngeneic BALB/c mice, an H-2Kb gene transfer provides only a limited antitumor effect after syngeneic (BALB/c→BALB/c) HSCT. By contrast, the H-2Kb gene transfer caused significant tumor suppression in the alloHSCT recipients, and this suppression was evident not only in the gene-transduced tumors but also in simultaneously inoculated distant tumors without gene transduction. In vitro cytotoxicity assay showed specific tumor cell lysis by donor T cells responding to the H-2Kb gene transfer. Graft-versus-host disease was not exacerbated serologically or clinically in the treated mice, demonstrating that alloMHC gene transfer enhances the antitumor effects of alloHSCT without exacerbating graft-versus-host disease. This combination strategy has important implications for the development of therapies for human solid cancers.

Allogeneic hematopoietic stem cell transplantation (alloHSCT) often leads to a significant graft-versus-tumor (GVT) effect, and has proved to be an effective therapeutic approach for several types of leukemia, particularly acute and chronic myelogenous leukemia. Recently, alloHSCT has been applied not only for hematologic malignancies but also for solid cancers, such as renal and breast cancers (1–8). However, the benefit of the GVT effect is often offset by the occurrence of graft-versus-host disease (GVHD), a potentially fatal adverse effect primarily mediated by donor T cells. It is commonly believed that in MHC-matched alloHSCTs, the target antigens for a GVT effect include tumor-associated antigens (TAA) and ubiquitously or tissue-specifically expressed minor histocompatibility antigens (mHA), whereas the targets for GVHD are mHAs. Therefore, efforts to selectively enhance a donor T-cell response to TAs may provide a means to augment antitumor activity without a concomitant increase in toxicity.

Intratumoral transfer of an allogeneic MHC (alloMHC) gene modifies tumor cells to express the alloMHC molecule, a highly immunogenic antigen that causes an allogeneic rejection response. In the process of this response, cytolytic T lymphocytes are generated not only against the modified tumor cells but also against unmodified tumor cells (9). A putative mechanism for the induction of specific tumor immunity is that the allogeneic response increases local production of cytokines, facilitates antigen presentation and T-cell accumulation, and consequently causes sensitization to previously unrecognized TAs. Clinical trials with direct intratumoral injection of the human leukocyte antigen-B7 gene–expressing plasmid DNA complexed with liposome revealed a higher safety profile and systemic response in substantial populations (10-15%) in patients with melanoma and head and neck cancer (10–16). The treatment seemed to be promising but showed a limited clinical efficacy, for which the immune tolerance established between tumor and host may be one of the plausible explanations (17).

We expect that an alloMHC gene transfer could enhance the GVT effect by promoting recognition of TAs by the donor immune system in alloHSCT recipients, and also that alloHSCT, on the other hand, could augment the therapeutic efficacy of an alloMHC gene transfer by providing a "fresh"
immune system in which tolerance to tumor cells is not yet induced. In this study, using an MHC (H-2d)–matched mouse alloHSCT model, we found that an intratumoral alloMHC (H-2Kb) gene transfer significantly enhanced the antitumor effects of alloHSCT against a murine colon cancer. Importantly, GVHD was not exacerbated in any of the treated mice, suggesting the augmentation of tumor-specific immunity of donor T cells by the H-2Kb gene transfer.

Materials and Methods

Animals and transplantation. Seven- to 9-week-old female BALB/c (H-2d, Ly-1.2) and DBA/2 (H-2d, Ly-1.1) mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan) and were housed under sterilized conditions. Nine- to 10-week-old BALB/c mice received a lethal dose (9 Gγ) of total body irradiation on the day of transplantation. The irradiated BALB/c mice were injected i.v. with 5 × 106 of T cell–depleted bone marrow cells and 2 × 105 splenic T cells from donor DBA/2 or BALB/c mice in a total volume of 0.2 ml. Dulbecco’s PBS solution, and the transplanted mice were designated as alloBMT or synBMT, respectively. Bone marrow cells were isolated from donors by flushing each femur and tibia with RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum (ICN Biomedicals, Inc., Irvine, CA), and splenic cells were prepared by macerating the spleens with a pair of tweezers. After lysis of the erythrocytes, the bone marrow and splenic cells were incubated with anti-Thy-1.2 immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°C for 15 minutes, followed by depletion and selection of T cells by AutoMACS (Miltenyi Biotec), respectively. More than 90% of T cells were depleted from the bone marrow cells.

Tumor cell lines. CT26 and Renca are weakly immunogenic BALB/c–derived colon and renal carcinoma cell lines, respectively, and were obtained from the American Type Culture Collection (Rockville, MD). Both cell lines were confirmed to express MHC class I molecules (H-2Kd and H-2Dd) abundantly by flow cytometry (data not shown). Cells were maintained in RPMI containing 10% fetal bovine serum, 2 mmol/L L-glutamine, and 0.15% sodium bicarbonate (complete RPMI). A CT26 cell line that stably expresses the H-2Kb gene was generated by retrovirus vector-mediated transduction and designated as CT26/H-2Kb.

Fluorescence-activated cell sorting analysis. FITC-conjugated monoclonal antibodies (mAb) to identify mouse H-2Kb, CD4, CD8, and phycoerythrin-conjugated mAb to CD3 were purchased from BD Pharmingen (San Diego, CA), and FITC-conjugated mAbs to Ly-1.1 and Ly-1.2 were purchased from Meiji Dairies Co. (Tokyo, Japan). Cells were incubated with the relevant mAbs in a total volume of 100 ml PBS with 5% fetal bovine serum for 30 minutes at 4°C. Cells were then washed twice with PBS containing 5% fetal bovine serum, suspended in PBS, and analyzed by FACS Calibur (BD Biosciences, San Jose, CA). Irrelevant IgG mAbs were used as a negative control. Ten thousand live events were acquired for analysis. Allogeneic donor (DBA/2) T-cell engraftment was determined by the percentages of Ly-1.1 cells among CD3+ cells.

Evaluation of GVHD. The degree of clinical GVHD in transplant recipients was assessed weekly by a scoring system that sums changes in five variables: weight loss, posture, activity, fur texture, and skin integrity (maximum index, 10) as previously described (18). In some recipients, selected serum chemistry was also examined for evaluation of GVHD.

In vitro cell proliferation assay. CT26/H-2Kb and CT26 cells were seeded at 2 × 103 per well in 96-well flat plates (Nunc A/S, Roskilde, Denmark). Cell numbers were assessed by a colorimetric cell viability assay using a water-soluble tetrazolium salt (Tetracolor One; Seikagaku, Tokyo, Japan). The absorbance was determined by spectrophotometry using a wavelength of 450 nm with 595 nm as a reference using ELISA Analyzer (Toyo Sokki, Tokyo, Japan). The assays (carried out in eight wells) were repeated a minimum of twice and the means ± SD were plotted.

In vivo tumor inoculation and alloMHC gene transfer. Tumor cells were prepared in a total volume of 50 ml PBS and injected s.c. on the right or left leg. A plasmid DNA expressing the H-2Kb gene under the control of the Rous sarcoma virus long terminal repeat promoter was used for intratumoral gene transfer. The same vector plasmid DNA without a transgene was used as a negative control. Plasmid DNA-liposome complex per mouse was prepared by addition of 10 μg plasmid DNA into a total of 25 μl PBS, followed by the addition of 25 μl of 1.5 mmol/L DMRIE-DOPE [2-(+/-)-N-(2-hydroxyethyl)N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide/dioleoylphosphatidylethanolamine], which was provided from Vical, Inc. (San Diego, CA). The mixture solution was incubated at room temperature for 15 minutes, and then injected directly into the s.c. tumor. In previous report, presensitization of mice with alloMHC gene–expressing CT26 cells could maximize the therapeutic efficacy of alloMHC gene transfer (9). In this study also, preimmunization with CT26/H-2Kb followed by the treatment with H-2Kb DNA-liposome complex was done in some of the transplanted mice as an optimized therapeutic control. As a preimmunization treatment, 1 × 106 of 30 Gy–irradiated CT26/H-2Kb cells were i.p. injected twice (at 14 and 7 days) before tumor inoculation. The shortest (r) and longest (l) tumor diameters were measured at indicated days and the tumor volume was determined as r 2l/2. Data are presented as means ± SD.

Cytotoxicity assays. Using 24-well plates (Nunc), responder splenocytes (5 × 105/ml) were cultured with 30 Gy–irradiated CT26/H-2Kb stimulators (3-4 × 105/ml) in a complete RPMI containing 50 μmol/L 2-mercaptoethanol for 5 days. The responder cells were then collected and used as effector cells in a 4-hour chromium release assay against indicated target cells. Concanavalin A lymphoblasts were prepared by stimulating the splenocytes of naive BALB/c mice for 3 days with 5 μg/ml concanavalin A at 2 × 105/ml in the complete RPMI containing 2-mercaptoethanol. Indicated target cells were labeled by combining 5 × 106 cells with 50 μCi 51Cr (Perkin-Elmer Japan Co., Kanagawa, Japan) in a total volume of 0.2 ml complete RPMI for 1 hour at 37°C, followed by washing thrice with plain RPMI. For the chromium release assay, 5 × 105 effector cells were mixed with 2 × 104 target cells (effector-to-target ratio, 25) in a total volume of 0.2 ml complete RPMI in 96-well round-bottom plates (BD Biosciences). To evaluate the relative contributions of CD4+ and CD8+ T cells to tumor cell lysis, effector cells were incubated with mAb to mouse CD4 (L343; Pharmingen), CD8 (Ly-2; Pharmingen), or both for 30 minutes at 37°C before mixture with target cells. Supernatants were harvested with the Skatron harvesting system (Skatron, Sterling, VA) and counted in a gamma counter (Packard Bioscience Company, Meriden, CT). Percentage of cytolysis was calculated as [experimental cpm – spontaneous cpm] / [maximum cpm – spontaneous cpm] × 100. Spontaneous cpm was obtained from targets cultured in medium alone, and maximum cpm was obtained from targets incubated in 1% NP40. Each assay was done in triplicate.

In vivo depletion of T-cell function. To deplete specific immune effector cell subsets before and during treatment with H-2Kb gene transfer, the transplanted mice received i.p. injections of 0.3 mg mAbs from the anti-CD4+ hybridoma (clone GK1.5, rat IgG2b) and/or anti-CD8+ hybridoma (clone Lyt-2.1, mouse IgG3, ref. 19). Injections started 5 days before the inoculation with CT26 cells and the treatment repeated every 5 to 6 days throughout the entire experimental period to ensure depletion of the targeted cell type. CD4+ and CD8+ T-cell depletion was confirmed by flow cytometry of splenic suspensions at the time of tumor injection and weekly afterward.

Statistical analysis. Comparative analyses of the data were done by the Student’s t test using SPSS statistical software (SPSS Japan, Inc., Tokyo, Japan). P < 0.05 was considered as a significant difference.
Results

**AlloHSCT causes GVHD and GVT effects.** We first assessed the posttransplant immune reconstitution of T cells and donor chimerism of splenic CD3+ T cells in alloBMT recipients (DBA/2 → BALB/c). To exclude biases related to transplant procedures, including lethal irradiation, we conducted synBMT (BALB/c → BALB/c) as a control. The reconstitution of both CD4+ and CD8+ T cells was delayed in alloBMT recipients compared with that in synBMT recipients at 8 weeks posttransplantation (Fig. 1A), which was consistent with other reports (20–22). The early (≤2 weeks) posttransplant mortality, most likely due to acute GVHD or graft failure, was usually <15% in transplant recipients. Analysis of donor engraftment showed 95.7 ± 1.5% donor type in alloBMT recipients (n = 3) at 8 weeks posttransplantation.

We then examined whether our alloHSCT models generate any GVHD and GVT effects. The clinical score of GVHD severity at 8 weeks was ~4 in alloBMT recipients, whereas it was <1 in synBMT recipients (Fig. 1B). Death from GVHD was rarely observed during the first 3 months after the transplantation. For evaluation of GVT effects, 1 × 10^6 CT26 or 2 × 10^6 Renca cells were s.c. inoculated into the mice 8 to 9 weeks posttransplantation. The growth of the tumors was substantially, although not statistically significantly, suppressed in the alloBMT recipients compared with that in the synBMT recipients (Fig. 1C). Our alloHSCT model was shown to constantly cause GVHD and a limited but detectable level of the GVT effect, which is highly similar to a clinical setting after allogeneic HSCT.

**AlloMHC gene–transduced tumor cells cause an immune response in vivo.** To determine whether alloMHC gene–transduced CT26 cells could induce an immune response in
mice, the tumorigenicity of CT26/H-2Kb cells was compared with that of unmodified CT26 cells. Flow cytometric analysis confirmed the expression of the H-2Kb molecule in CT26/H-2Kb cells (Fig. 2A), and the in vitro proliferation of CT26/H-2Kb cells was compatible with that of unmodified CT26 cells (Fig. 2B). However, when injected s.c. into naïve BALB/c mice, CT26/H-2Kb cells did not form a tumor mass over 3 weeks (n = 5), whereas unmodified CT26 cells developed a rapidly growing tumor during the same period (n = 5; Fig. 2C), suggesting that the alloMHC gene–transduced tumor cells are highly antigenic and cause an immune response in vivo. No apparent toxicity was observed in the CT26/H-2Kb cell-inoculated mice.

AlloMHC gene transfer induces synergistic antitumor effect with alloHSCT. To examine whether alloMHC gene transfer could enhance the antitumor effects of alloHSCT, the mice were inoculated s.c. with 1 × 10^6 CT26 cells at 8 weeks posttransplantation, and H-2Kb–expressing plasmid DNA complexed with liposome was injected into the tumor thrice (at 5, 7, and 9 days) after the inoculation. The tumor volumes at day 5 were ~60 to 100 mm^3. Preimmunization with irradiated CT26/H-2Kb cells was carried out in a group of transplanted mice as a positive control to maximize the effect of the H-2Kb immune gene therapy (9). A single injection of plasmid DNA-liposome complexes showed ~1% gene transduction efficiency in vivo (data not shown). The control empty vector was not immunogenic in vivo because the tumor volume of the negative control group without any intratumoral injections was similar to that of the empty plasmid–injected group (Fig. 3A). In the CT26/H-2Kb–preimmunized mice, H-2Kb gene transfer caused significant tumor suppression after either type of transplantation, synBMT or alloBMT (Fig. 3A). In non-preimmunized mice, H-2Kb gene transfer showed only a limited suppressive effect in synBMT recipients, which was probably due to the aggressive tumorigenicity of CT26 cells in vivo, whereas significant suppression of tumor growths was recognized in alloBMT recipients (Fig. 3A). The results showed that alloMHC gene transfer can augment the antitumor effects of donor immune cells in the context of alloHSCT.
than CD8+ T cells (Fig. 3D). One possible explanation is that as direct cytotoxicity. The reason that tumor inhibition in mice enhanced of cellular immunity by interacting with antigen-
roles in the generation of antitumor immunity. Whereas CD8+
contributive to tumor cell lysis in alloBMT recipients (Fig. 3C).

In vivo cell populations treated with anti-CD4 or anti-CD8 antibodies to deplete these antitumor effect of alloMHC gene transfer, either with or without exposure to wild-type CT26 cells, still led to significant tumor suppression (Fig. 5), indicating that, at least in this HSCT model, the presence of tumor cells at the time of transplantation does not induce immune tolerance to tumor cells and thus does not reduce the efficacy of subsequent alloMHC gene transfer.

AlloMHC gene transfer does not exacerbate GVHD. Although the in vitro cytotoxicity assay showed induction of a tumor-specific cytolytic T-lymphocyte response by H-2Kb gene transfer, alloMHC expression in tumor cells could theoretically promote a donor T-cell response not only against TAAs but also against mHAs shared by tumor and normal cells, which might result in GVHD exacerbation. We thus examined serum chemistry and the clinical GVHD score in the transplanted mice with the H-2Kb gene transfer. Albumin, total bilirubin, aspartate

Fig. 4. Suppression of tumors at distant sites by the intratumoral injection of H-2Kb vector in alloBMT mice. CT26 cells were s.c. inoculated on both legs of alloBMT or synBMT recipients, and H-2Kb vector were injected into only the right leg tumor thrice without preimmunization. Tumor volume at each leg was compared between the treatment groups at 12 days after the completion of vector injection (n = 7-11). *, P < 0.05. NS, not significant. Representative of at least three independent experiments.

In vitro cytotoxicity assay was done to assess antitumor cytolytic T-lymphocyte responses induced by alloMHC gene transfer in the non-preimmunized mice. The splenocytes derived from mice bearing H-2Kb-transduced tumors recognized and lysed unmodified CT26 cells as well as CT26/H-2Kb cells but not concanavalin A blasts, and the cytotoxic activity was higher in the alloBMT recipients than in the synBMT recipients (Fig. 3B). Splenocytes showed higher cytolytic response to CT26/H-2Kb than to CT26 cells, indicating that the donor T cells recognize the H-2Kb molecule as an allogeneic antigen.

Both CD4+ and CD8+ T cells contribute to the antitumor immunity. Then, in the in vitro blocking assays of lymphocyte cytotoxicity with antimurine CD4 and CD8 antibodies, CD8+ T cells were shown to be the dominant effector in synBMT recipients, whereas both CD4+ and CD8+ T cells were apparently contributive to tumor cell lysis in alloBMT recipients (Fig. 3C). To further explore the role of CD4+ and CD8+ T cells in antitumor immunity, the preimmunized alloBMT mice were treated with anti-CD4 or anti-CD8 antibodies to deplete these cell populations in vivo. The antitumor effect of H-2Kb gene transfer was completely cancelled in the transplanted mice with depletion of both CD4+ and CD8+ T cells, whereas the animals depleted of CD4+ or CD8+ T cells showed significant tumor growth inhibition (Fig. 3D). This in vivo depletion study indicated that the CD4+ and CD8+ cytotoxic T cells play central roles in the generation of antitumor immunity. Whereas CD8+ T cells were more contributive to in vitro tumor cell lysis than CD4+ T cells in the in vitro cytotoxicity assay (Fig. 3C), CD4+ T cells might be more contributive to in vivo tumor inhibition than CD8+ T cells (Fig. 3D). One possible explanation is that CD4+ T cells play a variety of roles in vivo, which include enhancement of cellular immunity by interacting with antigen-presenting cells and maintenance of immune memory, as well as direct cytotoxicity. The reason that tumor inhibition in mice depleted of only CD8+ T cells was compatible with that in mice without any T-cell depletion might be that the antitumor effect induced by the cooperative effect of CD4+ and CD8+ cytotoxic T cells was already saturated in the CT26 xenograft model.

AlloMHC gene transfer causes growth suppression of both local and distant tumors in alloHSCT recipients. Next, to evaluate the therapeutic efficacy of alloMHC gene transfer for tumors at distant sites, transplant recipients were s.c. inoculated with 1 × 10^6 CT26 cells on the right leg and, 5 days later, inoculated with 5 × 10^5 CT26 cells on the left leg. On the right leg, tumor was then transduced with H-2Kb gene thrice. In alloHSCT recipients, significant tumor suppression of the treated tumor on the right leg and the untreated tumor on the opposite leg was observed (Fig. 4), which showed that alloMHC gene transfer causes a systemic antitumor immunity in alloHSCT recipients.

Presence of tumor cells at the time of transplantation does not reduce therapeutic efficacy of alloMHC gene transfer. In these experiments, the recipient immune system was reconstituted in the absence of any tumor. To simulate a clinical situation in which patients have residual tumors at the time of alloHSCT, we injected 1 × 10^6 of 200 Gy–irradiated CT26 cells i.p. into alloBMT recipients at the time of transplantation (day 0) and also at 7 and 14 days posttransplantation. The mice were inoculated with 1 × 10^6 wild-type CT26 cells at 8 weeks after the transplantation and then injected with H-2Kb plasmid complexed with liposome. It was conceivable that, in this condition, the donor-derived immune system might acquire tolerance to TAAs during its reconstitution. Nonetheless, H-2Kb gene transfer, without any T-cell depletion might be more contributive to tumor cell lysis than to CT26 cells, indicating the donor T cells recognize the H-2Kb molecule as an allogeneic antigen.

//Insert Figure 5 here.//

Fig. 5. Preservation of antitumor activity of H-2Kb gene transfer in alloBMT recipients with tumor present during the immune reconstitution. Irradiated CT26 cells were i.p. injected at the time of transplantation, and 8 weeks after the transplantation the CT26 s.c. tumor was injected with H-2Kb vector thrice in alloBMT recipients. Tumor volume was compared between the groups at 11 days after the completion of vector injection (n = 6-8). *, P < 0.05.
Tumor-specific immunity induced by alloMHC gene transfer is long-lasting in vivo. The CT26 s.c. tumors disappeared in 10% to 20% of alloBMT mice by alloMHC gene transfer. To examine in vivo longevity of the tumor-specific immunity, a total of 11 alloBMT recipients who survived the initial CT26 challenge with complete tumor remission by H-2Kb gene transfer were again inoculated with CT26 cells on the right leg and Renca cells on the left leg. Of the 11 mice, eight had been i.p. injected with irradiated CT26 cells at the time of transplantation and three had not. Renca cells formed a tumor mass in all the mice, whereas CT26 cells were rejected in 10 of 11 mice (91%; Table 2), suggesting that TAAs and/or tissue-restricted mHAs are different between CT26 and Renca cells and that the H-2Kb gene transfer to CT26 cells increases the recognition of CT26-specific antigens by donor T cells. In vitro cytotoxicity assay also showed lysis of CT26 but not Renca cells or concanavalin A lymphoblasts (data not shown). The results showed that the tumor-specific immunity induced by alloMHC gene transfer is potentially long lasting in HSCT recipients.

**Table 1.** Selected serum chemistry and clinical GVHD score

<table>
<thead>
<tr>
<th></th>
<th>CT26/H-2Kb → H-2Kb</th>
<th>→ H-2Kb</th>
<th>→ Control</th>
<th>P</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AlloBMT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td>3.04 ± 0.22</td>
<td>3.2 ± 0.14</td>
<td>3.06 ± 0.28</td>
<td>NS</td>
<td>2.95 ± 0.23</td>
</tr>
<tr>
<td>TB (mg/dL)</td>
<td>0.052 ± 0.03</td>
<td>0.036 ± 0.009</td>
<td>0.05 ± 0.014</td>
<td>NS</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>239 ± 47</td>
<td>269 ± 56</td>
<td>314 ± 83</td>
<td>NS</td>
<td>81 ± 11</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>153 ± 33</td>
<td>177 ± 43</td>
<td>171 ± 52</td>
<td>NS</td>
<td>41 ± 14</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>428 ± 140</td>
<td>448 ± 110</td>
<td>397 ± 93</td>
<td>NS</td>
<td>365 ± 46</td>
</tr>
<tr>
<td>GVHD score</td>
<td>5.6 ± 0.5</td>
<td>5.4 ± 0.5</td>
<td>5.4 ± 0.9</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td><strong>SynBMT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td>3 ± 0.32</td>
<td>3.02 ± 0.25</td>
<td>2.92 ± 0.11</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>TB (mg/dL)</td>
<td>0.068 ± 0.023</td>
<td>0.046 ± 0.019</td>
<td>0.04 ± 0.024</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>210 ± 34</td>
<td>163 ± 57</td>
<td>213 ± 43</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>88 ± 32</td>
<td>94 ± 44</td>
<td>110 ± 38</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>267 ± 95</td>
<td>286 ± 60</td>
<td>295 ± 86</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>GVHD score</td>
<td>2.2 ± 0.8</td>
<td>2 ± 0.7</td>
<td>2.2 ± 0.8</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: H-2Kb-expressing or control vector was intratumorally injected thrice in alloBMT or synBMT recipients with or without preimmunization, and 15 days later the serum chemistry and clinical GVHD score were evaluated (n = 5 per group). "CT26/H-2Kb → H-2Kb" and "→ H-2Kb" indicate H-2Kb gene transfer with and without preimmunization, respectively.

Abreviations: ALB, albumin; TB, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; NS, not significant.

**Table 2.** Tumorigenicity of CT26 and Renca cells in alloBMT recipients cured of initial CT26 challenge

<table>
<thead>
<tr>
<th>Exposure of CT26 cells</th>
<th>CT26(right leg)</th>
<th>Renca(left leg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>1/8</td>
<td>8/8</td>
</tr>
<tr>
<td>−</td>
<td>0/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Total</td>
<td>1/11 (9%)</td>
<td>11/11 (100%)</td>
</tr>
</tbody>
</table>

NOTE: Eleven alloBMT recipients who survived the initial CT26 challenge with complete tumor remission by H-2Kb gene transfer were again inoculated with CT26 cells and Renca cells. Of the 11 mice, eight had been i.p. injected with irradiated CT26 cells at the time of transplantation and three had not.

**Discussion**

Several experimental investigations have been made thus far to separate a desirable GVT effect for hematologic or solid malignancies from an undesirable GVHD in alloHSCT recipients. Delayed donor leukocyte infusions into the recipients with mixed chimerism (23, 24), expansion and reinfusion of GVT-specific donor T-cell clones (25, 26), posttransplant immunization of the recipients with tumor cell vaccines (27–29), and pretransplant tumor antigen–specific immunization of the donors (30) were reported to have the potential to selectively enhance the GVT effect. On the other hand, administration of interleukin-11 (31), infusion of CD4+CD25+ regulatory T cells (32, 33), blockade of the function of donor antigen-presenting cells (34), and blockade of the GVHD-specific cytotoxic pathway (Fas-Fas ligand or perforin-granzyme; ref. 35) were shown to be capable of preventing GVHD.
while maintaining a GVT effect. In the present study, we showed that intratumoral alloMHC gene transfer could enhance the GVT effect without exacerbating GVHD by inducing systemic tumor-specific immunity in MHC-matched alloHSCT recipients.

Although there have been several animal studies showing the potential efficacy of a combination of alloHSCT and gene-based immunotherapy, such as a tumor vaccine using granulocyte macrophage colony-stimulating factor and interleukin 2/herpes simplex thymidine kinase genes (27–29), the reports only showed that the antitumor immune activity of tumor vaccines could be reproduced in the context of allogeneic transplantation. To our knowledge, ours is the first report that showed a synergistic antitumor effect of immune gene therapy combined with alloHSCT.

The mechanism for the synergism is yet unclear, but a mixed allogeneic and rejection reactions induced by the combination therapy may be of major importance in creating an environment strongly supporting the activation of an antitumor response. Although the recognition of tumor antigens by donor T cells was not strong enough to induce a significant antitumor immune response in the mice treated with alloHSCT alone, the combination with the alloMHC gene transfer may enhance (a) trafficking of immune cells into the tumor, (b) local production of various cytokines at the tumor site, and (c) presentation of tumor antigenic peptides on antigen-presenting cells through uptake of apoptotic tumor cell bodies induced by the alloMHC gene expression. These effects may facilitate the increased recognition of previously unrecognized or weakly recognized tumor antigens by donor T cells, which leads to a significant tumor specific immunity (36, 37). Furthermore, several murine bone marrow transplantation models have shown that CD4+ and CD8+ T cells both contributed to GVHD through their cytolytic activity (31, 38, 39), and in this study also, although the CD8+ T cells seemed to be major effectors of antitumor immunity by the H-2Kb gene transfer in naïve and synBMT mice (Fig. 3C; ref. 9), the combination of alloHSCT and allo-MHC gene transfer was able to induce effective cooperation of CD4+ and CD8+ cytotoxic T cells in tumor cell killing (Fig. 3C and D). The CD4+ T cells may have an important role in the antitumor immunity as well as GVHD in allogeneic HSCT.

In this study, the tumor cells were inoculated into the mice 8 weeks after the transplantation because the CT26 cell–injected BALB/c mice could not survive >2 months due to the aggressive tumorigenicity of the cell. On the other hand, we need at least a 6-week interval between alloHSCT and the immune therapy to allow a sufficient immune reconstitution necessary for the evaluation of the immune therapy. This experiment model does not exactly replicate the clinical situations in which the recipients usually harbor the relapse or residual cancer cells at the time of HSCT, and one could argue that the immune reconstitution in the presence of tumor cells might induce the acquisition of tolerance to tumor antigens. Therefore, we injected the irradiated CT26 cells at the initial phase after transplantation, and confirmed that the H-2Kb gene transfer elicited effective tumor suppression even in the HSCT recipients exposed to tumor cells during immune reconstitution, suggesting that the alloMHC gene transfer with alloHSCT is a promising therapeutic strategy in clinical setting. As a next step, a combination with other approaches, such as donor lymphocyte infusion and preimmunization with an alloMHC gene–expressing plasmid, might be examined for whether they can further enhance the antitumor effects of alloMHC gene transfer with alloHSCT.

The expression of alloMHC in tumor cells could theoretically promote a donor T-cell response not only for TAAs but also for mHAs shared by tumor and normal host cells, which may cause GVHD. However, in our study, the alloMHC gene transfer did not exacerbate serum enzymes and clinical GVHD scores in alloHSCT recipients. It is possible that much of the immune response was directed against nonimmunodominant mHAs with restricted tissue distribution or possibly even against TAAs, not against immunodominant mHAs. Other reports have also shown that immunization of alloHSCT recipients with a tumor cell vaccine substantially increased GVT activity of donor lymphocytes without exacerbating GVHD (27–30). Luznik et al. hypothesized that the immunogenic antigen-presenting cells at the vaccine site capture both TAAs and mHAs from tumor cells and promote tumor-specific immunity and GVHD, whereas away from the vaccine site, resting host antigen-presenting cells presenting mHAs induce tolerance in or exhaust alloreactive donor T cells (29, 40). Alloreactive T cells that have been activated at the vaccine site may subsequently become unresponsive to the immunodominant mHAs following an encounter with resting host antigen-presenting cells, whereas tumor-specific T cells would be expected to persist in activated or long-lived memory states. Another possible explanation for the GVH preference over GVHD might be that a major part of potential immunogenic antigens on CT26 tumor cells is TAAs, and indeed it was reported that CT26 cells express high levels of an H-2Ld-restricted peptide (AH1) from an endogenous retrovirus, and induce a robust AH-1-specific T-cell response (29, 41). Although we have shown an advantage and safety of the combination therapy in the preclinical study, CT26 cells may not be representative of all clinical human cancers and the occurrence or exacerbation of GVHD should be evaluated carefully in the future stage of the therapeutic development in a clinical study.

Because myeloablative conditioning is associated with a considerable risk of morbidity and mortality and because solid tumors, such as renal cancer, are typically refractory to chemotherapy, the nonmyeloablative alloHSCT is clinically applied against solid tumors (1, 2, 4). Nonmyeloablative alloHSCT often results in a mixed T-cell chimerism, and a donor leukocyte infusion is done for patients with mixed chimerism to achieve exclusively donor-derived hematopoiesis because the establishment of a complete donor chimerism is considered to be crucial for drawing the optimal GVT effect (42, 43). In this study, we used a myeloablative conditioning model to constantly achieve the full donor chimerism, because our primary purpose was to determine the effect of the immune gene therapy on alloHSCT and the full-blown effect after alloHSCT (i.e., complete chimerism). In the clinical setting, the nonmyeloablative conditioning may be optimal to test the potential of the combination therapy to reduce the regimen-related toxicity.

**Acknowledgments**

We thank Dr. Gary J. Nabel (Vaccine Research Center, National Institutes of Health, Bethesda, MD) for providing the H-2Kb expression plasmid and for useful discussions, Tomomi Ikeda for technical support, and Vical Incorporated for providing the liposome DMRIE/DOPE.
References


Allogeneic MHC Gene Transfer Enhances Antitumor Activity of Allogeneic Hematopoietic Stem Cell Transplantation without Exacerbating Graft-versus-Host Disease
