Allogeneic MHC Gene Transfer Enhances an Effective Antitumor Immunity in the Early Period of Autologous Hematopoietic Stem Cell Transplantation

Akihiko Kobayashi,1,3 Hidehiko Hara,1 Masaki Ohashi,2 Takeshi Nishimoto,1 Kimiko Yoshida,1 Nobuhiro Ohkohchi,3 Teruhiko Yoshida,2 and Kazunori Aoki1

Abstract

Purpose: In autologous hematopoietic stem cell transplantation (HSCT), lymphopenia-induced homeostatic proliferation of T cells is driven by the recognition of self-antigens, and there is an opportunity to skew the T-cell repertoire during the T-cell recovery by engaging tumor-associated antigens, leading to a break of tolerance against tumors. However, the homeostatic proliferation – driven antitumor responses seem to decline rapidly in association with tumor growth. We hypothesized that a tumor-specific immune response induced by an immune gene therapy could enhance and sustain homeostatic proliferation – induced antitumor immunity.

Experimental Design: The antitumor effect of allogeneic MHC (alloMHC) gene transfer was examined at the early phase of the immune reconstitution after syngeneic HSCT.

Results: Syngeneic HSCT showed significant tumor growth inhibition of syngeneic colon cancer cells within a period of 30 days; however, the tumor then resumed rapid growth and the survival of the mice was not prolonged. In contrast, when the alloMHC plasmid was intratumorally injected at the early phase after syngeneic HSCT, the established tumors were markedly regressed and the survival of recipient mice was prolonged without significant toxicities, whereas no survival advantage was recognized in recipient mice injected with a control plasmid. This tumor suppression was evident even in the other tumors that were not injected with the alloMHC plasmid. The antitumor response was characterized by the development of tumor-specific T cell – and natural killer cell – mediated cytotoxicities.

Conclusion: The results suggest the efficacy and safety of integrating intratumoral alloMHC gene transfer with an autologous HSCT for the treatment of solid cancers.

The central objective of cancer immunotherapy is to induce and sustain a tumor-specific immune response; that is, an in vivo generation of a large number of highly reactive antitumor lymphocytes that are not restrained by normal or cancer-induced tolerance mechanisms. One of the most promising approaches to achieve this objective is based on the recognition of tumor-associated antigens (TAA) by T cells (1).

It is well known that lymphopenia is followed by spontaneous expansion of the remaining T cells in the periphery to restore the original T-cell pool size and maintain homeostasis. Lymphopenia-induced homeostatic proliferation of T cells is driven by the recognition of self-MHC/peptide ligands in the absence of foreign antigens or inflammatory signals (2). Although TAAs are usually a poor target under lymphocyte-sufficient conditions because they are often self-antigens with low-affinity epitopes, lymphopenic conditions seem to be able to create an environment to mount an efficient antitumor immunity through the homeostatic proliferation – induced expansion of autoreactive T cells as shown in a variety of animal tumor models (3–7). However, several studies have also shown that the integration of other immunotherapeutic strategies might be necessary to successfully eradicate preexisting malignant tumors, because homeostatic proliferation – driven antitumor responses seem to rapidly decline in association with tumor growth (8).

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 TAAs. The first critical step of this immune gene therapy is to induce a robust immune reaction at the tumor sites. Because ~1% to 10% of T cells are considered to be alloreactive and the expression of alloMHC class I gene could induce strong allogeneic reactions in the injected tumor site, the transgene suits the aim of this therapy. Clinical trials with direct intratumoral injection of the human leukocyte antigen-B7 gene-expressing plasmid DNA complexed with liposome revealed an excellent safety profile and an objective systemic response in a substantial fraction (10-15%) of patients with melanoma and head and neck cancer (10–14). The treatment thus seemed to be promising, but at the same time it turned out to have some limitations of clinical efficacy (15).

Recently, we reported that the antitumor effect of intratumoral alloMHC gene transfer significantly augmented the graft-versus-tumor effect of allogeneic hematopoietic stem cell transplantation (HSCT) without exacerbating graft-versus-host disease. However, the graft-versus-host disease still remained as a severe adverse effect in the treated mice (16). On the other hand, autologous HSCT has several important advantages over allogeneic HSCT, such as safety and independence of donor availability. In this study, we examined whether homeostatic proliferation-induced antitumor activity can be enhanced by a strong exposure of TAAs to donor T cells by alloMHC gene transfer during the physiologic immune reconstitution after lymphocyte depletion. Furthermore, tumors often establish an immunotolerant environment with immunoinhibitory cytokines and regulatory T cells (17). The conditioning of HSCT with irradiation and/or immunosuppressive reagents can destroy such immunotolerance mechanisms deployed by the tumor. The “resetting” and rapid expansion of a fresh and re-established immune system, in which tolerance to tumor cells is not yet induced, may be able to augment the efficacy of immune therapies.

**Materials and Methods**

**Animals and transplantation.** Seven to 9-week-old female BALB/c (H-2d, Ly-1.2) mice were purchased from Charles River Japan, Inc., and were housed under sterilized conditions. Animal studies were carried out according to the Guideline for Animal Experiments of the National Cancer Center Research Institute and approved by the Institutional Committee for Ethics in Animal Experimentation. BALB/c mice received a lethal dose (9 Gy) of total body irradiation on the day of transplantation. The irradiated mice were injected i.v. with 5 × 10⁶ of T cell–depleted bone marrow cells and 2 × 10⁶ splenic T cells from donor BALB/c mice in a total volume of 0.2 mL Dulbecco’s PBS solution (Nissui Pharmaceutical Co.). Bone marrow cells were isolated from donors by flushing each femur and tibia with RPMI 1640 (Nissui Pharmaceutical Co.) supplemented with 5% fetal bovine serum (ICN Biomedicals, Inc.), 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) at 4°C for 15 min, followed by depletion or selection of T cells by AutoMACS (Miltenyi Biotec), respectively. More than 90% of the T cells were depleted from the bone marrow cells.

**Tumor cell lines.** CT26 and Renca cells are weakly immunogenic BALB/c-derived colon and renal cancer cell lines, respectively, and were obtained from the American Type Culture Collection. 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 (16).

**In vivo alloMHC gene transfer and preimmunization.** Tumor cells were prepared in a total volume of 50 μL 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 (empty) was used as a negative control. Plasmid DNA-liposome complex per mouse was prepared by the addition of 10 μg plasmid DNA into a total of 25 μL PBS, followed by the addition of 25 μL of 0.15 mmol/L DMRIE/DOPE ([+/-]-N,N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide/dioleoyl-phosphatidylethanolamine), which was provided from Vical, Inc. The mixture solution was incubated at room temperature for 15 min, and then injected directly into the tumor.

A preimmunization treatment with the plasmid DNA expressing H-2Kb gene to prime the allogeneic antigen-reactive cytolytic T-lymphocyte was done either by i.p. injection of 1 × 10⁶ of 30 Gy-irradiated CT26/H-2Kb cells twice before tumor inoculation (at 14 and 7 days), or by i.m. injection of 10 μg of H-2Kb–expressing plasmid DNA suspended with 50 μL of PBS twice before tumor inoculation (at days 14 and 7 or at days 7 and 0) or after tumor inoculation (at days 0 and 7). Based on the previous report (9), preimmunization with CT26/H-2Kb followed by treatment with H-2Kb DNA-liposome complex was done in some of the transplanted mice as a positive control to discover the best possible therapeutic effect. The shortest (r) and longest (l) tumor diameters were measured at indicated days and the tumor volume was determined as r²/l/2. Data are presented as mean ± SD.

**Reverse transcription-PCR of H-2Kb gene in s.c. tumors.** To examine the in vivo expression of the H-2Kb gene, total RNA was extracted from s.c. CT26 tumors at 3, 7, 10, and 14 days after the injection of 10 μg of H-2Kb plasmid complexed with liposome. Reverse transcription–PCR amplification was carried out using total RNA from the tumors in a 50 μL PCR mixture with the following primer set: H-2Kb upstream (5′-ACTATTACGGTGATCTCTCGT-3′) and downstream (5′-TTATTC- TTCAGCTCTGCTGTGAG-3′) primers, and β-actin upstream (5′-GATATTCCGCTGCGCTGGTCGT-3′) and downstream (5′-GTTGATCTTCTTACGGTTG-3′) primers. In total, 35 cycles (β-actin: 25 cycles) of the PCR were carried out at 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min. The PCR products were fractionated on 2.0% agarose gel.

**Cytotoxic assays.** Splenocytes (5 × 10⁶/mL) were cultured for 3 days with 30 Gy-irradiated CT26 stimulators (2.5 × 10⁶/mL) in a complete RPMI containing 2-mercaptoethanol. The responder cells were then cultured and used as effector cells in a 4-h chromium release assay against indicated target cells. Concanavalin A (ConA) lymphoblasts were prepared by stimulating the splenocytes of naive BALB/c mice for 3 days with 5 μg/mL concanavalin-A at 2 × 10⁶/mL in the complete RPMI containing 2-mercaptoethanol. Indicated target cells were labeled by combining 5 × 10⁶ cells with 50 μCi ⁵¹Cr (PerkinElmer Japan Co.) in a total volume of 0.2 mL complete RPMI for 1 h at 37°C, followed by washing thrice withplain RPMI. For the chromium release assay, 6 × 10⁵ effector cells were mixed with 2 × 10⁵ target cells in a total volume of 0.2 mL complete RPMI in a 96-well round-bottom plate (BD Biosciences). To evaluate the relative contributions of natural killer (NK) cells, CD4+ and CD8+ T cells for the tumor cell lysis, effector cells were incubated with monoclonal antibodies (mAb) against mouse CD4 (L3T4; BD Pharmingen), CD8 (Ly-2; BD Pharmingen), or NK (anti-asialo GM1, Wako Pure Chemical Industries, Ltd.) for 30 min at 37°C before mixing with target cells. Supernatants were harvested with the Skatron harvesting system (Skatron) and counted in a gamma counter (Packard Bioscience Company). Percentage of cytotoxicity was calculated as [(experimental cpm - spontaneous cpm) / (maximum cpm - spontaneous cpm)] × 100. Spontaneous cpm was obtained from targets cultured in the medium alone, and maximum cpm was obtained.
from targets incubated with 1% NP40. Each assay was done in triplicate.

**ELISpot assays.** IFN-γ ELISpot kits (BD Biosciences) were used according to the manufacturer’s instructions. Briefly, splenocytes (1 × 10⁶) and 30 Gy-irradiated CT26 (5 × 10⁶) cells were cocultured in 96-well plates precoated with mouse IFN-γ (BD Biosciences) for 20 h at 37°C in complete RPMI medium in triplicate. After aspirating the cell suspension and washing wells with deionized water, biotinylated anti-mouse IFN-γ antibody (2 μg/mL) was added and incubated for 2 h at room temperature. After extensive washing, a streptavidin–horseradish peroxidase solution was added and incubated for 1 h at room temperature. After washing, an aminoethyl carbazole substrate solution was added, and the plate was incubated for 15 min. Spots were counted under a stereomicroscope after washing the plate.

**Cell surface marker and intracellular cytokine staining.** Phycoerythrin-conjugated mAb to identify mouse IFN-γ and FITC-conjugated mAb to CD4, CD8, and CD49b were purchased from BD PharMingen. Splenocytes (1 × 10⁶) were incubated with medium alone (control) or CT26 (1 × 10⁶) cells for 10 h; brefeldin-A (10 μg/mL) was then added for a 2-h incubation. After washing, cells were incubated with the CD4, CD8, or CD49b mAbs in a total volume of 100 μL PBS with 5% fetal bovine serum for 30 min at 4°C, and then fixed and permeabilized with a permeabilization buffer (BD Biosciences). Cells were finally stained with antibody to IFN-γ for 15 min at room temperature, washed again, and analyzed by FACSCalibur (BD Biosciences). Irrelevant IgG mAbs were used as a negative control. Ten thousand live events were acquired for analysis.

**In vivo depletion of T and NK cells.** 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 mAb from the anti-CD4 T cell hybridoma (clone GK1.5, rat IgG2b) or anti CD8+ hybridoma (clone Lyt-2.1, mouse IgG2b; ref. 18) or 0.5 mg of anti-asialo GM1 antibody (Wako Pure Chemical Industries, Ltd). Injections started on the inoculation day of CT26 cells, and the treatment repeated every 5 to 6 days throughout the entire experimental period to ensure depletion of the target cell type. The ~80% of CD4+, ~60% of CD8+ T cells, and ~100% of NK cells were depleted in the antibody-treated mice by flow cytometry (data not shown).

**Immunohistochemistry.** Immunostaining was done using the streptavidin-biotin-peroxidase complex techniques (Nichirei). Consecutive cryostat tissue sections (5 μm) were mounted on glass slides and fixed in cold acetone (-20°C) for 5 min. After blocking with normal rat serum, the sections were stained with rat anti-mouse CD4 and CD8 antibodies (BD PharMingen). Parallel negative controls without primary antibodies were examined in all cases. The sections were counterstained with hematoxylin.

**Statistical analysis.** Using statistical software StatView for Windows (SAS Institute, Inc.), comparative analyses of the data were done by the Student’s t test and survival was analyzed using the Kaplan-Meier method with the log-rank test. P < 0.05 was considered as a significant difference.

**Results**

**Growth suppression of colon cancer in lymphopenic hosts.** We first examined whether homeostatic proliferation of T cells could induce antitumor immunity in lymphopenic hosts. On the day of lethal (9 Gy) irradiation, BALB/c mice were injected s.c. with CT26 colon cancer cells shortly after the irradiation, and then bone marrow and T cells were infused. The growth of tumors was significantly suppressed in the syngeneic HSCT recipients (Fig. 1A). Although the lymphocyte infusion into nonirradiated naive mice showed some antitumor effect at day 14 after the tumor injection, the tumors rapidly grew and caught up with the tumor growth in the control mice at day 24 (Fig. 1A). Sublethal irradiation (6.5 Gy) is a common means of inducing lymphopenia in mice. The infusion of T cells without marrow cells suppressed s.c. tumor growth in sublethally irradiated mice also (Fig. 1A), suggesting that T cells accompanying the graft could participate in antitumor immunity in lymphopenic recipients.

In the clinical autologous HSCT, the grafts were prepared from tumor-bearing patients. The influence of the tumor-bearing condition of the donor on the antitumor effect of the syngeneic HSCT was examined by using the BALB/c donor mice that had been injected with CT26 cells 4 weeks before the HSCT. The growth of tumors was substantially suppressed in the mice that received HSCT from a tumor-bearing donor compared with a naive donor (Fig. 1B), suggesting that some fraction of donor lymphocytes from tumor-bearing mice are primed in response to TAAs and remain responsive upon transplantation.

**AlloMHC gene transfer induces synergistic antitumor effect at early phase after syngeneic HSCT.** We sought to determine the time point after HSCT when alloMHC gene transfer could enhance the antitumor effects in syngeneic HSCT recipients. When we assessed the posttransplant immune reconstitution of T cells in the spleen, the CD4+ T-cell number returned to a normal level by the 8th week, whereas the CD8+ cell counts were increasing but remained below normal at 8 weeks (Supplementary Fig. S1). To compare the antitumor response by alloMHC gene transfer at various time points after the syngeneic HSCT, transplantations were staggered at weekly intervals, followed by the s.c. injection of 1 × 10⁶ CT26 cells. Then, for each experiment, the H-2Kb gene–expressing plasmid DNA complexed with liposome was injected thrice into the tumor at 5, 7, and 9 days after the tumor inoculation. The tumor volumes at day 5 were 60 to 100 mm³. A single injection of plasmid DNA–liposome complexes showed ~1% gene transduction efficiency *in vivo* (data not shown). The H-2Kb mRNA was detected up to 10 days after the intratumoral injection of H-2Kb plasmid/liposome complex by reverse transcription-PCR (Supplementary Fig. S2). 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 vector–injected group (data not shown). AlloMHC gene transfer at 8 weeks after transplantation did not enhance the antitumor immunity, whereas a substantial antitumor effect was observed by H-2Kb gene transfer at early phases (3-5 weeks) after the transplantation (Fig. 2A). These results suggested that intratumoral alloMHC gene transfer during immune reconstitution can induce a synergistic antitumor effect.

Next, we examined whether a preimmunization with i.m. injection of H-2Kb plasmid could enhance the antitumor effect of intratumoral H-2Kb gene transfer. In this experiment, an 8-week gene transfer model was used, because the strong antitumor effect of 3 to 5-week gene transfers would make the effect of preimmunization unclear. Preimmunization with irradiated CT26/H-2Kb cells was also carried out as a positive control, because it was expected to represent the maximum possible effect of the H-2Kb immune gene therapy (9). The preimmunization with the H-2Kb plasmid DNA induced a considerable suppression of the tumor growth (Fig. 2B), suggesting that the priming of immune cells with an alloMHC molecule augments the allogeneic rejection reaction
in gene-transfected tumors, leading to a strong antitumor effect. Because the i.m. injection of plasmid DNA is technically much more feasible compared with the making of alloMHC-expressing autologous tumor cells for each patient, this finding has important implications for the application of combining i.m. preimmunization and intratumoral gene transfer in the clinical setting.

**AlloMHC gene transfer prolongs the survival of syngeneic HSCT recipients.** We next examined the combined antitumor activity of intratumoral alloMHC gene transfer with i.m. preimmunization at an early time point of immune reconstitution in syngeneic HSCT recipients from a tumor-bearing donor. To avoid the influence of irradiation on tumor cells, recipient mice were inoculated s.c. with the CT26 cells shortly after lethal irradiation, followed by the infusion of the syngeneic bone marrow and T cells. The H-2Kb plasmid was i.m. injected on the left leg twice and then the H-2Kb plasmid–liposome complex was thrice injected into the tumor on the right leg (Fig. 3A). Although significant tumor suppression was observed up to 30 days after the tumor inoculation in the syngeneic HSCT recipients injected with control plasmid–liposome (Fig. 3A), mouse survival was not extended due to the subsequent rapid tumor growth (Fig. 3B). The tumors might establish the effective immunotolerant environment against the reconstituted immune system in the absence of the alloMHC transfer. In contrast, intratumoral H-2Kb gene transfer with preimmunization enhanced the antitumor effects in syngeneic HSCT recipients and the s.c. tumors disappeared in ~50% of the mice, leading to a prolongation of survival (Fig. 3A and B) without major side effects (data not shown). The disappearance of the tumors and the strong tumor suppression due to the sustainable antitumor immune reaction after the alloMHC gene transfer may be important factors to prolong mouse survival. The antitumor activity of combination therapy with gene transfer and preimmunization was also evident against syngeneic Renca renal cancer cells in the syngeneic recipient mice (Fig. 3C). Although we did not follow the treated mice until their final survival days, the three of three empty plasmid–injected mice showed steady tumor growth and one mouse already died for the tumor by day 60, whereas the s.c. tumors in two of four H-2Kb plasmid–injected mice disappeared up to the last observation day (day 96), at which point all the mice of the group were alive, suggesting improved survival (Fig. 3C, right).

The tumor growth suppression experiment was repeated by inoculating the CT26 cells 1 day before irradiation as a preexisting tumor model. Although the tumor growth might be retarded by irradiation per se, the combination therapy with gene transfer and preimmunization induced a strong suppression of the growth of the CT26 tumor (Fig. 3D).

**Expansion of tumor-specific T cells after intratumoral alloMHC gene transfer in syngeneic HSCT recipients.** To examine the
immune reaction to the alloMHC gene transfer in syngeneic HSCT recipients, the frequency of tumor-reactive T cells was determined by intracellular cytokine staining and flow cytometry on splenocytes. In nontransplanted mice that received the control vector, only 0.16% of splenocytes produced IFN-\(\gamma\) in response to the stimulation of CT26 cells. The IFN-\(\gamma\)-producing cells were 0.56% of the splenocytes in syngeneic HSCT recipients treated with control plasmid, and they increased to 1.3% in the syngeneic recipients injected with \(H-2K^b\) vector (Fig. 4A). The percentages of CD4+ and CD8+ T cells stimulated to produce IFN-\(\gamma\) in response to CT26 cells were also significantly higher in the recipient mice injected with the \(H-2K^b\) vector, and there was also an increase in the percentage of the IFN-\(\gamma\)-producing NK cells (Fig. 4A).

The ELISpot assay also showed that average numbers of IFN-\(\gamma\)-secreting spots were clearly increased in response to CT26 stimulation but not to lymphocytes in syngeneic HSCT recipients, and alloMHC gene transfer further increased the spot numbers for CT26 cells (Fig. 4B).

An in vitro cytotoxic assay was done to examine antitumor cytotoxic immune responses induced by alloMHC gene transfer in syngeneic HSCT recipients. The splenocytes derived from the transplanted mice recognized and lysed CT26 cells but not ConA blasts, and \(H-2K^b\) gene transfer significantly enhanced the cytolysis to CT26 cells (Fig. 4C, left) but not to ConA blasts (Fig. 4C, right). These results suggest that the alloMHC gene transfer-mediated exposure and recognition of TAAs induced expansion of the tumor antigen–specific T cells in a lymphopenic host.

\[CD4^+\] T cells, \[CD8^+\] T cells, and NK cells contribute to antitumor immunity. In the in vitro blocking assays of lymphocyte cytotoxicity with anti-murine CD4, CD8, and anti-asialo GM1 antibodies, all of the CD4+, CD8+ T cells, and NK cells were shown to contribute to the tumor cell lysis in syngeneic HSCT recipients treated with \(H-2K^b\) gene transfer (Fig. 5A). The flow cytometry detected no MHC class II expression on CT26 cells (data not shown). Several murine bone marrow transplantation models have shown that CD4+ T cells as well as CD8+ T cells are major effectors to induce the graft-versus-host disease and also graft versus tumor effects against MHC II–negative tumor cells, and the inflammatory cytokines such as tumor necrosis factor-\(\alpha\), IFN-\(\gamma\), and interleukin-1 produced by the T cells might contribute to the CD4-mediated killing effect in the MHC class II–independent manner (16, 19, 20). To further explore the role of immune cells in antitumor immunity in vivo, the mice were administered with anti-CD4, anti-CD8, and anti-asialo GM1 antibodies to deplete these cell populations. The antitumor effect was completely canceled in the mice with depletion of all of the CD4+ and CD8+ T cells and NK cells. Depletion of CD4+ T cells showed some growth advantages but still resulted in significant tumor growth inhibition, whereas the antitumor...
activities were inhibited in animals depleted of either CD8+ T cells or NK cells (Fig. 5B), suggesting that CD4+ T cells have a certain antitumor activity in vivo, but CD8+ cytotoxic T cells and NK cells play a major role in the in vivo antitumor immunity. In addition, the depletion of regulatory T cells (21–23) and making space for proliferation of CD8+ T cells may explain the difference in the antitumor effect of CD4+ T-cell depletion in vitro and in vivo.

Immunohistochemical staining showed that CD4+ and CD8+ T cells infiltrated into the CT26 s.c. tumors in syngeneic HSCT recipients, and that the H-2Kb gene transfer increased the number of these cells within the tumor (Supplementary Fig. S3). The average counts of CD4+ and CD8+ T cells increased at 1 week after the H-2Kb gene transfer on the HSCT recipient mice, and a significant number of cells were still detected until at least 3 weeks after the gene transfer. On the other hand, few of these...
cells were detected in tumors injected with the control plasmid at 3 weeks after the injection (Fig. 5C). Although the H-2Kb mRNA was not detected at day 14 after the intratumoral injection of H-2Kb plasmid (Supplementary Fig. S2), the significant numbers of CD4+ and CD8+ T cells were detected in the tumors until at least 3 weeks after the gene transfer (Fig. 5C), suggesting that a long-term expression of the transgene does not seem to be necessary to induce a sustainable activation of antitumor immunity.

**AlloMHC gene transfer during immune reconstitution causes growth suppression of both local and distant tumors.** To evaluate the therapeutic efficacy of alloMHC gene transfer for tumors at a distant site, CT26 cells were s.c. inoculated on the left leg of syngeneic HSCT recipients 3 days after the injection of H-2Kb vector into the tumors on the right leg. The H-2Kb gene transfer significantly suppressed the growth of the newly inoculated, untreated tumor on the left leg, as well as the vector-injected tumor on the right leg (Fig. 6A). The infiltration of CD4+ and CD8+ T cells was examined in the untreated tumors at the opposite site by immunohistochemical staining. Although the CD4+ and CD8+ cells infiltrated significantly into the untreated tumors 16 days after the tumor inoculation in the HSCT recipients with H-2Kb plasmid injection (Fig. 6B), the average number of CD8+ T cells were lower in the untreated tumors than the H-2Kb vector-injected tumors (Fig. 5C). The intratumoral

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**Fig. 4.** A larger number of IFN-γ-producing cells are induced by H-2Kb gene transfer in the syngeneic transplant mice. A, intracellular cytokine staining of IFN-γ-producing cells in response to stimulation of CT26 cells. The splenocytes (1 × 10⁶) from treated mice were incubated with CT26 (1 × 10⁵) and stained by phycoerythrin anti-mouse IFN-γ and counted (Overall). The activated cell fractions were analyzed by staining with FITC–anti-mouse CD4, CD8, and CD49b (NK) (n = 1–4). B, ELISPOT assay of IFN-γ-producing cells in response to stimulation of CT26 cells. Splenocytes (1 × 10⁵) from treated or control mice were cocultured with CT26 (5 × 10⁵) or control lymphocytes and stained with biotinylated anti-mouse IFN-γ antibody to detect captured IFN-γ (n = 3). C, in vitro cytotoxicity assay of transplant mice with alloMHC gene transfer. Splenocytes were collected from each treatment group 15 d after H-2Kb gene transfer, and their cytotoxicity was evaluated in a standard 4-h ⁵¹Cr release assay (effector/target ratio = 30) against CT26 cells (left) and BALB/c-derived ConA lymphoblasts (right) after stimulation with irradiated CT26 cells (n = 3). The CT26 killing at various effector to target (E/T) ratio in two groups (H-2Kb plasmid injection with preimmunization or empty plasmid injection without preimmunization) of HSCT recipients were also presented.
injection of H-2Kb plasmid may induce the infiltration of both alloreactive and tumor-reactive CD8+ T cells into the tumors, whereas the tumor-reactive T cells might mainly infiltrate into the untreated tumors at the opposite site.

As another model of distant metastasis, CT26 cells were inoculated into the peritoneal cavity 17 days after the H-2Kb gene transfer into the s.c. tumors. The injection of H-2Kb vector significantly improved the survival of the treated mice, whereas all the control vector–injected mice died by the 95th day after the i.p. injection of the tumor cells (Fig. 6C). All of the dead animals were confirmed to have disseminated tumors in the peritoneal cavity. These data showed that an intratumoral alloMHC gene transfer can induce a systemic antitumor immunity in the syngeneic HSCT recipients.

**Tumor immunity induced by alloMHC gene transfer is long-lasting in syngeneic HSCT recipients.** The CT26 s.c. tumors disappeared in 40% to 60% of the transplanted mice by the H-2Kb gene transfer. To examine the long-term duration of the tumor-specific immunity in vivo, a total of four mice that survived the initial CT26 injection with complete tumor

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**Fig. 5.** Antitumor immunity of alloMHC gene transfer after depletion of immune cells. A, blocking assay of in vitro cytotoxicity. Splenocytes from transplant mice treated with H-2Kb gene transfer were analyzed for their cytotoxicity in a 51Cr release assay (effector/target ratio, ref. 30) against CT26 cells with anti-CD4 or anti-CD8 or anti-asialo GM1 antibodies. B, antitumor effect of H-2Kb gene transfer after in vivo depletion of CD4+, CD8+ T cells, and NK cells. A group of transplant mice were treated with anti-CD4, anti-CD8, and anti-asialo GM1 antibodies to deplete these cell populations, and the CT26 tumors were injected with H-2Kb vector (n = 7-9). Empty, mice injected with the empty plasmid vector. Control, mice received no HSCT or gene transfer. Tumor volumes 10 d after the H-2Kb gene transfer were presented. C, count of CD4+ and CD8+ T cells in CT26 tumors. After H-2Kb gene transfer, s.c. tumors were processed for immunohistochemistry stained with anti-murine CD4 and CD8 antibodies. CD4+ and CD8+ cells were counted under the microscope in five high-power fields (×400) until 3 wk after the H-2Kb gene transfer. Points, mean; bars, SD. The immunohistochemistry data in the group with preimmunization followed by intratumoral alloMHC gene transfer was not available at day 32 because the tumor volumes were too small to process the staining.
regression were again inoculated with CT26 cells on the right leg and Renca cells on the left leg. At the time of the second tumor challenge, at least 8 weeks passed after the regression of the first tumor. In all the mice, Renca cells formed a tumor mass, whereas the CT26 cells were rejected (Supplementary Fig. S4), suggesting that TAAs are different between CT26 and Renca cells and that a H-2Kb gene transfer to CT26 tumor increases the recognition of CT26-specific antigens by immune cells. The results showed that the tumor-specific immunity induced by alloMHC gene transfer is potentially long lasting in the syngeneic HSCT recipients.

Discussion

Low-affinity self-antigens do not induce T-cell proliferation in the periphery of normal mice, but the same antigens
induce antigen-specific T-cell proliferation during immune reconstitution from lymphopenia, because the signal strength needed to trigger proliferation and activation of T cells is considerably reduced in a lymphopenia condition (8). This process could be used to break tolerance against tumor antigens, and indeed several studies in mouse models showed that the presence of tumor antigens during homeostatic T-cell proliferation leads to effective antitumor autoimmunity with specificity and memory (3, 24). In this study also, lymphopenia-driven T-cell proliferation significantly suppressed the growth of s.c. tumors. Baccala et al. hypothesized that an immunologically delicate environment decreases the activation threshold of tumor-specific T cells by TAA-presenting dendritic cells in the draining lymph nodes, leading to their preferential engagement and expansion (25). In clinical practice, until recently, the aim of the autologous HSCT for solid cancer has been to rescue the pancytopenia after high-dose chemotherapy (26–28). Although the treatment itself is well-established and can be done safely with a mortality rate of <5%, it is by no means satisfactory in improving patient survival (27, 29). One of the reasons for this regrettable outcome might be that the authors did not transfer T cells concomitantly with HSCs (28, 30). In addition, the antitumor immune reactions of homeostatic lymphocyte proliferation did not come to the front because focus was only on the therapeutic effects of chemotherapy.

Despite significant tumor suppression in syngeneic transplant recipients during the early posttransplant period, this response was not sustained in mice without gene transfer, because the tumor rapidly regained its growth (Fig. 3A and B). Borrello et al. (5) reported similar results: homeostatic proliferation–induced massive expansion and activation of tumor-specific T cells rapidly declines in association with tumor progression. The main reason for failure to fully eliminate the tumor might be T-cell tolerance mechanisms such as anergy and exhaustion, and this might be influenced by the relative quantity of antigens encountered (5, 31). To avoid a tolerogenic reaction, attempts at sustaining the host response through posttransplant immunization should be made before the establishment of tumor-specific T-cell tolerance; that is, long before full recovery of the host immune system (3, 5). Our data showed that the combination with alloMHC gene transfer significantly enhanced the antitumor immune response and prolonged the survival of treated mice. The alloMHC gene transfer can induce local allogeneic reactions, which consist of trafficking immune cells into a tumor, the presentation of tumor antigenic peptides on antigen-presenting cells, and the local production of various cytokines (9). These processes could greatly augment the exposure of TAAs to T cells during immune reconstitution in a lymphopenic host, leading to a significant homeostatic expansion of such tumor reactive T cells. In addition, the repeated immunization may serve to maintain the increased frequency of precursor cells and the expanded population and activation state of tumor-specific T cells (5).

There have been several animal studies showing the potential efficacy of gene- and cell-based immunotherapy in syngeneic transplant mice. A strong antitumor effect was observed for the mice vaccinated in the post–bone marrow transplantation period with autologous tumor cells that were retrovirally transduced with the granulocyte-macrophage colony stimulating factor gene (5). Another example of the combination of the immune therapy and HSCT was an immunization with dendritic cells pulsed with whole tumor cell lysates in the early phase of bone marrow transplantation, which led to efficient antitumor responses in a mouse breast tumor model (32). Adoptive transfer of tumor-specific T cells has also shown enhanced antitumor immune responses in lymphopenic mice compared with T-cell replete mice (8), and recently, Morgan et al. (33) reported the efficacy of a strategy composed of immunodepletion and adaptive cell transfer for patients with metastatic melanoma. However, because tumor-reactive T cells are mostly polyclonal and heterogeneous expressions of various TAAs coexist even in a tumor mass (34), the in vivo stimulation of multiple tumor-reactive lymphocytes might be of profound importance to the clinic (16). Compared with these approaches, one of the major advantages of the in vivo alloMHC gene transfer is that it does not need to manipulate and culture the immune and tumor cells ex vivo, making this strategy more feasible for many patients with solid cancers.

In some of the experiments in this study, the CT26 tumor cells were inoculated before the transfusion of bone marrow and T cells, considering the actual clinical situations, in which the recipients usually harbor relapse or residual cancer cells at the time of HSCT. This is an important point to consider in the experimental design, but most of the previous immunotherapeutic studies used a treatment model, in which tumor cells are inoculated after bone marrow transplantation. Our result that alloMHC gene transfer could significantly suppress the growth of preexisting tumor cells suggests that an alloMHC gene transfer with autologous HSCT is a promising therapeutic strategy in a clinical setting. As a next step, a combination with other approaches such as immune stimulatory cytokine might be examined for whether they can further enhance the antitumor effects of alloMHC gene transfer in autologous HSCT recipients.

Recently, it has been reported that the extent of lymphodepletion by conditioning influences the homeostatic proliferation–driven antitumor immunity (8, 35). Wrzesinski et al. (8) emphasized the potential of complete immunoaablation followed by autologous HSCT in treating solid cancer, because after the consumptive cellular cytokine “sinks” are removed, adoptively transferred T cells are able to exist in a less competitive environment and to access the cytokines. Our result that lethal irradiation seems to be more effective in tumor suppression than sublethal irradiation (Fig. 1A) might suggest the usefulness of complete lymphodepletion. On the other hand, high-intensity myeloablation can be associated with multiple toxicities, including mucositis, graft failure, engraftment syndrome, prolonged neutropenia-associated risk of infection, and early and late adverse effects of irradiation (27, 28, 36). Although there was little radiation-related mortality during the observation period in this study, a safer lymphodepleting regimen for future trials should be designed.

In conclusion, the combination of intratumoral alloMHC gene transfer with the autologous HSCT in the period of immune reconstitution showed significant enhancement in the cytotoxicity, tumor-specific release of cytokines, and an induction of long-lasting systemic tumor immunity without
major toxicity. The combination therapy–mediated complete eradication of some tumors and the overall survival of the animals was prolonged. The therapeutic strategy deserves an evaluation in future clinical trials for solid cancers.

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References

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