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Tumor Vaccination after Allogeneic Bone Marrow Cell Reconstitution of the Nonmyeloablative Conditioned Tumor-Bearing Murine Host

Margot Zöller

Allogeneic bone marrow cell reconstitution of the nonmyeloablative conditioned host is supposed to provide an optimized platform for tumor vaccination. We recently showed that an allogeneic T cell-depleted graft was well accepted if the tumor-bearing host was NK depleted. Based on this finding, a vaccination protocol in tumor-bearing, nonmyeloablative conditioned, allogeneically reconstituted mice was elaborated. Allogeneically reconstituted mice, bearing a renal cell carcinoma, received tumor-primed donor lymph node cells (LNC), which had or had not matured in the allogeneic host. Primed LNC were supported by tumor lysate-pulsed dendritic cells, which were donor or host derived. Optimal responses against the tumor were observed with host-tolerant, tumor-primed LNC in combination with host-derived dendritic cells. High frequencies of tumor-specific proliferating and CTLs were recorded; the survival time of tumor-bearing mice was significantly prolonged, and in >50% of mice the tumor was completely rejected. Notably, severe graft-vs-host disease was observed in reconstituted mice that received tumor-primed LNC, which had not matured in the allogeneic host. However, graft-vs-host was not aggravated after vaccination with tumor-primed, host-tolerant LNC. Thus, the LNC were tolerant toward the host, but not toward the tumor. The finding convincingly demonstrates the feasibility and efficacy of tumor vaccination after allogeneic reconstitution of the nonmyeloablative conditioned host. The Journal of Immunology, 2003, 171: 6941–6953.
provide a profound base for tumor vaccination (54–56). We speculated that newly emerging T cells: 1) should be tolerant toward the host, but not toward the tumor, and 2) should recognize tumor Ags in the context of the host MHC. In this study, we used BALB/c mice carrying a syngeneic renal cell carcinoma (RENCA) to provide experimental evidence that this hypothesis holds true and that a highly efficient antitumor response can be provoked by the transfer of donor-derived, host-tolerant T cells that have been primed against the tumor in the context of host-derived dendritic cells (DC).

Materials and Methods

Mice and tumors

BALB/c (H-2d) and 129SVEV (SVEV) (H-2b) mice were obtained from Charles River (Sulzdorf, Germany), or were bred at the central animal facilities of the German Cancer Research Center. Mice were used for experiments at the age of 8–10 wk. Where indicated, BALB/c mice were nonlethally (6 Gy) or lethally (8.5 Gy) irradiated. The BALB/c-derived renal cell carcinoma line RENCA (57) was used in vivo and in vitro. YAC cells were used as NK target. Both lines were maintained in vitro in RPMI 1640, supplemented with 10% FCS. Confluent RENCA cultures were trypsinized and split.

Antibodies

The hybridomas 33D1 (DC specific), 331.12 (anti-μ), 145-2C11 (anti-CD8), 7.4 (anti-CD4), and 14C11 (anti-NK1.1) were obtained from the American Type Culture Collection (Manassas, VA), and YTA3.2.1 (anti-CD4), YTS169 (anti-CD3), and PK136 (anti-NK1.1) were obtained from the American Type Culture Collection. Culture supernatants over protein G Sepharose 4B. Where indicated, purified mAb were biotinylated or FITC labeled. Anti-pan NK (DX5), biotinylated anti-cytokine Abs, FITC- or PE-labeled streptavidin, and anti-mouse and anti-rat IgG and IgM were obtained commercially.

Preparation of hemopoietic cells

Mice were killed by cervical dislocation. Spleen, lymph nodes, femura, and/or tibiae were removed. BMC were obtained by flushing the bones with 5 ml of PBS using a 21 G needle. Thymus, bone marrow, lymph nodes, and spleen were teased through fine gauze. Where indicated, BMC were T cell depleted. BMC were incubated with a mixture of anti-CD4 and anti-CD8 mAb, and Ab-coated cells were depleted by adherence to anti-rat IgG-coated petri dishes (59). The efficacy of T cell depletion was controlled by flow cytometry of the nonadherent population, which contained less than 2% CD4+ or CD8+ cells. DC were derived from BMC, which had been cultured in IMDM supplemented with 5% FCS, 8 ng/ml GM-CSF, and 2.5 ng/ml IL-4. After 2 days of culture, the supernatant (with nonadherent cells) was decanted and fresh medium was added. At day 5, nonadherent and loosely adherent DC clusters were collected and cultured for an additional 5 days in the same medium. Maturation of DC was evaluated by microscopy (veiled cells), and by flow cytometry (high expression levels of CD80, CD86, and MHC class II). Mature DC were loaded for 2 days with RENCA cell extract (extract of 105 cells/105 DC).

Flow cytometry and immunohistochemistry

BMC, spleen cells (SC), and thymocytes (TC) (5 x 105 cells) were stained according to routine procedures. For intracellular staining of cytokines, cells were fixed and permeabilized in advance. Negative controls were incubated with an isotype-matched control IgG and the secondary Ab. Analysis was performed with a FACSCalibur.

Long-term reconstitution, tumor implantation, and vaccination

Irradiated BALB/c mice received an i.v. injection of 2 x 106 T cell-depleted SVEV BMC. Where indicated, the host was treated with anti-asialo GM1 (Wako, Kyoto, Japan) 24 h before reconstitution. Simultaneously with the BMC reconstitution, RENCA tumor cells, 5 x 104, if not indicated otherwise, were s.c. injected. One week later, mice received an i.v. injection of 1 x 105 tumor lysate-pulsed DC, which were either host or donor derived, and/or 5 x 109 tumor-primed, donor-derived lymph node cells (LNC). Tumor lysates were obtained by freezing thawing three times. LNC were derived either from SVEV or BALB/c mice, which had been lethally irradiated and reconstituted with SVEV BMC (1 x 107/mouse). Mice were primed by intratrall injection of tumor cells in CFA. As far as reconstituted mice were primed, priming was done 8–10 wk after reconstitution. Draining lymph nodes were collected after 10 days.

GVHD, graft rejection, and tumor growth

In all experiments, the percentage of surviving mice and the repopulation with leukocytes were controlled. The percentage of donor and host cells was evaluated by flow cytometry. Evidence for GVHD was obtained by macroscopic inspection, particularly of skin, gut, and liver; weight loss; and the in vitro parameters indicated below. Graft rejection was defined by flow cytometric analysis of host- and donor-derived cells in central and peripheral lymphoid organs and the persisting absence of donor-derived cells. Tumor growth was controlled twice per week by measuring the mean tumor diameter and controlling for signs of rejection. Animals that had bloated or that became cachetic and had >25% weight loss were sacrificed. According to the immunoreactivity parameters described below, these mice were grouped as succumbing with graft rejection or GVHD. Mice were also sacrificed when developing a tumor with a mean diameter of 2 cm. The survival time was defined as the time between tumor cell inoculation and the development of a tumor with a 2-cm mean diameter.

Immunoreactivity

Immunoreactivity was evaluated by the analysis of inflammatory cytokine expression via flow cytometry, by determining the frequency of host-, donor-, and tumor-reactive proliferating T cells (Tprol) via limiting dilution (LD) and by evaluating the cytolytic activity of NK cells and CTL. For LD analysis, cells (24 replicates) were titrated from 11,200 to 100 cells/well in the presence of 105 irradiated stimulator lymphocytes or 104 irradiated tumor cells. 3HThymidine incorporation was determined after 8 days. The frequency of Tprol was calculated as C5 (fraction of nonresponding cultures) = e^-w, where w = c/w (number of c cells distributed in w wells) (60). GVH, host-vs-graft (HVG), and antitumor reactivity were also evaluated by defining the cytotoxic potential of SC after cells had been cultured for 10 days in the presence of 10 U/ml IL-2 plus irradiated host, donor, or tumor cells, respectively. Cytotoxic activity was evaluated using the JAM test (61). Lymphoblasts from BALB/c and SVEV mice and RENCA cells served as targets. A 10-fold excess of unlabelled BALB/c lymphocytes were added as cold targets when evaluating tumor-directed cytotoxicity. After 10 days of culture in the presence of only 10 U IL-2/ml, hardly any NK activity remained. This was confirmed by adding an excess of unlabeled YAC as cold targets (data not included). NK activity was tested in freshly harvested SC using YAC as target.

Statistical analysis

Significance of differences was calculated according to the Wilcoxon rank sum test (in vivo assays) or Student’s t test (in vitro studies). Functional assays were repeated at least three times. Mean values and SDs of in vivo experiments were derived from batches of three to five experiments. The individual experiments contained all groups of mice, which were compared. The individual groups in each experiments contained 10 and, occasionally, 20 mice. Thus, mean and SDs are derived from a total of 50–100 mice per group. Mean values of in vivo studies are based on three to four replicates.

Results

Optimizing the reconstitution protocol

We had described before that host NK depletion allows for reconstitution with T cell-depleted BMC without a significant decrease in engraftment, but a long lasting reduction in GvH reactivity (56). In this setting, animals were irradiated with 6.5 Gy and received 2 x 106 BMC. Despite the relatively high irradiation dose (lethal dose for BALB/c mice: 8.5 Gy), hemopoietic chimerism ranged between 80 and 90% of donor cells, but was never complete. Although 100% of 6.5 Gy irradiated mice, which were not reconstituted, survived, the question arose as to whether a milder conditioning would be favorable for reconstituted, tumor-bearing mice. We also wanted to know whether a higher dose of grafted BMC could facilitate the reconstitution process.

Lowering the dose of irradiation from 6 to 3 Gy was beneficial for the nonreconstituted, tumor-bearing host (Fig. 1A), but had only a minimal effect on the reconstituted host (Fig. 1B). In the
FIGURE 1. Impaired hemopoietic chimerism after very low dose irradiation conditioning. BALB/c mice were irradiated with 6, 5, 4, or 3 Gy and received an i.p. injection of anti-asialo GM1. One day thereafter, they received a s.c. inoculation of \(5 \times 10^4\) RENCA cells (A) or were reconstituted with \(2 \times 10^6\) SVEV BMC i.v. and received a s.c. inoculation of \(5 \times 10^4\) RENCA cells (B). The survival time of mice succumbing with a progressively growing tumor is shown. C, Tumor-bearing, reconstituted mice were sacrificed 2, 4, 6, and 8 wk after reconstitution to evaluate the number of BMC, TC, and SC. Mean values ± SD of three animals are shown. D, The percentage of donor- or host-derived cells was determined in BMC, TC, and SC of the mice described in C. The percentage of donor-derived cells is shown. C and D, Significant differences (\(p < 0.01\)) in comparison with 6 Gy irradiated mice are indicated by an asterisk.
reconstituted host, the mean survival time became prolonged from 58.5 ± 5.84 days (6 Gy) to 67.4 ± 8.42 days (5 Gy) (p = 0.013) to 71.0 ± 7.67 days (4 Gy) (p < 0.001) to 65.7 ± 15.12 days (3 Gy) (p = 0.177). Also, after irradiation with 3 or 4 Gy, a higher number of BMC and TC was recovered during the starting 4 wk and a higher number of SC at 4–8 wk after reconstitution (Fig. 1C). However, host cells dominated in the bone marrow until 6 wk after reconstitution and in spleen and thymus until 8 wk after reconstitution (Fig. 1D). The frequency of graft rejection was slightly (statistically, not significant) increased. No obvious differences in GVH reactivity were noted (data not shown).

Increasing the number of transferred BMC had no significant bearing on the mean survival time: 63.3 ± 15.3, 58.8 ± 11.2, 70.1 ± 27.9, and 69.6 ± 28.8 days after reconstitution with 2 × 10^6, 5 × 10^6, 10 × 10^6, and 20 × 10^6 BMC, respectively, the differences being statistically not significant (Fig. 2A). It should, however, be mentioned that 2 of 10 mice that received 10 × 10^6 or 20 × 10^6 BMC rejected the tumor. With increasing numbers of transferred BMC, repopulation of bone marrow and spleen was slightly accelerated, but the degree of hemopoietic chimerism was not significantly altered (Fig. 2, B and C). However, tolerance induction was clearly impaired. A significant reduction in host-reactive CTL was seen at 6 as compared with 2 wk after reconstitution when mice received 2 or 5 × 10^6 BMC. Instead, only a minor reduction was observed when mice received 1 or 2 × 10^7 BMC. Accordingly, in mice reconstituted with high numbers of BMC, hardly any reduction in the cytotoxic activity of SC, which were restimulated in vitro with host leukocytes, was observed at 6 as compared with 2 wk after reconstitution. Six weeks after reconstitution, with low numbers of BMC, cytotoxicity ranging between 12 and 18%, but reached nearly 60% after the transfer of 2 × 10^7 BMC.

The data are interpreted in the sense that a dominance of host cells (lowering the dose of irradiation) impedes the establishment of hemopoietic donor chimerism, while high numbers of grafted cells may more easily escape passage through the host thymus and tolerance induction. Therefore, we proceeded with 6 Gy irradiation and NK depletion of the host and the transfer of 2 × 10^6 allogeneic, T cell-depleted BMC.

Donor vs host cells for supporting the antitumor response

As shown before (56), NK-depleted, 6.5 Gy irradiated mice reconstituted with 2 × 10^6 T cell-depleted BMC developed an antitumor response rather late after reconstitution. To support the antitumor response, mice received, in addition, either SVEV LNC, which had been primed in vivo by an intratail application of tumor cells or tumor cell lysate in CFA, or DC, which had been loaded with tumor cell lysate to support the in vivo activation of tumor-specific T cells, or both primed LNC and Ag-pulsed DC.

In the first setting (Fig. 3A), tumor-primed SVEV LNC were transferred. The transfer of the allogeneic T cells was accompanied by a significant increase in lethal GVHD. A significant prolongation of the survival time by tumor-primed donor LNC was only seen in mice that had not been NK depleted. In the NK-depleted host, the survival time was only prolonged when mice received tumor-primed donor LNC plus tumor lysate-pulsed DC (Fig. 3B). The ex vivo analysis of host- and tumor-specific T cells provided evidence for a significant increase in the frequency of host-specific Tprol after the transfer of tumor-primed LNC. This increase, however, was clearly mitigated when mice received, in addition, tumor lysate-loaded DC (Fig. 3C). The frequency of tumor-specific Tprol increased most strongly when mice received the mixture of tumor lysate-primed LNC plus tumor lysate-loaded DC (Fig. 3D). The vaccination regimen had less impact on CTL activity. Host-directed cytotoxicity was consistently and throughout the observation period increased in mice that received LNC or LNC plus DC (Fig. 3E). In mice receiving LNC plus DC, tumor-directed cytotoxicity was noted already 2 wk after the transfer and remained elevated throughout the observation period. Thus, the application of allogeneic, primed LNC together with loaded DC was most efficient (Fig. 3F).

Taken together, the transfer of tumor-primed LNC facilitated a long lasting expansion/activation of tumor-specific Tprol and CTL, particularly, when applied together with tumor-pulsed DC. The vaccination protocol also sufficed for establishing an antitumor response early after enactment. However, a considerable proportion of the transferred cells reacted against the host rather than the tumor, which could culminate in lethal GVHD. Due to this deviation of the antitumor response, the overall benefit with respect to the survival time was minor. The problem could have been avoided by the generation of a RENCA-specific T cell clone. Yet, a RENCA-specific T cell clone would be either Th cell or a CTL clone and would only respond to a single immunogenic peptide. Alternatively, SVEV T cells tolerant toward the BALB/c host also should provide a clean source of RENCA-specific T cells. Because the latter approach is not burdened by the restriction to a single immunogenic peptide, and also, because, if successful, would be easier to transfer to the clinic, it appeared important to explore the efficacy of host-tolerant T cells in supporting tumor reactivity.

To gain BALB/c-tolerant SVEV T cells, BALB/c mice were lethally irradiated (8.5 Gy) and were reconstituted with high numbers (2 × 10^7) of SVEV BMC. After a period of 6–8 wk, >95% of lymphocytes were donor derived and lymph nodes were sufficiently repopulated (data not shown). These mice were primed with RENCA lysate in CFA. Draining LNC were collected after 10 days and were transferred into tumor-bearing BALB/c mice, which had been reconstituted 1 wk in advance. In vitro, the primed LNC exhibited cytotoxic activity against RENCA cells, but hardly against BALB/c blasts (Fig. 4A). After transfer into reconstituted tumor-bearing mice, BALB/c-passaged SVEV LNC did not induce lethal GVHD (Fig. 4B), but sufficed for a significant prolongation of the survival time (Fig. 4C). Ex vivo analysis confirmed an increase in RENCA-specific CTL activity and in the frequency of RENCA-specific Tprol (Fig. 4, D and E). The transfer of BALB/c-passaged, tumor-primed SVEV LNC had no impact on host-directed proliferative or cytotoxic activity. However, the efficacy of BALB/c-tolerant, RENCA-primed SVEV LNC against the tumor became weaker with time.

To further support maintenance of a tumor-specific response in the reconstituted host, mice received, in addition, 1 × 10^6 to 2 × 10^6 DC, which had been loaded with RENCA lysate. Because SVEV LNC were primed to recognize RENCA-derived peptides in the context of the BALB/c haplotype, host-derived DC were used. The setting should, in particular, provide an answer as to whether, by in vivo restimulation of RENCA-primed SVEV LNC with RENCA-pulsed BALB/c DC, the response would become more strongly directed against the tumor and deviated from the host.

Mice that received tumor lysate-pulsed BALB/c DC as only vaccine (Fig. 5) did not show a significantly improved proliferative or cytotoxic antitumor response, which could be due to the fact that the lymphoid system of the mice was still in the process of recreation. However, it should be mentioned that the transfer of host-derived tumor lysate-loaded DC was not accompanied by an increase in graft rejection (data not shown) or in HVG reactivities. Instead, tumor-specific Tprol and CTL expanded significantly.
when mice received host-tolerant, primed LNC together with tumor lysate-pulsed BALB/c-derived DC. Furthermore, the selective expansion of tumor-specific Tprol and CTL was accompanied by a more rapid decrease in host-specific Tprol and CTL.

Thus, host-tolerant, donor-derived, and tumor-primed LNC together with host-derived, tumor-pulsed DC appear to stimulate very efficiently the antitumor response without being accompanied by any signs of aggravated antihost reactions.

FIGURE 2. Delay in tolerance induction after the transfer of high numbers of allogeneic bone marrow cells. BALB/c mice were irradiated with 6 Gy and received an i.p. injection of anti-asialo GM1. One day thereafter, they were reconstituted with 2–20 × 10⁶ SVEV BMC i.v. and received a s.c. inoculation of 5 × 10⁴ RENCA cells. A, The survival time of mice succumbing with a progressively growing tumor is shown. B, Mice were sacrificed 2, 4, 6, and 8 wk after reconstitution to evaluate the number of BMC, TC, and SC. Mean values ± SD of three animals are shown. C, The percentage of donor- or host-derived cells was determined in BMC, TC, and SC of the mice described in B. The percentage of donor-derived cells is shown. D, The frequency of host-specific CTL in the spleen of the mice described in B was determined 2 and 6 wk after reconstitution. E, Host-specific cytotoxicity of in vitro restimulated SC of the mice described in B was evaluated 2 and 6 wk after reconstitution. The percentage of cytotoxicity at a SC:BALB/c blast ratio of 50:1 is shown. B–E, Significant differences (p < 0.01) in comparison with mice receiving 2 × 10⁶ BMC are indicated by an asterisk.
Tumor-primed LNC together with tumor-pulsed, host-derived DC suffice for tumor rejection in the allogeneically reconstituted, nonmyeloablatively conditioned mouse

To control the in vivo efficacy of the described tumor-specific proliferative and cytotoxic T cell response, nonmyeloablatively conditioned, allogeneically reconstituted mice, which had received donor-derived, host-tolerant, tumor-primed T cells and host-derived, tumor-pulsed DC were surveyed for tumor growth (Fig. 6, A–D). Thirty-one of 38 mice that had received T cell-depleted allogeneic BMC and $2 \times 10^6$ to $5 \times 10^6$ tumor cells finally succumbed with the progressively growing tumor. After vaccination with primed LNC and Ag-pulsed DC, mice receiving a tumor load of $2 \times 10^3$ were cured in 75% of cases. With increasing the starting tumor dose, complete tumor rejection was seen in 50% ($5 \times 10^3$ RENCA cells) and 60% ($2 \times 10^4$ and $5 \times 10^4$ RENCA cells) of mice.
Three features should be noted. First, initial tumor growth was observed in all mice, the rejection process mostly starting between 3 and 4 wk after the transfer. Depending on the initial tumor load, the tumor had a mean diameter of 0.5–1.5 cm (only shown for the inoculation of $2 \times 10^6$ RENCA cells). One week after reconstitution, mice received an i.v. injection of $2 \times 10^6$ BALB/c-tolerant SVEV LNC. BALB/c-tolerant SVEV LNC were collected from lethally irradiated BALB/c mice that had been reconstituted with $2 \times 10^6$ SVEV BMC. Seven days after reconstitution, these mice received an intratumoral injection of RENCA cells in CFA. Inguinal and paraaortic LNC were collected after 10 days. To evaluate the antitumor response after allogeneic reconstitution is transiently supported by the transfer of tumor-primed, host-tolerant LNC. BALB/c mice, which had or had not been primed with RENCA cells in CFA, were restimulated in vitro for 8 days with RENCA cell lysate. Blasts were collected to evaluate cytotoxic activity against BALB/c blasts and RENCA cells. The percentage of cytotoxicity (mean ± SD of triplicates) at E:T ratios of 80–10:1 is shown. A, LNC collected from lethally irradiated and SVEV BMC-reconstituted BALB/c mice, which had or had not been primed with RENCA cells in CFA, were restimulated in vitro for 8 days with RENCA cell lysate. Blasts were collected to evaluate cytotoxic activity against BALB/c blasts and RENCA cells. The percentage of cytotoxicity (mean ± SD of triplicates) at E:T ratios of 80–10:1 is shown. B, The percentage of mice rejecting the graft or succumbing with lethal GVHD in pooled experiments, each experiment comprising all indicated groups, is shown. Differences were not significant. C, The median survival time (+SD) of allogeneically reconstituted mice with progressively growing tumors is shown. Values of $p$ are derived from the comparison of mice receiving vs not receiving host-passaged SVEV LNC. D, Two, 4, 6, and 8 wk after reconstitution, the cytotoxic activity of CD8+ T cells toward BALB/c blasts and RENCA cells was evaluated. SC had been restimulated in vitro for 10 days. Tumor-specific cytotoxicity was evaluated in the presence of an excess of cold target BALB/c lymphocytes. Mean values ± SD of triplicate cultures are presented. E, Frequencies of host- and tumor-specific proliferating splenic T cells were evaluated under LD conditions at 2, 4, 6, and 8 wk after reconstitution. D and E, Significant differences ($p < 0.01$) in comparison with mice receiving only $2 \times 10^6$ T cell-depleted BMC are indicated by an asterisk.

As shown in Figs. 5A, 5B, and 6F, the efficacy of vaccination vanished with time; particularly the frequency of Tprol became similar to controls after 8 wk, and retardation of tumor growth became weak when tumor growth started with delay. Therefore, it was finally evaluated whether an additional challenge with tumor-pulsed BALB/c DC would rescue tumor-specific Th cells and, consequently, strengthen the cytotoxic tumor defense. The impact of a second application of host-passaged, tumor-primed LNC at 5 wk after grafting was also explored. Despite being passaged through BALB/c mice, a second transfer of tumor-primed SVEV LNC was unexpectedly accompanied by a remarkable aggravation of GVHD, 20% of mice dying within 2 wk after the second transfer. All these mice showed severe bleeding in and destruction of the gut as well as jaundice as an indication of hepatic failure (data not shown). Yet, when mice received a second challenge of tumor-primed BALB/c DC, the survival rate increased from 60 to 73%, and the mean survival time even of those mice that succumbed with the tumor was prolonged from 77 to 98 days (Fig. 7A). Tumor-directed cytotoxic activity remained stable for an additional 4

![Image](http://www.jimmunol.org/)

**FIGURE 4.** The antitumor response after allogeneic reconstitution is transiently supported by the transfer of tumor-primed, host-tolerant LNC. BALB/c mice were irradiated with 6 Gy and received, where indicated, an i.v. injection of anti-asialo GM1. One day thereafter, they were reconstituted with $2 \times 10^6$ SVEV BMC i.v. and received a s.c. inoculation of $5 \times 10^5$ RENCA cells. One week after reconstitution, mice received an i.v. injection of $2 \times 10^6$ BALB/c-tolerant SVEV LNC. BALB/c-tolerant SVEV LNC were collected from lethally irradiated BALB/c mice that had been reconstituted with $2 \times 10^6$ SVEV BMC. Six weeks after reconstitution, these mice received an intratumoral injection of RENCA cells in CFA. Inguinal and paraaortic LNC were collected after 10 days. As evaluated by flow cytometry, $>95\%$ of LNC were donor derived. A, LNC collected from lethally irradiated and SVEV BMC-reconstituted BALB/c mice, which had or had not been primed with RENCA cells in CFA, were restimulated in vitro for 8 days with RENCA cell lysate. Blasts were collected to evaluate cytotoxic activity against BALB/c blasts and RENCA cells. The percentage of cytotoxicity (mean ± SD of triplicates) at E:T ratios of 80–10:1 is shown. B, The percentage of mice rejecting the graft or succumbing with lethal GVHD in pooled experiments, each experiment comprising all indicated groups, is shown. Differences were not significant. C, The median survival time (+SD) of allogeneically reconstituted mice with progressively growing tumors is shown. Values of $p$ are derived from the comparison of mice receiving vs not receiving host-passaged SVEV LNC. D, Two, 4, 6, and 8 wk after reconstitution, the cytotoxic activity of CD8+ T cells toward BALB/c blasts and RENCA cells was evaluated. SC had been restimulated in vitro for 10 days. Tumor-specific cytotoxicity was evaluated in the presence of an excess of cold target BALB/c lymphocytes. Mean values ± SD of triplicate cultures are presented. E, Frequencies of host- and tumor-specific proliferating splenic T cells were evaluated under LD conditions at 2, 4, 6, and 8 wk after reconstitution. D and E, Significant differences ($p < 0.01$) in comparison with mice receiving only $2 \times 10^6$ T cell-depleted BMC are indicated by an asterisk.
wk (Fig. 7B), and the frequency of tumor-specific Tprol increased (Fig. 7C). Because of the latter, we speculated on a recruitment of nonadaptive defense mechanisms by the second transfer of tumor lysate-pulsed DC. In fact, inflammatory cytokine production was elevated (data not shown) and NK activity was significantly increased (Fig. 7D). Thus, allogeneic reconstitution can serve as a starting platform for active tumor immunotherapy.

**Discussion**

Clinical application of allogeneic bone marrow cell reconstitution after nonmyeloablative conditioning is expanding rapidly, because it is not burdened by severe side effects (2–4) and is considered as an immunotherapeutic option for hematological malignancies as well as for solid tumors (2, 62). Thus, the nascent tumor can support low zone tolerance induction due to a minimal tumor burden (63, 64) or T cells may be driven into a state of anergy by the contact with tumor-associated Ags in the absence of accessory molecules. Both phenomena unlikely play a role in the allogeneically reconstituted tumor patient. There will be higher amounts of tumor Ag and, more importantly, due to graft vs host reactions during the initial period of engraftment, some tumor cells most likely become destroyed and tumor Ags will be presented by professional APC (65, 66). Furthermore, many tumor-associated Ags are oncofetal Ags, and host, but not donor T cells may be tolerant by contact during development (67, 68). Finally, it is argued that allogeneic, host-tolerant T cells recognize tumor-associated Ags presented by the host MHC more efficiently than host-derived T cells (69, 70). For these reasons, it is argued that allogeneic reconstitution after nonmyeloablative conditioning should provide an optimal platform for active vaccination (39, 54–56, 62). The presented data clearly support this hypothesis. Nevertheless, the following features require discussion: 1) nonmyeloablative conditioning, which led to a partial reduction in host hemopoiesis, was superior to very mild conditioning; 2) tolerance induction was more readily achieved with suboptimal doses of transferred BMC; 3) host-passaged (tolerant), tumor-primed T cells were efficient in tumor rejection without aggravating GVHD; 4) host-derived DC efficiently presented tumor Ag to donor-derived T cells, which had matured in the host environment.

Several groups described that host NK depletion or blockade of NK cells greatly facilitates engraftment of allogeneic BMC (8, 21, 22, 23, 37–39), host NK cells potentially being very efficient in eliminating the graft due to the absence of NK inhibitor receptors on the allogeneic hemopoietic cells (43–46). Furthermore, we noted that even T cell-depleted BMC are engrafting, if the host is
NK depleted (39). As the transfer of T cell-depleted BMC has the advantages that GVHD are mitigated and that newly maturing donor T cells are host restricted and host tolerant (39, 50–53), this reconstitution protocol was used throughout.

However, patients mostly develop 100% of hemopoietic chimerism (6–8, 10, 11, 71–74), despite very mild conditioning (3, 6–8, 10, 11, 71, 72, 75–77). In mice, 100% hemopoietic chimerism was rarely seen even after conditioning with 6 Gy, which is a myeloreductive regimen. Nonetheless, one could argue that a non-myeloreductive conditioning may support the persistence of immune defense mechanisms. This was not the case in the tumor-bearing mouse. Although after non-myeloreductive irradiation tumor growth was delayed, tolerance induction was hampered due to a persisting dominance of host-derived T cells in the thymus. To my knowledge, it has not yet been investigated whether this finding is of clinical relevance.

FIGURE 6. Improved survival time and survival rate by the transfer of host-passaged donor lymphocytes and tumor lysate-pulsed host DC of allogeneically reconstituted tumor-bearing mice. A–F, BALB/c mice, conditioned by anti-asialo GM1 treatment and 6 Gy irradiation, received $5 \times 10^4$ (A, F), $2 \times 10^5$ (B, E, F), $5 \times 10^5$ (C, F), or $2 \times 10^6$ (D, F) RENCA cells, and 1 wk thereafter either tumor lysate-pulsed BALB/c DC ($1 \times 10^5$) or tumor lysate-pulsed BALB/c DC ($1 \times 10^5$) plus $5 \times 10^6$ LNC recovered from lethally irradiated BALB/c mice, which had been reconstituted with SVEV BMC and were primed, 6 wk thereafter, with RENCA cells in CFA. Before transfer, the LNC were cocultured with tumor lysate-pulsed BALB/c DC for 2 days. A–D, Survival time and survival rate of individual mice are shown. E, The mean tumor diameter of groups of 10 mice treated, as described above, and receiving $2 \times 10^5$ RENCA cells is shown. F, The median survival time $\pm$ SD of mice receiving between $2 \times 10^3$ and $5 \times 10^4$ RENCA cells is presented. Significance of differences in comparsion with mice receiving only T cell-depleted BMC is indicated.
The number of transferred BMC also differed from clinical settings that use higher doses (3, 6–8, 10, 71, 72, 78). In the murine model, the transfer of a higher dose of BMC had the advantage that some animals did not develop a tumor. Yet, tolerance induction was significantly delayed and not complete even 10 wk after reconstitution (data not shown). Furthermore, a second transfer of host-tolerant LNC was also accompanied by an increase in lethal GVHD, and these LNC, too, were derived from lethally irradiated mice that were reconstituted with high numbers of BMC. One could argue that the T cell depletion regimen was not vigorous enough and a small number of passaged T cells readily became stimulated and expanded in the allogeneic surrounding. However, the fact that 2 wk after reconstitution the frequency of host-specific CTL did not differ regardless of the number of transferred BMC, whereas it had significantly decreased at 6 wk after the transfer of low numbers of BMC, but not after the transfer of high number of BMC, argues rather for a partial failure in tolerance induction due to an overload in allogeneic cells accompanied by a higher percentage of T cells proceeding through extrathymic maturation and escaping tolerance induction toward the host.

Before debating the vaccination regimen, it should be briefly discussed why allogeneic reconstitution of the nonmyeloablative conditioned host mostly does not suffice by itself for tumor rejection, at least in animal transplantation tumors. As mentioned above, graft T cell depletion greatly reduces GVH reactivities. Yet, until graft-derived T lineage cells matured and repopulated the periphery, a large tumor mass has developed. Nevertheless and rather surprisingly, we noted abundant tumor necrosis and a striking shrinking of the tumor in the majority of mice, animals finally succumbing with a new tumor settled at the rim of the original tumor. It should also be mentioned that metastatic tumor growth, seen in roughly 50% of RENCA-bearing mice, was very rarely observed in allogeneically reconstituted mice. Thus, allogeneic reconstitution is a powerful weapon against solid tumors. However, a gradual loss in antitumor efficacy of the allogeneic T cells was observed. This could depend on the expansion of donor-derived APC, such that donor-derived Th cells will preferentially come into contact with tumor Ag presented by donor-derived APC. As could have been expected, the transfer of donor-derived, tumor-pulsed DC had no therapeutic effect,
whereas host-depleted tumor-loaded DC efficiently supported the antitumor response.

Because the RENCA tumor was transiently defeated in allogeneically reconstituted mice, it was evaluated whether a delayed transfer of tumor-primed, allogeneic LNC would strengthen the antitumor response. However, the transfer of tumor-primed LNC led to a strong increase in GVHD, which became lethal in 9% as compared with below 2% in mice, which received only allogeneic BMC. Furthermore, the frequency of host-specific Tprol and host-directed cytotoxic activity increased. Reactivity against the tumor was hardly improved. As already mentioned, reactivity against the tumor was also not improved by the transfer of tumor-pulsed allogeneic DC. Accordingly, the survival time was slightly prolonged. A possible explanation could be that allogeneic, tumor-primed T cells upon contact with the tumor lysate-depleted DC are partially deviated from recognizing host MHC molecules, such that GVH reactions become mitigated.

Taking into account that after reconstitution with a low number of T cell-depleted BMC newly matured donor T cells are mostly tolerant toward the host, the application of host-derived, donor-tolerant T cells appears most promising. To gain such T cells, BALB/c mice were lethally irradiated before reconstitution to guarantee full donor chimerism. The chimeric mice were vaccinated with RENCA tumor cells, and the draining LNC, which exhibited a good cytotoxic response against the tumor, were transferred into the reconstituted host. Lethal GVHD was not increased, the mean survival time was prolonged, and the frequency of tumor-specific Tprol and the lytic activity of tumor-specific CTL were increased. Thus, vaccination with host-tolerant, tumor-primed T cells was advantageous. Unfortunately, the effect vanished within 8 wk after reconstitution. The additional support by host-derived, tumor-pulsed DC strengthened and prolonged the antitumor effect without aggravating HVG reactivities. Thus, this protocol appeared to be most favorable for defeating a solid tumor. Surprisingly, application of this regimen in mice that had received graded numbers of tumor cells revealed that the efficacy of vaccination did not reversely correlate with the tumor dose, i.e., the effect became weaker with lower doses of RENCA cells, which did not form a palpable tumor for up to 8 wk or longer. Thus, one could argue that a boost injection of donor-derived, host-pasaged LNC or of host-derived DC may suffice to further increase survival time and rate. This has not been the case after a second transfer of host-tolerant LNC, which was accompanied by an increase in lethal GVHD, most likely due to incomplete tolerance induction, as outlined above. However, a challenge with tumor-primed host DC prolonged the survival time, increased the survival rate, and led to an increase in the tumor-specific cytotoxic potential as well as to a long lasting increase in tumor-specific proliferating T cells. It remains to be explored whether the latter accounted for the recruitment of nonadaptive defense, as apparent by high level of cytokine production (data not shown) and enhanced NK activity.

The vast majority of human tumors grow far more slowly than animal transplantation tumors. Taking this into account and the observation that none of the mice developed metastases, it could well be that the described vaccination protocol in combination with allogeneic reconstitution of the nonmyeloablative-conditioned host may not only lead to a significantly prolonged survival time, but may be curative in many instances. The findings indicate that: 1) creation of space by a nonlethal, but myeloreductive conditioning is favorable; 2) tolerance induction will be more easily achieved with the transfer of relatively low numbers of T cell-depleted BMC (or purified stem cells); and 3) host-derived APC, which can easily be collected before allogeneic reconstitution, together with donor-derived T cells collected from the reconstituted host do not aggravate HVG or GVH reactivities, but strongly increase the adaptive and nonadaptive response against the tumor.

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References


