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Treatment with GM-CSF secreting myeloid leukemia cell vaccine prior to autologous-BMT improves the survival of leukemia challenged mice

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Abstract

Vaccination with irradiated autologous tumor cells, engineered to secrete granulocyte macrophage colony stimulating factor (GM-CSF) (GM-tumor), can generate potent anti-tumor effects when combined with autologous bone marrow transplantation (BMT). That notwithstanding, the post-BMT milieu, characterized by marked cytopenia, can pose a challenge to the implementation of vaccine immunotherapies. To bypass this problem, partial post-BMT immune reconstitution has been allowed to develop prior to vaccination. However, delaying vaccination can also potentially allow the expansion of residual tumor cells. Other approaches have used re-infusion of "primed" autologous lymphocytes and multiple administrations of GM-tumor cells, which required the processing of large amounts of tumor. Utilizing the MMB3.19 murine myeloid leukemia model, we tested whether a single dose of GM-tumor cells, 7 days prior to syngeneic BMT, could be a curative treatment in MMB3.19-challenged recipient mice. This vaccination protocol significantly improved survival of mice by eliciting long-lasting host immune responses that survived lethal irradiation, and were even protective against post-BMT tumor rechallenge. Furthermore, we demonstrated that mature donor lymphocytes can also play a limited role in mounting the anti-tumor response, but our pre-BMT vaccination strategy obviated the need for either established de novo immune reconstitution or the use of multiple post-BMT immunizations.

Introduction

Allogeneic blood and marrow transplantation (BMT) constitutes one of the few potentially curative treatments for a number of hematological malignancies, including acute myeloid leukemia (AML) (1-3). Mature donor T cells administered with the graft play a critical role in enhancing engraftment, fighting opportunistic infections and mounting graft-versus-leukemia (GVL) responses to counteract residual tumor cells that survive intensive chemotherapy regimens. Although most transplants are performed between HLA-matched related or unrelated donors, allogeneic T cells can still mount immune responses against the different minor histocompatibility antigens (miHA) that are expressed by a number of host

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tissues, leading to graft-versus-host-disease (GVHD); one of the major complications associated with allogeneic BMT (4-6). An autologous transplant, on the other hand, offers the advantage of lower transplant-related morbidity and mortality as it does not cause GVHD or utilize immunosuppressive preconditioning drugs, but it is linked to higher relapse rates due to the absence of a sufficient GVL effect. Thus, a treatment modality capable of augmenting the anti-tumor immunity of autologous T cells represents an attractive alternative to pursue.

In this regard, a body of pre-clinical (7-10) and clinical studies (7,11,12) have shown that the use of cellular immunotherapy, in the form of vaccination with irradiated autologous tumor cells engineered to secrete granulocyte macrophage colony stimulating factor (GM-CSF) (GM-tumor), can generate a potent anti-tumor effect when combined with BMT. That notwithstanding, the post-BMT milieu, characterized by marked cytopenia, can pose a challenge to the implementation of vaccine immunotherapies (10),(13,14). In an effort to bypass this issue, partial post-BMT immune reconstitution for 30-35 days has been allowed prior to vaccination (12). Delaying vaccination, however, can potentially also allow the expansion of residual tumor cells. Another recent approach has been the use of “primed” autologous lymphocytes collected before transplantation for later re-infusions (7) along with multiple administrations of GM-tumor cells. Yet, the need for multiple injections can bound the widespread use of this immunotherapy since collection and processing of large amounts of autologous tumor cells can be a limiting factor.

To circumvent these issues, the current study utilizing the MMB3.19 murine leukemia model of C57Bl/6 (B6) origin (15), is designed to test the possibility that a single vaccination dose with GM-CSF secreting MMB3.19 (GM-MMB3.19) cells, 7 days prior to syngeneic BMT, could be a curative treatment against an MMB3.19 tumor challenge. Our results indicated that this vaccination protocol can significantly improve the survival rate of tumor challenged mice by eliciting long-lasting host immune responses that survive lethal irradiation conditioning, and are protective against post-BMT tumor rechallenge. Furthermore, in analyzing the mechanisms associated with the success of this protocol, we demonstrated that mature donor lymphocytes play only a limited role in the improved survival of GM-MMB3.19 treated mice, and that the need for either de novo immune reconstitution or the use of multiple immunizations can be avoided using this vaccination strategy.

Methods

Mice

Male C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The eGFP transgenic (eGFP-Tg) mice, on a B6 background, were obtained from the laboratory of Dr. Jon Serody (University North Carolina, Chapel Hill, NC) and bred in our colony. Donor and recipient mice between 8-12 wk of age were used in all experiments. Mice were kept in a pathogen-free environment in autoclaved microisolator cages and were provided with acidified (pH 2.5) water and autoclaved food ad libitum.

Syngeneic Bone Marrow Transplantation and Tumor Challenge

Phosphate-buffer saline (PBS) supplemented with 0.1% bovine serum albumin (BSA) was used for all preparative manipulations of donor cells. Donor lymphocytes (DL) were prepared as previously described (16) from pooled spleen (red blood cell lysed) and lymph node (LN) cell suspensions from B6 mice. T cell-depleted (anti-Thy-1.2 mAb-treated) bone marrow (ATBM) cells were prepared by flushing bone marrow cells from the femurs of donor mice, followed by incubation with J1j mAb and C’ for 45 min at 37°C. Recipients

were exposed to 8.5 Gy and 4 h later transplanted with either 2×10^6 ATBM cells alone or in combination with $4\text{-}5\times10^6$ DL, injected intravenously (i.v.) in PBS (DL numbers were adjusted to 2×10^6 $CD3^+$ T cells using flow cytometric (FC) analysis). On day 1 post-BMT, mice were challenged intraperitonealy (i.p.) with 1×10^5 B6-derived MMB3.19 myeloid leukemia cells, as previously described (17). Mice were monitored for morbidity and mortality until the termination of the experiment, 100 days post-BMT. Mice were autopsied at the completion of the experiment to confirm the absence of tumor masses.

Vaccine Preparation and Administration

GM-CSF secreting MMB3.19 cell (GM-MMB3.19) were produced using the MFG retroviral vector, as previously described (18). The GM-MMB3.19 cells were irradiated with 40 Gy, washed 2x with PBS and injected subcutaneously (s.c.) in the right flank or via i.p., as indicated (2×10^5 cells/0.4 ml) 7 days prior to BMT, or on day 1 post-BMT when indicated. GM-CSF secretion by GM-MMB3.19 was 100 ng/1×10^6 cells in 24 h, as determined by ELISA. In some experiments, mice were treated with 2×10^5, 40 Gy irradiated, non-GM-CSF secreting MMB3.19 cells (to be subsequently referred to as non-GM-MMB3.19 cells).

Phenotypic Analysis of Host vs. Donor Lymphocytes

B6 mice were vaccinated s.c. with either GM-MMB3.19 cells, non-GM-MMB3.19 cells or PBS (vehicle) and transplanted 7 days later with ATBM and DL from B6eGFP$^+$ mice. Three wk post-BMT, cell suspensions from the skin-draining inguinal LN were prepared in FC staining buffer (PBS, 0.5% BSA, 0.1% sodium azide) and analyzed in a Beckman Coulter FC500 flow cytometer (Brea, CA). FC analysis was conducted to determine $CD4^+/CD8^+$ T cell ratios in both donor ($eGFP^+$) and host ($eGFP^-$) populations, the percentage of memory ($CD44^+$) T cells, and the percentage of dendritic cells [as determined by the % $CD11c^+$ cells in the myeloid gate × (% size of the myeloid gate)/100], in both donor and host subsets. All mAb (PE or PE-Cy5-conjugated, or biotinylated mAb to mouse CD4, CD8, CD44 and CD11c) were purchased from BD Biosciences, (San Jose, CA)

Rechallenge Experiments

In some experiments, vaccinated mice that survived the initial tumor challenge with 1×10^5 MMB3.19 cells, were rechallenged at ≥ 12 wk post-BMT with 5×10^6 tumor cells. For control purposes, corresponding ATBM mice were also challenged with an equal amount of tumor cells. This tumor burden was chosen due to the fact that lower dosages of MMB3.19 cells can be rejected in immune-competent mice (data not shown). Mice were subsequently monitored for morbidity and mortality for an additional 100 days, until the termination of the experiment, when they were autopsied to confirmed the absence of tumor masses.

For rechallenge experiments following adoptive transfer, splenocytes, LN, and BM cells were harvested from vaccinated mice that survived initial tumor challenge or from their corresponding ATBM controls. Preparation of DL and ATBM cells was performed, as described above. Recipient mice were exposed to 8.5 Gy and 4 h later transplanted with 2×10^6 ATBM cells along with $4\text{-}5\times10^6$ DL (from either vaccinated or ATBM donors), followed by challenge on day 1 post-BMT, with 1×10^5 MMB3.19 cells.

Statistical Analysis

For statistical analysis of survival plots, Kaplan-Meier survival and the non-parametric Log-Rank (Mantel-Cox), or Wilcoxon Signed Rank test were performed. Data were pooled from 2-3 separate experiments consisting of 5-10 mice per group as indicated in each figured legend. Cox Proportional Hazards test was conducted to determine if there was interaction

between groups and time as previously described (19,20). Data was stratified by time for subsequent Log-Rank analysis, when interaction was statistically significant ($p < 0.05$). For statistical analysis of FC data, non-parametric Kruskal-Wallis ANOVA was used to compare all groups, followed by Dunn's multiple comparison test on individual pairs when ANOVA test was statistically significant ($p < 0.05$). Data were analyzed in GraphPad Prism (v. 4.0) or SAS (v. 9.2).

Results

GVL reactivity to MMB3.19 leukemia cells in mice vaccinated with GM-MMB3.19 cells or non-GM-MMB3.19 cells

The GVL effect of a single pre-BMT vaccination dose of either GM-MMB3.19 or (non-transduced) MMB3.19 cells was examined in combination with a syngeneic BMT, against a MMB3.19 tumor challenge. To this end, 8-12 wk old male B6 mice were vaccinated s.c. in the right flank with either 2×10^5 irradiated (40 Gy) GM-MMB.319 (secreting 100 ng/1×10^6 cells/24 h) or irradiated (40 Gy) MMB3.19 cells (Figure 1; Table 1). One wk post-treatment, all mice were exposed to lethal irradiation (8.5 Gy) and 4 h later transplanted with 2×10^6 syngeneic B6 ATBM cells along with $4\text{-}5\times10^6$ DL. On day 1, all mice (except ATBM; the engraftment control) were challenged i.p. with 1×10^5 viable MMB3.19 cells to mimic minimal residual tumor burden following a clinically relevant pre-conditioning regimen and BMT. The tumor-challenged mice that were transplanted with either ATBM alone (MMB3.19) or along with DL (DL) served as the unvaccinated control groups. Vaccinated groups (pre-BMT or post-BMT), all received ATBM + DL at the time of transplant and were tumor-challenged on day 1. The non-GM-MMB3.19 group had a MST of 21 days and 100% mortality by day 38 (Figure 1, Table 1). In comparison, the DL group had a small but significant increase in its survival rate (MST of 27 days and 12% 100-day survival, $p < 0.01$). Pre-BMT vaccination with irradiated MMB.319 cells provided a significant advantage (MST of 37.5 days and 25% survival) compared to unvaccinated DL mice ($p = 0.03$). On the other hand, DL transplantation along with post-BMT vaccination did not provide any increase in the MST or survival rate (14.3%) compared to the DL group ($p > 0.05$). Most notably, mice treated with GM-MMB3.19 cells 7 days pre-BMT exhibited a significantly greater survival rate of 52%, compared to that of either the DL, post-BMT vaccination, and non-GM-MMB3.19 cells pre-BMT groups ($p < 0.01$, $p < 0.01$ and $p = 0.03$, respectively; Table 1).

Role of syngeneic donor lymphocytes in the survival of pre-BMT vaccinated recipient mice

The results presented in Figure 1 suggested that syngeneic DL in combination with pre-BMT vaccination with GM-MMB3.19 significantly improved the survival rate of MMB3.19 tumor-challenged mice. In order to better understand the role of DL in the current GM-CSF secreting AML vaccine model, we compared the effects of vaccination in mice transplanted with ATBM cells alone or in combination with DL (Figure 2; Table 2). In addition, we also tested the GVL effects of irradiated, non-transduced MMB3.19 cells under these same conditions. As in the previous experiment, the unvaccinated control groups were transplanted with ATBM cells alone (MMB3.19) or ATBM and DL (DL) and challenged with MMB3.19 cells on day 1. The other four groups were vaccinated with irradiated GM-MMB3.19 or MMB3.19 cells 7 days prior to BMT and subsequently challenged with tumor on day 1 [i.e., GM-MMB3.19 pre-BMT + DL, non-GM-MMB3.19 pre-BMT + DL, GM-MMB3.19 pre-BMT (no DL) and non-GM-MMB3.19 pre-BMT (no DL)]. Comparisons between groups indicated that DL enhanced the effect of GM-MMB3.19 vaccination, as determined by the increased survival rate of the GM-MMB3.19 pre-BMT + DL group (53%) vs. GM-MMB3.19 pre-BMT (no DL) [33%] mice. This increase was statistically significant ($p = 0.044$), as determined by Wilcoxon Signed Rank test, which is best at detecting

differences that occur at early points in time (Table 2). Non-GM-MMB3.19 + DL mice also experienced significantly greater survival rates (25%) compared to their no DL counterparts (0%), using Log-Rank test, which is more sensitive to differences that occur at later time points. Furthermore, when the data was analyzed ≥ 28 days post-BMT (at which time point the survival curves no longer overlapped; Table 2), GM-MMB3.19 pre-BMT vaccinated (no DL) mice experienced significantly greater survival rates than the DL control group ($p < 0.01$), underscoring the potential contribution of non-DL (i.e., host elements) to the GVL effect of GM-CSF-secreting tumor in the current pre-BMT vaccination model. Survival of the irradiated MMB.319 pre-BMT (no DL) group on the other hand, was not improved, and actually was significantly less compared to that of control DL mice (0% vs. 10.5% respectively; $p = 0.02$). Finally, GM-MMB3.19 pre-BMT (no DL) mice had significantly increased survival compared to the non-GM-MMB3.19 pre-BMT (no DL) group (33% vs. 0% respectively; $p < 0.01$; Table 2), suggesting that the GVL effect of the latter group was dependent on the presence of DL.

Role of host T cells in the survival of pre-BMT vaccinated recipient mice

The improved survival rate exhibited by GM-MMB3.19 pre-BMT vaccinated recipient mice in the absence of DL suggested that host immune cells that survive irradiation must contribute to the anti-tumor response, an effect not present in mice vaccinated with non-GM-MMB3.19 cells that had 0% survival in the absence of DL (Figure 2). To further elucidate the mechanism associated with the increased survival in GM-MMB3.19 vaccinated mice, recipients were treated with 0.2 ml of GK1.5 (1:6 ratio) and 2.43 (1:50 ratio) mAb for depletion of $CD4^+$ and $CD8^+$ T cells, respectively, 4 days prior to vaccination with irradiated GM-MMB3.19 cells (11 days pre-BMT). Examination of spleen and LN cells using FC analysis indicated that, at time of vaccination, the depleted subsets constituted < 1% of the total lymphocyte population. In addition, we verified that administration of these mAb did not deplete the transplanted DL (data not shown). Upon vaccination, these recipients exhibited equivalent survival to that of non-vaccinated control mice (DL), suggesting that pre-BMT vaccination induced GVL effects were predominantly mediated by host T cells that survived irradiation conditioning. In these experiments, we also observed a significant difference in the survival of the GM-MMB3.19-vaccinated/non-T cell-depleted + DL group (50%) compared to GM-MMB3.19-vaccinated/T cell-depleted + DL recipients (6.7%), and between each of these groups and GM-MMB3.19/T cell-depleted (no DL) mice (0% survival) [$p < 0.01$ respectively; Figure 3; Table 3].

Phenotypic analysis of host vs. donor lymphocytes after vaccination with irradiated GM-MMB3.19 or Irradiated MMB.319 cells

In order to further examine the mechanism associated with the GVL effects of GM-MMB3.19 cells in the current model, pre-BMT s.c. vaccinated mice (treated with either irradiated GM-MMB3.19 cells, non-GM-MMB3.19 cells or vehicle PBS) were transplanted with ATBM + DL obtained from eGFP mice. In doing so, irradiation host immune cells surviving irradiation and not carrying the eGFP marker were easily distinguishable from DL given at time of transplant, as well as the de novo donor-derived cells. Three wk post-BMT, single cell suspensions from the draining inguinal LN were prepared from individual mice for FC analysis to compare CD4/CD8 ratios and expression of $CD44^{high}$ (a memory marker) on T cell subsets, as well as the percentage of $eGFP^-$ (host) and $eGFP^+$ (donor) DCs (as determined by the expression of CD11c in the myeloid gate). Statistical analysis showed that 3 wk post-BMT (4 wk post-vaccination) mice treated with irradiated GM-MMB3.19 cells presented with a significant increase in the mean expression of CD11c in the $eGFP^-$ population (1.97 ± 0.33%; Figure 4) compared to mice that received PBS (0.89 ± 0.37%; $p < 0.01$) or non-GM-MMB3.19 cells (1.31 ± 0.39%; $p < 0.05$). The CD11c expression in the non-GM-MMB3.19 treated group, although higher, was not statistically significant (p >

0.05) compared to PBS treated mice. On the other hand, the percentage of DCs in the donor cell population ($eGFP^+$) was comparable between the three groups (PBS = 0.26 ± 0.15% GM-MMB3.19 = 0.25 ± 0.2%, non-GM-MMB3.19 = 0.3 ± 0.26%). Likewise, $CD4^+/CD8^+$ ratios, and memory ($CD44^{high}$) expression in either T cell subset was found to be similar between groups (Table 4).

Effect of vaccination site on the survival rate of MMB3.19 myeloid leukemia challenged mice

The s.c. route was chosen as the preferred method of vaccination in the conducted studies for its clinical translatability. However, in lieu of the fact that in the current murine AML model, tumor cells were injected i.p., experiments were conducted to assess the effectiveness of i.p. vaccination, at the site of tumor challenge. To this end, mice were treated, as previously described, with either an i.p. or a s.c. injection of 1×10^5 irradiated GM-MMB3.19 cells 7 days pre-BMT (Figure 5; Table 5). The results from these experiments showed that animals receiving i.p. vaccination had a survival rate of 85% compared to 50% in s.c. vaccinated mice; a difference that was statistically different (p = 0.04). Both s.c. and i.p. vaccination routes significantly improved the survival rate of MMB3.19-challenged mice compared to the control DL group.

Rechallenge of surviving GM-MMB3.19 vaccinated mice

The long-lasting effects of vaccination were evaluated by either adoptive transfer of cells obtained from surviving mice, or by challenging vaccinated mice (i.e; GM-MMB3.19 treated recipients that survived the initial 1×10^5 MMB3.19 tumor challenge), and their corresponding ATBM controls (Figure 6), with a tumor burden of 5×10^6 cells. Mice that were transplanted with cells (ATBM and DL) from surviving mice that had been treated with GM-MMB3.19 and challenged on day 1 post-BMT with 1×10^5 MMB3.19 cells did not experience increased survival rates compared to mice that were transplanted with cells obtained from reconstituted ATBM mice. On the other hand, 84% of s.c. vaccinated mice survived tumor rechallenge, compared to 50% of i.p. vaccinated and 36% of control ATBM mice (p = 0.02 and $p < 0.01$ respectively; Table 6). No statistical difference (p = 0.52) was found between rechallenged (i.p.) and ATBM groups.

Discussion

As demonstrated in our studies, the successful implementation of a tumor vaccine relies on the delivery strategy selected. Understanding the mechanism driving the best immunization protocol could have widespread implications for the clinical translatability of this immunotherapeutic approach, and warrants further investigation. Using a murine model of AML, we developed a novel and simplified vaccination strategy that consisted of a single dose of GM-CSF-secreting autologous tumor, 7 days prior to BMT. Without the need for multiple post-BMT treatments (7,12), full donor-derived immune reconstitution (10,11), or infusion of “primed” lymphocytes, as previously described by others (7,9,13), we were able to increase the survival rate of MMB3.19 tumor-challenged mice. Survival rates increased from a maximum of 12% (achieved by transplantation of syngeneic DL alone) to 50-53% (Figure 1-3) following s.c., or 80% (Figure 5) following i.p. pre-BMT vaccination with GM-MMB3.19 cells. Post-BMT vaccination on day 1 (Figure 1), however, did not improve the survival rate of tumor-challenged mice. This result is in agreement with Borrello et al. (13) and Teshima et al. (10), who using two different murine models found that post-BMT donor reconstitution was needed prior to GM-CSF-secreting tumor immunotherapy in order to observe a significant anti-tumor response. Presumably, day 1-post-BMT vaccination failed because while it takes time for the immune response to peak following vaccination, the tumor cells have already started to expand during this time period. Thus, the tumor growth

may outpace the immune response and/or the tumor cells may inhibit the immune response by a variety of immune regulatory mechanisms (21). Likewise, and as demonstrated by others (7,12), multiple vaccinations may be required to generate optimal T cell responses when administration is done post-preconditioning and BMT.□□In addition, we also observed no difference in the survival rate of GM-MMB3.19 treated animals that received wild type ATBM or TCR KO bone marrow cells (unpublished results); further suggesting that de novo, thymic-derived immune cells may have little contribution to the GVL effect under the current vaccination protocol.

The GVL effect of pre-treatment with irradiated (non-GM-CSF secreting) MMB3.19 cells was tested as well, since it has previously been demonstrated that irradiated autologous tumor cells have anti-tumor capabilities (18). In our model, the use of non-GM-MMB3.19 cells provided only a small, albeit significant improvement, in survival (25%) of AML-challenged mice compared to mice that received DL alone (12%) [Figure 1, Table 1]. That notwithstanding, pre-BMT vaccination with GM-MMB3.19 proved to be a far superior immunotherapy (52% survival) [Figure 1, Table 1]. The survival rates of the GM-MMB3.19 vs. non-GM-MMB3.19 pre-treated mice suggested that at early time points (< 4 wk post-BMT) the two therapies yield similar outcomes; however the effects of GM-CSF immunotherapy were long-lasting, whereas the irradiated tumor cells did not elicit a sustained response, resulting in significant survival differences between these two groups at later post-BMT time points.

The contribution of DL to the overall GVL response, as summarized in Figure 2 and Table 2, revealed that DL improved the survival of GM-MMB3.19-vaccinated mice (53.3% in the presence of DL, vs. 33% in their absence), but that its synergistic effect had greater bearing soon after transplantation (< 35 days post-BMT), underscoring the limited, yet, significant anti-tumor effect of DL in our model. Similarly, the finding that survival of GM-MMB3.19 vaccinated (no DL) mice was significantly greater than that of the control DL group, only when the statistical comparison was performed at ≥28 days post-BMT (Figure 2, Table 2), also supported the idea, as suggested by others (13), that syngeneic mature DL and recent thymic emigrants may have a greater role at early post-BMT time points, when the number of residual tumor cells are low and anti-tumor T cells may be at a minimal proliferative state.

Interestingly, DL were actually indispensable to irradiated tumor-treated mice, as suggested by the significant increase in the survival rate of the pre-treated non-GM-MMB3.19 + DL group (25%) compared to the no DL counterpart (0% survival). An observation further supported by the fact that the survival of pre-treated irradiated MMB.319 (no DL) mice was significantly inferior to that of control mice receiving DL alone (0% vs. 10.5%, respectively; Figure 2 and Table 2). These observations supported the assertion that non-GM-CSF-secreting tumor cells were not capable of eliciting a sustained immune response; hence the greater significance of DL to the anti-tumor effect in non-GM-MMB3.19-treated mice.

The observation that a single pre-BMT vaccination was able to produce a significant GVL response in the absence of DL (Figure 2) led us to hypothesize that host immune cells surviving myeloablative pre-conditioning were key to this response. Indeed, depletion of host T cells (Figure 3) greatly compromised the benefits of GM-CSF secreting tumor vaccination in our model, as demonstrated by the fact that GM-MMB3.19 pre-treated/T cell depleted recipients only experienced 6.7% survival and were not significantly different from unvaccinated control (DL) mice. These results are in agreement with previously reported studies where depletion of T cells in melanoma-challenged mice severely reduced the systemic immunity provided by the vaccine (18). Taken together, the data from Figures 2 and 3 demonstrated that host T cells were critical to the GVL effect produced by pre-BMT

vaccination with GM-CSF-secreting tumor, and that syngeneic mature donor T cells may have had a greater effect in the early post-BMT period.

DCs are potent antigen presenting cells and upon appropriate stimulation, matured DCs migrate to the secondary lymphoid tissues where they can present tumor antigens to T cells and induce immune responses (22). Mach et al.(23) also demonstrated a dramatic increase of $CD11c^+$ cells in the spleen of GM-CSF-secreting tumor vaccinated mice, 14 days after treatment, and they went on to prove that these cells had higher levels of B7-1 and were more effective at eliciting systemic anti-tumor immunity. Here, we are the first to demonstrate increased expression of host-derived DCs at a later time point (4 wk post-vaccination) after BMT using this immunotherapy (Figure 4). Taken together, these results suggest that these cells are key to the observed long-lasting GVL effects induced by GM-MMB3.19 cells in our model. Additionally, given that we did not observe a statistically significant increase in the number of DCs between the mice treated with non-GM-MMB3.19 tumor cells and those treated with PBS this would suggest that irradiated tumor cells alone are less capable of producing a sustained immune response via DC expansion.

The existence of functional host-derived radioresistant T cells of both memory and naïve phenotype has been documented (24,25); thus we also wanted to determine if the improved survival of GM-MMB3.19-treated mice could be associated to differences in the $eGFP^-$/$eGFP^+$ (host/donor) T cell ratios or a greater percentage of memory T cells (as defined by the expression of $CD44^{high}$) in either the $eGFP^+$ or $eGFP^-$ compartments. Interestingly, all three groups had comparable amounts of these markers (Table 4). These results seemed to indicate a lack of increase in host-derived T cells (particularly of memory phenotype) in vaccinated mice; at least at the analyzed time point. It is possible that by this time point, three wk post-BMT, the number of anti-tumor/host-derived T cells was already diluted by homeostatic proliferation of DL and de novo thymic emigrants making it more difficult to quantify their potentially small presence. In fact, in support of this hypothesis, Borrello et al. demonstrated that T cell peripheral reconstitution ultimately diluted the percentage of tumor-antigen-specific T cells in their model (13). Further analysis at an earlier time point (3-5 days post-BMT), including other memory cell surface markers such as CD62L and CCR7, should determine if there are differences in the percentage of naïve, effector memory ($CD44^{high}CCR7^{high}CD62L^{low}$) and central memory ($CD44^{high}CCR7^{high}CD62L^{high}$) T cells (26-28) in either the donor and host populations among the experimental groups. Likewise, given that T cells (in particular those of host origin) were shown to be critical to the anti-tumor response in our model (Figure 3) and by others (18), we also hypothesize that in combination with the generation of long-lasting tumor antigen-presenting DCs, vaccination with GM-MMB3.19 cells best promote specific leukemia-reactive host T cells that remain viable after irradiation and greatly contribute to the GVL effect. Additional studies utilizing CDR3-size spectratype analysis (29) are currently underway in our laboratory to evaluate potential TCR Vβ repertoire diversity differences between GM-MMB3.19-treated and naive mice in order to evaluate anti-tumor reactivity.

Historically, GM-tumor vaccination has been administered via s.c. or intradermal injection. Since in our AML murine model, tumor challenge was via the i.p. route, we also tested the GVL effects of a single i.p. administration of GM-MMB3.19 cells (Figure 5). The results of these experiments indicated that i.p. vaccination generated a stronger GVL response than s.c. vaccination (80 vs. 50% survival, respectively). This difference was statistically significant (Table 5), potentially because circulating T cells encountered the tumor antigens and become activated in the peritoneal compartment, where the tumor grows. Interestingly however, when surviving mice were rechallenged 12 wk post-BMT (Figure 6), only mice that were injected s.c. survived the rechallenge at significantly greater rates than their ATBM counterparts. S.c. vaccination elicits the activation and maturation of dermal DCs

(dermal langerin(−) and dermal langerin(+) Dcs) and Langerhans cells (LCs; bone marrow-derived epidermal DCs) which then migrate to the regional LN to initiate systemic immune responses by presenting processed tumor antigens to T cells (30-32). FC analysis showed increased host-derived DC expression in the skin-draining inguinal LN to the injection site in s.c. GM-MMB3.19 vaccinated mice, observed even 4 wk post-vaccination (Figure 4). Similar observations were reported by Merad et al. who demonstrated that after a congenic BMT, no LC chimerism was detectable in the skin for more than 6 mon, despite rapid chimerism in BM, spleen and LN (33). In contrast to the skin, in other epithelia such as the gut and the airways, DCs were found to be quickly replaced by BM-derived precursors; thus demonstrating that the anatomical origin can affect not only the function but the life span of DCs (34,35). Finally, it has also been shown that despite the rapid turnover of individual mature DCs, some DC precursors are capable of transmitting internalized processed antigens to successive generations of their progeny during cell division, which can then present effectively to antigen-specific T cells (36). Taken together, these findings further substantiate the possibility that antigen-loaded DCs, present at the skin, persist over time in our model and are critical to the anti-tumor responses upon rechallenge associated with s.c. vaccination. Therefore, it is likely, that a combined regimen of vaccination near or at the site of tumor growth (an alternative that is more applicable to solid tumors) along with dermal (s.c. or intradermal) vaccination (9,12,18,37,38), could be a more effective strategy in order to initially eradicate residual tumor cells while providing sustained tumor immunity to prevent relapse.

The inability of adoptively transferred cells from the reconstituted vaccinated mice that survived tumor challenge to improve the survival rate of recipient mice (Figure 6); reiterated the concept that DL and bone marrow-derived cells are not adequately and/or sufficiently modulated by a single pre-BMT treatment. On the other hand, host immune cells, which constitute only a small percentage of the post-BMT reconstituted recipient after myeloablative conditioning (and most likely can't be adoptively transferred in sufficient quantities), are primary to this vaccination approach.

The fact that we successfully implemented this immunotherapy in a syngeneic BMT model is also clinically important because of the absence of allogeneic T cell-induced GVHD. However, in other murine models (9,10), and also in a recent clinical trial which involved the use of multiple administration of a GM-CSF-secreting leukemia vaccine *after* allogeneic BMT (days 30-35) (12), the approach was used both safely and efficaciously. For this reason, one can speculate that our pre-BMT vaccination strategy, which relies more on host T cells, could be used in combination with an allogeneic graft containing fewer DL, capable of further potentiating the GVL effect of the vaccine, while diminishing or eliminating the incidence of GVHD. Further studies using pre-BMT vaccination along with titrated numbers of allogeneic DL and BMT should provide additional understanding of the efficacy of such a combined therapy.

In summary, we demonstrated that a single pre-BMT injection of GM-MMB3.19 cells significantly increased the survival of syngeneic transplanted MMB3.19-challenged mice. These results are highly relevant to the clinical setting because harvesting large amounts of tumor for the preparation of multiple immunization dosages could be a difficult, time consuming and costly task; making this pre-BMT vaccination strategy more advantageous to achieve significant tumor immunity. Furthermore, because our methodology bypassed de novo immune reconstitution, it actually becomes highly attractive for elderly patients undergoing BMT who are likely to experience difficulties in reconstituting their T cell repertoire due to diminished thymic function. The improved survival rate of GM-MMB3.19 treated mice was shown to be strongly dependent on host T cells and was also most likely related to an increased presence of host-derived $CD11c^+$ DCs. Finally, the strength and

duration of the GVL response induced by this vaccination were subject to the delivery site, with more enduring protection obtained by s.c. injection.

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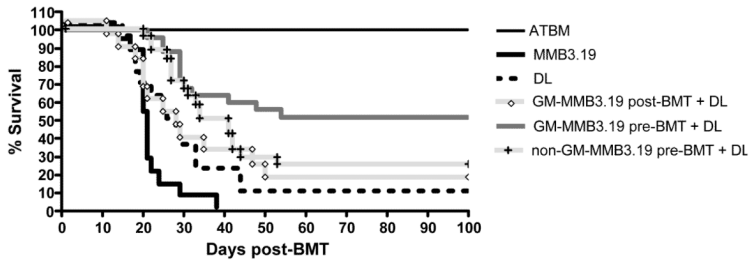

Figure 1.

Effect of pre-BMT vaccination with GM-MMB3.19 or non-GM-MMB3.19 cells on GVL responses to MMB3.19 leukemia challenge. B6 mice were vaccinated with a single s.c. dose of 2×10^5 GM-MMB3.19 or non-GM-MMB3.19 tumor cells. One week later, mice were exposed to 8.5 Gy and 4 h later transplanted with either B6 syngeneic 2×10^6 ATBM cells alone (ATBM and MMB3.19 groups) or in combination with $4\text{-}5\times10^6$ lymphocytes (DL, GM-MMB3.19 post and pre-BMT + DL and non-GM-MMB3.19 pre-BMT + DL groups). On day 1 post-BMT, all groups except the ATBM control were challenged with an i.p. injection of 1×10^5 MMB3.19 cells. The GM-MMB3.19 post-BMT group was vaccinated with a single dose of 2×10^5 GM-MMB3.19 cells on day 1 post-BMT. Data were pooled from 2-3 separate experiments consisting of 5-10 mice per group.

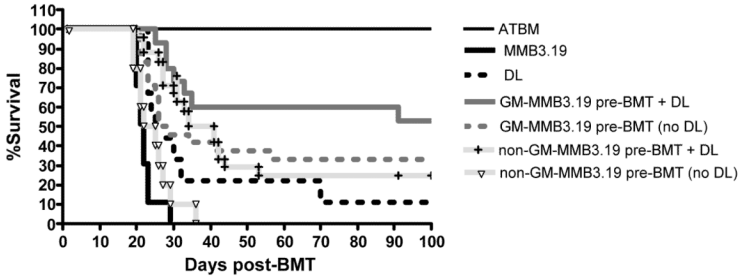

Figure 2.

Role of syngeneic DL in the GVL response to MMB3.19 leukemia challenge associated with GM-MMB3.19 and non-GM-MMB3.19 pre-BMT vaccination. B6 mice were vaccinated with a single dose of 2×10^5 GM-MMB3.19 or non-GM-MMB3.19 cells. One week later, mice were exposed to 8.5 Gy and 4 h later transplanted with either B6 syngeneic 2×10^6 ATBM cells alone [ATBM, MMB3.19, GM-MMB3.19 pre-BMT (no DL) and non-GM-MMB3.19 pre-BMT (no DL) groups] or in combination with $4\text{-}5\times10^6$ lymphocytes (DL, GM-MMB3.19 pre-BMT + DL and non-GM-MMB3.19 + DL groups). On day 1 post-BMT, all groups except the ATBM control were challenged with an i.p. injection of 1×10^5 MMB3.19 cells. Data were pooled from 2-3 separate experiments consisting of 5-10 mice per group.

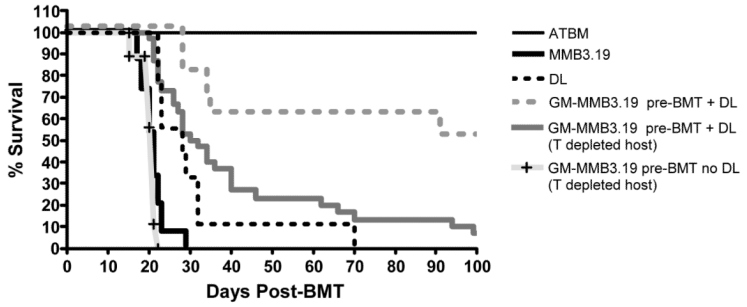

Figure 3.

Role of host T cells in the GVL effect following pre-BMT vaccination with GM-MMB3.19 cells. B6 mice were treated with 0.2 ml of GK1.5 (1:6) and 2.43 (1:50) mAb against $CD4^+$ and CD8+ T cells, respectively, 4 days prior to vaccination with a single s.c. dose of 2x105 GM-MMB3.19 cells. One week later (11 days after T cell-depletion), the mice were exposed to 8.5 Gy and 4 h later transplanted with either B6 syngeneic 2×10^6 ATBM cells alone (ATBM, MMB3.19 and GM-MMB3.19 pre-BMT no DL (T depleted host) groups] or in combination with $4\text{-}5\times10^6$ lymphocytes [DL, GM-MMB3.19 pre-BMT + DL, and GM-MMB3.19 pre-BMT + DL (T depleted host) groups]. On day 1 post-BMT, all groups except the ATBM control were challenged with an i.p. injection of 1×10^5 MMB3.19 cells. Data were pooled from 2-3 separate experiments consisting of 5-10 mice per group.

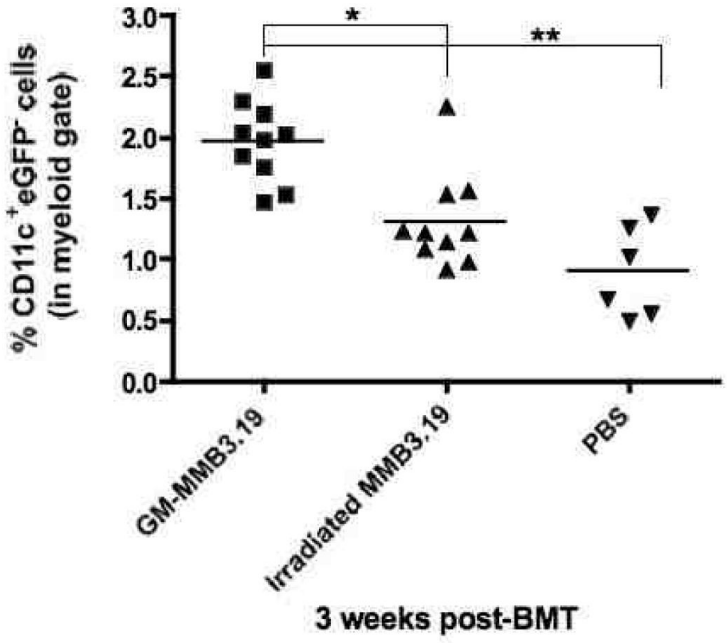

Figure 4.

Percentage of $CD11c^{+}eGFP^{-}$ cells in the skin-draining LN of vaccinated mice. B6 mice were vaccinated with a single dose of 2×10^{5} GM-MMB3.19, non-GM-MMB3.19 cells or PBS alone. One week later, mice were exposed to 8.5 Gy and 4 h later transplanted with ATBM and lymphocytes from $eGFP^{+}$B6 syngeneic mice in order to be able to differentiated between host ($eGFP^{-}$) and donor cells ($eGFP^{+}$). Three weeks post-BMT, cell suspensions from the skin-draining LN of individual mice were prepared for flow cytometric analysis to determine the percentage of $CD11c^{+}$ expressing cells. Data were pooled from 2 separate experiments consisting of 3-6 mice per group. Statistical difference between groups was determined using Kruskal-Wallis one-way ANOVA analysis ($p < 0.01$) followed by Dunn's multiple comparison test on individual pairs (*$p < 0.05$, ** $p < 0.01$).

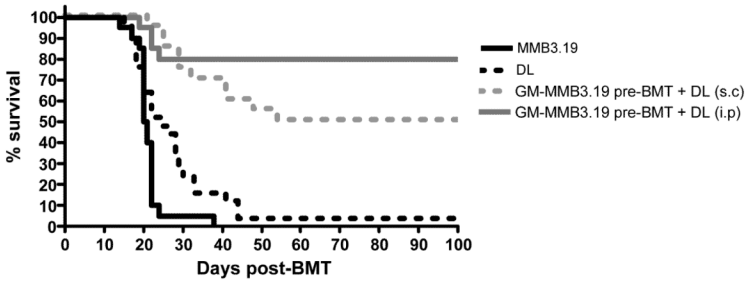

Figure 5.

Effect of vaccination site on the survival rate of MMB3.19 myeloid leukemia-challenged mice. B6 mice were vaccinated with a single s.c. or i.p. dose of 2×10^5 GM-MMB3.19 cells. One week later, mice were exposed to 8.5 Gy and 4 h later transplanted with either B6 syngenic 2×10^6 ATBM cells alone (ATBM and MMB3.19 groups) or in combination with $4\text{-}5\times10^6$ lymphocytes (DL, GM-MMB3.19 pre-BMT + DL s.c. and i.p. groups). On day 1 post-BMT, all groups except ATBM controls were challenged with an i.p. injection of 1×10^5 MMB3.19 cells. Data were pooled from 2-3 separate experiments consisting of 5-10 mice per group.

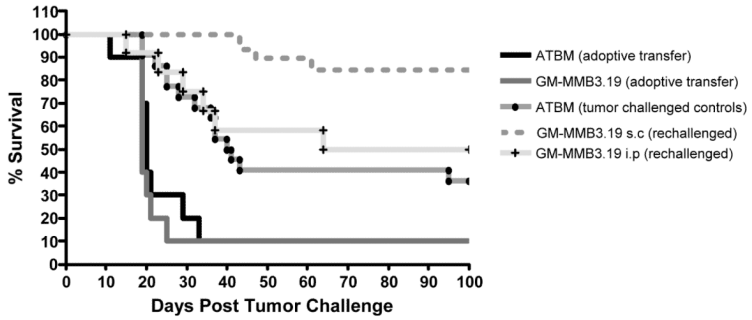

Figure 6.

Rechallenge of surviving GM-MMB3.19 vaccinated mice. Surviving GM-MMB3.19 vaccinated (i.p. or s.c.) mice were rechallenge with 5×10^6 MMB3.19 cells (i.p.) at ≥ 12 weeks post-BMT, or were used as donors for adoptive transfer of 2×10^6 ATBM cells along with $4\text{-}5\times10^6$ DL. For control purposes, their ATBM counterparts were also challenged with an equal dosage of tumor cells or were used as donors in adoptive transfer experiments. Adoptive transfer data were pooled from 2 different experiments of 10 mice per group. Re-challenge data were pooled from 4-5 different experiments consisting of 3-5 mice per group.

Table 1

Statistical comparison between groups from Figure 1

Groups compared	MST (days) and survival rate (%)	*P* value
DL vs. MMB3.19	27, 12% vs. 21, 0%	< 0.01
GM-MMB3.19 post-BMT + DL vs. DL	26.5, 14.3% vs. 27, 12%	NS (0.8)
GM-MMB3.19 pre-BMT +DL vs. DL	52% vs. 27, 12%	< 0.01
GM-MMB3.19 pre-BMT +DL vs. GM-MMB3.19 post-BMT	52% vs. 26.5, 14.3%	< 0.01
non-GM-MMB3.19 pre-BMT + DL vs. DL	37.5, 25% vs. 27, 12%	= 0.03
GM-MMB3.19 pre-BMT + DL vs. non-GM-MMB3.19 + DL	52% vs. 37.5, 25%	= 0.03 (stratified: ≥ 30 days)*

*Cox-proportional hazards test $p < 0.01$

Groups were compared using non-parametric Log-Rank test

Table 2

Statistical comparison between groups from Figure 2

Groups compared	MST (days) and survival rate (%)	*P* value
DL vs. MMB3.19	28, 10.5% vs. 21.5, 0%	< 0.01
GM-MMB3.19 pre-BMT + DL vs. DL	53.3, 0% vs. 28.0, 10.5%	< 0.01
GM-MMB3.19 pre-BMT (no DL) vs. DL	27.5, 33 % vs. 28.0, 10.5%	< 0.01 (stratified: ≥ 28 days)*
GM-MMB3.19 pre-BMT + DL vs. GM-MMB3.19 pre-BMT (no DL)	53.3% vs. 27.5, 33.0%	0.1 (Log-Rank) & 0.044†
non-GM-MMB3.19 pre-BMT + DL vs. non-GM-MMB3.19 pre-BMT (no DL)	37.5, 25% vs. 23.5, 0%	< 0.01
MMB3.19 + DL vs. non-GM-MMB3.19 pre-BMT (no DL)	28, 10.5% vs., 23.5, 0%	0.02
GM-MMB3.19 pre-BMT (no DL) vs. non-GM-MMB3.19 pre-BMT (no DL)	27.5, 33% vs. 23.5, 0%	0.01

*Cox Proportional Hazards test $p < 0.01$

Groups were compared using non-parametric Log-Rank test

†Comparison was performed using Wilcoxon Signed Rank test

Table 3

Statistical comparison between groups from Figure 3

Groups compared	MST (days) and survival rate (%)	*P* value
DL vs. MMB.319	28, 0% vs. 21, 0%	< 0.01
DL vs. GM-MMB3.19 pre-BMT + DL (T depleted host)	28, 0% vs. 31, 6.7%	NS (0.23)
DL vs. GM-MMB3.19 pre-BMT + DL (T depleted host)	95.5, 50% vs. 30.5, 6.7%	< 0.01
GM-MMB3.19 pre-BMT + DL (T depleted host) vs. GM-MMB3.19 pre-BMT + no DL (T depleted host)	30.5, 6.7% vs. 21, 0%	< 0.01

Groups were compared using non-parametric Log-Rank test

Table 4

Percentage expression of T cell subsets in the host ($eGFP^-$) and $eGFP^+$ populations

Phenotype	Control (PBS)	GM-MMB3.19	non-GM-MMB3.19
	(Mean percentage expression ± SD)		
$CD4eGFP^+/CD8eGFP^+$	2.29 ± 1.56	2.15 ± 1.07	2.03 ± 0.79
$CD4eGFP^-/CD8eGFP^-$	3.06 ± 1.37	3.82 ± 0.56	3.13 ± 0.91
$CD4^+CD44^{high}eGFP^+$	2.85 ± 1.15	2.77 ± 1.21	2.74 ± 1.05
$CD4^+CD44^{high}eGFP^-$	1.68 ± 0.91	1.49 ± 0.70	1.50 ± 0.58
$CD8^+CD44^{high}eGFP^+$	2.23 ± 2.03	2.38 ± 1.56	2.16 ± 1.56
$CD8^+CD44^{high}eGFP^-$	0.50 ± 0.35	0.46 ± 0.17	0.52 ± 0.20

Groups were compared using Kruskal-Wallis ANOVA test. No statistical differences were found for either phenotype

Table 5

Statistical comparison between groups from Figure 5

Groups compared	MST (days) and survival rate (%)	*P* value
DL vs. MMB.319	25, 4% vs. 20.5, 0%	= 0.013
GM-MMB3.19 pre-BMT + DL (s.c.) vs. MMB3.19 + DL	77, 50% vs. 25, 4%	< 0.01
GM-MMB3.19 pre-BMT + DL (i.p.) vs. MMB3.19 + DL	85% vs. 25, 4%	< 0.01
GM-MMB3.19 pre-BMT + DL (i.p.) vs. GM-MMB3.19 pre-BMT + DL (s.c.)	85% vs. 77, 50%	= 0.04

Groups were compared using non-parametric Log-Rank test

Table 6

Statistical comparison between groups from Figure 6

Groups compared	MST (days) and survival rate (%)	*P* value
GM-MMB3.19 s.c. (rechallenged) vs. ATBM controls	84% vs. 40.5, 36%	< 0.01
GM-MMB3.19 i.p. (rechallenged) vs. ATBM controls	82, 50% vs. 40.5, 36%	0.52
GM-MMB3.19 s.c. (rechallenged) vs. GM-MMB3.19 i.p. (rechallenged)	84% vs. 82, 50%	= 0.024
GM-MMB3.19 s.c. (adoptive transfer)vs. ATBM (adoptive transfer)	20, 10% vs. 20, 10%	NS

Groups were compared using non-parametric Log-Rank test