

Clinical relevance of recipient leukocyte infusion as antitumor therapy following nonmyeloablative allogeneic hematopoietic cell transplantation

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Objective. Graft-versus-leukemia effects of donor lymphocytes have been considered to be central to the therapeutic benefit of nonmyeloablative allogeneic hematopoietic cell transplantation (HCT) for malignant diseases. Surprisingly, some patients who reject donor grafts following nonmyeloablative HCT have sustained remissions of advanced, chemorefractory hematologic malignancies. In murine mixed chimeras prepared with nonmyeloablative conditioning, we previously showed that recipient leukocyte infusions (RLIs) induce loss of donor chimerism and mediate antitumor responses against host-type tumors. We assessed the clinical relevance of our mouse model.

Methods. Mixed chimeric mice were generated by a nonmyeloablative protocol and some of them received host-derived tumor cells and/or RLIs or donor lymphocyte infusion (DLI). We examined chimerism, graft-versus-host disease (GVHD), and tumor survival.

Results. RLI is still effective when the leukocytes are obtained from tumor-bearing mice. Established mixed chimerism is required prior to the induced rejection to achieve maximum antitumor effects. The antitumor effects of RLI are not dependent on a specific donor strain or conditioning protocol. In contrast to DLI, RLI leads to donor cell rejection without the risk of GVHD.

Conclusion. Together, these data reinforce the clinical potential of RLI therapy as a new HCT strategy that does not carry the risk of GVHD. © 2006 International Society for Experimental Hematology. Published by Elsevier Inc.

The antitumor potential of hematopoietic cell transplantation (HCT) is counterbalanced by its treatment-related toxicity, especially in relation to the development of graft-versus-host disease (GVHD) [1]. Less toxic, nonmyeloablative doses of chemotherapy and/or irradiation prior to allogeneic bone marrow transplantation (BMT) have recently been utilized in order to decrease transplant-associated complications [2–4]. Based on a murine model, we have developed a nonmyeloablative BMT regimen that includes in vivo T-cell depletion with pre- and posttransplant anti-thymocyte globulin or anti-CD2 monoclonal antibody (mAb; MEDI 507), thymic irradiation, pretransplant cyclophosphamide, and a short course of cyclosporine, which is discontinued by 5 weeks and followed by donor lymphocyte infusion

(DLI) [4]. Although all patients receiving these regimens developed initial mixed chimerism, a significant fraction of these patients subsequently lost chimerism. Remarkably, however, 20% of the patients who lost donor chimerism enjoyed sustained remissions of advanced hematologic malignancies, particularly lymphomas and multiple myeloma, without developing GVHD [5,6].

To better understand the mechanisms involved in this phenomenon, which could lead to the development of a new strategy for separating GVHD from graft-versus-leukemia (GVL) effects, we developed a mouse model. In mixed chimeras prepared with nonmyeloablative conditioning, we previously showed that recipient leukocyte infusions (RLIs) induced loss of donor chimerism and mediated antitumor responses against host-type tumors. Spontaneous loss of chimerism was also associated with antitumor effects, consistent with the clinical results described above. In the A20 BALB/c B-cell lymphoma model, RLI-mediated host-versus-graft reactions and tumor

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protection were mediated by distinct mechanisms involving interferon- γ -producing lymphocytes [7]. We now report studies aimed at further evaluating the clinical relevance of the use of RLI as an approach to achieving tumor responses.

Materials and methods

Animals

Female B10.BR, B10.RIII, and A.SW mice were purchased from the Jackson Laboratories (Bay Harbor, ME, USA). Female BALB/c, B10.A and A/J mice were purchased from the Frederick Cancer Research Facility, National Cancer Institute (Frederick, MD, USA). Mice were used in experiments at 8 to 12 weeks of age and housed in autoclaved microisolator environments. All procedures were performed in a laminar flow hood.

A20 cell line

A20 is a B-cell leukemia/lymphoma of BALB/c origin [8]. Cells were maintained in culture and administered intravenously at the lethal dose of 5×10^5 cells 7 days post-RLI, as described elsewhere [7].

Bone marrow transplantation

Mixed chimerism was induced in female BALB/c mice as previously described [9]. Mice were conditioned with depleting anti-CD4 and CD8 monoclonal antibodies (mAbs) on day -5, cyclophosphamide 200 mg/kg on day -1, and 7 Gy thymic irradiation or anti-CD40L (MR1) 0.5 mg on day 0 prior to transplantation of 25×10^6 allogeneic bone marrow cells. Some groups received RLI or DLI (3×10^7 spleen cells) 7 weeks post-BMT. Some RLI donor mice received BALB/c A20 B-cell lymphoma cells (1×10^5) 3 weeks before RLI. Some groups received RLI depleted of B cells by MACS column (Miltenyi, Biotech, Auburn, CA, USA) for purging of tumor cells. A20 cells (5×10^5) were given intravenously 1 week after RLI in chimeras or after allogeneic leukocyte infusion (3×10^7 B10.BR spleen cells) to untreated BALB/c mice.

Statistical analysis

Survival data were analyzed by the Kaplan-Meier technique using Prism software (Graphpad Software, San Diego, CA, USA) and significance was assessed using the log-rank test. A p value less than 0.05 was considered to be significant.

Results

Antitumor effects of RLI obtained from tumor-bearing hosts

Although we have observed antitumor effects in patients showing spontaneous rejection of the donor marrow graft, spontaneous rejection of the donor marrow only occurred in about 30% of patients treated with this protocol [5]. In order to assure rejection of the donor marrow in all patients, RLI would be needed. Because mixed chimerism induces tolerance to the donor, the nontolerant host lymphocytes must be collected before transplant and later used to induce

intentional rejection after establishing mixed chimerism, if this approach is applied in humans. In the mouse model, in contrast, rejection of the donor cells can be induced by injecting splenocytes from inbred, naive host-type mice. Because tumor-bearing hosts often demonstrate global immunosuppression [10], we therefore examined whether host-type leukocyte infusions were still effective when harvested from tumor-bearing mice. Splenocytes were harvested from BALB/c mice that had received a lethal dose of 1×10^5 A20 cells 3 weeks earlier. We have previously shown that these mice begin to die from tumor 4 weeks after injection of the tumor. Tumor cells were purged from the RLI using anti-B220 beads and MACS. Contamination with residual B220+ cells in the RLI was less than 0.1% (data not shown). As shown in Figure 1, recipients of RLI from tumor-bearing mice ($n = 10$) showed similar tumor survival compared with recipients of RLI from naive donors ($n = 10$; median survival time [MST] 53 vs 50 days, respectively; $p = 0.48$). Non-RLI BMT controls showed a shorter survival than recipients of RLI. Late recurrence of tumor was not observed in animals followed 200 days after RLI. Thus, RLI from tumor-bearing mice were as effective in mediating antitumor effects as RLI from non-tumor-bearing mice.

Antitumor effects of RLI from different BM donor strains

To examine whether these antitumor effects depend on the mouse strain used for allogeneic BMT, we compared the antitumor effect of RLI among recipients of BMT from different mouse strains. Longer survival was observed in the chimeras that received RLI compared with the chimeras not receiving RLI, regardless of donor strain ($p < 0.0001$ to $p < 0.05$; Fig. 2). However, some donor strains provided greater tumor protection than others. H2^a strains with two different genetic backgrounds (A/J and B10.A) showed a trend toward weaker protection than other strains with the same genetic background but different major histocompatibility complexes (MHCs); (A.SW, B10.BR, and B10.RIII). These data suggest that MHC differences may be of overriding importance in determining the strength of antitumor effects of RLI. Importantly, our results show that RLI-mediated antitumor effects are not dependent on a specific donor strain for BMT.

RLI induce antitumor effects in mixed chimeras prepared with different conditioning regimens

To determine whether or not the preparative regimen influences the antitumor effects of RLI, we used another protocol to induce stable mixed chimerism. Instead of 7 Gy thymic irradiation on day 0, we injected 0.5 mg of antagonistic anti-CD40L mAb (clone MR1) on day 0 just before BMC injection [11]. Although donor chimerism was lower in recipients of the MR1 protocol compared with mice receiving thymic irradiation, both MR1 and thymic irradiation protocols resulted in stable chimerism for up to 2

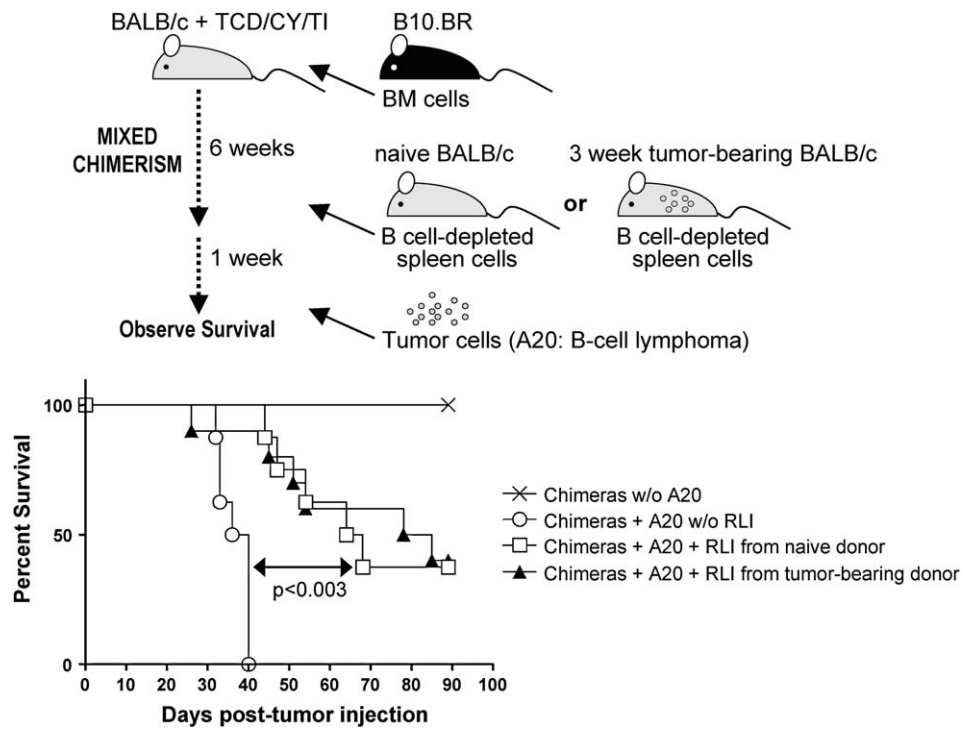


Figure 1. The antitumor effect of RLI from naive donors was compared with that from tumor-bearing donors. BALB/c (H-2^d) recipient mice were conditioned with anti-CD4 and anti-CD8 depleting mAbs intraperitoneally on day -5, CY 200 mg/kg intraperitoneally on day -1, and 7 Gy thymic irradiation on day 0. Mice were transplanted with 25×10^6 B10.BR BMC intravenously on day 0. Some groups received intravenous injection of 3×10^7 BALB/c spleen cells (RLI) intravenously on day +42 post-BMT and A20 BALB/c B lymphoma cells (5×10^5) on day +49 post-BMT. Survival is shown for nontumor controls (\times ; $n = 2$) and chimeras that received tumor without RLI (\circ ; $n = 8$), chimeric recipients of tumor plus RLI (\square ; $n = 8$), and for chimeric recipients of tumor plus RLI from tumor-bearing mice (\blacktriangle ; $n = 10$). p values are shown for chimeras without RLI vs chimeras with RLI or chimeras with RLI from tumor-bearing donors. Results from one of two similar experiments are shown. TCD, T-cell depletion; CY, cyclophosphamide; TI, thymic irradiation.

months without RLI (Fig. 3b). After RLI, donor chimerism was lost within 3 weeks, irrespective of which conditioning regimen had been used (Fig. 3b). Similar to observations with the thymic irradiation protocol, chimeras that received RLI (MST 97 days) had longer survival compared with those that did not (MST 57.5 days; $p < 0.01$; Fig. 3c). Interestingly, MR1 protocol-generated chimeras that did not receive RLI survived with tumor significantly longer than those generated using the thymic irradiation protocol (MST 57.5 vs 43 days, respectively; $p = 0.03$). A similar tendency was observed in chimeras that did receive RLI, but differences were not significant (MST 97 vs 76.5 days, respectively; $p = 0.17$; Fig. 3c). This experiment suggests that the antitumor effects of RLI are not dependent on the original conditioning regimen used to establish stable mixed chimeras.

Comparison of RLI with allogeneic leukocyte infusions in nonchimeric mice

Allogeneic leukocyte infusion alone has been reported to mediate antitumor effects against lymphomas in several clinical studies [12,13]. We therefore compared the antitumor effects achieved by rejection of allogeneic leukocytes vs that achieved in mice with established marrow allografts.

This was achieved by comparing the antitumor effect of allogeneic leukocyte infusion into naive mice with that of RLI given to mixed chimeras. As is shown in Figure 4, 5×10^5 A20 tumor cells were injected into mixed chimeras and naive BALB/c mice 1 week after RLI and allogeneic splenocyte infusion, respectively. Chimeras receiving RLI ($n = 13$) showed longer survival (MST 61 days) than naive mice receiving allogeneic leukocytes ($n = 10$; MST 39.5 days; $p < 0.01$). No survival benefit was observed in naive mice that received allogeneic leukocytes compared with chimeras that did not receive infusions, suggesting that rejection of allogeneic cells is insufficient and mixed chimerism is required prior to the induced rejection to achieve antitumor effects.

RLI mediate less powerful antitumor effects than DLI but do not carry the risk of GVHD

Next, we compared the magnitude of antitumor effects achieved with DLI vs RLI in mixed chimeras. We injected the same number of leukocytes from either donor-type or recipient-type mice into established mixed chimeras. Recipients of tumor plus RLI (3×10^7 splenocytes; $n = 11$) showed similar overall survival compared to recipients of DLI ($n = 11$; MST 53 vs 63 days, respectively; $p = 0.07$).

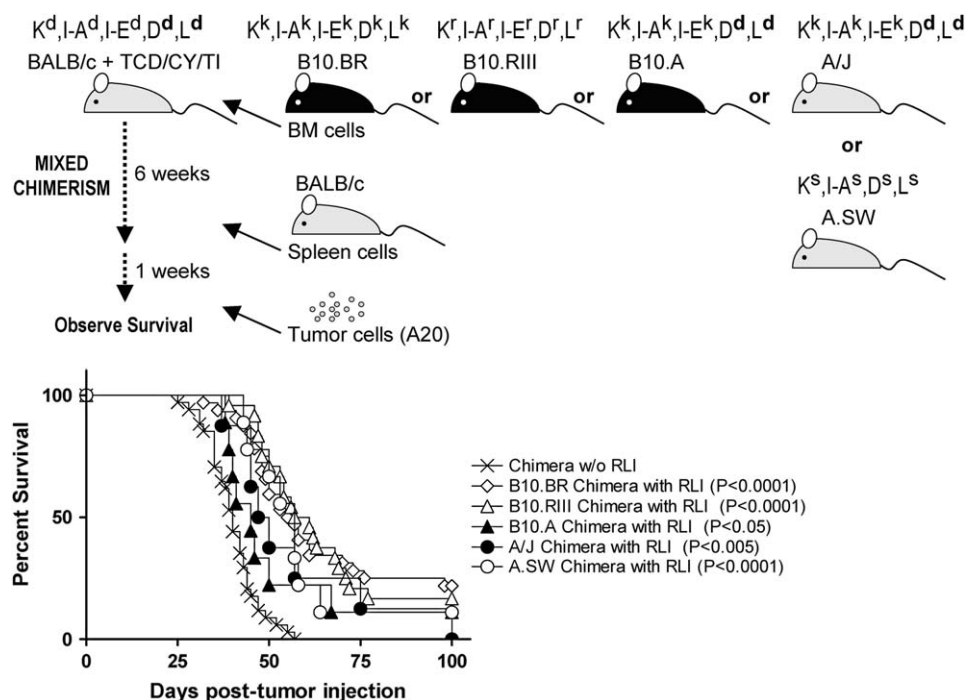


Figure 2. Antitumor effects were examined using different BM donor strains. Survival is shown for chimeras (with various marrow donor strains; all showed similar mortality) that received tumor without RLI (\times ; $n = 34$), for B10.BR \rightarrow BALB/c chimeric recipients of tumor plus RLI (\diamond ; $n = 31$), for B10.RIII \rightarrow BALB/c chimeric recipients of tumor plus RLI (Δ ; $n = 24$), for B10.A \rightarrow BALB/c chimeric recipients of tumor plus RLI (\blacktriangle ; $n = 9$), for A/J \rightarrow BALB/c chimeric recipients of tumor plus RLI (\bullet ; $n = 8$) and for A.SW \rightarrow BALB/c chimeric recipients of tumor plus DLI (\circ ; $n = 9$).

In contrast to studies involving smaller DLI administered to mixed chimeras at later time points [14], all recipients of DLI (3×10^7 splenocytes) showed histologic evidence of GVHD in this study (data not shown). They also showed loss of body weight and had no evidence of tumor on autopsy (Fig. 5 and data not shown). In contrast, recipients of RLI did not develop GVHD and converted to 100% host hematopoiesis (data not shown). These mice did have evidence of tumor on autopsy, with the exception of the long-term survivors (Fig. 5 and data not shown). Overall, though there were significantly stronger antitumor effects in mice receiving DLI, there was no survival benefit, due to increased death from GVHD.

Discussion

Currently, there is no curative therapy for chemorefractory leukemia/lymphoma except HCT. HCT, however, is not always effective and there is a serious risk of GVHD, which is the main cause of treatment-related mortality [1]. Many procedures derived from preclinical animal models have been proposed to separate GVL from GVHD, but currently no method has been widely applied in the clinical setting [15]. RLI provides a new approach to achieving antitumor effects without GVHD.

We have further explored here the clinical relevance of RLI. If this technique is to be used in the clinical setting,

it would be necessary to harvest leukocytes for RLI from tumor-bearing patients and purge the tumor cells from the harvest. This raises the concern that lymphocytes in tumor-bearing hosts may be unable to mount an appropriate antitumor response, due to tolerance [16] or nonspecific immunosuppression [17]. We have now demonstrated similar antitumor effects whether the RLI splenocytes are obtained from tumor-bearing mice or from naive syngeneic donors. Thus, although the primary tumor recipients in our study may have been tolerized [16] to the tumor or nonspecifically immunosuppressed [17], the RLI-induced alloresponse leading to rejection of the donor [18] was apparently sufficient to allow antitumor responses to develop. This result indicates that with a proper purging procedure, tumor-bearing recipient-derived RLI can be effective.

If this mouse model were only valid for specific donor and recipient combinations, its clinical applicability would be limited. Interestingly, we observed a trend toward reduced antitumor effects in two partially MHC class I-matched combinations of donor and recipient strains (Fig. 2), suggesting an important role for MHC disparity in these effects. Moreover, donor MHC appeared to be of greater importance than non-MHC background differences in determining the antitumor effect of RLI. However, we did see significant antitumor effects in all donor/recipient combinations used, indicating that the critical phenomenon is independent of donor strain.

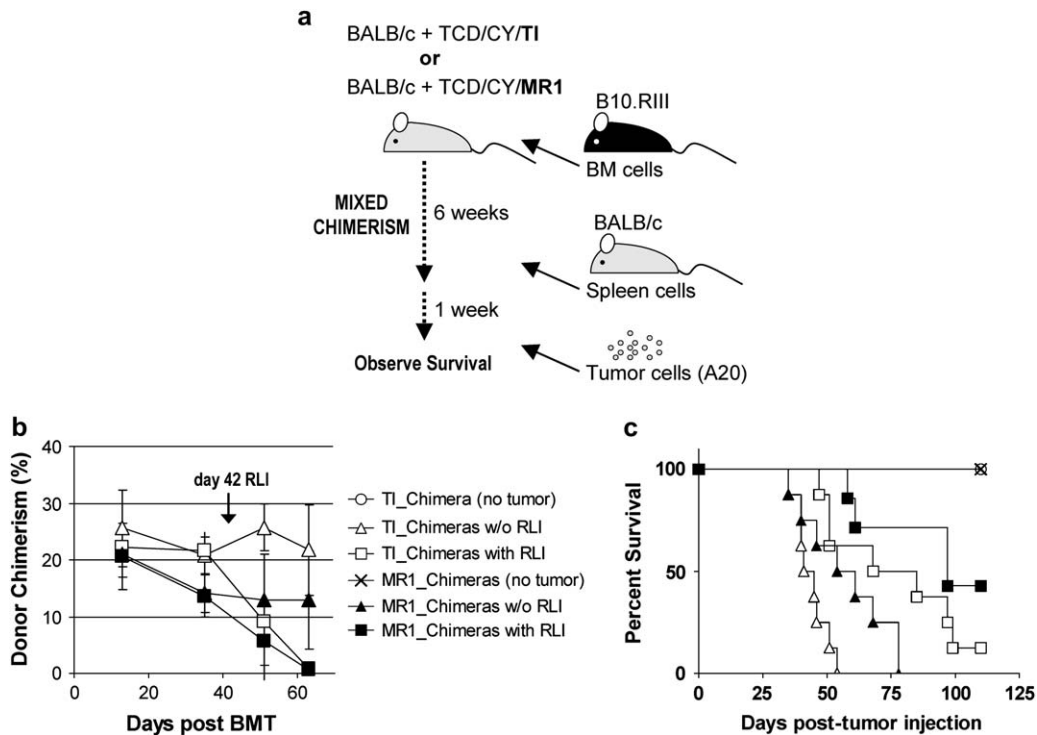


Figure 3. Antitumor effects were examined using different conditioning regimens. (a): Experimental scheme. (b): Mean (\pm SEM) Mac1⁺-cell chimerism in white blood cells at various times. (c): Survival is shown for chimeras that received the TI conditioning protocol (\circ ; $n = 3$), the MR1 conditioning protocol (\times ; $n = 3$), tumor plus the TI conditioning protocol without RLI (Δ ; $n = 8$), tumor plus the MR1 conditioning without protocol (\blacktriangle ; $n = 8$), tumor plus RLI with TI protocol (\square ; $n = 8$), and tumor plus RLI with MR1 protocol (\blacksquare ; $n = 7$).

Many clinical programs currently use various protocols for nonmyeloablative HCT. It was therefore important to determine whether or not the antitumor effects were regimen-specific. Our data suggest that they are not. It is surprising that MR1 protocol-generated chimeras that did not receive RLI survived significantly longer than those generated using the thymic irradiation protocol following tumor inoculation. Blockade of CD40-CD40L interaction by MR1 usually inhibits immune responses [19,20] and activation of CD40 signaling on dendritic cells (DC) has been reported to result in increased antitumor immunity [21]. However, MR1 has also been reported to prevent intrathymic deletion of self-reactive T lymphocytes [22], and T cells that escape deletion in the thymus may play a role in the antitumor effect in our model. Alternatively, it is possible that thymic irradiation is more immunosuppressive than MR1.

Two modalities being explored to treat hematologic malignancies include HCT followed by DLI, which can, under certain conditions, achieve GVL without GVHD [23–26] and allogeneic leukocyte infusion without HCT [12]. DLI following HCT is known to have potent antitumor effects but, especially in humans, can also cause GVHD [27]. Allogeneic leukocyte infusion without HCT is feasible but still experimental and its efficacy is not well established. We compared the efficacy of RLI therapy to these modalities. Our results predict that stronger antitumor effects

might be achieved from RLI therapy than has been achieved from allogeneic leukocyte infusion therapy without HCT in clinical trials. On the other hand, the antitumor effect of DLI is stronger than that of RLI, but survival may be counterbalanced by the risk of GVHD.

Our comparison between RLI in mixed chimeras and allogeneic leukocyte infusion in naive mice also suggests that the establishment of mixed chimerism greatly enhances the antitumor effect of hematopoietic cell rejection. Rejection of large numbers of allogeneic leukocytes in naive mice was not associated with antitumor effects in the aggressive A20 tumor model, in which RLI in mixed chimeras led to significant effects. One major difference between the two groups may relate to the distribution and presentation of donor antigen (Ag) by donor-derived DCs. DCs generated ex vivo can be efficiently loaded with antigen, but after reinjection few DCs traffic to secondary lymphoid organs, the critical sites for antigen presentation [28]. This problem can be avoided by transducing hematopoietic stem-progenitor cells (HSCs) with a model tumor antigen and then transplanting the gene-modified cells into irradiated recipient mice, resulting in efficient expression of the transgene in a large proportion of DCs in lymphoid organs [29]. These reports and the difference in tumor survival between the allogeneic leukocyte infusion and RLI groups in our studies suggest that the widespread establishment of HSC-derived,

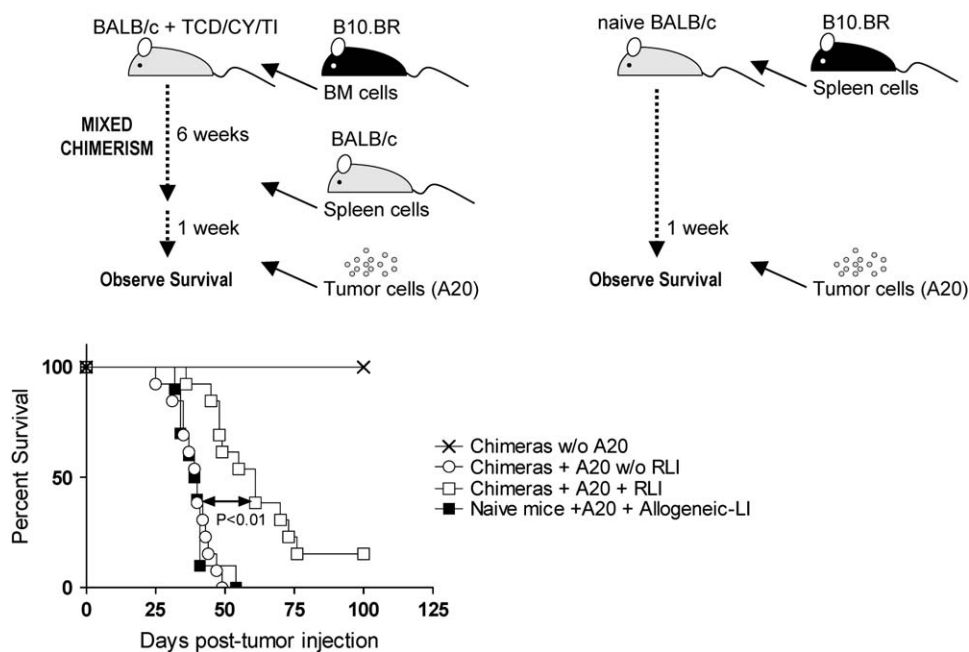


Figure 4. The antitumor effect of allogeneic leukocyte infusion into naive mice was compared with that of RLI given to mixed chimeras. Survival is shown for nontumor controls (x; n = 4), for chimeras that received tumor without RLI (o; n = 13), for chimeric recipients of tumor plus RLI (□; n = 13), and for naive mice that received tumor plus allogeneic leukocyte infusion (■; n = 10). Allogeneic-LI, allogeneic leukocyte infusion.

donor DCs in recipient lymphoid organs may be critical for this antitumor effect. We hypothesize that such established DCs initiate a cascade of immune responses beginning with activation of directly alloreactive T cells, with subsequent activation of indirectly alloreactive T cells and host antigen-presenting cells that also present tumor Ag, promoting

the generation of tumor-specific cytotoxic T lymphocyte responses.

In the treatment of slowly progressive yet lethal chemorefractory tumors such as follicular lymphoma or multiple myeloma, the risk-benefit ratio of a procedure such as allogeneic HCT raises many questions. The choice must be

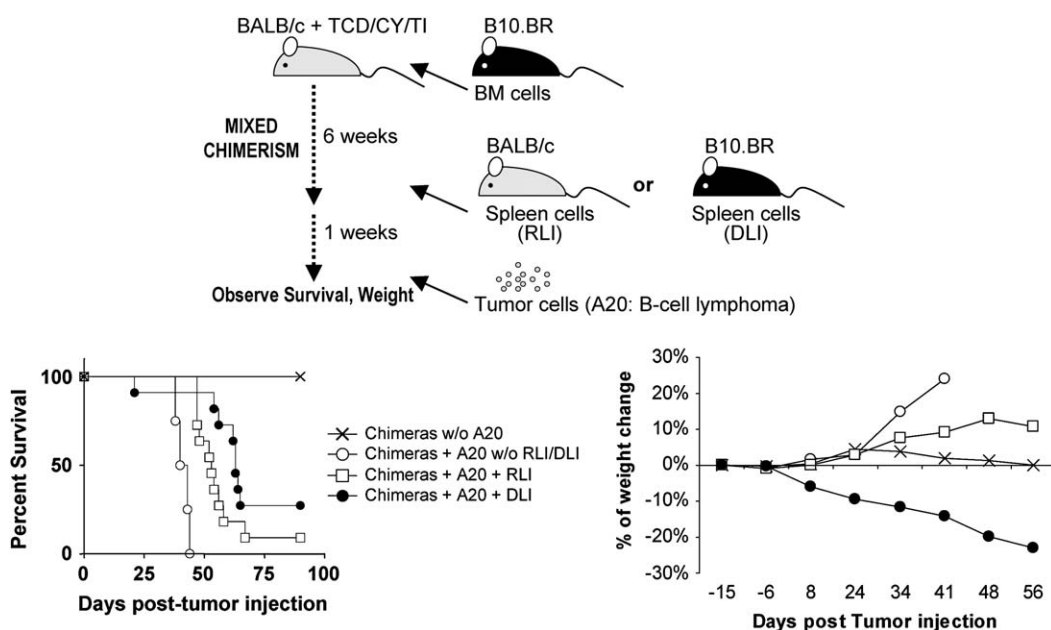


Figure 5. Antitumor effects and GVHD induced by DLI were compared with those of RLI given to mixed chimeras. Survival (bottom left) and weight changes (bottom right) are shown for nontumor controls (x; n = 2) and for chimeras that received tumor without RLI (o; n = 5), for chimeric recipients of tumor plus RLI (□; n = 11), and for chimeric recipients of tumor plus DLI (●; n = 11).

made between years of progressive deterioration in quality of life, and ultimate death 100% of the time, or taking a chance on a potentially curative procedure that may be fatal within the year. Together with our previous report [7] showing antitumor effects using other tumor cell lines, this study suggests that induction of an antitumor effect through rejection of the donor graft may be generalizable and that analyzing the mechanism will lead to an entirely novel approach to achieving antitumor effects without the risk of GVHD.

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