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Peritransplantation Vaccination with Chaperone-Rich Cell Lysate Induces Antileukemia Immunity

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ABSTRACT

We have reported that chaperone-rich cell lysate (CRCL) is an effective anticancer vaccine in immunocompetent mice. In this study, we explored the therapeutic applicability of CRCL in the context of syngeneic hematopoietic cell transplantation (HCT) to treat preexisting leukemia. Our results demonstrate that tumor growth is significantly delayed in mice receiving syngeneic HCT from 12B1 tumor CRCL-immunized donors compared with animals receiving HCT from nonimmunized donors. CRCL immunization after immune HCT further hindered tumor growth when compared with immune HCT without posttransplantation vaccination. The magnitude of the immune response was consistent with the antitumor effects observed in vivo. Rechallenge of surviving mice with 12B1 or A20 cells in opposite groins confirmed that mice had developed long-term tumor-specific immunity against 12B1 tumor cells. In addition, we documented that both T cells and natural killer cells contributed to the antitumor effect of CRCL vaccination, because depletion of either subset hampered tumor growth delay. Thus, our results indicate that CRCL is a promising vaccine capable of generating specific immune responses. This antitumor immunity can be effectively transferred to a host via HCT and further enhanced after HCT with additional tumor CRCL immunizations.
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KEY WORDS

Chaperone/heat shock proteins • BMT • CML

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is the only curative treatment for chronic myelogenous leukemia (CML) [1-3]. However, this therapy is generally limited to patients with matched donors and is usually not recommended for individuals over the age of 50 years because of the high risk of developing severe graft-versus-host–related complications. In contrast, autologous BMT for hematologic malignancies is associated with less toxicity and can be successfully used for elderly patients and those unable to find matched donors [4]. Nevertheless, autologous BMT is currently not recommended for patients with CML because of the unacceptably high relapse rates. Relapse results in part from residual *bcr-abl*$^+$ cells in the autologous graft and from the absence of a graft-versus-tumor effect [4,5]. Hence, efforts to augment host antitumor immunity in autologous BMT may provide a means to overcome leukemia relapse [6-9]. Adoptive transfer of autologous antigen-specific T cells from immunized hosts and/or active immunization after BMT is being explored in humans [10,11] and studied in animal models [7,8,12-14].

We have previously reported that in numerous animal models, tumor-derived chaperone-rich cell lysate (CRCL) that contains heat shock proteins (HSPs) 70 and 90, glucose regulate protein (GRP)94/glycoprotein (gp)96, and calreticulin can generate tumor-specific T-cell responses and protective antitumor immunity [15-20]. Tumor-derived CRCL constitutes an effective and abundant source of autologous tumor antigens, and, by virtue of its adjuvant properties, CRCL stimulates dendritic cells to mature and secrete interleukin 12 [19].

To investigate the effect of CRCL vaccination in an autologous hematopoietic cell transplantation (HCT) setting, we used an aggressive *bcr-abl*$^+$ murine leukemia, 12B1, that is resistant to total body irradi-

ation (TBI). We examined whether peritransplantation vaccination of mice with CRCL can be used effectively. Tumor growth was significantly reduced in mice receiving immune HCT compared with mice receiving HCT from nonimmunized donors. Additional CRCL vaccination in the early post-HCT period further delayed tumor growth. This therapy resulted in the activation of both tumor-specific T cells and natural killer (NK) cells. Peritransplantation vaccination with CRCL should be well tolerated and therefore may have applications in the autologous stem cell transplant setting for hematologic malignancies.

MATERIALS AND METHODS

Tumor Cell Lines

12B1 is a murine leukemia cell line derived by retroviral transformation of BALB/c bone marrow cells with the human *bcr-abl* (b3a2) fusion gene [21]. A20 is a B-cell leukemia/lymphoma that arose spontaneously in an old (>15 months) BALB/c mouse [22].

Tumor-Derived CRCL

Tumor-derived CRCL was generated as previously reported [16]. Briefly, tumor was homogenized in lysis buffer, and a 100 000*g* supernatant was obtained and quantified (BCA assay; Pierce Endogen, Rockford, IL). The high-speed supernatant was subjected to free-solution isoelectric focusing in a Bio-Rad Rotofor cell (Hercules, CA) for 5 hours at 15 W of constant power. Twenty fractions were harvested, and each fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot probing with specific antibodies for the chaperones HSP60, HSP70, HSP72 (inducible form of HSP70), HSP90, gp96, and calreticulin. Fractions from free-solution isoelectric focusing that contained substantial amounts of the above chaperone proteins, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting, were pooled and dialyzed stepwise out of urea and detergents. Pooled fractions were then concentrated using Centricon devices (Millipore, Bedford, MA). Detergents were removed by passage over an Extractigel matrix (Pierce Endogen). The fractions were reconstituted in phosphate-buffered saline (PBS), quantified as described previously, and stored at −70°C until use.

Mice

Female BALB/c (H-2^d) mice that were 6 to 10 weeks old (Harlan Sprague Dawley, Indianapolis, IN) were used for the experiments. The animals were housed in microisolator cages in a dedicated pathogen-free facility and cared for according to the University of Arizona Institutional Animal Care and Use Committee guidelines.

In Vivo HCT Experiments

On day −7, 6- to 8-week-old BALB/c mice were injected subcutaneously (SC) with 1×10^4 (10-fold the lethal dose) viable 12B1 cells. Tumor-bearing mice were given 900 cGy of TBI on day −1 by using a cobalt 60 radiation source at a rate of 100 cGy/min. Donor bone marrow cells and splenocytes were harvested from syngeneic mice receiving no immunization or 20 μg of 12B1 CRCL SC 5 and 11 days previously. After lysing the red cells with ammonium chloride potassium (ACK) lysing buffer, 2×10^7 bone marrow cells and 5×10^7 splenocytes were injected (on day 0) intravenously in a volume of 0.2 mL of PBS into the tail vein of tumor-bearing recipients. The animals that received transplants were maintained in sterile microisolator cages and received sterile food and water. The overall transplant-related mortality was <3%. Some HCT recipients also received post-transplantation vaccinations with 20 μg of 12B1 CRCL on days +1 and +6 (immune HCT + CRCL). Control animals consisted of mice injected with 10^4 12B1 cells without HCT (no HCT). In cell-depletion experiments, bulk splenocytes from CRCL-immunized donors were collected and subjected to negative immunoselection by using magnetic-activated cell-sorting microbeads and a pan–T-cell isolation kit (Miltenyi Biotec Inc, Auburn, CA). This procedure gives rise to 2 effector populations: CD3$^+$ T cells and CD3$^-$ non-T cells. The purity of CD3$^+$ T cells was >92%, and the percentage of CD3$^+$ cells in CD3$^-$ non-T cells was <5%. The CD3$^-$ non-T cells contain B cells, NK cells, dendritic cells, macrophages, and granulocytes. To evaluate whether T-cell depletion influences the antitumor effects, mice were injected with the CD3$^-$ non–T-cell population or CD3$^+$ T-cell population plus unfractionated bone marrow cells from immunized donors (T$^-$ immune HCT or T$^+$ immune HCT). To verify the effect of NK cell depletion, some donor mice that received CRCL immunization were given anti-asialo GM1 on days −6, −4, and −1 before their spleens were harvested (NK$^-$ immune HCT).

Tumor size was measured with calipers every other day once the tumors became palpable. Tumor volume was calculated by using the formula length × width^2 × π/6. Mice were killed when the tumor volume reached 4000 mm^3. Statistical comparisons in mean tumor volumes between groups were performed by using 1-way analysis of variance with Newman-Keuls multiple comparison post hoc tests, the Kaplan-Meier product-limit method was used to plot survival, and the log-rank statistic was used to test differences between groups [23,24]. Mice that rejected their tu-

mors were rechallenged with 3×10^3 12B1 or 10^6 A20 leukemia cells injected into the left or right groin 11 to 17 weeks after the first challenge. As controls, age-matched naive mice were given the same dose of 12B1 or A20 cells at the same time, and tumor growth was monitored.

The immune reconstitution of mice after HCT was determined by cell-surface staining of splenocytes with (anti-CD3, anti-CD4, and anti-CD8) specific mouse antibodies (PharMingen, San Jose, CA) and was analyzed by flow cytometry (FACScan; Becton Dickinson, San Jose, CA). The number of splenocytes was enumerated by trypan blue exclusion and reported as the mean cell yield.

In Vitro Assays

For all in vitro assays, the mice were manipulated in the same way as in the in vivo tumor growth experiments except that they had not been inoculated with 12B1 before HCT. Eight weeks after the HCT, splenocytes were harvested and assessed for proliferation, interferon (IFN)–γ production, and cytolytic activity.

For proliferation assays, the splenocytes were serially diluted and cultured in triplicate wells for 4 days in the presence or absence of 12B1 CRCL (10 μg/mL) followed by an 18-hour pulse with [^{3}H]thymidine (1 μCi per well; 0.037 MBq) as previously described [15]. Cells were harvested by using a 96-well Packard cell harvester, and radioactivity was measured on a β-counter (Packard Biosciences, Meriden, CT). Cell proliferation was expressed as counts per minute (CPM) of each experimental group stimulated with 12B1 CRCL minus the background CPM in the absence of 12B1 CRCL stimulation. Differences of CPM between groups were compared by analysis of variance.

For the IFN-γ production assay, splenocytes (1×10^6/mL) were cultured in 24-well plates in the presence or absence of 12B1 CRCL (10 μg/mL). The supernatants were collected 48 hours later, and IFN-γ was determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

Chromium 51 release assays were performed as described previously [25]. Splenocytes (2×10^6/mL) were stimulated with 12B1 CRCL (10 μg/mL) and cultured for 5 days. Interleukin 2 (20 U/mL) was added to the culture 72 hours later. Cytolytic activity against 12B1 and YAC-1 was determined in a standard 4-hour chromium 51 release assay at various effector-target ratios. Cytotoxicity was determined by the formula

$$\%\ \text{cytotoxicity} = (\text{exp mean cpm} - \text{spon release mean cpm})/(\text{max release mean cpm} - \text{spon release mean cpm}) \times 100$$

One lytic unit was defined as the number of effectors required to lyse 30% of targets, and cytotoxicity was presented as lytic units per 10^6 effector cells [26].

RESULTS

CRCL Immunization Delays Tumor Growth in the HCT Setting

We have previously described 12B1 as an aggressive leukemia: the lethal dose after SC injection is 10^3 cells [19,25]. SC inoculation of 10^4 cells (10-fold the lethal dose) induces palpable tumors at a median time of 10 days with *bcr-abl*$^+$ 12B1 cells infiltrating the spleen, lymph nodes, and bone marrow of tumor-bearing mice. To evaluate whether TBI followed by HCT is curative, BALB/c mice received 10^4 12B1 cells SC on day −7, a myeloablative dose of TBI (900 cGy) on day −1, and HCT on day 0. Because murine bone marrow is a poor source of lymphocytes, splenocytes were added to more closely mimic human autologous peripheral blood stem cell transplantation. We found that TBI followed by HCT delayed tumor growth (Figure 1) but extended the median survival time by only 6 days and failed to cure any mice of leukemia.

We have previously reported that 12B1-tumor CRCL immunization of immunocompetent mice was effective in suppressing tumor growth [16,18-20,26]. In this study, we examined whether CRCL vaccination would provide a therapeutic advantage in a syngeneic transplantation setting. Because 12B1-bearing mice die at a median time of 13 days after HCT (20 days from tumor induction), it was necessary to immunize mice in the first week after HCT, which clearly was not an optimal time to stimulate an immune response because CD4$^+$ and CD8$^+$ lympho-

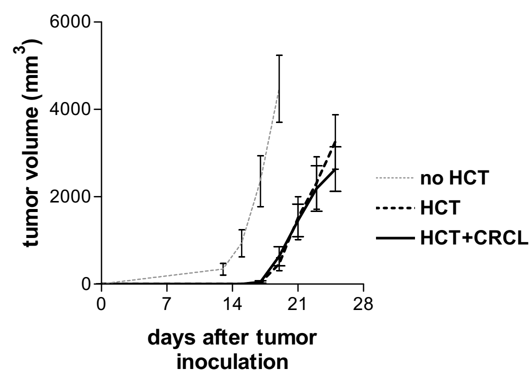

Figure 1. Active immunization with CRCL in the early reconstitution period after HCT. Mice (8 per group) bearing 6-day 12B1 tumors received either no treatment (no HCT) or TBI (day −1) followed by HCT (day 0) from nonimmunized donors. HCT consisted of 2×10^7 bone marrow cells plus 5×10^7 splenocytes. HCT recipients were vaccinated with 12B1 CRCL (HCT + CRCL) or PBS as control (HCT) on days +1 and +6 after HCT. Tumor growth was delayed in HCT groups regardless of post-HCT vaccination (either mock or CRCL vaccination) compared with the no-HCT group (day +23: $P < .001$, HCT versus no HCT). Active vaccination after HCT did not affect tumor growth (P = not significant; HCT + CRCL versus HCT). Representative data from 1 of 3 experiments are shown. Error bars represent SEM.

Table 1. *T-Cell Reconstitution after Hematopoietic Cell Transplantation (HCT)*

Time after HCT	Splenocytes		CD4$^+$ T Cells		CD8$^+$ T Cells	
	$\times 10^6$	%*	$\times 10^6$	%†	$\times 10^6$	%‡
Week 1	10.0	11.9	0.5	1.9	0.3	2.4
Week 2	50.5	58.7	2.4	9.2	1.0	7.9
Week 3	57.0	65.2	6.4	22.9	2.7	22.4
Week 5	94.0	92.2	21.5	65.8	6.1	43.4

*Percentage of total splenocytes compared with age-matched untreated controls.

†Percentage of CD4$^+$ T cells compared with age-matched untreated controls.

‡Percentage of CD8$^+$ T cells compared with age-matched untreated controls.

cytes were only beginning to reconstitute (Table 1). Mice were vaccinated on days +1 and +6 after HCT with 12B1-derived CRCL injected SC into the groin opposite the site of tumor implantation and were then monitored for tumor growth. Not surprisingly, there was no difference in tumor growth velocity between the 12B1 CRCL–vaccinated and PBS control mice (Figure 1), thus confirming that vaccination with CRCL during the early reconstitution period after HCT does not stimulate an immune response sufficient to affect tumor progression.

We next examined whether tumor-specific immunity generated in BALB/c donors can be adoptively transferred to HCT recipients and whether this can be further enhanced by CRCL vaccination after HCT. Donor BALB/c mice were immunized with CRCL 11 and 5 days before their bone marrow and spleens were harvested. HCT recipients of bone marrow and splenocytes from nonimmunized (HCT) or immunized (immune HCT) donors were vaccinated with CRCL (or PBS as control) on days +1 and +6 after HCT and monitored for tumor growth. Immune HCT recipients had significantly delayed tumor growth ($P < .05$, HCT versus immune HCT; Figure 2). CRCL immunization after immune HCT further hampered tumor growth when compared with immune HCT without posttransplantation vaccination ($P < .05$, immune HCT versus immune HCT + CRCL; Figure 2). Although we did not see apparent tumor regressions after an initial growth period, some tumors in the immune HCT + CRCL group seemed stable for some time. All mice in the no-HCT and HCT groups developed tumors, whereas 2 mice in the immune HCT group and 3 of 8 mice in the immune HCT + CRCL group rejected their tumors. These mice were followed up for >60 days after tumor inoculation. Taken together, these results indicate that pretransplantation antitumor immunity generated by CRCL vaccination can be effectively transferred to HCT recipients. In addition, CRCL vaccination, even when given during the early reconstitution period, can further delay tumor growth.

Mice Surviving after Immune HCT Have Long-Term Tumor-Specific Immunity

To evaluate whether surviving mice had developed long-term tumor-specific immunity, we rechallenged survivors (immune HCT and immune HCT + CRCL groups) from the experiments in Figure 2 (11 to 17 weeks after HCT) with a 3-fold lethal dose of 12B1 (3×10^3) and 1×10^6 A20 B-cell leukemia cells SC in opposite groins. Ten (83.3%) of 12 mice rejected 12B1 challenge, whereas only 3 (25%) of 12 mice rejected A20 leukemia, thus confirming that immunity was long lasting and tumor specific (Figure 3).

In Vitro Assays Demonstrate Persistent Immune Responses after HCT

To analyze the immune response generated by CRCL vaccination in the context of HCT, we evaluated proliferation, IFN-γ production, and the cytolytic activity of splenocytes harvested from posttransplantation mice after complete cell reconstitution after HCT (8 weeks). 12B1 CRCL–stimulated splenocytes from mice that received immune HCT followed by post-HCT CRCL vaccination displayed a significantly higher proliferation compared with spleen cells from naive HCT recipients ($P < .005$, immune HCT + CRCL versus HCT; Figure 4A). Splenocytes from immune HCT mice without posttransplantation immunization also showed substantial proliferation ($P < .05$, immune HCT versus HCT). The difference between immune HCT and immune HCT + CRCL was also significant ($P < .05$; Figure 4A). The num-

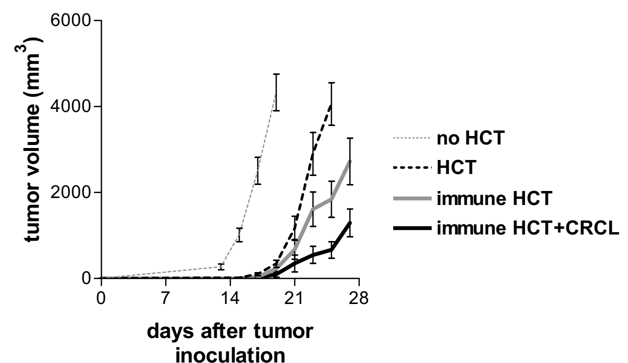

Figure 2. 12B1-specific immunity from immunocompetent donors can be adoptively transferred via syngeneic HCT. Mice bearing 6-day 12B1 tumors (8 per group) received no HCT (no HCT), HCT from nonimmunized donors (HCT), or HCT from 12B1 CRCL immunized donors (immune HCT); some of the immune HCT mice received additional CRCL vaccination on days +1 and +6 after HCT (immune HCT + CRCL). HCT consisted of 2×10^7 bone marrow cells plus 5×10^7 splenocytes. Immune HCT delayed tumor growth when compared with HCT (day 23 onward: $P < .05$, immune HCT versus HCT). Immune HCT followed by CRCL immunization further delayed tumor growth ($P < .05$, immune HCT + CRCL versus immune HCT). Representative data from 1 of 4 experiments are shown.

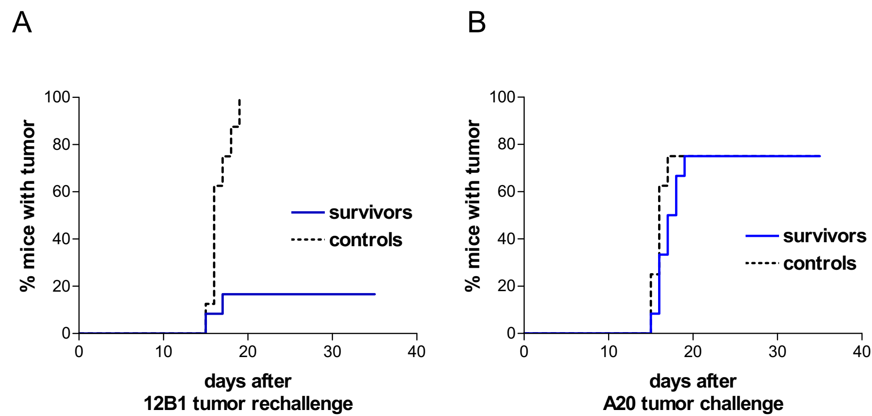

Figure 3. Mice surviving after immune HCT have long-term 12B1 tumor-specific immunity. Surviving mice (n = 12) from the experiments shown in Figure 2 were rechallenged 11 or 17 weeks after the initial tumor inoculation with both 3×10^3 12B1 cells and 1×10^6 A20 cells in opposite groins, as described in "Materials and Methods." Age-matched naive mice (controls; n = 8) were challenged with the same tumors. A, A total of 83.3% of survivors rejected rechallenge with 12B1, whereas all mice in the control group developed 12B1 tumors ($P < .01$, survivors versus controls). B, A20 cells grew in 75% of mice in both the survivor and control groups (P = not significant, survivors versus controls).

bers of T cells were comparable in the various groups shown in Figure 4.

We then determined IFN-γ secretion by spleen cells cultured in the presence of 12B1 CRCL. In vitro–stimulated splenocytes from mice that received immune HCT or immune HCT + CRCL produced

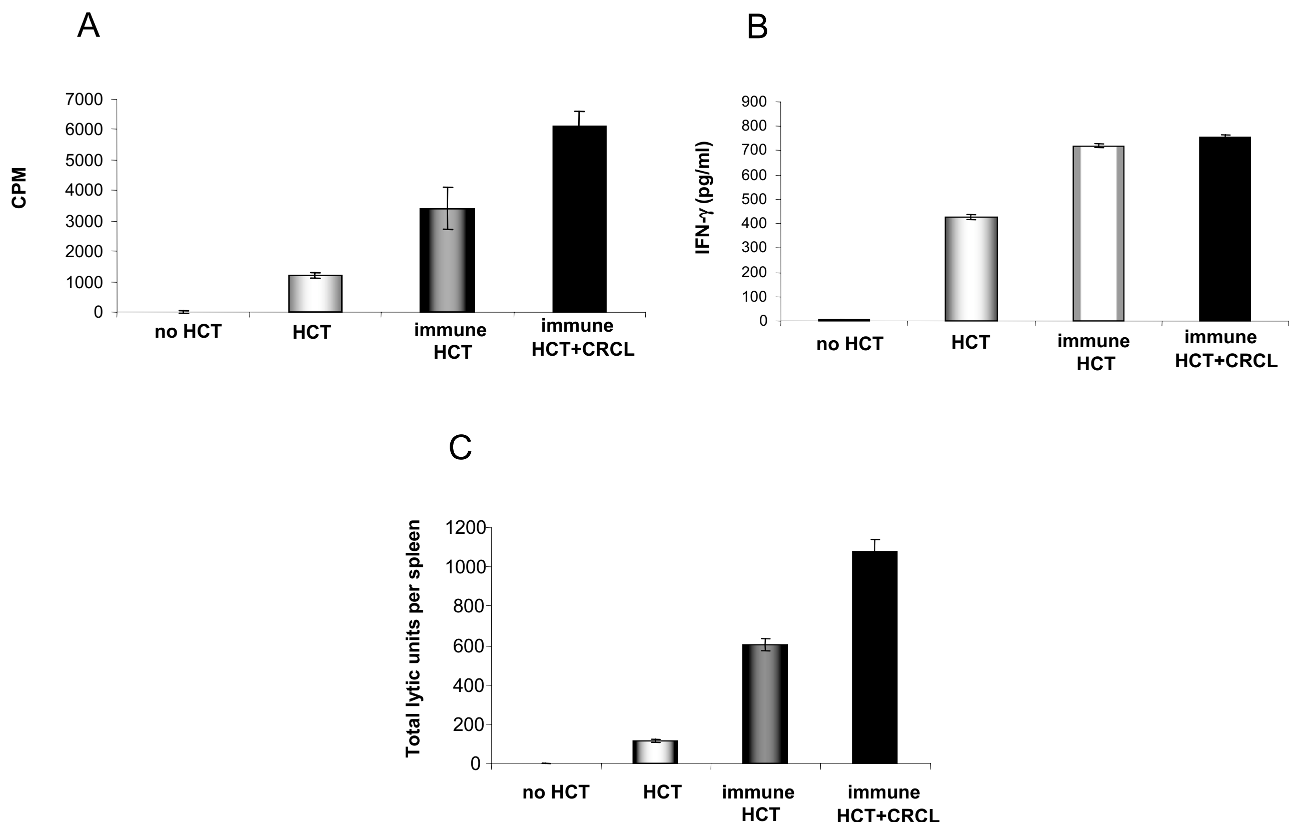

Figure 4. In vitro assays demonstrated persistent immune responses 8 weeks after HCT. Mice (4 per group) were treated as described in Figure 2 but did not receive tumor inoculation. Splenocyte proliferation, IFN-γ production, and cytotoxicity of splenocytes were assayed 8 weeks after HCT as described in "Materials and Methods." A, Splenocyte proliferation was expressed as counts per minute (CPM) of each experimental group stimulated with 12B1 CRCL minus the background CPM of the same group without 12B1 CRCL stimulation ($P < .05$, immune HCT versus HCT; $P < .05$, immune HCT + CRCL versus immune HCT). B, IFN-γ was measured by enzyme-linked immunosorbent assay ($P < .001$, immune HCT versus HCT; $P < .0001$, immune HCT + CRCL versus HCT; P = not significant, immune HCT + CRCL versus immune HCT). C, Cytotoxicity against 12B1 targets by effector splenocytes generated from HCT recipients. Cytotoxicity was expressed as total lytic units per spleen. All assays were performed at least 3 times.

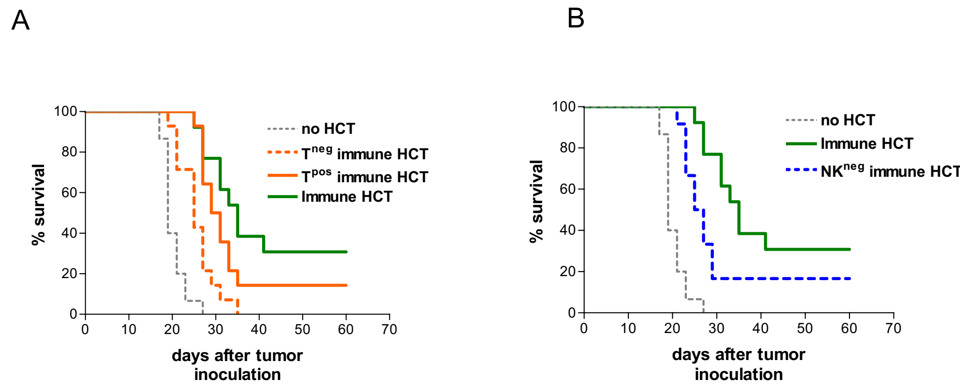

Figure 5. Both T and NK cells contribute to antitumor activity. A, Mice (14 to 16 mice per group) bearing 6-day 12B1 tumors received TBI and underwent transplantation with bone marrow from immunized donors, along with splenocytes that had been depleted of T cells (T^- immune HCT) or an enriched $CD3^+$ T-cell population (T^+ immune HCT), as described in "Materials and Methods." Mice that received no HCT or HCT from nonimmunized (HCT) or immunized (immune HCT) donors that had not been depleted of lymphocyte subpopulations were used as controls. T-cell depletion abrogated the antitumor effect of the adoptive transfer, as indicated by the reduction in tumor-free survival ($P < .05$, immune HCT versus HCT; $P < .05$, T^- immune HCT versus immune HCT; P = not significant, immune HCT versus T^+ immune HCT; P = not significant, HCT versus T^- immune HCT or T^+ immune HCT). B, HCT donor NK cells were depleted by intraperitoneal injection with anti-asialo GM1 on days −6, −4, and −1; the bone marrow and splenocytes from NK cell–depleted (NK^{neg} immune HCT) or nondepleted (immune HCT) donor mice were transfused to recipient mice bearing 7-day 12B1 tumor. Depletion of NK cells significantly impaired the antitumor effect of immune HCT ($P < .05$, NK^{neg} immune HCT versus immune HCT). Cumulative data of 2 experiments are shown.

significantly higher amounts of IFN-γ when compared with spleen cells from mice that received HCT ($P < .001$, immune HCT versus HCT; $P < .0001$, immune HCT + CRCL versus HCT; Figure 4B). In addition, splenocytes of mice that received immune HCT demonstrated significantly higher cytolytic activity against 12B1 compared with those from the naive HCT or non-HCT groups. CRCL vaccination after immune HCT further increased the killing by effector cells (Figure 4C).

T and NK Cells Contribute to the Adoptively Transferred Antitumor Activity after Syngeneic HCT

To assess the contribution of lymphocyte subpopulations in the antitumor effects observed after immune HCT, depletion of specific cell subsets was performed either in vitro or in vivo. As mentioned previously, donor mice were immunized with CRCL 11 and 5 days before cells were harvested. T cells were depleted or positively selected in vitro from splenocytes of immunized donors and infused along with bone marrow to recipient mice 1 day after TBI (T^- or T^+ immune HCT, respectively). The numbers of cells infused in the T^- and T^+ immune HCT groups were adjusted so that they were similar to the total number of $CD3^-$ T cells or $CD3^+$ T cells in the splenocyte preparation as immune HCT. Depletion of T cells from CRCL-immunized donors (T^- immune HCT) abrogated the effect of adoptive immunotherapy (Figure 5A). Mice that received T^+ immune HCT had a reduced antitumor effect when compared with those that received bulk splenocytes. This could be explained in part by the absence of other effectors, such as NK cells or macrophages. To address the role of NK cells, another donor group was treated in vivo with anti-asialo GM1 to deplete these cells after CRCL vaccination [27]. In vivo depletion of NK cells also resulted in reduced antitumor activity, thus confirming the active role of NK cells (Figure 5B). Overall, these results indicate that both T and NK cells contribute to the adoptively transferred antitumor activity.

To further analyze T and NK cell activity after HCT, we harvested spleen cells 8 weeks after mice received immune HCT. These splenocytes were restimulated in vitro with 12B1 CRCL for 5 days and then immunoselected into $CD8^+$ or $CD8^-$ effectors and tested against 12B1 for T-cell specificity and against YAC-1 for NK activity. Although the bulk splenocytes of mice that received immune HCT displayed a comparable killing activity against 12B1 and YAC-1, the $CD8^+$ and $CD8^-$ subsets showed different cytotoxicity against these targets. Indeed, the $CD8^+$ splenocytes were 75-fold more effective in killing 12B1 targets than their $CD8^-$ counterparts. In contrast, $CD8^-$ splenocytes were 3 times more potent than $CD8^+$ T cells in killing YAC-1 (Figure 6). Therefore, although immune HCT increased both cytotoxic T lymphocyte and NK activity in vivo, tumor-specific cytotoxic T lymphocytes could be further expanded in vitro with 12B1 CRCL stimulation.

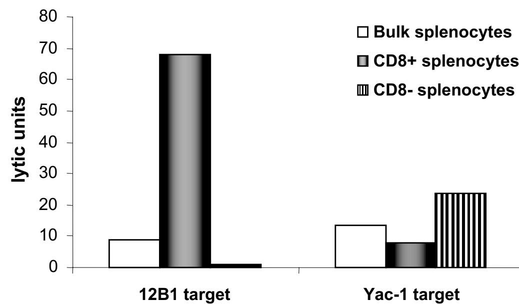

Figure 6. Both cytotoxic T lymphocyte and NK activities were enhanced in mice that received immune HCT. Mice received immune HCT as described in Figure 5. Eight weeks after HCT, splenocytes were harvested and cultured 5 days in vitro. The cytolytic activity of bulk splenocytes, immunoselected $CD8^+$ cells, and $CD8^-$ cells was assessed by chromium 51 release assay as described in "Materials and Methods." Lytic units per 10^6 effector cell subsets (bulk splenocytes, $CD8^+$ T cells, and $CD8^-$ cells separated from bulk splenocytes) were calculated. Representative data from 1 of 3 experiments are shown.

DISCUSSION

We demonstrated that immunity generated by tumor-derived CRCL immunization in syngeneic HCT donors can be effectively transferred to recipient mice via HCT. Adoptive transfer of CRCL immunity led to long-term tumor-free survival in a leukemia therapeutic model. This immunotherapeutic strategy was used for treatment of 12B1 leukemia that had been established for 6 days: a standard therapeutic protocol of TBI followed by HCT was unable to cure any mice. In our model system, adoptive transfer of CRCL immunity in the form of bone marrow and splenocytes from immunized donors (immune HCT) imparted a significant immunotherapeutic advantage over infusion of the same cells from naive donors (HCT). Moreover, adoptive immunity could be further augmented by active immunization with CRCL within 1 week after HCT, leading to long-term and specific protection against 12B1, from which the CRCL originated. In vivo priming of donors likely increased the percentage of tumor-specific memory T cells in the T-cell pool [28]. T-cell or NK cell depletion from donor splenocytes indicated that both lymphocyte populations contributed to the observed antitumor effect. Given that CRCL is composed of HSPs, which have been demonstrated to be capable of activating both the innate and adaptive immune systems [29-31], it is not surprising that we detected increased NK activity after CRCL treatment. HSPs have consistently been reported to activate NK cells by mechanisms that have not been well defined [30-34].

12B1 is an extremely aggressive leukemia model and resembles CML in blast crisis, a stage that is notoriously difficult to treat. Because of the rapid growth and metastatic spread of this tumor, it is very difficult to treat established disease even when immunotherapy is combined with imatinib as early as day 2 after tumor implantation [26]. Thus, the suppression of the tumor and prolongation of survival with immune HCT with or without CRCL in this model is noteworthy. Ours is not the first study in which HSP vaccines have been used in an HCT setting. Sato et al. [7] inoculated less aggressive A20 leukemia cells 10 days after BMT and started immunization of mice with HSP70 or gp96 before tumor induction. We have previously compared the CRCL vaccine with HSP70 and gp96 in numerous tumor models, including A20, and have found it to be at least as potent as and in some cases superior to the purified individual HSP vaccines [16,18]. In this study, we investigated the prospect of augmenting immunity in recipients with CRCL immunization at the early reconstitution period after HCT. From a quantitative aspect, the immune system generally returns to normal within 3 months after syngeneic BMT in mice, although the recovery of qualitative immune function may be longer [35]. Because of the aggressive and metastatic nature of 12B1, we were forced to immunize the HCT recipients within 1 week after HCT, a period in which the immune system is incompletely reconstituted. Several recent articles have demonstrated that homeostasis-driven T-cell proliferation in reconstituted lymphodepleted hosts improves the therapeutic efficacy of tumor vaccines [9,36-38]. We did not observe any enhancement of antitumor effect with CRCL immunization at this early reconstitution period after HCT from naive donors, possibly because the immunocompetence of mice that received lethal TBI was more severely impaired than the lymphopenia induced by sublethal chemotherapy or low-dose TBI [39-41]. Similarly, Borrello et al. [42] reported that vaccination of BALB/c mice 1 to 2 weeks after BMT with irradiated A20 lymphoma cells genetically modified to produce granulocyte-macrophage colony-stimulating factor failed to induce tumor rejection, whereas an anti-A20 effect was observed when the same vaccination was performed >4 weeks after BMT.

Adoptive transfer of tumor-specific immunity from BMT donors has been used to treat malignant disease in both animal and clinical studies [8,11-14,43]. In these studies, however, investigators used well-defined tumor antigens such as tumor-derived idiotype or influenza nucleoprotein [11-14,43]. Unfortunately, for most tumors there is a lack of well-defined, tumor-specific antigens. CRCL, which contains at least 4 known immunogenic HSPs, as well as potentially the entire repertoire of immunogenic peptides derived from tumor, would be an appropriate candidate for induction of tumor-specific immune responses [16,18-20,26].

In summary, we have shown that tumor-derived CRCL is an effective vaccine that can be used in

the transplantation setting to enhance antitumor responses. This study, along with our previous reports that CRCL vaccination is effective against a variety of tumors, supports the application of CRCL in stem cell transplantation for leukemia and other malignancies.

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