Bone Marrow Transplantation (2010) 45, 550–557
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ORIGINAL ARTICLE

Vector prime protein boost vaccination in the setting of myeloablative-induced lymphopenia suppresses growth of leukemia and solid tumors

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We have developed a vaccine, which is designed to induce tumor-associated antigen (TAA)-specific T cells and antibodies in the setting of profound lymphopenia induced by myeloablative therapy and T-cell-depleted bone marrow transplantation. Test mice were injected subcutaneously (sc) with the 32DP210Bcr-Abl cell line, which is positive for the p210Bcr-Abl protein (Group 1). In Group 2, 7 days after injection of the 32DP210Bcr-Abl positive cell line, the mice received 900 cGy total body irradiation (TBI) followed in 1 h by the intravenous infusion of 10 million T-cell-depleted syngeneic bone marrow cells (TCDBMT) (Group 2). The leukemia-bearing group received an intravenous injection of 10 million spleen cells (donor lymphocyte infusions) from unvaccinated (Group 3) and TAA/ecdCD40L-vaccinated (Group 4) syngeneic mice 3 days after completion of the TBI and TCDBMT. Groups 3 and 4 mice received three additional sc vaccinations at 7-day intervals with the TAA/ecdCD40L vaccine, in which the TAA was taken from the junctional peptide of the P210bcr-Abl protein. The survival of Groups 3 and 4 mice was significantly longer than that in Groups 1 and 2 mice. Vaccinated mice from Group 4, which developed complete responses, survived up to 350 days post-injection of the leukemia cells without any evidence of leukemia regrowth.
Bone Marrow Transplantation (2010) **45,** 550–557; doi:10.1038/bmt.2009.185; published online 3 August 2009
Keywords: CD40L; vaccine; Bcr-Abl; E7; adenovirus

Introduction

The goal of cancer vaccination is to increase the frequency of tumor-associated antigen (TAA)-specific T cells, which are capable of recognizing and killing neoplastic cells bearing TAA. Unfortunately, most patients with solid tumor and hematopoietic neoplasms are diagnosed in the fifth and sixth decades of life when the response to vaccination is diminished, and they respond poorly to vaccination.[1–4] TAA are usually presented in Class II MHC. In addition, many factors released by tumor cells directly suppress the development of an adaptive immune response, including trophic factors that induce the expansion of negative regulatory T cells in the tumor tissue.

Our laboratory[5–8] attached the TAA to the extracellular domain (ecd) of the potent immunostimulatory signal CD40L to create a molecule, which activates dendritic cells by binding the CD40 receptor, and delivers the TAA to the inside of the dendritic cells in which it ultimately becomes presented in Class I MHC. This compensates for the absence of the CD40L on the CD4+ helper T cells in the older individuals.[9,10] We also used subcutaneous (sc) injections of the adenovirus carrying the TAA/ecdCD40L transcription unit to activate the innate immune response through the binding of the viral DNA-specific motifs to the Toll-like receptors on the dendritic cells. This further expands the magnitude of the adaptive immune response induced by the vaccine. The use of the TAA/ecdCD40L protein boost to the Ad-sig-TAA/ecdCD40L vector helps to overcome the quantitative deficiency of antigen naive T cells in the cancer host.

We have shown[5–8] that this Ad-sig-TAA/ecdCD40L vector prime-TAA/ecdCD40L protein boost vaccine (we call this the TAA/ecdCD40L VPP vaccine) can overcome the anergy to TAA present in the TAA/Tg mouse even in old mice in which the response to vaccination is usually diminished.

We have already shown that the induction of an immune response with the TAA/ecdCD40L VPP vaccine does not require the presence of CD4+ T cells.[5] Several authors have reported that the use of DNA-damaging agents (chemotherapy or radiation) before vaccination increases the magnitude of the increase in the antigen-specific T cells induced by the vaccination. The delivery of vaccination post-chemotherapy or radiation has the advantage that the factors otherwise released by the tumor cells, including factors that induce the expansion of negative regulatory

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Received 23 February 2009; revised 5 May 2009; accepted 5 June 2009; published online 3 August 2009

Table 1 Experimental plan for testing the effect of the hBcrAbl/ecdCD40L VPP vaccination on the suppression of P210Bcr-Abl32D cells by TBI, TCDBMT, and DLI

Testing group	*TBI+TCDBMT*	*DLI*	*Recipient vaccination*
Group 1	None	None	None
Group 2	Yes	Unvaccinated donor	None
Group 3	Yes	Unvaccinated donor	Yes
Group 4	Yes	Vaccinated donor	Yes

T cells, are decreased at the time of vaccination (because the cancer cells are decreased in number). In addition, vaccination at the time of a depleted or diminished T-cell mass may create a regulatory environment in which the sensitivity to the vaccine-induced expansion of the TAA-specific T cells is enhanced.

To test this hypothesis, we first injected test mice sc with 2 million cells derived from the 32Dp210Bcr-Abl leukemia cell line. The mice were then treated as outlined in Table 1. The mice that received donor lymphocyte infusions (DLI) from vaccinated donors and that received vaccination post-DLI developed complete responses in 44% of the treated mice and 75% of these mice were disease free in unmaintained remission for over 350 days after the injection of the leukemia cells. This shows that the Bcr-Abl/ecdCD40L vaccination can expand the number of Bcr-Abl-specific T cells in mice that are severely lymphodepleted. Similar results were obtained with mice that had been injected sc with the E7/ecdCD40L VPP vaccination of mice first injected with the HPV E7 positive TC-1 cell line,[11] and then given total body irradiation (TBI) and T-cell-depleted syngeneic bone marrow cells (TCDBMT) with and without DLI from E7/ecdCD40L VPP-vaccinated syngeneic donors.

Materials and methods

Cell culture and mice

The 32Dp210Bcr-Abl cells[12,13] were obtained from Dr Brian Druker (Oregon Health Sciences University, Portland); 293 and TC-1 cells were obtained from the ATCC. C3H/HeNHsd mice ($H2^K$) and C57BL/6J mice were obtained from Harlan Sprague Dawley. All mice were approximately 6 weeks of age at the time of transplantation.

Construction of Ad-sig-BcrAbl/ecdCD40L vectors and BcrAbl/ecdCD40L protein plasmid expression vectors

To identify immunogenic peptide sequences spanning the Bcr-Abl junction, we reviewed earlier experiments and software that focuses on predicting the binding affinity of peptides to MHC molecules.[14,15] We chose two junctional peptides SSKALQRPV and ATGFKQSSKA. For the sequence encoding a secretory signal sequence and two Bcr-Abl junctional peptides, a pair of DNA oligmers (bcrabl5 and bcrabl6) was synthesized. The sequence of these DNA oligomers (156 and 148 bases each) is available on request. Proper annealing of bcrabl5 and bcrabl6 generates a DNA sequence that encodes an Ig-κ-chain secretory signal sequence and two Bcr-Abl junctional peptides (METDTLLLWVLLLWVPGSTGDAAASSKA LQRPVAAATGFKQSSKAAA). This sequence also carries BglII and XhoI recognition sequences at the 5′ and 3′ ends, respectively. Those sites were used to insert the bcrabl junctional peptide-containing fragment into the corresponding site of pShuttle-CMV (Quantum Biotech, Inc., Carlsbad, CA, USA). The sequence encoding the ecd of the mouse CD40L (ecdCD40L) was amplified from pDC406-mVD40L[1] and inserted into the Xho1/Xba1 sites. This plasmid was designated pSCMVbcrablCD40L. The pAd-sig-BcrAbl/ecdCD40L or pAd-sig-E7/ecdCD40L adenoviral vectors were generated by homologous recombination of pSCMVbcrablCD40L in a bacterial strain BJ5183-pAd1 (Stratagene, Inc., San Diego, CA, USA) for the Ad-sig-BcrAbl/ecdCD40L as described earlier.[5,6] The Ad-sig-E7/ecdCD40L vector was generated as described in our earlier publication.[5]

Bacterial expression and purification of TAA/ecdCD40L fusion protein

The mouse CD40L was amplified by the following primers: forward primer 5′-CGGATATC G GTCGAAGAGGAA GTAAACCTTC-3′; reverse primer 5′-AC CTCGA GTCAGAGTTTGAGTAAGCCAAAAGATGAG-3′. The PCR fragment was cloned into the pTriex-2 vector by using EcoRV and Xho I sites. The resulting vector was designated pTriex-ecdCD40L. The E7 fragment was amplified by the following primers: forward primer 5′-AA CCA TCA CTC TTC TGG T ATG CAT GGA G AT ACA CCT AC-3′; reverse primer 5′-CGCCG GATATC TGG TTT CTG AGA ACA GAT GGG -3.

The E7 PCR fragment was inserted into pTriex-ecdCD40L using XcmI and EcoRv sites.

The TAA/ecdCD40L cDNA was amplified from pSCMVTAACD40L and inserted into the BglII/Xho1 site of pTriEx-2 Hygro (Novagen, North Ryde, Australia). The resulting vector (pTriEx-2hygroTAA/ecdCD40L) was introduced into Rosetta(DE3)pLacI competent cells (Novagen). Transformed cells were grown and the expression of the protein was induced using Overnight Express Autoinduction System 2 (Novagen). The bacterial cell lysate was prepared by the CellyticB Plus kit (Sigma-Aldrich, St Louis, MO, USA). The TAA/ecdCD40L protein, to which was attached a hexahis tag, was purified from the soluble fraction by the HIS-select Nickel Affinity Gel column fractionation (Sigma-Aldrich). The protein was eluted by increasing concentrations of imidazole. Then, the protein was concentrated and desalted by centrifugation through an Ultra-free-15 Biomax-50 filter (Millipore) and eluted with PBS. The purified TAA/ecdCD40L protein appeared as a single dominant band on molecular weight PAGE gel electropheresis.

Treatment scheme for the BcrABl/ecdCD40L VPPP vaccine

The treatment scheme is summarized in Table 1 and in Figure 1. At 900 cGy, TBI (from our local Cobalt source irradiator) produced irreversible myelosuppression and 100% mortality in mice not receiving bone marrow

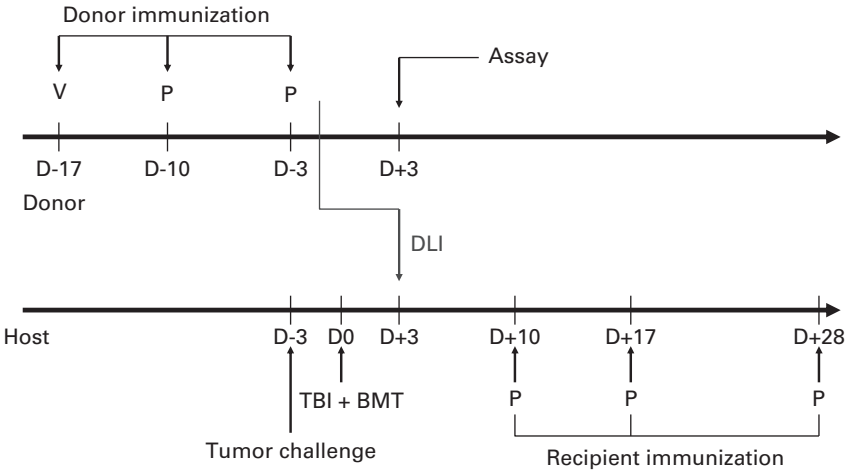


Figure 1 Treatment schema.

transplantation, but which could be rescued in 100% of the test mice, which received 10 million T-cell-depleted bone marrow cells.

Donor mice for BMT and DLI for the BcrAbl/ecdCD40L VPP vaccine

Ten male C3H/HeNHsd mice were vaccinated by administering an sc injection of the Ad-sig-BcrAbl/ecdCD40L vector. One and 2 weeks later, the mice were given sc 10 μg injections of the BcrAbl/ecdCD40L protein. Five of those mice were prepared for collection of spleen cells for use in the DLI (five mice), and at the same time, the other five mice were killed for collection of spleen cells to be used in cytotoxicity and ELISPOT assays. Another 10 male unvaccinated C3H/HeNHsd mice were killed for collection of bone marrow cells. The experiments for the E7/ecdCD40L VPP vaccination of the lymphocyte donors were carried out in a similar manner except that C57BL/6J mice were used.

Recipient mice for the TAA/ecdCD40L VPP vaccine

A total of 40 recipient female C3H/HeNHsd mice were used for this study. Among them, 10 mice were used for control Group 1 (see Table 1). Another 10 mice were used for the treatment Group 2 (see Table 1), which received TBI + TCDBMT without vaccination of the DLI donors or the recipients. In treatment Group 3 (see Table 1), the mice received TBI, TCDBMT, and DLI from unvaccinated donors, but the recipient mice received the BcrAbl/ecdCD40L vaccination themselves. In treatment Group 4, the mice received TBI, TCDBMT, and DLI from BcrAbl/ecdCD40L VPPP-vaccinated donors, and these recipient mice received vaccination with three sc injections of the BcrAbl/ecdCD40L protein. The E7/ecdCD40L VPP vaccination of the recipients was carried out in similar manner except that only one sc injection of the E7/ecdCD40L was given to the recipients 7 days after the DLI. ELISPOT and cytoxicity assays were used to characterize the number and functional status of the TAA-specific CD8+ T cells in vaccinated mice using the methods published earlier.[6–8]

Bone marrow harvesting from donor mice and TBI plus BMT administered to recipient mice

Donor bone marrow was T-cell depleted by incubation with CD90 (Thy 1.2) MicroBeads (Miltenyi Biotec Inc., Gladbech, Germany) followed by removal of the cells bound to the antibody conjugated beads. Over 95% of the T cells were removed by this procedure.

Statistical analysis

All parameters were analyzed using Student *t*-test or analysis of variance, followed by the Scheffé procedure for multiple comparisons as *post hoc* analysis. All data shown are presented as mean ± s.e.m. Survival was estimated using the Kaplan–Meier product-limit method. Survival rates were compared for statistical differences by using log-rank analysis. *P*-values >0.05 were considered as statistically significant and all *P*-values correspond to two-sided significance tests.

Results

Study of the effect of the Ad-sig-BcrAbl/ecdCD40L vector prime Bcr-Abl/ecdCD40L protein boost on the level of the Bcr-Abl-specific CD8 T cells in vaccinated vs unvaccinated mice

On the basis of earlier experiments and software models that predict binding affinity of peptides to the MHC-binding pocket,[14,15] we chose two junctional peptides SSKALQRPV and ATGFKQSSKA. These peptides from the Bcr-Abl junctional domain were expected to bind effectively to Class I MHC molecules of mouse haplotype H2k that corresponds to the Bcr-Abl-expressing 32D cells (32Dp210Bcr-Abl). C3H/HeNHsd mice ($H2^k$) were injected sc once with the Ad-sig-BcrAbl/ecdCD40L vector followed by two sc injections of the BcrAbl/ecdCD40L protein boost. Seven days after the completion of the vaccination, the mice were killed and the spleen cells disaggregated into a single cell suspension. As shown in Figure 2a, the BcrAbl/ecdCD40L VPPP vaccination induced an increase in the

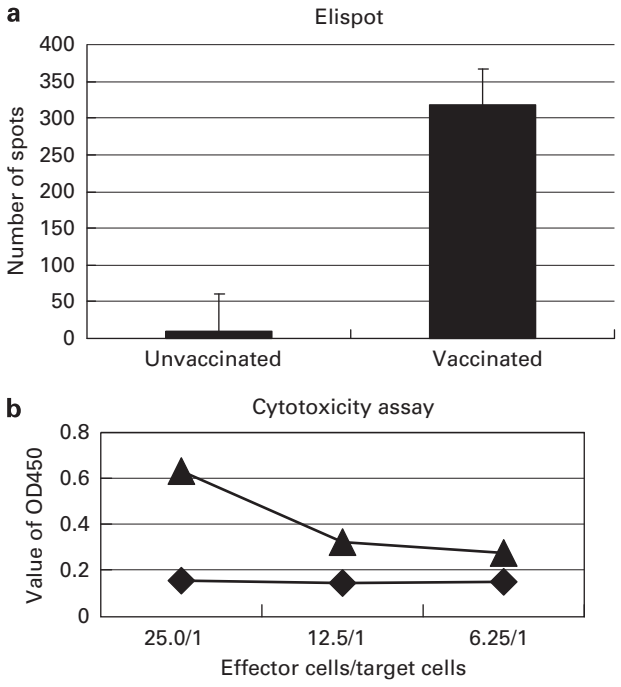


Figure 2 (**a**) The results of ELISPOT assay. The mice were injected sc once with the Ad-sig-BcrAbl/CD40L vector followed in 7 and 14 days with sc injections of the BcrAbl/ecdCD40L protein boost (vaccinated). The spleen cells from vaccinated and unvaccinated mice were harvested 3 weeks after the last vaccination. A total of 1×10^5 spleen cells were exposed to 10 μg of the Bcr-Abl junctional peptides for 48 h and the cells were counted for interferon-γ staining. Data are presented as mean ± s.e.m. (**b**) The results of cytotoxicity assay. The mice were injected sc once with the Ad-sig-BcrAbl/CD40L vector followed in 7 and 14 days by sc injections of the BcrAbl/ecdCD40L protein boost (vaccinated). The spleen cells from vaccinated and unvaccinated mice were incubated with Bcr-Abl-specific peptides in the presence of IL-2 for 5 days. The CD8+ effector cells were then isolated and incubated with 32Dp210Bcr-Abl cells in the effector/target ratios as indicated. The cytotoxicity was measured by LDH release as described in Materials and methods.

number of Bcr-Abl-specific cells >10-fold in vaccinated donor mice.

It is possible to induce an increase in the level of antigen-specific T cells assayed by ELISPOT and yet find out that they are functionless in one or more assays of T-cell function. Therefore, we compared the cytotoxic function of CD8+ effector cells collected from Ad-sig-BcrAbl/ecdCD40L vector prime-BcrAbl/ecdCD40L protein boost vaccinated vs unvaccinated mice. As shown in Figure 2b, the lysis of the p210Bcr-Abl positive 32Dp210Bcr-Abl target cells was significantly higher in the effector/target mixtures, which contained CD8+ T cells from vaccinated mice. These experimental results show that the vaccination not only resulted in an increase in the level of Bcr-Abl-specific CD8+ T cells, but also these cells were functionally cytotoxic to p210Bcr-Abl positive target cells (32Dp210Bcr-Abl).

Effect of the BcrAbl/ecdCD40L VPPP vaccine on regrowth of P210Bcr-Abl positive 32D myeloid cells after TBI, TCDBMT, and DLI

We next carried out the experiment outlined in Figure 1. As shown in Figure 3a and Tables 2–4, the average volume

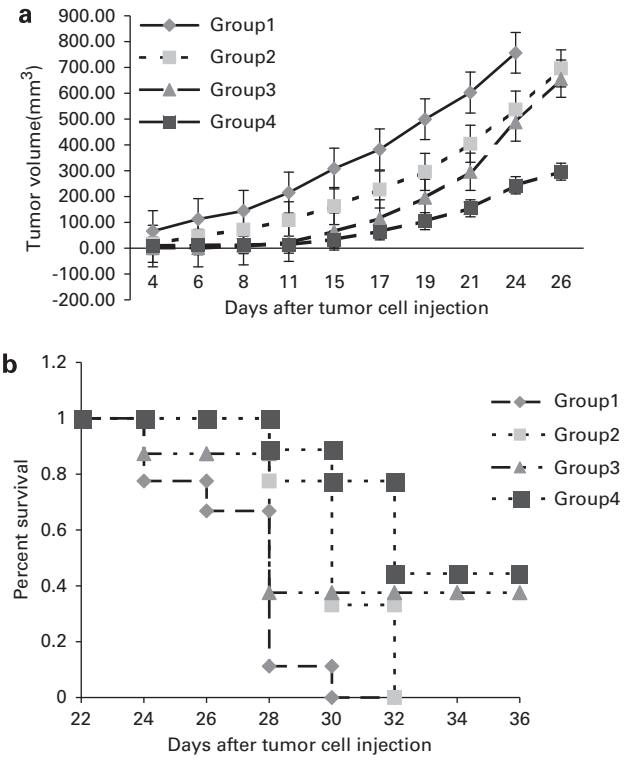


Figure 3 (**a**) Tumor volume after treatment. Two million 32Dp210Bcr-Abl cells were injected sc in C3H/HeNHsd mice. After 3 days, the mice were administered 900 cGy of TBI. One hour later, each mouse was given 10 million T-cell-depleted bone marrow cells by tail vein injection. Three days later, the mice were given intravenous injection 10 million spleen cells from unvaccinated (Groups 2 and 3) or vaccinated mice (Group 4). Mice in Groups 3 and 4 then received three sc injections of the BcrAbl/ecdCD40L protein on 10, 17, and 24 days after the TBI and TCDBMT as described in Materials and methods. Group 1 received sc injection of 2 million 32Dp210Bcr-Abl cells without TBI, TCDBMT, or DLI. The mean of the tumor volumes of Group 4 mice was statistically different from the volumes of tumors in other groups at the $P = 0.003$. (**b**) Survival. Two million 32Dp210Bcr-Abl cells were injected sc in C3H/HeNHsd mice. After three days, the mice were administered 900 cGy of TBI. One hour later, each mouse was given 10 million bone marrow cells, which were depleted of T cells by tail vein injection. Three days later, the mice were given intravenous injection 10 million spleen cells from unvaccinated (Groups 2 and 3) or vaccinated mice (Group 4). Mice in Groups 3 and 4 then received three sc injections of the BcrAbl/ecdCD40L protein on 10, 17, and 24 days after the TBI and TCDBMT as described in Materials and methods. Group 1 received sc injection of 2 million 32Dp210Bcr-Abl cells without TBI, TCDBMT, or DLI. The mice were monitored for cumulative survival. The mice in Group 3 had a survival, which differed statistically significantly from the other groups at the $P = 0.00001$.

of the sc tumor was lowest in Group 4 (the group receiving TBI, TCDBMT, and DLI from the BcrAbl/ecdCD40L VPP-vaccinated lymphocyte donors, and three sc injections of BcrAbl/ecdCD40L booster protein into the recipient). The mean of the tumor volumes of Group 4 mice was statistically significantly lower than the mean volumes of sc tumors in mice in Groups 1 and 2 at all time points at the $P = 0.003$ level by Student's *t*-test at 28 days after sc injection of the cell line. This shows that the vaccination of the donors of the DLI with the Bcr-Abl/ecdCD40L VPPP vaccine induced an immune response that suppressed the growth of the 32DP210Bcr-Abl leukemia cells more than the TBI and TCDBMT alone or more than TBI and

Table 2 Growth of sc deposits of 32Dp210Bcr-Abl leukemia cells

Days[a]	*Group1*	*Group2*	*Group3*	*Group4*
4	65.86	16.81	0	9.01
6	112.35	46.67	0	10.68
8	144.76	72.21	7.79	12.37
11	215.47	108.23	21.17	13.48
15	308.61	162.95	64.72	33.79
17	382.47	226.93	114.69	65.28
19	499.40	295.41	194.81	104.95
21	602.78	404.23	295.8	154.58
24	756.87	537.23	486.79	243.45
26		696.93	656.36	296.2

Data listed under Groups 1, 2, 3, and 4 is volume of the sc nodule at the site of sc injection of the 32Dp210Bcr-Abl leukemia cells.
[a]Days after TBI and T-cell-depleted bone marrow transplantation.

Table 3 Days of survival of mice following injection with 32Dp210Bcr-Abl leukemia cells

Treatment group	*Percent alive*
Group 1	0% at 32 days
Group 2	0% at 34 days
Group 3	38% at 34 days
Group 4	44% at 34 days
Group 4	100% of complete responders at 150 days
Group 4	75% of complete responders at 350 days

Table 4 Number of mice surviving divided by the total number of mice in each experimental group at various days after TBI and TCDBMT

Days[a]	*Group1*	*Group2*	*Group3*	*Group4*
24	7/9	8/9	7/8	8/9
26	2/3	8/9	7/8	8/9
28	1/9	7/9	3/8	8/9
30	0	1/3	3/8	7/9
32		0	3/8	4/9
34			3/8	4/9
36			3/8	4/9

[a]Days after TBI and T-cell-depleted bone marrow transplantation.

TCDBMT followed by DLI from unvaccinated donors. As shown in Figure 3a and Table 2, the mean of the tumor volumes of Group 3 mice (received DLI from unvaccinated donors, but then the recipients of DLI received three sc injections of the vaccine) was significantly lower than tumor volumes in Groups 1 and 2 from 6 to 21 days (see Figure 3a and Table 2).

As shown in Figure 3b and Tables 3 and 4, all mice were dead in Groups 1 and 2 by day 32. In contrast, 38% of Group 3 mice were alive at day 32. Seventy-eight percent of the Group 4 mice were alive at day 32. Forty-four percent of the Group 4 mice developed complete responses and 75% of these completely responding mice in the vaccinated Group 4 mice survived disease free over 350 days in unmaintained remission after sc injection of the leukemia cells (Table 3). The difference between survival of Group 4 mice and the survival in mice belonging to Groups 1 and 2 was statistically significant at the $P = 0.00001$ level by the log-rank test.

Effect of the E7/ecdCD40L VPP vaccine on regrowth of E7 positive TC-1 cells after TBI, TCDBMT, and DLI

To extend the findings described above for Bcr-Abl leukemia cells to solid tumor neoplasms, we tested the effect of adding DLI from E7/ecdCD40L VPP-vaccinated donors and E7/ecdCD40L vaccination of the recipient of TBI and TCDBMT for a solid tumor malignancy: cervical cancer. The first task was to show that the Ad-sig-E7/ecdCD40L vector prime-E7/ecdCD40L protein boost strategy would work for E7. As shown in Figure 4a, the levels of the E7-specific splenic CD8 T cells of the vaccinated mice increased as one proceeded from V to VP to VPP and to VPPP (all pair wise comparisons among VP, VPP, and VPPP are significantly different at the $P<0.05$ level). As shown in Figure 4b, the levels of the E7-specific serum antibodies against the E7 B cell epitope EIDGPAG-QAEPDRAHYNIVTFCCKCD of the vaccinated mice increased significantly with 1 (VP), 2 (VPP), and 3 (VPPP) protein boosts after the initial vector injection. At a dilution factor of 1/1000, the difference of the serum antibody levels in the vaccinated vs the control group was statistically significantly different at the $P = 0.004$ level for VP mice, at the $P<0.001$ level for the VPP mice, and at the $P<0.0001$ for the VPPP mice (Figure 4b).

The donor for post-TCDBMT DLI was first vaccinated with the Ad-sig-E7/ecdCD40L vector prime-E7/ecdCD40L protein boost VPP vaccine before collection of the DLI. The recipient C57BL/6J mice were injected sc with 100 000 E7 TC-1 cells on day −7 (D-7); 1200 cGy of TBI were administered on day 0, 7 days after injection of the tumor cells. This dose is different from the one used for the BcrAbl/ecdCD40L VPPP experiment outlined above. The irradiator at our center was modified between the two experiments accounting for the differences in the dose. One hour after the TBI, the mice were transplanted with a single intravenous injection of 10 million T-cell-depleted bone marrow cells from a syngeneic donor (D0). Spleen cells (50 million) collected 7 days after completion of the E7/ecdCD40L VPP vaccination of a syngeneic donor were given to mice on 3 days after TBI and TCDBMT. The recipient also received one sc injection of the E7/CD40L protein boost vaccination 1 week after the DLI.

As shown in Figure 5a, the administration of the TBI and TCDBMT 7 days post-injection of the TC-1 cells, DLI from a E7/ecdCD40L VPP-vaccinated donor 3 days after transplant (10 days after the E7 positive TC-1 tumor injection), and a single E7/ecdCD40L protein boost sc vaccination 1 week thereafter, resulted in a growth rate of the E7 positive tumor cells, which was less than the mice in the control group (injection of tumor cells without TBI/TCDBMT or DLI), or the animals in which the sc injection of the E7 positive tumor cells was followed in 7 days by TBI and TCDBMT without DLI or vaccination of the recipient. These data shows that the addition of DLI from an E7/ecdCD40L VPP-vaccinated donor to a TBI and TCDBMT, followed by E7 VPP vaccination of the recipient, generates more suppression of E7 positive tumor than the same TBI/TCDBMT without DLI from an E7/ecdCD40L VPP-vaccinated donor and vaccination of the recipient (see Figure 5b).

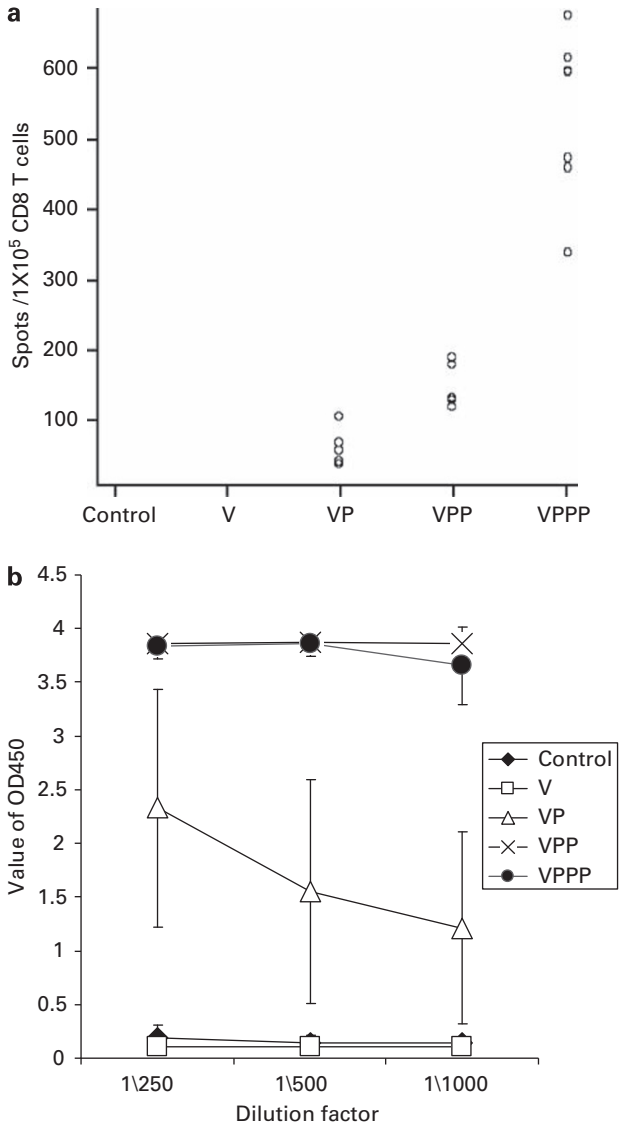


Figure 4 (**a**) ELISPOT assay of E7-specific T cells post-E7/ecdCD40L VPPP vaccine. The donor mice were exposed to the following E7/ecdCD40L vaccinations: V, VP, VPP, and VPPP. The cells were harvested 7 days post-completion of the vaccination and then exposed for 48 h to the E7 positive TC-1 cell line, which had been mitomycin C treated. Then, the ELISPOT assay was carried out as described in Materials and methods. There were 10 mice per group. (**b**) ELISA assay of E7-specific T-cells post-E7/ecdCD40L VPPP vaccine. The donor mice were exposed to the following E7/ecdCD40L vaccinations: V, VP, VPP, and VPPP. The peripheral blood sera were harvested 7 days post-completion of the vaccinations. Then, the ELISA assay was carried out as described in Materials and methods. There were 10 mice per group.

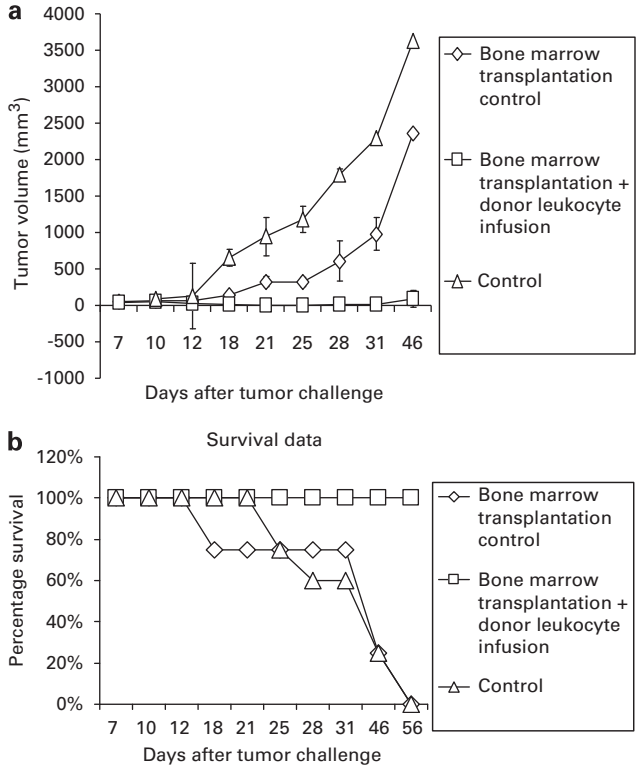


Figure 5 (**a**) Growth of E7 positive TC-1 cells in recipients of TBI, TCDBMT, and DLI from vaccinated and unvaccinated donors followed by E7/ecdCD40L vaccination. The recipients were injected sc with 100 000 TC-1 cells on day −7 and then received 1200 cGy TBI 1 h later and TCDBMT (10 million cells) 3 days later. The T-cell depletion of the BM was monitored by flow cytometry of the non-adherent cells after the treatment with antibodies *in vitro*, which were directed to CD4+ and CD8+ T cells. The level of these cells was reduced to <2% by the *in vitro* antibody treatment. Three days after the TBI and TCDBMT, the recipients received, where indicated, 50 million spleen cells from the tumor-free-vaccinated donors. Recipients were also given, where indicated, sc injections of 10 μg of the E7/ecdCD40L protein. Tumor growth at the site of the sc injection of the tumor cell line was monitored by measurement with calipers. Group 1 received sc injection of 100 000 TC-1 cells without TBI, TCDBMT, or DLI. Group 2 received TC-1 cells followed in 7 days by TBI and TCDBMT. Group 3 received 100 000 TC-1 cells followed in 7 days by TBI, TCDBMT, and DLI from E7/ecdCD40L VPP-vaccinated donors, and then one sc injection of the E7/ecdCD40L protein. (**b**) Survival of E7 positive TC-1 cells in recipients of TBI, TCDBMT, and DLI from vaccinated donors followed by E7/ecdCD40L vaccination. The methods were the same as outlined in (**a**).

Discussion

Vaccine for cancer hosts

P210Bcr-Abl has been identified as a TAA in CML.[16–18] We applied this result to our adenoviral vaccine platform,[5–8] which had been engineered to circumvent the defects acquired in the aged immune system and to improve presentation of P210Bcr-Abl-specific junctional peptides in Class I MHC. We further tried to increase the magnitude of the immune response to the vaccine by the infusion of T cells from syngeneic donors after vaccination with the TAA/ecdCD40L VPP vaccine in a lymphodepleted syngeneic recipient and/or recipient vaccination. Although several experiments showed that tumor vaccines can be effectively used in the early post transplant period,[19] we first report here the trial of adenoviral vaccine against p210Bcr-Abl during periods of lymphopenia, which follow TBI and TCDBMT.

As shown in Figure 3, the only group in which the growth of the p210Bcr-Abl32D cells was completely suppressed (in five of the nine treated mice) was Group 4 (which received spleen cells from vaccinated donors and were vaccinated in the setting of lymphodepletion after TBI and TCDBMT).

As shown in Tables 2–4, mice in Groups 1 and 2 (unvaccinated) were all dead by 33 days after tumor cell injection. When the DLI were derived from BcrAbl/ecdCD40L VPPP-vaccinated donors and the recipients were vaccinated after being given DLI from vaccinated donors (Group 4), four of the nine mice so treated were completely free of regrowth by the 32Dp210Bcr-Abl cells for >350 days after the injection of the p210Bcr-Abl cells. One of the nine mice, which were free of disease started to lose weight at >120 days after tumor cell injection and was found to have myeloid hyperplasia in the bone marrow on post-mortem examination.

We can now ask how this information could be applied to a treatment for leukemia and cancer in human subjects. One possible way is to vaccinate donors for allografts with the TAA/ecdCD40L VPP vaccine before the collection of the bone marrow and the peripheral blood T cells for DLI for use in TCDBMT post-preparative therapy. This would be especially useful in the minitransplant in which the relapse frequency is higher than with myeloablative regimens. An increase in the frequency of TAA-specific T cells in the DLI post-mini-allograft (induced by vaccination of the donors) would allow a reduction in the dose of the DLI thereby reducing the risk of GVHD. The increased level of TAA-specific T cells would decrease the frequency of relapse. In addition, vaccinations for viruses associated with fatal opportunistic infections (such as CMV) could be carried out. The use of the vaccines for normal allodonors would have to be restricted to TAA, which are foreign antigens (such as antigens from CMV or HPV), or which are unique tumor antigens (such as epitopes defined by junctional peptides from Bcr-Abl) for safety reasons.

Our experiments have implications for new directions in immunotherapy of cancer, which extend way beyond the allograft area. The TAA/ecdCD40L VPP vaccine could also be given to cancer patients whose disease is not responding to conventional treatment. After the completion of vaccination, peripheral blood mononuclear cells could be collected at the time of maximal trafficking of the TAA-specific effector T cells in the peripheral blood (7 days after completion of the vaccination), and then returned to the cancer patient when that patient had been rendered lymphodepleted by either myeloablative or non-myeloablative therapy to 'create space' in the lymphoid compartment. The existence of this 'additional space' in the lymphoid compartment has been reported to create an environment, which is unusually conducive to expansion of TAA-specific T cells induced by vaccination. Additional vaccination of the patient could then be carried out after the infusion of the autologous lymphocytes collected after the original vaccination. The vaccination of the patient in the lymphodepleted state after infusion of lymphocytes collected after the original vaccination could dramatically increase the magnitude of the antigen-specific T cells in the patient, thereby inducing control of the growth of the TAA positive neoplastic cells.

The selection of myeloablative vs non-myeloablative therapy would depend on the cancer or hematopoietic neoplasms for which the vaccine was being selected. For indolent diseases, non-myeloablative therapy would be used to create space, whereas for more rapidly proliferating neoplasms such as Burkitts lymphoma, ablative levels of chemotherapy would be used, not only to create space for T-cell expansion in the lymphoid compartment, but also to stem the growth of the rapidly advancing neoplasm.

The TAA/ecdCD40L VPP vaccine is unique in that it has been engineered to circumvent the defects, which limit the magnitude of the immune response to vaccines in the aged immune system. For the majority of patients with epithelial cancers or hematopoietic neoplasms who are in the fifth or sixth decade of life, the use of the TAA/ecdCD40L VPP vaccination would constitute an advantage.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The SKCC and AD thank the Kaye and Richard Woltman Foundation for support of the development of post-allograft vaccination methods designed to reduce the post-allograft incidence of recurrence and opportunistic viral infections. AD recognizes support of the vaccine program from the Breast Cancer Research Foundation, the DOD (BCRP program (BC022063)), and a grant from the State of California Breast Cancer Research Program (CBCRP12IB-0159). Authors thank Dr Brian Druker for 32Dp210Bcrabl cells. Dr Tae Hae Han recognizes support from SBRI grant B-A7-204 and SRC grant from Korea Science and Engineering Foundation.

References

1 Jefferson T, Rivetti A, Rudin M, Di Pietrantonj D, Demicheli V. Efficacy and effectiveness of influenza vaccines in elderly people: a systematic review. *Lancet* 2005; **366**: 1165–1174.
2 Deng Y, Jing Y, Campbell AE, Gravenstein S. Age-related impaired type 1 T cell responses to influenza: reduced activation *ex vivo*, decreased expansion in CTL culture *in vitro*, and blunted response to influenza vaccination *in vivo* in the elderly. *J Immunol* 2004; **172**: 3437–3446.
3 Castle SC. Clinical relevance of age-related immune dysfunction. *Clin Infect Dis* 2000; **31**: 578–581.
4 Saurwein-Teissl M, Lung TL, Marx F, Gschosser C, Asch E, Blasko I *et al*. Lack of antibody production following immunization in old age: association with CD8+CD28− T cell clonal expansions and an imbalance in the production of Th1 and Th2 cytokines. *J Immunol* 2000; **168**: 5893–5899.
5 Zhang L, Tang Y, Akbulut H, Zelterman D, Linton PJ, Deisseroth A. An adenoviral vector cancer vaccine that delivers a tumor-associated antigen/CD40-ligand fusion protein to dendritic cells. *Proc Natl Acad Sci USA* 2003; **100**: 15101–15106.
6 Tang Y, Zhang L, Yuan J, Akbulut H, Linton PJ, Deisseroth A. Multistep process through which adenoviral vector vaccine overcomes anergy to tumor-associated antigens. *Blood* 2004; **104**: 2704–2713.
7 Tang Y, Akbulut H, Koziol J, Linton PJ, Deisseroth A. Vector prime/protein boost vaccine that overcomes defects acquired during aging and cancer. *J Immunol* 2006; **177**: 5697–5707.
8 Akbulut H, Tang YC, Akbulut KG, Maynard J, Zhang L, Deisseroth A. Antitumor immune response induced by i.t. injection of vector activated dendritic cells and chemotherapy

suppresses metastatic breast cancer. *Mol Cancer Ther* 2006; **5**: 1975–1985.
9 Eaton SM, Burns EM, Kusser K, Randall TD, Haynes L. Age-related defects in CD4 T cells cognate helper function lead to reductions in humoral responses. *J Exp Med* 2004; **200**: 1613–1622.
10 Dong L, Mori I, Hossain J, Liu B, Kimura Y. An immunostimulatory oligodeoxynucleotide containing a cytidine-guanosine motif protects senescence-accelerated mice from lethal influenza virus by augmenting the T helper type 1 response. *J Gen Virol* 2003; **84**: 1623–1628.
11 Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J *et al*. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. *J Natl Cancer Inst* 1995; **87**: 796–802.
12 Rhodes J, York RD, Tara D, Tajinda K, Druker BJ. CrkL functions as a nuclear adaptor and transcriptional activator in Bcr-Abl-expressing cells. *Exp Hematol* 2000; **28**: 305–310.
13 La Rosche P, Johnson K, O'Dwyer ME, Druker BJ. *In vitro* studies of the combination of imatinib mesylate (Gleevec) and arsenic trioxide (Trisenox) in chronic myelogenous leukemia. *Exp Hematol* 2002; **30**: 729–737.
14 Yasukawa M, Ohminami H, Kojima K, Hato T, Hasegawa A, Takahashi T *et al*. HLA class II-restricted antigen presentation of endogenous bcr-abl fusion protein by chronic myelogenous leukemia-derived dendritic cells to CD4+ T lymphocytes. *Blood* 2001; **98**: 1498–1505.
15 http://bio.dfci.harvard.edu/Tools/rankpep.html.
16 Pinilla-Ibarz J, Cathcart K, Korontsvit T, Soignet S, Bocchia M, Caggiano J *et al*. Vaccination of patients with chronic myelogenous leukemia with bcr-abl oncogene breakpoint fusion peptides generates specific immune responses. *Blood* 2000; **95**: 1781–1787.
17 Sun JY, Krouse RS, Forman SJ, Senitzer D, Sniecinski I, Chatterjee S *et al*. Immunogenecity of a p210BCR-ABL fusion domain candidate DNA vaccine targeted to dendritic cells by a recombinant adeno-associated virus vector *in vitro*. *Cancer Res* 2002; **62**: 3175–3183.
18 Zeng Y, Graner MW, Thompson S, Marron M, Katsanis E. Induction of BCR-ABL-specific immunity following vaccination with chaperon-rich cell lysates derived from BCR-ABL+ tumor cells. *Blood* 2005; **105**: 2016–2022.
19 Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer* 2008; **8**: 299–308.