Completely mismatched allogeneic CD3/CD28 cross-linked Th1 memory cells elicit anti-leukemia effects in unconditioned hosts without GVHD toxicity

M. Har-Noy [a,c,*], M. Zeira [a], L. Weiss [a], S. Slavin [b]

[a] *Department of Bone Marrow Transplantation and Cancer Immunotherapy, Hadassah-Hebrew University Medical Center, Jerusalem 91120, Israel*
[b] *International Center for Cell Therapy and Cancer (ICTC), Tel Aviv Medical Center, Tel Aviv 64239, Israel*
[c] *Immunovative Therapies, Ltd., POB 974, Shoham 60850, Israel*

Abstract

Fully allogeneic CD3/CD28 cross-linked Th1 cells were found to elicit host-mediated anti-leukemia effects without GVHD toxicity. Mice inoculated with a lethal dose of BCL1 leukemia demonstrated significantly enhanced survival after allogeneic Th1 treatment. Cure rates of 12.5% with a single allogeneic cell infusion and 31.25% with multiple infusions were demonstrated. Cured mice were able to reject rechallenge with a lethal dose of tumor without further treatment. These results suggest that use of intentionally mis-matched, Th1 memory cells infused with cross-linked CD3/CD28 could represent a novel clinical approach to eliciting potent anti-tumor effects in patients without conditioning and without GVHD toxicity.
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1. Introduction

The graft vs. leukemia (GVL) immune reaction that follows allogeneic bone marrow transplant (BMT) is arguably the most powerful anti-tumor immunotherapy in clinical use. GVL is a curative therapy for hematological malignancy [1,2]. The curative GVL effect can also be elicited by non-myeloablative conditioning followed by allogeneic stem cell transplant [3] and upon donor lymphocyte infusion (DLI) in patients with prior allogeneic BMT [4]. GVL is effective against chemotherapy/radiation resistant hematological malignancies [5] as well as solid/metastatic tumors [6,7]. The GVL effect that occurs after DLI is currently the only curative therapy for recurrent CML patients [8].

However, the clinical application of the GVL effect is severely limited due to graft vs. host disease (GVHD) toxicity. Therefore, it is an important therapeutic goal to investigate and develop methods to preserve the beneficial GVL effect while eliminating the detrimental GVHD toxicity [9]. Separation of the GVL/GVHD effects is difficult because the effects are interrelated [10,11], both mediated by donor T-cells [12,13] and both mediated by similar mechanisms [14].

If these effects are coupled because GVHD serves as an adjuvant for the GVL effect, then we could hypothesize that if these effects were mediated by the host immune system, rather than the graft, they could remain coupled without the toxicity of GVHD. For example, an allogeneic cell infusion could elicit a host vs. graft (HVG) rejection effect which would serve as an adjuvant to a coupled host vs. leukemia

* Corresponding author at: Hadassah-Hebrew University Medical Center, Department of Bone Marrow Transplantation and Cancer Immunotherapy, POB 12000, Jerusalem 91120, Israel. Tel.: +972 54 232 7077; fax: +972 3 970 2090.

E-mail address: harnoy@immunovative.co.il (M. Har-Noy).

(HVL) effect. Unlike GVHD, HVG rejection of allogeneic cells is a non-toxic event. Thus, coupling a non-toxic HVG effect to an HVL effect would enable the same anti-tumor effect of allogeneic BMT to occur without the toxicity of GVHD.

There is evidence to support that host rejection of a grafted immune system (HVG) can be coupled to host-mediated anti-tumor immune responses against syngeneic tumors (HVL) [15]. The HVL effect in this model was correlated with the intensity of the HVG rejection response and was found not to be due to bystander killing of tumor cells or by alloactivated host T cells [15,16].

In chimeric tumor-bearing mice, the infusion of donor lymphocytes (DLI) causes donor immune cell rejection of the host immune system and conversion to full donor chimerism. This process elicits the expected coupled GVL/GVHD effects. However, infusion of host lymphocytes derived from syngeneic mice into a chimeric mouse causes rejection of the resident allogeneic donor cells (HVG) coupled to a host-mediated anti-tumor effect (HVL) [17,18]. The magnitude of the GVL vs. HVL antitumor effects observed in these mice were not significantly different, however the HVL effect occurred without GVHD toxicity [19].

These experiments support the concept that an allogeneic rejection response can be coupled to an anti-tumor effect regardless of whether the effectors are allogeneic or syngeneic to the tumor. However, these coupled HVL/HVG effects were demonstrated only in chimeric hosts infused with host derived cell infusions. Clinically, it would be desirable to elicit coupled HVL/HVG effects in immunocompetent refractory patients, especially in patients that were not candidates for allogeneic transplant or did not have a matched donor.

Past immunotherapy attempts aimed at stimulating a host-mediated immune attack against cancer have had very disappointing results in the clinic [20]. This is thought to be due to the ability of tumors to evoke several mechanisms to avoid an immune attack [21]. We have hypothesized that fully allogeneic Th1 memory cells infused in an activated state so they produce IFN-γ and express CD40L will elicit host-mediated anti-tumor immunity and that the rejection of these cells will result in an inflammatory cytokine environment that will overcome the tumor immunoavoidance which has hampered past immunotherapy protocols [22].

Here we report evidence supporting this hypothesis in the BCL1 mouse model of B-cell leukemia/lymphoma. We report that infusion of fully allogeneic, CD3/CD28-cross-linked, memory Th1 cells caused significant host-mediated anti-leukemia effects, providing significantly extended survival and curative immune responses with long-term protective tumor-specific memory. These results suggest that use of intentionally mis-matched, Th1 memory cells infused with cross-linked CD3/CD28 could represent a novel clinical approach to eliciting potent anti-tumor effects in patients without conditioning and without GVHD toxicity.

2. Materials and methods

2.1. Mice

Five to six week old female Balb/c (H-2$^{d/d}$) and male C57BL/6 (H-2$^{b/b}$) mice were purchased from the Hebrew University-Hadassah Medical School Animal Facility, Jerusalem, Israel. All mice were kept under specific pathogen-free (SPF) conditions and given acidified water and food *ad libitum*. The study was approved by the Animal Ethical Committee of the Hebrew University Medical School (Approval #MD-97.28-4). All mice were 6–8 weeks old when placed on experiment. The Balb/c mice weighed between 15 and 20 g when placed on experiment.

2.2. Tumor models

BCL1 is a spontaneous B-cell leukemia/lymphoma of Balb/c origin [23] that resembles human CLL. The line is maintained *in vivo* by serial passages in Balb/c recipients. In these experiments, animals were infused intravenously through the tail vein with 2000 BCL1 cells on day 0 and were treated with intravenous cell infusions of various types within the window from day 0 to day 14. This BCL1 dose is lethal in 100% of the mice. Mice consistently succumb to disease between 19 and 22 days post tumor inoculation. Upon infusion, the BCL1 tumor clone initially traffics to the spleen. Following 14–18 days, marked splenomegaly is evident. High leukocyte counts in excess of 500,000/mm^3 are routine after day 15. This model is designed to resemble the clinical setting of minimal residual disease after induction chemotherapy.

In some experiments, the 4T1 tumor was utilized. The 4T1 cell line was derived from a spontaneously arising Balb/c mammary tumor [24]. Intravenous infusion of 1×10^4 4T1 cells produces reproducible metastasis to lungs, liver, bone and brain and is 100% lethal.

2.3. Preparation of allogeneic Th1 memory cells

Spleen cells from male C57BL/6 mice were harvested and treated with ammonium chloride-potassium (ACK) buffer for lysis of red blood cells. Approximately 70–100 million cells were isolated per spleen. CD4+ T-cells were then purified by positive selection (purity > 97%) using CD4 immunomagnetic particles on an MS column (Miltenyi Biotec, Germany), approximately 7–12 million CD4 cells were isolated with a yield of 50–60%. Th1 memory cells were generated by expansion with anti-CD3 and anti-CD28–coated paramagnetic beads (CD3/CD28 T-cell expander beads, Dynal/Invitrogen) at an initial bead:CD4 cell ratio of 3:1. The purified CD4 cells were incubated with 20 IU/mL recombinant mouse (rm)IL-2, 20 ng/mL rmIL-7, 10 ng/mL rmIL-12 (Peprotech, New Jersey) and 10 μg/mL antimurine IL-4 mAb (Becton Dickenson) in RPMI 1640 media containing 10% FBS, penicillin–streptomycin–glutamine, non-essential amino acids (NEAA) (Biological Industries, Israel) and 3.3 mM *N*-acetyl-cysteine (NAC; Sigma) (complete media). Additional cytokine-containing complete media with rmIL-2 and rmIL-7 was added to the CD4 cultures daily from days 3–6 to maintain the cell concentration between 0.5 and 1×10^6 cells/mL. Additional CD3/CD28 beads were also added daily from day 3 to day 6. The number of beads added was calculated to maintain a 1:1 bead:cell ratio as the cells expanded. After 6 days in culture, the CD4

cells expanded approximately 80–100-fold and were harvested and debeaded by physical disruption and passage over a magnet. The phenotype of the harvested cells used in experiments were >95% CD4+, CD45RB$^{lo/int}$, CD62L^{lo}, CD44^{hi} and are thus referred to as "memory cells".

2.4. CD3/CD28 nanobead preparation

Biotinylated mouse anti-CD3 and anti-CD28 mAbs (BD Pharmingen) were mixed at a 1:1 ratio with Strepavidin-coated nanobeads (Miltenyi, Germany). These CD3/CD28-coated nanobeads were not able to activate naïve T-cells, but could activate T-cells that had been previously activated. Therefore, the nanobeads were titered against harvested Th1 memory cells that had been previous activated with CD3/CD28 T-cell expander beads (Dynal, Norway). While there were slight variations per batch, generally 20 μL/10^7 cells was found to provide optimal activation of previously activated Th1 memory cells.

2.5. CD3/CD28 cross-linking

In experiments that required the infusion of activated Th1 memory cells, the fresh harvested Th1 cells were incubated with a pre-titered concentration of CD3/CD28-conjugated nanobeads prior to infusion. For optimal activation, the cells had to be incubated with the nanobeads for a 4–8 h prior to infusion. Optimal activation caused production of IFN-γ and upregulation of CD40L and FasL on the cell surface. For these experiments, all infusions of CD3/CD28 cross-linked Th1 memory cells occurred after 4–8 h of pre-incubation. Cells were thoroughly washed prior to infusion to remove any unassociated nanobeads. CD3/CD28 cross-linked Th1 memory cells used in these experiments expressed FasL and CD40L on the cell surface and produced in excess of 5000 pg/mL/10^6 cells/6 h IFN-γ and less than 20 pg/mL IL-4 per 10^6 cells/6 h. Th1 memory cells without CD3/CD28 cross-linking did not produce cytokines or express FasL or CD40L.

2.6. Activated cell infusions

Either Balb/c (syngeneic) or C57BL/6 (allogeneic) mice served as donors for activated cell infusions. Spleens were removed and single cell suspensions of splenocytes isolated after ACK lysis of RBC were made. The splenocytes were cultured at 37 °C in RPMI 1640 complete media at 0.5 × 10^6 cells/mL. For activation, some cells were activated with 100 IU of rmIL-2 (Peprotech, New Jersey) and other groups with 0.5 μg/mL PHA (Sigma). The cells were harvested and washed after 15h of activation and infused immediately. CD3/CD28-activated splenocytes were generated by culturing isolated splenocytes with T-cell expander beads (Dynal/Invitrogen) at a 3:1 bead:cell ratio in complete media and 20 IU rmIL-2 for 9 days. The cultures were fed daily from day 3 to day 9 with fresh complete media containing rmIL-2 to maintain the cell density at 0.5 × 10^6 cells/mL, no additional beads were added to the culture. The cells expanded approximately 90-fold over the culture period. The phenotype of the cells upon harvest at 9 days was predominately CD8+ lymphocytes (70–85%) and the cultures produced both IFN-γ and IL-4 upon activation. Prior to infusion, the beads were removed by physical disruption and passage over a magnet.

2.7. Cytokine production

BCL1 infused Balb/c mice were treated with complete media (control) or various doses of allogeneic CD3/CD28 cross-linked Th1 memory cells (treated). Mice were anesthetized on day 15 and bled through the eye orbit vein and plasma was collected for cytokine analysis. In some experiments, the mice were then sacrificed and the spleens were harvested. CD4 and CD8 T-cell subsets were isolated from splenocyte single cell suspensions by positive selection (Miltenyi Biotec, Germany). The T-cell subsets were cultured for 5 days with syngeneic adherent antigen presenting cells (APC) and irradiated BCL1 tumor cells at a T cell:tumor cell ratio of 10:1. rmIL-2 (Peprotech) was added to the cultures at 20 IU/mL on day 3 and maintained until day 5. Cells for each test condition were counted on the day of supernatant harvest. The cell-free supernatant was collected to measure cytokine levels on days 2 and 5. Cytokines were quantified using the following enzyme-linked immunosorbent assay (ELISA) kits: IL-4, IL-10 and IFN-γ (R & D Systems, Minneapolis, MN). Results were indexed to cytokine production per million T-cells in culture.

2.8. Y chromosome analysis

Sex-mismatched cell infusions were used in these studies with male donor cells into female hosts. A sensitive PCR method described previously was used to detect the male cells within the female hosts [25]. This assay can detect one male cell in 1 × 10^6 female cells.

2.9. Statistical analysis

Survival data are depicted as Kaplan-Meier curves. Significant survival benefit was determined by time to event (death) analysis using the logrank test [26] and by Hazard Ratio analysis [27] with mice surviving >90 days censored from the analysis. Median survival is reported as the time at which half the mice have died and half are still alive. Differences in cytokine levels were analyzed by Student's *T*-test.

3. Results

3.1. Allogeneic rejection coupled anti-tumor effect

To determine whether rejection of allogeneic cells alone was sufficient to elicit a host-mediated anti-tumor effect, Balb/c mice were inoculated via tail vein with 2000 BCL1 tumor cells on day 0. On day 1, the mice were infused via the tail vein with either 0.2 mL complete media as control or 1 × 10^6 C57BL/6 derived: splenocytes; purified CD4 cells; purified CD8 cells; IL-2 activated splenocytes; PHA activated splenocytes; ex-vivo differentiated and expanded Th1 memory cells; ex-vivo differentiated and expanded Th1 cells memory activated with CD3/CD28 nanobeads; CD3/CD28-microbead expanded splenocytes or IL-2 expanded splenocytes. All infusions were in 0.2 mL complete media. Eight animals were inoculated per treatment group. The results are shown in Fig. 1A.

Control mice had a median survival of 20.5 days. Only the ex-vivo differentiated and expanded Th1 memory cells activated by cross-linking with CD3/CD28 nanobeads demonstrated any anti-tumor activity. Mice treated with ex-vivo differentiated and expanded Th1 memory cells activated with CD3/CD28 nanobeads had a significantly extended survival at a median of 28.5 days compared to a median survival of 20.5 days for the control mice ($P < 0.0001$ logrank; hazard ratio = 4.143). The same ex-vivo differentiated and expanded Th1 memory cells without CD3/CD28 antigen cross-linking did not exhibit an anti-tumor effect, demonstrating that cross-linking was a critical component of the observed effect.

To determine if the observed extended survival was related to the allogeneic nature of the cell infusion, the study above was repeated using donor cells derived from syngeneic Balb/c mice ($n = 6$ for each treatment), rather than allogeneic. No groups showed significant survival compared to control when the infusions were syngeneic (Fig. 1B), including the Th1 memory cell group with CD3/CD28 cross-linking. This demonstrates that the allogeneic nature of the CD3/CD28 cross-linked Th1 memory cells was a critical component of the observed anti-tumor effect.

3.2. Dose–response

Since anti-tumor activity was detected in the allogeneic CD3/CD28 cross-linked Th1 memory cell treatment group at a dose of 1×10^6 cells, studies were conducted to determine if this response was repeatable and whether the activity had a dose–response relationship. Balb/c mice were inoculated with 2000 BCL1 cells on day 0. On day 1, mice were injected intravenously with either 0.2 mL complete media (control), 1×10^5, 1×10^6 or 1×10^7 C57BL/6-derived CD3/CD28 cross-linked Th1 memory cells ($n = 8$ for each group) in 0.2 mL of complete media. The experiment was repeated 4 times. Because the control mice in each of the 4 separate experiments survived the same median of 20.0 days, the results were pooled and shown in Fig. 2A.

The pooled results demonstrated an apparent inverse dose–response. The lowest dose of 1×10^5 cells ($5–7 \times 10^6$ cells/kg) extended survival to a median of 35.5 days compared to 20 days for the control mice (logrank test $P < 0.0001$; hazard ratio = 4.602). The 1×10^6 cell dose ($5–7 \times 10^7$ cells/kg) extended survival a median of 28 days (logrank test $P < 0.0001$; hazard ratio = 3.526). The 1×10^7 cell dose ($5–7 \times 10^8$ cells/kg) resulted in a median survival of 20 days, the same as the control.

While the median survival of the mice treated with 1×10^5 cells was 35.5 days compared to median survival of 28 days in the mice treated with the 1×10^6 dose, the survival curves were not statistically different. However, the 1×10^5 dose had an increased cure rate (12.5%) compared to the 10^6 dose (6.25%).

3.3. Dose ranging

Because an inverse dose–response was initially observed, the dose–response experiments were repeated to include lower doses to determine if these lower doses might improve the efficacy of the treatment. Balb/c mice were infused intravenously with 2000 BCL1 cells on day 0. On day 1, mice were injected intravenously with either 0.2 mL complete media (control), 1×10^3, 1×10^4, 1×10^5, or 1×10^6 C57BL/6-derived CD3/CD28 cross-linked Th1 memory cells ($n = 10$ for each group) in 0.2 mL of complete media. These results are shown in Fig. 2B.

The median survival for the control mice was 20 days. All tested doses resulted in statistically significant survival benefit compared to control. The 1×10^3 dose extended survival to a median of 22 days (logrank $P < 0.0035$; hazard ratio = 2.601). The 1×10^4 dose extended survival to a median of 29.5 days (logrank $P < 0.0001$; hazard ratio = 4.282). The 1×10^5 dose extended survival to a median of 45 days (logrank $P < 0.0001$; hazard ratio = 4.692). The 1×10^6 dose extended survival to a median of 34 days (logrank $P < 0.0001$; hazard ratio = 4.595). The 1×10^5 dose resulted in a 20% "cure" rate and the 1×10^6 dose resulted in a 10% "cure" rate. No "cures" were observed in any other doses tested. Rather than a true inverse dose–response, these results indicate that the optimal therapeutic efficacy of the treatment occurs within a therapeutic window between 1×10^5 ($5–7 \times 10^6$ cells/kg) and 1×10^6 ($5–7 \times 10^7$ cells/kg) cell dose with the 1×10^5 cell dose providing the optimal response in terms of both survivability and cure rate of all the doses tested.

Previous studies had demonstrated the requirement for host-derived IFN-γ in mediating anti-tumor effects in recipient lymphocyte infusions in chimeric mice [17]. In order to determine if IFN-γ levels correlated with the observed anti-tumor effect of the allogeneic CD3/CD28 cross-linked Th1 memory cells, the dose–response experiment was repeated with doses of 1×10^3, 1×10^4, 1×10^5, 1×10^6 and 1×10^7. Mice ($n = 8$) were bled on day 15 via the eye orbit vein and the plasma was analyzed for IFN-γ by ELISA. The results are shown in Fig. 2C. IFN-γ levels in the plasma were significantly elevated compared to controls for all doses except the 1×10^7 dose ($P < 0.05$). The 1×10^7 dose resulted in a significant decrease in plasma IFN-γ compared to control ($P < 0.05$). These results correlated with the survival data, with all doses tested resulting in significant survival advantage except the 1×10^7 dose.

3.4. Allogeneic rejection response

In order to determine if the allogeneic cells were rejected by the host, five of the mice that were infused with 1×10^5 cells and five mice infused with 1×10^6 cells were bled via tail vein at 24 h after infusion. Since the allogeneic infusions were derived from male mice and the host mice were
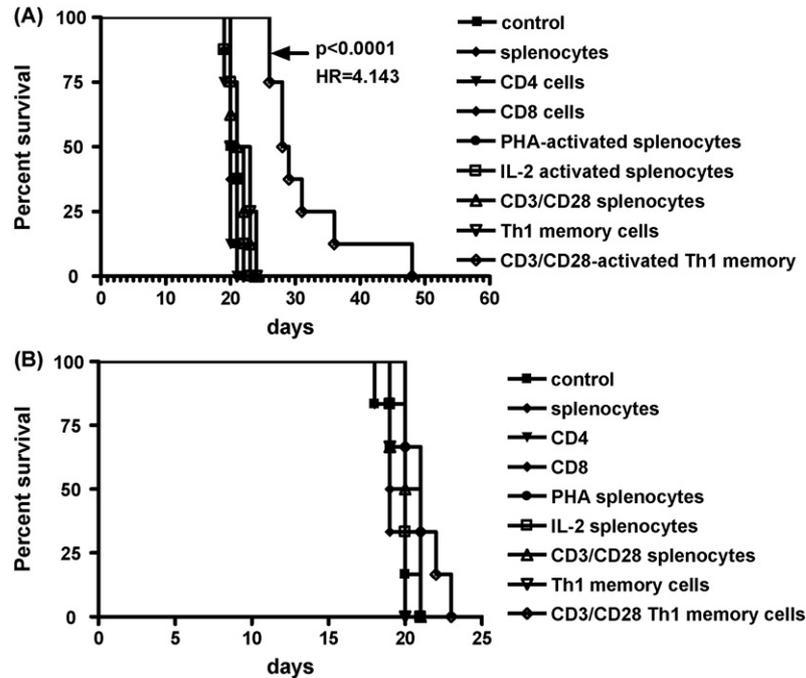
Fig. 1. (A and B) The anti-tumor effect elicited by allogeneic (A) or syngeneic (B) adoptive transfer of various immune cell types. Balb/c mice ($n = 8$ per treatment group) were inoculated with 2000 BCL1 cells via tail vein on day 0. On day 1, mice were injected with 1×10^6 B57BL/6-derived immune cells (allogeneic) or 1×10^5 Balb/c-derived immune cells (syngeneic) either: fresh splenocytes alone; purified CD4 T-cells; purified CD8 T-cells; splenocytes activated with PHA for 15 h; splenocytes activated with IL-2 for 15 h; CD3/CD28 bead activated and expanded splenocytes; ex-vivo differentiated and expanded Th1 memory cells; or ex-vivo activated and expanded Th1 memory cells infused with CD3/CD28 nanobeads. All infusions were made in 0.2 mL complete media. Control mice received complete media alone. The results are presented as Kaplan Meier survival curves. The survival curves for each group were compared to the control group using the logrank test and by hazard ratio. Only the allogeneic CD3/CD28 cross-linked Th1 memory cells demonstrated a significant survival advantage compared to control (logrank $P < 0.0001$; hazard ratio = 4.143).

female, an assay to detect the presence of Y chromosome in the blood was used to determine the amount of allogeneic cells resident in the host at 24 h. The results shown in Fig. 3 indicate that there was no detectable male allogeneic cells in the host peripheral blood at 24 h at either dose tested.

3.5. Dose timing

To determine the effect of the treatment timing, Balb/c mice were infused intravenously with 2000 BCL1 on day 0 and treatment was administered on different days. BCL1 is known to remain resident in the spleen until about day 15, after which a spike in blood counts occurs. 1×10^5 C57BL/6 CD3/CD28 cross-linked Th1 memory cells were infused intravenously on either day 0, 1, 7 or 14 in 0.2 mL of complete media. Control mice were infused with 0.2 mL complete media on day 0. Eight mice were included in each treatment group.

All treatments resulted in statistically significant survival benefit (see Fig. 4). The day 0 treatment resulted in a 25% "cure" rate with 2 of 8 animals surviving >90 days. Treatment at day 1 and day 3 both resulted in a 12.5% "cure" rate. The later doses at day 7 and day 14 did not result in any animals surviving 90 days.

3.6. Development of tumor-specific immunity

12 animals surviving >90 days after treatment with C57BL/6-derived CD3/CD28 cross-linked Th1 memory cells were evaluated for development of tumor-specific immunity. In order to determine the presence of immunologic memory to BCL1, these "cured" animals were rechallenged with a lethal dose of BCL1 tumor. As shown in Table 1, all animals previously cured from BCL1 rejected subsequent challenges with intravenous infusion of 2000 BCL1 cells ($n = 8$), but not a challenge from the unrelated 4T1 tumor ($n = 4$). All control mice that were previously untreated died of progressive tumor, indicating that the injected BCL1 ($n = 6$) and 4T1 ($n = 6$) cell lines were viable.

This immunologic memory could be transferred by the intravenous injection of a single cell suspension of 1×10^7 splenocytes from cured mice into naive (i.e., non-tumor-bearing and untreated) Balb/c mice. Twenty-four hours after the splenocyte transfer, these mice were challenged with a lethal dose of BCL1. None of the recipient mice developed tumor over a 30-day period when rechallenged with the same tumor from which the donor mice were cured, but developed tumors when challenged with the different 4T1 tumor. All of the naive control animals died of progressive tumor.
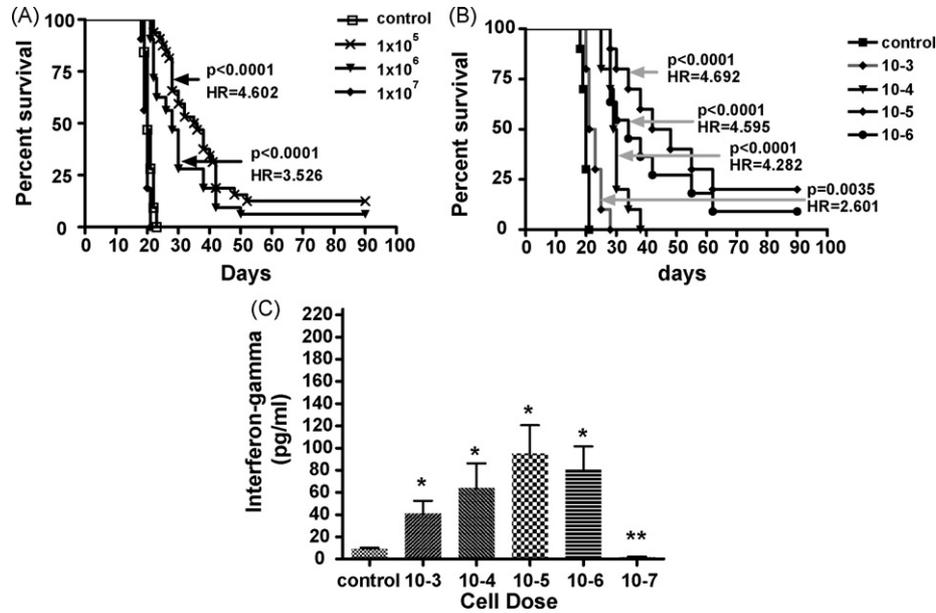
Fig. 2. (A–C) Dose–response to allogeneic CD3/CD28 cross-linked Th1 memory cells. Balb/c mice were inoculated via tail vein with 2000 BCL1 tumor cells on day 0. In the first experiment set, the mice ($n = 8$ per group) were infused intravenously with CD3/CD28-cross-linked Th1 memory cells derived from allogeneic C57BL/6 mice on day 1 at cell doses of either 1×10^5, 1×10^6 or 1×10^7 cells in 0.2 mL of complete media. Complete media alone was infused as control. The results of 4 pooled experiments ($n = 32$) are shown in subpart (A) as Kaplan Meier survival curves. The survival curves for each group were compared to the control group using the logrank test and by hazard ratio. Animals surviving greater than 90 days were censored from the analysis. Both the 1×10^5 and 1×10^6 cell doses resulted in significant survival compared to control, but were not statistically different compared to each other. Subpart (B) shows the Kaplan Meier survival curves of a separate experiment ($n = 10$) testing cell doses of either 1×10^3, 1×10^4, 1×10^5 or 1×10^6 cells in 0.2 mL of complete media. Complete media alone was the control. All doses resulted in significant survival compared to control. In an additional experiment, cell doses of 1×10^3, 1×10^4, 1×10^5, 1×10^6 and 1×10^7 cells in 0.2 mL of complete media were infused on day 1. Complete media alone was the control. The mice were bled on day 15 and the plasma analyzed for interferon-gamma level by ELISA. The results are shown in subpart (C). The 1×10^3, 1×10^4, 1×10^5 and 1×10^6 doses resulted in statistically significant increased levels of interferon-γ ($^*P < 0.05$ by Student's *T*-test). The 1×10^7 dose resulted in significantly suppressed levels of interferon-γ ($^{**}P < 0.05$).

3.7. Th1/Th2 immune deviation

Sustained Type 1 cytokine production is necessary to downregulate tumor Type 2 cytokines [28]. Type 1 cytokines such as IFN-γ and granulocyte macrophage colony-stimulating factor (GM-CSF), correlate with *in vivo* tumor eradication, whereas the Type 2 cytokines, such as IL-10, suppress antitumor reactivity [29]. Tumors actively produce Type 2 cytokines as a strategy to avoid immune elimination [30]. Therefore, switching the cytokine environment in cancer patients from Type 2 domination to Type 1 is an essential requirement for eliciting anti-tumor immunity [31].

To determine if treatment with allogeneic CD3/CD28 cross-linked Th1 memory cells resulted in a shift in the Th1/Th2 balance in the plasma and in the tumor-specific T-cell response, control mice ($n = 10$) were infused with 2000 BCL1 intravenously on day 0 and treated mice ($n = 10$) received 2000 BCL1 intravenously on day 0 and 1×10^5 allogeneic CD3/CD28 cross-linked Th1 memory cells on day 1. On day 15, the mice were anesthetized and bled through the eye orbit vein. Plasma samples were prepared and stored at $-80\,^\circ$C until they were analyzed. All mice were then sacrificed and spleens were removed. CD4 and CD8 T-cell subsets were purified from the splenocytes by positive selection. The subsets were stimulated with irradiated BCL1 tumor cells

incubated with syngeneic APC or irrelevant irradiated 4T1 tumor as control and cultured for 5 days. Supernatants from these cultures were obtained on day 2 and day 5 of the culture and stored at $-80\,^\circ$C for cytokine analysis by ELISA.
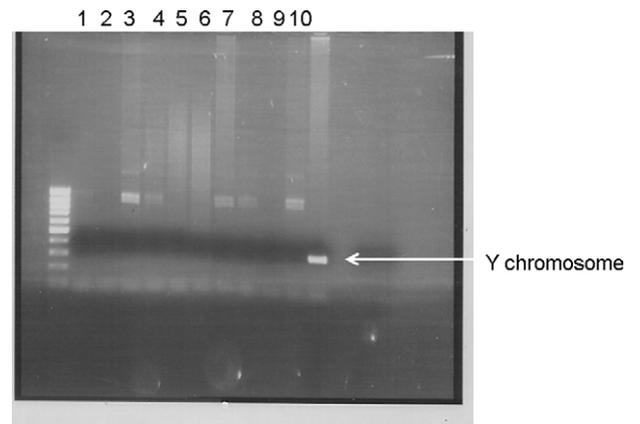

Fig. 3. Allogeneic rejection response. Male C57BL/6 CD3/CD28 cross-linked Th1 memory cells were infused intravenously via tail vein in female Balb/c mice. At 24 h, blood samples were taken and DNA prepared and analyzed by PCR to detect presence of the Y-chromosome. Lanes 1–5 represent infusion of 1×10^5 allogeneic cells. Lanes 6–10 represent infusion of 1×10^6 allogeneic cells. The far left lane is the 100 bp ladder. The arrow indicates detection of Y-chromosome in the male mouse as a positive control.
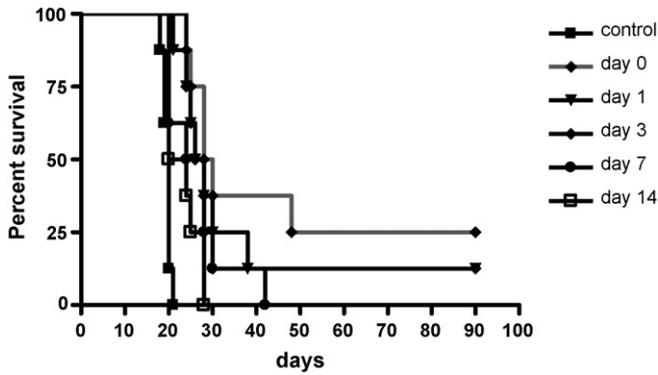
Fig. 4. Effect of the timing of allogeneic CD3/CD28 cross-linked Th1 memory cell treatment. Balb/c mice were inoculated with 2000 BCL1 tumor cells in 0.2 mL of complete media on day 0. C57BL/6 derived allogeneic CD3/CD28 cross-linked Th1 memory cells at a dose of 1×10^5 cells were inoculated either together with the BCL1 cells on day 0, or separately on day 1, day 3, day 7 or day 14 in 0.2 mL of complete media. Complete media alone on day 1 was the control. There were 8 mice in each group tested. The results are presented as Kaplan Meier survival curves. The survival curves for each group were compared to the control group by hazard ratio analysis. Animals surviving greater than 90 days were censored from the analysis. All groups demonstrated significant survival advantage compared to control. Day 0 (hazard ratio = 4.722); Day 1 hazard ratio = 4.224; Day 3 hazard ratio = 4.418; Day 7 hazard ratio = 2.728; and day 14 hazard ratio = 2.397.

The results shown in Fig. 5 indicate a Th2 → Th1 shift in the treated mice in both the plasma and in the BCL1-specific response in both T-cell subsets. Mice treated with allogeneic CD3/CD28 cross-linked Th1 memory cells had a significant

Table 1
Rechallenge and cross challenge experiments

Test group	Number	Rechallenge (no. of cells)	Tumor incidence
BCL1 cured	8	BCL1 (2000)	0/8
Naive	6	BCL1 (2000)	6/6
BCL1 cured	4	4T1 (1×10^4)	4/4
Naive	6	4T1 (1×10^4)	6/6

Mice that survived greater than 90 days after a lethal BCL1 infusion after treatment with activated allogeneic Th1 memory cells (BCL1 cured) were rechallenged with a second lethal injection of BCL1 or a lethal injection of 4T1 tumor with no additional treatment and observed for recurrence of tumor for 60 days. Naïve mice that were not previously treated received lethal BCL1 or 4T1 infusion without treatment as controls.

reduction in serum levels of IL-10 compared to control. The source of the IL-10 is likely from the BCL1 clone, as this clone was previously found to produce substantial amounts of IL-10 [23], a cytokine known to suppress cellular immunity and used as a mechanism for tumor immunoavoidance. However, Tr1 cells have been described [32] which also produce IL-10 and are found in B-cell malignancy [33], which may have contributed to the IL-10 levels found in BCL1 bearing mice.

3.8. Prime-boost protocol

Mice that have rejected an allogeneic cell infusion can develop anti-allogeneic immunity that can be recalled by subsequent exposure to the alloantigens. To determine if such a
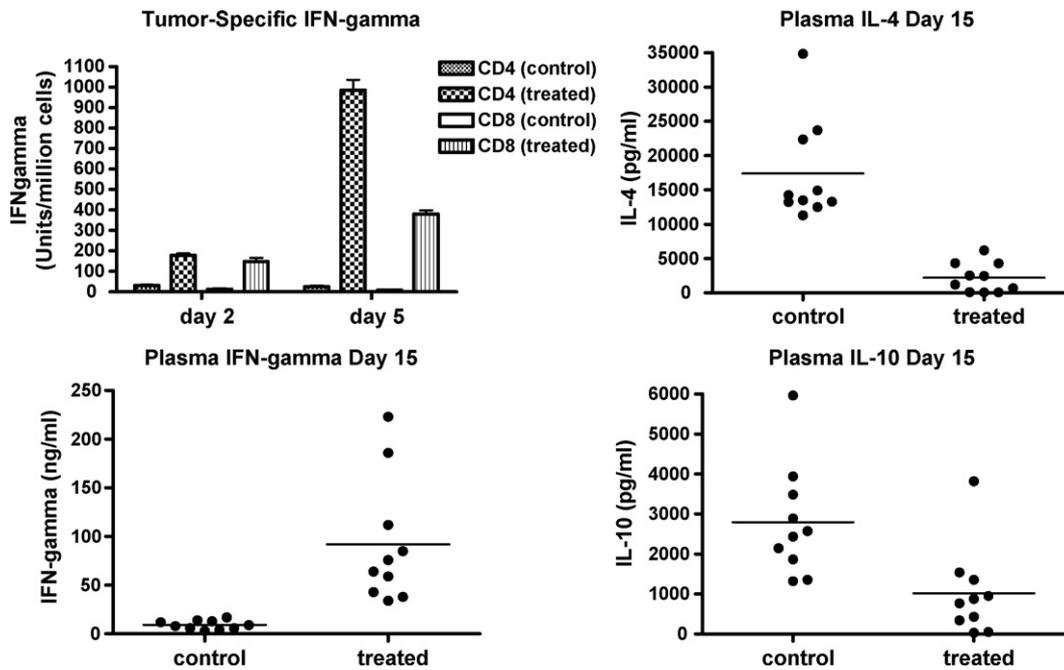

Fig. 5. Th1/Th2 balance analysis. The tumor-specific Th1/Th2 response to BCL1 tumor was evaluated in untreated mice (control) inoculated with 2000 BCL1 compared to mice inoculated with 2000 BCL1 treated with 1×10^5 allogeneic CD3/CD28 cross-linked Th1 memory cells (treated) on day 1. On day 15, CD4 and CD8 T-cells were purified from the mice and incubated for 5 days with syngeneic adherent APC and irradiated BCL1 or irradiated 4T1 (control). Results were normalized per one million T-cells in culture and the background 4T1 cytokine production subtracted. Supernatants were analyzed by ELISA on day 2 and day 5. Also on day 15, plasma samples were analyzed by ELISA for IFN-γ, IL-4 and IL-10. 10 animals were evaluated in each group. Statistical analysis by Student's *T*-test indicated that in all cases the treated groups were significantly different than the control ($P < 0.05$).
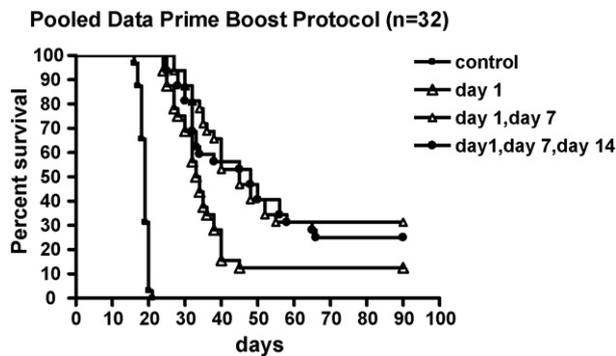
Fig. 6. Prime-boost protocol. The pooled results of 4 experiments with 8 mice per group. All mice were inoculated with 2000 BCL1 intravenously in 0.2 mL of complete media on day 0. On day 1, all mice were treated with intravenous 1×10^5 allogeneic CD3/CD28 cross-linked Th1 memory cells in 0.2 mL complete media. Control mice were treated with 0.2 mL complete media only. Mice received booster inoculations of 1×10^4 allogeneic CD3/CD28 cross-linked Th1 memory cells on day 7 or on days 7 and 14. The single infusion on day 1 resulted in a significant survival advantage compared to control (logrank $P < 0.0001$; hazard ratio = 4.81). The booster infusion on days 7 resulted in a significant survival advantage compared both to the control and to the single infusion on day 1 (logrank $P < 0.0001$; hazard ratio = 5.216). The booster infusions on days 7 and 14 resulted in a significant survival advantage compared to control, but did not differ significantly from a single booster infusion.

recall response could enhance the efficacy of the allogeneic CD3/CD28-cross-linked Th1 memory cell infusions, mice primed with an initial 1×10^5 intravenous dose on day 1 were provided booster infusions of 1×10^4 cells on day 7 or on both day 7 and day 14. The pooled results of 4 separate experiments ($n = 8$ for each treatment in each experiment) are shown in Fig. 6.

The results indicate that increased frequency of infusion can significantly increase survival and the cure rate. A single infusion on day 1 resulted in median survival of 33.5 days compared to a 19-day median survival in the control mice (logrank $P < 0.0001$; hazard ratio = 4.81) and a 12.5% cure rate (4 of 32 mice). A booster infusion on day 7 extended survival to a median of 45.0 days (logrank $P < 0.0001$; hazard ratio = 5.216 compared to control) and a cure rate of 31.25% (10 of 32 animals). The prime plus two booster infusions on day 7 and day 14 resulted in a median survival of 48 days and a cure rate of 25% (8 of 32 animals). The prime plus a single booster at day 7 was not statistically different than the prime plus two booster protocol, one on day 7 and another on day 14.

4. Discussion

We hypothesized that immunological rejection of an allogeneic cell infusion could be coupled to a host-mediated anti-tumor immune effect in immunocompetent hosts. We tested the adoptive transfer of a variety of activated allogeneic immune cell types in mice injected with a lethal dose of tumor.

Only CD3/CD28 cross-linked allogeneic Th1 memory cells elicited an anti-tumor effect. These cells differed from the other immune cells tested in that they expressed high density CD40L and produced high amounts of IFN-γ. Allogeneic Th1 memory cells without CD3/CD28 cross-linking or the same cells cross-linked with CD3/CD28 but derived from syngeneic, rather than allogeneic, CD4 precursors failed to elicit an antitumor effect. This demonstrates that both the CD3/CD28 activation component and the allogeneic component (rejection) of the Th1 memory cells were required for the observed anti-tumor effects.

The mechanism of the anti-tumor effect of activated allogeneic Th1 cell infusions remains to be further investigated. The requirement for the Th1 memory cells to be both activated and allogeneic in order to elicit anti-tumor effects may be related to Type 1 cytokine release and CD40L expression. Allogeneic rejection can upregulate production of Type 1 cytokines [34], and tumor regression has been found to correlate with dominating Type 1 cytokine levels [35]. In the presence of IFN-γ, CD40:CD40L interaction can further enhance the release of Type 1 cytokines [36] and Type 1 cytokines can upregulate CD40L expression on host T-cells [37,38].

CD40L mediates anti-tumor immunity through multiple mechanisms and its expression on the infused cells and its possible upregulation on host cells could contribute to the observed anti-tumor effects. CD40L activates innate NK cells [39] and up-regulates co-stimulatory molecules [40]. CD40L and IFN-γ provide the two signals necessary for maturation of DC [41] and their production of large amounts of IL-12 [42]. Mature DC that produce IL-12 are the link between innate and adaptive Type 1 immunity [43]. CD40L has also been shown to have direct anti-tumor effects both by suppressing tumor growth and by inducing extensive tumor death [44–46]. CD40L can also enhance CTL-mediated lysis of tumors [47]. CD40L is considered one of the strongest inducers of Th1 responses and CD40L stimulation also abrogates the suppressive effect of Treg cells [48].

The observation that the allogeneic Th1 memory cells only mediated anti-tumor effects if they were activated 4–8 h prior to infusion also supports a critical role for CD40L in the anti-tumor mechanism. Memory cells rapidly express CD40L upon activation from pre-formed storage [49]. Upon activation, Th1 memory cell expression of CD40L starts at 1 h to 2 h and peaks at 6 h [50] which correlates with the 4–8 h pre-activation requirement for these cells to demonstrate anti-tumor effects.

The infused allogeneic CD3/CD28 cross-linked Th1 memory cells could not be detected in the peripheral blood of animals at 24 h, supporting that the observed anti-tumor effects were the result of a host immune response and not mediated by the donor cells. However, we cannot rule out that residual donor cells were resident in secondary lymphoid organs such as the spleen and were not detected by our assay. If donor cells remained resident in the hosts, a portion of the observed anti-tumor response may be due to

a direct cytolytic effect of the allogeneic graft cells against the host tumor cells (GVL). Residual donor cells mediating GVL effects might explain why the cure rates were doubled when the allogeneic cells were infused at the same time as the tumor (day 0) compared to infusion a day later (day 1), as co-infusion would enhance detection of a direct killing mechanism.

Allogeneic immune cells contained in blood transfusions are 99.9% cleared by immunocompetent patients within 2 days post-transfusion. However there still is a transient 1-log increase in the circulation that can occur after 3–5 days [51]. Transient allogeneic cell increases mediating GVL effects might explain, in part, why booster injections of allogeneic cells at 7 days post lethal tumor inoculation was effective but not different than booster injections on both days 7 and 14.

While it is possible that residual donor lymphocytes are responsible for some of the anti-tumor effects demonstrated, it is unlikely they were the major contributor to the observed phenomenon, as the fully allogeneic nature of the donor cells would not have resulted in lasting engraftment in the non-conditioned animals. Allogeneic bone marrow transplantation in immunocompetent patients has always required matched donors and cytoreductive treatment of recipients with irradiation or cytotoxic drugs to achieve lasting engraftment. Only syngeneic marrow engraftment has been achieved in unconditioned hosts.

The mice that survived >90 days with an apparent total regression of tumor developed a tumor-specific immune response that could protect against re-challenge with lethal doses of tumor. This protective immunity could be transferred to naïve animals by adoptive transfer of host splenocytes to naïve hosts, demonstrating that the protection resides in the host cellular immune compartment and is not a result of residual donor cells.

However, this tumor-specific immunity was only demonstrated in a minority of the treated mice. The majority of the mice demonstrated enhanced survival in response to the allogeneic Th1 memory cell infusions, but eventually succumbed to disease. A possible explanation for this observation is that the majority of mice were stimulated to elicit only innate immunity against the tumors resulting in increased survival but not cure. In order to elicit a cure, the immunological cascade elicited by the treatment had to transition from innate to adaptive immunity. The 19–22 day lifespan of the tumor inoculated mice may not have been long enough to develop an adaptive immune response in all the mice.

Anti-tumor immunity is mediated by adaptive and innate components of cellular immunity [52–54]. The initiation of an innate immune response and the transition from innate to adaptive immunity is controlled by the nature of the cytokine environment. The innate component has been largely attributed to natural killer (NK) cells which can lyse tumor cells that have downregulated expression of MHC molecules to evade immune detection [55]. Thus, innate immune activation can result in reduction of tumor burden and increased survival as observed.

The adaptive component of immunity depends mainly on CD4+ and CD8+ T cells which are primed by DC that have processed tumor antigens and matured in a Type 1 cytokine environment. Tumor killing by adaptive immune cells is enhanced in the context of Type 1 cytokines, which serve to downregulate tumor immunoavoidance mechanisms, upregulate tumor immunogenicity and counter regulate tumor immunoavoidance mechanisms.

Our results demonstrate that the efficacy of the activated allogeneic Th1 memory cells infusions varies as a function of cell dose, dose timing and dosing frequency. Further studies may be required to optimize the timing of cell infusions in order to upregulate the expression of Type 1 cytokines to correspond with the timing of DC maturation, DC presentation of tumor antigens to cognate T-cells in the lymph nodes, and to the phase of cytolytic effector cell attack. This optimization may serve to increase the number of mice that transition from a putative innate immune response to curative adaptive immune responses.

The activated allogeneic Th1 memory cell infusions were shown to mediate an immunomodulatory mechanism, resulting in a shift from Th2 to Th1 dominance in the tumor-bearing mice, both in the plasma and the T-cell tumor-specific immune response. Type 1 immunity has been shown to be critical for immune-mediated protection against tumors as well as tumor eradication [56–58], and deviation of the tumor-specific immune response to Type 2 is a tumor strategy for immunoavoidance and survival [59]. A Th2 immune response against BCL1 has been associated with a fatal outcome, while a Th1 immune response has been shown to be protective [60]. This immunomodulatory mechanism is a significant finding, as the Balb/c mouse has a genetic predisposition to Th2 immunity in response to antigens [43,61].

The observed anti-tumor effects mediated by the activated allogeneic Th1 memory cell infusions occurred only at low cell doses and were abrogated at a high cell dose. The high dose resulted in a suppressed expression of IFN-γ, while all other doses tested resulted in increased IFN-γ plasma levels at day 15 post-infusion. This observation is consistent with previous observations that low doses of alloantigens favor Th1 responses while high doses favor Th2 responses. Th2 predominance is known to occur at high antigen doses in response to some model antigens and to some infectious antigens [62,63].

These findings demonstrate for the first time that an allogeneic cell infusion can elicit anti-tumor effects in an immunocompetent host, providing extended survival and curative immunity. These anti-tumor effects correlated with a Th2 to Th1 immune deviation. Translation of these results to the clinic may provide a strategy to extend remission in post-induction chemotherapy patients and may also benefit patients that are candidates for allogeneic BMT but unable to tolerate the procedure or are without a matched donor.

Conflict of interest

References

[1] van Rhee F, Szydlo RM, Hermans J, Devergie A, Frassoni F, Arcese W, et al. Long-term results after allogeneic bone marrow transplantation for chronic myelogenous leukemia in chronic phase: a report from the Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation. Bone Marrow Transplant 1997;20:553.

[2] Sohn SK, Kim DH, Kim JG, Sung WJ, Baek JH, Lee NY, et al. Outcome of allogeneic peripheral blood stem cell transplantation using matched sibling donors in patients with high-risk hematological diseases. Eur J Haematol 2004;72:430.

[3] Slavin S, Nagler A, Shapira M, Panigrahi S, Samuel S, Or A. Non-myeloablative allogeneic stem cell transplantation focusing on immunotherapy of life-threatening malignant and non-malignant diseases. Crit Rev Oncol Hematol 2001;39:25.

[4] Slavin S, Morecki S, Weiss L, Or R. Donor lymphocyte infusion: the use of alloreactive and tumor-reactive lymphocytes for immunotherapy of malignant and nonmalignant diseases in conjunction with allogeneic stem cell transplantation. J Hematother Stem Cell Res 2002;11:265.

[5] Giralt S, Khouri I, Champlin R. Non myeloablative "mini transplants". Cancer Treat Res 1999;101:97.

[6] Childs R, Barrett J. Nonmyeloablative stem cell transplantation for solid tumors: expanding the application of allogeneic immunotherapy. Semin Hematol 2002;39:63.

[7] Bay JO, Fleury J, Choufi B, Tournilhac O, Vincent C, Bailly C, et al. Allogeneic hematopoietic stem cell transplantation in ovarian carcinoma: results of five patients. Bone Marrow Transplant 2002;30:95.

[8] Dazzi F, Szydlo RM, Cross NC, Craddock C, Kaeda J, Kanfer E, et al. Durability of responses following donor lymphocyte infusions for patients who relapse after allogeneic stem cell transplantation for chronic myeloid leukemia. Blood 2000;96:2712.

[9] Fowler DH. Shared biology of GVHD and GVT effects: potential methods of separation. Crit Rev Oncol Hematol 2006;57:225.

[10] Weiden PL, Flournoy N, Thomas ED, Prentice R, Fefer A, Buckner CD, et al. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. N Engl J Med 1979;300:1068.

[11] Sullivan KM, Storb R, Buckner CD, Fefer A, Fisher L, Weiden PL, et al. Graft-versus-host disease as adoptive immunotherapy in patients with advanced hematologic neoplasms. N Engl J Med 1989;320:828.

[12] Kernan NA, Collins NH, Juliano L, Cartagena T, Dupont B, O'Reilly RJ. Clonable T lymphocytes in T cell-depleted bone marrow transplants correlate with development of graft-v-host disease. Blood 1986;68:770.

[13] Ferrara JL, Deeg HJ. Graft-versus-host disease. N Engl J Med 1991;324:667.

[14] Champlin R, Giralt S, Gajewski J. T cells, graft-versus-host disease and graft-versus-leukemia: innovative approaches for blood and marrow transplantation. Acta Haematol 1996;95:157.

[15] Rubio MT, Kim YM, Sachs T, Mapara M, Zhao G, Sykes M. Anti-tumor effect of donor marrow graft rejection induced by recipient leukocyte infusions in mixed chimeras prepared with nonmyeloablative conditioning: critical role for recipient-derived IFN-gamma. Blood 2003;102:2300.

[16] Kraus AB, Shaffer J, Toh HC, Preffer F, Dombkowski D, Saidman S, et al. Early host CD8 T-cell recovery and sensitized anti-donor interleukin-2-producing and cytotoxic T-cell responses associated with marrow graft rejection following nonmyeloablative allogeneic bone marrow transplantation. Exp Hematol 2003;31:609.

[17] Rubio MT, Saito TI, Kattleman K, Zhao G, Buchli J, Sykes M. Mechanisms of the antitumor responses and host-versus-graft reactions induced by recipient leukocyte infusions in mixed chimeras prepared with nonmyeloablative conditioning: a critical role for recipient CD4+ T cells and recipient leukocyte infusion-derived IFN-gamma-producing CD8+ T cells. J Immunol 2005;175:665.

[18] Saito TI, Rubio MT, Sykes M. Clinical relevance of recipient leukocyte infusion as antitumor therapy following nonmyeloablative allogeneic hematopoietic cell transplantation. Exp Hematol 2006;34:1271.

[19] Rubio MT, Zhao G, Buchli J, Chittenden M, Sykes M. Role of indirect allo- and autoreactivity in anti-tumor responses induced by recipient leukocyte infusions (RLI) in mixed chimeras prepared with nonmyeloablative conditioning. Clin Immunol 2006;120:33.

[20] Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med 2004;10:909.

[21] Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol 2007;25:267.

[22] Har-Noy M, Slavin S. The anti-tumor effect of allogeneic bone marrow/stem cell transplant without graft vs. host disease toxicity and without a matched donor requirement? Med Hypotheses 2008;70(6):1186.

[23] Slavin S, Strober S. Spontaneous murine B-cell leukemia. Nature 1978;272:624.

[24] Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 1992;52:1399.

[25] Pugatsch T, Oppenheim A, Slavin S. Improved single-step PCR assay for sex identification post-allogeneic sex-mismatched BMT. Bone Marrow Transplant 1996;17:273.

[26] Bland JM, Altman DG. The logrank test. Bmj 2004;328:1073.

[27] Spruance SL, Reid JE, Grace M, Samore M. Hazard ratio in clinical trials. Antimicrob Agents Chemother 2004;48:2787.

[28] Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Altern Med Rev 2003;8:223.

[29] Aruga A, Aruga E, Tanigawa K, Bishop DK, Sondak VK, Chang AE. Type 1 versus type 2 cytokine release by Vbeta T cell subpopulations determines *in vivo* antitumor reactivity: IL-10 mediates a suppressive role. J Immunol 1997;159:664.

[30] Zhou J, Ye F, Chen H, Lv W, Gan N. The expression of interleukin-10 in patients with primary ovarian epithelial carcinoma and in ovarian carcinoma cell lines. J Int Med Res 2007;35:290.

[31] Ikeda H, Chamoto K, Tsuji T, Suzuki Y, Wakita D, Takeshima T, et al. The critical role of type-1 innate and acquired immunity in tumor immunotherapy. Cancer Sci 2004;95:697.

[32] Levings MK, Roncarolo MG. T-regulatory 1 cells: a novel subset of CD4 T cells with immunoregulatory properties. J Allergy Clin Immunol 2000;106:S109.

[33] Marshall NA, Christie LE, Munro LR, Culligan DJ, Johnston PW, Barker RN, et al. Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. Blood 2004;103:1755.

[34] Simeonovic CJ, Townsend MJ, Karupiah G, Wilson JD, Zarb JC, Mann DA, et al. Analysis of the Th1/Th2 paradigm in transplantation: interferon-gamma deficiency converts Th1-type proislet allograft rejection to a Th2-type xenograft-like response. Cell Transplant 1999;8:365.

[35] Conrad R, Remberger M, Cederlund K, Hentschke P, Sundberg B, Ringden O, et al. Inflammatory cytokines predominate in cases of tumor regression after hematopoietic stem cell transplantation for solid cancer. Biol Blood Marrow Transplant 2006;12:346.

[36] De LKT, Whiting CV, Bland PW. Proinflammatory cytokine synthesis by mucosal fibroblasts from mouse colitis is enhanced by interferon-

gamma-mediated up-regulation of CD40 signalling. Clin Exp Immunol 2007;147:313.

[37] Kilinc MO, Aulakh KS, Nair RE, Jones SA, Alard P, Kosiewicz MM, et al. Reversing tumor immune suppression with intratumoral IL-12: activation of tumor-associated T effector/memory cells, induction of T suppressor apoptosis, and infiltration of CD8+ T effectors. J Immunol 2006;177:6962.

[38] Kim EY, Lee EN, Lee J, Park HJ, Chang CY, Jung da Y, et al. Two-signal blockade with anti-CD45RB and anti-CD154 monoclonal antibodies inhibits graft rejection via CD4-dependent mechanisms in allogeneic skin transplantation. Exp Mol Med 2006;38:284.

[39] Tomihara K, Kato K, Masuta Y, Nakamura K, Tanaka T, Hiratsuka H, et al. Gene transfer of the CD40-ligand to human dendritic cells induces NK-mediated antitumor effects against human carcinoma cells. Int J Cancer 2007;120:1491.

[40] Hunter TB, Alsarraj M, Gladue RP, Bedian V, Antonia SJ. An agonist antibody specific for CD40 induces dendritic cell maturation and promotes autologous anti-tumour T-cell responses in an in vitro mixed autologous tumour cell/lymph node cell model. Scand J Immunol 2007;65:479.

[41] Snijders A, Kalinski P, Hilkens CM, Kapsenberg ML. High-level IL-12 production by human dendritic cells requires two signals. Int Immunol 1998;10:1593.

[42] Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J Exp Med 1996;184:747.

[43] Steinman RM. Linking innate to adaptive immunity through dendritic cells. Novartis Found Symp 2006;279:101.

[44] Georgopoulos NT, Steele LP, Thomson MJ, Selby PJ, Southgate J, Trejdosiewicz LK. A novel mechanism of CD40-induced apoptosis of carcinoma cells involving TRAF3 and JNK/AP-1 activation. Cell Death Differ 2006;13:1789.

[45] Georgopoulos NT, Merrick A, Scott N, Selby PJ, Melcher A, Trejdosiewicz LK. CD40-mediated death and cytokine secretion in colorectal cancer: a potential target for inflammatory tumour cell killing. Int J Cancer 2007.

[46] Tong AW, Papayoti MH, Netto G, Armstrong DT, Ordonez G, Lawson JM, et al. Growth-inhibitory effects of CD40 ligand (CD154) and its endogenous expression in human breast cancer. Clin Cancer Res 2001;7:691.

[47] von Leoprechting A, van der Bruggen P, Pahl HL, Aruffo A, Simon JC. Stimulation of CD40 on immunogenic human malignant melanomas augments their cytotoxic T lymphocyte-mediated lysis and induces apoptosis. Cancer Res 1999;59:1287.

[48] Loskog A, Totterman TH. CD40L—a multipotent molecule for tumor therapy. Endocr Metab Immune Disord Drug Targets 2007;7: 23.

[49] Koguchi Y, Thauland TJ, Slifka MK, Parker DC. Preformed CD40 ligand exists in secretory lysosomes in effector and memory CD4+ T

cells and is quickly expressed on the cell surface in an antigen specific manner. Blood 2007;110(October (7)):2520 [Epub 2007 Jun 26].

[50] Casamayor-Palleja M, Khan M, MacLennan IC. A subset of CD4+ memory T cells contains preformed CD40 ligand that is rapidly but transiently expressed on their surface after activation through the T cell receptor complex. J Exp Med 1995;181:1293.

[51] Lee TH, Donegan E, Slichter S, Busch MP. Transient increase in circulating donor leukocytes after allogeneic transfusions in immunocompetent recipients compatible with donor cell proliferation. Blood 1995;85:1207.

[52] Zitvogel L, Casares N, Pequignot MO, Chaput N, Albert ML, Kroemer G. Immune response against dying tumor cells. Adv Immunol 2004;84:131.

[53] Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 2001;410:1107.

[54] Borg C, Terme M, Taieb J, Menard C, Flament C, Robert C, et al. Novel mode of action of c-kit tyrosine kinase inhibitors leading to NK cell-dependent antitumor effects. J Clin Invest 2004;114:379.

[55] Whiteside TL, Herberman RB. The role of natural killer cells in immune surveillance of cancer. Curr Opin Immunol 1995;7:704.

[56] Nishimura T, Nakui M, Sato M, Iwakabe K, Kitamura H, Sekimoto M, et al. The critical role of Th1-dominant immunity in tumor immunology. Cancer Chemother Pharmacol 2000;46(Suppl.):S52.

[57] Chamoto K, Kosaka A, Tsuji T, Matsuzaki J, Sato T, Takeshima T, et al. Critical role of the Th1/Tc1 circuit for the generation of tumor-specific CTL during tumor eradication *in vivo* by Th1-cell therapy. Cancer Sci 2003;94:924.

[58] Winter H, Hu HM, Poehlein CH, Huntzicker E, Osterholzer JJ, Bashy J, et al. Tumour-induced polarization of tumour vaccine-draining lymph node T cells to a type 1 cytokine profile predicts inherent strong immunogenicity of the tumour and correlates with therapeutic efficacy in adoptive transfer studies. Immunology 2003;108:409.

[59] Stremmel C, Greenfield EA, Howard E, Freeman GJ, Kuchroo VK. B7-2 expressed on EL4 lymphoma suppresses antitumor immunity by an interleukin 4-dependent mechanism. J Exp Med 1999;189: 919.

[60] Lee PP, Zeng D, McCaulay AE, Chen YF, Geiler C, Umetsu DT, et al. T helper 2-dominant antilymphoma immune response is associated with fatal outcome. Blood 1997;90:1611.

[61] Hsieh CS, Macatonia SE, O'Garra A, Murphy KM. T cell genetic background determines default T helper phenotype development in vitro. J Exp Med 1995;181:713.

[62] Hosken NA, Shibuya K, Heath AW, Murphy KM, O'Garra A. The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. J Exp Med 1995;182: 1579.

[63] Bretscher PA, Wei G, Menon JN, Bielefeldt-Ohmann H. Establishment of stable, cell-mediated immunity that makes "susceptible" mice resistant to Leishmania major. Science 1992;257:539.