Induction of Graft-versus-Leukemia (GVL) Effect without Graft-versus-Host Disease (GVHD) by Pretransplant Donor Treatment with Immunomodulators

Shoshana Morecki, Elena Yacovlev, Yael Gelfand, Yehudit Shabat, Shimon Slavin*

Pretransplant donor treatment with immunomodulators such as complete Freund’s adjuvant (CFA) or oligodeoxynucleotide sequences expressing CpG motifs (CpG), was applied in sublethally irradiated host mice inoculated with murine models of mammary carcinoma (4T1) or B cell leukemia (BCL1). Spleen cells or IL-2 activated splenocytes (lymphokine activated killer [LAK]) derived from donor mice treated with CpG emulsified in incomplete Freund’s adjuvant (IFA), (CpG

1
IFA) did not cause graft-versus-host disease (GVHD), but were not efficient enough to induce a significant graft-versus-tumor (GVT) response against 4T1 cells. In contrast, an efficient graft-versus-leukemia (GVL) effect was evident in BCL1-bearing mice inoculated with spleen cells from donors pretreated with CFA or CpG

1
IFA. Pretransplant donor treatment with CFA prolonged survival to a median of 62 days with 3 of 27 mice remaining GVHD- and leukemia-free for >200 days, compared to GVHD-related death of all mice inoculated with naive cells (median 17 days), or leukemia-related death of all mice inoculated with leukemia cells (median of 27 days). Pretransplant donor treatment with CpG + IFA exerted a more efficient GVL effect with reduced GVHD resulting in 12 of 26 GVHD- and leukemia-free survivors for >200 days. Our results suggest that it may be possible to prevent GVHD while sparing an efficient GVL effect by using pretransplant donor treatment with immunomodulators prior to allogeneic stem cell transplantation and/or donor lymphocyte infusions in hematologic malignancies.


KEY WORDS: Graft-versus-leukemia, Graft-versus-host, CpG, Donor treatment

INTRODUCTION

The major benefit of hematopoietic stem cell transplantation (HSCT) and allogeneic cell therapy (alloCT) is the induction of graft-versus-leukemia (GVL) or graft-versus-tumor (GVT) effects. Unfortunately, the alloreactivity directed against the host’s malignant cells often causes a severe reaction against normal host cells, known as graft-versus-host disease (GVHD) [1-6].

Although clinically significant, GVT effects may occur without GVHD [7,8], other clinical observations have indicated a reduced incidence of malignant relapse in patients with GVHD, reflecting the shared biology between GVHD and antitumor activity [9,10]. Because GVHD can develop into an uncontrolled life-threatening form, it has become an important goal of many research and clinical studies to define conditions in which GVL/GVT can be achieved without manifestation of the irreversible harmful symptoms that occur in GVHD. Many potential ways to separate these graft effects have been suggested and tested including: T cell depletion, reduced intensity conditioning (RIC), the use of defined cell subsets such as T or allogeneic natural killer (NK) cells, elimination of activated or antigen specific T cells, and application of various biologic agents or pharmacologic components that can modify the T helper (Th)1/Th2 balance and/or induce cellular and humoral immunoregulatory substances [11,12].

During the past decade, we have tried to enhance the benefits of alloCT by various strategies directed toward minimizing the risk of GVHD. We have...
reported previously that elimination of activated allo-reactive donor lymphocytes in the host mice prior to HSCT resulted in significant GVL/GVT effects in 40% to 80% of the mice with no clinical GVHD symptoms [13,14]. Recently, we showed that pretransplant donor treatment with immunomodulators such as complete Freund’s adjuvant (CFA) or oligodeoxynucleotide sequences expressing CpG motifs (CpG), prevented the development of GVHD efficiently in >77% of mice inoculated with donor splenocytes following nonlethal total body irradiation (TBI) [15]. We, therefore, decided to test this approach in tumor-bearing mice as part of our attempts to control GVHD while sparing the anti-malignancy activity of the allograft. In the current study pretransplant donor treatment with CFA or CpG was tested in a murine model of mammary carcinoma (4T1) and B cell leukemia (BCL1) using spleen cells or IL-2 activated splenocytes.

MATERIALS AND METHODS

Mice

Female BALB/c H-2d (BALB), C57BL/6 H-2b (C57), and (BALB/c × C57BL/6)F1 H-2d/b (F1) mice aged 10 to 12 weeks, weighing 22 to 24 g, were used in this study. All mice were purchased from Harlan, Israel, and maintained in the animal facility of the Hadassah University Hospital in full compliance with the regulations for the protection of animal rights.

Donor Pretreatment

CFA, incomplete Freund’s adjuvant (IFA) (Difco Laboratories, Detroit, MI), 100 μg CpG (ODN #1826) or 100 μg non-CpG control (ODN #2138) (Coley Pharmaceutical Group, Kanata, Canada) alone or as emulsions with IFA, were injected subcutaneously (s.c.) into 2 sites in naïve C57 mice (0.1 mL/site). Splenocytes were harvested 6 days following CpG treatment or 10 days following CpG and CFA treatment, based on previously reported data concerning (1) number of spleen cells, (2) enriched CD11b+GR-1+ myelogenous suppressor cells, and (3) their effect on GVHD inhibition [15,16].

Preparation of Lymphokine Activated Killer (LAK) Cells

Naïve or pretreated C57 splenocytes were cultured at a concentration of 1.2 × 10^6/mL with 6000 IU/mL rhIL-2 (Proleukin, Chiron, Amsterdam, The Netherlands) in 1000 mL Lifecell bags (Nexell Therapeutics Inc, Irvine, CA) for the preparation of LAK cells. The culture medium was RPMI 1640 containing 2 mM glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, 0.25 μg/mL amphotericin B, 1% minimal essential medium (MEM) nonessential amino acids (Biological Industries, Beit Haemek, Israel), 10% fetal bovine serum (FBS) (Invitrogen-GIBCO, Paisley, Scotland, UK), and 5 × 10^-3 M 2-mercaptoethanol (Sigma-Aldrich, Israel). Cells were harvested after 4 days in a 5% CO₂ incubator at 37°C.

Tumor Cells

BCL1 is a spontaneous B cell leukemia/lymphoma of BALB/c (H-2b) origin maintained by intravenous (i.v.) passage of blood from a tumor-bearing mouse [17]. 3B3 is a cell line of BCL1 origin that grows in vitro as a cell suspension [18]. 4T1 is a tumor cell line established from a spontaneous mammary tumor of BALB/c (H-2b) origin [19]. 4T1 cells propagate in vitro as a monolayer and are harvested by using 0.25% trypsin in 0.05% EDTA (Biological Industries, Beit Haemek, Israel). YAC-1(H-2b) is a lymphoma cell line used as a target for testing NK activity in cytotoxicity assays [20]. All cell lines were kept in a 5% CO₂ incubator at 37°C in RPMI 1640 medium containing 2 mM glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, 0.25 μg/mL amphotericin B, 1% MEM nonessential amino acids, and 10% FBS.

Experimental Design for GVHD Induction

F1 recipient mice were conditioned with 5.5 Gy TBI, using a 6 MEV linear accelerator at a dose rate of 1.9 Gy/min. These nonlethally irradiated recipients were inoculated i.v. 48 hours later with 30 × 10^6 splenocytes or 20 × 10^6 LAK cells from naïve or pretreated C57 mice.

Chimerism Assay

Recipient mice were anesthetized (ketamine 100 mg/kg and dihydrobenzperidol 1.2 mg/kg administered intraperitoneally) and blood was taken from the retro-orbital sinus of the eye. Peripheral blood mononuclear cells (PBMCs) were isolated using lympholyte-M gradient (Cedar Lane Laboratories, Ontario, Canada) and a percentage of donor cells was determined by fluorescent-activated cell sorting (FACS) analysis (see below) using phycoerythrin (PE) anti-H-2d antibodies (BD Pharmingen, San Diego, CA). Following haploidentical donor inoculation, the percentage of H-2b donor cells was determined by measuring the disappearance of host cells carrying H-2d antigen, according to the following formula: 100% – %H-2d- positive cells × %H-2b- positive cells.

Experimental Design for Induction of GVL and GVT

F1 recipients were conditioned as for GVHD induction. One day after irradiation, mice were inoculated i.v. with 5 × 10^6 4T1 tumor cells or 10^6 BCL1 cells. Twenty-four hours later mice were injected i.v. with 30 × 10^6 naïve or pretreated C57 splenocytes or 20 × 10^6 LAK cells from naïve or pretreated C57 mice.

Magnetic Cell Sorting

Magnetic cell separation was carried out on suspensions of splenocytes derived from either naïve or pretreated C57 mice. T cells were isolated by depletion of non-T cells (negative selection) with the Mouse Pan T cell Isolation Kit (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). T cell-depleted fractions (TCD) (negative selection) were obtained by T cell depletion with mouse CD90 (Thy 1.2) MicroBeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Phenotypic analysis of TCD isolated fraction showed 0.6% CD3⁺ cells and enrichment of CD11b⁺Gr-1⁺ myeloid suppressor cells (15%). All isolations were carried
out according to the manufacturers’ protocols. Briefly, for isolated T cells and TCD fractions, splenocytes were labeled with the corresponding antibody and then loaded onto a MACS LS column, which was placed in the magnetic field of a MACS Separator.

Adoptive Transfer (AT) Experiments

The 10^7 splenocytes from F1 recipients inoculated with BCL1 and treated with either naïve or pretreated C57 splenocytes or LAK cells, were adoptively transferred i.v. into naïve BALB/c hosts 14 or 21 days after BCL1 inoculation, as specified in each experiment.

Follow-up

In all experiments, mice were checked daily for the appearance of GVHD symptoms such as hunched posture, ruffled fur, diarrhea, and cachexia, as assessed by weekly body weight measurements. BCL1 leukemia was detected by means of peripheral blood counts (data not shown) and spleen enlargement. 4T1 lung metastases were detected in postmortem examinations. Mice were checked for survival over a follow-up period as specified in each experiment.

Cytotoxicity Test

Cytotoxicity was measured by the standard 51Cr release assay as previously described [21]. Briefly, LAK cells from naïve or pretreated C57 mice were diluted to 5 x 10^6 cells/mL and incubated for 4 h at different ratios with 5000 target naïve or pretreated C57 mice already labeled for 1 hour with 51Cr sodium chromate (GE Healthcare-Amersham, UK). Percentage of cytotoxicity was calculated by the following formula: (cpm of sample – cpm of spontaneous release)/ (cpm of target cells treated with 1% Triton – cpm of spontaneous release) x 100.

Flow Cytometry Analysis

For FACS analysis, cells were stained with the following antibodies: PE Pan NK cells (DX5) to detect CD49b^+^ cells, fluorescein isothiocyanate (FITC) anti-mouse-CD3 and allopheocyanin (APC) antimouse-CD8a to detect T cells (BD-Pharmingen, San Diego, CA).

Prior to fluorescence staining, splenocytes were lysed with mouse erythrocyte lysis buffer (R&D Systems, Mineapolis, MN). Fluorescein staining was carried out as follows: 5 x 10^5 splenocytes were incubated for 5 minutes with mouse Fc blocker (CD32/16) antibody (eBioscience, San Diego, CA) to prevent nonspecific staining, subsequently stained with the relevant antibodies for 20 minutes in ice, washed with 1% bovine serum albumin (BSA), 0.03% azide in phosphate-buffered saline (PBS), and resuspended in PBS for reading. Samples were analyzed with BD FACScalibur using the Cell Quest program (BD, San Diego, CA).

Statistical Analysis

The Kaplan-Meier method was used to calculate the probability of tumor-free survival, a function of time after cell or tumor inoculation [22]. The statistical significance between Kaplan-Meier curves was evaluated using the Log-rank test [23]. The statistical significance of cytotoxic assays, phenotypic analysis, and body weight was evaluated by the standard 2-tailed, unpaired Student’s t-test.

RESULTS

Effect of CpG + IFA Donor Treatment on GVHD Induced by LAK Cells

Splenocytes from CpG + IFA-treated donor’s shows the same frequency of CD3^-^ or CD8^-^ cells as naïve LAK cells, a nonsignificant increase in CD11b^-^Gr-1^-^ cells, and a slight significant reduction of NK1.1^+^ cells, compared to naïve LAK cells (Figure 1b). Following LAK generation, the phenotypic changes induced by CpG + IFA treatment in the spleen are no longer fully maintained (Figure 1c), and distribution of the LAK cell subset population is fairly similar to that of the LAK cell subset population identified in naïve cells.

The differentially reduced LAK activity in vitro led us to test its correlation to the ability to induce GVHD and to exert GVL/GVT effects in vivo. A murine model of GVHD was set up and LAK cells from CpG + IFA-treated C57 donor mice were inoculated into sublethally irradiated F1 host mice. As shown in Figure 2, naïve C57-derived LAK cells caused marked GVHD-related body weight loss of about 4 g over 10 days. A similar body weight loss was also seen in F1 host mice inoculated with LAK cells derived from splenocytes of C57 mice pretreated with IFA, CpG alone, non-CpG, or CpG + IFA. In contrast, donor-derived LAK cells prepared from C57 mice pretreated with CpG emulsified in IFA, did not induce GVHD-related symptoms such as hunched posture, ruffled fur, or cachexia as assessed by serial measurements of body weight. All control groups of mice inoculated with donor cells treated with IFA, CpG alone, non-CpG, non-CpG emulsified in IFA, or naïve donor cells, died of severe GVHD in a median of 10 to 14 days following LAK cell inoculation. Of 29 mice postmortem examinations. Mice were checked for survival over a follow-up period as specified in each experiment.
inoculated with LAK cells from C57 donor mice pre-treated with CpG emulsified in IFA, 18 mice survived GVHD-free for 200 days (Table 1). It is worth noting that these mice retained the H-2b donor-derived LAK cells for 80 days following LAK inoculation (Table 2).

**Effect of CpG-Treated Donor LAK Cells on GVT Response**

Sublethally irradiated F1 mice inoculated with 4T1 mammary carcinoma cells were treated with allogeneic LAK cells. LAK cells derived either from naive C57 mice or from C57 mice treated pretransplant with IFA or non-CpG + IFA caused severe GVHD, which led to the death of all host mice in a survival median of 17, 13, and 13 days, respectively. In contrast, LAK cells from donors treated pretransplant with CpG + IFA, did not cause GVHD, but almost all the hosts died of tumor between days 24 and 60, with a median of 27 days, the same as hosts inoculated with tumor cells only (Table 3). Overall, no statistically significant difference was observed in a comparison of hosts inoculated with LAK cells from C57 donor mice pre-treated with CpG emulsified in IFA, 18 mice survived GVHD-free for >200 days (Table 1). It is worth noting that these mice retained the H-2b donor-derived LAK cells for >80 days following LAK inoculation (Table 2).

**Figure 2.** Effect of LAK cells derived from CpG-treated donor mice on GVHD induction, as measured by body weight. Sublethally (5.5 Gy) irradiated (BALB x C57) F1 mice were inoculated with 20 x 10^6 LAK cells derived from either naive or CpG- or non-CpG-treated C57 donor mice 6 days before spleen harvest, and CpG + IFA- or non-CpG + IFA-treated C57 donor mice 10 days before spleen harvest. Results represent 1 of 2 experiments. P < .012 for the comparison of body weight on day 10 following donor treatment with CpG + IFA versus donor treatment with CpG, non-CpG, non-CpG + IFA, IFA, or no treatment of naive donor splenocytes.

**Figure 1.** Cyotoxic activity and phenotypic analysis of LAK cells derived from CpG + IFA-treated donors. Splenocytes were harvested from C57 naive mice or mice treated with non-CpG + IFA or CpG + IFA 10 days previously and incubated with rIL-2 for 4 days. Cytotoxicity (A) was tested by 51Cr release assay. Results are presented as mean of % specific lysis ± SE measured at a 25:1 cell ratio of effector: target. P = .001 for the comparison of cytotoxicity directed to 3B3 cells by LAK cells derived from naive versus CpG + IFA-treated donors. Results represent 1 of 2 experiments. Phenotypic analysis (B) was carried out by using flow cytometry. Data were pooled from 3 experiments, and results are presented as mean of % positive cells ± SE. P = .014 for the comparison of %NK1.1^+ LAK cells following CpG + IFA donor treatment versus %NK1.1^+ in LAK cells derived from naive mice. P < .001 for the comparison of %NK1.1^+, %CD11b^+ GR-1^+, %CD8^+, and %CD3^+ in splenocytes versus LAK cells derived from mice treated with CpG + IFA (C).
Table 1. Effect of Pretransplant Donor Treatment with CpG on GVHD Induction by LAK Cells

<table>
<thead>
<tr>
<th>Donor Pretreatment</th>
<th>Survival (Days)</th>
<th>GVHD-Related Death</th>
<th>Disease-Free Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>12 (7-21)</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>IFA</td>
<td>14 (7-17)</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>CpG</td>
<td>11 (7-14)</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>non-CpG + IFA</td>
<td>10 (10-13)</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>CpG + IFA</td>
<td>&gt;200 (10-200)</td>
<td>29</td>
<td>11</td>
</tr>
<tr>
<td>non-CpG + IFA</td>
<td>13 (7-17)</td>
<td>14</td>
<td>14</td>
</tr>
</tbody>
</table>

GVHD indicates graft-versus-host disease; CpG, CpG motifs; LAK, lymphocyte-activated killer cell.

Spleen cells derived from C57BL/6 donor mice treated with CpG + IFA 10 days previously were inoculated into F1 sublethally irradiated (5.5 Gy) mice. LAK cells were prepared by 4 days’ IL-2 activation of spleen cells with rIL-2. Adoptive transfer of splenocytes from either naïve donors or donors inoculated s.c. 10 days previously with IFA, CpG + IFA or non-CpG + IFA did not lead to a significant number of tumor- and GVHD-free survivors in 4T1 tumor-bearing mice. Therefore, we tested the GVT effect exerted by nonactivated spleen cells from donor mice that were treated pretransplant with various immunomodulators.

Donor treatment with CFA or CpG alone or emulsified in IFA resulted in delayed tumor-related death (median of 47, 43, and 42 days, respectively) of F1 host mice that were inoculated with 4T1 cells and C57 pretransplant-treated donor splenocytes (Table 4). The control group of mice inoculated with 4T1 only died of tumors in a median of 32 days, and the control group of mice given naïve C57 donor cells died mainly of GVHD in a median of 13 days. Despite the statistically significant difference between controls and experimental groups of mice inoculated with pretransplant-treated splenocytes, the donor treatment led to a moderate GVT effect. The best pretransplant donor treatment was CFA, which resulted in 11% (6 of 52) of disease-free survivors (DFS) over a follow-up period of 250 days.

Table 2. Induction of Chimerism by CpG + IFA Pretransplant Donor Treatment

<table>
<thead>
<tr>
<th>Donor Cell Source*</th>
<th>% Donor Cells (H-2b+)</th>
<th>Mean ± SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen cells</td>
<td>96 ± 4</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>LAK cells</td>
<td>88 ± 11</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

CpG indicates CpG motif; IFA, incomplete Freund’s adjuvant; LAK, lymphocyte killer.

*Splenocytes derived from C57BL/6 donor mice treated with CpG + IFA 10 days previously, were inoculated into F1 sublethally irradiated (5.5 Gy) mice. LAK cells were prepared by incubation of spleen cells with rIL-2 for 4 days before inoculation into F1 sublethally irradiated mice.

The Effect of CpG-Treated Donor LAK Cells on GVL Response

Spleen cells from either naïve donors or donors inoculated s.c. 10 days previously with IFA, CpG + IFA or non-CpG + IFA did not lead to a significant number of tumor- and GVHD-free survivors in 4T1 tumor-bearing mice. Therefore, we tested the GVT effect exerted by nonactivated spleen cells from donor mice that were treated pretransplant with various immunomodulators.

Donor treatment with CFA or CpG alone or emulsified in IFA resulted in delayed tumor-related death (median of 47, 43, and 42 days, respectively) of F1 host mice that were inoculated with 4T1 cells and C57 pretransplant-treated donor splenocytes (Table 4). The control group of mice inoculated with 4T1 only died of tumors in a median of 32 days, and the control group of mice given naïve C57 donor cells died mainly of GVHD in a median of 13 days. Despite the statistically significant difference between controls and experimental groups of mice inoculated with pretransplant-treated splenocytes, the donor treatment led to a moderate GVT effect. The best pretransplant donor treatment was CFA, which resulted in 11% (6 of 52) of disease-free survivors (DFS) over a follow-up period of 250 days.

Table 3. Effect of LAK Cells Derived from CpG-Treated Donors on Graft versus Mammary Carcinoma Cells (4T1)

<table>
<thead>
<tr>
<th>Donor Pretreatment</th>
<th>C57 LAK Cells</th>
<th>Survival</th>
<th>GVHD-Related Death</th>
<th>Tumor-Related Death</th>
<th>Disease-Free Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>27 (24-27)</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>—</td>
<td>+</td>
<td>17 (12-17)</td>
<td>7</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>IFA</td>
<td>+</td>
<td>13 (12-17)</td>
<td>9</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>CpG + IFA</td>
<td>+</td>
<td>27 (24-60)</td>
<td>9</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Non-CpG + IFA</td>
<td>+</td>
<td>13 (12-17)</td>
<td>9</td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>

GVHD indicates graft-versus-host disease; LAK, lymphokine activated killer; IFA, incomplete Freund’s adjuvant; CpG, CpG motif.

Sublethally irradiated F1 mice inoculated with BCL1 cells were treated with allogeneic LAK cells derived from either naïve C57 mice or C57 mice treated with non-CpG + IFA or CpG + IFA. All mice developed severe GVHD and died between days 10 and 17 (data not shown). Adoptive transfer of splenocytes harvested from these LAK-treated hosts on day 14 following tumor inoculation, revealed that all the LAK-treated hosts, including hosts treated with naïve or pretransplant-treated donor cells, exerted a strong and efficient GVL effect for 14 days. None of the secondary hosts inoculated with the adoptively
transferred splenocytes, developed leukemia over a follow-up period of >200 days (Table 5).

The Effect of CFA-Treated Donor Splenocytes on GVL

F1 mice inoculated with BCL1 cells and treated with allogeneic C57 splenocytes from mice previously treated with CFA, survived for a median of 62 days following leukemic cell inoculation in comparison with a survival median of 17 days for mice inoculated with naive C57 spleen cells or a survival median of 27 days for control mice inoculated with BCL1 only. Three of a total of 27 F1 mice inoculated with C57 splenocytes from CFA-treated mice were DFS without any evidence of GVHD or leukemia for >200 days (Figure 3). Evidence of a GVL effect was seen in AT experiments in which CFA pretreated spleen cells taken 14 or 21 days following tumor inoculation did not cause leukemia in any of the new hosts for a follow-up period of >230 days. In contrast, AT of splenocytes taken 14 or 21 days following tumor inoculation from control mice inoculated with BCL1 cells only (Table 6), led to onset of leukemia, and all these mice died of leukemia in a median of 42 and 24 days, respectively. It is worth noting that AT of splenocytes taken on day 14 from mice inoculated with naive spleen cells did not cause leukemia in the secondary hosts. AT on day 21 following tumor inoculation could not be carried out, because by that time all the mice inoculated with naive cells had died. In summary, our results showed an apparent advantage of CFA- donor spleen cells over naive donor cells in exerting a GVL effect.

Table 4. The Effect of Donor-Treated Splenocytes on Mammary Carcinoma Cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Splenocytes</th>
<th>Survival (Days)</th>
<th>GVHD-Tumor-Related Deaths</th>
<th>Disease-Free Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>--</td>
<td>31 (18-47)</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>b</td>
<td>--</td>
<td>13 (10-59)</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>c</td>
<td>CFA</td>
<td>47 (11-&gt;250)</td>
<td>52</td>
<td>10</td>
</tr>
<tr>
<td>d</td>
<td>CpG</td>
<td>43 (28-&gt;95)</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>e</td>
<td>non-CpG</td>
<td>13 (11-18)</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>f</td>
<td>CpG + IFA</td>
<td>42 (24-&gt;250)</td>
<td>27</td>
<td>11</td>
</tr>
<tr>
<td>g</td>
<td>non-CpG + IFA</td>
<td>23 (14-&gt;250)</td>
<td>18</td>
<td>13</td>
</tr>
</tbody>
</table>

GVHD indicates graft-versus-host disease; LAK, lymphocyte killer; IFA, incomplete Freund's adjuvant; CpG, CpG motif.

Sublethally irradiated (5.5 Gy) (BALB/C x C57 Bl/6) F1 mice were inoculated i.v. with 5 x 10^4 4T1 tumor cells 1 day following irradiation. Twenty-four hours later, naive, CFA, CpG, or CpG + IFA-treated C57BL/6 donor splenocytes (3 x 10^6) were inoculated i.v. Unless otherwise noted, all donor treatments were given s.c. 10 days before spleen harvesting. CpG and non-CpG were given to donor mice 6 days before harvesting. Data were collected from 5 experiments for groups a, b, and c, 1 experiment for groups d and e, and 3 experiments for groups f and g. P < .001 for the comparison of control of tumor only versus donor pretreatment with CFA, CpG, or CpG + IFA. P > .05 for the comparison of CpG versus CpG + IFA and for the comparison of CpG versus non-CpG and P > .05 for the comparison of CpG versus IFA versus non-CpG + IFA.

The Effect of CpG-Treated Donor Splenocytes on GVL

Spleen cells from naive or CpG-treated C57 mice were injected into sublethally irradiated F1 mice that were inoculated with BCL1 cells (Figure 4). Almost all mice inoculated with naive splenocytes died of severe GVHD in a median of 19 days, whereas CpG pretreated splenocytes significantly prolonged survival up to a median of 64 days. It is worth noting that most of these F1 host mice died of late GVHD (10 of 13) and only 3 of 13 died of leukemia (Figure 4A). C57 splenocytes from CpG + IFA-treated mice exerted a much

Table 5. Effect of LAK Cells Derived from CpG-Treated Donors on Leukemia Cells (BCL1) in Adoptive Transfer (AT) Experiments

<table>
<thead>
<tr>
<th>LAK</th>
<th>Donor Pretreatment</th>
<th>Survival (Days)</th>
<th>Tumor-Related Deaths</th>
<th>Disease-Free Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>--</td>
<td>--</td>
<td>31 (28-&gt;63)</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>+</td>
<td>--</td>
<td>&gt;200</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>+</td>
<td>CpG + IFA</td>
<td>&gt;200</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>+</td>
<td>non-CpG + IFA</td>
<td>&gt;200</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

LAK indicates lymphocyte killer; IFA, incomplete Freund’s adjuvant; CpG, CpG motif.

The 10^6 splenocytes were adoptively transferred into naive secondary BALB hosts 14 days following BCL1 inoculation into sublethally irradiated (5.5 Gy) BALB x C57 F1 mice that were treated with C57 LAK cells derived from either naive or CpG + IFA pretreated donor mice. CpG + IFA and non-CpG + IFA were inoculated s.c. 10 days before spleen harvesting. Data represent 1 experiment. P < .001 for the comparison of each experimental group versus group of mice inoculated with BCL1 only without cell therapy.
more significant GVL effect and prolonged survival time up to a median of 166 days, with 12 of 26 disease-free F1 host mice surviving for 200 days following BCL1 inoculation. Only 3 of 26 F1 host mice died of late leukemia (between days 101 and 122), 4 of 26 died of GVHD between days 14 and 49, and 7 of 26 mice died of late GVHD (between days 66 and 172) in this experimental group (Figure 4B). In 6 of 6 mice taken as a sample from the 12 of 26 DFS mice, >95% C57 donor cells were present on day 100 following cell inoculation (data not shown).

In an attempt to overcome the late GVHD-related death observed in BCL1 inoculated mice, we designed another protocol to achieve more efficient removal of BCL1 cells while ensuring that the presence of the BCL1 cells would not neutralize the benefit of pretransplant donor treatment with CpG + IFA. Accordingly, mice inoculated with a lethal dose of BCL1 cells were treated with splenocytes from C57 donor mice pretreated with CpG + IFA or with its control of non-CpG+IFA. Eight days later, mice were injected with C57 naive T cells plus TCD cell fraction of splenocytes derived from C57 donors pretreated 10 days previously with either CpG + IFA or with its control non-CpG + IFA. As seen in Figure 5, the addition of naive T cells + TCD splenocytes did not allow leukemia to develop, but 5 of 9 mice died of late GVHD (between days 62 and 146). The remaining 4 of 9 mice were DFS for >200 days with full donor chimerism (data not shown). Control groups of mice treated with C57 naive splenocytes only, or inoculated with 2 treatments of non-CpG + IFA derived cells, died of acute GVHD (aGVHD) in a median of 16 and 23 days, respectively (Figure 5). The addition of a second cycle of cell therapy consisting of naive T cells + TCD fraction following CpG + IFA treatment was not statistically different (P < .05) from 1 cycle of treatment with whole spleen cells following CpG + IFA. Overall, our results show the efficacy of CpG + IFA donor treatment in inducing a GVL response, and the ability to maintain a large part of the experimental group GVHD- and leukemia-free for a long time.

**DISCUSSION**

Pretransplant donor treatment with immunomodulators that enables efficient prevention of GVHD was tested for its ability to exert GVL/GVT effects.

Donor treatment with CpG + IFA efficiently prevented GVHD induced by parental LAK cells inoculated into sublethally irradiated F1 mice. A detailed phenotypic analysis of splenocytes derived from mice

---

**Table 6. Effect of Pretransplant Donor Treatment with CFA on Leukemia Cells (BCL1) in Adoptive Transfer (AT) Experiments**

<table>
<thead>
<tr>
<th>Splenocytes</th>
<th>Day of AT after BCL1 Inoculation</th>
<th>Survival (Days)</th>
<th>Tumor-Related Death</th>
<th>Disease-Free Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57 + CFA</td>
<td>14</td>
<td>12</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>C57 + +</td>
<td>14</td>
<td>&gt;230</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>C57 + +</td>
<td>14</td>
<td>&gt;230</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>C57 + +</td>
<td>21</td>
<td>24 (16-48)</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>C57 + +</td>
<td>21</td>
<td>&gt;230</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>C57 + +</td>
<td>21</td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

CFA indicates complete Freund’s adjuvant; GVHD, graft-versus-host disease.

The 10⁵ splenocytes were adoptively transferred into naive secondary BALB hosts 14 or 21 days following BCL1 (10⁴) inoculation into sublethally (5.5 Gy) irradiated (BALB × C57) F1 mice that were treated with naive or CFA pretreated C57 splenocytes. CFA was inoculated s.c. into C57 donor mice (200 μL), 10 days before spleen harvesting. P < .001 for the comparison of each experimental group versus group of mice inoculated with BCL1 only without cell therapy. Data represent 1 experiment.

*A = mice treated with naive C57 splenocytes died of acute GVHD before the AT experiment on day 21.

---

**Figure 4. Effect of Splenocytes from CpG-Treated Donors on GVL.** Sublethally (5.5 Gy) irradiated (BALB × C57) F1 mice were inoculated with 10⁴ BCL1 cells 24 hours following irradiation. On the day after, mice were injected i.v. with 30 × 10⁶ C57 splenocytes derived from naive mice, or from mice pretreated 6 days previously with CpG, or non-CpG (A); or from mice pretreated 10 days previously with CpG + IFA or non-CpG + IFA (B). All pretreatments were carried out by s.c. inoculation (100 μg). Data were pooled from 2 experiments. P < .001 for the comparison of treatment with CpG versus non-CpG, for the comparison of CpG + IFA versus non-CpG+IFA and for the comparison of CpG + IFA versus CpG.
treated with various immunomodulators and their effect on GVHD induction across semiallogeneic or fully mismatched allogeneic barriers, was previously reported by us [15,16]. Inoculation of splenocytes or LAK cells following CpG + IFA treatment resulted in efficient engraftment, as evidenced by the high percentage of donor cells present in peripheral blood samples taken >80 days following cell inoculation. This rules out the possibility that the absence of GVHD is because of donor cell rejection. Although pretransplant donor treatment with CpG alone prevented splenocyte-induced GVHD in 89% of the mice [15], it did not prevent severe GVHD induced by LAK cells. This might be related to our recent reports [16] showing the higher enrichment of myelogenous CD11b+Gr-1+ suppressor cells achieved following treatment of CpG + IFA compared to donor treatment with CpG alone. These CD11b+Gr-1+ cells were elevated as well following CFA treatment [15]. It is worth noting that following IL-2 activation of CpG + IFA pretreated splenocytes (LAK), there was a reduction in these myelogenous cells accompanied by an increase in CD3+, CD8+ CD16+, and NK1.1+ cells. These enriched cell subsets were previously identified by others in naïve LAK cells [24].

The phenotypic changes incurred can explain their reduced protective effect against GVHD induction following inoculation of CpG + IFA-treated LAK cells (our current manuscript) versus inoculation of CpG + IFA-treated splenocytes [15]. Because no changes in CD4+CD25+ or CD3+CD56+ cells were observed following CpG + IFA treatment, immunomodulation by CpG + IFA treatment could not be ascribed to these T regulatory or NKT cells [15].

To test the effect of pretransplant donor treatment on the induction of GVL and GVT responses, alloCT was carried out in mice inoculated with BCL1 or 4T1 cells, respectively. The GVL effect was tested by means of AT experiments carried out 14 days following leukemia (BCL1) inoculation. No evidence of residual leukemic cells was detected in the secondary hosts following injection of spleen or LAK donor cells derived from naïve mice or mice pretreated with immunomodulators. The advantage of pretransplant donor treatment with immunomodulators became apparent when AT was carried out on day 21; at that time, none of the mice treated with naïve splenocytes were alive and available for AT, whereas spleen cells from CFA-treated donor mice were available for transfer and did not cause leukemia in any of the naïve secondary hosts for >200 days of follow-up. Despite the long-lasting efficacy of pretransplant donor treatment to prevent GVHD in mice that were not inoculated with tumor cells, in the presence of BCL1, a number of the primary host mice developed late GVHD. In contrast to many malignant cells, BCL1 cells express major histocompatibility (MHC) class I and II antigens on their surface as well as the costimulatory B-7 molecule, which is critical for the induction of an immune reaction [25,26]. Residual BCL1 cells that may have survived the aGVHD while not causing leukemia in the primary hosts, may have served as antigen-presenting cells (APCs), which present host MHC alloantigens to donor cells, thereby leading to the development of late GVHD when the myelogenous suppressor cells from the CpG + IFA-treated donor no longer exist to exert their suppressive activity. In accordance with this, it is worth noting that BCL1 cells, unlike other malignant B cells, are good stimulators in mixed lymphocyte reactions (MLRs) in vitro (data not shown) probably because of the expression of the important molecules necessary for efficient T cell activation. We attempted to prevent late GVHD by removing leukemia cells more efficiently with a second cycle of cell therapy: naïve T cells given together with myelogenous suppressor cells (TCD fraction derived from CpG + IFA-treated donors), were administered a week after the first dose of alloCT. Unfortunately, late GVHD still developed in some of the mice, which suggests that the second cycle of cell therapy, consisting of the naïve T cells + TCD fraction, should be given after a longer time lapse.
Although no apparent GVT effect was achieved following CpG + IFA donor treatment, the splenocytes had a slight advantage over the LAK-derived cells. This might be because of the different cell subset population identified by phenotypic analysis, longer survival of the splenocytes exerting the GVT effect, as well as the possibility that the GVT effect required a specific effector mechanism as opposed to the non-specific killing effect provided by the LAK cells.

The ability to achieve a GVL effect with no apparent GVT effect might be explained by the fact that leukemia cells (eg, BCL1) circulate systemically and are therefore more easily accessible to donor cells than locally residing solid tumors (eg, 4T1 cells). The difference might also be related to the type and/or source of the targeted malignant cells, for example, hematopoietic cells versus cells of epithelial origin. The GVL effect was not a result of direct cytotoxicity, as can be seen from the significantly reduced cytotoxic activity of CpG + IFA-treated LAK cells against leukemic cells (3B3), compared to their unchanged cytotoxicity against 4T1 cells. Pretransplant donor treatment with immunomodulators significantly reduced the number of mice that died of GVHD compared to naïve or non-CpG-treated donors, but the majority of mice inoculated with 4T1 cells died of tumor, as opposed to BCL1 inoculated mice that died mainly of late GVHD. The discrepancy between GVL and GVT effects might possibly reflect the differential capability of an effector mechanism, which is able to prevent leukemia development, but is not activated by malignant epithelial cells. Generally, and in accordance with our observations, donor lymphocyte infusion (DLI) and HSCT aiming to exert a graft-versus-malignancy effect, have proved to be a treatment of choice for a number of hematologic malignancies, whereas there have been fewer reported cases of solid tumors in experimental models and clinical trials [14,27-33]. The disparity in the efficacy of alloCT directed against hematopoietic malignant cells versus carcinoma cells is confusing, and needs further intensive investigation.

CpG has been administered as an adjuvant to stimulate a Th1-type response [34], but it has also been able to mediate an anti-inflammatory response and to confer protection from arthritis through a T cell-independent increased level of interferon (IFN)-γ [35].

A similar model of action might play a role in the CpG immunomodulatory effect observed in our experiments, as shown by the elevated IFN-γ level detected following CpG + IFA treatment [16].

Pretransplant donor treatment with CpG + IFA was more effective than treatment with CFA for induction of long-lasting GVL effects. This finding gives our strategy of pretransplant donor treatment with immunomodulators a legitimate rationale for future clinical application. Replacing the mycobacterium component in the CFA with CpG allows for safer use and avoidance of the toxic granulomatous reaction that occurs following injection of this adjuvant [36].

Previously, we reported and discussed the advantage of pretransplant donor treatment over host treatment in preventing GVHD [15,16]. Now, we have tested the antileukemia response in our strategy of pretransplant donor treatment in inducing the antileukemia effect. Indeed, we have demonstrated that regulation of allogeneic response in GVHD, while sparing a GVL effect, could be achieved in a significant number of mice inoculated with pretransplant-treated donor splenocytes. The ability to achieve a GVL effect without GVHD was especially obvious in a significant number of mice for at least 100 days following alloCT.

We could not ascribe an essential role to NK cytotoxicity in our experimental model, and the major effect of pretransplant donor treatment with CpG in our study was the enrichment of the CD11bGR-1 cells, which successfully control an allogeneic reaction in the host within a narrow time window, when only a few leukemic cells are present. Similar to our previous [15,16] and current findings, a recent study showed that the CpG immunoregulatory activity was not mediated via NK cells but rather by activation of APCs, especially CD11bGR-1 cells, when CpG was given to the host in a murine bone marrow transplantation model [37]. The allogeneic reaction, ensued under the CpG immunomodulatory activity, facilitated the prevention of severe systemic GVHD on 1 hand and simultaneously inhibited leukemia cell propagation efficiently in a state of minimal leukemia disease. Because of the controversial issue of whether GVL/GVT effects can be achieved without concomitant induction of GVHD and the fact that a clean separation of GVL effects from GVHD has been achieved only in a minority of experimental models [8,12,38-41], our findings are of crucial importance. This should encourage further attempts to strive toward more effective cell therapy by alloreactive lymphocytes to improve the outcome of allogeneic stem cell transplantation in patients with hematologic malignancies.

ACKNOWLEDGMENTS

Financial disclosure: The authors wish to thank the Danny Cunniff Leukemia Research Laboratory for its continuous support of our ongoing basic and clinical research.

REFERENCES

2. Sullivan KM, Weiden PL, Storb S, et al. Influence of acute and chronic graft-versus-host disease on relapse and survival after bone marrow transplantation from HLA-identical siblings as