Introduction

Hematopoietic stem cell transplantation (HSCT) is a potentially curative therapy for many hematological malignancies [1-3]. Successful leukemia treatment by different methods of HSCT is associated with the conversion of recipient into donor-type hematopoietic chimeras [3-6]. Donor lymphocyte infusions (DLI) are frequently used after HSCT for prevention and treatment of leukemia relapse. DLI may induce both cytogenetic and molecular remission of relapsed leukemia, but the risk of graft-versus-host disease (GVHD) has to be considered [7, 8].

In animal models graft-versus-leukemia (GVL) effects of DLI are relatively strong in mixed hematopoietic chimeras (MC), but decline in full hematopoietic chimeras (FC) [1, 3, 4]. Experimental imitation of leukemia relapse in FC revealed that neither resident donor lymphocytes (DL), nor additional DL infused as DLI were able to stop the disease [9-11].

Studies of several clinically relevant murine transplantation models showed that host anti-
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Gen presenting cells (APC) and host alloantigen expression on malignant cells play a predominant role in evoking GVL responses from the donor T cells contained in DLI [12-14]. Therefore it was postulated that the DLI-mediated anti-leukemia effect would be extinguished over time because host APC would have been replaced by donor APC following the conversion of MC into FC [4, 15].

In order to test this assumption we used for DLI in this study a mixture of donor and host or donor and third party type splenocytes. Such cell mixtures contained both donor and host or donor and third party lymphocytes and APC. Utilizing splenocytes labeled by a fluorescent dye we examined immune status of chimeras in vivo according to their ability to generate a cytotoxic response against host or third party lymphocytes co-transplanted with donor cells.

We revealed that DLI-treated FC exposed to host transplantation antigens and host APC reject host hematopoietic cells significantly slower than third party targets. The efficiency of immune response in FC was in both cases significantly lower than in naive mice. These results show that cytotoxic response is deficient in FC even when host APC are available.

Materials and methods

Animals

Inbred C57BL/6 (B6; H-2b), BALB/c (H2d), C3H/hej (C3H; H2k) and (C57BL/6 x BALB/c) F1 (F1) female mice were purchased from Harlan Laboratories (Ein Kerem, Israel). The two month old mice used in the study were kept under standard animal house conditions, and fed with mouse chow and water ad libitum. All experiments were carried out in accordance with the requirements of the National Committee for Animal Protection Rights.

Chimerism induction

Full allogeneic chimeras (B6 → BALB/c or BALB/c → B6) were established by our method of bone marrow transplantation (BMT) (16). Briefly, 3-24 hours after low dose total body irradiation (150 – 300cGr) recipient mice were intravenously (IV) injected with 3x10⁷ donor bone marrow (BM) cells. Twenty-four hours later they received an intraperitoneal (IP) injection of 100 mg/kg cyclophosphamide (CY, Baxter Oncology, Frankfurt, Germany) for selective depletion of recipient's donor-activated lymphocytes. A second dose of donor BM cells (3x10⁷) was given to recipients IV one day after CY administration in order to convert them into full allogeneic chimeras (FC).

Flow cytometric analysis and assessment of chimerism

Blood lymphocytes were isolated on a Histopaque gradient of 1.087 g/ml density (Sigma-Aldrich, St. Louis, MO, USA), depleted of remaining red blood cells by hypotonic lysis buffer (Biological Industries, Beit Haemek, Israel), stained with allophycocyanin-labeled anti-H2b antibodies (BioLegend, San Diego, CA, USA) and analyzed by fluorescence-activated cell sorting (FACS) analysis using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Mice confirmed by FACS analysis to be FC were used in experiments 2-6 months after BM transplantation.

In vivo NK cell depletion

FC were injected IV with 60 µg of rabbit anti-asialo GM1 antibodies (Cedarlane, Burlington, Ontario, Canada) diluted in 0.2 ml of saline 7, 4 and 1 days before the experiment. This NK cell depletion scheme was found optimal on the basis of flow cytometry analysis. Staining splenocytes of the treated and control mice with APC-conjugated anti-NK1.1 (clone PK136) and FITC-conjugated anti-Ly49D antibodies (clone 4E5) revealed that this treatment reduced the percentage of NK1.1+DX5+ cells from 0.8%+0.1 to 0.1%+0.1. NK1.1+DX5+ cell deficiency remained for at least 10 days. Staining with APC-conjugated anti-mouse CD4 (clone GK1.5) or anti-CD8 (clone 53-6.7) monoclonal antibodies (mAbs) revealed that this NK depletion protocol had no effect on percentage of CD4+ and CD8+ cells in the spleen. All antibodies were obtained from eBioscience (San Diego, CA, USA).

In vivo T cell depletion

BALB/c mice were given 25µg of anti-CD90.2 (Thy 1.2) mAb (clone 30-H12, BioLegend, San Diego, CA, USA) diluted in 0.2 ml of saline 4 and 2 days before sacrifice for cell transfer. Flow cytometry analysis with the same mAb conjugated to FITC revealed that this treatment re-
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Produced percentage of CD3ε+ cells in spleen 10 times.

In vivo immune response test

Splenocytes were incubated with 5mM carboxyfluorescein diacetate succinimydil ester (CFDA-SE, Molecular Probes, Invitrogen, Eugene, Oregon, USA) for 20 min, at room temperature. Fetal calf serum (final concentration 20%) was then added to stop the reaction. After washing, two kinds of CFDA-SE labeled cells (donor and recipient, or donor and third party) were mixed in a 1:1 ratio and injected IV to naïve B6 mice (4\times10^7 cells of each type). Mice were sacrificed at different time points (1, 3, 7, 10, or 14 days after injection), spleen lymphocytes suspensions were prepared, depleted of red blood cells, stained with allophycocyanin-labeled anti-H2β antibodies (BioLegend, San Diego, CA, USA), and analyzed using a FACS analyzer. The immune response was assessed according to the percentage of single positive CFDA-SE+/H2β- cells of all CFDA-SE+ cells (Figure 1).

Figure 1. A method for visualization of immune response in vivo. Fresh isolated B6(H-2b) and BALB/c(H-2d) splenocytes were labeled with 5mM carboxyfluorescein diacetate succinimydil ester (CFDA-SE), mixed in 1:1 ratio and injected IV to naïve B6 mice (4\times10^7 cells of each type). Animals were sacrificed 1 or 3 days after the cell injection, their spleen lymphocytes were stained with APC-labeled anti-H2β antibodies, and analyzed by FACS. The immune response was assessed according to the changing ratio between two types of CFDA-SE-labeled cells: CFDA-SE+ and H2β+B6 cells (upper right square) and CFDA-SE+ and H2β+ BALB/c cells (lower right square). Both types of CFDA-SE+ cells were present in the spleen on day +1 after infusion of the cell mixture. On day +3 injected BALB/c cells (CFDA-SE+ H2β+) were rejected by the recipient (lower right square is almost empty).

Statistical analysis

Results were analyzed according to the Student’s unpaired t-test.

Results

1. Analysis of anti-host immune response in FC after DLI

Our previous studies and the results of others showed that animals cured of leukemia by allogeneic BMT usually converted into FC. They remained disease-free for a prolonged time period, but were vulnerable to a new challenge of leukemia cells, and could not be saved by DLI (15–17). Therefore in our first experiment we checked whether FC could produce an immune response to host antigens after DLI, or whether they remained completely tolerant to the host. For this purpose we injected B6 → BALB/c FC, BALB/c → B6 FC and control naïve BALB/c and B6 mice with a mixture of CFDA-SE-labeled splenocytes of donor and recipient origin and followed the change in donor/recipient labeled cell ratio over time. The results presented in Figure 2 show that the naïve mice rejected the allogeneic component of the mixture completely within 3 days. FC also rejected the host component of the mixture. However, the rejection time of host lymphocytes in FC was noticeably long (7 – 10 days). These results show that FC exposed to host hematopoietic cells and APC developed an anti-host immune response after DLI. Our data also show how important a role chimerism plays in support of naïve donor cells infused after tolerance formation: while B6 → BALB/c chimeras rejected BALB/c splenocytes, BALB/c → B6 chimeras rejected B6 lymphocytes from the same mixtures.

2. Specific deficiency of anti-host immune response in FC

The aim of our next experiment was to ascertain whether slower rejection of host lymphocytes after donor and host cell infusion to FC was due to general immune suppression or specific deficiency of anti-host immune response. B6 → BALB/c chimeras and naïve B6 mice were injected with a mixture of CFDA SE-labeled spleen cells from B6 and C3H mice. Figure 3 shows that in B6 → BALB/c chimeras the C3H component of the mixture was rejected significantly faster than the BALB/c component. Vigorous rejection of third party hematopoietic cells by FC
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is in accordance with our previously published data (18) demonstrating that GVHD-free B6→BALB/c chimeras, BALB/c→B6 chimeras, and naïve BALB/c and B6 control mice were injected with a mixture of CFDA-SE-labeled splenocytes of donor and recipient origin (4x10^7 cells of each kind). Several animals in each group (5-7) were sacrificed at each time point, and the percentage of host type cells of all CFDA-SE^+ splenocytes was evaluated by FACS analysis. The results show rejection of the BALB/c component (CFDA-SE^+ H2^b-) by B6→BALB/c chimeras and naïve B6 mice, and rejection of the B6 component (CFDA-SE^+ H2^b+) by BALB/c→B6 chimeras and naïve BALB/c mice.

3. Analysis of the impact of resident immunocompetent donor cells and donor cells provided by DLI on the anti-host immune response of FC

In the experiments described above mice were injected with a mixture of two types of immunocompetent cells which were able to react to each other because they were derived from naïve animals. Thus, rejection of host or third party hematopoietic cells in FC resulted from a cumulative anti-host or anti-third party response of resident immunocompetent donor cells in FC, and from immunocompetent donor type cells provided by DLI. To identify the role of resident donor lymphocytes and DLI-derived donor lymphocytes from naïve donor type mice we "canceled" the influence of the DLI-derived donor cells. For this purpose we injected B6→BALB/c FC with a mixture of CFDA-SE-labeled splenocytes from naïve B6 mice and B6→BALB/c FC. Another group of FC was injected with a mixture of CFDA-SE-labeled splenocytes from naïve B6→BALB/c FC and C3H mice. The immune response of these two groups of FC was compared to the immune response of FC injected with the mixtures containing naïve donor cells (Figures 4A and 4B). We noted no difference in the anti-host immune response generated by resident and naïve donor lymphocytes soon after transplantation (Figure 4A, days 1 and 3). However, later (days 7-14), the kinetics of host cell rejection was significantly slower in FC that had received CFDA-SE-labeled donor component of the mix-
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Figure 4 Impact of the resident and DLI-derived donor immunocompetent cells on the anti-host immune response of full chimeras. A. Full B6 → BALB/c chimeras and naïve B6 mice were injected with a mixture of CFDA-SE labeled splenocytes derived from naïve B6 and BALB/c mice, and another group of B6 → BALB/c full chimeras was injected with a mixture of CFDA-SE labeled splenocytes derived from full B6 → BALB/c chimeras and from naïve BALB/c mice. Several animals in each group (5-7) were sacrificed at each time point, and the percentage of labeled host type cells (CFDA-SE+, H-2b+) of all CFDA-SE+ cells was evaluated. Although the kinetics of host cell rejection was significantly slower in full chimeras that received donor cell component from similar chimeras, they were able to reject most CFDA-SE labeled host (BALB/c) lymphocytes. B. Full B6 → BALB/c chimeras and naïve B6 mice were injected with a mixture of CFDA-SE labeled splenocytes derived from naïve B6 and C3H mice, and another group of B6 → BALB/c FC was injected with a mixture of CFDA-SE labeled splenocytes derived from B6 → BALB/c FC and from naïve C3H mice. Several animals in each group (5-7) were sacrificed at each time point and the percentage of labeled C3H cells (CFDA-SE+, H-2b+) of all CFDA-SE+ cells was evaluated by FACS analysis. The kinetics of third party (C3H) hematopoietic graft rejection was similar in full chimeras transplanted with donor splenocytes derived from naïve mice and from B6→BALB/c full chimeras.

4. Analysis of the impact of resident NK cells on the anti-host immune response of FC

In order to understand the role that NK cells play in rejection of host-type lymphocytes we compared survival of T-cell depleted BALB/c lymphocytes in B6→BALB/c stable full chimeras, similar chimeras depleted of NK cells, and both allogeneic components were rejected within 3 days.
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In the first experiment CFDA-SE labeled T-cell depleted BALB/c splenocytes and splenocytes obtained from NK-cell depleted B6→BALB/c stable FC were co-transplanted into similar FC treated or untreated to deplete NK+ cells. The results presented in Figure 5A show that in both groups recipients rejected most host type splenocytes within 7 days.

In the second experiment we transplanted CFDA-SE labeled mixture of F1 and T-cell depleted BALB/c splenocytes into naïve F1 recipients. In this case both donor cell mixture and recipients were completely deprived of BALB/c (H-2\(^d\)) specific T cell clones but possessed relevant NK cells. The results presented in Figure 5B demonstrate prolonged survival of CFDA-SE labeled BALB/c graft in F1 mice. This result indicates that the role of NK cells in BALB/c lymphocytes rejection is modest in this line combination.

Thus, based on the observations presented above, we consider that residual donor-specific T cell clones in FC play a major role in rejection of host lymphocytes.
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Discussion

The aim of our work was to analyze the kinetics of anti-host immune response of FC and DLI treated FC provided with host hematopoietic and antigen-presenting cells. Our experimental model was based on an existing clinical challenge of ineffective immunotherapy for leukemia relapse after successful HSCT in those recipients who initially became disease-free FC [3-6]. Using an in vivo cytotoxicity visualization test we demonstrated that 2-6 months after allogeneic BMT, FC remain both generally deficient in their ability to respond against transplantation antigens expressed on hematopoietic cells and specifically deficient in their ability to react against host hematopoietic cells. Nevertheless, some host-reactive clones remain in FC. Due to their presence in clinically GVHD-free FC, the animals persistently maintained full hematopoietic chimerism and were able to reject newly transplanted host lymphocytes. These results confirm other experimental data demonstrating that host hematopoietic cell infusions can not break transplantation tolerance in FC generated by transplantation of non-T-cell depleted allogeneic bone marrow [19].

The kinetics of host hematopoietic cell rejection in FC was accelerated by infusion of DLI-derived donor T cells. DLI-induced facilitation of anti-host response denote against major involvement of regulatory T cells in the suppression of anti-host immunity in FC. No evidence of antigen-specific T regulatory cells was found also in long-term FC by the others [15]. Naïve donor cells engrafted in FC after DLI comprise only a small portion of hematopoietic compartment of FC. Therefore even after DLI, the cumulative anti-host response of FC remained relatively slow (about 1/4 of injected CFDA-SE labeled host cells stayed in FC for 7 days after donor and host splenocyte infusion). Naïve donor type mice, which can be regarded as a standard for resistance to allogeneic lymphocyte or leukemia cell engraftment, rejected the same amount of host cells within 3 days. The analysis of BCL1 leukemia cell growth after IV injection into BALB/c recipients revealed that BCL1 leukemia blasts doubling time is about 3 days [20]. In context of these data it is evident that full chimeras in contrast to donor-type mice are capable of rejecting only small amounts of leukemia blasts.

It is difficult to relate the slow rejection of host lymphocytes in FC to insufficient numbers of host APC transferred within the host and donor splenocyte cell mixture, because FC were able to reject the same amount of third party splenocytes much faster. No third party CFDA-SE labeled cells remained in FC 7 days after co-infusion with splenocytes of FC.

We demonstrated that although naïve donor lymphocytes facilitated host component rejection, they had no influence on the kinetics of third party cell elimination. Evidently, the contribution of DLI donor lymphocytes to the response against third party targets was masked by a more significant response of resident donor cells.

Depletion of NK cells both from donor and recipient chimeras had no considerable influence on kinetics of recipient splenocyte rejection. Moreover, prolonged persistence of T-cell depleted BALB/c splenocytes in F1 recipients proves that in the absence of host-specific T cell clones donor NK cells have minimal effect on host cell lymphocyte survival (at least in line combination used in the study).

Our previous experiments revealed that B6 BM transplant used to convert leukemia bearing BALB/c recipients into B6—BALB/c FC could eliminate the disease in 100% recipients [17] though the graft contained ten times less T cells than B6 DLI splenocyte graft used in this study. This comparison definitely demonstrates that environment plays a significant role in immune response intensity. Donor anti-host response soon after transplantation is vigorous due both to the abundance of host targets and to the post-conditioning lymphopenic drive to hematopoietic cell proliferation.

In conclusion, visualization of the degree of cytotoxic immune response in vivo demonstrated that clinically GVHD-free FC are able to reject hematopoietic host cells. This process is relatively slow due to significant deficiency in host-specific T cell clones in GVHD-free FC. It can be accelerated by infusion of the immunocompetent donor lymphocytes obtained from naive mice. However DLI is not efficient enough to protect FC against rapidly proliferating leukemia cells. Possible ways out of this "deadlock" may be transplantation of donor inoculate enriched with leukemia-reactive clones [21], or lessening the tumor burden prior to DLI by chemotherapy [22]. The method of the cytotoxicity
visualization used in this study may provide a quick preliminary validation of new DLI protocols for eradication of residual malignant cells after allogeneic stem cell transplantation.

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