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ORIGINAL ARTICLE

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Interleukin 2 regulation following semi-allogeneic bone marrow transplantation in mice

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Abstract Success of allogeneic and autologous bone marrow transplantation (BMT) is hampered by susceptibility to infection during the first two post-treatment years. Further, in treating malignant diseases, impaired anti-host reactivity for donor cells may contribute to a high rate of relapse. Both complications are a consequence of immune deficiency involving B and T lymphocytes. The present study evaluates several key parameters of the immunologic reconstitution mechanism in mice subjected to myeloablative total body irradiation following semi-allogeneic (parental) BMT. This resulted in a gradual reduction of splenic CD3, CD4 and CD8 cells until day 45 post-BMT. Concomitantly, there was an increase in monocytes and $CD4^+/CD8^+$ (double positive) cells, accompanied by a persistent elevation in the percentage of B lymphocytes. The total thymic and splenic T cell populations were reduced until day +30. The cellular reduction correlated with the poor proliferative response of the thymic and splenic cells. A decrease occurred in IL-2 mRNA expression in thymic cells during days 15–20 post-transplant, corresponding with the low level of IL-2 secretion in the spleen and thymus of the transplanted mice. In conclusion, following semi-allogeneic BMT, there was an overall immune down-regulation in the cells, gene and protein levels. Reduced immunological responsiveness following BMT reinforces the need for improving the immune dysfunction by immunotherapy post-BMT.

Keywords Interleukin 2 · Marrow transplantation · Immune function · Thymus · Spleen · Mice

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Introduction

Hematopoietic growth factors are known to accelerate hematopoiesis after high-dose myeloablative therapy [28, 23]. This is offset, however, by the slow kinetics of immunopoiesis, after allogeneic, and even autologous, bone marrow transplantation (BMT). The cause(s) of the tardiness of the immune reconstitution is not well understood, which implies that effective correction of the dysregulated immune system is not yet feasible [18, 10]. An important contributing factor to the immune deficient state post-allogeneic BMT is the development of graft-versus-host disease (GVHD) that is encountered in the majority of patients post-transplantation [28]. Immunosuppressive agents required for prevention and/or treatment of GVHD are contributing factors as well [26].

The risk of acute or chronic GVHD in recipients of HLA-identical and non-identical bone marrow (BM) transplant has been reduced by the use of T cell depletion (TCD) of the graft [2, 21]. However, the beneficial effects of complete TCD are counterbalanced by an increased rate of infections and relapse due to lack of adequate graft-versus-leukemia (GVL) reaction [12, 6, 25]. Attempts to overcome the risk of PAN T cell depletion using selective depletion of $CD8^+$ or $CD4^+$ T cells resulted in limited success [11, 15, 24]. Elucidation of the mechanisms of immune reconstitution of T cell-mediated functions may ultimately result in new strategies to improve the balance between GVL and GVHD after allogeneic BMT and post-transplant T cell-mediated immunotherapy. Investigations of the mechanisms

that underlie the GVL effect point towards the active role of $CD8^+$ and their cytokines [24, 27, 17].

Interleukin 2 (IL-2) is a major growth regulatory factor for T lymphocytes; its differential expression appears to be indicative of T cell differentiation and the type of immune effector function that develops in vivo. Transcription of the IL-2 gene is closely linked to receptor-mediated T cell activation, and it has a dual signal requirement. The first signal is provided by the T cell receptor (TCR) activation due to its interaction with the antigen-presenting cells in an MHC-dependent interaction, while the second signal is supplied by MHC-independent interaction embracing accessory molecules and/or cytokines [7, 19, 8].

In the present animal model, we define the role of IL-2 in generating alloreactive donor cytotoxic T cells after semi-allogeneic BMT for the treatment of leukemia. In this study, we aimed to establish the basic immunological incompetence after BMT, which will serve as platform for new therapeutical interventions to accelerate immune reconstitution.

Materials and methods

Animals

Female (BALB/c × C57BL/6) F1 ($H\text{-}2^{d/b}$) recipient (F1) mice (age 10–16 weeks) and male C57BL/6 ($H\text{-}2^{b/-b}$) donor mice (age 6–8 weeks) were purchased from the Hebrew University—Hadassah Medical School Mouse Colony. The mice received a regular diet and acidified water without antibiotics. All mice were maintained under standard conditions without isolation. Experiments were approved by the Institutional Ethical Committee for animal care.

Bone marrow transplantation

Bone marrow cells (BMC) were prepared by flushing RPMI 1640 medium (Beit Ha'emek, Israel) through the shafts of the femora and humeri of the donors, using a 25-gage needle. F1 (recipient) mice were conditioned with lethal total body irradiation (TBI) with 900 cGy using a Phillips X-ray apparatus (250 kV, 10 mA, 1 mm Cu filter) at a source-to-skin distance of 50 cm. BMC (10^7) plus spleen cells (2×10^6) in 0.25 ml medium were infused into the lateral tail vein of the recipients 24 h after termination of TBI.

Assay for chimerism

Chimerism was confirmed by typing F1 peripheral blood lymphocytes with B6 or BALB/c specific antisera, using in vitro complement-dependent microcytotoxicity assay with specific alloantisera and rabbit complement to determine the percentage of host or donor type cells. Specific alloantisera (BALB/c-anti-B6 and B6-anti-BALB/c) were prepared 6 and 12 weeks following inoculation of mice with 10^7 bone marrow cells plus spleen cells (2×10^6). Mice were bled and the sera stored at −70°C until used. Lymphocytes obtained from F1 recipients showed 100% cytotoxicity with both BALB/c-anti-B6 and B6-anti-BALB/c antisera, whereas lymphocytes obtained from chimeras were lysed by BALB/c-anti-B6 and not by B6-anti-BALB/c antisera. Percent chimerism was therefore calculated by the formula: % donor type B6 cells = cells lysed following treatment with BALB/c-anti-B6 antiserum, minus % cells lysed with B6-anti-BALB/c antiserum, minus % cells lysed with complement only.

Cell cultures

Spleen and thymus cells were recovered on days 3, 7, 14, 21, 35 and 42 post-BMT, and single suspensions of mononuclear cells (MNC) were prepared. Cells were washed three times and monocytes/macrophages were removed by adherence in incubation at 37°C. MNC were cultured with complete medium, which consisted of RPMI 1640 supplemented with glutamine (2 mM), penicillin (100 U/ml). Streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS) to final concentrations of 2×10^5 (spleen) and 1×10^6 (thymus) cells, 0.2 ml/well. Mitogens were added to final concentrations of concanavalin A (ConA; Miles-Yeda, Rehovot, Israel) 5 μg/ml and phytohemagglutinin (PHA; Difco Lab, Detroit, MI, USA) 2 μg/ml. Assays were performed in 0.2 ml flat-bottomed 96-well microtiter plates (Nunc, Denmark). Cell cultures were incubated at 37°C in a humidified air incubator with 5% CO_2 for 3 days. Samples were pulsed with 1 μCi [3H]–thymidine (Nuclear Research Center, Negev, Israel) for 26–18 h. Cultures were harvested by a multiple cell harvester, and [3H]–thymidine incorporation was measured using a Beckman scintillation counter. Assays were performed in triplicate.

RNA isolation, probes and hybridization

After BMT, untreated controls and transplanted mice were killed at the indicated time intervals. Thymus and spleen cells were prepared (as mentioned in cell culture) and activated with PHA, following which they were frozen and kept at −80°C until analyzed. Total cellular RNA was isolated from 1 to 5×10^6 thymus cells of normal and transplanted mice using RNAzol (Cinna/Biotex Labs, Friendwood, TX). A 2 μg sample of total RNA was applied to Zeta probe nylon membrane (Bio Rad) using a slot-blot apparatus. It is well accepted to isolate and demonstrate the acceptable profile of total RNA (i.e., 28s, 18s, and 4s RNA in comparable amounts); the quality of RNA was analyzed by running the sample on

agarose gel. Either IL-2 or human β-actin cDNA (25 ng) labeled with [^{32}P] dCTP was prepared using a random priming labeling kit (Boehringer Mannheim, Germany). The membranes were hybridized for 18 h at 65°C in 1.5-fold SSPE (0.15 M NaCl and 10 mM NaH_2PO_4). Media containing 10% polyethylene glycol, 7% sodium dodecyl sulfate (SDS), and 125 μg/ml of nonhomologous DNA were hybridized and washed twice with medium containing 0.3 NaCl plus 0.03 M sodium citrate (2-fold SSC) and 0.1% SDS (15 min at room temperature) and washed twice with 0.1-fold SSC and 0.1% SDS (15 min at 65°C). Semiquantitive analysis was performed using a Scan Maker 600%, Microtex connected to a Macintosh II fx. Densitometer. HUT 102 cells were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS. Total RNA was isolated from the cells, as described for the mouse thymus and spleen cells.

Determination of IL-2 production

The amount of IL-2 in the supernatants of spleen (1×10^6/ml) cells harvested at 24, 48 and 72 h following activation with monoclonal anti-murine CD_3 antibody was measured using IL-2-dependent murine CTL lines. Cells (1×10^6/ml) were added to 1 ml complete medium for incubation of 24, 48 and 72 h. The amount of IL-2 activity in cell-free supernatant was determined and calculated as described elsewhere [13].

Phenotype analysis by flow cytometry

Cell phenotype was determined by direct immunofluorescence using a fluorescent-activated cell sorter (FACstarplus, Becton-Dickinson Immunofluoremetry Systems, Mountain View, CA, USA). Cells were analyzed on the indicated days post-transplantation using the appropriate FITC-conjugated monoclonal anti-CD3, anti-CD4, anti-MAC-1 and anti-IgM (μ) mAbs, and the PE-conjugated anti-CD8 mAb (Pharmingen, USA). Cells (1×10^6 were incubated on ice for 30 min with a 1:500 dilution of the relevant antibody, washed and passed at an approximate rate of 1,000 cells/s through a 70-μm nozzle, using saline as the sheath fluid. A 488 nM argon laser beam at 250 Mw served as the light source for excitation. Green (FITC conjugated) fluorescence was measured by means of a 530 30-nM band filter (350 MT voltage and logarithmic amplification). Red (PE conjugated) fluorescence was measured using a 630 22 nM-band filter (525 PMT voltage and linear amplification).

Statistic

The student t test was used to assess the differences in cell number and proliferative response ($P < 0.05$ is significant).

Results

Chimerism

Following infusion of BMC, chimerism was detected at 6 weeks following transplantation. Circulating and spleen cells of all recipients were 95% and 100% of donor origin in 3 and 6 weeks, respectively, as confirmed by microcytotoxic assay.

Recipients did not have any signs of graft-versus-host disease (GVHD) like weight loss, humped position, although no post-grafting anti-GVHD prophylaxis was administered.

Proliferative response of T cells post-BMT

Evaluation of T cell dependent responses to mitogenic stimuli showed that splenic MNC obtained from BMT recipients was unresponsive to PHA 10 and 30 days following BMT as compared with normal spleen cells (Fig. 1a). Similar results were obtained with ConA and

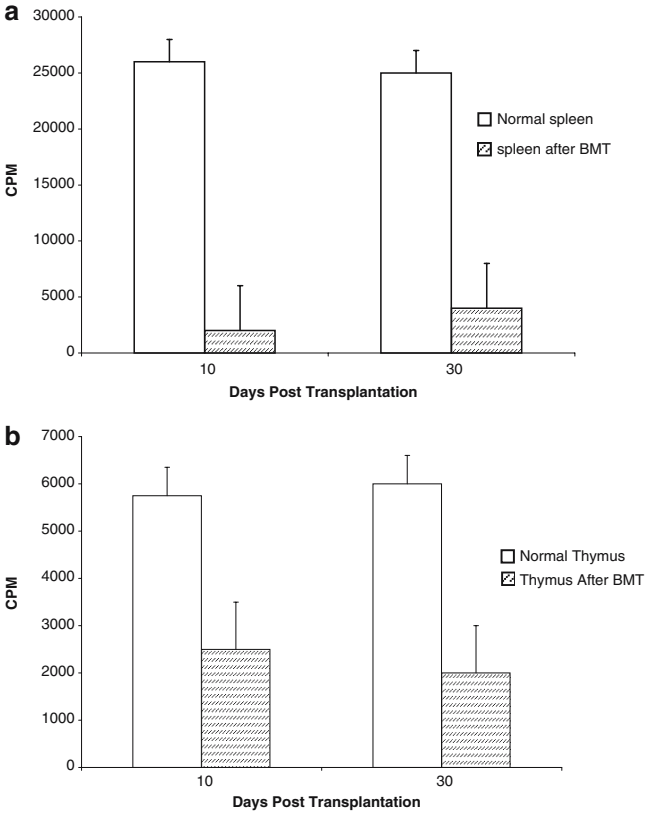

Fig. 1 T cell-dependent proliferation of splenic MNC following semi-allogeniec BMT. A total of 2×10^5 MNC spleen cells/well (**a**), and 1×10^6 thymocyte cells/well (**b**) from normal or transplanted mice were incubated with PHA, and [^{3}H]TdR incorporation was determined at the indicated days post-BMT, as described in Materials and methods. Each value represents the mean ± SD for three separate experiments

anti-CD3-induced proliferation (data not shown). Statistically significance results were obtained for thymic cells (Fig. 1b).

IL-2 mRNA expression in thymic and splenic cells

IL-2 mRNA levels in response to PHA activation were measured in spleen and thymic cells of normal and transplanted mice. On different days, post-transplantation total RNA was prepared from frozen cells at 0, 12, 24, 48, 72, 96 h after PHA activation. This experiment was repeated twice. The RNA preparations were analyzed by slot-blot hybridization with a specific probe for human IL-2. At peak levels, IL-2 mRNA in spleen cells of transplanted mice equaled the controls. In contrast, in the thymus we obtained a significant decrease in IL-2 mRNA levels ($P < 0.05$; Fig. 2). This unresponsiveness of the thymic cells to express the cytokine's mRNA was noted throughout the experimental period.

Secretion of IL-2

Since neither thymic nor splenic cells responded to PHA activation, the ability of these cells to produce IL-2 in vitro was investigated. Low levels of IL-2 were secreted in the culture supernatants ($P < 0.05$; Fig. 3). It should be mentioned that the proliferation of thymic and splenic cells was diminished in all transplanted mice post-BMT (Fig. 4).

Analysis of cell surface phenotype

Splenic evaluation Splenic cells from marrow recipient mice and controls were analyzed for their percentages of $CD3^+$, $CD4^+$, $CD8^+$ cells (Fig. 5a–c) to characterize T cell subtypes in the spleen after BMT, utilizing subset-specific monoclonal antibodies. A reduction in the percentage of all three cell groups was noted. In comparison, an increase in the $CD4^+/CD8^+$ (double positive) population occurred between days 5 and 45 post-BMT (Fig. 6). Percentage of monocytes increased until day +15, in comparison with normal levels, and somewhat decreased subsequently. The percentage of B cells in the spleen, which was initially low, increased until day +30 and reached normal levels (Fig. 7a, b, respectively). As is the case following myeloablative stem cell transplantation, there is an early post-transplant increase of the proportions of non-B non-T cells.

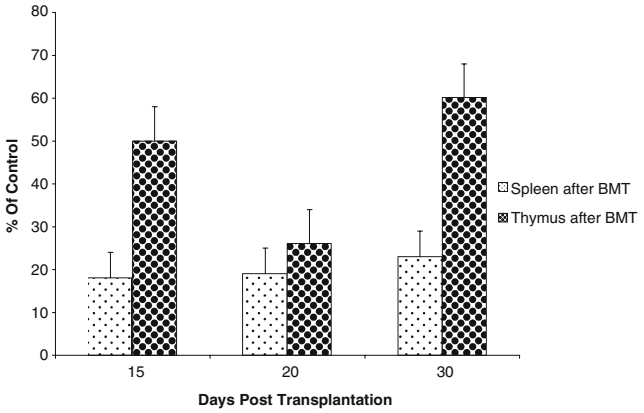

Fig. 3 IL-2 production by splenic mononuclear cells at different time intervals following semi-allogeneic BMT. MNC (1×10^6) were coated with monoclonal anti-CD3 antibodies and incubated in 24-well plates for 24, 48, and 72 h. The results of the 48 h incubation products are represented here. After 48 h, supernatants were collected and serially diluted and IL-2 activity was assayed using the IL-2 dependent CTL cell lines. Results represent 1 of 3 represented experiments

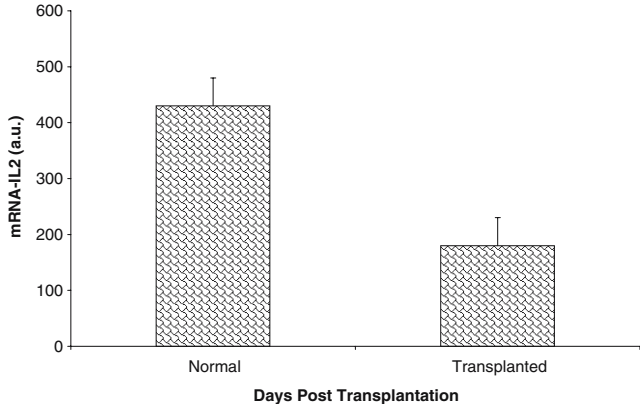

Fig. 2 IL-2 mRNA expression in normal and transplanted mice following PHA activation. Analysis as described in Materials and methods. Total RNA was isolated and subjected to slot-blot hybridization analysis as described in Materials and methods. IL-2 mRNA expression in normal thymus cells and in thymus transplanted mice. Peak level at day 30 post-transplant. Hybridization of slot blots with beta-acti probe demonstrated comparable amounts of RNA loaded in cell wells. Each value represents the mean ± SD for three separate experiments

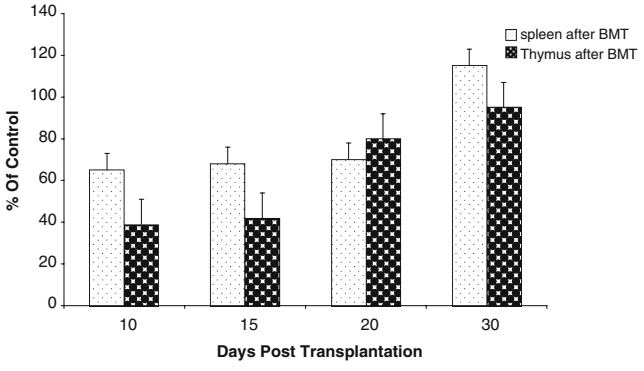

Fig. 4 Proliferation of thymic and splenic mononuclear cells following semi-allogeneic BMT. A total of 10^6 thymocytes/well, and 2×10^5 splenic cells/well were incubated with PHA at the indicated days post-BMT, and [^{3}H]TdR incorporation was determined in parallel with [^{3}H]TdR incorporation by normal thymocytes (8000 ± 1200 cpm). Each value represents the mean ± SD of the ration of [^{3}H]TdR of experimental versus normal thymocytes for three separate experiments. Similar results were obtained with ConA and monoclonal anti-CD3 antibodies (data not shown)

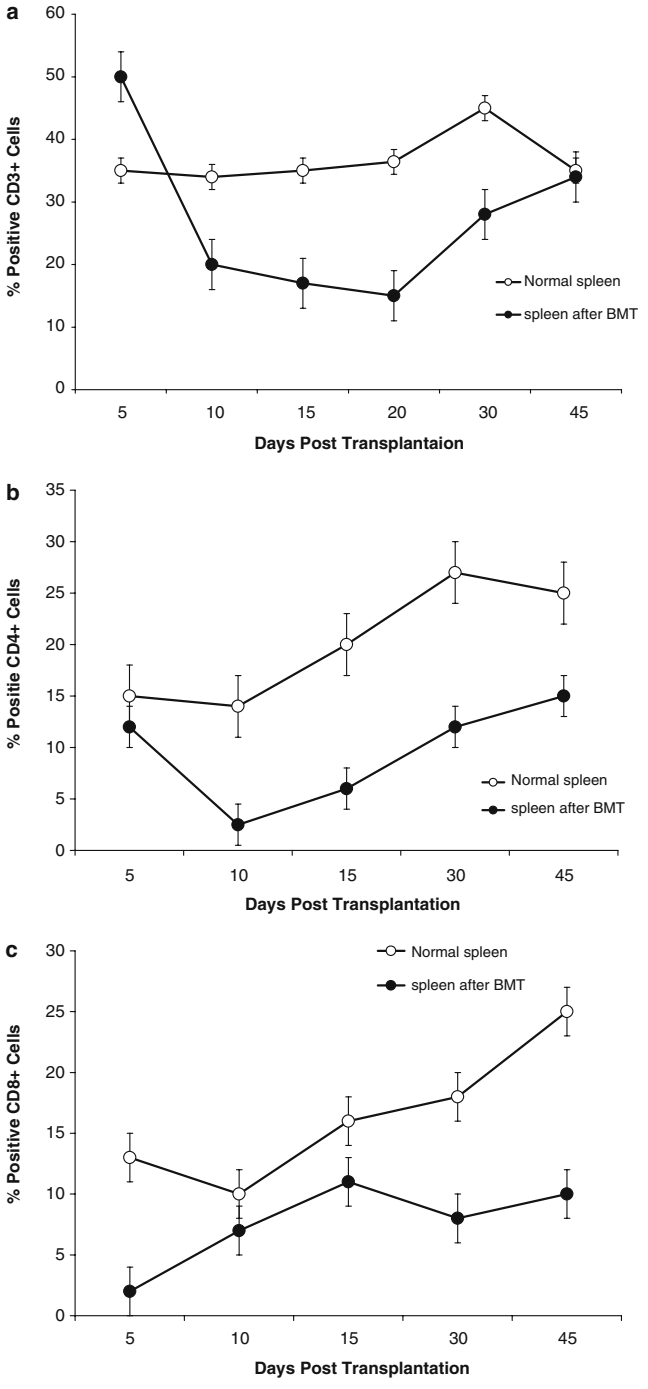

Fig. 5 Recovery of $CD3^+$, $CD4^+$, $CD8^+$ spleen cells following semi-allogeneic BMT. Splenic MNC cells were prepared from transplanted and normal mice at the indicated days post-BMT. The percentage of $CD3^+$ (**a**); $CD4^+$ (**b**); $CD8^+$ (**c**) spleen cells shown here represents three similar experiments. Results are presented as mean ± SD

Thymic evaluation Thymic cells showed a similar pattern to that observed for spleen cells, except for the $CD4^+$ cells which reached a higher level than in the control mice on day 10 post-BMT (data not shown).

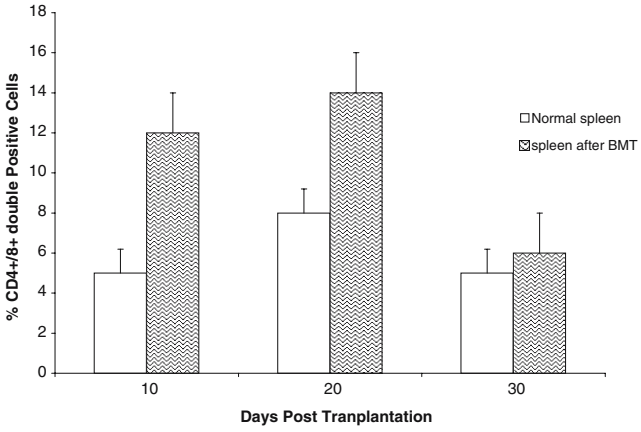

Fig. 6 Percentage of double-positive $CD4^+/CD8^+$ cells in the spleen following semi-allogeneic BMT. Splenic MNC of normal and experimental mice were obtained on the indicated days post-BMT. The percentage of $CD4^+/CD8^+$ double-positive cells presented here is representative of three similar experiments. Results are presented as mean ± SD

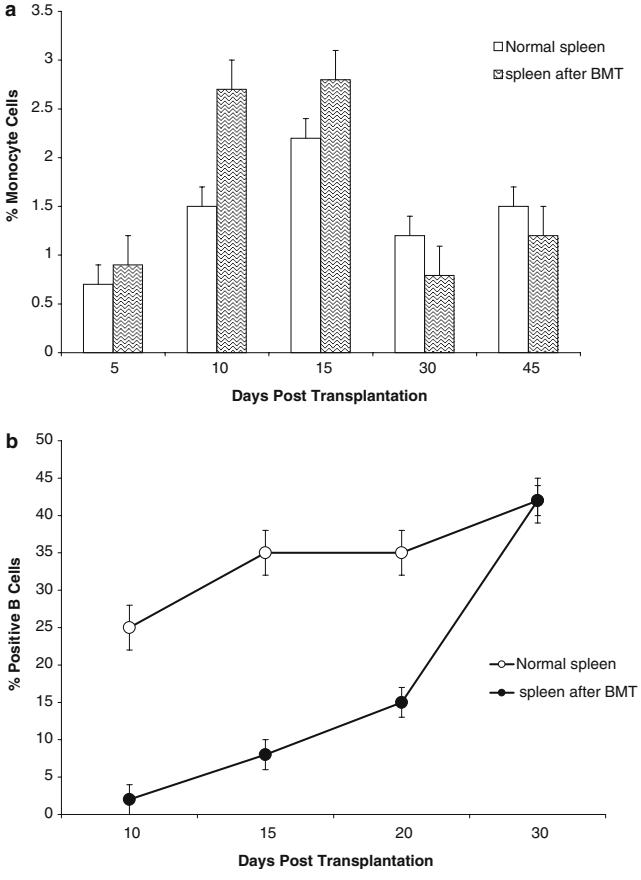

Fig. 7 Recovery of monocytes and B cells in the spleen at different time intervals following semi-allogeneic BMT. Splenic MNC were prepared from normal and experimental mice at the indicated days post-BMT. The percentage of monocytes (**a**) and B cells (**b**) presented here is representative of two additional experiments with similar results. Results are presented as mean ± SD

Discussion

This study evaluated the reconstitution of spleen and thymus lymphocytes in F1 mice exposed to lethal doses of radiation and subsequent semi-allogeneic BMT, in correlation with RNA expression and secretion.

As shown by FACS analysis, all recipients were reconstituted with donor type cells, and no overt clinical signs of GVHD were observed, probably due to the fact that mice were transplanted across semi-allogeneic barriers on the one hand, and due to the fact that the percentage of T cells in the murine bone marrow is low [16]. Full hematopoietic reconstitution of recipients with no clinical signs of GVHD on the one hand and no post-transplant administration of immunosuppressive agents, gave us the opportunity to observe the immune reconstitution of such chimeras with no interference by immunosuppressive medications used for prevention or treatment of GVHD.

The study focused on follow up of immune recovery post-BMT in correlation with status of IL-2 mRNA expression, the level of the protein, and the cellular ability to respond to mitogenic stimuli leading to cell proliferation in parallel with analysis of the percentage of lymphocytic subsets following PHA activation. The data presented here demonstrate a delay in recovery of both splenic and thymic cells in response to T cell dependent mitogenic stimuli post-semi-allogeneic BMT, as witnessed by the very low PHA proliferative capacity of these cells. In the thymus, transplanted mice showed a reduced capacity to respond to PHA activation (46.6% of the normal response). Moreover, this partial recovery is achieved only 30 days post-transplant.

As can be seen from the data presented, albeit full reconstitution of chimeras with 100% donor hematopoetic cells, no overt GVHD and no influence of immunosuppressive agents, reduced number and depressed proliferative functions were observed with failure of reconstitution, even at the mRNA level.

The immunosuppressive state following BMT is the rule and probably the reason why such patients are susceptible to infectious complications post-transplantation. Although much of the immunosuppressive state post-transplantation in clinical practice results from slow immunological reconstitution starting with stem cells, immunosuppressive treatment to prevent or to treat GVHD and direct consequences of GVHD, seem to augment the post-transplant immunosuppressive state, immunosuppression is also observed in patients undergoing autologous stem cell transplantation, consequently, independently of GVHD or anti-GVHD prophylaxis [4]. The immunosuppressive state following stem cell transplantation is probably mediated by a number of independent factors including time required for development of full immune maturity from uncommitted stem cells on the one hand, and chemoradiotherapy induced damage to bone marrow microenvironment and viral infections may also play an essential role in impaired immunohematopoietic reconstitution post-transplantation. Anti-host reactivity following allogeneic BMT may also participate in impairment of the function of host microenvironment.

Based on the aforementioned rationale, it seems that the immunosuppressive state following transplantation may be partially improved by using reduced intensity conditioning, as has been already suggested in clinical trials [14]. In parallel, enhancement of immune reconstitution may be accomplished by cytokines such as IL-7 that may facilitate thymic reconstitution of T cells and systemic reconstitution of B cells [3, 1, 5] or factors that may help reconstitute thymic microenvironment, such as keratinocyte growth factors (KGF) [9, 20].

A better understanding of the causes of immunosuppression post-transplantation and developing of new approaches for facilitation of immune reconstitution following transplantation, are of paramount importance in order to improve the outcome of recipients of allogeneic stem cells in clinical practice, especially in recipients of haploidentically mismatched stem cells. Transplantation of haploidentically mismatched stem cells requires pre-transplant T cell depletion for prevention of uncontrolled GVHD, however, such recipients usually require no post-grafting immunosuppression, since no GVHD results following adequate T cell depletion [26, 22]. Nonetheless, albeit overcoming the risk of graft rejection and GVHD, severe impairment of the immune function post-transplantation represents the major cause of failure following haploidentically mismatched transplantation and many patients succumb to viral, bacterial, or fungal infection, despite successful engraftment of donor hematopoietic cells and no GVHD. Interestingly, no such effects on mRNA were observed using a similar experimental model system following transplantation of syngeneic stem cells [29].

The difference between the pattern of reconstitution of mRNA and immunological reconstitution between recipients of syngeneic and allogeneic and mismatched allografts may suggest that in adequate interactions between stem cells and their adjacent microenvironment, they play a crucial role in the signals that stem cells obtain from the microenvironment. Thus, future approaches to improve immunological reconstitution may depend on the ability to improve signals provided to stem cells by microenvironment of the bone marrow.

Based on the following study and considering relevant clinical problems related to delayed and impaired immune reconstitution following stem cell transplantation, it seems that innovative approaches to facilitate immune reconstitution following successful stem cell transplantation, are of paramount importance in order to improve the outcome of allograft recipients. Our animal model may thus provide a simple model for investigating new biological agents and new procedures to facilitate immune reconstitution following allogeneic stem cell transplantation across MHC, using simple parameters to follow the immune status of allograft recipients at different time intervals following transplantation.

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