Cell Transplantation, Vol. 15, pp. 835–846, 2006
Printed in the USA.

0963-6897/06 $90.00 + .00
E-ISSN 1555-3892
www.cognizantcommunication.com

Induction of Tolerance Across Fully Mismatched Barriers by a Nonmyeloablative Treatment Excluding Antibodies or Irradiation Use

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A mixed-chimerism approach is a major goal to circumvent sustained immunosuppression, but most of the proposed protocols need antibody treatment or host irradiation. Another promising experience involves busulfan combined with cyclophosphamide treatment. Additionally, recent publications demonstrated that, differing from busulfan, treosulfan administration does not present severe organ or hemato toxicities. Currently, Duchenne muscular dystrophy (DMD) patients are treated with chronic immunosuppression for muscle precursor cell transplantation (MT). We have developed a safe tolerance approach within this cellular allotransplantation therapy background. Thus, we have conditioned, prior to a donor BALB/c MT, the dystrophic mouse model C57Bl10J mdx/mdx, with our treatment based on a donor-specific transfusion, then a treosulfan treatment combined with single cyclophosphamide dose, and finally a donor bone marrow transplantation (TTCB). A first MT was performed in all mixed chimeric mice resulting from the TTCB treatment in the left tibialis anterior (TA) muscles. A second MT from the same donor strain was performed 100 days later in the right TA without any additional therapy. Results show that all treated mice developed permanent mixed chimerism. Long-lasting donor-positive fibers were present in both TAs of the mice, which received MT after the TTCB treatment. Only a basal level of infiltration was observed around donor fibers and mixed chimeric mice rejected third-party haplotype skin grafts. Thus, mixed chimerism development with this TTCB conditioning regimen promotes donor-specific stable tolerance, avoiding costimulatory blockade antibodies or irradiation use and side effects of sustained immunosuppressive treatments. This protocol could be eventually applied for MT to DMD patients or others tissue transplantations.

Key words: Treosulfan; Chimerism; Tolerance; Transplantation; Muscular dystrophy

INTRODUCTION

Rejection of organ or cell allotransplantation is currently prevented with immunosuppressive drugs such as cyclosporine, FK506, mycophenolate mofetil, etc. Although these drugs are very effective for this purpose, their nonspecific immunosuppression increases the recipient's risks of developing opportunistic infections and malignancies. Moreover, their use is associated with some toxicities (6,14,15,37,38,54). In addition, chronic rejection is not well controlled by these immunosuppressive agents. An alternative approach to permit graft acceptance by the host immune system is to induce immunological tolerance. The establishment of mixed hematopoietic chimerism has several advantages over other tolerance-inducing methods. In mixed chimeras, tolerance to both host and donor is permanently established, leading to the absence of both graft-versus-host and host-versus-graft diseases (44,61). Many protocols to induce mixed chimerism have been investigated, but most of them required antibody treatment or irradiation of the host (9,12,28). An interesting protocol based on stable multilineage chimerism involved a nonmyeloablative treatment with susulfan (Busilvex®) in mice primed with allogeneic spleen cells followed by a single dose of an immunosuppressive drug, cyclophosphamide (Procytox®) (56). However, busulfan administration is associated with severe organ and hematotoxicity, which may not be avoided, even after reduced intensity conditioning (31,40,42,51,53).

A busulfan analog already used in clinics is treosulfan (Ovastat®), a prodrug of a bifunctional alkylating cytotoxic agent. It is indicated for oral or IV treatment of human advanced ovarian cancer (13,16,35,36). Re-

Received November 18, 2005; final acceptance June 30, 2006.
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cently, a successful combination of treosulfan with cyclophosphamide or fludarabine has been tested as a new preparative regimen before allogeneic hematopoietic stem cell transplantation in several patients with hematological malignancies (5,10). Furthermore, in the last years, a few groups have obtained mixed chimerism and tolerance-specific induction in mouse models with nonmyeloablative conditionings including treosulfan (39,57,58). However, these protocols still required depleting antibodies, which are not always available or accepted for clinical use.

In the context of muscle precursor cell allotransplantation (MT) as a clinical approach for Duchenne muscular dystrophy (DMD), a sustained FK506 immunosuppression is required to prevent a MT rejection in mice (8), monkeys (30,47), and humans (49). DMD is a fatal neuromuscular genetic recessive disease characterized by widespread muscle degeneration throughout the body. No cure is currently available for this disease, which is caused by the deficiency of a subsarcolemmal protein called dystrophin. MT consists to improve the strength of at least some muscles in DMD patients (49). Briefly, normal muscle precursor cells (MPC) harvested from a healthy donor are injected in skeletal muscles of DMD patients, fuse with host muscle fibers, introduce their nuclei containing the normal dystrophin gene, and thus restore dystrophin expression (29,45–49). As in all allotransplantation conditions, one of the problems facing this therapy is the specific immune reaction directed against transplanted cells and hybrid muscle fibers that they form (17–21).

The goal of the present study is thus to test whether an antibody and radiation-free tolerance induction protocol, supported by a mild myeloablative agent regimen conditioning, permits a stable allotransplantation such as MT for DMD.

To avoid the rejection of BALB/c myogenic cells without a constant FK506 administration, C57Bl10J mdx/mdx mice were treated with our tolerance-inducing protocol called TTCB (i.e., a donor specific transfusion, followed by a treosulfan treatment combined with single cyclophosphamide dose and a donor bone marrow transplantation). We have demonstrated that without antibodies or radiation conditioning, all TTCB-treated mice developed stable multilineage mixed chimerism and stable immunological tolerance towards fully allogeneic myogenic cells and skin.

MATERIALS AND METHODS

Animals

BALB/c mice (H-2d; Jackson Laboratory, Bar Harbor, ME, USA) were used as fully allogeneic donors. C57BL/10 J mdx/mdx mice (H-2b), which lack dystrophin expression, were used as hosts. C3H mice (H-2k; Jackson Laboratory) were used as third-party haplotype donors. All experiments were conducted in accordance of the Laboratory Animal Care and Use Ethics Committee of Laval University.

Donor-Specific Transfusion (25) and Drug Treatments

A DST consisting of a single dose of male and female adult BALB/c splenocytes (1×10^8, IV) was done in mdx recipients 3 days before the BALB/c bone marrow transplant (BMT). Briefly, the spleens were harvested and crushed in a cell strainer. The cell suspension was filtered through a 70-µm nylon mesh (Becton Dickinson, Franklin Lakes, NJ, USA) before assessing cell viability using trypan blue. Cells were suspended in RPMI-1640 medium (Gibco, Burlington, ON, Canada) before IV injection. Treosulfan (500 or 650 mg/kg) graciously obtained from Medac research (Medac, Wedel, DE, USA) was dissolved in 37°C sterile water and administered IV (100 µl/mouse, at days −3, −2, −1 before BMT). Cyclophosphamide (200 mg/kg) was dissolved in sterile water and administered IP (day −1 before BMT).

BMT

Bone marrow cells (BMC) were obtained by flushing femoral and tibialis bones from donor BALB/c mice with Hank's balanced salt solution (HBSS; Sigma, St. Louis, MO, USA). BMCs were T cells depleted by a treatment with anti-CD90/Thy-1 (RT-550-PABX; Medicorp, Montreal, QC, Canada) and with complement (low-tox M rabbit complement, Cedarlane, Hornby, ONT, Canada). BMT (day 0) consisted of a 40×10^6 freshly harvested adult BALB/c BMC IV injection.

MPC Culture

Newborn BALB/c mice (H-2d; Jackson Laboratory) were used for primary MPC cultures as previously described (11). Briefly, arm and leg muscles were dissociated with collagenase (600 IU/ml; Sigma) and dispase II (2 mg/ml; Roche Diagnostics, Mannheim, Germany). The cell suspension was grown in DMEM (Gibco) supplemented with 15% fetal calf serum and a mixture of penicillin G (10,000 IU/ml) and streptomycin (10 mg/ml). After 2 days in culture, cells were harvested and frozen in 40% DMEM, 50% fetal bovine serum (FBS), and 10% dimethyl sulfoxide, until MT.

MPC Transplantation (MT)

The hind legs of mdx mice, which did or did not receive the TTCB treatment, were irradiated (20 Gy) to inhibit host MPC proliferation and thus reduce competition with grafted MPCs (59). Prior to MT, 1×10^6 BALB/c MPCs were suspended in 10 µl of notexin (5 µg/ml) to induce myofiber necrosis without affecting the

blood vessels and peripheral nerves (23). The tibialis anterior (TA) of untreated or treated mdx mice (H-2b) were injected in several sites with these fully MHC allogeneic BALB/c MPCs (H-2d).

A positive control group for MT received a sustained immunosuppression during 1 month with FK506 (2.5 mg/kg/day, IM) (Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan).

Skin Transplantation

Full thickness tail skin ~1 cm^2 from BALB/c (H2-b) and third-party (C3H; H2-k) mice were grafted on the dorsum of the mixed chimeric mice 30 days after the BMT. Similar skin grafts were performed on untreated or FK506-treated mdx/mdx mice and represented negative and positive control groups, respectively. Skin grafts were considered rejected when less than 10% of the graft remained viable.

Blood and Muscle Collection

Mouse blood was collected on days 30 and 130 post-BMT. TAs injected with MPCs were dissected, placed in a 30% sucrose solution, embedded in OCT (Miles Inc., Elkhart, IN, USA), frozen in liquid nitrogen, and serially sectioned at 12 µm using a Microm cryostat.

Immunohistochemical Detection of Dystrophin

Cryostat sections were incubated with a rabbit anti-mouse dystrophin antibody (9), followed by a goat anti-rabbit IgG conjugated with Alexa 488 or Alexa 546 (Molecular Probes, Eugene, OR, USA).

CD4 or CD8 T Cells

Cryostat sections were incubated with a rat anti-CD4 antibody (clone GK 1.5; ATCC, Rockville, MD, USA) or a rat anti-CD8 antibody (clone YTS 169; gift from Dr. Waldmann, Oxford University, Oxford, UK). Sections were then incubated with a biotin-conjugated rabbit anti-rat antibody (clone E0468, Dakocytomation, Mississauga, ONT, Canada), followed by incubation with streptavidin-Cy3 (Sigma).

For all immunohistochemical detections, nonspecific binding sites were blocked with FBS 10% in PBS and staining was observed with a UV lamp microscope using the appropriate filter.

ELISPOT

Spleen cells from untreated or mixed chimeric mice were harvested and used as effectors. Spleen cells from BALB/c or C3H mice were harvested and used as stimulators. Cells were purified by Ficoll (Ficoll-Paque Plus, 17-1440-03, Amersham Bioscience AB, Uppsala, Seden) in order to isolate peripheral blood mononuclear cells. Stimulator cells were irradiated (20 Gy) and cocultured (ratio 1:1) with effector cells for 48 h. Roughly 1×10^4 cells were placed on the coated plates of INF-γ ELIS POT kit (Immuno-assay kit, KME1235, Biosource International, CA, USA) and incubated for 48 h. The spots were then revealed with the reagent from the ELISPOT kit. Resulting spots were counted in each well with a microscope. The mean number of spots in wells containing mixed chimeric mouse lymphocytes alone was evaluated and corresponded to the basal level of activity. The mean level of activation was also calculated in the other wells containing both effector and stimulator cells. The relative activity was the level of activation divided by the basal activity level.

Flow Cytometry

Blood samples of the treated mdx mice were harvested 30 days and 3 months post-MT to determine the level of microchimerism. Blood samples of mdx and BALB/c mice were also collected as negative and positive controls for H-2d haplotype. Briefly, blood cells were stained with an anti-MHC class I H-2d haplotype (clone 34-2-12, BD Biosciences, Mississauga, ONT, Canada) specific for the donor BALB/c strain. The chimerism level in different hematopoietic cell populations was determined with a phycoerythrin-coupled anti-CD90 (clone RM5504-3, Cedarlane), and Cy5-coupled anti-CD4 (clone L3T4, BD Biosciences) or a Cy5-coupled anti-CD8 (clone 53-67, BD Biosciences). An anti-Fcγ IIIR (clone 2.4G2, BD Biosciences) was added to the first incubation to block nonspecific FcγR binding of labeled antibodies.

A three-color FACS analysis was performed to analyze the expression of TCRs on the host T cells. Blood cells from treated or untreated mdx mice were harvested at 3 months post-BMT. Cells were labeled with phycoerythrin-conjugated anti-Vβ11 or anti-Vβ8.1/8.2 mouse antibody (clone CTVB11 or KJ16, respectively; Cedarlane), and Cy5-CD4 mouse antibody (L3T4, BD Biosciences). The percentage of mdx-derived CD4 cells that were Vβ11 or Vβ8.1/8.2 represents the number of CD4+ and Vβ+ cells gated on a total event of 1×10^4 leukocyte cells (discriminated by FACS with size scatter and forward scatter) divided by the total CD4+ cell population gated with the same method. The labeling was analyzed with flow cytometry (Epics XL, Coulter, Miami, FL, USA).

Statistical Analysis

A Fisher PLSD test was used for statistical analysis using the Stat View software (SAS Institute Inc., Cary, NC, USA) and $p < 0.05$ was considered statistically significant.

RESULTS

Cyclophosphamide/Treosulfan Combination Permits Bone Marrow Engraftment

The conditioning regimen, called TTCB, was done to mdx/mdx recipients as shown in Figure 1. Blood samples were collected 30 and 230 days after the BMT, to determine the peripheral chimerism level using cell-specific markers for CD90, CD4, and CD8 (Fig. 2). Thirty days after the BMT all TTCB-treated mice had significant levels of mixed chimerism for leukocytes (3–81%), CD4 lymphocytes (1–51%), and CD8 lymphocytes (1–29%). Significant levels of mixed chimerism were also observed 230 days after the BMT. However, leukocyte mean mixed chimerism decreased from 28% to 13% ($p < 0.05$) between 30 and 230 days post-BMT (Fig. 2A). As shown in Figure 2D, TTCB mice were classified by function of their CD90 mixed chimerism level measured 30 and 230 days post-BMT. Thirty days after the BMT, most of mice (69%) had a leukocyte mixed chimerism level between 15% and 35%. This distribution changed 230 days after the BMT, because an equal number of mice were distributed between 1.5% and 15% and between 15% and 35%level of leukocyte chimerism. This observation coincides with the decrease of the mean mixed chimerism mentioned above.

Two mice (numbers 5 and 13) died between days 30 and 230 after the BMT. We cannot exclude that these mice developed a GVHD because they both presented a significant level of mixed chimerism. However, no evident clinical sign of GVHD, such as a sudden loss of weight, was observed (data not shown).

Clonal Deletion of Alloreactive T Cells Occurred in Response to BMT After the TTCB Treatment

Vβ T-cell receptors (TCR) were used to evaluate antidonor reactivity and verify whether our protocol resulted in deletion of host cells bearing antidonor reactivity, an evaluation of central tolerance (11,23,24,55,56, 60). Two hundred days after the BMT, a deletion of Vβ11 TCR-bearing CD4+ T cells was observed in the peripheral blood of mice that received the TTCB tolerigenic treatment (Fig. 3). Thus, the percentages of Vβ11 TCR CD4+ T cells were significantly lower in TTCB-treated mdx mice than in untreated mdx mice ($p < 0.01$), and were similar to the levels seen in untreated BALB/c mice. As a control, the percentage of Vβ8, which should not be deleted in any strain of mice, was also examined. No significant differences in the percentage of CD4 cells expressing Vβ8 were detected in mdx mice before and after receiving the TTCB treatment, demonstrating the specificity for deletion of Vβ11 TCR-bearing CD4+ T cells and involving a central tolerance establishment.

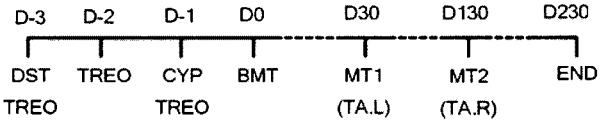

Figure 1. Tolerance induction protocol and MPC transplantation chronology. Time course of a nonmyeloablative conditioning treatment (called TTCB) and of MPC transplantations (MT1 and MT2, 1×10^6 BALB/c MPCs). The TTCB treatment includes: treosulfan (TREO, 3 doses of 500 mg/kg), BALB/c donor-specific transfusion (DST, 1×10^8 spleen cells), cyclophosphamide (CYP, 1 dose of 200 mg/kg), and BALB/c donor bone marrow transplantation (BMT, 40×10^6 CD90-depleted bone marrow cells). The level of mixed chimerism was analyzed at days 30 and 230. Mice were sacrificed 230 days after the BMT; blood and both grafted tibialis anterior (TA.L and TA.R) were collected.

The TTCB Treatment Led to Long-Term Survival of Fully Allogeneic MPC Transplantation (MT)

Mdx mice received the TTCB treatment followed 30 and 130 days later by two MTs in the left TA (MT1 in TA.L) and in the right TA (MT2 in TA.R). To assess MT survival and fusion with the remaining muscle fibers of mdx mice, both injected TAs were harvested 100 days after MT2 (Fig. 1). Dystrophin expression (Fig. 4) in whole TA section of a grafted muscle was determined by immunohistochemistry (of note, MPCs do not express dystrophin before their fusion with muscle fibers). An average of 340.91 ± 63.03 dystrophin-positive fibers were observed in left TA muscles (MT1 in TA.L) of TTCB-treated mice. Rare dystrophin-positive fibers (~5-10 fibers/muscle section) were present in muscles of naive mdx mice. Similarly, a low frequency (6.6 ± 3.7 fibers/muscle section) of dystrophin-positive fibers was observed in muscles of mdx control mice, which did not received the TTCB treatment but received a MT (Figs. 4B and 5). A high number of dystrophin-positive fibers (277 ± 41 fibers) was observed in mdx mice immunosuppressed daily with FK506 (Figs. 4A and 5). This shows that similar high-level engraftment of MPCs did occur when immunological rejection was prevented either with FK506 or with the TTCB treatment. Importantly, mice that received the TTCB treatment had significant long-term MPC engraftment as evidenced by high levels of dystrophin expression 200 days after MT1 (Fig. 4C and 5) in MPC-grafted TA.L.

Because several successive MTs will be required to treat several muscles in DMD patients, the immunological tolerance induced by the TTCB treatment should resistant to several donor stimulations. Therefore, MT2 was done as a challenge to assess the persistence of the tolerance. Dystrophin expression was examined in both injected TA muscles 100 days after the MT2. Interestingly (Figs. 4D and 5), the mean number of dystrophin-positive fibers was not significantly different in both

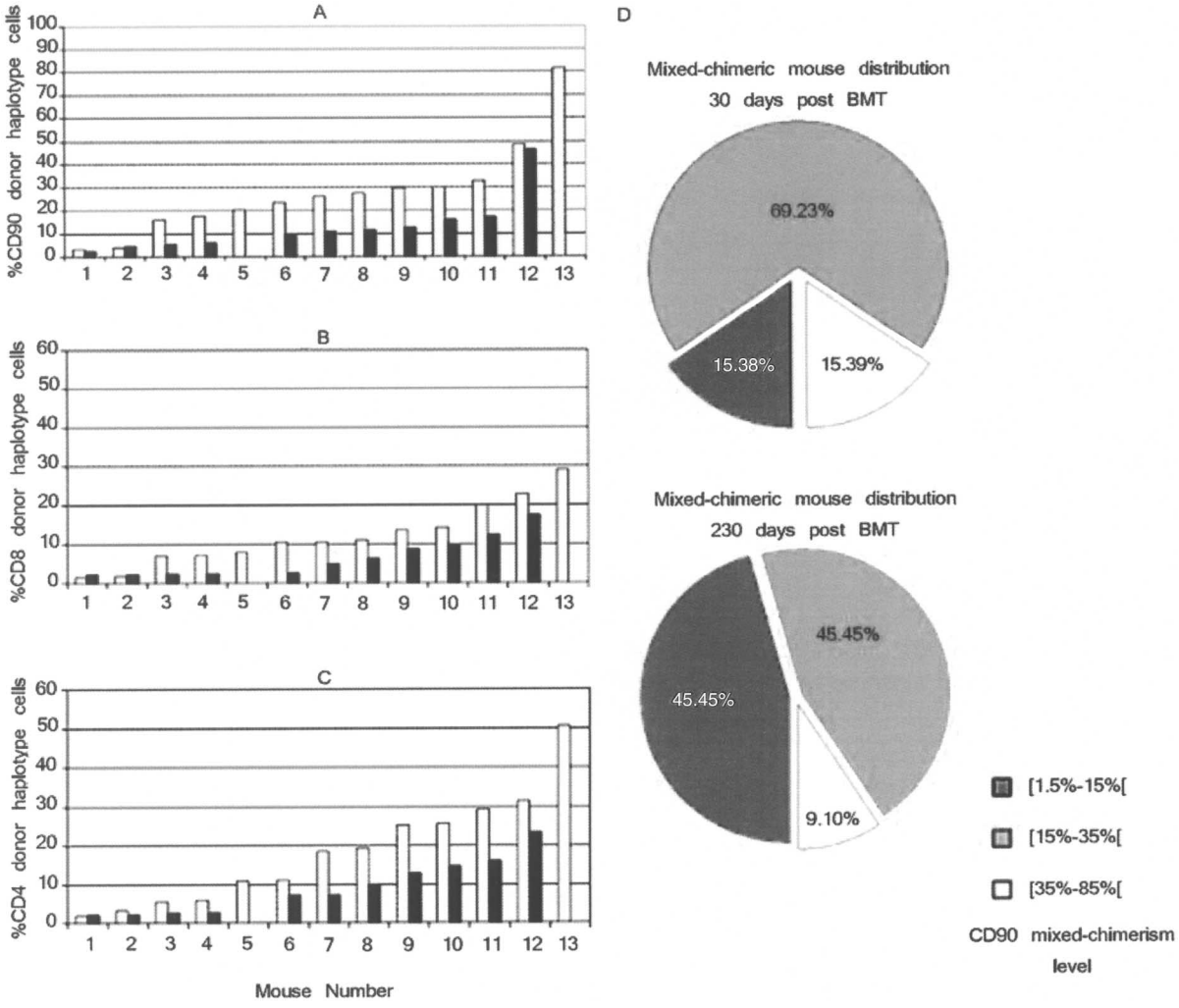

Figure 2. Peripheral mixed chimerism level evaluation. Levels of peripheral blood mixed chimerism were evaluated in mice that received the TTCB conditioning regimen. The level of mixed chimerism at 30 and 230 days after the BMT are represented in white and black columns, respectively. This analysis was performed for the leukocyte cell population and the CD8 or CD4 T-cell population, respectively (A, B, C). Note that mice 5 and 13 died between days 30 and 230. The distribution of mixed chimeric mice at 30 and 230 days after the BMT according to the percentage of donor haplotype CD90 cells is represented (D).

grafted TA (TA.L: 340.91 ± 63.03 fibers; TA.R: 331 ± 39.42 fibers), demonstrating that a second allograft did not affect the survival of the original allograft. Moreover, the number of muscle fibers expressing dystrophin in TA.L or TA.R was similar to that observed after a sustained FK506 administration.

No Increase of T-Cell-Mediated Activity Was Observed in the Grafted TAs of the TTCB-Treated Mice

Signs of a cellular immune reaction against the transplanted MPCs and against the muscle fibers resulting from their fusions were also assessed. This includes assessment of infiltration by CD4 and CD8 lymphocytes. Therefore, TA sections of mixed chimeric mice, which received MT, were evaluated for signs of muscle fiber invasion by cytotoxic cells. The presence of CD4 and CD8 lymphocytes around the dystrophin-positive fibers was investigated by immunohistochemistry. TAs from untreated mdx/mdx mice grafted with BALB/c MPCs were the positive controls for lymphocyte staining. High numbers of CD4 and CD8 lymphocytes infiltrated these positive control muscles (Fig. 6). Nongrafted TAs from untreated mdx/mdx were used as negative controls for lymphocyte staining and exhibited low-level patchy focal lymphocytic infiltrates containing both CD4 and CD8 cells (Fig. 6). Such a lymphocyte infiltration has previously been reported in the mdx muscles and attributed to the permanent inflammatory reaction present in these muscles where fibers are frequently in degeneration/regeneration (52). Comparable low levels of CD4 and CD8 infiltrations were observed in the MPC-grafted TA muscles of the mice that previously received the

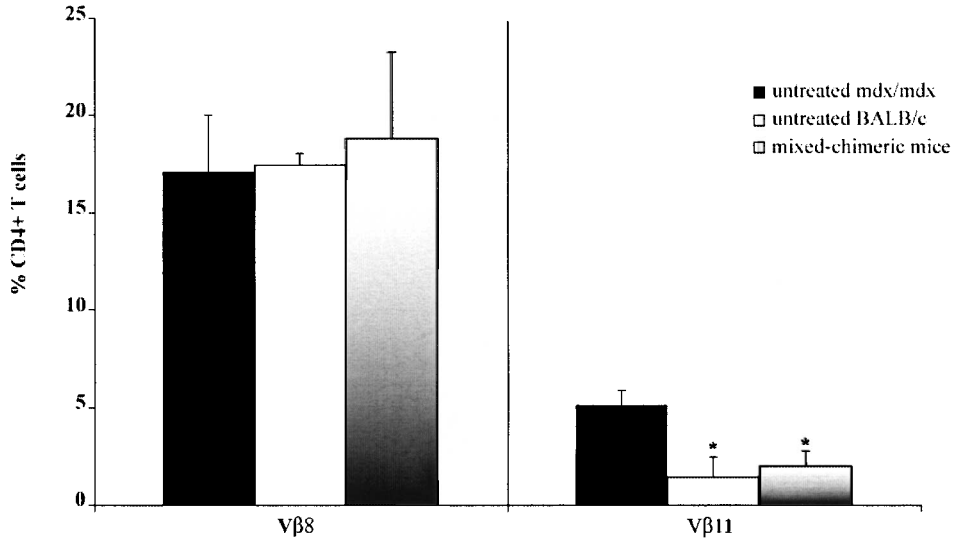

Figure 3. Clonal deletion of alloreactive T cells occurs in response to TTCB conditioning. Clonal deletion level of donor-reactive CD4+ Vβ11+ cells was assessed in peripheral blood samples of mixed chimeric mice ($n = 11$). The mixed chimeric mouse group was compared with untreated donor (BALB/c, $n = 5$) and recipient (mdx, $n = 5$) mouse strains. Peripheral CD4+ cells in mixed chimeric mice were assessed on day 230 post-BMT for Vβ8 (control) or Vβ11+ (donor reactive) T-cell receptor (TCR) (*$p < 0.001$ compared with untreated mdx mice).

TTCB treatment. Interestingly, the second allograft (TA.R) had no effect in the stability of this specific immune unresponsiveness against MT because no increased infiltration was observed.

The TTCB-Treated Mice Developed a Donor-Specific Tolerance

The antidonor immune response was also investigated in the mixed chimeric mice. To test this, all the mice that received the TTCB treatment and that had been successfully grafted with BALB/c donor MPCs were sacrificed 200 days after MT1. Spleen cells were collected, and the frequency of reactive T cells producing IFN-γ, a type 1 cytokine, was measured by a modified ELISPOT assay. Spleen cells obtained from untreated mdx/mdx mice were used as positive controls. T cells from chimeric mice did not react in presence of stimulator cells of a BALB/c donor (Fig. 7). In contrast, T cells of chimeric mice showed an increased IFN-γ production in the presence of stimulator spleen cells of a third-party allogeneic (C3H) mouse. These data indicate that tolerance to the BALB/c MT induced in TTCB-treated mice was strictly associated with the abrogation of anti-BALB/c type 1 immune response. T cells from TTCB-treated mice consistently showed strong stimulation and secretion of IFN-γ in the presence of stimulator cells of a third-party haplotype. Finally, the level of IFN-γ production was equivalent when T cells from TTCB-treated mice were in the presence of C3H T-cells and when T cells from untreated mdx mice were in the presence of either BALB/c T-cells or C3H T cells. This result indicated that the immune system of the TTCB mice conserved its capacity to respond to an allogeneic stimulation as well as the immune system of untreated mdx mice.

In order to support these in vitro results, an additional group of 6 mdx/mdx mice received the TTCB treatment. However, considering the variation and the time-dependent decrease of mixed chimerism level observed between the 13 TTCB-treated mice of the first experiment, the dose of treosulfan dose was increased from 500 mg/kg (first experiment) to 650 mg/kg. Their levels of mixed chimerism were also quantified as described above 30 and 230 days after the BALB/c donor BMT. Interestingly, as illustrated in Figure 7B, 30 days after the BMT all mice that received a TTCB treatment, including a higher dose of treosulfan, developed high and homogeneous mixed chimerism levels for leukocyte ($46.55 \pm 3.74\%$), CD4+ ($34.7 \pm 6.37\%$), and CD8+ ($16.92 \pm 4.39\%$) lymphocyte populations. Moreover, in contrast to the first experiment, 230 days after the BMT mixed chimerism level remained homogeneous between the TTCB-treated mice and increased for leukocyte ($68.64 \pm 3.74\%$), CD4+ ($68.34 \pm 11.43\%$), and CD8+ ($65.91 \pm 8.1\%$) lymphocyte populations ($p < 0.01$). These mixed chimeric mice then received allogeneic skin graft from both BALB/c and C3H donors. Untreated and FK506-treated mdx/mdx mice were also similarly grafted

and corresponded to our negative and positive control groups, respectively. As showed in Figure 7C, untreated mice promptly rejected both skin grafts (1–3 weeks postgrafting). FK506-treated mice preserved skin grafts until the end of the FK506 treatment period and then rejected the graft (1–4 weeks after the end of the FK506 treatment). TTCB-treated mice quickly rejected third-party donor skin graft (1–2 weeks postgrafting). Importantly, however, this rejection of C3H donor skin did not precipitate rejection of donor-specific BALB/c skin graft, which survived throughout the follow-up period (i.e., 10 weeks). Thus, the development of mixed chimerism by the TTCB treatment allowed a long-lasting donor-specific tolerance.

DISCUSSION

Sustained immunosuppression increases infection and cancer risks (6,14,15,37,38,54). An alternative method to permit graft acceptance by the host is the induction of immunological tolerance. An established approach depends on central immune system tutoring through the development of mixed hematopoietic chimerism (44,61).

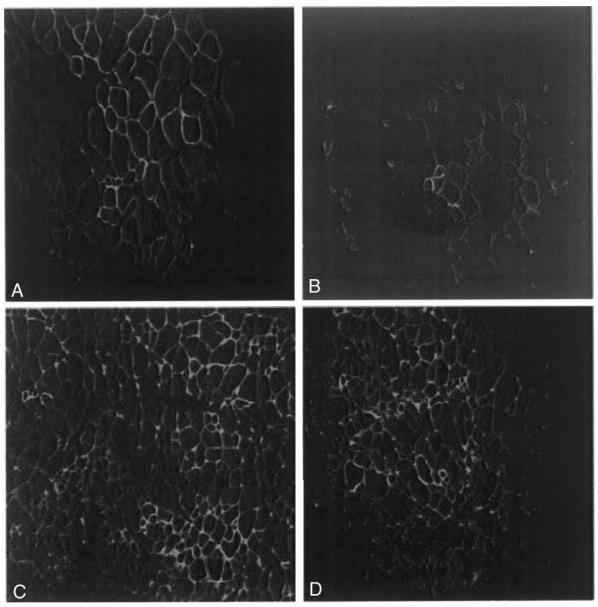

Figure 4. Stable mixed chimerism led to long-term survival of several fully allogeneic MPC transplantations. Dystrophin expression was detected by immunohistochemistry using an anti-dystrophin antiserum in cryostat sections of the TA muscles of mdx mice grafted with BALB/c MPCs (magnification ×100). (A) Mdx mice chronically immunosuppressed with FK506 shown 30 days after MPC injection. (B) Untreated mdx mice 40 days after MPC injection. (C) Mixed chimeric mdx mice shown 200 days after the first MPC injection (MT1) in left TA (TA.L). (D) Mixed chimeric mdx mice shown 100 days after the second MPC injection (MT2) in right TA (TA.R).

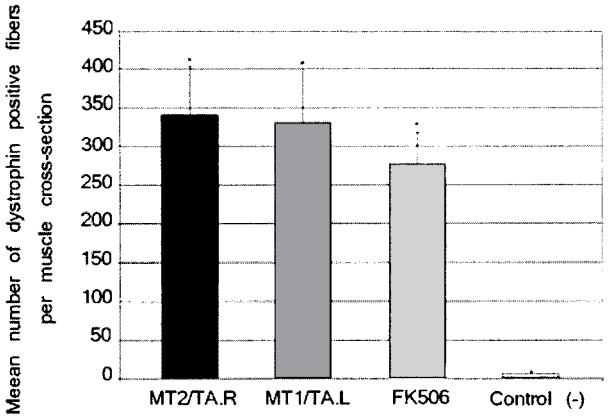

Figure 5. The success of MPC transplantation in mixed chimeric mice is similar to that observed in FK506 chronically immunosuppressed mice. Mean number of dystrophin-positive fibers in TA muscle sections of mice that received allogeneic BALB/c MPC transplantation. Dystrophin-positive fibers were individually counted in photographs of cryostat section of the muscle of the mixed chimeric mice TA ($n = 11$) 200 days after the first MPC injection in left TA (MT1/TA.L) or 100 days after the second (challenge) MPC transplantation in right TA (MT2/TA.R). A similar procedure was used for chronically FK506 immunosuppressed mdx mice ($n = 5$; FK506) and for untreated mdx mice [$n = 5$; control (-)]. TAs of FK506 or control (–) group were harvested 40 days after the MPC injection. $^*p < 1 \times 10^{-7}$ compared with control (–).

Several strategies for tissue or organ transplantations, developed in many animal models such as mouse or monkey, already allow permanent or transient mixed chimerism development. However, most of them involve irradiation conditioning associated with depleting or blocking antibodies (9,12,28,39,57,58). Moreover, the mAbs used for these treatments are not available or not already approved for clinical use. A promising alternative method proposed the combination of alkylators, busulfan, and cyclophosphamide, and a single donor spleen cell transfusion (56). All mice treated with this conditioning regimen developed stable multilineage chimerism. Furthermore, Tomita et al. demonstrated in that publication that all components of this regimen were required for the establishment of a firm mixed chimerism (56). However, busulfan toxicity compromises this therapy finality (10,31,40–42,51,53).

This study proposes a conditioning regimen called TTCB, which is based on treosulfan administration. The low dose of treosulfan (Figs. 2 and 7) used in this TTCB treatment should be considered as a nonmyeloablative conditioning regimen (39). This new conditioning regimen is well tolerated as expected because none of treated mice developed any signs of GVHD, and only 2 of 19 mice died after the beginning of protocol for unknown causes. As demonstrated following the TTCB

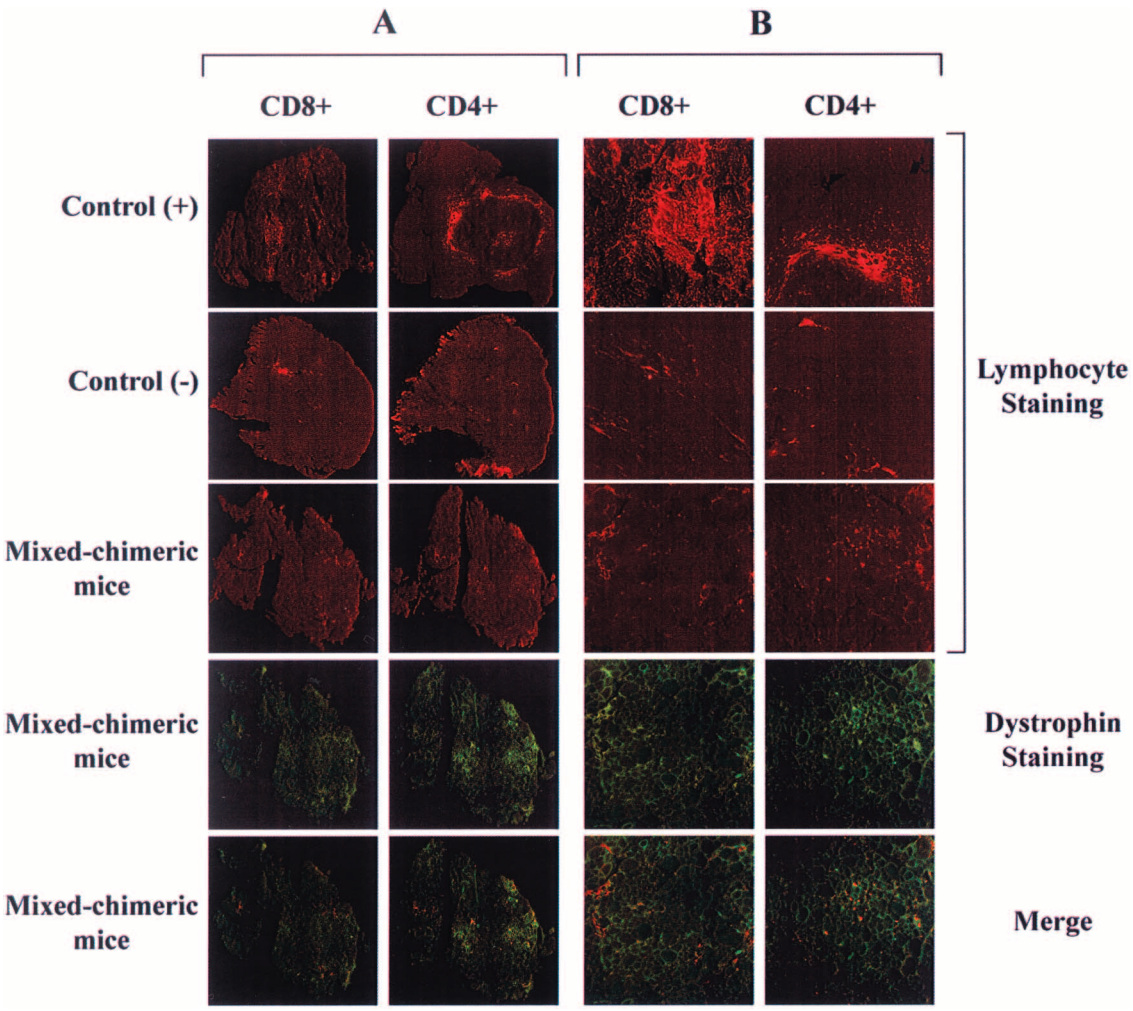

Figure 6. No increase of T-cell-mediated activity was observed in the grafted TA of mixed chimeric mice. Signs of a cellular immune reaction against the transplanted MPCs and against the muscle fibers resulting from their fusion were assessed by immunohistochemistry. This included assessment of infiltration by CD4 and CD8 T cells with a red immunofluorecence staining (Streptavidine-Cy3) and localization of dystrophin-positive fibers with green immunofluorecence staining (Alexa 488). Whole TA muscle sections (A) and 10× magnifications of the same sections (B) are represented. TA of untreated mdx mice grafted with donor BALB/c MPCs and collected 7 days after the MPC injection were considered as positive control [Control (+)]. Nongrafted TA muscles of untreated mdx mice were the negative controls [Control (−)]. Mixed chimeric mice injected with BALB/c donor MPCs for a second time in the right TA (MT2/TA.R) were sacrificed 100 days after this second MPC injection and both TAs were collected. Both stainings were performed on TA sections, combining dystrophin (dys) and CD4 staining or dystrophin and CD8 staining. The merges represent the respective staining associations (i.e., dys/CD4 or dys/CD8).

treatment, a stable multilineage mixed chimerism was obtained for leukocytes and especially for both CD4 and CD8 T-cell populations. In the first experiment (Fig. 2), the mixed chimerism levels were variable in TTCB-treated mice. However, this variability did not prevent the establishment of central tolerance (Fig. 3). In addition, all treated mice presented comparable stable clonal deletion level, 230 days after the BMT, independently of their mixed chimerism level, indicating that all of them had acquired central tolerance. In the second experiment, the TTCB-treated mice (Fig. 7) showed elevated and uniform mixed chimerism levels 30 and 230 days post-BMT, probably due to the higher dose of treosulfan administered (3×650 mg/kg instead of 3×500 mg/kg). In this second experiment, increased mixed chimerism levels were observed in the TTCB-treated mice between days 30 and 230 post-BMT ($p < 0.01$). A previous study in mice, involving T-cell depletion with an anti-CD3 mAb combined with a treosulfan treatment, demonstrated that similar uniform levels of mixed chimerism were obtained. However, this protocol required not only the use of a monoclonal antibody but also

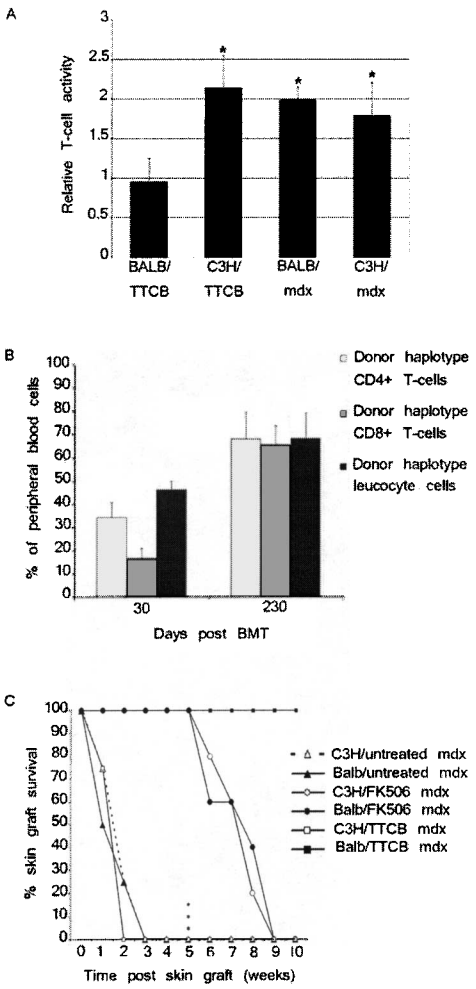

Figure 7. Mixed chimeric mice developed a stable donor specific tolerance. T-cell activity of mixed chimeric mice in the presence of donor BALB/c or third-party C3H spleen cells was evaluated by the INFγ ELISPOT method (A). Spleen cells of diverse mice were mixed in a 1:1 ratio. Spleen cells of mice that developed mixed chimerism following the TTCB treatment were mixed with BALB/c or C3H spleen cells (BALB/TTCB, $n = 10$ or C3H/TTCB, $n = 6$, respectively). Spleen cells of mdx mice were mixed with BALB/c or C3H spleen cells (BALB/mdx, $n = 5$ or C3H/mdx, $n = 5$, respectively). As reference of basal T-cell activity, the mean number of spots/well was calculated for spleen cells alone of mixed chimeric mice ($n = 6$). The ratios of the mean number of spots/well is illustrated for each cell combination and for basal T-cell activity (*$p < 0.001$ compared with BALB/TTCB combination). Levels of peripheral blood mixed chimerism were evaluated in 6 mice that received the TTCB treatment (B). The levels of mixed chimerism 30 and 230 days after the BMT are represented for the leukocyte cell population and for the CD8 and CD4 T-cell populations. Skin graft survival was determined for mice that received the TTCB regimen (C). Untreated mdx mice ($n = 5$) or FK506-treated mdx mice ($n = 5$) received simultaneously BALB/c and C3H skin grafts. Similar skin grafts were performed 30 days after BALB/c BMT on mice, which received the TTCB regimen ($n = 6$). ***Marks the end of FK506 administration.

higher doses of treosulfan (i.e., three consecutive administrations of 1500 mg/kg) (57).

The second aim of this study was to evaluate the application of a protocol of tolerance induction in the context of an allotransplantation for a cellular therapy. Currently, no effective treatment for DMD exists and MT represents a potential treatment because normal MPCs, carrying the wild-type dystrophin gene, can fuse with and provide the preexisting muscle fibers with a normal dystrophin gene. However, MT faces several technical problems. Because MPCs have a weak motility in vivo, multiple injections are required to reach a good incorporation of the donor MPCs throughout a given skeletal muscle (47). Because of this problem, many MTs at different times would be needed to efficiently restore the dystrophin expression in several muscles of a DMD patient. Therefore, strong and stable tolerance is essential to avoid a repeated tolerigenic treatment before each MT. Our present results are important in this context because, for the first time, a regimen exclusively based on drugs currently approved for human use allowed tolerance induction for allogeneic MT. Indeed, in agreement with the potential clinical application requiring several consecutive MTs in DMD patients, all mice receiving the TTCB regimen demonstrated long-term engraftment and donor-specific tolerance to a rechallenge with allogeneic MPCs.

Even without any allogeneic MT, the presence of activated lymphocytes ($CD44^{high}$, CD4, or CD8) was increased in mdx mouse muscles compared to those of the nondystrophic allelic strain (i.e., C57BL10J mice) (52). However, the frequency of activated T cells was not elevated in mdx lymph nodes, suggesting a muscle-specific T-cell activation. Moreover, it has been demonstrated that T cells promote mdx pathology and suggested that immune-based therapies may provide benefit to DMD patients (52). A previous tolerigenic protocol, by our group, using costimulatory blockade with anti-CD45Rb and anti-CD154, revealed an increased lymphocyte infiltration in MPC-grafted muscles of mixed chimeric mdx/mdx mice even though dystrophin-positive fibers formed by the transplanted MPCs persisted (9). Our present results showed (Fig. 6) that in TTCB-treated mice the presence of CD4 or CD8 T cells was not increased in grafted TA compared with the mdx/mdx standard TA. Thus, our protocol avoided a muscle-specific response that may have been present with the costimulatory blockade protocol.

Finally, the TTCB-treated mice significantly maintained reactivity to unrelated antigenic challenge as demonstrated by third-party skin graft rejection. The tolerance specificity was confirmed by the inactivity of the T cells of the TTCB-treated mice towards BALB/c spleen cells, while in contrast a high reactivity was pres-

ent against the spleen cells of a third party (C3H) (Fig. 7A). Additionally, the reactivity level of the C3H-activated T cells was similar for TTCB-treated or native mdx mice, suggesting that the specific immune system of TTCB-treated mice had conserved the original capacity to respond to alloantigens.

The present study demonstrates that treosulfan, in a protocol requiring no irradiation and using only drugs already approved for clinical use, permitted to obtain a safe and sustained donor-specific immunological tolerance for MT in mice. Moreover, given that treosulfan is already reported to have a low toxicity profile in hematopoietic stem cell transplantations, the merits of including treosulfan in nonmyeloablative regimen in the clinical setting, such as for dystrophic patients, should be seriously evaluated (5,10,43).

This study also shows that multiple MPC injections after a drug-induced tolerance leads to stable dystrophin expression in a large number of muscle fibers (Fig. 5). The level of dystrophin expression obtained in the present study has been previously shown to improve strength and decrease exercise-induced injury (1,3,4,7,26,27,34). Moreover, some of the transplanted MPCs will not fuse immediately and will remain as quiescent satellite cells, which will be able to repair subsequent damage induced by normal muscle activity (2,22,32,33). Additionally, we have shown that excellent distribution of MPCs can be achieved even when transplanted into larger muscles of nonhuman primates with up to 70% of the fibers expressing a donor gene (47). Finally, a recent patient study showed a successful MT that restored around 50% of dystrophin expression in the grafted area (50). Associated with the monkey and murine studies noted above (1,3,4,7,26,27,34,47), MT coupled with TTCB treatment could be expected to improve strength, quality of life, and perhaps the life span of DMD patients. Moreover, this approach may be useful to improve TTCB as a potential tool for other cell, organ, or tissue transplantations.

ACKNOWLEDGMENTS: This work was supported by ROTRF (Roche Organ Transplantation Research Fondation) and AFM (Association Française contre les Myopathies). We thank Dr. D. Skuk for revisions.

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