Why Do Cultured Transplanted Myoblasts Die In Vivo? DNA Quantification Shows Enhanced Survival of Donor Male Myoblasts in Host Mice Depleted of CD4+ and CD8+ Cells or NK1.1+ Cells

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Overcoming the massive and rapid death of injected donor myoblasts is the primary hurdle for successful myoblast transfer therapy (MTT), designed as a treatment for the lethal childhood myopathy Duchenne muscular dystrophy. The injection of male myoblasts into female host mice and quantification of surviving male DNA using the Y-chromosome-specific (Y1) probe allows the speed and extent of death of donor myoblasts to be determined. Cultured normal C57BL/10Sn male donor myoblasts were injected into untreated normal C57BL/10Sn and dystrophic mdx female host mice and analyzed by slot blot using a 32P-labeled Y1 probe. The amount of male DNA from donor myoblasts showed a remarkable decrease within minutes and by 1 h represented only about 10–18% of the 2.5 × 10^7 cells originally injected (designated 100%). This declined further over 1 week to approximately 1–4%. The host environment (normal or dystrophic) as well as the extent of passing in tissue culture (early “P3” or late “P15–20” passage) made no difference to this result. Modulation of the host response by CD4+/CD8+-depleting antibodies administered prior to injection of the cultured myoblasts dramatically enhanced donor myoblast survival in dystrophic mdx hosts (15-fold relative to untreated hosts after 1 week). NK1.1 depletion also dramatically enhanced donor myoblast survival in dystrophic mdx hosts (21-fold after 1 week) compared to untreated hosts. These results provide a strategic approach to enhance donor myoblast survival in clinical trials of MTT.

Key words: Myoblast transfer therapy; Survival; CD4+/CD8+/NK1.1 depletion; DNA quantification

INTRODUCTION

The X-linked recessive disease Duchenne muscular dystrophy (DMD) is caused by defects in the gene encoding the 427-kDa protein dystrophin, which is located beneath the sarcolemma of skeletal muscle fibers ([31, 50,102]; reviewed in [9,20]). The absence of a functional dystrophin molecule in humans leads to muscle fiber necrosis and progressive muscle weakness. The progression of the disease usually results in patients becoming wheelchair-bound by the age of 12 and death due to respiratory and/or cardiac failure by 20 years (87). The mdx mouse is also dystrophin deficient, but does not develop the severe pathology of DMD due to its apparent capacity to regenerate muscle fibers after repeated cycles of necrosis without replacement by fibrous connective tissue (15,64,66,79,86).

Transplantation of muscle precursor cells, widely referred to as myoblasts, from normal donors into dystrophic host muscle is the basis of myoblast transfer therapy (MTT). The observation that transplantation of minced muscles between mice resulted in the formation of hybrid myofibers composed of a syncytium of donor and host nuclei (63) supported the use of myoblasts grown in vitro as a cellular therapy for hereditary muscle diseases (29,41,46,53,67,95,96). The aim of this cell transplantation strategy is to introduce high numbers of normal donor nuclei that have the capacity to produce dystrophin and the potential to fuse with existing dystrophic host myofibers. The resulting hybrid myofiber will therefore be able to restore a functional dystrophin molecule to the syncytium and maintain muscle fiber integrity (65,67,90). Ideally, MTT would involve the survival of individual normal donor myoblasts after injection (followed by their replication and migration from the site), fusion with dystrophic host myofibers, and successful long-term expression of a functional dystrophin molecule.

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Major clinical trials were carried out on DMD patients in North America and Italy, but have widely been accepted as unsuccessful (26,57,89,90). Nevertheless, subsequent studies using animal models have yielded vital information about the limitations, as well as the potential, of MTT.

While MTT has been successfully demonstrated in animal models, the vast majority of early studies were conducted using conditions that were not comparable to those in the clinical MTT trials (26). For example, myoblasts were injected into either immuno compromised or immunodeficient mice that were unable to mount an immune response (16,56,67); into immuno incompatible hosts (52,53); into host muscle that had been irradiated, thereby impairing host myoblast proliferation and preferentially exaggerating the contribution made by donor myoblasts (33,58). In other situations, the conditions used in animal studies could potentially be applied clinically, such as transplantation into tolerant hosts (96, 97), or into hosts that were immunosuppressed (33,40, 49,80,95). The rejection of organ and tissue grafts is widely known to be prevented by the use of powerful immunosuppressants. FK506 and its analogue rapamycin have been used successfully to prevent the rejection of donor myoblasts in MTT (47,49,92), and continuous use of cyclosporin A prolongs myoblast survival, although rapid rejection results if withdrawn even 4 weeks after transplantation (39,100). The use of monoclonal antibodies that deplete specific T-cell subsets is an attractive alternative to the continued use of such drugs that have deleterious side effects (77). Depleting antibodies have been shown to induce long-term tolerance to MHC mismatched grafts after withdrawal of immunosuppressive treatment (93), and it is known that CD4+ and CD8+ T cells are essential in allograft rejection (13,61,94). The importance of CD4+ and CD8+ T-cell depletion has been shown in the prolonged long-term survival of injected muscle grafts implanted into mdx host mice (22,81). CD4+ and CD8+ T cells have also been observed in dystrophic (mdx) muscle (1,19,84) and have been attributed a role in the rejection of donor myoblasts in MTT (8). Pure myogenic cell lines (such as C2C12) have also been used in many studies. These cells can move readily throughout host muscle and fuse with host myofibers. However, they differ from primary cultures of myoblasts in that they are transformed cells that have the propensity to form tumors in vivo, and their growth requirements and proliferation in vitro are different (3,18,38,45,59,74,99).

The major obstacle to the success of MTT is donor myoblast survival. After injection into the host, cultured donor myoblasts undergo rapid and massive cell death. This was shown to be extensive by 2 days (23,75) and is now known to occur even more rapidly (4–6,72). Numerous studies in animal models attribute this to the host immune response, although the exact mechanism involved is not yet known. The rapid and massive cell death of cultured donor myoblasts after injection is in marked contrast with the excellent long-term survival (up to 1 year) of donor myoblasts using intact or sliced muscle grafts implanted into “untreated” dystrophic mdx hosts (21,22,81,82), which points to a crucial role for cell isolation and culture in the death of the transplanted cells.

Whether freshly cultured donor myoblasts have a significantly different potential for survival and successful fusion after injection into host mdx muscle than myoblasts passaged numerous times in culture, or indeed myoblasts taken from stored frozen stocks, has not yet been quantitatively assessed. Myoblasts are grown in culture in order to expand the numbers for injection and so the requirement of even a small amount of time in culture is unavoidable. Reliable procedures have been developed for growing primary cultures of myoblasts in vitro (74,98) and show an enrichment for the percentage of myogenic cells with time (since contaminating fibroblasts decrease with time). Culture conditions themselves (such as media components and serum factors) may alter the antigenicity of the donor myoblasts (3,37), and contaminating fibroblasts may compound any immune response mounted by the host because they express a large amount of MHC class II antigens (44,71).

Finally, the origin of donor myogenic cells used for primary culture may influence their survival after injection into host muscle. The analysis of myoblast populations from limb and masseter muscles shows an increased regenerative capacity in vivo and a faster growth rate in vitro for limb-derived myoblasts (68). Different myoblast populations isolated from fast and slow twitch muscle fibers may also follow a preferential difference in fate after injection (35,72,73).

This study uses a Y-chromosome-specific probe (62) to quantify the survival of male donor myoblasts into muscles of female host mice. It compares: a) the survival in untreated normal versus dystrophic mdx host mice, b) early versus late passage-cultured myoblasts, and c) dystrophic hosts depleted of CD4+CD8+ T cells and NK1.1 cells (regimes designed to modulate the host response). The answers to these fundamental questions provide a strong foundation upon which to develop strategies designed to enhance MTT in the clinical situation.

**MATERIALS AND METHODS**

**Animals**

Mdx mice were obtained from the Australian Neuromuscular Research Institute. C57BL/10Sn (the normal parental strain for mdx) were obtained from the Animal Resource Centre, Western Australia. All animal proce-
dures were carried out in strict accordance with National Health and Medical Research Council of Australia guidelines.

**Tissue Culture**

Skeletal muscles were taken from the hind limbs and lower back of 4–6-week-old donor male C57BL/10Sn mice. Myoblasts were isolated from these muscles by enzymic digestion and filtration through 100-μm nylon gauze as described previously (23,54). The resulting primary culture was maintained in Ham’s F10 medium (Trace) supplemented with 20% (v/v) fetal calf serum (FCS) (Trace), 4 mM L-glutamine (Sigma), 100 IU/ml penicillin, 100 μg streptomycin (Sigma), and 25 ng/ml basic fibroblast growth factor (bFGF) (Synergen). Medium was replaced every other day and cells were grown to 70–80% confluency before harvesting by digestion with 0.1% (w/v) trypsin (ICN-Flow). Cells were reseeded at approximately 1–2 × 10^5 cells per 75-cm^2 flask [precoated with 1% (w/v) gelatin] in fresh medium and passaged successively in this way. Fresh primary cultures (up to 3 passages) or late passage (15–20 passages) were also fixed with 4% (w/v) paraformaldehyde and subjected to immunocytochemistry using an anti-desmin polyclonal antibody (BioGenex) followed by detection with a rabbit anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Dakopatts) and o-phenylenediamine or 4-chloro-naphthol substrate in order to estimate the percentage of myoblasts within the population.

**Myoblast Injection**

Either fresh primary cultures (P3) or late passage (P15–20) cultures were adjusted to a concentration of 2.5 × 10^7/10 μl in phosphate-buffered saline (PBS, pH 7.2) and kept on ice. Ten microliters of this cell suspension was injected longitudinally into each tibialis anterior (TA) of 6–8-week-old female host mice using a Hamilton syringe with a 29-gauge needle. The needle was retracted carefully as the cells were injected in order to minimize the physical trauma of injection. Host mice used for 0 h time points were injected after surgically exposing the TA muscle, so that the sample was ready for immediate removal.

**Immunodepletion**

CD^+/-CD^8^- Depletion. Host mice were injected IP with 100 μl (15 mg) of each neat monoclonal antibody (in serum-free medium) over a period of 5 days prior to MTT, in order to deplete the host of CD^+ and CD^8^- cells. The YTS191 (rat anti-mouse CD^+^-) and YTS169 (rat anti-mouse CD^8^-) antibodies, both kindly provided by Dr. T. Scalzo (Dept. of Microbiology, UWA), were injected on day −5, day −3, and day −1, as well as a final injection at least 4 h (day 0) before MTT.

**FACS Analysis.** In order to confirm that mdx host mice had been depleted of their CD^+^ and CD^8^- T cell populations, fluorescence-activated cell sorting (FACS) analysis was performed on cells isolated from the spleens and thymi of depleted and control mice. Briefly, spleens and thymi were removed from each mouse and cells isolated and washed in PBS. All cells were kept on ice throughout the procedure. After adjusting the cell number to approximately 1 × 10^7 cells/ml, multiple samples were incubated on ice for 1 h with a range of dilutions of FITC-conjugated rat anti-mouse CD^4^- (L3T4, Pharmingen) and rat anti-mouse CD^8^- (53-6.7, Pharmingen) antibodies, both individually and in combination. Cells were washed again in ice-cold PBS. Controls of cells from undepleted spleens and thymi were also tested. Samples were analyzed with a Becton Dickinson FACSCalibur. Sample acquisition and file analysis was performed using CELLQuest. Files were collected using a gate to count lymphocytes, as defined by forward and side scatter measurements, to ensure that each file contained 10,000 lymphocytes for analysis.

**NK1.1+ Depletion.** Host mice were injected IP with 100 μl of anti-NK1.1+ ascites fluid (PK136, kindly provided by Dr. T. Scalzo, Dept. Microbiology, UWA) on day 5, 3, and 1 before MTT in order to deplete hosts of their NK1.1+ cells.

**Chromium-51 Release Assay.** In order to confirm that mdx host mice had been depleted of their NK1.1+ cell population, a ^51^Cr release cytotoxicity assay was performed on splenocytes isolated from control mdx hosts. Briefly, YAC-1 target cells were grown in tissue culture and incubated with ^51^Cr (1 μCi/ml in 200 μl) for 4 h in RPMI medium plus 10% (v/v) FCS. Cells were washed in medium, resuspended, and plated into 96-well plates at 4 × 10^3 cells per well. Effector cells (splenocytes) were added in the ratio of 80:1, 40:1, 20:1, 10:1, and 5:1 in triplicate. Triton X-100 (100 μl) was added to target cells (for maximum ^51^Cr release, designated T_active). Control target cells (for spontaneous release into medium, designated S_0) and negative control cells (no ^51^Cr incubation) were also included in triplicate. Effector and target cells were incubated together for another 4 h at 37°C before harvesting. Cells were pelleted by centrifugation and the supernatant placed into scintillation vials. The number of ^51^Cr particles released per minute (cpm) was counted. Control and test samples were designated as cpm test. Percentage lysis was calculated using the following formula: % lysis = (cpm_0 - cpm_S_0)/(cpm_T_active - cpm_S_0) × 100%.
DNA Quantification

Female host mice (mdx or normal C57BL/10Sn) were sacrificed at 0, 1, 4, 8, 24, 48, or 168 h (1 week) after infection and TA muscles isolated. Each muscle was cut into 2–3 mm³ pieces and homogenized in DNA isolation buffer [50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA (pH 8)] using an Ultra-Turrax homogenizer (Janke and Kunkel). After adding SDS to a final concentration of 0.2% (w/v) and incubating at 65°C for 20 min, proteinase K (Boehringer Mannheim) was added to a final concentration of 200 μg/ml and the samples incubated overnight at 37°C. Samples were extracted once in phenol (Tris-HCl buffered, pH 7.4–7.9) (Gibco BRL), once in 25:24:1 phenol/chloroform/isoamyl alcohol, once in 24:1 chloroform/isoamyl alcohol, and then ethanol precipitated overnight at −20°C. DNA pellets were resuspended in double distilled water overnight at 4°C and quantified by measuring the absorbance at 260/280 nm. DNA integrity was also checked visually under UV illumination after electrophoresis in 1% (w/v) agarose in the presence of ethidium bromide. The remainder of the DNA was applied to Hybond-N+ nylon membrane (Amersham) using a slot blot apparatus (Bio Rad) according to the manufacturer’s instructions. Donor male DNA was quantified by hybridization with the Y-chromosome-specific Y1 probe (23,25,62) that was random prime-labeled using [α-32P]dCTP (DuPoint). Quantification was performed by densitometric analysis after exposure to phosphorimaging screens (Fuji) using the MacBas 2500 phosphorimaging system (FujiFilm) and Image Reader Version 1.5E/Image Gauge V3.0 software. Statistical analysis of quantitative DNA data was performed using Minitab software (Minitab Inc.). Probe specificity and sensitivity was confirmed by hybridization to male and female genomic DNA and dilutions of Y1 plasmid DNA as well as to different amounts of DNA isolated from donor primary myoblasts grown in tissue culture. A detailed series of experiments was carried out to determine whether the presence of female DNA “masked” the amount of male DNA present in each sample.

RESULTS

Numbers of Myoblasts in Early and Late Passage Primary Cultures

Fresh primary cultures from normal donor male C57BL/10Sn muscles, which had not exceeded 3 passages in tissue culture, were shown to contain approximately 75% desmin-positive myoblasts. Late passage cultures, whose passage number in tissue culture ranged between 15 and 20, were shown to contain 95–100% desmin-positive myoblasts, as determined by immunocytochemical staining (data not shown). Irrespective of passage number, cells were maintained in HAMS F10 medium supplemented with 20% (v/v) FCS and 25 ng/ml basic fibroblast growth factor (bFGF) and kept in a logarithmic phase of growth (i.e., 60–75% confluent) in order to optimize their growth status at the time of harvesting for injection into host muscles. Donor myoblast viability was assessed to be 93–97% before and after harvesting (prior to injection) using trypan blue staining. Donor myoblast viability was also assessed to be 95–97% 1 h after injection back into tissue culture (following harvesting) using trypan blue staining (data not shown).

Detection of Donor Male Myoblast DNA on Slot Blots Using the Y1 Probe

Donor male myoblast survival is represented as the percentage of male (Y-chromosome-specific) DNA recovered from samples compared to that obtained from 2.5 × 10⁶ cultured donor male myoblasts (designated as 100%) (Fig. 1). Injection of donor myoblasts back into tissue culture flasks followed by DNA quantification showed that 100% of male DNA is recoverable and detectable. The minimum amount of male DNA (Y1 fragment) visibly detectable on slot blots was approximately 1 pg, which corresponds to the amount of male DNA detectable in approximately 1 × 10⁴ myoblasts. This level of sensitivity is reliable, accurate, and reproducible. The amount of female DNA present in each sample was found not to “mask” the detection of male DNA: as little as 1 pg of male DNA was reliably detected in up to 2–5 μg of female DNA isolated from the whole TA muscle (data not shown). The amount of donor male DNA recovered from injected female host TA muscles was always compared to control samples of DNA obtained from 2.5 × 10⁶ cultured donor male myoblasts (100%). The amount of donor male myoblast DNA in each sample was quantified by calculating the percent-

<table>
<thead>
<tr>
<th>Donor Male Myoblast DNA</th>
<th>Y1 Sequence</th>
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<tbody>
<tr>
<td>100% Injected</td>
<td>1ng</td>
</tr>
<tr>
<td>50% Injected</td>
<td>100pg</td>
</tr>
<tr>
<td>10% Injected</td>
<td>10pg</td>
</tr>
<tr>
<td>100% = 2.5 x 10⁵ cells</td>
<td>1pg</td>
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</tbody>
</table>

Figure 1. Quantification of donor male myoblast DNA. Male DNA isolated from injected female host TA muscles was quantified using a Y-chromosome-specific (Y1) probe on slot blots. Dilutions of Y1 sequence and male myoblast DNA controls were used in order to quantify percentage of donor myoblast survival compared to 100% of injected (2.5 × 10⁶) cells.
age of radioactive signal compared to that obtained from $2.5 \times 10^5$ cultured donor male myoblasts (designated as 100%).

**Time Course of Myoblast Survival Following Injection of Late and Early Passage Normal Male Donor Myoblasts Into Untreated Normal and Dystrophic Host Mice**

Quantification of male DNA on slot blots using the Y1 probe showed a remarkably rapid and massive loss of male DNA after injection of normal C57BL/10Sn donor male myoblasts into both normal C57BL/10Sn and dystrophic mdx untreated female host mice. At time 0 h (representing a maximum of 2–5 min after injection) only about 10% of the injected donor cells had survived in C57BL/10Sn female hosts, irrespective of whether early (P3) or late (P15–20) passage myoblasts were used. The percentage of surviving donor myoblasts continued to decline over time (Fig. 2A). Less than 10% remained after 1 h, approximately 5% after 4 and 8 h, and less than 5% remained after 1 day. By 1 week (168 h) only about 1% of late passage and approximately 5% of early passage donor myoblasts had survived. Two-way ANOVA showed no statistical difference between the survival of early or late passage donor myoblasts at each time point after injection into normal C57BL/10Sn host mice, apart from at 1 week after injection ($p < 0.005$) where significantly more (approximately 4 times) early passage (P3) myoblasts were present.

In dystrophic mdx female hosts (Fig. 2B) the average percentage of surviving donor myoblasts at 0 h was about 18% of that originally injected if late (P15–20) myoblasts were used, or 13% if early (P3) passage myoblasts were used. Again, the percentage of surviving donor myoblasts continued to decline over time: less than 10% remained after 1 h, approximately 5% after 4 and 8 h, less than 4% after 1 day, and by 1 week only about 1% of late passage and approximately 4% of early passage donor myoblasts had survived. Two-way ANOVA analysis showed no statistical difference between the survival of early or late passage C57BL/10Sn donor male myoblasts at each time point after injection into dystrophic mdx host mice. However, at 1 week (168 h) after injection significantly more (approximately 4 times; $p < 0.005$) early passage myoblasts survived relative to late passage cells.

Comparison of the survival of early passage donor myoblasts in C57BL/10Sn and mdx hosts (Fig. 3A) showed no statistical difference between host strains at each time point after injection, apart from at 48 h where nearly 3 times the amount of donor myoblast DNA was present in C57BL/10Sn hosts. However, a comparison of the survival of late passage donor myoblasts between strains showed statistically less survival at time 0 h ($p < 0.005$) in C57BL/10Sn hosts with only 10% of donor myoblasts surviving compared to 18% in mdx hosts (Fig. 3B). After this time, the number of surviving donor myoblasts declined steadily, with no statistical difference in donor myoblast survival between strains at each time point. When the data for survival of both early and late passage donor myoblasts were combined for each strain (normal and dystrophic) and plotted once more (Fig. 4), there was no statistical difference between the survival of donor myoblasts (average of early and late passage) between strains at any time point after injection.

Host TA muscles that leaked injected myoblasts were rejected as they would bias any quantification of donor myoblast survival. This was a rare event and clearly visible, particularly in 0 h time points where the skin was opened and the TA already surgically exposed. All injected host mice were carefully examined for leakage of injected myoblasts. Again, this rare event was visible due to either direct leakage through the skin from the site of injection as the needle was removed, or as prominent swelling beneath the skin over the TA. Injection of 10 μl volumes of 1% (w/v) Evans blue dye in preliminary experiments confirmed that leakage after injection was a rare and easily detectable event (data not shown). The massive initial death of injected donor myoblasts could therefore not be readily attributed to leakage of injected myoblasts from the site of injection.

**Effect of CD4°/CD8° T-Cell Depletion in mdx Host Mice**

Prior to analyzing the effect of leukocyte depletion on the survival of transplanted myoblasts, assays for the effectiveness of T-cell depletion were carried out. FACS analysis of the spleens of mdx hosts showed that there was effective depletion (approximately 80–90%) of CD4° and CD8° T cells in these animals prior to injection of normal donor male myoblasts (Fig. 5). However, the population of CD4° and CD8° T cells in the thymus remained relatively high (over 90%), even 4 h after the final injection of depleting antibodies, and returned to normal levels within 24 h (data not shown). The repopulation of CD4° and CD8° T cells in both spleens and thymi of depleted host mice up to 3 weeks after MTT was also followed using FACS analysis. Host mice injected with depleting antibodies showed approximately 21% survival (79% depletion) of CD4° cells and approximately 13% (87% depletion) of CD8° cells in the spleen, respectively, at the time of myoblast injection. Only 60% of CD8° T cells (compared to nondepleted hosts) were present up to 3 weeks after MTT compared to 91% of CD4° T cells, indicating that the former had been more effectively depleted (Fig. 5).
Survival of Late Passage Donor Myoblasts in CD4<sup></sup>/CD8<sup></sup>-Depleted mdx Host Mice

There was a dramatic increase in survival of donor myoblasts after injection into dystrophic female hosts pretreated with anti-CD4<sup></sup> and anti-CD8<sup></sup> antibodies. The results are shown in Figure 6 and also summarized in Table 1. In CD4<sup></sup>/CD8<sup></sup>-depleted hosts there was nearly a threefold increase in average donor myoblast survival at 0 h (to 43.3% from 15.8%) compared with untreated hosts. At 4 h after injection, the difference was fivefold (to 25.7% from 4.8%) and after 24 h it was eightfold (to 16.8% from 2.1%). At 48 h the difference was 24-fold (to 16.8% from 0.7%) and by 1 week there was still an eightfold increase in average donor myoblast survival (to 14.7% from 1.8%). There was considerable variation between samples in each treated group, although ANOVA analysis showed that there was significant difference ($p < 0.05$) of donor myoblast survival between CD4<sup></sup>/CD8<sup></sup>-depleted and untreated host mice at 0 h, 48 h, and after 1 week. The significant decrease in numbers of donor male myoblasts in CD4<sup></sup>/CD8<sup></sup>-depleted mdx hosts over 1 week suggests that although antibody depletion did increase donor myoblast survival initially, it was not effective at sustaining this.

Effect of NK1.1<sup></sup> Cell Depletion in mdx Host Mice

Prior to analyzing the effect of leukocyte depletion on the survival of transplanted myoblasts, assays for the effectiveness of NK1.1<sup></sup> cell depletion were carried out. $^{51}$Cr release assays using NK-sensitive YAC-1 target cells and spleen cells isolated from NK1.1<sup></sup>-depleted mdx hosts (as effector cells) showed an almost total
depletion of cytotoxic activity, with average $^{51}$Cr release values indicating a range of only 5–14% above spontaneous control levels at 0 h. Using splenocytes isolated from mdx hosts at 1 week (168 h) after depletion, average $^{51}$Cr release values ranged from 77% to 87% of those obtained from control (undepleted) mdx splenocytes (data not shown).

**Survival of Late Passage Donor Myoblasts in NK1.1$^{-}$-Depleted mdx Host Mice**

There was also a dramatic increase in survival of donor myoblasts after injection into dystrophic mdx female hosts pretreated with antibody depletion of NK1.1$^{-}$ cells. Again, the results are shown in Figure 6 and summarized in Table 1. In NK1.1$^{-}$-depleted hosts, there was nearly a fourfold increase in average donor myoblast survival at 0 h (to 56.5% from 15.8%) compared with untreated hosts. At 4 h there was over an eightfold increase in survival (to 40.5% from 4.8%) and at 24 h there was a 15-fold increase in average donor myoblast survival (to 30.5% from 2.1%). After 48 h there was approximately a 59-fold increase in average donor myoblast survival (to 41% from 0.7%) and by 1 week there was still a 21-fold increase in average donor myoblast survival (to 38.8% from 1.8%) compared with untreated hosts. While there was some variation between samples in treated hosts, ANOVA analysis showed that the differences between untreated and NK1.1$^{-}$-depleted hosts
were significant ($p < 0.05$) at each time point. There was no statistical difference in the survival of donor myoblasts over the first week (0–168 h), indicating that the survival of donor myoblasts was significantly enhanced and sustained in NK1.1−-depleted mdx host mice.

**DISCUSSION**

**Quantification of Donor Myoblast Survival**

The quantification of surviving male donor myoblast DNA using the Y-chromosome-specific (Y1) probe has characterized the speed and extent of myoblast death after injection into untreated female host mice, and hosts depleted of specific immune cells. The results emphasize the importance of designating the preinjected population as 100% and then measuring the percentage survival of donor myoblasts at specific time points based on this accurate and reliable quantitation method.

In agreement with previous studies (4–6,23,27,34) we observed a rapid and massive loss of cultured donor myoblasts in the first hour after injection. This typical

**Figure 4.** Pooled data for early (P3) and late (P15–20) passage myoblasts to compare donor male myoblast survival in female C57Bl/10Sn and mdx host mice. Standard deviation is shown for each sample set ($n = 4$ at 0 h, $n = 6$ for all other time points).

**Figure 5.** Flow cytometry analysis of CD4+ and CD8+ cells in the spleens of CD4+/CD8−-depleted mdx host mice. The graph indicates the recovery of CD8+ and CD4+ T cells in the spleens of depleted hosts, respectively, after MTT relative to untreated controls. Standard deviation is shown ($n = 3$).
Figure 6. Survival of late passage (P15–20) donor male myoblasts after injection into untreated CD4+/CD8- depleted, and NK1.1-depleted female mdx host mice. Standard deviation is shown for each sample set (n = 4 at 0 h, n = 6 for all other time points). *Statistical significance of p < 0.05 compared to untreated controls at each time point.

A "death curve" is present in both untreated normal C57BL/10Sn hosts as well as the dystrophic mdx female hosts. This indicates that the pathology of dystrophic muscle does not play a critical role in donor myoblast survival. Beauchamp et al. (4) conducted a similarly detailed study of donor male myoblast loss in nude (athymic) mdx mice. While the overall picture is similar, Beauchamp et al. reported survival values of around 85% at time 0, while the present study shows average values of between 15.8% for untreated mdx hosts and 56.5% for NK1.1-depleted mdx hosts at this time. The significance of these early differences is not immediately apparent. This may be accounted for by the fact that nude mice were used by Beauchamp et al. (4) compared with the immunologically competent hosts used in the present study. The differences between the two studies are restricted to time 0 only and data for all later time points agree. We consider that the massive initial loss of injected donor myoblasts is due to cell death, but cannot exclude the possibility that some "undetectable" leakage may have occurred. However, every care was taken to minimize leakage including trial injection into TA muscles where the skin was opened and no leakage was observed. Furthermore, only small variations were seen between multiple samples for each time point, plus the antibody depletion regimes "rescued" myoblasts from the initial "death," which argues strongly against significant leakage.

Viability assays before and after injection of donor myoblasts back into tissue culture confirmed that the process of injection through the needle itself did not result in cell death. Comparable levels of cell death have also been observed in donor myoblasts that were transplanted into host muscles within fibrin clots (6) that

Table 1. Average Percentage Survival of Late Passage C57BL/10Sn Donor Male Myoblasts After Injection Into Untreated, CD4+/CD8- Depleted, and NK1.1-Depleted Female mdx Host Mice

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Untreated</th>
<th>CD4+/CD8- Depleted</th>
<th>NK1.1-Depleted</th>
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<tr>
<td>0</td>
<td>15.8 ±9.1</td>
<td>43.3 ±9.4</td>
<td>56.5 ±33.4</td>
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<tr>
<td>4</td>
<td>4.8 ±2.7</td>
<td>25.7 ±20.4</td>
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<tr>
<td>24</td>
<td>2.1 ±1.1</td>
<td>16.3 ±16.2</td>
<td>30.5 ±26.5</td>
</tr>
<tr>
<td>48</td>
<td>0.7 ±1.0</td>
<td>16.8 ±10.3</td>
<td>41.0 ±17.6</td>
</tr>
<tr>
<td>168</td>
<td>1.8 ±1.6</td>
<td>14.7 ±5.7</td>
<td>38.8 ±31.9</td>
</tr>
</tbody>
</table>

Standard deviation shown in parentheses; n = 4 at 0 h and n = 6 for all other time points.
avoided the potential trauma of injection in vivo. Myo-
blast death was not considered to be apoptotic, because
electron microscopy showed no evidence of extensive
condensed chromatin or nuclear blebbing of donor myo-
blasts in TA muscle sections taken at 0, 1, 4, and 24 h
after injection (data not shown). Furthermore, DNA
isolated from injected TA muscles did not show any
DNA ladder characteristic of apoptosis (17,88) after
electrophoresis in agarose gels (data not shown).

Our data strongly suggest that it is the process of iso-
lolation of donor myoblasts from skeletal muscles, or ex-
posure of these myoblasts to tissue culture conditions
(83), that renders them susceptible to this rapid and mas-
sive cell death after transfer to the in vivo environment.
The significantly better survival of donor male myo-
blasts when slices of donor muscle (rather than isolated
cultured myoblasts) are transplanted (21,23,81) is inter-
esting to note in this context.

Survival of Early vs. Late Passage Myoblasts

There is essentially no difference in the survival of early
(P3) or late (P15–20) passage donor myoblasts after injection, suggesting that, at least in these experi-
ments, the length of time spent by donor myoblasts in
tissue culture is not critical to their initial survival after
transplantation. However, there appears to be a slight
repopulation of early passage (P3) myoblasts in both
C57BL/10Sn and mdx hosts between 48 h and 1 week
after injection, which may be due to proliferation of
these cells. This is in accord with the results of Beau-
camp et al. (4), which showed a slight increase in male
donor myoblast (using the Y1 probe as a measurement
of Y chromosome content) from 48 to 96 h after trans-
plantation. This is important, as it is often convenient to
use cultures that have been expanded over many pas-
sages as the source of donor myoblasts in MTT experi-
ments. However, there may be long-term consequences
for the capacity of myoblast replication after extended
passaging in culture, because nearly 4% of early passage
donor myoblasts survived after 1 week compared to 1%
of late passage donor myoblasts, in both normal and
dystrophic hosts. If, indeed, expansion of myoblast pop-
ulations in tissue culture does subsequently restrict their
capacity to proliferate in vivo (after injection), this ar-
gues strongly against their use in clinical trials. Ex-
tended time in tissue culture may also lead to transforma-
tion of mouse myoblasts, resulting in the formation of
aberrant muscle tissue and/or tumors after transplanta-
tion in vivo (T. Partridge, personal communication).

Preliminary data using frozen stocks of early and late
passage myoblasts show no difference in the percentage
of donor myoblast survival between frozen and nonfro-
zen stocks after injection into normal or dystrophic host
mice, even after approximately 25 passages in culture
(S. Hodgetts, unpublished results). It is important to
know that there are apparently no adverse effects associ-
ated with freezing myoblasts, as some MTT experiments
may use large numbers of donor myoblasts that can be
conveniently accumulated and frozen until required and
then pooled for injection.

Manipulation of the Host Immune Response

Depletion of Host CD4+ and CD8+ T Cells. Dystro-
phic female mdx hosts depleted of CD4+ and CD8+ T
cells showed an improved (threelfold) survival of cul-
tured injected donor male myoblasts immediately after
injection. Although the average percentage of surviving
myoblasts decreased with time in CD4+/CD8+-depleted,
hosts, after 1 week there was still an eightfold increase
in survival compared to myoblasts transplanted into un-
treated control hosts. These results suggest that CD4+
and CD8+ T cells play a role in myoblast survival after
transplantation.

Activation of CD4+ T cells generally leads to the pro-
duction of cytokines that are extremely effective immu-
noregulatory molecules, while activation of CD8+ T cells
leads to cytotoxic activity (reviewed in (30,85)). CD4+
and CD8+ T cells recognize foreign antigens via an asso-
ciation with class II and class I major histocompatibility
complex (MHC) molecules, respectively, and this inter-
action is required for processing and subsequent T-cell
activation. The importance of CD4+ and CD8+ T-cell
depletion has been shown in the prolonged long-term
survival of donor myoblasts that have migrated out of
sliced muscle grafts implanted into mdx host mice
(22,82). A role for T cells is also supported by the
success of MTT experiments performed in immuno-
compromised or immunodeficient (16,56,67), immuno-
 suppressed (33,40,48,69,95), and tolerized host mice
(96, 97).

Our data show that CD4+ and CD8+ T-cell depletion
was not 100% complete in the spleen compared to that
reported by Spencer et al. (84) for CD8+ depletion in
mdx mice. Blocking antibodies may also be required to
affect total CD4+ and CD8+ T-cell depletion (12,14).
However, our data show that CD4+/CD8+ depletion
(even if incomplete) dramatically enhanced donor myo-
blast survival after injection. The repopulation of CD4+
and CD8+ T cells after their initial depletion also sug-
gests that they might continue to be involved in donor
myoblast death for up to 3 weeks after injection, as the
reemergence of normal numbers of CD4+ and CD8+ T
cells mirrors the continuing decline in surviving donor
male myoblasts. It seems likely that, even if CD4+ and
CD8+ T cells are not the main effector of the host
immune response, their contribution to pro-inflamma-
tory cytokine production would have an important
and marked influence on the survival of cultured donor
myoblasts. Our data suggest that complete depletion of these cells may be required to sustain the enhanced long-term survival of donor myoblasts after injection.

Depletion of Host NK1.1+ Cells. Dystrophic female hosts depleted of NK1.1+ cells also showed a dramatic enhancement of cultured donor male myoblast survival (fourfold at 0 h and nearly 22-fold 1 week after injection). Although the average percentage of surviving donor myoblasts decreased slightly, there was no statistical significance in donor myoblast survival between time points, indicating that the enhanced survival was maintained for at least 1 week. Cytotoxicity assays using the NK-sensitive YAC-1 target cells showed that there was effective NK1.1+ cell depletion (5–14% above spontaneous controls) at 0 h and NK activity (as measured by 51Cr release) had recovered to approximately 77–87% of control (undepleted) values after 1 week. This suggests that NK1.1+ cells may play a more crucial role in the initial death of cultured donor myoblasts but are less critical in their later death.

Natural Killer (NK) cells are large, granular lymphocytes that play an important role in the innate as well as the adaptive immune systems, employing cytosis and cytokine production as major effector mechanisms (7). They are probably activated by a variety of factors such as interferons, cytokines (e.g., TNFα, IL2, IL12, IL15), and chemokines. IL12 is itself produced by macrophages, dendritic cells, and B cells, and directly enhances cytolytic activity in T cells, NK cells, and lymphokine-activated killer cells (LAKs), while TNFα is an important cofactor in NK cell activation and induces NK cells to secrete interferon-γ (10,60). NK cells recognize target cells via a mechanism involving several surface receptors that are responsible for activation in a poorly understood combination with receptors specific for MHC class I molecules that inhibit their activity (51, 101). NK cells have also been reported to participate in xenogeneic organ rejection and are found at high frequency in cellular infiltrates associated with xenograft rejection (2,24,43,91). NK cell depletion in combination with immunosuppression has been found to slightly prolong xenograft survival, although at least one study suggested that IL2 production by T cells may also be required [reviewed in (2)].

Dendritic cells (DC) have also been shown to express the NK cell receptor protein 1, express NK1.1, and to possess cytotoxic activity (42,55,76,78). DC are resident in skeletal muscle (70) and their major function is processing exogenous antigen for MHC class I and II presentation (78). The significance of potential NK1.1 expression on DC within skeletal muscle and the enhanced donor myoblast survival following NK1.1 depletion is unclear at this stage.

Transient immunosuppression with monoclonal antibodies to ICAM-1 and LFA-1 has been shown to increase the retention of allogeneic myoblasts up to 4 months after MTT (69). Similarly, donor myoblast survival (100% was measured by β-Gal staining at 1 h after MTT) was also enhanced for up to 1 month using anti-LFA-1 antibody to modify the inflammatory reaction that accompanies MTT (28). LFA-1 is an adhesion molecule present on the surface of neutrophils, macrophages, NK cells, and lymphocytes (27). The inflammatory reaction after MTT was characterized by a massive infiltration of neutrophils, basophils, eosinophils, LFA-1-positive cells, and MAC-1+ cells (macrophages and NK cells) (27). In addition, cytokotic T cells and NK cells have been shown to rapidly damage developing myotubes (32). It is also of interest that monoclonal antibodies to NK cells react with neural cell adhesion molecule (N-CAM), which is a surface glycoprotein present in muscle satellite cells and regenerating or newly dervated muscle fibers (36). As development proceeds, N-CAM disappears from developing myofibers, as does MHC class I antigen. This alteration of MHC class I expression is involved in the development of an immune response. Expression of class I MHC antigens on pancreatic islet cells [which are target cells for immune rejection in insulin-dependent diabetes mellitus (11)] is altered by effectors such as interferon-γ and TNFα. Target islet cells with altered MHC class I molecules on their surface, or those that lack them entirely, are quickly destroyed by NK cells (11). Perhaps NK cells are activated to destroy myoblasts because of an alteration of class I MHC expression on their surface as a direct consequence of their exposure to tissue culture conditions. It is tempting to speculate that the generation of neoantigens on myoblasts due to tissue culture conditions (37) might cause an aberrant recognition of class I MHC by NK cell receptors, followed by their subsequent activation and the destruction of donor myoblasts.

Long-term studies are in place to confirm the impact of each of these depletion strategies for optimizing the success of MTT.

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REFERENCES


42. Josien, R.; Haslan, M.; Soulillou, J. P.; Cuturi, M. C. Rat spleen dendritic cells express natural killer cell receptor protein 1 (NKR-P1) and have cytotoxic activity to select targets via a Ca2+-dependent mechanism. J. Exp. Med. 186:467–472; 1997.


52. Law, P.; Goodwin, T.; Wang, M. Normal myoblast injec-


69. Pavlath, G. K.; Rando, T. A.; Blau, H. M. Transient immunosuppressive treatment leads to long-term retention
81. Smythe, G. M.; Fan, Y.; Grounds, M. D. Immunosuppressants enhance the migration of donor myoblasts into dystrophic and normal host muscle. Cell Transplant. 8; 1994.