INNATE INFLAMMATORY CELLS ARE NOT RESPONSIBLE FOR EARLY DEATH OF DONOR MYOBLASTS AFTER MYOBLAST TRANSFER THERAPY

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Background. Myoblast transfer therapy (MTT) is a cell-based gene therapy representing a potential treatment for Duchenne muscular dystrophy. The rapid disappearance of donor myoblasts from transplanted muscles after MTT is one of the most controversial and significant obstacles facing research in this area. Dystrophin-deficient muscles show constitutively high levels of inflammation, thus necessitating an examination of whether inflammatory cells, specifically natural killer (NK) cells, neutrophils, and macrophages, within dystrophic muscle are responsible for poor graft survival.

Methods. Female mdx mice were treated with RB6-8C5 monoclonal antibody, PK136 monoclonal antibody, or clodronate liposomes to systemically deplete neutrophils, NK cells, and macrophages, respectively. At each depletion regimen, the mice and age-matched controls received 5.0×10⁶ male myoblasts injected longitudinally into each tibialis anterior muscle. Donor myoblast survival was assessed by Y-chromosome specific quantitative real-time polymerase chain reaction analysis.

Results. The systemic depletion of host neutrophils and NK cells resulted in a transient improvement in donor myoblast survival at 72 hr and 7 days post-MTT, respectively. Systemic depletion of macrophages had no significant beneficial effect on myoblast survival. Overall, the number of detectable male donor myoblasts was similar at time 0 and 1 hr post-MTT; however, there was significant loss by 24 hr (~50%-70%) followed by a continual decline in donor cell numbers.

Conclusions. Neutrophils and macrophages do not seem to play a major role in the rapid death of donor myoblasts after transplantation into dystrophic muscle. NK cells similarly seem to have no significant effect, contrary to earlier findings reported by our group.

Duchenne muscular dystrophy is a myopathy caused by mutations in the dystrophin gene. The lack of dystrophin, which plays a role in maintaining muscle plasma membrane integrity and stability, results in progressive weakness leading to death by the third decade (1). Myoblast transfer therapy (MTT) is a potential cell-based gene therapy that aims to restore dystrophin expression in the muscles of patients with Duchenne muscular dystrophy. Human clinical trials have been unsuccessful with low levels of donor cell survival (2).

Some studies report rapid and massive myoblast death (~90%) within hours of transplantation (3–5), whereas others report no significant loss of the transplanted cells during this period (6, 7). There are several areas of dispute that may be explained by important differences between the studies, the most pertinent being the different reference standards used to assess 100% donor myoblast survival and differences in donor myoblast detection methods. The fate of donor myoblasts after transplantation is still a controversial issue. Whereas the acquired immune response has been largely excluded as a factor involved in the initial events after MTT, the innate cellular immune response has received more attention in this regard. In severe combined immunodeficient mice, myoblast death rules out an acquired immune response as the major cause of early myoblast death (6, 9). Although the acquired immune response is an important factor at later time points after transplantation, the occurrence of graft rejection after myoblast transplantations performed in the muscles of histocompatible or immunosuppressed mice, which minimizes the acquired immune reaction, highlights the importance of other (innate) factors in graft rejection (10–12).

Although groups agree that the innate immune response is likely to be important in the early death of donor myoblasts (13, 14), it remains unclear what the important factors are. Whole-body irradiation of mice before myoblast transplantation has been reported to enhance myoblast survival, indicating that host immune cells are indeed contributing directly or indirectly to myoblast death (15). The expression of the anti-inflammatory chemokine transforming growth factor-β has also been shown to improve myoblast survival by decreasing polymorphonuclear cell and macrophage infiltration into the transplanted muscle (16). Both neutrophils (8) and natural killer (NK) cells (5, 17) have been implicated as important cellular mediators of myoblast death after transplantation. Guerette et al. (8) demonstrated a potential role for neutrophils after observing the infiltration of polymorphonuclear cells in muscle tissue within 1 hr of a myoblast transplant; this event was followed by an infiltration of Mac-1+ and LFA-1+ cells, both being surface markers expressed on neutrophils. A rapid inflammatory reaction, characterized by a neutrophil infiltration, has also been demonstrated in the muscles of dogs after myoblast transplantation.
(18). Guerette et al. (8) also showed that death of injected myoblasts was reduced by approximately 58% by depleting the neutrophil population, achieved by treating recipients with an anti-LFA-1 monoclonal antibody (mAb) before transplant. Immuno-depletion was also applied to determine a role for NK cells in the myoblast rejection process with Hodgetts et al. (5) reporting that depletion of NK1.1+ cells resulted in an approximate fourfold increase at 0 hr, which increased to 59-fold by 48 hr posttransplant. The role of NK cells was further supported by the use of beige mice, which lack functional NK cells, and transfusion of donor myoblasts with m144, a murine homolog of major histocompatibility complex class I that protects the cell from NK-mediated attack (17).

A recent study characterizing the cellular immune reaction to myoblast transplantation (7) shows that although neutrophils are the first inflammatory cells to be present at the injection site and may contribute to donor myoblast death, the net survival of transplanted myoblasts can be independent of the neutrophil response. Furthermore, these authors did not detect NK cells at the site of injection in significant numbers before day 6 posttransplant, suggesting that, in contrast with earlier studies (5, 17), NK cells are not important in the early host response to the donor cells (7). Also reported in the study was the early and sustained presence of macrophages at the graft site. Macrophages have been implicated as important factors controlling long-term graft survival in a study examining cardiac xenotransplantation (19) and are present in significant numbers in mdx muscle (13, 20). Macrophages may be beneficial to graft survival by phagocytosing dead cell debris and releasing growth factors to enhance proliferation (21), but may also contribute to the specific immune response against grafted cells by performing antigen presentation functions.

The present study was conducted to further investigate whether the presence of host neutrophils, macrophages, and NK cells influenced the immediate and medium-term survival of normal male donor myoblasts injected intramuscularly into female dystrophic mdx host mice. The total amount of male donor DNA was quantitated using real-time polymerase chain reaction (PCR), and the issue of an appropriate 100% reference standard for myoblast survival was also addressed.

**MATERIALS AND METHODS**

**Preparation of Primary Murine Myoblasts**

Myoblasts were prepared from neonatal C57BL/10Sn male mice according to a modified preplate protocol established by Qu-Petersen et al. (22). Myoblasts from preplate 6 (pp6) cultures were expanded and used in the transplants.

**Cell Lines and Reagents**

Monoclonal antibodies to deplete NK1.1+ cells and neutrophils, respectively, were generated from PK136 (αNK1.1) (23) and RB6-8C5 (αGr-1) (24, 25) hybridomas cultured in CD-hybridoma medium (protein-free). To deplete macrophages, elongate containing liposomes (26) and control phosphate-buffered saline (PBS) containing liposomes were used.

**Monoclonal Antibody Purification**

Monoclonal antibodies (mAbs) were purified from the hybridoma culture supernatant by addition of an equal volume of 80% saturated ammonium sulphate, added drop-wise and stirred overnight at 4°C. The precipitate was collected by centrifugation at 1000g for 30 min at 4°C, and the pellet was drained and resuspended in PBS (5% of the original supernatant volume). The solution was dialyzed against daily changes of PBS for 3 days. The dialyzed solution was filter sterilized (0.8-μm filter) and stored in single-use aliquots at −20°C. The protein content of the mAb preparations was determined by spectrophotometry at 280 nm using a Varian DMS 70 spectrophotometer.

**Myoblast Transfer Therapy**

Female host mdx mice (C57BL/10Sn-<sup>mmd</sup>) were anesthetized by intraperitoneal (IP) injection (10 μL/kg body weight) of ketamine (1 mg/mL) and xylazine (10 μg/mL), 0.0%<sup>10</sup> ppm male myoblasts (2.5×10<sup>6</sup> for the NK depletion experiment), and 5 μL HAM's F-10 medium, were injected longitudinally into the tibialis anterior (TA) muscle using a 10-μL Hamilton syringe.

**DNA Extraction and Quantitation Procedure**

DNA was extracted from frozen TA muscle samples and myoblasts using a QiAamp DNA Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Each DNA sample was quantitated using a Fluorescent DNA Quantitation Kit (BioRad, Hercules, CA) according to the manufacturer's instructions.

**Y-Chromosome Quantitative Real-Time Polymerase Chain Reaction**

DNA extracted from transplanted TA muscles was assessed by Y-chromosome-specific real-time quantitative (Q)-PCR with a PE Applied Biosystems 7700 Sequence Detector (Foster City, CA), as described by Byrne et al. (27). SYBR-green amplification reactions were prepared using the QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer instructions. Q-PCR product specificity was ensured by assessment of dissociation curves for each sample. Male DNA quantification was calculated in comparison with a titrated standard curve constructed from male myoblast DNA. Samples were standardized by correction for total DNA quantity.

**Collection and White Blood Cell Isolation from Peripheral Blood**

Mice were anesthetized with Penthrane, and blood was collected through cardiac puncture. The blood was combined with equal volumes of 3 mg/mL EDTA/PBS and 2% Dextran T-500, and the red blood cells (RBCs) were allowed to sediment for 30 min at 37°C. The upper, white blood cell (WBC), fraction was collected and centrifuged for 2 min at 400g. RBCs were lysed by incubation in 0.15 M NaCl in HCl-buffered Tris (pH 7.65) for 5 min at room temperature with occasional inversion, and the cell suspension was centrifuged for 2 min at 400g. Purified WBCs were washed twice in PBS and resuspended once in PBS (supplemented with 5% fetal calf serum and 5 mM Na<sub>2</sub>EDTA) before staining for flow cytometric analysis.

**Preparation of Spleen Lymphocyte Suspensions**

Spleens were collected and pressed through fine wire mesh into Roswell Park Memorial Institute (RPMI)-1640. RBCs were lysed, as described previously, lysis was stopped by the addition of 5 mL RPMI-1640 (Gibco BRL, Gaithersburg, MD) supplemented with 20% newborn calf serum (Gibco BRL), and cells were washed twice in RPMI-1640. WBC pellets were resuspended in RPMI-1640 and counted in a Neubauer chamber using trypan blue exclusion to visualize viable cells.

**Fluorescence-Activated Cell Sorter Staining of White Blood Cell Suspensions**

1×10<sup>6</sup> WBCs were pelleted by centrifugation at 1,000 rpm for 5 min at 4°C and incubated in 50 μL primary antibody (1/100 dilution) (anti-NK1.1-phycocerythrin conjugated [PK136]; anti-Gr-1-fluorescein isothiocyanate conjugated [RB6-8C5]; anti-T-cell receptor β antigen-presenting cell conjugated [H57-557]), BD Pharmingen, San
Diogo, CA) for 20 min at 4°C. Cells were washed twice in 1× PBS (supplemented with 5% fetal calf serum and 5 mM NaN₃) and analyzed using a FACScalibur flow cytometer with CELLQuest software (Becton Dickinson, Franklin Lakes, NJ).

**Immunohistochemistry**

Frozen sections (10 μm) were blocked for 20 min with 10% normal goat serum and 0.003% H₂O₂ in PBS buffered saline (PBS). Macrophages were demonstrated by incubation with P480 hybridoma supernatants (HB-196) for 90 min, biotinylated goat anti-rat immunoglobulin-G (L/200) (BD Pharmingen) for 30 min, and streptavidin HRP (BD Pharmingen) for 30 min. Detection was by the addition of diaminobenzidine substrate (Sigma, St. Louis, MO). All incubations were at room temperature in a humid chamber. Sections were counterstained with 20% hematoxylin. Negative controls omitted the primary antibody.

**RESULTS**

**Defining 100% Donor Cell Survival**

The initial definition of 100% donor myoblast survival has varied in previous studies: Some groups base this quantity on the amount of male DNA extracted from an equivalent number of male donor myoblasts (pretransplantation), and other groups define 100% donor myoblast survival based on the male DNA recovered from an injected muscle immediately after transplantation (i.e., at time 0). Our results demonstrate that there are indeed significant differences in the amount of male DNA that can be extracted in these two situations.

The amount of male DNA obtained from 5.0×10⁶ male myoblasts alone and the amount of male DNA obtained from the same number of male myoblasts when extracted in the presence of a whole female TA muscle was assessed by Q-PCR. The amount of male DNA measured from extractions containing female muscle tissue were approximately 10% of the amount of male DNA extracted from myoblasts alone (Fig. 1A). This 10-fold reduction in the amount of male DNA detected when compared with myoblasts alone is reproducible and highlights the need for an appropriate reference standard for 100% survival of male DNA. If a myoblast standard alone is used, a 90% loss of male DNA would appear instan-

taneously, a scenario that has been interpreted as rapid death (5, 17). It is therefore appropriate to set 100% recovery of male DNA as the amount that can be recovered in the presence of female muscle tissue at time 0. No male DNA signal resulted from extraction of DNA from female TA muscle.

Further experimentation assessed the issue of masking of the male DNA by the excess female DNA present in the MTT extraction mixture. A standard curve of male DNA (0.01--100 ng) extracted from cultured myoblasts was mixed with constant excess amounts of female DNA (100 ng). Samples of these mixtures were then assessed by Q-PCR to determine the specificity of the PCR for the male DNA and the difference between the amounts of male DNA detected in the mixture with female DNA when compared with the amount detected in standard curves generated from male DNA alone. No significant difference in the amount of male DNA detected was observed when mixed with female DNA or when assessed alone (Fig. 1B).

**Systemic Neutrophil Depletion Does Not Improve the Survival of Donor Myoblasts after Myoblast Transfer Therapy**

Neutrophils were depleted from recipient mdx mice by mAb therapy, using the anti-Gr-1 mAb purified from the RB6-8C5 hybridoma cell line. Host neutrophil depletion was achieved by IP injection of 40 μg of the mAb administered 24 hr before MTT and continued every 48 hr until day 5 post-MTT. Control mice received an injection of isotype control antibody in the same regimen. Depletion was assessed by flow cytometric and histologic analyses (Fig. 2A–C). WBCs isolated from peripheral blood and splenocytes of anti-Gr-1 mAb-treated and control mice were stained with fluorescein isothiocyanate-conjugated Gr-1 mAb, and neutrophil depletion was analyzed by flow cytometry (Fig. 2A,B). Positive identification of the depleted population was performed by histology of cytospin preparations of fluorescence-activated cell sorted cells (Fig. 2C). Hematoxylin-eosin staining of the sorted high side-scatter and high GR-1 population confirmed that the cells being targeted by the depletion regimen

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**Figure 1.** Presence of female muscle tissue, but not female DNA, alters the quantitation of male DNA after myoblast transfer therapy (MTT). (A) Real-time quantitative polymerase chain reaction (Q-PCR) assessment of male DNA in total DNA extractions from female muscle, female muscle with 5.0×10⁶ male myoblasts, and 5.0×10⁶ male myoblasts only. (B) Female DNA does not mask the detection of male DNA in the real-time Q-PCR reaction. Comparison of standard curves constructed from male DNA alone (○) or varying amounts (0.01–100 ng) of male DNA mixed with of a constant amount (100 ng) of female DNA (●). Cycle threshold is the number of cycles in the PCR reaction at which specific product is detected during the real-time PCR.

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were indeed neutrophils on the basis of their characteristic morphology, showing multi-lobed and “doughnut” nuclei. Examination of peripheral blood smears by light microscopy confirmed the neutropenia in the anti-Gr-1–treated mice where neutrophil numbers decreased by more than 80% within 12 hr of the first anti-Gr-1 treatment compared with numbers in the isotype-treated controls (data not shown). The duration of neutrophil depletion after the first injection of anti-Gr-1 mAb was 5 days, after which time neutrophil numbers rapidly rebounded to slightly higher than pretreatment levels (~120%) before returning to normal circulating levels (data not shown). The transient neutropenia induced by the anti-Gr-1 treatment is consistent with findings of earlier studies (24, 25).

Twenty-four hours after the commencement of mAb therapy, MTT was performed and muscles were sampled at time 0, 1 and 24 hr, and 3 and 5 days posttransplantation for Q-PCR analysis of extracted DNA (Fig. 2D). Myoblast survival after transplantation into neutrophil-depleted recipients was not improved in comparison with control-treated recipients. At 3 days posttransplant, a small statistically significant increase in male DNA recovered was noted \( P=0.0154 \) in the neutrophil-depleted group, which was not sustained at 5 days. Notably, no significant difference in myoblast survival (as measured by male DNA) was noted between time 0 and time 1 hr posttransplantation in the control or treated mice. These results do not support the belief of rapid death and loss of male cells within minutes of transplantation. However a 40% to 46% decrease in recovered male DNA, in relation to the 1-hr time point, is clearly apparent 24 hr posttransplantation.

**Systemic Macrophage Depletion Does Not Improve the Survival of Donor Myoblasts after Myoblast Transfer Therapy**

Systemic macrophage depletion can be achieved by treatment with cladronate liposomes (26). Recipient host mice...
were injected with clodronate or PBS (control) liposomes IP (200 μL) or through the footpad (20 μL) 5 days before MTT. Such a depletion regimen resulted in a systemic clearance of macrophages for up to 3 weeks. The depletion was confirmed by immunohistochemistry of spleen (Fig. 3) and lymph node tissue (data not shown). Sections were prepared from frozen spleen and lymph node tissue and stained with F4/80 mAb to visualize macrophages. In PBS liposome-treated sections, strong F4/80 positive staining was observed within the red pulp of the spleen (Fig. 3A), whereas after clodronate liposome treatment, few F4/80 positive cells were observed (Fig. 3B), indicating efficient clearance of macrophages from these tissues. The systemic depletion of macrophages after treatment with clodronate liposomes is consistent with other studies (26, 28).

MTT was performed at day 5 after liposome treatment, and muscles were sampled for Q-PCR analysis at time 0, 1 hr, 24 hr, 3 days, 7 days, and 3 weeks posttransplant (Fig. 3C). No significant difference in myoblast survival was observed between clodronate liposome-treated and PBS liposome-treated groups at any of the time points investigated. Again, there was no significant myoblast death within the first hour after MTT; however, by 24 hr post-MTT, a clear reduction in the amount of male myoblast DNA recovered in comparison with the 1-hr time point.

**Systemic Natural Killer Cell Depletion Does Not Improve the Survival of Donor Myoblasts after Myoblast Transfer Therapy**

NK cells were depleted from recipient mdx mice by mAb therapy with the anti-NK1.1 mAb purified from the PK-136 hybridoma cell line. Depletion was achieved by IP injection of 770 μg of the mAb administered at 48 and 24 hr before MTT and continued every 48 hr until day 5 post-MTT. Control mice received an injection of isotype control antibody in the same regimen. Depletion was assessed by flow cytometric analysis of splenocytes isolated from control and anti–NK1.1-treated mice stained with phycocyanin-conjugated anti-NK1.1 and antigen-presenting cell-conjugated anti–T-cell receptor β mAbs to stain for NK and NKT cells. Depletion of more than 90% NK cells and more than 75% NKT cells (Fig. 4) occurred within 48 hr of anti-NK1.1 treatment, compared with the isotype control-treated group, and was maintained for at least 7 days (data not shown).

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**Figure 3.** Systemic macrophage depletion does not improve donor myoblast survival after MTT. Macrophages were depleted from recipient female mdx mice by both IP (200 μL) and footpad (20 μL) injection of clodronate liposomes, administered 5 days before MTT. Control mice were administered phosphate-buffered saline (PBS) liposomes in the same regimen. Depletion was assessed by immunohistochemistry of frozen tissue sections (×100 magnification) using F4/80 mAb to identify macrophages in the spleens of clodronate liposome-treated (A) and PBS liposome-treated (B) mice. Groups of female recipient mdx mice (n=5) treated with clodronate liposomes or PBS liposomes were injected with 5.0×10⁶ donor male myoblasts into each TA muscle. Donor myoblast survival was determined by real-time Q-PCR analysis of male DNA extracted from injected muscles collected post-MTT at 0, 1, 24, and 72 hr (T). The 1-week and 3-week time points post-MTT (Twk) (C). Error bars represent the SEM.
Figure 4. Systemic natural killer (NK) cell depletion does not improve donor myoblast survival after MTT. NK cells were depleted from recipient female mdx mice by IP injection of 770 µg purified PK-136 mAb administered at 48 and 24 hr before MTT and every 48 hr after the initial dose up to day 5 post-MTT. Control mice received a similar regimen of isotype control. Depletion was assessed by flow cytometric analysis of NK1.1 expression of splenic WBCs at 48 hr post-antibody administration in control (A) and PK-136–treated (B) mice. NK cells (i.e., NK1.1high/T-cell receptorlow) (R2 rectangle); NKT cells (i.e., NK1.1high/T-cell receptorhigh) (R3 rectangle). Groups of female recipient mdx mice (n=5) treated with NK1.1 mAb or isotype control antibody were injected with 2.5×10^6 donor male myoblasts into each TA muscle. Donor myoblast survival was determined by real-time Q-PCR analysis of DNA extracted from injected muscles collected at 0, 1, 24 hr, 3 and 7 days, and 3 weeks post-MTT (C). The 1- and 3-week time points post-MTT (Twk) (C). Error bars represent the SEM.

MTT was performed 48 hr after initiation of mAb, and muscles were sampled at time 0, 1 hr, 24 hr, 3 days, 7 days, and 3 weeks posttransplant for Q-PCR analysis (Fig. 4C). No significant difference in myoblast survival was observed between control and NK1.1-depleted mice at the immediate time points after transplantation (0 and 1 hr). A small but significant increase in donor myoblast numbers was observed at day 7 posttransplant (P=0.0299); however, at the 3-week time point this difference was no longer apparent.

DISCUSSION

We showed that in the presence of female muscle tissue, extraction of DNA from the donor male myoblasts is significantly compromised (Fig. 1A) compared with the efficiency of donor DNA extraction from the donor myoblasts alone. It therefore follows that MTT data should be compared with a time 0 control in which the donor myoblasts are extracted in the presence of female muscle tissue. The data generated in these studies indicate that there is no rapid and massive loss of donor myoblasts within minutes of the transplantation. In fact, we observed no significant loss of donor myoblasts within 1 hr post-MTT. However, in agreement with many other reports (4, 5, 17) our studies did show a significant loss of the donor cells by 24 hr post-MTT and a continual decline of donor cells remaining in the host muscles over our experimental time frame of 3 weeks post-MTT.

Disparity over the role inflammatory cells play in the loss of donor myoblasts also exists in the literature (7). Depletion of LFA-1, a surface marker expressed on neutrophils, has been shown to increase myoblast survival after transplant (8, 29). Furthermore, neutrophils are one of the first populations of cells present at the graft site after transplantation, peaking in number at 6 hr posttransplant (7). In the present study, depletion of more than 90% of circulating neutrophils persisted for 5 days before numbers returned to normal levels. A significant improvement on donor myoblasts survival (as determined by quantitation of male DNA) occurred at 72 hr post-MTT. However, by 120 hr post-MTT there was no
longer a significant difference between the control and depleted groups, which may be attributable to the transient nature of the mAb-induced neutropenia and the rapid rebound of circulating neutrophils that follows 5 days after initiation of mAb treatment (data not shown). This rebound may result in a significant, albeit delayed, infiltration of neutrophils into the transplanted muscle, thus negating any survival advantage that the donor cells may have had under the depletion regimen at earlier time points after MTT. It should also be noted that our Y-chromosome quantitative analysis does not account for donor cell proliferation, which may complicate the interpretation of differences between treatment and control groups, particularly at later time points when surviving donor cell numbers are low and can more significantly affect donor cell proliferation. The use of tritiated thymidine as a quantitative marker of total donor cells has the virtue that it is not increased by proliferation (unlike the Y-chromosome) (4). However, labeling of donor cells with tritiated thymidine does not accurately quantitate the long-term success of the procedure in repopulating the tissue with donor myoblasts, which is the overall aim of the present experiments.

Macrophages have been shown to be important in transplant rejection with their depletion improving long-term graft survival in a cardiac xenotransplantation model (19). The systemic depletion of macrophages with clodronate liposome chemotherapy was clearly demonstrated in the present study (Fig. 3A,B); however, this had little effect on the survival of donor myoblasts at any of the time points investigated. It is therefore unlikely that the presence of macrophages within the muscles of transplanted mice is a significant contributing factor to the early death of transplanted myoblasts.

Previous results by our research group proposed that NK cells play an important role in the initial death of donor myoblasts (5, 17). In the present study, however, systemic depletion of NK cells did not dramatically improve myoblast survival. Although a small increase in donor myoblast survival was observed in the NK cell-depleted mice at 7 days posttransplant, this increase was not sustained at the 3-week time point (Fig. 4). The differences between these previous reports and the present NK depletion data are significant. Although the NK1.1 mAb depletion regime was common, other aspects of the present study are different. These experimental differences include the transplantation of neonatal pp6 myoblasts (rather than adult early plating primary myoblast cultures), inclusion of immunoglobulin isotype control-treated groups, and major changes to the extraction and quantitation methodologies for male donor cell DNA. The reevaluation of time 0 values in the light of marked differences in DNA extraction efficiency between myoblasts and transplanted muscle tissue (as discussed) was not appreciated previously and further complicated comparisons. Except for the issue of defining 100% survival, our earlier work, using southern analysis (slot-blot apparatus) to quantitate male donor DNA, did not correct for the relative extraction efficiencies of each sample, did not quantitate donor male DNA in comparison with tetrated male DNA standards, and did not run all samples on the same membrane with a single batch of labeled probe. This examination of our southern analysis methodologies has raised concerns about previously published results implicating NK cells in the early death of donor myoblasts (5, 17). The current data support the immunochemical observation by Skuk et al. (7) that few NK cells infiltrate the graft site before day 6 posttransplant, suggesting that the NK cell population is unlikely to play a role in myoblast death until at least this time point posttransplantation.

The real-time PCR–based quantitation used in the present study is more rapid and convenient than the earlier slot-blot–based methodology and offers increased reliability, particularly at later time points when male donor DNA concentrations approach the lower limit of southern analysis sensitivity. A detailed comparison of these two methodologies for male DNA quantitation is the subject of a separate publication (30).

Overall, systemic neutrophil depletion resulted in transient improvement in donor cell survival at 72 hr, whereas NK depletion produced transient improvement at 1 week post-MTT. These transient increases in donor cell survival may reflect the timing of each cell population's infiltration into the graft site and the ensuing period of time in which they exert some effect on donor myoblast survival. However, regardless of the approach taken within this study to improve the survival of donor myoblasts, no significant improvement in donor myoblast survival was observed at early time points (<24 hr), was sustained over multiple time points, or was evident at 3 weeks post-MTT. Inflammation is a complex condition, mediated by cellular and humoral components. It is possible that a combination of factors may require modulation before a significant effect on donor myoblast survival can be sustained. Indeed, others have reported improvements in cardiac xenotransplant survival after Dexamethasone treatment, a nonspecific anti-inflammatory therapy, which significantly reduced the infiltration of NK cells, macrophages, and granulocytes, whereas the specific depletion of NK cells and macrophages did not reproduce the effect (31). Less-specific anti-inflammatory strategies may prove to be more beneficial in enhancement of donor myoblast survival. However, the importance of inflammation itself in myoblast death remains unclear.

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