De Novo Circulating Antidonor’s Cell Antibodies During Induced Acute Rejection of Allogeneic Myofibers in Myogenic Cell Transplantation: A Study in Nonhuman Primates

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**Background.** Transplantation of myogenic cells has potential applications in the treatment of muscle pathologies. Excluding purely autologous cell transplantation, graft viability depends on an adequate control of acute rejection (AR). To contribute in understanding AR in this context, we analyzed whether de novo circulating antibodies against donor’s cells are detected during induced AR of graft-derived myofibers in nonhuman primates. **Methods.** We allotransplanted satellite cell-derived myoblasts in macaques immunosuppressed with tacrolimus. To induce AR of graft-derived myoblasts, we administered tacrolimus for 4 weeks to allow complete myofiber formation, and then we stopped tacrolimus administration. Cell-grafted sites were biopsied at tacrolimus withdrawal and then every 2 weeks and analyzed by histology until AR completion. Blood samples were taken before immunosuppression, at tacrolimus withdrawal and then every 2 weeks and analyzed by histology until AR completion. Blood samples were taken before immunosuppression, at tacrolimus withdrawal and then every 2 weeks and analyzed by histology until AR completion. **Results.** There was an increase of antibodies against the donor’s cells related to AR in all monkeys. This increase was variable in intensity, and preceded, coincided or followed the histological evidence of AR (focal accumulations of lymphocytes) and/or the loss of myofibers of donor origin, and remained until the end of the follow-up (up to 8 weeks after tacrolimus withdrawal). **Conclusions.** Flow cytometry detection of de novo circulating antibodies against the donor’s cells was consistently associated with AR. A clear increase in this antibody detection indicated current or recent AR. Smaller increases in comparison to the preimmunosuppression values were not associated with AR.

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Transplantation of myogenic cells (that is, mononuclear cells with the capacity to form multinucleated myofibers) has potential applications in the treatment of skeletal muscle diseases.1-4 Excluding autologous transplantation, graft viability depends on the control of acute rejection (AR). Because the permanent control of AR is not fully guaranteed in clinical practice due to the limits imposed by the toxicity of the immunosuppression drugs, monitoring of AR is essential to treat it if it occurs, so as to preserve the graft. We previously defined in nonhuman primates the histological features of AR in muscle biopsies after allotransplantation of myogenic cells.5 However, an aspect that until now has not deserved specific studies is the humoral response in this context. Flow cytometry detection of circulating antidonor cell antibodies was used since the early studies of myogenic cell transplantation in mice,6-9 dogs,10 monkeys,11-13 and human patients14-17 to determine the existence or not of AR. However, we lack elements to affirm that there is a relationship between AR and the detection of circulating antidonor cell antibodies in this context.

In the present study, we wanted to contribute in understanding the value of circulating antidonor cell antibodies in the diagnosis of AR of the myofibers formed by the allotransplantation of myogenic cells, using nonhuman primates.18,19 To induce rejection of myofibers, we immunosuppressed monkeys of the genus Macaca with optimal levels of tacrolimus for 4 weeks (to allow complete myofiber formation by the grafted cells) and then we discontinued tacrolimus administration to trigger AR. To monitor the graft by histology in some
monkeys, we labeled the cells with a LacZ reporter gene. To confirm that the immune findings were due to the allo-
genetic context and not to the expression of β-galactosidase (β-Gal), we grafted in other monkeys cells with no genetic 
modification. To monitor the graft in this case, we transplanted 
cells from male donors into females and we detected the Y 
chromosome in the cell-grafted muscles by polymerase chain 
reaction (PCR). Cells used for transplantation were the only 
ones that so far proved to be myogenic in nonhuman pri-
 mates and clinical trials, that is, satellite cell-derived myo-
blasts.3 For sake of simplicity, the word muscle in the rest of the article will be used as equivalent of skeletal muscle.

**MATERIALS AND METHODS**

**Animals**

Seven cynomolgus monkeys (Macaca fascicularis) (Table 1) 
received transplantation of allogeneic myoblasts. For 
transplantation and biopsies, they were kept under general 
anesthesia using isoflurane (1.5%-2% in oxygen) after 
induction with intramuscular ketamine (10 mg/kg) and 
glycopyrrolate (0.03 mg/kg). Buprenorphone (0.01 mg/kg twice 
a day for 3 days) was given for postoperative analgesia. 
Monkeys were controlled daily, and weight was measured 
one a week. Hematological and biochemical analyses were 
performed as needed. Because many muscular biopsies had 
to be performed throughout the study, euthanasia was done 
at the end of the experiment by intravenous administration 
of a pentobarbital overdose (120 mg/kg) after anesthesia using 
intramuscular ketamine (15 mg/kg). The Laval University 
Animal Care Committee authorized these procedures.

**Cell Culture**

For transplantation of β-Gal+ myoblasts we used frozen 
cells transduced with the LacZ reporter gene and previously 
obtained in our laboratory from a cynomolgus monkey. An-
other cell line was proliferated without genetic manipulation 
from a muscle biopsy performed in one of the male monkeys 
included in the study (Table 1, monkey 3). In both cases, 
muscle samples were minced with fine scissors into fragments 
of less than 1 mm3 and then dissociated with 0.2% 
collagenase (Sigma, St. Louis, MO) in Hank balanced salt 
solution (HBSS) (Gibco, Grand Island, NY) for 1 hour, 
followed by another dissociation in 0.125% trypsin (Gibco) 
in HBSS for 45 minutes. The cells were subcultured in 
molecular, cellular, and developmental biology-120 culture 
medium29 with 15% fetal bovine serum (FBS) (Hyclone, 
Logan, UT), 10 ng/mL basic fibroblast growth factor (Feldan, 
St Laurent, Canada), 0.5 mg/ml bovine serum albumin 
(Sigma), 1.0 μM dexamethasone (Sigma), and 5 μg/mL 
human insulin (Sigma). β-Gal + cells were produced by 
in vitro infection with a replication-defective retroviral vector 
LNPoZC721 (gift from Dr. Constance Cepko, Harvard 
University, Boston, MA), encoding a LacZ reporter gene 
and a neomycin-resistance gene, and including a viral long 
terminal repeat and an internal poliovirus 5′-nontranslated 
region. They were selected with 600 μg/mL Geneticin 
(Invitrogen, Burlington, Canada), proliferated until confluence, 
and frozen for storage in liquid nitrogen. Myoblasts 
without genetic modification were stored in liquid nitrogen 
after the first passage. In preparation for transplantation, 
frozen cells were thawed and proliferated during 1 or 2 
passages in culture. A sample of cells was incubated with a 
phycoerythrin-coupled antilcluster of differentiation (CD)56 
antibody (Beckman Coulter, Fullerton, CA) and analyzed 
by flow cytometry. Ninety-nine percent (β-Gal+) and 98% 
(unlabeled cells) cells were CD56+ (a marker of myoblasts), 
respectively.

For transplantations, cells were detached from the flasks 
using 0.1% trypsin in HBSS and washed 3 times with HBSS. 
Final cell pellets were resuspended in HBSS for injection. Cell 
viability was verified in a sample of cells with a trypan blue 
exclusion method. We added 0.1 mL of a 0.4% trypan blue 
(Sigma) solution to 1 mL of the cell suspension, which was 
loaded in a hemocytometer and examined under an inverted 
microscope. The cell viability was in a range of more than 
99% to 94.1%.

**Cell Transplantation**

Cell transplantation was performed percutaneously by 
parallel equidistant injections placed approximately 1 mm 
apart, perpendicular to the surface of the muscle, using 
27-gauge needles of 0.5 inches. Most transplantations were 
performed with a 250-μL syringe (Hamilton, Reno, NV) 
at-tached to a PB600-1 repeating dispenser (Hamilton)22 in 
sites of approximately 1 cm3. Only one biceps brachium 
in monkey 1 was transplanted with myoblasts throughout 
its volume, using a specific device for repetitive intramuscu-
lar injections.23 The amount of cells injected per cm3 of 
muscle (Table 1) varied with the total number of cells

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**TABLE 1.**

Details of the monkeys included in the study and of the cell transplantations

| Monkey number | Sex   | Age, monthsa | Weight, kga | Origin of | Reporter gene | Amount of living cells injected per site (x 106)b | Total amount of living cells grafted (x 106) | No. sites biopsied/sites of 1 cm3 grafted |
|---------------|-------|--------------|-------------|-----------|--------------|-----------------------------------------------|-----------------------------------------------|------------------------------------------|
| 1             | Male  | 54           | 3.5         | NS19      | LacZ         | 21.5                                          | 343.5                                         | 7/5 + 1 biceps                            |
| 2             | Male  | 54           | 3.4         | NS19      | LacZ         | 26.5                                          | 212                                           | 7/8                                       |
| 3             | Male  | 51           | 7.8         | NS19      | LacZ         | 17.3                                          | 207.6                                         | 7/12                                     |
| 4             | Male  | 63           | 6.6         | NS19      | LacZ         | 23.5                                          | 258.5                                         | 7/11                                     |
| 5             | Female| 46           | 2.4         | Monkey 3  | non          | 29.7                                          | 326.7                                         | 9/11                                     |
| 6             | Female| 44           | 2.4         | Monkey 3  | non          | 29.1                                          | 291                                          | 7/10                                     |
| 7             | Female| 46           | 2.5         | Monkey 3  | non          | 26.6                                          | 266                                          | 9/10                                     |

a Values at transplantation.

b Evaluated by a trypan blue exclusion test before transplantation.

NS19, identification of the cynomolgus monkey donor of the cells.
produced at the time of transplantation. Each injection consisted of needle penetration to full depth and delivery of 5 μL of cell suspension in the needle trajectory. An OpSite sterile transparent dressing with a 5-mm grid (Smith & Nephew, Hull, United Kingdom) was adhered to the skin to control the injection pattern. To identify the injected sites during biopsies, 2 stitches of inert nonabsorbable polypropylene 4.0 suture (Prolene, Ethicon Inc, Somerville, NJ) were placed ~ 5 mm on both sides of each site. Muscles used for transplantations were the left and right biceps brachii, quadriceps femoris, and gastrocnemius. One to 3 sites were grafted per muscle, depending on the muscle size, separated by 0.5 to 1.5 cm.

**Immunosuppression**

An intramuscular formulation of tacrolimus for animal use (a generous gift from Astellas Pharma Inc., Osaka, Japan) was administered intramuscularly once a day, beginning 5 to 7 days before transplantation. Injections were performed in the posterior compartment of the thigh, alternating between one limb and the other. Blood samples were taken by femoral venipuncture at 1-week intervals to quantify tacrolimus blood levels using an IMx tacrolimus II kit for microparticle enzyme immunoassay (Abbott, Wiesbaden, Germany). We targeted the tacrolimus blood levels needed to control AR during the first month after myoblast allotransplantation in macaques (>40 ng/mL). Four weeks after transplantation, tacrolimus administration was discontinued to induce rejection. Because the threshold of blood tacrolimus detection in our clinical laboratory is 3 ng/mL, reports indicating less than 3 ng/mL were considered 0 for calculations.

**Muscle Sampling**

Biopsies were performed in the cell-grafted sites at 4 weeks posttransplantation and then every 2 weeks until no β-Gal+ myofibers (ie, myofibers from donor origin) were observed in at least 2 consecutive biopsies. Each biopsy sampled a different grafted site, alternating the muscles from 1 biopsy to another. Biopsies were mounted in embedding medium and snap frozen in liquid nitrogen. Each biopsy was cut completely in a cryostat, obtaining serial cross-sections of 10 to 15 μm. In each case, 1 of every 20 to 25 sections were placed in glass microscopic slides so as to have a complete biopsy sample, and stored at −80°C for subsequent histological analysis. In biopsies of female monkeys transplanted with male-derived cells, the sections that were not placed on slides were collected in microtubes and stored at −80°C for subsequent deoxyribonucleic acid (DNA) isolation.

**Histological Analysis**

Sections were stained with hematoxylin and eosin (H&E). For histochemical detection of β-Gal, sections were fixed 3 minutes in 0.25% glutaraldehyde, rinsed with phosphate-buffered saline (PBS), incubated 24 hours at room temperature in a solution containing 0.4 mM X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) (Boehringer Mannheim, Vienna, Austria) from a 20-mg/mL stock in dimethylformamide, 1 mM magnesium chloride, 3 mM potassium ferrocyanide, and 3 mM potassium ferricyanide in PBS. Immunodetection of CD8+ and CD4+ lymphocytes was done, respectively, with a mouse antihuman CD8 monoclonal antibody (clone RPA-T8; BD Biosciences, Mississauga, ON, Canada), and a mouse antihuman CD4 monoclonal antibody (clone L200; BD Biosciences). Cross-reactivity with cynomolgus was known by previous studies. Nonspecific binding was blocked by a 30-minute incubation with 10% FBS in PBS. Sections were incubated 1 hour with the primary antibody (1/100), followed by a 30-minute incubation with a biotinylated antimonos antibody (1/150, Dako, Copenhagen, Denmark) and then a 30-minute incubation with streptavidin-Cy3 (1/700, Sigma). Antibodies and streptavidin were diluted in PBS, pH 7.4, containing 1% FBS. Incubations were at room temperature. Muscle sections were analyzed using an Axiohot microscope with epifluorescent and bright field optics (Zeiss, Oberkochen, Germany). The analysis was not blinded.

**PCR Detection of Y Chromosome**

We extracted the total DNA from cryostat sections of the muscle biopsies performed in the 3 female macaques grafted with male myoblasts non–β-Gal-labeled. We also extracted the total DNA from cryostat sections of a muscle biopsy performed on a male macaque as a positive control and a muscle biopsy performed on a female macaque (non grafted site) as a negative control. PCR analysis was done using the following oligonucleotide primers: J130, 5′- CGTGTCTTTTCCCCATGGCTC-3′ (forward) and macaque CDY-2r, 5′-GTTTACCATGGATTCGACCC-3′ (reverse), engineered to amplify a 1610-bp region of the cynomolgus Y chromosome. PCR conditions were as follows: one cycle at 95°C for 10 minutes; then 30 cycles at 95°C for 1 minutes, 66°C for 1 minute, and at 72°C for 1 minute, and one cycle at 72°C for 10 minutes. The PCR products were loaded on 1% agarose gel, and the DNA stained with ethidium bromide was scanned with an AlphaImager digital imaging system, avoiding saturation. RAG gene DNA was detected to control the quality of the extracted DNA. Water (instead of DNA) was also used as a negative control.

**Detection of Antibodies Against the Donor’s Cells**

Blood samples were taken by femoral venipuncture before the beginning of tacrolimus administration (baseline), 4 weeks after transplantation (at the moment of the first biopsy and last tacrolimus administration), and then every 2 weeks during 12 to 14 weeks. The serum was extracted and stored at −20°C to be analyzed at the same time at the end of the experiment. For these tests, myoblasts from the same batch as those transplanted were resuspended in Roswell Park Memorial Institute medium with 10% FBS. A 40-μL sample of this suspension was incubated 1 hour with 20 μL of serum from the transplanted monkey. Thereafter, cells were washed in 4 mL of cold PBS and incubated for 15 minutes at 4°C with 200 μL of 1/40 goat antimouse antibody conjugated to fluorescein isothiocyanate (Immunonjugate, Tilburg, The Netherlands). After several rinses in PBS, labeled cells were analyzed in a flow cytometer at 488 nm.

**Quantitative Analysis**

To quantify the graft in monkeys grafted with LacZ-labeled cells, we estimated the percentage of the sectional area of the biopsy that was β-Gal+. To quantify the lymphocyte infiltration, we quantified the percentage of the sectional area of the biopsy occupied by lymphocyte accumulations. These areas were measured using ImageJ software (NIH Image) J.49, Bethesda, MD). To quantify the level of circulating antidonor cell antibodies in the hosts’ sera, we used the P2 population percentage of the parent population (P1),
gating P2 at 5% (±0.1%) of P1 in the plots corresponding to the baseline. To assess the probability of significant differences, we used an unpaired t test, defining statistical significance as P value less than 0.05. We also assessed whether there was a correlation between the level of circulating antidonor cell antibodies and different variables in relation to the graft or the cellular AR. We represented these values as point cloud graphs, estimating the Pearson correlation coefficient.

RESULTS

Tacrolimus Blood Levels

Tacrolimus blood levels remained above 40 ng/mL between the transplantation and the time of tacrolimus withdrawal 4 weeks later (Figure 1). Thereafter, tacrolimus blood levels gradually decreased as a downward curve until complete disappearance 7 to 9 weeks later (Figure 1, Figure 2).

Evidence of AR

In macaques that received LacZ-labeled myoblasts (monkeys 1 to 4), there was first a period of 4 to 6 weeks after tacrolimus withdrawal in which the myofibers of donor origin were maintained within quite similar values in each monkey (Figures 2 and 3). The mean values for this period (in surface of the grafted region that was β-Gal+) were: 12.3% ± 2% (monkey 1, weeks 0 to 4), 30.4% ± 2.8% (monkey 2, weeks 0 to 6), 32.7% ± 2% (monkey 3, weeks 0 to 4), and 22.9% ± 3.7% (monkey 4, weeks 0 to 4). There was a more or less dramatic decrease of β-Gal at 6 or 8 weeks after tacrolimus withdrawal (Figure 2 and 3). With respect to the average of the preceding β-Gal + surface values, this decrease was of 69.5% (monkey 1, week 6), 82.5% (monkey 2, week 8, illustrated in Figure 2), 41.6% (monkey 3, week 6), and 73% (monkey 4, week 6). The average of β-Gal loss was of 66.6% ± 17.6%. This was followed by a complete absence of β-Gal 2 weeks later, that is, at 8 or 10 weeks after tacrolimus withdrawal (Figures 2 and 3).

In female macaques that received unlabeled male myoblasts, a PCR product corresponding to the Y chromosome was observed up to 6, 8, or 10 weeks after tacrolimus withdrawal (Figure 2, monkeys 5 to 7). The last band detected was more or less weaker than the preceding, and the Y chromosome was not detected in subsequent biopsies. The control PCRs confirmed the presence of an intense band at the same height in the male macaque muscle biopsies (positive control) and the absence of labeling in the female (nongrafted) macaque muscle biopsies (Figure 2).

Considering the 7 monkeys, the graft remained stable 4 to 8 weeks after tacrolimus withdrawal (Figure 2). The lowest tacrolimus blood levels at which the amount of β-Gal+ myofibers and the Y chromosome PCR product were similar to those observed in the first biopsy (thus, a still preserved graft) were in the range of 5.1 to 13.4 ng/mL (mean, 8.7 ± 3.4 ng/mL). There were no significant difference (P = 0.58) between monkeys grafted with β-Gal+ cells (mean, 9.4 ± 4.3 ng/mL) and monkeys grafted with unlabeled cells (mean, 7.8 ± 1.6 ng/mL). Tacrolimus blood levels at which there was significant decrease of the graft were in the range of less than 3 ng/mL (3 monkeys) to 7.2 ng/mL (mean, 2.8 ± 2.8 ng/mL, considering < 3 ng/mL as 0). Tacrolimus blood levels at which the graft was absent were in the range of less than 3 ng/mL (6 monkeys) to 3.4 ng/mL (monkey 2).

Flow Cytometry Detection of Circulating Antidonor Cell Antibodies in the Recipient’s Sera

Analyzing the bar graphs that plot the P2 population in the flow cytometry of the circulating antidonor cell antibodies (red graphs in Figure 2), it can be seen that all monkeys presented 2 phases during the period that started with the tacrolimus withdrawal. In the first phase, values fluctuated at a low level, corresponding, in the first 4 weeks or later, with high tacrolimus blood levels, stable graft, and absence of lymphocyte accumulations. These values were similar, higher or lower than the baseline, and remained in a quite narrow range for each monkey (Figure 2 and Figure 4A). In the second phase, there was an increase in the values of the P2 population, which preceded (2 to 6 weeks), coincided, or followed (2 to 4 weeks) the loss of myofibers of donor origin and/or the peak of lymphocyte infiltration. This increase was more or less intense according to the animal, and remained until the end of the follow-up (up to 8 weeks after the first increase). The first increase of antibody detection in the second phase coincided in some cases with the peak of antibodies and in other cases preceded it (Figure 4). As can be seen in Figure 2 and by the SD in Figure 4A, the values that followed the first increase of antibody detection remained in a quite narrow range for each monkey.

Regardless of whether or not the beginning of the second phase matched chronologically the morphological or PCR evidence of AR, it is clear that in all cases there was a humoral immune response (HIR) involving antibody production against the donor’s cells that was linked to AR. Therefore, we will refer to both phases as pre-HIR and HIR.
FIGURE 2. Correlation of several transplantation parameters in the 7 monkeys of the study. For each monkey (in columns), it is displayed the tacrolimus blood levels (A), the evolution of the graft (B), the lymphocyte accumulations in the biopsies (C), and the detection of antibodies against the donor's myoblasts in the recipient's serum (D). Y-axes are at the same scale for each parameter. X-axes indicate the week after tacrolimus withdrawal. "Pre" is the preimmunosuppression value, that is, before tacrolimus administration. We illustrate flow cytometry results for the presence of antibodies against the grafted myoblasts in the serum of each monkey (E), corresponding to the preimmunosuppression serum ("Pre", in red) and the serum corresponding to the highest value in the graphics (blue, the number inside the histogram indicates the week in the graphic). We show also the value at week 0 in three monkeys in which there were significant differences with preimmunosuppression values. To illustrate the evolution of the graft in monkeys transplanted with β-Gal+ myoblasts (B in monkeys 1 to 4), we estimated the percentage of the sectional area of the muscle fascicles that was β-Gal+. To illustrate the evolution of the graft in monkeys transplanted with unlabeled myoblasts (B in monkeys 5 to 7), we show the Y chromosome (Y chr) PCR products detected by electrophoresis. A control PCR (corresponding to monkey 5) is shown at the right end to confirm the accuracy of Y chromosome detection, ♀, female nongrafted muscle, ♂, male muscle. To quantify the amount of lymphocyte infiltration in the biopsies, we estimated the percentage of the sectional area of the grafted muscle biopsy occupied by lymphocyte accumulations (graphics in C). In monkeys that received transplantation of β-Gal+ myoblasts, the peak of lymphocyte infiltration correlated with the decrease or the disappearance of β-Gal+ myofibers. In monkeys that received allotransplantation of unlabeled myoblasts, the peak of infiltration correlated with a decrease in the intensity of the band corresponding to the Y chromosome or its disappearance. Focal lymphocyte accumulations correlated with the loss of myofibers of donor origin in the 7 monkeys. All monkeys showed an increase of antibodies against the donor's myoblasts, taking as a reference the preimmunosuppression and prerejection values. This increase was highly variable in intensity, and preceded (~2 weeks), coincided or followed (~2 weeks) the focal lymphocyte accumulations and/or the loss of myofibers of donor origin, and remained high thereafter. Levels of antibody detection after the start of immunosuppression were the same or slightly higher or lower than the preimmunosuppression values.
Considering that some pre-HIR values were somewhat higher than baseline (1.9 ± 0.5 fold in monkey 1 and 2.9 ± 0.6 fold in monkey 4), we wondered which difference in the P2 population could be considered as indicative of HIR and potentially diagnostic of AR and whether it was best to take as reference the baseline (preimmunosuppression) values or the posttransplant period before HIR. Normalizing the baseline to 1, the pre-HIR values varied over a range of 0.23 ± 0.07 (monkey 7) and 2.94 ± 0.58 (monkey 4) (mean = 1.35 ± 0.9, Figure 4B). The mean rejection values varied over a range of 4.89 ± 0.36 (monkey 1) and 17.33 ± 5.77 (monkey 5) (mean = 10.52 ± 4.82, Figure 4B). The first HIR value varied over a range of 3.38 (monkey 7) and 17.02 (monkey 3) (mean = 7.84 ± 5.26, Figure 5B). The insets in Q and R show some myofibers surrounded by CD8+ or CD4+ lymphocytes at higher magnification. The myofiber with an asterisk (Q) exhibits invasion by a CD8+ lymphocyte. Scale bars = 0.5 mm (showed in G and N; they apply to A to N since magnification is the same for these images) and 100 μm (O to R).

The strongest correlation ($\rho = 0.784$) was found in the monkeys grafted with β-Gal+ cells when we compared the maximum amount of circulating antidonor cell antibodies in each monkey with the amount of β-Gal+ myofibers in the prerejection period (Figure 5A). A similar correlation ($\rho = 0.762$) was found if the amount of β-Gal+ myofibers was multiplied by the number of grafted sites that were still not biopsied at the time of AR beginning (Figure 5B), which could be approximately extrapolated as the amount of allogeneic myofibers present at the time of AR. The correlation was very weak or null when the maximum amount of circulating antidonor cell antibodies in each monkey was compared with the maximal lymphocyte infiltration ($\rho = 0.23$, Figure 5C), the amount
of viable cells transplanted per kilogram of body weight ($p = 0.11$), the amount of cell-grafted sites of $1 \text{ cm}^3$ ($p = -0.32$), and the amount of cell-grafted sites of $1 \text{ cm}^3$ remaining after the previous biopsies at the beginning of AR ($p = -0.32$). Otherwise, the maximum amount of circulating antidonor cell antibodies in each monkey was not different between monkeys grafted with β-Gal+ cells or unlabeled cells (Figure 5D).

**DISCUSSION**

Because our objective in cell transplantation research is to provide elements of clinical utility, we prioritize studies in nonhuman primates. This is especially important in studies of transplantation immunology, because the functional similarities between human and macaque immune systems are well documented, in contrast with the many differences in the immune system of mice and humans. Importantly, AR in nonhuman primates is driven by the same immune elements than humans, showing comparable histological features and cadence. Indeed, studies in nonhuman primates were important to define the clinical pathology of AR in limb transplantation, in contrast to rats, rabbits, dogs, or pigs.

The primary goal of myogenic cell transplantation is the integration of the grafted cells into the recipient’s myofibers to induce genetic complementation, or the formation of new myofibers. Consequently, our focus was AR of myofibers...
totally or partially derived from the cell graft. The formation of graft-derived myofibers depends on the process of muscle regeneration, which, in macaques, is completed in 4 weeks. Therefore, to allow myofiber regeneration by the grafted myoblasts, we administered tacrolimus for 4 weeks. Then, we discontinued tacrolimus to activate AR. In fact, discontinuation of tacrolimus did not imply a rapid absence of immunosuppression, because tacrolimus blood levels remained elevated and controlled AR for at least 4 weeks more, until they fall below a level unable to inhibit AR. At that point, loss of myofibers of donor origin occurred in all monkeys. Because we observed no differences between monkeys grafted with β-Gal cells and monkeys grafted with unlabelled cells, the study suggests that β-Gal expression did not induce AR reactions different from those due to the allogeneic context of transplantation in this study.

As previously reported, loss of myofibers of donor origin coincided with a characteristic histological picture: focal lymphocyte accumulations composed of CD8+ and CD4+ cells, essentially endomysial, typically surrounding myofibers (partially or completely), and frequently invading some of them. In addition, all monkeys exhibited an HIR consisting of the appearance of de novo circulating antidonor cell antibodies in the body flow cytometry analysis. The amount of these antibodies was very variable from one monkey to another but was always present, which could lead to infer that circulating antidonor cell antibodies production is constant during myofiber AR in myogenic cell transplantation.

Taking into account the large variation in the level of circulating antidonor cell antibodies between the different monkeys, we wanted to see if there was a correlation with graft-related factors or the intensity of the cellular AR. The only correlation that we found (although a larger sample would have been more conclusive) was with (1) the amount of β-Gal + myofibers, that is, myofibers that integrated the grafted cells and expressed allogeneic proteins by genetic complementation and (2) the amount of β-Gal + myofibers multiplied by the number of grafted sites that were still not biopsied at the time of rejection beginning (which was used as a way of quantifying approximately the amount of allogeneic myofibers present at the time of rejection). In the first instance, this could mean that the level of de novo circulating antidonor cell antibodies, in the event of rejection, will depend on the amount of allogeneic myofibers present in the host. A factor that we were unable to study was the correlation with the histocompatibility disparity between hosts and donors. This absence is frequent in transplantation studies in nonhuman primates, which is explained by the complexity of the major histocompatibility complex in macaques.

Therefore, the present study supports that the detection of circulating antidonor cell antibodies by flow cytometry could be a reliable test to monitor AR of the graft-derived myofibers after myogenic cell allotransplantation. This is an easy test to perform in a cell transplantation context, because a sample of the grafted cells can be kept frozen after their preparation for transplantation, and thawed before each test to be briefly proliferated in vitro for flow cytometry analysis. Beyond the potential interest to monitor AR in this context, it would be interesting to investigate in the future whether the circulating antidonor cell antibodies are harmful to the myogenic cell graft, that is, whether there is an antibody-mediated rejection. In solid organ transplantation, antibodies are considered as a major component of graft failure, depending mostly in the interaction with endothelial cells. This causes cell death by complement-dependent and -independent pathways, leading to loss of vascular integrity in the transplanted organ and subsequent injury. Because in myogenic cell transplantation the vasculature remains that of the host, this antibody-mediated vasculopathy a priori would not occur, and therefore the only effect of the antidonor antibodies, if any, should be on the myofibers as well as on the satellite cells that can derive from the grafted myoblasts. In the absence of vasculopathy, we can assume that in this cell transplantation, the problem of chronic rejection would occur, which, however, remains to be confirmed. Another issue that would be interesting to investigate is whether the development of de novo circulating antidonor cell antibodies may prevent further successive cell transplants in a sensitized recipient. We plan future studies to analyze these topics in nonhuman primates.

REFERENCES

1. Vilquin JT, Catelain C, Vauchez K. Cell therapy for muscular dystrophies: advances and challenges. Curr Opin Organ Transplant. 2011;16: 640–649.
2. Briggs D, Morgan JE. Recent progress in satellite cell/myoblast engraftment—relevance for therapy. Febs J. 2013;280:4281–4293.
3. Skuk D, Tremblay JP. Cell therapy in muscular dystrophies: many promises in mice and dogs, few facts in patients. Expert Opin Biol Ther. 2015: 1–13.
4. Negroni E, Bigot A, Butler-Browne GS, et al. Cellular therapies for muscular dystrophies: frustrations and clinical successes. Hum Gene Ther. 2016; 27:117–126.
5. Skuk D. Acute rejection of myoblasts in nonhuman primates: key histopathologic features. J Neuropathol Exp Neurol. 2012;71:398–412.
6. Vilquin JT, Kinosita I, Roy R, et al. Cyclophosphamide immunosuppression does not permit successful myoblast allotransplantation in mouse. Neuromuscul Disord. 1996;5:511–517.
7. Vilquin JT, Wagner E, Kinosita I, et al. Successful histocompatible myoblast transplantation in dystrophin-deficient mdx mouse despite the production of antibodies against dystrophin. J Cell Biol. 1995;131:975–988.
8. Boulanger A, Asselin I, Roy R, et al. Role of non-major histocompatibility complex antigens in the rejection of transplanted myoblasts. Transplantation. 1997;63:893–899.
9. Vilquin JT, Asselin I, Guerette B, et al. Myoblast allotransplantation in mice: degree of success varies depending on the efficacy of various immunosuppressive treatments. Transplant Proc. 1994;26:3372–3373.
10. Ito H, Vilquin JT, Skuk D, et al. Myoblast transplantation in non-dystrophic dog. Neuromuscul Disord. 1998;8:95–110.
11. Kinosita I, Roy R, Dugre FJ, et al. Myoblast transplantation in monkeys: control of immune response by FK506. J Neuropathol Exp Neurol. 1990;55:687–697.
12. Skuk D, Goulet M, Roy B, et al. Efficacy of myoblast transplantation in nonhuman primates following simple intramuscular cell injections: toward defining strategies applicable to humans. Exp Neurol. 2002;175:112–126.
13. Skuk D, Goulet M, Roy B, et al. Myoblast transplantation in whole muscle of nonhuman primates. J Neuropathol Exp Neurol. 2000;59:197–206.
14. Huard J, Roy R, Bouchard JP, et al. Human myoblast transplantation between immunohistocompatible donors and recipients produces immune reactions. Transplant Proc. 1992;24:3049–3051.
15. Tremblay JP, Bouchard JP, Malouin F, et al. Myoblast transplantation between monozygotic twin girl carriers of Duchenne muscular dystrophy. Neuromuscul Disord. 1993;3:583–592.
16. Skuk D, Goulet M, Roy B, et al. Duchenne muscular dystrophy patients after high-density injections of normal myogenic cells. J Neuropathol Exp Neurol. 2006;65:371–386.
17. Miller RG, Sharma KR, Pavlath GK, et al. Myoblast implantation in Duchenne muscular dystrophy: the San Francisco study. Muscle Nerve. 1997;20:469–478.
18. Kitchens WH, Adams AB. Nonhuman primate models of transplant tolerance: closer to the holy grail. Curr Opin Organ Transplant. 2016;21: 50–53.
19. Kean LS, Singh K, Blazar BR, et al. Nonhuman primate transplant models finally evolve: detailed immunogenetic analysis creates new models and strengthens the old. *Am J Transplant*. 2012;12:812–819.

20. Ham RG, St. Clair JA, Webster C, et al. Improved media for normal human muscle satellite cells: serum-free clonal growth and enhanced growth with low serum. *In Vitro Cell Dev Biol*. 1988;24:633–844.

21. Adam MA, Ramesh N, Miller AD, et al. Internal initiation of translation in retroviral vectors carrying picornavirus 5′ nontranslated regions. *J Virol*. 1991;65:4985–4990.

22. Skuk D, Goulet M, Tremblay JP. Use of repeating dispensers to increase the efficiency of the intramuscular myogenic cell injection procedure. *Cell Transplant*. 2006;15:659–663.

23. Richard PL, Gosselin C, Laliberté T, et al. A first semiautomatic device for clinical intramuscular repetitive cell injections. *Cell Transplant*. 2010;19:67–78.

24. Kostova E, Rottger S, Schempp W, et al. Identification and characterization of the cynomolgus monkey chromodomain gene cynCDY, an orthologue of the human CDY gene family. *Mol Hum Reprod*. 2002;8:702–709.

25. Slierendregt BL, van Noort JT, Bakas RM, et al. Evolutionary stability of transspecies major histocompatibility complex class II DRB lineages in humans and rhesus monkeys. *Hum Immunol*. 1992;35:29–39.

26. Geluk A, Ellerink DG, Slierendregt BL, et al. Evolutionary conservation of major histocompatibility complex-DR/peptide/T cell interactions in primates. *J Exp Med*. 1993;177:979–987.

27. Jaeger EE, Bontrop RE, Lanchbury JS. Structure, diversity, and evolution of the T-cell receptor VB gene repertoire in primates. *Immunogenetics*. 1994;40:184–191.

28. Levivison G, Hughes AL, Letvin NL. Sequence and diversity of rhesus monkey T-cell receptor beta chain genes. *Immunogenetics*. 1992;35:75–88.

29. Rose SM, Blustein N, Rotrosen D. Recommendations of the expert panel on ethical issues in clinical trials of transplant tolerance. National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Transplantation. 1998;66:1123–1125.

30. Kirk AD. Transplantation tolerance: a look at the nonhuman primate literature in the light of modern tolerance theories. *Crit Rev Immunol*. 1999;19:349–388.

31. Kirk AD. Crossing the bridge: large animal models in translational transplantation research. *Immunol Rev*. 2003;196:176–196.

32. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol*. 2004;172:2731–2738.

33. Gibbons DL, Spencer J. Mouse and human intestinal immunity: same ballpark, different players; different rules, same score. *Mucosal Immunol*. 2011;4:148–157.

34. Cendales LC, Xu H, Bacher J, et al. Composite tissue allotransplantation: development of a preclinical model in nonhuman primates. *Transplantation*. 2005;80:1447–1454.

35. Skuk D, Tremblay JP. Intramuscular cell transplantation as a potential treatment of myopathies: clinical and preclinical relevant data. *Expert Opin Biol Ther*. 2011;11:359–374.

36. Skuk D, Goulet M, Tremblay JP. Electroporation as a method to induce myofiber regeneration and increase the engraftment of myogenic cells in skeletal muscles of primates. *J Neuropathol Exp Neurol*. 2013;72:723–734.

37. Skuk D, Tremblay JP. Necrosis, sarcolemmal damage and apoptotic events in myofibers rejected by CD8+ lymphocytes: observations in nonhuman primates. *Neuromuscul Disord*. 2012;22:997–1005.

38. Garces JC, Giusti S, Staffeld-Coit C, et al. Antibody-mediated rejection: a review. *Ochsner J*. 2017;17:46–55.

39. Gloor J, Cosio F, Lagier DJ, et al. The spectrum of antibody-mediated renal allograft injury: implications for treatment. *Am J Transplant*. 2008;8:1367–1373.

40. Skuk D, Paradis M, Goulet M, et al. Intramuscular transplantation of human postnatal myoblasts generates functional donor-derived satellite cells. *Mol Ther*. 2010;18:1689–1697.