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

Daniel Skuk, MD and Jacques P. Tremblay, PhD

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 myofibers, 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 to detect antibodies against the donor's cells by flow cytometry. **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 patients[14-17] 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

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Axe Neurosciences, Research Center of the CHU de Quebec-CHUL, Quebec, QC, Canada.

Correspondence: Daniel Skuk, MD, Axe Neurosciences, P-09300, CHUL, 2705 Blvd., Laurier, Québec, Canada G1V 4G2. (daniel.skuk@crchudequebec.ulaval.ca).

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monkeys, we labeled the cells with a *LacZ* reporter gene. To confirm that the immune findings were due to the allogeneic context and not to the expression of ß-galactosidase (ß-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 transplantations were the only ones that so far proved to be myogenic in nonhuman primates and clinical trials, that is, satellite cell-derived myoblasts.[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 isofluorane (1.5%-2% in oxygen) after induction with intramuscular ketamine (10 mg/kg) and glycopyrrolate (0.05 mg/kg). Buprenorphine (0.01 mg/kg twice a day for 3 days) was given for postoperative analgesia. Monkeys were controlled daily, and weight was measured once 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 ß-Gal+ myoblasts we used frozen cells transduced with the *LacZ* reporter gene and previously obtained in our laboratory from a cynomolgus monkey. Another 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 mm^3 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 medium[20] 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). ß-Gal + cells were produced by in vitro infection with a replication-defective retroviral vector LNPoZC7[21] (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 anticluster of differentiation (CD)56 antibody (Beckman Coulter, Fullerton, CA) and analyzed by flow cytometry. Ninety-nine percent (ß-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) attached to a PB600-1 repeating dispenser (Hamilton)[22] in sites of approximately 1 cm^3. Only one *biceps brachium* in monkey 1 was transplanted with myoblasts throughout its volume, using a specific device for repetitive intramuscular injections.[23] The amount of cells injected per cm^3 of muscle (Table 1) varied with the total number of cells

TABLE 1.

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

Monkey number	Sex	Age, months[a]	Weight, kg[a]	Origin of grafted cells	Reporter gene	Amount of living cells injected per site ($\times 10^6$)[b]	Total amount of living cells grafted ($\times 10^6$)	No. sites biopsied/sites of 1 cm^3 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).[5] 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 as 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 –20°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 ß-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-ß-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.[5] 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 antimouse 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 Axiophot 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–ß-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 with the following oligonucleotide primers[24]: J130, 5′-CGTGTCTTTCCTCATGGCTTC-3′ (forward) and macaque CDY-2r, 5′-CTTTACCATGGATTCGACCC-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 antimonkey antibody conjugated to fluorescein isothiocyanate (Immunoconjugate, 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 ß-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 ImageJ 1.49v, 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 ß-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 ß-Gal at 6 or 8 weeks after tacrolimus withdrawal (Figure 2 and 3). With respect to the average of the preceding ß-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 ß-Gal loss was of 66.6% ± 17.6%. This was followed by a complete absence of ß-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 ß-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 ß-Gal+ cells (mean, 9.4 ± 4.5 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).

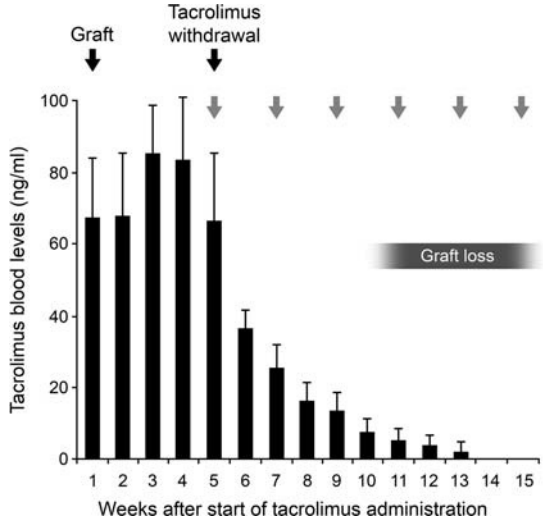

FIGURE 1. Tacrolimus blood levels and graft rejection in the study. Bars correspond to the mean of n = 7 monkeys + 1 SD. During the first 5 weeks, target levels were above 40 ng/mL, in accordance with our previous experience with this type of transplant in monkeys. Tacrolimus blood levels gradually declined after cessation of tacrolimus administration, until disappearing 9 weeks later in all monkeys (for this graph, the values reported as < 3 ng/mL were considered as 0). Gray arrows indicate the first 6 biopsies and blood samplings for antibody detection. The zone in which the graft was lost (determined by the decrease and posterior absence of ß-Gal + myofibers in monkeys 1 to 4 and of Y chromosome PCR product in monkeys 5 to 7) is indicated approximately.

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.

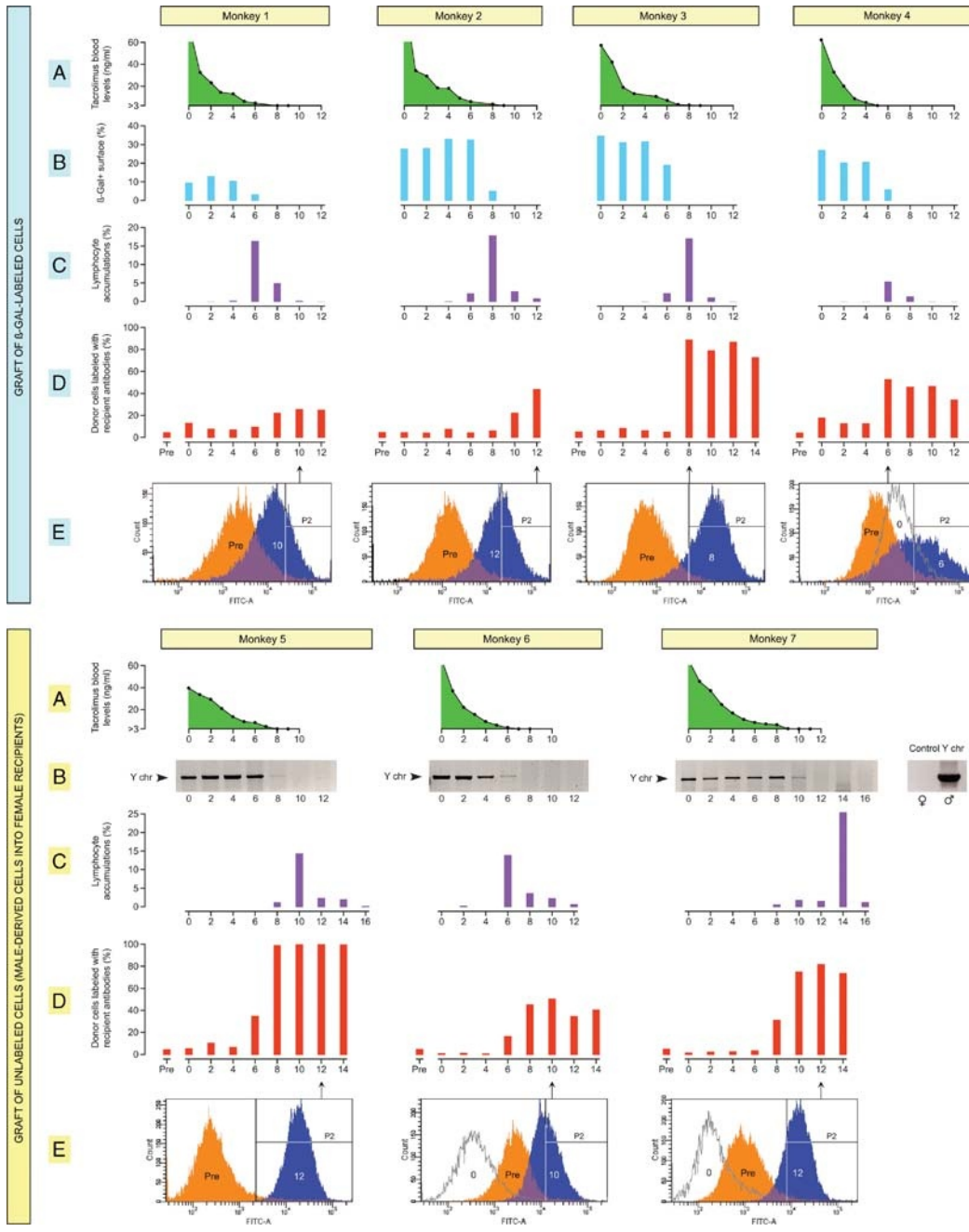

FIGURE 2. Correlation of several transplantation parameters in the 7 monkeys of the study. For each monkey (in columns), it is displayed the tacrolimus blood levels (A), the evolution of the graft (B), the lymphocyte accumulations in the biopsies (C), and the detection of antibodies against the donor's 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 ß-Gal+ myoblasts (B in monkeys 1 to 4), we estimated the percentage of the sectional area of the muscle fascicles that was ß-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 ß-Gal+ myoblasts, the peak of lymphocyte infiltration correlated with the decrease or the disappearance of ß-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.

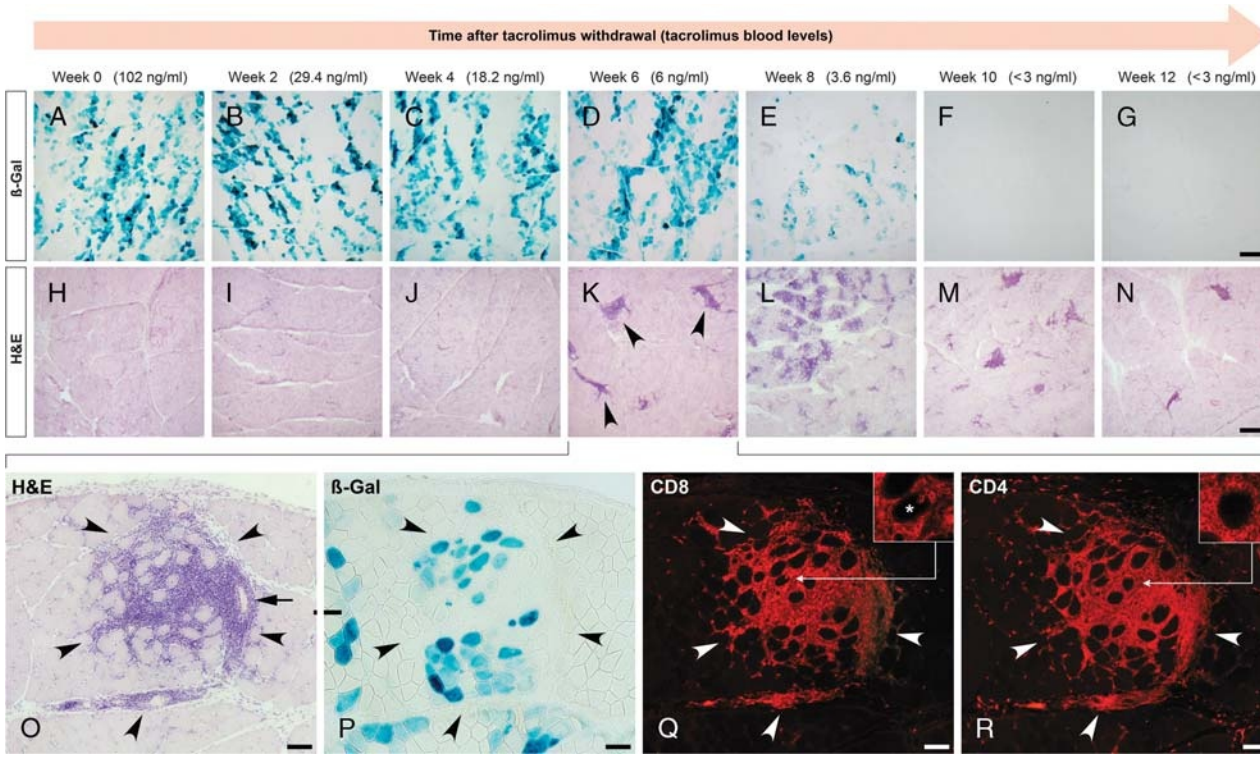

FIGURE 3. Histological demonstration of AR in a monkey transplanted with ß-Gal+ cells (monkey 2), through the complete sequence of biopsies, showing cross-sections of the cell-grafted sites (A to N). The weeks after tacrolimus withdrawal are indicated in the top with the tacrolimus blood levels at each period. For each period, serial sections are shown stained for demonstration of ß-Gal+ myofibers in the first row (A to G) and with hematoxylin and eosin (H&E) to observe the focal accumulations of lymphocytes characteristic of myofiber rejection in the second row (H to N). Many ß-Gal+ myofibers are observed from week 0 to week 6 (A to D). Focal lymphocyte accumulations are absent from week 0 to 4 (H to J) and appear at week 6 (K, basophilic spots, arrowheads). The amount of ß-Gal+ myofibers decreased noticeably in week 8 (E) concomitantly with a strong infiltration by focal lymphocyte accumulations (L, basophilic spots). No ß-Gal+ myofibers were observed at weeks 10 and 12 (F and G), whereas some focal lymphocyte accumulations remain, decreasing from week 10 to 12 (M and N, basophilic spots). The lower row (O to R) shows serial cross-sections of a region corresponding to the biopsy taken at week 6, to illustrate the main characteristics of the focal lymphocyte accumulations associated to rejection (between arrowheads) at higher magnification. Sections are stained with H&E (O), for ß-Gal histochemical detection (P) and for red fluorescent immunodetection of CD8+ (Q) and CD4+ (R) lymphocytes. As observed with H&E (O), the focal lymphocyte infiltration is linked to perimysial blood vessels (one indicate with an arrow) and infiltrates a muscle bundle surrounding completely or partially several myofibers, among which several are ß-Gal+ in the serial section (P). These lymphocytes are CD8+ (Q) and CD4+ (R). 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).

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 = 7.58 ± 4.75, Figure 4B). If baseline is taken as reference, the highest value among the first pre-HIR values (at tacrolimus withdrawal) represented a 3.61-fold increase (monkey 4), whereas the lowest first increase in the HIR period was of 3.39-fold (monkey 7).

Normalizing the average of the prerejection values to 1, the mean rejection values varied over a range of 2.66 ± 0.52 (monkey 4) and 37.9 ± 5.95 (monkey 7) (mean, 15.26 ± 13.6). The first rejection value varied over a range of 2.35 (monkey 1) and 14.65 (monkey 7) (mean, 7.84 ± 5.26).

Correlation Between Antibody Detection and the Graft or the Cellular AR

We wanted to analyze if there was a correlation between the intensity of the circulating antidonor cell antibodies increase and factors related to the graft or the cellular AR. The strongest correlation ($\rho = 0.784$) was found in the monkeys grafted with ß-Gal+ cells when we compared the maximum amount of circulating antidonor cell antibodies in each monkey with the amount of ß-Gal+ myofibers in the prerejection period (Figure 5A). A similar correlation ($\rho = 0.762$) was found if the amount of ß-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

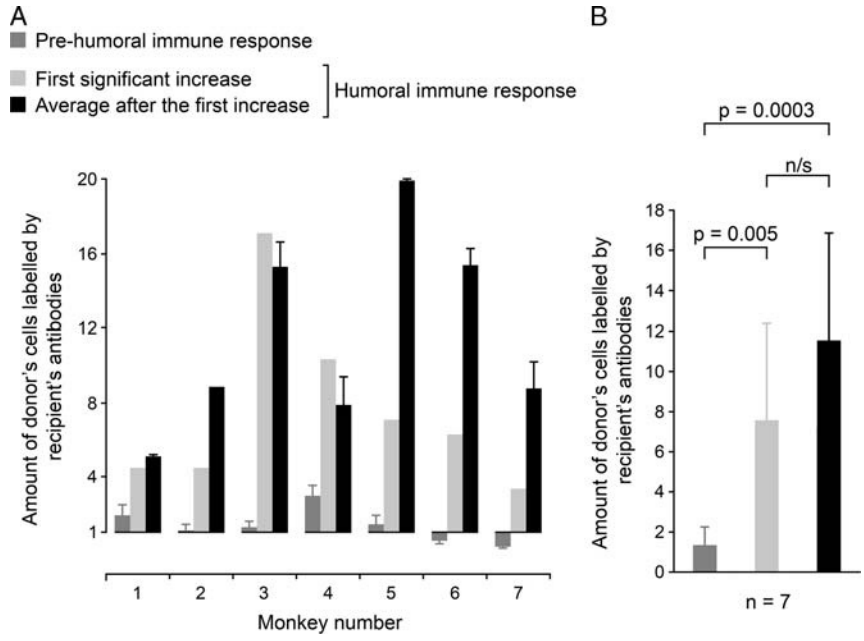

FIGURE 4. Bar graphs illustrating the different levels of antibodies against the donors' cells detected by flow cytometry in the recipients' sera, quantified by the P2 population percentage of the parent population, during the period that started with the tacrolimus withdrawal. Graph in A separates in each monkey the values corresponding to the "prerejection" period (mean of n = 3 to 5 + 1 SD), the first significant increase of the "rejection" period (single value), and the average of the rest of the rejection period (mean of n = 3 to 4 + 1 SD, except monkey 2 in which is a single value). The baseline was normalized to 1 and the values in the graph are shown consequently. Values corresponding to the "prerejection" period fluctuate in a low level, remained in a fairly constant range and were similar, higher or lower than the baseline. The values that followed the first significant increase of antibody detection remained in a fairly constant range for each monkey. The graph in B summarizes the previous values as a mean + 1 SD of the averages or single values in A. The values of the periods considered "prerejection" and "rejection" constitute 2 variable but significantly different populations.

of viable cells transplanted per kilogram of body weight ($\rho = 0.11$), the amount of cell-grafted sites of 1 cm^3 ($\rho = -0.32$), and the amount of cell-grafted sites of 1 cm^3 remaining after the previous biopsies at the beginning of AR ($\rho = -0.32$). Otherwise, the maximum amount of circulating antidonor cell antibodies in each monkey was not different between monkeys grafted with ß-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,[25-31] in contrast with the many differences in the immune system of mice and humans.[32,33] Importantly, AR in nonhuman primates is driven by the same immune elements than humans, showing comparable histological features and cadence.[31] 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.[34]

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.[35] Consequently, our focus was AR of myofibers

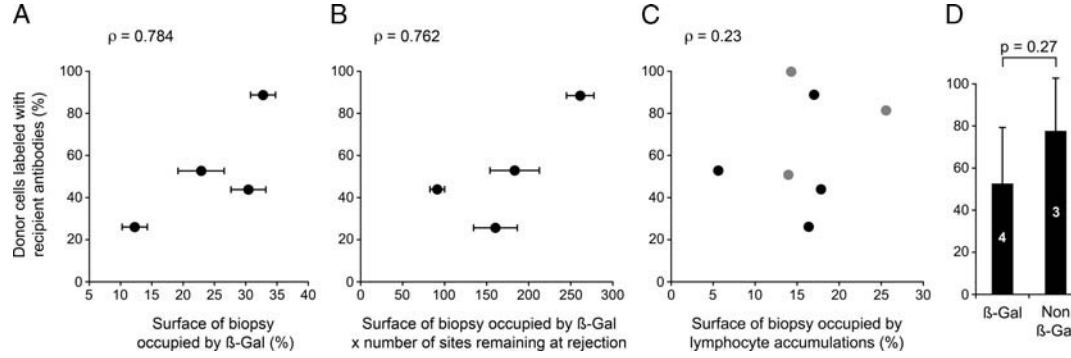

FIGURE 5. Correlation between the level of antibody detection and factors related to the graft or the cellular AR. The strongest correlation was found comparing the maximum amount of antibodies detected in the monkeys grafted with ß-Gal+ cells and the amount of ß-Gal+ myofibers in the prerejection period (A). A similar correlation was found if the amount of ß-Gal+ myofibers was multiplied by the number of grafted sites that were still not biopsied at the time of rejection beginning (B). As an example of weak correlation, we show the maximum amount of antibodies detected in each monkey compared with the surface of biopsy occupied by lymphocyte accumulations (C). In A and B, we indicate ± 1 SD for the ß-Gal+ surface. There was no difference in the maximum amount of antibodies between monkeys grafted with or without ß-Gal+ cells (D).

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.[36] 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 unlabeled 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,[5,37] 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 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 ß-Gal + myofibers, that is, myofibers that integrated the grafted cells and expressed allogeneic proteins by genetic complementation and (2) the amount of ß-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.[19]

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.[38] This causes cell death by complement-dependent and -independent pathways,[39] 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.[40] 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.

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