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INTERFERON GAMMA IMPROVES LONG-TERM EFFICACY OF ALLOGENEIC MESENCHYMAL STEM CELL THERAPY AFTER ACUTE MYOCARDIAL INFARCTION

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Contents

Summary

Introduction: Cardiac cell therapy using allogeneic mesenchymal stem cells (MSCs) is a promising approach after myocardial infarction. Preclinical data suggest a loss of efficacy in the long term, probably due to immune rejection of the cells. Our working hypothesis is that pretreatment with IFN-γ of allogeneic MSCs improves cardiac cell therapy as compared to untreated allogeneic MSCs. This study aims to determine: (1) whether pretreatment of allogeneic MSCs by interferon gamma (IFN-γ) provides a long-term cardiac functional benefit, (2) whether effect of the pretreatment with IFN-γ is specific to allogeneic cell therapy, (3) and whether the pretreatment with IFN-γ modulates the alloimmunization induced by MSCs.

Methods: Autologous or allogeneic MSCs, pretreated or not with IFN-γ, were injected intramyocardially in a rat model of myocardial infarction induced by coronary artery ligation. Cardiac function was assessed by echocardiography for 4 to 6 months. Bimonthly blood tests enabled to detect humoral or cell alloimmunization. At 4 or 6 months follow-up, rat hearts were removed for histological analysis of cardiac repair.

Results: Allogeneic MSCs pretreated with IFN-γ improved cardiac repair and cardiac function in the long-term, with benefits comparable to autologous MSCs. In contrast, pretreatment with IFN-γ did not modify functional benefits obtain with autologous MSCs. Injection of allogeneic MSCs pretreated or not with IFN-γ induced both humoral and cellular alloimmunization.

Conclusion: Our study shows a beneficial effect of IFN-γ pretreatment of MSCs on cardiac repair after cardiac cell therapy, that is specific to the context of allogeneic cell therapy and suggests a favorable immune modulation induced by IFN-γ as the mechanism of this effect.

Key words

Cardiac cell therapy; myocardial infarction; allogeneic mesenchymal stem cells; interferon gamma; alloimmunization

Abbreviations

µCi:	Microcurie
µm:	Micrometers
BSL:	Baseline
D:	Day
DNA:	Deoxyribonucleic Acid
EDTA:	Ethylenediaminetetraacetic Acid
FGF 2:	Fibroblast Growth Factor 2
FOXP3:	Forkhead Box P3
IDO:	Indoleamine 2,3-Dioxygenase
IFN-γ:	Interferon Gamma
Ig:	Immunoglobulin
IL:	Interleukin
LVEF:	Left Ventricular Ejection Fraction
LVVd:	Left Ventricular Diastolic Volume
LVVs:	Left Ventricular Systolic Volume
M:	Month
MHC:	Major Histocompatibility Complex
MI:	Myocardial Infarction
MLR:	Mixed Lymphocyte Reaction
MSCs:	Mesenchymal Stem Cells
NK:	Natural Killer
NO:	Nitric Oxide
PBMC:	Peripheral Blood Mononuclear Cells
PBS:	Phosphate Buffered Saline
TCR:	T Cell Receptor
TGF-β:	Transforming Growth Factor Beta
TNF-α:	Tumor Necrosis Factor Alpha
Vd:	Diastolic Volume
Vs:	Systolic Volume

Introduction

Ischemic heart disease is the leading cause of death worldwide, particularly due to myocardial infarction (MI) and its frequent progression to heart failure.

Human heart has only a small cardiomyogenesis capacity and currently there is no routine treatment to regenerate necrotic cardiomyocytes after MI (1). Heart transplantation remains the only therapeutic option in the advanced stages of heart failure, but shortage of graft availability is an important limitation of this therapy.

Cardiac cell therapy is a promising approach to prevent the evolution of MI toward heart failure (2). First generations trials using autologous stem cells, including bone marrow mononuclear cells, showed mixed results. Use of Mesenchymal stem cells (MSCs) is an alternative with important current development.

Many preclinical studies have shown that autologous MSCs injection after MI improves cardiac function and preserves myocardial remodeling (3,4). These positive effects seem to be mainly related to paracrine mechanisms acting on the inflammatory component of the post MI remodeling (5–7).

However, autologous MSCs therapy has important limitations, including the need to prepare a cell therapy product from each patient and the difficulty of obtain cells with sufficient quality and quantity to be used at the acute phase of MI (8,9).

This would be a clear clinical advantage if cells from healthy donors, prepared in advance and ready "off the shelf", could be used as allogeneic therapy without immunosuppressive treatment. In this matter, the choice of MSCs seems relevant because they are known to have immunomodulatory properties, allowing them to escape the immune recognition system (10).

Several preclinical and clinical studies have shown good tolerance and short-term effectiveness of allogeneic MSCs administered into ischemic myocardium , at the acute phase of MI or in the setting of chronic ischemic heart failure (11–15).

However, the effectiveness of such treatment remains to be determined in the long term. . Indeed, a study in rats showed a loss of the initial gain of cardiac function after several months of follow-up, in animals injected with allogeneic MSCs as compared to those injected with autologous MSCs (13). This suggest that despite the immunomodulatory properties of MSCs, the host immune response would result in rejection and destruction of injected MSCs by the receiver after few months. Recent *in vitro* work, confirmed that MSCs have both immunomodulatory and immunogenic properties (16–18).

MSCs preactivation by interferon gamma (IFN-γ) results in the activation of various MSCs immunosuppression channels (19–21). These immunosuppressive properties allow MSCs to inhibit the proliferation and function of the main immune cell populations (T and B lymphocytes, NK cells), to modulate dendritic cells activity, and to induce regulatory T cells (20). In this context, it has been shown, *in vivo*, the decrease of immune response against allogeneic MSCs pretreated with 100 IU/ml IFN-γ 24 hours prior to intravenous injection in healthy rats (16).

Our working hypothesis is that the therapeutic efficacy of allogeneic MSCs pretreated with IFN-γ is improved as compared to untreated allogeneic MSCs. Mechanisms suspected to explain these effects are: (1) an amplification of the beneficial actions of MSCs on cardiac repair, allowing a more favorable remodeling and a higher functional gain, (2) a favorable immunomodulation/immunogenicity ratio for pretreated MSCs leading to immune tolerance and reduced rejection by the host.

To meet these assumptions, our study was conducted in two steps.

A first step which aim was to evaluate the *in vivo* long-term improvement of post MI heart function after intramyocardial injection of allogeneic MSCs pretreated with IFN-γ, as compared to use of untreated allogeneic MSCs, and autologous MSCs.

A second step which aim was: (1) to validate first step results in a MI model with larger infarct size, (2) to seek if the beneficial functional effect of IFN-γ pretreatment was specific to allogeneic therapy by analyzing the functional effect of IFN-γ in the context of autologous MSCs therapy, (3) to seek if the treated animals develop immunization against allogeneic MSCs by studying humoral and cellular responses, and if pretreatment with IFN-γ reduces this alloimmunization.

Methods

A. Animals

Syngeneic Lewis (LEW/OrlRj, Janvier France livestock, CMH RT1^{l}) female rats and syngeneic Brown Norway (BN/OrlRJ, Janvier France livestock, CMH RT1^{n}) female rats were used. As these rat strains were chosen with distinct MHC (RT1) and represent an already described relevant model allogeneic transplantation studies (22,23), we speculated that it would also represent a relevant model for comparison of autologous and allogeneic cell therapies.

The experimental protocol was in accordance with Guide for the Care and Use of Laboratory Animals published by U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) (24). It was approved by "*Comité régional d'éthique en expérimentation animale des Pays de la Loire » (referred as CEEA-2009-07).*

B. Isolation and culture of rat mesenchymal stem cells

Animals used were aged of 8 to 12 weeks and weighed 150 to 200 g. They were anesthetized by inhalation of isoflurane (Forene®, Abott) and then sacrificed by intraperitoneal injection of 1 ml pentobarbital. After dissection of femurs and tibias, bone marrow was recovered by flushing culture medium through femoral and tibial diaphysis. After centrifugation, resuspension, and counting, cells were cultured at 37°C and 21% O2.

A homogeneous population of fibroblastoid cells was obtained. The cell phenotype was assessed by flow cytometry (markers CD34-, CD45-, CD29+ and CD90+) (25). When cells reached confluency, they were detached, counted and frozen.

One week before each series of surgery, MSCs were thawed and returned to culture. MSCs pretreatment consisted of adding IFN-γ (Recombinant rat IFN-γ 400-20, Peprotech®), in the culture medium at a final concentration of 100 IU/ml of medium, 24 hours prior to injection. On the day of surgery, MSCs were detached, counted, washed, concentrated to $25*10^6$ cells per ml of PBS (Phosphate Buffered Saline), and stored on ice until injection.

C. Induction of myocardial infarction by coronary artery ligation

The experiment was performed on female Lewis rats. Animals were 9-week-old and weighted 180-200 g on the day of surgery.

Rats were anesthetized with isoflurane in an induction chamber, and placed on a heating pad to maintain their body temperature at 37°C. After tracheal intubation, rats were ventilated with a small animal respirator (Harvard rodent ventilator®, Harvard Apparatus).

Left thoracotomy was performed at the fourth intercostal space and gave access to heart. The left coronary artery was ligated with non-absorbable ligature (6.0 Prolene®, Ethicon). Depending on study step, ligation level was chosen to obtain required infarct size. Occlusion of the coronary artery was monitored visually by the rapid blanching of the left ventricular region downstream of the ligation.

Using a 26G (gauge) needle, 3 intramyocardial injections of 50 µl each ($3.75*10^6$ cells in total) were carried out at the border zone of the infarcted area. According to the treatment group, injection consisted of one of the following:

- Allogeneic MSCs (Brown-Norway)
- Allogeneic MSCs treated with 24 100 IU/ml of IFN-γ,
- Autologous MSCs (Lewis)
- Autologous MSCs treated with 24 100 IU/ml of IFN-γ,
- Cell injection solution (PBS).

The thorax and the skin incisions were closed, with a 4.0 suture (Dafilon® 4.0., B. Braun). After surgery, animals remained on life support until wake-up. Once extubated, animals were returned to their cage, under a heat lamp until recovery of their usual motricity.

Analgesia was achieved by systematic subcutaneous injection of 10µg nalbuphine (opioid analgesic; Nubain®, Serb), before and after surgery, followed by optional injections depending pain monitoring.

D. Echocardiographic assessment

Cardiac function was assessed by echocardiography with a longitudinal follow up, performed for each animal one week before surgery (baseline), and postoperatively:

- at 1, 7 and 15 days (D1, D7 and D15) and 1, 2, 4 and 6 months (M1, M2, M4 and M6) for step 1 protocol and
- at 1 and 7 days (D1 and D7) and 1, 2, 3 and 4 months (M1, M2, M3 and M4) for Step 2 protocol.

A General Electric Vivid 7VR (GE Medical System®; Milwaukee, WI, USA, http://www.gehealthcare.com) system equipped with a 10 MHz probe was used.

Animals were anesthetized with isoflurane and placed on a heating pad. All examinations were performed in animals with a heart rate greater than 300 beats per minute. Subcutaneous electrodes were positioned to monitor electrocardiogram during the exam.

The variables measured were diastole and systole left ventricular surfaces (S) and lengths (L) (bidimensional long-axis plane), and heart rate.

Diastolic (LVVd) and systolic (LVVs) left ventricular volumes were calculated using the ellipsoid monoplane formula: volume = $(8S^2)/(3\pi L)$.

The ejection fraction of the left ventricle (LVEF) was calculated using the formula: LVEF = (LVVd – LVVs) / LVVd

Results were obtained from an average of 3 consecutive measurements per animal. The data review and analysis were performed by an observer blinded to treatment group.

E. Immunological assessment

1. Blood samples

Blood samples were obtained every two weeks from one month post-MI to sacrifice. Samples of 1 ml each were obtained by retro-orbital puncture.

Animals were anesthetized with isoflurane andreceived subcutaneous injection of 10 µg nalbuphine (Nubain®, Serb). A microcapillary tube was introduced into the inner canthus of the eye until penetration in the retro-orbital sinus. After blood sampling, a manual pressure was applied on the eyelids until hemostasis was obtained.

For all animals, blood samples were collected 1 (M1), two (M2), 3 (M3) and 4 months (M4) after the coronary ligation. Serum was prepared from collected blood and was used to detecthumoral alloimmunization.

The animals from groups that received allogeneic MSCs, underwent 4 additional blood sample collections, at 1 and a half (M1.5), 2 and a half (M2.5), 3 and a half (M3.5), and 4 months after MI. The peripheral blood mononuclear cells (PBMC), extracted from whole blood, were used for cell alloimmunization detection.

2. Cell alloimmunization

Cell alloimmunization detection was performed by mixed lymphocyte reactions (MLR) according to an established technique (26).

PBMC were extracted from heparinized blood samples by Ficoll method (FicollPaque More GE Healthcare; ref 17-1440-02). Lymphocytes from naive Lewis rat (negative controls) were isolated identically.

Spleens from naive Brown Norway rats were dilacerated then filtered through a 70 µm grid. Splenocytes were isolated by the Ficoll method, and then irradiated (35 Gy).

MLR were performed by first mixing *in vitro* PBMC of receiver animals (Lewis rats, MHC haplotype $RT1^{l}$) and irradiated allogeneic splenocytes (Brown Norway rats, MHC haplotype $RT1^{n}$).

After a 4 days of incubation, 1 µCi of tritiated thymidine was incorporated per well. After further 16 hour incubation, the reaction was stopped by storing the plate at 4°C.

Then, a camera measured the radioactivity in each well in counts per minute. Data collected were:

- the medians of triplicates for each reaction in counts per minute,
- the proliferation index of MLR was then calculated as:

$$\frac{\text{receiver animal MLR} - \text{Irradiated splenocytes control}}{\text{Control of the same receiver animal}}$$

(alloimmunization was defined as positive if the proliferation index > 3).

3. Humoral alloimmunization

Splenocytes from Naive Brown Norway rat were prepared as described above.

Blood samples were kept at room temperature for one hour, and then centrifuged 20 minutes at 2000 rounds per minute. Sera were recovered and frozen.

The experiment was conducted using an established technique (27). Sera of the receiver animals (Lewis rats, MHC haplotype RT1l) were incubated with allogeneic splenocytes (Brown Norway rats, MHC haplotype RT1n) for 30 minutes. Splenocytes recognized by IgG were revealed using a mixture of secondary antibody against lymphocytes and monocytes.

Flow cytometry analysis allowed quantification of the different marked cell types. The mean fluorescence percentage and intensity of IgG positive lymphocytes were measured. Similarly, the mean fluorescence percentage and intensity of positive IgG monocytes were

measured. For each sample and each cell type, a positivity score was calculated as the product of mean fluorescence intensity by the percentage of positive cells.

F. Animal sacrifice and histological analysis

Rats were euthanized at 4 or 6 months postoperatively by an intraperitoneal overdose of pentobarbital.

Histological analysis was performed only for the step 2 protocol.

Rat hearts were recovered, washed in PBS and fixed in 10% formalin. Hearts were then embedded in paraffin and 6 µm sections were cut from the apex to the level just below the ligation site. From each heart, 3 sections equally distributed between apex and base were selected. Sections were stained with Hemalun - Eosine - Safran, scanned and transformed into a NDP format file usable by the Nano Zoom Digital Pathology software. The digital photographs were independently analyzed by two reviewers blinded to the treatment groups using Image J software.

Infarct size was measured by calculating the percentage of infarct zone area in the left ventricle. The circumferential extent of the scar to total LV tissue was measured using the midline length measurement technique. The LV myocardial midline was drawn at equidistance between the epicardial and endocardial surfaces. Midline infarct length was taken as the midline of the length of infarct that included greater than 50% of the whole thickness of the myocardial wall. Infarct size was calculated by dividing the sum of midline infarct lengths from all 3 sections by the sum of midline circumferences from all sections (28).

The relative scar thickness was also quantified. Scar and remote zone thickness were measured and relative scar thickness was calculated as the mean scar thickness divided by the mean remote zone thickness. For each heart sections, two to three measurements of myocardial wall thickness were performed in the remote zone and three to five measurements were performed in the infarct zone (28).

G. Statistical analysis

Continuous and discrete data were presented as means with standard error of the mean. Categorical data were presented as percentages.

To validate the use of parametric tests, normality test (Kolmogorov – Smirnov test) was systematically applied before analysis.

For the echocardiographic data, a first analysis by 2-factor ANOVA with repeated measures was carried out, followed by a *post hoc* Bonferroni test. Intra-group longitudinal analyzes were performed to compare data from different time-points using a paired t test. Inter-group comparisons at selected time-points were compared by one-way ANOVA test followed by *post hoc* Bonferroni test.

For immunological data, nonparametric tests were used due to non-normal distribution of the data. For the flow cytometry data, inter-group comparisons were performed at each time-point by the Kruskal-Wallis test followed by a *post-hoc* Dunns test. MLR data was presented as percentage of immunized animals since J0 for each group. Groups were compared at each time-point by Fisher exact test.

For histological data, the infarct size was compared between treatment groups by one-way ANOVA. Concerning the relative thickness of the infarcted wall, data were compared between groups by two-way ANOVA to include variability between histological section levels.

The statistical significance threshold was defined as $p<0.05$ for all tests. Data were analyzed using the GraphPrism 4.0 software for Windows.

H. Experimental protocol

Figure 1.

Results

A. Surgery

1. Step 1

A total of 54 rats were operated for this study. Surgical results are summarized in Table I.

Intraoperative mortality was 21% (n = 23). Among the rats surviving to surgery, 86% (n = 43) displayed a significant MI size with a LVEF <70% at D1. Such animals were therefore included in the longitudinal monitoring protocol of cardiac function by echocardiography. There was no postoperative mortality during the follow-up.

Eventually, 4 treatment groups were obtained:

- 11 animals formed the group receiving allogeneic MSCs (Allo)
- 11 animals formed the group receiving allogeneic MSCs pretreated with IFN-γ (IFN Allo)
- 10 animals formed the group receiving autologous MSCs (Auto)
- 11 animals formed the group receiving PBS (PBS).

2. Step 2

A total of 109 rats were operated for this study. Surgical results are summarized in Table II.

Intraoperative mortality was 21% (n = 23). Among rats the surviving to surgery, 70% (n = 60) showed a significant MI size with a LVEF <50% at D1. Such animals were therefore included in the longitudinal monitoring protocol of cardiac function by echocardiography. Postoperative mortality was 10% (n = 9).

Eventually, 5 treatment groups were obtained:

- 10 animals formed the group receiving allogeneic MSCs (Allo)

- 10 animals formed the group receiving allogeneic MSCs pretreated with IFN-γ (IFN Allo)
- 12 animals formed the group receiving autologous MSCs (Auto)
- 10 animals formed the group receiving autologous MSCs pretreated with IFN-γ (IFN Auto)
- 9 animals formed the group receiving PBS (PBS).

B. Echocardiography

1. Step 1

A longitudinal echocardiographic follow-up was performed for each animal. A total of 372 echocardiograms were performed and analyzed blinded to treatment group.

Figure 2 shows the evolution of the mean LVEF of each animals group.

Animals had a mean preoperative LVEF of 90±3%, then at D1 postoperatively, a mean LVEF of 56±5%. Myocardial infarctions were significant with a ~35% reduction of LVEF. There was no statistical intergroup LVEF difference preoperatively and at D1.

LVEF of the groups treated with cells increased between D1 and D7 with an average of 72±4% at D7 and no significant difference between groups.

Postoperatively, the LVEF in the control group (PBS) was continuously deteriorating from day 1 to M6, decreasing by 53%±9 to 43±6% ($p=0.0003$). In contrast, LVEF of animals, receiving pretreated allogeneic MSCs and autologous MSCs, was maintained until M6 with no significant difference between D7 and M6. LVEF at M6 was 71±4% ($p>0.9999$ *versus* D7) and 68±3% ($p>0.9999$ *versus* D7), respectively. For the group of animals receiving untreated allogeneic MSCs, LVEF was maintained until M1 and then deteriorated to reach an average of 53±6% at M6 ($p<0.0001$ *versus* D7).

At the end of the sixth months of echocardiographic monitoring, LVEF of animals that received pretreated allogeneic MSCs or autologous MSCs, did not differ ($p=0.6338$) and was significantly higher *as compared to* control group animals ($p<0.0001$ for both) and as compared to the group of animals that received allogeneic MSCs without pretreatment ($p<0.0001$ for both). Higher LVEF was observed for the group of animals that received allogeneic MSCs without pretreatment as compared to the control group ($p<0.0001$).

2. Step 2

A longitudinal echocardiographic follow-up was performed for each animal. A total of 357 echocardiograms were performed and analyzed blinded to treatment group.

Figure 3 shows the evolution of the mean LVEF of each animals group.

Animals had a mean preoperative LVEF of 84±5%, then at D1 postoperatively, a mean LVEF of 37±5%. Mi size was larger than in step 1 protocol, with a 50% reduction of LVEF (figure 4). There was no statistical LVEF intergroup difference preoperatively (p=0.62) and at D1 (p=0.84).

Postoperatively, the LVEF in the control group (PBS) was continuously deteriorating from day 1 to M4, decreasing from 39±6% to 26±2% (p=0.0003). In contrast, LVEF of animals injected with pretreated allogeneic MSCs and pretreated or not autologous MSCs, was maintained until M4 with no significant difference between D1 and M4. Their LVEF at M4 was 37±8% (p=0.88 *versus* D1), 37±8% (p=0.91 *versus* D1) and 38±4% (p=0.96 *versus* D1), respectively. For the group of animals receiving untreated allogeneic MSCs, LVEF gradually deteriorated to reach an average of 28±4% at M4 (p=0.0005 *versus* D1).

At the end of the four months of echocardiographic monitoring, LVEF of animals that received pretreated allogeneic MSCs and pretreated or not autologous MSCs, did not differ (p>0.99) and was significantly higher as compared to LVEF of control group animals (p=0.0014, p=0.0012 and p=0.0003, respectively) and to group of animals that receive allogeneic MSCs without pretreatment (p=0.0087, p=0.0072 and p=0.002, respectively). LVEF of the control group and of the group that received allogeneic MSCs without pretreatment did not differ (p>0.99).

C. Immunological study

A longitudinal immunological blood monitoring was carried out for each animal,. A total of 408 blood samples were analyzed blinded to treatment group.

1. Humoral alloimmunization

In both groups of animals that received allogeneic cells, the anti-donor antibody response was maximal from the first measure, at 1 month postoperatively (Figure 5).

Animals from control group and from groups that received autologous MSCs, with or without pretreatment, didn't show significantly different lymphocytic and monocytic scores ($p>0.05$) for all time-points, indicating the absence of humoral alloimmunization.

In contrast, animals groups that received allogeneic MSCs pretreated or not, developed humoral alloimmunization with lymphocytic and monocytic scores significantly higher than the control group scores ($p<0.05$ for all measurement time). Moreover, animals receiving allogeneic MSCs pretreated with IFN-γ seemed to display higher scores but without significant difference.

The anti-donor antibody response profile for lymphocytes and monocytes appeared identical.

2. Cell alloimmunization

The measure of lymphocyte reactivity in animals that received allogeneic MSCs treated or not, showed a cell alloimmunization (Figure 6).

MLR data showed a similar cellular alloimmunization pattern in both groups. M1.5 data suggested a weaker immunization in the pretreated group but the difference was not statistically significant as compared to the non-pretreated group ($p=0.1827$).

D. Histological study

Infarct size did not differ between treatment groups (p=0.4227) (Figure 7).

In contrast, the relative thickness of infarct area was significantly higher in groups of animals that received autologous MSCs pretreated or not and allogeneic MSCs, as compared to the control group (p=.0171, p=0.0089 and p=0.0167 respectively) and to the group that received unpretreated allogeneic MSCs (p=0.0439, p=0.0148 and p=0.0257, respectively). The relative thickness of the infarct area did not differ between control and not pretreated allogeneic MSCs groups (p=0.8087), as well as between the other three treatment groups (Auto *versus* AutoINF p=0.3371; Auto *versus* Allo INF p=0.9785 and; AlloINF *versus* AutoINF p=0.0375) (Figure 8).

Discussion

Our study aimed at to evaluating the immunological response and the functional impact of the injection of allogeneic MSCs pretreated with IFN-γ after MI in immunocompetent rat.

The main result of this study is that injection of allogeneic MSCs, pretreated or not by IFN-γ, in a rat model of acute MI induced by coronary artery ligation, generates both an humoral and cellular alloimmunization,. In parallel, the echocardiographic monitoring of animals demomstrated the effectiveness of allogeneic MSCs pretreated with IFN-γ on the long-term on cardiac repair with a gain of cardiac function stable over time and comparable to improvement due to treatment with autologous MSCs. In contrast, IFN-γ pretreatment of autologous MSCs did not provide higher functional benefits as compared to the use of autologous MSCs not pretreated.

At last, these results show a beneficial effect of IFN-γ that is specific to the setting of allogeneic cell therapy and suggest a favorable immune modulation induced by IFN-γ.

Allogeneic cell therapy and immune response

MSCs have repetitively been described as immunoprivileged (20,29). In theory, these properties allow them to escape the immune system and therefore, would allow them to be used for allogeneic cell therapy without need of immunosuppressive treatment.

Overall, the *in vitro* studies found an immune privilege of the MSCs, both in animals (30,31) and in men (10,32).

The *in vivo* data are more heterogeneous. In an experimental study in rat, Imanishi *et al.* found a transient accumulation of macrophages at the injection site, without activation of T lymphocytes but with a short follow-up. However, they found few remaining injected MSCs at 7 days (11).

In contrast, many studies, in agreement with our results, have shown immune rejection of allogeneic MSCs in animals (13,16,31,33,34). Our study showed a maximum humoral alloimmunization from the first measurement at 1 month after surgery persistent during the 4 month follow-up. A cellular alloimmunization was also observed for all animals treated with allogeneic cells at 4 months follow up. Accordingly, Poncelet *et al.* observed, in pigs, humoral alloimmunizaton response to allogeneic MSCs therapy at 14 days post injection and a cell alloimmunization at 1 month post injection (31). In a rat model of allogeneic MSCs injection after MI, a humoral immune response was detected five weeks after infarction (13). Shu *et al.* found the presence of allo-antibodies from 14 days after allogeneic MSCs injection (16). However, in these two last studies, authors didn't notice cell alloimmunization, possibly because of too early detection (7 days and 48 hours respectively).

In humans, Hare *et al.* have looked for a humoral alloimmunization in a phase I/II clinical study. They have not found a more important immunization in the group that received allogeneic MSCs (15). In a phase II study, Perin *et al.* have not observed cell alloimmunization but a humoral alloimmunization directed against class 1 MHC in 11% of patients receiving allogeneic MSCs (35).

Several hypotheses are discussed to explain this secondary alloimmunization. For Huang *et al.,* alloimmunization was caused by the differentiation of MSCs into cardiac cells, explainingthe biphasic nature of immune response against MSCs in cardiac cell therapy. Undifferentiated MSCs were immunoprivileged, therefore tolerated until their differentiation and the transition to an immunogenic phenotype, which made them susceptible to immune

rejection by the host (13). For Shu *et al.*, alloimmunization was related to allogeneic MSCs injection in an inflammatory environment. In this context, the upregulation of class I and II MHC and VCAM-1 adhesion molecule, promoted contact between MSCs and lymphocytes T. This proximity between cells could outweigh the immunosuppressive capacities of MSCs (16). Alltogether, these two studies suggest that overexpression of MHC, linked to differentiation or to inflammation, trigger the immune rejection of MSCs and hence, trigger the loss of their effectiveness. In our study, the presence of IgG targeting lymphocytes and monocytes confirms overexpression, by allogeneic MSCs, of class I MHC and possibly class II MHC (36,37). However, this overexpression did not correlate with a loss of efficacy when allogeneic MSCs have been pretreated with IFN-γ. A limitation of our study is the absence of MSCs tracking, in order to demonstrate that alloimmunization does not necessarily result in immune rejection of MSCs.

Furthermore, Shu *et al.* suggest that inflammation would act as an important part in the interaction between MSCs and immune system. However, role of inflammation still remains unclear in this setting. Some authors suggested that inflammation may be the cause of immune rejection (16), while others suggested that inflammation can guide MSCs to damage areas (38) and activate their immunomodulatory properties (39). In our study, MSCs injection was performed in the acute MI setting, *i.e.* in an inflammatory environment, which may explain immunization. On the contrary, Hare *et al.* didn't find humoral alloimmunization, perhaps because they treated patients with chronic ischemic heart disease in which cardiac muscle is a less inflammatory environment (15).

In the perspective of use of allogeneic cardiac cell therapy in humans, target population, providing maximum efficacy of this therapy, includes patients suffering from ischemic heart disease with significant left ventricular dysfunction (40). These target patients are potential candidates for heart transplant if their clinical status deteriorates.

Alloimmunization due to an earlier cardiac cell therapy would result in access to a reduced numbers of grafts. One solution would be to design allogeneic cell therapy products from patients with rare MHC. Patients immunized against a rare MHC, would therefore remain compatible with the majority of heart grafts. This strategy can be perfectly applied. Banks of immunocompatible stem cells for the majority of the population can now be created. Therefore, proceeding inversely, it would be possible to create banks of allogeneic MSCs incompatible with the majority of the population (41).

Role of interferon gamma

Our study showed that improvement of cardiac function, induced by the cell therapy with allogeneic MSCs pretreated with IFN-γ, was maintained in the long term, with comparable results to autologous MSCs therapy. In contrast, cardiac function of animals that received untreated allogeneic MSCs, declined over time to reach function level similar to the control group, as described by Huang *et al.* (13). Several theories compete to explain IFN-γ's action and its mechanisms.

The hypothesis that IFN-γ might increase repair potential of MSCs, independently of the immune response, is not supported by our study. Indeed, improvement of cardiac function observed for the group that received autologous MSCs pretreated with IFN-γ and for the group that received untreated autologous MSCs did not differ. Thus, IFN-γ's action seems specific to allogeneic MSCs and is likely to be related to the immune response.

It as been suggested that IFN-γ may change the immunogenic potential of allogeneic cells to induce their tolerance in the long term (16,19,20). However, our results showed that the use of allogeneic MSCs pretreated or not was responsible for humoral and cellular alloimmunization. Therefore, the functional advantage provided by pre-treatment with IFN-γ would not correspond to immunosuppression but to immunomodulation

This immunological advantage could be related to regulatory T cells. Indeed, MSCs can induce the differentiation of CD4+/CD25+ T cells, displaying a regulatory phenotype FOXP3+ (42). The mechanisms to induce these regulatory T cells are not completely solved yet but MSCs activation by pro-inflammatory molecules, including IFN-γ (43) are known to promote regulatory T cells differentiation. This IFN-γ-induced MSCs activation allows interaction with the immune cells, by cell-cell contact and by production of soluble factors, that drive metabolic manipulation of the microenvironment leading to upregulation of indoleamine 2,3-dioxygenase (IDO) (44). IDO is an inducible enzyme that can induce the transition from naive CD4+ and CD8+ T cells to a regulatory phenotype (45). A recent *in vivo* study confirmed that MSCs could promote renal allograft tolerance by induction of IDO pathway and production of regulatory T cells (46). Furthermore, other factors are secreted by MSCs, like TGF-β1, prostaglandins E2 and IL-10, and these factors can also induce activation of regulatory T cells, in addition to their immunosuppressive function (44). For example, Dhingra *et al.* showed that maintaining high extracellular matrix levels of prostaglandin E2 preserved immune privilege after MSCs differentiation, preventing rejection of implanted MSCs, and restoring cardiac function (47).

Other immunomodulation pathways, regulated by MSCs under the influence of IFN-γ, are currently under study, such as shift of cytokine profile in the Th1/Th2 balance toward an anti-inflammatory phenotype Th2 (48), B cell immunomodulation (39), or reduction of lysing capacity of NK cells by overexpression of MHC class I (20).

Eventually, the beneficial effects of MSCs are now recognized and several current works aim at modulating MSCs therapeutic efficacy in order to potentiate cardiac repair and functional improvement. Zhang *et al.* have used MSCs exosomes implanted in cardiac stem cells showing myocardial fibrosis reduction and LVEF improvement as compared to controls (49). Likewise, Williams *et al.* have combined human MSCs and human cardiac stem cells

which were injected into immunosuppressed pigs after myocardial infarction. They showed a significant decrease in the infarct size as compared to controls and a restoration of the cardiac systolic function (50). In our study, allogeneic cells pretreated with IFN-γ has also resulted in better cardiac repair, evidenced by a greater myocardial thickness, and in improvement of functional benefit, validated by LVEF monitoring.

Conclusion

Our study shows the effectiveness of allogeneic MSCs on the long-term cardiac repair when pretreated with IFN-γ, in a rat model of acute MI. Thus, this pretreatment resulted in an improvement in cardiac repair and in cardiac function with an improvement similar to autologous MSCs therapy. However, IFN-γ pretreatment doesn't allow an additional functional benefit when used with autologous MSCs. Our study also shows that pretreatment with IFN-γ doesn't prevent both humoral and cellular alloimmunization, but could favorably modulate immune reaction.

Eventually, these results show a beneficial effect of IFN-γ specific to the context of allogeneic cell therapy and suggest a favorable immune modulation induced by IFN-γ. To confirm these results, it would be important to determine the influence of pretreatment with INF-γ: (1) on allogeneic MSCs survival using cell tracking experiments, (2) and on immune response, in particular on the shift of the T cell cytokine profile toward an anti-inflammatory T cell profile or a regulatory T cell profile.

In comparison to autologous approaches and their limitations, these results could have a significant impact on allogeneic cell therapy strategy currently in development in humans. Indeed, for all allogeneic cell therapy products, and for most targeted organs, the question of immune tolerance and long-term effectiveness will arise.

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Tables

Tableau I: Surgical data step 1

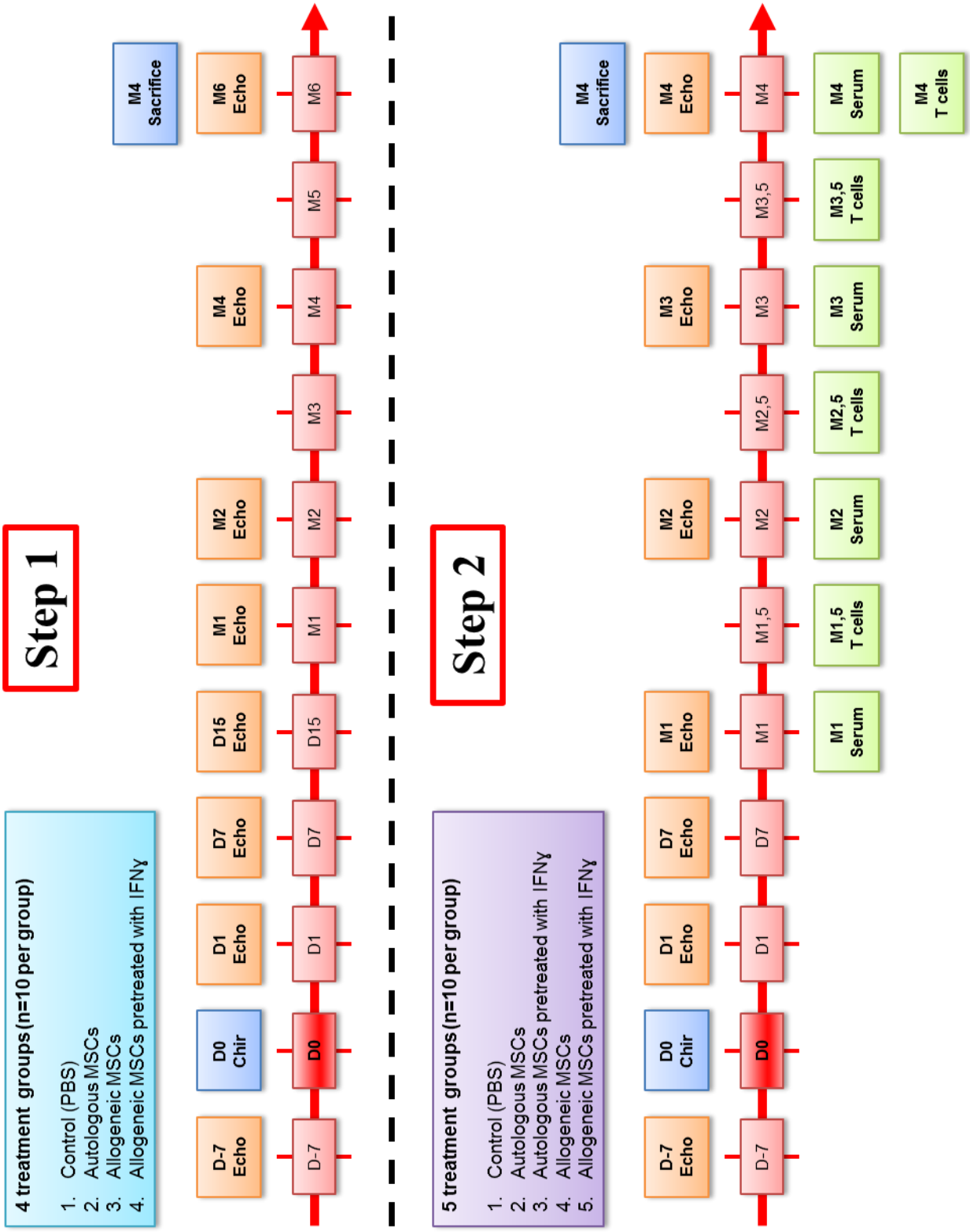
Treatment group	Allo	Allo INF	Auto	PBS	TOTAL
Operated rats, n	13	12	11	18	54
Intraoperative mortality, n (%)	1 (8)	0 (0)	1 (9)	2 (11)	4 (7)
Postoperative survival, n	12	12	10	16	50
Rats included in the monitoring, n (%) - (FEVG < 70% à J1)	11 (92)	11 (92)	10 (100)	11 (69)	43 (86)
Mortality during follow-up, n (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Rats with complete follow-up	11	11	10	11	43

Tableau II: Surgical data step 2

Treatment group	Allo	Allo INF	Auto	Auto INF	PBS	TOTAL
Operated rats, n	23	18	21	22	25	109
Intraoperative mortality, n (%)	6 (26)	5 (28)	2 (10)	7 (32)	3 (12)	23 (21)
Postoperative survival, n	17	13	19	15	22	86
Rats included in the monitoring, n (%) - (FEVG < 50% à J1)	12 (71)	11 (85)	13 (68)	11 (73)	13 (59)	60 (70)
Mortality during follow-up, n (%)	2 (12)	1 (8)	1 (5)	1 (7)	4 (18)	9 (10)
Rats with complete follow-up	10	10	12	10	9	51

Figures

Figure 1: Experimental protocol



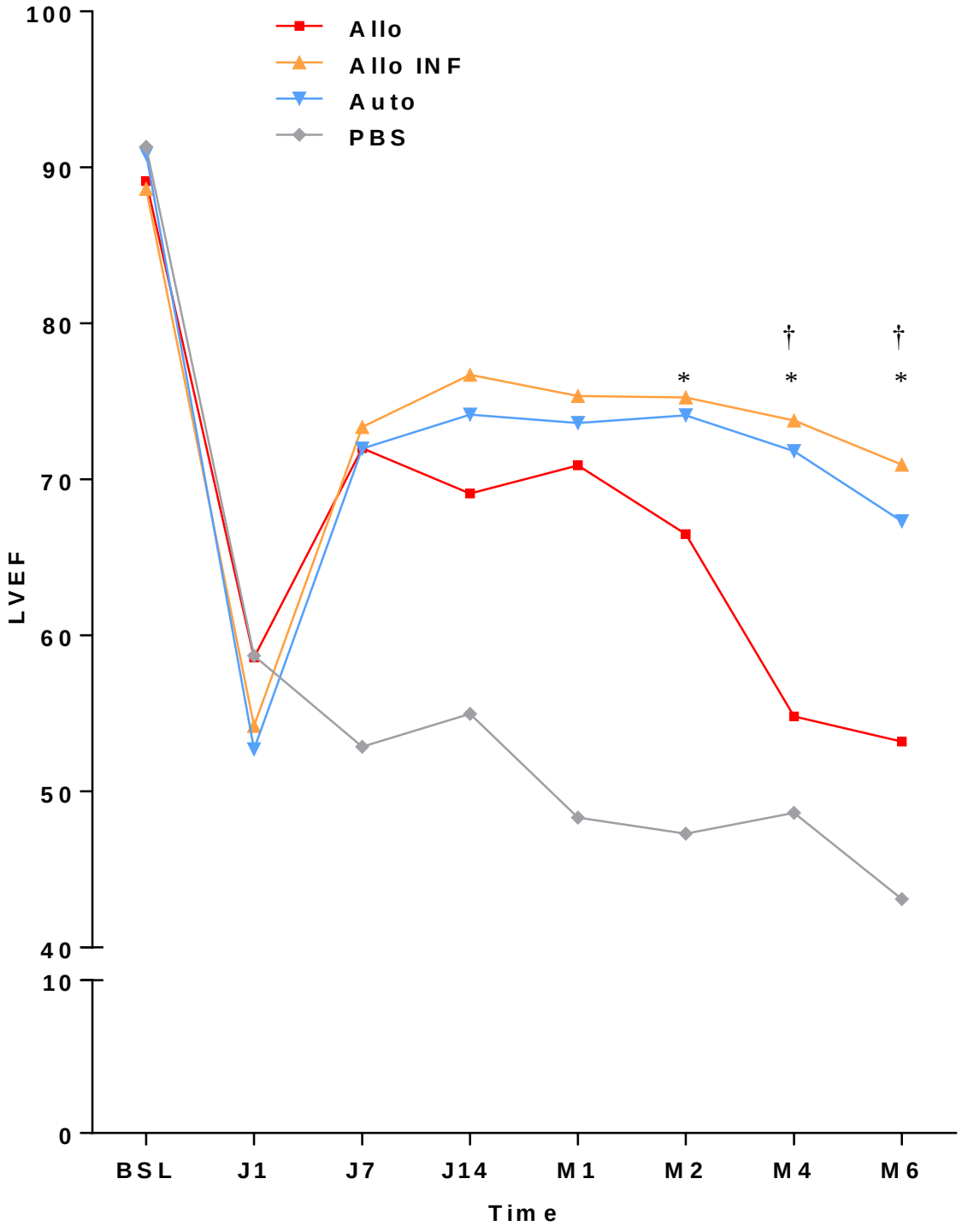


Figure 2: Evolution of the left ventricular ejection fraction (LVEF) over time for each treatment group. Statistical analysis by two-factor ANOVA with repeated measures (p=0.001 for interaction).
*: p<0.01 (*post-hoc* test Allo *versus* Allo INF)
†: p<0.01 (*post-hoc* test Allo *versus* Auto)

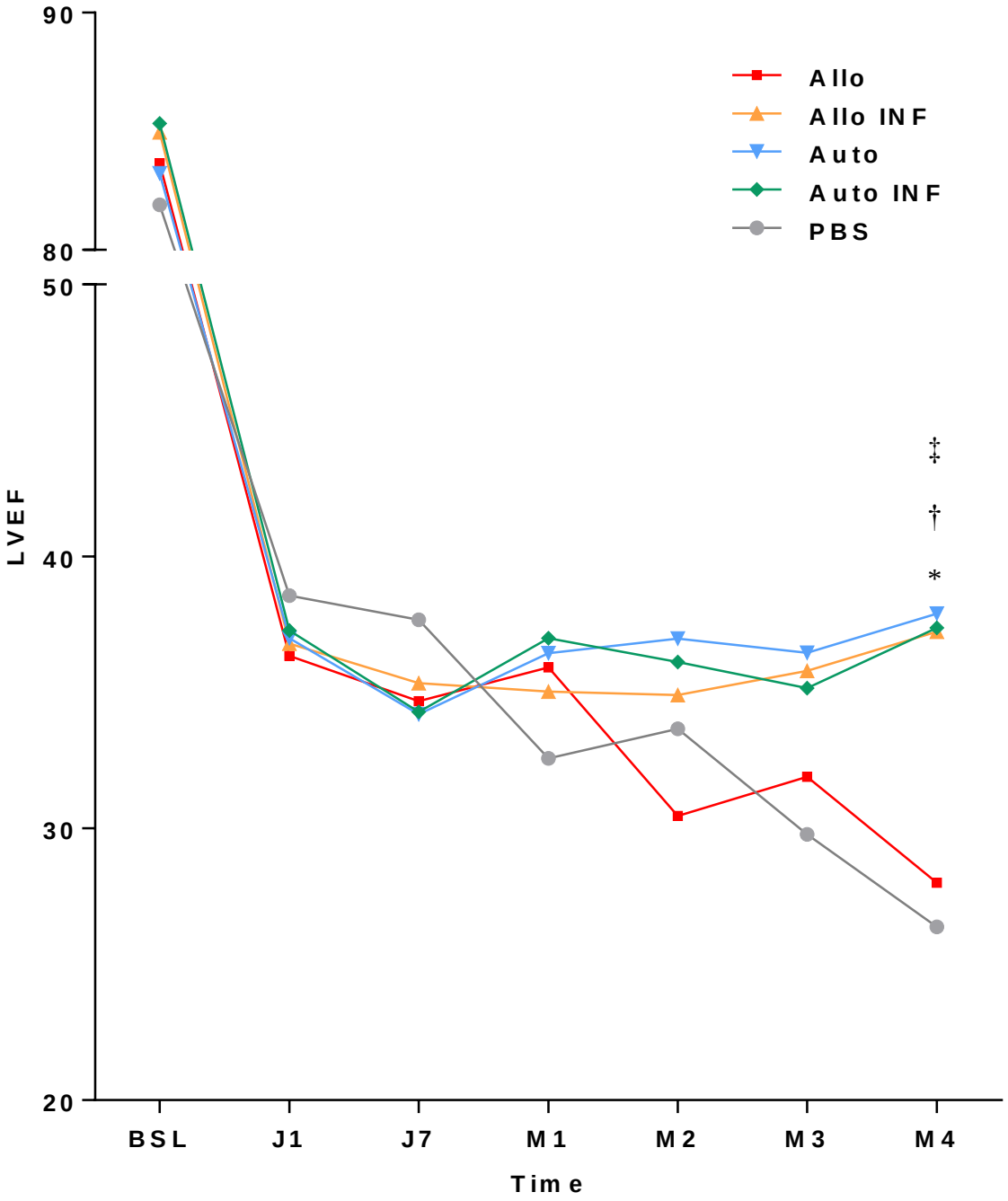


Figure 3: Evolution of the left ventricular ejection fraction (LVEF) over time for each treatment group. Statistical analysis by two-factor ANOVA with repeated measures (p=0.0005 for interaction).
*: p<0.01 (*post-hoc* test Allo *versus* Allo INF)
†: p<0.01 (*post-hoc* test Allo *versus* Auto INF)
‡: p<0.01 (p*ost-hoc* test Allo *versus* Auto)

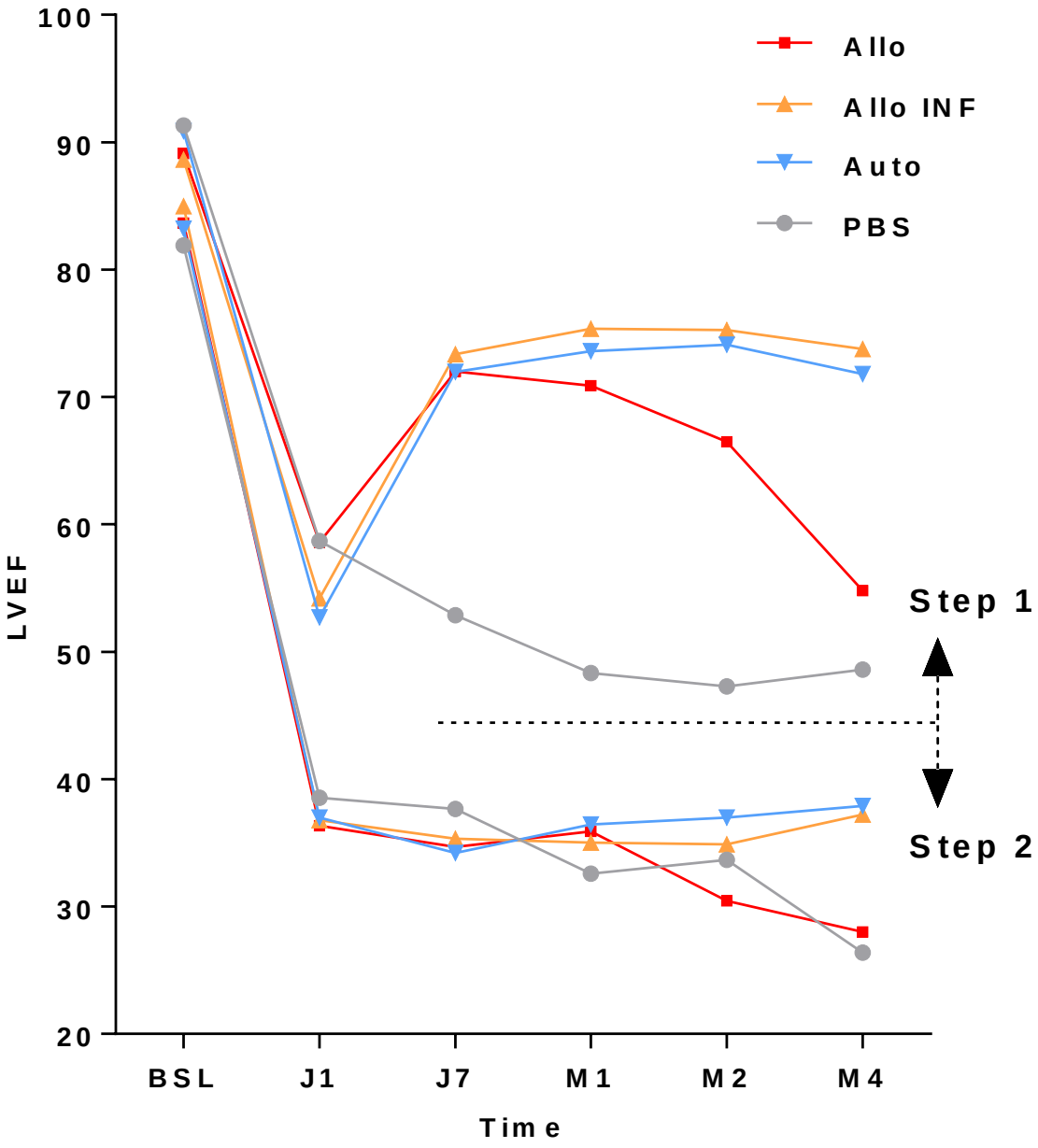


Figure 4: Evolution of the left ventricular ejection fraction (LVEF) over time for each treatment group. Comparison between results in steps 1 and 2.

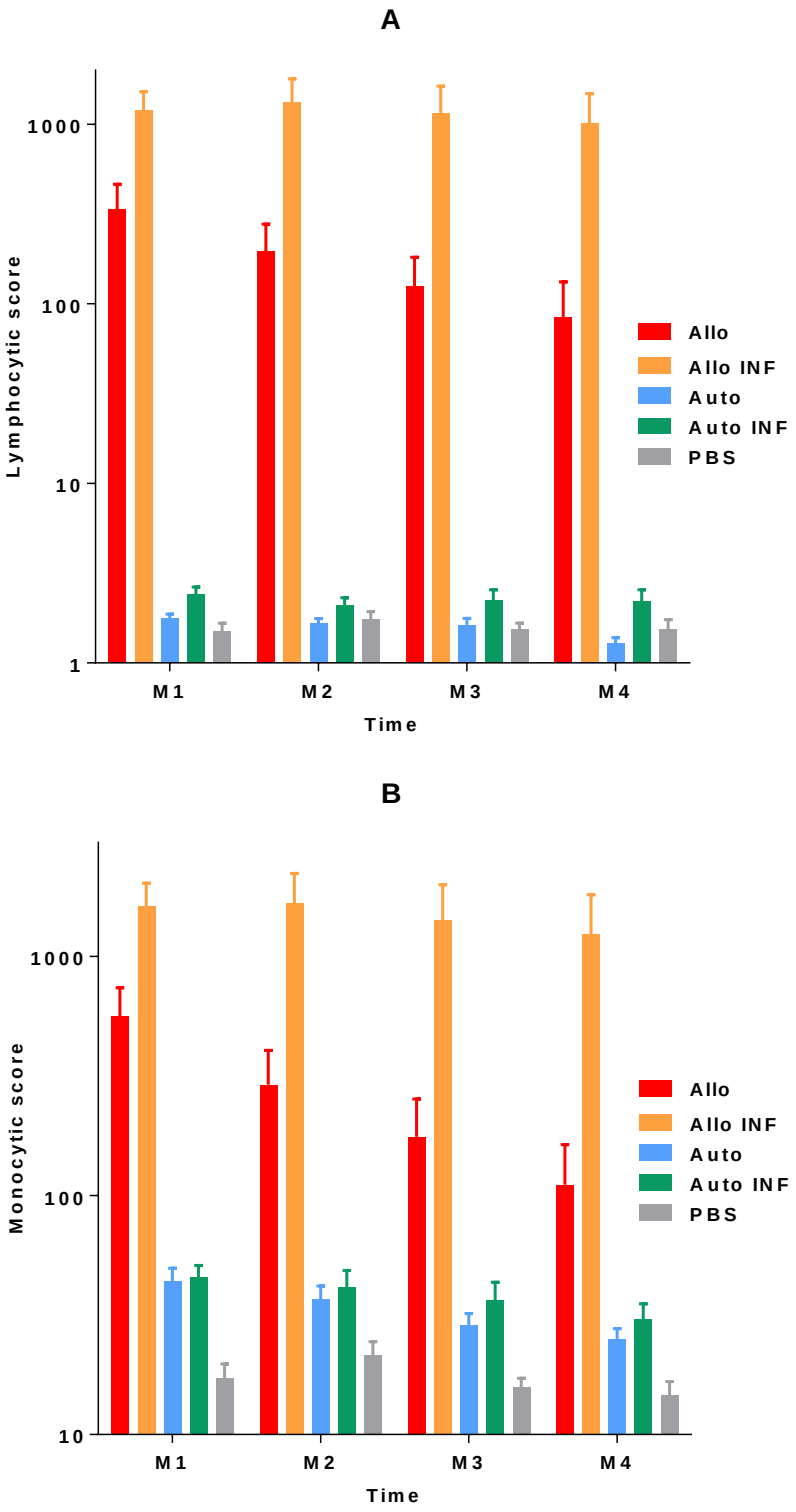


Figure 5: Evolution of anti-donor immune response on lymphocytes (A) and monocytes (B), over time and between groups.
Lymphocytic score:
percentage of labeled lymphocytes * labeling intensity (mean fluorescence intensity)
Monocytic score:
percentage of labeled monocytes * labeling intensity (mean fluorescence intensity).

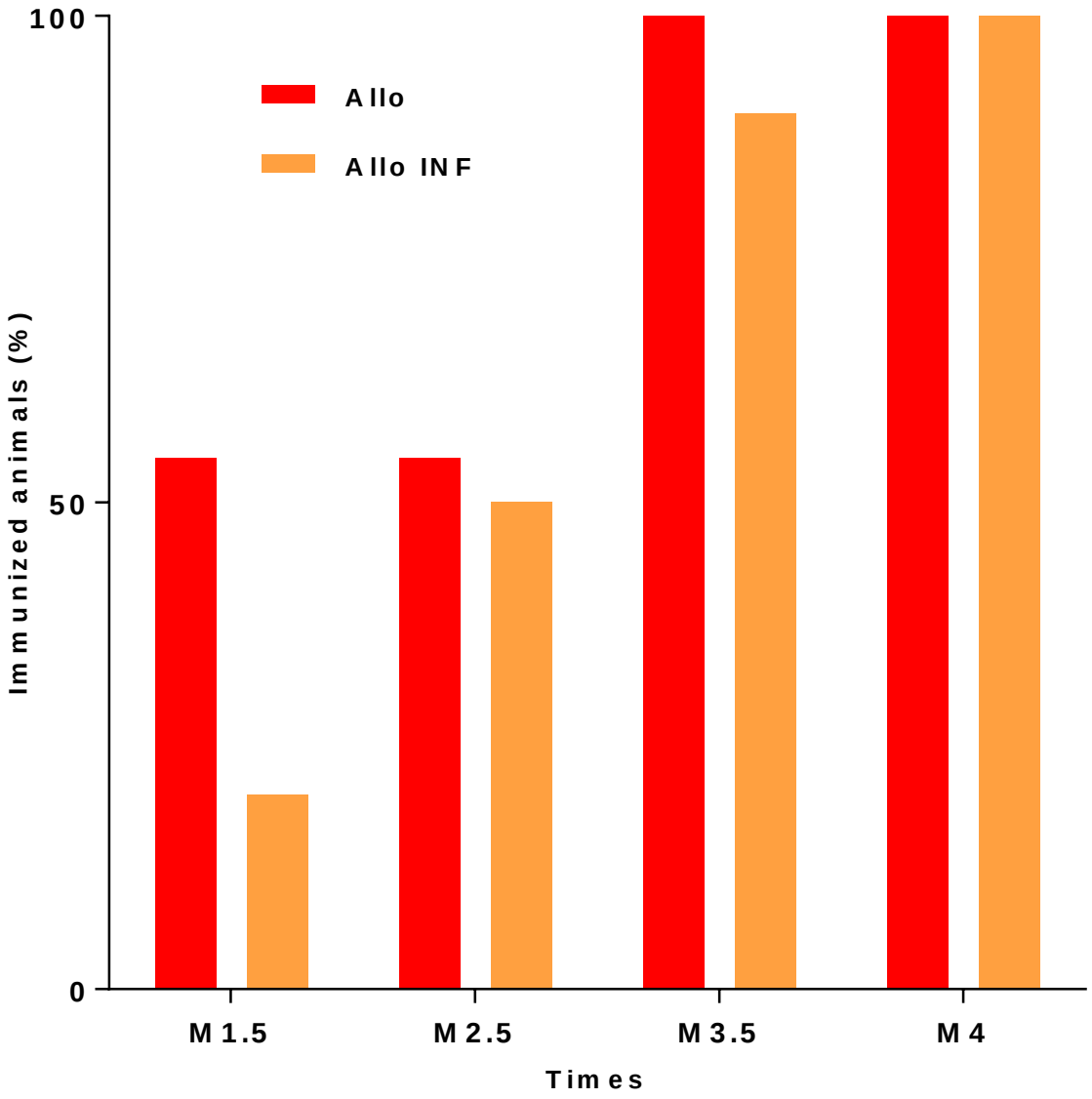


Figure 6: Evolution of immunization of animals receiving allogeneic MSCs pretreated (Allo INF) or not (Allo) over time.
p>0.05 (comparison Allo *versus* Allo INF)

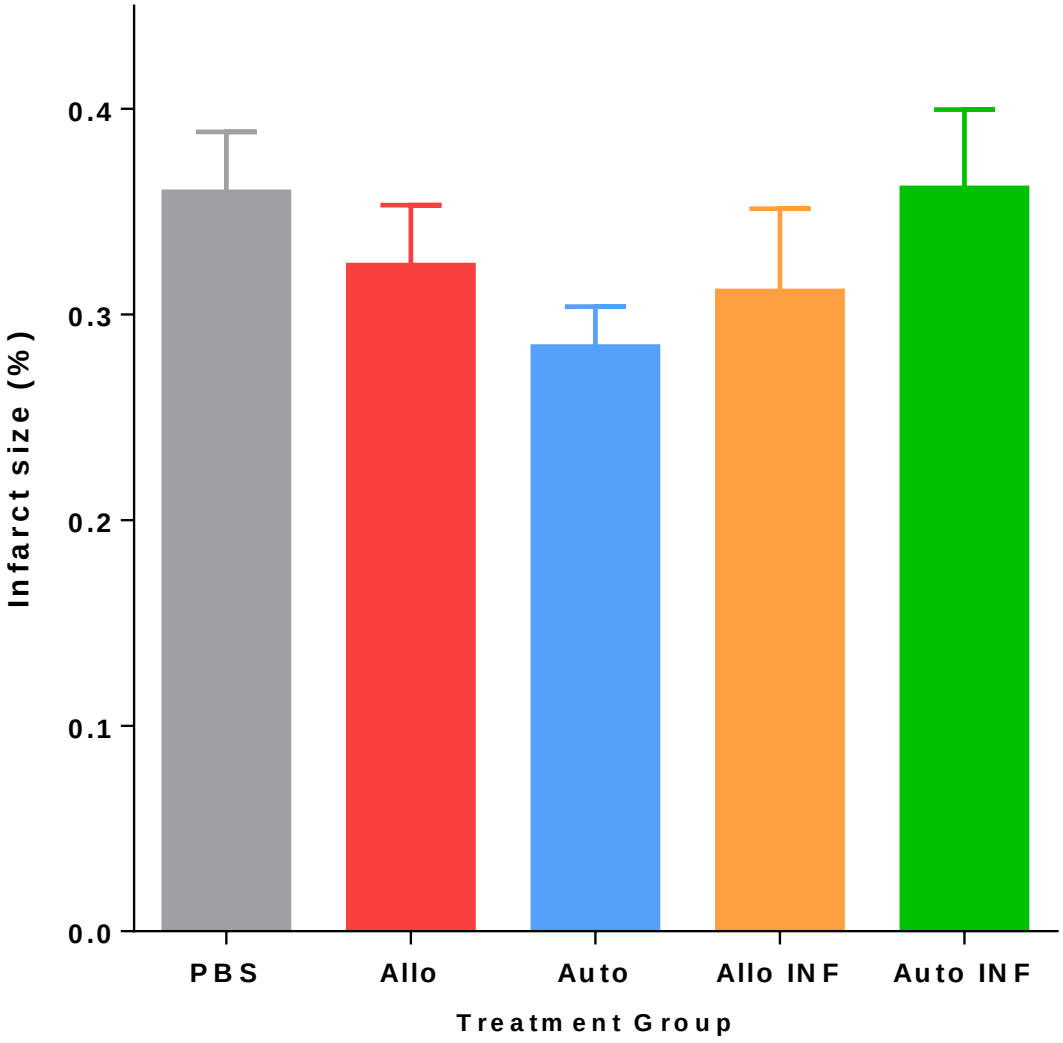


Figure 7: Comparison of the infarction size between the different treatment groups.

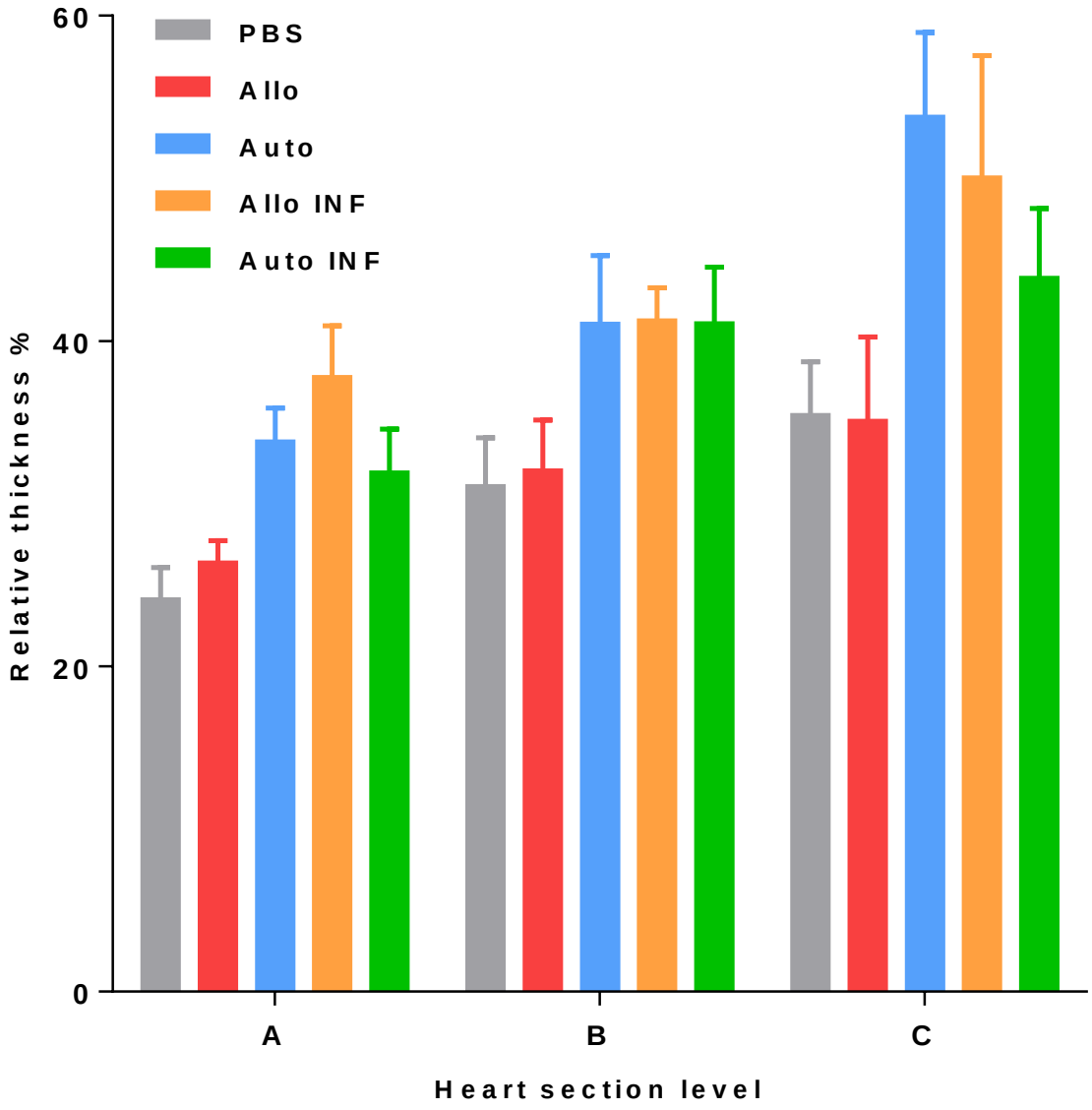


Figure 8: Comparison of the relative scar thickness, between treatment groups, by heart section level.

Vu, le Président du Jury,
(tampon et signature)

Vu, le Directeur de Thèse,
(tampon et signature)

Vu, le Doyen de la Faculté,
(tampon et signature)

NOM : **TERUIN** PRENOM : **Pierre**

Titre de Thèse:

INTERFERON GAMMA IMPROVES LONG-TERM EFFICACY OF ALLOGENEIC MESENCHYMAL STEM CELL THERAPY AFTER ACUTE MYOCARDIAL INFARCTION

RESUME

Cardiac cell therapy using allogeneic mesenchymal stem cells (MSCs) loses efficacy on the long term, probably by immune rejection of the cells. Our working hypothesis is that pretreatment with IFN-γ of allogeneic MSCs improve cardiac cell therapy.

In a rat model of allogeneic MSCs injection in post myocardial infarction, we compare the effect of autologous or allogeneic MSCs, pretreated or not with IFN-γ. Cardiac function was assessed by echocardiography, alloimmunization by blood tests and cardiac repair by histological study.

Despite a humoral and cellular alloimmunization, allogeneic MSCs pretreated with IFN-γ improve cardiac repair and cardiac function in the long-term, similarly to autologous MSCs.

Our study shows a beneficial effect of IFN-γ specific to the context of allogeneic cell therapy and suggests a favorable immune modulation induced by IFN-γ as the mechanism of the effect.

MOTS-CLES

Cardiac cell therapy; myocardial infarction; allogeneic mesenchymal stem cells; interferon gamma; alloimmunization