Intravenous infusion of donor apoptotic leukocytes before transplantation delays allogeneic islet graft rejection through regulatory T cells

F. Mougel a,b,c,d,*, F. Bonnefoy a,b,c, S. Kury-Pau lin a,b,c,d, S. Borot a,b,c,d,e, S. Perruche a,b,c, B. Kanteli p f, A. Penfornis d,e, P. Saas a,b,c, F. Kleinclauss a,b,c,g

Abstract

Aim. – This study describes the ability of intravenous donor apoptotic leukocyte infusion before islet transplantation to delay allogeneic graft rejection and implicates regulatory T cells ($T_{\text{reg}}$) in the effect.

Methods. – Allogeneic FVB (Friend virus B-type) islet transplants were placed under the kidney capsule of BALB/c recipient mice rendered diabetic by streptozotocin. Apoptotic donor leukocytes were infused intravenously 7 days before transplantation. Foxp3/DTR/GFP transgenic C57BL/6 mice were used as recipients to show depletion of $T_{\text{reg}}$ after apoptotic cell infusion. Control mice received islet transplants without apoptotic cells.

Results. – The graft median survival time (MST) in recipient mice was 15 ± 1.5 days when apoptotic cells were infused 7 days prior to transplantation of a 1000-islet-containing allograft and 6 ± 0.5 days in the control mice ($P < 0.01$). The same effect was observed using a 500-islet allograft, with an MST of 9 ± 1.1 days vs. 3 ± 0.8 days with and without (controls) apoptotic cells, respectively ($P < 0.01$). This immunomodulatory effect was not observed when apoptotic cell administration was performed on the day of transplantation. Specific $T_{\text{reg}}$ depletion in Foxp3/DTR/GFP recipient mice inhibited the beneficial effect of apoptotic cell infusion with an MST of 8 ± 1.5 days after apoptotic cell infusion vs. 2 ± 0.2 days when $T_{\text{reg}}$ were depleted ($P < 0.01$). Furthermore, $T_{\text{reg}}$ were specifically detected in the islet grafts of mice infused with apoptotic cells prior to islet transplantation.

Conclusion. – Infusion of donor apoptotic cells 7 days before allogeneic transplantation delays islet allograft rejection through a process involving $T_{\text{reg}}$.

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Keywords: Islet transplantation; Immunomodulation; Apoptotic cells; Regulatory T cells

Résumé

L’administration intraveineuse de leucocytes apop totiques issus du donneur avant transplantation retarde le rejet de l’allogreffe d’îlots par un mécanisme dépendant des lymphocytes T régulateurs ($T_{\text{reg}}$).

Objectif. – Décrire la capacité des leucocytes apoptotiques issus du donneur à retarder le rejet de l’allogreffe d’îlots et l’implication des lymphocytes T régulateurs ($T_{\text{reg}}$) dans cet effet.

Méthodes. – Des transplantations d’îlots allogéniques issus de souris Friend virus B-type (FVB) sont réalisées sous la capsule rénale des souris receveuses BALB/c rendues diabétiques par streptozotocine. Les leucocytes apoptotiques issus du donneur sont injectés par voie intraveineuse sept jours avant la transplantation. Des souris transgéniques C57BL/6 Foxp3/DTR/GFP utilisées comme receveuses nous ont permis d’éliminer spécifiquement les $T_{\text{reg}}$.

Abbreviations: BMT, bone marrow transplantation; DAMP, damage-associated molecular pattern; DTR, diphtheria toxin receptor; GFP, green fluorescent protein; GVHD, graft-versus-host disease; i.v., intravenous; MST, median graft survival time; NOD, non-obese diabetic; $T_{\text{reg}}$, regulatory T cells.

* Corresponding author. Tel.: +33 3 81 66 93 92; fax: +33 3 81 66 83 75.

E-mail address: francoismougel@gmail.com (F. Mougel).
tic strategies are needed to improve islet engraftment and to reduce the side-effects of immunosuppressive drugs. Indeed, it has previously been established that transfusion of apoptotic splenocytes from the donor 7 days before transplantation prolonged the MST of the heart graft. In contrast to BMT, they demonstrated that the allograft-protective effect of transfused apoptotic cells was donor-specific [15]. Wang et al. [16] gave an injection of donor apoptotic splenocytes 7 days after transplantation and observed a significant increase in survival of mouse cardiac allograft, whereas no effect was detected after therapy with necrotic donor splenocytes [16]. In these heart transplantation models, recipient phagocytes played an important role in the effect [15,16]: the long-term tolerated heart allografts were found to be infiltrated by Treg (Foxp3+ CD4+ T cells secreting interleukin [IL]-10 and TGF-β) [16]. All these studies suggest that Treg induction participates in the graft-facilitating effect of apoptotic cells. However, the precise mechanism remains to be demonstrated.

For this reason, it was decided to evaluate the consequences of i.v. apoptotic leukocyte administration on the engraftment of islet allografts without the use of immunosuppressants. After validation of our experimental islet transplantation model using syngeneic grafts, allogeneic transplants were performed. Donor apoptotic splenocytes were infused, or not, into diabetic recipient mice at different times (7 days before transplantation or at time of transplantation) and using different numbers of apoptotic splenocytes. It was also decided to perform tests at the time of transplantation to allow comparisons with previous data obtained in BMT settings [12–14] and at 7 days before transplantation to compare with data from the literature [15]. The MSTs of the islet grafts in each group were also compared. Thus, based on specific Treg depletion in Foxp3/diphtheria toxin receptor (DTR)/green fluorescent protein (GFP) recipient mice and immunohistochemical staining of the Treg, the role of this T cell subset in the apoptotic cell effect was assessed.
2. Materials and methods

2.1. Animals

Female 6- to 10-week-old BALB/cJRj (H-2d) recipients and FVB/NRj (H-2b) donor mice were obtained from Janvier (Le Genest-Saint-Isle, France). C57BL/6 (H-2b) Foxp3/DTR/GFP transgenic mouse recipients (kindly provided by A.Y. Rudensky in Seattle, WA, USA) were bred in our animal facility. Experimental protocols were performed according to our institutional guidelines and local ethics committee, and in accordance with the principles of laboratory animal care (as described in the US National Institutes of Health publication No. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm).

2.2. Induction of diabetes and blood glucose monitoring

Diabetes was induced in the recipient mice by a single injection of streptozotocin (200 mg/kg body weight; Sigma-Aldrich Chemie, Steinheim, Germany) and confirmed by measuring blood glucose from samples obtained by tail-snipping. Mice with two consecutive non-fasting blood glucose levels greater than 250 mg/dL were considered diabetic and used as recipients.

2.3. Islet isolation and transplantation

Islet donors were anaesthetized. After laparotomy and proximal pancreatic duct occlusion, the pancreas was distented by intraductal injection of 3 mL of cold Liberase RI (0.33 mg/mL; Roche Applied Science, Mannheim, Germany). The distended pancreas was excised and digested by Liberase RI, and islets purified on a Ficoll gradient; 1000 or 500 islets were then harvested by handpicking under a microscope.

In recipient mice, these 1000 or 500 islets were transplanted under the kidney capsule using silicone tubing. Glycosuria and blood glucose were monitored each day after allogeneic transplantation. Primary graft function was defined as blood glucose levels less than 250 mg/dL at day 1 post-transplantation, while rejection was defined as two successive blood glucose determinations greater than 250 mg/dL after a period of normoglycaemia.

2.4. Apoptosis induction

The spleen was dissociated mechanically. Splenocytes were irradiated (35 Gy) and cultured for 6 h to allow apoptotic changes to occur, as described elsewhere [12]. That corresponded to early-stage apoptotic leukocytes, as confirmed by annexin V and propidium iodide (Sigma) staining [12]. Apoptotic cells (5 × 10^6 or 50 × 10^6/mouse) were infused i.v. into the recipient mice.

2.5. Regulatory T cell depletion

C57BL/6 Foxp3/DTR/GFP transgenic mice allowed short-term Treg ablation in vivo through daily intraperitoneal injections of 50 μg of diphtheria toxin/kg body weight for seven consecutive days [17]. Treg depletion was confirmed by flow cytometry (using CD25 monoclonal antibody and GFP staining in CD4+ T cells).

Diphtheria toxin does not interact with murine receptors. Indeed, this mouse model carries a transgene encoding a simian DTR–GFP fusion protein under control of the murine Foxp3 promoter [18].

2.6. Histopathological analysis

Recipient kidneys grafted with islets were explanted, and 4-μm sections stained with haematoxylin–eosin and safranin. Immunoperoxidase staining was also performed with anti-insulin antibodies (Roche Diagnostics, Meylan, France).

To determine Treg infiltration into islet allografts, Foxp3/DTR/GFP recipient mice were killed 2 days after transplantation; the kidneys containing grafts were immediately frozen in nitrogen, after which 6-μm frozen sections were taken. Fluorescent immunohistochemical staining, using DAPI (4′, 6′-diamidino-2-phenylindole), fluorescein isothiocyanate (FITC)-conjugated CD3 (anti-mouse CD3 FITC; BioLegend, San Diego, CA, USA) and R-phycoerythrin (PE)-conjugated Foxp3 antibodies (anti-mouse Foxp3 PE; eBioscience, San Diego, CA, USA), was performed to determine the presence of intragraft Treg.

2.7. Statistical analyses

Analyses were performed using StatView software version 5.0 (SAS Institute, Cary, NC, USA). MSTs were compared using the log-rank test (Kaplan-Meier survival analysis), and P values < 0.05 were considered statistically significant.

3. Results

3.1. Islet transplant model validation by syngeneic islet grafts in BALB/c mice

All recipient mice (n = 5) rendered diabetic by streptozotocin were normoglycaemic at day 1 post-transplantation with 500 syngeneic BALB/c islets (Fig. 1A). That correction was sustained for 60 days. At 2 months post-transplantation, left nephrectomy was performed and rapid recurrence of diabetes was observed in all grafted mice (n = 5), confirming the role of islet transplantation in the diabetes cure. Using conventional pathology analysis, islet grafts were observed between the kidney capsule and cortex (Fig. 1B). Insulin immunostaining confirmed insulin secretion in situ, demonstrating that the model is functional (Fig. 1B).

3.2. Infusion of apoptotic leukocytes before transplantation delayed islet allograft rejection

Five million donor apoptotic cells (FVB, H-2b) were infused intravenously 7 days before (n = 5) and on the day (n = 5) of allogeneic islet allograft transplants (consisting of 1000 FVB islets)
in BALB/c recipient mice. The MST over the 7-day interval was 15 ± 1.5 days (range: 10–18 days), which was significantly longer than for same-day infusion (10 ± 0.8 days, range: 6–10 days) or untreated grafted control mice (6 ± 0.5 days, range: 5–8 days; \( P < 0.01 \); Fig. 1C). There was no survival difference between the 0-day interval group and the untreated mice group. Thus, only i.v. apoptotic cell infusion 7 days before transplantation significantly increased allogeneic islet graft survival.

This effect was subsequently confirmed using the same experimental design, but with a smaller number of allogeneic islets (500), which was consistent with the principle of the ‘3Rs’ (replacement, reduction and refinement) of animal experimentation [19]. In this case, normoglycaemia was achieved at day 1 post-transplantation. In addition, when \( 5 \times 10^6 \) or \( 50 \times 10^6 \) apoptotic leukocytes were infused 7 days before islet allograft in recipient mice, a delay was observed in islet rejection whatever the number of donor apoptotic cells infused: MST was 9 ± 1.1 days (range: 5–12 days) for \( 5 \times 10^6 \) and 8 ± 1 days (range: 6–14 days) for \( 50 \times 10^6 \) apoptotic leukocytes vs. 3 ± 0.8 days (range: 2–6 days; \( P < 0.01 \)) with no donor apoptotic cell infusion (Fig. 1D).

3.3. Regulatory T cells found in islet allografts only after apoptotic cell infusion

Islet allografts were then performed in Foxp3/DTR/GFP recipient diabetic (H-2b) mice with 500 FVB (H-2k) islets. Of these mice, one group received apoptotic cell infusion 7 days before transplantation while a second group did not. The mice were killed on day 2 after transplantation while the graft was functional (Fig. 2A). Immunohistochemical analyses of DAPI, CD3 and Foxp3 confirmed that 2 days after islet transplantation, Foxp3+ T cells were only detected in the grafts of mice that had undergone i.v. apoptotic cell infusion 7 days prior to transplantation (Fig. 2B), whereas such cells were not detectable in the grafts of mice not receiving apoptotic cells or in those given saline buffer under the kidney capsule (controls). This excluded the possibility of Foxp3 expression due to surgical stress, and normoglycaemia within 1 day of transplantation that lasted throughout the 60-day graft function-monitoring period post-transplantation. Left nephrectomy induced rapid recurrence of diabetes. Tx: transplantation. B. Histological analysis, using haematoxylin–eosin and safranin stains (×10 magnification of 1) and insulin immunostaining (×20, ×40 and ×63 magnification of 2, 3 and 4, respectively), of a syngeneic islet graft under the kidney capsule 60 days post-transplantation show functional grafted islets as brown. C. Effect of intravenous (i.v.) apoptotic splenocyte infusion on islet engraftment with apoptotic cell infusion performed at day 7 and day 0. Graft survivals for the 7-day interval (7 days before allogeneic transplantation, open squares), 0-day interval (day of allogeneic transplantation, black squares), without apoptotic splenocytes (open circles) and with syngeneic transplantation (black circles), \( n = 5 \) per group. D. Effect of i.v. apoptotic splenocyte infusion on islet engraftment, using two different quantities of donor apoptotic cells infused 7 days prior to transplantation, as shown by graft survival rates with \( 5 \times 10^6 \) (black triangles) and \( 50 \times 10^6 \) (open triangles) apoptotic FVB donor spleen cells administered to BALB/c (H-2k) recipient mice 7 days before FVB islet allografts, without apoptotic splenocyte administration (open circles) and for syngeneic grafts (black circles), \( n = 5 \) per group.
suggested the role of $T_{reg}$ in delaying islet graft rejection induced by apoptotic cell infusion.

### 3.4. Regulatory T cell depletion prevented apoptotic cell infusion benefits

To further assess the functional role of $T_{reg}$ in the beneficial effect of apoptotic cells, C57BL/6 Foxp3/DTR/GFP recipient mice were rendered diabetic. This transgenic model allows transient $T_{reg}$ ablation in vivo and, indeed, using flow cytometry, it was found that no CD25+ GFP+ cells were detected after diphtheria toxin treatment (Fig. 3A). To deplete $T_{reg}$ in Foxp3/DTR/GFP recipient mice, diphtheria toxin had been administered intraperitoneally over the 7 days prior to transplantation, and donor FVB apoptotic cells were infused 7 days before transplantation of 500 FVB islets under the recipient’s kidney capsule (Fig. 3B).

Infusion of $5 \times 10^6$ apoptotic cells 7 days prior to transplantation significantly delayed islet rejection. The MST was $8 \pm 1.5$ days (range: 6–15 days) and $3 \pm 0.3$ days (range: 3–4 days) with and without the cell therapy ($P < 0.01$), thereby confirming another donor/recipient combination wherein i.v. infusion of apoptotic cells delayed islet rejection. Furthermore, depletion of $T_{reg}$ by diphtheria toxin injections abolished the benefits of i.v. apoptotic cell infusion: the MST was $8 \pm 1.5$ days (range: 6–15 days) with cell infusion and $2 \pm 0.2$ days (range: 2–3 days) with $T_{reg}$ depletion ($P < 0.01$). Yet, in the absence of apoptotic cell infusion, $T_{reg}$ depletion had no significant effect (MSTs were $2 \pm 0.6$ days [range: 2–4 days] and $3 \pm 0.3$ days [range: 3–4 days] for allogeneic grafts only; Fig. 3C). Thus, it
appears that i.v. apoptotic cell infusion delayed allogeneic islet transplantation through some process involving T_{reg}.

4. Discussion

In humans, allogeneic islet graft survival is limited by immunosuppressant-regimen toxicity, alloimmune responses and autoimmunity recurrence. To modulate allogeneic immune responses after transplantation, a cell-based therapeutic approach was developed based on the immunomodulatory properties of i.v. donor apoptotic cell infusion. In allogeneic BMT settings, our team previously reported that i.v. apoptotic leukocyte infusion performed at the same time as allogeneic bone marrow grafts favoured haematopoietic engraftment, prevented alloimmunization and delayed GVHD [12–14]. Others showed that i.v. donor apoptotic cell infusion 7 days before or after transplantation delayed allogeneic heart rejection [15,16]. In the present islet allograft model, the therapy delayed rejection when given 7 days before transplantation whatever the number of cells tested (five million or 50 million). In our experiments, the graft MST was significantly higher after i.v. infusion of donor apoptotic leukocytes 7 days prior to transplantation. On immunohistochemical analyses, Foxp3+ T cells were specifically present within allografts only if apoptotic cell therapy was infused prior to transplantation. Furthermore, specific T_{reg} depletion in recipient mice abolished the beneficial effect of the cell therapy. Thus, our transplantation model again confirms the ability of donor apoptotic cell infusions to modulate alloimmune responses.

A possible explanation for the discrepancy observed between infusion of apoptotic leukocytes at the time of and 7 days before transplantation is the impact of streptozotocin administered 2 days before islet allograft. Indeed, streptozotocin is responsible for extensive apoptosis of pancreatic beta cells [24] that could generate secondary necrotic cells and the subsequent release of damage-associated molecular patterns (DAMP) [25]. These DAMP might then activate host innate immune cells [25] and counteract the immunomodulatory effect of i.v. apoptotic leukocyte infusion. Indeed, streptozotocin has been shown to up-regulate major histocompatibility complex (MHC) class II molecular expression on macrophages in the endocrine pancreas [26] and to stimulate Th1 responses as well as cytotoxic T cells [27].

Previously, our team reported that i.v. apoptotic cell infusion was associated with a CD4+CD25+ T_{reg} increase early after BMT [13]. The T_{reg} expressed high levels of Foxp3 transcription and exerted potent suppressive activity ex vivo through a cell-to-cell contact mechanism [13]. T_{reg} induction by apoptotic cells in vivo was also suggested by Wang et al. [16] and confirmed in other settings, including after anti-CD3 antibody-induced apoptosis [20] as well as apoptosis in the intestine [21]. Xia et al. [22,23] showed that transfusion of apoptotic cells (beta cells and, more recently, splenic stromal cells) protected non-obese diabetic (NOD) mice from developing T1DM. Intra-venous apoptotic cell infusion suppressed anti-beta cell antibody development and induced ‘immunosuppressive’ T cells [22]. Moreover, limited apoptosis of pancreatic beta cells, obtained by low-dose streptozotocin, induced the development of T_{reg} and protected NOD mice against autoimmune diabetes [24].

Yet, T_{reg} induction after i.v. apoptotic cell infusion was prevented in several models by TGF-β neutralization [13,20], and whether this mechanism was involved in the present study remains to be determined. In addition, the direct link between apoptotic cells, delayed graft rejection and T_{reg} has yet to be elucidated. In our present study of an islet transplantation model, it was demonstrated that the immunomodulatory effect induced by apoptotic cells was prevented by specific T_{reg} depletion, thus supporting the role of T_{reg} induction in the immunomodulatory effect of apoptotic cells.

However, before such an approach can be used in clinical settings, it is important to study how immunosuppressant drugs influence apoptotic cell immunomodulatory effects. It was previously shown that cyclosporin A prevented the graft-facilitating effect of apoptotic cells in a BMT model [28]. In contrast, other drugs such as sirolimus, used in the Edmonton immunosuppressant protocol [3], exerted a synergistic effect [28]. Whether any other immunosuppressant drugs used in the Edmonton protocol can interfere with i.v. donor apoptotic cell infusion also remains to be determined.

Nevertheless, this cell-based therapy using donor apoptotic cells could be proposed to extend islet transplantation to more patients with T1DM. Currently, the main source of islets is cadaveric donors. In our present model as in other solid-organ transplantation models [15], donor apoptotic cells must be infused 7 days before transplantation to exert any immunomodulatory effect, thereby requiring cadaveric donor islet culture for 7 days before transplantation. Nowadays, the isolation of islets is performed through mechanically enhanced enzymatic digestion of pancreas. During the process, islets are exposed to a number of insults such as oxidative stress, ischaemia and cytokine injury that may result in cellular damage and functional impairment that, in turn, could lead to a reduction in the viable islet mass recovered [29] as well as release of DAMP [25]. This means that islet culture for 7 days prior to transplantation could increase the loss of viable islets and the immunogenicity of these islets through DAMP release. However, human islet allografts are performed via the portal vein and, thus, the graft is introduced directly into the liver, an immune-privileged organ in which antigen-presenting cells appear to be biased towards inducing tolerability [30]. In contrast, our study involved grafting islet allografts under the kidney capsule, a conventional rejection site. After i.v. infusion, some apoptotic cells were eliminated in the liver [15] and that could have delayed human islet allograft rejection.

In conclusion, based on our present data overall, i.v. apoptotic cell infusion before islet transplantation appears to both decrease non-specific immunosuppression and its side-effects, and prevent autoimmunity recurrence in transplanted islets.

Disclosure of interest

The authors declare that they have no conflict of interest concerning this article.
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References


