Cytokine mobilization of bone marrow cells and pancreatic lesion do not improve streptozotocin-induced diabetes in mice by transdifferentiation of bone marrow cells into insulin-producing cells

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Abstract

Objective. – Transdifferentiation of bone marrow cells (BMC) into insulin-producing cells might provide a new cellular therapy for type I diabetes, but its existence is controversial. Our aim was to determine if those cells could transdifferentiate, even at low frequency, into insulin-producing cells, in testing optimized experimental conditions.

Methods. – We grafted mice with total BMC, genetically labeled either ubiquitarily, or with a marker conditionally expressed under the control of the insulin β-cell specific promoter. We treated some of the recipients with an agent toxic to β-cells (streptozotocin) and with cytokines stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF).

Results. – The contribution of grafted cells could be detected neither for natural turnover (n = 6), nor for β-cell regeneration after pancreatic lesion (n = 7), 90 days post-transplantation. Cytokine mobilization of BMC in the blood stream, reported to favor their transdifferentiation into cardiac and neural cells, had never been tested before for β-cell generation. Here, we showed that injection of SCF and G-CSF did not lead to a detectable level of transdifferentiation (n = 7).

Conclusions. – We conclude that BMC cannot spontaneously transdifferentiate into insulin-producing cells in vivo, even after β-cell lesion and mobilization induced by cytokines. Interestingly, however, treatment by cytokines may have beneficial indirect effects on STZ-induced hyperglycaemia.

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Résumé

La mobilisation des cellules souches de la moelle osseuse en cellules β par des cytokines ou par lésion pancréatique n’améliore pas le diabète induit chez la souris par la streptozotocine par transdifférenciation des cellules souches en cellules productrices d’insuline.

Objectif. – La transdifférenciation de cellules souches de la moelle osseuse en cellules bêta (β) reste controversée. Notre objectif était de déterminer si cette transdifférenciation est possible, dans des conditions expérimentales favorables.


Résultats. – Aucune contribution au renouvellement naturel des cellules β (n = 6) ni à une régénération après lésion par la streptozotocine (n = 7), n’a pu être mise en évidence, jusqu’à 90 jours après transplantation. La mobilisation dans le sang des cellules médullaires, obtenue par les cytokines (connues pour favoriser la transdifférenciation des cellules souches en cellules cardiaques ou nerveuses), ne permet pas la transdifférenciation à un niveau détectable (n = 7).

Conclusion. – Les cellules souches médullaires ne se transdifférencient pas spontanément in vivo en cellules insulinosécrétrices, même dans un contexte de lésion pancréatique et de mobilisation par des cytokines. Ces dernières cependant pourraient avoir des effets bénéfiques indirects sur l’hyperglycémie induite par la streptozotocine.

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Abbreviations: BMC, bone marrow cell; CK, cytokines; G-CSF, granulocyte-colony stimulating factor; SCF, stem cell factor.

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Mots clés : Pancréas ; Thérapie cellulaire ; Transdifférenciation ; Greffe de moelle osseuse ; Cytokines ; Cellules β

1. Introduction

Type 1 diabetes, characterized by the destruction of insulin-producing β-cells [1], needs new therapeutic solutions. Replacement of pancreatic cells by pancreas or islet allotransplantation was recently improved [2], but is limited by the scarcity of donors. We and others reported that the use of porcine materials (xenotransplantation) could reduce the incidence of diabetes in mice [3–5], but posed immune problems [6–9] and sanitary risks for the recipient [10–12]. To overcome these hurdles, a solution would consist in generating new β-cells. As β-cell proliferation is limited in vitro [13,14], an alternative strategy would be to induce differentiation of stem cells into β-cells. Differentiation of embryonic stem (ES) cells into insulin-producing cells has been reported [15–17], but poses safety risks [18,19], as well as ethical concerns [20,21]. Differentiation of adult stem cells could be another approach. Different adult pancreatic stem cells have been described [22–25]. However, these cells have to be better characterized, and their in vitro multiplication, and differentiation into β-cells greatly improved before being considered to be of clinical use.

Alternatively, stem cells from other adult tissues could be used. Adult bone marrow-derived stem cells present an unsuspected plasticity and seem able to transdifferentiate into cells from all three germ layer cell types [26–29]. Concordant studies also specifically reported possible transdifferentiation of these cells into myocytes [30], cardiomyocytes [31], neurons [32], or hepatocytes [33,34]. Taking into account 1/ this reported ability of BMC to transdifferentiate into hepatocytes, 2/ the close relationship between the pancreas and the liver during embryogenesis, and 3/ the ability of liver cells to transdifferentiate into insulin-producing cells [35], it has been suggested that BMC may also be used to replace β-cells. These cells have interesting characteristics which may be useful for a cellular therapy for type 1 diabetes: they are available in large amounts, and could allow auto-transplantation which would circumvent immunological problems [36–38].

Recent studies reported that some BMC are able to “transdifferentiate” into insulin-producing cells, in vitro [39–41]. After in vivo injection, these cells may improve the state of diabetic mice. BMC injection was even reported to directly contribute to new β-cells in mice [42,43]. However, those results are controversial. Different authors did not observe any transdifferentiation of BMC into “β-like” cells in “physiological” conditions, nor after β-cell lesion [44–46]. Finally other authors, who also found no BMC transdifferentiation into β-cells, suggest a possible indirect benefit of such treatment [47].

In light of these conflicting results, our aim was to directly address the question of BMC transdifferentiation into insulin-producing cells, using an original in vivo model and combining several not yet tested experimental conditions, that were reported to favor BMC transdifferentiation at least for cardiac or neural cells [48,49]. We grafted BMC from knock out (KO) insulin-2 mice, carrying the LacZ gene under the control of the insulin-2 promoter, into KO insulin-1 irradiated recipients. Donor cells will only express β-galactosidase after activation of the insulin-2 promoter, allowing to detect a possible BMC transdifferentiation into new insulin-producing cells, even at very low frequency. First, we determined if BMC were involved in the natural turnover of β-cells. Secondly, we looked at whether they could contribute to the generation of new insulin-producing cells, after induction of diabetes by streptozotocin (STZ) injection. Finally, we evaluated if BMC mobilization in peripheral blood by cytokine treatment could enhance transdifferentiation and improve diabetes.

2. Materials and methods

2.1. Mice

Donor homozygote KO Ins2/LacZ and recipient KOIns1 mice, of C57BL/6 genetic background, KO for insulin-2 and insulin-1 genes, respectively, were generously provided by Dr. Carel (Inserm U661, Paris, France) [50]. KO Ins2/LacZ+ mice also carry the LacZ gene under the control of the insulin-2 promoter, and only express β-galactosidase in cells where the insulin-2 promoter is activated. Transdifferentiation of BMC cells into insulin-producing cells may thus be easily detected after grafting KOIns2/LacZ cells into KOIns1 recipients, by detection of mRNA or protein β-galactosidase (“KOIns2/LacZ model”).

A second complementary model was used to detect grafted cells even in the absence of transdifferentiation into insulin-producing cells (“ROSA26 model”). Cells of homozygote B6.129S-Gt(Rosa)26Sor mice (ROSA26 mice), constitutively expressing LacZ gene, were grafted into corresponding non-transgenic B6129SF2/J controls (B6129 mice, the Jackson Laboratory, Bar Harbor, ME, USA).

All principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed.

2.2. BMC isolation and transplantation

BMC were isolated from the femurs of 5-week-old male ROSA26 or KOIns2/LacZ mice. After filtration (40 μm), red blood cells were depleted using ice-cold ammonium chloride (0.17 M). Either 5 × 10⁶ ROSA26 or KOIns2/LacZ cells were injected, after cell viability control (mortality < 5%), into the tail vein of 5-week-old irradiated (9.5 Gy) female B6129 or KOIns1, respectively, in different protocols (Fig. 1).
2.3. STZ treatment and glycaemia control

In some protocols, STZ (115 mg/kg, Sigma-Aldrich, Saint Quentin Fallavier, France) was injected 52 days post-transplantation (Fig. 1b, c). This STZ dose was optimized to induce a limited hyperglycaemia in order to keep local conditions allowing a possible regeneration. Control irradiated grafted mice were injected with citrate buffer. Glycaemia (glucose oxidase reaction test, Biomerieux, Lyon, France) and body weight were measured once a week after STZ injection. To be able to detect a slight improvement of β-cell function, oral glucose tolerance tests (OGTT) were carried out on mice 10 and 38 days after STZ injection. After an overnight fast, mice were fed with glucose (2 g/kg) dissolved in water. Blood samples were taken from the retro-orbital sinus at 0, 15, 30, 60 and 180 min post-glucose ingestion. As hyperglycaemia has been reported to be toxic for β-cell regeneration, hyperglycemic animals were treated with insulin for the duration of the experiment.

2.4. Cytokine treatment

Six weeks post-irradiation and transplantation, some mice were injected subcutaneously with recombinant murine cytokines: stem cell factor (SCF), and granulocyte-colony stimulating factor (G-CSF) (250 μg/kg per day, R&D Systems, Lilles, France), once a day for 5 days (Fig. 1c). Controls consisted of irradiated grafted mice injected with citrate buffer and cytokines, and also of irradiated grafted mice injected with STZ and PBS. To check the efficiency of hematopoietic cell mobilization by cytokines, numerations were performed on white blood cells from cytokine-injected versus control mice after depletion of red blood cells.

2.5. Detection and quantification of Y chromosome sequence by PCR

DNA was extracted from blood samples collected from the recipient retro-orbital sinus and from other tissues, with the QIAamp DNA blood mini-kit (Qiagen, Coutarboeuf, France). Classical PCR amplification of the Y chromosome specific sequence was performed on GeneAmp PCR System 9700 (PE Applied Biosystem, Foster City, CA, USA) with 100 ng of DNA, in the following 50 μl reaction mix: 200 μmol/l of each dNTP (Promega, Charbonnières, France), 2.5 U Red-Taq polymerase (Sigma-Aldrich) and 0.5 μmol/l of each primer (Table 1, Invitrogen, Cergy Pontoise, France). PCR conditions were: 94 °C for 5 min; 40 cycles with 94 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s. A final elongation step was performed for 10 min at 72 °C.

Y chromosome sequence was also amplified by real-time quantitative PCR to determine the percentages of grafted cells in the pancreas, liver and brain of female recipients. To avoid the bias induced by DNA dosage, a reference gene: Islet Antigen 2 (IA2) coding for a tyrosine phosphatase, was also amplified. Quantification of Y and IA2 sequences, using a

Fig. 1. Experimental designs of BMC injection. a. Injection of KOIns2/LacZ or ROSA26 BMC in irradiated mice. b. Induction of β-cell lesion by STZ in irradiated mice grafted with Ins2/LacZ BMC. c. Mobilization of BMC by cytokines in irradiated mice grafted with KOIns2/LacZ BMC and treated with STZ.
fluorescence-based real-time GeneAmp 5700 sequence detection system (Applied Biosystem), was performed in 25 μl, with the 2X real-time Sybr Green mix (Applied Biosystem) and 300 nmo/l of each primer (Invitrogen, Table 1). A 100 ng DNA amount was used in each test (performed in triplicate). Cycling parameters were 2 min at 50 °C, 10 min at 95 °C, 15 s at 95 °C and 1 min at 60 °C for 40 cycles. For each test, the threshold cycle (Ct) was recorded and a relative quantification of the Y chromosome sequence was performed by calculating the parameter 2[Ct(Y) - Ct(IA2)] (Applied Biosystem).

To correlate this value with a percentage of male donor cells, results were compared with a standard curve established with diluted samples of known male/female cell ratio (Fig. 3a). In that aim, female and male BMC were isolated and mixed in different proportions. DNA was extracted from these different mixes and quantitative PCR was performed.

2.6. Flow cytometric analysis

In the ROSA26 model, the spleen was harvested and mechanically separated into a single cell suspension. Spleen cells (10^5) were stained with the 7-AAD (Sigma-Aldrich) to detect viable cells and analyzed for the presence of β-galactosidase-expressing donor cells by flow cytometry (FACScalibur™, BD Biosciences), using the FluoroReporter LacZ Flow Cytometry kit (Molecular Probes, Eugene, OR, USA). Splenocytes of ROSA26 mice were used as positive controls for FACS analysis.

2.7. Detection of β-galactosidase and insulin-1 mRNA by RT-PCR

PCR was performed on GeneAmp PCR System 9700 (PE Applied Biosystem, Foster City, CA, USA). β-Galactosidase amplification used 5 μl of cDNA templates in a 50 μl reaction volume: 200 μmol/l of each dNTP (Promega), 2.5 U Red-Taq polymerase (Sigma-Aldrich) and 0.5 μmol/l of each primer (Invitrogen, Table 1). The PCR conditions for β-galactosidase amplification were: 94 °C for 5 min, followed by 35 cycles with 94 °C for 30 s, 60 °C for 1930 s and 72 °C for 30 s. A final elongation step was performed at 72 °C for 10 min. To evaluate the sensitivity of our LacZ PCR test, we performed serial dilutions (up to 1/640) of a mixture of 100 KOIns2/LacZ blood-derived mononuclear cells (PBMC) into 2 × 10^6 KOIns1 PBMC. LacZ PCR were still positive with DNA corresponding to the equivalent of 0.2 KOIns2 cells in a background of KOIns1 cells (data not shown).

For insulin-1 amplification, PCR was performed with a “hot-start” polymerase (2.5 U Jump-Start polymerase, Sigma) to prevent primer annealing on the insulin-2 gene (Table 1). The same PCR conditions as for β-galactosidase amplification were used, except for a 68 °C annealing temperature. KOIns2/LacZ and KOIns1 pancreatic mRNA were used as positive or negative controls, respectively, for insulin-1 RT-PCR. Both KOIns2/LacZ and ROSA26 pancreatic mRNA were used as positive controls for β-galactosidase RT-PCR.

Complementary DNA samples were controlled by actin sequence PCR amplification with a 57 °C annealing temperature (Table 1).

2.8. Detection of beta-galactosidase expression in recipient pancreases

Pancreases from recipients were frozen in isopentan and cryopreserved in cryomatrix embedding medium (Thermo Shandon, PA, USA) at −80 °C. Frozen pancreatic sections of 10 μm were fixed with 0.5% glutaraldehyde (Sigma-Aldrich) and incubated with X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside, Promega) overnight at 37 °C. The presence of beta-galactosidase-expressing cells was investigated by observing sections at ×400 magnification. Pancreatic slides of both ROSA26 and KOIns2/LacZ mice were used as positive controls for beta-galactosidase expression.

<table>
<thead>
<tr>
<th>Target sequence Primer sequence</th>
<th>Size of the amplified product (bp)</th>
<th>Sequence reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insl (“classical” PCR)</td>
<td>5′-ATGGGCGCTGTGGTGCACTTTCC-3′</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>5′-TTAGTTGCACTTCTCAGCTGGG-3′</td>
<td></td>
</tr>
<tr>
<td>Beta-galactosidase (“classical” PCR)</td>
<td>5′-ATCCCTCGATGTGACGGTC-3′</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>5′-AGACGATTGATGGCCACAT-3′</td>
<td></td>
</tr>
<tr>
<td>Y (“classical” PCR)</td>
<td>5′-GACAAGTTTTGGGACTGGTA-3′</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td>5′-ATCTCTGTGGCTCTGGGAAA-3′</td>
<td></td>
</tr>
<tr>
<td>Actin (“classical” PCR)</td>
<td>5′-AGCCATGTAGTGACCATCCT-3′</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>5′-CTTCTGAGTGGTGGGAAA-3′</td>
<td></td>
</tr>
<tr>
<td>Y (“real-time quantitative” PCR)</td>
<td>5′-CATGCAAATACAGAGATCACGGA-3′</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>5′-TTTTTTTGCGTCTCTGAAAGCT-3′</td>
<td></td>
</tr>
<tr>
<td>IA2 (“real-time quantitative” PCR)</td>
<td>5′-CAGCGCCCCTCTTACAAGTC-3′</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>5′-GACATGATGTGCGAGCA-3′</td>
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</tbody>
</table>
2.9. Statistical analysis

Data are presented as mean values ± S.E.M. Statistical significance of differences was evaluated using Kruskall Wallis and Dunn’s multiple comparison tests with Prism software (GraphPad Software, www.graphpad.com). A $P$ value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Control of BMC engraftment in recipient tissues

Male KOIns2/LacZ or ROSA26 BMC were grafted either into irradiated KOIns-1 female recipients (“KOIns2/LacZ model”) or C57 non-transgenic female recipients (“ROSA26 model”). In order to provide short-term reconstitution after lethal irradiation, whole BMC were injected (Fig. 1a).

KO Ins2/LacZ mice carry the LacZ gene encoded under the control of the insulin-2 promoter. They only express β-galactosidase in cells which have the capacity to activate the insulin-2 promoter. The KOIns2/LacZ model was thus used to directly evaluate the ability of BMC to transdifferentiate into insulin-producing cells, by detection of mRNA or protein β-galactosidase (see below). As cells of ROSA26 mice constitutively express the LacZ gene, the “ROSA26 model” was used complementarily to detect grafted cells, even in the absence of transdifferentiation into insulin-producing cells.

Before looking for BMC transdifferentiation, we first controlled hematopoietic engraftment as a function of time. In both models, grafted cells were detected in peripheral blood cells by PCR amplification of a Y chromosome sequence, in all recipients tested (Table 2). Secondly, the ROSA26 model was used to further control and quantify hematopoietic engraftment by FACS analysis of β-galactosidase producing-cells in recipient spleens. Results showed a high percentage of grafted β-galactosidase-expressing cells in spleens (97% ± 2.68, 90 days post-transplantation, $n=5$), and confirmed the engraftment success.

Initially, in both models, we confirmed the presence of persistence of donor cells in the pancreas and liver, by PCR detection of male KOIns2/LacZ or ROSA26 donor cells, in tissues of all female recipients tested (Table 2). Moreover, in a convergent way, β-galactosidase-expressing cells were detected on all pancreatic sections of all recipients grafted with ROSA26 cells tested ($n=6$). Positive cells were observed near blood vessels (Fig. 2a), among acini (Fig. 2b), but never in islets.

To be able to quantify the level of engraftment in the pancreas, we set up an original method. By real-time quantitative PCR, the Y chromosome sequence and a reference gene were amplified in recipient pancreases and in other samples corresponding to different known proportions of male and female cells (as described in Section 2, Fig. 3a). The engraftment level was determined for the KOIns2/LacZ model. Results showed that engraftment of transplanted cells was higher in the pancreas (1.09% ± 0.58, 30 days post-transplantation, $n=5$) and the liver (1.96% ± 0.42, $n=5$) than in the brain (0.27% ± 0.05, $n=2$; $P<0.05$). Moreover, the number of grafted cells increased in both tissues with time. It reached a mean of 9.15 ± 6.32% ($n=6$) in the pancreas, 90 days post-transplantation (Fig. 3b). The brain blood barrier may prevent medullar cells from entering this tissue, but it hardly seems to be the case as the presence of grafted cells in the brain has already been reported after irradiation and bone marrow transplantation [32,51].

3.2. BMC do not contribute to natural β-cell turnover and to β-cell regeneration after STZ lesion

To evaluate the ability of BMC to transdifferentiate into insulin-producing cells, we used the “KOIns2/LacZ model”. It has been suggested that heart and liver lesion may be neces-

Table 2

<table>
<thead>
<tr>
<th>Days post-transplantation</th>
<th>Peripheral blood cells</th>
<th>Pancreas</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ROSA26</td>
<td>KOIns2/LacZ</td>
<td>ROSA26</td>
</tr>
<tr>
<td>60</td>
<td>8/8</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>90</td>
<td>20/20</td>
<td>15/15</td>
<td>6/6</td>
</tr>
</tbody>
</table>

NT: non-tested.

Fig. 2. Typical results of blue β-galactosidase-expressing cells detection, on pancreatic sections, of an irradiated non-STZ-treated recipient grafted with ROSA26 BMC (Fig. 1). Donor cells were observed near blood vessels (a), and among exocrine cells (b).
sary for transdifferentiation of BMC into cardiac and hepatic cells, respectively [52,53]. Then, we tested if a toxic pancreatic damage induced by STZ could favor transdifferentiation of BMC into

\[ \beta \]-cells. About 2 months after transplantation, recipients grafted with KOIns2/LacZ cells were injected with STZ (“STZ group”), or with citrate buffer (“citrate buffer control group”, Fig. 1b).

The injection of 115 mg/kg STZ-induced a limited hyperglycaemia (Fig. 5b), compared to higher tested doses (data not shown). We chose this dose to induce a limited destruction of \( \beta \)-cell in recipient mice, to keep conditions allowing a possible regeneration of \( \beta \)-cells beyond STZ injection. We indirectly verified the limited impact of this treatment in controlling the glycaemia of recipient mice, and confirmed insulin-2 mRNA expression (\( n = 5 \)) by RT-PCR 40 days after the STZ treatment (data not shown).

To determine whether the insulin promoters of donor BMC had been activated in recipient pancreases, we looked for \( \beta \)-galactosidase and insulin-1 mRNA by RT-PCR, and for \( \beta \)-galactosidase protein by histochemistry.

Concerning \( \beta \)-galactosidase expression, transcripts were not detected in pancreases of non-STZ-treated mice (controls), nor in pancreases of STZ-treated recipients (\( n = 6 \) control recipients, \( n = 7 \) STZ-treated recipients, 90 days post-transplantation), whereas transcripts were detected in KOIns2/LacZ control pancreases. Signals were also undetected in the brain (\( n = 6 \) control recipients, \( n = 7 \) STZ-treated recipients), the spleen (\( n = 6 \) control recipients, \( n = 7 \) STZ-treated recipients) and the liver (\( n = 6 \) control recipients, \( n = 7 \) STZ-treated recipients, Fig. 4). Concurrently, \( \beta \)-galactosidase activity could not be detected in the pancreas (\( n = 5 \) control recipients, \( n = 5 \) STZ-treated recipients, 30 sections per pancreas), although \( \beta \)-cells from KOIns2 pancreases were strongly labeled. Concerning insulin-1 expression, transcripts were detected in none of the tested pancreases (\( n = 6 \) control recipients, \( n = 7 \) STZ-treated recipients, data not shown).

3.3. BMC do not transdifferentiate into insulin-producing cells even after stem cell mobilization by cytokines

To determine if BMC could transdifferentiate into insulin-producing cells even at low frequency, we used new experimental conditions reported to favor transdifferentiation into neural and cardiac cells, but never yet tested for \( \beta \)-cells (Fig. 1c). Stem cells are very rare in peripheral blood cells and it has been suggested that their mobilization by cytokines, out of the bone marrow, increases transdifferentiation frequency. We tested this hypothesis for transdifferentiation into \( \beta \)-cells by injecting SCF and G-CSF, at the time of pancreatic lesion, in some of the recipients grafted with KOIns2/LacZ
cells ("CK-STZ" group). Control recipients were not injected with STZ ("CK-citrate buffer" group) or not injected with cytokines ("PBS-STZ" group).

First, cytokine impact on grafted cell mobilization was indirectly evaluated by counting the number of peripheral blood nucleated cells. As expected, cytokines induced a rapid transient increase of the number of circulating blood cells (five-fold increased on the fifth day of injection compared to day 3 before the first injection, $P < 0.05$, Fig. 5a). Then recipient glycaemia was measured from day 5 post-STZ injection, glycaemia was significantly higher in the PBS-STZ group than in the CK-citrate buffer group (Fig. 5b). Surprisingly, glycaemia was not significantly different between the CK-STZ and the CK-citrate buffer groups. It tended to be lower in the CK-STZ group (mean = 2.32 g/l ± 1.52 between day 4 and day 40 post-STZ) than in the PBS-STZ group (mean = 3.03 g/l ± 1.73). This tendency was not linked to an undernourishment of the recipients as their weight...
tended to be higher than in PBS-STZ mice (data not shown). In the same way, OGGT performed 10 and 38 days post-STZ injection showed that the PBS-STZ group was significantly less tolerant to glucose than the CK-citrato buffer group. On the contrary, CK-STZ mice were not significantly less tolerant than CK-citrato buffer mice but tended to be more tolerant to glucose than PBS-STZ mice (Fig. 5c).

Next, we looked if glycemia tended to be lower because of a transdifferentiation of BMC in insulin-producing cells. However, neither significant insulin-1 and \( \beta \)-galactosidase transcriptions, nor \( \beta \)-galactosidase protein activity was detected in the pancreases of CK-STZ recipients (\( n = 7 \), data not shown). We also controlled the absence of transdifferentiation in control mice (PBS-STZ and CK-citrato buffer groups, data not shown).

4. Discussion

Bone marrow cells (BMC) were suggested to be able to transdifferentiate in vivo or in vitro into insulin-producing cells [39, 40, 42] and may provide a new cellular therapy for type 1 diabetes. However, contradictory results have also been reported [44–47, 54, 55]. Our aim was to determine if BMC could transdifferentiate into insulin-producing cells, even at low frequency, in experimental conditions designed to favor transdifferentiation. As the phenotype of BM stem cell able to transdifferentiate is currently not elucidated (MSC, HSC, SP…), we used whole BMC in order not to exclude any kind of stem cells.

To be able to trace BMC, we grafted irradiated recipient mice with genetically labeled allogeneic total BMC. We used either ROSA26 BMC, constitutively expressing the LacZ gene, or KOIns2/LacZ cells, expressing \( \beta \)-galactosidase under the control of insulin-2 promoter. In the two different models, donor cells were detected either by flow cytometry, histochemistry or PCR in peripheral blood, spleen, liver and pancreas, confirming the engraftment of recipients. We also showed that pancreatic chimerism increased with time. This may be related to either the multiplication of grafted cells or to a preferential organ tropism or both mechanisms. However, transdifferentiation did not occur, as no evidence of insulin promoter activity was detected either by histochemistry or RT-PCR. We also tested if a pancreatic lesion by STZ could induce local signals favoring BMC migration and transdifferentiation. However, in STZ-treated recipients, \( \beta \)-cells derived from grafted cells could not be detected either. Those results suggest that, in our hands, BMC do not contribute spontaneously and directly to \( \beta \)-cell turnover or replacement.

Finally, since bone marrow-derived stem cells are naturally extremely rare in peripheral circulation, transdifferentiation may be enhanced by increasing the amount of medullar progenitors in peripheral blood with cytokine treatment. However, even after mobilization of BMC by SCF and G-CSF injection, transdifferentiation was not observed, even when diabetes was induced by STZ, at the time of BMC mobilization.

Results related to BMC contribution to \( \beta \)-cell turnover presented in this study, are in accordance with those of Choi et al. [44] who did not report any transdifferentiation into insulin-producing cells, in the absence of \( \beta \)-cell lesions, in a similar model of genetic-labeled BMC transplantation. Since liver and heart lesions have been reported to induce BMC transdifferentiation into hepatic and cardiac cells, recipients were treated with STZ. However, signals induced by STZ did not induce transdifferentiation either in Choi’s model or in ours. This lack of effect for pancreas might be related to a too high STZ dosage, leading to a complete destruction of \( \beta \)-cell and the corresponding disappearance of conditions compatible with \( \beta \)-cell regeneration. This does not seem to be the case because Choi et al. used mild multiple low dose of STZ. Moreover, we verified in our model the limited impact of our 115 mg/kg STZ (compared to higher doses) on glycemia and the persistence of insulin mRNA expression several weeks after STZ treatment. This indirectly suggest that both STZ treatments may not preclude regeneration signals to occur.

Our results are also in accordance with those of Mathews et al., obtained in a similar system. As hyperglycaemia has been reported to limit \( \beta \)-cell regeneration in vitro [56, 57] and in vivo [58–60], hyperglycaemic mice were treated with daily insulin injections in this study and in the study of Mathew et al. One can argue however that insulin might obviate signals that could promote endogenous \( \beta \)-cell regeneration. However, insulin treatment cannot be the only reason for the lack of transdifferentiation in our studies, as BMC transdifferentiation was not observed in STZ-injected mice, even in the absence of insulin treatment [44–47]. The lack of transdifferentiation seems irrespective to the type of lesion generated. Indeed, Lechner et al. [45] reported that partial pancreatectomy do not induce transdifferentiation. Moreover a recent report also showed that transdifferentiation did not occur in STZ-induced diabetic RIP/IGF-1 mice, although this model allow conditions for a strong \( \beta \)-cell regeneration [54]. No significant transdifferentiation was detected in diabetic NOD mice either [55].

Our results and those obtained by others are in accordance with an absence or extremely low frequency (if any) of BMC to \( \beta \)-cell transdifferentiation [44–47, 54]. However, this conclusion differs from the results of Ianus et al. [42]. After bone marrow transplantation, these authors found that up to 3% of islet \( \beta \)-cells were of bone marrow origin, even in the absence of \( \beta \)-cell lesion and BMC mobilization. Experimental conditions may explain those discrepancies, as the end-point of Ianus’ study is 2 months earlier than in our study. Then, it is possible that BMC transdifferentiated into insulin-producing cells immediately after transplantation as observed by Ianus et al. and disappeared thereafter in our model. Since we showed that the colonization of grafted cells increased between 4 and 13 weeks (Fig. 3b), we would have to admit that transdifferentiated cells, unlike other grafted cells, disappeared during the course of the experiment. In that case, the clinical interest of BMC transplantation would be very limited. Another major difference lies in the labeling of transplanted cells. Ianus et al. also used donor cells with a reporter gene produced only when the insulin promoter is activated (Cre-Lox/GFP system). But in this system, once cells have activated the insulin promoter, they irreversibly express EGFP. It implies that all cells which transiently activate the insulin promoter are marked. It has been shown that in different contexts the insulin promoter can be activated [43]. Ianus et al. quantified BMC contribution...
to islet cells by flow cytometry analyses. The culture of islet cells before analysis could have for instance led to an artificial activation of the insulin promoter and irrelevant β-cell marking.

Thus, in accordance with most authors, we showed that in the absence of mobilization, BMC do not transdifferentiate in vivo into insulin-producing cells. The low frequency of bone marrow stem cells in peripheral circulation could be a limiting factor in our studies. For instance, transdifferentiation into neural and cardiac cells has been reported to be increased after BMC mobilization by SCF and G-CSF [48,49]. As far as we know, this combination of cytokines had never been tested for β-cells. Here, we showed that even after a mobilization of BMC, no evidence of transdifferentiation was detected. Our results are in accordance with the results of Taneera et al. [61] who were unable to detect any transdifferentiation with a G-SCF treatment. This confirm the hypothesis that the spontaneous transdifferentiation of BMC into insulin-producing cells is, at most, an extremely rare event. Although BMC may be able to contribute to cardiac or neural cell regeneration, it hardly seems the case for β-cells.

Those conclusions are in accordance with other studies about BMC plasticity. Krause et al. showed that hematopoietic stem cells could transdifferentiate into all three embryonic lineages after implantation in a blastocyst. However, they did not report any contribution to pancreatic tissue. In a similar way, MAPC derived ex vivo from mesenchymal cells have not report any contribution to pancreatic tissue. In a similar lineages after implantation in a blastocyst. However, they did

In spite of any evidence of transdifferentiation, it is noteworthy that our results suggest that cytokine treatment may have a beneficial effect on the state of diabetic mice, compared to their corresponding non-cytokine treated controls (as suggested for both glycaemia and OGTT). Many of the grafted cells present in the pancreas of our recipient mice are of the hematopoietic lineage (CD31 and CD117 positive by FACS analyses, data not shown). Hess et al. [47] suggest that BM cells could induce pancreatic regeneration by differentiation into endothelial cells. Concordantly, GM-CSF mobilization of BMC resulted in higher angioblasts in peripheral circulation, associated with higher islet engraftment [62]. Our results obtained with cytokines could then be attributed to BMC differentiation into endothelial cells. Endothelial cell factors, such as VEGF, may trigger generation of new β-cells [64]. Other factors may be of interest, such as IGF-1 [65,66]. Recently, the involvement of the cMet/HGF pathway has been implicated between BMC and the generation of new insulin-producing cells [67]. It would be of interest in another study to test other combinations of growth factors, and to analyze the phenotype of BM derived cells localized in pancreas. However, the aim of our work was to determine if BMC could transdifferentiate into β-cells, and our tools and animal models (KOIns) were designed to answer this precise question.

In conclusion, data presented in this report support the concept that the transplantation of unpurified BMC, although technically appealing, is not associated with transdifferentiation, and is, as such, probably unlikely to be of any clinical relevance to directly produce insulin-producing cells. However our results also suggest that cytokine BMC mobilization may exert a possible positive impact on the diabetes in mice. Mobilization of BMC in addition to cytokines and/or growth factors could be an exciting approach favoring β-cells neogenesis.

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