Received: 7 February 2019 | Revised: 28 May 2019 | Accepted: 19 June 2019

DOI: 10.1002/term.2925

RESEARCH ARTICLE

WILEY

Daily bone marrow cell transplantations for the management of fast neurodegenerative processes

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Funding information
University of Salamanca; Centre for Regenerative Medicine and Cell Therapy of Castile and Leon; Regional Government of Castile and Leon, Grant/Award Number: SA030P17; Spanish Ministry of Economy and Competitiveness (MINECO), Grant/Award Numbers: SAF2013-41175-R and SAF2016-79668-R

Abstract

Cell therapy has been proven to be a promising treatment for fighting neurodegenerative diseases. As neuronal replacement presents undeniable complications, the neuroprotection of live neurons arises as the most suitable therapeutic approach. Accordingly, the earlier the diagnosis and treatment, the better the prognosis. However, these diseases are commonly diagnosed when symptoms have already progressed towards an irreversible degenerative stage. This problem is especially dramatic when neurodegeneration is aggressive and rapidly progresses. One of the most interesting approaches for neuroprotection is the fusion between healthy bone marrow-derived cells and neurons, as the former can provide the latter with regular/protective genes without harming brain parenchyma. So far, this phenomenon has only been identified in Purkinje cells, whose death is the cause of different diseases like cerebellar ataxias. Here we have employed a model of aggressive cerebellar neurodegeneration, the Purkinje Cell Degeneration mouse, to optimize a cell therapy based on bone marrow-derived cell and cell fusion. Our findings show that the substitution of bone marrow in diseased animals by healthy bone marrow, even prior to the onset of neurodegeneration, is not fast enough to stop neuronal loss in time. Conversely, avoiding bone marrow replacement and ensuring a regular supply of healthy cells through continuous, daily transplants, the neurodegenerative milieu of PCD is enough to attract those transplanted elements. Furthermore, in the most affected cerebellar regions, more than a half of surviving neurons undergo a process of cell fusion. Therefore, this method deserves consideration as a means to impede neuronal cell death.

KEYWORDS

busulfan, cell transplants, cerebellum, Purkinje cell degeneration, Purkinje cells, radiation

1 | INTRODUCTION

Neurodegenerative diseases are characterized by a progressive neuronal loss that leads to cognitive, sensorial, and/or motor impairments. Although their early detection and prevention is optimum, these maladies are generally diagnosed when neuronal death has already begun, and sometimes with a progression that is extremely fast (Arneson, Zhang, Yang, & Narayanan, 2018; Laforce et al., 2018; Zhang, Han, & Wang, 2018). Cell therapy is one of the most promising strategies for dealing with neurodegenerative diseases, by palliating or slowing down neuronal death. Bone marrow-derived cells (BMDC) are among the

Abbreviations: BMDC, bone marrow-derived cells; CB, calbindin; DAPI, 4'-6-diamidino-2-phenylindole; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PCD, Purkinje cell degeneration

different cell types potentially used for such therapy, as they reach physiologically into the encephalon and integrate as both neurons and glial cells (Eglitis & Mezey, 1997; Brazelton, Rossi, Keshet, & Blau, 2000; Mezey, Chandross, Harta, Maki, & McKercher, 2000; Recio et al., 2011). As a result, in the last two decades, several research groups have dedicated their efforts to unravel the mechanisms that underlie such integration. In this sense, cell fusion is one of the most remarkable ways for the encephalic integration of BMDC and has been proposed as a therapeutic approach against neurodegenerative diseases (Álvarez-Dolado, 2007). A neuron fused with a BMDC forms a structure called heterokaryon that contains two nuclei expressing its particular genetic material (Johansson et al., 2008; Weimann, Johansson, Trejo, & Blau, 2003). The transplant of healthy, genetically competent bone marrow in individuals carrying a genetic deficit has been proposed as a suitable system for cell/genetic therapy (Álvarez-Dolado, 2007; Kemp et al., 2018). Moreover, this phenomenon has sparked a great deal of interest with respect to the treatment of neurodegenerative diseases, because an inflammatory environment potentiates the cell fusion of BMDC into the brain (Johansson et al., 2008).

Intriguingly, Purkinje cells are almost the only neuronal type that undergoes cell fusion (Álvarez-Dolado et al., 2003; Recio et al., 2011; Díaz, Recio, Weruaga, & Alonso, 2012). Therefore, the study of animal models suffering from cerebellar impairments presents an opportunity for studying the potential use of cell fusion as a therapeutic approach against neurodegenerative diseases. More precisely, certain cerebellar diseases, such as spinocebellar ataxias, are good examples of a fast-onset process of neurodegeneration (Anheim, Tranchant, & Koenig, 2012; de Assis Franco et al., 2018; Karakaya et al., 2019; Shashi et al., 2018; Sheffer et al., 2019).

There are different murine mutations that lead to Purkinje cell death such as staggerer, reeler, Lurcher, weaver, or Purkinje Cell Degeneration (PCD) among others (Lalonde & Strazielle, 2007). In particular, PCD mice suffer a dramatic Purkinje cell loss that begins around postnatal day 18 (P18) and progresses rapidly over the next 2 weeks, until virtually all of these neurons have disappeared at around P40 (Mullen, Eicher, & Sidman, 1976; Wang & Morgan, 2007). Moreover, the *pcd* mutation causes the lack of expression of the *Agtpbp1* gene (also known as *Nna1* or *Ccp1*), which can origin early-onset and aggressive human cerebellar impairments (Karakaya et al., 2019; Shashi et al., 2018; Sheffer et al., 2019). Therefore, the PCD mouse offers a suitable model for the study of fast neurodegeneration that can be used as a demanding trial for cell therapies.

Previous works have reported the potential benefits of the transplant of embryonic neural (cerebellar) stem cells into the cerebellar parenchyma of PCD mice, with the aim to substitute the Purkinje cell population, even in completely degenerated cerebella (Carletti & Rossi, 2005; Triarhou, Zhang, & Lee, 1995, 1996; Zhang, Lee, & Triarhou, 1996). Although some behavioral amelioration has been achieved, the integration of new neurons in the adult central nervous system is far away from the complete restoration of standard synaptic connections. In addition, the direct injection of cells into the cerebellum presents the problem of causing even more harm to the cerebellar parenchyma, as well as the localized effect of the injection in a context of massive degeneration (Díaz, Muñoz-Castañeda, Alonso, & Weruaga, 2015).

Consequently, bone marrow transplantation, aimed at either neuronal rescue or delaying degeneration, is a much more conservative alternative that should be considered (Kemp et al., 2018). However, to date, the most promising results obtained using this type of therapy and PCD mouse have been related to a different type of degeneration (Díaz, Lepousez, et al., 2012). Hence, the use of cell fusion for rescuing Purkinje cells still remains elusive. One reason for this could be that host bone marrow ablation and subsequent healthy bone marrow transplantation are generally performed around P20. As a result, the substitution of the hematopoietic system and the integration of BMDC into the cerebellum occur more slowly than the fast progression of the degeneration (Díaz et al., 2018; Recio et al., 2011). Additionally, although a degenerative/inflammatory milieu can foster the arrival of bone marrow-derived cells into the brain (Johansson et al., 2008), this environment seems unable to attract healthy BMDC in time. These issues should be kept in mind, especially with respect to neurodegenerative processes that develop with extremely fast cell death.

As a consequence, there are two strategies a priori for achieving the cell fusion of Purkinje neurons and BMDC in scenarios of rapid neurodegeneration: to carry out the transplantation as soon as possible and to increase the attraction of cells to the cerebellum. This was precisely the aim of this work, which included three different variations of cell therapy with BMDC: perinatal transplants, perinatal transplants with radiation, and daily intravenous transplants.

2 | EXPERIMENTAL PROCEDURES

Green fluorescent protein (GFP) transgenic BALBc mice (Mus musculus, L. 1758; Jackson Bar Harbor, ME) were used as donors of bone marrow cells. Both wild-type and PCD mice of the C57BL/DBA strain (Jackson) were used as the recipients of the bone marrow transplants. The mice were also sorted for carrying out three different methods (perinatal transplants, perinatal transplants with radiation, and daily intravenous transplants). In total there were six groups containing four animals each (three in the case of PCD mice, which were subjected daily to intravenous transplants). The mice were housed at the Animal Facilities of the University of Salamanca at constant temperature and humidity, with a 12/12-hr photoperiod and were fed ad libitum with water and special rodent chow (Rodent toxicology diet, B&K Universal G.J., S.L. Molins de Rei, Barcelona, Spain). All animals were housed, manipulated, and sacrificed in accordance with current European (2010/63/UE and Recommendation 2007/526/CE) and Spanish legislation (Law 32/2007 and RD 53/2013). The experiments were approved by the Bioethics Committee of the University of Salamanca.

The mice were genotyped as previously described (Valero, Berciano, Weruaga, Lafarga, & Alonso, 2006). Briefly, DNA was extracted from a tissue sample taken from the tail of offspring from heterozygous (+/pcd) parental mice. The wild-type and *pcd* alleles were identified by amplifying the associated microsatellite regions

D13Mit250 and D13Mit283 by PCR. The primers used (web resource of Jackson Laboratories) can be found at http://www.informatics.jax.org/searches/probe.cgi?38700 and http://www.informatics.jax.org/searches/probe.cgi?41581.

The PCR products of the D13Mit250 and D13Mit283 regions have different sizes for both associated alleles, which differentiates the genotype of the mice. These differences were visualized on a 3% (w/v) agarose gel after electrophoresis.

To ablate the bone marrow of the recipients (in both cases for perinatal transplants), busulfan (Sigma-Aldrich, St. Louis, MO, USA) injections were performed intraperitoneally in a pregnant female at embryonic days E17 and E18, as previously described (Espejel, Romero, & Álvarez-Buylla, 2009), in order to have offspring lacking bone marrow.

In the experiments involving radiation, the recipient animals were irradiated at P2 (for one variation of perinatal transplants) or P19 (for daily transplants) with a ^{137}Cs source suitable for mice (model Gammacell 1000 Elite, MDS Nordion, Ottawa, Canada). This device provides a radiation rate of 243 cGy/min, with energy of 0.662 MeV. The doses administered were 4 (perinatal transplants) or 3 Gy (daily transplants).

The cells for carrying out the transplants were harvested as previously described (Díaz, Recio, et al., 2012). Briefly, donors were sacrificed by cervical dislocation and the femurs and tibias were dissected. Bone marrow extraction was performed using an Iscove's Modified Dulbecco's Medium injection medium (Iscove's Modified Dulbecco's Medium; Invitrogen; Carlsbad, CA, USA) at both epiphyses. The wash was filtered through a 70-μm pore size filter (Becton Dickinson; Franklin Lakes, NJ, USA) and centrifuged at 1,500 rpm for 5 min. The supernatant was removed, and the pellet was resuspended in lysis buffer (140 mM NH4Cl, 17 mM Tris-base, pH 7.4) for 5 min to break up the erythrocytes. The reaction was finished by adding 45 ml of 0.1 M phosphate-buffered saline, pH 7.4 (PBS), to each collection tube. The number of cells in each tube was estimated from an aliquot using a Thoma chamber, and the cell suspension was centrifuged again at 1,500 rpm for 5 min. For transplants in newborns, the pellet was directly resuspended in PBS for transplantation into the recipient animals. For daily transplants, the pellet was resuspended in a 1:1 (v/v) mixture of Iscove's Modified Dulbecco's Medium and dimethyl sulfoxide (Probus, Badalona, Spain), aliquoted, and quickly frozen at -20 °C until use.

Cell transplants for newborn mice, treated prenatally with busulfan, were carried out intrahepatically (Espejel et al., 2009) either at P0 or at P4 (for those previously irradiated). Each mouse received a single injection of 7.5 million bone marrow cells from GFP animals. For daily transplants, each day a frozen aliquot was quickly thawed, the cell suspension was centrifuged (1,500 rpm for 5 min) and resuspended in PBS. Then, the mice were subjected to injections in the tail vein from P20 to P34 (1 million cells each day) with a 30 G insulin syringe.

Prior to sacrifice, the level of donor engraftment in perinatal-transplanted mice was assessed by flow cytometry of peripheral blood (FACS Calibur, BD Biosciences; NJ, USA). Peripheral blood was subjected to red blood cell lysis by treatment with ammonium chloride (see above), washed, and resuspended in PBS. Cytometric analysis was performed to determine the donor chimerism by means of the percentage of GFP cells.

Perinatal-transplanted mice were sacrificed at P30 and daily-transplanted animals at P35. The mice were deeply anaesthetized with 1 μl/g b.w. of chloral hydrate (Prolabo, Fontenay-St.-Bois, France). Then, the animals were perfused intracardially with 0.9% NaCl (w/v) to wash their blood vessels, followed by 15 min of Somogyi's fixative (4% w/v depolymerized paraformaldehyde and 15% v/v saturated picric acid in phosphate buffer 0.1M pH 7.4). After perfusion, the brains were dissected out, postfixed for 2 hr with the same fixative, and rinsed with phosphate buffer.

Tissue blocks were cryoprotected by immersing them in 30% (w/v) sucrose in phosphate buffer and frozen. The cerebella were sectioned in 40-μm-thick parasagittal slices using a freezing-sliding microtome (Jung SM 2000, Leica Instruments, Nussloch, Germany) attached to a freezing unit (Frigomobil, Leica Instruments).

Immunofluorescence was performed by washing the tissue slices in PBS and incubating them overnight at room temperature under continuous rotary shaking in a medium containing 0.2% (w/v) Triton X-100, 5% (v/v) normal donkey serum, the primary anti-GFP polyclonal goat antibody (1:2,000; Abcam, Cambridge, UK), and another primary antibody (to characterize the transplant-derived cells) in PBS. The second primary antibody was either an anti-calbindin D28k (CB) monoclonal mouse antibody (1:2,000; Swant, Bellinzona, Switzerland) for labeling Purkinje cells, an anti-glial fibrillary acidic protein polyclonal mouse IgG (1:1,000; Sigma-Aldrich) for labeling astrocytes, or an anti-Iba1 polyclonal rabbit IgG (1:1,000; Wako, Osaka, Japan) for labeling microglia. Next, the slices were washed in PBS and incubated in a second medium with Cy2-conjugated donkey anti-goat fluorescent antibody (1:500; Jackson Laboratories, West Grove, PA, USA) and Cy3-conjugated donkey anti-mouse or anti-rabbit fluorescent antibody (1:500; Jackson) in PBS for 2 hr at room temperature under continuous rotary shaking. Thirty minutes before the end of the incubation, 4'-6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), 1:2000 v/v, was added to the medium to counterstain the cell nuclei. Finally, sections were rinsed in PBS and mounted with coverslips and a freshly prepared anti-fade medium.

The cells were analyzed using the Neurolucida (V8.23, MicroBrightField, Colchester, VT, USA) and Neuroexplorer programs (V4.70.3, MicroBrightField), and co-localization was analyzed using a confocal microscope (Leica TCS SP2, Leica Instruments). To carry out the statistical analyses, the data obtained from wild-type and PCD animals were compared using the Mann Whitney's U test of the SPSS 22.0 for Windows program (SPSS, Chicago, IL, USA).

Image processing was restricted to adjusting the brightness and contrast of the figures, when necessary.

3 | RESULTS

Our first attempt at achieving cell fusion of BMDC and Purkinje neurons in PCD mice was based on carrying out the transplantation as

soon as possible. For this reason, we decided to ablate the bone marrow of the recipients with busulfan and to transplant new cells intrahepatically at P0, approximately 18 days before the onset of cerebellar degeneration.

The levels of blood chimerism at the age of sacrifice during this first experiment (P30) revealed a donor engraftment of around 35%, with no differences between the wild-type and PCD groups (p >.05; Figure 1a). This result indicated that genotype does not affect such integration.

Then, we analyzed the integration of BMDC into the encephalon of animals. The only cell type positive for GFP found in both wild-type and PCD mice was microglia (Figures 1c-1e), at low abundance in both genotypes. In addition, no Purkinje cells derived from the transplant were detected in wild-type or in PCD animals. Moreover,

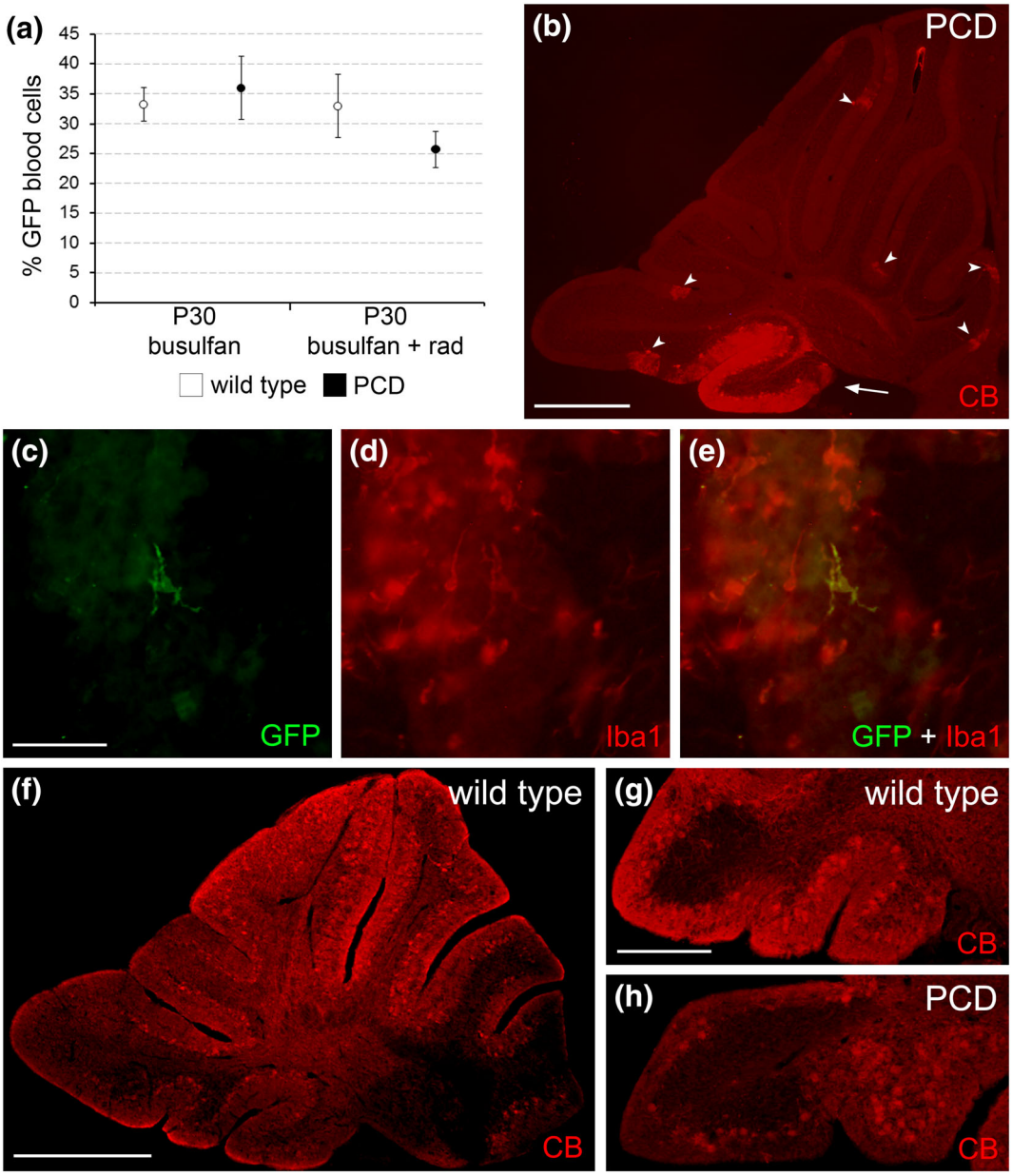

FIGURE 1 Transplants after bone marrow ablation with busulfan. (a) Chart showing the percentage of green fluorescent protein (GFP)-positive cells in peripheral blood of wild-type and PCD animals at P30 (before sacrifice) after bone marrow transplantation; the animals were either subjected to only busulfan treatment or in combination with radiation (rad); no differences were detected between the experimental groups. (b) Panoramic view of the vermis of a PCD mouse at P30 subjected to bone marrow transplantation; note the advanced degenerative process because only few calbindin-labelled Purkinje cells remain alive (CB, red, arrowheads), with the lobule X (arrow) being the most degeneration-resistant region. (c-e) Bone marrow-derived microglia labeled with GFP (green) and also positive for Iba1 (red). (f) Panoramic view of the vermis of a wild-type mouse at P30 subjected to radiation at P2 and bone marrow transplantation; note the aberrant appearance of Purkinje cells labelled with calbindin (CB, red), as well as the regions lacking those neurons. (g and h) Images of cerebellar regions of both wild-type and PCD animals after radiation and transplantation; note that the Purkinje cells (CB, red) do not form a defined layer. Scale bar: 500 μm for b; 50 μm for c-e; 500 μm for f; 200 μm for g and h [Colour figure can be viewed at wileyonlinelibrary.com]

the pattern of cerebellar neurodegeneration in PCD mice was similar to what has been described in standard mutant animals at P30: only lobule X presented a high density of Purkinje cells, which were scarce in the rest of the cerebellum (Figure 1b; Mullen et al., 1976; Baltanás et al., 2011; Baltanás et al., 2013). This result indicated that this initial experiment was not fast or efficient enough to rescue Purkinje cells.

In our second approach, we decided not only to carry out early perinatal transplants but to also increase the possible attraction of BMDC into the cerebellum. Previous reports have shown that at perinatal stages, low doses of radiation harm the cerebellar cortex, thus creating an inflammatory milieu that increases the rate of cell fusion in a dose-dependent manner (Espejel et al., 2009). Accordingly, newborn mice, whose bone marrow was previously ablated with busulfan, were subjected to a radiation dose of 4 Gy at P2. These animals were subsequently transplanted intrahepatically at P4.

The levels of blood chimerism at P30, when the animals were sacrificed, were statistically similar to those of the previous experiment, where no differences between wild-type and PCD mice ($p > .05$; Figure 1a) were found.

Signs of tissular damage caused by the radiation were detected in both wild-type and PCD animals when the cerebellum was analyzed. The cerebellum of the wild-type animals was smaller than nonirradiated P30 mice. In addition, their Purkinje cells presented an aberrant distribution that was displaced and the cerebellar cortex showed in some regions a clear loss of these neurons (Figures 1f and 1g). In the case of PCD mice, the cerebellar degeneration associated with the mutation was even greater when radiation was administered (Figure 1h). In addition, the only neural cell type derived from the transplant was microglia (data not shown), and no GFP-positive Purkinje cells were detected in any of the experimental groups. It could be concluded that the combination of both chemical bone marrow ablation and cell attraction by radiation damage was not fast enough to foster cell fusion before PCD cerebellar degeneration. Moreover, this second strategy increased the damage in an already affected environment and was thus discarded.

The data obtained from the previous two studies indicated that the restitution of bone marrow is an excessively long process and hampers the arrival of BMDC into the cerebellum prior to Purkinje cell death in PCD mice. Consequently, we decided to test a new strategy that bypassed the process of bone marrow ablation and reconstitution. Previous findings have suggested that a single intravenous injection of stem cells without bone marrow ablation can generate heterokaryons (Kemp et al., 2011). However, a continuous supply of bone marrow cells into the cerebellar cortex is also optimum for maximizing the number of fusion events that would ameliorate the severity and fast progression of the cerebellar degeneration. Taking this into account, we injected 1 million bone marrow stem cells daily in the tail vein of mice during 15 days from P20 to P34. This time frame coincided with cerebellar degeneration in the PCD animals. Moreover, 24 hr before the first injection (at P19), the mice were irradiated with a low dose of 3 Gy in order to facilitate the access of cells through the blood brain barrier (Díaz et al., 2015).

Flow cytometry was not carried out because the experiment did not involve the substitution of bone marrow. The analysis of the cerebellum revealed small, round GFP-positive cells in both genotypes. These cells did not present any glial or neuronal process and were not positive for any of the neural markers analyzed (data not shown). No additional cell types were detected in any of the four wild-type animals used for this experiment. By contrast, in the three PCD mice undergoing daily transplants, several nucleated elements in the Purkinje cell layer, positive for both CB and GFP, appeared throughout the entire cerebellum (Figures 2 and S1 in the Supporting Information) and were not restricted to the lobule X of the vermis, as standard Purkinje cells of PCD mice at P35 (Baltanás et al., 2011). These cells presented a soma smaller than standard Purkinje neurons and lacked their characteristic dendrites (Figures 2 and S1). However, certain CB- and GFP-positive elements appeared as the remainder of a degenerated dendritic arborization in close proximity to some of these cells in the molecular layer (Figures 2e-2h and S1a-S1f). In addition, the soma of these cells presented two lobes, both containing a nucleus, which was probably the result of cell fusion (Figures 2a and 2g and S1g).

Then, we calculated the proportion of Purkinje cells that had undergone cell fusion among the total number of surviving neurons. Thus, we quantified both the standard Purkinje cells and those neurons undergoing cell fusion (positive for both calbindin and GFP staining) in eight sagittal sections of the cerebellum of each PCD animal (Table 1). To have a comprehensive estimate of the therapeutic potential of this third transplant method, we restricted these quantifications to the cerebellar regions most affected by the *pcd* mutation. That is to say, we excluded the lobule X, where not many events of cell fusion were detected and quantifying Purkinje cells was much more problematic, because this region is neuro-resistant around P30-P35 (Baltanás et al., 2011). Our results showed that more than half (mean of 59.5%) of the surviving Purkinje cells external to lobule X had been fused with transplanted cells (Table 1), which supports the therapeutic potential of this third and final method.

4 | DISCUSSION

The present work has addressed different methods for the transplantation of bone marrow stem cells with the aim of achieving their fusion with Purkinje neurons in PCD mice.

Previous reports employing bone marrow ablation (by radiation) and the subsequent transplantation of healthy bone marrow have demonstrated neuroprotective effects in the olfactory bulb of this same model (Díaz, Lepousez, et al., 2012) and in other models of cerebellar degeneration (Chen et al., 2011). However, the neuroprotection of Purkinje cells in PCD mice using this method has not yet been achieved either by cell fusion or by the secretion of neuroprotective substances. Reasons for this could be associated with the extreme quickness of Purkinje cell death and that the time between bone marrow substitution and the arrival of their derived cells into the cerebellum is too long (Recio et al., 2011). Consequently, early transplantation

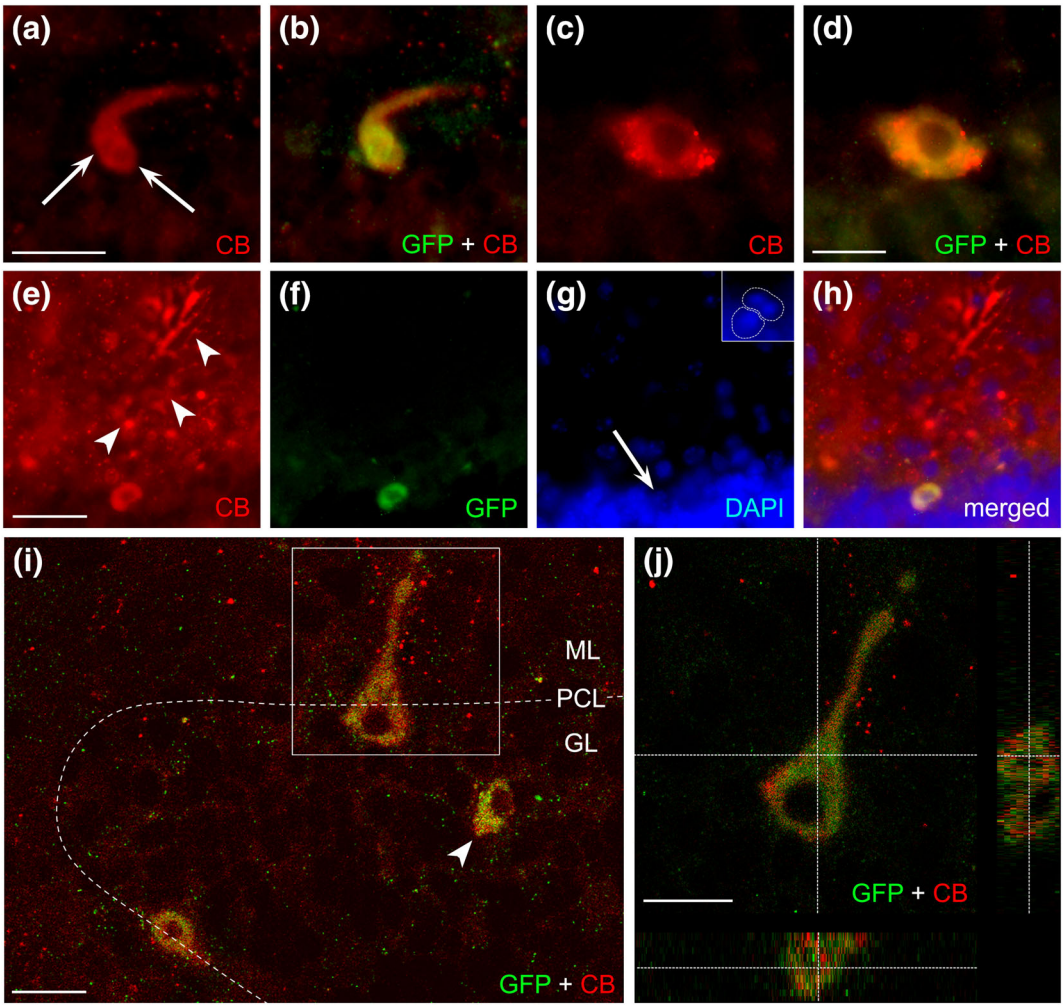

FIGURE 2 Daily transplants in PCD mice. (a-d) Two examples of bone marrow-derived Purkinje cells labeled with calbindin (CB, red) and positive for green fluorescent protein (GFP; green); the beginning of the dendritic tree can be seen in the first neuron (a and b) and the probable location of the two nuclei derived from the process of cell fusion (arrows). (e-h) Purkinje cell derived from a fusion process labeled with calbindin (CB, red) and positive for GFP (green); the degenerative stage of this neuron is evident, with a small soma and the vestiges of their dendritic arborization (arrowheads); in c and d nuclei are shown with DAPI counterstain (blue); note in c the position of the two nuclei of the neuron (arrow), which are magnified and delimited (dotted line) in the inset. (i) Focal plane of the cerebellar cortex, in which Purkinje cell layer is highlighted (dashed line); here three neurons (probably Purkinje cells) are labeled with both calbindin (CB, red) and GFP (green) and one of them is displaced from its natural position (arrowhead). (j) Stack of a Purkinje cell shown in i, positive for both calbindin (CB, red) and GFP (green); note the beginning of the dendritic tree. GL: granular layer; ML: molecular layer; PCL: Purkinje cell layer. Scale bar: 20 μm for a and b and I; 10 μm for c and d; 25 μm for e-h [Colour figure can be viewed at wileyonlinelibrary.com]

could be a way to rescue Purkinje cells by cell fusion. This is why the first two experiments were designed to transplant bone marrow cells at the perinatal stage of development, as previously described (Espejel et al., 2009).

To determine the efficiency of the bone marrow transplant itself, and thus to ensure the possibility of neuronal rescue, we determined the percentage of blood chimerism by flow cytometry. The percentage of GFP-positive blood cells after transplantation did not differ between PCD and control mice, as previously described (Díaz et al., 2018; Recio et al., 2011), which suggests the genotype of the recipients seems not to affect bone marrow substitution. Conversely, the percentages of blood chimerism in all experimental groups oscillated between 30 and 35%. Previous reports have shown higher chimerisms, even as high as in donors (Díaz et al., 2011; Díaz et al., 2018). However, in these works the bone marrow ablation of the recipient was performed by radiation, which can foster bone marrow substitution (Díaz et al., 2015; Nygren et al., 2008). In addition, the survival times of mice from these two first types of transplant were 30 (transplanted at P0) and 26 days (irradiated at P2 and transplanted at P4) after transplant, and longer times are necessary for higher percentages of blood chimerism (Díaz et al., 2011; Díaz et al., 2015; Recio et al., 2011). In any case, previous reports have considered percentages of blood chimerism around 30% as suitable for detecting neural BMDC (Massengale, Wagers, Vogel, & Weissman, 2005), which validates the first part of this method and has permitted the cerebellum to be further analyzed.

When the cerebellum of mice transplanted at P0 was analyzed, only a few neural BMDC were detected. The double immunolabeling

TABLE 1 Purkinje cell counts in PCD animals (excluding lobule X)

Animal	Standard Purkinje cells	Heterokaryons	Total Purkinje cells	% heterokaryons
PCD #1	28	37	65	56.9
PCD #2	7	22	29	75.8
PCD #3	13	11	24	45.8
			Mean	59.5

Note. The number of both standard neurons and heterokaryons in eight sections for each animal were recorded. The contribution of cell fusion to neuronal survival has been estimated as the percentage of heterokaryons of the remaining Purkinje cells.

Abbreviation: PCD: Purkinje cell degeneration.

showed that these cells were microglia, which constitutes the main bone marrow-derived neural elements, as previously described (Eglitis & Mezey, 1997; Brazelton et al., 2000; Mezey et al., 2000; Recio et al., 2011; Díaz, Recio, et al., 2012; Díaz, Lepousez, et al., 2012, Díaz et al., 2015; Díaz et al., 2018). Additionally, some of the double-labeled cells for Iba1 and GFP could be microglia that had phagocyted the debris from other GFP-positive dead cells. If this was indeed the case, such debris would be observed as "patches" in the cytoplasm of microglia, corresponding to phagosomes (Neher et al., 2011). Although this labeling pattern was not detected, and the GFP staining of microglia appeared uniformly distributed throughout the entire cytoplasm, we cannot discard this additional possibility. Also, we failed to detect any Purkinje cells derived from the transplant, which explained the general cerebellar degeneration in transplanted PCD mice. Some studies have reported the rapid arrival of bone marrow-derived glia into the cerebellum in only 3 days (Eglitis & Mezey, 1997), but their fusion with Purkinje neurons requires at least 2 months (Álvarez-Dolado et al., 2003; Nygren et al., 2008) or more (Chen et al., 2011; Díaz, Recio, et al., 2012; Recio et al., 2011). Although cerebellar damage has been reported to attract BMDC and foster cell fusion (Díaz, Recio, et al., 2012; Johansson et al., 2008; Magrassi et al., 2007), it seems not enough to provoke cell fusion in the time elapsing from P0 (transplants in newborns) to P30.

In line with the aim of improving transplantation techniques, the next experiment was based on other studies that show that radiation in newborns increases cell fusion in the cerebellum in a dose-dependent manner (Espejel et al., 2009). Then, we irradiated the animals at P2 with 4 Gy, thus in the suggested time frame and with the most effective dose reported (Espejel et al., 2009). Surprisingly, in wild-type mice no cell fusion events were detected at P30, which appears to contradict the results obtained by Espejel et al. (2009). However, in this previous work, BMDC fusion with Purkinje neurons was detected 3 months after transplant and no shorter survival times were analyzed. Therefore, the perinatal radiation (in experiments that involve bone marrow ablation and restoration) seems not to be enough to accelerate cell fusion up to P30. Consequently, and aside from the stimulant effects of the radiation in newborns, it seems that cell fusion between BMDC and Purkinje cells takes longer (Álvarez-Dolado et al., 2003; Chen et al., 2011; Díaz, Recio, et al., 2012; Nygren et al., 2008; Recio et al., 2011), which could explain the inconsistencies between our work and that of Espejel.

With respect to the mutant mice, it was expected that the combination of radiation and PCD degeneration would trigger cell fusion in cerebellum in a shorter time, at least up to P30 (because of the additional promoting cerebellar impairments; Díaz, Recio, et al., 2012 ; Johansson et al., 2008 ; Magrassi et al., 2007). However, this was not the case and no GFP-positive Purkinje cells were either detected in PCD mice. Interestingly, a few bone marrow-derived microglia were observed. It may have been possible that radiation caused temporary inflammation (Díaz et al., 2011) prior to transplantation or before new bone marrow was restored; as a result, bone marrow-derived microglia would not have been stimulated by such inflammation. Similarly, the degeneration of PCD mice has been shown to attract bone marrow-derived microglia (Recio et al., 2011), but at longer periods of time and once the bone marrow had been completely restored. Focusing in Purkinje cells, it seems that the cerebellar damage caused by radiation most certainly requires longer periods of time to foster cell fusion with BMDC (Espejel et al., 2009). Finally, the cerebellar damage caused by radiation at perinatal stages is unacceptable as a side effect of a putative cell therapy.

Thus, we can conclude that cell therapy comprising bone marrow substitution is not suitable for fast neurodegenerative processes such as that experienced by PCD mice.

As indicated by the results of the first two experiments, bone marrow ablation and substitution are techniques that delay a possible therapeutic effect of BMDC in the cerebellum of PCD mice. Therefore, the last method tested in this work involved daily intravenous transplants aimed at providing a continuous supply of bone marrow cells (1 million at day) as a means to avoid such bone marrow ablation and substitution. When the cerebella of mice were analyzed, some of the detected BMDC were small, round GFP-positive cells without any supplementary labeling. This suggested that they were probably BMDC that went into cerebellar parenchyma without any further differentiation up to the time when the mice were sacrificed, as previously proposed (Massengale et al., 2005; Wagers, Sherwood, Christensen, & Weissman, 2002). This finding is consistent with a daily supply of cells, in the absence of bone marrow ablation, and consequently, without the homing effect of an empty niche (Díaz et al., 2015).

In parallel, our data also showed that bone marrow cells injected daily can fuse with Purkinje cells. Therefore, it seems that bone marrow cells can directly fuse with Purkinje neurons, avoiding the need for derivation from a restored bone marrow, as suggested in previous works (Kemp et al., 2011). In addition, GFP-positive Purkinje cells were detected only in the PCD animals, but not in wild-type mice, indicating that the neurodegenerative milieu of these mutant mice is necessary and sufficient to foster cell fusion without the need for additional neural damage (Kemp et al., 2011). Furthermore, perinatal cerebellar damage (Espejel et al., 2009) seems insufficient to

accelerate BMDC fusion with Purkinje cells around P30, even in PCD mice, using a protocol involving bone marrow ablation and restitution (see the discussion of the second experiment above). By contrast, if this protocol is bypassed, the cerebellar damage of PCD mice can independently promote cell fusion between transplanted cells and Purkinje neurons, as previously described (Magrassi et al., 2007; Johansson et al., 2008; Díaz, Recio, et al., 2012), even in a short time. Thus, bypassing the protocol, combined with a daily support of bone marrow cells, raises as a key point in relation to the use of putative therapies against fast and aggressive neuronal degeneration like the one occurring in our model.

The heterokaryons found in PCD mice presented a "bi-lobulated" soma, corresponding to the two regions where the nuclei of both Purkinje and bone marrow cells were located (Figure 2a and 2g and S1g), as previously described (Díaz, Recio, et al., 2012; Johansson et al., 2008; Weimann et al., 2003). Purkinje cells with similar features were also identified in other experiments involving direct grafts of neural progenitors into the cerebellum (Cedikova et al., 2014), a result that supports our findings. However, it is necessary to note that this process occurred in neurons that probably had already started to die, exhibiting a small size (even with two nuclei) and a degenerated dendritic tree, as previously described (Baltanás et al., 2013; Inoue et al., 2001; Yoshida et al., 2014). The analysis of the survival of these heterokaryons is beyond the scope of this work, but a damaged dendritic tree would not allow Purkinje neurons to function normally as those of wild-type mice. One possible solution for this problem could be the implementation of an adjuvant/synergic therapy that could stabilize the dendritic arbor of Purkinje cells complementarily to their putative rescue. In this sense, some chemicals like oleic acid or oleylethanolamide have been proven to have such effect (Agarwal, Yadav, & Chaturvedi, 2017; Bento-Abreu, Tabernero, & Medina, 2007; Fidaleo, Fanelli, Cerù, & Moreno, 2014).

In any case, to our knowledge, this is the first time that cell fusion between bone marrow cells and Purkinje neurons has been achieved in a model presenting fast and severe neurodegeneration such as that observed in the PCD mouse. Moreover, the method employed involving intravenous injections is not invasive as long as it does not additionally harm the cerebellar parenchyma, a measure that should be considered when dealing with neurodegenerative diseases (Díaz et al., 2015). Our results showed that more than a half of the surviving Purkinje neurons in the most affected region of the cerebellum in PCD mice (out of the lobule X) corresponded with heterokaryons. Therefore, the third method employed has a significant influence on delaying the death of Purkinje cells in PCD mice. Furthermore, it is necessary to keep in mind that the survival of only a few Purkinje cells can lead to notable improvements in behavior (Bae et al., 2007; Jones et al., 2010; Chen et al., 2011).

Taken together, all of these findings allow us to propose that the daily transplant of bone marrow stem cells could be a promising method for cell therapy against aggressive neurodegenerations, and of interest to those researchers studying this type of disease. Moreover, this therapeutic approach can be especially interesting for dealing with infantile human cerebellar impairments, also sharing the genotypic features and symptoms of PCD mice (Karakaya et al., 2019; Shashi et al., 2018; Sheffer et al., 2019). However, further work is required to improve the use of this strategy for cell rescue, either by cell fusion or by the release of neuroprotective factors.

ACKNOWLEDGEMENTS

The authors also express their gratitude to E. Keck for editing the English and to P. Fernández for the technical support.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

D.D., C.d.P. and E.W. performed the bone marrow extraction and transplants; D.D., J.C. and C.d.P. performed the histological analysis; D.D., and E.W. carried out the statistical analyses; and D.D. and J.R. A. wrote the manuscript and prepared the figures. All of the authors revised the manuscript.

FINANCIAL SUPPORT

This work was supported by grants SAF2013-41175-R and SAF2016-79668-R obtained from the Spanish Ministry of Economy and Competitiveness (MINECO), grant SA030P17 from the Regional Government of Castile and Leon, the Centre for Regenerative Medicine and Cell Therapy of Castile and Leon, and the University of Salamanca.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Daily transplants in PCD mice. **A-F**; additional examples of bone marrow-derived Purkinje cells labelled with calbindin (CB, red) and positive for GFP (green); note the degenerative stage of these neurons, which exhibit a small soma and the vestiges of their dendritic arborization (arrowheads). **G**; bone marrow-derived Purkinje cell positive for both calbindin (CB, red) and GFP (green); arrows point to the position of its two nuclei, counterstained with DAPI (blue). **H**; stack of a Purkinje cell soma positive for both calbindin (CB, red) and GFP (green). Scale bar: 25 μm for A-F; 10 μm for G; 20 μm for H.

How to cite this article: Díaz D, del Pilar C, Carretero J, Alonso JR, Weruaga E. Daily bone marrow cell transplantations for the management of fast neurodegenerative processes. *J Tissue Eng Regen Med*. 2019;13:1702–1711. https://doi.org/10.1002/term.2925