Original Article

Engraftment of autologous bone marrow cells into the injured cranial cruciate ligament in dogs

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Abstract

Current research indicates that exogenous stem cells may accelerate reparative processes in joint disease. However, no previous studies have evaluated whether bone marrow cells (BMCs) target the injured cranial cruciate ligament (CCL) in dogs. The objective of this study was to investigate engraftment of BMCs following intra-articular injection in dogs with spontaneous CCL injury. Autologous PKH26-labeled BMCs were injected into the stifle joint of eight client-owned dogs with CCL rupture. The effects of PKH26 staining on cell viability and PKH26 fluorescence intensity were analyzed in vitro using a MTT assay and flow cytometry. Labelled BMCs in injured CCL tissue were identified using fluorescence microscopy of biopsies harvested 3 and 13 days after intra-articular BMC injection.

The intensity of PKH26 fluorescence declines with cell division but was still detectable after 16 days. Labelling with PKH26 had no detectable effect on cell viability or proliferation. Only rare PKH26-positive cells were present in biopsies of the injured CCL in 3/7 dogs and in synovial fluid in 1/7 dogs. No differences in transforming growth factor-β1, and interleukin-6 before and after BMC treatment were found and no clinical complications were noted during a 1 year follow-up period. In conclusion, BMCs were shown to engraft to the injured CCL in dogs when injected into the articular cavity. Intra-articular application of PKH26-labeled cultured mesenchymal stem cells is likely to result in higher numbers of engrafted cells that can be tracked using this method in a clinical setting.

Keywords: Bone marrow cells; Cranial cruciate ligament; Dog; Mesenchymal stem cells, PKH26; Transplantation.
Introduction

The cranial cruciate ligament (CCL) is essential for stifle joint stability and its rupture leads to functional impairment, meniscal lesions and early onset of osteoarthritis (Arnoczky and Marshall, 1977; Korvick et al., 1994). In dogs, CCL injury is common and has been treated using a variety of different surgical techniques since 1952 (Paatsama, 1952), but no single treatment option has been shown to be clearly superior. The goals of most reported techniques are to alleviate pain, decrease instability and minimize osteoarthritis (OA) (Moore and Read, 1995), but residual lameness is frequent and OA is a common sequel (Elkins et al., 1991; Innes et al., 2004; Rayward et al., 2004). The lack of ideal outcome following surgery has prompted interest in exploring new adjunctive treatment options, such as regenerative stem cell therapy. Knowledge of the benefits of these treatment strategies would be useful both for the treatment of spontaneous canine CCL injury and for investigations into degenerative anterior cruciate ligament (ACL) disease in humans using the dog as a model.

Mesenchymal stem cell (MSC) therapy is a newly developing therapeutic approach in OA that has proven useful in cartilage repair in a variety of animal models (Black et al., 2007, 2008; Chong et al., 2007; Guercio et al., 2012; Jorgensen and Noel, 2012; Khan et al., 2010; Kirkby and Lewis, 2012; Koga et al., 2008; Mokbel et al., 2011a, b; Murphy et al., 2003). Bone marrow-derived MSCs hold particular promise for tissue repair because of their ability to engraft into tissues and differentiate into the target tissue cell type, including fibroblasts, osteocytes, adipocytes, chondrocytes and myocytes (Chamberlain et al., 2007; Pittenger et al., 1999). In recent studies, MSCs were found to accelerate healing of transected ligaments in animal models (Agung et al., 2006; Kanaya et al., 2007; Kim et al., 2011) and evidence suggests that fresh whole bone marrow cells (BMCs) may have superior effects compared to purified MSCs, presumably because of an additional benefit of hematopoietic stem cells (Oe
et al., 2011). Indeed, injured rat ACLs treated with BMCs had more mature fibroblasts and
tighter collagen bundles compared to those treated with MSCs, leading the authors to
conclude that bone marrow (BM) transplantation is an effective treatment for ACL injury (Oe
et al., 2011). To date, no studies have investigated the potential benefit of stem cell adjunctive
treatment in dogs with experimental or spontaneous CCL injury.

The purpose of the present study was to assess the engraftment potential of autologous
BMCs injected into the articular cavity in dogs with partial or complete CCL rupture and to
determine whether PKH26 red fluorescent labelling is a safe and effective way to track canine
BMCs.

Material and methods

Animals

Client-owned dogs presented for surgical treatment of spontaneous partial or complete
CCL rupture to the Division of Small Animal Surgery and Orthopaedics of the University of
Berne were considered for inclusion in the study (see Appendix: Supplementary data for
details). Informed client consent was obtained for each dog. Study inclusion criteria were
diagnosis of partial or complete CCL rupture confirmed by arthroscopy and unremarkable
results of routine haematological and serum biochemical analyses. Dogs were excluded if
there was a recent history of illness other than pelvic limb lameness or if they had undergone
previous intra-articular application of any substance or previous surgery on the affected limb.
Dogs were screened using an orthopaedic examination with various parameters: lameness,
functional disability, range of motion and pain on manipulation. For each parameter a score
was recorded at first time of presentation and 3 month after surgery. The scores assigned were
based on a 4-point scale, 0 (no/best) to 4 (worst) (For details see appendix: Supplementary material).

All animal experiments were reviewed and approved by the Commission of Animal Experimentation of the Canton of Berne, Switzerland (BE42/12; date of approval, 30/4/2012).

Isolation of bone marrow cells

Bone marrow was harvested from the proximal humerus in each dog using a 13-G BM biopsy needle connected to a 10-mL syringe containing 2 mL of heparin solution (3,000 U/mL). A total of 15 mL BM was aspirated and immediately injected into a transfer bag containing 7 mL citrate phosphate dextrose adenine solution. The BM aspirate was passed through a blood transfer filter set into a 20-mL syringe, and cells were separated by density gradient centrifugation at 445 g for 35 min. The interface with the nucleated cell fraction was transferred and washed twice in phosphate buffered saline (PBS) before counting and partitioning for PKH26 labelling, intra-articular injection, and cultivation (See Appendix: Supplementary material for details).

Flow cytometric characterization of cells

Freshly isolated cells and cultured cells were evaluated by flow cytometry for the specific MSC markers, CD90 (YKIX337.217, eBioscience) and CD44 (FAB5449A, R&D), and for the hematopoietic stem cell marker, CD45 (YKIX716.13, eBioscience). Data were analyzed using a flow cytometer (LSR II, BD Bioscience) and commercial software (FACSDiva, BD Bioscience).
Labelling of cell membranes was performed using the PKH26 Red Fluorescence Kit (Sigma-Aldrich) according to the manufacturer’s instructions. After staining, a portion of PKH26-BMCs was resuspended in PBS at a concentration of $1 \times 10^7$ cells/mL for intra-articular injection. In addition, stained cells were suspended in complete medium for evaluation of dye cytotoxicity, growth characteristics and fluorescence intensity.

**Evaluation of PKH26 cytotoxicity**

The effect of PKH26 labelling on cell viability was tested using a colorimetric MTT assay. For this, MSCs from the second passage were stained with PKH26 dye at $2 \times 10^{-6}$ M/$10^6$ cells and $4 \times 10^{-6}$ M/$10^6$ cells. The assay was conducted in replicate with MSCs from four dogs as described elsewhere (Waldherr et al., 2012). Cell viability in each well, measured as the optical density (OD), was calculated as follows: $100 \times (\text{OD of sample well} – \text{OD of blank well})/(\text{OD of control well} – \text{OD of blank well})$. Mean values of repeated measurements were used for analysis.

**Population doubling time**

Growth characteristics of MSCs at the first and second passages unstained and stained with PKH26 ($2 \times 10^{-6}$ M and $4 \times 10^{-6}$ M) from six dogs were investigated. The MSCs were seeded into a 24-well plate at a density of $2.1 \times 10^3$ cells/cm$^2$. After a recovery time of 48 h, three wells were detached daily for a period of 8 days and cell numbers were counted in a hemocytometer. The population doubling time was computed using an online calculator.$^1$

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PKH26 fluorescence intensity

Fluorescence intensity was assessed in freshly isolated BMCs and MSCs during cell proliferation over 16 days as described in detail in the Appendix: Supplementary material.

In vivo experimental protocol

The experimental schedule is summarized in Fig. 1. On day 0, dogs presenting with signs of CCL injury were clinically examined. BM was harvested and pre-operative radiographs were performed under general anaesthesia. BMCs were isolated and labelled with PKH26 (final concentration: $2 \times 10^{-6} \text{ M PKH26 and } 1 \times 10^7 \text{ cells/mL}$) within 3 h of harvesting. Synovial fluid was first aspirated and a total of $1 \times 10^7$ PKH26-labeled BMCs diluted in 1 mL PBS was injected immediately afterwards through the same needle under aseptic conditions. An aliquot of remaining BMCs was used for microbiological quality control. The dogs were then presented again for stifle arthroscopy and tibial plateau levelling osteotomy (TPLO) either 13 days (Group 1) or 3 days (Group 2) following intra-articular BMC injection. Prior to arthroscopy, synovial fluid was again collected.

Tissue collection

During arthroscopy immediately prior to surgical treatment by TPLO, the gross appearance of the stifle joint was evaluated and biopsies of the damaged CCL and synovial membrane were excised. Synovial membrane was harvested craniomedially and craniolaterally to the optic port that was located lateral to the patellar ligament halfway between patella and tibial tuberosity. Tissues were snap frozen on dry ice in O.C.T.
compound (Tissue-Tek). Each block was cut into 5 µm sections at 10 µm intervals and placed on specimen slides. Sections were stored at -80 °C pending fluorescence microscopy.

Fluorescence microscopy of harvested samples

Slides were examined for PKH26 fluorescence using a confocal laser scanning fluorescence microscope (FluoView FV1000, Olympus) after counterstaining with TOTO-3 iodide (Life Technologies). Sections were defined as positive if a clear cell structure with spindle-shaped fibroblast-like morphology was detected showing at least partial red fluorescence in the membrane and far red fluorescence of the nucleus. Synovial fluid samples were examined for PKH26 fluorescence after centrifugation in a 96-well plate.

Synovial fluid analyses

Because of a possible effect of BMCs on cytokine production and immune cell attraction, synovial fluid obtained before and after intra-articular BM injection was examined cytologically, and transforming growth factor (TGF)-β1 and interleukin (IL)-6 were quantified using a commercial ELISA (canine TGF-B1, IL-6 Quantikine ELISA Kit, R&D) according to the manufacturer’s protocol.

Follow-up examinations

Dogs were discharged from hospital 1 day after surgery with a soft-padded bandage on the operated leg for 3 days and administered carprofen (Rimadyl, Pfizer, 4 mg/kg PO once daily for 7 days). Owners were instructed to restrict activity initially to leash walks, followed by a gradual increase in activity. Dogs were re-examined 14 days and 3 months after surgery. The same clinician carried out both initial and follow-up orthopaedic and clinical
examinations. In addition, a final follow-up inquiry with the owners was performed by telephone 12 months after surgery. Complications, including infection, pain or worsening of articular function were recorded during follow-up examinations.

Statistical analyses

For each donor and experimental condition at least triplicate samples were used for each assessment unless otherwise stated. Statistical analysis was performed with NCSS 2007 software. Differences in population doubling time, TGF-ß1 and IL-6 were evaluated using a paired samples t-test after testing for normality. A P-value of <0.05 was considered as significant.

Results

Animals

Eight cases were initially included, but one was subsequently excluded because it was not presented for arthroscopy as scheduled. Four dogs underwent arthroscopy 13 days after injection. Because of a low rate of detected PKH26 positive cells the interval between BMC transplantation and tissue harvesting was shortened, therefore the second group of three dogs went to surgery 3 days after injection (Fig. 1).

Isolation, cultivation and flow cytometric characterization of cells

The mean value of nucleated cell fraction recovered after BM aspiration (eight dogs) and density gradient centrifugation was $23.0 \times 10^7$ cells (range, $0.35-33.2 \times 10^7$ cells). Freshly isolated BMCs stained largely positive for CD45 with only a small proportion (<1%) of cells
negative for CD45 and double positive for CD44 and CD90. Cultured cells were adherent within 2 to 3 days showing spindle-shaped fibroblast-like morphology generating subsequently colony-forming units. After 8 to 15 days in culture, colonies became confluent and were passaged for the first time. Primary cultured cells (second and fifth passages) stained on average 92% ± 5% positive for CD44, 45% ± 3% double positive for CD44 and CD90, and 100% negative for CD45 on flow cytometry, confirming phenotype consistent with MSCs in most cells. Lack of expression of CD45 on cultured cells indicated that cells of haematopoietic origin had been excluded during cell culture.

**Evaluation of PKH26 cytotoxicity**

The colorimetric MTT assay performed on MSCs from the 2nd passage in four dogs revealed that the mean relative number of viable MSCs 24 h after PKH26 staining compared to unstained MSCs was 93.4% ± 3.5% at 2 x 10^{-6} M and 98.6% ± 9.2% at 5 x 10^{-6} M PKH26.

**Population doubling time**

Mean population doubling time of unlabelled MSCs and PKH26-labeled MSCs from 6 dogs was 146.7 ± 63.5 h (range, 44.5-238.0 h) and 107.2 ± 37.5 h (range, 43.0-173.0 h), respectively. No significant difference was found between these population doubling times by using a paired samples \( t \)-test (\( P = 0.43 \)).

**PKH26 fluorescence intensity**

Labelled BMCs plated in culture dishes attached efficiently and showed uniformly distributed red fluorescence on microscopy. The labelling rate of BMCs assessed by flow cytometry was 97.3 ± 3.3% and labelling intensity decreased to 67.5 ± 8.3% at the end of the
16-day observation period. The labelling rates of second passage MSCs assessed by flow cytometry on days 0 and 16 were 94.0 ± 2.1% and 15.1 ± 4.6%, respectively, with a mean number of cell divisions after 16 days of 8 ± 3.

Fluorescent microscopy of harvested samples

Fluorescence microscopy of control CCL tissue co-cultivated with PKH26-labelled BMCs revealed adhesion and migration of BMCs based on numerous red fluorescent cells located superficially and within the tissue (Fig. 2; positive control). Tissue of CCL and synovial membrane were obtained from seven dogs after BMCs transplantation. A total of 280 sections (40 sections per dog) of CCL and synovial membrane were examined for PKH26 fluorescence. Positive cells were only detected in eight slides from three dogs of which one were sampled 3 days after BMC injection and two were sampled 13 days after BMC injection (Fig. 3). Positive cells were located within the organized CCL tissue and arranged predominantly as single cells and occasionally in groups. However, the numbers of positive cells was extremely small with no more than 10 per section. The intensity of PKH26-positive cells was clearly less in these samples than in ex vivo CCL samples co-cultured with PKH26-labeled BMCs. A single synovial sample (harvested on day 3) showed PKH26 positive cells.

Synovial fluid analyses

Cytological examination of synovial fluid taken both prior to BMC injection and prior to arthroscopy exhibited less than 5% neutrophils. No significant difference was found in TGF-β1 ($P = 0.21$) and IL-6 ($P = 0.29$) concentrations between samples harvested prior to and those harvested after BMCs treatment using a paired samples $t$-test (Fig. 4).
Clinical assessment and follow-up of study dogs

Pre-operative arthroscopy of BMC-treated dogs revealed no gross changes in the stifle joint other than those generally observed in dogs assessed for partial or complete CCL rupture. Bacteriological cultures of the remaining portion of injected BMC preparations revealed no growth in all samples.

Follow-up examination performed 3 months after surgery revealed mild lameness and swelling of the knee joint in one dog. The other dogs showed improvement of lameness, function, and pain on manipulation (see Appendix: Supplementary material). Four dogs had improved scores when testing the range of motion but withdrew the affected leg at full range manipulation. A final inquiry was performed in all dogs 12 months after surgery. Normal activity was reported by all owners during these inquires. The owners of three dogs indicated slight stiffness in the morning. No complications associated with the intra-articular injection of the PKH26-labeled BMCs were observed in any of the dogs.

Discussion

Several previous studies have investigated the effects of intra-articular stem cell application on cartilage in dogs with OA (Black et al., 2007, 2008; Guercio et al., 2012; Mokbel et al., 2011a). However, most previous reports have focused on clinical outcome. This study evaluated engraftment of fluorescent-labelled BMCs into injured ligaments when applied into the articular cavity in dogs with spontaneous CCL injury. The decision to use BMCs instead of MSCs was based on ease of processing, making the procedure practical for future use in a clinical setting, as well as the potential additional benefit of BMCs based on studies in a rat model (Oe et al., 2011).
Several pre-conditions of harvested cells were tested in order to ensure that the selected study design was applicable regarding dye and cells. Separated BMCs of the eight dogs comprise haematopoetic stem cells showing CD45+ which were the main fraction with around 99% and a very small part of mesenchymal stem cells which showed a phenotype of CD45- CD44+ and CD90+. These findings are in accordance with the findings of Alvarez-Viejo et al. (2013).

Following cultivation, cells were replaced by a homologous layer of adherent cells expressing putative surface specific antigens, such as CD44+ and CD90+ as markers for MSCs and lacking the haematopoetic stem cell marker CD45. They presented MSC characteristics as reported in other studies (Csaki et al., 2007; Kisiel et al., 2012). Lack of expression of CD45+ on cultured MSCs indicated that cells of haematopoietic origin had been excluded during the cell culture process. Cell numbers recovered following gradient centrifugation varied between samples, but were largely similar to those previously reported in dogs (Nishida et al., 2012; Sato et al., 2011). Likewise, in vitro growth potential varied between dogs, demonstrated by the wide range in population doubling time. These results are consistent with previously published data for humans and may reflect patient variability (Bertolo et al., 2013), age-related replicative senescence (Mareschi et al., 2006; Zhou et al., 2008), as well as variation in individual MSC differentiation potency (Ding et al., 2013).

The ability to track cells is undoubtedly necessary to evaluate the potential of cell migration and new tissue transformation after in vivo transplantation. Differentiation between graft and host cells after transplantation requires a method that labels cells of interest and identifies them after harvesting at a later time. Previous studies used BMCs or MSCs
expressing green fluorescent protein (GFP) injected into the injured stifle joints of dogs (Mokbel et al., 2011a), donkeys (Mokbel et al., 2011b) and goats (Murphy et al., 2003), as well as GFP transgenic animals (Oe et al., 2011). An advantage of using GFP is its potential use in long-term studies as daughter cells adopt GFP gene expression and, with it, fluorescence is multiplied after several cell divisions. However, use of GFP requires a gene transfer agent, such as a virus that is non-integrating or, preferably from a biosafety point of view, a non-viral vector. These, however, are limited by the requirement of large cell numbers, high levels of cell death and low transfection efficiency (Bakhshandeh et al., 2012).

The transformation procedures for GFP labelling are time consuming, making its use impractical for implantation of autologous freshly isolated cells. Given this and some ethical considerations as to potential adverse effects of GFP-modified cells, its use was considered inappropriate for cell tracking in client-owned dogs. Instead, we used PKH26 red fluorescent dye, a lipophilic cell membrane stain that has previously been used for tracking of a variety of different cell types (Wisenberg et al., 2009). Data in the present study showed that cell viability and population doubling time of MSCs in culture was not significantly affected by PKH26 staining, corroborating findings of a previously reported study in which no effect on cell growth or proliferation was observed (Shao-Fang et al., 2011). Furthermore, we demonstrated that PKH26 labelling was highly effective and fluorescence was strong in BMCs co-cultured with CCL tissue for 16 days. However, progressive loss of fluorescence was observed in cultured MSCs over the same time frame. These findings suggest that BMCs attached to tissue ex vivo do not proliferate as quickly as in monolayers due to their incipient phenotype differentiation prior to replication.
The findings of the present study, using the dog as a model of spontaneous CCL rupture, confirm previous observations in rats with transected ACL, showing that BMCs injected into the articular cavity engraft to the injured site of the ACL (Oe et al., 2011). In rats, GFP-transduced cells were found to be present in high numbers in the transected ligaments and seemed to be involved in appreciable neoligamental tissue transformation after 4 weeks. However, we were only able to detect a very small number of PKH26 positive cells in a few CCL biopsies in 3/7 dogs. This may be due to the very low numbers of MSCs, estimated as 1 MSC per 10^4 mononuclear cells, which can differentiate into tissue in the transplanted BM as well as a low survival rate after in vivo transplantation (Pittenger et al., 1999; Wexler and Donaldson, 2003).

Currently, no data are available with regard to the numbers of cells that can be safely transplanted into articular cavities in the dog. In previous studies, numbers of injected cells varied between 1.4 and 5 × 10^6 MSCs in different articular cavities in dogs (Black et al., 2007, 2008; Guercio et al., 2012; Mokbel et al., 2011a) and 1 × 10^7 MSCs were injected into goat articular cavity without adverse effects (Murphy et al., 2003). Based on this empirical data, we injected 1 × 10^7 BMCs in the hope of achieving sufficient numbers of MSCs without untoward effects. Larger numbers of BMCs may result in higher numbers of engrafted MSCs, but further studies are needed to assess the safety and efficiency of larger transplants in dogs.

Furthermore, a limitation in the evaluation of tissue samples in our study was the inability to obtain full-thickness biopsies in clinical patients. The small size of biopsies may therefore have led to some false negative results. Finally, in contrast to GFP expression, the fluorescence of PKH26 labelling decreases with cell division. The numbers of cells and time point at which labelled cells are assessed is therefore crucial for cell tracking. However, we
did not find a significantly greater number of PKH26 positive biopsies harvested 3 days compared to those harvested 13 days after intra-articular BM injection.

Previous findings of enhanced ACL healing were associated with increased TGF-ß1 concentrations in synovial fluid and ACL material in rats treated with intra-articular BMCs (Oe et al., 2011). Secretion of TGF-ß1 is directly influenced by the transplanted cells (Kuroda et al., 2000). TGFb1 plays an anabolic role in the healing of ligaments by accelerating proteoglycan synthesis and cell proliferation. However, the concentrations of TGF-B1 measured prior to and after intra-articular BMC treatment in dogs in the present study were not significantly different. Moreover, a possible inflammatory response after cell transplantation was tested by analysing IL-6. IL-6 produced by several cell types functions to increase the number of inflammatory cells in synovial tissue and its production is stimulated by IL-1 and/or TNF (Venn et al., 1993). Our results revealed no inflammatory response after BMC injection. IL-6 levels were no different prior to and after transplantation. For the interpretation of the data, however, it should be noted that our results are based on a small sample size.

Conclusions

Fresh BMCs injected into the articular cavity in dogs with spontaneous CCL injury can engraft in the injured CCL, but were only rarely detected using this procedure. The low recovery of transplanted cells implies that application of MSCs may be more useful for cell tracking after PKH26 labelling in a clinical setting. Overall, BMC transplantation into the stifle joint was well tolerated and showed no undesirable clinical effects on dogs followed for up to 1 year. The clinical procedure was found to be practical and safe, but a decrease in fluorescence with cell division renders the method inadequate for cellular tracking.
Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix A: Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:…

References


Figure legends

Fig. 1. Timetable of the study design. Group 1 underwent the long procedure, group 2 the shortened procedure. TPLO, Tibia plateau levelling osteotomy.

Fig. 2. Fluorescence photomicrographs of control tissues showing PKH26 positive (red) and TOTO-3 positive (grey) cells (B, D); A and C are corresponding photomicrographs merged with tissue. Control samples were made from tissues of CCL obtained from dogs undergoing surgical treatment for CCL disease without intra-articular BM injection. Positive control tissues were made by co-cultivation of tissue with PKH26-labeled BMCs at a density of $1 \times 10^6$ cells/cm$^2$ in 12-well plates containing complete medium, harvested after 8 (C, D) and 16 (A, B) days and processed in an identical manner as study samples. Labelled cells were associated with the surface (C, D) and were also integrated within the CCL explant (A, B). Magnification: $\times$ 100.

Fig. 3. Fluorescence photomicrographs showing PKH26 positive (red) and TOTO-3 positive (grey) cells (B, D); merged with tissue (A, C). In vivo specimens of torn CCLs from two dogs at day 13 after transplantation of PKH26-labelled autologous BMCs. Labelled cells were detected within the CCLs. Magnification: $\times$ 100.

Fig. 4. Scatter blot of concentrations of TGF-ß1 and IL-6 in synovial fluid sampled prior to and post BMC transplantation in seven dogs. Samples of synovial fluid were collected from all dogs on day 0 before BMC transplantation, additionally from dogs of group 1 (plotting symbol: circle, black) on day 13 and of group 2 (plotting symbol: triangle, grey) at day 3 after BMC transplantation. A median smooth line of the seven dogs is presented.