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Non-clinical assessment of safety, biodistribution and tumorigenicity of human mesenchymal stromal cells

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ABSTRACT

Guidelines regulating the development of advanced therapy medicinal products (ATMPs) request nonclinical data for toxicity, biodistribution and tumorigenicity before mesenchymal stromal cell (MSC) products can be administered in large clinical trials. We assessed the biodistribution/persistence, safety and tumorigenicity of MC0518, a human allogeneic MSC product from pooled bone marrow mononuclear cells of eight healthy, adult, unrelated donors, which is currently investigated for the treatment of steroid-refractory acute Graft-versus-Host Disease (aGvHD) after hematopoietic stem cell transplantation. In our GLP studies, immuno-deficient mice were administered repeat doses of MC0518 (once weekly for 6 weeks, i.v.) at doses exceeding the proposed human clinical dose 20-60-fold. No signs of toxicity were observed in the combined biodistribution/toxicity study. Human MSCs in mouse tissues were detected by quantitative PCR (qPCR) and in situ hybridization (ISH). MC0518 showed initial trapping in the lung, occasional distribution into other organs and low tissue persistence beyond 24 h after application. No MSC-induced tumors of human origin were identified after a follow-up of six months. Additionally, we found that the combination of different detection methods (qPCR and ISH) is crucial for a reliable interpretation of biodistribution results. Our data suggest that MC0518 is safe for use in human.

1. Introduction

Human mesenchymal stromal cells (MSCs) are widely recognized for their immuno-modulatory and anti-inflammatory properties and their capacity to stimulate repair and regeneration of diseased or damaged tissue. MSCs have demonstrated efficacy in a broad range of experimental animal disease models, not only for regenerative medicine but also in the restoration of dysregulated immune systems. Their clinical potential is currently tested in numerous clinical trials and multiple indications [1,2].

MC0518 is an ex vivo expanded, allogeneic MSC product derived from pooled bone marrow (BM) mononuclear cells (MNCs) of eight unrelated, adult healthy donors, and is currently investigated for the treatment of steroid-refractory aGvHD (SR-aGvHD) [3]. The cells fulfill the criteria defined by ISCT for MSCs in respect to expression of cell surface markers like CD105, CD73 and CD90, trilineage differentiation potential (chondrocytes, osteoblasts, adipocytes) and progression into replicative senescence at later passages of in vitro cultivation [4,5]. The final MC0518 clinical product is harvested at passage 3 and is relatively young compared to other MSC products under clinical investigation e.g. remestemcel-L [6]. MSCs in MC0518 were genomically stable, and showed a normal karyotype and diploid pattern in the vast majority of the cells, with no post-senescence proliferation and no expression of the proto-oncogenes c-myc and hTERT [3]. MSCs in MC0518 exerted a higher allosuppressive potential in vitro than the mean allosuppressive potential of the MSCs generated from the same donors individually [3].

Although clinical tolerability of MC0518 was good after treatment of patients with SR-aGvHD as observed under a national hospital exemption in Germany (PEI.A.11748.01.1) [7], non-clinical biodistribution, toxicity and tumorigenicity studies were requested for MC0518 before

Abbreviations: MSC, mesenchymal stromal cell; ATMPs, advanced therapy medicinal products; MNCs, mononuclear cells; (SR)-aGvHD, (steroid-refractory) acute graft-versus-host disease; NSG mouse, NOD/SCID/IL2Rγnull mouse.

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starting a larger Phase III clinical trial, conform to the guideline on quality, non-clinical and clinical development for investigational ATMPs (EMA/CAT/852602/2018).

For this reason, a combined biodistribution/toxicity study and a tumorigenicity study were performed in NOD/SCID/IL2Rγnull (NSG) mice. NSG mice are immuno-compromised lacking mature T, B, and natural killer cells, are deficient in multiple cytokine signaling pathways and have many defects in innate immunity [8]. They are an often used model for studying human cell therapies as persistence of the human cells is expected to be longer than in immunocompetent animals, which will reject a xenotransplant quickly [9]. This allows longer observations and assessment of engraftment, biodistribution and tumor formation of the injected MSCs.

We here report the results of two GLP repeat-dose studies with pooled bone marrow-derived MSCs: a biodistribution/toxicity and a tumorigenicity study in a mouse model, which allows sufficient persistence, and with a reasonable follow-up time of 6 months to enable tumorigenicity assessment. We used a dose multiple of at least 20-times the human clinical dose as repeated administrations, covering the maximum human dose administration frequency. The tumor formation potential was assessed after 3 and 6 months, a time during which standard cells with tumorigenic potential would have formed tumors in this mouse model [10,11]. Human MSCs were detected in the mouse background using validated qPCR and in situ hybridization methods. Publications of GLP repeat-dose toxicity studies for bone marrow-derived human MSCs are sparse; the studies reported here are intended to fill this gap.

2. Materials and methods

2.1. MC0518 cell preparation

The generation of the MC0518 cell bank and the cultivation of MC0518 clinical-grade product have been described previously by Kuci et al. [3]. Briefly, bone marrow was collected from 8 healthy, adult human donors after written informed consent and with approval of the local Ethics Committee. Frozen bone marrow MNC samples were thawed, pooled and cultivated for 14 days in tissue culture flasks. Plastic-adherent MSCs were isolated by medium exchange. The adherent cells were harvested as MSCs-passage 1 and cryopreserved in aliquots at concentrations of 1.5×10^6 cells/vial to be used as cell bank. To generate the clinical grade cell product MC0518, an aliquot of the MSC cell bank was thawed and cultured in CellSTACK culture chambers using optimized culture medium containing 10% platelet lysate until the end of passage 2, resulting in a product passaged 3 times in total. The obtained adherent MSCs were harvested, resuspended in cryopreservation medium (0.9% NaCl with 5% human serum albumin and 10% DMSO) at $1-2 \times 10^6$ cells/mL, and stored frozen in the vapor phase of liquid nitrogen until use. All manufacturing steps were carried out according to good manufacturing practices (GMP).

2.2. Animals and dosing

NSG mice were obtained from Jackson Laboratories, Bar Harbor, USA. Mice were maintained in microisolators in ventilated racks to reduce potential exposure to pathogens. The animals (approx. 9 weeks of age at the start of treatment) were injected intravenously (i.v., slow bolus) into the tail vain once per week for 6 weeks with 1×10^6 MSCs/animal in 0.25 mL vehicle (cryopreservation medium: 10% DMSO and 5% human serum albumin in 0.9% saline) using a 27 G needle.

The studies were performed at Charles River Laboratories, Laval, Canada, under the OECD principles of Good Laboratory Practice (GLP) in an AAALAC-accredited animal research facility. The studies were approved by the Institutional Animal Care and Use committee (IACUC) and were conducted in accordance with Canadian Guidelines for animal Care and Use of Laboratory Animals.

2.3. Combined biodistribution/toxicity study

The biodistribution and persistence, and the toxicity of MC0518 mesenchymal stromal cells were assessed after repeated i.v. slow bolus injections in NSG mice (7 groups, 12 males and 12 females per group). Doses of 1×10^6 cells/animal (40×10^6 cells/kg body weight (bwt), for a 25 g mouse (average weight)) were given once weekly in six consecutive weeks (6 doses in total, covering the maximum number of doses given in clinical practice); vehicle control groups were dosed with freezing medium and assessed concurrently. The study design is shown in Table 1. Animals were approximately 9 weeks of age at the onset of dosing and body weights ranged from 25−30 g and 17−23 g for males and females, respectively. Animals in the biodistribution study groups were investigated 24 h (Day 37), 1 week (Day 43), and 4 weeks (Day 64) after the 6th dose for analysis of human cells in mouse background, using quantitative PCR (qPCR) and in situ hybridization (ISH). This allowed the analysis of cell persistence in mouse tissues. For qPCR assessment, tissues of the 20 major organs were tested (for list see results); of these, several selected tissues were also assessed by ISH, including PCR-positive and negative samples.

Animals in the toxicity group were assessed for clinical signs, body weight and food consumption, functional and behavioral changes and clinical pathology (hematology parameters: hematocrit, hemoglobin, mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, hemoglobin distribution width, platelet count, red blood cell count, red cell distribution width, reticulocyte counts (absolute and relative), white blood cell count (WBC), WBC differential (absolute and relative), plateletcrit/thrombocrit); clinical chemistry parameters: A/G ratio (calculated), alanine aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, bilirubin (total), calcium, chloride, cholesterol (total), creatinine, globulin (calculated), glucose, phosphorus (inorganic), potassium, protein (total), sodium, triglycerides, urea; urinalysis: blood, pH, glucose, protein, urobilinogen, ketones, bilirubin, color and appearance, specific gravity, volume). At the end of the observation time, macroscopic

Table 1
Study design of the biodistribution/toxicity study.

Group	Treatment	Dose level [cells/animal]	Duration of observation [days]	Number of animals	
				Males	Females
1 (Toxicity)	Control: vehicle*	0	43	12	12
2 (Toxicity)	Control: vehicle*	0	64	12	12
3 (Biodistribution)	MC0518	1×10^6	37	12	12
4 (Toxicity)	MC0518	1×10^6	43	12	12
5 (Biodistribution)	MC0518	1×10^6	43	12	12
6 (Toxicity)	MC0518	1×10^6	64	12	12
7 (Biodistribution)	MC0518	1×10^6	64	12	12

* Vehicle consisting of freezing medium (10% DMSO and 5% human serum albumin in 0.9% saline).

Table 2
Study design of the tumorigenicity study.

Group	Treatment	Dose level [cells/animal]	Duration of observation [days]	Number of animals	
				Males	Females
1	Control: vehicle*	0	92 (3 months)	12	12
2	MC0518	1×10^6	92 (3 months)	12	12
3	HL-60 cells	2×10^6	42 (1.5 months)	5	5
4	Control: vehicle*	0	183 (6 months)	12	12
5	MC0518	1×10^6	183 (6 months)	12	12

* Vehicle consisting of freezing medium (10% DMSO and 5% human serum albumin in 0.9% saline).

changes and organ weights were recorded and organs subjected to histopathology examination.

Animals of the toxicity study part were assessed for mortality, clinical behavioral changes, body weight, food consumption, neurological examination (Functional Observational Battery, after the 1st dose), clinical pathology (hematology, clinical chemistry and urinalysis) and macroscopic and microscopic and histopathological changes were assessed at study termination 1 week and 4 weeks after the last dose in a broad number of tissues in haematoxylin eosin stained tissue sections.

2.4. Tumorigenicity study

The tumorigenicity of MC0518 mesenchymal stromal cells was assessed after repeated i.v. slow bolus injections in NSG mice (12 males and 12 females per group) after 3 and 6 months (Day 92 and Day 183). Doses of 1×10^6 cells/animal (40×10^6 cells/kg bwt, for a 25 g mouse (average weight)) were given once weekly in six consecutive weeks (6 doses in total, covering the maximum number of doses proposed for clinical use); a vehicle control group for each time point (12 males and 12 females) and a positive control group (5 males and 5 females) treated with tumor-inducing HL-60 cells (single dose of 2×10^6 HL-60 cells/animal) were assessed concurrently. The study design is shown in Table 2. Animals were approximately 9 weeks of age at the onset of dosing and body weights ranged from 24−31 g and 18−25 g for males and females, respectively. During the in-life phase, all animals were assessed for mortality, clinical examinations with mass palpation, body weight and food consumption. At necropsy, 3 and 6 months after first dosing, the control and MSC-treated group each were assessed for organ weight changes, macroscopic and microscopic changes, and tumor masses. The HL-60 positive control group was sacrificed after 42 days (approx. 1.5 months). After this time, most of the animals had developed (palpable) tumors. All identified masses of control and MSC-treated groups were isolated and assessed by histopathology. The origin of all masses was determined by human and mouse-specific qPCR and ISH.

2.5. Quantitative PCR for human DNA detection

Human MSCs were quantified in mouse tissues using a qPCR method adapted from Becker et al. [12]. Prior to measurement of study samples, the method was validated using tissue samples spiked with defined numbers of MSCs. The validation parameters were assay range, intra-day and inter-day accuracy and precision, linearity, matrix effects and freeze-thaw and storage stability of tissue homogenates and DNA isolates. The determined assay range was: lower limit of quantification (LLOQ): 100 MSCs/200 µL tissue homogenate (containing 5–20 mg tissue), upper limit of quantification (ULOQ): 5×10^4 MSCs/200 µL tissue homogenate and limit of detection (LOD): 50 MSCs/200 µL tissue homogenate.

For qPCR measurement, genomic DNA was extracted from tissue homogenates using the Nucleospin® 96 Tissue Core Kit (Macherey Nagel, Germany) following the manufacturer's instructions. Dependent on the organ size and consequently the amount of tissue available, DNA was isolated from homogenates containing between 5 and 20 mg tissue. The amount of human DNA in each sample was quantified by amplification of a human-specific DNA fragment of the satellite DNA on chromosome 17. For quality control of the DNA extraction, a qPCR specific for genomic mouse DNA was performed. The following primers and probes were used:

human-specific forward: 5′-GGGATAATTTCAGCTGACTAAACAG-3′
human-specific reverse: 5′-AAACGTCCACTTGCAGATTCTA-3′
human specific Probe: 5′-FAM-CACGTTTGAAACACTCTTTTTGCA-BHQ-1-3′
mouse-specific forward: 5′-TACCTGCAGCTGTACGCCAC-3′
mouse-specific reverse: 5′-GCCAGGAGAATGAGGTGGTC-3′
mouse-specific Probe: 5′-TAMRA-CCTGCTGCTTATCGTGGCTG-BHQ-2-3′

3 µL of the DNA eluate (corresponding to 0.25–1 mg tissue) extracted from the different tissues was amplified using the GoTaq Probe qPCR Master Mix PCR system (Promega, USA) according to the manufacturer's instructions, and evaluated using the ViiA™ 7 Dx Real-Time PCR system (Applied Biosystems, USA). Calibration curves with MSC-spiked samples and defined numbers of MSC cells were run in parallel to determine MSC numbers in tissue samples. MSC numbers per gram tissue were calculated.

2.6. In situ hybridization for detection of intact human cells

Cells of human origin were detected in mouse cell background using the RNAscope® Technology, which stains species-specific messenger RNAs on tissue sections. The method was validated using human (tonsil) and mouse (spleen) tissues before analysis of study samples. Nuclear staining with the human probe was observed in nearly 100% of the cells in human control tissue and no staining was observed in the matching negative control tissues, rendering the method suitable for human cell detection.

Formalin-fixed paraffin-embedded tissue sections were hybridized and stained using a Ventana Discovery Ultra automat.

For detection of human cells, the human Alu Detection Probe RNAscope® 2.5 VS Probe – Hs-Alu-Rep-SxJ (442859) was used, for detection of mouse cells a probe for mouse PPIB (peptidyl-prolyl cis-trans isomerase B) was employed (RNAscope® 2.5 VS Positive Control – Probe_Mm-PPIB (313919)). As negative control, a probe for the bacterial gene dapB (dihydrodipicolinate reductase, bacterial) was used (RNAscope® 2.5 VS Negative Control – Probe_dapB (312039)), which is not expected to generate a signal in eukaryotic cells.

The tissue sections were hybridized and stained according to the RNAscope® manufacturer's protocol. In brief, the sections were deparaffinized and pre-treated by heating to 97 °C in RNAscope® VS Universal Target Retrieval buffer (323740) for 16 min, and subsequently incubated with a protease for additional 12 min at 37 °C. The probes of

Table 3
Biodistribution Study: Incidence of Human DNA Positive qPCR Results in Different Tissues of MC0518 Treated Animals.

Organs	Number of samples with positive qPCR results (%) Min. – Max. [cells/g tissue]		
	Day of sampling (Time after last cell application)		
	Day 37 (24 h)	Day 43 (1 week)	Day 64 (4 weeks)
Brain	3/24 (13%)	0/24 (0%)	0/23 (0%)
	9.12×10^3 – 24.8×10^3	n.a.	n.a.
Heart	12/23 (52%)	2/24 (8%)	0/23 (0%)
	5.21×10^3 – 17.8×10^3	6.35×10^3 – 21.3×10^3	n.a.
Spleen	0/24 (0%)	1/23 (4%)	0/23 (0%)
	n.a.	104×10^3	n.a.
Pancreas	0/24 (0%)	1/24 (4%)	0/23 (0%)
	n.a.	12.6×10^3	n.a.
Liver	0/24 (0%)	1/24 (4%)	0/23 (0%)
	n.a.	35.6×10^3	n.a.
Lung	24/24 (100%)	6/24 (25%)	2/23 (8%)
	116×10^3 – 1440×10^3	7.15×10^3 – 36.7×10^3	6.99×10^3 – 20.1×10^3
Injection site (tail)	4/24 (17%)	5/24 (21%)	1/23 (4%)
	34.4×10^3 – 171×10^3	10.7×10^3 – 42.5×10^3	28.2×10^3

Biodistribution and persistence of MSCs from MC0518 was assessed in immuno-compromised NSG mice at 24 h (Day 37), 1 week (Day 43) and 4 weeks (Day 64) after the last of 6 once weekly doses of 1×10^6 cells/animal (40×10^6 cells/kg bwt, for a 25 g mouse (average weight)).
n.a.: not applicable.

interest were hybridized at 43 °C for 2 h. The amplification and detection systems (RNAscope® VS Universal HRP Detection reagent – 323210) were applied following the manufacturer's recommendations. For counterstaining, the sections were incubated in hematoxylin for 4 min and in bluing reagent for an additional 4 min. After washing in soap water and running water, the slides were mounted for scoring under a light microscope (Olympus BX61). The intensity of staining was graded as: - negative, 1: minimal, 2: slight, 3: moderate, 4: marked, 5: strong. Pictures were taken with an Olympus camera (DP74) using the Cell Sens software.

2.7. Statistics

Data acquisition was carried out using the Provantis data capture system 9.3.0.0. Statistical analysis was performed using SAS 9.2 and 9.3.

Numerical data obtained in the biodistribution/toxicity study were analyzed excluding groups with less than three observations or with zero variance. For each data set, a two-sample *t*-test was performed and the residuals were tested for normality using a Shapiro-Wilk test. When the distribution of the residuals was significantly different from a normal one ($p \leq 0.05$), a two-sample *t*-test using square root transformed data was performed. When a data set included negative values, the absolute lowest observed value was added to the data before the transformation. When the Shapiro-Wilk test was not significant ($p > 0.05$), a Levene test was performed on the residuals to assess the homogeneity of the treatment variances. When differences between variances were not found to be significant ($p > 0.05$), the results from the related *t*-test were retained to assess the treatment mean comparison. When the Shapiro-Wilk test on the residuals was significant or when heterogeneous treatment variances ($p \leq 0.05$) were revealed by the Levene test, the *t*-test results were discarded and the treatments were compared using a non-parametric Wilcoxon rank-sum test.

Numerical data obtained in the tumorigenicity study were analyzed using a one-way analysis of variance (ANOVA) and the residuals were tested for normality using a Shapiro-Wilk test switching to square root transformed data as described above in case of significance. When a data set included negative values, the absolute lowest value was added to the data before the transformation. A Levene test was performed as described above. When differences between treatment variances were not found to be significant ($p > 0.05$), the results from the related ANOVA were retained. When significant differences among the treatment means were indicated by the ANOVA overall F-test ($p \leq 0.05$), a *t*-test on least-squares means was used to perform all pairwise treatment mean comparisons. The non-parametric Kruskal-Wallis test was used in case of significant Shapiro-Wilk or Levene test. When the Kruskal-Wallis test was significant ($p \leq 0.05$), a Wilcoxon sum-rank test was used to perform all pairwise treatment comparisons.

Non-numerical data such as incidence of masses, incidence of tumors of any origin or incidence of tumors of MSC origin was compared between groups.

Each pairwise comparison was conducted via a two-sided test at the 5% significance level.

3. Results

3.1. Biodistribution

Biodistribution and persistence of MC0518 MSCs was assessed in NSG mice at 24 h, 1 week and 4 weeks after the last of six once weekly doses of 1×10^6 cells/animal/administration (dose multiple: 20 to 60-fold the envisaged clinical dose). Quantitative PCR for detection of human DNA in a background of mouse tissue was performed on 20 major organs (brain, bone marrow, bone, kidney, lung, lymph node, liver, mammary gland, jejunum, heart, skeletal muscle, skin, spleen, testes, uterus, prostate, pancreas, ovaries, blood, and injection site). The presence of human DNA was found in seven of these tissues (Table 3).

The highest incidence (number of samples positive for MC0518) and derived concentration of MC0518 cells was observed in the lung at 24 h after the last dose administration. The remaining positive tissues had concentrations of MC0518 cells which were 10−100-fold lower than the highest lung value on day 37 (24 h after the last dose), and were of much lower incidence. A time-dependent decrease in cell concentration and incidence was noted with substantial reduction 1 week after the last dose in lung, brain and heart, while incidental positive samples were observed for spleen, pancreas and liver independent of the sampling time. At the injection site, several samples positive for human DNA were found 24 h and 1 week after the last injection of MC0518, however the incidence decreased at the 4 week sampling time point, and in most tissues no human DNA was detected 4 weeks after the last injection (Table 3).

Several qPCR positive and negative tissues were selected to assess the presence of human MSCs on cellular level in mouse organs by ISH of human Alu and mouse PPIB messenger RNA. The selected organs were lung, heart, liver, kidney, brain, spleen, pancreas, ovary, uterus, prostate, and injection site. In general, tissues/samples with positive results for human DNA in qPCR were chosen. In cases where no tissue sample

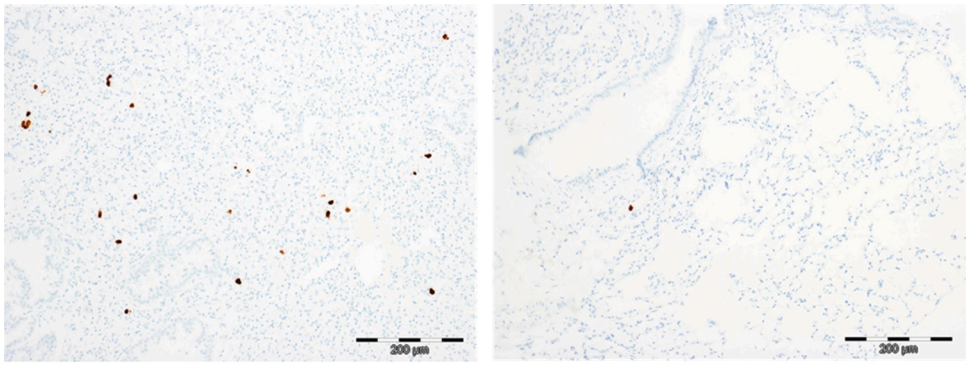


Fig. 1. Lung sections hybridized with hAlu probe detecting human MSCs in mouse tissue. Brown spots are positive cells within the haematoxylin stained mouse tissue (blue). (A) Tissue isolated 24 h after the last MSC dose, (B) tissue isolated 1 week after the last dose. Magnification 10×.

was found qPCR positive, samples from animals were selected randomly.

Evaluation of the lung tissue (Fig. 1) showed a good correlation of ISH with PCR results in terms of incidence and signal strength (Table 4). In the remaining selected tissues, most PCR-negative samples were also hAlu-negative in ISH. Many samples with a weak PCR-positive signal (10^3-10^4 cells/g tissue) did not show any human cells at ISH (Table 4). This might be either attributed to the limited number of slides examined or the higher sensitivity of the qPCR method which can also detect traces of airborne human DNA contamination.

3.2. Toxicity

MC0518 was well tolerated after repeat-dose administration of 1×10^6 cells/animal/administration (40×10^6 cells/kg bwt, for a 25 g mouse (average weight)). A dose multiple of 20 to 60-fold the envisaged clinical dose was achieved. There were no treatment-related deaths, clinical signs or neurological changes during the observation period up to 4 weeks after the last of six doses. Also, body weight, body weight gains and food consumption were unaffected by treatment. Hematology parameters, clinical chemistry parameters and urinalysis (blood, pH, glucose, protein, urobilinogen, ketones, bilirubin, color and appearance, specific gravity, volume) were unchanged when compared to controls (see Supplemental Table A.1). The few statistically significant differences between groups were considered incidental and unrelated to treatment based on incidence and distribution. At necropsy, 1 and 4 weeks after the last dose, no treatment-related organ weight changes, or macroscopic and microscopic organ changes were observed. The increased mean absolute prostate weight (approx. 20% vs controls) 1 week after the last dose in the MC0518 treated group was not observed at the later sampling time point 4 weeks after the last dose, and did not correlate with relevant histopathological changes. This finding was therefore considered incidental and non-adverse. Histopathological changes of decreased lymphoid cells in spleen and thymus were observed in all groups and are associated with the NOD/SCID/IL2Rγnull background. In the lung, a slightly higher incidence of macrophage infiltration was seen in the MSC treated group 1 week after the last dose as compared to controls (3/24 animals in the MC0518 treated group versus 0/24 animals in the control group). However, a comparable incidence of this finding was observed in treated and control animals at the 4 week time point (2/24 in each group). The finding was not considered adverse and might indicate the clearance of the MSCs in this organ at the early time point. Thrombi were not detected in this study. Lesions at the injection site were observed at comparable incidence in control and MC0518 treated animals and were considered procedure-related (Supplemental Tables A.2 and A.3). These data demonstrate the good tolerability of MC0518.

3.3. Tumorigenicity

Six once weekly administrations of MC0518 to NSG mice (1×10^6 cells/animal/administration; dose multiple: 20 to 60-fold the envisaged clinical dose) were well tolerated and not associated with tumorigeniciy for up to 6 months after administration. There were no MC0518-related mortalities during the observation period. Two animals injected with the positive control HL-60 cells had to be prematurely euthanized due to deteriorating condition, likely associated with the presence of tumor masses. Also, in this control group several clinical signs were observed that were attributed to the occurrence of masses.

In the vehicle and MC0518-treated groups no palpable masses were detected. No adverse clinical signs were observed and body weights, food consumption, organ weights and macroscopic and microscopic assessment revealed no MC0518-related findings when compared to control animals (see Supplemental Tables A.4, A.5 and A.6). Histopathological changes of decreased lymphoid cells in spleen and thymus were again observed in all groups and are associated with the NOD/SCID/IL2Rγnull background. As seen in the toxicity study at earlier time points, non-adverse infiltration of macrophages in the lung was observed in both control and MC0518-treated animals at 3 months and 6 months after treatment. The incidence was comparable in control and MC0518 treated animals, which rather suggests a correlation with the strain-specific background of the mice than with treatment or MSC clearance.

At necropsy, 3/5 male and 5/5 female animals administered HL-60 cells were found with one or multiple masses. When evaluated by qPCR, all masses were strongly positive for human DNA, with cell counts generally above the limit of quantification. In addition, selected samples showed strong positivity for human Alu mRNA sequences by ISH assessment (Fig. 2).

Few spontaneous masses were found in vehicle control and MC0518-treated animals, mostly at the 6 months necropsy time point. Four masses were identified in control animals and 4 masses were found in MC0518-treated animals. As the incidence was comparable between vehicle control and MSC-treated animals, they were considered likely unrelated to MC0518 administration. When assessed by qPCR analysis, some masses from control animals as well as from MC0518-treated animals showed a positive signal for human DNA. Derived human cell counts were generally between 100 to 10,000-fold lower compared to HL-60 cell-derived tumors. The positive PCR signal, especially in the vehicle control groups, was highly unexpected, as the animals did not receive any human cells. For this reason, all masses were also assessed by ISH. This hybridization did not reveal any human Alu positivity, but a strong mouse PPIB signal in vehicle control as well as MC0518 treated animals, confirming the murine origin of these masses (Fig. 3). Comparative data are shown in Table 5. Given the much lower qPCR

Table 4
Biodistribution Study: Correlation between qPCR and ISH Results.

Day of sampling (Time after last cell application)	Animal No.	qPCR (MSCs/g tissue)	hAlu
Tissue: Lung			
Day 37 (24 h)	3001	1440×10^3	+++
	3012	300×10^3	++
	3506	1310×10^3	++
	3510	116×10^3	++
Day 43 (1 week)	5005	36.7×10^3	—
	5007	7.15×10^3	+ [a]
	5503	12.4×10^3	—
	5507	<LLOQ	+ [b]
Day 64 (4 weeks)	7001	20.1×10^3	—
	7009	6.99×10^3	+ [b]
	7501	<LOD	—
	7502	<LOD	—
Tissue: Heart			
Day 37 (24 h)	3007	10.3×10^3	—
	3009	5.32×10^3	—
	3512	8.01×10^3	—
	3509	17.8×10^3	—
Day 43 (1 week)	5001	6.35×10^3	—
	5002	<LOD	[a]
	5505	21.3×10^3	—
	5506	<LOD	—
Day 64 (4 weeks)	7001	<LOD	—
	7002	<LOD	—
	7501	<LOD	—
	7502	<LOD	—
Tissue: Brain/Cerebellum			
Day 37 (24 h)	3003	9.12×10^3	— / —
	3005	<LOD	+ [c] / —
	3508	15.7×10^3	— / —
Day 43 (1 week)	3509	24.8×10^3	— / —
	7001	<LOD	— / —
	7002	<LOD	— / —
Day 64 (4 weeks)	7501	<LOD	— / —
	7502	<LOD	— / + [d]
Tissue: Injection site (two tissue pieces per animal)			
Day 37 (24 h)	3008	69.6×10^3	+ [e] / —
	3507	171×10^3	— / —
Day 43 (1 week)	5010	34.1×10^3	— / + [f]
	5511	42.5×10^3	— / —
Day 64 (4 weeks)	7001	28.2×10^3	+ [e] / ++ [g]
	7501	<LOD	— / —
Tissue: Kidney			
Day 37 (24 h)	3001	<LLOQ	—
	3506	<LOD	—
Tissue: Prostate			
Day 37 (24 h)	3001	<LOD	—
	3012	<LOD	—
Tissue: Uterus			
Day 37 (24 h)	3506	<LOD	—
	3510	<LOD	—
Tissue: Ovary			
Day 64 (4 weeks)	7501	<LOD	—
	7502	<LOD	—
Tissue: Spleen			
Day 43 (1 week)	5006	104×10^3	—
Tissue: Pancreas			
Day 43 (1 week)	5504	12.6×10^3	—
Tissue: Liver			
Day 43 (1 week)	5506	35.6×10^3	+ [h]

Biodistribution and persistence of MSCs from MC0518 was assessed in immunocompromised NSG mice at 24 h (Day 37), 1 week (Day 43) and 4 weeks (Day 64) after the last of 6 once weekly doses of 1 × 106 cells/animal (40×10^6 cells/kg bwt, for a 25 g mouse (average weight)).
hAlu: human Alu, LLOQ: lower limit of quantification (100 cells), LOD: limit of detection (50 cells).
-: no staining, +: minimal, rare positive cells (1–10 per field; 20× magnification) ++: slight, few positive cells (approx. 10–20 per field; 20x magnification), +++: moderate; some positive cells (more than 20 per field; 20× magnification).
[a] Single cell.
[b] Single cell within the alveolar wall.
[c] Single cell on brain section.
[d] Single cell on cerebellum section.
[e] Two cells.
[f] A spot of approx. 5 cells.
[g] A focus of approx. 20 cells.
[h] Few cells in the liver sinusoids.

signal compared to the HL-60 group and the absence of any human Alu positive cells by ISH, the positive PCR signal from the masses of the vehicle control and MC0518-treated groups was considered to be due to contamination with human DNA during processing. Hence, no MSC-induced tumors were identified after repeat-dose dose i.v. administration of 1×10^6 MC0518/dose and a follow-up of six months.

4. Discussion

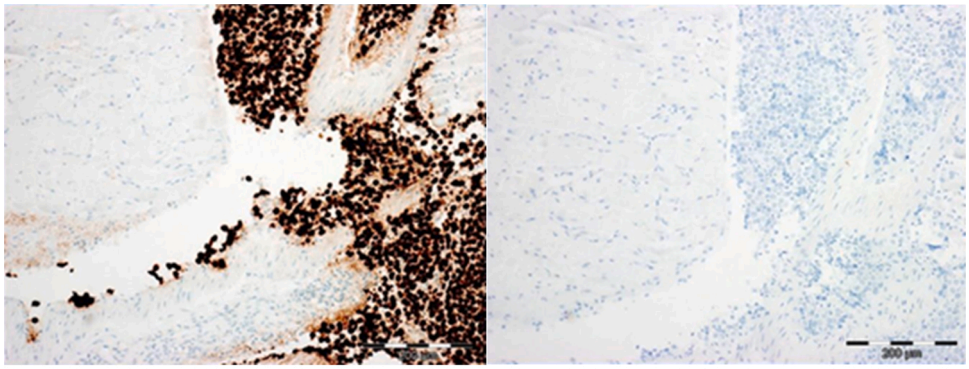
Well-designed GLP-compliant toxicity studies with sufficiently large group sizes and sufficiently long observation times are essential before the start of clinical trials. Our nonclinical data for MC0518 obtained in GLP-compliant studies in NSG mice demonstrate their fast clearance, safety and lack of tumor formation. The data are in line with previously published results for other MSC preparations. However, GLP repeat-dose toxicity studies for bone marrow-derived human MSCs are sparse in literature; our studies are intended to fill this gap. The major risks identified so far for therapeutic cell products are immunogenicity, hemoincompatibility (e.g. thrombotic events), persistence in tissues and subsequent ectopic tissue formation, and malignant transformation during culturing, resulting in tumor formation in the recipient [13,14]. The two studies presented here assessed the major complications of hemoincompatibility as well as the persistence and tumor formation potential of MC0518.

4.1. Biodistribution/toxicity

Multiple nonclinical studies investigated the biodistribution and persistence of MSCs of different sources and administered via different routes. Recent reviews have been published by Salvadori and colleagues [15] and Brooks and co-workers [16]. The authors suggest that MSCs distribute primarily to the lung when administered intravenously, while the vessel diameter influences trapping of the cells in the lung. Distribution of MSCs into organs other than the lung is low, but sites of injury or inflammation might trigger distribution into these regions. The authors suggest that the viability of MSCs after systemic administration is short and persistence low. Similar results were observed by Allers and co-workers (single i.v. dose in nude mice) [17], Kyriakou and colleagues (fluorescently labelled human MSCs in NSG mice) [18], Eggenhofer and colleagues and Niyibizi and co-workers (allogeneic mouse MSCs in mice) [19,20] and Gao and co-workers (rat MSCs in rats) [21]. Zhuang and colleagues [22] investigated an additional detection method using fluorescent nanodiamond (FND) tracking of placenta choriodecidual membrane-derived MSCs in healthy mice. Also in this study a pulmonary first-pass effect and a time-dependent decline was observed.

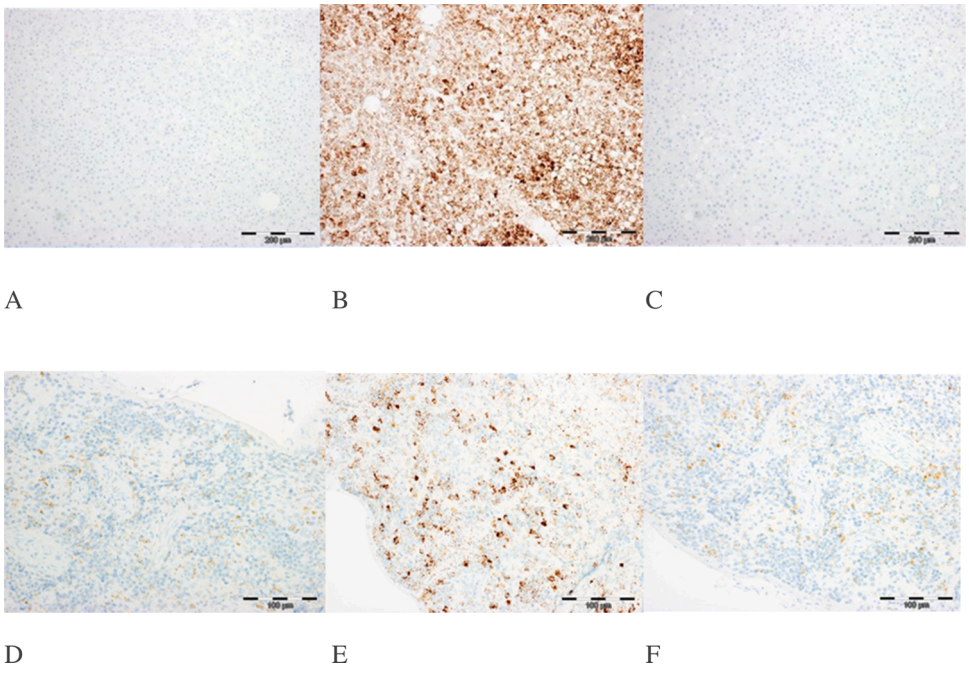
The limitations in the above mentioned studies were the sometimes short observation times, the low doses generally given as single dose and the manipulation of cells for labelling. For regulatory safety assessment of cell therapeutics, however, unchanged clinical product should be tested and validated methods used.

In our study we used repeat-dose treatments and up to 4 weeks observation time after the last dose. The biodistribution and persistence results, however, were similar, which confirms fast clearance and the low survival of MSCs in vivo. MC0518 cells distributed primarily to the



A B

Fig. 2. Tissue section of a mass isolated from a mouse treated with the positive control HL-60 cells hybridized with hAlu (A) and dapB (B). The brown staining in (A) indicates the human origin in the haematoxylin stained mouse tissue (blue). Magnification 10×.



A B C

D E F

Fig. 3. Tissue section of a mass isolated from a control mouse (A-C) and a mouse treated with MC0518 (D-F) hybridized with hAlu (A and D), mPPIB (B and E) and dapB (C and F). The dark brown staining with mPPIB indicates the mouse origin of the mass in the haematoxylin stained sections (blue). The yellowish staining in sections (A) and (C) are hemosiderin pigments. Magnification 10× for upper panel and 20× for lower panel.

lung with 100% qPCR positive samples at 24 h after administration, and a time-dependent decline during the next four weeks. This was reflected in the number of positive samples as well as in the declining numbers of MSCs in the samples. A similar decline of MSCs was observed in the heart, in the brain, and at the injection site in the tail. Occasional positivity was found in spleen, pancreas and liver, which suggests either incidental cells but could also be due to contamination.

Only few toxicity studies with human bone marrow-derived MSCs have been published so far in literature. Aithal and colleagues and Rengasamy and coworkers [23,24] report good safety and tolerability in rats and rabbits after single or multiple doses of human MSCs, however the experiments were performed in immunocompetent animals, in which early destruction of the cells might impact toxicity assessment.

The data of our study suggest good tolerability with no target organs of toxicity in an animal model that allows survival and potential persistence of the MSCs. In humans, the cells have the potential to survive for several days in the recipient, as they are considered immune-evasive due to their low potential for the induction of allo-reactive antibodies [25]. In the chosen NSG mouse model the situation is similar insofar as the cells are not rejected by an intact immune system. The data are also in line with the current clinical experience in GvHD patients treated with our MSC product that describes good tolerability and only few adverse events [7,26].

4.2. Tumorigenicity

The risk of tumorigenicity of MSCs is derived from their potential of tissue persistence and of becoming aberrant by acquisition of chromosomal abnormalities during in vitro expansion. For this reason, clinically used MSC products are tested in vitro for senescence and genetic stability and in vivo for persistence and tumor formation.

Immuno-compromised NSG mice are often used as model for the growth of patient-derived tumor xenografts [10] and are therefore also suitable for assessing MSCs' potential of tumor formation. The animals have a low predisposition for the development of spontaneous tumors, and the naturally occurring neoplastic lesions are usually not

Table 5
Tumorigenicity Study: Correlation between PCR and ISH Results for Human Cell Detection.

Group (Duration)	Animal No.	qPCR [MSCs/g tissue]	In Situ Hybridization (ISH)	
			hAlu	mPPIB
Group 1 (vehicle control at 3 months post dose)	1008	2340×10^3	—	++++
Group 2 (MC0518 at 3 months post dose)	No masses	NA	NA	NA
Group 3 (HL-60 positive control group) selected samples	3004	>ULOQ	+++++ [a]	++ [b]
	3501	>ULOQ	+++++ [a]	+ [b]
	3504	>ULOQ	+++++ [a]	++ [b]
Group 4 (vehicle control at 6 months post dose)	4501	0.648×10^3	—	++++
	4504	0.165×10^3	—	++++
	4510	<LOD	—	++++
Group 5 (MC0518 at 6 months post dose)	5003	0.505×10^3	—	+++
	5004	0.297×10^3	—	+++
	5009	<LOD	—	++++
	5603	<LOD	—	+++

Biodistribution and persistence of MSCs from MC0518 was assessed in immuno-compromised NSG mice at 24 h (Day 37), 1 week (Day 43) and 4 weeks (Day 64) after the last of 6 once weekly doses of 1×10^6 cells/animal (40×10^6 cells/kg bwt, for a 25 g mouse (average weight)). hAlu: human Alu, mPPIB: mouse peptidyl-prolyl cis-trans isomerase B, NA: not applicable, ULOQ: upper limit of quantification (50,000 cells), LOD: limit of detection (50 cells).
-: no staining, +: minimal, few cells with positive dots, ++: slight, some cells with positive dots, +++: moderate; several cells with positive dots, ++++: marked, many cells with positive dots, +++++: strong, more than 90 % of positive cells.
[a] Mass-more than 90% cells.
[b] Interstitial cells.

contributing to morbidity [8]. The most common neoplastic lesions in ageing NSG mice (median age 52 weeks) were mammary gland neoplasms, neoplastic lesions in the female reproductive tract, liver tumors and cutaneous neoplasms [27].

When patient-derived tumors or tumor cell lines are transplanted in NSG mice, tumors typically engraft within 2–4 months after transplantation [10,11]. An observation time of 6 months in our tumorigenicity study with MC0518 was therefore considered sufficient for being able to detect potential MSC-derived tumors.

In the HL-60 cell control group, a total of 19 masses were found in 8/10 (80%) animals either prematurely sacrificed due to deteriorating health or at terminal sacrifice 42 days after injection. This frequency and time of onset of tumor growth is in the normal range for HL-60 tumor induction and confirmed the suitability of the model used [28].

At the 6 months necropsy, four masses each were identified in control animals and in MC0518-treated animals. As the incidence was comparable between control and MSC-treated animals, they were considered likely unrelated to MC0518 administration. The incidence of 4/24 animals (16%) at the age of 35 weeks is slightly higher than the published values of Moyer and colleagues, who report 16/365 (4.4%) or Shultz and co-workers, who observed 3/34 animals with tumors (9%) [8,29]. The higher background incidence in our study might be caused by recording all masses while not distinguishing between benign and malignant tumors.

When assessed by qPCR analysis, some masses from control animals as well as from MC0518-treated animals showed a positive signal for human DNA. Derived human cell counts were generally between 100 to 10,000-fold lower compared to HL-60 results, but in most of the cases well above the limit of quantification. The positive PCR signal especially in the vehicle control groups was highly unexpected as they did not receive any human cells. All assay controls tested negative for human cells, which suggests the absence of a false-positive background signal of the assay. For this reason we suspected a contamination in the sample and as follow-up, the masses were assessed by ISH to detect intact cells. The hybridization did not reveal any positive human cells but a strong mouse PPIB signal, confirming the murine origin of the masses. Given the much lower qPCR signal compared to the HL-60-derived tumors and absence of any human Alu positive signal by ISH in tumors of control/MC0518-treated animals, we conclude that the positive PCR signal from the masses was likely due to accidental contamination with human DNA during processing. It is known that PCR techniques are associated with a high contamination risk [30]. In summary, no MSC-induced tumors were identified after repeat-dose dose i.v. administration and a follow-up of six months. Since MSCs in MC0518 were also genomically stable and showed a normal karyotype and diploid pattern in the vast majority of the cells, our data suggest that MC0518 bears a low risk for tumor formation. As both detection methods (qPCR and ISH) have advantages and disadvantages, we believe that a combination of both gives the most robust and reliable interpretation of results. While the lower limit of quantification is 100 cells/mg tissue for the qPCR, ISH allows the detection of a single cell. On the other hand, for qPCR analysis, the whole organ or at least 0.25 mg is analyzed, while ISH is based on tissue sections with a limited number of cells. But since ISH detects intact living cells, it is not susceptible to contamination like the qPCR method. The DNA detected in a tissue sample via qPCR can also stem from DNA contamination during processing of the sample. For this reason we consider qPCR and ISH as complementary methods, which helped to identify false positive samples in our tumorigenicity study. In the biodistribution study, the strong qPCR signals correlated with multiple human cells in the tissue sections. However, in tissues with weaker PCR signals, human cells could not always be detected, and even in several qPCR negative samples incidental positive cells on sections were observed. This shows the limitation of both methods and the benefit of combining the two bioanalytical approaches.

5. Conclusion

In conclusion, we investigated the biodistribution/persistence, safety and tumorigenicity of MC0518 in NSG mice. MC0518 represents a human MSC product from pooled bone marrow mononuclear cells of eight human, healthy, adult donors. Our data suggest that MC0518 is safe for the intended use in patients with SR-aGvHD. We used qPCR as well as ISH as complementary methods for the detection of MC0518 DNA or cells, respectively. We were able to show the presence of intact

MC0518 cells in tissue sections and observed a good correlation between qPCR and ISH results in organs with high MC0518 cell numbers. The additional use of ISH analysis also allowed identifying possible false positive qPCR results. Hence, we believe that the combination of these detection methods is crucial for obtaining robust and reliable tissue distribution results.

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Author contributions

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Matthias Müller: Investigation, Writing – review and editing.

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All authors have approved the final article.

Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The data are available at the authors' institution.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2021.11.016.

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