Maintaining osteoclastogenesis following allogeneic hematopoietic stem cell transplantation for osteopetrosis: evidence from *in vitro* testing

Osteopetrosis (OP) is a rare progressive disorder caused by several different gene defects and associated with significant morbidity and mortality.[1] Allogeneic hematopoietic stem cell transplantation (allo HSCT) can be curative for infantile OP.[2] Deficiency of osteoclast (OCL) development and/or function would lead to dense bone structure, and lack of space for medullary hematopoiesis. We investigated the origin of OCL in two allo HSCT recipients with OP after the first recipient had a significant decline in peripheral blood (PB) donor myeloid chimerism by short tandem repeat (STR) analysis, which concerned us about the maintenance of osteoclastogenesis and its potential effect on patient outcome.

Case 1. We previously reported an infant with severe combined immunodeficiency (SCID) due to interleukin-2 receptor γ chain gene mutation, congenital chromosomally-integrated herpesvirus-6 (HHV-6) infection, hemophagocytic syndrome and OP.[3] The patient tested negative for known OP mutations by whole-exome and genome sequencing. Bone biopsy report emphasized the lack of osteoclastic activity. At 5 months of age, the patient underwent an allo HSCT from a matched unrelated donor (MUD) conditioned with fludarabine, busulfan and rabbit anti-thymocyte globulin, graft-*versus*-host disease (GvHD) prophylaxis with mycophenolate mofetil and tacrolimus and veno-occlusive disease (VOD) prophylaxis with defibrotide.

Early days of transplant were complicated by liver VOD and thrombotic microangiopathy. The patient achieved timely engraftment and continues to have normal peripheral blood counts without any GVHD, normal immunoglobulin levels and T-cell function, has developed anti-HHV-6 antibodies, but continues to have low level HHV-6 viremia due to chromosomal integration of the virus without clinical consequences.[4] Skeletal X-ray findings are almost normalized. Thirty-four months after HSCT, the patient has mild azotemia with small-for-age kidneys not requiring intervention, also has autism spectrum disorder without any neurodegeneration in serial brain magnetic resonance imaging studies or abnormalities in ophthalmologic and audiologic examinations. Peripheral blood cell counts recovered and STR analysis revealed "donor cells only" status on day +100. However, while donor chimerism was 93% in lymphocytes, donor myeloid chimerism declined to 43% at +13 months and 27% at +20 months. Further decline in PB donor myeloid

chimerism to 11% was observed at +22 months. (Table 1)

Case 2. A 7-month-old Caucasian male with infantile malignant osteopetrosis due to double heterozygous *TCIRG1* exon 12 mutations (1. C.1312C>T and 2. C.1320_1321delTC) underwent MUD allo HSCT at 7 months of age. He presented to our hospital at the age of 4 months with difficulty in feeding, congestion, harsh breathing, a recessed chin, obstructive sleep apnea, failure to thrive, right-sided optic atrophy and strabismus, hepatosplenomegaly, anemia, thrombocytopenia, leukoerythroblastic reaction in PB and findings consistent with OP in the skeletal survey.

A MUD HSCT was performed following fludarabine 40 mg/m^{2}/dose on days -6 to -3, busulfan on days -5 to -2 targeting a cumulative area under the curve of 20,000 micromole x min/L and rabbit anti-thymocyte globulin 2.5 mg/kg/dose on days -8 to -6 conditioning with a mononuclear cell dose of 14.3×10^8/kg and CD34+ cell dose of 21.2×10^6/kg and GVHD prophylaxis with mycophenolate mofetil and tacrolimus. Neutrophil engraftment was established on day +13. Post-HSCT period was complicated with VOD despite defibrotide prophylaxis and skin GVHD for which he was treated on steroids. He has complete PB count recovery 3 months from HSCT and is doing well. One month post-HSCT, STR analysis showed donor chimerism of 64% in lymphocytes, and 100% in myeloid cells.

Osteoclast culture, characterization, and short tandem repeat analysis

Osteoclast culture methods proposed by Abdallah *et al.* were used with modifications to generate the OCL precursors (OCLp) and OCL from the PB mononuclear cells (PBMC).[5] In preliminary experiments to optimize the osteoclast culture conditions, we incubated Ficoll-Hypaq density gradient-separated PBMC in RPMI medium 1640 Glutamax™, supplemented with 10% of fetal bovine serum (FBS) charcoal-stripped (v/v) and various concentrations of recombinant human macrophage colony-stimulating factor (M-CSF) and/or recombinant human RANK-L (Sigma Aldrich, St. Louis, MO) in multiple wells in 24-well culture plates replacing with fresh culture medium every 48 hours. Cells were harvested using Accutase (Sigma Aldrich, St. Louis, MO) from different conditions and phenotyping was done to assess cell surface expression of markers CD14, HLA-DR (DR), CD45 (Beckman Coulter, Brea, CA) , CD51/61, CD68, osteopontin, CD115 (M-CSF receptor) (Biolegend, San Diego, CA), calcitonin receptor,

CD265 (hRANK) (R&D systems, Minneapolis, MN), TRAP(D-3) (Santa Cruz Biotechnology Dallas, Texas), using 10-color Gallios flow cytometer (Beckman Coulter, Brea, CA) on days 6, 9, 13 and 17.

Since OCL are multinucleated large cells, they cannot pass through the flow cytometry chambers intact. Thus, OCL flow cytometric analysis cannot be performed. However, OCLp are much smaller in size and therefore, can be studied by flow cytometry. Decrease in CD14 and DR expression was observed as cultured cells progressed towards OCLp or OCL formation in a subset of cells. Cytospin slides were prepared, stained with Write-Giemsa stain, and reviewed under microscope for the presence of OCL. Based on the cytospin cell morphology and CD14/DR expression in our preliminary experiments, day 14 was decided as an optimal time to harvest and isolate OCLp.

Osteoclastogenesis occurs by fusion of monocyte precursors derived from hematopoietic stem cells in presence of RANK and M-CSF (CD115).

It has been demonstrated in the time-course parabiosis experiments in mice with osteopetrotic phenotype that fusion of donor and recipient monocytic cells circulating in the blood results in expression of a donor-derived gene by recipient osteoclasts, and transfusion of monocytic cells can result in transfer of genes in osteoclasts for an extended period of time even in the absence of HSC chimerism.[6] Since monocyte origin and maintenance of adult life osteoclastogenesis has been demonstrated, we used magnetic-activated cell sorting (MACS)-isolated CD14+ monocytes, which also reduced lymphocyte contamination in our optimized approach (Figure 1).[5–7] Peripheral blood samples were collected at 20 and 22 months in the first patient and 1 month after allo HSCT in the second patient. The first case had shown decreasing donor myeloid chimerism but maintained stable lymphoid donor chimerism above 90%. Thus, there was a concern, if complete loss of donor myeloid engraftment leading to diminished production of healthy OCL and resurgence of OP findings was in process. In the second case, donor-derived osteoclast generation in vitro was investigated at 1 month following HSCT.

Monocytes were suspended in RPMI medium 1640 Glutamax™, supplemented with 10% of FBS charcoal-stripped (v/v), 25 ng/mL of recombinant human M-CSF and 100
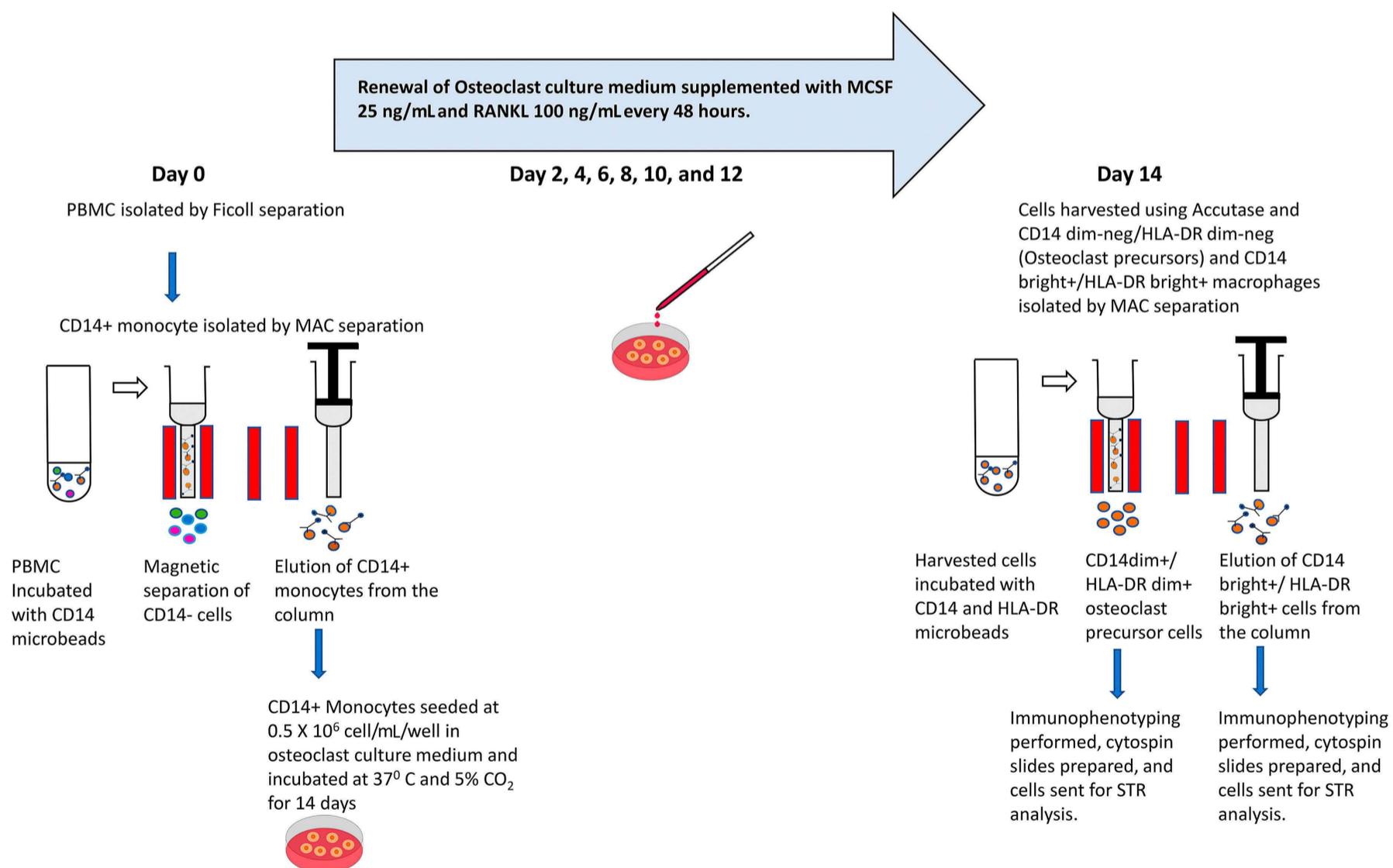

Figure 1. Experimental steps used in *in vitro* generation and characterization of osteoclasts and their precursors. Osteoclast precursors (OCLp) were generated by culture of magnetic-activated cell sorting (MACS)-isolated peripheral monocytes from peripheral blood mononuclear cells (PBMC). Peripheral blood monocytes were supplemented with fresh osteoclast culture medium containing recombinant human M-CSF at 25 ng/mL and recombinant human RANKL at 100 ng/mL every 48 hours for 14 days to stimulate differentiation into OCLp. On day 14, harvested cells were separated into CD14-DR- OCLp and CD14+DR+ macrophages by MACS for further evaluation by flow cytometry, short tandem repeat (STR) analysis and preparation of cytospin slides.

ng/mL of recombinant human RANK-L and seeded into two wells of a 24-well plates at the density of 0.5×10^6 cells/mL per well. The cells were incubated at 37°C, in humidified atmosphere containing 5% CO_2 over time. The medium was changed every 48 hours. On day 14, cells were harvested gently using Accutase and assessed for viability with trypan blue. The harvested cells were enriched by MACS separation using CD14 and DR microbeads (Miltenyi Biotec, Auburn, CA) into two separate populations, CD14-negative/DR-negative cells (OCLp) and CD14-bright/DR-bright cells (macrophages). Purity of enriched cells was checked by flow cytometry. Immunophenotyping was performed to assess the expression of CD14, CD115, HLA-DR, and osteopontin (Figure 2). Cytospin slides were prepared using suspension cells from both CD14-negative/DR-negative and CD14-bright/DR-bright populations, stained with Wright-Giemsa stain and reviewed under microscope. Cells from both populations were tested for donor chimerism by STR analysis.

Mature OCL and OCLp express CD45, CD51/61, osteopontin, CD115, calcitonin receptor, CD265 (hRANK), and TRAP(D-3) similar to macrophages.[8-12] We observed variable expression intensity patterns in these surface markers among different cases in OCLp and macrophages. While multinucleated giant OCL at different stages of maturation were seen on stained cytospin slides from OCLp, smaller cells with single nucleus were observed in the macrophage population under microscope (Table 1; Figure 2).

STR analysis of culture-grown OCLp showed the presence of 26% and 12% donor cells at 20 and 22 months following HSCT in the first case, respectively (Table 1). Donor OCLp percent values paralleled PB donor myeloid chimerism values indicating that both recipient and donor monocytes have the capacity to develop OCLp/OCL in vitro with the proper stimulation. We demonstrated that PB monocytes were induced to develop OCLp/OCL in vitro prior to HSCT in the second case; however, this was not performed in the first case. Despite declining donor myeloid chimerism, skeletal X-ray findings continue to resolve without any evidence of extramedullary hematopoiesis in the first case. Three of 14 patients with bone X-ray finding improvements following allo HSCT were reported to have mixed donor chimerism without specific numbers provided.[13]

We previously speculated that in utero HHV-6 viremia on a fetal severe combined immune deficiency background might have suppressed OCL development from hematopoietic cells leading to OP phenotype in the first patient.[3] As emphasized above, HHV-6 viremia inevitably continues following successful allogeneic HSCT, though at a much lower level, and is controlled by immune surveillance. Thus, HHV-6 cannot affect neither donor nor recipient hematopoietic stem cells anymore. It could be further speculated that OCL of recipient origin are also functional and help maintain osteoclastogenesis in the first case. Alternatively, as demonstrated in this study, 12% donor

Table 1. Peripheral blood, culture-grown macrophage and osteoclast precursor donor chimerism status and surface marker expression patterns.

Case	Post–HSCT in months	Cells	Donor Chimerism, N (%)	Flow Cytometry (MFI)	
				CD14	DR
1	3	PB-myeloid cells	Donor cells only		
		PB-lymphocytes	Donor cells only		
	13	PB-myeloid cells	43		
		PB-lymphocytes	93		
	20	Culture-grown-MΦ	26		
		Culture-grown-OCLp	26		
		PB-myeloid cells	27		
		PB-lymphocytes	93		
	22	Culture-grown-MΦ	12	99	136
		Culture-grown-OCLp	12	17	8
		PB-Myeloid cells	11		
		PB-Lymphocytes	93		
2	1	Culture-grown-MΦ	100	153	230
		Culture-grown-OCLp	100	8	17
		PB-myeloid cells	100		
		PB-lymphocytes	64		

Surface marker expression mean fluorescence intensities (MFI) evaluated by flow cytometry in culture-grown macrophages (MÞ) and osteoclast precursors (OCLp) and donor chimerism assessment done by short tandem repeat assay on peripheral blood lymphocytes and myeloid cells and MÞ and OCLp populations. HSCT: hematopoietic stem cell transplantation; mo: months; PB: peripheral blood.
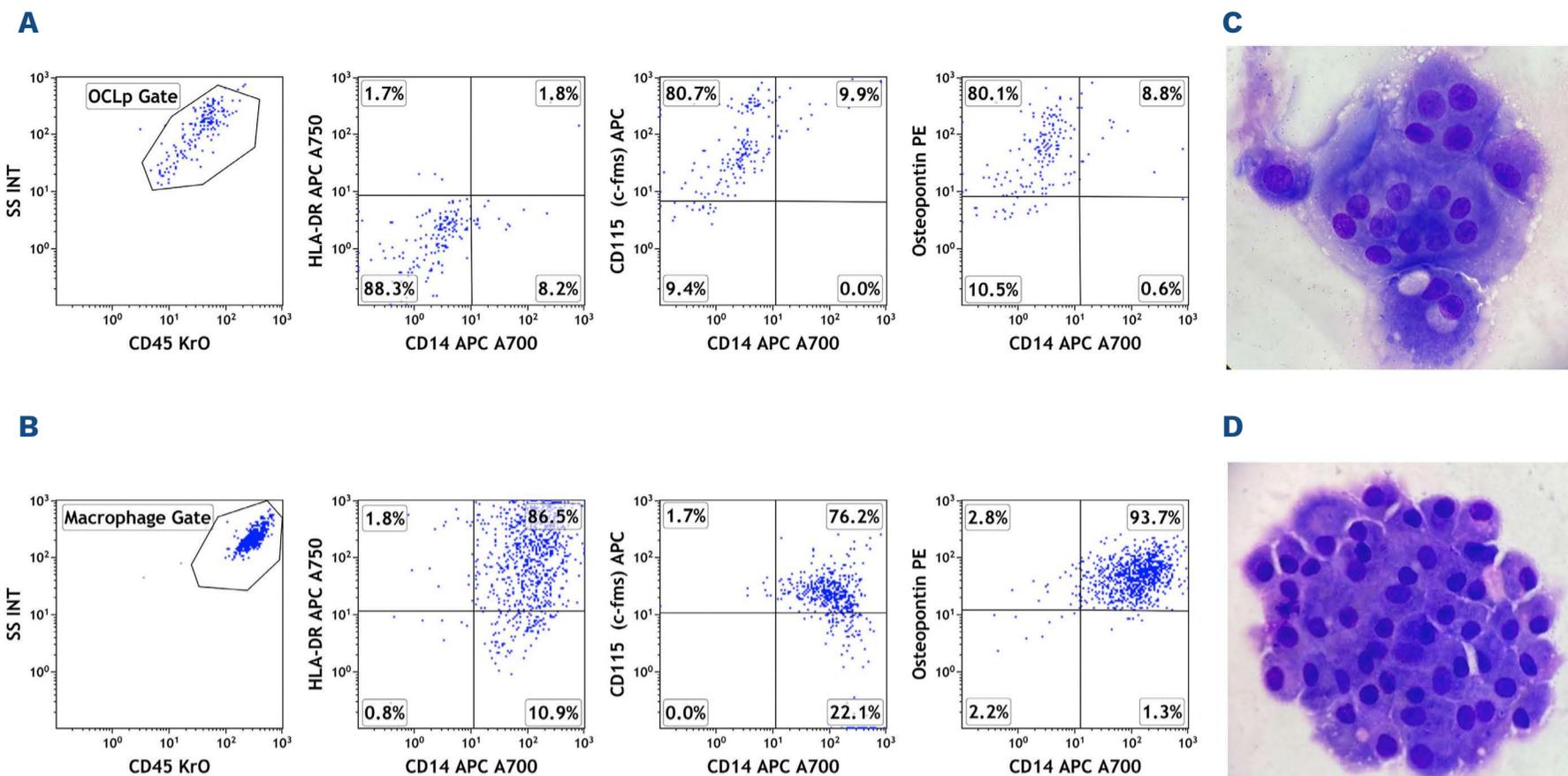
Figure 2. Immunophenotypic and morphologic characterization of culture-grown osteoclast precursors and macrophages. (A) Representative flow cytometric dot plots of culture-grown and isolated by magnetic-activated cell sorting osteoclast precursors (OCLp) characterized by CD14-negative/DR-negative staining and CD115 and osteopontin expression in the first case. (B) Representative flow cytometric dot plots of culture-grown CD14-positive/DR-positive macrophage (MΦ) with expression of CD115 and osteopontin in the second case. (C) Wright-Giemsa-stained cytospin slide image of isolated CD14-negative/DR-negative cells showing predominantly OCLp characterized by their large size and multi-nucleation on day 14 of culturing (100x). (D) Wright-Giemsa-stained cytospin slide image of isolated CD14-positive/DR-positive cells showing predominantly MΦ characterized by their comparatively smaller size and single-nucleation on day 13 of culturing (100x).

myeloid cells are capable of producing sufficient number of donor-derived OCL maintaining proper OCL function and bone health for this patient.

We were able to demonstrate *in vitro* development of OCLp as well as young and mature OCL from peripheral blood monocytes supported by morphology and confirmed by CD14−/DR−/CD45+/CD51/61+/CD115+/calcitonin receptor+/CD265+/TRAP+ OCLp phenotype using flow cytometry in these two allo HSCT recipients with OP. Continued monitoring of donor myeloid chimerism might shed further light onto discovering the minimum required donor myeloid cell presence to maintain osteoclastogenesis in allo HSCT recipients with OP. Since we could not study multinucleated mature OCL due to their size by flow cytometry and perform STR analysis, we cannot comment on the potential occurrence of gene transfer from donor monocytes into OCL as elegantly demonstrated in mice by Jacome-Galarza CE *et al.*[6] The approach used in this report may be tested further in future studies.

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Disclosures
No conflicts of interest to disclose.

Contributions
SS designed the project and experiments, obtained cytospin images,

Data-sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request

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