Indian J Med Res 135, June 2012, pp 853-872

A novel autologous stem cell procedure for the treatment of aplastic anaemia using reprogrammed mature adult cells: a pilot study

Ilham Saleh Abuljadayel, Dipika Mohanty[*] & Rajendar K. Suri[**,+]

TriStem UK Limited, London, England, [*]National Institute of Immunohaematology (ICMR), Mumbai & [**]GG Haemhealth India, New Delhi, India

Received July 8, 2010

***Background & objectives*: Aplastic anaemia is a life threatening rare bone marrow failure disorder. The underlying haematopoietic cellular deficit leads to haemorrhage, infection and severe anaemia. The treatment of choice for this haematological condition is allogeneic bone marrow transplantation from fully matched HLA sibling. Though this procedure is curative in the majority of young patients with aplastic anaemia, extending this benefit to older patients or those lacking a family donor remains a major challenge. Herein, the safety and efficacy of infusing autologous retrodifferentiated haematopoietic stem cells (RHSC) into four patients with aplastic anaemia without the use of any pre- or post-conditioning regimen including immunosuppression is described.**

***Methods*: Un-mobilized, mononuclear cells were harvested from four patients with acquired aplastic anaemia by aphaeresis. Mononuclear cells of patients were cultured with purified monoclonal antibody against the monomorphic regions of the beta chain of MHC class II antigens (Clone CR3/43) for 3 h, to obtain autologous RHSC. Autologous RHSC were washed and infused into the four patients without the use of any pre- or post-conditioning regimen. Thereafter, the efficacy (engraftment) of autologous RHSC was assessed in these patients.**

***Results*: Following single infusion of the autologous RHSC, two of the four patients with aplastic anaemia become transfusion independent for more than seven years. Karyotyping and G-banding analysis prior and post-procedure in all patients remained the same.**

***Interpretation & conclusions*: The findings of this pilot study demonstrated the functional utility of reprogrammed fully differentiated adult cells into pluripotent stem cells with extensive repopulation potentials in a human setting and without any pre- or post-conditioning regimen, including immunosuppression. This autologous approach of stem cell creation may broaden the curative potentials of stem cell therapy to a wider population of patients with aplastic anaemia, including many patients suffering from other haematological and non-haematological disorders.**

Key words Aplastic anaemia - autologous stem cells - induced pluripotent stem cells - leukocytes - reprogrammed mature adult cells - retrodifferentiation

[+]*Present address*: 177, Sector 7, Panchkula 134 109, Haryana, India

Aplastic anaemia[1] is a rare, albeit, fatal bone marrow failure disorder, more common in developing countries than the Western world[2]. The hallmark of this haematological condition is pancytopenia and hypocellular bone marrow. Earlier a variety of ill defined causative agents were suggested to be responsible for the acquired form of the disease ranging from viruses to chemical toxins including antibiotics and radiation. However, with advances in cell biology and immunology increasing evidences suggest immune-mediate pathophysiology. Clinical and laboratory studies suggest that most aplastic anaemia is secondary to immunologically mediated destruction of haemopoietic cells by cytotoxic lymphocytes (CTL) and their cytokine products[3,4] . Immunosuppression with antithymocyte globulin (ATG) and cyclosporine is effective in restoring blood cell counts in the majority of patients[5], but relapse with evolution of clonal haematopoietic cells including renal failure and opportunistic infections remain a serious drawback[6,7].

Undoubtedly, the majority of young aplastic anaemia patients can be cured with stem cell transplantation (SCT) obtained from HLA-matched siblings[8], though, extending this approach to older patients or those who lack family donors remains a challenge. Despite the reasonable survival rate after HLA-matched allogeneic SCT, the procedure carries some potentials risks due to the immunosuppressive regimen used to prevent graft versus host disease (GVDH). For example, high dose cyclophosphamide with or without ATG leads to prolonged period of immunosuppression and predisposes the patient to opportunistic infections. Other potential risk is graft failure which may ensue weeks or months after SCT[9,10]. Moreover, the risk of graft failure increases with the number of blood transfusions received prior to SCT.

Syngeneic bone marrow transplant is an ideal though, a rare option during which reduction in treatment related mortality and improvement in overall survival rate are achieved when compared to transplantation from HLA-identical sibling, though relapse rate is comparable[11,12]. Alternatively, somatic cell reprogramming to a pluripotent haematopoietic stem cell states in mature adult cells such as leukocytes[13-16] offers an attractive option to treat a variety of congenital and acquired haematological diseases in an autologous or allogeneic setting, respectively. In this protocol the conversion process is 100 per cent efficient and results in the production of unprecedented levels of pluripotent stem cells[13] capable of xenogeneic engraftment[14]. This process which has been termed retrodifferentiation, induces somatic cell reprogramming in leucocytes, resulting into a variety of pluripotent stem cell classes, in response to ligation of the monomorphic region of the beta-chain of MHC class II antigens. Simply, the conversion or the retrodifferentiation process is achieved by exposure of leukocytes to a specific culture condition containing clone CR3/43 monoclonal antibody without the insertion of any potentially hazardous genetic modifiers, such as those used to generate induced pluripotent stem cells (IPS)[17]. The haematopoietic inductive culture condition in the retrodifferentiation protocol leads to the generation of large quantities of pluripotent stem cells from the blood of either patients or HLA-matched donor. This safe approach, may allow the escalation of immunosuppression or cytoreductive therapy combination to achieve faster recovery of the haematopoietic system. In addition, speedy autologous engraftment leads to increased survival and unprecedented cure rates due to abrogation or amelioration of GVHD, sometime encountered even following syngeneic bone marrow transplant[18].

Here we describe in this pilot study the efficacy of a single infusion of 3 h autologous retrodifferentiated haematopoietic stem cells (RHSC) following exposure to haematopoietic inductive culture condition into patients with acquired aplastic anaemia without any pre- or post-conditioning including immunosuppression regimen.

Material & Methods

Patients: The protocol of this pilot clinical study was approved by the ethical committee of the King Edward Memorial Hospital, Mumbai, and was performed in joint collaboration with the Institute of Immunohaematology [IIH, now National Institute of Immunohaematology, NIIH, (ICMR), Mumbai]. Following counselling, fulfilling the clinical criteria (Table I) and signing the consent form provided by the ICMR/NIIH, four (3 males & 1 female) patients with severe and hypo-plastic anaemia, respectively, were enrolled into the study during May 2004. These four patients were selected and monitored by NIIH/KEM staff. Patient's clinical details, treatment history and CD34+ cells infusion dosage are given in Table II. All patients were negative for paroxysmal nocturnal haemoglobinuria (PNH) as determined by CD59 expression using flow cytometry and Ham's test[19].

Aphaeresis and retrodifferentiation procedure: Following obtaining consent and subsequently prior

Table I. Patient inclusion criteria

1. Absolute neutrophils count < $0.5X10^9$ /l
2. Platelet count <$20X10^9$ /l
3. Anaemia with corrected reticulocyte <1%
And at least one of the following
4. Bone marrow cellularity <25%
5. Bone marrow cellularity <50% with fewer than 30% haematopoietic cells

aphaeresis patients were transfused with 2 units of irradiated packed red blood cells and 4 units platelets to maintain their haemoglobin level above 8 g/dl and platelets counts above 50 x 10^9/l. Apheresis involved jugular and anticubetal venous catheterization with single lumen catheter for venous access. Patient were apheresed by processing 2-3 times their total blood volume using the Cobe Spectra aphaeresis machine and the white blood cells separation kit (both from Gambro BCT, USA). It should be noted that in all patients aphaeresis was performed in the

Table II. Clinical and treatment history of aplastic anaemia patients up to autologous RHSC infusion including number of CD34 cells infused

Patient	Clinical history up to RSC infusion	Treatment up to RSC infusion	CD34+ cells received/ kg,bw
No.1 25 yr male Severe aplastic anaemia Wt: 48 kg Ht: 152 cm DOI:6/7/04	Diagnosed with SAA in 2002. Presented with symptoms of weakness and dysponoea. Frequent episodes of rectal and gum bleeding and vomiting. Receives 4 and 2 units of blood and platelets respectively, every month. Appear jaundiced with severe eye congestion	Received a trial of anabolic steroid (Tab Menabol) for 8 months without any significant improvement	$11.7x10^6$
No.2 26 yr female Hypoplastic anaemia Wt: 38 kg Ht: 153 cm DOI: 14/7/04	Diagnosed with hypopalstic anaemia in January 2004. Presented with symptoms of polymenorrhagia of 6 months duration. Only prior RSC infusion patients received 4 and 6 units, respectively of packed red blood cells and platelets, respectively	Received haematics since 3 months prior infusion of RSC with no response	$20x10^6$
No.3 19 yr male Very severe aplastic anaemia Wt: 52 kg Ht: 166 cm DOI: 27/7/04	Diagnosed with very severe aplastic anaemia in 2003. Presented with anaemia, weakness, dysponoea on exertion. Fever with chills lasting 10-15 days due to severe neutropenia. Multiple episodes of infections, vomiting and purpuric spots. Gluteal abscess. Fainted twice due to brain haemorrhage. Receives 5 and 2 units of blood and platelets, respectively every 2 wk	Received cyclosporine therapy for 6 months which ended in March 2003 with no effect	$25x10^6$
No.4 35 yr male Very severe aplastic anaemia Wt: 52.5 kg Ht: 160 cm DOI: 17/8/04	Anaemia, weakness, dysponoea. Fever with chills due to severe neutropenia and multiple episodes of infections and bleeding. Diagnosed 3 yr ago. Receives 4 and 2 units of blood and platelets, respectively every month	Received cyclosporine and anti-tuberculosis therapies. Did not respond to 6 months of immunosupression	$23x10^6$

absence of administering any growth factor. Prior the retrodifferentiation procedure an aliquot of cells was collect aseptically for CD34 analysis following collection of 150-200 ml of buffy coat. Thereafter, the buffy coat was subjected to retrodifferentiation under haematopoietic inductive culture condition as described earlier[13]. Briefly, the procedure involved the addition of 1000 µg of purified CR3/43 (specially prepared by DakoCytomation, Denmark, for TriStem Corp) diluted in 30 ml of Iscove's modified Dulbecco's medium (GIBCO, UK), aseptically into the white blood cell bag. The bag was then incubated in a sterile tissue culture incubator maintained at 37°C and 5 per cent CO_2 for 3 h. A three hour incubation period has been demonstrated to be sufficient to make haematopoietic stem cells with long term repopulation potential[14,15]. Following completion of the retrodifferentiation reaction the converted cells were analysed for CD34+ cell content. Thereafter, cells were washed twice with saline solution using the Cobe cell processor 2991 (USA). Upon agitation and re-suspension in saline solution, the cell suspension was infused into the patients via the jugular vein under gravity using an infusion set. Vital signs including CBC counts of patients were continuously monitored before and following infusion of the autologous retrodifferentiated cells.

Clinical monitoring of patients: The clinical monitoring was carried out by the NIIH/KEM staff. Transfusion requirement was determined prior and post-infusion from transfusion records obtained following receiving any unit of blood product. The transfusions were given after getting consent from patients and immediate relatives as witness. Prior transfusing, all blood products were irradiated at the Tata Memorial Hospital, Mumbai. Patients were also asked questions regarding their well being prior and post-infusion of the retrodifferentiated stem cells. All patients carried a copy or original records of their conditions and all laboratory and clinical follow up. The duration of monitoring of these patients was planned initially for 2 years but extended it further. For the first month post-infusion patients were kept in hospital in sterile positive pressure Hepa filter rooms.

Immunophenotyping of apheresed buffy coat prior and post the retrodifferentiation process, including bone marrow and peripheral blood samples of patients: One million cells were stained according to the manufacturer's instructions with the following panels of monoclonal antibodies (all from DakoCytomation, Denmark). Qualitative cell viability was assessed using trypan blue and a haemocytometer[20].

Panel 1 consisted of isotype negative control IgG1-FITC, IgG1-PE-Cy5 and IgG1-RPE conjugates

Panel 2 consisted of anti-human CD45-FITC and CD34-RPE-Cy5

Panel 3 consisted of anti-human CD38-FITC and CD34-RPE-Cy5

Panel 4 consisted of CD61-FITC and CD34-RPE-Cy5

Panel 5 consisted of CD33/13 RPE and CD7-FITC

Cell analysis was performed with a FACSCalibur system (BD Bioscience, USA) using the BD cell Quest software and the FCS Express 4 Flow, Denovo software, USA.

Clonal analysis: For clonal assay, bone marrow mononuclear cells (MNC) of patient prior and post-infusion of the retrodifferentiated stem cells were seeded into methocult GFH4434 supplemented with recombinant growth factors according to the manufacturer's instructions (Stem Cell Technologies, Mumbai, India). Differentiation into haematopoietic cell colonies was assessed and scored with time using phase contrast inverted microscope (Sony, Japan).

Complete blood counts (CBC) including white blood cell (WBC) differentials, liver enzymes and haemoglobin variants: Patients' CBC, liver enzymes and haemoglobin variants were continuously monitored before and post-procedure. Following discharge from hospital, patients' CBC, liver enzymes haemoglobin variants and peripheral blood karyotyping and G banding were also monitored by an independent laboratory (Super Religare Laboratories, Mumbai, India) for reconfirmation purposes. These tests were performed frequently following infusion of the autologous retrodifferentiated stem cells.

Karyotyping and G-banding: Peripheral blood samples and bone marrow cells were analyzed before and following infusion of the autologous retrodifferentiated cells. This test was repeated on a six monthly basis for the first year and on a yearly basis following two years post initiation of autologous retrodifferentiated stem cell therapy. Also retrodifferentiated cells were analyzed prior to infusion to look into the stability of the cells, which was also performed following the 3 h conversion step as well as post-establishment of a maximum one month long term culture of the converted cells. Karyotyping and G banding was also monitored by a third independent laboratory (Super Religare Laboratories, India).

Bone marrow smears and trephine sections analysis: Bone marrow smears and trephine section were performed before and post-infusion of the autologous retrodifferentiated stem cells at different intervals. This test was performed 14-20 days post-infusion of the RHSC and thereafter on a yearly basis. All smear and trephine sections were scanned using a microscope hooked to a camcorder (Sony, Japan) before and following infusion of the RHSC to assess and keep record of engraftment.

Results

All four patients tolerated the aphaeresis and the single RHSC infusion procedure with no adverse event. Patients 1 and 4 became transfusion independent for nearly seven years now, post-single infusion of RHSC (Table III). In these two patients platelets and neutrophils counts including haemoglobin concentration increased with time over a period of nearly seven years and in the absence of any medication given.

Table III. Neutrophils and platelets counts including total and foetal haemoglobin concentrations with liver enzymes in patients with aplastic anaemia prior and post-infusion of RHSC

Blood test	Date of blood test					
Patient no.1	7/6/2004 Nadir prior infusion	19/7/2004 Post-infusion	26/5/2005 Post-infusion	7/3/2006 Post-infusion	5/1/2008 Post-infusion	17/5/2010 Post-infusion
HB g/dl	2.6	7.1	9	12	14	14.1
Platelets 10^3/µl	18	26	37	58	189	198
Neutrophils 10^3/µl	0.32	1.000	1.054	1.184	1.994	2.385
HBF g/dl	ND	1.2	2.4	2.6	1.82	1.17
SGOT U/l	150	127	74	32	30	33
SGPT U/l	145	181	49	37	44	51
Patient no.2	8/6/2004 Nadir prior infusion	15/7/2004 Post-infusion	21/7/2004 Post-infusion	26/7/2004 Post-infusion	3/8/2004 Post-infusion	19/8/2004 Post-infusion
HB g/dl	6	9.5	10.4	11.4	9.1	6.6
Platelets 10^3/µl	10	65	27	29	36	10
Neutrophils 10^3/µl	1.4	5.7	2.6	2.1	2.3	1.7
HBF g/dl	ND	ND	3.9	4	ND	ND
Patient no.3	13/7/2004 Nadir prior infusion	28/7/2004 Post-infusion	30/7/2004 Post-infusion	2/8/2004 Post-infusion	10/8/2004 Post-infusion	16/8/2004 Post-infusion
HB g/dl	3.6	6	5.6	7.2	8.2	8.5
Platelets 10^3/µl	5	74	44	28	14	18
Neutrophils 10^3/µl	0.12	0.3	0.3	0.2	0.3	0.5
HBF g/dl	0	ND	ND	ND	ND	0.2
Patient no. 4	16/7/2004 Nadir prior infusion	31/8/2004 Post-infusion	30/5/2005 Post-infusion	7/3/2006 Post-infusion	5/1/2008 Post- infusion	6/7/2010 Post-infusion
HB g/dl	3	11.1	7.7	11.5	13	13.9
Platelets 10^3/µl	5	30	20	25	68	93
Neutrophils 10^3/µl	0.1	0.72	0.50	1.0	1.24	1.785
HBF g/dl	0.11	0.2	0.38	0.9	0.42	0.42
SGOT U/l	160	46	46	69	34	27
SGPT U/l	150	57	32	60	46	55

HBF, foetal haemoglobin; SGOT, serum glutamic-oxaloacetic transminase; SGPT, serum glutamic-pyruvic transminase

Patients 2 and 3 remained transfusion independent for 36 and 21 days, respectively, post-infusion of RHSC (Table III). On day 36, haemoglobin count of patient no.2 dropped due to menorrhagia. Subsequently, this patient was started on hormonal replacement therapy and became transfusion dependent. Patient no.3 became transfusion dependent on day 21 post-infusion of HRSC. On day 36, this patient was admitted with high grade fever, mucosal bleeding and purpura. Patient was treated with antibiotics (Inj Augmentin, Inj Ceftazidime and Inj Amikacin) along with whole blood and platelets transfusion. Patient nos.2 and 3 died 2 years and six months post-infusion of RHSC, respectively**.** Sustained foetal haemoglobin switching was noted only in patient 1 and 4 (Table III). Prior infusion, liver enzymes were elevated in patient nos.1 and 4 despite being negative for HCV (by ELIZA). Liver enzymes started to normalize post-infusion of RHSC and reached normal levels four years post-infusion of RHSC. The two patients who exhibited persistent Hb F switching showed long term engraftment post single infusion of the autologous RHSC now approaching seven years. Further, patients 1 and 4 did not receive any medication post-infusion of the RHSC and are doing well and working.

Immunophenotyping of apheresed mononuclear cells prior and 3 h post-induction of retrodifferentiation: Flow cytometry of apheresed mononuclear cells (MNC) before and 3 h post-induction of haematopoietic retrodifferentiation in the four patients are shown in Figs 1, 2, 3 and 4a. The number of CD34 positive cell generated post haematopoietic retrodifferentiation is listed in Table II. Flow cytometry of apheresed mononuclear before and post-haematopoietic retrodifferentiation showed an increase in the number of CD34 positive cells with and without expression of CD45, CD38 and CD61 (Figs 1, 2, 3 and 4a). Upon retrodifferentiation the number of primitive haematopoietic stem cells, CD34+CD38-, increased in treated MNC samples of patients 1, 2 and 3 only. On the other hand, the number of CD34+CD45- cells only increased in treated MNC samples of patients 1 and 4. B lymphocytes count as marked by CD19 expression fell in treated MNC samples of the four patients (Figs 1 and 2 a and 3 and 4b). This decrease was always accompanied by an increase in the number of myeloid progenitors co-expressing CD33&13 with CD7 or CD34 with CD61 (Figs 1 and 2a and 3 and 4b).

Interestingly, in RHSC the highest CD34 count was obtained when measured with conjugated monoclonal antibody panel CD34 and CD61 in all treated MNC of the four patients, when compared to measurement of CD34 count using panel CD45CD34 or CD34CD38 (Figs 1, 2, 3, 4a). No significant cell death occurred in all sample analysed by flow cytometry when assessed using trypan blue viability testing. However, large numbers of platelets and precursors were generated post-induction of retrodifferentiation, which was confirmed by flow cytometry and immunostaining for platelets antigen CD61 and CD34 (Fig. 5).

Immunophenotyping of peripheral blood samples of patients with acquired aplastic anaemia before and after infusion of RHSC: On infusion of the RHSC into patients, sequential peripheral blood analysis showed circulation of the CD34 cells in peripheral blood for several days post-infusion. Thereafter, differentiation into myelocyte was noted as depicted by an increase in cells expressing CD33&13 with and without CD7 and CD61, with and without CD34, having high forward and side scatter (Figs 1 b&c, 2 b&c, 3 a&b and 4 a&b). An increase in CD61 low expressing cells not in platelets gate was noted in peripheral blood sample of patients 2 and 3 (Figs 2b day 20 and 3a day 6). Myelocyte redifferentiation was observed in all patients though was not sustainable in patients 2 and 3.

Clonal analysis, bone marrow smear and trephine section: Hardly any colonies formed from patients bone marrow aspirate before infusion of the autologous RHSC following seeding into methylcellulose cell culture (Fig. 5a). Clonal assay only in patient no.2 who was suffering from hypoplastic anaemia, showed depressed haematopoiesis. However, 14 to 20 days post-infusion all bone marrow aspirates obtained from patients gave rise to normal range of a variety of haematopoietic colonies with mild elevation in the number of burst forming unit -erythroid (BFU-erythroid). Bone marrow smears and trephine sections (Fig. 6a) 14-20 days post-infusion of autologous of RHSC showed an increase in the number of myelocytes at various stages of differentiation with mature and immature megakaryocytes in all patients when compared to baseline. Erythroid hyperplasia at various stages of differentiation was also noted in all samples.

Karyotyping and G banding: No changes in Karyotyping and G banding pattern in peripheral blood or bone marrow samples observed before and after infusion (in patients 1 and 4 for up to more than 4 years) of autologous RHSC in all patients.

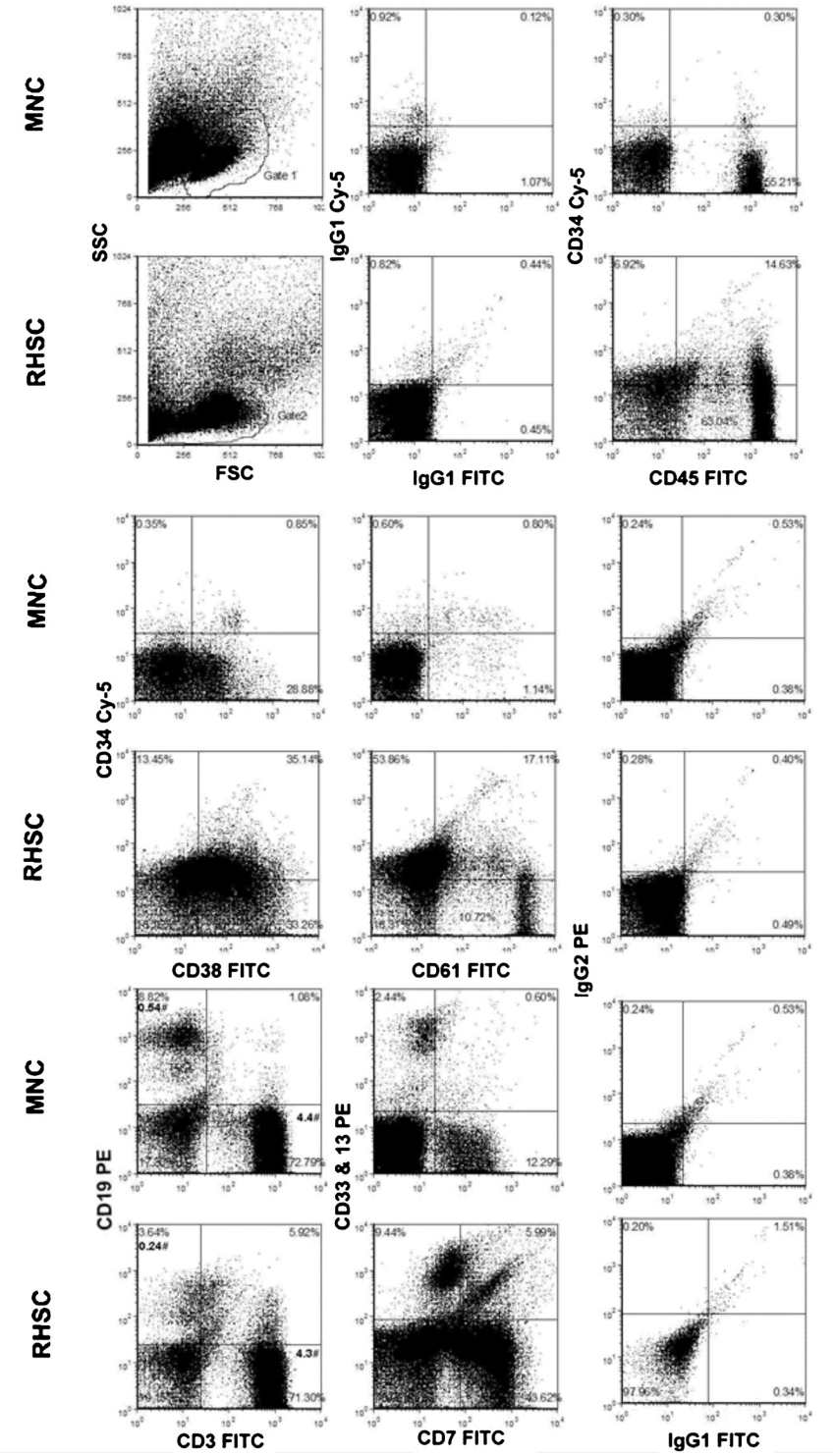

Fig. 1a. Immunophenotyping of aphaeresed mononuclear cells of patient no.1 with very severe aplastic anaemia before (MNC) and after induction of retrodifferentiation (RHSC). Cells were labelled with monoclonal antibodies conjugated to RPE Cy-5 or PE (vertical legends) for IgG1 isotype control, IgG2 isotype control, CD34. CD 19, CD33 & 13. Cells were also stained for CD45, CD38, CD61, CD7, CD3 and IgG1 isotype control monoclonal antibodies conjugated to FITC (horizontal legends). Forward and side scatter are also shown (top left plots). Absolute B and T lymphocyte counts (10^6/ml) are shown and denoted as # on dot plots.

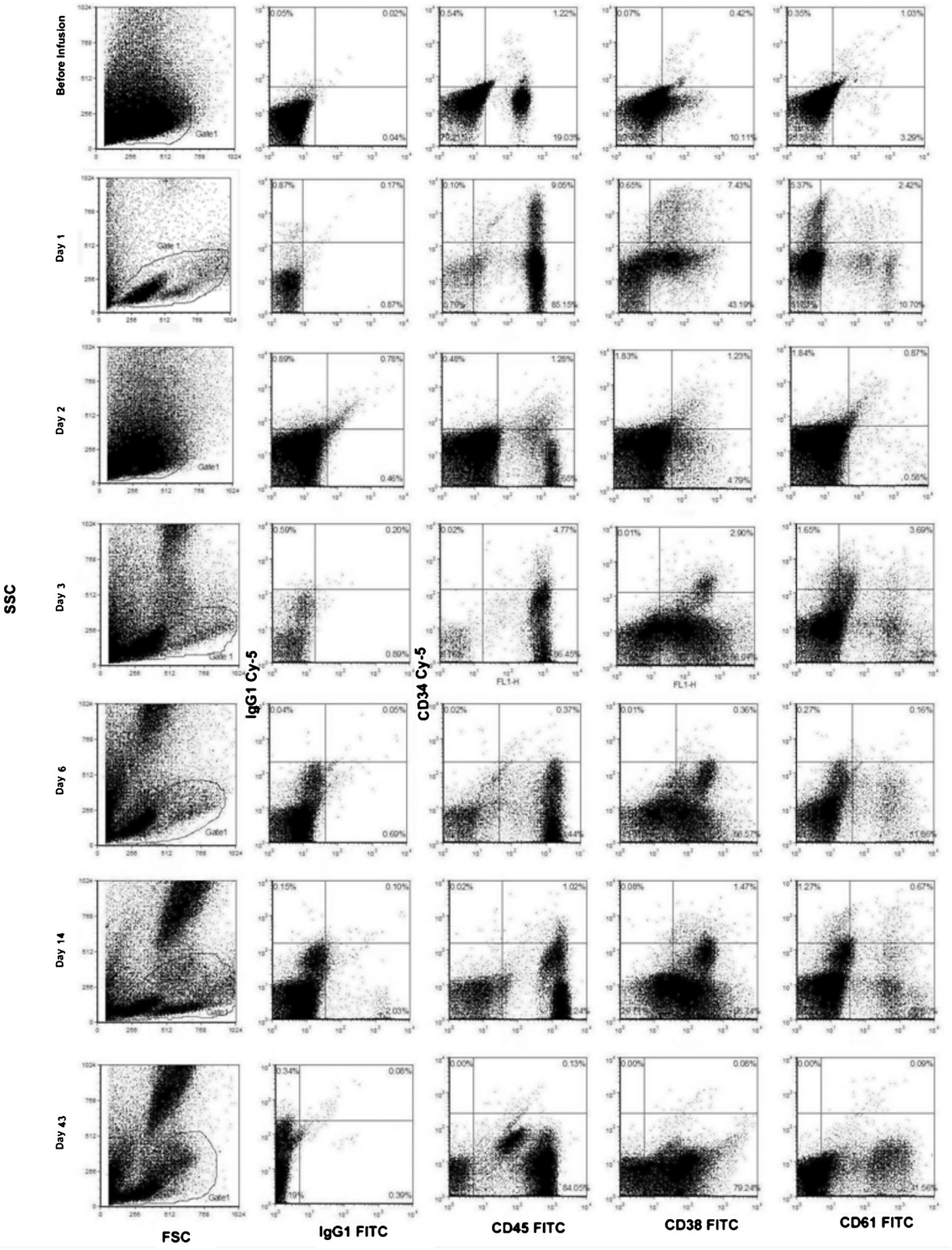

Fig. 1b. Sequential immunophenotyping of peripheral blood samples of patient no.1 with very severe aplastic anaemia before (before infusion) and following infusion of autologous HRSC (post days 1, 2, 3, 6 14 and 43, horizontal). Cells were labelled with monoclonal antibodies against CD34, IgG1 isotype control (vertical legends) and CD45, CD38, CD61 and IgG1 isotype control (horizontal legends). The left vertical panel represents forward and side scatter.

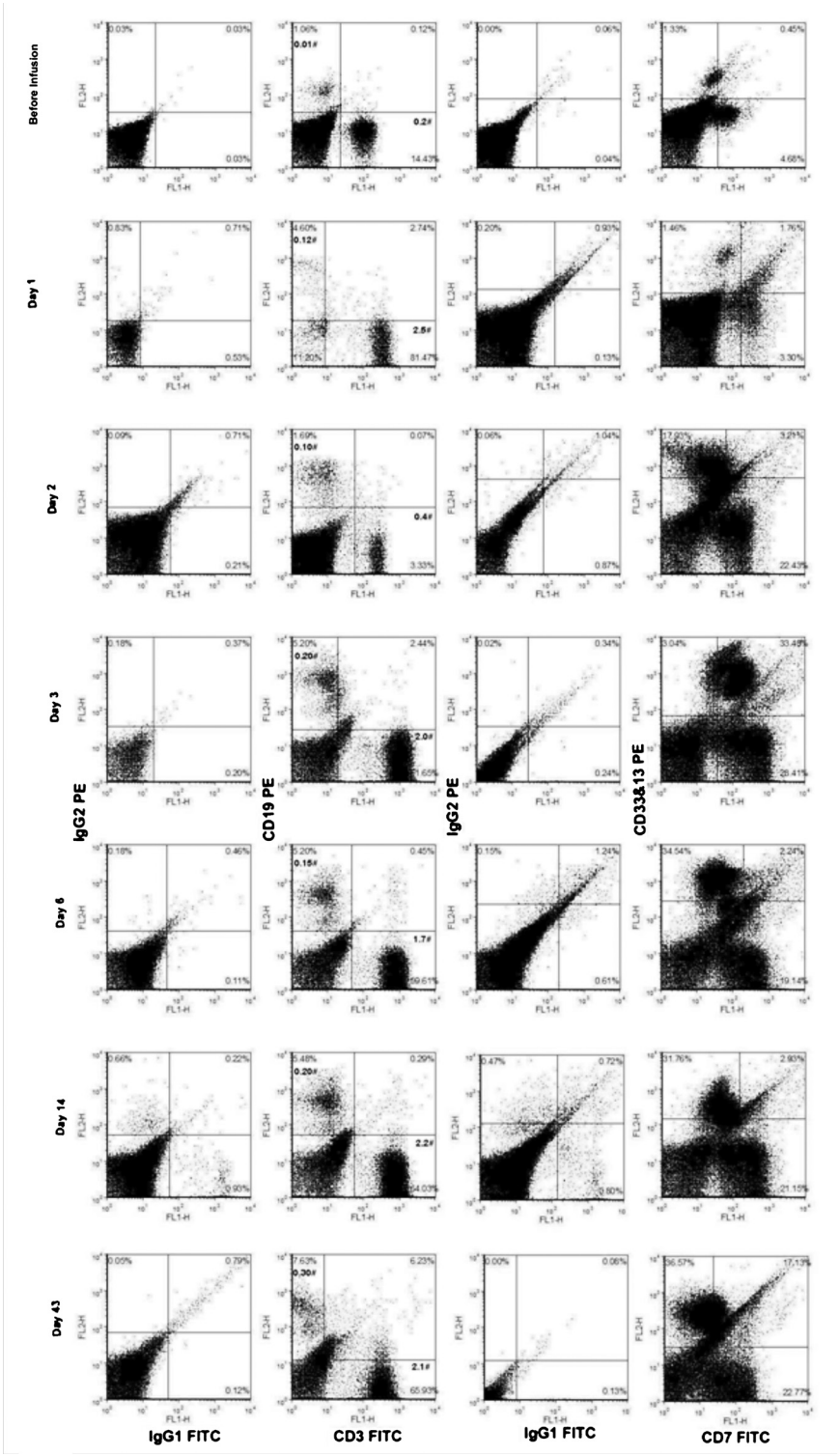

Fig. 1c. Sequential immunophenotyping of peripheral blood samples of patient no.1 with very severe aplastic anaemia before (before infusion) and following infusion of autologous RHSC (post days 1, 2, 3, 6, 14 and 43 post-infusion, horizontal). Cells were labelled with monoclonal antibodies IgG1 isotype control, CD3 and CD7 (horizontal legends) and CD 19, IgG2 isotype control and CD33 & 13 and CD7 (vertical legends). Absolute B and T lymphocyte counts (10^6/ml) are shown and denoted as # on dot plots.

Discussion

Acquired (idiopathic) aplastic anaemia is a rare bone marrow disorder resulting from haematopoietic cellular deficit of the stem cell compartment which increasingly appears to be immune- mediated in most cases[1,3,4]. The first line of treatment in the majority of young patients is bone marrow transplant from HLA matched sibling. The second line of treatment involves immunosuppression with or without transfusion and this only applies when bone marrow transplant technology is not available or in the absence of a suitable donor. Syngeneic hematopoietic stem cell transplant[11,12] for haematological conditions is a rare event. Somatic cell reprogramming technologies may offer alternative to the unique syngeneic stem cell setting with the added potential abrogation of syngeneic graft versus host disease.

In this pilot study, findings of a novel procedure involving infusion of autologous reprogrammed or retrodifferentiated[13-16] haematopoietic stem cells, not involving any genetic manipulation to attain pluripotency[17], in four patients with aplastic anaemia are

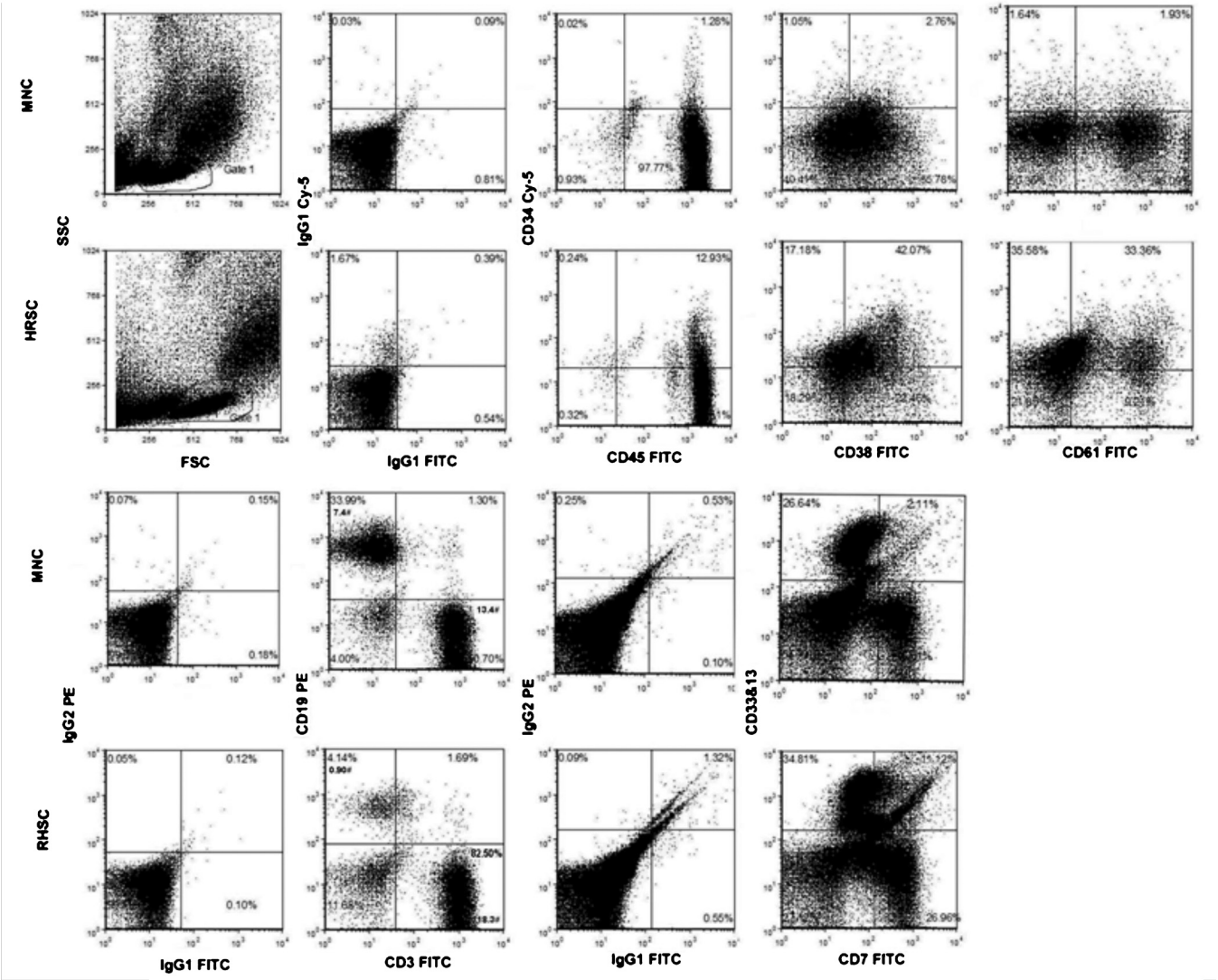

Fig. 2a. Immunophenotyping of apheresed mononuclear cells of patient no.2 with hypoplastic anaemia before (MNC) and after induction of retrodifferentiation (RHSC). Cells were labelled with monoclonal antibodies conjugated to RPE Cy-5 or PE (vertical legends) for IgG1 isotype control, IgG2 isotype control CD34. CD 19, CD33 & 13. Cells were also stained for CD45, CD38, CD61, CD7, and IgG1 isotype controls monoclonal antibodies conjugated to FITC (horizontal legends). Forward and side scatter are shown (top left plots). Absolute B and T lymphocyte counts (10^6/ml) are shown and denoted as # on dot plots.

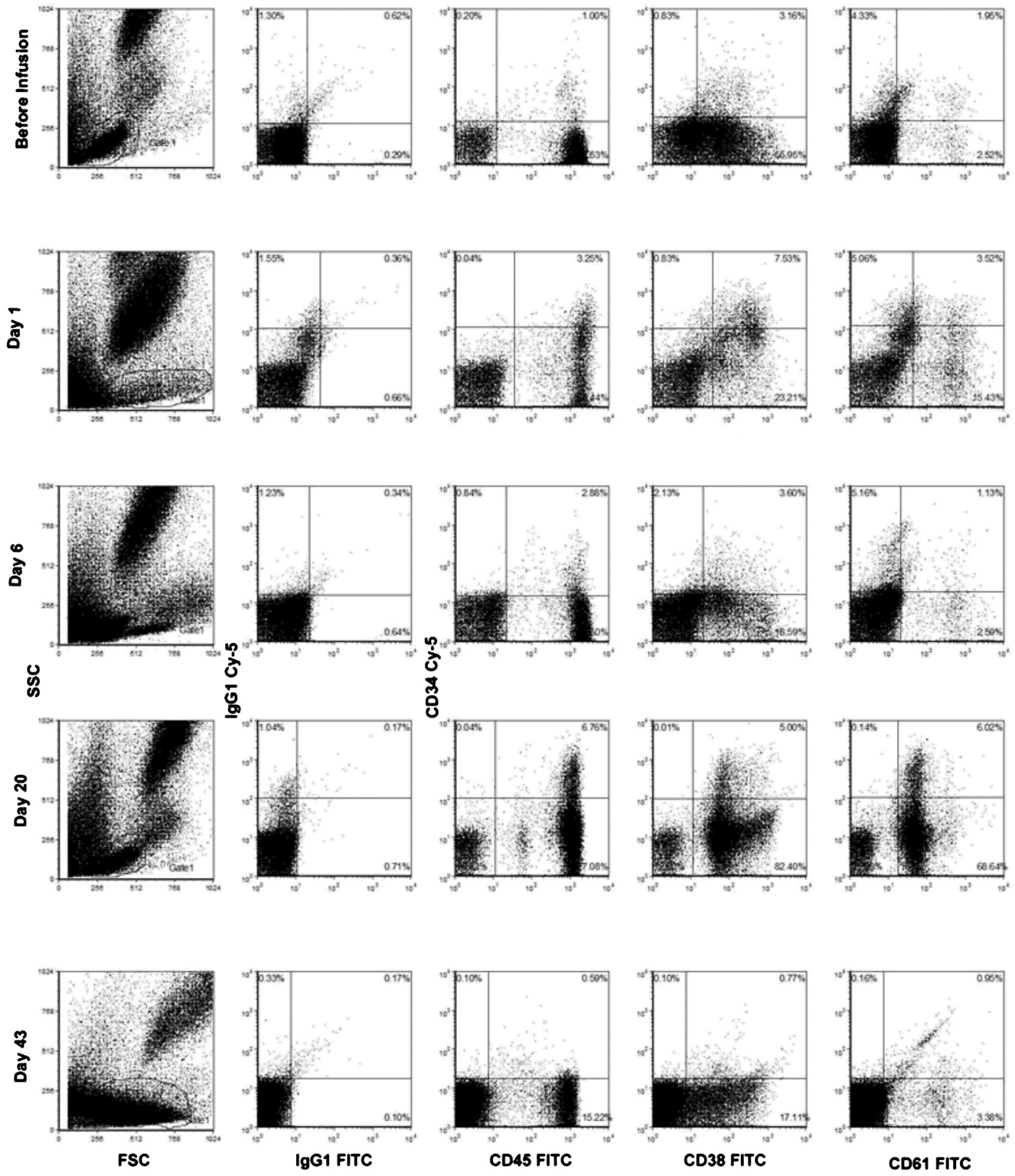

Fig. 2b. Sequential immunophenotyping of peripheral blood samples of patient no.2 with hypoplastic anaemia before (before infusion) and following infusion of autologous HRSC (post days 1, 6, 20 and 43 post-infusion, horizontal). Cells were labelled with monoclonal antibodies against CD34 IgG1 isotype control (vertical legends) and CD45, CD38, CD61 and IgG1 isotype control (horizontal legends). The left vertical panel represents forward and side scatter.

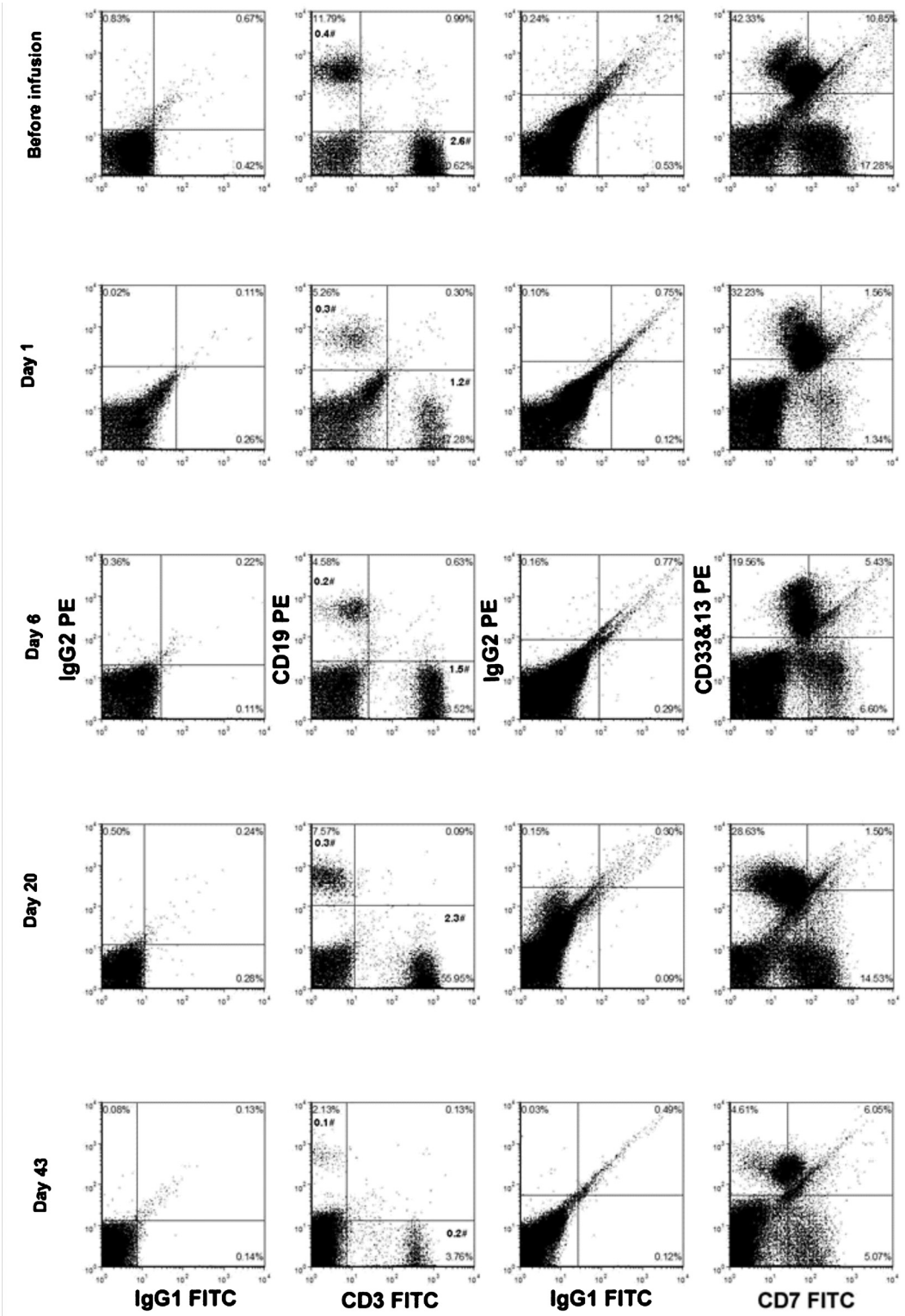

Fig. 2c. Sequential immunophenotyping of peripheral blood samples of patient no.2 with hypoplastic anaemia before (before infusion) and following infusion of autologous RHSC (post days 1, 6, 20 and 43 post-infusion, horizontal). Cells were labelled with monoclonal antibodies IgG1 isotype control, CD3 and CD7 (horizontal legends) and CD 19, IgG2 isotype control and CD33 & 13 and CD7 (vertical legends). Absolute B and T lymphocyte counts (10^6/ml) are shown and denoted as # on dot plots.

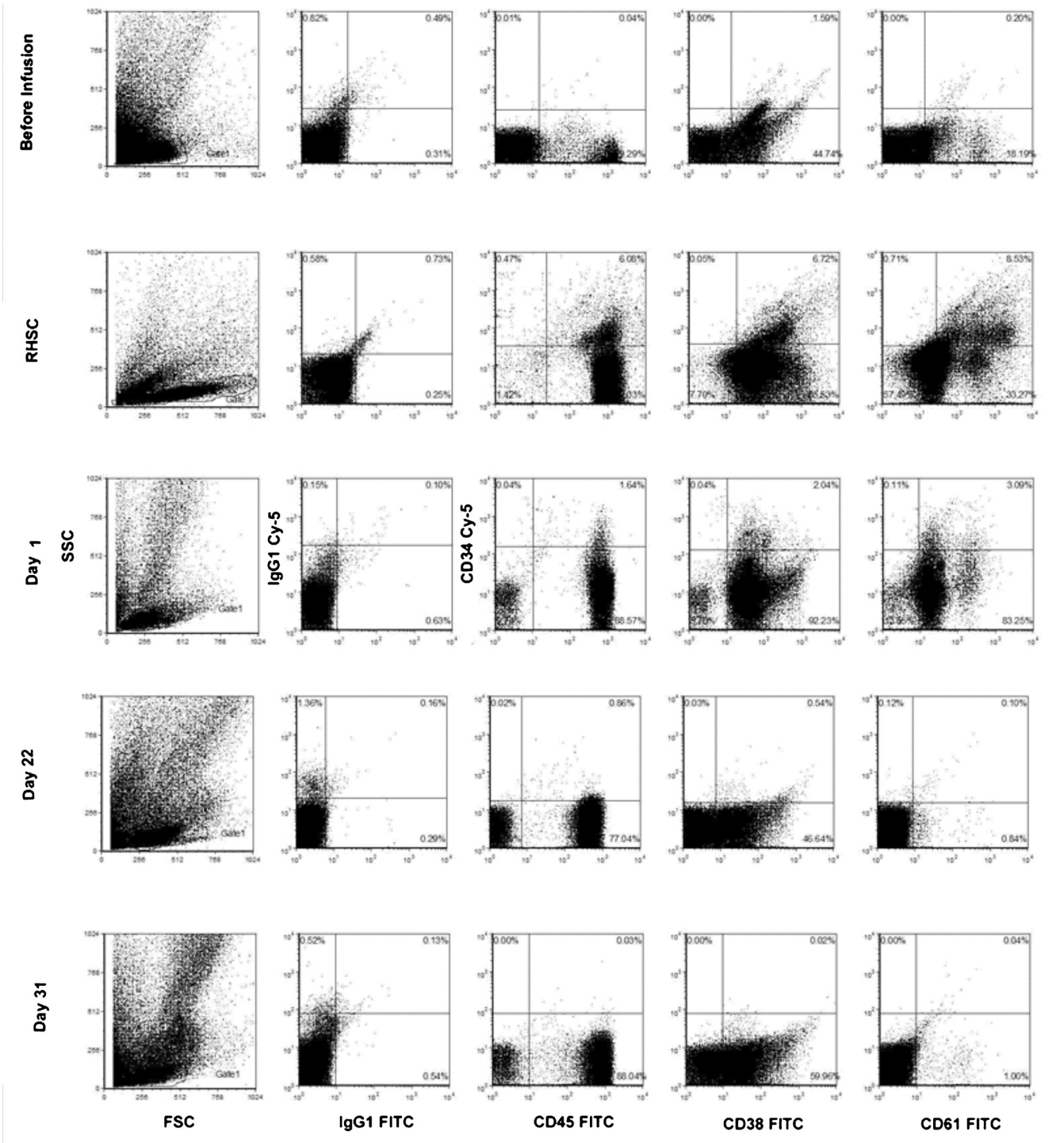

Fig. 3a. Immunophenotyping of peripheral blood samples of patient no.3 with very severe aplastic anaemia before (before infusion), after induction of retrodifferentiation in apheresed MNC (RHSC) and post-infusion of RHSC (days 6, 22 and 31 post-infusion, horizontal). Cells were labelled with monoclonal antibodies against CD34 IgG1 isotype control, (vertical legends) and CD45, CD38, CD61 and IgG1 isotype control (horizontal legends). The left vertical panel shows forward and side scatter.

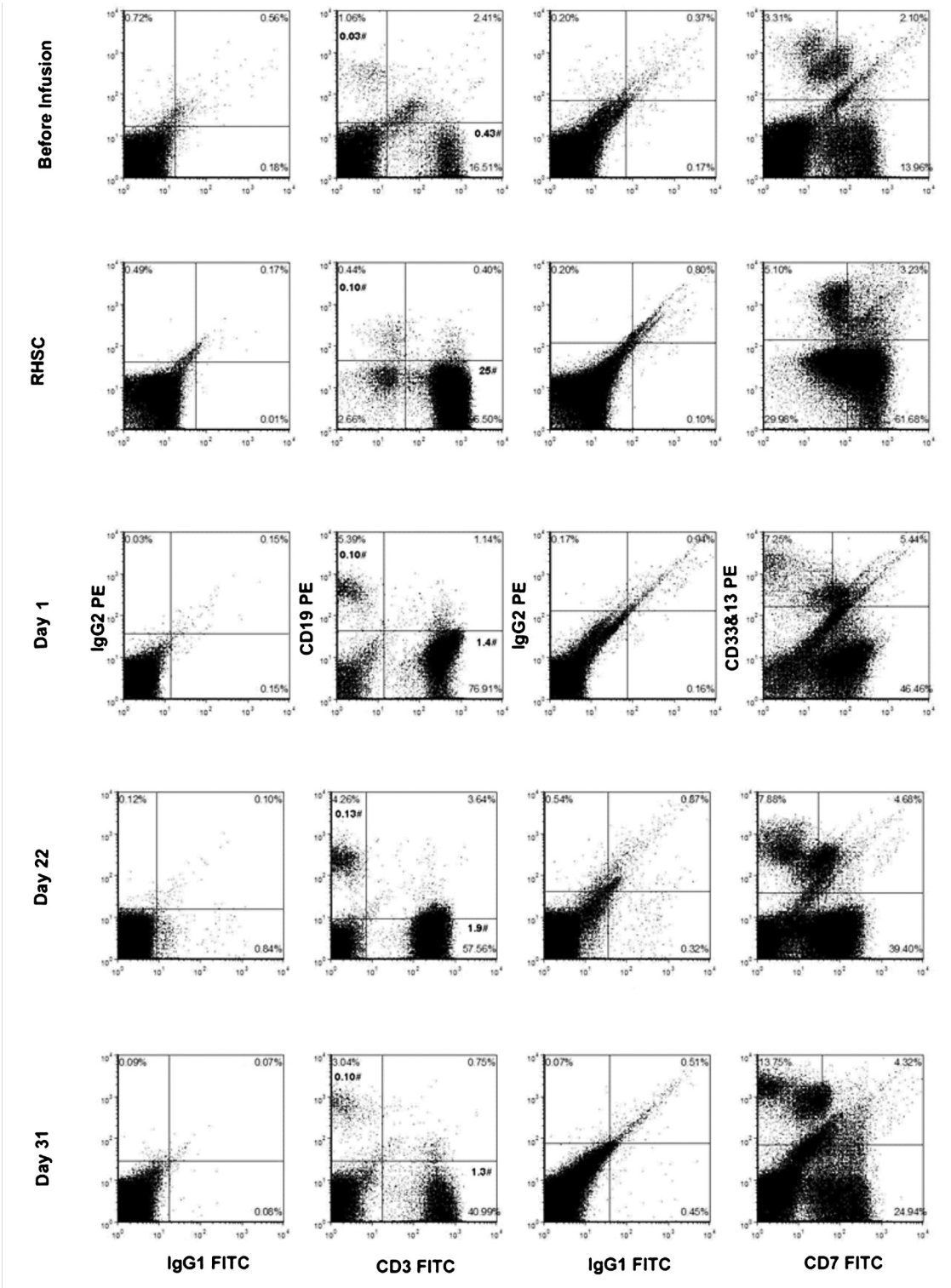

Fig. 3b. Sequential immunophenotyping of peripheral blood samples of patient no.3 with very severe aplastic anaemia before (before infusion), after induction of retrodifferentiation in apheresed MNC (RHSC) and post-infusion of RHSC (days 6, 22, 31 post-infusion, horizontal). Cells were labelled with IgG1 isotype control, CD3 and CD7 (horizontal legends) and IgG2 isotype control, CD19 and CD33 & 13 (vertical legends). Absolute B and T lymphocyte counts (10^6/ml) are shown and denoted as # on dot plots.

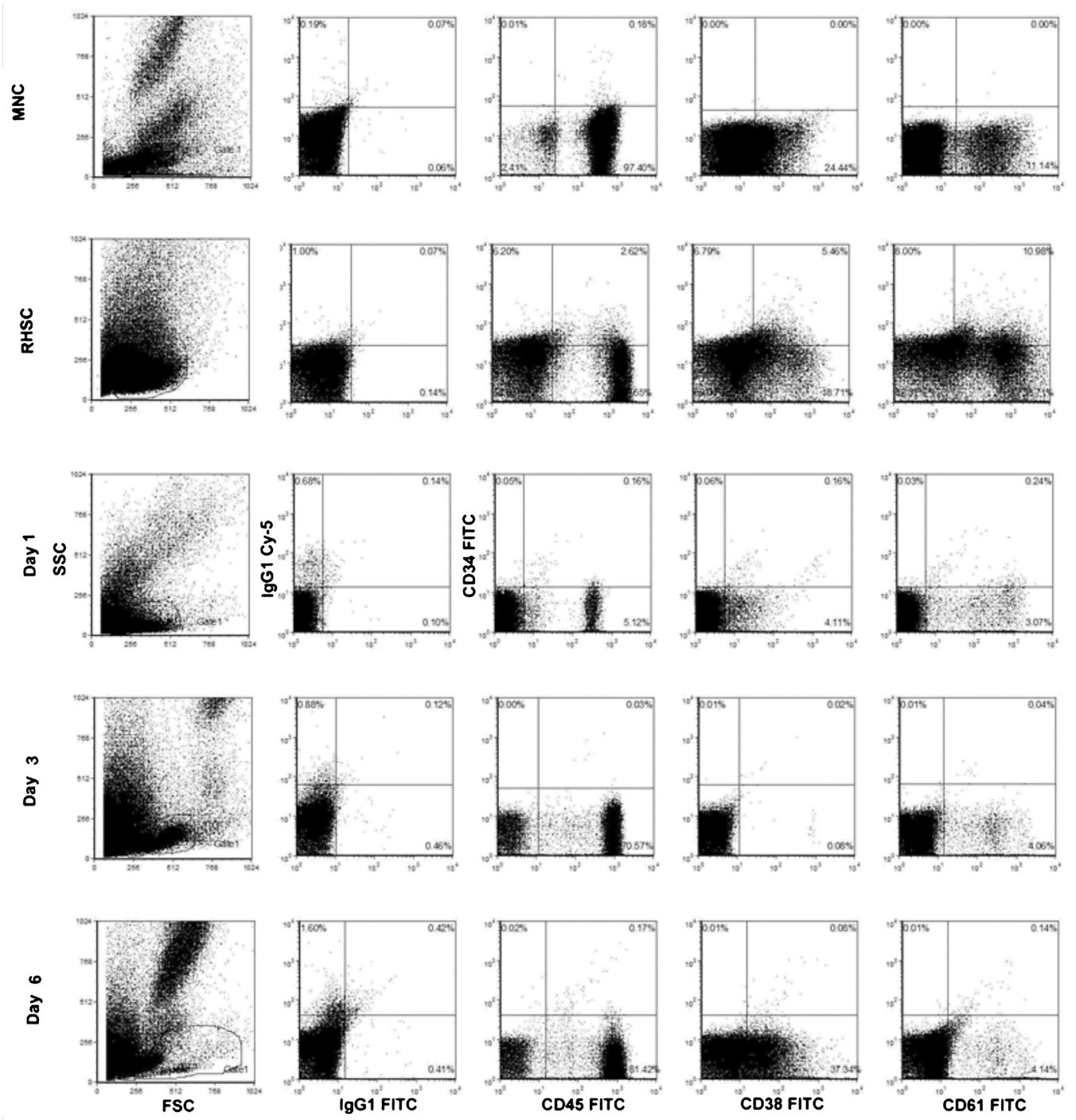

Fig. 4a. Immunophenotyping of peripheral blood sample of patient no.4 with very severe aplastic anaemia before induction of retrodifferentiation in apheresed MNC (MNC), after induction of retrodifferentiation in MNC (RHSC) and post-infusion of RHSC (days 1, 6, 14, post-infusion, horizontal). Cells were stained with IgG1 isotype control, CD45, CD38, CD61 (horizontal legends) and IgG1 isotype control and CD34 (vertical legends). Forward and side scatter are shown in left vertical panel.

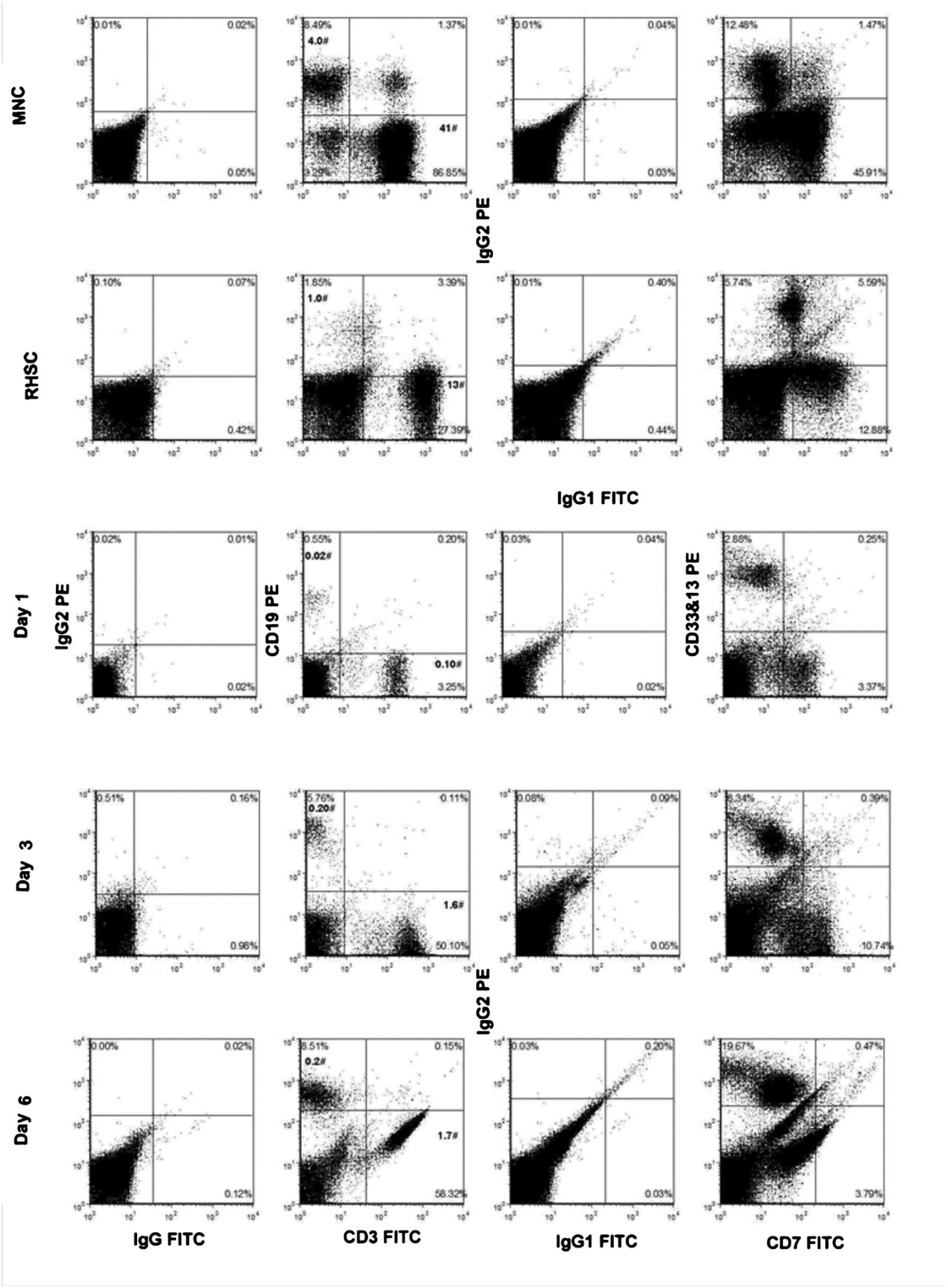

Fig. 4b. Immunophenotyping of peripheral blood sample of patient no.4 with very severe aplastic anaemia before induction of retrodifferentiation in apheresed MNC (MNC), after induction of retrodifferentiation in apheresed MNC (RHSC) and post infusion of RHSC (days 1, 6, 14, post-infusion, horizontal). Cells were stained with IgG1 isotype control, CD3, CD7 (horizontal legends) and IgG2 isotype control, CD19 and CD33 & 13 (vertical legends). Absolute B and T lymphocyte counts (10^6/ml) are shown and denoted as # on dot plots.

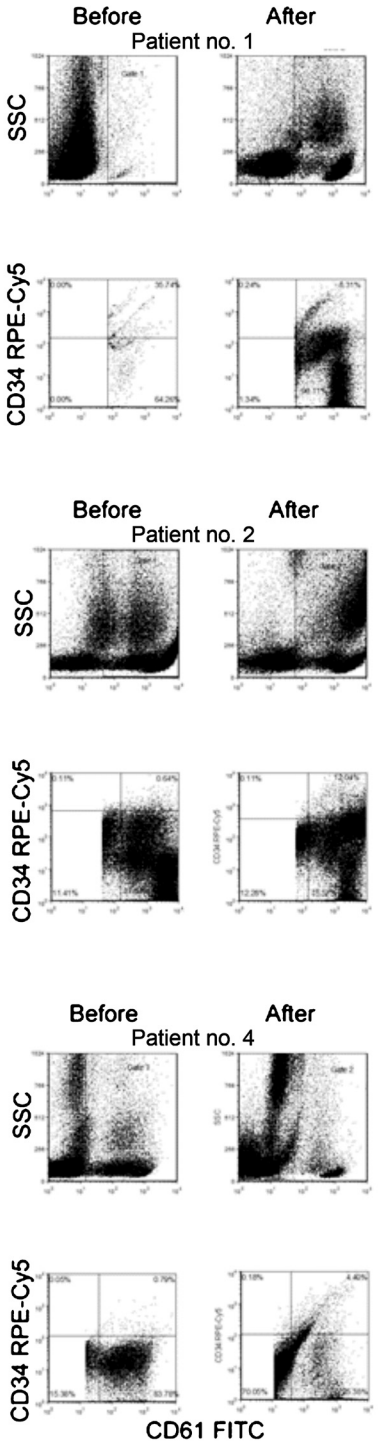

Fig. 5. Immunophenotyping of aphaeresed mononuclear cells of patients 1, 2 and 4 with aplastic anaemia before (MNC) and after induction of retrodifferentiation (RHSC). Cells were labelled with monoclonal antibody conjugated to RPE Cy-5 (vertical legends) CD34 and CD61 monoclonal antibody conjugated to FITC (horizontal legends). Cells were gated according to side scatter, CD61 expression and corresponding isotype control.

described. On 3 h post-initiation of retrodifferentiation, CD34 with and/or without CD38, CD61 expression increased in treated apheresed MNC samples obtained from the patients with aplastic anaemia. This increase in CD34 cells was always accompanied by decrease in CD19 (a B-cell marker) expressing cells concomitant to increase in cells expressing myeloid antigens such CD33&13, CD33&13CD7 and CD61. The reason for the highest CD34 counts in the MNC samples post-induction of retrodifferentiation obtained with panel CD34CD61 could have been due to platelets adherence to leukocyte which may have hindered CD34 staining when using panel CD34CD45 and CD34CD38[18].

Post-infusion of the autologous HRSC into aplastic anaemia patients, myeloid, erythroid and megakaryocytic engraftment ensued which resulted initially in transfusion independence in all patients but was long term in two patients only. Most likely primitive RHSC having CD34+CD38- immunophenotype[21] was responsible for the long term engraftment in these two patients. The mode of engraftment was unprecedented in patients 1, 2 and 4, which could have been due to the infusion of mega doses of CD34+ RHSC containing significant numbers of both committed (CD34+CD38+, CD34+CD61+ and CD33&13+CD7+) and non-committed (CD34+CD38- and CD34+CD45-) stem cells. Only RHSC of patient no.3 suffering from very severe aplastic anaemia did not contain significant numbers of primitive CD34+CD38- stem cells and, therefore, long term engraftment was not attainable. As to why patient no.2 who was suffering from hypoplastic anaemia and received RHSC containing large amount of CD34+CD38- primitive stem cells did not show similar long term engraftment remained unanswered. May be a partially intact bone marrow has hindered seeding of such primitive haematopoietic stem cells. The two patients who have shown long term engraftment received an RHSC infusate containing significant numbers of CD34+CD45- cells. This cellular immunophenotyping which could have been mesenchymal precursors in origin are pivotal for long term survival of the RHSC. Mesenchymal cells are extremely important accessory cells of the bone marrow microenvironment or stroma, necessary for haematopoietic stem cells proliferation and differentiation as well as homing[22].

Post-infusion CD34 cell circulated peripheral blood for several days and was followed by gradual appearance of cells with high forward and side scatter expressing myeloid progenitors immunophenotype

such as CD33&13 with and without CD7 expression and CD61 with and without CD34 expression in all four patients. Failure to demonstrate long-term engraftment in two patients (nos.2 and 3) was associated with increase in the number of CD61 cells not in platelets gate. This cellular immunophenotype emerged just prior these patients became transfusion dependent. Platelet antigens in association with leukocytes have been shown to be involved in graft rejection[23].

Hb F switching seen in patients 1 and 4 confirmed the reprogramming capabilities of the infused RHSC towards juvenile Hb phenotype and hence engraftment and reconstitution as observed with cord blood stem cells transplant[24,25]. As to whether Hb F switching can be used as indicator of successful outcome, remains to be seen. However, in another study involving five patients with aplastic anaemia successful engraftment of HRSC appeared to be associated with Hb F switching (unpublished data).

Transcriptional profiling of RHSC prior and post-infusion would have been valuable to understand the exact mechanism of engraftment in these four patients.

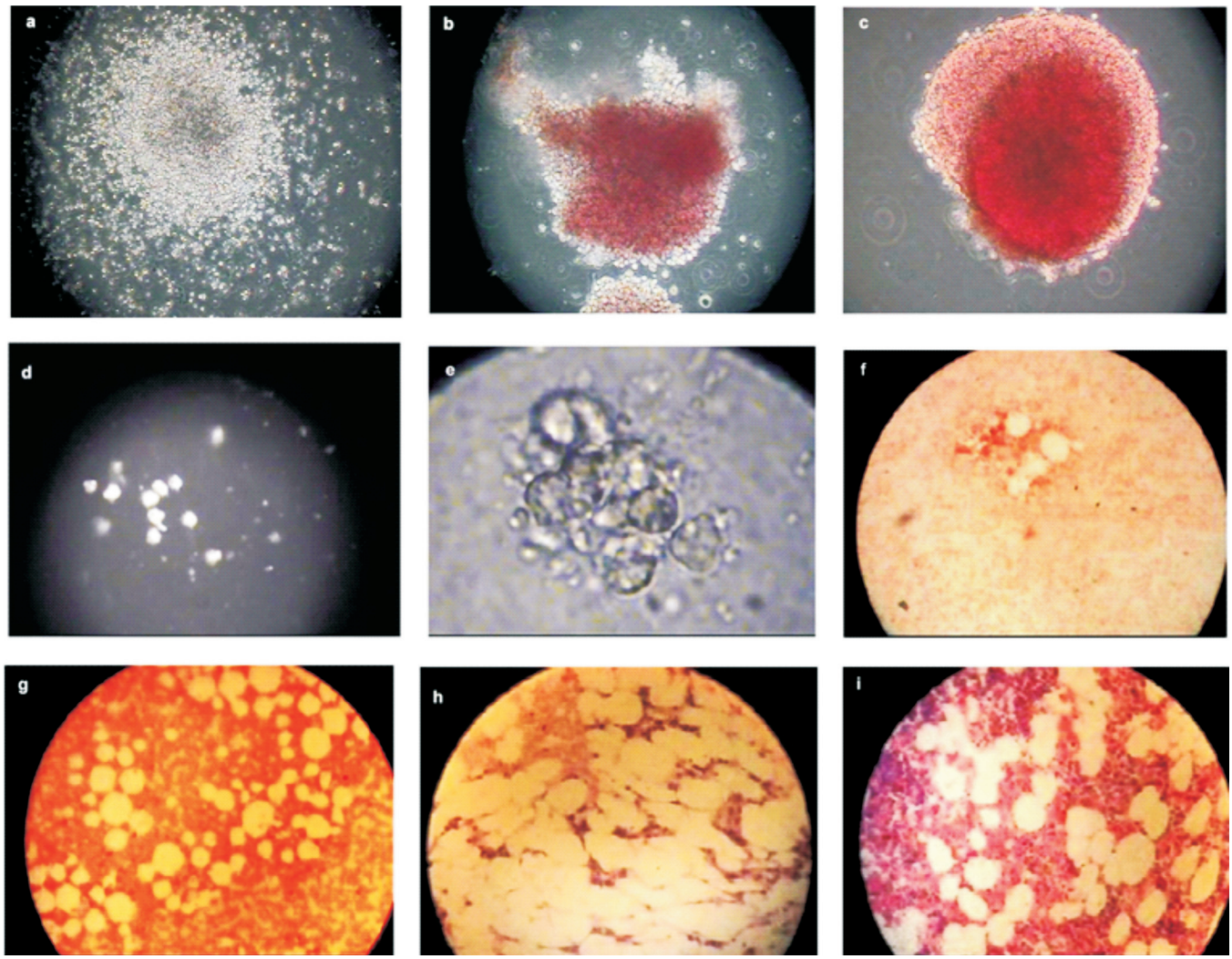

Fig. 6a. Bone marrow analysis of a patient with very severe aplastic anaemia (patient no.4) post 14 days infusion of RHSC. Clonal analysis of bone marrow following infusion of RHSC shows growth of colony forming unit granulocyte and macrophage CFU-GM **(a)**, colony forming unit granulocyte, erythrocyte, monocyte and megakaryocyte CFU-GEMM **(b)**, Burst forming unit erythroid BFU-E **(c)** colony forming unit monocyte CFU-M **(d)** and colony forming unit megakaryocyte CFU-Meg **(e)**. Also shown bone marrow smear for red blood cells before and after therapy **(f** and **g)** and trephine section before and after therapy **(h** and **i)**. Fig **f-i** are displayed on TriStem webpage: *htp//www.tristemcorp.com/HumanStudies02.htm*.

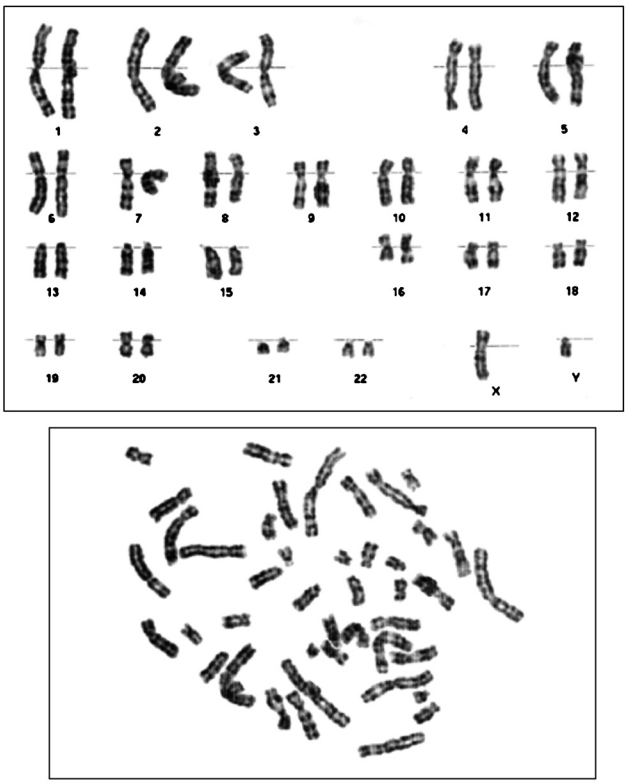

Fig. 6b. Karyotyping and G banding of peripheral blood sample of patient no.1 with severe aplastic anaemia following four years infusion with autologous RHSC showing normal male karyotype.

In a separate study, transcriptional profiling of RHSC obtained from healthy donors or patients not suffering from aplastic anaemia showed significant upregulation of primitive stem cell markers including embryonic and foetal erythropoiesis accompanied by the upregulation of many genes involved in immunoregulation and tolerance (unpublished observations). The haematopoietic engraftment in the two patients (1 and 4) who responded to the treatment, was similar to those obtained following transfusion of haematopoietic stem cell from identical twins without the use of any conditioning regimen[11]. These two patients had elevated liver enzymes prior to the autologous HRSC therapy and were serologically negative for hepatitis C virus. However, it remains to be seen whether they were also negative for HCV by quantitative PCR[26] which have may shown serological negativity due the underlying improvised immunity in these critically blood transfused patients.

The long term engraftment with preservation of chromosome number and banding reflects the safety of infusing the RHSC in a haematological condition where clonal evolution is not a rare event with conventional therapies[27]. It remains to be seen whether a second or repeat infusion of the autologous HRSC in the other two patients who died, with or without adjunct use of immunosuppression would have had eventually achieved engraftment.

Prior to infusion, hardly any haematopoietic colonies were generated from bone marrow aspirate of severe aplastic anaemia patients apart from patient no.2 who was suffering from hypoplastic anaemia. However, post-infusion all bone marrow samples gave rise to variety of haematopoietic cell colonies with elevation in the number of erythroid progenitors as observed in their trephine section, indicating thereby that there was homing of RHSC in bone marrow. Probably these RHSC did not repopulate very well in the two patients (nos. 2 and 3) due to defective microenvironment of the individual for restoration of normal haemopoiesis.

Like forward differentiation, the elucidation of the exact mechanism behind retrodifferentiation will yield valuable information regarding normal and aberrant cell development. The postulated mechanism behind retrodifferentiation[16] or dedifferentiation involves gradual loss of lineage associated markers in a population of more committed cells, at the genetic and protein level. Just like differentiation, the re-winding of ontogeny to a stem cells state is hierarchical. For example, less mature cells of a specific lineage will reach a stem cell state before mature ones. At this switch stage in development less mature cells will first re-gain pluripotency before mature ones and, therefore, are enabled to transdifferentiate or redifferentiate. This consecutive cascade of orderly reverse differentiation leads to either transdifferentiation or redifferentiation into a variety of specialised cells at various stages in development giving rise to either, altogether different or more rejuvenated tissue, respectively. These preliminary findings showed that the autologous RHSC were capable of long term engraftment and survival rate in a subset of severe aplastic anaemia patients without use of any pre-conditioning or immunosuppression regimen, just like those seen with syngeneic stem cells. This novel approach of stem cell therapy whether, autologous or allogeneic may achieve better cure rates alone or in conjunction with current immunosuppression or cytoreductive therapies[28] in many fatal haematological and non-haematological disorders due to abrogation of GVHD.

Acknowledgment

Authors acknowledge Prof. Nirmal K. Ganguly, former Director-General of the ICMR for leadership and vision that made

this project possible, including involvement in Clinical Protocol Design and Dr Vasantha Muthuswamy, former Senior Deputy Director General for facilitating protocol design, and thank Drs Farah Jijina and Manisha Madgaikar for provision, selection, counselling and clinical monitoring of patients and Ms Maya Gupta for technical assistance.

References

1. Young NS, Calado RT, Scheinberg P. Current concepts in the pathophysiology and treatment of aplastic anemia. *Blood* 2006; *108* : 2509-19.
2. Young NS, Kaufman DW. The epidemiology of acquired aplastic anemia. *Haematologica* 2008; *93* : 489-92.
3. Bacigalupo A. Aplastic anemia: pathogenesis and treatment. *Hematology Am Soc Hematol Educ Prorgam* 2007; *1* : 23-8.
4. Solomou EE, Rezvani K, Mielke S, Malide D, Keyvanfar K, Visconte V, *et al*. Deficient CD4+ $CD25^+$ $FOXP3^+$ T regulatory cells in acquired aplastic anemia. *Blood* 2007; *110* : 1603-6.
5. Locasciulli A, Oneto R, Bacigalupo A, Socie G, Korthof E, Bekassy A, *et al*. Severe Aplastic Anemia Working Party of the European Blood and Marrow Transplant Group. Outcome of patients with acquired aplastic anemia given first line bone marrow transplantation or immunosuppressive treatment in the last decade: a report from the European Group for Blood and Marrow Transplantation (EBMT). *Haematologica* 2007; *92* :11-8.
6. Tisdale JF, Maciejewski JP, Nunez O, Rosenfeld SJ, Young NS. Late complications following treatment for severe aplastic anemia (SAA) with high-dose cyclophosphamide (Cy): follow-up of a randomized trial. *Blood* 2002; *100* : 4668-70.
7. Rosenfeld SJ, Kimball J, Vining D, Young NS. Intensive immunosupression with antithymocyte globulin and cyclosporine as treatment for severe acquired aplastic anemia. *Blood* 1995; *85* : 3058-65.
8. Camitta BM, Thomas ED, Nathan DG, Santos G, Gordon-Smith EC, Gale RP, *et al*. Severe aplastic anemia: A prospective study of the effect of early marrow transplantation on acute mortality. *Blood* 1976; *48* : 63-70.
9. Gottdiener JS, Appelbaum FR, Ferrans VJ, Deisseroth A, Ziegler J. Cardiotoxicity associated with high-dose cyclosphosphamide therapy. *Arch Intern Med* 1981; *141* : 758-63.
10. Sanders JE. The impact of marrow transplant preparative regimens on subsequent growth and development. The Seattle Marrow Transplantation Team. *Semin Hematol* 1991; *28* : 244-9.
11. Hinterberger W, Rowlings PA, Hinterberger-Fischer M, Gibson J, Jacobsen N, Klein JP, *et al*. Results of transplanting bone marrow from genetically identical twins into patients with aplastic anemia. *Ann Intern Med* 1997; *126* : 116-22.
12. Hwang WL, Yang Y, Chen GR, Tsai CS, Jour JH. Syngeneic peripheral blood stem cell transplantation with brief immunosuppression for severe aplastic anemia. *Bone Marrow Transplant* 2000; *25* : 337-9.
13. Abuljadayel IS. Induction of stem cell-like plasticity in mononuclear cells derived from unmobilised adult human peripheral blood. *Curr Med Res Opin* 2003; *19* : 355-75.
14. Abuljadayel IS, Afghan RK, McCaffrey TA, Lundergan C, Hawley TS, Hawley RG, *et al*. SCID repopulating cells derived from unmobilised adult human peripheral blood. *Curr Med Res Opin* 2004; *20* : 87-100.
15. Abuljadayel IS, Quereshi H, Ahsan T, Rizvi S, Ahmed T, Khan SM, *et al*. Infusion of autologous retrodifferentiated stem cells into patients with beta-thalassemia. *Scientific World J* 2006; *6* : 1278-97.
16. Abuljadayel IS. Harnessing pluripotency from differentiated cells: A regenerative source for tissue-specific stem cell therapies. *Curr Stem Cell Res Ther* 2006; *1 :* 325-31.
17. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichiska T, Tomoda K, *et al*. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; *131* : 861-72.
18. Dercksen WM, Gerritsen WR, Rodenhuis S, Dirkson MKA, Slaper-Cortenbach ICM, *et al*. The value of flow cytometric analysis of platelet glycoprotein expression on CD34+ cells measured under conditions that prevent P-selectin- mediated binding of platelets. *Blood* 1995; *86* : 3771-82.
19. Gupta PK, Charan VD, Kumar H. PNH revisited: Clinical profile, laboratory diagnosis and follow-up. *Indian J Pathol Microbiol* 2009; *52* : 38-41.
20. Katsares V, Petsa A, Felesakis A, Paparidis Z, Nikolaidou E, Gargani S, *et al*. A rapid and accurate method for the stem cell viability evaluation. The case of the thawed umbilical cord blood. *Lab Med* 2009; *40* : 557-60.
21. Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. *Nat Med* 1998; *4* : 1038-45.
22. Chamberlain G, Fox J, Ashton B, Middleton J. Mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* 2007; *25* : 2739-49.
23. Kirk AD, Morrell CN, Baldwin WM 3rd. Platelets influence vascularized organ transplants from start to finish. *Am J Transplant* 2009; *9* : 14-22.
24. Elhasid R, Ben Arush MW, Pollack S, Tavor K, Streichman S, Postovsky S, *et al*. Immune and hematopoietic reconstitution after transplantation of cord blood progenitor cells: case report and review of the literature. *Leukemia 2000; 14* : 931-4.
25. Locatelli F, Maccario R, Comoli P, Bertolini F, Giorgiani G, Montagna D, *et al*. Hematopoietic and immune recovery after transplantation of cord blood progenitor cells in children. *Bone Marrow Transplant* 1996; *18* : 1095-101.
26. Pol S, Thiers V, Driss F, Devergie A, Berthelot P, Bréchot C, *et al*. Lack of evidence for a role of HCV in hepatitis-associated aplastic anaemia. *Br J Haematol* 1993; *85* : 808-10.
27. Bakhshi S, Gupta A, Kumar L. Acute myeloid leukemia with severe aplastic anemia following immunosuppressive therapy. *Indian J Pediatr* 2006; *73* : 1033-5.

Reprint requests: Dr Ilham Saleh Abuljadayel, TriStem UK Limited, 571 Finchley Road, Unit 320, London NW3 7BN, England
e-mail: iabuljadayel@tristemcorp.com