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ORIGINAL RESEARCH

Longitudinal Evaluation of Immune Reconstitution and B-cell Function After Hematopoietic Cell Transplantation for Primary Immunodeficiency

Alessia Scarselli[1] · Silvia Di Cesare[1] · Claudia Capponi[2] · Simona Cascioli[3] · Maria L. Romiti[1,6] · Gigliola Di Matteo[1] · Alessandra Simonetti[1] · Paolo Palma[1] · Andrea Finocchi[1] · Barbarella Lucarelli[4] · Rita M. Pinto[4] · Ippolita Rana[4,7] · Giuseppe Palumbo[1,4] · Maurizio Caniglia[4,8] · Paolo Rossi[1] · Rita Carsetti[2,3] · Caterina Cancrini[1] · Alessandro Aiuti[1,5]

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Abstract
Purpose Hematopoietic cell transplantation (HCT) provides a curative therapy for severe forms of primary immunodeficiencies (PID). While the timing and extent of T-cell reconstitution following transplant for PID has been studied in depth, less is known about the kinetics of B-cell development and long-term restoration of humoral functions, which been often reported to be suboptimal after HCT.
Methods We studied longitudinally B-cell development and function in a cohort of 13 PID patients transplanted between 1997 and 2010, with a follow-up ranging from 0.7 to 15 years. Flow cytometric analysis of naïve and antigen-experienced B-cell subsets and in vitro functional responses to CpG were compared with data from healthy children and correlated with the degree of B-cell chimerism and in vivo antibody production.
Results We found that total memory B-cells count remained below normal levels for the first 2 years of follow up and progressively normalized. Switched memory B-cells (CD19+ CD27+IgD-IgM-) were restored early and better than IgM memory B-cells (CD19+CD27+IgD+IgM+), which remained significantly reduced long-term. The recovery of memory B-cells correlated with good in vivo humoral function and normalization of CpG-response. A complete B-cell reconstitution was usually associated with donor B-cells chimerism and pre-transplant conditioning. Donor source and the underlying genetic defect represented also important variables.
Conclusion Monitoring of phenotypic and functional changes on B-cells following HCT may prove clinically relevant to tailor patients' care. In particular the analysis of IgM memory and switched memory B-cells in addition to in vitro B-cells stimulation are recommended before Ig replacement therapy (IgRT) discontinuation.

Rita Carsetti, Caterina Cancrini and Alessandro Aiuti contributed equally to this manuscript.

✉ Caterina Cancrini
cancrini@med.uniroma2.it

✉ Alessandro Aiuti
a.aiuti@hsr.it

[1] University Department of Pediatrics, DPUO, Unit of Immune and Infectious Diseases, Bambino Gesù Children's Hospital, Rome, Italy

[2] Department of Laboratories, Children Hospital Bambino Gesù, Rome, Italy

[3] Immunology and Pharmacotherapy Area, Bambino Gesù Children's Hospital, Rome, Italy

[4] Department of Pediatric Hematology and Oncology Bambino Gesù Children's Hospital, Rome, Italy

[5] San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Milan, Italy

[6] Servizio Prevenzione e Protezione, University of Rome Tor Vergata, Rome, Italy

[7] General Pediatrics and Infectious Diseases, Bambino Gesù Children's Hospital, Rome, Italy

[8] Pediatric Hematology and Oncology, S. Maria della Misericordia, Perugia, Italy

Keywords Primary immunodeficiency · hematopoietic stem cell transplantation · immune reconstitution · B-Lymphocytes · conditioning · follow up studies · child

Introduction

Hematopoietic cell transplantation (HCT) provides a curative therapy for primary immunodeficiencies (PID), a heterogeneous group of inherited disorders affecting the immune system and leading to recurrent severe infections and predisposition to autoimmunity and cancer [1–3]. The outcome of HCT has improved considerably due to better supportive care, graft versus host disease (GVHD) prevention and use of the reduced intensity conditioning regimen and novel strategies for stem cell manipulation and purification [4–8]. While the timing and extent of T-cell reconstitution has been studied in depth [9–14], less is known about B-cell development and functions after transplant for PID. Previous studies suggested that B-cell recovery can be slower than T-cell reconstitution and often suboptimal, and in these patients immunoglobulin replacement therapy (IgRT) is still required [15–18]. It has been suggested that delayed or partial humoral reconstitution is often associated to the persistence of autologous defective B lymphocyte [19–21]. The use of myeloablative conditioning facilitates establishment of donor B lymphocyte chimerism [22–24], but this benefit may be outweighed by the increased toxicity and some patients may require IgRT even after pre-transplantation conditioning [25, 26]. Moreover, humoral reconstitution is also influenced by the molecular type of PID, degree of HLA compatibility, source of grafts, and post-transplant course (e.g. infections and GVHD) [25, 27–29].

Here, we studied long-term B-cell reconstitution in a small cohort of PID patients who received different sources of HCT. We found that the kinetic of recovery of naïve and antigen-experienced B cells was variable and mainly influenced by the degree of donor chimerism, type of donor source and the underlying disease. Our data suggest that monitoring of phenotypic and functional changes on B-cells following HCT may allow to identify early predictors of efficient humoral function.

Methods

Patients' Characteristics and Data Collection

We collected data on immune reconstitution and clinical status after HCT of 16 patients who consecutively underwent HCT at Bambino Gesù Children's Hospital (OPBG) in Rome between 2002 and 2010 and one patient who was transplanted in 1997 at the Necker-Enfants Malades Hospital and followed at OPBG. Data on immune reconstitution and clinical status after HCT were collected until the end of 2012. PID was diagnosed based on clinical findings, immune phenotype and function, and confirmed by genetic analysis. In 2 patients no mutations were identified. In vivo T cell depletion has been used in 12 procedures (all but one mismatched related donor – MMRD-, all matched unrelated donor- MUD- and two matched sibling donor –MSD- transplants) by anti-thymocyte globulin or Alemtuzumab or anti-CD2 anti-leukocyte function atigen-1. In addition, Pt 11 received a donor graft enriched for CD34+ cells. Patients underwent HCT from different donor sources (BM, n=14, CB, n=3), with (n=11) or without (n=6) conditioning treatment. The median age at transplantation was 11 months (range 5–144 months), patients' follow-up ranged from 0,7 to 15 years, with an overall survival of 77 %. Two SCID patients (Pt 2, 3) died early post HCT due to disseminated infections present at time of HCT, a patient affected by Wiskott-Aldrich syndrome (WAS) (Pt 13) died 9 months after HCT due to a EBV-positive diffuse large B-cell lymphoma, whereas one patient with Familial Haemophagocytic Lymphohistiocytosis (FHLH) (Pt 15) died 6 months after the rejection of the third HCT transplant (Table 1). Patients' family signed informed consent for immunological studies under a research protocol approved by Ethic Committee of OPBG.

Chimerism and T Cell Function

Whole blood and sorted peripheral cell subsets (CD3+, CD19+, CD14+, CD16+56+) were studied for chimerism at 1, 3, and 6 months after HCT and then yearly. Chimerism was analyzed by fluorescence in situ hybridization for donor–recipient sex mismatches or by polymerase chain reaction amplification of short tandem repeats in case of donor-recipient sex-matching. Immunological reconstitution of T, B and NK populations were monitored by flow cytometry on peripheral blood mononuclear cells (PBMCs) according to standard protocols with a FACS CANTO II flow cytometer and monoclonal murine antibodies conjugated to FITC or PE specific for CD3, CD4, CD8, CD16, CD19, CD45RO, and CD45RA (30). Pt17 was analyzed at 0.7 years before he returned to his country, and data were grouped with the 1 year follow up. The function of T lymphocytes was assessed by lymphocyte proliferation upon mitogens, T-cell repertoire and thymic activity (TREC) as previously described [30, 31].

B Cell Function

Reconstitution of B-cell compartment was analyzed through multiple approaches including the analysis of naïve and antigen-experienced B-cell subsets (anti-CD27, anti-IgD and anti- IgM were used to identify different stages of B-cell maturation), the determination of immunoglobulin isotype, the IgG subclasses and the ability to generate antibodies to protein

Table 1 Characteristics of patients and HCT

Pt	Gender	Diagnosis	Age at HCT	Conditioning Regimen	GvHD prophylaxis	Donor type	Source	Clinical events	Follow up
1	M	SCID γ-chain	7 months	nd	CsA/Methyl-pred/ATG	MMRD (1AgMM)	BM	warts 7 years post HCT	10 year
2	M	SCID γ-chain	7 months	nd	CsA/Methyl-pred/ATG	MMRD (1AgMM)	BM	CMV encephalitis	Died at 17 days post HCT
3	M	SCID γ-chain	6 months	nd	nd	MMRD	BM	fungal sepsis	Died at 5 weeks post HCT
4	M	SCID Jak3	13 months	BU/Cy	CsA/Methyl-pred/ATG	MMRD	BM	GVHD III gut; VOD	8 years
5	F	SCID Jak3	7 months	nd	nd	MSD	BM		8 years
6	M	ADA-SCID	5 months	nd	nd	MSD	BM	HHV-6 viremia	6 years
7	F	SCID T-B+	11 months	nd	nd	MSD	BM	BCG reactivation, immune reconstitution inflammatory syndrome	1.2 years
			12 months	nd	nd	MSD	BM		
8	F	OS (RAG1)	7 months	BU/FLU/ Thiotepa	CsA/Methyl-pred/ATG	MUD	UCB	GVHD III skin	3 years
9	F	OS (RAG1)	8 months	Treo/FLU/Thiotepa	CsA/Mycoph/ATG	MUD	BM	GVHD II skin	2 years
10	M	OS (RAG2)	7 months	Thiotepa/Treo/FLU	CsA/Methyl-pred/ATG	MUD	UCB	GVHD III/IV skin and gut; cGVHD, haemolytic syndrome; PCJ pneumonia	2 years
11	M	EDA-ID (IkBα)	12 months	BU/Cy	Anti CD2 anti-LFA1	MMRD	BM	seizures; PCJ pneumonia; chronic broncho-pneumopathy	15 years
12	F	DCML deficiency	8 years	Treo/ FLU/Thiotepa	CsA/Mycoph/ATG	MUD	BM	GvHD I skin	3 years
13	M	WAS	3 years	Thiotepa/BU/ FLU	CsA/Mycoph/PDN/ATG	MUD	UCB	EBV-positive diffuse large B-cell lymphoma	Died at 9 months after HCT
14	M	XLP	7 years	BU/Cy	CsA/MTX/ Methylpred/ ATG	MUD	BM	GVHD I skin; EBV reactivation controlled by Rituximab	3 years
15	M	FHLH (MUNC 13–4)	12 years	FLU/Melphalan/ Alemtuzumab	CsA/Methyl-pred	MSD	BM	hemolytic anemia; HCT rejection	Died 6 months after the 3rd HCT
			12 years	Thiotepa/Treo/FLU	CsA/MTX	MSD	BM		
			13 years	nd	ATG	MSD	BM		
16	M	CGD	4 years	BU/Cy	CsA	MSD	BM	mild hemorrhagic cystitis	7 years
17	M	CGD	6 years	Thiotepa/Treo/ FLU	CsA/MTX	MSD	BM	Pseudomonas A. sepsis	0.7 years

nd not done, *SCID* Severe Combined Immunodeficiency, *ADA* Adenosine deaminase, *OS* Omenn Syndrome, *EDA-ID* anhidrotic ectodermal dysplasia with immunodeficiency, *DCML* DC, monocyte, B and NK lymphoid cells, *WAS* Wiskott-Aldrich syndrome; *XLP* X-linked lymphoproliferative disease, *FHLH* Familial hemophagocytic lymphohistiocytosis; *CGD* Chronic granulomatous disease, *BU* Busulphan, *Cy* Cyclophosphamide, *Flu* Fludarabine, *Treo* Treosulfan, *Mycoph* Mycophenolate mofetil, *anti-LFA1* anti-leukocyte function atigen-1; *PDN* prednisone; *Methylpred* methylprednisolone; *MTX* Methotrexate, *1AgMM*=1-antigen mismatch, *MMRD* mismatched related donor; *MSD* Matched Sibling Donor, *MUD* Matched Unrelated Donor; *BM* bone marrow; *UCB* umbilical cord blood; *VOD* veno-occlusive disease, *PCJ* pneumocystis jiroveci; *GVHD* graft versus host disease, *sdr* syndrome

and polysaccharide antigens after tetanus toxoid, hepatitis B, Haemophilus influenzae and pneumococcal immunization. B-cell subsets references for controls were obtained from 81 healthy children at different age being referred to our hospital for elective procedures (14 children of 1 year, 7 of 2 years, 14 between 3 and 5 years, 46 between 6 and 15 years). We calculated the 5° and 95° percentile for each group of age and compared them to the values of patients according to the years following HCT. The ability of B-cells to differentiate, proliferate and produce immunoglobulins was tested by stimulation with bacterial DNA (CpG). Briefly, PBMC obtained through ficoll from peripheral blood of patients, were labeled at the final concentration of 0.1 μg/ml CMFDA (5-chloromethylfluorescein diacetate, CellTracker; Molecular Probes) and cultured at 5×10^5 cells per well in 96-well plates in complete RPMI 1640 (InvivoGen) supplemented with 10 % FBS (HyClone Laboratories). Human CpG oligodeoxynucleotides (Hycult Biotechnology) was used at the optimal concentration of 2.5 μg/ml. Cell proliferation and differentiation into plasma cells, were measured on day 7 by FACSCanto II flow cytometer (BD Biosciences). Secreted Igs were detected at by ELISA [32].

Results

Patients' Treatment and Donor Cell Engraftment

We studied longitudinally the immune reconstitution in 13 surviving PID patients in a cohort of 17 patients who received allogeneic HCT. Among them 6 patients had long-term follow-up (>5 years). Characteristics of patients and details about transplants are summarized in Table 1. Conditioning regimens varied based on patient's underlying disease and clinical condition at transplantation. Successful hematopoietic engraftment was observed in all 13 survivors with a median time of 17 days (range 12–29 days) for neutrophils (ANC>500/ul) and 20 days (range 12–55 days) for platelets (>50,000/ul). Four patients developed grade II-III acute Graft versus Host Disease (GvHD), and one experienced chronic GvHD. In unconditioned SCID patients, nearly full donor chimerism was detected in the T-cell lineage whereas B-cell and monocytes remained mostly or totally of recipient origin. Specifically, 2 out of 4 non-conditioned patients had an absent donor B, monocyte and NK-cell engraftment (Fig. 1a). In contrast, all patients who received cytoreductive conditioning regimens achieved stable full donor chimerism in all lineages, except one patient (Pt 8) with mixed chimerism on CD14+ cells. Three patients showed substantial fluctuations in donor B-cell contribution over time and in two of them an increase of donor B-cells occurred very late (>4 years) during follow up (Fig. 1b). Interestingly, Pt5 (JAK-3 deficiency) and Pt6 (ADA-SCID) who received unconditioned transplants from siblings, showed normal humoral function despite the prevalence of host B-cells. When switched memory B-cells were sorted from these patients, molecular analyses of chimerism revealed that the majority of them were of donor origin (60 and 95 %, respectively, data not shown).

Effective Long-Term T-Cell Reconstitution

T-cell immune reconstitution was evaluated at yearly follow up in 13 surviving patients. Complete T-cell count recovery was obtained in all except two patients who experienced severe clinical complications (GvHD, immune reconstitution syndrome associated to BCG). One year after HCT total CD3+, CD4+ and CD8+ cell counts were normal in the majority of patients. NK cell count was normal in all patient except one affected by γ-chain SCID and treated with HCT from unconditioned mismatched related donor. Evaluation of thymic function revealed normal CD4+CD45RA+ naïve T-cell counts and T-cell receptor excision circle (TREC) copies, in a high proportion of patients starting the first year after transplantation (Table 2) [33]. Of note, patients with reduced TREC values had received unconditioned transplants (data not shown). T-cell diversity analysis by T-cell repertoire spectratyping showed a polyclonal distribution in the CD4+ compartment and to a lower extent in CD8+ T-cells 12 months after transplantation, which was maintained or improved during follow up. T-cell proliferation in responses to both OKT3 and PHA achieved normal levels in 54 % of patients within 1 year after HCT. In 5 out of 6 long term-survivors (follow up >5 years) T-cell reconstitution was sustained over time. In Pt1 a decline in T cell count and functionality with skewed TCR profile, low response to PHA, undetectable TRECs, and appearance of face warts was observed 7 years after HCT.

B-Cell Development After HCT

One year after HCT proportions and absolute B-cell counts were significantly reduced as compared to age matched controls (Fig. 2a); they reached normal value for age after 2 years, remaining in the normal range long-term (>5 years). B-cell subsets were examined longitudinally in depth by multiparameter flow cytometry in 11 evaluable patients after transplant. We found a significant increase in the proportion of transitional B-cells (CD19+CD38++IgM++) at 1 year follow up, which normalized over time (Fig. 2b). Total memory B-cells (CD19+CD27+) were significantly below normal at 1 and 2 years and increased starting 3–5 years of follow up (Fig. 2c). Remarkably, switched memory B-cells (CD19+CD27+IgD-IgM-) were restored earlier and better then IgM memory B-cells (CD19+CD27+IgD+IgM+), which remained significantly reduced in the long-term cohort (Fig. 2d, e). B-cell absolute counts and percentage did not differ between MSD and MMRD transplant in long-term surviving patients, but the

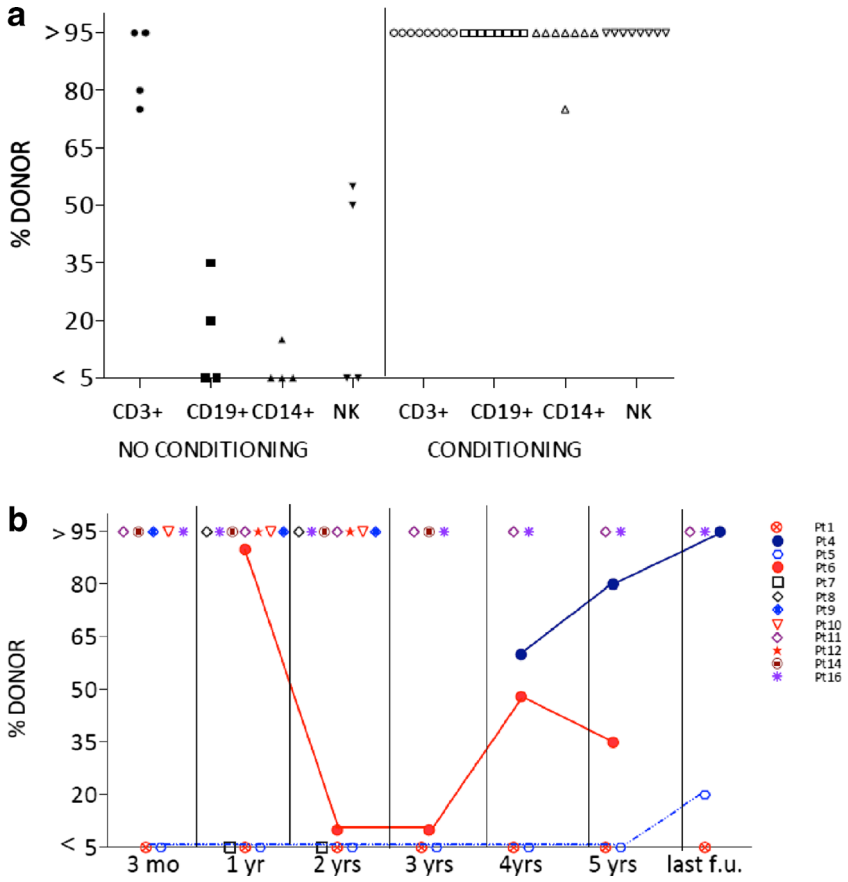

Fig. 1 **a** Donor chimerism in different lineages Percentage of donor cells in peripheral blood CD3+,CD19+, CD14+ and NK analyzed by in situ hybridization or STR analysis in unconditioned and conditioned patients at latest follow up. b Percentage of donor cells in peripheral blood CD19+ during the follow up. Donor chimerism was >95 % in patients 8,9,10,11,12,14,16, <5 % in Patients 1, 7. Patients 4,5,6 had intermediate chimerism and changed over time. Chimerism on sorted cells were not performed in Pt 17, chimerism on PBMC was 100 % donor

latter group displayed a reduced frequency of memory B-cells (data not shown).

In Vivo Antibody Production

The majority of patients discontinued IgRT between the first and second year after HCT and maintained normal values of IgG, IgA, IgM and IgG subclasses during the follow up [34] (Fig. 3). Four patients required long-term IgRT substitution therapy. Pt1 (SCID γ-chain deficiency) did not attain donor B-cell engraftment whereas Pt11 (EDA-ID patient) was maintained on IgRT in spite of B-cell engraftment due to very low antibody response for S. pneumoniae antigens. Both patients showed very low frequency of memory B-cells and almost absent

Table 2 Proportion of transplanted patients with normal immune parameters after transplant

	1 year post HCT % n	2 years post HCT % n	Long-term follow-up (>5 years) % n
Normal CD3+	76 % (10/13)	80 % (8/10)	100 % (6/6)
Normal CD4+	76 % (10/13)	80 % (8/10)	83 % (5/6)
Normal CD8+	76 % (10/13)	80 % (8/10)	100 % (6/6)
Normal CD4+CD45RA+	69 % (9/13)	80 % (8/10)	60 % (4/6)
Normal NK cell	92 % (12/13)	90 % (9/10)	83 % (5/6)
Normal T-cell function	54 % (6/11)	66 % (6/9)	83 % (5/6)
Polyclonal TCRVβ CD4+	72 % (8/11)	83 % (5/6)	83 % (5/6)
Polyclonal TCRVβ CD8+	54 % (6/11)	83 % (5/6)	66 % (4/6)

Normal CD3+ cell count at different years post HCT: 1 year>1000/mcl, 2 years>1400/mcl, >5 years>1000/mcl; Normal CD4+ cell count at different years post HCT: 1 year>500/mcl, 2 years>650/mcl, >5 years>450/mcl; Normal CD8+ cell count at different years post HCT: 1 year>300/mcl, 2 years>400/mcl, >5 y>300/mcl; Normal CD4+CD45RA+cell count>200/mcl; Normal NK cell count >50 /mcl.Normal reference values according to (33). Normal T cell function measured by proliferative response to mitogens (PHA>30.000 cpm and OKT3> 20.000 cpm, according to internal reference range)

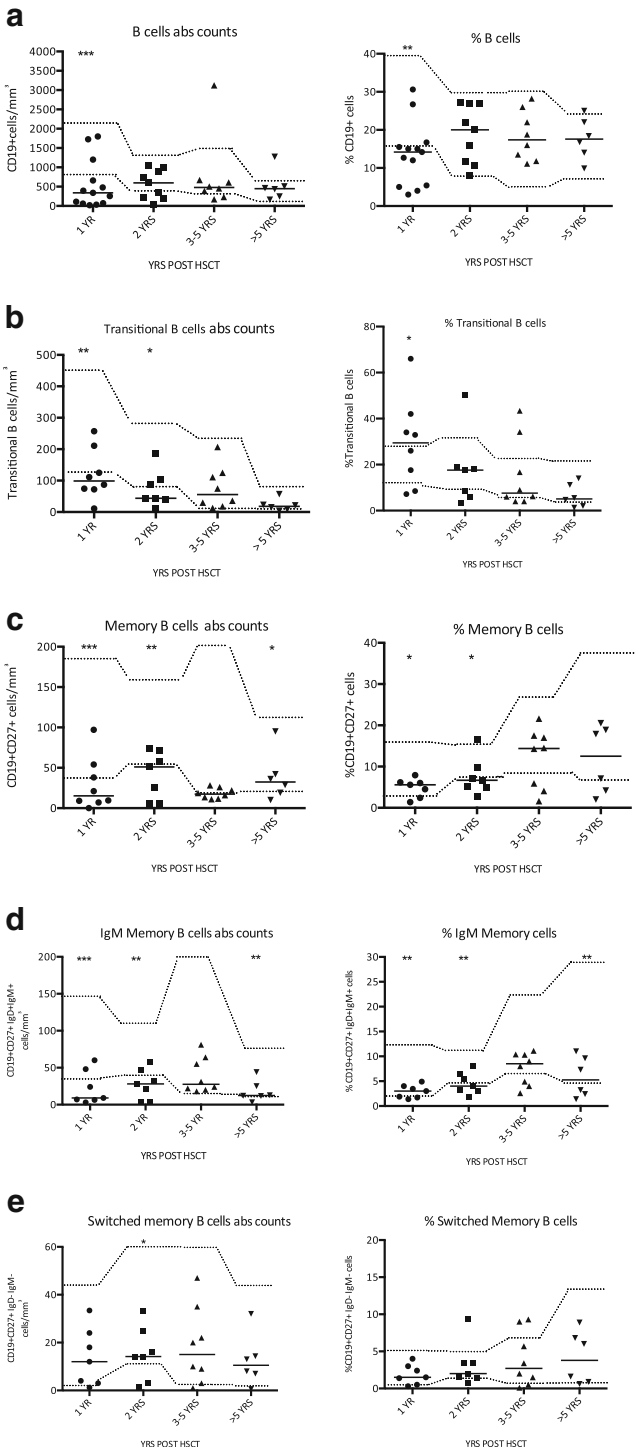

Fig. 2 **a**–**e** B-cell subsets at different time points after HCT Lines represent median values. B-cell are defined as CD19+ (A), transitional as CD19+38++24++ (**b**), memory B-cell as CD19+27+ (**c**), IgM memory as CD19+27+IgD+IgM+ (**d**), switched B-cell as CD19+27+IgD-IgM- (E). Relative numbers in percentage (*right*) and absolute counts in cells/mm^3 (*left*). Significant differences between median values at different time points after HCT are indicated by asterisks: * $p<0{,}01$, ** $p<0{,}001$,*** $s<0{,}0001$. Dotted lines represent 5° percentile and 95° percentile of healthy controls as specified in the methods

switched B-cells for several years after HCT. Two other patients (Pt 7 and Pt 10) experienced severe clinical complications and required IgRT longer than 2 years after HCT. The nine patients in which IgRT was discontinued received vaccinations according to European Blood Marrow Transplantation (EBMT) recommendations [35]. Protective antibody titers developed following vaccination in almost all tested patients. One patient (Pt 4) required an additional hepatitis B booster three years after initial doses, one patient (Pt 14) required a fourth dose of conjugate pneumococcal vaccine and one patient (Pt 5) showed low titer anti-hepatitis B and anti-Pneumococcus 5 years after the last vaccinations. Isohemagglutinins appropriate for the blood group were found at significant titers in 7 patients evaluated beyond 1 year post HCT (Table 3).

In Vitro B-cell Functions After HCT

To better study restoration of B-cell functions in transplanted patients we studied B-cell proliferation, maturation and Ig production in vitro. B cells isolated from peripheral blood were stimulated with human CpG oligodeoxynucleotides (unmethylated bacterial DNA), an agonist of TLR9 expressed in human on plasmocitoid dendritic and B-cells and at high levels on memory B-cells [32, 36]. Under these conditions memory B-cells proliferate and differentiate to immunoglobulin–secreting cells in response to CpG through T-independent signalling. All 3 patients who underwent transplantation from a MSD showed an optimal and complete B-cell response. In the MUD and MMRD cohort, B-cell proliferation, differentiation and antibody production were present in most patients but overall reduced as compared to MSD patients. In particular, 2 MMRD still requiring IgRT, failed to produce IgG and IgA in response to CpG (Table 4).

Discussion

HCT is an established curative treatment for the most severe PID variants. However, reduced or poor humoral immune function can persist in a significant proportion of patients requiring lifelong IgRT and leading to a higher frequency of clinical complications such as infections, autoimmunity and chronic-GVHD [33, 37, 38]. In our cohort of long-term surving patients, the majority of patients were clinically well and did not experience severe late HCT-related complications. In patients with long-term follow up (6–15 years) T-cell function was retained in all but one γ-chain patient who received a non-conditioned mismatched related HCT. In this case we observed a gradual decline of T-cell counts and functionality. Four patients experienced main clinical events including chronic GvHD requiring immunosuppressive therapy, chronic bronchopneumopathy, BCG reactivation, and warts. Of

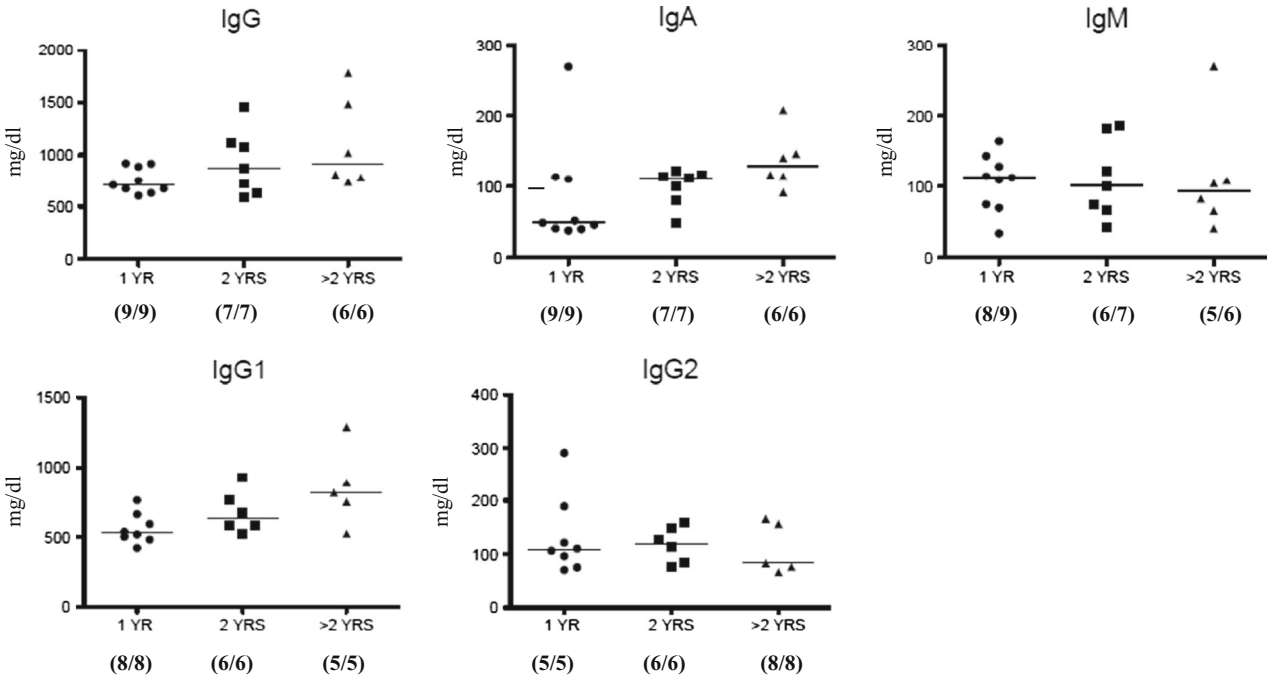

Fig. 3 Serum Ig level in transplanted patients Levels of IgA, IgM, IgG, IgG1, IgG2 are reported at 1 year, 2 years and at latest follow up (>2 years) after IgRT discontinuation. Subjects with normal values according to age are also indicated between brackets

notice, all these patients required IgRT supplementation and 3 of them displayed low T-cell numbers and functions, indicating that long-term complications were associated with suboptimal immune reconstitution. We found that a good humoral function was usually associated with the presence of donor B-cell chimerism and promoted by myeloablative conditioning regimen. Indeed, all patients who received conditioning (n=9) showed a full donor B-cell chimerism and the majority (n=7; 78 %) discontinued IgRT 1–2 years after HCT maintaining protective levels of serum Igs and specific antibody titers (Table 5).

Our data indicate that the development of the B-cell pool is a slow process after HCT for PID and that quantitative defects may persist in the long-term. The relative expansion of the transitional B-cell compartment in the first year after HCT is

Table 3 Antibody titers to vaccination and isohemagglutinins at last follow up

Pt	Tetanus *UI/L*	Time from last immunization	Haemophilus I. *mg/L*	Time from last immunization	Hepatitis B *mUI/ml*	Time from last immunization	Pneumococcus *mg/L*	Time from last immunization	Anti-A	Anti-B
1*	n.e.		n.e.		n.e.		n.e.		1:256	n.e.
4	0,7	4 yrs	>9	4 yrs	256	4 weeks	65	1 yr	1:64	n.e.
5	0,1	5 yrs	0,2	5 yrs	Undetectable	5 yrs	7,8	5 yrs	0	1:16
6	0,6	3 yrs	4,5	3 yrs	284	6 weeks	61	2 yrs	n.e.	1:8
7*	n.e.		n.e.		n.e.		n.e.		n.e	1:2
8	n.t.		9	8 mo	n.t.		131	6 mo	1:64	1:4
9	1,1	4 weeks	>9	4 weeks	n.t.		180	4 weeks	n.t.	n.t.
10*	n.e.		n.e.		n.e.		n.e.		n.t.	n.t.
11*	n.e.		n.e.		n.e.		n.e.		0	0
12	1	1 yr	0,1	1 yr	25	1 yr	57	1 yr	n.e.	n.e.
14	0,2	2 yrs	0,5	2 yrs	11	2 yrs	63	1 yr	0	1:32
16	1,8	3 yrs	>9	3 yrs	539	3 yrs	41	2 yrs	1:64	n.e.
17	n.t.		n.t		n.t		n.t.		n.t	n.t

Patients with * are on *IgRT*; Haemophilus I. = Haemophilus Influenzae. The following range are reported for antibodies responses to vaccination: Tetanus: <0,03 absent; 0,03-0,1 not warranted; 0,1-0,5 present; 0,6-1 sufficient; 1,1-5,5 long-term; >5 very high; Haemophilus Influenzae: <0,03 absent; 0,15 present; 0,15-1,0 sufficient; 1,1-5,5 long-term; >9 very high; Pneumococcus>35 mg/l present; Hepatitis B≥11 mUI/ml present; n.e. not evaluable; n.t. not tested; *yrs* years; *mo* months

Table 4 Proliferation, differentiation and in vitro Ig production after CpG stimulation

	Patient n°	Follow up at time of analysis	B-cell Proliferation	Differentiation to Plasma cells	In vitro Ig production		
					IgM	IgA	IgG
MSD	5	8 years	+	++	+++	+	++
	6	6 years	+++	+++	++	+	+
	16	7 years	+	+	++	+	+
MUD	8	3 years	+	+/–	+	+/–	+/–
	9	2 years	+/–	+/–	+	+/–	+
	12	3 years	+/–	+/–	+	+/–	+
	14	3 years	+	+	+	+/–	-
MMRD	1	10 year	+/–	+/–	+/–	–	–
	4	8 years	+	+/–	++	+/–	+
	11	15 years	+/–	+/–	+/–	–	–

Data form patients' at latest follow-up are shown. The degree of functional responses are indicated by a scale from negative (−) to highly positive (+++)

in line with previous reports showing that post-HCT the majority of B-cells are immature and develop recapitulating B-cell ontogeny [39–43]. Patients who achieved a good humoral function in vivo and in vitro showed a progressive increase in switched memory B cells, which was paralleled by a recovery of CpG response. Despite the normalization of the in vitro functional response we noticed a delay in the recovery of the IgM memory subset. The reduced number of IgM memory B-cells, that are thought to be generated in a T-independent manner, could reflect the inability of host defective B-cells or even donor B-cells to properly differentiate in the host environment. This defect in T-cell-independent antibody response could be responsible of the recurrent infections with encapsulated bacteria and poor responses to polysaccharides vaccines which occur even in presence of antibody production (41, 42) and remain relevant late complications after HCT. The establishment of the switched B-cell pool, generated in germinal center (GC)-dependent manner, reflects the T-cell reconstitution occurring from the first year post HCT and mirrors the ability to effectively respond to antigen and develop serological memory [44, 45]. Thus, a complete T-cell reconstitution is necessary for an appropriate B-cell function in the germinal center. Indeed, in the 9 patients who had discontinued IgRT and had a relevant specific antibody after vaccination, a progressive increase of both IgM and switched B-cell occurred and response to CpG normalized twelve months after HCT. In contrast, within the group of patients still requiring IgRT, two patients were severely lymphopenic (Pt7, Pt10) and two had

Table 5 Summary of immune recovery at last follow up in surviving patients

Pt	Immunophenotype					Chimerism (% of donor cells)			IgRT
	CD3 (/mm^3)	CD4 (/mm^3)	CD8 (/mm^3)	CD19 (/mm^3)	CD16/56 (/mm^3)	CD3	CD19	CD14	
1	1277	531	740	246	20	>95	<5	<5	Yes
4	2198	1156	678	508	315	>95	>95	>95	No
5	2584	1139	1212	434	110	80	20	15	No
6	1396	613	629	160	238	75	35	75	No
7	155	109	43	36	166	>95	<5	<5	Yes
8	4190	2648	1033	607	798	>95	>95	75	No
9	2555	1480	810	730	314	>95	>95	>95	No
10	173	160	13	32	63	>95	>95	>95	Yes
11	3357	1683	1603	1272	326	>95	>95	>95	Yes
12	2588	1255	1290	1006	185	>95	>95	>95	No
14	2206	1064	896	574	240	>95	>95	>95	No
16	1671	945	525	462	95	>95	>95	>95	No
17	730	283	436	340	436	>95	>95	>95	No

almost undetectable switched B-cells (Pt1 0.6 %, Pt 14 0.9 %) and poor response to CpG despite the normalization of T-cell counts. B-cell reconstitution was also dependent on the nature of the underlying genetic defect. Pt11, affected by EDA-ID, despite a complete full donor chimerism including T, monocytes and B-cells showed an altered B-cell phenotype and mostly IgM production after CpG stimulation without switched B-cells. Since B-cells derive from healthy transplanted maternal stem and progenitor cells, the absence of memory B-cells is not due to an intrinsic alteration of the B-cells. We hypothesize that developmental abnormalities of the secondary lymphoid organs, caused by the IkBα genetic defect, are responsible for the altered B-cell development and function in vivo [46]. The use of pre-conditioning was generally associated with a better humoral reconstitution with adequate production of immunoglobulins. On the other hand, humoral reconstitution was achieved in 2 patients (ADA-SCID and JAK3) who did not receive pre-conditioning regimen and, despite low proportion of donor B-cell engraftment, showed the presence of normal values of switched B-cell and appropriate in vitro and in vivo B-cell functions. The observation in the ADA-SCID patient (Pt6) is in agreement with the recent retrospective analyses on HCT in ADA-SCID patients showing that humoral immunity was present even after unconditioned transplant [47]. On the other hand, a side by side comparison with a relative of Pt6 who underwent HCT following reduced chemotherapy showed better thymopoiesis and faster B-cell and metabolic reconstitution following conditioning [48]. In the JAK3-deficient (Pt5) patient most B-cells remained of host origin and thus non-functional. In the small fraction of donor B-cells γc-dependent cytokine signaling was intact and the cells were perfectly functional, able to differentiate in switched B-cell and produce immunoglobulin. Indeed, in both patients purified switched memory B-cell were predominant of donor origin indicating a strong selective pressure for normal cells during final stages of B-development. According to our experience, if the method of detecting donor chimerism is not sufficiently sensitive [21], it could understimate the presence of chimerism in small subsets. Thus, for genetic defects expected to lead to selective advantage, it is important to investigate the percentage of donor-derived B-cells in sorted switched cell subsets. In addition to the underlying genetic disease, the donor source has been an additional factor associated to humoral recovery that is influenced by the use of non-HLA-identical donors. We did not find major difference between MSD and MUD in terms of B-cell recovery kinetic, although the two group are too small to draw definitive conclusions. The worse outcome was detected in 2 patients who underwent transplant from a MMRD and showed persisting low memory B-cell (both IgM memory and switched B-cell), did not respond properly to CpG and needed IgRT. In this regard, the use of alternative methods of T-cell depletion has greatly improved the outcome of MMRD transplant and kinetics of T-cell reconstitution and could provide also enhanced humoral immunity long-term [6].

Conclusions

B-cell reconstitution remains a significant issue after HCT for PID with important implications. Since the recovery of switched B-cells and their capacity to produce Ig in vitro corresponds to ability to mount an effective antibody response to antigens and to vaccinations, in patients with absent memory B-cells or abnormal functional in vitro tests would be safer postpone the IgRT discontinuation. Even if our cohort is too small to give general reccomandations more thorough analyses of B cell function and distinct B-cell phenotype (IgM memory and switched memory) are recommended. Moreover, due to the delay in IgM memory recovery, the patients could benefit from long-term antibiotic prophylaxis and a careful anti-pneumococcus immunization. Multicenter studies exploiting standardised methodology seem therefore necessary to better define the determinants of B-cell recovery in different form of PID and allow the development of new algorithms for management and follow-up of these critical children.

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References

1. Fischer A. Human primary immunodeficiency diseases. Immunity. 2007;27(6):835–45.
2. Porta F, Forino C, De Martiis D, Soncini E, Notarangelo L, Tettoni K, et al. Stem cell transplantation for primary immunodeficiencies. Bone Marrow Transplant. 2008;41 Suppl 2:S83–6.
3. Rappeport JM, O'Reilly RJ, Kapoor N, Parkman R. Hematopoietic stem cell transplantation for severe combined immune deficiency or what the children have taught us. Hematology/Oncol Clin North America. 2011;25(1):17–30.
4. Gennery AR, Slatter MA, Grandin L, Taupin P, Cant AJ, Veys P, et al. Transplantation of hematopoietic stem cells and long-term survival for primary immunodeficiencies in Europe: entering a new century, do we do better? J Allergy Clin Immunol. 2010;126(3):602–10 e1-11.

5. Veys P. Reduced intensity transplantation for primary immunodeficiency disorders. Immunol Allergy Clin North Am. 2010;30(1):103–24.
6. Bertaina A, Merli P, Rutella S, Pagliara D, Bernardo ME, Masetti R, et al. HLA-haploidentical stem cell transplantation after removal of alphabeta+T and B cells in children with nonmalignant disorders. Blood. 2014;124(5):822–6.
7. Rao K, Amrolia PJ, Jones A, Cale CM, Naik P, King D, et al. Improved survival after unrelated donor bone marrow transplantation in children with primary immunodeficiency using a reduced-intensity conditioning regimen. Blood. 2005;105(2):879–85.
8. Antoine C, Muller S, Cant A, Cavazzana-Calvo M, Veys P, Vossen J, et al. Long-term survival and transplantation of haemopoietic stem cells for immunodeficiencies: report of the European experience 1968–99. Lancet. 2003;361(9357):553–60.
9. Burroughs L, Woolfrey A. Hematopoietic cell transplantation for treatment of primary immune deficiencies. Cell Ther Transplant. 2010;2(8) : [Author Manuscript 1–22].
10. Friedrich W, Honig M, Muller SM. Long-term follow-up in patients with severe combined immunodeficiency treated by bone marrow transplantation. Immunol Res. 2007;38(1–3):165–73.
11. Slatter MA, Brigham K, Dickinson AM, Harvey HL, Barge D, Jackson A, et al. Long-term immune reconstitution after anti-CD52-treated or anti-CD34-treated hematopoietic stem cell transplantation for severe T-lymphocyte immunodeficiency. J Allergy Clin Immunol. 2008;121(2):361–7.
12. Borghans JA, Bredius RG, Hazenberg MD, Roelofs H, Jol-van der Zijde EC, Heidt J, et al. Early determinants of long-term T-cell reconstitution after hematopoietic stem cell transplantation for severe combined immunodeficiency. Blood. 2006;108(2):763–9.
13. Cavazzana-Calvo M, Carlier F, Le Deist F, Morillon E, Taupin P, Gautier D, et al. Long-term T-cell reconstitution after hematopoietic stem-cell transplantation in primary T-cell-immunodeficient patients is associated with myeloid chimerism and possibly the primary disease phenotype. Blood. 2007;109(10):4575–81.
14. Patel DD, Gooding ME, Parrott RE, Curtis KM, Haynes BF, Buckley RH. Thymic function after hematopoietic stem-cell transplantation for the treatment of severe combined immunodeficiency. N Engl J Med. 2000;342(18):1325–32.
15. Avanzini MA, Locatelli F, Dos Santos C, Maccario R, Lenta E, Oliveri M, et al. B lymphocyte reconstitution after hematopoietic stem cell transplantation: functional immaturity and slow recovery of memory CD27+ B cells. Exp Hematol. 2005;33(4):480–6.
16. D'Orsogna LJ, Wright MP, Krueger RG, McKinnon EJ, Buffery SI, Witt CS, et al. Allogeneic hematopoietic stem cell transplantation recipients have defects of both switched and igm memory B cells. Biol Blood Marrow Transplant. 2009;15(7):795–803.
17. Griffith LM, Cowan MJ, Kohn DB, Notarangelo LD, Puck JM, Schultz KR, et al. Allogeneic hematopoietic cell transplantation for primary immune deficiency diseases: current status and critical needs. J Allergy Clin Immunol. 2008;122(6):1087–96.
18. Griffith LM, Cowan MJ, Notarangelo LD, Kohn DB, Puck JM, Pai SY, et al. Primary Immune Deficiency Treatment Consortium (PIDTC) report. J Allergy Clin Immunol. 2014;133(2):335–47.
19. Liu A, Vosshenrich CA, Lagresle-Peyrou C, Malassis-Seris M, Hue C, Fischer A, et al. Competition within the early B-cell compartment conditions B-cell reconstitution after hematopoietic stem cell transplantation in nonirradiated recipients. Blood. 2006;108(4):1123–8.
20. White H, Thrasher A, Veys P, Kinnon C, Gaspar HB. Intrinsic defects of B cell function in X-linked severe combined immunodeficiency. Eur J Immunol. 2000;30(3):732–7.
21. Recher M, Berglund LJ, Avery DT, Cowan MJ, Gennery AR, Smart J, et al. IL-21 is the primary common gamma chain-binding cytokine required for human B-cell differentiation in vivo. Blood. 2011;118(26):6824–35.
22. Haddad E, Le Deist F, Aucouturier P, Cavazzana-Calvo M, Blanche S, De Saint BG, et al. Long-term chimerism and B-cell function after bone marrow transplantation in patients with severe combined immunodeficiency with B cells: A single-center study of 22 patients. Blood. 1999;94(8):2923–30.
23. Mazzolari E, Forino C, Guerci S, Imberti L, Lanfranchi A, Porta F, et al. Long-term immune reconstitution and clinical outcome after stem cell transplantation for severe T-cell immunodeficiency. J Allergy Clin Immunol. 2007;120(4):892–9.
24. Haddad E, Leroy S, Buckley RH. B-cell reconstitution for SCID: should a conditioning regimen be used in SCID treatment? J Allergy Clin Immunol. 2013;131(4):994–1000.
25. Buckley RH. B-cell function in severe combined immunodeficiency after stem cell or gene therapy: a review. J Allergy Clin Immunol. 2010;125(4):790–7.
26. Railey MD, Lokhnygina Y, Buckley RH. Long-term clinical outcome of patients with severe combined immunodeficiency who received related donor bone marrow transplants without pretransplant chemotherapy or post-transplant GVHD prophylaxis. J Pediatr. 2009;155(6):834–40. 6.
27. Xie M, Fu HX, Chang YJ, Xu LP, Liu DH, Zhang XH, et al. Characteristics and influencing factors of CD19+ B cell reconstitution in patients following haploidentical/mismatched hematopoietic stem cell transplantation. Int J Hematol. 2012;96(1):109–21.
28. Chan WY, Roberts RL, Moore TB, Stiehm ER. Cord blood transplants for SCID: better B-cell engraftment? J Pediatr Hematol Oncol. 2013;35(1):e14–8.
29. Buckley RH, Win CM, Moser BK, Parrott RE, Sajaroff E, Sarzotti-Kelsoe M. Post-transplantation B cell function in different molecular types of SCID. J Clin Immunol. 2013;33(1):96–110.
30. Finocchi A, Romiti ML, Di Cesare S, Puliafito P, Pensieroso S, Rana I, et al. Rapid T-cell receptor CD4+ repertoire reconstitution and immune recovery in unrelated umbilical cord blood transplanted pediatric leukemia patients. J Pediatr Hematol Oncol. 2006;28(7):403–11.
31. Aiuti A, Cattaneo F, Galimberti S, Benninghoff U, Cassani B, Callegaro L, et al. Gene therapy for immunodeficiency due to adenosine deaminase deficiency. N Engl J Med. 2009;360(5):447–58.
32. Capolunghi F, Cascioli S, Giorda E, Rosado MM, Plebani A, Auriti C, et al. CpG drives human transitional B cells to terminal differentiation and production of natural antibodies. J Immunol. 2008;180(2):800–8.
33. Neven B, Leroy S, Decaluwe H, Le Deist F, Picard C, Moshous D, et al. Long-term outcome after hematopoietic stem cell transplantation of a single-center cohort of 90 patients with severe combined immunodeficiency. Blood. 2009;113(17):4114–24.
34. Ugazio AGDM, Notarangelo LD, Plebani A, Porta F. Il bambino immunodepresso. Perché lo è e come va difeso. 2nd ed. Casa Editrice Ambrosiana: Milan; 1995.
35. Ljungman P, Engelhard D, de la Camara R, Einsele H, Locasciulli A, Martino R, et al. Vaccination of stem cell transplant recipients: recommendations of the Infectious Diseases Working Party of the EBMT. Bone Marrow Transplant. 2005;35(8):737–46.
36. Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood. 2003;101(11):4500–4.
37. Corre E, Carmagnat M, Busson M, de Latour RP, Robin M, Ribaud P, et al. Long-term immune deficiency after allogeneic stem cell transplantation: B-cell deficiency is associated with late infections. Haematologica. 2010;95(6):1025–9.
38. Greinix HT, Pohlreich D, Kouba M, Kormoczi U, Lohmann I, Feldmann K, et al. Elevated numbers of immature/transitional CD21- B lymphocytes and deficiency of memory CD27+ B cells identify patients with active chronic graft-versus-host disease. Biol Blood Marrow Transplant. 2008;14(2):208–19.

39. Brigida I, Sauer AV, Ferrua F, Giannelli S, Scaramuzza S, Pistoia V, et al. B-cell development and functions and therapeutic options in adenosine deaminase-deficient patients. J Allergy Clin Immunol. 2014;133(3):799–806. 3.
40. Marie-Cardine A, Divay F, Dutot I, Green A, Perdrix A, Boyer O, et al. Transitional B cells in humans: characterization and insight from B lymphocyte reconstitution after hematopoietic stem cell transplantation. Clin Immunol. 2008;127(1):14–25.
41. Suryani S, Tangye SG. Therapeutic implications of advances in our understanding of transitional B-cell development in humans. Expert Rev Clin Immunol. 2010;6(5):765–75.
42. Bemark M, Holmqvist J, Abrahamsson J, Mellgren K. Translational Mini-Review Series on B cell subsets in disease. Reconstitution after haematopoietic stem cell transplantation - revelation of B cell developmental pathways and lineage phenotypes. Clin Exp Immunol. 2012;167(1):15–25.
43. Storek J, Ferrara S, Ku N, Giorgi JV, Champlin RE, Saxon A. B cell reconstitution after human bone marrow transplantation: recapitulation of ontogeny? Bone Marrow Transplant. 1993;12(4):387–98.
44. Kruetzmann S, Rosado MM, Weber H, Germing U, Tournilhac O, Peter HH, et al. Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen. J Exp Med. 2003;197(7):939–45.
45. Tangye SG, Tarlinton DM. Memory B cells: effectors of long-lived immune responses. Eur J Immunol. 2009;39(8):2065–75.
46. Mooster JL, Le Bras S, Massaad MJ, Jabara H, Yoon J, Galand C, et al. Defective lymphoid organogenesis underlies the immune deficiency caused by a heterozygous S32I mutation in IkappaBalpha. J Exp Med. 2015.
47. Hassan A, Booth C, Brightwell A, Allwood Z, Veys P, Rao K, et al. Outcome of hematopoietic stem cell transplantation for adenosine deaminase-deficient severe combined immunodeficiency. Blood. 2012;120(17):3615–24. quiz 26.
48. Cancrini C, Ferrua F, Scarselli A, Brigida I, Romiti ML, Barera G, et al. Role of reduced intensity conditioning in T-cell and B-cell immune reconstitution after HLA-identical bone marrow transplantation in ADA-SCID. Haematologica. 2010;95(10):1778–82.