Type 1 regulatory T cells are associated with persistent split erythroid/lymphoid chimerism after allogeneic hematopoietic stem cell transplantation for thalassemia

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ABSTRACT

Background
Thalassemia major can be cured with allogeneic hematopoietic stem cell transplantation. Persistent mixed chimerism develops in around 10% of transplanted thalassemic patients, but the biological mechanisms underlying this phenomenon are poorly understood.

Design and Methods
The presence of interleukin-10-producing T cells in the peripheral blood of eight patients with persistent mixed chimerism and five with full donor chimerism was investigated. A detailed characterization was then performed, by T-cell cloning, of the effector and regulatory T-cell repertoire of one patient with persistent mixed chimerism, who developed stable split erythroid/lymphoid chimerism after a hematopoietic stem cell transplant from an HLA-matched unrelated donor.

Results
Higher levels of interleukin-10 were produced by peripheral blood mononuclear cells from patients with persistent mixed chimerism than by the same cells from patients with complete donor chimerism or normal donors. T-cell clones of both host and donor origin could be isolated from the peripheral blood of one, selected patient with persistent mixed chimerism. Together with effector T-cell clones reactive against host or donor alloantigens, regulatory T-cell clones with a cytokine secretion profile typical of type 1 regulatory cells were identified at high frequencies. Type 1 regulatory cell clones, of both donor and host origin, were able to inhibit the function of effector T cells of either donor or host origin *in vitro*.

Conclusions
Overall these results suggest that interleukin-10 and type 1 regulatory cells are associated with persistent mixed chimerism and may play an important role in sustaining long-term tolerance *in vivo*. These data provide new insights into the mechanisms of peripheral tolerance in chimeric patients and support the use of cellular therapy with regulatory T cells following hematopoietic stem cell transplantation.

Key words: thalassemia, hematopoietic stem cell transplantation, persistent mixed chimerism, tolerance, type 1 regulatory T (Tr1) cells.

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Introduction

β thalassemia major is a genetic disease characterized by the absence or reduced production of the hemoglobin β chain. At present, allogeneic hematopoietic stem-cell transplantation (HSCT) represents the only cure for this and other hemoglobinopathies.[1-3] The incidence, early after the transplant (3-6 months after HSCT), of complete donor chimerism, with the presence of only donor-derived cells in the bone marrow and peripheral blood of transplanted patients, is around 70%. If this condition remains stable for at least 1 year after HSCT, the engraftment status of patients with complete chimerism never subsequently changes.[1,2]

The presence of mixed chimerism, i.e., the co-existence of donor and host cells in the recipient, is not a rare event following a successful transplant.[4] Although mixed chimerism represents a risk factor for graft rejection if it occurs within the first months after the transplant (transient mixed chimerism),[5] when the co-existence of donor and host cells persists for a period longer than 2 years (persistent mixed chimerism [PMC]), patients remain blood-transfusion independent, similar to those with full donor engraftment. Approximately 10% of thalassemic patients undergoing HLA-identical HSCT will develop PMC, with the proportion of residual host cells ranging from 10% to 75%.[5,6] To date, the mechanisms responsible for the induction and maintenance of PMC after HSCT are unknown.

We previously described that mixed chimerism is not always sustained by clonal deletion[7,8] and peripheral mechanisms of tolerance, mediated by regulatory T cells (Treg), have often been associated with the maintenance of post-transplant homeostasis despite the allogeneic disparities.[9] Several kinds of Treg cells have been described, such as $CD4^+CD25^+$, Tr1, Th3, $CD3^+CD4^-CD8^-$, $CD8^+$, and natural killer T cells.[10-12] Among the $CD4^+$ T-cell subset, $CD4^+CD25^+$ Treg cells and type 1 regulatory T (Tr1) cells are the best defined. Natural $CD4^+CD25^+$ Treg cells arise in the thymus and play an important role in the mechanism of self-tolerance;[13,14] many reports indicate that these cells are also involved in transplantation tolerance.[15] Tr1 cells differentiate in the periphery upon priming of naïve T-cell precursors with antigen in the presence of interleukin (IL)-10. They produce high levels of IL-10, transforming growth factor (TGF)-β, and IL-5; low amounts of interferon (IFN)-γ and IL-2; and no IL-4. Tr1 cells suppress both naïve and memory T-cell responses *in vivo* and *in vitro*, through the secretion of IL-10 and TGF-β.[16,17] The first suggestion that human Tr1 cells are involved in maintaining peripheral tolerance *in vivo* came from studies in patients with severe combined immunodeficiency (SCID) successfully transplanted with HLA-mismatched allogeneic fetal liver stem cells. In the absence of immunosuppressive therapy, these patients did not develop graft-versus-host disease (GvHD). Interestingly, high levels of IL-10 mRNA were detected *in vivo* in T cells and monocytes of these patients, and a significant proportion of donor-derived T cells, which were specific for host HLA-antigens and produced high levels of IL-10, could be isolated *in vitro*.[7] These findings initially prompted us to hypothesize about a possible IL-10–mediated regulatory role for these T cells *in vivo*. However, the presence of a high number of IL-10–producing T cells was not detected following HLA-haploidentical bone marrow HSCT.[8] Therefore, it remained to be clarified whether the source of stem cells or the absence of post-transplant immunosuppression was responsible for the induction of these T cells in the SCID human split chimera. The correlation between IL-10 and the absence of GvHD or transplant-related complications after HSCT[18-20] and the importance of IL-10–producing T cells for the development of tolerance to the graft in solid organ transplantation[21,22] has been widely demonstrated since then.

In this study, we measured the levels of IL-10 produced by peripheral blood mononuclear cells (PBMC) of several thalassemic patients with PMC and characterized the effector T and Tr1 cells of one thalassemic patient who developed PMC after an HLA-matched unrelated HSCT.

Design and Methods

Patients

Fourteen non-consecutive patients undergoing HSCT between 1998 and 2004 for transfusion-dependent β-thalassemia were analyzed. The patients' characteristics and indications for transplantation are shown in Table 1. Their median age was 3 years and 8 months (range, 2-8 years). Risk class according to Pesaro[2] was class I in eight patients, class II in three and class III in three others. Twelve patients were transplanted from an HLA genotypically identical sibling, one from an HLA phenoidentical related donor and one from a 12/12 allele-level HLA-matched unrelated donor. All patients received a

Table 1. Characteristics of patients, donors and transplants.

Patient N.	Risk class[a]	Host/ donor gender	Donor[b]	Time post-transplant (years)	Outcome[c]	Donor cells in PB (%)[d]	GvHD
1	2	M/M	MUD	8	PMC	50	no
2	1	M/M	Pheno	6	PMC	50	Gr.3 (day 15)
3	1	F/M	Sib	2	PMC	70	no
4	1	F/F	Sib	9	PMC	75	no
5	1	M/M	Sib	4	PMC	90	no
6	1	F/F	Sib	2	PMC	76	no
7	1	F/M	Sib	3	PMC	44	no
8	1	F/M	Sib	3	PMC	87	no
9	3	F/F	Sib	10	PMC	75	no
10	3	M/F	Sib	1	CC	100	Gr.2 (day 28)
11	3	M/F	Sib	1	CC	100	no
12	2	M/F	Sib	1	CC	100	Gr.2 (day 30)
13	1	M/F	Sib	2	CC	100	no
14	2	F/M	Sib	1	CC	100	Gr.1 (day 15)

[a]According to the Pesaro classification.[b]The source of donor stem cells was bone marrow for all the patients. PB: peripheral blood; GvHD: graft-versus-host disease; MUD: matched unrelated donor; Pheno: phenoidentical family donor; Sib: sibling HLA identical donor.[c]PMC: persistent mixed chimerism; CC: complete chimerism; [d]determined by short tandem repeat typing.

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myeloablative conditioning regimen followed by infusion of unmanipulated bone marrow cells. Patients in risk class I-II (including patient #1 transplanted from an unrelated donor) were given conditioning based on oral busulfan 14 mg/kg and cyclophosphamide 200 mg/kg. In addition to this, patients aged less than 4 years old were conditioned with thiotepa 10 mg/kg. Patients in risk class III were conditioned with busulfan 14 mg/kg associated with reduced-dose cyclophosphamide 160 mg/kg. Patient #11 received a HSCT conditioned with busulfan 14 mg/kg, cyclophosphamide 200 mg/kg, thiotepa 10 mg/kg and antithymocyte globulin (Thymoglobulin; Genzyme- Sangstat, Lyon, France) 8 mg/kg following rejection of a first HSCT. Post-HSCT GvHD prophylaxis consisted of cyclosporine, methylprednisolone and a short course of methotrexate.[23] Cyclosporine was started at a dose of 5 mg/kg intravenously on day -2 through to day +5 then reduced to 3 mg/kg and tapered from day +60 by 5%/week until complete withdrawal at day +365. The desired plasma range was 150-250 ng/mL. Methylprednisolone was started at a dose of 0.5 mg/kg intravenously on day -1 and stopped at day +30. Short-course methotrexate was given at a dose of 10 mg/m^2 intravenously (on days +1, +3 and +6) with folinic acid rescue. Chimerism was determined and the frequency of IL-10-producing T cells analyzed in the peripheral blood of patients over a wide period of time (from 1 to 10 years after the transplant). Three out of five (60%) patients with complete chimerism and only one out of nine (11%) patients with PMC developed GvHD. The study was approved by the Ethical Committee of the Policlinico Tor Vergata, Rome. Informed consent from patients was obtained according to institutional guidelines.

Evaluation of mixed chimerism

Short tandem repeat typing for nucleated cells

Recipient and donor DNA samples were typed by short tandem repeat loci and the amelogenin locus using the AmpFISTR Profiler Plus kit (Applera, Foster City, CA, USA). Amplification reactions were carried out using 1-2 ng of DNA following the manufacturer's instruction. Poymerase chain reaction (PCR) products were electrophoresed on an ABI Prism 3130xl Genetic Analyzer (Applera). Informative loci in post-transplant samples were screened to quantify the percentage of donor cells in mixed chimerism. A method based on the ratio between peak areas of donor and recipient alleles was used for this quantitative determination.[24]

Fluorescence-activated cell sorting analysis of erythrocytes

Differences in the blood groups of the patients and donors were evaluated by sequence-specific primed PCR analysis in Rhesus alleles using specific primers. The presence of donor and/or recipient erythrocytes was determined by indirect flow cytometry analysis using a series of monoclonal antibodies directed against Rhesus antigens - D, C, c, E, e - (Institute Jacques Boys SA, Reims Cedex, France) in fresh peripheral blood following the manufacturer's instructions.

Establishment of T-cell clones

PBMC from patients and normal donors were isolated by centrifugation over Ficoll-Hypaque gradients (Nycomed Amersham, Uppsala, Sweden). CD4$^+$ T cells were purified from PBMC by negative selection using the CD4$^+$ T-cell isolation kit (Miltenyi Biotech, Auburn, CA, USA) according to the manufacturer's instructions. T-cell clones were obtained from CD4$^+$ cells by limiting dilution at 0.3 cells/well in the presence of a feeder cell mixture and soluble anti-CD3 monoclonal antibody (1 μg/mL, OKT3, Jansen-Cilag, Raritan, NJ, USA) in X-vivo 15 medium (BioWhittaker, Verviers, Belgium) supplemented with 5% pooled human AB serum (BioWhittaker), 100 U/mL penicillin/streptomycin (Bristol-Myers Squibb, Sermoneta, Italy). At day 3, IL-2 (40 U/mL, Chiron Italy, Milan, Italy) was added. T-cell clones were restimulated every 14 days with feeder cell mixture and soluble anti-CD3 monoclonal antibody (1 μg/mL). Between stimulations with feeder cells, T-cell clones were expanded with IL-2 (40 U/mL). Once the T-cell clones had been established, at every change of medium IL-15 (5 ng/mL, R&D System, Minneapolis, MN, USA) was added as a Tr1 growth factor.[25]

Cytokine detection

To determine the cytokine production after polyclonal activation, T-cell clones (1×10^6 cells/mL) were activated with immobilized anti-CD3 (10 μg/mL) and soluble anti-CD28 (1 μg/mL, PharMingen, San Diego, CA, USA) monoclonal antibodies. Supernatants were collected after 24 h for assessment of IL-2, and after 48 h for IL-4, IL-10, and IFN-γ. Cytokine production was determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (PharMingen). To test cytokine production after antigen-specific activation, T-cell clones (1×10^6 cells/mL) were plated with 1×10^5/mL mature allogeneic dendritic cells. Cytokine levels in cell culture supernatants were detected using the human Th1/Th2 cytokine cytometric bead array system (BD Biosciences, San Jose, CA, USA). For intracellular cytokine production, T-cell clones were activated with phorbol myristate acetate (10 ng/mL, Calbiochem) and anti-CD3 monoclonal antibody (10 μg/mL) for 6 h. Brefeldin A (10 mg/mL, Sigma) was added for the final 3 h. Cells were fixed with formaldehyde, permeabilized in saponin buffer [phosphate-buffered saline (PBS) 2% fetal calf serum (FCS) and 0.5% saponin, Sigma, Italy] and stained with phycoerythrin (PE)–labeled anti–IL-2, anti–IL-4, anti–IL-10 and fluorescein isothiocyanate (FITC)–labeled anti–IFN-γ monoclonal antibodies (BD Pharmingen). Total PBMC were activated with phorbol myristate acetate and ionomycin (150 ng/mL, Sigma) for 12 h in the presence of brefeldin A. Cells were fixed and permeabilized with FOXP3 Fix/Perm buffer set (Biolegend) and stained with PE-labeled anti-IL-10 monoclonal antibody. When appropriate, data were analyzed by Student's t test.

Flow cytometry analysis

For the detection of cell surface antigens, T cells were stained with monoclonal antibodies against CD3, CD4, CD8, CD25, CD16, CD56, CD19, and CD14

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(PharMingen or BD Biosciences). Cells were incubated with the monoclonal antibody for 20 min at 4°C in PBS 2% FCS, washed twice and fixed with 0.2% formaldehyde. To determine the expression of granzyme-A and granzyme-B (BD Bioscience and PharMingen), after surface staining, cells were fixed, permeabilized in saponin buffer and stained with granzyme-A and granzyme-B monoclonal antibodies. Intracytoplasmic staining for human Foxp3 was performed using the anti-Foxp3 staining kit (Biolegend, San Diego, CA, USA), according to the manufacturer's instructions.

Enzyme-linked immunospot assay

IL-10-secreting T cells were enumerated by a enzyme-linked immunospot (ELISPOT) assay. Fresh PBMC were plated at 10^5/well in ELISPOT plates (Millipore, Bedford, MA, USA) coated with an anti–IL-10 capture monoclonal antibody (clone M010, Endogen, Pierce, Rockford, USA). After 48 h of incubation, plates were washed and IL-10-producing cells were detected by the anti–IL-10 detection monoclonal antibody (clone M011B, Endogen, Pierce). Spots were counted by a KS ELISPOT system (Zeiss Vision, Göttingen, Germany).

Suppression assays

Responder cells were stimulated alone or in the presence of T-cell clones (1:1 ratio) in 96-well round-bottom plates with immobilized anti-CD3 (10 μg/mL) and soluble anti-CD28 (1 μg/mL) monoclonal antibodies. Patients' PBMC, used as responder cells, derived from unseparated post-transplant samples, therefore included both host and donor cells. After 5 days of culture, supernatants were collected for analysis of IFN-γ and TNF-α production using the cytrometric bead assay system (BD Biosciences). To test the suppressive capacity of Tr1 cell clones by flow cytometry, patients' PBMC were labeled with 5-(and-6)-carboxy fluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA) before stimulation with anti-CD3 and anti-CD28 monoclonal antibodies. After 5 days of culture, proliferation of CFSE-labeled PBMC was determined by flow cytometry, gating the responder cells for $CD4^+$ or $CD4^-$ cells.

To test the role of endogenous IL-10 in inhibiting T-cell proliferation, PBMC were stimulated with allogeneic mature dendritic cells (ratio of T cells to dendritic cells, 10:1) with or without anti-IL10R monoclonal antibody (50 μg/mL, 3F9, R&D Systems) in complete medium in 96-well round-bottom plates. After 4 days of culture, wells were pulsed for 16 h with 1 μCi/well of ^{3}H-thymidine.

Results

High frequency of interleukin-10-producing T cells in the peripheral blood of patients with persistent mixed chimerism

The cytokine production profile of freshly isolated PBMC from transplanted patients with PMC, was determined and compared to that of patients who developed complete donor chimerism following HSCT. Five out of eight patients with PMC were analyzed early after the establishment of chimerism (from 2 to 4 years after the transplant), whereas the other PMC patients were tested at later time points (from 6 to 10 years after the transplant). The percentage of $CD4^+$ IL-10-producing T cells after phorbol myristate acetate/ionomycin stimulation is shown in Figure 1A. The proportion of IL-10-producing T cells detected in patients with PMC (n=7) was higher

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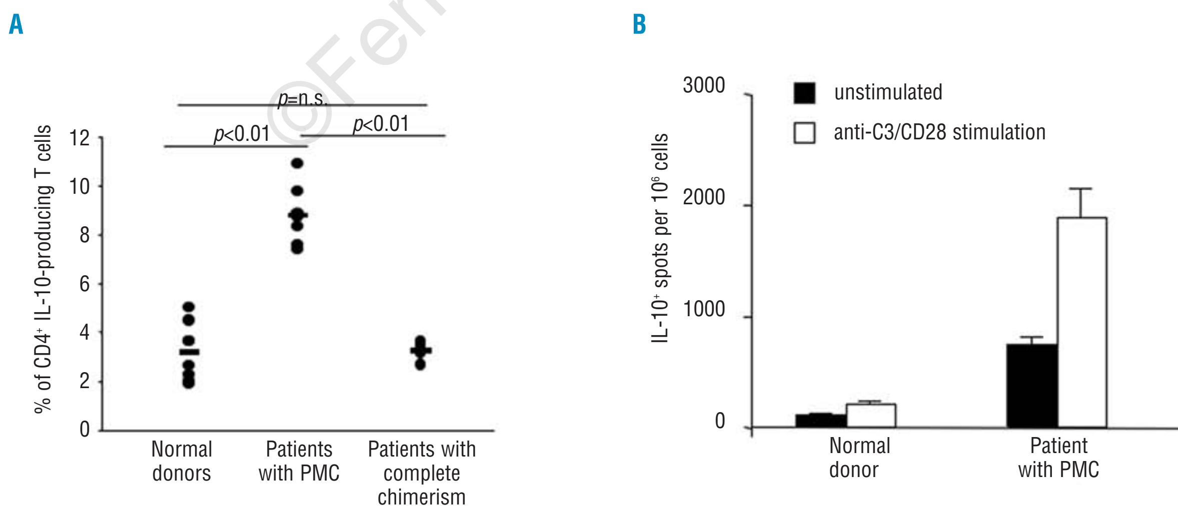

Figure 1. IL-10 production by T cells of transplanted thalassemic patients with persistent mixed chimerism (PMC). (A) Cytokine production by PBMC of patients with PMC (n=7), patients with complete chimerism (n=5), and normal donors (n=8), was determined by intracytoplasmic staining after polyclonal activation with phorbol myristate acetate/ionomycin. The percentages of IL-10-producing T cells within the gated $CD4^+$ T cells for each patient and normal donors (●), and the corresponding averages (▬) are indicated in the graph. (B) IL-10-producing cells of a patient with PMC and of a normal donor were counted by ELISPOT. The IL-10-positive spots were measured in the unstimulated cultures and after anti-CD3/CD28 stimulation.

than that in patients with complete chimerism (n=5) and in normal donors (n=8), with a statistically significant difference between the IL-10 levels measured in PMC patients and in patients with compete chimerism ($p<0.01$) or in normal donors ($p<0.01$). No significant differences in the production of other cytokines (IL-2, IL-4, and IFN-γ) were observed between patients with PMC or complete chimerism or normal donors (*data not shown*).

A higher number of IL-10-producing T cells could also be detected prior to stimulation, and upon T-cell receptor (TCR)-mediated activation, in a PMC patient than in a normal donor, as shown in Figure 1B. These data indicate that constitutive and induced high IL-10 production could be detected specifically in tolerant transplanted chimeric patients, independently of the time after the transplant.

Kinetics of persistent mixed chimerism

We further characterized the peripheral T-cell repertoire in patients with PMC following HSCT. One patient with PMC, who had received a transplant from a matched unrelated donor 8 years earlier, was extensively studied for the presence and function of IL-10–producing Tr1 cells. The patient was in good clinical condition, with stable normal values for white and red blood cell counts ($8.450\times10^9\pm2.069\times10^9$/L and $4\times10^{12}\pm0.17\times10^{12}$/L, respectively), hemoglobin concentration (11.4±0.1 g/dL), and percentage of reticulocytes (1.5±0.3), over the past 4 years. The absolute lymphocyte count and the proportions of T cells ($CD3^+$, $CD4^+$, $CD8^+$), B cells ($CD19^+$), monocytes ($CD14^+$), and NK cells ($CD16^+/CD56^+$), determined 76, 91, and 101 months after the transplant, were comparable to those in normal donors (*data not shown*). In addition, $CD4^+CD25^+$ and $CD4^+CD25^+Foxp3^+$ T cells were present in normal proportions (18% and 5%, respectively) (*data not shown*). Shortly after the HSCT, the patient showed complete donor chimerism, but 1 year following the transplant, the donor cells began to decrease both in the bone marrow and in the peripheral blood, declining to 50% by 36 months after HSCT and remaining stable 8 years following the transplant (Figure 2A). In parallel with the presence of this large proportion of residual host cells in the peripheral blood, the amount of donor β globin remained stable between 80% and 100% (Figure 2A) and the α/non-α globin ratio ranged between 1.21–1.7 (*data not shown*). The proportion of donor T lymphocytes, both in the $CD4^+$ and $CD4^-$ sub-populations, ranged between 40% and 50%, 76 and 91 months after the transplant. These results were similar to those observed 101 months after HSCT, with the proportions of donor $CD3^+$, $CD19^+$, and $CD56^+$ cells being 50%, 25%, and 40%, respectively (Figure 2B). Interestingly, despite the low percentage of donor cells in the lymphoid lineages, the proportion of donor erythrocytes was 85% (Figure 2B). This indicates a predominant donor chimerism in the erythrocyte compartment, associated with split, long-term chimerism in the lymphoid cells.

Characterization of T-cell clones isolated from the peripheral blood of a patient with persistent mixed chimerism

T-cell clones were isolated from two blood samples, taken 76 and 91 months after HSCT, obtained from the PMC patient, from a normal donor and from a thalassemic patient prior to transplantation. Based on their cytokine production profile,[26,27] different subsets of T-cell clones could be identified. The proportions of Th0 ($IL-2^+$, $IL-4^+$, $IL-10^+$, and $IFN-\gamma^+$), Th1 ($IL-2^+$ and $IFN-\gamma^+$), and Th2 ($IL-4^+$ and $IL-10^+$) cell clones were comparable in the patient with PMC, in the normal donor, and in the thalassemic patient prior to HSCT (Figure 3A). According to data from the literature[17,25] and from our laboratory, Tr1 cell clones were defined by a high ratio of IL-10 to IL-4 production. In the present study, we classified a T-cell clone as Tr1 when the IL-10/IL-4 ratio was at least 8. In contrast to the Th cell clones, the proportion of Tr1 cell clones obtained from the T-cell clonings of the PMC patient was consistently higher (28% and 24%) than that from the normal donor (9%)

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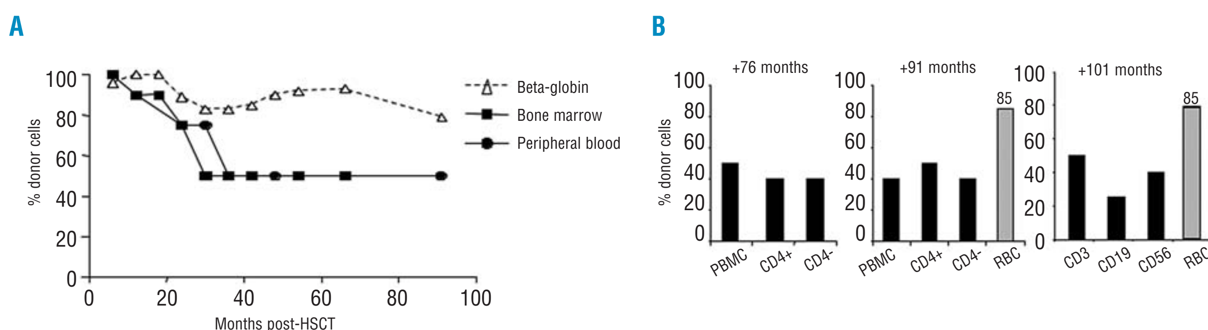

Figure 2. Kinetics of engraftment of a patient with PMC 8 years after HSCT. (A) Long-term stable mixed chimerism was evaluated by short tandem repeat analysis in the peripheral blood and in the bone marrow of the patient. High levels of the adult donor β-globin were found at all time points, as determined by high performance liquid chromatography. (B) Mixed chimerism was evaluated in PBMC and in different lymphoid sub-populations ($CD4^+$, $CD4^-$, $CD3^+$, $CD19^+$, $CD56^+$) isolated from the peripheral blood of the PMC patient, at three different time points: 76 months, 91 months and 101 months after HSCT. Chimerism in the erythroid compartment was determined 91 and 101 months following the transplant.

and the thalassemic patient prior to HSCT (9%) (Figure 3A). The cytokine production by the PMC patient's (upper panel for the first T-cell cloning and central panel for the second) and the normal donor's (lower panel) T-cell clones following TCR-mediated activation is illustrated in Figure 3B. Overall, a higher amount of IL-10 was produced by T-cell clones isolated from the patient with PMC than by those from the normal donor, together with a higher frequency of T-cell clones with elevated IL-10/IL-4, and IL-10/IFN-γ ratios.

Moreover, the absolute amount of IL-10 produced by the Tr1 cell clones of the patient was greater (median level of 7,855 pg/mL, range 967 to 16,996 pg/mL for the first T-cell cloning; median level of 14,668 pg/mL, range 1,208 to 42,732 pg/mL for the second T-cell cloning) than the amount of IL-10 secreted by the Tr1 cell clones

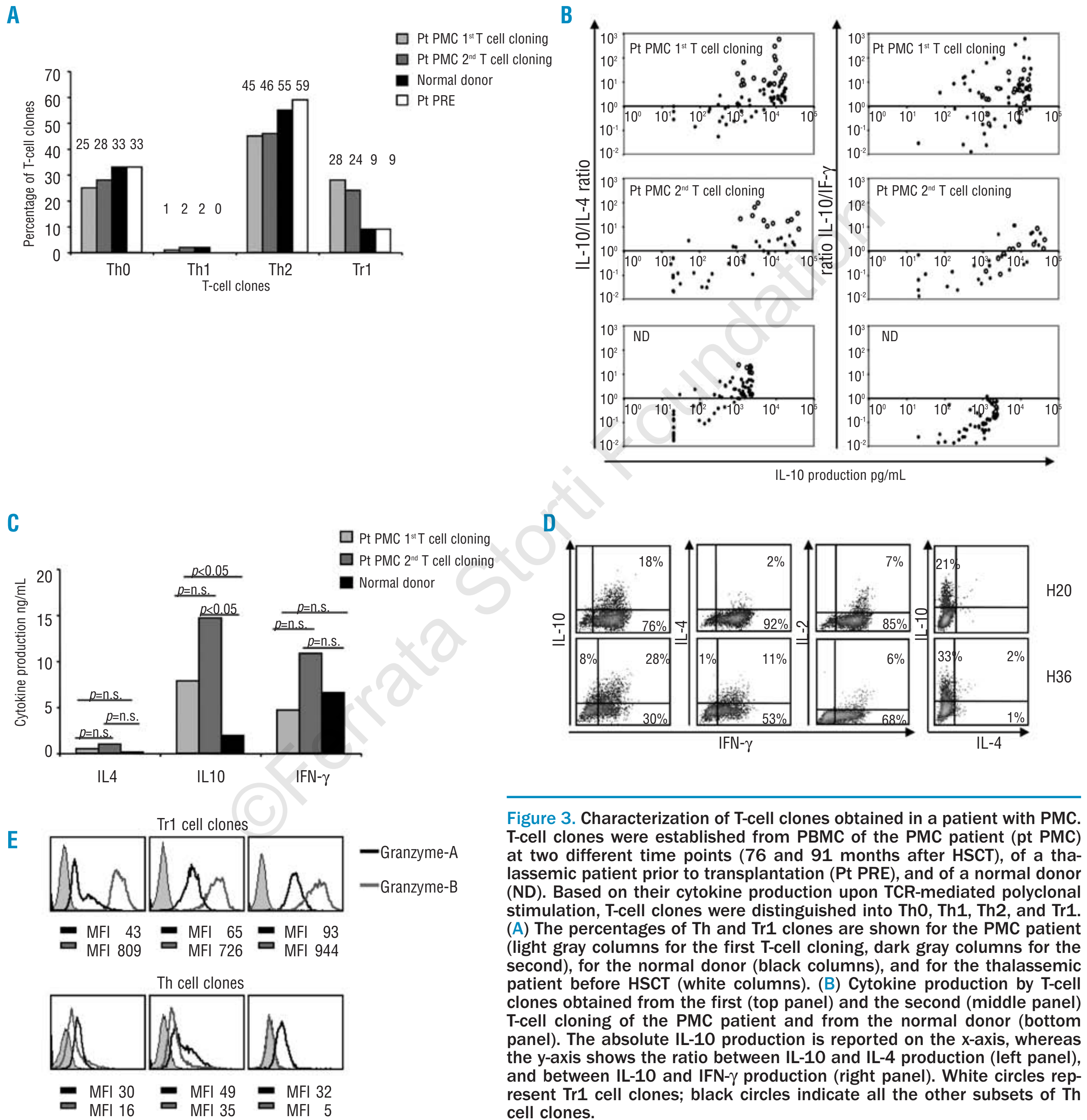

Figure 3. Characterization of T-cell clones obtained in a patient with PMC. T-cell clones were established from PBMC of the PMC patient (pt PMC) at two different time points (76 and 91 months after HSCT), of a thalassemic patient prior to transplantation (Pt PRE), and of a normal donor (ND). Based on their cytokine production upon TCR-mediated polyclonal stimulation, T-cell clones were distinguished into Th0, Th1, Th2, and Tr1. (A) The percentages of Th and Tr1 clones are shown for the PMC patient (light gray columns for the first T-cell cloning, dark gray columns for the second), for the normal donor (black columns), and for the thalassemic patient before HSCT (white columns). (B) Cytokine production by T-cell clones obtained from the first (top panel) and the second (middle panel) T-cell cloning of the PMC patient and from the normal donor (bottom panel). The absolute IL-10 production is reported on the x-axis, whereas the y-axis shows the ratio between IL-10 and IL-4 production (left panel), and between IL-10 and IFN-γ production (right panel). White circles represent Tr1 cell clones; black circles indicate all the other subsets of Th cell clones.

(C) Average values of IL-4, IL-10 and IFN-γ produced by Tr1 cell clones of the PMC patient (light gray columns for the first T-cell cloning [n=20], dark gray columns for the second [n=11]) and of the normal donor (black columns [n=7]). (D) Intracellular cytokine production by Tr1 cell clones of the patient with PMC, following TCR-mediated polyclonal activation. Two representative Tr1 cell clones from the second T-cell cloning of the patient are shown. (E) Intracellular expression of granzyme-A (black line) and granzyme-B (gray line) in the patient's T-cell clones. Filled histograms represent the isotype control. The mean fluorescence intensity (MFI) for granzyme-A and granzyme-B is shown. Three representative Tr1 cell clones (top panel) and three Th cell clones (bottom panel) are shown.

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from the normal donor (median level of 1,897 pg/mL, range 1,036 to 2,256 pg/mL), with a statistically significant difference ($p<0.05$) between the first/second T-cell cloning and the normal donor (Figure 3C). Importantly, there were no statistically significant differences among the IL-2, IL-4, and IFN-γ levels secreted by the Tr1 cell clones from the patient and the normal donor (p = n.s.) (Figure 3C), indicating that only IL-10 production was higher in the patient's T-cell clones than in the normal donor's ones. The cytokine production profile of the patient's Tr1 cell clones was confirmed at a single-cell level. Two representative Tr1 cell clones are shown in Figure 3D. A high proportion of cells positive for IL-10 alone or for IL-10 and IFN-γ was observed, while all the cells positive for IL-10 were negative for IL-4 (Figure 3D, right dot plot). Overall, the percentage of cells producing IL-4 and IL-2 was very low.

Further characterization of the patient's Tr1 cell clones showed that expression of membrane CD25 and expression of intracellular Foxp3 were upregulated following TCR-mediated activation, to levels comparable to those detected in activated Th0 and Th2 cell clones (*data not shown*). Interestingly, the levels of granzyme-B expressed by resting Tr1 cell clones were much higher than those expressed by Th2 and Th0 cell clones (Figure 3E), confirming results previously reported by Grossman *et al*. on human Tr1-like cell lines.[28,29] In contrast, we did not observe any difference in granzyme-A expression in either resting Tr1 or Th2/Th0 cell clones (Figure 3E). Comparable numbers of donor- and host-derived T-cell clones were isolated from both patient's T-cell clonings (57% donor-derived and 43% host-derived for the first, 44% donor-derived and 56% host-derived for the second T-cell cloning), consistent with the mixed chimerism detected in the peripheral lymphoid compartment. Interestingly, T-cell clones of donor and host origin were also equally represented in the Tr1 cell subset (55% donor-derived and 45% host-derived within the Tr1 subset for both T-cell clonings), suggesting the presence of both host- and donor-derived Tr1 cells *in vivo*.

Antigen specificity of the patient's T-cell clones

To investigate the antigen specificity of the T-cell clones, mature dendritic cells were used *in vitro* as allogeneic host/donor antigen-presenting cells since HLA matched monocytes are not able to induce T-cell activation and response *in vitro* in the context of matched unrelated compatibility (*Giorgia Serafini, unpublished observation*). In addition, compared with proliferation, cytokine production resulted in a more sensitive read-out when detecting T-cell responses in HLA-identical unrelated donors. Both host-derived T-cell clones reactive to donor mature dendritic cells (Figure 4A, upper panel) and donor-derived T-cell clones reactive to host mature dendritic cells (Figure 4B, upper panel) could be identified. Out of 19 T-cell clones analyzed, 11 did not respond to the dendritic cells, while eight T-cell clones produced several cytokines after antigen-specific activation. Of these eight T-cell clones, four (50%) showed a cytokine production profile typical of Tr1 cell clones. None of the T-cell clones, except one, reactive to host/donor mature dendritic cells produced cytokines in response to third-party mature dendritic cells, indicating their specificity to host or donor allo-antigens (Figure 4A-B lower panel). Although the absolute amount of cytokines produced following antigen-specific stimulation was much lower than that produced by stimulation

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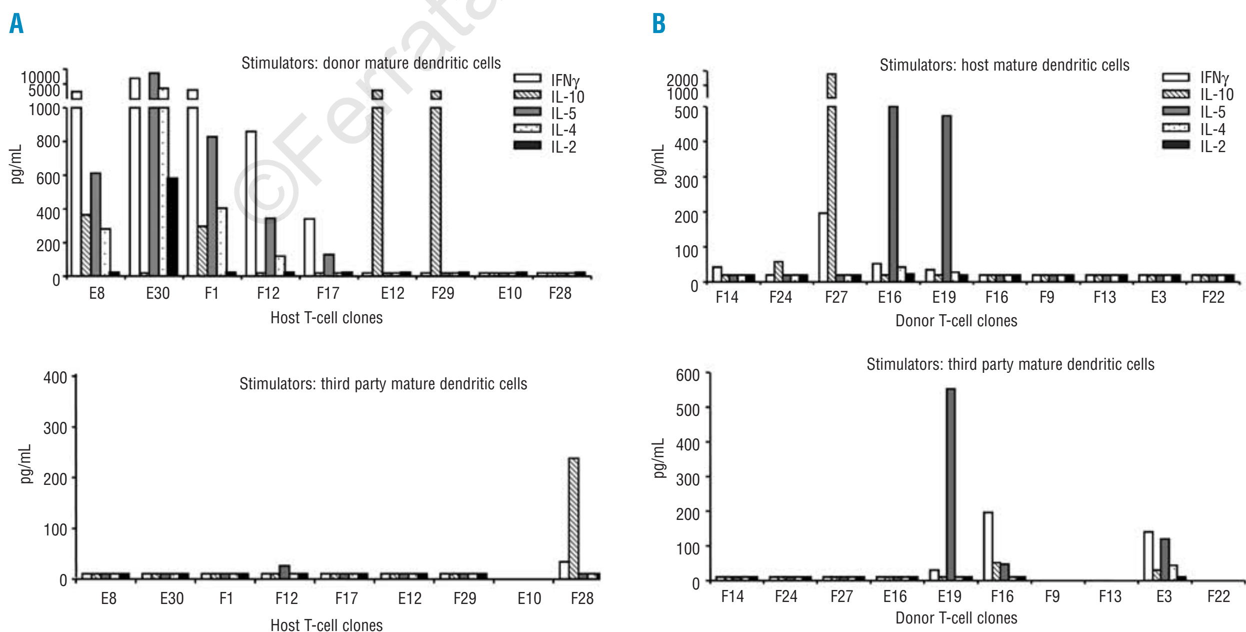

Figure 4. Allo-antigen specificity of the PMC patient's T-cell clones. Cytokine production profile of the patient's host/donor-derived T-cell clones in response to allogeneic donor/host or third party mature dendritic cells. (A) Host-derived T-cell clones were stimulated with donor-derived mature dendritic cells (top panel) and with third party mature dendritic cells (bottom panel). (B) Donor-derived T-cell clones were stimulated with host-derived mature dendritic cells (top panel) and with third party mature dendritic cells (bottom panel).

with anti-CD3 and anti-CD28 monoclonal antibodies, the cytokine profile, useful for defining Tr1 or Th cell clones, was maintained after both types of stimulation (Table 2) in all but one of the T-cell clones tested.

Table 2A. Cytokine production by a patient's T-cell clones after antigen-specific and TCR-mediated polyclonal activation.

Host-derived T-cell clones	Stimulation	IL-10[c] (pg/mL)	IL-4[c] (pg/mL)	IL-2[d] (pg/mL)	IFN-γ[c] (pg/mL)
E8	mDC[a]	364	280	<20	2888
	TCR[b]	17270	9376	4394	2360
E30	mDC[a]	<20	4144	580	8024
	TCR[b]	480	2762	4342	37225
F1	mDC[a]	296	404	<20	3520
	TCR[b]	4750	961	<20	2042
F12	mDC[a]	<20	120	<20	860
	TCR[b]	1973	1111	63	<20
F17	mDC[a]	<20	<20	<20	340
	TCR[b]	13434	1395	<20	6271
E12	mDC[a]	3360	<20	<20	<20
	TCR[b]	1398	21	<20	729
F29	mDC[a]	2988	<20	<20	<20
	TCR[b]	1326	112	0	2786
E10	mDC[a]	<20	<20	<20	<20
	TCR[b]	13703	1315	<20	2490
F28	mDC[a]	<20	<20	<20	<20
	TCRb	9198	30	<20	1975

[a]mature dendritic cells (mDC) are donor-derived cells; [b]TCR indicates polyclonal activation via anti-CD3/28 monoclonal antibodies; [c]cytokines tested after 48 h of stimulation; [d]cytokine tested after 24 h of stimulation; < 20 means under the limit of detection.

Table 2B. Cytokine production by patient's T cell clones after antigen-specific and TCR-mediated polyclonal activation.

Donor-derived T-cell clones	Stimulation	IL-10[c] (pg/mL)	IL-4[c] (pg/mL)	IL-2[d] (pg/mL)	IFN-γ[c] (pg/mL)
F14	mDC[a]	<20	<20	<20	42
	TCR[b]	15388	1080	26	1181
F24	mDC[a]	58	<20	<20	< 20
	TCR[b]	16996	865	<20	3407
F27	mDC[a]	1826	<20	<20	196
	TCR[b]	967	43	<20	344
E16	mDC[a]	<20	42	<20	52
	TCR[b]	324	1380	<20	37
E19	mDC[a]	<20	28	<20	34
	TCR[b]	7584	4560	<20	7229
F16	mDC[a]	<20	<20	<20	< 20
	TCR[b]	8940	1049	<20	1130
F9	mDC[a]	<20	<20	<20	<20
	TCR[b]	16666	4128	117	8491
F13	mDC[a]	<20	<20	<20	<20
	TCR[b]	6934	806	<20	1326
E3	mDC[a]	<20	<20	<20	<20
	TCR[b]	16300	4344	<20	174
F22	mDC[a]	<20	<20	<20	<20
	TCR[b]	14474	4747	137	5950

[a]mature dendritic cells (mDC) are host-derived cells; [b]TCR indicates polyclonal activation via anti-CD3/28 monoclonal antibodies; [c]cytokines tested after 48 h of stimulation; [d]cytokine tested after 24 h of stimulation; <20 means under the limit of detection.

Importantly, the amount of IL-10 produced by Tr1 cell clones and the IL-10/IL-4 ratio remained elevated following either antigen-specific or TCR-mediated polyclonal activation. Only two IL-10–producing T-cell clones (F28 and F16) could be identified that were not specific to host or donor antigens but that were able to produce IL-10 upon not-antigen-specific stimulation. No cytokine production was detected in the supernatants of resting T-cell clones (*data not shown*). These results indicate that alloreactive T-cell clones specific to donor and host antigens were isolated from the patient with long-term mixed chimerism, and that half of them were Tr1 cell clones.

Suppressive function of the patient's Tr1 cell clones

We next tested the capacity of host- and donor-derived Tr1 cell clones isolated from the PMC patient to suppress donor or recipient cell responses following TCR-mediated activation.

Two host-derived Tr1 cell clones (E12 and F29) inhibited the production of IFN-γ by the recipient and donor PBMC used as responder cells (Figure 5A). The grade of inhibition was 75% and 68% for clone E12, and 23% and 27% for clone F29, for the recipient and donor PBMC, respectively. The other host-derived Tr1 cell clones did not show any suppressive activity (Figure 5A). Similar results were observed measuring the TNF-α production (*data not shown*). The host-derived Tr1 cell clones that could inhibit the patient's PBMC production of IFN-γ and TNF-α were also able to suppress the proliferative responses of recipient cells (Figure 5C). Interestingly, as detected by surface staining of CFSE-labeled proliferating cells, the Tr1 cell clone E12 displayed suppressive activity against both the patient's $CD4^-$ and $CD4^+$ cells (43% and 36% suppression, respectively), whereas Tr1 cell clone F29 was able to suppress only the proliferation of the patient's $CD4^-$ cells (70% suppression) and was ineffective at inhibiting $CD4^+$ proliferation. The addition of Th0 and Th2 cell clones to the cell cultures did not inhibit but rather induced an increase in cytokine production and proliferation of responder cells (*data not shown*).

In parallel, we tested the suppressive capacity of Tr1 cell clones against the donor and host effector T-cell clones used as responder cells. Among the three host-derived Tr1 cell clones, only F29 was able to inhibit IFN-γ production by both host- and donor-derived (Figure 5B) T-cell clones, confirming the suppressive capacity shown against recipient and donor PBMC. Among the donor-derived Tr1 cell clones, two (F24 and F27) were able to inhibit cytokine production by the autologous donor-derived T-cell clones (45% inhibition for F24 and 53% for F27) (Figure 5B). In addition, T-cell clone F27 also inhibited IFN-γ production by host-derived T cell clones (55% inhibition) (Figure 5B). These results were confirmed by measuring inhibition of TNF-α production. The remaining two donor-derived Tr1 cell clones did not show any suppressive capacity.

In summary, we demonstrated that four Tr1 cell clones, two host-derived (E12 and F29) and two donor-derived (F24 and F27), were able to suppress both host- and donor-activated responder T cells. These Tr1 cell

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clones produced high levels of IL-10 upon host or donor antigen activation (Figure 4A-B). Our results indicate that host and donor alloreactive Tr1 cell clones are functional *in vitro* and are able to suppress proliferation and cytokine production of recipient and donor cells.

Indeed, in the presence of anti-IL10R monoclonal antibody to neutralize the effect of endogenous IL-10, proliferative responses of PBMC from the patient with PMC consistently increased in primary mixed lymphocyte reactions towards host and donor mature dendritic cells (44% towards host dendritic cells and 51% towards donor dendritic cells) as shown in Figure 5D. In contrast, the increase in proliferation versus third party mature dendritic cells was low (12%) and comparable to that detected in primary mixed lymphocyte reactions between unrelated normal donor responder and stimulator cells (mean increase of two normal donors tested was 11% and 7% towards host and donor mature dendritic cells, respectively) (Figure 5D). Moreover, as expected, the proliferative responses of the patient's PBMC towards both host and donor mature dendritic cells were lower than to third-party dendritic cells, given the minimal degree of HLA disparity between the host and the donor (Figure 5D). When the patient's PBMC were stimulated with host or donor mature dendritic cells in the presence of anti-IL10R monoclonal antobody, the amount of IL-10 in the supernatant ranged between 300-386 pg, whereas the normal donor's PBMC produced between 54-146 pg of IL-10 upon allogenic mature dendritic cell stimulation.

Discussion

In this study we provide evidence that the association of Tr1 phenotype with post-transplant PMC is a consistent and general phenomenon. Considering IL-10 as the hallmark of Tr1 cells, we first found a high percentage of IL-10-producing T cells among the CD4+ T cells of thalassemic patients with short or long-term mixed chimerism but not in those with complete donor chimerism after HSCT; secondly, a high proportion of IL-10-producing Tr1 cell clones was found in the peripheral blood of a patient with long-term PMC; and thirdly, endogenous IL-10 inhibited alloantigen-specific responses towards both host and donor cells, in the peripheral blood of the patient with PMC. We investigated the engraftment of this transplanted thalassemic patient at two different times after chimerism had been well established and showed that the early predomi-

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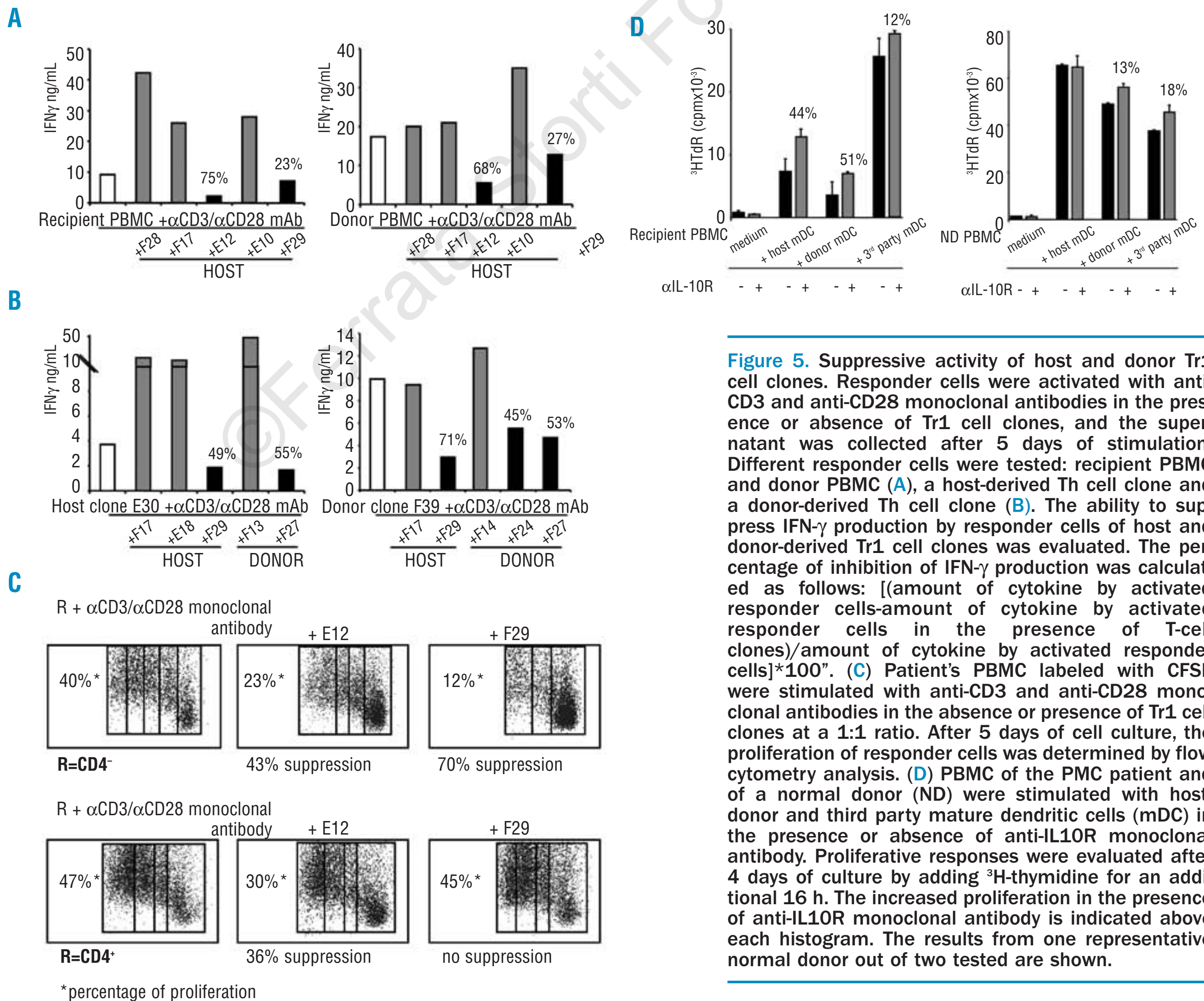

Figure 5. Suppressive activity of host and donor Tr1 cell clones. Responder cells were activated with anti-CD3 and anti-CD28 monoclonal antibodies in the presence or absence of Tr1 cell clones, and the supernatant was collected after 5 days of stimulation. Different responder cells were tested: recipient PBMC and donor PBMC (A), a host-derived Th cell clone and a donor-derived Th cell clone (B). The ability to suppress IFN-γ production by responder cells of host and donor-derived Tr1 cell clones was evaluated. The percentage of inhibition of IFN-γ production was calculated as follows: [(amount of cytokine by activated responder cells-amount of cytokine by activated responder cells in the presence of T-cell clones)/amount of cytokine by activated responder cells]*100". (C) Patient's PBMC labeled with CFSE were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies in the absence or presence of Tr1 cell clones at a 1:1 ratio. After 5 days of cell culture, the proliferation of responder cells was determined by flow cytometry analysis. (D) PBMC of the PMC patient and of a normal donor (ND) were stimulated with host, donor and third party mature dendritic cells (mDC) in the presence or absence of anti-IL10R monoclonal antibody. Proliferative responses were evaluated after 4 days of culture by adding ³H-thymidine for an additional 16 h. The increased proliferation in the presence of anti-IL10R monoclonal antibody is indicated above each histogram. The results from one representative normal donor out of two tested are shown.

nant donor chimerism remained stable for years in the erythroid compartment, whereas the proportion of host lymphoid cells gradually increased, giving rise to long-term, mixed lymphoid chimerism. However, the patient is in a good clinical condition, blood-transfusion independent, and cured from thalassemia following the allogeneic HSCT from a matched unrelated donor. Single-cell characterization of alloantigen–specific T cells showed that both regulatory and effector T-cell clones, able to respond to host and donor HLA-antigen, were present in the peripheral blood. Notably, the Tr1 cells were both host- and donor-derived, indicating that the induction of regulatory T cells occurred independently of their origin. Consistent with our previous observations, obtained in studies performed at a clonal level in representative chimeric patients,[7] in this study we confirm that, despite *in vivo* tolerance, host- and donor-reactive effector T cells can be isolated *in vitro*, indicating that post-transplant deletional mechanisms, if present, are only partially effective. In addition, we demonstrate that T cells producing high amounts of IL-10 suppress both host- and donor-specific responses.

Studies in chimeric mouse models showed that the dominant mechanism for the maintenance of tolerance following bone marrow transplantation is intrathymic deletion of donor-reactive thymocytes.[30] In additional studies, no indications of peripheral mechanisms were found in these mixed chimeras.[31,32] However, we have previously demonstrated that, in SCID patients, long-term tolerance is not due to clonal deletion of alloreactive T cells, but rather to peripheral regulatory mechanisms.[7,8] We now demonstrate, in a thalassemic patient with PMC, the persistence of T cells reactive against both the host and the graft HLA-antigens in the direction of GvHD and graft rejection, respectively. Similarly, the isolated Tr1 cells were of both host and donor origin and functionally active in both directions (graft or host).

The simultaneous presence of cells of donor and host origin often occurs in patients transplanted to cure thalassemia and hematologic malignancies, especially when reduced-intensity pre-transplant conditioning regimens are used.[2,33-35] The co-existence of donor and host cells soon after the transplant, even at very low percentages, leads to a chronic allo-antigenic exposure that could contribute to the induction of IL-10–producing regulatory T cells during the early post-transplant period. Indeed, chronic antigen exposure has been described as a crucial event in the generation of IL-10–producing T cells *in vivo*.[36,37] It has also been demonstrated that the presence of mixed chimerism after HSCT might induce a status of immunological tolerance prior to a second solid organ transplant from the same donor.[38-40]

Several studies have shown that patients who develop mixed chimerism after bone marrow transplantation for malignant[41-43] and non-malignant hematologic diseases, such as severe aplastic anemia[44] and β-thalassemia,[45] have significantly lower incidences of acute and chronic GvHD in comparison to patients with full donor chimerism. Similarly, in thalassemic patients the incidence of GvHD was lower in patients with PMC than in those with complete donor chimerism.

The multiple allogeneic blood transfusions that thalassemic patients receive during their lives as supportive treatment could be considered as a source of chronic antigen stimulation favoring IL-10 production and Tr1 induction, even in the pre-transplant period. Indeed, several studies suggest that allogeneic blood transfusions in cancer and trauma patients correlate with the induction of microchimerism and with downregulation of the immune response, due to repeated exposure to foreign antigens.[46,47] However, in our study the proportion of Tr1 cell clones detected before the transplant was comparable to that in normal donors, suggesting that multiple transfusions prior to the transplant should not have contributed to the induction of IL-10 production. Since not all thalassemic patients with mixed chimerism early after the transplant develop PMC,[5,6] it is possible that multiple factors are involved in the establishment of long-term tolerance. Recent genetic studies indicate that individual genetic variants of key molecules implicated in immune regulation are associated with a favorable transplant outcome. For example, specific IL-10 polymorphisms[48,49] and HLA-G polymorphisms[50] of the donor and recipient have been correlated with a lower risk of acute GvHD.

The Tr1 cells that we isolated from the peripheral blood of the patient with PMC phenotypically resemble the *bona fide* Tr1 cells previously described.[17] In particular, they showed very high levels of IL-10 production, which had previously been found only in Tr1 cells induced *in vivo*.[7,25] Importantly, in our functional *in vitro* assay, the cells showed suppressive activity in both directions, inhibiting IFN-γ and TNF-α production of donor and recipient T cells. The mechanism by which Tr1 cell clones suppress the effector function of responder cells is still being investigated. Grossman *et al.* recently demonstrated that human Tr1-like and natural $CD4^+$ $CD25^+$ Treg cells can develop considerable cytotoxic activity, through the production of granzyme-B and granzyme-A granules, respectively, in a perforin-dependent manner.[28] Here, we show that the patient's Tr1 cell clones expressed very high levels of granzyme-B compared with the patient's Th0 and Th2 cell clones. These results suggest that degranulation and lysis of the target cells might play an important role in the suppressive activity of Tr1 cells, together with the production of IL-10. Indeed, preliminary results show that the presence of IL-10 can aspecifically increase the intracellular expression of granzyme-B, suggesting a functional link between IL-10 and granzyme-B.

It remains to be shown whether the establishment of an active tolerance mechanism in the lymphoid compartment contributed to the high engraftment of the donor's erythroid compartment, primarily facilitated by the selective advantage of normal red blood cells from the donor over the thalassemic erythroid cells of the host. A recent report described the presence of Rhesus peptide–specific IL-10–secreting Treg cell clones in the spleen of a patient with autoimmune hemolytic anemia.[51] These findings, together with the isolation of activated autoreactive T cells specific to human red blood cell auto-antigens,[52] demonstrate the existence of both regulatory and effector T cells specific to erythroid antigens.

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In conclusion, the results of our study indicate the presence of a large number of IL-10-producing T cells and, specifically, high percentages of Tr1 cells in patients with PMC, suggesting that these cells could be responsible for the induction and maintenance of long-term tolerance. The presence of Tr1 cells could inhibit not only GvHD but also the occurrence of graft rejection, the incidence of which is high in transplanted thalassemic patients. An important clinical implication emerging from this observation could be the use of conditioning regimens favoring mixed chimerism soon after the transplant. Ultimately, these data support the rationale for possible cellular therapy with Tr1 cells to prevent not only GvHD but also rejection in the context of HSCT for thalassemia.

Authorship and Disclosures

GS designed and performed experiments and wrote the paper; MA organized blood sample collection and contributed to writing the paper; MB and AB performed molecular and erythroid research experiments; EB contributed to the patients' immunological studies; MGR supervised the research and revised the paper; RB designed and supervised experiments and wrote the paper; KF, SM, MT, and GL revised the paper.

The authors reported no potential conflicts of interests.

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