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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 (HSCT). Persistent mixed chimerism (PMC) develops in around 10% of transplanted thalassemic patients, but the biologic mechanisms underlying this phenomenon are poorly understood.

Design and Methods
The presence of IL-10 producing T cells in the peripheral blood of eight patients with PMC and five with full donor chimerism has been investigated. A detailed characterization was then performed, by T cell cloning, of the effector and regulatory T cell repertoire of one PMC patient, who developed a stable split erytroid/lymphoid chimerism after an HLA-matched unrelated HSCT.

Results
High levels of IL-10 by PBMCs of patients with PMC in comparison to those of patients with complete donor chimerism or normal donors were detected. From the peripheral blood of the PMC patient, T cell clones of both host and donor origin could be isolated. Together with effector T cell clones reactive against host or donor alloantigens, regulatory T cell clones with a cytokine secretion profile typical of Tr1 cells were identified at high frequencies. Tr1 cell clones, both donor and host derived, were able to inhibit the function of effector T cells of either donor or host origin in vitro.

Conclusions
Overall these results suggest that IL-10 and Tr1 cells are associated with PMC and may play an important role in sustaining long-term tolerance in vivo. These data provide new insights regarding the mechanisms of peripheral tolerance in chimeric patients and support the use of cellular therapy with regulatory T cells following HSCT.

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 for other hemoglobinopathies. The incidence, early after the transplant (5-6 months after HSCT), of a complete donor chimerism (CC), with the presence of only donor-derived cells in the bone marrow (BM) and in the peripheral blood (PB) of transplanted patients, is around 70%. If this condition remains stable for at least one year after HSCT, patients with CC never change their engraftment status over the time.

The presence of mixed chimerism (MC), i.e., the coexistence of donor and host cells in the recipient, is not a rare event following a successful transplantation. Although MC represents a risk factor for graft rejection if it occurs within the first months after the transplant (transient mixed chimerism [TMC]), when the coexistence of donor and host cells persists for a period longer than two years (persistent mixed chimerism [PMC]), patients remain blood-transfusion independent, similar to those with full donor engraftment. Approximately 10% of thalassemic patients receiving HLA-identical HSCTs will develop a status of PMC, with a proportion of residual host cells (RHCs) ranging from 10% to 75%. 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 and peripheral mechanisms of tolerance, mediated by regulatory T cells (Treg), have been often associated with the maintenance of post-transplant homeostasis despite the allogeneic disparities. Several kinds of Treg cells have been described, such as CD4+CD25+, Tr1, Th3, CD3+CD4+CD8+, CD8-, and NKT cells. Among the CD4+ T cell subset, CD4+CD25+ Treg cells (nTreg) 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. Many reports indicate that these cells are also involved in transplantation tolerance. T1 cells differentiate in the periphery upon priming of naïve T cell precursors with the antigen (Ag) in the presence of IL-10. They produce high levels of IL-10, TGF-b, and IL-5; low amounts of IFN-α and IL-2; and no IL-4. Tr1 cells suppress naïve and memory T cell responses in vivo and in vitro, through the secretion of IL-10 and TGF-β. The first suggestion that human Tr1 cells are involved in maintaining peripheral tolerance in vivo came from studies in severe combined immunodeficient (SCID) patients 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-AgS and produced high levels of IL-10, could be isolated in vitro. 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. Therefore, it remained to be clarified whether the source of stem cells or the absence of post-transplant immunosuppression were responsible for the induction of these T cells in the SCID human split chimeras. The correlation between IL-10 and the absence of GVHD or transplant-related complications after HSCT has been demonstrated since then. The importance of IL-10–producing T cells for the development of tolerance to the graft in solid organ transplantation has been widely demonstrated since then.

In this study, we demonstrated high levels of IL-10 produced by PBMCs of several thalassemic PMC patients and we characterized the effector T and Tr1 cells of a thalassemic patient who developed PMC after an HLA-matched unrelated HSCT. Results demonstrate that IL-10 and Tr1 cells are associated with PMC and may play a pivotal role in sustaining long-term tolerance, independently from the transplant source or the disease setting.

Design and Methods

Patients

Fourteen non-consecutive patients undergoing HSCT between 1998 and 2004 for transfusion dependent β-thalassemia were analysed. Patients’ characteristics and indications for transplantation are shown in Table 1. Median age was 3 years and 8 months (range 2-8 years). Risk class according to Pesaro [2] was class I (8), II (3) and III (3). Twelve patients were transplanted from a HLA genotypically identical sibling, 1 from an HLA phenotypically related donor and 1 from a 12/12 allele-level HLA-matched unrelated donor (MUD). All patients received a myeloablative conditioning regimen followed by infusion of unmanipulated bone marrow cells. Patients in class I-II (including #1 transplanted from an unrelated donor) were given a conditioning based on oral busulfan (Bu) 14 mg/kg and cyclophosphamide (Cy) 200 mg/kg. In addition to this, patients aged less than 4 years were conditioned with thiotepa (TT) 10 mg/kg. Patients in class III were conditioned with Bu 14 mg/kg associated with reduced dose cyclophosphamide (Cy) 160 mg/kg. Patient #11 received a HSCT conditioned with Bu 14, Cy 200, TT 10 mg and antithymocyte globulin (Thymoglobulin; Genzyme- Sangstat, Lyon, France) 8 mg/kg following rejection of a first HSCT. Post-HSCT GvHD prophylaxis consisted of cyclosporine, metilprednisolone and short methotrexate. Cyclosporine was started at 5 mg/kg intravenously day -2 through day +5 then reduced to 5 mg/kg and tapered from day +60 of 5%/week till a complete stop at +365. The desired plasma level was 150-250 ng/ml. Metilprednisolone was started at 0.5 mg/kg iv at -1 and stopped at +30. Short methotrexate was given at 10 mg/m2 intravenously on days +1, +3 and +6 with folinic rescue. The determination of chimerism and the
analysis of the frequency of IL-10 producing T cells in the peripheral blood of patients, were performed in a wide period of time (from 1 to 10 years after the transplant). Three out of five (60%) patients with CC 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.

**Mixed chimerism evaluation**

**STR typing for nucleated cells**

Recipient and donor DNA samples were typed by STR and 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 manufacturer’s instruction. PCR products were electrophoresed on an ABI Prism 3130xl Genetic Analyzer (Applera). Informative loci for post-transplant samples were screened for quantification of donor cell percentage in mixed chimerism. For quantitative determination was applied a method based on the ratio between peak areas of donor and recipient alleles.  

**Erythrocytes FACS analysis**

Differences in the blood groups of the patient and donor were evaluated by PCR-SSP analysis in Rhesus alleles using specific primers. The presence of donor and/or recipient erythrocytes was determined by an indirect flow cytometry analysis using a series of monoclonal antibodies (mAbs) 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.

### Table 1. Characteristics of patients (pt), donors and transplants.

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<th>Outcome</th>
<th>Donor cells in PB (%)</th>
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*According to Pesaro classification. °°The source of donor stem cell has been bone marrow for all the patients. MUD: matched unrelated donor. Phenotypeidential family donor; Sib: sibling HLA identical donor. PMC: persistent mixed chimerism; CC: complete; °°determined by STR typing. PB: Peripheral Blood.

### Establishment of T cell clones

PBMCs from patients and normal donor were isolated by centrifugation over Ficoll-Hypaque gradients (Nycomed Amersham, Uppsala, Sweden). CD4+ T cells were purified from PBMCs by negative selection using the CD4+ T cell isolation kit (Miltenyi Biotech, Auburn, CA) according to manufacturer’s instructions. T cell clones were obtained from CD4+ cells by limiting dilution at 0.5 cell/well in presence of a feeder cell mixture and soluble anti-CD3 mAb (1 mg/mL, OKT3, Jansen-Cilag, Raritan, NJ, USA) in X-vivo 15 medium (BioWhittaker, Verviers, Belgium) supplemented with 5% pooled AB human 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 mAb (1 mg/mL). Between stimulations with feeder cells, T cell clones were expanded with IL-2 (40 U/mL). Once T cell clones were established, every change medium was added IL-15 (5 ng/mL, R&D System, Minneapolis, MN, USA) as Tr1 growth factor.

### Cytokine detection

To determine the cytokine production after polyclonal activation, T cell clones (1×10⁶ cells/mL) were activated with immobilized anti-CD3 (10 mg/mL) and soluble anti-CD28 (1 mg/mL, Pharmingen, San Diego, CA) mAbs. Supernatants were collected after 12 hours in the presence of brefeldin A (10 mg/mL, Sigma) for 12 hours. Cells were fixed with formaldehyde, permeabilized in saponin buffer (PBS 2% FCS and 0.5% saponin, Sigma, Italy) and stained with PE-labeled anti–CD3, anti–CD28 (10 µg/mL, BD Pharmingen), anti–IL-10 (10 µg/mL, BD Pharmingen), anti–IL-2 (10 µg/mL, BD Pharmingen), anti–IL-4 (10 µg/mL, BD Pharmingen), and anti–IFN-γ (10 µg/mL, BD Pharmingen). Total PBMCs were activated with TPA and ionomycin (150 ng/mL, Sigma) for 12 hours in the presence of brefeldin A. Cells were fixed and permeabilized with FoxP3 Fix/Perm buffer set (Biolegend) and stained with PE-labeled anti-CD3 mAb. When appropriate, data were analyzed by Student’s t test.

### Flow cytometry analysis

For the detection of cell surface Ags, T cells were stained with mAbs against CD3, CD4, CD8, CD25, CD16, CD56, CD19, and CD14 (Pharmingen or BD Biosciences). Cells were incubated with the mAb for 20 min at 4°C in PBS 2% FCS, washed twice and fixed with 0.2% formaldehyde. For the expression of granzyme-A (GZ-A) and granzyme-B (GZ-B) (BD Cedex, France) in fresh peripheral blood following the manufacturer’s instructions.
Bioscience and Pharmingen), after surface staining, cells were fixed, permeabilized in saponin buffer and stained with GZ-A and GZ-B mAbs. Intracytoplasmic staining for human Foxp3 was performed using the anti-Foxp3 staining kit (Biolegend, San Diego, CA, USA), according to the manufacturer’s instructions.

**ELISPOT assay**

IL-10 secreting T cells were enumerated by enzyme-linked immunospot (ELISPOT) assay. Freshly PBMCs were plated at 10^5/well in ELISPOT plates (Millipore, Bedford, MA) coated with anti–IL-10 capture mAb (clone M010, Endogen, Pierce, Rockford, USA). After 48 hours of incubation, plates were washed and IL-10 producing cells were detected by anti–IL-10 detection mAb (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 mg/ml) and soluble anti-CD28 (1 mg/ml) mAbs. Patient PBMCs, used as responder cells, derived from un-separated post-transplant samples, therefore include both host and donor cells. After 5 days of culture, supernatants were collected for analysis of IFN-α and TNF-α production using the CBA system (BD Biosciences). To test the suppressive capacity of Tr1 cell clones by flow cytometry, patient’s PBMCs were labeled with 5-(and-6)-carboxy fluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) before the stimulation with anti-CD3 and anti-CD28 mAbs. After 5 days of culture, proliferation of CFSE-labeled PBMCs 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, PBMCs were stimulated with allo-geneic mature DC (10:1, T cells/DC) with or without anti-IL10R (50 mg/mL, 3E9, R&D Systems) mAb, in complete medium in 96-well round-bottom plates. After 4 days of culture, wells were pulsed for 16 hours with 1 mCi/well of [%H]-thymidine.

**Results**

**High frequency of IL-10 producing T cells in the peripheral blood of patients with PMC**

Cytokine production profile of freshly isolated PBMCs from transplanted patients with persistent mixed chimerism (PMC), was tested and compared to that of patients who developed complete donor chimerism (CC) post-HSCT. Five out of eight PMC patients 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 TPA/ionomycin stimulation is shown in Figure 1A. A higher proportion of IL-10 producing T cells could be detected in PMC patients (n=7) compared to CC patients (n=5) and to normal donors (n=8), with a statistically significant difference between the IL-10 levels measured in PMC patients and in CC patients (p<0.01) or in ND (p<0.01). No significant differences in the production of other cytokines (IL-2, IL-4, and IFN-γ) were observed between PMC patients and CC patients or ND (data not shown).

A higher number of IL-10 producing T cells could also be detected prior to stimulation and upon TCR mediated activation, in a PMC patient compared to ND, 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 from the time after the transplant.

**Kinetics of persistent mixed chimerism**

We further characterized the peripheral T cell repertoire in patients with PMC following HSCT. A PMC
A patient who received a MUD transplant eight 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 (8450±2069 cell/mL and 4x106±1.7x106 cell/mL, respectively), hemoglobin concentration (11.4±0.1 g/dL), and percentage of reticulocytes (1.5±0.3), over the past four years. The absolute lymphocyte number 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 with those of normal donors (data not shown). In addition, CD4+CD25+ and CD4+CD25+Foxp3+ T cells were present in normal proportion (18% and 5%, respectively) (data not shown). Shortly after the HSCT, the patient showed complete donor chimerism, but one year following the transplant, the donor cells began to decrease both in the bone marrow (BM) and in the peripheral blood (PB), declining up to 50%–56 months after HSCT and maintaining stable levels eight years following the transplant (Figure 2A). In parallel with the presence of this large proportion of residual host cells (RHCs) in the total PB, the amount of donor beta globin remained stable between 80% and 100% (Figure 2A) and the alfa/non-alfa ratio ranged between 1.21–1.7 (data not shown). The proportion of donor T lymphocytes, both in the CD4+ and CD4- subpopulations, ranged between 40% and 50%, 76 and 91 months after the transplant. These results were similar to those obtained 101 months after HSCT, with proportions of donor CD3+, CD19+, and CD56+ cells of 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 a split, long-term chimerism in the lymphoid cells.

**Characterization of T cell clones isolated from PBMCs of the patient with PMC**

T cell clones were isolated from two distinct blood samples, 76 and 91 months after HSCT, obtained from the PMC patient (pt PMC), from a normal donor (ND) and from a thalassemic patient before the transplant (pt PRE). Based on their cytokine production profiles different subsets of T cell clones could be identified. The proportion of Th0 (IL-2+, IL-4+, IL-10+, and IFN-α+), Th1 (IL-2+ and IFN-α+), and Th2 (IL-4+ and IL-10+) cell clones was comparable in the patient with PMC, in the ND, and in the patient pre-HSCT (Figure 3A). According to data from the literature 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-fold. In contrast with the Th cell clones, the proportion of Tr1 cell clones obtained from the T cell clonings of PMC patient was consistently higher (28% and 24%) compared with that of the ND (9%) and of the patient pre-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 ND’s (lower panel) T cell clones following TCR-mediated activation is reported in Figure 3B. Overall, a higher amount of IL-10 was produced by T cell clones isolated from the patient with PMC compared to ND, together with a higher frequency of T cell clones with an elevated ratio between IL-10/IL-4, and IL-10/IFN-γ.

Moreover, the absolute amount of IL-10 produced by the Tr1 cell clones of the patient was higher (median level of 7,855 pg/mL, ranging from 967 to 16,996 pg/mL for the first T cell cloning and median level of 14,668 pg/mL, ranging from 1208 to 42,732 pg/mL for the second T cell cloning) compared to the IL-10 secreted by the Tr1 cell clones of the ND (median level of 1,897 pg/mL, ranging from 1056 to 2256 pg/mL), with a statistically significant difference (p<0.05 between the first/second T cell cloning and the ND) (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 ND (p = n.s.) (Figure 3C), indicating that IL-10 production only was higher in the patient’s T cell clones compared to the ND’s. The cytokine production profile of the patient’s Tr1 cell clones was confirmed at 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 CD127, CD2, and CD4 was high in the majority of the cells (Figure 3E). In contrast, expression of CD3 and CD8 was low in the majority of the cells (Figure 3F). The expression of CD25 and CD127 was consistent with the CD4+CD25+Foxp3+ phenotype of Tr1 cells. Moreover, the majority of CD4+CD25+ Foxp3+ T cells expressed the Foxp3 transcription factor (Figure 3G). The expression of CD4 and CD8 was low in the majority of the cells (Figure 3H). In contrast, expression of CD25 was high in the majority of the cells (Figure 3I). Finally, the expression of CD127 was low in the majority of the cells (Figure 3J). The expression of CD4 and CD8 was low in the majority of the cells (Figure 3K). In contrast, expression of CD25 was high in the majority of the cells (Figure 3L). The expression of CD127 was low in the majority of the cells (Figure 3M).
expression of intracellular Foxp3 were upregulated following TCR-mediated activation, at levels comparable with those detected in activated Th0 and Th2 cell clones (data not shown). Interestingly, resting Tr1 cell clones expressed much higher levels of granzyme-B (GZ-B) in comparison with 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 (GZ-A) expression in either resting Tr1 or Th2/Th0 cell clones (Figure 3E). A comparable number 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, respectively), 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 patient's T cell clones**

To investigate the antigen specificity of the T cell clones, mDCs were used in vitro as allogeneic host/donor APCs 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 mDCs (Figure 4A, upper panel) and donor-derived T cell clones reactive to host mDCs (Figure 4B, upper panel) could be identified. Out of 19 T cell clones analysed, 11 did not respond to mDC, while 8 T cell clones produced several cytokines after Ag-specific activation. Of these 8 T cell clones, 4 (50%) showed a cytokine production profile typical of Tr1 cell clones. All the T cell clones, except one, reactive to host/donor mDCs did not produce cytokines in response to third-party mDCs, indicating their specificity to host or donor allo-Ags (Figure 4A-B lower panel). Although the absolute amount of

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

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*mDC are donor-derived cells; TCR indicates a polyclonal activation via anti-CD3/28 mAbs; cytokines tested after 48h of stimulation; ‡ cytokine tested after 24h of stimulation < 20 means under the limit of detection.

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

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<th>Donor-derived T cell clones</th>
<th>Stimulus</th>
<th>IL-10‡ (pg/mL)</th>
<th>IL-4‡ (pg/mL)</th>
<th>IL-2§ (pg/mL)</th>
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*mDC are host-derived cells; TCR indicates a polyclonal activation via anti-CD3/28 mAbs; cytokines tested after 48h of stimulation; ‡ cytokine tested after 24h of stimulation < 20 means under the limit of detection.
cytokines produced upon Ag-specific stimulation was much lower than that produced upon anti-CD3 and anti-CD28 mAbs stimulation, the cytokine profile, meaningful to define Tr1 or Th cell clones, was maintained after the two stimulations (Table 2) in all but one T cell clones tested. Importantly, IL-10 produced by Tr1 cell clones and the ratio IL-10/IL-4 remained elevated following either Ag-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 Ags but were able to produce IL-10 upon not-Ag-specific stimulation. No cytokine production was detected in the supernatants of resting T cell clones (data not shown). These results indicate that allo-active T cell clones specific to donor and host Ags could be isolated from the patient with long-term chimerism, and that half of them were Tr1 cell clones.

**Suppressive function of 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 PBMCs 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 PBMCs, respectively. These results indicate that allore-active T cell clones specific to donor and host Ags could be isolated from the patient with long-term chimerism, and that half of them were Tr1 cell clones.

**Figure 5.** Suppressive activity of host and donor Tr1 cell clones. Responder cells were activated with anti-CD3 and anti-CD28 mAbs in the presence or absence of T cell clones, and the supernatant was collected after five days of stimulation. Different responder cells were tested: recipient PBMCs and donor PBMCs (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: \[ \frac{\text{amount of cytokine by activated Responder cells-amount of cytokine by activated Responder cells in the presence of T cell clones}}{\text{amount of cytokine by activated Responder cells}} \times 100\% \]. (C) Patient’s PBMCs labeled with CFSE were stimulated with anti-CD3 and anti-CD28 mAbs in the absence or presence of Tr1 cell clones at a 1:1 ratio. After five days of cell culture, the proliferation of responder cells was determined by flow cytometry analysis. (D) PBMCs of the PMC patient and of a ND were stimulated with host, donor and third party mDC in the presence or absence of anti-IL10R mAb. Proliferative responses were evaluated after 4 days of culture by adding ‘H-thymidine for an additional 16 hours. The increased proliferation in the presence of anti-IL10R mAb is indicated above each histogram. One representative ND out of two tested is shown.
PBMCs, 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). Moreover, the host-derived Tr1 cell clones that could inhibit 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, Tr1 cell clone E12 displayed suppressive activity against both patient’s CD4 and CD8 cells (45% and 56% suppression, respectively), whereas Tr1 cell clone F29 was able to suppress only the proliferation of patient’s CD4 (70% suppression) and was ineffective at inhibiting CD8 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 PBMCs. Among the donor-derived Tr1 cell clones, two of them (F24 and F27) were able to inhibit cytokine production by the autologous donor-derived T cell clones (45% of inhibition for F24 and 53% for F27) (Figure 5B). In addition, T cell clone F27 also inhibited IFN-γ produced by host-derived T cell clones (55% of 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 clones produced high levels of IL-10 upon host or donor Ag 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 mAb to neutralize the effect of endogenous IL-10, proliferative responses of PBMCs of the patient with PMC consistently increase in primary MLR towards host and donor mDC (44% towards host mDC and of 51% towards donor mDC, respectively) as shown in Figure 5D. On the contrary, the increase in proliferation versus third party mDC was low (12%) and comparable to that detected in primary MLR between unrelated normal donor responder and stimulator cells (mean increase of two ND tested was of 11% and 7% towards host and donor mDC, respectively) (Figure 5D). Moreover, as expected, the proliferative responses of the patient’s PBMCs towards both host and donor mDC were lower than to third-party mDC, given the minimal degree of HLA disparity between the host and the donor (Figure 5D). When patient’s PBMC were stimulated with host or donor mDC in the presence of anti-IL10R mAb, IL-10 in the supernatant ranged between 300-386 pg, whereas the ND PBMC produced an amount of IL10 ranging between 54-146 pg upon allogenic mDC 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 demonstrate first, that a high percentage of IL-10 producing T cells could be detected in 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, that a high proportion of IL-10 producing Tr1 cell clones could be found in the peripheral blood of a long-term PMC patient; and thirdly, that endogenous IL-10 inhibits allo-Ag-specific responses towards both host and donor cells, in the peripheral blood of patient with PMC. We investigated the engraftment of this transplanted thalassemic patient at two different time points after the chimerism was well established and showed that the early predominant donor chimerism remained stable for years in the erythroid compartment, whereas the proportion of host lymphoid cells gradually increased, giving rise to a long-term, mixed lymphoid chimerism. However, the patient is in good clinical condition, blood-transfusion independent, and cured from thalassemia following allogeneic MUD-HSCT. Single-cell characterization of allo-Ag–specific T cells showed that both regulatory and effector T cell clones, able to respond to host and donor HLA-Ags, could be isolated from the peripheral blood. Notably, the Tr1 cells were both host and donor derived, indicating that the induction of regulatory T cells occurred independent of their origin. Consistent with our previous observations, obtained in studies performed at clonal level in representative chimeric patients, 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 the intrathymic deletion of donor-reactive thymocytes. In additional studies, no indications of peripheral mechanisms were reported in these mixed chimerocytes. 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. We now demonstrate, in a thalassemic PMC patient, the persistence of T cells reactive against both the host and the graft HLA-Ags, 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 tha-
lassemia and hematological malignancies, especially when reduced-intensity pre-transplant conditioning regimens are used. The coexistence 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 Ag exposure has been described as a crucial event in the generation of IL-10-producing T cells in vivo. 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 transplantation from the same donor.

Several studies showed that patients who developed mixed chimerism after bone marrow transplantation for malignant and not-malignant hematologic diseases, such as severe aplastic anemia and β-thalassemia, had a significantly lower incidence of acute and chronic GvHD in comparison to patients with full donor chimerism. Similarly, in thalassemic patients with PMC the incidence of GvHD was lower than in those with CC.

The multiple allogeneic blood transfusions that thalassemic patients receive during their lives as supportive treatment, could be considered as a source of chronic Ag stimulation that favor 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 the downregulation of the immune response, due to repeated exposure to foreign Ags. However, in our study the proportion of Tr1 cell clones detected before the transplant was comparable to that of ND, 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, 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 and HLA-G polymorphisms 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. In particular, they show very high levels of IL-10 production, which had previously been found only in Tr1 cells induced in vivo. Importantly, in our functional in vitro assay, they show 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 nTreg cells can develop considerable cytotoxic activity, through the production of GZ-B and GZ-A granules, respectively, in a perforin-dependent manner. Here, we show that patient's Tr1 cell clones expressed very high levels of GZ-B compared with 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 GZ-B, suggesting a functional link between IL-10 and GZ-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 study describes the presence of Rh peptide-specific IL-10–secreting Treg cell clones in the spleen of a patient with autoimmune hemolytic anemia. These findings, together with the isolation of activated autoreactive T cells specific to human red blood cell auto-Ags, demonstrate the existence of both regulatory and effector T cells specific to erythroid Ags.

In conclusion, the results of our study indicate the presence of high 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, which has a high incidence 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 a possible cellular therapy with Tr1 cells to prevent not only GvHD but also rejection in the context of HSCT for thalassemia.

Authorship and Disclosures

G.S. designed and performed experiments and wrote the paper; M.A. organized blood sample collection and contributed to writing the paper; M.B. and A.B. performed molecular and erythroid research experiments; M.G.R. supervised the research and revised the paper; R.B. designed and supervised experiments and wrote the paper; K.E., S.M., M.T., and G.L. revised the paper. The authors reported no potential conflicts of interests.

References

Tr1 cells in thalassemia


41. Lim ZY, Pearce L, Ho AV, Barber L, Ingram W, Ussia M, et al. Delayed attainment of full donor chimaerism following alemtuzumab-based reduced-intensity conditioning haematopoietic stem cell transplantation for acute myeloid leukemia and myelodysplastic syndromes is...


