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IL-10 mediated immunological tolerance: preclinical and clinical studies

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INDEX

ABSTRACT

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 biological mechanisms underlying this phenomenon are poorly understood. IL-10 is an immunomodulant cytokine that plays a central role in controlling inflammation, down-regulating immune responses, and inducing immunological tolerance. Detection of high levels of IL-10, produced by PBMCs of patients with PMC, in comparison to those of patients with complete donor chimerism (CC) or normal donors (ND), prompted us to characterize the T cell repertoire in a thalassemic patient with long-term tolerance following HSCT. 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, we identified regulatory T cell clones, with a cytokine secretion profile typical of type 1 regulatory T cells (Tr1), 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*, suggesting a contribution by these regulatory T cells to the maintenance of PMC *in vivo*.

In parallel, we demonstrated that IL-10 could inhibit primary allogeneic proliferation of total PBMCs and could induce antigen-specific T-cell hyporesponsiveness. The use of tolerogenic dendritic cells (DC) differentiated in the presence of IL-10 (DC-10), as compared to monocytes+IL-10, allowed a more consistent anergy induction, even in the context of HLA-matched donors. Importantly, both monocytes+IL-10-anergized

and DC-10-anergized T cells preserved their ability to respond to nominal and third party antigens.

All together these results provide first, new insights regarding the mechanisms of peripheral tolerance in transplanted thalassemic patients, and secondly, offer a strong rationale for the development of a clinical protocol for the use of *ex-vivo* monocytes+ IL-10/DC-10-anergized T cells of donor origin as cellular therapy to promote immune-reconstitution and prevent graft-versus-host disease after HSCT.

RIASSUNTO

La Talassemia Major è una patologia che può essere curata con il trapianto di cellule staminali. In circa il 10% dei pazienti talassemici, dopo il trapianto si sviluppa una condizione immunologica chiamata chimerismo misto persistente, ma i meccanismi biologici alla base di questo fenomeno sono ancora poco conosciuti. L'interleuchina IL-10 è una citochina immunomodulante che gioca un ruolo centrale nel controllare i fenomeni infiammatori, abbassare le risposte immunitarie e indurre tolleranza immunologica. L'osservazione di alti livelli di IL-10, prodotta dalle PBMCs di pazienti con chimerismo misto persistente, in confronto ai livelli di IL-10 prodotta da pazienti con chimerismo completo o da donatori sani, ci ha spinto a caratterizzare il repertorio delle cellule T di un paziente talassemico con tolleranza a lungo termine dopo trapianto di midollo osseo. Dal sangue periferico del paziente con chimerismo misto persistente sono stati isolati cloni T di origine sia del donatore che del ricevente. Insieme a cloni T effettori, reattivi nei confronti di allo-antigeni del donatore e del ricevente, sono stati isolati ad alte frequenze cloni T regolatori con un profilo citochinico tipico delle cellule T regolatorie di tipo 1 (Tr1). I cloni Tr1, di origine sia del ricevente che del donatore, si sono dimostrati capaci di inibire le funzioni delle cellule effettrici di entrambi le origini *in vitro*, suggerendo un contributo da parte di queste cellule regolatorie nel mantenimento del chimerismo misto persistente *in vivo*.

In parallelo, abbiamo dimostrato che l'interleuchina IL-10 può inibire la proliferazione primaria allogenica delle PBMCs, e può down-regolare la risposta delle cellule T antigene-specifica. L'uso di cellule dendritiche differenziate in presenza di IL-10 (DC-10), in paragone all'uso di monociti+IL-10, induce uno stato di anergia più

consistente, perfino nel contesto di donatori HLA-identici. Le cellue anergizzate, sia in presenza di monociti+IL-10, sia in presenza di DC-10, mantengono la loro capacità di rispondere ad antigeni scorrelati.

Questi risultati, forniscono nuove conoscenze riguardo i meccanismi che regolano la tolleranza periferica in pazienti talassemici trapiantati, e successivamente offrono un forte razionale per lo sviluppo di un protocollo clinico basato sull'uso di cellule T del donatore anergizzate *ex vivo* in presenza di monociti+IL-10/DC-10, come terapia cellulare per promuovere l'immuno-ricostituzione e prevenire la "malattia da trapianto contro l'ospite" (GvHD) dopo trapianto di midollo osseo.

LIST OF PAPERS

I. Giorgia Serafini, Marco Andreani, Manuela Testi, MariaRosa Battarra, Andrea Bontadini, Katharina Fleischhauer, Sarah Marktel, Guido Lucarelli, Maria Grazia Roncarolo, and Rosa Bacchetta. "Type 1 regulatory T cells are associated with persistent split erythroid/lymphoid chimerism after allogeneic hematopoietic stem cell transplantation for thalassemia." Submitted to Haematologica Journal- Accepted.

II. Silvia Gregori, Giorgia Serafini, Claudia Sartirana, Ute Schulz, Elisabetta Zino, Khatarina Fleishhauer, Stefan Tomiuk, Uwe Janßen, Maria Grazia Roncarolo and Rosa Bacchetta. "Molecular and functional characterization of alloantigen-specific anergic T-cell suitable for cell therapy". Manuscript in preparation.

ABBREVIATIONS

TM	Thalassemia major
RBC	Red blood cell
TT	Thalassemia intermedia
HSCT	Hematopoietic stem-cell transplantation
BU	Busulfan
CY	Cyclophosphamide
GvHD	Graft versus host disease
CC	Complete chimerism
MC	Mixed chimerism
RHCs	Residual host cells RHCs
TMC	Transient mixed chimerism
PMC	Persistent mixed chimerism
PHA	Phytohemagglutinin
PBMC	Peripheral blood mononuclear cell
Tr1	Type 1 regulatory T cells
IBD	Inflammatory bowel disease
EAE	Experimental autoimmune encephalomyelitis
RA	Rheumatoid arthritis
Ag	Antigen
NOD	Nonobese diabetic
SIT	Specific immunotherapy
APC	Antigen presenting cells

SCID	Severe combined immunodeficient
Treg	Regulatory T cells
GZ	Granzyme
DC	Dendritic cells
MHC	Major histocompatibility complex
T1DM	Type 1 diabetes
HL	Hodgkin lymphoma
HNSCC	Head and neck squamous cell carcinoma
TIL	Tumor infiltrating lymphocytes
MLR	Mixed lymphocyte reaction
iDC	Immature DC
mDC	Mature DC
LPS	Lipopolysaccharide
NHP	Nonhuman primates
DLI	Donor lymphocyte infusions
GVL	Graft-vs.-leukemia activety
GVT	Graft-vs.- tumor activity

INTRODUCTION

Thalassemias

Thalassemia is a genetic disease characterized by defects in the hemoglobin production. Although originally endemic to the tropics and subtropics, it is now found worldwide as a result of migration and has become an important part of clinical practice in Europe, US and Australasia. Moreover, it is estimated that thalassemia is among the most common genetic disorders: 4.83 percent of the world's population carry globin variants, including 1.67 percent of the population who are heterozygous for α-thalassemia and β-thalassemia (1).

The thalassemias are named according to the globin chain affected, therefore, α-globin gene mutations give rise to α-thalassemia and β-globin mutations cause β-thalassemia. Normal human hemoglobin is composed of two α-like and two β-like globin chains. Adult hemoglobin consists of hemoglobin A ($\alpha_2\beta_2$) plus small amounts of hemoglobin A_2 ($\alpha_2\delta_2$) and hemoglobin F ($\alpha_2\gamma_2$). Genetic mutations in one of the globin genes (α or β) result in decreased or absent production of that globin chain and a relative excess of the other. These mutations can result in no globin production (β° or α°) or decreased globin production (β^+ or α^+). In addition, thalassemias can be classified by the clinical severity. Thalassemia major (TM) syndrome requires more than eight red blood cell (RBC) transfusions per year while thalassemia intermedia

(TI) requires no or infrequent transfusions. Untreated TM is fatal in the first few years of life; in addition, TM and severe TI can lead to considerable morbidity affecting nearly all organ systems (2).

Beta-Thalassemia

The β-globin gene resides on the chromosome 11. More than 200 mutations on this gene are known to result in a phenotype of β-thalassemia, while, in contrast to the α-thalassemias, the disease is rarely caused by deletions. As indicated above, there are two main varieties of β-thalassemia alleles: β^0 thalassemia in which no β-globin is produced, and β^+ thalassemia in which some β-globin is produced, but less than normal (3). The clinical manifestations of β-thalassemia, corresponding to the degree of expression of the two β-globin genes that encode β-globin, are extremely various. The most severe condition is characterized by the results of the trasmission of two β^0 thalassemia alleles (TM). This condition causes severe ineffective erythropoiesis, massive erythroid hyperplasia in the bone marrow and extramedullary sites, and hemolytic anemia necessitating chronic transfusions. When only one β-globin gene is affected, the resulting phenotype is milder, depending on the degree of gene expression and relative imbalance of globin chain (2).

In patients with β-thalassemia major both ineffective erythropoiesis and chronic transfusion therapy inevitably lead to iron overload resulting in multiple organ damage which causes endocrine deficiencies, liver disease, and cardiac disease with poor quality of life and increased mortality. Regular blood transfusion and iron chelation have improved both survival and quality of life and have changed a previously fatal disease with early death to a chronic, although progressive disease

compatible with prolonged survival (4). Up to date, allogeneic hematopoietic stem-cell transplantation (HSCT) represents the only cure for this and for other hemoglobinopathies (4-6).

Hematopoietic Stem Cell Transplantation for Thalassemia

The first two HSCT procedures for thalassemia were carried out in December 1981, in Seattle (WA) and in Pesaro (Italy) with marrow from HLA-identical relatives (5, 7). Since Pesaro group collected large experience during these years and became one of the Italian reference group for transplantation in thalassemia, I will focus my attention on the patients treated with the Pesaro protocols for bone marrow transplantation.

Prof. Lucarelli classified patients into three risk classes based on clinical characteristics and on liver biopsy (Table 1). The three risk factors that were considered for the classification were: iron chelation, hepatomegaly more than 2 cm and liver fibrosis.

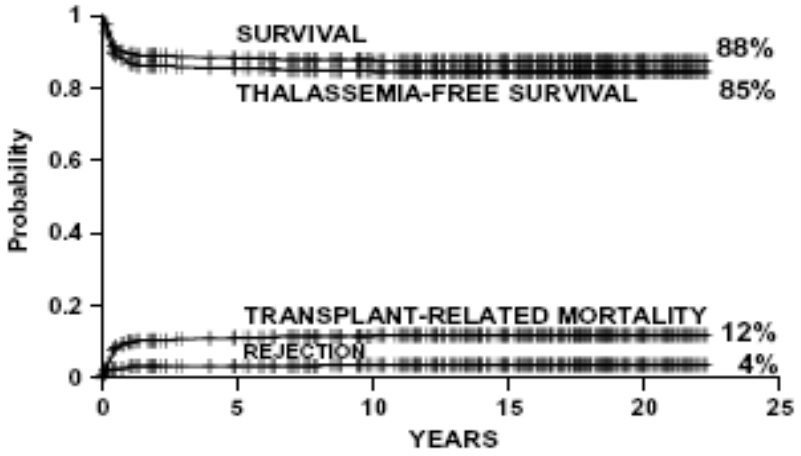
Table 1 Risk factors and risk classes.

Risk factors	Risk classes		
	Class 1	Class 2	Class 3
Chelation	Regular	Regular/ Irregular	Irregular
Hepatomegaly >2 cm	No	No/Yes	Yes
Liver fibrosis	No	No/Yes	Yes

From Lucarelli et al., Blood Reviews, 2008

Table 1. Risk factors and risk classes for HSCT in thalassemia

Class 1 patients had none of these adverse risk factors, class 3 patients had all three and class 2 patients had one or two adverse risk factors (Table 1). For the classes 1 and 2 the transplant protocol was similar, based on busulfan (BU) for the eradication of the hematopoietic system, and on cyclophosphamide (CY) for the suppression of the immune system. Graft versus host disease (GvHD), that could be a complication of the transplant, was prevented by the prophylactic administration of cyclosporine with or without methotrexate after the HSCT (6). The group of Prof. Lucarelli transplanted five hundred and fifteen class 1 and class 2 patients with median age of 7 years (range from 1 to 16 years) between October 1985 and August 2007. The thalassemia-free survival obtained was of 85%, the transplant-related mortality of 12%, and the incidence of rejection of 4% (4) (Figure 1).

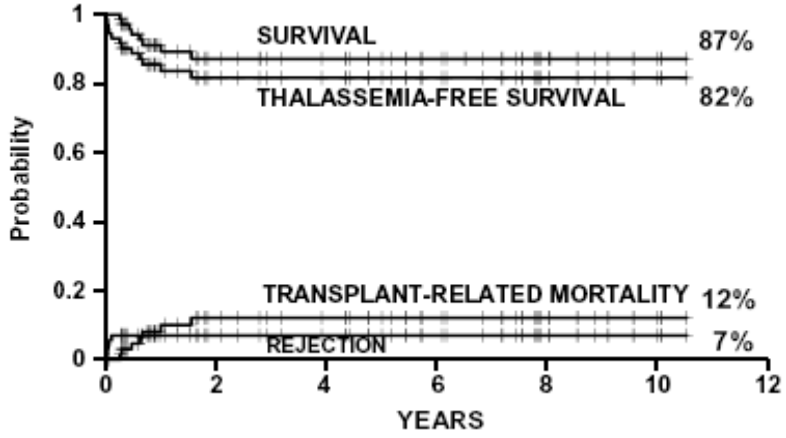


From Lucarelli et al., Blood Reviews, 2008

Figure 1. Estimates of survival, thalassemia-free survival, non-rejection mortality and rejection for 515 class 1 and class 2 patients younger than 17 years.

The protocol for class 3 patients has required more experience and time. After

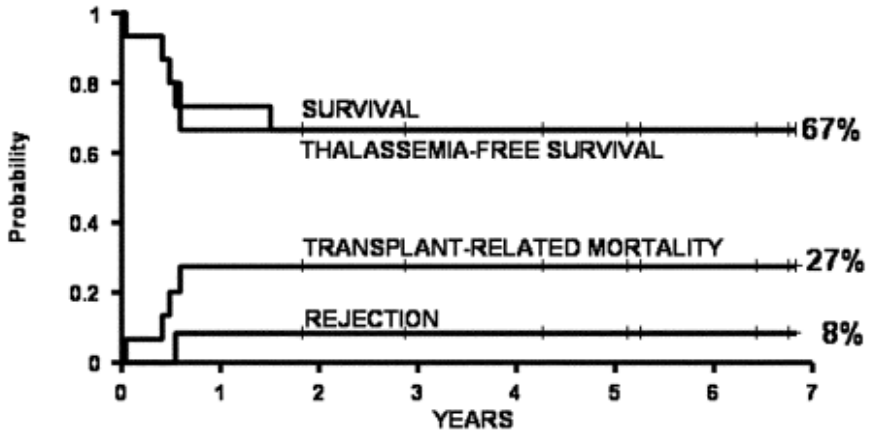
different procedures, in April 1997 a new regiment, called Protocol 26, was adopted for class 3 patients younger than 17 years. The survival, thalassemia-free survival, rejection and non-rejection mortality in 73 class 3 patients, aged less than 17 years with median age of 11 years (range 4–16 years), treated with Protocol 26 were 87%, 82%, 7% and 12%, respectively (4, 8) (Figure 2).



From Lucarelli et al., Blood Reviews, 2008

Figure 2. Estimates of survival, thalassemia-free survival, non-rejection mortality and rejection for 73 class 3 patients younger than 17 years who were treated with Protocol 26.

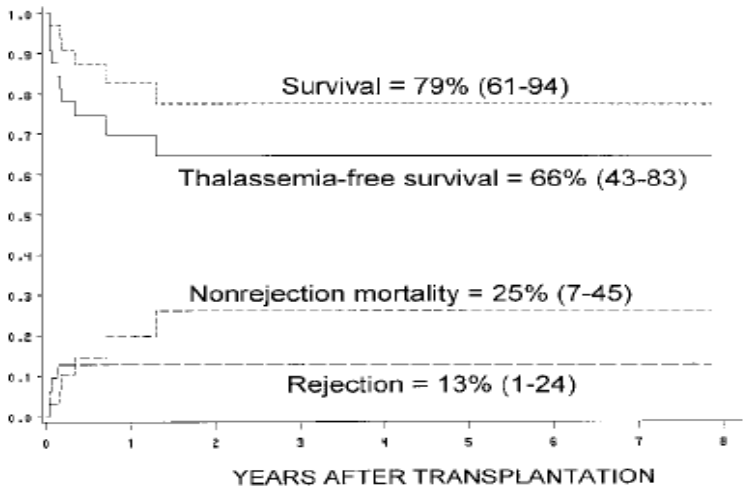
A regiment similar to the Protocol 26 was adopted for the Adult group. The probability of survival, thalassemia-free survival, rejection and non-rejection mortality in 15 high risk group patients were 67%, 67%, 8% and 27% respectively (9) (Figure 3).



From Gaziev et al, Ann. N.Y.Ac. Sci.,2005

Figure 3. Estimates of survival, thalassemia-free survival, non-rejection mortality and rejection in 15 adult patients treated with Protocol 26 regimen.

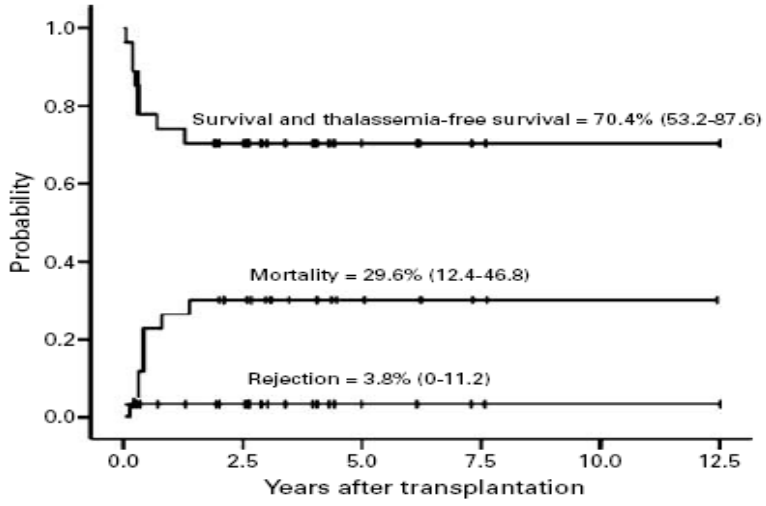
The majority of thalassemic patients (70%) do not have an HLA-identical donor within the family, therefore alternative stem cell donations have to be developed. In the last years the number of HSCT from unrelated donors has increased progressively, due to the higher number of volunteer donors worldwide. Moreover, thanks to the introduction of high-resolution molecular techniques for histocompatibility testing, the outcome of the transplant has gradually improved, reaching results comparable to those obtained from HLA-matched family donors. An analysis of the outcome of HSCT of 32 thalassemic patients transplanted with unrelated donors showed a survival, thalassemia-free survival, rejection and non-rejection mortality of 79%, 66%, 13% and 25%, respectively (10) (Figure 4) .



From La Nasa et al, Blood, 2002

Figure 4. Estimates of survival, thalassemia-free survival, non-rejection mortality and rejection for 32 thalassemia patients who received transplants from HLA-matched unrelated donors (between parenthesis: 95% confidence limits at 2 years).

More recently, in another study of HSCT from unrelated donors, it has been obtained comparable results with the probability of survival, thalassemia-free survival, rejection and transplant-related mortality of 70%, 70%, 4% and 30%, respectively (11) (Figure 5).



From La Nasa et al, BMT, 2005

Figure 5. Estimates of survival, thalassemia-free survival, non-rejection mortality and rejection in 27 adult class 3 thalassemia patients transplanted from HLA-matched unrelated donors (between parenthesis: 95% confidence limit).

Persistent mixed chimerism

It is well known that when complete chimerism (CC) of the marrow donor cells is established, a successful HSCT occurred. However, the presence of mixed chimerism (MC), i.e., the coexistence of donor and host cells in the recipient, is not a rare event following transplantation. The group of Pesaro established three different levels of MC based on the percentage of residual host cells (RHCs) present in the patient after the transplant. Patients with RHCs less than 10% were included in the level 1, between 10% and 30% in the level 2, and patients with more than 30% of RHCs in the level 3. Nesci reported that the incidence of MC at 2, 6 and 12 months after the transplant was 36,5%, 34,7% and 16,7%, respectively in Pesaro experience, and that the majority of the patients at 2 and 6 months after HSCT had a level 1 of MC (less than 10% of RHCs). Moreover, they showed that the probability of rejection for patients with MC levels 1 and 2 was 19% and 17% respectively, while all the patients with MC level 3 rejected the transplant within the first year after HSCT (12). Therefore, MC represents a risk factor for graft rejection if it occurs within the first months after the transplant (transient mixed chimerism [TMC]) (13).

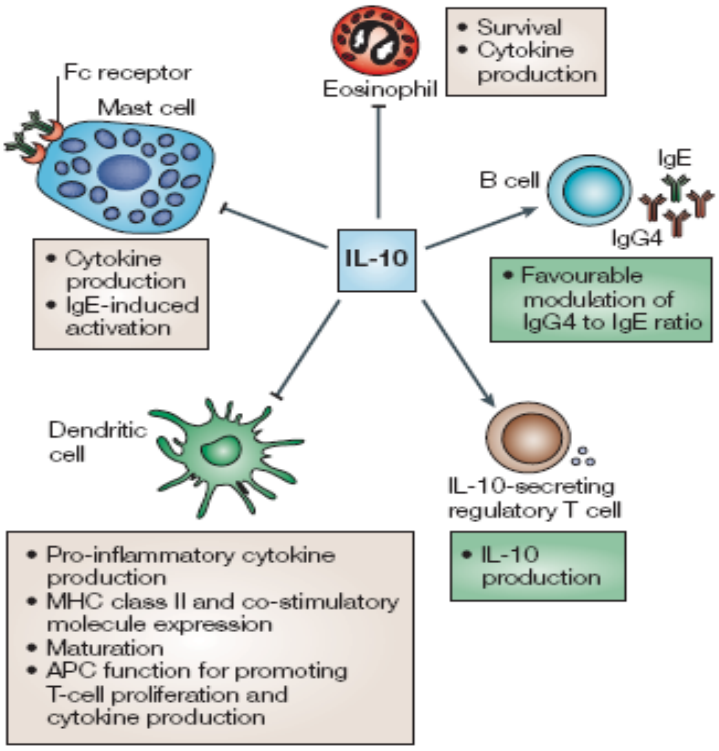
Nevertheless, when the coexistence of donor and host cells persisted in the marrow and in the peripheral blood for a period longer than two years (persistent mixed chimerism [PMC]), patients had a functional graft and remained blood-transfusion independent with hemoglobin levels ranging from 8.3 g/dl to 14.7 g/dl, similarly to

those of patients with full donor engraftment (14). These observations suggest that few engrafted donor cells may be sufficient to correct the disease phenotype in patients with thalassemia major once the tolerance between host and donor cells has been established. Battaglia analyzed the T-cell repertoire of 3 PMC patients by measuring the CDR3 length of TCR Vβ families. She showed a profound skewed repertoire with small number of specific T-cell clonotypes in the peripheral blood of these patients. After *in vitro* stimulation with phytohemagglutinin (PHA), the normal Gaussian distribution TCR CDR3 sizes were reestablished. This showed that the skewed repertoire in the peripheral blood mononuclear cell (PBMC) was associated with an expansion of specific T cells but not with a collapse of the whole repertoire and it could be related to an active mechanism of peripheral tolerance (15).

Up to date the mechanisms responsible for the induction and maintenance of PMC after HSCT are unknown.

IL-10

Since its discovery in 1990 (16) (17), IL-10 has gained increasing importance as a potent immunomodulatory cytokine that plays a central role in controlling inflammatory processes, suppressing T-cell responses and maintaining immunological tolerance (18). IL-10 directly reduces T-cell proliferation by inhibiting IL-2, TNF-α and IL-5 production (19-21), and down-regulates production of inflammatory cytokines as well as expression of costimulatory molecules on monocytes/macrophages (22). IL-10 enhances the cytotoxicity and the IFN-γ production of NK cells (23), and may have inhibitory and stimulatory effects on human $CD8^+$ T depending on their state of activation (18) (24). IL-10 has also an antiapoptotic effect on B cells, is involved in B-cell isotype switching and plays a role in autoimmune diseases with underlying B-cell dysregulation (25). Moreover, IL-10 not only induces long-lasting anergy in both $CD4^+$ (26) and $CD8^+$ T cells (24) but also the differentiation of type 1 regulatory T (Tr1) cells both in humans and mice (27) (Figure 6).



From Hawrylowicz CM et al. Nat Rev Immunol 2005

Figure 6. Functions of interlukin-10. IL-10 is a pleiotropic cytokine that has been shown to inhibit activation of, and cytokine generation by, mast cells, as well as survival of, and cytokine production by, eosinophils. It also inhibits antigen-presenting cell function, including the maturation of dendritic cells, the expression of MHC class II and co-stimulatory molecules and the activation of T-helper-2 cells. IL-10 has been shown to enhance immunoglobulin isotype switching in B cells, and it might promote the induction of IL-10-secreting regulatory T cells.

IL-10 *in vivo* in mouse model

The role of IL-10 in controlling the immune response and inducing tolerance has been demonstrated in several animal models. IL-10 deficient mice lack regulatory T cells, develop inflammatory bowel disease (IBD) (28) and a severe clinical experimental autoimmune encephalomyelitis (EAE) (29). IL-10 has been shown to be effective in inhibiting allergic inflammation in animal models. Injection of IL-10 before allergen

treatment induced an hapten-specific T cell tolerance in mice, inhibiting the production of proiflammatory cytokines (30). Moreover, adoptive transfer of $CD4^+$ T cells engineered to produce IL-10, effectively inhibited the capacity of effector cells to induce allergic airway inflammation (31).

These results provided a strong rational for the use of IL-10 *in vivo* to prevent autoimmune diseases and allograft rejection through the induction of peripheral tolerance. Unfortunately, after the *in vivo* administration of IL-10 in different experimental models of autoimmune disease and transplantation, contrasting effects have been described. In models of autoimmune diseases mediated by Th1 cells, such as rheumatoid arthritis (RA), IL-10 administration before and/or after induction of the disease reduced joint inflammation (32). In an adoptive transfer model of EAE, intravenous injection of IL-10 failed to abrogate the disease (33), while the intraperitoneal treatment could inhibited antigen(Ag)-induced EAE in mice if administered at the time of immunization (34). In nonobese diabetic (NOD) mouse, daily subcutaneous administration of IL-10 before 9-10 weeks of age, prevented cellular infiltration of islet cells, delayed the onset of disease and significantly reduced the incidence of diabetes (35). Conversely, IL-10 transgenic NOD mice, in which IL-10 was expressed in the pancreatic islets, had severe insulitis and prominent ductal proliferation. In addition, the onset of diabetes in NOD transgenic mice was earlier than that of 14 weeks of age at the earliest in non-transgenic NOD mice (36).

Similarly, in mouse model of bone marrow transplantation, Blazar showed that IL-10 had a dose-dependent effect on the GvHD lethality, because high doses accelerated lethality, while low amounts protected mice from GvHD (37). In a mouse model of heart allograft, the effects of exogenous IL-10 on organ allograft survival were dependent on timing and dosage. IL-10 treatment prior to the transplant increased

heart survival while the administration at the time of the graft or after the graft, had little beneficial effect or even promoted rejection (38).

These controversial data showed that the several effects of IL-10 on the modulation of autoimmune diseases and graft rejection, depended on the site and time of administration and importantly, on the dose of cytokine injected.

IL-10 *in vivo* in human

Similarly to the mouse, the role of IL-10 in the control of immune response in human is well accepted. Several reports showed that IL-10 modulated many cells and effector functions associated with allergy. Lim described an inverse association between IL-10 levels and the severity of allergic and asthmatic disease, demonstrating that in asthma, the low IL10-producing haplotype was more likely to be associated with severe disease (39). In the nasal mucosa from allergic rhinitis patients, IL-10 was expressed with large variation between individuals that correlates with allergic symptoms. Patients with lower epithelial IL-10 levels had severe allergic rhinitis in comparison to patients with mild symptoms (40). Moreover, it has been demonstrated that IL-10 mRNA expression, by purified T cells of children with allergic and non-allergic asthma and children with atopic dermatitis, was strongly decreased as compared with that of healthy controls (41). Several reports indicated that IL-10 could play a pivotal role also in allergen-specific immunotherapy (SIT), efficiently used in allergy to insect venoms and allergic rhinitis. SIT was found to be associated with a decrease in IL-4 and IL-5 production and an increased in IL-10 and/or TGF-β production (42). Akdis showed that during the early phase of the bee venom-SIT, intracellular IL-10 significantly increased in the Ag-specific T cell population and activated $CD4^+$ T

lymphocytes, while B cells and monocytes were responsible for IL-10 production at later time points. The data indicated that the anergic state in T cells was induced by IL-10, initially produced by the peripheral T cells themselves after high-dose of allergen administration and then followed by the modulated antigen-presenting cell (APC) population, required to maintain tolerance (43). The proliferative and cytokine responses could be reconstituted by *ex vivo* neutralization of endogenous IL-10, indicating that this cytokine was actively involved in promoting tolerance after allergen-SIT (43). Moreover, it has been demonstrated that therapies useful for the treatment of allergy and asthma, such as glucocorticoids, promoted the increase of IL-10 secretion by T cells (44).

In bone marrow transplantation, several works focalized their attention on the correlation between IL-10 and the absence of GvHD after HSCT. The importance of endogenous IL-10 production has been clearly demonstrated 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 developed GvHD, despite the HLA disparity. Interestingly, high levels of IL-10 mRNA were detected *in vivo* in T cells and monocytes of these patients (45). Moreover, it has been demonstrated a positive correlation between high spontaneous IL-10 production by PBMC of patients prior to allogeneic bone marrow transplantation and a subsequent low incidence of GvHD and transplant-related mortality, compared to patients with low or intermediate IL-10 production. In patients with high cellular IL-10 production, there was also an increased in IL-10 serum levels, indicating a simultaneous occurrence of cellular as well as systemic IL-10 production (46, 47).

Type 1 Regulatory T cells

The first type of IL-10-producing Regulatory T cells (Tregs) described at the clonal level was designated Tr1 cells by Groux and colleagues. These cells are characterized by their capacity to produce high levels of IL-10 and TGF-β, and by their ability to suppress Ag-specific effector T-cell responses *in vitro* and *in vivo* via a cytokine-dependent mechanism mediated by IL-10 and TGF-β (27). Tr1 cells can be generated *in vitro* and *in vivo* upon priming of naïve T cell precursors with the Ag in the presence of IL-10. Tr1 cells can be distinguished from Th1 and Th2 cells, because they produce high levels of IL-10, TGF- β, and IL-5; low amounts of IFN-γ and IL-2; and no IL-4 (Table 2).

	$CD4^+$ T-cell subsets			
Cytokine	Th0	Th1	Th2	Tr1
IL-2	+++	+++	+/−	+/−
IFN-γ	++	+++	+/−	++
IL-4	++	+/−	+++	−
IL-5	++	+/−	+++	++
IL-10	+	+	++	+++
TGF-β	++	++	++	+++

From Roncarolo et al., Immunol. Reviews, 2001

Table 2. The typical cytokine production profile of human $CD4^+$ T cell subsets

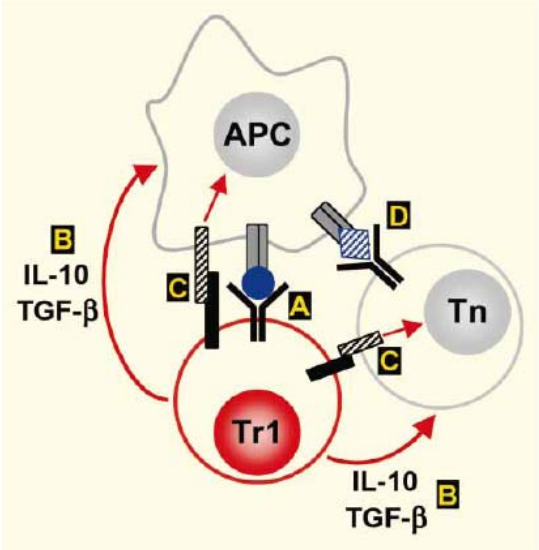
Tr1 cells secrete IL-10 very soon after activation as compared to others T cell subsets. IL-10 is detectable 4 hours after activation and the highest concentration is reached 12–24 h after activation (45, 48). Depending on the experimental conditions used for

their induction, Tr1 cells can produce different amounts of TGF-β, IFN-γ, and/or IL-5, but their levels of IL-10 production, which represents the true hallmark of Tr1 cells, are invariably high.

Tr1 cells are anergic upon polyclonal-mediated or Ag-specific activation (27, 45). These cells do not expand significantly under standard culture conditions, this could be due to the autocrine production of IL-10, since addition of anti-IL-10 monoclonal antibody partially restores the proliferation (27, 45). Even if Tr1 cells display low proliferative capacity, they can expand in the presence of IL-2 and IL-15. Long-term culture in IL-15 does not alter the phenotype or function of Tr1 cell clones, although it enhances production of IFN-γ (49). This observation is support by the fact that activated Tr1 cells express the IL-2 receptor (IL-2R) α chain, and high levels of the IL-15Rα chain, together with both the IL-2/15Rβ and IL-2Rγ chains. Upon TCR-mediated activation Tr1 cells also express activation markers such as CD40L, CD69, CD28, cytotoxic T-lymphocyte antigen-4 CTLA-4 and HLA-DR (49). In the resting phase, human Tr1 cells express both Th1-associated (CXCR3 and CCR5) and Th2-associated (CCR3, CCR4, and CCR8) chemokine receptors (50). CCR8 is expressed at higher levels compared with Th2 cells, and upon activation, human Tr1 cells migrate preferentially in response to I-309, a ligand for CCR8 (50). It has been recently demonstrated that in a model of helminth infection the expression of CCR8 was strongly associated with IL-10-producing $CD4^+$ T cells, which resemble Tr1 cells, suggesting a relevant role of CCR8 *in vivo* (51). Several reports showed that Tr1 cells do not constitutively express the transcription factor forkhead box protein 3 (Foxp3), but they can upregulated Foxp3, upon activation, to levels similar to those observed in activated $CD4^+CD25^-$ T cells (52, 53). Although significant efforts have been made to identify molecules expressed by Tr1 cells to discriminate them from

other Treg subsets and from effector T cells, no specific marker for Tr1 cells has been reported so far. The low proliferative capacity of Tr1 cells is a major limitation and has hampered progress in characterizing this T cell subset *in vitro*.

Tr1 cells are involved in down-regulation of immune responses due to their ability to produce high levels of the immunosuppressive cytokines IL-10 and TGF-β. They suppress both naıve and memory T-cell responses *in vivo* and *in vitro* (27, 45, 54). Ag-specific Tr1 cells need to be activated via the TCR to exert their suppressive function; but, once activated, they can mediate bystander suppressive activity against other Ags by the release of IL-10 and TGF-β (55, 56) (Figure 7).



From Roncarolo et al., Immunol. Reviews, 2001

Figure 7. Possible mechanisms of action of Tr1 cells. (A) Tr1 cells release IL-10 and TGF-b into the microenvironment after activation via the TCR. (B) These cytokines act on APCs and/or naive (Tn) or memory (Th) $CD4^+$ T cells in close proximity, resulting in indirect and direct suppression of T-cell-mediated responses. (C) Tr1 cells may also upregulate inhibitory receptors, whose ligands are expressed on APCs and/or T cells. (D) Tr1 cells mediate bystander suppression, and thus do not necessarily share the same Ag specificity as the target T cells.

The suppressive effects of Tr1 cells are reversed by addition of anti-IL10 and anti-TGF-β neutralizing monoclonal antibodies (27, 45, 52), but additional mechanisms may also contribute. For example, Grossman recently demonstrated that human Tr1 cells generated *in vitro* by cross-linking of CD3 and CD46, a complement regulatory protein, and nTreg cells, can develop considerable cytotoxic activity, through the production of granzyme (GZ) B and GZ-A granules, respectively, in a perforin-dependent manner (57-59).

Tr1 cells *in vivo*

As indicated previously, the importance of IL-10 in controlling immune response has been widely demonstrated. Furthermore, up to now several works provided evidence that Tr1 cells are involved in many T cell-mediated diseases and could play a pivotal role in the regulation of transplantation tolerance *in vivo*.

In the setting of bone marrow transplantation, the first suggestion that human Tr1 cells were involved in maintaining peripheral tolerance *in vivo* came from studies in SCID patients successfully transplanted with HLA-mismatched allogeneic stem cells. From the peripheral blood of these patients it would be possible to isolate a high proportion of donor-derived T cells, specific for the host HLA Ags and able to produced high levels of IL-10 (45).

Interesting is the finding reported by Weston, who showed a correlation between high number of donor T cells producing IL-10 towards recipient allo-Ags and the absence of acute GvHD after HSCT. He suggested that a condition where IL-10 was present during repetitive presentation of the Ag *in vivo* could therefore predispose to less or absent grades of acute GvHD (60). Moreover, the importance of IL-10 and TGF-β

producing $CD4^+$ T cells has been described in solid organ transplantation (61-62), and correlates with the spontaneous development of tolerance to kidney or liver allograft.

Regarding the role of Tr1 cells in allergy, Nickel-specific Tr1 cell clones could be isolated from both peripheral blood and lesional skin of patients allergic to nickel (Ni). These Ni-specific Tr1 cells inhibited in an IL-10 dependent manner the functions of monocytes and dendritic cells (DC) and directly suppress Ni-specific Th1 responses. The results showed that Tr1 cells may have a crucial role in limiting excessive tissue reaction to haptens by blocking DC and monocytes functions and Th1 cell response. The high frequency of Tr1 cells in nonallergic donors also suggests that these cells may be important in determining whether silent immune responses or manifest allergic disease can develop (63). Akdis demonstrated an increased frequency of allergen-specific Tr1 cells with a concomitant decreased of allergen-specific Th2 cells in non-allergic subjects compared to allergic individuals. These results clearly indicated that the balance between allergen-specific Tr1 and Th2 cells was a key determinant in the development of allergic responses in humans (64).

Several works showed that Tr1 cells regulate responses to self-Ags controlling autoimmunity. Indeed, self-major histocompatibility complex (MHC)-reactive Tr1 cell clones, functionally distinct from Ag-specific T cell clones, could be isolated from the peripheral blood of healthy individuals. These cell clones inhibited proliferation of primary $CD4^+$ T cells and tetanus toxoid-specific T-cell clones via IL-10 and TGF-β, suggesting that activated self-MHC-reactive T cells with the cytokine phenotype of Tr1 cells, may be important regulatory cells that mediate peripheral tolerance and prevent the development of autoimmunity (65). Similarly, Arif showed that autoreactive T cells specific for HLA class II molecules associated with type 1 diabetes (T1DM) development, exhibited extreme polarization toward a pro-

inflammatory Th1 phenotype, producing predominantly IFN-γ, in patients with T1DM. On the contrary, autoreactive T cells from non-diabetic individuals, carrying HLA class II molecules associated with T1DM, were present but polarized to produce IL-10 in response to islet peptides. The authors concluded that development of T1DM depended on the balance of autoreactive Th1 and Treg cells, that islet destruction was characterized by pro-inflammatory autoreactive T cells, while the tolerant state was characterized by autoreactive IL-10 secreting T cells (66).

Finally, some reports indicated the presence *in vivo* of Tr1 cells specific for tumor Ags. In the context of Hodgkin lymphoma (HL), it has been demonstrated that Hodgkin lymphoma infiltrating lymphocytes (HLIL), not only were anergic, but also could profoundly inhibit effector T cell responses. HLIL were highly enriched in IL-10-secreting Tr1 and $CD4^+CD25^+$ Treg cells, which induced a profoundly immunosuppressive environment and so provided an explanation for the ineffective immune clearance of cancer cells (67). Strauss showed that in patients with head and neck squamous cell carcinoma (HNSCC), tumor infiltrating lymphocytes (TIL) were enriched in Treg cells. These subset of cells expressed a unique phenotype distinct from that of Treg in PBMC, they produced IL-10 and TGF-β and did not require cell-to-cell contact to responder cells for suppression. Furthermore, their presence in the tumor was linked to a poor prognosis in subjects with HNSCC (68).

In vitro induction of Tr1 cells

Since the importance of IL-10 and Tr1 cells in controlling immune responses and inducing peripheral tolerance *in vivo* has been widely demonstrated, several protocols have been developed to generate Tr1 cells *in vitro*.

Groux demonstrated that human $CD4^+$ T cells, activated by allogeneic monocytes in a primary mixed lymphocyte reaction (MLR) in the presence of exogenous IL-10, failed to proliferate after restimulation with the same allo-Ags. The anergic state induced by IL-10 was long-lasting (26). During the culture in the presence of IL-10, T cells became not only anergic, but differentiated in a distinct population of IL-10 producing T cells. Activation of both human and murine $CD4^+$ T cells in the presence of IL-10 generated T cell clones with a cytokine production profile typical of Tr1 cells and different from that of Th1 and Th2 cells (27).

Although IL-10 has been shown to be indispensable for Tr1 cell induction, it is not sufficient for the differentiation of Tr1 cells in the absence of APC *in vitro* (69). In mouse model, it has been demonstrated that IL-10 synergized with TGF-β to promote allo-Ag hyporesponsiveness in murine $CD4^+$ T cells. These anergized T cells were Ag-specific since they proliferated in response to nominal Ags, and *in vivo* transfer of these cells resulted in the prevention of lethal GvHD after bone marrow transplantation (70). In other studies, it has been also shown that Ag-specific regulatory T cells developed via the ICOS–ICOS-ligand pathway and that both the induction and the function of Treg cells depended on the presence of IL-10 and ICOS pathway. These cells produced IL-10 and had potent *in vivo* and *in vitro* inhibitory activity; when transferred into sensitized mice they blocked the development of allergen-induced airway hyperreactivity (71).

In human, it has been demonstrated that endogenous or exogenous IL-10 in combination with IFN-α, but not TGF-β, promoted the differentiation of $CD4^+$ T cells derived from cord blood, into T cells with the cytokine profile and the biological activities of Tr1 cells. In parallel, naive $CD4^+$ T cells derived from peripheral blood required both exogenous IL-10 and IFN-α for Tr1 cell differentiation (72).

Kemper showed that co-engagement of CD3 and the complement protein CD46 in the presence of IL-2 induced a Tr1-specific cytokine phenotype in human $CD4^+$ T cells. These CD3/CD46-stimulated IL-10-producing $CD4^+$ cells proliferated strongly, suppressed activation of bystander T cells and acquired a memory phenotype (59).

Finally, Barrat showed that vitamin D3 and Dexamethasone (Dex) could induced human and mouse naive $CD4^+$ T cells to differentiate *in vitro* into regulatory T cells. The development of these Tr1 cells was enhanced by neutralization of the Th1 and Th2 inducing cytokines IL-4, IL-12, and IFN-γ. Tr1 cells were induced *in vitro* stimulating naïve T cells with immunosuppressive drugs in the presence of APC, but the authors demonstrated that Vit D3 and Dex also could induce the expansion of IL-10 producing T cells when naive $CD4^+$ T cells were stimulated with immunosuppressive drugs plus anti-CD3 and anti-CD28 only. The injection of these Tr1 cells in a mouse model of experimental autoimmune encephalomyelitis (EAE), prevented EAE with absolute abrogation of disease onset and absence of clinical signs in an IL-10 dependent manner (54).

Induction of Tr1 cells by immature or semi-mature dendritic cells

DC are the most effective APC that regulate the balance between effector T and Treg cells on the basis of the expression of specific immunogenic or tolerogenic molecules

and of the secretion of inflammatory or modulatory cytokines (73). Several studies indicated that Treg cells could be induced by DC both in an immature or semi-mature stage. In the steady state, presentation of the Ag by immature DC (iDC) lacking of costimulatory stimuli and unable to stimulate immunity to the self-Ags they have captured, results in tolerance (74).

Jonuleit compared the influence of different maturational states of DC on the differentiation of naive $CD4^+$ T cells, showing that iDC and mature DC (mDC) induced completely different T cell phenotypes. Repetitive stimulation of naïve cord blood $CD4^+$ T cells with allogenic iDC resulted in the differentiation of IL-10 producing Treg. These T cells lost their ability to produce IFN-γ, IL-2, or IL-4 and became non-proliferating T cells able to inhibit the Ag-driven proliferation of Th1 cells in a contact- and dose-dependent, but Ag-nonspecific manner (75).

Similarly, it has been reported that repeated stimulation of naïve peripheral blood $CD4^+$ T cells with allogeneic iDC could induce the differentiation of human Tr1 cells *in vitro*. In this system, after three rounds of stimulation with iDC, T cells became profoundly anergic and acquired regulatory function. These T cells secreted high levels of IL-10 and TGF-β, significant amounts of IFN-γ and IL-5, low IL-2, and no IL-4, and they suppressed T-cell responses by the production of IL-10 and TGF-β̃. The induction of these Treg cells could be blocked by anti-IL10R monoclonal antibody, indicating that they were Tr1 cells generated through autocrine production of IL-10 by iDC (52).

Similarly, the stimulation of iDC with TNF-α in the absence of danger signals, resulted in incompletely matured DC (semi-mature DC). The maturation by TNF- α induced high levels of MHC class II and costimulatory molecules on DC, but they remained weak producers of proinflammatory cytokines. Therefore, these semi-

mature DC led to the induction of peptide-specific predominantly IL-10 producing $CD4^+$ T cells *in vivo* and provided complete protection from EAE (76).

Induction of Tr1 cells by tolerogenic dendritic cells

It has been demonstrated that not only immature or semi-mature DC, but also DC rendered tolerogenic with biological or pharmacological agents, could drive the differentiation of Treg cells (48, 77).

IL-10 modulates the function of APC, including DC (22, 78). After IL-10 treatment, DC display reduced surface expression of MHC class I and class II molecules and costimulatory molecules of the B7 family (79), and the release of pro-inflammatory cytokines by DC, such as IL-1β, IL-6, TNF-α and most markedly IL-12, is abolished (80).

Immature DC differentiated and treated *in vitro* with exogenous IL-10 displayed reduced allo-stimulatory capacity and were shown to induce anergic Ag-specific T cells (81). Furthermore, DC matured in the presence of exogenous IL-10 for the last two days of culture, showed a strongly reduced capacity to stimulate a $CD4^+$ T cell response in allogeneic MLR in a dose-dependent manner and induced Ag-specific anergic T cells (78). These anergic T cells were able to suppress activation and function of T cells in an Ag-specific manner through a cell-cell contact mechanism (82).

Gregori recently demonstrated that IL-10 induced the differentiation of a unique subset of myeloid DC (DC-10), which were $CD14^+CD11c^+CD11b^+CD83^+HLA\text{-}DR^+CD71^+CD1a^-$, displayed a myeloid mature phenotype ($CD80^+CD86^+$) and expressed ILT2, ILT3 and ILT4. These cells secreted significantly higher levels of IL-

10 compared with iDC, whereas the amounts of IL-12 were low and comparable to those produced by iDC. Interestingly, this ratio of cytokine production was maintained upon activation with lipopolysaccharide (LPS) and IFN-γ. DC-10 were potent inducers of Tr1 cells in a allogenic system *in vitro*, more powerful than iDC, since they reproducibly induced anergic T-cell lines, with strong suppressive activity, in a single round of stimulation (Gregori et al., 2008, manuscript submitted).

A variety of immunosuppressive agents currently used in the treatment of autoimmune diseases and in the control of allograft rejection, including glucocorticoids, mycophenolate mofetil and sirolimus, have been shown to induce DC with tolerogenic phenotype and function. These agents impair DC maturation and inhibit up-regulation of costimulatory molecules, secretion of proinflammatory cytokines, in particular IL-12, and allostimulatory capacity. On the other hand, controversial effects of calcineurin inhibitors, like cyclosporine A and tacrolimus, have been reported on DC maturation, although these drugs have a clear inhibitory effect on DC, decreasing their cytokine production and allostimulatory capacity (77).

Several studies reported that DC loaded with tumor Ags could also induce the differentiation of Tr1 cells. For example, exposure of DCs to myeloma cells (83), or to an adenoviral vector expressing a prostate-specific antigen (PSA) (84) could prime DC to produce IL-10. These DC primed the differentiation of Tr1 cells, which inhibit effector T cells specific for PSA.

Finally, DCs could be engineered to express tolerogenic molecules, such as IL-10, TGF-β, CTLA-4, and drove the differentiation of Tregs that mediate Ag-specific tolerance (85).

I*n vivo* induction of Tr1 cells

The induction *in vivo* of Tr1 cells, in addition to their *ex vivo* generation, is certainly an attractive goal.

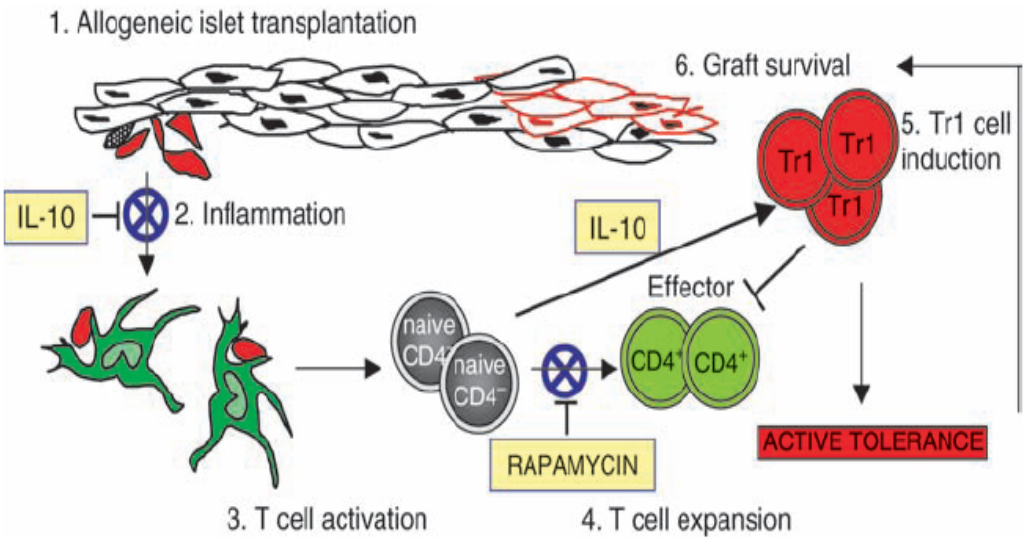
Treatment of mice with a killed Mycobacterium vaccae-suspension gave rise to allergen-specific regulatory T cells which conferred protection against airway inflammation. This inhibition was mediated by IL-10 and TGF-β, since antibodies against IL-10 and TGF-β completely reversed the inhibitory effect of Tr1 cells (86).

The possibility to induce *in vivo* Tr1 cells specific for allo-Ags has been investigated by our and other groups in models of pancreatic islet transplantation (87) (88). Asiedu demonstrated that peritransplant treatment of multiple nonhuman primates (NHP) with anti-CD3 immunotoxin and deoxyspergualin induced stable rejection-free tolerance to MHC-mismatched allografts, associated with sustained elevations in serum of IL-10. In this regimen, anti-CD3 immunotoxin depleted effector T cells, whereas deoxyspergualin arrested the production of proinflammatory cytokines and the maturation of DCs. An increase in the frequency of Tr1 cells was found in the peripheral blood of long-term tolerant NHP recipients in comparison with controls. Moreover, also an increase in $CD4^+$ $CD25^+Foxp3^+$ Tregs frequency was detected in tolerant NHP. In summary, the study showed an expansion of Treg populations in tolerant NHP recipients, suggesting that these variations should be involved in maintenance of stable tolerance (87).

Similarly, Battaglia et al. demonstrated that an *in vivo* treatment with rapamycin + IL-10 was able to prevent allograft rejection in diabetic mice transplanted with allogeneic pancreatic islets. This treatment not only prevented acute allograft rejection but also led to active long-term tolerance via induction of Ag-specific Tr1 cells.

Administration of IL-10 alone was not able to prevent rejection. Even if IL-10 administration should down-regulated inflammation and allowed induction of Tr1 cells, it might not be sufficient to block the expansion of effector T cells. The combination of IL-10 with a compound able to down-modulate the effector phase of the immune response was required for the induction of operational tolerance in this model. Rapamycin blocked IL-2-induced expansion of effector T cells, without preventing Tr1 cell induction via TCR stimulation in the presence of IL-10 (Figure 8) (88).

Both studies suggested that induction of Ag specific long-term tolerance needed the synergistic effect of drugs that down-modulate inflammation, block effector T cells, and differentiate Tr1 cells.



From Roncarolo et al., Immun. Reviews, 2006

Fig. 8. Prevention of allograft rejection in vivo by Tr1 cells induced with rapamycin and interleukin-10. To prevent allograft rejection, the massive inflammation caused by the transplant must be modulated in order to reduce the recruitment and maturation of professional APC. (1). Expansion of T effector cells (Teff) must be tightly controlled and avoided, while conditions that permit Treg development and function should be preserved. Accordingly, treatment with rapamycin and interleukin-10 (IL-10) leads to allograft tolerance in diabetic mice transplanted with allogeneic pancreatic islets. Rapamycin and IL-10 have a general anti-inflammatory effect (2). Rapamycin, without inhibiting T-cell activation (3), blocks the early expansion of alloreactive T cells (4) and allows induction of Ag-specific Tr1 cells through IL-10 (5). With this combined therapy, graft survival and transplantation tolerance via induction of Tr1 cells is efficiently achieved (6).

Cellular therapy with Tr1 cells

At present, allogeneic HSCT is the treatment of choice for several disorders of the hematopoietic system, such as hematologic malignancies (leukemia, lymphoma, and multiple myeloma) and genetic diseases (β-thalassemia and primary immunodeficiencies) (89). Immunologic mechanisms mediated by donor derived T cells, contained along with the graft or administered separately as donor lymphocyte infusions (DLIs), make a major contribution to the eradication of malignant cells after HSCT, since donor cells not only accelerate immune reconstitution but also eliminate residual neoplastic cells [graft-vs.-leukemia activity (GVL) and graft-vs.- tumor activity (GVT)]. Unfortunately, acute and chronic GvHD which is also mediated by donor T cells and has been linked to tumor regression, remains one of the main clinical complications after allogeneic HSC transplantation (90). Thus, several studies has been made trying to suppress GvHD with maintenance of GVT activity.

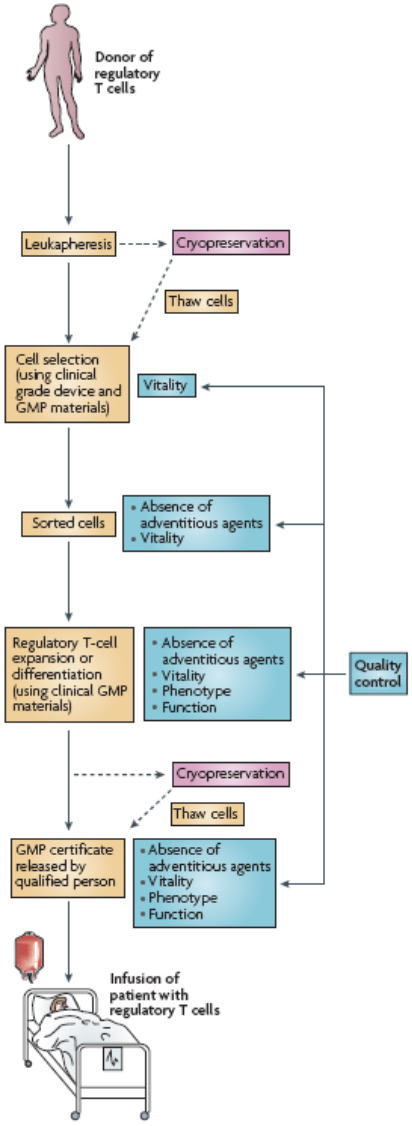
The use of Treg cells as a cellular therapy after allogeneic HSC transplantation, represent a promising approach to separate the two effects. The role of natural occurring $CD4^+CD25^+$ Tregs has been extensively explored in preclinical murine models of cellular therapy (91). Edinger showed that, in a murine model of HSCT followed by engraftment of leukemia cells, $CD4^+CD25^+$ Tregs were able to prevent acute GvHD by suppressing alloreactive T-cell expansion without abrogating the anti-tumor effector functions mediated by these cells (92).

In view of the fact that Tr1 cells can be differentiate *ex-vivo*, the possibility to use this T cell subset as cellular therapy to prevent GvHD *in vivo* has been explored. The first demonstration that Tr1 cells differentiated *ex-vivo* with IL-10 could modulate immune responses *in vivo* came from Groux's observation that Tr1 cell clones suppressed Th1-

mediated colitis induced in SCID mice by transfer of naïve $CD4^+CD45RB^{hi}$ cells. He demonstrated that OVA-specific Tr1-cell clones were functional only if mice were treated with the specific OVA peptide, indicating that Tr1 cells had to be activated via the TCR in order to exert their regulatory effects (27).

In mouse model, IL-10 synergies with TGF-β to promote allo-Ag hyporesponsiveness in murine $CD4^+$ T cells. Donor $CD4^+$ T cells, anergized *ex vivo* and transferred in a preclinical model of bone marrow transplantation, were markedly impaired in inducing GvHD alloresponses to MHC class II mismatched recipients (70).

All together these results indicated that Ag-specific Tr1 cells generated *ex vivo* with different approaches can be used in cellular therapy to efficiently prevent GvHD in human *in vivo* (Figure 9).



From Roncarolo et al., Nature Rev. Immunol., 2007

Figure 9. Development and assessment of human cell therapy products. The steps necessary for the development of regulatory T cells as a medicinal product are shown. From cell collection to cell processing, expansion and differentiation, and final infusion into the patient, clinical good manufacturing practice (GMP) procedures need to be performed. Various quality controls are required in each manufacturing step and the final product can be released and infused into the patient only after approval by a qualified person.

PATIENTS, MATERIAL AND METHODS

Patients

Fourteen non-consecutive patients undergoing HSCT between 1998 and 2004 for transfusion dependent beta-thalassemia were analysed. Patients' characteristics and indications for transplantation are shown in Table 3. Median age was 3 years and 8 months (range 2-8 years). Risk class according to Pesaro (6) was class I (8), II (3) and III (3). Twelve patients were transplanted from a HLA genotipically identical sibling, 1 from an HLA phenoidentical 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. 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. Pt #11 received a BMT 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 BMT. Post-HSCT GvHD prophylaxis consisted of cyclosporine, metilprednisolone and short methotrexate (93). Cyclosporine was started at 5 mg/kg intravenously day -2 through day +5 then reduced to 3 mg/kg and tapered from day +60 of 5%/week till a complete stop at +365. The desired plasmatic range 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/m^2 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, was performed in an extensive period of time (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.

Pt no.	Risk Class*	Host:donor gender	Donor°	Time post-transplant (year)	Outcome`	Donor cells in PB (%)°°	GvHD
1	2	M/M	MUD	8	PMC	50	no
2	1	M/M	Pheno	6	PMC	50	Gr.3 (d.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 (d.28)
11	3	M/F	Sib	1	CC	100	no
12	2	M/F	Sib	1	CC	100	Gr.2 (d.30)
13	1	M/F	Sib	2	CC	100	no
14	2	F/M	Sib	1	CC	100	Gr.1 (d.15)

*According to Pesaro classification.
°The source of donor stem cell has been bone marrow for all the patients. MUD: Matched unrelated donor; Pheno: Phenoidentical family donor; Sib: Sibling HLA identical donor.
`PMC: Persistent mixed chimerism; CC: Complete chimerism.
°°Determined by STR typing. PB: Peripheral Blood.

Table 3. Characteristics of patients (pt), donors and transplants

Mixed Chimerism evaluation:

A - 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 (94).

B – 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 Ags - D, C, c, E, e - (Institute Jacques Boys SA, Reims Cedex, France) in fresh peripheral blood following the manufacturer's instructions.

TCR repertoire analysis by TcLandscape

The TCR repertoire analysis of the peripheral T cells of the patient was provided by TcLand company, Nantes, France.

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 0.3 cell/well in presence of a feeder cell mixture and soluble anti-CD3 mAb (1 μg/ml, OKT3, Jansen-Cilag, Raritan, NJ) 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) as Tr1 growth factor (49).

Cytokine detection

To determine the cytokine production after polyclonal activation, T cell clones (1x106 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 24 h for IL-2, and 48 h for IL-4, IL-10, and IFN-γ. Cytokine production was determined by ELISA according to manufacturer's instructions (PharMingen). To test cytokine production after Ag-specific activation, T cell clones ($1x10^6$ cells/ml) were plated with $1x10^5$/ml mature allogeneic DCs. Cytokine levels in cell culture supernatants were detected using the human Th1/Th2 cytokine cytometric bead array (CBA) system (BD Biosciences, San Jose CA, USA). For intracellular cytokine production, T cell clones were activated with phorbol myristate acetate (TPA, 10 ng/ml, Calbiochem) and anti-CD3 (10 μg/ml) mAb for 6 hours. Brefeldin A (10 mg/ml, Sigma) was added for the final 3 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–IL-2, anti–IL-4, anti–IL-10 and FITC–labeled anti–IFN-γ mAbs (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-IL-10 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, CD25, CD28, CD14, and HLA-DR (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 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), according to the manufacturer's instructions.

ELISPOT assay

IL-10 secreting T cells were enumerated by enzyme-linked immunospot (ELISPOT) assay. Fresh 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, US). 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. After 5 days of culture, supernatants were collected for analysis of IFN-γ 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 allogeneic mature DC (10:1, T cells/DC) with or without anti–IL-10R (50 μg/mL, 3F9, 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 μCi/well of [3H]-thymidine.

DC differentiation

$CD14^+$ monocytes were isolated as the adherent fraction after an incubation of 1 hour in RPMI 1640 (Biowhittaker) with 5% pooled AB human serum (HS) (BioWhittaker), 100 U/ml penicillin/streptomycin (Bristol-Myers Squibb), and 2mM L-glutamine (GIBCO BRL, Gaithersburg) (DC medium) at 37°C. After different washing, adherent monocytes were cultured in 10 ng/ml rhIL-4 (R&D Systems) and 100 ng/ml rhGM-CSF (R&D Systems) in DC medium alone or in the presence of 10 ng/ml of rhIL-10 (BD Bioscience, Mountain View, CA) for 5 days. After 5 days, DC differentiated in the absence of IL-10 were matured with lipopolysaccharide from E.

Coli (LPS) (1 μg/ml, Sigma Chemicals, St Louis, MO) for additional 2 days. At day 7, DC generated in the presence of IL-10 (DC-10), and mature DC (mDC) were collected, irradiated at 6000 Rads and used to stimulate T cells. The purity and maturation state of DC were checked by flow cytometry to determine expression of CD1a, CD14, CD83 and HLA-DR.

Mixed lymphocyte cultures (MLR) and proliferation assay

Human peripheral blood was obtained from healthy donors, haploidentical donors, and HLA-matched unrelated donors in accordance with local ethical committee approval. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation over Lymphoprep (Nycomed Amersham, Uppsala, Sweden). Total PBMC were used as responder cells and CD3-depleted cells or DC as stimulators. When CD3-depleted cells were used as stimulators, responder cells were plated at 1:1 ratio: $5x10^5$/well with the same number of stimulator cells in a final volume of 1 ml, in 24-well-plates (Costar, Cambridge, MA), or $1x10^5$/well in a final 200μl in round bottomed 96-well-plates, in the presence or absence of rhIL-10 (10 ng/ml) (Figure 5A). When DC were used as stimulators, responder cells were plated at 10:1 ratio: 10^6/well PBMC with 10^5/well of DC (mDC or DC-10) in a final volume of 1 ml, in 24-well-plates (Costar), or 10^5/well PBMC with 10^4/well of DC in a final 200μl in round bottomed 96-well-plates (Figure 5B). To evaluate secondary responses, primary cultures were carried on in 24-well plates for 10 days, in the presence or absence of IL-10. At day 7 half of the medium, with or without cytokines, was replaced with fresh one. At day 10 cells were collected, washed and plated in 96-well plates with newly prepared stimulator cells (at 1:1 ratio for CD3-depleted cells or 10:1 ratio for mDC), without the addition of cytokines. After the indicated time, cells

were either pulsed for 16 hours with 1 μCi/well ^{3}H-thymidine or supernatants were collected for analysis of IFN-γ production.

Antigen-specific responses

After 10 days of culture, cells were harvested, washed and plated at 10^{5}/well in 96-well plates with 10^{5}/well autologous irradiated CD3-depleted cells in the presence of Candida Albicans (10^{6}/well heat-inactivated spores, generously provided by Proff.ssa L. Romani, University of Perugia, Italy) or Tetanus Toxoid at 5 μg/ml (Alexis Biochemicals, San Diego, CA). In parallel cells were stimulated with TPA (10 ng/ml; Calbiochem, Biosceince, La Jolla, CA) plus Ionomycin (150 ng/ml; Sigma). After the indicated time, cells were either pulsed for 16 hours with 1 μCi/well ^{3}H-thymidine.

Statistical analysis

All analysis for statistically significant differences were performed with the student's *t* test or non-parametric Wickoxon test. *p* values less than 0.05 were considered significant.

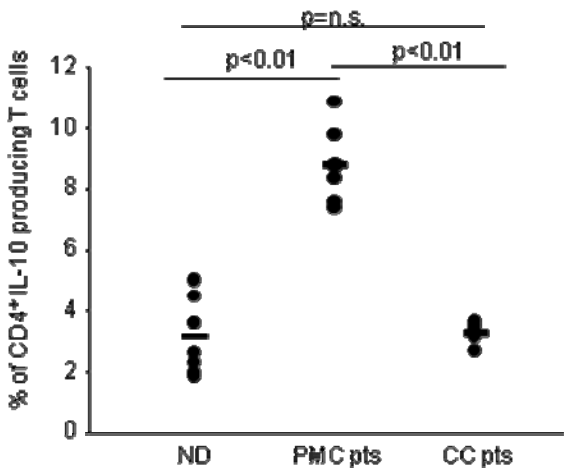
RESULTS PART 1

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 (2 to 4 years after the transplant), whereas the other PMC patients were tested at later points (6 to 10 years after the transplant) (Table 3). The percentage of $CD4^+$ IL-10 producing T cells after TPA/ionomycin stimulation is shown in Figure 10A. 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 10B. 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.

A



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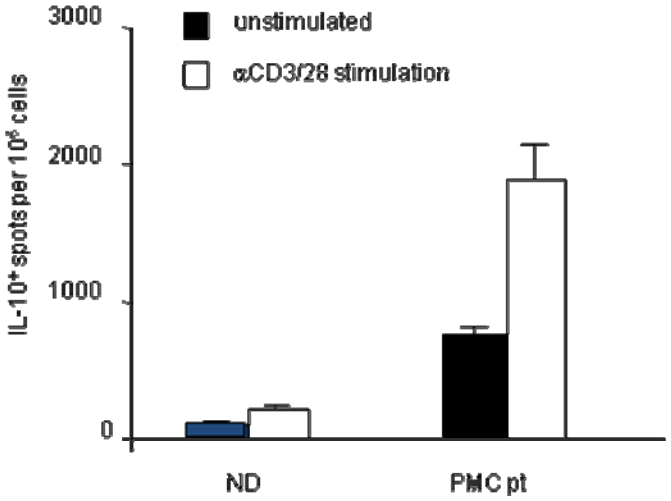


Figure 10. IL-10 production by T cells of transplanted thalassemic patients with persistent mixed chimerism.

(A) Cytokine production by PBMCs of patients with PMC and normal donors was determined by intracytoplasmic staining after polyclonal activation with TPA/ionomycin. The percentages of IL-10 producing T cells within the gated $CD4^+$ T cells for each patient and ND are indicated in the graph. (B) IL-10 producing cells of a PMC patient and of a ND were counted by ELISPOT. The IL-10 positive spots were measured in the unstimulated cultures and after αCD3/αCD28 stimulation.

Kinetics of persistent mixed chimerism

We further characterized the peripheral T cell repertoire in patients with PMC following HSCT. A PMC 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, hemoglobin concentration and percentage of reticulocytes, over the past four years (Table 4).

	Mean*	sd*
WBC (cell/μl)	8450	2069
RBC (cell/μl)	4×10^6	$1{,}7 \times 10^5$
Hb (g/dl)	11.4	0.1
RTC (%)	1.5	0.3

*Data represent means of values from +5 to +8 years after the transplant.
WBC indicates white blood cells; RBC, red blood cells; Hb, hemoglobin; RTC, reticulocytes.

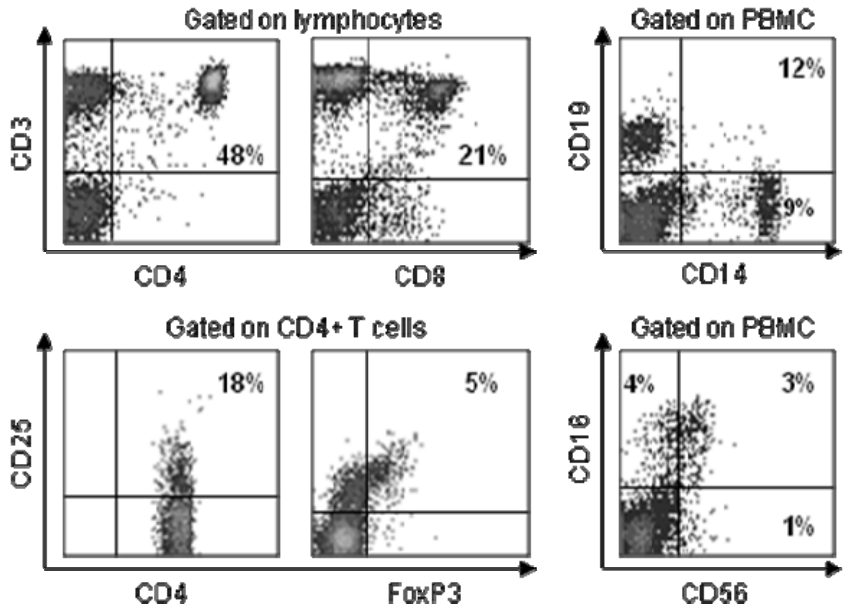
Table 4. Mean blood cell counts of patient with PMC

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 (Figure 11A). In addition, $CD4^+CD25^+$ and $CD4^+CD25^+Foxp3^+$ T cells were present in normal proportion (18% and 5%, respectively) (Figure 11A).

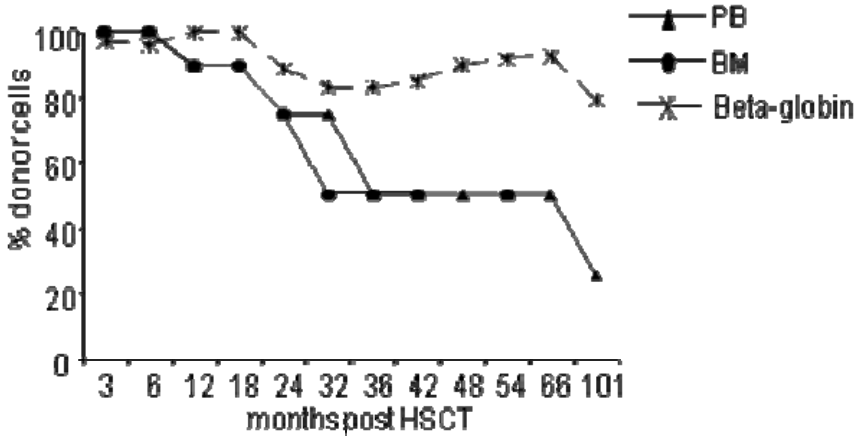
Shortly after the HSCT, the patient showed complete donor chimerism, but one year following the transplant, the donor cells began to decrease both in the bone marrow (BM) and in the peripheral blood (PB), declining up to 50% 36 months after HSCT and remaining stable eight years following the transplant (Figure 11B). 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 11B) 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 observed 101 months after HSCT, with proportions of donor $CD3^+$, $CD19^+$, and $CD56^+$ cells of 50%, 25%, and 40%, respectively (Figure 11C). Interestingly, despite the low percentage of donor cells in the lymphoid lineages, the proportion of donor erythrocytes was 85% (Figure 11C). This indicates a predominant donor chimerism in the erythrocyte compartment, associated with a split, long-term chimerism in the lymphoid cells.

Studies of the Vβ repertoire of the peripheral T cells of the patient, performed at two time points following HSCT, revealed a stable skewed repertoire in multiple Vβ families, which clearly differed from the TCR repertoire of the donor. In particular, the expansion of a small number of specific T cell clonotypes was detected, resulting in the presence of a marked oligoclonality in the Vβ families 5.1, 7, 9, 15, 17, and 24 (Figure 11D). Importantly, after *in vitro* stimulation with PHA, the normal gaussian distribution in all the patient's Vβ families was reestablished, despite the skewed repertoire of freshly isolated T cells (data not shown).

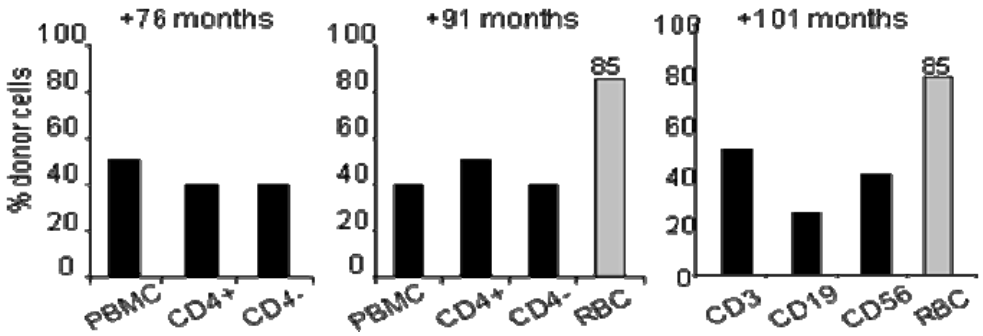
A


Gated on lymphocytes
CD3
48%
21%
CD4
CD8
Gated on PBMC
CD19
12%
9%
CD14
Gated on CD4+ T cells
CD25
18%
5%
CD4
FoxP3
Gated on PBMC
CD16
4%
3%
1%
CD56

B


PB
BM
Beta-globin
% donor cells
100
80
60
40
20
0
3 6 12 18 24 32 36 42 48 54 66 101
months post HSCT

C


+76 months
+91 months
+101 months
% donor cells
100
80
60
40
20
0
85
PBMC
CD4+
CD4-
RBC
CD3
CD19
CD56

D

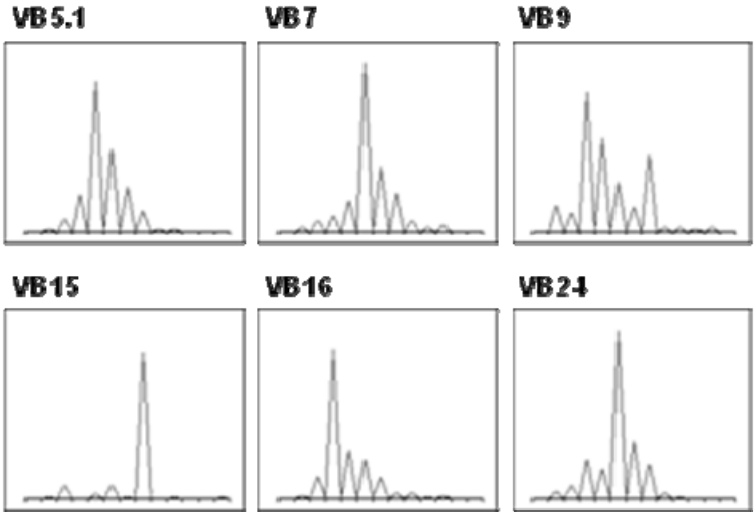


Figure 11. Kinetics of engraftment and immunological characterization of a patient with PMC eight years after HSCT.

(A) Freshly isolated patient's PBMCs were stained with mAbs to identify T cells ($CD3^+$, $CD4^+$, $CD8^+$), B cells ($CD19^+$), monocytes ($CD14^+$), and NK cells ($CD16^+$/ $CD56^+$) 91 and 101 months after the transplant. Anti-CD25 and anti-Foxp3 mAbs were used to identify $CD4^+CD25^+Foxp3^+$ regulatory T cells. A representative staining at 101 months post-HSCT is shown. (B) Long-term stable mixed chimerism was evaluated by short tandem repeat (STR) analysis in the peripheral blood (PB) and in the bone marrow (BM) of the patient. High levels of the adult donor β-globin chain were found at all time points, as determined by HPLC. (C) Mixed chimerism in patient's PBMC subpopulations was evaluated 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. (D) The TCR-Vβ repertoire was evaluated in the PBMCs of the patient 91 months after the transplant. The histograms show the TCR profiles of six representative Vβ families showing a skewed distribution.

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

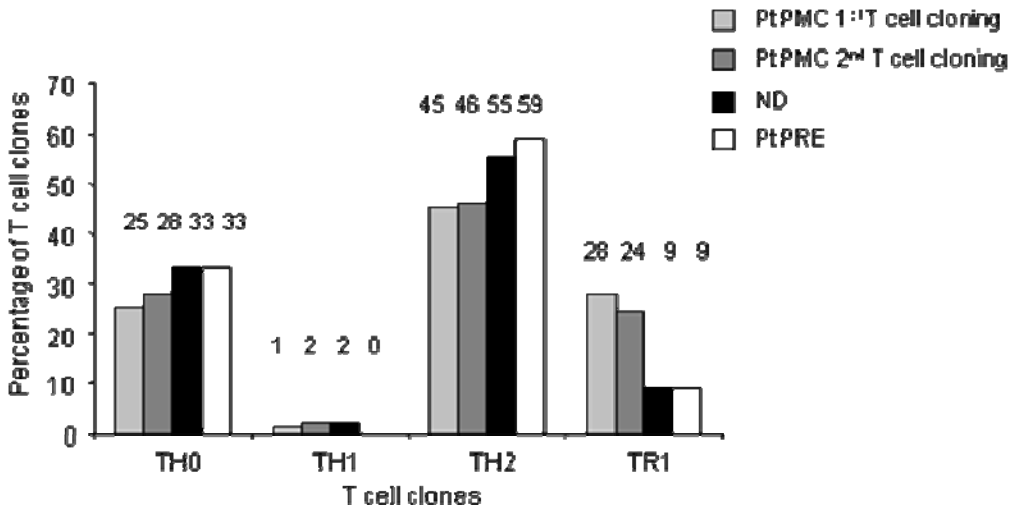
T cell clones were isolated from two distinct blood samples, 76 and 91 months after HSCT, obtained from the PMC patient (pt PMC), from a normal donor (ND) and from a thalassemic patient before the transplant (pt PRE). Based on their cytokine production profile (95, 96), different subsets of T cell clones could be identified. The proportion of Th0 (IL-2+, IL-4+, IL-10+, and IFN-γ+), Th1 (IL-2+ and IFN-γ+), 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 12A). According to data from the literature (27, 49) 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 12A). 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 12B. 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 16996 pg/ml for the first T cell cloning and median level of 14,668 pg/ml, ranging from 1208 to 42732 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 1036 to 2256 pg/ml), with a statistically

significant difference ($p < 0.05$ between the first/second T cell cloning and the ND) (Figure 12C). 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 12C), indicating that IL-10 production only was higher in the patient's T cell clones compared with 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 12D. 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 12D, 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, 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 12E), confirming results previously reported by Grossman et al. on human Tr1-like cell lines (57, 58). In contrast, we did not observe any difference in granzyme-A (GZ-A) expression in either resting Tr1 or Th2/Th0 cell clones (Figure 12E). 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 (Table 5). 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) (Table 5),

suggesting the presence of both host- and donor-derived Tr1 cells *in vivo*.

A



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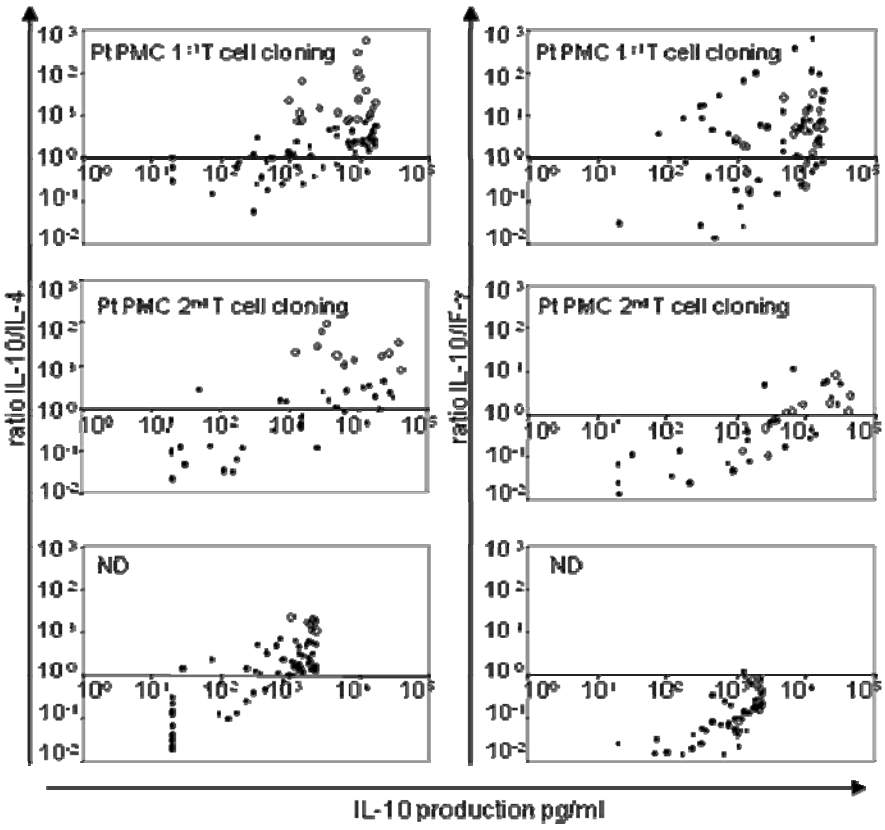

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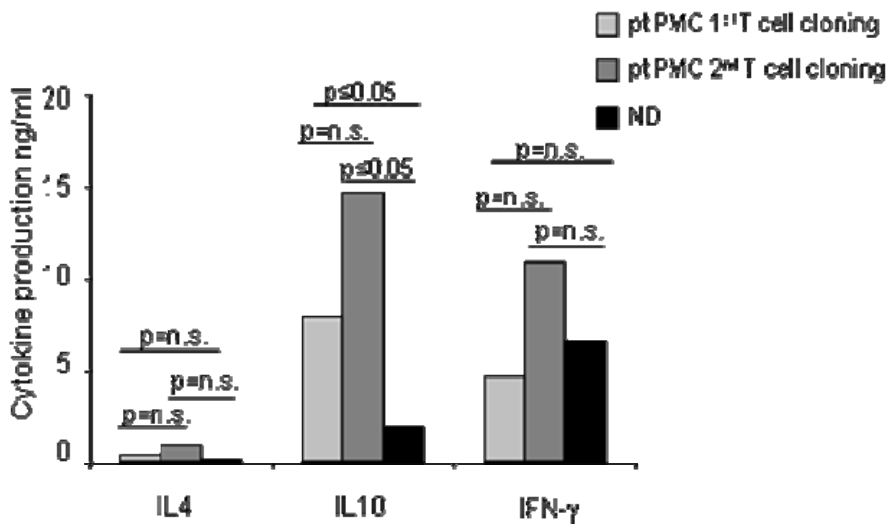
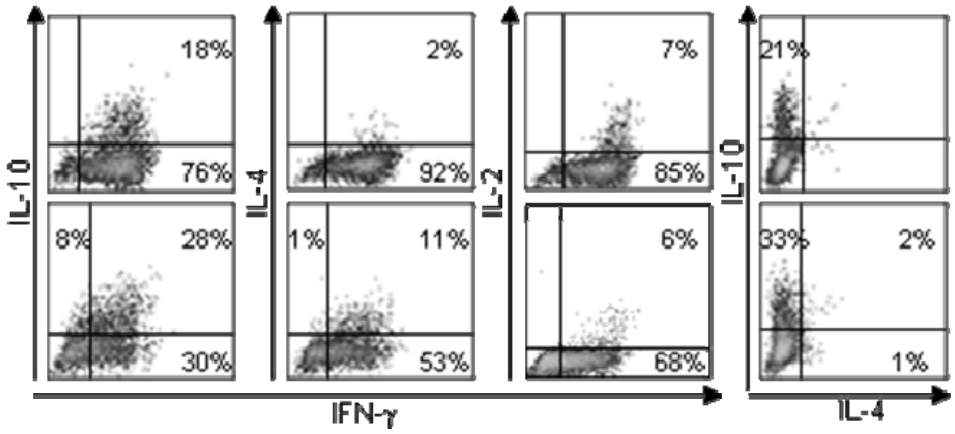

pt PMC 1st T cell cloning
pt PMC 2nd T cell cloning
ND
Cytokine production ng/ml
20
15
10
5
0
p≤0.05
p=n.s.
p≤0.05
p=n.s.
p=n.s.
p=n.s.
p=n.s.
p=n.s.
IL4
IL10
IFN-γ

D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IL-4
IL-2
IL-10
IFN-γ
IL-4
18%
76%
8%
28%
30%
2%
92%
1%
11%
53%
7%
85%
6%
68%
21%
33%
2%
1%

E

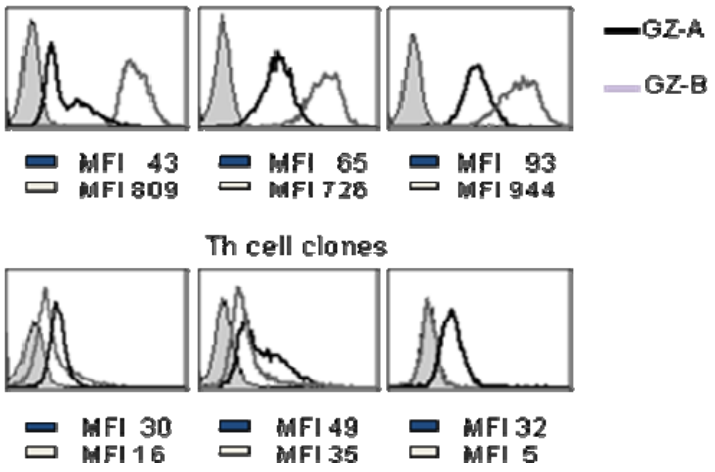


Figure 12. Characterization of T cell clones obtained in a patient with PMC.

T cell clones were established from PBMCs of the PMC patient at two different time points (76 and 91 months after HSCT), of a thalassemic patient before the transplant, and of a normal donor. Based on their cytokine production upon TCR-mediated polyclonal stimulation, T cell clones were distinguished in Th0, Th1, Th2, and Tr1. (A) The percentage of Th and Tr1 clones is shown in the PMC patient (light grey columns for the first T cell cloning, dark grey columns for the second), in the ND (black columns), and in the 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 ND (bottom panel), is shown. On the x-axis, the absolute IL-10 production is reported, whereas on the y-axis, the ratio between IL-10 and IL-4 production (left panel), and the ratio between IL-10 and IFN-γ production (right panel), are shown. 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 grey columns for the first T cell cloning [n=20], dark grey columns for the second [n=11]) and of the ND (black columns [n=7]) are shown. (D) Intracellular cytokine production by Tr1 cell clones of PMC patient, following TCR-mediated polyclonal activation, is shown. 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 (grey line) in patient's T cell clones. Filled histograms represent the isotype control. The mean fluorescence intensity (MFI) for GZ-A and GZ-B is shown. Three representative Tr1 cell clones (top panel) and three Th cell clones (bottom panel) are shown.

	1st T cell cloning		2nd T cell cloning	
ORIGIN	n° clones	n° Tr1	n° clones	n° Tr1
HOST	12	5*	26	5*
DONOR	16	6*	20	6*
TOTAL	28	11	46	11

*Number of Tr1 cell clones from the host/donor derived T cell clones characterized

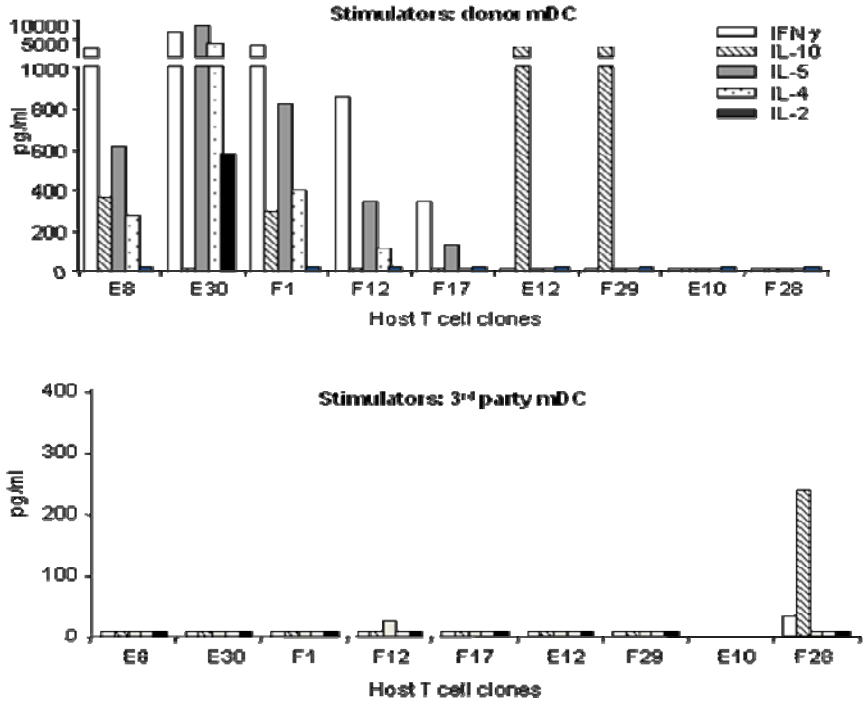
Table 5. Origin of T cell clones of patient with PMC

Antigen specificity of patient's T cell clones

To investigate the Ag 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, manuscript in preparation). 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 13A, upper panel) and donor-derived T cell clones reactive to host mDCs (Figure 13B, 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 13A-B lower panel).

Although the absolute amount of 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 6) 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 alloreactive 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.

A



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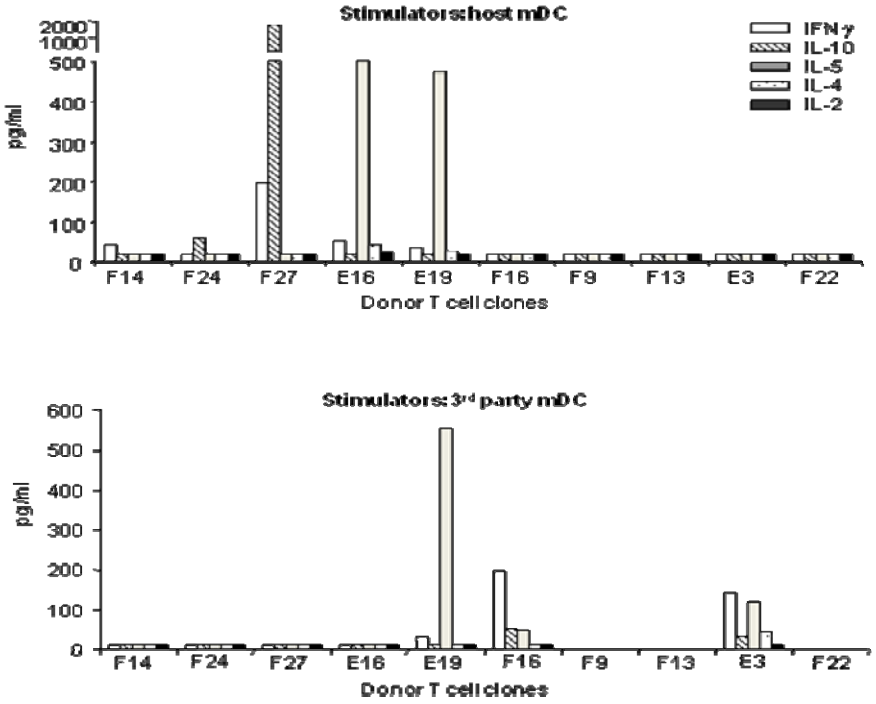


Figure 13. Allo-Ag specificity of PMC patient's T cell clones.

Cytokine production profile by patient's host/donor-derived T cell clones in response to allogeneic donor/host or 3rd party mDCs is shown. (A) Host-derived T cell clones were stimulated with donor-derived mDCs (top panel) and with 3rd party mDCs (low panel). (B) Donor-derived T cell clones were stimulated with host-derived mDCs (top panel) and with 3rd party mDCs (low panel).

Host-derived T cell clones	stimulation	IL-10‡	IL-4‡	IL-2§	IFN-γ‡
		pg/ml			
E8	mDC*	364	280	< 20	2888
	TCR†	17270	9376	4394	2360
E30	mDC	< 20	4144	580	8024
	TCR	480	2762	4342	37225
F1	mDC	296	404	< 20	3520
	TCR	4750	961	< 20	2042
F12	mDC	< 20	120	< 20	860
	TCR	1973	1111	63	< 20
F17	mDC	< 20	< 20	< 20	340
	TCR	13434	1395	< 20	6271
E12	mDC	3360	< 20	< 20	< 20
	TCR	1398	21	< 20	729
F29	mDC	2900	< 20	< 20	< 20
	TCR	1326	112	40	2786
E10	mDC	< 20	< 20	< 20	< 20
	TCR	13703	1315	< 20	2490
F28	mDC	< 20	< 20	< 20	< 20
	TCR	9198	30	< 20	1975

*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

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

*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

Table 6. Cytokine production by patient's T cell clones after Ag-specific and TCR-mediated polyclonal activation

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.

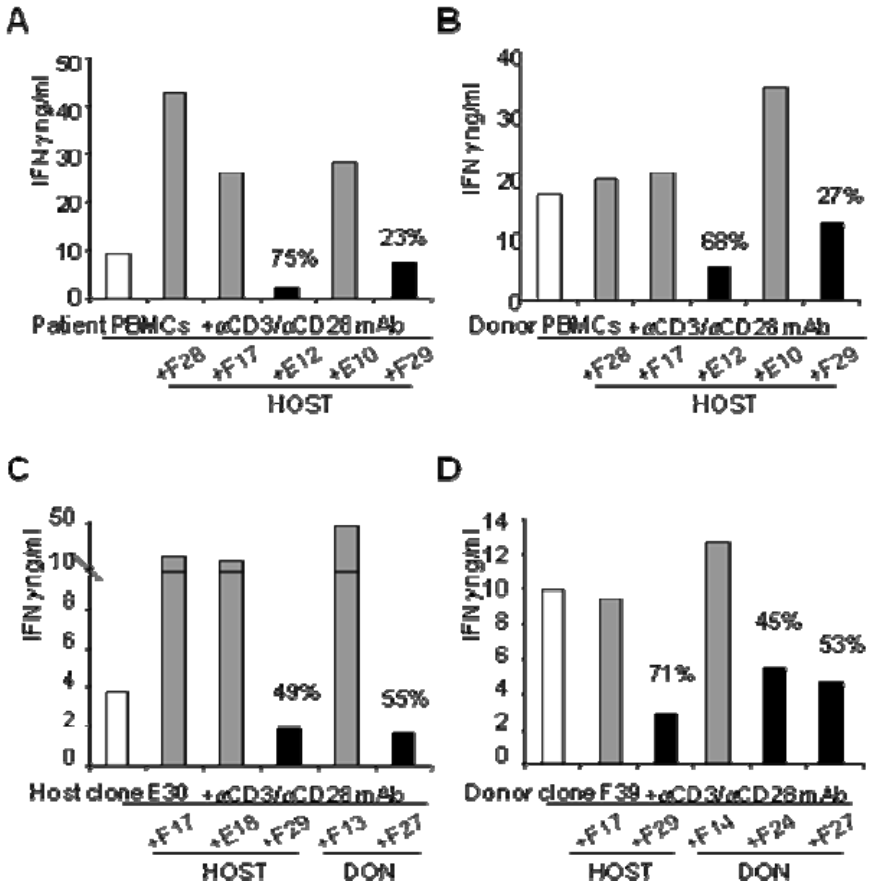
Two host-derived Tr1 cell clones (E12 and F29) inhibited the production of IFN-γ by the recipient and donor PBMCs used as responder cells (Figure 14A–B). The grade of inhibition was 75% and 68% for clone E12, and 23% and 27% for clone F29, for the recipient and donor PBMCs, respectively. The other host-derived Tr1 cell clones did not show any suppressive activity (Figure 14A–B). 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 14E). Interestingly, as detected by surface staining of CFSE-labeled proliferating cells, Tr1 cell clone E12 displayed suppressive activity against both patient's $CD4^-$ and $CD4^+$ cells (43% and 36% 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 $CD4^+$ 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- (Figure 14C) and donor-derived (Figure 14D) 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

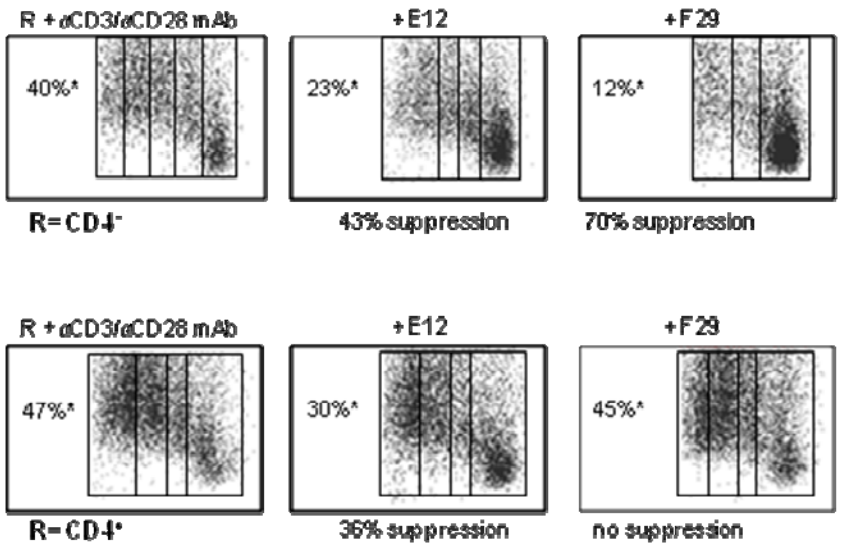
the autologous donor-derived T cell clones (45% of inhibition for F24 and 53% for F27) (Figure 14D). In addition, T cell clone F27 also inhibited IFN-γ produced by host-derived T cell clones (55% of inhibition) (Figure 14C). These results were confirmed by measuring inhibition of TNF-α production. The 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 13A–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 5F. On the contrary, the increase in proliferation versus third party mDC was low and comparable to that detected in primary MLR between unrelated normal donor responder and stimulator cells (Figure 5F). 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 5F).



E



*percentage of proliferation

F

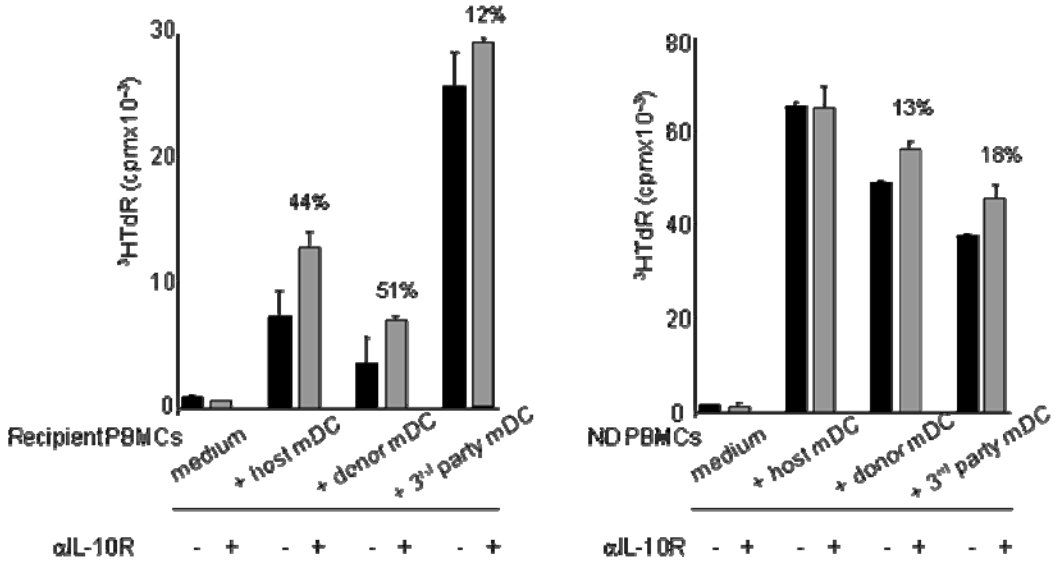


Figure 14. Suppressive activity of host and donor Tr1 cell clones.

Responder cells were activated with anti-CD3 and anti-CD28 mAbs in the presence or absence of Tr1 cell clones, and the supernatant was collected after five days of stimulation. Different responder cells were tested: recipient PBMCs (A), donor PBMCs (B), a host-derived Th cell clone (C), and a donor-derived Th cell clone (D). The ability to suppress IFN-γ production by responder cells of host and donor-derived Tr1 cell clones was evaluated. 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. (E) 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. (F) 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-IL-10R mAb. Proliferative responses were evaluated after 4 days of culture by adding 3H-thymidine for an additional 16 hours. The increased proliferation in the presence of anti-IL-10R mAb is indicated above each histogram.

DISCUSSION PART 1

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 alloAg-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 (45), 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 (97). In additional studies, no indications of peripheral mechanisms were reported in these mixed chimeras (98, 99). 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 (45, 100). 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 thalassemia and hematological malignancies, especially when reduced-intensity pre-transplant conditioning regimens are used (6, 101-103). 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 (43, 63). 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 (104-106).

Several studies showed that patients who developed mixed chimerism after bone marrow transplantation for malignant (107-109) and not-malignant hematologic disesases, such as severe aplastic anemia (110) and β-thalassemia (111) had a significant 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 (112, 113). 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 (9, 14), 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 (114, 115) and HLA-G polymorphisms (116) 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 (27). In

particular, they show very high levels of IL-10 production, which had previously been found only in Tr1 cells induced in vivo (45, 49). 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 (57). 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.

In recent years, the importance of nTreg cells in the maintenance of peripheral tolerance to self and foreign Ags has been established (117). In the peripheral blood of the patient we describe here and in other patients (data not shown) with long-term PMC, CD4+CD25+Foxp3+ T cells were detected at the same percentages as in normal donors. However, the proportion of nTreg cells soon after the transplant was not determined. Overall, according to data we obtained from PMC patients, Tr1 cells are likely to be the main Treg subset involved in long-term peripheral regulation, but it is possible that nTreg cells intervene early on after transplantation. The respective role of both Tr1 and nTreg cells at different time points following HSCT, needs to be further investigated in a large cohort of transplanted thalassemic patients.

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 (118). These findings, together with the isolation of activated autoreactive T cells specific to human red blood cell auto-Ags (119), 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.

RESULTS PART 2

IL-10 inhibits primary MLR proliferation and induces T-cell anergy

The presence of exogenous IL-10 inhibited primary MLR proliferation of PBMC in response to both mismatched and haploidentical monocytes. A mean percentage of reduction in proliferation of 79% ± 14 ($n=19$, $p<0.00002$) and of 74% ± 15 ($n=9$, $p<0.0037$) was observed in PBMC primed in the presence of IL-10 with mismatched or haploidentical allo-Ags respectively, compared to control PBMC primed in the absence of IL-10 (Figure 15A). T cell cultures previously primed with allo-Ags in the presence of IL-10 become unresponsive to subsequent stimulation with the same allo-Ag, showing a highly significant decrease in proliferation in secondary MLR. An average reduction of 81% ± 15 in mismatched donors ($n=20$, $p<0.005$) and of 75% ± 13 in haploidentical donors ($n=10$, $p<0.0007$) was observed in proliferation of MLR/10 cells compared to MLR cells (Figure 15B). These results suggest that moctes+IL-10 promoted T-cell hyporesponsiveness that we defined as anergy.

A comparable percentage of anergy induced by monocytes+IL-10 could be obtained among pairs with different HLA disparities. Optimal effect was observed when the exogenous IL-10 was added at the concentration of 10 ng/ml at day 0 and the cultures were incubated for 10 days (data not shown). However, in some donors (15% of the ones tested) IL-10 was not able to promote T-cell anergy at any concentration used or in prolonged cultures. A significantly higher percentage of $CD14^+$ cells was observed in cultures obtained in the presence of exeogenous IL-10 compared to cultures

generated in the absence of IL-10. The proportion of $CD4^+$ and $CD8^+$ T cells at the end of ten days culture in the presence of exogenous IL-10 was comparable to that present in total PBMC and to that of cultures obtained in the absence of IL-10. However, monocytes+IL-10-anergized cultures showed a significant decrease in the expression of $CD25^+$ and of HLA-DR^+ on T cells compared to those of control cells, indicating an impairement in T-cell activation.

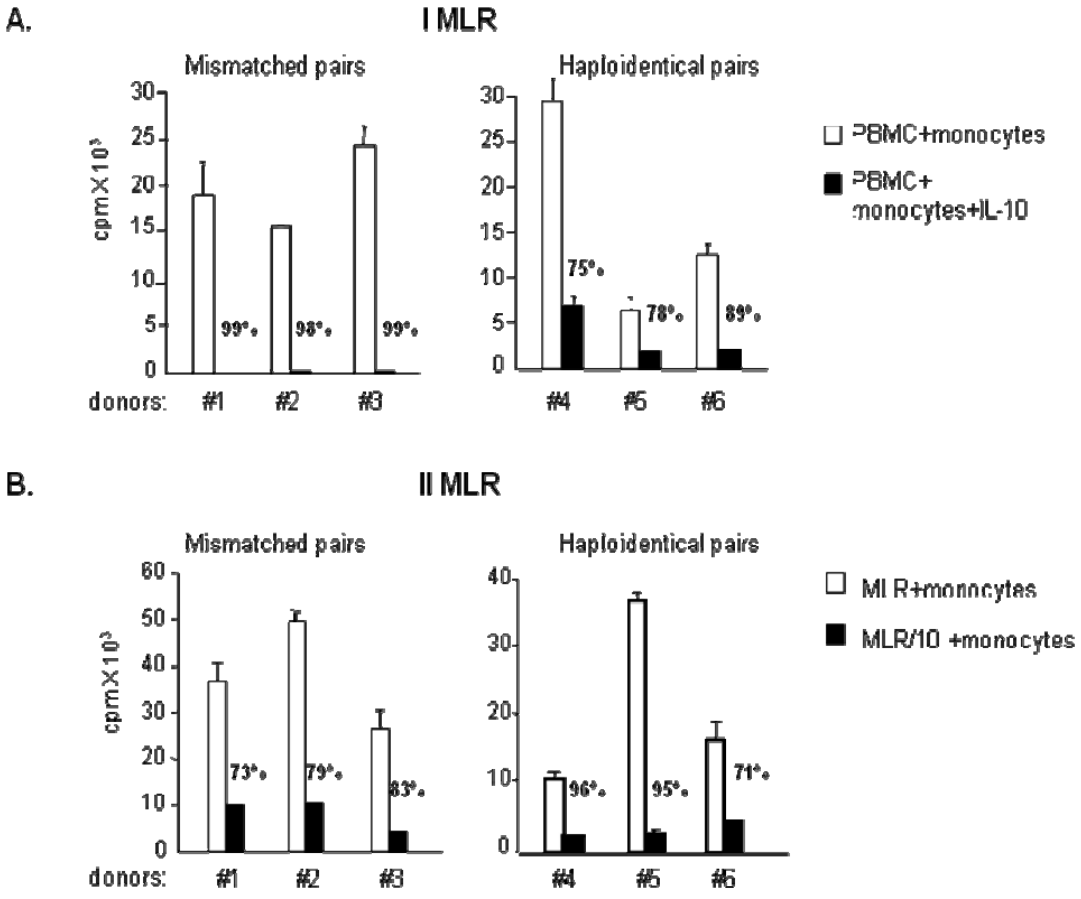


Figure 15. Effect of IL-10 on primary and secondary allo-specific proliferation.
(A) PBMC were stimulated with allogeneic monocytes in the absence (white column, MLR) or presence of IL-10 (10 ng/ml) (black column, MLR/10). Proliferative responses were evaluated after 4 days of culture by adding ^{3}H-thymidine for an additional 16 hours. (B) PBMC were stimulated with allogeneic monocytes without (white column, MLR) or with IL-10 (10 ng/ml) (black column, MLR/10) for 10 days. At the end of stimulation, T cells were collected and tested for proliferative responses to the same allogeneic monocytes used in the primary stimulation. Proliferative responses were evaluated after 48 hours of culture by adding ^{3}H-thymidine for an additional 16 hours. Results represent the mean of triplicates. Three mismatched donor (#1, #2, #3) and three haploidentical donor (#4, #5, #6) pairs are shown. Numbers indicate % of inhibition of proliferation in the primary stimulation (A) and % of anergy in the secondary stimulation (B), calculated as follows: (MLR cpm-MLR/10 cpm)/MLR*100.

It has also demonstrated that monocyets+IL-10-anergized cultures, which were anergic towards allo-Ag encountered during T cell priming, showed a preserved ability to proliferate in response towards Tetanus Toxoid (TT), Candida Albicans, or third party Ags (Figure 16). In addition, monocyets+IL-10-anergized cultures proliferated vigorously to polyclonal activators such as TPA+ionomycin, indicating their preserved viability and proliferative potential (data not shown). These results suggest that monocyets+IL-10-anergized cells retain the immunoprotective potential of untreated cells despite the fact that they acquire, at the same time, the unresponsiveness towards a specific allo-Ag.

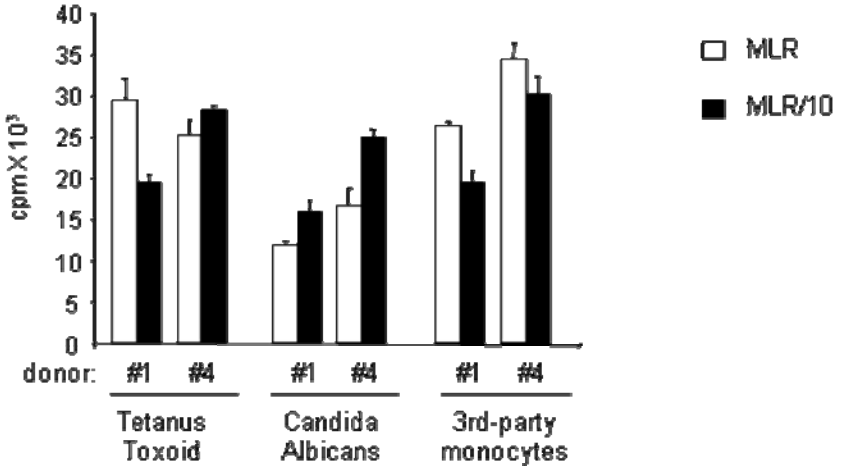
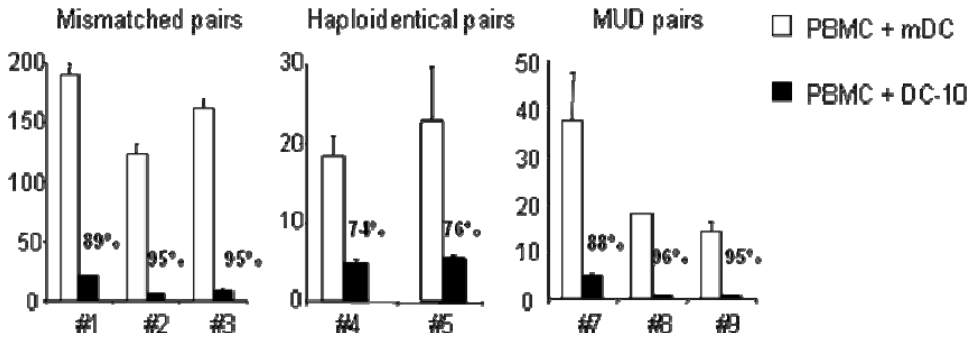


Figure 16. Monocytes+IL-10 anergized T cells preserve the ability to proliferate towards nominal or third-party Ags. PBMC were stimulated with allogeneic monocytes without (MLR) or with IL-10 (10 ng/ml) (MLR/10) for 10 days. (A) After culture, MLR (white bars) and MLR/10 (black bars) were tested towards Tetanus Toxoid, Candida Albicans and 3rd-party alloAg. Proliferative responses were evaluated after 4 days of culture by the addition of ^{3}H-thymidine for an additional 16 hours. Results represent the mean of triplicated cultures tested from one mismatched and one haploidentical pair.

DC-10 inhibit primary MLR proliferation

DC-10 have been demonstrated to be very efficient in priming naïve T cells to become anergic alloAg-specific Tr1 cells *in vitro*, since they are a source of endogenous IL-10 and express tolerogenic markers (Gregori et al, manuscript submitted). As expected, proliferative responses of PBMC induced by allogeneic DC-10 were consistently lower compared to that elicited by fully mature DC (mDC). The inhibition of proliferation was observed without the addition of exogenous IL-10 and not only using mismatched or haploidentical cells as stimulator, but also matched-unrelated (MUD) cells (Figure 17A). PBMC primed with mismatched DC-10 displayed a significantly lower proliferative response with a reduction in proliferation of 90% ± 8 ($n=14$, $p<0.00005$) when compared to PBMC primed with mDC. Similarly, a significant inhibition of proliferation was observed in haploidentical and MUD pairs with a reduction of proliferation of 72% ± 18 ($n=5$, $p=0.02$) and of 86% ± 16 ($n=10$, $p=0.0061$) in comparison to PBMC primed with mDC, respectively. IFN-γ production by PBMC stimulated with DC-10 in the three different setting was significantly reduced when compared to production by PBMC primed with mDC (a mean reduction of 93% ± 14, of 95% ± 7, and of 90% ± 15 in mismatched, haploidentical, and MUD pairs, respectively) (Figure 17B).

A



B

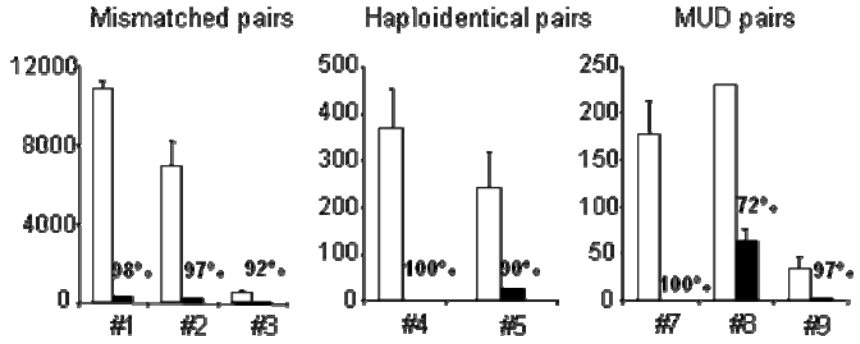
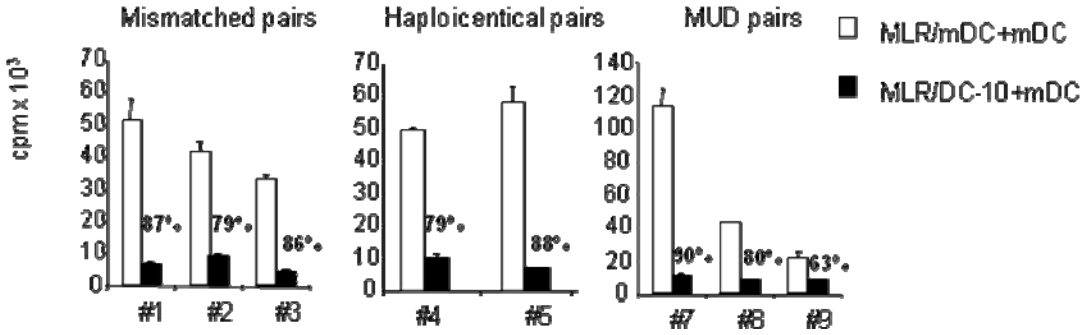


Figure 17. DC-10 induce T-cell hyporesponsiveness. (A) PBMC were stimulated with allogeneic mDC (white column, MLR/mDC) or with DC-10 (black column, MLR/DC-10). Proliferative responses were evaluated after 4 days of culture by adding ^{3}H-thymidine for an additional 16 hours. (B) In parallel, IFN-γ production was evaluated in culture supernatants by ELISA.

DC-10 promote T-cell anergy

DC-10 promote T-cell anergy, since PBMC activated with DC-10 become unable to proliferate when restimulated with mDC from the same donor. After ten days of culture, T cells primed with allogeneic DC-10 (MLR/DC-10) were profoundly hyporesponsive to re-activation with mDC, whereas PBMC stimulated with mDC (MLR/mDC) were not. PBMC primed with DC-10 from mismatched, haploidentical, and MUD donors, showed a reduction in Ag-induced proliferation of 77% ± 13 (n=18, $p<0.0005$), of 78% ± 8 (n=4, p=0.009), and of 78% ± 14 (n=3, p=n.s.), respectively, in comparison to T cells primed with mDC (MLR/mDC) (Figure 18A). Comparable results were obtained when IFN-γ production by cultures re-challenged with mDC was measured (92% ± 14 (n=14), 90% ± 12 (n=3), and 99% ± 2 (n=3) reduction compared to MLR) (Figure 18B). Similarly to cultures with monocytes+IL-10, DC-10-anergized cultures, which were hyporesponsive towards alloAg encountered during T-cell priming, were fully responsive to other Ags, such as TT, candida, and third party Ag (data not shown).

A



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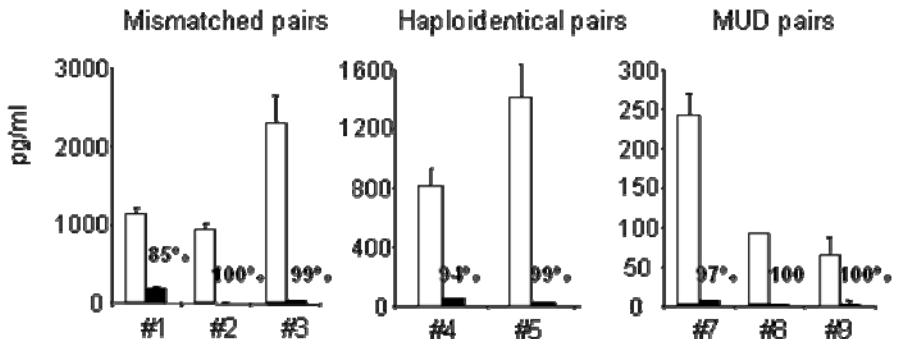


Figure 18. DC-10 induce T-cell anergy. (A) PBMC were stimulated with allogeneic mDC (white column, MLR/mDC) or with DC-10 (black column, MLR/DC-10) for 10 days. At the end of stimulation, T cells were collected and restimulated with the mDC from the same donor used in the primary stimulation. Proliferative responses were evaluated after 48 hours of culture, by adding ^{3}H-thymidine for 16 hours. (B) In parallel, IFN-γ production was tested in culture supernatants by ELISA. Results represent the mean of triplicates. Three mismatched donor (#1, #2, #3), two haploidentical donor (#4, #5), and three MUD donor (#7, #8, #9) pairs are shown. Numbers indicate % of inhibition of proliferation and of anergy calculated as follows: (MLR/mDC - MLR/DC-10) / MLR/mDC*100.

Taken together these results indicate that DC-10 are efficient in promoting T-cell anergy independently from the degree of HLA disparities. Importantly, the use of DC as stimulator when MUD pairs are used, is required, since in HLA-matched monocytes (CD3-depleted cells) are unable to elicit an allogeneic response detectable in vitro (Figure 19A).

A

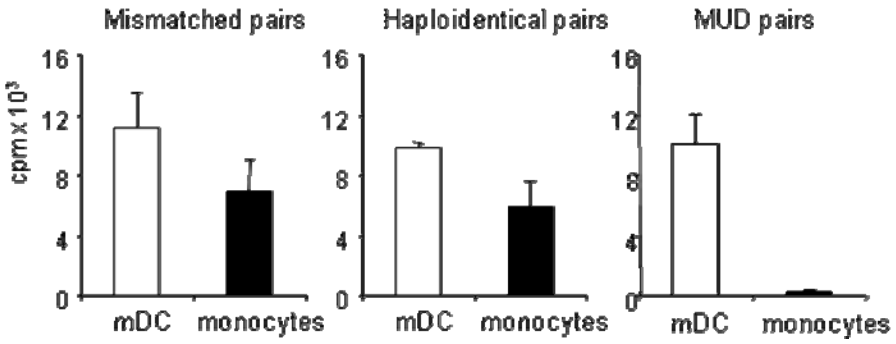


Figure 19. DC-10 are required to promote proliferation in MUD pairs.

PBMC were stimulated with allogeneic mDC (white column, MLR/mDC) and with monocytes (black column, MLR). Proliferative responses were evaluated after 4 days of culture by adding ^{3}H-thymidine for an additional 16 hours. Results represent the mean of triplicates.

Comparison between monocytes+IL-10 and tolerogenic DC-10 in promoting T-cell anergy

T-cell anergy induced by DC-10 was comparable to that induced by monocytes+IL-10 in mismatched pairs, with an average inhibition of proliferation of 81 ± 15 (n=20) and of 77 ± 13 (n=18), respectively. DC-10 were comparable to monocytes+IL-10 in inducing anergy also in haploidentical pairs. However, DC-10 were able to promote anergy, with an inhibition of proliferation of 68% and 88% when compared to that of PBMC primed with mDC, in haploidentical pairs in which monocytes+IL-10 promoted a limited T-cell hyporesponseveness upon re-challenge with the same allo-Ag (inhibition of proliferation of 35%, and 36% when compared to that of control MLR) (Table 7). These results indicate that, although monocytes+IL-10 are as good as DC-10 in promoting T-cell anergy, DC-10 are able to induce T-cell anergy in pairs in which monocytes+IL-10 are not efficient.

Moreover, the frequency of IL-10-producing T cells in anergized cultures obtained with DC-10 was higher compared to that obtained in the presence of monocytes+IL-10 (Figure 20).

	Donor #1	Donor #2	Donor #3	Donor #4
MLR/10	82%	99%	35%	36%
MLR/DC-10	79%	75%	68%	88%

Table 7. DC-10 are powerful inducer of T cell anergy in haploidentical pairs

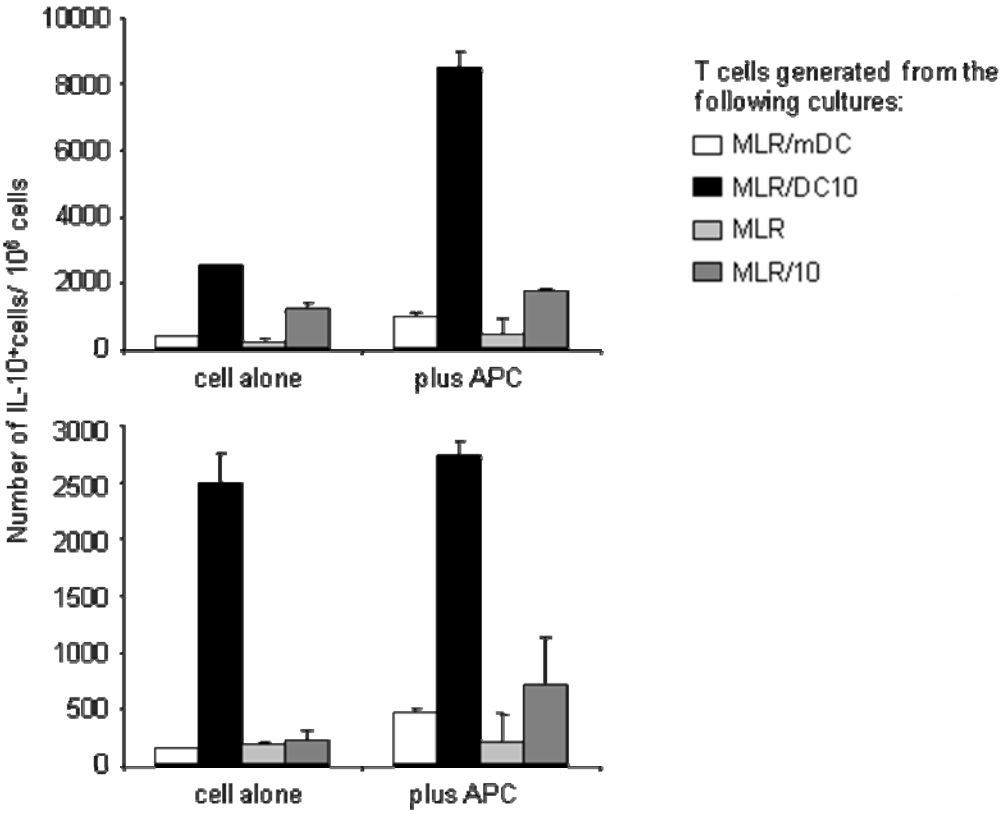


Figure 20. Higher proportion of IL-10 producing cells in MLR/DC-10 anergized cultures than in MLR/10 cultures.

PBMC were stimulated with allogeneic mDC (white column, MLR/mDC), DC-10 (black column, MLR/DC-10) or with CD3-depleted cells in the absence (brigh grey column, MLR) or presence of IL-10 (10 ng/ml) (grey column, MLR/10) for 10 days. At the end of stimulation, T cells were collected and restimulated with the mDC or CD3-depleted cells from the same donor used in the primary stimulation. Specific IL-10 producing T cells present in the cultures were counted by ELISPOT. Two different experiments are shown.

Gene expression profile of monocyets+IL-10 and DC-10 anergized T cells.

The differential gene expression profile of anergized cultures, either by monocytes+IL-10 (MLR/10) or by DC-10 (MLR/DC-10) and their respective control cultures (MLR and MLR/mDC), was analyzed by RNA microarray. Cultures obtained from three different donors in which PBMC used as responder cells were collected from a single donor and APC (DC or monocytes) from a single allogeneic donor were evaluated. Genes with a consistent differential expression of at least 1.7 fold in all three donors were considered meaningful. Overall, 43 genes were found to be up-regulated in the anergized cultures in comparison to control cultures, independently from the anergizing conditions (Table 8). Interestingly, gene up-regulated are involved in wound repearing, inflammatory responses, defence responses, in cell migration. Among the genes strongly and consistently up-regulated, the scavenger receptor CD163, whose expression is induced by IL-10 (120) (121), c-type lectins (CLEC7A or Dectin-1 and CLEC4E) involved in stimulating anti-tumor and anti-microbial activity in vivo (122), and chemokines (CCL2 and CXCL1) responsible for cell mobility, were detected (Fig. 21A). On the contrary, 14 genes were found to be significantly down-regulated in both anargized culture conditions (Fig. 21B and Table 7). Among them, we identified genes involved in cell cycle and cell division pathways (CENPF, CKAP2, NUSAP1) (123) (124), IL2RA (CD25), and Lag-3 associated protein (CENPJ) were detected. In addition, we identified genes that were up-regulated in MLR/10 vs MLR but that were down-regulated in MLR/DC-10 vs MLR/mDC (n=10), and genes that were up-regulated in MLR/DC-10 vs MLR/mDC but down-regulated in MLR/10 vs MLR (n=6) (Table 8).

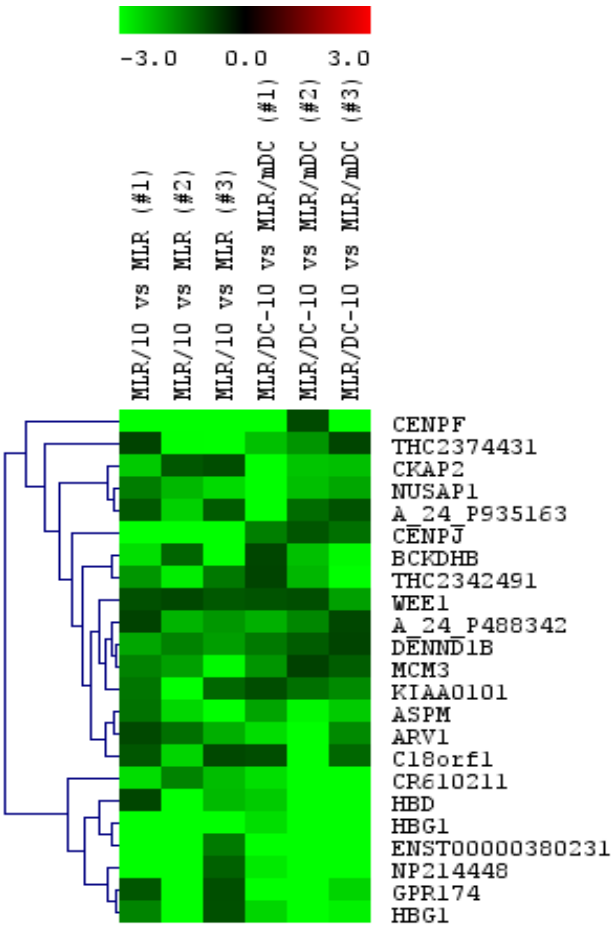
For example, the ZN174 (zinc finger protein 174), a transcriptional repressor, was

found to be up-regulated in MLR/DC-10 but down-regulated in MLR/10 (Fig. 21C). Conversely, ZN526 (zinc finger protein 526), involved in transcriptional regulation, and SLAM7, protein involved in lymphocyte adhesion, were up-regulated in MLR/10 but down-regulated in MLR/DC-10 (Fig.21D). These results indicate that anergized cultures obtained with monocytes+IL-10 or with DC-10 displayed a number of common genes up- and down-regulated. However, some levels of differences in gene expression profile by using monocytes+IL-10 or DC-10 are detectable likely due to a different kinetic of T cell response to activation by monocytes vs DC.

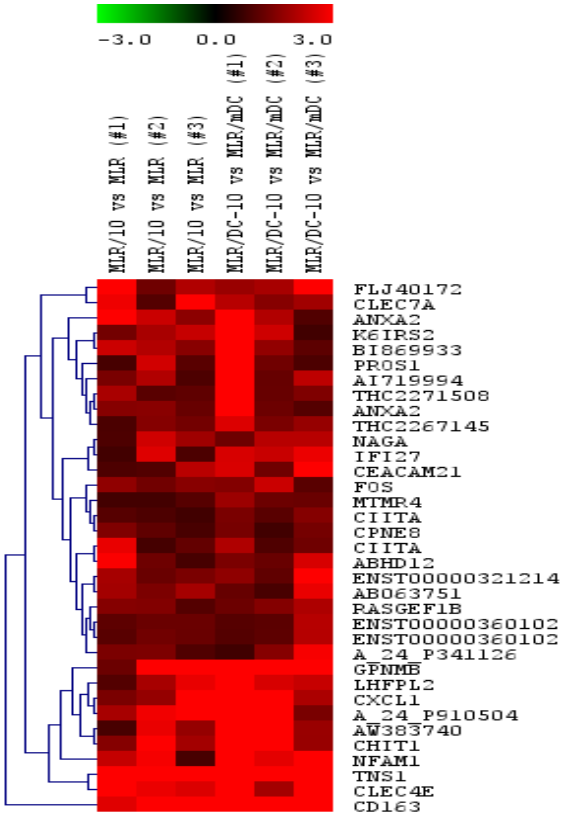
Differential expression	no. of reporters	sheet
Downregulated both in MLR/10 and MLR/DC10	14	Down in both
Upregulated both in MLR/10 and MLR/DC10	43	Up in both
Upregulated in MLR/10, downregulated in MLR/DC10	10	Up in 1, down in 2
Downregulated in MLR/10, upregulated in MLR/DC10	6	Down in 1, up in 2

Table 8. Genes up- and down- regulated in MLR/10 and MLR/DC-10.

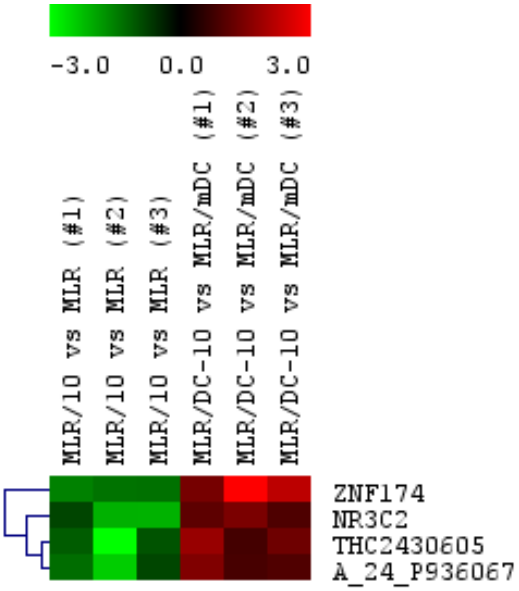
A


-3.0
0.0
3.0
MLR/10 vs MLR (#1)
MLR/10 vs MLR (#2)
MLR/10 vs MLR (#3)
MLR/DC-10 vs MLR/mDC (#1)
MLR/DC-10 vs MLR/mDC (#2)
MLR/DC-10 vs MLR/mDC (#3)
CENPF
THC2374431
CKAP2
NUSAP1
A_24_P935163
CENPJ
BCKDHB
THC2342491
WEE1
A_24_P488342
DENND1B
MCM3
KIAA0101
ASPM
ARV1
C18orf1
CR610211
HBD
HBG1
ENST00000380231
NP214448
GPR174
HBG1

B


-3.0
0.0
3.0
MLR/10 vs MLR (#1)
MLR/10 vs MLR (#2)
MLR/10 vs MLR (#3)
MLR/DC-10 vs MLR/mDC (#1)
MLR/DC-10 vs MLR/mDC (#2)
MLR/DC-10 vs MLR/mDC (#3)
FLJ40172
CLEC7A
ANXA2
K6IRS2
BI869933
PROS1
AI719994
THC2271508
ANXA2
THC2267145
NAGA
IFI27
CEACAM21
FOS
MTMR4
CIITA
CPNE8
CIITA
ABHD12
ENST00000321214
AB063751
RASGEF1B
ENST00000360102
ENST00000360102
A_24_P341126
GPNMB
LHFPL2
CXCL1
A_24_P910504
AW383740
CHIT1
NFAM1
TNS1
CLEC4E
CD163

C



D

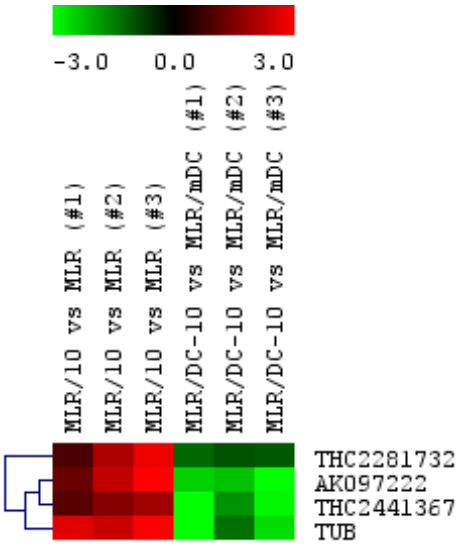


Figure 21. Gene profile

DISCUSSION PART 2

It is well known that IL-10 induces T-cell anergy (26) and the differentiation of Tr1 cells in vitro (27). In the present study we demonstrated the consistency, the strength, and the Ag-specificity of anergy induced by monocyte+IL-10. Moreover, we showed that in human T cells tolerogenic DC are more efficient than monocyte+IL-10 in promoting T-cell anergy in haploidentical pairs and are required in MUD pairs. Independently from the APC use as stimulator, the IL-10-anergized cells display a specific gene signature.

Our results represent the first clear evidence that IL-10-induced anergy can be obtained in total unselected PBMC from fully mismatched and haploidentical pairs. T-cell anergy can be induced when T cells are activated in the presence of IL-10 (26, 27, 49). This phenomenon is partially due to the ability of IL-10 to modulate T cells. Indeed, analysis of monocytes+IL-10-anergized cultures evidenced that although the proportion of $CD4^+$ and $CD8^+$ T cells was comparable to that observed in control cultures, activation markers, including CD25 and HLA-DR, were significantly lower, indicating a direct inhibitory effect of IL-10 on T cell activation. Accordingly, differential gene expression profile of anergized cultures obtained with both monocyte+IL-10 and DC-10 revealed that genes involved in cell cycle, cell division, mitosis, and T cell activation were significantly down-regulated in anergized cultures independently from the anergizing condition. For instance, mRNA expression CKAP2, cytoskeleton-associated protein 2, was consistently down-regulated in

anergized cultures. CKAP2 is only expressed in actively dividing cells and not in cells in G0/G1 phase, and silencing of CKAP2 resulted in a significant reduction in cell proliferation (125), indicating that it is clearly involved in controlling T cell proliferation. CENPJ, proteins belonging to the centromere protein family, that plays an important role in cell division and centrosome functions and CENPF, protein associated with the centromere-kinetochore complex, involved in chromosome segregation (123), have been also consistently down-regulated in anergized cultures. On the other hand, genes associated with IL-10 and with inhibition of immune mediated T-cell responses have been found to be constistently down-regulated in all anergized cultures independently from the anergizing condition. The hemoglobin scavenger receptor CD163, that is up-regulated by IL-10 and IL-6 (120, 121), was indeed constistently up-regulated in both monocyets+IL-10- and DC-10- anergized cultures. C-type lectins, such as Dectin-1 (CLEC7A), a receptor for α-glucan that is important for innate recognition of fungi and contributes to the phagocytosis {Heinsbroek, 2005 #38;Steele, 2003 #39;Gantner, 2005 #40} was also found up-regulated in all anergized cultures. The parallel induction of an Ag-specificity and a gene signature of anti-inflammation and anti-proliferation confer to anergized cells, in a short-term culture, a specific interest for their potential clinical application. This consideration is particular relevant taken into account that a single stimulation of naïve $CD4^+$ T cells with tolerogenic allogeneic DC-10 is sufficient to induce a population of anergic T cells that cointains up to 15% of allo-specific IL-10-producing Tr1 cells *in vitro* (Gregori et al, submitted). These cells suppress primary T cell responses in a dose-dependent manner in vitro. Moreover, anergic Tr1 cells generated with tolerogenic DC-10 are hyperesponsive to restimulation with the same Ag used to generate them but maintain their ability to respond to nominal Ag. The

effect that tolerogenic DC-10, not only can be differentiated *in vitro* from peripheral monocytes in the presence of exogenous IL-10, but are also present *in vivo* suggest that the direct and indirect role of IL-10 in modulating immune responses is operational in vivo. The present work demonstrates the potency and the applicability of tolerogenic DC-10 in promoting T-cell hyporesponsiveness in PBMC independently from HLA disparities between responder and stimulator cells. The use of tolerogenic DC-10 represents an advantage when HLA-A disparity is very low such as in MUD pairs.

T-cell anergy has been demonstrated using different systems. Typically can be obtained by TCR ligation in the absence of full costimulation (126). Many studies underline the importance of costimulatory molecules. Blockade of the CD28/B7 or CD40/CD40L pathway alone has been shown to be sufficient to prolong the survival of cardiac allograft in rodents (127-129). The treatment with anti-CD40L mAb and CTLA-4Ig (130) or anti-CD40L mAb alone in rhesus monkeys prolongs the survival of. However, clinical trails with CD40L were stopped for thromboembolic complications (131). Current approaches to block CD40-CD40L interaction, are based on CD40 blockade. A phase-I study in patients with Crohn's disease has been performed using-CD40 mAb (132). Moreover, a new anti-CD40/CD86-fusion protein might be a good tool for clinical application (133). In comparison to other approaches aimed to modulate immune responses, the use of IL-10 has the advantage to be Ag-specific and therefore does not interfere with the possibility to mount a normal immune response to bacterial and viral Ags. These data offer a strong rationale for the use of anergized cells as cell therapy for the modulation of immune responses and induction of tolerance. Indeed, a clinical protocol using transfer of *ex-vivo* monocytes+IL-10-anergized cells of donor origin into patients undergoing

haploidentical HSCT, which is presently ongoing at the San Raffaele Hospital (48) in order to reduce incidence of acute GvHD. Overall, we developed a method to generate Ag-specific anergic T cells, using either monocyte+IL-10 or tolerogenic DC-10, to be used as cell therapy to promote/re-establish tolerance in T-cell mediated diseases. The method can be established using either donor or host cells as responder cells to be anergized and, therefore, it could be applicable to inhibit GvHD after bone marrow transplantation or to prevent organ rejection, respectively. Furthermore, it can be envisaged that Ag-specific anergic T cells induced by monocyte+IL-10/DC-10 might be achived for any given Ag, including allergens or self-Ags.

CONCLUSIVE REMARKS

In this work we clearly demonstrate the role of IL-10 and Tr1 cells in inducing and maintaining a state of long-term tolerance after bone marrow transplantation, by the evidence that Tr1 cell clones can be isolated *in vivo* from the peripheral blood of PMC patients and are able to inhibit the function of effector T cells, of host and donor origin, *in vitro*.

These findings have two important implications for future development.

First, our results raise the question whether high IL-10 production and high frequency of Tr1 cells can be used as predictive tools for the establishment of long term tolerance. Therefore, we think that it would be important to study the role of IL-10 and Tr1 cells in the induction of chimerism early after HSCT and, in parallel, to analyze their presence in the peripheral blood of the donor or of the recipient before the transplant.

Secondly, these data offer a strong rationale for the use of Tr1 cells, induced *in vitro* by the stimulation of total PBMCs with tolerogenic DC-10, as cellular therapy for the modulation of immune responses and induction of tolerance after HSCT.

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