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Type 1 Regulatory T cells: Lytic Activity and Molecular Signature

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"The fairest thing we can experience is the mysterious. It is the fundamental emotion which stands at the cradle of true art and true science. He who knows it not and can no longer wonder, no longer feel amazement, is as good as dead, a snuffed-out candle."

Ideas and Opinions, Albert Einstein

TABLE OF CONTENTS

Chapter 1

GENERAL INTRODUCTION

Immunological tolerance

Immune system has the unique feature to identify and mount a specific adaptive immune response to eliminate harmful pathogens, while simultaneously minimizing reactions against self and non-harmful antigens (Ag). The regulation of self- and innocuous Ag-reactive T-cell response is achieved by a series of mechanisms that occur both during the generation of T lymphocytes from lymphoid progenitors in the thymus and during the differentiation of mature T cells in the periphery (Figure 1). Both the mechanisms of thymic central and peripheral tolerance concur to the establishment and maintenance of immunological tolerance. Mice deficient for Forkhead box P3 (Foxp3), known as Scurfy mice, exhibit lethal autoimmunity with massive proliferation and severe inflammatory infiltration of multiple organs, in particular the lungs, liver, and skin. Addition of mutation in the Autoimmune regulator (Aire) gene exacerbates the immunological defect, as the $Aire^{-/-}Foxp3^{-/-}$ double knock out mice showed more rapid and extensive disease compared to the single knock out mice[1].

Central tolerance is induces in the thymus, where developing thymocytes carrying high affinity T cell Receptor (TCR)s for self-peptide-Major Histocompatibility Complex (MHC) are deleted, a process known as negative selection or clonal deletion[2]. Peripheral tolerance controls T cell responses to self-Ags that are not expressed in the thymus, and to foreign Ags that are encounter in peripheral tissues, such as food and environmental ones.

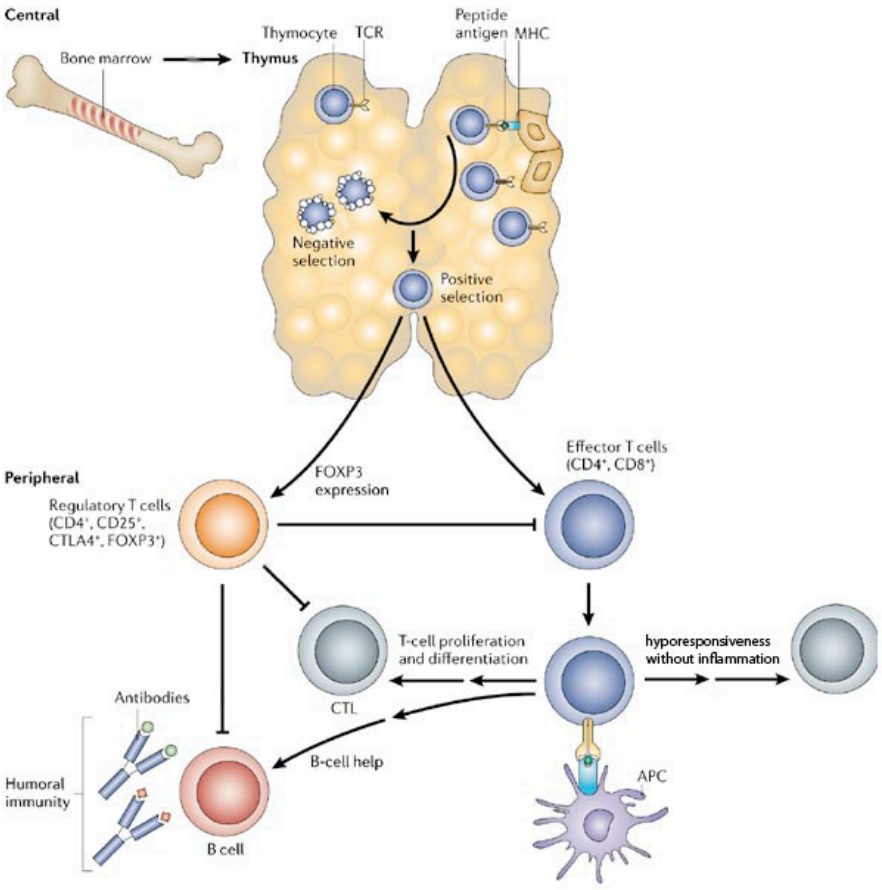

Figure 1: Central and peripheral tolerance mechanisms. Central tolerance takes place in the thymus (top panel) where thymocytes undergo a maturation and selection process that deletes self-reactive thymocytes (negative selection), preserving functional thymocytes (positive selection). In the cortex, thymocytes interact with MHC/peptide complex and receive positive signals whether they are specific and reactive. Conversely, weak reactive thymocytes die by apoptosis. Strongly self-reactive thymocytes are then deleted in the medulla when they recognize tissue-restricted self-peptides in complex with MHC that are transcribed by the gene AIRE expressed by medullary thymic epithelial cells. Mature thymocytes develop into effector $CD4^+$ and $CD8^+$ T cells and migrate in the periphery where they are controlled by several mechanisms of peripheral tolerance. These mechanisms include suppression by regulatory T cells and

hyporesponsiveness when lymphocytes recognize specific Ag in the absence of costimulation originated by inflammation *(Modified from Gregersen P.K. & Behrens T.W., Nature Reviews Genetics, 2006)*

Several mechanisms concur in inducing and maintaining peripheral tolerance, including ignorance, clonal exhaustion and deletion, and active suppression by regulatory T (Treg) cells (Figure 2).

The concept of ignorance deals with the fact that potentially self-reactive T cells are not activated since the specific Ag is expressed in low concentration or at immune privileged sites, such as eyes, Central Nervous System (CNS), and testis. In the latter situations, anatomical barriers separate the potentially autoreactive T cells from the cells that express the tissue-specific Ag. Autoreactive T cells engaged by self peptide/MHC complexes become functionally inactivated or die by apoptosis in periphery, a mechanism known as clonal deletion[3]. Presentation of the Ags in the absence of co-stimulation not only fails to prime T cells but can also delete them[4, 5]. Additionally, peripheral deletion results from the lack of growth factors and death of T cells mediated by pathway involving tumour necrosis factor (TNF) receptor superfamily (Fas) engagement by Fas ligand (L) interaction and the mitochondrial apoptosis pathway which is regulated by Bim-dependent triggering of B-cell lymphoma (Bcl)2 and Bcl-xL[6, 7].

Persistent presence of Ag without infection induces a status of hyporesponsiveness in T cells, called clonal T-cell anergy[8]. Clonal T-cell anergy is associated with a block in cell cycle progression and with defects in the cytokine production and effector function. Anergy is reversible with strong stimuli, such as high levels of Interleukin-(IL)- 2 or CD28 signaling.

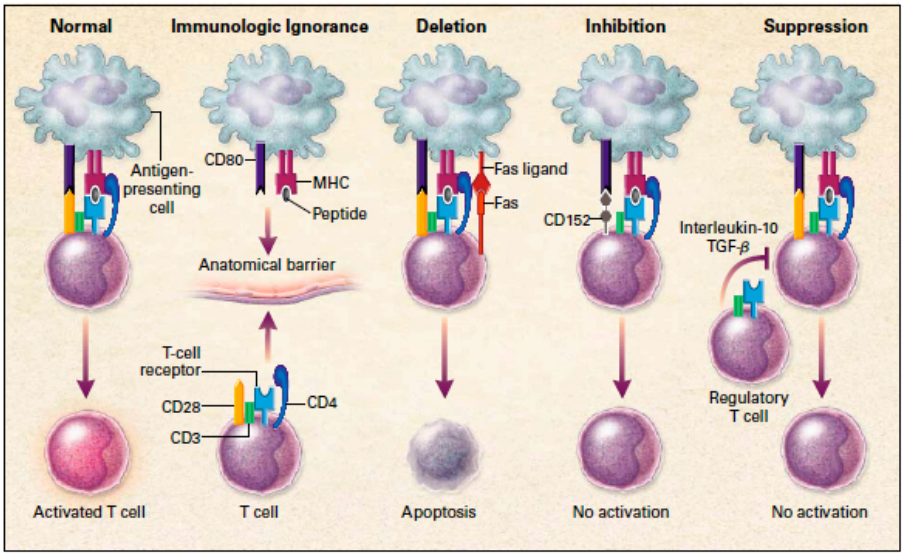

Figure 2: Peripheral tolerance mechanisms. T cells that are physically separated from their specific Ag cannot become activated, a circumstance referred as immunological ignorance. Fas-expressing T cells can undergo apoptosis by clonal deletion upon signals from cells that expressed FasL. Activation of T cells can be inhibited by antigen presenting cells (APCs) *via*, for instance, CD152/CD80 interaction. Regulatory T cells can suppress other cells, through different mechanisms, including production of inhibitory cytokines *(Modified from Mackay I.R. & Rosen F.S., The New England Journal of Medicine, 2001)*

A critical role in the determining the outcome of T cell responses is mediated by dendritic cells (DC). Depending on their lineage, stage of maturation, Ag dose, and on pathogen-derived products and cytokines to which are exposed, DCs either activate T cells or promote anergy. Microbial products, necrosis and proinfammatory cytokines induce DC maturation, and, thus, down-regulation of the Ag uptake and processing, up-regulation of MHC class II and costimulatory molecules. Mature DCs migrate from the periphery to the lymph node,

and promote activation of naïve T cells[9] (Figure 3). Conversely, immature DC (iDC)s as well as by DC render tolerogenic with biologic agents induce an anergic state in naïve T cells. In steady state, iDCs constitutively uptake apoptotic cells that, unlike necrotic ones, induce the expression of the ligand for c-mer proto-oncogene tyrosine kinase (MerTK). MerTK triggering promotes the expression of suppressors of cytokine signaling (SOCS)1 and SOCS3, two proteins that interfere with activation of DC[10, 11] (Figure 3). Not only myeloid iDCs but also immature plasmacytoid DC (pDC)s can induce anergy in human Ag-specific $CD4^+$ T cell lines[12]. Moreover, DCs could become tolerogenic by expressing a variety of immune modulatory molecules and cytokines, such as IL-10[13-16], inducible T-cell co-stimulator (ICOS)[17], programmed cell death (PD)- 1L[18, 19], Immunoglobulin-like transcript (ILT)3 and ILT4[20], and indoleamine 2,3-dioxygenase (IDO)[21, 22]. Signaling in T cells by these molecules is crucial to start the molecular events that give rise to the functional inactivation of self-reactive T cells. In addition, autoreactive T cells control themselves by expression of counter-regulatory receptors, such as CTLA-4[23-25], a structural homolog of CD28 that binds the costimulatory molecules CD80 and CD86, and PD1[26], the ligand of PD-L1 and PD-L2.

Anergy may have widespread consequences, since certain subsets of anergic T cells can secrete immune-suppressive cytokines, such as IL-10, which suppress activation of effector T cells. Thus, when anergic T cells actively suppress immune responses are referred as regulatory T cells (Treg) lymphocytes[27]. Cells with regulatory function exist within all major T, Natural Killer (NK)T [28-30], NK[31, 32], and B

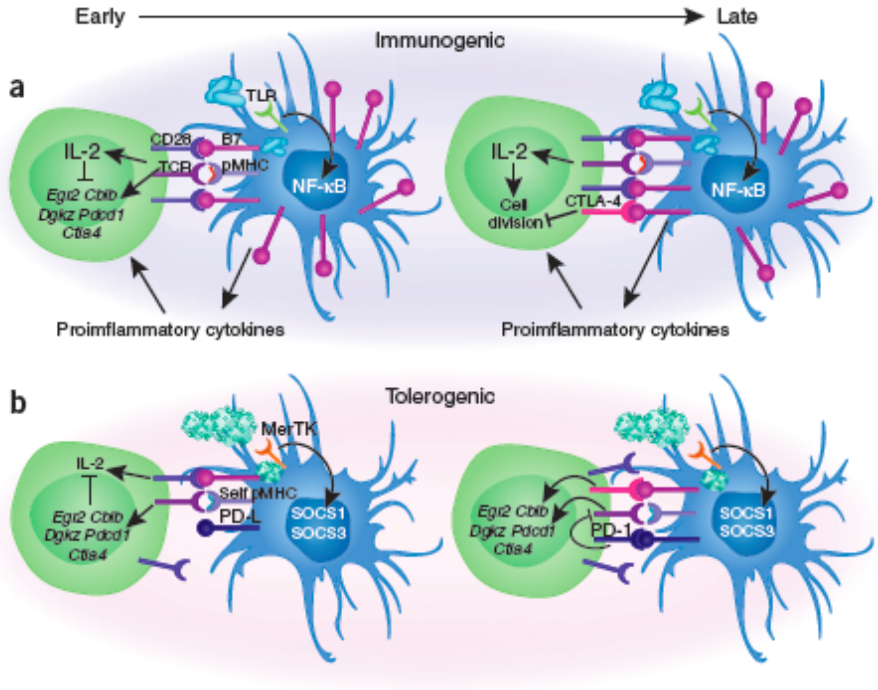

Figure 3: Control of immune response by DC. a) Mature DCs trigger activation of T cells, with induction of autocrine growth factors, such as IL-2, promoting cell cycle progression and inhibiting the transcription of anergy factors (*Egr2, Cblb, Dgkz, Pdcd1* and *Ctla4*). b) Tolerogenic DCs induce the transcription of anergic factors in T cells, such as PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA)-4. *(Modified from Gregersen P.K. & Behrens T.W., Nature Reviews Genetics, 2006)*

cell subsets[33-36], although most attention has been focused on Treg cells with a $CD4^+$ phenotype. Regulatory T cells suppress *via* several mechanisms, including inhibitory receptors, secretion of immuno-modulatory cytokines such as Il-10 and TGF-β, and cytotoxicity.

Regulatory T cell

Tregs are specialized cell subsets that regulate immune responses to pathogens, self- and not-harmful Ags and promote and maintain immune tolerance in periphery. Different T cell subsets with regulatory functions have been described starting from the 1990's (reviewed in[37]). They have been subdivided in natural (matured in the thymus) and adaptive (inducible from naïve T cells in the periphery) populations on the base of their ontogeny (Figure 4).

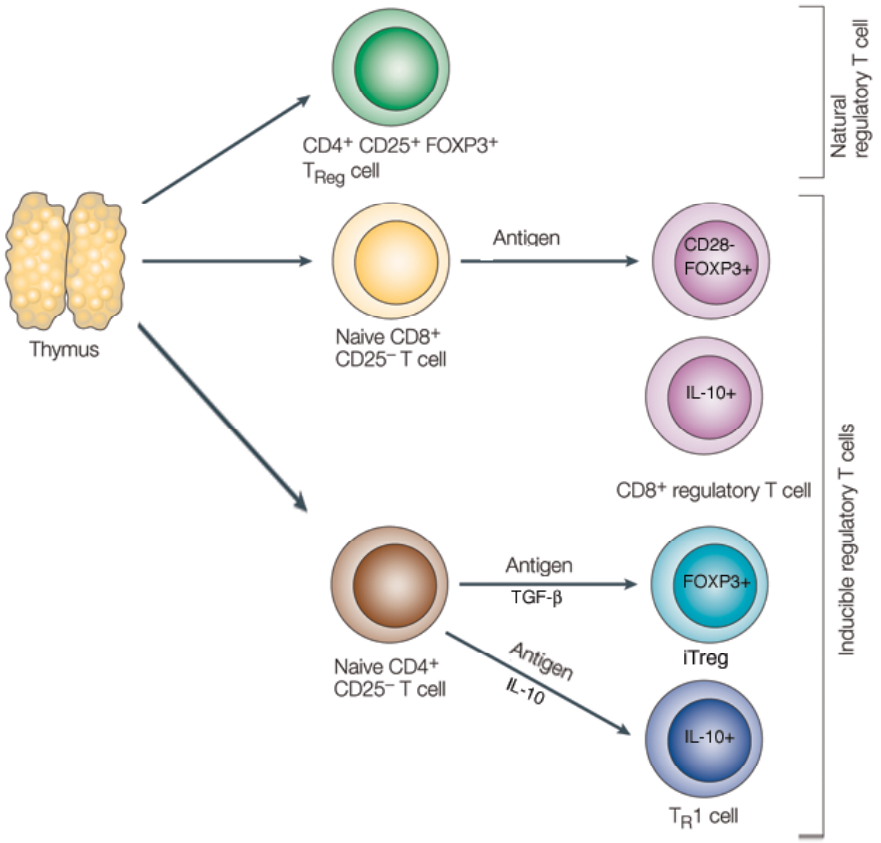

Figure 4: Natural and inducible regulatory T cells in humans. $CD4^+$ natural $FOXP3^+CD4^+CD25^+$ Treg (nTreg) cells mature and migrate from the thymus; they express the cell-surface marker CD25 and the transcription factor FOXP3. Adaptive

regulatory T cells can be induced from naïve $CD4^+CD25^-$ or $CD8^+CD25^-$ T cells in the periphery upon encounter with their specific Ag in a tolerogenic milieu, such as in the presence of transforming growth factor (TGF)-β and IL-10. The inducible populations of regulatory T cells include: IL-10-producing Type 1 T regulatory (Tr1), inducible $FOXP3^+CD4^+CD25^+$ Treg (iTreg), and $CD8^+$ regulatory T cells that can secrete IL-10 *(Modified from Mills K. H. G., Nature reviews Immunology, 2004)*.

$CD4^-$ adaptive Treg cell subsets were described, such as $CD4^-CD8^-$ T cells[38], $CD8^+$ γδ T cells[39] and $CD8^+CD28^-$ T cells[40] that, resembling the $CD4^+$ IL-10-producing Treg cells, secrete both IL-10 and TGF-β. Natural $FOXP3^+CD4^+CD25^+$ Treg (nTreg) cells erase from the thymus and are present in the individual since birth[41]. Conversely, adaptive IL-10-producing Type 1 T regulatory (Tr1)[42] cells and TGF-β-secreting T helper 3 (Th3)[43] cells arise from naïve T cells in the periphery after priming by DC in particular tolerogenic milieu (Figure 5). In addition, adaptive Treg cells resembilng the nTreg phenotype could be generated from both murine and human $CD4^+$ T cells in the presence of TGF-β[44, 45](Figure 4).

Feature	Mouse	Human
$CD4^+CD25^+$ naturally occurring regulatory T cells		
Phenotype	$CD25^{hi}CD127^{low/-}FOXP3^+$	$CD25^{hi}CD127^{low/-}FOXP3^+$
	Anergic *in vitro*	Anergic *in vitro*
	IL-2-dependent *in vivo* proliferation	ND
	Granzyme-B expression on activation	Granzyme-A and B expression on activation
FOXP3	One isoform	Two isoforms
	Lack of FOXP3 expression by $CD4^+CD25^-$ T cells activated *in vitro*	FOXP3 expression by $CD4^+CD25^-$ T cells activated *in vitro*
	Deletion of *Foxp3* in scurfy mice	Mutations in *FOXP3* in patients with IPEX
Suppressive mechanism	Cell–cell contact *in vitro*	Cell–cell contact *in vitro*
	Cytokine mediated in some *in vivo* models	ND
	Downregulation of *Il2* transcription in target T cells	Downregulation of *IL2* transcription in target T cells
	Apoptosis of effector T cells via a granzyme-B-dependent and perforin-independent pathway	Killing of monocytes, DCs, $CD4^+$ T cells, $CD8^+$ T cells and B cells via a granzyme-A-, B-, and perforin-dependent pathway
	Suppression of innate immunity	ND
T_R1 cells		
Phenotype	$IL\text{-}4^-IL\text{-}5^+IL\text{-}10^+IFN\gamma^-TGF\beta^+$	$IL\text{-}4^-IL\text{-}5^+IL\text{-}10^+IFN\gamma^{low}TGF\beta^+$
	$CD4^+CD25^-CD45RB^{low}$ ($IFN\gamma R^+IL\text{-}10R^+$)	$CD4^+CD25^-$
	Anergic *in vitro*	Anergic *in vitro*
FOXP3	Constitutively $FOXP3^-$	Constitutively $FOXP3^-$
	ND	Upregulation on activation *in vitro*
Differentiation *in vitro*	Exogenous IL-10	Exogenous IL-10 with IFNα and APCs
Suppressive mechanism	IL-10 and TGFβ mediated	IL-10 and TGFβ mediated
	ND	Killing via a granzyme B- and perforin- dependent pathway

Figure 5: Differences between mouse and human regulatory T cells *(modified from Roncarolo M. G., Nature reviews Immunology, 2007).*

Natural Regulatory T cells

nTreg cells were first identified in the mid-1990s by Sakaguchi S., who demonstrated that a minor population of $CD4^+$ T cells coexpressing the IL-2 receptor (R) α chain (IL-2Ra, CD25) were crucial to maintain immunological self tolerance in mice[46]. Subsequently, human nTreg cells counterpart have been identified being the population characterized by the bright expression of CD25 that represent the 2-4% of peripheral blood of healthy individuals[47, 48]. Both human and mouse nTreg cells are characterized by constitutive high expression of the transcription factor FOXP3[49-51]. FOXP3 is considered the master regulator for nTreg cell development and function. High and stable levels of FOXP3 are required for acquisition and maintenance of regulatory capacity by T cells and for the development of normal nTreg population in humans[52, 53]. Lack of FOXP3 in mice leads to autoimmunity, such as in the Scurfy mouse, whereas in humans causes the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), a severe early-onset autoimmune disease, in which nTreg cells are absent or profoundly disfunctional[54]. However, FOXP3 expression in human $CD4^+$ T cells is not expressed only on Treg cells but also at low levels in activated $CD4^+$ T cells, upon TCR-mediated activation in the presence of IL-2, is transient and never reaches levels observed in Treg cells[51, 55, 56]. nTreg cells have a central memory phenotype, being $CD62L^+$, $CD45RO^+$, $CD45RA^-$[48, 57]. Additional markers are expressed by human $CD4^+CD25^{bright}$ Treg population, including MHC class II, CD95, glucocorticoid-induced TNF receptor family-related gene (GITR), and CTLA4 (reviewed in [58]). Additionally, low levels

of CD127 (IL-7Ra) on the surface of $CD25^+$ T cells has been recently proposed as marker for the identification of Treg cells[59, 60]. Overall, although all the above mentioned markers are important for the isolation of human nTreg cells, they are also expressed by activated effector T (Teff) cells.

Treg cells are anergic and suppress mainly though still not completely elucidated contact-dependent, and cytokine-mediated mechanisms[61] (reviewed in [62]). The different mechanisms of suppression mediated by nTreg cells can be grouped into four basic modes of action: i. metabolic disruption, ii. modulation of DCs, iii. cytolysis, and iv. secretion of inhibitory cytokines (Figure 6).

Treg cells can suppress by depriving Teff cells of proliferation-supporting IL-2[46, 63]. Expression of the ectoenzymes CD39 and CD73 has been shown to generate pericellular adenosine, which suppresses Teff cell function *via* the type 1 purinergic adenosine $A2_A$ receptor[64-66]. Alternatively, Treg cells suppress Teff cell function directly by transferring the inhibitory second messenger cyclic AMP (cAMP) into Teff cells through membrane gap junctions[65]. Treg cells inhibit Teff cells via CTLA4, as blockade of CTLA4 abrogates Treg suppressive activity both *in vitro* and *in vivo*[67-69].

Treg cells exert suppressive function by repressing APC maturation and/or function (reviewed in [70]). Treg cells condition DCs to express IDO, which induces the catabolism of tryptophan into pro-apoptotic metabolites, *via* a CTLA4:CD80/CD86-dependent mechanism[71]. Lymphocyte activation gene (LAG)3, which is required for maximal Treg function[72], is expressed by Treg cells,

interacts with MHC class II, and has been suggested to modulate DC maturation.

Both murine and human nTreg cells express high levels of granzyme A (GZA) and granzyme B (GZB)[73-77], have cytolytic activity, and kill target cells in a GZB/perforin (PRF)- dependent manner[75, 78, 79].

nTreg cells can secrete a panel of immuno-regulatory cytokines, including IL-10, TGF-β, and IL-35. IL-10 and TGF-β display intrinsic suppressive activity (reviewed in [80] and [81]). Their primary role in the generation of specific subsets of inducible Treg cells is well defined[82, 83], but their contribution to thymus-derived nTreg functions is still debated (reviewed in [37]). IL-10 production by Treg cells is essential for the prevention of colitis in IBD models[84], but on the other hand IL-10 production by Treg cells is not necessary for the suppression of *in vivo* allergic inflammation and airway hyper-reactivity after allergen challenge in the lung[85]. Similar to IL-10, the contribution of TGF-β to nTreg suppressive activity is controversial, since several studies showed requirement of TGF-β for Treg activity, especially in the gut[86], but it is not yet clear whether TGF-β is produced by nTreg or by locally induced Treg cells. Membrane-bound TGF-β has been shown to mediate suppression by Treg cells in a contact-dependent manner[87, 88]. A novel inhibitory cytokine, IL-35, has been recently described to contribute significantly to murine Treg cell functions[89]. IL-35 is a member of the IL-12 family of cytokines and is constituted by Epstein-Barr-virus-induced gene 3 (Ebi-3) and IL-12 α/p35 subunits. Both subunits are highly expressed by mouse $FOXP3^+$ Treg cells but not resting or activated T

eff cells[89]. Treg cells knock out for either Ebi-3 or p35 have significantly reduced regulatory activity both *in vitro* and *in vivo*[89]. Conversely, ectopic expression of IL-35 confers regulatory activity to naïve $CD4^+$ T cells[89]. Although this cytokine seems to play an important role in Treg-mediated suppression of immune responses in mice, recent data indicate that IL-35 does not substantially contribute to the suppressive mechanism of human Treg cells[90, 91].

Overall, Treg cells possess multiple mechanisms to mediate their suppressive activity. The different suppressor mechanisms might be integrated and used predominantly, depending on the nature of the immune response, the eliciting agent, the effector cell subset to suppress and the tissue compartment.

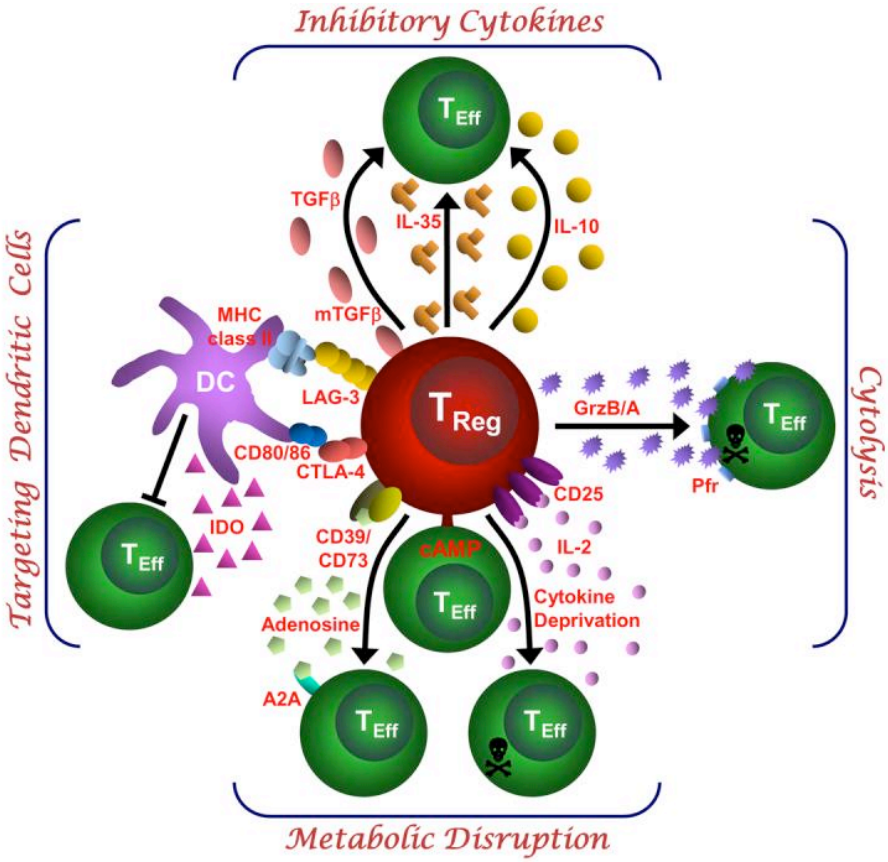

Figure 6: Suppression mechanisms of Treg cells. $CD4^+$ $FOXP3^+CD4^+CD25^+$ Tregs suppress immune-response by distinct mechanisms here grouped in four basic mode of action. Metabolic disruption by IL-2-deprivation-mediated apoptosis, by cAMP- mediated inhibition, and by adenosine-mediated suppression. Targeting DC by inhibiting their maturation via LAG3-MHC-class-II. GZA-, GZB- and PRF- dependent killing. Inhibitory cytokines such as IL-10, TGF-β, and IL-35 *(Modified from Vignali D. A., Nature reviews Immunology, 2008).*

Type 1 Regulatory T cells

Biological features

The adaptive IL-10-producing regulatory T cell subset knows as Tr1 cells was originally showed by Groux and colleagues[92] and was the first Treg cell population described at the clonal level. Tr1 cells are characterized by their specific cytokine production profile, which is distinct from that of Th1 and Th2 cells. Tr1 produce high amounts of IL-10, considerable levels of interferon (IFN) -γ, TGF-β, and IL-5, but low amounts of IL-2 and no IL-4[92]. Tr1 cells suppress immune response *via* IL-10 and TGF-β-dependent mechanisms.

Tr1 cells are characterized by low proliferative response upon TCR-mediated stimulation[93, 94]. Tr1 cells normally up-regulate activation markers, such as Human leukocyte Ag (HLA)-DR, CD25, CTLA4, CD69 and CD40L, when they are TCR-stimulated[94]. Their anergic phenotype is at least due to the autocrine production of IL-10, since anti-IL-10 monoclonal (m)Abs partially restores their proliferative responses[94]. Although Tr1 cells proliferate poorly *in vitro*, they can be expanded in the presence of exogenous IL-15 and low amounts of IL-2 without loosing their intrinsic feature to secrete immuno-modulatory cytokines[94], as Tr1 cells express high levels of CD122 (IL-2/IL-15Rβ) and CD132 (IL-2R common γ chain)[94]. After the discovery of FOXP3 as master regulator of nTreg function, FOXP3 expression by Tr1 cells has also been investigated. Although Tr1 cells do not constitutively express FOXP3, they can up-regulate FOXP3 expression upon activation to levels similar to those observed in activated Teff cells[95, 96].

Human Tr1 cells from allergic patients express both PD-1 and CTLA-

4[97]. Human Tr1-like cells induced upon crosslinking with CD3 and CD46 mAbs[76, 98] and Tr1 cell clones expressed GZB[99].

In resting phase, human Tr1 cells express both Th1-associated (CXCR3 and CCR5) and Th2-associated (CCR3, CCR4, and CCR8) chemokine receptors[100]. CCR8 is expressed at higher levels compared to Th2 cells, and upon activation, human Tr1 cells migrate preferentially in response to I-309, a ligand for CCR8[100]. In a model of helminth infection the expression of CCR8 was strongly associated with IL-10-producing $CD4^+$ T cells, which resemble Tr1 cells[101]. In addition, Tr1 cells express the gut-associated homing receptor CCR9, suggesting that Tr1 cells are present in the gut[102].

CD18 and CD49b can be used to identify a subset of human IL-10-producing $CD4^+$ T cells that suppress responder cell proliferation in an IL-10- and TGF-β-dependent manner[103]. Interestingly, an enhanced frequency of $CD4^+CD18^+CD49b^+$ T cells is observed in asthmatic patients but, in contrast to those from healthy donors, those cells lack IL-10 production[103]. In addition, $CD4^+CD18^+CD49b^+$ T cells are enhanced in recurrent hepatitis C after liver transplantation[104]. The corresponding subset in the mouse system is the $CD4^+CD49b^+$ T cells, which secrete IL-10 and suppress ongoing arthritis when adoptively transferred[105].

Recently, it has been identified a subset of human blood $CD4^+$ T cells that are $CD45RA^-$, $CD25^-$ and $CD127^-$ with characteristic similar to Tr1 cells[106]. About the 9% of the $CD25^-CD127^-$ T cell population secrete high levels of IL-10 together with IFN-γ; they are anergic, $FOXP3^-$, and suppress naïve and memory T cells in an IL-10-dependent manner[106]. $CD4^+CD45RA^-CD25^-CD127^-$ cells are

Bcl2^{low}, KI67^{high}, and ICOS^{high}, all markers of recently activation *in vivo*, suggesting that persistence of these cells *in vivo* depends on their chronic TCR stimulation[106]. Upon activation *ex vivo* isolated IL-10-producing CD4^{+} T cells from human peripheral blood expressed CD69, CD25 but not CD127[107]. However, it is not clear whether they are Tr1 cells since they suppress in an IL-10 independent contact-dependent manner and do not express FOXP3 upon activation.

CD4^{+}CD25^{-}LAG3^{+} regulatory T cells have been recently described in the mouse system[108]. They express IL-10 under the control of the transcription factor Early growth response (Egr)- 2. IL-10-producing LAG-3^{+} Treg cells have been described also in humans but these cells are distinct from conventional Tr1 cells since are FOXP3^{+}, and suppress *via* an IL-10-independent contact-dependent mechanisms[109].

Mouse Tr1 clones expressed the transcriptional repressor of GATA-3 (ROG)[110]. However, it has to be noticed that ROG is not specific for Tr1 cell since is expressed also by activated T cells.

None of the abovementiones markers have been confirmed in different experimental systems, and thus, so for no molecular marker that specifies Tr1 cells have been found.

Mode of induction:

IN VITRO

Tr1 cells arise from naïve precursors in specialized microenvironments. Several experimental protocols have been developed to generate Tr1 cells *in vitro* from naïve precursors. Groux demonstrated that mouse ovalbumin-specific T cells repeatedly stimulated with splenic APCs and ovalbumin peptide in the presence of IL-10 proliferate poorly in response to antigenic stimulation. Transfer of these cells *in vivo* prevented colitis in SCID mice reconstituted with $CD4^{+}CD45RB^{high}$ splenic T cells[92].

In vitro priming of human T cells with allogeneic monocytes in the presence of exogenous IL-10 induces a population of anergized T cells, containing Tr1 cell precursors. IL-10-anergized cultures preserve the ability to respond to nominal and viral Ags, and suppress Teff cells with the same Ag-specificity([15, 94] and Figure 7).

Although IL-10 has been shown to be indispensable for Tr1 cell induction, it is not *per se* sufficient for the differentiation of Tr1 cells in the absence of APCs *in vitro*[111]. In the mouse model, it has been demonstrated that IL-10 synergize with TGF-β to promote allo-Ag hyporesponsiveness in murine $CD4^{+}$ T cells stimulated with T cell-depleted MHC class II disparate splenic stimulators. IL-10 plus TGF-β treatment do not affect $CD4^{+}$ T cells response to nominal Ags, and adoptive transfer of these cells resulted in the prevention of lethal Graft versus Host Disease (GvHD) after bone marrow transplantation[112]. We developed an *in vitro* system using artificial APCs expressing high levels of CD58 and CD80 to generate Tr1 cells. Addition of exogenous IL-10 results in a relatively small increase in

IL-10-producing Tr1 cells. Co-addition of IFN-α, a cytokine crucial for the clearance of viral infections, but not TGF-β, can further promote autocrine IL-10 production, resulting in efficient differentiation of human $CD4^+$ Tr1 cells from naïve $CD4^+$ T cells from cord or peripheral blood *in vitro*[113].

Activation of $CD4^+$ T cells with artificial APCs bearing CD2 has been shown to promote IL-10-producing Tr1 cells. Signaling *via* CD2, the ligand for CD58, inhibits $CD4^+$ T cell proliferation in an IL-10-dependent way, and enhances the induction of Ag-specific Tr1 cells[114].

Activation in the presence of vitamin D3 (Vit D3) and dexamethasone (Dex) can induce human and mouse Tr1 cell differentiation *via* stimulation of autocrine IL-10 production[95, 115]. The induction of Tr1 cells *via* Vit D3- and Dex- is enhanced by neutralization of the Th1 and Th2 inducing cytokines IL-4, IL-12, and IFN-γ. In a mouse model of experimental autoimmune encephalomyelitis (EAE) *in vivo* injection these Tr1 cells prevented disease in an IL-10-dependent manner[115].

Cross-linking of $CD4^+$ T cells with anti-CD3 and anti-CD46 mAbs, a complement regulator protein, in the presence of IL-2 induces IL-10-producing Tr1-like cells. However, it is still unclear whether these CD3/CD46-stimulated T cells are *bona fide* Tr1 cells, since they proliferate strongly, and do not suppress T-cell responses when directly co-cultured with responder cells[116].

Chimeric anti-human CD45RO/RB monoclonal antibody is a potent immunomodulant that induces Ag-specific anergic T cells, which

display a Tr1 phenotype and suppress IFN-γ production and proliferation of Teff cells via IL-10 and TGF-β[117].

IL-27 has recently been proposed as a novel differentiation factor for the generation of murine and human Tr1 cells. IL-27 promotes the expression of the transcription factor v-maf musculoaponeurotic fibrosarcoma oncogene homolog (c-Maf), IL-21, and ICOS[118, 119]. It has been proposed that IL-27 differentiates also human Tr1 cells but it is unclear whether these cells could be considered *bona fide* Tr1 cells. Actually, it has been recently published that IL-27 induces the generation of T cells that secrete large amounts of IL-10. However, their supernatants, but not the cells alone, suppress T cell proliferation in an IL-10-dependent manner[120].

DCs both in an immature or semi-mature stage are potent inducers of Tr1 cells. Repetitive stimulation of naïve cord blood $CD4^+$ T cells with allogeneic monocyte-derived iDC results in the differentiation of IL-10-producing Treg. These T cells loose their ability to produce IFN-γ, IL-2, or IL-4 and become anergic T cells able to inhibit the Ag-driven proliferation of Th1 cells in a contact- and dose-dependent, but Ag-non-specific manner[121]. We demonstrated that repeated stimulation of naïve peripheral blood $CD4^+$ T cells with allogeneic iDCs induces the differentiation of human Tr1 cells *in vitro*. In this system, after three rounds of stimulation with iDCs, T cells become profoundly anergic and acquire regulatory functions. iDC-induced T cells secrete high levels of IL-10 and TGF-β, significant amounts of IFN-γ and IL-5, low IL-2, and no IL-4, and suppress T-cell responses by the production of IL-10 and TGF-β. The induction of these Treg

cells could be blocked by anti-IL10R monoclonal antibody, indicating that autocrine production of IL-10 by iDC is required for Tr1 cell induction (Figure 7)[96].

Several biological or pharmacological agents can modulate DC[82, 122]. Among the compounds able to induce tolerogenic DC, we can list cytokines, such as IL-10[13, 123, 124], TGF-β[125], IFN-α[126], and TNF-α[127], ligands for inhibitory receptors, such as HLA-G[128] and ICOS-L[129], immunosuppressive drugs, such as glucocorticoids, mycophenolate mofetil (MMF), rapamycin[130], or immuno-modulatory compounds, like Vit D3 and Dex[131, 132]. These agents impair DC maturation and inhibit up-regulation of costimulatory molecules, secretion of proinflammatory cytokines, in particular IL-12, and allostimulatory capacity. Controversial effects of immuno-suppressive 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[130].

After IL-10 treatment, DCs display lower levels of MHC class II molecules and costimulatory molecules of the B7 family[133], reduced release of pro-inflammatory cytokines by DCs, such as IL-1β, IL-6, TNF-α and most markedly IL-12[134]. Priming of T cells by allogeneic iDCs treated *in vitro* with exogenous IL-10 induces anergic Ag-specific T cells[135]. Furthermore, DCs matured in the presence of exogenous IL-10 for the last two days of culture show a strongly reduced capacity to stimulate a $CD4^+$ T cell response in allogeneic mixed lymphocyte reaction (MLR) in a dose-dependent manner and induce Ag-specific anergic T cells[13]. These anergic T cells are able

to suppress activation and function of T cells in an Ag-specific manner through a cell-cell contact mechanism[124].

Recently, we identified and characterized a novel population of human tolerogenic DCs, termed DC-10, that is present *in vivo* in peripheral blood and in secondary lymphoid tissues and can be differentiated *in vitro* from peripheral blood monocytes in the presence of IL-10 (Figure 7)[16]. DC-10 are $CD14^{bright}CD16^{+}$, display a mature myeloid phenotype, and express high levels of the tolerogenic molecules ILT2, ILT3, ILT4, and membrane-bound HLA-G1. DC-10 secrete high levels of IL-10 but low amounts of IL-12, display a low stimulatory capacity, and induce, by a single stimulation of naïve T cells, the differentiation of a population of cells containing a significant proportion of IL-10-producing Tr1 cells (Figure 7). Differentiation of Tr1 cells by DC-10 is dependent on IL-10, HLA-G1 and ILT4, but not ILT2, since neutralizing anti-IL-10R, anti-ILT4, or anti-HLA-G mAbs reverts the anergic status and the suppressive ability of the differentiated T cells (Figure 8)[16].

DCs treated with Vit D3 and/or Dex, which also modulate DC maturation, prime T cells to become anergic and suppressive[131, 132]. Multiple restimulation of T cells with Vit D3 or Dex-treated DC gives rise to a subpopulation of Tr1 cells, by arresting DC maturation.

ICOS-L expressing DCs induce a population of IL-10-producing Tr1 cells that have potent *in vivo* and *in vitro* inhibitory activity; when transferred into sensitized mice, they blocked the development of allergen-induced airway hyper-reactivity. The induction of Tr1 cells in this model depends on the presence of IL-10 and ICOS pathway[129].

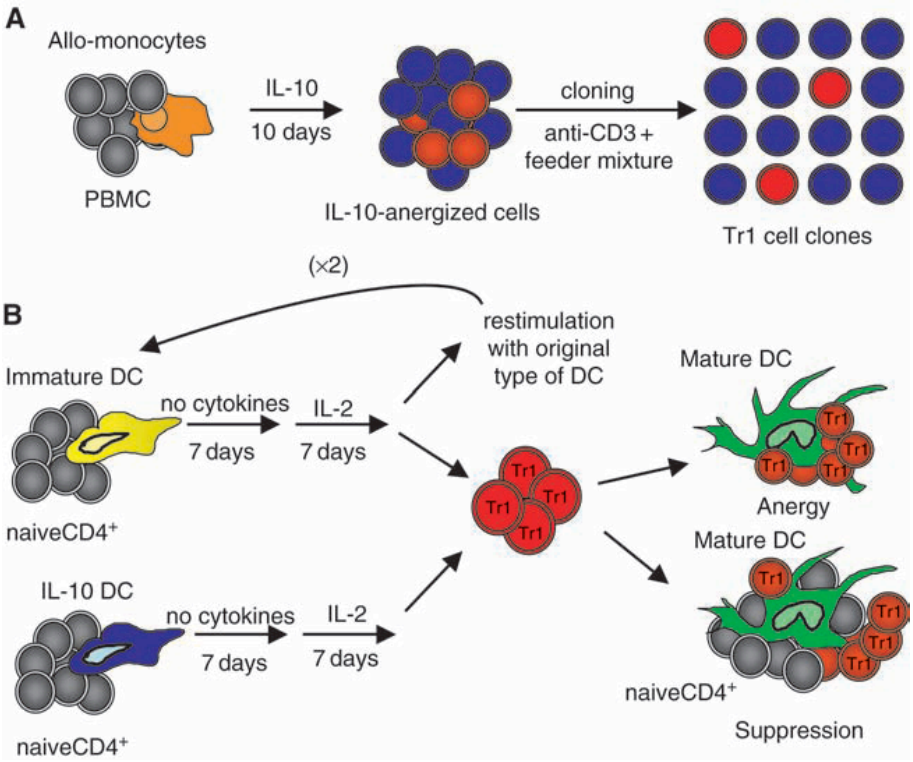

Figure 7: *In vitro* differentiation of Tr1 cells using monocytes or DC. a) Peripheral blood mononuclear cells (PBMC) are stimulated *in vitro* with irradiated allogeneic monocytes in the presence of recombinant human IL-10 (primary MLR). Cloning of the obtained IL-10-anergized cultures in the presence of feeder mixture consisting of allogeneic PBMC, Epstein-Barr virus lymphoblastoid cells (EBV-LC), and anti-CD3 mAb demonstrated that the original population is enriched of Tr1 cells. b) Peripheral blood naïve $CD4^+CD45RO^-$ T cells are stimulated *in vitro* with irradiated allogeneic iDC at 10:1 ratio in the presence of low amount of IL-2. After 7 days, T cells are restimulated twice with iDC from the original donor. Alternatively, peripheral blood naïve $CD4^+CD45RO^-$ T cells are stimulated *in vitro* with irradiated allogeneic DC-10 at 10:1 ratio for 7 days and expanded for additional 7 days in the presence of low amount of IL-2. The resulting Tr1 cell lines are anergic towards

mDCs from the same donor and suppress autologous effector T cells activated with mDC with the same Ag-specificity *(modified from Roncarolo M. G., Immunological reviews, 2006)*

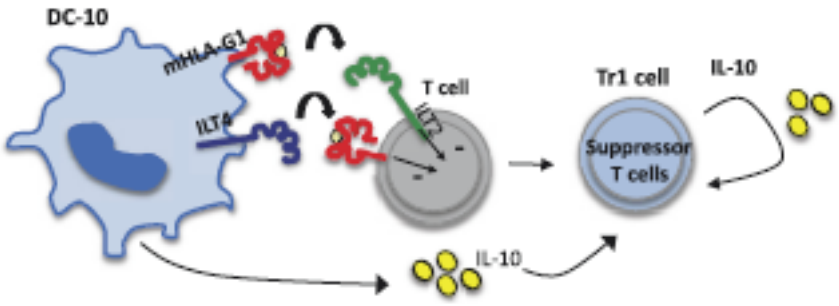

Figure 8: Molecular mechanism underneath Tr1 cell induction by DC-10. Naïve $CD4^{+}CD45RO^{-}$ T cells stimulated with DC-10 promote the induction of Tr1 cell lines by HLA-G1/ILT4 pathway. Interaction between ILT4 and HLA-G1 drives signals on DC that sustains the tolerogenic phenotype on T cells and promote Tr1 cell induction *(modified from Gregori S., Human Immunology, 2009)*

Induction of IL-10-producing T cells *via* ICOS-L/ICOS interaction seems to be a mechanism shared by different subsets of DC since activated human pDCs express ICOS-L and generate IL-10-producing regulatory T cells[136].

DC loaded with tumour Ags including myeloma cells[137], or to an adenoviral vector expressing a prostate-specific antigen (PSA)[138] could prime the differentiation of Tr1 cells. Similarly, activation of DCs with allergens or pathogens, such as Bordetella pertussis, Lactobacillus reuteri, L. casei render them tolerogenic and induce the differentiation of Tr1 cells[139].

It has to be noticed that depending on the experimental conditions used for their induction, levels of IL-10 produced by Tr1 cells are

invariably high and IL-4 remains constantly undetectable, but amounts of TGF-β, IFN-γ, and/or IL-5 can vary[82, 92].

IN VIVO

Adaptive Tr1 cells arise in the periphery upon chronic Ag-stimulation in the presence of IL-10 form naïve T cell precursors[92]. The first identification at the single cell level of Tr1 cells was *in vivo* in a severe combined immunodeficient (SCID) patient who develops long-term tolerance after HLA-mismatched haematopoietic transplantation[93]. Then, Tr1 cells was associated with persistent mixed chimerism (PMC) after allogeneic haematopoietic stem cell transplantation (HSCT) for thalassemia[99]. The co-existence of donor and host cells soon after the transplant, leads to a chronic allo-antigenic exposure that contribute to the induction of IL-10–producing regulatory Tr1 cells during the early post-transplant period. Indeed, chronic antigen exposure has been described as a crucial event in the generation of IL-10–producing T cells *in vivo*[140, 141]. It has been suggested that Tr1 cells could be also converted from Th1 or Th1 cells upon chronic stimulation, maintaining only the ability to secrete IL-10 and not the other cytokines[142].

Tr1 can be generated *in vivo* in mice and humans infected with certain pathogens, such as Bordetella Pertussis[139], or following immunization with self or foreign antigens using protocols previously shown to generate immune tolerance, such as multiple intranasal immunizations with soluble peptides from myelin basic protein (MBP)[143, 144]. Treatment of mice with a killed Mycobacterium vaccae-suspension gives rise to allergen-specific regulatory T cells.

These regulatory T cells confer protection against airway inflammation mediated by IL-10 and TGF-β[145]. Consecutive injections of peptide-pulsed semi-mature DCs, obtained by stimulation of iDC with TNF-α in the absence of danger signals, lead to the induction of peptide-specific IL-10 producing $CD4^+$ T cells *in vivo* and provide complete protection from EAE[127]. In addition, mature pulmonary DCs in the bronchial lymph nodes of mice exposed to respiratory allergen induced the development of Tr1 cells, in a process that required ICOS-ICOS-ligand pathway[129]. Furthermore, DC pulsed with keyhole limpet hemocyanin (KLH) in the presence of CT *in vitro* and adoptively transferred into naïve mice generated KLH-specific Tr1 cells[146].

We and others explore the possibility to induce *in vivo* allo-Ags-specific Tr1 cells in models of pancreatic islet transplantation[147, 148]. Peritransplant treatment of multiple nonhuman primates (NHP) with anti-CD3 immunotoxin and deoxyspergualin induces long-term stable rejection-free tolerance to MHC-mismatched allograft, associated with high levels of IL-10 in serum and elevated Tr1 and $CD4^+CD25^+$ Treg cells. In this regimen, anti-CD3 immunotoxin deplete Teff cells, whereas deoxyspergualin arrest the production of proinflammatory cytokines and the maturation of DCs. Of note, depletion of Treg restores anti-donor responses in tolerant NHP[147].

In vivo treatment with rapamycin combined with IL-10 prevents allogeneic pancreatic islet transplantation rejection in non-obese diabetic (NOD) mice and promotes long-term tolerance *via* the induction of Ag-specific Tr1 cells. The combination of IL-10 with a rapamycin which down-modulates the effector phase of the immune

response is required for the induction of operational tolerance in this model. Rapamycin indeed blocks IL-2-induced expansion of Teff cells, without preventing Tr1 cell induction *via* TCR stimulation in the presence of IL-10 (Figure 9)[148]. In a stringent islet transplant model, addition of a depleting agent to the IL-10 and rapamycin such as anti-CD45RB mAb is fundamental for generation of Tr1 cells *in vivo*[149].

Thus, induction of Ag specific long-term tolerance mediated by Tr1 cells requires the synergistic effect of drugs that down-modulate inflammation, and block Teff cells.

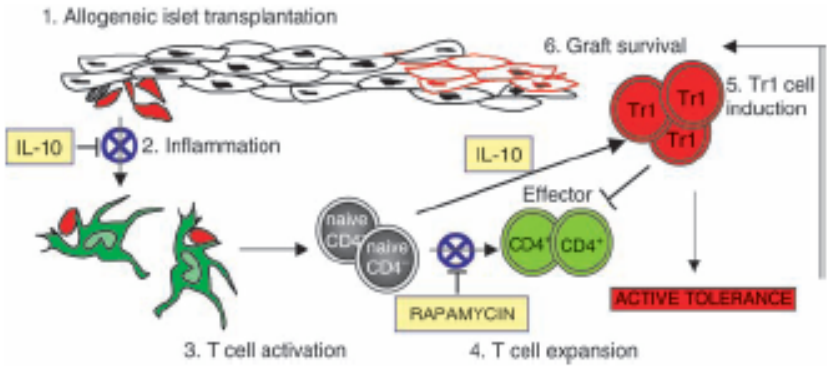

Figure 9: Prevention of allograft rejection *in vivo* by Tr1 cells induced with rapamycin and IL-10. To prevent allograft rejection, the massive inflammation caused by the transplant must be modulated in order to reduce the recruitment and maturation of professional APCs. 1) Expansion of Teff cells must be tightly controlled and avoided, while conditions that permit Treg development and function should be preserved. Accordingly, treatment with rapamycin and 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) *(modified from Roncarolo M. G., Immunological reviews, 2006)*

Recently, it has been reported that exogenous IL-27 induced the differentiation of Tr1 cells *in vivo*, reducing the severity of adoptively transferred EAE by an IL-10-dependent mechanism[118]. Activation of the transcription factor aryl hydrocarbon receptor (AhR) induces Tr1 cells[150]. AhR bounds to c-Maf and promoted transactivation of the IL-10 and IL-21 promoters, which resulted in the generation of Tr1 cells and the amelioration of experimental autoimmune encephalomyelitis[151].

Mechanisms of Suppression

The chief mechanism by which Tr1 cells down-regulate immune responses is secretion of high levels of the immunosuppressive cytokines IL-10 and TGF-β[92, 93, 115, 152]. Notably, Ag-specific Tr1 cells need to be activated *via* their TCR in order to exert their suppressive functions. Tr1 cells, once activated, they can mediate bystander suppressive activity against other Ags. The bystander suppression is mediated by the release of IL-10 and TGF-β[92] which act on both APC and T cells (Figure 10).

IL-10 limits the magnitude of immune response, as proved by mice lacking IL-10 that exhibit spontaneous enterocolitis. IL-10 down-regulate the expression of costimulatory molecules, such as CD80, CD86, and MHC Class II, and pro-inflammatory cytokine production by APCs and inhibits the secretion of IL-2 and TNF-α by T

cells[153]. Tr1 supernatants diminish the capacity of monocytes to stimulate Th1 responses, block the differentiation and maturation of DC *via* an IL-10-dependent mechanism[141].

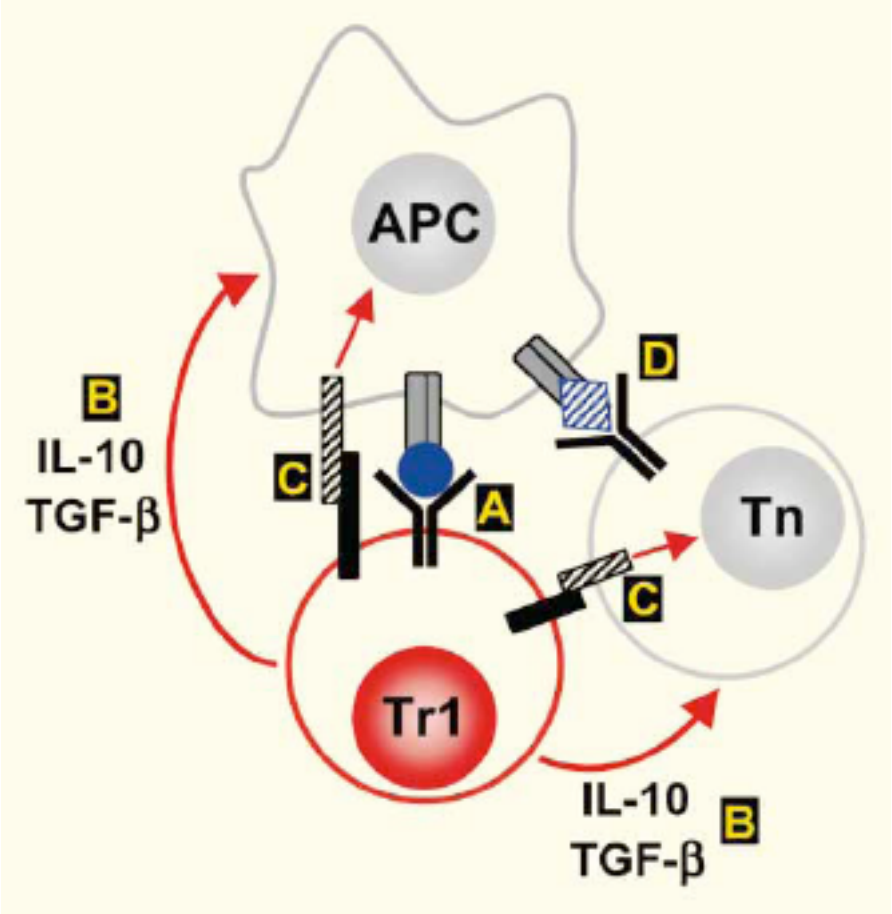

Figure 10: Putative suppressive mechanisms of Tr1 cells. a) Upon Ag-specific activation *via* TCR, Tr1 cells secrete IL-10 and TGF-β. b) These cytokines act on APCs and naïve T (Tn) or memory T cells and inhibit T-cell-mediated response directly and indirectly. c) Tr1 cells may also up-regulate inhibitory receptors that drive signals to APCs and/or T cells. d) Once activated, Tr1 cells mediate bystander suppression of APC and/or T cells that do not necessary shared the same Ag-

specificity of Tr1 cells *(modified from Roncarolo M. G., Immunological reviews, 2001)*

TGF-β down-regulates APCs functions[154] and inhibits the proliferation and cytokine production by T cells[155].

Suppressive effects of Tr1 cells can be patially reversed by addition of anti-IL10 and anti-TGF-β neutralizing monoclonal Abs[92, 93, 96], indicating the additional mechanisms may also contribute to Tr1 cell-mediated suppression.

Allergen-specific suppression mediated by human Tr1 cells from allergic patients is dependent on IL-10, TGF-β, and PD-1 and CTLA-4[97]. PD-1 is an immunoreceptor tyrosine-based inhibitory motif–containing receptor expressed upon T cell activation that binds members of the B7 family, PD-ligand (L)1 and PD-L2. PD-1 deficient mice develop autoimmune diseases, suggesting an inhibitory role for PD-1 in immune responses[156]. PD-1/PD-L engagement on murine $CD4^+$ and $CD8^+$ T cells results in inhibition of proliferation and cytokine production[157].

Human Tr1-like cells generated *in vitro* by cross-linking of CD3 and CD46 can kill target cells of different origins through a GZB- and PRF- mediated mechanism[76, 98].

In addition to suppress T cell responses, human Tr1 cells induce IgG4 and suppress IgE production in PBMC and B cells, *via* a GITR- and IL-10- dependent mechanisms[158, 159].

Relevance of Tr1 cells in diseases

The importance of Tr1 cells in controlling immune response has been further proved by several works that claim their involvement in many T cell-mediated diseases and in the regulation of tolerance *in vivo*.

The first suggestion that human Tr1 cells are involved in maintaining peripheral tolerance *in vivo* came from studies in SCID patients that developed long-term tolerance after HLA-mismatched allogeneic stem cells transplantation without the need of immunosuppression. Tolerance achieved in these patients is associated to high proportion of donor-derived T cell clones specific for the host HLA Ags and able to produced high levels of IL-10[93]. Recently, IL-10 and Tr1 cells have been associated to the induction of PMC after HSCT in β-thalassemic patients, a status of long-term tolerance *in vivo* in which donor and host cells co-exist for a period longer than 2 years. High frequency of IL-10-producing T cells in the PBMC of PMC patients are observed compared to the controls. Notably, Tr1 cell clones, of both donor and host origins, are isolated from a PMC patient and are able to inhibit the proliferation and the IFN-γ production of effector T cells of either donor or host origin[99]. The importance of IL-10 and TGF-β-producing $CD4^+$ T cells with suppressive functions has been also described in kidney or liver allograft, and correlates with the spontaneous development of tolerance[160]. Together this data demonstrate the key role of Tr1 cells in inducing tolerance after bone marrow and solid organ transplantation.

Tr1 cells can regulate responses to self-Ag. We and others previously reported that myelinHuman self-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 inhibit 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 displayed a Tr1-like phenotype may be important regulatory cells that mediate peripheral tolerance and prevent the development of autoimmunity[161]. Tr1 cells specific for Desmoglein 3 are present in healthy individuals whereas are rarely detected in patients affected by Pemphigus Vulgaris (PV), a severe autoimmune disease associated with circulating autoAbs against Desmoglein 3[152]. Similarly, autoreactive T cells specific for HLA class II molecules associated with type 1 diabetes are polarized toward a proinflammatory Th1 phenotype, producing predominantly IFN-γ in diabetic patients, whereas are polarized to produce IL-10 in non-diabetic individuals[162]. In rheumatoid arthritis (RA) patients, the frequency of IL-10-producing $CD4^+$ T cells in the peripheral blood and synovial tissue is significantly lower than in control patients and is inversely correlated with the frequency of Th1 cells, disease activity score, and the degree of lymphocyte infiltration in rheumatoid synovium[163]. Upon stimulation *in vitro* with the RhD protein, the major red blood cell autoAg, IFN-γ- or IL-10- producing T cells are found in patients with autoimmune haemolytic anaemia[164].

Tr1 cells are also involved in controlling allergic diseases. Nickel (Ni)-specific Tr1 cell clones could be isolated from both peripheral blood and lesional skin of patients allergic to nickel. These Ni-specific Tr1 cells inhibit in an IL-10-dependent manner the functions of monocytes and DC and directly suppress Ni-specific Th1 responses. [141]. The balance between Tr1 and Th2 cell compartments plays a

crucial role in the development of the allergic responses. Allergen-specific effector Th2 and suppressor Tr1 cells exist in both healthy and allergic individuals. However, an increased frequency of allergen-specific Tr1 cells, with a concomitant decrease of allergen-specific Th2 cells, is present in healthy individuals compared to allergic subjects [97]. Interestingly, continuous exposure of high dose bee venom exposure in non-allergic beekeepers by natural bee stings induce T cell tolerance, by expansion of IL-10-producing Tr1 cells[165]. $CD4^+$ T cells from patients with severe asthma that fail to demonstrate clinical improvement upon glucorticoid therapy show defective production of IL-10 when stimulated *in vitro* in the presence of Dex compared to glucorticoid-sensitive counterparts[166]. Administration of Vit D3 to glucorticoid resistant patients enhances subsequent responsiveness to Dex for induction of IL-10-producing regulatory T cells, suggesting that induction of Tr1 cells contribute to the clinical efficacy of the therapy[167]. Tr1 cells are involved in suppressing the immune response not only toward allergens, but also to food Ags. Gliadin-specific Tr1 cells have been isolated from the intestinal microflora of celiac patients in remission[168].

Finally, some reports indicated the induction *in vivo* of Tr1 cells in chronic infectious diseases and tumours. Treatment of mice with a killed Mycobacterium vaccae-suspension gives rise to allergen-specific $CD4^+CD45RB^{Low}$ regulatory T cells, which confer protection against airway inflammation through IL-10 (IL-10) and TGF-β[145]. Cholera toxin also promotes the generation of Tr1 cells. T cells from mice immunized with antigen in the presence of Cholera toxin induce a population of Ag-specific $CD4^+$ T cells that produced IL-10 in the

absence of IL-4, and inhibit Ag-specific proliferation as well as IFN-γ production by Th1 cells *via* a cell contact-independent manner[146, 169]. Pathogen-specific Tr1 cells are induced in the respiratory tract by Bordetella pertussis[139]. Differentiation of Tr1 *in vivo* is also promoted by infection with Leishmania major[170], hepatitis C[171], helminths[172], and Helicobacter hepaticus[173]. In addition, the streptococcal ligands for the complement receptor CD46, the M proteins M5 and M22, induce T cells to develop into the IL-10-producing Tr1-like phenotype[174]. In the context of tumours, and, in particular in Hodgkin lymphoma (HL), it has been demonstrated that Hodgkin lymphoma infiltrating lymphocytes are anergic, inhibit effector T cell responses, and are highly enriched in IL-10-secreting Tr1 cells. The presence of Tr1 cells induces a profoundly immunosuppressive environment and so provides an explanation for the ineffective immune clearance of cancer cells[175]. Similarly, in patients with head and neck squamous cell carcinoma, tumour infiltrating lymphocytes are enriched in Treg cells that produced IL-10 and TGF-β and do not require cell-to-cell contact to responder cells for suppression[176].

Overall these data suggest that Tr1 cells play a key role in the induction of long-term tolerance *in vivo* and support their clinical application.

Tr1 cells as therapeutic tool

Induction of Tr1 cells *in vivo* or *ex vivo* has been exploited as therapeutic strategy in immune-mediated diseases[111, 177]. The benefits of the use of Tr1 cell therapy over conventional treatments are the lack of general immune suppression, the possible induction of a long-term and stable tolerance, the limited side effects due. Open questions regarding Treg-based approached are whether Tr1 or nTreg cells can be used. The main advantages of inducible Tr1 cells are, their Ag-specificity and thus suppress specific reponses and the comncominantly possibility to down-regulate the immune responses *via* bystander suppression. In preclinical mouse model we demonstrated that infusion of polyclonal Tr1 cells promoted graft tolerance only in the non-stringent model of islet transplantation. Conversely, injection of allo-specific Tr1 cells was required to induce tolerance in the stringent model[149], thus suggesting that antigen specificity may be required for a more efficient and safe result.

Transplantation is one of the applications in which cellular therapy with Treg will be feasible and a promising tool. In particular, allogeneic HSCT is the treatment of choice for several disorders of the haematopoietic system, such as haematologic malignancies (leukaemia, lymphoma, and multiple myeloma) and genetic diseases (β-thalassemia and primary immunodeficiencies)[178]. Donor T cells, contained along with the graft or administered separately as donor lymphocyte infusions (DLIs), are crucial for providing immune reconstitution without recurrent life-threating infections, and, in the case of haematological malignancies, eradicate malignant cells after HSCT, eliminating residual neoplastic cells *via* the graft-versus-

leukaemia (GVL) and graft-versus-tumour activity (GVT). Unfortunately, donor T cells mediated also acute and chronic GvHD, which remains one of the main clinical complications after allogeneic HSC transplantation. Thus, several studies have been made trying to suppress GvHD with maintenance of GVT activity. The use of Treg cells as a cellular therapy after allogeneic HSCT represents a promising approach to separate the two effects. The role of nTreg has been extensively explored in preclinical murine models of cellular therapy[179]. Cell therapy trials with nTreg, freshly isolated or *in vitro* expanded, are ongoing in allogeneic HSCT recipients to prevent GvHD[180-183].

The rationale for developing a clinical protocol for transfer Tr1 cells to treat GvHD and promote immune reconstitution after HSCT was provided by pre-clinical studies demonstrated that *in vivo* transfer of *in vitro* induced IL-10-producing Tr cells controlled GvHD following bone-marrow transplantation[112], and by the development of suitable methods to generate human Tr1 cells *in vitro.* In this system, priming of T cells with allogeneic monocytes in the presence of exogenous rIL-10 (MLR10) or, alternatively, with IL-10-producing DC-10 (MLR-DC10) induces a population of anergized T cells, containing allo-Ag-specific Tr1 cells that preserves the ability to respond to nominal and viral Ags[15, 16]. A clinical phase I-II trial based on cell therapy of *ex vivo* allo-Ag-specific IL-10-anergized cells *via* the MLR10 and, subsequently, MLR-DC10 protocol to treat GvHD and promote immune reconstitution after HLA-haploidentical HSCT has been performed in recipients with high-risk haematological cancers(ALTEN, San Raffaele Hospital, Milan, Italy, Figure 11).

Rapid immune reconstitution in the absence of adverse effects or severe GvHD indicates that Ag-specific Tr1 cells generated *ex vivo* can be used in cellular therapy to efficiently prevent GvHD in human *in vivo*(Bacchetta R., manuscript in preparation). Clinical grade production of antigen-specific Tr1 cells has been developed by the group of Foussat[184] and a phase I/II trial in patients displaying severe Crohn's disease has been completed proving the safety of the cell-therapy. A similar trial has been planned in patients with rheumatoid arthritis by the same group (http://www.txcell.com).

DC-10 generated from children with allergy to house dust mites (HDM) pulsed with Derp2, the major HDM Ag, induced Tr1 cells, indicating that modulation of allergen-specific T cell responses could be achieved by the use of tolerogenic DC in clinic[185].

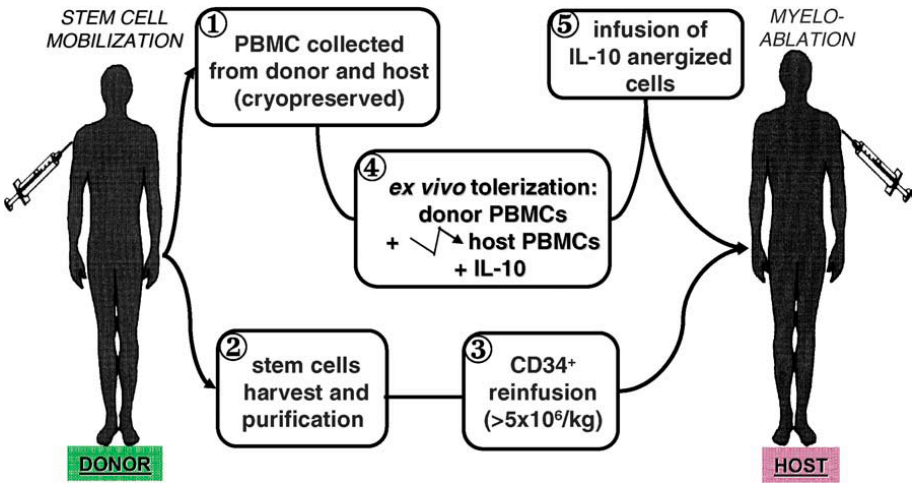

Figure 11: Clinical protocol with IL-10-anergized cells. 1) PBMC are collected and cryopreserved from the HLA-haploidentical donor prior to mobilization and from the host prior conditioning. 2) Stem cells are harvested after donor mobilization and (3) purified $CD34^+$ cells are infused into the recipient who underwent myelo-ablation. 4) After the first signs of neutrophyl reconstitution, donor PBMC are cultured with irradiated host PBMC or DC-10 in the presence of IL-10. 5) After 10 days of culture, the IL-10 anergized donor cells are infused in the patients *(modified from Battaglia M., Seminars in Immunology, 2006)*

Scope of the thesis

The aim of my thesis was to further characterized the mechanism of suppression mediated by Tr1 cells and to define the Tr1 gene signature. In particular, we aimed at identifying specific molecular markers of Tr1 cells, master regulator genes, and genes involved in their effector function.

A lot of progress has been made on the characterization of Tr1 cells and their involvement in immune mediated diseases, but little is still known on the molecules involved in their induction, activation, and effector function. In addition to cytokine-mediated suppression of Teff cells, other mechanisms have been hypothesized to concur to their suppressive activity. The cellular and molecular mechanisms underlying the suppression of APCs by Tr1 cells are still not completely elucidated. It has been previously reported that CD3/CD46 Tr1-like cells express GZB and mediate cytotoxicity. However, it is not already clear the target specificity and the molecular mechanisms underlying their cytotoxicity.

To date no specific biomarkers that would help in the isolation and characterization of Tr1 cells have been described. The combinations of the markers $CD4^+CD18^+CD49b^+$[103] and $CD4^+CD45RA^-CD25^-CD127^-$[106] have been used for this purpose, but are not widely accepted. In addition, the identification of molecules involved in the generation of Tr1 cells will be highly beneficial for designing protocols that efficiently induce differentiation and expansion of Ag-specific Tr1 cells *in vitro* and/or *in vivo*. The induction of Tr1 cells will be a major step forward for their use as cellular therapy to control undesired immune responses, including immune response against

vector-derived and therapeutic proteins. Moreover, the discovery of specific master regulator gene or set of genes determining the conversion of a naïve and, possibly, memory T cells in Tr1 cells will open new perspectives in the generation of homogeneous population of Tr1 cells using gene therapy approach. Finally, the identification of novel mechanisms of suppression mediated by Tr1 cells will be instrumental in defining appropriate immuno-modulatory therapies.

Specifically, two major lines of research have been investigated:

- **Characterization of the molecular and cellular mechanisms underlying cytotoxicity mediated by Tr1 cells (chapter 2):**
  We investigated the molecular mechanisms and the target specificity of Tr1 and the relationship between granzyme B and IL-10 expression.

- **Define the Tr1 cell signature (chapter 3):**
  Genes differentially expressed by Tr1 cells clones in comparison with Th0 cell clones were performed using DNA microarrays technology. Gene expression profiles of human unstimulated (t0) and TCR-activated (6 and 16 hours) T cell clones were analyzed. Genes emerging from this study are genes differentially expressed in Tr1 cells in the resting state and after activation, and represent potential targets for therapeutical approaches of immune mediated diseases.

- In **chapter 4**, all the results presented in this thesis are briefly discussed as well as the future perspectives of this research in molecular and translational medicine.

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Chapter 2

KILLING OF MYELOID APC *VIA* HLA CLASS I, CD2 AND CD226 DEFINES A NOVEL MECHANISM OF SUPPRESSION BY HUMAN Tr1 CELLS

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Killing of myeloid APC *via* HLA Class I, CD2 and CD226 defines a novel mechanism of suppression by human Tr1 cells

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Key words: Type 1 regulatory T (Tr1) cells, Cytotoxicity, Immune regulation, granzyme B

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Abbreviations used in this paper:

Granzyme B: GZB

Granzyme A: GZA

Healthy Donor: HD

Hematopoietic stem cell transplantation: HSCT

Ionomycin: IONO

Killer cell Ig-like Receptors: KIR

Lymphocyte Function-associated Antigen: LFA

Patient: pt

Perforin: PRF

Persistent mixed chimerism: PMC

Summary

IL-10-producing $CD4^+$ type 1 regulatory T (Tr1) cells, defined based on their ability to produce high levels of IL-10 in the absence of IL-4, are major players in the induction and maintenance of peripheral tolerance. Tr1 cells inhibit T cell responses mainly *via* cytokine-dependent mechanisms. The cellular and molecular mechanisms underlying the suppression of APC by Tr1 cells are still not completely elucidated. Here, we defined that Tr1 cells specifically lyse myeloid APC through a granzyme B (GZB)- and perforin (PRF)-dependent mechanism that requires HLA class I recognition, CD54/Lymphocyte Function-associated Antigen (LFA)-1 adhesion, and activation *via* CD2. Notably, interaction between CD226 on Tr1 cells and their ligands on myeloid cells, leading to Tr1 cell activation, is necessary for defining Tr1 cell target specificity. We also showed that high frequency of GZB expressing $CD4^+$ T cells is detected in tolerant patients and correlates with elevated occurrence of IL-10-producing $CD4^+$ T cells. In conclusion, the modulatory activities of Tr1 cells are not only due to suppressive cytokines but also to specific cell-to-cell interactions which lead to selective killing of target cells and possibly bystander suppression.

Introduction

$CD4^+$ type 1 regulatory (Tr1) cells are adaptive IL-10-producing Treg cells known to be fundamental in controlling immune responses and in inducing peripheral tolerance both in humans and mice[1-3]. The first indication that Tr1 cells mediate peripheral tolerance *in vivo* came from SCID patients (pts) who developed long-term tolerance to stem cell allograft[1]. After that, Tr1 cells have been found to be induced in a variety of *in vivo* settings[4]. Tr1 cells have been recently associated to the induction of persistent mixed chimerism (PMC) in β-thalassemic pts after HLA identical hematopoietic stem cell transplantation (HSCT)[5].

Tr1 cells are induced in the periphery upon chronic Ag stimulation in the presence of IL-10 derived from tolerogenic APC[4]. No specific cell markers for Tr1 cells have been identified so far. Therefore, Tr1 cells can be characterized based on their specific cytokine production profile ($IL\text{-}10^{++}$, $TGF\text{-}\beta^+$, $IL\text{-}4^-$, $IL\text{-}2^{low}$, $IFN\text{-}\gamma^{low}$). Tr1 cells are Ag-specific, hypo-responsive, and suppress effector T cells mainly by the release of IL-10 and TGF-β[2, 6]. It has been hypothesized that a cell-contact-dependent mechanism cooperates with the release of immunosuppressive cytokines in inhibiting immune responses by Tr1 cells, since addition of neutralizing antibodies against IL-10R and TGF-β did not completely revert suppression mediated by Tr1 cells[7].

Murine $CD25^+$ Treg cells express granzyme B (GZB)[8, 9], and induce apoptosis of T cells[10], indicating that GZB-dependent killing of T cells represents one of the mechanisms responsible for Treg-mediated suppression. In line with these findings, $CD25^+$ Treg cells

isolated from GZB-deficient mice have reduced suppression ability compared to $CD25^+$ Treg cells from wild type mice[10].

Human naturally occurring Treg cells or adaptive IL-10-producing Treg cells, depending on the mode of activation/generation, can express both granzyme A (GZA) and GZB[11, 12]. nTreg cells express GZA or GZB when activated in the presence of low or high concentrations of IL-2, respectively[11, 12]. IL-10-producing Treg cells generated *in vitro* by activating $CD4^+$ T cells with anti-CD3 and anti-CD46 mAb express only GZB[11], whereas IL-10-producing Treg cells induced by HSV stimulated human plasmacytoid DC express both GZA and GZB[13]. nTreg cells activated with CD3/CD28 and IL-10-producing Treg cells activated with CD3/CD46 were shown to kill different target cells through the adhesion of CD18[11].

In the present study, we investigated the cellular and molecular mechanisms underneath Tr1-mediated cytotoxicity. Results show that polarized Tr1 cell lines and Tr1 cell clones express and release high levels of GZB in an IL-10-dependent manner, and lyse APC *via* GZB and PRF. Lysis mediated by Tr1 cells requires HLA class I recognition, Lymphocyte Function-associated Antigen (LFA)-1-mediated adhesion, and stimulation *via* CD2 and CD226, and consequently is restricted to myeloid APC that express high levels of the ligands of LFA-1 (CD54), of CD2 (CD58), and of CD226 (CD155). GZB^+CD4^+ T cells are detected in the periphery of multiple-transfused β-thalassemic pts and in PMC β-thalassemic pts in whom Tr1 cells are present at high frequency, supporting the hypothesis that GZB is relevant also for the *in vivo* function of Tr1 cells.

Results

Human Tr1 cells express and release high levels of GZB.

Tr1 polarized cell lines (defined as Tr1 cell lines) expressed significantly higher levels of GZB compared to Th0 cell lines (97.3% vs. 12.9%, $p<0.0001$, Figure 1A). Notably, IL-10-producing Tr1 cells represent 10-15% of the polarized population, thus GZB expression is not restricted to this population of cells (Figure 1B). Tr1 cell lines express also significantly higher levels of GZA compared to Th0 cell lines (58.7% vs. 9%, $p<0.0001$, not shown), nevertheless its expression was consistently lower than that of GZB. Tr1 cell lines contained also a significantly higher percentage of PRF^+ cells compared to Th0 cell lines before (8.8% vs. 1.8%, $p=0.015$) and after stimulation (13.3% vs. 5.1%, $p=0.007$, not shown).

Tr1 cell clones, isolated from peripheral blood of two distinct healthy donors (HD) and defined based on IL-10/IL-4 ratio ≥8 (Table 1), expressed higher levels of GZB compared to Th0 cell clones (Figure 1C). The MFI of GZB expression was variable among both Tr1 and Th0 cell clones, but it correlated with the amounts of IL-10 produced by Tr1 but not Th0 cell clones (Figure 1D), suggesting a relationship between the presence of IL-10 in Tr1 cell culture and GZB expression.

Tr1 cell lines spontaneously released significantly higher levels of GZB compared to Th0 cell lines ($39x10^3±14$ vs. $0.5x10^3±0.3$ Spot Forming Units (SFU)/10^6 cells, in Tr1 and Th0 cell lines, n=5, $p=0.006$, Figure 1E). GZB released by Tr1 cell lines was further increased upon activation (Figure 1E). Purified IL-10-producing Tr1 cells secreted significantly higher amounts of GZB compared to the original Tr1 cell lines ($132x10^3±54$ vs. $32x10^3±20$ SFU/10^6 cells, n=3,

p=0.05, Figure 1F), indicating that IL-10-producing Tr1 cells are the main GZB producers in culture.

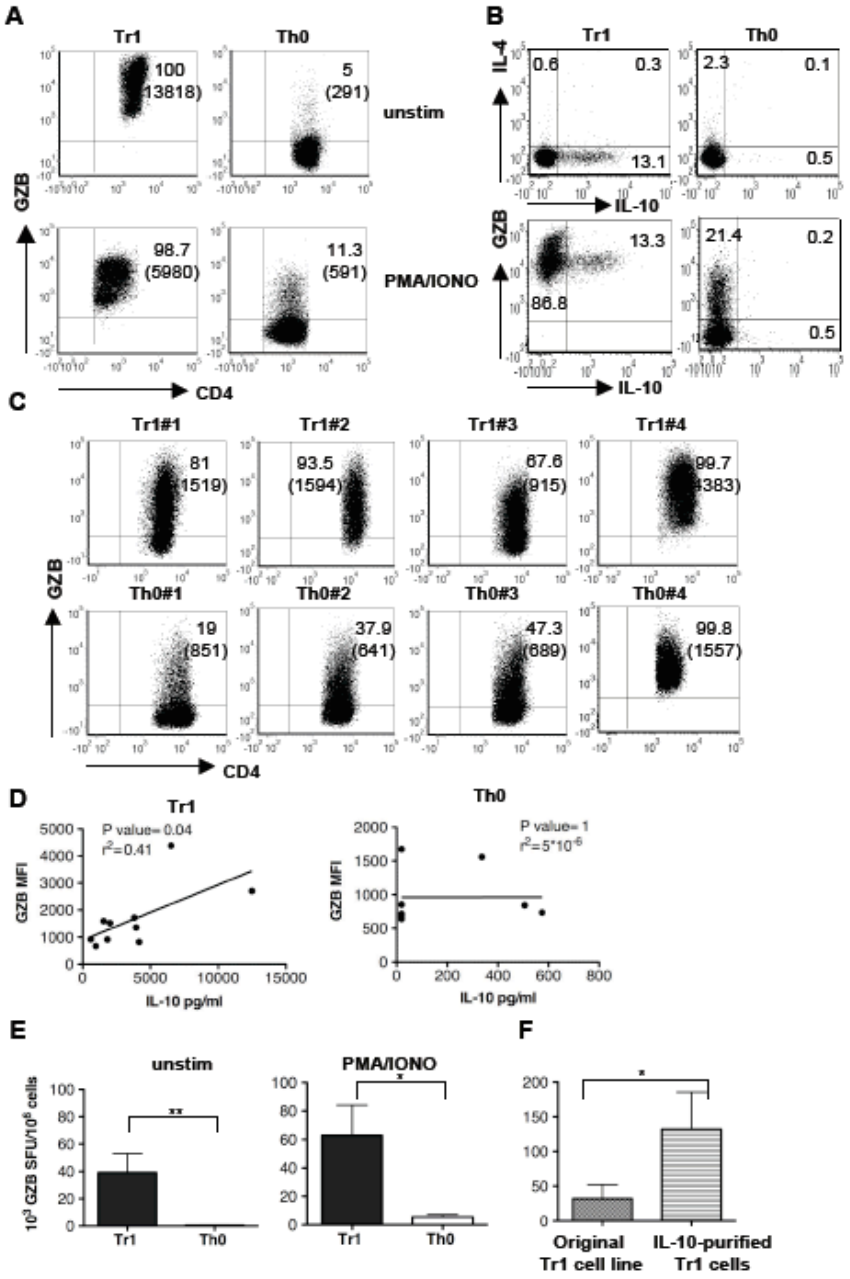

Figure 1. Tr1 cells express and release GZB. GZB expression was determined in Th0 and Tr1 T cell lines unstimulated (unstim) or stimulated with PMA (10 ng/ml; Sigma) plus IONO (150 ng/ml; Sigma) for 6 h. One donor out of 11 unstimulated donors and of 5 stimulated donors tested is shown. Numbers represent percentage of positive cells and MFI in bracket. (B) Alternatively, IL-10, IL-4, and GZB expression was determined upon stimulation with Leukocyte Activation Cocktail (BD Pharmingen) for 5 h. One donor out of 6 donors tested is shown. Numbers represent percentage of positive cells. (C) GZB expression was determined in 4 unstimulated Tr1 and Th0 cell clones. Numbers represent percentage of positive cells and MFI in bracket. (D) Plot represents IL-10 production expressed as pg/ml vs. MFI of GZB expression in each of 10 Tr1 cell clones and of 8 Th0 cell clones tested. The line represents the linear regression. The P value of the correlation and the coefficient of determination (r^2) are reported (two-tailed test). (E) GZB release by unstimulated (unstim) and stimulated with PMA/IONO Tr1 and Th0 cell lines was measured by ELISPOT. (F) GZB release by original Tr1 cell lines, and purified IL-10-producing cells was measured by ELISPOT. Mean±SE of GZB spots normalized to 10^6 cells of 5 (unstim), 3 (PMA/IONO), and 3 (F) independent experiments performed in duplicate is shown. SFU = Spot Forming Units *P≤0.05, and **P≤0.005 (one-tailed test)

These data demonstrate that Tr1 cells expressed and released high levels of GZB, and that non IL-10-producing T cells present in the polarized Tr1 cell lines expressed GZB as result of IL-10 exposure.

	IL-2 pg/ml	IL-4 pg/ml	IL-10 pg/ml	IFN-γ pg/ml	IL-17 pg/ml
Tr1#1	48	< 9	2015	2205	< 30
Tr1#2	< 15	37	1558	7942	< 30
Tr1#3	185	< 9	1818	11015	67
Tr1#4	< 15	728	6528	7093	< 30
Tr1#5	< 15	< 9	590	766	< 30
Tr1#6	126	97	3808	6187	218
Tr1#7	< 15	22	3941	1796	59
Tr1#8	< 15	< 9	4160	52684	90
Tr1#9	< 15	757	12500	3723	< 30
Tr1#10	170	127	959	967	< 30
Th0#1	22	> 3000	< 19	469	< 30
Th0#2	105	1446	< 19	1895	< 30
Th0#3	132	2105	< 19	3099	< 30
Th0#4	< 15	145	336	3124	< 30
Th0#5	294	> 3000	< 19	2615	< 30
Th0#6	> 1000	> 3000	574	2567	< 30
Th0#7	527	2523	506	2833	< 30
Th0#8	1211	2597	< 19	3099	< 30

Table 1. Cytokine profile of T cell clones[a)]

Tr1 and Th0 cell clones were stimulated with immobilized anti-CD3 mAb and soluble anti-CD28 mAb. Culture supernatants were collected after 24 h (IL-2) and 48 h (IL-4, IL-10, IFN-γ, IL-17) and levels of cytokines were measured by ELISA.

Tr1 cells specifically kill myeloid cells.

Tr1 cell lines efficiently lysed U937 cells, a monocytic cell line, but not K562 cells, a erythroleukemic cell line (Figure 2A), or Daudi, a B lymphoblast cell line, or Jurkat, a T leukemic cell line (Supplemental Figure 1). Th0 cell lines exerted limited lytic activity on the cell lines tested (Figure 2A and Supplemental Figure 1).

When Tr1 cells were co-cultured with U937 cells, high percentage of $CD107a^+GZB^+$ cells was observed (on average 26% of $CD107a^+GZB^+$ cells in Tr1 cell lines cultured with U937 cells compared to 3% in Tr1 cell lines alone, p=0.0002, Figure 2B), consistent with the lysis assessed by ^{51}Cr assay (Figure 2A). As expected, percentages of $CD107a^+GZB^+$ cells within the Tr1 cells co-cultured with K562, Daudi, or Jurkat cells were low and similar to those observed in Tr1 cells cultured alone (Figure 2B and not shown). Despite the ability of Th0 cell lines to degranulate when activated with U937 cells, as demonstrated by CD107a staining, they were unable to lyse these target cells. The percentages of $CD107a^+GZB^+$ cells were indeed similar to those of observed in Tr1 cell cultures alone (Figure 2B).

Four different Tr1 cell clones efficiently lysed U937 but not K562 cells (Figure 2C-D and not shown). Tr1 clone #4 that displayed the highest lytic ability (Figure 2C) had also the highest GZB expression (99.7%, MFI 4383, Figure 2D left panel) and secreted the highest level of IL-10 (6528 pg/ml, Table 1), whereas Tr1 clone #5 had the lowest lytic ability (Figure 2C), the lowest GZB expression (39%, MFI 798, Figure 2D left panel) and secreted the lowest levels of IL-10 (590 pg/ml, Table 1). Tr1 clones #1 and #3 killed U937 cells similarly (Figure 2C) and had similar levels of GZB (81%, MFI 1519, and

67.6%, MFI 915, respectively, Figure 2D left panel) and IL-10 production (2015 pg/ml and 1818 pg/ml, respectively, Table 1). The percentages of $CD107a^+GZB^+$ cells in Tr1 cell clones were higher (Figure 2D) compared to Tr1 cell lines (Figure 2B), consistent with the fact that Tr1 cell clones are an homogeneous population of IL-10-producing T cells. Notably, Th0 cell clones lysed U937 cells but cytotoxicity was independent from the levels of GZB expression and from their ability to secrete IL-10 (data not shown). These data demonstrated that GZB expression by Tr1 cells correlates not only with IL-10 production but also with their lytic activity against myeloid cell line.

Tr1 cell lines lyse freshly isolated $CD14^+$ and $CD1c^+$ cells, but not $CD19^+$ and $CD3^+$ cells (both allogeneic and autologous, Figure 3 and Supplemental Figure 2). The mean percentages of $CD107a^+GZB^+$ cells in Tr1 cell lines co-cultured with autologous $CD14^+$ cells or $CD1c^+$ cells were 27% and 18%, respectively. Similar results were obtained when Tr1 cell lines were co-cultured with allogeneic $CD14^+$ cells (21%) and with allogeneic $CD1c^+$ cells (18%).

Overall these findings demonstrate that Tr1 cell lines specifically kill both autologous and allogeneic cells of myeloid origin.

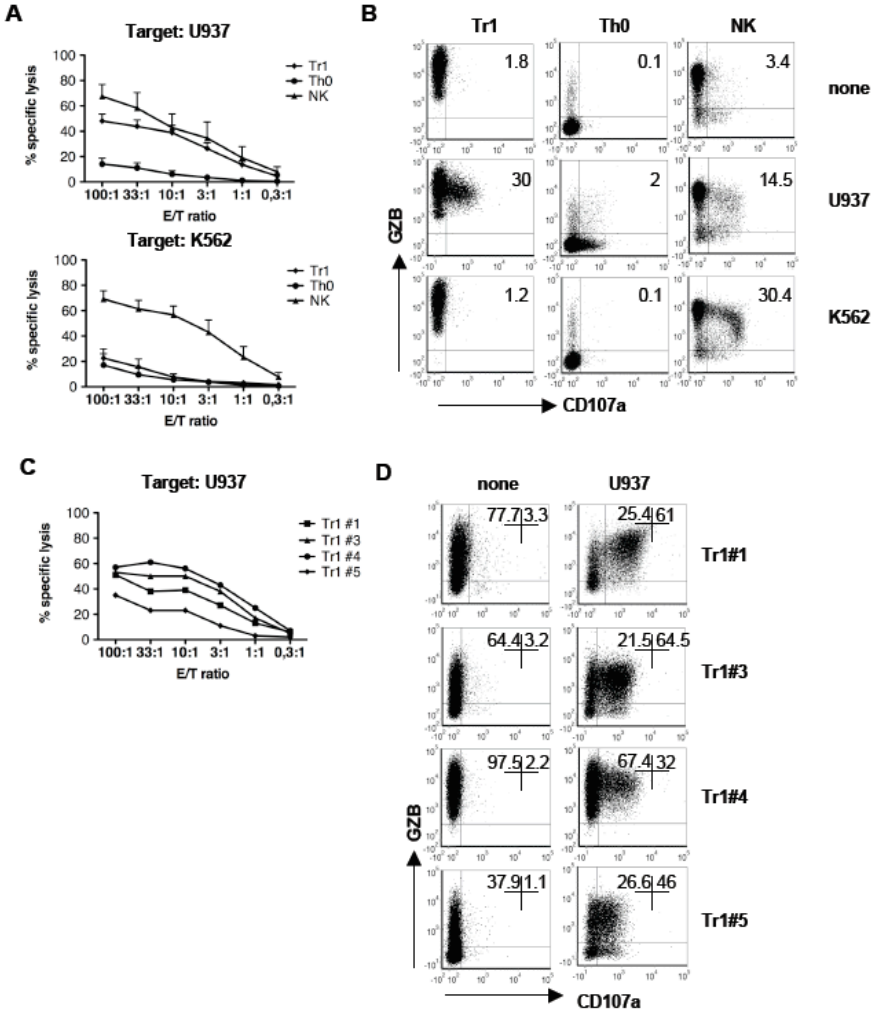

Figure 2. Tr1 cell lines and Tr1 cell clones specifically lyse target cell of monocytic origin. (A) The cytotoxic activity of Tr1 and Th0 cell lines against U937 and K562 target cell lines was determined by ^{51}Cr release. As positive control NK cell lines from the same HD were used. Mean±SE of 6 donors for U937 and of 5 for K562 performed in duplicate are reported. (B) Tr1 and Th0 cell lines were co-cultured with U937 and K562 target cell lines at 10:1 E:T ratio. Cytotoxicity was measured by co-expression of CD107a and GZB in $CD4^+$ T cells. As positive control NK cell lines from the same HD were used. One donor out of 10 donors performed in 8 independent experiments for U937 and of 4 donors performed in 3 independent experiments for K562 is shown. Numbers represent percentage of

$CD107a^+GZB^+$ cells. (C) Cytotoxic activity of 4 Tr1 cell clones against U937 target cell line was determined by ^{51}Cr release. (D) In parallel, Tr1 cell clones were co-cultured with U937 target cell line at 10:1 (E:T) ratio and cytotoxicity was measured by co-expression of CD107a and GZB in $CD4^+$ T cells. Numbers represent percentage of $CD107a^+GZB^+$ cells.

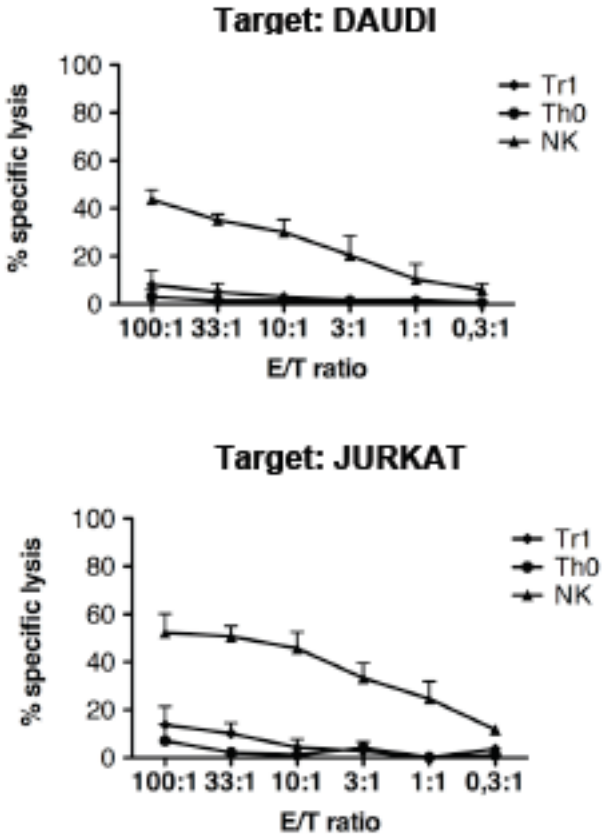

Figure S1. Tr1 cell lines do not lyse Daudi and Jurkat cells. Cytotoxic activity of Tr1 and Th0 cell lines against Daudi and Jurkat target cell lines was determined by ^{51}Cr release standard assay. As positive control NK cell lines from the same HD were used. Mean ± SE of 3 independent donors for Daudi and 4 for Jurkat performed in duplicate are reported.

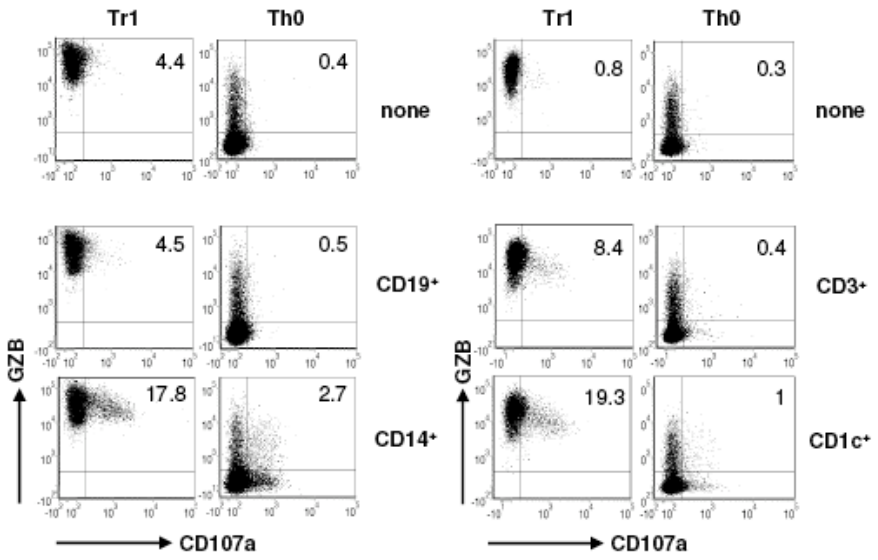

Figure 3. Tr1 cell lines specifically lyse primary monocytes and myeloid DC. Tr1 and Th0 cell lines were co-cultured with allogeneic freshly isolated $CD19^+$, $CD14^+$, $CD3^+$, and $CD1c^+$ cells at 10:1 (E:T) ratio, and cytotoxicity was measured by co-expression of CD107a and GZB in $CD4^+$ T cells. One donor out of 4 ($CD19^+$), 3 ($CD14^+$), 3 ($CD3^+$), and 4 ($CD1c^+$) donors performed in 2 independent experiments is shown. Numbers represent percentage of $CD107a^+GZB^+$ cells.

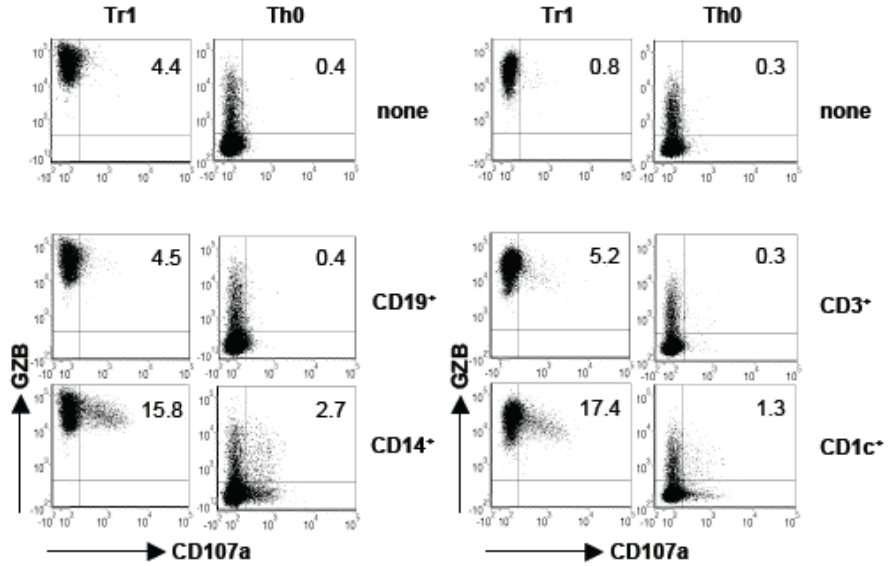

Figure S2. Tr1 cell lines specifically lyse primary autologous monocytes and myeloid DC. Tr1 and Th0 cell lines were co-cultured with autologous freshly isolated $CD19^+$, $CD14^+$, $CD3^+$, and $CD1c^+$ cells at 10:1 (E:T) ratio, and cytotoxic activity was measured by co-expression of CD107a and GZB in $CD4^+$ T cells. One representative donor out of 3 ($CD19^+$), 3 ($CD14^+$), 4 ($CD3^+$), and 3 ($CD1c^+$) performed in 2 independent experiments is shown. Numbers represent percentage of $CD107a^+GZB^+$ cells.

Tr1-mediated cytotoxicity is dependent on GZB and PRF, and requires HLA class I recognition.

The role of GZB in the lytic activity mediated by Tr1 cells was demonstrated by the addition of Z-AAD-CMK, a specific inhibitor of GZB, which completely abrogated the cytotoxic activity of Tr1 cell lines in a dose-dependent manner (Figure 4A). Similarly, the Tr1-mediated cytotoxicity was nearly abolished when CMA, a specific PRF inhibitor, was added (Figure 4B), indicating that both GZB and PRF are required for killing mediated by Tr1 cells.

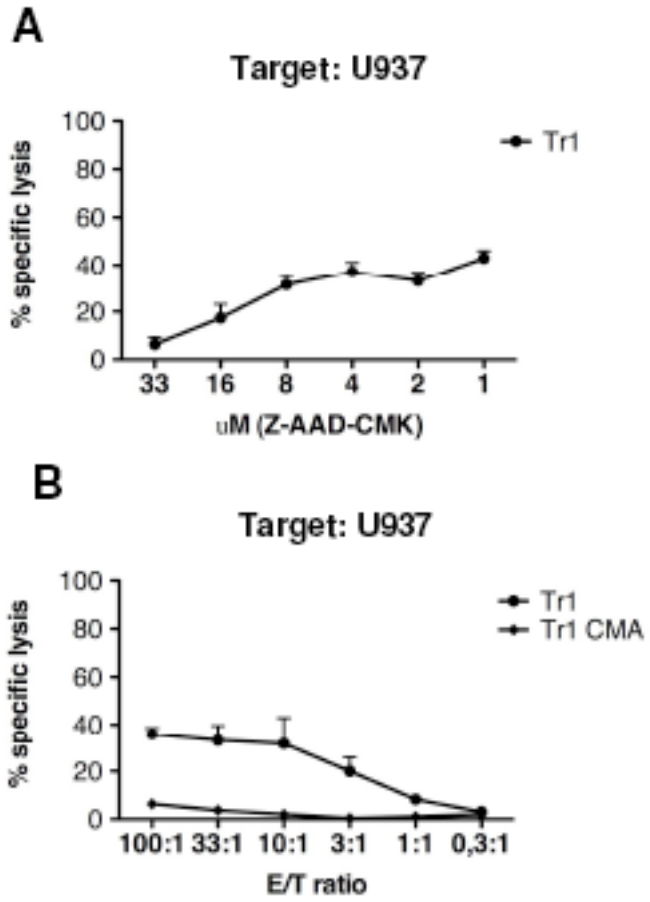

Figure 4. Tr1-mediated cytotoxicity is GZB- and PRF-dependent. Naive Tr1 cell lines were co-cultured with U937 target cell line at 100:1 (E:T) ratio in the presence of Z-AAD-CMK at the indicated concentrations (A) or in the presence of CMA (100nM) (B) and cytotoxicity was determined by ^{51}Cr release. Mean±SE of 3 donors are reported.

Addition of a pan anti-HLA-I mAb (clone W6/32) significantly inhibited, in a dose-dependent manner, the killing of U937 cells, and of $CD14^+$ and $CD1c^+$ cells (autologous and allogeneic) by Tr1 cell lines and by three distinct Tr1 cell clones (Figure 5A-C and not shown).

Tr1 cells express a variety of stimulatory Killer cell Ig-like Receptors (KIR) including KIR2DS2, KIR2DS3, KIR3DS1, and KIR2DL4, the ligand specific for HLA-G (data not shown). Addition of neutralizing anti-HLA-G mAb (clone 87G) partially inhibited, in a dose-dependent manner, the killing of U937 cells, and of $CD14^+$, and $CD1c^+$ cells by both Tr1 cell lines (Supplemental Figure 3A-B), and Tr1 cell clones (Supplemental Figure 3C), supporting the contribution of stimulatory KIR in promoting killing of target cells.

Thus, similar to the NK-mediated killing, Tr1 cells, despite the fact that they are $CD4^+$ T cells, require Ag-nonspecific HLA class I recognition and activation *via* KIR to lyse target cells.

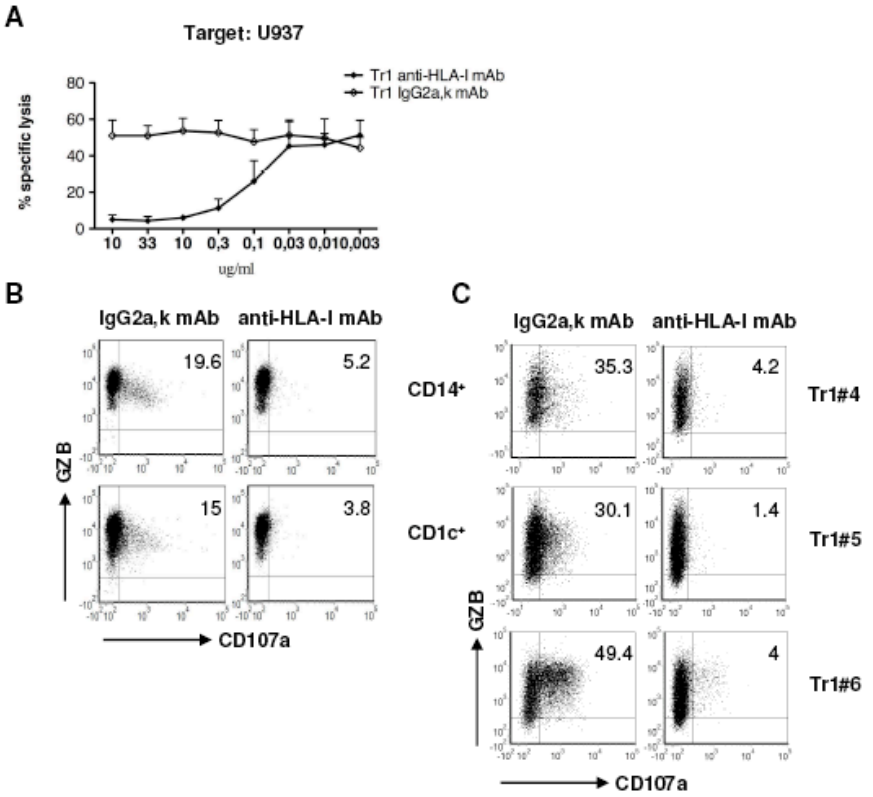

Figure 5. Tr1-mediated cytotoxicity is HLA class I-dependent. (A) Tr1 cell lines were co-cultured with U937 target cell line at 100:1 (E:T) ratio in presence of anti-HLA class I mAb or IgG2a,k isotype control at the indicated concentrations, and cytotoxicity was determined by ^{51}Cr release. Mean±SE of 4 donors performed in 2 independent experiments are reported. (B) Tr1 cell lines were co-cultured with freshly isolated autologous $CD14^+$ and $CD1c^+$ cells at 10:1 (E:T) ratio in the presence of anti-HLA-I mAb or IgG2a,k isotype control, and cytotoxicity was measured by co-expression of CD107a and GZB in $CD4^+$ T cells. One donor out of 3 donors performed in a single experiment is shown. Numbers represent percentage of $CD107a^+GZB^+$ cells. (C) Tr1 cell clones were co-cultured with U937 target cell line at 10:1 (E:T) ratio in the presence of anti-HLA-I mAb or IgG2a,k isotype control, and cytotoxicity was measured by co-expression of CD107a and GZB in $CD4^+$ T cells. Numbers represent percentage of $CD107a^+GZB^+$ cells.

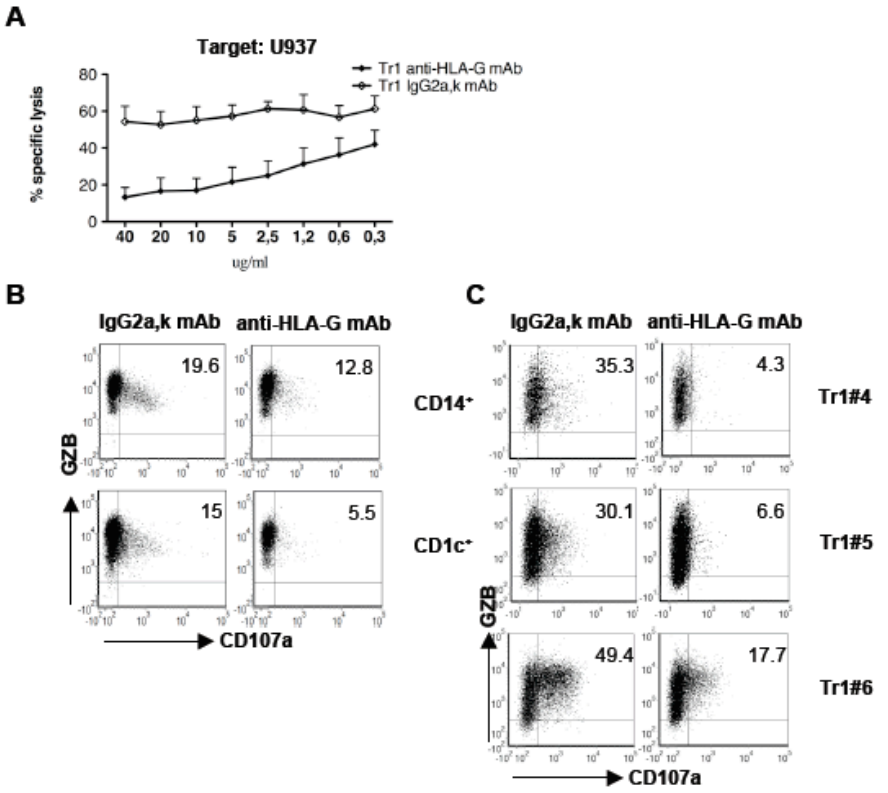

Figure S3. Cytotoxicity mediated by Tr1 cells is partially HLA-G-dependent. (A) Tr1 cell lines were co-cultured with U937 target cell line at 100:1 (E:T) ratio in presence of anti-HLA-G mAb or IgG2a,k isotype control mAb at the indicated concentrations, and cytotoxic activity was determined by ^{51}Cr release. Mean ± SE of 3 donors performed in 2 independent experiments are reported. (B) Tr1 cell lines were co-cultured with freshly isolated autologous $CD14^+$ and $CD1c^+$ cells at 10:1 (E:T) ratio in the presence of anti-HLA-G mAb or IgG2a,k isotype control, and cytotoxic activity was measured by co-expression of CD107a and GZB in $CD4^+$ T cells. One representative donor out of 3 donors performed in a single experiment is shown. Numbers represent percentage of $CD107a^+GZB^+$ cells. (C) Tr1 cell clones were co-cultured with U937 target cell lines at 10:1 (E:T) ratio in the presence of anti-HLA-G mAb or IgG2a,k isotype control (20 μg/ml), and cytotoxic activity was measured by co-expression of CD107a and GZB in $CD4^+$ T cells. Numbers represent percentage of $CD107a^+GZB^+$ cells.

Tr1-mediated killing of myeloid cells is dependent on CD54-mediated adhesion and activation *via* CD2 and CD226.

To dissect the molecular mechanism underlying the target specificity of Tr1 cells, we investigated adhesion and signaling molecules involved in Tr1-myeloid APC interaction. Both $CD14^+$ and $CD1c^+$ cells expressed higher levels of CD54 compared to T and B cells, and Tr1 cells expressed LFA-1 (CD18/CD11a), the CD54 ligand (not shown). Addition of neutralizing anti-CD18 mAb completely blocked the killing of $CD14^+$ or $CD1c^+$ cells mediated by Tr1 cell lines (Figure 6A). CD54 expression on target cells is specifically involved in the formation of a stable immunological synapse essential for cytotoxicity mediated by NK and CTLs[14]. Therefore, our findings suggest that the high expression of CD54 on myeloid cells is responsible for the formation of a stable and prolong interaction with Tr1 cells, leading to the target cell lysis.

We next investigated whether CD2, which is implicated not only in the adhesion between NK cells and its target cells, but also in the activation of NK cells[15], contributes to Tr1 cell activation. Anti-CD2 bearing p815 cells promoted a strong Tr1 cell degranulation and GZB-dependent lysis of target cells (Figure 6B), whereas induced degranulation of Th0 cell lines in the absence of GZB. Notably, CD58 is expressed at high levels in both $CD14^+$ and $CD1c^+$ cells (not shown). These results demonstrate that activation of Tr1 cells *via* CD2 is required for cytotoxic activity mediated by Tr1 cells and suggest that CD58/CD2 interaction plays a key role in the killing of myeloid cells.

DNAM-1 (CD226) is an adhesion/signaling molecule that contributes to the NK-mediated lysis of DC[16]. Tr1 cell lines and Tr1 cell clones express high levels of CD226 (not shown), and CD155, the ligand of CD226, is specifically expressed on myeloid APC[17]. We thus investigated whether CD226 contributed to Tr1-mediated lysis of myeloid cells. Addition of anti-CD226 mAb significantly increased the killing of $CD14^+$ cells mediated by Tr1 cell lines (21% vs. 16% of $CD107a^+GZB^+$ cells in cultures in the presence or absence of anti-CD226, respectively, p=0.0016, Figure 6C). Similar results were obtained when $CD1c^+$ cells were used as target cells (18% vs. 15%, of $CD107a^+GZB^+$ cells in cultures in the presence or absence of anti-CD226, respectively, p=0.02, Figure 6C). These results indicate that specific interaction between CD226 on Tr1 cells and CD155 on myeloid cells leads to Tr1 cell activation.

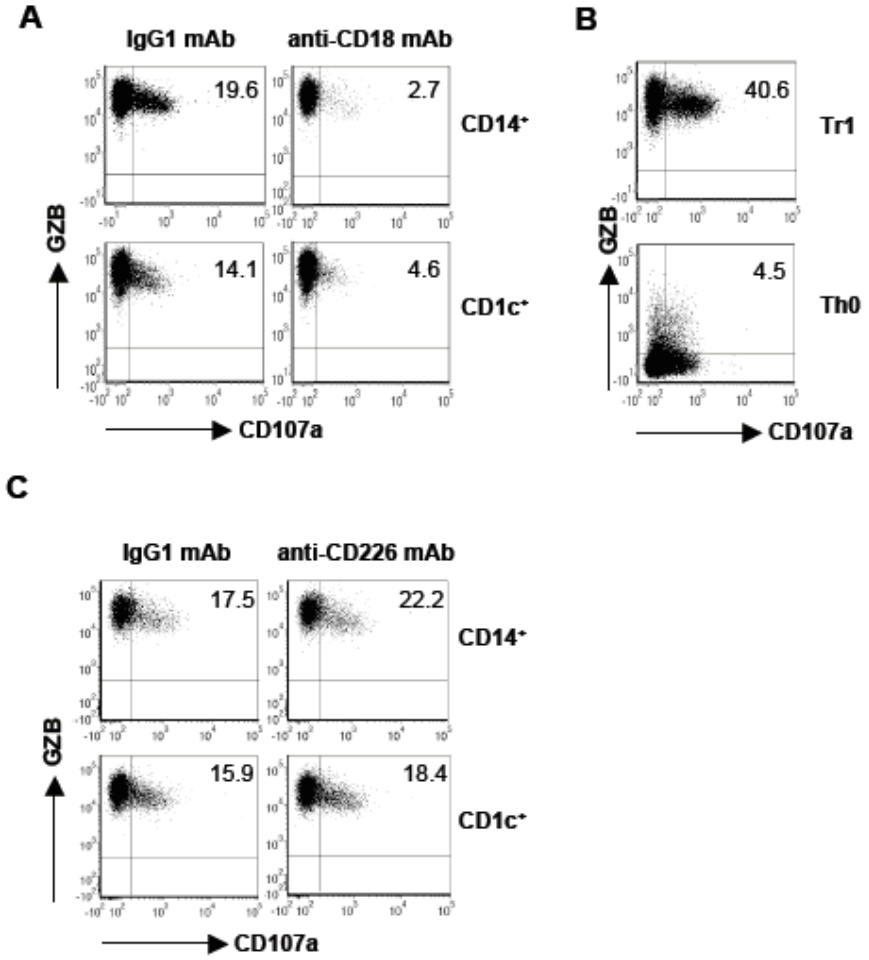

Figure 6. Tr1-mediated cytotoxicity requires CD18 adhesion and activation *via* CD2 and CD226. (A) Tr1 cell lines were co-cultured with freshly isolated allogeneic $CD14^+$ and $CD1c^+$ cells at 10:1 (E:T) ratio in the presence of anti-CD18 mAb or IgG1 isotype control, and cytotoxicity was measured by co-expression of CD107a and GZB in $CD4^+$ T cells. One donor out of 3 donors tested in 2 independent experiments is shown. Numbers represent percentage of $CD107a^+GZB^+$ cells. (B) Tr1 and Th0 cell lines were co-cultured with p815 cell line pre-incubated with anti-CD2 mAb at 10:1 (E:T) ratio, and cytotoxicity was determined by co-expression of CD107a and GZB in $CD4^+$ T cells. One donor out of 3 donors performed in 2 independent experiments is shown. Numbers represent percentage of $CD107a^+GZB^+$ cells. (C) Tr1 cell lines were co-cultured with $CD14^+$ or CD1c cells

at 10:1 (E:T) ratio in the presence of anti-CD226 mAb or IgG1 isotype control, and cytotoxicity was measured by co-expression of CD107a and GZB in $CD4^+$ T cells. One donor out of 6 donors for $CD14^+$ cells and of 4 donors for $CD1c^+$ cells tested in 3 independent experiments is shown. Numbers represent percentage of $CD107a^+GZB^+$ cells.

High frequencies of GZB-expressing $CD4^+$ T cells correlate with elevated percentages of IL-10-producing $CD4^+$ T cells *in vivo*.

We next investigated whether the relation between GZB expression and IL-10-producing Tr1 cells occurs also *in vivo*. We showed that IL-10 production and presence of Tr1 cells correlate with PMC in β-thalassemic pts after HSCT ([5] and not shown). In peripheral blood of these PMC pts, the percentage of $CD4^+GZB^+$ T cells was higher compared to HD (7.9% vs. 3.9%, p=0.04, Figure 7A). Activation of PBL with PMA/IONO further increase the proportion of $CD4^+GZB^+$ T cells in PMC pts (24% vs. 3.5%, p=0.0001, Figure 7A). Importantly, a correlation between the percentages of $CD4^+GZB^+$ T cells and the frequencies of IL-10-producing $CD4^+$ T cells in PMC pts was observed[5] (Figure 7B), suggesting that IL-10 modulates GZB expression also *in vivo*. We also investigated the presence of both $CD4^+GZB^+$ and $CD4^+IL\text{-}10^+$ T cells in β-thalassemic pts tolerized after repetitive exposure to allo-Ag during multiple transfusions and prior-HSCT. The frequency of $CD4^+GZB^+$ T cells was similar in β-thalassemic pts prior-HSCT and HD (Figure 7C). Upon activation of PBL with PMA/IONO a significantly higher proportion of $CD4^+GZB^+$ T cells was detected in β-thalassemic pts compared to HD (15% vs. 3.8%, p=0.009, Figure 7C). Interestingly, the proportion of IL-10-, but not IL-2-, IL-4-, and IFN-γ-producing $CD4^+$ T cells was also

significantly higher in β-thalassemic pts compared to HD (3.7% vs. 1.2%, p=0.02, Supplemental Figure 4 and not shown), and correlated with the frequency of $CD4^+GZB^+$ T cells (Figure 7D).

Overall, these results indicate that in two different clinical conditions associated with tolerance, the presence of IL-10-producing cells correlates with GZB-expressing cells.

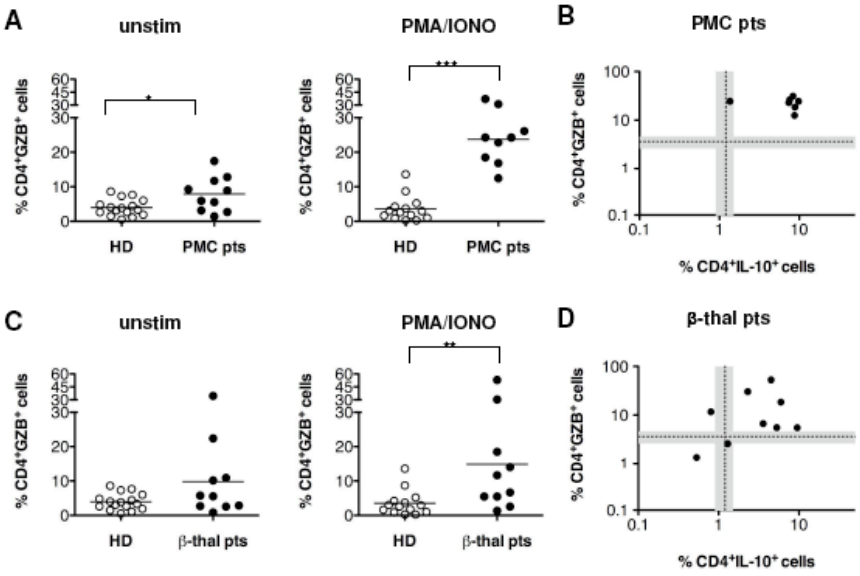

Figure 7. High frequencies of GZB-expressing $CD4^+$ T cells correlated with elevated percentages of IL-10-producing $CD4^+$ T cells *in vivo*. PBMC from PMC pts, β-thalassemic pts prior HSCT and HD were activated with PMA (10 ng/ml; Sigma) and IONO (150 ng/mL; Sigma) or left unstimulated for 12 h in the presence of brefeldin A (10μg/ml; Sigma). (A) Percentages of $CD4^+GZB^+$ T cells in each of 10 PMC pts and 16 HD tested for unstimulated condition and in each of 9 PMC pts and 14 HD tested for PMA/IONO are presented. Black lines represent the mean percentages of $CD4^+GZB^+$ T cells. (B) Plot represents percentages of $CD4^+IL\text{-}10^+$ T cells vs. percentages of $CD4^+GZB^+$ T cells in each of 7 PMC pts tested after activation with PMA/IONO. Dashed lines represent the mean percentages of $CD4^+IL\text{-}10^+$ T cells of 12 HD and $CD4^+GZB^+$ T cells of 14 HD and the grey bar ±

SE. (C) Percentages of $CD4^+GZB^+$ T cells in each of 10 β-thalassemic pts prior HSCT and 16 HD tested for unstimulated condition and in each of 10 β-thalassemic pts and 14 HD tested for PMA/IONO are presented. Black lines represent the mean percentages of $CD4^+GZB^+$ T cells. (D) Plot represents percentages of $CD4^+IL\text{-}10^+$ T cells vs. percentages of $CD4^+GZB^+$ T cells in each of 9 β-thalassemic pts prior HSCT tested after activation with PMA/IONO. Dashed lines represent the mean percentages of $CD4^+IL\text{-}10^+$ T cells of 12 HD and $CD4^+GZB^+$ T cells of 14 HD and the grey bar ± SE. * P≤0.05, **P≤0.005, and ***P≤0.0005 (two-tailed test)

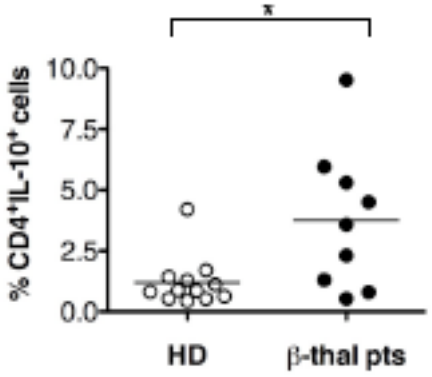

Figure S4. High percentage of $IL\text{-}10^+CD4^+$ T cells in β-thalassemic pts prior-HSCT. PBMC from β-thalassemic pts prior HSCT and HD were activated with PMA (10 ng/ml; Sigma) and IONO (150 ng/mL; Sigma) for 12 h in the presence of brefeldin A (10μg/ml; Sigma). Percentages of $CD4^+IL\text{-}10^+$ T cells in each of 9 β-thalassemic pts and 12 HD tested after activation with PMA/IONO are presented. Black lines represent the mean percentages of $CD4^+IL\text{-}10^+$ T cells. * P≤0.05, (two-tailed test)

Discussion

In this study, we define the cellular and molecular mechanisms underlying the cytotoxicity mediated by human Tr1 cells. We demonstrate that human Tr1 cells, *in vitro* generated and *ex vivo* isolated, express and release high levels of GZB, and specifically lyse target cells of myeloid origin, but not T and B lymphocytes. The mechanism of Tr1-mediated cytotoxicity is dependent on GZB and PRF, requires HLA class I recognition, LFA-1-mediated adhesion, and activation *via* CD2 and CD226. GZB expression correlates with the amounts of IL-10 produced by Tr1 cells *in vitro* and *in vivo,* since high frequencies of IL-10-producing $CD4^+$ T cells in tolerant β-thalassemic pts are associated with elevated occurrence of $CD4^+GZB^+$ T cells. These results demonstrate that, in addition to cytokine-mediated suppression of effector T cells, a key function of Tr1 cells is the GZB-dependent killing of myeloid cells.

Tr1 cell clones isolated from PMC β-thalassemic pts express higher levels of GZB compared to Th0 cell clones[5]. Furthermore, IL-10-producing Treg cells generated by CD3/CD46 cross-linking, preferentially express GZB and lyse different target cells[11]. In the present study, we show that IL-10-producing Tr1 cells, generated *in vitro* or *ex vivo* isolated, not only express, but also secrete GZB, which mediates killing of cells of myeloid origin.

Despite the fact that Tr1 cells are $CD4^+$ T cells, they require recognition, and activation, *via* HLA class I molecules expressed on target cells to lyse myeloid cells, indicating that cytotoxicity mediated by Tr1 cells is Ag-independent. This mechanism of target recognition by Tr1 cells resembles the Ag-nonspecific-mediated recognition and

activation of NK cells[18, 19], and it is opposed to the Ag-specific activation of $CD8^+$ and $CD4^+$ CTLs[20, 21]. In line with this finding, Tr1 cells express different stimulatory KIR involved in $CD4^+$ T cell activation and consequent lytic activity[22-27].

The myeloid cell killing by Tr1 cells is attributable to the high levels of CD54, CD58, and CD155 expression on $CD14^+$ and $CD1c^+$ cells. CD54/LFA-1 and CD58/CD2 interactions not only contribute to a stable adhesion leading to the formation of lytic immunological synapse by NK cells[28], but also participate in their activation[29-31]. Neutralizing mAb against CD18 efficiently abrogates the Tr1-mediated cytotoxicity, and activation of Tr1 cells through CD2 leads to GZB release independently from TCR engagement. This effect concurs with the activation of Tr1 cells *via* CD226-mediated signaling, and results in the polarized degranulation and release of both GZB and PRF, and in myeloid cell killing. CD226 is known to be critically involved in the NK-mediated killing of myeloid APC, since CD155, one of the ligands of CD226, is specifically expressed by monocytes and myeloid DC[16, 17, 32-34]. Since Tr1 cells express LFA-1, CD2, and CD226, we can speculate that their target specificity depends upon the array and density expression of their ligands, CD54, CD58, and importantly, CD155 on myeloid cells. The combination of signaling through these receptors, in association with activation by KIR, is necessary to achieve the threshold required to properly activate Tr1 cell lytic activity.

The expression of GZB by Tr1 cell lines and cell clones correlates, and it is intrinsically associated, with the presence of IL-10 in culture. The direct role of IL-10 in promoting and maintaining GZB

expression in $CD4^+$ T cells *in vitro* is supported by previous reports[13, 35] and by results obtained with PBL cultured in the presence of IL-10, which up-regulated GZB (Serafini G., unpublished observations), and with human $CD4^+$ T cells transduced with a lentiviral vector encoding for human IL-10, which constitutively express high levels of GZB (Andolfi G., submitted for publication). Here we demonstrate that GZB expression by Tr1 cell clones is dependent on their autocrine IL-10 production. Tr1 cells are Ag-specific and produce IL-10 upon TCR stimulation[4]. Thus, GZB expression by Tr1 cells is specific and occurs primarily when Tr1 cells are activated. This effect is independent of TGF-β and opposed to that observed in murine $CD8^+$ CTLs[36]. In line with these *in vitro* observations are concomitant high frequency of IL-10-producing T cells[5] and $CD4^+GZB^+$ T cells observed in PMC and poly-transfused β-thalassemic pts. Thus, these findings indicate that GZB is associated with IL-10 *in vitro* and *in vivo*, and can be used as surrogate marker for Tr1 cells *in vivo*.

Tr1 cells suppress T-cell responses mainly *via* IL-10 and TGF-β, secreted upon Ag-specific TCR activation. These immunomodulatory cytokines directly inhibit effector T cell proliferation and expression of HLA class II and costimulatory molecules on APC, which indirectly suppress effector T cells[4]. We now provide evidences that IL-10 produced by Tr1 cells upon TCR activation also directly induces GZB expression in Tr1 cells, which in turn acquire the ability to kill monocytes and myeloid DC in an Ag-nonspecific manner. Based on these findings, we propose that selective depletion of

myeloid APC by Tr1 cells, during an active immune response, represents an additional bystander mechanism of suppression.

Materials and Methods

Cell isolation and purification

Human peripheral blood was obtained from HD upon informed consent in accordance with local ethical committee approval (TIGET PERIBLOOD) and with the World Medical Association's Helsinki Declaration. PBMC were isolated by centrifugation over Lymphoprep Ficoll gradients (Fresenius Kabi Norge AS, Halden, Norway). $CD4^+$ T lymphocytes were purified using the untouched $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotech, Auburn, CA) according to manufacture's instructions. Naïve $CD4^+CD45RO^-$ T lymphocytes were purified by CD45RO MicroBeads (Miltenyi Biotech). NK cells were purified by negative Dynabeads selection for $CD3^+$, $CD19^+$, and $CD14^+$ cells (Dynal, Oxoid, Italy), as previously described [37]. $CD14^+$ and $CD1c^+$ cells were purified using CD14 MicroBeads (Miltenyi Biotech) or using CD1c (BDCA-1) Dendritic Cell Isolation Kit (Miltenyi Biotech), respectively, according to manufacture's instructions. $CD3^+$ were enriched by negative Dynabeads selection for $CD19^+$, and $CD14^+$ cells and $CD19^+$ cells by positive Dynabeads selection for $CD19^+$ (Dynal, Oxoid, Italy) according to manufacture's instructions. IL-10-producing T cells were purified from polarized Tr1 cell lines stimulated for 4 h with immobilized anti-CD3 mAb (10 μg/mL; Jansen-Cilag, Raritan, NJ, USA) and PMA (10 ng/ml; Sigma) using the IL-10-secretion assay (Miltenyi Biotech), according to the manufacturer's instruction.

Patients

Nineteen pts affected of β-thalassemia with age ranged from 2 to 17 y

have been transplanted from HLA-identical sibling donors at the San Raffaele Scientific Institute since 2005 and at the Istituto Mediterraneo IME since 2004. Before transplantation, the pts were subjected to not-leucodepleted multiple blood transfusions to overcome the inefficient synthesis of hemoglobin. We analyzed ten pts who remained in a state of PMC, in which patient and donor cells co-exist for longer than 2 y after HSCT. The study was approved by the Ethical Committee of the Policlinico Tor Vergata, Rome and by the Ethical Committee of San Raffaele Scientific Institute, Milan. Informed consent from pts was obtained according to institutional guidelines and to the Helsinki Declaration.

T cell differentiation

Tr1 and Th0 cell lines were differentiated using murine L cells transfected with hCD32, hCD80, and hCD58 and supplemented with anti-CD3 mAb (100 ng/ml; OKT3, Jansen-Cilag) as previously described[7].

Establishment of T cell clones

T cell clones were obtained from $CD4^+$ cells by limiting dilution at 0.3 cells/well in the presence of a feeder cell mixture and soluble anti-CD3 mAb (1 μg/mL; OKT3, Jansen-Cilag) in X-VIVO 15 medium (BioWhittaker, Verviers, Belgium) supplemented with 5% pooled human AB serum (BioWhittaker), 100 IU/mL penicillin/streptomycin (BioWhittaker) as previously described[5, 38]. Clones were classified according their cytokine production profile [39, 40].

Cytokine detection

10^6 cells/ml were stimulated with immobilized anti-CD3 mAb (10 μg/mL; Jansen-Cilag) and soluble anti-CD28 mAb (1 μg/mL; BD Pharmingen). After 24 (IL-2), and 48 h (other cytokines), culture supernatants were harvested and levels of cytokines were determined by ELISA according to the manufacturer's instruction (BD Biosciences). The limits of detection were: IFN-γ: 60 pg/ml; IL-10: 19 pg/ml; IL-4: 9 pg/ml; IL-2: 15 pg/ml; IL-17: 30 pg/ml.

Flow cytometry analysis

T cells were tested for the expression of GZB (Caltag MedSystem, Buckingham, UK), GZA (BD Pharmingen), PRF (Biolegend), IL-10 (BD Pharmingen) and IL-4 (BD Pharmingen). For intracytoplasmic staining, T cells were stained with anti-CD4 mAb (BD Pharmingen) before fixation, permeabilization (Fixation/Permeabilization Solution Kit, BD Bioscience, San Diego, CA, USA) and incubation with mAb. Samples were acquired using a BD FACS Canto flow cytometer (BD Biosciences), and data were analyzed with FCS express (De Novo Software, Los Angeles, CA). Quadrant markers were set accordingly to unstained controls.

ELISPOT assay

GZB-releasing T cells were evaluated by GZB ELISPOT kit (eBiosciences, San Diego, CA, USA) according to the manufacturer's instruction. Briefly, T cells were plated at the starting concentration of $0.5x10^6$ T cells/ml in 200 μl of RPMI (BioWhittaker) supplemented with 10% FCS (BioWhittaker), 100 IU/ml penicillin/streptomycin

(BioWhittaker), 2 mM L-Glutamine (BioWhittaker). The test was performed in duplicate for 6 consequence dilutions 1:2 and incubated for 48 h. The spots were counted by KS ELISPOT system (Zeiss Vision, Göttingen, Germany).

Cytotoxic assay

Cytotoxicity was assessed in a standard 4h ^{51}Cr release assay, as previously described [41]. U937, K562, Daudi, and Jurkat target cell lines used was kindly provided by Dr K. Fleischhauer. In some experiments, concanamycin A (CMA, Sigma-Aldrich, St Louis, MO, USA), Z-AAD-CMK (Calbiochem, San Diego, CA, USA), anti-HLA-I mAb (clone W/632, BioLegend), anti-HLA-G mAb (clone 87G, Exbio Praha, Nad Safinou, Czech Republic), and isotype controls (IgG2a,k, BD Pharmingen, San Diego, CA, USA) were added at the indicated concentrations.

CD107a/GZB mobilization assay

T cell degranulation was evaluated in a CD107a flow cytometric assay, according to a protocol adapted from Alter et *al.* [42]. Briefly, 10^5 cells from T cells were plated in IMDM (BioWhittaker) supplemented with 10% FCS (BioWhittaker), 100 IU/ml penicillin/streptomycin (BioWhittaker), 2 mM L-Glutamine (BioWhittaker), with anti-CD107a mAb (20 μL/mL; BD Pharmingen), in 96-well round-bottom plates, in the presence or absence of 10^4 cell lines or freshly isolated target cells at 37°C. After 3h, monensin A (Sigma-Aldrich) was added (30 μg/mL). After additional 3h of incubation, cells were washed and stained with anti-CD4, and anti-

GZB mAb. In some cultures anti-HLA-I mAb (20 μg/mL, clone W6/32, BioLegend), anti-HLA-G mAb (20 μg/mL, clone 87G, Exbio Praha), anti-CD18 mAb (25 μg/mL, clone TS1/18, BioLegend), anti-CD226 (10 μg/mL, clone 102511, R&D Systems, Minneapolis, MN, USA), and Ig isotype controls (IgG2a,k, and IgG1, respectively, BD Pharmingen) were added. $5x10^5$ p815 were incubated 30 minutes with 5 μg/mL anti-CD2 mAb (clone RPA-2.10, BD Pharmingen) and subsequently washed, before being used as target cells.

Statistical analysis

Mean values were reported as Mean±SE. Mann Whitney test was used to determine the statistical significance of the data. Two-tailed analysis was performed, unless not specified in the text. Significance was defined as *P≤0.05; **P≤0.005; and ***P≤0.0005. Statistic calculations were performed with the Prism program 5.0 (GraphPad Software, Inc.).

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Conflict-of-interest disclosure:

The authors declare no competing financial interests.

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Chapter 3

GENE SIGNATURE OF HUMAN Tr1 CELLS

(Manuscript in preparation)

Gene signature of human Tr1 cells

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Abbreviations used in this manuscript:

Antibody: Ab

Antigen: Ag

Antigen presenting cells: APC

β2-microglobulin: B2M

Differentially expressed gene: DEG

Expression system tag: EST

Granzyme B: GZB

Graft versus Host Disease: GvHD

Healthy donors: HD

Hour: h

Hypoxanthine phosphoribosyltransferase 1: HPRT

Monoclonal: m

Mean fluorescent intensity: MFI

Overnight: on

Peripheral blood: PB

Reverse Transcription Polymerase Chain Reaction: RT-PCR

Type 1 T regulatory: Tr1

Regulatory T: Treg

Transcriptional repressor of GATA-3: ROG

Summary

Adaptive type 1 regulatory T (Tr1) cells are suppressor cells characterized by the production of IL-10 in the absence of IL-4 and by the ability to suppress immune responses mainly by the release of IL-10 and TGF-β. Despite several efforts for the detection of molecular markers of Tr1 cells have been made, so far Tr1 cell identification relies on cytokine production profile. Moreover, to date no master regulator gene for Tr1 cells have been defined. To identify Tr1 cell specific surface biomarkers, master regulator genes, and molecules involved in their suppressive functions, we performed a gene expression profiling to compare the gene expression of *ex vivo* isolated Tr1 cell clones compare to Th0 cell clones, in resting state and upon TCR activation. Results demonstrated that Tr1 cells signature is of anti-inflammation, anti-proliferation, and immuno-modulation. In addition, we identified surface molecules such as CD49b, CD226, and CD29 that could be useful to identify Tr1 cell population. Interestingly, among the genes up-regulated in Tr1 cell clones, we found MGAT5 that encodes an enzyme that initiates GlcNAc β1,6 branching on N-glycans and modulates T cell activation and function. Finally, two transcription factors TRERF1 and ETV7 and the transcriptional regulator RBPMS resulted differentially expressed in Tr1 cells compared to Th0 cells, which may represent master regulators of Tr1 cells.

Introduction

Type 1 T regulatory (Tr1) cells play a critical role in promoting immunological tolerance *in vivo*[1, 2]. Tr1 cells secrete high levels of IL-10 and TGF-β, intermediate amount of IFN-γ and IL-2, no IL-4, and are induced in the periphery upon antigen (Ag) exposure in the presence of IL-10[3]. Tr1 cells are anergic when stimulated *via* their TCR[3, 4] but can be expanded in the presence of IL15[5]. Tr1 cells inhibit effector T cells[3, 4, 6, 7] and modulate antigen presenting cells (APC) function[8], mainly by the release of IL-10 and TGF-β. Suppression requires stimulation *via* TCR; however, once activated, Tr1 cells acquire non-specific bystander suppressor activity[3]. We recently demonstrated that Tr1 cells selective kill myeloid cells *via* a granzyme B (GZB)- dependent mechanism (Magnani CF., under revision). Several advantages in the comprehension of the mechanisms underlying immune-regulation mediated by Tr1 cells has been made, however it still remains to determine whether additional pathways are involved.

Tr1 cells are critically involved in promoting tolerance in different settings, including colitis[3], atherosclerosis[9], type 1 diabetes[10], and graft-vs-host disease (GvHD)[11]. Morevoer, adoptive cell therapy with Tr1 cells has been exploited as therapeutic strategy in immune-mediated diseases[12, 13]. Despite this significant progress, studies of Tr1 cells have been hampered by the lack of specific surface molecular markers, which would help identifying Tr1 cells *in vivo*, limiting their study in human diseases. Some markers have been previously suggested for Tr1 cells, such as PD-1[14], or CD49b[15, 16]. We recently demonstrated that GZB can be used as surrogate

marker for Tr1 cells *in vivo*, since it is associated with IL-10 (Magnani CF., under revision). $CD45RA^{-}CD25^{-}CD127^{-}$ $CD4^{+}$ T cell subset of human blood have been proposed to be Tr1 cells[17]. In addition, transcriptional profile that mouse Tr1 clones expressed the transcriptional repressor of GATA-3 (ROG)[18]. However, these markers have not been corfirmed in different experimental settings and thus specific markers of Tr1 cells are still elusive.

Further advance in our knowledge of the biology of Tr1 cells could lead to a more effective and powerful application in the modulation of the immune responses in clinical settings. Until now, gene expression analysis on Tr1 cells was restricted to murine system[18, 19]. In the present study, we performed a gene wide expression profile of human Tr1 cell clones compared to Th0 cell clones. 114 genes are differentially expressed in resting Tr1 cell clones compared to resting Th0 cell clones; 87 gene probesets are differentially expressed in Tr1 vs Th0 subsets at 6 and 16 hours (h) after activation; 63 gene probesets are differentially expressed both in resting and activated state. Among the genes differentially expressed, we found genes encoding for proteins related to anti-inflammatory pathways, anti-proliferative responses, and immuno-modulation, defining the Tr1 cell subset-specific gene expression signature.

Materials and methods

Cell isolation and purification

Human peripheral blood (PB) was obtained from healthy donors upon informed consent in accordance with local ethical committee approval (TIGET PERIBLOOD) and with the Helsinki Declaration. PBMC were isolated by centrifugation over Lymphoprep Ficoll gradients (Fresenius Kabi Norge AS, Halden, Norway). $CD4^+$ T lymphocytes were purified from PBMC by negative selection using the untouched $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotech, Auburn, CA) according to manufacture's instructions. Naïve $CD4^+CD45RO^-$ T lymphocytes were purified from $CD4^+$ T lymphocytes by CD45RO MicroBeads (Miltenyi Biotech). The proportion of $CD4^+CD45RO^-CD45RA^+$ was consistently greater than 90%.

Establishment of T cell clones

T cell clones were obtained from $CD4^+$ cells by limiting dilution at 0.3 cells/well in the presence of a feeder cell mixture and soluble anti-CD3 mAbs (1 μg/mL, OKT3, Jansen-Cilag, Raritan, NJ, USA) in X-vivo15 medium (BioWhittaker, Verviers, Belgium) supplemented with 5% pooled human AB serum (BioWhittaker), 100 U/mL penicillin/streptomycin (BioWhittaker). At day 3, IL-2 (40 U/mL; Chiron, Italia, Milan, Italy) was added. T-cell clones were restimulated every 14 days with feeder cell mixture and soluble anti-CD3 mAbs (1 μg/mL). Between stimulations with feeder cells, T cell clones were expanded with rhIL-2 (40 U/mL). Once the T cell clones had been established, at every change of medium rhIL-15 (5 ng/mL, R&D System, Minneapolis, MN, USA) was added as a Tr1 cell

growth factor[5, 20]. We classified the clones based on the cytokine production profile[21, 22]. Tr1 cell clones were defined when the ratio between IL-10 and IL-4 was higher than 8, as we have previously described[3, 5, 20]. All cell clones were tested in a suppression assay to assess their regulatory activity. We used as responder total PBMC labelled with CSFE stimulated with immobilized anti-CD3 monoclonal Antibody (mAb, 10 µg/mL; Jansen-Cilag) and soluble anti-CD28 mAb (1 µg/mL, BD Pharmingen, San Diego, CA, USA). Cells were labelled with 1 µM 5-(and 6)- Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE or CSFE, Molecular Probes, Invitrogen, Carlsbad, CA, USA) just before stimulation. In the cell, esterases cleave the acetyl group, leading to the fluorescent diacytylated CFSE. Cell division accompanied by CFSE dilution was analyzed by flow cytometry. Analysis was done by gating on both the $CD4^+CSFE^+$ population and the $CD8^+CSFE^+$ population.

RNA isolation and DNA microarray experiments

Tr1 and Th0 cell clones from two distinct healthy donors (HD) were left unstimulated (t0) or, alternatively, stimulated (6 and 16 hours) with immobilized anti-CD3 mAb (10 µg/mL; Jansen-Cilag) and soluble anti-CD28 mAb (1 µg/mL, BD Pharmingen) in complete medium at a concentration of 10^6 T cells/ml. Total RNA was extracted with RNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Total RNA (100 ng) was used for GeneChip analysis. Preparation of terminal-labelled cDNA, hybridization to the whole-transcript GeneChip® Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA,

USA) and scanning of the arrays was carried out according to manufacturer's protocols (https://www.affymetrix.com). Raw data was preprocessed with RMA algorithm. Clustering analysis was performed with unsupervised hierarchical methods with different distance (correlation and euclidean) and linkage (average and centroid). In order to detect differentially expressed genes, Welch t-test without p-value correction were performed. Genes are considered differentially expressed if gene expression is at least 2 times different with p-value <0.05. All this steps are performed using R and Bioconductor (http://www.bioconductor.org/).

Real-time Quantitative PCR analysis

Total RNA was extracted with RNeasy Mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized with high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Real time analysis was performed using ABI Prism 7500/SDS2.2.1 software. Levels of mRNA were quantified using Assay on Demand quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) kits (Applied Biosystems) with TaqMan Universal PCR Master Mix (Applied Biosystems). Samples were run in duplicate or triplicate, and relative expression was determined by normalizing to hypoxanthine phosphoribosyltransferase 1 (HPRT) and β2-microglobulin (B2M) expression in each set of samples to calculate a fold-change in value and by comparing the relative amount to calibrator (expression level of a pool of $CD4^+$ T cell lines from 4 distinct HD). Analysis were

performed with the qBase v1.3.5 software (Jan Hellemans & Jo Vandesompele).

Gene expression assay identification code from Applied Biosystems (Assay ID) and nucleotide sequence of the probe (Probe Sequence 5' to 3') used for each tested gene (gene symbol)

AssayID	Gene Symbol
Hs00158127_m1	ITGA2
Hs00170832_m1	CD226
Hs00159136_m1	MGAT5
Hs00199302_m1	RBPMS
Hs00925242_m1	STOM
4326321E	HPRT1
Hs99999907_m1	B2M

Cytokine detection

10^6 T cells/ml were stimulated with immobilized anti-CD3 mAb (10 µg/mL; Jansen-Cilag) and soluble anti-CD28 mAb (1 µg/mL, BD Pharmingen) in complete medium. To measure IL-2, IL-4, IL-10, IFN-γ, and IL-17 production, culture supernatants were harvested after 24 (for IL-2 detection), and 48 h (for other cytokines) of culture and levels of cytokines were determined by capture ELISA according to the manufacturer's instruction (BD Biosciences). The limits of detection were as follows: IFN- g: 60 pg/ml; IL-10: 19 pg/ml; IL-4: 9 pg/ml; IL-2: 15 pg/ml; IL-17: 30 pg/ml.

Flow cytometry analysis

To detect CD49b, CD226, CD29, and CD161 T cell clones were stained with anti-CD4 (BD Pharmingen), anti-CD49b (Biolegend, San Diego, CA, USA), anti-CD226 (Biolegend), anti-CD29 (BD Pharmingen), anti-CD161 (BD Biosciences) mAbs. Samples were acquired using a BD FACS Canto flow cytometer (BD Biosciences), and data were analyzed with FCS express (De Novo Software). Quadrant markers were set accordingly to unstained controls.

Statistical analysis

Average values are reported as Average±SE. Mann Whitney test was used to determine the statistical significance of the data. Significance was defined as *P≤0.05; **P≤0.005; and ***P≤0.0005. Statistic calculations were performed with the Prism program 5.0 (GraphPad Software, Inc.).

Results

Characterization and Hierarchical Clusterization of Tr1 cell clones from healthy donors.

RNA samples from Tr1 and Th0 cell clones *ex vivo* isolated from the PBMC of two distinct healthy donors (HD) were processed and analyzed by using the whole transcript Affymetric chips that monitor the expression of 28869 human genes and expression system tags (ESTs). Analysis was performed on T cell clones unstimulated (t0) or anti-CD3/anti-CD28 mAbs stimulated (6 h and overnight, on). Tr1 cell clones with IL-10/IL-4 ratio higher than 8 (Table 1) and displaying suppressive activity (Figure 1) were defined as Tr1. Analysis were performed in 5 Tr1 and 10 Th0 unstimulated cell clones, one of which performed in duplicate, and 4 Tr1 and 6 Th0 stimulated cell clones. Based on the correlation ward method, hierarchical clustering analysis was performed to explore the relationship between the samples, according to the degree of similarity present in the global gene expression data. Dataset contains expression levels of unique genes filtered for affymetrix control probes represented by 36 cDNA from either unstimulated, or stimulated T cell clones.

Global hierarchical clustering analysis reveals two distinct clusters, each of them subdivided in two groups (Figure 2). Samples unstimulated and stimulated clustered in two separated main branches, with the exception of the sample Th0#1 on, confirming previous microarray analysis in which T cell activation induced significant changes in the transcriptome[23, 24].

Table 1. Cytokine profile of T cell clones[a)]

		IL-2 pg/ml	IL-4 pg/ml	IL-10 pg/ml	IFN-g pg/ml	IL-17 pg/ml
HD_A	**Tr1#1**	48	< 9	2015	2205	< 30
HD_A	**Tr1#2**	< 15	< 9	590	766	< 30
HD_A	**Tr1#3**	< 15	< 9	1515	302	27
HD_A	**Th0#1**	362	211	< 19	1799	< 30
HD_A	**Th0#2**	< 15	36	< 19	580	< 30
HD_A	**Th0#3**	< 15	786	636	3218	52
HD_A	**Th0#4**	< 15	> 1200	2337	> 15000	< 30
HD_A	**Th0#5**	18	96	168	626	< 30
HD_B	**Tr1#4**	< 15	757	> 12500	3723	< 30
HD_B	**Tr1#5**	185	< 9	1818	11015	67
HD_B	**Th0#6**	294	> 3000	< 19	2615	< 30
HD_B	**Th0#7**	72	1283	< 19	9808	< 30
HD_B	**Th0#8**	< 15	2342	784	3811	< 30
HD_B	**Th0#9**	259	> 3000	466	> 15000	< 30
HD_B	**Th0#10**	53	2690	289	10418	< 30

a) Tr1 and Th0 cell clones were isolated from peripheral blood (PB) of two HD (HD_A, and B) using limiting dilution and polyclonal stimulation. T cell clones were stimulated with immobilized anti-CD3 mAb and soluble anti-CD28 mAb. Culture supernatants were collected after 24 h (IL-2) and 48 h (IL-4, IL-10, IFN-γ, IL-17) and levels of cytokines were measured by ELISA.

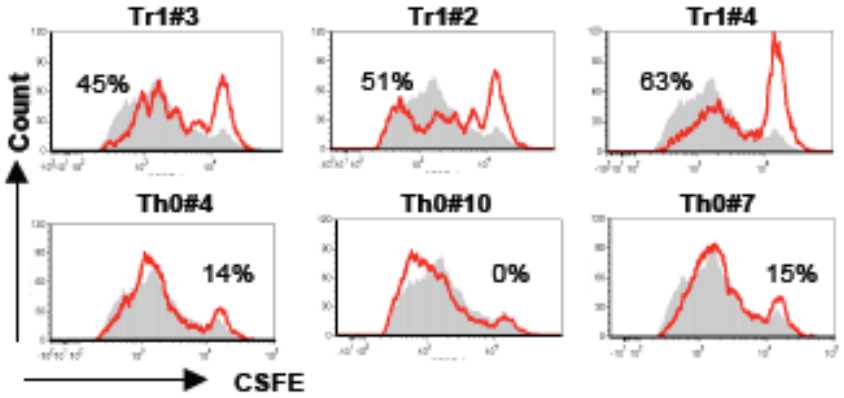

Figure 1. Tr1 cell clones suppress proliferation of responder PBMC. Tr1 and Th0 cell clones were isolated from PB of two HD using limiting dilution and polyclonal stimulation. Suppressive activity of three representative Tr1 and Th0 cell clones (red line) toward responder $CD4^+$ (grey solid) was determined by CSFE standard assay. Percentages of suppression are reported.

Unstimulated samples from the two HDs clustered separately, with the exception of the sample Tr1#5 t0 from HD_B, revealing a great similarity between the global gene expression profile of unstimulated Tr1 and Th0 cell clones. Conversely, samples from activated T cell clones clustered in two distinct branches, one containing activated Tr1 cell clones and one containing activated Th0 cell clones. These findings are in line with the concept that the distinct cytokine profile and functions of Tr1 cells is TCR-dependent. Similar results were obtained also with the method of Euclidean complete (Figure 3).

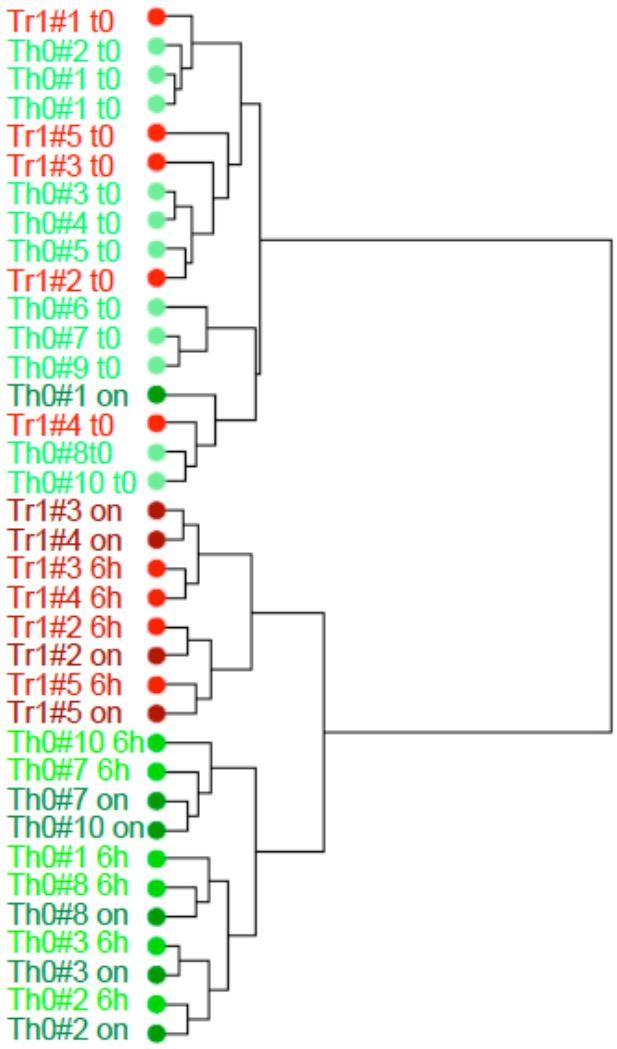

Figure 2. Hierarchical clustering of Tr1 and Th0 cell clones. Tr1 and Th0 cell clones, isolated from PB of two HD using limiting dilution and polyclonal stimulation, were left unstimulated (t0) or, alternatively, stimulated (6h and on) with immobilized anti-CD3 and soluble anti-CD28 mAb. Results from hierarchical structure formed by clustering data of the microarray from Gordon, A. D.[25]. This schematized dendrogram reflects the process of clustering microarray samples according to the similarity of their gene expression profiles as measured by the correlation ward method. Distances between array sample clusters are approximated

(not to scale) by the vertical axis. Along the left side of the dendrogram are the microarray tissue samples from T cell clones.

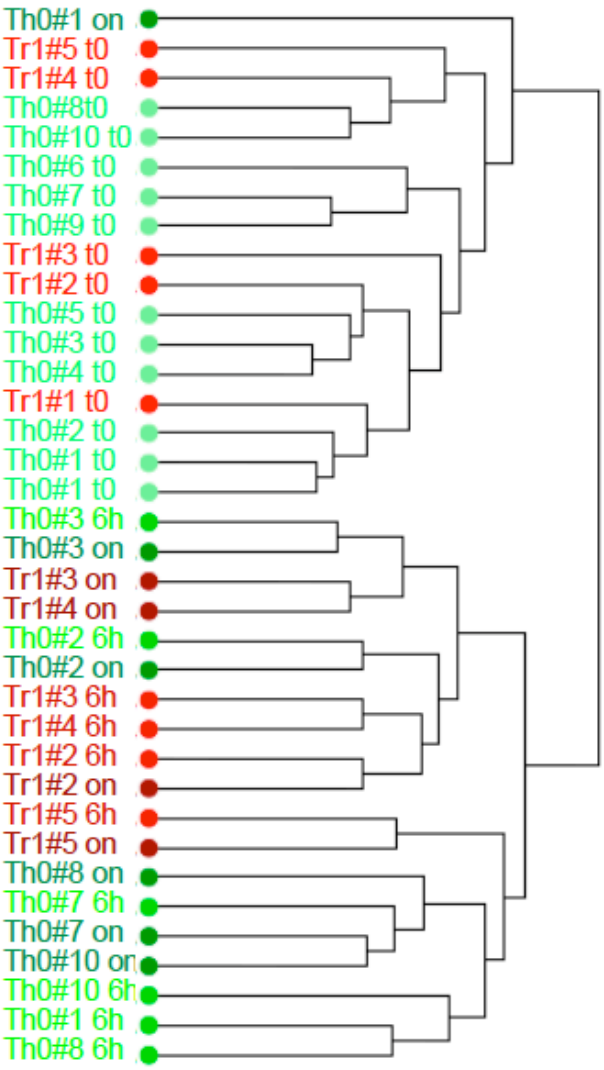

Figure 3. Hierarchical clustering of Tr1 and Th0 cell clones. Tr1 and Th0 cell clones, isolated from PB of two HD using limiting dilution and polyclonal stimulation, were left unstimulated (t0) or, alternatively, stimulated (6h and on) with immobilized anti-CD3 and soluble anti-CD28 mAb. Results from hierarchical structure formed by clustering data of the microarray from Gordon, A. D.[25]. This

schematized dendrogram reflects the process of clustering microarray samples according to the similarity of their gene expression profiles as measured by the Euclidean complete method. Distances between array sample clusters are approximated (not to scale) by the vertical axis. Along the left side of the dendrogram are the microarray tissue samples from T cell clones.

Analysis of the expression of genes already known to be specifically expressed or down-regulated in Tr1 cells, demonstrated that expression of mRNA IL-10 was indeed up-regulated in Tr1 cell clones after activation. Both Tr1 and Th0 cell clones expressed TGF-β at high mRNA levels. Surprisingly, despite lower levels of IL-4 produced by Tr1 cell clones compared to Th0 cell clones, IL-4 mRNA levels from Tr1 cell clones were comparable to those of Th0 cell clones. This discrepancy can be attributable to the levels of detection of the arrays. IL-5 had a decreased mRNA levels in Tr1 cell clones before and after activation (Figure 4A). TNF-α and IFN-γ were expressed at high mRNA levels in both the T cell subsets, the second slightly up-regulated in Tr1 cell clones, whereas IL-2 and IL-17 at a lower mRNA levels (not shown). Consistent with these data, the mRNA expression of the Th1-cell- and Th17-cell- specific transcription factors, T-bet, and the RAR-related orphan receptor (ROR) C, were similar, whereas the mRNA expression of Th2-cell-specific transcription factor GATA-3 was down-regulated in Tr1 compared to Th0 cell clones. As expected, FOXP3 mRNA expression increased in Tr1 cell clones after activation, remaining, however, lower compared to Th0 cell clones (Figure 4B). Notably, no differences were observed between mRNA levels of the IL-10-

associated transcription factors c-MAF, IKAROS and EGR-2 in Tr1 and Th0 cell subsets (Figure 4B and data not shown).

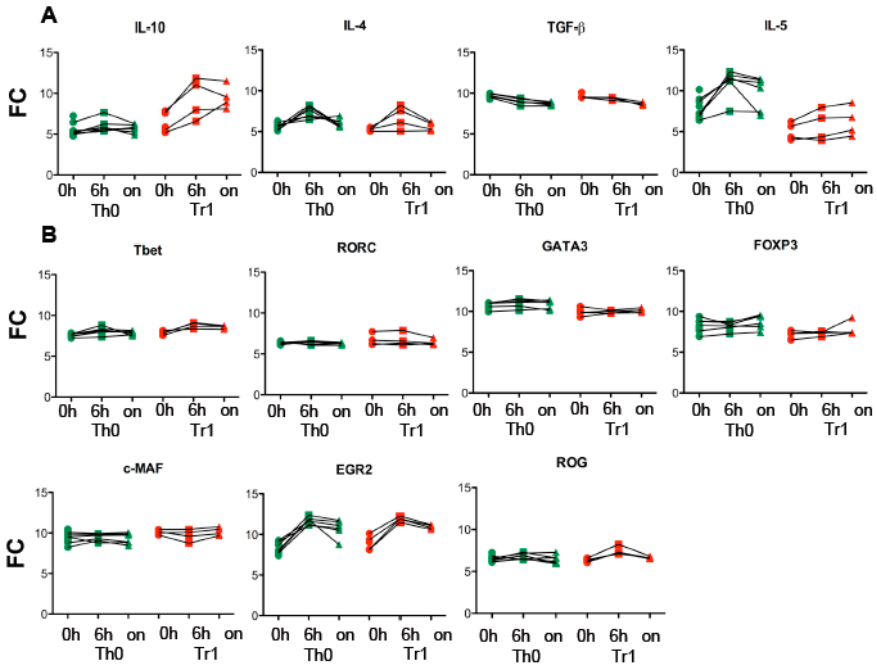

Figure 4. Cytokine profile and expression of transcription factors of Tr1 and Th0 cell clones. Tr1 and Th0 cell clones, isolated from PB of two HD using limiting dilution and polyclonal stimulation, were left unstimulated (t0) or, alternatively, stimulated (6h and on) with immobilized anti-CD3 and soluble anti-CD28 mAb. (A) Cytokine profile as analyzed by the DNA microarrays analysis carried out in Tr1 and Th0 cell clones. Numbers represent arbitrary units. (C) Expression of transcription factors as analyzed by the DNA microarrays analysis carried out in Tr1 and Th0 cell clones. Numbers represent arbitrary units. FC = Fold change

Tr1-associated markers GZB[20, 26] and PD-1[14] were significantly up-regulated in Tr1 cell clones (Figure 5), whereas the transcriptional repressor of GATA-3 (ROG)[18], identified as specifically expressed by mouse Tr1 cell clones, was not (Figure 4B). Finally, as previously

published[5], upon TCR stimulation, Tr1 cell clones up-regulated mRNA levels of activation markers similar to Th0 cell clones (not shown).

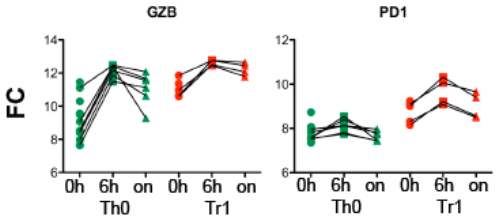

Figure 5. Expression of markers of Tr1 and Th0 cell clones. Tr1 and Th0 cell clones, isolated from PB of two HD using limiting dilution and polyclonal stimulation, were left unstimulated (t0) or, alternatively, stimulated (6h and on) with immobilized anti-CD3 and soluble anti-CD28 mAb. Expression of Tr1 cell markers as analyzed by the DNA microarrays analysis carried out in Tr1 and Th0 cell clones. Numbers represent arbitrary units. FC = Fold change

Data analysis

Data were normalized using the standard RMA normalization protocol, and analysis were performed with the t test welch without the FDR correction that might lead to false negative results. Only genes with P-values < 0.05 and a minimum two fold difference (log2FC > 1) were considered significantly regulated.

Th0 cell clones RNA was used as common reference RNA, i.e. gene expression levels in Tr1 cell clones were related to the levels detected in Th0 cell clones. Therefore, genes were considered up-regulated when their average expression in Tr1 cell clones was increased

compared with the average expression in Th0 cell clones and down-regulated when their expression was decreased. The differentially expressed genes (DEG) are classified in functional families using KEGG (Kyoto Enciclopedia of Genes and Genome) and Gene Ontology databases. We selected the most interesting genes using basically two criteria: 1. the top 10 ranked probes according to the p-value, 2. probes selected according to multiparametric analysis consisting in p-value, Log2FC, consistency among donors, kinetic of expression, protein localization and functions.

Our experimental design allows two main different possible and significant analyses:

- Horizontal analysis in which Tr1 and Th0 cell population are compared at the three different activation status and DEG genes are selected when are differentially expressed in all the three time points. Our goal in the application of this analysis was to identify specific markers and putative master regulators differentially expressed between the two population, regardless the activation status, and to define the Tr1 cell signature.
- Vertical analysis to reveal changes during activation. Tr1 and Th0 cell populations are compared at two time points following TCR activation and DEG genes are selected when are differentially expressed in all the two time points. This comparison would allow the selection of genes that may be involved in the effector phase, such as molecules with putative effector functions and master regulators expressed after activation.

We retained some surface or intracellular molecules that were significantly differentially expressed especially in the resting T cell clones that may be specific markers or functional molecules in the steady state.

Up- and down- regulated genes in Tr1 cells

In unstimulated clones, 63 genes (72 probes) were found to be up-regulated, and 51 genes (52 probes) were found to be down-regulated in Tr1 cell clones compared to Th0 cell clones. Upon TCR activation, 228 genes (249 probes) were found to be up-regulated and 74 genes (102 probes) down-regulated at 6 hours and 151 genes (163 probes) up-regulated and 93 genes (98 probes) down-regulated on in Tr1 compared to Th0 cell clones (Figure 6A). A wide variety of genes classified concerning the cellular component are represented, including membrane receptors, secreted extracellular proteins, nuclear, lysosomal, and cytoplasmic molecules, and proteins with unknown localization (Figure 6B).

Comparison of intersection of the three time points revealed an of 63 gene probesets differentially expressed, with 40 probes up-regulated (Figure 6C) and 23 probes down-regulated (Figure 6D) in Tr1 compared to Th0 cell clones (Figure 6C). A detailed description of these genes is summarized in Table 2A and visualized as heatmap in Figure 7 and 8. Upon TCR stimulation in Tr1 compared with Th0 cell clones, a set union of only 87 probes were significantly differentially expressed at both 6h and on, but not in resting state, with 66 probes up-regulated (Figure 6C) and 21 probes down-regulated (Figure 6D, Table 2B, Figure 7 and 8).

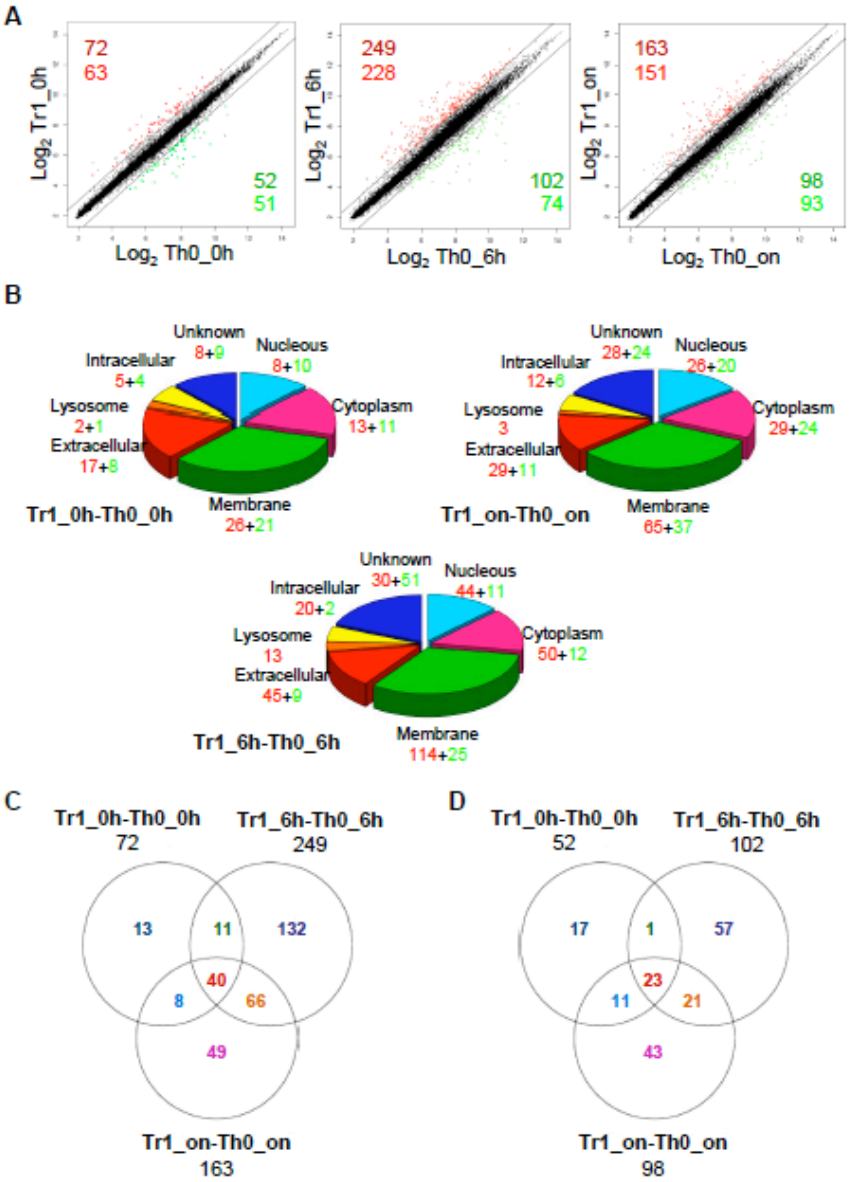
A
72
63
52
51
Log2 Tr1_0h
Log2 Th0_0h
249
228
102
74
Log2 Tr1_6h
Log2 Th0_6h
163
151
98
93
Log2 Tr1_on
Log2 Th0_on
B
Unknown 8+9
Nucleous 8+10
Intracellular 5+4
Lysosome 2+1
Extracellular 17+8
Cytoplasm 13+11
Membrane 26+21
Tr1_0h-Th0_0h
Unknown 28+24
Nucleous 26+20
Intracellular 12+6
Lysosome 3
Extracellular 29+11
Cytoplasm 29+24
Membrane 65+37
Tr1_on-Th0_on
Unknown 30+51
Nucleous 44+11
Intracellular 20+2
Lysosome 13
Extracellular 45+9
Cytoplasm 50+12
Membrane 114+25
Tr1_6h-Th0_6h
C
Tr1_0h-Th0_0h
72
Tr1_6h-Th0_6h
249
13
11
132
40
8
66
49
Tr1_on-Th0_on
163
D
Tr1_0h-Th0_0h
52
Tr1_6h-Th0_6h
102
17
1
57
23
11
21
43
Tr1_on-Th0_on
98

Figure 6. Micoarray comparison of Tr1 and Th0 cell clones. Tr1 and Th0 cell clones, isolated from PB of two HD using limiting dilution and polyclonal stimulation, were left unstimulated (t0) or, alternatively, stimulated (6h and on) with immobilized anti-CD3 and soluble anti-CD28 mAb. **(**A) Normalized expression values for profiles directly comparing Tr1 versus Th0 cell clones at t0 (left), 6h (centre) and on (right) after stimulation. The diagonal line indicates equal expression in conditions on both the vertical and horizontal axes. Dark numbers indicate the number of probes, whereas light numbers indicate the number of genes whose expression differed by more or less than twofold. In the top left quadrants (points above the diagonal lines), probes up-regulated in Tr1 cell clones are represented as red dots. In the bottom right quadrants (points below the diagonal lines), probes down-regulated in Tr1 cell clones are represented as green dots. (B) Pie charts represents the distribution of the DEG in Tr1 versus Th0 cell clones at t0 (left), 6h (centre) and on (right) after stimulation, concerning the protein localization from the cellular component information of the Gene Ontology database http://www.geneontology.org/. Red numbers represents the number of probes up-regulated in Tr1 versus Th0 cell clones, whereas green numbers represents the number of probes down-regulated. (C) and (D) Venn diagrams of microarray results for Tr1 cell clones at 0h, 6h, and on. Th0 cell clones RNA was used in all comparisons as a common reference. (C) Up-regulated genes: up-regulated in Tr1 compared with Th0 cell clones. (D) Down-regulated genes: down-regulated in Tr1 compared with Th0 cell clones. Dark numbers (upper) represent the number of probes, whereas light numbers (lower) represent the number of genes.

Table 2A. DEG between Tr1 and Th0 cell clones at t0, 6h, on

Category	Gene Title	Gene Symbol	PublicID
up	protein tyrosine phosphatase, non-receptor type 13	PTPN13	NM_080683
	RNA binding protein with multiple splicing	RBPMS	NM_001008711
	interferon, gamma	IFNG	NM_000619
	protein tyrosine phosphatase, receptor type, M	PTPRM	NM_001105244
	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	ITGA2	NM_002203
	CD86 molecule	CD86	NM_175862
	chemokine (C-C motif) receptor 1	CCR1	NM_001295
	glycine dehydrogenase (decarboxylating)	GLDC	NM_000170
	V-set and transmembrane domain containing 3	VSTM3	BC101291
	STON1-GTF2A1L	STON1-GTF2A1L	NM_172311
	coagulation factor II (thrombin) receptor	F2R	NM_001992
	sprouty homolog 1, antagonist of FGF signaling (Drosophila)	SPRY1	NM_005841
	T-cell lymphoma invasion and metastasis 1	TIAM1	NM_003253
	CDC42 effector protein (Rho GTPase binding) 3	CDC42EP3	NM_006449
	KIAA0888 protein	KIAA0888	NM_015566
	protein S (alpha)	PROS1	NM_000313
	protein S (alpha)	PROS1	NM_000313
	carcinoembryonic antigen-related cell adhesion molecule 1	CEACAM1	NM_001712
	chromosome 4 open reading frame 26	C4orf26	NM_178497
	amyloid beta precursor protein-binding, family A, member 1	APBA1	NM_001163
	low density lipoprotein-related protein 12	LRP12	NM_013437
	stomatin	STOM	NM_004099
	death-associated protein kinase 2	DAPK2	NM_014326
	NA	NA	NA
	SET binding protein 1	SETBP1	NM_015559
	plexin B2	PLXNB2	NM_012401
	spermine oxidase	SMOX	NM_175839
	sphingosine-1-phosphate phosphotase 2	SGPP2	NM_152386
	SHC (Src homology 2 domain containing) family, member 4	SHC4	NM_203349
	cytochrome b-561	CYB561	NM_001915
	coiled-coil domain containing 50	CCDC50	NM_178335
	DCN1, defective in cullin neddylation 1, domain containing 3	DCUN1D3	NM_173475
	vitamin D (1,25- dihydroxyvitamin D3) receptor	VDR	NM_001017535
	trichorhinophalangeal syndrome I	TRPS1	NM_014112
	CD226 molecule	CD226	NM_006566
	parathymosin	PTMS	NM_002824
	family with sequence similarity 3, member C	FAM3C	NM_014888
	programmed cell death 1	PDCD1	NM_005018
	family with sequence similarity 3, member C	FAM3C	NM_014888
	LATS, large tumor suppressor, homolog 2 (Drosophila)	LATS2	NM_014572
down	prostaglandin D2 synthase, hematopoietic	PGDS	NM_014485
	chemokine (C-C motif) receptor 4	CCR4	NM_005508
	NEL-like 2 (chicken)	NELL2	NM_006159
	interleukin 5 (colony-stimulating factor, eosinophil)	IL5	NM_000879
	ectonucleotide pyrophosphatase/phosphodiesterase 3	ENPP3	NM_005021
	formin-like 2	FMNL2	NM_052905
	phospholipase A2, group IVA (cytosolic, calcium-dependent)	PLA2G4A	NM_024420
	prostaglandin-endoperoxide synthase 2	PTGS2	NM_000963
	interleukin 17 receptor B	IL17RB	AF208111
	arginase, type II	ARG2	NM_001172
	titin	TTN	NM_133378
	coiled-coil domain containing 141	CCDC141	NM_173648
	ferric-chelate reductase 1	FRRS1	BC029438
	aldo-keto reductase family 1, member C3	AKR1C3	NM_003739
	choline dehydrogenase	CHDH	NM_018397
	SLAM family member 7	SLAMF7	NM_021181
	chromosome 6 open reading frame 192	C6orf192	NM_052831
	cysteinyl leukotriene receptor 2	CYSLTR2	AK291739
	family with sequence similarity 102, member A	FAM102A	NM_001035254
	RNA binding motif, single stranded interacting protein	RBMS3	NM_001003793
	connector enhancer of kinase suppressor of Ras 2	CNKSR2	NM_014927
	PTPRF interacting protein, binding protein 1 (liprin beta 1)	PPFIBP1	NM_003622
	RAB6 interacting protein 1	RAB6IP1	NM_015213

Table 2B. DEG between Tr1 and Th0 cell clones at 6h, on

Category	Gene Title	Gene Symbol	PublicID
up	interleukin 21	IL21	NM_021803
	plastin 3 (T isoform)	PLS3	NM_005032
	solute carrier family 26, member 4	SLC26A4	NM_000441
	SH2 domain containing 1B	SH2D1B	NM_053282
	interleukin 8	IL8	NM_000584
	5'-nucleotidase, ecto (CD73)	NT5E	NM_002526
	granulysin	GNLY	NM_012483
	hydroxyprostaglandin dehydrogenase 15-(NAD)	HPGD	NM_000860
	A kinase (PRKA) anchor protein 5	AKAP5	M90359
	protease, serine, 23	PRSS23	NM_007173
	fer-1-like 3, myoferlin (C. elegans)	FER1L3	NM_013451
	NA	NA	ENST00000376495
	interleukin-1 receptor-associated kinase 2	IRAK2	NM_001570
	cyclin A1	CCNA1	NM_003914
	polo-like kinase 2 (Drosophila)	PLK2	NM_006622
	CD244 molecule, natural killer cell receptor 2B4	CD244	NM_016382
	tumor necrosis factor (ligand) superfamily, member 9	TNFSF9	NM_003811
	G protein-coupled receptor 56	GPR56	NM_201524
	adenosine A2a receptor	ADORA2A	NM_000675
	interleukin 13 receptor, alpha 1	IL13RA1	NM_001560
	SMAD family member 1	SMAD1	NM_005900
	phospholipase D1, phosphatidylcholine-specific	PLD1	NM_002662
	myosin, heavy chain 10, non-muscle	MYH10	NM_005964
	sortilin 1	SORT1	NM_002959
	ubiquitin associated and SH3 domain containing, B	UBASH3B	NM_032873
	F-box protein 32	FBXO32	NM_058229
	lymphocyte-activation gene 3	LAG3	NM_002286
	neurocalcin delta (NCALD), transcript variant 1, mRNA	NCALD	ENST00000311028
	hypothetical protein DKFZp667G2110	DKFZp667G2110	NM_153605
	endoplasmic reticulum metallopeptidase 1	ERMP1	NM_024896
	golgi membrane protein 1	GOLM1	NM_016548
	interleukin 1 receptor accessory protein	IL1RAP	NM_002182
	purinergic receptor P2Y, G-protein coupled, 8	P2RY8	NM_178129
	purinergic receptor P2Y, G-protein coupled, 8	P2RY8	NM_178129
	calcium/calmodulin-dependent protein kinase ID	CAMK1D	NM_153498
	neuropilin (NRP) and tolloid (TLL)-like 2	NETO2	NM_018092
	NA	NA	ENST00000372922
	immunoglobulin-like domain containing receptor 1	ILDR1	NM_175924
	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	NM_078467
	TNF receptor-associated factor 5	TRAF5	NM_145759
	hypothetical protein FLJ11151	FLJ11151	NM_018340
	frizzled homolog 6 (Drosophila)	FZD6	NM_003506
	cellular repressor of E1A-stimulated genes 1	CREG1	NM_003851
	fibroblast growth factor binding protein 2	FGFBP2	NM_031950
	TBC1 domain family, member 4	TBC1D4	NM_014832
	tight junction protein 2 (zona occludens 2)	TJP2	NM_004817
	tyrosylprotein sulfotransferase 2	TPST2	NM_003595
	pleckstrin homology domain containing, family A member 5	PLEKHA5	NM_019012
	transcriptional regulating factor 1	TRERF1	NM_033502
	colony stimulating factor 1 (macrophage)	CSF1	NM_000757
	receptor tyrosine kinase-like orphan receptor 2	ROR2	NM_004560
	ankyrin repeat domain 10	ANKRD10	NM_017664
	ets variant gene 7 (TEL2 oncogene)	ETV7	NM_016135
	chromosome 9 open reading frame 95	C9orf95	NM_017881
	ectodysplasin A2 receptor	EDA2R	NM_021783
	anthrax toxin receptor 2	ANTXR2	NM_058172
	metaxin 3	MTX3	NM_001010891
	osteoclast stimulating factor 1	OSTF1	BC007459
	prostaglandin F2 receptor negative regulator	PTGFRN	NM_020440
	TIMP metallopeptidase inhibitor 1	TIMP1	NM_003254
	potassium calcium-activated channel, subfamily N, member 4	KCNN4	NM_002250
	cathepsin B	CTSB	NM_147780
	HOP homeobox	HOPX	NM_032495

Table 2B. Continued

Category	Gene Title	Gene Symbol	PublicID
up	GPRIN family member 3	GPRIN3	NM_198281
	cortactin	CTTN	NM_005231
	phosphatase and tensin homolog	PTEN	NM_000314
down	dispatched homolog 2 (Drosophila)	DISP2	AL359580
	interleukin 7 receptor	IL7R	NM_002185
	sestrin 3	SESN3	NM_144665
	NIMA (never in mitosis gene a)-related kinase 6	NEK6	NM_014397
	purinergic receptor P2Y, G-protein coupled, 5	P2RY5	BC045651
	killer cell lectin-like receptor subfamily G, member 1	KLRG1	NM_005810
	G protein-coupled receptor 64	GPR64	NM_001079858
	death-associated protein kinase 1	DAPK1	NM_004938
	family with sequence similarity 105, member A	FAM105A	NM_019018
	NA	NA	DQ658414
	NLR family, pyrin domain containing 2	NLRP2	NM_017852
	protein tyrosine phosphatase type IVA, member 3	PTP4A3	NM_032611
	similar to cyclin I	FLJ16793	NM_001039780
	dehydrogenase/reductase (SDR family) member 3	DHRS3	NM_004753
	FLJ45983 protein	FLJ45983	NM_207423
	lysosomal associated protein transmembrane 4 beta	LAPTM4B	NM_018407
	chromodomain helicase DNA binding protein 7	CHD7	NM_017780
	calsequestrin 1 (fast-twitch, skeletal muscle)	CASQ1	NM_001231
	hypothetical protein FLJ38359	FLJ38359	AK095678
	male sterility domain containing 1	MLSTD1	NM_018099
	NAD(P)H dehydrogenase, quinone 1	NQO1	NM_000903

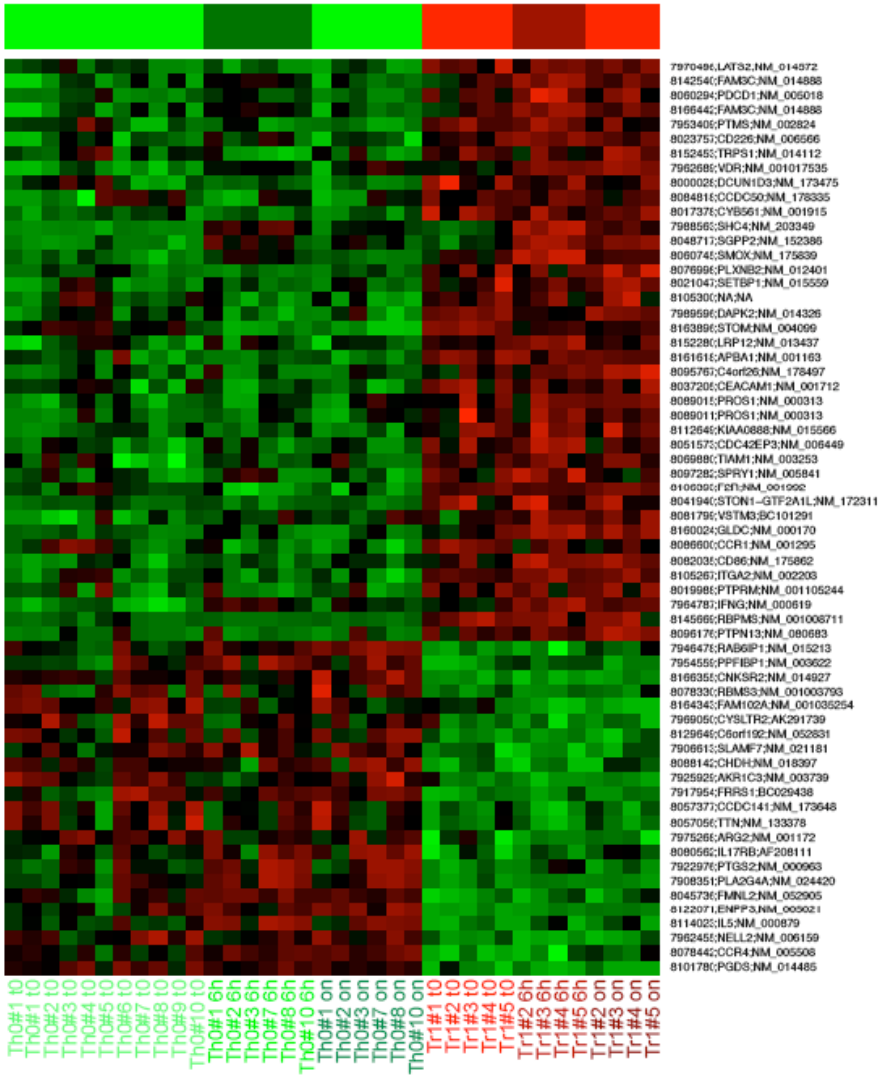

Figure 7. DEG between Tr1 and Th0 cell clones at t0, 6h, on.

Tr1 and Th0 cell clones, isolated from PB of two HD using limiting dilution and polyclonal stimulation, were left unstimulated (t0) or, alternatively, stimulated (6h and on) with immobilized anti-CD3 and soluble anti-CD28 mAb. Differential expression of 28869 genes was investigated by whole transcript Affymetric chips. Following data normalization by standard RMA protocol and statistical analysis (t test welch without the FDR correction), Tr1 and Th0 cell population are compared at the three different activation status and DEG genes are selected when are differentially expressed in all the three time points. Two-dimensional hierarchical

clustering analysis yielded the displayed transcriptional pattern, which consists of 40 probes up-regulated and 23 probes down-regulated in Tr1 compared to Th0 cell clones. Each row represents a gene probe, whereas each column a sample. Red indicates genes that are expressed at higher levels compared with the mean signal intensities of all experiments, whereas down-regulated genes are coloured in green and black indicates signal intensities near the mean expression level. The rows are scaled to have mean zero and standard deviation one.

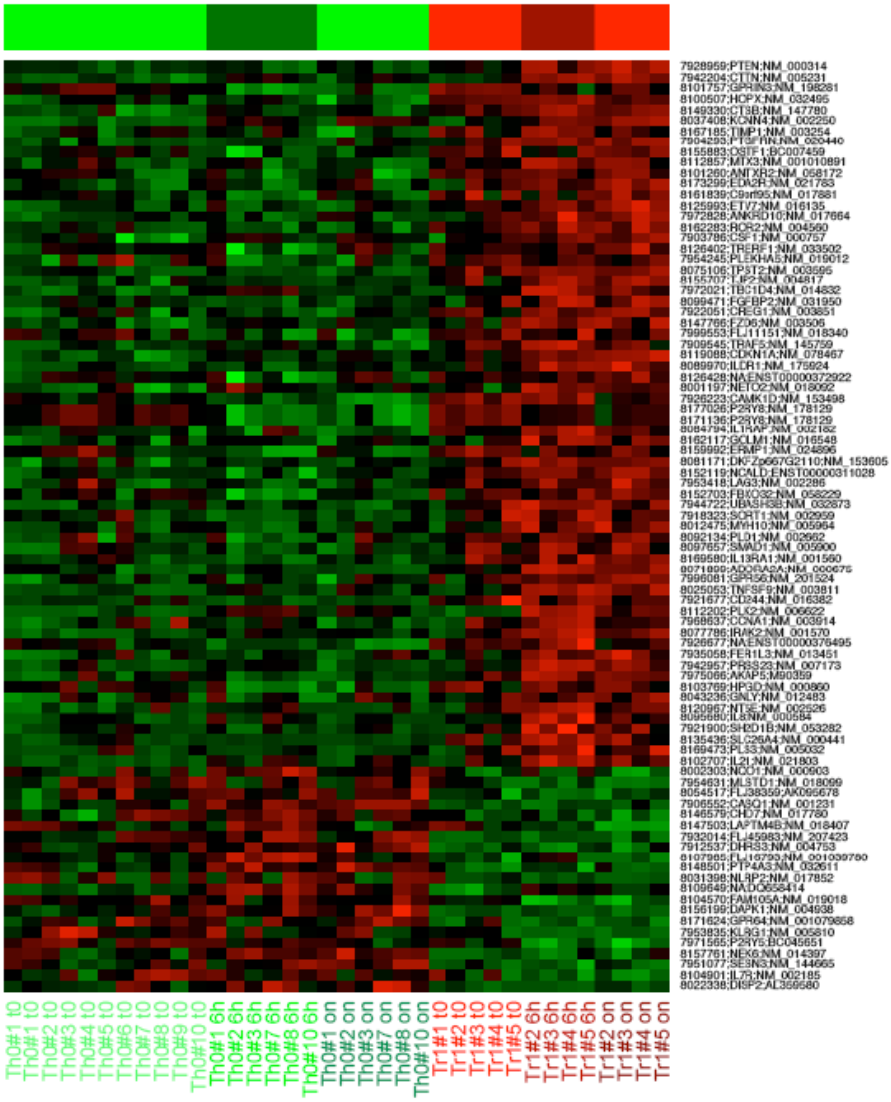

Figure 8. DEG between Tr1 and Th0 cell clones at t0, 6h, on.

Tr1 and Th0 cell clones, isolated from PB of two HD using limiting dilution and polyclonal stimulation, were left unstimulated (t0) or, alternatively, stimulated (6h and on) with immobilized anti-CD3 and soluble anti-CD28 mAb. Differential expression of 28869 genes was investigated by whole transcript Affymetric chips. Following data normalization by standard RMA protocol and statistical analysis (t test welch without the FDR correction), Tr1 and Th0 cell populations are compared at two time points following TCR activation and DEG genes are selected when are differentially expressed in all the two time points. Two-dimensional hierarchical clustering analysis yielded the displayed transcriptional pattern, which consists of 66 probes up-regulated and 21 probes down-regulated in Tr1 compared to Th0 cell clones. Each row represents a gene probe, whereas each column a sample. Red indicates genes that are expressed at higher levels compared with the mean signal intensities of all experiments, whereas down-regulated genes are coloured in green and black indicates signal intensities near the mean expression level. The rows are scaled to have mean zero and standard deviation one.

To validate the accuracy of our microarray data in more detail, real-time RT-PCR (reverse transcription polymerase chain reaction) was performed using the original samples. Additional two Tr1 and two Th0 cell clones *ex vivo* isolated from a third HD were added to RT-PCR analysis, in order to confirm the results observed in the first two HD (Table 3).

Table 3. Cytokine profile of T cell clones[a)]

		IL-2 pg/ml	IL-4 pg/ml	IL-10 pg/ml	IFN-g pg/ml	IL-17 pg/ml
HD_C	**Tr1#6**	59	49	1140	539	< 30
HD_C	**Tr1#7**	741	12	3218	3643	61
HD_C	**Th0#11**	1198	2644	140	300	< 30
HD_C	**Th0#12**	10	3152	334	510	< 30

a) Tr1 and Th0 cell clones were isolated from peripheral blood the HD_C using limiting dilution and polyclonal stimulation. T cell clones were stimulated with immobilized anti-CD3 mAb and soluble anti-CD28 mAb. Culture supernatants were collected after 24 h (IL-2) and 48 h (IL-4, IL-10, IFN-γ, IL-17) and levels of cytokines were measured by ELISA.

We selected 5 of the DEG (*CD49b*, *CD226*, *RBPMS, STOMATIN, MGAT5*) and confirmed their Tr1 cell specific expression by quantitative real-time RT-PCR (Figure 9). Real time RT-PCR results correlated with the differential gene expression data obtained by microarray. Variability in gene expression was observed between RT-PCR data of the samples from the HD_C and the samples from HD_A and _B, but the up-regulation in Tr1 cell clones was consistent, lending confidence to the reliability of the microarray results.

Overall, these data provided greater credence and reliability to the numerous additional genes that we found differentially expressed by the microarray analysis.

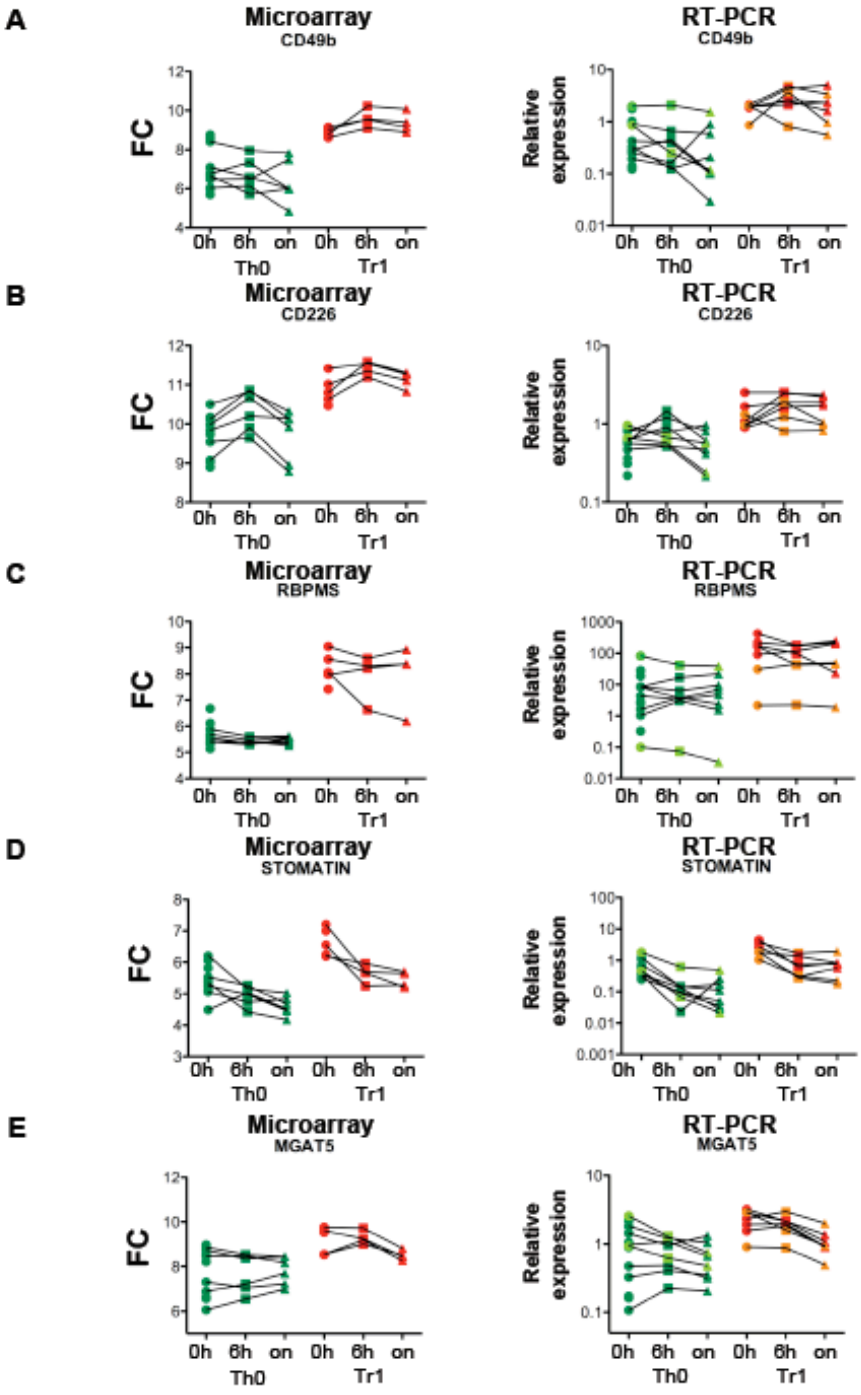
A
Microarray
CD49b
FC
RT-PCR
CD49b
Relative expression
B
Microarray
CD226
FC
RT-PCR
CD226
Relative expression
C
Microarray
RBPMS
FC
RT-PCR
RBPMS
Relative expression
D
Microarray
STOMATIN
FC
RT-PCR
STOMATIN
Relative expression
E
Microarray
MGAT5
FC
RT-PCR
MGAT5
Relative expression
0h 6h on 0h 6h on
Th0 Tr1

Figure 9. Confirmation of microarray results by RT-PCR.

Real-time RT-PCR was performed for (A) *CD49b*, (B) *CD226*, (C) *RBPMS,* (D) *STOMATIN* (E) *MGAT5* expression in the original samples form Tr1 (red) and Th0 (green) cell clones and additional two Tr1 (orange) and two Th0 (light green) cell clones *ex vivo* isolated from a third HD. Following normalization to HPRT and B2M, relative mRNA amounts of T cell clones were adjusted to corresponding expression levels in of a calibrator (pool of $CD4^+$ T cell lines from 4 distint HD). Real-time RT-PCR results (right) were compared with fold changes arising from the microarray (left). Numbers represent arbitrary units. FC = Fold change

Selection of candidate genes

According to the criteria described aboved, we selected genes encoding for surface molecules with the final goal to identified marker or a set of markers that can discriminate Tr1 cells from other T cells including helper and regulatory T (Treg) cells. In addition, we selected genes encoding for transcription factors with the aim to identify the putative master regulator of Tr1 cells. Among genes that we selected, many were surface proteins, or were related to anti-proliferative responses, anti-inflammatory responses, and immuno-modulation. In addition, some unknown or not associated with T cell transcriptional profile were selected.

Putative surface specific markers

Among genes encoding for surface molecules, we identified 9 surface and 1 intracellular genes that can be used to distinguish Tr1 cell subset from all other T cells (Table 4).

Table 4. Putative surface markers

Gene	*0 h*	*16 h*	*on*	*Molecular Function*
CD49b	↑	↑	↑	α2 integrin; cell adhesion to collagen
CD29	↑	-	-	β1 integrin; cell adhesion to collagen and laminin
CD161	↑	-	-	Receptor of LLT1; Th17 cell marker
CD203c	↓	↓	↓	hydrolysis of extracellular nucleotides
CCR4	↓	↓	↓	Receptor of MIP-1, RANTES, MCP-1
CCR1	↑	↑	↑	Receptor of MIP-1α, RANTES, MCP-3, MPIF-1
CD226	↑	↑	↑	Receptor of CD155 or CD112
LAG3	-	↑	↑	MHC-class-II-binding CD4 homolog
CD127	-	↓	↓	IL7Rα
GZB	↑	-	-	serine esterase/mediates cytotoxicity

CD49b in conjuntion with CD18 has been proposed to be a marker of IL-10-producing $CD4^+$[15]. The expression profile observed here confirm these observation, since expression of CD49b (ITGA2), the α integrin subunit of the very-late-activation antigen (VLA) -2 integrin, was increased in both resting and activated Tr1 cells clones compared to Th0 cell clones. Interestingly, the expression of integrin β1 subunit

of VLA-2, CD29 (ITGB1), resulted also increased in resting Tr1 cell clones, suggesting that VLA-2 may represent a marker of Tr1 cells.
Additional interesting surface molecules were differentially expressed between Tr1 and Th0 cell clones. Specifically, CD226 (DNAX Accessory Molecule-1, DNAM-1), a glycoprotein expressed on NK and a subset of T cells involed in cellular adhesion and specific killing of DC bearing its ligands, CD112 and CD155[27, 28], was higher expressed in resting and activated Tr1 cell clones compared to Th0 cell clones. Converselly, CD203c (ENPP3), the ectonucleotide pyrophosphatase/phosphodiesterase 3 that is involved in hydrolysis of extracellular nucleotide and is a marker of basophils, was lower expressed in resting and activated Tr1 cell clones compared to Th0 cell clones.

Overall expression of chemokines and chemokines receptors was similar between Tr1 and Th0 cells, since few differences were observed. CCR4, known to be expressed by Th2, Th17 and Treg cells[29, 30] was lower express by Tr1 cell clones, whereas CCR1, expressed by a broad spectrum of leukocytes, including neutrophils, monocytes, eosinophils, and lymphocytes[31], was up-regulated.
Interestingly, CD161 (KLRB1), a NK cell receptor recently identified as a specific markers of Th17 cells[32], was up-regulated in resting Tr1 cell clones, indicating a possible correlation between Th17 and Tr1 cells. As already mentioned, expression of GZB was significantly higher in resting Tr1 compared to Th0 cell clones, confirming previous observations [20, 33](Magnani CF., submitted). Finally, lymphocyte-activation gene 3 (LAG3), an MHC-class-II-binding CD4

homolog, marker of murine IL-10-producing Treg[34], was expressed at higher levels in activated Tr1 cells compared to Th0 cell clones, suggesting that its expression might be modulated by IL-10

The expression of CD49b, CD161, CD29, and CD226 was evaluated by flow cytometry (Figure 10). Around 30% of Tr1 cell clones expressed CD49b whereas only very few Th0 cell clones displayed it on their cell surface (on average 30.1% of $CD4^+CD49b^+$ cells in Tr1 cell clones compared to 6.7% in Th0 cell clones, p=0.039, Figure 10). Similar results were found for CD161 (on average 40.8% of $CD4^+CD161^+$ cells in Tr1 cell clones compared to 7.2% in Th0 cell clones, p=0.004, Figure 10).

Although both Tr1 and Th0 cell clones expressed CD29 and CD226, their expression resulted significantly higher in Tr1 cell clones compared to Th0 cell clones as demonstrated by the mean fluorescent intensity (MFI, on average 3705 of $CD4^+CD29^+$ cells in Tr1 cell clones compared to 2372 in Th0 cell clones, p=0.04, and on average 1283 of $CD4^+CD226^+$ cells in Tr1 cell clones compared to 875 in Th0 cell clones, p=0.01, Figure 10).

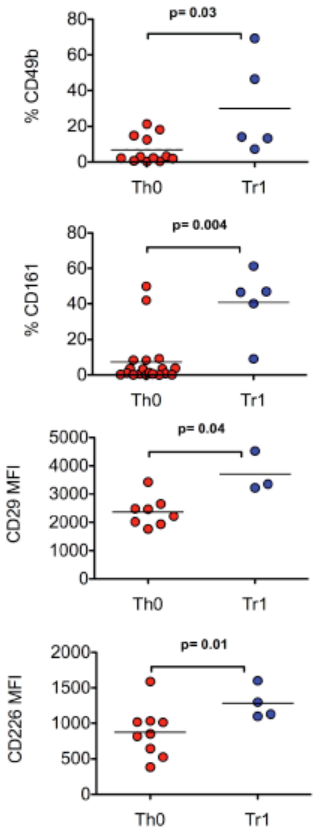

Figure 10. Tr1 cell clones have an increased protein expression of the surface molecules CD49b, CD29, CD226, and CD161. Tr1 and Th0 cell clones were *ex vivo* isolated from PB of three HDs using limiting dilution and polyclonal stimulation. T cell clones were analyzed for surface expression of CD49b, CD29, CD226, and CD161. Percentages in 5 Tr1 (blue dots) and 12 Th0 (red dots) cell clones are shown for CD49b, in 5 Tr1 (blue dots) and 19 Th0 (red dots) cell clones for CD161. Mean of the mean fluorescent intensity (MFI) in 3 Tr1 (blue dots) and 8 Th0 (red dots) cell clones is shown for CD29, in 4 Tr1 (blue dots) and 9 Th0 (red dots) cell clones for CD226. p = p-value

Genes associated with anti-proliferative response

Tr1 cells proliferate poorly upon TCR-mediated stimulation, and are anergic[4, 5]. As expected, much of the changes in genes associated with cell cycle, cell growth and anergy occurred in Tr1 cells upon TCR activation (Table 5).

Table 5. Genes associated with cell signaling, cell cycle and TCR pathway

Gene	*0h*	*16 h*	*on*	*Molecular Function*
CDC42EP3	↑	↑	↑	negatively regulates CDC42 and TC10
CNKSR2	↓	↓	↓	positive regulator of Ras signaling
RASGRF2	↓	-	-	activates Ras
P21	-	↑	↑	blocks G1/S progression; associated with anergy
PTEN	-	↑	↑	blocks AKT/PKB pathway
CREG-1	-	↑	↑	transcriptional corepressor; may control cell growth and differentiation
NEK6	-	↓	↓	kinase required for mitotic progression
LATS2	↑	↑	↑	blocks G2/M and G1/S
STS-1	-	↑	↑	phosphatase; negatively regulates TCR signaling
MGAT5	↑	↑	-	negatively regulates TCR by increasing GlcNAc branching

The expression of proteins that positively regulate Ras signaling, including Connector enhancer of kinase suppressor of Ras 2 (CNKSR2) and Ras protein-specific guanine nucleotide-releasing factor 2 (RASGRF2), was down-regulated in Tr1 cell clones.

Conversely, CDC42 effector protein 3 (CDC42EP3), that negatively regulates the Ras homologs CDC42 and TC10, and phosphatase and tensin homolog (PTEN) that blocks AKT/PKB pathway and is associated to anergy, were up-regulated in Tr1 cells.

In addition, we observed an up-regulation of the expression of genes associated to inhibition of the cell cycle and cell growth, such as the cyclin-dependent kinase inhibitor activity 1 A (CDKN1A), that encodes the cell cycle inhibitor p21/Cip1 and is associated to anergy, large tumor suppressor homolog 2 (LATS2) that encodes a serine/threonine protein kinase required for the transition from the G2 to the mitotic phase, and the cellular repressor of E1A-stimulated genes (CREG-1) that has been suggested to contribute to the transcriptional control of cell growth and differentiation. In contrast, the expression of RNA of nucleus serine/threonine kinase 6 (NEK6), that control initiation of mitosis and is required for mitotic progression, was down-regulated in activated Tr1 cell clones compared to Th0 cell clones.

Tr1 cell clones also expressed high levels of mRNA encoding for molecules that regulates TCR activation, such as UBASH3B that encodes for the suppressor of T-cell receptor signaling 1 (STS-1) and the α-1,6-mannosylglycoprotein 6-β-N-acetylglucosaminyltransferase (MGAT5).

Genes encoding for anti-inflammatory response

Many changes in our microarray data was related to molecules involved in the inflammatory process and to cytokines (Table 6). This was unexpected but is in line with the immunoregulatory activity of

Tr1 cells. Interestingly, the expression of genes associated to the arachidonic acid pathway, such as PLA2G4A (phospholipase A2, group IVA) and PTGS2 (prostaglandin-endoperoxide synthase 2) also known as COX2, was down-regulated in resting and activated Tr1 cell clones. Upon activation Tr1 cell clones expressed higher levels of mRNA encoding for genes associated with IL-1R signaling pathway: IRAK2 (IL-1 receptor associated kinase 2) and IL1RAP (IL-1 receptor accessory protein).

Table 6. Genes associated with inflammation and cytokines

Gene	*0h*	*16 h*	*o n*	*Molecular Function*
PLA2G4 A	↓	↓	↓	Phospholipase A2; hydrolysis of membrane phospholipid to arachidonic acid
PTGS2/ COX2	↓	↓	↓	Prostaglandin. Endoperoxidase synthase 2; from arachidonic acid to prostanoids
IRAK2	-	↑	↑	IL-1R associated kinase 2
IL1RAP	-	↑	↑	IL-1R accessory protein; mediated IL-1 dependent activation of NFkB/decoy receptor
F2R/PAR -1	↑	↑	↑	Activates signaling pathway (vav1, Lck, ZAP70) in T cells after ligation with thrombin
IL-8	-	↑	↑	Chemokine that attracts neutrophils, basophils, and T cells; binds to CXCR1 and CXCR2
IL-21	-	↑	↑	Regulates proliferation of B and T cells; promotes IL-10 production

F2R/PAR1 (coagulation factor II receptor) that activates signaling pathways in T cells after ligation of thrombin was also up-regulated in resting and activated Tr1 cell clones as compared to Th0 cell clones.

Finally, IL-8 and IL-21, that promotes IL-10 production and induces the differentiation of Tr1 cells[35], exhibited an increased expression in Tr1 cell clones followed activation. Finally, IL-8 and IL-21, that promotes IL-10 production and induces the differentiation of Tr1 cells[35], exhibited an increased expression in Tr1 cell clones followed activation.

Genes associated with Immuno-modulation

The expression of a number of genes encoding for proteins involved in immune response regulation were differentially expressed in Tr1 cells (Table 7). mRNA for Vitamin D Receptor (VDR) was up-regulated in resting and activated Tr1 cell clones, suggesting a role for vitamin D3 signaling not only in Tr1 cell induction[6, 36, 37] but also in the maintenance of their biological features and effector functions. In addition, mRNA for ectoenzymes CD39, CD73 and type 1 purinergic adenosine $A2_A$ receptor ($A2_AR$) were expressed at higher levels in activated Tr1 cell clones compared to Th0 cell clones. CD39 and CD73 are surface markers of Treg cells that impart a specific biochemical signature characterized by adenosine generation that has functional relevance for cellular immuno-regulation[38-40].

Genes related to the TGF-β/SMAD (mothers against decapentaplegic homologue) signaling pathway, including RBPMS (RNA-binding protein with multiple splicing), TGF-βR1, and TGF-βR2, were also highly expressed in Tr1 cell clones. RBPMS, that enhances Smad-dependent transcriptional activity in TGF-β-dependent manner[41], and is RNA-binding protein with putative role in post-transcriptional regulation, was significantly higher expressed in resting and activated

Tr1 cell clones as compared to Th0 cell clones.

mRNA for SMAD1 that mediates the signals of the bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, was up-regulated in activated Tr1 cells. BMP signalling pathway is involved in several biological activities including cell growth, apoptosis, morphogenesis, development, and immune responses[42]. It is therefore possible that effector functions mediated by Tr1 cells involved BMT-mediated signalling.

Finally, TNFSF9 (tumor necrosis factor (ligand) superfamily, member 9) that encodes CD137L, also known as 4-1BB-L, a costimulatory receptor molecule in T cells, was increased in Tr1 compared to Th0 cell clone upon activation.

Table 7. Genes associated with Immuno-modulation

Gene	*0h*	*16 h*	*o n*	*Molecular Function*
VDR	↑	↑	↑	controls immune response/role in Tr1 cell induction
CD73	-	↑	↑	catalyzes the conversion of AMP to adenosine
$A2_A$ R	-	↑	↑	receptor for adenosine; signaling promotes anergy
RBPMS	↑	↑	↑	SMAD-dependent signaling/post-transcriptional control
SMAD1	-	↑	↑	mediates signaling pathway (TGF-β superfamily pathway)
4-1BB-L	-	↑	↑	4-1BB ligand, a costimulatory receptor

Selection of genes ancoding for putative master regulators

Transcription factors are fundamental proteins for lineage specific differentiation pathways. They are able to influence the choice of the appropriate lineage by driving the expression of genes specific of the different cell types, also inhibiting inappropriate gene expression programs. Our analysis revealed two transcription factors (Table 8) which were significantly up-regulated in activated Tr1 cells: TRERF1, a zinc-finger transcription factor, that interacts with the CREB binding protein, CBP/p300 and regulates gene expression[43] and cell proliferation[44], and ETV7, known as TEL2, a member of the ETS family of transcription factors that play an important role in development and differentiation, and oncogenesis. TEL2 is expressed in hematopoietic tissues, associates with TEL1, a transcription factor required for the development of natural Treg cells[45], and has been suggested to be an important hematopoietic regulatory protein[46]. We plan to investigate the function of these transcription factors, together with the transcriptional regulator RBPMS, with the aim to identify factors that contribute to the regulation of Tr1 cell development and transcriptional program.

Table 8. Putative master regulators

Gene	*0h*	*16 h*	*o n*	*Molecular Function*
TRERF1	-	↑	↑	Transcriptional regulator of gene expression and cell proliferation
ETV7	-	↑	↑	ETS family; transcriptional regulator of development, differentiation, and oncogenesis

Discussion and future aims

In the present study, we used the microarray technology to define the signature of Tr1 cells and to identify specific surface markers, master regulators and novel molecules involved in the effector functions of Tr1 cells. The overall Tr1 cell signature is of anti-proliferation, anti-inflammation, and immuno-modulation. Our analysis reveals a number of surface proteins that can be used in combination to identify and isolate Tr1 cells *in vivo*. Moreover, genes encoding for protein that may represent master regulator of Tr1 cell differentiation were identified. Some genes were validated by RT-PCR, and we generally found a reasonable concordance between microarray expression data and PCR results. Thus, we are confident that the global analysis we performed is valid and that the expression data of the additional genes are reliable. Among the genes identified as up-regulated in Tr1 cells, we found the surface molecules CD49b, CD29, and CD226 that could be useful to isolate and follow Tr1 cell population and the enzyme MGAT5 that probably play a crucial role in the maintenance of the anergic status of Tr1 cells.

It is not surprising that Tr1 and Th0 cells display similar gene expression profiles, as they are both memory T cell subsets primed *in vivo*. Nevertheless, 114 genes were found to be consistently differentially expressed in Tr1 cells compared to Th0 cells, with 63 up-regulated and 51 down-regulated, suggest that the different signals driving specific polarization of the two cell subsets influence the transcription machinery, despite maintaining the global transcriptome of the memory lineage. Notably, the global gene expression profile of Tr1 and Th0 cells becomes different upon TCR triggering, indicating

that during an active immune response Tr1 cells display a distinctive phenotype.

Similar to Th cells that express specific transcription factors that control differentiation of Th1, Th2, Th17, such as Tbet, GATA3, and RORC[47, 48], Tr1 cells expressed the above mentioned transcription factors. Notably, our analysis did not reveal the selective expression of the transcription factor c-MAF, associated with IL-10 production in macrophages, Th17, Th1, and IL-27-induced Tr1 cells[35, 49-51], or of IKAROS[52], a zinc finger DNA-binding protein that was shown to regulate IL-10 in murine $CD4^+$ T cells, or of EGR-2, a transcription factor that plays a key role in anergy induction in T cells and controls IL-10 production in $CD4^+CD25^-LAG3^+$ Treg cells in mice[34, 41]. The fact that C-MAF, IKAROS, and EGR-2 are expressed at similar levels and kinetic in Tr1 compared to Th0 cells does not exclude the possibility that these transcription factors are involved in Tr1 cell development and function. Indeed activated Tr1 cells expressed increased levels of IL-21, whose expression was shown to be transactived by c-MAF and necessary for IL-10 production in IL-27-induced Tr1 cells [35].

One of the goals of this study was the identification of specific cell surface molecules to track Tr1 cells *in vivo*. Several genes that encode surface protein were identified and validated, including CD49b, CD226, CD29, and CD161. CD49b was already associated to IL-10-producing T cells, both in humans and mice[15, 16, 53]. Our data provide an additional demonstration that CD49b could be used as specific marker to identify Tr1 cells *in vivo*. Our results indicate that in addition to CD49b, additional markers, including CD45RO,

CD226, and CD29 should be used to identifying Tr1 cells. Surprising Tr1 cells expressed high levels of CD161, the Th17-cell specific marker[32]. It would be interesting to define whether CD161 is a marker shared by Th17 and Tr1 cells or if there is an overlap between the two subsets.

The patterns of genes expressed in Tr1 cells identifies a specific Tr1 cell signature that is consistent with the fact that Tr1 cells represent a specialized subset of Treg cells, since several differences were closely correlated with the distinct functional properties of Tr1. Notably, Tr1 cells differentially expressed genes related to anti-proliferative response, anti-inflammatory response, and immuno-modulation. The specific expression of the signaling molecules CNKSR2, RASGRF2, CDC42EP3, PTEN and of the cell cycle regulators p21/Cip1, LATS2, CREG-1, NEK6 is in line with the anergic status of Tr1 cells, dampening the responses to the signals that drive cell cycle progression and proliferation. Indeed, we recently showed that genes involved in signal transduction, cell cycle, cell division are significantly down-regulated in anergized cultures of MLR/10 and MLR/DC-10 containing Tr1 cells[54]. Similar cell cycle and cell division modulation was observed in murine IL-10/TGF-β anergized cultures[55]. In addition, it has been reported that the low proliferative response of IL-10-producing Tr1-like cells generated *in vitro* by CD3/CD46 cross-linking depended on the altered expression of different cell cycle-associated proteins, such as p27/Kip1, and a specific defective activation of Akt[56]. Mouse Treg induced by IL-10-modulated DC (IL-10-DC) have a elevated activation of p38 MAPK that promote the induction of p27/kip1[57]. We did not

observed a significant difference in the expression of the specific cyclin-dependent kinase (CDK) inhibitor p27/Kip1, but of p21/Cip1, suggesting that different mechanisms could be responsible of the same impairment in cell cycle progression seen in IL-10-DC Treg, Tr1-like, and Tr1 cell population. In line with the findings is the up-regulation of the expression of STS-1 and MGAT5, two proteins known to be key regulators of TCR activation. In particular, MGAT5 enzyme initiates GlcNAc β1,6 branching on N-glycans, thereby increasing N-acetyllactosamine, the ligand for galectins. MGAT5-modified N-glycans on the TCR complex bind to galectin-3, sequestering TCR within a multivalent galectin-glycoprotein lattice, resulting in a higher T-cell activation threshold. MGAT5-deficient mice showed kidney autoimmune disease, enhanced delayed-type hypersensitivity, and increased susceptibility to experimental autoimmune encephalomyelitis[58, 59]. The expression of MGAT5 in Tr1 cells is increased compared to Th0 already in resting state and at 6 hours after activation. Thus, induction of MGAT5 expression in Tr1 cells may be necessary to lower TCR signaling, promoting their anergic status.

Expression of genes associated with inflammation, such as PLA2G4A, PTGS2, encoding enzymes for the synthesis of inflammatory mediators of the arachidonic acid pathway, and IRAK2, IL1RAP, proteins associated to IL-1R signaling pathway, and of the coagulation factor II receptor (F2R/PAR1) were differentially expressed in Tr1 compared to Th0 cells. Both IL-1R and proteinase-activated receptors (PARs) can activate T-cell signaling pathway and, in particular, it was previously shown that IL-1β is required for development of Th17 cells in mice[60, 61] and humans[29], whereas tethered ligand-derived

peptides of PAR3 activate F2R/PAR1 and PAR2 in Jurkat T cells[62]. It is possible that Tr1 cells use these signaling pathways in order to sense the inflammatory environment. The impact of serine proteinases and IL-1 on Tr1 cell functions, in inflammatory milieu, merits further investigations.

Tr1 cell signature included genes encoding molecules associated with immuno-modulatory pathways, such as Vitamin D, TGF-β/SMAD, 4-1BB/4-1BB-L, and adenosine pathways. The role of these pathways in Tr1 cell function should be further investigated. Interestngly, the up-regulation of CD73 and $A2_A$ R observed in Tr1 cells suggests that the adenosine pathway may represent an additional mechanism of suppression mediated by Tr1 cells.

In conclusion, we have used an extensive analysis of gene expression profiles to identify genes expressed selectively in Tr1 cells. The gene expression data presented here may yield important insights into the functional activity of Tr1 cells, as well as their identification markers, and master regulators. In addition, the strategy used in this study clearly identifies a gene signature specific for Tr1 cells in the resting state and after activation.

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Chapter 4

FINAL DISCUSSION: MOLECULAR AND CLINICAL CONSIDERATIONS

Summary

The Ph.D. project in which I was engaged has being carry on at the San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET). The subject of the project focused on the genetic and molecular mechanisms underneath immunological tolerance mediated by regulatory T cells. In order to generate new advanced diagnostic and therapeutical applications in the field of transplantation, autoimmune diseases, allergy, chronic infection and tumours, our work is focus on the translation of basic and applied research into therapeutic advances. In particular, I worked on the characterization of $CD4^+$ Type 1 Regulatory T cells (Tr1), a peripherally induced subset of IL-10-producing regulatory T cells that suppress immune responses both in humans and in mice. The aim of my thesis was to elucidate the molecular mechanisms involved in the lytic function of Tr1 cells and to characterize their gene signature with the final goal to find novel genes whose expression is crucial for their phenotype and functions.

With this purpose we characterized the cytotoxic activity in human Tr1 cell lines differentiated *in vitro* from naïve $CD4^+$ T cell precursors and Tr1 cell clones isolated *ex vivo*, in terms of target specificity, cytotoxic molecules implicated in the mechanism of lysis, molecular mechanisms of recognition of the target, and of activation of Tr1 cells. We demonstrated that Tr1 cells express and release granzyme B (GZB) and that the expression of GZB is correlated to the level of IL-10 in culture. Tr1 cells specifically kill myeloid antigen presenting cells (APCs), whereas preserve an erythroid cell line and lymphocytes, such as B and T cells. The mechanism of killing is dependent on the lytic molecules of the granule pathway GZB and

perforin (PRF), is Ag-nonspecific, and needs the recognition of HLA class I on target cells. The myeloid APC specificity of Tr1 cells depends on the formation of a stable and prolong interaction between APC and Tr1 cells that allows the activation of the cytotoxic machinery of Tr1 cells. In particular, CD54/LFA-1-mediated adhesion, and activation *via* CD58/CD2 and CD155/CD226 interactions are the molecular mediators of the specific adhesion and activation of Tr1 cells. Finally, we showed that GZB expression on $CD4^+$ T cells is increased in tolerant patients *in vivo* and correlates with the expression of IL-10, indicating that GZB can be used as surrogate marker for Tr1 cells. The specific killing of myeloid APCs represents a novel bystander mechanism of suppression mediated by Tr1 by which the priming of Teff cells is reduced and the immune response is dampened (Figure 1).

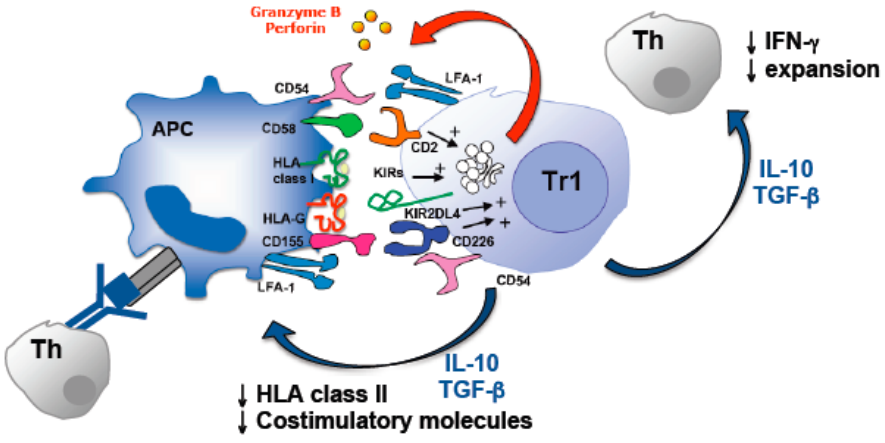

Figure 1. Tr1 cells use the cytotoxic pathway as strategy to suppress immune response. Tr1 cells suppress immune-response by releasing inhibitory cytokines such as IL-10, and TGF-β when they are TCR activated by Ag-specific triggering. IL-10 and TGF-β directly inhibit effector T cell (Th) proliferation and cytokine production, and APC cell functions by down-regulating HLA class II and costimulatory molecules. In addition, IL-10 secreted in the milieu acts as autocrine factor to induce GZB expression on Tr1 cells. GZB^+ Tr1 cells become able to kill myeloid APCs in an Ag-nonspecific manner by recognition of HLA class I on target cells and engagement of LFA-1 adhesion molecules and specific activatory receptors, such as CD226 and CD2. Selective elimination of myeloid APCs prevents the priming of effector T cells, acting as an additional bystander mechanism of suppression of immune response mediated by Tr1 cells.

Thus far, genomic studies on Tr1 cells were restricted to murine systems, and their phenotype, the genetic requirements for their development, and mode of action in humans are poorly defined. To perform a global characterization of human Tr1 cells, we analyzed the whole genome expression data of resting or activated (TCR-activated at 6 and 16 hours) Tr1 cell clones compared to Th0 cell clones isolated *ex vivo* from two different healthy donors (HDs). Hierarchical clustering analysis revealed that the patterns of gene expression of resting Tr1 and Th0 cells were similar, whereas were quite distinct upon activation. Statistical analysis identified 114 differentially regulated genes (DEG) in Tr1 compared to Th0 cells in the resting state, 63 DEGs despite the activation status, and 87 DEGs upon activation. Among differentially expressed genes, several genes that are known to be associated to Tr1 cells, such as IL-10, GZB, and PD-1, were recovered. Real-time RT-PCR of selected genes validated the microarray results. Tr1 cells expressed a gene signature of anti-

proliferation, anti-inflammation, and immuno-modulation. The comprehensive set of genes identified provides a starting point for further studies to unravel the biological and functional features of Tr1 cells, and, in particular, to select molecular markers for Tr1 cells identification and selection, master regulators of their development and function, and functional molecules defining their specific features.

Conclusion and future prospectives

The clinical relevance

Association between presence of Tr1 cells *in vivo* and tolerance induction has been demonstrated in different clinical setting, such as allogeneic stem cell transplantation[1, 2], kidney and liver allografts[3], asthma[4], and celiac disease[5]. Conversely, decreased frequency of Ag-specific Tr1 cells was observed in autoimmune diseases such as Pemphigus Vulgaris[6], type 1 diabetes[7], rheumatoid arthritis[8], and allergy[9]. Even though recent progresses in the characterization of the mechanism of differentiation and function of Tr1 cells have supported their clinical application, an improved understanding of their phenotype and biological features will provide clues for a safer and more efficient clinical use.

Clinical application of Tr1 cells has been limited by the absence of specific surface markers, the unfeasibility to obtain a homogeneous population of Tr1 cells, and the incomplete characterization of their biological features. Two clinical trials based on transfer of Tr1 cells to induce tolerance in haematological cancer patients undergoing HLA-haploidentical HSCT (ALTEN, San Raffaele Hospital, Milan, Italy) and in patients with severe and refractory Crohn's disease (CATS,

TxCell, Sophia-Antipolis, France), started few years ago, demonstrate the safety, efficacy, and tolerability of cell based therapy with Tr1 cells.

The mechanism underneath the induction of stable and long term tolerance by Tr1 cells is, at least in part, mediated by the release of the immunomodulatory cytokines IL-10 and TGF-β. Additional mechanisms that could cooperate in the tolerance induction mediated by Tr1 cells have been hypothesized[10].

Tr1 cells regulates immune response by killing APC

The rationale to use Tr1 cells in clinic was provided few years ago when Blazar and co-workers showed that IL-10- and TGF-β-anergized T cells prevent Graft versus Host Disease (GvHD) in murine HLA class II disparate recipients. The mechanism underneath the regulatory activity of IL-10- and TGF-β-anergized mixed lymphocyte reaction (MLR) was not demonstrated[11]. It is now generally accepted that Tr1 cells suppress T cell responses mainly *via* the secretion of IL-10 and TGF-β, secreted upon Ag-specific TCR activation[12]. Our results demonstrate that specific killing of myeloid APCs is a novel bystander mechanism of suppression mediated by Tr1 cells and imply further considerations in the setting of clinical application. We believe that Ag-nonspecific killing of APCs mediated by Tr1 cells amplifies the tolerogenic loop induced by IL-10 and TGF-β. The elimination of APCs has been considered a crucial mechanism for the regulation of T cell responses[13]. Several reports indicate that $CD8^+$ T and NK cells can lyse dendritic cells (DCs), both in the peripheral tissues by extravasating effector cells, both in lymph

node by effector memory cells generated in situ or re-entering through afferenting lymphatic vassels[14-17]. Our study has shown that killing of APCs is not only a classical mechanism of feedback regulation by effector T cells but is a specific suppressive function of a subset of regulatory T cells.

Tr1 cells need to be activated in Ag-specific manner to exert their suppression functions. Thus, regarding the clinical application based on adoptive cell transfer, we can hypothesize that Tr1 cells once *in vivo* encounter the specific Ag in the lymph node presented by APCs and are activated. At the same time, effector T cells are primed and migrate to periphery, starting immune response towards the specific Ag. Activated IL-10-secreting Tr1 cells up-regulate GZB expression, reach the periphery where they inhibit effector T cells by releasing IL-10 and TGF-β and kill myeloid APCs, blocking further amplification of T cell priming. This mechanism may occur in the contest of prevention of GvHD, where adoptively transferred donor Tr1 cells specific for host-Ags are primed by donor APCs. Conversely, in the treatment of autoimmune diseases, adoptively transferred autologous Ag-specific Tr1 cells are activated by host APCs. This model of prevention of T cell stimulation could explain why we found a streight anti-inflammation signature by microarray analysis of patients enrolled in the ALTEN trial (Bacchetta R., submitted). Furthermore, Tr1 cells are able to lyse U937, a myeloid leukaemia cell lines. We can speculate that the cytotoxic activity could be not only a crucial mechanism to inhibit GvHD but also a way to contribute to the GvL effect. Further studies are necessary to prove a specific killing activity against tumours of myeloid origin.

Finally, we showed a correlation between GZB^+ $CD4^+$ T cells and IL-10-producing Tr1 cells in tolerant patient, indicating that GZB could be used as surrogate marker of Tr1 cell *in vivo*. Since the possibility to monitor Tr1 cell development after cell therapy could be useful to predict the outcome of the patients, GZB expression in $CD4^+$ T cells can be easily followed at different time points after the infusion. Actually, microarray analysis of ALTEN patients' PBMC showed an up-regulation of the mRNA expression of GZB at late time points following the infusion(Bacchetta R., submitted).

Tr1 cell signature

The use of Tr1 cells in clinic is hampered by the lack of a specific cell surface marker that render difficult the tracking of these cells *in vivo*, the isolation *ex vivo*, and the characterization *in vitro*. In addition, only a low frequency of *bona fide* IL-10-producing Tr1 cells can be obtained under the different protocols established in the last 10 years[10, 12, 18-21]. The contaminating effector T cells remain a limitation for their use for *in vivo* applications. Methods based on sorting of $CD49b^+CD18^+$[22] or, alternatively, $CD45RO^+CD127^-CD25^-$[23] have been suggested but the frequency of IL-10-producing cells has not been evaluated in the first study and was around the 10% in the second one.

An alternative strategy for generation of a homogeneous population of Tr1 cells is the gene transfer of a master regulator gene promoting the commitment to Tr1 cell lineage. Moreover, a complete characterization of the functions and behaviour of Tr1 cells will provide more robust rationale for the clinical application.

The microarray analysis employed in our study is, to our knowledge, the first global approach to analyze specific gene expression of human Tr1 cells. We found a variety of surface proteins specifically up- or down- regulated in Tr1 compared to Th0 cell clones. Future studies would address the issue to select the best panel of markers to define Tr1 cells in peripheral blood and tissues. Combination of markers already known and newly identified by this analysis will be used to sort $CD4^+$ T cells from PBMC and the frequency of IL-10^+ cells, the proliferative and suppressive functions of the sorted population will be evaluated. Further validation in tolerant patients will prove the possibility to use the specific gating strategy to follow Tr1 cells in clinical settings.

In order to find factors acting as regulator of development and/or function of human Tr1 cell subset, we select some transcription factors and molecules with post-transcriptional regulatory activity. Overexpression and interference of these molecules would be useful to understand whether they regulate the conversion of $CD4^+$ T in a homogeneous population with regulatory phenotype and functions.

An additional implication of our work on the signature of Tr1 cells is to better characterize the effector function of Tr1 cells. As we showed for the cytotoxic activity, additional mechanisms could be involved in the immune-regulation mediated by Tr1 cells *in vivo*. One example can be the gene MGAT5.

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