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The cellular and molecular mechanisms of immuno-suppression by human type 1 regulatory T cells

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The immuno-regulatory mechanisms of IL-10-producing type 1 regulatory T (Tr1) cells have been widely studied over the years. However, several recent discoveries have shed new light on the cellular and molecular mechanisms that human Tr1 cells use to control immune responses and induce tolerance. In this review we outline the well known and newly discovered regulatory properties of human Tr1 cells and provide an in-depth comparison of the known suppressor mechanisms of Tr1 cells with FOXP3$^+$ T_{reg}. We also highlight the role that Tr1 cells play in promoting and maintaining tolerance in autoimmunity, allergy, and transplantation.

Keywords: IL-10, Tr1 cells, immunotolerance, FOXP3$^+$ T_{reg}

INTRODUCTION

The original study that led to the discovery of interleukin (IL)-10-producing type 1 regulatory T (Tr1) cells began in the late eighties when the immune system of a severe combined immunodeficiency (SCID) patient, who developed long-term mixed chimerism after HLA-mismatched fetal liver hematopoietic stem cell transplant (HSCT), was analyzed to understand how tolerance was acquired. This patient did not develop graft-versus-host disease (GvHD) in the absence of immunosuppressive therapy, despite having host-specific CD4$^+$ T helper and CD8$^+$ cytolytic T cells of donor origin, suggesting that an allo-regulatory suppressor mechanism(s) was responsible for maintaining tolerance (Roncarolo et al., 1988). Interestingly, allo-reactive CD4$^+$ T cell clones isolated from this transplanted patient had a cytokine profile distinct from both type 1 and type 2 helper T cells (Th1 and Th2, respectively), since they produced IL-5, IFN-γ, and GM-CSF, low levels of IL-2, but not IL-4 (Bacchetta et al., 2002), suggesting that these T cell clones were a unique population of T cells. Pursuant to the cloning of human IL-10 (Vieira et al., 1991), a second tolerant SCID patient who underwent HLA-mismatched HSCT was found to have high plasma levels of IL-10, and a significant proportion of donor-derived CD4$^+$ T cell clones specific for the host HLA antigen (Ag)s that produced IL-10 at high levels (Bacchetta et al., 1994). A few years later, Groux et al. (1997) demonstrated in mice and humans that IL-10-producing CD4$^+$ T cells, are indeed, a distinct subset of T cells that are Ag-specific and immunosuppressive and can mediate immune-tolerance. Together, these findings led to a new classification of IL-10-producing CD4$^+$ T cells appropriately named Tr1 cells. Since their seminal discovery, a finding that predates the identification of the more widely studied CD4$^+$CD25$^+$ regulatory T cells (T_{reg}) (Sakaguchi et al., 1995), Tr1 cells have proven to be important in mediating tolerance in several T cell mediated diseases (review in Roncarolo et al., 2006, 2011; Roncarolo and Battaglia, 2007).

Apart from being detected in SCID patients after allogeneic HSCT (Bacchetta et al., 1994), Tr1 cells have been described in β-thalassemic patients who developed persistent mixed chimerism (Serafini et al., 2009). These tolerant β-thalassemic patients had high levels of IL-10 in peripheral blood and an increased percentage of IL-10-producing CD4$^+$ T cells compared to normal donors. Moreover, allo-specific Tr1 cell clones isolated from patients' peripheral blood suppressed alloAg-specific T cell proliferation and cytokine production in an IL-10 dependent manner (Serafini et al., 2009). Overall, the results from tolerant HSCT patients indicate that Tr1 cells are associated with long-term chimerism, possibly induced through chronic exposure to alloAgs after transplantation.

Interleukin-10-producing Tr1 cells have also been demonstrated in an array of different immune-mediated diseases. For example, Tr1 cells specific for self-Ags such as desmoglein 3 and islet Ags have been described in pemphigus patients (Veldman et al., 2004) and diabetes patients (Tree et al., 2010), respectively. Furthermore, Tr1 cells specific for the foreign-Ags gliadin in celiac disease (Gianfrani et al., 2006), and for nickel (Cavani et al., 2000), Derp 1 (Akdis et al., 2004), and bee-venom (Meiler et al., 2008)

in allergic patients have been reported. Conversely, the absence of Tr1 cells in acute viral infections correlates with clearance of the virus, while the presence of Tr1 cells is associated with viral persistence in chronic viral infections such as HIV, HCV, and HBV (Granelli-Piperno et al., 2004; Ha et al., 2008), and in bacterial infections such as *Bordetella pertussis* (McGuirk et al., 2002) and *Mycobacterium tuberculosis* (Boussiotis et al., 2000; McGuirk et al., 2002).

In this review we provide an overview of the similarities and differences between human Tr1 cells and forkhead box P3 (FOXP3)-expressing $CD4^+CD25^+$ T_{reg} cells ($FOXP3^+$ T_{reg}) by summarizing their cellular and molecular suppressive mechanisms and describe the current knowledge of their safety and efficacy in clinical trials.

T_{reg} SUBSETS: DIFFERENT CELLS SHARING SIMILAR MARKERS

Regulatory T cells are a fundamental component of a healthy immune system since they play a vital role in fine-tuning the balance between effector and tolerogenic immune responses. It is well documented that a deficiency in T_{reg} frequency or number, or a defect in their function can lead to inflammation and/or autoimmune diseases (Roncarolo and Levings, 2000; Roncarolo and Battaglia, 2007; Sakaguchi et al., 2008). Over the years, several types of T_{reg} populations have been identified: TGF-β secreting, Type 3 helper cells (Th3; Miller et al., 1992), $CD8^+CD28^-$ T cells (Liu et al., 1998), HLA-E-specific $CD8^+$ T cells (Jiang et al., 2010), etc. but, to date, the best characterized are the $FOXP3^+$ T_{reg} (Hori et al., 2003; Khattri et al., 2003) and the $CD4^+$ IL-10-producing Tr1 cells (Groux et al., 1997; Barrat et al., 2002; Akdis et al., 2004). Their distinct intracellular and surface markers and cytokine expression profile distinguish $FOXP3^+$ T_{reg} and Tr1 cells from one another. $FOXP3^+$ T_{reg} are identified by standard flow cytometry techniques based on their constitutively high expression of CD25 and the transcription factor FOXP3 (Sakaguchi, 2005). $FOXP3^+$ T_{reg} can be subcategorized into naturally occurring $FOXP3^+$ T_{reg} (Yagi et al., 2004), which are selected in the thymus, and adaptive $FOXP3^+$ T_{reg} (Fantini et al., 2004; Tran et al., 2007; Horwitz et al., 2008; Lu et al., 2010) that are induced in the periphery, based on the surface expression of Helios, which is found only on the former population (Thornton et al., 2010). In addition, the naturally occurring $FOXP3^+$ T_{reg} are identified and distinguished from activated $CD4^+$ T cells by the expression of low levels of CD127 (Liu et al., 2006; Seddiki et al., 2006), and of CD49d (Kleinewietfeld et al., 2009), and by the DNA demethylation of a specific region of the FOXP3 gene called T_{reg}-specific demethylated region (TSDR; Baron et al., 2007). Furthermore, the expression of CD45RA distinguishes naïve from activated $FOXP3^+$ T_{reg} and from activated conventional $CD4^+$ T cells (Hoffmann et al., 2006; Miyara et al., 2009).

Type 1 regulatory T cells, on the other hand, are a more discrete population of T_{reg} that are induced in the periphery which, to date, lack a define cell surface signature. Similar to other human effector T cells (Allan et al., 2007; Passerini et al., 2008), Tr1 cells transiently express FOXP3 upon activation (Levings et al., 2005, and S. Gregori and M. G. Roncarolo, personal communication); however, FOXP3 expression in Tr1 cells is not maintained after activation and never reaches the high expression levels characteristic of $FOXP3^+$ T_{reg}. Furthermore, FOXP3 is not required for Tr1 cell induction or function since suppressive Tr1 cells can be generated or isolated from peripheral blood of patients with immunedysregulation polyendocrinopathy enteropathy X-linked (IPEX), a disease condition due to FOXP3-mutations, even in those patients with complete deletion of FOXP3 (Passerini et al., 2011). The rapid onset of autoimmune-mediated wasting disease after birth in IPEX patients indicates that, although Tr1 cells can be induced they are not present or sufficient to control aggressive autoimmunity early in life. The naturally occurring $FOXP3^+$ T_{reg}, which are present from birth, are immediately effective especially to modulate self-reactivity, whereas Tr1 cells are induced in the periphery and are involved in regulation later on in life. Therefore, Tr1 cells and naturally occurring $FOXP3^+$ T_{reg} in humans are distinct subsets of cells with regulatory activity that co-operate in promoting and controlling tolerance *in vivo*.

Type 1 regulatory T cells are currently identified by their unique cytokine profile consisting of high levels of IL-10, TGF-β, low levels of IL-2, variable levels of IL-5 and IFN-γ, in the absence of IL-4 after stimulation (Groux et al., 1997). The reliance on a cytokine profile to distinguish Tr1 cells from other T cell subsets complicates their identification and study since Tr1 cells are not the only T cell subset that secretes IL-10. Other T cell subsets such as $FOXP3^+$ T_{reg} (Ito et al., 2008), Th1 and Th2 cells (Yssel et al., 1992; Del Prete et al., 1993; Chang et al., 2007; Saraiva et al., 2009), Th9 (Veldhoen et al., 2008), and Th17 (McGeachy et al., 2007) can also express IL-10, depending on stimulation and environmental conditions. For example, the strong stimulation of Th1 cells via T cell receptor (TCR) in the presence of high levels of IL-12p70 can result in the induction of IL-10 production (Gerosa et al., 1996); however it is unclear if the induced IL-10 expression remains fixed once the environmental stimuli is removed. Tr1 cell clones display a distinct kinetics of IL-10 secretion, since IL-10 is detectable in culture supernatants at high concentrations early after Ag-specific activation and is maintained over the life of the cell (Bacchetta et al., 1994), whereas IL-10 production by either Th cell clones or peripheral blood cells occurs late after stimulation and never reaches the level of Tr1 cells (Yssel et al., 1992; de Waal Malefyt et al., 1993). The reason why Tr1 cells are able to secrete high levels of IL-10 shortly after activation is unknown; however, it is possible that the high levels of IL-10 secretion is a result of chromatin remodeling at the IL-10 locus, which allows Tr1 cells to readily secrete IL-10 upon activation and to bypass IL-10 repression (Trinchieri, 2007). Answering this question will help to determine if Tr1 cells are a truly fixed lineage of cells or if Tr1 cells are plastic like the other T cell subsets such as $FOXP3^+$ T_{reg}.

Several groups, including ours, aimed at identifying markers specific for Tr1 cells that distinguish them from other $CD4^+$ T cell subsets. Thus far, a number of candidate molecules have been proposed, but unfortunately, most of them are not specific for Tr1 cells. For example, a recent report indicated that the surface expression of lymphocyte activation gene-3 (LAG-3) on $CD4^+$ T cell correlates with IL-10 production and possibly Tr1 cells (Okamura et al., 2009); however, LAG-3 has been shown to be

Table 1 | Proposed markers of human Tr1 cells.

Molecule	Tr1 cells			Other cell types
	Resting	Activated	Specific	
LAG-3	Yes	Yes ↑	No	FOXP3$^+$ T_{reg} (Camisaschi et al., 2010)
ICOS	Yes	Yes ↑	No	FOXP3$^+$ T_{reg} (Ito et al., 2008)
PD-1	Yes	Yes ↑	No	FOXP3$^+$ T_{reg} (Raimondi et al., 2006)
				Tfh (Yu et al., 2009)
CD49b	Yes	Yes	?	Th17 (Boisvert et al., 2010)
p-STAT3	Yes	Yes ↑	No	Th17 (de Beaucoudrey et al., 2008)
				Tfh (Schmitt et al., 2009)
C-MAF	Yes	?	No	Th2 (Rani et al., 2011)
				Th17 (Hiramatsu et al., 2010)
AhR	Yes	?	No	Th17 (Veldhoen et al., 2008)
				Th22 (Trifari et al., 2009)
FOXP3	No	Yes ↑	No	FOXP3$^+$ T_{reg} (Hori et al., 2003)

↑, *high level of expression; ?, indicates that data is not determined.*

associated with FOXP3$^+$ T_{reg} (Camisaschi et al., 2010), making LAG-3 an undependable marker for distinguishing Tr1 cells from other T_{reg} subsets. Similar to LAG-3, other proposed biomarkers for Tr1 cells, such as inducible co-stimulatory molecule (ICOS; Haringer et al., 2009), and programmed death receptor 1 (PD-1; Akdis et al., 2004) have been associated with IL-10-producing T cells, but, here again, they are also found on other T cell subsets. Another report described the combined expression of the integrins CD18^{high} and CD49b as Tr1 cell-specific markers in peripheral blood of healthy donors (Charbonnier et al., 2006; Rahmoun et al., 2006). Unpublished results from our group also show that Tr1 cell clones isolated from normal donors express higher levels of CD49b compared to Th0 clones, whereas CD18 is expressed at similar levels (Gagliani and Gregori, unpublished). In addition to surface molecules, transcription factors known to regulate IL-10 expression such as STAT3 (Levings et al., 2001a), C-MAF (Pot et al., 2009), and aryl hydrocarbon receptor (AhR; Apetoh et al., 2010) have been used to identify human IL-10-producing T cells, but it is still unclear if these transcription factors can be considered master regulators and *bona fide* markers for Tr1 cells (**Table 1** for summary of proposed markers). These data indicate that several markers correlate with IL-10-producing T cells, but the search for a unique and specific marker for human Tr1 cells has been as of today not fruitful. Several studies, including gene expression profiling using *ex vivo* isolated Tr1 cell clones and *in vitro* generated Tr1 cells, are ongoing to identify Tr1 specific markers.

T_{reg} SUBSETS: DIFFERENT CELLS SHARING SIMILAR MECHANISMS OF SUPPRESSION

MECHANISMS OF FOXP3$^+$ T_{reg} SUPPRESSION

Both naturally occurring and induced FOXP3$^+$ T_{reg} suppress immune responses through several mechanisms, which can be grouped into five basic modes of action: (i) cell-to-cell contact, (ii) modulation of dendritic cells (DC), (iii) secretion of inhibitory cytokines, (iv) metabolic disruption, and (v) cytolysis (review in Vignali, 2008).

FOXP3$^+$ T_{reg} suppress effector T cell responses through still not completely elucidated cell-to-cell contact dependent mechanisms (Takahashi et al., 1998; Thornton and Shevach, 1998). Nonetheless, it has been demonstrated that FOXP3$^+$ T_{reg} requires TCR activation (Thornton and Shevach, 2000) and IL-2 (Thornton et al., 2004b) to exert their suppressive functions. The means by which FOXP3$^+$ T_{reg} suppress is through the transcriptional inhibition of IL-2 in effector T cells (Thornton and Shevach, 1998; Thornton et al., 2004a) and IL-2 consumption (de la Rosa et al., 2004). The molecular mechanisms underlying the suppression of IL-2 mRNA in effector T cells is still unknown; however, it is independent of TGF-β/IL-10 or IL-2 consumption (Oberle et al., 2007).

Modulation of DC function is another means by which FOXP3$^+$ T_{reg} control T cell proliferation. FOXP3$^+$ T_{reg} regulate the catabolic enzyme indoleamine 2,3-dioxygenase (IDO) on DC through the interaction of Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) with CD80/86 (Fallarino et al., 2003; Munn et al., 2004). IDO inhibits effector T cell proliferation by reducing tryptophan, which is necessary for cell division. In addition, FOXP3$^+$ T_{reg} can prevent DC activation by sending inhibitory signals to the DC through the interaction of LAG-3 with the MHC class II molecules (Liang et al., 2008).

FOXP3$^+$ T_{reg} secrete regulatory cytokines IL-10 (Ito et al., 2008), TGF-β (Levings et al., 2001b; Stockis et al., 2009; Wang et al., 2009), and IL-35 (Chaturvedi et al., 2011). While IL-10, TGF-β, and IL-35 expression have been shown to be involved in suppression mediated by human FOXP3$^+$ T_{reg} *in vitro*, it remains to be defined whether these cytokines contribute to their suppressive function *in vivo*.

FOXP3$^+$ T_{reg} can also generate adenosine *via* the enzymatic hydrolysis of extracellular ATP by the ectoenzymes CD39 and CD73 (Deaglio et al., 2007), which disrupt the metabolic state of effector T cells. Adenosine upon binding to its receptor, the adenosine-specific A2A receptor (Borsellino et al., 2007), suppresses inflammatory cytokine secretion by effector T cells in a cyclic AMP (cAMP) dependent fashion (Bopp et al., 2007).

Killing of target cells *via* the secretion of granzymes (Gzs) is a well known feature of natural killer (NK) and cytotoxic $CD8^+$ T cells (Cullen and Martin, 2008), but it is now evident that also $CD4^+$ T cells, including $FOXP3^+$ T_{reg}, can use Gzs to kill cells. Upon activation $FOXP3^+$ T_{reg} express GzA and can lyse target cells of both myeloid and lymphoid origin. Lysis mediated by $FOXP3^+$ T_{reg} requires CD18-mediated adhesion (Grossman et al., 2004a).

MECHANISMS OF Tr1-MEDIATED SUPPRESSION

Although multiple mechanisms of Tr1-mediated suppression have been identified (Roncarolo et al., 2006), the relative importance of each mechanism *in vivo* remains to be defined since most of the studies regarding human Tr1 cell activity have been done *in vitro*. The main mechanism by which Tr1 cells mediate immune suppression and promote tolerance is cytokine-mediated. However, new evidences suggest that Tr1 cells are multifaceted suppressors that use several modes of immune regulation to achieve tolerance. Tr1 cells indeed can inhibit T cell responses by cell contact dependent mechanisms (Akdis et al., 2004), metabolic disruption (Mandapathil et al., 2010), and cytolysis (Grossman et al., 2004b; Magnani et al., 2011; see **Table 2** for a summary of proposed Tr1 cells suppressive functions).

CYTOKINE-MEDIATED MECHANISMS OF SUPPRESSION BY Tr1 CELLS

The chief mechanism by which Tr1 cells control immune responses is through the secretion of high levels of the immunosuppressive cytokines IL-10 and TGF-β (Bacchetta et al., 1994; Groux et al., 1997; Barrat et al., 2002; Veldman et al., 2004). IL-10 directly suppresses T cell responses by inhibiting IL-2, IFN-γ, and GM-CSF production by T cells (Vieira et al., 1991) and by preventing proliferation (Taga and Tosato, 1992). Similarly, TGF-β has potent immune-modulatory effects that directly inhibit T cell responses (Gorelik and Flavell, 2002; Gorelik et al., 2002; **Figure 1A**). Together, IL-10 and TGF-β secreted by Tr1 cell lines or clones can inhibit IFN-γ production by T effector cells and limit their proliferation (Cavani et al., 2000; Levings et al., 2005; Meiler et al., 2008; Serafini et al., 2009; Gregori et al., 2010). Whether IL-10 and TGF-β expressed by Tr1 cells modulate other effector T cells such as Th9 (Veldhoen et al., 2008), Th17 (Park et al., 2005), Th22 (Trifari et al., 2009), and T follicular helper (Tfh) cells (Crotty, 2011) has yet to be elucidated.

Type 1 regulatory T cells also suppress effector T cells indirectly by inhibiting inflammatory factors and activating tolerogenic pathways of antigen-presenting cells (APC; Roncarolo et al., 2006). Many of the molecules hindered by IL-10 on APC, like MHC class II, co-stimulatory molecules (de Waal Malefyt et al., 1991), and pro-inflammatory cytokines (Fiorentino et al., 1991a; Mosser and Zhang, 2008) are important mediators of adaptive immune responses. IL-10 treated APC, which up-regulate a number of tolerogenic molecules, including immunoglobulin-like transcript (ILT)-3 and 4 (Manavalan et al., 2003; Gregori et al., 2010), and the non-classical HLA-G (Moreau et al., 1999; Gregori et al., 2010), become regulatory cells capable of dampening immune responses and inducing T_{reg} (Morelli and Thomson, 2007; Gregori, 2011; **Figure 1A**). For example, IL-10-treated macrophages or DC have reduced abilities to secrete IL-12 after LPS activation and to induce Th1 responses (Fiorentino et al., 1991b). It was demonstrated that in allergic patients nickel-specific Tr1 cells inhibit DC to produce IL-12 after LPS stimulation and nickel-specific Th1 cell responses (Cavani et al., 2000).

B cells are targets of IL-10 and can promote tolerance by secreting specific immunoglobulin subclasses (Scott-Taylor et al., 2010). For example, activation of B cells in the presence of IL-10 prevents apoptosis, enhances their proliferation, differentiation, and MHC class II expression, and promotes immunoglobulin switching (Go et al., 1990; Sabat, 2010). IL-10 derived from Tr1 cells promotes IgG4 production by B cells (Satoguina et al., 2005), and in an allergy setting, allergen-specific Tr1 cells induce IgG4 and suppress IgE production *via* an IL-10-mediated mechanisms (Meiler et al., 2008). Therefore, Tr1 cells promote tolerance also by modulating B cell functions.

Interleukin-10 produced by Tr1 cells contributes to the generation of T_{reg}. Our group showed that Ag stimulation of human naïve $CD4^+$ T cells in the presence of IL-10 promoted the induction of Tr1 cells *in vitro* (Bacchetta et al., 2002, 2010; Gregori et al., 2010). Signaling through the IL-10 receptor (IL-10R) can induce IL-10 production by $CD4^+$ T cells (Barrat et al., 2002), promote T cell anergy in $CD4^+$ and $CD8^+$ T cells (Groux et al., 1996, 1998), and Tr1 cell differentiation (Groux et al., 1997) in a STAT3-dependent manner. The importance of activating the STAT3 pathway to induce Tr1 cells is highlighted by the fact that other STAT3 activating cytokines such as IL-27 or IFN-α alone (Awasthi et al., 2007; Fitzgerald et al., 2007; Murugaiyan et al., 2009) or in combination with IL-10 (Levings et al., 2001a) promote Tr1 cells *in vitro*. Notably, a difference between the induction

Table 2 | Mode of suppression mediated by Tr1 cells and/or $FOXP3^+$ T_{reg}.

	Tr1 cells	$FOXP3^+$ T_{reg}
CYTOKINES		
IL-10	Yes	Still controversial (Ito et al., 2008)
TGF-β	Yes	Yes (Levings et al., 2001b)
IL-35	Undefined	Yes (Chaturvedi et al., 2011)
CELL-TO-CELL CONTACT		
CTLA-4	Yes	Yes (Fallarino et al., 2003)
PD-1	Yes	Undefined
LAG-3	Undefined	Yes for murine cells (Liang et al., 2008) Undefined for human cells
ICOS	Undefined	Undefined
LAP	Undefined	Yes (Levings et al., 2001b; Stockis et al., 2009; Wang et al., 2009)
METABOLIC DISRUPTION		
CD39 and CD73	Possible	Yes (Borsellino et al., 2007)
cAMP	Undefined	Yes for murine cells (Bopp et al., 2007) Undefined for human cells
CYTOLYSIS		
Granzyme A/perforin	No	Yes (Grossman et al., 2004b)
Granzyme-B/perforin	Yes	No (Grossman et al., 2004a)

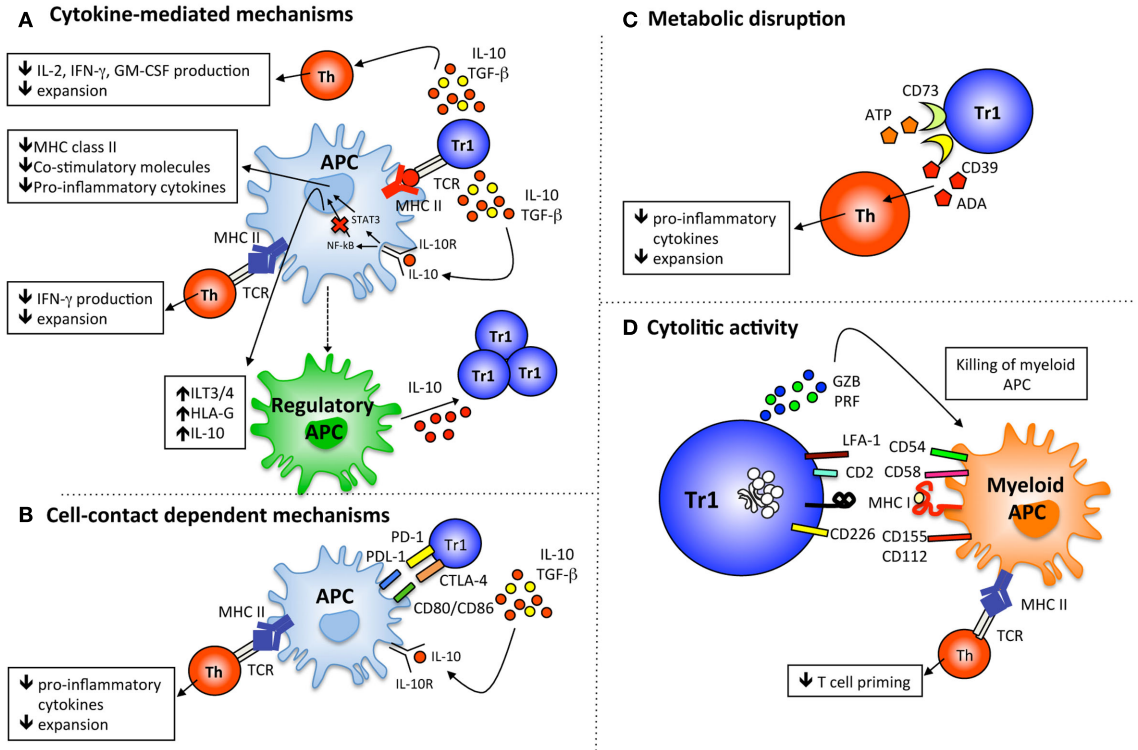

FIGURE 1 | Mechanisms of suppression mediated by Tr1 cells. (A) Tr1 cells upon Ag-specific activation secrete IL-10 and TGF-β that directly inhibit the function of effector T cells and indirectly modulate APC, which in turn limit effector T cell proliferation and cytokine production. IL-10 produced by Tr1 cells up-regulates the expression of tolerogenic molecules (ILT3, ILT4, and HLA-G) on APC and their ability to produce IL-10, and become regulatory APC able to promote *de novo* induction of Tr1 cells. **(B)** CTLA-4 and PD-1 on Tr1 cells down-regulate or prevent the up-regulation of MHC class II and co-stimulatory molecules on APC upon interaction with their ligand, which limits effector T cell activation. **(C)** Catalytic inactivation of extracellular ATP by the ectoenzymes CD39 and CD73 represents an anti-inflammatory mechanism used by Tr1 cells to prevent effector T cell proliferation and cytokine production. **(D)** IL-10 produced by Tr1 cells upon activation promotes autocrine granzyme-B (GZ-B) that in association with perforin (PRF) allows an Ag-non-specific Tr1-mediated killing of myeloid APC. Myeloid-specific killing by Tr1 cells requires HLA class I activation and the expression of CD54, CD58, CD112, and CD155 on myeloid cells.

of IL-10 in Tr1 cells and other IL-10-producing T cells is that IL-10 expression in non-Tr1 cells is STAT3 independent. For instance, IL-10 production by Th1 cells requires strong TCR activation and IL-12p70-induced STAT4 activation (Trinchieri, 2007; Saraiva et al., 2009). Thus, IL-10 production by Tr1 cells is induced under specific conditions compared to other IL-10-producing T cells and STAT3 activation may represent a key signaling pathway that allows these cells to maintain the expression of IL-10 at high levels.

The binding of IL-10 to its tetrameric, transmembrane IL-10R composed of two IL-10R1 and two IL-10R2 molecules induces the phosphorylation of Jak1 and Tyk2 within the intracellular domain of IL-10R1, which acts as docking sites for STAT3 (Donnelly et al., 1999). After STAT3 docking and phosphorylation (p-), p-STAT3 homodimerizes in the cytoplasm and then translocates to the nucleus, where it binds to STAT3-binding elements in the promoters of various genes, including IL-10 (Donnelly et al., 1999), and transcriptional repressors (i.e., SOCS3). STAT3 acts by selectively inhibiting gene transcription of pro-inflammatory cytokines such as TNF-α and IL-12p40 in APC (Murray, 2005). Moreover, IL-10-induced SOCS3, which exerts negative regulatory effects on various cytokine genes by inhibiting STAT and JAK2 activation, is functioning in both T cells and APC (Mosser and Zhang, 2008). In T cells, IL-10 also activates SOCS1, which negatively regulates IFN-induced gene transcription (i.e., IP-10 and ISG-4; Asadullah et al., 2003), whereas in APC, IL-10 selectively induces p50 nuclear translocation while blocks the translocation of the classical NF-kB heterodimer p65/p50 by inhibiting IKK activity (Wang et al., 1995; Clarke et al., 1998). Together the molecular mechanism of IL-10 to dampen activation of both T cells and APC plays a major part in regulating immune responses.

The broad immune-modulatory effects of IL-10 prompted investigators to perform clinical trials using human recombinant(r) IL-10 to treat inflammatory diseases. The earliest studies performed in patients with psoriasis were encouraging; IL-10 was tolerated well, and local subcutaneous administration of rIL-10 induced significant clinical benefits at the site of injection (Asadullah et al., 1998). In a subsequent study in patients with more severe forms of psoriasis the local delivery of rIL-10 to all lesions became a major challenge, thus systemic administration of rIL-10 was used but resulted in only temporary and local clinical improvement (Asadullah et al., 2001). In addition, in patients with Chron's disease, and rheumatoid arthritis, subcutaneous administration of rIL-10 was well tolerated but did not result in disease remission (Keystone et al., 1998; Schreiber et al., 2000). Unfortunately,

patients from another Crohn's disease trial using daily subcutaneous administration for 28 days of high doses of rIL-10 had enhanced inflammatory T cell responses resulting in higher levels of TNFα and IFNγ production (Tilg et al., 2002), suggesting that high levels of systemic IL-10 can have a immune-stimulatory effects. Taken together, these clinical trials indicate that IL-10 is most effective when delivered locally to the site of inflammation. In this scenario the use of IL-10-producing Tr1 cells, which produce high levels of IL-10 upon Ag-specific stimulation, represents an alternative approach to the delivery of IL-10 at the site of Ag-specific inflammation.

Type 1 regulatory T cells indeed require TCR activation by their cognate Ag to produce high amounts of IL-10 and to induce bystander tolerogenic effects. Allergen-specific immunotherapy (SIT) induces a significant increase of Ag-specific IL-10-producing Tr1 cells and elevated levels of IL-10 and TGF-β in peripheral blood and allergic tissues in patients (Akkoc et al., 2011; Jutel and Akdis, 2011). The induction of Ag-specific Tr1 cells can lead to bystander suppression in patients after SIT. Two independent groups demonstrated that peptide-specific Tr1 cells induced after SIT could down-regulate an established inflammatory response driven by multiple T cell epitopes (Grindebacke et al., 2009; Yamanaka et al., 2009). These bystander effects, however, do not lead to systemic immuno-suppression, since tolerant patients with high frequency of Tr1 cells and patients adoptively transferred with Tr1 cells have normal immune responses to pathogens (Bacchetta et al., 2009; Serafini et al., 2009; Roncarolo et al., 2011; Bacchetta et al., submitted).

CELL CONTACT-DEPENDENT MECHANISMS OF SUPPRESSION BY Tr1 CELLS

Type 1 regulatory T cells express inhibitory receptors including CTLA-4 (Bacchetta et al., 2002; Akdis et al., 2004), PD-1 (Akdis et al., 2004), and ICOS (Haringer et al., 2009) that are known to modulate T cell functions. CTLA-4 is a negative regulator of T cell activation (Rudd et al., 2009) known to be expressed on FOXP3$^+$ T$_{reg}$ (Takahashi et al., 2000). Akdis et al. demonstrated that allergen-specific Tr1 cells inhibit allergen-specific Th2 cells *via* CTLA-4 (Akdis et al., 2004; Meiler et al., 2008) and suggested that CTLA-4 co-operates with IL-10 and TGF-β in the suppression of allergic responses mediated by Tr1 cells. PD-1 is a receptor that is critical for the regulation of T cell activation and function during immunity and tolerance. PD-1 interactions with its ligands PD-Ligand (L)1 and PD-L2 inhibit T cell effector functions in an Ag-specific manner (reviewed in Fife and Pauken, 2011). It has been shown that neutralization of PD-1 partially inhibits Tr1-mediated suppression of allergen-specific T cells (Akdis et al., 2004; Meiler et al., 2008), suggesting that PD-1 plays a role in the suppression mediated by Tr1 cells (**Figure 1B**). Despite these results it remains to be defined to what extent Tr1 cells exploit cell-to-cell contact mechanisms to control immune responses.

ROLE OF METABOLIC DISRUPTION IN Tr1 CELL-MEDIATED SUPPRESSION

Like FOXP3$^+$ T$_{reg}$, Tr1 cells can express the ectoenzymes CD39 and CD73 (Bergmann et al., 2007; Mandapathil et al., 2010) and are believed to use these enzymes to generate the immunosuppressive molecule adenosine. Bergmann et al. (2007) showed that *in vitro* generated IL-10-producing T cells in the presence of cyclooxygenase 2 (COX2)-expressing cells and immature DC express CD39 and CD73. Furthermore, Mandapathil et al. (2010) showed that adenosine generated by Tr1 cells suppresses proliferation and cytokine production of effector T cells upon binding with its receptor (**Figure 1C**). We have found that human Tr1 cell clones also express significantly higher levels of mRNA encoding for CD39 compared to Th0 cell clones (Gregori et al., unpublished data). These results suggest that metabolic disruption of conventional T cells might represent an additional mechanism used by Tr1 cells to mediate suppression.

CYTOLYTIC ACTIVITY OF Tr1 CELLS

Human Tr1 cells, depending on the mode of activation/generation, can express both GzA and GzB and selectively kill target cells (Grossman et al., 2004b; Kawamura et al., 2006; Efimova and Kelley, 2009; Czystowska et al., 2011; Magnani et al., 2011). We recently demonstrated that human Tr1 cells, generated *in vitro* and isolated *ex vivo*, express and release high levels of GzB, and specifically lyse cells of myeloid origin, but not other APC or T and B lymphocytes (Magnani et al., 2011). Tr1-mediated cytotoxicity of myeloid APC is Ag-independent and requires recognition and activation *via* HLA class I molecules expressed on target cells (**Figure 1D**). This differs from NK cells, which kill target cells lacking HLA class I molecules. Specific killing of myeloid APC by Tr1 cells depends on the high expression levels of CD54, CD58, CD155, and CD112 on myeloid cells, which, upon interaction with their ligands on Tr1 cells, mediate stable Tr1/APC adhesion and Tr1 specific activation (Magnani et al., 2011). Killing of myeloid cells by Tr1 cells represents an additional indirect mechanism of suppression, which may contribute to bystander suppression. It is tempting to speculate that upon encounter with their cognate Ag Tr1 cells up-regulate GzB expression and kill myeloid cells in an Ag-non-specific manner. This indirect effect could result in a reduction of allo-reactive APC in the case of transplantation, or APC that present self-Ags in the case of autoimmune diseases, limiting priming and expansion of effector T cells.

COMPARISON BETWEEN THE SUPPRESSIVE ACTIVITIES OF FOXP3$^+$ T$_{reg}$ AND Tr1 CELLS

FOXP3$^+$ T$_{reg}$ and Tr1 cells have distinct and shared mechanisms of suppression (see **Table 2**). The main distinction is that Tr1 cells suppress immune responses primarily *via* IL-10, whereas FOXP3$^+$ T$_{reg}$ regulate effector T cells principally *via* T:T cell contact mediated mechanisms. TGF-β is used by both cell subsets to modulate T cell responses, but Tr1 cells secrete TGF-β upon activation *via* their TCR, while FOXP3$^+$ T$_{reg}$ express TGF-β on the cell surface in a complex with latent associated peptide (LAP; Nakamura et al., 2001). More recently, it has been shown that GARP, an orphan toll-like receptor, is selectively expressed by activated FOXP3$^+$ T$_{reg}$ and is required for LAP expression (Stockis et al., 2009; Tran et al., 2009). GARP by interacting with LAP in activated FOXP3$^+$ T$_{reg}$ delivers LAP to their cell surface allowing suppression (Battaglia and Roncarolo, 2009; Stockis et al., 2009). It remains to be defined whether human Tr1 cells express LAP and GARP upon activation, and if they use LAP to suppress T cell responses.

Induction of tolerogenic APC by FOXP3$^+$ T_{reg} and Tr1 cells is a common mechanism of suppression. Since both T cell subsets express CTLA-4 it is likely that they induce similar tolerogenic signals in APC; however, it is still elusive whether the interaction between Tr1 cells and APC will lead to IDO expression as it has been demonstrated for FOXP3$^+$ T_{reg} (Fallarino et al., 2003). Moreover, additional studies are needed to define if the interaction between CTLA-4 on Tr1 cells and CD86 on APC synergizes with IL-10 to inhibit up-regulation of co-stimulatory molecules on APC.

Metabolic disruption of effector T cells by FOXP3$^+$ T_{reg} and Tr1 cells represents an additional common mechanism of immune suppression. Both FOXP3$^+$ T_{reg} and Tr1 cells express CD39 and CD73 and produce adenosine. On the contrary cytolysis mediated by FOXP3$^+$ T_{reg} and Tr1 cells is distinct: FOXP3$^+$ T_{reg} lyse a wide array of immune cells (Grossman et al., 2004b), whereas Tr1 cells specifically kill myeloid cells (Magnani et al., 2011).

PRE-CLINICAL MODELS TO TEST SAFETY AND EFFICACY OF HUMAN T_{reg}-BASED THERAPY

Adoptive transfer of T_{reg} to restore or induce tolerance toward self-Ags or allo-Ags is an efficient means to prevent or cure several T cell mediated diseases, including GvHD, allograft rejection, autoimmunity, and chronic inflammatory diseases in pre-clinical mouse models (Bluestone et al., 2007; Roncarolo and Battaglia, 2007). To evaluate the efficacy of human FOXP3$^+$ T_{reg} therapy *in vivo* humanized mouse models of xeno-GvHD and of allogeneic T cell responses have been developed. Kleinewietfeld et al. (2009) showed that adoptive transfer of human CD4$^+$CD27$^-$CD49d$^-$FOXP3$^+$ T_{reg} is an effective means to prevent xeno-GvHD induced by human PBMC in Rag2$^{-/-}\gamma c^{-/-}$ mice. Similarly, injection of *in vitro* expanded human FOXP3$^+$ T_{reg} with artificial APC expressing CD86 and CD64 in the presence of anti-CD3 mAbs, IL-2 and rapamycin could block xeno-GvHD induced by human PBMC in NOD/scid IL2Rγc^{null} mice (Golovina et al., 2008), while only partially delayed xeno-GvHD in NOD/scid mice (Hippen et al., 2011). In addition, preliminary results by our research group showed that human T cells engineered to stably over-express FOXP3 suppress xeno-GvHD in humanized NOD/scid mice (Passerini and Bacchetta, unpublished).

The efficacy of T_{reg}-based cell therapy with human FOXP3$^+$ T_{reg} to suppress allogeneic T cell responses *in vivo* has been recently evaluated. In this study Sagoo et al. (2011) demonstrated in a humanized mouse allo-skin graft model that adoptive transfer of expanded alloAg-specific human CD4$^+$CD25^{high}CD127$^{low/-}$ T_{reg}, but not expanded polyclonal CD4$^+$CD25^{high}CD127$^{low/-}$ T_{reg}, significantly improved protection of human skin damage. In addition, the group of K. Wood demonstrated that transfer of *in vitro* expanded polyclonal CD4$^+$CD25^{high}CD127$^{low/-}$ T_{reg} (Nadig et al., 2010) or *in vitro* induced FOXP3$^+$ T_{reg} (Feng et al., 2011) prevented ateroesclerosis in a humanized model. Taken together, adoptive transfer of human FOXP3$^+$ T_{reg} is a promising means to control T cell-mediated diseases in humanized mouse models suggesting that the efficacy of human T_{reg}-based therapy may mirror the data garnered from the numerous pre-clinical mouse studies.

The effectiveness of Tr1-based cell therapy to control xeno-GvHD is under investigation by our group. We have recently demonstrated in a humanized mouse model that human IL-10-producing CD4$^+$ Tr1 cells, generated by transducing CD4$^+$ T cells with a lentiviral vector encoding for IL-10, suppress xeno-GvHD and promote survival when co-transferred with allogeneic PBMC (Andolfi and Fousteri, submitted). Additional studies are needed to define which are the mechanisms of *in vivo* suppression mediated by FOXP3 T_{reg} and Tr1 cells.

CLINICAL TRIAL WITH T_{reg} CELLS

Results obtained in pre-clinical studies and the evidence that T_{reg} are critically involved in promoting peripheral tolerance prompted investigators to use adoptive transfer of T_{reg} as a therapeutic. Proof-of-principle clinical trials in allogeneic HSCT demonstrated the safety of T_{reg}-based cell therapy with both FOXP3$^+$ T_{reg}, *ex vivo* isolated (Trzonkowski et al., 2009; Di Ianni et al., 2011; Edinger and Hoffmann, 2011) or *in vitro* expanded (Brunstein et al., 2010), and Tr1 cells (Bacchetta et al., 2009; Bacchetta, submitted). Furthermore, a phase I/II trial showed that cell therapy with Tr1 cell clones in patients displaying severe Crohn's disease is safe and does not lead to general immuno-suppression[1]. One of

[1]http://www.txcell.com

Table 3 | Clinical trials with T_{reg}-based cell therapy.

Trial	Disease	Methods of generation			Ag-specificity
		In vitro induction	*In vitro* expansion	*Ex vivo* isolation	
TR1 CELLS					
Allo-specific (Bacchetta et al., 2009)	Allo-HSCT	Yes	No	No	Yes
OVA-specific (http://www.txcell.com)	Crohn's disease	No	Yes	No	Yes
T_{REG} CELLS					
CD25^{high} cells (Di Ianni et al., 2011)	Allo-HSCT	No	No	Yes	No
CD25^{high} cells (Brunstein et al., 2010)	Allo-HSCT	No	Yes	Yes	No
CD25^{high} cells (Trzonkowski et al., 2009)	Allo-HSCT	No	Yes	Yes	No
CD25^{high} cells (Edinger and Hoffmann, 2011)	Allo-HSCT	No	No	Yes	No
CD25$^+$CD127$^{low/-}$ cells (ClinicalTrials.gov Identifier: NCT01210664)	T1D	No	Yes	Yes	No

the advantages of using Tr1 cells over FOXP3$^+$ T_{reg}, is that Tr1 cells are inducible *ex vivo* and, therefore, can be generated and expanded *in vitro* against the required Ag-specificity. Taken together, results from these clinical trials suggest that: (i) FOXP3$^+$ T_{reg} may prevent GvHD without inhibiting prompt immune reconstitution in allo-HSCT (Brunstein et al., 2010; Di Ianni et al., 2011; Edinger and Hoffmann, 2011), (ii) allo-specific Tr1 cells promote faster immune reconstitution (Bacchetta et al., 2009; Bacchetta, submitted), (iii) infusion of autologous Ag-specific Tr1 cell clones in patients with Chron's disease has beneficial effects (see text footnote 1). Several open questions regarding T_{reg}-based therapy in humans remain: how long do T_{reg} survive *in vivo* after transfer, what is their mechanism of suppression *in vivo*, and whether Ag-specificity is required to induce long-term tolerance (see **Table 3**). Caution for Tr1-based cell therapy should be used considering the adverse effects of excessive levels of systemic IL-10 demonstrated in rIL-10 clinical trials (Tilg et al., 2002; Mosser and Zhang, 2008).

Future clinical trials planning to test the safety and efficacy of T_{reg}-based therapy in patients after allogeneic organ transplant (THE ONE study)[2] as well as in patients with autoimmune diseases, such as type 1 diabetes (ClinicalTrials.gov Identifier: NCT01210664) and rheumatoid arthritis (see text footnote 1) will define if T_{reg} can suppress allograft rejection under the umbrella of immune suppression, and whether T_{reg} can suppress self-Ag specific T cell responses, and can regulate an ongoing immune response. Overall, these studies will define the efficacy of T_{reg}-based therapy as therapeutic option to restore or induce tolerance in T cell mediated diseases.

CONCLUSION

Since the discovery of Tr1 cells nearly two decades ago, research has firmly established their role in controlling immune homeostasis and modulating a wide variety of diseases. While a great deal of progress has been made in understanding the mechanisms of suppression by Tr1 cells, a number of questions still remain. First, a unique set of surface or intracellular marker(s) for Tr1 cells remains to be identified. Second, although it is well established that FOXP3$^+$ T_{reg} and Tr1 cells are distinct populations sharing similar mechanism of suppression, additional research is needed to define what the relationship is between these T_{reg} subsets. Third, since there are several mechanisms of suppression used by Tr1 cells, it needs to be determined whether these mechanisms are operational *in vivo* and whether they are specific to a particular disease state. Answering these questions will not only bring us closer to understanding how Tr1 cells function, but also how to exploit or modulate their suppressive activity for targeted therapy against a wide variety of diseases.

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[2]http://www.onestudy.org/

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