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THE MOLECULAR MECHANISMS OF REGULATORY T CELL IMMUNOSUPPRESSION

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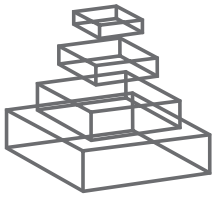

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THE MOLECULAR MECHANISMS OF REGULATORY T CELL IMMUNOSUPPRESSION

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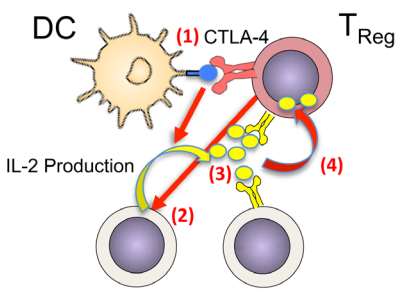

Image from Gasteiger G and Kastenmuller W (2012) Foxp3+ regulatory T-cells and IL-2: the Moirai of T-cell fates? *Front. Immun.* 3:179. doi: 10.3389/fimmu.2012.00179

Ever since Regulatory T cells (T-Regs) were first defined as peripheral CD4+ T cells that express the interleukin-2 (IL-2) receptor alpha chain (IL-2Rα), there have been intensive efforts to determine the molecular mechanisms whereby this minor subset of CD4+ T cells (~5–10%) nonspecifically suppresses all potential effector T cells, whether reactive to self or non-self antigens.

Multiple possible molecular mechanisms have been implicated, including the scavenging of IL-2 via the expression of high densities of IL-2Rs, the inhibition of antigen presentation via CTLA-4 molecules leading to decreased IL-2 production, the activation of intracellular cAMP thereby suppressing both IL-2 production and action, and the production of suppressive cytokines such as IL-10 and Tumor Growth Factor-beta, to list a few. However, the field has thus far failed to come to a consensus, such that some investigators have now asserted that many molecular mechanisms may be operative, in fact that perhaps all of the described mechanisms may account for the suppressive effects of these cells, acting either simultaneously or sequentially. Thus, this Research Topic is focused on articles that can shed some new light on the molecular mechanisms responsible for T-Reg immunosuppression.

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EDITORIAL
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The molecular mechanisms of regulatory T cell immunosuppression

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Fifty years ago Jacques Miller devised a technique to thymectomize neonatal mice to explore the hypothesis that the thymus played a role in the development of immunity. He found that if thymectomized by day 3 postpartum, the mice would develop normally for the first month, but thereafter they underwent a runting syndrome similar to that observed during Graft vs. Host Disease (GvHD) (Miller, 1962). During the second month of life the mice would lose weight and suffer from a dermatitis and generalized lymphadenopathy and splenomegaly followed by premature death. A more detailed examination of the immune system revealed that early after thymectomy the mice were lymphopenic and immunocompromized, unable to reject allogeneic or even xenogeneic skin grafts, and incapable of generating antibodies to routine antigens. Miller correctly interpreted his findings as evidence that the thymus appeared to be critical in the first few weeks of life for the development of a mature functional immune system, but he did not speculate on the cause of the later enigmatic development of lymphoproliferation and apparent autoimmunity.

Twenty years after Miller's seminal observations, Shimon Sakaguchi reported that the lymphoproliferative/autoimmune diseases of immunocompromized day-3 thymectomized (d3Tx) could be transferred to neonatal mice with Thy-1+, Lyt-1+, Ly-23- splenocytes from the afflicted animals (Sakaguchi et al., 1982b). Furthermore, the autoimmune syndrome that developed in d3Tx mice could be completely prevented by a single intraperitoneal injection of Thy-1+, Lyt-1+, Lyt-23- splenocytes or thymocytes taken from normal adult mice (Sakaguchi et al., 1982a). Prior to these experiments, alloantisera reactive with Lyt-1 were thought to mark the helper T cell subset (Th cells) (Cantor and Boyse, 1975; Kisielow et al., 1975). However, Lyt-1 alloantigens were subsequently found on all T cells to a varying degree and therefore could not be the murine equivalent to the T4 (CD4) determinants that specifically identified human Th cells, restricted to antigen recognition with MHC class II molecules (Reinherz et al., 1979b; Ledbetter et al., 1980).

Additional progress in the molecular understanding of the regulation of adaptive immunity was required before it was possible to make further progress in the dissection of these phenomena, especially the molecular mechanism(s) responsible for the apparent suppressive activities of mature T cells vs. neonatal T cells. Thus, T cell clones (Baker et al., 1979) were necessary to define the molecular nature of the T cell antigen receptor (TCR) complex, including the roles of the accessory molecules CD4 and CD8 as facilitating recognition of antigenic peptides bound to MHC class II and class I, respectively (Reinherz et al., 1979a, 1980a), as well as the role of the CD3 molecules as triggers of antigen recognition (Reinherz et al., 1980b), found to be mediated by the disulfide-linked heterodimeric α and β chains (Meuer et al., 1983). Thus, antigen-specific recognition by the TCR complex leads to the expression of antigen-non-specific cytokines, such as interleukin-2 (IL-2) and its receptors (Meuer et al., 1984), so that the tempo, magnitude, and duration of immune responses came to be understood to depend upon antigen non-specific hormone-like molecules (Cantrell and Smith, 1984; Smith, 1988). Inherent in these concepts was the demonstration that IL-2 interacts with specific receptors that satisfied all of the characteristics of true hormone receptors, i.e., high affinity, stereospecificity, saturability, and physiologic relevance (Robb et al., 1981).

Given these findings, a totally unexpected result of the deletion of the IL-2 gene was reported by Ivan Horak's group (Schorle et al., 1991). Mice developing with the total absence of IL-2 were remarkably similar to Miller's neonatal thymectomized mice. Intitially, the IL-2(−/−) mice grew normally and as expected were immunocompromized (Kundig et al., 1993). However, as they aged there occurred a lymphoroliferative syndrome with the accumulation of activated T cells in secondary lymphoid organs and even invasion of non-lymphoid organs that culminated in premature death due to autoimmune hemolytic anemia and inflammatory bowel disease (Horak et al., 1995; Sadlack et al., 1995).

Concurrent with these publications, Sakaguchi and his colleagues reported that a critical subset of CD4+ T cells that express the IL-2R α-chain, ∼10% of mature peripheral CD4+ T cells, could prevent autoimmune diseases of immunodeficient *nu/nu* mice injected with immunocompetent CD4+ T cells depleted of IL-2Rα+ cells (Sakaguchi et al., 1995). Subsequently, the inhibitory molecule CTLA-4 was found to play a major role in the regulatory function of CD4+IL-2Rα+ cells (Takahashi et al., 2000).

The finding that CD4+IL-2Rα+CTLA-4+ cells express the transcriptional regulator FOXP3 helped to explain the phenotype of regulatory T cells (T-Regs) (Fontenot et al., 2003; Hori et al., 2003; Walker et al., 2003). Moreover, IL-2 was found to be required for FOXP3 expression and the normal development of FOXP3+ cells (Zorn et al., 2006; Burchill et al., 2007). Also, FOXP3 was found to inhibit IL-2 expression, which accounted

for T-Reg anergy, and led to the conclusion that IL-2 activates a negative-feedback loop via FOXP3 that limits T cell proliferative expansion during an immune reaction (Popmihajlov and Smith, 2008). However, the FOXP3-induced increase in the expression of both CTLA-4 and IL-2Rα chains did not immediately translate into mechanisms that could readily explain immunosuppression (Wu et al., 2006).

A seminal breakthrough in understanding the molecular mechanisms of T-Reg immunosuppression was contributed by Pushpa Pandiyan and Michael Leonardo and their co-workers, who detailed how T-Reg cells, incapable of producing IL-2, are very efficient in binding and degrading IL-2, thereby leading to cytokine deprivation apoptosis of T-Effector cells (T-Eff) (Pandiyan et al., 2007), as well as T-Regs themselves (Pandiyan and Lenardo, 2008).

Thomas Hofer's group (Busse et al., 2010) and independently, Gregoire Altan-Bonnet's group (Feinerman et al., 2010), using both theoretical and experimental approaches, reported that during an immune response there is a competition for IL-2 between T-Regs and activated effector T cells (T-Effs). Moreover, Altan-Bonnet showed that the IL-2 up-regulation of the IL-2Rα+ chain, first noted soon after the IL-2Rα+ chain was discovered (Leonard et al., 1982; Smith and Cantrell, 1985), can result in a 1000-fold increase in the affinity of IL-2 binding to the trimeric IL-2R. Consequently, T-Regs can rapidly respond to the initial IL-2 produced by T-Effs, and up-regulate IL-2Rα+ chains, which will favor IL-2 binding and degradation much more efficiently than T-Effs, which require several hours before they can express IL-2Rα+ chains upon antigen stimulation. Thus, the "strength" of the initial antigenic stimulation, which determines the amount of IL-2 produced initially, can be overcome by T-Regs when the antigens are of low affinity or at low concentrations (i.e., "weak"), but cannot be competed successfully by T-Regs if the antigenic stimulus is "strong" (i.e., high affinity or at high concentrations). Assuming autoantigens to be "weak" and non-self antigens to be "strong," this system could account for self–non-self recognition.

With this brief chronology as background, readers will find many of the contributions to this volume remarkable, in that many of the field leaders, but not all, have reached a consensus that the major molecular mechanism whereby T-Regs suppress T-Effs revolves around their capacity to regulate the availability of IL-2 as well as other cytokines.

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Role of dendritic cell maturity/costimulation for generation, homeostasis, and suppressive activity of regulatory T cells

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Tolerogenicity of dendritic cells (DCs) has initially been attributed exclusively to immature/resting stages, while mature/activated DCs were considered strictly immunogenic. Later, all different subsets among the myeloid/conventional DCs and plasmacytoid DCs have been shown to bear tolerogenic potential, so that tolerogenicity could not be attributed to a specific subset. Immunosuppressive treatments of immature DC subsets could prevent re-programming into mature DCs or upregulated inhibitory surface markers or cytokines. Furthermore, the different T cell tolerance mechanisms anergy, deletion, immune deviation, and suppression require different quantities and qualities of costimulation by DCs. Since expansion of regulatory T cells (Tregs) has been shown to be promoted best by fully mature DCs the role of CD80/B7-1 and CD86/B7-2 as major costimulatory molecules for Treg biology is under debate. In this review, we discuss the role of these and other costimulatory molecules on myeloid DCs and their ligands CD28 and CD152/CTLA-4 on Tregs for peripheral conversion from naive $CD4^+$ T cells into the major subsets of $Foxp3^+$ Tregs and $Foxp3^-$ IL-10^+ regulatory type-1 T cells (Tr1) or Tr1-like cells and their role for peripheral maintenance in the steady state and after activation.

Keywords: regulatory T cells, Foxp3, IL-10, dendritic cells, costimulation

SUBSETS OF Tregs

Regulatory T cells can be generated in the thymus and are released as so-called natural regulatory T cells (nTregs) or by extrathymic induction from naive T cells (iTregs) in secondary lymphoid organs (Horwitz et al., 2008; Sakaguchi et al., 2008; Yamazaki and Steinman, 2009). Several Treg subsets or functional states, depending on their anatomical origin or mode of generation, have been described (Sakaguchi et al., 2008) but a common signature or distinct sublineages may not exist (Feuerer et al., 2010). Two major subtypes of iTregs can be distinguished on the basis of their Foxp3 expression. Many authors refer only to the $Foxp3^+$ subtypes as iTregs, while the $Foxp3^-$ IL-10^+ T cells are mostly named Tr1 (T regulatory type-1) or Tr1-like cells (**Figure 1**). Of note, most activated Tregs seem to release IL-10, independent of their Foxp3 expression (**Figure 1**). Since these Treg subsets have been in focus the last years, there is ample information about their interaction with DCs and this will be discussed here. In contrast, the less well investigated IL-35^+ Tregs and TGF-β-producing Th3 or $CD8^+$ Tregs are reviewed elsewhere (Filaci and Suciu-Foca, 2002; Wang, 2008; Bettini and Vignali, 2009; Weiner et al., 2011). Since the generation of nTregs in the thymus seems not to depend on DCs but B7 costimulation on medullary epithelial cells and other APCs, we also refer to other reviews (Kyewski and Klein, 2006; Ohkura and Sakaguchi, 2010).

The best studied costimulatory molecules are CD80 (B7-1) and CD86 (B7-2). Both B7 molecules are the ligands of CD28 and CTLA-4 receptors on T cells (Lenschow et al., 1993). Whereas signaling by B7/CD28 pathway is crucial for enhancing T cell activation and survival, signaling by B7/CTLA-4 mainly regulates inhibitory T cell responses (Sansom and Walker, 2006). Moreover, selectivity for CD28 to CD86 and CTLA-4 to CD80 interactions may have to be considered due to stoichiometric and affinity measurements *in vitro* (Collins et al., 2002).

Abbreviations: APC, Antigen-presenting cells; DC, dendritic cell; iTreg, induced regulatory T cells; nTreg, natural regulatory T cells; Tr1, T regulatory type-1; Th1, T helper 1; Th2, T helper 2; Th17, T helper 17.

ROLE OF COSTIMULATION FOR THE GENERATION OF Tr1 CELLS

MOLECULAR FACTORS DRIVING IL-10 IN T CELLS

Initial reports defined Tr1 cells as IL-10 producing T cells which developed from naive T cells and acquired suppressive activity in the presence of IL-10. They adapted a particular cytokine expression profile distinct from Th1 or Th2 effector T cells (Groux et al., 1997). However, ever since, many IL-10 producing Tregs have been described that could be induced under various experimental conditions (**Table 1**) and as reviewed in Hawrylowicz and O'Garra (2005), Roncarolo et al. (2006). Interestingly, high production of IL-10 and acquisition of regulatory function can also occur as a result of chronic stimulation of differentiated T helper cells thereby gradually losing production of effector cytokines such as IFN-γ or IL-4 (O'Garra et al., 2004). The question remains whether all $Foxp3^-$ IL-10^+ iTregs cells that are either derived from naive T cell precursors (Tr1) or from chronically stimulated effector T cells (Tr1-like) develop via similar IL-10- and costimulation-dependent signaling mechanisms. To date this remains unclear. However, for Th1-like cells common signaling pathways have been reported.

Saraiva et al. identified the mitogen-activated protein kinases Erk1 and Erk2 along with strong T cell receptor (TCR) triggering

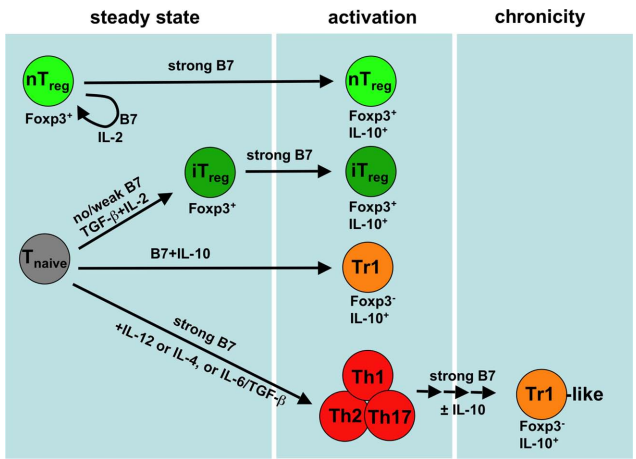

FIGURE 1 | Generation and maintenance of different Treg subsets and their costimulation requirements. During the steady state thymus derived Foxp3$^+$ nTregs require IL-2 and B7 (CD80/CD86) costimulation to persist. The conversion of iTregs from naive T cells into the Foxp3$^+$ subtype prefers the absence of B7 molecules but requires the presence of TGF-β and IL-2. Strong B7 costimulation favors the activation of both Foxp3$^+$ nTregs and Foxp3$^+$ iTregs such as provided by mature DCs. Generation of Tr1 cells from naive T cells requires the presence of costimulation and IL-10. The appearance of Tr1-like cells is preceded by immunogenic Th1 (by IL-12), Th2 (by IL-4), or Th17 (by TGF-β/IL-6) induction and subsequent short interval restimulations under the same immunogenic conditions that will result in a loss of the Th phenotype and a gain of IL-10 production capacity. Of note, all events indicated by arrows require TCR signals in addition.

and activation of the respective signal transducer and activator of transcription (STAT) as a common signaling pathway for the production of IL-10 by Th1, Th2, or Th17 effector T cells (Saraiva et al., 2009; Saraiva and O'Garra, 2010). Motomura et al. (2011) further supported the concept of a common signaling cascade for the appearance of IL-10 by effector T cells by identifying the central transcription factor E4 promoter-binding protein 4 (E4BP4), also known as NFIL3, essential for the regulation of both the IL-13 and IL-10 expression in chronically stimulated Th1 cells and other innate cells. Further research is needed to clarify the signaling pathways in T cells upstream of E4BP4 and the role DC-derived costimulatory molecules in this process.

IL-10 AND THE DEVELOPMENT OF Tr1 CELLS

Whereas the role of DC maturation and costimulation for the generation of Tr1 cells has remained poorly investigated, the requirement of IL-10 is well documented (Groux et al., 1997; Levings et al., 2005; Roncarolo et al., 2006). Indeed, differentiation of Tr1 by means of immunosuppressive drugs such as dexamethasone and vitamin D3 is inhibited even in APC-free conditions in the presence of anti-IL-10R antibodies (Barrat et al., 2002). Other studies on Tr1 differentiation by human immature DCs suggested that DC-derived IL-10 is critical for the generation of Tr1 cells (Levings et al., 2005; Gregori et al., 2010; van der Aar et al., 2011). The use of IL-10 reporter mice indicated that mouse Tr1 cells can develop *in vivo* in the absence of IL-10 (Maynard et al., 2007). It has been discussed whether IL-10 may only be required to maintain T cell anergy of Tr1 cells (Roncarolo et al., 2006). In this scenario IL-10 modulates the DC or APC maturation phenotype rather than through direct activity on T cells (Wakkach et al., 2001). Indeed, IL-10 is a potent down-modulator of MHC II and costimulatory molecule expression on DCs (Moore et al., 2001; Sato et al., 2002; Gabrysova et al., 2009; Gregori et al., 2010). Immature DCs cultured in the presence of additives such as IL-10, TGF-β, glucocorticoids or vitamin D3 analogs or low doses of GM-CSF can acquire even a maturation-resistant DC phenotype, thus remaining strictly immature and generating anergic T cells *in vitro* (Lutz, 2006). However, not all such anergic T cells gain regulatory capacity (Berger et al., 2009). It remains open whether this can be explained by the fact that T cell anergy can result either in complete absence of CD80/CD86 signals or by CD80/CD86 engagement of CTLA-4 (Greenwald et al., 2001; Macian et al., 2004).

COSTIMULATION THROUGH CD80, CD86, CD58, AND ICOS-L

The initial report used murine splenic APCs or human monocytes to differentiate Tr1 cells (Groux et al., 1997). Low levels of CD80/CD86 molecules are provided by both cellular sources. However, the requirement for CD28 or CTLA-4 signals for Tr1 cell generation have initially not been addressed. Several reports demonstrated that Tr1 cells typically express large amounts of CTLA-4 regardless of the effector T cell type the cells were originally derived from (**Table 1**). Indeed, Perez et al. (2008) demonstrated that preferential ligation of CTLA-4 by CD80 on mature DCs resulted in anergic T cells bearing suppressive activity and producing high amounts of IL-10 upon restimulation. Although the presence of anti-IL-10R antibodies abolished the differentiation of iTregs in this culture set-up, IL-10 production itself appeared largely dependent on CTLA-4 signaling (Perez et al., 2008). A role of CD58–CD2 interaction for human Tr1 has been proposed on the basis of studies using CD58-transfected artificial APCs (Levings et al., 2001; Wakkach et al., 2001). Since CD58 is not a ligand for CD2 in the mouse, this pathway may not follow a general rule for Tr1 generation.

Others claimed that only immature DCs contributed to the differentiation of IL-10 producing Tr1 cells (Jonuleit et al., 2000; Levings et al., 2005). However, those human immature monocyte-derived DCs (although largely CD83 negative) express substantial CD80 and CD86 on their cell surface (Jonuleit et al., 2000; Gregori et al., 2010) and appear thereby rather semi-mature (Lutz and Schuler, 2002), whereas these markers can be hardly detected on freshly isolated Langerhans cells (McIlroy et al., 2001), splenic DCs (Berthier-Vergnes et al., 2001), or liver perfusate DCs (Bosma et al., 2006) from human donors. Only human DCs isolated from thymus or tonsils showed substantial expression of costimulatory markers (Summers et al., 2001; Vandenabeele et al., 2001). In addition, human Tr1 cells could be differentiated in the presence of exogenous IL-10 and monocyte-derived DCs, which did not produce IL-12p70 but expressed high levels of costimulatory molecules on their surface (Gregori et al., 2010). Thus, costimulation through CD80/CD86 as provided by semi-mature DCs, but not by truly immature DCs, is required to generate IL-10$^+$ T cells from naive precursors (**Figure 1**).

Secretion of IL-10 for both Foxp3$^+$ Treg or Tr1 cells has been linked to high ICOS surface expression (Hutloff et al., 1999; Akbari et al., 2002; Herman et al., 2004). Costimulation via ICOS-L (CD275) on the surface of mature myeloid DCs (Witsch et al., 2002) or mature plasmacytoid DCs (Ito et al., 2007) stimulates

Table 1 | Generation and phenotype of Tr1 and Tr1-like cells.

Th origin	Cytokine origin	Cytokine shift toward	Antigens/DC system	Route	Anergy	CTLA-4	*In vitro/ in vivo*	Human/ rodent	References
Th1	IL-10^{mid} IFN-γ^{hi} IL-4^{lo} IL-13^{lo}	IL-10^{hi} IFN-γ^{lo} IL-4^{lo} IL-13^{lo}	High dose bee venom (bee stings subcutaneously)	s.c. (skin)	+	+	*In vivo*	Human	Meiler et al. (2008)
?	?	IL-10^{hi} IFN-γ^{lo} IL-4^{lo}	Aeroallergens	Inhalation	+	+	*In vivo*	Human	Akdis et al. (2004)
Th2	IL-10^{hi} IFN-γ^{lo} IL-4^{hi}	IL-10^{hi} IFN-γ^{lo} IL-4^{lo}	OVA protein (by pulmonary DC expressing high B7 costimulation and ICOS-L and producing IL-10)	i.n.	+	?	*In vivo*	Rodent	Akbari et al. (2001, 2002)
Th1	IL-10^{lo} IFN-γ^{hi} IL-4^{lo}	IL-10^{hi} IFN-γ^{lo} IL-4^{hi}	LPS-matured DC and anti-CD86 antibodies	(–)	+	+	*In vitro*	Rodent	Perez et al. (2008)
Th1	IFN-γ^{hi} IL-4^{lo} IL-5^{lo}	IL-10^{hi} IFN-γ^{lo} IL-4^{neg}	High affinity peptides	i.n.	+	+	*In vivo*	Rodent	Burkhart et al. (1999), Sundstedt et al. (2003), Gabrysova et al. (2009)
Th0	(Naive) (IL-4^{lo} only Barrat et al., 2002)	IL-10^{hi} IFN-γ^{neg} IL-5^{neg} (IL-4^{lo} only Barrat et al., 2002)	Immunosuppressive drugs conditioned DC	(–)	+	+	*In vitro*	Both	Barrat et al. (2002), Vieira et al. (2004)
Th0	(Naive)	IL-10^{hi} IFN-γ^{lo} IL-5^{hi} IL-4^{neg}	Splenic APC, OVA peptide, and exogenous IL-10	(–)	+	?	*In vitro*	Both	Groux et al. (1997)

(Continued)

Table 1 | Continued

Th origin	Cytokine origin	Cytokine shift toward	Antigens/DC system	Route	Anergy	CTLA-4	*In vitro/ in vivo*	Human/ rodent	References
Th0	(Naive) Cord versus peripheral blood $CD4^+$ T cells	IL-10^{hi} IFN-γ^{lo} IL-4^{neg} IL-5^{neg} (Jonuleit et al., 2000)	Repetitive stimulation with allogeneic monocyte-derived DC (CD80 and CD86 high; CD83 neg)	(–)	+	+ (Jonuleit et al., 2000)	*In vitro*	Human	Jonuleit et al. (2000), Levings et al. (2005)
Th0	(Naive)	IL-10^{hi} IFN-γ^{lo} IL-5^{hi} IL-4^{neg}	Artificial APC as L cell line transduced with CD58 and CD80 in addition of exogenous IL-10 and IFN-α	(-)	+	?	*In vitro*	Human	Levings et al. (2001)
Th0	(Naive)	IL-10^{hi} IFN-γ^{hi}	Splenic DC with high dose OVA peptide and exogenous IL-12	(–) s.c.	?	?	*In vitro/ in vivo*	Rodent	Saraiva et al. (2009)
Th1	IFN-γ^{hi} IL-4^{neg} IL-5^{neg} IL-13^{neg}	IL-10^{hi} IFN-γ^{hi} IL-13^{hi}	Splenic APC and Th1 skewing conditions	(–)	?	?	*In vitro*	Rodent	Motomura et al. (2011)
Th0	(Naive)	IL-10^{hi} IFN-γ^{neg} IL-5^{hi} IL-4^{hi} IL-13^{hi}	Long-term culture of mature DC and "diffDC" or stroma conditioned DC	(–)	+	–	*In vitro*	Rodent	Xu et al., 2011

IL-10 release by Tr1 or Tr1-like cells, respectively. Enhanced expression of ICOS on *in vitro* generated Tr1 cells has also been attributed to the cytokine IL-27 (Pot et al., 2009). IL-27 can be produced by DCs upon interaction with Foxp3$^+$ Tregs, which acquire a particular plasmacytoid-like DC phenotype expressing costimulatory molecules able to generate Tr1 cells (Awasthi et al., 2007). Human immature DCs specifically upregulate PD-L1 in the presence of exogenous IL-27 (Karakhanova et al., 2011). Further research is needed to define the role of a DC maturation phenotype in differentiation of Tr1 cells driven by exogenous IL-27 as most studies are performed under APC-free culture conditions.

Thus, some extent of costimulation by DCs seems necessary for the generation of Tr1 cells despite the fact that a variety of approaches have used "immature" DCs to generate Tr1 cells *in vitro* (**Table 1**).

COSTIMULATION FOR Tr1-LIKE CELLS BY CHRONIC STIMULATION OF EFFECTOR T CELLS OR DURING INFECTION

PLASTICITY OF T CELLS FOR PRODUCTION OF CYTOKINES OTHER THAN IL-10

The concept of classical lineage commitment into Th1, Th2, and others has been challenged by recent work and it is suggested now that T cells show substantial functional plasticity in their cytokine secretion (O'Shea and Paul, 2010). As an example, IL-13 and IL-10 production by Th1 cells could be demonstrated upon chronic stimulation through TCR and CD28 pathways (Motomura et al., 2011). The Tr1 cells described by Roncarolo and colleagues require IL-10 for their generation and then produce IL-10, some IFN-γ and IL-5 but no IL-4 (Groux et al., 1997; Levings et al., 2001). Originally IL-10 (but also IL-5 and IL-13) was defined as a classical Th2 cytokine, however, the mechanisms and/or costimulatory signals initiating IL-10 production in Th2 cells remained unclear (Moore et al., 2001). Expression of IL-10 by Th2 memory cells has been attributed to repetitive TCR receptor signaling and continuous IL-4 signals (Lohning et al., 2003; Chang et al., 2007). Alternatively, it is well established that the differentiation of IL-10 and IL-4 producing Th2 cells is CD28-signaling dependent but inhibited by CTLA-4 (Bour-Jordan et al., 2003; Coquerelle et al., 2009; Hunig et al., 2010). Indeed, injection of anti-CTLA-4 antibodies in the presence of splenic DCs induced ICOS$^+$ Tregs producing both IL-4 and IL-10 (Coquerelle et al., 2009). In contrast, triggering CTLA-4 signaling on Th1 cells by CD80 from mature DCs seems to promote IL-10 secretion and Tr1 differentiation (Perez et al., 2008). These observations not only emphasize the necessity of DC costimulation for the induction of IL-10 secretion in effector T cells but in addition suggest that CTLA-4 and CD28 might have opposing functions dependent on the initial differentiation program that shaped the original Th1 or Th2 cells. Furthermore, since DC-derived signals are essential in directing differential T helper cell phenotypes, it is tempting to speculate that the common signature regulating IL-10 expression in T cells is in fact driven by DC-derived costimulatory signals.

Tr1-LIKE CELLS GENERATED BY TNF OR β-CATENIN STIMULATED SEMI-MATURE DCs

Many groups, including ours, searched intensively for the DC maturation phenotype that differentiates IL-10$^+$ Tr1-like cells from either Th1 or Th2 cells and whether this would result in tolerance or immunity (Lutz and Schuler, 2002; Lutz, 2006; Tarbell et al., 2006). Tr1-like cell generation might be initiated by triggering the β-catenin pathway in DCs, at least when induced by disrupting E-cadherin mediated homotypic interactions between DCs or after inflammatory stimulation of the DCs (Menges et al., 2002; Spörri and Reis e Sousa, 2005; Jiang et al., 2007). These DCs show a particular semi-mature phenotype characterized by high expression of MHC II and B7 costimulatory molecules but absence of cytokine secretion (Menges et al., 2002; Spörri and Reis e Sousa, 2005). Repetitive injections of such semi-mature DCs prevented the induction of experimental autoimmune encephalomyelitis (EAE) by inducing IL-10$^+$ Tr1-like cells (Menges et al., 2002; Jiang et al., 2007) but also IL-4$^+$ and IL-13$^+$ T and NKT cells (Wiethe et al., 2007). Such TNF-stimulated semi-mature DCs were also protective in models of thyroiditis (Verginis et al., 2005) but failed to prevent CD8-mediated type-1 diabetes (Kleindienst et al., 2005). Surprisingly, semi-mature DCs deficient for the coinhibitory ligand PD-L1 (CD274) showed an increased induction of IL-10 and IL-13 secreting CD4$^+$ T cells *in vivo*, indicating that PD-1 signals counteract Tr1-like cell induction (Brandl et al., 2010).

It remains open at this point whether DC maturation/costimulation profiles inducing either Th2 effector cell differentiation or tolerogenic Tr1-like cells are different or identical.

Tr1-LIKE CELLS DERIVED FROM Th2 CELLS

Although repetitive injections of antigen-loaded semi-mature DC were able to prevent Th1-mediated diseases by inducing increasing numbers of Th2-derived Tr1-like cells with each injection (Menges et al., 2002), the same regimen failed to suppress Th2-mediated footpad swelling in a *Leishmania major* infection model (Wiethe et al., 2008). However, Th1-derived Tr1-like cells effectively suppressed immunity in a Th1-polarized, chronic, non-healing *Leishmania major* infection model (Anderson et al., 2007). Whether the differences in protection depend on the model or on differences between Th1- or Th2-derived Tr1-like cells remains unresolved. In murine parasitic infection models such as *Trypanosoma brucei, Schistosoma mansoni*, and *Echinococcus multilocularis* a cytokine shift to anti-inflammatory IL-10, IL-4, and IL-13 has been reported which is believed to protect the host from extensive tissue damage (Perona-Wright et al., 2006; Stijlemans et al., 2007; MacDonald and Maizels, 2008; Vuitton and Gottstein, 2010). The Th2/Tr1-like profile induced by helminth-derived secretory products of *S. mansoni* eggs or *Nippostrongylus brasiliensis* excretory/secretory antigens have been shown to result in partial DC maturation (Balic et al., 2004; Perona-Wright et al., 2006). Together, parasites or their products result in immature or semi-mature DC signatures, which trigger mild TCR and costimulatory signaling cascades and result in Th2/Tr1-like polarizations. However, how DC maturation signatures regulate the master switch leading to IL-10 production in Th2 or Tr1-like cells remains to be shown.

Tr1-LIKE CELLS DERIVED FROM Th1 CELLS

The ability of Th1 differentiated T cells to produce IL-10 upon chronic stimulation is very well documented (**Table 1**). DCs direct Th1 differentiation as fully mature DCs by providing high TCR and costimulatory signaling in the presence of IL-12p70 cytokine

secretion (Kapsenberg, 2003) or via CD70 signals (Soares et al., 2007). As a difference between Th1- or Th2-derived Tr1-like cells, chronic Th1 stimulation seems to be required for Tr1-like cell generation, while IL-10 release by Th2 effector T cells may occur immediately (Lohning et al., 2003; Motomura et al., 2011). Several reports indicated that the presence of IL-12 released by DCs is essential for IL-10 production in Th1 cells mediated by sustained STAT-4 signaling (Chang et al., 2007; Rutz et al., 2008; Saraiva et al., 2009). Also a high antigen dose favors their IL-10 production (Saraiva et al., 2009). IL-10 induction in Th1 cells was abrogated when DC were deficient in IL-12p40 production suggesting that IFN-γ produced by Th1 cells induced continuous IL-12 production in DCs by a feedback loop (Saraiva et al., 2009).

Alternatively, complement factors, which could be produced locally as a result of cognate DC-T cell interactions, regulated the expression of MHC II, costimulatory molecules and IL-12p70 production (Heeger and Kemper, 2011). Repetitive engagement of the complement regulator proteins CD46 or CD55 on human T cells induced IL-10 production from Th1 cells although addition of exogenous IL-2 was required (Capasso et al., 2006; Cardone et al., 2010), similar as for the generation of human Tr1 cells *in vitro* (Groux et al., 1997; Jonuleit et al., 2000; Levings et al., 2005). It remains open how the CD55 receptor CD97 is regulated on the DC surface. Of note, Th1 cells, which acquire the ability to produce IL-10, gradually lose their expression of IL-2 (Gabrysova et al., 2009; Saraiva et al., 2009). Sustained IL-12 driven STAT-4 signaling is also required for the IL-10 production in IFN-γ-secreting Th1 cells induced by Notch signaling (Rutz et al., 2008). Hence, it is tempting to speculate that chronic stimulations by DCs shift the intrinsic T cell polarization profile from IFN-γ to IL-10 production. Radbruch and colleagues showed that Th1 cells up-regulate the transcription factor Twist1, which requires IL-12 induced STAT-4 signaling for suppression of the Th1-effector cytokines IFN-γ, IL-2, and TNF (Niesner et al., 2008). It is worth to speculate whether this shift might be supported through CTLA-4 signals since gradual CTLA-4 upregulation by Tr1-like cells differentiated from Th1 cells has been observed regularly (**Table 1**). In a rejection model for organ transplantation, Th1 cells adapt to persistent antigen stimulation by upregulation of inhibitory surface molecules and cytokines such as CTLA-4, Twist1, and IL-10 (Noval Rivas et al., 2009). Earlier studies demonstrated that T cells exposed to chronic antigen stimulation down-modulate effector cytokine secretion during the process of so-called adaptive tolerance (Singh and Schwartz, 2003).

Together, IL-12 production by DCs seems to be critical for the induction of IL-10 in Th1 cells by triggering sustained STAT-4 signaling. Whether the low expression of CD80/CD86 on immature DCs is sufficient or rather semi-mature or mature DCs stages are required remains open.

COSTIMULATION FOR DIFFERENT Foxp3$^-$ IL-10$^+$ iTreg TYPES *IN VIVO*

LUNG

The generation of Tr1-like cells has been reported also in the absence of overt infections (**Table 1**). However, Tr1-like cell induction may require some commensal stimulation during steady state conditions through TLRs for both Th1 and Th2 responses. The mucosal application route may favor a Th2 outcome as supported by experiments where exogenous antigens were administered intranasally. The antigens could enter the respiratory but also gastrointestinal tracts and migratory DC transported the intranasally applied antigens to the draining lymph nodes to induce Th2 responses (Constant et al., 2000; Piggott et al., 2005; Derbyshire et al., 2011). Studies by Umetsu and colleagues showed that high dose antigen exposure through intranasal routes caused pulmonary DCs to mature and migrate into the draining lymph nodes where they induced IL-10 and IL-4 secreting Tr1-like cells protecting mice from airway hyper-reactivity (Akbari et al., 2001, 2002). Moreover, Akdis et al. (2004) demonstrated that a fine-tuned balance between aeroallergen-specific Tr1-like and Th2 cells exists in allergic or healthy patients, which may regulate allergy to common environmental proteins. How DCs or lung epithelium contribute to the generation of Th2 effector cells in lung-draining lymph nodes has been studied intensively (Hammad and Lambrecht, 2008). A mature DC signature has been revealed only by one study (Akbari et al., 2001), possibly resulting from minute amounts of endotoxin to allow DC maturation and indicating that costimulation would be required to induce Tr1-like cells.

INTESTINE

In contrast, the oral tolerance phenomenon seem to be dependent largely on DCs present in the lamina propria and mesenteric lymph nodes as shown upon administration of sugar-modified antigens, which trigger differentiation of IL-10 and IFN-γ producing Tr1-like cells (Worbs et al., 2006; Zhou et al., 2010). It has been suggested that the presence of gut microbiota and hence, the particular IL-10$^+$ semi-mature DC signature present under these conditions, contributes to the IL-10 production also of the T cells (Hrncir et al., 2008; Monteleone et al., 2008). TLR-2 has been previously reported to promote IL-10 production by DCs and respective Treg cell induction (Manicassamy et al., 2009). In addition, the β-catenin signaling pathway, although independent of commensal triggering, appeared critical for the tolerogenic phenotype of lamina propria DCs by promoting IL-10 production (Manicassamy et al., 2010). Okamura et al. (2009) identified lymphocyte activation gene-3 (LAG-3)-expressing Tr1-like cells in the intestine, which developed on environmental microbiota. Induction of IL-10 production by Foxp3$^+$ iTregs resulted in response to polysaccharide A from the human commensal *Bacteroides fragilis* and was largely dependent on TLR-2-signaling (Round and Mazmanian, 2010). Thus, the generation of IL-10$^+$ Tr1-like cells but also Foxp3$^+$ iTregs in the gut under steady state conditions is heavily influenced by the local microbiological stimulation leading to DC maturation and induction of costimulation via β-catenin, TLRs, or presumably other signals.

INTRAVENOUS ANTIGEN, CONVERSION FROM ANERGIC T CELLS

In case of intravenous peptide-induced tolerance anergic T cells or Tregs develop from naive precursors upon TCR receptor signaling mostly in the absence of or with weak costimulation under these steady state conditions (Thorstenson and Khoruts, 2001; Jeon et al., 2004; Safford et al., 2005). Intravenous antigen applications induce Foxp3$^+$ Tregs as well as Foxp3$^-$ Tr1 or Tr1-like cells both of which require high affinity TCR agonists (Gabrysova and Wraith, 2010;

Gottschalk et al., 2010). While single injections of low doses of peptides suffice to generate Foxp3$^+$ Tregs (Gottschalk et al., 2010), their Tr1 or Tr1-like counterparts are formed after several repetitive injections of high doses of peptide (Gabrysova and Wraith, 2010). Whether antigen doses and application regimens are decisive in this process is unclear. Most likely local environmental differences such as the availability of TGF-β and all-trans retinoic acid might further contribute to Foxp3 induction (Maynard et al., 2007, 2009). In any case, these protocols follow antigen applications in the absence of inflammatory or microbial stimulation, indicating that B7 costimulation may not be involved. Whether the resulting IL-10$^+$ cells in these assays are Tr1 or Tr1-like cells or another subtype is unclear. On one hand they may be considered as Tr1 cells because they are derived from naive T cells under low costimulation conditions and not from polarized immunogenic Th cells. On the other hand several rounds of stimulation with strong antigenic and costimulatory conditions are more similar to Tr1-like cells. Clear genetic markers would be needed to discriminate between Tr1 cells, Tr1-like cells, and these IL-10$^+$ T cells described here to clarify this point.

ROLE OF COSTIMULATION FOR THE EXTRATHYMIC *DE NOVO* CONVERSION OF Foxp3$^+$ iTregs

Other major subtypes of Tregs are characterized by their expression of Foxp3. While natural Foxp3$^+$ nTregs are directly released from the thymus independent of DCs (Kyewski and Klein, 2006; Ohkura and Sakaguchi, 2010), conversion of Foxp3$^+$ iTregs occurs in peripheral lymphatic organs by interactions with DCs. The basal requirements for the development of Foxp3$^+$ iTregs *in vitro* and *in vivo* are defined by TCR signaling and the presence of the cytokines IL-2 and TGF-β (**Figure 1**; Fantini et al., 2004; Davidson et al., 2007; Curotto de Lafaille and Lafaille, 2009). In addition, costimulatory and coinhibitory molecules on the surface of DCs importantly regulate the differentiation and functions of iTregs from naive T cells (Bour-Jordan et al., 2011). Although more and more costimulatory and coinhibitory molecules of the B7 and TNF-families have been identified on DCs, which also affect peripheral T cell tolerance, the definite requirements for CD80/CD86 costimulation for Foxp3$^+$ Treg biology are still under debate and therefore will be discussed here. Also PD-L1/PD-L2 heavily contribute to Treg induction and function, which is discussed in more detail elsewhere (Francisco et al., 2010) but will be briefly touched below.

CD80/CD86 MOLECULES FOR Foxp3$^+$ iTreg CONVERSION

Initial evidence that these costimulatory molecules might play a role in Treg development reveal from studies using mice deficient for CD80/CD86 or CD28 or treated with the respective blocking antibodies. In both cases, mice exhibited markedly reduced numbers of Foxp3$^+$ Tregs in the thymus and the periphery (Salomon et al., 2000; Bour-Jordan et al., 2004, 2011; Tang et al., 2004). However, these studies did not discriminate between effects of CD80/CD86 molecules on nTreg or iTreg development. First indications for peripherally induced iTregs come from investigations on the conversion of naïve CD4$^+$ CD25$^-$ T cells into CD4$^+$ CD25$^+$ Tregs after adoptive transfer into CD80/CD86$^{-/-}$ mice (Liang et al., 2005). Under these conditions, naive CD4$^+$ CD25$^-$ T cells are unable to convert into Tregs, suggesting a role of CD80/CD86 molecules in iTreg induction (Liang et al., 2005). Although in this study Tregs were not defined by expression of Foxp3, these findings are in line with our *in vivo* conversion data using a transgenic skin-antigen model and adoptive transfer of antigen-specific naive CD25$^+$ Foxp3$^-$ T cells (Azukizawa et al., 2011). In this model, elevated levels of costimulatory molecules were found on DCs migrating and transporting self-antigen under these steady state conditions, thus representing a semi-mature phenotype. These steady state migratory DCs induced peripheral conversion of naive T cells into Foxp3$^+$ iTregs, dependent on TGF-β/latency-associated peptide (LAP) on the DC surface (Azukizawa et al., 2011). This has been also described for specific tolerogenic DC subsets in the intestine (Coombes et al., 2007; Sun et al., 2007) and spleen (Yamazaki et al., 2008). Furthermore, mature bone marrow-derived DCs could promote iTreg differentiation *in vitro*, but only in the presence of exogenous TGF-β (Yamazaki et al., 2007). These reports suggest that DCs of all maturation stages are able to induce Foxp3 expressing iTregs, but the T cells prefer no or low CD28 signals. A strict requirement seems TGF-β, which can be either produced by DCs or is recruited from the environmental milieu.

CD28 VERSUS CTLA-4 FOR iTreg CONVERSION

Several studies indicate that the maturation of DCs and therefore a high costimulation is counteracting iTreg generation (Kretschmer et al., 2005; Yamazaki et al., 2007; Wang et al., 2008). Kretschmer et al. (2005) found that DC maturation *in vivo* upon injection of agonist anti-CD40 antibodies diminished their potential to promote iTreg conversion. *In vitro* Treg conversion assays with splenic DCs from CD80$^{-/-}$/CD86$^{-/-}$ mice indicated that both molecules on DCs are not directly required for the differentiation of Foxp3$^+$ iTregs (Yamazaki et al., 2007; Wang et al., 2008) but they might promote the production of IL-2 by effector T cells, which in turn enhances iTreg generation and maintenance (Yamazaki et al., 2007). On the other hand, two studies using agonistic anti-CD28 antibodies and CD80$^{-/-}$/CD86$^{-/-}$ mice demonstrated that at least a low CD28 costimulation was required for naive T cells to become iTregs, whereas strong CD28 interaction induces the differentiation of Teff cells (Benson et al., 2007; Semple et al., 2011). In this context it has to be taken into account that both effector T cells and Tregs themselves upregulate CD80 and CD86 on their surface after activation and CTLA-4$^+$ Tregs even suppress effector T cells by ligating their CD80/CD86 molecules (Paust et al., 2004). In this scenario also T–T cell communication through CD28/CTLA-4 and CD80/CD86 can occur in the absence of APCs and may contribute to Treg conversion. Together, CD28 costimulation appears to counter-regulate TGF-β dependent iTreg conversion and survival *in vitro* but also *in vivo* (**Figure 1**).

Unlike CD28 costimulation the interaction between CD80/CD86 molecules on DCs and CTLA-4 on T cells initially does not seem to play a role in the development of Tregs, since CTLA-4$^{-/-}$ mice exhibit normal numbers of Foxp3$^+$ T cells (Tang et al., 2004; Kataoka et al., 2005; Wing et al., 2008). Two groups further analyzed the role of CTLA-4 in the differentiation of iTregs using CTLA-4 blocking antibodies and naive CTLA-4$^{-/-}$ T cells in Treg conversion assays *in vitro* (Zeng et al., 2006; Wang et al., 2008).

Both groups found a reduced induction of Foxp3 expressing iTregs despite the presence of costimulation provided by anti-CD28 antibodies or splenic DCs (Zeng et al., 2006; Wang et al., 2008). These results cannot be explained simply by a different expression of the CTLA-4 ligands CD80/CD86 by DCs, since high costimulatory levels by anti-CD28 antibodies and low costimulatory levels by immature splenic DCs were used (Zeng et al., 2006; Wang et al., 2008). Therefore, further investigations are needed to clarify the role of CTLA-4 in iTreg development.

In this context the question remains, whether semi-mature steady state migratory DCs are tolerogenic because of or despite their costimulatory capacity. In agreement with previous results, Benson et al. (2007) could show in *ex vivo* studies that CD80/CD86 molecules expressed by splenic DC inhibit iTreg conversion, but administration of IL-2 and TGF-β in the absence or presence of retinoic acid can overcome the inhibition of iTreg differentiation, indicting a dominant role for IL-2 and TGF-β despite CD80/CD86 cosignaling. IL-2 production by DCs can hardly be detected under steady state conditions (Granucci et al., 2003). However, steady state migratory DCs employ endogenous TGF-β or the TGF-β binding partner LAP to induce Treg conversion (Azukizawa et al., 2011) and since these DCs are skin-derived and in close contact with TGF-β producing keratinocytes, the environmental milieu in which such migratory DCs were embedded might also affect their tolerogenic potential.

Together, these observations suggest that immature costimulatory^{low} DCs have the highest capacity to induce iTregs *in vivo* and *in vitro*, but costimulatory^{int} semi-mature and costimulatory^{high} mature DC are still able to induce iTregs. The semi-mature DC phenotype might reflect an evolutionary compromise between partial activation to upregulate CCR7 and MHC II to enable their migration to the lymph nodes and improve presentation of peripheral self-antigens with only moderate CD80/CD86 upregulation. In fact studies on human migratory DC indicated that they might maintain an immature phenotype with respect to HLA-DR and CD80/CD86 expression while CCR7 was upregulated (Geissmann et al., 2002; Verbovetski et al., 2002).

PD-L1 AND PD-L2

PD-L1 is constitutively expressed by most hematopoietic cells and can be further upregulated upon activation. In contrast, PD-L2 is inducible on DCs, macrophages, and B cells. PD-L1 and PD-L2 both are the ligands of PD-1, which is expressed on activated B and T cells as well as resting Tregs (Francisco et al., 2010). The role of PD-L1 and PD-L2 for iTreg generation has been first studied by Noelle and colleagues, which showed that blocking of PD-L1, but not of PD-L2, negatively affects the induction of Foxp3$^+$ iTregs by immature splenic DCs *in vitro* (Wang et al., 2008). The same group also established a tumor antigen model demonstrating that DC-derived PD-L1 was required for tumor-induced iTreg conversion (Wang et al., 2008). PD-L1$^{-/-}$ mice used in this study show significant changes in their expression of other costimulatory molecules such as CD80, CD86, and CD40 (Wang et al., 2008). PD-L1 and TGF-β play a synergistic role in the differentiation of iTregs, since both factors alone induce less iTregs *in vitro* than in combination (Francisco et al., 2009). In addition, mice deficient for PD-L1, PD-L2, and Rag1 have a decreased capacity to induce iTregs in comparison with WT Rag1$^{-/-}$ mice (Francisco et al., 2009).

In sum, these data suggest a pivotal role for DC-derived PD-L1 on iTreg development. All studies described above were performed under steady state conditions or with isolated immature DC indicating that the constitutive level of PD-L1 expressed by DC is sufficient for iTreg generation.

OTHER COSTIMULATORY AND COINHIBITORY MOLECULES FOR iTregs

Despite the importance of CD80/CD86 and PD-L1/PD-L2, other regulatory molecules significantly influence iTreg conversion. Since further detailed review will be beyond the scope of this article only two examples will be mentioned. Consistent with findings from blocking CD80 and CD86 *in vitro*, CD40 blockage on splenic DCs increased iTreg generation (Wang et al., 2008), supporting the inhibitory effect of costimulation on Treg differentiation. *In vitro* conversion assays with splenic DCs treated with an agonistic GITR antibody showed a diminished induction of Foxp3$^+$ T cells, indicating also a costimulatory role of GITR in Treg conversion (Wang et al., 2008).

ROLE OF DC COSTIMULATION FOR Foxp3$^+$ nTreg MAINTENANCE

The TCR-specificities of natural Treg and effector T cell repertoires are overlapping between 10 and 40%, depending on the study (Hsieh et al., 2004; Pacholczyk et al., 2006; Wong et al., 2007). Among the Tregs some recognize self-antigens and may continuously undergo activation in those lymph nodes where steady state migratory DCs transport and present peripheral tissue antigens to specific T cells (Fisson et al., 2003), whereas others are specific for foreign antigens derived from pathogens (Belkaid and Rouse, 2005; Kretschmer et al., 2005).

CD80/CD86

Dendritic cells can influence the expansion and survival of Tregs by different mechanism. The interaction of CD80/CD86 molecules with CD28 on Tregs is a major requirement for the thymic development and the peripheral homeostasis of Tregs (Salomon et al., 2000; Tang et al., 2003). Previous data by Bar-On et al. (2011) support this idea since CD80/CD86 provided by DC was crucial for maintenance of the peripheral Treg pool. In this study bone marrow chimeras lacking CD80/CD86 specifically on DCs were used, which result in a significantly reduced frequency and number of Tregs in the periphery, but not in the thymus (Bar-On et al., 2011). Thus, the costimulatory molecules CD80 and CD86 on DC are required to mediate Treg homeostasis. In agreement with the above described observations costimulatory^{high} mature antigen-bearing DCs induce effectively Treg expansion *in vitro* and *in vivo* (Yamazaki et al., 2003; Fehervari and Sakaguchi, 2004b). The proliferation of Tregs in those studies was dependent on CD80/CD86 expressed by DCs and small amounts of IL-2 (Yamazaki et al., 2003, 2007; Yamazaki and Steinman, 2009), which was mainly produced by effector T cells and not DCs (Fehervari and Sakaguchi, 2004b). Of note, costimulatory^{low} immature DCs are less effective than mature DCs in promoting Treg expansion *in vitro* (Fehervari and Sakaguchi, 2004a). However, under steady state conditions only immature and semi-mature DCs with basal to intermediate levels of costimulatory molecules are present in secondary

lymphoid organs (Azukizawa et al., 2011). Fully mature DC phenotypes appear upon infection or inflammation (Lutz and Schuler, 2002) and therefore cannot play a role in Treg homeostasis. Rather mature DCs are suggested to be required for the induction of protective effector T cell responses and its subsequent control by Treg in two ways. First, mature DCs secrete pro-inflammatory cytokines like IL-6, which inhibit the suppressive activity of Tregs (Fehervari and Sakaguchi, 2004a). Second, by interacting of CD80/CD86 with CD28 on Tregs mature DCs may induce expansion of Tregs at the peak of the immune response resulting in the suppression of effector T cells. It remains to be determined how semi-mature DCs in comparison to immature DCs affect peripheral Treg numbers.

In this context it has to be considered that under steady state conditions lymph node resident immature DCs and migratory semi-mature DCs present self-antigens of different sources (Lutz et al., 2010; Azukizawa et al., 2011). While migratory DC take up low doses of soluble or cell-associated self-antigens in peripheral tissues, lymph node resident immature DC can only capture and present high doses of soluble antigens transported from the conduit system (Sixt et al., 2005).

IL-2

As mentioned above, DCs are also required for Treg homeostasis by inducing IL-2 production in effector T cells (Yamazaki et al., 2003, 2007; Fehervari and Sakaguchi, 2004a). IL-2, rather than IL-7 and IL-15, is an essential growth factor for Tregs to survive and expand in the periphery (**Figure 1**; Almeida et al., 2002; Malek et al., 2002; D'Cruz and Klein, 2005; Setoguchi et al., 2005). To induce IL-2 expression in effector T cells, B7/CD28 interaction between DCs and effector T cells is required (Tang et al., 2003; Yamazaki et al., 2003, 2007). However, the contribution of DC-derived IL-2 in Treg homeostasis is controversial. Whereas several *in vitro* studies have revealed that IL-2 secreted by DCs plays no role in Treg maintenance (Yamazaki et al., 2003, 2007; Fehervari and Sakaguchi, 2004a), another study indicated that DC-Treg interaction via CD40/CD40L induces IL-2, which is required for Treg expansion (Guiducci et al., 2005). However, DCs have been shown to produce IL-2 only upon microbial activation but not under steady state conditions (Granucci et al., 2003). Thus, the question remains whether immature and semi-mature DCs under steady state conditions are capable to produce IL-2 and if so, what is its relevance for Tregs.

Fms-LIKE TYROSINE KINASE 3 LIGAND

The control of Treg homeostasis has also been linked to DC numbers and the level of Fms-like tyrosine kinase 3 ligand (Flt3L; Liu et al., 2003; Darrasse-Jeze et al., 2009; Swee et al., 2009; Suffner et al., 2010). Flt3L is a hematopoietic growth factor supporting proliferation and differentiation of DC precursors (Karsunky et al., 2003; Liu et al., 2003). Repetitive injections of Flt3L into mice resulted not only in an increased number of DCs, but also enhanced the proliferation of peripheral nTregs (Swee et al., 2009). *In vitro* experiments by the same group indicated that Flt3L-mediated nTreg expansion was directly dependent on DC contact and IL-2, but not on TCR engagement (Swee et al., 2009). In contrast, earlier studies showed that fully mature DC generated from bone marrow by GM-CSF most efficiently induced Treg expansion (Yamazaki et al., 2003), while both immature and mature Flt3L-generated bone marrow-derived DCs had a similar capacity to promote Treg proliferation (Swee et al., 2009). Others claimed that a feedback loop exists, which regulates the homeostasis of both Tregs and DCs in a Flt3L- and MHC II-dependent manner (Darrasse-Jeze et al., 2009; Hochweller et al., 2009).

TONIC SIGNALS FROM MHC II MOLECULES

Under steady state conditions T cells scan self-MHC molecules to achieve basal activation state enabling a faster subsequent response upon recognition of foreign antigens (Stefanova et al., 1989). A recent study by Hochweller et al. (2010) revealed that the responsiveness of T cells toward their cognate antigen specifically depends on the recognition of self-MHC molecules on DCs but not other APC. Since Tregs express a TCR with a higher affinity for self-MHC molecules as compared to effector T cells, it was found by two-photon microscopy *in situ* that antigen-presenting DCs interact for longer periods with TCR transgenic antigen-specific Tregs than effector T cells (Tadokoro et al., 2006; Tang et al., 2006). It would be interesting to investigate if such tonic TCR signals also play a role in Treg homeostasis *in vivo*. An initial study showed that inactivation of Lck in Tregs and thereby abrogating their TCR signaling results in an impaired proliferation and homeostatic expansion of those Tregs (Kim et al., 2009). These data indicate that DC–T cell interaction via TCR and MHC II molecules affect the maintenance of Tregs.

In sum, these observations strongly support a crucial role of DCs in Treg homeostasis and demonstrate that DCs affect the proliferation of Tregs by several mechanisms such as their expression of costimulatory molecules, surface TGF-β/LAP, the induction of IL-2 production and their frequency in lymphoid organs.

SUPPRESSIVE ACTIVITY OF Tregs ON DCs

Despite the fact that numerous effector mechanism for Treg-mediated suppression have been described (Roncarolo et al., 2006; Sakaguchi et al., 2008; Shevach, 2009; Yamazaki and Steinman, 2009; Weiner et al., 2011), the question remains, whether Tregs can act directly on effector T cells or all regulation occurs indirectly through DCs or other APCs? Clearly, Tregs can directly suppress T cell responses *in vitro* since APC-free co-cultures of Tregs and effector T cells, stimulated by CD3 and CD28 antibodies, show suppressive activities (Dieckmann et al., 2002). On the other hand major suppression mechanisms can also act on DCs, as described below. The relative contribution of both Treg targeting strategies *in vivo* is less well understood (Miyara and Sakaguchi, 2007; Shevach, 2009). Our own findings indicated that both direct and indirect mechanisms occur simultaneously in the spleen by using adoptively transferred DCs and $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs. In this setting the indirect effect on DC exceeded the direct suppression of effector T cells (Hänig and Lutz, 2008).

COMPETITION FOR DC

Some published data suggest that Tregs interfere with the ability of DCs to activate effector T cells, either by physically obstructing effector T cells to DCs contact as shown *in vitro* (Onishi et al., 2008). However, competition could not be confirmed *in vivo* during effector T cell responses or under regulatory conditions

even using high numbers of adoptively transferred mature DCs, Tregs and effector T cells and then following the cluster formation and sizes in spleens of mice (Hommel and Kyewski, 2003; Hänig and Lutz, 2008). Most probably, the three dimensional and highly dynamic T cell turnover in lymphatic organs cannot be reflected *in vitro* where DCs highly aggregate homotypically via E-cadherin interaction (Jakob and Udey, 1998; Riedl et al., 2000; Jiang et al., 2007).

TREGS PREVENT DC MATURATION

Tregs preclude upregulation of costimulatory molecules such as CD80, CD86, PD-L1, PD-L2, and CD40 but also the number of MHC-peptide complexes. In addition Tregs may reduce the production of inflammatory cytokines by DCs such as IL-12, IL-1β, IL-6, and IL-8 (Fallarino et al., 2003; Misra et al., 2004; Feunou et al., 2007; Larmonier et al., 2007; Hänig and Lutz, 2008; Wing et al., 2008; Andre et al., 2009). Many of these functions remained unexplained or could be attributed to soluble factors like IL-10 or TGF-β production by Tregs as discussed above for IL-10 by Tr1 and Tr1-like cells.

Treg SURFACE MARKERS: LAG-3, CD39

Onishi et al. (2008) showed that DC-Treg aggregation occurs as a first step where LAG-3, a CD4-related transmembrane protein, expressed by Tregs and activated effector T cells binds MHC II molecules on DCs, followed by CTLA-4-dependent prevention of CD86 upregulation. Additional function of LAG-3 has been described, proving that LAG-3 expressed on the Tregs surface leads to MHC II molecule crosslinking and consequential Erk-mediated recruitment of SHP-1 responsible for the inhibition of DC maturation l (Liang et al., 2008).

Another surface molecule with immunosuppressive activity expressed by Tregs is CD39 (nucleoside triphosphate diphosphohydrolase-1), an ectoenzyme that degrades ATP to AMP. Extracellular ATP has an adjuvant feature in inflammation (Gallucci et al., 1999), whereas its degradation product AMP acts anti-inflammatory (Kumar and Sharma, 2009). CD39 expression is constitutive in Tregs and controlled by Treg master transcription factor Foxp3. After TCR ligation, the activity of membrane-bound CD39 increases (Borsellino et al., 2007).

Treg SOLUBLE MEDIATORS: IL-10, TGF-β, IDO, GLUTHATHIONE

In the early nineties it has been demonstrated that exogenous or Th cell-produced IL-10 can inhibit costimulatory function of macrophages, Langerhans cells, and DCs (Ding and Shevach, 1992; Caux et al., 1994; Péguet-Navarro et al., 1994). In most of the studies exogenous IL-10 was used, although Ding and Shevach (1992) designated a Th2 subset as a source of this cytokine. Activated Tr1 or Tr1-like cells are the source of IL-10 and as such prevent DC maturation (see above). Similar effects were observed with TGF-β (Epstein et al., 1991; Bonham et al., 1996). Soon after Tregs were discovered, it was found that this cell population represents the source for both cytokines and can inhibit DC maturation in a similar manner as exogenous cytokines (Hara et al., 2001; Suri-Payer and Cantor, 2001; Belghith et al., 2003; Li et al., 2007).

Tregs express high level of CTLA-4 and several CTLA-4-dependent mechanisms exist through which Tregs inhibit DC function. DCs incubated with CTLA-4$^+$ Tregs or CTLA-4–Ig fusion proteins triggering CD80/CD86 lead to indoleamine 2,3-dioxygenase (IDO) production (Fallarino et al., 2003; Feunou et al., 2007). The major effect of IDO is tryptophan degradation, causing its depletion, and the production of proapoptotic metabolites.

Another effect of the CTLA-4 interaction is Treg-mediated redox perturbation. In this case, Treg-DC contact results in CTLA-4-dependent diminished glutathione (GSH) synthesis in DCs via decreased expression of γ-glutamylcysteine synthetase, the limiting enzyme for GSH synthesis (Yan et al., 2010).

Altogether, Treg-modified DCs maintaining an immature or only semi-mature status are incapable of eliciting strong, productive immune responses, and lead to induction of tolerance.

DOWNREGULATION OF COSTIMULATORY MOLECULES FROM THE MATURE DC SURFACE

While prevention of DC maturation could be a significant regulatory mechanism to avoid unwanted immunity, the question remains to which extent are mature/activated DCs sensitive to Treg cell inhibitory action? This topic has been partially assessed by several groups. Some authors claim that strong TLR-stimuli completely abrogate auto-regulation (Pasare and Medzhitov, 2003; Iwasaki and Medzhitov, 2004; Kabelitz et al., 2006). Furthermore, inflammatory cytokines produced by activated DCs seem to impede Treg functions (King and Segal, 2005; Valencia et al., 2006; Wan et al., 2007). On the other hand, although not directly addressing this question in the light of time kinetics, Veldhoen et al. (2006) could show that the repertoire of cytokine production of the LPS- and CpG-matured DCs changes from inflammatory to immunosuppressive depending on the presence of Tregs in the cultures. This result partially opposes the previous claim that strong TLR-stimuli completely disqualify Treg effects. Our own group and that of Santamaria could show that mature DCs are only partially sensitive to Tregs by modulating CD80/CD86 and PD-L1/PD-L2 but no other markers, since the Treg effect could be abrogated by CD40 licensing (Serra et al., 2003; Hänig and Lutz, 2008). Although not using additional stimuli for DC maturation beside KLH and OVA, a study of Oldenhove et al. (2003) presented convincing set of data pointing to the ability of Tregs to attenuate Th1 responses initiated by transfer of mature DCs. Remarkably, Treg depletion in the same model reflected negatively on Th2 development. Recently, a mechanism for the disappearance of CD80 and CD86 molecules on ApPCs was provided by a mechanism called trans-endocytosis, meaning the removal of individual CD80/CD86 molecules but not whole membrane patches (trogocytosis) out of the APC membrane by CTLA-4 molecules (Qureshi et al., 2011). The captured molecules are then rapidly internalized, explaining the known high turnover and predominant intracellular expression of CTLA-4.

CONCLUSION

All available data indicate that DCs interact by many means with T cells not only for induction of effector T cells but also to generate and maintain Tregs and in turn also to be controlled by Tregs. While TCR-specific activation is required for all Treg suppressor functions so far, the induction of Tr1 or Tr1-like cells

clearly requires also CD80/CD86 costimulation. Tr1 or Tr1-like cell generation may be further supported by IL-10. In contrast, Foxp3$^+$ iTreg generation occurs more efficient without CD80/CD86 signals and requires TGF-β. The maintenance and activation of nTreg is strongly supported by high levels of CD80/CD86 and IL-2. Finally a major control mechanism of mature, but not CD40-licensed, DCs occurs through CTLA-4-dependent activity on CD80/CD86 molecules by trans-endocytosis and IDO induction at the DC-Treg interface. Future research activities, which have to consider also the CD80/CD86 expression on activated Tregs and effector T cells, will lead to a more complete understanding of these molecules and how this can be exploited to develop new therapies.

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The molecular mechanisms of regulatory T cell immunosuppression

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$CD4^+CD25^+Foxp3^+$ T lymphocytes, known as regulatory T cells or T_{regs}, have been proposed to be a lineage of professional immune suppressive cells that exclusively counteract the effects of the immunoprotective "helper" and "cytotoxic" lineages of T lymphocytes. Here we discuss new concepts on the mechanisms and functions of T_{regs}. There are several key points we emphasize: 1. Tregs exert suppressive effects both directly on effector T cells and indirectly through antigen-presenting cells; 2. Regulation can occur through a novel mechanism of cytokine consumption to regulate as opposed to the usual mechanism of cytokine/chemokine production; 3. In cases where $CD4^+$ effector T cells are directly inhibited by T_{regs}, it is chiefly through a mechanism of lymphokine withdrawal apoptosis leading to polyclonal deletion; and 4. Contrary to the current view, we discuss new evidence that T_{regs}, similar to other T-cells lineages, can promote protective immune responses in certain infectious contexts (Chen et al., 2011; Pandiyan et al., 2011). Although these points are at variance to varying degrees with the standard model of T_{reg} behavior, we will recount developing findings that support these new concepts.

Keywords: tregs, Th17, immune suppression mechanism, BIM cytokine deprivation death, apoptosis, regulatory T cells, Foxp3, cytokine consumption

INTRODUCTION

The immune system maintains a delicate balance between adaptive lymphocyte responses to foreign antigens that efficiently counteract a myriad of microbial assaults, but maintain unresponsiveness or "tolerance" to a large variety of harmless self-antigens. This requires distinguishing harmless "self" (that also includes commensal microbes) from harmful "non-self" throughout the lifetime of the host. When this balance is disturbed, it can lead to inflammatory disorders, autoimmunity, and allergy. Although immunity involves panoply of different cell types, we will focus principally on the control of T lymphocytes, which serve to orchestrate most immune responses. Tolerance is achieved in part by selecting against self-reactive T lymphocytes during ontogeny in the thymus, a powerful mechanism termed as central tolerance (Gershon and Kondo, 1971; Bonomo et al., 1995; Zheng et al., 2003; Kyewski and Klein, 2006; St Clair et al., 2007). However, there are "peripheral" tolerance mechanisms that divert immune responses away from self and toward appropriate pathogens (Miller and Morahan, 1992; Arnold et al., 1993; Anderson and Chan, 2004; Anderson et al., 2005; St Clair et al., 2007; Moraes-Vasconcelos et al., 2008).

Peripheral tolerance can be achieved through multiple mechanisms affecting the survival, differentiation, and function of effector T lymphocytes (Sprent, 1995). In this review, we will focus on the programmed death of T cells which in the periphery, accounts generally for propriocidal or autoregulatory deletion of self-reactive cells upon chronic restimulation by self-antigens (deletional tolerance; Lenardo et al., 1999; Sprent and Surh, 2001). Peripheral deletion can be mediated by cell death pathways involving Fas, TNF-α, granzymes, or passive cytokine deprivation pathways driven by pro-apoptotic members of the Bcl-2 family such as Bim (Chen et al., 1995; Stockinger, 1999; Strasser et al., 2000; Bidere et al., 2006). Peripheral deletion pathways are typically clonal in that specific engagement of antigen receptors in the T-cells activity on internal death program leading to deletion (Lenardo, 1991; Hornung, 1997). Other mechanisms of peripheral tolerance not discussed in this review include rendering self-specific cells inactive (anergy), deviating the immune system to innocuous or protective responses (immune deviation), adjusting lymphocyte responses so that available antigen levels are below the threshold of detection (clonal ignorance) or suppression of local responses by biological mediators such as CTLA-4, IL-10, and TGF-β (suppression or regulation; Schwartz, 2003; Anderson and Chan, 2004).

Recently, a great deal of experimental work has centered on a form of dominant tolerance mediated by a class of thymus-derived $CD4^+$ $CD25^{hi}$ CTLA-4^{hi} $Foxp3^+$ T lymphocytes, known as regulatory T cells or T_{regs}, that appear to suppress effector T-cell function (Sakaguchi et al., 2008). There are long-standing antecedents to that concept. T lymphocytes regulate immune responses both positively and negatively. Historically, a binary paradigm, which categorized $CD4^+$ cells as "helpers" and CD8 cells as "suppressors" was put forward, as soon as T cells could be distinguished by cell surface markers. This paradigm has had a powerful influence on the literature of immunology (Janeway et al., 1975; Damle, 1986). However, this concept lost ground when CD8 cells were found to mediate protective anti-viral cytolysis and other problems arose in the characterization of "suppressors" (Shevach, 2011). A similar binary paradigm has been reincarnated in the past decade for CD4 cells such that Th1, Th2, and Th17 cells

mediate immunoprotective helper (and autoimmune) functions and CD4+CD25+Foxp3+T_{reg} cells mediate suppressive or anti-helper functions (Zhu and Paul, 2010). The main functions of the helper cells are that Th1 cells express interleukin-2 (IL-2), interferon- (IFN)-γ, and lymphotoxin (LT) or TNF-α, Th2 cells produce IL-4 and IL-5, and Th17 cells produce IL-17A, -17F, IL-22, and TNF-α (Bettelli et al., 2007). On the other hand, T_{regs} express IL-10, TGF-β, CD25, and Foxp3 but do not produce inflammatory cytokines under most situations (Fontenot and Rudensky, 2005). Severe atopic autoimmune conditions in *scurfy* mice and immune dysregulation polyendocrinopathy X-linked (IPEX) syndrome patients with mutations in FOXP3, highlight the functional importance of T_{regs} in suppressing severe autoimmune reactions in mice and humans (Bennett et al., 2001).

However, less attention has been focused on the fact that immunoprotective functions are defective when FOXP3 and presumably T_{reg} cells are lacking (Costantino et al., 2008; Coutinho and Carneiro-Sampaio, 2008). IPEX is a primary immunodeficiency because severe infections involving bacteria, viruses, and fungi such as *Candida* sp. affecting a variety of organ systems contribute to early mortality in IPEX patients (Moraes-Vasconcelos et al., 2008). The immunoprotective functions of Fox P3 and presumably T_{regs} have not been elucidated in detail. Recent research has provided important new insights into the regulator behavior of T_{regs}, both as suppressors and immune stimulators. Here we discuss the molecular mechanisms governing immune responses, especially T lymphocyte effector functions by T_{regs}, with an emphasis on polyclonal deletional tolerance (Pandiyan et al., 2007). Furthermore, we review the recent descriptions of immunoprotective functions of T_{regs} including evidence for functional plasticity in T_{regs}, depending on the immune environment (Pandiyan et al., 2011). Finally, we suggest an alternative model of immunoregulation and immunoprotection functions being distributed among all CD4+ T lymphocyte subtypes and each subset has important positive and negative immunoregulatory roles.

T_{regs} – DISCOVERY AND FUNCTIONS

$CD4^+CD25^+Foxp3^+$ T_{regs} [natural (n) T_{regs}] are thymus derived, and constitute 5–10% of peripheral CD4 T cells. They are said to be "anergic" because they do not express the IL-2 gene and proliferate poorly when stimulated alone under most conditions. The suppressive capacity of T_{regs} was identified *in vivo* by seminal experiments showing that day 3 thymectomized mice and mice depleted of T_{regs} succumbed to systemic autoimmunity (Sakaguchi et al., 1982, 1996). The ability of T_{regs} to restrain auto-aggressive immune reactions, led to the idea that these cells represented a type of specialized suppressor cells. However, other *in vitro* data show that addition of exogenous IL-2 and α-CD28 combined with antigen-presenting cells (APC) not only breaks the anergic state and promotes T_{regs} proliferation, but also largely abrogates the suppression effect (Takahashi et al., 1998; Thornton et al., 2004b). This opened the door to possible immunological roles for T_{regs} besides suppression, but until recently this has been largely unexplored.

The plurality of mechanisms that have been postulated by different groups to explain nT_{regs} function in different *in vitro* and *in vivo* settings is remarkable (Shevach, 2002; Von Boehmer, 2005; Rudensky and Campbell, 2006; Tang and Bluestone, 2008). Many investigators detect immune regulatory effects by culturing a mixture of T_{regs} and effector T cells *in vitro* (Takahashi et al., 1998; Thornton and Shevach, 1998; Von Boehmer, 2005; Pandiyan et al., 2007; Tran et al., 2009a). The manner in which such mixtures are prepared and examined often leads to varied conclusions but mainly interpreted to reinforce the preconceived binary paradigm of helpers/suppressors. For example, the presence or absence of α-CD28 antibodies, APC, cell density, and types of target cells in such mixtures have led to different interpretations by different investigators (Takahashi et al., 1998, 2000; Thornton and Shevach, 1998; Pandiyan et al., 2007). Failing to take into account the experimental details, such as the target cells that are suppressed, cell density, TCR activation strength, has caused confusion and even misleading information regarding the regulatory action of T_{regs} (Takahashi et al., 1998, 2000; Thornton and Shevach, 1998; Tran et al., 2009b). This is illustrated in the following examples.

First, one *in vitro* study sought to establish that T_{reg} cells serve as "professional" suppressor cells that shut off IL-2 gene transcription when present during the stimulation of effector T cells, ostensibly supporting the helper/anti-helper paradigm (Thornton and Shevach, 1998). In this study, however the fate (cell death) of the effector T cells was not examined. Later studies showed that consumption of IL-2 and not IL-2 production suppression by the T_{reg} cells led to lymphokine withdrawal death of the effector cells indicating that decreased thymidine incorporation was due to death of the effector cells (Pandiyan et al., 2007). This is explained by the fact that, in this conventional proliferation assay, the indicator cells are the proliferating cells themselves and if these cells are dying, then no conclusions can be drawn regarding the "suppression" of proliferation. IL-2 is well-known to be regulated at both transcriptional and message stability levels (Zhu et al., 2010). Since the effector T cells die under these conditions, less steady-state mRNA was found, but when examined, no direct suppressive effect on the IL-2 promoter or gene transcription was found (Klein et al., 2003; Pandiyan et al., 2007). Finally, the conclusion drawn from these assays was that T_{reg} cells were "professional suppressor cells" that are solely suppressive, but no experiments were done to assess their helper functions. The later discovery that T_{reg} cells can promote the differentiation and cytokine production by Th17 cells in an immunoprotective setting (described below) undermines the binary concept of "professional" helpers and suppressors in the CD4 T lineages. This is important because current investigations involve injecting T_{reg} cells as therapy for human disease based on the professional suppressor notion. However, current findings indicate that the injected T_{reg} cells may have unpredictable effects or even exacerbate disease owing to their plasticity (see below; Zhou, 2008; Zhou et al., 2009a; Zhu and Paul, 2010; Pandiyan et al., 2011).

Similarly, *in vivo* assays have led different investigators to explain immunosuppressive effects by a variety of molecules such as IL-10, TGF-β, IL-35, and CTLA-4 among others. IL-10 produced by T_{regs}, seems to be suppressive *in vivo*, keeping immune responses in check at environmental interfaces of the host, such as the colon and lungs (Klein et al., 2003; Kearley et al., 2005; Rubtsov et al., 2008). However, it is puzzling that IL-10 production by T_{reg} cells is not required for the control of T cells *in vitro*

(Thornton and Shevach, 1998; Takahashi et al., 2000; Rubtsov et al., 2008). Similarly, CD4 effector T cells from TGF-β receptor II (RII) deficient mice have been shown to be resistant to T_{reg}-mediated suppression *in vivo*. On the other hand, evidence showing that TGF-β deficient T_{regs} suppress T cells in *in vitro* assays and autoimmune colitis *in vivo* clearly, which seems to exclude TGF-β mediated regulation as the principle suppressive mechanism of T_{regs} (Shevach et al., 2001). One alternative explanation is that TGF-β RII knockout (KO) T cells are intrinsically hyperactive and less suppressible by T_{regs} (Wan and Flavell, 2007). TGF-β RII KO CD4 cells may produce abnormally high levels of cytokines causing them to be refractory to cytokine deprivation death. Thus, TGF-β seems unlikely to directly mediate T_{reg} suppression. TGF-β produced by natural T_{regs} may convert conventional CD4 T cells into Foxp3 expressing "induced" T_{regs} (iT_{regs}; Andersson et al., 2008; Pandiyan and Lenardo, 2008; Shevach et al., 2008). These iT_{regs} have suppressive functions and may confer "infectious tolerance" since they can be induced or recruited to spread tolerance (Shevach et al., 2008). However, the exact mechanism of how they suppress is unknown. Similarly, IL-35, a novel member of the IL-12 family, also contributes to T_{reg}-mediated infectious tolerance by converting conventional T cells into T_{regs} (Collison et al., 2010). However, the presence and function of IL-35 in human CD4+ Foxp3+ T_{regs} is controversial (Bardel et al., 2008; Chaturvedi et al., 2011; Liu et al., 2011). Moreover, EBI-3 (IL-35) KO mice do not exhibit gross autoimmunity similar to that displayed by Foxp3, IL-2 or CTLA-4 deficient mice, raising a question about its physiological role in T_{regs} function (Bardel et al., 2008; Chaturvedi et al., 2011; Dokmeci et al., 2011; Liu et al., 2011). Finally, although CTLA-4 was originally claimed not to be required for the suppressive mechanism of T_{regs} *in vitro*, as described below, new evidence clearly supports a role in modulating APC function which will indirectly inhibit T-cell activation under certain circumstances (Wing et al., 2008; Qureshi et al., 2011). The contradictions in these data appear to result from the classic "blind men and the elephant" effect. Different groups have examined only selected aspects of the multi-step process of suppression. Taken together, T_{regs} deploy different mechanisms of regulation depending on the immunological context, location, conditions of T-cell activation and differences in target cells (Shevach, 2002; Von Boehmer, 2005; Rudensky and Campbell, 2006; Tang and Bluestone, 2008).

Recently, a few mechanisms of T_{regs} action have become increasingly clear. We will briefly describe two negative effects on T effector responses – mediated by the high affinity IL-2 receptor (IL-2R) and the CTLA-4 molecule – and one positive effect on T effector responses mediated by the high affinity IL-2R.

NEGATIVE EFFECTS OF T_{reg} CELLS

The negative effects are mediated, not surprisingly, by two cell surface "receptors" that are expressed extraordinarily highly on T_{reg} cells – IL-2R (CD25) and the CD152 (CTLA-4) receptors. In order to understand the immunosuppressive effect of $T_{regs\ in\ vitro}$, it is important to distinguish the direct impact on T-cells mediated by IL-2R and indirect inhibitory effects on APC mediated by CTLA-4 (Pandiyan et al., 2007; Sakaguchi and Wing, 2011; Wing et al., 2011).

IL-2 CONSUMPTION AND CYTOKINE DEPRIVATION APOPTOSIS IN CD4 T CELLS

A defining feature used in the initial isolation of T_{reg} cells was a characteristically high level of expression of CD25. It was therefore surprising that TCR stimulation of T_{reg} cells led to no production of IL-2 under most circumstances. This led us and others to hypothesize that the CD25 (IL-2R) served an important regulatory function for T_{reg} cells. One of the most important characteristics of nTregs is the requirement of IL-2 for their suppressive functions (Scheffold et al., 2005). Scheffold and Stockinger's groups demonstrated that T_{regs} consume IL-2 to exert their suppressive functions (Barthlott et al., 2003; De La Rosa et al., 2004). Starting from these observations, we investigated whether consumption of IL-2 is an actual suppressive mechanism for immune responses by T_{regs}. We also recognized that none of the studies on T_{reg} suppression had determined the fate of the "suppressed" CD4 effector cells, especially in the *in vitro* assay systems, However, apoptosis and cell survival were well-known immune regulatory mechanisms (Lenardo et al., 1999). Therefore, we examined the direct effects of T_{regs} on CD4 T cells and the fate of the "suppressed" T cells (Pandiyan et al., 2007). We observed that T_{regs} consume a large fraction of the IL-2 that is produced by the stimulated CD4 responder cells (effector cells). T_{regs} produce no IL-2 themselves even though their survival depends on it. Close proximity of a large number of T_{reg} cells deprives the effector CD4 cells of this primary growth factor causing proliferation arrest and apoptosis of both cell populations (Pandiyan et al., 2007). This effect could quantitatively explain the drop in proliferation measured by tritiated thymidine incorporation described in earlier studies (Thornton and Shevach, 1998) and did not require the presence of APCs. Thus, T_{regs} directly regulate the IL-2 producing Th0 effector cells through a chain of events leading to IL-2 deprivation and polyclonal deletion (PCD). The deletion is "polyclonal" at least according to *in vitro* experiments, because the suppression does not depend on any clonotype specificity of T_{regs} and target cells consistent with prior evidence that T_{regs} "suppression" is not antigen specific in most settings (Thornton and Shevach, 2000; Sakaguchi et al., 2008).

Cytokine deprivation happens at two levels. First, T_{regs} directly consume growth cytokines secreted at early phase of activation by effector T cells that are in the immediate vicinity at early phase of activation. Close juxtaposition is crucial for interception of IL-2 in its autocrine loop of production and utilization by effector T cells. Second, they cause failure of activated cells to produce cytokines at a later phase of activation by removing a critical IL-2 positive feedback (Pandiyan et al., 2007). In Th0 cells, a powerful positive feedback loop is initiated by IL-2 production during early T-cell activation, in which autocrine IL-2 induces the high affinity IL-2R expression rendering activated T cells to react more efficiently with IL-2. This leads to the production of other T-cell cytokines and proliferation. Signaling by early IL-2 is essential for later optimal cytokine production and effector functions. By consumption of IL-2, T_{regs} can effectively interrupt these processes by down-regulating IL-2R expression and dampening further cytokine expression and proliferation. Thus, suppression of proliferation and death due to cytokine deprivation may have a more pronounced effect on effector and effector

memory T-cell populations. Further investigations elucidated the importance of TCR stimulation strength in the processes of IL-2 consumption and T_{reg} suppression (Tran et al., 2009b). These experiments demonstrated that human T_{regs} consume mouse IL-2, which inhibits mouse effector T cells at mid range but not at very strong stimulation conditions. We also find that in human T_{regs}, consumption causes PCD under low dose α-CD3 stimulations and not at high α-CD3 concentrations (Pandiyan and Lenardo, unpublished results). However, the minimum amount of IL-2 that must be consumed to obtain PCD, how TCR signal strength affects IL-2 consumption, other cytokines that might alter the IL-2 consumption rate and the suppression processes, and how IL-2 consumption may affect APCs, remain to be investigated. It seems intuitive that low strength TCR stimulation is more reflective of an immune response to the low-affinity self-antigens *in vivo* and therefore, PCD may play a predominant role in such an immune context. In addition to direct cytokine consumption, whether Bim-dependent apoptosis may also be caused by TGF-β remains to be addressed (Ramesh et al., 2008; Houde et al., 2009; Sanjabi et al., 2009; Tinoco et al., 2009). However, more work is needed to address these important questions, which will facilitate our understanding of how T_{regs} and PCD can be therapeutically deployed.

CYTOKINE DEPRIVATION VERSUS OTHER DELETION MECHANISMS IN CD4 T CELLS

The consumption of available IL-2 by T_{regs} plays a key role in PCD of responder T cells but other cytokines *in vitro* and *in vivo* may be involved (Pandiyan et al., 2007). The biochemical events were PI-3 kinase inactivation, Akt/BAD dephosphorylation and Bim-dependent apoptosis. This was verified by removing the Bim apoptosis gene in effector T cells, and examining their response to suppressive effects in co-cultures with wild-type T_{regs}. The Bcl-2 family member Bim is a central mediator of intrinsic apoptosis pathways, orchestrating cytokine withdrawal apoptosis in various hematopoietic cells that depend on trophic cytokines for growth and survival (Hildeman et al., 2002). In particular, it mediates lymphocyte death caused by cytokine deprivation generally at the end of immune responses (Hildeman et al., 2002). Bim knockout (KO) mice suffer with lupus-like autoimmunity and kidney disease due to defective apoptosis of T and B cells (Strasser, 2005). We found that Bim-KO effector T-cells clearly resist suppression by fully active T_{regs} and are not impaired in late-phase cytokine production. Hence, "suppression" was abrogated by removal of an apoptosis gene in the effector T cells that were the target of wild-type Tregs. Unaffected cytokine production in Bim-KO cells implies that the late-phase cytokine suppression resulted from the death of cytokine producers. These data validate the hypothesis that T_{reg} cells can regulate cytokine dependent homeostatic proliferation of effector T cells by killing them in an antigen non-specific manner *in vivo*. This was demonstrated by the ability of T_{reg} cells to suppress inflammatory bowel disease (IBD) in a mouse model, in which the disease is partially driven by homeostatic proliferation of naïve CD4 cells in a lymphopenic environment (Powrie et al., 1994). However, T_{reg} cells failed to suppress IBD caused by Bim-KO cells. This indicates that direct cytokine deprivation and BIM-dependent apoptosis by T_{regs} plays a significant role in their ability to regulate IBD.

Polyclonal deletion mediated by natural T_{reg} cells apparently does not involve direct cytolytic mechanisms such as activation of Fas or cell contact-dependent, granzyme-mediated cell death (Takahashi et al., 2000; Pandiyan et al., 2007). We found no evidence of death mechanisms mediated by TNF receptor family members or perforin and/or granzymes. Moreover, our data showing that PCD of the CD4 T-cells occurred slowly (3–4 days after activation) argues against the direct cytolysis mechanism, which usually kills the target cells within 24 h. Our studies also support the idea that close proximity, not necessarily direct physical contact, is crucial for T_{reg} cells to compete effectively for cytokines produced by the effector T cells or APCs and thereby block the autocrine and paracrine loops that are essential for effector T-cell survival, proliferation, and cytokine production (Scheffold et al., 2005; Busse et al., 2010). Although the exact distance required between target cells and T_{reg} cells for efficient IL-2 consumption is currently unknown, we believe that the closer the proximity of the two cells, the more likely it will be that the IL-2Rs on the T_{regs} will compete better to capture the IL-2 produced by the effector T cells before it can interact with the IL-2Rs on the T effector cells themselves.

It has been shown that T_{reg} cells can block the autoimmune disease that occurs in IL-2 KO mice (Schimpl et al., 2002). Along with our results showing that T_{reg} cells kill IL-2 KO T cells *in vitro*, it raises a possibility that T_{reg} cells can cause PCD by consuming cytokines besides IL-2. In fact, at this point it is unclear consumption of which cytokine contributes the most to the immunoregulatory properties of Tregs. Because T_{reg} cells are themselves heavily dependent on common gamma (γ_c)-chain cytokines, it is likely that in the absence of IL-2, they will consume/regulate other γ_c-chain cytokines to gain control of activated CD4 cells (Pandiyan and Lenardo, 2008). We found that the addition of γ_c-chain cytokines such as IL-4, IL-15, and others to the T_{reg}/effector T-cell co-culture can completely rescue effector T cells from apoptosis, confirming that a variety of cytokines can tip the survival balance (Pandiyan et al., 2007). Cytokine/s that drive the proliferation of the IL-2 KO CD4 T cells in the absence of IL-2, how that cytokine is regulated by T_{regs}, and how APCs are affected in cytokine deprived situations *in vivo*, are questions that remain to be answered.

Although T_{regs} mediate the PCD of activated effector $CD4^+$ T cells, extensive experimentation shows they do not suppress priming or initiation of T-cell receptor (TCR) activation and/or early IL-2 production in T cells (Thornton et al., 2004a; Oberle et al., 2007; Pandiyan et al., 2007; Esquerre et al., 2008). This contradicts a tenet of T_{reg} suppression proposed in previous studies (Thornton and Shevach, 1998). Early IFNγ and IL-2 gene expression is not affected by mouse T_{regs} in conventional co-cultures, so that the simplistic paradigm of helper/anti-helper competition to control early activation events fails to explain T effector cell regulation by T_{regs} (Pandiyan et al., 2007). This conclusion is reinforced by the fact that in human T cells, early TCR signaling events and calcium mobilization in effector T cells are unaffected by T_{reg} cells (Esquerre et al., 2008). Further experiments have demonstrated that the proliferation (and survival) of previously activated effector T cells are susceptible to T_{reg} suppression, indicating that T_{reg}

cells do not need to affect initial priming of mouse T cells in order to suppress them *in vitro* (Pandiyan et al., 2007; Esquerre et al., 2008). Moreover, cytokine deprivation and Bim regulates the survival of T_{regs} themselves, and, in the absence of gamma chain cytokines, they die by apoptosis (Pandiyan and Lenardo, 2008). During an immune response, early production of IL-2 drives activated T cells into proliferation and at the same time makes the proliferating T-cells totally dependent on IL-2 (Lenardo et al., 1999). Intact early activation events and normal effector T-cell proliferation are therefore essential for the IL-2 consumption made of PCD by T_{reg} (Klein et al., 2003). Although T_{regs} can modulate APCs, we have found that potent T_{reg} suppression of effector T cells by PCD occurs in the absence of APCs *in vitro*. Taken together, direct induction of PCD in activated and cycling T-cells plays a major role in the T_{reg}-mediated immune suppression but not direct impairment of naïve T cells or any direct effect on early T-cell activation.

MODULATION OF APC BY T_{regs} ALSO CONTRIBUTES TO SUPPRESSION

A second mode of negative regulation is the effect of T_{regs} on APC such as dendritic cells. When T_{regs} are mixed with APCs and then the APCs are isolated and used to stimulate T cells, these APCs function suboptimally in many conditions. Hence, T_{reg} effects on APC's could indirectly affect T-cell responses. Consistent with the idea of APC regulation, CTLA-4, which is constitutively expressed on Tregs, has been found to indirectly regulate T cells by down-regulating co-stimulatory molecules such as CD80 and CD86 on APC (Wing et al., 2008). Initially, this effect was somewhat mysterious, but elegant studies showed that CTLA-4 could capture its ligands from opposing cells, by a process of trans-endocytosis. This reduces the level of CD80 and CD86 on the APCs in the immediate vicinity, which will reduce co-stimulation provided to the effector T cells (Qureshi et al., 2011). T_{reg}-specific CTLA-4 deficiency impairs *in vivo* and *in vitro* suppressive function of T_{regs} (Wing et al., 2008). *In vitro* blocking experiments with anti-CTLA-4 antibodies give less clear-cut results because of low/transient CTLA-4 binding at the cell surface. The role of APC modulation is further supported by the studies demonstrating that T_{reg} cells could regulate the contact and clustering between APC and T cells (Tadokoro et al., 2006; Tang et al., 2006; Qureshi et al., 2011). Thus, strong evidence now indicates that T_{regs} inhibit immune responses by directly blocking the survival and effector functions of CD4 cells and indirectly by down-modulating APC co-stimulation. These new observations provide a compelling mechanism, but further validation by other groups and more information about the specificity and kinetics of the CTLA-4 transcytosis will be necessary. However, CTLA-4 and IL-2-dependent suppression can be regarded as core mechanisms of T_{reg}-mediated suppression, explained by the high levels of CTLA-4 and CD25 (IL-2R) characteristically expressed by T_{regs} (Sakaguchi et al., 2009).

COORDINATION BETWEEN DELETION OF T CELLS AND OTHER MECHANISMS OF SUPPRESSION *IN VIVO*

Consumption of cytokines and PCD of effector T cells by T_{regs} plays an important role in reducing the strength of the immune response *in vivo* (Pandiyan et al., 2007). We speculate that in the initial phase of an immune response, the effector T:T_{reg} ratio is higher because effector T cells are proliferating faster than T_{regs}. However as the response progresses, T_{regs} consume IL-2 from activated cells, proliferate, and cause accelerated apoptosis of IL-2-dependent effector T cells. This begins to shift the effector T:T_{reg} balance. Bim-dependent PCD of a substantial number of cytokine producing CD4 cell progenitors in the initial phase of the response inhibits further cytokine production by affecting differentiation of CD4 effector T cells into Th1, Th2, or Th17 effectors (due to poor expression of cytokines and cytokine receptors; **Figure 1**). These forces all act together to reduce the number of specific effector T cells, to dampen the immune response.

Thus, PCD directly reduces the intensity of an immune response by tapering the T-cell population, and the diminished population of effector cells is rendered more susceptible to other suppression mechanisms (Tang and Bluestone, 2008; **Figure 1**). The suppressive factors could directly be produced by T_{regs}, or indirectly induced in other cells by T_{regs}. Cytokine consumption ensures loss of effector functions in the remaining effector population making them immunologically inactive and restores T_{reg} numbers for future regulation. Even though we believe that suppression of late cytokine production is chiefly caused by the "disruption of cytokine positive feedback loop," the effects of other postulated mechanisms could be important. Cytokines produced during effector response may further potentiate T_{regs} to produce other suppressive or even non-suppressive factors (see below). For example, IL-2 may trigger transcriptional events leading to the expression of IL-10, TGF-β, EBI-3, or IL-35 in T_{regs} (Maloy and Powrie, 2005). These suppressive molecules further reduce inflammation by inhibiting effector CD4 cells, as well as modulating APC and other inflammatory cells. Furthermore, T_{reg} dependent Foxp3 and IL-35 induction in effector T cells may also contribute to their intrinsically impaired cytokine production at later phases (Andersson et al., 2008; Pandiyan and Lenardo, 2008; Collison et al., 2010). The specific role of each mechanism in suppressing a heterogeneous population of effector Th1, Th2, and Th17 cells *in vivo* remains to be seen. Deprivation of cytokines and induction of apoptosis by T_{regs} could differentially affect target cell functions, depending on whether the immune response is pro- or non-inflammatory, or whether the immune cells are responding to microbial infections or self-antigens. The phase (early versus late) of the immune response and the corresponding cytokine environment also likely dictate the functions of T_{regs}. Thus, these suppressive mechanisms may play their distinct roles in the multi-step process of immune suppression at different stages.

ARE $Foxp3^+$ $CD4^+$ CELLS SOLELY DEVOTED TO TOLERANCE?

There is great deal of literature correlating Foxp3 expression in CD4 cells with active immune suppression. However, recent evidence indicates that Foxp3+ cells may play other immunological roles (Komatsu et al., 2009; Zhou et al., 2009b; Chen et al., 2011; Pandiyan et al., 2011). Although Foxp3+ CD4+ T_{regs} have suppressive capability, Foxp3 expression in CD4 cells may not always indicate suppression. T_{regs} may have positive immune functions and this possibility has been explored much less because of the preconception that they are simply "suppressor" cells. Transient

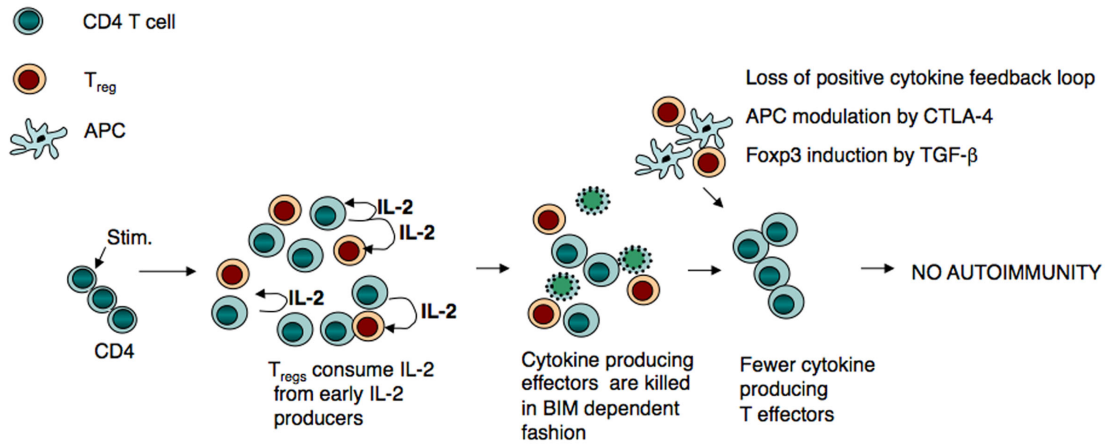

FIGURE 1 | T_{reg}-mediated apoptosis plays a major role in limiting Th07Th1/Th2 cell responses. Under steady-state conditions, $CD4^+$ T cells upon priming, produce IL-2 and /or other growth factors and expand responding to these cytokines. If T_{regs} are present during this early phase of IL-2 production, $CD4^+$ T cells are deprived of IL-2 leading to $CD4^+$ T-cell death. However, cells that are not in direct/close proximity to T_{reg5}, may remain in the system and continue to expand. Because of the low levels of IL-2 in their milieu and inactivation of APC by CTLA-4, their later effector function and cytokine production are impaired. This limits autoimmunity, perhaps under homeostatic conditions involving self-antigens.

expression of Foxp3 in a subset of activated cells or in TGF-β-induced iT_{regs} does not necessarily mean that these cells can function like-thymus-derived suppressive "natural" T_{reg} cells (Hoffmann et al., 2009; Huehn et al., 2009; Komatsu et al., 2009; Lahl et al., 2009; Thornton et al., 2010). Also, Foxp3 alone does not control all the elements of T_{reg} signature phenotype (Zhou et al., 2008). The presence of Foxp3+ cells *in vivo* may reflect an inflammation that has induced Foxp3 expression in activated cells (iT_{regs}) or proliferation of natural T_{regs}, and not necessarily associated with active suppression of immune responses. Similar to T_{regs} induced *in vitro* (iT_{regs}), a subset of natural T_{regs} might represent a population with unstable Foxp3 expression *in vivo*. Given the plasticity of T_{regs} and Foxp3 expression in activated cells, care should be taken when interpreting the data from experiments involving Foxp3 positive cells in disease conditions.

Although controversial, recent accumulating evidence shows that Foxp3+ T_{reg} cells can themselves become effector cells or produce cytokines under strong pro-inflammatory conditions (Komatsu et al., 2009; Zhou et al., 2009b; Rubtsov et al., 2010). In some instances they are even shown to exacerbate pathogenic immune responses (Zhou et al., 2009a,b). It has been observed in an experimental allergic encephalomyelitis (EAE) model, that Ag specific T_{regs} accumulate in brain but fail to control inflammation (Korn et al., 2007). This raises an interesting possibility that T_{reg} functions are not limited solely to suppression but also to other effector functions, including protection against infection. Evidence to support this idea emerged with the demonstration that T_{regs} play an important role in controlling lethal West Nile virus (Lanteri et al., 2009). Those functions may be largely dependent on cytokine milieu or innate immune responses.

We and others have shown that under Th17 milieu, IL-2 consumption by T_{regs} actually leads to an enhanced proliferation and cytokine production of early Th17 cells *in vitro* and in the context of oral *Candida* infection or inflammation *in vivo* (Chen et al., 2011; Pandiyan et al., 2011). IL-2 has been known to inhibit Th17 differentiation. By enhancing Th17 cells, these non-suppressive T_{regs} reduce the fungal burden in mice (Pandiyan et al., 2011; **Figure 2**). Moreover, our preliminary studies show that T_{regs} themselves lose Foxp3 in Th17 inflammatory conditions *in vitro*, which also likely contributes to the loss of their suppressive properties (unpublished results). These destabilized T_{regs} (Ex-T_{regs}) are only present transiently, because in a chronic Th17 IBD model, such Ex-T_{regs} can regain suppressive functions and show a delayed, but strong potential to suppress Th17 inflammation (Pandiyan et al., 2011). The mechanism of reacquisition of suppressive properties by T_{regs} and the mechanism of the delayed suppression of Th17 cells remain to be investigated. The loss of suppressive functions in T_{regs} during infections might be associated with certain toll like receptor (TLR) ligands and strong responses through massive production of inflammatory cytokines (Pasare and Medzhitov, 2003). This mechanism might allow protective immune responses against microbes to proceed normally. Thus, Foxp3+ T_{regs} could be principally suppressive when inflammatory cytokines are low, such as during late-phase responses, which prevents immunopathology in the host. However, they may not be destined solely for suppressive functions, because they also act as helper cells to promote certain immune responses in cytokine-rich environments, such as during infections (Pandiyan et al., 2004; Zhou, 2008; Lanteri et al., 2009). Taken together, Foxp3+ cells may represent a subset of protective immune cells like T helper cells, but with elaborate suppressive capacities.

Even T helper cells such as Th1, Th2, and Th17 cells can be regarded to possess regulatory potentials. These cells along with Th22 cells and inducible regulatory T cells such as Foxp3+ iT_{regs} and iTr35 (the inducible regulatory T-cells expressing IL-35) polarize under specific cytokine milieu along with TCR stimulation. (Yang et al., 2008; Bluestone et al., 2009; Lohr et al., 2009; Bending et al., 2011; Hirota et al., 2011). For example, under a Th1 skewing condition, IFN-γ and IL-12 activate signal transducer and activator of transcription 4 (STAT4) and STAT1, leading to the activation

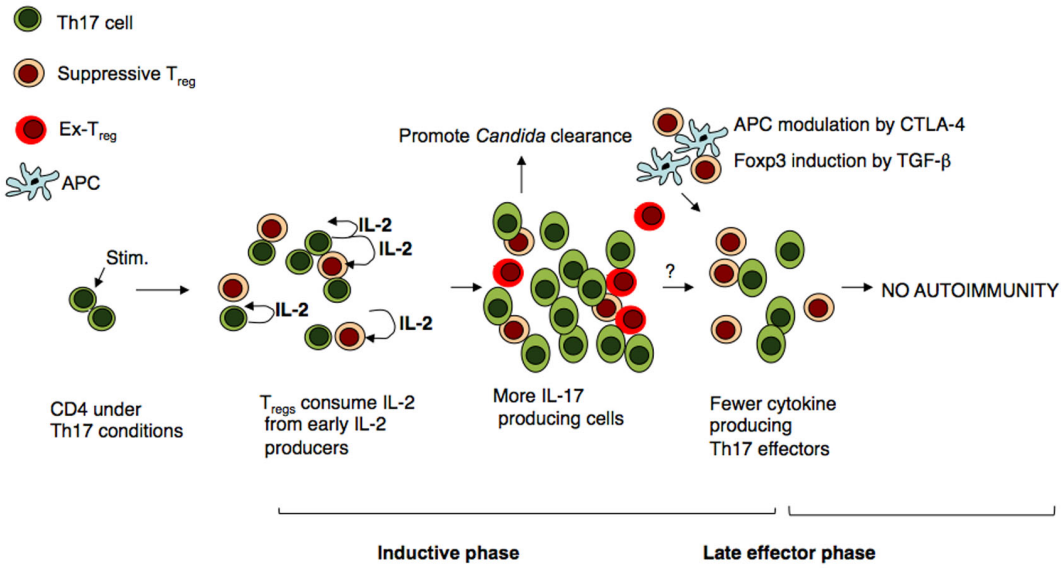

FIGURE 2 | T_{reg} cells promote Th17 cells at early phase and modulate inflammatory Th17 responses at later phases. Under Th17 inflammatory conditions, $CD4^+$ T cells and expand responding to factors such as IL-21 and IL-6. T_{regs} do not inhibit the survival or proliferation of those cells because Th17 cells produce excess of IL-2, IL-21, IL-6, and TNF-α, all of which overcome the suppression by cytokine deprivation. Some T_{regs} also lose Foxp3 expression (Ex-T_{reg}), which also likely contributes to the absence of suppression. However, T_{regs} enhance IL-17 production by consuming IL-2. This promotes clearance of pathogens that require protective Th17 responses. However when the cytokine levels drop at later phases, T_{regs} limit Th17 cells, likely by other mechanisms such as TGF-β and APC modulation by CTLA-4.

of T-bet, a transcription factor and a master regulator for Th1 differentiation (Ashkar et al., 2000; Glimcher and Murphy, 2000; Szabo et al., 2000). Along with Runx3 activation, a positive feedback loop forms and accelerates the expression of the Th1 hallmark cytokine IFN-γ. Meanwhile, activated T-bet and Runx3 can suppress GATA3 signaling, causing the expression of the Th2-specific IL-4 gene to be inhibited (Hegazy et al., 2010; Zhu and Paul, 2010). Inhibition of Th2 factors is critical for the maintenance of Th1 cells. On the other hand, IL-4 and IL-2 are the major cytokines that promote Th2 differentiation (Ben-Sasson et al., 1990; Le Gros et al., 1990). Through stimulating respectively STAT6 and STAT5, IL-4 (also produced by DC) and IL-2 activate GATA3, a master regulator of Th2 cell differentiation, and then promote IL-4 gene expression (Ouyang et al., 2000; Hofer et al., 2002; Tykocinski et al., 2005). Activated GATA3 also inhibits Runx3 and thereby turns off IFN-γ gene expression in order to suppress Th1 and augment Th2 differentiation (Yagi et al., 2010). This phenomenon, where one type of cytokine milieu favors a lineage of T helper cell differentiation, is always accompanied by suppression of the other lineages. (Mosmann and Coffman, 1989a,b; Paul and Seder, 1994; Seder and Paul, 1994; Zhu and Paul, 2010). This process can also be called as cross regulation. The induction versus suppression of differentiating CD4 T-cells appears to be dependent on available cytokines in the microenvironment of any given immune response. Although most of the available data indicate that inhibition of transcription factors during Th differentiation occurs in the same cell, we speculate that it is likely to occur through cytokines at intercellular level as well. For example, differentiated Th1 cells may suppress Th2 cells and *vice versa*. It will be interesting to study the cross regulation of well-differentiated Th1 or Th2 cells. In essence, while the differentiated helper T cells can assist in mounting appropriate immune responses, the same "helper" cells can exercise functions of suppression, at least on some differentiating T cells. Thus, most immune cells may harbor both immunosuppressive and immune promoting functions and immune suppression function may not be confined to specific subsets.

A recent example that supports this tenet is the identification of Foxp3+ suppressive macrophages (Zorro Manrique et al., 2011). Historically, macrophages are regarded as effector cells, classical APCs and one of the first responders in an immune response. Now, it has been shown that under certain conditions they can be suppressive in function. More importantly, we have also shown that even effector CD4 T cells with no Foxp3 expression can suppress CD4 T cells by consuming IL-2. These "pseudo-T_{regs}," mimic nT_{regs} in expressing CD25 and being dependent on IL-2, but being unable to produce IL-2 (Pandiyan et al., 2007). Therefore, "helpers" and "suppressors" should be regarded as relative terms; suppressive properties can reside in cells that are conventionally conceived as effector/helper cells. The dose of antigens, site of action, timing and cytokine milieu may dictate the function of the immune cells, whether "immunosuppressive" or "immune promoting." This explains why $Foxp3^+$ $CD4^+$ cells regulate Th1 and Th2 functions by cytokine deprivation but promote other types of responses such as the Th17 response, which in and of themselves may regulate each other. Thus, the binary model in which $Foxp3^+$ $CD4^+$ cells are exclusively suppressive and other effector T helper cells function exclusively to promote immune responses, may not explain all immune functions. Ultimately, coordination between suppression of certain cells and enhancement of certain other effector cells leads to appropriate immune responses.

CONCLUSION

In an optimal immune response by CD4 cells, mixture of Th1, Th2, and Th17 effector cells are generated and the dominant population in a given response varies. T_{reg} cells are probably equipped mainly to kill IL-2-dependent Th0/Th1 cells but not all the effector cells equally. Importantly, T_{regs} promote (and clearly do not suppress) Th17 responses during acute inflammation. Thus the function and stability of T_{regs} can depend largely on the immunological milieu in which they are operating. These parameters are complex and therefore it is important to identify the signals that regulate the function of Foxp3+ T_{regs} and other Th cells and determine how they cross regulate each other at the molecular level. Regarding the overall suppression, PCD of T-cells combined with suppression of APC by CTLA-4 and other suppressive mechanisms ensures more stringent control of different T-cells subsets. The cytokine milieu and the target effector cells that are suppressed determine the predominant mechanism employed by T_{regs} in a particular immune response. The temporal and spatial parameters that determine which mechanism predominates suppression by T_{regs} and what is the sequence in which the core mechanisms, i.e., cytokine deprivation and regulation by CTLA-4 are employed remain to be studied. Relative contribution of each of the mechanism of suppression by T_{regs} and when T_{regs} lose their suppressive properties remain to be the important questions for future investigations. When planning strategies for intervention of immune-related disorders using T_{regs}, cellular and cytokine networks as a whole, and not the function of a single subset of cells, should be taken into consideration. This is a fascinating area of research and warrants vigorous investigation, given its potential implications in chronic inflammation.

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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.

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)

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

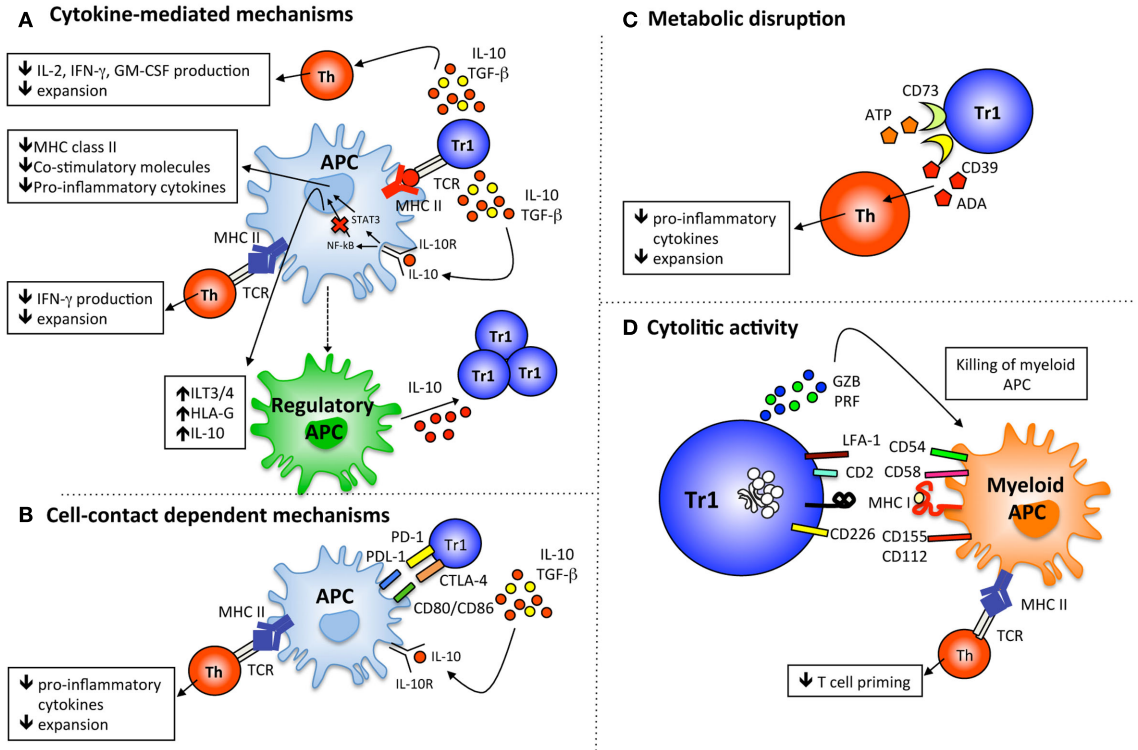

FIGURE 1 | Mechanisms of suppression mediated by Tr1 cells. (A) Tr1 cells upon Ag-specific activation secrete IL-10 and TGF-β that directly inhibit the function of effector T cells and indirectly modulate APC, which in turn limit effector T cell proliferation and cytokine production. IL-10 produced by 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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Molecular mechanisms of Treg-mediated T cell suppression

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$CD4^+CD25^{high}Foxp3^+$ regulatory T cells (Tregs) can suppress other immune cells and, thus, are critical mediators of peripheral self-tolerance. On the one hand, Tregs avert autoimmune disease and allergies. On the other hand, Tregs can prevent immune reactions against tumors and pathogens. Despite the importance of Tregs, the molecular mechanisms of suppression remain incompletely understood and controversial. Proliferation and cytokine production of $CD4^+CD25^-$ conventional T cells (Tcons) can be inhibited directly by Tregs. In addition, Tregs can indirectly suppress Tcon activation via inhibition of the stimulatory capacity of antigen presenting cells. Direct suppression of Tcons by Tregs can involve immunosuppressive soluble factors or cell contact. Different mechanisms of suppression have been described, so far with no consensus on one universal mechanism. Controversies might be explained by the fact that different mechanisms may operate depending on the site of the immune reaction, on the type and activation state of the suppressed target cell as well as on the Treg activation status. Further, inhibition of T cell effector function can occur independently of suppression of proliferation. In this review, we summarize the described molecular mechanisms of suppression with a particular focus on suppression of Tcons and rapid suppression of T cell receptor-induced calcium (Ca^{2+}), NFAT, and NF-κB signaling in Tcons by Tregs.

Keywords: Treg, TCR signaling, suppression mechanisms

INTRODUCTION: DISCOVERY AND PROPERTIES OF REGULATORY T CELLS

Regulatory T cells (Tregs) play an indispensable role in the immune system as they are involved in the prevention of autoimmune diseases, allergies, infection-induced organ pathology, transplant rejection as well as graft versus host disease (GvHD) by suppression of effector T cells and other immune cells (Sakaguchi, 2004). However, Tregs can also dampen immune responses against tumors, as described for various types of cancer (Zou, 2006; Curiel, 2008). Conversely, in certain situations, Tregs can also protect against cancer by controlling cancer-associated inflammation (Gounaris et al., 2009). Therefore, Tregs are subject to intense investigations.

The history of Tregs begins in 1970 (Gershon and Kondo, 1970). Unfortunately, due to lack of a molecular marker, research on suppressor T cells was stopped, until many years later Sakaguchi et al. (1995) identified CD25 as a phenotypic marker for suppressive $CD4^+$ T cells in mice. These suppressive T cells were named Tregs and were later also found in humans within the $CD4^+CD25^{high}$ T cell population (Baecher-Allan et al., 2001; Dieckmann et al., 2001; Jonuleit et al., 2001; Levings et al., 2001; Stephens et al., 2001; Taams et al., 2001). Constitutive expression of CD25 is restricted to Tregs in naïve mice while in humans, only cells that highly express CD25 designate Tregs, and activated T cells acquire intermediate levels of CD25 (Sakaguchi et al., 1995; Baecher-Allan et al., 2001). Importantly, Foxp3 was identified as a lineage-defining transcription factor for Tregs in mice (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003) and humans (Yagi et al., 2004; Roncador et al., 2005). In mice, up to 90% of Tregs express Foxp3, while resting as well as activated conventional T cells do not show detectable levels of Foxp3. Analogous to mice, the majority of human $CD4^+CD25^{high}$ Tregs also express Foxp3. However, in contrast to mice, human conventional T cells (Tcons) transiently express intermediate levels of Foxp3 upon activation (Pillai et al., 2007), questioning Foxp3 as a specific marker for human Tregs. While the necessity of Foxp3 for suppressive function of Tregs is undisputed, contradictory reports leave it unclear whether (temporary) Foxp3 expression is sufficient to confer suppressive abilities to human activated T cells. Some studies demonstrated suppressive capacities of $Foxp3^+$ activated human T cells (Walker et al., 2003; Pillai et al., 2007) while others did not (Gavin et al., 2006; Wang et al., 2007; Allan et al., 2007). However, activation-induced transient Foxp3 induction in human Tcons was not observed in other studies (Yagi et al., 2004). These discrepancies might be due to differences in culture conditions, such as strength of T cell receptor (TCR) stimulation and presence of IL-2 or TGF-β. For example, Shevach's group found that TCR-induced Foxp3 expression in human naïve Tcons is indeed dependent on TGF-β, but does not result in a suppressive phenotype (Tran et al., 2007). In addition, his group claimed that a certain antibody clone used for staining of human Foxp3 results in unspecific staining of activated T cells, although this is controversial (Pillai and Karandikar, 2008). Conflicting results may also be ascribed to differences in kinetics and level of Foxp3 expression and, consequently, expression of Foxp3 target genes (Allan et al., 2008; Zheng et al., 2008).

$Foxp3^+$ Tregs are not a uniform population but rather comprise several subphenotypes. The majority of human Tregs displays

a CCR7$^-$CD45RA$^-$ effector/memory phenotype (Beyer and Schultze, 2007). However, only the naïve CD45RA$^+$ Treg subset maintains Treg phenotype and function upon *ex vivo* expansion (Hoffmann et al., 2006). A detailed characterization of the human Foxp3$^+$ Treg repertoire suggested a further grouping into CD45RA$^+$Foxp3^{low} resting Tregs, CD45RA$^-$Foxp3^{high} activated Tregs, and CD45RA$^-$ Foxp3^{low} cytokine-secreting Tregs, the latter being non-suppressive (Miyara et al., 2009). Further, several subphenotypes of murine Tregs with distinct transcriptional signatures were found in different anatomical locations (Feuerer et al., 2009, 2010). Diverse Treg subpopulations may apply different suppression mechanisms and specifically control certain effector cell types. In this review, we mainly focus on naturally occurring Tregs (nTregs), which are thymus-derived. Yet peripheral tolerance is not only ensured by nTregs but also involves various induced Treg populations (iTregs), which seem to play a role mainly in the intestine (Curotto de Lafaille and Lafaille, 2009).

MOLECULAR MECHANISMS OF Treg-MEDIATED SUPPRESSION

Tregs can suppress a variety of immune cells including B cells, NK cells, NKT cells, CD4$^+$, and CD8$^+$ T cells, as well as monocytes and dendritic cells (DCs). In the following, we will focus on suppression of CD4$^+$CD25$^-$ conventional T cells (Tcons).

Upon cell–cell contact, Tregs inhibit TCR-induced proliferation and IL-2 transcription of Tcons, as shown for murine Tregs already in 1998 (Thornton and Shevach, 1998). Suppression of murine (Ermann et al., 2001) or human (Dieckmann et al., 2001) Tcon proliferation by Tregs can occur directly, i.e., in the absence of antigen presenting cells (APCs). This direct suppression can involve immunosuppressive cytokines or other factors; however, contact-dependent direct suppression has also been described. In addition, Tregs can inhibit Tcons indirectly by influencing the activation status of APCs and therefore activation of Tcons. Based on these properties, a standard assay to assess Treg function is inhibition of responder Tcon proliferation upon stimulation via the TCR in the presence of APCs. A main function of Tregs is suppression of activation and expansion of naïve Tcons, but they can also inhibit activated effector T cells and memory CD4$^+$ (Levings et al., 2001) and CD8$^+$ (Suvas et al., 2003) T cells. To be suppressive, Tregs themselves have to be TCR-activated in the presence of IL-2 (Takahashi et al., 1998; Thornton and Shevach, 1998; de la Rosa et al., 2004; Thornton et al., 2004a,b) while costimulation via CD28 is dispensable (Takahashi et al., 2000). However, a more recent study questioned the requirement for Treg activation: Szymczak-Workman et al. (2009) showed that TCR-transgenic Tregs were able to suppress Tcons with different antigen specificity in the absence of the Treg-cognate antigen. Differences in the type of APCs used in the assays, the transgenic system, or "pre-activation" by the cell purification procedure might provide an explanation for the controversy. Yet, the study is in line with other reports that show that Tregs, once active, can suppress Tcons independently of antigen, leading to so-called bystander suppression (Thornton and Shevach, 2000; Karim et al., 2005).

Different mechanisms of Treg-mediated suppression have been described, mostly on the basis of *in vitro* suppression assays. These mechanisms may also operate *in vivo* depending on the target cell type and activation status as well as the location and cytokine and microorganism milieu of the immune reaction. Thus, the contribution of suppressive mechanisms might be interpreted differently depending on the cell types and their activation state used in *in vitro* suppression assays. In addition, differences may occur depending on the readout, as suppressing the production of certain effector cytokines or the release of cytotoxic granules *in vivo* and *in vitro* can occur without concomitant suppression of proliferation (Mempel et al., 2006; Schmidt et al., 2011; Sojka and Fowell, 2011). For example, using another *in vivo* model, it was shown that Tcon expansion in response to antigen was suppressed by Tregs relatively late, yet the remaining Tcons still cycled and produced effector cytokines at these late time points (Klein et al., 2003). It was suggested that activation and/or expansion of antigen-specific Tregs may be a prerequisite for suppression of Tcons *in vivo*, as it might result in sufficient Treg numbers to enable contact with target Tcons at the site of the immune response (Klein et al., 2003).

The described mechanisms of Treg-mediated T cell suppression are summarized in **Figure 1** and will be described in more detail in the following sections.

MODULATION OF APC FUNCTION BY Tregs THROUGH CTLA-4 AND OTHER MOLECULES

The coinhibitory molecule CTLA-4 is constitutively expressed in murine and human Tregs and exposed on the cell surface upon activation (Read et al., 2000; Takahashi et al., 2000; Dieckmann et al., 2001). A role for CTLA-4 in suppression *in vivo* has been suggested, since CTLA-4 deficiency or blockade in mice results in spontaneous autoimmunity, which can be ameliorated by Tregs (Bachmann et al., 1999; Takahashi et al., 2000). In addition, CTLA-4 blockade abrogates the protective effects of Tregs in murine colitis models (Read et al., 2000). Also *in vitro* suppression of murine Tcon proliferation (in the presence of APCs) was shown to be abrogated in some studies by CTLA-4 blockage (Takahashi et al., 2000; Tang et al., 2004). However, CTLA-4 deficient Tregs could still suppress through compensatory mechanisms involving TGF-β and IL-10 *in vitro* and *in vivo* (Tang et al., 2004; Read et al., 2006). Similarly, studies with human Tregs do not show a uniform picture: some *in vitro* studies did not find an involvement of CTLA-4 in Treg-mediated suppression of Tcon proliferation (Baecher-Allan et al., 2001; Levings et al., 2001), while others could partially abrogate suppression by CTLA-4 blockage (Birebent et al., 2004). These discrepancies may be explained by the involvement of CTLA-4 in some but not all aspects of suppression. Our results showed that rapid Treg-mediated suppression of cytokine transcription in human Tcons was unaffected by CTLA-4 blockage, irrespective of the presence or absence of APCs, while suppression of proliferation in the presence of APCs was partially dependent on CTLA-4 (Schmidt et al., 2011).

Regarding CTLA-4 blockade in long-term experiments, it has to be considered that CTLA-4 is also expressed by activated Tcons after 1–2 days of TCR stimulation (Walunas et al., 1994) and thus cell-intrinsic negative signals are involved. These include competition with the costimulatory molecule CD28 for binding to the B7 molecules CD80 (B7.1) and CD86 (B7.2; van der Merwe et al.,

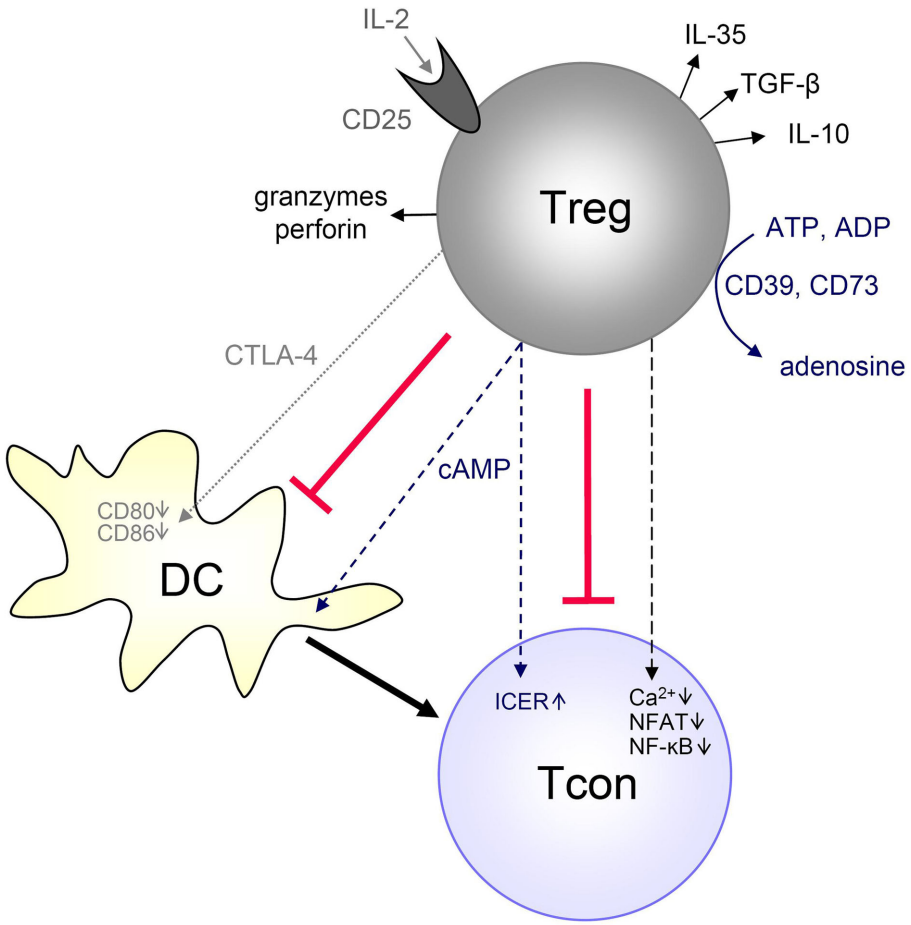

FIGURE 1 | Mechanisms of Treg-mediated Tcon suppression. Tregs have been described to suppress Tcons by different mechanisms, depending on the experimental setup, site and type of immune response. Tregs can generate immunosuppressive adenosine or transfer cAMP to Tcons. Tregs can rapidly suppress TCR-induced Ca^{2+}, NFAT, and NF-κB signaling. Tregs can also produce immunosuppressive cytokines (IL-10, TGF-β, IL-35), and they can suppress by IL-2 consumption or induce effector cell death via granzyme and perforin. Furthermore, Tregs can suppress Tcons indirectly by downregulating costimulatory molecules on APCs (such as DCs) via CTLA-4. Details are described in the text.

1997), transduction of negative signals that induce cell cycle arrest and prevent IL-2 secretion (Krummel and Allison, 1996; Walunas et al., 1996), and limiting T cell contact with APCs (Schneider et al., 2006). Recently, the molecular mechanism of Treg-mediated suppression via CTLA-4 was elucidated in more detail. Downregulation of CD80/CD86 expression on APCs by Tregs (Cederbom et al., 2000) was shown to be partly dependent on CTLA-4 and the adhesion molecule LFA-1, thereby indirectly inhibiting Tcon activation by APCs *in vitro* (Oderup et al., 2006; Onishi et al., 2008) and *in vivo* (Wing et al., 2008). Mechanistically, downregulation of CD80/CD86 on target cells by CTLA-4-expressing cells can involve capture of these ligands by CTLA-4, a process called trans-endocytosis (Qureshi et al., 2011). Another study supports the importance of CTLA-4 in *in vivo* suppression without direct effects on the responder T cells (Friedline et al., 2009). Strikingly, mice with Treg-specific ablation of CTLA-4 spontaneously develop fatal autoimmune disease (Wing et al., 2008), underlining the importance of CTLA-4 expression on Tregs for the maintenance of peripheral tolerance.

In contrast to the studies showing necessity of CTLA-4 for Treg-mediated suppression, Tran et al. (2009) applied a mixed coculture system with murine and human cells and showed that human Tregs can suppress proliferation of murine Tcons by targeting murine DCs through LFA-1:ICAM-1 interaction between Tregs and DCs. Suppression was only evident in the presence of APCs, and it was not abrogated when human CTLA-4 was blocked. Interestingly, murine Tregs could not suppress human Tcons in the presence of human APCs. These results argue for species-specific differences in suppressive molecules, especially regarding CTLA-4.

Certain human and murine DC subsets express the enzyme indoleamine 2,3-dioxygenase (IDO) that catalyzes degradation of tryptophan to kynurenine, leading to starvation of effector T cells and also to direct cell cycle arrest. At the same time, IDO leads to iTreg generation (Fallarino et al., 2003, 2006; Curti et al., 2007). Tregs themselves can, via CTLA-4-induced signaling, further increase IDO expression in DCs. Additionally it was shown that murine Tregs diminish glutathione synthesis in DCs, presumably CTLA-4-mediated, which leads to a redox environment

unfavorable for Tcon proliferation (Yan et al., 2009, 2010). In conclusion, the importance of CTLA-4 in Treg-mediated suppression is undisputed, but it does not seem to be the only suppressive mechanism, and also species differences may exist. Importantly, CTLA-4 blockage has already shown promising outcomes in clinical trials for metastatic melanoma, presumably due to its effects on both effector T cells and Tregs (Peggs et al., 2009). Despite the prominent role for CTLA-4 in Treg-mediated inhibition of DC activation, involvement of the costimulatory molecule CD40, the deubiquitinase A20 acting as antigen-presentation attenuator, and the surface receptor neuropilin-1 has also been described (Hanig and Lutz, 2008; Sarris et al., 2008; Song et al., 2008). Further, the adhesion molecule LAG-3 is expressed on Tregs, binds to MHC class II molecules and was proposed to be involved in suppression, since deficiency or blockage of LAG-3 resulted in disturbance of T cell homeostasis *in vivo* and reduced suppressive activity of Tregs *in vitro* (Huang et al., 2004; Workman and Vignali, 2005). These effects might be mediated by LAG-3-induced inhibition of DC activation (Liang et al., 2008). However, LAG-3 deficient mice do not show signs of autoimmune disease. Further, MHC class II expression on an activated human Treg subpopulation that exerts rapid contact-dependent suppression seems to be important (Baecher-Allan et al., 2006) as blockage of MHC class II on activated Tregs was shown to abrogate suppression (Peiser et al., 2007).

Tregs do not only reduce antigen presenting activity of APCs but also support an immunosuppressive cytokine milieu by reducing IL-6 while increasing IL-10 production by DCs (Veldhoen et al., 2006). In addition to these mechanisms, Tregs can inhibit Tcon activation by reduction of contact formation between Tcons and DCs (Tadokoro et al., 2006). As already mentioned, there is mutual interplay between Tregs and tolerogenic DCs, the latter acting in induction and expansion of Tregs.

SUPPRESSION VIA THE IMMUNOSUPPRESSIVE CYTOKINES TGF-β, IL-10, AND IL-35

The role of immunosuppressive cytokines in Treg-mediated suppression is still incompletely understood. Despite the importance of TGF-β and IL-10 in several *in vivo* models, these cytokines seem to be dispensable for other disease models such as autoimmune gastritis (Suri-Payer and Fritzsching, 2006) as well as for most *in vitro* systems. Treg-mediated suppression via cytokines is described in more detail below.

TGF-β

TGF-β1 deficient mice develop T cell-mediated autoimmunity within several weeks after birth (Li et al., 2006b). A similar phenotype is observed in mice lacking TGF-β responsiveness specifically in T cells (Li et al., 2006a; Marie et al., 2006). Tregs can produce high amounts of membrane-bound and soluble TGF-β, and blocking TGF-β partially abrogated suppression of T cell proliferation *in vitro* using murine or human T cells (Nakamura et al., 2001, 2004; Levings et al., 2002), suggesting that Treg-produced TGF-β controls autoimmunity. However, other groups did not find involvement of TGF-β in Treg-mediated suppression of T cells (Baecher-Allan et al., 2002; Piccirillo et al., 2002; Godfrey et al., 2005; Oberle et al., 2007). Although TGF-β deficient Tregs could suppress Tcon proliferation *in vitro*, TGF-β1 production by Tregs was necessary to prevent colitis in several studies (Read et al., 2000; Izcue et al., 2009). In addition, TGF-βRII knockout Tcons could not be suppressed *in vivo* in colitis models, but TGF-β produced by other cells than Tregs was likely involved in these models (Mamura et al., 2004; Fahlen et al., 2005). Along that line, TGF-β produced by DCs in the gut was critical to prevent colitis by Treg induction (Travis et al., 2007). Nevertheless, mice with T cell-specific TGF-β1 deficiency showed enhanced Th1 and Th2 responses and immunopathology including colitis, underscoring the importance of T cell-produced TGF-β; however, these mice were resistant to the induction of experimental autoimmune encephalitis (EAE) likely due to impaired Th17 induction (Li et al., 2007). Therefore, the role of TGF-β in Treg-mediated suppression might depend very much on the type of effector cell and the site of the immune response, and TGF-β may even promote proinflammatory Th17 responses. Interestingly, human Tregs could confer suppressive activity to target Tcons in a cell-contact-dependent manner (so-called infectious tolerance). Suppressive cells generated by infectious tolerance use a mechanism involving TGF-β (Jonuleit et al., 2002). A more recent study shows that infectious tolerance in mice requires membrane-bound TGF-β on Tregs (Andersson et al., 2008). Thus, TGF-β function *in vivo* probably does not only involve direct suppression of effector T cell signaling but also induction of Tregs, consistent with lethal autoimmune disease and reduced peripheral Treg numbers in TGF-β1 deficient mice (Marie et al., 2005).

IL-10

The cytokine IL-10 exerts mainly immunosuppressive effects on various cell types (Moore et al., 2001). IL-10 plays an important role in Treg-mediated suppression of intestinal inflammation, since blocking IL-10 or using IL-10 deficient Tregs abrogates the protective effect of Tregs on T cell transfer-induced colitis (Asseman et al., 1999). Importantly, while the control of memory/antigen-experienced T cells during prevention or cure of colitis required IL-10, Treg-dependent prevention of naïve T cell-mediated colitis does not require IL-10 (Asseman et al., 2003; Uhlig et al., 2006), highlighting the involvement of different suppression mechanisms depending on the activation status of the target cell. The importance of IL-10 in Treg function *in vivo* has been extended to infection and EAE models (McGeachy and Anderton, 2005; Belkaid, 2007). Further, another study showed that Treg-derived IL-10 is important for control of inflammation at environmental interfaces but seems to be dispensable for control of systemic autoimmunity (Rubtsov et al., 2008). Along that line, IL-10 or IL-10 receptor deficient mice do not develop autoimmunity, but are susceptible to colitis in the presence of triggering flora (Kuhn et al., 1993; Spencer et al., 1998). Importantly, the IL-10 locus was identified as a susceptibility locus in ulcerative colitis (Franke et al., 2008), a form of human inflammatory bowel disease (IBD). Furthermore, IL-10RA or IL-10RB mutations result in severe early onset IBD (Glocker et al., 2009). Although IL-10 can suppress various immune cells including DCs, direct effects of IL-10 on effector/memory T cells are important in prevention of T cell-mediated colitis (Kamanaka et al., 2011). Two recent studies further delineate that IL-10R signaling is needed in Tregs as well as in Th17 cells in order to suppress colonic Th17

responses (Chaudhry et al., 2011; Huber et al., 2011). Adding to the complexity of IL-10 function in Treg-mediated suppression, it was recently shown that Tregs need IL-10 (and not IL-35 and TGF-β) to control IFN-γ production by T cells in the inflamed skin, while IL-10 was dispensable for regulation of IFN-γ and T cell expansion in the lymph node (Sojka and Fowell, 2011).

Of note, anti-inflammatory IL-10 might not be produced only by T cells including Tregs and Foxp3$^-$ T regulatory 1 (Tr1) cells (Roncarolo et al., 2006), but also by other cells such as regulatory B cells (Mauri and Ehrenstein, 2008) and macrophages. The latter were shown to indirectly influence suppression by acting on Tregs to maintain Foxp3 (Murai et al., 2009).

IL-35

IL-35 is a recently discovered cytokine implicated in Treg-mediated suppression and was shown to directly inhibit Tcon proliferation (Collison et al., 2007). Tregs deficient in one of the IL-35 chains had reduced suppressive ability *in vitro* and *in vivo* in an IBD model, although these mice did not show autoimmune disease. In contrast to murine Tregs, human Tregs do not constitutively express IL-35 (Bardel et al., 2008). Nevertheless, IL-35 may play a role also in human immunosuppression, as treatment of naïve human or mouse T cells with IL-35 induced a so-called iT$_R$35 regulatory population that mediated suppression via IL-35 but did not require IL-10, TGF-β, or Foxp3 (Collison et al., 2010). These iT$_R$35 were strongly suppressive in several *in vivo* mouse models. Although naïve human Tregs did not express high amounts of IL-35, long-term activation of human Tregs led to upregulation of the IL-35 subunits starting at 3 days of activation (Chaturvedi et al., 2011). These long-term activated Tregs exerted contact-independent *in vitro* suppression in an IL-35-dependent manner and also induced iT$_R$35 cells. Thus, IL-35 may contribute to infectious tolerance.

SUPPRESSION OF EFFECTOR CELLS BY IL-2 CONSUMPTION AND APOPTOSIS INDUCTION

One efficient way to repress immune responses would be killing of effector cells by Tregs, which was indeed observed in certain settings. It was shown that upon CD3/CD46 activation, human nTregs express the serine protease granzyme (Gzm) A and kill CD4$^+$ T cells and other target cells in a perforin-dependent manner, yet independent of the death receptor CD95 (Grossman et al., 2004). In another report, contact-dependent suppression of Tcon proliferation by activated murine Tregs *in vitro* was described to be partially GzmB-dependent; however, in contrast to the results of Grossman et al. perforin was not involved here (Gondek et al., 2005). As suggested from mouse studies, GzmB-mediated suppression might be important *in vivo* to maintain transplant tolerance (Gondek et al., 2008), but it might counteract tumor clearance by killing tumor-reactive NK cells and CD8$^+$ T cells (Cao et al., 2007). Involvement of the death receptor TRAIL in Treg-mediated suppression of activated mouse Tcons was shown *in vitro* and *in vivo* in a transplantation model, but required strong pre-activation of Tcons and Tregs (Ren et al., 2007).

The importance of IL-2 consumption by Tregs due to their high CD25 expression remains controversial. A study by Lenardo's group proposed that Tregs induce IL-2 deprivation-mediated apoptosis in mouse Tcons, which was dependent on close proximity between the cells (Pandiyan et al., 2007). However, the authors did not observe suppression of IL-2 production during the first 48 h of coculture, which is not in line with many other studies (Thornton and Shevach, 1998; Barthlott et al., 2005; Sojka et al., 2005). Pandiyan et al. (2007) found that T cells deficient in the proapoptotic protein Bim could not be suppressed *in vitro* and *in vivo* in a T cell transfer-induced IBD model. In contrast to these results, a recent study by Vignali's group did not find involvement of cell death induction by Tregs (Szymczak-Workman et al., 2011). Szymczak-Workman et al. used responder Tcons that are resistant to cytokine withdrawal-induced apoptosis due to Bim deficiency or Bim/Puma double knockout or Bcl-2 overexpression and performed *in vitro* and *in vivo* assays to conclude that Tregs do not suppress via induction of apoptosis in responder Tcons. In addition to these mouse studies, we (Oberle et al., 2007) and others (Vercoulen et al., 2009) also did not observe apoptosis induction in human Tcons by human Tregs.

IL-2 does not only promote survival but also proliferation of T cells, and IL-2 consumption seems to be involved in reducing the positive effects of IL-2 on proliferation and IL-2 expression, because exogenous IL-2 could abrogate Treg-mediated suppression of proliferation and/or of IL-2 production *in vitro* (Thornton and Shevach, 1998; Baecher-Allan et al., 2001; de la Rosa et al., 2004). However, during inhibition of murine Tcons by human Tregs, IL-2 consumption was not causative for suppression of proliferation, as blocking human CD25 did not abrogate suppression under optimal stimulation conditions for the human Tregs (Tran et al., 2009). Of note, under suboptimal stimulation conditions, human Tregs required CD25 signals in order to be functionally suppressive, yet the suppression mechanism under these conditions remains elusive and may or may not involve IL-2 consumption (Tran et al., 2009). We found that, although exogenous IL-2 partially abrogated suppression of human Tcon proliferation by human Tregs, rapid suppression of IL-2 transcription by human pre-activated Tregs was not affected (Oberle et al., 2007), which argues for different suppression mechanisms for proliferation and cytokine transcription. Here, IL-2 itself led to increased IL-2 mRNA expression (Oberle et al., 2007), however, for later time points, negative feedback of IL-2 on IL-2 mRNA and protein expression was described (Villarino et al., 2007). These differences in the effect of IL-2 on IL-2 expression may depend on time point, species, amount of IL-2 and cellular activation status and may contribute to the inconsistent results on the role of IL-2 consumption in Treg-mediated suppression. Together, the role of IL-2 consumption remains controversial and may depend on the specific setting and stimulation conditions of the Tregs. IL-2 consumption by Tregs may even enhance certain effector T cell reactions, which was shown for Th17 responses (Pandiyan et al., 2011). In addition, the link of IL-2 consumption to apoptosis is unclear, as exogenous IL-2 could rescue proliferation of suppressed human Tcons without reducing background apoptosis (Vercoulen et al., 2009).

Together, it can be concluded from these studies that IL-2 consumption by Tregs and apoptosis induction in suppressed target cells may only play a role in suppression under certain circumstances.

SUPPRESSION VIA EXPRESSION OF EFFECTOR T CELL-SPECIFIC TRANSCRIPTION FACTORS

Tregs may be specialized to suppress a certain $CD4^+$ T cell subset in particular by expression of the hallmark transcription factor of this very subset. In this regard, it was shown that a Treg subset upregulated T-bet in response to IFN-γ, which was essential for the control of Th1-mediated inflammation (Koch et al., 2009). Similarly, IRF-4 (which is typically made by Th2 and Th17 cells) expression in Tregs was required for suppression of Th2 responses (Zheng et al., 2009). Moreover, expression of STAT3 (which drives Th17 differentiation) in Tregs was increased and crucial during control of Th17-mediated intestinal pathology (Chaudhry et al., 2009). Adding to that list, loss of the Th2-associated transcription factor GATA-3 in Tregs led to autoimmune disease, in that case accompanied by destabilization of Foxp3 expression and elevated Th1, Th2, and Th17 cytokine levels (Wang et al., 2011). GATA-3 deficient Tregs tended to convert into a Th17 phenotype, and it was further shown that GATA-3 was upregulated upon stimulation in murine as well as human Tregs (Wohlfert et al., 2011). However, the mechanism through which Tregs suppress via expression of effector T cell-specific transcription factors is currently unclear but might involve competition for limiting factors. The transcription factors could directly interact with Foxp3, as described for IRF-4, STAT3, and RORγt. Expression of the effector T cell-specific transcription factors and their regulated genes such as chemokine receptors might also enable Tregs to migrate to and proliferate at the site of immune response. Accordingly, suppressor activity of IRF-4 or STAT3 knockout Tregs *in vitro* was shown to be unimpaired, and GATA-3 was selectively expressed by Tregs at environmental interfaces.

In contrast, expression or activation of Th-specific transcription factors in Tregs might lead to a loss of Foxp3 expression and suppressive function. For example, STAT3 was described to mediate downregulation of Foxp3, and intriguingly, STAT3 is activated by several cytokines which negatively regulate Tregs and Foxp3, such as IL-6, IL-23, or IL-27 (Yang et al., 2007, 2008; Yao et al., 2007; Huber et al., 2008; Ahern et al., 2010). Thus, loss of STAT3 expression in T cells during T cell transfer-induced colitis and systemic inflammation increased Treg induction and ameliorated disease (Durant et al., 2010).

Plasticity between $CD4^+$ T cell subsets might be higher than originally anticipated, although the stability of the Treg lineage *in vivo* is highly controversial (Zhou et al., 2009; Rubtsov et al., 2010). Yet several lines of evidence indicate that plasticity is particularly pronounced between Th17 cells and iTregs (Lee et al., 2009), and an intermediate state with expression of both RORγt and Foxp3 at the same time was observed (Lochner et al., 2008; Zhou et al., 2008). In certain cytokine milieus, even induction of IL-17 was described in murine and human Tregs, which might retain suppressive function (Xu et al., 2007; Koenen et al., 2008; Osorio et al., 2008; Beriou et al., 2009; Voo et al., 2009).

ADENOSINE AS IMMUNOSUPPRESSIVE MOLECULE

Hydrolysis of extracellular ATP to ADP or AMP by the ectoenzyme CD39, expressed by all murine Tregs and by about 50% of human Tregs, represents another Treg-mediated anti-inflammatory mechanism (Borsellino et al., 2007). CD39 knockout Tregs showed reduced suppressive capacities *in vitro* and *in vivo* (Deaglio et al., 2007). In human Tregs, CD39 expression was suggested to identify a highly suppressive Treg subset (Mandapathil et al., 2009), and suppression of Tcon proliferation by this subset could be partially abrogated by blockage of ectonucleotidase activity (Mandapathil et al., 2010). CD73, also expressed by Tregs, further degrades AMP to adenosine (Kobie et al., 2006). Adenosine signals via the A2A adenosine receptor and may inhibit DCs as well as activated T cells, e.g., by elevation of cyclic AMP (cAMP; Ernst et al., 2010). In human but not mouse T cells, ATP can also directly increase cAMP levels through the $P2Y_{11}$ receptor (Abbracchio et al., 2006). *In vivo*, A2A receptor signaling might induce anergy and promote iTreg generation (Zarek et al., 2008). In conclusion, generation of adenosine seems to be important for the suppressive function of certain Treg subsets.

SUPPRESSION VIA cAMP, ICER, AND NFAT

Tregs produce high levels of cAMP and cAMP amounts were shown to rise in suppressed Tcons upon contact with Tregs, presumably via transfer through gap junctions from murine Tregs to Tcons (Bopp et al., 2007). Accumulation of high cAMP amounts in Tregs might require Treg pre-activation (Bazhin et al., 2010). cAMP seems to be an important component of Treg-mediated suppression, since suppression of IL-2 transcription and proliferation could be partly abrogated by a cAMP antagonist or a gap junction inhibitor (Bopp et al., 2007). The same group recently also showed that induction of cAMP in murine DCs upon Treg coculture contributes to suppression of DCs (Fassbender et al., 2010). The authors suggest that in suppressed Tcons, cAMP acts by inducing expression of the inducible cAMP early repressor (ICER), which functions as a repressor, e.g., at the IL-2 and IL-4 gene loci (Bodor et al., 2000). ICER protein and mRNA were previously shown to be increased in suppressed mouse T cells after 17–20 h of Tcon:Treg coculture stimulation (Bodor et al., 2007a) which correlated with an increase in cAMP (Bopp et al., 2007). In these studies, an increase in ICER and cAMP was not yet detectable after 4–5 h. In human Tcons, we also did not observe ICER upregulation upon coculture with Tregs compared to coculture with Tcons within 5 h (Oberle et al., 2007; Oberle and Schmidt, unpublished data). Since NFAT1/4 double knockout Tcons cannot be fully suppressed by Tregs in long-term suppression assays, such as proliferation or IL-2 mRNA and protein expression after 18 h (Bopp et al., 2005), it was proposed that NFAT forms inhibitory complexes on cytokine promoters with transcriptional repressors such as ICER, PPRγ, or $p21^{SNFT}$ (Bandyopadhyay et al., 2007; Bodor et al., 2007a,b). However, by calculating the percentage of suppression, partial inhibition can be observed although NFAT1/4 double knockout T cells display hyperproduction of IL-2, suggesting that NFAT1/4 double knockout Tcons are less susceptible, but not completely resistant to Treg-mediated suppression. Furthermore, although ICER failed to accumulate in suppression assays with B7 deficient responder T cells (as ICER induction in suppressed T cells was CTLA-4/B7 and GITR-dependent), residual suppression was still detectable (Bodor et al., 2007a). These results argue for additional suppressive mechanisms together with those mediated by CTLA-4 and ICER:NFAT. More recently, it was proposed that NFAT2 together with ICER translocates to the nucleus

to confer repression of the IL-2 promoter in murine T cells upon coculture with Tregs (Vaeth et al., 2011). However, depending on the experimental setting, either increased or decreased NFAT2 nuclear translocation was observed in suppressed compared to control mouse T cells, while ICER clearly translocated into the nucleus upon Treg-mediated suppression (Vaeth et al., 2011).

Together with a report showing that Cbl-b deficient T cells are less sensitive to suppression by Tregs (Wohlfert et al., 2004), data on inhibitory NFAT complexes imply that Treg-suppressed Tcons display features of anergy, as both NFAT1 and Cbl-b are crucial in anergy induction (Macian et al., 2002; Soto-Nieves et al., 2009). Anergy induction in suppressed Tcons might occur later than inhibition of cytokine transcription since we did not detect accumulation of ICER mRNA or other anergy-related genes up to 5 h after TCR stimulation in suppressed compared to control human Tcons (Oberle et al., 2007). By comparing gene expression of suppressed and anergized murine Tcons, Sukiennicki and Fowell (2006) found only partial overlap of NFAT-dependent gene expression while most genes did not overlap. However in their study, changes in gene expression were not yet pronounced 12 h after coculture, but only evident after 36 h. This is in contrast to the gene array performed with human T cells in our group, in which, e.g., NFAT and NF-κB target genes were already suppressed in human Tcons within 1.5–3 h of coculture; however, this gene array was performed with pre-activated Tregs (Schmidt et al., 2011). Thus, discrepancies may be due to Treg pre-activation or species.

In contrast to its role in anergy induction, NFAT also plays an important activating role in TCR-induced transcription of T cell effector cytokines. This rapid TCR-induced NFAT activation in Tcons is suppressed by Tregs, which will be described in the next section.

RAPID SUPPRESSION OF TCR-INDUCED CALCIUM, NFAT AND NF-κB SIGNALING AND CONSEQUENTLY CYTOKINE TRANSCRIPTION IN Tcons UPON SUPPRESSION BY Tregs

Signaling events and in particular TCR signaling in responder Tcons upon suppression by Tregs has not been studied extensively so far. Yet detailed knowledge might be important for therapeutic interventions in certain T cell-mediated diseases, as resistance of effector T cells to Treg-mediated suppression frequently occurs in autoimmune diseases (Buckner, 2010). However, kinetics of cytokine suppression has been analyzed in detail. Our group previously showed that inhibition of cytokine transcription occurs rapidly within 1–3 h (Oberle et al., 2007). This suppression of cytokine transcription was also observed when stronger TCR/CD28 stimulation was applied and occurred within 30–45 min when pre-activated Tregs were used (Schmidt et al., 2011). Thornton and Shevach (1998) showed potent inhibition of IL-2 mRNA production in murine Tcons by Tregs, though the authors did not analyze earlier than 15 h. Further experiments with murine Tcon:Treg cocultures showed that IL-2 mRNA suppression starts between 5 and 6 h of coculture stimulation (Barthlott et al., 2005) or even later, after 6–12 h (Sojka et al., 2005). In a subsequent paper, the latter group did not observe IL-2 mRNA suppression after 12 h of coculture (Sukiennicki and Fowell, 2006). In contrast to our experiments, these studies were conducted in the presence of APCs and with murine T cells. Thus, discrepancies in IL-2 mRNA suppression kinetics might be due to species differences or experimental setup regarding strength of stimulation and presence of APCs. There may also be differences in responder Tcon susceptibility to suppression, which could be influenced by the cell purification procedure and resting time between purification and initiation of cocultures. In addition, we used pre-activated Tregs, while the others did not. Nevertheless, when Sojka et al. used pre-activated Tregs, they still observed suppression of IL-2 secretors not earlier than 4–6 h after initiation of coculture. However, at earlier points in time, the induction of IL-2 also was still very low (Sojka et al., 2005).

To analyze Treg-mediated effects on Tcons, we favored an *in vitro* cell culture system without APCs. Thus, we could analyze direct effects of Tregs on Tcons and we obtained pure populations of responder Tcons after coculture. However, it is still a matter of debate whether the main targets of Treg-mediated suppression *in vivo* are T cells or rather APCs. It remains possible that Tregs utilize distinct mechanisms to directly suppress Tcons or APCs, and both mechanisms might play a role *in vivo*. Although several studies have shown that murine as well as human Tregs inhibit TCR-induced proliferation of Tcons in the absence of APCs (Dieckmann et al., 2001; Ermann et al., 2001), others found that murine Tregs fail to inhibit Tcons stimulated by anti-CD3 antibodies (Thornton and Shevach, 2000). In general, significant suppression in the absence of APCs is mostly observed at high (1:1) Treg to Tcon ratios, which makes cell density very important to control. Nevertheless, as APC-containing cocultures may represent a more physiological setting than Tcon:Treg cocultures without APCs, we also used another *in vitro* cell culture system to show that rapid suppression of cytokine transcription can occur in the presence of APCs (Schmidt et al., 2011).

Transcription of several effector T cell cytokines is initiated upon TCR stimulation and costimulation by the cognate antigenic peptide presented on a mature APC or, artificially, by agonistic anti-CD3 and anti-CD28 antibodies. As we observed rapid suppression of cytokine transcription, we questioned whether Tregs directly influence TCR signaling. Until recently, there was no comprehensive study on TCR signaling events in Tcons upon suppression by Tregs. TCR stimulation leads to a cascade of protein phosphorylation and dephosphorylation events, which ultimately activates transcription factors to initiate a distinct transcriptional program (Lin and Weiss, 2001; Smith-Garvin et al., 2009). The most important transcription factors for the induction of cytokine expression are NF-κB, NFAT, and AP-1, which together mediate activation of several cytokine genes, e.g., IL-2 and IFN-γ (Jain et al., 1995; Young, 1996; Sica et al., 1997). Before we describe our results regarding TCR signaling in Treg-suppressed Tcons, we will give a brief introduction into TCR signaling.

OVERVIEW: TCR SIGNALING

Activation of the NF-κB, NFAT, and AP-1 signaling pathways starts with TCR stimulation-induced phosphorylation of TCR-associated CD3 chains by the Src family kinases Fyn and Lck, leading to binding of ZAP-70. ZAP-70 phosphorylates the adapter protein LAT, which is critical for recruitment of important signaling proteins, leading to activation of Phospholipase Cγ1 (PLCγ1;

Qi and August, 2007). Active PLCγ1 cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG mediates its effects via membrane recruitment and, thus, activation of signaling proteins, whereas IP_3 induces calcium (Ca^{2+}) influx into the cytosol. Activation of both NF-κB and NFAT requires the activity of PLCγ1, while the AP-1 pathway is largely PLCγ1-independent. These signaling pathways are summarized in **Figure 2** and will be described in more detail, although still simplified, below.

Ca^{2+} signaling

The main pathway to initiate a Ca^{2+} signal in T cells is via IP_3 binding to IP_3 receptors (IP_3Rs) in the endoplasmatic reticulum (ER) membrane, which leads to release of Ca^{2+} from the ER into the cytoplasm. Depletion of the ER Ca^{2+} store induces Ca^{2+} influx from outside the cell by a process called store-operated calcium entry (SOCE). The stromal interaction molecule (STIM) senses ER Ca^{2+} store depletion and in turn triggers opening of calcium release-activated calcium (CRAC) channels in the plasma membrane, leading to Ca^{2+} entry from the extracellular space into the cytoplasm (Quintana et al., 2005; Feske, 2007).

T cells express three types of IP_3Rs to varying degrees depending on their stage of differentiation (Grafton and Thwaite, 2001). It is not clear which IP_3R is the most important receptor in peripheral T cells, and knowledge of regulation of these receptors by molecules other than IP_3 is very limited. IP_3-mediated Ca^{2+} store depletion appears to be the main mode of Ca^{2+} entry in lymphocytes (Oh-Hora and Rao, 2008). However, Ca^{2+} release from intracellular stores can be initiated not only by IP_3, but cyclic ADP ribose (cADPR) seems to be important for long-lasting release of Ca^{2+} from stores (Guse et al., 1999; Schwarzmann et al., 2002). Further, nicotinic acid adenine dinucleotide phosphate (NAADP) was described to act prior to IP_3 and cADPR to provide sufficient Ca^{2+} amounts to sensitize IP_3Rs (Gasser et al., 2006). Regarding plasma membrane Ca^{2+} channels, there is evidence that CRAC channels are most critical for TCR signal transduction (Feske et al., 2005), but the L-type voltage-gated Ca^{2+} channel $Ca_V1.4$ also seems to be important for TCR signaling and T cell homeostasis (Omilusik et al., 2011). STIM1 seems to be the predominant player

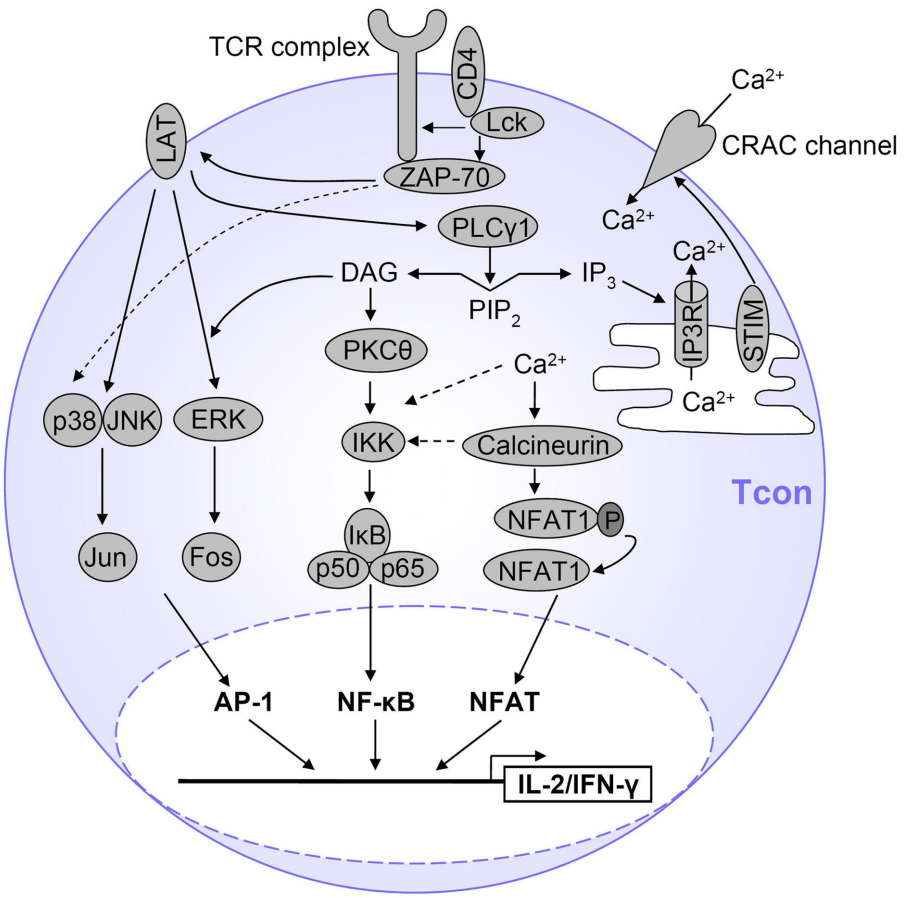

FIGURE 2 | Simplified view of TCR signaling in T cells. After TCR stimulation, several pathways are initiated which ultimately result in T cell activation, proliferation and expression of cytokines such as IL-2 and IFN-γ. The main transcription factors involved are AP-1, NFAT, and NF-κB. AP-1 is activated by a MAPK cascade. NF-κB is activated by PKCθ-dependent activation of the IKK complex and subsequent IκB degradation, and Ca^{2+} also contributes to NF-κB activation. NFAT is activated by Ca^{2+} influx which activates the NFAT-dephosphorylating phosphatase calcineurin. Only the major pathways are displayed here and represent a simplified view of TCR signaling. Details are described in the text.

of SOCE in T cells although both STIM1 and STIM2 can reconstitute SOCE in STIM1 deficient T cells (Oh-Hora et al., 2008). However, STIM1 as well as STIM2 knockout T cells show severely impaired Ca^{2+} entry, impaired NFAT activation and cytokine production. Of note, these cells can still proliferate *in vivo* (Oh-Hora et al., 2008). Interestingly STIM1, which is very abundant in lymphocytes but not in excitable cells, inhibits voltage-gated $Ca_V1.2$ channels while it activates the CRAC constituent ORAI (Park et al., 2010; Wang et al., 2010).

NFAT activation

NFAT activation in T cells is dependent on the generation of a Ca^{2+} signal, which activates the Ca^{2+}:calmodulin-dependent phosphatase calcineurin. Calcineurin dephosphorylates NFAT, thus unmasking its nuclear localization sequence and enabling transport of NFAT into the nucleus. The NFAT family consists of five members, three of which are expressed by T cells: NFAT1 (also called NFATp or NFATc2), NFAT2 (NFATc or NFATc1), and NFAT4 (NFATx or NFATc3; Macian, 2005), and each of them appears in several isoforms. The constitutively expressed NFAT1 represents the main NFAT isoform in resting T cells (Macian et al., 2002). Although NFAT can act as a transcription factor alone or in homodimers, many transcription factors interact with NFAT to activate or inhibit transcription, amongst them AP-1. Various cytokine promoters, e.g., IL-2, IL-4, IL-5, IFN-γ, and TNF-α contain cooperative NFAT:AP-1 binding sites which greatly enhance transcription of the respective cytokine (Rao et al., 1997; Chinenov and Kerppola, 2001; Macian et al., 2001; Hermann-Kleiter and Baier, 2010). Of note, AP-1 binding to the IL-2 promoter becomes undetectable in the absence of NFAT (Jain et al., 1993). Interestingly, it could be shown that differences in Ca^{2+} influx change the number of IL-2 expressing human $CD4^+$ T cells rather than the amount of IL-2 expression per cell, suggesting an all-or-nothing response in Ca^{2+}-induced IL-2 (and IFN-γ) production. NFAT was identified as the molecular switch being either in or outside the nucleus, while nuclear translocation of NF-κB and expression of exclusive NF-κB target genes was graded (Podtschaske et al., 2007).

NF-κB activation

In resting T cells, the NF-κB heterodimer is associated with the cytosolic inhibitor of κB (IκB), which masks its nuclear localization sequence (Vallabhapurapu and Karin, 2009). Several NF-κB and IκB family members exist, from which the p65:p50:IκBα complex is the most prominent NF-κB:IκB complex in T cells. After TCR triggering, DAG is important for localization and, thus, activation of PKCθ (Isakov and Altman, 2002). PKCθ activates the NF-κB pathway by modulation of the CARMA1:Bcl-10:MALT1 (CBM) complex, which then activates the trimeric IKK complex comprising the catalytic subunits IKKα and IKKβ as well as the regulatory subunit IKKγ. The catalytic subunits of the active IKK complex phosphorylate IκBα, leading to IκBα polyubiquitination and subsequently its proteasomal degradation, allowing for translocation of NF-κB to the nucleus. Importantly, NF-κB activation is also influenced by Ca^{2+} (see below).

The NF-κB complex itself can be phosphorylated and acetylated, which regulates its transcriptional activity and association with other transcription factors (Li and Verma, 2002; Perkins, 2006). For example, phosphorylation of p65 on serine (Ser)529 and Ser536 in its transactivation domain by IKKβ may enhance activity (Sakurai et al., 1999).

The AP-1 pathway

The AP-1 pathway is initiated by a MAPK cascade leading to activation of MAPKs, such as ERK, JNK, and p38 (Dong et al., 2002). LAT recruits certain proteins, which are important for MAPK activation via Ras and Rac. Recent data led to the assumption that in T cells, p38 is not only activated by the classical MAPK cascade but also, and primarily, via an alternative pathway characterized by direct phosphorylation of p38 by ZAP-70 and subsequent p38 autophosphorylation (Mittelstadt et al., 2009). MAPKs promote expression, phosphorylation, and activation of Fos and Jun, which together comprise the AP-1 transcription factor (Johnson and Lapadat, 2002). In contrast to NF-κB and NFAT, the AP-1 pathway is largely independent of PLCγ, but DAG can enhance Ras recruitment and activation and, thus, ERK1/2 signaling. In addition, calcineurin can contribute to JNK activation (Werlen et al., 1998), however, JNK1/2 seem to be dispensable for primary activation and IL-2 production of T cells (Sabapathy et al., 2001).

TCR SIGNALING IN Tcons UPON Treg-MEDIATED SUPPRESSION

Previously, our group showed that rapid suppression of Th1 cytokine transcription in human Tcons by Tregs occurs immediately after induction of mRNA transcription (Oberle et al., 2007). The suppression mechanism was independent of TGF-β and IL-10, IL-2 consumption, initiation of apoptosis, and induction of anergy-related genes in suppressed Tcons. As Tcon inhibition was detected very rapidly, we asked whether Tregs may directly disrupt key components of TCR signaling which could impede Tcon cytokine transcription and, consequently, Tcon function. Indeed, our recent data show that pre-activated human Tregs rapidly suppressed TCR-induced signaling events in Tcons (Schmidt et al., 2011). In detail, TCR-induced Ca^{2+} release from intracellular stores and, consequently, Ca^{2+} influx was blocked immediately after TCR stimulation in suppressed Tcons. Inhibition of Ca^{2+} signals resulted in decreased NFAT1 dephosphorylation and NFAT target gene expression. Also NF-κB activation, as detected by phosphorylation of IKK, IκBα, and p65 as well as target gene expression, was strongly and rapidly inhibited. Ca^{2+} suppression appears to be causative for suppression of NFAT, NF-κB, and IL-2, as suppression of either one could be abrogated by artificial Ca^{2+} store depletion. In contrast, Ca^{2+}-independent events such as TCR-proximal signaling and the AP-1 pathway were not affected in suppressed Tcons. Interestingly, PLCγ1 phosphorylation as well as IP_3 generation were not altered in suppressed Tcons. Therefore, Tregs inhibit Ca^{2+} store depletion in Tcons via so far unknown mechanisms.

A summary of these results is shown in **Figure 3** and discussed in more detail below.

TCR-proximal and AP-1 signaling

To our knowledge, there are no studies regarding phosphorylation of TCR signaling molecules in $CD4^+$ Tcons upon suppression by Tregs. An approach using human $CD8^+$ T cells demonstrated that CD3ζ and ZAP-70 phosphorylation was unaffected by Tregs, though activation of ERK was inhibited (Baatar et al., 2009). In

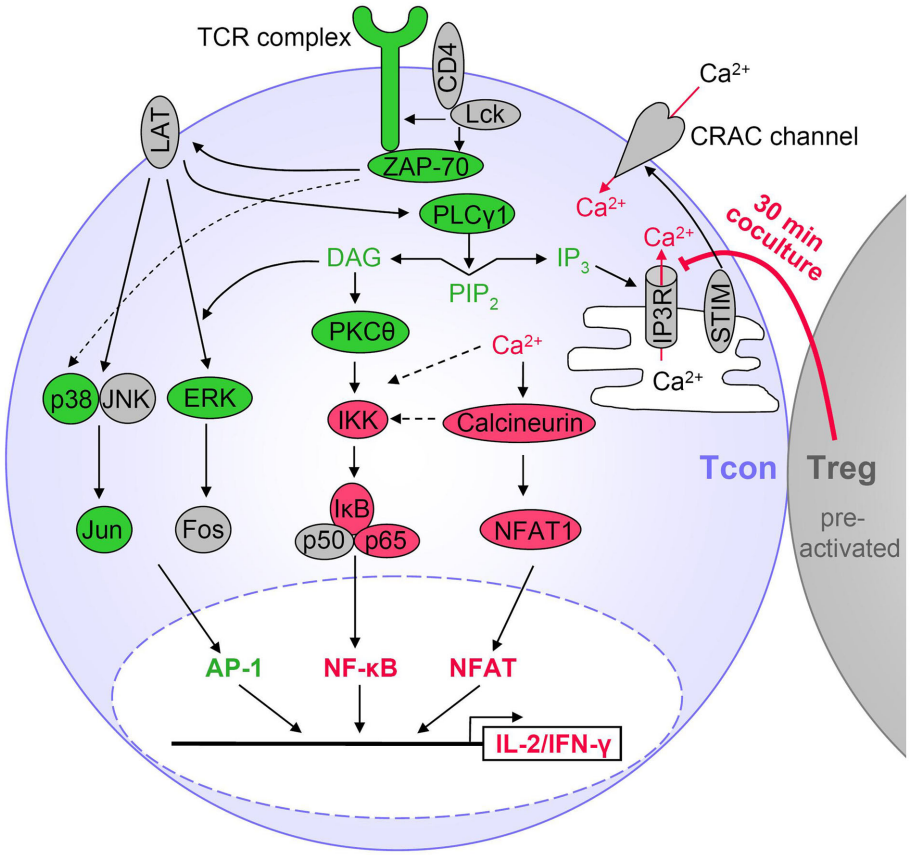

FIGURE 3 | Tregs suppress particular TCR signaling pathways in Tcons. Activated Tregs rapidly suppress cytokine expression in Tcons via inhibition of Ca^{2+} signals and consequently reduced NF-κB and NFAT activation. Suppression by Tregs requires at least 30 min of coculture, is favored by cell contact and sustained after Treg removal. Red indicates Treg-mediated suppression of TCR-induced activation in Tcons. Molecules displayed in green were not inhibited during Treg-mediated rapid suppression of cytokine transcription. Molecules in gray were not analyzed or TCR-induced activation was not detectable.

contrast, during rapid suppression of $CD4^+$ Tcons, we did not find inhibition of the AP-1 pathway. This discrepancy might be due to differences between $CD4^+$ and $CD8^+$ T cells or to the coculture setup as Baatar et al. used non-pre-activated Tregs and cocultures were set up 6 h before stimulation.

Suppression of calcium signaling in Tcons

At first glance, our results seem to be in contrast with two other studies which claim that Tregs do not inhibit initial TCR signaling in target T cells. However, the experimental setup differs. Tang and Krummel (2006) analyzed TCR-induced Ca^{2+} signals in suppressed murine T cells in contact with freshly isolated Tregs by microscopic Ca^{2+} measurements. The authors used a TCR-transgenic system and stimulated with peptide-pulsed B cells. They did not observe suppression of Ca^{2+} signals in Tcons and concluded that Tregs do not influence TCR-proximal and Ca^{2+} signaling. However, using non-pre-activated Tregs, Ca^{2+} suppression may not be detectable as Tregs might not be sufficiently activated within the short time period of these experiments to obtain suppressive capacities (unpublished observation). In addition, species differences might account for the discrepancy between these results and ours. Another study found that sustained Ca^{2+} increase in human T helper cells was not affected by Tregs when they were stimulated with superantigen-pulsed B cells (Esquerre et al., 2008). A main difference between these experiments and ours is that the authors explored the effect of expanded Tregs on the effector phase of the immune response and, thus, as target cells they used pre-activated T helper cells which were expanded at least 14 days *in vitro*. These cells might be less susceptible to Treg-mediated Ca^{2+} suppression and/or more reactive to stimulation than resting $CD4^+CD25^-$ Tcons as used in our study. In addition, stimulation by superantigen-pulsed B cells might be stronger than the anti-CD3/anti-CD28 antibody stimulation used by us. As the authors do not provide data on the initial stimulation-induced increase in Ca^{2+} concentrations in T helper cells but focus on the sustained Ca^{2+} signal, it cannot be discussed whether Tregs would influence the initial increase in Ca^{2+} immediately after being stimulated by the APC, as we observe for Tcons stimulated with crosslinked anti-CD3/anti-CD28 antibodies. Of note, we also detected rapid suppression of NF-κB signaling and IL-2 transcription when we used APCs plus anti-CD3 antibodies for stimulation, although we did not perform Ca^{2+} analyses

in this setting. Interestingly, Esquerre et al. found that human Tregs (which were *in vitro* expanded for at least 14 days) inhibited polarization of T helper cell IFN-γ toward the APC in a TGF-β-dependent manner, while inhibition of proliferation and IFN-γ production was not affected by TGF-β blocking. Together, these data again emphasize that Tregs seem to employ different mechanisms of suppression, depending on the activation state of the Tregs themselves as well as of the responder T cells and suggest differences in inhibition of priming versus inhibition of effector function.

We found that Tregs inhibited Ca^{2+} signaling without involvement of TCR-proximal signaling molecules, including PLCγ1. Similarly, a report by Gri et al. (2008) shows Treg-mediated suppression of Ca^{2+} signals in murine mast cells in a PLCγ2-independent fashion. However, suppression of Ca^{2+} signals in mast cells did not affect Ca^{2+} store depletion but rather involved a cAMP-dependent suppression of Ca^{2+} influx through the plasma membrane. The authors proposed that cAMP alters the membrane potential through unknown mechanisms and decreases permeability of Ca^{2+} channels, as demonstrated for mast cells previously. This suppression was dependent on interaction of OX40 on Tregs with OX40L on mast cells. In our setup, OX40 is likely not involved since OX40L is not expressed by T cells, but mainly by APCs. Though suppression of Ca^{2+} signals might be a general mechanism of Treg action, we describe a cAMP-independent rapid suppression of Ca^{2+} store depletion in human T cells. This Ca^{2+} suppression resulted in immediate inhibition of NF-κB and NFAT activation and, consequently, IL-2 transcription. However, we still observed a late effect with a cAMP antagonist on IL-2 mRNA suppression upon stimulation for approximately 5 h (unpublished observation), which is in line with results by others using murine Tcons (Bopp et al., 2007). Interestingly, we found that IFN-γ suppression was not affected by the cAMP antagonist (unpublished observation). In general, suppression mechanisms might be diverse regarding different cytokines, e.g., by the influence of promoter-specific repressors such as ICER.

The exact mechanism through which Tregs inhibit Ca^{2+} store depletion in Tcons remains elusive. Since IP_3 amounts were unaltered, IP_3 metabolism seems unchanged. Treg pre-activation was required to observe this rapid suppression, yet it is unknown which molecular changes take place in Tregs during pre-activation. Furthermore, the receptor for Tregs on Tcons remains elusive; however, our results indicate that triggering of this unknown receptor must lead to a suppressive signal within approximately 30 min. It is tempting to speculate that this rapid process involves signaling via phosphatases or kinases in Tcons. Suppression might involve yet unknown modifications of the IP_3R which hinder its interaction with IP_3. Future studies will be needed to address these questions and decipher the mechanism how Tregs inhibit Ca^{2+} signals in Tcons. In addition, it would be interesting to analyze potential differences in Ca^{2+} suppression when distinct Treg subsets are used. Further, there may be differences in suppression of diverse target T cell types. Although Tregs can suppress Th1, Th2, and Th17 cytokines, one could speculate that Ca^{2+} suppression may be irrelevant for Th17 suppression, since IP_3R-mediated Ca^{2+} release is needed for initial IL-2 and IFN-γ production, but negatively regulates IL-17 production (Nagaleekar et al., 2008). Of note, several studies found that Th17 cells tend to be refractory to Treg-mediated suppression (Stummvoll et al., 2008; Chauhan et al., 2009; Vercoulen et al., 2009), although the underlying mechanism remains to be investigated.

NFAT1 suppression

In addition to the rapid suppression of NFAT1 dephosphorylation we described, it is possible that after several hours of Treg:Tcon interaction, NFAT also exerts a repressive role by forming inhibitory complexes with other transcription factors on cytokine promoters, as described for NFAT:ICER complexes. Different roles of NFAT depending on the experimental readout are also implied by data from Vaeth et al. (2011) who observed increased NFAT2 nuclear translocation upon contact to Tregs in an adoptive T cell transfer model while in an *in vitro* coculture assay of murine Tcons and Tregs in the presence of APCs, they show decreased NFAT2 nuclear translocation. The latter result corresponds to our data for human NFAT1. Vaeth et al. analyzed NFAT2/ICER translocation not earlier than 24 h after stimulation. We could not detect NFAT2 in primary human Tcons upon short-term stimulation (unpublished observation), in line with reports describing that NFAT2/αA, the most prominent NFAT2 isoform in peripheral T cells, is only weakly expressed in the naïve state and induced following CD3/CD28 stimulation (Serfling et al., 2006). In conclusion, NFAT2:ICER complexes may play a role in suppression of pre-activated T cells or within long-term suppression assays, but do not seem to be involved in resting T cells. In addition, other NFAT family members may have a dual role in early versus late suppression mechanisms.

Suppression of NF-κB signaling in Tcons downstream of PKCθ

To our knowledge, except for our report (Schmidt et al., 2011) there are no publications comparing IKK and IκBα phosphorylation or NF-κB activation in suppressed and control Tcons. Yet one study showed that Tregs inhibit TLR-induced NF-κB activation in murine macrophages, suggesting suppression of NF-κB as a common mechanism of Treg-mediated suppression (Li et al., 2010). However, Li et al. cannot exclude that NF-κB suppression is a secondary effect due to increased IL-10 and TGF-β levels in the suppressed macrophages.

As artificial Ca^{2+} store depletion could abrogate Treg-mediated suppression, we hypothesize that suppression of Ca^{2+} signals causes suppression of NF-κB. However, the exact mechanism through which Ca^{2+} influences NF-κB in T cells and how Tregs interfere here remains to be resolved. Calcineurin, which is activated by Ca^{2+}, synergistically contributes to degradation of IκBα when PKCθ is active (Frantz et al., 1994; Feske, 2007). As shown in Jurkat and primary T cells, this effect is probably due to activation of IKKβ by calcineurin and PKCθ (Trushin et al., 1999). The effects of Ca^{2+} on the CBM complex are less clear and involve positive as well as negative regulation. On the one hand, the Ca^{2+}:calmodulin-dependent kinase CaMKII was shown to enhance NF-κB activation by phosphorylation of CARMA1 (Ishiguro et al., 2006) and Bcl-10 (Oruganti et al., 2011). Furthermore, calcineurin was recently reported to enhance CBM complex formation by dephosphorylation of Bcl-10 [at (a) different phosphosite(s)] (Frischbutter et al., 2011; Palkowitsch et al., 2011). On

the other hand, Ca^{2+}:calmodulin binding to Bcl-10 was shown to reduce association of Bcl-10 with CARMA1 (Edin et al., 2010). Interestingly, we have indications pointing to an unknown and calcineurin-independent role for IKK in rapid TCR-mediated and Ca^{2+}-dependent NF-κB activation. In Tcons which were treated with the calcineurin inhibitor cyclosporin A, NFAT activation could be completely abrogated while IKK phosphorylation was not and IκBα phosphorylation was only slightly affected (unpublished observation). In contrast, when we treated Tcons with the Ca^{2+} chelator EGTA, IKK and IκBα phosphorylation was markedly reduced, similar to what we observe upon coculture with Tregs (Schmidt et al., 2011). Yet calcineurin inhibition might add to NF-κB suppression, as p65 phosphorylation was shown to be sensitive to calcineurin inhibitors (Frischbutter et al., 2011).

It remains possible that NF-κB suppression is not only mediated by Ca^{2+} suppression, but other components of the NF-κB pathway might be inhibited as well. Our data indicate that PKCθ activity is unchanged as PKCθ (Thr538) phosphorylation was not suppressed. Thr538 phosphorylation is important for PKC activity, interaction with IKK, and NF-κB activation. This phosphorylation is generated by the kinase GLK, which is activated downstream of the TCR (Chuang et al., 2011), and also involves PDK1 activation downstream of the costimulatory receptor CD28 (Liu et al., 2002; Lee et al., 2005). However, other phosphorylation sites in PKCθ have been described. Furthermore, PKCθ might also act upstream of PLCγ (Manicassamy et al., 2006a) and may also influence Ca^{2+} flux though there are controversies in two different studies with PKCθ deficient mice (Sun et al., 2000; Pfeifhofer et al., 2003; Manicassamy et al., 2006b). It should be subject to further investigations whether PKCθ recruitment and/or activity or formation of the CBM complex might be changed in suppressed T cells.

Regarding PKC recruitment, one study demonstrated reduced recruitment of PKCθ to the immunological synapse in suppressed murine Tcons (Sumoza-Toledo et al., 2006), yet this was only true when the Treg and the Tcon were of the same antigen specificity and had contact to the same APC. Even when a Treg and a Tcon with different antigen specificity had contact to the same APC, which presented both antigens, no suppression of PKCθ recruitment was observed. However, findings of Sumoza-Toledo's group are not in accordance with many other studies in that they observed suppression of IL-2 and proliferation only when Treg and Tcon were of the same antigen specificity and had contact to the same APC (Tanchot et al., 2004). We used polyclonal human Tcons and Tregs in the absence of APCs, which represents a different setup than the system used by Sumoza-Toledo et al. and renders it unlikely that suppression of PKCθ recruitment is involved in our study regarding direct Treg-mediated rapid cytokine suppression. Accordingly, DAG generation, which is crucial for PKCθ recruitment, was unaffected in suppressed Tcons (Schmidt et al., 2011).

CONTACT-DEPENDENT CYTOKINE SUPPRESSION IS RETAINED AFTER REMOVAL OF Tregs

Direct cell contact seemed to be required for suppression of TCR signaling in Tcons, yet the inhibited state of Tcons was sustained after removal of Tregs from the coculture (Schmidt et al., 2011). To be suppressed, Tcons must have had cell contact with pre-activated Tregs for only about 45 min. This implies that Tregs, despite being present in lower numbers than Tcons *in vivo*, may have the capacity to suppress several Tcons in a sequential fashion. Interestingly, we found that suppression of TCR signaling, IL-2 and IFN-γ transcription as well as IFN-γ secretion was retained upon Treg removal, while suppression of proliferation was not. Also Sojka et al. (2005) found that murine Tregs can be removed from the coculture with Tcons and still render Tcons suppressed, yet in contrast to our results, suppression of proliferation was observed. However, pre-cocultures were done in the presence of APCs, so additional effects of Tregs on B7 expression of APCs cannot be excluded. Our human Tcon:Treg pre-cocultures were done without APCs for 30–90 min. In the study by Sojka et al., 2 h of coculture was the shortest time period investigated, which already resulted in approximately 50% suppression while pre-coculture of ≥12 h resulted in 90% suppression of proliferation.

SUPPRESSION OF PROLIFERATION VERSUS CYTOKINE PRODUCTION – DIFFERENT MECHANISMS?

As suggested by others (Tang and Bluestone, 2008), we propose that different mechanisms of suppression might be used by Tregs depending on their activation state, the site of inflammation, effector cell types and point in time of suppression. Furthermore, direct suppression of T cells or indirect suppression via APC inhibition both may be relevant *in vivo*. Along that line, in addition to suppression of T cell priming and proliferation through inhibition of APCs by Tregs as described by many groups, direct suppression of $CD8^+$ T cell effector function independently of repressed proliferation *in vivo* has been described (Mempel et al., 2006), underscoring the relevance of direct T cell suppression by Tregs. Further, it was shown that Treg-mediated suppression of IFN-γ production by $CD4^+$ T cells can occur without concomitant suppression of proliferation *in vivo* (Sojka and Fowell, 2011). However, the importance of direct suppression of TCR signaling and, consequently, rapid suppression of IL-2 and IFN-γ transcription *in vivo* remains elusive to date.

Our data might suggest that at least three different mechanisms of human Tcon suppression may operate. First, rapid suppression of Ca^{2+}, NFAT, and NF-κB signaling results in inhibition of cytokine production, which requires Treg pre-activation, is favored by cell contact, retained upon Treg removal and seems to be independent of CTLA-4, APCs, and IL-2 deprivation (Oberle et al., 2007; Schmidt et al., 2011). Second, based on our data, we propose that direct suppression of human Tcon proliferation seems to require prolonged contact to Tregs and may be independent of Ca^{2+} and cytokine suppression. In line with that, mice with $CD4^+$ T cell-specific double deficiency of STIM1 and STIM2 display massive T cell proliferation despite undetectable store-operated Ca^{2+} influx (Oh-Hora et al., 2008). In addition, a marked decrease in Treg numbers contributes to lymphoproliferation in these mice. However, alternative modes of Ca^{2+} entry might compensate for the absence of STIM, e.g., Ca_v calcium channels might operate when their inhibition by STIM1 is lacking (Park et al., 2010; Wang et al., 2010). It remains to be shown whether, despite suppression of IL-2 transcription, residual IL-2 might be sufficient to drive proliferation several days after Treg removal. Depending on the strength of stimulation, IL-2 amounts and, thus, the effect on

proliferation may differ. Third, suppression of APCs by Tregs via CTLA-4 contributes to suppression of Tcon proliferation in the presence of APCs.

IS CD28 SIGNALING AFFECTED IN Tcons UPON SUPPRESSION BY Tregs?

Proliferation and activation of T cells does not only require TCR stimulation but also a second, costimulatory signal (Acuto and Michel, 2003). CD28, the most prominent costimulatory receptor, is expressed on naïve and activated T cells and is triggered by its ligands CD80 and CD86, which are expressed on activated APCs and at low levels on long-term activated T cells. Phosphoinositide 3-kinase (PI3K), which generates phosphatidylinositol 3,4,5-trisphosphate (PIP_3), is one of the key effectors downstream of CD28 and facilitates activation of PDK1 and one of its targets, the kinase Akt (protein kinase B, PKB). PTEN is a lipid phosphatase that negatively regulates the PI3K/Akt pathway by dephosphorylation of PIP_3 into PIP_2. TCR-induced activation of the transcription factors AP-1, NFAT, and in particular NF-κB is directly influenced and amplified by CD28 signaling.

Thus far, the influence of Tregs on CD28 signaling in Tcons is unclear, although several hints from the literature support the hypothesis that Tregs might suppress CD28 signaling in addition to TCR signaling: strong activation or strong CD28 costimulation can (partially) overcome suppression of Tcons by Tregs. This was shown for suppression of IL-2 mRNA and protein as well as for proliferation of murine T cells (Thornton et al., 2004a; Sojka et al., 2005), and also for proliferation and IFN-γ secretion of human Tcons (Baecher-Allan et al., 2002), all performed in the presence of APCs. We used CD28 costimulation to achieve detectable phosphorylation of TCR signaling proteins and still observed Treg-mediated suppression. Yet, we have indications that suppression of Ca^{2+} and of proliferation was abrogated upon increasing the strength of stimulation (unpublished observation).

Reversal of Treg-mediated suppression by CD28 costimulation might involve activation of PI3K/Akt, since different studies imply that hyperactivation of the PI3K/Akt pathway renders Tcons resistant to Treg-mediated suppression (Wohlfert and Clark, 2007). For example, T cell-specific TRAF6 deletion led to hyperactivation of the PI3K/Akt pathway, resistance to Treg-mediated suppression and T cell-mediated autoimmune disease (King et al., 2006). However, PI3K/Akt-induced autoimmune disease might also be attributed to impaired CD95-mediated T cell apoptosis (Ohashi and Yeh, 2006). In addition, partial resistance to Treg-mediated suppression was conferred by expression of constitutively active Akt in T cells (Pierau et al., 2009) or PI3K/Akt activation by IL-15 (Ben Ahmed et al., 2009). These authors did not find involvement of apoptosis in contrast to Pandiyan et al. (2007) who claim that Tregs deprive responder Tcons of IL-2 or IL-15 and, thus, reduced Akt activation and survival of murine Tcons was observed. Similarly, a slight downregulation of Akt phosphorylation could be demonstrated in $CD8^+$ T cells upon coculture with Tregs, while ZAP-70 was unaffected (Kojima et al., 2005). Furthermore, it was found that effector T cells from juvenile arthritis patients are resistant to suppression due to Akt hyperactivation (Wehrens et al., 2011). In addition, Cbl-b knockout T cells are resistant to suppression (Wohlfert et al., 2004), which might involve functions of Cbl-b in anergy as well as its negative influence on CD28/PI3K signaling. Also NFAT1/4 double knockout T cells, which are partially resistant to suppression (Bopp et al., 2005), do not require CD28 costimulation to be activated (Ranger et al., 1998), suggesting PI3K/Akt hyperactivation in these cells.

Since the PI3K/Akt pathway crosstalks with all main TCR signaling pathways, it might be involved in Treg-mediated rapid suppression. It remains to be analyzed whether hyperactivation of the PI3K/Akt pathway also abrogates Treg-mediated suppression of Ca^{2+}, NF-κB, and NFAT. Further, it remains to be shown whether Tregs might act via inhibition of PI3K/Akt signaling in human Tcons. However, it is unclear how this would directly translate into reduced Ca^{2+} store depletion without concomitant reduction of, e.g., PLCγ activity. Analyses of known and unknown pathways downstream of CD28 in Tcons upon suppression by Tregs remain subject to further investigation.

CONCLUDING REMARKS

A growing body of evidence indicates that Tregs do not use only one universal mechanism of suppression, but rather an arsenal of different ones. So far, it is unclear how a Treg "decides" which mechanism to apply, and whether it can switch from one to the other and/or apply several modes of suppression simultaneously. Mechanisms of suppression as well as target T cell susceptibility to suppression likely differ depending on tissue site, cell types involved and activation status of target cell and Treg. Furthermore, different Treg subsets exist and further research should reveal whether these are specialized on a particular suppressive mechanism.

In the commonly used *in vitro* suppression assays, not all aspects of suppression can be detected with a single readout or point in time, as suppression of proliferation does not necessarily reflect suppression of effector cytokine production. In addition, effects of Tregs on APCs have to be addressed if these are included in the assay. Furthermore, it was shown by several groups that Tregs have to be TCR-activated in order to be suppressive, which might be particularly important for assays assessing rapid suppression and, thus, leaving little time for the Tregs to get activated during stimulation of the coculture. Therefore, it is important to control for potential effects of the pre-activating reagent itself. A possible control for effects of the pre-activating reagent is the use of pre-activated Tcons in comparison with pre-activated Tregs, however, human pre-activated Tcons themselves might upregulate Foxp3 and/or acquire suppressive function. Another possibility is the use of pre-activating reagents which can be removed completely after Treg pre-activation, such as covalently platebound anti-CD3 antibodies.

The knowledge of molecules and signaling pathways affected in Tcons upon suppression by Tregs might be crucial for their therapeutic manipulation in the future. In cancer, suppression of effector T cells is deleterious and breaking the suppressive state is highly desirable. In contrast, a suppressed state of autoreactive T cells is warranted during autoimmune disease. Further research is required to elucidate which mechanisms of Treg-mediated suppression or of target T cell resistance to suppression are most important in a particular disease, and possible therapeutic interventions have to be performed extremely carefully.

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Quorum-sensing in CD4$^+$ T cell homeostasis: a hypothesis and a model

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Homeostasis of lymphocyte numbers is believed to be due to competition between cellular populations for a common niche of restricted size, defined by the combination of interactions and trophic factors required for cell survival. Here we propose a new mechanism: homeostasis of lymphocyte numbers could also be achieved by the ability of lymphocytes to perceive the density of their own populations. Such a mechanism would be reminiscent of the primordial quorum-sensing systems used by bacteria, in which some bacteria sense the accumulation of bacterial metabolites secreted by other elements of the population, allowing them to "count" the number of cells present and adapt their growth accordingly. We propose that homeostasis of CD4$^+$ T cell numbers may occur via a quorum-sensing-like mechanism, where IL-2 is produced by activated CD4$^+$ T cells and sensed by a population of CD4$^+$ Treg cells that expresses the high-affinity IL-2Rα-chain and can regulate the number of activated IL-2-producing CD4$^+$ T cells and the total CD4$^+$ T cell population. In other words, CD4$^+$ T cell populations can restrain their growth by monitoring the number of activated cells, thus preventing uncontrolled lymphocyte proliferation during immune responses. We hypothesize that malfunction of this quorum-sensing mechanism may lead to uncontrolled T cell activation and autoimmunity. Finally, we present a mathematical model that describes the key role of IL-2 and quorum-sensing mechanisms in CD4$^+$ T cell homeostasis during an immune response.

Keywords: CD4$^+$ T cells, regulatory T cells, IL-2, autoimmunity, immune-therapy, homeostasis, quorum sensing, mathematical modeling

INTRODUCTION TO CD4$^+$ T CELL HOMEOSTASIS

As in other organs and tissues of the body, the number of cells of the immune system is typically maintained throughout the adult life of an individual. This type of control is akin to the mechanisms that act to maintain nutrient levels or temperature, which were first identified by Bernard (1865) and later termed homeostasis (Cannon, 1932). T cell numbers are essentially maintained at a stable level throughout adult life, despite their daily production and export from the thymus and their peripheral division. The contribution of the thymus to the establishment and maintenance of T cell numbers in peripheral pools has been quantified (Metcalf, 1965; Miller, 1965; Leuchars et al., 1978; Berzins et al., 1998; Almeida et al., 2001). The degree of thymus restoration is determined by the availability of competent precursors, but the number of peripheral T cells is largely independent of the number of thymus precursor cells, for example, mice with reduced thymus production showed replete peripheral compartments (Almeida et al., 2001). These experimental findings have indicated that T cell numbers are controlled by peripheral mechanisms, and lead to the suggestion that competition is an important factor in maintaining T cell homeostasis. Indeed, in normal mice with full thymus production, if the generation of new T cells exceeds the minimal requirements to fill the peripheral pools and their overall peripheral number is kept constant, it follows that each newly generated cell must compete for survival with other newly produced or resident cells (Freitas et al., 1996; Freitas and Rocha, 2000). Therefore, lymphocyte survival in peripheral pools is not a passive phenomenon, but rather a continuous active process driven by highly selective environmental cues (Freitas et al., 1996; Freitas and Rocha, 2000). However, all T cells are not equal; the peripheral T cell pool is composed of a diverse set of subpopulations that play different essential roles in establishing immune responses and immune-competence. Thus, the mechanisms responsible for guiding the survival and homeostasis of peripheral T cell numbers must include a qualitative dimension, allowing for the coexistence of different T cell populations (Almeida et al., 2005). How is this achieved?

The peripheral αβ TCR$^+$ T cell pool can be divided into CD4$^+$ "helper" and CD8$^+$ "cytotoxic" T cells, and each of these two major subpopulations consists of naïve (not having encountered antigen) and activated/memory (antigen-experienced) pools. These populations represent the potential to react to newly encountered

antigens, and to provide fast and efficient responses to recurrent antigens, respectively. For naïve T cells, the trophic survival signals are contingent on TCR-mediated signals upon interactions with MHC Class-I or MHC Class-II molecules, and on γc-dependent cytokines, namely IL-7 (Surh and Sprent, 2008). The roles of TCR signals and TCR-MHC interactions were demonstrated by transfer of T cells to MHC-devoid hosts (Takeda et al., 1996; Brocker, 1997; Kirberg et al., 1997; Tanchot et al., 1997) and by ablation of TCRs (Labrecque et al., 2001); both approaches resulted in the loss of T cells. IL-7-dependency was shown upon either transferring T cells into IL-7-deficient animals (Schluns et al., 2000; Tan et al., 2001) or treatment of host animals with anti-IL-7 antibodies (Vivien et al., 2001); again, both approaches resulted in T cell death. IL-7 also modulates MHC Class-II expression in dendritic cells, establishing a relationship between cytokines and TCR-mediated signals in naïve $CD4^+$ T cell homeostasis (Guimond et al., 2009). IL-7 is present in the secondary lymph nodes (LN), where it is produced by fibroblastic reticular cells (FRCs; Link et al., 2007). Expression of the CD62L and CCR7 homing receptors allow T cells to follow gradients of CCL19 and CCL21 chemokines made by the LN stromal cells, and thus access IL-7. Interestingly, the transcription factor Foxo1 regulates expressions of IL-7R, CD62L, and CCR7 in naïve T cells (Freitas and Rocha, 2009; Kerdiles et al., 2009; Ouyang et al., 2009), establishing a close link between lymphocyte migration and survival. Migration ensures the distribution of lymphocytes between different environments and allows the cells to find the appropriate niche for survival.

Memory T cells have less stringent requirements for survival. $CD8^+$ memory T cells can survive in absence of the restricting MHC Class-I element (Tanchot et al., 1997), while the survival of $CD4^+$ memory T cells is MHC Class-II independent (Swain et al., 1999; Polic et al., 2001). Thus, memory T cell survival seems mostly dependent on IL-7 and IL-15 (Surh and Sprent, 2008). While there is some degree of overlap in survival signals, naïve, and memory T cells do not seem to compete, allowing for the coexistence of these populations. This was shown in studies where animals manipulated to have only naïve $CD8^+$ T cells showed halved total $CD8^+$ T cell numbers, as well as in studies where animals with only memory T cells were unable to fill up the entire T cell pool (Tanchot and Rocha, 1995; Freitas and Rocha, 2000). Coexistence of these populations is ensured through a mechanism of niche segregation; separated control of these populations ensures that both are maintained, and avoids competition between them (Tanchot et al., 1997; Freitas and Rocha, 2000; Almeida et al., 2005).

The above-described division, however, fails to fully account for the complexity of peripheral T cell pools. Several other major subpopulations should be considered, including populations of $CD4^+FOXP3^+$ regulatory T cells (Sakaguchi, 2004), $CD4^+$ and $CD8^+$ effector T cells, and the division of memory pools into central-memory and effector-memory, which relies on differences in migratory behavior and T cell differentiation status (Sallusto et al., 2004).

REGULATORY $CD4^+CD25^+FOXP3^+$ T CELLS: AN ESSENTIAL POPULATION OF THE PERIPHERAL T CELL POOLS

The physiologic function of regulatory $CD4^+$ T (Treg) cells is central in establishing self-tolerance and in controlling autoimmune diseases. These cells were initially identified in models of autoimmune disease or inflammatory bowel disease (IBD) following the adoptive transfer of naïve T cells into lymphopenic animals, due to their ability to prevent the provoked autoimmune manifestations (Powrie et al., 1993; Sakaguchi et al., 1995; Asano et al., 1996; Sakaguchi, 2000; Maloy and Powrie, 2001). Treg cells specifically express the transcription factor FOXP3, which is strictly required for their development (Fontenot et al., 2003; Hori et al., 2003). Indeed, carriers of spontaneous FOXP3 mutations – e.g., the IPEX (immunodysregulation, polyendocrinopathy, enteropathy, x-linked) syndrome in humans (Wildin et al., 2001) and scurfy mice (Blair et al., 1994; Bennett et al., 2001) – develop rapid and lethal autoimmune syndromes that can be rescued upon transfer of Treg cell populations (Fontenot et al., 2003; Hori et al., 2003). Similar results were obtained in FOXP3-deficient animals generated by homologous recombination (Fontenot et al., 2003) or upon the selective ablation of Treg cells in adult mice (Kim et al., 2007a; Lahl et al., 2007). However, $CD4^+$ Treg cell actions can also counter-productively suppress immune responses against tumors and viral infections (Antony et al., 2005; Belkaid and Rouse, 2005), indicating that regulatory T cells are capable of influencing all T cell-mediated responses (Sakaguchi, 2004).

Others and we have shown that $CD4^+$ Treg cells can prevent IBD and autoimmune diseases caused by transfer of naïve $CD4^+$ T cells into lymphopenic animals, as well as control the expansion and regulate the total number of these T cells (Annacker et al., 2000; Almeida et al., 2002). The critical role of $CD4^+$ Treg cells on peripheral T cell homeostasis was further demonstrated in experiments with mouse chimeras reconstituted with bone marrow cells from $CD25^{-/-}$ (Almeida et al., 2002) or $FOXP3^{-/-}$ (Fontenot et al., 2003) donors; the co-transfer of small numbers of $CD4^+CD25^+$ Treg cells rescued these mice from massive lymphoproliferation, autoimmunity, and death. Importantly, the rescued chimeras exhibited normal absolute numbers of recovered T cells, and relative proportions of naïve, memory, and effector $CD4^+$ and $CD8^+$ T cells were restored (Almeida et al., 2002; Fontenot et al., 2003). These observations demonstrated that the lymphoid hyperplasia observed in $CD25^{-/-}$ or $FOXP3^{-/-}$ mice was not cell autonomous, and that restoring the $CD4^+$ Treg population sufficed to restore the equilibrium of the total peripheral T cell pool. Furthermore, mice transgenic for FOXP3 overexpress the "scurfin" protein and show an increased fraction of Treg cells within reduced numbers of peripheral T cells (Khattri et al., 2001). We conclude from these studies that the presence of $CD4^+$ Treg cells is essential to the homeostasis of naïve, memory, and effector T cells. Two important questions arise: (1) what mechanisms do regulatory T cells use to control $CD4^+$ T cell numbers, and (2) how is homeostasis of these regulatory T cells achieved?

FUNCTION OF $CD4^+$ Treg CELLS: THE POSSIBLE ROLE OF IL-2

Previous studies have identified a vast number of putative mechanisms of suppression used by $CD4^+$ Treg cells (Shevach, 2009). Notably, these include both cell-contact dependent and independent mechanisms, and while initial studies pointed to a segregation into those categories according to the experimental system used, with *in vitro* studies falling into the first category and *in vivo* studies in the second, later studies have shown that both direct and

indirect mechanisms may occur *in vivo* (Shevach, 2000). Indeed, strong experimental evidence indicates that *in vivo* the suppressive activity of Treg cells may be cell-contact dependent and APC-mediated. Treg cells from mice deficient in LAG-3 (a CD4-related molecule that binds MHC Class-II) show reduced regulatory activity (Huang et al., 2004). More importantly, recent findings using Treg cells with a specific CTLA-4 deficiency indicate that CTLA-4 is required for Treg cells to suppress immune responses by disrupting the ability of antigen-presenting cells to activate other T cells (Wing et al., 2008). A hallmark feature of $CD4^+FOXP3^+$ Treg cells is the expression of CD25, the α-chain of the high-affinity IL-2 receptor (Nelson and Willerford, 1998); therefore, it has also been proposed that the suppressive function of Treg cells could be due to their higher ability to use available IL-2, which could prevent IL-2 utilization by other naïve or effector cells that lack expression of the high-affinity IL-2 receptor (Barthlott et al., 2003; de la Rosa et al., 2004).

This concept of suppression via IL-2 consumption was based on the belief that IL-2 is a critical essential factor for T cell responses and expansion, as supported by *in vitro* evidence (de la Rosa et al., 2004). The dissociation kinetics, the directionality of IL-2 production within the immunological synapse, and the TCR-dependency of both IL-2 production and IL-2R expression all contributed to the notion of a requirement for proximity between producers and users of IL-2, suggesting a role of IL-2 consumption in the mechanism of suppression (Malek and Castro, 2010). However, the view that IL-2 acts as a major growth factor for *in vivo* T cell proliferation has since been challenged. Indeed, IL-$2^{-/-}$, IL-$2R\alpha^{-/-}$, and IL-$2R\beta^{-/-}$ mice develop lymphoproliferative syndromes associated with both autoimmunity and immunodeficiency (Kundig et al., 1993; Suzuki et al., 1995; Willerford et al., 1995; Almeida et al., 2006b). In these mice the strong activation state of T cells renders them refractory to further *in vitro* stimulation and is responsible for an "immunodeficiency" state. In IL-2R-deficient mice disease can be prevented by the transfer of relatively small numbers of Treg cells (Almeida et al., 2002; Malek et al., 2002). The increased lymphocyte proliferation and the progressive accumulation of considerable numbers of T cells observed argue against the strict requirement of IL-2 for T cell expansion *in vivo*. It also suggests that other γc-chain dependent cytokines, i.e., IL-15 and IL-7 or IL-7 alone in the case of the IL-$2R\beta^{-/-}$ mice, could induce extensive T cell proliferation *in vivo*. Nevertheless, it has also been shown that $CD4^+$ T cell expansion in response to antigen can occur in the absence of IL-2Rγ-chain (γc) expression (Lantz et al., 2000). Moreover, mice deficient in STAT5, the transcription factor downstream of the IL-2R signaling that has been reported to be critical for lymphocyte cell cycle progression *in vitro* (Moriggl et al., 1999), also develop extensive lymphoproliferation (Snow et al., 2003; Burchill et al., 2007; Yao et al., 2007), suggesting that TCR signals alone or associated with other γc-independent cytokines suffice to elicit T cell expansion *in vivo*. Overall these observations make apparent the discrepancies between *in vitro* and *in vivo* studies and clearly demonstrate that *in vivo*, TCR signals can bypass IL-2 requirements for $CD4^+$ T cell proliferation and expansion. It should be pointed out that although IL-2 does not seem to be strictly required for T cell proliferation and primary responses *in vivo*, as T cell responses have been shown to resolve infection in the absence of IL-2 (Malek, 2008), it appears to be critical in secondary responses, in particular for the differentiation and development of CD8 T cell memory cells (Williams et al., 2006) hinting to an impact of IL-2 in the quality of the immune response rather than to an absolute requirement for IL-2 in T cell responses. Thus the precise quantitative contribution of IL-2 for CD4 T cell proliferation *in vivo* remains yet to be established.

The observations that *in vitro* $FOXP3^+$ Treg cells lacking CD25 retain intact suppressive capacities (Fontenot et al., 2005a) and, more importantly, that a FOXP3 transgene restored Treg cell function and protected against the onset of autoimmunity in IL-$2R\beta^{-/-}$ mice (Soper et al., 2007), indicate that the suppressive activity of Treg cells can occur in the complete absence of IL-2 signals. Thus, it is likely that the suppressive activity of Treg cells can occur both by inhibiting naïve T cell activation and IL-2 production and possibly by influencing the response via IL-2 consumption. Indeed, recent reports have suggested suppression via IL-2 consumption by $CD4^+CD25^+$ Treg cells as a mechanism of controlling immune responses *in vivo* (Chen et al., 2011; Pandiyan et al., 2011). These studies may also be interpreted as suggesting that Treg cells modify environmental factors and immune responses to favor specific TH17 mimicking suppression of other T cell fates. Nevertheless, there exists a vast body of evidence demonstrating other non-IL-2 related mechanisms of Treg-mediated suppression, including IL-10-mediated regulation of gut IL-17 responses (Chaudhry et al., 2011; Huber et al., 2011), implying that not all Treg function is translated into TH17 responses by the suppressed effectors. It remains to be established if these effects can occur simultaneously and what cues determine the triggering of these diverse mechanisms.

HOMEOSTASIS OF REGULATORY $CD4^+$ T CELLS: THE ROLE OF IL-2

Our investigation of $CD4^+$ Treg cell homeostasis revealed that the absolute number of Treg cells and the ratio of non-Treg/Treg $CD4^+$ T cells are tightly regulated under steady-state *in vivo* conditions (Almeida et al., 2006b). Adoptive transfer of a limited number of $CD25^+$ T cells rescues $CD25^{-/-}$ bone marrow (BM) chimeras (Almeida et al., 2002), neonatal $FOXP3^{sf}$ mice (Fontenot et al., 2003), and IL-$2R\beta^{-/-}$ mice (Malek et al., 2002) by reconstituting a normal $CD4^+CD25^+$ Treg pool that persists indefinitely without new thymus output. Our strategy involved mixed BM chimeras that were reconstituted with precursor cells from WT donors (that were competent to generate $CD4^+CD25^+$ Treg cells) diluted at different ratios with precursor cells from $CD25^{-/-}$ donors (that were incompetent to generate $CD4^+CD25^+$ Treg cells). We found that similar numbers and proportions of peripheral $CD4^+CD25^+$ Treg cells were recovered regardless of the fraction of precursor BM cells that were competent to generate $CD4^+CD25^+$ Treg cells (Almeida et al., 2006b). These findings and the constancy of Treg cell numbers and proportions demonstrated that these cells occupy a limited and specialized niche in the peripheral T cell pool. The expression of CD25 (IL-2Rα) by the vast majority of $CD4^+$ Treg cells suggests a major role of IL-2 in $CD4^+$ Treg biology. Indeed, mice that are genetically deficient in IL-2

(Schorle et al., 1991; Sadlack et al., 1995; Wolf et al., 2001), IL-2Rα (Willerford et al., 1995), IL-2Rβ (Suzuki et al., 1995; Malek et al., 2000), or STAT5 (the transcription factor downstream of the IL-2R signaling; Snow et al., 2003; Burchill et al., 2007; Yao et al., 2007) each lack sizeable populations of $CD4^+$ Treg cells and, consequently, develop lethal lymphoid hyperplasia and autoimmune diseases. Moreover, IL-2-dependent signaling is involved in the maintenance of FOXP3 expression and in the maintenance of the FOXP3-dependent gene signature, even at low levels of IL-2Rβ-dependent signaling (Gavin et al., 2002; Hill et al., 2007; Yu et al., 2009). Others and we have shown that IL-2 is essential for the peripheral survival and expansion of $CD4^+CD25^+$ Treg cells. Thus, Treg cells from IL-2-deficient donors fail to survive in IL-$2^{-/-}$ hosts (Almeida et al., 2006b) or to accumulate in the periphery in the absence of IL-2R signals (Almeida et al., 2002, 2006b; Fontenot et al., 2005b), and blocking IL-2R or neutralizing IL-2 reduces Treg cell numbers (Bayer et al., 2005; Setoguchi et al., 2005).

The observation that $CD4^+CD25^+$ T cell numbers and proportions are remarkably stable does not explain how $CD4^+CD25^+$ Treg homeostasis is achieved or the mode of action of IL-2. Parsimony suggests that the expression of high-affinity IL-2Rα and the dependence on IL-2 for survival are critical and connected clues to understanding the homeostasis of the Treg cell subpopulation. We showed that expression of high levels of high-affinity IL-2Rα specializes the $CD4^+CD25^+$ Treg cells for exploiting the IL-2 resource *in vivo*. In fact, in the $CD4^+$ T cell pool of mouse chimeras reconstituted with a mixture of precursors differing only in their potential to exploit IL-2, the WT $CD4^+$ T cells that are able to express CD25 have a noticeable seeding advantage over the $CD25^{-/-}CD4^+$ cells (Almeida et al., 2006b). Thus, CD25 expression by Treg cells permits them to occupy a niche defined by the IL-2 resource, and allows them to avoid direct competition with other $CD4^+$ T cells that do not express the high-affinity IL-2Rα. This explains how the immune system ensures the presence of this subpopulation of cells, but it fails to explain their constancy or their relative proportion.

We have shown that complementation chimeras reconstituted with a mix of donor BM from $CD25^{-/-}$ and IL-$2^{-/-}$ animals present a normal peripheral T cell pool, including a normal proportion of $CD4^+CD25^+$ Treg cells (Almeida et al., 2002). This indicates that paracrine IL-2 is sufficient for the peripheral survival of $CD4^+CD25^+$ Treg cells. We also reported that, in steady-state conditions, establishment of a fully sized and functional Treg cell population that is capable of preventing development of autoimmune syndromes only occurs in the presence of IL-2-competent αβ T cells (Almeida et al., 2006b). This suggests that αβ T cells (particularly activated $CD4^+$ T cells) represent the major source of the IL-2 required for the survival of a functional population of Treg cells in the peripheral pools (Curotto de Lafaille et al., 2004; Setoguchi et al., 2005; Almeida et al., 2006b). We have also used transgenic mice overexpressing IL-7 to greatly increase peripheral T cell numbers recovered at equilibrium time-points, and we found that the numbers of $CD4^+CD25^+$ Treg cells increased proportionally to the absolute number of $CD4^+$ T cells (Almeida et al., 2006b). Since $CD4^+$ Treg cells are unable to produce IL-2 (Shevach, 2000) due to active FOXP3-dependent repression of the *il2* gene (Wu et al., 2006; Ono et al., 2007), the corollary conclusion from these observations is that Treg cells are reliant on other IL-2-producing T cells for survival. To demonstrate this point, we reconstituted irradiated Rag2$^{-/-}$IL-2$^{-/-}$ hosts with mixes of IL-2-sufficient and IL-2-deficient BM cells, and we found a direct correlation between the numbers of IL-2-competent cells and $CD4^+$ Treg cells (Almeida et al., 2006b). We concluded that the size of the Treg niche corresponds to the available quantity of IL-2, meaning that the number of $CD4^+CD25^+$ Treg cells is tied to the number of IL-2-producing $CD4^+$ T cells. This explains why the relative proportion of the two populations is stably maintained. Importantly, the size of the IL-2 niche is not fixed by factors external to the overall T cell pool; instead, it is mainly dependent on IL-2 produced by other T cells (Almeida et al., 2006b).

This mechanism of homeostasis implies that the survival of a given Treg and the capacity for expansion will be dependent on the amounts of IL-2 produced by other T cells and, thus, Treg cells are capable of extended expansion and renewal when faced with an empty Treg niche. Indeed, when very low numbers of Treg cells are co-transferred with IL-2-producing $CD4^+$ T cells into lymphopenic animals, they expand considerably and tend to occupy the vacant $CD4^+$ Treg niche (Almeida et al., 2006b; Komatsu et al., 2009). A similar situation occurs in the first days of life, as the first wave of $CD4^+$ Treg cells expand considerably between days 3 and 7 (Bayer et al., 2005). Interestingly, the capacity of expansion of "converted" or $CD25^{low}$-expressing $FOXP3^+$ contaminants in transfers of $CD4^+CD25^-$ T cells is greatly affected by the presence of $CD4^+CD25^+$ co-transferred cells, suggesting strong competition for the occupancy of the Treg niche and further demonstrating that initial CD25 expression is a major competitive advantage in IL-2 usage (Almeida et al., 2006a).

QUORUM-SENSING AND $CD4^+$ T CELL HOMEOSTASIS

It is believed that control of lymphocyte numbers may be determined by cellular competition for a limited amount of resources, e.g., trophic factors required for survival (Freitas and Rocha, 2000). However, it is not clear what mechanisms are used to control expanding lymphocyte numbers in situations where resources are not limiting, e.g., during immune responses or an excess of self-antigens or cytokines. It remains unanswered how lymphocyte populations "count" the number of their individuals and how do they "know" when to stop growing?

Many species of bacteria use quorum-sensing mechanisms to coordinate their gene expression according to their population density (Miller and Bassler, 2001; Diggle et al., 2007). A similar quorum-sensing-like mechanism may play a critical role in lymphocyte homeostasis, if lymphocytes can assess the number of molecules with which they interact and respond accordingly once a threshold number of molecules is detected.

We propose that control of lymphocyte numbers could be achieved by the ability of lymphocytes to perceive their own population density (de Freitas, 2009). The correlation between the numbers of $CD4^+CD25^+FOXP3^+$ Treg cells and of activated IL-2-producing T cells indeed suggests the presence of a quorum-sensing-like mechanism. In this case $CD4^+$ T cell populations use the Treg cell subset expressing the high-affinity IL-2Rα-chain to

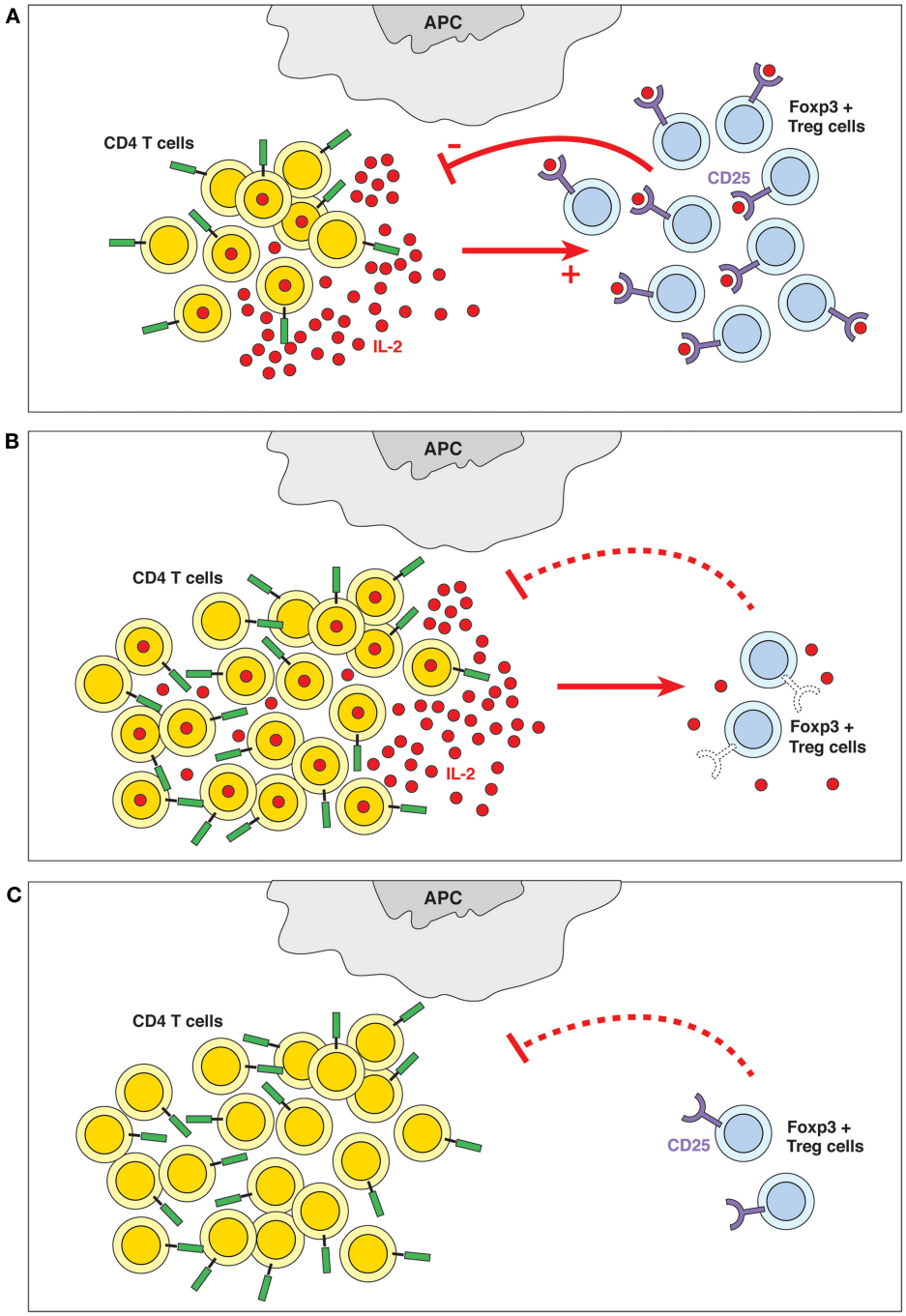

FIGURE 1 | (A) Quorum-sensing. The presence of IL-2 and the ability of the Treg cells to detect its levels are crucial to the homeostasis of the immune system. Quorum-sensing in this case is defined as an indirect feedback loop where the IL-2 produced by a subpopulation of activated T cells (among others) is detected (sensed) by a subpopulation of $CD4^+$ Treg cells expressing the high-affinity IL-2Rα-chain; these cells contribute to controlling the number of $CD4^+$ T cells. In other words, the overall $CD4^+$ T cell populations sense the produced quantities of IL-2 and adapt their behavior accordingly. **(B)** Failure of quorum-sensing by defective sensor molecule. The inability to detect IL-2 because of defects in IL-2R expression (in IL-2Rα$^{-/-}$ or IL-2Rβ$^{-/-}$ mice) or signaling (in STAT5$^{-/-}$ mice) leads to lymphoid hyperplasia and autoimmune disease. **(C)** Failure of quorum-sensing due to absence of the sensed molecule. In the absence of IL-2, Treg cells do not survive, which causes lymphoid hyperplasia and autoimmune pathology.

monitor the number of activated $CD4^+$ T cells by detecting (sensing) the IL-2 produced, and they adapt their collective behavior according to the quantities of IL-2 sensed (**Figure 1A**). In this manner, $CD4^+$ T cells may be able to control not only the number of activated IL-2-producing $CD4^+$ T cells, but also the size of the total $CD4^+$ T cell pool. This mechanism prevents uncontrolled

lymphocyte proliferation and maintains a constant pool size. Thus, according to the quorum-sensing hypothesis, IL-2, rather than being a mere growth factor required for the proliferation of some T cells, represents a key molecule playing a central role in $CD4^+$ T cell homeostasis.

We hypothesize that failure of this quorum-sensing mechanism results in uncontrolled $CD4^+$ T cell activation and autoimmune disease. The inability of $CD4^+$ T cells to detect IL-2 – due to defects of IL-2R expression (Willerford et al., 1995; Malek et al., 2000) or IL-2 signaling (Burchill et al., 2007), or due to failure to produce IL-2 (Schorle et al., 1991) – leads to lymphoid hyperplasia and lethal autoimmune disease (**Figures 1B,C**). Other autoimmune diseases are also linked to defects in the IL-2/IL-2R signal pathways. Autoimmunity in NOD mice seems to be dependent on lower IL-2 production resulting from genetic defects that map to the *il-2* region (Yamanouchi et al., 2007). Polymorphisms linked to IL-2 receptors, IL-2Rα and IL-2Rβ, are associated with several autoimmune diseases, such as type 1 diabetes, multiple sclerosis, celiac diseases, and rheumatoid arthritis (Gregersen and Olsson, 2009; Todd, 2010). It has also been reported that Dicer, the enzyme responsible for generating functional miRNAs, enhances Treg cell development (Cobb et al., 2006). FOXP3 regulates miR155 expression and optimizes Treg cell responses to limiting quantities of IL-2 by targeting suppressor of cytokine signaling 1 (SOCS1) expression (Lu et al., 2009; Stahl et al., 2009). Consequently, the selective disruption of miRNAs in the Treg cell lineage results in lethal autoimmunity (Liston et al., 2008; Zhou et al., 2008). However, it should be noted that genetic risk for autoimmunity might be more complex than the IL-2 regulation of Treg cell function, as it may also involve increased responses of the self-reactive clones to IL-2.

By modifying IL-2 levels it should be possible to increase Treg cell numbers and control auto-reactive immune responses. However, IL-2 also contributes to the effector arm of immune responses by driving $CD8^+$ T cell proliferation and differentiation into effector functions (Kalia et al., 2010; Pipkin et al., 2010; Zaragoza et al., 2011) and development of $CD8^+$ T cell memory (Williams et al., 2006). Treg cells have a major competitive advantage for IL-2 usage (Almeida et al., 2006b); this is due to the constitutive expression of the high densities of the IL-2Rα chain that confers a up to 1000-fold increase of affinity of binding to the IL-2Rα, β, γ trimer (Feinerman et al., 2010), the fact that even a low level of IL-2-dependent signaling sufficiently ensures maintenance of FOXP3 expression (Gavin et al., 2002; Hill et al., 2007; Yu et al., 2009), and because FOXP3 expression itself optimizes Treg cell responses to limiting quantities of IL-2 (Lu et al., 2009; Stahl et al., 2009). Therefore, Treg cells should be particularly susceptible to small increases in IL-2 availability. Indeed, administration of low doses of IL-2 (Tang et al., 2008; Grinberg-Bleyer et al., 2010) or inducible adenovirus bearing IL-2 genes (Goudy et al., 2011) can reverse disease or prevent diabetes in NOD1 mice by increasing Treg cell numbers in the target organs. Some anti-IL-2/IL-2 immune complexes have been used *in vivo* to prolong the half-life of the administered cytokine (Boyman et al., 2006), and can selectively favor Treg cell expansion by blocking the IL-2 binding site to the IL-2Rβ (Boyman et al., 2006; Webster et al., 2009). Differences in the IL-2R signaling pathways between Treg and T effector cells may also allow manipulation of responses to either favor or antagonize Treg function (reviewed by Malek and Castro, 2010).

Feedback loops between interacting effector and regulatory cells have been proposed to occur in the course of immune responses (Knoechel et al., 2005; Fehervari et al., 2006; Carneiro et al., 2007). However, these previous models describing regulatory feedback loops between Teff/Treg cells did not take into consideration the protagonist part of IL-2 and Treg cells in overall lymphocyte homeostasis. The quorum-sensing hypothesis extends beyond the scope of specific immune responses and predicts the behavior of the global T cell pool, including maintenance of the naïve pool and control of the size of the activated T cell pool. Indeed, the effects of the absence of IL-2 and/or of the Treg cell subset extend to all subpopulations on the $CD4^+$ T cell pool suggesting that this homeostatic mechanism acts to maintain total T cell numbers rather than the size of effector and regulatory T cells only. The quorum-sensing hypothesis, besides its proposed overall role on T cell homeostasis, also embodies a feedback mechanism to control T cell activation while allowing immune responses to occur; this likely plays a part in the contraction phase of the $CD4^+$ T cell responses. In the initial stages of an immune response, the presence of antigen perturbs the immune system equilibrium and incites proliferation of antigen-specific cells. Upon activation, the number of IL-2-producing cells (T and non-T) and the IL-2 concentrations increase; Treg cells respond with proliferation, and eventually re-establish a steady-state by suppressing further T cell activation and proliferation, thus decreasing the number of IL-2-producing cells. Such a mechanism might have evolved to control peripheral cross-reactive or auto-reactive T cell clones, while allowing controlled increases in overall peripheral T cell number. This mechanism definitively enables $CD4^+$ T cells to limit their expansion when in presence of an excess of resources.

In conclusion our quorum-sensing model provides new mechanistic insights and has implications for understanding autoimmune disease and will create a new way of thinking about the homeostasis of lymphocyte populations.

PROPOSED MATHEMATICAL MODEL FOR THE ROLE OF "QUORUM-SENSING" IN $CD4^+$ T CELL HOMEOSTASIS

Based on the suggested quorum-sensing hypothesis we here propose a mathematical model that is intended to describe in a quantitative way the role of IL-2 and quorum-sensing mechanisms in $CD4^+$ T cell homeostasis. This model describes $CD4^+$ T cell dynamics, that is the time evolution of four different $CD4^+$ T cell subsets: naïve (n_1), IL-2-producing (n_2), activated/memory non-IL-2-producing (n_3), and regulatory $CD4^+$ T cells (n_4). The model assumes that cells can divide, differentiate, or die. The assumptions of the model are as follows.

THYMUS OUTPUT

We model thymus output as occurring at a constant rate, independent of the number of cells in the peripheral $CD4^+$ T cell population. We only consider thymus output for naïve and

regulatory T cells. These terms take the form

$$+ \nu_i,$$

where $i = 1, 4$.

NATURAL DEATH

We assume that $CD4^+$ T cells possess a natural death rate and that this constant death rate depends on the T cell subset. Thus, the death terms are proportional to the population size of each subset, namely

$$-\mu_i n_i,$$

where $i = 1, 2, 3$. We have already discussed the requirement for IL-2 for the survival of regulatory T cells. This can be encoded as an IL-2-dependent death term for the subset of regulatory T cells. We do not directly include a dynamical equation for IL-2 levels, but we assume that the IL-2 levels are proportional to the number of IL-2-producing cells. The death term for the regulatory T cells takes the form

$$-\mu_4 \frac{\kappa_2}{\kappa_2 + n_2} n_4,$$

where κ_2 is the number of IL-2-producing cells at which the death rate for regulatory cells is half its value in the absence of any IL-2.

TCR/IL-7-INDUCED PROLIFERATION

We assume that all T cell subsets proliferate in response to TCR- and/or IL-7-mediated signals; thus, we include them as logistic growth terms with carrying capacity κ. In this way, we impose a limit on the population size that these signals can sustain, which is consistent with the idea that T cells must compete for such signals in the periphery. The proliferation due to TCR and IL-7 takes the form

$$+\lambda_{i_{\mathrm{TCR}}} n_i \left(1 - \frac{n_i}{\kappa}\right)$$

where $i = 1, 2, 3$.

IL-2-INDUCED PROLIFERATION

In addition to growth terms due to TCR and/or IL-7 stimulus we also include proliferation terms to model T cell responses to IL-2. For a given T cell subset (except for naïve T cells), the growth rate due to IL-2 signals is assumed to be proportional to the number of IL-2-producing cells. We model IL-2-induced proliferation with quadratic terms of the form

$$+\lambda_{i_{\mathrm{IL-2}}} n_2 n_i$$

where $i = 2, 3, 4$.

DIFFERENTIATION OF NAÏVE T CELLS

Naïve T cells can be activated in response to their specific antigen. We model this differentiation by assuming naïve T cells become IL-2-producing cells at a constant rate α_{12}. We also assume naïve T cells differentiate into memory cells at a constant rate α_{13}. These assumptions are modeled by terms of the form

$$\pm\alpha_{1i} n_1$$

where $i = 2, 3$.

DIFFERENTIATION OF IL-2-PRODUCING CELLS

We assume IL-2-producing cells stop producing IL-2 at a constant rate α_{23} and thereafter differentiate to the activated/memory pool. This term is written as

$$\pm\alpha_{23} n_2.$$

REACTIVATION OF MEMORY

We assume that memory T cells can become IL-2-producing cells in response to further stimulation by their specific antigen. This assumption is modeled by a term of the form

$$\pm\alpha_{32} n_3.$$

SUPPRESSION

We encode regulatory T cell suppression as follows: regulatory T cells give a signal to activated IL-2-producing T cells that causes them to stop producing IL-2. We model this as a contact term proportional to the number of regulatory T cells, with the assumption that IL-2-producing cells may become memory cells after having received this signal. The assumption is modeled by a term of the form

$$\pm\beta n_2 n_4.$$

SYSTEM OF ODEs

Based on the assumptions introduced above, we can write a system of ordinary differential equations (ODEs) that fully describes the model. The ODEs are given by

$$\begin{aligned}
\frac{dn_1}{dt} &= \nu_1 - \mu_1 n_1 + \lambda_{1_{TCR}} n_1 \left(1 - \frac{n_1}{\kappa}\right) - \alpha_{12} n_1 - \alpha_{13} n_1, \\
\frac{dn_2}{dt} &= -\mu_2 n_2 + \lambda_{2_{TCR}} n_2 \left(1 - \frac{n_2}{\kappa}\right) + \lambda_{2_{IL-2}} n_2 n_2 + \alpha_{12} n_1 \\
&\quad - \alpha_{23} n_2 + \alpha_{32} n_3 - \beta n_2 n_4, \\
\frac{dn_3}{dt} &= -\mu_3 n_3 + \lambda_{3_{TCR}} n_3 \left(1 - \frac{n_3}{\kappa}\right) + \lambda_{3_{IL-2}} n_2 n_3 + \alpha_{13} n_1 \\
&\quad + \alpha_{23} n_2 - \alpha_{32} n_3 + \beta n_2 n_4, \\
\frac{dn_4}{dt} &= \nu_4 - \mu_4 \frac{\kappa_2}{\kappa_2 + n_2} n_4 + \lambda_{4_{IL-2}} n_2 n_4.
\end{aligned}$$

We note that a detailed mathematical analysis of this system of ODEs (existence and stability of steady-states, for example) is beyond the scope of this paper and will be explored in a separate publication.

The mathematical model introduced in this section assumes that the limiting factors mediating the expansion of the $CD4^+$ Treg population are not only the available levels of IL-2, but also the number of IL-2-producing cells. Implicit in this assumption

is that upon the encounter of an IL-2-producing T cell and a Treg cell, the strength of the signal which can be delivered via IL-2 is non-limiting; indeed the limiting factor is the rate at which such encounters occur. The quorum-sensing mechanism described here can be understood as follows: Treg cells "count" and regulate the numbers of activated T cells through the detection of the IL-2 and the number of interactions between these two populations, of which a specified proportion (encoded within the parameters of the model) leads to cellular events such as a division, survival, or suppression. Previous mathematical models have explored the consequences of different mechanisms for the action of Tregs on IL-2-producing cells (usually called effector T cells). In the model presented here, the suppression mechanism (represented by the term proportional to β) is mathematically encoded as a non-linear density dependent term in the equation of motion for the IL-2-producing cells. Naive T cells, at a rate proportional to the number of Treg cells and to β, become activated/memory cells. Such a suppressive mechanism is in contrast to previously studied mechanisms where the action of Treg cells is to consume available IL-2, thus limiting the number of survival or proliferative signals cells within the $CD4^+$ T cell population can receive. We believe that our approach is more robust since the action of Treg cells is assumed to limit the number of cells which are activated and produce the resource IL-2, rather than try to limit the IL-2 resource itself by consumption of IL-2 post-production. Whilst it has been extensively shown that Treg cells are critically dependent on IL-2 for their survival and proliferation, it is not so clear whether IL-2-producing cells are solely dependent on IL-2 for their own expansion. In our model, we encode two possible mechanisms of expansion for the IL-2-producing (effector) population, one of which is IL-2 independent. In such a scenario, the action of Treg cells focused around consumption of IL-2 would only serve to limit one of these two mechanisms for expansion of the IL-2-producing T cell population. By assuming that the suppressive action of Treg cells prevents overall T cell activation and proliferation, the degree of expansion through either pathway is controlled, and thus both populations, are indexed to each other.

Table 1 | Parameter values and dimensions for a quorum-sensing model of CD4 T cell homeostasis.

Parameter	Value	Units
μ_1	1×10^{-3}	h^{-1}
μ_2	1×10^{-2}	h^{-1}
μ_3	1×10^{-2}	h^{-1}
μ_4	1×10^{-2}	h^{-1}
$\lambda_{1_{TCR}}$	2×10^{-2}	h^{-1}
$\lambda_{2_{TCR}}$	2×10^{-2}	h^{-1}
$\lambda_{3_{TCR}}$	5×10^{-2}	h^{-1}
$\lambda_{2_{IL-2}}$	5×10^{-5}	$Cell^{-1}\ h^{-1}$
$\lambda_{3_{IL-2}}$	2×10^{-5}	$Cell^{-1}\ h^{-1}$
$\lambda_{4_{IL-2}}$	1×10^{-4}	$Cell^{-1}\ h^{-1}$
κ	1×10^{3}	Cell
κ_2	1×10^{1}	Cell
α_{12}	1×10^{-1}	h^{-1}
α_{13}	1×10^{-2}	h^{-1}
α_{23}	1×10^{-2}	h^{-1}
α_{32}	1×10^{-3}	h^{-1}
β	2×10^{-4}	$Cell^{-1}\ h^{-1}$

PLOTS

We conclude with a series of plots of the deterministic trajectories of the above system of equations as well as with realizations of a stochastic version of the deterministic model. It must be noted that we neglected thymus output terms ($\nu_1 = 0$ and $\nu_4 = 0$). Initial conditions were 100 naïve $CD4^+$ T cells and 10 Treg cells. We choose parameter values to simulate the dynamics of a clone of $CD4^+$ T cells in response to antigenic challenge, starting at time $t = 0$. The parameter values are given in **Table 1**. In order to make the mathematical model more realistic we have also assumed that naïve T cells differentiate progressively with cell division, i.e., they differentiate after they have divided at least three times. Additionally, we assumed that IL-2-producing cells only differentiate into activated/memory cells if they have divided at least three times. Suppression still acts across every generation of IL-2-producing cells, and memory cells are free to become IL-2-producing cells without any requirements for prior division. In the simulations this is achieved by keeping track of the generation each cell belongs to (by writing an ODE for each respective generation) and keeping parameter values constant across all generations with the exception of those parameters, which are generation-dependent. The deterministic solutions were simulated over periods of 2 and 8 weeks, respectively, are shown in **Figure 2**. We also present realizations from a stochastic version of the same deterministic model (**Figure 3**), with simulations run over the same time windows as the deterministic trajectories. The stochastic realizations shown here were produced using the Gillespie algorithm. The system of ODEs was solved using a fourth-order Runge–Kutta scheme. Initially, the model shows slow growth in the number of T cells; during the first 2 days, the total cell population approximately doubles from around 100 cells to 200 cells, with the immune response reaching a peak and at around day 7. At days 4–5 there is a sharp increase in the number of IL-2-producing cells, followed by a subsequent increase in the number of antigen-experienced cells, which are not producing IL-2. Notably, this leads to a large increase in regulatory cells around day 7, which is immediately followed by a sharp contraction phase that lasts around 2 days. A slower contraction phase that lasts around 3 weeks follows before a homeostatic equilibrium is reached. We note that for this parameter set the regulatory T cell subset takes longer to reach equilibrium than the memory T cell subset.

At equilibrium, the model predicts that the size of the memory T cell population is limited only by the availability of TCR/IL-7 signals, which is included in the model as the carrying capacity κ. This population acts as a source for the population of IL-2-producing cells, so that there is a small but constant supply of IL-2-producing cells at equilibrium. This source of IL-2 is then sufficient to maintain a regulatory T cell population, which in turn keeps the IL-2-producing population from expanding. The deviation away from this ratio of regulatory T cells and IL-2-producing cells allows the model to reproduce the key

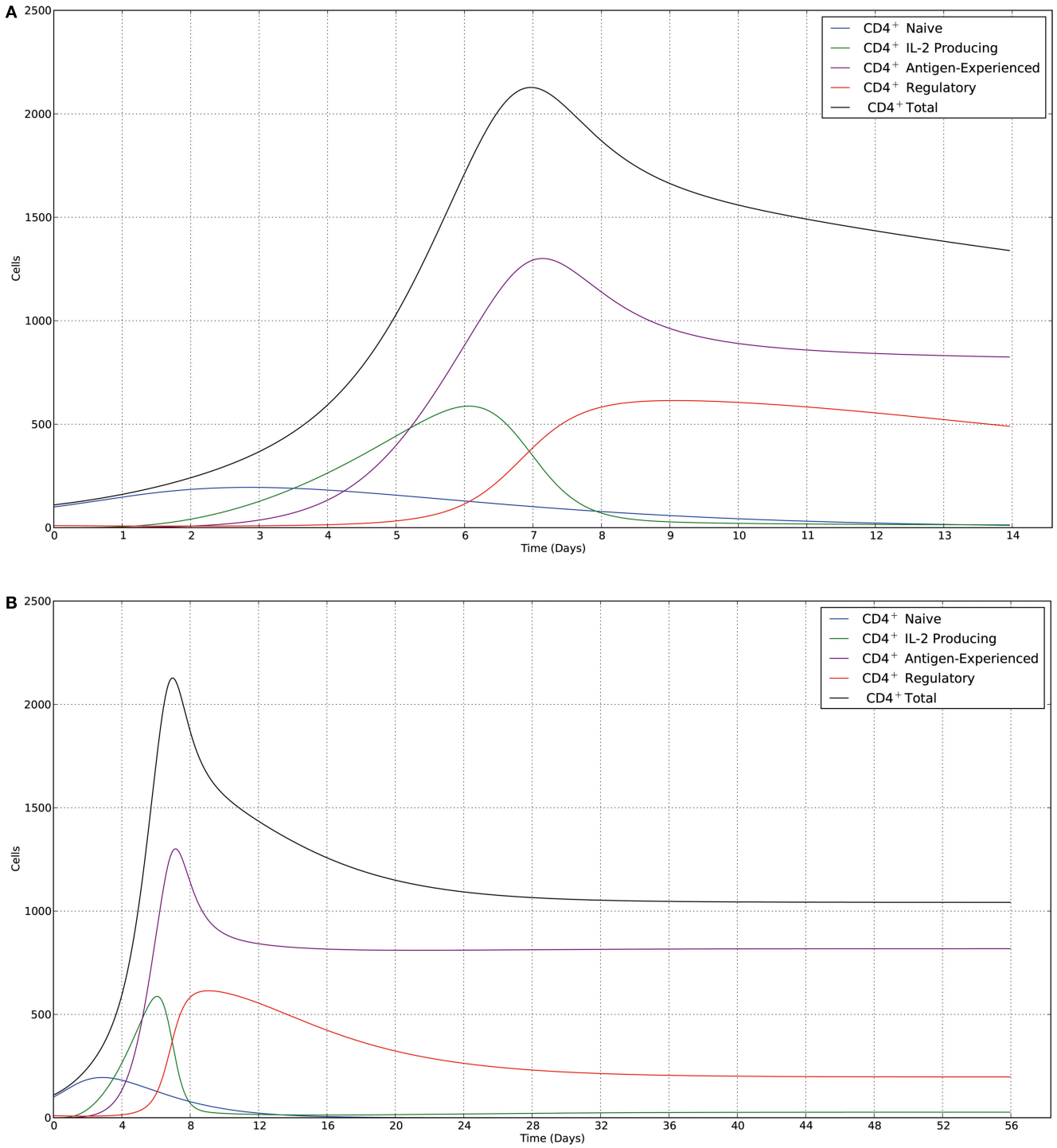

FIGURE 2 | Trajectories of the deterministic model for the parameter set given in Table 1, with the following initial conditions: 100 naïve cells (blue) and 10 regulatory cells (red). (A) Data shown over a time course of 2 weeks. The immune response peaks at ∼7 days, then declines to a homeostatic equilibrium dominated by memory cells. **(B)** Data shown over a time course of 8 weeks. The contraction phase occurs over an extended time period of ∼20 days for memory cells and ∼40 days for regulatory cells.

characteristics of the immune peak and the contraction phase before settling down to equilibrium. Indeed, it is possible to simulate a larger immune peak by specifying a larger ratio between naïve cells and regulatory cells in the initial conditions, or to dampen the immune peak by changing the ratio in the opposite manner.

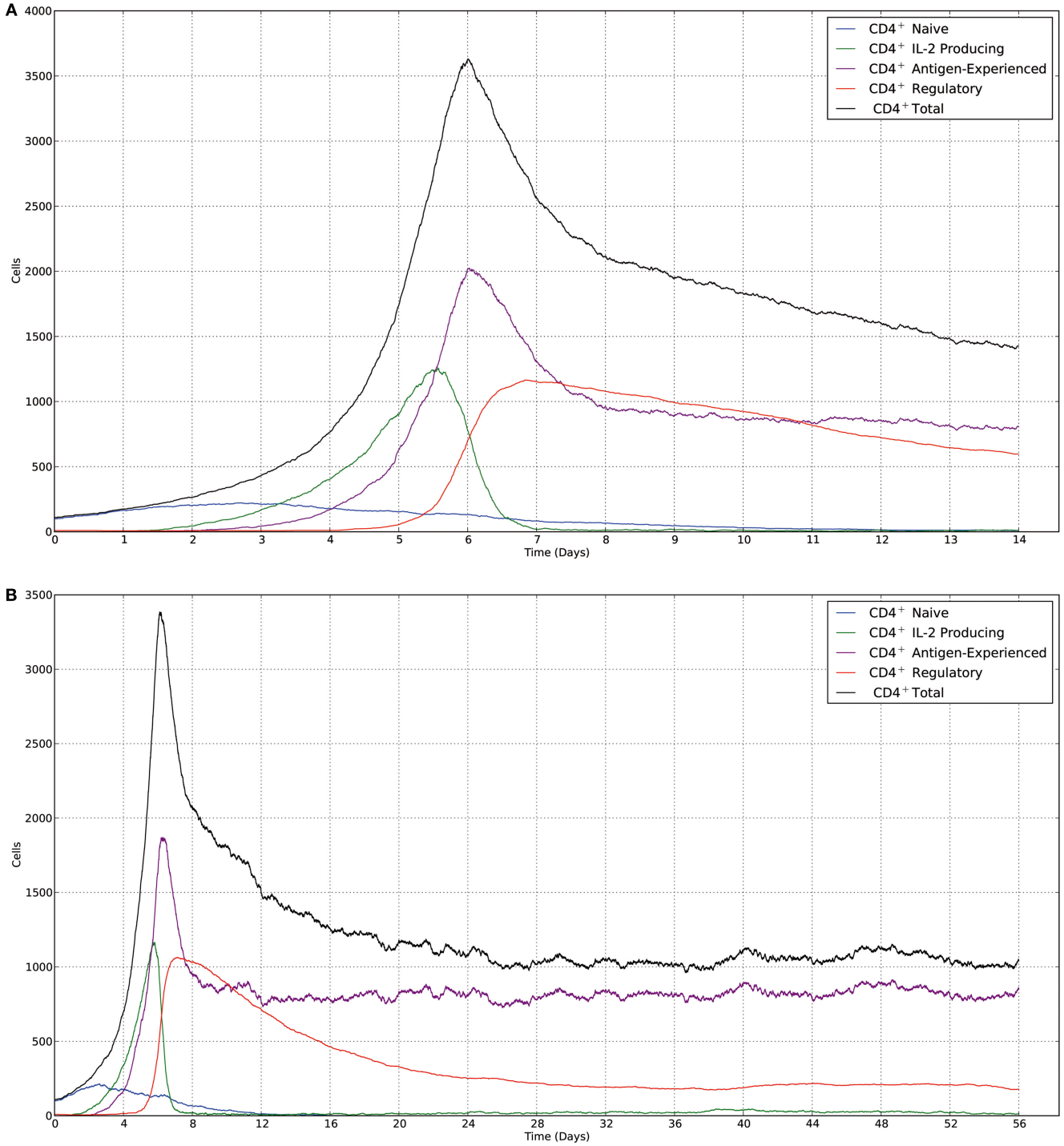

FIGURE 3 | Realizations of the stochastic model carried out using the Gillespie algorithm. All parameters and initial conditions are the same as in the deterministic model, with **(A,B)**, respectively, correlating to **(A,B)** from **Figure 2**. There is a higher degree of noise at equilibrium for memory cells, which is due to memory cells repeatedly receiving antigen-mediated signals to become IL-2-producing, then reverting back to a non-IL-2-producing state.

In our model, we assumed that antigen is constant (encoded in the proliferation parameter $\lambda_{i_{\mathrm{TCR}}}$) and non-limiting; however, an effective immune response should serve to remove antigen. We could address this issue by either introducing time-dependent rates for the activation of naïve or memory cells, or by introducing within the rates a dependence on a number of cells that would be linked to the antigen-clearance rate, e.g., effector cells. We have also assumed that naïve T cells may only be activated

after having proceeded through at least three divisions, and equivalently IL-2-producing cells are assumed to only revert back to a non-IL-2-producing state if they have gone through three divisions. In reality, the division/proliferation-linked differentiation program is unlikely to be so clear-cut. We could refine the model by introducing a probabilistic differentiation based on generation, where cells are increasingly likely to differentiate as they progress through more divisions. The model we introduce here has been applied only to the pool of specific T cells that can play a part in response to their cognate antigen. The model could be extended to mirror the effect this immune response would have on a bystander population of T cells that is not involved in the immune response. We note that our model can also be applied to model T cell homeostasis with a different set of parameter values. However, this is beyond the scope of what we wish to present here.

Based on single-cell *in vitro* experiments, Feinerman et al. (2010) recently developed a quantitative model for the regulation of immune responses by IL-2 – in particular, for the competition for IL-2 between effector and regulatory $CD4^+$ T cells during an immune response. They discuss how binding of IL-2 leads to IL-2Rα up-regulation in both effector and regulatory cells. Regulatory cells express higher basal IL-2Rα levels, which gives them a distinct advantage over effector cells in the ability to scavenge IL-2. This advantage reportedly allows regulatory cells to critically limit the amount of IL-2 available for consumption by effector cells. The authors develop a biochemical computational model of IL-2R and STAT5 signaling, which takes into account the cell-to-cell variability in IL-2 receptor expression levels, as well as a dynamical model of IL-2 competition between regulatory and effector T cells. Similarly, Busse et al. (2010) introduced a reaction-diffusion model to describe the spatio-temporal dynamics of IL-2, and regulatory and helper T cells. Their model captures the interplay between IL-2 production, binding, internalization, degradation, and recycling after antigen stimulation; it is based on the positive correlation between IL-2 binding and cell-surface number of IL-2Rα. The authors conclude that IL-2-induced up-regulation of high-affinity IL-2R establishes a positive feedback loop of IL-2 signaling. Furthermore, under conditions of limiting IL-2, their results indicate that regulatory T cells can deprive moderately stimulated helper T cells of IL-2 (Busse et al., 2010). It is important to note, however, that antigen-specific $CD4^+$ T cells expand in the absence of the IL-2Rγ-chain (Lantz et al., 2000) and that a FOXP3 transgene restored Treg cells and protected against autoimmunity in Il-$2R\beta^{-/-}$ mice (Soper et al., 2007), both observations indicating that expansion of effector cells can occur in the absence of IL-2 and the suppressive activity of Treg cells can occur in the absence of IL-2 consumption and signaling.

In contrast, the model that we propose here does not include competition for IL-2 at the receptor/molecular level; however, in choosing the parameters, we imposed $\lambda 4\text{IL-}2 > \lambda 2\text{IL-}2$. Implicit in this inequality is the fact that regulatory T cells are more sensitive to IL-2 than IL-2-producing cells (Gavin et al., 2002; Almeida et al., 2006b; Hill et al., 2007; Lu et al., 2009; Stahl et al., 2009; Yu et al., 2009), but we do not assume that this limits the proliferative capacity of IL-2-producing cells. In our model, the limiting factor of proliferation for IL-2-producing cells is the number of cells themselves, for which regulatory cells play a major role through the suppressive term β.

Recent experimental work by Quiel et al. (2011) and mathematical work by Bocharov et al. (2011) provide a model based on the observation that the number of antigen-specific cells after immunization is not proportional to the number of precursors, and that the factor of expansion decreases as the number of precursors increases. The authors speculate that this phenomenon is due to crowding effects, and use a deterministic model with four $CD4^+$ T cell populations that differ in their maturation and differentiation states (Bocharov et al., 2011). The authors assume that differentiated non-dividing T cells impose a feedback mechanism on less mature dividing T cells, which acts to increase the differentiation rates of these less mature cells, thereby limiting their proliferative capacity. This differs from our model, where proliferation will continue "unchecked" in the absence of regulatory cells.

There is already a large body of work devoted to the development of mathematical and computational models to study the dynamic interactions between the populations of Treg cells and effector $CD4^+$ T cells. Early work focused on investigating different potential mechanisms of interaction between Treg cells and effector cells (Leon et al., 2003; Carneiro et al., 2007), occurring at conjugation sites present on the surface of antigen-presenting cells (APCs; Tang et al., 2006). Thus, Treg cells are assumed to be reliant on paracrine cytokines for their clonal expansion, and it is thought that IL-2 is the critical cytokine required for this expansion (Almeida et al., 2002, 2006b; Malek et al., 2002; Curotto de Lafaille et al., 2004; de la Rosa et al., 2004; Setoguchi et al., 2005). The "crossregulation model" explores the mathematical consequences of several of these assumptions, particularly the mechanisms by which Treg cells limit the proliferative capacity of effector cells (Carneiro et al., 2007).

Finally, we should mention other mathematical modeling efforts that have included bystander T cell activation (Kim et al., 2007b; Burroughs et al., 2011). Burroughs et al. (2011) make use of a dynamical systems approach to model a regulatory T cell population, a population of effector T cells that have received APC-mediated antigenic stimulus, and a bystander T cell population that is unresponsive to the antigen. The authors conclude that autoimmune conditions can arise following a primary immune response due to a bystander T cell population and that the equilibrium of the immune system depends on a balance between effector and regulatory T cell populations.

In conclusion, we have introduced a quantitative mathematical model of quorum-sensing for $CD4^+$ T cell homeostasis. The model includes thymus output, cellular proliferation (TCR/IL-7 and IL-2-induced), differentiation and suppression, as well as death, linked to the levels of IL-2-producing cells in the case of regulatory T cells. The model has been used to explore the dynamics of $CD4^+$ T cells during an immune response. Our results (**Figures 2** and **3**) show that there is a sharp increase in the number of IL-2-producing cells at days 4–5, followed by an increase in the number of activated/memory non-IL-2-producing cells. This leads to a large increase, around day 7 (which corresponds to the

peak of the response) in the number of regulatory T cells, which is then followed by a sharp contraction phase that lasts around 2 days. Steady-state conditions or homeostasis are reached after a slower contraction phase that lasts around 3 weeks. Key features of the model are: (i) IL-2-producing cells, which correlate with IL-2 levels, dictate the proliferation rate of regulatory T cells and (ii) regulatory T cells control IL-2-producing cell differentiation to non-IL-2-producing cells. These two terms guarantee that $CD4^+$ T cells are able to restrain their own proliferation by "sensing" the number of IL-2-producing cells, and thus preventing unbounded lymphocyte division as well as controlling the number of activated T cells and the total $CD4^+$ T cell population.

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The development and immunosuppressive functions of CD4$^+$ CD25$^+$ FoxP3$^+$ regulatory T cells are under influence of the adenosine-A2A adenosine receptor pathway

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The A2A adenosine receptor (A2AR)-mediated immunosuppression is firmly implicated in the life-saving down-regulation of collateral tissue damage during the anti-pathogen immune response and in highly undesirable protection of cancerous tissues during anti-tumor immune response. Therefore, depending on specific clinical situation there is a need to either weaken or strengthen the intensity of A2AR signal. While the A2AR-mediated immunosuppression was shown to be T cell autonomous in studies of effector T cells, it was not clear how A2AR stimulation affects regulatory T cells (Treg). Here we show in parallel assays that while A2AR stimulation on T cells directly inhibits their activation, there is also indirect and longer-lasting T cell inhibitory effect through modulation of Treg. A2AR stimulation expanded CD4$^+$ CD25^{hi} FoxP3$^+$ cells, which also express CD39, CD73, and CTLA-4. Treg cultured with A2AR agonist showed increased expression of CTLA-4 and stronger immunosuppressive activity. There was a significant increase of Treg cell number after A2AR stimulation. The CD4$^+$ FoxP3$^+$ population contained those induced from CD4$^+$ CD25$^-$ cells, but CD4$^+$ FoxP3$^+$ cells predominantly derived from CD4$^+$ CD25$^+$ natural Treg. Thus, A2AR stimulation numerically and functionally enhanced Treg-mediated immunosuppressive mechanism. These data suggest that the A2AR-mediated stimulation of lymphocytes using A2AR agonists should be considered in protocols for *ex vivo* expansion of Treg before the transfer to patients in different medical applications.

Keywords: regulatory T cells, adenosine, immunosuppression, A2A adenosine receptor, cancer, autoimmune, transplantation

INTRODUCTION

It is now no longer controversial and it is now widely accepted that there are professionally immunosuppressive regulatory T cells (Treg), which have been first identified and characterized by observations of autoimmunity in mice depleted of CD4$^+$ CD25$^+$ T cell subpopulation (Sakaguchi et al., 1982, 1985). The mechanisms of development and immunoregulatory functions of Treg have been subjects of extensive investigation (Lu and Rudensky, 2009; Ohkura and Sakaguchi, 2011; Rudensky, 2011; Sakaguchi, 2011). Treg are also of great interest due to their potential to treat immunological diseases and control physiological and pathological immune responses. However, there are still important and yet to be answered questions about the influence of the microenvironments in lymphoid and inflamed tissues in the development and immunoregulatory functions of Treg.

Here we investigated modulation of Treg-dependent immunosuppressive activities by the adenosine-A2AR signaling which was shown to represent the powerful physiological immunosuppressive mechanism (Ohta and Sitkovsky, 2001; Lukashev et al., 2004; Sitkovsky et al., 2004; Belikoff et al., 2011) that protects both normal (Ohta and Sitkovsky, 2001; Thiel et al., 2005; Ohta et al., 2007) and cancerous tissues (Ohta et al., 2006) from inflammatory damage. It is believed that the adenosine-A2AR pathway has evolved as a negative feed-back immunosuppressive mechanism that limits the extent of the collateral tissue damage by activated immune cells during anti-pathogen responses (Sitkovsky and Lukashev, 2005; Sitkovsky and Ohta, 2005). This mechanism may regulate the other major, but evolutionary younger immunosuppressive mechanisms including Treg (Pouliot et al., 2002; Cadieux et al., 2005; Sitkovsky, 2009).

But is there really a relation between Treg and immunosuppressive effect of extracellular adenosine? There are several lines of converging suggestive evidence that Treg activity is mediated by the accumulation of extracellular adenosine. The extracellular adenosine was first implicated in Treg activity during the unbiased screening of differential expression of surface antigens on Treg revealing that Treg express high levels of the extracellular adenosine-generating enzymes CD73 ecto-enzyme, an 5′-nucleotidase (Kobie et al., 2006) and the upstream ecto-enzyme CD39 apyrase (ecto ATPase/ADPase; Deaglio et al., 2007). These studies suggested that the CD39 and CD73 ecto-enzymes on Treg play a role in immunosuppressive loops generating extracellular adenosine that down-regulates T cell activation (Whiteside, 2012).

However, there is still paucity of data that may firmly implicate the adenosine and A2AR in functions of Treg. It was suggested that the A2AR stimulation might promote the induction of adaptive regulatory T cells, but this claim is lacking direct evidence. The ability of A2AR agonist to increase the expression of foxP3 mRNA was studied (Zarek et al., 2008), but effects of A2AR agonist on the number and immunosuppressive activity of Treg are not known.

In this study, we provide evidence that the engagement of A2AR results in expansion of Treg and promotes immunoregulatory activity of Treg. These data support the overall model of the adenosinergic regulation of Treg functions (Sitkovsky, 2009).

MATERIALS AND METHODS

MICE

C57BL/6 (Thy1.2$^+$) and BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). B6.PL-Thy1a/CyJ mice (Thy1.1$^+$ C57BL/6 mice) were purchased from Jackson Laboratory. A2AR$^{-/-}$ mice were backcrossed for 12 times to C57BL/6 mice (Chen et al., 1999). Mice were used at 8–12 weeks of age. The experiments were approved by the Northeastern University Institutional Animal Care and Use Committee and were carried out in accordance with the institutional animal care guidelines.

MIXED LYMPHOCYTE CULTURE (MLC)

Spleen cells from C57BL/6 mice (responder; H-2^b) were stimulated with allogenic spleen cells. As stimulators, spleen cells from BALB/c mice (H-2^d) were pretreated with mitomycin C (Sigma, St. Louis, MO). Responders (6×10^6 cells) and stimulators (2×10^6 cells) were cultured for 5 days in the presence or absence of A2AR agonist, 1 μM CGS21680 (CGS) or 1 μM 5′-N-ethylcarboxamidoadenosine (NECA). A2AR antagonist ZM241385 (ZM) was added at 1 μM to some samples. The concentrations of compounds are optimal to stimulate or antagonize A2AR according to our previous study (Ohta et al., 2009). The activated cells were restimulated with mitomycin C-treated BALB/c spleen cells for 2 more days in the same condition. NECA was obtained from Sigma (St. Louis, MO). CGS and ZM were from Tocris (Ellisville, MO).

FLOWCYTOMETRY

The resulted cells after MLC were analyzed by flowcytometry. Following antibodies were used to label surface molecules: PE-conjugated anti-CD4, anti-CD25, anti-CD39, anti-CD73, and FITC-conjugated anti-CD8, anti-H-2K^b and allophycocyanin (APC)-conjugated anti-CD4 antibodies. For the analysis of Treg, the cells were subsequently fixed and permeabilized using FoxP3 staining buffer set (eBioscience, San Diego, CA), and were labeled with APC-conjugated anti-FoxP3 and PE-conjugated anti-CTLA-4 antibodies. All antibodies were from BD Biosciences (San Diego, CA) except for anti-FoxP3, anti-CD39 (eBioscience) and anti-CD25 (Miltenyi Biotec, Auburn, CA) antibodies. The data were acquired using FACSCalibur (BD Biosciences).

MLC IN THE ABSENCE OF CD8$^+$ CELLS

To enrich Treg after the culture, MLC was set up using CD8$^+$-depleted C57BL/6 spleen cells. CD8$^+$ cells were labeled with FITC-conjugated anti-CD8 mAb (BD Biosciences) and anti-FITC microbeads (Miltenyi Biotech) and removed using AutoMACS separator (Miltenyi Biotec). These responder cells were cultured with mitomycin C-treated stimulator cells as described above.

CELL PROLIFERATION ASSAY USING CFSE-LABELED CELLS

The extent of T cell proliferation was monitored by the stepwise dilution of fluorescence in CFSE-labeled cells. To label with CFSE (Molecular Probes, Eugene, OR), cells were washed with PBS and incubated with 1 μM CFSE for 8 min. To remove excess CFSE, the cells were washed twice with fetal calf serum.

REGULATORY ACTIVITY OF TREG

After MLC using CD8$^+$-depleted responders for 7 days (2 days after restimulation), the regulatory activity was evaluated according to the inhibition of effector T cell proliferation. CD8$^+$-depleted spleen cells from Thy1.1-expressing C57BL/6 mouse were labeled with CFSE and used as the source of responder T cells (Tresp). Tresp (2.5×10^4 CD4$^+$ cells) were co-cultured with the product of MLC, which contains Treg, so that the ratio of CD4$^+$ cells in Tresp and CD4$^+$ FoxP3$^+$ cells in the MLC would be constant between groups. Tresp cell proliferation was induced with anti-CD3 mAb (0.1 μg/ml 145-2C11; BD Biosciences) for 2 days in a round-bottomed 96-well plate, and the extent of Tresp proliferation was analyzed after gating for Thy1.1$^+$ CD4$^+$ cells.

TREG FROM CD4$^+$ CD25$^-$ CELLS

To start MLC in the absence of natural Treg, CD25$^+$ cells were removed from the responder cells prior to the culture. Spleen cells were labeled with PE-conjugated anti-CD25 and anti-CD8 mAbs, and the labeled cells were depleted using anti-PE microbeads (Miltenyi Biotec) and AutoMACS. After 7-days MLC as described above, the appearance of CD4$^+$ FoxP3$^+$ cells was tested by flowcytometry.

NATURAL TREG

CD4$^+$ CD25$^+$ cells were purified from spleen cells of Thy1.1-expressing C57BL/6 mice as described (Nagahama et al., 2007). CD24$^+$ cells and CD8$^+$ cells were removed from the spleen cells using FITC-conjugated antibodies and anti-FITC microbeads. Subsequently, CD25$^+$ cells were retrieved by positive selection using PE-conjugated anti-CD25 antibody and anti-PE microbeads. This procedure achieves 95–98% pure CD4$^+$ CD25$^+$ cells. Responder cells of MLC were reconstituted by mixing Thy1.1$^+$ CD4$^+$ CD25$^+$ cells (6×10^4) with Thy1.2$^+$ spleen cells depleted of CD8$^+$ and CD25$^+$ cells (3×10^6). After 7-days MLC as described above, the origin of CD4$^+$ FoxP3$^+$ cells was separately analyzed for natural Treg-derived Thy1.1$^+$ cells and CD4$^+$ CD25$^-$ cells-derived Thy1.1$^-$ cells.

cAMP INDUCTION IN TREG

Purified CD4$^+$ CD25$^+$ cells (1.6×10^5) were incubated with NECA or CGS for 15 min at 37°C. The concentration of

A2AR agonists was 10 μM, and 1 μM for A2AR antagonist, ZM241385. cAMP levels were determined by ELISA (GE Healthcare, Buckinghamshire, UK).

STATISTICS

Data represent mean ± SD. Statistical calculations were performed using Student's *t*-test. Statistical significance was accepted for p values less than 0.05.

RESULTS

Immunosuppressive effects of extracellular adenosine are at least in part due to the inhibition of T cell activation. We have shown that stimulation of A2AR inhibits activation of effector T cells and their effector functions (Ohta et al., 2009). In agreement with our previous studies, A2AR agonists, CGS21680 (CGS) and NECA, blocked upregulation of CD25 on $CD8^+$ T cells during MLC suggesting impaired activation of the effector T cells in response to allogenic stimulation (**Figure 1** top panels). Interestingly, however, the proportion of $CD25^+$ $CD8^-$ cells was found to rather increase when CGS or NECA was added to the culture. This prominent increase of $CD25^+$ cells by A2AR stimulation belonged to $CD4^+$ population (**Figure 1** middle panels). Most $CD4^+$ $CD25^+$ cells after treatment with CGS and NECA were distinct in their higher expression of CD25. Since A2AR stimulation is generally immunosuppressive, the increase of $CD4^+$ $CD25^+$ cells was not likely to represent activation of $CD4^+$ effector T cells. Indeed, massive increase of $FoxP3^+$ cells suggested that what appeared as $CD4^+$ $CD25^{hi}$ cells after A2AR stimulation could be regulatory T cells (**Figure 1** bottom panels). Statistically significant changes were observed on day 5 of MLC and became more prominent on day 7 (**Figures 2A,B**). The decrease of $CD8^+$ $CD25^+$ cells and the increases of $CD25^+$ and $FoxP3^+$ proportions in $CD4^+$ cells by the addition of CGS and NECA were all blocked by A2AR antagonist ZM241385 (**Figures 1** and **2**). A2AR-dependence of these changes was also confirmed by experiments using $A2AR^{-/-}$ responder cells in which CGS and NECA failed to block $CD8^+$ cell activation and to induce CD25 and FoxP3-expressing $CD4^+$ cells (**Figure 2C**).

We further characterized A2AR-mediated increase of $CD4^+$ $CD25^+$ population. The increased $CD4^+$ cells expressed not only CD25 and FoxP3 but also CD39, CD73 (**Figure 3A**) and CTLA-4 (**Figure 3B**), which are closely relevant to immunoregulatory

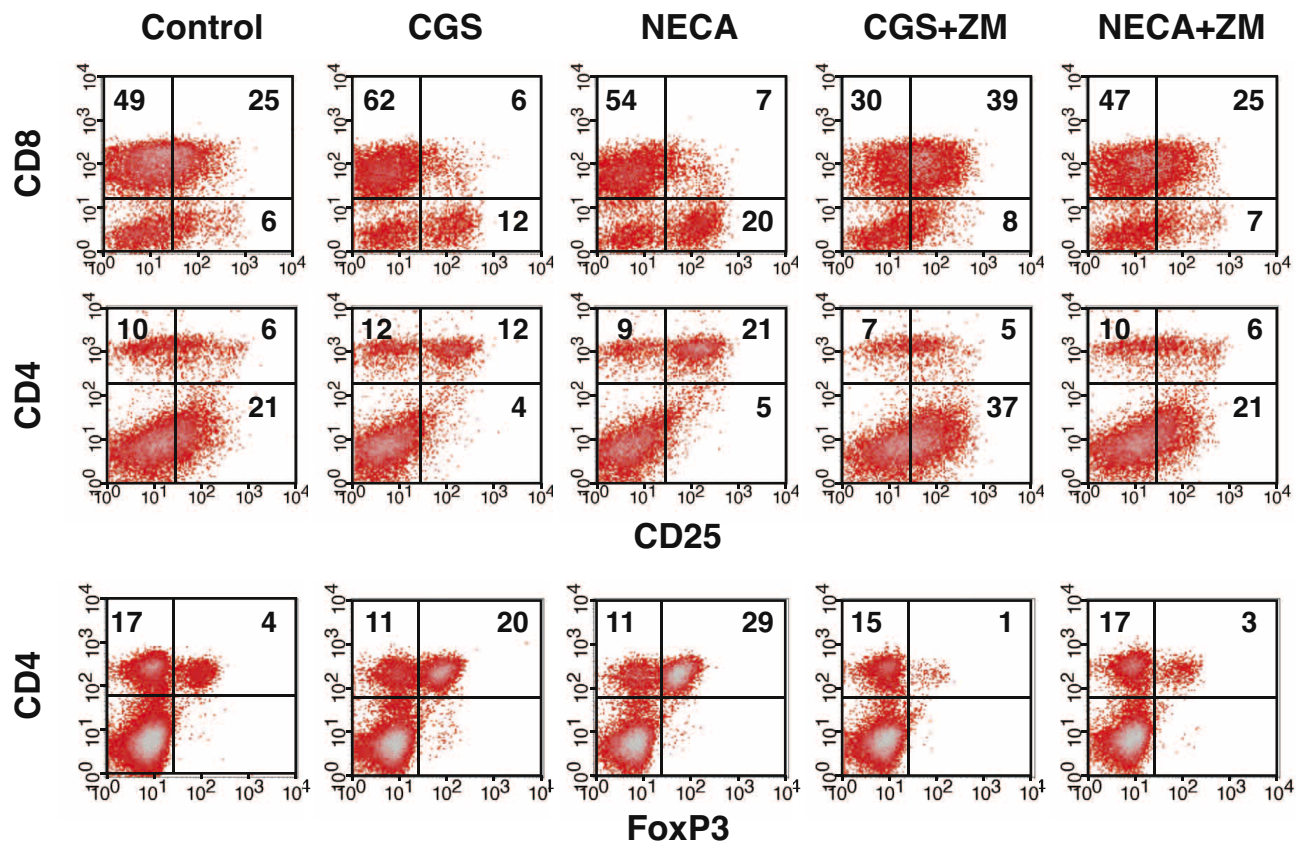

FIGURE 1 | Increase of Treg population by the stimulation of A2AR. Mixed lymphocyte culture (MLC) was set up in the presence of A2AR agonist, CGS21680 (1 μM), or NECA (1 μM). After 5 days, the cultured cells were restimulated with the same allogenic stimulator cells for 2 more days in the same condition. A2AR stimulation inhibited CD25 expression in $CD8^+$ cells (top row), whereas the population of $CD4^+$ $CD25^+$ cells was rather increased in the same culture (middle row). The change in $CD4^+$ $CD25^+$ cells correlated well with an increase of FoxP3-expressing $CD4^+$ cells (bottom row). The addition of A2AR antagonist ZM241385 (1 μM) reversed the changes. Numbers in the panels represent percentages in each quadrant. The data shown here represent four separate experiments with similar results.

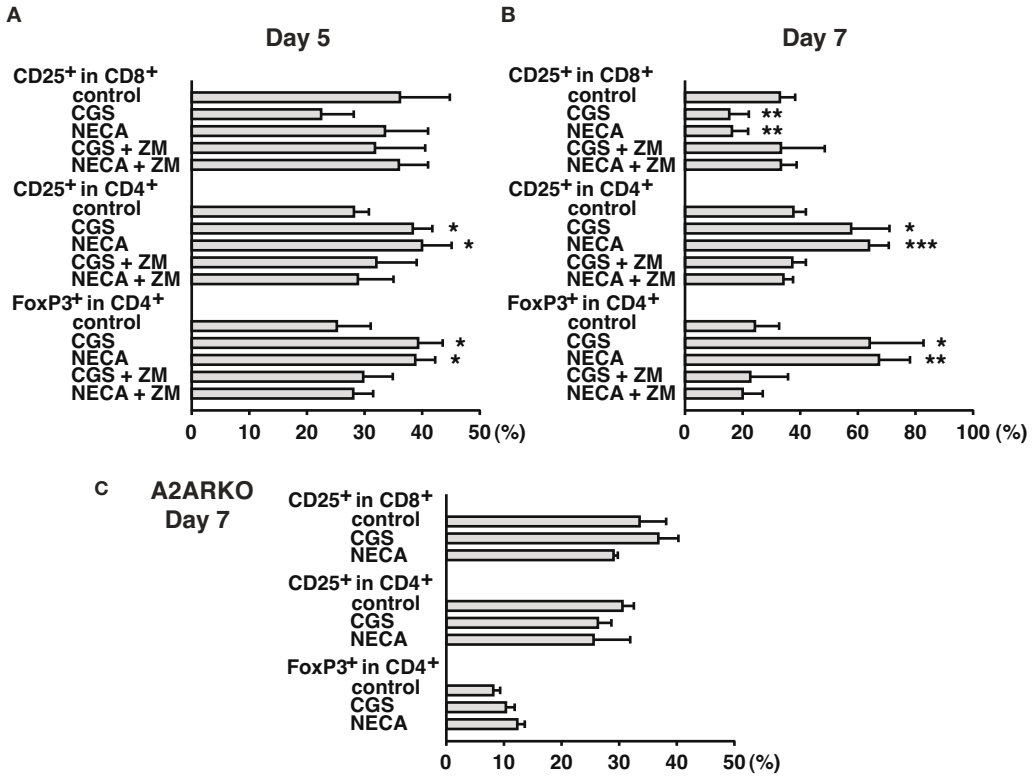

FIGURE 2 | Time-dependent changes of Treg increase during MLC with A2AR agonist. Cell culture was done as described in **Figure 1**. Spleen cells from wild-type **(A,B)** and A2AR$^{-/-}$ mice **(C)** were used as responder cells. Cells were analyzed by flowcytometry on day 5 **(A)**, and day 7 **(B,C)**. A2AR agonists inhibited CD8$^+$ T cell activation and enhanced CD25/FoxP3 expression in CD4$^+$ cells from wild-type mice, but not A2AR$^{-/-}$ mice. Data represent average ± SD of 3–4 separate experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control MLC.

activity of Treg (Kobie et al., 2006; Deaglio et al., 2007; Sakaguchi et al., 2009). These results further implied that the emerging CD4$^+$ CD25$^+$ cells in the culture with A2AR agonists were Treg. Moreover, MLC in the presence of CGS or NECA upregulated CTLA-4 levels in CD4$^+$ FoxP3$^+$ cells (**Figures 3C,D**). CTLA-4 is constitutively expressed in Treg and plays an important role in the regulatory activity (Wing et al., 2008; Sakaguchi et al., 2009; Pandiyan et al., 2011). Therefore, it was also speculated that A2AR stimulation might induce CD4$^+$ cells having enhanced regulatory activity.

Accordingly, we sought to examine regulatory activity of these cells. Since activated CD8$^+$ T cells are predominant in regular MLC, which is inconvenient as the source of Treg in the assay of regulatory activity, we set up MLC after depletion of CD8$^+$ responder cells. In this CD4$^+$ MLC, the proportion of FoxP3$^+$ cells was confirmed to increase by A2AR agonists (**Figure 4** top panels). Immunoregulatory activity of these CD4$^+$ FoxP3$^+$ cells was evaluated by the inhibitory effect on T cell proliferation. The assay was normalized so that the same number of CD4$^+$ FoxP3$^+$ cells would be added to the constant number of CFSE-labeled CD4$^+$ T cells. Comparing to the uninhibited control (Tresp alone), the addition of CD4$^+$ FoxP3$^+$ cells dose-dependently inhibited T cell proliferation. When the product of control MLC was added at 2:1 (Tresp: Treg), these Treg caused modest decrease of responder T cell proliferation (**Figure 4** left). Such degree of T cell inhibition, however, was observed when the product of CGS or NECA-treated MLC was added at 8:1 (**Figure 4** middle and right, **Figure 5**). Similarly, a larger number of control Treg (1:1) caused more significant reduction of proliferation, while this pattern corresponded to the result with CGS or NECA-treated MLC at 4:1 (**Figures 4** and **5**). A higher number of CGS or NECA-treated MLC product (2:1 and 1:1) inhibited T cell proliferation even stronger. This result confirmed that A2AR stimulation resulted in emergence of Treg and their regulatory activity was approximately 4-times stronger than that of control Treg.

A2AR stimulation enhanced not only regulatory activity of Treg but also the number of Treg. While flowcytometric analysis showed the increased *proportion* of Treg in cultures treated with A2AR agonist, it does not necessarily indicate a *numerical* increase of Treg, especially because A2AR agonists can suppress activation of effector T cells. Total cell number in the culture was counted, and the numbers of CD4$^+$ FoxP3$^+$ and CD4$^+$ FoxP3$^-$ cells were calculated from their proportions in the flowcytometric analysis. The result showed a massive increase of CD4$^+$ FoxP3$^+$ cells and a statistically insignificant decrease of CD4$^+$ FoxP3$^-$ cells by A2AR agonists (**Figure 6**).

Thus, A2AR stimulation was found to enhance immunoregulation by Treg in both qualitative and quantitative means. The quantitative change, a numerical increase of Treg, could

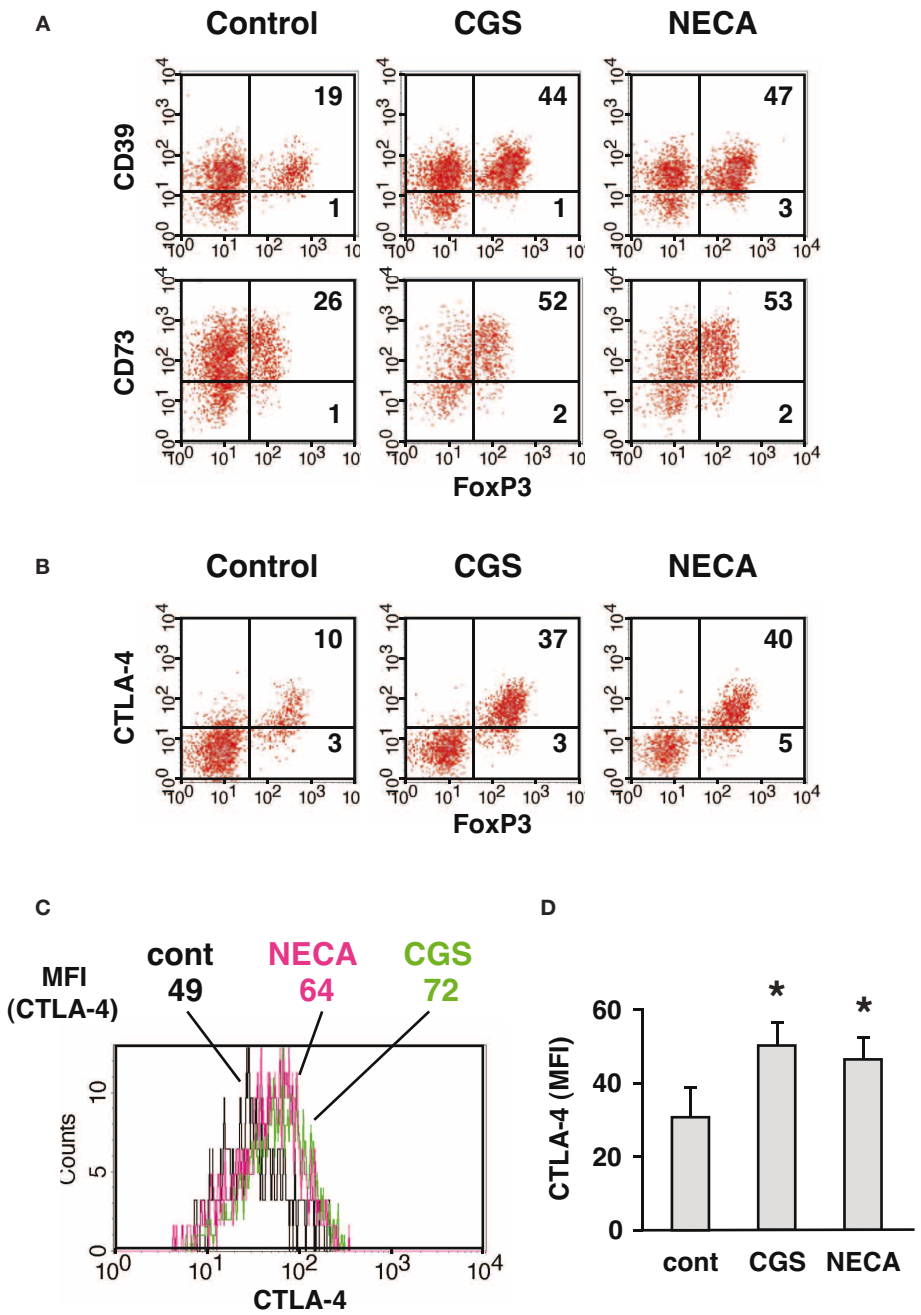

FIGURE 3 | Increase of CD4$^+$ cells expressing CD39, CD73, and CTLA-4 when cultured in the presence of A2AR agonist. MLC was done as described in **Figure 1**. **(A)** CD39 and CD73 expression in CD4$^+$ FoxP3$^+$ cells. The data was gated for H-2K^{b+} CD4$^+$ cells. **(B)** CTLA-4 expression was analyzed by intracellular staining together with FoxP3. The data was gated for H-2K^{b+} CD4$^+$ cells. Numbers in the panels represent percentages in each quadrant. **(C)** Histogram plots of CTLA-4 intensity in CD4$^+$ FoxP3$^+$ cells. Numbers represent mean fluorescence intensity (MFI) of CTLA-4. The data shown here represent five separate experiments with similar results. **(D)** Statistically significant increase of CTLA-4 levels by treatment with A2AR agonists. Data represent average ± SD of five separate experiments. *$P < 0.05$ vs. control MLC.

result from the proliferation of preexisting natural Treg (nTreg) and/or the induction of new Treg. A2AR agonist was previously shown to upregulate FoxP3 mRNA in activated T cells (Zarek et al., 2008). Therefore, to monitor the appearance of new Treg, we started MLC with responder cells depleted of nTreg. The depletion of CD25$^+$ cells got rid of most FoxP3$^+$ cells from the culture (**Figure 7A**). Some CD25$^-$ CD4$^+$ FoxP3$^+$ cells remained in the culture, but these cells accounted for only 0.5–0.6% of CD4$^+$ cells. Control MLC using such responder cells resulted in the induction of CD4$^+$ FoxP3$^+$ cells to 4.5% of CD4$^+$ cells (**Figure 7B**). A2AR agonists gave rise to further induction of CD4$^+$ FoxP3$^+$ cells to approximately

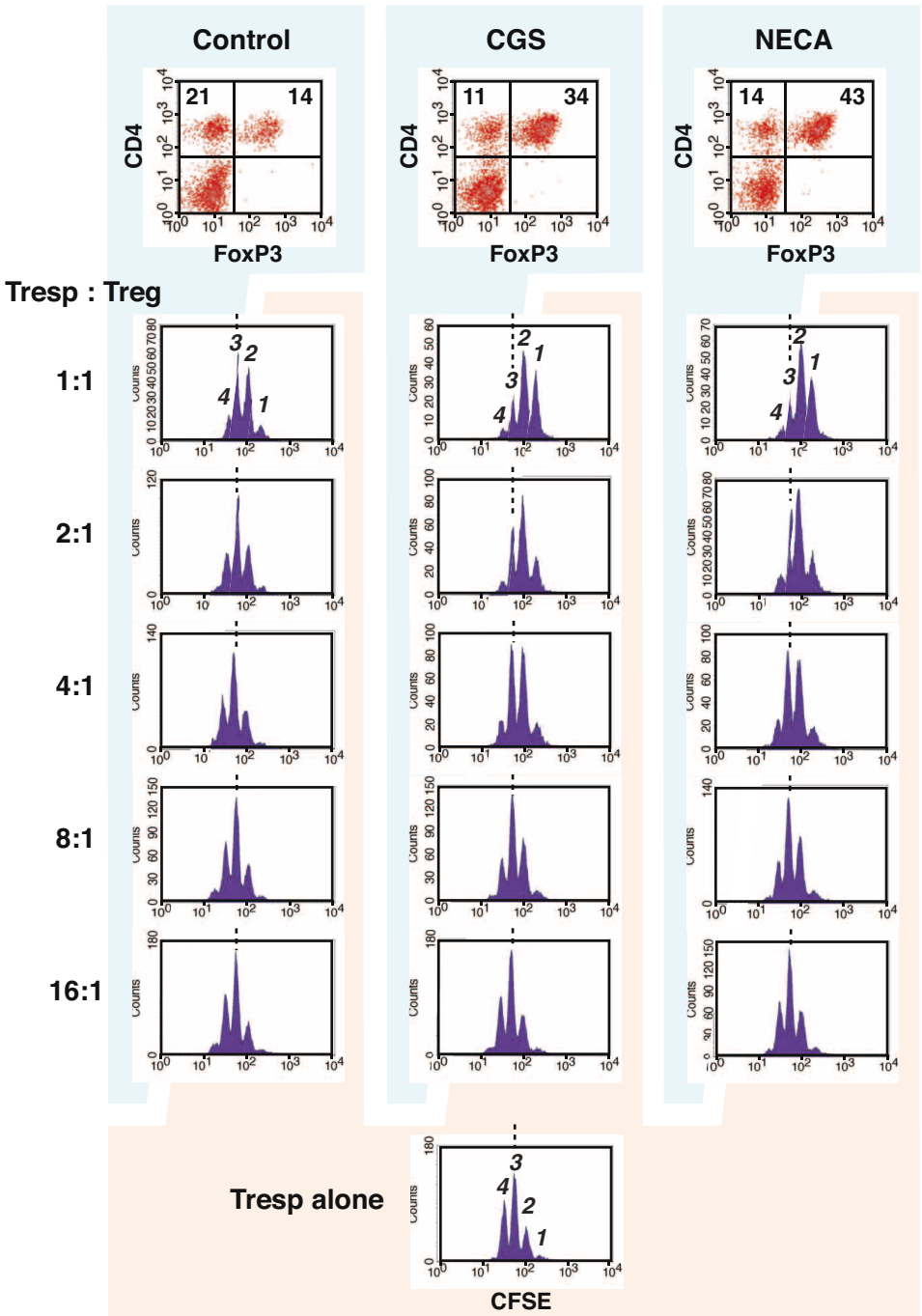

FIGURE 4 | A2AR stimulation promoted regulatory activity of Treg. To enrich Treg in MLC, $CD8^+$ cells were depleted from responder cells prior to the culture. It was confirmed that the treatment with CGS and NECA increased Treg population in this culture condition (top panels). The regulatory activity on T cell proliferation was determined by CFSE assay. $CD8^+$-depleted spleen cells from Thy1.1-expressing C57BL/6 mouse were labeled with CFSE and used as responder cells (Tresp). Tresp containing 2.5×10^4 $CD4^+$ cells were co-cultured with the product of MLC, which contains Treg. Tresp:Treg in the figure is the ratio of $CD4^+$ cells in Tresp to $CD4^+$ $FoxP3^+$ cells in the MLC. The extent of $CD4^+$ Tresp cell proliferation was analyzed 2 days after the stimulation with anti-CD3 mAb. The histograms were gated for $Thy1.1^+$ $CD4^+$ cells. Broken lines indicate the same peak (peak 3). The data shown here represent three separate experiments with similar results.

12% of $CD4^+$ cells. It was also confirmed that the increase of $CD4^+$ $FoxP3^+$ proportion accompanied significant increase in the absolute number of $CD4^+$ $FoxP3^+$ cells (**Figure 7C**). This result suggested that Treg could be derived from $CD4^+$ $CD25^-$ cells during MLC, and A2AR stimulation promoted this increase.

Next, we compared contribution from nTreg and $CD4^+$ $CD25^-$ cell-derived Treg in the A2AR-mediated increase of

$CD4^+$ $FoxP3^+$ cells. To distinguish preexisting nTreg from $CD4^+$ $CD25^-$ cell-derived Treg produced during culture, $CD4^+$ $CD25^+$ cells were purified from $Thy1.1^+$ mice. In the purified cells, $CD4^+$ $CD25^+$ cells were 95–98% and FoxP3-expressing cells were up to 96% of the purified cells (**Figure 8A**). Purified Treg produced cAMP in response to CGS and NECA, and the increase was blocked by the addition of ZM suggesting functional expression of A2AR on Treg (**Figure 8B**). These Thy1.1-expressing nTreg were added to $CD8^+$, $CD25^+$-depleted $Thy1.2^+$ spleen cells, which were prepared as in **Figure 7A**, in order to reconstitute MLC responders. MLC of these responder cells in normal condition yielded $CD4^+$ cells predominant with $FoxP3^-$ effectors (**Figure 8C**). These $CD4^+$ $FoxP3^-$ cells were mostly from $Thy1.1^-$ cells as expected, while most of $CD4^+$ $FoxP3^+$ cells were $Thy1.1^+$. There were also some $Thy1.1^-$ $CD4^+$ $FoxP3^+$ cells, but these accounted for only a minor portion (20%) of total $CD4^+$ $FoxP3^+$ cells (**Figure 8C**). Treatment with A2AR agonists strongly reduced proportion of $FoxP3^-$ effectors and increased $CD4^+$ $FoxP3^+$ cells. $CD4^+$ $CD25^-$ cell-derived Treg emerged from $Thy1.1^-$ cells were found to increase by A2AR stimulation ($FoxP3^+$ within $Thy1.1^-$ cells: 7% in control, 29% in CGS and 16% in NECA); however, induction of iTreg had a limited contribution to the increase of total $CD4^+$ $FoxP3^+$ cells (**Figure 8C**). The $CD4^+$ $FoxP3^+$ population after A2AR stimulation was again mostly $Thy1.1^+$ cells, which accounted for almost 90% of total $CD4^+$ $FoxP3^+$ cells. These data suggest that A2AR stimulation may promote $CD4^+$ $CD25^-$ cell-derived Treg and expansion of nTreg, but the latter mechanism may play a major role in the numerical increase of Treg.

We further analyzed proliferation of nTreg using CFSE-labeled $Thy1.1^+$ $CD4^+$ $CD25^+$ cells. In the reconstituted MLC, nTreg were found to enter massive proliferation (**Figure 8D**). $CD4^+$ $CD25^+$ cells proliferated well even in the presence of CGS and NECA, but these A2AR agonists did not further promote proliferation. Interestingly, a large proportion of nTreg lost FoxP3 expression in control MLC, while nTreg with A2AR agonists maintained FoxP3 expression better (**Figure 8D**). These results suggest that A2AR stimulation can, at least in part, increase the number of Treg by preventing down-regulation of FoxP3.

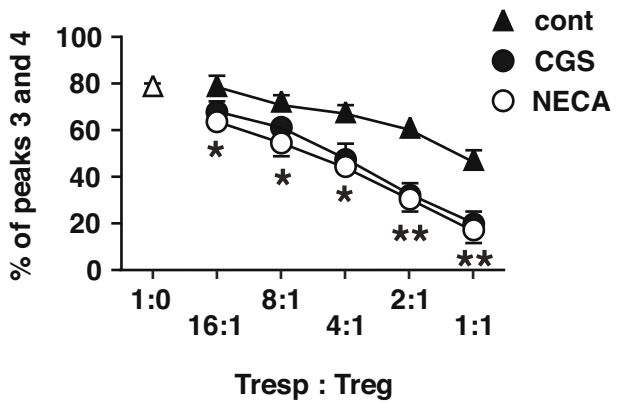

FIGURE 5 | Statistically significant enhancement of T cell inhibitory activity of Treg cultured with A2AR agonists. The data of CFSE assay was presented as proportion of cells which entered into extensive proliferation. Numbers represent combined percentages of peaks 3 and 4 in **Figure 4**. Data represent average ± SD of three separate experiments. $^*P < 0.01$; $^{**}P < 0.001$ for both control vs. CGS and control vs. NECA.

DISCUSSION

Adenine, and by extension adenosine, may have been one of the oldest organic compounds on the earth whose appearance preceded the first life form by many 100 million years according to interpretation of life origin experiments (Miller, 1953; Miller and Urey, 1959; Oró and Kimball, 1961). Maybe utilization of adenosine belongs to the oldest and most ancient group of mechanisms regulating immune system in organisms.

Immune cells express A2AR at high levels, and stimulation of A2AR strongly suppresses various immune functions (Ohta and Sitkovsky, 2001; Lukashev et al., 2004; Sitkovsky et al., 2004; Thiel et al., 2005; Belikoff et al., 2011). Interaction of A2AR

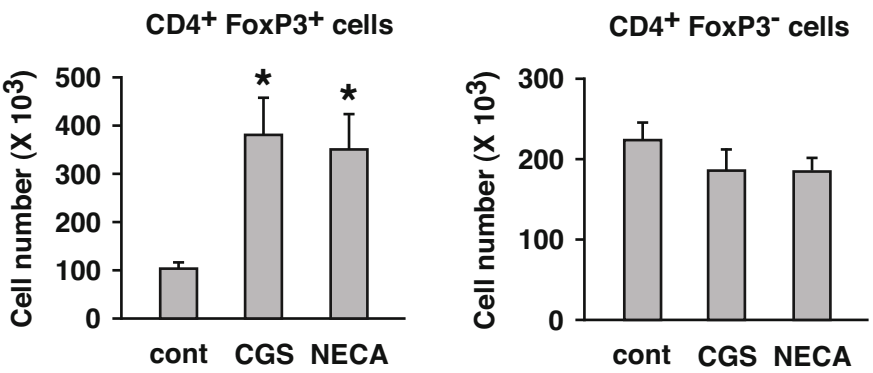

FIGURE 6 | Numerical increase of Treg after T cell activation in the presence of A2AR agonists. MLC was set up in the absence of $CD8^+$ cells. Two days after restimulation (7 days after initial stimulation), total live cells were enumerated under a microscope. Numbers of $H\text{-}2K^{b+}$ $CD4^+$ $FoxP3^+$ and $H\text{-}2K^{b+}$ $CD4^+$ $FoxP3^-$ cells were estimated from the result of flowcytometric analysis. The number of $H\text{-}2K^{b+}$ $CD4^+$ cells in the beginning of MLC was approximately 1×10^6 cells including approximately 1×10^5 $H\text{-}2K^{b+}$ $CD4^+$ $FoxP3^+$ cells. Data represent average ± SD of three separate experiments. $^*P < 0.01$ vs. control MLC.

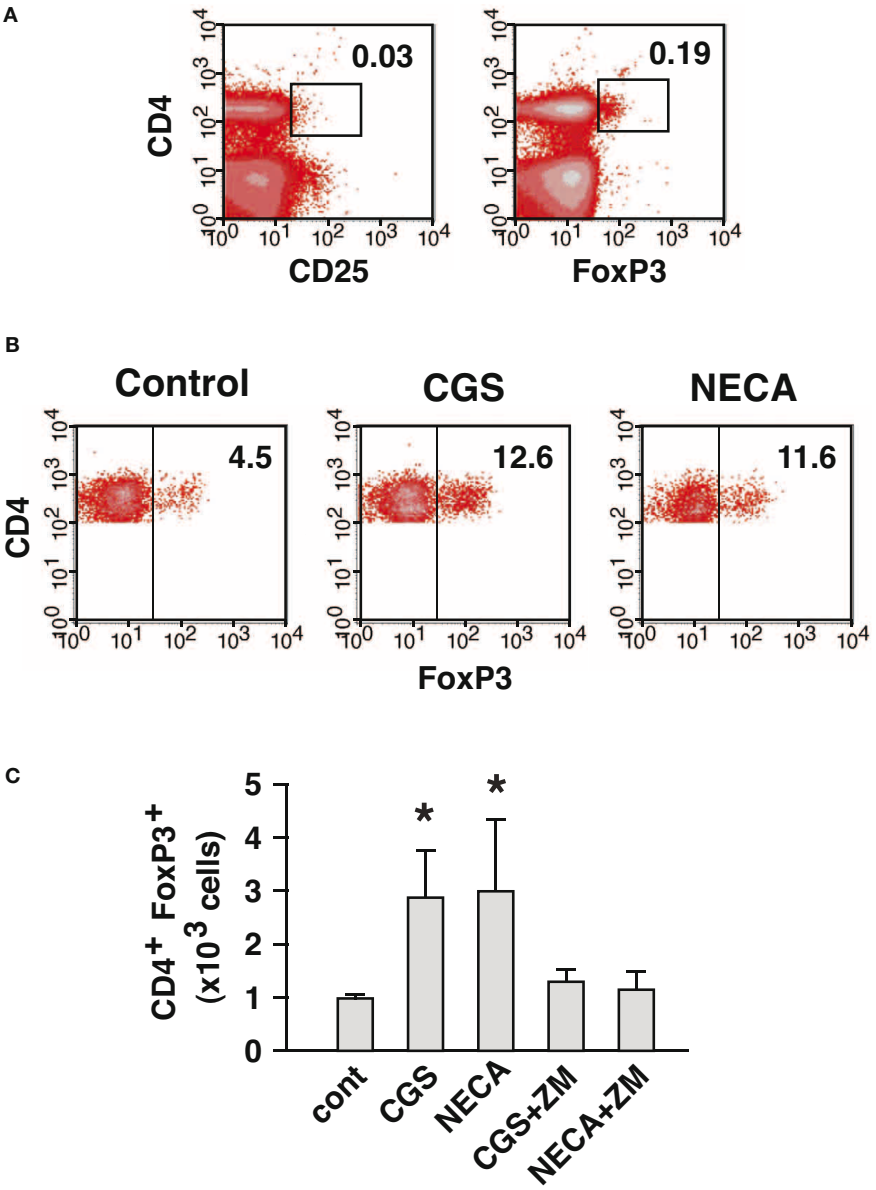

FIGURE 7 | A2AR stimulation can enhance the induction of CD4$^+$ FoxP3$^+$ cells from CD4$^+$ CD25$^-$ cells. (A) MLC responder cells after the depletion of CD25$^+$ cells. The numbers indicate percentages of CD4$^+$ CD25$^+$ and CD4$^+$ FoxP3$^+$ cells. The percentage of CD4$^+$ cells was approximately 30% because of co-depletion of CD8$^+$ cells. **(B)** Induction of CD4$^+$ FoxP3$^+$ cells from the cells in **(A)**. After 5 (primary stimulation) plus 2 (restimulation) days of MLC, the increase of CD4$^+$ FoxP3$^+$ cells in control was further enhanced in the presence of A2AR agonists. The data were gated for CD4$^+$ cells. The numbers represent percentages of FoxP3$^+$ cells in CD4$^+$ population. The data shown here represent three separate experiments with similar results. **(C)** Numerical increase of CD4$^+$ FoxP3$^+$ cells derived from CD4$^+$ CD25$^-$ cells. Total live cells were enumerated after 7 days, and numbers of H-2K^{b+} CD4$^+$ FoxP3$^+$ cells were calculated as in **Figure 6**. Data represent average ± SD of three separate experiments. $^*P < 0.05$ vs. control.

with endogenously produced adenosine serves as a self-limiting mechanism of inflammation. Immunosuppression through the adenosine-A2AR pathway seems to be critical in maintaining inflammation to proper levels. This is because the lack of A2AR led to exaggeration of proinflammatory responses and subsequent inflammatory tissue damage (Ohta and Sitkovsky, 2001; Thiel et al., 2005; Ohta et al., 2006).

Activation of CD4$^+$ and CD8$^+$ T cells is under control of the adenosine-A2AR pathway. Our previous results have shown A2AR-mediated inhibition of T cell activities such as proliferation, cytokines production and cytotoxicity (Huang et al., 1997; Ohta et al., 2009). This inhibitory effect of adenosine and its analogs is based on a direct action via A2AR expressed on T cells. It is consistent with the interruption of T cell receptor signaling by cAMP increase after A2AR stimulation (Vang et al., 2001; Linnemann et al., 2009). In addition to the inhibitory effect on T cell priming, A2AR stimulation produced activated T cells with impaired effector function. Indeed, T cells activated in the

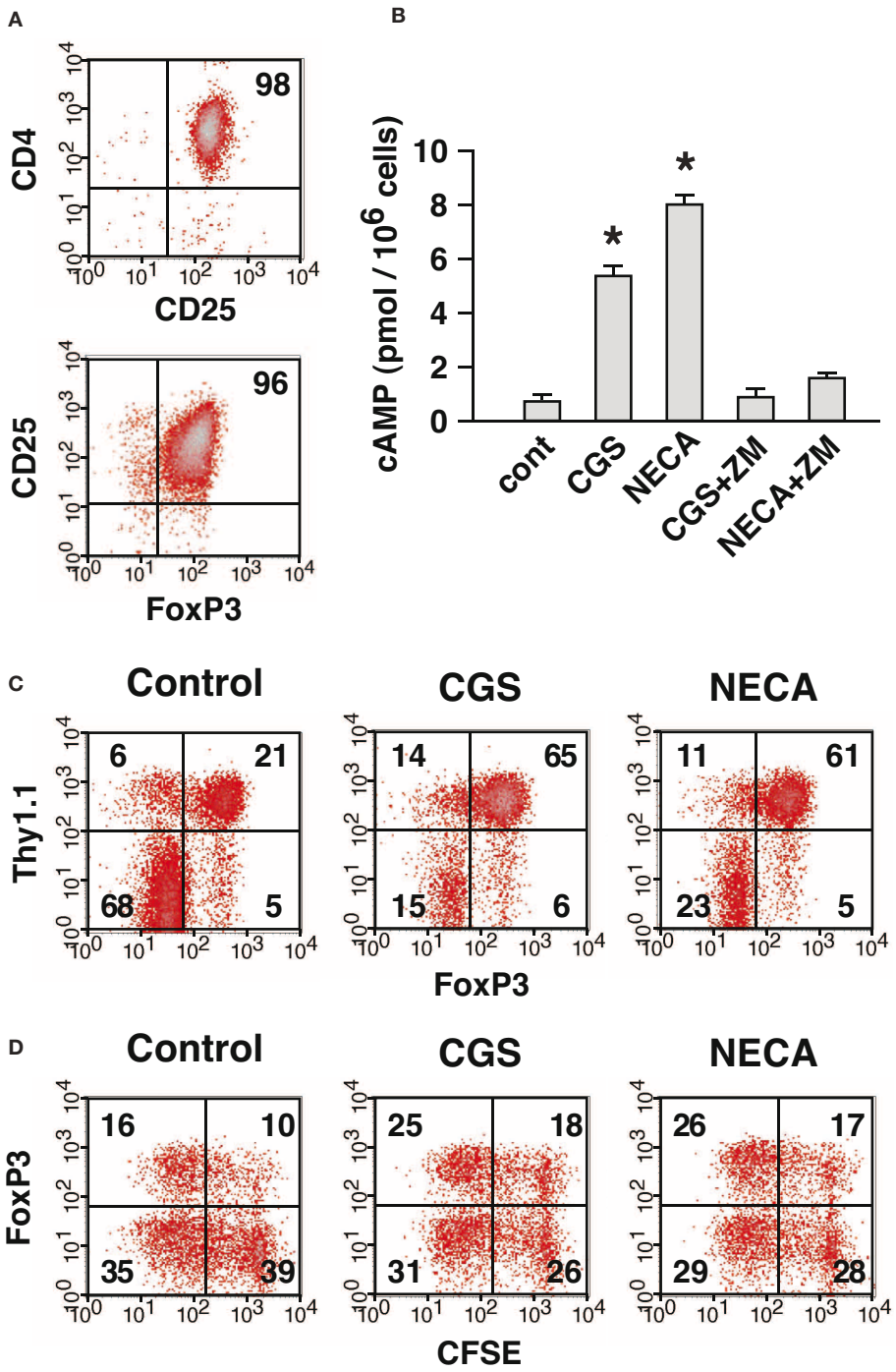

FIGURE 8 | The expansion of Treg in the presence of A2AR agonist was mostly due to the proliferation of preexisting nTreg. (A) Purified $CD4^+$ $CD25^+$ cells represent nTreg. The numbers indicate percentages of $CD4^+$ $CD25^+$ and $CD25^+$ $FoxP3^+$ population in the purified cells. These $CD4^+$ $CD25^+$ cells were obtained from Thy1.1-expressing mice and mixed with Thy1.2-expressing MLC responder cells depleted of $CD4^+$ $CD25^+$ cells, which were prepared as described for **Figure 7A**. Thus, MLC responder cells were reconstituted so that only nTreg were expressing Thy1.1. **(B)** Treg express functional A2AR. Incubation of purified $CD4^+$ $CD25^+$ cells with A2AR agonist induced cAMP production, which was blocked by ZM, A2AR antagonist. Data represent average ± SD of 3 samples. $^*P < 0.001$ vs. control. **(C)** A large part of A2AR-mediated increase of Treg was derived from nTreg. On day 7, the MLC was analyzed for the expression of FoxP3. The data were gated for $CD4^+$ cells. The numbers represent percentages of each quadrant. $Thy1.1^+$ (upper quadrants) and $Thy1.1^-$ (lower quadrants) cells were mostly $CD4^+$ $CD25^+$ nTreg and $CD4^+$ $CD25^-$ non-Treg cells in the beginning of culture, respectively. **(D)** Active proliferation of nTreg in MLC and well-maintained FoxP3 expression by treatment with A2AR agonist. MLC responders were reconstituted as in **(C)** except that $CD4^+$ $CD25^+$ cells from Thy1.1-expressing mice were labeled with CFSE. The panels show CFSE fluorescence and FoxP3 expression in $Thy1.1^+$ cells on day 4. The data shown here represent three separate experiments with similar results.

presence of A2AR agonist showed persistently lower cytokine-producing activity even after the removal of A2AR agonist (Zarek et al., 2008; Ohta et al., 2009).

In our studies on CTL development, we noticed the uniqueness of MLC, where massive expansion of Teff and increase of Treg were observed in the same culture. This may mimic immune responses *in vivo*: i.e., promotion of proinflammatory activities and compensatory anti-inflammatory responses to prevent excessive tissue destruction. Such responses were not observed in artificial stimulation of T cells with anti-CD3 mAb as Teff strongly overwhelm the culture. The current study revealed that A2AR stimulation inhibited activation of effector T cells and, at the same time, increased the number of Treg (**Figures 1**, **2,** and **6**). $CD4^+FoxP3^+$ cells from the MLC system express CD25, CTLA-4, CD39, and CD73 at high levels and have immunoregulatory activities as it has known for Treg induced by other methods (**Figures 3–5**). Our data suggests that A2AR not only directly inhibits T cell activation but also produces immunosuppressive cellular environment by inducing massive increase of Treg. Therefore, immunosuppressive concentration of extracellular adenosine may provide a long-lasting immunoregulatory effect even after the disappearance of adenosine.

The A2AR-mediated numerical change of Treg was due to increase of both nTreg and $CD4^+$ $CD25^-$ cell-derived Treg. MLC after the depletion of nTreg showed development of Treg from $CD4^+$ $CD25^-$ cells and its enhancement by A2AR stimulation (**Figure 7**). The increase of $CD4^+$ $CD25^-$ cell-derived Treg in normal MLC, in the presence of nTreg, required distinction for the origin of $CD4^+$ $FoxP3^+$ cells in the product. Reconstitution of MLC responders using Thy1.1-expressing nTreg made this distinction possible, and the A2AR-mediated increase of $CD4^+$ $CD25^-$ cell-derived Treg was confirmed in regular MLC (**Figure 8**). The increase of new Treg is consistent with a previous paper reporting mRNA upregulation of FoxP3 and LAG-3 in CGS-treated T cell culture (Zarek et al., 2008). Although the increase of Treg from $CD4^+$ $CD25^-$ cells was significant, these Treg accounted for only a minor portion of A2AR-mediated increase of Treg. Most of Treg after MLC was found to derive from nTreg (**Figure 8**). Purified nTreg was reported to proliferate when cultured with allogenic dendritic cells and IL-2 (Yamazaki et al., 2006). IL-2 was not added in our culture system, but activated $CD4^+$ cells might have produced IL-2 and supported proliferation of Treg. Indeed, massive proliferation of nTreg was observed in the current study (**Figure 8D**). Other possible reasons for A2AR-mediated enhancement of nTreg include prevention of FoxP3 down-regulation and cell death, which have been observed during Treg culture (Strauss et al., 2007; Hoffmann et al., 2009). In the current study, a number of nTreg were found to lose their FoxP3 expression after activation, while A2AR stimulation considerably prevented FoxP3 loss (**Figure 8D**). This mechanism may be partly responsible for the increase of $CD4^+$ $FoxP3^+$ cells treated with A2AR agonists. In addition, A2AR agonists are known to block activation-induced cell death of $CD4^+$ T cells (Himer et al., 2010).

Not only A2AR agonist increased the number of Treg, it also enhanced their immunoregulatory activity. T cell suppression assay showed that the same number of Treg from CGS/NECA-treated MLC had 4-times stronger regulatory activity than that from control MLC (**Figure 3**). The mechanism how Treg suppress T cell activation is still unclear, but CTLA-4 and CD25 represent important candidates. Pathogenesis of autoimmune disorders in mice with Treg-specific CTLA-4 deficiency demonstrated the importance of CTLA-4 in their regulatory activity (Wing et al., 2008; Sakaguchi et al., 2009). CD25 expression on Treg at high levels is considered to withdraw IL-2 from the microenvironment and induce effector cell death because of IL-2 deprivation (Pandiyan et al., 2007, 2011). The A2AR-mediated upregulation of CTLA-4 and CD25 on Treg (**Figures 1–3**) may support the enhancement of regulatory activity.

We assumed that it would be very effective immunosuppression if the activities of Treg were additive to or synergistic with other immunosuppressive mechanisms in the microenvironment. Accordingly, it was hypothesized that the immunosuppressive cytokines and molecules such as CTLA-4 would be increased on Treg by the same mechanism that mediates the hypoxia-adenosinergic immunosuppression (Sitkovsky, 2009). The detailed studies of hypoxia response element (HRE)/hypoxia-inducible factor-1α (HIF-1α) and cAMP response element (CRE)/cAMP response element binding protein (CREB) have been implicated in Treg activities only by circumstantial set of data (Sitkovsky, 2009) and the direct studies of CRE and HRE in Treg are still to be performed.

Tumors contain high levels of extracellular adenosine (Blay et al., 1997; Ohta et al., 2006). Adenosine in tumor microenvironment may be one of the important immunosuppressive mechanisms, which discourage anti-tumor immune responses, because A2AR-deficient mice could efficiently eradicate tumors, while wild-type mice could not (Ohta et al., 2006). One important reason for adenosine-mediated inactivation of anti-tumor immune responses would be a direct action at A2AR on T cells. However, the present data also suggest that the adenosine-A2AR signaling may enhance Treg in tumors. Tumors contain overwhelming number of Treg to suppress effector T cells (Turk et al., 2004; Antony et al., 2005; Sitkovsky et al., 2008; Facciabene et al., 2011). There might be a contribution from intratumoral high concentration of adenosine to the increase of Treg. Moreover, A2AR stimulation may enhance the regulatory activity of Treg in tumors and further inactivate anti-tumor immune responses. Physiological control of Treg activity *in vivo* by extracellular adenosine is yet to be determined.

While Treg is a target to be discouraged for the improvement of tumor immunotherapy, transfusion of Treg is a promising approach for the treatment of autoimmune diseases and allogenic reaction after transplantation (Riley et al., 2009; Matsuoka et al., 2010). Because large doses of Treg are necessary to suppress GVHD, Treg require massive expansion *ex vivo* before transfer, but the expansion of Treg is somewhat challenging. It is difficult to start the expansion from a large number of Treg because of low frequency of Treg in peripheral blood. In addition, since both Treg and activated effector T cells share $CD4^+$ $CD25^+$ phenotype, polyclonal activation of T cell could result in considerable contamination by effector T cells (Riley et al., 2009). Treatment with

A2AR agonist induces expansion of Treg, while it suppresses activation of effector T cells. Such a culture condition favoring Treg outgrowth may be suitable for the expansion of Treg. Effects of A2AR agonists on human Treg will need to be examined for numerical increase and enhancement of regulatory activity. Dependent on the nature of human Treg, optimization of culture condition is expected to improve the recovery of functionally enhanced Treg.

In conclusion, we found that T cell activation in the presence of A2AR agonist resulted in expansion of Treg. After the A2AR-mediated expansion, Treg acquired stronger immunoregulatory activity. The quantitative and qualitative enhancement of Treg by the adenosine-A2AR pathway may be relevant to the establishment of longer-lasting immunomodulation. This mechanism may be utilized in the expansion of Treg for treatment of autoimmune diseases and GVHD.

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Mechanisms of T_{reg} suppression: still a long way to go

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How do natural, thymus-derived T_{regs} work? This question has preoccupied and perplexed T cell biologists for nearly two decades (Tang and Bluestone, 2008; Vignali et al., 2008; Workman et al., 2009). As with all important questions, there are some issues where there is a general consensus and others where there is still considerable disagreement. I would venture that most would agree with the following four basic tenets related to T_{reg} function.

First, T_{regs} are a critical peripheral tolerance mechanism that maintains immune homeostasis and prevents widespread autoimmunity (Ramsdell, 2003; Sakaguchi, 2003; Fontenot and Rudensky, 2005). Thus, determining how T_{regs} work is a very important goal.

Second, T_{regs} can suppress or modulate the function of a wide variety of cell populations, in diverse anatomical locations and in multiple disease situations (Tang and Bluestone, 2008).

Third, T_{regs} have an extensive arsenal and can utilize multiple contact-dependent and contact-independent mechanisms (Shevach et al., 2006; Vignali et al., 2008). This may be important because not all cell populations will be sensitive to all T_{reg} mechanisms.

Fourth, in addition to natural, thymus-derived T_{regs}, there are several induced T_{reg} (iT_{reg}) populations that can be generated *in vitro* or directly by T_{regs} via one of several inhibitory cytokines (TGFβ, IL-10, IL-35; Shevach, 2006; Collison and Vignali, 2008). The mechanism of suppression used by these populations seems less complicated with each appearing to depend on one key inhibitory cytokine. Thus our discussion here will focus on natural T_{regs}.

However, I would argue that we know far less than we think we do about natural T_{reg} function and there is probably more contention than consensus. It is likely that continued, extensive analysis, and unique tools and approaches are likely to be required before a clear picture emerges. Some of these issues can be encapsulated around three key questions that pertain to T_{reg} function.

Which mechanisms are most important? This remains a contentious issue with each mechanism having its set of protagonists and antagonists. The mechanisms utilized by natural T_{regs} that have probably been examined and discussed the most are inhibitory cytokines (TGFβ, IL-10, IL-35), inhibitory receptors (CTLA4, LAG-3), cytotoxicity (Granzyme/Perforin) and metabolic disruption (IL-2 deprivation-mediated apoptosis, adenosine; Shevach et al., 2006; Tang and Bluestone, 2008; Vignali et al., 2008). Of course there may be several mechanisms that are important, with each contributing significantly in different disease scenarios, anatomical locations or against diverse cell types. Whether this is reality or appeasement remains to be resolved.

Before one determines if a mechanism is important, one would first need to obtain convincing *in vivo* evidence that a particular mechanism has a clearly definable physiologic impact. In my view, too many strong conclusions have been derived from exclusively or predominantly *in vitro* studies. Even when *in vivo* experiments have been performed, they are either limited to one model system or have potential, inherent weaknesses or caveats. It is hard to define what is "sufficient" but it seems reasonable to propose that data should be derived from multiple *in vivo* models (at least three) using (1) mice harboring conditionally (and ideally temporally) deleted alleles and (2) *in vivo* blockade/neutralization with a specific monoclonal antibody. Indeed, one could argue that no proposed T_{reg} mechanism has been extensively assessed *in vivo* thus far. Although we have championed the potential importance of IL-35 using five *in vivo* model systems with neutralizing antibodies and mutant T cell populations targeting the cytokine and its receptor (Collison et al., 2007, 2010, 2012), I would be the first to admit that more remains to be determined regarding the physiological importance of IL-35. So for mechanisms that have not reached this bar, further analysis is clearly required. Of course a major challenge is that some of these mechanisms may utilize molecules that also contribute to T_{reg} development or homeostasis. Thus it may be very difficult, or even impossible, to divorce their roles in T_{reg} function versus development/homeostasis. Furthermore, some mechanisms may be hard to target without affecting other cellular processes.

The current debate regarding the importance of one proposed T_{reg} mechanism illustrates many of these issues. T_{regs} express high levels of the high affinity IL-2 receptor (CD25; Sakaguchi et al., 1995). It was initially proposed that T_{regs} may act as a "sink" absorbing IL-2 from the local environment, thereby depriving recently stimulated T cells from the IL-2 required to initiate proliferation and subsequent differentiation. Subsequent analysis of mice lacking the capacity to make IL-2 or lacking CD25 expression revealed that IL-2 plays a critical role in maintaining peripheral T_{reg} homeostasis (D'Cruz and Klein, 2005; Fontenot et al., 2005). It was suggested that these data refuted the idea that T_{regs} act as an IL-2 "sink," although T_{reg} function *in vivo* was not directly examined (Maloy and Powrie, 2005). More recently, it has been suggested that IL-2 deprivation-mediated apoptosis, facilitated by high CD25 expression, is a prominent mechanism of suppression used by T_{regs} (Pandiyan et al., 2007). These conclusions were supported predominantly by the observation that T cells targeted by T_{regs} die by apoptosis in *in vitro* assays but are resistant if they lack Bim, a pro-apo-

ptotic Bcl-2 family member. Bim binds to Bcl-2 in response to stress signals, such as growth factor deprivation, thereby priming the mitochondrial pathway of apoptosis (Kuwana et al., 2005). $Bim^{-/-}$ T cells are resistant to apoptosis induced by cytokine or growth factor withdrawal, particularly IL-2 (Bouillet et al., 1999). Curiously, Pandiyan and colleagues also showed that T_{regs} do not affect early activation or proliferation of effector T cells which is at odds with the importance of IL-2-induced STAT5 activation during these early stages (Rawlings et al., 2011).

The importance of this mechanism remains controversial as several studies have suggested that IL-2 depletion alone is not required for the suppression of human T cells (Tran et al., 2009; Vercoulen et al., 2009). More recently, we have revisited this issue to ask if T_{regs} suppress via programmed cell death pathways (Szymczak-Workman et al., 2011). Contrary to the findings of Pandiyan and colleagues, we clearly showed that T_{reg}-mediated suppression of $Bim^{-/-}$, $Bim^{-/-}Puma^{-/-}$, and Bcl-2 transgenic T cells is comparable to controls using a variety of *in vitro* and *in vivo* assays (Szymczak-Workman et al., 2011). Our use of $Bim^{-/-}Puma^{-/-}$ mice was particularly revealing as they have been shown to be completely resistant to cytokine withdrawal (You et al., 2006; Cho et al., 2009), and yet could be readily suppressed by T_{regs}.

Even though *in vitro* assays can be very informative, too often strong conclusions are drawn from data obtained primarily from such assays, as highlighted above. When T cells are stimulated *in vitro*, substantial T cell apoptosis can occur if IL-2 becomes limiting, and so T cell death can be readily observed *in vitro* in the absence of T_{regs}. The paradox here is that T_{reg} assays can be established where suppression is observed and either there is significant effector T cell death or almost no death, questioning whether the latter has anything to do with T_{reg} function. Although Pandiyan and colleagues suggested that $Bim^{-/-}$ T cells were resistant to T_{reg}-mediated suppression in an inflammatory bowel disease (IBD) model (Pandiyan et al., 2007), we showed in two *in vivo* models that $Bim^{-/-}$ T cells could be effectively suppressed by T_{regs} and that $Bim^{-/-}$ T cells had a far greater propensity to convert into iT_{regs} *in vivo* (Szymczak-Workman et al., 2011; Wang et al., 2012).

Regardless of which side of this debate your views lie, it is clear that further analysis, especially using more sophisticated *in vivo* analysis and model systems, will be required to provide further insight into this controversy. While we do not claim that T_{regs} cannot mediate suppression by any cell death pathway under any circumstances, I would respectfully argue that there is not yet sufficient data to conclude that IL-2 deprivation-mediated apoptosis is a prominent mechanism of T_{reg}-mediated suppression (Pandiyan et al., 2007). Given the inextricable link between the role of CD25 in controlling T_{reg} homeostasis and its possible role in mediating suppression via IL-2 deprivation-mediated apoptosis, definitive conclusions may be hard to reach.

Given these issues, the debate over the importance of other mechanisms is likely to conjure similar discussions. Nevertheless, we need to persevere dissecting what is clearly a very important issue. A final thought on this question (sobering or exciting depending on your point of view). It remains possible that some key T_{reg} mechanisms have yet to be identified. Indeed, we have yet to define the function of many of the genes that are upregulated in T_{regs}, especially when derived from disease locations (Vignali et al., 2008).

How many mechanisms do T_{regs} need? If we accept that T_{regs} may utilize many mechanisms to mediate suppression, especially at sites of substantial inflammation, it would seem important to determine how many are required to sustain credible T_{reg} function. T_{reg} depletion or absence results in the development of severe autoimmunity, but this is not matched by the disruption of any known mechanism (Fontenot and Rudensky, 2005; Vignali, 2008). This suggests that either key mechanisms have yet to be identified or multiple mechanisms work in concert to mediate T_{reg} function. I have discussed this topic in the past (Vignali, 2008), and the bottom line is that we do not know the minimum number of mechanisms required for T_{reg} function or how many can be lost before T_{regs} become fully dysfunctional.

We recently began to address this issue with surprising results. We assessed the suppressive capacity of IL-10/IL-35 double-deficient T_{regs} anticipating that they would exhibit functional defects that were greater than their single knockout counterparts. Surprisingly, IL-10/IL-35 double-deficient T_{regs} were fully functional *in vitro* and *in vivo*, essentially indistinguishable from wild type T_{regs} (Pillai et al., 2011). Thus, they seemed to have gained rather than lost function. Subsequent analysis revealed that the loss of IL-10 and IL-35 was compensated for by the concurrent increase in cathepsin E (CTSE) expression. This appeared to be required to facilitate the expression and/or release of TRAIL, a member of the TNF superfamily that can mediate apoptosis, programmed necrosis (necroptosis) or suppress proliferation via its surface bound form or as a soluble trimer (Wang and el-Deiry, 2003; Schaefer et al., 2007). Importantly, this rendered IL-10/IL-35 double-deficient T_{regs} functionally dependent on TRAIL *in vitro* and *in vivo* (Pillai et al., 2011). These data highlight two important concepts. First, the loss of certain regulatory mechanisms may result in unforeseen molecular changes which facilitate functional compensation by the upregulation of another inhibitory mechanism. Second, this study revealed that unappreciated cross-regulatory pathways may exist which control the utilization of certain suppressive mechanisms. Collectively this may serve to facilitate T_{reg} functional plasticity. Whether such mechanisms operate *in vivo* in the absence of genetic manipulation remains to be determined. However, it is possible that the mechanisms that are dominant differ in T_{regs} from different genetic backgrounds. Indeed, TRAIL is not a major mechanism used by C57BL/6 T_{regs}, which express low levels of CTSE, but may be a more dominant mechanism used by Balb/c T_{regs}, which coincidentally express high levels of CTSE (Pillai et al., 2011). Additional studies will clearly be required to determine the prevalence of T_{reg} functional plasticity caused by divergent genetic backgrounds and/or altered environmental circumstances.

Is T_{reg} function altered in response to their environment? While this was addressed in part in the previous section, there are two other important points to make here. First, several recent and exciting studies have highlighted the importance of specific transcription factors in shaping T_{reg} migration and function toward the control of particular Th responses (Chaudhry et al., 2009; Koch et al., 2009; Zheng et al., 2009). However, the precise molecular mechanism behind these observations remains obscure and an

important priority for future research. Also, one wonders if other transcription factors play similar functions in facilitating T_{reg} control of diverse cell types and environments. Second, we have previously postulated that T_{regs} receive molecular cues from their target population and local environment that potentiates or boosts their regulatory capacity (Vignali et al., 2008). Indeed, we have shown that T_{reg}:T cell target interaction can substantially boost T_{reg}-mediated suppression (Collison et al., 2009; Chaturvedi et al., 2011). While the molecular mechanism of T_{reg} functional potentiation remains to be determined, it is particularly intriguing that the transcriptional landscape of "boosted" T_{regs} versus "activated" T_{regs} is very different, suggesting that a lot remains to be discovered.

In closing, while we have made substantial progress in our quest to determine how T_{regs} work, it seems that much still remains to be discovered and clarified.

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Multiple Treg suppressive modules and their adaptability

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Foxp3$^+$ regulatory T cells (Tregs) are a constitutively immunosuppressive cell type critical for the control of autoimmunity and inflammatory pathology. A range of mechanisms of Treg suppression have been identified and it has not always been clear how these different mechanisms interact in order to properly suppress autoimmunity and excessive inflammation. In recent years it has become clear that, while all Tregs seem to share some core suppressive mechanisms, they are also able to adapt to their surroundings in response to a variety of stimuli by homing to the sites of inflammation and exerting ancillary suppressive functions. In this review, we discuss the relevance and possible modes of Treg adaptability and put forward a modular model of Treg suppressive function. Understanding this flexibility may hold the key to understanding the full spectrum of Treg suppressive behavior.

Keywords: Tregs, suppression, transcription factors, adaptability, CTLA-4

INTRODUCTION

T-regulatory cells (Tregs) are a suppressive subset of T cells, which make up around 10% of CD4$^+$ cells, crucial to the proper maintenance of immune self-tolerance and homeostasis. Natural Tregs are produced in the thymus but some Tregs may also be induced in the periphery (Sakaguchi et al., 2008). Since the discovery that CD25-expressing T cells in the normal immune system have suppressive function (Sakaguchi et al., 1995), the field of regulatory T cells has advanced with the discovery that the transcription factor Foxp3 is critical to the suppressive function of Tregs as illustrated by the finding that ectopic expression of Foxp3 can induce regulatory function in naïve T cells (Fontenot et al., 2003; Hori et al., 2003). A loss of Foxp3 function results in Treg deficiency or dysfunction and is responsible for the autoimmune disorders seen in both Scurfy mice (Brunkow et al., 2001; Khattri et al., 2003) and the human immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) syndrome (Wildin et al., 2001). Foxp3 regulates expression of a large number of genes including those responsible for key features of Tregs, such as an absence of IL-2 production and high expression of cytotoxic T lymphocyte antigen-4 (CTLA-4) and CD25. While it has been clear for some time that Tregs are critical to the maintenance of immune self-tolerance and homeostasis, the exact mechanisms by which they are able to do this has been a source of considerable debate (Rudensky and Campbell, 2006; Sakaguchi et al., 2009; Shevach, 2009).

CORE MECHANISMS OF Treg SUPPRESSION

A number of different Treg suppressive mechanisms have been proposed. It seems that in part this is due to Tregs being able to adapt to environmental cues and alter their suppressive mechanisms to fit the circumstances. However, while there is some heterogeneity of function, all Foxp3-expressing Tregs may share a set of core suppressive mechanisms. As discussed below, CTLA-4 and IL-2 are most stably activated or repressed, respectively, by Foxp3 and appear to be involved in such core mechanisms.

CTLA-4-DEPENDENT SUPPRESSION

CTLA-4 is constitutively expressed by Foxp3$^+$ Tregs and competes with the closely related CD28 for binding with the co-stimulatory molecules, CD80 and CD86, on the surface of APCs. While CTLA-4 is also expressed on activated T-effector cells, mice with a Treg specific CTLA-4 depletion suffer from fatal lymphoproliferative disease, similar to that seen in totally CTLA-4-deficient mice, despite the development of Tregs in normal numbers (Waterhouse et al., 1995; Wing et al., 2008). CTLA-4-deficient Tregs also show impaired *in vitro* suppressive activity (Wing et al., 2008), while mice with mixed bone marrow chimeras of CTLA-4-deficient and sufficient donors do not develop autoimmune disease, demonstrating cell extrinsic function (Bachmann et al., 1999). For some time the mechanisms of CTLA-4 function have been obscure but recent work has elegantly demonstrated that CTLA-4-mediated trans-endocytosis and degradation of CD80/86 from the surface of APCs is a key mechanism of CTLA-4 function (Qureshi et al., 2011). However, this may not be the sole mechanism of CTLA-4 function as CTLA-4 ligation of CD80/86 also induces nuclear translocation of the transcription factor Foxo3 inhibiting dendritic cell (DC) expression of IL-6 and TNFα, while Foxo3 KO mice have enhanced CD80/86 expression (Dejean et al., 2009). In addition, CTLA-4 engagement may cause DCs to express the immunosuppressive tryptophan catabolizing enzyme IDO (Grohmann et al., 2002). These findings collectively suggest that CTLA-4 is critical to the suppressive function of Tregs and appears to act primarily

via cell extrinsic effects on APCs (Wing et al., 2008) in which Treg expression of CTLA-4 reduces APC expression of CD80 and 86 thus reducing their co-stimulatory capacity (Wing et al., 2011).

IL-2-DEPENDENT SUPPRESSION

Several cardinal features of Foxp3$^+$ Tregs are their absence of IL-2 production, constitutively high expression of the alpha chain receptor for IL-2 (CD25) and consequent ability to bind IL-2 with high affinity (Sakaguchi et al., 2007). A central role for IL-2 in Treg biology has been demonstrated by *in vivo* studies of mice either deficient in IL-2 production (Schorle et al., 1991) or IL-2 receptor (Suzuki et al., 1995;Willerford et al., 1995): both suffer from fatal lymphoproliferative disease in a manner similar to Foxp3-deficient scurfy mice. Tregs are able to suppress both IL-2 mRNA and protein production by responder T cells (Takahashi et al., 1998; Thornton and Shevach, 1998), most likely indirectly due to a CTLA-4-mediated reduction in CD80/86 co-stimulation from APCs and also consume IL-2, thus denying it to local T-effector cells (de la Rosa et al., 2004). This may in turn lead to arrest of proliferation and apoptosis of T-effector cells (Pandiyan et al., 2007). Since Tregs are dependent on exogenous IL-2 for their survival and function (Furtado et al., 2002; Setoguchi et al., 2005) but suppress its production by T cells, it seems that IL-2 is used as a feedback mechanism to prevent Treg overgrowth that might otherwise result in excessive immunosuppression.

Foxp3 regulated features of Treg biology such as high expression of CTLA-4, CD25 and an absence of IL-2 production are critical to the function of Tregs, efficiently controlling APC-dependent activation and proliferation of T cells. As a result, knockout of either CTLA-4 or CD25 expression replicates the phenotype of Foxp3-deficient mice. However, in addition to this "core module" of suppression, Foxp3$^+$ Tregs are also able to adapt to their environment and take on additional suppressive functions in order to fit the immune context they find themselves in. This may be of particular importance in the more inflammatory conditions found at barrier sites such as the gut in which CTLA-4 and IL-2-based suppression may be sometimes overwhelmed due to an excess of inflammatory signals leading to a requirement for additional mechanisms such as IL-10 (Rubtsov et al., 2008; Yamaguchi et al., 2011).

ADAPTABILITY OF SUPPRESSIVE MECHANISMS AND INDUCTION OF ADDITIONAL TRANSCRIPTION FACTORS

CTLA-4-dependent suppression is not the only mechanism by which Tregs are able to suppress the activity of other cells. *In vitro* experiments give a clear indication of this: while normal Tregs suppress T-effector proliferation in a CTLA-4-dependent manner, Tregs derived from total CTLA-4-deficient mice also retain *in vitro* suppressive capacity. In this case it seems likely that Tregs derived from CTLA-4-deficient mice are highly activated due to the ongoing autoimmune pathology and as such may have induced suppressive functions such as IL-10 and TGFβ secretion. A wide range of other suppressive molecules have been identified in Tregs including: IL-10, TGFβ, Galectin, Neuropilin, IL-35, cAMP, Granzyme-B, perforin, and cAMP (Sakaguchi et al., 2009; Shevach, 2009). Many of these molecules are upregulated in effector CD44^{high} Tregs.

In addition to adopting an effector phenotype in response to stimulation, recent work has demonstrated that Tregs are able to adapt to environmental signals and further differentiate from their base state by expression of transcription factors normally associated with other T cell subsets (Campbell and Koch, 2011). It seems likely that Tregs share much of the same molecular machinery as conventional CD4$^+$ T cells and respond to the same differentiation stimuli. This induction/co-opting of additional transcription factors seems to be critical to Treg suppression of particular T cell subsets and their associated immunopathology.

T-bet – Th1

Koch et al. (2009) have demonstrated that the transcription factor T-bet, normally required for the linage development of Th1 cells, is upregulated in Tregs in response to IFNγ, suggesting that the induction of this Treg subset occurs in reaction to a Th1 immune response (Koch et al., 2009). T-bet causes the Th1-associated chemokine receptor CXCR3 to be upregulated on the surface of Tregs and a small increase in IFNγ expression was also observed. CXCR3 homes the cell to the sites of Th1 inflammation via its IFNγ-induced ligands CXCL9, CXCL10, and CXCL11, allowing Tregs to provide *in situ* suppression. T-bet-deficient Tregs are unable to accumulate at the sites of Th1 inflammation and properly control the Th1 immune response (Koch et al., 2009). In addition, T-bet-deficient Tregs retain their *in vitro* suppressive activity (Bettelli et al., 2004) and *in vivo* have only a modest (approximately 50%) reduction in mRNA expression of the effector molecules, IL-10 and TGFβ (Koch et al., 2009). This suggests that the primary role of T-bet in Treg suppression of Th1 responses may be the control of CXCR3 expression and Treg localization.

IRF4 AND GATA-3 – Th2

The transcription factor interferon regulatory factor 4 (IRF4) is critical for the differentiation of Th2 cells and may also play a role in the differentiation of Th17 cells (Lohoff et al., 2002; Brustle et al., 2007). Foxp3 is able to bind to the promoter region of IRF4 and enhance its expression (Zheng et al., 2009). Additionally, Treg-specific deletion of *Irf4* prevents Tregs from efficiently suppressing Th2 immune responses leading to an uncontrolled Th2 immune response characterized by significantly enhanced IL-4 and IL-5 production, but not IFNγ, IL-2, or IL-17 (Zheng et al., 2009). Additionally, IRF4-deficient Tregs have decreased expression of the genes encoding the suppressive molecules IL-10 and Granzyme B, while also reducing CCR8 expression, the chemokine receptor important for migration to the sites of Th2 inflammation (Zheng et al., 2009).

Cretney et al. (2011) demonstrated a wider role for IRF4 expression with the finding that IRF4 may also be required for T-bet and Blimp-1 expression. Blimp-1 is a transcriptional repressor normally associated with plasma cell differentiation, but in Tregs it appears to be crucial to the expression of IL-10 (Cretney et al., 2011). This would suggest that IRF4 expression by Tregs may also be critical to IL-10-dependent effector mechanisms. As a result it seems likely that part of the reason for the failure of IRF4-deficient Tregs to regulate Th2 responses may be due to a lack of IL-10 production.

It has also become clear that the canonical Th2 transcription factor, GATA-3, may also expressed by Tregs (Campbell, 2011; Wang et al., 2011; Wohlfert et al., 2011). In GATA-3-deficient Tregs, expression of Foxp3 and constitutively expressed Treg markers/effector molecules such as CLTA-4, GITR, and CD25 were all reduced both at the mRNA and protein levels. GATA-3-deficient Tregs are unable to prevent the induction of systemic lymphoproliferative disease and increased production of Th1, Th2, and Th17 cytokines (Wang et al., 2011). Thus, unlike STAT3 and T-bet, whose expression in Tregs appears to be largely linked to the control of the T cell subtypes with which they are associated, it seems that GATA-3 has a wider function in the maintenance of immunity. This is perhaps not surprising as while GATA-3 is often primarily associated with Th2 cells, it is also expressed in a range of other cell types and is required for thymocyte development (Pai et al., 2003).

BCL-6 – T-FOLLICULAR

During a T-dependent immune response $CD4^+$ T cells are able to express the transcription factor BCL-6 that converts them to a $PD\text{-}1^+$ $CXCR5^+$ T-follicular helper subtype (Tfh; Crotty, 2011). These cells then home to the follicle, due to the action of the chemokine receptor CXCR5 guided by CXCL13 produced by follicular stromal cells, and specialize in assisting B cell affinity maturation and memory cell formation within the germinal center (Crotty, 2011). Recent work has demonstrated that thymically derived Tregs are also able to effectively mimic this phenotype by induction of BCL-6 giving a $CXCR5^+$ $PD\text{-}1^+$ phenotype that home to the follicular region. These cells appear to differentiate from thymically produced Tregs in response to the same SLAM-associated protein (SAP), CD28, and B cell-dependent signals that induce Tfh differentiation (Chung et al., 2011; Linterman et al., 2011). This follicular Treg subset then travels to the germinal center and controls the germinal center reaction (Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011). At this time the primary suppression mechanisms used by follicular Tregs are not clear, although CTLA-4 protein levels and IL-10 mRNA expression is increased, while Granzyme B is reduced in comparison to normal Tregs (Linterman et al., 2011). Surprisingly, given their expression of BCL-6, normally a mutual repressor of Blimp-1, these cells also express high levels of *prdm1* mRNA encoding the transcriptional repressor Blimp-1 (Chung et al., 2011; Linterman et al., 2011) crucial to the expression of IL-10.

STAT3 AND RORγt – Th17

The transcription factor STAT3 is essential for the proper development of Th17 cells (Mathur et al., 2007). More recently a role for STAT3 in Treg biology has been demonstrated by the finding that STAT3-deficient Tregs are unable to prevent fatal Th17-mediated colitis (Chaudhry et al., 2009). Interestingly, these Tregs retain *in vitro* suppressive capacity, presumably due to intact CTLA-4 function, but have reduced expression of IL-10, IL-35, and CCR6 (Chaudhry et al., 2009). STAT3 phosphorylation by Tregs is induced in an IL-10-dependent manner with IL-10 receptor-deficient Tregs failing to phosphorylate STAT3 and restrain Th17 responses and the development of colitis (Chaudhry et al., 2011). Additionally, IL-10 receptor (IL10R) expression by Th17 cells is critical for prevention of their inflammatory pathology (Huber et al., 2011).

A number of groups have reported that Tregs may be able to simultaneously express Foxp3 and the Th17 defining transcription factor RORγt, and may produce IL-17 (Zhou et al., 2008; Voo et al., 2009; Hovhannisyan et al., 2011). In humans, Tregs have been observed to lose suppressive function while producing IL-17 but then regain suppressive function, suggesting that this could be a temporary loss of suppressive capability in order to allow the immune response sufficient time to effectively fight pathogens before suppressive function is re-established (Beriou et al., 2009). Interestingly, only CCR6-expressing Tregs expressed IL-17 (Beriou et al., 2009). This is of relevance since CCR6 controls Treg accumulation at the sites of Th17-mediated inflammation due to the IL-17-mediated release of its ligand CCL20 by epithelial cells (Hirota et al., 2007; Yamazaki et al., 2008).

SYNTHESIS

While all Tregs can be characterized as $Foxp3^+$ $CTLA\text{-}4^+$ and lacking IL-2 production, it seems that from this base state a range of transcription factors may be induced/co-opted in order to allow the Treg to adapt to inflammatory cues. It seems likely that Tregs share much of the same molecular machinery as conventional $CD4^+$ T cells and thus respond to the same stimuli to differentiate into Thχ-like Tregs.

In this sense there is a core module of Treg suppression driven by Foxp3 expression that acts to prevent the activation, proliferation, and as a result the differentiation, of T-effector cells. This core module may be augmented by induction of additional modules, driven by transcription factors normally associated with other T cell subsets, allowing Tregs to adapt to become "Thχ-like" Tregs and deliver suppression to diverse sites within the body (**Figure 1**).

While the possibility that some Tregs may lose Foxp3 expression and become proinflammatory "ex-Tregs" is highly controversial (Zhou et al., 2009; Rubtsov et al., 2010; Hori, 2011; Miyao et al., 2012), we should perhaps not be surprised to find some evidence of proinflammatory cytokine expression by Thχ-like Tregs that broadly retain suppressive function. While Foxp3 is a master regulator it does not totally repress the loci associated with Th1, Th2, and Th17 cytokines (Wei et al., 2009; Duhen et al., 2012). As a result it is not necessarily a contradiction to find Tregs-expressing proinflammatory cytokines and it will be interesting to see if in some settings there are benefits to Treg expression of proinflammatory cytokines. For example, Treg expression of IFNγ has been reported in several settings; during infection with *Toxoplasma gondii* IFNγ-producing Tregs retained their *in vitro* suppressive capacity (Oldenhove et al., 2009) while IL-12-induced IFNγ-producing Tregs retain both *in vitro* suppressive capacity and the ability to prevent colitis in an IFNγ-dependent manner (Feng et al., 2011). Treg produced IFNγ may also have a protective role in transplant tolerance. Alloreactive Tregs are able to prevent rejection of a BALB/c skin graft in C57/B6 mice, while Tregs from IFNγ-deficient mice were significantly less protective (Sawitzki et al., 2005).

Thχ-like Tregs appear to have an effector Treg phenotype, with clear roles for IL-10 in STAT-3 and IRF4 driven suppression, while

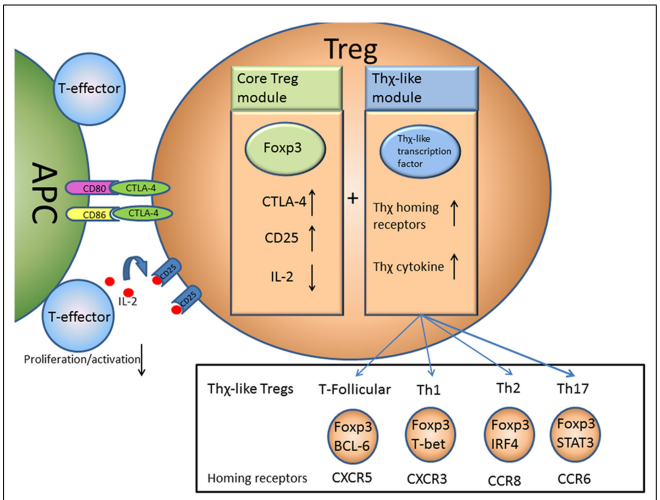

FIGURE 1 | A two module model of Treg function. Foxp3 acts to control the core module of Treg suppressive function by regulating expression of a number of key molecules such as CTLA-4 and CD25. This allows Tregs to suppress T-effector activation and proliferation by suppressing APC function via CTLA-4 and possibly depriving IL-2 from other T cells. An additional Thχ-like module of suppressive function may also be induced that causes Tregs to express Thχ-like transcription factors allowing the Tregs to take on some of the properties of Th1, Th2, Th17, or follicular T cells, travel to the same sites and deliver *in situ* suppression.

T-bet and BCL-6-expressing Tregs also seem to have upregulated expression of IL-10. While IL-10 is not critical to the *in vitro* function of Tregs (Takahashi et al., 1998; Rubtsov et al., 2008), *in vivo* experiments have demonstrated IL-10 to be important in the maintenance of immune homeostasis in the gut, with IL-10-deficient mice spontaneously developing intestinal inflammation (Kuhn et al., 1993), while Treg-specific deletion of *il10* leads to colitis and inflammation at the lung surface (Rubtsov et al., 2008), suggesting that its role may be to control excessive inflammation from microbial stimulation at mucosal surfaces rather than maintenance of self-tolerance. At this time it is not clear if the suppressive mechanisms employed by Thχ-like Tregs are different from previously defined effector Tregs, or if the differences are primarily due to cell localization. Additionally, it seems likely that, on close examination of their transcription factor expression, there may be substantial overlap between previously identified effector Tregs and Thχ-like Tregs. As it stands, no clear mechanisms of suppression that are truly specific to a particular Thχ-like Treg have been identified. As a result, it seems likely that these are specialized subsets of effector Tregs and the primary role of the induction of these transcription factors is to allow Tregs to respond to the same stimuli as conventional T cells, travel to the same sites, and enhance or upregulate existing effector Treg suppressive mechanisms such as CTLA-4 and IL-10.

CONCLUSION

While Tregs have a core module of suppression driven by Foxp3 expression, they are also able to adapt to changes in their environment and harness additional modules by the expression of transcription factors normally associated with other T cell subtypes in order to better control immunopathology. Particularly in the light of ongoing work examining either transfer or depletion of Tregs in clinical settings it is critical that we understand both the core functions of Tregs and their range of flexibility that allows them to modulate their mechanisms of suppression and cell localization in response to specific stimuli.

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Foxp3+ regulatory T-cells and IL-2: the Moirai of T-cell fates?

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Regulatory T-cells (T_{reg}) have emerged as a crucial cellular checkpoint acting to prevent potentially harmful immune responses. Due to its highly diverse activities, regulating the immune system requires more than general suppression. Particularly, during the developing immune response to infection, T_{reg} need to balance the host reaction to achieve both an effective response against the invading pathogen as well as to prevent immunopathology from excessive or inappropriate activity. In this opinion article we discuss the dual roles of IL-2 as both a key inducer of T_{reg} activity and also a target of T_{reg} control during the acute phase of infection. We propose a model in which Foxp3+ regulatory T-cells dynamically "measure" IL-2 availability and restrict its access to effector T-cells, thereby controlling differentiation of these useful but potentially harmful cells.

CD4+ T-lymphocytes play a central role in orchestrating immune responses by modifying the functionality of other immune cells and guiding the qualitative features of a response to one optimal for resisting a particular microorganism. Besides augmenting both innate and adaptive immune responses, CD4+ T-cells limit excessive immune activation and immunopathology during infections. Among CD4+ T-cells, Foxp3+ regulatory T-cells (T_{reg}) are essential for the maintenance of peripheral tolerance (Kim et al., 2007; Lahl et al., 2007). These cells also modulate the responses to pathogens (Belkaid and Tarbell, 2009). A plethora of mechanisms for how T_{reg} exert their function have been suggested (Shevach, 2009; Josefowicz et al., 2012). However, there is still an ongoing debate as to which functions of T_{reg} are essential under which circumstances. Likely, T_{reg} function can be attuned to specific conditions and distinct "rules" govern T_{reg} behavior in the steady-state versus inflammatory environments, secondary lymphoid (SLO) versus peripheral organs, developing versus ongoing immune responses, or acute versus chronic infections, for example. In this article we focus on the function of T_{reg} in the SLO during a developing acute infection and, although several cytokines are relevant, we concentrate on IL-2 as a central platform that enables effective immune control, as it (1) links activation of effector and regulatory responses, (2) establishes a feed-back loop for T-cell expansion, and (3) allows control over T-cell differentiation and fate decisions, preserving memory formation.

IL-2 IS A CENTRAL CYTOKINE FOR T-CELL ACTIVATION

IL-2, originally discovered as a mitogenic factor for T-cells, is bound as a quaternary complex with CD25 (IL2Rα-chain), CD122 (IL2Rβ-chain), and CD132 (common γ-chain). The α/β-heterodimer facilitates IL-2 capture with high affinity and, further stabilized by the γ-chain, forms a very stable complex which is terminated via receptor internalization rather than ligand dissociation (Smith, 2006). Upon activation by TCR interactions and additional co-stimulation via CD80/CD86, conventional T-cells (T_{conv}) produce IL-2 and upregulate CD25 expression, which enhances IL-2 capture and consequently IL-2 signaling, further promoting CD25 expression, T-cell activation, and proliferation. This feed-forward loop can lead to activation-induced cell death, but highly activated, proliferating T-cells also undergo apoptosis when acutely deprived of IL-2 signals. Therefore, IL-2 is a master regulator of T-cell activation, proliferation, and death, excellently reviewed in Malek and Castro (2010), Boyman and Sprent (2012).

ACTIVATION OF T_{REG} THROUGH IL-2: ANTICIPATION AND SENSING OF EFFECTOR RESPONSES

In contrast to conventional T-cells (T_{conv}), T_{reg} constitutively express CD25 (Sakaguchi et al., 1995) and have STAT5 phosphorylation in the steady-state, arguing for continuous or high frequency intermittent IL-2 signaling in the absence of infection. Indeed, IL-2 signals seem to be pivotal for T_{reg} survival because animals that lack IL-2, CD25, or CD122 are largely devoid of peripheral T_{reg} and suffer from severe autoimmunity (Sadlack et al., 1993; Suzuki et al., 1995; Willerford et al., 1995; Fontenot et al., 2005). T_{reg} do not produce IL-2 themselves when stimulated through the TCR and therefore rely on paracrine IL-2 for their maintenance. T_{conv} produce IL-2 upon activation and then gradually upregulate CD25. Since T_{reg} constitutively express CD25, they can sense and signal via IL-2 as soon as it is produced, assuming that these T_{reg} are within suitable proximity to the cytokine secreting cells. Because IL-2 signaling further upregulates CD25, T_{reg} can even increase their ability to capture IL-2 as compared to T_{conv} which need to initiate CD25 expression post TCR-mediated activation (Feinerman et al., 2010). Indeed, it has been shown *in vivo* that T_{reg} are the first cells to respond to IL-2 upon antigenic challenge of T_{conv} (O'Gorman et al., 2009). As the amount of IL-2 produced by T-cells correlates with the extent of co-stimulation from DC *in vitro* (Shahinian et al., 1993) and *in vivo* (Kastenmuller et al., 2011), it might reflect the magnitude of pathogen burden and the extent of innate stimulation. Therefore, T_{reg} "sense" the initiation of an adaptive immune response in a qualitative and potentially quantitative manner when responding to IL-2 signals derived from adaptive effectors.

T_{REG} CONTROL THE AVAILABILITY OF IL-2

Given the relative abundance of T_{reg} in SLO, where adaptive responses are initiated and IL-2 is being produced, it seems likely that the "sensing" of IL-2 by T_{reg} consumes a significant amount of the totally available IL-2. In this scenario, the mere presence of T_{reg} could reduce IL-2 availability and limit T_{eff} responses, without a need for active regulation (cytokine-sink model). Indeed, the presence of T_{reg} leads to substantial competition for IL-2, resulting in impaired proliferation of T_{eff} cells *in vitro*. Competition was further demonstrated *in vivo*, with a primary effect on the survival of T_{eff} and not on their proliferation (Pandiyan et al., 2007; Kastenmuller et al., 2011). T_{reg} also control IL-2 production (Thornton and Shevach, 1998), either by directly acting on T-cells (Bodor et al., 2007; Vaeth et al., 2011) or indirectly, through DC (Onishi et al., 2008). The latter concept is based on *in vitro* evidence of a positive correlation between IL-2 production by Teff, the strength of ConA stimulation, and the amount of CD28 expression (Shahinian et al., 1993). T_{reg} express significant levels of surface CTLA-4 on their surface and this molecule can directly block co-stimulatory molecules and CD28-CD80/86-interactions, or, via trans endocytosis, modulate the amount of CD80/86 that is displayed by DC (Wing et al., 2008; Qureshi et al., 2011). Importantly, the amount of CTLA-4 expressed on T_{reg} is again regulated by IL-2 signals. Consequently, T_{reg} control the level of co-stimulation through CD80/CD86 surface expression not only during steady-state (Schildknecht et al., 2010), but, importantly, also during highly inflammatory processes such as viral infection (Kastenmuller et al., 2011).

Therefore, DC appear to constitute a platform on which both stimulation and regulation of conventional T-cells is executed, with IL-2 being a central mediator that activates both T_{eff} and T_{reg}. Feed-back loops involving the constitutive high levels of CD25 on T_{regs} and the IL-2-promoted upregulation of CTLA-4 on these cells operate in concert to restrict IL-2 availability to activated T_{eff}, controlling their expansion, differentiation, and survival (**Figure 1**).

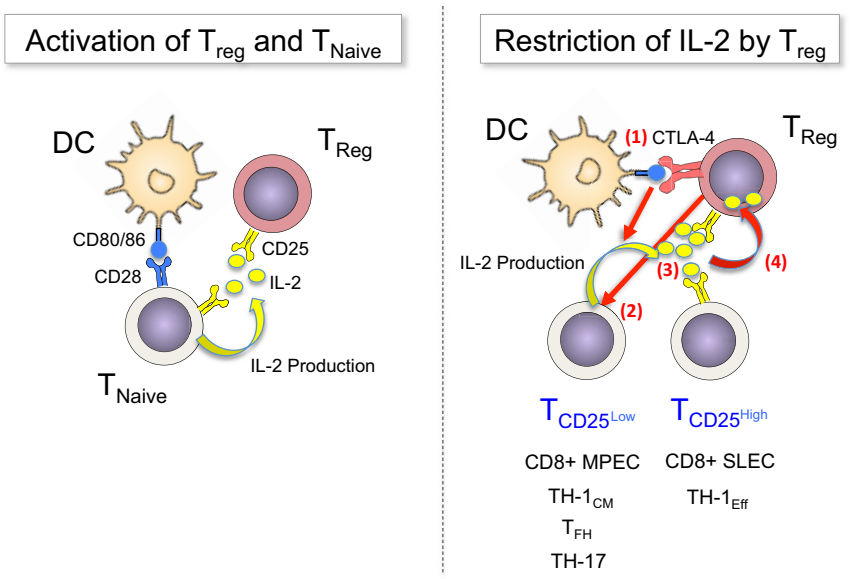

FIGURE 1 | T_{reg} sense IL-2 early during immune responses and restrict cytokine availability to control T-cell fates. *Left panel:* TCR signals (not depicted) and co-stimulatory signals via CD80/86 activate naïve T-cells to upregulate CD25 and produce IL-2. T_{reg} have constitutive expression of CD25, which enables them to "sense" early (d0) production of IL-2 and become activated. *Right panel:* Once activated T-cells divide, they become heterogeneous for CD25 expression (d2–4): a fraction of cells downregulates CD25, avoiding further IL-2 signals – and potentially regulation through T_{reg} – in order to differentiate into long-lived memory cells (MPEC or TH-1_{CM}), follicular helper cells or TH-17 cells, or they maintain CD25 expression for prolonged IL-2 signals to undergo terminal effector differentiation. T_{reg} can balance this differentiation processes by regulating the availability of IL-2 via multiple mechanisms, among them (1) the blocking and reduction of CD80/86 via CTLA-4, (2) the direct inhibition of IL-2 production by T-cells, (3) the competition for IL-2, and (4) the consumption and degradation of IL-2.

T_{REG} SELECTIVELY REGULATE EFFECTOR T-CELL RESPONSES BUT PRESERVE MEMORY DEVELOPMENT

Recently, the effects of IL-2 during acute infection have been further refined through analysis of the role of this cytokine in the various stages of CD8+ T-cell differentiation. After their initial activation and induction of CD25 expression, a subpopulation of CD8+ T-cells decrease CD25 levels and become unresponsive to further IL-2 signals, and, at the same time, upregulate the IL-7Rα-chain and develop into long-lived memory cells. This is in contrast to T-cells that are exposed to IL-2 for a prolonged period and maintain CD25 expression, undergo enhanced expansion, but differentiate into short-lived effector cells (SLEC) that are prone to apoptosis and severe population contraction after the peak of the response (Kalia et al., 2010; Obar et al., 2010; Pipkin et al., 2010). The recent development of genetic models allowing the specific depletion of $Foxp3^+$ T_{reg} (Kim et al., 2007; Lahl et al., 2007) without blocking IL-2/CD25 interactions (Murakami et al., 2002; Suvas et al., 2003; Toka et al., 2004; Heit et al., 2008) enabled asking whether T_{reg} differentially affect these CD8+ T-cell subpopulations. Indeed, manipulating the numbers of activated T_{reg} *in vivo* impacted CD25 expression on activated CD8+ T-cells, indicative of altered IL-2 availability. This resulted in specific changes in numbers of SLEC while leaving the memory CD8+ T-cell compartment largely unaltered (Kastenmuller et al., 2011; McNally et al., 2011). Interestingly, the "window of opportunity" for the regulation of CD8+ T-cell responses by T_{reg} overlapped with the time of CD25 expression on CD8+ T-cells: depletion of T_{reg} cells as late as d2–3 post infection enhanced antigen-specific T-cell numbers in a viral infection model, but depletion later than d5 (when CD8+ T-cells do not express CD25) failed to do so (Kastenmuller et al., 2011). In addition, enhanced expansion of SLEC in the absence of T_{reg} was abrogated through the neutralization of IL-2 (McNally et al., 2011). Therefore, by controlling the availability of IL-2, T_{reg} cells can act as rheostats that balance the differentiation and expansion of pathogen-specific CD8+ effector T-cells. Importantly, by selectively regulating SLEC over memory precursor T-cells, which rapidly seem to become independent of IL-2 signals (d2–3), T_{reg} can limit the extent of acute effector responses

without blunting the development of long-lived memory (Kastenmuller et al., 2011). However, once memory is formed and the host is re-challenged with a pathogen, T_{reg} can again control the expansion of secondary effector cells adapted to the extent and requirements of the current infection.

T_{REG} MEDIATED RESTRICTION OF IL-2 AS A GENERAL MECHANISM TO REGULATE FATE DECISIONS IN T-CELLS

Beyond CD8+ T-cells, IL-2 likely serves as a central element that allows T_{reg} to regulate population size and differentiation of T-cells in general. A series of recent studies have established the role of IL-2 in CD4+ T-cell differentiation into T_{H1}, T_{H2}, T_{H17}, and T_{FH} cells (Liao et al., 2011; Boyman and Sprent, 2012). As with CD8+ T-cells discussed above, CD4+ T-cells segregate into $CD25^{high}$ and $CD25^{low}$ cells within the first days of a response to an acute infection, and prolonged IL-2 signals in $CD25^{high}$ cells leads to terminal differentiation and Blimp1 and T-bet upregulation in CD4+ effector cells. In contrast, $CD25^{low}$ cells gave rise to long-lived $CXCR5^{high}CCR7^{high}$T-bet^{low} precursors of central memory cells, as well as $CXCR5^{high}Bcl6^{high}$ follicular T-helper cells (T_{FH}; Choi et al., 2011; Pepper et al., 2011). In line with this, T_{reg} limit the expansion of antiviral CD4+ during acute infection, yet do not influence the generation of neutralizing antibodies (Kastenmuller et al., 2011).

Therefore, by limiting excessive IL-2, T_{reg} might not only blunt T_{eff} response but also ensure the generation of T_{FH} cells and consequently the development of appropriate humoral immunity early during acute infection, as IL-2 signals negatively regulate T_{FH} differentiation (Ballesteros-Tato et al., 2012; Johnston et al., 2012). By restricting IL-2 during acute infection, T_{reg} might additionally enhance mucosal immunity and regeneration (potentially preventing superinfection) through promotion of T_{H17} generation, because production of this class of effector cells is inhibited by IL-2 (Chen et al., 2011; Pandiyan et al., 2011).

SUMMARY AND PERSPECTIVE

In summary, we propose a model in which IL-2 availability is a central factor that controls the magnitude and shapes the character of adaptive immune responses. T_{reg} control access of other T-cells to this crucial cytokine by limiting its production through interference with co-stimulatory molecule availability on DC, as well as by reducing its abundance through consumption. Importantly, this does not act to simply blunt the overall immune response but selectively impacts on T-cell fates that require larger amounts of IL-2. In contrast, other T-cell subpopulations, such as memory-precursors or T_{FH}, are not suppressed, allowing for the generation of cellular and humoral immunological memory to protect the host from future pathogen encounters (**Figure 1**). T_{reg} are therefore not merely immunosuppressive, they actively participate in guiding the differentiation and fate decisions of other T-cells by regulating the availability of IL-2 in SLO. In this regard, T_{reg} controlling IL-2 availability remind us of the three Moirai (the incarnation of destiny in greek mythology) who controlled the thread of life and thereby directed the fate of individuals.

In addition to this early regulation of effector responses through IL-2, T_{reg} can undergo functional specialization that parallels the differentiation of conventional CD4+ T-cells in terms of transcription factor usage and expression of chemokine receptors important for homing to peripheral sites (Chaudhry et al., 2009; Koch et al., 2009; Zheng et al., 2009; Chung et al., 2011; Linterman et al., 2011). This differentiation might facilitate T_{reg} control of fully differentiated effector cells in infected tissues, which is likely to involve mechanisms distinct from regulating or competing for IL-2 (Soper et al., 2007), such as the production of immunosuppressive cytokines (Rubtsov et al., 2008) or cytotoxic molecules (Cao et al., 2007; Loebbermann et al., 2012).

Based on the emerging picture of selective control of effector T-cell fates, we speculate that interfering with T_{reg} function will help to optimize short-term immunotherapeutic approaches, but might be less promising to increase the efficacy of prophylactic vaccines aiming at the induction of long-term memory through T- and B-cells.

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Competition for IL-2 between regulatory and effector T cells to chisel immune responses

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In this review we discuss how the competition for cytokines between different cells of the immune system can shape the system wide immune response. We focus on interleukin-2 (IL-2) secretion by activated effector T cells (T_{eff}) and on the competition for IL-2 consumption between T_{eff} and regulatory T cells (T_{reg}). We discuss the evidence for the mechanism in which the depletion of IL-2 by T_{reg} cells would be sufficient to suppress an autoimmune response, yet not strong enough to prevent an immune response. We present quantitative estimations and summarize our modeling effort to show that the tug-of-war between T_{reg} and T_{eff} cells for IL-2 molecules can be won by T_{reg} cells in the case of weak activation of T_{eff} leading to the suppression of the immune response. Or, for strongly activated T_{eff} cells, it can be won by T_{eff} cells bringing about the activation of the whole adaptive immune system. Finally, we discuss some recent applications attempting to achieve clinical effects through the modulation of IL-2 consumption by T_{reg} compartment.

Keywords: computational modeling, cytokine competition, IL-2, regulatory T cells, systems immunology

INTRODUCTION

Recent studies, specifically on the role of IL-2 in the regulation of immune responses, have highlighted how cytokine competition may be a critical determinant to arbitrate the balance between tolerance and response, and/or to channel the activation of lymphocytes toward specific differentiation paths.

Cytokines are ubiquitous in immunology as mediators of cell–cell communications. Most knock-out mouse models of cytokines (with the notable exception of IFNγ) display critical and often deadly pathologies. For example, IL-2 knock-out mice are riddled with systemic autoimmune disorders (Horak et al., 1995), explained by the abrogated development and maintenance of regulatory T cells in peripheral lymphoid organs. On the other hand, there exist few clinical protocols whereby perturbations of cytokine pathways lead to clinical therapies. This review aims at presenting the need of quantitatively understanding cytokine function as a basis for more targeted therapeutic manipulation. We will discuss how the balance between cytokine secretion and consumption by multiple cell types fine-tunes the immune response.

This review is organized in four parts. First, we review recent experimental work addressing the role of cytokine consumption. Second, we present basic quantitative facts that highlight the explosiveness of cytokine secretion as well as the importance of cytokine consumption for lymphocyte–lymphocyte communication. We focus on IL-2 secretion and uptake as the best modeled case of cytokine competition, and also because it is a "self-contained" regulatory system (secreted by T cells, consumed by T cells). Third we summarize recent theoretical studies that addressed the role of IL-2 competition as a mechanism of suppression by regulatory T cells. Finally, we discuss the biological relevance of these theoretical efforts toward better understanding immunological regulations.

THREE EXPERIMENTAL EVIDENCES AGAINST A ROLE FOR IL-2 DEPLETION AS A MECHANISM FOR T_{reg} SUPPRESSION CAN BE MITIGATED

The original identification of the CD4+CD25+ compartment as a key population to enforce peripheral tolerance (Sakaguchi et al., 1995) led to the conjecture that IL-2Rα played a critical role in T_{reg} function. Given the critical role of IL-2 for T cell survival and proliferation *in vitro*, many researchers originally conjectured that IL-2 depletion by T_{reg} cells would be a critical mechanism to enforce their suppressive capabilities. However, the autoimmunity observed in IL-2 knock-out mice drew into question an activating function for IL-2 for T cell immune responses *in vivo* (Kundig et al., 1993). A partial resolution of this perceived conundrum came from the observation that the development and maintenance of T_{reg} cells depends on IL-2, so that IL-2 was attributed an immunosuppressive – rather than an activating – function *in vivo*. However, careful studies that went beyond the constitutive IL-2 knock-out model have since demonstrated that the action of IL-2 on both CD4+CD25− and CD8+ T cells supports immune response in multiple ways (e.g., by sustaining different modes of proliferation of CD8+ T cells; Kundig et al., 1993; Williams et al., 2006; Cho et al., 2007), furthering the survival of CD4+ T cells (Dooms et al., 2004), and driving CD4 effector and memory cell differentiation

(Yamane et al., 2005; Chen et al., 2011; Pandiyan et al., 2011). Thus IL-2 serves dichotomous functions in the suppression and enhancement of adaptive immunity.

While these studies open the possibility of an immunosuppressive role for IL-2 consumption by T_{reg} cells, experiments at the turn of the millenium have further challenged this idea. For example, experiments with a knock-out genetic model for the IL-2Rβ chain (CD122) delivered negative results regarding the role of IL-2 depletion by T_{regs} as a mechanism of suppression of autoimmune response. Specifically, Malek et al. (2002) relied on a thymic-transgenic expression of wild-type IL-2Rβ to drive the development of CD4+CD25+ T cells in the thymus then abrogate expression of IL-2Rβ in the periphery. These mice, whose peripheral lymphoid tissues contained CD4+IL-2Rα+IL-2Rβ− T_{reg} cells, were devoid of autoimmune disorders, while mice from straight IL-2Rβ−/− models lacked CD4+CD25+ cells and suffered systematic autoimmune attacks, analogously with IL-2Rα−/− models. This experimental observation was further analyzed and interpreted in terms of functional suppressive capabilities among IL-2Rβ-deficient T_{reg} cells, challenging the role of IL-2 signaling for T_{reg} suppression. However, more recent work mitigated this conclusion with the observation that peripheral T_{reg} cells from these IL-2Rβ thymic-transgenic knock-out mice retain their capabilities, albeit diminished, to respond to IL-2 (Bayer et al., 2007). These experimental inconsistencies may be explained by the recycling and long-term stability of the IL-2Rβ receptor, even after abrogation of its expression in the periphery. Hence, the accurate peripheral tolerance and lack of autoimmune disorders in IL-2Rβ thymic-transgenic knock-out mice can no longer be interpreted as a complete rejection of IL-2 depletion as a necessary mechanism for T_{reg} suppression.

The discovery of FoxP3 as the transcription factor that identifies unequivocally the T_{reg} lineage clarified the field, by offering a proprietary marker for T_{reg} that distinguishes them from transiently expressing CD4+CD25+ effector T cells. Work by the Rudensky lab (Fontenot et al., 2005) clearly established the critical role of IL-2 signaling for T_{reg} development and maintenance. On the other hand, this study showed that FoxP3+CD4+ cells from Il2ra−/− mice were as suppressive as T_{reg} from Il2ra-sufficient mice, at least in the classical *in vitro* proliferation assay: this observation (among others) again led to the conclusion that IL-2 signaling is dispensable for suppression. On the other hand, IL-2 signaling for Il2ra−/− FoxP3+ cells was not quantified and it is possible that compensatory mechanisms – e.g., upregulation of beta and gamma chains of the IL-2R receptors (Li et al., 2001) – would enable these IL-2Rα deficient cells to maintain their ability to respond and deplete IL-2. In particular, IL-2Rα−/− T cells have been shown to respond to IL-2, albeit at higher concentrations (1 nM instead of the characteristics 10 pM): this could explain why IL-2Rα−/− mice (that have FoxP3+ peripheral cells but at lower frequency than IL-2Rα sufficient mice) still suffer from systemic autoimmune disorders (a hallmark of defective suppression by T_{reg}) but with less intensity than IL-2−/− mice (these mice are completely devoid of FoxP3+ cells).

A third line of experiments has previously been used to reject cytokine depletion as a mechanism for T_{reg} suppression and have led to the dogma that cell–cell contact between T_{reg} cells and T_{eff} cells is absolutely required for suppression. In the past, many groups (Shevach et al., 1998; Takahashi et al., 1998; Nakamura et al., 2001; Dieckmann et al., 2002; Xu et al., 2003) have used the classical transwell assay whereby T_{reg}-T_{eff} contacts are forbidden by a membrane separation and found that this abolishes suppression of T_{eff} proliferation by T_{reg} cells. Unfortunately, this setup can potentially generate false-negative results – as originally suggested in Scheffold et al. (2005), Pandiyan et al. (2007). Indeed, as pointed out by Shevach (2009) in a recent review, "*It should be emphasized that the failure to observe suppression when T_{reg} cells are separated from the responder cells by a membrane does not rule out the possibility that T_{reg} cells secrete an as yet uncharacterized cytokine that functions in a gradient fashion and requires proximity between suppressor and responder.*" In this regard, it is noteworthy that the transwell geometry typically separate T_{reg} and T_{eff} cells by 4 mm when one uses the same Corning Costar transwell within a 24-well plate as described in Thornton and Shevach (1998). This is a very large distance to be bridged by diffusion. For IL-2 (a globular protein of 17 kDa), the coefficient of diffusion D in solution is of the order of 1×10^{-6} cm^2/s (Weidemann et al., 2011). Thus the characteristic diffusion time τ_D across $d = 4$ mm is: $\tau_D = d^2/(2D) = 40000\,\text{s} = 11\,\text{h}$. Hence, the physical separation imposed by the transwell geometry implies a large time-delay between the secretion of soluble molecules by T_{eff} cells and their potential sensing (and scavenging) by T_{reg} cells (this time-delay might in fact be even larger, $\tau_{mixing} > 20$ h, due to the low porosity of the transwell membrane). Under these conditions, paracrine and autocrine consumption of IL-2 by the T_{eff} population, rather than competitive take-up by the very distant T_{reg} cells will dominate (Scheffold et al., 2007; Busse et al., 2010; Feinerman et al., 2010). Thus abrogation of suppression in a T_{eff}-T_{reg} transwell setup (Takahashi et al., 1998; Thornton and Shevach, 1998) does not rule out cytokine competition as one of the mechanisms of T_{reg} action (Scheffold et al., 2007). All in all, there is consensus in the field of regulatory T cells, regarding IL-2, that this cytokine is critical for the development and maintenance of this subpopulation. On the other hand, the three main lines of evidence dismiss the functional significance of IL-2 signaling in terms of the T_{reg} cells' suppressive capacities may not be definitive.

FUNCTIONAL EVIDENCE FOR IL-2 COMPETITION AS ONE MECHANISM FOR T_{reg} SUPPRESSION

The renaissance for IL-2 depletion as a mechanism for T_{reg} suppression came with studies from the Scheffold and Stockinger groups (de la Rosa et al., 2004; Barthlott et al., 2005; Brandenburg et al., 2008). Both groups documented how IL-2 depletion or blockage phenocopied the effect of T_{reg} cells on antigen-activated T cells. Conversely, either the exogenous addition of IL-2 or the blocking IL-2 uptake by T_{reg} cells only – and not by T_{eff} cells – was sufficient to abrogate suppression *in vitro*. Hence, IL-2 was conjectured to be a limiting factor for T_{eff} cell expansion *in vitro*. These groups then demonstrated that T_{eff} cells do produce IL-2 (despite reduced transcription of the IL-2 gene). An early predictor of suppressed T_{eff} cell expansion in these experiments was the lack of strong IL-2Rα expression on T_{eff} cells, accompanied in a reciprocal manner by further upregulation of IL-2Rα on T_{reg} cells. This behavior is readily explained by competitive IL-2 consumption

through T_{reg} cells, as pSTAT5 drives IL-2Rα upregulation in both cell types. This reciprocal regulation of IL-2Rα in T_{eff} and T_{reg} cells has also been observed *in vivo* (Klein et al., 2003; Barthlott et al., 2005). Of note, IL-2 has been shown to prime T_{reg} cells for later expression of the immunosuppressive cytokine IL-10 *in vitro* and *in vivo* (Barthlott et al., 2005; Brandenburg et al., 2008).

These papers were followed by the comprehensive study by the Lenardo group (Pandiyan et al., 2007) that focused on the enhanced apoptosis among activated effector cells, when common-gamma chain (γ_c) cytokines are missing because of their depletion by T_{reg} cells. All the hallmarks of cytokine deprivation-induced apoptosis (loss of phosphorylation of AKT, phosphorylation of BAD, membrane blebbing, resistance to death in Bim−/− mutants) were observed in the suppression assay *in vitro*. Moreover, Pandiyan et al. (2007) reported the measurements of reduced IL-2 concentration in supernatants of T_{eff}-T_{reg} cocultures compared to T_{eff}-only cultures: this was assigned to IL-2 consumption by T_{reg} cells rather than to reduced IL-2 production by T_{eff} cells. The measured IL-2 concentrations (around 1 unit/ml, i.e., 10 pM) were exactly in the range where maximal functional impact would be expected (see following section for details). Finally, a model of inflammatory bowel disease (IBD) was used as an *in vivo* assay of T_{reg} function: upon adoptive co-transfer of CD4+CD25+ T cells in SCID mouse, the onset of IBD was abrogated and colitogenic T_{eff} cells were shown to undergo apoptosis. Vice versa, when mice were not injected with CD4+CD25+ T cells, adoptively transferred CD45 T cells would proliferate and trigger IBD. Hence, Pandiyan et al. (2007) made a convincing case that depletion of IL-2 by T_{reg} cells constitute a critical mechanism to account for T_{reg} suppression.

QUANTITATIVE ASPECTS OF CYTOKINE ACCUMULATION AND CONSUMPTION

Addressing the role of cytokine depletion in enforcing suppression by T_{reg} cells depends on the quantitative understanding of the dynamics of cytokine accumulation and consumption in the extracellular medium of lymphoid organs. Of note, most cytokines are functional in concentration ranges (below 100 pM) that are unusual for most biological systems. Indeed, most ligand-receptor interactions, most hormones and growth factors operate in 10 nM to 10 μM range. Hence, there are specific challenges of the biophysics of cell–cell communication in the pM range that we need to address. In this section, we summarize the numbers related to IL-2 secretion and uptake, and estimate the kinetics of IL-2 accumulation in a lymph node. We note that the experimental uncertainties for kinetic rates and receptor numbers are rather large, so the correct parameter values might be within a factor of 2–3 from the stated value. In addition the receptor numbers and secretion rates are broadly distributed within the population and depend on the experimental protocols of measurement. Accordingly, we will keep our estimations simple, favoring clarity while aiming to stay within an order of magnitude of the actual parameter values.

First, we will briefly describe the kinetic steps involved in IL-2 signaling and consumption by T cells following the model presented in Feinerman et al. (2010). In general, IL-2 consumption by T cells proceeds in three steps (Feinerman et al., 2010):

1) Free IL-2 molecules reversibly bind α chains (IL-2Rα) of IL-2 receptors with characteristic on- and off-rates of $k_{weak}(+) = 1.4\ 10^7/(M^*s)$, $k_{weak}(-) = 0.4/s$.

   $$\text{IL-2} + \text{IL-2R}\alpha \leftrightarrow \text{IL-2}^*\text{IL-2R}\alpha$$

2) IL-2*IL-2Rα locks into a tight complex with available IL-2Rβ and γ_c chains of the IL-2 receptor with characteristic on- and off-rates of $k_2(+) = 3 \times 10^{-4}/s$, $k_2(-) = 2.3 \times 10^{-4}/s$, forming a complete IL-2*IL-2R receptor.

   $$\text{IL-2}^*\text{IL-2R}\alpha + \text{IL-2R}\beta^*\gamma_c \leftrightarrow \text{IL-2}^*\text{IL-R}$$

   The assembly of the tetrameric cytokine/receptor complex triggers the phosphorylation of the transcription factor STAT5 into pSTAT5. pSTAT5 molecules dimerize and enter the cell nucleus where they regulate a variety of genes, among them many genes associated with cell survival and proliferation. Importantly within the context of IL-2 communication, pSTAT5 upregulates the expression of IL-2Rα and downregulates the secretion of IL-2.
3) IL-2*IL-R complex is internalized by the cells with the rate of $k_{endocytosis} = 1.1 \times 10^{-3}/s = (15\ min)^{-1}$ (Duprez and Dautry-Varsat, 1986; Duprez et al., 1988, 1991; Hemar et al., 1995). We assume further that upon internalization the IL-2R receptor chains return to the cell membrane. This constitutes a simple way to model the conditions at the cell membrane as quasi-stationary. In experiments, the quasi-stationary conditions are supported by the fact that cytokine consumption dynamics happens at the time scales of several minutes, while the numbers of receptor chains remain stable over the course of several hours.

For the kinetic rates and numbers of receptors chains typical for T cells, the two-step model for IL-2 binding and uptake gives the number N_R of assembled IL-2*IL-R complexes per cells as:

$$N_R \approx \frac{N_{\beta\gamma}}{1 + \frac{K}{N_\alpha [IL-2]}} \tag{1}$$

and the IL-2 consumption rate by the cell of

$$J_{cons} = k_{endocytosis} N_R \approx \frac{k_{endocytosis} N_{\beta\gamma}}{1 + \frac{K}{N_\alpha [IL-2]}} \tag{2}$$

where $K = \frac{(k_{2(-)} + k_{endocytosis}) k_{weak(-)}}{k_{weak(+)} k_{2(+)}}$ and N_α, $N_{\beta\gamma}$ are the numbers of IL-2Rα and of IL-2Rβ*γ_c complexes per cell respectively.

The first main consequence of this model is that the EC50 for IL-2 signaling (defined as the concentration of IL-2 that yields 50% of the pSTAT5 response) is inversely proportional to the number of IL-2Rα chains per cell. In particular, for a moderately activated T cell with $N_\alpha \approx 10^4$, EC50 ∼10 pM, while for a strongly activated T cell with $N_\alpha \approx 10^5$, EC50 ∼1 pM. The second consequence of the model is that at high IL-2 concentrations ([IL-2] ≫ EC50), IL-2 signaling and consumption reach saturation and are limited by the number of IL-2Rβ*γ_c complexes that has been estimated

to be ∼300 in the naïve T cell and rises to ∼1000 upon activation (Feinerman et al., 2010). In general, this number appears to be limited by the number of available γ_c chains: although there typically ∼5000–8000 of them per cell, they are shared between many types of different cytokine receptors: the best estimates for the number of available γ_c came from fitting IL-2 consumption by T_{reg} cells (Feinerman et al., 2010). Notice that the consumption rate being rather low $k_{endocytosis} = 1.1 \times 10^{-3}$/s, a strongly activated cell exposed to saturating concentrations of IL-2 would be able to consume at most one molecule of IL-2/s.

IL-2 secretion starts only upon activation of naïve T cells with foreign antigens. Within hours of activation through their TCR signaling pathways, effector T cells start secreting IL-2 at an average rate of ∼10 molecules/s (our unpublished data). As IL-2 is secreted in the extracellular medium, it diffuses away from the secreting cell and the IL-2 field around the cell is established with the maximal concentration near the cell with a characteristic decay length of about cell radius R. The IL-2 concentration near the cell c_0 is set by the balance between IL-2 secretion and its diffusion:

$$J_{sec} = 4\pi R^2 D \nabla c \approx 4\pi R^2 D \frac{c_0}{R}$$
$$c_0 \approx \frac{J_{sec}}{4\pi RD} \quad (3)$$

where D is the diffusion coefficient for IL-2. Its value in an aqueous buffer is $\sim 100\,\mu m^2$/s, but the diffusion coefficient within a lymph node is not known. Assuming that the viscosity of the extracellular matrix is similar to that of the cell cytoplasm (∼6 times higher than that of aqueous solution), we could estimate $D \sim 16\,\mu m^2$/s. Then using $R \approx 5\,\mu m$, we arrive at $c_0 \approx 20$ pM, which is larger than EC50 of even moderately activated T cells. Thus an activated secreting cell should be able to sense IL-2 it produces in an autocrine manner. However, at the onset of activation T cells lack IL-2Rα chain and would need concentrations of IL-2 larger than 1 nM to respond to it. The expression of IL-2Rα is initiated upon cell activation, and within 24–48 h the number of IL-2Rα reaches $\sim 10^4$–10^5 per cell lowering EC50 to ∼1–10 pM of IL-2 and allowing for cell sensing its own field of IL-2.

However, even a strongly activated cell would consume only a small fraction of secreted IL-2 in an autocrine matter. Indeed, as discussed above a strongly activated T cell can consume at most ∼1 IL-2 molecule/s while producing 10 molecules/s on average. The rest of the molecules would in principle accumulate within the lymph node and contribute to communication between different T cells and to coordination and strengthening of the system immune response. Assuming there are ∼200 activated T cells in a draining lymph node ∼48 h after immunization (there are typically few hundreds T cells in a body tuned to activate to a particular peptide, not all of them will reach the lymph node and those activated in the node will experience 1–2 divisions during that time) and taking the volume of a lymph node to be 1 μl, we estimate that the IL-2 concentration in the node would reach 48 h × (3600 s/h) × (10 molecules/s/cell) × (200 cells)/1 μl/$N_{Avogadro} \sim 600$ pM within 48 h of secretion. Such IL-2 concentration should indeed allow for strong signaling and cross communication between activated T cells. Specifically, even weakly activated cells (i.e., cells with lower levels of IL-2Rα would be able to phosphorylate STAT5: cell–cell communication through IL-2 would be rather unspecific and universal for all T cells in a lymphoid organ.

However, such accumulation of IL-2 is prevented by regulatory T cells. Unlike naïve T cells, T_{reg} cells express constitutively all of the chains of IL-2R even in homeostasis ($\sim 10^4$ of IL-2Rα and ∼300 of IL-2Rβ*γ_c) and can consume IL-2 from the onset of an immune response. Thus concentration of IL-2 will be established by the balance of overall secretion rate by T_{eff} cells and the consumption rate by T_{reg} cells. There are ∼5 10^6 T cells in a lymph node and ∼5% among them are T_{reg} cells, i.e., ∼2.5 10^5 cells. Comparing their consumption rate from Eq. 2 to the secretion rate of 200 activated T cells (i.e., 2000 molecules/s), we estimate that in first 24–48 h, IL-2 concentration does not exceed 0.15 pM. This level is too low for signaling to occur, so there will be almost no cross talk between different T_{eff} cells. Similarly, with the exception of cells in the immediate vicinity of a secreting T_{eff} cell, the majority of T_{reg} cells do not activate their pSTAT5 and downstream genes.

For an efficient cross talk to occur, the IL-2 concentration has to rise about tenfold to ∼1 pM, which is achieved when the number of activated T cells reaches ∼2000, which could take another 24–48 h. Well activated T_{eff} cells (with EC50 ∼1 pM) signal much more efficiently than the majority of T_{regs} (EC50 ∼10 pM) at these IL-2 concentrations. As described above, IL-2 signaling leads to upregulation of IL-2Rα expression and therefore to yet stronger signaling by T_{eff} cells allowing them from that stage onward to win over T_{regs} in the competition for IL-2. Only a minority of T_{reg} cells, those in the immediate vicinity of a secreting cell, have chances to keep up with T_{eff} cells. The number of such T_{reg} cells can be estimated from the probabilistic argument: since in a close packed situation each cell has ∼12 neighbors and 5% of those are T_{regs}, the number of strongly signaling T_{reg} cells should be ∼60% of activated T helper cells. However, as T_{eff} further proliferate the importance of IL-2 consumption by T_{regs} becomes negligible (Sojka et al., 2005, 2008). IL-2 secreted by the weakly activated (e.g., autoimmune) T_{eff} cells would be consumed mostly by T_{reg} cells; the T_{eff} cells would not be able to cross-communicate and their response would be suppressed. Yet T_{eff} cells strongly activated by the foreign proteins will be able to eventually overcome the suppression, and to exchange IL-2 cytokines and coordinate their response.

In addition to the competition between T_{eff} and T_{reg} cells, there exists competition for IL-2 within the T_{eff} system. As noted above, IL-2 signaling pathway leads to both upregulation of the expression of IL-2Rα and downregulation of IL-2 secretion. This means that T_{eff} cells that got more IL-2Rα signal stronger than others, which leads them to express more IL-2Rα and achieve yet stronger signaling capabilities. At the same time, they produce less and less of IL-2. Thus these two feedbacks are two mechanisms that bring about the split of T_{eff} population into two subpopulations: "consumers" and "producers." "Consumers" have shut down their IL-2 secretion, but have plenty of IL-2Rα that allows them to "steal" IL-2 from "producers." The "producers" are stuck in IL-2 secretion state since they have few IL-2Rα and so little chances of starting signaling and changing their state as their IL-2 is being stolen by the "consumers." This consideration implies that a continuum of IL-2Rα levels within the T_{eff} cell population may

yields to a digital split between IL-2 consumers and producers, with functional consequence in terms of differentiation (effector vs. memory phenotypes). Note that the heterogeneity between producers and consumers is not exclusively due to negative feedback in IL-2 secretion, as production is heterogeneous from the outset (Hollander et al., 1998; Podtschaske et al., 2007). This has been particularly well studied in the context of other cytokines (Mariani et al., 2010), but results to be published will confirm this endogenous variability in IL-2 production. Ultimately, competition for cytokines and heterogeneity in T cell signaling are critical contributors for T cell diversification during differentiation. For example, Catron et al. (2006) found that antigen-specific CD4+ cells arriving late into the draining lymph node divide less and their progeny is more likely to become memory cells than that of resident cells.

MODELING THE IL-2 TUG-OF-WAR BETWEEN T_{reg} AND T_{eff} CELLS: A SYSTEMS IMMUNOLOGY CHALLENGE

A conspicuous feature of IL-2 and other T cell cytokines is their action as both autocrine and paracrine messengers. Thus a key question in understanding the physiological effects of cytokines is: which cells actually receive the cytokine signal? Does the secreting cell consume most of it in an autocrine manner, or is it quickly distributed to neighboring cells – and if so, how far does the signal travel in space? Moreover, cytokine signaling is likely to be highly dynamic in time and space since cytokines regulate the expression of genes involved in the signaling – most prominently cytokine genes themselves as well as cytokine receptor genes.

To understand how the rates of cytokine production, diffusion, and cellular consumption as well as the feedback regulation of cytokine signaling shape the biological action of a cytokine, mathematical models provide an appropriate tool. Such models incorporate in a systematic way the kind of arguments made in the previous section, in order to simulate the dynamics of cytokine signaling for populations of secreting consuming and signaling cells. Two recent studies have employed a combination of experiments and mathematical modeling to dissect IL-2 signaling between antigen-activated, IL-2 secreting CD4 T cells and regulatory T cells (Busse et al., 2010; Feinerman et al., 2010). These studies provide mechanistic insight into how a single cytokine can serve as a messenger to two opposing T cell subsets.

Our two theoretical models describe the binding of IL-2 to IL-2 receptors, downstream signal transduction via the Stat5 pathway and activation of IL-2Rα gene expression (**Figure 1A**). Feinerman et al. (2010) provide a detailed description of signal transduction and its cell-to-cell heterogeneity and also include feedback repression of the IL-2 gene. Busse et al. (2010) study the spatial aspect of signaling in dense cell assemblies that is governed by the interplay between IL-2 diffusion and competitive uptake. Various experimental measurements were used by our two groups to parameterize these models and make testable predictions.

Both quantitative models consistently show that there exist three very different biological outcomes of IL-2 secretion by antigen-stimulated responder T cells, depending on two parameters: (1) the rate of IL-2 secretion and (2) the presence (or absence) of proximal T_{reg} cells. In the absence of T_{reg} cells, even weak antigenic stimulation of responder T cells and, correspondingly, low IL-2 secretion rates are sufficient to upregulate expression of IL-2Rα and trigger the IL-2/Stat5 pathway in the responder T cells. By contrast, in the presence of T_{reg} cells that constitutively express IL-2Rα, the T_{reg} cells will completely deprive responder T cells of their own IL-2 and prevent Stat5 activation. Thus, with a stimulation at low concentration of antigens, the outcome of IL-2 signaling is dichotomic: either the responder T cells receive the signal (no T_{reg} cells present) or it is completely consumed by the T_{reg} cells (**Figure 1B**). IL-2 deprivation by T_{reg} cells will therefore quench spurious activation events and sharpen the antigen activation threshold (**Figure 1C**). However, strongly antigen-stimulated responder T cells will produce sufficient IL-2 so that both the responder T cells and nearby T_{regs} will share the signal. As IL-2 primes T_{reg} cells for later IL-10 expression *in vivo* (Brandenburg et al., 2008), this sharing of IL-2 can both support an immune response and initiate a delayed negative feedback loop (Scheffold et al., 2005; Yamaguchi et al., 2011). Taken together, these findings provide a mechanistic underpinning for IL-2 competition as a suppressive mode of T_{reg} cells that depends on the strength of the antigen stimulus (de la Rosa et al., 2004; Barthlott et al., 2005; Pandiyan et al., 2007; McNally et al., 2011).

The positive feedback regulation of IL-2Rα chain expression by IL-2/Stat5 signaling plays a critical role in this regulatory network. In responder T cells, full IL-2Rα expression and formation of high-affinity IL-2 receptors requires, in addition to the antigen stimulus, phosphorylation of Stat5. Hence T_{reg} cells, which constitutively express IL-2Rα, will deprive of IL-2 weakly stimulated responder T cells (here, cells that fail to upregulate IL-2Rα sufficiently) and keep them from expressing high-affinity IL-2 receptors, thus inflicting a "double hit" (Feinerman et al., 2010). However, if IL-2 is abundant (strongly stimulated) responder T cells upregulate their own IL-2Rα expression due to positive feedback. These activated cells do no longer suffer from IL-2 deprivation by T_{regs} because they have themselves become efficient sensors and consumers of the cytokine. Under conditions where IL-2Rα is limiting for the formation of high-affinity IL-2 receptors, the IL-2–IL-2Rα positive feedback can function as a digital switch that converts graded changes in the antigen stimulus into an all-or-nothing decision for cell proliferation at the single-cell level (Busse et al., 2010). Quantitation of the IL-2R subunit expression and resulting Stat5 phosphorylation in responder T cells shows that both IL-2Rα and IL-2Rβ levels control the responsiveness of a cell to IL-2. Moreover, cell-to-cell variability in the expression of both receptor subunits results in a broad distribution of IL-2 sensitivities in a cell population (Feinerman et al., 2010).

Interestingly, a recent systems-biology study of an unrelated cytokine pathway, Epo signaling in erythroid progenitors, has shown a key role for signal processing of cytokine consumption by rapid receptor turnover (Becker et al., 2010). However, what makes the IL-2–IL-2R system ideally suited for cytokine competition is the positive feedback regulation of receptor (IL-2Rα) expression in both responder T cells and T_{reg} cells. Through this self-amplification of IL-2 signaling and consumption, rather subtle initial differences in strength and timing of antigen stimulation can lead to clear-cut biological outcomes (Feinerman et al., 2010).

An important question that has already been introduced in the discussion of the transwell assay is how far a cytokine signal can

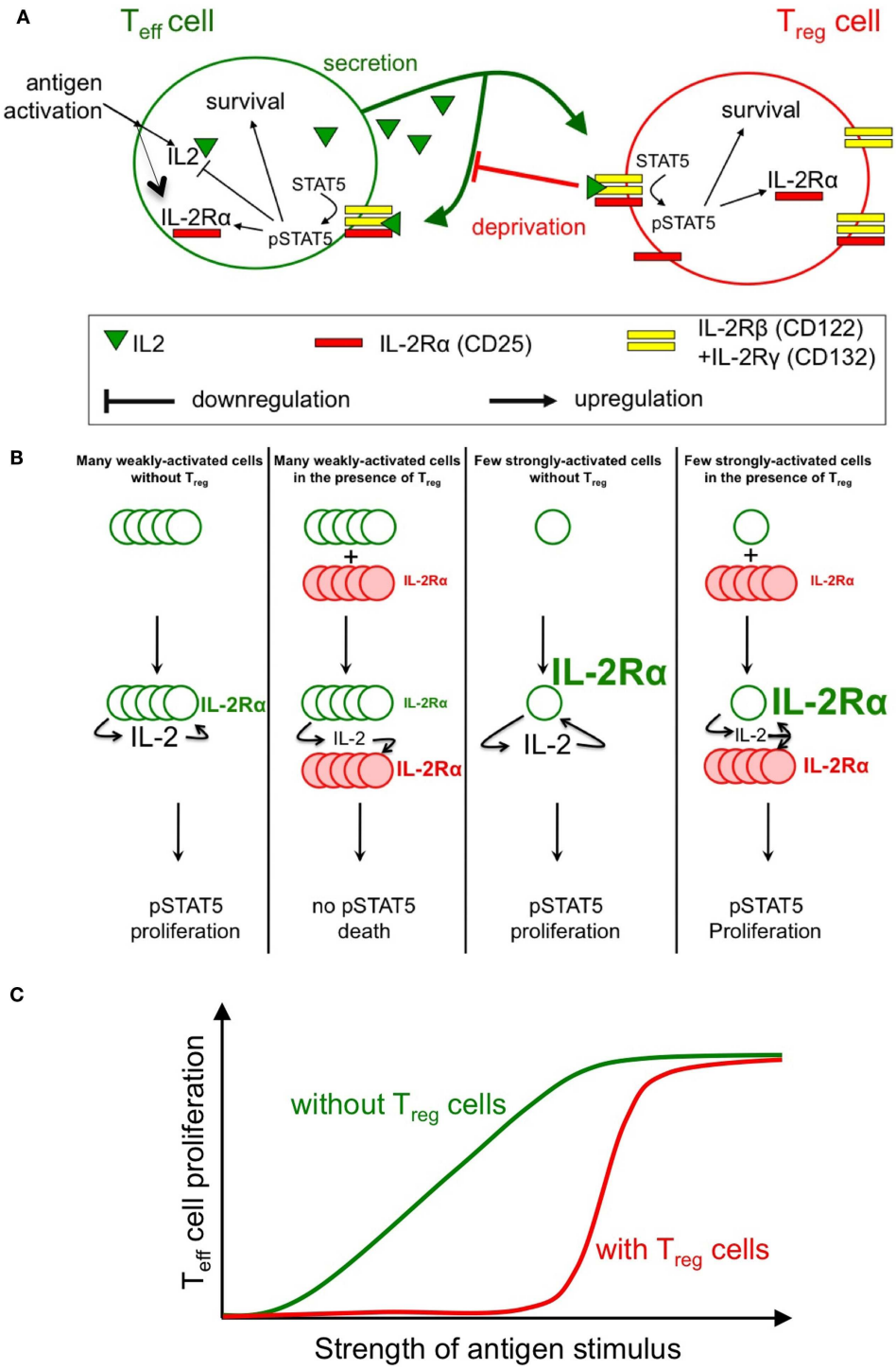

FIGURE 1 | Sketch of the feedback involved in the regulation of IL-2 response. Antigenic engagement of T_{eff} cells leads to their activation, with IL-2Rα upregulation and IL-2 production **(A)**. Studies by our groups (Busse et al., 2010; Feinerman et al., 2010) quantified the strength of feedback regulations on IL-2 signaling and secretion. In particular, the role of T_{reg} cells (whose constitutive expression of IL-2Rα allows early IL-2 depletion) in regulating STAT5 phosphorylation in T_{eff} cells was modeled *in silico*. Three main predictions from our models were validated experimentally: (1) T_{reg} can impact a double suppressive hits on T_{eff} cells by blocking IL-2Rα upregulation and IL-2 accumulation hence abrogating STAT5 phosphorylation **(B)**; (2) This suppression is highly dynamic and variable: in particular, T_{reg} cells can rely on the competition for IL-2 to block the proliferation of weakly activated T_{eff} cells, while allowing strongly activated T_{eff} cells to mount an immune response **(C)**; note how the presence of T_{reg} cells reduce the overall proliferation of T_{eff} cells but, as well, sharpen the dose responsive curve for proliferation vs. antigen strength; (3) Complex spatio-temporal coupling allow T_{reg} cells to modulate and regulate the extent of suppression in crowded environments (e.g., *in vivo*).

travel in space – Section "*Three Experimental Evidences Against a Role for IL-2 Depletion as a Mechanism for T_{reg} Suppression can be Mitigated.*" Clearly, diffusion over mm-range distances, with diffusion times of several hours (as in the transwell setup), is prohibitive for efficient communication. However, a biologically more relevant question is whether cytokine gradients occur on a smaller scale that could compartmentalize cell-to-cell signaling in lymphoid organs. For *in vitro* experiments, Feinerman et al. (2010) estimated that diffusion through the supernatant would not allow steep concentration gradients to develop (and moreover, convection, which is much faster than diffusion over larger distances, is also likely to occur in typical *in vitro* setups). By contrast, the explicit modeling of diffusion in rather dense cell assemblies (where extracellular space and total cell volume are of comparable magnitude) show that competitive IL-2 uptake under conditions of limited supply can cause strong concentration gradients (Busse et al., 2010). This is particularly evident for T_{reg} cells, which due to their high constitutive IL2-Rα expression function as potent sinks of the cytokine. As a consequence, T_{reg} cells can absorb the IL-2 secreted by localized, weakly stimulated responder T cells and thus prevent the paracrine spread of this signal to other responder T cells in the neighborhood. On the other hand, when IL-2 secretion is strong (owing to a high fraction of secreting responder T cells and/or high secretion rates), T_{reg} cells will become saturated and the IL-2 signal could pervade an entire lymph node [as shown previously for IL-4 (Perona-Wright et al., 2010)]. In summary, the modeling suggests that T_{reg} cells also control the spatial propagation of IL-2 signals in lymphoid organs.

By iterating between modeling and experiments, the studies by Busse et al. (2010) and Feinerman et al. (2010) have revealed an unexpected plasticity of the IL-2 cytokine network, where quantitative parameters (secretion rate, diffusion, and competitive uptake) shape the biological outcome. Further theoretical studies have modeled T cell population dynamics and IL-2 signaling (Burroughs et al., 2006), making experimentally testable predictions. As several other cytokines of the adaptive immunity share principle features of the IL-2 system, especially competitive uptake by different cell populations and feedback regulation of signaling, we expect that similar "behavioral" plasticity will be found also in other cytokine networks.

PERTURBING THE IL-2 TUG-OF-WAR TO MAXIMIZE IMMUNOTHERAPEUTIC IMPACT

At the same time as the issue of cytokine competition was being revisited, pre-clinical and clinical studies have put forward the possibility of applying IL-2 treatments to manipulate the T_{reg} compartment and impact on clinical outcomes (Murphy et al., 2012). Specifically, using antibody to cross-link IL-2 and to increase its lifetime *in vivo* (bare IL-2, because of its low molecular weight, gets filtered out of the system, mostly in the kidneys), researchers discovered that the T_{reg} compartment could be expanded. For example, Boyman and colleagues achieved proliferation of CD4+FoxP3+ lymphocytes in mice, increasing the frequency of T_{reg} cells by 10-fold, 3 days post-injection of the cytokine/antibody complex (Webster et al., 2009). The functional significance of this observation was immediately tested on a model of experimental autoimmune encephalomyelitis (EAE) whose induction could be abrogated after such robust T_{reg} cell expansion.

Similar observations (whereby the T_{reg} compartment is expanded with high levels of IL-2Rα and enhanced suppressive capacities) have been reported in other models. The Bluestone group applied a low-dose regiment of IL-2 to NOD mice, and obtained a strong delay or complete abrogation of diabetes (Tang et al., 2008): concomitantly, they reported that T_{reg} cells harvested from the pancreatic islet were more abundant (27% instead of 7% among CD4+ T cells) and expressed higher levels of IL-2Rα (20-fold higher). Similar results were obtained in a clinical setting, whereby low-dose regimen of IL-2 was demonstrated to be sufficient to delay the onset of Graft-vs.-Host-disease in allogeneic bone marrow transplant settings (Koreth et al., 2011). Again, these results should be analyzed quantitatively to test whether the subtle balance between immune response (autoimmune attack of the pancreatic islet or allogeneic activation of graft T cells) and immune tolerance (suppression of activation and proliferation) could result from the boosted ability of T_{reg} cells to compete for cytokines. A model for such immunotherapeutic intervention has already been proposed and makes further experimental testing critical. IL-2 regimen clearly upregulates IL-2Rα levels (with enhanced ability to bind and deplete IL-2), but it could also trigger other suppression mechanisms (e.g., secretion of IL-10 or upregulation of CTLA4).

Further quantitative modeling of this cytokine competition within clinical settings will thus be necessary to test and optimize cytokine competition, mostly to block autoimmune disorders using natural suppressive capabilities, but also to boost cytotoxic impact in cancer immunotherapies (2012). Although, this may prove difficult as direct and repeated probing of the tissue of interest (skin in GvHD, pancreatic islets in diabetes), we conjecture that mathematical models will become more and more critical to interpret functional changes as measured among accessible peripheral blood mononuclear cell (e.g., IL-2Rα upregulation in an expanded T_{reg} compartment) and extrapolate them to the tissue of relevance. Note that accurate measurements of *in vivo* concentrations of cytokines would go a long way toward resolving issues about cytokine communications in the immune system: this remains a challenging task given the low levels of free cytokine within tissues, but technical developments (ELISA miniaturization and the use of physiological reporters for cytokines) will solve this problem in the coming years. Ultimately, mouse pre-clinical models will be particularly useful to fine-tune the blood-to-tissue quantitative interpolation of immune responses.

CONCLUSION

We presented a review of recent efforts in Systems Immunology that aim at addressing the role of cytokine competition as one mechanism of immune suppression by T_{reg} cells. Modeling quantitatively how cytokine is secreted by effector T cells undergoing activation, and how much gets scavenged by regulatory T cells remains challenging because of the dynamic complexity of the system. However, computational models have already highlighted the spatio-temporal intricacies of IL-2 competition: depending on the speed of IL-2Rα upregulation among T_{eff} cells, there exists a time window when T_{reg} cells deplete the extracellular medium of

the secreted IL-2 and "snuff" this critical cytokine for differentiation. Spatially, the tight-packed space of lymphoid organs as well as the high density of polyclonal T_{reg} cells with high tonic level of IL-2Rα expression can limit the spatial extent at which T_{eff} cells can communicate through IL-2 sharing. This in turns, can act as a differential regulatory mechanisms to discriminate between activation of T_{eff} cells with low or high concentrations of antigens. Future work will need to extend the modeling framework introduced for IL-2 to other cytokines (e.g., IL-4, IL-10, TGFβ) as well as other costimulatory signals (e.g., B7/CTLA4). Further modeling effort will need to deal with cell proliferation and homeostasis, as proposed in a recent study (Burroughs et al., 2011; Almeida et al., 2012). These quantitative approaches will contribute greatly to assess the relevance of these varied mechanisms of T_{reg} cell suppression *in vitro* and *in vivo*. Beyond fundamental immunology, such quantitative insight may open new avenues of cytokine perturbation, to maximize immunotherapeutic impact in clinical settings.

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IL-2 expression in activated human memory FOXP3$^+$ cells critically depends on the cellular levels of FOXP3 as well as of four transcription factors of T cell activation

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The human CD4$^+$FOXP3$^+$ T cell population is heterogeneous and consists of various subpopulations which remain poorly defined. Anergy and suppression are two main functional characteristics of FOXP3$^+$Treg cells. We used the anergic behavior of FOXP3$^+$Treg cells for a better discrimination and characterization of such subpopulations. We compared IL-2-expressing with IL-2-non-expressing cells within the memory FOXP3$^+$ T cell population. In contrast to IL-2-non-expressing FOXP3$^+$ cells, IL-2-expressing FOXP3$^+$ cells exhibit intermediate characteristics of Treg and Th cells concerning the Treg cell markers CD25, GITR, and Helios. Besides lower levels of FOXP3, they also have higher levels of the transcription factors NFATc2, c-Fos, NF-κBp65, and c-Jun. An approach combining flow cytometric measurements with statistical interpretation for quantitative transcription factor analysis suggests that the physiological expression levels not only of FOXP3 but also of NFATc2, c-Jun, c-Fos, and NF-κBp65 are limiting for the decision whether IL-2 is expressed or not in activated peripheral human memory FOXP3$^+$ cells. These findings demonstrate that concomitant high levels of NFATc2, c-Jun, c-Fos, and NF-κBp65 lead in addition to potential IL-2 expression in those FOXP3$^+$ cells with low levels of FOXP3. We hypothesize that not only the level of FOXP3 expression but also the amounts of the four transcription factors studied represent determining factors for the anergic phenotype of FOXP3$^+$ Treg cells.

Keywords: cytokine expression, transcription factors, T cell activation, IL-2 expression, lymphocyte, flow cytometry, human Treg cells, memory Th cells

INTRODUCTION

FOXP3-expressing Treg cells are essential for the maintenance of immunological self-tolerance and immune homeostasis. CD4$^+$FOXP3$^+$ Treg cells are able to suppress the activation, proliferation, and effector functions of many different immune cells such as Th cells, cytotoxic T cells, NK cells, and APCs. These Treg cells play a central role in preventing autoimmune diseases and allergies as demonstrated in human diseases and animal models. Compared to murine FOXP3$^+$ T cells, human FOXP3$^+$ T cells are more heterogeneous concerning their phenotypical and functional properties resulting in various FOXP3$^+$ subpopulations (reviewed in Sakaguchi et al., 2010; Miyara and Sakaguchi, 2011). Furthermore, not all peripheral human CD4$^+$FOXP3$^+$ cells are suppressive (Baecher-Allan et al., 2001). This high complexity of human FOXP3$^+$ T cells causes some confusion in the field. Therefore, many efforts were undertaken to discriminate between suppressive and non-suppressive subpopulations among the *ex vivo* isolated CD4$^+$FOXP3$^+$ human T cells. Most promising so far is the division of FOXP3$^+$ cells into three subpopulations based on FOXP3 and CD45RA or CD45RO expression: (i) naïve Treg cells (CD45RA$^+$FOXP3^{low}); (ii) memory effector Treg cells (CD45RA$^-$FOXP3^{high}); and (iii) memory non-suppressive Th cells (CD45RA$^-$FOXP3^{low}; Miyara et al., 2009).

A detailed knowledge of human FOXP3$^+$ cell subsets is essential for a better understanding of the regulation of human FOXP3 expression, the study of abnormalities among the FOXP3$^+$ subpopulations in autoimmune diseases and allergies, and the selection and purification of the most promising subpopulation(s) of Treg cells for *in vitro* expansion and adoptive transfer (Fujii et al., 2011; Miyara and Sakaguchi, 2011).

In this study we aimed at a better characterization of human memory FOXP3-expressing T cell subpopulations (CD4$^+$CD45RO$^+$FOXP3$^+$) concerning anergy defined by the inability to produce proinflammatory cytokines upon TCR/CD28 stimulation. Anergy and suppression are two main functional characteristics of FOXP3$^+$ Treg cells (Gavin et al., 2007; Wan and Flavell, 2007; Williams and Rudensky, 2007). Therefore, we compared IL-2-expressing and IL-2-non-expressing FOXP3$^+$ memory T cells using Treg cell markers and the expression levels of five

transcription factors. We used measurement of cytokine expression, in particular IL-2, by T cells as a substitute for suppression assays, because it was hard to get enough pure cells after sorting of the subpopulations. Performing a combination of flow cytometric measurements with statistical analysis (Bendfeldt et al., 2012) we studied the impact of low, medium low, medium high, and high levels of the transcription factors FOXP3, NFATc2, c-Jun, c-Fos, or NF-κBp65 on the decision whether IL-2 is expressed or not in activated memory FOXP3$^+$ cells. Results of our data analysis suggest that low levels of FOXP3 expression in combination with high levels of NFATc2, c-Jun, c-Fos, or NF-κBp65 are critically required for IL-2 expression of FOXP3$^+$ T cells.

MATERIALS AND METHODS

HUMAN T CELL ISOLATION, STIMULATION, AND STAINING

Peripheral blood mononuclear cells from healthy volunteers were prepared using Ficoll PAQUE gradients from leukocyte concentrates obtained from the blood bank of the red cross. Positively selected CD4$^+$ cells were depleted of CD45RA$^+$ Th cells to a purity of >97% CD4$^+$CD45RO$^+$ memory Th cells (MACS Separation Reagents; Miltenyi Biotech). After resting over night cells were cultured in RPMI supplemented with 10% fetal calf serum and stimulated either with 10 ng/ml PMA (Sigma) and 1 μg/ml Ionomycin (Sigma) or with anti-CD3/CD28 antibody-coupled beads (25 μl per 1 × 106 cells; Dynal Beads, Invitrogen). Brefeldin A (Sigma) was added 30 min after stimulation to inhibit IL-2 secretion and to trap the expressed IL-2 within the cells. For cytometric analysis cells were stained for surface molecules with anti-CD69-APC (Miltenyi) and anti-CD25-PE-Cy7 antibodies (BD). For intracellular staining cells were fixed and permeabilized using the FOXP3 staining buffer set (eBioscience) and stained with antibodies against FOXP3-FITC (eBioscience), IL-2-APC (BD), IL-4-PE (BD), IL-17-PE (eBioscience), IFN-γ-Pacific orange (own in-house antibody), NFATc2-FITC (own antibody, BD), c-Fos-A488 (Santa Cruz Biotechnology), phospho-c-Jun-A488 (Santa Cruz Biotechnology), NF-κBp65-A488 (Santa Cruz Biotechnology), GITR-PE (Miltenyi), or Helios-PE (Biolegend). Subsequently, cells were analyzed using a LSR II Fortessa (BD). The MEK1/2 inhibitor U0126 (Biomol GmbH) was pre-incubated with Th cells for 20 min before stimulation and was used in concentrations of 0.01–250 μM.

ISOLATION OF CD25$^-$CD127$^+$ CELLS BY FACS

Sorted CD4$^+$CD45RO$^+$ memory Th cells were stained with antibodies against CD25-PE-Cy7 (BD) and CD127-PE (Beckman Coulter) and sorted using a FACS Aria (BD).

DATA ANALYSIS AND MATHEMATICAL MODELING

The free software R was used for data analysis. Sorted memory Th cells (CD4$^+$CD45RO$^+$) were gated for FOXP3 positive cells. Quantification was performed by transforming the fluorescence intensities of the cells into a linear scale using the asinh function. The range of transcription factor expression levels was divided either into four bins with equal cell numbers or into six bins with equidistant expression levels resulting in uneven cell number distribution. For each bin the number of IL-2-producing cells was calculated. Changes in mean expression levels were tested for significance using a paired Student *t*-test.

Mathematical modeling was done using Matlab. For each data set the parameters of the heuristic model were estimated by minimizing the distance from the simulated data to the experimental data (least square fitting). To compare models with different number of parameters we used Akaike's information criterion with correction for finite sample sizes (AICc), which takes into account the distance between model and data as well as considering the complexity of the model based on the number of parameters.

RESULTS

UP TO ONE-FIFTH OF PERIPHERAL HUMAN MEMORY FOXP3$^+$ CELLS ARE ABLE TO EXPRESS IL-2

Comparing transcription factor levels and cytokine expression within the memory CD4$^+$ T cell population of healthy donors (Bendfeldt et al., 2012) we discovered that there are always 8–20% IL-2-expressing FOXP3$^+$ Th cells, in spite of the fact that FOXP3$^+$ cells are widely considered to be incapable of producing IL-2 and other effector cytokines (Hori et al., 2003). The IL-2-expressing FOXP3$^+$ cells were induced by stimulation with PMA/ionomycin as well as with anti-CD3/CD28 antibodies (**Figure 1A**). On the one hand, we confirmed that this population did not emerge from stimulation-dependent up-regulation of FOXP3 in FOXP3$^-$ memory Th cells. First, the frequency of FOXP3$^+$ T cells did not change within the studied time frame of 5 h after stimulation (**Figure 1B**). Second, sorted memory Th cells (CD25$^-$CD127$^+$) that are depleted of Treg cells (CD25^{high}CD127^{low}), did not up-regulate FOXP3 expression after stimulation with PMA/ionomycin for 5 h (**Figure 1C**). On the other hand, staining of the activation marker CD69 demonstrated that all FOXP3$^+$ T cells were activated equally under the stimulation conditions employed (**Figure 1D**).

We already knew that approximately 35% (+5%) of IL-2-expressing activated memory Th cells are co-expressing IFN-γ (Podtschaske et al., 2007 and data not shown). Here, we observed similar frequencies of IFN-γ$^+$ cells (approximately 30%) within the IL-2$^+$FOXP3$^+$ cell population (data not shown). Among the whole entity of FOXP3$^+$ cells there were only low frequencies of cells expressing IFN-γ (between 5 and 10%) and IL-17 as well as IL-4 (both under 5%) after stimulation (**Figure 1E**).

IL-2-EXPRESSING FOXP3$^+$ CELLS EXPRESS A LOWER LEVEL OF PHENOTYPIC MARKERS OF TREG CELLS

To discover phenotypic differences between IL-2-non-expressing and IL-2-expressing CD4$^+$ FOXP3$^+$ T cells we analyzed the levels and frequencies of the Treg cell markers CD25, GITR, and Helios in both subpopulations and compared them both with memory Th cells (**Figure 2**). As expected IL-2$^-$ FOXP3$^+$ cells express CD25, GITR, and Helios (orange line), whereas all memory FOXP3$^-$ T cells have a lower level of CD25 and GITR and were negative for Helios (light and dark green lines). IL-2$^+$ FOXP3$^+$ showed an intermediate expression level for CD25 and GITR and were negative for Helios expression (red line) indicating a cellular phenotype between Treg and memory Th cells.

Specifically, to study whether Helios expression is dependent on the level of FOXP3 per cell we divided the FOXP3 fluorescence

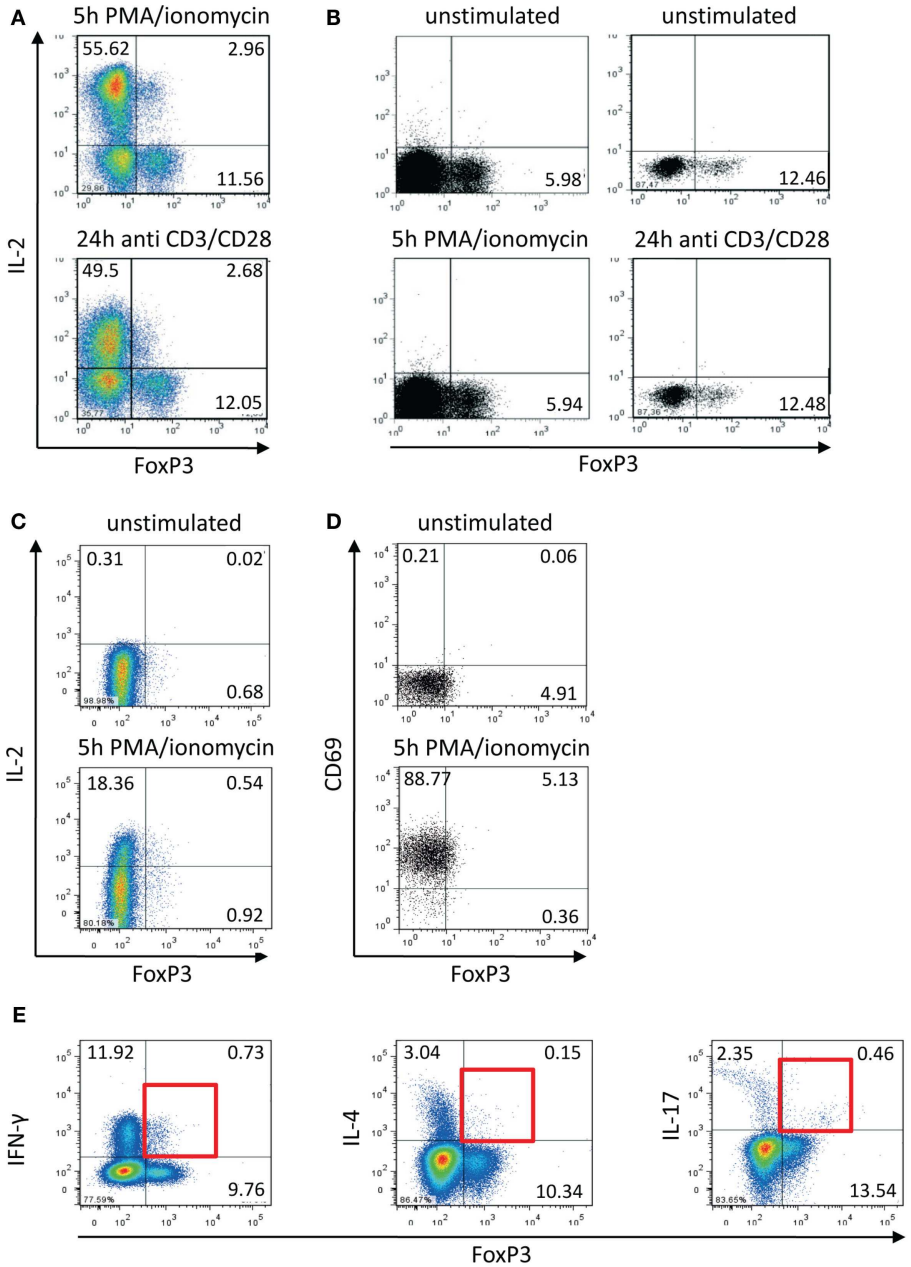

FIGURE 1 | Activation-induced expression of IL-2 within the FOXP3$^+$ memory T cell population. Human CD4$^+$CD45RO$^+$ T cells were isolated using magnetic cell-sorting. **(A,B)** Cells were stimulated with PMA/ionomycin and antibodies against CD3/CD28 for 5 and 24 h, respectively, and subsequently stained with fluorophore-coupled antibodies and analyzed by flow cytometry. **(C)** CD4$^+$CD45RO$^+$ T cells were stained with fluorophore-coupled antibodies against CD25 and CD127. Subsequently CD25$^-$CD127$^+$ cells were sorted by FACS, stimulated with PMA/ionomycin for 5 h, and subsequently fixed, stained, and analyzed by flow cytometry. **(D,E)** PMA/ionomycin-stimulated CD4$^+$CD45RO$^+$ T cells (5 h) were fixed, stained with fluorophore-coupled antibodies, and analyzed by flow cytometry. Data are representative out of two **(C–E)** or more than three **(A,B)** independent experiments.

intensity (FI) of the IL-2$^-$FOXP3$^+$ subpopulation into quartiles. Each quartile contained the same number of cells (**Figure 2B**, left). Subsequently, the Helios expression in each quartile was plotted into a histogram (**Figure 2B**, right). The level of FOXP3 per cell showed a clear correlation with the number of Helios-expressing FOXP3$^+$ cells.

IL-2-EXPRESSING FOXP3$^+$ CELLS EXPRESS MORE NFATc2 AND AP-1 BUT LESS FOXP3 THAN IL-2-NON-EXPRESSING FOXP3$^+$ CELLS

Recently, we discovered that sorted human memory Treg cells (CD25^{high}CD127^{low}) cells express lower levels of NFATc2, c-Fos, NF-κBp65, and c-Jun than Treg-depleted memory Th cells (CD25^{low}CD127^{high}) by western blotting (Bendfeldt et al., 2012).

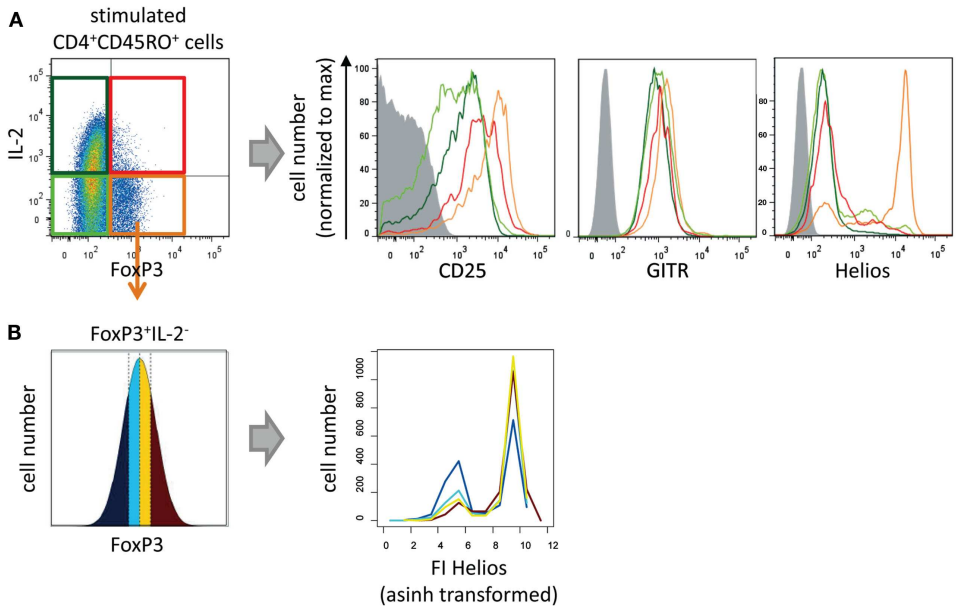

FIGURE 2 | Phenotypical characterization of IL-2-expressing FOXP3+ cells. Sorted human CD4+CD45RO+ T cells were stimulated with PMA/ionomycin for 5 h and subsequently fixed, stained with fluorophore-coupled antibodies, and analyzed by flow cytometry. **(A)** Histograms show overlays of CD25, GITR, or Helios expression of gated IL-2−FOXP3− (light green), IL-2+FOXP3− (dark green), IL-2−FOXP3+ (orange), and IL-2+FOXP3+ cells (red). **(B)** Within the gated FOXP3+ IL2− cell population, the range of FOXP3 expression level per cell was divided into quartiles (with the same cell number). The effect of the FOXP3 expression levels on Helios expression is shown in histogram overlays. Results of one representative experiment are shown (**A,B**: $n = 2$).

These differences motivated us to examine whether the low transcription factor expression observed in Treg cells is also a general feature of IL-2-non-expressing, as compared with IL-2-expressing, FOXP3+ cells.

Established co-stainings for IL-2 and the respective transcription factors (Bendfeldt et al., 2012) enabled us to directly compare quantitative differences in the expression levels of NFATc2, c-Fos, NF-κBp65, p-c Jun, and c-Fos in IL-2+ and IL-2− FOXP3+ cells (**Figure 3**, upper part). Flow cytometric analysis of PMA/ionomycin-stimulated memory Th cells from five to seven different donors showed a higher expression of NFATc2 ($p = 0.0105$), c-Fos ($p = 0.0076$), and p-c-Jun ($p = 0.0197$) in IL-2-expressing than in IL-2-non-expressing FOXP3+ cells (**Figure 3**, lower part). No significant differences were observed in the expression of NF-κBp65 between IL-2+ and IL-2− FOXP3+ cells ($p = 0.9988$). In order to stain c-Jun, we had to use a phospho-specific antibody because co-staining of c-Jun together with IL-2 and FOXP3 was not possible so far. Interestingly, the expression of FOXP3 was much lower ($p = 0.0030$) in IL-2+ than in IL-2− FOXP3+ cells ($n = 6$; **Figure 3**). CD3/CD28-stimulated memory Th cells showed similar results (data not shown).

Several reports revealed defects in the proximal TCR-signaling cascade in FOXP3+ Th cells (Hickman et al., 2006; Carson and Ziegler, 2007). Recently, we could rule out that these defects have an impact on the activation of the main transcription factors at strong stimulation, and therefore that they play a decisive role for IL-2 expression in FOXP3+ T cells under these conditions (Bendfeldt et al., 2012). Using co-staining and flow cytometric measurement of nuclei we demonstrated that the activation of NFATc2, c-Fos, and NF-κBp65 is unaffected after stimulation of FOXP3+ cells, both with PMA/ionomycin and CD3/CD28 stimulation. Therefore, the repression of IL-2 expression in FOXP3+ cells is not due to impaired activation of NFATc2, c-Fos, c-Jun, and NF-κBp65.

LOW LEVELS OF FOXP3 AND HIGH LEVELS OF NFATc2, c-JUN, c-FOS, AND NF-κBP65 ARE IMPORTANT FOR IL-2 DECISION MAKING IN FOXP3+ CELLS

Testing the hypothesis that physiological levels of FOXP3 are limiting for IL-2 expression in primary Treg cells usually requires over-expression or knock-down of FOXP3. However, such manipulations of primary Th cells often lead to a different *status quo* not only of one transcription factor but also of the complete transcription factor network compared to unmodified *ex vivo* cells.

Therefore, we decided to circumvent these difficulties, using a novel approach combining flow cytometry data analysis with statistical evaluation of single cell data. Within the FOXP3+ cell population of sorted and activated memory Th cells (CD4+CD45RO+) of six healthy donors, we correlated the physiological level of five transcription factors with IL-2 expression per single cell. Specifically, for each transcription factor we divided the FI, as a measure of the protein level per cell, into quartiles. Each quartile contained the same number of cells (**Figure 4**, upper left). Subsequently, the IL-2 expression in each quartile was plotted into a histogram (**Figure 4**). The level of the four transcription factors NFATc2, c-Fos, c-Jun, and NF-κBp65 showed an impressive correlation with the number of IL-2-expressing FOXP3+ cells. This is in contrast to the observed data concerning memory FOXP3− cells where only c-Fos and to a lower amount NFATc2 but not c-Jun and NF-κBp65 limit IL-2 expression (Bendfeldt et al., 2012).

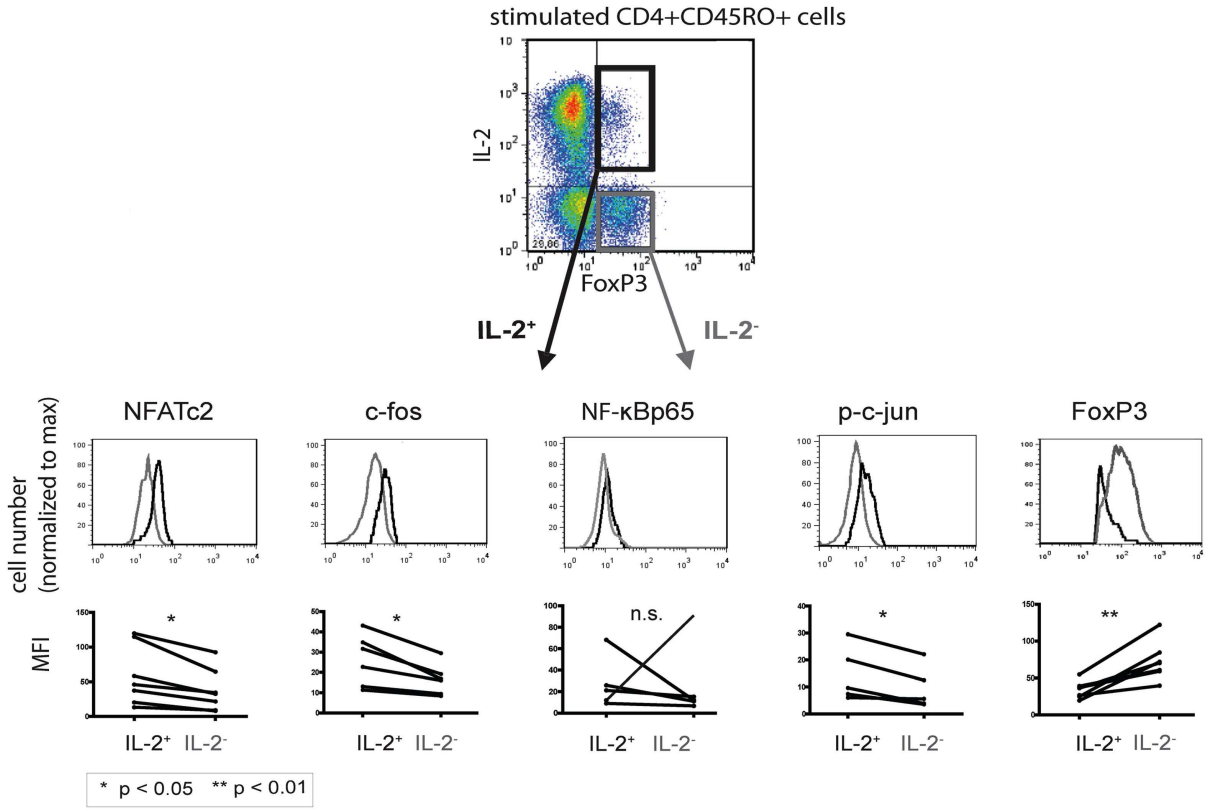

FIGURE 3 | Comparison of the transcription factor expression levels between IL-2-expressing and -non-expressing FOXP3+ cells. PMA/ionomycin-stimulated human CD4+CD45RO+ T cells were fixed, stained with fluorophore-coupled antibodies, and analyzed by flow cytometry. IL-2+ (black) and IL-2− (gray) FOXP3+ T cells were gated and analyzed for the respective transcription factors. Displayed are histogram overlays of the transcription factor expression in the two subpopulations of one representative donor ($n = 5$ or 6). Differences in the mean fluorescence intensity (MFI) of the transcription factors between IL-2-expressing and -non-expressing FOXP3+ Th cells in five or six different donors are shown as graphs. Significance was determined using a paired student *t*-test.

FOXP3 itself exhibits a clear negative correlation with the number of IL-2-producing FOXP3+ cells. As expected, quartiles with higher levels of FOXP3 (yellow and ruby color) have lower numbers of IL-2-expressing cells. In contrast, quartiles with very low (dark blue color) and relatively low (blue color) levels of FOXP3 per cell exhibit the highest numbers of IL-2 -expressing cells, namely 28.7 ± 7.9 and $12.4 \pm 8.0\%$, respectively (**Figure 4**, insets).

To verify that the level of the studied transcription factors NFATc2, c-Jun, c-Fos, and NF-κBp65 correlates with IL-2 expression in FOXP3+ cells, we divided the FI of each transcription factor into six bins and depicted the cell numbers as well as the frequencies of IL-2 expression in each bin (**Figure 5**, lower part). Obviously, the ratio of FOXP3 and the respective transcription factor is important for a high probability of IL-2 expression (arrows are depicted in the bins with more than 30% IL-2-expressing cells). Comparison of cell numbers of memory FOXP3+ with FOXP3− cells (**Figure 5**, upper part) revealed that the whole population of FOXP3+ cells shifted to a lower level of transcription factors, clearly visible for NFATc2, c-Fos, and NF-κBp65. Higher cell numbers are in bins (darker bins) with a lower level of these transcription factors. However, with this kind of studies we cannot answer the question whether the reduced expression of these transcription factors is a direct or indirect effect of FOXP3.

A minimal heuristic model estimates the balance of transcription factors regulating IL-2 production in FOXP3+ cells

To better understand the underlying mechanisms of IL-2 expression in human memory FOXP3+ cells we combined our experimental data with a minimal model of transcriptional regulation.

Most models assume that the rate of transcription of a certain gene is a function of the concentrations of the active transcription factors. The cytokine IL-2, however, is expressed in an all-or-none fashion (Podtschaske et al., 2007; Smith and Popmihajlov, 2008). Therefore, the level of IL-2 per cell remains constant after successful activation. Consequently, we modeled the number of IL-2-expressing cells instead of IL-2 expression level as a function of transcription factor concentration.

To quantify the experimental findings, we used a heuristic model with one activating variable x_1 and one inhibiting variable x_2

$$y = \frac{f \cdot x_1 + b}{1 + c_1 \cdot x_1 + c_2 \cdot x_2}, \quad (1)$$

where b and f correspond to the basal and the maximal response level, respectively. The parameters c_1 and c_2 reflect the effects of

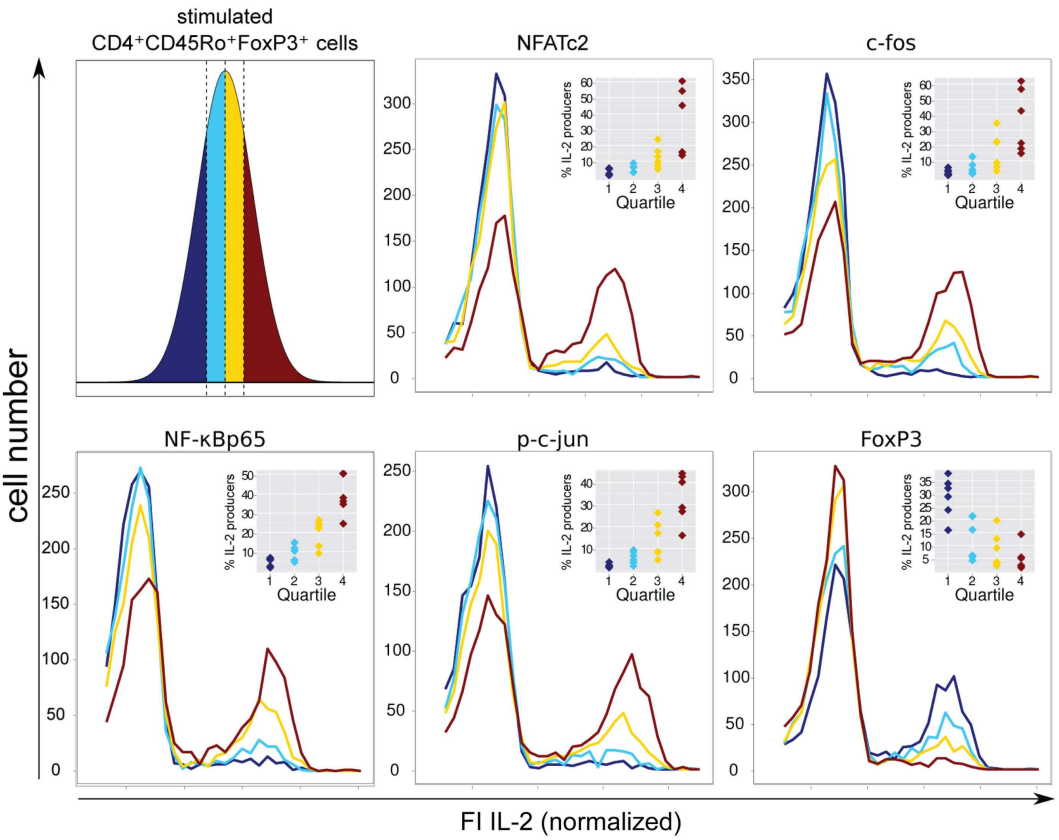

FIGURE 4 | Negative correlation of FOXP3 and positive correlation of NFATc2, c-Fos, NF-κBp65, and c-Jun expression level with IL-2 expression within the FOXP3+ population. Sorted human CD4+CD45RO+ T cells were stimulated with PMA/ionomycin for 5 h, fixed and stained with fluorophore-coupled antibodies against FOXP3, intracellular IL-2 and in parallel with one of the transcription factors NFATc2, c-Fos, NF-κBp65, or p-c-Jun. Data from the gated FOXP3+ cell population were transformed into a linear scale using the asinh function. Subsequently, the range of transcription factor expression level was divided into quartiles (with the same cell number) according to the level of expression of each transcription factor at single cell level (upper left). The effect of different transcription factor expression levels on IL-2 expression is shown in histogram overlays of one experiment. The inserts show the frequencies of IL-2-expressing FOXP3+ cells within the quartiles from six different donors.

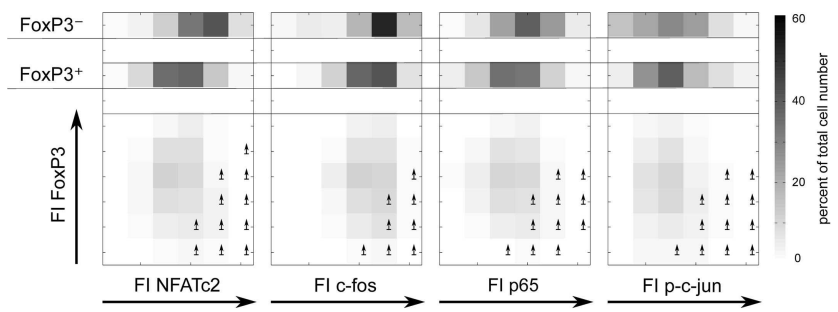

FIGURE 5 | The ratios of FOXP3 and NFATc2, c-Fos, NF-κBp65, or c-Jun expression level are important for a high probability of IL-2 expression. Sorting, stimulation, and staining of the cells were identical as described in **Figure 4**. The range of the fluorescence intensity (FI) of each transcription factor was divided into six bins. The cell numbers are depicted in a gray scale. Arrows label the bins that exhibit more than 30% of IL-2-expressing cells.

the activating and the inhibiting transcription factors. The activating variable x_1 represents the concentrations of the transcription factors c-Fos, NFATc2, NF-κBp65, and p-c-Jun and the inhibiting variable x_2 corresponds to the concentration of FOXP3. The dependent variable y reflects the fraction of cells that are able to express IL-2 and therefore should be in the range between 0 and 1. For simplicity, the parameters b (=0) and f (=1) were kept constant, whereas c_1 and c_2 were estimated by minimizing the distance

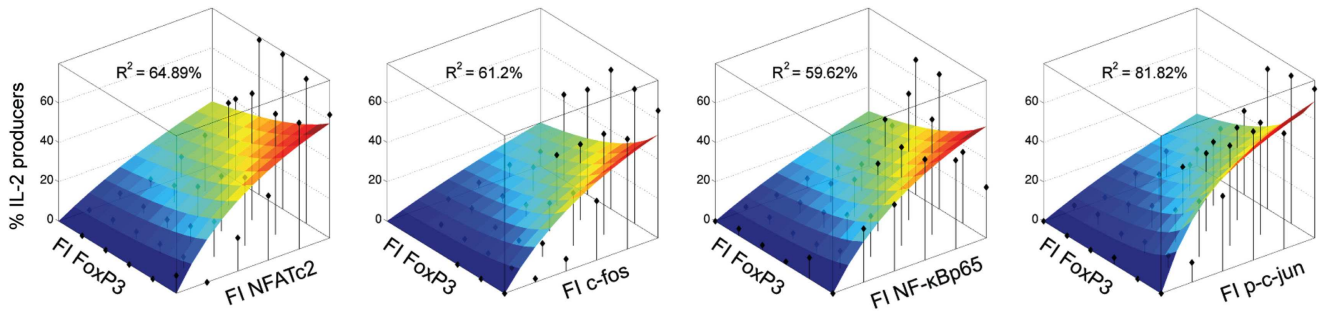

FIGURE 6 | Modeling confirms that NFATc2, c-Fos, c-Jun, and NF-κBp65 have an activating effect on the frequency of IL-2-expressing cells within the FOXP3+ population. Sorted human Treg cells stimulated with PMA/ionomycin for 5 h were analyzed in parallel for IL-2, FOXP3, and one activating transcription factor per cell by flow cytometry. The normalized expression levels of the transcription factors were divided into 2D-bins. The fraction of IL-2-expressing cells was calculated for each bin, which contained at least five cells (black diamonds). A heuristic model including an activating and an inhibiting term was fitted to the data. The landscape of the model for each data set (each TF from one donor) is superimposed on the data and the R^2 value is shown.

from the simulated data to the experimental data (least square fitting) for each combination of transcription factors available. The explained variance R^2 is a measure for the goodness of a fit and describes the proportion of variability (or variance) of a data set, which is accounted by a model. This simple model successfully explains up to 55% of the variance of the data within one donor, leaving the remaining variance open to experimental and technical noise. Relaxing the model constraints to allow f to be fitted as well, leads to similar explained variance, but the Akaike's value as a measure of goodness of fit favors the two-parameter model.

The modeling confirms that the studied transcription factors NFATc2, c-Fos, c-Jun, and NF-κBp65 have indeed an activating effect on the frequency of IL-2-expressing cells within the FoxP3$^+$ cell population. The model and corresponding data are shown in **Figure 6**. Interestingly, the model predicts that the contribution of all four activating transcription factors for IL-2 expression is similar, because the fitted parameter c_1 is comparable between them.

MANIPULATION OF C-FOS EXPRESSION CONFIRMS THE CAUSAL RELATIONSHIP BETWEEN THE LEVEL OF c-FOS AND THE PROBABILITY OF IL-2 PRODUCTION PER CELL IN THE FOXP3+ POPULATION

In order to confirm that the transcription factor c-Fos is limiting for IL-2 production, we manipulated the physiological c-Fos expression levels using U0126, a specific MEK1/2 small molecular inhibitor. Recently, we confirmed that U0126 does not inhibit the expression of NFATc2, c-Jun, and NF-κBp65 under the conditions used (Bendfeldt et al., 2012). U0126 inhibits *de novo* synthesis of c-Fos after T cell stimulation in a dose-dependent manner (**Figure 7**, upper part). Simultaneously, the frequency of IL-2 producers declines in both, memory FOXP3$^-$ and FOXP3$^+$ subpopulation of sorted human CD4$^+$ T cells (**Figure 7**, lower part).

DISCUSSION

A detailed knowledge of human FOXP3$^+$ cell subsets is essential for a better understanding of the regulation of human FOXP3 expression, the study of abnormalities among the FOXP3$^+$ subpopulations in autoimmune diseases and allergies, and the selection and purification of the most promising subpopulation(s) of Treg cells for *in vitro* expansion and adoptive transfer (Fujii et al., 2011; Miyara and Sakaguchi, 2011). Recent studies from different groups have demonstrated the heterogeneity of human FOXP3$^+$ cells and have begun to define and characterize possible subsets (Baecher-Allan et al., 2001; Ito et al., 2008; Thornton et al., 2010; Akimova et al., 2011; Bianchini et al., 2011; Schuler et al., 2011; Solstad et al., 2011; Duhen et al., 2012).

In our studies, we depicted the IL-2-expressing cell subset within the memory FOXP3$^+$ population for further characterization. Our procedure is based on the observation that cytokine production (in particular IL-2 and IFN-γ) and suppression are mutually exclusive functional programs within the FOXP3$^+$ T cell population (Gavin et al., 2007; Wan and Flavell, 2007; Williams and Rudensky, 2007). Therefore, we used measurement of anergic behavior as a substitute for suppression assays.

In agreement with others we found that up to 20% of all memory FOXP3$^+$ T cells (Miyara et al., 2009; Solstad et al., 2011) and up to 35% of memory FOXP3^{low} T cells express IL-2 after stimulation. The present comparison of IL-2-expressing vs. IL-2-non-expressing cell subsets of the memory FOXP3$^+$ population revealed the following main phenotypical and functional differences: IL-2-expressing memory FOXP3$^+$ cells have (i) lower levels of FOXP3, (ii) higher levels of NFATc2, c-Fos, c-Jun, and NF-κBp65, (iii) lower levels of CD25 and GITR, and (iv) almost no Helios expression. In contrast to memory Th cells, IL-2 expression is not only limited by endogenous cellular amounts of c-Fos and NFATc2 but in addition by c-Jun and NF-κBp65.

Due to the physiological importance and possible clinical applications of human Treg cells, there have been many studies examining discrimination and characterization of different subsets. So far, the most promising discrimination strategy of human FOXP3$^+$ Th cell subsets was proposed by Miyara et al. (2009). The CD45RA$^-$FOXP3^{low} subset in particular is still very heterogeneous concerning the phenotypical and functional properties. The memory FOXP3^{low} fraction reveals intermediate characteristics of memory Treg and memory Th cells.

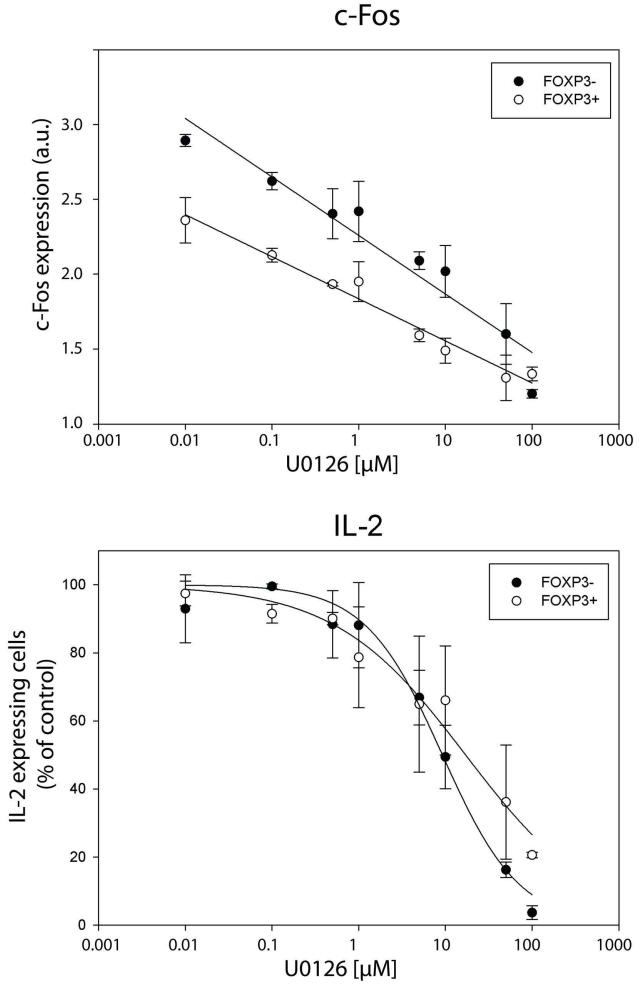

FIGURE 7 | Inhibition of c-Fos expression diminishes IL-2 expression per cell in both, FOXP3$^-$ and FOXP3$^+$ subpopulation. Sorted human CD4+ T cells were pre-incubated with different concentrations of the MEK1/2 inhibitor U0126 for 20 min and subsequently stimulated with PMA/ionomycin for 5 h. Gated memory Th cells (CD45RO$^+$ CD45RA$^-$) were divided into FOXP3$^-$ and FOXP3$^+$ cells for analyzing c-Fos and IL-2 expression in these two subpopulations. C-Fos expression and frequencies of IL-2-expressing cells are depicted at different concentrations of U0126. Shown are mean values and the standard deviation of two independent experiments with three replicates each. Regression lines serve for guiding the eye.

Why is it interesting to subdivide the memory FOXP3^{low} population into further subpopulations? On one hand, this population is very large, comprising approximately 40% of the FOXP3$^+$ cells (Miyara et al., 2009). On the other hand this population might contain both specific Th cells and specific Treg cells. The specific CD4$^+$ T cells with low FOXP3 expression could be recently activated Th cells (Gavin et al., 2006; Allan et al., 2007; Tran et al., 2007; Wang et al., 2007). The specific memory Treg cells with low FOXP3 expression could be exTreg (former Treg) cells, as suggested by recent publications on murine Th cells (Miyao et al., 2012). The activation-induced up-regulation of FOXP3 in *ex vivo* stimulated human Th cells seems to depend on the strength of stimulation (Gavin et al., 2006; Allan et al., 2007; Wang et al., 2007). ExTreg cells were recently identified in mice (Miyao et al., 2012). These cells represent a minor population of non-regulatory FOXP3$^+$ cells losing FOXP3 expression but retaining its memory. ExTreg cells are characterized by a lower expression of certain Treg cell markers, such as FOXP3, CD25, GITR, and Helios, and a higher frequency of IL-2- and IFN-γ-expressing cells than those cells that retain a high FOXP3 expression. Approximately 30% of these exTreg cells regain FOXP3 expression in a TCR-dependent manner. Whether there are human exTregs among the FOXP3^{low} cell population has to be studied.

However, irrespective of the suppressive capacities of "true" FOXP3$^+$Treg cells, FOXP3$^+$CD25$^+$ anergic T cells might play a role in regulating Th cell proliferation, and differentiation anyway by not producing IL-2 on the one side and consuming IL-2 on the other side.

FOXP3 is neither a specific marker for human Treg cells nor is it sufficient to induce stable Treg cells. Ectopic expression of both human FOXP3 isoforms in human CD4$^+$ T cells exhibited a profound suppression of IL-2 and IFN-γ production and a partial up-regulation of several Treg cell-associated markers. However, over-expression of FOXP3 did not lead to acquisition of significant suppressor activity *in vitro* (Allan et al., 2005). FOXP3 deletion (Gavin et al., 2007; Williams and Rudensky, 2007) or reduction (Wan and Flavell, 2007) in mice partially recovered the ability of IL-2 and IFN-γ production of Treg cells.

FOXP3 is directly acting on several genes as a transcriptional repressor or activator such as CD25 and CTLA-4, respectively. Our recent data (Bendfeldt et al., 2012) and the present data illustrated in **Figures 3** and **5** suggest that the studied transcription factors NFATc2, c-Fos, c-Jun, and NF-κBp65 are downregulated in the FOXP3$^+$ population. However, so far it is not known whether these are direct or indirect effects of FOXP3. Interestingly, within the memory FOXP3$^-$ population, only the endogenous level of c-Fos and, to a lesser extent, NFATc2 are limiting for IL-2 expression (Bendfeldt et al., 2012). The transcription factors c-Jun and NF-κBp65 occur in such physiological ranges that they do not reduce the probability of IL-2 expression in memory Th cells. This is in contrast to the memory FOXP3$^+$ cells described here. In these cells, not only are c-Fos and NFATc2 limiting for IL-2 expression but in addition c-Jun and NF-κBp65. We hypothesize that the concentration of c-Jun and NF-κBp65 shifted from saturation for IL-2 expression in memory FOXP3$^-$ cells into a region of limiting concentrations in memory FOXP3$^+$ cells. All our studies do not rule out the importance of other transcription factors in the regulation of IL-2 expression such as c-Rel or NFATc1 (Serfling et al., 1989; Randak et al., 1990; Briegel et al., 1991; Hentsch et al., 1992; Kontgen et al., 1995; Rao et al., 2003).

Computational models of transcriptional regulation combined with comprehensive experimental data are helpful in understanding the underlying molecular mechanisms of gene expression. In the present report, we modeled the relation between transcription factor concentration and the fraction of IL-2-expressing cells

based on a simple activation and inhibition form. Even with this basic approach we were able to describe the experimental data very well. More importantly, the estimated parameters are in a similar range for each transcription factor, suggesting that their regulatory strength is similar.

Altogether, our results indicate that human IL-2-expressing compared to IL-2-non-expressing FOXP3$^+$ cells exhibit a lower level of phenotypical properties of Treg cells, a lower concentration of FOXP3, and higher concentrations of NFATc2, c-Fos, c-Jun, and NF-κBp65. We suggest that these two FOXP3+ cell subsets behave also differently *in vitro* and *in vivo*. Therefore, it is important to further characterize these two subpopulations concerning their proliferative behavior and suppressive capacity as well as in the context of autoimmune diseases (Miyara and Sakaguchi, 2011), if it is possible to separate distinct phenotypic subpopulations within the FOXP3^{low} cell population in sufficient amounts.

AUTHORS CONTRIBUTION

Hanna Bendfeldt, Manuela Benary, and Tobias Scheel conducted the experiments and analyzed the data; Hanspeter Herzel and Ria Baumgrass supervised the project; Andreas Radbruch and Kerstin Steinbrink provided essential advice; Ria Baumgrass wrote the paper. All authors read and commented on the draft versions of the manuscript.

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