Boosting regulatory T cell function by CD4 stimulation enters the clinic

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Understanding tolerance mechanisms at the cellular and molecular level holds the promise to establish novel immune intervention therapies in patients with allergy or autoimmunity and to prevent transplant rejection. Administration of mAb against the CD4 molecule has been found to be exceptionally well suited for intentional tolerance induction in rodent and non-human primate models as well as in humanized mouse models. Recent evidence demonstrated that regulatory T cells (Treg) are directly activated by non-depleting CD4 ligands and suggests Treg activation as a central mechanism in anti-CD4-mediated tolerance induction. This review summarizes the current knowledge on the role of Treg in peripheral tolerance, addresses the putative mechanisms of Treg-mediated suppression and discusses the clinical potential of harnessing Treg suppressive activity through CD4 stimulation.
constitutively express the two co-inhibitory membrane-bound suppression is most likely of an indirect nature.

Next to the transfer of cAMP through gap junctions, production of extracellular adenosine has been suggested as an alternative mechanism in cAMP-dependent suppression by Treg (Deaglio, 2007). Extracellular nucleotides are anti-inflammatory mediators produced by a variety of cell types including Treg (Deaglio, 2007), Mandapathil et al. (2009) and Th17 cells (Chalmin et al., 2012). Physiologically, extracellular nucleotide production represents a protective mechanism in response to tissue injury (Fredholm, 2007). In Treg suppression adenosine formation through the ectonucleotides CD93 and CD39, expressed by murine Treg and a subpopulation of human Treg (Mandapatil, 2009), has been assumed to induce cAMP production in conventional T cells or DCs upon binding to the A2A receptor (Deaglio, 2007; Ernst, 2010). However, the role of adenosine as a major suppressive mechanism employed specifically by Treg is questionable. Blockade of cAMP generation in Treg coincides with increased susceptibility to Treg-mediated suppression (Klein et al., 2012). In addition, A2A receptor expression is detectable on T cells 4 days after stimulation (Deaglio et al., 2007) while T cells are susceptible to Treg suppression exclusively within the first 24 h after stimulation (Hagness et al., 2012). Finally, Blockage of ectonucleotidase activity only slightly abrogates suppression of human T cells by CD39 expressing Treg (Mandapatil et al., 2009). Thus, while nucleotides certainly affect numerous cellular functions – including de novo cAMP generation in Treg – their role in Treg suppression is most likely of an indirect nature.

Interestingly, cAMP up-regulation in Treg coincides with another cell contact-dependent mechanism of suppression: Treg constitutively express the two co-inhibitory membrane-bound molecules CTLA-4 and TIGIT (Read et al., 2000; Takahashi et al., 2000) which are believed to provide inhibitory signals. In mice CTLA-4 deficiency (Bachmann et al., 1999), CTLA-4 blockade (Takahashi et al., 2000), and CTLA-4-specific ablation of CD84 (Wing et al., 2008) resulted in spontaneous autoimmunity. Yet, CTLA-4 deficient Treg remain suppressive in vitro and in vivo (Tang et al., 2008; Read et al., 2010) suggesting additional mechanisms to be involved. Studies on human Treg in vitro revealed only a minor role of CTLA-4 in Treg suppression (Birebent et al., 2004) or firmly excluded CTLA-4 as a suppressive mechanism (Bascsher-Allen et al., 2001; Jonuleit et al., 2001; Leving et al., 2001). However, discrepancies regarding the importance of CTLA-4 in Treg suppression might in part be due to the use of different target cells. While the role of CTLA-4 in suppression of T cells remains uncertain, it is unequivocally required in the suppression of APC. Suppression of DCs by Treg via CTLA-4 has been shown to induce the downregulation of CD80 and CD86 (Cederbom et al., 2000) preventing effector T cell activation by the APC in vitro (Oderup et al., 2006) and in vivo (Wing et al., 2008). Notably, elevated cAMP levels in T cells have been shown to increase CTLA-4 expression (Vendetti et al., 2006) and cAMP and CTLA-4 expression are simultaneously up-regulated in Treg upon activation (Becker et al., 2009).

While a majority of studies firmly excluded soluble factors in Treg suppression in vitro, there is growing evidence that cytokines substantially add to the immune regulatory function of Treg in vivo. In particular, transforming growth factor-β (TGF-β) and IL-10 seem to be indispensable for sustained tolerance induction by Treg. A role for TGF-β in maintenance of peripheral tolerance was initially suggested by its importance in infectious tolerance (Chen et al., 2001) particularly its long-lasting production by CD4+ T cells from tolerant mice in long-term acceptance of allografts (Daley et al., 2007). However, in order to exert its biological functions, TGF-β needs to be converted from its latent (bound to latency associated peptide, LAP) into its active conformation by proteolytic cleavage (Khalil, 1999). Yet, there are multiple mechanisms of activating TGF-β from its latency (Lawrence, 2001; Annes et al., 2001) and it is unclear how TGF-β is activated in vivo.

Although repeatedly observed in disease models (Nakamura et al., 2001) a direct contribution of TGF-β in Treg suppression remains controversial because anti-TGF-β antibodies and soluble TGF-βRII failed to affect the suppressive function of Treg (Andersson et al., 2008). Recently, “glycoprotein A repetitions predominant” (GARP) expressed on the surface of Treg upon activation (Wang et al., 2008, 2009; D’Alise et al., 2011) has been shown to act as a receptor for the TGF-β/LAP complex (Stoakis et al., 2009). Reminiscent of infectious Treg suppression (Jonuleit et al., 2002; Stassen et al., 2004) latent TGF-β bound to GARP on the surface of activated Treg has been demonstrated to convert responder T cells into induced Treg (Andersson et al., 2008). Thus, apart from acting as a soluble modulator of immune cells, TGF-β supposedly helps Treg to execute their contact-dependent suppressive activity by binding to GARP (Battaglia and Roncarolo, 2009).

IL-10 has been unequivocally shown to form another important mediator in Treg suppression in vitro ( Kearley et al., 2003; Collison et al., 2007) particularly in suppression of pathogenic Th17 cells (Chaudhry et al., 2011; Huber et al., 2013). Correspondingly, TGF-β-specific ablation of IL-10 leads to inflammation (Rubtsov et al., 2008). In contrast to general Treg deficiency, however, Treg-specific IL-10 paucity leads to mucosal but not systemic autoimmunity, suggesting mucosal restriction of IL-10-mediated Treg tolerance induction. This view is supported by our previous observation that human Treg expressing gut-homing β7 integrin preferentially induce IL-10 production in converted secondary T helper suppressor cells (Stassen et al., 2004).

Due to their far-ranging tolerizing capability Treg have become key targets in the development of tolerance-inducing therapies (Wing and Sakaguchi, 2010). Like other T cells, Treg require activation for their function. Attempts to exploit Treg for therapeutic purposes therefore depend on Treg activation, either by antigen or polyclonal stimulation (Jordan et al., 2001). Current efforts to increase the frequency and potency of Treg in vivo include the use of cytokines (Tawara et al., 2010), antigen targeting to immature DCs (Mahade et al., 2003), and monoclonal antibodies (mAb) against surface molecules (Belghith et al., 2003). As a whole population Treg are biased toward recognition of self-antigens
(Hsieh et al., 2004), however, because antigenic specificities of Treg in diseases have not been elucidated, potential clinical applications have mainly focused on polyclonal Treg activation methods (Hoe witz et al., 2004).

**CORECEPTOR ENGAGEMENT AND PERIPHERAL TOLERANCE**

Tcell surface molecules that participate in T cell receptor-mediated stimulation have a significant influence on T cell function. mAb against coreceptors have been successfully shown to allow intentional tolerance induction in rodents and non-human primate models (Krieger et al., 1996). One particularly well-established regimen of tolerance induction is the administration of anti-CD4 mAb (Waldmann and Cobbold, 1998). Although the mechanisms underlying tolerization by anti-CD4 mAb are not yet fully understood, the activation of Treg has been recognized as the entering wedge to successful tolerance induction (Becker et al., 2009; Kendal et al., 2011; Martin et al., 2012).

CD4, a 55-kDa glycoprotein with four extracellular domains (Littman, 1987), recruits the protein kinase p56\(^{lck}\) (Rudd et al., 1988; Veillette et al., 1988) to the TCR complex (Heldorf et al., 2004; Kim et al., 2003; Naka et al., 2010) and strengthens the contact between T cells and APCs through its interaction with non-polymorphic regions of MHC class II molecules (Greerstein et al., 1984; Doyle and Strominger, 1987; Konig et al., 1992, 1995). CD4 molecules on T cell surface have been shown to preferentially form disulfide-linked dimers and tetramers (110 and 220 kDa; Li et al., 1996; Moldovan et al., 2002) and mutations disabling dimerization completely abrogate its coreceptor function (Vignali and Vignali, 1999). CD4 expression on mature T cells is uniform with the exception of polarized T helper 2 cells (Ish et al., 2003) and Treg (Brey et al., 2001) which both show decreased CD4 expression supposedly entailing altered proximal TCR signaling (Hannier et al., 2002; Ish et al., 2003; Tang et al., 2006).

Through its interaction with tyrosine kinase p56\(^{lck}\), CD4 engagement alone can induce TCR-independent signaling events in T cells (Zhou and Konig, 2003). Selective engagement of the CD4 coreceptor by certain mAb raises intracellular calcium and IL-2 production (Carrel et al., 1991), whereas other anti-CD4 mAb prime T helper cells to activation-dependent cell death triggered by subsequent TCR/CD3-mediated signals (Newell et al., 1995; Tamama et al., 1997). Comparing mAb against different CD4 epitopes, Baldari and colleagues suggested that the geneactivating and proapoptotic potential of different anti-CD4 mAb may be associated with different epitopes (Baldari et al., 1995; Di Somma et al., 1995; Milia et al., 1997). However, a similar range of divergent responses can be induced through a single CD4 epitope as demonstrated for the CD4-binding (Lasky et al., 1987) human immunodeficiency virus-1 (HIV-1) envelope protein gp120 (Liebler and Sites, 1994; Westendorp et al., 1995; Masci et al., 1999). It is therefore tempting to speculate that the functional outcome of CD4-stimulation mainly depend on the functional state of the T cell addressed rather than on a specific CD4 epitope. However, the functional state is believed to affect the formation of CD4 oligomers, which, in turn, regulate the activation of the CD4 cytoplasmic tail-associated tyrosine kinase p56\(^{lck}\), by trans-phosphorylation (Veillette et al., 1989).

Even before the role of the CD4 molecule in T cell activation had been fully recognized, three groups reported that short courses of anti-CD4 mAb application induce long-term tolerance to foreign proteins (Benjamin and Waldmann, 1986; Benjamin et al., 1986; Goronyu et al., 1986; Gustein et al., 1986). Subsequent studies revealed that anti-CD4-mediated tolerance induction was not based on T cell depletion but rather an activation of regulatory mechanisms (Benjamin et al., 1988; Carteron et al., 1988, 1989, 1990). Further, tolerance could not only be induced to foreign proteins but also to various transplanted alloantigens (Shizuru et al., 1987; Qin et al., 1989; Davies et al., 1996), demonstrating that the tolerizing potential of anti-CD4 mAb is not restricted to a particular type of antigen. Immunoregulatory mechanisms initially suggested to operate in anti-CD4 induced tolerance include a predisposition of developing T cells to selective deletion, or anergy in the thymus (Arima et al., 1997), immune deviation (Scilly et al., 1997), receptor blockade (Vehrsen et al., 2002; Harding et al., 2002) modulation of CD4 expression (Portoles et al., 1999); and transmission of negative signals (Chermule et al., 1999). However, none of these – not mutually exclusive – processes could reasonably explain the "infectious tolerance" phenomenon (Qin et al., 1993). Rather than being substantive, anti-CD4 induced tolerance relied on dominant immune suppression by T cells activated in presence of the antibody. In regard to the dominant suppressive T cell type in charge several functionally and phenotypically different anti-CD4 mAb-induced tolerogenic CD4\(^{+}\) T cell populations have been proposed (Bushell et al., 2003; Chen et al., 2003; Cobbold et al., 2004; Karim et al., 2005). However, whether these had been directly or indirectly induced by anti-CD4 treatment remained undefined at first. The impressive capacity of Treg and their ability to confer regulatory properties upon suppressed T cells (Jonuleit et al., 2002; Melsen et al., 2004; Andersson et al., 2008) in particular, strongly suggested a role of Treg in anti-CD4-mediated "infectious tolerance" induction. In support of this assumption administration of non-depleting anti-CD4 mAb into mice had been shown to result in pre-activation of Treg in vivo (Karim et al., 2005; Yang et al., 2007). Eventually, using B6. Fas(+/hCD2) mice to ablate Treg with an anti-hCD2 mAb Kendal et al. (2011) formally demonstrated that Treg are crucial for infectious tolerance induced by non-ablative anti-T cell mAb.

Motivated by the description of activated Treg in murine anti-CD4 tolerance models we previously analyzed the effect of anti-CD4 binding agents on human Treg. Comparing numerous anti-CD4 mAb we found that certain anti-CD4 mAb have the potential to induce the suppressive function of isolated human Treg in a supposedly T cell receptor-independent manner (Becker et al., 2007). In addition, we and others observed that the CD4-binding HIV-1 surface protein gp120 activates the suppressive function of Treg (Nilsson et al., 2004; Kinter et al., 2007) in vivo and in two humanized mouse models in vivo (Becker et al., 2009; Li and Clod, 2009) signifying that stimulation via the CD4 receptor represents an efficient Treg activating pathway with potential to induce immunological tolerance in humans. Difference between anti-CD4 mAb to trigger Treg suppressive activity could not be related to a particular CD4 epitope. However, comparing the Treg activating potential of different anti-CD4
mAb and CD4 binding virus envelopes we observed that one crucial event that separates Treg activating and non-activating CD4 ligands consists in up-regulation of the second messenger cAMP (Becker et al., 2009 and unpublished results). Moreover, the binding affinity of CD4 ligands seems to play a role as suggested by the fact that weak CD4 binding viral envelopes from HIV-2 (gp105) and SIV (gp130) did not activate human Treg in vitro and in vivo.

However, apart from these general observations the signaling events initiated by separate ligation of CD4 on Treg so far remain unexplored. In particular, it is unclear whether CD4 stimulation of Treg is truly independent of TCR signals, whether and how both pathways resemble or differ from another, and, most important, whether CD4-mediated signals are differently or similarly handled in Treg and conventional CD4\(^+\) T effector cells. The latter question is of particular interest since Treg are believed to maintain an activated phenotype through constant stimulation by self antigens, yet, require additional stimulation to become suppressive. Future insights into how TCR and CD4 signaling pathways drive the suppressive activity of Treg will undoubtedly help to understand Treg biology and discover alternative intervention points for functional manipulation of Treg suppressive activity.

As summarized in Figure 1 at least three different immune mechanisms can be distinguished that help to explain the tolerizing effect of CD4-specific agents: First, a general Treg-independent mechanism that consists in interference with proper CD4 coreceptor function resulting in induction of T cell anergy or T cell depletion (Figure 1A). This effect seems to depend either on CD4/MHC class II binding blockade or additional TCR-independent signaling. Second, by modulating antigenic stimulation, individual CD4 mAb induce differentiation of naive T cells into adaptive Tregs (Oliveira et al., 2008), which are suggested to control pathogenic effectors through TGF-\(\beta\) (Oliveira et al., 2011) or IL-10 release (Figure 1B). Finally, and crucially important for tolerance induction, CD4-specific mAb activate the suppressive function of Treg (Becker et al., 2007; Kendal et al., 2011), which, upon activation, exert control on pathogenic T cells
by direct and linked suppression (Figure 1C). These different effects of CD4 stimulation are intrinsic functions of individual anti-CD4 mAb.

**CLINICAL APPROACHES TO Treg-MEDIATED TOLERANCE INDUCTION**

Current immunosuppressive therapies are efficient in preventing acute transplant rejection and dampening inflammation in autoimmune diseases such as rheumatoid arthritis or lupus. Nevertheless, immune suppression remains inadequate, as it comprises significant side effects such as organ toxicity and hypersuppression disabling protective immune responses against pathogens and enhancing the risk of chronic infections. Hence, there is a clinical need for novel immunotherapeutic drugs with the ability to rebalance the immunologic tolerance network without persistently affecting immune function. In contrast to pharmacological immune suppression, re-induction of tolerance through the exploitation of evolutionarily established tolerance mechanisms is expected to offer a parentally operative cure. Among mechanisms operative in self-tolerance, the immune-suppressive activity of Treg appears to be exceptionally well suited for therapeutic exploitation for several reasons: First, activated Treg dampen the function of a wide range of immune cells including T cells (Pandiyana et al., 2007), B cells (Lim et al., 2005), DC (Misra et al., 2004; Larmontier et al., 2007), and monocytes (Taams et al., 2005) and affect a broad range of immune contexts including cardiovascular disease (Att-Onifalla et al., 2006) and obesity-induced insulin resistance (Fourrier et al., 2009). Second, the activation of Treg is antigen-specific defined by the selected T cell receptor repertoire in the thymus. However, once activated the suppressive mechanisms of Treg operate in an antigen-non-specific manner, sidestepping the need to identify disease-specific antigens to affect a particular Treg population. Prime examples of the Treg immune-dampening potential are experiments demonstrating that Treg can be expanded and re-infused to limit immune responses (Hoffmann et al., 2002) preventing GvHD induction without causing toxicity. While persistent polyclonal Treg activation would lead to general immune hyporesponsiveness, a short-term Treg activation – as established for tolerance induction with non-depleting anti-CD4 mAb in mice – is expected to induce (or re-induce) antigen-specific regulatory networks that maintain antigen-specific tolerance when Treg activity has returned to normal levels.

Based on the evidence for Treg activation by CD4 ligands as outlined above, anti-CD4 mAb seem to represent ideal compounds for Treg-mediated tolerance induction. However, although animal studies have provided a compelling basis for clinical application of anti-CD4-mediated tolerance induction, this approach has been remarkably unsuccessful when transferred to the clinic. Although short interventions with particular mAb have been shown to offer quick symptomatic relief, improvements supposedly caused by inactivation and depletion of CD4+ T cells (Koo et al., 2003; Choy et al., 2002) remained transient. Failure to establish an anti-CD4-based tolerogenic therapy in humans is most likely due to difficulties in translating the timing and dosage used in animal models for human application. Importantly, in contrast to animal models, mAb are administered at late disease stages in clinical studies. Whereas the immature immune system seems to dependably allow tolerance induction with anti-CD4 mAb, it seems more difficult to tolerate the experienced immune system in patients, in part due to the presence of effector and memory T cells resistant to the suppressive action of Treg (Yang et al., 2007). In fact, Treg-based therapies have been found to be generally less effective in models of autoimmune diseases. Withers et al. (2011) for example observed that functionally active Treg failed to control hyperactivated T effector cells in rheumatoid arthritis patients with ongoing inflammation but prevented autoimmune immune responses in non-inflammatory arthritis. Impaired Treg suppression under inflammatory conditions has been mainly ascribed to the influence of TNF-α, IL-1, and IL-6, which turn effector T cells resistant to Treg-mediated suppression (Walker, 2009; Goodman et al., 2011). Certainly, resistance to Treg-mediated suppression can be overcome by blockade of IL-6 (Chen et al., 2009) and supposedly, the beneficial effects of anti-TNF-α treatment include a similar effect too (Ehrenstein et al., 2004; Valencia et al., 2006). Thus, provided Treg can be sufficiently activated in the host, their suppressive efficiency might depend on the disease stage, which strongly argues for a combination of Treg enhancing strategies with biologicals that reverse Treg resistance in autoimmune T effector cells. As exemplified with anti-CD3 mAb already in the clinic evacuation of T effectors cells and concomitant enhancement of Treg activity can form a very effective treatment (Chatenoud and Bluestone, 2007).

With regard to anti-CD3-mediated tolerance induction in humans, it is important to emphasize again that anti-CD4 mAb vary in their capacity to activate Treg (Becker et al., 2007) and antibodies used in clinical trials so far have not been analyzed with regard to their Treg activating potential. However, clinical trials with Treg enhancing agents such as the anti-CD4 mAb Tegalizumab in rheumatoid arthritis have been initiated to investigate the efficacy of Treg-based anti-CD4-mediated tolerance induction in patients with autoimmune diseases.

**CONCLUDING REMARKS**

In summary, polyclonal activation of Treg through their surface molecules by biologicals that enhance their intracellular cAMP level are effective to induce the suppressive function of Treg for reinduction of tolerance in small animal models and in humanized mice. It is therefore expected that polyclonal Treg activation forms a rational for tolerance induction in humans. However, both the exact conditions, efficiency in different stages of disease and cooperation with additional treatment regiments to diminish T effector cells need to be thoroughly explored. Moreover differential signals in Treg versus T effector cells are far from being clear. In addition to deepening our understanding of Treg biology investigation of the latter holds the key to define alternative entry points for therapeutic manipulation of Treg function.

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