

Mucosally induced Immunological Tolerance, Regulatory T Cells and the Adjuvant Effect by Cholera Toxin B Subunit

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Abstract

Induction of peripheral immunological tolerance by mucosal administration of selected antigens (Ags) ('oral tolerance') is an attractive, yet medically little developed, approach to prevent or treat selected autoimmune or allergic disorders. A highly effective way to maximize oral tolerance induction for immunotherapeutic purposes is to administer the relevant Ag together with, and preferably linked to the non-toxic B subunit protein of cholera toxin (CTB). Oral, nasal or sublingual administration of such Ag/CTB conjugates or gene fusion proteins have been found to induce tolerance with superior efficiency compared with administration of Ag alone, including the suppression of various autoimmune disorders and allergies in animal models. In a proof-of-concept clinical trial in patients with Behcet's disease, this was extended with highly promising results to prevent relapse of autoimmune uveitis. Tolerization by mucosal Ag/CTB administration results in a strong increase in Ag-specific regulatory CD4⁺ T cells, apparently via two separate pathways: one using B cells as APCs and leading to a strong expansion of Foxp3⁺ Treg cells which can both suppress and mediate apoptotic depletion of effector T cells, and one being B cell-independent and associated with development of Foxp3⁻ regulatory T cells that express membrane latency-associated peptide and transforming growth factor (TGF- β) and/or IL-10. The ability of CTB to dramatically increase mucosal Ag uptake and presentation by different APCs through binding to GM1 ganglioside (which makes most B cells effective APCs irrespective of their Ag specificity), together with CTB-mediated stimulation of TGF- β and IL-10 production and inhibition of IL-6 formation may explain the dramatic potentiation of oral tolerance by mucosal Ags presented with CTB.

Introduction

It is well established that mucosal administration, e.g. by the oral, sublingual or nasal routes, of many (but not all) antigens (Ags) can induce peripheral tolerance, often referred to as oral tolerance, which is characterized by a decreased immune response to systemic immunization with the same Ag [for recent reviews see 1, 2]. Mucosally induced tolerance, which appears to be a physiologically important way of avoiding development of allergic or other harmful immunologic reactions to ingested or inhaled food or environmental Ags, has for a long time been recognized as a promising approach to prevent or treat allergic or autoimmune disorders. Yet despite its attractive features and promising results in animal mod-

els, there are no products available for clinical use based on this principle with the recent exception for a few sublingual (s.l.) products for sublingual immunotherapy against allergic rhinitis [3].

It seems clear that to achieve this goal more broadly, there is a need for development of improved Ag formulations and administration regimens with enhanced tolerance-inducing potency [2]. Therefore, it represents a promising invention with great potential for immunotherapeutic development that we and others have shown that mucosal administration of relevant Ags when conjugated to the non-toxic B subunit protein of cholera toxin (CTB) induce oral tolerance with exceptionally high efficiency [4] and in experimental models suppress the development of autoimmune diseases in an Ag-specific

manner [5–9]. The superior tolerizing effect of Ag conjugated to CTB has been shown to extend to other immunopathological reactions [10], including IgE-mediated allergies [11], infection-induced tissue-damaging inflammation [12, 13] and graft rejection reactions [2]. These findings were recently extended to patients with Behcet's disease in a proof-of-concept clinical trial [14].

Here, we discuss the oral tolerance induced by mucosally administered Ags, especially of Ags linked to CTB or its close analogue LTB (the B subunit protein of *Escherichia coli* heat-labile enterotoxin), both with regard to the immunotherapeutic effects in different immunopathological conditions and with regard to the underlying mechanisms.

Oral tolerance immunotherapy: superior efficacy using B subunit-linked Ags

For use in immunotherapy in e.g. autoimmune disorders or allergy, mucosally induced immunological tolerance should be effective in patients in whom a disease process is already established and/or in whom potentially tissue-damaging immune cells already exist. However, current protocols for mucosal tolerance induction have had limited success in suppressing already established inflammation caused by systemic immunological sensitization [1, 2, 10]. This may partly explain the disappointing results of clinical trials that explored oral tolerance treatment in patients with multiple sclerosis and rheumatoid arthritis [1, 2].

To enhance the immunotherapeutic potential, efforts have been devoted to the construction of improved formulations to induce mucosal tolerance, especially by identifying useful 'adjuvants' to co-administer with a specific Ag. The most promising adjuvants to date are CTB and the closely related protein LTB, which are particularly effective when used as carriers for mucosal delivery of tolerogenic proteins or peptides linked to the adjuvant.

As we first described in 1994 [4], oral administration of various Ags chemically conjugated to recombinant CTB can very efficiently induce tolerance to a subsequent immunization with the same Ags even when the latter is given together with Freund's complete adjuvant. Conjugation to CTB minimized by several hundred-fold the amounts of Ags required and also drastically reduced the number of doses needed in other protocols of orally induced tolerization; even a single oral dose of Ag/CTB conjugate was effective.

Of great importance, this strategy appears to be applicable for suppressing the expression also of already established states of systemic immune sensitization. As shown with many different Ags, cell-mediated delayed-type hypersensitivity (DTH) reactions are nearly completely suppressed, and in contrast with previous regimens, the treatment also efficiently reverses DTH reactions in

previously sensitized animals. Furthermore, promising results have been obtained in preventing or suppressing IgE mediated allergen hypersensitivity in animal models. Similar to the effects of chemical Ag/CTB conjugates, oral administration of peptides of relevant protein Ags genetically fused to CTB can also suppress DTH-mediated inflammation and disease or IgE-mediated allergy in animal models. Below, we discuss the results of studies that have used this approach of immunotherapy [5–18].

Protection against experimental autoimmune disorders

Mucosal administration of relevant auto-Ags linked chemically or by genetic fusion to CTB has been shown to prevent or suppress disease progression of autoimmune diseases such as experimental allergic encephalitis (EAE), diabetes, arthritis and uveitis in animal models [5–10, 14].

In EAE, oral administration of microgram amounts of myelin basic protein (MBP) conjugated to CTB not only prophylactically prevented disease development but also efficiently protected against progression to 'clinical' disease when given to animals with already induced 'preclinical' EAE inflammation [5, 8]. Similarly, nasal treatment with myelin proteolipid protein (PLP) genetically fused to CTB significantly suppressed the further progression of early EAE in SJL mice, associated with inhibition of both PLP peptide-specific DTH responses and leucocyte infiltration into the spinal cord [19]. Oral administration of 10 µg of a fusion protein comprising an immunodominant peptide of the neuronal protein synapsin and LTB was reported to induce cross-tolerance to the encephalitogenic effect of immunization with MBP in rats; treatment prior to EAE induction diminished disease incidence, DTH reactions and central nervous system inflammation in response to MBP [20].

Similarly, in female non-obese diabetic (NOD) mice, oral treatment with a CTB-insulin conjugate suppressed the development of type I diabetes [6] even when the treatment was started as late as 15 weeks after birth, at which time all female NOD mice normally have pancreatic inflammation. When adult NOD mice were fed small amounts (2–20 µg) of human insulin conjugated to CTB, beta cell destruction and clinical diabetes were suppressed. The protective effect could be transferred to untreated NOD female mice by CD4⁺ T cells from CTB/insulin-treated animals and was associated with reduced lesions of insulinitis [21–24].

Furthermore, various CTB/insulin gene fusion proteins and even directly fed CTB-insulin fusion protein-containing plants were shown to induce oral tolerance to insulin and prevent diabetes development [15–19], and this has later been extended to many additional different insulin-CTB fusion proteins produced in plants, viruses and silkworm larvae [25–28].

Our studies in a collagen-induced arthritis model in mice showed it was possible to effectively prevent joint inflammation and tissue destruction by nasal administration of a collagen II Ag/CTB conjugate and also to protect against further disease progression by starting treatment with this conjugate in the early stages of manifest arthritis [7]. Similarly, Kim *et al.* [29] reported that whilst feeding B10.RIII (H-2r) mice with a high dosage of a collagen II peptide (CB11) provided partial protection against chondritis, oral administration of even a tiny amount of CB11/CTB conjugate suppressed the disease much more effectively.

Finally, in a rat uveitis model of experimental Bechet's disease (BD), Phipps *et al.* [9] showed in an extensive study in Lewis rats that uveitis induced by heat shock protein 60 (HSP60) immunization was decreased by 75% ($P < 0.0001$) by oral pretreatment with the uveitogenic HSP60 peptide 336–351 linked to CTB.

In conclusion, all these studies indicate that self protein Ags or peptides linked to CTB or LTB have a protective role. If the effects observed in the experimental systems would extend to clinical disease in the approach humans could provide a promising therapeutic tool for medical intervention in various autoimmune diseases.

Prevention of type I allergy

Mucosal administration of ovalbumin (OVA) can induce partial tolerance in an experimental model of asthma in mice, so therefore the possibility of enhancing the treatment effect by mucosal administration of OVA linked to CTB or LTB has been tested in a mouse model of OVA-induced allergic reactions. Mice given OVA conjugated to *E. coli* LTB nasally prior to allergic sensitization showed suppressed serum IgE antibody responses to the inhaled allergen and also suppressed cutaneous anaphylaxis responses to OVA; treated mice also showed markedly decreased anaphylactic responses to intravenously administered OVA [30]. These results were confirmed and extended by studies in which OVA was conjugated to CTB and given orally, resulting in suppression of IgE allergic responses also in already sensitized mice [11].

Also treatment with Ags mixed with rather than conjugated to CTB have been found to prevent allergic inflammation in animal models. Guinea pigs with experimentally induced allergic conjunctivitis had significantly reduced clinical allergic reaction scores and fewer histological findings (e.g. eosinophilic infiltration) than the control group when treated with topical instillation of OVA mixed with CTB [31]. Topical application of CTB together with OVA onto mouse skin also suppressed OVA-specific IgE responses in serum [32]. Furthermore, Cuburu *et al.* [30, 33] recently found that s.l. administration of CTB alone or admixed with or (for stronger effect) linked to OVA in an Ag-specific manner markedly

reduced IgE and other Th2 type responses and eosinophil infiltration in target tissues in an experimental model of allergic asthma.

The tolerogenic property of allergen/CTB conjugate may be related to the nature of the coupled allergen. Wiedermann *et al.* [35] reported that oral administration of OVA/CTB prior to sensitization with OVA led to a significant decrease of Ag-specific IgE antibody levels and lymphoproliferative responses in a mouse model of aerosol OVA-sensitization. They initially failed to get the same results in an aerosol birch pollen allergy model when they used a chemical rBet-v1/CTB conjugate for treatment [35]; however, in a recent study similar oral treatment with a fusion protein between Bet-v1 and CTB did effectively suppress the birch pollen allergic response in this model [18].

Takagi *et al.* [36] recently expressed a gene fusion protein of CTB with T-cell epitopes of Cry j 1/2, the major allergen in Japanese cedar pollen, in rice seed and showed that feeding mice with even low amounts of the transgenic rice seed efficiently suppressed allergen-specific IgE responses and pollen-induced clinical symptoms.

Taken together, the findings in the autoimmune and allergy models show that under certain conditions, mucosal administration of a protein allergen together with an immunomodulating mucosal vector such as CTB or LTB can quite efficiently suppress both systemic Th1- and Th2-driven responses also in already sensitized individuals [18–20]. Of particular note is also, as first suggested by us [37] and recently experimentally nicely demonstrated by Smits *et al.* [34], that for immunotherapy of allergy it is of special importance that mucosal treatment with Ag/CTB, in addition to inducing peripheral tolerance also at the same time can strongly promote protective mucosal IgA antibody formation, which can effectively synergize with tolerance in the suppression of allergy and asthma.

Clinical proof of principle

Oral tolerance induction by Ag conjugated to CTB was recently found to extend to humans in a proof-of-concept pilot clinical trial in patients with Behcet's disease (BD). BD is a condition characterized by uveitis, ulcers in the mouth and the genitals, and often also arthritis, symptoms which all of them may be mainly due to an autoimmune reaction causing vasculitis in affected organs [14]. BD patients characteristically have what appear to be pathogenic T cells that specifically react with peptide p331–351 of HSP60, a peptide shown to be uveitogenic in animals [9]. In an open but well-controlled phase I/phase II clinical study, BD patients were treated orally with an HSP60p336–351/CTB conjugate three times a week for 12 weeks and were during this period gradually withdrawn from all immunosuppressive drugs. The

patients were monitored by clinical and ophthalmological examinations and by extensive immunological investigations. Before the p336–351/CTB conjugate treatment, at least one attempt had been made in the previous years to reduce the immunosuppressive treatment but had in all cases led to acute relapse of uveitis even when a 25% reduction in immunosuppressive dosage had been tried so it was considered that each of the enrolled patients were on their minimal effective immunotherapy dosage to avoid uveitis relapse. By contrast, during oral p336–351/CTB conjugate treatment there was no uveitis relapse in five of eight BD patients in whom all immunosuppressive therapy was withdrawn, and three of these five patients remained free of relapsing uveitis for 10–18 months after cessation of all treatment. The five ‘success’ cases were all among the six patients that were free of any disease activity prior to the tolerization regimen; in two individuals who had remaining BD symptoms (arthritis) although not uveitis the oral p336–351/CTB conjugate treatment had no effect inasmuch as these patients rapidly got uveitis when the immunosuppressive treatment was reduced. Immunological analyses showed that the five successfully treated patients all of them had a marked reduction in p336–351 specific T-cell proliferative, IL-2 and IFN- γ responses and instead a strong increase in IL-10 production by p336–351 stimulated CD4⁺ T cells, effects which were lacking in the three patients who did not respond clinically to treatment [9]. In no case, the oral p336–351/CTB conjugate treatment was associated with any adverse reactions. These promising findings give hope that in humans this novel strategy may be a useful treatment tool in Behcet’s disease, and indicate that further clinical trials of this strategy are warranted both in patients with BD and with other autoimmune or inflammatory disorders.

Oral tolerance mechanisms with special reference to mucosal tolerization by Ag/CTB conjugates

Results from the studies described indicate that tolerance induced by mucosal administration of relevant Ags conjugated or fused to CTB, similar to but more efficiently than by administration of Ag alone, is mediated by more than one type of mucosally induced regulatory T cells and is associated with increased IL-10 and/or transforming growth factor (TGF- β) cytokine production. Consistent with this, tolerization by Ag/CTB conjugate administration has been found to result in pronounced increases in Ag-specific regulatory T cells in both draining and peripheral lymph nodes (LNs) together with a strong induction of TGF- β and often also IL-10 production. Our work has also revealed that deletion/anergy and active regulation may be different aspects of the same tolerogenic process. Thus, the peripheral immunological tolerance induced by mucosal administration of a CTB-

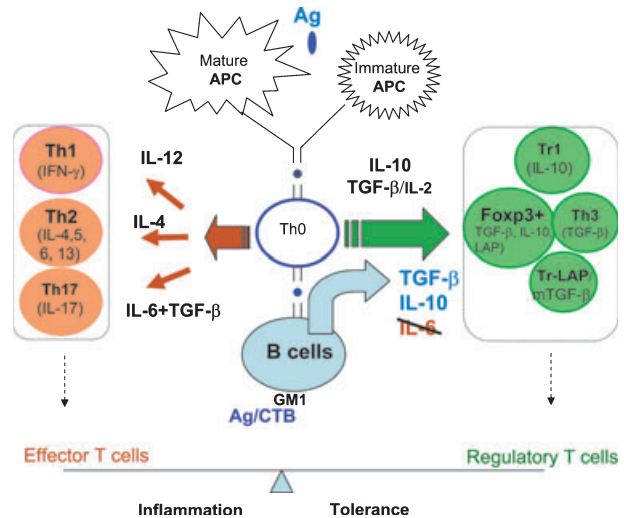


Figure 1 Factors influencing the mucosal induction of regulatory (Treg) and effector (Teff) CD4 T cells. Antigen (Ag) presentation by mucosal immature APCs under non-inflammatory conditions preferentially induce TGF- β and/or IL-10 driven differentiation of naive T (Th0) cells into Treg cells such as Tr1, Th3, Foxp3⁺ and/or Foxp3⁺LAP⁺ or CD25⁺LAP⁺ Treg cells. Ag presentation by mature APCs (or immature APCs after exposure to proinflammatory conditions) instead preferentially induce IL-12, IL-4 and/or IL-6 driven development of different Teff cells including Th1, Th2 and/or Th17 cells. A dramatic potentiation of oral tolerance by mucosal Ags conjugated or fused to CTB (Ag/CTB) may be explained by the ability of CTB, through binding to GM1 ganglioside, to dramatically increase mucosal Ag uptake and presentation by different APCs together with CTB-induced stimulation of TGF- β , IL-10 and/or IL-2 production and inhibition of IL-6 production. These effects appear to be of special importance in making B cells stimulated by Ag/CTB to become especially prominent as tolerogenic APCs for induction of Treg cells and mucosal tolerance.

conjugated Ag mechanistically appears to comprise the induction of Ag-specific regulatory CD4⁺ T cells, both Foxp3⁺ and Foxp3[−] cells, which can both functionally suppress effector T cells as well as induce Ag-specific apoptosis and deletion of such cells [38, 39]. In the subsections below, we will further discuss these effects and mechanisms also outlined schematically in Fig. 1, and also our recent findings indicating an especially important role of B cells as antigen-presenting cells (APCs) by mucosal CTB-linked Ag treatment in oral tolerance induction and Treg cell development [40].

Modulation of cytokine production

Mucosal treatments with Ag/CTB conjugates inducing immunological tolerance have consistently been found to modulate the production of cytokines by different T-cell subsets in an Ag-specific manner.

As mentioned in regard to the observed suppression of EAE induced by MBP/CTB conjugate, the expression of Th1 cytokines (IFN- γ , IL-12 and TNF- α) as well as of Th1 attracting chemokines, such as monocyte chemotactic protein (MCP)-1 and RANTES, are markedly reduced

in the spinal cord; in contrast, TGF- β -expressing CD4 T cells are strongly increased in the spinal cord of animals treated orally with the MBP/CTB conjugate [8]. Similarly, oral delivery of CTB conjugated to HSP60p336–351, which effectively prevented mucosally induced uveitis in rats, was associated with enhanced IL-10 and TGF- β and reduced IL-12 and IFN- γ production [9], and as mentioned successful treatment of patients with Behcet's disease with such a conjugate was likewise associated with increased production of IL-10 and reduced IL-2 and IFN- γ production (TGF- β was not monitored in this clinical study) (14). A similar change in cytokine pattern together with an increased CD4⁺ regulatory T-cell migration into pancreatic islets was described after oral administration of CTB-insulin conjugate, which prevented diabetes development in NOD mice [6, 21–23]. In the arthritis model where a CTB-collagen II conjugate induced Ag-specific tolerance and suppression of disease development, these effects were associated with suppression of both Th1- and Th2-mediated cytokine responses (e.g., IL-2, IFN- γ as well as IL-6 were markedly decreased) [7, 41].

Recently, Th17 cells producing IL-17 have been identified as contributing to the pathogenesis of autoimmune disease in animal models [42]. This has led to an interest in examining the effects of oral tolerance on Th17 cells. Recent studies have shown that after oral administration of Ags, Ag-specific peripheral tolerance was associated with a reduction of Th17 cells through effects dependent on suppression of IL-6 [43] and with suppression of both Th1 and Th17 cytokines in an arthritis model [44]. In mice with EAE induced by immunization with myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 together with CFA, we have also noted that after tolerization with the same peptide conjugated to CTB, production of IL-17 was significantly suppressed in draining LNs compared with untreated mice [JB Sun and J Holmgren unpublished data].

Mucosal generation of different types of regulatory T cells

Different types of regulatory T cells are important mediators of oral tolerance, these comprise, e.g. Tr1 cells, which secrete IL-10 [45], and Th3 cells, which secrete TGF- β [46]. Recently, CD25⁺CD4⁺ regulatory T cells (Treg) that characteristically express the forkhead transcription factor Foxp3 have been found in Peyer's patches (PP) and mesenteric LN (MLN) shortly after oral administration of high-dose OVA [47]. Treg play a crucial role in maintaining immune tolerance to self-Ag [48] and can produce either or both of TGF- β and IL-10 [49] and they also express CTLA-4 [50]. Importantly, these cells may also confer suppressor activity ('infectious tolerance') on various CD4⁺ T cells by inducing the expression of the Foxp3 gene, which appears to be critical for the genera-

tion and suppressive function of many but not all regulatory T cells (e.g. IL-10-producing Tr1 cells appear to maintain a Foxp3[−] status) [50].

Recently, we investigated the extent to which 'oral tolerance' after mucosal OVA/CTB conjugate administration is mediated by Foxp3⁺CD25⁺CD4⁺ Treg cells [38]. Our results demonstrate that oral tolerance induced by CTB-conjugated Ag is associated with an increase in TGF- β and in both increased numbers and increased per-cell suppressive activity of Foxp3⁺ and CTLA-4⁺ CD25⁺ Treg cells, together with the generation of either Foxp3⁺ or Foxp3[−] CD25[−]CD4⁺ regulatory T cells. These findings are in line with our findings showing that feeding mice with an influenza haemagglutinin (HA) peptide-CTB fusion protein promotes the development and expansion of Ag-specific CD25⁺ Treg cells with gut-homing properties [51]. Collectively, these results indicate that tolerance induced by Ag/CTB conjugates is associated with increased numbers of CD25⁺ Treg cells and with increased suppressive activity and Foxp3 activation in more than one population of regulatory T cells.

TGF- β plays an important role in the induction and suppressive action of natural Treg cells as well as in the generation of Ag-induced peripheral Treg cells [52]. We found that oral tolerance induction by mucosal administration of OVA/CTB led to a markedly elevated level of TGF- β 1 in serum in association with an increase in Treg cells in MLNs. Conversely, *in vivo* depletion of TGF- β by specific antibody treatment completely suppressed the generation of CD25⁺ Treg cells in response to the oral OVA/CTB treatment and effectively removed most, if not all, of the ability of oral OVA/CTB treatment to induce functional suppression of T-cell responses [38]. It is notable that mucosal Ag/CTB treatment both increases TGF- β production [8, 9, 33, 38, 53, 54] and almost completely suppresses proinflammatory IL-6 production [7]. Betteli *et al.* [42] recently reported that TGF- β in the absence of IL-6 strongly promotes Treg generation; however, when combined with IL-6, TGF- β instead induces IL-17-producing, so-called Th17 effector T cells. We have proposed that the combined increase in TGF- β and decrease in IL-6 may explain or contribute to the remarkably efficient oral tolerance induction and Treg generation by Ag/CTB treatment [38]. This suggestion is further supported by the observations that cholera holotoxin, which opposite to the CTB protein efficiently inhibits oral tolerance induction and Treg development, induces IL-6 together with IL-1 β production [55], while, as mentioned, Ag/CTB treatment suppresses IL-6 formation [7].

Yet, it should be noted that not only typical Foxp3⁺ Treg are induced and active in oral tolerance induced by Ag/CTB. Instead, our findings indicate that mucosal treatment with OVA/CTB in addition to strongly suppressive Foxp3⁺CD25⁺ Treg also give rise to CD25[−]CD4⁺ T cells that acquire regulatory-suppressive function without

activation of the Foxp3 gene system [38]. Our results in this regard agree with those of another study from this laboratory in which the induction of tolerance after oral administration of an influenza virus HA peptide/CTB gene fusion protein was reduced but not abolished by depletion of CD25⁺ T cells [51].

Mucosal Ag/CTB-generated regulatory T cells induce apoptosis and depletion of effector T cells

Recent work by us and others suggests that, what was previously thought to be separate forms of tolerance after mucosal administration of Ag, anergy/deletion versus regulatory T cells, may indeed overlap [44]. Thus, we showed that mucosal (s.l.) administration of a CTB-conjugated Ag (OVA) induced peripheral immunological tolerance that comprised the induction of Ag-specific regulatory T cells that mechanistically, in addition to their ability to functionally suppress effector T cells, also induced progressive Ag-specific apoptosis and depletion of such cells [39].

By studying adoptively transferred CFSE-labelled OVA TCR Tg T cells from various LNs 2 or 3 days after s.l. OVA/CTB administration by flow cytometry, we found that the s.l. treatment had stimulated a rapid cell division and expansion of both Treg cells (defined as Foxp3⁺CD25⁺CD4⁺ T cells) and effector T (Teff) cells (defined as Foxp3⁻CD25⁺CD4⁺ cells) in draining cervico-mandibular LNs [39]. In peripheral LNs, such as MLNs in non-sensitized or popliteal LNs (PLNs) in sensitized mice, the Teff cells showed signs of Ag-specific apoptosis and cell depletion already a few days after the s.l. OVA/CTB treatment with further progression of these effects over the next one or two weeks. Adoptive transfer experiments indicated that the induction of apoptosis was dependent on Ag-specific Treg cells (CD25⁺CD4⁺ T cells) but, as tested in IL-10-KO mice, not on the ability of the recipient mice to produce IL-10 [39]. The precise mechanisms for Treg mediated suppression and apoptosis of Teff cells are not well defined. In a recent study Pandiyan *et al.* [56] described, similar to our findings [39], that CD4⁺Foxp3⁺ Treg cells can induce apoptosis of CD4⁺ Teff cells, and further showed that this could be explained by cytokine deprivation. Very recently, Wang *et al.* [57] described another novel possible mechanism of tolerance induction by CTB in that receptor GM1 expressed on CD25⁺ Teff cells appears to be the primary target of galectin-1 expressed by CD25⁺ Treg and that CTB-mediated co-cross-linking and associated TRPC5 channel activation may contribute importantly to the mechanism of autoimmune suppression.

Recently, we could also describe what seems to be yet another distinct regulatory CD4⁺ T-cell population in oral tolerance induced by mucosal Ag/CTB administration; these cells are characterized by being Foxp3⁻ but

instead expressing the latency-associated peptide (LAP) on the cell surface [40]. The presence and role of these cells in oral tolerance became evident when we studied oral tolerance induction in B-cell deficient μ MT^{-/-} mice and found that B cells are the most important APCs for generation of Treg cells in response to mucosal administration of CTB-conjugated Ag [40]. We found that effective Ag-specific suppression of the peripheral Teff cell response to parenteral immunization by prior mucosal treatment with Ag/CTB conjugate could be achieved also in μ MT^{-/-} mice lacking B cells, even though these mice were unable to mount a significant Foxp3⁺CD25⁺CD4⁺ Treg cell response. Others have also described effective oral tolerance induction in μ MT^{-/-} mice [58]. As mentioned, our previous work has shown that besides Foxp3⁺ Treg cells also Foxp3⁻CD4⁺ T cells with regulatory-suppressive function on Teff cells are induced by oral OVA/CTB administration mice [38], and we have now found that many of these Foxp3⁻ suppressive T cells express LAP on the surface. It has been suggested that the expression of LAP/TGF- β on APCs and CD4⁺ T cells is in fact required for the generation of Foxp3⁺ Treg cells [59, 60], and consistent with this, we have found that in naive WT mice s.l. treated with OVA/CTB the increase in Foxp3⁺ Treg cells and apoptotic Teff cells is correlated with the number of LAP⁺/TGF- β ⁺CD4⁺ T cells [JB Sun and J Holmgren, unpublished data]. In this context it is striking that in μ MT^{-/-} mice the same OVA/CTB treatment induced a significantly stronger increase in such Foxp3-LAP⁺CD4⁺ T cells than in the WT mice and we believe that this increase may at least in part compensate for the weak Foxp3⁺ Treg cell response in these mice. Although recently LAP was described to have anti-inflammatory immunomodulating activity in its own right [61], the main immunomodulatory activity of LAP⁺ on either CD25⁺ or CD25⁻ T cells is usually ascribed to TGF- β complexing with LAP on the cell surface [62]. The increased LAP⁺ regulatory T cells in μ MT^{-/-} mice in response to mucosal Ag/CTB treatment could explain the observed suppression of Ag-specific Teff cell proliferation in PLNs after s.c. injection of OVA together with (proinflammatory) CFA. In fact, under inflammatory conditions, such LAP⁺/TGF- β ⁺ cells have been reported to suppress activated (not resting) Teff cells with much greater efficiency than Foxp3⁺ Treg cells [59, 62, 63].

Induction of tolerogenic APCs

Dendritic cells (DC) play a pivotal role in the induction and early steering of the immune response to an encountered Ag. Recent findings indicate that the development of tolerance or inflammation is to a large extent determined by the DC phenotype presenting Ag to T cells in draining LNs (Fig. 1). One possibility is therefore that

mucosally administered Ag/CTB conjugate preferentially is taken up by tolerogenic subsets of mucosal DC or other APCs. Mucosal DC differ in several respects from DC in systemic LNs and play a critical role in the induction of the 'default' peripheral immune tolerance process to food proteins and most other non-inflammatory environmental Ags [64]. Several studies have shown that immature DC in the intestinal mucosa are the critical cells that take up dietary proteins and migrate to the draining MLNs, where they induce regulatory CD4 T-cell stimulation and differentiation [65, 66]. The induction of Ag-specific Foxp3⁺ Treg cells in mucosal tissues has been attributed to the interaction of T cells with Ag-presenting CD103⁺ DC in MLNs after oral Ag administration through a process dependent on both TGF- β and retinoic acid (vitamin A) [65]. Recently, D'Ambrosio *et al.* [67] suggested that CTB promotes the induction of regulatory T cells by preventing human DC maturation, this was based on their finding that CTB could partially prevent LPS-induced maturation of monocyte-derived DC, decrease their IL-12 production, and in co-cultured T cells promote IL-10 production associated with a reduced IFN- γ production.

The efficient generation and functional activation of CD25⁺ Treg cells by mucosal treatment with Ag/CTB conjugates is probably explained both by effects of the conjugates on gut mucosal epithelial cells and on mucosal APCs. Since CTB effectively facilitates the binding and uptake of the linked Ag by intestinal epithelial cells, Ag presentation by epithelial cells may preferentially induce tolerance due to their inherent inability to provide costimulation unless they also receive an inflammatory signal [2, 10]. The conjugation of Ag to CTB, which binds with high affinity to GM1 ganglioside receptors present both on mucosal epithelial cells and on all known professional APC [2], would also greatly facilitate Ag uptake and MHC class II-restricted Ag presentation by DC as well as by other types of APC, including intestinal B cells, macrophages and mast cells [68]. The intestinal epithelium normally produces large amounts of immunosuppressive cytokines, especially TGF- β or IL-10, and these cytokines probably also promote intestinal DC to preferentially induce regulatory T cells, including both Treg and Tr1 [66].

The level of APC activation in parallel with induction of distinct cytokines is also critical for the expression of Treg suppressive function, since signals that activate DC also are free from Treg control and permit the induction of effector immune responses instead as indicated in Fig. 1 [69]. This could explain how mucosal administration of Ag together with CT, rather than CTB, in all systems tested to date has effectively prevented tolerization (even of co-administered Ag/CTB conjugates) and instead acted as an exceptionally potent mucosal stimulus to promote mucosal and systemic effector immune responses

(Th1, Th2 and Th17). In contrast with CTB, which induces TGF- β and IL-10 and suppresses IL-6 formation, CT instead induces strong production of IL-6 and IL-1 β from both DC and other APCs and from the intestinal epithelium. We suggest that this converts the normally tolerogenic gut environment and makes the intestinal APCs become mainly immunostimulatory, which explains why CT so efficiently promote the induction of immune responses rather than tolerance and is especially potent in stimulating strong secretory IgA antibody responses which are known to be specifically promoted by a combination of IL-1, IL-6, TGF- β and retinoic acid [38, 55, 65, 66].

Plasmacytoid DC (pDC) may have an especially important role in sustaining tolerance to innocuous Ags at mucosal sites [70] as they can induce development of anergic T cells or T cells with regulatory functions *in vitro*. Recently, Anjuere *et al.* [71] reported on a selective increase in this DC subset in MLNs following Ag/CTB feeding and also the ability of this DC population from Ag/CTB-fed mice to support the expansion and differentiation of CD4⁺ Ag-specific regulatory T cells producing TGF- β and IL-10. The properties of the mucosal pDC might be influenced by the microenvironment, including the locally produced high concentrations of IL-10 and TGF- β [71]. In agreement with this, we had earlier reported that adoptively transferred DC from PP/MLN of mice fed with Ag/CTB could suppress Ag-specific DTH and T-cell proliferative response [72]. Similar to Anjuere *et al.*, we also observed an increase of CD11c⁺CD8 α ⁺B220⁺ DC in parallel with an increased frequency of suppressive Treg cells in PPs and MLNs a few days after oral treatment with OVA/CTB [JB Sun and J Holmgren, unpublished data].

Induction of tolerogenic B cells

Our recent studies have drawn special attention to B cells as being important APCs in oral tolerance induction after mucosal administration of CTB-linked Ag [40, 58]. Several studies had found that B cells are important in processing of autoantigens [73] and in natural protection against autoimmune tissue inflammation and disease [74–77]. However, the role of B cells in oral tolerance induction had been much less studied and also given inconclusive results [73, 78, 79]. We therefore tested the importance of B lymphocytes for the induction of oral tolerance after s.l. administration of OVA/CTB as a model system for mucosally induced tolerance by CTB-linked Ag. Similar to other findings, our results indicate that B cells are efficient tolerogenic APCs, especially for the development of Ag-specific Foxp3⁺ Treg cells [40, 80, 81], production of IL-10 and TGF- β [80, 82, 83] and that the coupling of Ag to CTB dramatically potentiates the mucosal tolerizing effect of B cells [40]. Indeed, since

CTB provides efficient binding to GM1 ganglioside receptors, the majority of all B cells irrespective of their BCR specificity can become effective APCs for any contacted Ag coupled to CTB, thus increasing Ag uptake and presentation more than 10,000-fold [68].

Recently, Smits *et al.* [34] have also shown attention to the role of B cells in the treatment of allergic asthma in a mouse model, and especially in promoting allergen-specific protective mucosal IgA antibody formation. They found that CTB strongly suppressed the salient features of asthma, in which the tolerance was only transferable to other mice by tolerized B cells, and mediated by the CTB-induced TGF- β -dependent rise in Ag-specific IgA in the airway luminal secretions. Still, however, B cells are not indispensable for induction of functional peripheral T-cell tolerance after mucosal treatment with OVA/CTB inasmuch as μ MT $^{-/-}$ mice lacking B cells could (partly compensatory) develop regulatory mechanisms independent of Foxp3 $^{+}$ Treg cells including the ability to expand LAP/TGF- β expressing Foxp3 $^{-}$ CD4 $^{+}$ regulatory T cells [40]. Our results indicate that mucosal tolerance comprises at least two separate pathways: one being B cell-dependent and associated with expansion of Foxp3 $^{+}$ Treg cells and Treg mediated suppression and apoptotic depletion of Teff cells, and one being B cell-independent and associated with development of Foxp3 $^{-}$ TGF- β $^{+}$ regulatory T cells. Since also *in vitro* the exposure of B cells to Ag/CTB conjugates render the B cells efficiently tolerogenic also *in vivo* when transferred to Ag-sensitized recipients, the use of *in vivo* cell therapy with *in vitro*-Ag/CTB pretreated B cells represents an attractive additional potential tool for immunotherapy in e.g. autoimmune diseases. This approach has already proven effective in animal models of both MOG-induced EAE and collagen II-induced arthritis [JB Sun, C Czerkinsky, J Holmgren, unpublished data; T Jin, JB Sun, J Homgren, unpublished data].

Concluding remarks

The mucosal immune system comprises ca 80% of all immunocytes and has developed effective means for protecting the body both against mucosal infections and harmful immune responses to ingested or inhaled antigens. With regard to the latter aspect, mucosally induced tolerance ('oral tolerance') is a promising yet still incompletely understood and medically not well-developed form of immunomodulation for treating certain autoimmune diseases and allergies. We and later also many other groups have shown that the mucosal administration of relevant Ags together with, and preferably linked to the non-toxic B subunit protein of cholera toxin (CTB) by either oral, sublingual or intranasal administration represents a highly effective way to maximize oral tolerance induction for immunotherapeutic purposes, being superior to administration of Ag alone.

The dramatic potentiation of oral tolerance by mucosal Ags presented with CTB is probably explained both by the ability of CTB to dramatically increase mucosal Ag uptake and presentation by different APCs through binding to GM1 ganglioside (which makes most B cells effective APCs irrespective of their Ag specificity) and by ability of CTB to promote TGF- β and IL-10 production while at the same time inhibiting IL-6 formation.

Encouraged by the promising results in a recent proof-of-concept phase II clinical trial in patients with Behcet's disease, where oral treatment with an autoimmune T-cell target Ag peptide linked to CTB without any adverse reactions in the majority of cases effectively controlled autoimmune uveitis associated with suppression of autoreactive T-cell activity *in vitro*, we propose that oral tolerization by Ag/CTB conjugates or fusion proteins should be further tested clinically in expanded trials in patients with Behcet's disease. Other proposed targets for immunotherapy based on this principle are those autoimmune diseases which have a strong autoimmune T-cell component, such as type I diabetes, rheumatoid arthritis or multiple sclerosis, where in all cases mucosal treatment with Ag/CTB have also been effective in animal models.

Yet another category recommended for clinical trials are mucosal respiratory or gastrointestinal allergies, where again there are promising findings in animal models and where it would appear to be especially attractive that mucosal Ag/CTB treatment can efficiently induce not only peripheral tolerance but also at the same time induce a strong protective mucosal IgA antibody response, which can synergize with the tolerization effect.

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