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Accumulating evidence suggests that the dichotomy between tolerance and active IgA immunity in mucosal immune responses is regulated at the APC level. Therefore, immunomodulation of the APC could be an effective mechanism to control the two response patterns. In this study, we demonstrate that ADP-ribosylation controls the outcome of tolerance or active effector T cell immunity to an internal peptide p323–339 from OVA inserted into the cholera toxin (CT)-derived CTA1-OVA-DD adjuvant. We found that a single point mutation, CTA1R7K-OVA-DD, resulting in lack of enzymatic activity, promoted peptide-specific tolerance in TCR transgenic CD4⁺ T cells following a single intranasal (i.n.) treatment. The CTA1R7K-OVA-DD–induced tolerance was strong, long-lasting, and impaired the ability of adoptively transferred naive peptide-specific CD4⁺ T cells to respond to Ag-challenge, irrespective of whether this was given i.p. or i.n. The tolerance correlated with induction of regulatory T cells of the regulatory T type 1 (T(Reg)) type in mice exhibiting unimpaired CD4⁺ T cell responsiveness to recall Ag irrespective of whether they were untreated (PBS) or treated with CTA1R7K-OVA-DD. Thus, for the first time, we provide unequivocal proof that ADP-ribosylation can control the outcome of mucosal Ag exposure from tolerance to an enhanced effector CD4⁺ T cell response. The exploitation of this system for clinical treatment of autoimmune diseases is discussed.

Mucosal immune responses to foreign Ag typically are characterized by tolerance induction, which is a naturally occurring protective mechanism to prevent inflammatory reactions to food Ags or the bacterial microflora (1, 2). Understanding the mechanisms that control mucosal tolerance might provide a means to avoid tolerance, allowing for better and more effective mucosal vaccines, or, alternatively, it could even be exploited for the treatment of autoimmune diseases and common allergies (3–6). Not only is feeding of Ag effective at inducing tolerance, but also nasal or sublingual administrations of Ag have been found equally effective at tolerance induction (7, 8). Whereas there are numerous examples of mucosal tolerance induction in rodents that have prevented disease development in models of autoimmunity, few successful clinical examples have been reported (9). A possible explanation for this could be the lack of a detailed understanding of the mechanisms responsible for induction of tolerance or failure to develop clinical protocols or formulations effective at stimulating mucosal tolerance.

Accumulating evidence in the mouse model suggests that the dichotomy between tolerance and/or active effector immune responses involving IgA production is regulated at the APC level (10, 11). Therefore, immunomodulation of the APC could be an effective mechanism for the control of the two response patterns. Cholera toxin (CT) or the closely related Escherichia coli heat labile toxin are known to profoundly affect the APC and strongly promote effector T cell responses and IgA immunity to adixed Ags (12–14). However, when Ag is conjugated to the B subunit of the holotoxin, such as the CTB subunit (CTB) conjugates, it primarily induces T cell tolerance following mucosal administration (6, 15, 16). The tolerogenic property of CTB is dependent on the ganglioside monosialoganglioside (GM1) binding, a receptor present on the membrane of all nucleated cells (16, 17). Whether it is the ADP-ribosylating property of the holotoxin or a structural element in the AB5 complex that exerts the immunoenhancing function is presently not known, because enzymatically inactive mutants, such as the CTE112K, have been found to have retained adjuvant function (18, 19). Thus, both the ADP-ribosylating and/or the structural property of the holotoxin could be responsible for breaking tolerance following mucosal administration.

To circumvent the toxicity of the CT holotoxin, we previously developed the CTA1-DD gene fusion protein (20). We found that this molecule, composed of the ADP-ribosylating A1-subunit of CT (CTA1) genetically linked to a dimer of fragment D from Staphylococcus aureus protein A, was nontoxic and as efficient as CT in its adjuvant function when simply admixed with protein Ags (20). This property of CTA1-DD was dependent on the ADP-ribosylating function of CTA1, because single site-directed mutations that killed the enzyme resulted in loss of adjuvant effect (21). Today, numerous studies with CTA1-DD have proven its strong adjuvant function when given by the intranasal (i.n.) route together with, for example, Helicobacter pylori lysate, rotavirus viruslike particles, HIV Env proteins, Chlamydia trachomatis major outer membrane protein, influenza A virus M2e peptide, and many more Ags (13). Originally, the CTA1-DD was designed to specifically target B cells via DD binding to the Ig receptor, but recent studies have proven that the adjuvant function is also present in mice lacking B cells (22, 23). Contrary to CT, the CTA1-DD adjuvant is nontoxic and cannot
bind to the GM1-ganglioside receptor (24). Therefore, it is not taken up by nerve cells and does not accumulate in the central nervous tissues, olfactory bulb, or nerve, following i.n. administration, which is unfortunately a risk with all GM1-ganglioside–binding holotoxins or toxin derivatives (24, 25).

The present study was undertaken to investigate whether the ADP-ribosylating property of CTA1 can be responsible for changing the outcome following mucosal exposure to a foreign protein from tolerance into an active effector T cell immune response. To this end, we constructed two gene fusion proteins that differed by only a single point mutation in the CTA1, effectively killing ADP-ribosylation. We inserted the MHC class II-restricted OVA peptide, p323–329, into the fusion proteins and used this to determine their ability to induce tolerance or effector T cell immunity following i.n. administration of a single dose of CTA1-OVA-DD or CTA1R7K-OVA-DD. Thus, the two molecules differed with regard to one single amino acid replacement in the CTA1 moiety only. The outcome of the i.n. treatment was assessed as suppression or enhancement of Cd4+ T cell responses to recall Ag in vitro or in vivo using wild-type BALB/c mice adoptively transferred with p323–339 peptide-specific TCR transgenic (Tg) Cd4+ T cells from DO11.10 mice.

To our knowledge, the current study is the first to unequivocally address whether ADP-ribosylation can be responsible for controlling the outcome of mucosal tolerance or augmented effector T cell responses. In this study, we provide compelling evidence that ADP-ribosylation, is, indeed, a unique mechanism for breaking tolerance and enhancing effector T cell immunity. CTA1R7K-OVA-DD induced regulatory T type 1 (Tr1)-like cells that exerted global Ag-specific suppression of naive Cd4+ T cell responses via production of IL-10. The potential of CTA1R7K-OVA-DD as a new, highly effective vector for inducing tolerance and its potential use for treatment of autoimmune conditions is discussed.

Materials and Methods

Mice

Female BALB/c (H-2d), C57BL/6 (H-2b), or nude mice (nu/nu H-2b) were obtained from Taconic Farms (Ry, Denmark) and Charles River Laboratories (Sulzfeld, Germany). DO11.10 (H-2b) mice with a TCR transgene specific for the OVA peptide 323–339/L-A1 or hemagglutinin (HA) TCR for the HA peptide 111–119/L-A1 from influenza virus and IL-10-/- (H-2b) mice were bred at the Department of Experimental Biomedicine (University of Gothenburg, Göteborg, Sweden) (26–28). Adaptive transfer was performed with DO11.10 splenocytes by i.v. injection of 10^7 cells to age-matched female BALB/c mice. All mice were kept under specific pathogen-free conditions. Sex-matched mice were used at 6–12 wk of age. The studies were approved by the local ethics committee for animal experimentation.

Ags and immunomodulators

Chicken OVA (fraction V) was obtained from Sigma-Aldrich (Stockholm, Sweden), the OVA323–339 peptide was obtained from KJ Ross-Petersen (Hamar, Norway), and the HA peptide was solubilized by treatment with 8 M urea. After addition of distilled water to allow refolding, the fusion protein was purified by affinity chromatography on IgG-Sepharone (Pharmacia Biotech, Up psala, Sweden) (20). Following concentration and sterile filtration, the purified fusion proteins were stored at neutral pH in PBS at −80°C until use. The preparations were tested for ADP-ribosylation using the NAD-agaros e test (20). Briefly, the ADP-ribosyltransferase activity of the fusion proteins was determined by assessing incorporation of [U-14C] adenine into [U-14C] adenine-labeled ADP-ribosyl-agmatine were collected for determinations of radioactivity. The cpm activity of the fusion proteins was calculated from a standard curve generated by dilutions of intact CT (List Biological Laboratories, Cambell, CA). The fusion proteins were also assayed for binding to rat IgG by using an ELISA, screened for purity by Western blotting, and, finally, the presence of endotoxin was assessed (LAL Endochrome, Charles River Endosafe, Charleston, SC) (20). The preparations contained very low levels of endotoxin: <80 EU/ml protein.

Induction of tolerance or immunity

Mice were given a single i.n. treatment with indicated doses of CTA1R7K-OVA-DD or CTA1-OVA-DD in 20 μl PBS. In most experiments, we used 5 μg per dose, which is equivalent to 187.5 ng OVA323–339 peptide. Control mice received PBS only or when indicated OVA323–339 peptide was given i.n. at all doses. After adoptive immunization with 200 μg OVA in Ribi Adjuvant (TriChem, Copenhagen, Denmark) in a total volu m of 200 μl. All experiments used groups with 5–10 mice. In vivo tolerance was monitored following intranasal treatment with the fusion proteins in BALB/c mice, and then CFSE-labeled (Fluka Chemie, Buchs, Switzerland) naïve splenocytes or lymph node (LN) cells from TCR-Tg mice were injected. One day later, recipient mice were then challenged i.p. by OVA in Ribi Adjuvant (TriChem), and T cell responses in vivo were assessed 3 d later by gating on Cd4+ T cells in the FACS analysis.

In vitro cultures

Freshly isolated splenocytes or highly enriched KJ-126/Cd4+ T cells (>98%) after sorting on an FACSaria device (BD Biosciences, San Jose, CA) were cultured in triplicate in round-bottomed 96-well tissue culture plates (Nunc, Roskilde, Denmark) in Iscove’s medium (Biochrom, Berlin, Germany), supplemented with 10% heat-inactivated FCS (Biochrom), 50 μg/ml NE-2 ME (Sigma-Aldrich), 1 mM L-glutamine (Biochrom), and 50 μg/ml gentamycin (Sigma-Aldrich), and cultured at 37°C in 5% CO2 either alone or together with recall Ag (OVA323–339 or HA111–119) at 1 μM with or without the addition of recombinant IL-2 at 7.5 ng/ml (R&D Systems, Abingdon, U.K.) or anti–IL-10 mAb at 10 μg/ml (BD Biosciences) (24). Highly enriched KJ-126/Cd4+ T cells (DO11.10) were cultured at 10,000 cells/well in triplicate in the presence of 100,000 irradiated APCs from syngeneic mice or naive mouse spleen. Briefly, splenocytes were labeled with anti–Cd4–FITC and anti–KJ-126–PE (BD Biosciences) and enriched using a two-step procedure using AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-PE MACS beads followed by sorting on an FACSaria (BD Biosciences) according to the manufacturer’s instructions. Ag-specific cell proliferation to recall Ag was assessed after 72 h of culturing by the addition of 1 μCi/well [3H]thymidine (Amersham Bio science, Uppsala, Sweden) for the last 6 h. The [3H]thymidine uptake was determined using a scintillation counter (Beckman, Bromma, Sweden). Data was expressed as mean cpm ± SD of 5–10 mice per group in each experiment. Cytokine-containing supernatants were collected after 96 h of cell culturing and stored at −70°C until analyzed. The presence of IFN-γ, IL-4, and IL-10 was determined by ELISA. CFSE (Fluka Chemie) labeling was performed according to the manufacturer’s protocols, and cell divisions were assessed by FACS on gated Cd4+ T cell populations.

Ab and cytokine determinations

Detection of anti-OVA-specific Abs in serum was performed by ELISA as follows: 96-well, flat-bottomed plates (Nunc) were coated with 200 μg/ml OVA (Sigma-Aldrich). After blocking with 0.1% BSA/PBS, serial dilutions of serum were added to the plates, followed by incubation overnight. Alkaline phosphatase-conjugated anti-mouse IgG-specific Abs (SouthernBiotech, Birmingham, AL) were added. The phosphatase substrate (1 mg/ml Sigma-Aldrich) was added to the plates, and the enzyme reaction was terminated by using a Titertek Multiscan (Labsystems, Helsinki, Finland) at 405 nm. Anti-OVA titers were defined as the interpolated OD readings on the linear part of the curve with an absorbance of 0.4 mm above background levels. Cytokine determinations were done as follows: 96-well, flat-bottomed plates (Nunc) were coated with 2.5 μg/ml rat anti-mouse IFN-γ (BD Biosciences) or 1–5 μg/ml Abs against IL-4 or IL-10 using the DuoSets for ELISA (R&D Systems) according to the manufacturer’s instructions. Serial 3-fold dilutions of supernatants (1/5) and standard recombinant IFN-γ, IL-4, or IL-10 (R&D Systems) were added to appropriate subwells, and the plates were incubated overnight. Bound cytokines were detected by sequential incubations with alkaline phosphatase-conjugated polyclonal rabbit anti-mouse IFN-γ, anti-IL-4, or anti-IL-10 mAbs as described (29). The phosphatase substrate (1 mg/ml Sigma-Aldrich) was added to the wells, and the enzymatic reaction was analyzed using a Titertek Multiscan (Labsystems) at 405 nm. Cytokine concentrations were expressed as mean pg/ml ± SD of each group as calculated from the plotted standard curves of serial dilutions of the recombinant cytokines. The sensitivity for detection of the respective cytokine was 5 pg/ml.
FACS analysis
Lymphocytes from spleen or LNs were suspended in PBS supplemented with 0.1% BSA and 0.02% EDTA and labeled with Abs specific for CD25-FITC, CD4-APC, CD4-FITC, KJ-126-PE (BD Biosciences), KJ-126-FITC, or IL-10-PE (BD Biosciences) or forhead box P3 (Foxp3)-PE mAbs (eBioscience, San Diego, CA), after fixing and permeabilizing cells using the FoxP3 staining set (eBioscience). To ensure single-cell suspensions, the cells were filtered through a MACS preseparation filter (Miltenyi Biotec). The cells were kept in PBS supplemented with 0.1% BSA and 0.02% EDTA and analyzed on an LSR-II flow cytometer (BD Biosciences) using DiVa software (BD Biosciences). The data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

Statistical analysis
Significant differences between treatment groups were assessed by one-way ANOVA test. The analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL). * indicates p < 0.05.

Results
ADP-ribosylation controls the outcome of mucosal tolerance or active immunity
Previous studies have documented tolerance-inducing properties in CTB, whereas CT holotoxin is associated with adjuvant-augmenting effects when given at mucosal sites (3, 4, 13, 16). To specifically address whether the ADP-ribosylating property of the CT holotoxin could be the key element responsible for changing tolerance into active effector cell immunity, we made two gene fusion proteins, differing by one amino acid replacement only. The CTA1-DD adjuvant molecule was redesigned to host the MHC class II-restricted OVA peptide 323–339, CTA1-OVA-DD, and by site-directed mutagenesis, we killed the ADP-ribosylating property of CTA1 in the mutant, CTA1R7K-OVA-DD (Fig. 1A). These molecules were analyzed by Western blotting and then screened for protein chemical properties, such as enzymatic activity and IgG binding. As predicted, the two fusion proteins were identical except for the lack of ADP-ribosylating activity in CTA1R7K-OVA-DD (Fig. 1B). The ADP-ribosylating ability of CTA1-OVA-DD was comparable to that of CT at a molar level, whereas the CTA1R7K-OVA-DD mutant was devoid of enzymatic activity, as tested in the NAD-agmatine assay (C, left panel). Thus, both T and B cell responses were impaired in CTA1R7K-OVA-DD–treated mice (p < 0.05), whereas the enzymatically active molecule enhanced (p < 0.05) the anti–OVA-specific IgG response (Fig. 1D, inset). This, the ADP-ribosylating property of CTA1-OVA-DD prevented development of tolerance of both T cell- and B cell-mediated responses to OVA, and hence, it appeared that a single amino acid replacement could control the outcome of tolerance or active immunity of this fusion protein following mucosal immunization. The enzymatically inactive CTA1R7K-OVA-DD fusion protein was clearly an effective mucosal tolerogen when given at 5 μg i.n., and subsequent experiments established that even doses 10-fold lower had some tolerance-inducing effect (data not shown).

Induction of tolerance in TCR-Tg T cells
The previous experiment did not reveal at what level the impaired T cell response in treated BALB/c mice was established. For example, deletion of Ag-specific T cells would result in lack of specific T cells in the cultures. Therefore, we extended our analysis using the same protocol, but now we exploited BALB/c mice adoptively transferred with naive OVA p323–339 peptide-specific TCR-Tg CD4+ T cells from DO11.10 mice (30). This enabled us to assess, prior to culture, the presence of potential responder T cells...
and allowed us to label these cells with CFSE to follow their proliferation to recall Ag in vitro. Using the clonotypic Ab KJ-126, which distinctly identifies OVA-specific T cells, we found that OVA-specific T cell numbers did not significantly differ in the respective cultures (Fig. 2A). However, mice treated with CTA1R7K-OVA-DD showed reduced proliferation and production of IFN-γ in response to peptide in vitro as compared with T cells from untreated (PBS) mice (Fig. 2B). In fact, the responses to recall Ag were as low as in naive mice after adoptive transfer without i.p. challenge immunization (Fig. 2B). For comparison, mice given OVA or p323–339 peptide in equimolar or 20-fold higher doses as a single treatment i.n. demonstrated no reduction in T cell responses compared with that seen with cells from untreated (PBS) mice (Fig. 2B). Moreover, CFSE-labeled cells gated on KJ-126+ CD4+ T cells largely failed to enter cell division when derived from i.n. CTA1R7K-OVA-DD–treated mice, as illustrated in Fig. 2, whereas cells from untreated or peptide-treated mice demonstrated strong unimpaired proliferation to recall Ag in vitro (Fig. 2C). Thus, the CTA1R7K-OVA-DD molecule provided a unique and effective formulation for the induction of peptide-specific tolerance. Interestingly, the unresponsiveness in isolated T cells extended not only to spleen and cervical LN, but involved all peripheral LNs, including the mesenteric LNs, indicating a state of global tolerance in these cells following i.n. treatment (data not shown).

In vitro restoration of responsiveness in tolerized T cells

To follow-up on the poor in vitro proliferative response to recall Ag in KJ-126+CD4+ T cells from CTA1R7K-OVA-DD–treated mice, we investigated the potential to restore responsiveness by changing culture conditions and stimuli. Freshly isolated splenocytes were taken from i.n.-treated and i.p.-challenged adoptively transferred BALB/c mice and cultured in the presence or absence of peptide and IL-2 or anti–IL-10 mAbs. We found that the addition of IL-2 completely restored proliferative responses in cultures with tolerized T cells, whereas it had no effect in untreated (PBS) cells, suggesting anergy in the former T cells (Fig. 3A) (31). Furthermore, the addition of neutralizing anti–IL-10 mAbs also restored proliferative responses to peptide in the CTA1R7K-OVA-DD–treated CD4+ T cells, suggesting that IL-10 exerted a regulatory function in the cultures. These proliferative responses were fully reflected in the increased production of IFN-γ, indicating that T cell effector functions could also be restored in CTA1R7K-OVA-DD–tolerized CD4+ T cells by the addition of IL-2 or anti–IL-10 mAbs (Fig. 3B).

Intranasal CTA1R7K-OVA-DD induces IL-10–producing regulatory T cells

Given that whole spleen CD4+ T cell responses to recall Ag stimulation were reduced if mice had been treated i.n. with CTA1R7K-OVA-DD, we next FACS-sorted KJ-126+CD4+ T cells to specifically analyze the response in this target population. Highly enriched (≈98%) OVA-specific CD4+ T cells from untreated (PBS) or i.n.-treated mice were cultured in the presence of peptide and irradiated splenic APCs from T cell-deficient nu/nu mice. Cell proliferation and cytokine production were assessed. We observed that the enriched KJ-126+CD4+ T cells from the spleen of i.n. CTA1R7K-OVA-DD–treated mice demonstrated severely impaired proliferation and reduced IFN-γ and IL-4 responses to recall Ag as compared with that of similarly enriched T cells from untreated (PBS) control mice (Fig. 4). By contrast, the production of IL-10 in these cultures was significantly enhanced compared with KJ-126+CD4+ T cell cultures from untreated (PBS) mice (Fig. 4). The latter finding indicated that CTA1R7K-OVA-DD–tolerized KJ-126+CD4+ T cells largely failed to proliferate or produce IL-4 or IFN-γ as a consequence of the presence of regulatory T cells (Tregs) producing IL-10, thereby effectively preventing Th1- or Th2-type responses. Subsequent phenotypic analysis by FACS of the sorted KJ-126+CD4+ T cells failed to detect CD25+ or FoxP3+ CD4+ T cells, but clearly detected a subpopulation expressing high levels of IL-10, arguing in favor of induction of the Tr1 type of Tregs by i.n. treatment with CTA1R7K-OVA-DD (Fig. 4) (32). Importantly, no significant

![FIGURE 2. Induction of tolerance in TCR-Tg T cells. BALB/c mice were adoptively transferred with DO11.10 TCR-Tg splenocytes 1 d before i.n. treatment with 5 μg CTA1R7K-OVA-DD. Control mice were treated i.n. with PBS. 1 μg OVA323–339 peptide (20-fold higher dose of peptide than the fusion protein), or an equimolar dose of OVA at 6 μg. Ten days later, mice were challenged i.p. with 200 μg OVA in Ribi Adjuvant (TriChem). Mice were sacrificed 16 d after i.n. administration, and the percentage of KJ-126+CD4+ T cells in freshly isolated splenocytes was determined by FACS prior to culture with recall Ag (A). Whole splenocytes were cultured in trypitope and stimulated with recall 1 μM OVA323–339 peptide. Ag-specific cell proliferation after 72 h was assessed by [3H] TdR uptake, and values are given as mean cpm ± SD of five individually analyzed mice in the indicated groups (B, left panel). IFN-γ production was assessed after 96 h of culturing with recall Ag, and the values are given as mean cytokine concentrations in pg/ml ± SD and represent five individually analyzed mice in the indicated groups (B, right panel). These values are from a representative experiment of three giving similar results. Prior to culturing, the freshly isolated splenocytes from treated mice were labeled with CFSE and cultured for 3 d with recall Ag and analyzed by FACS for cell divisions (C). *p < 0.05.]
induction of IL-10–producing KJ-126+ T cells was achieved by i.n. treatment with the enzymatically active CTA1-OVA-DD as assessed by FACS of sorted T cells (Fig. 4C).

Failure to induce Tregs by i.n. CTA1R7K-OVA-DD in IL-10–deficient mice

Next, we asked whether Tregs were induced following i.n. treatment with CTA1R7K-OVA-DD in IL-10–deficient mice. We treated both wild-type C57BL/6 and IL-10−/− mice i.n. with a single dose of CTA1R7K-OVA-DD or PBS. Following i.p. challenge with OVA plus Ribi Adjuvant (TriChem), we isolated splenocytes and cultured these cells in the presence or absence of recall Ag. Strikingly, whereas tolerance was induced in wild-type mice, no tolerance was induced in IL-10−/− mice, and both proliferation and IFN-γ production were unaltered or even enhanced (Fig. 5). These findings supported the notion that i.n. treatment of mice with CTA1R7K-OVA-DD effectively stimulated IL-10–producing Tregs of the Tr1 type.

CTA1R7K-OVA-DD tolerance is Ag-specific, long-lived, and global in nature

To assess to what extent the tolerance induced by i.n. treatment with the mutant fusion protein was maintained in vivo and affected newly arriving naive cells, we undertook the following experiment: BALB/c mice were treated i.n. with a single dose of CTA1R7K-OVA-DD or PBS, and 10 d later mice were challenged i.p. with 200 μg OVA in Ribi Adjuvant (TriChem). Whole splenocytes were cultured in triplicate and stimulated with recall 1 mM OVA323-339 peptide in the presence or absence of 7.5 ng/ml recombinant IL-2 or 10 μg anti–IL-10 mAb. Ag-specific cell proliferation after 72 h was assessed by [3H] TdR uptake, and values are given as mean cpm ± SD of five individually analyzed mice in the indicated groups (A). IFN-γ production was assessed after 96 h of culturing with recall Ag, and the values are given in pg/ml as means ± SD and represent five individually analyzed mice in the indicated groups (B). All values are given with background cytokine levels subtracted. This is one representative experiment of four giving similar results. *p < 0.05.

Discussion

To our knowledge, this is the first study to unequivocally demonstrate that ADP-ribosyltransferase activity may control responsiveness to a protein administered at mucosal membranes. We
found that a single amino acid replacement in CTA1R7K-OVA-DD, which killed the enzymatic activity of CTA1, changed the outcome from augmenting CD4+ T cell priming to inducing mucosal tolerance in the target T cell population. The nature of this tolerance was long-lasting and appeared to be global, because naive nontolerant T cells adoptively transferred to tolerized mice were unable to respond to a challenge immunization regardless of whether this was given systemically (i.p.) or mucosally (i.n.). The CTA1R7K-OVA-DD protein possessed unique tolerogenic properties, because whole OVA or the p323–339 peptide at doses at least 20-fold higher had no tolerance-inducing ability at all. The tolerance was mediated by Tregs of the Tr1 type making IL-10, which was also further corroborated by our finding that i.n. treatment with CTA1R7K-OVA-DD failed to induce tolerance in IL-10−/− mice (33, 34).

FIGURE 4. CTA1R7K-OVA-DD–tolerized TCR-Tg CD4+ T cells host Tr1-like properties. Following i.n. treatment with PBS or 5 μg CTA1R7K-OVA-DD and an i.p. challenge with OVA plus Ribi Adjuvant of BALB/c mice, previously adoptively transferred with OVA-specific TCR-Tg cells, KJ1-26+CD4+ T cells were sorted by MACS and FACS into high purity (>98%) on day 16 posttolerization (A). The sorted T cells (10,000 cells/well) were cultured together with irradiated splenic APCs from nu/nu mice (100,000 per well) in the absence or presence of 1 mM OVA323–339 peptide, and the proliferative and cytokine responses were detected at 72 h and 96 h, respectively (B). Proliferation was assessed by [3H] TCR uptake and given as mean cpm ± SD of five mice in each group. Cytokine production and levels of IFN-γ, IL-4, and IL-10 were assessed by ELISA and given in pg/ml as means ± SD of five mice in each group. All values are given with unstimulated background cytokine levels subtracted. This is one representative experiment of three giving similar results. Following PBS, 5 μg CTA1-OVA-DD, or 5 μg CTA1R7K-OVA-DD i.n. treatment of adoptively transferred mice and an i.p. challenge with OVA plus Ribi Adjuvant (TriChem), KJ1-26+CD4+ T cells were analyzed directly or after 24 h of culturing for expression of CD25, FoxP3, and intracellular IL-10 by FACS (C). Histograms are representative examples from three independent experiments, showing that CTA1R7K-OVA-DD stimulates Tr1-like cells (IL-10–producing), whereas CTA1-OVA-DD fails to do so (C, right panel). *p < 0.05.

FIGURE 5. IL-10–deficient mice fail to develop tolerance after i.n. treatment with CTA1R7K-OVA-DD. IL-10−/− or wild-type (C57BL/6) mice were treated with PBS (untreated) or 5 μg CTA1R7K-OVA-DD followed by an i.p. challenge with OVA in Ribi Adjuvant (TriChem) 10 d later. The splenic CD4+ T cell responses to recall Ag (1 mM OVA323–339 peptide) were assessed on day 16, and cell proliferation was determined in the cultures at 72 h by [3H] TCR uptake and given as mean cpm ± SD (A). Production of IFN-γ in response to recall Ag was assessed after 96 h of culturing, and values are given in pg/ml and means ± SD of five mice in each group after subtraction of background (unstimulated) values (B). This is one representative experiment of two giving similar results. *p < 0.05.
At least three mechanisms have been used to explain immune tolerance induced by mucosal Ag administration (1). These are: deletion, anergy, or the generation of Tregs (2). The dose of Ag, duration of treatment, and route of administration are crucial factors that determine which of these mechanisms will be involved. Whereas low doses of Ag favor development of Tregs, high Ag loads promote deletion of target T cells. By employing advanced cell sorting, we could determine that in vivo treatment with CTA1R7K-OVA-DD i.n. stimulated IL-10–producing regulatory Tr1 type of cells. The KJ-126/CD4+ T cells making IL-10 were CD25+ and FoxP3+, which corresponds well to previously described Ag-induced Tregs following mucosal Ag delivery (32, 35). Both the Th1 and Th2 cell functions of KJ-126 peptide-specific CD4+ T cells were impaired when stimulated with recall Ag in vitro, whereas IL-10 production was high in these cultures. The addition of anti–IL-10 dramatically restored the in vitro proliferation and Th1 and Th2 functions, supporting that i.n. treatment with CTA1R7K-OVA-DD induced Tr1-like cells. These findings agree well with those of Maynard et al. (36), who reported that in vivo-isolated Tr1-like FoxP3+ Tregs, enriched in the GALT, were partially impaired in their regulatory function by the addition of anti–IL-10R mAb. Our finding of Tr1-like activity following i.n. treatment with CTA1R7K-OVA-DD is also consistent with the original reports from Groux et al. (37) on in vitro-generated Tr1 cells. Moreover, our evaluation of activation requirements in vitro revealed that the tolerized T cells were in a state of anergy, a hallmark of Tregs (38), because exogenous IL-2 could restore cell division and Th1 and Th2 cytokine production (31).

The mechanism by which IL-10 exerts inhibitory control over T effector cell functions is still much debated (35, 39). Most investigators agree that these mechanisms are STAT3-dependent, but whether the IL-10 blocks APC functions exclusively or whether direct effects on the T cells involving TCR signal transduction could be affected by IL-10 has not been completely resolved (40–42). It is well established that IL-10 prevents APC function by impairing costimulation and cytokine production and, thereby, indirectly inhibits Th1 and Th2 development (43–45). However, some studies also point to direct effects via IL-10 receptors on the T cells, such as blocking of IL-2 production, and, more recently, Taga et al. and Naundorf et al. (46, 47) have reported inhibitory effects by IL-10 directly on IFN-γ production in memory CD4+ T cells.

It is well documented that high doses of protein given orally, i.n., or at other mucosal sites stimulate tolerance against the protein itself. However, we achieved tolerance with nanogram doses of peptide in CTA1R7K-OVA-DD, whereas equimolar or 20-fold higher doses of either whole OVA or peptide failed to stimulate tolerance. This is remarkable, because most other systems require 1000–10,000-fold higher doses to induce tolerance (48, 49). One exception is the conjugates between Ag and the CTB molecule, or the closely related E. coli heat-labile toxin (LT) B, which are both excellent tolerogens when delivered at mucosal sites (50). It has been thought that the binding ability of CTB to the ganglioside receptors present on all nucleated cells is important for this function. This is partly supported by the lack of immunomodulating properties in the G33 mutant of the B subunit, which disrupts the ganglioside-binding ability completely (51). Contrary to CTB or LT, CTA1R7K-OVA-DD cannot bind to ganglioside receptors, and, subsequently, it is an example of a molecule with tolerogenic properties independent of the ganglioside receptor-binding ability (24). This fact may be critical for future studies aiming at unraveling the mechanism for the tolerogenic effect of these molecules, because our findings with CTA1R7K-OVA-DD indicate that ganglioside receptor-independent pathways for mucosal tolerance induction using low Ag doses also exist.

A difference between the CTB conjugates and the CTA1R7K-OVA-DD system is the dominant Treg population that is responsible for the tolerance. Whereas we found induction of the Tr1 type of Tregs, CTB conjugates have been found to stimulate primarily CD25+FoxP3+ Tregs, but FoxP3+CD25+ Tregs were also found (52). Recent findings have suggested that B cells were...
critical for the induction of the FoxP3^{+}CD25^{+}CD4^{+} Tregs, whereas the less prominent B cell-independent pathway generated FoxP3^{+}CD25^{+}CD4^{+} Tregs following CTB-conjugated OVA given sublingually (53). Although the CTA1-DD fusion protein was designed to bind to B lymphocytes via the Ig receptors (BCR), and it has also been shown to be taken up via the BCR, we have yet to investigate if the tolerance-inducing ability of CTA1R7K-OVA-DD is dependent on this pathway. At variance with the tolerogenic CTB conjugates, we found only Tregs of the FoxP3^{+}CD25^{+}CD4^{+} phenotype, which were independent of B cells in the report by Sun et al (53). Furthermore, CTA1-DD has been found to function as an adjuvant in the complete absence of B cells, hence dendritic cell (DC)-mediated induction of Ag-specific effector T cell tolerance could be a possibility (23).

Ongoing comparative studies focus on these differences, whereas the prospects of combining the CTB-X conjugate and the CTA1-X-DD systems would perhaps provide two distinctly different and complementary systems for induction of mucosal tolerance. If this is the case, it needs to be better verified. However, because of the problem with ganglioside binding, the safety of CTB conjugates may be compromised, as happened with an E. coli LT-adjuvanted i.n. influenza vaccine that was taken off the market because of several cases of Bell’s palsy (facial paralysis) reported in vaccinees (54). Experimental data in mice have shown accumulation in the olfactory nerve and bulb following i.n. administration of ganglioside-binding toxins or toxin derivatives (25). This side effect may be precluding the use of CTB conjugates in the clinic. In contrast, the mutant CTA1R7K-X-DD molecule would provide a safe and effective platform for the successful therapy of several autoimmune conditions. We have evidence that if we replace the OVA p323–339 peptide with the p259–274 from collagen type II, we can prevent disease from developing in a majority of mice with collagen-induced arthritis (55). This promising result showed a strong correlation between serum IL-10 levels and protection against disease, demonstrating the feasibility of using the mutant CTA1R7K-COL-DD platform for treatment of an autoimmune disease.

Several previous studies have demonstrated that oral tolerance can induce IL-10–producing CD4^{+} Tregs that were FoxP3^{+}CD25^{+} (34, 35, 53, 56). In yet other studies of mucosal tolerance, the Tregs have been found to be CD4^{+}CD25^{+} FoxP3^{+} cells producing IL-10 (36). Thus, both natural CD25^{+} and inducible CD25^{+} Tregs may be involved in curbing autoimmune diseases, as reflected in the collagen-induced arthritis model mentioned above. In the current study, we have evidence that i.n. treatment with CTA1R7K-OVA-DD promotes such CD4^{+}CD25^{+} FoxP3^{+} T~1r~ type of cells. We therefore conclude that it appears that i.n. CTA1R7K-OVA-DD treatment stimulates Treg development, which controls CD4^{+} effector T cell functions, including Th1 and Th2 cells. The mechanism for the tolerance-inducing effect of i.n. CTA1R7K-OVA-DD can only be a matter of speculation at this time. Noteworthy is that a single amino acid was changed (R7K) in the CTA1-DD vector to cause the loss of ADP-ribosylation and the complete change of outcome from an augmenting effect on anti-OVA peptide immunity (CTA1-OVA-DD) to tolerance (CTA1R7K-OVA-DD). This is a remarkable finding that suggests that the immunomodulating effect on the APC may be the key to understanding the tolerogenic mechanism. It is well known that immature DCs can induce Tregs in both experimental animals and in humans (57). This has also been used to dampen collagen-induced arthritis (58–60). ADP-ribosylation is an effective means to drive DCs into maturation, as we have shown with CT (29). Hence, a plausible explanation is that CTA1-DD effectively promotes maturation of the APC, which could be the DC, whereas CTA1R7K-DD fails to do so. Whereas both vectors deliver the OVA to the DC, CD4^{+} T cells are primed, but imprinting of T cell function favors Treg development rather than Th1 or Th2 in the absence of ADP-ribosylation. In this way, a stable and long-term effect of i.n. CTA1R7K-OVA-DD is achieved. Ongoing studies are addressing these possibilities. A particular focus will be given to the potential to treat autoimmune conditions with CTA1R7K-X-DD, where the X represents a known peptide from a disease-associated autoantigen, such as the proinsulin or myelin-basic protein involved in type I diabetes and experimental autoimmune encephalitis, respectively. Finally, the enhancing effect of CTA1-DD was clearly dependent on the enzymatic activity, whereas the tolerogenic effect could be the effect of the DD fragment or a feature of the whole CTA1R7K-OVA-DD molecule. The present study was not designed to explore these possibilities. However, future studies will address this question by introducing mutations in the DD fragment that obliterates the binding ability of this moiety as well as constructs that lack the CTA1R7K moiety.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


