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CTLA-4 Signaling Regulates the Intensity of Hypersensitivity Responses to Food Antigens, but is Not Decisive in the Induction of Sensitization

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Although food allergy has emerged as a major health problem, the mechanisms that are decisive in the development of sensitization to dietary Ag remain largely unknown. CTLA-4 signaling negatively regulates immune activation, and may play a crucial role in preventing induction and/or progression of sensitization to food Ag. To elucidate the role of CTLA-4 signaling in responses to food allergens, a murine model of peanut allergy was used. During oral exposure to peanut protein extract (PPE) together with the mucosal adjuvant cholera toxin (CT), which induces peanut allergy, CTLA-4 ligation was prevented using a CTLA-4 mAb. Additionally, the effect of inhibition of the CTLA-4 pathway on oral exposure to PPE in the absence of CT, which leads to unresponsiveness to peanut Ag, was explored. During sensitization, anti-CTLA-4 treatment considerably enhanced IgE responses to PPE and the peanut allergens, *Ara h 1*, *Ara h 3*, and *Ara h 6*, resulting in elevated mast cell degranulation upon an oral challenge. Remarkably, antagonizing CTLA-4 during exposure to PPE in the absence of CT resulted in significant induction of Th2 cytokines and an elevation in total serum IgE levels, but failed to induce allergen-specific IgE responses and mast cell degranulation upon a PPE challenge. These results indicate that CTLA-4 signaling is not the crucial factor in preventing sensitization to food allergens, but plays a pivotal role in regulating the intensity of a food allergic sensitization response. Furthermore, these data indicate that a profoundly Th2-biased cytokine environment is insufficient to induce allergic responses against dietary Ag. *The Journal of Immunology,* 2005, 174: 174–179.

Food allergy, which results from adverse immune responses to dietary Ag, affects ~2% of the American population (1). Peanut allergy is responsible for the majority of fatal food-induced allergic reactions (2), and the prevalence of peanut allergy seems to be increasing. Recently, progress has been made in the treatment of clinical symptoms in peanut allergic patients (3), but mechanisms responsible for the induction of sensitization to food allergens remain largely unknown.

Mucosal immune responses to orally ingested food Ag are generally based on Ag exclusion and suppressive immunity leading to "oral tolerance" (reviewed in Ref. 4). In the case of allergic hypersensitivity to food Ag, oral tolerance is abrogated. Because costimulation is required for effective immunity, signals delivered by different costimulatory pathways—stimulatory or suppressive—may play a critical role in the balance between oral sensitization and tolerance induction.

The interaction between CD28 on T cells, and its two ligands B7-1 (CD80) and B7-2 (CD86) on APC is considered to be the master costimulatory pathway for optimal T cell responses (5). In contrast to the stimulatory signals provided by CD28, interaction of CD80/CD86 with the CD28 homologue CTLA-4 provides signals that down-modulate T cell activation. Antagonizing CTLA-4 signaling enhances T cell proliferation (6), whereas cross-linking of CTLA-4 in vitro inhibits anti-CD3-induced T cell proliferation (7). Furthermore, CTLA-4-deficient mice develop a fatal lymphoproliferative disease (8), showing a role for CTLA-4 in the establishment of peripheral self tolerance, as well as regulation of active immunity.

Although very little is known about the role of the CD28-CTLA-4/B7 pathway in food allergy, anti-CTLA-4 treatment has been shown to abrogate systemic hyporesponsiveness to orally administered OVA in oral tolerance models (9, 10). This suggests that CTLA-4 signaling might be decisive in oral tolerance development. In addition, recent studies in murine models of allergic asthma (a comparable Th2-induced mucosal hypersensitivity response) suggest an indispensable role for the CTLA-4 signaling pathway in controlling the production of Th2 cytokines, and in the development of airway hyperresponsiveness. As such, inhibition of CD28-B7 interaction ameliorated (11), and even reversed asthmatic manifestations (12), whereas the blockade of CTLA-4 enhanced allergic sensitization (13). In line with these observations, we hypothesized that CTLA-4 is involved in suppressing the induction and progression of sensitization to food proteins.

To test this hypothesis, an established oral peanut sensitization model (with minor modifications) was used. In this model, exposure to peanut extract (PPE)[2] in the presence of the mucosal adjuvant cholera toxin (CT) induces sensitization to peanut, characterized by PPE- and peanut allergen (*Ara h*)-specific IgE serum Ab and cytokine production, and subsequent clinical reactions (14). In

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[2] Abbreviations used in this paper: PPE, peanut protein extract; AP, alkaline phosphatase; CT, cholera toxin; DIG, digoxigenin; MLN, mesenteric lymph node; mmcp-1, mouse mast cell protease-1; Tr, T regulatory.

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the present study, mice were treated with the mAb 4F-10 to inhibit the CTLA-4 signaling pathway, and the effects on parameters in an early and later phase of sensitization were explored. In agreement with our hypothesis, anti-CTLA-4 treatment resulted in enhanced sensitization to PPE and the purified peanut allergens *Ara h 1*, *Ara h 3*, and *Ara h 6*, characterized by a profound elevation of specific-IgE production, and increased clinical reaction upon an oral challenge.

Additionally, the effect of anti-CTLA-4 treatment on oral exposure to PPE without adjuvant, which results in unresponsiveness to peanut Ag (no PPE- or allergen-specific IgE), was investigated. Despite the induction of Th2 cytokines, activation of APC, and an elevation in total serum IgE levels, anti-CTLA-4 treatment failed to induce PPE-specific or *Ara h*-specific IgE Abs or mast cell degranulation.

These results suggest that CTLA-4 signaling plays an important role in regulation of the intensity of hypersensitivity responses to food proteins, but is not decisive in preventing the induction of sensitization to dietary Ag. Moreover, current data challenge the pivotal role of a Th2 cytokine environment in the induction phase of food allergy.

Materials and Methods

Mice

Female, specific, pathogen-free C3H/HeOuJ Ico mice, 4 wks of age, were purchased from Charles River Laboratories and were maintained under barrier conditions in filter-topped macrolon cages with wood chip bedding, at mean temperature of 23 ± 2°C, 50–55% relative humidity, and a 12-h light/dark cycle. Drinking water and standard laboratory food pellets were provided ad libitum. The experiments were approved by the Animal Experiments Committee of the Faculty of Veterinary Medicine, Utrecht University.

Chemicals and reagents and mAbs

Chemicals were obtained from Sigma-Aldrich unless stated otherwise. Cells producing anti-CTLA-4 (4F-10) were obtained from American Type Culture Collection and Abs were purified using thiophilic agarose (SeaKem). Purified hamster IgG (Rockland Immunochemicals) was used as an isotype control. For cytokine measurements, IL-4, IL-5, and IFN-γ capture and detecting Abs, and IL-10 ELISA kit were obtained from BD Pharmingen and streptavidin-HRP from Sanquin. BD Pharmingen was the supplier of rat anti-mouse IgE and the digoxigenin (DIG)-coupling kit was obtained from Boehringer Mannheim and anti-DIG-peroxidase Fab from Roche Diagnostics. Alkaline phosphatase (AP)-conjugated goat anti-mouse, human adsorbed IgG1, IgG2a, and AP-conjugated rat anti-mouse IgE were purchased from Southern Biotechnology Associates. Campina Melkuni was the supplier of milk powder used in blocking buffer. Mouse Ig reference serum with 2 μg/ml monoclonal DNP-specific IgE was obtained from Valeant. Medium that was used for the in vitro restimulation was complete RPMI 1640 with Glutamax-I (Invitrogen Life Technologies) supplemented with 10% FCS (Valeant) and 2% penicillin-streptomycin.

Preparation of PPE and purification of allergens

Peanuts from the Runner cultivar (Cargill) were kindly provided by Imko Nut Products. The PPE was prepared as described by Koppelman et al. (15). Briefly, peanuts were ground and the protein was extracted by mixing 25 g of ground peanut with 20 ml of 20 mM Tris buffer (pH 7.2). After 2 h stirring at room temperature, the aqueous fraction was collected by centrifugation (3000 × *g* for 30 min) and subsequently centrifuged at 10000 × *g* for 30 min to remove residual traces of fat and insoluble particles. The extract contained 30 mg/ml protein as determined by Bradford analysis with BSA as a standard. SDS-PAGE analysis confirmed the presence of proteins migrating at the same molecular weights as the purified allergens *Ara h 1*, *Ara h 3*, and *Ara h 6* (data not shown). The allergens, *Ara h 1* and *Ara h 3*, were purified as described by Koppelman et al. (15), and *Ara h 6* was purified from a side fraction.

Treatment protocol

Mice ($n = 6$) were orally exposed to PPE plus CT, PPE alone, or were left untreated. Oral exposure to PPE was performed by intragastric dosing of 6 mg of roasted PPE on 3 consecutive days, followed by weekly dosing of 6 mg of PPE (4 wks). CT (10 μg) was coadministered on days 1, 2, 3, 8, 15, and 21. Equivalent groups ($n = 6$) were additionally injected i.p. with 100 μg of anti-CTLA-4 mAb on days 1, 3, 7, 14, and 20 during the oral-dosing regime. PPE-exposed mice were also treated with an appropriate isotype control, which had no effect on any of the measured (data not shown). At day 30, mice received an oral challenge with 12 mg of PPE. Mice were killed by cervical dislocation on day 7 or 31 days after the onset of exposure.

Measurement of serum IgG1, IgG2a, and IgE Abs

Blood was collected at weekly intervals and levels of PPE and *Ara h*-specific Abs were measured by ELISA (IgG1 and IgG2a) or sandwich ELISA (IgE). Plates (highbond 3590; Costar) were coated overnight with 20 μg/ml PPE or *Ara h* (for IgG1 and IgG2a detection) or with 1.5 μg/ml purified rat anti-mouse IgE (for Ag-specific and total serum IgE detection) in carbonate buffer (pH 9.6), followed by 1-h blocking (37°C) with PBS-Tween/3% milk powder. Each test serum was titrated starting at 1/8 or 1/16 dilution and incubated for 1 h (37°C). A presera pool was used as reference value (dilution 1/4) for Ag-specific Ab levels, and a standard curve of a reference serum (2 μg/ml IgE) was used to determine total serum IgE levels. For detection of IgG1 and IgG2a and total serum IgE Abs, AP-conjugated Abs were added (1 h at 37°C). Subsequently, 1 mg/ml *p*-nitrophenylphosphate in diethanolamine buffer was used for the color reaction, which was stopped with a 10% EDTA solution, and absorbance was measured at 405 nm using an ELISA reader ELX800 (BIOTEK Instruments). To measure Ag-specific IgE Abs, serum was incubated for 2 h and subsequently a PPE- or *Ara h*-DIG conjugate solution was added (1 h at 37°C). The coupling of DIG to PPE or the *Ara h*'s was performed according to the manufacturer's instructions. Briefly, the coupled proteins were separated on a sephadex G-25 column and labeling efficiency was determined spectrophotometrically at 280 nm. After incubation (1 h at 37°C) with peroxidase-conjugated anti-DIG fragments, a tetramethylbenzidine substrate (0.1 mg/ml) solution was used and the color reaction was stopped with 2 M H_2SO_4. Absorbance was measured at 450 nm. The reciprocal of the furthest test serum dilution resulting in an extinction higher than the reference value was read as a titer.

Cell culture and cytokine measurement

Spleen and mesenteric lymph node (MLN) single-cell suspensions (150 μl of 2.5×10^6 cells/ml in complete RPMI 1640) were incubated in the presence or absence of PPE (200 μg/ml) in 96-well plates for 96 h at 37°C, 5% CO_2. After centrifugation for 10 min at 150 × *g*, supernatant was collected and stored at −20°C until analysis.

In the culture supernatants, IFN-γ, IL-4, IL-5, and IL-10 were determined by sandwich ELISA. Plates (highbond 3590; Costar) were coated overnight with 1.5 μg/ml rat anti-mouse IL-4, IL-5, or IFN-γ, and the following day, plates were blocked with PBS-Tween/3% milk powder for 4 h at room temperature. Samples and cytokine standards were added in several dilutions and incubated overnight at 4°C. Plates were incubated with 0.25 μg/ml rat-anti-mouse IL-4, IL-5, or IFN-γ conjugate for 1 h at room temperature, followed by streptavidin-HRP incubation for 45 min. Finally, tetramethylbenzidine substrate (0.1 mg/ml) was added and the color reaction was stopped with 2 M H_2SO_4. Absorbance was measured at 450 nm. The IL-10 ELISA was performed in accordance with the manufacturer's instructions.

Measurement of serum mouse mast cell protease-1 (mmcp-1)

Blood was collected before, and 1 h after, an oral challenge with PPE at day 30, and serum levels of mmcp-1 were determined with an ELISA kit (Moredun). The ELISA was performed according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using SigmaStat statistical software package (SPSS, Chicago, IL). For serum Ab levels, cytokine levels, and mmcp-1 serum levels, the differences between group means (log-transformed data) were determined by using one-way ANOVA with Bonferroni as post hoc test. Values of $p < 0.05$ were considered statistically significant.

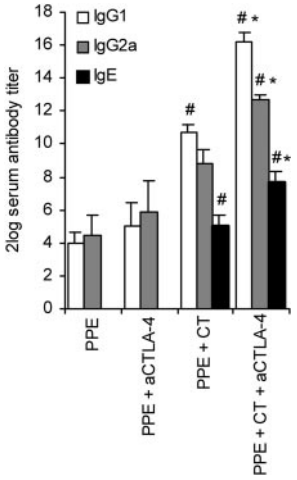

FIGURE 1. Effect of anti-CTLA-4 treatment on PPE-specific Ab production. PPE-specific Th1-associated (IgG2a) and Th2-associated (IgG1 and IgE) serum Ab levels were determined after 4 wks of oral PPE exposure in the presence or absence of CT and with or without anti-CTLA-4 mAb (aCTLA-4) treatment. The data are presented as the mean 2log Ab titer ± SEM of six mice per group. *, Significantly different ($p < 0.01$) from the corresponding non-CTLA-4-treated group. #, Significantly different ($p < 0.01$) from the corresponding non-CT-exposed group.

Results

Different effects of antagonizing CTLA-4 on PPE-specific Ab levels in the absence or presence of an adjuvant

Mice were orally exposed to PPE in the presence or absence of the mucosal adjuvant CT. Additionally, both PPE- and PPE plus CT-exposed mice were treated with anti-CTLA-4 mAb or did not receive mAb treatment.

The group receiving 4 wks of oral treatment with PPE plus CT showed PPE-specific serum IgG1, IgG2a, and IgE (Fig. 1). Blockade of CTLA-4 during the oral sensitization protocol increased ($p < 0.01$) both Th2-related (IgG1 and IgE) and Th1-related (IgG2a) PPE-specific Ab levels compared with PPE plus CT treatment group.

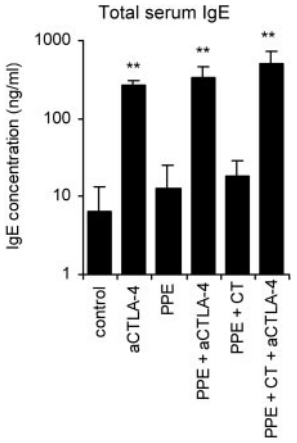

FIGURE 2. Effect of anti-CTLA-4 treatment on total serum IgE levels. Mice were orally exposed to PPE with or without CT or were left untreated (control). Indicated groups received blocking anti-CTLA-4 mAb (aCTLA-4) during the oral exposure protocol. Total serum IgE levels were measured by ELISA after 4 wks of dosing. The data are presented on a log scale as the group mean ± SD of six mice per group. **, Significantly different ($p < 0.01$) from corresponding non-anti-CTLA-4-treated group.

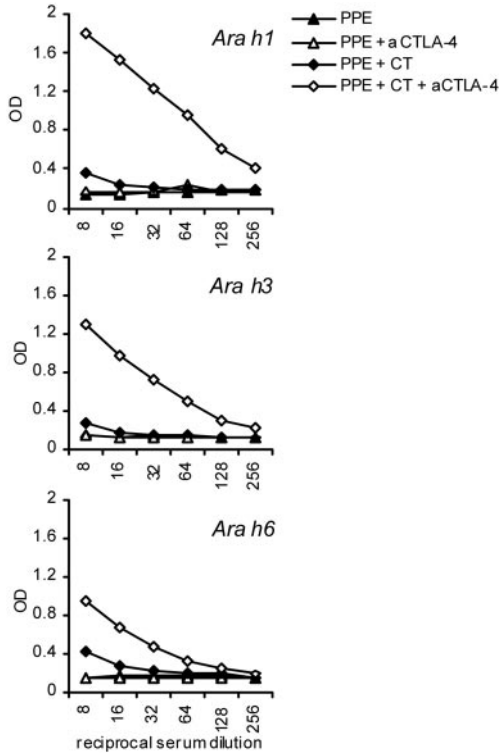

FIGURE 3. Effect of CTLA-4 blockade on allergen-specific IgE Ab responses. Serum IgE Ab levels against the purified peanut allergens *Ara h 1*, *Ara h 3*, and *Ara h 6* were determined after 4 wks of oral PPE exposure in the presence or absence of CT. Indicated groups received blocking anti-CTLA-4 mAb (aCTLA-4) during the oral exposure protocol. *Ara h*-specific IgE Ab levels were measured in pooled sera (six mice per group) using a sandwich ELISA.

The group that was exposed to PPE without CT produced low levels of PPE-specific IgG1 and IgG2a and no IgE. In the similarly exposed group, blockade of CTLA-4 had no effect on Ab levels, demonstrating that hyporesponsiveness to peanut upon oral PPE exposure without adjuvant is not abrogated by anti-CTLA-4 treatment.

Anti-CTLA-4 treatment increases total serum IgE levels

In contrast to PPE-specific IgE levels, anti-CTLA-4 treatment clearly augmented total IgE production in both PPE-exposed groups (irrespective of the presence of CT, see Fig. 2). Mice treated with anti-CTLA-4 without oral dosing of PPE showed a similar significant elevation of total IgE serum levels compared with control mice that received no treatment at all. These data demonstrate a prominent role for T cells expressing CTLA-4 in regulating total circulating IgE levels.

Anti-CTLA-4 treatment increases Ara h*-specific IgE serum levels in sensitized mice*

The remarkable differences between PPE-specific IgE and total IgE levels following CTLA-4 blockade warranted further evaluation of Ag-specific IgE responses.

PPE consists of different peanut proteins, and we have earlier demonstrated that it is possible to determine specific serum IgE Abs against individual proteins from the extract (16). Several peanut proteins are recognized by serum IgE of peanut allergic patients and these allergens are referred to as *Ara h 1-7* (17). In the current study, we used the purified allergens *Ara h 1*, *Ara h 3*, and the minor allergen *Ara h 6* to measure allergen-specific serum IgE

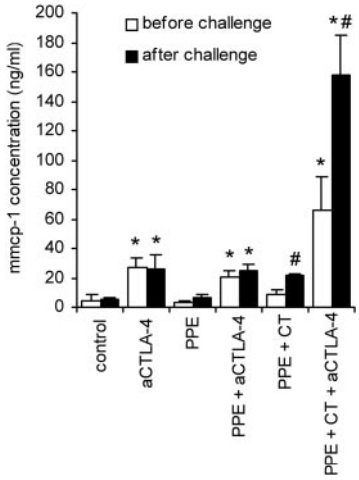

FIGURE 4. Effect of anti-CTLA-4 treatment on mmcp-1 serum levels upon an oral challenge with PPE. Mice were orally exposed to PPE with or without CT or were left untreated (control). Indicated groups received blocking anti-CTLA-4 mAb (aCTLA-4) during the oral exposure protocol. Four weeks after the initial exposure all groups received an oral challenge with 12 mg of PPE. Levels of mmcp-1 were determined by ELISA in blood samples collected before and 1 h after the oral challenge. Levels are expressed as the group mean ± SD of six mice per group. *, Significantly different ($p < 0.05$) from corresponding non-anti-CTLA-4-treated group. #, Significant difference ($p < 0.05$) between levels before and 1 h after oral challenge.

responses upon oral PPE exposure. Treatment with PPE plus CT for 4 wks induced low levels of allergen-specific IgE (Fig. 3). Blockade of CTLA-4 in this group resulted in a profound increase of allergen-specific IgE levels with the highest responses against *Ara h 1*, followed by *Ara h 3* and *Ara h 6*.

No allergen-specific IgE was found after oral exposure to PPE alone. Treatment with anti-CTLA-4 mAb during PPE exposure in the absence of adjuvant did not provoke IgE responses against *Ara h 1*, *Ara h 3*, or *Ara h 6*, confirming that CTLA-4 blockade does not overcome the IgE unresponsiveness to peanut allergens in mice that are orally exposed to PPE without an adjuvant.

Anti-CTLA-4 treatment enhances mast cell degranulation upon oral challenge with PPE

Clinical symptoms in peanut allergic patients are caused by mast cell mediators that are released upon ingestion of peanut. In the current murine model, mast cell degranulation upon an oral challenge with PPE was determined by measuring serum levels of the mast cell mediator mmcp-1 before and after challenge. Treatment with PPE plus CT induced a significant elevation of mmcp-1 serum levels after challenge compared with control and PPE-treated mice (Fig. 4).

Anti-CTLA-4 treatment induced higher base levels of serum mmcp-1 before oral challenge compared with levels in non-anti-CTLA-4-exposed animals. Blockade of CTLA-4 only induced an increase in mmcp-1 serum concentration after oral challenge in the PPE plus CT treatment group, with mmcp-1 levels being four times higher than in the PPE plus CT treatment group. These results demonstrate that the challenge response to PPE is exacerbated by anti-CTLA-4 treatment.

Enhancement of Th2-related cytokine production upon anti-CTLA-4 treatment

The cytokine environment plays an important role in the development of sensitization to food proteins, including the production of

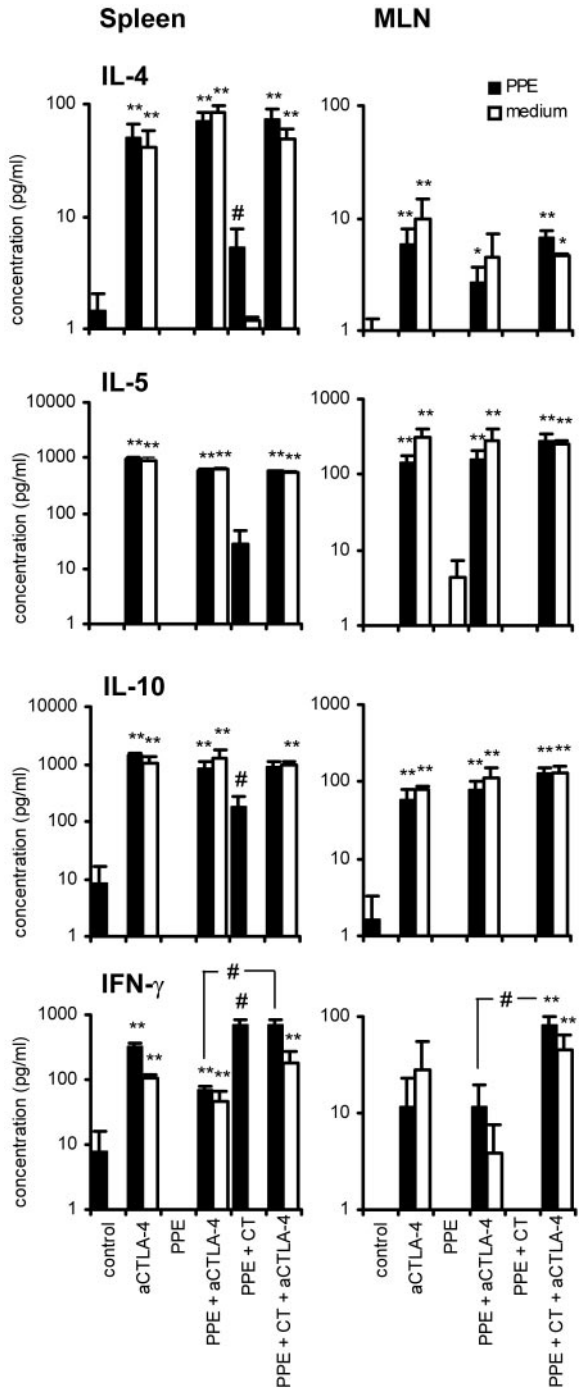

FIGURE 5. Effect of CTLA-4 blockade on cytokine levels in splenocyte and MLN cell culture supernatants. Mice were orally exposed to PPE with or without CT or were left untreated (control). Indicated groups received blocking anti-CTLA-4 mAb (aCTLA-4) during the oral exposure protocol. Spleen and MLN cells were removed 4 wks after the initial oral exposure and were cultured (2.5×10^6 cells/ml) for 96 h in the presence or absence of PPE (200 μg/ml). Culture supernatants were collected and cytokine levels of IL-4, IL-5, IL-10, and IFN-γ were determined by ELISA. Levels of cytokines induced by restimulation with an irrelevant protein BSA were similar to levels induced in cultures with medium alone (data not shown). Data are presented on a log scale as the group mean ± SEM of 6 mice per group. *, Significantly different (*, $p < 0.05$, **, $p < 0.01$) from the corresponding non-anti-CTLA-4-treated group. #, Significantly different ($p < 0.05$) from the corresponding non-CT-treated group.

Ag-specific Abs. Therefore, the effect of CTLA-4 blockade on the production of IL-4, IL-5, IL-10, and IFN-γ was investigated, 4 wks after the onset of exposure to PPE, by culturing single-cell suspensions in the presence or absence of PPE. Treatment with anti-CTLA-4 significantly enhanced Th2-associated (IL-4 and IL-5) and T regulatory (Tr)-related (IL-10) cytokine production in both MLN and spleen independent of PPE or CT administration (Fig. 5). Cells cultured in the presence or absence of PPE produced comparable amounts of IL-4, IL-5, and IL-10, indicating that the stimulation of cytokine production was polyclonal. This implies that a Th2 cytokine environment is not the key factor in sensitization to PPE.

Levels of the Th1-associated cytokine IFN-γ were also elevated in the anti-CTLA-4-treated groups (Fig. 5). However, coadministration of the adjuvant CT significantly elevated IFN-γ levels compared with levels in the non-CT-exposed groups. Furthermore, dosing with PPE plus CT induced Ag-specific IFN-γ, illustrated by significantly higher levels in cultures restimulated with PPE.

Because development of sensitization to food proteins occurs already in an early phase of exposure, we additionally examined the effect of CTLA-4 blockade early (7 days after the primary exposure to PPE) in the response. Also in an early stage, anti-CTLA-4 treatment strongly induced IL-5, IL-10, and IFN-γ cytokine production in cultures of both spleen and MLN compared with non-mAb-injected groups (data not shown).

Conclusively, CTLA-4 blockade induces non-PPE-specific IL-4, IL-5, and IL-10 production in both spleen and MLN, whereas the adjuvant CT seems to be the most important factor for the induction of IFN-γ production.

Discussion

In the current study, the involvement of CTLA-4 signaling in immune responses to oral food allergens was investigated and it was demonstrated that CTLA-4 regulates the intensity of a food allergic response but is not decisive in the induction of sensitization.

Blockade of functional CTLA-4 signaling during oral sensitization to PPE plus CT resulted in enhanced IgE responses against PPE and the peanut allergens, *Ara h 1*, *Ara h 3*, and *Ara h 6*, leading to a more pronounced mast cell degranulation response upon oral challenge with PPE. Disease exacerbation was accompanied by an increased production of Th2-associated cytokines (IL-4, IL-5, and IL-10) and the Th1-associated cytokine IFN-γ in both spleen and MLN.

CTLA-4 signaling thus seems to play a pivotal role in regulating the intensity of food allergic responses. Similarly, Hellings and colleagues (13) demonstrated an aggravation of Th2-mediated allergic airway disease during anti-CTLA-4 treatment. Furthermore, in humans, CTLA-4 promoter polymorphism has been linked to total serum IgE levels (18) and bronchial hyperresponsivity (19) in asthmatic patients, meaning CTLA-4 is implicated in regulating the intensity of allergic disease. Several nonmutually exclusive mechanisms can be proposed that may underlie regulation of an allergic response by CTLA-4 signaling. CTLA-4 engagement may have a direct suppressive effect on Th2 cells as shown by Oosterwegel and colleagues (20). Furthermore, CTLA-4 is also expressed on B cells, and CTLA-4 signaling on these cells has been shown to inhibit IL-4-driven isotype switching, thereby possibly preventing allergen-specific IgG1 and IgE production (21). Thus, in the present study, anti-CTLA-4 treatment may have directly affected T and B cell responses, resulting in increased Th2-associated responses against PPE allergens and concomitant aggravated food allergic disease. CTLA-4 inhibition may additionally have an indirect effect via $CD4^+CD25^+$ Tr cells that down-regulate immune responses in a polyclonal fashion. In the mouse intestine, this population of Tr cells has been shown to constitutively express CTLA-4 and is able to control intestinal inflammation (22). Cross-linking of CTLA-4 (resulting in CTLA-4 signaling) can induce TGF-β production by murine $CD4^+$ T cells (23) and the suppressive effect of these Tr cells has been demonstrated to be TGF-β dependent (22). The disruption of Tr cell function by anti-CTLA-4 treatment may have played an important role in the increased immune responses against peanut allergens that were observed in the present study.

Besides the role of CTLA-4 in regulating the intensity of an allergic response, it was investigated whether abrogation of CTLA-4 signaling also resulted in the induction of active immunity against oral Ag. It has been suggested that CTLA-4 is required for oral tolerance induction. However, this remains controversial because it has been reported that CTLA-4 blockade can abolish oral tolerance induced by oral exposure to OVA (9, 10, 24), while others have reported that CTLA-4 blockade failed to overcome development of oral tolerance to OVA in a similar model (25). In the present model, mice orally exposed to peanut without adjuvant do not develop PPE-specific IgE and only very low IgG responses. We hypothesized that inhibition of CTLA-4 would abrogate this "unresponsiveness" to peanut proteins and would induce a productive PPE-specific humoral response. However, anti-CTLA-4 treatment failed to induce PPE- or *Ara h*-specific IgE Ab responses following PPE exposure, suggesting that CTLA-4 signaling is not decisive for the induction of sensitization to food proteins. Accordingly, in contrast to the severity of asthma, no significant association between incidence of asthma or atopy and the CTLA-4 polymorphic loci has been found in humans (18).

Because we demonstrated that an allergic response to food Ag is not initiated by CTLA-4 blockade, the question remains which factors are responsible for the development of sensitization to food proteins. In the present model, coadministration of the mucosal adjuvant CT turned out to be crucial for the induction of sensitization to PPE. CT is thought to stimulate Th2-dependent immune responses to a bystander Ag by provoking Th2 cytokine production (26). This phenomenon was also observed—-to a much larger extent—upon anti-CTLA-4 treatment. Thus, our results suggest that excessive Th2 cytokine production per se is insufficient for the development of active immunity against food proteins and challenge the concept of Th2-bias as the key factor for food allergy (27).

For the induction of sensitization, a significant proportion of the intact protein has to be absorbed by the intestine and in food-allergic patients increased protein absorption has been reported. Thus, an alternative explanation for the impact of CT may be the influence on (systemic) availability of bystander Ag for the immune system. The most prominent effect of CT in the present experiments was PPE-induced IFN-γ production. One of the effects attributed to IFN-γ is the induction of changes in epithelial permeability and intestinal integrity (28). Additionally, CT itself promotes dendritic cell activation (29, 30) and migration (31), which facilitates Ag presentation to the immune system.

Anti-CTLA-4 treatment (with or without PPE) induced a profound increase in total serum IgE levels and polyclonal Th2 cytokine production. Blocking CTLA-4/B7 interaction promotes Th2 differentiation (20), which may explain the observed excessive Th2 cytokine production and IgE levels. It has also been shown that CTLA-4 plays a major role in down-regulating autoaggressive T cells (32, 33) and that CTLA-4-deficient mice develop a fatal lymphoproliferative autoimmune disorder (8). Concurring, in the present study, higher levels of IgG Abs against ssDNA-specific were found in the serum of anti-CTLA-4-treated mice compared

with control mice (data not shown), suggesting autoantibody formation upon anti-CTLA-4 exposure.

The elevated levels of the mast cell mediator mmcp-1 that were observed upon anti-CTLA-4 treatment (in the absence of oral Ag) demonstrate that mast cells are also affected by anti-CTLA-4 treatment. The high production of IL-4 and IL-10 cytokines and serum IgE Abs in anti-CTLA-4-treated mice may have enhanced mast cell proliferation and differentiation as described by Lukacs and colleagues (34). In addition, the CTLA-4 ligands CD80 and CD86 are expressed on mast cells (35). It is unknown whether ligation of CTLA-4 with these molecules may have a direct suppressive effect on mast cells, but it might be another explanation for the observed mast cell degranulation upon anti-CTLA-4 treatment.

Taken together, these results suggest that in food allergy CTLA-4 signaling is involved in regulating the intensity of the hypersensitivity responses, but is not decisive for induction of sensitization to orally ingested Ag. Accordingly, CTLA-4 may represent a potential target for treatment of food allergic disorders. Furthermore, these findings imply that the initiation of food allergy requires another crucial factor in addition to a Th2 cytokine environment.

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