

The CD28/CTLA-4-B7 Signaling Pathway Is Involved in Both Allergic Sensitization and Tolerance Induction to Orally Administered Peanut Proteins

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Dendritic cells are believed to play an essential role in regulating the balance between immunogenic and tolerogenic responses to mucosal Ags by controlling T cell differentiation and activation via costimulatory and coinhibitory signals. The CD28/CTLA-4-CD80/CD86 signaling pathway appears to be one of the most important regulators of T cell responses but its exact role in responses to orally administered proteins remains to be elucidated. In the present study, the involvement of the CD28/CTLA-4-CD80/CD86 costimulatory pathway in the induction of allergic sensitization and oral tolerance to peanut proteins was investigated. In both an established C3H/HeOuJ mouse model of peanut hypersensitivity and an oral tolerance model to peanut, CD28/CTLA-4-CD80/CD86 interactions were blocked using the fusion protein CTLA-4Ig. To examine the relative contribution of CD80- and CD86-mediated costimulation in these models, anti-CD80 and anti-CD86 blocking Abs were used. In the hypersensitivity model, CTLA-4Ig treatment prevented the development of peanut extract-induced cytokine responses, peanut extract-specific IgG1, IgG2a, and IgE production and peanut extract-induced challenge responses. Blocking of CD80 reduced, whereas anti-CD86 treatment completely inhibited, the induction of peanut extract-specific IgE. Normal tolerance induction to peanut extract was found following CTLA-4Ig, anti-CD86, or anti-CD80 plus anti-CD86 treatment, whereas blockade of CD80 impaired the induction of oral tolerance. We show that CD28/CTLA-4-CD80/CD86 signaling is essential for the development of allergic responses to peanut and that CD86 interaction is most important in inducing peanut extract-specific IgE responses. Additionally, our data suggest that CD80 but not CD86 interaction with CTLA-4 is crucial for the induction of low dose tolerance to peanut. *The Journal of Immunology*, 2007, 178: 6894–6900.

Food allergy has emerged as a major health problem in westernized countries and peanut allergy is one of the most serious of the hypersensitivity reactions to foods in terms of severity and persistence. At present no therapy is available and elucidation of the underlying mechanisms of allergic sensitization may contribute to the development of novel therapeutic strategies.

Under normal circumstances, oral administration of food proteins leads to a state of systemic immunologic hyporesponsiveness, known as oral tolerance (1). Impaired oral tolerance induction or a failure to maintain oral tolerance may result in the development of hypersensitivity responses to food Ags (2). Dendritic cell (DC)²-T cell interactions, leading to either activation or suppression of T cells, represent an important event in controlling the delicate bal-

ance between sensitization and tolerance. Activation of T cells requires, besides TCR-MHCII/peptide complex recognition, additional secondary signals provided by costimulatory molecules expressed on APCs. The interaction between CD28 on T cells, and its two ligands B7-1 (CD80) and B7-2 (CD86) on APCs is considered to be the master costimulatory pathway for optimal T cell responses (3). CD86 is constitutively expressed on APCs at low levels and rapidly up-regulated upon stimulation, whereas CD80 is inducibly expressed later than CD86. In contrast to the stimulatory signals provided by CD28, interaction of CD80 or CD86 with the CD28 homolog CTLA-4 induces signals that down-regulate T cell activation. CTLA-4 is constitutively expressed only on CD4⁺CD25⁺ regulatory T cells and is induced on activated T cells and we have previously shown that CTLA-4 signaling plays an important role in regulating the intensity of allergic disease (4).

Type I food allergy is characterized by Th2-associated responses resulting in increased levels of allergen-specific IgE. CD28-B7 engagement has been shown to be required for Th2 cell development (5, 6). Studies in murine models also suggest a critical role for CD28/CTLA-4-B7 interactions in the production of Ag-specific IgE responses and the development of allergic asthma (7–9). However, very little is known about the requirement of CD28-B7 costimulation in the development of hypersensitivity responses to orally administered proteins. Although it seems likely that costimulation will play a role in orally induced immune responses regarding the data on airway allergy, the question remains to what extent the CD28-B7 pathway plays a role, and whether CD80 and CD86 are equally involved.

Furthermore, it is still controversial whether B7-mediated costimulation is also involved in oral tolerance induction. It is well

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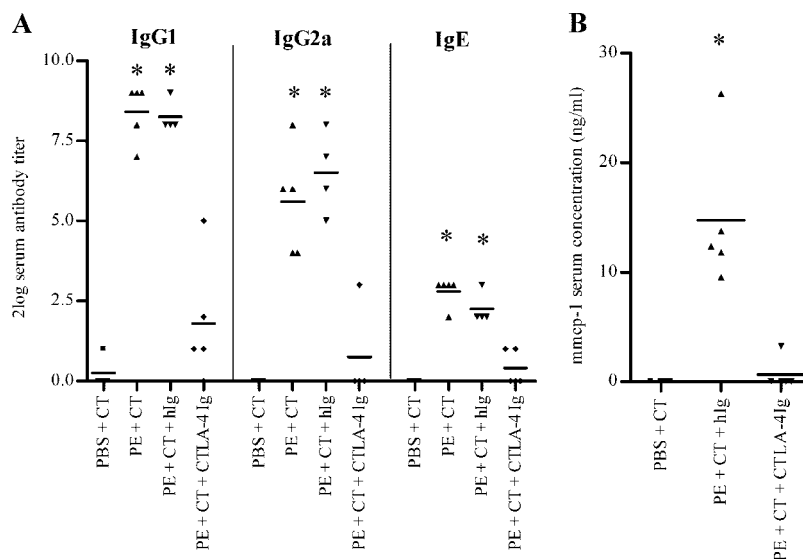
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² Abbreviations used in this paper: DC, dendritic cell; DIG, digoxigenin; CT, cholera toxin; Treg, regulatory T cell.

FIGURE 1. Effect of CTLA-4Ig treatment on peanut extract-specific Ab responses and mast cell degranulation. Mice were orally exposed for 4 wk to PBS plus CT (control) or peanut extract plus CT (sensitization). During oral exposure indicated groups were treated with CTLA-4Ig or control Ab (hIgG). **A**, Peanut extract-specific Th1-associated (IgG2a) and Th2-associated (IgG1 and IgE) serum Ab levels after 4 wk of exposure. The data are presented as the mean $^2\log$ Ab titer of 4–5 mice per group (symbols indicate individual animals). **B**, mmcp-1 serum levels upon an oral challenge with peanut extract. Levels of mmcp-1 were determined in blood samples collected 45 min after the oral challenge. The data are presented as the group mean (symbols indicate individual animals) of 4–5 mice per group. *, Significantly different ($p < 0.05$) from control (PBS + CT) group.



established that in the absence of costimulation, tolerance is induced by clonal T cell anergy (10). However, when low levels of B7 are expressed, they might actually engage CTLA-4 (which has a much higher affinity for both B7 receptors than CD28), thereby contributing to oral tolerance. It has accordingly been demonstrated by Liu et al. (11) that CD86 but not CD80 interaction is essential for low dose oral tolerance induction to OVA. Interestingly, other evidence points to a possible role for CD80 signaling in tolerance induction (12).

In the present study, we have investigated the involvement of CD28/CTLA-4-B7 pathway in the development of sensitization and tolerance to orally administered peanut proteins. For this purpose, CTLA-4Ig fusion protein, which prevents interactions of CD28/CTLA-4 on T cells with B7 on APCs, and the antagonizing mAbs anti-CD80 and anti-CD86 were used in an oral tolerance model and in an established model of peanut hypersensitivity.

We show here that CTLA-4Ig treatment completely inhibits the development of hypersensitivity responses to peanut and that CD86 interaction is most important in inducing optimal peanut-specific IgE responses. Additionally, our data indicate that CD80 but not CD86 plays an essential role in the induction of low dose oral tolerance to peanut proteins.

Materials and Methods

Mice

Female specific pathogen-free C3H/HeOuJ Ico mice (4–5 wk of age) purchased from Charles River were maintained under barrier conditions in filter-topped macrolon cages. Drinking water and peanut-free 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, reagents, and mAbs

Peanuts from the Runner cultivar (Cargill Peanut Products) were provided by Imko Nut Products and peanut extract was prepared as previously described (4, 13). The extract contained 30 mg/ml protein as determined by Bradford analysis (Pierce) with BSA as a standard.

Cells producing anti-CD80 (HB-301; 16-10A1), anti-CD86 (HB-253; GL-1), and anti-CTLA-4 (4F-10) were obtained from American Type Culture Collection. Total hamster IgG and total rat IgG (Rockland Immunochemicals) were used as appropriate isotype controls for anti-CD80 and anti-CD86/anti-CTLA-4, respectively. Cells producing the pan-specific TGF- β neutralizing Ab 1D11 were obtained from ATCC and mouse IgG1 (provided by P. van Kooten, Utrecht) was used as an appropriate isotype control. All Abs were purified using thiophilic agarose (Seakem). The anti-murine IL-10 mAb (clone Jes5-2A5) was purchased from Bioexpress, and

total rat IgG was used as a control. The neutralizing capacity of anti-IL-10 and TGF- β was confirmed in vitro.

Murine CTLA-4-IgG fusion protein (CTLA-4Ig), a fusion protein of the murine extracellular transmembrane and cytoplasmic domain of CTLA-4 and the hinge and Fc regions of human IgG1 H chain, was prepared as previously described (8) and provided by Dr. A van Oosterhout (University Medical Center Groningen, Groningen, The Netherlands). Human IgG (hIg; ICN Biochemicals) was used as a control for CTLA-4Ig.

Chemicals were obtained from Sigma-Aldrich unless stated otherwise.

Treatment protocols

Oral sensitization to peanut extract. Mice ($n = 5$) were orally exposed to PBS plus cholera toxin (CT) (control) or peanut extract plus CT (sensitization). Oral exposure was performed by intragastric dosing of 6 mg of peanut extract plus 10 μ g of CT on 3 consecutive days, followed by weekly dosing of peanut extract plus CT (days 8, 15, and 22). During sensitization, mice were injected i.p. with CTLA-4Ig (200 μ g), anti-CD80 (100 μ g), anti-CD86 (100 μ g), anti-CD80 (100 μ g) plus anti-CD86 (100 μ g), or the same dose of an appropriate isotype control Ab (human IgG, hamster IgG, or rat IgG) on days 1, 3, 7, 14, and 21. At day 31, all groups received an oral challenge with 12 mg of peanut extract and mice were sacrificed on day 32.

Oral tolerance induction to peanut extract. Mice ($n = 5$) were intragastrically exposed to 1 mg of peanut extract (to induce oral tolerance) or PBS on 3 consecutive days (days 1–3). Before oral exposures (day 0), mice received an i.p. injection with CTLA-4Ig (200 μ g), anti-CD80 (100 μ g), anti-CD86 (100 μ g), anti-CD80 (100 μ g) plus anti-CD86 (100 μ g), anti-TGF- β (100 μ g), anti-IL-10 (1 mg), anti-TGF- β (100 μ g) plus anti-IL-10 (1 mg), anti-CTLA-4 (200 μ g), or the same dose of an appropriate isotype control Ab (human IgG, hamster IgG, mouse IgG1, or rat IgG). Mice were immunized i.p. with 100 μ g of peanut extract in alum (25 mg/ml), 21 and 35 days after the last oral exposure and mice were sacrificed at day 49.

Measurement of serum IgG1, IgG2a, and IgE Ab levels

Blood samples were collected at various time points and stored at -20°C until analysis. Levels of *Ara h*- and peanut extract-specific Abs were determined by ELISA (IgG1 and IgG2a) or sandwich ELISA (IgE) as previously described (4). In brief, plates (highbond 3590; Costar) were coated overnight with 20 μ g/ml peanut extract, *Ara h*1, or *Ara h*3 (for IgG1 and IgG2a detection) or with 1.5 μ g/ml purified rat anti-mouse IgE (BD Pharmingen) in carbonate buffer (pH 9.6), followed by 1 h blocking with PBS-Tween/3% milk powder. Each test serum was titrated (at least 8 serial dilutions) starting at 1/8 or 1/16 dilution and incubated for 2 h. A presera pool was used as reference value (dilution 1/4). For detection of IgG1 and IgG2a, AP-conjugated Abs (Southern Biotechnology Associates) were added (1 h at room temperature). 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. To measure Ag-specific IgE Abs, serum was incubated for 2 h and subsequently an Ag-digoxigenin (DIG) conjugate solution was added (1 h at room temperature). The coupling of DIG to the Ag was performed according to the manufacturer's instructions (Boehringer Mannheim). After incubation (1 h at

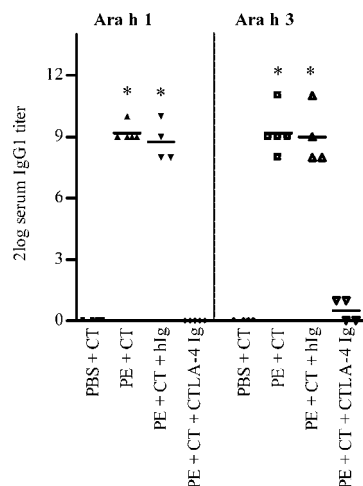


FIGURE 2. Effect of CTLA-4Ig treatment on allergen-specific IgG1 levels. *Ara h 1* and *Ara h 3*-specific serum IgG1 levels were determined after 4 wk of oral exposure to PBS plus CT or peanut extract plus CT. Indicated groups were treated with CTLA-4Ig or control Ab (hIgG) during the oral exposure protocol. The data are presented as the mean $2\log$ Ab titer of 4–5 mice per group (symbols indicate individual animals). *, Significantly different ($p < 0.05$) from the control (PBS + CT) group.

room temperature) with peroxidase-conjugated anti-DIG fragments (Roche Diagnostics), 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 measurements

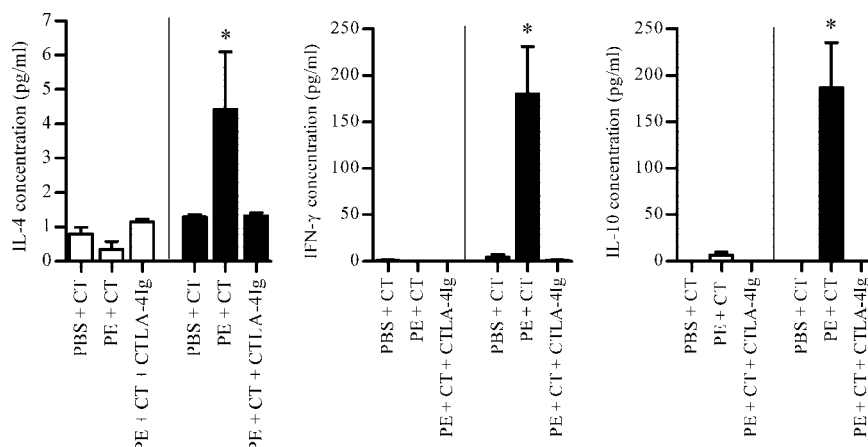
Spleen single cell suspensions (3.75×10^5 cells in 200 μ l of complete RPMI 1640 (Invitrogen Life Technologies) containing 10% FCS (ICN Pharmaceuticals)) were incubated in the presence or absence of 200 μ g/ml peanut extract in 96-well plates for 96 h at 37°C, 5% CO_2 . After centrifugation for 10 min at $150 \times g$, supernatant was collected and stored at $-20^\circ C$ until analysis.

In the culture supernatants, levels of IFN- γ , IL-4, IL-5, and IL-10 were determined by sandwich ELISA. IFN- γ , IL-4, and IL-5 capture and biotin-conjugated Abs were obtained from BD Pharmingen, and the ELISAs were performed as previously described (4). The IL-10 (BD Pharmingen) ELISA (BioSource International) was performed in accordance with the manufacturer's instructions.

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

Blood was collected 45 min after oral challenge with peanut extract, and serum levels of mmcp-1 were determined using an ELISA kit (Moredun). The ELISA was performed according to the manufacturer's instructions.

FIGURE 3. Effect of CTLA-4Ig treatment on cytokine levels in splenocyte culture supernatants. Mice were orally exposed for 4 wk to PBS plus CT (control) or peanut extract plus CT (sensitization). During oral exposure, indicated groups were treated with CTLA-4Ig or control Ab (hIgG). After 4 wk of oral exposure, spleens were removed and single cell suspensions were cultured in the presence (■) or absence (□) of peanut extract and IL-4, IFN- γ and IL-10 cytokine levels were measured by ELISA. Data are presented as the group mean $\pm$ SEM of 5 mice per group. *, Significantly different ($p < 0.05$) from the control (PBS + CT) group.



Statistics

Data were analyzed using SigmaStat statistical software package (SPSS). In the oral sensitization study, multiple comparisons of group means were analyzed using one-way ANOVA with Bonferroni as post hoc test. In the oral tolerance study, the differences between PBS and peanut extract-exposed group means were determined by using independent samples t test procedure. For cytokine levels and mmcp-1 serum levels, statistical analysis was performed following logarithmic transformation to achieve normal distribution. A value of $p < 0.05$ was considered statistically significant.

Results

CTLA-4Ig treatment during oral sensitization inhibits the development of peanut extract- and allergen-specific Abs and peanut extract-induced challenge responses

To investigate the involvement of the CD28/CTLA-4:CD80/CD86 signaling pathway in hypersensitivity responses to peanut proteins, this costimulatory pathway was blocked with CTLA-4Ig during the oral sensitization protocol. After 4 wk of exposure to peanut extract plus CT, mice developed peanut extract-specific IgG1, IgG2a and IgE Ab responses. CTLA-4Ig treatment was associated with a profound inhibition of both Th1-associated (IgG2a) and Th2-associated (IgG1 and IgE) peanut extract-specific Ab levels compared with nontreated or human Ig-treated mice (Fig. 1A). Furthermore, CTLA-4Ig-treatment prevented the release of mmcp-1 upon an oral challenge with peanut extract (Fig. 1B).

Peanut extract consists of a large variety of different proteins but only a number of them have been identified as allergens and are recognized by serum IgE of peanut allergic patients. Therefore, the effect of CTLA-4Ig treatment on IgG1 Ab responses against the purified major peanut allergens *Ara h 1* and *Ara h 3* was evaluated. Exposure to peanut extract plus CT resulted in profound *Ara h*-specific IgG1 responses in non-mAb-treated or human Ig-treated mice, these allergen-specific responses were completely inhibited in CTLA-4Ig-treated mice (Fig. 2).

CTLA-4Ig treatment during oral sensitization inhibits peanut extract-induced cytokine production

To analyze the effect of CTLA-4Ig on T cell responses, spleen cells were cultured in the presence or absence of peanut extract and cytokine levels were determined in the culture supernatants. In agreement with previous studies, significant levels of IL-4, IL-10, and IFN- γ were found upon restimulation with peanut extract in culture supernatants of peanut extract-sensitized mice. peanut extract-restimulated spleen cell cultures derived from CTLA-4Ig-treated mice showed significantly reduced levels of IL-4, IL-10,

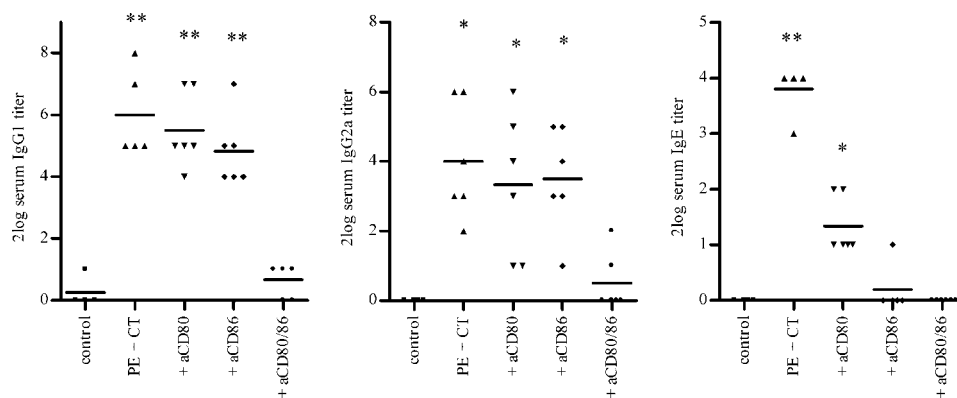


FIGURE 4. Involvement of CD80 and CD86 in peanut extract-specific Ab responses. Peanut extract-specific IgG1, IgG2a, and IgE serum Ab levels were determined after 4 wk of oral exposure to PBS plus CT (control) or peanut extract plus CT. Mice were treated with anti-CD80, anti-CD86, or with isotype control (peanut extract plus CT represents both rat IgG- and hamster IgG-treated groups). The data are presented as the mean $2\log$ Ab titer of 4–6 mice per group (symbols indicate individual animals). *, Significantly different (*, $p < 0.05$ and **, $p < 0.01$) from the control (PBS + CT) group.

and IFN- γ production compared with levels found in cell cultures of human Ig-treated mice (Fig. 3).

Either CD80 or CD86-signaling is required for the production of peanut extract-specific IgG Abs, but CD86 ligation is most important for optimal IgE responses

Because CTLA-4Ig inhibits the interaction of both CD28 and CTLA-4 to both CD80 and CD86, we set out to investigate the specific roles of the individual B7 molecules in the induction of allergic sensitization to peanut proteins. For this purpose, mice were treated with blocking mAbs (anti-CD80, anti-CD86, or a combination of both) during the entire Ag sensitization period.

Treatment with either mAb alone had no significant effect on the production of peanut extract-specific IgG1 or IgG2a Abs in sensitized mice (Fig. 4). However, treatment with the combination of both mAbs during the sensitization period resulted in a large reduction in both IgG1 and IgG2a Ab levels. Interestingly, inhibition of CD80 ligation reduced, but did not completely inhibit peanut extract-specific IgE production, whereas inhibition of CD86 ligation completely prevented peanut extract-specific IgE responses in 5 of 6 animals. When both CD80 and CD86 were blocked, no peanut extract-specific serum IgE was found. These results suggest that either CD80 or CD86 ligation is sufficient to induce IgG1 or

IgG2a responses to orally administered peanut extract, and that CD86 ligation is most important for the induction of systemic IgE responses.

CD80 but not CD86 ligation plays a role in oral tolerance induction

Because CD80 and CD86 can also act as coinhibitors by engagement with the high-affinity ligand CTLA-4, they may additionally be involved in the induction of tolerance to orally administered proteins. To investigate this, mice were treated with a single i.p. injection of blocking mAb or control Ab before oral exposure to peanut extract or PBS (days 1–3). Mice were immunized i.p. with peanut extract plus alum 21 and 35 days later and mice were sacrificed at day 49. Spleen cells were cultured in the presence or absence of peanut extract to measure cytokine production, and peanut extract-specific Ab levels were determined in the serum. As shown in Fig. 5, multiple low dose peanut extract exposures significantly suppressed peanut extract-specific IgG1 and IgE levels compared with PBS-exposed mice. Anti-CD86, anti-CD80/86, and CTLA-4Ig treatment had no effect on the reduced peanut extract-specific IgG1 and IgE levels found in isotype control-treated mice following oral peanut extract pre-exposure. However, in peanut extract-exposed anti-CD80-treated mice, peanut extract-specific

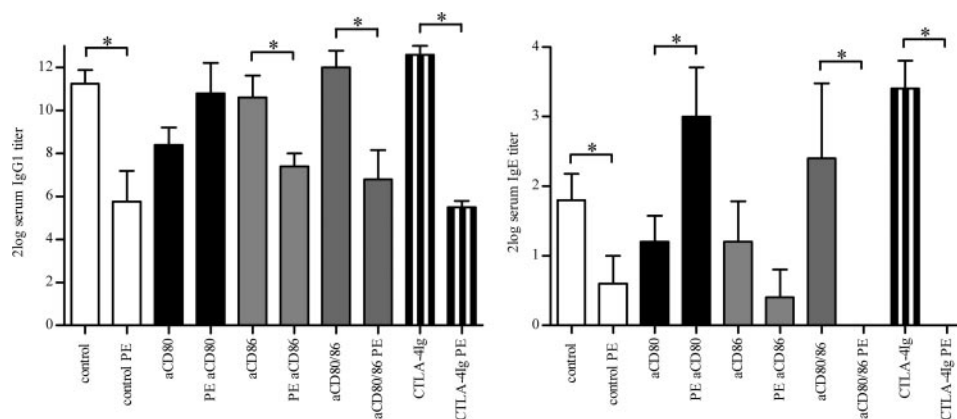


FIGURE 5. Effect of CD80 and CD86 blockade on Ab production in an oral tolerance model. Mice were orally exposed for 3 consecutive days with PBS or peanut extract. Before oral exposure mice were treated with anti-CD80, anti-CD86, anti-CD80/anti-CD86, CTLA-4Ig, or isotype control (the presented control group is representative for the rat IgG-, hamster IgG-, and human IgG-treated groups). Mice were subsequently immunized i.p. with peanut extract plus alum (2 times with a 2-wk interval) and peanut extract-specific IgG1 and IgE Abs were measured 2 wk after the last i.p. injection. Data are presented as the group mean $\pm$ SEM of 5 mice per group. *, Significantly different ($p < 0.05$) from indicated group.

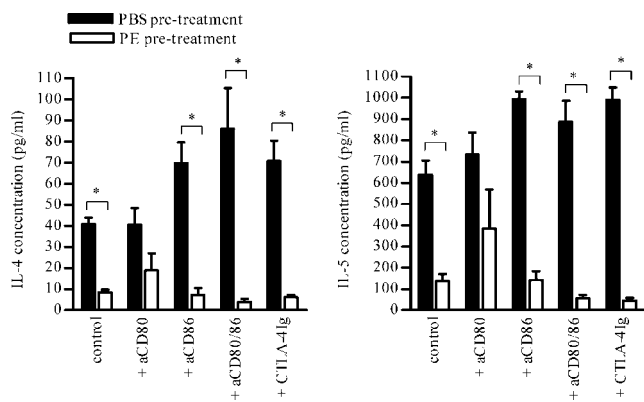


FIGURE 6. Effect of CD80 and CD86 blockade on cytokine production in an oral tolerance model. Mice were orally exposed for 3 consecutive days with PBS (□) or peanut extract (■). Before oral exposure, mice were treated with anti-CD80, anti-CD86, anti-CD80/anti-CD86, CTLA-4Ig, or isotype control (the control group is representative for the rat IgG-, hamster IgG-, and human IgG-treated groups). Mice were subsequently immunized i.p. with peanut extract plus alum (2 times with a 2-wk interval), and 2 wk after the last i.p. injection, splenic single cell suspensions were cultured in the presence or absence of peanut extract. In the supernatant IL-4 and IL-5 cytokine levels were determined by ELISA. In cultures restimulated in the absence of peanut extract no cytokine production was observed (data not shown). Data are presented as the group mean $\pm$ SEM of 5 mice per group. *, Significantly different ($p < 0.05$) from indicated group.

IgG1 and IgE levels were comparable or even significantly higher (IgE) than levels found in PBS-exposed controls, indicating that oral tolerance was abrogated by anti-CD80 treatment. As shown in Fig. 6, oral tolerance induction following low dose peanut extract exposure is also associated with a decrease in Th2 cytokine (IL-4

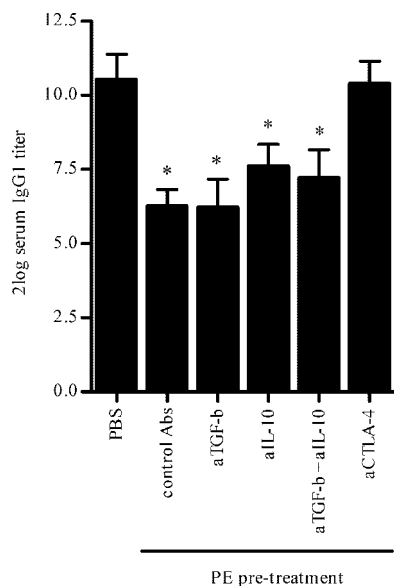


FIGURE 7. Effect of CTLA-4, TGF- β , or IL-10 blockade on oral tolerance induction. Mice were orally exposed for 3 consecutive days with PBS or peanut extract. Before oral exposure, mice were treated with anti-CTLA-4, anti-TGF- β , anti-IL-10, anti-TGF- β /anti-IL-10, or isotype control (the presented Ab control group is representative for the rat IgG- and mouse IgG-treated groups). Mice were subsequently immunized i.p. with peanut extract plus alum (2 times with a 2-wk interval), and peanut extract-specific IgG1 Abs were measured 2 wk after the last i.p. injection. Data are presented as the group mean $\pm$ SEM of 5 mice per group. *, Significantly different ($p < 0.05$) from the PBS-treated control group.

and IL-5) production in spleen cell cultures restimulated with peanut extract. In all mAb-treated groups a significant reduction of IL-4 and IL-5 production was found following oral peanut extract pre-exposure, except for the anti-CD80-treated group in which the reduction was not significant, suggesting that CD80 blockade partly inhibits T cell suppression related with oral tolerance induction.

Oral tolerance induction is impaired following CTLA-4, but not TGF- β or IL-10 blockade

The involvement of CD80 in oral tolerance induction may be mediated by several mechanisms. Engagement of CD80 with the inhibitory molecule CTLA-4 may lead to direct contact-mediated suppression of T cells or to the production of suppressive cytokines such as TGF- β and IL-10. To examine these possibilities, mAbs were used to block CTLA-4 interaction or to neutralize TGF- β and/or IL-10 during oral tolerance induction to peanut extract. Whereas TGF- β and/or IL-10 neutralization had no effect on the induction of oral tolerance, CTLA-4 blockade abrogated the suppression of peanut extract-specific Ab levels induced by oral pretreatment with peanut extract (Fig. 7). These data indicate that oral tolerance induction to peanut extract might depend on contact-mediated suppression via CTLA-4.

Discussion

The CD28/CTLA-4-CD80/86 signaling pathway is the best characterized T cell costimulatory pathway, but it is complex because of the dual specificity of CD80 and CD86 for the stimulatory receptor CD28 as well as the inhibitory receptor CTLA-4. In the current study, we investigated the involvement of the CD28/CTLA-4-CD80/86 signaling pathway in oral sensitization and oral tolerance to peanut. Blockade of the CD28/CTLA-4-CD80/86 pathway by treatment with CTLA-4Ig inhibited all Ag-specific T cell, B cell, and challenge responses in an oral model of peanut hypersensitivity, illustrating the essential role of CD28 costimulation in food allergic responses. The present findings are in agreement with previous reports, which have demonstrated a critical role for CD28-CD80/86 signaling in Th2 differentiation and the development of allergic airway responses by treatment with the same CTLA-4Ig fusion protein (7–9). The strong effect of CTLA-4Ig treatment may not solely be explained by the preventing CD28-CD80/86 interaction; recent studies have demonstrated that CTLA-4Ig ligation of CD80/CD86 on DCs may induce tolerance by stimulating DC tryptophan catabolism via B7 signaling (14, 15).

In the current study, results on the individual roles of CD80 and CD86 in the hypersensitivity model showed that signaling through either CD80 or CD86 was sufficient to generate peanut extract-specific IgG responses, whereas CD86 costimulation was most important for the induction of allergic peanut extract-specific IgE responses. These data are consistent with results obtained in mouse models of allergic airway inflammation, demonstrating that CD86 and not CD80 interaction is essential for the development for systemic IgE responses (16, 17).

Although expression of CD80 and CD86 on APCs and T cells is increased upon encounter with inflammatory stimuli, both CD80 and CD86 are constitutively expressed in small amounts on resting DCs (18, 19). It has been hypothesized that in noninflammatory conditions the B7 molecules may play a role in maintaining aspects of immune tolerance (12). Our data clearly indicate that CD80 but not CD86 is involved in the induction of low dose oral tolerance to peanut proteins. In agreement, Lohr et al. (20) have

demonstrated that low constitutive expression of B7 on DCs potentially limits T cell activation and functions to maintain self-tolerance. This tolerance induction seems to be mediated by interaction with CTLA-4 constitutively expressed on CD4⁺CD25⁺ regulatory T cells (Tregs) (12, 20, 21). Importantly, in the present oral model blockade of CTLA-4 resulted in impaired oral tolerance induction, and we have recently shown that CD4⁺CD25⁺ Tregs play an essential role in the induction of oral tolerance to peanut extract (unpublished data). Together these data suggest that CD80 engagement with CTLA-4 (probably expressed by CD4⁺CD25⁺ Tregs) may also play an important role in oral tolerance. The suppressive effect seemed to be solely contact-dependent whereas the induction of oral tolerance did not depend on the effects of the suppressor cytokines IL-10 and TGF- β . However, it remains to be elucidated whether in vivo CD80-mediated suppression involves T cell-T cell interactions and/or T cell-APC interactions.

The current finding that CD80 is the major ligand involved in oral tolerance induction by interaction with CTLA-4 is (indirectly) supported by several observations. Physicochemical studies revealed that CD80 has a much higher CTLA-4 binding affinity and avidity than CD86 (22) and crystallographic data show that it is unlikely that CD86 is able to form stable dimers necessary for CTLA-4 binding (23). Furthermore, CD80 is the major ligand mediating CTLA-4 localization, whereas CD86 is the main ligand for CD28 concentration at the immunological synapse (24). Additionally it has been recently shown that only in the presence of CD80 (and not CD86) CTLA-4 engagement and inhibition of T cell proliferation is observed (25, 26). Finally, in several mouse models of autoimmunity, administration of anti-CD80-blocking Abs has been associated with exacerbation of the disease (27, 28). However, in contrast, a study of low dose oral tolerance showed that CD86 but not CD80 is indispensable for the induction of tolerance to OVA using TGF- β and IFN- γ cytokine production as the readout for oral tolerance (11). These contradictory results may be due to the use of a completely different oral tolerance and mAb treatment protocol with different readout parameters compared with the current study. Indeed, timing of anti-B7 Ab treatment has been shown to be critical for the effects that are found (28). For example anti-CD86 therapy prevents the onset and early progression of diabetes in NOD mice, but is not effective during the late phase of disease progression (28). Nevertheless, CD80 and CD86 appear to have distinct as well as overlapping roles in regulating responses to orally administered Ags, and the functional outcome likely depends on the context, including relative expression levels of CD80, CD86, and CTLA-4, the presence of adjuvants, and the dose of Ag.

Blockade of the CD28/CTLA-4-CD80/86 costimulation pathway has been proposed as an effective treatment in allergic disease. In the hypersensitivity model, we found that 4 wk of CTLA-4Ig treatment not only prevented the production of Ag-specific IgE Abs but also strongly reduced total serum IgE levels (data not shown). These data suggest that existing elevated serum IgE levels in atopic patients may be targeted with CTLA-4Ig. However, the complex dual role of this pathway has significant implications for developing clinical treatment strategies. Under certain circumstances, CD80, and to a lesser extent CD86, provides a critical interaction with CTLA-4 to maintain or induce tolerance (as shown in the present manuscript) or to down-regulate immune responses. Temporal and tissue-specific expressions complicate the prediction of the consequences of B7 blockade. In addition, whereas CTLA-4-B7 interactions (but not CD28-B7 interactions) seem to play an important role in CD4⁺CD25⁺ T cell function, recent data indicate that CD28-B7 interactions (but not CTLA-4-B7 interactions) are essential in the development of CD4⁺CD25⁺ Tregs and for their survival in the periphery (29,

30). Accordingly it has been shown that CD28^{-/-} and B7-1/B7-2^{-/-} NOD mice develop accelerated diabetes and have markedly reduced numbers of CD4⁺CD25⁺ Tregs (31). Interestingly, in the current study a significant 2.5-fold decrease in CD4⁺CD25⁺ Tregs was found following 4 wk of CTLA-4Ig treatment (data not shown), confirming that also CTLA-4Ig therapy may result in complicated outcomes, especially when treatment is stopped and CD4⁺CD25⁺ Treg levels may not be restored instantly.

In summary our data extend the knowledge on the involvement of the CD28/CTLA-4-B7 signaling pathway in responses to orally administered proteins and clearly show the different roles of CD80 and CD86. The data indicate that this costimulatory pathway is indispensable for the induction of oral sensitization and IgE-mediated hypersensitivity to peanut, with CD86 being the most important ligand. In contrast, CD80 but not CD86 interaction is crucial for the induction of low dose tolerance to peanut, probably by engaging CTLA-4.

Disclosures

The authors have no financial conflict of interest.

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