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Role of CTLA-4, IL-18 and IL-10 on the Induction of Low Dose Oral Tolerance

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Abstract

Oral Tolerance is the temporary loss of systemic immunological responsiveness to a specific soluble antigen after ingestion of that antigen. Results from our lab and others indicated that CTLA-4 and lack of IL-12 played a role in the induction of low dose oral tolerance at the Th1 cell level. Previous literature suggested that IL-18 also played a role in preventing oral tolerance induction while the cytokine IL-10 had been shown to be a factor contributing to suppressed immune responses. To determine the role of CTLA-4 in conjunction with either IL-18 or IL-10 in low dose oral tolerance induction, anti-CTLA-4 mAb and either IL-18 or anti-IL-10 mAb were administered concurrently to mice fed either ovalbumin (OVA) or water. Results showed that the PLN cell proliferation of mice treated with anti-CTLA-4 mAb and IL-18 remained significantly suppressed compared with water-fed controls, while a partial abrogation of suppressed IL-4 and IFN- γ levels were observed. In contrast, mice treated with anti-CTLA-4 mAb and anti-IL-10 mAb exhibited a reversal of PLN cell proliferation and IL-4 suppression; however, IFN- γ levels remained suppressed. Results suggest that IL-10, IL-18 and CTLA-4 play roles in the induction of oral tolerance at the cell proliferation and cytokine level.

Keywords

oral; tolerance; anti-CTLA-4; IL-10; IL-18

INTRODUCTION

Soluble antigen that is delivered orally often results in suppressed immune responses to subsequent immunogenic delivery of that same antigen. The development of this phenomenon, known as oral tolerance, may have the evolutionary advantage of preventing hypersensitivity reactions to ingested food proteins (Kagnoff 1987; Strobel and Mowat 1998). In addition, oral tolerance may aid in reducing the risk of inducing autoimmune reactions via cross-reactivity of ingested proteins with self-antigens (Kagnoff 1987). While oral tolerance has been observed and studied for years, the mechanism responsible for inducing this suppressed immune state remains to be fully elucidated.

Results from previous studies suggested that suppression of T cell responses might be due to an interaction of B7 on the antigen presenting cell (APC) with CTLA-4 on the T cell, rather than with the co-stimulatory molecule, CD28 (Perez et al. 1997). Studies had shown that tolerance induced by oral administration of antigen was at least partially prevented by *in vivo* administration of anti-CTLA-4 mAb, as indicated by decreased antibody secretion,

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lymphocyte proliferation, and cytokine secretion (Samoilova et al. 1998). Further studies by our laboratory and others had shown that suppression of IFN- γ levels and IgG2a in tolerized mice could be completely prevented if the pro-inflammatory cytokine IL-12 was administered concurrently with anti-CTLA-4 mAb at the time of oral tolerance induction (Van Parijs et al. 1997; Barone et al. 2002). These results were consistent with those of others suggesting that decreased levels of IL-12 must be present during oral tolerance induction to prevent the development of a Th1 cell response (Claessen et al. 1996; Marth et al. 1996; Karpus et al. 1998).

Recently it has been suggested that the cytokine IL-18, in combination with IL-12, may play a role in preventing the induction of oral tolerance at the Th1 cell level (Eaton et al. 2003). IL-18, produced by macrophage cells, keratinocytes, intestinal epithelial cells, adrenal cortex cells, and osteoblasts (Conti et al. 1997; Matsui et al. 1997; Stoll et al. 1997; Torigoe et al. 1997) is similar to IL-12 in that it primes the immune response and enhances IFN- γ production in various cells including natural killer cells and most T cells (Okamura et al. 1995; Ushio et al. 1996; Xu et al. 1998). In addition, IL-18 is believed to up-regulate IL-12R, induce transcription of IFN- γ and, in combination with IL-12, upregulate CD80 expression on dendritic cells (Walker et al. 1999; Chang et al. 2000; Eaton et al. 2003).

In contrast with IL-18, IL-10 is an anti-inflammatory cytokine produced by a number of cells, including T cells, macrophages and dendritic cells (Moore et al. 1993). It is thought to exert its effects by acting on the APC, primarily by down-regulating molecules that are involved in T cell co-stimulation, including CD80 expression and IL-12 production (Moore et al. 1993). Results from recent studies support the hypothesis that IL-10 plays a critical role in the induction of oral tolerance. Mice administered antigen orally exhibited increased levels of IL-10 compared with water-fed control mice (Chen et al. 1997; Gonnella et al. 1998; Marth et al. 2000), and administration of IL-10 at the time of feeding enhanced tolerance (Slavin et al. 2001). However, administration of anti-IL-10 mAb has been effective at inhibiting suppression in some but not other oral tolerance experimental models (Aroeira et al. 1995; Rizzo et al. 1999), and suppression in IL-10^{-/-} mice has been variable in response to fed antigen (Aroeira et al. 1995). These results suggest that IL-10 alone is not responsible for all of the phenomena observed in oral tolerance induction.

Given our previous studies indicating that anti-CTLA-4 mAb in combination with IL-12 was capable of preventing suppression of Th1 cell responses, it was of interest to determine if either IL-18 or anti-IL-10 mAb, in combination with anti-CTLA-4 mAb, would also be able to prevent the induction of low dose oral tolerance. To assess this, mice were treated *in vivo* with anti-CTLA-4 mAb and either anti-IL-10 mAb or IL-18 at the time of oral administration of OVA; subsequently, mice were assessed for their ability to generate immune responses. Our results indicate that treatment with anti-CTLA-4 mAb and IL-18 is able to partially reverse tolerance with respect to suppression of both IL-4 and IFN- γ levels, while treatment with anti-CTLA-4 mAb and anti-IL-10 mAb is capable of preventing oral tolerance induction at the proliferative and IL-4 levels.

MATERIALS AND METHODS

Mice

Female 6-to 8-week old BALB/c mice were obtained from Harlan-Sprague Dawley (Indianapolis, IN) and were housed in the animal facility at Thomas More College in accordance with guidelines outlined by the American Association for Laboratory Animal Care.

Antigen, Antibodies and Cytokines

Chicken egg albumin (OVA), Grade V, was obtained from Sigma Chemical Co. (St. Louis, MO). Rat IgG was purchased from Sigma Chemical Co. Anti-CTLA-4 mAb was obtained from culture supernatants of hybridoma UC10-4F10-11 cells (ATCC, Rockville, MD). Hybridomas were cultured in 1% Nutridoma-SP serum-free medium (Boehringer Mannheim, Indianapolis, IN), and the secreted monoclonal antibodies were partially purified by ammonium sulfate precipitation. The antibody preparation was then dialyzed against PBS. Sample purity and antibody concentration were determined by SDS-PAGE analysis using rat IgG (Sigma Chemical Co.) as a standard. Murine rIL-18 was obtained from MBL International Corporation (Woburn, MA). Anti-IL-10 (JES2A5) was obtained from Pharmingen Division of BD Biosciences (San Diego, CA).

In Vivo Antibody and Cytokine Treatment of Mice

Mice were injected i.p. with 120 µg/mouse of anti-CTLA-4 mAb, 150 µg of rat IgG Ab, 0.5 µg of IL-18 and 40 µg of anti-IL-10 mAb. Mice were treated on days 0, +1, and +2.

Induction of Tolerance, Immunization, and Collection of Tissue Samples

Mice were orally tolerized by feeding 1 mg of OVA/mouse for 3 consecutive days; antigen was delivered in 0.5 ml of water by gastric intubation. Mice were fed on days 0, +1, and +2. Ten days after the last feeding (day +12), all mice were immunized in the foot pad and tail base with OVA (10 µg/mouse) emulsified in IFA. Eleven days after immunization, mice were sacrificed by cervical dislocation; popliteal lymph nodes (PLN) were then removed and single cell suspensions prepared.

Cells were cultured in supplemented RPMI 1640 (Gibco BRL) containing 5% heat inactivated FCS, 25 mM Hepes, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 5×10^{-5} M β 2-mercaptoethanol, Pen/Step (100 units of penicillin, and 100 mg of streptomycin) and 5 mg/ml gentomycin (cRPMI). Cultures were maintained at 37°C in humidified 5% CO₂ atmosphere.

In Vitro Lymphocyte Proliferative Responses

Cells were aliquoted at 5×10^5 cells/well in 96-well flat-bottom plates along with OVA at final concentrations of 1000 µg/ml (Barone et al. 1998; Barone et al. 2002). All cultures were then incubated at 37°C in humidified 5% CO₂ for 48 h. Proliferative responses were assayed using the BrdU cell proliferation colorimetric kit (Boehringer-Mannheim), per manufacturer's recommendations. Briefly, BrdU was added to cultures, and the cells were incubated for 8 h at 37°C; BrdU was then removed by centrifugation. After air-drying for 1 h, fixdent solution was added to each well and cells were incubated for an additional 30 min at room temperature (RT). Fixdent was removed and anti-BrdU mAb was added; cells were incubated for 1 h at RT. Plates were then washed three times and substrate (TMB) was added for an additional 30 min. Absorbance was measured at a wavelength of 450 nm using an automated microplate reader (BioTek Instruments, Inc.). Addition of 100 µg/ml of an unrelated antigen, BSA, was added to control wells to assess non-specific proliferation.

Cytokine Analysis

Cells were aliquoted at 4×10^6 cells/well in 24-well flat-bottom plates along with either cRPMI alone or 1000 µg/ml OVA; total volume of 2 ml (Barone et al. 1998; Barone et al. 2002). Cells were then incubated at 37°C in humidified 5% CO₂ for 48 h. Supernatants were harvested and stored at -70°C until assayed. IL-4 and IFN γ production was quantified using murine IL-4 and IFN γ ELISA sets, respectively (BD Biosciences – Pharmingen, San Diego, CA). All ELISAs were carried out per the manufacturer's recommendation. Briefly, coating antibody was added

to 96 well plates for 24 h at 4°C and then washed. Plates were blocked for 1 h at RT using BSA buffer. Next, standards and samples were added and allowed to incubate for 2 hr at RT. After washing, biotinylated detecting antibody plus horseradish peroxidase conjugate was added for 1 h at RT. Plates were again washed, and TMB substrate was added for 30 min; the reaction was terminated by the addition of 2N sulfuric acid solution. Absorbance was measured on an automated microplate reader set at 450 nm. The following ranges of concentrations were used to generate standard curves: IL-4 (7.8 pg/ml to 500 pg/ml) and IFN- γ (31.3 pg/ml to 2000 pg/ml). The concentration of experimental samples was determined using the KC Junior computer software program (BioTek Instruments, Inc.).

Statistical Analysis

For proliferation and cytokine assays, pooled samples for each group were assayed in either quadruplicate (proliferation) or duplicate (cytokine) and expressed as OD or pg/ml $\pm$ standard deviation. Statistical significance of differences among experimental values was determined using Student's *t*-test for multiple comparisons. Values of $P \leq 0.05$ were considered significant. All experiments were repeated at least once.

RESULTS

Cell Proliferation Responses: Effect of Anti-CTLA-4 mAb, IL-18 and/or anti-IL-10 mAb Treatment

Initial experiments were performed to assess the role of CTLA-4, IL-18 and IL-10 in the suppressed cellular proliferation response of mice fed low doses of OVA. As expected, control (rat IgG-treated) mice fed low doses of OVA exhibited decreased levels of proliferation compared with water-fed mice. In agreement with earlier findings from our lab (Barone et al. 2002), treatment with anti-CTLA-4 mAb alone during feeding of antigen failed to prevent this suppression (Figure 1). The suppression index (SI) for PLN from rat-IgG-treated, OVA-fed mice was 0.75, while that of anti-CTLA-4 mAb-treated, OVA-fed mice was 0.66. Suppression indices were calculated by dividing values of OVA-fed mice by those of the corresponding water-fed mice.

As seen in Figure 1, the co-administration of IL-18 along with anti-CTLA-4 mAb failed to reverse suppression of cellular proliferation. However, suppression of proliferation was abrogated in PLN's from orally tolerized mice treated with both anti-CTLA-4 mAb and anti-IL-10 mAb. The SI for PLN from anti-CTLA-4 mAb and IL-18-treated, OVA-fed mice was 0.52, while that of anti-CTLA-4 mAb and anti-IL10 mAb –treated, OVA-fed mice was 1.05.

Cytokine Responses: Effect of anti-CTLA-4 mAb, IL-18 and/or anti-IL-10 mAb Treatment

The effect of co-administration of CTLA-4 mAb with either IL-18 or anti-IL-10 mAb on the cytokine response was assessed. Results showed that PLN from both rat IgG and anti-CTLA-4 mAb OVA-fed mice exhibited reduced IL-4 (Table 1) and IFN- γ (Table 2) levels compared with water-fed mice. Interestingly, mice treated with anti-CTLA-4 mAb and IL-18 exhibited a partial abrogation of tolerance with respect to both PLN IL-4 (Table 1) and IFN- γ (Table 2) levels, although both of these cytokines continued to remain significantly suppressed compared to water-fed control mice (SI of 0.86 and 0.72, respectively). Treatment with IL-18 alone did not result in increased IL-4 or IFN- γ levels (data not shown). In contrast, IL-4 levels in PLN from OVA-fed mice treated with anti-CTLA-4 mAb and anti-IL-10 mAb exhibited a complete abrogation of suppression and were no longer inhibited compared with water-fed control mice (Table 1; SI of 0.97). IFN- γ levels remained significantly suppressed in anti-CTLA-4 mAb and anti-IL-10 mAb-treated OVA-fed mice.

DISCUSSION

The mechanisms underlying the induction of low dose oral tolerance have been long studied and appear to be due to a multifaceted process. The precise procedure utilized can vary and may include deletion and anergy of antigen-specific CD4 cells as well as the production (or lack of production) of various cytokines. The growing consensus among scientists in this field is that low dose oral tolerance is most likely a combination of these various mechanisms (Garside and Mowat 1997; Barone et al. 1998).

Previous research on oral tolerance induction has implicated interaction of B7 on the APC with CTLA-4, a molecule that is expressed on the surface of T cells 24-72 hr post-activation (June et al. 1994) and binds to B7 with 20- to 50-fold higher affinity than does CD28 (June et al. 1990). Once bound, CTLA-4 is thought to deliver a negative or down-regulatory signal (Walunas et al. 1994, 1996; Krummel and Allison 1995) manifested by inhibited IL-2 receptor expression (Walunas 1996) and restriction of normal cell cycle progression (Krummel and Allison 1996). Various laboratories, including our own, have shown that blocking this molecule with anti-CTLA-4 mAb can play a role in preventing the induction of oral tolerance. However, for complete reversal of Th1 cell suppression, IL-12 needed to be administered concurrently with anti-CTLA-4 mAb at the time of feeding (Barone et al. 2002). It was proposed that restoration of this response was due to blocking of CTLA-4, thus allowing B7-CD28 interaction to occur. In addition, the presence of IL-12 may have increased IL-2R expression on T cells, thus promoting the proliferative response, as well as allowing for the differentiation of Th0 cells into IFN- γ producing Th1 cells.

Recently, studies have shown that suppression of IFN- γ and DTH responses could be prevented in orally tolerized animals that were treated with both IL-12 and IL-18 at the time of feeding (Eaton et al. 2003). Results from this study indicate that the presence of these two cytokines allowed for the production of Th0 cells and subsequent differentiation into Th1 cells, perhaps due to the ability of IL-18 in combination with IL-12 to up-regulate B7 (CD80) expression on APC and the ability of IL-12 to induce the differentiation of Th0 cells into Th1 cells. Experiments were thus performed in our laboratory to assess the ability of anti-CTLA-4 mAb and IL-18 to prevent the induction of Th1 oral tolerance. Our data indicated that this treatment did not reverse suppression of PLN proliferation in OVA-fed mice. This may have been due to the inability of IL-18 treatment alone to increase B7 expression (Eaton et al., 2003) and thus allow for optimal B7-CD28 interaction and full T cell activation to occur. However, this same treatment did result in a partial reversal of both Th1 (IFN- γ) and Th2 (IL-4) cytokine levels in orally tolerized mice.

It is possible that despite our inability to detect a restoration in the proliferative response of tolerized, anti-CTLA-4 mAb/IL-18 treated mice, levels of B7-CD28 interaction were sufficient to permit some, albeit minimal, Th0 cell development. That some activation may have occurred is supported by results from Samoilova et al. (1998) who showed that anti-CTLA-4 mAb treatment alone was sufficient to prevent suppression of the proliferative response in a high dose oral tolerance model. In our model, the presence of IL-18 in tolerized mice may have permitted the differentiation of Th0 cells into Th1 cells (and thus IFN- γ production) by increasing their sensitivity to endogenous IL-12 via up-regulation of IL-12 receptors. It should be noted that it is unlikely that the increase in IFN- γ detected was due to a direct stimulation by IL-18 of NK cells or other innate cells, because treatment with IL-18 alone resulted in continued suppression of IFN- γ levels in OVA-fed mice (SI of 0.1; data not shown). With respect to the partial reversal of suppressed IL-4 levels observed, recent studies indicate that IL-18 has the potential to induce Th2 cytokines in addition to its well documented ability to generate Th1 cytokines (Rodriguez-Galan et al., 2005). Alternatively, Th2 cell cytokines could have been produced if the putative default pathway (i.e., in the absence of IL-12) was utilized,

with at least some Th0 cells differentiating into Th2 cells (Sadick et al. 1990; Patton et al. 2001).

Results from a number of studies support the hypothesis that IL-10 plays a critical role in the induction of oral tolerance (Chen et al. 1997; Gonnella et al. 1998; Marth et al. 2000), including the down-regulation of B7 expression on the surface of APC's (Moore et al. 1993). Lower levels of B7 on APC's would thus favor engagement with the high affinity CTLA-4 molecule on the T cell (resulting in suppression) rather than CD28 (resulting in activation). Therefore, it was of interest to see if low dose oral tolerance induction could be prevented if anti-CTLA-4mAb and anti-IL-10 mAb were co-administered to mice at the time of feeding. Our results indicated that mice treated in this manner no longer exhibited suppressed proliferative responses, perhaps due to the blocking of CTLA-4 along with the up-regulation of B7 on APC's; such results might allow for sufficient B7-CD28 binding to promote T cell activation. In addition, tolerized mice treated with both anti-CTLA-4 mAb and anti-IL-10 mAb produced levels of IL-4 similar to that of non-tolerized mice, while IFN- γ levels remained suppressed. Feeding has been shown to result in lower IL-12 levels (Marth et al 1996; Karpus et al. 1998) and our treatment did not entail or allow for the production of IL-12, a necessary component for Th1 differentiation. In addition, unlike treatment with IL-18, anti-IL-10 mAb treatment has not been shown to increase IL-12R, thus negating the possibility that Th0 cells might be more sensitive to low levels of endogenous IL-12. However, the restoration of the IL-4 response in treated, tolerized mice might be explained if the activated, proliferating T cells differentiated primarily into Th2 cells. As mentioned previously, IL-18 has been shown to induce the differentiation of Th2 cells (Rodriguez-Galan et al., 2005) and a number of studies have shown that in the absence of IL-12, T cells will differentiate into Th2 cells via a "default pathway" (Sadick et al. 1990; Patton et al. 2001). Alternatively, requisite but minimal secondary signal interaction (i.e., B7-CD28) could result in a decreased state of dendritic cell activation, a situation that has been shown to favor Th2 over Th1 development (Chaussabel et al. 2003; Reis e Sousa, 2004). It also should be noted that the increase in IL-4 levels in tolerized, treated mice could be due to cytokine secretion by Th0 or other non-T cells and is an area of future investigation.

Interest in discovering the underlying mechanism of specific immune suppression has intensified recently due to the therapeutic potential it holds for the treatment of certain autoimmune diseases (Faria and Weiner 2006). Additional studies assessing CTLA-4, IL-12 and IL-10 utilization will help to further clarify the complicated process of oral tolerance induction.

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LITERATURE CITED

- Aroeira LS, Cardillo F, De Albuquerque DA, Vaz NM, Mengel J. Anti-IL-10 treatment does not block either the induction or the maintenance of orally induced tolerance to OVA. *Scandinavian Journal Immunology* 1995;41:319–323.
- Barone KS, Tolarova DD, Ormsby I, Doetschman T, Michael JG. Induction of Oral Tolerance in TGF β 1 null mice. *Journal of Immunology* 1998;161:154–160.
- Barone KS, Herms B, Karlosky L, Murray S, Qualls J. Effect of in vivo administration of anti-CTLA-4 monoclonal antibody and IL-12 on the induction of low-dose oral tolerance. *Clinical and Experimental Immunology* 2002;130:196–203. [PubMed: 12390306]
- Chang JT, Segal BM, Nakanishi K, Okamura H, Shevach EM. The costimulatory effect of IL-18 on the induction of antigen-specific IFN-gamma production by resting T cells is IL-12 dependent and is

- mediated by up-regulation of the IL-12 receptor beta2 subunit. *European Journal of Immunology* 2000;30:1113–1119. [PubMed: 10760800]
- Chaussabel D, Semnani RT, McDowell MA, Sacks D, Sher A, Nutman TB. Unique gene expression profiles of human macrophages and dendritic cells to phylogenetically distinct parasites. *Blood* 2003;102:672–681. [PubMed: 12663451]
- Chen Y, Inobe J, Weiner L II. Inductive events in oral tolerance in the TCR transgenic adoptive transfer model. *Cellular Immunology* 1997;178:62–68. [PubMed: 9184699]
- Claessen AME, Von Blomberg BME, De Groot J, Wolvers DAE, Kraal G, Scheper RJ. Reversal of mucosal tolerance by subcutaneous administration of interleukin-12 at the site of attempted sensitization. *Immunology* 1996;88:363–367. [PubMed: 8774351]
- Conti B, Jahng JW, Tinti C, Son JH, Joh TH. Induction of interferon-gamma inducing factor in the adrenal cortex. *Journal of Biological Chemistry* 1997;272:25737–25742. [PubMed: 9325300]
- Eaton AD, Xu D, Garside P. Administration of exogenous interleukin-18 and interleukin-12 prevents the induction of oral tolerance. *Immunology* 2003;108:196–203. [PubMed: 12562328]
- Faria AM, Weiner HL. Oral tolerance: therapeutic implications for autoimmune diseases. *Clinical and Developmental Immunology* 2006;13:143–57. [PubMed: 17162357]
- Garside P, Mowat AM. Mechanisms of Oral Tolerance. *Critical Reviews of Immunology* 1997;17:119–137.
- Gonnella PA, Chen Y, Inobe J, Komagata Y, Quartulli M, Weiner HL. In situ immune response in gut-associated lymphoid tissue (GALT) following oral antigen in TCR-transgenic mice. *Journal of Immunology* 1998;160:4708–4718.
- June CH, Ledbetter A, Linsley PS, Thompson CB. Role of the CD28 receptor in T-cell activation. *Immunology Today* 1990;11:211–216. [PubMed: 2162180]
- June CH, Bluestone A, Nadler LM, Thompson CB. The B7 and CD28 receptor families. *Immunology Today* 1994;5:321–331. [PubMed: 7522010]
- Kagnoff, MF. Antigen handling by intestinal mucosa: Humoral and cell-mediated immunity, tolerance and genetic control of local immune responses. In: Marsh, MN., editor. *Immunopathology of the Small Intestine*. John Wiley and Sons Ltd.; 1987.
- Karpus WJ, Kennedy KJ, Kunkel SL, Lukacs NW. Monocyte chemotactic protein 1 regulates oral tolerance induction by inhibition of T helper cell-related cytokines. *Journal of Experimental Medicine* 1998;187:733–741. [PubMed: 9480983]
- Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *Journal of Experimental Medicine* 1995;182:459–465. [PubMed: 7543139]
- Krummel MF, Allison JP. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *Journal of Experimental Medicine* 1996;183:2533–2540. [PubMed: 8676074]
- Marth T, Strober W, Kelsall BL. High dose oral tolerance in ovalbumin TCR-transgenic mice: systemic neutralization of IL-12 augments TGF β secretion and T cell apoptosis. *Journal of Immunology* 1996;157:2348–2357.
- Marth T, Ring S, Schulte D, Klensch N, Strober W, Kelsall BL, Stallmach A, Zeitz M. Antigen-induced mucosal T cell activation is followed by TH1 T cell suppression in continuously fed ovalbumin TCR-transgenic mice. *European Journal of Immunology* 2000;30:3478–3486. [PubMed: 11093167]
- Matsui K, Yoshimoto T, Tsutsui H, Hyodo Y, Hayashi N, Hiroishi K, Kawada N, Okamura H, Nakanishi K, Higashino K. *Propionibacterium acnes* treatment diminishes CD4⁺ NK1.1⁺ T cells but induces type I T cells in the liver by induction of IL-12 and IL-18 production from Kupffer cells. *Journal of Immunology* 1997;159:97–106.
- Moore K, O'Garra A, deWaal M, Viera RP, Mosmann T. Interleukin 10. *Annual Review of Immunology* 1993;11:165–190.
- Okamura H, Tsutsi H, Komatsu T, Yutsudo M, Hakura A, Tanimoto T, Torigoe K, Ocurra T, Nubada Y, Hattori K. Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* 1995;378:88–91. [PubMed: 7477296]
- Patton EA, Brunet LR, La Flamme AC, Pedras-Vasconcelos J, Kopf M, Pearce EJ. Severe schistosomiasis in the absence of interleukin-4 (IL-4) is IL-12 dependent. *Infectious Immunity* 2001;69:589–592.

- Perez VL, Van Parijs L, Biuckians A, Zheng XX, Strom TB, Abbas AK. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity* 1997;6:411–417. [PubMed: 9133420]
- Reis e Sousa C. Activation of dendritic cells: Translating innate into adaptive immunity. *Current Opinions in Immunology* 2004;16:21–25.
- Rizzo LV, Morawetz RA, Miller-Rivero NE, Choi R, Wiggert B, Chan C-C, Morse HC III, Nussenblatt RB, Caspi RR. IL-4 and IL-10 are both required for the induction of oral tolerance. *Journal of Immunology* 1999;162:2613.
- Rodríguez-Galan MC, Bream JH, Farr A, Young HA. Synergistic Effect of IL-2, IL-12 and IL-18 on Thymocyte Apoptosis and Th1/Th2 Cytokine Expression. *Journal of Immunology* 2005;174:2796–2804.
- Sadick MD, Heinzel FP, Holaday JJ, Pu RT, Dawkins RS, Locksley RM. Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T cell-dependent, interferon gamma-independent mechanism. *Journal of Experimental Medicine* 1990;171:115–127. [PubMed: 2104918]
- Samoilova EB, Horton JL, Zhang H, Khoury J, Weiner HL, Chen Y. CTLA-4 is required for the induction of high dose oral tolerance. *International Immunology* 1998;10:491–498. [PubMed: 9620605]
- Slavin AJ, Maron R, Weiner HL. Mucosal administration of IL-10 enhances oral tolerance in autoimmune encephalomyelitis and diabetes. *International Immunology* 2001;13:825–833. [PubMed: 11369711]
- Stoll S, Muller G, Kurimoto M, Saloga J, Tanimoto T, Yamauchi H, Okamura H, Knop J, Enk AH. Production of IL-18 (IFN-gamma-inducing factor) messenger RNA and functional protein by murine keratinocytes. *Journal of Immunology* 1997;159:298–302.
- Strobel S, Mowat AM. Immune responses to dietary antigens: oral tolerance. *Immunology Today* 1998;19:173–181. [PubMed: 9577094]
- Torigoe K, Ushio S, Okura T, Kobayashi S, Taniai M, Kunikata T, Murakami T, Sanou O, Kojima H, Fujii M, Ohta T, Ikeda M, Ikegami H, Kurimoto M. Purification and characterization of the human interleukin-18 receptor. *Journal of Biological Chemistry* 1997;272:25737–25742. [PubMed: 9325300]
- Ushio S, Namba M, Okura T, Hattori K, Nukada Y, Akita K, Tanabe F, Konishi K, Micallef M, Fujii M, Torigoe K, Tanimoto T, Fukuda S, Ikeda M, Okamura H, Kurimoto M. Cloning of the cDNA for human IFN-gamma-inducing factor, expression in *Escherichia coli*, and studies on the biologic activities of the protein. *Journal of Immunology* 1996;156:4274–4279.
- Van Parijs L, Perez VL, Biuckians A, Maki RG, London CA, Abbas AK. Role of interleukin 12 and costimulators in T cell energy in vivo. *Journal of Experimental Medicine* 1997;186:1119–1128. [PubMed: 9314560]
- Walker W, Aste-Amezaga M, Kastelein RA, Trinchieri G, Hunter CA. IL-18 and CD28 use distinct molecular mechanisms to enhance NK cell production of IL-12 induced IFN-gamma. *Journal of Immunology* 1999;162:5894–901.
- Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, Thompson CB, Bluestone JA. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1994;1:405–413. [PubMed: 7882171]
- Walunas TL, Bakker CY, Bluestone JA. CTLA-4 ligation blocks CD28-dependent T cell activation. *Journal of Experimental Medicine* 1996;183:2541–2550. [PubMed: 8676075]
- Xu D, Chan WL, Leung BP, Hunger D, Schulz K, Carter RW, McInnes IB, Robinson JH, Liew FY. Selective expression and functions of interleukin 18 receptor on T helper (Th) type 1 but not Th2 cells. *Journal of Experimental Medicine* 1998;188:1485–92. [PubMed: 9782125]

Figure 1.

Effect of *in vivo* anti-CTLA-4 mAb, IL-18 and anti-IL-10 mAb administration on cell proliferation in orally tolerized mice. Mice were treated *in vivo* as indicated and fed either water (black bars) or OVA (grey bars). Ten days after feeding, mice were immunized with OVA in the foot pad and tail base and sacrificed 11 days later. PLN cells were removed and assessed for proliferation (see Material and Methods section). The error bars represent standard deviations calculated from quadruplicate samples. Asterisks indicate Student *t*-test values that significantly differ ($P \leq 0.05$) from water-fed controls.

Table 1

Effect of anti-CTLA-4 mAb, anti-IL-10 mAb and/or IL-18 on IL-4 secretion in PLN

Treatment	Fed	In Vitro Stimulus	[IL-4] (pg/mL)	IL-4 SP
Rat IgG	H ₂ O	1000 µg OVA	26.92 ± 0.77	
Rat IgG	OVA	1000 µg OVA	7.81 ± 1.34	0.29*
Rat IgG	H ₂ O	Medium	0.87 ± 1.50	
Rat IgG	OVA	Medium	0.0 ± 0.0	0
Anti-CTLA-4	H ₂ O	1000 µg OVA	120.30 ± 13.71	
Anti-CTLA-4	OVA	1000 µg OVA	34.19 ± 7.46	0.28*
Anti-CTLA-4	H ₂ O	Medium	21.07 ± 4.44	
Anti-CTLA-4	OVA	Medium	2.02 ± 1.07	0.10*
Anti-CTLA-4/anti-IL-10	H ₂ O	1000 µg OVA	57.25 ± 4.67	
Anti-CTLA-4/anti-IL-10	OVA	1000 µg OVA	55.44 ± 9.17	0.97
Anti-CTLA-4/anti-IL-10	H ₂ O	Medium	8.43 ± 3.44	
Anti-CTLA-4/anti-IL-10	OVA	Medium	5.43 ± 1.64	0.64*
Anti-CTLA-4/IL-18	H ₂ O	1000 µg OVA	51.57 ± 7.89	
Anti-CTLA-4/IL-18	OVA	1000 µg OVA	44.43 ± 10.26	0.86*
Anti-CTLA-4/IL-18	H ₂ O	Medium	18.11 ± 6.82	
Anti-CTLA-4/IL-18	OVA	Medium	3.50 ± 1.10	0.19*

J Suppression indices were calculated by dividing values of OVA-fed mice by those of the corresponding water-fed mice.

* Student *t*-test values differ significantly ($P \leq 0.05$) from water-fed controls

Table 2Effect of anti-CTLA-4 mAb, anti-IL-10 mAb and/or IL-18 on IFN- γ secretion in PLN

Treatment	Fed	In Vitro Stimulus	[IFN γ] (pg/mL)	IFN γ SP
Rat IgG	H ₂ O	1000 μ g OVA	4911.25 $\pm$ 42.77	
Rat IgG	OVA	1000 μ g OVA	813.89 $\pm$ 66.19	0.17*
Rat IgG	H ₂ O	Medium	1243.62 $\pm$ 87.83	
Rat IgG	OVA	Medium	5.67 $\pm$ 9.82	0.00*
Anti-CTLA-4	H ₂ O	1000 μ g OVA	4951.04 $\pm$ 47.11	
Anti-CTLA-4	OVA	1000 μ g OVA	481.77 $\pm$ 50.53	0.10*
Anti-CTLA-4	H ₂ O	Medium	1280.76 $\pm$ 82.58	
Anti-CTLA-4	OVA	Medium	55.22 $\pm$ 31.35	0.04*
Anti-CTLA-4/anti-IL-10	H ₂ O	1000 μ g OVA	9344.96 $\pm$ 62.20	
Anti-CTLA-4/anti-IL-10	OVA	1000 μ g OVA	187.73 $\pm$ 62.20	0.02*
Anti-CTLA-4/anti-IL-10	H ₂ O	Medium	47.77 $\pm$ 28.64	
Anti-CTLA-4/anti-IL-10	OVA	Medium	0 $\pm$ 0	0.00*
Anti-CTLA-4/IL-18	H ₂ O	1000 μ g OVA	4517.05 $\pm$ 230.04	
Anti-CTLA-4/IL-18	OVA	1000 μ g OVA	3245.88 $\pm$ 502.65	0.72*
Anti-CTLA-4/IL-18	H ₂ O	Medium	924.24 $\pm$ 34.43	
Anti-CTLA-4/IL-18	OVA	Medium	514.66 $\pm$ 28.71	0.56*

/ Suppression indices were calculated by dividing values of OVA-fed mice by those of the corresponding water

* Student *t*-test values differ significantly ($P \leq 0.05$) from waterfed controls