Brief Definitive Report

CTLA-4 Ligands Are Required to Induce an In Vivo Interleukin 4 Response to a Gastrointestinal Nematode Parasite

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Summary

The costimulatory signal provided to T cells through CTLA-4–ligand interactions is required for T cell activation resulting in increased interleukin 2 (IL-2) production in vitro, but its role in the production of IL-4 and other cytokines is unclear and few in vivo studies have been performed to confirm results of in vitro experiments. We have examined the in vivo effects of blocking CTLA-4 ligands on the T helper cell 2 (Th2)-associated mucosal immune response that follows oral infection of mice with the nematode parasite, *Heligmosomoides polygyrus*. CTLA-4Ig administration inhibited *H. polygyrus*–induced increases in mesenteric lymph node (MLN) B cell major histocompatibility complex class II expression and size and T cell–derived IL-4 gene expression. In addition, CTLA-4 immunoglobulin (Ig) partially blocked increased IL-3, IL-5, and IL-9 cytokine gene expression in Peyer's patch (PP) and MLN 8 d after primary inoculation of mice with the parasite. Increases in the number of IL-4– but not IL-5–secreting cells were also inhibited by CTLA-4Ig. *H. polygyrus*–induced elevations in serum IgE levels but not blood eosinophils, were markedly inhibited by CTLA-4Ig. These results suggest that stimulation of CD28 and/or CTLA-4 is required for T cell priming leading to IL-4 cytokine production, B cell activation, and IgE secretion during a Th2-like, mucosal immune response to a nematode parasite.

During the primary in vivo immune response, $CD4^+$ T cells use the CD3-associated TCR-α/β complex to recognize Ag bound to self-class II MHC on the surface of the APC. In addition to signals through the TCR, costimulatory signals are also required for T cell activation. The interaction of B7 (both B7-1 and B7-2) on APCs with CD28 on T cells has been found to be necessary for T cell activation leading to IL-2 production in vitro (1–4). In vitro studies have demonstrated that, in the absence of exogenous IL-2, Th1 clones and fresh T cells require CD28 signaling in addition to TCR signaling for activation and IL-2 production (5, 6), and that in the absence of such costimulation, Th1 cells are anergized (7). In contrast, some Th2 clones produce IL-4 upon engagement of the TCR, but are not responsive to IL-4 unless provided with costimulatory signals, in particular IL-1 (8–10) or CD28 (11). Furthermore, CTLA-4Ig inhibits alloantigen-specific responses and IL-2 and IFN-γ gene expression in MLCs but increased IL-4 gene expression and some proliferation persist (12).

These findings suggest that CTLA-4–ligand interactions may be required for IL-2 production in vitro but that differentiation towards IL-4 production may be induced by other costimulatory signals. In this study, we have examined the role of the CTLA-4 ligand costimulatory pathway in priming for T cell IL-4 production during an in vivo Th2-like immune response to the nematode parasite, *Heligmosomoides polygyrus*. This parasite has a strictly enteral life cycle in which infective third-stage larvae invade the intestinal mucosa <24 h after ingestion and develop there into mature adults (13). The resulting mucosal immune response is associated with elevations in $CD4^+$ T cell IL-4 gene expression and protein secretion, T cell–dependent and –independent elevations in IL-3, IL-5, and IL-9 mRNA, eosinophilia, and also a vigorous B cell response resulting in marked increases in serum IgE (13, 14, and our unpublished results).

Our results show that CTLA-4Ig profoundly inhibits IL-4 gene expression and IL-4 secretion, B cell size, surface MHC class II expression, and serum IgE levels, and document the

requirement of CTLA-4–ligand interactions for both the T and B cell primary in vivo response to this enteric pathogen. In contrast, cytokine production by non-T cells, in particular IL-5, is still elevated and can support the development of eosinophilia in the absence of CTLA-4–ligand–mediated T cell activation.

Materials and Methods

Animals. BALB/c female mice were purchased from the Small Animal Division of the National Institutes of Health ([NIH] Bethesda, MD) and were used at age 8–12 wk. The experiments herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education and Welfare (NIH) 78-23.

Abs. Abs and reagents used include mouse CTLA-4Ig fusion protein produced and purified as described previously (15, 15a), and purified human-mouse chimeric mAb L6, a gift of Ingegerd and Karl Eric Hellstrom (Bristol-Myers Squibb Pharmaceutical Research Institute). For FACS® analysis (Becton Dickinson, Mountain View, CA), we used the anti-MHC class II (MKD6) (16), anti-CD4 (GK1.5) (17), anti-TCR-α/β (Pharmingen, San Diego, CA), and anti-B220 (6B2) Abs, and for the ELISPOT we used a pair of anti-IL-4 Abs (BVD41D112 and BVD6.24G2.3 [18]) and a pair of anti-IL-5 Abs (TRFK-4 and TRFK-5 [19]).

Parasitological Parameters. Infective, ensheathed third-stage larvae of *H. polygyrus* (specimens on file at the U.S. National Parasite Collection, U.S. National Museum Helminthological Collection, U.S. National Museum Helminthological Collection no. 81930, Beltsville, MD) were propagated and stored at 4°C until used. Mice were inoculated orally with 200 larvae using a ball-tipped feeding tube (14).

Treatment with CTLA-4Ig Fusion Protein. After *H. polygyrus* inoculation, mice were injected intravenously via the tail vein on days 0 and 1 with 100 μg murine CTLA-4Ig or the control fusion protein L6.

Cell Labeling and Analysis. Mesenteric lymph node (MLN) cells were washed and then stained with appropriate dilutions of FITC–anti-MHC class II antibodies and biotinylated anti-B220 Abs followed by PE-avidin as previously described (20). Dual-color flow cytometric analysis and sorting was accomplished with an Epics Elite flow cytometer (Coulter Electronics, Miami, FL). An argon laser was used as the excitation source for both FITC and PE. Sorted CD4$^+$, TCR-α/β^+ cells were >97% pure.

Quantitation of Serum Ig and Eosinophil Counts. Serum IgE levels were determined by a micro ELISA (21). Eosinophils were counted from fresh blood samples with the Unopette Test (Becton Dickinson, Rutherford, NJ).

ELISPOT. The frequency of IL-4– and IL-5–producing cells was determined by an ELISPOT assay as previously described (22). The anticytokine "capture" Abs that were bound to the polystyrene plates were either BV41D112 for IL-4 or TRFK-5 for IL-5. The biotinylated anticytokine Abs which were used to bind cytokine antigen captured from cytokine-secreting cells by the plate-bound Ab were BVD6.24G2.3 for IL-4 and TRFK-4 for IL-5.

RT-PCR. The coupled RT-PCR reaction was used as previously described to quantitate differences in cytokine gene expression between treatment groups (20, 23).

Results

CTLA-4Ig Inhibits H. polygyrus–induced Cytokine Gene Expression. Previous studies have shown that an early T-independent increase in IL-3, IL-5, and IL-9 mRNA occurs in the Peyer's patch (PP) during the first several days after *H. polygyrus* inoculation. By day 6, elevations in these three cytokines are at least partly T cell dependent. IL-4 gene expression and protein secretion are, in contrast, first elevated at 6–8 d after inoculation. Cell sorting analyses have shown that the primary source (>99%) of IL-4 mRNA elevation and protein secretion is CD4$^+$, TCRα/β^+ cells (14, and our unpublished data).

To determine whether blocking CTLA-4 ligands could inhibit increases in Th2-associated cytokines, mice were injected with 100 μg i.v. of either murine CTLA-4Ig or the control fusion protein L6 at days 0 and 1 after oral inoculation with 200 third-stage *H. polygyrus* larvae. 8 d after inoculation, PP and MLN were removed from mice (five per group) and in-

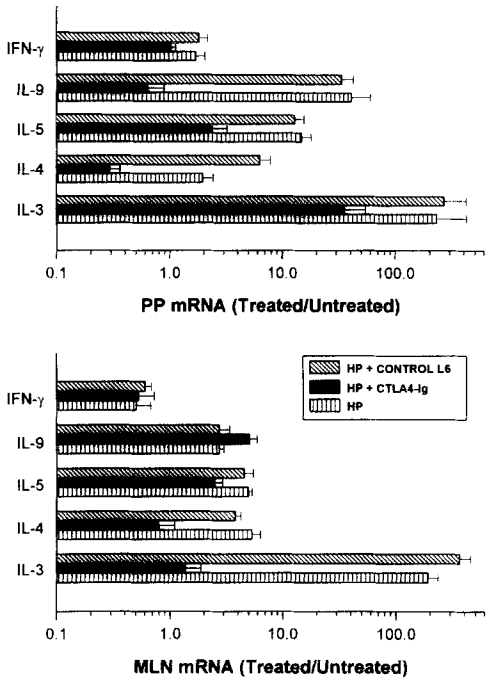

Figure 1. CTLA-4Ig administration inhibits elevations in IL-4 and IL-9 and partially inhibits elevations in IL-3 and IL-5 in the Peyer's patch (*PP*) and inhibits elevations in IL-4 and IL-3, but not in IL-5 in the mesenteric lymph node (*MLN*) at day 8 after *H. polygyrus* inoculation. IFN-γ was not consistently elevated in this response and IL-9 was not consistently elevated in the MLN. CTLA-4-Ig (100 μg) or the control fusion protein (*L6*) (100 μg) was administered at days 0 and 1 after oral inoculation with 200 third-stage larvae. Tissues were collected at day 8 after inoculation and cytokine gene expression levels were determined by a quantitative RT-PCR. The mean and SE derived from PP or MLN of five individual BALB/c mice are shown for each treatment group. All data were individually normalized to the endogenous internal standard, HPRT, which did not show greater then two- to threefold changes throughout the experiment. The means are expressed relative to the mean of the uninfected control, which was arbitrarily given a value of 1. Similar results were obtained in two independent experiments.

dividually analyzed for cytokine gene expression. CTLA-4Ig blocked IL-4 elevations in both the PP and the MLN (Fig. 1). Increases in IL-3 were completely blocked by CTLA-4Ig in the MLN and partially blocked in the PP whereas increases in IL-5 were partially blocked in both the PP and the MLN. IL-9, which increases in PP but usually not MLN, was inhibited by CTLA-4Ig. IFN-γ was not elevated in either the PP or the MLN of *H. polygyrus*–inoculated mice and was not affected by CTLA4-Ig.

Blocking CTLA-4 Ligands Completely Suppresses H. polygyrus–induced Increases in IL-4– but not IL-5-secreting MLN Cells. The complete inhibition of *H. polygyrus*–induced IL-4, but not IL-5 gene expression by CTLA4-Ig suggested that CTLA4-Ig might have similar effects on cytokine secretion. ELISPOT assays were used to examine this possibility. The number of IL-4–secreting cells/10^6 MLN cells was markedly elevated at 8 d after *H. polygyrus* inoculation of mice given control L6. CTLA-4Ig treatment of *H. polygyrus*–inoculated mice inhibited the number of IL-4–secreting cells to levels close to those of uninfected mice (Fig. 2). Similarly, sorted $CD4^+$, TCR-α/β^+ cells from *H. polygyrus*–inoculated mice given L6 showed marked increases in IL-4–secreting cells whereas almost complete inhibition occurred in $CD4^+$, TCR-α/β^+ cells from *H. polygyrus*–inoculated mice given CTLA-4Ig (data not shown). Although a marked increase in IL-5–secreting cells was also detected in the MLN of *H. polygyrus*–inoculated mice, blocking CTLA-4 ligands did not decrease the number of IL-5–secreting cells (Fig. 2) consistent with the less than twofold inhibition by CTLA-4Ig of IL-5 cytokine gene expression in *H. polygyrus*–inoculated mice. Cell sorting showed that the major source of the IL-5–secreting cells in *H. polygyrus*–inoculated mice was $CD4^-$ cells (data not shown).

CTLA-4Ig Blocked Serum IgE Elevations and Partially Suppressed Blood Eosinophil Counts. The decreases observed in both IL-4 gene expression and secretion suggested that IgE production, which is IL-4 dependent (24), might be reduced by blocking CTLA-4 ligands during the *H. polygyrus* response. Because circulating IgE levels are not consistently elevated until 2 wk after *H. polygyrus* inoculation, an independent experiment was performed in which sera were collected at that time. Mice administered L6 on the day of and one day after *H. polygyrus* inoculation showed marked increases in serum IgE whereas IgE levels in *H. polygyrus*–inoculated mice treated with CTLA-4Ig at the same time points were only slightly greater than those of untreated control mice (Fig. 3). In the same experiment, the *H. polygyrus*–induced increase in blood eosinophil counts was, like the increase in PP and MLN IL-5 gene expression, only partially inhibited by CTLA4-Ig treatment (Fig. 3).

B Cell Activation Is Blocked by Administration of CTLA-4Ig Fusion Protein. The inhibition of serum IgE levels 14 d after *H. polygyrus* inoculation suggested that blocking CTLA-4 ligands may affect B cell activation or differentiation to Ig-secreting cells. To examine whether B cells were activated in *H. polygyrus*-inoculated, CTLA-4Ig-treated mice, MLN cell suspensions from these mice were stained simultaneously with anti-MHC class II and B cell–specific anti-B220 (6B2).

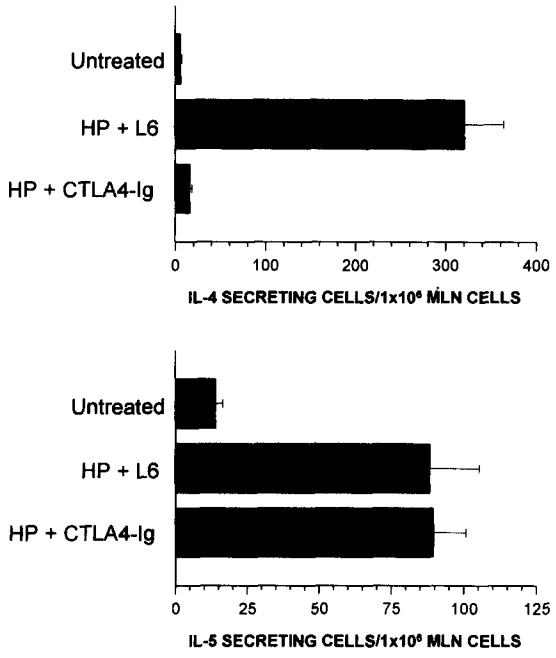

Figure 2. Elevations in the number of IL-4– but not IL-5–secreting cells from the MLN of *H. polgyrus*–inoculated mice are blocked by CTLA-4-Ig administration. CTLA-4Ig treatment and *H. polygyrus* inoculation are as described in Fig. 1. The numbers of IL-4– and IL-5–secreting cells/10^6 MLN cells were determined in an ELISPOT assay without restimulation. The mean and SE derived from PP of five individual BALB/c mice are shown for each treatment group.

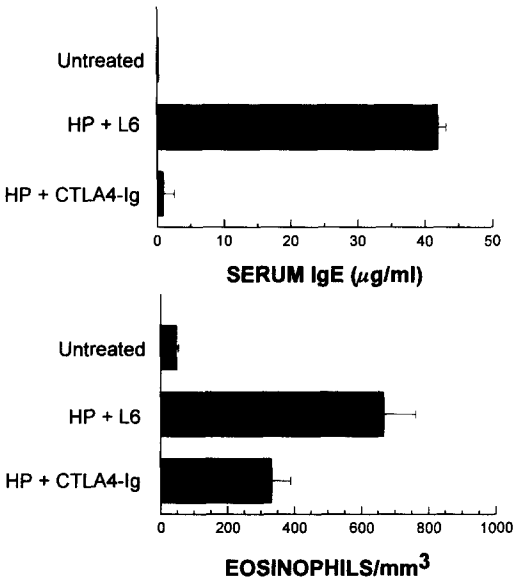

Figure 3. Blocking CTLA-4 ligands inhibits elevations in serum IgE levels but only partially inhibits blood eosinophil elevations at day 14 after *H. polygyrus* inoculation. CTLA-4Ig treatment and *H. polygyrus* inoculation are as described in Fig. 1. Mice were bled at day 14 after inoculation and serum IgE levels and blood eosinophil counts were determined.

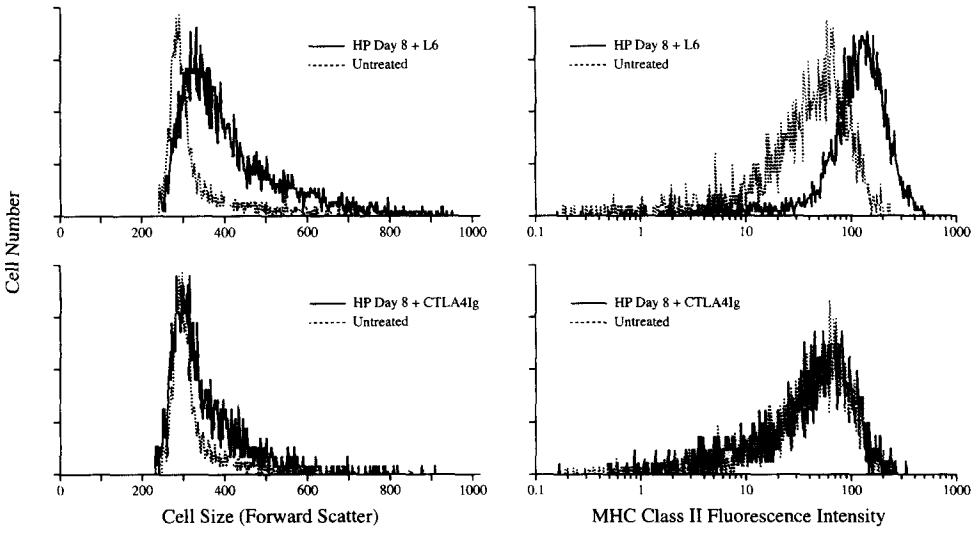

Figure 4. Increases in B cell MHC class II expression and B cell size are inhibited by blocking CTLA-4 ligands during the immune response to *H. polygyrus*. CTLA-4-Ig (100 μg) or the control fusion protein (*L6*) was administered at days 0 and 1 after oral inoculation with 200 third-stage *H. polygyrus* larvae. MLN tissue was collected at day 8 after inoculation and cell suspensions from five individual BALB/c mice per treatment group were pooled and dual stained with FITC-anti-MHC class II and biotinylated anti-B220 (6B2) followed by PE-avidin. Single histogram analyses of B cell ($B220^+$) MHC class II expression and cell size are shown.

MHC class II expression and forward light scatter (a correlate of cell size) on $B220^+$ cells was markedly increased in *H. polygyrus*–inoculated mice administered L6, whereas *H. polygyrus*–inoculated mice given CTLA-4Ig showed MHC class II and forward light scatter profiles similar to B cells from untreated mice (Fig. 4).

Discussion

This investigation demonstrates that CTLA-4 ligands play a key role in the development of the Th2-like enteric immune response to the nematode parasite, *H. polygyrus*. This vigorous immune response is restricted to the enteric region and is characterized by marked elevations in both serum IgE and blood eosinophils (24) and elevations in IL-3, IL-4, IL-5, and IL-9 gene expression (14). Elevations in IL-4 are derived from $CD4^+$ T cells and elevations in IL-3, IL-5, and IL-9 are at least partially T cell independent (14). Our studies show that administration of CTLA-4Ig at early stages of the infection inhibits development of both the T and B cell responses, but only partially inhibits eosinophilia.

Previous studies have not been performed on the role of CTLA-4 ligands in the development of an IL-4–dominant in vivo immune response. However, in vitro studies have suggested that Th2 cells can utilize costimulatory signals besides CD28, in particular IL-1 (8–10, 25). McArthur and Raulet (11) have shown that either IL-1 or CD28 can serve as costimulatory signals mediating responsiveness to IL-4 in Th2 clones, but that CD28 is not required for IL-4 production. Our studies extend these findings to an in vivo model where IL-4 is produced in the early stages of a primary response by T cells in the absence of increased IL-2 gene expression (14). Our findings show that CTLA-4–ligand interactions are necessary for elevations in IL-4 gene expression and IL-4 production in this system and suggest that other costimulatory signals, such as IL-1, the heat stable antigen (26), or LFA-1 and CD2 (27, 28), are either not available or cannot substitute for CTLA-4–ligand interactions.

In contrast to our observations, mice transgenic for soluble murine CTLA-4-Hγ1 exhibit normal T cell priming and cytokine production after immunization with a T cell–dependent antigen (29, 30). One possible reason for this difference from our results is that these transgenic mice lack CD28/CTLA-4 costimulatory signals from birth and may have compensated during ontogeny by using other signals. Alternatively, mCTLA-4-Hγ1 may not be produced at sufficient levels to block CTLA-4–ligand interactions required for the induction of T cell responses. Our results clearly show that blocking CTLA-4 ligands by administration of CTLA-4Ig in normal mature animals completely inhibits elevations in T cell–derived cytokine gene expression and IL-4 protein secretion. Consistent with this observation, we have also observed that T cell cytokine production is blocked in the spleen of goat anti-mouse IgD-immunized mice treated with CTLA-4Ig (Lu, P., X. Zhou, S. J. Chen, M. Moorman, A. Sconøvøld, S. Morris, F. D. Finkelman, P. Linsley, E. Claassen, and W. C. Gause, manuscript submitted for publication).

Although high circulating IgE levels are detected 14 d after *H. polygyrus* inoculation, CTLA-4Ig administration at day 0 and 1 after inoculation strongly inhibited serum IgE levels. Previous studies (29–31) have also demonstrated CTLA-4Ig inhibition of a primary in vivo antibody response. Our further observation that increases in B cell size and MHC class II expression are blocked by CTLA-4Ig administration is consistent with a requirement for T cell help for B cell activation during this in vivo mucosal response. Cytokine production by non-T cells and costimulatory signals provided to B cells by mast cells and basophils expressing gp39 (32) are either themselves T cell dependent or cannot substitute for the Th cell stimulation that is mediated by CTLA-4–ligand interactions. In contrast, our finding that blocking CTLA-4–ligand interactions only partially inhibited elevations in IL-5 gene expression and blood eosinophilia suggests partial T cell independence of these responses and is consistent with the prior observations that anti-CD4 mAb only partially inhibits blood eosinophilia (Urban, J. F., unpublished data).

Studies from in vitro systems have suggested that IL-2 may

be required for IL-4 production (33, 34) and that blocking CTLA-4 ligands, in the absence of exogenous IL-2, may inhibit IL-4 production by initially blocking IL-2 production (35). However, IL-2 gene knockout mice have been shown to have constitutively high levels of IgG1, which is at least partially IL-4 dependent, and to make in vivo T cell–dependent immune responses to some pathogens that are comparable to those of control mice, suggesting that IL-2 is not required for in vivo T cell–mediated immune responses (36, 37). It is also unlikely that IL-2 plays a major role in the *H. polygyrus* system since no increase is detected in IL-2 gene expression in the PP or the MLN during the course of the response to *H. polygyrus* (14) and administration of anti-IL-2 Abs to *H. polygyrus*–infected mice does not affect serum IgE production (Finkelman, F. D., and J. F. Urban, unpublished observations), although the same anti-IL-2 Abs block immune responses in another system (38). Thus, we believe that a CTLA-4–ligand interaction is important for the generation of an IL-4 response even in an IL-2–independent system.

Our findings are also important from a therapeutic viewpoint since they suggest that the T cell development pathway leading to IL-4 production in the mucosal region can be inhibited by blocking CTLA-4–ligand interactions. One potential therapeutic approach relies on the premise that blocking CTLA-4–ligand interactions during an immune response to an allergen or other antigen might induce tolerance, resulting in unresponsiveness to subsequent challenges. Our findings that the primary T cell response to *H. polygyrus* is dependent on B7 costimulation suggest an experimental in vivo model for future studies directed towards determining whether T cell anergy will occur in the absence of CTLA-4–ligand costimulatory signals during a Th2-like response.

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