Protective Immunity to Nematode Infection Is Induced by CTLA-4 Blockade

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Summary

The recent observation that neutralization or genetic deletion of the T lymphocyte receptor CTLA-4 allows enhanced T cell reactivity offers new opportunities for immunotherapy against infectious agents. We used a neutralizing antibody to block CTLA-4 interaction with its ligands CD80 and CD86 during infection of mice with the nematode, *Nippostrongylus brasiliensis.* CTLA-4 blockade greatly enhanced and accelerated the T cell immune response to *N. brasiliensis*, resulting in a profound reduction in adult worm numbers and early termination of parasite egg production. The ability of CTLA-4 blockade to accelerate primary immune responses to a protective level during an acute infection indicates its potential as an immunotherapeutic tool for dealing with infectious agents.

The T cell molecule CTLA-4 is expressed at high levels on the surface 48–72 h after T cell activation (1) and functions as a negative regulator of activation (2–5). The genetic deletion of CTLA-4 in mice results in a profound lymphoproliferative disease with some of the hallmarks of autoimmunity (6, 7). Similarly, blockade of CTLA-4 interaction with its ligands CD80 and CD86 on antigen-presenting cells with either whole anti–CTLA-4 mAb or monovalent Fab fragments results in enhanced T cell expansion to either peptide (8) or super antigen (9). However, it is not clear whether T cell effector function is altered, or whether the observed increases in T cell expansion give rise to an enhanced immune response. We studied the effects of CTLA-4 blockade on the immune response to the parasite *Nippostrongylus brasiliensis* (Nb)[1] as changes in T cell effector function and subsequent effects on protective immunity could be easily monitored.

Primary infection of mice with Nb is characterized by migration first to the lung then small intestine (days 2–4) where final maturation, copulation, and egg production (days 5–10) occur (10, 11). The adult worms are eventually expelled from the gut by day 12–14 after infection. Subsequent cycles of worm infection come under increasingly effective immune attack resulting in poor worm maturation and inhibition of egg production (12). The major immune response which is mounted is a CD4^{+} Th2-type response characterized by IL-4 and IL-5 cytokine production with resulting eosinophilia, mastocytosis, and IgE production (12–15).

Materials and Methods

Antibody Treatment. Anti–CTLA-4 mAb (4F10, hamster IgG) and control hamster IgG were purified from hybridoma supernatant and normal hamster serum, respectively, using protein G affinity columns. C57BL/6 mice were injected intraperitoneally with 1 mg/wk of either anti–CTLA-4 mAb to block CTLA-4 signaling or hamster IgG as control. Antibody treatment always started at day 0 of parasite infection. The concentration of circulating anti–CTLA-4 mAb was directly measured by a sandwich ELISA using plate-bound mCTLA-4Ig as capture and anti-hamster IgG-biotin to detect anti–CTLA-4 mAb in the serum. Using this method anti–CTLA-4 mAb was present in high levels in the serum 7 d after treatment but was not detectable in any of the treated mice 14 d after the last treatment. In the secondary challenge experiment, mice were inoculated with infective larvae 47 d after the final injection of anti–CTLA-4 mAb to ensure no residual effect of CTLA-4 blockade on the memory response.

N. brasiliensis Inoculation and Quantitation of Worms and Eggs. *N. brasiliensis* was maintained by passage through Lewis rats. C57BL/6 mice (6–10 wk old) were inoculated with third-stage infective larvae by either subcutaneous (750 L3) or intraperitoneal (1,000 L3) injection. Parasite egg numbers were determined from group samples of feces collected daily. Adult worm numbers were determined per mouse by removing small intestine, slicing open longitudinally, cutting into small sections, and suspending in a gauze bag submerged in PBS at 37°C to allow worms to migrate out and settle to the bottom. Microscopic analysis of the small intestine revealed any worms that had remained attached to the intestinal mucosa.

Cytokine Production and ELISA. Mediastinal, mesenteric, or inguinal lymphocytes were cultured at 1×10^6 cells/200 μl/well in the presence of rIL-2 (200 U/ml) in 96-well flat-bottom microplates coated with anti-CD3 (2C11, 10 μg/ml). After 60 h incubation, supernatants were harvested and kept frozen until analysis. A sandwich ELISA was used to measure cytokines, using TRFK5 and TRFK4-biotin conjugate (anti-IL-5), and 11B11 and BVD6-24G2-biotin conjugate (anti–IL-4) as capture and detecting reagents, respectively. In brief, polyvinyl chloride 96-well plates were coated overnight at 4°C with 5 μg/ml capture mAb and blocked with 10% BSA in PBS for 60 min at room temperature. Appropriate dilutions of test supernatants, or mIL-4 or mIL-5 internal stan-

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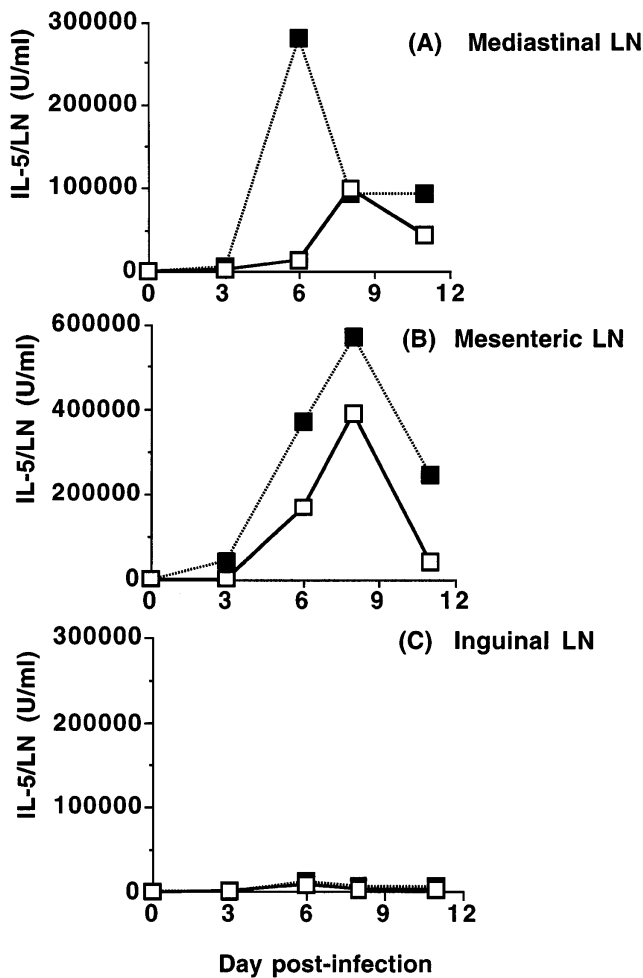[1] *Abbreviation used in this paper:* Nb, *Nippostrongylus brasiliensis.*

Figure 1. Treatment with anti–CTLA-4 greatly enhances IL-5 production in lymph nodes draining the sites of infection with Nb. C57BL/6 mice were inoculated i.p. with 1,000 Nb L3 larvae and treated with control hamster IgG (*open squares*) or hamster anti–mouse CTLA-4 mAb (*filled squares*) at 1 mg/week beginning at day 0. Total lymph node cells were stimulated in vitro with anti-CD3 and IL-5 levels in the supernatant measured by ELISA. Values represent the mean concentration of IL-5 produced from 1×10^6 cells from 4 mice and adjusted to IL-5 production per lymph node based on the total lymph node cell number. Results shown are representative of three separate experiments. (*A*) IL-5 production from the draining mediastinal lymph node. (*B*) IL-5 production from the draining mesenteric lymph node. (*C*) IL-5 production from the inguinal lymph node. This lymph node does not drain any site of Nb infection.
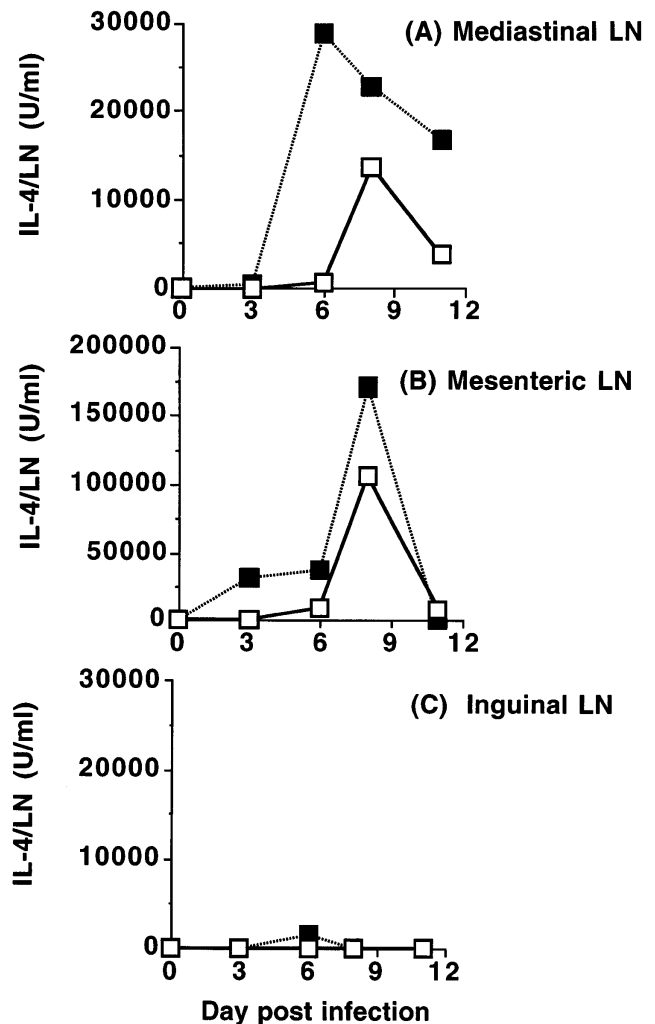
Figure 2. Treatment with anti–CTLA-4 greatly enhances IL-4 production in lymph nodes draining the sites of infection with Nb. C57BL/6 mice were inoculated i.p. with 1,000 Nb L3 larvae and treated with control hamster IgG (*open squares*) or hamster anti–mouse CTLA-4 mAb (*filled squares*) as in Fig. 1. Total lymph node cells were stimulated in vitro as described in Fig. 1 and IL-4 levels in the supernatant measured by ELISA. Values represent the mean concentration of IL-4 produced from 1×10^6 cells from four mice and adjusted to IL-4 production per lymph node based on the total lymph node cell number. Results shown are representative of three separate experiments. (*A*) IL-4 production from the draining mediastinal lymph node. (*B*) IL-4 production from the draining mesenteric lymph node. (*C*) IL-4 production from the inguinal lymph node. This lymph node does not drain any site of Nb infection.

dards, were added and incubated for 2 h at room temperature. Appropriate dilutions of detecting Ab and then peroxidase-labeled streptavidin were added for 1 h at room temperature. Freshly made 1 mM ABTS (100 μl) in citrate phosphate buffer, pH 9.2, and 0.03% H_2O_2 was added to each well to develop the reaction. The reaction was stopped by adding 100 μl 2 mM NaN_3 and the plates read at 414 nM using an Anthos Hill plate reader. Cytokine production is expressed in Genzyme U/ml; the limit of detection for the IL-4 and IL-5 ELISA was >0.2 U/ml and >70 U/ml, respectively.

Results and Discussion

Anti–CTLA-4 Treatment Enhances IL-4 and IL-5 Production In Vivo. To establish whether T cell effector function is altered by blockade of CTLA-4 signaling, we followed IL-4 and IL-5 production in the draining mediastinal and mesenteric lymph nodes of mice infected with Nb and receiving weekly injections of anti–CTLA-4 neutralizing mAb. We found that anti–CTLA-4 mAb treatment induced a profound 20-fold increase in IL-5 production (Fig. 1 *a*) and a massive 40-fold increase in IL-4 production (Fig. 2 *a*) from the draining mediastinal lymph node at day 6 after infection compared to mice given control antibody. Significantly, the peak mediastinal cytokine production of both IL-5 and IL-4 during CTLA-4 blockade occurred earlier
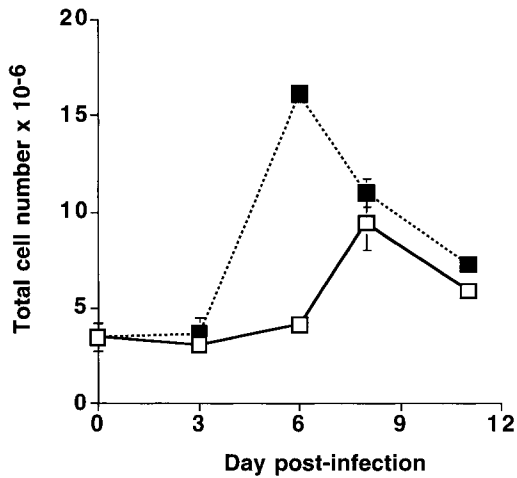
Figure 3. Treatment with anti–CTLA-4 increases total lymphocyte numbers in mediastinal lymph nodes of Nb-infected mice. C57BL/6 mice were inoculated i.p. with 1,000 Nb L3 larvae and treated with control hamster IgG (*open squares*) or hamster anti–mouse CTLA-4 mAb (*filled squares*) as in Fig. 1. Values represent the mean cell number from four mice ± SE and results are representative of three separate experiments.

than that in control mice. Increased cytokine production in response to CTLA-4 blockade was due to increased lymphocyte numbers per mediastinal lymph node as well as increased cytokine production. The mediastinal lymph node obtained from mice given anti–CTLA-4 mAb had nearly fourfold more lymphocytes 6 d after infection than control mice (Fig. 3). Cytokine production in mesenteric lymph nodes was also significantly increased with anti–CTLA-4 mAb treatment (IL-5; Fig. 1 *b*, IL-4; Fig. 2 *b*) but it is interesting that in this lymph node peak cytokine production occurred at the same time in control and anti–CTLA-4–treated mice. Increased cytokine production from this lymph node reflected increased IL-4 and IL-5 production only as total mesenteric lymph node cell numbers were not significantly different between groups (data not shown). The observation that increased cellularity and cytokine production only occurred in the lymph nodes draining the site of infection and not the inguinal nodes indicated that the effect of CTLA-4 blockade was antigen driven (IL-5; Fig. 1 *c*, IL-4; Fig. 2 *c*).

Blockade of CTLA-4 Signals Decreases Parasite Egg Production. To examine whether the accelerated and increased response during anti–CTLA-4 mAb treatment could enhance immunity to the parasite Nb, we examined fecal egg output throughout a primary infection of Nb (Fig. 4 *a*). CTLA-4 blockade nearly abolished parasite egg production compared to the tens of thousands of eggs produced per day in control mice. Surprisingly, the numbers of eggs produced during treatment with anti–CTLA-4 mAb more closely resembled the low numbers observed after a secondary infection with Nb (Fig. 4 *b*). Importantly, blockade of CTLA-4 signaling during a primary Nb infection did not adversely affect development of memory to a subsequent infection with Nb (Fig. 4 *b*). This observation differs from prediction
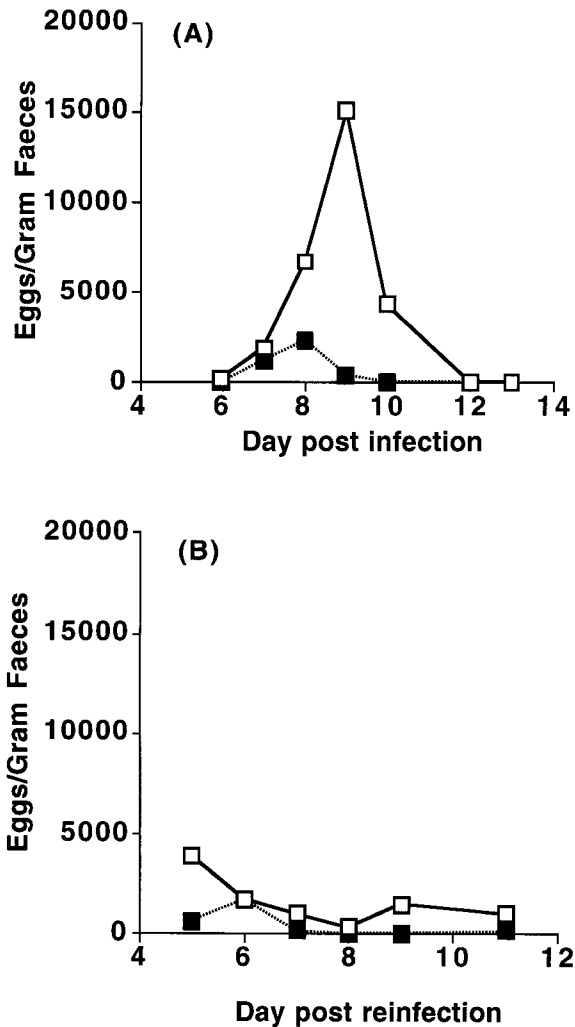

Figure 4. Treatment with anti–CTLA-4 decreases parasite egg output in a primary Nb infection and does not affect development of memory to a subsequent infection with Nb. (*A*) C57BL/6 mice were inoculated i.p. with 1,000 L3 Nb larvae and left untreated (*open squares*) or treated with anti–CTLA-4 neutralizing mAb (*filled squares*) as described in Fig. 1. Feces were collected daily from groups of mice (*n* = 8) starting day 6 after infection. (*B*) Mice from *A* were inoculated i.p. with 1,000 L3 Nb larvae 68 d after primary Nb infection. During the primary infection mice were untreated (*open squares*) or treated with anti–CTLA-4 mAb (*filled squares*) as above. The last injection of mAb was given 21 d after primary infection. Feces were collected daily from groups of mice (*n* = 8) starting day 5 after challenge.

by others that CTLA-4 signaling is required to facilitate the generation of memory T cells (16). Both groups of mice show strong memory responses against Nb as evidenced by the very low level of fecal egg production.

Anti–CTLA-4 Treatment Decreases the Peak Intestinal Worm Burden. The size and characteristics of the adult worm population found in the small intestine during the peak of infection is often used as an indicator of the efficacy of the protective immune response. We found that the number of adult worms residing in the small intestine at the peak of infection was reduced to a 24% take in mice treated with anti–CTLA-4 mAb compared with a 54% take in control

185   McCoy et al.

Table 1. *Anti–CTLA-4 mAb Treatment Decreases Worm Colonization of the Small Intestine*

	Worms inoculated	Peak worm burden (Day 7)	Development take
			%
Control mice	750	406	54
Anti–CTLA-4 mAb–treated mice	750	181	24

C57BL/6 mice were infected subcutaneously with 750 Nb L3 larvae and treated with either anti–CTLA-4 mAb or control hamster IgG (1 mg/week, i.p.). The small intestine was removed 7 d after infection and adult worm numbers determined per mouse by slicing open the intestine longitudinally, cutting into small sections, and suspending in a gauze bag submerged in PBS at 37°C to allow worms to migrate out and settle to the bottom. Microscopic analysis of the small intestine revealed any worms that had remained attached to the intestinal mucosa.

mice (Table 1). Microscopic analysis of the worms resident in the small intestine revealed that most of the female worms present in the control mice contained the expected 20–30 eggs, whereas many of the female worms present in the anti–CTLA-4 treated mice contained few or no visible eggs. These data suggest that CTLA-4 blockade enhances the immune response against the migratory larvae resulting in a reduction in the number of healthy adults that reach the small intestine, thus reducing both the worm burden and overall fecundity.

It has been suggested that in vivo blockade of the proposed negative signaling function of CTLA-4 could allow greater expansion of antigen-specific T cells and possibly prolong an immune response. Here we report that treat-ment of mice with anti–CTLA-4 neutralizing mAb during an acute infection with the extracellular parasite Nb allows greater expansion and activation of parasite-specific T cells and this results in greatly enhanced protective immunity similar to that observed during a memory response. In addition to the increase in the magnitude of T cell reactivity, an important aspect of the response induced during CTLA-4 blockade is that the kinetics of the anti-parasite immune response is advanced to the point where it is protective. We postulate that the profoundly enhanced pulmonary response observed with anti–CTLA-4 treatment leads to immune damage of the migrating larvae before they reach the intestine, resulting in a lower worm burden and decreased fecundity. This is in concordance with a previous suggestion that damage to migratory larvae while passing through the lungs may be an important factor in determining the health of the adult worm population (17). The greatly enhanced pulmonary immune response also has the potential to cause increased tissue damage to the lung. However, we did not observe any gross increases in pulmonary damage in mice treated with anti–CTLA-4 mAb. If mice are inoculated with a higher concentration of infective larvae, many of them die at the time that the worms pass through the lungs, presumably due to pulmonary damage. Anti–CTLA-4 mAb treatment did not lead to an increase in the number of deaths observed therefore, if there was any increase in tissue damage it did not equal that obtained by a higher infection dose. Furthermore, although serum IgE levels in the control infected mice increase more than 100-fold, serum IgE levels in anti–CTLA-4 mAb–treated mice were only very moderately increased (approximately twofold) above that observed in the control animals (data not shown). Blockade of CTLA-4 function may therefore provide an important mechanism for enhancing protective immunity against many infectious agents.

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