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Nippostrongylus brasiliensis Can Induce B7-Independent Antigen-Specific Development of IL-4-Producing T Cells from Naive CD4 T Cells In Vivo

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Th2 immune responses to a number of infectious pathogens are dependent on B7-1/B7-2 costimulatory molecule interactions. We have now examined the Th2 immune response to Nippostrongylus brasiliensis (Nb) in B7-1/-/- mice and show that Th2 effector cells develop that can mediate worm expulsion and produce substantial Th2 cytokines comparable with wild-type infected mice; however, in marked contrast, B cell Ag-specific Ab production is abrogated after B7 blockade. To examine the mechanism of T cell activation, OVA-specific DO11.10 T cells were transferred to recipient mice, which were then immunized with a combination of Nb plus OVA or either alone. Only the combination of Nb plus OVA triggered T cell differentiation to OVA-specific Th2 cells, suggesting that Nb acts as an adjuvant to stimulate Ag-specific naive T cells to differentiate to effector Th2 cells. Furthermore, using the DO11.10 TCR-transgenic T cell adoptive transfer model, we show that blocking B7-1/-/- interactions does not impair nonparasite Ag-specific DO11.10 Th2 cell differentiation; however, DO11.10 T cell cycle progression and migration to the B cell zone are inhibited.


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4 Abbreviations used in this paper: Nb, Nippostrongylus brasiliensis; RAG2−/−, recombination-activating gene-deficient; WT, wild type; MLN, mesenteric lymph node; PNA, peanut agglutinin; GC, germinal center.
dependent on CD4+ T cells producing Th2 cytokines (18–20). Surprisingly, CTLA4Ig administration of Nb-infected mice did not impair Th2 cell-mediated worm expulsion; however, Th2 cytokine production was impaired after in vitro restimulation (21). In another study, mesenteric lymph node (MLN) cell suspensions from Nb-infected CD28−/− mice showed reduced Th2 and Th1 cytokine production after prolonged in vitro restimulation (22). However, differences have been detected in costimulatory molecule requirements between T cells restimulated in vitro and more physiological measurements of T cell cytokine production and associated host protection (23). The possible B7-independence of the Th2 immune response to Nb suggested that this parasite may function as a potent adjuvant to trigger Ag-specific Th2 cell differentiation, although it was also possible that a previously primed T cell population was cross-reactive with an Nb Ag or, alternatively, that T cell activation occurred through a non-Ag-specific mechanism. B cell activation is often polyclonal in parasite infections (24, 25); however, whether T cell activation is also polyclonal has not been carefully examined.

The development of the adoptive transfer model in which a small number of CD4+, OVA323-339-I-A^d-specific T cells from the DO11.10 TCR-transgenic mouse line are transferred into normal BALB/c recipients, which are then immunized with OVA peptide plus a Th1-inducing adjuvant, such as LPS or CFA, has been widely used to examine Ag-specific naïve T cell differentiation and migration in vivo (26, 27). However, this in vivo model has not yet been examined in the context of a Th2 response to determine whether pathogens can also act as adjuvants to trigger naive DO11.10 T cells to differentiate to Th2 cells.

In the studies presented herein, we examined the development of Th2 cells in vivo during Nb infection and the role of B7 interactions in this response. In studies with B7-1/B7-2−/− mice, we found a profound difference between the ability of the differentiated Th2 cells to produce cytokines resulting in worm expulsion and their capacity to provide help for B cell Ab production. Using the aforementioned adoptive transfer model, we determined that Nb can act as an adjuvant which, in the presence of specific Ag, can promote the differentiation of DO11.10 naïve nonparasite Ag-specific T cells to Th2 cells even when B7 interactions are blocked.

Materials and Methods

Mice

B7-1/B7-2−/− mice (backcrossed for 10 generations) and the DO11.10 TCR-transgenic mice on an inbred BALB/c background were obtained from Dr. A. Sharpe. The DO11.10 mice contain a large population of CD4+ T cells that express a TCR specific for chicken OVA323-339-I-A^d complexes. This TCR is uniquely recognized by the KJ1-26 anti-clonotypic mAb (28). All the mice were maintained in a specific pathogen-free, virus Ab-free facility during the experiments. The studies reported here conformed to the principle for laboratory animal research outlined by the Animal Welfare Act and the Department of Health, Education and Welfare (National Institutes of Health) guidelines for the experimental use of animals.

Adoptive transfers

Peripheral lymph nodes and spleen were harvested from DO11.10 TCR-transgenic mice that were age and sex matched to the adoptive transfer recipients. Single-cell suspensions were prepared by pressing tissue through nylon strainer (BD Biosciences, San Jose, CA). OVA-specific KJ1-26 CD4+ T cells (5 × 10^6) were injected i.v. into recipient mice. The number of KJ1-26+ cells injected was calculated based on multiplying the percentage of KJ1-26+ CD4+ cells measured by flow cytometry times the number of live cells obtained. In some experiments, the DO11.10 T cells were incubated with anti-CD4 beads and further purified by passing through an LS column (Miltenyi Biotec, Auburn, CA). Purified CD4+ T cells (with purity of ~99% as determined by FACS) were resuspended at 5 × 10^7 cells/ml in PBS containing 0.1% BSA. A final concentration of 10 μM fluorescent dye CFSE (Molecular Probes, Eugene, OR) was added, and the cells incubated for 10 min at 37°C. The labeled cells were washed twice in cell culture medium containing 10% FCS (Life Technologies, Gaithersburg, MD) before transfer. In some cases, parallel experiments were performed with transferred cells from BALB/c DO11.10 recombination-activating gene-deficient (RAG2−/−) mice, generously provided by Dr. M. Jenkins (University of Minnesota, Minneapolis, MN).

Parasite infection, CD4 depletion, and OVA immunization

Mice were inoculated s.c. with infective third-stage Nb (L3). Parasite egg numbers and adult worm numbers were evaluated as described previously (29). In several experiments, CD4 T cells were depleted in vivo by i.v. administration of 1 mg of anti-CD4 mAb (clone GK1.5, purified from ascites) on the day of inoculation. This dose has previously been shown to effectively deplete CD4+ T cells in vivo (30). HPLC-purified OVA323-339 with the sequence IQAVHHAAHINEGR-COOH was synthesized by Biomedical Instrumentation Center at University Services University. In some experiments, third-stage Nb and 30 μg OVA peptide were injected intracutaneously in the ear of DO11.10 T cell transfer recipient mice. In some cases, mice immunized with peptide plus Nb were given i.v. 200 μg of murine CTLA4Ig or control fusion protein L6 on days 0 and 1 after immunization.

Quantitation of serum IgS

Total serum IgE, IgG1, and IgG2a levels were quantitated by ELISA. Ag-specific IgG1 level was measured using a modified ELISA. Briefly, individual wells of Immulon IV plates (Thermo Labsystems, Franklin, MA) were coated with diluted (5 μg/ml) Nb excretory/secretory Ag. After 4°C overnight incubation, 1% PBS plus 0.1% sodium azide was used for blocking. Serum samples were added to the plates in 4-fold serial dilutions and incubated for 2 h at room temperature after blocking. Then anti-mouse IgG1–alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL; 1:2000 dilution) was added to individual wells, and the plates were incubated for 30 min at room temperature. The substrate (5% 4-methylumbelliferyl phosphate-folate-acid solution) was then added, and fluorescence of the samples was quantitated using a MicroFLUOR Reader (Dynatech Laboratories, Chantilly, VA).

Immunohistochemical staining

The procedure used for germinal center (GC) staining was as described previously (4). Briefly, 8-μm frozen MLN tissue sections were stained with HRP conjugated to peanut agglutinin (PNA; ICN Biomedicals, Aurora, OH), washed, and then incubated with the peroxidase substrate, 3-amino-9-ethylcarbazole (Sigma-Aldrich, St. Louis, MO). All photographs of the tissue sections were taken at the same magnification (×125).

Cell sorting and cytokine gene expression by RT-PCR

For sorting, MNL cells were labeled with anti-CD4 beads and passed through LS columns (Miltenyi Biotec). The CD4+ and CD4− populations were collected and assessed for purity using FACS analysis. The CD4+ population was >98% pure, and the CD4− population was >95% pure in all sorts described. For RT-PCR, total RNA was extracted from purified cell populations with the RNA Isolation Kit (Stratagene, Cedar Creek, TX), specially developed for isolating small RNA quantities, and from tissue as previously described (31). Total RNA was then reverse transcribed as previously described (31). Real-time PCR kits (Applied Biosystems, Foster City, CA), a specific for individual cytokines or rRNA, were used to quantify differences in gene expression, and all data were normalized to constitutive rRNA values. The Applied Biosystems 7700 sequence detector was used for amplification of target mRNA, and quantitation of differences between treatment groups was calculated according to the manufacturer’s instructions.

Cell cultures and cytokine secretion

Single-cell suspensions were prepared from the MLN, and cells were placed in RPMI 1640 supplemented with 10% FCS that had been heat-inactivated for 30 min at 57°C, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μM HEPES. The anti-CD3 mAb restimulation assay was based on previously described techniques (32), with some modifications. Briefly, MLN cells (2 × 10^6 cells/well) from either Nb-infected or uninfected mice were cultured in triplicate wells of 96-well round-bottom plates coated with anti-CD3 mAb (2C11, 10 μg/ml; BD PharMingen, San Diego). After 72 h, the supernatants of each well were collected and stored at −70°C for cytokine production analysis. Cytokine production in the supernatants of cultured cells was measured using commercial ELISA kits (R&D Systems, Minneapolis, MN).
ELISPOT

Two different ELISPOT assays were used. The first was as previously described (3, 33). Briefly, single-cell lymph node suspensions were prepared in RPMI 1640 containing 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine (all from Life Technologies). Cells (0.5 × 10^6) were seeded into each well of an anti-IL-4 (clone BVD4-1D11.2, a gift from Dr. Finkelman)-coated Immulon IV 96-well microtiter plate (Microtiter, Chantilly, VA). After short term culture (5–12 h), the plate was washed several times with PBS followed by washes with PBS-Tween 20. Secondary biotinylated anti-IL-4 Ab was diluted in PBS, 0.05% Tween, 5% FCS; added at 100 μl/well; and incubated overnight at 4°C. Plates were then washed, and a 1/2000 dilution of streptavidin-APK (Jackson ImmunoResearch, West Grove, PA) was added. Plates were developed, and results were counted as described. The second ELISPOT assay was modified to include the capability to quantitate IL-4-producing cells following in vitro restimulation with OVA peptide. Lymph node cells were cultured with 10 μg/ml OVA peptide for 3 days on anti-IL-4-coated plates, before being washed away with PBS and PBS-Tween. Secondary anti-IL-4 Ab was next added, and subsequent steps were identical with those described for the first ELISPOT assay.

Flow cytometry

Lymph node cells were harvested, and 1 × 10^6 cells were blocked with Fc Block (BD PharMingen) and then incubated with anti-CD4-Cy-Chrome (BD PharMingen), anti-CD69-PE (BD PharMingen), and KJ1-26-FITC (Caltag Laboratories, Burlingame, CA) or anti-MHCII-PE and anti-B220-FITC (BD PharMingen). After washes, cells were fixed with 1% paraformaldehyde (Fisher, Pittsburgh, PA) and analyzed by flow cytometry using an EPICS XL-MCL (Beckman Coulter, Fullerton, CA). For CFSE-labeled cells, the CD4, CD8, and KJ1-26-PE (Caltag) were used to distinguish the DO11.10 T cells.

Ex vivo intracellular cytokine measurement

For intracellular cytokine detection, the cervical draining lymph nodes of the ear were harvested 2 h after OVA peptide challenge i.v., and 5 × 10^6 cells were incubated for 5 h with plate-binding anti-CD3 mAb (2C11, 5 μg/ml; BD PharMingen) and Golgi-Stop (BD PharMingen). Lymphocytes were harvested and incubated with Fc Block (2.4G2; BD PharMingen) plus 10% rat serum (Sigma-Aldrich) for 20 min at room temperature. Cell surface markers were stained by anti-CD4-Cy-Chrome (BD PharMingen) and KJ1-26-FITC mAb (Caltag Laboratories). Cells were fixed in 4% paraformaldehyde (Fisher) and permeabilized in 0.5% saponin (Sigma-Aldrich) before staining with PE-conjugated rat anti-mouse IL-4 or anti-IFN-γ mAb (BD PharMingen). Over 200,000 lymphocyte-gated events were collected, to obtain >2,000 KJ1-26-CD4+ gated events.

Immunofluorescent microscopy

Draining cervical lymph nodes were harvested from sacrificed mice and frozen in liquid nitrogen. Cryostat-cut tissue sections (8 μm) were fixed in acetone and stained as described previously (4, 34) with the following reagents: PE-conjugated KJ1-26 (Caltag Laboratories); biotinylated anti-B220 (BD PharMingen) or biotinylated PNA (Sigma-Aldrich); and streptavidin-Alex 487 (Molecular Probes). Sections were mounted in Fluormount G (Southern Biotechnology Associates) and viewed with a fluorescence microscope (Axioskop; Zeiss, Oberkochen, Germany). Images were acquired on a digital camera and were processed with SlideBook software (Intelligent Imaging, Denver, CO).

Results

Host protection and mucosal cytokine production are not inhibited in Nb-infected B7-1/B7-2+ /− mice

Previous studies have suggested that worm expulsion is intact and Th2 cell cytokine production occurs but is significantly impaired in the MLN after CTLA4Ig treatment of Nb-infected mice (21). To examine whether the host protective Th2 response was intact in mice deficient in B7-1 and B7-2, B7-1/B7-2−/− and B7-1/B7-2+/− BALB/c mice were inoculated with 500 Nb L3. Mice (five per treatment group) were killed at days 8 and 14 after parasite inoculation, and adult worm survival and fecundity were determined. As shown in Fig. 1, by day 14 after Nb inoculation, adult worms were not detectable in either B7-1/B7-2−/− or B7-1/B7-2+/− BALB/c mice. Furthermore, worm expulsion was inhibited and worm fecundity was pronounced in Nb-infected B7-1/B7-2+/− or B7-1/B7-2−/− mice treated in vivo with anti-CD4 Ab. In a further experiment, B7-1/B7-2−/− mice were inoculated with different doses (500, 250, and 50) of Nb L3 to investigate whether B7 interactions may be required at lower Ag concentrations. All worms were expelled at all doses in both B7-1/B7-2−/− and B7-1/B7-2−/− BALB/c controls (data not shown), indicating that the dose of Ag did not influence the B7 dependence of the Th2 immune response.

Real-time quantitative RT-PCR was used to assess changes in IL-4 mRNA gene expression. At days 8 and 14 after Nb inoculation, MLN and Peyer’s patches were collected. Pronounced increases in IL-4 mRNA were detected in Nb-infected B7-1/B7-2−/− mice that were at least as elevated as and in some cases more elevated than IL-4 mRNA levels in Nb-infected wild-type (WT) B7-1/B7-2+/− BALB/c mice. Anti-CD4 Ab treatment blocked elevations in IL-4 in both Nb-infected B7-1/B7-2−/− BALB/c mice and Nb-infected B7-1/B7-2−/− controls (Fig. 2a). CD4− and CD4+ T cells were also isolated from MLN suspensions of B7-1/B7-2−/− mice at day 8 after Nb inoculation. CD4+ T cells accounted for >90% of the IL-4 mRNA detected in two separate experiments (Fig. 2b). On day 8 after Nb inoculation, MLN lymphocyte suspensions were collected (five mice per group) and individually analyzed for IL-4 secretion by ELISPOT assay, a method that measures the number of IL-4-secreting cells and requires only a short term in vitro culture without restimulation. Longer in vitro restimulation assays detected markedly reduced levels of IL-4 in MLNs from Nb-infected B7-1/B7-2−/− mice compared with Nb-infected B7-1/B7-2−/− BALB/c mice (data not shown). As shown in Fig. 2c, pronounced and comparable increases in the number of IL-4-secreting CD4 cells were detected in Nb-infected B7-1/B7-2−/− mice. These data suggested that mice genetically deficient in B7-1 and B7-2 do not exhibit inhibition in IL-4 gene and protein expression when more physiological measurements of in vivo cytokine expression are used. The pronounced increases in Th2 cytokine gene and protein expression suggested potential expansion, perhaps non-Ag-specific, of CD4 T cells after Nb inoculation. However, increases in total CD4 T cell activation, as measured by CD69 up-regulation, were not observed after Nb inoculation of B7-1/B7-2−/− or B7-1/B7-2+/− mice (Fig. 2d), suggesting that a large subpopulation of T cells was not activated. In contrast, the Th2 primary response, including IL-4 elevations, is inhibited in B7−1/B7−2−/− BALB/c mice inoculated with H. polygyrus (35) and in CTLA4Ig-treated H. polygyrus-inoculated B7−1/B7−2+/− BALB/c mice (3). These latter findings
indicate pronounced differences in B7 dependence in the Th2 response to different parasites and confirm and extend earlier studies suggesting that the Nb immune response is B7-1/B7-2 independent (21).

The humoral immune response is abrogated in Nb-inoculated B7-1/B7-2-/- mice

Our findings that the development of IL-4-producing T cells was unimpaired in Nb-inoculated B7-1/B7-2-/- mice suggested that a humoral immune response should occur. Previous studies have shown that elevations in total serum IgE are decreased but not blocked after CTLA4Ig treatment of Nb-inoculated mice (21). In our studies, elevations in serum IgE levels were completely blocked in Nb-infected B7-1/B7-2-/- mice, whereas in the same experiments CD4-dependent increases in serum IgE levels were detected in Nb-infected WT controls. Nb Ag-specific IgG1 titers were absent in infected B7-1/B7-2-/- mice but were pronounced in B7-1/B7-2+/- mice at day 14 after inoculation (Fig. 3a). GC formation is an important CD4 T cell-dependent microenvironment that contributes to Ig class switching and memory B cell development. Immunohistochemical analysis showed an almost complete absence of GC formation in the mesenteric lymph nodes of Nb-inoculated B7-1/B7-2-/- mice in contrast to pronounced increases in GC formation observed in Nb-inoculated WT controls (Fig. 3b). Increased B cell MHCII expression is IL-4 dependent during nematode infections and is used as an indicator of B cell IL-4R signaling and activation in vivo (3, 36). MLN cell suspensions from Nb-inoculated B7-1/B7-2+/- and Nb-inoculated B7-1/B7-2-/- mice were stained simultaneously with anti-MHC class II and B cell-specific anti-B220 (6B2) Abs. MHC class II expression and B cell-specific CD69 expression on MLN CD4+ T cells was determined by FACS analysis at day 8 after inoculation.

These findings suggest that although IL-4-producing Th2 cells can develop that mediate worm expulsion in B7-1/B7-2-/- mice, they cannot deliver IL-4 signals to B cells to mediate increases in MHC class II.

Parenteral inoculation with Nb also triggers a B7-1/B7-2-independent Th2 response

A number of studies have suggested that the mucosal immune response favors the development of Th2 cells (37–39). The mucosal microenvironment may thus be permissive for the development of a potent Th2 response that can occur in the absence of B7-1/B7-2 interactions. To test whether Nb could induce a Th2 response in a nonmucosal milieu and, if so, whether the response remained B7 independent, a novel immunization model was developed in which Nb L3 were injected intracutaneously in the ear. Studies of the kinetics of IL-4 and IL-13 mRNA gene expression in the draining ear lymph node showed that peak levels were attained at day 7 after inoculation with 300 Nb L3 (data not shown). As shown in Fig. 4, pronounced increases in both IL-13 and IL-4, but not IFN-γ mRNA, were detected in the draining ear lymph node at day 7 after inoculation of either B7-1/B7-2-/- or B7-1/B7-2+/- mice. These findings demonstrated that Nb could induce a B7-independent Th2 immune response in a nonmucosal lymphoid environment.

Nb stimulates Ag-specific Th2 cell differentiation

The unusual B7-independent Th2 cell differentiation pathway that occurs after Nb inoculation may involve activation of T cells by Nb through an Ag-specific or an Ag-nonspecific, perhaps bystander or polyclonal, T cell activation mechanism. To distinguish between these alternative mechanisms of T cell differentiation,
5 × 10⁶ DO11.10 OVA-specific T cells from DO11.10 WT or DO11.10 RAG2⁻/⁻ mice were transferred to WT recipients (five per treatment group) through i.v. injection. In initial experiments, DO11.10 RAG2⁻/⁻ transferred cells were also used to confirm that endogenous TCR from the DO11.10 WT mice was not significantly contributing to the activated donor T cell population. Two days after adoptive transfer, recipient WT mice (five per treatment group) were inoculated in the ear with 300 Nb L3 and 30 μg OVA. Seven days later, mice were killed, and cervical ear lymph nodes collected for analysis. The lymph node cell suspensions were dual-stained for CD4 and KJ1-26 (anti-DO11.10 Ab). As shown in Fig. 5a, the total number of DO11.10 T cells per cervical lymph node was little increased over untreated mice in treatment groups immunized in the ear with either OVA or Nb alone. However, recipient mice immunized with the combination of OVA plus Nb showed marked expansion of DO11.10 T cells. To detect IL-4-secreting cells, an OVA-specific ELISPOT assay was developed and used as described in Materials and Methods. Recipient mice immunized with Nb alone triggered background IL-4 levels in the OVA-stimulated group similar to that observed in cells stimulated with medium alone, whereas cells from recipient mice immunized with Nb plus OVA exhibited marked increases in the number of IL-4-secreting cells (Fig. 5b). Taken together, these results indicate that a combined immunization with OVA plus Nb is required to trigger DO11.10 T cell activation and differentiation to Th2 cells. The observation that Nb immunization alone cannot stimulate DO11.10 cells indicates that during this nematode parasite infection Ag-nonspecific mechanisms of T cell activation, such as bystander T cell activation, are not sufficient to activate the naive DO11.10 T cells. Nb can thus act as an adjuvant to promote Ag-specific Th2 cell differentiation in vivo.

FIGURE 4. Nb can induce a B7-independent Th2 response after parenteral inoculation. Three hundred Nb L3 were injected intracutaneously in the ear of B7-1/B7-2⁻/⁻ or B7-1/B7-2⁻/⁻ BALB/c mice (five per treatment group). On day 7 after inoculation, cervical lymph nodes were removed, and RNA was purified. IL-4, IL-13, and IFN-γ gene expression was determined by real-time PCR of cDNA reverse transcribed from total RNA. This experiment was repeated twice with similar results. tr/untr, Treated vs untreated.

FIGURE 3. Serum Ig elevations, GC formation, and increased MHC class II expression are inhibited in B7-1/B7-2⁻/⁻ BALB/c mice compared with B7-1/B7-2⁺/+ BALB/c mice after Nb inoculation. a, B7-1/B7-2⁺/+ and B7-1/B7-2⁻/⁻ BALB/c mice were bled on days 8 and 14 after Nb inoculation, and total serum IgE and Nb-specific IgG1 levels were determined by ELISA. The mean and SE derived from five individual mice are shown for each treatment group. b, MLNs were collected from B7-1/B7-2⁺/+ and B7-1/B7-2⁻/⁻ BALB/c mice (five per treatment group) at day 14 postinfection and sectioned and stained with PNA to detect GC formation. c, On day 14 after Nb inoculation, MLN B cell MHC class II expression was determined by FACS analysis of pooled samples from five mice per treatment group. ND, Not detectable.
B7-1/B7-2 interactions are not required for Ag-specific DO11.10 Th2 cell differentiation after Nb plus OVA immunization

The observation that transferred DO11.10 T cells differentiated to Th2 cells in vivo after immunization with OVA plus Nb suggested that this would be a useful model to examine B7-independent Ag-specific Th2 cell differentiation in vivo. In these experiments, rather than using B7-1/B7-2-deficient mice, CTLA4Ig was used to block B7-1/B7-2 interactions in WT B7-1/B7-1+/+ BALB/c mice in vivo, so that B7-1/B7-2 expression on transferred DO11.10 T cells could also be inhibited. Five million sorted DO11.10 CD4+ T cells were transferred to BALB/c recipients. At day 2 after adoptive transfer, recipient mice were given 200 µg of murine CTLA4Ig (five mice per treatment group) or control fusion protein L6 (five mice per treatment group) and immunized in both ears with 300 Nb L3 plus 30 µg OVA. At day 3, an additional dose of 200 µg of CTLA4Ig or L6 was administered. This dose has previously been shown to block the Th2 immune response to H. polygyrus in BALB/c mice (3). At day 7 after Nb plus OVA immunization, mice were immunized i.v. with OVA and killed 2 h later, and individual cervical ear lymph nodes were collected and prepared for FACS analysis. In vivo restimulation with OVA was necessary because the in vitro OVA-specific restimulation ELISPOT assay did not detect increases in IL-4 in recipient mice treated with CTLA4Ig (data not shown). Instead of ELISPOT, cytoplasmic staining, as described in Materials and Methods, was used to detect IL-4 elevations in KJ1-26+ CD4+ T cells. As shown in Fig. 6, pronounced increases in CD69 expression and IL-4 production, but not IFN-γ, were detected in DO11 T cells from the cervical lymph node of BALB/c mice given either CTLA4Ig or L6. These findings suggest that Nb can act as an adjuvant that supports B7-independent nonparasite Ag-specific Th2 cell activation and differentiation.
DO11.10 T cell cycling is reduced following B7 blockade after Nb plus OVA immunization, and DO11.10 T cell trafficking to the B cell zone is inhibited

The observation that DO11.10 T cells can develop into IL-4 T cells, although B7 interactions are blocked, did not preclude the possibility that the DO11.10 Th2 cells may show impaired cell cycle progression. Five million sorted DO11.10 CD4⁺ T cells were stained for CFSE, as described in Materials and Methods, and transferred to BALB/c recipients. Two days after adoptive transfer, mice were immunized in both ears with Nb plus OVA and treated with CTLA4Ig (five per treatment group) or L6 (five per treatment group) at days 0 and 1 after immunization. At day 7, mice were killed, both draining cervical lymph nodes were collected, and cell suspensions were prepared from one lymph node for FACS analysis whereas the other was imbedded in OCT and frozen in liquid nitrogen for immunofluorescent analysis. Cell populations were stained for KJ1-26 and assessed for CFSE staining. As shown in Fig. 7, cell cycling was detectable up to nine generations in DO11.10 T cells from immunized mice given either L6 (Fig. 7b) or CTLAA4Ig (Fig. 7c). In both treatment groups, considerable DO11.10 cell cycling was detected compared with untreated controls (Fig. 7a). However, the frequency of DO11.10 T cells that cycled nine or more generations was markedly reduced in immunized mice given CTLA4Ig compared with immunized mice given L6. Thus, although Nb can support differentiation to IL-4-producing T cells, subsequent cell cycle progression is reduced.

To examine DO11.10 T cell trafficking in the cervical lymph node of immunized mice, frozen sections were stained from recipient mice injected intracutaneously in the ear with Nb plus OVA or OVA alone. As shown in Fig. 8, the combination of Nb plus OVA triggered markedly increased expansion and migration of T cells to the B cell zone of the draining cervical lymph node compared with mice immunized with OVA alone (Fig. 8b). Furthermore, in another experiment, mice given Nb plus OVA plus CTLA4Ig showed a pronounced decrease in DO11.10 T cell migration to the B cell zone compared with the control group given Nb plus OVA plus L6 (Fig. 8, c and d). These results suggest that the combination of Nb plus OVA is required for DO11.10 T cell expansion and migration to the B cell zone and that B7 blockade inhibits such trafficking. In an additional experiment, DO11.10 T
cells were transferred into B7-1/B7-2−/− recipient BALB/c mice before inoculation in the ear with Nb. CTLA4Ig was also administered using the same protocol already described. The results were very similar to those obtained with Nb-inoculated B7-1/B7-2+/+ BALB/c DO11.10 T cell recipient mice treated with CTLA4Ig with respect to both cytokine expression and cell cycling (data not shown).

Discussion

Our findings demonstrate that B7-1/B7-2 blockade during the primary immune response to the nematode parasite, Nb, inhibits humoral immunity but does not inhibit the development of IL-4 producing T cells that can mediate worm expulsion. Furthermore, Nb L3 can act as an adjuvant that induces nonparasite Ag-specific Th2 cell differentiation of naive DO11.10 T cells in vivo and that, if B7 interactions are blocked, naive DO11.10 T cells can still differentiate to Th2 cells.

B7-1/B7-2 costimulation is generally considered a requirement for the development of Th2 cells from naive T cells. The surprising observation that after B7 blockade CD4 T cell effector function was sufficiently intact to mediate host protection during the immune response to Nb suggested that this parasite elicits an alternative pathway for the development of Th2 cells (21). Although these previous studies had suggested that Th2 cell cytokine production was inhibited after in vitro restimulation assays, our findings show that T cell IL-4 production is comparable in B7-1/B7-2−/− and B7-1/B7-2+/+ Nb-inoculated mice when assay systems are used that more directly assess cytokine gene and protein expression in vivo. As described in the results, we also found that the B7 independence of the Th2 response was not dose dependent given that low inoculums induced similar Th2 responses in B7-1/B7-2−/− and B7-1/B7-2+/+ mice. In contrast, the primary Nb Th2 response to a different nematode parasite, H. polygyrus, which is also associated with pronounced T cell IL-4 production, is blocked in B7-1/B7-2−/− mice (35) and in WT mice treated with CTLA4Ig (3). The Th2 response is also blocked after CTLA4Ig administration in T. muris-infected BALB/c mice (5).

There are several possible mechanisms that might explain the difference in B7 dependence between the immune response to Nb and both T. muris and H. polygyrus. Both T. muris and H. polygyrus are strictly enteric, whereas Nb migrates from the skin to the lungs and finally to the small intestines. Nb may thus encounter and activate cell populations distinct from those activated in the intestine by either T. muris or H. polygyrus, some of which may mediate B7-independent T cell activation. It is also possible that Nb expresses a particularly effective adjuvant that can support B7-independent T cell activation. Previous studies have shown that CD4 T cell maturation in some Th1 responses can occur without B7 signaling (5, 6, 8). In these cases, microbial adjuvants may trigger the release of cytokines (40–42), which then provide sufficient signaling to circumvent a B7 requirement for Th1 cell activation. It is possible that Nb produces an analogous microbial adjuvant, which when recognized by the immune system can trigger the rapid development of a host-protective B7-independent Th2 response. Recent studies suggest that soluble Nb excretory-secretory proteins can stimulate IL-4 production and polyclonal IgE synthesis, although the response is considerably reduced compared with live parasite infection (43); it is possible that Nb excretory-secretory proteins and/or other structures associated with Nb may be responsible for triggering the B7-independent development of the Th2 response. Th2 adjuvants have also been identified on Schistosoma mansoni egg Ags, and recent studies indicate that lacto-N-fucopentaose(III), the predominate carbohydrate in S. mansoni egg Ags, can stimulate Th2 responses, including Ag-specific IgE production, when conjugated to human serum albumin (44, 45).

The development of an immunization model system to examine whether live Nb can similarly promote a nonmucosal Th2 immune response allows for the direct comparison of the immune response after enteric vs parenteral inoculation with the same live pathogen. Our results demonstrate that a similarly potent and highly polarized nonmucosal Th2 immune response is induced and that this response is B7 independent. In further studies, we examined whether Nb can function as an adjuvant to drive the development of nonparasite Ag-specific Th2 cells by transferring DO11.10-transgenic T cells specific for OVA peptide to recipient mice subsequently inoculated with Nb plus OVA. Previous studies with this adoptive transfer model for studying Ag-specific T helper cell differentiation have used Th1-inducing adjuvants, including LPS and CFA, to trigger DO11.10 Th1 cell differentiation in vivo (26, 27). Our findings show that Nb can analogously act as an adjuvant that instead drives the development of DO11.10 Th2 cells in vivo. Thus, naive T cells with the same specificity and affinity for Ag can rapidly develop in vivo into either Th1 or Th2 cells when sufficiently strong, polarizing microbial adjuvants are available, suggesting that under these circumstances TCR signal strength is not a major factor influencing Th cell cytokine production.

It was possible that during the Nb response, Th2 cells were activated to produce IL-4 through a bystander T cell activation mechanism, as has been observed in other immunization systems (1), particularly given the lack of requirement for B7 costimulatory signals. However, the observation that Nb alone did not significantly activate adoptively transferred DO11.10 T cells even as late as day 7 after inoculation suggests that bystander naive T cells (in this case DO11.10 T cells), which lack specificity for Ags associated with Nb, do not play a major role in this response. Thus, the mechanism of Nb-induced Th2 cell responses is probably restricted to the augmentation of Ag-dependent naive T cell differentiation. Considering that the B cell response is frequently polyclonal in parasitic infections (24, 25), it is possible that the contrasting stringent regulation of Ag-specific T cell activation during this parasitic infection plays an important role in controlling the specificity of the response. There was also the possibility that endogenous TCRs, expressed by the DO11.10 transgenic T cells, may recognize Ags associated with Nb. However, the observation that there was little difference between stimulation of transferred DO11.10 T cells from DO11.10 RAG2−/− mice and DO11.10 RAG2−/− mice in any of the treatment groups showed that endogenous TCRs expressed on T cells from DO11.10 RAG2−/− mice had little effect, indicating that among the transferred cells naive T cells, specific for OVA, were the major activated population.

The finding that, in the context of the Nb in vivo immune response, naive DO11.10 T cells could differentiate to IL-4-producing T cells, although B7 interactions were inhibited, suggests that the in vivo adjuvant properties of this parasite extend to the activation of B7-independent nonparasite Ag-specific Th2 cell differentiation from naive T cells. Previous in vitro studies have suggested that filarial excretory-secretory products may act as adjuvants that promote Th2 cell development; however, T cell differentiation to cytokine production remained B7 dependent, and restimulation with potent mitogens was required to observe the adjuvant effect (46). Other studies have suggested that in vivo a “default” pathway may develop in the absence of microbial adjuvants, which leads to the development of Th2 responses (10–12). Previous studies with adult Nb excretory-secretory products have shown that excretory-secretory products can augment B cell IgE challenge responses to nonparasite Ags (43). Our findings, using transferred DO11.10 T cells, now show that Nb promotes in vivo
Ag-specific naive T cell differentiation during a primary response, even when the TCR is specific for a nonparasite Ag, suggesting that this parasite has structures that can function as a microbial adjuvant to stimulate Ag-specific Th2 cell differentiation in vivo.

In marked contrast to the sustained development of cytokine producing T cells and associated worm expulsion, the humoral response was abrogated in Nb-inoculated B7-1/B7-2−/− mice, consistent with previous studies that elevations in total IgE were substantially inhibited after CTLA4Ig administration of Nb-inoculated mice (21). Our observation that up-regulated B cell surface MHC class II expression, which is IL-4 dependent (3, 36), was also inhibited suggests that although B7-independent IL-4-producing T cells could develop that could mediate worm expulsion, their ability to interact with B cells was severely compromised. Our further finding that, after immunization with Nb plus OVA, adoptively transferred D011.10 T cells produced IL-4 but showed reduced migration to the B cell zone indicates that the development of IL-4-producing T cells is separable from their differentiation to T helper cells that can migrate to the B cell zone and provide B cell help. These results suggest that IL-4-producing T cells first develop in the T cell zone and then, after additional differentiation stages, migrate to the B cell zone where they contribute to B cell differentiation and GC formation. In vivo cell cycling was reduced in Nb-inoculated mice, when B7 interactions were blocked. Because the majority of the D011.10 cells that had migrated to the B cell zone in Nb-inoculated mice had undergone multiple cell divisions, as determined by their reduced or undetectable CFSE staining (see Fig. 7), it is possible that T cell migration to the B cell zone requires differentiation stages that are cell cycle dependent and that occur after Th cell differentiation to IL-4 production. These results are consistent with findings suggesting that Th2 cell differentiation (although not necessarily IL-4) may be controlled by the cell cycle (47). It is also possible that B-T interactions, which were inhibited in the absence of B7, promote T cell proliferation; several studies have suggested that B cells are important in Ag presentation during Th2 responses (48, 49).

Taken together, our results demonstrate that after Nb immunization naive T cells can differentiate in the absence of B7-1/B7-2 interactions to Th2 effector cells that can mediate worm expulsion. Furthermore, Nb act as an adjuvant to induce nonparasite Ag-specific Th2 cell differentiation in vivo.

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