Identification and Characterization of *Neospora caninum* Cyclophilin That Elicits Gamma Interferon Production

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Gamma interferon (IFN-\(\gamma\)) response is essential to the development of a host protective immunity in response to infections by intracellular parasites. Neosporosis, an infection caused by the intracellular protozoan parasite *Neospora caninum*, is fatal when there is a complete lack of IFN-\(\gamma\) in the infected host. However, the mechanism by which IFN-\(\gamma\) is elicited by the invading parasite is unclear. This study has identified a microbial protein in the *N. caninum* tachyzoite *N. caninum* cyclophilin (NcCyP) as a major component of the parasite responsible for the induction of IFN-\(\gamma\) production by bovine peripheral blood mononuclear cells (PBMC) and antigen-specific CD4\(^+\) T cells. NcCyP has high sequence homology (86%) with *Toxoplasma gondii* 18-kDa CyP with a calculated molecular mass of 19.4 kDa. NcCyP is a secretory protein with a predicted signal peptide of 17 amino acids. Abundant NcCyP was detected in whole-cell *N. caninum* tachyzoite lysate antigen (NcAg) and *N. caninum* tachyzoite culture supernatant. In *N. caninum* tachyzoite culture supernatant, three NcCyP bands of 19, 22, and 24 kDa were identified. NcAg stimulated high levels of IFN-\(\gamma\) production by PBMC and CD4\(^+\) T cells. The IFN-\(\gamma\)-inducing effect of NcAg was blocked by cyclosporine, a specific ligand for CyP, in a dose-dependent manner. Furthermore, cyclosporine abolished IFN-\(\gamma\) production by PBMC from naïve cows as well as PBMC and CD4\(^+\) T cells from infected/immunized cows. These results indicate that the *N. caninum* tachyzoite naturally produces a potent IFN-\(\gamma\)-inducing protein, NcCyP, which may be important for parasite survival as well as host protection.

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Gamma interferon (IFN-\(\gamma\)) production in response to intracellular microbial exposure is critical to the development of a host protective immunity (25). During neosporosis (7), a disease caused by the intracellular protozoan parasite *Neospora caninum*, IFN-\(\gamma\) induced by *N. caninum* tachyzoites plays a pivotal role in control of the acute phase of the disease (2, 18, 20). It has been clearly shown that the complete lack of IFN-\(\gamma\) in the IFN-\(\gamma\)−/− mouse model renders the host highly susceptible to infection (often fatal) by *N. caninum* (21). Continued production of *N. caninum*-elicited IFN-\(\gamma\) production during infection will lead to a chronic state of neosporosis in cattle with no obvious acute symptoms of the disease. However, infection by environmental *N. caninum* oocysts or reactivation of chronic infection, leading to the release of tissue bradyzoites from the tissue cyst in infected animals, will elicit high levels of IFN-\(\gamma\), which may be an important cause for bovine abortion (13, 15). Presently, it remains unknown how host IFN-\(\gamma\) production is regulated by the invading parasite.

Cyclophilins (CyPs) are ubiquitous cytosolic proteins and have been described in prokaryotic as well as eukaryotic organisms (9). CyP was discovered for its peptidyl-prolyl cis-trans isomerase (PPIase) activity and its high binding affinity to cyclosporine, an immunosuppressant drug commonly used to prevent graft rejection (8–10, 19, 27). The PPIase activity is cyclosporine binding has been shown to block the effect of the PPIase activity of CyP. A large number of cyclosporine binding proteins have been reported belonging to the CyP family, most of which have been shown to function as mediators of intra- and intercellular communications (5). In particular, *Toxoplasma gondii* 18-kDa CyP (T. gondii C-18) has been recently shown to act as a molecular mimic to bind the cysteine-cysteine chemokine receptor CCR5 on murine dendritic cells and stimulate interleukin-12 (IL-12) production (1). The production of IL-12 by murine dendritic cells was blocked by cyclosporine in vitro and in vivo, indicating the role of CyP in regulating IL-12 (1). In an attempt to search for *N. caninum* immunodominant antigens using bovine CD4\(^+\)-T-cell lines, the present study has identified *N. caninum* CyP (NcCyP) using mass spectrometry from *N. caninum* tachyzoite lysate that was associated with T-cell antigenic stimulatory activity (30). NcCyP has the highest protein sequence homology with T. gondii C-18. The *N. caninum* tachyzoite lysate antigen (NcAg) induced high levels of IFN-\(\gamma\)-production by peripheral blood mononuclear cells (PBMC) from naïve and exposed/infected cows as well as CD4\(^+\) T cells established from immunized/challenged cows. Abundant NcCyP was detected in *N. caninum* tachyzoite whole-cell lysate or tachyzoite culture supernatant. The CyP ligand cyclosporine was able to block IFN-\(\gamma\) production by bovine PBMC or CD4\(^+\) T cells in response to NcAg stimulation, suggesting that the ability of NcAg to elicit IFN-\(\gamma\) production may be due largely to NcCyP. NcCyP may play an important role in the development of host protective immunity as well as in the induction of abortion when high levels of IFN-\(\gamma\) are elicited. Our results indicate that the study of NcCyP may be crucial to the understanding of host
proteic immunity to *N. caninum* infection and will facilitate the development of a subunit vaccine against neosporosis.

**MATERIALS AND METHODS**

**Animals.** Holstein dairy cows were immunized subcutaneously with killed, whole-cell *N. caninum* tachyzoite lysate in ImmunoMax SR (Zomagen, Woodlands, TX) and challenged intravenously with culture-derived live *N. caninum* (NC-1 strain) tachyzoites (6). The cows were bled from a jugular vein weekly using Vacutainers containing EDTA as anticoagulant. The cows were maintained at the U.S. Department of Agriculture (USDA) Dairy facility at Beltsville, MD. Animal use and care were approved by the USDA/Agricultural Research Service Beltsville Agricultural Research Center Animal Use and Care Committee.

**Reagents.** Cyclosporine (99% pure) was purchased from LC Laboratories (Woburn, MA). Bovine antiserum against NcAg was collected from cows that were previously immunized with whole-cell NcAg and challenged with live *N. caninum* (29). Rabbit antiserum against *N. caninum* tachyzoites was generated by injecting rabbits with live *N. caninum* tachyzoites (16). Rabbit antiserum against *T. gondii* tachyzoites was a generous gift from Alan Sher (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) (1). A bovine IFN-γ enzyme-linked immunosorbent assay kit (BOVIGAM) was purchased from Pfizer Animal Health (Omega, NE).

**T. gondii tachyzoite propagation and lysate preparation.** *T. gondii* tachyzoites were obtained from the peritoneal cavities of mice inoculated intraperitoneally with tachyzoites of the RH strain of *T. gondii*. Tachyzoites were passed through a 27-gauge needle, filtered with a 3-μm-pore-size filter, and washed twice with phosphate-buffered saline (PBS; pH 7.4). *T. gondii* tachyzoites were then subjected to three freezing-thawing cycles and centrifuged at 10,000 × g at 4°C for 15 min. Protein concentration of the supernatant was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, Illinois) and stored at −70°C until use.

**N. caninum tachyzoite propagation and NcAg preparation.** *N. caninum* tachyzoites and NcAg were prepared as described elsewhere with modifications (3, 29). *N. caninum* (NC-1 isolate) tachyzoites were cultured on bovine mono

**RESULTS**

Immunodominant antigens were recognized with bovine anti-NcAg sera or rabbit anti-*N. caninum* tachyzoite sera. In our laboratory previously, a band of proteins was identified by high-pressure liquid chromatography to be associated with CD4+ T-cell stimulatory activity (30). In the present study, Western blotting revealed that immunodominant antigens migrate at 17 to 19 kDa on 4 to 12% NuPAGE gels, which was recognized by both sera from NcAg-immunized/challenged dairy cows (Fig. 1A, lane 1) and *N. caninum* tachyzoite-infected rabbits (Fig. 1B, lane 1). The 17- to 19-kDa proteins were also visualized by both silver (Fig. 1C) and Coomassie blue staining (Fig. 1D). This protein band was excised from the Coomassie blue-stained gel and submitted for mass spectrometric analysis.

**Identification of N. caninum cyclophilin by mass spectrometry.** A total of 11 distinctive genes and proteins were matched to the peptide sequences identified in the 17- to 19-kDa N. caninum cyclophilin. After the first week in culture and weekly thereafter, all cell lines were subcultured with irradiated autologous PBMC as a source of APCs. T cells were analyzed for phenotype by flow cytometry and tested for antigen-dependent proliferation 7 days following the last antigen stimulation. The phenotypes of T-cell lines were analyzed for surface expression of CD3, CD4, and the delta chain of the γδ T-cell receptor by using bovine-specific monoclonal antibodies (Washington State University Monoclonal Center, Pullman, WA) in a flow cytometer (Beck
c

**Culture of PBMC and CD4+ T cells in the presence of NcAg and cyclosporine.** PBMC (2 × 10^6 cells per well) and CD4+ T cells (1 × 10^6 cells per well) plus APCs (2 × 10^5 cells per well) were cocultured in triplicate wells with complete medium, NcAg (1 μg/ml), or host cell protein (1 μg/ml) in a total volume of 100 μl complete medium per well using 96-well plates. Cells were cultured for 18 h of culture with 0.25 μCi/well [3H]thymidine (PerkinElmer), harvested using a semiautomated cell harvester (Tomtec, Orange, CT), and counted with a liquid scintillation counter (Wallace 1450 MicroBeta Trilux liquid scintillation & lumino

**Lympohocyte proliferation assay.** The proliferation assay was performed as described elsewhere (28). T cells were cultured in 100 μl complete medium alone, NcAg (1 μg/ml) alone, or NcAg (1 μg/ml) in the presence of increasing concentrations (0.1 to 1.000 nM) of cyclosporine in 10-fold serial dilutions. Cells were incubated for 48 h at 37°C in a 5% CO2 and 95% air atmosphere. Supernatants were collected and centrifuged at 1,000 × g for 15 min at 4°C and stored at −25°C until analyzed for IFN-γ concentration. Cyclosporine was solubilized in 100% ethanol at 100 mM and stored at −25°C until used. Final concentration of ethanol in culture was less than 0.001% (vol/vol).

**Nucleotide sequence accession number.** The protein sequence data reported in this paper will appear in the Swiss-Prot and TrEMBL data banks under accession number P64343.

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Neospora caninum proteins by mass spectrometry (Table 1). Since the objective of the present study was to identify and characterize N. caninum proteins that regulate host IFN-γ production, we then focused our effort on the N. caninum CyP (Tables 1 and 2) whose homolog from T. gondii was shown to modulate the production of IFN-γ (1). Five nonoverlapping peptide sequences were obtained from the mass spectrometric analysis (Table 2), which were matched perfectly to the deduced protein sequences of nine identical, but different in length, CyPs (Table 2), which were matched perfectly to the deduced protein sequences. The protein sequence data reported in this paper will appear in the Swiss-Prot and TrEMBL data banks under accession number P84343.

Multiple sequence alignment revealed that NcCyP has the highest homology (86%) with T. gondii CyP (C-18) (Fig. 2C). The NcCyP protein sequence was also compared to those of other species, including both prokaryotic and eukaryotic organisms (Fig. 2C). Surprisingly, in addition to T. gondii CyP, NcCyP is more similar (62%) to the CyP of the zebra fish (Brachydanio rerio) than to CyPs from other species (Fig. 2C). The sequence homology between the NcCyP and CyPs of other species including Homo sapiens (human), Mus musculus (house mouse), Theileria parva, Gallus gallus (chicken), Bos taurus (cattle), Sus scrofa domestica (domestic pig), Dro...

**Table 1.** N. caninum genes and proteins that matched peptides identified within the 18- to 19-kDa-molecular-mass range of N. caninum proteins by mass spectrometric analysis

<table>
<thead>
<tr>
<th>Gene accession no.</th>
<th>Protein</th>
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<tr>
<td>CF371177</td>
<td>GRA1</td>
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<tr>
<td>BF716434</td>
<td>Unknown</td>
</tr>
<tr>
<td>BF225290</td>
<td>Peptidyl-prolyl cis-trans isomerase</td>
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<td>BM132349</td>
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<td>Histone H2A.Z</td>
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<td>CD537951</td>
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<td>60S ribosomal protein L32</td>
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<tr>
<td>AI180389</td>
<td>Unknown</td>
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* This table lists the significant hits of Mascot search results (Matrix Science, United Kingdom) from all EST databases. The EST databases were used because the N. caninum genome has not been sequenced yet and most of the N. caninum sequences available now are ESTs. None of these sequences have been published, but references can be found in GenBank.

**Table 2.** Peptides identified by mass spectrometry and matched to N. caninum cyclophilin cDNA sequences

<table>
<thead>
<tr>
<th>Sequence no.</th>
<th>Peptide amino acid sequence</th>
<th>Corresponding amino acid positions</th>
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<tbody>
<tr>
<td>1</td>
<td>KAFMDIEIDGESAGR</td>
<td>24–38</td>
</tr>
<tr>
<td>2</td>
<td>NFIGLFDKYK</td>
<td>53–63</td>
</tr>
<tr>
<td>3</td>
<td>VIADFMIOQGDFENHGTTGHSIYG</td>
<td>69–105</td>
</tr>
<tr>
<td></td>
<td>PRFEDENFTLK</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>GVISMANAGPTNGSQQFITTVK</td>
<td>108–131</td>
</tr>
<tr>
<td>5</td>
<td>ITNDSWPTVQIAEALGSSGGRPSKI</td>
<td>144–169</td>
</tr>
</tbody>
</table>

* These peptides cover 61% (109 of 178 amino acids) of the entire NcCyP sequence. The protein sequence data reported in this paper will appear in the Swiss-Prot and TrEMBL data banks under accession number P84343.

**Table 3.** EST cDNA sequences in GenBank that matched the N. caninum peptide sequences identified in the present study

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>EST cDNA length (bp)</th>
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<td>466</td>
<td>Partial</td>
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<td>CF940514</td>
<td>551</td>
<td>Partial</td>
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</table>

* Sequences listed in this table were generated by Cole et al. in the USDA-Washington University Neospora EST Project, Washington University School of Medicine, St. Louis, MO, and obtained from the GenBank database (unpublished data). The EST sequence CF422590 was used to infer the rest of the amino acid sequence of NcCyP.

* Coding sequence for NcCyP.
sophila melanogaster (fruit fly), Felis catus (cat), Lycopersicon esculentum (tomato), Trypanosoma cruzi, and Plasmodium yoelii yoelii ranged from 52 to 57% at the protein level (Fig. 2C). Apparently, the differences in length of CyPs from different species are generated mostly from the amino termini of the CyP (Fig. 2C). For the selected amino acids compared in this study, only 2 amino acid additions and/or deletions were observed at the carboxyl termini (Fig. 2C). NcCyP is 1 amino acid shorter than the T. gondii CyP at the carboxyl terminus. Overall, the cyclophilin type peptidyl-prolyl cis-trans isomerase signature sequence is conserved across selected species at 50% homology. The protein sequence homology between NcCyP and T. gondii CyP within the cyclophilin type peptidyl-prolyl cis-trans isomerase signature sequence is 83%, suggesting that NcCyP may also possess PPIase activity, which is being confirmed in our laboratory.

**Detection of N. caninum CyP by rabbit antibodies against T. gondii CyP.** It has previously been demonstrated in multiple studies that N. caninum and T. gondii are highly related organisms, which is reflected not only by their morphology but also by the high degrees of gene sequence homologies shared by these two organisms (4, 11, 15). Since N. caninum CyP and T. gondii CyP are highly similar at the protein level (Fig. 2B), a rabbit antiserum against T. gondii CyP (C-18) was used to detect CyP in lysates of T. gondii and N. caninum tachyzoites (Fig. 3A) and culture supernatant of N. caninum tachyzoites (Fig. 3B). T. gondii CyP with an apparent molecular mass of 19 kDa was detected in T. gondii tachyzoite lysate (Fig. 3A, lane 2), which is consistent with the molecular mass of T. gondii C-18 reported previously (1, 12). NcCyP was detectable in whole-cell NcAg with the apparent molecular masses of 19 and 24 kDa (Fig. 3A, lane 3). In addition to the 19- and 24-kDa bands, a minor band of 22 kDa was also detected in N. caninum tachyzoite culture supernatants obtained by incubating Percoll-purified N. caninum tachyzoites in PBS (pH 7.4) at 37°C for 1 h (Fig. 3B).

**Dose-dependent cyclosporine inhibition of NcAg-induced IFN-γ production by bovine PBMC and T cells.** T. gondii C-18 has been shown to stimulate dendritic cells to produce IL-12 (1), which in turn elicits the production of IFN-γ by T cells (24). The NcAg stimulation of IFN-γ production by PBMC and T cells and the inhibitory effect of cyclosporine on NcAgelicited IFN-γ production by PBMC and T cells as an indirect indication of IL-12 production by dendritic cells are demonstrated in Fig. 4. The NcAg (1 μg/ml) alone stimulated high levels of IFN-γ production by the PBMC of infected cows (Fig. 4). Cyclosporine exhibited a dose-dependent inhibitory effect on NcAg-stimulated IFN-γ production by the PBMC of N. caninum-exposed cows. A dose range of 100 to 1,000 nM for cyclosporine was effective to completely suppress the IFN-γ production stimulated by 1 μg/ml of whole-cell NcAg (Fig. 4A and B).

Since CyP elicits IL-12 production by dendritic cells in an antigen-nonspecific manner (1), we have further determined the effect of CyP on IFN-γ production by PBMC from naive cows or antigen-specific T cells established from N. caninum-infected cows (Fig. 5A to D). PBMC isolated from the N. caninum-nonexposed cows used in this experiment did not proliferate in response to NcAg (data not shown). NcAg-specific T-cell lines composed of primarily the CD4+ T cells used for this experiment proliferated highly to NcAg stimulation (Fig. 5A and B). Again, NcAg alone stimulated high levels of IFN-γ production by both PBMC from naive cows and T cells.
from infected cows (Fig. 5C and D). In the presence of 100 nM cyclosporine, the production of IFN-γ induced by NcAg was abolished (Fig. 5C and D).

### DISCUSSION

Our previous study demonstrated that immunodominant antigens within the molecular mass range of 17 to 19 kDa were identified by antibodies against *N. caninum* tachyzoites. These antigens were present only in high-pressure liquid chromatography fractions that highly stimulated NcAg-specific CD4+ T-cell proliferation (30). Consequently, these results led to the identification of NcCyP in the present study. The findings of the present research have demonstrated that NcCyP is present in *N. caninum* tachyzoites and induces IFN-γ production. The cyclosporine-blocking experiments indirectly indicate that NcCyP is the major component responsible for the induction of IFN-γ production by PBMC and T cells. Since IL-12 up-regulates IFN-γ production by T cells and NK cells and *T. gondii* C-18 (highly similar to NcCyP) induces IL-12 in the mouse (1), we hypothesized that IFN-γ production by either PBMC or T cells in this study is due to the effect of IL-12 induced by NcCyP. Our results confirmed the previous report that CyP is a critical *T. gondii* tachyzoite constituent that...
accountable for the induction of IL-12 production by dendritic cells in the mouse model (1). These results support the notion that the presence of IFN-γ-inducing factors, such as CyP, may be important for the control of acute neosporosis by eliciting high levels of IFN-γ, leading to the latent disease state which does not pose health problems unless animals become preg-

FIG. 3. Western blot analysis of NcCyP in lysates of *T. gondii* and *N. caninum* tachyzoites (A) and *N. caninum* tachyzoite culture supernatant (B) using a rabbit antibody against *T. gondii* C-18. Fifteen μg total proteins was loaded per lane. Lanes 1, molecular weight markers (MagicMark); lane 2 (panel A), *T. gondii* tachyzoite lysate; lane 2 (panel B), *N. caninum* tachyzoite culture supernatant incubated in PBS at 37°C for 1 h; lane 3, whole-cell NcAg.

FIG. 4. Dose-dependent inhibition by cyclosporine (CsA) of NcAg-stimulated IFN-γ produced by bovine PBMC. Freshly isolated PBMC were cocultured for 48 h at 37°C with (+) or without (−) NcAg (1 μg/ml) in the presence or absence (−) of increasing concentrations of cyclosporine (0.1 to 1,000 nM). Data represent results from two *N. caninum*-positive cows (with high anti-*N. caninum* tachyzoite antibody titers [data not shown]), cows 1816 (A) and 2443 (B), in two separate experiments. Values are means of ng/ml IFN-γ, and error bars represent standard errors of the means.

FIG. 5. Cyclosporine (CsA) suppression of NcAg-elicited IFN-γ production by PBMC from *N. caninum*-positive cows and bovine *N. caninum*-specific CD4+ T cells established from *N. caninum*-positive cows. Specific proliferation of the *N. caninum*-specific CD4+ T-cell lines in response to NcAg stimulation (A), phenotypes of these T cells (B), and CsA inhibition of NcAg-stimulated IFN-γ production by PBMC (C) and by CD4+ T-cell lines (D) of infected cows are shown. Values in panel A are means of counts per minute (cpm), in panel B are means of percent total T cells, and in panels C and D are means of ng/ml IFN-γ. Error bars represent standard errors of the means. Host cell proteins were prepared from the host cells used to propagate *N. caninum* tachyzoites in vitro.

nant (13). On the other hand, as speculated (1), production of CyP may be one of the survival strategies of the parasite, which intends not to kill the infected intermediate hosts.

NcCyP is highly similar to *T. gondii* C-18 in that they both
are secretory proteins containing a number of conserved functional motifs. One of the significant differences between T. gondii C-18 and NcCyP is that NcCyP is 1 amino acid residue shorter than T. gondii C-18 at the C terminus. However, it is unknown how such a change in NcCyP would affect its function. Since cyclosporine has effectively inhibited NcCyP function to upregulate IFN-γ, it may be reasonable to speculate that the amino acid residue at the C terminus is not critical to the IFN-γ-inducing effect of NcCyP or cyclosporine binding to CyP. In fact, most CyP sequences lack one to two of the amino acid residues at the C termini in comparison to T. gondii C-18, indicating that such a deletion may not have a significant effect on their PPIase and cyclosporine binding activities. The other difference between NcCyP and T. gondii C-18 is that NcCyP had an additional predicted N-linked glycosylation site (a total of three N-linked glycosylation sites) compared to T. gondii C-18 (a total of two N-linked glycosylation sites). This may explain the apparent molecular weight differences between NcCyP and T. gondii C-18 demonstrated in this study. The presence of an extra N-linked glycosylation site in NcCyP may explain the slight increase in molecular weight of NcCyP detected in Western blot analysis. The effect of such a glycosylation on CyP is unknown in comparison to T. gondii C-18. Recombinant T. gondii C-18 has been shown to have potent PPIase and IL-12-inducing activities (1), suggesting that additional N-linked glycosylation may have little effect on CyP function.

Our results indicated that cyclosporine inhibits NcAg-induced IFN-γ production in a dose-dependent manner without a noticeable effect on cell survival. Interestingly, cyclosporine was able to inhibit NcAg-elicted IFN-γ production by PBMC from naive animals as well as PBMC and T cells derived from infected or immunized animals. For PBMC from naive cows, the production of IFN-γ by naive T cells and NK cells may rely largely on IL-12 induced by NcCyP in NcAg. Therefore, IFN-γ production may be abolished once the source for IL-12 is blocked. For PMBC and CD4+ T cells in the presence of APCs from infected or immunized cows, IFN-γ production by antigen-specific T cells may depend on both antigen-specific interactions between the APCs and CD4+ T cells and the IL-12 present at the antigen-priming stage. Apparently, as shown in this study, IL-12 production may be very critical for IFN-γ production by antigen-specific CD4+ T cells since the specific blockade of NcCyP, an IL-12-inducing microbial component, led to the elimination of IFN-γ. These results suggest that the lack of or reduced levels of NcCyP will influence the cytokine milieu at antigen priming and affect vaccination outcome. This further suggests that the lack of critical microbial regulatory components in vaccination regimes will have a major impact on vaccine efficacy. This may be particularly important for subunit vaccines in which only one or a few characterized proteins are included. The omission of important inflammatory cytokine-inducing microbial factors, in the case of intracellular pathogens, will explain why many of the subunit vaccines had no effect on protection or reduced efficacy (22, 23, 26). The study of such a potent microbe-derived IFN-γ inducer will facilitate our understanding of mechanisms by which the invading parasite manipulates host immune response for the purpose of long-term survival of the species. The knowledge obtained from these studies may be used to develop vaccines against parasitic diseases by using the parasite’s strategies that have evolved over millions of years.

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