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Requirement for CD40-CD40 Ligand Interaction for Elimination of *Cryptosporidium parvum* from Mice

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Mice with disrupted genes for CD40 and CD40 ligand (CD40L) are unable to clear infection with *Cryptosporidium parvum* and develop cholangitis. Parasites are present in the gut, gall bladder, and biliary tree, and biliary epithelial cells express CD40 on the cell surface. SCID mice infected with *C. parvum* for >1 month can clear the infection after reconstitution with spleen cells from CD40, but not CD40L, knockout mice. In an in vitro model, *C. parvum*-infected HepG2 cells were triggered to apoptosis when incubated with a CD40L-CD8 fusion protein. The requirement for CD40-CD40L interactions for immunity to *C. parvum* indicated by our results may entail the triggering of apoptosis in infected cells, in addition to the known role of CD40L-CD40 interactions in stimulating cytokine production and promoting T-cell responses.

Cryptosporidium parvum is a highly infectious protozoan parasite responsible for cryptosporidiosis in humans and many animals (6). *C. parvum* oocysts discharge sporozoites which then attach to and replicate in the intestinal epithelium, causing changes in electrolyte handling (8). Immunocompetent subjects experience a transient diarrhea, while individuals with impaired immunity, such as AIDS patients, are unable to clear the infection. The severity of the diarrhea (14) and cholangitis (5), which *C. parvum* causes in immunodeficient patients, has focused increasing attention on mechanisms of cryptosporidial immunity. Immunocompetent hosts respond to infection with antibody production, and the secreted antibodies appear to reduce parasite numbers in the intestine. Nevertheless, antibodies to *C. parvum* do not seem to be able to protect AIDS patients from heavy parasite burdens (6), and it seems likely that cell-mediated immunity is important for recovery from *C. parvum* infection. We recently identified *C. parvum* as an important pathogen in boys with the hyper-immunoglobulin M (hyper-IgM) syndrome (9), in which mutations of the CD40 ligand (CD40L) gene prevent the expression of a T-cell ligand important for T-cell immunity and for immunoglobulin isotype switching (16).

Animal models provide the strongest evidence that cell-mediated immunity plays a major role in recovery from *C. parvum* infection. SCID and nude mice, for example, can be infected with *C. parvum* with colonization of the ileum and gall bladder (15, 20), and they are partially protected by cytokines such as gamma interferon (IFN-γ) (3) and interleukin-12 (IL-12) (22). Although CD40L defects clearly predispose humans to *C. parvum* infections and soluble CD40L can interfere with *C. parvum* infection of hepatocellular cells (9), the mechanisms for this increased susceptibility are not understood. CD40L is a tumor necrosis factor-like molecule that is transiently expressed on T cells after activation and which has recently been implicated in the host defense against vaccinia virus (18), herpes simplex virus, and *Pneumocystis carinii* (26). CD40, the natural ligand of CD40L, is expressed on the bile duct epithelial (BDE) cells, to which cryptosporidia can attach (9). Exploration of the role of CD40-CD40L interactions in the host defense against cryptosporidia requires an animal model, so we tested mice with disrupted genes for CD40 and CD40L for their ability to eliminate cryptosporidia.

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MATERIALS AND METHODS

Animals and infection. The C57BL/6 CD40L knockout mice (7) were supplied by R. Flavell, and the C57BL/6 CD40 knockout mice (12) were supplied by H. Kikutani. These and the C57BL/6 SCID animals were bred in our specific-pathogen-free mouse facility, were housed in microisolator cages, and received sterile food, water, and bedding. Eight milliliters of trimethoprim-sulfamethoxazole antibiotic solution was added to drinking water in alternate weeks for *P. carinii* prophylaxis. Animals (approximately equal numbers of males and females) were transferred to a biohazard facility for infection with *C. parvum* at between 4 and 5 weeks of age, and stool specimens were collected weekly to monitor infection status.

C. parvum oocysts were obtained from McKesson Bioservices (catalog no. 1372) through the AIDS Research and Reference Reagent Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Before use, they were washed in phosphate-buffered saline (PBS) to remove the potassium dichromate buffer and then sterilized in a 1:10 sodium hypochlorite solution in PBS followed by two washes in PBS to remove bleach (21). Animals were infected by gavage one time with 10^6 oocysts in 0.1 ml of Hanks balanced salt solution.

Characterization of knockout animals. Offspring of the homozygous CD40L knockout and heterozygous CD40 knockout parents were bled once at 4 weeks. The serum was tested for IgG in a radial immunodiffusion assay (catalog no. RA272; Binding Site, Birmingham, United Kingdom) according to the manufacturer's instructions. Animals with serum IgG levels of below 15 mg/dl were verified as CD40L knockout mice on the basis of their inability to switch immunoglobulin subclasses from IgM to IgG.

Blood mononuclear cells were stained for CD40 with a fluorescein isothiocyanate (FITC) conjugate of the clone 3/23 (no. 09964D; Pharmingen, San Diego, Calif.), and likely knockout mice were identified by reduced CD40 expression. Characterization of mice as homozygous CD40 knockout mice was confirmed by PCR amplification of DNA extracted from blood by using DNAstat (Tel-Test, Friendship, Tex.). The primers and PCR conditions are described in references 7 and 12. The PCR products were resolved on a 1% agarose gel in the presence of 5 μg of ethidium bromide per ml to identify the 900-bp products in the $CD40^{+/+}$ and $CD40^{+/-}$ mice, with a 2.1-bp product from the neomycin cassette in the $CD40^{-/-}$ mice.

ELISA. Stool specimens were tested for the presence of cryptosporidium-specific antigen (CSA) by using an enzyme-linked immunosorbent assay (ELISA) kit (Prospect-T catalog no. 540-96; Alexon, Sunnyvale, Calif.). Stool specimens were stored at −70°C until use and then thawed and resuspended by vortexing in 300 μl of sample dilution buffer. The suspension was spun at 250 × *g* for 10 min to remove solid debris, and then 200 μl of the supernatant along with positive and negative controls supplied with the kit were transferred to the wells of the 96-well anti-CSA ELISA plate. Plates were incubated for 1 h at room temperature, washed, incubated for 30 min with enzyme conjugate, washed, and

incubated for 10 min at room temperature with substrate; stop buffer was then added, and results were read on a Dynatech plate reader at 450 nm.

The presence of oocysts in stool samples was also verified by staining cytocentrifuged samples of resuspended stool, staining with a monoclonal antibody (8C12) to oocyst antigens followed by anti-mouse IgG–phycoerythrin, and examining with a fluorescence microscope. The ELISA and immunofluorescence tests were concordant when infected and uninfected animals were compared.

Tissue specimens. Livers and gall bladders were harvested from all mice, and small intestines were harvested from representative animals. Tissue for CD40 immunostaining was snap frozen in the vapor phase of liquid N_2, and tissue for electron microscopy was fixed in 2.5% glutaraldehyde and postfixed in osmium tetroxide. Tissue for routine histology was fixed in 10% buffered formalin and paraffin embedded for sectioning and hematoxylin-and-eosin staining by standard methods. Representative intact gall bladders, each from a different mouse, were also preserved by each of the above-described methods.

Immunohistochemistry. Frozen sections of liver were thawed and air dried, and endogenous peroxidase was blocked with 6% H_2O_2 in PBS with 20% rat serum. One hundred microliters of a 1:20 dilution of rat anti-mouse CD40 (catalog no. RM6800; Caltag, San Francisco, Calif.) in PBS with 2% rat serum was added to each section and incubated at 4°C for 1 h in a humid chamber. After being washed, sections were treated with 100 μl of a 1:200 dilution of rat anti-mouse–horseradish peroxidase (cat R40007, Caltag) for 30 min at 4°C. Sections were incubated for 5 min with diaminobenzidine and then counterstained with hematoxylin. Specimens were blinded and scored as positive or negative for the presence of CD40 on BDE. Slides were viewed on a Leitz Ortholux microscope with incident UV and transmitted phase-contrast optics. Images were captured with an LG3 camera and processed with Adobe software.

TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) staining. HepG2 cells were cultured on glass coverslips in six-well tissue culture plates and infected with 10^6 *C. parvum* oocysts as previously described (9). The cells were then treated with culture supernatant containing CD40L fusion protein (13) for 18 h, and then cells were processed for detection of DNA fragmentation characteristic of apoptosis by using the Apotag kit (Oncor, Ltd., Gaithersburg, Md.) according to the manufacturer's instructions.

Reconstitution experiments. Donor mice were killed at 6 weeks of age by CO_2 inhalation, and spleen cell suspensions were prepared at 5×10^7 cells/ml. Cells were washed and resuspended in Hanks balanced salt solution, and 5×10^6 splenocytes were injected into the peritoneal cavity of each recipient mouse. *C. parvum*-infected SCID recipients were infected at 4 weeks of age as described above and were used as recipients at 8 weeks, after two stool samples had tested positive for *C. parvum* infection by ELISA. At various intervals after reconstitution, blood of reconstituted SCID mice was stained with FITC-conjugated monoclonal antibodies to murine CD4 and CD8 (L3/T4 [Caltag catalog no. RM2401-3] and Ly 2 [Caltag catalog no. RM2204], respectively) and viewed on a Coulter Elite cytometer to confirm T-cell reconstitution.

RESULTS

Persistence of *C. parvum* infection in CD40 and CD40L knockout mice. The ELISA test for *C. parvum* antigen became positive with stool samples from all animals in the week following *C. parvum* infection. ELISA positivity was accompanied by the shedding of oocysts identified in stool smears by immunofluorescence microscopy and by acid-fast staining (not shown). Because the ELISA test with stool from uninfected animals was consistently negative while results from infected animals were consistently positive, we elected to use this test to monitor the course of *C. parvum* infection in the knockout animals. Results from the CD40 and CD40L knockout mice showed an increase in stool ELISA optical density (OD) over the first 2 weeks, with OD levels above 0.1 being maintained for 8 weeks or more (Fig. 1). Littermates with wild-type CD40 and CD40L genes also became infected as judged by their stool ELISA OD, but results of this test reverted to control values in the 4 weeks that followed infection.

Tissue evidence for infection. The anti-CSA ELISA measures an antigen released during the course of infection but gives no indication of the location of infection or the severity of tissue damage. To address these issues, the livers, gall bladders, and intestines of infected and control mice were harvested for histology. Figure 2 shows the gall bladder wall of an uninfected control (Fig. 2a) and *C. parvum* associated with the gall bladder epithelium of an infected CD40L knockout mouse (Fig. 2b, arrow). The occurrence of *C. parvum* in the gall bladder was consistently associated with the presence of a mononuclear infiltrate in the underlying tissue. *C. parvum* was also seen attached to the crypts and villi of the jejenum and ileum (not shown); there was no inflammatory infiltrate in the lamina propria or muscularis coats of the bowel wall. Infection of small bile ducts within the liver was documented by electron microscopy (Fig. 2c, arrow). In the liver, *C. parvum* infection was associated with an inflammatory response in both the CD40 and CD40L knockout animals. We previously showed that CD40 was expressed on the BDE of boys with CD40L deficiency and cholangitis, so it was of interest to determine whether CD40 was also expressed on mouse bile ducts. The results of immunoperoxidase staining (Fig. 2d) show positive CD40 staining of a *C. parvum*-infected CD40L knockout animal. Portal veins (Fig. 2e) and adjacent small bile ducts were surrounded by a mixed cellular infiltrate comprising predominantly mononuclear cells but with some neutrophils. Sclerosis and obliteration of bile ducts were also seen (Fig. 2f, arrow).

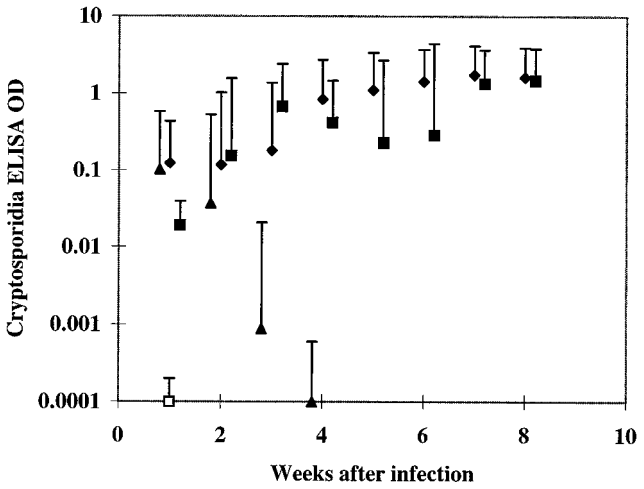

FIG. 1. ELISA test for *C. parvum* in feces from homozygous CD40L knockout mice (■), homozygous CD40 knockout mice (◆), and wild-type B6 controls (▲). □, results for uninfected animals. Results are the log means + 1 standard deviation for groups of six to eight animals.

CD40L knockout effector cells fail to eliminate *C. parvum* from SCID mice. Adoptive transfer of spleen cells from the knockout animals into an environment containing CD40 was used to explore the role of CD40 and CD40L in the elimination of *C. parvum*. Thus, B6 SCID mice with intact CD40 expression were infected with *C. parvum*, and infection was verified by stool positivity in the ELISA test. These mice were then reconstituted with 5×10^6 spleen cells from uninfected CD40 and CD40L knockout donors. The SCID recipients of CD40 knockout T cells cleared the infection in the ensuing 7 weeks (Fig. 3), while the recipients of the CD40L knockout cells remained with positive stool ELISA tests.

Apoptosis in *C. parvum*-infected cells in vitro induced by CD40L. To determine whether CD40L could interact directly with CD40 on a *C. parvum*-infected target cell, we used the CD40-positive cell line HepG2. We had previously determined that this line could be infected in vitro with *C. parvum* and that infection was reduced when a culture supernatant containing CD40L fusion protein was added (9). To determine whether apoptosis contributed to this effect, we cultured control and *C. parvum*-infected HepG2 cells with and without CD40L fusion protein and then stained them for DNA fragmentation by

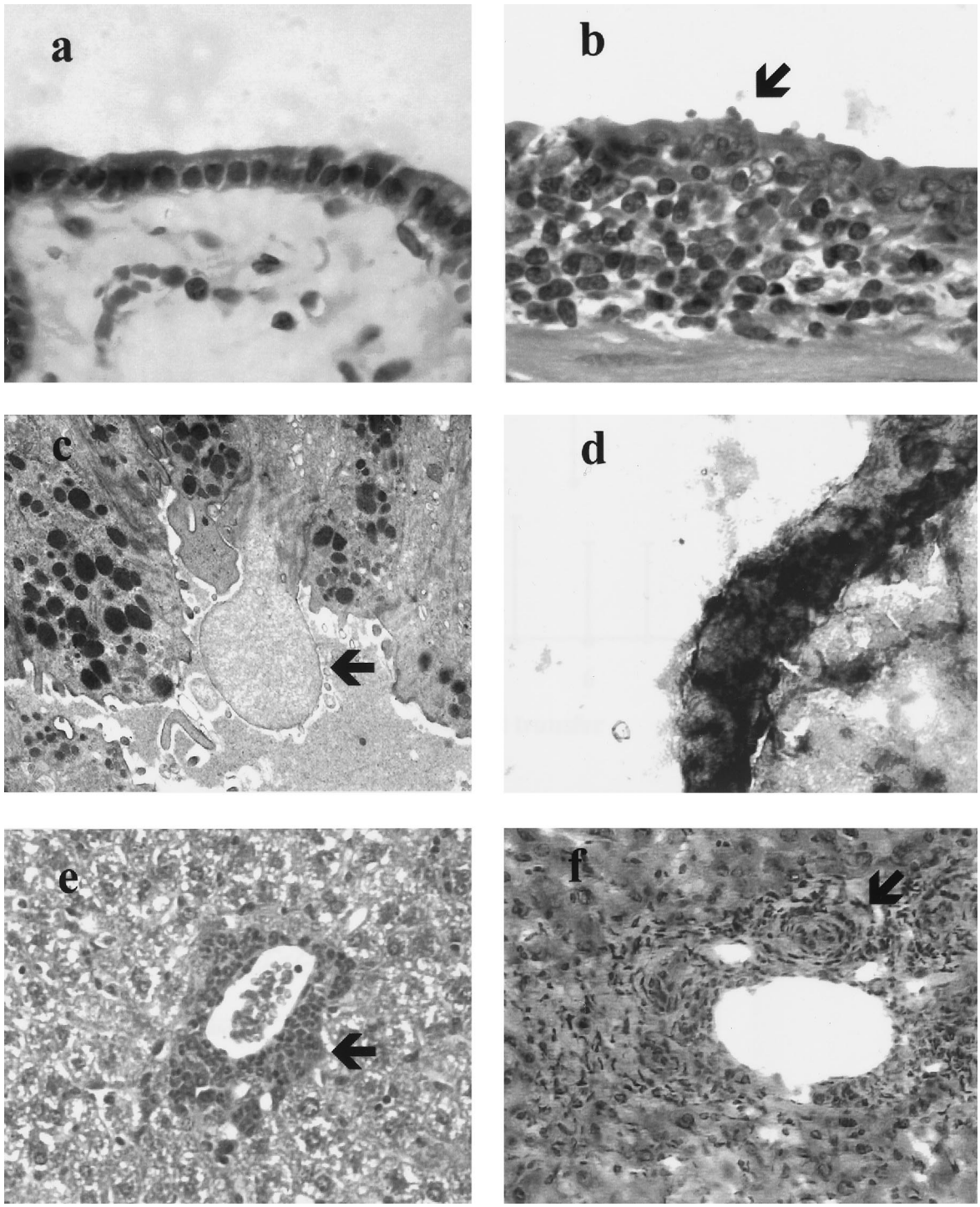

FIG. 2. Histologic features of *C. parvum* infection in CD40L knockout mice. (a) Uninfected control gall bladder. Magnification, ×400. (b) to (f) Infected animals. (b) Gall bladder. Magnification, ×400. Arrow, *C. parvum*. (c) Transmission electron micrograph. Arrow, *C. parvum*. Magnification, ×10,000. (d) CD40 expression on BDE. Magnification, ×1,000. (e) Mononuclear infiltrate (arrow) surrounding a small portal vein. Magnification, ×400. (f) Extensive fibrosis and bile duct sclerosis (arrow) surrounding a portal vein after >12 weeks of infection. Magnification, ×400.

the TUNEL technique. No nuclear staining was observed in the HepG2 cells cultured with *C. parvum* or the CD40L fusion protein alone (not shown). Apoptotic cells were seen (Fig. 4) in cultures of HepG2 cells which were first infected with *C. parvum* and then exposed to CD40L for 18 h.

DISCUSSION

Our data indicate that CD40L and CD40 are necessary for mice to clear a *C. parvum* infection. This result is consistent with the finding that boys with CD40L deficiency, who have the

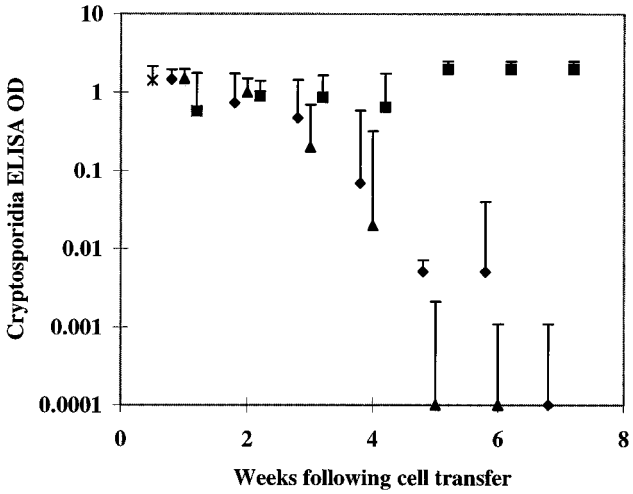

FIG. 3. ELISA test with feces from SCID mice infected with *C. parvum* before reconstitution (×) and after reconstitution with 10^6 spleen cells from homozygous CD40L knockout mice (■), homozygous CD40 knockout mice (◆), and wild-type B6 controls (▲). Results are the log means + 1 standard deviation for groups of six to eight animals.

hyper-IgM syndrome, are susceptible to severe and protracted cryptosporidium infections. A role for CD40-CD40L interactions in immunity to other intracellular pathogens, including *Leishmania* spp. (2, 11, 19) and *P. carinii* (26), has recently been reported, and our results indicate that *C. parvum* may be similarly handled. There are several mechanisms by which CD40-CD40L interactions may be required for immunity to intracellular parasites. CD40L can contribute to T-cell activation by binding to CD40 on macrophages, stimulating IL-12 production and the upregulation of costimulatory molecules such as B7 (11). CD40L might also convey an afferent signal to a T cell, allowing $CD4^+$ cells which have bound antigen through the T-cell receptor to become more efficient helpers of B cells (23) and perhaps to advance through the activation pathway (7). An inability to make CD40L would therefore be expected to interfere with T-cell activation and hence with the clonal expansion of antigen-specific T cells (1), which is required for the expression of cell-mediated immunity. In the context of immunity to *Leishmania*, CD40 was important for conveying an afferent signal for macrophages that enhanced their production of IL-12 (11, 25), which in turn permitted a greater TH1 component of the T-cell response. This interpretation is consistent with our finding that spleen cells from CD40 knockout donors were able to clear *C. parvum* infection from SCID recipients, while they were not able to clear the infection from an environment devoid of CD40. Nevertheless, the afferent signal to T cells through CD40L is not obligatory for all T-cell-mediated responses. For example, CD40L-deficient mice are able to generate substantial CD8 and some CD4 T-cell responses to viruses such as lymphocytic choriomenigitis virus (17, 25), even though their $CD4^+$ cells cannot receive a CD40L-mediated signal. Similarly, CD40L-deficient boys do not in general have severe herpesvirus or fungal infections (16), even though they are unable to switch the isotype of their antibody responses from IgM to IgG or IgA because they cannot make CD40L.

Another, and not necessarily exclusive, explanation for the persistence of *C. parvum* in CD40 and CD40L knockout mice is that *C. parvum* cannot be eliminated from the infected target cell unless a CD40-CD40L-mediated signal is delivered. CD40L is known to deliver effector signals to B cells, causing an isotype switch, and can also trigger an apoptotic pathway in other cells, such as fibroblasts (10). We had previously shown that inflamed human BDE expressed CD40, and in our present study we show that mouse BDE from *C. parvum*-infected animals is also positive for CD40. In an in vitro model we found previously that soluble CD40L sufficed to trigger DNA condensation in the CD40-positive HepG2 cell line when it was infected with *C. parvum*, and we have now used the more specific TUNEL technique to show that this condensation is associated with DNA fragmentation typical of apoptosis. HepG2 cells have many similarities with BDE cells (4), raising the possibility that their responses may serve as a model for the consequences of CD40L binding to CD40 on a *C. parvum*-infected BDE cell.

Other host defense factors thought to contribute to *C. parvum* immunity include IFN-γ and IL-12, and these cytokines are also known to interact with the CD40-CD40L pathway. IFN-γ, for example, upregulates expression of CD40 (24), among other cell surface determinants, and IL-12 boosts the IFN-γ component of T-cell responses. Treatment with IL-12 prior to infection with *C. parvum* prevented or greatly reduced infection in neonatal SCID and normal mice. However, established infections were not ameliorated by IL-12 treatment (22). CD40L induces macrophages to produce inflammatory cytokines, including tumor necrosis factor alpha, IL-1β, IL-6, and IL-8, many of which can affect BDE cells (24). Given the range of T-cell interactions which can lead to the production of cytokines which might in turn regulate CD40 expression on *C. parvum*-infected cells, it is not surprising that immunity to *C. parvum* could fail in certain primary and secondary immunodeficiency states.

Experimental approaches using knockout animals can simplify study design by reducing the number of variables. In the context of *C. parvum* infection, the CD40L knockout mice may well serve as an animal model for *C. parvum* infection of the biliary tree in boys with X-linked immunodeficiency with hyper-IgM syndrome. To define the role of CD40L in *C. parvum* immunity and to distinguish between the potential contributions of the afferent and efferent signaling pathways of CD40L, further adoptive transfer experiments are likely to be necessary.

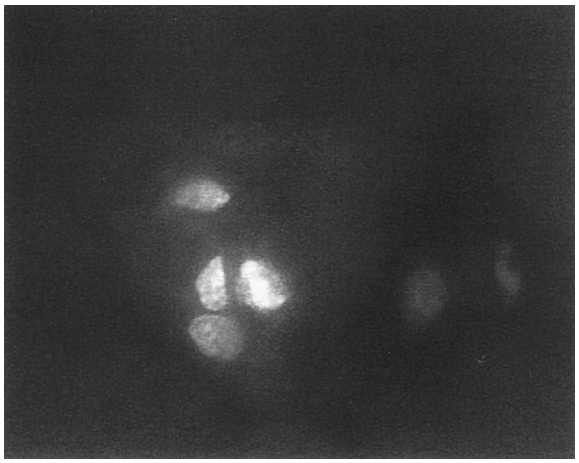

FIG. 4. TUNEL staining of HepG2 cells infected with *C. parvum* for 72 h and then incubated with CD40L fusion protein for 18 h. The stained nuclei are visualized by virtue of their binding FITC-conjugated antidigoxigenin antibody.

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