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Role of Tumor Necrosis Factor Alpha in Development of Immunity against *Cryptosporidium parvum* Infection

I-Sarah Lean,[1] Sonia Lacroix-Lamandé,[2] Fabrice Laurent,[2] and Vincent McDonald[1]*

Centre for Gastroenterology, Institute of Cell and Molecular Science, Barts and the London School of Medicine, Turner Street, London E1 2AD, United Kingdom,[1] and Laboratoire Contrôle et Immunologie des Maladies Entériques du Nouveau-né, UR1282, IASP213, IFR136, INRA de Tours, 37380 Nouzilly, France[2]

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Tumor necrosis factor (TNF-α) significantly reduced *Cryptosporidium parvum* development in a murine enterocyte cell line, and a key mechanism of action appeared to be inhibition of parasite invasion. However, TNF-α-deficient mice controlled infection as effectively as wild-type mice. This suggests that TNF-α might have only a redundant role for establishing immunity against *C. parvum*.

The protozoan *Cryptosporidium parvum* is an important agent of cryptosporidiosis that infects different host species, including cattle and humans (2). The entire development of *Cryptosporidium*, comprising asexual reproduction, gametogony, and formation of oocysts containing four sporozoites, occurs within the same host and infection is transmitted in a fecal-oral manner by oocysts (2). The disease is characterized primarily by diarrhea that is normally of limited duration, but infections in immunologically immature or immunocompromised hosts can be severe and fatal. Chemotherapeutic agents are often ineffectual, but some immunotherapies can reduce parasite reproduction (2).

Immunologically mediated elimination of *Cryptosporidium* requires $CD4^+$ T cells (6, 18, 20), and this occurs readily in the absence of either $CD8^+$ T cells or B cells (reviewed in references 6 and 18). IFN-γ is crucial in the development of innate and adaptive immunity. A potent IFN-γ-dependent innate immunity has been described in T-cell-deficient mice, with the most likely source of the gamma interferon (IFN-γ) being NK cells (6, 20). Clearance of infection in humans, mice, and cattle, however, appears to require a Th1 response in which $CD4^+$ T cells produce IFN-γ (6, 8, 18, 20, 22). IFN-γ-deficient mice are highly susceptible to *C. parvum* infection, but whereas C57BL/6 (B.6) IFN-$\gamma^{-/-}$ mice usually die, BALB/c IFN-$\gamma^{-/-}$ mice survive infection, although the IFN-γ-independent immune mechanisms are unclear (5, 17, 18). One important protective role for IFN-γ may be activating antimicrobial killing mechanisms by enterocytes (11).

TNF-α often plays a key role in inflammation and it can also be involved in immunoprotective mechanisms against intracellular infections (7, 9, 23). In humans infected with *C. parvum*, tumor necrosis factor alpha (TNF-α) levels in the intestine were elevated (12). Also, the cytokine was expressed in *C. parvum*-infected human intestinal xenografts in SCID mice (15). During infection of B.6 wild-type mice, TNF-α mRNA was highly expressed in the intestine, but in B.6 IFN-$\gamma^{-/-}$ mice that experience a rapidly developing fulminant infection, the expression was poor (3). In contrast, TNF-α expression by splenocytes of infected BALB/c IFN-$\gamma^{-/-}$ mice increased during infection (17). We observed that administration of TNF-α to B.6 IFN-$\gamma^{-/-}$ mice ameliorated infection (3), and in addition, the cytokine inhibited *C. parvum* infection of the human enterocyte cell line HT29, although no antimicrobial killing mechanism was established (11).

These studies suggest that TNF-α might be involved in immune mechanisms that control *C. parvum* infection. The aim of the current investigation, therefore, was to employ in vitro and in vivo infection models to better characterize the role of TNF-α in the host protective immune response against *C. parvum*.

TNF-α prevents establishment of *C. parvum* infection in enterocytes. The possibility that TNF-α could inhibit *C. parvum* sporozoite invasion of enterocytes in vitro was investigated. The CMT-93 cell line was employed, as it is derived from the B.6 mice used in our in vivo experiments and it readily supports *C. parvum* infection and also because small young trophozoites (~1-μm diameter) can be easily detected after Giemsa staining (4). The cells were grown in 24-well plates and infected as described previously (4). Some monolayers were treated with murine recombinant TNF-α (Peprotech, United Kingdom) for 24 h before infection; this treatment did not affect cell viability. Two hours after the addition of 2×10^5 oocysts (Moredun isolate), the monolayers were fixed and stained for microscopic examination. Cells that had been pretreated with 20 ng/ml TNF-α had 79% fewer trophozoites than control cells (466 ± 36 compared with 98 ± 37; $P < 0.0001$ by analysis of variance) (Fig. 1). Even the lowest employed concentration of TNF-α reduced parasite numbers by 67%. These findings confirm our earlier result (using a human cell line) that TNF-α inhibits *C. parvum* development in enterocytes (11) and, in addition, suggest that an important mechanism of cytokine action is to prevent parasite invasion. Interestingly, TNF-α has a variable capacity to directly affect development of intracellular parasites. It inhibited reproduction of *Leishmania* in macrophages (19) but not development of two organisms related to *Cryptosporidium*: *Toxoplasma gondii* in neuronal cells (13) and *Plasmodium yoelii* sporozoites in hepatic cells (10). A direct inhibitory effect on *C. parvum* development of

* Corresponding author. Mailing address: Barts and the London School of Medicine, Institute of Cell and Molecular Science, Centre for Gastroenterology, Turner Street, London E1 2AD, United Kingdom. Phone: (44) 20 7882 7202. Fax: (44) 20 7882 2192. E-mail: v.mcdonald@qmul.ac.uk.

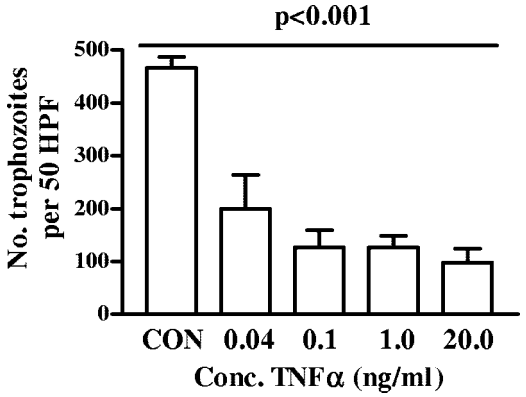

FIG. 1. Effect of treatment of CMT-93 cells with TNF-α on invasion by *C. parvum* sporozoites. TNF-α was added to monolayers of CMT-93 cells 24 h before the addition of oocysts. Two hours postinfection parasites were counted microscopically in 50 high powered fields (HPF). Four samples were prepared for each treatment, and the data represent mean parasite numbers ± standard deviations. Results show that significantly fewer trophozoites were present in cells treated with TNF-α ($P < 0.001$; analysis of variance).

cells may partly explain our observation that TNF-α treatment of B.6 IFN-$\gamma^{-/-}$ mice reduced parasite reproduction (3).

B.6 TNF-$\alpha^{-/-}$ mice are less able than wild-type mice to express intestinal IFN-γ at the peak of *C. parvum* infection but express IL-1α normally. The involvement of TNF-α in the host response to *C. parvum* was studied using B.6 wild-type and TNF-$\alpha^{-/-}$ mice (Jackson Laboratory, Bar Harbor, Maine). Neonatal mice were infected at 7 days of age by oral gavage with 1×10^4 *C. parvum* oocysts. As immunity in neonatal mice had correlated with high intestinal expression of IFN-γ mRNA on day 7 postinfection (5), measurements of the cytokine were made at this time by RT-PCR. Cytokine expression was measured in the colonic tissue and was normalized to the expression of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) housekeeping gene as described previously (5). IFN-γ was detected only in infected mice (Fig. 2A) but was at a higher level of expression than in wild-type mice ($P < 0.002$). On day 10, similar lower levels of expression were obtained in each strain. The day 7 findings were in agreement with results of a study involving *L. major*, in which the IFN-γ response was delayed in TNF-α-deficient mice (21). This suggests that TNF-α may promote the IFN-γ response by NK and T cells early in infection, and it is known, for example, that TNF-α enhances expression of the IFN-γ-inducing cytokine interleukin-12 (IL-12) (16).

Expression of another proinflammatory cytokine, IL-1α, which has some overlapping functions with TNF-$\alpha^{-/-}$, was also examined (the forward primer for IL-1α was AAGTTTG TCATGAATGATTCCCTC, and the reverse primer was GT CTCACTACCTGTGATGAGT). On day 7 of infection there was a significant increase in the amount of IL-1α in wild-type and mutant mice compared with their controls (*P* values were <0.002 and <0.02, respectively) (Fig. 2B), but no difference in expression was obtained between the strains. By day 10, the expression had descended to the control level. Thus, TNF-α deficiency did not affect the expression of IL-1α during *C. parvum* infection.

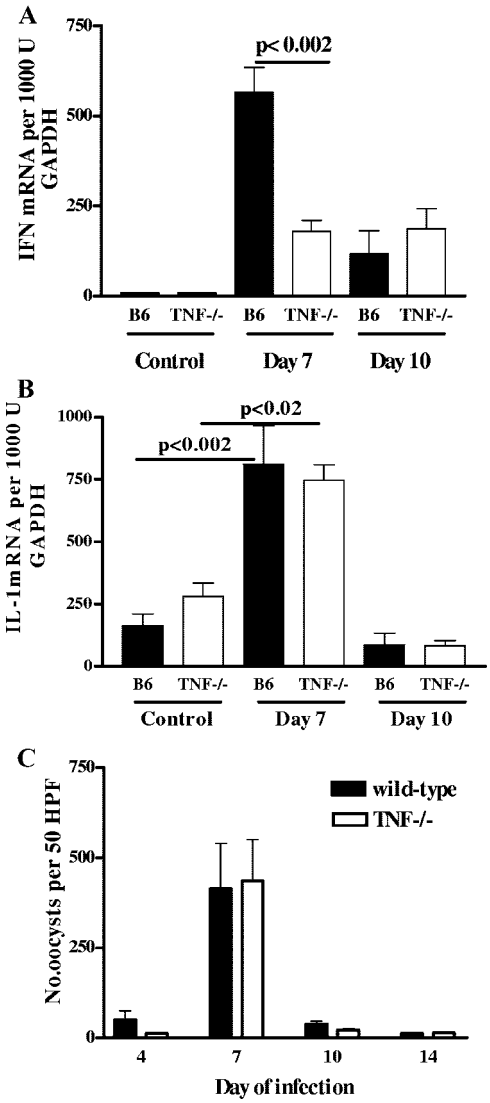

FIG. 2. (A through C) Expression of cytokine mRNA and oocyst production on different days of *C. parvum* infection of B.6 wild-type and TNF-$\alpha^{-/-}$ mice. Panels A and B show the mean ± standard error band intensities normalized to GAPDH for IFN-γ and IL-1α mRNA expression, respectively, on days 7 and 10. On day 7, IFN-γ expression was greater in wild-type mice than in TNF-$\alpha^{-/-}$ mice ($P < 0.002$; Mann-Whitney U test). Levels of IL-1α expression at the peak of infection were similar in wild-type and mutant mice, and the values were greater than those for uninfected controls ($P < 0.02$). Panel C shows oocyst reproduction in neonatal B.6 wild-type and TNF-$\alpha^{-/-}$ mice infected with *C. parvum*. Oocyst numbers in acid-fast stained smears of cecal contents were counted microscopically for six mice on each day of infection shown. No differences were observed in parasite counts (means ± standard errors) between conventional and mutant mice.

TNF-α deficiency does not increase susceptibility to infection. Oocyst excretion patterns in B.6 TNF-$\alpha^{-/-}$ and wild-type neonatal mice were compared at key stages of infection (Fig. 2C). Oocyst numbers in acid-fast stained smears of colon contents were measured microscopically in groups of six mice (5). However, no differences in mean oocyst production were observed between the two strains. Also, a histological study of

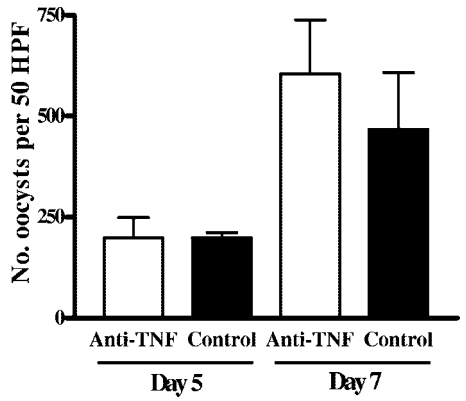

FIG. 3. Effect of anti-TNF-α-neutralizing MAbs on development of *C. parvum* in neonatal BALB/c IFN-$\gamma^{-/-}$ mice. Seven-day-old mice were injected subcutaneously with 100 μg hamster anti-mouse TNF-α IgG MAb 2E2 in PBS prior to infection with *C. parvum*. Controls received the same volume of PBS. Oocyst counts in cecal contents of eight mice per group were performed on days 5 and 7. No significant differences were found between mean oocyst counts in antibody-treated and control mice on either day.

hematoxylin/eosin-stained intestinal sections indicated no differences between the two mouse strains either in endogenous parasite numbers or in extent of inflammation (results not shown). Thus, TNF-$\alpha^{-/-}$ mice, despite having a reduced capacity to express IFN-γ at the site of infection, were able to control infection normally, indicating that TNF-α activity is not crucial for development of immunity to *C. parvum*. In contrast to this result, TNF-α deficiency in mice led to a loss of resistance against *Mycobacteria*, *Listeria*, *Leishmania*, and *Toxoplasma* spp. (7, 14, 21, 23). Also, infection in the absence of TNF-α could cause severe inflammation, but this was not evident in our study. Our findings do not discount the possibility that TNF-α has a redundant protective role so that in its absence, other proinflammatory cytokines, e.g., IL-1α, might become more prominent.

IFN-γ-independent immune mechanisms do not require TNF-α. We considered the possibility that in the absence of IFN-γ-dependent mechanisms of immunity, TNF-α might take on a more important protective role. To test this, neonatal BALB/c IFN-$\gamma^{-/-}$ mice were injected subcutaneously with 100 μg of hamster anti-mouse TNF-α immunoglobulin G-neutralizing monoclonal antibody (MAb) 2E2 (kindly provided by the National Cancer Institute, Rockville, MD) in phosphate-buffered saline (PBS) or with PBS only, prior to infection. The amount administered was derived from the study of Brieland et al. (1), in which the neutralizing effect in adult mice was evident for at least 6 days. Following infection, oocyst production was measured on days 5 and 7. There was no significant difference between parasite numbers obtained from control animals and anti-TNF-α-treated mice on either day, however (Fig. 3). This experiment was performed three times, with similar results being obtained, indicating that the IFN-γ-independent mechanisms of immunity in BALB/c mice do not require TNF-α activity. It has been reported that IL-4 was not involved either, as BALB/c wild-type but not IFN-γ-deficient mice given IL-4-neutralizing MAbs had an exacerbated acute infection (5). Thus, the nature of IFN-γ-independent immune mechanisms against *C. parvum* remains elusive.

In conclusion, the results of this study indicate that TNF-α can inhibit *C. parvum* reproduction in enterocytes by prevention of host cell invasion, but clearance of the infection in the murine host may still be readily accomplished without the involvement of this cytokine.

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