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Cryptosporidium parvum: The contribution of Th1-inducing pathways to the resolution of infection in mice

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Abstract

The contribution of cytokines IL-12, IL-18, IL-23, and IFN-γ, and Stat1 signaling molecules involved in Th1 responses associated with host resistance to *Cryptosporidium parvum* infection was investigated in adult IL-12p40$^{-/-}$mice. Host resistance to *C. parvum* infection was assessed in different mouse strains lacking IL-12, IL-18, and IL-23 genes. We found that as in IL-12p40$^{-/-}$ mice (which lack both IL-12 and IL-23), IL-12p35$^{-/-}$ mice (which lack IL-12) and IL-18 deficient mice were also susceptible to infection with *C. parvum.* Varied levels of resistance were observed when mice were treated with cytokines like IL-18, IL-23 and IFN-γ. Mice treated with IL-12, as expected, were completely resistant to infection until day 5 post infection, and had significantly decreased (85%) parasite loads at peak infection (day 7), whereas rIL-23 had a lesser effect, decreasing parasite load by approximately 45%. Interestingly, IL-18 appears to play a significant role in initial immune response, even in the absence of IL-12, since treatment with IL-18 in IL-12p40$^{-/-}$ knockout mice decreased parasite load by approximately 70%. In addition, the establishment of *C. parvum* infection in mice lacking the Stat1 gene demonstrated the involvement of this pathway in resolution of infection. These observations indicate a strong requirement for Th1 response in the development of immunity to *C. parvum* in the adult IL-12p40$^{-/-}$ mice, information that will be essential to further investigate the immune responses during infections and in the development of potential vaccine candidates.

Index Descriptors and Abbreviations: Cryptosporidium parvum; Protozoa; Oocyst; IL-12p40-knockout; Mouse; Interleukin (IL)-18; Interferon (IFN)-γ; Stat1; Host resistance

1. Introduction

Cryptosporidiosis is a gastrointestinal disease caused by parasitic protozoans from the *Cryptosporidium genus* with *Cryptosporidium parvum* the most widespread species affecting a variety of mammalian species, including mice and humans. These intracellular parasites infect epithelial cells in the intestine and are a significant cause of diarrhea in individuals with compromised immune systems, which has been associated with some morbidity and mortality (Egger et al., 1990; Guerrant, 1997; Hoxie et al., 1997; Riggs, 2002). Presently, effective chemotherapeutic agents, especially for immunocompromised individuals are lacking. Treatments that augment the immune response, such as cytokine therapy or vaccination could be employed to better safeguard against opportunistic infections.

Several studies have shown that cytokines are induced in response to *Cryptosporidium* infection (Tilley et al., 1995; Canals et al., 1998; Gomez-Morales et al., 1999; Robinson et al., 2000, 2001; Smith et al., 2000). For example, in a recent study from our laboratory, we reported changes in the gene expression of IFN-γ, IL-4, IL-15, IL-18, TNF-α and TGF-β during primary *C. parvum* infection of mice with a targeted disruption of the gene for IL-12p40. In addition, antigen-specific proliferation and IL-6 and IFN-γ production were also observed in MLN cells from these mice (Ehigiator et al., 2005). Of these cytokines, IFN-γ is believed to

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play a significant role in host resistance to infection with *C. parvum* as demonstrated by the fact that mice lacking the IFN-γ gene are more susceptible (Theodos et al., 1997; Griffiths et al., 1998; Mead and You, 1998; Campbell et al., 2002). Many investigators have reported the critical role for IL-12 in the differentiation of Th1 cells and subsequent production of IFN-γ (Hsieh et al., 1993; Gazzinelli et al., 1994; Urban et al., 1996; Cooper et al., 1997; Decken et al., 1998). However, other pathways for IFN-γ production involving IL-18, IL-23 and possibly IL-27 have also been reported (Qureshi et al., 1999; Oxenius et al., 1999; Wei et al., 1999; Kawakami et al., 2000; Cooper et al., 2002; Takeda et al., 2003; Artis et al., 2004; Lieberman et al., 2004a). For example, IL-18 has been shown to potentiate the differentiation of Th1 cells but does not induce the response (Cai et al., 2000a). However, IL-18 can induce host resistance against infection with intracellular parasites in the absence of IL-12 through innate immunity mediated by the production of IFN-γ by Natural killer cells (Bohn et al., 1998; Cai et al., 2000a; Kawakami et al., 2000). Other studies have demonstrated the involvement of IL-23 as an additional mechanism of resistance to intracellular organisms (Lieberman et al., 2004a). These studies clearly demonstrate the complexity and redundancy involved in the mechanism of recovery and resistance to infection with intracellular pathogens. While the involvement of these cytokines in immunity or host resistance to infections with intracellular pathogens such as *Toxoplasma gondii*, *Mycobacterium* spp., and *Cryptococcus neoformans* have been reported (Bohn et al., 1998; Cai et al., 2000a; Kawakami et al., 2000; Cooper et al., 2002; Lieberman et al., 2004a; McDonald et al., 2004), a definitive role for these cytokines involved in Th1-mediated responses has not been determined in the recently described adult IL-12p40$^{-/-}$ model of *C. parvum* infection (Campbell et al., 2002; Ehigiator et al., 2003, 2005). Thus, to advance the mechanisms associated with host resistance to *C. parvum* infection in adult IL-12p40$^{-/-}$ mice, the contribution of different cytokines and intracellular signaling molecules involved in Th1 responses associated with intracellular pathogen infections was investigated.

2. Materials and methods

2.1. Animals

Six-to-eight week-old female IL-12p40$^{-/-}$ (which lack both IL-12 and IL-23), IL-18$^{-/-}$ mice, C57BL/6 strain and Stat4$^{-/-}$ (C.129S2-*Stat4*^{tm1Gru}/*J*; Kaplan et al., 1996) BALB/c strain (Jackson Laboratories, Bar Harbor, ME), and Stat1$^{-/-}$ (129S6/SvEvTac-*Stat*^{tm1Rds}; Meraz et al., 1996) Agouti strain (Taconic Laboratories, Hudson, NY) mice were used in these experiments. The knockout mice were fed sterile food and water and kept in HEPA filtered barrier-isolated facilities. Manipulations for these mice were performed in HEPA filtered biological containment hoods. All mice were housed at the Veterans Affairs Medical Center (Decatur, GA) animal facility.

2.2. Oocyst treatment and mice inoculation

The *C. parvum* isolate used for this study was the IOWA bovine isolate. Oocysts were collected and purified through discontinuous sucrose and cesium chloride gradients as previously described (Arrowood and Donaldson, 1996). Purified oocysts were stored at 4 °C in 2.5% potassium dichromate ($K_2Cr_2O_7$) aqueous solution. Oocyst inocula were prepared by washing purified oocysts (stored < 6 months) with PBS (pH 7.2) to remove potassium dichromate. To establish infection in different mouse groups, IL-12p40$^{-/-}$ and IL-18 knockout mice were inoculated with 10^3 oocysts by oral gavage, while the Stat1$^{-/-}$ Agouti strain and Stat4$^{-/-}$ BALB/c strain were inoculated with 10^6 oocysts.

2.3. Recombinant cytokine reagents

Murine rIL-12 and rIL-18 were obtained from BioSource (Camarillo, CA). Recombinant mouse IL-23 and IFN-γ were obtained from *e*Bioscience (San Diego, CA). All lyophilized cytokines were reconstituted in PBS, aliquoted and stored as recommended by the suppliers.

2.4. In vivo cytokine treatment

IL-12p40$^{-/-}$ mice were treated with different murine recombinant cytokine to assess their contribution to *C. parvum* infection dynamics in our standardized mouse model. Mice groups were injected intraperitoneally with PBS (Control), IL-12 (0.5 μg), IL-18 (1 μg), IL-23 (0.5 μg) or IFN-γ (1 μg), daily starting at day minus 1 before infection with oocysts and every day thereafter until the third or fourth day post infection with 10^3 *C. parvum* oocysts. Fecal samples were collected every 2–3 days, 0–17 days post infection, from the different groups ($n = 5$ mice), processed and analyzed as described below.

2.5. Quantitation of parasite load in fecal samples

Fecal samples were collected from the mice at 2 or 3-day intervals and assessed for parasite load by flow cytometry as previously described (Arrowood et al., 1995). Briefly, fecal pellets were homogenized in adjusted volumes of 2.5% $K_2Cr_2O_7$. The samples were vortexed and allowed to stand for large debris to settle. Supernatant aliquots were overlaid onto micro-scale sucrose gradients that were prepared by underlaying a 1.103-specific gravity sucrose solution beneath a 1.064-specific gravity sucrose solution in 2.0-ml micro-centrifuge tubes. Each tube was centrifuged at 1000*g* for 20 min. The interface between the two sucrose solutions was then collected, washed with saline, and suspended in PBS supplemented with 0.1% bovine serum albumin. The partially purified stool concentrate was incubated for 30 min at 37 °C with 5 μl of an oocyst-specific monoclonal antibody conjugated with fluorescein isothiocyanate (OW50-FITC) and then analyzed by flow cytometry as pre-