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Immune response to *Encephalitozoon cuniculi* infection

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Abstract

Microsporidia are obligate intracellular parasites, which can cause complications in immunocompromised individuals. Very little is known about the host immune response generated against these infectious agents. *Encephalitozoon cuniculi* is the best studied microsporidian and the protective immune response against this parasite is mediated by cytotoxic $CD8^+$ T cells.

1. Introduction

Microsporidia are a ubiquitous group of eukaryotic, intracellular parasites that infect an extremely wide range of hosts in the animal kingdom. They are unique enough to be placed in a separate phylum, Microspora, and are characterized by the polar filament, which injects the sporoplasm into a host cell [1]. The species of microsporidia that infect mammals are unicellular, Gram-positive organisms with mature spores 0.5–2 × 1–4 μm in diameter [2].

Microsporidia are commonly found in laboratory animals such as mice, rabbits and hamsters. Amongst over 140 genera in the phylum Microspora, several different genera have been demonstrated in human disease; in particular, *Encephalitozoan* spp. were found in many mammals including man [3].

Encephalitozoon cuniculi, which was previously observed in laboratory animals, is considered to be a zoonotic infection [4]. Complications due to *E. cuniculi* infection have been reported in immunocompromised patients [5]. AIDS patients with peritonitis and hepatitis induced by *E. cuniculi* infection have been documented. HIV-infected patients with *E. cuniculi* infection have a wide range of organ involvement including renal failure, pneumonitis, sinusitis and granulomatous liver necrosis [6]. In a recent report, autopsy findings in a patient with AIDS showed disseminated *E. cuniculi* infection in the brain [7]. In a separate study, a female AIDS patient died from necrotizing microsporidial infection in adrenal glands and kidneys [8]. In this case IFA staining and molecular analysis identified the microsporidian as *E. cuniculi*. These investigators suggest inclusion of *E. cuniculi* in the differential diagnosis of disseminated opportunistic infection in AIDS patients. Based on these observations, the National Institute of Allergy and Infectious Diseases has recently classified *E. cuniculi* as an emerging infectious agent [9].

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2. Host immune response to *E. cuniculi* infection

Little is known regarding host immunity to microsporidia, and in particular *E. cuniculi*. *E. cuniculi* was the first mammalian microsporidian successfully grown in vitro [10]. It infects epithelial and endothelial cells, fibroblasts, and macrophages in a variety of mammals, including rabbits, rodents, carnivores, monkeys and humans [4, 11, 12]. In an experimental model, normal mice infected with *E. cuniculi* usually express few clinical signs of disease [13]. Sensitive and resistant strains of mice exist, as defined by the percentage of macrophages parasitized following intraperitoneal infection, suggesting a genetic basis for innate resistance [13].

Following infection, many mammals have a chronic infection with *E. cuniculi*, as evidenced by a persistent high antibody titer and ongoing inflammatory process (e.g., persistent encephalitis and kidney lesions in rabbits [14], and congenital disease in carnivores such as foxes [15]. In murine *E. cuniculi* infection, ascitis develops and then clears in immunocompetent mice. If such mice are then immunosuppressed by corticosteroids they will redevelop ascitis, consistent with a latent persistence of microsporidia in these animals [16]. Other immunodeficient hosts, such as athymic or SCID mice, develop lethal disease after experimental infection [17, 18], usually manifested by ascitis with dissemination of spores. SCID mice are deficient in T and B cells but possess intact natural killer (NK) cell function [19]. Although enhanced NK cell activity in *E. cuniculi*-infected mice has been reported it does not seem to offer significant in vivo protection [20]. Infected SCID mice reveal numerous microsporidia in visceral and parietal peritoneum as well as in the liver and spleen. Adoptive transfer of sensitized syngeneic T-enriched spleen cells protected athymic or SCID mice against *E. cuniculi* challenge [21, 22]. Transfer of naive lymphocytes or hyperimmune serum failed to protect or prolong the survival of these mice. Studies by Didier have shown that cytokines released by sensitized T cells activate macrophages to kill *E. cuniculi* in vitro [23]. These findings suggest that a protective immune response to *E. cuniculi* is likely dependent on cytokine-producing immune T cells.

2.1. Role of cytokines

Th1 cytokines like IFN-γ and IL-12 are important for protective immunity against a number of intracellular viral, bacterial and parasitic infections [24–26]. Studies with *Encephalitozoon intestinalis*, a parasite closely related to *E. cuniculi*, have reported that mice lacking the IFN-γ gene are unable to clear the infection [27]. Based on earlier in vitro observations, it was suggested that IFN-γ also plays an important role in protective immunity against *E. cuniculi* infection [23, 28] However, the importance of IFN-γ in natural *E. cuniculi* infection in vivo was not confirmed until recently [29]. In these studies treatment of *E. cuniculi*-infected mice with neutralizing antibody against IFN-γ or IL-12 resulted in increased mortality for these animals. The use of gene knockout mice further validated the importance of Th1 cytokines in the immune response to *E. cuniculi*. Both $p40^{-/-}$ mice (which are unable to produce IL-12) and IFN-$\gamma^{-/-}$ animals succumbed to infection upon *E. cuniculi* challenge.

Minimal Th2 cytokine production has been observed during *E. cuniculi* infection [29]. The mRNA for IL-4, a prominent Th2 cytokine, was undetectable in the splenocytes of the infected animals. Similarly, no circulating IL-4 was detected in the sera of infected mice. Nevertheless, an increase in the mRNA for IL-10, another Th2 cytokine, was seen in the splenocytes of such infected animals. As IL-10 has been reported to be involved in the regulation of Th1 immune response in other infectious disease models [30, 31], it is possible that it plays a similar role in *E. cuniculi* infection.

Cytokine-activated murine peritoneal macrophages can inhibit the replication of *E. cuniculi* in vitro [32]. This was reported by the studies which demonstrated that inhibition of nitric oxide synthesis prevented parasite growth. However, mice deficient in inducible nitric oxide synthase ($NOS2^{-/-}$) showed no mortality during *E. cuniculi* infection [29]. Thus, nitric-oxide-induced killing of *E. cuniculi* by macrophages or other cells does not appear to be a critical mechanism for control of this infection.

2.2. T-cell subtypes induced in *E. cuniculi*-infected mice

As stated previously, immune T cells are critical for protection against *E. cuniculi* infection in the normal host. The role of individual T-cell subtypes during *E. cuniculi* infection has been recently reported [33]. Phenotypic analysis of the spleen cells from infected animals revealed an increase in the $CD8^+$ T-cell population starting at day 10 postinfection. This rise in the $CD8^+$ T-cell population continued until day 17 p.i. when a > 3-fold increase in the cell type over uninfected controls was observed. Subsequent analysis for activation markers suggested that $CD8^+$ T cells are activated as early as day 3 post-*E. cuniculi* infection. No significant increase in $CD4^+$ T cells during the course of infection was observed.

To determine whether $CD8^+$ T cells are critical for host survival during *E. cuniculi* infection gene knockout animals were utilized. All mice deficient in the $CD8^+$ gene succumbed to parasite challenge, showing signs of severe sickness (lethargy, development of ascitis) just before the time of death. Histopathological analysis of the tissues from these animals demonstrated disseminated infection in the spleen and liver. In contrast, *E. cuniculi* infection did not result in any mortality in $CD4^{-/-}$ mice. Similarly to wild-type controls none of these animals died or demonstrated signs of clinical illness in response to *E. cuniculi* challenge.

3. Cytotoxic T-cell response to *E. cuniculi* infection

$CD8^+$ T cells play an important role in the number of intracellular infections [34–36]. The protective effect of $CD8^+$ T cells is mediated by their ability to produce cytokines [37]. Alternatively, $CD8^+$ T cells can reduce the parasite load by killing the infected targets in the host tissue [38]. The major killing mechanism exhibited by $CD8^+$ T cells during *E. cuniculi* infection is via the perforin pathway [39]. Mice lacking the perforin gene, similar to $CD8^{-/-}$ animals, succumb to *E. cuniculi* infection. These observations indicate the importance of the cytotoxic T-cell response in *E. cuniculi*-infected animals. The kinetics of induction of the $CD8^+$ CTL response in *E cuniculi*-infected mice has been studied [33]. The cytotoxic effect of splenocytes from infected animals was obvious at day 17 p.i. as measured by an in vitro cytotoxicity assay. At this time point effector to target ratio of 20:1 caused 50–60% lysis of *E. cuniculi*-infected macrophages in vitro. In vitro depletion of $CD8^+$ T cells in the effector cell population resulted in complete loss of cytotoxic activity [33]. These observations suggest that $CD8^+$ T cells mediate the cytolytic effect within the effector immune population. This has been further confirmed by our recent findings in which minimal cytotoxic activity in the splenocytes of $CD8^{-/-}$ mice was detected (Moretto et al., unpublished observations).

3.1. Regulation of $CD8^+$ T-cell immunity in *E. cuniculi*-infected animals

3.1.1. Role of $CD4^+$ T cells—In a majority of cases, $CD8^+$ T cells are primed via IL-2-producing $CD4^+$ T cells [40]. However, in certain viral infections a normal in vivo $CD8^+$ T-cell response, in the absence of $CD4^+$ T cells, can be induced [41]. As mentioned earlier [33] lack of $CD4^+$ T cells did not affect the outcome of *E. cuniculi* infection in knockout animals. In-depth analysis of the $CD8^+$ T-cell response to *E. cuniculi* infection in $CD4^+$ T-cell-deficient animals was performed. Interestingly, a normal antigen-specific $CD8^+$ T-cell response to *E. cuniculi* infection was observed in $CD4^{-/-}$ mice. The lack of $CD4^+$ T cells

did not alter the magnitude of the antigen-specific cytotoxic response and cytokine pattern of $CD8^+$ T cells during *E. cuniculi* infection. *E. cuniculi* infection thus offers an example, amongst intracellular parasitic infections, of how $CD8^+$ T cells can be induced in the absence of $CD4^+$ T cells.

3.1.2. Role of γδ T cells—The importance of γδ T cells in response to infectious diseases has been increasingly evident [42] with recent reports indicating that these T cells may be involved in establishing primary immune responses [43]. Studies with *Listeria monocytogenes*, an intracellular bacteria have demonstrated that an early rise in IFN-γ-producing γδ T cells is followed by an increase in IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells [44]. A many-fold increase in the γδ T-cell population was observed within several days of *E. cuniculi* infection (Moretto and Khan, unpublished observations). Mice deficient in γδ T cells exhibited susceptibility to *E. cuniculi* when challenged with very high parasite doses. However, unlike $CD8^{-/-}$ or αβ T-cell-deficient mice, $\delta^{-/-}$ animals were able to survive a low-dose infection (Moretto and Khan, unpublished observations). The susceptibility of $\delta^{-/-}$ mice can be attributed to downregulation of $CD8^+$ T-cell immunity in these animals, as we observed a significant decrease in the antigen-specific $CD8^+$ T-cell immune response to *E. cuniculi* infection in these mice (Moretto and Khan, unpublished observations). Based on these observations it appears that γδ T cells play a prominent role in priming of the $CD8^+$ T cell during *E. cuniculi* infection. The induction of $CD8^+$ T-cell immunity by γδ T cells may be due to their ability to release early IFN-γ necessary from priming the $CD8^+$ T-cell response as reported in other models [43].

4. Humoral immunity in microsporidial infections

Adoptive transfer of immune B lymphocytes into athymic BALB/c (nu/nu) or SCID mice does not protect these animals from death following *E. cuniculi* infection [45]. Passive transfer of hyperimmune serum into athymic BALB/c (nu/nu) mice does not prevent lethal infection [17]. Nonetheless, during *E. cuniculi* infection there is a strong antibody response to different antigens of this organism and many of these antibodies are cross-reactive with other microsporidia [17, 46]. In humans, microsporidia antibodies have been found in from 5 to 50% of survey populations, with the highest incidence in those patients from the tropics or who had visited the tropics [47, 48]. In random blood donors the seroprevalence is 5% [49]. Data exist showing that maternal antibodies may protect new-born rabbits from infection with *E. cuniculi* during the first 2 weeks of life [50]. In addition, in vitro infectivity of microsporidia is reduced by treatment with immune serum and complement [21], monoclonal antibody (mAb 3B6) to spore coat [45] or monoclonal and polyclonal antibodies to polar tube protein (Weiss, unpublished data). Thus, it is likely that antibodies play a role in limiting infection in the host, although they are clearly not sufficient to prevent mortality or to cure infection.

5. Conclusion

Cell-mediated immunity is critical for the survival of *E. cuniculi*-infected hosts. Over the last several years significant progress has been made in understanding the cellular immune responses to *E. cuniculi* infection. A working hypothesis of the immune response to *E. cuniculi* is illustrated in figure 1. Similar to other intracellular infections, *E. cuniculi* may result in a strong burst of IL-12 production by host macrophages or dendritic cells [51]. Early IL-12 release results in the polarization towards Th1 cytokines manifested by high levels of IFN-γ in the circulation and tissues. γδ T cells, which are increased at early stages of infection, are probably important sources of IFN-γ production. Th2 cytokines such as IL-4, are not detectable throughout the course of infection. The increase in IFN-γ production is known to cause upregulation of class I molecules on the infected cells [52]. This most

likely leads to antigen-specific $CD8^+$ T-cell proliferation. $CD8^+$ CTLs are primary effector cells responsible for keeping parasite replication under control [53]. The role of $CD4^+$ T cells in protective immune response to *E. cuniculi* is somewhat ambiguous. $CD4^+$ T-cell-deficient mice show normal protection against parasite challenge [33] and the $CD8^+$ T-cell immune response in these knockout mice is not compromised (Moretto et al., unpublished observations). Based on these findings, it appears that the $CD8^+$ CTL response during *E. cuniculi* infection can be launched independently of $CD4^+$ T cells. The presence of γδ T cells appears to be crucial for the induction of optimal $CD8^+$ T-cell immunity.

Based on the information available, the following hypothesis can be put forward: *E. cuniculi* infection induces a strong $CD8^+$ CTL response, which restricts parasite growth by lysing the infected cells via a perforin-dependent mechanism. The induction of $CD8^+$ CTLs is at least partially regulated by γδ T cells. This regulation may be dependent on the ability of γδ T cells to produce cytokines like IFN-γ. The role of IFN-γ in inducing and maintaining $CD8^+$ T-cell immunity against infectious agents has been clearly demonstrated [54]. There are still, however, important questions which remain to be addressed: is IFN-γ critical for the induction of the $CD8^+$ T response during *E. cuniculi* infection? What is the mechanism of γδ T-cell induction of the $CD8^+$ CTL response? Ongoing research in our laboratory should provide answers to these critical questions and refine our understanding of the host immune response to these emerging pathogens.

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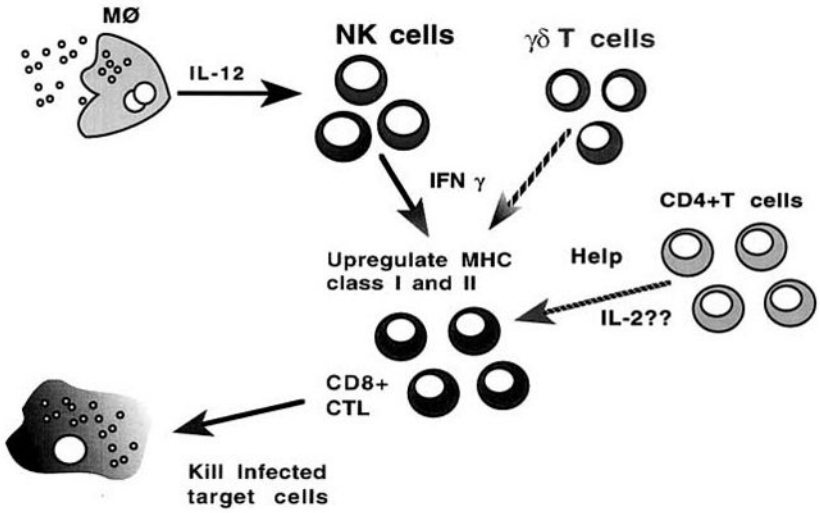

Figure 1.

Overall picture of immune response to *E. cuniculi* infection. $CD8^+$ T cells play a critical role in host defense against *E. cuniculi* infection. The primary $CD8^+$ CTL response can be induced in the absence of IL-2-secreting $CD4^+$ T cells. IFN-γ-producing NK cells or γδ T cells may play a role in the induction of early $CD8^+$ T-cell immunity.