Published in final edited form as:

Semin Immunopathol. 2015 May ; 37(3): 281–287. doi:10.1007/s00281-015-0482-8.

Effector CD8 T cell immunity in microsporidial infection: A lone defense mechanism

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Abstract

Microsporidia are a group of pathogens, which can pose severe risks to the immunocompromised population such as HIV infected individuals. The expertise to diagnose these pathogens is limited and therefore their prevalence is believed to be much higher than what is currently known. In a mouse model of infections, it has been reported that CD8 T cells are the primary effector cells responsible for protecting the infected host. As the infection is acquired via per-oral route, CD8 T cells in the gut compartment apparently act as a first line of defense against the pathogens. Thus, generation of a robust CD8 T cell response that exhibits polyfunctional ability is critical for host survival. In this review, we describe the effector CD8 T cells generated during microsporidial infection and underline the factors that may be essential for the elicitation of protective immunity against this understudied but significant pathogen. Overall, this review will highlight the necessity for a better understanding of the development of the CD8 T cell response in gut associated lymphoid tissue (GALT) and provide some insights into therapies that may be used to restore defective CD8 T cell functionality in an immunocompromised situation.

Keywords

Microsporidia; CD8 T cells; CD4 T cells; IFN γ ; cytotoxicity; intraepithelial lymphocytes; intestinal immunity

Introduction

First discovered by Pasteur in 1870, Microsporidia are understudied parasites that can infect a wide range of hosts including humans. These newly emerging opportunistic infections remained atypical until the onset of the HIV epidemic. Still largely under-diagnosed, they have recently been associated with various severe immunocompromised conditions. To this day, there are very few studies related to the immune response generated by these pathogens. The purpose of this review is to present them as well as some potentially new research interests. The majority of the immunological studies with microsporidia have been conducted with *Encephalitozoon cuniculi*, a microsporidia species of human importance which can be easily cultured in laboratory. Moreover, experiments in mice, to a large extent, mimic human disease.

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The importance of adaptive immunity in the protection against microsporidial infection was reported very early, as athymic and SCID animals (which lack T cells) were unable to control infection unlike immunocompetent mice [1,2]. During the later years, it was reported that the CD8 subset is the primary effector population [3,4]. One important finding was the generation of a strong cytotoxic T-lymphocyte (CTL) response, and its importance in host protection, which is unusual in a non-viral pathogen [5]. Lack of perforin (a cytotoxic granule) compromised the ability of the knock out animals to clear infection [5,6]. The other interesting observation is the dichotomous role of CD4 T cells based on the route of infection. During intra-peritoneal infection, CD4 response was dispensable for the host protection or the development of CD8 T cell immunity [5,7], conversely, the subset apparently played a synergistic role during per-oral infection [8]. As stated above, findings related to the mechanisms responsible for the generation of CD8 effector immunity is essential for understanding immunoprotection against this pathogen. Moreover, it can serve as a valuable model to study the gut CD8 T cell response against other pathogens acquired via oral route.

Microsporidia

Microsporidia are obligate, spore forming, intracellular parasites related to fungi that infect a wide range of hosts. They are ubiquitous and amongst the 1200 species characterized, 14 species have been detected in humans [9]. The species most commonly associated with human infections include *Enterocytozoon bienersi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem* and *Encephalitozoon cuniculi*. Transmission is believed to occur primarily via fecal-oral route, mainly by ingestion of contaminated water or food [9] such as drinking water, wastewater and recreational water [10]. In fact, a large waterborne outbreak involving a lake contamination has been reported [11] and the very first acknowledged foodborne outbreak associated with microsporidia was recently released [12]. Because of their waterborne transmission potential, microsporidia are included in both the drinking water contaminant candidate lists of the U.S. Environmental Protection Agency (EPA) (<http://www2.epa.gov/ccl/contaminant-candidate-list-3-ccl-3>) and the category B Priority Pathogens list of the Biodefense and Emerging Infectious Diseases from the National Institute of Allergy and Infectious Diseases (<http://www.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/Pages/CatA.aspx>).

Depending on the species, microsporidiosis can be localized or disseminated and is often associated with a broad range of symptoms including, but not limited to diarrhea, hepatitis, encephalitis, nephritis and keratoconjunctivitis [13]. Microsporidia are opportunistic parasites predominantly associated with severely immuno-suppressed AIDS patients [14]. To this day, the incidence of microsporidia in HIV patients remains elevated in places like Russia, Venezuela and Thailand, where microsporidia prevalence amongst AIDS patients can range from 13 to 80% [15–17]. Additionally, in the past two years, there have been increasing reports of microsporidiosis associated with solid organ transplant recipients [18–20]. Occurrence of microsporidiosis has also been reported in travelers, children and the elderly population [21–23]. Due to their small size and lack of standardized lab diagnosis, microsporidia are often overlooked and certainly under-diagnosed. However, increased awareness and improved detection methodology has shown that microsporidia prevalence in

immunocompetent individuals is greater than once estimated. In fact, microsporidia screening of healthy Slovak Roma children revealed that 30% of the children tested were excreting microsporidia spores [23]. Likewise, a Japanese study showed that over 30% of healthy individuals expressed microsporidia specific IgM [24]. Unexpectedly, the frequency of specific IgM was significantly lower in the adult population compared to subjects less than 20 years of age. A recent report also showed that healthy individuals who had occupational exposure to various animals were at greater risk [25]. In this study, specific anti microsporidial antibodies were detected in fourteen out of fifteen people tested although none of the individuals exhibited any clinical symptoms.

Evidences have recently emerged suggesting that microsporidiosis is a latent infection. In an animal model, corticosteroid induced immunosuppression of mice with parasite burden below detectable level led to the reactivation and dissemination of the parasite [26]. This report confirms older data documenting the frequent relapse of infection in HIV patients who discontinued therapy once microsporidiosis was cleared [27,28]. Similarly, several studies have shown that cancer patients receiving chemotherapy, which can cause immunosuppression, can also trigger latent intestinal microsporidiosis [29,30]. Microsporidia spores were detected in 21.9% of the stool samples of cancer patients, but remarkably, there was no difference between the individuals undergoing chemotherapy and those who were newly diagnosed and had yet to start treatment [29]. These reports underline that the maintenance of robust long-term immunity is critical to keep this widespread pathogen under control.

CD8 T cell response to microsporidia infection

Given that microsporidia is an intracellular parasite, predictably, very early reports demonstrated that protective immunity was primarily dependent on T cells [31]. Of the most significant species of microsporidia that infect humans, all but *E. bieneusi* can be propagated in cell culture and *E. cuniculi* is widely used to study the immune response in an animal model. Effectively, earlier studies involving athymic and SCID mice have shown that these immunodeficient animals are highly susceptible to *E. cuniculi* infection [2,1] and adoptive transfer of immune T cells conferred protection against a lethal challenge [32]. As expected, hyper-immune antiserum transfer failed to protect or prolong survival of the recipients, ruling out the protective role of humoral immune response. Studies, including those conducted in our laboratory, have reported severe susceptibility of CD8^{-/-} mice to i.p. infection. Moreover, adoptive transfer of immune CD8 T cells to immunodeficient mice protected them against the pathogen, suggesting that protection against intraperitoneal challenge was primarily dependent on CD8 T cells [4,3,33]. In contrast, CD4 T cells played a minimal role in the protective immunity against *E. cuniculi* infection administered via i.p. route, as the knock out mice did not exhibit any susceptibility to infection and adoptive transfer of immune CD4 T cells also failed to protect immunocompromised animals. It is important to note that mice lacking CD4 T cells generated a normal CD8 response, thus excluding a critical helper role during i.p. challenge [7]. Interestingly, gamma delta TCR deficient mice developed a sub-optimal CD8 T cell response and these animals also exhibited partial susceptibility to i.p. challenge [34]. Adoptive transfer of immune CD8 T cells isolated from these mutant animals failed to transfer protection to susceptible hosts.

These findings are rather unique as, to the best of our knowledge, the helper role of $\gamma\delta$ T cells in the elicitation of effector CD8 T cell immunity against any pathogen/diseases has not yet been described. These findings may be useful in HIV infection where, in the absence of optimal CD4 immunity, $\gamma\delta$ T cells may be important targets for maintaining a robust CD8 T cell immunity against this opportunistic infection.

In immunocompetent mice, *E. cuniculi* infection induces a robust antigen specific CD8 T cells effector response, which a few years ago were referred to as short lived effector CD8 T cells (SLEC). The SLEC are phenotypically identified by expression of KLRG1, which is absent in memory cells. In recent years, polyfunctional ability of CD8 T cells has been recognized as one of the hallmarks of a robust protective immunity against intracellular pathogens, especially in viral infections [35–37]. Similarly, studies conducted in our laboratory suggest that the SLEC population exhibit polyfunctional characteristics during acute *E. cuniculi* infection, underlining their importance in controlling the infection [38]. They are highly polyfunctional, as shown by their ability to exhibit simultaneous up-regulation of granzyme B, IFN γ and TNF α in response to antigenic stimulation [38]. However, it is essential to note that very early, our laboratory recognized the ability of immune CD8 T cells to kill *E. cuniculi* infected targets as a fundamental function, since mice lacking perforin gene (an critical cytolytic protein responsible for killing infected targets) succumb to *E. cuniculi* infection due to the accumulation of high pathogen load in these animals [4,6]. Nevertheless, it seems that besides their critical cytolytic ability, other functions of CD8 T cells like secretion of IFN γ and TNF α may play a synergistic role in controlling the dissemination of the pathogen.

Gut CD8 T cell response against *E. cuniculi* infection

The majority of the studies related to protective immunity against *E. cuniculi* infection mentioned above were carried out using an intraperitoneal route of infection. As the pathogen is acquired via per-oral route, studies evaluating the gut immunity are critical for understanding the immuno-protection against the pathogen. Interestingly, we observed that intestinal CD4 T might be playing a synergetic role along with CD8, as mortality of infected animals was only observed when both T cell subsets were depleted [8,39]. These findings were further supported by the studies carried with *E. intestinalis*, another microsporidia species, as SCID mice receiving either CD4 or CD8 T cell depleted splenocytes survived the infection [39]. However, these immunocompromised recipients succumbed to infection when reconstituted with splenocytes depleted of both CD4 and CD8 T cells. Intraepithelial lymphocytes (IEL) represent one of the first lines of defense against gut pathogens and are an important component of the GALT. IEL are a heterogenous population comprised predominantly of CD8 T cells (CD8 $\alpha\alpha$ and CD8 $\alpha\beta$) and a minor CD4 population [40]. We observed that CD8 IEL localized in the lining of the gut were recruited very early during *E. cuniculi* infection and displayed strong polyfunctional properties against pathogen infected targets [8]. This strong antigen specific response was characterized by both IFN γ expression and cytotoxic response and was able to confer partial protection to immunosuppressed host against a lethal challenge [8]. The protective ability amongst the CD8 IEL was attributed to the CD8 $\alpha\beta$ subset as adoptive transfer partially controlled the infection in immunocompromised host. [6]. Similar to their splenic counterparts, CD8 IEL are able to

up-regulate several markers of the cytotoxic response (perforin, CD95L and granzyme B) at day 7 post infection. Moreover, the mechanism of protection is predominantly dependent on perforin, as IEL from knock out mice failed to protect immunocompromised animals against lethal challenge [6]. A recent report showed that gut flora, via engagement of the toll like receptor 9 (TLR9), played an essential role in the maintenance of a robust gut immune response to *E. cuniculi* [41] and a healthy intestinal homeostasis. Furthermore, mice lacking TLR9 gene displayed increased frequencies of CD4⁺Foxp3⁺ regulatory T cells leading to impaired immune response to microsporidia. Future studies related to the microbiota of the gut and the effect of Tregs on the development of polyfunctional CD8 IEL should provide interesting information about the development and maintenance of IEL immunity in this tissue.

Although latency of microsporidial infection was not considered earlier, as stated above recent reports are emerging that suggest that infection can subsist in a latent form [26] putting the elderly population at risk. Murine studies in our laboratory have demonstrated that older mice exhibited increased susceptibility due to a defective mucosal CD8 T cell response [42]. At 9 months of age, murine T cells displayed lower proliferative and cytotoxic capabilities than cells from younger animals. Also, adoptive transfer of immune CD8 T cells from MLN of aged animals failed to confer protection to SCID mice. This defective mucosal T cell immunity was linked to suboptimal dendritic cells response. Unlike their splenic counterparts, intestinal DC failed to up-regulate IL-12 expression as well as co-stimulatory molecules after *in vitro* stimulation with *E. cuniculi* [42]. In a very recent study, we observed that aged animals have a severe defect in the generation SLEC response and the cells with increasing age lose their polyfunctional capability [38]. Remarkably, defect in the SLEC response is not cell intrinsic as adoptive transfer of SLEC (KLRG1⁺ CD8 T cells) from aged mice exhibited normal polyfunctional ability when transferred to young recipients.

Role of cytokines in the development of CD8 T cell immunity against *E. cuniculi*

As mentioned previously, IFN γ a signature Th1 cytokine, is critical for protection against microsporidia infection as both knock out mice as well as antibody depleted wild type animals succumbed to infection, regardless of the route of challenge [4,43,6]. Infection with *E. cuniculi* induced IFN γ producing CD4 and CD8 T cells both systemically as well as locally [7,8]. Essentially, this cytokine was detected in the serum and also in splenic CD8 and CD4 T cells around day 14 post-infection, which corresponds to the peak of the effector CD8 T cell response [44,4]. IFN γ was critical for the generation of a robust CD8 T cell response and blockade of cytokine signaling abrogated its beneficial effect [45]. Expectedly, adoptive transfer of CD8 T cells from IFN γ deficient mice failed to protect susceptible host against a lethal challenge. Interestingly, CD8 T subset was dependent on $\gamma\delta$ T cells ability to produce IFN γ [34]. This was further emphasized by the observation that exogenous administration of this recombinant cytokine was able to protect gamma delta TCR deficient animals apparently by restoring CD8 T cell immunity. We observed that DC population isolated from IFN γ knock out mice was not able to up-regulate IL-12 in response to *E.*

cuniculi stimulation [6]. *In vitro* studies demonstrated that when cultured with IFN γ ^{-/-} DC, CD8 IEL exhibited poor functional abilities and were unable to express the homing receptors needed for re-location to the gut mucosal sites. Furthermore, adoptive transfer of IEL isolated from IFN γ deficient infected animals failed to protect immunocompromised host against a lethal challenge [6].

IL-12, a third signal cytokine produced by antigen presenting cells and a key regulator of the Th1 cell-mediated immune response, is responsible for the upregulation of the transcription factor T-bet which is critical for IFN γ production and therefore plays an essential role in the initiation of the CD8 T cell response [46]. In the case of microsporidia, IL-12 plays a dichotomous role depending on the route of infection. Early report from Khan *et al.* demonstrated that IL-12p40^{-/-} mice were unable to withstand intraperitoneal infection but subsequent studies reported that these animals were able to survive a per-oral infection with either *E. cuniculi* or *E. intestinalis* [4,43]. As expected, IL-12p40 knockout animals displayed serious defects in expansion of CD8 T cell response to intraperitoneal *E. cuniculi* infection [45]. Addition of exogenous IL-12 corrected this defect but unexpectedly the mechanism was independent of IFN γ , since exogenous treatment with the cytokine failed to correct the defect. The fact that IL-12 deficient animals can survive oral infection suggests that this cytokine might not be as critical as once believed. These reports also point to the possibility that another yet unknown third signal cytokine may be replacing IL-12 during the priming of the CD8 T cell response.

Another cytokine of high interest during *E. cuniculi* infection is TGF β . A very recent report from our laboratory has established the immunosuppressive role of this cytokine in an aged model of infection [38]. In older animals, loss of CD8 T cell polyfunctionality was attributed to highly elevated levels of TGF β 1. Intrinsic blockade of this cytokine signaling cascade restored the CD8 T cell polyfunctionality in aged animals to the levels observed in young mice. Interestingly, the production of TGF β in the *E. cuniculi* infected animals was independent of T cells and treatment with anti-CD3 antibodies did not alter the levels of cytokine. Obviously, further studies still need to be conducted to identify the source of TGF β in the aged population. Furthermore, it will be very interesting if these observations can be extended to other infections where morbidity in the elderly population is linked to sub-optimal CD8 T cell immunity.

Role of CD4 help in the development of CD8 T cell immunity against *E. cuniculi* infection

CD4 T cell help for optimal CD8 T effector cell generation and maintenance has been described in several models but, the role of this subset in generation or maintenance of a robust CD8 T cell response in a microsporidia model is still lacking. The fact that depletion of both CD4 and CD8 T cell subsets is necessary for triggering susceptibility to the infection demonstrate the importance of the interactions between these two subsets in experimental per oral microsporidiosis [8]. Indeed, unpublished data from our laboratory showed that CD8 T cell response in the gut is hampered in wild type animals depleted of CD4 T cells (Harrow D, unpublished data). This defect was characterized by lower cytotoxicity and IFN γ

expression by CD8 T cells in that tissue. The nature of the help provided by CD4 T cells and the mechanism involved are currently being investigated.

CD4 T cell help, although considered to be important in the generation of CD8 T cell response, is still debated in several infectious models. After infection with intracellular pathogens, naïve CD4 T cells differentiate into Th1 cells, which can secrete a wide array of cytokines, and in turn induce the expansion of differentiated CD8 T cell, enhancing their cytolytic ability and improving their survival [47]. Also, the CD4 T cells are involved in the “licensing” of antigen presenting cells via CD40-CD40L interactions, increasing their ability to deliver the costimulatory signals during priming of the CD8 T cell response. During microsporidia infection, CD4 T cells produce IFN γ in response to antigenic stimulation [7] and this cytokine was proven to be critical for protection of infected hosts [4,43]. Although CD4 T cells are also well known for producing IL-2, a key cytokine that can potentially promote the activation and proliferation of CD8 T cells [48], this cytokine was undetectable for at least the first 30 days following oral infection (unpublished data). Another major cytokine produced by CD4 T cells is the recently described IL-21, a member of the common gamma chain family that is closely related to IL-2 [49]. Using a Lymphocytic Choriomeningitis virus model, several studies showed that mice lacking IL-21 signaling can develop an initial CD8 T cell response that subsequently crashes when antiviral CD8 T cells begin to lose their effector capabilities [50–52]. Data from our laboratory recently showed that IL-21 was up-regulated during the peak of the effector CD8 T cell immune response to *E. cuniculi* infection and absence of signaling for this cytokine led to impaired CD8 T cell immunity (manuscript under preparation). Our findings are supported by recent observations, which demonstrated that under IL-2 deprivation conditions, IL-21 might act as the major survival factor promoting the T cell immune response [53]. In addition, it has been reported that IL-21 can synergize with two other common gamma chain members, IL-7 and IL-15, in order to promote expansion and in some cases anti tumor functions of CD8 T cells [54,55]. However, similar to IL-2, these cytokines were not detected after *E. cuniculi* infection (Moretto M. unpublished data). A more probable mechanism by which IL-21 affects the CD8 T cell response in microsporidia most likely involves T-bet, a master transcription factor for CD8 T cells. Recently, Sutherland *et al.* demonstrated that IL-21 can up-regulate T-bet expression in CD8 T cells which, in turn, differentiated into IL-21 dependent cytotoxic CD8 T cells [56]. IL-21 signaling can also drive the expression of BLIMP1, a transcription factor involved in CD8 T cell terminal differentiation [57]. Moreover, IL-21 can inhibit IL-2 production, which could explain why we were unable to detect this cytokine after *E. cuniculi* infection [58]. Our unpublished findings strongly suggest that IL-21 plays an important role in the development of the effector CD8 T cell response during microsporidia infection, however, the mechanisms involved still need to be evaluated (fig. 1). The role of this cytokine in the development of the polyfunctional effector CD8 T cell immunity should to be investigated in the peripheral and gut compartment. These findings will shed a new light on the precise role of the CD4 T cell immunity in controlling a per-oral infection.

Conclusions and future perspectives

Evidently, CD8 T cell effectors are essential front line soldiers that keep the microsporidial infection under control and prevent its spread to other tissues. It is also apparent that due to their ability to produce cytokines, CD4 T cells are important for the development of such response. In a typical immunocompromised situation when CD4 T cells counts are significantly lower, like advanced AIDS, the CD8 T cell immunity is severely compromised, leading to dissemination of the pathogen. Understanding the nature of the CD4 T cell help involved in the generation of a robust CD8 T cell immune response against this significant group of opportunistic pathogens is critical for developing future immunotherapeutic agents.

Acknowledgments

This work was supported by NIH grants AI096978 and AI102711 to IAK.

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Fig. 1.

CD8 T cell immunity to *E. cuniculi*.

IL-12 and IFN γ producing dendritic cells are responsible for priming the CD8 T cell immunity against *E. cuniculi* infection. This response, in the case of an oral infection, is apparently dependent on CD4 T cell help. The mechanism most likely involves IL-21, a member of the common gamma-chain cytokine family, which affects the CD8 T cell response needed for controlling *E. cuniculi* infection. The cytokine possibly plays a critical role in CD8 T cells generation, expansion, or increased functionality (IFN γ and cytotoxicity) via upregulation of T-bet and Blimp1.