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Echinococcus multilocularis immunomodulatory strategies in the intermediate host

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SAMMANFATTNING

Echinococcus multilocularis är en helmint med indirekt livscykel. Parasiten lever i tarmen på sin huvudvärd, framför allt räv, som utsöndrar ägg med faeces. Äggen tas upp av en mellanvärd, migrerar till levern och utvecklas där till ett larvstadium kallat metacestod. Mellanvärdar är framför allt vilda smågnagare, men även andra djur inklusive människor kan infekteras. Hos mellanvärden ger *E. multilocularis* upphov till en sjukdom kallad alveolär echinococcos. Den ger en typisk bild med cystbildning i levern, och ibland även andra organ. Värdens immunförsvar försöker i den mån det är möjligt att bromsa infektionen genom bildning av granulom runt cystorna. Detta har visats ha bromsande effekt på metacestodens tillväxt, men kan även leda till problem hos värden om svaret blir allt för kraftigt.

För att säkerställa sin egen överlevnad och fortplantning måste parasiter dels undvika att bli neutraliserade av värdens immunförsvar, men samtidigt undvika att orsaka onödigt kraftiga skador som dödar värddjuret. Detta arbete tar upp några aspekter på immunsvaret mot *E. multilocularis* som kan tolkas som exempel på att parasiten utvecklat undvikande och immunomodulerande strategier.

Det har visats att CD4+ T-celler är nödvändiga för att granulom ska bildas runt metacestoderna och därmed för förmåga att begränsa parasitens spridning. Medan det tidigaste T-cellssvaret är av Th1-typ så övergår det successivt till ett Th2-svar. Detta skifte i svarsprofilen tros skydda kroppen mot överdrivet kraftig inflammation, men kan också vara mindre effektivt i att bekämpa parasiten. Det har föreslagits att parasiten genom att modulera värdens immunförsvar kanske bidrar till skiftet mot Th2, och även senare mot ett regulatoriska T-cellssvar. Hos kroniskt infekterade möss sker bland annat en nedreglering av uttrycket av major histocompatibility complex class II (MHC-II) på antigenpresenterande celler. Det antas leda till att T-cellspopulationen genomgår ett skifte mot T-regulatoriska celler på grund av bristande på stimulering. Detta skulle i sin tur vidare nedreglera immunsvaret, och kunna främja parasitens fortlevnad.

Parasitens larvstadium har ett yttre skyddande lager (laminated layer, LL), som skyddar den mot skada från immunförsvaret. LL består till största delen av en kolhydratkomponent kallad Em2(G11), som har visat sig vara ineffektiv när det kommer till att stimulera T-cellssvar.

Dessutom har antigen från metacestoden visats ha proteolytisk verkan på eotaxin, vilket skulle kunna leda till minskad rekrytering av eosinofiler till infekterad vävnad.

Dessa faktorer skulle tillsammans kunna leda till att immunsvaret i mellanvärden försvagas, och övergår till en form som parasiten lättare överlever.

SUMMARY

Echinococcus multilocularis is a helminth with an indirect life cycle. The adult parasite lives in the intestines of its definitive host, and excretes eggs with the host's faeces. The eggs are taken up by an intermediate host, and migrate to the liver where they develop into a larval stage called a metacestode. The intermediate hosts are primarily wild rodents; however other animals including humans can be infected. In the intermediate host, *E. multilocularis* gives rise to a disease called alveolar echinococcosis, which has a typical clinical picture with cysts in the liver, and sometimes in other organs. The immune system of the host responds to the infection, through formation of granulomas around the cysts. This has been shown to slow the growth of cyst, but can also lead to problems in the host if the response is too strong.

To secure its own survival and reproduction, parasites has to avoid being killed by the hosts defenses, whilst at the same time make sure not to cause unnecessary harm to its host. This essay will focus on some of the immunomudulatory and evasive strategies employed by *E. multilocularis* in order to achieve this goal.

It has been shown that CD4+ T-cells are necessary for the formation of granulomas and thereby for limiting parasite growth and spreading. While the initial response is of the Th1-type, it progressively shifts into a Th2 response. This shift in the cytokine profile is believed to be host protective by limiting inflammation, but can on the other hand be less effective at controlling the infecting parasite. It has been suggested that the parasite, through modulation of the host's immune responses, might contribute to the shift towards a Th2, response and later even to a T-regulatory response. In chronically infected mice the expression of major histocompatibility complex class II (MHC-II) on antigen presenting cells is down-regulated. This is assumed to cause a shift in the T-cell population towards a T-regulatory type, due to lack of stimuli. This would in turn further lower the immune response, and may contribute to the parasites survival.

During the larval stage, the parasite possesses an external protective laminated layer (LL), which protects it from the host's immune system. The LL is made up of primarily a large carbohydrate component known as Em2(G11), which has proven ineffective in stimulating a T-cell response.

Additionally, antigens from the metacestode have been shown to have a proteolytic effect on eotaxin, which could lead to a lower level of eosinophiles in the infected tissues.

These factors together could compromise the immune response of host, and modulate it into a form that the parasite has an easier time surviving.

INTRODUCTION

Echinococcus multilocularis is a cestode parasite, with different host species at different stages of its life cycle i.e. a definitive host for the adult form, and an intermediate host for the the larval stages. The adult worm resides in the intestine of the definitive host, where it attaches its head (scolex) to the mucosal lining, using hooks and suckers, and produces its eggs. The adult form of *E. multilocularis* lacks any form of digestive tract, having only a scolex and egg producing proglottids. The eggs are excreted via the feces, and are then ingested by an intermediate host; usually through grazing of contaminated plants. Inside the intermediate host the egg hatches in the small intestine, releasing what is called an oncosphere. This oncosphere will then pass through the intestinal wall, and enter the intermediate host's bloodstream from which it will travel to its primary site of infection (mainly the liver and/or lungs). When arriving at its destination the oncosphere will grow into a cyst. This cyst will develop a protective outer laminated layer (LL), and the inside layer will start to germinate and produce protoscolices (sing. protoscolex), and more cysts will bud from the original cyst. At this stage the larva is called a metacestode. Transmission to a definitive host takes place through ingestion of the cyst-infected organs by the primary host, in which the protoscolices develop into mature worms in the intestine (Vuitton, 2012).

The definitive host of *E. multilocularis* is the fox, but dogs, cats and wolves may also become infected. The intermediate hosts are rodents, although humans may be also be infected, and serve as an intermediate host. However this is usually incidental, as humans are not natural prey for canines, and thus we do not spread the parasite onward (Vuitton, 2012).

The disease caused by *E. multilocularis is* called alveolar echinococcosis (AE), and is characterized by a very specific clinical picture with multiple hydatid (fluid filled) cysts in the liver or other organs. About 15% of these cysts contain protoscolices (Vuitton, 2012).

The cysts are surrounded by granulomatous tissue contributed by the host, which encapsulate the cyst. The granulomas are a response by the host's immune system to the infection and that response also results in fibrosis and necrosis in the affected organs. These reactions serve to protect the host, but can also cause problems. The fibrosis serves to contain and encapsulate the infection but can constrain and exert pressure on blood vessels and bile ducts, and the necrotic foci may serve as breeding ground for different kinds of bacteria (Vuitton & Gottstein, 2010). The larvae indefinitely stay in the proliferative stage, and continue to expand and grow more cysts, which can invade other organs or tissue through direct infiltration or via heamatogenous spreading. In that sense, the disease is similar to cancer in the way that it spreads and infiltrates surrounding tissue (Bresson-Hadni et al., 2008).

E. multilocularis occurs locally throughout Europe. The heaviest concentrations tend to be found in central Europe, with as many as 50% of wild foxes being infected in and around the Alps. In Sweden the parasite was first found in 2011, and appears to be spread throughout various parts of the country. However the prevalence is estimated to be low. Out of 2985 examined foxes only three were infected, giving a prevalence of 0.13% (Statens veterinärmedicinska anstalt, 2012).

The question asked in this essay is: “In what way does the body of the intermediate host attempt to neutralize the parasite, and which immunomodulatory strategies are employed by *E. multilocularis* in order to avoid them?”

MATERIALS AND METHODS

A review on previously published literature within the research field was conducted, in which focus was placed on articles containing information about the immunological and pathological reactions that occur in the intermediate host during infection with *E. multilocularis*. The primary source of scientific articles was the online publishing database PubMed, however certain entries from Google Scholar and Science Direct were also used. Search terms include, but are not limited to, “Echinococcus multilocularis immune response” + “intermediate host”.

LITERATURE REVIEW

General immune responses to helminths

The raison d’etre for any invading parasite is to survive long enough in the host to produce offspring. In the case of a parasitic larva infecting an intermediate host the goal is to reach maturity. This will in turn allow it to infect the definitive host and there produce offspring. However there is also a secondary objective of keeping the host alive and as undamaged as possible, since if the host dies the parasite usually dies with it. In order to achieve this goal of mutual survival all parasites must strike a delicate balance in the parasite-host interactions. A mutual tolerance is required for this system to work, wherein the parasite modulates the host's immune system in order to be left alone, and also makes sure not to cause unnecessary harm to the host organism, either through direct tissue damage or via immune reactions leading to excessive pathology. This goal is often accomplished by tricking the host into developing an ineffective immune response (Vuitton & Gottstein, 2010; Anthony et al., 2007).

In general the response against helminthic infection is referred to as a T-helper (Th) 2 response, which is characterized by a cytokine profile predominated by high levels of interleukin (IL)-4, increased activity of CD4+ Th2-cells, B-cell activation with secretion of immunoglobulin (Ig) E and eosinophilia. During infection peptide antigens from the parasite are presented by dendritic cells (DC) on major histocompatibility complex class II (MHC-II) and with co-stimulatory molecules CD80/86. The naïve CD4+ T-cells react to this stimulus, and differentiate into Th2-cells, which secrete IL-4 and other Th2-associated cytokines (Anthony et al., 2007; Tizard, 2008). IL-4 is the signaling molecule responsible for the differentiation of naive CD4+ T-cells into Th2-cells, class switching of B-cells to IgE and an increase in the expression of MHC-II on antigen-presenting cells (Tizard, 2008). The activated T-cells then secrete more IL-4, and other cytokines (IL-5, IL-9 and IL-13), which affects the body in several ways, for example increasing the motility of the gut, in order to expel intestinal parasites (Anthony et al., 2007).

In contrast, the Th1 response is a reaction towards microbial infections, and relies heavily on interferon (IFN)-γ and the effect of cytotoxic CD8+ T-cells. These two responses generally work against one another, and an increase in either one down-regulates the effect of the other. There have been suggestions that the Th2-response in some cases is used as a control measure by the body, in order to down-regulate the Th1-response that can otherwise turn pathological (Anthony et al., 2007).

In many helminthic infections the formation of granulomas around the parasitic lesion is a main component of successful containment of the invader. This formation is a reaction by neutrophils and macrophages towards a nidus of foreign organisms or material and is often attributed to a Th1 response. However in many helminthic infections it is suggested that the formation of granulomas is mediated by CD4+ Th2-cells, and the macrophages take the alternative activation pathway as a consequence of stimulation by Th2-cytokines (primarily IL-4). This is in contrast to the classically activated macrophages normally associated with infection by microorganisms. These alternatively activated macrophages are suggested to down-regulate Th1 responses, for example through expression of transforming growth factor beta (TGF-β), and might play a role in wound healing (Anthony et al., 2007). TGF-β is a regulatory cytokine, associated with the T-regulatory (T_{reg}) response. TGF-β blocks the activation of lymphocytes and monocyte derived phagocytes, as well as stimulates the growth and development of T_{reg} cells. T_{reg} cells can down-regulate immune responses by the expression the CTLA-4 surface molecule, which is an inhibitory factor that interacts with the CD80/86 receptor (Tizard, 2008).

As mentioned above, the Th2-component might be necessary to avoid excessive damage to the body. For example, during infection with *Schistosoma mansoni,* mice failing to shift from the initial Th1 response to a Th2 -response show a massive growth of granulomatious tissue around the parasite eggs. If a Th2-response develops the granulomas are smaller and well circumscribed by eosinophils, macrophages and fibrin. Uncontrolled granulomas expand, and cause damage to the surrounding tissue, which suggests that the shift towards a Th2 response sometimes serves the purpose of protecting the body against excessive harm (Anthony et al., 2007).

The immune response towards *E. multilocularis*

As mentioned above, during infection with tissue-dwelling helminths the body reacts in order to protect itself with formation of granulomas around the parasites, and the key effector cell of this granulomatous formation is the CD4+ T-cell (Anthony et al., 2007). This is also true in the case of *E. multilocularis* as shown by Dai et al. (2004). They based their reasoning on the findings that strains of mice deficient of this cell type did not show formation of granulomas around the parasitic lesions, and had markedly larger parasitic masses following experimental infection when compared to wild type controls. In contrast parasite growth and the number of granulomatous lesions in mice lacking CD8+ T-cells or B-cells did not differ from wild type mice.

In addition to the granulomas, the clinical picture also comprises heavy fibrosis and necrosis (Vuitton & Gottstein, 2010). This fibrosis and necrosis is considered characteristic of AE in human patients, and is believed to be caused partly by immune responses triggered by the presence of the parasite. The fibrosis is believed to be caused by endogenous cytokines, namely TGF-β. The diffusion pattern of the lesions, which can be seen even far from the parasitic vesicles, suggests this rather than them being caused by the parasite in itself or it's secreted substances. However it was also stated that it has not yet been experimentally examined. In addition, this fibrosis may actually serve as an indirect defensive mechanism for the parasite in the sense that it cannot be reached by the effector cells of the immune system. This could also explain the low levels of anaphylactic reactions in the host. Furthermore, they suggest that the necrosis is influenced by an increased tumor necrosis factor alpha (TNF-α) production in macrophages at the periphery of the parasitic granuloma (Vuitton & Gottstein, 2010).

As mentioned earlier, the Th2-response is associated with secretion of antibodies, and in particular with elevated IgE levels. However the study by Dai et al. in 2004 pointed towards the fact that antibody secretion is not the key method of controlling *E. multilocularis*. They showed that B-cell deficient mice did not have higher rates of parasitic proliferation than the wild type controls. This points towards the T-cells' important role in containing and fighting off the parasitic infection.

When examining peritoneal T-cells of chronically experimentally infected mice, it was discovered that these cells showed high levels of IL-4, and low levels of IFN-γ (Mejri et al., 2011a; Mejri et al., 2011b). This suggests a dominating Th2-response. However, during the early stages of *E. multilocularis* infection, a cytokine profile consistent with a Th1-response has been observed, namely higher levels of IL-12, IFN-γ and TNF-α. This has been suggested to have a protective role during the earliest stages of infection (Vuitton & Gottstein, 2010).

The infection model used in the most of the studies referred to in this essay is based on intraperitoneal inoculation of mice with parasitic vesicles grown *in vitro*. This mimics to some extent the natural infective stage of the parasite, and explains why peritoneal T-cells are examined. However the parasitic vesicles are fully grown when injected, and as such the protective LL around the cysts is already in place. During normal infection the infecting oncosphere has not yet developed the protective layer, and is thus more susceptible to attack by the immune system.

The laminated layer and its protective properties

The cyst-wall of the larvae of *E.multilocularis* consists of two layers; One inner germinal layer (GL), which comprises the metacestode, and the outer acellular LL. It has been suggested in studies that this outer LL protects the inner GL against both detection by, and the effects of, the host's immune system. The LL is made up of a large number of different carbohydrate components, and it has been suggested to shield the metacestode from cytotoxic CD8+ T-cells (Dai et al., 2004). Dai et al. (2004) reason that an intact LL is vital for the survival of the metacestode during experimental infection. If the LL is damaged and the

interior of the metacestode is exposed to immune cells or antibodies the parasite can easily be killed. This would partially mimic the initial stages of natural infection before the LL has developed.

As mentioned the LL is also suggested to shield from discovery. A study was made by Dai et al. in 2001, where different antigens from the metacestode were examined in regards to whether or not they generated a T-cell response. The research team isolated the carbohydrate antigen Em2(G11), which is part of the LL, from *in-vitro* cultured metacestode vesicles. First they showed, by intracellular immunoflourescent staining of macrophages incubated in a Em2(G11) solution, that the carbohydrate component was taken up by macrophages, a prerequisite for antigen presentation. In spite of this it was also found through studies of T-cell proliferation *in vitro*, that this antigen did not generate an antigen specific T-cell response in the host. *In vitro* T-cell proliferation tests are performed in order to examine whether or not the infected host has generated an immune response against a certain antigen. This is done by taking out spleen cells and subjecting them to the antigen. T-cells that recognize the antigen proliferate, and an increase in cell numbers can be detected. As a possible explanation for the lack of T-cell response the authors suggested that the Em2(G11) antigen is not recognized by the T-cell receptor when presented on MHC molecules (Dai et al., 2001).

They also noticed production of only low avidity IgG against Em2(G11), independent of CD40-CD40L interaction. CD40 is a molecule on the antigen-presenting cell (APC), which binds to the CD40 ligand (CD40L) that is present on T-cells. This causes the APC to be activated, and in the case of B-cells it contributes to isotype switching and differentiation into antibody producing plasma cells. Based on this, and results of experimental infection in T-cell deficient mice (Tizard, 2008). Dai et al. (2001) concluded that the carbohydrate component Em2(G11) of the LL acts as a T-cell independent (TI) antigen. This means the antigen would not help generate a substantial T-cell mediated immune response, even when taken up and presented by APC. Dai et al. (2001) summarize that the LL is the parasites main form of protection, seeing as the major component of said layer only acts as an inefficient TI-antigen and therefore avoids an immune attack.

Down-regulatory effects on the immune system

In addition to the protection offered by the LL, *E. multilocularis* also uses more direct down-regulation of the immune system as a survival strategy. One study showed lowering of the expression of MHC-II on the surface of DCs from infected mice compared to uninfected controls. This seemed to be caused through down regulation of gene expression associated with MCH-II synthesis and peptide complex formation. It was found that the expression of CD80 and CD86 on the surface of the DCs from infected mice was lowered as well. These same DC were also been shown to express high levels of TGF-β mRNA. In addition it was found in *in vitro* experiments that parasite antigens, both in excretory/secretory products and vesicle fluid, could affect MHC-II molecules expressed on the surface of DCs, perhaps through proteolytic effects (Mejri et al., 2011a; Mejri et al., 2011b).

Mejri et al., (2011b) reason that the parasite modulates the immune system for it's own benefit. They suggest, based on their findings, a gradual shift in the cytokine profile. They claim the IFN-γ and other cytokines from the Th1-group are slowly replaced by cytokines from the Th2-group, amongst others IL-4 and TGF-β. This suggests that infection with *E. multilocularis* yields a Th2-shifted immune response later on when infection has turned chronic, a thesis that was also suggested by Dai et al. in 2001.

According to Dai et al. (2004), the Th2-response is easier for the parasite to survive. Mejri et al (2011b) have also suggested that the down regulation of co-stimulatory molecules on the surface of the DCs causes insufficient stimuli of the CD4+ T-cells. This lack of stimuli, combined with the high levels of TGF-β and IL-4 would promote the T-cells into becoming T_{reg} cells. These T_{reg}-cells would then contribute to the down regulation of the host's immune system

Proteolysis of eotaxin

Eosinophilia is considered a hallmark of helminthic infections in tissues, where the eosinophiles contain granules filled with toxic molecules, which are often harmful to the invading parasite (Vuitton & Gottstein, 2010). It has later been suggested that antigens from the metacestode, both in excretory/secretory products and vesicle fluid, has a proteolytic effect on eotaxin, a CC-chemokin responsible for chemotaxis (the attraction of immune response cells to the site of infection (Tizard, 2008)) and mobilization of eosinophils. This might be the reason for the markedly lower level of eosinophils in blood, as well as around the parasites, than could be expected, which in turn could be part of the events maintaining the low level of inflammation in the host (Mejri & Gottstein, 2009).

DISCUSSION

Based on the studies examined in this essay, it would appear that the way in which the parasite *E. multilocularis* manages to elude and inactivate the host's immune system are not fully understood. There appears to be several different mechanisms whose complex interactions contribute to create a suitable climate for the parasite, in regard to immune status in the host.

The section about general response during helminthic infection is largely based on a review article by Anthony et al. (2007) which is not based on the parasite *E. multilocularis*, but rather *Schistosoma masoni*. Seeing as both are tissue-dwelling parasites, the mechanisms should be applicable to infection by *E. multilocularis* as well. However this might constitute a source of error in this essay.

It has been suggested by Mejri et al. (2011a, 2011b) that infection by *E. multiloclaris* yields a shift in the cytokine profile from the Th1- to the Th2-variant during later stages of the infection, when it has become chronic. In addition, they also suggest that by down-regulating the function and activity of dendritic cells, a lack of antigen presentation and stimulation might cause T-cells to shift into the down regulating T_{reg}-cells.

In shifting the immune response to the less effective Th2-response, and later to a down-regulatory cytokine profile, it seems clear that the parasite has developed strategies for avoiding the immune system. Seeing as the later shift towards a down-regulatory cytokine profile seems to occur due to a lack of stimuli on the immune cells, one can also assume that the parasite not only suppresses the immune system actively, but also have evolved to use the immune systems own mechanisms against it.

Also the parasite's LL seems to play a large role in its defense against the host's immune system. In a study by Dai et al. (2004) it is suggested that the LL protects against the effects of CD8+ T-cells, and that a defective LL will render the parasite vulnerable. One might reason that once the parasite has formed its LL, it is very resilient, however it can still be fought off before that. Previously this research group had shown that an antigen from the LL, namely Em2(G11) did not generate any detectable T-cell response. However the antigen did generate a low avidity IgG-response, suggesting it works as a T-cell independent antigen (Dai et al 2001). This in conjunction with their later finding that B-cells did not seem to play an important role in protection (Dai et al., 2004) suggests that the LL serves a large role in protecting the parasite from the effects of the immune system; probably from antibodies and perhaps from the effects of cytotoxic CD8+ T-cells. This would appear a very important component in the parasite’s survival strategy.

Dai et al. (2004) also claim that the formation of granulomas around the parasitic vesicles is a very important mechanism in constraining the infection, and also suggests the CD4+ T-cell as a major component in that formation. In addition, based on further findings, they suggest that B-cells and antibodies play a very small role in the general defense. This puts focus on the T-cells as the most important immune effector cells when it comes to fighting off infection by *E. multilocularis*.

A theory has been submitted by Mejri & Gottstein (2009) in which it is suggested that certain metabolites from the metacestode can have a proteolytic effect on eotaxin. This would lead to a lower eosinophil count than what could otherwise be expected, and seeing as eosinophiles are considered to be an important component of the defense against helminthic infections, this is most likely a key factor as well.

In conclusion, it appears as if the immune responses by the intermediate host during infection by *E. multilocularis* are still not fully understood. The parasite uses both protective and down-regulatory mechanisms in order to further its own survival. The LL acts as a shield, protecting it from both harm and detection. It is also suggested that the parasitic antigens acts as general down-regulators on the immune system; amongst others through the inhibition of MHC-II formation and the proteolysis of eotaxin. This down-regulation of the immune system creates a downward spiral, in which lack of stimuli further diminishes the response. The immune situation during infection by *E. multilocularis* clearly needs further investigation, and more research should be conducted on the subject in order to establish a clearer picture.

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