MUCOSAL IMMUNITY IN *TOXOPLASMA GONDII* INFECTION

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Summary:
Toxoplasma gondii is an intracellular parasite that frequently infects a large spectrum of warm-blooded animals. This parasite induces abortion and establishes both chronic and silent infections, particularly in the brain. Parasite penetration into the host activates a strong anti-parasite immune response. In the present paper, we will discuss the interplay between innate and adaptive immunity that occurs within the infected intestine to clear the parasite and to maintain intestinal homeostasis despite the exacerbation of an inflammatory immune response.

KEY WORDS : *Toxoplasma gondii*, immunity, mucosal.

The gastrointestinal tract is the largest mucosal area of the body in contact with the external environment. The intestinal epithelium protects the host against microbial infection not only by forming a physical barrier, but also by active participation in host innate defence via the production of cytokines, chemokines and antimicrobial peptides. The gastrointestinal tract is populated by a resident microflora, essential for immunological intestinal homeostasis and as a source for nutrients. Maintenance of resident bacterial number and simultaneous protection against potential pathogens, including *Toxoplasma gondii*, acquired by the oral route, is provided by numerous non immunological and immunological factors. In the present review the role of gut associated immune system in protection against *T. gondii* is discussed.

INNATE IMMUNITY

Epithelial cells provide the first line of innate immunological host defense against oral *T. gondii* infection. During natural infections, *Toxoplasma* initially crosses the intestinal epithelium, disseminates into the deep tissues and traverses biological barriers in the placenta, the blood-brain and the blood-retina barrier (Barragan, 2003). The tight junctions that provide complex enterocyte-enterocyte interaction constitute a physical barrier against the penetration of intestinal microorganisms. However, despite this physical barrier, *Toxoplasma* actively crosses polarized cell monolayers (such as intestinal epithelium) and this ability is linked to parasite motility and virulence in the mouse model (Barragan, 2002). Parasite transmigration required viable and actively motile parasites but the integrity of host cell barriers is not altered during parasite transmigration. In all likelihood, enterocytes play a crucial role as sentinel against parasite invasion. Alpha-defensins (or Cryptdins – Crps) are a group of cationic antimicrobial peptides, harbouring a broad spectrum of microbicidal activity against microbes. In mouse small intestine, epithelial cells and more specifically Paneth cells produce Crps as a component of secretory granules released in the lumen. Infection of B6 mice with *T. gondii*, can regulate Crps mRNAs expression by intestinal epithelial cells. This response appears mediated via TLR9-dependant production of IFN-β that may be involved in the blockade of *T. gondii* host penetration (David Fourreau, manuscript in preparation).

T. gondii infection of the intestine following oral challenge in certain strains of inbred and outbred mice as well as rodents, pigs and non-human primates can induce a severe form of intestinal inflammation. In C57BL/6 mice, this pathology shares both morphologic and histologic characteristics with human IBD, such as loss of intestinal epithelial architecture, shortened villi, massive influx of inflammatory cells into the lamina propria and scattered patches of necrosis. When unregulated, this inflammatory process results in the early mortality of the susceptible hosts (Lisenfeld, 1996).

Migration of $CD11c^+$ and $CD11b^+$ monocytes, DCs, macrophages and PMNs into the lamina propria at day 7 after infection has been reported following oral infection with parasite tissue cysts (Courret, 2005).

At day 5 after infection with *T. gondii* most of these lamina propria DCs are mature, as indicated by high-

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level expression of MHC class II, CD40, CD80, and CD86. It has been proposed that DCs from the lamina propria might gain access to the intestinal contents by using unique proteins to separate the tight junctional border between the enterocytes without disrupting the monolayer integrity (Rescigno, 2001). This process would allow for the direct sampling by DCs of pathogens within the gut lumen. Alternatively pathogens that cross the epithelium may also be captured directly by the DCs that process antigen for presentation. An additional way for the DCs from the lamina propria to sample the antigens is via the infected enterocytes. In this model, apoptotic enterocytes are digested by the DCs and processed for antigen expression.

When hosts, including humans, ingest tissue cysts or oocysts containing *T. gondii*, free parasites are released in the gut lumen. They subsequently enter enterocytes where they multiply and initiate the infection. Enterocytes loaded with parasites secrete chemokines that recruit leukocytes in the lamina propria (LP) extravascular space. Parasites then disseminate to several distant tissues including the brain, a major site supporting parasite latency (Dubey, 1997). This event has important clinical implications since *T. gondii* as a chronic infection is associated with the encysted bradyzoite that slowly replicates under the control of unique host dependent immune signals. *T. gondii* can efficiently enter and survive within DCs (Channon, 2000). The functional plasticity as well as the migratory property of DCs (mostly $CD11c^+$ cells) can then be utilized by pathogens for dissemination through the body.

Studies in one of our laboratories (DBG) have demonstrated that following intragastric inoculation of cysts in mice, $CD11c^+$ dendritic cells from the intestinal lamina

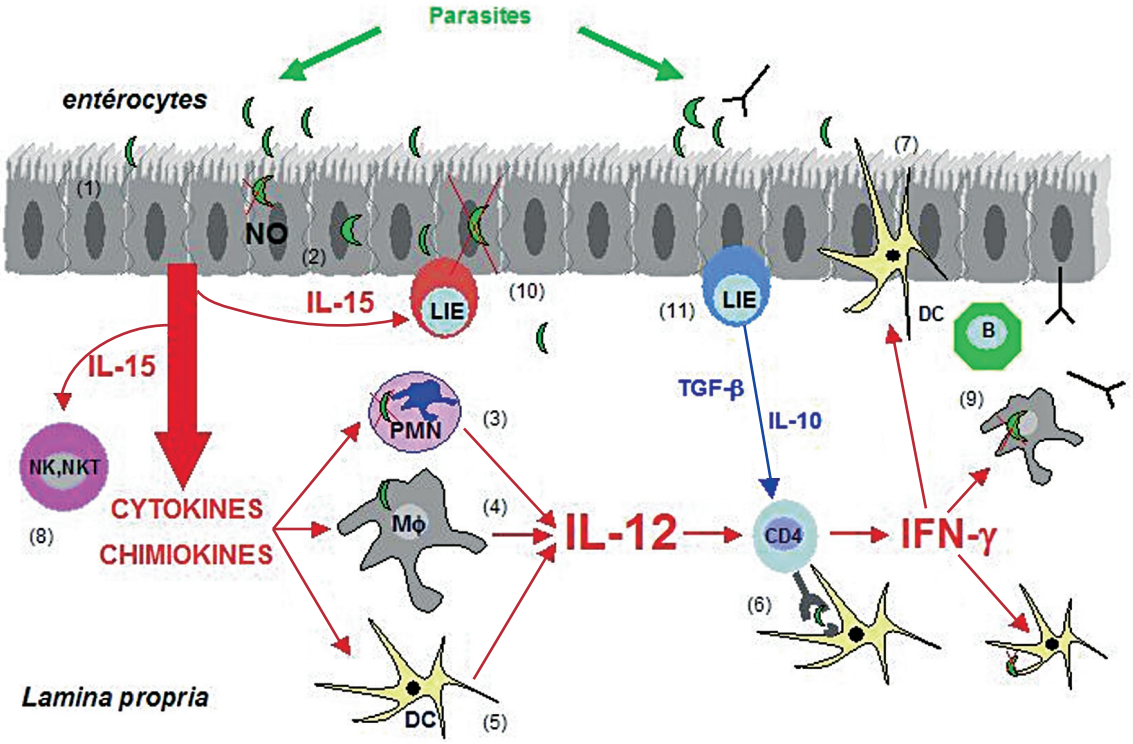

Fig. 1. – A model of the GI mucosal immune response to *Toxoplasma gondii*.

When parasites invade the mucosal intestinal epithelium, they first face a physical barrier brought by the enterocytes bound together by tight junctions (1).

Parasites have developed multiple strategies to adhere, sometimes to invade the enterocytes and to spread beyong the epithelium. When enterocytes are infected by the parasites, physiological and morphological disturbances occur, and enterocytes might secrete cytotoxic molecules such as nitric oxyde (NO) (2).

In addition, enterocytes respond to the infection by secretion of chemokines and cytokines that attract polymorphonuclear leukocytes (PMNs) (3), macrophages MΦ (4) and dendritic cells (DCs) (5).

When stimulated, these cells from the innate immune system can be directly microbicidal. They are also source of cytokines such as IL-12 that triggers the adaptive CD4 immune response (6).

To be elicited specific immune response needs antigen presentation, mainly through DCs. DCs sample the antigen by different pathways, one of them, is direct antigen capture into the lumen by elongation of the dendrites through the tight junctions (7).

Activated T cells, in addition to NK and NKT cells (8) stimulated by cytokines produced by infected enterocytes such as IL-15, secrete IFN-γ that activates MΦ, DCs, enterocytes for parasite clearance.

B cells (9) are also triggered to secrete antibodies that can cross the epithelial barrier by active transcytose and reach parasite into the lumen. Besides microbicidal activities, IFN-γ if not controlled, might damage the intestinal integrity.

Intraepithelial lymphocytes (IEL) (10) are cytotoxic for infected enterocytes and might produce TGF-β that limits IFN-γ production (11).

propria, the Peyer's patches and the mesenteric lymph nodes were parasitized whereas parasites were associated with the $CD11c^-$ $CD11b^+$ monocytes in the peripheral circulation. These parasitized cells are involved in disease induction in the brain of naive recipient mice as demonstrated by adoptive transfer experiment. *Ex vivo* analysis of parasitized cells showed that single tachyzoites, non-replicating parasites could be identified at the cell periphery often surrounded by the host cell plasma membrane. By several approaches including vital staining of leukocytes, antibody labeling or chimeric mice in which the hematopoietic cells expressed the green fluorescent protein, it was determined that *T. gondii* infected $CD11b^+$ leukocytes can traffic to the brain extra-vascular space (Courret, 2005). Additional studies identified $CD11c^+$ and $33D1^+$ cells localized at inflammatory sites in infected brain (Fisher, 2000).

Parasite infection of enterocytes results in the upregulation and expression of CD40, CD1d, Class II suggesting that enterocytes may act as APCs and be involved in the activation of lymphocytes. Because of the limited expression of CD80 and CD86 mandatory for efficient lymphocyte stimulation, DCs and macrophages that infiltrate the infected epithelium are likely to be principally involved as APCs for $CD4^+$ T cells activation from the lamina propria. In addition to their crucial role as APC, DCs display an anti microbial function. IFN-γ activation of DCs triggers oxygen dependant inhibition of *T. gondii* (Aline, 2002).

In addition to PMNs, both DCs (Aliberti, 2003) as well as macrophages (Oliviera, 2000) produce IL-12 following parasite infection. Interleukin-12 (IL-12) is the major cytokine triggering IFN-γ synthesis by NK and T lymphocytes during *T. gondii* infection. IL-12 is the major initiation signal for host resistance to the parasite. IL-12 is also assumed to be responsible for T-helper 1 (Th1) effector choice in *T. gondii* infection.

CD40/CD154 interaction is involved in the regulation of macrophage production of interleukin 12 (IL-12) and T-cell production of IFN-γ. Infection of C57BL/6 mice with *T. gondii* results in an upregulation of CD40 expression on accessory cell populations at local sites of infection as well as in lymphoid tissues. CD40/CD154 ligation is essential to initiate the intestinal inflammatory response observed after oral infection of C57BL/6 mice (Li, 2002) and CD40/CD40L interaction is crucial in resistance to *T. gondii* (Reichmann, 2000; Andrade, 2005).

Aside from being the precursors of the antibody secreting cells, B cells are engaged in other immune functions such as antigen presentation to T cells or cytokine production. These functions may contribute to the pathogenic role of B cells in a wide range of autoimmune diseases. We demonstrate that B cells acquire the capacity to amplify IFN-γ production by CD4 and CD8 T cells during the course of the Th1 inflammatory response to *T. gondii* infection. Using two different strategies: *i)* reconstitution of B cell-deficient mice with B cells expressing an alloantigen different from the recipients, and *ii)* adoptive transfer of B and T cells into RAG-/-mice, we observed that B cells from *T. gondii*-infected mice, but not from naïve mice, induce higher IFN-γ expression by splenic host T cells. *In vitro* assays allowing the physical separation of T cells and B cells demonstrate that antigen-primed B cells enhance IFN-γ production by T cells in a contact-dependent fashion. Using an OVA-transgenic strain of *T. gondii* and OVA-specific CD4 T cells, we observed that the pro-inflammatory effect of B cells is neither antigen-specific nor requires MHCII expression. However, TNF-α expressed on the surface of B cells appears to mediate in part the upregulation of IFN-γ by the effector T cells (Mennard, 2007).

Because of their major role in microbial recognition, the involvement of receptor pathways involving the Toll-like receptor (TLR)/IL-1R superfamily in triggering both DC IL-12 production and host resistance to *T. gondii* has recently been addressed. Innate immune recognition relies on a limited number of germ-line encoded receptors, such as TLRs that recognize Pathogen Associated Molecular Patterns (PAMPs) of microbial origin. Although TLRs are expressed in a broad range of tissues the greatest variety of TLR mRNAs is found in professional APCs suggesting a key role of TLRs in innate immunity that is essential in the development of the acquired immune response characterized by polarization of naive $CD4^+$ helper T cells toward the TH1 or TH2 phenotype. Mice lacking myeloid differentiation factor 88 (MyD88), an adapter molecule used by all TLRs as well as IL-1R and IL-18R exhibited a near complete abrogation of the parasite-induced IL-12 response, and when challenged with *T. gondii*, the knockout (KO) animals displayed a loss in resistance to infection equivalent to that of IL-12-deficient mice (Scanga, 2002). Recently we have identified a role for TLR9 in the Th1-type inflammatory response that ensues following oral infection with *T. gondii*.

Following oral infection with *T. gondii*, susceptible B6 but not $TLR9^{-/-}$ (B6 background) mice develop a Th1 dependent acute ileitis compared to $TLR9^{-/-}$ mice (B6 background) that are free of gut inflammation. $TLR9^{-/-}$ mice have higher parasite burdens than control WT mice suggesting depressed IFN-γ dependent parasite killing. IL-12 producing DCs were reduced in $TLR9^{-/-}$ mice compared with the WT controls which corresponded with a reduction in total T cell frequency as well as IFN-γ producing T cells from the Lamina Propria. Infection of chimeric mice deleted of TLR9 in either the hematopoietic or non-hematopoietic compartments indicates that TLR9 expression in both compartments is involved (Minns, 2006).

In addition to IL-12, macrophages and dendritic cells produced IL-15. IL-15 exhibits pleiotropic functions at the interface between innate and adaptive immunity. IL-15 is a 14 kD cytokine that shares common features with IL-2 and exhibits stimulatory effects on T cell proliferation. However, IL-15 can be distinguished from IL-2 by its broad cell-type distribution, its potent *in vivo* anti-apoptotic effects and as most relevant to this review a bridge between the innate and adaptive immune response. IL-15 is necessary for the differentiation and/or homeostatic maintenance of the three subsets of lymphocytes linked to innate immune response, NK, NK/T and CD8αα intraepithelial lymphocytes (IEL). IL-15 is also mandatory for the survival of memory CD8 T lymphocytes. IL-15 induces the effector functions of NK and CD8 T lymphocytes, promotes the selection of high avidity cytotoxic CD8 T cells and their expression of the co-receptor CD8αβ (reviewed in Fehinger, 2001). Finally, IL-15 stimulates the maturation of dendritic cells and thereby promotes antigen presentation.

The role of IL-15 produced by hematopietic cells is controversial after infection by *T. gondii* (Doherty, 1996; Lieberman, 2004; Khan, 2002). In contrast, we have recently observed, using hematopoietic chimeric mice that IL-15 produced by infected enterocytes, initiates the inflammatory immune response that leads to the development of the lethal ileitis in C57BL/6 mice (Julie Shulthess, manuscript in preparation). IL-15 is critical for the differentiation and or homeostasis of several murine innate immune cell subsets, including natural killer, NK/T cells and CD8αα intraepithelial lymphocytes as well as the generation and maintenance of specific memory CD8 TCRαβ cells. In addition, IL-15 plays redundant functions with other cytokines to promote maturation of dendritic cells, proliferation of T and B cells, cytotoxicity of NK and $CD8^+$ T cells and production of proinflammatory cytokines.

Among cells targeted by IL-15, NK and NKT cells plays a major role during the early phase of the *T. gondii* infection. Natural killer T cells represent a minor subset of T lymphocytes that share receptor structures with conventional T cells and NK cells. Murine NKT cells express intermediate levels of a TCR using a semi-invariant Vα14-Jα281 TCR-chain paired with a limited number of β chain such as Vβ8, -7, or -2 TCR together with NK cell receptors (NKR-P1, Ly-49, and NK1.1 in C57BL/6 mice). These cells are located mainly in the liver, spleen, thymus, and bone marrow and recognize Ag in the context of the monomorphic CD1d Ag-presenting molecule. Our findings suggest a potentially critical role for these early responder cells in the initiation and regulation of the lethal inflammatory process. The implication of NKT cells was demonstrated by the observation that NKT cell-deficient mice ($J\alpha281^{-/-}$) are more resistant than C57BL/6 mice to the development of lethal ileitis. $J\alpha281^{-/-}$ mice failed to overexpress IFN-γ in the intestine early after infection. This detrimental effect of NKT cells is blocked by treatment with α-galactosylceramide, which prevents death in C57BL/6, but not in $J\alpha281^{-/-}$, mice. This protective effect is characterized by a shift in cytokine production by NKT cells toward a Th2 profile and correlates with an increased number of mesenteric Foxp3 lymphocytes. These results highlight the participation of NKT cells in the parasite clearance by shifting the cytokine profile toward a Th1 pattern and simultaneously to immunopathological manifestation when this Th1 immune response remains uncontrolled and give the evidence that NKT cells are important in regulation of Th1/Th2 differentiation (Ronet, 2005). The parasite antigen able to trigger NKT function is not yet determined, but it is well known that NKT recognize Ag in the context of the monomorphic CD1d Ag-presenting molecule. CD1d and the invariant TCR-chain are essential for the normal development of NKT. CD1 molecules present hydrophobic lipid Ags. However in contrast to mice that are genetically impaired for NKT cell ($J\alpha281^{-/-}$ mice) and that exhibit resistance to the development of lethal ileitis in C57B6 mice, CD1d deficient mice were more susceptible to the infection and apparently do not control their inflammatory response (Smiley, 2005). In C57BL/6 mice, $CD4^+$ cells can cause intestinal pathology during *T. gondii* infection. Compared with WT mice, infected CD1d-deficient C57BL/6 mice had higher frequencies and numbers of activated (CD44high) $CD4^+$ cells in mesenteric lymph nodes. Depletion of $CD4^+$ cells from CD1d-deficient mice reduced weight loss and prolonged survival, demonstrating a functional role for $CD4^+$ cells in their increased susceptibility to *T. gondii* infection. An other explanation might be that in addition to NKT depletion, regulatory cells, such as IEL and B cells, are also reduced in $CD1d^{-/-}$ mice (Allez, 2004; Mizoguchi, 2002).

INTESTINAL ADAPTIVE IMMUNE RESPONSE

Activation of the innate immune system results in antigen presentation and activation of the antigen specific T and B cell intestinal response. Intraepithelial lymphocytes (IELs) are located inbetween epithelial cells, below the intercellular tight junctions. Most of the IELs are $CD8^+$ T lymphocytes and bear an oligoclonal repertoire of T-cell antigen receptor (TCR) and express the unusual integrin αEβ7, which is involved in adherence to epithelial cells by binding to E-cadherin.

Infection of the gut with mucosal pathogens can result in the migration and activation of IELs. IEL migration towards the *T. gondii* infected enterocytes requires the expression of the chemokine receptor CCR5 in response to the secretion of MIP-1α (Luangsay, 2003).

IELs provide a number of important immunological functions, including cytotoxic activity, secretion of cytokines and modulation of epithelial cell death and regeneration. *T. gondii* antigen-primed IELs are cytotoxic for *T. gondii*-infected enterocytes (Chardès, 1994; Buzoni-Gatel, 1997). We showed that IL-15KO mice failed to develop the lethal ileitis after infection by the parasite. Our preliminary data indicate that IEL boosted by IL-15 might participate to the deleterious role of this cytokine in the loss of intestinal homeostasis although parasite killing might require IL-15 (Schulthess, manuscript in preparation).

However IELs may display a dual role depending on the phenotype. Adoptive transfer of antigen primed CD8 α/β TCR α/β IELs into naive mice prior to infection, rescues the recipient mice from death (Buzoni-Gatel, 2001) in contrast to IELs that exhibit αTCR γ/δ phenotype. *T. gondii* antigen-primed IELs produce substantial amounts of TGF-β that down regulate the production of IFN-γ from the CD4 lymphocytes in the lamina propria through a Smad 2, 3 dependant pathway (Mennechet, 2004).

In intestinal toxoplasmosis the development of a Th1 like T cell response, orchestrated by IFN-γ producing CD4 T cell from the lamina propria leads to the inhibition of parasite replication, but also may damage the intestinal barrier. CD4 T cells from the *T. gondii* infected lamina propria produce copious amount of IFN-γ and TNF-α that enhance the production of chemokines by infected enterocytes and increase the inflammatory response (Mennechet, 2002).

Studies indicate that production of secretory IgA antibodies are associated with early infection in mice (Chardès, 1992, 1993). The lamina propria is indeed populated with numerous B cells that differentiate into IgA plasmacytoid cells. In addition the natural presence of TGF–β into the intestine contributes to IgA switch. However, the protective role of these secretory IgA are still debated. Specific antibodies are not considered to be the major factor in recovery from infection, although they may play a role in protection against re-infection and are useful for an early diagnostic.

PARASITE ANTIGENS THAT TRIGGER THE INNATE RESPONSE

The role of specific Toxoplasma antigens in the induction of the innate response is only partially understood. The surface of *T. gondii* comprises of a family of developmentally regulated glycosylphosphatidylinositol (GPI)-linked proteins (SRSs), of which surface antigen 1 (SAG1) is the prototypic member. SAG1 protein is exclusively expressed on the tachyzoite. The biological role for this superfamily of surface proteins remains mostly enigmatic although there is evidence for a role in parasite attachment. SAG1 induces the dominant antibody response during infection and a strong, systemic Th1-like T cell response characterized by high-titer IFN-γ production by CD4 and CD8 T lymphocyte.

A SAG1 null mutant was engineered by homologous recombination and used to infect C57BL/6 mice. This mutant was shown *in vitro* to adhere and to replicate in fibroblasts at the same or even at a better rate than the control parental strain. *In vivo*, we were able to demonstrate that this antigen deficient parasite is unable to induce ileitis following intraluminal infection. Although this mutant can replicate in both the host and *in vitro* cell culture, infection is associated with a decrease in both innate and adaptive inflammatory immune responses (Rachinel, 2004).

Parasite penetration into the host activates a strong anti-parasite immune response, but is also used by the parasite to chronically persist. John Boothroyd reports (Saeij, 2007) the molecular cross talk between the parasite rhoptry proteins and the host cell. During host cell invasion, rhoptries participate to the constitution of the mobile junction that drives the parasite into the host cell, while building the parasitophorus vacuole in which the parasite grows (El Hajj, 2007). Some soluble rhoptries, such as Rop16, are shed into the cytoplasm, and then reach the nucleus where they can eventually impact different signalling pathways such as STAT3/6, key molecules in the immune response establishment. Whatever the signals and the pathways used to shift the immune response, a Th1 like immune response is absolutely necessary to control parasite replication. If left unmodulated this Th1 like immune response can lead to lethal host damage such as the ileitis seen in C57BL/6 mice.

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