

CD40, autophagy and *Toxoplasma gondii*

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Toxoplasma gondii represents a pathogen that survives within host cells by preventing the endosomal-lysosomal compartments from fusing with the parasitophorous vacuoles. The dogma had been that the non-fusogenic nature of these vacuoles is irreversible. Recent studies revealed that this dogma is not correct. Cell-mediated immunity through CD40 re-routes the parasitophorous vacuoles to the lysosomal compartment by a process called autophagy. Autophagosome formation around the parasitophorous vacuole results in killing of the *T. gondii*. CD40-induced autophagy likely contributes to resistance against *T. gondii* particularly in neural tissue.

Key words: macrophage - lysosome - endosome - IFN- γ - nitric oxide

Toxoplasma gondii is an excellent example of a pathogen of clinical importance that avoids eradication by the host. Indeed, *T. gondii* causes a chronic infection in humans despite the onset of an immune response. This infection can reactivate under conditions that impair cell-mediated immunity leading to disease (toxoplasmosis). One of the mechanisms utilized by the parasite to avoid eradication relies on its residence within a compartment - the parasitophorous vacuole - that avoids fusion with late endosomes-lysosomes. This is a key feature of the biology of the parasite because *T. gondii* cannot survive within the lysosomal compartment.

The parasitophorous vacuole is formed during the process of active invasion of host cells. The membrane of the vacuole is extensively modified because *T. gondii* excludes many host cell proteins and inserts proteins of parasite origin (Hakansson et al. 2001, Joiner & Roos 2002). Host endocytic structures are recruited to the parasitophorous vacuole and delivered intact into the vacuolar space (Coppens et al. 2006). However, no release of endosomal contents into the vacuole takes place. The dogma had been that once the parasitophorous vacuole is formed, its non-fusogenic nature could not be changed (Joiner et al. 1990, Mordue & Sibley 1997). Thus, a critical question in the interaction between *T. gondii* and the immune system has been whether there is a way to change the non-fusogenic nature of the parasitophorous vacuole. This question is not only relevant to *T. gondii* but also to the large number of pathogens that survive by avoiding phago-lysosomal fusion. We recently demonstrated that this is achieved through CD40 stimulation (Andrade et al. 2006, Subauste et al. 2007a).

CD40 and the immune response against *T. gondii*

IFN- γ is essential for protection against *T. gondii* in mice. IFN- γ -deficient mice die of fulminant infection even when challenged with an avirulent strain of the parasite (Suzuki et al. 1988, 1989, Gazzinelli et al. 1991) and this cytokine is required for resistance against toxoplasmic encephalitis and ocular toxoplasmosis (Suzuki et al. et al. 1989, Gazzinelli et al. 1993, 1994). TNF- α , NOS2 and the production of nitric oxide are other components of the immune response critical to control the parasite in the brain and eye (Gazzinelli et al. 1993, 1994, Hayashi et al. 1996a, b, Scharton-Kersten et al. 1997, Deckert-Schluter et al. 1998, Yap et al. 1998, Roberts et al. 2000). These findings are in agreement with the synergistic role of IFN- γ and TNF- α for induction of NOS2.

Although IFN- γ /TNF- α -NOS2 are key for resistance against *T. gondii* in neural tissue, it is likely that there are other components of cellular immunity that are required for control of the parasite in these sites. This possibility is particularly relevant to humans because of their restricted expression of NOS2 and because patients with a mutation in IFN- γ R1 that prevents recruitment of STAT1 do not develop disease after *T. gondii* infection while STAT1^{-/-} mice die acutely after challenge with the parasite (Janssen et al. 2002, Gavriulescu et al. 2004, Lieberman et al. 2004).

CD40 is a member of the TNF receptor superfamily expressed on antigen presenting cells and various non-hematopoietic cells (van Kooten & Banchereau 2000). Its counter receptor, CD154 (CD40 ligand) is expressed primarily on activated CD4⁺ T cells (van Kooten & Banchereau 2000). The interaction between CD40 and CD154 regulates several aspects of cellular and humoral immunity (van Kooten & Banchereau 2000). Many studies reported that this pathway mediates resistance against *T. gondii* in humans and mice. Patients with X-linked Hyper IgM syndrome (X-HIGM, a congenital immunodeficiency caused by lack of functional CD154) are susceptible to toxoplasmosis (Leiva et al. 1998, Subauste et al. 1999). The CD40 - CD154 pathway restricts the growth of *T. gondii* in peripheral tissues during the acute phase of infection (Subauste & Wessendarp 2006). How-

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ever, the role of this pathway is particularly important for control of the parasite in the brain. CD154^{-/-} mice are susceptible to toxoplasmic encephalitis (Reichmann 2000).

The CD40 - CD154 pathway promotes a type 1 cytokine response against *T. gondii* in humans (Subauste 1999, Subauste & Wessendarp 2000) and mice (Subauste & Wessendarp, unpublished observations). However, studies in mouse models of *T. gondii* infection indicate that this pathway also activates mechanisms of host resistance that act independently of IFN- γ (Reichmann 2000). CD154^{-/-} mice develop toxoplasmic encephalitis despite upregulation of IFN- γ in the brain that is similar to that of infected wild-type mice (Reichmann et al. 2000). These findings raise the possibility that the CD40 - CD154 pathway triggers an alternate pathway to promote resistance to *T. gondii* in neural tissue.

CD40 transforms the parasitophorous vacuole into a compartment that fuses with late endosomes-lysosomes

Macrophages and microglia are effectors of resistance against *T. gondii* (Gazzinelli et al. 1993, Robben et al. 2005, Deckert et al. 2006). Macrophages and T cells infiltrate the brain and the retina in cerebral and ocular toxoplasmosis (Schluter et al. 1991, Gazzinelli et al. 1994). Therefore, CD154⁺ *T. gondii*-reactive activated CD4⁺ T cells likely trigger CD40 signaling in macrophages and resident microglia. CD40 stimulation of macrophages allows them to acquire toxoplasmicidal activity (Andrade et al. 2003, 2005a, b, 2006). This effect is not only mediated by recombinant CD154 or an agonistic anti-CD40 mAb but also by CD154 expressed on the membrane of activated CD4⁺ T cells (Andrade et al. 2005a). Killing of *T. gondii* tachyzoites induced by CD40 does not require IFN- γ , or effector molecules downstream of this cytokine: NOS2 and Immune Related GTPases (IRG) (Andrade et al. 2003, 2005a, Subauste & Wessendarp 2006). In addition, killing of the parasite is not mediated by the oxidative pathway or starvation for tryptophan (Andrade et al. 2005a).

CD40 causes fusion of parasite-containing vacuoles with late endosomes-lysosomes (Andrade et al. 2006, Subauste et al. 2007a). A critical question was whether parasitophorous vacuoles, which by definition have been considered non-fusogenic, fused with late endosomes-lysosomes. The formation of parasitophorous vacuoles involves secretion of contents of parasite organelles located in the apex of the organism (Archbarou et al. 1991, Cesbron-Delauw 1994, Carruthers & Sibley 1997). The use of transgenic parasites that express a fluorescent protein targeted to the dense granules allowed to demonstrate that vacuoles where contents of the dense granules are secreted into the vacuolar lumen fuse with late endosomes-lysosomes (Andrade et al. 2006). Moreover, preformed parasitophorous vacuoles still fuse with late endosomes-lysosomes even if CD40 is engaged 18 h after infection (Andrade et al. 2006). These studies demonstrated that the immune system through CD40 alters a fundamental aspect of the biology of *T. gondii*: avoidance of lysosomal degradation.

CD40 induces killing of *T. gondii* through vacuole-lysosome fusion because pharmacologic inhibition and genetic manipulation of molecules key for vesicular trafficking and lysosomal degradation [lysosomal enzymes, vacuolar ATPase, class III phosphatidylinositol 3-kinase (PI3K) or hVps34, Rab7] abrogate killing of *T. gondii* induced by CD40 (Andrade et al. 2006). These studies uncovered a new paradigm where interaction between CD154 on T cells and CD40 expressed on macrophages leads to killing of an intracellular pathogen via the induction of vacuole-lysosomal fusion (Andrade et al. 2006, Subauste et al. 2007a). Vacuole-lysosome fusion induced by CD40 likely contributes to host protection because CD40 stimulation in vivo induces macrophage toxoplasmicidal activity and reduces the parasite load (Subauste & Wessendarp 2006).

CD40 induces vacuole - lysosomal fusion and toxoplasmicidal activity through autophagy

Studies of expression of endosomal-lysosomal markers revealed that while late endosomal-lysosomal markers (Mannose 6-phosphate receptor, Rab7, LAMP-1, LAMP-2, CD63, cathepsin D) are recruited to the parasitophorous vacuole, this is not preceded by recruitment of the early endosomal markers Rab5, EEA1 (Andrade et al. 2006). These findings suggested that CD40 re-routes the parasitophorous vacuole to the endosomal-lysosomal compartment using a mechanism that differs from the classical pathway of phago-lysosomal fusion.

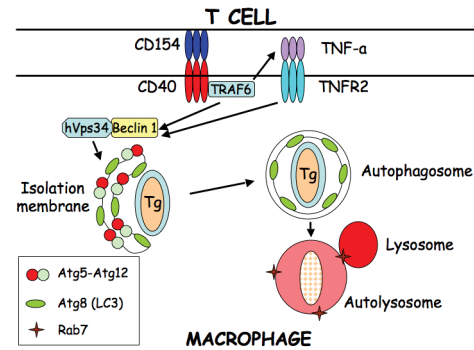
Autophagy is a process that directs cytoplasmic material and organelles to the lysosomes (Mizushima et al. 2002, Levine & Klionsky 2004). This is a ubiquitous and highly conserved process present in eukaryotic cells. In response to starvation and other forms of stress, an isolation membrane is produced by polymerization of autophagy proteins (Atg) that belong to two ubiquitin-like conjugation systems: Atg8 (LC3) and Atg12-Atg5 (Ohsumi 2001). This process is driven by the complex of the Atg6 (Beclin 1) with class III PI3K (hVps34) (Kihara et al. 2001). The isolation membrane engulfs portions of cytosol and organelles leading to the formation of autophagosomes (Dunn 1994, Mizushima et al. 2002, Levine & Mizushima 2004). Fusion between autophagosomes and endosomes-lysosomes culminates in the formation of autolysosomes and degradation of their contents (Dunn 1994). Thus, autophagy represents an alternate route to the lysosomal compartment. Autophagy is a homeostatic process that allows cells to adapt to environmental changes and that enables cells to degrade damaged or surplus organelles. Recent studies revealed that autophagy has a much wider role that includes regulation of aging and development, protection against cancer and neurodegeneration (Ravikumar et al. 2002, Melendez et al. 2003, Qu et al. 2003).

Autophagy has recently been identified as an innate mechanism that leads to degradation of pathogens such as *Streptococcus pyogenes*, metabolically arrested *Listeria monocytogenes*, *Salmonella enterica* (Rich et al. 2003, Nakagawa et al. 2004, Birmingham et al. 2006), *Shigella flexneri* and Herpes simplex virus 1 (HSV-1) that lack the virulence factors IscB and ICP34.5, respec-

tively, are also targeted by autophagosomes (Talloczy et al. 2002, Ogawa et al. 2004). Autophagy induced by starvation or rapamycin also kills *Mycobacteria* (Gutierrez et al. 2004, Singh et al. 2006). A critical question was to determine whether adaptive immunity activates autophagy to kill pathogens. IFN- γ induces autophagosomes that target *Mycobacterium tuberculosis* (Gutierrez et al. 2004, Singh et al. 2006) and it has been proposed that IFN- γ kills the pathogen through autophagy. CD40 stimulation of *T. gondii*-infected macrophages causes recruitment of the highly specific autophagy marker Atg8 (LC3) around the parasitophorous vacuole (Andrade et al. 2006). This phenomenon precedes recruitment of LAMP-1 around the parasitophorous vacuole suggesting that autophagy may be responsible for vacuole-lysosomal fusion (Andrade et al. 2006). Indeed, knock-down of the autophagy molecule-Atg6 (Beclin 1) revealed that autophagy mediates fusion of the parasitophorous vacuole with late endosomes-lysosomes and killing of *T. gondii* in CD40-activated macrophage (Andrade et al. 2006). These studies established that autophagy can be activated by adaptive immunity to kill a pathogen.

Recent studies revealed that CD40 signals through adapter proteins to trigger autophagy (Subauste et al. 2007a). TNF Receptor Associated Factors (TRAF) are adapter proteins recruited to the intra-cytoplasmic tail of CD40 (Bishop et al. 2007). CD40 has two binding sites that directly recruit TRAF2 and TRAF3 and a binding site that directly recruits TRAF6 (Ishida et al. 1996, Pullen et al. 1998, Lu et al. 2003). The TRAF6 binding site plays a dual role in the autophagic killing of *T. gondii*: it enhances autocrine production of TNF- α (Mukundan et al. 2005) and TRAF6 signaling downstream of CD40 synergizes with TNF- α to activate autophagy (Subauste et al. 2007a). As a result, autophagosomes are recruited around the parasitophorous vacuole and this is followed by Rab7-dependent fusion with late endosomes-lysosomes and killing of *T. gondii* (Andrade 2005b, 2006) (Figure).

IFN- γ also induces recruitment of autophagosomes around *T. gondii* (Ling et al. 2006). However, this phenomenon follows disruption of the parasitophorous vacuole membrane (Ling et al. 2006), a mechanism by which IFN- γ has been reported to kill *T. gondii* (Martens et al. 2005). Therefore, autophagosome formation in IFN- γ -treated macrophages is unlikely to mediate anti-microbial activity against the parasite but rather is a response to removal of altered intracellular structures: disrupted parasitophorous membranes and dead tachyzoites. Importantly, studies in human and mouse macrophages demonstrated that pharmacological inhibition of lysosomal enzymes, vacuolar ATPase, PI3K (including the autophagy inhibitor 3-methyl adenine), knockdown of hVps34, expression of dominant negative Rab7, knockdown of Beclin 1 did not inhibit anti-microbial activity induced by IFN- γ but ablated CD40-induced toxoplasmicidal activity (Andrade et al. 2006). The different mechanisms used by CD40 and IFN- γ to kill *T. gondii* may contribute to the cooperation observed between CD40 and IFN- γ to promote control of the parasite (Andrade et al. 2003).



Model of autophagy-dependent killing of *Toxoplasma gondii* induced by CD40. *T. gondii*-specific CD4⁺ T cell acquires expression of CD154 after interaction with infected macrophages. CD40-CD154 binding results in recruitment of TNF Receptor Associated Factors 6 (TRAF6) to the intracytoplasmic tail of CD40 that in turn enhances TNF- α production. TRAF6 signals downstream of CD40 and TNF- α signaling synergize to trigger autophagy. Beclin 1 together with hVps34 promotes recruitment of autophagosome to the parasitophorous vacuole that contains the pathogen (Tg). Subsequent recruitment of Rab7 controls fusion with the lysosomes and killing of *T. gondii*.

Potential relevance of CD40-induced autophagy in *T. gondii* infection

Although autophagy appears to be involved in the clearance of the HSV-1 (Orvedahl et al. 2007), it has not been proven that autophagy mediates host protection in vivo. It is likely that autophagy is protective against *T. gondii* particularly in neural tissue. The role of macrophages/microglia as effector cells against *T. gondii*, the fact that mice deficient in the CD40 - CD154 pathway are susceptible to cerebral toxoplasmosis despite unimpaired upregulation of IFN- γ together with the demonstration that CD40 induces autophagic killing of *T. gondii* independently of IFN- γ /NOS2 strongly suggest a paradigm where two arms of immunity: one dependent on IFN- γ /NOS2 and another dependent of CD40-induced autophagy are required to control *T. gondii* in the brain. This paradigm can explain why IFN- γ is insufficient for control of *T. gondii* in neural tissue (Yap et al. 1998, Reichmann et al. 2000).

Mouse models of *T. gondii* infection do not fully mimic immunity against *T. gondii* as it occurs in humans. Although IFN- γ is clearly indispensable to control the parasite in mice, IFN- γ -independent mechanisms of resistance against *T. gondii* appear to be more effective in humans. Children with an autosomal dominant defect in IFN- γ R1 that causes a deletion in the STAT1 binding site do not develop disease when infected with *T. gondii* (Janssen et al. 2002). In marked contrast, STAT1^{-/-} mice die within one week after infection with the parasite (Gavriulescu et al. 2004, Lieberman et al. 2004). The differences between the immune response in humans and mice also apply to the downstream effectors of IFN- γ . NOS2 in humans is more tightly regulated than in rodents and the production of nitric oxide appears to be weaker in humans than in mice (Kroncke et al. 1998). In addition, while mice have 23 IRG genes of which *Irgm1* (LRG-

47), *Irgm3* (IGTP) and *Irga6* (IIGPI) mediate anti-*T. gondii* activity in mouse cells (Collazo et al. 2001, 2002, Martens et al. 2005), IRG in humans have been reduced a truncated gene *IRGM* (syntenic with the mouse gene *Irgm1*) and *IRGC*, both of which lack an IFN inducible element (Bekpen et al. 2005). CD40-induced autophagic killing of *T. gondii* may be an important contributor to control of *T. gondii* in humans because CD40 induces killing of *T. gondii* independently of IFN- γ , STAT1, NOS2 and IRG (Andrade et al. 2005a, 2006, Subauste & Wessendarp 2006, Subauste et al. 2007a). Defects in the CD40 pathway are likely relevant to at least three groups of patients that develop cerebral and/or ocular toxoplasmosis: patients with X-linked Hyper IgM syndrome who lack functional CD154 (Subauste et al. 1999), newborns since neonatal CD4⁺ T cells exhibit impaired expression of CD154 (Durandy et al. 1995, Nonoyama et al. 1995, Julien et al. 2003, Han et al. 2004, Kaur et al. 2007) and neonatal dendritic cells have reduced levels of CD40 (Kaur et al. 2007). Impaired CD154 induction is particularly more pronounced in preterm babies (Kaur et al. 2007), a finding relevant to toxoplasmosis in newborns since this is an infection acquired prior to birth. The CD40-dependent pathway of host protection is also relevant to HIV-1⁺ patients because these individuals exhibit a defect in CD154 induction in their CD4⁺ T cells (Subauste et al. 2001, 2004, 2007b, Zhang et al. 2004).

The studies on CD40 uncovered a mechanism by which adaptive immunity utilizes autophagy to modify the fusogenic potential of pathogen-containing vacuoles leading to lysosomal dependent microbial killing. These results shed new light on the spectrum of mechanisms of resistance against *T. gondii* and are likely relevant to the pathogenesis of this disease as it occurs in humans. Not all CD40⁺ cells kill *T. gondii* after CD40 stimulation (Andrade et al. 2006). Future studies should identify the mechanisms activated by the parasite to avoid autophagy as well as determine whether manipulation of CD40 and autophagy signaling can enhance pathogen eradication.

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