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Differences among the three major strains of *Toxoplasma gondii* and their specific interactions with the infected host

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Toxoplasma gondii **is one of the most successful protozoan parasites owing to its ability to manipulate the immune system and establish a chronic infection. There are many *T. gondii* strains but the majority identified in Europe and North America falls into three distinct clonal lineages. Many studies have investigated the ability of *T. gondii* to manipulate its host but few have examined directly whether the three lineages differ in this ability.**

Focus on *Toxoplasma*

Toxoplasma gondii is an obligate intracellular parasite capable of infecting virtually any warm-blooded animal [1]. In humans, *Toxoplasma* infections are widespread and can lead to severe disease in individuals with an immature or suppressed immune system. Other genera of the phylum Apicomplexa also include important pathogens including *Plasmodium*, *Eimeria*, *Neospora*, *Babesia*, *Theileria* and *Cryptosporidium*. Unlike most of these organisms, *T. gondii* is an especially tractable model with which to study intracellular Apicomplexan parasitism because the parasite can be cultured, easily transfected both transiently and stably, crossed, and studied genetically *in vitro* and in diverse animal models [2]. Acute infection, associated with the rapidly dividing form or 'tachyzoite', is normally controlled by the immune system, with a major role for interferon-γ (IFN-γ)-dependent mechanisms. However, the parasite can differentiate into a 'bradyzoite' state, which can persist in the host within cysts that are apparently refractory to the robust immune response induced by tachyzoites [3,4]. Persistent bradyzoites can then initiate a new infection upon ingestion by a predator or scavenger. Theoretically, *T. gondii*, unlike other Apicomplexa, can be perpetuated asexually in this manner.

Sexual reproduction occurs only in the gut of members of the *Felidae* family, and genetic exchange between different strains can only occur in the rare event of a feline becoming infected simultaneously with more than one strain. In this case, however, millions of extremely stable oocysts, which upon maturation will contain eight haploid sporozoites each, are excreted in the feces of the cat. As a result of meiosis, many of those highly infectious oocysts will contain different sporozoite genotypes, and thus a huge number of new, genetically distinct organisms could potentially be generated from a single infected cat. Animals ingesting these oocysts can subsequently function to select the most successful of these genotypes. There is evidence that such a selection indeed occurs because currently the majority of *T. gondii* strains in Europe and North America seems to fall into just three distinct clonal lineages (Box 1).

There is considerable interest in determining how particular *T. gondii* genotypes might differ in their capacity to induce pathology or occurrence in a particular animal species. Understanding the genetic factors that influence *T. gondii* virulence and the mechanism of genotype selection according to host species could contribute to the development of therapeutics designed to eliminate transmission or cure disease. Unfortunately, however, most *T. gondii* strains have been genotyped with only a small number of markers that are shared among many different strains. As a result, only provisional designation of particular isolates can be made using such methods. The fact that isolates of nominally the same genotype differ in virulence [5], ability to cause encephalitis [6] or cytokine induction [6] makes more thorough genetic analysis essential before any real conclusions can be drawn. For this reason, this review focuses on the extensively characterized type I strains RH and GT-1, the type II strain ME49 [and its derivatives (PDS, and PLK, PTg)] and the type III strains CEP (CTg) and VEG; these strains were either completely or partially sequenced (ME49; RH; see ToxoDB at http://www.toxodb.org), were part of *T. gondii* expressed sequence tag projects (ME49, RH, VEG), and/or have been used as the parents in genetic crosses and subsequently genotyped with more than 130 markers (GT-1, CEP, ME49; see *Toxoplasma* Genome Map at http://www.toxomap.wustl.edu).

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Box 1. Population biology of *Toxoplasma gondii*

Understanding the population biology of *T. gondii* could enable prediction of the outcome of infection based on the genotype of the infecting organism [7]. For example, not all seropositive AIDS patients develop TE; the ones that do might be infected with a particular subset of parasite strains. Similarly, seroconversion during pregnancy does not always lead to infection of the fetus; this might be a result of variability in the ability of different strains to cross the placental barrier.

Several studies have concluded that, in Europe and North America, the majority of *T. gondii* strains comprises three distinct clonal lineages [53,54] that differ genetically by 1% or less [55]. Population genetic analyses suggest that recombination(s) between two discrete ancestral gene pools produced recombinant progeny, of which a small number have recently come to dominate over most other strains in many parts of the world. As a result, only two alleles exist for a majority of genes in a majority of strains. *T. gondii* strains with atypical or novel combinations of alleles have been isolated from nondomestic animals [56], or in other continents, such as South America or Africa [57–60], and from patients with unusual clinical presentations [8,61]. Unfortunately, many genotyping studies on *T. gondii* strains have examined only one (mainly SAG2) or two markers [11,62,63]. Given that many strains share identical alleles at many loci, such analyses cannot give a definitive identification of strain, although they can exclude some genotypes from the possibilities. Thus, a multilocus and multichromosome genotyping strategy is preferable; for example, CL19 and CL11, two progeny from a type II×III cross [64], have 127 and 113 type III loci, respectively, out of 139 markers analyzed (see *Toxoplasma* Genome Map at http://www.toxomap.wustl.edu), and looking at only two to three markers might have easily led to misclassification of these strains as more examples of the type III clonal type.

Toxoplasma gondii genotype and virulence

Toxoplasma virulence is normally defined based on the LD_{50} in mice. Little is known, however, about the correlation between *T. gondii* virulence in mice compared with other species. Several studies have investigated the correlation between *T. gondii* genotype and disease manifestation in humans (for review, see Boothroyd *et al.* [7]). Notably, only type I and some unusual strains were found in immunocompetent individuals suffering from severe, atypical ocular toxoplasmosis in the USA [8]. This is unexpected because type II strains seem otherwise to dominate human infections in this country [9]. Type I strains have also been disproportionately associated with severe congenital toxoplasmosis in Europe [10,11]. Although the role of individual genes in pathogenesis can be investigated using reverse genetics (e.g. knockouts in the surface antigen SAG3 [12], dense granule antigen GRA2 [13], carbamoyl phosphate synthetase II [14], and microneme proteins MIC1 and MIC3 [15] all decrease *T. gondii* virulence in mice), genetic crosses are much more powerful and much less biased ways of approaching this question. Hence, by examining the virulence of recombinant progeny generated by crossing *Toxoplasma* strains that differ in their virulence, genomic regions important for pathogenesis have been identified (Box 2).

Box 2. Use of genetic crosses to identify virulence genes

Quantitative trait loci analysis of crosses between type I (acutely virulent in mice) and type III strains identified a major virulence locus on chromosome VIIa. A minor association with virulence around the ROP1 marker (which is now known to be on chromosome XI) was also found. The intermediate virulence of several recombinant progeny indicates that, as expected, acute virulence is multigenic. Interestingly, a separate cross between the relatively avirulent type II and type III strains produced one progeny that was three orders of magnitude more virulent than either parent (i.e. LD_{50} ~1 instead of >1000), demonstrating that a new combination of alleles is all that is needed for a dramatic increase in virulence. The genome map (see *Toxoplasma* Genome Map at http://www.toxomap.wustl.edu) for *T. gondii* will aid in the fine-mapping of these virulence genes. Although the relatively large map unit (104 kb per cM) in *T. gondii* makes it relatively easy to map a phenotype to a general chromosomal region, it hinders the precise mapping by linkage analysis because many progeny are required to find informative crossovers [27]. Although sequence variation in genes common to all strains could be the key to virulence, the absence or presence of genes in certain strains cannot be ruled out; for example, using transverse alternating field electrophoresis, Sibley *et al.* [65] noted strain-specific differences in the migration of certain chromosomes, indicating significant differences (>100 kb) in size and perhaps gene content. Whether these are simply interchromosomal translocations or real differences in gene content has yet to be determined.

Migration and virulence

Crossing biological barriers such as gut epithelia, the blood–brain barrier or the placenta is important for successful *T. gondii* infection. Therefore, in addition to invading cells that circulate in the body, active parasite motility could also provide an effective means of dissemination. For example, different migration capacities might contribute to strain-specific differences in parasite dissemination and access to immunoprivileged sites such as the central nervous system. This might be especially true for transplacental migration because maternal cells do not routinely traffic to the fetus [16]. Barragan and Sibley [17] demonstrated that a subpopulation of type I parasites display enhanced migration *in vitro*, compared with type II and type III strains. When these parasites are cloned, many have this long-distance migration (LDM) phenotype. Furthermore, transmigration across polarized Madin–Darby canine kidney (MDCK) cells, migration across the extracellular matrix, and *ex vivo* penetration of lamina propria and submucosa were all enhanced in type I strains, compared with type II or III strains. *In vivo*, LDM RH parasites migrate more effectively to the spleen than do type II or III parasites. Genetic analysis supports the association of migratory capacity and virulence: analysis of recombinant progeny from a type I with type III cross revealed that the LDM phenotype maps to a quantitative trait locus on chromosome VIIa, the same chromosome region previously linked to acute virulence [16].

Growth rate and virulence

Growth rate is a common virulence trait in protozoan pathogens [18–20], and the correlation between *T. gondii* virulence and growth rate has been extensively described [21,22]. The *T. gondii* parasite burden is a major contributor to *Toxoplasma* pathogenesis in mice [23,24], related to an overstimulation of the immune system [23–25] leading to high levels of T helper cell type 1 (Th1) cytokines, increased apoptosis and organ damage. Although one tachyzoite of a type I strain is sufficient to generate high parasite loads and high levels of Th1 cytokines, high parasite loads of a type II strain, generated by increased

inoculation, will lead to equally high levels of cytokines and pathology. This immune pathology, therefore, could simply be a reflection of high antigenic load rather than other qualitative differences between the different strains.

These studies do not address what gives a type I strain the ability to reach high parasite loads even after low parasite inoculation. Such an increased parasite burden *in vivo* could be the result of an inherently shorter doubling time (Td), enhanced resistance to the host immune system and/or a decreased conversion to the slower growing bradyzoite form. Although several studies have noted that type I strains grow faster than type II or III strains [26], it is important to differentiate among time to host cell lysis, the number of parasites in a culture and Td. We routinely observe that the type I strain RH completely lyses a flask of cultured cells much faster than the type II or III strains but we (J.P.J. Saeij *et al.*, unpublished) and others [27] have not observed significant differences in Td between the strains within the context of a single round of intracellular growth. Hence, the discrepancy in overall growth rate might be explained by a higher reinvasion rate of type I parasites. In support of this hypothesis, we have found that extracellular type I parasites remain infectious for a longer time compared with the type II or III strains, and so they might be able to disseminate more efficiently to new cells after an infected cell is lysed (J.P.J. Saeij *et al.*, unpublished).

The Td of *T. gondii* seems to be related to the developmental stage of the parasite. Jerome *et al.* [28] described that tachyzoites emerging from cells infected with type III sporozoites grow rapidly for ~20 divisions (Td=6 h), after which their growth slows (Td=15 h). The decrease in tachyzoite growth rate after emerging from sporozoite-infected cells was correlated with the appearance of bradyzoite markers such as bradyzoite antigen (BAG)1; the proportion of tachyzoites expressing this marker increased from zero to 5% when assayed five and 15 days following sporozoite inoculation. Interestingly, a spontaneous mutant, MS-J, maintained its initial rapid growth rate *in vitro*, did not express BAG1 at any time and its virulence was 1000 times that of the parental VEG strain. Radke *et al.* [29] have described a similar change in growth rate in tachyzoites emerging from bradyzoite-infected cells. This difference in growth rate *in vitro* is not without phenotypic consequence. When IFN-$\gamma^{-/-}$ mice were infected with MS-J tachyzoites, or VEG tachyzoites derived from sporozoites (either three or eight days after sporozoite culture initiation), the time to death was found to correlate with the growth rate *in vitro* [28] (MS-J-infected mice died 12 days postinfection (dpi), mice infected with VEG tachyzoites derived from sporozoites three days after sporozoite culture initiation died 14 dpi and mice infected with VEG tachyzoites derived from sporozoites eight days after sporozoite culture initiation died 16 dpi).

Similarly, we recently employed luciferase-expressing parasites to demonstrate that a virulent progenitor (named S23) from a type II×III cross had a higher *in vivo* growth rate and better dissemination than an avirulent progenitor (named S22) [30] (Figure 1). cDNA microarray analysis demonstrated that the avirulent strain, when grown under tachyzoite conditions, was shifted towards a bradyzoite pattern of gene expression, whereas the virulent strain remained firmly on the tachyzoite end of the continuum (J.P. Boyle *et al.*, unpublished). Thus, there seems to be an inverse correlation between the virulence of *Toxoplasma* strains and the expression of bradyzoite genes ('bradyzoiteness') under normal culture conditions.

Toxoplasma gondii modulation of pathways leading to proinflammatory cytokine secretion

The effects of *T. gondii* infection on nuclear factor-κB (NF-κB) and mitogen-activated protein kinase signaling cascades have recently been reviewed [31,32], so discussion here will be limited to strain differences in the manipulation of these pathways. The transcription factor NF-κB has a central role in regulating the inflammatory,

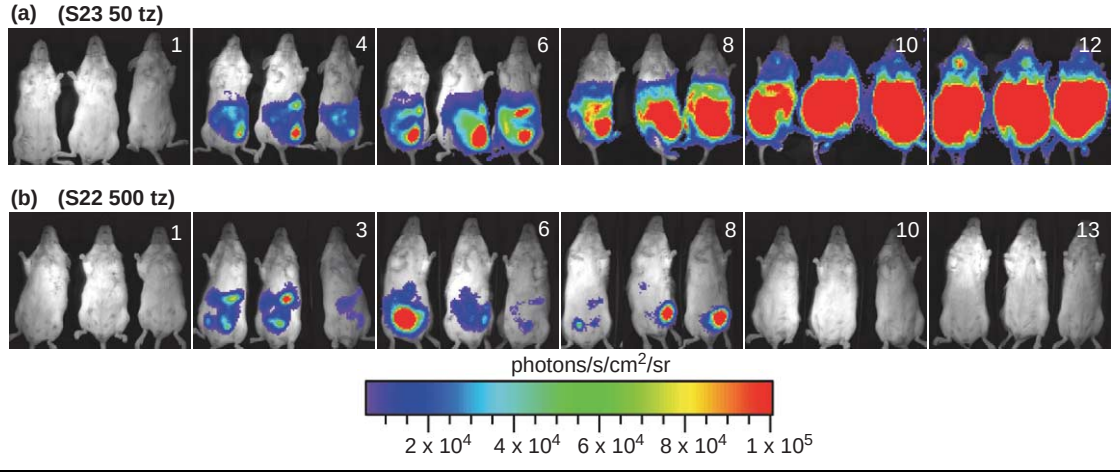

Figure 1. Luminescent *Toxoplasma gondii*. **(a)** Typical course of i.p. infection of mice infected with *T. gondii* strain S23-luc7 (50 parasites). **(b)** Typical course of i.p. infection of mice infected with *T. gondii* strain S22-luc2 (500 parasites). Tachyzoites were isolated from human foreskin fibroblasts by syringe lysis, and Balb/C mice were infected with S23-luc7 [50 tachyzoites (tz)] or S22-luc2 (500 tz). Mice were imaged ventrally, starting one day after infection, and data are representative of two experiments ($n=3$ per group). For all images shown, the color scale ranges from blue [just above background noise, set to 6000 photons/s/cm^2/steradian (sr)] to red (at least 10^5 photons/s/cm^2/sr). Parasites were imaged at the indicated dpi (top right of each image) with an IVIS™ Imaging System (http://www.xenogen.com/ivistech.html). Reproduced, with permission, from Ref. [30].

immune and anti-apoptotic responses. The activation of NF-κB translocation by *T. gondii* is an area of controversy. *T. gondii* can transiently (during the first 6 h postinfection) block NF-κB translocation in mouse macrophages and human fibroblasts, thereby inhibiting the transcription of genes involved in the proinflammatory response, such as those encoding interleukin (IL)-12p40 and tumor necrosis factor-α [33]. By contrast, *T. gondii*-infected mouse fibroblasts display clear nuclear translocation of NF-κB, and the transcription factor has a role in the induction of anti-apoptotic genes [34]. Interestingly, in infected mouse fibroblasts, the phosphorylated inhibitor of NF-κB (IκB)α localizes to the parasitophorous vacuole, and a *T. gondii* kinase could be involved in the IκBα phosphorylation, thereby enabling NF-κB to translocate to the nucleus [35]. Discrepancies between these studies [33,34] might be due to the use of different cell types and/or host species but not, in theory, to a difference in strain type because these studies all used the virulent (type I) RH strain. In other work, however, differences in the ability to translocate NF-κB to the nucleus have been associated with strain type: the type II strain ME49, but not the type I strain RH, induces translocation of NF-κB to the nucleus of mouse splenocytes [36] and mouse bone marrow-derived macrophages [37]. Further work is needed to determine the molecular basis of all of these observations.

IL-12 is a proinflammatory cytokine that stimulates the production of IFN-γ, drives the differentiation of Th1 cells and forms a link between innate and adaptive immunity [38]. Hence, differences in the ability to induce IL-12 could lead to differences in the immune response against various *Toxoplasma* strains. There seem to be no differences among *T. gondii* strains in their ability to induce IL-12p40 production by neutrophils [39] and dendritic cells [37]. However, Robben *et al.* [37] demonstrated that thioglycolate- or peptone-elicited peritoneal exudate cells or bone marrow-derived macrophages infected with type II parasites, but not type I or type III parasites, produce high levels of IL-12p40 in a myeloid differentiation primary response gene 88 (MyD88)-dependent and Toll-like receptor (TLR)2- and TLR4-independent fashion. Interestingly, soluble tachyzoite antigen preparations from type I or type II strains did not show any difference in IL-12p40 induction when added to macrophages (although these preparations induced much less IL-12p40 compared with live type II tachyzoites). Active host-cell invasion was required for high IL-12p40 induction by type II parasites, and this induction could not be inhibited by previous infection with type I strains, suggesting that high IL-12 production is due to specific induction in type II-infected cells rather than inhibition of IL-12 production by type I parasites. Type II strains also induced higher levels of IL-10, IL-1β and IL-6. This study has been corroborated by two other studies; early after intraperitoneal (i.p.) infection (two dpi), type II strains induce more IL-12p40 and IFN-γ than type I strains [40], and a type II strain (DX) induced more IL-12 production by thioglycolate-elicited macrophages than a type I strain (BK) [41]. Schade *et al.* [41] also noted that macrophages isolated from mouse strains susceptible to *T. gondii* produce more IL-12p40 than those from mouse strains resistant to *T. gondii*. In contrast to these studies, Butcher *et al.* [42] state that neither type II nor type I strains induce tumor necrosis factor-α or IL-12p40 production in thioglycolate-elicited macrophages or in macrophage cell lines. Thus, the ability of the type II strain ME49 to induce translocation of NF-κB to the nucleus of macrophages might be related to its capacity to induce proinflammatory cytokine release and IL-12 production by these cells.

The potent proinflammatory response of macrophages to infection with type II parasites might explain the ability of type II strains to cause pathology after oral infection of Th1-prone mice such as C57BL/6 because this pathology has been associated with IL-12 production, and IL-10$^{-/-}$ mice die after infection with the type II strain ME49 as a result of high production of IL-12 and IFN-γ [43–45]. However, there are no studies comparing the pathology induced by different *T. gondii* strains after oral infection of C57BL/6 mice. Also, toxoplasmic encephalitis (TE) is caused by chronic infection with ME49, and is associated with high levels of inflammatory cytokines in mice and elevated IL-12 levels in human patients [46]. These elevated levels of inflammatory cytokines could be the result of a capacity of this strain to induce translocation of NF-κB to the nucleus. Interestingly, the type III strain VEG does not cause TE in mice [5], and it remains to be seen if differences among the three main *T. gondii* strains to cause TE are related to their different abilities to induce translocation of NF-κB to the nucleus or to induce IL-12 production.

Attraction of different cell types

Differences among *T. gondii* strains in their ability to attract particular cell types to the site of infection might explain differences in their ability to disseminate, replicate or survive. Several studies have noted that i.p. infection induces a rapid recruitment of inflammatory cells (especially neutrophils) to the site of injection [40,47,48]. The importance of neutrophil recruitment for host resistance is demonstrated by studies showing that depleting neutrophils [47] or impairing the ability of neutrophils to reach the site of infection (using CXCR2$^{-/-}$ [48] or CCR1$^{-/-}$ [49] knockout mice) leads to impaired resistance and enhanced growth of *T. gondii*. Paradoxically, Mordue *et al.* [40] demonstrated that, although type I strains attract more neutrophils than do type II strains, after i.p. injection of an equal number of parasites, type I strain parasites are found in higher numbers in the peritoneum than type II strain parasites. Interestingly, they noted that, although parasites are rarely found inside neutrophils (an observation also noted by others [48]), significantly more type I parasites were found within these cells than was seen for type II. Thus, the differing abilities of various *T. gondii* strains to grow in (or infect) neutrophils might explain the difference in their ability to reach the parasite burden associated with host pathology and death. Alternatively, although neutrophils are required for host protection, an excess could exacerbate disease independently of any role for them as potential host cells. A role for neutrophils in immunopathology is supported by the finding that D-galactosamine-sensitized

mice infected with *T. gondii* die from cytokine-mediated lethal shock that is dependent on the presence of granulocytes [50]. Exactly how macrophages and neutrophils inhibit *T. gondii* growth and entry early in infection is unclear. Nitric oxide production does not seem to have a role early in infection [51] but there is evidence that reactive oxygen species (ROS) might be important in this period. Macrophages from mice lacking uncoupling protein-2, a protein that uncouples respiration from energy production in mitochondria, produce higher levels of ROS and have increased toxoplasmacidal activity [52]. To date, there are no published reports on the susceptibility of the various *T. gondii* strains to the different toxoplasmacidal activities (e.g. nitric oxide, ROS, tryptophan starvation and iron deprivation) of immune cells.

Concluding remarks

From the literature, it is clear that significant differences in the host response to different *T. gondii* strains exist. No studies, however, have directly compared the host response to the three main *T. gondii* strains in concurrent experiments. Such experiments will be necessary if we are to understand how these three clonal lineages have come to dominate over other strains and how they lead to different pathologies. Simultaneous microarray analysis of host and parasite gene expression of cells infected with different *T. gondii* strains would provide a broad, unbiased survey of parasite and host genes that are differentially expressed or induced between the different *T. gondii* strains. When differences between strains are found, progeny of a cross between these strains can be used to map genetically these differences to a particular part of a chromosome. We have recently shown (J.P.J. Saeij *et al.*, unpublished) that this approach is feasible and we have mapped differences between parasite strains to manipulate the NF-κB and STAT (signal transducer and activator of transcription) pathways.

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