Advances in the Development of Anti-*Toxoplasma gondii* Vaccines: Challenges, Opportunities and Perspectives

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Abstract

Important progress has been made in understanding how immunity is elicited against *Toxoplasma gondii* - a complex pathogen with multiple mechanisms of immune evasion. Many vaccine candidates have been tested using various strategies in animal models. However, none of these strategies has delivered as yet, and important challenges remain in the development of vaccines that can eliminate the tissue cysts and/or fully block vertical transmission. In this review, we provide an overview of the current understanding of the host immune response to *T. gondii* infection and summarize the key limitations for the development of an effective, safe and durable toxoplasmosis vaccine. We also discuss how the successes and failures in developing and testing vaccine candidates have provided a roadmap for future vaccine development.

Why Do We Need a Toxoplasmosis Vaccine?

Toxoplasma gondii is an obligate intracellular protozoan of global importance, causing toxoplasmosis in humans and animals [1,2]. This parasite places a major health and socioeconomic burden on society, with more than one-third of the global population at risk of infection [1,2]. The complex life-cycle of *T. gondii* involves sexual reproduction in the intestine of members of the family Felidae, the only definitive host, and asexual propagation in a broad spectrum of intermediate hosts (Figure 1). *T. gondii* has the ability to infect all warm-blooded animals and humans, using multiple routes of transmission (Figure 1). Three developmental stages (**tachyzoites** (see Glossary), tissue **cysts** containing **bradyzoites**, and **oocysts**) are infectious to hosts and *T. gondii* can be transmitted horizontally or vertically [1,2].

Most infections in immunocompetent people are asymptomatic and remain undetected with only mild flu-like, myalgia, or other nonspecific symptoms occurring. However, when primary, or reactivated infection occurs during pregnancy or around the time of conception, serious health consequences may occur, such as abortion or severe congenital defects in the affected fetus including mental retardation, blindness, and hydrocephaly [3]. Reactivation or primary infection in immunocompromised patients (e.g. HIV-infected patients, organ transplant recipients, cancer patients) may cause severe complications, such as encephalitis or pneumonitis [4]. In addition to causing disease in humans, *T. gondii* may induce abortion and stillbirth in livestock especially in sheep and goats, posing a serious threat to sustainable agricultural economies [2].

In humans, the primary therapeutic regimen involves the combined use of the antifolate drugs, pyrimethamine and sulfadiazine, however failure rates remain significant [5]. Other

regimens including pyrimethamine together with clindamycin, clarithromycin, azithromycin, or atovaquone, or monotherapy with trimethoprim-sulfamethoxazole (TMP-SMX), have been used for treating toxoplasmosis, however none have been found to be superior to the pyrimethamine-sulfadiazine combination [5]. All treatment regimens used in clinical practice are active against the rapidly dividing tachyzoite stage of the parasite, but do not exert any significant activity against the slow-dividing bradyzoites within the tissue cysts, and cannot cure persistent infections [5]. Also, of concern with current therapeutics are the side effects that may occur in the treated host [5]. Vaccines offer better alternatives for efficient long-term disease control, while reducing the reliance on, and consequences of, chemical therapeutics. Currently, only one commercially available vaccine (Toxovax®, MSD Animal health) is licensed for use in sheep to prevent abortion while this vaccine has several drawbacks (*see* below) [6]. The development of an effective, safe and durable vaccine against *T. gondii* infection remains a necessity to facilitate the control of toxoplasmosis.

Mechanisms of Immunity to Toxoplasmosis

Protective immunity to *T. gondii* is complex, involves the innate and adaptive immune response, and is primarily dependent on T helper 1 (Th1) cell-mediated immunity driven by production of high levels of interleukin-12 (IL-12) and interferon-γ (IFN-γ) [7-9]. Following infection with *T. gondii*, innate immune cells including dendritic cells (DCs), macrophages and neutrophils migrate to the site of infection where they detect the parasite, mainly via toll-like receptors (TLR), and secrete IL-12, to stimulate $CD4^+$ and $CD8^+$ T cells, and natural killer (NK) cells to produce IFN-γ (Figure 2). IFN-γ limits the parasite proliferation and the progression of infection

through multiple intracellular mechanisms, such as stimulating the expression of **guanylate-binding proteins (GBPs)** and **immunity-related GTPases (IRGs)** to degrade the **parasitophorous vacuole (PV)**, increasing the expression of major histocompatibility complex (MHC) and antigen presentation genes, and the upregulation of other anti-parasitic factors such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) [7-9]. IL-12 and IFN-γ are critical cytokines for clearance of tachyzoites during acute infection, they are also essential for inducing tissue cyst formation and sustaining latent infection [7-9]. In addition, IL-1, IL-2, IL-4, IL-6 and tumor necrosis factor alpha (TNF-α) play a protective role in *T. gondii* infection. The anti-inflammatory cytokines IL-4, IL-10, IL-13, IL-27 and transforming growth factor (TGF-β) are responsible for minimizing damage caused by an excessive immune-inflammatory response [7-9].

Adaptive immunity depends largely on antigen-presenting cells (APCs), such as DCs and macrophages, and their ability to present *T. gondii* antigen, and activate B cells, $CD4^+$ and $CD8^+$ T cells in secondary lymphoid organs. Activation of B cells, $CD4^+$ and $CD8^+$ T cells requires both the recruitment of APCs from the infection site to secondary lymphoid organs, and the activation of resident APCs through exposure to *T. gondii* antigens. Once activated, B cells serve as APCs to stimulate $CD4^+$ T cells, and secrete cytokines such as IFN-γ and IL-12 [10]. Activation of T cells elicits immunity to *T. gondii*, and adoptive transfer of activated T cells can confer resistance to naive hosts [11]. $CD4^+$ and $CD8^+$ T cells are essential for long-term survival of the host, acting synergistically to prevent reactivation of cysts, and are important for the development of protective immunity against re-infection [12].

Humoral immunity is also essential for the control of toxoplasmosis [2,13]. Infection with *T. gondii* increases the levels of circulating immunoglobulins: IgA, IgE, IgM, and IgG, which exert their protective effects through multiple mechanisms, such as opsonization of the parasites by phagocytosis; inhibiting the parasite's attachment to host cells; blocking parasite invasion; and activating the classical complement pathway [10].

A Historical Account of Toxoplasmosis Vaccines

Considerable efforts have been made to develop a vaccine against toxoplasmosis over the last 30 years. The toxoplasmosis vaccine research has advanced considerably in recent times, with a large number of vaccine candidates having been tested using various vaccination strategies. Among these, inactivated vaccines, live-attenuated vaccines, DNA vaccines, protein vaccines, epitope vaccines, and live vector-based vaccines have been investigated in animal models to determine their ability to induce protective immune responses against *T. gondii* infection. However, none of these vaccination approaches have been able to eliminate the tissue cyst and/or block vertical transmission [14-16].

Vaccines based on killed organisms, lysate antigen, excretory-secretory (ES) products, subunit proteins and DNAs, are particularly effective against *T. gondii* infection as they can induce strong cytotoxic T lymphocytes and activate a number of lymphocytes. Among the tested antigens, studies have focused on the surface antigens (SAGs), rhoptry antigens (ROPs), microneme antigens (MICs), dense granule antigens (GRAs) and other antigens secreted by tachyzoites and bradyzoites, which play critical roles in *T. gondii* motility, attachment, invasion, replication and manipulation of the host's immune response [14-16]. Multi-antigen vaccines,

expressing several proteins or peptides representing different stages of the parasite, seem more powerful and efficient than single-antigen vaccines [14-16]. The components of the vaccine (e.g., peptides and adjuvants) seem to play roles in the efficacy of the vaccine. Several adjuvants, such as Freund's adjuvant, interleukins, CpGDNA, monophosphoryl lipid A, MHC-restricted adjuvant, have been incorporated into vaccines to enhance immune responses [14-16]. For example, co-administration of IL-15 and IL-21 with MIC8 stimulated specific humoral and cellular immune responses to acute and chronic *T. gondii* infection [17].

Live nonpathogenic microbes (bacteria, protozoa and virus) are the preferred vectors for delivering *T. gondii* antigen due to their ability to mimic the intracellular niche of *T. gondii* and induce an immune response without an additional adjuvant [18-20]. Immunization using a combined virus-like particle (VLP) vaccines expressing ROP18 and MIC8 induced robust humoral and cellular immune responses, and reduced parasite burden [19]. Also, heterologous prime-boost immunization strategies could induce high levels of specific humoral and cellular immune responses, providing a basis for the development of preventative and therapeutic interventions against *T. gondii* infection. For example, prime immunization using recombinant influenza viruses expressing SAG2 antigen followed by a booster vaccination using recombinant adenovirus encoding the same antigen induced strong immune responses and protected mice with an 85% reduction in parasite burden [20].

Live-attenuated vaccines are among the most efficient approaches that confer immune protection because they mimic natural infection, providing an approximation of the microenvironment for antigen processing and presentation. Live-attenuated vaccines based on mutants, such as T-263, TS-4 and S48, have shown high efficacy in the prevention of shedding

oocysts from cats, reduction of the incidence of abortion in sheep, and reduction of tissue cyst formation in food-producing animals [14-16]. However, only one commercial vaccine, Toxovax®, based on the live-attenuated tachyzoite S48 strain is licensed in New Zealand, UK and other European countries, and is used to reduce the incidence of abortion in sheep [6]. This live commercial vaccine (Toxovax®) S48 tachyzoites was originally isolated from an aborted lamb in New Zealand, and with repeated passage in excess of 3000-times in mice, has lost its capacity to form tissue cysts or produce oocysts [21]. However, Toxovax® has a relatively short shelf life and cannot fully eliminate the parasite. Furthermore, because the molecular basis of this live-attenuated tachyzoite S48 strain is not fully understood, spontaneous mutations may raise concerns due to the possibility of reversion to a pathogenic phenotype, which can pose a high risk to vulnerable individuals [21].

New trends in toxoplasmosis vaccine development

Live attenuated vaccines based on gene editing

In recent years, with the advances in genetic engineering methods, various approaches have been used to develop an attenuated toxoplasmosis vaccine [22]. Several genetically attenuated parasite strains, generated by deletion of certain genes, have shown good efficacy against toxoplasmosis (Table 1). The protective efficacy of genetically attenuated parasites depends on the challenge dose and challenge time, as well as the parasite background and host susceptibility. Not all genetically attenuated parasites have the potential to induce a strong immunity to wild-type *T. gondii* challenge. There is evidence that the apical secretion of some rhoptry proteins is

a key element for the induction of long-lasting sterile protective immunity [23]. On the other hand, *T. gondii* has been under selection pressure due to their interaction with their hosts' immune response; and it is possible to genetically alter the parasite in a manner that prevents inhibition of, and maybe even boosts, immune responses. This may be achieved by overexpression of some vital protective antigens, or by deletion of some important genes that the parasite uses to manipulate its host cell, or deletion of some key genes involved in controlling bradyzoite differentiation. For example, tachyzoites engineered to constitutively express bradyzoite-specific SRS9 protein, trigger a stronger SRS9-specific immune response than the wild type tachyzoite [24]. Live-attenuated toxoplasmosis vaccines can provide more protection than vaccines prepared by other approaches. However, before a licensure of an efficacious live attenuated vaccine can be sought, there is a need for evaluation systems to validate vaccine safety and efficacy with higher predictive values in the preclinical and clinical studies in order to prioritize promising candidate strains and guide the development of the much needed vaccines.

Nanoparticle-based vaccines

Recombinant subunit vaccines, containing one or multiple target antigens from a pathogen, are a safer alternative to live-attenuated vaccines, but they are less immunogenic and thus less effective at eliciting protective immunity [14-16]. Recent advances in nanomaterial engineering have opened up new avenues for the development of novel vaccines [25]. Nanoparticles (NPs) have significant potential as a delivery system. First, antigen encapsulation within NPs or via covalent attachment to the NPs, protect the integrity of the antigen against enzymatic

degradation, prolonging their systemic circulation time and enhancing the likelihood of presentation to immune cells [25]. Second, NP-based antigen systems can deliver antigens to certain cells, such as DCs, by coupling NPs with antibodies that bind with specific receptors on the surface of target cells [26]. Third, NPs use lower doses of antigens and adjuvant, thus reducing the risk of toxicity and side effects [27]. Some NPs are innately immunogenic and can serve as an adjuvant for activation of the immune system [28].

Many types of NPs have been used as a potent immunostimulatory delivery system to improve the induction of an effective immune response against *T. gondii* infection [29]. For example, immunization of mice using multi-epitope recombinant *T. gondii* vaccine containing predicted T and B cell epitopes of SAG1, AMA1, ROP2, and GRA4 proteins and its encapsulation in poly lactic-co-glycolic acid NPs as a delivery vehicle enhanced Th1 immune response and significantly reduced the parasite load, prolonging the survival of infected mice [30]. Immunization of mice with cocktail DNA vaccines, containing ROM4 and GRA14 together with calcium phosphate nanoparticles (CaPNs), enhanced immune responses to acute toxoplasmosis [31]. A pioneering study used an adjuvant-free modified dendrimer nanoparticle vaccine, which simultaneously carries multiple replicon RNAs encoding GRA6, ROP2A, ROP18, SAG1, SAG2A and AMA1, protected mice against lethal *T. gondii* challenges [32]. Although the protective efficacy of NP vaccine is higher than conventional subunit vaccines, it is still less effective than live-attenuated vaccines.

Exosomes-based vaccines

Exosomes are nano-sized (~30-150 nm in diameter) membrane-bound vesicles, released by most eukaryotic cells through fusion of multivesicular bodies with the plasma membrane [33]. They can transfer mRNA, miRNA and proteins between cells, representing a new way of intercellular communication, which is crucial to the development of immune homeostasis [34]. The exosomes also contain pathogen-derived antigens and represent a novel tool for vaccination against infectious diseases [35]. Immunization of mice with exosomes derived from *T. gondii*-pulsed DCs, induced a specific and protective T-cell response to congenital toxoplasmosis [36]. Recent research has shown that exosomes isolated from *T. gondii* can induce humoral and cellular immune responses, which are protective against acute infection in mice [37]. These studies may motivate further exploration of the potential of exosome as a new vaccine delivery system to support research efforts in quest for toxoplasmosis vaccine.

Carbohydrate-based vaccines

The surface of many pathogens contains carbohydrates such as polysaccharides, oligosaccharides and glycans, which are used by pathogens as receptors for attachment to and invasion of host cells [38]. Carbohydrates on the pathogen surface are recognized by the host innate immune receptors, leading to the production of anti-glycan antibodies. This makes the surface carbohydrates of pathogens attractive candidates for vaccine development [39,40]. In *T. gondii*, glycosylphosphatidylinositols (GPIs) can be recognized by TLR-2 and TLR-4 to induce inflammatory response, such as TNF-α production [41]. Two major *T. gondii* GPI glycoforms have been evaluated as potential vaccine candidates. Although antibodies were induced, immunization did not protect mice against challenge with the virulent *T. gondii* RH

strain. Mouse serum antibodies were more specific to the linker used to attach the carbohydrate to the carrier protein, than the desired carbohydrate side-branch of the parasite GPI [42]. Given the proven success of carbohydrate vaccines to combat several microbial infections, further selection of more immunogenic carbohydrate antigens and leveraging the potential of glycan array technologies may facilitate the development of an effective carbohydrate-based anti-*T. gondii* vaccine.

Hurdles That May Limit Development of Effective Vaccines

Despite more than three decades of a critical mass of basic and preclinical research into toxoplasmosis vaccine development, and despite employing various approaches to test many antigens and various formulations, none of the tested vaccine can eliminate the tissue cysts and/or fully block vertical transmission. Several challenges for the development of an effective vaccine exist, such as the genetic diversity among *T. gondii* strains, the multi-stage life-cycle of the parasite; the challenge of clinical translation, the remarkable parasite's ability to evade and subvert host immunity, the ability of *T. gondii* to establish latent infection, the lack of standardized vaccination protocol, and the influence of co-infection with other pathogens on the host response to vaccination.

The impact of strain variations on immunogenicity

Genetic diversity studies of *T. gondii* isolates from North America and Europe, have revealed that *T. gondii* has a clonal structure where the majority of *T. gondii* strains are classified into three predominant lineages, type 1, type 2 and type 3 [43]. These three lineages are all similar

(only ~ 1-2% divergence at the nucleotide level) and probably originated from a common ancestor around 10,000 years ago [44]. Despite the low genetic diversity between these three lineages, they differ markedly in their pathogenicity. Type 1 strains are lethal in laboratory mice, with an infectious dose of a single viable organism, whereas type 2 and 3 strains are considerably less virulent. Type 1 strains also exhibit increased migratory capacity *in vivo* and *in vitro* and have a higher growth rate *in vitro* than type 2 and type 3 [45]. Other differences between the three predominant lineages include the frequency of differentiation, induction of intestinal pathology during acute infection, development of central nervous system pathologies during chronic infection and manipulation of host cell signaling (*see* below) in laboratory mice [45].

Further analysis of the global population structure of *T. gondii* strains have revealed that the majority of *T. gondii* strains are divided into six major clades, which contain more than 16 haplo-groups with distinct geographical patterns [46,47]. Compared with strains from Europe and North America, *T. gondii* isolates from South America are more genetically diverse with no predominant genotypes, and show stronger evidence of genetic recombination. Like type 1 strains, the majority of *T. gondii* strains from South America are highly virulent to mice [46,47]. Genome analysis of 62 globally distributed *T. gondii* strains revealed a diversification of secretory proteins that are important determinants in host-pathogen interactions, highlighting crucial differences between major clades of *T. gondii* [48]. Expansion of polymorphic genes encoding secretory proteins that interact with and modulate the host signaling pathways have made *T. gondii* strains efficient modulators of host immune responses [48]. Previous studies have shown that some genetically distinct strains may have the ability to superinfect the same

host, suggesting insufficient cross protection from immunization with a single *T. gondii* strain [49,50].

A complex, multi-stage, life-cycle

T. gondii has a multi-stage life-cycle, wherein it expresses many different proteins during the stage to stage progression [51]. Therefore, immune responses produced against a specific life-cycle stage (i.e. tachyzoite, bradyzoite or oocyst) of *T. gondii* may fail to protect against infection caused by other stages, making vaccine design for this parasite more challenging than for simpler organisms, such as most viruses and bacteria. Vaccines that target a stage-specific antigen or based on single or two antigens may elicit immune response that lacks sufficient cross-protection. The complex life-cycle of this parasite, coupled with significant genetic variations, as discussed above, raise doubts that single-antigen vaccines could confer the desired broad-spectrum protection. Combining immunogenic antigens from the different forms of *T. gondii* life-cycle in a multi-stage-specific cocktail may offer a more robust approach towards the development of a toxoplasmosis vaccine capable of inducing sterile immunity and effectively preventing infection.

Species barriers to clinical translation

T. gondii can infect almost all warm-blooded animals and its pathogenesis has been studied in different animal models. Sheep, goats, pigs, and even chickens have been used to model toxoplasmosis. However, the laboratory mouse is considered the most widely used model to study the pathogenesis and immunological events involved in the control or prevention of *T.*

296 *gondii* infection [52]. Hundreds of vaccination studies over the last 30 years have been
297 performed in laboratory mice, yielding important insights into toxoplasmosis vaccine
298 development. However, the use of mouse model to study the development of toxoplasmosis
299 vaccine has several drawbacks.

300 Cross-species differences create barriers for translational research which may impede the
301 success of clinical trials. Rodents are the natural intermediate host of *T. gondii* and frequently
302 encountered this parasite over the course of their evolution and this long-term interaction might
303 have shaped the immune system of mice to handle *T. gondii* in a way that is different from an
304 accidental intermediate host, such as humans and food-producing animals [52]. Therefore,
305 mechanisms of innate response to *T. gondii* infection are expected to vary between rodents and
306 humans, although both rely on IFN-γ and STAT1 signaling pathways [7-9]. In mice, TLR11
307 and TLR12 are the innate sensors of *T. gondii*, where they recognize parasite profilin, an actin
308 binding protein (Figure 2) [53]. TLR11 is a pseudogene, and TLR12 is absent from the human
309 genome, and the sensors involved in *T. gondii* recognition by human innate immune cells
310 remain unknown [7-9]. In addition, the IRGs, a family of IFN-γ-inducible proteins, are essential
311 for the mouse resistance to *T. gondii* infection [54]. There are 23 members of this family in the
312 mouse genome while humans lack most IRGs and only one IRG homologue, IRGM, is present
313 in the human genome. Furthermore, IRGM is severely truncated and is not regulated by IFN-γ,
314 possibly not functioning as it does in mice, another example of human/mouse differences in
315 IFN-γ-mediated immunity to *T. gondii* [54]. Therefore, antigens identified in mice may not
316 protect humans or food-producing animals, and more vaccine studies should be performed in
317 food-producing animals, such as sheep and pigs.

Manipulation of host cell signaling pathways

As an obligate intracellular parasite, *T. gondii* has evolved several strategies to fine-tune the cellular microenvironment to promote its own growth and persistence inside host cells thereby increasing the spread of infection and chance of transmission to new hosts. After invasion of the host cell, *T. gondii* employs a repertoire of effector proteins secreted from dense granules and rhoptry organelles to manipulate host gene expression and signaling pathways [55].

Some of these effector proteins secreted from dense granules organelle traffic to the host cell nucleus (GRA16, GRA24 and TgIST), whereas others translocate to the **PV membrane (PVM)** (GRA15) or partially interact with the host cell cytosol while exhibiting a vacuole-restricted localization (GRA6). In all *T. gondii* strains, GRA16 upregulates the expression of host genes involved in cell cycle progression, metabolism and the p53 tumor suppressor pathway [56]. GRA24 prolongs auto-phosphorylation and nuclear translocation of host cell p38α, which correlates with the activation of transcription factors such as Egr-1 and c-Fos, and the production of pro-inflammatory cytokine IL-12 and chemokine monocyte chemoattractant protein 1 (MCP-1) [57]. TgIST blocks IFN-γ response by blocking STAT1-dependent pro-inflammatory gene expression [58,59]. GRA6 activates the host transcription factor NFAT4 (nuclear factor of activated T cells 4) in a strain-specific manner, promoting the production of chemokines, such as Cxcl2 and Ccl2 [60]. GRA15 is another strain-specific effector that activates the NF-κB pathway and induces more IL-12 secretion in type 2, compared to type 1 or type 3 strains [61].

Some effector proteins secreted from rhoptry organelle can also alter the host's transcriptional response. For example, rhoptry kinase ROP16 injected into the host cell nucleus can maintain constitutive activation of signal transducer and activator of transcription (STAT)-3 and STAT6 in a strain-specific manner; which results in decreased secretion of IL-12, skewing the response to Th2 [62,63]. In addition, polymorphic ROP5/17/18 complexes can phosphorylate IRGs and protect the parasite from clearance [64]. Whether the identified polymorphic effector proteins have similar or different effects across different host species remain unknown and the diverse mechanisms used by *T. gondii* to evade and manipulate host immunity hinder the development of an effective vaccine.

Establishment of latent infection

A key factor in the pathogenesis and transmission of *T. gondii* is the ability to convert from a rapidly replicating stage (tachyzoite) to a slow-dividing stage (cyst-containing bradyzoites). This tachyzoite-bradyzoite transformation occurs in response to selective pressure imposed by the host immune system. The tachyzoite-bradyzoite transformation and protection offered by the highly glycosylated cyst-wall allows *T. gondii* to avoid immune-mediated destruction and persist, effectively invisible, for years within the infected host, increasing the chances of transmission [65]. Significant changes in bradyzoite gene expression are associated with tissue cyst development, and the **apicomplexan AP2 (ApiAP2)** transcription factors are a crucial mechanism regulating this developmental transcriptome. A total of 67 ApiAP2 repressors and activators located in the *T. gondii* genome are responsive to stress signals and are active in tachyzoite-bradyzoite differentiation [66-68]. For example, the *Api2IV-4* gene normally

expressed by tachyzoites has a role in inhibiting bradyzoite gene expression. Deletion of Api2IV-4 caused the expression of bradyzoite-specific proteins in tachyzoites and the mis-timing of bradyzoite antigens in Δ*Api2IV-4* tachyzoites induced a potent host immune response that effectively eliminated the parasite and prevented brain tissue cyst formation [68]. However, the mechanisms that allow *T. gondii* to adapt to different tissue environments and permit long-term persistence as dormant tissue cyst remain to be fully elucidated. Therefore, a better understanding of the molecular mechanisms that drive the tachyzoite-bradyzoite interconversion is required to manage transmission and prevention of *T. gondii.*

The lack of a standardized vaccination protocol

Considerable efforts by many research groups have been put into designing and testing various vaccination strategies against toxoplasmosis. However, there has been a lack of synergy between toxoplasmosis vaccine studies because they vary in parasite strain used, infective stage, infection dose or route of parasite infection and host species [14-16]. *T. gondii* parasites have a diverse population structure; however, the strains frequently used to evaluate the protective efficacy of a toxoplasmosis vaccine are type 1 RH, and type 2 (Pru and ME49); all of which have been maintained in laboratories for many years and may no longer represent the naive isolates, for example, RH strains have lost the ability to form oocysts or tissue cysts [69]. In the natural setting, toxoplasmosis is transmitted by oral or congenital routes. However, intraperitoneal challenge has been frequently used in vaccine experiments, and may not reflect a natural infection route. Although oral challenge has been used to mimic infection, most studies have focused on the oral administration of tissue cysts, with few studies using infective oocysts.

These inconsistencies in vaccination regimens could hinder the accurate evaluation of the vaccination efficacy. The genetic background of experimental animals also affects the outcome of any vaccination. Immunity conferred by vaccination in mouse models may not reflect the mechanisms implicated in the protective immune response in food-producing animals or humans. Therefore, future vaccination studies should use different *T. gondii* strains, administered through natural infection route, in different animal models, particularly domestic cats and food-producing animals.

Co-infection with other pathogens

In the natural settings, a host may be simultaneously infected with several pathogens (parasites, viruses and bacteria). Coinfections alter the host's immune response compared to a single pathogen infection [70]. *T. gondii* infection induces a Th1-type immune response; whereas other pathogens, such as helminthes, induce a Th2-type immune response [71]. Whether or not Th2 type immunity induced by prior or concurrent helminth infection can antagonize a protective Th1 type immune response of toxoplasmosis vaccines remains to be determined. Understanding the immune response to mixed infections should be a part of future vaccine development.

Perspectives for Toxoplasmosis Vaccines

Although substantial progress has been made in the search for and the development of vaccines to prevent *T. gondii* infection, there is still no effective and safe toxoplasmosis vaccine to eliminate the tissue cysts and/or fully block vertical transmission. Technology-oriented research

is the way forward to identify and isolate more immunogenic vaccine candidates. For example, **reverse vaccinology** and **immunomics** offer enormous opportunities to enable targeted antigen discovery, and rational vaccine design (Figure 3) [72-74]. Reverse vaccinology aims to identify the complete repertoire of antigens that a pathogen secretes or expresses on its surface; while immunomics aims to elucidate sets of proteins or peptides that interact with the host immune system and the potential mechanisms involved in these interactions [72-74]. Recent technological advances in computational analysis, and availability of genomics resources of both host and *T. gondii* genomes have facilitated the identification of novel antigen vaccine candidates [75]. **Structural vaccinology** which focuses on understanding the structure of immune silent and immune-dominant antigens, is increasingly applied to the rational structure-guided design of immunogens to optimize their immunogenicity, stability and safety [76]. The application of these genome-based reverse vaccinology techniques to the development of toxoplasmosis vaccines may increase the chance of success.

Concluding Remarks

Decades of toxoplasmosis vaccine development have resulted in the identification and testing of various candidate antigens; however, none have shown durable and adequate protective efficacy. Potential impediments and solutions to the development of an effective toxoplasmosis vaccine have been discussed. There is still much to be learned about the immunopathogenesis of toxoplasmosis, and the identification of better immune-correlates and vaccination candidates (see Outstanding Questions). Successful vaccine development depends on full understanding of the immunopathogenesis of *T. gondii* infection, choosing appropriate candidate antigens and

selecting the right adjuvant or delivery vehicle, as well as testing the efficacy in suitable animal models in order to enable a transition from preclinical testing to food-producing animal and domestic cat trials. The future of toxoplasmosis vaccines appears bright, and the clinical data and resources generated by genomics, transcriptomics, proteomics, metabolomics and other technologies, are likely to build substantially on, and invigorate, basic research to develop effective and safe vaccines against toxoplasmosis.

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Disclaimer Statement

The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1. The two-host, predator-prey, life-cycle of *Toxoplasma gondii*. Members of the family Felidae (domestic and wild cats) are the only known definitive hosts of *T. gondii* **(A)**. They become infected after ingestion of viable tissue cysts **(B)**. Cats shed unsporulated oocysts in their feces into the environment **(C)**. After shedding, oocysts mature in the environment into infective (sporulated) oocysts **(D)**. Oocysts are able to survive in the environment for long periods of time and the sporulated oocysts can contaminate soil, water, vegetables and fruits **(E)**, providing a route of infection to a wide range of intermediate hosts **(F)** and cats **(G)**. *T. gondii* infection is initiated in humans by ingestion of oocysts in contaminated food or water **(H)** or ingestion of viable tissue cysts in raw or undercooked meat **(I)**. Other means of infection include contact with cats **(J)**, mother-to-child transmission **(K)** or by organ transplantation **(L)**. Shortly after infection, infective oocysts and tissue cysts transform to fast-replicating tachyzoites, the parasite stage responsible for acute infection. As the infection progresses, tachyzoites differentiate into slow-replicating bradyzoites enclosed within tissue cysts, which mark the latent form of infection and can persist throughout the lifetime of the affected host.

Figure 2. Host immune responses against *Toxoplasma gondii*. Chemokines secreted from cells at the site of infection trigger the recruitment of neutrophils, dendritic cells (DCs), monocytes and macrophages to the site of infection. The interaction of *T. gondii* derived profilin protein with Toll-like receptor 11 (TLR11) and TLR12 on DCs is required for the secretion of interleukin-12 (IL-12) in mice. In addition to stimulating IL-12 secretion, tumor necrosis factor

alpha (TNF-α) are also produced by macrophages, in response to TLR2- and TLR4-mediated sensing of *T. gondii* glycosylphosphatidylinositol (GPI)-anchored proteins. IL-12 stimulates natural killer (NK) cells, and $CD4^+$ and $CD8^+$ T cells to produce IFN-γ. Neutrophil is an important source for IFN-γ; however, the mechanisms that regulate neutrophil-derived IFN-γ remain incompletely known. The regulatory IL-10 cytokine plays an important role in the modulation of immune pathways and can prevent the lethal overproduction of T helper 1 type pro-inflammatory cytokines. IFN-γ has pleiotropic effects on the course of infection. IFN-γ amplifies a signal through a surface IFN-γ receptor to activate signal transducer and activator of transcription 1 (STAT1). In response to activation of STAT1, monocytes and macrophages upregulate their expression of the indoleamine 2,3-dioxygenase (IDO) enzyme and inducible nitric oxide synthase (iNOS) and reactive oxygen species (ROS). IDO depletes tryptophan, an essential amino acid for *T. gondii* growth. iNOS, in addition to production of nitric oxide (NO), controls parasite replication by depleting arginine, another essential amino acid for *T. gondii* growth. ROS has been implicated in the IFN-γ-mediated control of *T. gondii* in a cell type- and species-specific manner. Both hematopoietic and non-hematopoietic cells upregulate two families of defense proteins called guanylate-binding proteins (GBPs) and immunity-related GTPases (IRGs), which accumulate at the parasitophorous vacuole (PV) and contribute to the parasite clearance.

Figure 3. An integrated strategy for the discovery and testing of vaccine candidates in the post-genomics era. (A) Discovery of novel vaccine *T. gondii* candidates. Vaccine candidates can be identified through analysis of the *T. gondii*'s genome (the complete genetic content of

T. gondii), transcriptome (the complete set of RNA transcripts expressed by *T. gondii* under a certain condition), proteome (the complete set of proteins expressed by *T. gondii* under a certain condition), secretome (the complete set of proteins secreted by *T. gondii* under a certain condition) and surfome (the subset of proteins that are surface exposed). In parallel, immunoproteome (the set of antigens that interact with the host immune system) can provide a panoramic view of the spectrum, intensity and dynamics of antibodies binding to *T. gondii* proteins, revealing their immunogenicity potential. **(B)** Toxoplasmosis biomarkers and immune-correlates of protection across vulnerable host species. Further characterization of protective immune response is required in order to identify novel unambiguous markers that correlate with and predict vaccine-induced protective immunity against *T. gondii* infection. **(C)** Computational (*in silico*) analysis can facilitate all aspects of the preclinical and clinical vaccine development. **(D)** The structural vaccinology (the 3D structure of proteins, in particular the structural epitopes of immunogenic antigens) can be a useful aid in any future *T. gondii* vaccine development. **(E)** Experimental infection models are crucial for testing the clinical efficacy of *T. gondii* vaccine. **(F)** Animal-free (*in vitro*) systems can provide preclinical proof-of-concept in support of vaccine development.

Glossary

Apicomplexan AP2 (ApiAP2): a family of apicomplexan proteins with a 60 amino acid DNA binding domain related to Apetala-2 (AP2) family in plants that are linked to stress responses.

Bradyzoites: are slowly-replicating form of *Toxoplasma gondii*, found within tissue cysts, and are associated with latent infection.

Cysts: are thick-walled intracellular vacuolar structures that contain quiescent bradyzoites and can persist for the life of the host predominantly in muscle and neuronal tissues.

Guanylate-binding proteins (GBPs): a family of large 65-75 kDa GTPases induced by interferon and contribute to immune control of *Toxoplasma gondii*.

Immunity-related GTPases (IRGs): a family of GTPases, about 47 kDa, stimulated by interferon gamma. They are important in the control of *Toxoplasma gondii* in mice. Only one present in humans and not responsive to interferon.

Immunomics: an integrated immunology, genomics, proteomics, transcriptomics and bioinfomatics approach aimed at discovery of immunogenic antigens by studying the global interaction between host immune system and pathogens.

Oocysts: are thick-walled environmental forms of *Toxoplasma gondii* that are excreted in the feces of cats and can survive for lengthy periods outside the host under various ecological conditions.

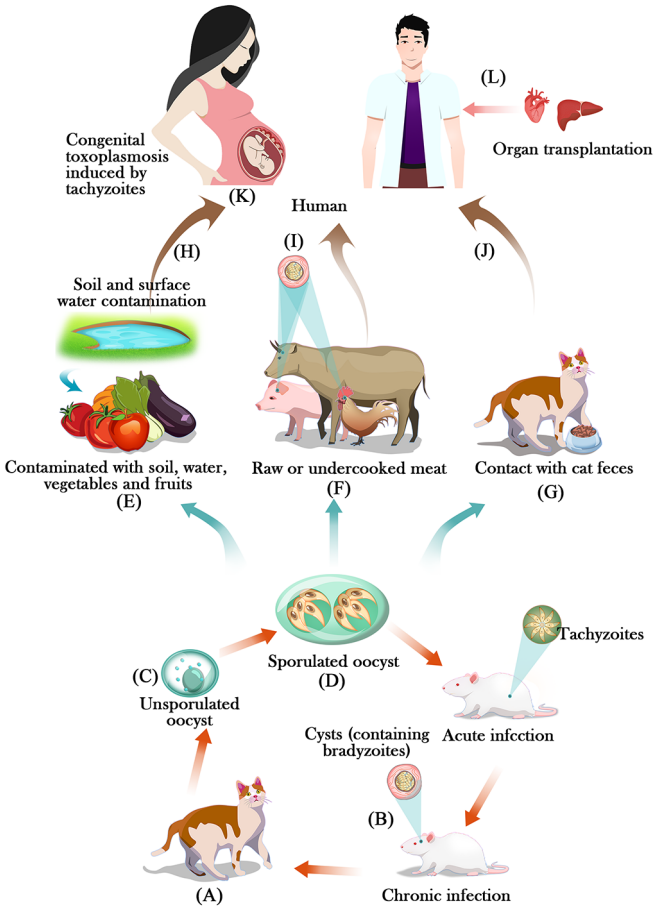
Parasitophorous vacuole (PV): a special membrane-bound intracellular vacuolar compartment formed during host cell invasion by the parasite and serves as a surrogate niche where the parasites divide through a special process known as endodyogeny.

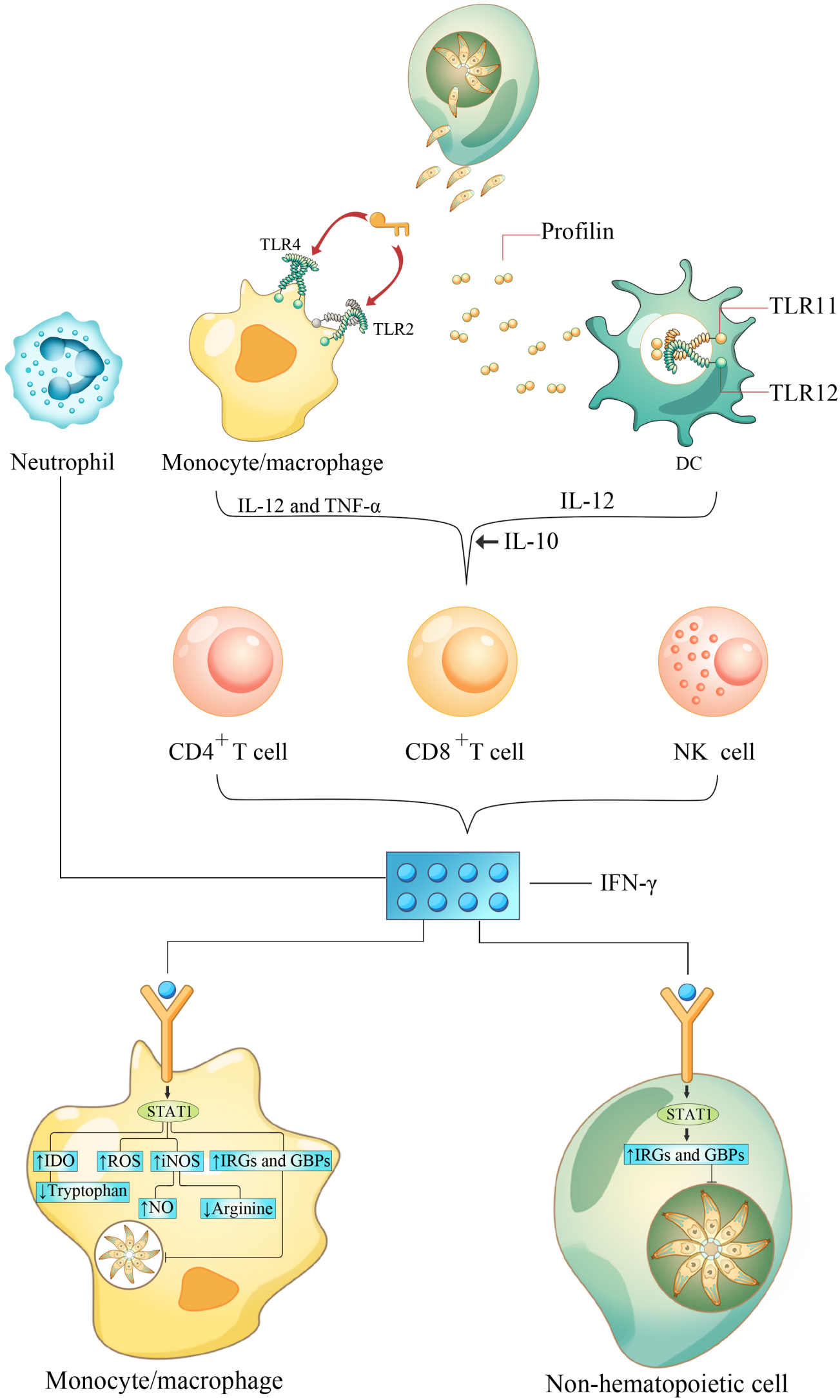
Parasitophorous vacuole membrane (PVM): a physical membrane that surrounds the parasitophorous vacuole and is modified by the parasite to facilitate nutrient acquisition and avoid host defenses.

Reverse vaccinology: an approach used for the discovery of candidate vaccine antigens from genomic information.

Structural vaccinology: the use of knowledge of protein molecular structure to identify protective antigens.

Tachyzoites: are fast-replicating form of *Toxoplasma gondii* and are associated with acute phase of infection.


Congenital toxoplasmosis induced by tachyzoites
(K)
(L)
Organ transplantation
Human
(H)
(I)
(J)
Soil and surface water contamination
Contaminated with soil, water, vegetables and fruits
(E)
Raw or undercooked meat
(F)
Contact with cat feces
(G)
Sporulated oocyst
(D)
Tachyzoites
(C)
Unsporulated oocyst
Cysts (containing bradyzoites)
Acute infcction
(B)
(A)
Chronic infection


TLR4
TLR2
Profilin
TLR11
TLR12
Neutrophil
Monocyte/macrophage
DC
IL-12 and TNF-α
IL-12
IL-10
CD4+ T cell
CD8+ T cell
NK cell
IFN-γ
STAT1
↑IDO
↑ROS
↑iNOS
↑IRGs and GBPs
↓Tryptophan
↑NO
↓Arginine
Monocyte/macrophage
STAT1
↑IRGs and GBPs
Non-hematopoietic cell

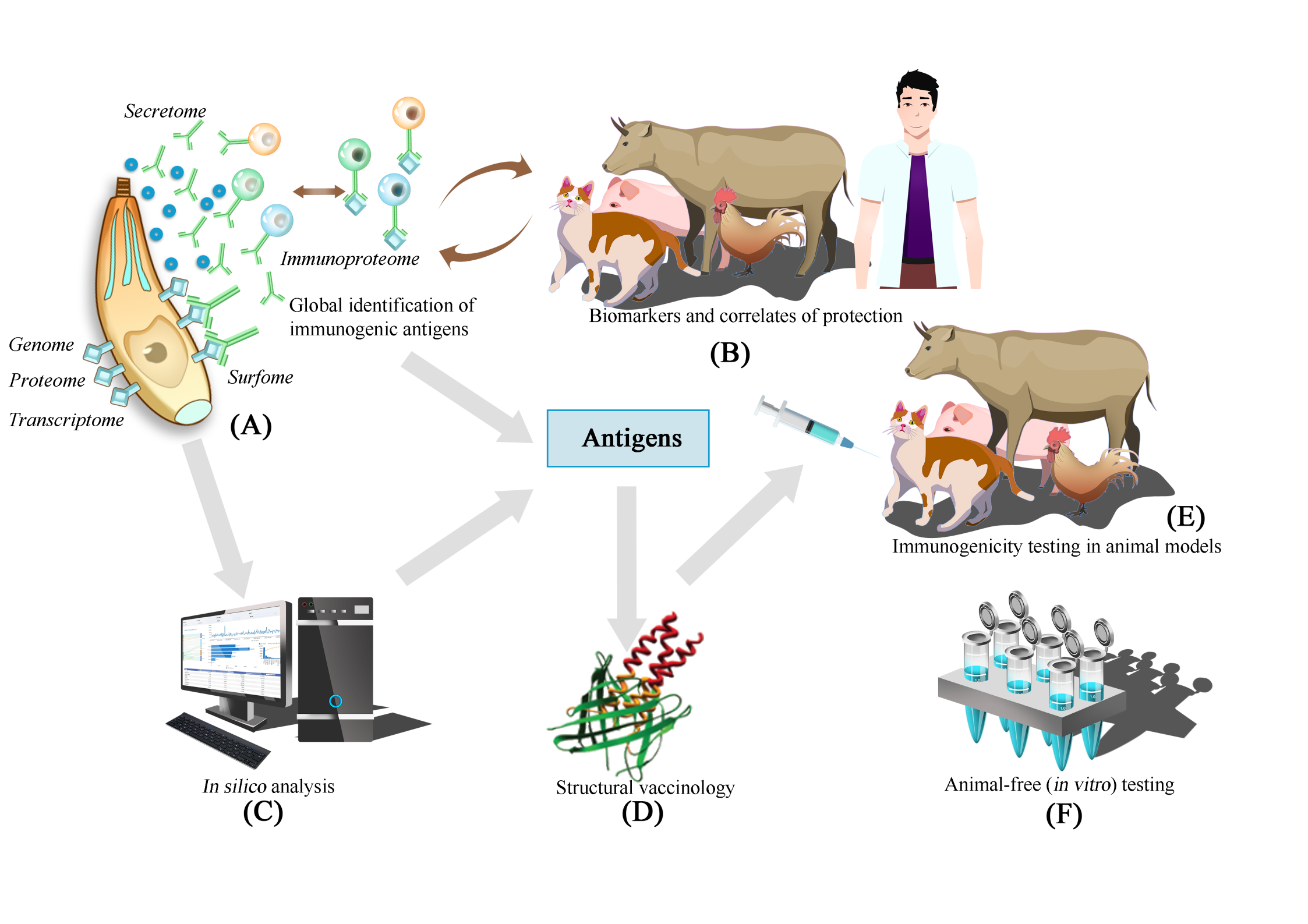

Secretome
Immunoproteome
Global identification of immunogenic antigens
Genome
Proteome
Transcriptome
Surfome
(A)
Biomarkers and correlates of protection
(B)
Antigens
Immunogenicity testing in animal models
(E)
In silico analysis
(C)
Structural vaccinology
(D)
Animal-free (in vitro) testing
(F)

Table 1. Selected List of Attenuated Live Vaccines Tested Against Experimental *Toxoplasma gondii* Infection

Targeted gene	Parasite strain	Dosage	Animal model	Spectrum of protective efficacy	Refs
CPSII	RH	10^7 tachyzoites	BALB/c, C57BL/6 mice	Acute and chronic infection	11, 77
MIC1-3	RH	20 tachyzoites	Swiss OF1 mice	Acute, chronic and congenital infection	78
OMPDC	RH, Pru	10^7 tachyzoites	C57BL/6 mice	Acute and chronic infection	79, 80
AMA1	RH	10^6 tachyzoites	BALB/c, C57BL/6, CD-1 mice	Acute and chronic infection	81
PTS	RH	5×10^3 tachyzoites	C57BL/6 mice	Acute and chronic infection	82
GRA17	RH	5×10^4 tachyzoites	Kunming mice	Acute, chronic and congenital infection	83
CDPK2	Pru	5×10^2 tachyzoites	Kunming mice	Acute, chronic and congenital infection	84
MIC1-3	RH	10^5 tachyzoites	Sheep	Congenital infection	85

Highlights

There is a compelling need to develop a safe and effective toxoplasmosis vaccine.

Successful vaccination of domestic cats is the key step in reducing *T. gondii* transmission to humans and food-producing animals.

An effective toxoplasmosis vaccine must be able to induce both humoral and cellular immune responses, directed against multiple different proteins, at different stages of the parasite life-cycle.

Live attenuated *T. gondii* strains offer good protection against toxoplasmosis, but the possibility of reversion to the virulent type remains possible.

There is a need to identify more immunogenic antigens, adjuvants and antigen delivery systems together with defining robust immuno-correlates of protection.

Having a standardized protocol for assessment of vaccine efficacy can facilitate the synergy between the results obtained by various research groups.

"Omics" technologies have revolutionized our understanding of the pathophysiology of toxoplasmosis, paving the way for development of a safe and effective vaccine.

Outstanding Questions

Can candidate vaccine antigens identified in mice protect humans or food-producing animals?

Can a humanized animal model predict protective efficacy of toxoplasmosis vaccine reflective of human responses?

What can be done to improve the efficacy and safety of live attenuated vaccines?

How many immunogenic candidates remain to be discovered and how can we modify existing antigens to make them more immunogenic?

How do coinfections with other pathogens influence the efficacy of vaccination against toxoplasmosis?

How many mechanisms are used by *T. gondii* to evade host immune responses and establish persistent infection?

What new technologies are having the greatest impact on *T. gondii* immunobiology research, and why?