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13. ABSTRACT *(Maximum 200 Words)*
We are testing the hypothesis that a model parasite gene therapy vector can be genetically altered to safely, specifically and effectively target breast cancer cells *in vitro* and *in vivo*. The primary purpose and scope of this IDEA award project is to experimentally examine approaches to safely target the *Toxoplasma gondii* parasite gene therapy vector to breast cancer tissue using *in vitro* and *in vivo* models. We found that cytosine deaminase (CD) and thymidine kinase (TK) markers expressed in *T. gondii* produce a significant bystander killing effect on both human fibroblasts and SKBR3 tumor cells *in vitro*. We report the construction of a highly attenuated uracil auxotrophic *T. gondii* parasite strain for safely targeting gene therapy to breast cancer. Using trifunctional enzyme expression plasmids we co-expressed TK and CD markers in transgenic *T. gondii*. An approach for selectively targeting the TK and CD transgenes to HER2/neu overexpressing cells was examined. We found that a bi-specific antibody with specificity to Her2/neu and a mjor *T. gondii* surface antigen provided a small but detectable increase in the fraction of Her2/neu cells infected by the targeting parasite, and sensitization of tumors cells to killing by ganciclovir or 5-fluorocytosine therapy. Improved technology will need to be developed to improve the specificity of the targeting strategy in this model.

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Introduction

We are testing the hypothesis that a model parasite gene therapy vector can be genetically altered to safely, specifically and effectively target breast cancer cells *in vitro* and *in vivo*. We have developed a novel strategy to establish the protozoan parasite *T. gondii* as the next generation vector for breast cancer gene therapy. The significant innovative aspect of this approach is the promise of this strategy to deliver a novel vector for breast cancer gene therapy that is superior to the current vectors under current development and refinement. The primary purpose and scope of this IDEA award project is to experimentally examine approaches to target the *Toxoplasma gondii* parasite gene therapy vector to breast cancer tissue using *in vitro* and *in vivo* models.

Body

We have attempted several types of plasmid constructs for developing a cytosine deaminase (CD) and thymidine kinase (TK) gene fusion construct that will stably express both enzyme activities in *T. gondii*. Direct fusions of CD-TK or TK-CD failed to yield expression of both enzyme activities. These experiments were complicated by the absence of a positive selectable marker in transfecting these constructs into *T. gondii*. Thus we are unsure if the difficulty is the lack of selection, the failure of the construct to express both enzyme activities, the overall efficiency, or a combination of these potential problems. It should be noted that in theory, it should be possible to co-express separate single TK and single CD gene constructs in the same transgenic parasite. However, in the absence of a selection procedure it would require a significant effort to score, screen and empirically obtain clones with high sensitivity to the two prodrugs in use.

In a second approach we attempted to construct quadfunctional DHFR-TK-CD-TS plasmids based on our success at expressing the trifunctional DHFR-TK-TS, or DHRF-CD-TS enzymes. This approach did work....albeit with limited success due to the apparent low expression of the markers from this plasmid construct. To circumvent these problems we have solved the problem posed by Task 1 by co-transfecting our successful trifunctional DHFR-TK-TS and DHFR-CD-TS plasmids into *T. gondii*. Selection in pyrimethamine produced transgenic parasites and upon subcloning we found that approximately 20% of clones had acquired sensitivity to both 50 μM 5-fluorocytosine and 5 μM ganciclovir.

We have now examined the bystander effect for transgenic *T. gondii* expressing thymidine kinase (TK) or bacterial cytosine deaminase in SKBR3 cells that overexpress HER2/neu. Assessment by the DEAD red cell assay and by Trypan blue exclusion assays showed that infection of approximately 10 to 20% of the cell population with TK or CD expressing *T. gondii*, followed by treatment of the cell culture with 5 μM ganciclovir (Fig. 1) , or 50 μM 5-fluorocytosine (Fig. 2), respectively, resulted in greater than 90% killing of the uninfected cell population after a 24 h incubation.

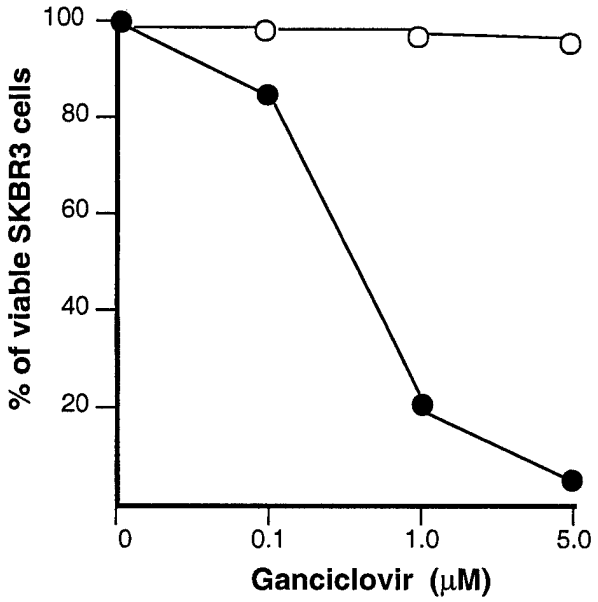

Figure 1. SKBR3 cells were infected at a MOI of 0.2 with TK-transgenic *T. gondii* and exposed at 4h to various concentrations of ganciclovir. 24 h later cultures were assayed for viability by DEAD red or trypan blue exclusion.

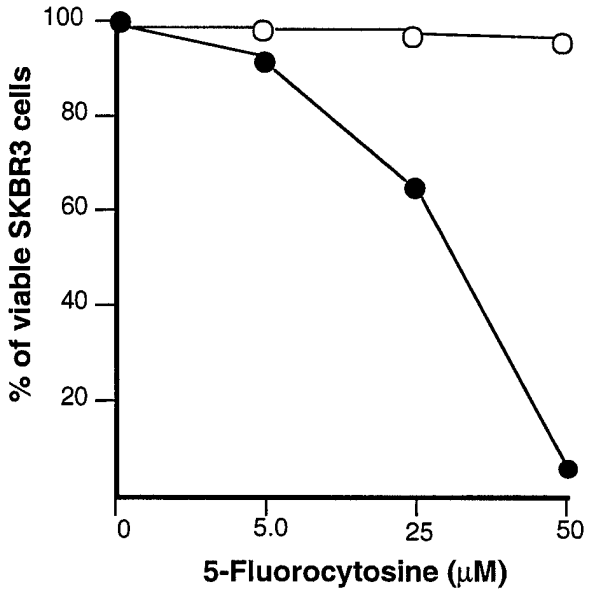

Figure 2. SKBR3 cells were infected at a MOI of 0.2 with CD-transgenic *T. gondii* and exposed at 4h to various concentrations of 5-fluorocytosine. 24 h later cultures were assayed for viability by DEAD red or trypan blue exclusion.

We have obtained transgenic *T. gondii* that stably express both enzyme activities in *T. gondii*. These parasites are sensitive to both prodrugs (ganciclovir and 5-fluorocytosine). As expected, we find that the bystander effect is greater in the parasites which express both suicide enzymes. We found that comparable killing could be obtained at reduced MOI's using dual treatment. Only 5 to 10% of the SKBR3 cells need to be infected in order to kill >90% of the cells after treatment with ganciclovir and 5-fluorocytosine in the *in vitro* assays.

Related to the task of constructing transgenic *T. gondii* expressing both TK and CD is the development of a "safe" or "safer" strain of *T. gondii* which could be more appropriately used and controlled in the *in vitro* and *in vivo* targeting studies. We report that we have created a uracil auxotroph mutant *of T. gondii* that has properties which make it an ideal vector for targeting cancer cells. This mutant is completely attenuated in both immune competent and immunocompromised mice [1]. The mutant invades host cells normally and will express proteins for several days; however, this mutant does not replicate *in vitro* or *in vivo* in the absence of uracil supplementation (Appendix reprint). We developed of the

expression of TK and CD markers, at useful levels, in the uracil auxotroph mutant strain of *T. gondii.*

We have made significant progress on examining the proposed targeting strategy. A bi-specific targeting antibody was constructed. One arm of the antibody recognizes Her2/neu, while the other arm of the bi-specific antibody recognizes P30, the major surface antigen of *T. gondii.* In mixed culture experiments we prelabeled SKBR3 cells then mixed non-tumor HFF cells into the culture in a 50:50 ratio. Cultures were infected with bi-specific antibody opsonized, or unopsonized,TK and CD transgenic *T. gondii* at a MOI of 0.2 and the fractions of infected SKBR3 or HFF cells was determined at 1 h or 6 h by fluorescent and light microscopy. At 1 h, we observed that, approximately 62% of infected cells were SKBR3 (Her2/neu+), while 38% of infected cells were HFF (Her2/neu-). In unopsonized infected cultures approximately 53% of infected cells were SKBR3 and 47% of infected cells were HFF. At 6h we observed similar results. These data suggest that opsonization provides a short window of slightly enhanced targeting of *T. gondii* to Her2/neu positive tumor cells.

Key Research Accomplishments to date

- Co-expression of cytosine deaminase and thymidine kinase in *T. gondii*
- TK transgenic *T. gondii* exhibit the bystander effect with ganciclovir treatment
- CD transgenic *T. gondii* exhibit the bystander effect with 5-fluorocytosine treatment
- CD and TK transgenic *T. gondii* exhibit the bystander effect on HER2/neu overexpressing SKBR3 tumor cells
- A uracil auxotroph mutant strain of *T. gondii* was developed and found to be completely avirulent in both immune competent as well as in severely immune devifient mice
- High avidity/affinity IgG antibody to the major surface protein of *T. gondii* was obtained and purified
- Bi-specific antibody to Her2/neu and *T. gondii* P30 surface antigen was engineered and validated.
- Opsonization with bi-specific antibody was shown to slightly, but detectably enhance the targeting of TK or CD transgenic *T. gondii* selectively to Her2/neu overexpressing tumor cells.

Reportable Outcomes

Fox, BA & Bzik DJ. (2002) De novo pyrimidine synthesis is required for virulence of *Toxoplasma gondii*. **Nature** 415: 926-929.

Conclusions

Both the thymidine kinase and cytosine deaminase genetic markers have been expressed in transgenic *T. gondii* and express the "bystander" effect in both human fibroblast and HER2/neu overexpressing SKBR3 cell lines. That is, that neighboring cells to those expressing CD or TK can be killed by the toxic products formed following treatment with 5-fluorocytosine or ganciclovir, respectively. Transgenic *T. gondii* have been obtained that co-express the TK and CD transgenes. A targeting strategy employing bispecific antibody or stable genetic equivalents was developed to target the TK and CD activities to breast cancer tissue(s) *in vitro* and *in vivo*. Targeting was determined, however, the model was not robust and improved technologies would need to be developed to improve the efficacy of this approach. A *T. gondii* uracil auxotroph was developed and found to be avirulent in immune competent and immune deficient mice. The parasite vector may be an ideal vector, or ideal prototype for our targeting strategies. This avirulent vector should provide a "safer" vector for use in the targeting studies, as well as a parasite that may be more useful in a variety of ways. This attenuated mutant is important when considering the *in vivo* studies. We can now perform studies in immunocompromised mice, or normal mice, and we can have significant latitude on timing of drug delivery or targeting effects. In addition, studies at direct inoculation of mice tumors is now possible using this avirulent mutant.

References

1. Fox, BA & Bzik DJ. (2002) De novo pyrimidine synthesis is required for virulence of *Toxoplasma gondii*. **Nature** 415: 926-929.

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1. Fox, BA & Bzik DJ. (2002) De novo pyrimidine synthesis is required for virulence of *Toxoplasma gondii*. **Nature** 415: 926-929.

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Appendices

De novo pyrimidine biosynthesis is required for virulence of *Toxoplasma gondii*

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Toxoplasma gondii **is a ubiquitous protozoan parasite that is responsible for severe congenital birth defects and fatal toxoplasmic encephalitis in immunocompromized people[1]. Fundamental aspects of obligate intracellular replication and pathogenesis are only now beginning to emerge for protozoan parasites. *T. gondii* has a fragmented pathway for salvaging pyrimidine nucleobases derived from the parasite or host cell, and this limited pyrimidine salvage capacity is funnelled exclusively through uracil phosphoribosyltransferase[2,3]. Disrupting the function of this enzyme does not affect the growth of *T. gondii* tachyzoites[4], which suggests that the *de novo* pyrimidine biosynthesis pathway may be necessary for growth. We have examined the virulence of *T. gondii* mutants that lack carbamoyl phosphate synthetase II (uracil auxotrophs) to determine whether *de novo* pyrimidine biosynthesis is required *in vivo*. Here we show that *T. gondii* uracil auxotrophs are completely avirulent not only in immune-competent BALB/c mice but also in mice that lack interferon-γ. A single injection of the uracil auxotroph into BALB/c mice induces long-term protective immunity to toxoplasmosis. Our findings indicate the significance of the *de novo* pyrimidine biosynthesis pathway for the virulence of parasitic protozoa, and suggest routes for developing vaccines and chemotherapy.**

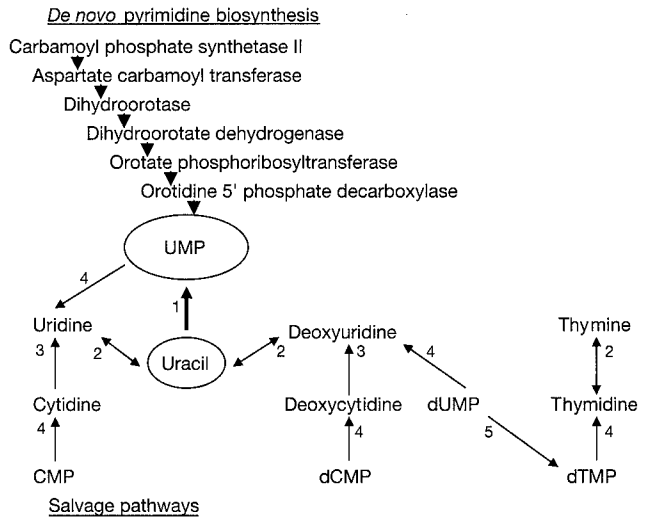

Figure 1 Pyrimidine salvage and *de novo* pyrimidine biosynthesis pathways in *T. gondii*. Pathways that produce UMP, the precursor of all pyrimidines used by *T. gondii*, are shown. The six steps of the *de novo* pyrimidine biosynthesis pathway and their corresponding enzymes are shown. Salvage pathway[2,3] steps that have been detected in *T. gondii* are shown in solid lines. Double arrowheads indicate that the activity is capable of both conversions. Enzyme activities: uracil phosphoribosyltransferase (1); nucleoside phosphorylase (2); nucleoside deaminase (3); nucleoside 5′-monophosphate phosphohydrolase (4); and thymidylate synthase (5; part of the bifunctional DHFR-TS[6,19]). Thymidylate synthase is not considered a salvage enzyme; it is a key enzyme in the interconversion of pyrimidine nucleotides. Many potential salvage enzyme activities have not been detected in *T. gondii*[3].

Because of its genetic accessibility[5–7] and natural virulence in mice, *T. gondii* is an excellent model for the discovery and evaluation of auxotrophic mutants that capitalize on differences in metabolism between protozoan parasites and their hosts. Like its host, *T. gondii* has an intact pathway for *de novo* pyrimidine biosynthesis, but differs in that it has only a limited pyrimidine salvage pathway (Fig. 1). In *T. gondii*, as in other parasites of the phylum Apicomplexa, the key regulatory enzyme of *de novo* pyrimidine biosynthesis is carbamoyl phosphate synthetase II (CPSII), a parasite enzyme that has distinctive properties compared with the CPSII activity of the mammalian host cell[8]. In addition, apicomplexan parasites have a monofunctional CPSII domain fused onto the same polypeptide as the glutamine amidotransferase domain, which produces an enzyme architecture that is not observed in bacteria, fungi or mammals[9,10]. These unique features of *T. gondii* CPSII make this activity an attractive target to disrupt to obtain null mutants.

We cloned a 6.6-kilobase (kb) *Hin*dIII *CPSII* genomic DNA fragment from strain RH that contains exons with significant similarity to CPSII from fungi, plants and mammals (Supplementary Information). The single chromosomal copy of this 6.6-kb *Hin*dIII fragment was disrupted to obtain uracil auxotroph strains *cps1-1* and *cps2-1*. Neither strain had detectable CPSII activity compared with the activity measured in the wild-type RH strain (57 nmol h^{-1} mg^{-1}). Without uracil supplementation the parasite invaded normally, but it failed to replicate once intracellular. Essentially, no growth was observed at uracil concentrations lower than 20 μM (Fig. 2a). Normal parasite growth was seen between about 0.2 and 0.6 mM uracil; however, growth was suppressed in uracil concentrations higher than 0.8 mM, which indicates that normal regulation of pyrimidine pools or salvage mechanisms may be disrupted in the auxotrophic mutants. The ability of added uracil to rescue this nonreplicating uracil auxotroph declined with time (Fig. 2b).

We verified that disruption of the *CPSII* gene was the genetic lesion responsible for the uracil auxotrophy of *cps1-1* and *cps2-1*. Transfection of *cps1-1* and *cps2-1* with a linearized 6.6-kb *CPSII Hin*dIII fragment from the wild-type strain RH rescued significant numbers of parasites (scored as plaques), which then grew normally in the absence of uracil (Table 1). Rescued isolates (*cps1-1rs*) all had the wild-type RH CPSII genotype and phenotype on the basis of Southern blot (data not shown), CPSII activity (61 nmol h^{-1} mg^{-1} protein), and growth responses in uracil (Fig. 2a). In contrast, transfection of *cps1-1* or *cps2-1* with only plasmid DNA, or with the Δ6.6-kb *CPSII Hin*dIII plasmid containing an internal 1.1-kb *Bam*HI-generated deletion failed to rescue any additional plaques in the absence of uracil (Table 1).

We tested the virulence of the *T. gondii CPSII* gene knockout mutants in a BALB/c mouse model of lethal toxoplasmosis. The parental RH strain is hypervirulent in all mice strains, with an estimated 100% lethal dose of fewer than 10 parasites[11]. Mice injected intraperitoneally with only a low dose of the parental wild-type RH strain succumb rapidly to the infection (median survival of 9 d). In contrast, mice injected with the *cps1-1* or *cps2-1* mutant survived the infection at inoculating doses of 10^3, 10^4, 10^5, 10^6 (data not shown) and 10^7 tachyzoites (Fig. 3a). Parasites could not be recovered from the intraperitoneal cavity of mice at 3 weeks after inoculation. Mice inoculated with the uracil auxotroph mutants survived longer than 12 months with no evidence of any phenotype of parasite persistence (tachyzoites or brain cysts). In contrast, parasites such as *cps1-1rs*, which were rescued by transfection of the uracil auxotroph mutants with the wild-type 6.6-kb *CPSII Hin*dIII fragment (Table 1), were highly virulent in BALB/c mice (Fig. 3a).

We examined the virulence of *cps1-1* in homozygous interferon-γ (IFN-γ) knockout (*gko*) mice on a BALB/c background. Because the cytokine IFN-γ is necessary for host control of *T. gondii* infection, IFN-γ knockout mice rapidly succumb to toxoplasmosis even after infection with normally avirulent strains[12]. As expected, *gko* mice rapidly succumbed to the RH strain (median survival of 9 d; Fig. 3b). Unexpectedly, *gko* mice injected with the *cps1-1* mutant survived the

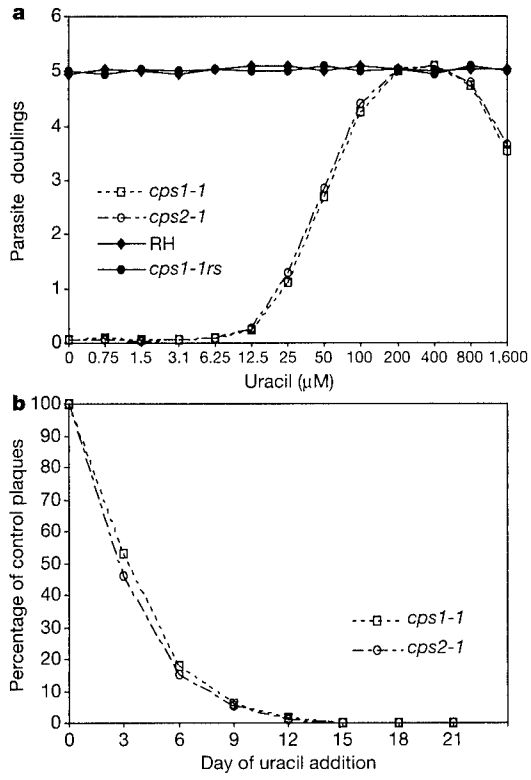

Figure 2 Growth and viability of uracil auxotrophs of *T. gondii*. **a**, Growth rate of uracil auxotroph mutants was measured as a function of the uracil concentration of the infection medium. Parasites were allowed to invade HFFs for 3 h, the cells were then washed and incubated with infection medium containing various concentrations of uracil. Host cells were microscopically inspected 36 h later to identify individual vacuoles containing parasites. The average number of tachyzoites per vacuole was determined by scoring 50 independent vacuoles for each data point. The average number of parasites per vacuole was converted into 'parasite doublings' (for example, 1 parasite doubling = 2 parasites per vacuole, and 5 parasite doublings = 32 parasites per vacuole). Growth responses for auxotrophic mutants *cps1-1* and *cps2-1* are compared with that of the RH parent and a representative clone (*cps1-1rs*) of a rescued parasite obtained after transfection of *cps1-1* with the wild-type 6.6-kb *CPSII Hin*dIII fragment (see Table 1). **b**, Ability of added uracil to rescue the nonreplicating intracellular parasites was determined by a plaque assay (Methods). For uracil addition on day 15, 18 and 21, plaques from the auxotrophic mutants (*cps1-1* and *cps2-1*) were reduced to about 0.2%, 0.02% and 0.003%, respectively, of the control p.f.u.

Table 1 Frequency of rescue of uracil auxotroph mutants after transfection of plasmid DNA

Plasmid DNA	Parasite	p.f.u. rescued (× 10^{-7})
6.6-kb *CPSII Hin*dIII	*cps1-1*	24
6.6-kb *CPSII Hin*dIII	*cps2-1*	21
Δ6.6-kb *CPSII Hin*dIII	*cps1-1*	< 1
Δ6.6-kb *CPSII Hin*dIII	*cps2-1*	< 1
pBluescript	*cps1-1*	< 1
pBluescript	*cps2-1*	< 1
Mock	*cps1-1*	< 1
Mock	*cps2-1*	< 1

Auxotrophic mutants were transfected with plasmid DNA and plaques (p.f.u.) were scored in uracil-free medium (Methods). Either *Hin*dIII-digested plasmid DNA (20 μg) was transfected, or no plasmid was transfected (mock). Parasites (1.2×10^7) were transfected as indicated and were cultured in a single large HFF flask. Plaques were scored 8 d after transfection by visual and microscopic inspection of plaques. Data shown are the mean from three independent experiments. In other experiments, the spontaneous *in vitro* reversion frequency of *cps1-1* and *cps2-1* was determined to be about 2.8×10^{-7}.

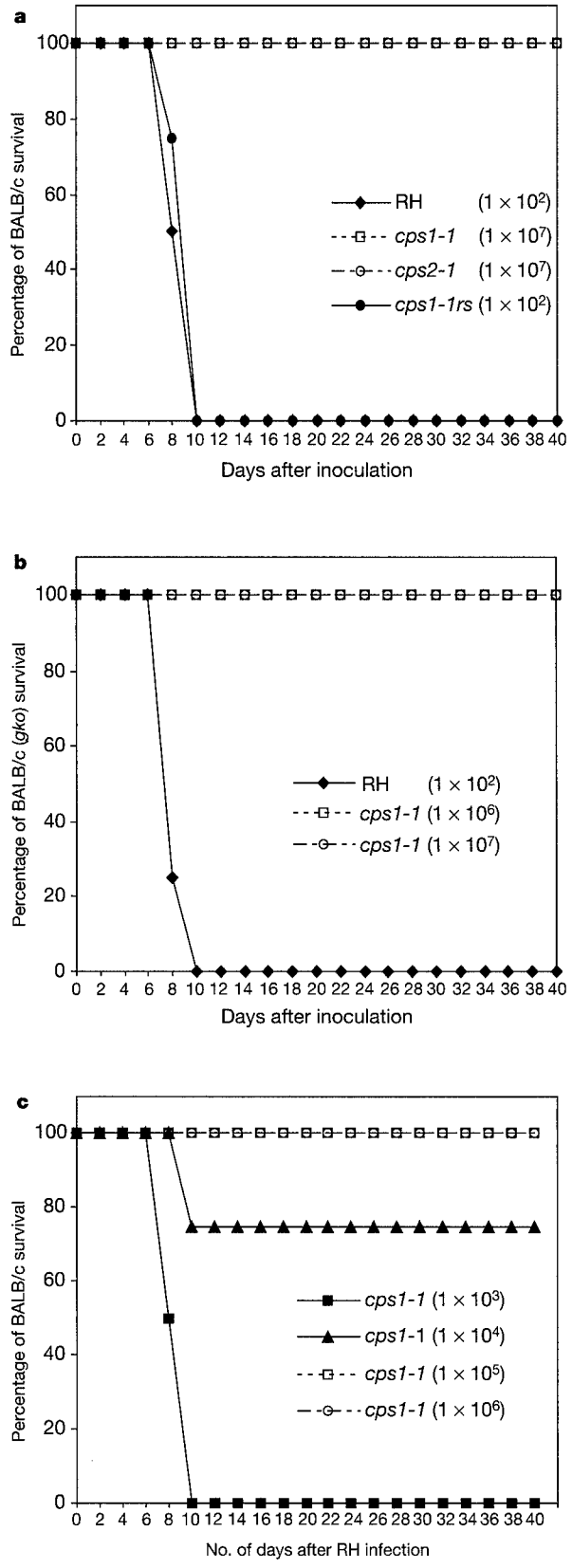

Figure 3 Uracil auxotroph mutants are avirulent in mice and confer long-term protective immunity. **a**, Tachyzoites of the indicated strains were injected i.p. into BALB/c mice ($n = 4$) and monitored for more than 40 d (Methods). The inoculation doses are shown. **b**, Tachyzoites of the indicated strains were injected i.p. into homozygous IFN-γ knockout (BALB/c background) mice ($n = 4$) and monitored for more than 40 d. The inoculation doses are shown. **c**, Various doses of tachyzoites of *cps1-1* were injected i.p. into BALB/c mice ($n = 4$). Mice were challenged 40 days later with 200 p.f.u. of wild-type strain RH tachyzoites administered by the i.p. route.

infection at inoculating doses of 10^3, 10^4, 10^5 (data not shown), 10^6 and 10^7 tachyzoites (Fig. 3b). The *cps1-1*-inoculated *gko* mice showed long-term survival (> 6 months), with no apparent phenotype of parasite persistence.

We examined whether the avirulent uracil auxotrophic mutants could protect mice from a lethal toxoplasma challenge infection. Groups of BALB/c mice were injected once with various doses of *cps1-1* tachyzoites. These mice were injected 40 d later with a lethal challenge dose of strain RH. Parasite doses greater than 10^4 *cps1-1* tachyzoites were highly effective in inducing a long-term protective immunity in BALB/c mice (Fig. 3c).

Our data indicate that the obligate intracellular *T. gondii* parasite may have evolved to have a strict reliance on its own *de novo* pyrimidine biosynthesis pathway *in vivo*. The parasite's pyrimidine salvage pathway cannot apparently salvage significant amounts of pyrimidines from its host cell. This adaptation of the protozoan parasite may have arisen as a consequence of the apparent low availability of pyrimidines in animal tissues[13,14]. Notably, the pyrimidine starvation phenotype of the *cps1-1* and *cps2-1* mutants results in a complete block of parasite replication *in vitro* (Fig. 2) and *in vivo* (Fig. 3). Identifying compounds that limit the *de novo* biosynthesis of pyrimidines in *T. gondii* might be a route for antiparasite drug design. The auxotrophic mutants described here are remarkably avirulent and can also induce long-term protective immunity to toxoplasmosis. To our knowledge, these are the first reported mutants of *T. gondii* that do not kill *gko* mice[15]. *T. gondii* elicits a strong cell-mediated immune response that controls its own growth and that can stimulate nonspecific resistance to unrelated pathogens and tumours[16,17]. Consequently, these auxotrophic mutants may offer even greater promise as a strategy for vaccine development. Developing pyrimidine auxotrophs in other protozoan parasites might verify drug targets in *de novo* pyrimidine biosynthesis and might also provide a broad-ranging approach to obtaining protozoan parasite mutants that are severely attenuated in their virulence. □

Methods

Parasite culture and phenotypic analysis

Human foreskin fibroblasts (HFFs) were cultured in EMEM medium in 10% fetal bovine serum (FBS) at 37 °C in 95% air/5% CO_2. We cultured parasites in EMEM in 1% FBS at 37 °C in 95% air/5% CO_2. Plaques were visualized by fixing HFF monolayers in 50% (v/v) methanol and 7% (v/v) glacial acetic acid, and staining with saturated Coomassie blue dissolved in fixative. The CPSII enzyme assays were done as described[8]. We isolated parasite DNA and carried out Southern analysis as described[18].

To determine whether added uracil could rescue intracellular mutants, replicate sets of flasks of confluent HFF cells were inoculated with 2×10^5, 2×10^4, 2×10^3 or 2×10^2 plaque forming units (p.f.u.) of uracil auxotroph tachyzoites. After a 3-h incubation at 37 °C in medium lacking uracil for attachment and invasion, the remaining extracellular parasites were removed ($t = 0$) by washing the monolayer with PBS, and cultures were incubated in medium lacking uracil. At various times, medium was removed from duplicate sets of the inoculated flasks and was replaced with medium containing 0.2 mM uracil. We stained monolayers and scored plaques by visual inspection 8 d after uracil addition.

Mutant construction and analysis

A genomic DNA fragment of the *T. gondii CPSII* gene (strain RH) was cloned using a polymerase chain reaction (PCR) signature homology strategy. A *CPSII* PCR DNA fragment of the expected size of 450 base pairs (bp) was isolated and subcloned into pBluescript. We examined 30 individual clones, each of which had an identical sequence with a high amino-acid similarity to other CPSII species. We used the 450-bp *CPSII* fragment to identify a single-copy 6.6-kb *Hin*dIII fragment of genomic DNA by Southern blot analysis and to screen a *T. gondii Hin*dIII plasmid library constructed in pBluescript to obtain the 6.6-kb *CPSII Hin*dIII clone. The Δ6.6-kb *CPSII Hin*dIII plasmid was constructed by creating a 1.1-kb *Bam*HI deletion (deleting bp 2,261 to 3,347) in the central part of the 6.6-kb *CPSII Hin*dIII clone.

We made the targeting plasmid by inserting a dihydrofolate reductase/herpes simplex virus thymidine kinase/thymidylate synthase positive/negative selection marker[18] adjacent to the 3′ *CPSII* sequences in the Δ6.6-kb *CPSII Hin*dIII plasmid. This plasmid was integrated into the *CPSII* locus by transfection[18] of strain RH and selection in pyrimethamine with uracil supplementation. We screened clones derived from this selection for ones that grew normally in medium with 0.2 mM uracil, but had disrupted CPSII activity and could not plaque in medium lacking uracil. These *cps1* and *cps2* mutants were then subjected to negative selection in ganciclovir in the presence of uracil. This strategy

selected parasites that lost expression of the integrated thymidine kinase marker[18]. We subcloned ganciclovir-resistant and pyrimethamine-sensitive parasites from this selection to produce *T. gondii* strains, *cps1-1* and *cps2-1*. Uracil auxotrophs *cps1-1* and *cps2-1* have two tandem copies of the targeting plasmid integrated into the *CPSII* locus. Only the *CPSII* locus was disrupted, and integration was achieved by homologous recombination in *CPSII* sequences on the 5′ side of the *Bam*HI sites of the 6.6-kb *CPSII Hin*dIII fragment (data not shown). We maintained the uracil auxotrophs in culture in medium supplemented with 0.2 mM uracil.

Murine virulence assay

We obtained tachyzoites by allowing infected HFF monolayers to lyse completely. Tachyzoites were purified by filtration through sterile 3-μm nuclepore membranes, washed in PBS and collected by centrifugation. We resuspended tachyzoites pellets in PBS and counted them under the microscope. Tachyzoites were injected intraperitoneally (i.p.) in 0.2 ml into mice aged 6–8 weeks. The actual p.f.u. in the inoculum was determined by plaque assay. In all of the mouse injection experiments and for all of the parasite strains tested, the p.f.u. to parasite ratio was between 0.4 and 0.6. We used four mice per parasite dose with each strain. Experiments with groups of mice were repeated twice. Mice were monitored for 40 d, and in some experiments for more than 1 year. We cared for mice according to NIH guidelines.

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Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to D.J.B. (e-mail: David.J.Bzik@Dartmouth.edu). The sequence of the 6.6-kb *CPSII Hin*dIII fragment has been deposited with GenBank (accession code AY059630).