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Toxoplasma secreting Cre recombinase for analysis of host-parasite interactions

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Abstract

We describe the use of site specific recombination to study the host-parasite interactions of *Toxoplasma gondii*. We present a *Toxoplasma* strain that efficiently injects a Cre fusion protein into host cells. In a Cre-reporter cell line, a single parasite invasion induced Cre-mediated recombination in 95% of infected host cells. By infecting Cre reporter mice with these parasites, host cell infection could also be monitored *in vivo*.

Site specific recombination (SSR) is a well established system for genetically modifying targeted cells and has been used to spatially and temporally control the expression of selected genes. SSR should offer the same advantages to the study of intracellular pathogens as it does to the study of developmental or cancer biology[1,2]. Engineered intracellular pathogens that introduce Cre into host cells would permit the use of SSR to examine the consequences of removing specific host genes only in the infected cells. We sought to extend the use of the Cre-*loxP* system to studying the host-pathogen interactions of the eukaryotic parasite *Toxoplasma gondii* by creating a *Toxoplasma* strain that could inject Cre into host cells.

Toxoplasma is an obligate intracellular pathogen that has unique organelles – the rhoptries and dense granules – that are known to secrete effector proteins into host cells during invasion[3–5]. It is a natural pathogen for mice which thus serve as an excellent model for the

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Author's Contributions: A.E.F. and A.A.K. generated the SeCreEt parasites. A.A.K. analyzed the SeCreEt parasites. M.B.L. developed the plasmid for making toxofilin a partner for injecting fusion proteins into host cells. O.K. created the Cre-reporter cell line. H.M.B. oversaw the work of O.K. and J.C.B. oversaw the work of A.A.K., A.E.F., and M.B.L.

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study of human infection[6,7]. Hence, the combination of a Cre-secreting strain of *Toxoplasma* and the many mouse strains that have already been engineered to carry *loxP*-flanked ("floxed") genes represents a powerful tool for studying *Toxoplasma*-host interactions *in vitro* and *in vivo*.

A *Toxoplasma* strain that secretes Cre has been previously described[8] but the released Cre did not efficiently enter host cells: only 1–2% of infected reporter cells showed evidence of Cre-mediated recombination. We engineered a fusion between Cre and toxofilin (Fig. 1a), a rhoptry protein[3,9] that is introduced into host cells during invasion[10]. We electroporated parental parasites with a plasmid encoding HA-tagged toxofilin-Cre and a separate cassette for a drug-selectable marker[11], and selected clones by limiting dilution.

We confirmed appropriate organelle targeting of toxofilin-Cre in individual clones by immunofluorescence microscopy, for which we co-stained the parasites for HA and ROP2/4, a well studied rhoptry protein (Fig. 1b). We established the integrity of the fusion protein by probing Western blots of whole parasite lysates using anti-HA antibodies (Fig. 1c) which revealed a protein of the appropriate size (~65 kDa). Using anti-toxofilin antibodies, the expression level of the fusion protein was found to be approximately equivalent to that of the endogenous protein (data not shown). We selected a clone with all of these properties for further characterization. We refer to this parasite strain as the SeCreEt (Secreted Cre, Epitope-tagged) strain.

To develop Cre-reporter cells, we transduced immortalized mouse fibroblast precursor cells with a retrovirus derived from a Cre reporter plasmid (Fig. 2a). The reporter cells constitutively express DsRed unless Cre-mediated recombination occurs, in which case eGFP is expressed. We isolated clones stably expressing red fluorescent protein (DsRed) by fluorescence-activated cell sorting (FACS).

To evaluate the efficiency and time course of Cre-mediated recombination by SeCreEt parasites, we synchronously infected Cre reporter cells, fixed the infected cultures at 2, 4, 6, 10, 18, 24 and 34 hours after infection, and then examined the cells for eGFP expression. SeCreEt parasites, but not parental parasites, triggered Cre-mediated recombination leading to eGFP expression in infected reporter cells (Fig. 2b,c); staining for the *Toxoplasma* protein SAG-1 was used to confirm infection of cells. In fact, invasion by a single SeCreEt parasite, as defined by a single parasitophorous vacuole in the host cell, led to eGFP expression in 95% ± 2% s.e.m. ($n = 3$) of infected reporter cells (Fig. 2d) at 24 hours post-infection. In addition, we first noted eGFP expression in ~13% of the infected cells at 4 hours post-infection. As expected, the percentage and intensity of eGFP expression increased over time until reaching a plateau at 18–24 hours (Fig 2d). No eGFP expression was seen in reporter cells incubated with heat-killed SeCreEt parasites, with the lysate of freeze-thaw ruptured SeCreEt parasites, or with media from cultured SeCreEt parasites (data not shown). eGFP expression was also not seen in reporter cells transfected with the toxofilin-Cre plasmid, in which toxofilin:Cre expression is driven by a *Toxoplasma* specific promoter (data not shown). Overall, these data suggest that live SeCreEt parasites introduce toxofilin-Cre at or around the time of invasion and that the fusion protein rapidly and efficiently produces Cre-mediated recombination *in vitro*. Interestingly, we also observed a number of eGFP-positive,

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uninfected cells exclusively in the cultures infected with live SeCreEt parasites. This population of cells could be due to some combination of abortive invasion events and cells that divided after infection (yielding one infected and one uninfected daughter cell). The origin of these cells will require further investigation.

To determine whether the SeCreEt parasites could be used to analyze host-parasite interactions *in vivo*, we utilized a Cre-reporter mouse line that has firefly luciferase in the *Gt(Rosa)26Sor* locus but with a floxed triple polyadenylation site between the firefly luciferase-coding region and the promoter[12]. As a result, luciferase expression should only occur after Cre-mediated recombination. We inoculated these mice intraperitoneally with live SeCreEt parasites, live parental parasites or a lysate of 3.2×10^6 freeze-thawed SeCreEt parasites. We then examined the mice daily by bioluminescence imaging (BLI), as previously described[13]. Infection with live SeCreEt parasites activated the luciferase as early as 5 days post infection (dpi) while infection with the parental strain or the freeze-thaw lysate never yielded a luciferase signal (Fig. 3 and Supplemenatry Fig. 1 online).

To verify that the majority of the luciferase signal coming from the SeCreEt-infected animals was generated from parasite-infected cells, we infected Cre-reporter mice with SeCreEt parasites that also expressed mCherry, collected the peritoneal exudate cells (PECS) at 7 dpi, and then used FACS to separate infected (mCherry-positive) from uninfected cells (mCherry-negative). As anticipated in this acute stage of the disease, the FACS indicated that ~60% of the PECS were infected, which we confirmed by using fluorescence microscopy to inspect the pre-FACS PECS. We performed quantitative PCR on cDNA obtained from infected and uninfected cells, and observed that the infected cell population expressed four times the amount of luciferase mRNA compared to the uninfected population (see Supplementary Fig. 2 online). The fact that we observe luciferase mRNA also in uninfected cells is consistent with the *in vitro* results described above, and suggests that there may also be abortive invasion events and/or division of host cells after infection *in vivo*. Nevertheless, these results clearly indicate that the SeCreEt parasites also function effectively *in vivo*.

There are several advantages to utilizing a secreted Cre-based system to investigate host-parasite interactions of *Toxoplasma*. First, Cre mediated recombination leads to a permanent change in host cells which is particularly germane for organisms such as *Toxoplasma* that have prolonged latent states. Hence, once activated, the reporter gene will be expressed throughout the life of the host cell, allowing for *in vivo* analysis of latent toxoplasmosis at any time point, even months or years, after invasion. Second, SSR allows the detection of miniscule amounts of *Toxoplasma*-derived effector proteins introduced into host cells[14]. Finally, the greatest advantage of the SeCreEt parasites may be their ability to conditionally activate or inactivate individual genes only in cells that have had intimate contact with *Toxoplasma*. This circumvents several problems: it allows the study of essential genes, deletion of which would otherwise lead to embryonic lethality, it circumvents unknown compensatory changes that may result from germline deletions even of non-essential genes, and it restricts gene ablation to predominantly infected cells, which would remain challenging even in conditional or inducible knockouts. Thus the role of a given host gene in the host-parasite interaction can be studied on a cell-by-cell level.

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There are, of course, limitations to this system. First, a small fraction of uninfected cells are permanently genetically modified, as described above. Second, 100% *in vitro* recombination efficiency was never achieved, even when we prolonged the length of time after invasion and increased the multiplicity of infection to 4 (data not shown). Third, an inevitable lag will exist between invasion, excision of the target gene and loss of its pre-existing product through decay. Hence, host processes that are launched immediately and irrevocably upon invasion (e.g., signals detected by the innate immune system) may not be affected by the excision of the triggering gene. Even with these limitations, and in view of the large number of existing floxed cell lines and mice, the SeCreEt parasites are a highly efficient system for detecting infected host cells and for determining the function of a specific host gene at the level of a single infected cell *in vitro* and *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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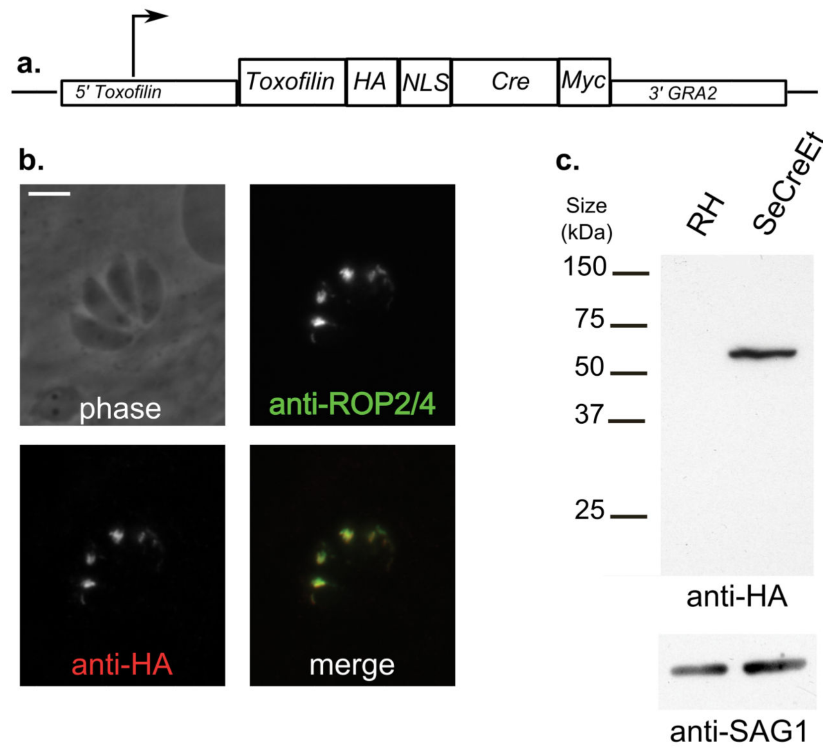

Figure 1.

SeCreEt parasites. (**a**) Schematic of the toxofilin-Cre fusion construct. Arrow indicates the transcription start. (**b**) Phase contrast and fluorescence images of SeCreEt parasites stained as indicated. Scale bar, 5 μm. (**c**) Western Blot of lysate from parental strain (RH) or SeCreEt strain probed anti-HA antibody. SAG-1 serves as the loading control.

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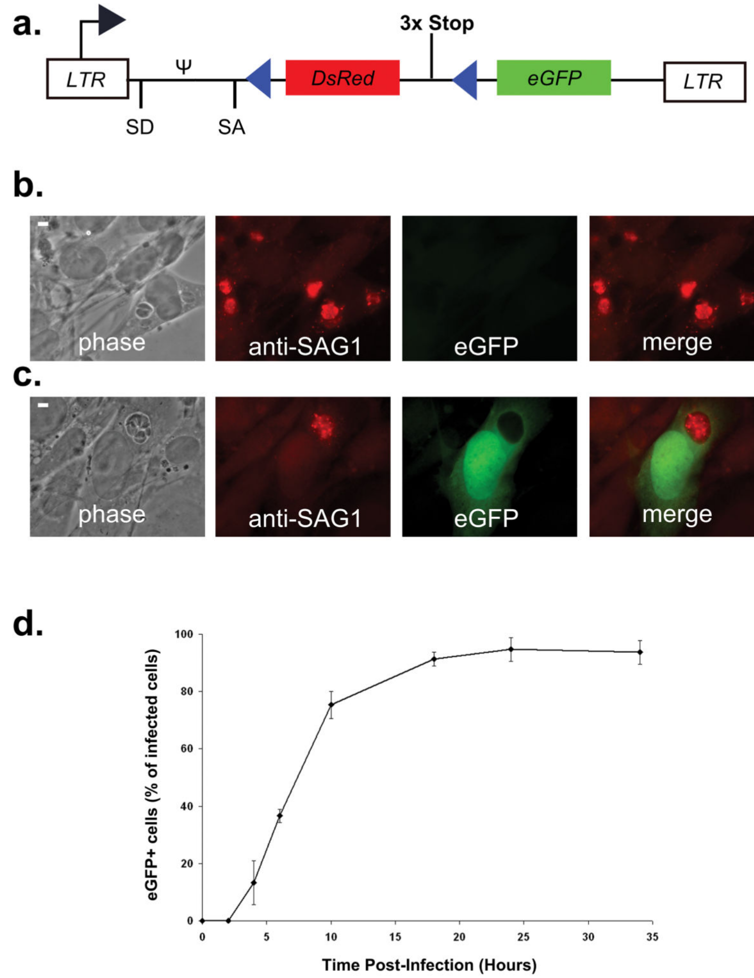

Figure 2.

SeCreEt parasites activate eGFP in reporter cell lines. (**a**) Schematic of the retroviral construct used to produce the Cre-reporter cell line. The DsRed and eGFP coding regions are driven by a promoter in the long terminal repeat (LTR) but have three stop codons separating them, thus preventing expression of eGFP. Arrow indicates transcription start. Blue triangles indicated *loxP* sites and their orientation. SD is splice donor, SA is splice acceptor, and Psi (ψ) is a retroviral packaging signal. (**b**,**c**) Cre-reporter cells 24 hours after infection with parental strain (**b**) or SeCreEt parasites (**c**). Panels from left to right: phase, red shows both DsRed expression and staining with *Toxoplasma*-specific anti-SAG1 antibody, green shows eGFP expression, merge. Scale bars, 5 μm. (**d**) The percentage of SeCreEt parasite-infected Cre-reporter cells that showed eGFP expression is plotted at the indicated time points.

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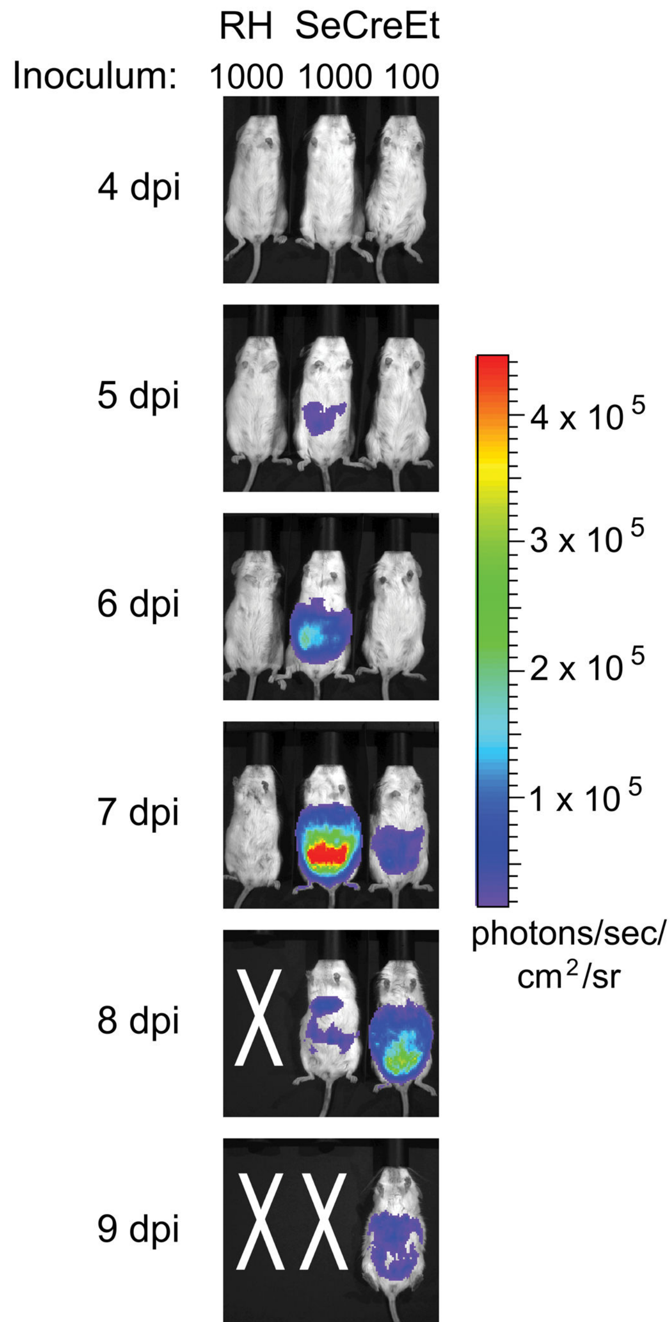

Figure 3.

Analysis of SeCreEt parasites *in vivo*. Bioluminescence imaging of Cre-reporter mice with a "floxed stop luciferase" after inoculation with the indicated numbers of parental (RH) or SeCreEt parasites. Ventral images from 4 to 9 days post-inoculation (dpi) are shown. An "X" indicates no further data could be obtained due to death of the animal.