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Motile invaded neutrophils in the small intestine of *Toxoplasma gondii*-infected mice reveal a potential mechanism for parasite spread

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Toxoplasma gondii **infection occurs through the oral route, but we lack important information about how the parasite interacts with the host immune system in the intestine. We used two-photon laser-scanning microscopy in conjunction with a mouse model of oral *T. gondii* infection to address this issue. *T. gondii* established discrete foci of infection in the small intestine, eliciting the recruitment and transepithelial migration of neutrophils and inflammatory monocytes. Neutrophils accounted for a high proportion of actively invaded cells, and we provide evidence for a role for transmigrating neutrophils and other immune cells in the spread of *T. gondii* infection through the lumen of the intestine. Our data identify neutrophils as motile reservoirs of *T. gondii* infection and suggest a surprising retrograde pathway for parasite spread in the intestine.**

neutrophil motility | dynamic imaging | gut | mucosal immunology

Toxoplasma gondii infects around a third of humans worldwide and is widely dispersed in other warm-blooded hosts. Although clinical manifestations in the brain, eye, and developing fetus receive the most attention, *T. gondii* is an oral pathogen and first enters the body and establishes infection in the small intestine. Infection follows consumption of cyst-containing meat or oocyst-contaminated water and produce and is associated with the development of small intestinal pathology in a variety of nonhuman hosts (1). Most notably, experimental infection of C57BL/6 mice by the oral route results in an inflammation of the small intestine that shares immunological features with inflammatory bowel disease (2). This model is useful to further our understanding of host–pathogen interactions in the intestine and of common mechanisms underpinning the development of inflammatory bowel disease (3). Nevertheless, we have limited understanding of how and in which cells infection is established in the intestine, the extent to which the parasite replicates and spreads within the intestine, and how these factors contribute to the development of pathology (2, 4–9). The ability to label living parasites fluorescently and track them in the tissues of infected hosts provides an important tool for investigating these questions (10–14).

Starting in the small intestine, *T. gondii* must travel long distances and surmount a variety of biological barriers to establish chronic infection in the brain. These barriers include the mucus, the intestinal epithelium, and the blood–brain barrier (7, 15). Cells of the immune system are often highly motile and represent attractive transport vessels for pathogens seeking to reach and enter tissues while being protected from the external environment. Consequently, recent studies have focused on the role of immune cells in transporting parasites between tissues (4, 16–23). For example, cluster of differentiation 11b-positive ($CD11b^+$) cells have been implicated in the dissemination of *T. gondii* through the blood and across the blood–brain barrier (4, 19). Following oral infection, it is thought that the initial invasion or traversal of intestinal epithelial cells by ingested parasites is followed by parasite replication in tissue and the transport by host cells to other tissues. However, our understanding of how the parasite enters and disseminates through the intestine itself and of the role played by immune cell populations in this initial phase of infection is extremely limited.

Two-photon microscopy provides important spatial and dynamic information to further our understanding of how pathogens interact with their hosts in complex natural tissue environments (10). However, such techniques rarely have been applied to the intestine, particularly in the context of infection (14, 24–28). Here we used a physiologically relevant oral infection model in conjunction with two-photon microscopy to reveal that neutrophils in the lumen of the small intestine are motile reservoirs of live *T. gondii*. Our findings implicate these cells in an unexpected luminal pathway for the spread of infection.

Results

Spatial and Temporal Pattern of *T. gondii* Infection in the Intestine of Orally Infected Mice Suggests Parasite Spread Via the Intestinal Lumen. Surprisingly little is known about how *T. gondii* behaves in the small intestine of orally infected hosts. There are isolated reports of dividing parasites in intestinal tissue 1 d after infection (dpi), and the parasite increases in number in the small intestine between 3 and 7 dpi (2, 4–6, 9). However, we lack basic information concerning the distribution of parasites in the small intestine during the first week of infection and how this distribution

Significance

Toxoplasma gondii **infection occurs following consumption of infected meat or contaminated water and produce. As a result, the parasite first enters the body in the intestine, but we understand surprisingly little about how it behaves there. In this study, we show that *T. gondii* can invade neutrophils in the intestine directly, hitching a ride in these cells as they migrate out of the intestinal tissue into the lumen. Our findings implicate neutrophils and other immune cells in a surprising luminal pathway for the spread of infection and suggest new targets for therapeutic intervention in oral infection.**

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relates to the presence of immune cell populations. Therefore we infected mice orally with tissue cysts generated from RFP-expressing parasites and assessed the distribution of *T. gondii* by immunofluorescence microscopy (13). In preliminary experiments, we established that parasites could be reliably detected in the intestine by microscopy only beginning 5 dpi. Therefore we focused our analysis on the distal third (including ileum) of the small intestine and on time points of 5 dpi and later. At 5–6 dpi, a small proportion of villi contained *T. gondii*, which was observed most frequently as an isolated, single parasite surrounded by uninfected villi (Fig. 1 *A*–*C*). Interestingly, we occasionally observed a focus of infection with >10 parasites at these early time points, pointing to the existence of rare "hot spots" of parasite replication (Fig. 1*B*). Alternatively, hot spots may occur as a result of simultaneous infection by multiple bradyzoites released locally from a tissue cyst. By 7–8 dpi, the proportion of villi containing single parasites had increased, as had the number of larger foci of infection (Fig. 1 *A*–*C*). We also noted that parasite density often was highest at the tips of the villi (Fig. 1*D*). This pattern of parasite spread over time is difficult to reconcile with a model in which parasites replicate and spread within intestinal tissue but fits well with a model in which hot spots of parasite replication or invasion at the tips of villi result in the release of parasites into the lumen. Released parasites then may colonize neighboring villi, increasing the size of the foci of infection, or infect new villi at distant sites, accounting for the unexpected increase in villi harboring single parasites at later time points.

Neutrophils and Inflammatory Monocytes, but Not Dendritic Cells, Accumulate Near Foci of *T. gondii* Infection in the Intestine of Orally Infected Mice. *T. gondii* is thought to use the migratory capacity of immune cell populations to spread both within and

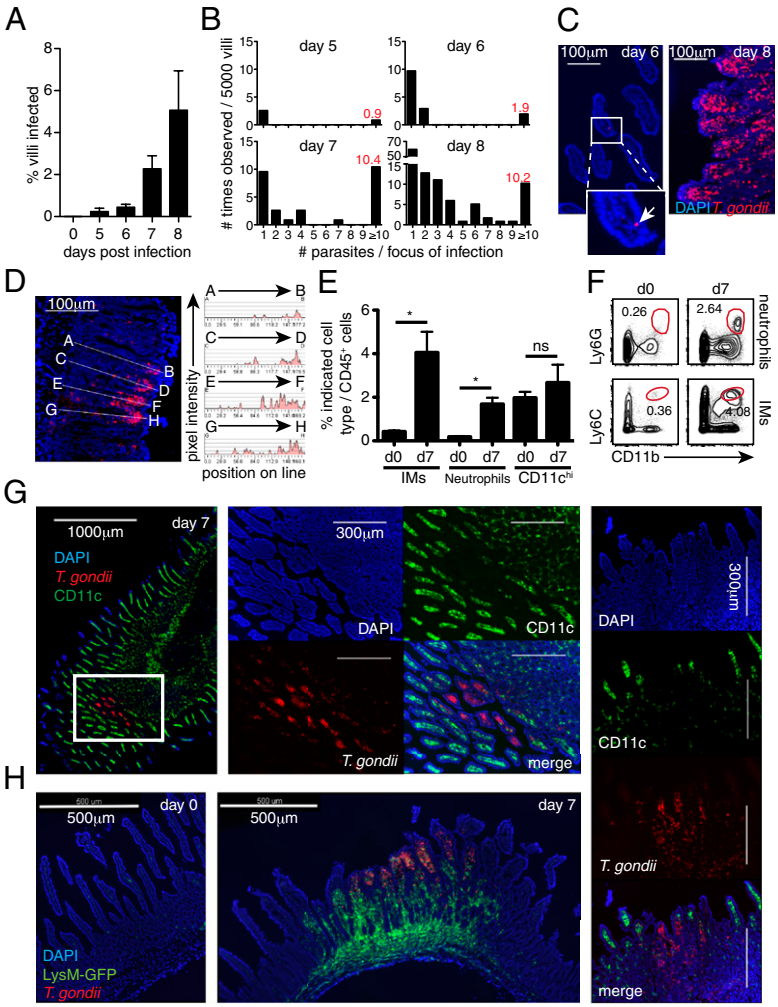

Fig. 1. *T. gondii* establishes discrete, concentrated foci of infection in the small intestine after oral infection. Mice were orally infected with 50 cysts of RFP-expressing *T. gondii*, and the small intestine was analyzed at the indicated time points post infection. (*A*) The percentage of infected villi in the distal third of the small intestine, as determined by immunofluorescence microscopy. Data shown represent the mean ± SEM of six tissue sections from two mice per time point analyzed over two independent experiments. (*B*) Quantification of the number of parasites in each focus of infection at the indicated time point. Data are normalized to the total number of villi analyzed and are pooled from two independent experiments. Red numbers indicate the number of foci containing >10 parasites for every 5,000 villi analyzed. (*C*) Representative images showing *T. gondii* (red) in villi (visualized by DAPI staining, blue). An enlarged view of the boxed region in the left panel is presented. (*D*) The intensity profile of the RFP (*T. gondii*) channel along the indicated line is shown. Each line extends along the length of a single villus. (*E*) Flow cytometric analysis showing the proportion of IMs (Ly6C^{high}CD11b^{+}), neutrophils (Ly6G^{+}CD11b^{high}), and CD11c^{high} cells among CD45^{+} cells isolated from the small intestinal lamina propria. Dead cells, doublets, and CD45^{-} cells were excluded from the analysis. Data shown are mean ± SEM, n = 6–11, pooled from four independent experiments. *$P < 0.05$; ns, not significant. (*F*) Representative flow cytometry plots. (*G*) Representative images showing foci of *T. gondii* infection (red) in the small intestine of CD11c-YFP reporter mice (green). The boxed region in the left panel is enlarged in the middle panel. (*H*) Representative images depicting recruitment of LysM-GFP–expressing cells to foci of *T. gondii* infection (red) in the small intestine.

between tissues. For example, dendritic cells (DCs) have been implicated in spread between tissues, transporting *T. gondii* from the intestine to the draining mesenteric lymph node early after infection (4). We speculated that immune cells also may be involved in spread within the intestine by facilitating exit of the parasite into the intestinal lumen and the reinfection of new villi. To gain insight into which cells may be involved in the spread of infection in the intestine, we assessed the recruitment of immune cells to foci of infection. At 7 dpi the proportion of neutrophils ($Ly6G^+CD11b^{high}$) and inflammatory monocytes (IMs, $Ly6C^{high}CD11b^+$), but not $CD11c^{high}$ DCs/macrophages, had increased among $CD45^+$ cells isolated from the small intestinal lamina propria (Fig. 1 *E* and *F*). To assess the spatial relationship of these cell types to foci of infection, we used genetically engineered mice that express fluorescent reporters in either DCs/macrophages [CD11c-YFP mice (29)] or neutrophils and IMs/macrophages [LysM-GFP mice (30)]. We performed epifluorescence microscopy on tissue sections from the intestines of orally infected CD11c-YFP or LysM-GFP reporter mice at 7 dpi. Although CD11c-YFP^{bright} cells were distributed uniformly throughout the lamina propria of uninfected tissue, we observed a striking absence of these cells from concentrated foci of infection (Fig. 1*G*). This absence may reflect prior bulk migration of activated or invaded $CD11c^{high}$ DCs from the site of infection to the draining lymph node and would be consistent with their proposed role in the early dissemination of *T. gondii* away from the intestine (4). Conversely, LysM-GFP^{bright} cells were recruited specifically to foci of infection and the underlying muscularis but not to adjacent tissue (Fig. 1*H*). In the small intestine of *T. gondii*-infected LysM-GFP mice, both neutrophils ($Ly6G^+CD11b^{high}$) and IMs ($Ly6C^{high}CD11b^+$) express high levels of GFP (Fig. S1*A*). Analysis of tissue sections from infected LysM-GFP mice revealed that only a subset of LysM-GFP^{bright} cells stained with an antibody to the neutrophil marker Ly6G, indicating that both neutrophils and IMs are recruited to foci of infection (Fig. S1*B*). The close proximity of neutrophils and IMs to parasites suggests that these cells, rather than CD11c-YFP^{bright} cells, may play a role in the spread of infection through the intestine at later time points.

Oral *T. gondii* Infection Elicits Transepithelial Migration of Neutrophils and Inflammatory Monocytes. For parasite spread to occur through the lumen of the intestine, invaded cells would have to be capable of migrating across the intestinal epithelium. We therefore investigated whether neutrophils or IMs could undergo transepithelial migration after oral infection. Analysis of the epithelial cell layer and luminal contents by flow cytometry revealed an infection-induced increase in the proportion of both neutrophils ($Ly6G^+CD11b^{high}$) and IMs ($Ly6C^{high}CD11b^+$) in these compartments (Fig. 2 *A* and *B*). The presence of neutrophils/IMs in the epithelial cell layer was confirmed by staining tissue sections from the intestines of orally infected LysM-GFP reporter mice with an antibody to the epithelial cell marker, epithelial cell adhesion molecule (EpCAM) (Fig. 2*C*). This analysis showed LysM-GFP^{bright} cells with elongated morphology appearing to squeeze between epithelial cells while crossing the epithelial layer. To confirm the presence of neutrophils/IMs in the lumen of the small intestine, we orally infected LysM-GFP reporter mice and explanted small pieces of tissue from the small intestine for two-photon microscopy. The epithelial layer was revealed by labeling the tissue with a membrane dye added to the medium surrounding the explant. We observed numerous GFP^{bright} cells, not only within the villi but also in the lumen of the intestine (Fig. 2*D*). Therefore, oral infection with *T. gondii* results in the recruitment of neutrophils and IMs across the epithelial cell layer and into the luminal space, raising the possibility that *T. gondii* could use this pathway to spread through the intestine.

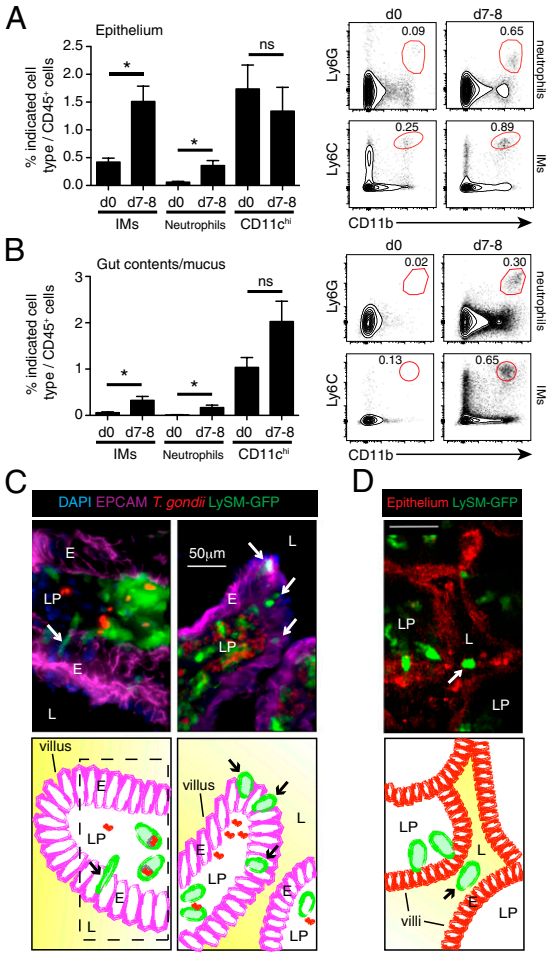

Fig. 2. Oral *T. gondii* infection elicits transepithelial migration of neutrophils and inflammatory monocytes. C57BL/6 mice were orally infected with 50 cysts of RFP-expressing *T. gondii*, and the small intestine was analyzed at the indicated time points post infection. (*A*) Flow cytometric analysis showing the proportion of IMs ($Ly6C^{high}CD11b^+$), neutrophils ($Ly6G^+CD11b^{high}$), and $CD11c^{high}$ cells among $CD45^+$ cells isolated from the small intestinal epithelium. Dead cells, doublets, and $CD45^-$ cells were excluded from the analysis. Pooled analysis (mean ± SEM, n = 7–14, pooled from five independent experiments) and representative flow cytometry plots are shown. $*P < 0.05$; ns, not significant. (*B*) Flow cytometric analysis showing the proportion of IMs ($Ly6C^{high}CD11b^+$), neutrophils ($Ly6G^+CD11b^{high}$), and $CD11c^{high}$ cells among $CD45^+$ cells isolated from the small intestinal lumen. Dead cells, doublets, and $CD45^-$ cells were excluded from the analysis. Pooled analysis (mean ± SEM, n = 5–10, pooled from four independent experiments) and representative flow cytometry plots are shown. $*P < 0.05$; ns, not significant. (*C*) Tissue section from the small intestine of an LysM-GFP mouse stained with an antibody to the epithelial cell marker, EpCAM. (*Left*) An LysM-GFP–expressing cell (green) traversing the small intestinal epithelium (EpCAM staining, purple) is depicted. The position of this image on a villus is shown in the schematic beneath. (*Right*) Multiple LysM-GFP–expressing cells (green) are present in the epithelial cell layer (purple). E, epithelium; L, lumen; LP, lamina propria. (*D*) Two-photon microscopy of a small intestinal explant showing an LysM-GFP expressing cell (green) in the lumen. The epithelium is labeled with a membrane dye added to the medium surrounding the explant and is shown in red. The schematic beneath is provided for orientation. E, epithelium; L, lumen; LP, lamina propria.

Parasites Are Preferentially Associated with Neutrophils. *T. gondii* can invade and replicate within almost any nucleated cell, including cells of the immune system. We used flow cytometry to investigate which immune cells contained fluorescent parasites, focusing our attention on neutrophils and IMs. Strikingly, 10% of neutrophils in the lamina propria contained parasites (Fig. 3*A*).

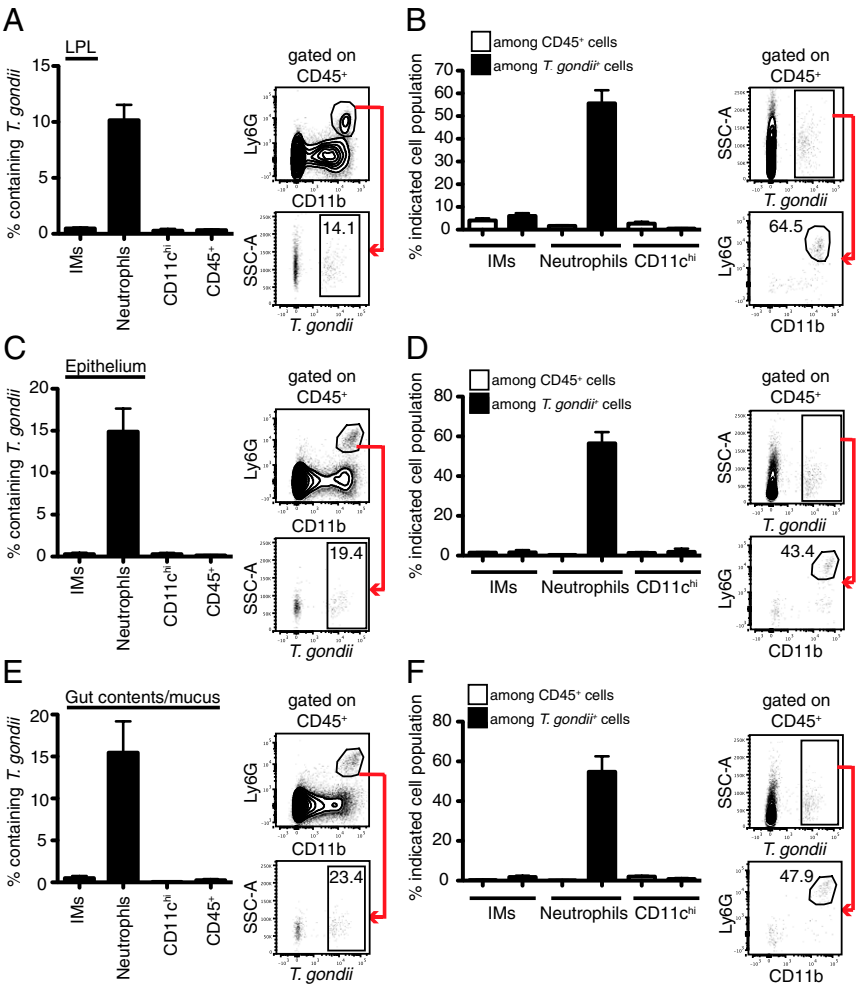

Fig. 3. *T. gondii* is preferentially associated with neutrophils in the small intestine of orally infected mice. C57BL/6 mice were orally infected with 50 cysts of RFP-expressing *T. gondii*, and the small intestine was analyzed 7–8 dpi. (*A*) Flow cytometric analysis showing the proportion of *T. gondii*-containing (RFP$^+$) cells among IMs (Ly6C^{high}CD11b$^+$), neutrophils (Ly6G$^+$CD11b^{high}), and CD11c^{high} cells isolated from the small intestinal lamina propria. Dead cells, doublets, and CD45$^-$ cells were excluded from the analysis. Pooled data (mean ± SEM, n = 7–10, pooled from four independent experiments) and gating strategy are shown. (*B*) Flow cytometric analysis showing the proportion of IMs (Ly6C^{high}CD11b^{high}), neutrophils (Ly6G$^+$CD11b^{high}), and CD11c^{high} cells among CD45$^+$ *T. gondii*-containing (RFP$^+$) cells isolated from the small intestinal lamina propria (black bars). The proportion of these cell types among all CD45$^+$ cells is replicated from Fig. 1 for direct comparison (white bars). Pooled data (mean ± SEM, n = 7–10, pooled from four independent experiments) and gating strategy are shown. (*C* and *D*) As in *A* and *B* but showing analysis of CD45$^+$ cells isolated from the small intestinal epithelium (mean ± SEM, n = 7, pooled from two independent experiments). (*E* and *F*) As in *A* and *B* but showing analysis of CD45$^+$ cells isolated from the contents of the lumen of the small intestine (mean ±SEM, n = 7, pooled from two independent experiments). Gating strategies are for illustrative purposes. In some cases gates were drawn on *T. gondii*$^+$ cells while displayed against a different parameter.

Furthermore, neutrophils represented ~50% of parasite-containing CD45$^+$ cells, despite representing <2% of all CD45$^+$ cells (Fig. 3*B*). In contrast, fewer than 0.5% of IMs contained parasites, and they represented only 6% of the parasite-containing cells (Fig. 3 *A* and *B*). Finally, consistent with the absence of CD11c-YFP^{bright} cells in foci of infection, fewer than 0.3% of CD11c^{high} cells contained parasites, and they represented less than 0.5% of parasite-containing cells (Fig. 3 *A* and *B*). Very similar results were obtained after analysis of the epithelial cell layer and the luminal contents (Fig. 3 *C*–*F*). Therefore, compared with other leukocytes, neutrophils preferentially harbor parasites in the infected intestine and account for the bulk of parasite-containing host immune cells in the gut after oral infection.

***T. gondii* Directly Invades Neutrophils in the Small Intestine.** Neutrophils may contain parasites as a result of phagocytosis or active invasion. These processes can be distinguished by the presence of a parasitophorous vacuole, which surrounds only parasites that actively invade cells. To determine whether parasites within neutrophils had actively invaded the cell or were phagocytosed, we enriched neutrophils/IMs from the lamina propria of infected mice and spun them onto slides. *T. gondii*-containing neutrophils were identified as cells containing RFP fluorescence and staining with an antibody to Ly6G. The parasitophorous vacuole was identified by staining for the parasite-dense granule protein GRA6. The majority of neutrophils harbored parasites that appeared intact and were surrounded by GRA6 staining, suggesting that neutrophils had been actively invaded and harbored live parasites (Fig. 4*A*, *Upper*; 46/62 cells). In a minority of observed cells, GRA6 staining appeared as puncta within the parasite itself, or the parasite appeared partially degraded (Fig. 4*A*, *Lower*; 16/62 cells). This latter category likely consists of neutrophils that had been invaded recently as well as those that phagocytosed parasites.

As an alternative method of detecting actively invaded cells, we used a previously described reporter system to detect parasite

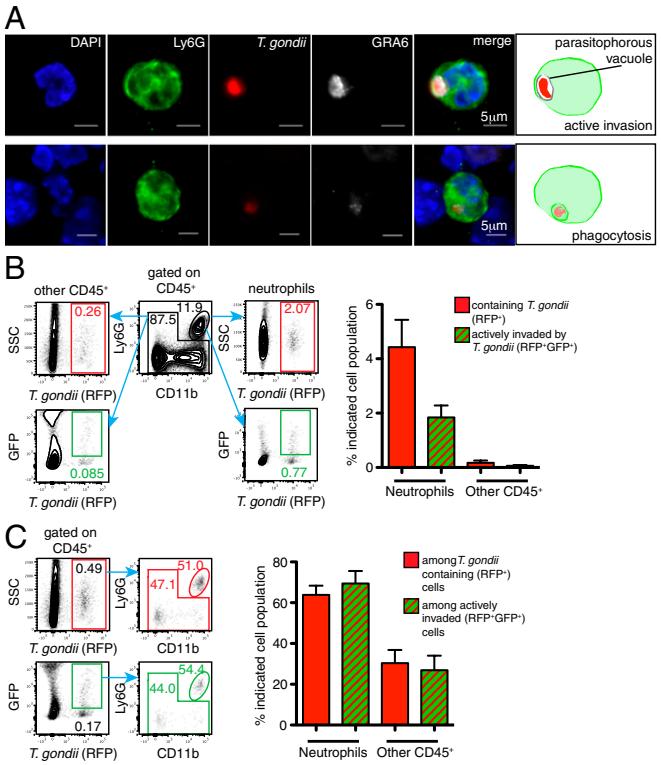

Fig. 4. *T. gondii* can actively invade neutrophils in the small intestine. (*A*) C57BL/6 mice were orally infected with 50 cysts of RFP-expressing *T. gondii*. Ly6B2$^+$ cells were enriched from the lamina propria of the small intestine, spun onto slides, and stained with antibodies to Ly6G (green) and GRA6 (white). (*Upper*) An example of a parasite surrounded by GRA6 staining, indicative of the presence of a parasitophorous vacuole and active invasion. (*Lower*) An example of a parasite with loss of fluorescence and a degraded appearance. (*B* and *C*) Rosa26-GFP Cre reporter mice were orally infected with 50 cysts of Cre- and RFP-expressing *T. gondii* and were analyzed 7–8 dpi (mean ± SEM, $n = 4$, pooled from two independent experiments). (*B*) Flow cytometric analysis showing the percentage of neutrophils (Ly6G$^+$) or other CD45$^+$ cells that contain *T. gondii* (RFP$^+$, red gates and bars) or are actively invaded by *T. gondii* (RFP$^+$GFP$^+$, green gates and green/red bars). Gating strategy and pooled data are shown. (*C*) Flow cytometric analysis showing the percentage of neutrophils (Ly6G$^+$) or other CD45$^+$ cells among *T. gondii*-containing cells (RFP$^+$, red bars) or among actively invaded cells (RFP$^+$GFP$^+$, green/red bars). Gating strategy and pooled data are shown.

secretion into host cells in vivo. In this system mice harboring a Cre recombinase activatable GFP gene are infected with RFP-expressing *T. gondii* engineered to inject a Cre fusion protein into host cells (31, 32). GFP is expressed by host cells in the reporter mice only after Cre-mediated recombination. Therefore, actively invaded cells will display both RFP and GFP fluorescence, whereas cells that have phagocytosed parasites or that were invaded relatively recently will display only RFP fluorescence. Cells displaying only GFP fluorescence are thought to result from parasites injecting proteins into cells that they do not subsequently invade (33). Using this model, we demonstrated that ~2% of lamina propria neutrophils, compared with <0.1% of other CD45$^+$ cells, expressed GFP and therefore were actively invaded by *T. gondii* (Fig. 4*B*). This result is likely to be an underestimate because recently invaded cells may not have had sufficient time to express GFP. Nevertheless, by combining both these methods we can conclude that a large proportion of parasite-containing neutrophils in the small intestine were actively invaded and likely harbor live parasites. Furthermore, when we more broadly scored which cells are actively invaded during intestinal infection, neutrophils accounted for >50% of actively invaded leukocytes (Fig. 4*C*).

The preferential association of live parasites with neutrophils could reflect an impaired ability of neutrophils to respond to cues, such as IFN-γ, that stimulate parasite killing. In agreement with this possibility, in vitro infection of IFN-γ–treated bone marrow cells resulted in an overrepresentation of neutrophils among parasite-invaded cells, whereas infection of untreated bone marrow cells led to equivalent parasite invasion of neutrophils and other cells (Fig. S2). Thus, the impaired killing ability of neutrophils relative to other host cells likely contributes to their preferential invasion within the intestine of infected mice.

Neutrophils Transport Parasites Across Biological Barriers and Remain Motile in the Mucus-Filled Intestinal Lumen. The preponderance of neutrophils among actively invaded cells in the intestine of infected mice, together with their ability to migrate across the intestinal epithelium, suggested that neutrophils may be involved in the spread of *T. gondii* infection through the lumen of the intestine. To explore this possibility further, we sought to determine whether invaded neutrophils remain motile within intestinal tissue. We explanted small pieces of intestine from orally infected LysM-GFP mice and performed time-lapse imaging using two-photon microscopy. We then compared the motility of parasite-containing GFP^{bright} cells (~90% neutrophils, Fig. S1*A*) with that of other GFP^{bright} cells (neutrophils and IMs) that did not contain any parasite fluorescence. There was no difference in the average speed or confinement index between these populations (Fig. 5 *A* and *B* and Movie S1). Furthermore, parasite-containing cells participated in typical neutrophil swarming behavior (Fig. 5*C* and Movie S2) (13). Therefore, invasion by *T. gondii* does not appear to alter neutrophil motility significantly.

To show that neutrophils can transport parasites across the intestinal epithelium into the lumen, we used either tissue autofluorescence or a membrane dye to identify the luminal surface of villi during two-photon imaging. Over the course of three independent imaging days we observed eight LysM-GFP^{high} cells adopting an elongated morphology as they traversed the epithelial cell layer to reach the lumen. Of these, five cells contained parasites (Fig. 5*D* and Movie S3).

Once the parasitized neutrophil crosses the epithelium, infection may be spread to new locations in the intestine as a result of active neutrophil migration through the lumen of the intestine or by passive transport of neutrophils with the contents of the intestine. To provide evidence in support of the former pathway, we assessed the motility of LysM-GFP^{high} cells contained within the lumen of the intestine. We first confirmed the presence of mucus on intestinal explants by overlaying fluorescent beads and performing two-photon microscopy. As expected, we observed a clear separation between the beads and tissue of up to 100 microns, characteristic of an intact mucus layer (Fig. S3) (34). Interestingly, LysM-GFP^{high} cells displayed motility in the lumen similar to that in the tissue, regardless of whether they contained parasites (Fig. 5 *E* and *F* and Movie S4). These data are in line with previous reports that neutrophils can migrate through mucus and also can "swim" in solution (35–37). Together these findings confirm that parasite-containing neutrophils can traverse the intestinal epithelium and migrate through the lumen of the intestine, supporting a role for neutrophils in the spread of infection to neighboring or distant villi via the intestinal lumen.

Parasites Within Neutrophils Are Invasion Competent. For neutrophils to transmit *T. gondii* from villus to villus through the lumen of the small intestine, the parasite within the neutrophil must retain its capacity to infect new cells. To test this capacity, we purified parasite-containing neutrophils (Ly6G$^+$CD11b^{high}) or other parasite-containing CD45$^+$ cells from the luminal

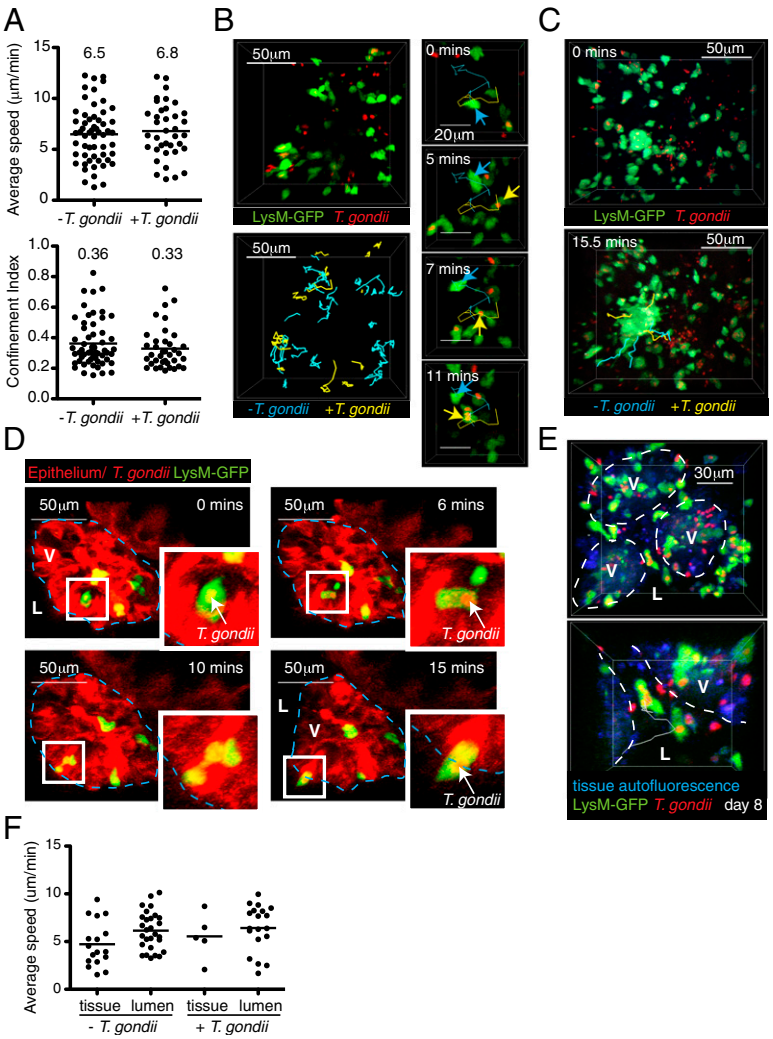

Fig. 5. LysM-GFP$^+$ cells harboring parasites remain motile and can cross the intestinal epithelium to reach the lumen. LysM-GFP mice were orally infected with 50 cysts of RFP-expressing *T. gondii*. Pieces of small intestine were explanted 8 dpi and imaged by two-photon microscopy. (*A*) Average speed and confinement index of *T. gondii*-containing LysM-GFP$^+$ cells, compared with other LysM-GFP$^+$ cells in the same imaging volume. Data are pooled from four imaging volumes acquired on four separate imaging days. (*B*) (*Upper*) A single time point from a representative two-photon movie. LysM-GFP$^+$ cells are green, and *T. gondii* is red. (*Lower*) Tracks of *T. gondii*-containing LysM-GFP$^+$ cells (yellow) and other LysM-GFP$^+$ cells (blue). The right panels are a cropped view of a region from the same imaging volume. The blue arrow highlights a LysM-GFP$^+$ cell and the yellow arrow highlights a parasite-containing LysM-GFP$^+$ cell. (See Movie S1.) (*C*) Two-photon images showing the migration of both *T. gondii*-containing LysM-GFP$^+$ cells (green cells containing red fluorescence, yellow tracks) and other LysM-GFP$^+$ cells (green cells, blue tracks) into swarms. (See Movie S2.) (*D*) Two-photon images showing a *T. gondii*-containing LysM-GFP$^+$ cell (green, parasite indicated by arrow) migrating across the intestinal epithelium (labeled with a membrane dye, red). L, lumen; V, villus. (See Movie S3.) (*E*) Two-photon images depicting the presence of a motile, *T. gondii*-containing, LysM-GFP cell in the lumen of the small intestine (green cell containing red parasite fluorescence, white track). Villi are identified by tissue autofluorescence (blue). L, lumen; V, villus. The lower panel is a close-up view of the upper panel. (See Movie S4.) (*F*) Comparison of the average speed of *T. gondii*-containing LysM-GFP$^+$ cells and other LysM-GFP$^+$ cells in the tissue or mucus of the same imaging volume. Cell location was determined using tissue autofluorescence or a membrane dye. Data are pooled from three imaging volumes acquired on three separate imaging days. Some datasets overlap with those shown in *A*.

contents of the small intestine 8–9 d after oral infection (Fig. 6 *A* and *B*). The sorted cell populations were seeded onto confluent human fibroblast cultures, and plaques were counted 7 d later (Fig. 6*C*). Both populations formed plaques with similar plating efficiency (Fig. 6*C*). We also tested the ability of the sorted cell populations to establish infection in naive C57BL/6 mice after i.p. injection. Eleven to 15 d after injection, parasites were detected in the spleen and mesenteric lymph nodes of mice that received parasite-containing neutrophils (Fig. 6*D*). The ability to transmit infection was not an exclusive property of luminal neutrophils but also occurred after transfer of other parasite-containing CD45$^+$ cells (Fig. 6*D*). However, the predominance of neutrophils among parasite-containing cells increases the likelihood that they would contribute to the luminal spread of infection in vivo.

Luminal spread of infection could be achieved by the lysis of infected immune cells in close proximity to epithelial cells, allowing the parasite to infect or traverse the epithelium. Additionally, the parasite-containing cell itself may migrate across the epithelial cell layer of an uninfected villus, establishing infection. This latter activity may be facilitated by the ongoing inflammatory process, which diminishes the integrity of the epithelial cell layer. To test this possibility, we enriched neutrophils from the bone marrow of naive mice and infected them with *T. gondii* tachyzoites in vitro. These were seeded onto the luminal surface of small intestinal explants prepared from an orally infected mouse at

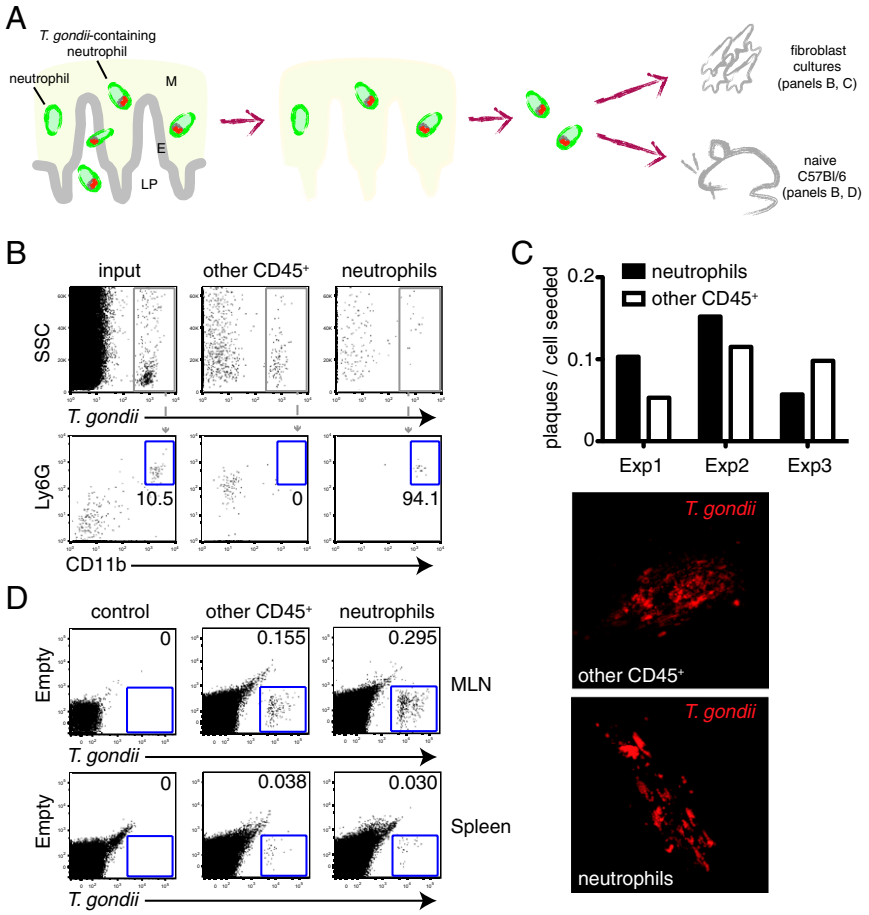

Fig. 6. Spread of infection by *T. gondii*-containing neutrophils in the lumen of the small intestine. (*A*) Mice were orally infected with 50 cysts of RFP-expressing *T. gondii*. At 8–9 dpi the contents of the small intestine were removed, and parasite-containing neutrophils or other CD45$^+$ cells were enriched and seeded onto fibroblast monolayers or injected intraperitoneally into naive C57BL/6 mice. (*B*) Flow cytometric analysis of the purity of the sorted cell populations. (*C*) Quantification of plaque number (relative to the number of parasite-containing cells seeded onto the fibroblast monolayer) (*Upper*) and representative images of plaques (*Lower*). (*D*) Representative flow cytometry plots showing spleen and mesenteric lymph nodes isolated 11–15 d after injection of parasite-containing neutrophils or other parasite-containing CD45$^+$ cells. Data are representative of two independent experiments.

6 dpi. After 2 h, explants were fixed, stained with phalloidin to reveal the structure of the villi, and imaged using two-photon microscopy. *T. gondii*-containing bone marrow neutrophils were observed in villi with intact epithelial cell layers, indicating that neutrophils are capable of transmitting *T. gondii* from the lumen of the intestine into the lamina propria (Movie S5).

Discussion

Infection with *T. gondii* occurs either through ingestion of cysts in the tissues of intermediate hosts or ingestion of oocysts shed in cat feces. It was thought that ingested parasites initially enter the host by traversing or invading intestinal epithelial cells before replicating locally and spreading to other tissues in host cells. However, we have limited information about how the parasite behaves and spreads within the primary site of infection, the small intestine. Here we used a mouse model of oral *T. gondii* infection in conjunction with two-photon microscopy to address this question. We describe a pattern of infection consistent with parasite spread through the lumen of the intestine and demonstrate that neutrophils are recruited to foci of infection in the small intestine and preferentially harbor parasites compared with other leukocytes. Parasite-containing neutrophils were observed migrating across the epithelium into the lumen of the intestine and, along with other immune cells, were capable of establishing new infection both in vitro and in vivo. Our data provide evidence for a role for neutrophils in the spread of infection in the small intestine and suggest a surprising retrograde pathway for parasite dissemination via the intestinal lumen.

T. gondii can infect any nucleated cell type, with neutrophils at first glance appearing to be a relatively inhospitable option. Nevertheless, we demonstrate a striking overrepresentation of neutrophils among *T. gondii*-invaded leukocytes in the small intestine. Consistent with this finding, *T. gondii* has been shown to survive and replicate in human neutrophils, and a high proportion of neutrophils in *T. gondii*-infected lymph nodes contain parasites (13, 38, 39). These findings may reflect direct targeting of neutrophils by the parasite, for example through release of parasite-derived neutrophil chemotactic factors (40). Alternatively, it may reflect an intrinsic or parasite-induced impairment in the ability of neutrophils to destroy parasites. In support of the latter possibility, we find that the preferential invasion of neutrophils relative to other immune cells can be recapitulated in vitro but only when bone marrow cells are treated with IFN-γ to induce parasite-killing activity. To survive within a phagocytic cell, pathogens must evade or modify the phagosome, resist cellular antimicrobial mechanisms, contend with the potential for host cell death, and ensure access to an adequate supply of nutrients. *T. gondii* invades host cells through an active process and subsequently occupies a nonfusogenic parasitophorous vacuole. The parasite protects this niche by delivering proteins that

modulate the activation of host-protective pathways into the host cytosol. For example the *T. gondii* rhoptry protein, ROP18, inhibits the destruction of the parasitophorous vacuole by host immune-regulated GTPases (41, 42). In addition, *T. gondii* may delay apoptosis to protect its intracellular niche (43, 44). Any combination of these mechanisms could allow *T. gondi*i to reside in neutrophils. Interestingly, neutrophils also are the predominant infected cells after *Leishmania* infection in the ear dermis, with neutrophil recruitment having a beneficial effect on parasite survival (12).

T. gondii is thought to use migrating leukocytes to disseminate both within and between tissues (4, 16–21). Therefore it was interesting to note that parasite-containing neutrophils in the small intestine remained motile and were observed crossing the epithelial cell layer and migrating in the lumen. These findings, together with the pattern of infection observed in the small intestine, suggest a model in which parasitized neutrophils transmit infection from one villus to another through the lumen of the intestine. An important assumption of this model is that parasites residing in luminal neutrophils remain able to infect new targets. We demonstrate that they do, building on earlier demonstrations that intestinal contents can transmit *T. gondii* infection (45, 46). Neutrophil transepithelial migration is a common feature of intestinal inflammation, raising the possibility that *T. gondii* simply hijacks an existing migratory pathway of neutrophils to reach the lumen of the intestine. However, *T. gondii* induces a hypermotility phenotype in DCs and macrophages, increasing in vitro transmigration and potentially contributing to in vivo dissemination (17). It is possible that a similar mechanism operates in neutrophils, potentiating their transepithelial migration. In this way, the parasite could acquire both transportation across the intestinal epithelium and protection from the inhospitable environment of the gut lumen. Although the predominance of neutrophils among actively invaded cells increases the likelihood that they would contribute to the spread of infection, other $CD45^+$ cells also were capable of performing this function.

Myeloid-derived cells are recruited to sites of *T. gondii* infection in vivo and can either contribute to host protection or exacerbate pathology. Replication of *T. gondii* in the small intestine is controlled by IMs, but the contribution of neutrophils remains less clear (47, 48). Neutrophils produce IL-12 and generate neutrophil extracellular traps in response to *T. gondii* infection, suggesting a host-protective role (49–51). Antibody-mediated neutrophil depletion and genetic disruption of neutrophil recruitment support this notion (52–54). However, it was demonstrated recently that the depleting antibody used in some of these studies also eliminated IMs, which are critical for control of parasite growth (47). Instead, it was suggested that neutrophils contribute to the development of small intestinal pathology (47). In light of this finding, elucidation of the exact role played by neutrophils in different experimental settings likely will require a more nuanced approach than complete eradication of the population. Future work will address the mechanism by which infected immune cells traverse the intestinal epithelium so that this pathway can be blocked specifically and the effect on parasite spread investigated.

Our data raise the question of whether other gut-colonizing pathogens use a similar pathway to spread through the intestine. Several pathogens, including *Salmonella* and *Listeria*, are released back into the lumen of the intestine as part of their life cycle, allowing reinfection of other villi or Peyer's patches (55, 56). Whether immune cells have a role in this process is not yet clear. Both *Salmonella* and *Listeria* can use myeloid cells to spread between host tissues, in some cases through active stimulation of cell motility (57–59). In addition, *Salmonella* infection stimulates the transepithelial migration of DCs and neutrophils into the gut lumen, and it has been proposed that $CD18^+$ cells transport *Salmonella* from the intestinal lumen to the bloodstream (14, 57, 58, 60). Using an in vivo model of oral *T. gondii* infection, we extend these findings by directly visualizing the migration and transepithelial passage of parasite-containing neutrophils and demonstrating that these cells can establish infection in new hosts. Should this pathway be applicable to other oral pathogens, it may be possible to design therapies that specifically target host cell migration in the gut lumen, thereby reducing the burden of infection in the tissue.

In conclusion, our data provide insight into how *T. gondii* interacts with the intestinal immune system and point to an unusual leukocyte-mediated pathway for the spread of infection through the lumen of the intestine. This finding is important because it conceptually allows even a very low initial infection to establish multiple foci of infection along the length of the intestine, increasing opportunities for the parasite to spread from the intestine to other tissues. The ability to visualize neutrophil migration in the intestine will provide valuable insight not only into how infection spreads but also into the development of intestinal inflammation and in vivo mechanisms of neutrophil transepithelial migration.

Materials and Methods

Mice. C57BL/6 mice were purchased from Jackson Laboratory or were bred in-house. CBA/J mice and B6.Cg-Gt(ROSA)26Sor$^{tm6(CAG-ZsGreen1)Hze}$/J Cre reporter mice were purchased from Jackson Laboratory. LysM-GFP mice were a gift from T. Graf (Albert Einstein College of Medicine, Bronx, NY) (30). CD11c-YFP mice were a gift from M. Nussenzweig (Rockefeller University, New York) (29). Mice were housed under specific pathogen-free conditions at the Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility in the Life Science Addition, University of California, Berkeley. Animal experiments were approved by the Animal Care and Use Committee of the University of California, Berkeley, CA.

***T. gondii* Infections.** Type II Prugniaud (Pru) parasites engineered to express tdTomato (an RFP variant) and ovalbumin (Ova) were maintained, purified, and used for oral infection as described previously (13). These parental Pru-tomato-Ova parasites, which lack the endogenous gene for hypoxanthine xanthine guanine phosphoribosyl transferase (HPT), then were engineered to secrete Cre recombinase into host cells as previously described (31). In brief, the parental parasites were electroporated with the previously described toxofilin-Cre vector, which expresses the selectable marker HPT and the epitope-tagged rhoptry protein toxofilin fused to Cre (31). The parasites then were subjected to several rounds of selection for the expression of HPT using medium containing 25 μg/mL mycophenolic acid and 50 μg/mL xanthine before being cloned by limiting dilution (61). To confirm the secretion of a functional toxofilin:Cre fusion protein, single clones that were HPT^+ were tested for efficacy in causing Cre-mediated recombination (31). Mice were infected with 50–80 cysts by oral gavage.

Cell Isolation and Flow Cytometry. Leukocytes were isolated from the small intestinal lamina propria, epithelium, and gut contents/mucus using a modified version of an existing protocol (62). Briefly, small intestines were removed into ice-cold HBSS/2% (vol/vol) FBS/15 mM Hepes, and the Peyer's patches were removed. The intestines then were butterflied open, and the contents and mucus were removed by gentle swirling followed by gentle scraping with a glass slide. The intestinal contents were loosened by pipetting, passed through a 70-μm filter, and washed. Intestinal tissue was cut into 0.5-cm lengths and was washed briefly three times in ice-cold HBSS/2% (vol/vol) FBS/15 mM Hepes. To remove the epithelial cell layer, the tissue was incubated in RPMI/10% (vol/vol) FCS/5 mM EDTA at room temperature for 15 min with gentle shaking. This step was repeated four times, with supernatants being collected for isolation of intraepithelial leukocytes. The pieces of small intestine were washed for 10 min in RPMI/10% (vol/vol) FCS/15 mM Hepes, minced into small pieces, and incubated in RPMI/10% (vol/vol) FCS/15 mM Hepes/1 mg/mL Type VIII Collagenase (Sigma Aldrich) at 37 °C for 1 h with gentle shaking. Supernatants were collected and filtered, and the digestion step was repeated for an additional hour. Epithelial and lamina propria cell fractions were resuspended in 30% Percoll and carefully layered over a 40% (vol/vol) and 75% (vol/vol) Percoll gradient (GE Healthcare Life Sciences). After centrifugation, cells were collected from the 40%/75% interface and prepared for flow cytometry.

For in vitro assays, freshly isolated bone marrow cells were preincubated overnight with 1 ng/mL recombinant IFN-γ (EBioscience) and then were infected for 8 h with live parasites [multiplicity of infection (MOI) = 1].

Bone marrow cultures and lamina propria, epithelial and gut contents/mucus fractions were stained with the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Molecular Probes, Invitrogen) and were blocked using 2.4G2 supernatant. Antibodies to mouse CD11b (M1/70), CD11c (N418), Ly6C (HK1.4), and CD45 (30-F11) were obtained from Ebioscience; the antibody to mouse Ly6G (1A8) was from BD Biosciences. Data were acquired using a BD LSR II (BD Biosciences) and were analyzed using FlowJo software (Tree Star). In most cases, gating proceeded as follows: leukocytes (FSC vs. SSC-A), singlets (SSC-A vs. SSC-H), viable $CD45^+$ cells (LIVE/DEAD Aqua vs. CD45).

Two-Photon Imaging. Two-photon imaging was performed on 1-cm lengths of small intestine that were explanted, butterflied open, immobilized muscle side down on a glass coverslip, and perfused in warmed oxygenated medium (63). In some experiments, the intestinal epithelium was labeled by staining the explanted tissue with the styryl dye FM 4–64 (Molecular Probes) for 1 min on ice immediately before imaging. The presence of mucus on explants prepared in the same way was determined by overlaying the tissue with 2-μm fluorescent beads for 5 min before imaging.

Images were acquired using a custom-built microscope with a 20×/0.95 Nikon objective and Spectra-Physics MaiTai laser tuned to 920 nm. Emission light was separated with 495 and 510 or 560 dichroics and was collected with three photomultiplier tube detectors. A bandpass filter, HQ 450/80 M, was used to minimize spectral overlap. Typical imaging volumes measured 172 × 143 × 50–110 μm and were scanned every 30 s for 10–30 min. In some cases, imaging data were processed further after acquisition to limit spectral crosstalk or background signal. Spectral unmixing was performed in MATLAB, or nonspecific background signal was subtracted using a digital mask generated on an unrelated channel using Imaris software. The *x*, *y*, *z* coordinates of individual cells over time were obtained with Imaris Bitplane software. Motility parameters were calculated with MATLAB (code available upon request).

The locations of individual villi and the epithelial surface were determined using tissue mapping, tissue autofluorescence, and the membrane dye, FM 4–64 (Molecular Probes, Invitrogen).

Fluorescence Microscopy. For most experiments, the distal third of the small intestine was butterflied open and fixed in 4% (vol/vol) formaldehyde and 10% (wt/vol) sucrose in PBS for 1 h, then placed in 10%, 20%, and 30% sucrose for 12–16 h each. Tissues were coiled, embedded in optimum cutting temperature (OCT) compound (VWR), frozen over dry ice, and stored at −80 °C. Then 20-μm sections were cut using a cryostat and were mounted on slides. For immunofluorescence staining, sections were brought to room temperature and fixed in acetone at −20 °C for 10 min. Sections were air dried, rehydrated in PBS, blocked with 10% normal donkey serum for 1 h at room temperature, stained with combinations of anti-Ly6B2-biotin (7/4; AbD Serotec), anti-Ly6G FITC (1A8; BD Biosciences), and purified anti-EpCAM (G8.8) for 2 h at room temperature or overnight at 4 °C. After extensive washing in PBS, sections were incubated with fluorophore-conjugated, species-specific secondary antibodies or streptavidin obtained from Jackson ImmunoResearch. Sections were washed again extensively in PBS before air drying and mounting with Vectashield. Images were acquired using a Nikon Eclipse E800, 10× air (Plan Fluor 10×/0.3) or 40× oil (Plan Fluor 40×/1.3) objectives and Slidebook 3I implementation of deconvolution or using a Nikon Eclipse TE2000-E and 10× air Plan Fluor objective. Further analysis was performed using Imaris Bitplane and ImageJ software.

To quantify infection in the small intestine,10×-magnification maps of three tissue sections per mouse (separated by at least 100 μm) were generated. The proportion of villi containing *T. gondii* and the number of parasites in each focus of infection then were determined for each tissue section. Foci were defined as single, isolated, infected villi or as groups of infected villi in which any two infected villi were separated by no more than one uninfected villus.

For GRA6 staining, $Ly6B2^+$ cells were enriched from the lamina propria fraction of WT mice using the EasySep Biotin Positive Selection Kit according to the manufacturer's instructions (Stemcell Technologies), spun onto slides, and stained with anti-Ly6G-FITC and for GRA6 as previously described (20).

Purification and Adoptive Transfer of Infected Neutrophils from Intestinal Contents. Small intestines were placed in ice-cold HBSS/2% (vol/vol) FBS/15 mM Hepes and were butterflied open, and the contents and mucus were removed by gentle swirling followed by gentle scraping with a glass slide. The intestinal contents were loosened by pipetting, filtered, washed, resuspended in 30% (vol/vol) Percoll, and carefully layered over a 40–75% Percoll gradient. After centrifugation, cells were collected from the 40/75% and 30/40% interfaces, incubated with 2.4G2 supernatant, and stained with antibodies to mouse CD11b, Ly6G, and CD45. Cells were sorted into $CD45^+CD11b^{high}Ly6G^+RFP^+$ (infected neutrophils) or $CD45^+Ly6G^-RFP^+$ (other infected $CD45^+$) populations on a BD Influx cell sorter. For the neutrophil fraction, >90% of sorted RFP^+ cells were $Ly6G^+CD11b^+$.

Neutrophil Reverse Transepithelial Migration Assay. $Ly6G^+$ cells were enriched from the bone marrow of WT mice using the EasySep Biotin Positive Selection Kit according to the manufacturer's instructions (Stemcell Technologies). Ly6G-enriched cells were infected for 1 h with live tachyzoites (MOI = 1) and labeled with the Vybrant CFDA SE Cell Tracer Kit (Molecular Probes, Invitrogen). Small intestine was harvested 6 d after oral infection, flushed with ice-cold complete RPMI, and rested for 15 min on ice. Then 3-cm lengths of intestine were butterflied open and were pinned lumen side down on a silicone plug, and the muscle layer was dissected away gently. Then 1-cm^3 pieces from the center of the tissues were transferred, lumen side up, to a 0.4-μm organotypic cell-culture insert (BD Biosciences) over 1.5 mL of complete RPMI in a six-well plate. A small plastic ring was placed on the surface of the tissue and was filled with a suspension of 0.5×10^6 infected neutrophils, ensuring that only the luminal face of the tissue was accessible to the neutrophils. Neutrophils were allowed to migrate into the slice for 2 h in a 37 °C incubator, after which the tissue was fixed in 2% (vol/vol) paraformaldehyde, rinsed in PBS, and stained with Alexa Fluor 647-conjugated phalloidin (Molecular Probes, Invitrogen) for 1 h at room temperature. Tissue was immobilized lumen side up on glass coverslips, placed in cold PBS, and single time-point images were acquired using two-photon microscopy.

Statistics. Values are expressed as mean ± SEM. Levels of significance were calculated by the unpaired Student *t* test using GraphPad Prism software.

Note Added in Proof. During review of this manuscript, another study reported the use of fluorescent parasites to study *T. gondii* infection in the intestine (64).

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