The *Toxoplasma* effector GRA28 promotes parasite dissemination by inducing dendritic cell-like migratory properties in infected macrophages

**Graphical abstract**

**Highlights**

- *Toxoplasma* effector protein GRA28 drives a pro-migratory transcriptional remodeling
- In parasitized macrophages, GRA28 cooperates with host chromatin modifiers
- Parasitized macrophages upregulate CCR7 GRA28-dependently and chemotax
- GRA28 facilitates systemic migration of adoptively transferred macrophages in mice

**Authors**

Arne L. ten Hoeve, Laurence Braun, Matias E. Rodriguez, ..., Jeroen P.J. Saeij, Mohamed-Ali Hakimi, Antonio Barragan

**Correspondence**

mohamed-ali.hakimi@univ-grenoble-alpes.fr (M.-A.H.), antonio.barragan@su.se (A.B.)

**In brief**

*Toxoplasma gondii* exploits the trafficking of phagocytes for dissemination. Ten Hoeve et al. show that parasitized macrophages acquire dendritic cell-like CCR7-dependent migratory properties via parasite effector GRA28-mediated transcriptional remodeling of the host cell, which facilitates *T. gondii* dissemination. This work highlights a remarkable migratory plasticity in differentiated mononuclear phagocytes.
The Toxoplasma effector GRA28 promotes parasite dissemination by inducing dendritic cell-like migratory properties in infected macrophages

Arne L. ten Hoeve,1,5 Laurence Braun,2,5 Matias E. Rodriguez,1 Gabriela C. Olivera,1 Alexandre Bougdour,2 Lucid Belmudes,3 Yohann Couté,3 Jeroen P.J. Saeij,4 Mohamed-Ali Hakimi,2,* and Antonio Barragan1,6,*

1Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, 106 91 Stockholm, Sweden
2Institute for Advanced Biosciences, INSERM U1209, CNRS UMR5309, Université Grenoble Alpes, Grenoble, France
3Univ. Grenoble Alpes, INSERM, CEA, UMR BioSanté U1292, CNRS, CEA, FR2048, 38000 Grenoble, France
4Department of Pathology, Microbiology, and Immunology, University of California Davis, Davis, CA 95616, USA
5These authors contributed equally
6Lead contact
*Correspondence: mohamed-ali.hakimi@univ-grenoble-alpes.fr (M.-A.H.), antonio.barragan@su.se (A.B.)
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SUMMARY

Upon pathogen detection, macrophages normally stay sessile in tissues while dendritic cells (DCs) migrate to secondary lymphoid tissues. The obligate intracellular protozoan Toxoplasma gondii exploits the trafficking of mononuclear phagocytes for dissemination via unclear mechanisms. We report that, upon T. gondii infection, macrophages initiate the expression of transcription factors normally attributed to DCs, upregulate CCR7 expression with a chemotactic response, and perform systemic migration when adoptively transferred into mice. We show that parasite effector GRA28, released by the MYR1 secretory pathway, cooperates with host chromatin remodelers in the host cell nucleus to drive the chemotactic migration of parasitized macrophages. During in vivo challenge studies, bone marrow-derived macrophages infected with wild-type T. gondii outcompeted those challenged with MYR1- or GRA28-deficient strains in migrating and reaching secondary organs. This work reveals how an intracellular parasite hijacks chemotaxis in phagocytes and highlights a remarkable migratory plasticity in differentiated cells of the mononuclear phagocyte system.

INTRODUCTION

The mononuclear phagocyte system plays pivotal roles in immune responses and comprises principally monocytes, macrophages and dendritic cells (DCs) (Guilliams et al., 2014). The identification of DC-restricted precursors indicates ontological differences between DCs and macrophages (Sathe et al., 2014). While DCs arise from bone marrow-derived common DC precursors, various tissue-resident macrophages develop from yolk-sac or erythro-myeloid progenitors during fetal development (Guilliams et al., 2014). Macrophages and mono-ocyte-derived DCs (Mo-DCs) also differentiate from monocytic precursors (Helft et al., 2015).

Ontology and differences in tissue localization are reflected in the transcriptomes of macrophages and DCs, which can be distinguished by transcriptional signatures (Miller et al., 2012). Marked by the expression of ZBTB46 (Satpathy et al., 2012), conventional DCs (cDCs) develop in vivo in response to STAT3-activating growth factor FLT3L (Laouar et al., 2003) and under the control of IRF8, BATF3, or IRF4 (Amon et al., 2019). Similarly, Mo-DCs depend on IRF4 for their differentiation, while monocytes divert toward macrophages independently of IRF4 (Briseño et al., 2016). Together, STAT3, BATF3, IRF4, and IRF8 form part of the regulatory network that controls cDC development (Lin et al., 2015).

The study of host-pathogen interactions provides a powerful approach to gain insight into cellular processes in immune cells. The protozoan Toxoplasma gondii commonly infects humans and other warm-blooded vertebrates, for example, rodents. It is estimated that one third of the global human population encounters T. gondii during a life-time (Pappas et al., 2009). Following oral infection, T. gondii disseminates widely from the intestine to reach peripheral organs, such as the central nervous system. Although principally asymptomatic in healthy individuals, infection can cause life-threatening encephalitis in immune-compromised persons, severe neurological manifestations in the developing fetus, and recurrent ocular disease in immune-competent individuals (Montoya and Liesenfeld, 2004; Schlüter and Barragan, 2019).

The disseminating stage of T. gondii—the tachyzoite—is obligate intracellular. Thus, active invasion of host cells, including leukocytes, is essential for parasite survival (Dobrowolski and...
Figure 1. Expression of DC-associated transcription factors in T. gondii-challenged macrophages
(A) Representative micrograph shows primary bone marrow-derived macrophages (BMDMs) stained for F-actin (red) and nuclei (blue), with replicating intra-cellular GFP-expressing T. gondii tachyzoites (green) 18 h post-challenge. Scale bars, 20 μm.
Invasion of host cells encompasses the discharge of secretory organelles, called rhoptries, into the cytosol and the secreted proteins (ROPs) modulate cellular responses of parasitized cells (Frickel and Hunter, 2021; Hakimi et al., 2017). The virulence-associated rhoptry kinase ROP16 activates STAT signaling in infected cells, which results in inhibition of inflammatory cytokine secretion (Butcher et al., 2011; Saeij et al., 2007). Further, a secretory machinery (MYR) ensures transport of dense granule proteins (GRAs) across the intracellular parasitophorous vacuole where the parasite resides (Franco et al., 2016).

Shortly after oral infection, T. gondii crosses the intestinal epithelium (Barragan and Sibley, 2003; Dubey, 1997). Systemic spread from the intestine to peripheral organs via the blood circulation is largely mediated by parasitized CD11c+ and CD11b+ leukocytes (Couret et al., 2006). DCs can act as “Trojan horses” for T. gondii dissemination in mice (Lambert et al., 2006) in a parasite genotype-related fashion (Lambert et al., 2009). Upon active invasion by T. gondii, DCs are induced to migrate via activation of non-canonical GABAergic signaling and mitogen-activated protein (MAP) kinase activation (Bhandage et al., 2020; Kanatani et al., 2017; Olafsson et al., 2019, 2020). This migratory activation, termed hypermigratory phenotype (Weidner and Barragan, 2014), implicates secreted parasite effectors (Drewry et al., 2019; Sangaré et al., 2019; Weidner et al., 2016) and does not rely on chemotaxis (Fuks et al., 2012; Weidner et al., 2013).

Intestinal macrophages are present at higher densities along the villi and are located more closely to the epithelial cells than DCs (Schulz et al., 2009). Although macrophages and DCs share many functions, they differ in their migration under steady-state and inflammatory conditions. Upon inflammation, DCs migrate from peripheral tissues via lymphatic vessels to lymph nodes, for which the upregulation of the chemokine receptor CCR7 is indispensable (Alvarez et al., 2008; Förster et al., 1999). By and large, macrophages can be distinguished from DCs by their lack of Ccr7 expression (Miller et al., 2012). In line with this, macrophages along the intestinal epithelium are sessile and do not normally migrate to draining lymph nodes upon Toll-like receptor (TLR) stimulation, unlike neighboring DCs (Schulz et al., 2009).

Here, we show that T. gondii imparts a DC-like transcriptional signature on macrophages and that parasitized macrophages upregulate CCR7 expression with the onset of chemotaxis. Our data highlight a mechanism by which T. gondii exploits the migratory plasticity of parasitized macrophages to potentiate its dissemination.

RESULTS

T. gondii induces expression of DC-associated transcription factors in parasitized macrophages

The transcription factor IRF4 was found to be essential for the differentiation of monocytes to DCs but not macrophages (Briese et al., 2016; Goudot et al., 2017). Because T. gondii encounters mononuclear phagocytes in tissues and modulates their migratory properties (Bhandage et al., 2020), we investigated the impact of infection on transcriptional signatures that define DCs and macrophages. Interestingly, upon challenge with live T. gondii tachyzoites (Figure 1A), an elevated expression of Irf4, similar to that in bone marrow-derived DCs (BMDMs), was measured in resident peritoneal macrophages (PEMs), macrophage-like phorbol 12-myristate 13-acetate (PMA) bone marrow-derived cells (PMA-BMCs), and bone marrow-derived macrophages (BMDMs) (Figure 1B). In contrast, neither challenge with tachyzoite lysate nor LPS altered Irf4 expression. Flow cytometry analyses confirmed markedly elevated IRF4 protein expression in T. gondii-infected BMDMs but not in bystander BMDMs (Figure 1C). IRF4 expression in infected BMDMs reached levels comparable with expression in BMDMs (CD11c+MHCIId+) (Figure S1A), and with prominent nuclear staining (Figure S1B).

Additional DC signature genes include the transcription factors Zbtb46, Batf3, Nr4a3, and Runx3 (Miller et al., 2012), and ChIP data suggest that these genes are targeted by IRF4 (Figure S1C; Boulet et al., 2019). In line with this, challenge with T. gondii resulted in a significant increase in Zbtb46, Batf3, Nr4a3, and Runx3 mRNA in macrophages, approaching or surpassing their relative expression in BMDMs (Figures 1D–1F and S1D). In contrast, expression was non-significantly altered by T. gondii lysate or LPS. Elevated ZBTB46 protein expression in T. gondii-challenged macrophages was confirmed by western blotting (Figure 1G). Notably, challenge with T. gondii minorly impacted expression of the pDC-associated transcription factor Spiib, receptor Flt3, and Ly6C (Figures S1E–S1G), while expression of the M-CSF receptor CD115 was reduced on both BMDMs and PEMs, in the absence of a measurable bystander effect (Figures 1H, 1I, and S1H). We confirmed that Zbtb46 and Irf4 are induced by a variety of T. gondii strains (Figure S1I). Additionally, we could largely confirm the induction of Zbtb46, Batf3, Nr4a3, and Irf4 by T. gondii in previously published transcriptomics data (Figures S2A and S2B). However, comparing...
Figure 2. Expression of Ccr7 and chemotaxis toward CCL19 by *T. gondii*-challenged macrophages

(A) qPCR analyses of Ccr7 cDNA from BMDCs, PEMs, PMA-BMCs, and BMDMs challenged for 18 h with *T. gondii* type I tachyzoites (RH), tachyzoite lysate, LPS, or left unchallenged (unchall.). Relative expression (2−ΔΔCq) is displayed as mean + SE and individual measurements (n = 3–4).

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T. gondii-induced transcriptional changes with Mo-DC differentiation and gene expression differences between BMDMs and BMDCs, we found no broad similarities. We conclude that the challenge of different types of macrophages with T. gondii induces the expression of transcription factors associated with cDCs, but not with DC differentiation per se.

**T. gondii-infected macrophages perform chemotaxis in response to CCL19**

Because Zbtb46, Batf3, Nr4a3, and Irf4 mediate DC development and differentiation, including associations to CCR7-mediated DC migration (Bajaña et al., 2012), we addressed whether infection impacted chemotaxis in macrophages. First, challenge with T. gondii tachyzoites induced a significantly superior transcriptional expression of Ccr7, related to LPS or MOI-equivalent doses of parasite lysate, and similar or superior to BMDCs (Figures 2A and S2C). BMDMs infected with T. gondii displayed elevated CCL19 receptor expression over unchallenged macrophages (Figure 2B), all consistent with the relative expression profiles of DC-associated transcription factors (Figure 2C). Next, in chemotaxis assays with the CCR7-ligand chemokine CCL19, T. gondii-infected BMDM displayed a distinct migratory response toward the CCL19 source. Similarly, BMDCs chemotaxed (Figures 2D and 2E), as described (Helft et al., 2015), with undetectable chemotaxis by non-infected bystander BMDMs (Figure 2E) and LPS-challenged macrophages (Figure 2F). Additional control characterizations excluded chemokinin or haptokinetic effects by CCL19 (Figures S2D and S2E). Of note, parasitized macrophages exhibited hypermotility, in line with previous results in monocytes and DCs (Bhandage et al., 2020), and this component of the migratory response was independent of CCL19 and was absent in bystander macrophages (Figures S2D and S2E). Further, we found that T. gondii-infected BMDMs expressed significantly higher levels of CD86 and MHCII, but not CD40 and CD80 (Figures 2G, 2H, and S2F), consistent with the impact of T. gondii on macrophage phenotypic maturation, cytokine responses, and proliferative responses in leukocytes (Figures S2G–S2J). While noting an increase in CD11c+MHCII+-expressing cells among resident CD11b+CD19+ peritoneal cells (PECs) infected in vitro (Figure 2I), we also found increases of CD86 and MHCII on T. gondii-infected PEMs (Figure 2J). We conclude that challenge with T. gondii elevates the expression of CCR7 in parasitized macrophages with chemotactic responses to CCL19, in absence of a detectable bystander effect.

**The T. gondii MYR1 secretory pathway and ROP16-STAT signaling differently impact chemotaxis and migration-associated transcription in infected macrophages**

Because the migratory responses indicated effects related to intracellularly located parasites, we assessed two main pathways utilized by T. gondii to modulate host cell responses and transcriptome: rhoptry (ROP) protein-mediated activation of STAT signaling (Saeij et al., 2007) and effector protein secretion via the MYR1 pathway (Franco et al., 2016; Naor et al., 2018). Importantly, chemotaxis was abrogated in macrophages infected with MYR1-deficient parasites (TgΔmyr1) but maintained upon challenge with ROP16-deficient parasites (TgΔrop16) (Figure 3A). Consistently, Ccr7 expression was reduced (TgΔmyr1) and elevated (TgΔrop16), respectively, upon challenge with the mutant parasites (Figure 3B). In line with this, contrasting effects were observed on Zbtb46, Irf4/IRF4, Batf3, and Nr4a3 expression (Figures 3B, S3A, and S3B). Moreover, the downregulation of CD115 was abrogated and partially reduced by MYR1 and ROP16 deficiency, respectively (Figure S3C). Consistent with the downmodulation of inflammatory cytokine responses by ROP16 (Jensen et al., 2013), macrophages challenged with TgΔrop16 expressed higher levels of Ifng and Cd86, and elicited higher Ifng expression in mixed leukocyte reaction (MLR) (Figures 3C, 3D, and S3D). STAT inhibition confirmed the implication of STATs in the observed transcriptional changes (Figure S3E). In sharp contrast, Ifng and Cd86 expression was significantly reduced upon challenge with TgΔmyr1 parasites (Figures 3C and 3D). To confirm that the observed phenotypic effects were linked to the MYR1 secretory pathway and not MYR1 expression itself, we assessed two additional mutants (TgΔrop17, TgΔgra45) associated with this secretory pathway (Panas et al., 2019; Wang et al., 2020) with similar effects on Ccr7, Ifng, Zbtb46, Irf4, and Irf4 expression (Figures 3E and S3F). In contrast, a mutant deficient in TgWIP, a rhoptry protein associated with non-chemotactic hypermotility of parasitized DCs (Sangaré et al., 2019), maintained elevated expression of Ccr7 and chemotaxis of macrophages, similar to wild type (Figures 3E, 3F, and S3F). Jointly, these data show that the expression of DC-associated transcripts and markers in T. gondii-challenged macrophages depends on...
Figure 3. Impact of parasite-derived secreted effectors on macrophage chemotaxis
(A) Motility plots depict displacement of BMDMs challenged with T. gondii type I (RH, Tg) wild-type, MYR1-deficient (Δmyr1), or ROP16-deficient (Δrop16) tachyzoites for 12 h in a collagen matrix with a CCL19 gradient (scale in μm). Directional migration (μm/min) toward the CCL19 source and speed (μm/min) of...
both parasite-derived ROP16 and MYR1-mediated secretions, which partly have opposite effects. ROP16 inhibited phenotypical maturation of macrophages with the reduction of Ccr7 expression but with maintained CCL19-dependent chemotaxis. In contrast, the MYR1 secretion system heavily impacted pro-inflammatory cytokine response, elevated Ccr7 expression, and mediated CCL19-dependent chemotaxis.

The MYR1-associated parasite effector GRA28 mediates CCR7-dependent chemotaxis of macrophages

Given the impact of the MYR1 secretion system on chemotaxis and transcription, we hypothesized that MYR1-secreted effector molecules that target the host cell nuclei mediated the observed phenotypes in macrophages. GRA28, a 200 kDa highly disordered dense granule protein, was initially suspected because the protein is exported to the host cell nuclei in a MYR1- and ASP5-dependent manner (Figures 4A and 4B; Nadipuram et al., 2016) and, like other exported GRAs, contributes to the re-orchestration of host gene expression in macrophages but uniquely triggers CCR7 gene expression (Figures 4C–4E, S4A, and S4B). Comparing the agra28 mutant to the parental strain, we found that the expression of a relatively small number of host genes was indeed upregulated in a GRA28-dependent manner in human and murine macrophages, including CCL22 (Figures 4E, S4C, and S4D), a previously described GRA28-regulated gene (Rudzki et al., 2021). Reintroduction of one copy of the gene into gra28-deficient parasites was sufficient to restore the expression of these genes (Figures 4E and S4B–S4D). Although transcriptomics clearly identified GRA28 as a transcriptional activator in infected cells, it also showed that the protein triggers repression of a substantial fraction of host genes (Figures 4C and 4D). Quantitative reverse transcription PCR of mRNA from four of the repressed genes (i.e., Cxcl3, Cxcl5, Vcam1, and Iil1a) documented a robust and persistent pattern of GRA28-mediated cytokine and Vcam1 repression (Figure S4E).

Having identified GRA28 as a regulator of CCR7 expression, we investigated its role in the chemotaxis phenotype by comparing it with GRA24, another effector known to regulate the expression of chemokines and their receptors (Braun et al., 2013). Notably, while macrophages challenged with GRA24-deficient T. gondii (Tgggra24) maintained Ccr7 expression and chemotaxis, challenge with GRA28-deficient tachyzoites (Tgggra28) dramatically reduced Ccr7 expression, with abrogated chemotaxis to CCL19 (Figures 4F and 4G). Consistent with these results, ablation of GRA28, but not GRA24, abrogated the induction of Il12p40 (Figure 4H) and the expression of CD86 (Figure 4I), compared with wild type. In contrast, a downregulation of the M-CSF receptor CD115 (Figure 1H) was maintained for both mutants (Figure S4F). Finally, we confirmed the differential effects of GRA24- and GRA28-deficiency of transcription factors. In particular, the GRA24-dependent early growth response 1 (Egr1) upregulation (Braun et al., 2013; ten Hoeve et al., 2019) was maintained upon GRA28-deficiency (Figure 4J). Further, Zbtb46 expression was reduced by both GRA24- and GRA28-deficiency compared with wild-type parasites, whereas Irf4 expression was maintained or increased (Figure 4J). Consistently, the induction of Nr4a3 and Runx3, but not Batf3, was also partially dependent on GRA28 (Figure S4G). Taken together, these results show that GRA24 and GRA28 differently impact transcription and effector functions of macrophages and that the MYR1-mediated induction of Ccr7 and chemotaxis in parasitized macrophages is dependent on GRA28 secretion.

GRA28 forms versatile complexes with chromatin modifiers with antagonistic activities

GRA28 is an inherently unstructured protein (Hakimi et al., 2017) and, in this respect, has no discernible protein motifs or defined domains that might indicate specific interaction partners in the host cell nucleus, where it accumulates after infection. To gain functional insight into how GRA28 regulates host gene expression, we searched for host cell partners by applying conventional and affinity chromatography to extracts from murine macrophages infected with tachyzoites expressing a HAFlag-tagged version of GRA28 (Figures 5A and 5B). Silver staining analysis of the eluate showed that GRA28 binds to multiple proteins under very stringent wash conditions (0.5 M NaCl and 0.1% NP40), forming two distinct complexes with apparent molecular weights of approximately 2 MDa (complex 1, fractions F14–F19) and 500–600 kDa (complex 2, fractions F20–F22) once resolved in size-exclusion chromatography (Figure 5B). These partners were subsequently elucidated by proteomics based on mass spectrometry (Table S1), suggesting that GRA28 forms versatile complexes with (1) the multi-subunit NuRD repressor complex, which includes chromatin-remodeling ATPase (CHD4) and deacetylation enzymes (HDAC1,2) (Xue et al., 1998), and (2) the SWI/SNF (BAF) chromatin remodeler, a 2 MDa multi-subunit complex characterized by, among others, its core members SMARCB1, SMARCC1/SMARC2, and SMIACA4 (also known as BRG1), which use energy from ATP hydrolysis to mobilize nucleosomes (Cairns et al., 1998; Kingston and Narlikar, 1999; Figure 5C). Of note, GRA28 binds to a NuRD complex distinctly from that of Tgl1ST in that it does not contain STAT1, nor the transcriptional corepressors CtBP1 and CtBP2 (Figure S5A; Gay et al., 2016).
These data suggest the intriguing possibility that once GRA28 enters the host cell nucleus, it cooperates with NuRD and SWI/SNF to repress and activate host gene expression, respectively. Accordingly, GRA28 is expected to bind to chromatin. ChiP analyses on samples of human fibroblasts infected with HFlag knockin tachyzoites showed that GRA28 was highly enriched in a specific manner near 1,976 genes (10-fold enrichment; Figure S5B; Table S2) compared with control GRAX, which also accumulated in host nuclei after infection but was rarely bound to chromatin (137 genes, 10-fold enrichment) (Figure S5B; Table S2). GRA28 is bound to upstream and intergenic regions, and at the promoter level, the protein is enriched upstream and downstream of the transcription start site (TSS) (Figures 5D, 5E, and SSC). Introns were also highly represented (33%) among GRA28-enriched regions, as reported for BRG1 in human cells (Attanasio et al., 2014). Of note, GRA28 was clearly enriched at the CCR7 locus (Figure 5F), and its induction may be mediated by the recruitment of SWI/SNF (BAF) by GRA28, a chromatin remodeler known to activate transcription by promoting nucleosome repositioning. Chromatin occupancy of GRA28 downstream and upstream of CCR7, sometimes far from TSS, suggests that GRA28 cooperates with chromatin remodelers to increase DNA accessibility by creating an open chromatin conformation over long distances that favors transcription factor binding to site-specific sequences and RNA polymerase progression (Figure 5F).

**Role of macrophage migratory activation on the dissemination of *T. gondii* in vivo**

To address the impact of *T. gondii*-induced migratory activation of BMDMs on dissemination in vivo, we designed separate approaches in mice. For the different conditions, equivalent numbers of pre-treated and pre-labeled cells were simultaneously adoptively transferred intraperitoneally (i.p.) to mice in competition assays. 14–18 h post-inoculation, organs were harvested and cells were characterized by flow cytometry (Figures 6A and S6A–S6D). First, unchallenged and *T. gondii*-challenged BMDMs were assessed for migration. Interestingly, challenged BMDMs (CMTMR+GFP+) migrated to the omentum, mesenteric lymph nodes (MLNs), and spleen at a higher rate than unchallenged BMDM (Deed Red+), and this difference was further accentuated in infected (CMTMR+GFP+) cells (Figure 6B). Next, when BMDMs challenged with wild-type or MYR1-deficient (TgΔmyr1) *T. gondii* were allowed to compete, relatively lower numbers of Δmyr1-infected BMDMs were retrieved in organs (Figure 6C). Similarly, BMDMs challenged with GRA28-deficient *T. gondii* (TgΔgra28) migrated at a relative lower frequency compared with BMDMs challenged with wild-type *T. gondii* (Figure 6D), with a relative re-elevation of migration in BMDMs challenged with reconstituted parasites (TgΔgra28 + GRA28) (Figure 6E). Jointly, this indicated an implication of MYR1-associated GRA28 secretion in the migration of parasitized BMDMs.

These results, together with a previously observed enhanced migration of parasitized BMDCs (Bhandage et al., 2020; Lambert et al., 2006), motivated a comparison of the migratory behaviors of parasitized BMDCs and parasitized BMDMs. Both cell populations exhibited a comparable high migration rate, with BMDMs relatively enriched in spleen and omentum (Figure 6G), indicative of a migration capability by parasitized BMDMs. Finally, we found that the migration frequencies of CCR7-deficient BMDMs were reduced related to wild-type BMDMs (Figure 6G), confirming a relative dependency of parasitized BMDMs on CCR7 for migration. As in in vitro assays (Figure 3D), wild-type infected adoptively transferred BMDMs collected from spleen expressed significantly higher levels of CD86 (Figure 6H), and chemotaxis phenotypes were confirmed in vitro for all conditions (Figures S6E and S6F). Altogether, the data show that *T. gondii*-infected BMDMs can traffic to secondary lymphoid organs, with an impact of the MYR1 pathway, secreted effector GRA28, and
Figure 5. GRA28 binds to chromatin and partners with chromatin remodelers NuRD and SWI/SNF
(A and B) GRA28-associated proteins were purified by FLAG chromatography from protein extracts of the murine RAW macrophage line infected with RHku80 GRA28-HA-FLAG. Fractions from size-exclusion chromatography of GRA28-containing complexes after Flag-affinity selection were analyzed by silver staining and then by mass spectrometry-base proteomics to detect GRA28 and the aforementioned partners.
(C) Heatmap showing the abundance ranks of each protein in each purified fraction derived from extracted iBAQ values. The identity of the proteins is indicated.
(legend continued on next page)
homing receptor CCR7. We conclude that parasitized BMDMs exhibit enhanced systemic migration and can potentiate dissemination of intracellularly located T. gondii.

Impact of T. gondii effectors on transcriptional activation and chemotaxis of human monocytes and macrophages

Because hypermigratory responses upon T. gondii challenge were also observed in infected human mononuclear phagocytes (Bhandage et al., 2020), we sought to confirm our key findings in human peripheral blood monocytes and monocyte-derived macrophages. Interestingly, parasitized human macrophages chemotaxed in a CCL19 gradient, unlike bystander macrophages (Figure 7A). Extending data in murine macrophages, Tgmyr1 parasites, and LPS treatments failed to significantly induce chemotaxis (Figure 7A). Consistent with these findings, challenge with T. gondii wild-type induced CCR7 expression in both monocytes and macrophages, in contrast with Tgmyr1 (Figure 7B). Despite undetectable chemotactic responses under these conditions, LPS challenge induced CCR7 expression (Figures 7A, 7C, and 7D). As in murine macrophages, we found that IRF4 and BATF3 were upregulated by T. gondii challenge in monocytes and macrophages, but not by tachyzoite lysates or LPS (Figures S7A–S7C). Furthermore, the induction of IRF4 and BATF3 expression in human monocytes and macrophages was dependent on MYR1 (Figure S7D), while the induction of BATF3 expression in human cells was in line with findings in murine macrophages (Figure S7E), and ZBTB46 was not significantly affected (Figure S7F). Altogether, these data corroborate and extend our findings on the MYR1-dependent induction of chemotactic migratory activation to human monocytes and macrophages.

DISCUSSION

In peripheral tissues, the encounter of T. gondii with mononuclear phagocytes can result in neutralization of the parasite or in infection of the host cell. Here, we addressed the impact of infection on the migratory responses of macrophages. The data demonstrate that infection with T. gondii allows normally sessile macrophages to acquire migratory features typically attributed to DCs. First, infection led to transcriptional signatures consistent with the acquisition of DC-like migratory properties. Second, we identified the parasite effector GRA28 as the mediator of upregulated CCR7 expression and chemotaxis in parasitized macrophages. Third, the migratory responses of macrophages impacted parasite dissemination in mice.

We report that challenge of separate types of murine and human macrophages with T. gondii induces the expression of transcription factors typically associated with cDCs. Importantly, T. gondii infection, but not challenge with parasite lysates or LPS, induced expression of IRF4, ZBTB46, Batf3, and Nr4a3. These responses coincided with CCR7-mediated chemotaxis by infected macrophages. Notably, most responses impacted infected cells but not non-infected bystander cells, indicating direct effects of parasitization. In DCs, BATF3 is essentially associated with the development of cDC1 (Grajales-Reyes et al., 2015), while IRF4 and NR4A3 are required for mo-DC differentiation and CCR7-mediated migration (Bajarla et al., 2012; Boulet et al., 2019; Briseño et al., 2016). Moreover, ectopic expression of ZBTB46 rescues cDC differentiation from IRF8-deficient bone marrow cells (Satpathy et al., 2012). However, despite the transcription factors induced being strongly associated with DCs, we found no evidence indicating differentiation of macrophages to DCs. Thus, rather than a global transcriptional reprogramming, intracellular T. gondii selectively targeted transcription, with alterations in the migratory responses, activation, and cytokine responses of macrophages. Moreover, the phenotypical effects of T. gondii infection were not limited to specific types of mouse macrophages, with generally a similar activation in human monocytes and monocyte-derived macrophages. Yet, in diverse cell types, host-cell-type-specific effects are to be expected (Swierzy et al., 2017).

We identify the secreted dense granule protein GRA28 as a mediator of CCR7 upregulation and chemotaxis of T. gondii-infected macrophages. GRA28 is exported to the host cell nucleus in a MYR1- and ASP5-dependent manner (Nadipuram et al., 2016; Figure 4B) and was recently linked to the transcriptional regulation of the placenta-associated chemokine CCL22 (Rudzki et al., 2021). GRA28 is a 200-kDa intrinsically disordered protein (IDR) expected to be structurally flexible and able to adopt multiple conformations to interact with SWI/SNF and NuRD, probably via independent short linear motifs (SLIMs), usually 3 to 11 residues, which remain to be defined. We can predict a prion-like structure (Zhang et al., 2013) in a central region of GRA28 that is known to phase-separate to form weaker multivalent interactions. In this regard, GRA28 can function as scaffold proteins to recruit chromatin remodelers and form membraneless condensates capable of dynamically exchanging components with opposing functions, i.e., SWI/SNF and NuRD, thereby selectively accelerating or inhibiting host gene expression.

GRA28 has features in common with the dense granule protein Tg IST that it binds to the NuRD repressor complex, but the nucleosome deacetylation and ATP-dependent chromatin-remodeling functions of NuRD can be hijacked by both in different ways. Tg IST may co-opt NuRD-associated HDACs that compete with HATs (e.g., p300/CBP) to prevent STAT1 acetylation and DNA dissociation, thereby impairing STAT1 recycling and IFN-γ responsiveness (Gay et al., 2016; Olias and Sibley, 2016). Tg IST was also shown to prolong STAT1 occupancy on chromatin and allosterically impairs its ability to recruit the acetyltransferase p300/CBP (Huang et al., 2022). GRA28, which binds to chromatin over long distances, may use the ATP-dependent chromatin-remodeling enzymes CHD3 and CHD4 of NuRD to generate a nonpermisive chromatin state by altering the positioning of nucleosomes, but this remains to be shown. In this

(D) The pie chart shows the distribution of GRA28 peaks in the genome of human fibroblasts relative to gene features.
(E) The average distribution of GRA28 and the control GRAX in gene promoters. Average-signal profiles of each protein were plotted over a region from −5 kb to +5 kb relative to the TSS of each gene. Average tag count of the enrichment is shown on the y axis.
(F) IGB images of the gene CCR7 and ChIP-seq signal peaks for GRA28 and GRAX. See also Figure S5.
regard, the diversification of effectors in *T. gondii*, as exemplified by the interaction of TgIST and GRA28 with the NuRD complex, is likely an evolutionary strategy of the parasite to adapt to multiple hosts and their different cell and organ types. Similarly, TgIST and TgNSM were shown to cooperate and to signal through chromatin by binding to their respective corepressors, NuRD and NCoR/SMRT (Gay et al., 2016; Olías and Sibley, 2016; Rosenberg and Sibley, 2021).

In sharp contrast to the effects of Δmyr1 and Δgra28 mutants, challenge with a Δrop16 mutant parasite conversely elevated Ccr7 expression, with maintained chemotaxis. Because the secreted rhoptry protein ROP16 is a known down-modulator of inflammatory cytokine responses by acting on STAT3/6 (Jensen et al., 2011; Saeij et al., 2007; Yamamoto et al., 2009), our findings phenotypically corroborate the recently described counteracting transcriptional effects of MYR1-dependent and MYR1-independent effectors (Naor et al., 2018; Rastogi et al., 2020). However, the data indicate that Ifr4/IRF4 and Batf3 induction are MYR1/GRA28-independent, while MYR1/GRA28-deficient parasites failed to upregulate Ccr7 expression. This suggests that BATF3 and IRF4 may act in concert to remodel the chromatin in *T. gondii*-infected macrophages, jointly with MYR1-related factors such as GRAs and Egr1 (Braun et al., 2013; ten Hoeve et al., 2019) that ultimately enable transcription at the Ccr7 locus. However, the precise contributions of IRF4 and BATF3 to the migratory phenotype remain to be addressed in future studies under IRF4- and BATF3-deficient conditions. Consistent with the above considerations, the IL-12 pro-inflammatory response and the expression of the costimulatory molecule CD86 generally coincided with CCR7 upregulation and downregulation. Jointly, the data demonstrate that *T. gondii* orchestrates the modulation of macrophage chemotaxis with both a pro-migratory, pro-inflammatory effector axis (MYR1-dependent GRA secretion) and an alternative activation-mediating effector axis (ROP16) that downmodulates IL-12 responses. This establishes the CCR7-dependent pro-migratory axis as distinct from the M2-like signature driven by ROP16 (Jensen et al., 2011; Patil et al., 2014). Despite CCR7 responses being observed for all strains tested, the existing polymorphisms of effectors between strains are likely of relevance because of their link to virulence in type II strains and to dissemination (Barragan and Sibley, 2002; Hitziger et al., 2005; Sibley and Boothroyd, 1992).

For example, type I ROP16 activates STAT3 more potently than type II ROP16, which may partly explain these differences (Yamamoto et al., 2009). Consequently, the proinflammatory effect of GRA28 is likely partly counteracted by ROP16 in a genotype-related fashion. Also, although GRA28 predominantly mediated CCR7 responses, it is likely that additional GRA proteins contribute to the equilibrium of other responses (Merce et al., 2020; Mukhopadhyay et al., 2020). For example, the M1-associated effector GRA15 in type II strains (Jensen et al., 2011) modulates leukocyte adhesion in relation to hypermigration (Ross et al., 2021, 2022). Additionally, systemic responses mediated by PAMPs/PRRs are central for immunity in toxoplasmosis (Frickel and Hunter, 2021; Scanga et al., 2002) and MyD88-independent responses in infected cells also mediate protective immunity (Merce et al., 2020; Mukhopadhyay et al., 2020). Here, GRA24 (type I) suppressed II12p40 expression, consistent with a previously reported IL-12-inhibiting role of p38 MAP kinase (Katholign et al., 2013). This finding is, however, in contrast to previous findings on GRA24 in type II *T. gondii* (Braun et al., 2013; ten Hoeve et al., 2019). Similarly, GRA6-dependent NFAT4 activation impacts immune responses in a strain-dependent manner (Ma et al., 2014). Altogether, this highlights the complex host-pathogen interplay mediated by polymorphic ROPs and GRAs in different parasite strains and host cells, leading to differences in CCR7 and IL-12 regulation.

We report a central role for the MYR1 secretory pathway in the chemotactic and pro-inflammatory responses of macrophages. This was further corroborated with mutants of GRA45 and ROP17, which are constituents of the MYR1 secretory pathway (Cygan et al., 2020). Interestingly, ROP17 has also been
implicated in hypermigration of monocytic cells (Drewry et al., 2019), indicating dual or alternative modes of action by ROP17 on hypermigration. In contrast, chemotaxis and the IL-12 response were not abrogated upon deletion of the rhoptry protein TgWIP, implicated in the migratory activation of DCs (Sangaré et al., 2019). However, the velocity of chemotaxing cells was diminished, indicating overlapping effects on cell locomotion. Indeed, hypermigration and chemotaxis jointly promote the migration of infected phagocytes \textit{in vitro} (Fuks et al., 2012; Weidner et al., 2013) and, while the two migratory phenotypes are activated by separate signaling pathways, they commonly share a dependence on calcium signaling and MAP kinase signaling (Bhandage et al., 2020; Ölafsson et al., 2020). Thus, based on the data at hand, rather than being antithetical, we postulate that both signaling cues cooperatively potentiate the migration of infected phagocytes and parasite dissemination. It is also noteworthy that, under the conditions applied, LPS treatment did not induce chemotaxis in human or mouse macrophages. This is in contrast to LPS responses by DCs (Fuks et al., 2012; Weidner et al., 2013) and indicative of the strong activation and rapid onset of chemotaxis upon \textit{T. gondii} infection of macrophages.

Figure 7. Expression of DC-associated transcription factors and chemotaxis in \textit{T. gondii}-challenged human monocytic cells

(A) Motility plots and analyses (n = 3) of human monocyte-derived macrophages challenged with \textit{T. gondii} type I (RH, Tg) wild type, Tg\textsuperscript{Δmyr1} or LPS for 12 h in a CCL19 gradient as indicated under methods details.

(B) qPCR analysis of CCR7 cDNA from human macrophages and monocytes challenged for 18 h as in (A). Relative expression (\(2^{-\Delta Cq}\)) is shown (mean + SE, n = 4).

(C and D) qPCR analysis of CCR7 cDNA from human macrophages (C) and human monocytes (D) challenged for 18 h with \textit{T. gondii} type I tachyzoites (Tg), tachyzoite lysate, LPS. For reference cells were or left unchallenged (unchall.). Relative expression (\(2^{-\Delta Cq}\)) is shown (mean + SE, n = 4–5).

\* \(p \leq 0.05\), \*\* \(p \leq 0.01\), \*\*\* \(p \leq 0.001\), ns \(p > 0.05\) (B–D; ANOVA, Dunnett’s post-hoc test).

See also Figure S7.
Hypermigratory responses by myeloid mononuclear phagocytes (DCs, monocytes, macrophages, microglia) upon challenge with *T. gondii* appear to be a conserved feature across host species (human, mouse, bovine) and are also induced by the related coccidian *Neospora caninum* (Bhandage et al., 2020; Collantes-Fernandez et al., 2012; García-Sánchez et al., 2019; Lambert et al., 2009). Yet, measurable differences exist between parasite strains/lineages in the magnitude of induction of the hypermigratory phenotype *in vitro* (Weidner and Barragan, 2014) and *in vivo* in mice (Lambert et al., 2009). Here, using an *in vivo* adoptive transfer setup (Fuks et al., 2012; Kanatani et al., 2017), we report that parasitized macrophages can potentiate the dissemination of *T. gondii* in a MYR1- and GRA28-dependent fashion. Consistent with these data, we previously showed that MYR1-deficient parasites had no defect in peritoneal survival but a defect in organ colonization (Sangaré et al., 2019). Interestingly, adoptive transfer of CCR7-deficient infected macrophages significantly reduced, but did not abolish, migration to omentum, MLNs, and spleen, indicating a partial dependency on CCR7-mediated chemotaxis for systemic migration. Consistent with this, *in vitro*, CCR7-deficiency abolished chemotaxis of infected macrophages toward CCL19 while hypermotility was maintained. Thus, synergistically with the onset of GABA-mediated hypermigration (Bhandage et al., 2020; Lambert et al., 2006, 2011), the acquired sensitivity of parasitized macrophages and other monocyteic cells to CCL19/CCL21 gradients could carry replicating tachyzoites to lymph nodes and beyond, thereby potentiating systemic dissemination. Importantly, the MYR1-dependent CCR7 responses were corroborated in human macrophages and monocytes, advocating for conserved signaling across species. Yet, differences in the transcriptional regulation were also noted, indicating that, despite the conserved migratory plasticity of macrophages, regulatory response differences exist between human and murine cells. Altogether, the data highlight the implication of host-cell-related and parasite-genotype-related components in “Trojan horse”-mediated dissemination, which requires further investigation.

It is becoming increasingly clear that bacterial, viral, and fungal microorganisms use elaborate strategies to thrive inside macrophages and other phagocytes (Mercer and Greber, 2013; Sansonetti and Di Santo, 2007; Tan and Russell, 2015). To do this, pathogens need to sense, respond to, and induce alterations in host cells that benefit their survival. Recent findings show that *Mycobacterium tuberculosis* manipulates alveolar macrophage trafficking for rapid localization to the lung interstitium (Loveday et al., 2022). Similarly, *Leishmania* parasites impact transcription and epigenetic regulation in the host macrophage, thereby modulating immune responses (Lecoëur et al., 2022), including the inhibition of macrophage motility (de Menezes et al., 2017). Following oral infection, *T. gondii* encounters sessile macrophages in the intestine and other peripheral tissues. Dissemination of *T. gondii* from the intestine is rapid, asymptomatic, and without generalized inflammation (Gregg et al., 2013). Our study provides a framework for how *T. gondii* manipulates the migration of mononuclear phagocytes to disseminate. The findings reveal a remarkable migratory plasticity (Friedl and Weigelin, 2008) in normally sessile macrophages, which can acquire migratory properties typically attributed to the more migratory DCs upon infectious challenge with *T. gondii*. The data also highlight a transcriptional plasticity linked to the migratory responses of differentiated monocytic cells, which obligate intracellular parasites may utilize to their advantage. Because macrophages and monocytes outnumber DCs in tissues, a migratory activation of these cells may favor the dissemination of *T. gondii*. The findings unveil putative alternative pathways by which mononuclear phagocytes can be made migratory or activated, which could—by extension—be exploited, for example, in cell therapies.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.chom.2022.10.001.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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## Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** | | |
| PE anti-mouse CCR7 (clone 4B12) | eBioscience | Cat 12-1971-82; RRID:AB_465905 |
| PE-Cyanine7 anti-mouse CD11c (clone N418) | eBioscience | Cat 25-0114-82; RRID:AB_469590 |
| APC anti-mouse/human CD11b (clone M1/70) | BioLegend | Cat 101211; RRID:AB_312794 |
| anti-mouse CD16/CD32 (Mouse BD Fc Block) | BD Biosciences | Cat 553142; RRID:AB_394657 |
| anti-mouse CD16/CD32 (clone 93) | eBioscience | Cat 14-0161-82; RRID:AB_467133 |
| Super Bright 780 anti-mouse CD40 (clone 1C10) | eBioscience | Cat 78-0401-82; RRID:AB_2762674 |
| Super Bright 645 anti-mouse CD80 (clone 16-10A1) | eBioscience | Cat 64-0801-80; RRID:AB_2663120 |
| Super Bright 600 anti-mouse CD86 (clone GL1) | eBioscience | Cat 63-0862-80; RRID:AB_2662861 |
| Brilliant Violet 605 anti-mouse CD115 (clone AF98) | BioLegend | Cat 135517; RRID:AB_2562760 |
| Super Bright 702 anti-mouse MHCII I-A/1-E (clone M5/114) | eBioscience | Cat 67-5321-82; RRID:AB_2717173 |
| PE anti-mouse IRF4 (clone Q9-343) | BD Biosciences | Cat 566649 |
| PerCP-Cyanine5.5 anti-mouse IL-12/23 p40 (clone C17.8) | eBioscience | Cat 45-7123-80; RRID:AB_1107021 |
| PE anti-mouse Ly6C (clone HK1.4) | BioLegend | Cat 128007; RRID:AB_1186133 |
| PE anti-mouse ZBTB46 (clone U4-1374) | BD Biosciences | Cat 565832; RRID:AB_2739372 |
| APC/Fire 750 anti-mouse F4/80 (clone BM8) | BioLegend | Cat 123151; RRID:AB_2616724 |
| PE mouse IgG1 Kappa Isotype Control (clone MOPC-21) | BD Biosciences | Cat 551436; RRID:AB_394195 |
| PE rat IgG2a k Isotype Control (clone R35-95) | BD Biosciences | Cat 553930; RRID:AB_479719 |
| Anti-TATA binding protein | Abcam | Cat ab51841; RRID:AB_945758 |
| Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP | Thermo Fisher | Cat 32430; RRID:AB_1185566 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Brefeldin A Solution (1000x) | eBioscience | Cat 00-4506-51 |
| TPCA-1 STAT3 and IKK-2 inhibitor | MedChemExpress | Cat HY-10074 |
| AS1517499 STAT6 inhibitor | MedChemExpress | Cat HY-100614 |
| CellTracker Orange CMTMR Dye | Thermo Fisher | Cat C2927 |
| CellTracker Deep Red Dye | Thermo Fisher | Cat C34565 |
| CellTrace CFSE Cell Proliferation Kit | Thermo Fisher | Cat C34554 |
| LIVE/DEAD Fixable Far Red Dead Cell Stain Kit | Thermo Fisher | Cat L34973 |
| **Critical commercial assays** | | |
| Nile Blue Fluorescent Particles, 2.5-4.5 μm | BD Biosciences | Cat 556270 |
| CD14 MicroBeads, human, 2 mL | Miltenyi Biotec | Cat 130-050-201 |
| MiniMACS Separation columns, type MS | Miltenyi Biotec | Cat 130-042-201 |
| Lymphoprep | Axis Shield Poc As | Cat 1115754 |
| Direct-zol RNA Miniprep kit | Zymo Research | Cat R2052 |
| **Deposited data** | | |
| https://www.ebi.ac.uk/pride/login | N/A | |
| MS proteomics | ProteomeXchange Consortium | PXD032360 |
| **Experimental models: Cell lines** | | |
| Human foreskin fibroblasts HFF-1 | American Type Culture Collection | Cat SCRC-1041; RRID:CVCL_3285 |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Antonio Barragan (antonio.barragan@su.se).

Materials availability
New plasmids and materials generated in this study are available from the lead contact.

Data and code availability
- The MS proteomics data have been deposited to the ProteomeXchange Consortium through the PRIDE partner repository with the dataset identifier PXD032360. This paper analyzes existing, publicly available data. The NCBI GEO accession numbers for these datasets are GSE40727, GSE66899, GSE64767, GSE99837 and GSE27972. All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
Wild-type and CCR7-deficient C57BL/6 (6-10 week old, male and female) and wild-type BALB/c (6 week old, female) mice were used for the isolation of bone marrow. Bone marrow-derived macrophages (BMDMs), dendritic cells (BMDCs) and macrophage-like PMA bone marrow cells (PMA-BMC) were cultivated from bone marrow with M-CSF-containing conditioned medium, recombinant GM-CSF or PMA as indicated in the method details. Peritoneal cells were harvested from wild-type C57BL/6 mice (6-10 week old, male and female) via peritoneal lavage. C75BL/6 cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS), gentamicin (20 μg/mL) and glucose (2 mM) and BALB/c cells in Dulbecco’s modified Eagle’s medium, high glucose, with 50 μM 2-mercaptoethanol, 1X non-essential amino acids and 2% penicillin/streptomycin-glutamine at 37°C/5% CO2. C57BL/6 mice (5-18 week old, male and female) were used for adoptive transfer of BMDMs and BMDCs. The Regional Animal Research Ethical Board, Stockholm, Sweden, approved experimental procedures and protocols involving extraction of cells from mice (permit numbers 9707/2018, 14458/2019 and N 78/16), following proceedings described in EU legislation (Council Directive 2010/63/EU).

Primary human cells
Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from healthy adult donors at the Karolinska University Hospital Blood Center. The sex or age of these donors are unknown to the authors due to privacy law restrictions. CD14+ monocytes were obtained from PBMC via positive selection and used for generating macrophages with M-CSF. Cells were cultured at 37°C/5% CO2 in RPMI 1640 with 10% fetal bovine serum (FBS), gentamicin (20 μg/mL) and glucose (2 mM). The Regional Ethics Committee, Stockholm, Sweden, approved protocols involving human cells (application number 2006/116-31). All donors received written and oral information upon donation of blood at the Karolinska University Hospital Blood Center. Written consent was obtained for utilization of white blood cells for research purposes.

Cell lines
Human foreskin fibroblasts HFF-1 were cultured in Dulbecco’s modified Eagle’s medium, high glucose, (DMEM; Thermo Fisher scientific) with 10% fetal bovine serum (FBS; Sigma), gentamicin (20 μg/mL; Gibco or Sigma) and glucose (2 mM; Gibco). Human THP-1 (male) and murine RAW264.7 (male, BALB/c) cells were used for in vitro experiments and cultured at 37°C/5% CO2.

T. gondii strains
T. gondii tachyzoites were maintained by serial 2-day passages in human foreskin fibroblast HFF-1 monolayers. Freshly egressed tachyzoites were used for all infections. The different strains used are listed in the key resources table. All cell cultures used were periodically tested for mycoplasma and found to be negative.

METHOD DETAILS

Mouse cell culture
Cells from bone marrow of 6-10-week-old male or female wild-type or CCR7-deficient C57BL/6 mice (see key resources table) were cultivated in RPMI 1640 with 10% fetal bovine serum (FBS), gentamicin (20 μg/mL; Gibco or Sigma) and glucose (2 mM; Gibco), Human THP-1 (male) and murine RAW 264.7 (male, BALB/c) cells were used for in vitro experiments and cultured at 37°C/5% CO2. Mice were identified phenotypically from peritoneal lavage cells that were challenged in vitro as indicated. BALB/c BMDMs were generated as described previously (He et al., 2018). Briefly, bone marrow was isolated by flushing femurs and tibias of six-week-old female BALB/c mice (Janvier Labs) and cultured for 1 week in complete macrophage medium (DMEM supplemented with 10% heat-inactivated FBS (Invitrogen), 20% conditioned medium from macrophage-colony stimulating factor-secreting L929 fibroblasts (Aziz et al., 2009), 50 μM 2-mercaptoethanol, 1X non-essential amino acids, and 2% penicillin/streptomycin-glutamine). After 6 days, the cells were washed with PBS to remove non-adherent cells, harvested in ice-cold PBS supplemented with 1 mM EDTA and replated for the assay. RAW264.7 (RRID:CVCL80493, ATCC, cat. No. TIB-71) cell line was cultured in DMEM (Invitrogen), supplemented with 10% heat-inactivated FBS (Invitrogen), 10 mM Hepes buffer, pH 7.2, 2 mM L-glutamine, and 50 μg/ml penicillin and streptomycin (Thermo Fisher Scientific). THP1 (ATCC, cat. No. TIB-202) cell line was cultured in RPMI (Invitrogen), supplemented with 10% heat-inactivated FBS (Invitrogen), 10 mM Hepes buffer, pH 7.2, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 50 μg/ml penicillin and streptomycin (Thermo Fisher Scientific). Cell purity was confirmed by microscopy and flow cytometry (Figures 11 and S1H; Data S1).
Human cell culture
Human CD14+ monocytes were isolated from peripheral blood mononuclear cells (PBMC) after density gradient centrifugation on Lymphoprep with CD14 MicroBeads from buffy coats obtained from healthy donors at the Karolinska University Hospital Blood Center and cultured in complete medium. Macrophages were generated from CD14+ monocytes through culture for 6-7 days in complete medium supplemented with 50 ng/mL human recombinant M-CSF.

Infection challenges
Carry-over from routine *T. gondii* culture to experiments was minimized by repeated washing of the freshly egressed tachyzoites before preparation of crude tachyzoites lysates (prepared by repeated freeze-thaw) or challenge with live tachyzoites. For all qPCR and western blot experiments, C57BL/6 BMDMs, PEMs, PMA-BMCs and human monocytes and macrophages were challenged with freshly egressed *T. gondii* tachyzoites at MOI 2 for 18h. Tachyzoite lysates were used at amounts derived from equivalent tachyzoite numbers as indicated live parasites. LPS was used at a final concentration of 10 ng/mL. For qPCR experiments, BALB/c BMDMs, RAW and THP-1 cells were challenged with *T. gondii* tachyzoites for 24h as indicated. For flow cytometry assays, C57BL/6 BMDMs were challenged for 6h or 18h, as indicated, with GFP-expressing *T. gondii* tachyzoites (MOI 1) or with non-fluorescent protein-expressing tachyzoites (MOI 2). Peritoneal cells were challenged with GFP-expressing *T. gondii* type I tachyzoites (RH-LDM; MOI 0,5) for 18h for flow cytometry. Infection frequencies for GFP-expressing type I (RH) *T. gondii* tachyzoites (MOI 1 or 2) were determined by flow cytometry (Data S1). For chemotaxis experiments, cells were challenged for 12-14h with GFP-expressing or CMTMR-labeled *T. gondii* tachyzoites at MOI 1 before seeding in the chemotaxis chamber.

Plasmid construction
To construct vector pLIC-GRA28-HF, the coding sequence of GRA28 was amplified using the primers LIC-231960-F and LIC-231960-R and genomic DNA of Pru*Δku80* strain of *T. gondii* as the DNA template. The resulting PCR product was cloned into the pLIC-HF-dhfr vector using the ligation independent cloning (LIC) method.

The plasmid pTOXO_Cas9-CRISPR::sgGRA28 was generated as previously described (Curt-Varesano et al., 2016) to construct the *gra28* deletion in the RH*Δku80* and Pru*Δku80* strains. Briefly, primers GRA28-gRNA-Fwd and GRA28-gRNA-Rev containing the sgRNA targeting *GRA28* genomic sequence were phosphorylated, annealed and ligated into pTOXO_Cas9-CRISPR plasmid linearized with Bsal, leading to pTOXO_Cas9-CRISPR::sgGRA28.

To construct the pGRA28-GRA28-HA vector, the promoter sequence of GRA28 and the *GRA28* coding sequence were amplified by PCR using the primers LICF-PGRA28_F and LICR-GRA28_R. The resulting PCR product was cloned into the pLIC-3HA-DHFR. The chimeric construct pGRA28-GRA28-3HA-3DHFR was amplified using the primers UPRT-PGRA28_F and UPRT-3DHFR_R and inserted within the UPRT locus.

*T. gondii* transfection
Vectors were transfected into *T. gondii* strains by electroporation. Electroporation was conducted in a 2-mm cuvette at 1.100 V, 25 μF and 25 μF. Stable integrants were selected in media containing 1 μM pyrimethamine or 10 μM 5-fluorodeoxyuracil and single-cloned in 96 wells plates by limiting dilution.

Inhibitors
When indicated, cells were treated with inhibitors TPCA-1 or AS1517499 at 3 μM or vehicle (DMSO) initiated 1h prior to challenge.

Mixed leukocyte reaction (MLR)
Syngeneic MLR was performed as described (Nussenzweig and Steinman, 1980). Briefly, BMDM were challenged for 6h as indicated, washed and co-cultured with syngeneic un-fractionated splenocytes for 18h at 1:20 ratio. Splenocytes were freshly isolated from triturated spleens, filtered through a 40 μm cell strainer and subjected to red blood cell lysis (ammonium chloride).

Quantitative polymerase chain reaction (qPCR)
C57BL/6 BMDMs, PMA-BMCs and PEMs were cultured with complete medium or challenged with freshly egressed *T. gondii* tachyzoites of the indicated strains, lysate of egressed *T. gondii* tachyzoites (ME49) at MOI equivalents or LPS as indicated and lysed in TRI Reagent (Sigma-Aldrich). Total RNA was extracted according to the manufacturers’ protocol using the Direct-zol RNA Miniprep kit and reverse transcribed with Maxima H Minus Reverse Transcriptase (Thermo Fisher). Real time qPCR was performed SYBR® green PCR master mix (KAPA biosystems), specific forward and reverse primers at target-dependent concentrations (100 or 200 nM) and cDNA (10-30 ng) in a QuantStudio 5 System (Thermo Fisher) with ROX as a passive reference. qPCR results were analyzed using the ΔΔCq method and displayed as fold change relative to unchallenged or as ΔCq and relative to Importin-8 and TATA-binding protein (TBP) as housekeeping genes. BALB/c BMDMs and RAW and THP-1 cells were either uninfected or *T. gondii*-infected (MOI = 6) for 24 h. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific). Complementary DNA was synthesized with random hexamers using the High Capacity RNA-to-cDNA kit (Applied Biosystem). Samples were analysed by real-time quantitative PCR for *CCL2*, *CCL3*, *TNFα*, *CXCL1*, *CXCL3*, *CXCL5*, *Vcam1* and *Ilt1a* using the TaqMan gene expression master mix (Applied Biosystem) according to the manufacturer’s instructions. The internal control gene *TBP* was used for normalization. Primers are listed in the key resources table.
**Flow cytometry**

C57BL/6 BMDMs or BMDCs were challenged as indicated and stained with anti-CD11c, CD11b, MHCII I-A/I-E, CD86, CD115, IRF4, Ly6C, IL-12p40 or isotype control antibodies and live/dead far red stain. For intracellular staining cells were fixed (2% PFA) and then permeabilized in either Tween-20 (1%) in FACS buffer (PBS/0.5% FBS/1mM EDTA; IL-12p40) or Triton X-100 (1%) in PBS/5% BSA (IRF4/isotype) supplemented with anti-CD16/CD32 antibody prior to staining. For IL-12p40 staining cells were cultured in the presence of Brefeldin A (eBioscience) for the last 4h of challenge. Extracellular staining was performed on fixed (2% PFA) or live cells, blocked with anti-CD16/CD32 antibody, in FACS buffer. For bead phagocytosis, PMA-BMCs were mixed with Nile Blue Fluorescent Particles (2.5-4.5 μm, 10 particles/cell) and cultured for 1h on ice or at 37°C, washed and fixed (2% PFA). PMA-BMCs kept on ice were <1% bead+. C57BL/6 peritoneal lavage cells were challenged in vitro as indicated, stained with LIVE/DEAD Far Red stain, fixed (2% PFA) and then stained with anti-CD11c, CD11b, CD19, MHCII I-A/I-E, CD86, CD115, Ly6C and F4/80 antibodies. PEMs were defined as CD19 CD11b<CD11c (Figures 1I and S1H). Flow cytometry was performed on a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo X (FlowJo LLC).

**Western blot**

For Western blotting cells were challenged as indicated, harvested, washed with PBS and then lysed directly in Laemmli buffer for whole cell lysates. Proteins were separated using 8% or 10% SDS-PAGE, blotted onto a PVDF membrane and blocked (10% BSA in TBS/0.5%, Tween-20) followed by incubation with primary and secondary antibodies: anti-TATA-binding protein, anti-ZBTB46, antimouse IgG-HRP or anti-rat IgG-HRP in 10% BSA/0.5% Tween in TBS. Proteins were revealed by mean of enhanced chemiluminescence (GE Healthcare) in a BioRad ChemiDoc XRS+. Densitometry analysis was performed using ImageJ (NIH, MD, USA).

**Chemotaxis**

BMDCs, BMDMs or human macrophages were challenged as indicated, washed, resuspended in CM with 1 mg/mL bovine collagen type I (Sigma) and seeded into gelatin (1%)-coated or uncoated ibiTreat μ-slide chemotaxis chambers (ibidi, Martinsried, Germany). Collagen was allowed to polymerize for 45 minutes and media, inhibitors and 1,25 μg/mL murine (BMDCs, BMDMs) or human recombinant CCL19 (human macrophages) were added as indicated conform the manufacturer’s instructions (application note 23). Cells were then imaged every 2,5 (BMDCs) or 5 (BMDMs, human macrophages) min for 4h (BMDCs) or 8h (BMDMs, human macrophages; Zeiss Observer Z.1). Motility tracks for 35-60 cells per condition were analyzed using ImageJ software for each experiment.

**Immunofluorescence microscopy**

BMDMs were seeded on gelatin (1%)-coated glass coverslips, challenged freshly egressed T. gondii tachyzoites (RH1-1; MOI 2) for 18h and fixed with 2% PFA. Cells were then permeabilized with 0,1 or 1,0% Triton X-100 in PBS and stained with phalloidin Alexa Fluor 594 (Thermo Scientific) or anti-IRF4 and DAPI. Images were acquired on a Leica DMi8 with 63x objective.

**Adoptive transfers**

Adoptive transfers were performed as previously described (Bhandage et al., 2020). Briefly, BMDMs or BMDCs were stained with CellTracker CMTMR (2 μM) or Deep Red (1 μM) dyes (2,5x10^6 cells each), washed and challenged with freshly egressed T. gondii GFP-expressing or CFSE-stained RH tachyzoites (MOI 1,5) or left unchallenged in complete medium for 4h. Cells were then washed and injected i.p. into C57BL/6 mice. Mice were sacrificed 18h and fixed with 2% PFA. Cells were then permeabilized with 0,1 or 1,0% Triton X-100 in PBS and stained with phalloidin Alexa Fluor 594 (Thermo Scientific) or anti-IRF4 and DAPI. Images were acquired on a Leica DMi8 with 63x objective.

**Handling of publicly available datasets**

ChiP-seq data available from NCBI GEO series GSE40727 (IRF4), GSE66899 (H3K27ac), GSE64767 (H3K4me3), which are described elsewhere, was visualized in the UCSC genome browser (Glasmacher et al., 2012; Grajales-Reyes et al., 2015; Lin et al., 2015). Changes in gene expression by 1 or 2 day treatment of sorted monocytes with GM-CSF and IL-4 were determined from RNA-seq read counts in NCBI GEO serie GSE99837 (Boulet et al., 2019). Microarray data from NCBI GEO series GSE27972 was analyzed with R packages oligo and limma to derive differentially expressed genes upon 6h T. gondii (RH) challenge of BMDMs (Morgado et al., 2011).

**RNA-seq**

For each biological assay, 3 x 10^6 BMDMs were seeded per well in six-well tissue culture plates. Cells were left uninfected or infected with the PruΔku80 and PruΔku80 agra28 strains (MOI = 6) for 24 hr. Total RNAs were extracted and purified as described previously (Farhat et al., 2020). RNA-sequencing was performed by GENEWIZ (South Plainfield, NJ, USA) as described previously (Farhat et al., 2020).

**ChIP-seq assay**

HFF cells were infected (MOI = 6) for 24h with RHΔku80 GRA28–HA–FLAG or GRAx–HA–FLAG. Cells were then cross-linked with 1% formaldehyde for 10 min before quenching with 125 mM glycine for 5 min. The ChIP assay was performed by using the Transcription
Factor Chromatin Immunoprecipitation kit (Diagenode) according to the manufacturer’s protocol. In brief, fixed cells were sonicated to shear the cross-linked chromatin into an average DNA fragment size of 200–600 bp. We used 40 × 106 sorted nuclei in 300 μl immunoprecipitation buffer supplemented with fresh proteinase inhibitors. By using a Diagenode Bioruptor precooled to 4°C, shearing was achieved in 1.5-ml low-binding tubes in the appropriate tube adaptor with 18 high-energy cycles of 30 sec on and 30 s off. The aforementioned antibodies were used for immunoprecipitation. After an overnight incubation, the DNA–protein–antibody complex was eluted. The crosslinks were reversed by heating the samples at 65°C for 4 h. DNA was purified by using IPure kit (Diagenode) according to the manufacturer's protocol. 10 ng of your DNA samples were prepared for Illumina sequencing as the following steps: 1) DNA samples were blunt-ended; 2) A dA base was added to the 3’ end of each strand; 3) Illumina's genomic adapters were ligated to the DNA fragments; 4) PCR amplification was performed to enrich ligated fragments; 5) Size selection of ~200–1500bp enriched product using AMPure XP beads. The completed libraries were quantified by Agilent 2100 Bioanalyzer. The libraries were denatured with 0.1 M NaOH to generate single-stranded DNA molecules, captured on Illumina flow cell, amplified in situ. The libraries were then sequenced on the Illumina NovaSeq 6000 following the NovaSeq 6000 S4 Reagent Kit (300 cycles) protocol. After the sequencing platform generated the sequencing images, the stages of image analysis and base calling were performed using Off-Line Basecaller software (OLB V1.8). Sequence quality was examined using the FastQC software. After passing Solexa CHASTITY quality filter, the clean reads were aligned to human genome (UCSC HG19) using BOWTIE software (V2.2.7). Aligned reads were used for peak calling of the ChIP regions using MACS V1.4.2. Statistically significant ChIP-enriched regions (peaks) were identified by IP, using a p-value threshold of 10^-4. The peaks in samples were annotated by the nearest gene using the newest UCSC RefSeq database. The annotation of the peaks which were located within -2Kb to +2Kb around the corresponding gene TSS in samples can be found from the Peaks_Promoter_Annotation.xls file. The signal profile (at 10 bp resolution) with UCSC WIG file format was generated from ChIP-seq data, which can be visualized on UCSC genome browser or IGB browser (Integrated Genome Browser, Java Runtime Environment needed, http://www.bioviz.org/igb/). All wig flies and a brief guide of visualizing the profiles can be found in Data_Visualization folder.

Chromatographic purification of GRA28-containing complexes
Protein extracts from Raw cells infected with RHΔku80 GRA28-HA–FLAG-tachyzoites were incubated with anti-FLAG M2 affinity gel (Sigma-Aldrich) for 1 h at 4°C. Beads were washed with ten column volumes of BC500 buffer (20 mM Tris–HCl, pH 8.0, 500 mM KCl, 20% glycerol, 1 mM EDTA, 1 mM diithiothreitol, 0.5% NP-40 and protease inhibitors). The bound polypeptides were eluted stepwise with 250 μg ml−1 FLAG peptide (Sigma-Aldrich) diluted in BC100 buffer. For size-exclusion chromatography, protein eluates were loaded onto a Superose 6 HR 10/30 column equilibrated with BC500. The flow rate was fixed at 0.35 ml min−1 and 0.5-ml fractions were collected.

Mass spectrometry-based proteomics
Proteins contained in fractions from size-exclusion chromatography of GRA28-containing complexes purified by FLAG co-immunoprecipitation were solubilized in Laemmli buffer and stacked in the top of a 4-12% NuPAGE gel (Invitrogen). After staining with R-250 Coomassie Blue (Biorad), proteins were digested in-gel using trypsin (modified, sequencing purity, Promega) as previously described. The resulting peptides were analyzed by online nanoliquid chromatography coupled to MS/MS (Ultimate 3000 RSLCNano and Q-Exactive Plus, Thermo Fisher Scientific) using a 140 min gradient. For this purpose, the peptides were sampled on a precolumn (300 μm x 5 mm PepMap C18, Thermo Scientific) and separated in a 75 μm x 250 mm C18 column (Reposil-Pur 120 C18-AQ, 1.9 μm, Dr. Maisch). The MS and MS/MS data were acquired by Xcalibur (Thermo Fisher Scientific). Peptides and proteins were identified by Mascot (version 2.6.0, Matrix Science) through concomitant searches against the T. gondii database (ME49 taxonomy, v.30, downloaded from ToxoDB), the Uniprot database (Mus musculus taxonomy, August 2019 version), and a homemade database containing the sequences of classical contaminant proteins found in proteomic analyses (bovine albumin, keratins, trypsin, etc.). Trypsin/P was chosen as the enzyme and two missed cleavages were allowed. Precursor and fragment mass error tolerances were set at respectively at 10 ppm and 25 mmu. Peptide modifications allowed during the search were: Carbamidomethyl (C, fixed), Acetyl (Protein N-term, variable) and Oxidation (M, variable). The Proline software was used for the compilation, grouping, and filtering of the results (conservation of rank 1 peptides, peptide length ≥ 7, PSM score ≥ 25, false discovery rate of peptide-spectrum-match identifications < 1% as calculated on peptide-spectrum-match scores by employing the reverse database strategy, and a minimum of 1 specific peptide per identified protein group). Proline was then used to perform a compilation, grouping and MS1 quantification, based on razor and specific peptides, of the identified protein groups. Proteins from the contaminant database were discarded from the final list of identified proteins. Proteins in each sample were further quantified through calculation of their corresponding intensity-based absolute quantification values (iBAQ). The MS proteomics data have been deposited to the ProteomeXchange Consortium through the PRIDE partner repository with the dataset identifier PXD032360.

QUANTIFICATION AND STATISTICAL ANALYSIS
Western blot analysis was performed by band intensity quantification with ImageJ. qPCR results were analyzed using the ΔΔCq and ΔCq methods from Cq values provided by QuantStudio Design and Analysis Desktop Software. Cell tracks were obtained by manual tracking with ImageJ from image sequence files. Speeds and endpoints from individual cells were calculated and derived from these tracks with the Chemotaxis and Migration Tool and combined with track lengths calculated with R and RStudio. Flow
cytometry data in FCS files was analyzed with FlowJo X. Quantification of RNA-seq and proteomics data was performed as described in the method details. Details on the software used can be found in the key resources table.

**Statistical analyses**

Volcano plots, scatter plots, and histograms were generated with Prism. Statistical analyses were performed with R, RStudio and packages afex (repeated-measures ANOVA), emmeans (Dunnett’s and Holm-Bonferroni post-hoc), DAAG and rcompanion (one sample permutation). Statistical significance is defined as $p < 0.05$. The figure legends detail which hypothesis tests were employed, chosen based on experimental design, the hypothesis to be tested and data distribution, and which statistics are presented. All experiments were performed in biological replicates to allow for statistical analyses and in independent biological replicates as stated for each experiment in the manuscript. The number of $n$ denotes the number of biological replicates (adoptive transfer experiments) or independent experiments.