1   **Differential Impacts on Host Transcription by ROP and GRA Effectors from the**

2   **Intracellular Parasite *Toxoplasma gondii***

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24 **Differential Impacts on Host Transcription by ROP and GRA Effectors from the**

25 **Intracellular Parasite *Toxoplasma gondii***

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30 **ABSTRACT**

31 The intracellular parasite *Toxoplasma gondii* employs a vast array of effector

32 proteins from the rhoptry and dense granule organelles to modulate host cell biology;

33 these effectors are known as ROPs and GRAs, respectively. To examine the individual

34 impacts of ROPs and GRAs on host gene expression, we developed a robust, novel

35 protocol to enrich for ultra-pure populations of a naturally occurring and reproducible

36 population of host cells called uninfected-injected (U-I) cells, which *Toxoplasma* injects

37 with ROPs but subsequently fails to invade. We then performed single cell

38 transcriptomic analysis at 1-3 hours post-infection on U-I cells (as well as on uninfected

39 and infected controls) arising from infection with either wild type parasites or parasites

40 lacking the MYR1 protein, which is required for soluble GRAs to cross the

41 parasitophorous vacuole membrane (PVM) and reach the host cell cytosol. Based on

42 comparisons of infected and U-I cells, the host's earliest response to infection appears

43 to be driven primarily by the injected ROPs, which appear to induce immune and

44 cellular stress pathways. These ROP-dependent pro-inflammatory signatures appear to

45 be counteracted by at least some of the MYR1-dependent GRAs and may be enhanced

46 by the MYR-independent GRAs, (which are found embedded within the PVM). Finally,

47   signatures detected in uninfected bystander cells from the infected monolayers

48   suggests that MYR1-dependent paracrine effects also counteract inflammatory ROP-

49   dependent processes.

50

51   **IMPORTANCE**

52   This work performs the first transcriptomic analysis of U-I cells, captures the

53   earliest stage of a host cell's interaction with *Toxoplasma gondii*, and dissects the

54   effects of individual classes of parasite effectors on host cell biology.

55

56   **MAIN TEXT**

57   **Introduction**

58   The obligate intracellular parasite *Toxoplasma gondii* parasitizes a wide range of

59   avian and mammalian organisms, including humans (1). During the acute phase of

60   infection, this unicellular eukaryote rapidly expands within host tissues by penetrating

61   host cells, establishing and replicating within an intracellular parasitophorous vacuole

62   (PV), and simultaneously avoiding clearance by the host immune system (reviewed in

63   (2)). To orchestrate these events, *Toxoplasma* employs a vast repertoire of effector

64   proteins housed primarily in two secretory organelles, the rhoptries and dense granules

65   (Fig. 1A). The rhoptry organelle effectors (ROPs) are secreted by the parasite into the

66   host cytosol during or immediately prior to invasion by an as yet undefined mechanism

67   (reviewed in (3)). The handful of ROPs that have been characterized to date include

68   virulence factors that disrupt immune clearance mechanisms (4-7), remodel the host's

69   cortical actin cytoskeleton at the point of parasite penetration (8, 9), and co-opt the

70 STAT3 and STAT6 pathways (10-12). In contrast, the dense granule effectors (GRAs)

71 are thought to be secreted later during the invasion process and mostly after the

72 parasite has invaded the host cell (reviewed in (3)). Many GRAs remain in the PV lumen

73 or PV membrane (PVM) (13-17), but others traverse the PVM to reach the host cytosol

74 and often proceed to the host nucleus (18-24). The translocation of this latter class of

75 GRAs across the PVM is dependent on a group of PVM proteins called the MYR

76 complex, i.e., MYR1, MYR2, and MYR3, which are so-named because they are

77 required for parasite-dependent host c-Myc regulation (25-27). Host signaling pathways

78 modulated by the PVM-embedded, MYR-independent GRAs (MIGs) include the nuclear

79 factor kappa light chain enhancer of activated B cells pathway. Host pathways

80 modulated by MYR-dependent GRAs (MDGs) include the interferon gamma (IFN-

81 gamma), mitogen activated protein kinase, and p53 pathways, as well as the cyclin E

82 regulatory complex (22, 23, 28).

83 　　　The collective host response to ROPs, MDGs, MIGs, and other perturbations

84 during infection with *Toxoplasma gondii* have been well-documented in transcriptomic

85 studies that compare infected and mock-infected cells; however, the contribution of

86 individual parasite effector compartments to the overall picture is more poorly defined,

87 particularly because many parasite effectors are introduced during the earliest stages of

88 infection in very narrow time intervals. Specifically, invasion is a rapid process that takes

89 approximately 40 seconds (29), and deployment of the ROPs likely occurs during the

90 first third of invasion in one shot (30). In contrast, MDGs have been detected by

91 immunofluorescence assays in the nuclei of parasitized host cells at ~3 hours post-

92 invasion at the earliest (19), which suggests that they likely modulate host transcription

93   much later than ROPs. Parasite mutants that lack a functional MYR complex have

94   helped separate the impact of MDGs from those of other parasite effectors (25, 26, 31),

95   but the specific impact on host transcription by ROPs and MIGs has yet to be

96   determined. The rhoptry organelle's contribution is of particular interest given that ROP

97   injection is essential to parasite invasion, survival, and virulence, and that the functions

98   of most ROPs are unknown.

99   Here we document the impact of specific classes of parasite effectors, including

100   for the first time ROPs, by leveraging a rare population of host cells that parasites inject

101   with ROPs but subsequently fail to invade (32). These uninfected-injected (U-I) host

102   cells (Fig. 1B) do not arise as a result of parasite-derived exosomes delivering effectors

103   to host cells (32) or due to impending host cell death (Fig. 2B). They arise

104   spontaneously in tissue culture during *Toxoplasma* infection and may also arise *in vivo*

105   in the brains of mice chronically infected with *Toxoplasma* (32). To interrogate the host

106   response specifically to effectors injected before invasion, we developed a novel

107   pipeline to purify and perform single cell RNA sequencing (scRNA-seq) of U-I cells, as

108   well as of infected and uninfected controls from the same host cell monolayer. To

109   resolve the impact of effectors released during vs. after invasion, we also analyzed U-I

110   cells, infected cells, and uninfected cells from host cell monolayers infected with

111   parasites lacking MYR1, a component of the complex required for translocation of

112   MDGs into the host cytosol (25, 26). Our findings reveal new insight into the impact of

113   individual parasite effector compartments on the biology of *Toxoplasma*-infected host

114   cells.

115

116 **Results**

117 ***A Novel FACS-Based Single Cell RNA Sequencing Pipeline Captures***

118 ***Transcriptomic Signatures of Individual Effector Compartments***

119   According to the current model of parasite effector secretion (Fig. 1A), the host

120 response to an infection with *Toxoplasma* tachyzoites can be attributed to 5 parasite-

121 dependent stimuli: 1) the secretion of paracrine effectors into the extracellular milieu; 2)

122 injection of ROPs; 3) activity of MIGs; 4) secretion of MDGs; and 5) parasite invasion

123 and establishment of the PV. To resolve the individual impacts of each of these classes

124 of stimulus, we transcriptomically profiled infected, uninfected, and U-I host cells from

125 monolayers exposed to parasites. To this end, we devised a novel pipeline in which: 1)

126 infections were designed to generate a heterogeneous pool of host cells each impinged

127 upon by one of several combinations of parasite-dependent stimuli; 2) a fluorescence

128 activated cell sorting (FACS)-based protocol was employed to separate host cells of

129 interest from infected monolayers based on the parasite-dependent stimuli by which

130 they were affected; and 3) the host cell transcriptomes were profiled by full-length

131 scRNA-seq. Given the novelty and complexity of this pipeline, before we delineate our

132 biological findings, here we present an account of our strategy and technical findings

133 during pipeline development.

134

135 *FACS-Based Isolation of Host Cells for Single Cell RNA Sequencing Purifies*

136 *Uninfected-Injected Cells*

137   To generate host cells impacted by various combinations of parasite-dependent

138 stimuli for downstream RNA-seq, we subjected the host cells to infection with either wild

139　type RH (type I) strain parasites, mutant *Δmyr1* parasites lacking the MYR1 protein

140　(constructed from an RH *Δmyr1* mCherry parental strain (25); Fig. S1)), or a parasite-

141　free cell lysate (i.e., mock infection) from the feeder human foreskin fibroblast (HFF)

142　cells used to maintain both parasite lines. From here on, we designate host cells that

143　arose from the wild type, *Δmyr1*, and mock infections as Wt, Dmyr1, or Mock cells,

144　respectively. All parasites were engineered to express the ROP fusion protein toxofilin-

145　beta-lactamase (Tfn-BLA) as well as a constitutive red fluorescent protein. In addition,

146　we chose 10 T1/2 mouse fibroblasts as the preferred host cell type over conventionally

147　used HFFs, which we found unsuitable for single cell sorts due to their propensity to

148　remain clumped in FACS buffer.

149　　　As each true infection results in a heterogeneous monolayer containing U-I cells,

150　infected cells (designated I-I for being infected and injected), and uninfected cells

151　(denoted U-U for being uninfected and uninjected; Fig. 1B), the infection conditions

152　yielded seven key species of host cell: Wt U-I, Wt I-I, Wt U-U, Dmyr1 U-I, Dmyr1 I-I,

153　Dmyr1 U-U, and Mock. Each of these species was expected to be influenced by a

154　specific combination of parasite-dependent stimuli (Fig. 1C). By comparing the

155　transcriptomes of key pairs of these seven cell species (see Materials and Methods,

156　Data Availability), we expected to uncover the impact of previously unexamined

157　parasite-dependent stimuli, e.g., ROPs (summarized in Fig. 1D). Of note, these

158　experimental conditions enabled transcriptomic assessment of paracrine effects during

159　*Toxoplasma* infection (by comparison of U-U vs. mock-infected cells) and generated

160　pure I-I and U-I cell populations at extremely short infection durations, an improvement

161 over traditional methods that rely on high multiplicities of infection and long infection

162 times to distinguish infected vs. uninfected transcriptomic signatures.

163 To capture individual cells from each cell species, we devised a FACS-based

164 protocol that purified each species from the host cell monolayers (Fig. 2A). The pipeline

165 employed the toxofilin-beta-lactamase (Tfn-BLA) assay (8), in which the (mock)-infected

166 host cell monolayers were stained for 30 minutes with CCF2-AM, a reporter dye that is

167 taken up by live host cells and shifts from green to blue fluorescence when cleaved by

168 beta-lactamase. CCF2-AM staining occurred at 30, 90, or 150 minutes post-infection, to

169 yield cells at 1, 2, or 3 hours post-infection (hpi). In addition, CCF2-AM staining was

170 performed at room temperature (23 °C) to preserve the integrity of the CCF2-AM dye,

171 which decomposes at 37 °C. U-I cells were then FACS sorted based on their blue

172 (cleaved) CCF2-AM fluorescence and their absent red fluorescence (since they lacked

173 internalized parasites). In addition, I-I cells were sorted for their double positive blue

174 (from cleaved CCF2-AM) and red (from internalized parasites) fluorescence, and U-U

175 and Mock cells were sorted for their green (cleaved) CCF2-AM fluorescence. Single

176 cells and bulk populations of 50-100 cells of each cell species were collected.

177 To ensure confidence in the identity of the sorted cells and to limit the number of

178 parasites per I-I cell to one, we gated stringently during FACS, particularly for the "U-I

179 cells" gate and "parasite-associated" gate (which included I-I cells; Fig. 2B). We also

180 subjected all cells to extensive washes before FACS sorting, as debris from the human

181 feeder cells used to culture the parasites contaminates the U-I gate (Fig. S2), likely due

182 to retention of both CCF2-AM dye and the parasite fusion protein Tfn-BLA. Of note, the

183 debris posed significant challenges to downstream bioinformatic analyses of bulk U-I

184 cells, but they registered as single cells in the scRNA-seq pipeline and were

185 automatically discarded during quality control due to their high percentage of human

186 reads. At a multiplicity of infection of ~6, U-I cells constituted ~8.8% of the injected (i.e.,

187 U-I + I-I) cell population at 1 hpi, ~2.0% at 2 hpi, and ~3.4% at 3 hpi (Fig. 2C).

188 Abundances of sorted U-I, I-I, and U-U cells relative to all sorted cells are depicted in

189 Fig. 2D.

190   To validate U-I cells as appropriate models for host responses to injected

191 parasite effectors, we also limited the possibility of U-I cells arising by mechanisms

192 other than aborted invasion events. One such mechanism is host immune clearance of

193 internalized parasites. However, this route is an unlikely source of U-I cells in our

194 pipeline for two reasons: 1) immune clearance of intracellular pathogens in mouse cells

195 is activated by exogenous IFN-gamma, which we did not add to the host cells, and 2) all

196 known strains of *Toxoplasma* escape IFN-gamma-mediated pathogen clearance by

197 suppressing the IFN-gamma signaling pathway if they infect the host cell before it is

198 exposed to extracellular IFN-gamma (33, 34). In another potential mechanism, an

199 infected host cell may divide and produce an uninfected daughter (a U-Id cell, for U-I by

200 division). To limit the occurrence of U-Id cells, we serum starved all host cells for the 24

201 hours preceding infection to induce cell cycle arrest (Fig. S3A) and limited infection

202 durations to 3 hpi or less, as live video microscopy of 200 parasite-infected, serum-

203 starved 10 T1/2 host cells for 16 hours revealed that no infected cell divided before 3.67

204 hpi (Fig. S3B). Finally, a newly internalized parasite might spontaneously exit early from

205 the host cell, a rare and poorly characterized process that is therefore difficult to control.

206 Though we cannot exclude the possibility that a very small number of our U-I cells arose

207 from this mechanism of premature parasite egress, and though U-Id production cannot

208 be completely ruled out, an advantage of scRNA-seq is its ability to distinguish

209 differences between cells arising via different mechanisms (assuming they differ

210 transcriptomically).

211

212 *Technical Validation of Single Cell Sequencing for Cells Exposed to or Parasitized by*

213 Toxoplasma gondii

214 Given the limited duration of infection ($\leq$ 3 hours), we expected relatively subtle

215 transcriptional changes in U-I and I-I cells. To maximize the sensitivity of scRNA-seq

216 analyses to detect such signatures, we employed Smart-seq2 library preparation and

217 sequenced to a depth of 1 million reads per cell. All reads were aligned to a

218 concatenated mouse-*Toxoplasma* genome using the genomic sequence for the GT1

219 parasite strain, a clonal relative of RH (35, 36). In addition, we identified and discarded

220 43 mouse genes to which reads from extracellular RH parasites aligned (mostly

221 representing evolutionarily conserved genes; Table S1).

222 To ensure that poorly amplified or poorly sequenced host cells did not confound

223 downstream analysis, we filtered samples based on several quality metrics (Materials

224 and Methods), yielding 453, 2,026, and 2,875 cells at 1, 2, and 3 hpi, respectively, for

225 downstream analysis (Fig. S4A). Mapping efficiency of the analyzed cells was >85% in

226 all experimental trials (Fig. S4B). Characterization of measurement sensitivity based on

227 logistic regression modeling of ERCC spike-in standards revealed a 50% detection rate

228 of 31, 11, and 34 RNA molecules per cell at 1 hpi, 2 hpi, and 3 hpi, respectively (Fig.

229 S4C), amounting to a level of sensitivity comparable to that previously reported (37).

230   After filtering out genes with expression above the detection limit in <6 cells, we

231   normalized for sequencing depth across cells by dividing each read count by the

232   median read sum to yield counts in units of counts per median (cpm) (Materials and

233   Methods). Gene expression in the scRNA-seq dataset exhibited a strong positive

234   correlation to expression in bulk RNA-seq samples processed the same way specifically

235   for differentially expressed genes (DEGs) between experimental conditions (i.e., U-U, I-I,

236   and U-I cells; Fig. S4D). Furthermore, each bulk experiment's expression data exhibited

237   the best correlation with their cognate single cell data (Fig. S4E). Taken together, these

238   data demonstrate that scRNA-seq captures similar transcriptomic signatures in host

239   cells to those detected in bulk RNA-seq experiments, validating the scRNA-seq platform

240   as an approach to further characterize host cell transcriptomic responses during

241   *Toxoplasma* infection.

242

243   *Single Cell Resolution Reveals Cell-to-Cell Heterogeneity Inaccessible to the Bulk RNA*

244   *Sequencing Platform*

245   A key advantage of single cell resolution is that it enables identification and

246   separation of parasite-independent host cell heterogeneity. Accordingly, single cell

247   resolution facilitates an extra checkpoint to validate the identities of U-I, I-I and U-U cells

248   by quantifying the percentage of *Toxoplasma*-derived reads in each cell. As expected,

249   *Toxoplasma* read content across all cells exhibited a bimodal distribution, where most

250   uninfected cells contained <0.01% *Toxoplasma* reads, while most infected cells

251   possessed higher percentages, i.e., 0.5-4%. However, a small proportion of U-I cells

252   and I-I cells exhibited unexpectedly high (for U-I) or low (for I-I) *Toxoplasma* read counts

253 and were considered to be misclassified (Fig. S5A). This may have resulted from a low

254 rate of TdTomato or mCherry loss in some of the parasites (for cells misclassified as U-I)

255 or from attached but not fully invaded parasites being dislodged from the host cell at

256 some point between fluorescence detection and deposition of the cell into lysis buffer

257 (for cells misclassified as I-I). Such misclassified cells may have contributed significant

258 and potentially misleading signatures to their respective samples. To preclude this

259 possibility in our single cell dataset, we excluded all U-I cells with >0.04% *Toxoplasma*

260 reads and all I-I cells with <0.32% *Toxoplasma* reads from downstream analysis.

261   Next, because the experimental pipeline examined cells during their earliest

262 interaction with *Toxoplasma*, we expected the subtle, parasite-dependent transcriptional

263 signatures to be potentially eclipsed by intrinsic host processes such as cell cycle,

264 which still progressed in some cells even with serum starvation (Fig. S3A). To separate

265 host cell cycle from other biological processes the parasites could potentially modulate,

266 we determined the phases of all single cells (i.e., G1, S, or G2/M) based on expression

267 of 175 curated cell cycle marker genes (Fig. S5B; Table S2; Materials and Methods). A

268 breakdown of cell cycle phase composition for each experimental condition across all

269 time points revealed a consistent pattern at 2 hpi in which the proportion of cells in G2

270 or M phase increased in a manner that appeared dependent on injected effectors, i.e.,

271 from 13.3% in Mock to 33.9% in Wt U-I, 41.4% in Dmyr1 U-I, and 22.5% in CytD U-I; Fig.

272 S5C). These findings are consistent with potential induction of cell cycle arrest in U-I

273 cells by injected effectors, as previously reported (38-41).

274   To identify other sources of heterogeneity in the dataset, we used the *Uniform*

275 *Manifold Approximation and Projection* (UMAP) algorithm for dimensionality reduction

276 and to visualize relationships between all cells based on the most dispersed (i.e.,

277 variable) genes. Leiden clustering revealed three distinct clusters, designated herein as

278 populations 1, 2, and 3 (Fig. S5D). Curiously, cells from different time points exhibited

279 differences in the proportion of cells belonging to each population, where 2 hpi cells fell

280 entirely in population 1, and 3 hpi cells fell in all three populations. In conventional single

281 cell analysis, dimensionality reduction and cell clustering are used to identify novel cell

282 types. Though the 10 T1/2 host cell line used in our experiments is clonal, it is derived

283 from a pluripotent stem cell population that has a propensity to differentiate, such that

284 the three identified populations could conceivably represent distinct differentiation states

285 (42).

286   To limit cell-to-cell heterogeneity from overshadowing potentially subtle

287 transcriptomic signatures induced by parasite effector secretion and parasite invasion,

288 we limited our remaining analyses to host cells in G1 phase (to limit cell cycle

289 signatures) and from UMAP population 1 (to factor out potential cell type signatures).

290

291 ***Injected Parasite Effectors Drive Inflammatory Transcriptional Signatures***

292 ***Associated with Parasite Infection***

293   Having established a robust pipeline to isolate, RNA sequence, and

294 bioinformatically analyze the transcriptomes of individual cells from each of seven

295 relevant cell species (i.e., Wt U-I, Wt I-I, Wt U-U, Dmyr1 U-I, Dmyr1 I-I, Dmyr1 U-U, and

296 Mock cells; Fig. 1C), we next sought to determine host responses to each of five

297 individual classes of parasite-dependent stimuli, namely rhoptry proteins (ROPs), MYR-

298 dependent dense granule proteins (MDGs), MYR-independent dense granule proteins

299   (MIGs), parasite invasion, and paracrine effects. To isolate each parasite-dependent

300   stimulus, we used the Model-based Analysis of Single Cell Transcriptomics (MAST)

301   algorithm to perform differential gene expression analysis on key pairs of the seven

302   relevant cell species from the pool of G1 phase, UMAP population 1 cells. Across all

303   pairwise comparisons between conditions within each time point, 39, 2,252, and 10,995

304   differentially expressed genes (DEGs) were detected at 1 hpi, 2 hpi, and 3 hpi,

305   respectively (Table S3A). These results show that parasite effectors and invasion result

306   in almost no detectable transcriptional response at 1 hpi, a modest response involving

307   regulation of a core group of genes at 2 hpi, and ramping up of this response at 3 hpi in

308   which transcription in a much larger set of host cells is modulated. Because of the

309   negligible response at 1 hpi, we excluded data from this time point from the remaining

310   analyses.

311   For additional validation, we assessed the 2 hpi and 3 hpi datasets for their

312   agreement with two previous bulk RNA-seq studies that captured the host response to

313   infection at 6 hpi in nominally the same parasite strains and culture conditions, albeit in

314   HFFs instead of mouse 10 T1/2 fibroblasts (10, 31). Because these studies measured

315   the infection response by comparing infected vs. mock-infected cells, we examined

316   DEGs between Wt I-I and mock-infected (i.e., Mock) cells in our own datasets. Gene set

317   enrichment analysis (GSEA) of the resulting DEGs at 2 hpi and 3 hpi (Table S3B) using

318   the Molecular Signatures Database's Hallmark gene sets (43) revealed that nearly all

319   significantly enriched gene sets at 2 hpi and 3 hpi were previously identified in the Naor

320   et al. reference dataset, and many pertained to immune processes (Fig. 3; 31). Of note,

321   gene sets were considered to be significantly enriched if their false discovery rates were

322 < 0.25, a standard cutoff for GSEA given the lack of coherence in most transcriptional

323 datasets and the relatively low number of gene sets being analyzed. Nearly all gene

324 sets *not* preserved in the Naor et al. reference were enriched from genes

325 *down*regulated upon infection. The lack of agreement between our downregulated gene

326 sets and those obtained from the references likely reflects the infection response's

327 tendency towards gene *up*regulation, and to stochasticity in expression levels for the

328 substantially fewer downregulated genes detected, an interpretation corroborated by the

329 downregulated genes' higher p-values and lower fold changes. Overall these results

330 indicate that the 2 hpi and 3 hpi datasets capture well-known host responses to infection

331 with *Toxoplasma gondii.*

332   Next, to determine the host response to injection of parasite effectors, we

333 compared U-I cells from wild type parasite infection (i.e., Wt U-I cells) to uninfected cells

334 from the same monolayer (i.e., Wt U-U cells). At 2 hpi, only 10 DEGs were identified

335 (Table S3C), and GSEA of these DEGs revealed no significant enrichment of the

336 Hallmark gene sets. Therefore, injection of parasite effectors without invasion appears

337 to elicit only a trace response at 2 hpi. At 3 hpi, 156 DEGs were detected between Wt

338 U-I and Wt U-U cells (Table S3C), for which GSEA revealed at total of 17 gene sets (Fig.

339 4A); 14 corresponded to genes expressed higher in Wt U-I cells than in Wt U-U cells,

340 and several were associated with inflammation. In addition, 10 gene sets were common

341 to the infection response, i.e., also enriched in Wt I-I vs. Mock DEGs (Fig. 3), which

342 suggests that much of the early response to parasite infection is driven by the injection

343 of parasite effectors (likely ROPs) into host cells prior to invasion.

344   To confirm that the transcriptional signatures in Wt U-I cells originated from ROP

345   injection, we made two additional comparisons. For the first, we compared U-I cells

346   from *Δmyr1* parasite infection (i.e., Dmyr1 U-I cells) to uninfected cells from the same

347   monolayer (i.e., Dmyr1 U-U cells). As MDGs fail to traverse the PVM during *Δmyr1*

348   parasite infections, the absence of MYR1 should effectively limit the parasites' effector

349   repertoire to ROPs and MIGs; consequently, transcriptional signatures from Dmyr1 U-I

350   cells and Wt U-I cells should largely resemble one another. In the second comparison,

351   to account for the possibility that Wt U-I signatures originated from a pre-existing

352   difference in those host cells from their neighbors, rather than from injected effectors,

353   we collected U-U, U-I, and I-I cells from host monolayers exposed to wild type parasites

354   pre-treated with the invasion inhibitor cytochalasin D, which allows parasite attachment

355   but blocks subsequent invasion. As expected, cytochalasin D treatment increased the

356   proportion of U-I cells in infected monolayers by ~6-fold at 2 hpi and by ~9.3-fold at 3

357   hpi (Fig. S6), so at least 85-90% of the artificially induced U-I cells (i.e., CytD U-I cells)

358   presumably arose from drug-induced abortion of parasite invasion events, rather than

359   from parasite-independent host cell differences. We predicted that transcriptional

360   signatures detected in CytD U-I cells would be driven almost entirely by injected

361   parasite effectors and would mirror those identified in both Wt U-I and Dmyr1 U-I cells.

362   As expected, at 2 hpi very few DEGs (29 and 51, respectively) and no enriched

363   gene sets were identified for Dmyr1 and CytD U-I cells vs. their corresponding U-U cells,

364   while at 3 hpi substantially more DEGs (103 and 174 for Dmyr1 and CytD U-I vs. U-U

365   cells, respectively) and gene sets were identified (Table S3D; Table S3E). The 3 hpi

366   gene sets exhibited a strong degree of overlap with the original Wt U-I vs. Wt U-U

367 comparison, such that 14 of the 17 Wt U-I vs. Wt U-U gene sets were also identified in

368 one or both of the corresponding Dmyr1 and CytD comparisons (Fig. 4B). These data

369 are consistent with an inflammatory response to ROP injection that drives much of the

370 host cell's total response to parasite infection.

371   In light of the many shared genes and gene sets between the infection vs.

372 injection responses, we next sought to define the distinction between these responses

373 by comparing Wt U-I (injected) to Wt I-I (infected) cells using two complementary

374 approaches. In the first approach, we used MAST to identify DEGs between Wt U-I and

375 Wt I-I cells and subjected the DEGs to GSEA. To better compare the trajectories of Wt

376 I-I and Wt U-I gene expression, we also computed fold changes in DEG expression

377 between each of these conditions and Wt U-U cells (Table S3F). In the second

378 approach, we identified genes with significant differences between Wt U-I and Wt I-I

379 cells in their correlation to a quantity called the CCF2-AM ratio, i.e., the ratio of blue

380 (cleaved) to green (uncleaved) CCF2-AM indicator dye detected during FACS sorting.

381   In this second approach, the CCF2-AM ratio was used as a quantitative proxy for

382 the influence of parasite-dependent effectors on individual host cells. Briefly, in the

383 strictest sense, the CCF2-AM ratio is a biological readout for penetration of a given host

384 cell by the injected ROP fusion protein Tfn-BLA, as the intracellular CCF2-AM dye in our

385 pipeline is cleaved by the beta-lactamase in Tfn-BLA. Since the host cells should exhibit

386 more or less equal loading of the CCF2-AM substrate, we presumed that the extent of

387 conversion in CCF2-AM signal from green (uncleaved) to blue (cleaved) reflected both

388 the concentration of Tfn-BLA protein introduced into the cell and the amount of time it

389 had spent within the cell. In Wt U-I cells, Tfn-BLA penetration occurs concomitantly with

390 injection of the other ROP effectors; therefore, the CCF2-AM ratio can be interpreted as

391 a quantitative measure for ROP penetration in Wt U-I cells. Wt I-I cells, however, are

392 presumably penetrated not only by ROPs but also by MIGs, MDGs, and the parasites

393 themselves. Accordingly, since the amount of ROPs injected into cells and the time

394 ROPs spend inside cells likely track with the same quantities for the remaining parasite-

395 dependent stimuli, we used the CCF2-AM ratio in Wt I-I cells as a proxy for the

396 presence of all four parasite stimuli inside each cell. Next, we computed the Spearman

397 correlation between each gene's expression and the CCF2-AM ratio separately in both

398 Wt U-I cells and Wt I-I cells. Of note, because we calculated the correlation by

399 incorporating the CCF2-AM ratios from Wt U-U cells (which should be devoid of ROPs,

400 MIGs, MDGs, and parasites) as negative controls for both the Wt U-I and Wt I-I

401 analyses, the correlation data do not encapsulate the influence of parasite-dependent

402 stimuli that Wt U-U cells have in common with Wt U-I and Wt I-I cells, i.e., paracrine

403 factors. Finally, we identified genes that exhibited significant shifts between Wt U-I and

404 Wt I-I cells in their relationship to the CCF2-AM ratio (Table S4A) by first modeling a

405 Gaussian distribution of their Spearman correlations to the CCF2-AM ratio (Fig. 5A).

406 Genes that significantly deviated from the Gaussian distribution and that also exhibited

407 a sufficient difference in their CCF2-AM correlation scores in Wt U-I vs. Wt I-I cells were

408 interpreted to be associated with either ROP injection alone (in Wt U-I cells) or with all

409 secreted effectors plus parasites (in Wt I-I cells, in which ROP penetration should be

410 accompanied by all other parasite-induced insults).

411 Analysis of Wt I-I vs. Wt U-I cells at 2 hpi revealed no significant DEGs, pointing

412 to a profound similarity in host transcription in Wt I-I and Wt U-I cells at 2 hpi. However,

413 the corresponding CCF2-AM correlation data exhibited a higher sensitivity for detecting

414 differences between Wt I-I vs. Wt U-I cells and yielded 81 significant deviants from the

415 Gaussian distribution (Fig. 5A, left panel). Of the deviants, 73 (~90.1%) fell in regions of

416 the scatterplot where genes were either: 1) negatively correlated with the CCF2-AM

417 ratio in one condition and positively correlated in the other; or 2) had stronger positive or

418 negative correlations in Wt U-I cells than in Wt I-I cells as determined by a Gaussian

419 model (Fig. 5A, left panel, blue regions; Table S4A). This provides evidence for

420 downstream events in infection (i.e., release of dense granule proteins, parasite

421 invasion) suppressing effects of genes induced by ROP injection as early as 2 hpi.

422 At 3 hpi, 122 DEGs and 423 CCF2-AM correlation deviants were identified for Wt

423 I-I vs. Wt U-I cells, pointing to a divergence in the injection vs. infection responses at

424 this later time point. Of the DEGs, 100 (~82.0%) exhibited opposing trajectories in Wt I-I

425 vs. Wt U-I cells (Table S3F), i.e., they were either: 1) upregulated in one cell type and

426 downregulated in the other compared to a common Wt U-U standard; or 2) more

427 upregulated or downregulated in Wt U-I cells compared to the standard than in Wt I-I

428 cells. In addition, of the CCF2-AM correlation deviants, 333 (~79%) exhibited at least a

429 moderate negative correlation with the CCF2-AM ratio in Wt I-I cells, an observation

430 consistent with these genes being downregulated in response to the combination of all

431 parasite-derived insults and effectors during infection (Fig. 5A, right panel; Table S4A).

432 Furthermore, 282 (~67%) of the deviants fell in regions of the scatterplot in which the

433 impact of ROPs is dampened by dense granule proteins (GRAs) and parasite invasion

434 (Fig. 5A, right panel, blue regions; Table S4A), which is consistent with host responses

435 in Wt U-I cells being counteracted by parasite effectors that are operating in Wt I-I cells

436   only. This lends further support to the notion that effectors injected circa invasion (i.e.,

437   ROPs) are counterbalanced by subsequently introduced effectors (i.e., GRAs). Of note,

438   the remaining 141 CCF2-AM deviant genes at 3 hpi fell within the sections of the

439   scatterplot in which the effects of ROPs are enhanced by those of the GRAs and

440   parasite invasion (Fig. 5A, right panel, yellow regions; Table S4A). This suggests that

441   while GRA release and parasite penetration may counterbalance some ROP-induced

442   host responses, these events may also enhance other genes induced by ROP injection.

443   To discern the biological significance of the differences between the infection vs.

444   injection responses, we performed GSEA on Wt U-I vs. Wt I-I DEGs. The resulting

445   enriched gene sets (Fig. 5B) included many that were previously identified in the host

446   infection response, with gene sets enriched from genes expressed higher in Wt I-I cells

447   corresponding to more inflammatory processes. Because the vast majority of genes

448   with higher expression in Wt U-I cells than in Wt I-I cells exhibited evidence of ROP

449   effectors being counteracted by subsequently secreted effectors such as MDGs and

450   MIGs (84 out of 93 genes; Table S3F), these findings are consistent with injection-

451   associated inflammatory host processes ramping up upon parasite penetration (and

452   GRA release), while other injection-associated processes are dampened by these later

453   events.

454

455   ***Parasite Effectors Counteract One Another to Yield a Modest Host Response to***

456   ***Early Parasite Infection***

457   Next, we sought to determine the contribution of individual parasite effector

458   compartments to the difference between the host infection (in Wt I-I cells) vs. ROP

459   injection (in Wt U-I cells) responses. Because Wt U-I and Wt I-I cells are both injected

460   with ROPs and originate from the same monolayer, ROPs and paracrine effectors likely

461   do not explain the differences between these two cell types. Instead, MDGs, MIGs, and

462   the act of parasite penetration itself are, *a priori,* most likely to explain these differences.

463   Our dataset, which includes infected (I-I), bystander uninfected (U-U), and U-I cells

464   originating from monolayers infected with wild type and *Δmyr1* parasites, presents a

465   unique opportunity to examine the impacts of these compartments in isolation.

466   To determine how individual effector compartments contribute to the

467   transcriptomic differences between Wt I-I vs. Wt U-I cells, we made two key

468   comparisons. In the first comparison, we examined Dmyr1 U-I cells, which presumably

469   respond to ROP injection, and Dmyr1 I-I cells, which are thought to respond to ROP

470   injection plus MIGs and parasite penetration (Fig. 1C; Fig. 1D). We predicted that MIGs

471   and parasite penetration would account for the differences between Dmyr1 I-I and

472   Dmyr1 U-I cells, and for a subset of the differences between Wt U-I and Wt I-I cells. In

473   the second comparison, we examined Wt I-I vs. Dmyr1 I-I cells, which originate from

474   monolayers infected with wild type and *Δmyr1* parasites, respectively. Signatures

475   detected in Dmyr1 I-I cells likely reflect the host response to the combination of ROP

476   injection, MIG activity, parasite penetration of host cells, and paracrine effectors

477   secreted into the extracellular milieu, while those detected in Wt I-I cells likely reflect the

478   response to these elements plus MDG secretion (Fig. 1C). Therefore, we predicted that

479   comparing Dmyr1 I-I vs. Wt I-I would illustrate the impact of MDGs, as well as any

480   paracrine effects dependent on the presence of MYR1 (Fig. 1D), and that this would

481 account for a second subset of the differences between the infection vs. injection

482 response showcased in the Wt I-I vs. Wt U-I comparison.

483

484 *MYR-Independent GRAs and Parasite Penetration Collectively Enhance ROP-induced*

485 *Inflammatory Responses*

486   To examine the host response to the combination of MIGs and parasite

487 penetration, we compared Dmyr1 U-I cells, which should be penetrated by ROPs, and

488 Dmyr1 I-I cells, into which parasites should secrete ROPs and MIGs, but not MDGs (Fig.

489 1C; Table S3G). At 2 hpi, only 5 DEGs and no GSEA enriched gene sets were identified,

490 implying profound similarity between Dmyr1 U-I and Dmyr1 I-I cells at this time point (as

491 was also the case for Wt U-I vs. Wt I-I cells). At 3 hpi, 80 DEGs were identified, pointing

492 to a slight divergence between these two cell types at this time point. 48 (60%) of the

493 DEGs exhibited evidence of being influenced by effectors that counteract one another's

494 effects (i.e., their expression exhibited either: 1) opposing trends in Dmyr1 U-I vs.

495 Dmyr1 I-I cells when compared to an uninfected Dmyr1 U-U standard; or 2) a stronger

496 induction or suppression in Dmyr1 U-I cells than in Dmyr1 I-I cells), which suggests that

497 MIGs may play a role in neutralizing the effects of the ROPs preceding them. Note,

498 however, that we cannot exclude the possibility that this effect is attributable to stimuli

499 related to physical penetration by the parasites.

500   GSEA of the 3 hpi DEGs identified 15 enriched gene sets (Fig. 6), of which 9

501 were also found to be enriched in the Wt U-I vs. Wt I-I comparison (Fig. 5B). For gene

502 sets enriched from genes expressed higher in Dmyr1 I-I cells than in Dmyr1 U-I cells,

503 nearly all corresponded to gene sets already identified as part of the host response to

504 ROP injection and/or were associated with inflammatory processes. In contrast, none of

505 the gene sets enriched from genes expressed higher in Dmyr1 U-I cells were identified

506 as part of the injection response and instead corresponded to other processes, i.e.,

507 complement cascade, coagulation, MTORC1 signaling, and myogenesis. Taken

508 together, these results suggest that MIGs and parasite penetration do indeed account

509 for some of the difference between the infection response (in Wt I-I cells) vs. the

510 injection response (in Wt U-I cells). More specifically, genes corresponding to

511 inflammatory processes that are already induced upon ROP injection (in Wt U-I and

512 Dmyr1 U-I cells) appear to be further induced, i.e., enhanced, by the combination of

513 MIGs and parasite penetration, while genes for which the effect of MIGs + Parasites

514 dampen the influence of the ROPs appear to correspond to a different set of cellular

515 processes. Although this latter set of genes accounts for the majority (60%) of Dmyr1 U-

516 I vs. Dmyr1 I-I DEGs, the majority of the total enriched gene sets corresponds to cellular

517 processes enhanced rather than dampened by MIGs + Parasites, and these enhanced

518 gene sets collectively exhibit much higher statistical significance. This raises the

519 possibility that at least some processes dampened by MIGs + Parasites may not be

520 adequately captured by the Hallmark gene sets.

521

522 *MYR-Dependent GRAs Counterbalance Parasite Effectors Released Earlier in the Lytic*

523 *Cycle*

524   A previously published bulk RNA-seq experiment comparing host transcription in

525 monolayers infected with wild type vs. *Δmyr1* parasites at 6 hpi (31) revealed a set of

526 genes such that in the absence of MYR1, expression changes were unmasked, while in

527   the presence of MYR1 (i.e., during wild type infection), there was no net change. This

528   work implies that collectively, MDGs and associated paracrine effectors secreted during

529   infection with wild type but not *Δmyr1* parasites participate in counterbalancing

530   transcriptional signatures induced by prior parasite-dependent stimuli.

531   To determine whether MDGs and associated paracrine effects play a similar role

532   in the present single cell datasets, and to ascertain their contribution to the difference

533   between the infection vs. injection responses, we compared Dmyr1 I-I and Wt I-I cells.

534   MAST analysis of this comparison identified 46 and 367 DEGs at 2 hpi and 3 hpi,

535   respectively (Table S3H). While GSEA of the 2 hpi DEGs returned no significantly

536   enriched gene sets, GSEA at 3 hpi revealed 16 significantly enriched gene sets, of

537   which all but one were enriched from genes expressed higher in Dmyr1 I-I cells than in

538   Wt I-I cells (Fig. 7A). Furthermore, 8 gene sets were identified in the previously

539   published bulk RNA-seq experiment comparing infections with wild type vs. *Δmyr1*

540   parasites (31), while 6 corresponded to infection-associated gene sets (i.e., were

541   enriched from Wt I-I vs. Mock DEGs), and 8 were identified as part of the ROP injection

542   response (i.e., were enriched from Wt U-I vs. Wt U-U DEGs). Taken together, these

543   data are consistent with MDGs and/or their associated paracrine effects selectively

544   impinging on Wt I-I cells and dampening the effects of parasite effectors introduced into

545   host cells earlier in the lytic cycle. The overlap between these gene sets and those of

546   the injection response shows that some of the effectors whose responses were

547   dampened by MDGs and associated paracrine effects include injected effectors (i.e.,

548   ROPs), which was previously suspected (31) but never before explicitly demonstrated.

549   Though comparing Dmyr1 I-I vs. Wt I-I cells illustrates the collective impact of

550   MDGs and MYR-dependent paracrine effects on host transcription (Fig. 1D), this

551   comparison cannot distinguish the individual impacts of these two stimuli. To determine

552   the effect of MDGs alone, we performed CCF2-AM ratio correlation analysis on Wt I-I vs.

553   Dmyr1 I-I cells (Table S4B), as was performed for Wt I-I vs. Wt U-I. As was the case for

554   computation of both Wt I-I and Wt U-I CCF2-AM correlations, this type of analysis

555   excludes the impact of paracrine effects because each set of CCF2-AM correlation data

556   is computed using the CCF2-AM ratios of both I-I cells and U-U cells from the same

557   infected monolayer. 224 and 343 CCF2-AM ratio correlation deviants were identified

558   between Wt I-I and Dmyr1 I-I cells at 2 hpi and 3 hpi, respectively. 218 (~97.3%) and

559   297 (~86.6%) deviants at 2 hpi and 3 hpi, respectively, fell in the regions of the CCF2-

560   AM correlation scatterplot in which the impact of effectors found in Dmyr1 I-I cells (i.e.,

561   ROPs + MIGs) are dampened by effectors found in Wt I-I cells (i.e., MDGs; Fig. 7B, blue

562   regions; Table S4B), which implies that signatures induced by effectors released into

563   Dmyr1 I-I cells are counteracted specifically by MYR-dependent effectors released into

564   Wt I-I cells.

565

566   *MYR-Dependent Paracrine Effects Also Counterbalance Parasite Effectors Released*

567   *Earlier in the Lytic Cycle*

568   To determine the impact of specifically paracrine factors on the host response,

569   we examined two key comparisons: Wt U-U vs. Mock (which captures all paracrine

570   effects; Table S3I) and Dmyr1 U-U vs. Mock (which captures MYR-independent

571   paracrine effects; Table S3J). As illustrated in Fig. 1C and Fig. 1D, differences between

572   these two comparisons should be attributable to paracrine factors released from host

573   cells in a MYR-dependent fashion.

574   At both 2 hpi and 3 hpi, paracrine effects in *Δmyr1* parasite infections exhibited

575   robust differences from paracrine effects in wild type infections. At 2 hpi, ~196 and 54

576   DEGs were identified for the Dmyr1 and Wt cell comparisons, respectively; at 3 hpi, the

577   difference was even more pronounced, with 1,864 DEGs identified for the Dmyr1

578   comparison, vs. only 20 DEGs for Wt. In addition, GSEA of Dmyr1 U-U vs. Mock DEGs

579   exposed an abundance of gene sets, many of which corresponded to inflammatory

580   processes and other pathways found to be part of the infection response, whereas

581   GSEA of Wt U-U vs. Mock DEGs enriched for few, if any gene sets (3 and 0 at 2 hpi

582   and 3 hpi, respectively; Fig. 8A). Furthermore, the responses of these DEGs were

583   reproduced consistently between not only U-U cells vs. mock-infected cells, but also

584   between U-I or I-I cells vs. mock-infected cells (Fig. 8B), which establishes that these

585   trends affect all cell types within a given infected monolayer. Taken together, these

586   results suggest that gene expression trends induced during *Toxoplasma* infection (i.e.,

587   those encapsulated by the DEGs that arise from comparing Dmyr1 U-U and Mock cells),

588   including those corresponding to pathways that respond to injected ROPs, are

589   suppressed via a MYR-dependent paracrine mechanism. Accordingly, supernatants

590   taken from host cell cultures infected with wild type and *Δmyr1* could, in theory, exert

591   transcriptional influence on fresh host cell monolayers, an interesting avenue for future

592   investigation.

593

594 ***Model of Host Responses to Individual* Toxoplasma *Parasite-Dependent Stimuli***

595 ***and Effector Compartments***

596 The preceding analyses have accounted for host responses to 5 parasite-

597 dependent stimuli: 1) rhoptry protein (ROP) injection; 2) MYR-independent dense

598 granule (MIG) secretion; 3) MYR-dependent dense granule (MDG) secretion; 4)

599 paracrine effects (which can be further subdivided into MYR-independent vs. MYR-

600 dependent paracrine effects); and 5) parasite invasion. To succinctly represent the

601 interplay between these stimuli, we curated a list of gene sets that best represented the

602 expression trends captured in our analyses. Gene sets were included if they were

603 significantly enriched (false discovery rate < 0.25) from DEGs between a majority of the

604 following 8 key comparisons: 1) Wt U-I vs. Wt U-U (ROP injection); 2) Wt I-I vs. Dmyr1

605 I-I (MDGs + MYR-dependent paracrine effects); 3) Wt U-U vs. Dmyr1 U-U (MYR-

606 dependent paracrine effects); 4) Dmyr1 I-I vs. Dmyr1 U-I (MIGs + Parasite invasion); 5)

607 Dmyr1 U-U vs. Mock (MYR-independent paracrine effects); 6) Wt I-I vs. Wt U-I (MIGs +

608 MDGs + parasite invasion); 7) Wt I-I vs. Wt U-U (all stimuli except paracrine effects);

609 and 8) Wt I-I vs. Mock (all stimuli). The 12 gene sets selected included those pertaining

610 to immune responses, cell proliferation, cellular stress, and the complement pathway.

611 Expression trends for the DEGs within these gene sets are summarized in Fig. 9.

612 Based on our analyses, we propose the following model of parasite-driven effects

613 on host cell transcription. First, because Wt U-I cells appeared to induce DEGs in nearly

614 all the showcased gene sets compared to Wt U-U cells, ROP injection likely induces

615 many of the pathways these gene sets capture, particularly immune-related and cellular

616 stress pathways (Fig. 9A and Fig. 9B, ROPs; Fig. 9C, ROP injection). These signatures

617   may arise in response to the ROPs themselves, or due to cell trauma secondary to

618   perforation of the host cell membrane by the parasite during ROP injection. Next,

619   parasites penetrate the host cell and secrete MIGs into the PVM. The host response to

620   these two stimuli together is relatively mild, as evidenced by the high false discovery

621   rates and small number of genes per gene set, even at 3 hpi. Though MIGs + Parasites

622   appeared to counteract the effects of ROP injection at the *gene* level, their most

623   significant impact at the *gene set* level appears to be enhancement of inflammatory

624   transcriptional signatures induced by ROP injection (Fig. 9A and Fig. 9B, MIGs +

625   Invasion; Fig. 9C, MIG Secretion and Parasite Penetration). This does not exclude the

626   possibility that other host processes not covered by the Hallmark gene sets are

627   suppressed by MIGs + Parasites. Next, in cells infected with *Δmyr1* parasites, MYR-

628   independent paracrine factors secreted by neighboring infected cells appear to enhance

629   the effects of ROP injection even more so than MIGs + Parasites and may do so not

630   only for immune-related genes, but also for genes pertaining to cellular stress,

631   complement, and cellular proliferation (Fig. 9A and Fig. 9B, dmyr1UU-mock/P(MI)). In

632   contrast, during wild type infections, two additional parasite-dependent stimuli, MDGs

633   and MYR-dependent paracrine factors, both appear to rein in transcriptional signatures

634   induced by the other stimuli (Fig. 9A and Fig. 9B, MDGs + P(MD); Fig. 9C, MYR-

635   dependent stimuli; Fig. 7B). Together, all five parasite classes produce transcriptional

636   signatures that veer toward induction of genes pertaining to inflammation and cellular

637   stress but that are less pronounced than the response to injection of ROPs.

638

639   **Discussion**

640   In this study, we examined host responses to infection with the parasite

641   *Toxoplasma gondii* using scRNA-seq on *in vitro* infected, uninfected, and uninfected-

642   injected (U-I) host cells, the latter of which arise from aborted invasion events and that

643   have until very recently (44) been characterized primarily morphologically. Key fixtures

644   of our experimental pipeline included: 1) early time points, to limit isolating false positive

645   U-I cells arising from mechanisms besides aborted invasion; 2) FACS sorts, which

646   purified rare U-I cells and relatively rare infected cells at early time points; and 3) single

647   cell resolution, which enabled bioinformatic validation of all cells' infection status. The

648   level of confidence lent by these measures to the validity of the captured U-I cells

649   enabled interrogation of host responses specifically to ROP injection, an aspect of

650   parasite infection previously inaccessible to study due to the rapid kinetics of effector

651   secretion at the time of invasion. Note that while others have also used scRNA-seq to

652   measure host responses to *Toxoplasma* infection at a single cell level, these studies

653   analyzed cells from animals at many days post-infection, and therefore did not assess

654   the earliest impacts of infection or particularly ROP injection (45).

655   Because the experimental pipeline also leveraged infections with *Δmyr1*

656   parasites, our dataset is a comprehensive resource for the individual impacts of not only

657   ROPs, but also MIGs, MDGs, and paracrine stimuli on host transcription. Our analyses

658   revealed an early response to *Toxoplasma* infection with subtle yet clear signatures

659   overlapping with inflammatory and cellular stress signaling axes. Induction of these

660   axes appears to be 1) driven primarily by ROP injection; 2) enhanced somewhat by the

661   combination of MIGs, parasites, and MYR-independent paracrine factors; and 3)

662   counterbalanced by MDGs and downstream paracrine effects, i.e., factors secreted

663 during wild type but not *Δmyr1* infection. These findings substantiate previous evidence

664 that at least some MDGs suppress host responses induced by other parasite-driven

665 transcriptomic perturbations (31). They may also explain the recent finding that the

666 avirulent phenotype of *Δmyr1* parasites during *in vivo* mouse infections is rescuable by

667 co-infecting animals with both wild type and MYR1-deficient parasites (46); parasites

668 expressing MYR1 may induce host cells to secrete paracrine factors that suppress

669 transcription of inflammatory gene products that would otherwise limit *Δmyr1* parasite

670 infections. Note that while this manuscript was in preparation, a transcriptomic study of

671 U-I macrophages was published by Hunter and colleagues (44). While they used

672 different strains (Pru and CEP), looked at later time points (20-24 hpi), and did not look

673 at MYR1-dependent effects, their primary conclusions that U-I cells experience a major

674 impact of rhoptry effectors (in their case, specifically ROP16), and that paracrine effects

675 are also in play, are similar to the conclusions reached here.

676   Of note, the host response to MIGs + Parasites was especially subtle, given that

677 parasite invasion involves dramatic mechanical perturbations to host cells that might be

678 expected to trigger transcriptional responses. As we did not use host cells containing

679 MIGs and no parasites or vice versa, we could not discern the impact of the MIGs in

680 isolation. Nonetheless, it is tempting to speculate that the response to MIGs + Parasites

681 may reflect MIGs counteracting the effects associated with parasite penetration and

682 PVM formation, which may explain the subtle net inflammatory response.

683   In addition, the presence of ROPs and MDGs that respectively activate and

684 suppress certain host processes might be interpreted as energetically wasteful. Why

685 might *Toxoplasma*, an obligate intracellular organism likely under selective pressure for

686   transcriptional efficiency, expend extra resources on effectors that negate one other's

687   effects? One possibility is that ROPs may have undergone selection optimizing for

688   functions required to establish and protect the parasite's intracellular niche (4-9, 47-52),

689   but may also trigger unavoidable host responses detrimental to the parasite. In this

690   scenario, MDGs and paracrine effects could ameliorate such ROP-triggered side effects

691   while theoretically leaving processes beneficial to the parasite intact. Another possibility

692   is that ROPs and GRAs could grant *Toxoplasma* the ability to fine tune host responses

693   in terms of timing and/or magnitude, likely an advantage to an organism that must be

694   equipped to encounter a diversity of intracellular environments due to its extraordinarily

695   broad host range (1, 53, 54).

696   Our analysis also reveals a striking effect on the host cell cycle, in which U-I cells

697   exhibit enrichment of G2/M phase cells. Curiously, this enrichment appeared not to be

698   preserved at 3 hpi, which suggests that the responsible parasite factors exert only a

699   transient influence on host cell cycle-related genes. Of the parasite effectors currently

700   known to modulate the host cell cycle (19, 22, 23, 40, 55), ROP16 is most likely to

701   explain these results: ROP16 phosphorylates ubiquitin-like containing PHD and RING

702   finger domain 1 (UHRF1) in a manner that peaks at 3 hpi, which leads to epigenetic

703   silencing of cyclin B1 (40), a component of the cyclin B1/Cdk1 complex required for the

704   G2/M transition. However, ROP16 is likely not the only ROP to impinge on the host cell

705   cycle, as comparison of G1 phase U-I vs. U-U cells also revealed enrichment in the p53

706   Pathway and KRAS Signaling Up gene sets (Fig. 4; Fig. 9A and Fig. 9B, ROPs), whose

707   corresponding pathways promote cell cycle progression. Further analysis of U-I cells

708   from yet more time points and particularly those not in G1 may shed more light on this

709   interplay.

710   Finally, our analyses thus far reflect a fraction of the possible uses of our dataset,

711   which includes variables of time, parasite strain, infection status, cell cycle phases, and

712   UMAP populations. For example, our dataset includes reads not only from host cells but

713   also the *Toxoplasma* parasites, rendering the data a co-transcriptomic resource that will

714   likely illuminate novel host-parasite interactions. Furthermore, because of limiting

715   numbers, the analyses described here dealt primarily with G1 phase cells in UMAP

716   population 1 and largely excluded cells in the remaining cell cycle phases and UMAP

717   populations. The raw and processed transcriptomic data for these remaining cells,

718   including the results of differential gene expression analysis and of gene set enrichment

719   analysis, are publicly available (as are the data described thus far) on the Gene

720   Expression Omnibus under accession number GSE145800. Future analyses of such

721   cells may reveal roles for cell cycle phase, host cell type, or other host-dependent

722   processes.

723

724   **Materials and Methods**

725   Cell and Parasite Culture

726   All *Toxoplasma gondii* strains were maintained by serial passage in human

727   foreskin fibroblasts (HFFs) cultured at 37ºC in 5% carbon dioxide (CO_2) in complete

728   Dulbeco's Modified Eagle Medium (cDMEM) supplemented with 10% heat-inactivated

729   fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml

730   streptomycin.

731

Construction of Parasite Strains

RH Tfn-BLA TdTomato parasites were constructed by transfecting ~10^7 RH

Toxofilin-HA-beta-lactamase (RH Tfn-BLA) parasites (8) with the plasmid pSAT1::Cas9-

U6::sgUPRT (56) and with the linearized pCTR$_{2T}$ plasmid containing the construct to

express TdTomato (57) using the AMAXA Nucleofector 4D system (U-033 setting) and

the P3 primary cell 4D-nucleofector X kit with the 16-well nucleocuvette strip (Lonza,

V4XP-3032). Clones were obtained by FACS using the FACS Aria II sorter at the

Stanford Shared FACS Facility for the brightest red parasites and single cloning the

TdTomato+ enriched population by limiting dilution into 96-well plates.

A CRISPR-Cas9 strategy was used to construct RH *Δmyr1* mCherry Tfn-BLA

parasites from an RH *Δmyr1* mCherry parental strain (25). The parental strain was

transfected with the plasmid pSAG1::Cas9-U6::sgUPRT and a linear construct that

contained Toxofilin-HA-Beta-lactamase (Tfn-HA-BLA) expressed under *toxofilin*'s

endogenous promoter. The Tfn-HA-BLA construct was PCR amplified from the plasmid

SP3 (8) such that the final amplicon was flanked by 20 nucleotide (nt) homology arms to

the *UPRT* gene that are identical to those used to target constructs to the *UPRT* locus

in (56). 15 μg of pSAG1::Cas9-U6::sgUPRT and 3 μg of the Tfn-HA-BLA linear

amplicon were transfected into ~10^7 parental strain parasites using the AMAXA

Nucleofector 4D system as described above, except the setting on the nucleofector (T-

cell human unstimulated HE setting). Selection for parasites with 5 μM 5-

fluorodeoxyuridine (FUDR) began at 1 day post-transfection and proceeded for 3 lytic

cycles in monolayers of human foreskin fibroblasts (HFFs). The transfected, selected

754   parasite populations were subjected to two rounds of single cloning, one to generate

755   populations of parasites enriched for the presence of the Tfn-HA-BLA construct, and

756   one to purify for individual parasite clones containing the construct, where the readout

757   for the presence of the construct was cleavage of the BLA-cleavable FRET-based dye

758   CCF2-AM, which results in a fluorescence color shift.

759   IFA of monolayers infected with newly constructed RH *Δmyr1* mCherry Tfn-BLA

760   parasites was used to validate correct localization of the Tfn-HA-BLA construct to the

761   parasite rhoptry organelles and the absence of expression of host factors known to be

762   induced in the host nucleus as a result of MYR-dependent GRAs. Coverslips seeded

763   with confluent HFF monolayers were infected with putative RH *Δmyr1* mCherry Tfn-BLA

764   clones for ~24 hours, fixed in 4% formaldehyde for 20 minutes, permeabilized in 0.2%

765   Triton X-100 for 20 minutes, blocked in 1x PBS containing 3% bovine serum albumin (3%

766   BSA solution) for 1 hour at room temperature, stained with primary and secondary

767   antibodies in 3% BSA solution, and mounted onto glass slides using DAPI-containing

768   VectaShield (Vector Laboratories, H-1200) and sealed with colorless nail polish. For

769   Tfn-HA-BLA localization, primary antibodies were mouse-anti-ROP2/3/4 (1:250 dilution,

770   4A7, (58)) and rat anti-HA (1:500, clone 3F10, Sigma Aldrich, 11867431001), and

771   secondary antibodies were goat anti-mouse IgG-Alexa 647 (1:2000 dilution, Thermo

772   Fisher, A-21235) and goat anti-rat IgG-Alexa 488 (1:2000 dilution, Thermo Fisher, A-

773   11006). To verify the absence of the MYR1 protein, the primary antibody rabbit anti-c-

774   myc (1:600 dilution, Sigma, M5546) and the secondary antibody goat anti-rabbit IgG-

775   Alexa 594 (1:2000 dilution, Thermo Fisher, A-11012) were used, and coverslips were

776   seeded with either >3-week-old confluent HFF monolayers or with younger monolayers

777   that had been serum starved for at least 24 hours (using 0.5% FBS instead of 10% FBS

778   in the culture medium) to ensure no spurious induction of c-myc in uninfected host cells.

779   Only clones where the anti-HA and anti-ROP2/3/4 signal colocalized and where no

780   expression of c-myc was detected in the infected host nucleus were selected. RH Tfn-

781   HA-BLA TdTomato parasites (32) and the parental RH *Δmyr1* mCherry parasites were

782   also subjected to both protocols as positive and negative controls, respectively.

783   Coverslips were imaged either on the Stanford Neuroscience Imaging Service core's

784   Zeiss LSM 710 confocal microscope or on an Olympus BX60 upright fluorescence

785   microscope.

786   Western blot was used to verify for the correct size of the Tfn-HA-BLA fusion

787   protein expressed in RH *Δmyr1* mCherry Tfn-BLA parasite clones. Lysates were

788   generated by treatment of parasite pellets with SDS-PAGE loading dye containing 10%

789   beta-mercaptoethanol. The lysates were separated by SDS-PAGE and transferred to a

790   PVDF membrane, and the membrane was blocked with TBST (TBS, 0.05% Tween-20)

791   containing 5% milk for 1.25 hours. The membrane was incubated with horseradish

792   peroxidase (HRP)-conjugated rat anti-HA (clone 3F10) monoclonal antibodies (Roche,

793   Indianapolis, IN) at a dilution of 1:5,000 for 2 hours, and with 1:10,000 dilution rabbit

794   anti-SAG1 for 30 minutes followed by 1:20,000 dilution goat anti-rabbit-HRP for 30

795   minutes, and developed using the ECL Prime Western Blotting System (Sigma-Aldrich,

796   RPN2232).

797

798   Determination of Time to Division for Infected 10 T1/2 Cells

799   To determine the time post-infection at which infected 10 T1/2 cells divided, 10

800   T1/2 host cell monolayers were infected with RH Tfn-BLA TdTomato parasites, and the

801   infected monolayers were imaged by time lapse microscopy.

802   To prepare the parasites for the infection, the parasites were released from

803   heavily infected monolayers of HFFs by mechanical disruption of the monolayers using

804   sterile, disposable scrapers and passage at least 6 times through a 25 gauge syringe.

805   The parasites were washed by pelleting out HFF debris (133.5-208.5 x g for 5 minutes)

806   and resuspending the parasite pellet generated by spinning the remaining supernatant

807   469.2 x g in phenol red-negative low serum DMEM.

808   To prepare the host cell monolayers, 10 T1/2 cells approximately 1 week from

809   their date of thaw were seeded into 12-well tissue culture plates at approximately 6.0 x

810   10^4, 1.2 x 10^5, and 2.4 x 10^5 cells per well. The 10 T1/2 cells were then incubated for >2

811   hours in cDMEM, serum starved by incubation at 37°C in 5% CO_2 in low serum DMEM

812   (i.e., cDMEM containing 0.5% FBS instead of 10% FBS) for 23 hours, washed and

813   stained with 500 μl of Cell Tracker Green CMFDA (CTG-CMFDA; Thermo Fisher,

814   C2925) diluted 1:1000 in prewarmed PBS for 30 minutes at 37°C in 5% CO_2, and

815   washed and incubated in phenol red-negative low serum DMEM (Thermo Fisher,

816   31053028) for 30 minutes. At 24 hours post-infection, RH Tfn-BLA TdTomato parasites

817   were added to the monolayers at a multiplicity of infection (MOI) of 6.

818   Time lapse, epifluorescence images of the infected monolayers were acquired

819   over 16 hours in a controlled (37°C and 5% CO_2) environment using a Nikon Eclipse

820   inverted microscope (Julie Theriot lab). Images were acquired every 20 minutes using

821   100 ms of exposure at 25% power for the Phase, mCherry (to visualize parasites), and

822   GFP (to visualize host cytoplasmic CTG-CMFDA) channels. Cells that were uninfected

823   at the start of the time lapse were followed to determine whether they were infected by

824   the end of the time lapse, as determined by: 1) clearing of cytoplasmic CTG-CMFDA in

825   the exact position of the parasite, 2) disappearance of birefringence in the Phase

826   channel upon parasite invasion, and c) the parasite tracking with the cell at all time

827   points following presumed infection. For each cell for which the precise moment of

828   infection was captured in the live video footage, the time to division was determined

829   using the time the cell was infected as the start time and the time the cell divided (if

830   applicable) as the end time.

831

832   <u>FACS of Single Uninfected-Injected (U-I) and Control Cells for Single Cell RNA</u>

833   <u>Sequencing (scRNA-seq)</u>

834   *Preparation of Single Cells for FACS Sorting*

835   To generate U-I cells for FACS sorting, 10 T1/2 host cells approximately 1 week

836   from their date of thaw were seeded into 6-well tissue culture plates at a density of

837   approximately 2.6 x 10^5 cells per well, incubated for >2 hours in cDMEM, and serum

838   starved by incubation in low serum DMEM (i.e., cDMEM containing 0.5% FBS instead of

839   10% FBS) for 24 hours. 10 T1/2 host cells were chosen as they are monolayer-forming,

840   contact-inhibited fibroblasts that are also suitable for single cell sorting due to adequate

841   dissociation into individual cells by a combination of mechanical and chemical disruption.

842   Next, either RH Tfn-BLA TdTomato or RH *Δmyr1* mCherry Tfn-BLA parasites were

843   released from heavily infected monolayers of HFFs by mechanical disruption of the

844   monolayers using sterile, disposable scrapers and passage at least 6 times through a

845   25 gauge syringe. Parasite-free lysate was similarly generated by mechanical disruption

846   of uninfected HFFs. Parasites and parasite-free lysate were washed by pelleting out

847   HFF debris (133.5-208.5 x g for 5 minutes) and resuspending the parasite pellet

848   generated by spinning the remaining supernatant 469.2 x g in low serum DMEM

849   containing either 1 μM DMSO (for the wild type and *Δmyr1* conditions) or with 1 μM of

850   the invasion inhibitor cytochalasin D (cytD, for the cytD-treated wild type condition),

851   incubated at room temperature for 10 minutes, and applied to the serum starved 10

852   T1/2 monolayers at multiplicity of infection (MOI) = 6, which maximized the abundance

853   of U-I cells in tissue culture. All 10 T1/2 monolayers were spun at 469.2 x g for 5

854   minutes to synchronize parasite contact with the monolayer. Infections were allowed to

855   proceed for 30 minutes, 1.5 hours, or 2.5 hours at 37°C. Because cytD is a reversible

856   inhibitor, extra cytD-containing low serum DMEM was added to each of the drug-treated

857   infections to maintain the concentration of cytD at 0.5-1 μM for the entire infection

858   duration.

859   To identify host cells injected by parasite proteins, a 6x stock solution of the beta-

860   lactamase substrate CCF2-AM (Thermo Fisher, K1032) was added to the media over

861   10 T1/2 cells so that the final concentration of CCF2-AM was 1x. CCF2-AM treated

862   monolayers were incubated under foil (to protect from light) for 30 minutes at room

863   temperature (to prevent breakdown of CCF2-AM, which degrades at 37 °C), bringing up

864   the total infection duration to 1 h, 2 h, and 3 h.

865   To harvest the 10 T1/2 monolayers for subsequent FACS analysis, the

866   monolayers were: 1) washed 3 times in 1x phosphate buffered saline (PBS) to remove

867   any HFF debris adhering to the monolayer; 2) incubated in trypsin (prepared in plastic

868 vessels only) at room temperature for 6-10 minutes; 3) quenched in an equal volume of

869 FACS buffer (1 x PBS + 2% FBS + 50 mM MgCl$_2$*6H$_2$0 + 50 µg/ml DNase I); 4) passed

870 3 times through an 18 gauge syringe to break any residual cell clumps; and 5) washed

871 in FACS buffer to remove excess trypsin. Of note, DNase I and MgCl$_2$ were included in

872 the FACS buffer to prevent clumping of cells from cell death. The cells were then

873 stained with a viability dye and an extracellular parasite stain by 1) resuspending them

874 in 500 µl of chilled 4°C 1x PBS containing 3% bovine serum albumin (BSA) and 1:500

875 dilution rabbit anti-SAG2A primary antibody (gift of C. Lekutis) for 30 minutes on wet ice,

876 2) washing them in 5 ml of ice cold 1x PBS and spinning at 133.5 x g (lowest setting) at

877 4°C for 5 minutes, and 3) resuspending the pellets in chilled 1x PBS containing 3% BSA,

878 1:1000 dilution goat anti-rabbit IgG-Alexa 647 (Thermo Fisher), and 3 µl/ml near-

879 infrared live/dead fixable viability dye (Thermo Fisher, L94375) for 30 minutes on wet

880 ice. Samples were then washed as before, the pellets were resuspended in 1 ml of

881 chilled FACS buffer, and the cell suspension was transferred through a nylon filter cap

882 (Thomas Scientific, 4620F40) into polypropylene FACS tubes stored on wet ice and

883 protected from light until FACS sorting.

884

885 *FACS of Single Cells*

886   To prepare the multi-well lysis plates into which cells were deposited during

887 FACS sorting, lysis buffer was dispensed either by the Mantis liquid handling robot

888 (Formulatrix) at 0.4 µl per well into 384-well hard shell low profile PCR plates (Bio-rad)

889 for single cell RNA sequencing, or by hand at 5 µl per well into 96-well hard shell low

890 profile PCR plates (Bio-rad) for bulk RNA sequencing. Lysis buffer was prepared in

891 batches of 8 ml by mixing 5.888 ml water, 160 µl recombinant RNase inhibitor (Takara

892 Clonetech), 1.6 ml of 10 mM dNTP (Thermo Fisher), 160 µl of 100 µM oligo-dT (iDT),

893 1:600,000 diluted ERCC spike-in RNA molecules (Thermo Fisher), and 32 µl 10% Triton

894 X-100. All reagents were declared RNase free. Lysis plates were prepared the night

895 before each FACS sort, stored overnight at -80°C, and kept on dry ice during the FACS

896 sort.

897   All host cell samples were sorted at the Stanford Shared FACS Facility (SSFF)

898 by the BD Influx Special Order sorter using the following channels: forward scatter (488

899 nm blue laser, SSC detector), side scatter (488 nm blue laser, FSC detector), BV421

900 (405 nm violet laser, V460 detector, which detected cleaved CCF2-AM), BV510 (405

901 nm violet laser, V520 detector, which detected uncleaved CCF2-AM), mCherry (561 nm

902 yellow laser, Y610 detector, which detected parasite-associated cells), APC (640 nm

903 red laser, R670 detector, which detected the extracellular parasite stain vs. anti-SAG2A),

904 and APC-Cy7 (640 nm red laser, R750 detector, which detected dead cells). Gating

905 strategy used to obtain U-I, I-I, and U-U cells is indicated in Fig. 2B. More specifically,

906 cells without red fluorescence (i.e., parasite-free) but with enhanced signal from cleaved

907 CCF2-AM (i.e., injected) were sorted as U-I cells, while those with red fluorescence (i.e.,

908 parasite-associated), enhanced signal from CCF2-AM (i.e., injected), and low

909 extracellular parasite stain were sorted as I-I cells. Of note, the parasite-associated gate,

910 from which I-I cells were obtained, was intentionally kept narrow to ensure host cells

911 were each infected with approximately one parasite apiece, which limited confounding

912 downstream analysis with penetration of >1 parasite at two different time points. Single

913 color and colorless controls were used for compensation and adjustment of channel

914   voltages. Fluorescence data were collected with FACSDiva software and analyzed with

915   Flowjo software. Cells were index sorted such that each cell's fluorescence data were

916   recorded for subsequent analysis. For single cell experiments, cells were sorted into the

917   384-well lysis plates at 1 cell per well. For bulk experiments, cells were sorted into 96-

918   well lysis plates at 50-100 cells per well. Plates were sealed with foil plate sealers and

919   immediately placed on dry ice until the completion of the sort. Plates were then stored at

920   -80°C until library preparation.

921

922   cDNA Synthesis from Single Cell RNA, Library Preparation, and Sequencing

923   To convert the RNA obtained from single cells and bulk samples to cDNA, we

924   employed the Smart-seq2 protocol (59). For single cell library preparation, the liquid

925   handling robots Mantis (Formulatrix) and Mosquito (TTP Labtech) were employed to

926   transfer and dispense small volumes of reagents, and final reaction volume was 2 µl per

927   well. For bulk sample library preparation, liquid handling was performed with standard

928   multichannel pipets and final reaction volume was 25 µl per sample. cDNA was subject

929   to 19 round of pre-amplification and then quantified using EvaGreen and diluted in EB

930   buffer to obtain a final concentration of 0.4-0.8 ng/µl per sample. Library preparation

931   continued using in-house Tn5 tagmentation. For single cell libraries, we used custom

932   barcoded indices for each cell, and for bulk libraries, we used Nextera XT indices.

933   Libraries were submitted to the Chan Zuckerberg Biohub Genomics Core for

934   sequencing. Single cell libraries were sequenced on the NovaSeq 6000 by 2x150 base

935   pair paired end sequencing aiming at ~1 million reads per cell. Bulk libraries were

936   sequenced on the NextSeq by 2x150 base pair paired end sequencing at ~10 million

937   reads per sample.

938

939   Sequencing Alignment

940   Reads output from sequencing were aligned to a concatenated genome

941   composed of the mouse genome (GRCm build 38) and the GT1 *Toxoplasma gondii*

942   genome (ToxoDB version 36), which is the most complete reference for type I parasite

943   strains such as the RH strains used in this work. Alignment was performed using STAR,

944   and transcript counting was performed by Htseq-count, with standard parameters used

945   for both packages. A custom python script was used to sum transcript counts to yield a

946   final gene count matrix consisting of all sequenced cells and the number of read counts

947   detected for each gene.

948

949   Data Preprocessing

950   To filter out cells of poor quality from the analysis, we excluded cells based on

951   the following metrics: gene count, total read sum, percentage of reads that mapped to

952   the mouse-*Toxoplasma* concatenated genome, percentage of reads derived from

953   spiked in ERCC standards, and percentage of reads derived from ribosomal RNA.

954   The gene count matrices were then normalized as counts per median (cpm).

955   Briefly, we first calculated the sum of reads for all cells. We then divided the read counts

956   by the corresponding sum of reads in each cell and multiplied the fractional count by the

957   median of the sum of reads as a scaling factor. Normalized data were transformed to

958   log2 space after adding a pseudo-count of 1 for each gene of each cell.

959   To determine the detection limit of each experimental trial (e.g., the 50%

960   detection rate), we computed a logistic regression model from a plot of the detection

961   probability for spiked-in ERCC standards. We then excluded genes where less than 5

962   cells in the experimental trial expressed that gene at a level above the detection limit.

963   To identify host genes associated with infection, we first excluded mouse genes

964   to which *Toxoplasma* sequences erroneously map (Table S1) by aligning RNA

965   sequences obtained from single cell extracellular RH parasites to the concatenated

966   mouse-*Toxoplasma* genome and eliminating all mouse genes with an average

967     $\log_2(\text{cpm}+1)$ expression of 0.2 or greater.

968

969   Single Cell vs. Bulk Sample Correlation Analysis

970   To validate the single cell expression data, we plotted the log2 mean expression

971   of each differentially expressed gene, as identified by the MAST algorithm using the

972   single cell data, in single cells vs. bulk samples. The sklearn package was applied to

973   compute linear regression and the corresponding R^2 (coefficient of determination)

974   values.

975

976   Cell Cycle Analysis and Annotation

977   To predict the cell cycle phase of individual single cells, we curated a list of 175

978   cell cycle marker genes from the literature (60-67) and from the database CycleBase

979   3.0 (68). We computed the first two principle components of the gene count matrix using

980   principle component analysis (PCA) and projected the cells. We partitioned the cells

981   using K-Means clustering and assigned the clusters with their predicted cell cycle

982   phases (G1, S, and G2/M) based on the expression of 175 cell cycle marker genes

983   curated from the literature (Table S2).

984

985   Dimensionality Reduction

986   To visually represent relationships between single cells across all experimental

987   trials based on their transcriptional variation regardless of experimental conditions, we

988   identified and filtered for the top 1000 genes with the highest dispersion (i.e., the genes

989   with the most variable expression for their bin groups with similar expression level)

990   across all datasets, applied mutual nearest neighborhood batch correction (MNNPY) to

991   correct for batch effects, and projected the data onto two dimensional space with the

992   uniform manifold approximation and projection (UMAP) algorithm using default

993   parameters in Scanpy (69). Leiden clustering using the top 1000 dispsered genes

994   enabled partitioning of cells into three populations (1, 2, and 3), which we separated into

995   individual datasets for downstream analysis.

996

997   Infection Status Classification

998   We also determined the host infection load by quantifying the percent of reads

999   that mapped to *Toxoplasma* in a given sample. We filtered samples which were shown

1000   by FACS to exhibit one presumed infection status (based on red fluorescence from

1001   internalized parasites) but were determined to exhibit the opposite or an ambiguous

1002   infection status otherwise (based on percentage of reads derived from *Toxoplasma*).

1003

1004   Differential Expression Analysis

1005   To obtain differentially expressed genes (DEGs) between all pairs of conditions,

1006   we used the Model-based Analysis of Single Cell Transcriptomics (MAST) algorithm (70)

1007   to compute the results on all G1, correctly classified, and UMAP population 1 cells. We

1008   used the default settings except with an adaptive conditional mean of expression based

1009   on 20 number of bins, at least 30 genes in each bin, and we did not filter out any gene

1010   with non-zero expression frequency in the samples.

1011

1012   Gene Set Enrichment Analysis

1013   We performed gene set enrichment analysis (GSEA) on the lists of differentially

1014   expressed genes between all pairs of experimental conditions, ranked by their relative

1015   expression in each of the two conditions, for each individual experimental trial using the

1016   fast pre-ranked gene set enrichment analysis (fgsea) package (71). Genes were

1017   compared to the Molecular Signature Database's Hallmarks gene sets. Pathways with

1018   an adjusted p-value, i.e., false discovery rate (FDR) of < 0.25 were considered to be

1019   significantly enriched at the top or the bottom of the ranked list of differentially

1020   expressed genes.

1021

1022   Identification of Differentially Regulated Genes between Conditions Using CCF2-AM

1023   Ratio Correlation Analysis

1024   To identify host genes associated with injection, we first computed the CCF2-

1025   ratio for each cell, a metric that serves as a readout for parasite effector injection and

1026   that was calculated by dividing the log-transformed cleaved CCF2-AM fluorescence by

1027   the log-transformed uncleaved CCF2-AM fluorescence. Next, we excluded mouse

1028   genes below the detection limit and mouse genes to which *Toxoplasma* sequences

1029   erroneously map. Finally, for each infected or injected condition (i.e., Wt U-I, Wt I-I,

1030   Dmyr1 U-I, Dmyr1 I-I, CytD U-I, and CytD I-I), we computed the Spearman correlation of

1031   each of the remaining genes to the CCF2-AM ratio using cells from the condition of

1032   interest and cells from the cognate U-U condition (e.g., to calculate Spearman

1033   correlations for Wt U-I cells, we correlated gene expression to the CCF2-AM ratio in Wt

1034   U-I and Wt U-U cells).

1035   To identify genes differentially regulated between pairs of conditions using the

1036   CCF2-AM correlation data, we generated scatterplots where each data point

1037   represented a gene and its displacement on each of the x- and y-axes represented the

1038   Spearman correlation in each of the conditions. We modeled a Gaussian distribution

1039   using the Sklearn package with default parameters with settings "n_components=1" and

1040   "covariance_type='full'". Genes with a difference in CCF2-AM correlation score of >0.2

1041   or <-0.2 and whose probability densities were more than three standard deviations from

1042   the mean probability density were considered to exhibit significant differential

1043   expression between the pair of conditions under scrutiny.

1044

1045   <u>Generation of Strip Plots</u>

1046   Strip plots in Fig. 8B were generated using seaborn's catplot and boxplot

1047   packages to plot the normalized expression scores for each cell across 11 experimental

1048   conditions for differentially expressed genes (DEGs) between Dmyr1 U-U and Mock

1049   samples. The normalized expression score for each cell was calculated by 1)

1050   subtracting the minimum log2 cpm for that gene across all cells in the experiment, 2)

1051   dividing the difference by the maximum log2 cpm across all cells, such that the cell with

1052   the lowest count received a score of 0 and the cell with the highest count received a

1053   score of 1, and 3) computing the average of the normalized cpm's for each cell.

1054

1055   Generation of Bubble Plots

1056   Bubble plots in Fig. 9A and Fig. 9B were generated using a custom Python script

1057   in which columns indicated the gene set and rows indicated the comparison (taken to

1058   signify the impact of one to a few parasite-dependent stimuli on host transcription) from

1059   which the gene sets were enriched. The bubbles in each plot were color coded and

1060   sized based on one of two schemes. In the first scheme, bubble color indicated the

1061   normalized enrichment score, where scores were positive if the genes corresponding to

1062   a given gene set were expressed higher in the first member of the pair in the given

1063   comparison than in the second member of the pair, and bubble size indicated the

1064   significance (i.e., false discovery rate, or FDR) of the enrichment, where the absolute

1065   size of each bubble corresponded to the reciprocal of the FDR. In the second scheme,

1066   bubble color indicated the log2 normalized fold change in expression of DEGs from a

1067   given comparison that also corresponded to a given Hallmark pathway, where positive

1068   fold changes indicated genes were expressed higher in the first member of the pair in

1069   the comparison than in the second member, and bubble size indicated the number of

1070   DEGs used to calculate the fold change. To calculate fold change for each given

1071   comparison's gene set, DEGs from the comparison that fell under the gene set were

1072   identified, expression of these DEGs in cpm was normalized across all cells to a

1073   maximum value of 1 and averaged across all the cells in each condition, and average

1074   normalized expression in the first experimental condition was divided by average

1075   normalized expression in the second experimental condition.

1076

1077   Data Availability

1078   The RNA sequencing dataset produced in this study has been uploaded in its

1079   entirety to the National Center for Biotechnology Information (NCBI) Gene Expression

1080   Omnibus (GEO) database under accession number GSE145800. The dataset includes

1081   the raw fastq files, processed gene count files (in counts per median), an anndata file

1082   containing the processed gene count files and other metadata such as the cell cycle

1083   phase and percentage of *Toxoplasma*-derived reads for each cell, and results of

1084   differential gene expression analysis and gene set enrichment analysis for up to eleven

1085   distinct species of host cell (i.e., Wt U-I, Wt I-I, Wt U-U, Dmyr1 U-I, Dmyr1 I-I, Dmyr1 U-

1086   U, CytD U-I, CytD I-I, CytD U-U, Mock, and CytD Mock) at each of three time points (i.e.,

1087   1, 2, and 3 hours) post-infection. Here, Wt, Dmyr1, and CytD designate host cells

1088   arising from monolayers infected with wild type (RH Tfn-BLA TdTomato), *Δmyr1* (RH

1089   *Δmyr1* mCherry Tfn-BLA) or cytochalasin D-treated wild type parasites, respectively,

1090   while Mock and CytD Mock refer to cells arising from monolayers mock-infected with

1091   parasite-free lysate (where the lysate was pretreated with cytochalasin D in the CytD

1092   Mock condition).

1093

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1109

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1329 **MAIN TEXT FIGURE AND TABLE LEGENDS**

1330   **Figure 1.** Key experimental conditions and comparisons. **(A)** Simplified illustration of

1331   effector secretion during early tachyzoite infection. Blue curved arrows = modulation of

1332   host transcription, black curved arrows = transitions through stages of lytic cycle, blue

1333   jagged arrow = modulation without translocation to the nucleus, ROPs = rhoptry

1334   proteins, MIGs = MYR-independent dense granule proteins, MDGs = MYR-dependent

1335   dense granule proteins, MYR = putative translocation required for MDG penetration of

1336   host cytosol. **(B)** As a result of the events in (A), infected host cell monolayers produce

1337   infected-injected (I-I) cells when tachyzoite invasion proceeds as usual, uninfected-

1338   uninjected (U-U) bystander cells, and uninfected-injected (U-I) cells from aborted

1339   invasion after effector injection. **(C)** Parasite-dependent stimuli (i.e., effectors, paracrine

1340   factors) that influence each of seven cell species collected for RNA sequencing,

1341   according to the current model of effector secretion for *Toxoplasma* tachyzoites. Wt and

1342   Dmyr1 designate cells originating from host cell monolayers infected with wild type and

1343   D*myr1* parasites, respectively; Mock indicates cells from a mock-infected monolayer. **(D)**

1344   Parasite-dependent stimuli that explain the differences in expression trends between

1345   key pairs of collected cell species. PV = parasitophorous vacuole.

1346

1347   **Figure 2.** Experimental pipeline. **(A)** Pipeline to collect and analyze experimental

1348   conditions for single cell RNA sequencing. BLA = toxofilin-beta-lactamase ROP fusion

1349   protein, CCF2-AM = indicator dye that reveals injected (blue) vs. uninjected (green)

1350   cells, U-U = uninfected-uninjected bystander host cell, U-I = uninfected-injected host cell,

1351   and I-I = infected-injected host cell. **(B)** FACS gating strategy to obtain mock-infected,

1352   U-U, I-I, and U-I cells. For cells originating from the parasite-free gate, the distribution of

1353   sorted U-I (orange) and U-U (pink) cells from each of the 1 hpi, 2 hpi, and 3 hpi time

1354   points is shown. **(C)** Proportion of injected (U-I + I-I) cells that are U-I. **(D)** Abundances

1355   of U-U, I-I, and U-I cells during FACS, expressed as percentages of cells from the

1356   Live/Mock gate from which they were sorted.

1357

1358   **Figure 3.** Infection response recapitulates previously identified signatures of early

1359   infection. **(A)** Gene set enrichment analysis (GSEA) of the ranked list of differentially

1360   expressed genes between Wt I-I and mock cells at 2 hpi (FDR < 0.1, FC > 1.5x) using

1361   the Hallmark gene sets from the Molecular Signatures Database. **(B)** GSEA as in (A) of

1362   the ranked differentially expressed genes between Wt I-I and mock cells at 3 hpi. FDR =

1363   false discovery rate, NES = normalized enrichment score, and FC = fold change. Gray

1364   cells indicate pathways common to those identified with FDR <10^{-5} in Naor et al. 2018.

1365

1366   **Figure 4.** Host response to injected parasite effectors. **(A)** Gene set enrichment

1367   analysis (GSEA) of the ranked list of differentially expressed genes (DEGs; false

1368   discovery rate (FDR) < 0.1, fold change > 1.5x) between Wt U-I and Wt U-U cells at 3

1369   hpi using the Hallmark gene sets. Green rows indicate gene sets enriched from genes

1370   expressed higher in Wt U-I than in Wt U-U, and red rows indicate gene sets enriched in

1371   genes expressed lower in Wt U-I than in Wt U-U. Gene sets are designated as part of

1372   the infection response if they were also found to be enriched from DEGs between Wt I-I

1373   vs. Mock at 2 hpi or 3 hpi. **(B)** GSEA as in (A) between various U-I cells (i.e., from the

1374   Wt, CytD, and Dmyr1 infection conditions) and their cognate U-U cells at 3 hpi. Gene

1375   sets in bold gray text were also found to be enriched in the infection response. Positive

1376 normalized enrichment scores represent enrichment in genes expressed higher in U-I

1377 cells than in U-U cells.

1378

1379 **Figure 5**. Comparison of host response to injection vs. infection (i.e., signatures in Wt

1380 U-I vs. Wt I-I cells.) **(A)** Scatterplot comparing injection-associated genes in Wt I-I cells

1381 and Wt U-I cells, as determined by the Spearman correlation of gene expression to the

1382 CCF2-AM ratio in Wt I-I and Wt U-I cells. Contours reflect density of points, with central

1383 contours being in the area of highest density. Labeled points on the plot are the top 20

1384 genes with the lowest probability of belonging to a Gaussian model fit with parameters

1385 that best describe the observed Spearman correlation data. Yellow and blue shading

1386 mark regions of the scatterplots where gene expression trends in Wt U-I cells are either

1387 enhanced or dampened upon parasite invasion and release of dense granule effectors

1388 into the host cell (as seen in Wt I-I cells). **(B)** GSEA of the ranked list of differentially

1389 expressed genes between Wt U-I and Wt I-I cells at 3 hpi, where green rows denote

1390 gene sets enriched from genes expressed higher in Wt U-I cells than in Wt I-I cells, and

1391 red rows represent genes expressed higher in Wt I-I than in Wt U-I. FDR = false

1392 discovery rate, NES = normalized enrichment score.

1393

1394 **Figure 6**. GSEA of the ranked list of differentially expressed genes between Dmyr1 U-I

1395 and Dmyr1 I-I cells at 3 hpi, which illustrates the impact of MYR-independent dense

1396 granule proteins (MIGs) and parasite invasion on the host response to infection with

1397 *Toxoplasma.* Green rows denote gene sets enriched from genes expressed higher in

1398 Dmyr1 U-I cells than in Dmyr1 I-I cells, and red rows represent genes expressed higher

1399   in Dmyr1 I-I cells than in Dmyr1 U-I cells. FDR = false discovery rate, NES = normalized

1400   enrichment score.

1401

1402   **Figure 7**. Impact of MYR-dependent dense granule proteins (MDGs) and MYR-

1403   dependent paracrine effectors, as illustrated by comparing Dmyr1 I-I and Wt I-I cells. **(A)**

1404   Gene set enrichment analysis (GSEA) of ranked list of differentially expressed genes

1405   between Dmyr1 I-I and Wt I-I cells at 3 hpi. Green rows denote enrichment from genes

1406   expressed higher in Dmyr1 I-I cells than in Wt I-I cells, and red rows represent

1407   enrichment from genes expressed higher in Wt I-I than in Wt U-I. FDR = false discovery

1408   rate, NES = normalized enrichment score. **(B)** Scatterplot comparing injection-

1409   associated genes in Dmyr1 I-I cells and Wt I-I cells, as determined by the Spearman

1410   correlation of gene expression to the CCF2-AM ratio in Dmyr1 I-I and Wt I-I cells.

1411   Contours reflect density of points, with central contours being in the area of highest

1412   density. Labeled points on the plot are the top 20 genes with the lowest probability of

1413   belonging to a Gaussian model fit with parameters that best describe the observed

1414   Spearman correlation data. Yellow and blue shading mark regions of the scatterplots

1415   where gene expression trends in Dmyr1 I-I cells are either enhanced or dampened by

1416   MDGs or paracrine effects (as seen in Wt I-I cells).

1417

1418   **Figure 8.** Comparison of Dmyr1 U-U vs. Mock cell types reveals impact of MYR-

1419   dependent paracrine effects on host cell transcription during *Toxoplasma* infection. **(A)**

1420   Gene set enrichment analysis (GSEA) of the ranked list of differentially expressed

1421   genes between Dmyr1 U-U and Mock cells at 3 hpi. FDR = false discovery rate, NES =

1422   normalized enrichment score, and sc = the current single cell RNA-seq dataset. **(B)**

1423   Trends in expression of Dmyr1 U-U vs. Mock DEGs are conserved in U-I and I-I cells

1424   from Wt and Dmyr1 infections, as illustrated by strip plots depicting expression of DEGs

1425   between Dmyr1 U-U and Mock cells across all cell types. Each point represents a single

1426   cell, and its y-axis displacement reflects the average of the normalized expression

1427   scores for all DEGs between Dmyr1 U-U and Mock cells. The normalized expression

1428   score of a given gene is calculated by scaling the $\log_2$ count per median (cpm) for that

1429   gene, such that the cell with the lowest cpm receives a score of 0, and the cell with the

1430   highest cpm receives a score of 1.

1431

1432   **Figure 9.** Summary of host transcriptional response to parasite-dependent stimuli. **(A)**

1433   Bubble plot of parasite-dependent host transcriptional signatures at 3 hpi that depicts

1434   the behavior of 11 representative Hallmark gene sets from the Molecular Signatures

1435   Database (columns) across 8 key comparisons between experimental conditions (rows,

1436   indicated on left of each plot) that each encapsulates the impact of certain parasite-

1437   dependent stimuli on the 11 gene sets (indicated on right of each plot). Colors indicate

1438   normalized enrichment scores (NES) from gene set enrichment analysis (GSEA) of

1439   differentially expressed genes (DEGs) between each comparison. FDR = false

1440   discovery rate, where FDR < 0.25 is considered significant (as is standard for GSEA).

1441   Parasite-dependent stimuli include ROPs = rhoptry proteins, MDGs = MYR-dependent

1442   dense granule proteins, MIGs = MYR-independent dense granule proteins, P(MD) =

1443   MYR-dependent paracrine effects, P(MI) = MYR-independent paracrine effects, and All

1444   = ROPs + MDGs + MIGs + Invasion + P(MD) + P(MI). **(B)** Bubble plot as in (A) where

1445   colors indicate $\log_2$ normalized fold change in expression of DEGs that fall under each

1446   comparison and each gene set. **(C)** Model of individual impacts of parasite-dependent

1447   stimuli on host transcription, which include immune pathways (e.g., TNFA Signaling via

1448   NFKB, IL6 JAK STAT3 Signaling, Inflammatory Response, IL2 STAT5 Signaling,

1449   Interferon Gamma Response, and Allograft Rejection), cellular stress (e.g., Hypoxia,

1450   Apoptosis), and cell proliferation-associated pathways (i.e., KRAS signaling, p53

1451   pathway). Curved lines indicate modulation of host transcription, and jagged lines

1452   indicate modulation without effector translocation to the nucleus.

1453

1454   **SUPPLEMENTARY FILE FIGURE AND TABLE LEGENDS**

1455   **Figure S1.** Construction and validation of RH *Δmyr1* mCherry Toxofilin-HA-beta-

1456   lactamase (RH *Δmyr1* mCherry Tfn-HA-BLA). **(A)** CRISPR/Cas9 strategy to construct

1457   RH *Δmyr1* mCherry Tfn-HA-BLA by disrupting parasite UPRT (blue) in parental strain

1458   RH *Δmyr1* mCherry. The parental strain was transfected with plasmid pSAG1::- Cas9-

1459   U6::sgUPRT (pink) and a linear amplicon containing Tfn-HA-BLA (purple) and flanked

1460   with 20 nucleotide homology arms to UPRT (blue). The region of UPRT complementary

1461   to the guide RNA, which also contains the site at which Cas9 cleaves, is in pink. **(B)**

1462   Western blot for the Tfn-HA-BLA construct in RH *Δmyr1* mCherry Tfn-HA-BLA. All lanes

1463   were obtained from the same gel. **(C)** Immunofluorescence assay (IFA) for

1464   colocalization of the Tfn-HA-BLA protein product and ROP2/3/4. **(D)** FACS analysis of

1465   10 T1/2 cells infected with parasite-free lysate, RH Tfn-HA-BLA, and RH *Δmyr1*

1466   mCherry Tfn-HA-BLA. Cleavage of reporter dye CCF2-AM indicates injection of the Tfn-

1467   HA-BLA construct.

1468   **Figure S2.** Human foreskin fibroblast (HFF) feeder cell debris contaminates the

1469   uninfected-injected (U-I) FACS gate. In all panels, a host cell monolayer is treated with

1470   either a parasite-free lysate of HFFs or RH Tfn-HA-BLA parasites syringe released from

1471   HFFs and then incubated with CCF2-AM to reveal (un)injected cells. *Left:* Infection with

1472   RH Tfn-HA-BLA reveals uninjected and injected cell populations. *Middle:* Mock

1473   "infection" with para- site-free lysate reveals HFF feeder cell debris contaminating the

1474   "injected" cell population. *Right:* Infection with parasite-free lysate that was washed to

1475   remove HFF debris reveals a reduction in contamination of the injected population.

1476

1477   **Figure S3. (A)** Serum starvation for 24 h partially inhibits cell division of 10 T1/2 host

1478   cells, reducing the possibility of capturing U-I cells that arise from division of an infected

1479   host cell (U-Id cells) rather than from an aborted invasion event. Note that the S phase

1480   population in the bottom right panel (serum replete, infected cells) also contains G1

1481   phase cells containing parasites, as parasite nuclear content enhances the propidium

1482   iodide signal in these cells. **(B)** Histogram depicting the number of infected host cells

1483   that divided at various times post-infection, as determined by live video microscopy

1484   footage of 200 serum starved 10 T1/2 cells for which the precise moment of infection

1485   was captured on camera. Of the 200 infected 10 T1/2 cells, 53 divided over a 16 hour

1486   time course, and none divided at earlier than 3.67 hours post-infection.

1487

1488   **Figure S4**. Quality control metrics for single cell RNA sequencing data. **(A)** Comparison

1489   of gene counts (number of genes for which reads from each cell mapped to the

1490   concatenated mouse-*Toxoplasma* genome, y-axis) and read sum (total reads, x-axis)

1491 for all experimental trials. Cells that passed quality control are indicated in color. **(B)**

1492 Percentages of total reads that mapped to open reading frames (ORFs) in the mouse-

1493 *Toxoplasma* concatenated genome. **(C)** *Top panel:* Linear regression modeling of

1494 measurement accuracy fitted on ERCC spike-ins with abundance above the detection

1495 limit. The text within each subplot denotes the coefficient of determination for the

1496 regression fit. *Bottom panel:* Logistic regression modeling of detection limit based on

1497 ERCC spike-ins. The 50% detection rate is indicated with a black dotted line, and the

1498 text within each subplot indicates the detection limit for each experiment in absolute

1499 molecular counts. **(D)** Linear regression fitted to scatterplot of average gene counts of

1500 differentially expressed genes for single cell RNA sequencing data (x-axis) vs. bulk RNA

1501 sequencing data (y-axis). Each point represents a DEG. Text within each subplot

1502 denotes the coefficient of determination (R^2) for the regression. **(E)** The coefficients of

1503 determination for linear regression lines fit to scatterplots as in (D) for all possible

1504 combinations of single vs. bulk RNA-seq combinations.

1505

1506 **Figure S5.** Single cell resolution enables strategic partitioning of individual cells for

1507 downstream analysis. **(A)** Percentage of reads derived from *Toxoplasma* validates

1508 infection status of individual cells. Cells are scored as uninfected if left of the lower

1509 decision line (bold, dashed), infected if right of the upper decision line (dotted), and

1510 ambiguous if between the decision lines (cross-hatched section). **(B)** Principal

1511 component analysis (PCA) projection of cells based on 175 curated cell cycle markers

1512 and subsequent Leiden clustering enables partitioning of cells by predicted cell cycle

1513 states, G1 (green), S (gold), and G2/M (purple). **(C)** Proportion of cells from each

1514   experimental condition in each cell cycle phase. **(D)** Dimensionality reduction and

1515   projection of single cells using the *Uniform Manifold Approximation and Projection*

1516   (UMAP) algorithm reveals 3 putative cell populations. All panels are reproduced copies

1517   of the same projection, each of which is color coded by specific parameters. *Left:*

1518   Louvain clusters, used to assign the cells to populations 1, 2, and 3; *middle:* cell cycle

1519   phase; *right (3 panels):* experimental trial (1 hpi, 2 hpi, and 3 hpi).

1520

1521   **Figure S6.** Effect of cytochalasin D treatment on the proportion of 10 T1/2 host cells

1522   that are U-I cells. **(A)** U-U and U-I cells that arise from Wt and CytD infections at 2 hpi.

1523   **(B)** U-U and U-I cells that arise from Wt and CytD infections at 3 hpi.

1524

1525   **Table S1:** Mouse genes discarded due to reads from extracellular RH parasites

1526   mapping to these genes in the concatenated mouse-*Toxoplasma* genome.

1527

1528   **Table S2:** Curated list of genes used to assign cell cycle phases to single host cells.

1529

1530   **Table S3:** Differentially expressed genes (DEGs). In all tables, fdr = false discovery rate,

1531   and log2FC is the $\log_2$ fold change in expression of DEGs between the indicated

1532   conditions. **(A)** All differentially expressed genes at all time points. **(B)** Wt I-I vs. Mock

1533   Differentially Expressed Genes. **(C)** Wt U-I vs. Wt U-U Differentially Expressed Genes.

1534   **(D)** Dmyr1 U-I vs. Dmyr1 U-U Differentially Expressed Genes. **(E)** CytD U-I vs. CytD U-

1535   U Differentially Expressed Genes. **(F)** Wt U-I vs. Wt I-I Differentially Expressed Genes.

1536   **(G)** Dmyr1 U-I vs. Dmyr1 I-I Differentially Expressed Genes. **(H)** Wt I-I vs. Dmyr1 I-I

1537 Differentially Expressed Genes. **(I)** Wt U-U vs. Mock Differentially Expressed Genes. **(J)**

1538 Dmyr1 U-U vs. Mock Differentially Expressed Genes. For (F) and (G), a gene is

1539 designated as showing evidence of being acted upon by counterbalancing effectors

1540 (indicated in the "Evidence of Effectors in Wt I-I Cells Counteracting Effectors in Wt U-I

1541 Cells?" and "Evidence of Effectors in Dmyr1 I-I Cells Counteracting Effectors in Dmyr1

1542 U-I Cells?" columns, respectively) if it exhibits either: 1) upregulation in one cell type

1543 and downregulation in the other compared to a common Wt U-U standard, or 2) more

1544 upregulation or downregulation in Wt U-I cells compared to the standard than in Wt I-I

1545 cells.

1546

1547 **Table S4:** CCF2-AM correlation data for **(A)** Wt U-I vs. Wt I-I cells and **(B)** Dmyr1 I-I vs.

1548 Wt I-I cells. A gene is designated as showing evidence of being acted upon by

1549 counterbalancing effectors (indicated under the "Evidence of Effector

1550 Counterbalancing?" column) if it exhibits either: 1) negative correlation with CCF2-AM in

1551 one cell type and a positive CCF2-AM correlation in the other cell type, or 2) stronger

1552 positive or negative correlations in Wt U-I cells than in Wt I-I cells.
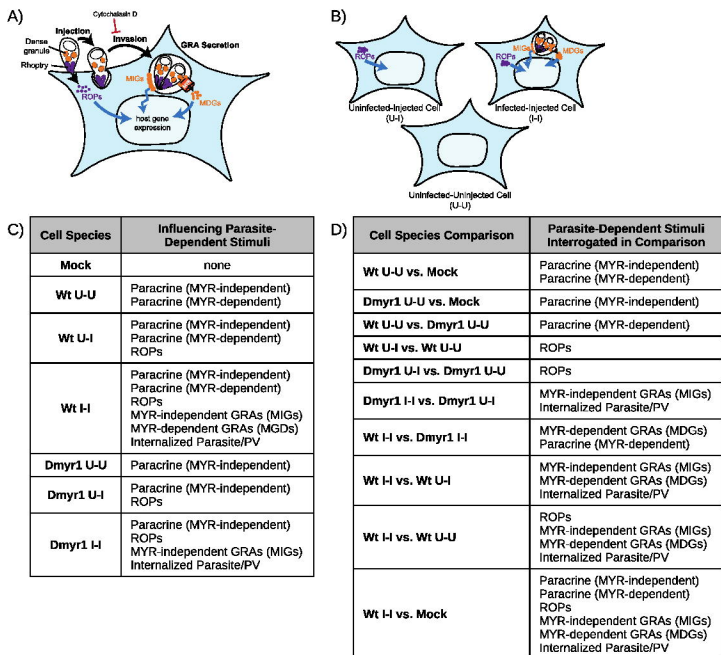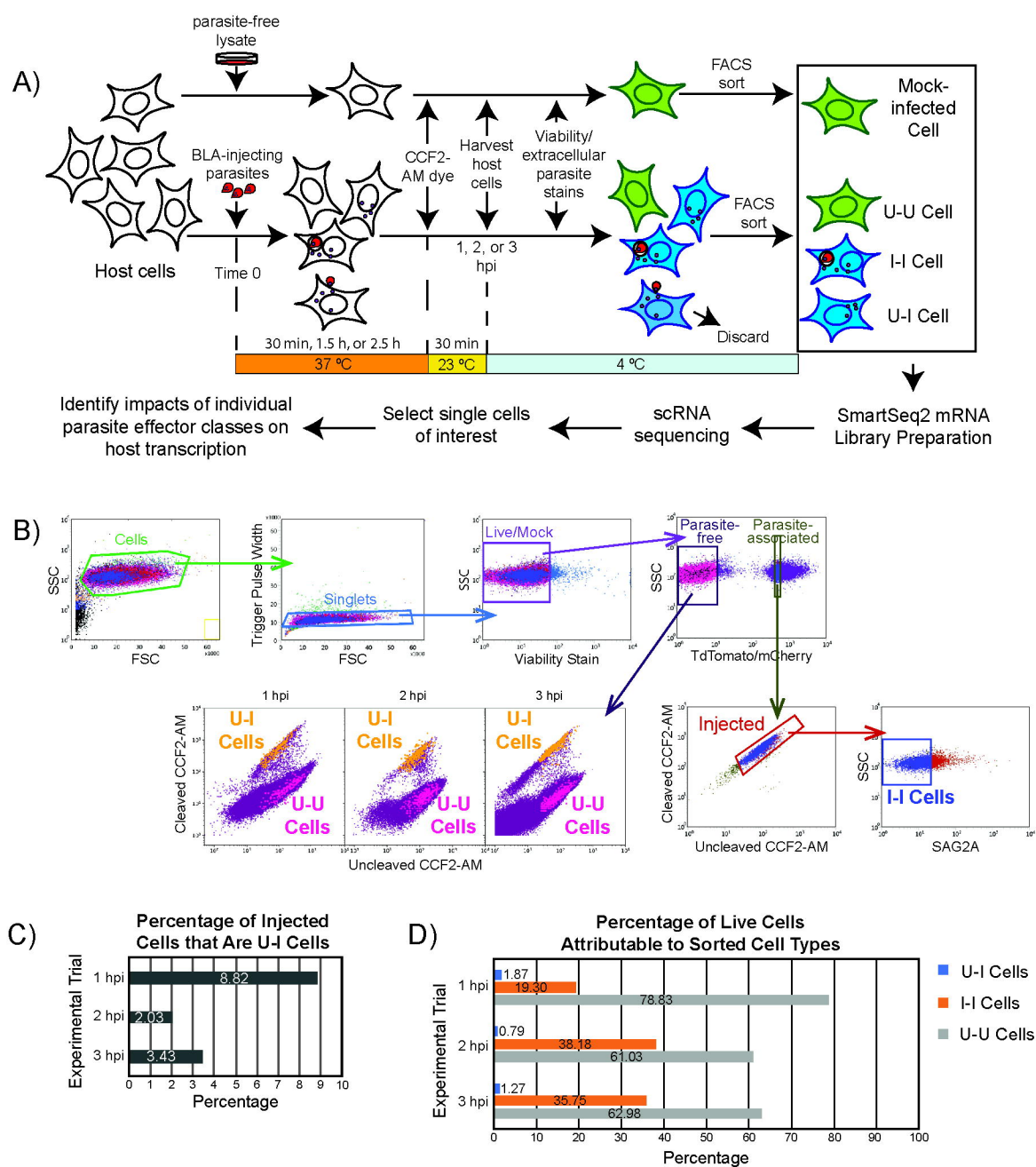
Figure 1

A) B)

C)

Cell Species	Influencing Parasite-Dependent Stimuli
Mock	none
Wt U-U	Paracrine (MYR-independent) Paracrine (MYR-dependent)
Wt U-I	Paracrine (MYR-independent) Paracrine (MYR-dependent) ROPs
Wt I-I	Paracrine (MYR-independent) Paracrine (MYR-dependent) ROPs MYR-independent GRAs (MIGs) MYR-dependent GRAs (MGDs) Internalized Parasite/PV
Dmyr1 U-U	Paracrine (MYR-independent)
Dmyr1 U-I	Paracrine (MYR-independent) ROPs
Dmyr1 I-I	Paracrine (MYR-independent) ROPs MYR-independent GRAs (MIGs) Internalized Parasite/PV

D)

Cell Species Comparison	Parasite-Dependent Stimuli Interrogated in Comparison
Wt U-U vs. Mock	Paracrine (MYR-independent) Paracrine (MYR-dependent)
Dmyr1 U-U vs. Mock	Paracrine (MYR-independent)
Wt U-U vs. Dmyr1 U-U	Paracrine (MYR-dependent)
Wt U-I vs. Wt U-U	ROPs
Dmyr1 U-I vs. Dmyr1 U-U	ROPs
Dmyr1 I-I vs. Dmyr1 U-I	MYR-independent GRAs (MIGs) Internalized Parasite/PV
Wt I-I vs. Dmyr1 I-I	MYR-dependent GRAs (MDGs) Paracrine (MYR-dependent)
Wt I-I vs. Wt U-I	MYR-independent GRAs (MIGs) MYR-dependent GRAs (MDGs) Internalized Parasite/PV
Wt I-I vs. Wt U-U	ROPs MYR-independent GRAs (MIGs) MYR-dependent GRAs (MDGs) Internalized Parasite/PV
Wt I-I vs. Mock	Paracrine (MYR-independent) Paracrine (MYR-dependent) ROPs MYR-independent GRAs (MIGs) MYR-dependent GRAs (MDGs) Internalized Parasite/PV

Figure 2

Figure 3

Wt I-I vs mock GSEA (2 hpi)

A)

	Gene Set	FDR	NES	Identified at 6 hpi (Naor et al 2018)
Higher in Wt I-I	TNFA SIGNALING VIA NFKB	0.078	1.888	Yes
	ALLOGRAFT REJECTION	0.086	1.634	Yes
	IL6 JAK STAT3 SIGNALING	0.108	1.692	Yes
	INTERFERON GAMMA RESPONSE	0.108	1.785	Yes
	ESTROGEN RESPONSE LATE	0.169	1.573	Yes
Higher in Mock	TGF BETA SIGNALING	0.199	-1.725	No
	UV RESPONSE DN	0.199	-1.594	Yes

Wt I-I vs mock GSEA (3 hpi)

B)

	Gene Set	FDR	NES	Identified at 6 hpi (Naor et al 2018)
Higher in Wt I-I	TNFA SIGNALING VIA NFKB	0.052	2.596	Yes
	INFLAMMATORY RESPONSE	0.052	2.065	Yes
	ALLOGRAFT REJECTION	0.052	2.062	Yes
	IL6 JAK STAT3 SIGNALING	0.052	1.997	Yes
	APOPTOSIS	0.059	1.85	Yes
	P53 PATHWAY	0.059	1.762	Yes
	IL2 STAT5 SIGNALING	0.071	1.803	Yes
	KRAS SIGNALING DN	0.089	1.703	Yes
	INTERFERON GAMMA RESPONSE	0.103	1.729	Yes
	UV RESPONSE DN	0.148	1.669	Yes
	UV RESPONSE UP	0.159	1.565	Yes
	HEME METABOLISM	0.163	1.574	Yes
	MTORC1 SIGNALING	0.163	1.525	Yes
	MITOTIC SPINDLE	0.185	1.504	No
	E2F TARGETS	0.243	1.458	Yes
Higher in Mock	APICAL SURFACE	0.052	-1.636	No
	OXIDATIVE PHOSPHORYLATION	0.052	-2.191	No
	KRAS SIGNALING UP	0.154	-1.639	No
	COAGULATION	0.159	-1.592	No
	ANGIOGENESIS	0.159	-1.602	No
	MYOGENESIS	0.163	-1.638	Yes

Figure 4

A)

GSEA for Wt U-I vs. Wt U-U at 3 hpi

	Gene Set	FDR	NES	Part of Infection Response?
	TNFA SIGNALING VIA NFKB	0.019	2.699	Yes
	INFLAMMATORY RESPONSE	0.019	2.483	Yes
	KRAS SIGNALING UP	0.019	2.113	No
	IL6 JAK STAT3 SIGNALING	0.019	2.021	Yes
	HYPOXIA	0.058	1.842	No
Higher in Wt U-I	IL2 STAT5 SIGNALING	0.06	1.823	Yes
	ALLOGRAFT REJECTION	0.06	1.809	Yes
	EPITHELIAL MESENCHYMAL TRANSITION	0.06	1.787	No
	INTERFERON GAMMA RESPONSE	0.06	1.766	Yes
	P53 PATHWAY	0.064	1.74	Yes
	COMPLEMENT	0.142	1.578	No
	KRAS SIGNALING DN	0.121	1.541	Yes
	APICAL JUNCTION	0.159	1.524	No
	UV RESPONSE DN	0.192	1.491	Yes
Higher in Wt U-U	NOTCH SIGNALING	0.115	-1.323	No
	UNFOLDED PROTEIN RESPONSE	0.216	-1.416	No
	OXIDATIVE PHOSPHORYLATION	0.075	-1.771	Yes

B)

GSEA for U-I vs. U-U Cells across Conditions at 3 hpi
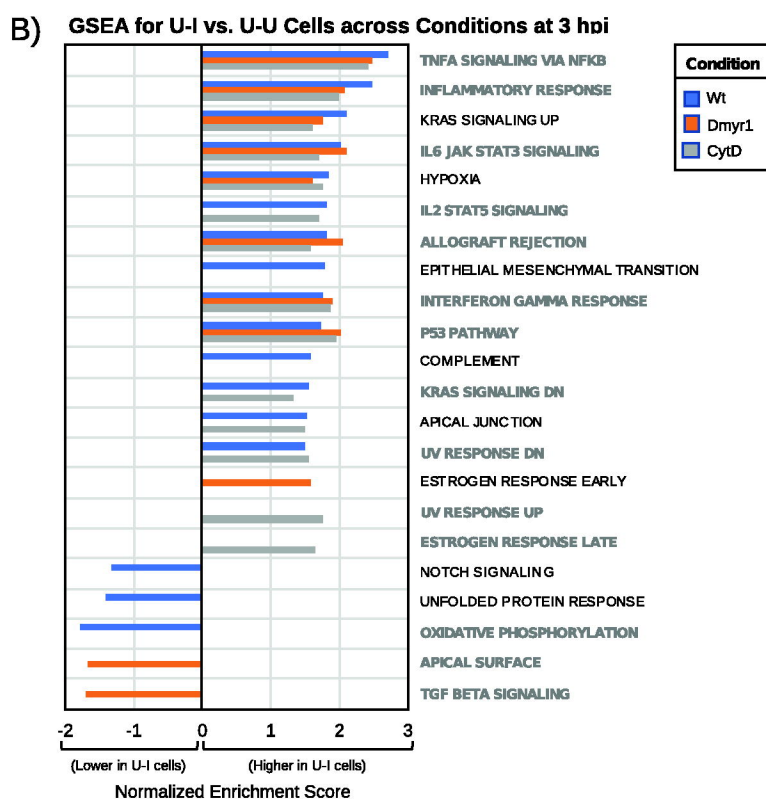
Figure 5

A)

Wt I-I vs Wt U-I CCF2-AM Correlation Plots
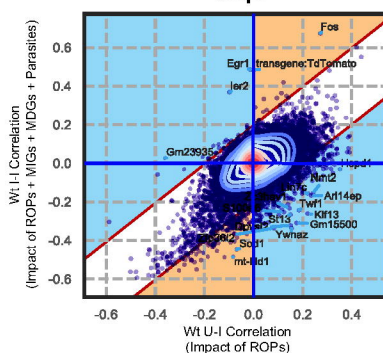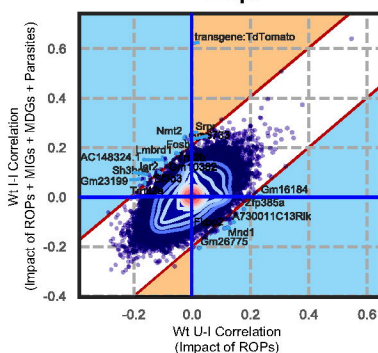
B)

	Gene Set	FDR	NES
Higher in Wt U-I	EPITHELIAL MESENCHYMAL TRANSITION	0.050	2.029
	MYOGENESIS	0.050	1.893
	COMPLEMENT	0.054	1.854
	COAGULATION	0.092	1.736
	PI3K AKT MTOR SIGNALING	0.100	1.645
	BILE ACID METABOLISM	0.100	1.574
	XENOBIOTIC METABOLISM	0.198	1.505
	ANDROGEN RESPONSE	0.198	1.487
	KRAS SIGNALING DN	0.198	1.407
	ANGIOGENESIS	0.232	1.334
Higher in Wt I-I	UV RESPONSE UP	0.05	-2.165
	TNFA SIGNALING VIA NFKB	0.081	-1.855
	IL6 JAK STAT3 SIGNALING	0.092	-1.934
	ALLOGRAFT REJECTION	0.100	-1.847
	ESTROGEN RESPONSE EARLY	0.186	-1.593
	INTERFERON GAMMA RESPONSE	0.186	-1.627
	ESTROGEN RESPONSE LATE	0.186	-1.636
	MITOTIC SPINDLE	0.232	-1.458

GSEA for Wt U-I vs. Wt I-I at 3 hpi

Figure 6

GSEA for Dmyr1 U-I vs. Dmyr1 I-I at 3 hpi

	Gene Set	FDR	NES	Part of Infection Response?	Part of Injection Response?	Differentially Regulated between Wt I-I and Wt U-I?
Higher in Dmyr1 U-I	COMPLEMENT	0.203	1.427	No	No	Yes
	COAGULATION	0.203	1.427	Yes	No	Yes
	MTORC1 SIGNALING	0.214	1.542	No	No	No
	MYOGENESIS	0.217	1.404	Yes	No	Yes
Higher in Dmyr1 I-I	IL6 JAK STAT3 SIGNALING	0.093	-1.900	Yes	Yes	Yes
	ALLOGRAFT REJECTION	0.126	-1.780	Yes	Yes	Yes
	UV RESPONSE UP	0.126	-1.806	Yes	No	Yes
	INTERFERON GAMMA RESPONSE	0.135	-1.711	Yes	Yes	Yes
	INFLAMMATORY RESPONSE	0.135	-1.738	Yes	Yes	No
	UV RESPONSE DN	0.135	-1.770	Yes	Yes	No
	TNFA SIGNALING VIA NFKB	0.135	-1.830	Yes	Yes	Yes
	P53 PATHWAY	0.189	-1.626	Yes	Yes	No
	KRAS SIGNALING DN	0.203	-1.335	Yes	Yes	No
	ESTROGEN RESPONSE EARLY	0.221	-1.497	No	Yes?	Yes
	ANGIOGENESIS	0.242	-1.312	No	No	No

Figure 7

GSEA for Dmyr1 I-I vs. Wt I-I at 3 hpi

A)

	Gene Set	FDR	NES	Identified at 6 hpi (Naor et al. 2018)	Part of Infection Response	Part of Injection Response
Higher in Dmyr1 I-I	KRAS SIGNALING UP	0.079	2.223	No	No	Yes
	IL2 STAT5 SIGNALING	0.080	2.000	Yes	Yes	Yes
	TNFA SIGNALING VIA NFKB	0.080	1.917	Yes	Yes	Yes
	INFLAMMATORY RESPONSE	0.080	1.891	Yes	Yes	Yes
	ANGIOGENESIS	0.080	1.852	No	No	No
	PI3K AKT MTOR SIGNALING	0.080	1.826	No	No	No
	INTERFERON GAMMA RESPONSE	0.080	1.821	Yes	Yes	Yes
	APOPTOSIS	0.119	1.710	Yes	Yes	No
	IL6 JAK STAT3 SIGNALING	0.083	1.706	Yes	Yes	Yes
	COMPLEMENT	0.153	1.589	No	No	Yes
	GLYCOLYSIS	0.169	1.563	No	No	No
	HYPOXIA	0.153	1.517	Yes	No	Yes
	WNT BETA CATENIN SIGNALING	0.080	1.516	No	No	No
	HEDGEHOG SIGNALING	0.169	1.511	No	No	No
	NOTCH SIGNALING	0.169	1.504	No	No	No
Higher in Wt I-I	MITOTIC SPINDLE	0.124	-1.635	Yes	No	No

B)

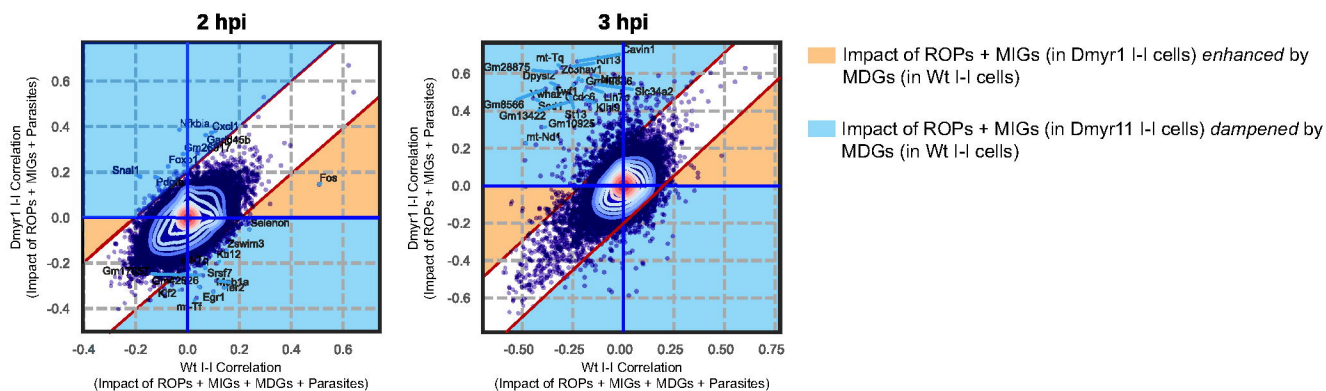Dmyr1 I-I vs Wt I-I CCF2-AM Correlation Plots

Figure 8

A)

GSEA for Dmyr1 U-U vs. Mock at 3 hpi

	Gene Set	FDR	NES	Part of Infection Response (sc)	Part of Infection Response (Naor et al. 2018)	Part of Injection Response (sc)
Higher in Dmyr1 U-U than in Mock	TNFA SIGNALING VIA NFKB	0.012	2.557	Yes	Yes	Yes
	HYPOXIA	0.012	2.000	No	Yes	Yes
	IL6 JAK STAT3 SIGNALING	0.012	2.070	Yes	Yes	Yes
	INFLAMMATORY RESPONSE	0.012	2.304	Yes	Yes	Yes
	P53 PATHWAY	0.012	2.100	Yes	Yes	Yes
	KRAS SIGNALING UP	0.012	2.143	Yes (downregulated)	Yes	Yes
	CHOLESTEROL HOMEOSTASIS	0.026	1.834	No	Yes	No
	ALLOGRAFT REJECTION	0.026	1.783	Yes	Yes	Yes
	APOPTOSIS	0.029	1.790	Yes	Yes	No
	MTORC1 SIGNALING	0.029	1.741	Yes	Yes	No
	INTERFERON GAMMA RESPONSE	0.044	1.733	Yes	Yes	Yes
	XENOBIOTIC METABOLISM	0.046	1.726	No	Yes	No
	UV RESPONSE DN	0.046	1.682	Yes	Yes	Yes
	IL2 STAT5 SIGNALING	0.104	1.517	Yes	Yes	Yes
	GLYCOLYSIS	0.133	1.540	No	Yes	No
	ESTROGEN RESPONSE EARLY	0.164	1.487	No	Yes	No
	MYC TARGETS V2	0.164	1.533	No	Yes	No
	MYOGENESIS	0.192	1.483	Yes (downregulated)	Yes	No
	PI3K AKT MTOR SIGNALING	0.199	1.444	No	No	No
	UV RESPONSE UP	0.247	1.418	Yes	Yes	Yes

B)

Expression of Dmyr1 U-U vs. Mock DEGs across All Cells

Higher in Dmyr1 U-U than in Mock

Higher in Mock than in Dmyr1 U-U

Figure 9