

1 **A genetic screen identifies a protective type III interferon response to**
2 ***Cryptosporidium* that requires TLR3 dependent recognition**

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21

22 **Abstract**

23 *Cryptosporidium* is a leading cause of severe diarrhea and diarrheal-related death in
24 children worldwide. As an obligate intracellular parasite, *Cryptosporidium* relies on
25 intestinal epithelial cells to provide a niche for its growth and survival, but little is known
26 about the contributions that the infected cell makes to this relationship. Here we
27 conducted a genome wide CRISPR/Cas9 knockout screen to discover host genes
28 required for *Cryptosporidium parvum* infection and/or host cell survival. Gene enrichment
29 analysis indicated that the host interferon response, glycosaminoglycan (GAG) and
30 glycosylphosphatidylinositol (GPI) anchor biosynthesis are important determinants of
31 susceptibility to *C. parvum* infection. Several of these pathways are linked to parasite
32 attachment and invasion and C-type lectins on the surface of the parasite. Evaluation of
33 transcript and protein induction of innate interferons revealed a pronounced type III
34 interferon response to *Cryptosporidium* in human cells as well as in mice. Treatment of
35 mice with IFN λ reduced infection burden and protected immunocompromised mice from
36 severe outcomes including death, with effects that required STAT1 signaling in the
37 enterocyte. Initiation of this type III interferon response was dependent on sustained
38 intracellular growth and mediated by the pattern recognition receptor TLR3. We conclude
39 that host cell intrinsic recognition of *Cryptosporidium* results in IFN λ production critical to
40 early protection against this infection.

41 **Author Summary**

42 *Cryptosporidium* infection is an important contributor to global childhood mortality.
43 There are currently no vaccines available, and the only drug has limited efficacy in
44 immunocompromised individuals and malnourished children who need it most. To
45 discover which host proteins are essential for *Cryptosporidium* infection, we conducted a
46 genome wide knockout screen in human host cells. Our results confirm the importance of
47 glycosaminoglycans on the surface of epithelial cells for attachment and invasion of the
48 parasite. We also found that host GPI anchor biosynthesis and interferon signaling
49 pathways were enriched by our screen. Examining the role of interferon signaling further
50 we found a type III interferon response, IFN λ , was generated in response to infection and
51 shown to be initiated in the infected cell. Utilizing mouse models of infection, we found
52 that the type III interferon response was important early during infection with its induction
53 likely preceding IFN γ , a key cytokine for the control of this infection. We also determined
54 that TLR3 was the pattern recognition receptor responsible for IFN λ production during
55 *Cryptosporidium* infection. Our work shows that IFN λ acts directly on the enterocyte and
56 its use in treating immunocompromised mice produced striking reductions in infection.

57 **Introduction**

58 *Cryptosporidium* is a leading cause of diarrheal disease. In the United States, this
59 apicomplexan parasite accounts for more than half of all waterborne disease outbreaks
60 and infection can be life-threatening in individuals with compromised immune function [1,
61 2]. Globally, the burden of this disease rests disproportionately on children under the age
62 of two and the parasite is an important contributor to early childhood mortality [3]. Children
63 can experience multiple episodes of infection, however, parasite and disease burden
64 diminish over successive infection and non-sterile immunity protects children from severe
65 illness as well as stunting [4].

66 It is well established that T cells are critical to protection from and the resolution of
67 infection with *Cryptosporidium* [5]. The production of interferon gamma (IFN γ) is
68 recognized to be one of the essential functions of T cells during *Cryptosporidium* infection
69 [6], but T cells are not the only source of IFN γ [7-9]. Numerous other chemokines and
70 cytokines produced by the enterocyte including IL-8, IL-18, TGF β , and RANTES and type
71 one and three interferons have been noted as well [10-14]. These can act directly on
72 enterocytes and/or stimulate responses by proximal immune cells in the intestinal
73 epithelium and adjacent tissues leading to the enhanced production of IFN γ , among other
74 responses. IL-18 was shown to be produced by the enterocyte and to signal to ILC1s
75 promoting IFN γ production [9]. New *in vitro* enteroid models of infection have also
76 revealed the presence of a type I IFN response through RNA sequencing [15, 16].
77 Additionally, type III interferon (IFN λ) production has been observed in response to *C.*
78 *parvum* infection in neonatal piglets and neonatal mice [12]. Type III interferons, the most
79 recently discovered members of the cytokine family, were shown to play unique roles at

80 mucosal sites that could not be compensated for by type I interferons [17] making their
81 role during *Cryptosporidium* infection of particular interest..

82 *Cryptosporidium* infection is typically restricted to the small intestine, but infection
83 of the biliary tree and respiratory involvement has also been reported [18, 19]. Within the
84 intestine, the infection is limited to epithelial cells in which the parasite occupies an
85 intracellular but extra-cytoplasmic niche at the brush border. A number of cytoskeletal and
86 membranous structures separate the parasitophorous vacuole from the bulk of the
87 infected enterocyte [20, 21]. While reorganization of the actin cytoskeleton is one of the
88 most prominent changes in host cell morphology, infection is also known to interfere with
89 the composition and function of tight junctions, to induce tyrosine phosphorylation, and to
90 activate PI3K signaling [22, 23]. Recent studies have identified parasite proteins that are
91 injected into the host cell during and after invasion [24, 25] but we know very little about
92 the specific components of the host cell that shape host-parasite interaction for
93 *Cryptosporidium*.

94 Here, we used a CRISPR-Cas9 knockout screen to identify host genes that impact
95 host cell survival during *Cryptosporidium* infection. The screen revealed the importance
96 of several pathways, with IFN signaling, sulfated GAGs, and GPI anchor synthesis most
97 prominent. We found that the interferon signaling pathway identified here was triggered
98 by robust production of type III but not type I interferon in human host cells. This response
99 required live infection and was initiated in infected cells. We investigated the molecular
100 recognition mechanism that leads to this response and studied its impact on the infection.
101 *In vivo* experiments showed IFN λ to limit parasite growth an effect that was independent

102 of the presence of IFN γ . Thus, we elucidate a mechanism of cell intrinsic recognition and
103 control of *Cryptosporidium*.

104

105

106 **Results**

107 **A screen for host genes that impact *Cryptosporidium* infection and host cell** 108 **survival**

109 How *Cryptosporidium* interacts with its host cell is poorly understood. The parasite
110 is thought to rely on pathogenesis factors exposed on its surface or secreted during and
111 after invasion [26, 27], however, host proteins are likely to play important roles in this
112 interaction as well. To identify such host factors, we conducted an unbiased genetic
113 screen. Since *Cryptosporidium* infection is limited to epithelial cells, we chose to screen
114 in HCT-8 cells, a colon-derived human adenocarcinoma cell line widely used for
115 experiments with this parasite [28]. In this *in vitro* culture system, parasites can only be
116 propagated for 72 hours and then growth ceases. First, we measured the survival of HCT-
117 8 cells over a range of infection conditions and found *C. parvum* to induce host cell death
118 in a dose-dependent fashion over the 72 hours (Fig 1A). We chose to move forward with
119 a 90% kill dose to impose strong selection for loss of function in genes required for
120 parasite growth or cell death as part of the host response to infection. Next, we generated
121 clonal HCT-8 cell lines that stably express Cas9 [29] and assessed activity in each clone
122 using an EGFP reporter assay [30]. Briefly, Cas9 expressing cells were transfected with
123 a lentiviral vector encoding EGFP as well as a single guide RNA (sgRNA) targeting the
124 EGFP gene and analyzed by flow cytometry. Cells with Cas9 activity show reduced
125 fluorescence when compared to the parental cell line and are shown normalized to a

126 control cell line expressing no EGFP (Fig 1B). Clones C, I, and K showed high activity
127 and served as three independent biological replicates in the subsequent screen. Using
128 the Brunello lentiviral CRISPR library, we targeted the full complement of human protein
129 coding genes with four sgRNAs each in addition to controls [31] for a total of 77,441
130 sgRNAs. 10^8 cells of each clone were transduced with the library at an MOI of 0.4 to
131 ensure each cell received only one sgRNA. Following seven days of puromycin selection,
132 cells were expanded to 4 T175 flasks, achieving roughly 500-fold coverage and infected
133 with *C. parvum* at a 90% kill dose. After 72 hours, the media was changed, and surviving
134 cells were allowed to expand. Cells were exposed to *C. parvum* for a total of three rounds
135 of infection and expansion to enrich for resistant host cells (Fig 1C). Genomic DNA was
136 extracted from the input population as well as following each round of infection.

137

138 **Genetic screen reveals genes required for infection and host response**

139 Deep sequencing of the integrated sgRNAs and comparison with the input
140 population revealed the progressive enrichment of a subset of sgRNAs with each round
141 of infection (Fig 1D). Using model-based analysis of genome-wide CRISPR/Cas9
142 knockout [32], we identified 35 significantly enriched genes (FDR < 0.05, Fig 1E). Among
143 these genes, gene set enrichment analysis (GSEA) reveals three distinct pathways each
144 supported by multiple genes. IFNAR2, IFNLR1, IL10RB, IRF9, STAT1, STAT2, JAK1,
145 and TYK2 cluster within the pathway of interferon (IFN) signaling. B3GAT3, B4GALT7,
146 EXT1, SLC35B2 are genes encoding enzymes in the biosynthesis of sulfated
147 glycosaminoglycans (GAG). In addition, the screen selected for nine enzymes required
148 for glycosylphosphatidylinositol (GPI) anchor biosynthesis (GPAA1, MGAT1, PGAP2,
149 PIGA, PIH, PIGL, PIGO, PIGP, PIGT). Beyond these pathways, a number of genes were

150 significantly enriched that were not members of a particular pathway or represented the
151 single representative of a pathway. Among those with known molecular function were
152 accessory proteins to ATP flippase (TMEM30A), tyrosine protein kinases (CSK), GTPase
153 activators (RALGAPB), G protein coupled receptor signaling regulators (PDCL), granule
154 biogenesis proteins (NBEAL2), transcriptional activators of apoptosis (RRP1B),
155 dehalogenases (IYD), tetraspanins (CD151), fibronectin domain proteins (FNDC3B),
156 chaperones (UNC93B1 and HSP90B1), transcription factors (OLIG1), serine protease
157 (TMPRSS3), and peptide hormone receptors (NPR3).

158 To validate the screening results, HCT-8 cells were transduced with siRNA
159 targeting a subset of the top candidates for 24 hours prior to infection. Knockdown was
160 assessed by qPCR and a decrease in transcripts was found to be typically 30% or greater.
161 48 hours after *C. parvum* infection, we assessed host cell viability using the MTT assay.
162 We found that many candidates when knocked down, show increased resistance to cell
163 death, no difference was noted in the absence of infection (S1 Fig).

164

165 ***Cryptosporidium parvum* infection induces an interferon response**

166 Interferon signaling was the most highly enriched pathway identified by our screen.
167 The critical role of IFN γ is well documented in humans [33] and mice [5] and there are
168 also reports of *Cryptosporidium* associated induction of type I and III interferons [12, 13,
169 15]. To examine the epithelial cell response to *C. parvum*, we infected 6 well cultures of
170 HCT-8 cells with 100,000 oocysts and performed RNAseq. 1600 genes were differentially
171 expressed (1.5-fold; adjusted p value < 0.05) by 48 hours post infection, compared to
172 naïve cells (Fig 2A). The majority of differentially expressed genes were upregulated in

173 the infected population compared to the uninfected control (689 genes downregulated).
174 GSEA identified significant enrichment of the interferon signaling pathway in infected
175 cultures compared to uninfected controls (Fig 2B). Other strongly enriched pathways are
176 related to interferon signaling, such as REACTOME: Antiviral Mechanism by IFN
177 Stimulated Genes.

178 To validate the observed interferon signature and to establish kinetics, we next
179 conducted a qPCR time course experiment for three selected interferon stimulated genes
180 (ISGs) over 72hours of *C. parvum* infection to determine when the interferon response is
181 initiated. We infected 96-well cultures with *C. parvum* and isolated RNA at 0, 12, 24, 48,
182 and 72 hours post infection. ISG transcripts were increased in the first sample taken at
183 12 hours post infection and peaked at 72 hours (Fig 2C). Binding of interferons to their
184 receptors initiates an intracellular signaling cascade that culminates in the
185 phosphorylation of the transcription factor STAT1 leading to transcription of ISGs. We
186 therefore assessed STAT1 phosphorylation by Western Blot using a modification specific
187 antibody in whole cell lysates. Phosphorylation of STAT1 was not detectable in uninfected
188 cells but was observed as early as 12 hours post infection (Fig 2D). We also observed an
189 increase in total STAT1 protein at 24 hours post infection indicating that STAT1 itself was
190 induced by the infection, in line with its classification as an ISG. We conclude that *C.*
191 *parvum* infection induces a strong interferon response in HCT-8 cells.

192

193 ***Cryptosporidium* infection preferentially induces a type III interferon response**

194 Next, we asked which interferon was responsible for the response observed.
195 There are three major interferon types; type II interferon, IFN γ , is only produced by certain

196 lymphocytes and thus absent from our cultures. In contrast, type I interferons, most
197 prominently IFN α and IFN β , and type III interferons, IFN λ 1-4, are known to be produced
198 by epithelial lineages including the HCT-8 cells used here [34] (S2 Fig). Our GSEA
199 analyses found enrichment signatures for type I and type III interferon in infected cultures
200 (S2 Fig), but because both types act through the same intracellular signaling cascade, it
201 is difficult to distinguish between them by the genes they induce [35]. To determine which
202 types of interferons are expressed - simultaneously or individually - in response to *C.*
203 *parvum*, we measured the transcript abundance of *IFNB*, *IFNL1*, and *IFNL2/3* by qPCR.
204 *IFNB* transcripts did not increase at early time points and remained comparably low at 72
205 hours (4-fold, Fig 2E). In contrast, at 12 hours, the first time point sampled, type III
206 interferon transcripts were already markedly elevated. Type III interferon transcripts
207 peaked at 48 hours (*IFNL1*: 35-fold, *IFNL2/3*:1200-fold). We also performed enzyme-
208 linked immunosorbent assays (ELISA) to directly measure protein levels
209 for IFN β and IFN λ . Only modest amounts of IFN β were detectable, peaking at 48 hours
210 post infection (185 pg/mL). IFN λ production was detected as early as 24 hours post
211 infection and continued to increase until 72 hours, exceeding IFN β levels by two orders
212 of magnitude (16,029 pg/mL, $p < 0.0001$, Two-way ANOVA, Fig 2F). The kinetics of the
213 induction of IFN λ protein followed that of parasite replication, with a large increase
214 between 24 and 48 hours, when the parasites were actively replicating, and a plateau
215 between 48 and 72 hours when parasites terminally differentiate to gametes and growth
216 ceases (Fig 2G and [36]). Taken together, these experiments demonstrate that type III,
217 rather than type I interferons are preferentially induced by *C. parvum* infection in HCT-8
218 cells.
219

220 **Live *C. parvum* infection is required to induce type III interferon production**

221 A variety of pathogen associated molecular patterns (PAMPs) have been shown
222 to induce a type III interferon response including many bacterial proteins, glycans and
223 lipids [37]. Oocysts used in our experiments were isolated from the feces of cows or mice;
224 therefore, we considered that inadvertent inoculation of cultures with bacterial PAMPS
225 rather than *C. parvum* infection may drive IFN λ production. To test this, we heated
226 oocysts to 95°C for 10 min prior to adding them to cells. This kills the parasite but does
227 not inactivate LPS [38]. Heat killed parasites failed to induce IFN λ at any timepoint
228 assessed, and at 72 hours post infection, the difference in IFN λ production compared to
229 controls was highly significant (Fig 3A, $p < 0.0001$, Two-way ANOVA). Consistent with
230 the lack of IFN λ production, we did not observe phosphorylation of STAT1 in cultures
231 inoculated with heat killed parasites (Fig 3B). To further assess the importance of parasite
232 replication for interferon induction, we used nitazoxanide, the only currently FDA
233 approved drug for the treatment of *Cryptosporidium* infection. Treatment of cultures led
234 to a 35-fold decrease in parasite infection as assessed by qPCR (Fig 3C). In the
235 nitazoxanide treated infected cultures, IFN λ induction was no longer observed (Fig 3D, p
236 < 0.05 , One-way ANOVA). In contrast, the induction of IFN λ using an agonist of interferon
237 signaling, Poly(I:C), was intact under nitazoxanide treatment, demonstrating that the
238 observed response is specific to parasite infection. We therefore conclude that live
239 parasites and active parasite replication are required to induce the type III interferon
240 response.

241

242 **IFN-lambda is initially produced by infected cells and signals in an autocrine**
243 **manner**

244 The parasite completes its replicative cycle within 12 hours and parasite egress is
245 accompanied by host cell lysis and the release of intracellular contents, including both
246 host and parasite molecules (Fig 3E). Both intracellular parasite growth, and/or host cell
247 lysis could trigger the interferon response. Furthermore, signaling, once initiated, results
248 in the secretion of interferons, which may act on both producing and surrounding cells in
249 an auto- as well as paracrine fashion. This amplifies the signal through a feedforward loop
250 rapidly leading to cytokine from essentially all cells, making it difficult to determine how
251 the cascade originates.

252 To determine the cells that initiate the type III interferon response, we infected
253 HCT-8 with a *C. parvum* strain marked by expression of tandem Neon green
254 fluorescent protein (Fig 3F). At 10 hours post infection, and prior to first parasite egress,
255 we sorted cells for green fluorescence and isolated Neon positive infected cells as well
256 as Neon negative bystander cells from the same culture (Fig 3G). Three biologically
257 independent samples were subjected to RNA sequencing for each population. Infection
258 resulted in significant differences in gene expression with 380 upregulated and 466
259 downregulated genes (1.5-fold; adjusted p value < 0.05, Fig 3H). We noted the induction
260 of IFNL1 and 126 additional ISGs as identified by Interferome DB [39]. Many of these
261 genes represent a subset of the interferon signature we observed in our 48-hour RNAseq
262 but the amplitude of expression was lower, likely a reflection of the early timepoint and
263 the lack of paracrine amplification. Importantly, at this timepoint induction of the interferon
264 pathway is exclusive to infected Neon positive cells. We conclude that the type III
265 interferon response is initiated during intracellular replication of *C. parvum* in a cell
266 intrinsic fashion.

267 **The type III interferon response is required for early in vivo host defense**

268 To understand the consequences of the type III interferon response on infection,
269 we turned to an *in vivo* model of infection that uses a *C. parvum* strain adapted to mice
270 by continued serial passage [9]. First, we asked whether and when type III interferons are
271 produced in response to *C. parvum in vivo*. At day 2 post infection of C57BL/6 mice, we
272 found an average 4-fold increase of *Ifnl2/3* transcripts in the small intestine, and at day 4,
273 the induction was approximately 2-fold. (Fig 4A). We also assessed IFN λ secretion during
274 *C. parvum* infection using an ELISA from punch biopsies of the ileum and found similar
275 kinetics. IFN λ secretion was increased at 2 days post infection and waned below
276 detectable levels after 4 days (Fig 4B).

277 Type I and III interferons initiate a similar intracellular signaling cascade but utilize
278 different receptors, IFNAR for type I and a heterodimer of IFNLR1 and IL10RB for type
279 III. We infected C57BL/6 wild type mice, mice lacking the type I interferon receptor, *Ifnar*
280 ^{-/-}, and mice lacking the type III interferon receptor, *Il28ra* ^{-/-}, with 50,000 *C. parvum*
281 oocysts. Infection was monitored by measuring parasite produced Nanoluciferase from
282 feces [40]. Surprisingly, loss of the type I interferon receptor consistently resulted in a 3-
283 fold reduction in shedding when compared to wild type mice (area under the curve (AUC),
284 Fig 4C). In contrast, loss of type III interferon signaling resulted in an overall 2.7-fold
285 increase in parasite shedding compared to wild type mice (AUC, Fig 4C). We note that
286 histopathology revealed no baseline differences between WT and *Il28ra* ^{-/-} mice (S3 Fig).
287 To further validate this finding independent of mouse mutants, we used antibody-based
288 depletion. C57BL/6 mice were intraperitoneally injected daily with 20 μ g of an anti-*Ifnl2/3*
289 antibody and infected with 50,000 *C. parvum* oocysts. Again, we observed an increase in

290 parasite shedding of about 2-fold (AUC, Fig 4D). We conclude that type III, but not type I
291 interferons contribute to the early control of *Cryptosporidium in vivo*.

292

293 **Exogenous IFN-lambda treatment protects mice from severe *Cryptosporidium*** 294 **infection**

295 Since mice lacking the type III interferon receptor exhibited an increase in early
296 susceptibility, we tested the impact of exogenous administration of IFN λ on
297 *Cryptosporidium* infection. *Ifng*^{-/-} mice were injected intraperitoneally with daily doses of
298 *Ifn* λ 2 ranging from 0-5 μ g for the first three days of infection. As little as 0.1 μ g per day (the
299 smallest amount tested) produced a marked reduction in shedding (4.3-fold decrease
300 AUC, Fig 4E), and increasing the dose beyond 1 μ g did not yield further enhancement.
301 To assess whether this effect could be maintained long term, *Ifng*^{-/-} mice were infected
302 with *C. parvum* and injected intraperitoneally with a daily dose of 1 μ g *Ifn* λ 2 for the duration
303 of the infection. This treatment resulted in 7.7-fold reduction of shedding when compared
304 to mock injected control infections (AUC, Fig 4F). In contrast to mock injected mice, we
305 did not observe mortality among treated mice.

306 We note that administration of IFN λ was protective in *Ifng*^{-/-} mice, suggesting that
307 this protection does not require IFN γ . However, IFN λ has been shown to promote IFN γ
308 production [41]. To examine this potential interaction further we tested the effect of IFN λ
309 in mice lacking cells known to produce IFN γ in response to *C. parvum*: T cells, NK cells
310 and ILCs [7, 9, 42]. BL6, *Rag2*^{-/-}, and *Rag2*^{-/-}*Il2rg*^{-/-} mice were infected and treated with
311 1 μ g of *Ifn* λ 2. This resulted in comparable reduction of parasite shedding (BL6: 2.4-fold,
312 *Rag2*^{-/-}: 4.4-fold, *Rag2*^{-/-}*Il2rg*^{-/-}: 2-fold, Fig 4G), again suggesting that the benefit of IFN λ

313 treatment does not require immune cells, but largely rests on an enterocyte intrinsic
314 response. Finally, we conducted experiments with mice in which the STAT1 gene was
315 specifically ablated from enterocytes using Cre recombinase under the control of the
316 *Villin1* promoter. Removing STAT1 from the enterocyte lineage alone abolished the
317 benefit of IFN λ treatment (1.1-fold AUC, Fig 4H). Taken together, these data suggest that
318 IFN λ protects mice and does so by acting directly on the intestinal epithelium.

319

320 **TLR3 detects *Cryptosporidium* infection leading to IFN-lambda production**

321 Enterocytes have been shown to use a range of pattern recognition receptors to
322 detect infection with different pathogens, many of which can lead to a type III interferon
323 response [43]. HCT-8 cells, as many other cancer-derived lines, no longer express the
324 full complement of innate immune recognition and cell death pathways [44], but the IFN λ
325 response to *Cryptosporidium* remains intact. We took advantage of this to narrow the list
326 of potential receptors. HCT-8 cells were treated with known agonists of different pattern
327 recognition receptors and IFN λ production was measured after 24 hours. Specifically, we
328 tested Poly(I:C) with or without lipofection (TLR3 and RLRs), mTriDAP (NOD1 and
329 NOD2), 5' triphosphate dsRNA (RIGI), HSV60 DNA (CDS), ssPolyU RNA (TLR7), or CpG
330 motif containing DNA (TLR9) [45]. As shown in Fig 5A, only Poly(I:C) and ssPolyU RNA
331 produced an IFN λ response. This suggests TLR3, TLR7, or the RLRs MDA5 and RIGI as
332 potential receptors. We next tested each of these candidates *in vivo* using suitable mouse
333 mutants. Mice lacking MAVS, the adapter protein to RLRs, and TLR7 showed no
334 difference in infection compared to wild type controls (Fig 5B and 5C). However, mice
335 lacking TLR3 were more susceptible, resulting in an 8-fold increase in parasite shedding

336 and an overall pattern of infection that was reminiscent of *Ii28ra*^{-/-} mice (AUC, Fig 5D). We
337 found the production of IL-18, an enterocyte derived cytokine induced by *Cryptosporidium*
338 infection, [46, 47] to be intact in the absence of TLR3 (Fig 5E). Next, we measured IFN λ
339 secretion from ileal punches of infected *Tlr3*^{-/-} mice and wild type controls at day 2 of
340 infection by ELISA. In the absence of TLR3, IFN λ production was reduced to the limit of
341 detection (Fig 5F, *p* value < 0.001, Unpaired t-test). Note this reduction occurs despite an
342 8-fold higher infection in *Tlr3*^{-/-}. We conclude that the production of type III IFN during
343 *Cryptosporidium* infection depends on TLR3 signaling.

344 **Discussion**

345 We conducted a whole genome knock out screen to identify human genes that
346 influence *Cryptosporidium* infection and host survival. We identified 35 genes with high
347 confidence, and they implicate multiple pathways.

348

349 **Parasite attachment and invasion**

350 Four of the genes enriched in our screen encode steps in the synthesis of
351 glycosaminoglycans. This provides further support for the notion that interactions
352 between a parasite C-type lectin and host glycosaminoglycans are critical to parasite
353 binding and invasion [48]. GPI anchor synthesis is also highly prominent among the
354 enriched genes. GPI anchored proteins are preferentially targeted to the apical
355 membrane of polarized cells [49], the membrane used by the parasite to invade. GPI
356 anchored proteins are thus exposed to the parasite. Among them are glypicans which
357 serve as the platform of apically displayed membrane associated glycosaminoglycans in
358 the intestine [50]. The screen also identified the tetraspanin CD151. Interestingly, in
359 infected cells this host protein is recruited to the host-parasite interface (S4 Fig). CD151
360 is critical to the uptake and intracellular trafficking of human cytomegalovirus and
361 papillomavirus [51], and the related protein CD81 is required for the invasion of
362 hepatocytes by *Plasmodium* sporozoites [52]. Tetraspanins act as scaffolds forming
363 membrane microdomains that mediate adhesion, signaling, fusion and fission, and
364 CD151 is well known for its role in integrin signaling [53]. Candidate FNDC3B contains a
365 fibronectin type III domain involved in interactions with integrins and knockdown of this
366 genes in HCT8 leads to a decrease in phosphorylation of PI3K [54]. Polymerization of

367 host actin is a prominent feature of *Cryptosporidium* invasion and host modification and
368 there is evidence for parasite engagement of host integrins and PI3K signaling [55, 56] in
369 this context.

370

371 **Cellular signaling and membrane trafficking**

372 The screen also identified the kinase CSK, a negative regulator of Src family
373 kinases. c-Src kinase was shown to play an important role in host actin polymerization
374 during *Cryptosporidium* infection [23]. Traditionally, this has been viewed as aiding
375 parasite infection; however, recent studies may suggest a more complex picture in which
376 the cortical cytoskeleton might also act in host defense [24, 57]. Src family kinases are
377 also critical to pattern recognition receptor mediated detection of pathogens leading to
378 the production of interferons and CSK is critical to tune this response [58, 59].

379 Multiple hits may impact membranes and their trafficking including NBEAL2, TMEM30A,
380 and RALGAPB. RALGAPB is an inhibitor of the small GTPases RalA and RalB, which in
381 turn activates the exocyst complex. In epithelial cells the exocyst is critical to exocytosis
382 as well as the dynamic remodeling of the actin cytoskeleton [60]. RalA activity is required
383 for membrane recruitment to the *Salmonella typhimurium* infection site [61]. TMEM30A is
384 an essential binding partner of P4 type ATPase flippases and directs the trafficking of the
385 catalytic subunits from the trans-Golgi to the plasma membrane [62]. Deletion of
386 TMEM30A leads to defects in both endocytosis and exocytosis due to a loss of the
387 asymmetric distribution of phospholipids across the plasma membrane [63]. NBEAL2 is
388 required for the formation of secretory granules in a variety of cells [64]. At least two hits
389 act on nucleotide signaling, PDCL or phosphatidylinositol-like G-protein, is a chaperone of G-

390 protein beta gamma dimers [65] and erythrocyte G-proteins have been shown to play a
391 role in *Plasmodium falciparum* invasion [66]. NPR3 is a G-protein coupled receptor which
392 binds polypeptide hormones termed natriuretic peptides. Binding to this receptor inhibits
393 adenylate cyclase and decreases cAMP [67].

394

395 **Innate immunity**

396 By far the most prominent pathway to emerge from our screen was interferon
397 signaling. As we demonstrate in this study, *Cryptosporidium* infection leads to the
398 production and rapid accumulation of high levels of IFN λ in HCT-8 cultures. The screen
399 selected for host cell growth and survival, and interferons are potent inducers of cell death
400 programs [68, 69]. Interferons can arrest the cell cycle of target cells and induce
401 apoptosis, necroptosis, and autophagy [70-73].

402 The role of IFN γ in cryptosporidiosis is well established, but there have also been
403 reports of interferons directly produced by the enterocyte during *Cryptosporidium*
404 infection. Barakat et al., described the production of type I interferons in response to *C.*
405 *parvum* infection in a mouse cell line [13]. Transcriptional profiling of infected organoids
406 from the lung and small intestine revealed a signature that was similarly interpreted as
407 response to type I IFN signaling [15, 16]. In contrast, Ferguson et al. recently reported
408 type III interferon production in response to *C. parvum* infection in neonatal piglets and
409 neonatal mice [12]. Their studies further suggest that IFN λ may block parasite invasion
410 and promote barrier integrity during *C. parvum* infection.

411 In this study we show pronounced production of type III, but not type I interferons
412 in human cells (Fig 6). We found the response to be initiated intrinsically in the infected

413 cell (Fig 3H) and amplified by an autocrine loop. Experiments *in vivo* demonstrated a
414 protective role for IFN λ that did not require IFN γ or adaptive immunity but relied
415 exclusively on signaling in enterocytes (Fig 4G, Fig 5H). Further, we discovered the
416 pattern recognition receptor, TLR3, to be required for type III interferon production. TLR3
417 recognizes dsRNA, and we used the synthetic analog Poly (I:C) to induce IFN λ
418 production. Previous studies have shown that injection of Poly (I:C) reduced *C. parvum*
419 infection [74].

420 TLR3 is known to recognize other protozoan parasites including Neospora [75]
421 and *Leishmania* [76] where it induces type I IFN production. For *Leishmania*, TLR3
422 recognition was dependent on the presence of a dsRNA virus found in certain parasite
423 isolates. Interestingly, *Cryptosporidium* is also host to a dsRNA viral symbiont which could
424 be a possible source of dsRNA recognized by TLR3 [77, 78]. In contrast to leishmaniasis
425 where type I IFN production exacerbates disease [79], the type III interferon response to
426 *C. parvum* is host protective. There have also been reports of *Cryptosporidium* derived
427 RNAs to be trafficked into the host cell nucleus during infection providing an additional
428 potential trigger for TLR3 [80]. While our screen did not identify TLR3 as a top candidate,
429 UNC93B1, a protein critical to proper trafficking of endosomal TLRs [81], was a highly
430 enriched gene. Cryptosporidiosis is most dangerous in children below the age of two
431 years and it is thus noteworthy that TL3 is poorly expressed in neonates, and that low
432 levels of TLR3 in the intestinal epithelium have been linked to the heightened
433 susceptibility of neonatal mice to rotavirus [82].

434 The increase in parasite burden in mice lacking TLR3 exceeded that of mice
435 lacking IFNLR1 (8-fold compared to 2.5-fold), potentially pointing towards TLR3 functions

436 beyond induction of type III interferon in epithelial cells. TLR3 is known to promote cross-
437 priming of CD8+ T cells via DC phagocytosis of virus infected cells [83]. The absence of
438 TLR3 signaling can impair the production of IL-12 by DCs [84] which is important for the
439 control of *Cryptosporidium* infection [85].

440 We recently reported that an enterocyte intrinsic NLRP6 inflammasome is
441 activated by *Cryptosporidium* infection leading to release of IL-18 [14]. This IL-18 in
442 conjunction with IL-12 then promotes downstream IFN γ production by ILCs [9]. Here we
443 describe a second parasite detection mechanism that depends on TLR3 and produces a
444 rapid IFN λ response that precedes the production of IFN γ by NK/ILCs and T cells. IFN λ
445 has been shown to augment the IFN γ response of NK cells via a mechanism involving IL-
446 12 [41]. Consistent with this idea treatment of Rag2^{-/-}Il2rg^{-/-} was repeatedly less effective
447 than that of Rag2^{-/-} alone (Fig 4G), suggesting a potential role for IFN λ in promoting IFN γ
448 production in NK/ILCs. However, loss of STAT1 exclusively in the enterocyte lineage led
449 to almost a complete abrogation of the effects of IFN λ treatment (Fig 4H), arguing that
450 the primary role of IFN λ is to act on the enterocyte. The protective effects of IFN λ
451 treatment even in immunocompromised mice were striking. Treatment resulted in control
452 in mice that are extremely susceptible to cryptosporidiosis [7, 9] and lack central elements
453 of innate and adaptive immunity. Treatment of immunocompromised individuals suffering
454 from cryptosporidiosis remains very challenging [86, 87]. Clinical trials for use of
455 pegylated IFN λ have shown promise for the treatment of viral infections [88, 89] and its
456 efficacy against *Cryptosporidium* warrants further study.

457

458 **Materials and Methods**

459 **Ethics Statement**

460 All *in vivo* experiments were performed in accordance with protocols for animal care
461 approved by the Institutional Animal Care and Use Committee at the University of
462 Pennsylvania (#806292).

463

464 **Mice**

465 C57/BL6J (stock no: 000664), B6129 (stock no: 101045), *Ifng*^{-/-} (stock no: 002287),
466 *Ifnar*^{-/-} (stock no: 028288), *Vil1 Cre* (stock no: 021504), *Mavs*^{-/-} (stock no: 008634), *Tlr3*^{-/-}
467 (stock no: 005217), *Tlr7*^{-/-} (stock no: 008380) mice were purchased from Jackson
468 Laboratories. C57/BL6 (Model no: B6NTac), *Rag2*^{-/-} (Model no: RAGN12), *Rag2*^{-/-} *Il2rg*^{-/-}
469 (Model no: 4111) mice were purchased from Taconic Biosciences. *Vil1-Cre* (stock
470 no:021504) were purchased and *STAT1* flox mice were generated as previously described
471 [90] and maintained in house. *Il28ra*^{-/-} mice (Bristol Meyers Squibb) were maintained in
472 house. Mice used in this study were males or females between 6-10 weeks of age. All
473 mice were sex and age matched for each experiment. No differences in infection were
474 observed between male and female mice.

475

476 **Cells, Parasites, and Infections**

477 HCT-8 cells (ATCC) were maintained in RPMI supplemented with 10% FBS at 37°C and
478 5% CO₂. Wild-type *Cryptosporidium parvum* oocysts used in this study were purchased
479 from Bunchgrass Farms (Dreary, ID). Parasites expressing Tandem mNeon were
480 generated in a previous study [25]. For *in vitro* infections oocysts were incubated in a (1:3)

481 bleach: water solution for 10 minutes at 4°C, centrifuged then resuspended in a 0.08%
482 solution of sodium deoxytaurocholate and incubated at 16°C for 10 minutes. Oocysts
483 were then washed in PBS and finally resuspended in infection media (complete RPMI
484 with 1% FBS) and added directly to host cells.

485 *C. parvum* oocysts used for all *in vivo* experiments are mouse adapted mCherry and
486 Nanoluciferase expressing [14]. Mice were infected with 50,000 *C. parvum* oocysts by
487 oral gavage unless otherwise noted.

488

489 **Killing Assay**

490 6 well cultures grown to 60% confluency were infected with 5×10^5 , 1.25×10^6 , 2.5×10^6 ,
491 3.75×10^6 , or 5×10^6 oocysts per well in biological duplicate. Following a 72-hour infection,
492 cells were trypsinized and incubated in a 1:4 solution of Trypan Blue. Cells were counted
493 and Trypan Blue exclusion used to determine viability.

494

495 **CRISPR Screen**

496 The Brunello CRISPR sgRNA library [31] was optimized for on-target activity and to
497 minimize off-target effects. Brunello contains four sgRNAs per protein coding gene in the
498 human genome. HCT-8 in media containing 1µg/mL polybrene were spininfected (2 hours,
499 30°C at 1,000xg) with lentivirus to produce a constitutively expressing Cas9 cell line
500 (lentiCas9-Blast, plasmid#52962, addgene). Following a 7-day selection with blasticidin
501 (1.5µg/mL), cells were diluted to generate clonal Cas9 expressing cell lines. To measure
502 Cas9 activity these cells were subjected to an EGFP reporter assay for Cas9 activity.
503 Cas9 expressing cells were spininfected with lentiXPR_011, encoding an EGFP and a

504 sgRNA targeting EGFP. 24 hours post spinfection cells were flow sorted to assess green
505 fluorescence. Cells lacking or stably expressing EGFP expressing were used as controls
506 for flow cytometry.

507 We sought to achieve 1000-fold coverage across multiple biological replicates of the
508 screen. Each replicate achieved 500-fold coverage. Per mL of the Brunello library there
509 were 4.2×10^7 lentiviruses/guides. We infected at an MOI of 0.4 therefore 1.02×10^8 cells
510 were transduced with the library. These cells were trypsinized and spinfected as before
511 into a total of six 6-well plates. Plates were incubated at 37°C, 5% CO₂ for 24 hours then
512 media was changed to include 1ug/mL puromycin for selection of transduced cells. Seven
513 days later, cells were trypsinized and expanded into 12 T-175 flasks. After expansion,
514 genomic DNA was isolated from four flasks as the input population and at least 4×10^7
515 cells (500-fold coverage) were passaged into 4 T-175. These 4 T-175 were then infected
516 with a 90% kill dose of *C. parvum* oocysts. After 72 hours media was replaced with fresh
517 media and cells were allowed to recover. Once confluent, cells were trypsinized and
518 seeded into new flasks to be re-infected while at least 4×10^7 cells were taken for genomic
519 DNA extraction. In total, the population was subjected to three rounds of successive *C.*
520 *parvum* infection.

521 Genomic DNA was extracted using the QIAamp DNA Blood Maxi kit (Qiagen). sgRNAs
522 were amplified by PCR as described [31]. Read counts were normalized to reads per
523 million in each condition.

524

525

526

527 **MAGeCK Analysis of CRISPR Screen**

528 Data from our CRISPR screen was analyzed using MAGeCKFlute in R [91]. MAGeCK
529 uses a negative binomial to test for differences in sgRNA abundance between conditions
530 [32]. The input population for Clone K was compared to the output of each round of
531 infection. For Clones C and I input was compared only to the final population. Results
532 shown are for the combined data of screens with Clone I and K. Clone C was excluded
533 due to poor sequencing depth. Genes with three or four sgRNAs positively ranked by the
534 robust ranking aggregation (RRA) algorithm and an FDR of less than 0.05 were
535 considered significantly enriched. Pathways identified by GSEA that included multiple
536 genes of the top candidates were displayed in Fig 1.

537

538 **RNAi Screen**

539 siRNAs targeting top screening candidates were purchased from Ambion (ThermoFisher
540 Scientific, Waltham, MA). Both scrambled non-targeting siRNAs and a positive
541 transfection control RNA targeting GAPDH were included. siRNAs were delivered to 96
542 wells at 50% confluency using Lipofectamine RNAiMax (ThermoFisher Scientific,
543 Waltham, MA) to a final concentration of 100nM per well. 24 hours later, wells were
544 infected with 25,000 *C. parvum* oocysts. At 48 hours post infection, cells were lysed and
545 RNA extracted using the Rneasy Mini Kit (Qiagen). Knockdown of target genes was
546 assessed by qPCR. Host cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-
547 yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, media was removed from all wells
548 and replaced with 100 μ L of fresh RPMI. 10 μ L of 12mM MTT solution was added to each
549 well. Plates were incubated at 37°C for 4 hours. Then all media was removed and

550 replaced with 50µL of DMSO (Sigma, St. Louis, MO) and mixed thoroughly by pipetting
551 up and down. Following a 10 minute incubation at 37°C, plates were read for absorbance
552 at 540nm.

553

554 **Immunofluorescence assay**

555 Infected HCT-8 coverslip cultures were fixed in 4% paraformaldehyde and permeabilized
556 with 0.1% Triton X-100 for 10 minutes each at room temperature. Samples were blocked
557 in 3% Bovine Serum Albumin (BSA) for 1 hour and primary antibodies were diluted in 3%
558 BSA. Anti-CD151 (ab33315, Abcam) was diluted 1:100 and anti-Tryptophan synthase
559 beta [40] was diluted 1:1000. Secondary antibodies (ThermoFisher) were diluted 1:1000
560 in 3% BSA. FITC conjugated phalloidin (F432, ThermoFisher) was included in the
561 secondary antibody incubation. Cell nuclei were labeled with Hoechst 1:10,000 for 5
562 minutes and coverslips were mounted using Vectashield (Vector Laboratories). Slides
563 were imaged using a Leica DM6000 Widefield microscope.

564

565 **RNA sequencing**

566 Total RNA was extracted using the RNeasy Mini (48-hour) RNeasy Micro (10-hour) kit
567 (Qiagen). cDNA was synthesized using the SMART-Seq v4 Ultra Low Input RNA Kit
568 (Takara Bio USA), and barcoded libraries were prepared using the Nextera XT DNA
569 Library Preparation Kit (Illumina). Total RNA and libraries were quality checked and
570 quantified on an Agilent TapeStation 4200 (Agilent Technologies). Samples were pooled,
571 and single-end reads were run on a NextSeq 500 (Illumina).

572 Reads were pseudo-aligned to the Ensembl *Homo sapiens* reference transcriptome v86
573 using kallisto v0.44.0 [92]. In R, transcripts were collapsed to genes using Bioconductor
574 tximport [93] and differentially expressed genes were identified using Limma-Voom [94,
575 95]. Gene set enrichment analysis (GSEA) was performed using the GSEA software and
576 the annotated gene sets of the Molecular Signatures Database (MSigDB) [96].

577

578 **qPCR**

579 RNA concentrations were measured by NanoDrop (ND-1000; Thermo Fisher Scientific,
580 Waltham, MA) for each sample and an equal amount of cDNA was prepared using
581 SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA).
582 Following reverse transcription, a 20 μ L reaction was loaded into a ViiA 7 Real Time PCR
583 system (Thermo Fisher Scientific, Waltham, MA). The following conditions were used:
584 Initial incubation 3 min at 95°C, 40 cycles of 95°C for 15 sec and 60°C for 30 sec. A single
585 melt curve and $\Delta\Delta$ Ct method was used to determine relative expression with GAPDH
586 used as the housekeeping gene. See Table 1 for list of primers.

587

588 **Western Blot**

589 24 well HCT-8 cultures grown to 60% confluency were infected with 2x10⁵ *C. parvum*
590 oocysts in RPMI containing 1% serum for the indicated time. Media was removed and
591 cells were lysed in PierceTM IP Lysis Buffer (ThermoFisher Scientific, Waltham, MA),
592 supplemented 1:100 with protease inhibitor cocktail (Sigma St. Louis, MO). Lysates were
593 incubated on ice 10 minutes, then spun at 20,000 g for 10 min at 4°C. The cleared lysate
594 was removed and flash frozen. Cleared lysates were thawed on ice and protein

595 concentration was assessed by BCA (23225, ThermoFisher Scientific, Waltham, MA).
596 18µg of sample was loaded per well diluted 1:1 with freshly prepared 2X Laemmli Sample
597 buffer (BioRad Hercules, CA) + β-Mercaptoethanol (1:20) (Sigma St. Louis, MO) and
598 boiled for 10 minutes at 95°C. 20 µL sample was loaded per each lane of an any KD Mini-
599 PROTEAN® TGX™ Precast Protein Gel (BioRad Hercules, CA) and run at 150 V for 1
600 hour. Wet transfer to a 0.45 µm pore size pre-cut Nitrocellulose membrane (ThermoFisher
601 Scientific Waltham, MA) was conducted at 20V for 2.5 hours at room temperature. The
602 Nitrocellulose membrane was blocked for 1 hour at room temperature using
603 Intercept®(TBS) Protein-Free Blocking Buffer (LI-COR Lincoln, NE). Primary antibody
604 was incubated at room temperature for 2 hours in Intercept®(TBS) Protein-Free Blocking
605 Buffer with 0.01% Tween®20 (Sigma, St. Louis, MO) using STAT1 1:1000 (#14994, Cell
606 Signaling Technology), phosphor STAT1 Y701 1:1000 (ab29045, Abcam) and alpha-
607 tubulin 1:5000 (ab7291, Abcam). The membrane was washed 3 times with PBS with
608 0.01% Tween®20 (Sigma, St. Louis, MO). Secondary antibody was incubated at room
609 temperature protected from light for 1 hour in Intercept®(TBS) Protein-Free Blocking
610 Buffer with 0.01% Tween®20 (Sigma, St. Louis, MO) using IRDye® 800CW Goat anti-
611 Mouse IgG secondary antibody at 1:10,000 (LI-COR Lincoln, NE) and IRDye® 680RD
612 Goat anti-Rabbit IgG secondary antibody at 1:10,000 (LI-COR, Lincoln, NE). After 3 PBS
613 + 0.01% Tween®20 (Sigma, St. Louis, MO) washes, the membrane was imaged on the
614 Odyssey Infrared Imaging System v3.0 (LICOR, Lincoln, NE).

615

616

617

618 **ELISA**

619 96 well HCT-8 cultures grown to 60% confluency were infected with 25,000 *C. parvum*
620 oocysts. At the indicated timepoint post infection, supernatants were removed and spun
621 at 1,000xg for 10 minutes to pellet debris. Supernatants were frozen at -80°C. IFN β and
622 IFN λ protein levels from HCT-8 cultures were measured by Human IFN-beta DuoSet
623 ELISA (DY814, R&D Systems) and Human IL29/IL28B (IFN-lambda 1/3) DuoSet ELISA
624 (DY1598B, R&D Systems). Protein levels of IFN λ from intestinal biopsies were measured
625 by Mouse IL28B/ IFN-lambda 3 DuoSet ELISA (DY1789B, R&D Systems). Protein levels
626 of IL-18 from intestinal biopsies were measured by ELISA (BMS618-3, ThermoFisher,
627 Waltham, MA). Assays were performed according to the manufacturer's instructions.

628

629 **Flow sorting of infected cells**

630 HCT-8 6 well cultures were infected with 1×10^6 *C. parvum* Neon oocysts. 10 hours later,
631 cells were trypsinized in TrypLE (ThermoFisher), washed with PBS, and passed through
632 a 40 μ m filter. Cells were sorted using a BD FACSJazz Sorter (BD Biosciences).
633 Uninfected HCT-8 were used to gate on singlets. 10,000 positive cells and 10,000
634 negative cells were sorted from three independent biological replicates directly into RLT
635 Lysis buffer (Qiagen).

636

637 **Ileal biopsies**

638 Three 5mm punch biopsies were taken from the distal small intestine of each mouse.
639 Punches were incubated in complete RPMI for 18 hours. Supernatants were then used
640 for ELISA.

641 For qPCR, punches were placed in RNAlater (Sigma) at 4°C until RNA was extracted
642 using the RNeasy Mini Kit (Qiagen)

643

644 **Nanoluciferase Assay**

645 To monitor infection *in vivo*, 20mg of fecal material was resuspended in 1mL of lysis
646 buffer. Samples were shaken with glass beads for 5min at 2,000 rpm. Samples were
647 briefly centrifuged to pellet any floating material and the cleared lysate was mixed 1:1 with
648 prepared Nanoluciferase solution (substrate: lysis buffer 1:50). Luminescence was
649 measured using a Promega GloMax plate reader.

650

651 **Cytokine neutralization and administration**

652 To neutralize IFN λ , 20 μ g of Anti IL28A/B (Clone 244716, MAB17892, R&D Systems,
653 Minneapolis, MN) was infected intraperitoneally one day prior and each day following
654 infection for the duration.

655 For administration of Ifn λ 2 (250-33, Peprotech, Cranbury, NJ), 1 μ g, unless otherwise
656 noted, was injected intraperitoneally daily beginning at 6-8 hours prior to infection and
657 then each day of the infection.

658

659 **Histology**

660 Tissue from the lower third of the small intestine was flushed with 10% neutral buffered
661 formalin (Sigma, St Louis, MO, USA), then 'swiss-rolled' and fixed overnight. Fixed
662 samples were paraffin-embedded, sectioned, and stained with hematoxylin and eosin for
663 detailed histologic evaluation. Slides were evaluated by a board-certified veterinary

664 pathologist in a blinded fashion for quantitative measurements of number of parasites,
665 villus/crypt architectural features, and semi-quantitative scores for villus epithelium
666 lesions as previously described [97].

667

668 **PRR agonist screen**

669 Agonists of pattern recognition receptors were purchased from Invivogen. Cells were
670 seeded into 96 well plates and at 60% confluency, cells were either infected with *C.*
671 *parvum* (25,000 oocysts per well) or treated with an agonist. 10µg/mL LMW Poly (I:C)
672 was either lipofected or added to the medium. The following agonists were delivered with
673 Lipofectamine: 5'ppp RNA (10µg/mL), mTriDAP (10µg/mL), HSV60 (5µg/mL), ssPolyU
674 RNA (10µg/mL), and CpG ODN (5µM). After 24 hours the media was removed for ELISA
675 and the cells were lysed and RNA extracted (RNeasy Mini Kit, Qiagen).

676

677 **Statistical Methods**

678 Mean +/- SD are reported. When measuring the difference between two populations, a
679 standard *t*-test was used. For datasets with 3 or more experimental groups, a one-way
680 ANOVA with multiple comparison's test was used. For datasets with 2 or more
681 experimental groups and an additional factor of time, a two-way ANOVA with multiple
682 comparison's test was used and simple linear regression was used to determine the
683 goodness of fit curve for host cell killing by *C. parvum*. *P* values of less than 0.05 were
684 considered significant. These tests were performed in GraphPad Prism or in R.

685

686

687 **Data Availability**

688 Data are within the manuscript and supporting information files and are accessible
689 through GEO accession number: GSE185247. All code used to process and analyze
690 the data is available through Code Ocean: <https://doi.org/10.24433/CO.1074647.v1>

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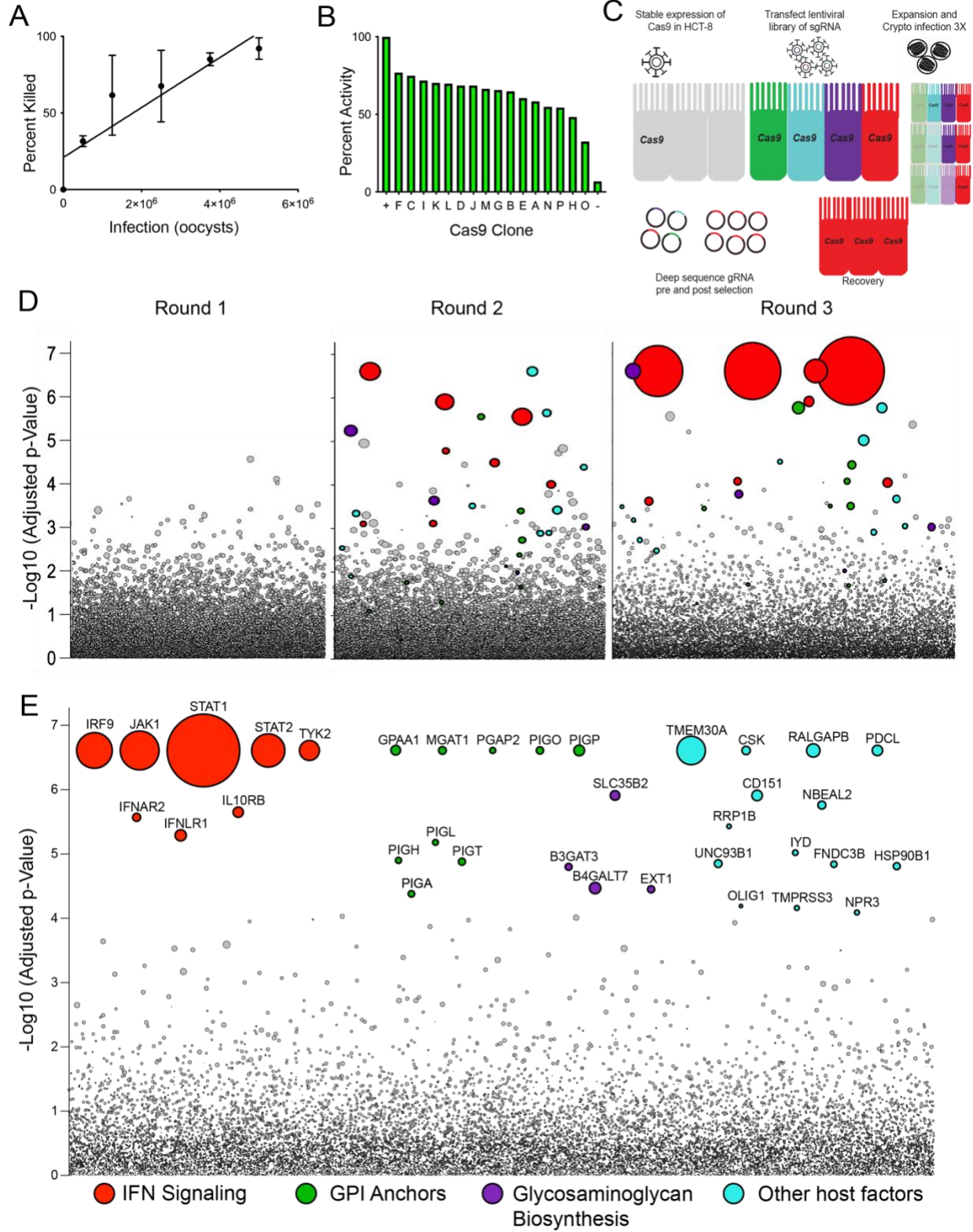
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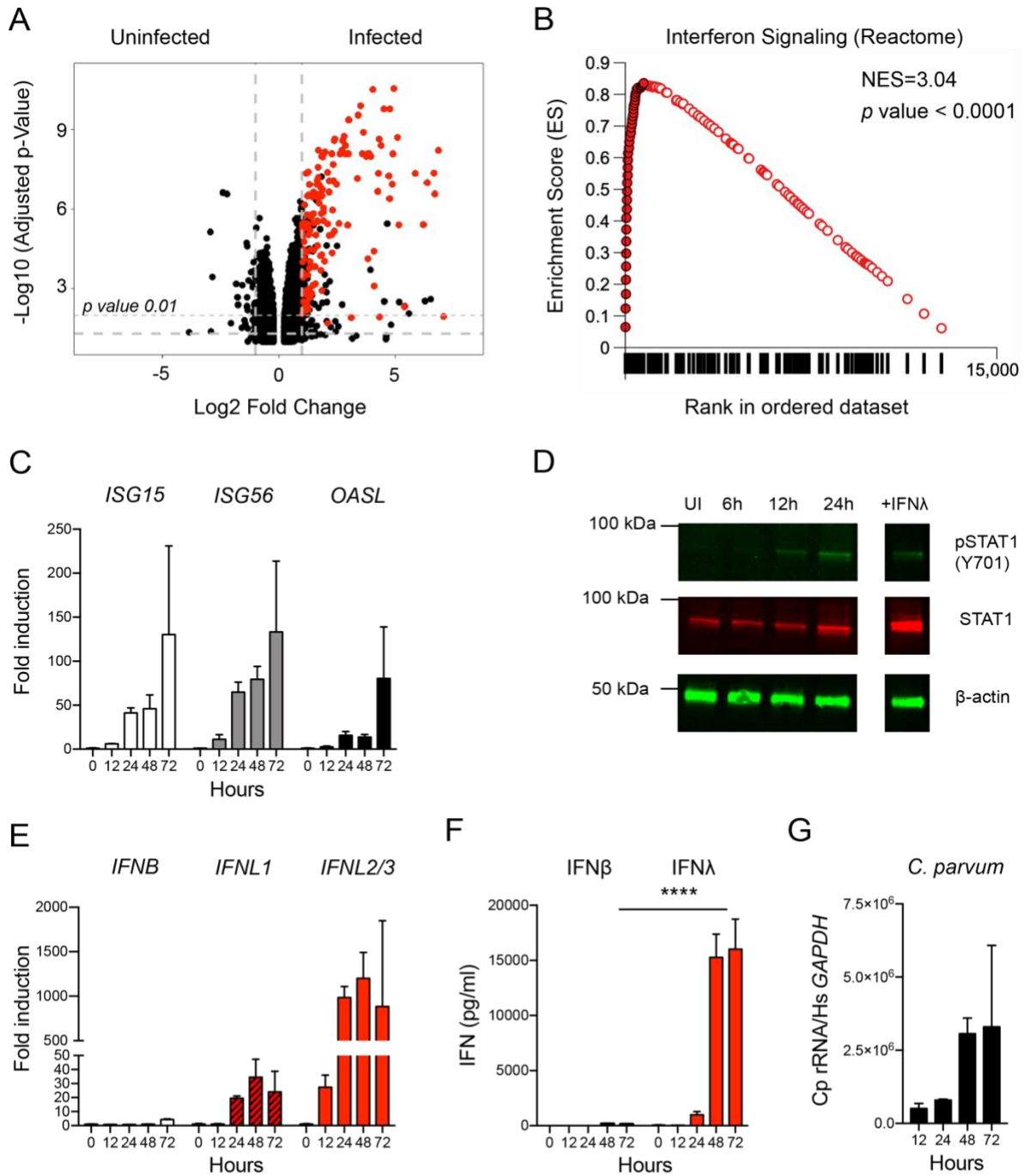
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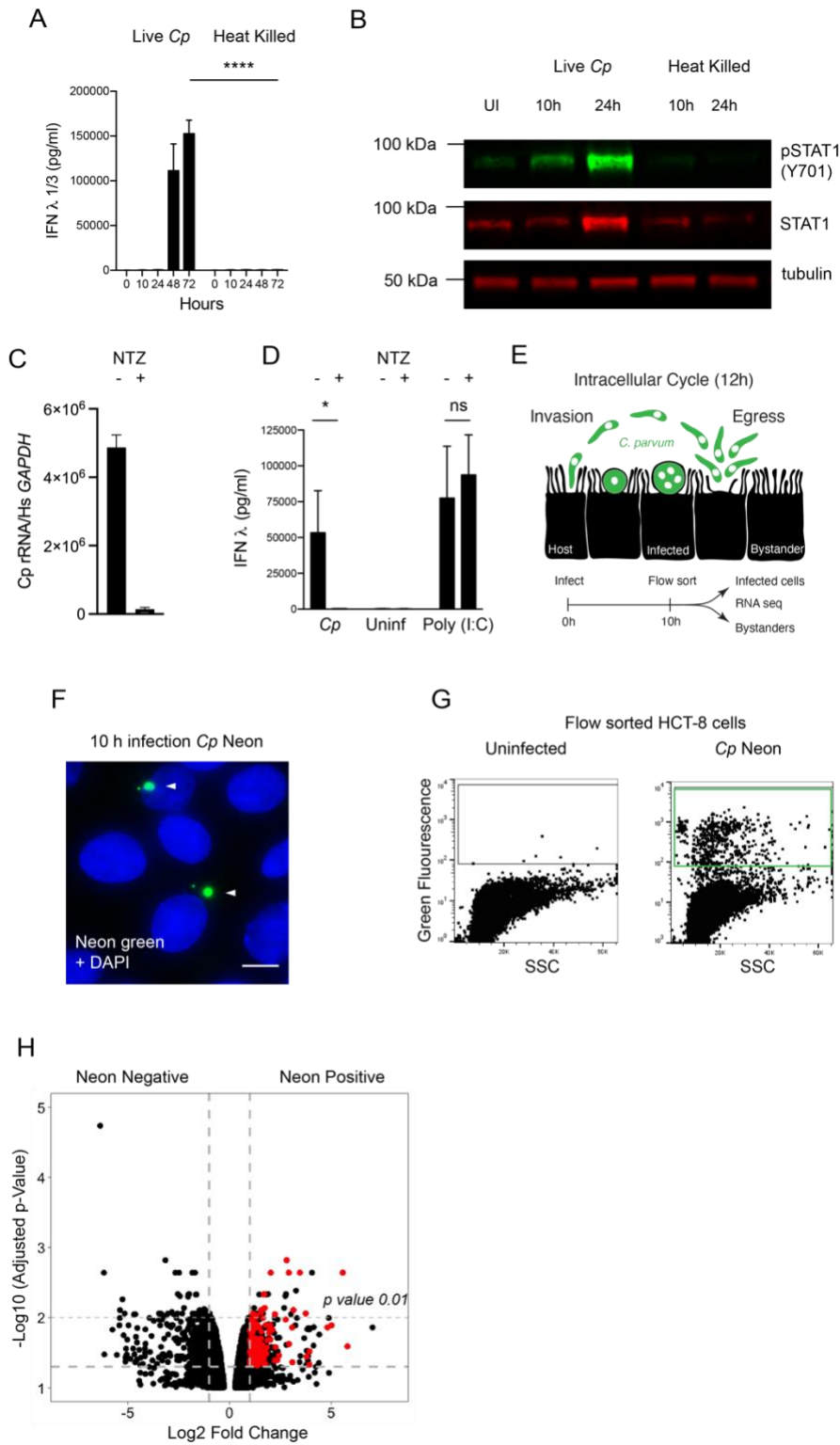
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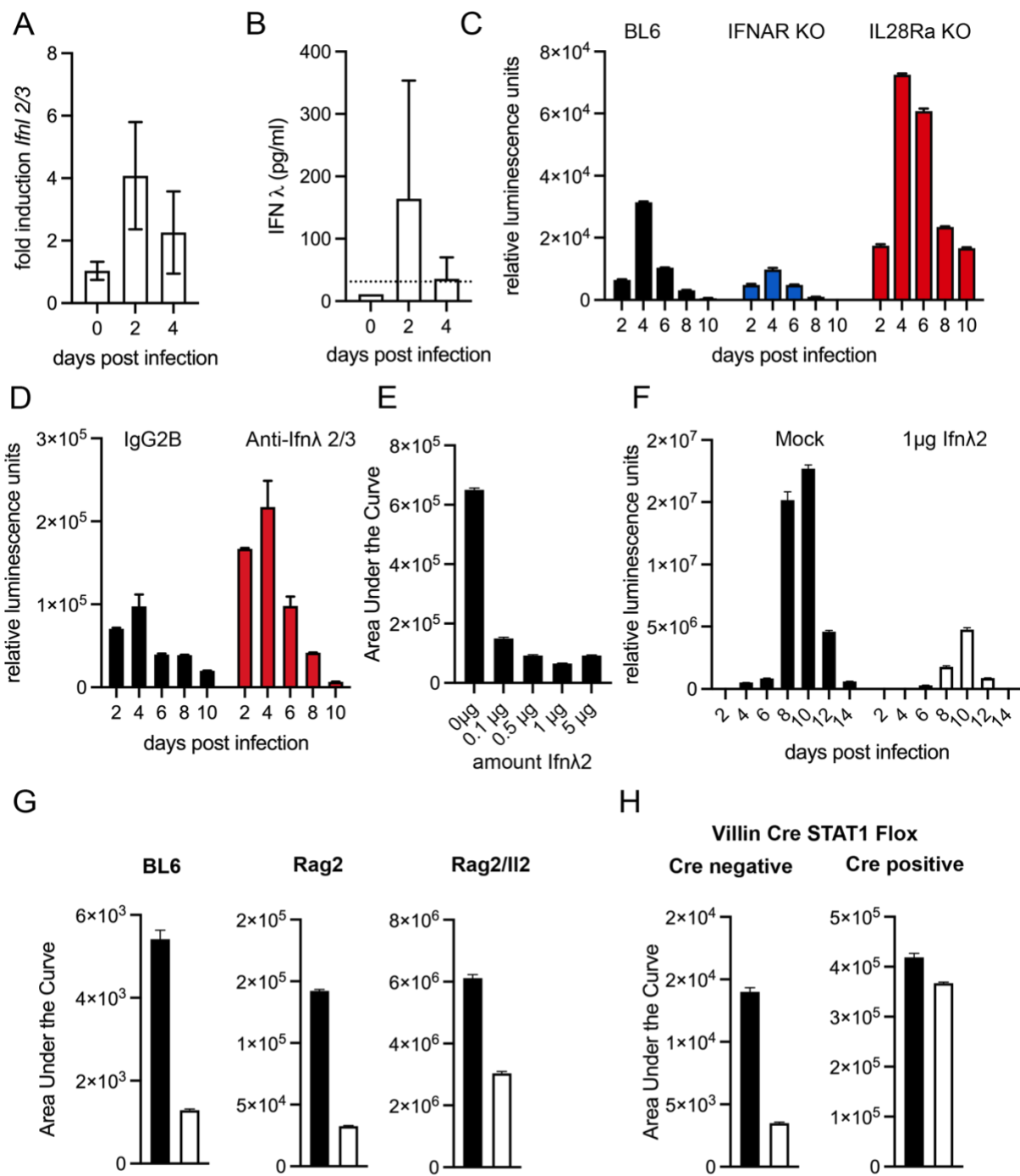
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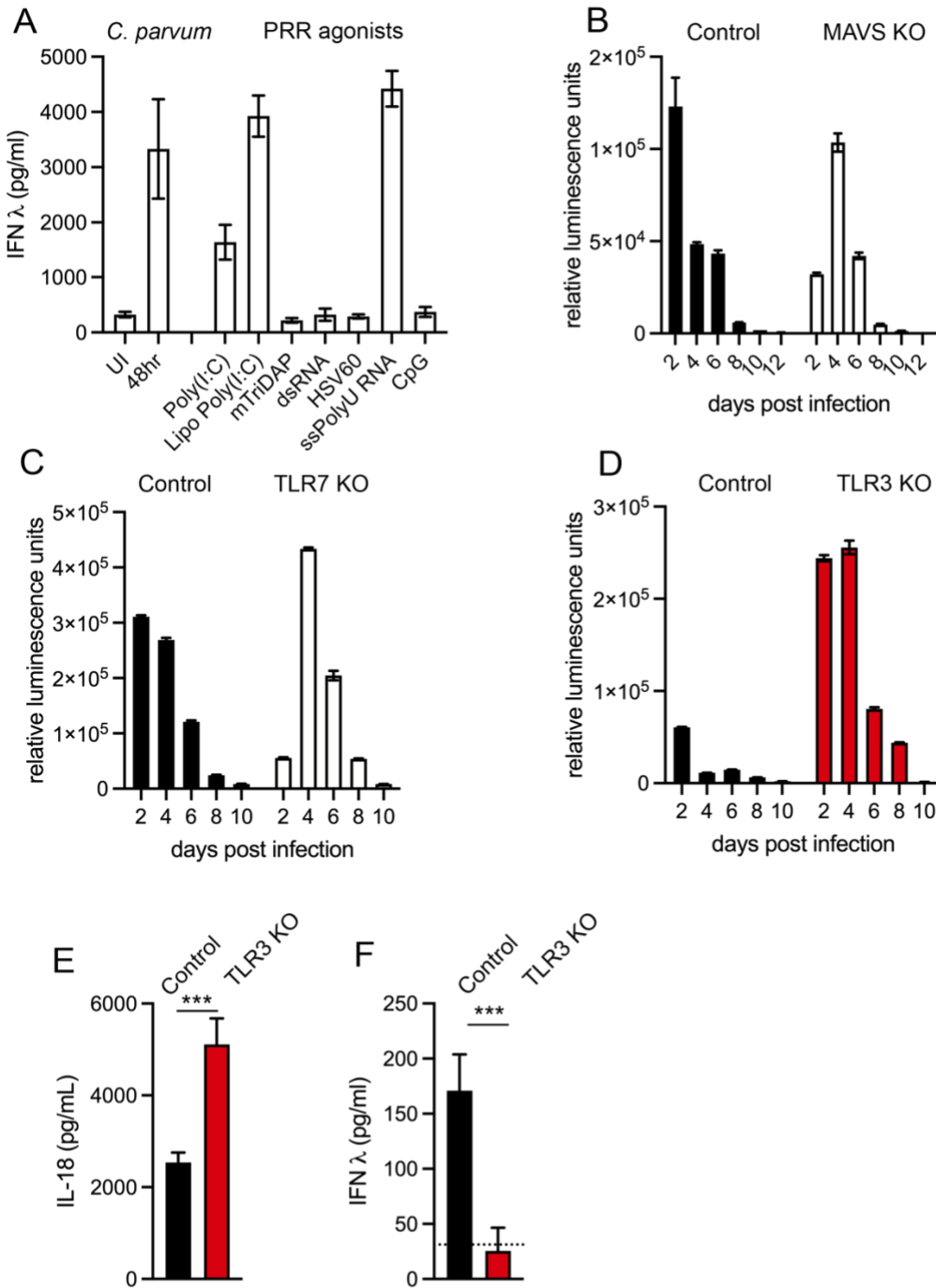




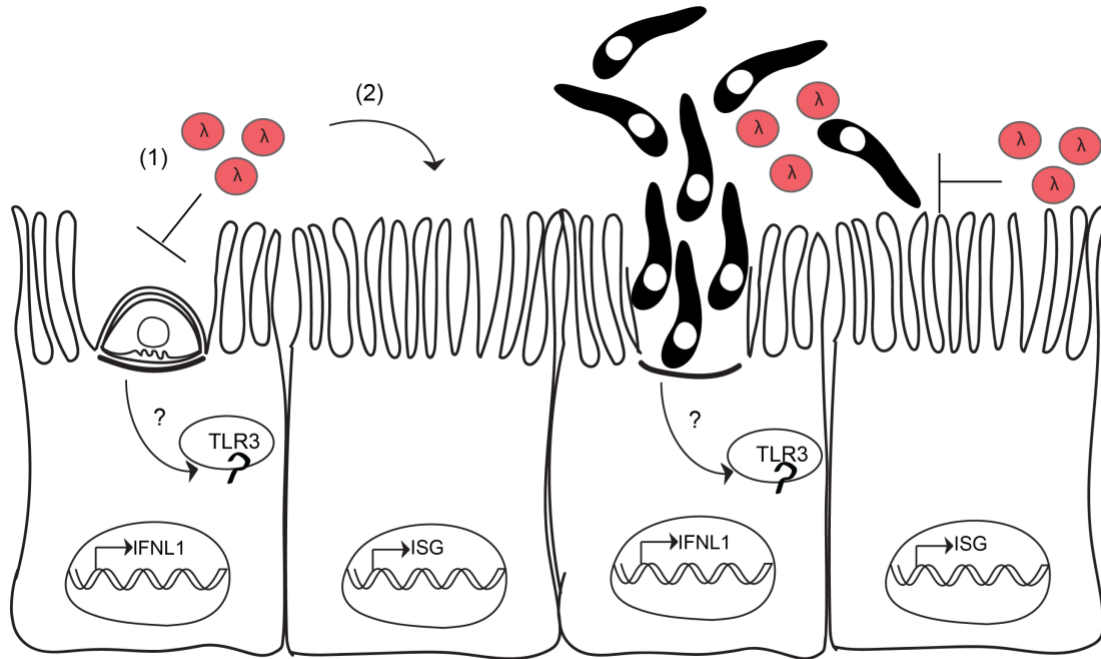
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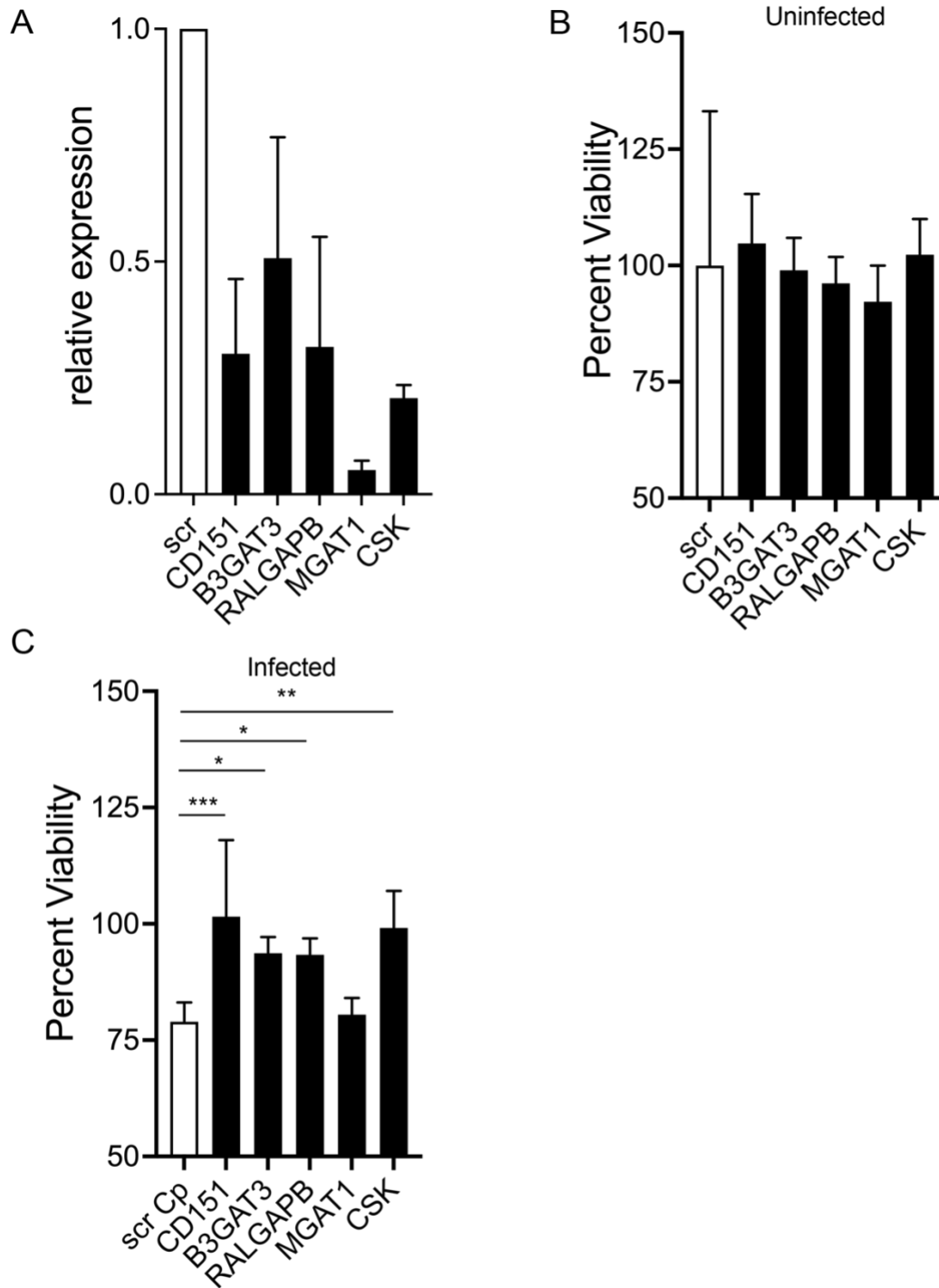
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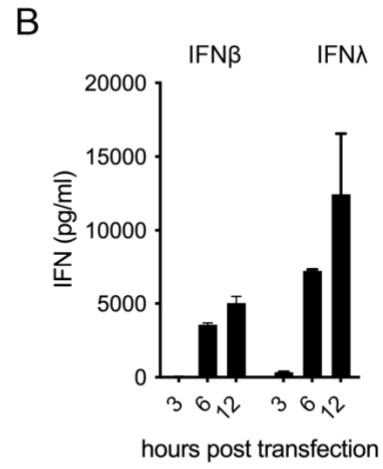
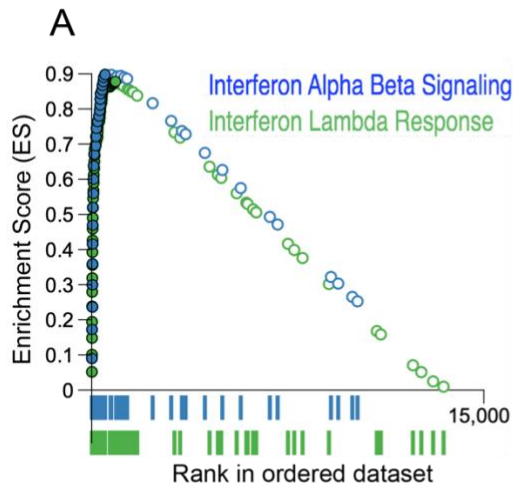


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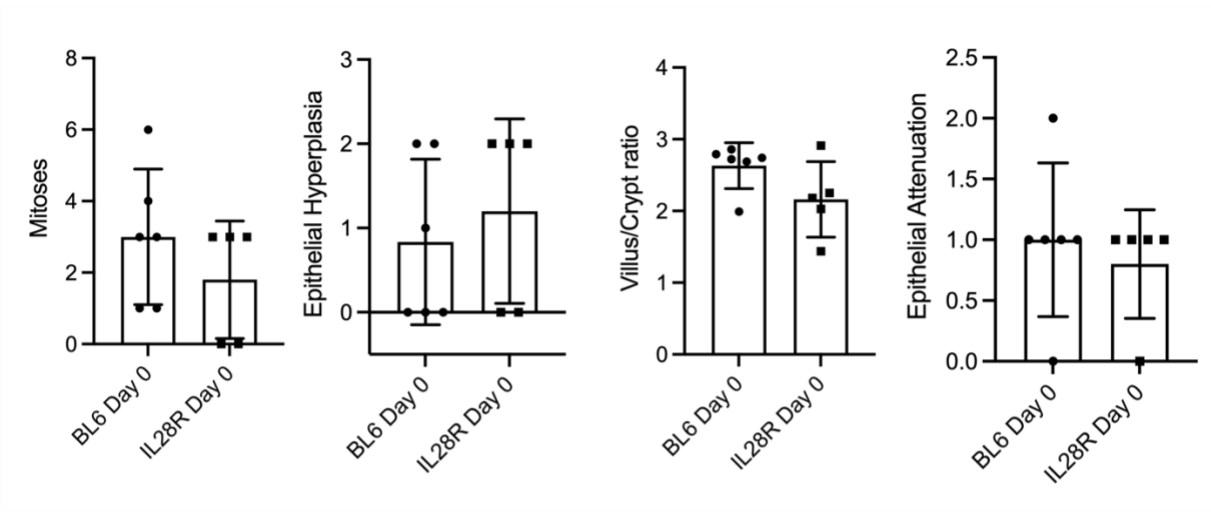
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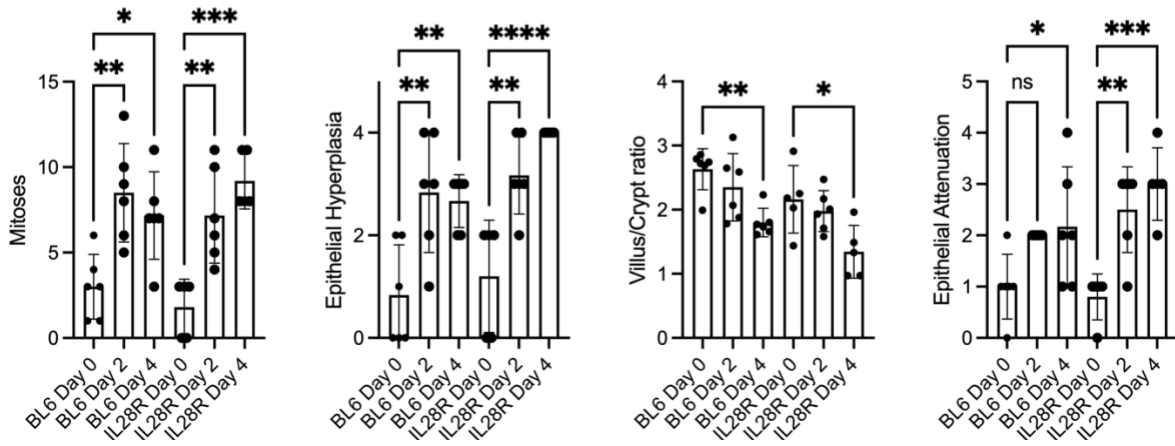


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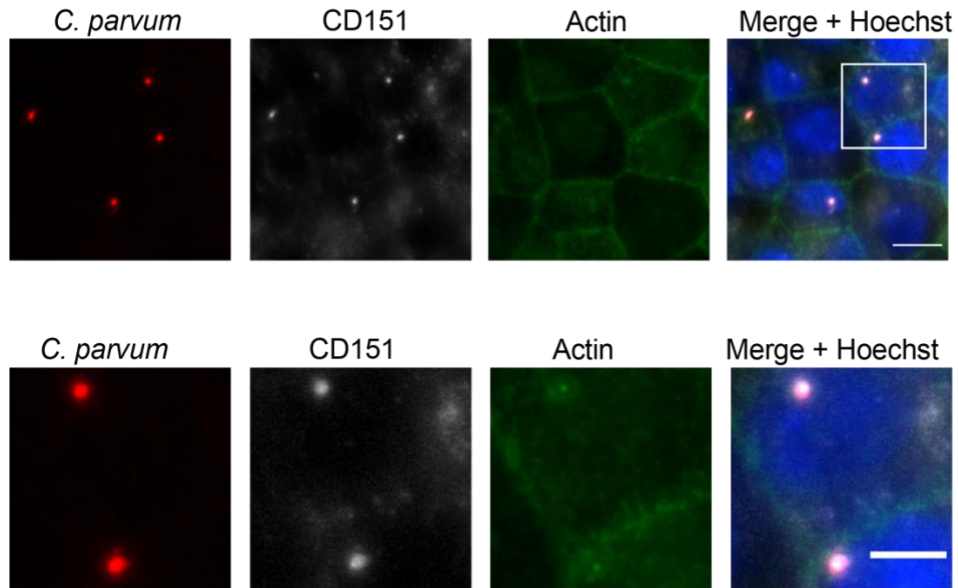


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1005 **Fig 1. A genome-wide screen reveals genes required for susceptibility to**
1006 ***Cryptosporidium* infection and host cell death**

1007 To identify host genes required for *Cryptosporidium* infection we performed a genome-
1008 wide knockout screen.

1009 (A) HCT-8 cells infected with increasing numbers of *C. parvum* oocysts are killed in a
1010 dose-dependent manner. Host cell viability was assessed by Trypan Blue exclusion.

1011 $R^2=0.7522$

1012 (B) Cas9 activity in different clones of Cas9 expressing HCT-8 cells assessed by flow
1013 cytometry normalized to a positive control set to 100 percent.

1014 (C) Schematic of CRISPR screen using *C. parvum* induced host cell death as selection.

1015 (D) Bubble plot of Cas9 expressing Clone K screen showing enrichment of specific
1016 genes with each round of selection by *C. parvum* infection. Each bubble represents a
1017 human gene and the size of bubbles corresponds to fold change. y-axis is the inverse of
1018 the adjusted *p* value.

1019 (E) Bubble plot of concatenation of Clones I and K comparing input to the final selection
1020 for 1000-fold coverage. The top 35 genes were colored and grouped based on function.
1021 Size of bubbles corresponds to fold change.

1022

1023 **Fig 2. *Cryptosporidium* infection induces a type III interferon response in human**
1024 **intestinal epithelial cells**

1025 We examined the response to *C. parvum* infection in HCT-8 to determine which specific
1026 interferons were induced.

1027 (A) HCT-8 cultures were infected with *C. parvum* oocysts, and RNA was isolated 48
1028 hours post infection from 3 biological replicates and matched uninfected controls.

1029 Volcano plot showing differentially expressed genes between uninfected and infected
1030 HCT-8 (n=3, biological replicates per group). Genes in red are part of the “REACTOME:
1031 Interferon Signaling” signature.

1032 (B) GSEA plot of “REACTOME: Interferon Signaling” signature identified at 48 hours
1033 post infection. Closed circles represent genes that make up the core enrichment of the
1034 signature. Net enrichment score=3.04, *p* value <0.0001

1035 (C) 96 wells HCT-8 cultures were infected with 25,000 *C. parvum* oocysts for 12-72
1036 hours. Transcript abundance of three representative interferons stimulated genes
1037 (ISGs) measured by qPCR is shown over a time course of *C. parvum* infection (n=3)

1038 (D) Immunoblot showing presence of phospho STAT1 and total STAT1 in uninfected
1039 cultures and following *C. parvum* infection. Treatment with IFN λ is used as a control for
1040 induction of phosphoSTAT1. One representative of 2 biological replicates is shown.

1041 (E) Samples as in (C). Induction of type I (IFN β) and type III interferon (IFN λ) transcripts
1042 as assessed by qPCR. Note peak of *IFNL1*: 35-fold, *IFNL2/3*:1200-fold at 48 hours
1043 while peak *IFNB*: 4-fold at 72 hours. *n*=3

1044 (F) Protein levels of type I and type III interferons as assessed by ELISA. Samples as in
1045 (C, E) At 48 and 72 hours post infection, the difference between IFN β and IFN λ was

1046 highly significant. Two-way ANOVA with Dunnett's multiple comparisons **** $p < 0.0001$.

1047 $n=3$

1048 (G) Relative abundance of *C. parvum* ribosomal RNA transcripts normalized to host

1049 *GAPDH*. $n=3$

1050

1051 **Fig 3. IFN-lambda production requires live infection and is initiated by infected**
1052 **cells**

1053 We examined the requirements and kinetics of initiation of the type III interferon
1054 response to *C. parvum*.

1055 (A) 24 well HCT-8 cultures were infected with 200,000 *C. parvum* live or heat killed
1056 oocysts and protein levels of IFN λ were assessed by ELISA. At 48 and 72 hours post
1057 infection the difference between live and heat killed was highly significant. Two-way
1058 ANOVA with Šídák's multiple comparisons test **** $p < 0.0001$. $n=3$

1059 (B) Immunoblot comparing induction of STAT1 phosphorylation by infection with live
1060 versus heat killed parasites. Phospho STAT1 is only detected in live infection. One
1061 representative example of two biological replicates is shown.

1062 (C) Following treatment with nitazoxanide (NTZ), infection is reduced 35-fold as
1063 assessed by relative abundance of *C. parvum* ribosomal RNA transcripts normalized to
1064 host *GAPDH*. $n=3$

1065 (D) Protein levels of IFN λ in HCT-8 infected with *C. parvum* compared to cultures
1066 stimulated with 10 μ g/mL lipofected Poly(I:C), both in the presence or absence of
1067 nitazoxanide. A decrease in IFN λ protein is observed only when NTZ is used in
1068 infection. One-way ANOVA with Šídák's multiple comparisons test * $p < 0.05$. $n=3$

1069 (E) Schematic of the 12-hour intracellular cycle of *C. parvum* and outline of a
1070 sequencing experiment to examine transcriptional differences between bystanders and
1071 infected cells from the same culture.

1072 (F) Immunofluorescence of HCT-8 infected with *Cp* Neon (green) at 10 hours post
1073 infection. Hoechst in blue. Scale bar 10 μ m

1074 (G) Flow cytometry dot plot of infected cells showing green fluorescence and side
1075 scatter. Three biological replicates were sorted for Neon positive to Neon negative
1076 comparison.

1077 (H) Volcano plot showing differentially expressed genes between Neon negative
1078 (bystander) and Neon positive (infected) HCT-8 at 10 hours post infection. Genes in red
1079 are ISGs as identified by Interferome DB.

1080

1081 **Fig 4. The type III Interferon response is host protective and epithelial cell**
1082 **intrinsic**

1083 We used a mouse model of infection to examine the role of type III interferon in
1084 *Cryptosporidium* infection *in vivo*. All mice were infected with mouse adapted *C.*
1085 *parvum*.

1086 (A) C57/BL6 (BL6) mice were infected with 50,000 *C. parvum* oocysts and relative
1087 abundance of IFN λ transcript is shown for ileal biopsies of infected mice, 4 mice per
1088 day, $n=2$

1089 (B) BL6 mice were infected with 50,000 *C. parvum* oocysts and secreted IFN λ protein
1090 from ileal biopsies was assessed by ELISA, 2 mice per day, $n=2$

1091 (C) Fecal luminescence measured every two days in BL6 wild type mice, mice lacking
1092 the type I IFN receptor *Ifnar*^{-/-}, and mice lacking the type III interferon receptor *Il28ra*^{-/-}
1093 following infection with 50,000 *C. parvum*. A reduction of 3-fold was observed in *Ifnar*
1094 and an increase of 2.7-fold was observed with *Il28ra*. 4 mice per group. Data shown is
1095 representative of 3 biological replicates. (*Ifnar*: -2.8, -2.9-fold, *Il28ra*: 2.3, 2.7-fold) $n=3$

1096 (D) C57/BL6 mice were treated with anti-*Ifn* λ 2/3 antibody or an isotype control daily via
1097 intraperitoneal (i.p.) injection and infected. Fecal luminescence was measured every
1098 two days. Representative of two biological replicates. An increase of 2-fold was
1099 observed in each replicate. $n=2$

1100 (E) *Ifng*^{-/-} mice were injected i.p. with indicated doses of *Ifn* λ 2 daily for days 0-3 of
1101 infection. Mice were infected with 20,000 *C. parvum* oocysts. The total area under the
1102 curve of fecal luminescence for the 3-day infection is shown. 2 mice per dose.

1103 Representative of 2 biological replicates (0.1µg: 4.3 and 5.9-fold; 0.5µg: 7 and 1.3-fold;
1104 1µg: 3.8 and 8.8-fold; 5µg: 7 and 18.6-fold). $n=2$

1105 (F) *Ifng*^{-/-} mice were injected i.p. with 1µg of *Ifn*λ2 beginning at day 0 and each day for
1106 the duration of the infection. Mice were infected with 20,000 *C. parvum* oocysts. Fecal
1107 luminescence measured every two days. A 7.7-fold decrease in shedding occurred
1108 upon treatment representative of 2 biological replicates (5-fold decrease) $n=2$

1109 (G) BL6 wild type mice, mice lacking T cells *Rag2*^{-/-}, and mice lacking NK cells, ILCs,
1110 and T cells *Rag2/Il2rg*^{-/-} were treated with 1µg of *Ifn*λ2 daily for the days 0-3 of infection.

1111 The total area under the curve of fecal luminescence for the 3-day infection is shown.

1112 (BL6: 2.4, 1.14, 1.22-fold; *Rag2*^{-/-} 4.4, 1.8, 16.3-fold; *Rag2/Il2rg*^{-/-} 2, 3.8, 6.5-fold)

1113 Representative of 3 biological replicates. $n=3$

1114 (H) Villin Cre STAT1 flox mice or littermate Cre negative controls were treated with 1µg
1115 of *Ifn*λ2 daily for the days 0-3 of infection. The total area under the curve of fecal

1116 luminescence for the 3-day infection is shown. Representative of 2 biological replicates

1117 (Cre negative: 4 and 2.2-fold; Cre positive: 0.85 and 1.14-fold) $n=2$

1118

1119 **Fig 5. TLR3 dependent recognition of *Cryptosporidium* infection**

1120 We sought to identify the pattern recognition receptor that is activated to induce IFN λ
1121 production in response to *Cryptosporidium* infection.

1122 (A) ELISA of HCT-8 cultures infected with *C. parvum* or treated with the indicated
1123 agonist for 24 hours to assess IFN λ production in response to stimulus against a variety
1124 of pattern recognition receptors, $n=3$. IFN λ is induced in response to the following
1125 stimuli: infection, Poly(I:C), lipofected Poly(I:C), and ssPolyU RNA

1126 (B) Infection of wild type control mice (B6129) compared to mice lacking MAVS. Fecal
1127 luminescence measured every 2 days. Representative of 2 biological replicates (1.18
1128 and 1.04-fold increase) $n=2$

1129 (C) Infection of wild type control mice (C57B6N/J) compared to mice lacking TLR7.
1130 Fecal luminescence measured every 2 days. Representative of 3 biological replicates
1131 (1.3, 0.70, 2.2-fold increase) $n=3$

1132 (D) Infection of wild type control mice (B6129) compared to mice lacking TLR3. Fecal
1133 luminescence measured every 2 days. An increase of 8-fold in oocyst shedding was
1134 observed. Representative of 3 biological replicates (8, 12.4, 3.8-fold respectively) $n=3$

1135 (E) IL-18 protein detected from ileal biopsies of infected mice wild type (B6129)
1136 compared to $Tlr3^{-/-}$ at day 2 post infection. Standard t-test *** $p < 0.001$. 11 mice per
1137 group, $n=2$

1138 (F) IFN λ protein detected from ileal biopsies of infected mice wild type (B6129)
1139 compared to $Tlr3^{-/-}$ at day 2 post infection. Standard t-test *** $p < 0.001$. 11 mice per
1140 group, $n=2$. Dotted line represents the limit of detection.

1141

1142 **Fig 6. Model**

1143 *Cryptosporidium* infection leads to sensing by the endosomal pattern recognition
1144 receptor TLR3. Following activation of TLR3, IFN λ transcription is induced. Once IFN λ
1145 is secreted, it acts first on the infected cell (1), and then on uninfected bystanders (2) to
1146 induce the transcription of hundreds of ISGs. Lysis of the host cell by parasite egress
1147 releases intracellular contents, including IFN λ , further amplifying the type III interferon
1148 response. The protective effects of IFN λ in mice require intact STAT1 signaling in the
1149 intestinal epithelial cell lineage. One proposed mechanism of action for IFN λ is to block
1150 invasion of the parasite.

1151

1152 **S1 Fig. Impact of siRNA knockdown of screening hits on host cell survival upon**
1153 ***C. parvum* infection.**

1154 We used siRNA treatment to knockdown transcripts of genes identified in our screen
1155 and assessed host cell viability following infection.

1156 (A) Relative expression of genes targeted for knockdown normalized to the scrambled
1157 (scr) siRNA control. $n=2$

1158 (B) Knockdown of top candidates does not affect host cell viability in the absence of
1159 infection. MTT assay normalized to uninfected scrambled (scr) siRNA control. $n=2$

1160 (C) Knockdown of candidates leads to an increase in host cell viability during *C. parvum*
1161 infection. MTT assay normalized to uninfected scrambled (scr) siRNA control. $n=2$

1162

1163

1164

1165 **S2 Fig. Type I and type III interferons in HCT-8**

1166 (A) GSEA plot showing Interferon Alpha Beta Signaling and Interferon Lambda

1167 Response signatures identified at 48 hours post infection. Closed circles represent

1168 genes that make up the core enrichment of the signature. Note that many of the genes

1169 overlap. Alpha Beta: Net enrichment score=2.9, p-value <0.0001, Lambda: Net

1170 enrichment score=3.04, p value <0.0001

1171 (B) Protein levels of IFN β and IFN λ following lipofection with 10 μ g/mL Poly(I:C) as

1172 measured by ELISA. Note the maximal production of IFN β is 27-fold higher than that

1173 observed during *C. parvum* infection

1174

1175 **S3 Fig. BL6 and IL28Ra KO mice exhibit similar baseline and post-infection**

1176 **intestinal pathology scores**

1177 (A) Histology scoring from uninfected BL6 wild type mice and mice lacking the type III

1178 interferon receptor Il28ra^{-/-}. No differences were observed.

1179 (B) Histology scoring from BL6 wild type mice and mice lacking the type III interferon

1180 receptor Il28ra^{-/-} infected with 50,000 *C. parvum* and uninfected controls. One-way

1181 ANOVA with Šídák's multiple comparisons test * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** p

1182 <0.0001. Differences between uninfected Il28ra^{-/-} mice compared to infection tended to

1183 be more statistically significant than those observed between uninfected and infected BL6

1184 mice.

1185

1186 **S4 Fig. CD151 localizes to the *Cryptosporidium* invasion site**

1187 Immunofluorescence of HCT-8 infected with *C. parvum* at 1 hour post infection. *C.*
1188 *parvum* (red), CD151 (gray), actin (green) Hoechst label nuclei. Scale bar 10 μ m in top
1189 panel, scale bar 5 μ m in bottom panel

1190

1191 **S1 Table:** List of primers

1192

1193 **S2 Table:** siRNA sequences

1194

1195 **S3 Table:** CRISPR screening data. Summary read count file used for analysis. Summary
1196 of MAGeCK analysis. GSEA for screening data

1197

1198 **S4 Table:** Differential gene expression and GSEA for RNAseq at 48 hours post infection

1199

1200 **S5 Table:** Differential gene expression and GSEA for RNAseq at 10 hours post infection

1201