Supporting Information

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SI Materials and Methods

The tests used to monitor cognitive behavior, the behavior of the mice exposed to ATCV-1, as well the procedures used for metagenomic sequencing, RNA extraction, microarray analysis, data normalization and statistical analysis for microarray transcriptomics, pathway analysis, and measurement of antibodies to ATCV-1 and related chloroviruses in mouse blood samples follow.

Cognitive Testing. All of the participants underwent a battery of cognitive tests, as previously described (1). These included the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) (2), Trail Making Test Part A (Trails A) (3), and the Information subtest of the Wechsler Adult Intelligence Scale (WAIS) III (4).

The RBANS consists of 12 subtests that are used to calculate five index scores and a total score. Test indices are Immediate Memory (comprising List Learning and Story Memory tasks), Visuospatial/Constructional (comprising Figure Copy and Line Orientation tasks), Language (comprising Picture Naming and Semantic Fluency tasks), Attention (comprising Digit Span and Coding tasks), and Delayed Memory (comprising List Recall, Story Recall, Figure Recall, and List Recognition tasks). Each index score is expressed as an age-adjusted standard score with a mean of ∼100 and an SD of ∼15. The index scores were combined to yield an RBANS Total score, which is a measure of overall cognitive functioning. Trails A requires an individual to draw lines sequentially connecting 25 encircled numbers distributed on a sheet of paper; the score is based on the time to complete the task. Trails A is a test of visual scanning and motor speed. The Information subtest of the WAIS is a test of general knowledge, including questions about geography and literature. For the latter two tests, scores are expressed as an age-adjusted scaled score with a mean of 10 and an SD of 3. For the purposes of calculating odds ratios, a low performance on the RBANS tests was defined as less than or equal to 80 and low performance on the other tests as performance below the 25th percentile (4, 5).

Monitor the Behavior of Mice. Behavioral tests were performed on mice inoculated with *Chlorella heliozoae*/ATCV-1 (exposed) and *C. heliozoae* (control) between 6 and 22 wk postinoculation. The tests were performed in the following order: novelty-induced activity in open field, Y-maze, object placement, dark–light box, new object recognition or location, prepulse inhibition (PPI) of the acoustic startle, and passive avoidance.

Novelty-induced activity. Novelty-induced activity in the open field was assessed over a 30-min period using activity chambers with infrared beams (San Diego Instruments Inc.), as previously described (6).

Spatial recognition in the Y-maze. Spatial recognition memory was evaluated in a Y-maze as described by Melnikova et al. (7). In brief, one arm of the maze was blocked and a mouse was allowed to freely explore the two open arms for 5 min. After a 20-min delay, the block was removed and the mouse was allowed to freely explore all three open arms for 5 min. The percentage of time and visits into the novel (previously blocked) arm during the first 2 min of the 5-min trial was analyzed.

Dark–light box. Anxiety was evaluated using a dark–light box (Colbourn Instruments). Mice were placed in the transparent side of the box and allowed to freely move between the dark and light chambers for 5 min. The latencies to cross between chambers were automatically recorded using Graphic State v 3.03.

Novel object recognition. The novel object recognition test was used to assess recognition memory (8). Briefly, mice were habituated for 4 d to an empty mouse cage (28.3 cm length × 17.4 cm width × 13 cm height) for 10 min each day as previously described (9, 10). On day 5, two identical objects were placed on opposite ends of the empty cage, and the mouse was allowed to freely explore the objects for 10 min. After a 1-h delay, during which the mouse was held in its home cage, one of the two familiar objects was replaced with a novel one, and the mouse was allowed to freely explore the familiar and novel object for 5 min. The percent time near the novel object was calculated as the time near the novel object divided by the total time near either object.

Novel location recognition. Novel location recognition was used to assess spatial recognition memory. Briefly, mice were habituated for 4 d to an empty mouse cage for 10 min each day. On day 5, two identical objects were placed on opposite ends of the empty cage, and the mouse was allowed to freely explore the objects for 10 min. After a 1-h delay, one object was moved to a different location in the cage, and the mouse was allowed to explore for 5 min. The percent time near the object at the novel location was calculated as the time near the novel location divided by total time near novel and old location.

Sensorimotor gating. Sensorimotor gating was assessed using PPI of the acoustic startle (San Diego Instruments Inc.). Mice were acclimatized to a 70-dB background noise for 5 min. They were then given 10 presentations each of a 120-dB pulse and 0-dB pulse. This was followed by 5–6 presentations in randomized order of a 120-dB pulse, 0-dB pulse, or the following prepulses followed by the 120-dB pulse: 74, 78, 82, 86, and 90 dB. The intervals between each presentation varied from 10 to 19 s. PPI% was calculated by [100 – (mean startle amplitude of each prepulse/mean startle amplitude of 120 dB pulse)] × 100. Mean PPI% was calculated by averaging all PPI% values for presentations of all prepulses for each experimental group.

Passive avoidance. Associative learning and memory were evaluated using a 2-d passive avoidance test (San Diego Instruments Inc.). On day 1, mice were placed in a lit compartment with the gate to the dark compartment closed. After a 30-s delay, the gate opened, allowing the mouse to cross to the dark compartment. Once the mouse crossed over, the gate automatically closed, and after a 3-s delay, a 0.3-mA shock was administered for 3 s. Twenty-four hours later, the mouse was again placed in the lit compartment with the gate shut. After a 5-s delay, the gate opened. The trial ended either when the mouse crossed to the dark compartment or once 10 min elapsed. On each day, the latency in seconds for the mouse to cross from the light to dark compartment was automatically recorded and used in the analysis.

Statistical Analyses of Behavioral Studies. The behavioral data were analyzed with one-way analysis of variance (ANOVA) for all tests except PPI. The PPI data were analyzed using two-way repeated measures ANOVA with treatment as a between-subject factor and PPI as a within-subjects factor. We did not use sex of animals as an independent variable, as our analyses detected no sex-dependent effects in the tests described. If the data did not pass tests for normality or equal variance, the data were rank-transformed before further statistical analysis.

Metagenomic Sequencing. DNA samples from 33 individuals (two independent experiments with 17 and 16 individuals) were analyzed by metagenomic sequencing. The demographic information on these individuals is reported in Table S1*A*. The method used

for the sequencing is presented as follows. A total of 75–100 ng of DNA was used for paired-end library generation using the Nugen Ultralow DR Multiplex System (NuGEN) following the manufacturer's instructions. The libraries were purified and analyzed on the Bioanalyzer (Agilent Technologies) to confirm size and concentration. The purified libraries were sequenced using Illumina Hi Seq, which generated ~200,000,000 paired-end reads of 100 nucleotides.

Sequence reads were filtered to remove low-quality sequences, resulting in a minimum length of 60 nucleotides. To evaluate putative viruses associated with the human throat sequence reads, human, bacteria, fungi, and parasite sequence reads were removed by bioinformatic filtering as follows: sequence reads with homology to human samples were removed in two stages. The first stage used the program Bowtie (bowtie-bio.sourceforge.net/index.shtml). A sliding window approach was used to align a 40-base-pair subsequence from the reads to the human genome Build 37 (www.snpedia.com/index.php/GRCh37). During each iteration of this procedure, reads mapping to the human genome were removed from the analysis and subsequences used for alignment were offset by five bases. The second filtering of human sequences used CLC Genomics Workbench Version 6 (www.clcbio.com) using a reference set of sequences based on the human genome Build 37 with the following settings: length fraction, 0.4; similarity, 0.4. Sequence reads that were not removed by this subtraction were filtered sequentially to remove bacterial, fungal, and protozoan sequences by matching to appropriate National Coalition Building Institute Reference Sequence (RefSeq) databases (www.ncbi.nlm.nih.gov/refseq/). The remaining sequences, which consisted of <1% of the starting sequences, were then mapped to the RefSeq complete set of viral genomes (ftp://ftp.ncbi.nlm.nih.gov/refseq/release/viral/) using CLC Genomics Workbench Version 6 with the following settings: length fraction, 0.8; similarity, 0.8. Sequences homologous to ATCV-1 (reference sequence NC_008724.1) by this analysis were further mapped to the ATCV-1 genome using CGView (11).

RNA Extraction. Following completion of the behavioral experiments, the mice were killed and brains were removed and placed on ice. The hippocampus was dissected, placed into RNAlater RNA stabilization reagent (Qiagen), and stored at −80 °C. Total RNA was isolated from either the left or right hippocampus using miRNeasy Qiagen mini kit (cat. no. 217004) following the manufacturer's protocol. To remove genomic DNA carryover, RNA samples were treated with DNase for 20 min at 37 °C using a TurboDNA-free kit from Ambion (cat. no. AM1907). Samples were assessed for RNA quality and concentration by TapeStation 2200 (Agilent Technologies). Based on these measurements, 24 samples were selected for analysis. These included samples from 16 mice gavaged with *C. heliozoae*/ATCV-1 and from eight mice gavaged with *C. heliozoae* alone.

Microarray Analysis. RNA transcript levels were quantified by microarray analyses. RNAs were amplified into cDNA and biotinylated by in vitro transcription with Affymetrix reagents, using the Whole Transcript Sense Target Labeling protocol as described in the Affymetrix manual (www.affymetrix.com/support/technical/product_updates/wt_1_1_assay.aff). Biotinylated cDNAs were purified, fragmented, and subsequently hybridized to Affymetrix GeneChip Mouse Gene 2.0 ST arrays.

Data Normalization and Statistical Analysis for Microarray Transcriptomics. Affymetrix CEL files, containing the raw GeneChip data, were prepared using GeneCp Command Console software. These data were extracted and normalized with Genomics Suite v6.6 (Partek Inc.) software using the Robust Multichip Analysis algorithm. One *C. heliozoae* inoculated control (ID no. 650) had RNA that was largely degraded and was omitted from the final analyses. To detect differentially expressed genes under various conditions, a single expression value was assigned for each transcript, including all its exons. One-way ANOVA was used to detect statistically significant changes in gene expression between samples from mice inoculated with *C. heliozoae*/ATCV-1 and control mice inoculated with *C. heliozoae* alone. Transcripts with a difference of at least 2 SDs in either direction between the ATCV-1–exposed and control mice were selected for pathway analyses (12).

Pathway Analysis. Network, function, and pathway analyses were generated using Ingenuity Pathways Analysis (Ingenuity Systems), which facilitates the interpretation of microarray data by grouping differentially expressed genes into known functional pathways. These analyses identified statistically increased representations of the differentially expressed genes in biologically relevant processes (13). Genes that showed differential expression of greater than 2 SDs between inoculated and control mice were compared with those genes that did not, using the Fisher's exact test to identify the differentially expressed genes' pathways for review of potential biological function. Based on its curated Knowledge Base (MAP Molecule Activity Predictor; www.ingenuity.com/products/ipa/ipa-summer-release-2014), Ingenuity Pathways Analysis further predicted whether the genes' observed levels of altered transcription were in accordance with regulatory relationships from the literature; for example, elevated expression of gene A (a known inhibitor of gene B) is observed together with reduced expression of gene B. Due to the exploratory nature of this study, pathways with P values ≤ 0.05 were selected for inclusion.

Measurement of Antibodies to ATCV-1 and Related Chloroviruses in Mouse Blood Samples.

Enzyme immunoassays. IgG antibodies to ATCV-1 were measured by ELISA using variations of previously described procedures. Highly purified virion stocks containing ~10^{11} PFU/mL were diluted 1:1,000 in 50 μL carbonate buffer and coated overnight at 4 °C on 96-well polystyrene flat bottom MaxiSorp plates (Nunc; Thermo Fisher Scientific). Plates were blocked for 1 h at 37 °C with Starting Block (Thermo Scientific). Plates were then incubated with a 1:1,000 dilution of the mouse test serum in duplicate wells, incubated for 1 h at 37 °C, washed, and incubated with peroxidase-conjugated goat–anti-mouse IgG for 45 min at 37 °C (Southern Biotech). A 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) hydrogen peroxide solution (KPL Protein Research Products) was added for color development, and absorbance was measured at 405 nm, with a reference wavelength of 490 nm, in an automated microtiter plate reader (Molecular Devices), with the results expressed as absorbance units. A sample was considered positive for antibodies to ATCV-1 if it generated a signal in the wells coated with ATCV-1 that gave an absorbance value of at least 0.4 units.

Measurement of antibodies by Western blotting. *C. heliozoae* infected with ATCV-1 and uninfected *C. heliozoae* were added to SDS Laemmli buffer and heated to 95 °C, diluted 1:10, and loaded on a precast NuPAGE Novex 4–12% Bis·Tris gel (Life Technologies). Proteins were resolved through SureLock gel electrophoresis, and the gels were stained with SimplyBlue SafeStain (Life Technologies) to visualize protein amounts. Proteins were transferred to nitrocellulose using iBlot dry transfer technology (Life Technologies). Membranes were incubated overnight at 4 °C with Starting Block (Thermo Scientific). Following 1 h incubation with a 1:1,000 dilution of the test sample of mouse serum, the blots were washed and incubated for 45 min with goat–anti-mouse IgG conjugated to alkaline phosphatase (Southern Biotech). Following another incubation, the blots were washed and developed with an alkaline phosphatase-based conjugation kit (BioRad Life Science). For reference, we also used a 1:500 dilution of a rabbit polyclonal antibody gener-

ated to recognize a recombinant GST-labeled major capsid protein A430L from chlorovirus PBCV-1. The same immunoblotting procedure was performed, except an anti-rabbit IgG secondary was applied.

1. Dickerson F, et al. (2008) Association between cognitive functioning, exposure to Herpes Simplex Virus type 1, and the COMT Val158Met genetic polymorphism in adults without a psychiatric disorder. *Brain Behav Immun* 22(7):1103–1107.
2. Randolph C (1998) *RBANS Manual—Repeatable Battery for the Assessment of Neuropsychological Status* (Psychological Corporation, San Antonio, TX).
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5. Dickerson F, et al. (2014) Association between cytomegalovirus antibody levels and cognitive functioning in non-elderly adults. *PLoS ONE* 9(5):e95510.
6. Pletnikov MV, et al. (2008) Inducible expression of mutant human DISC1 in mice is associated with brain and behavioral abnormalities reminiscent of schizophrenia. *Mol Psychiatry* 13(2):173–186, 115.
7. Melnikova T, et al. (2006) Cycloxygenase-2 activity promotes cognitive deficits but not increased amyloid burden in a model of Alzheimer's disease in a sex-dimorphic pattern. *Neuroscience* 141(3):1149–1162.
8. Bevins RA, Besheer J (2006) Object recognition in rats and mice: A one-trial non-matching-to-sample learning task to study 'recognition memory'. *Nat Protoc* 1(3): 1306–1311.
9. Kim D, Chae S, Lee J, Yang H, Shin HS (2005) Variations in the behaviors to novel objects among five inbred strains of mice. *Genes Brain Behav* 4(5):302–306.
10. St-Amour I, et al. (2014) IVIg protects the 3xTg-AD mouse model of Alzheimer's disease from memory deficit and Aβ pathology. *J Neuroinflammation* 11:54.
11. Stothard P, Wishart DS (2005) Circular genome visualization and exploration using CGView. *Bioinformatics* 21(4):537–539.
12. Marvanová M, Lakso M, Wong G (2004) Identification of genes regulated by memantine and MK-801 in adult rat brain by cDNA microarray analysis. *Neuropsychopharmacology* 29(6):1070–1079.
13. Xiao J, Jones-Brando L, Talbot CC, Jr, Yolken RH (2011) Differential effects of three canonical Toxoplasma strains on gene expression in human neuroepithelial cells. *Infect Immun* 79(3):1363–1373.

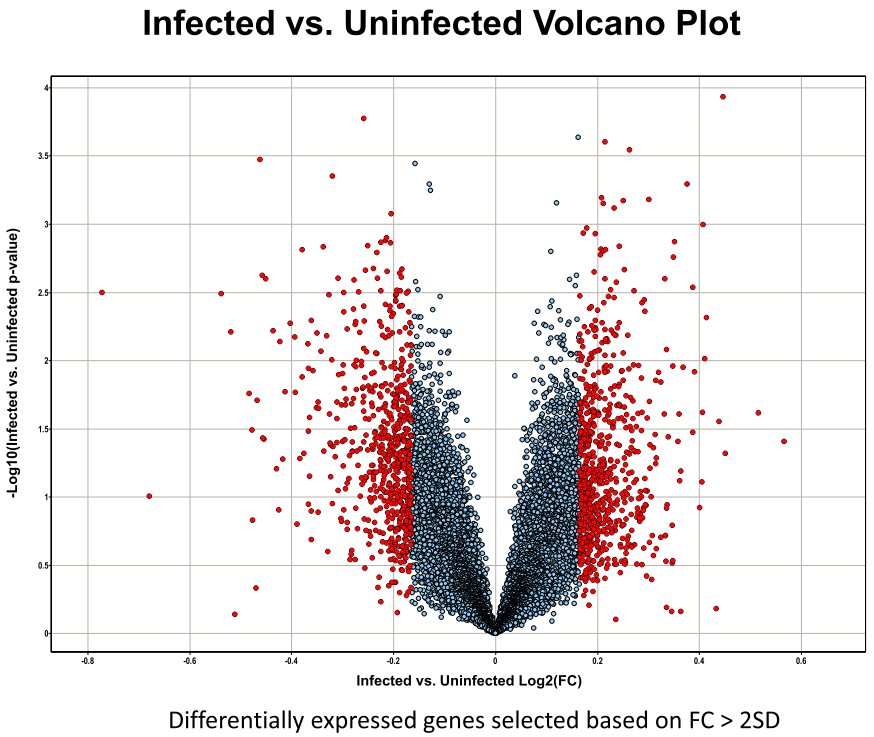

Fig. S1. Distribution plot of genes expressed in the hippocampus of mice gavaged with ATCV-1–infected *C. heliozoae* (n = 16) and control mice (n = 7) gavaged with *C. helliozoae* alone. The dots shown in red represent transcripts with expression differing by at least 2 SDs.

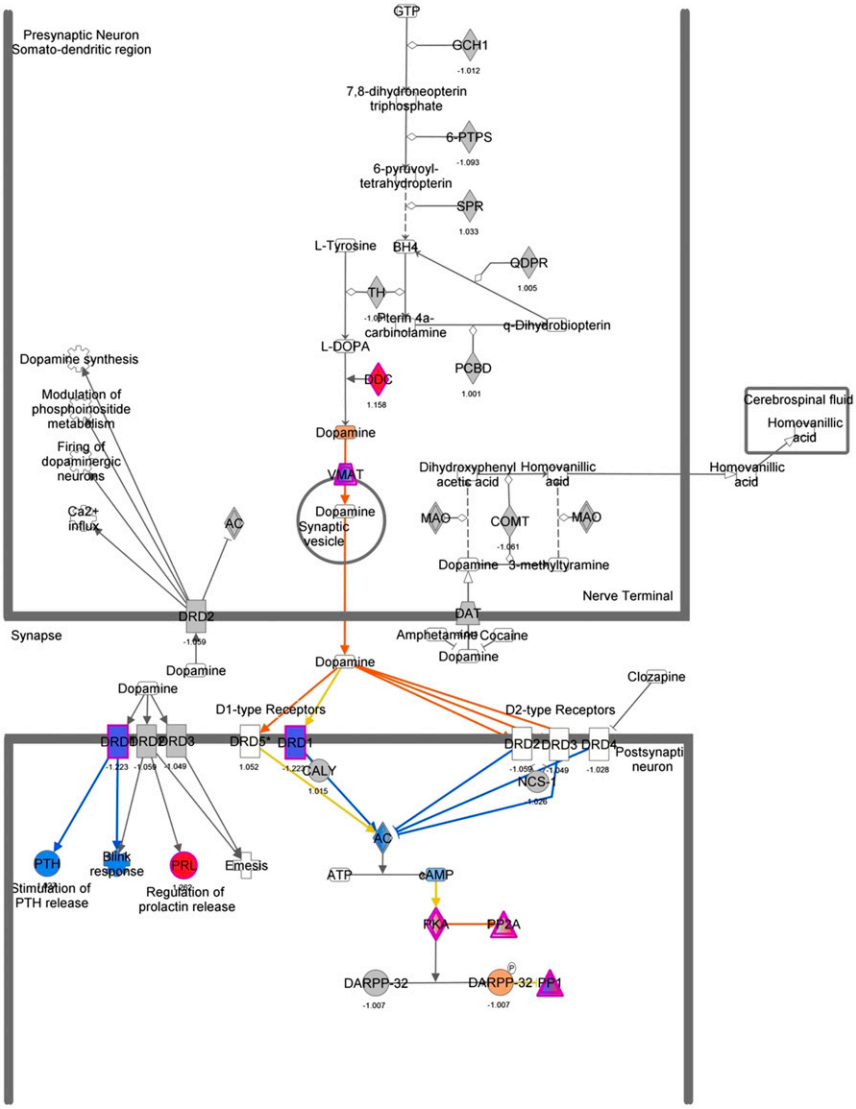

Fig. S2. Differences in the statistically significant dopamine receptor signaling pathway between mice exposed orally to ATCV-1–infected *C. heliozoae* and control mice exposed orally to *C. heliozoae* alone. The numbers shown represent the linear fold change of expression between the two groups. The legend for the color coding is presented in Fig. S8.

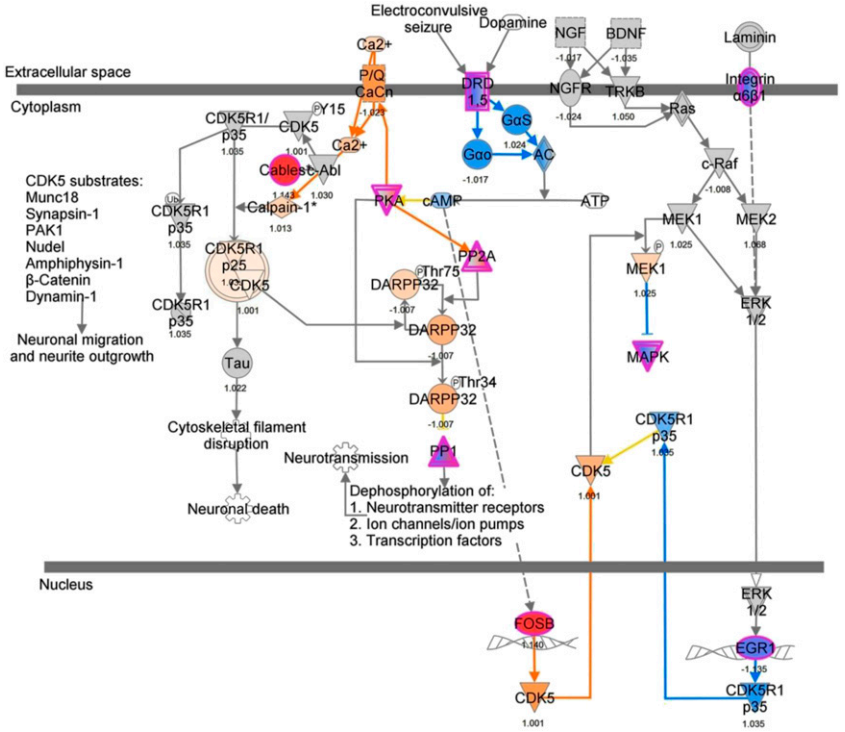

Fig. S3. Differences in the statistically significant CDK5 signaling pathway between mice exposed orally to ATCV-1–infected *C. heliozoae* and control mice exposed orally to *C. heliozoae* alone. The numbers shown represent the linear fold change of expression between the two groups. The legend for the color coding is presented in Fig. S8.

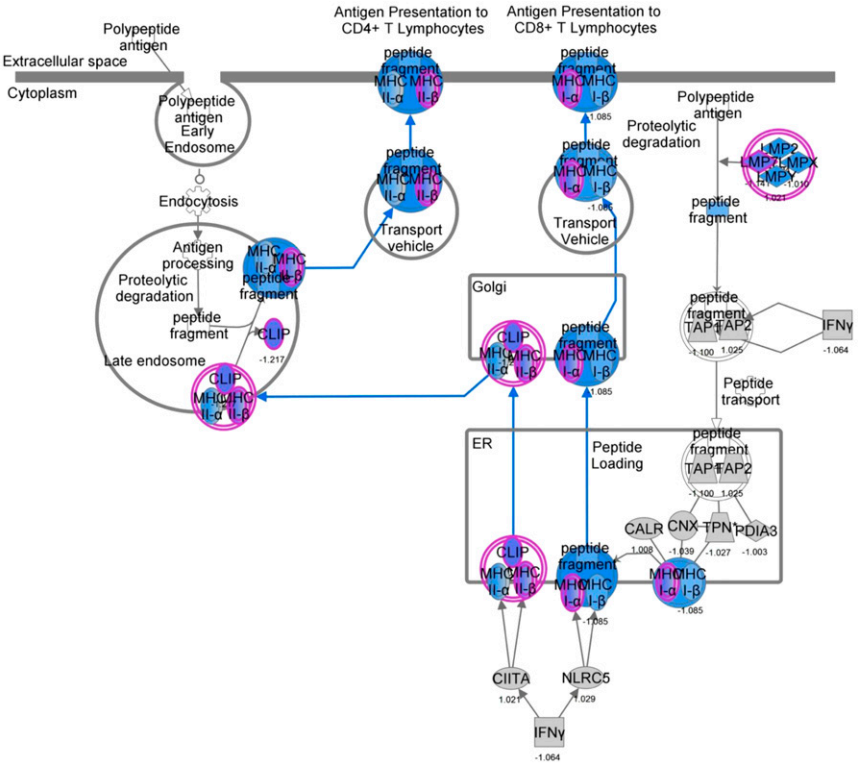

Fig. S4. Differences in the statistically significant antigen presentation pathway between mice exposed orally to ATCV-1–infected *C. heliozoae* and control mice exposed orally to *C. heliozoae* alone. The numbers shown represent the linear fold change of expression between the two groups. The legend for the color coding is presented in Fig. S8.

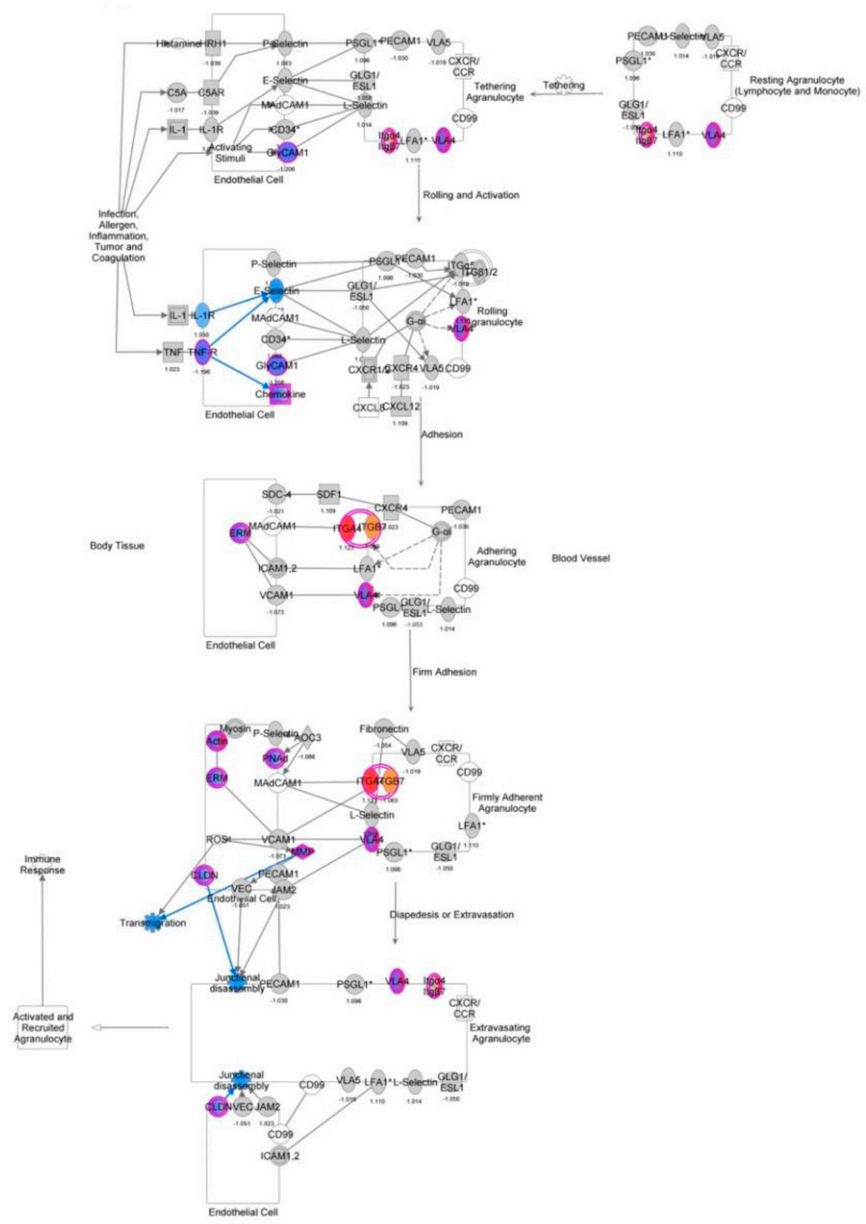

Fig. S5. Differences in the statistically significant agranulocyte cell adhesion pathway between mice exposed orally to ATCV-1–infected *C. heliozoae* and control mice exposed orally to *C. heliozoae* alone. The numbers shown represent the linear fold change of expression between the two groups. The legend for the color coding is presented in Fig. S8.

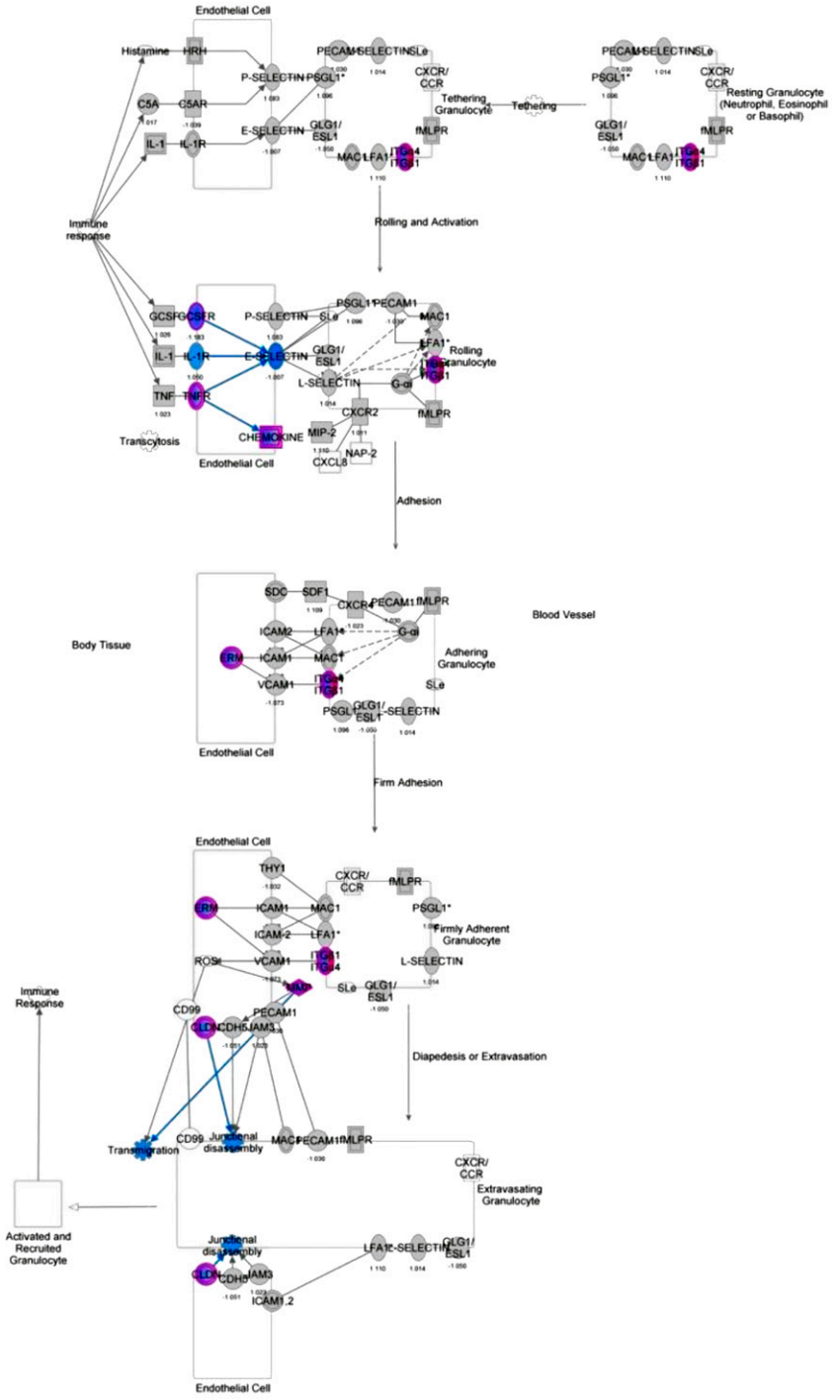

Fig. S6. Differences in the statistically significant granulocyte cell adhesion pathway between mice exposed orally to ATCV-1–infected *C. heliozoae* and control mice exposed orally to *C. heliozoae* alone. The numbers shown represent the linear fold change of expression between the two groups. The legend for the color coding is presented in Fig. S8.

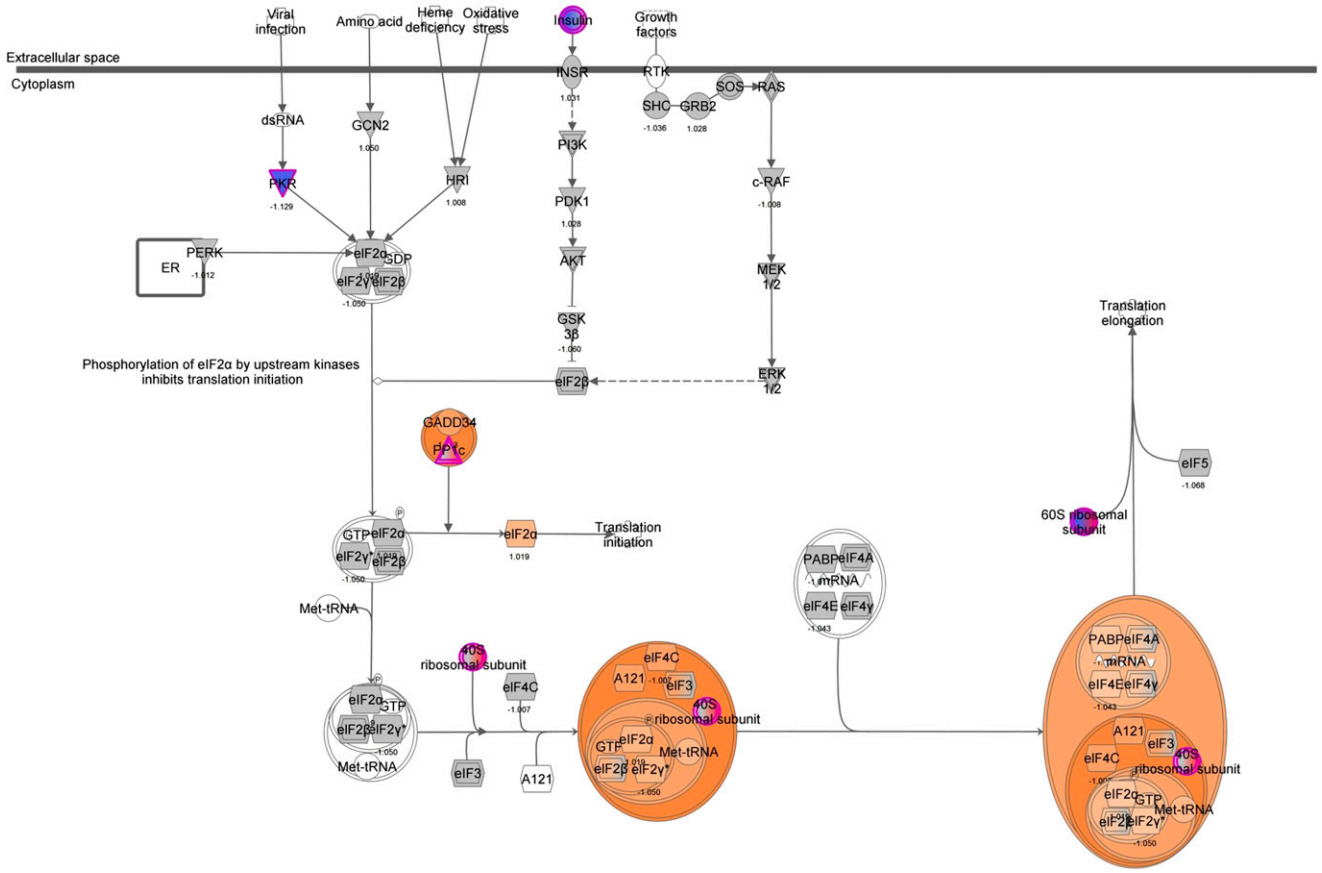

Fig. S7. Differences in the statistically significant eIF2 signaling pathway between mice exposed orally to ATCV-1–infected *C. heliozoae* and control mice exposed orally to *C. heliozoae* alone. The numbers shown represent the linear fold change of expression between the two groups. The legend for the color coding is presented in Fig. S8.

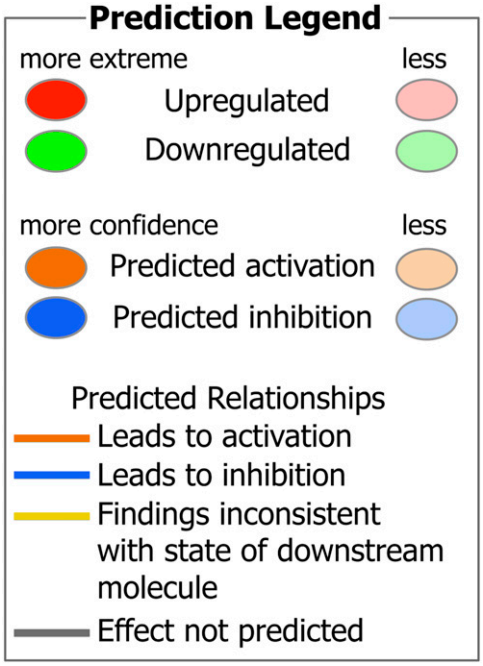

Fig. S8. The legend for the color coding in Figs. S2–S7.

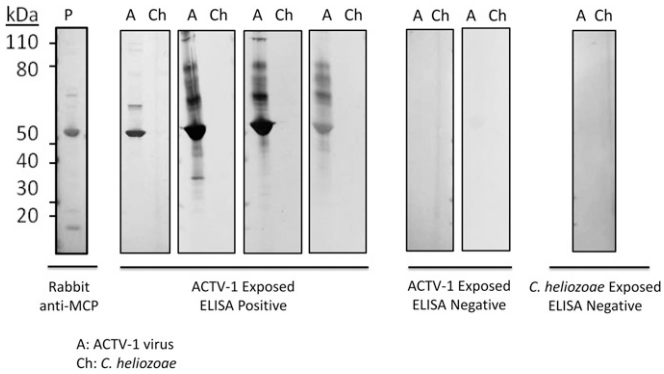

Fig. S9. Western blot assays performed with antigens derived from purified ATCV-1 (A) and *C. heliozoae* (Ch) prepared and reacted as described in the text. The first lane (labeled P) is from rabbit antibody prepared against the major capsid protein (A430L) of chlorovirus PBCV-1 as a reference. ATCV-1 exposed ELISA positive, reactivity of sera from mice exposed to ATCV-1 and reactive to ATCV-1 antigens by ELISA. ATCV-1 exposed ELISA negative, reactivity of sera from mice exposed to ATCV-1 and not reactive to ATCV-1 antigens by ELISA. *C. heliozoae* exposed ELISA negative, reactivity of serum from a mouse exposed to *C. heliozoae* in the absence of ATCV-1. All mice with this exposure were nonreactive by ELISA.

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)
Table S3 (DOCX)