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Article

miR-146a and miR-155 Delineate a MicroRNA Fingerprint Associated with *Toxoplasma* Persistence in the Host Brain

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SUMMARY

microRNAs were recently found to be regulators of the host response to infection by apicomplexan parasites. In this study, we identified two immunomodulatory microRNAs, miR-146a and miR-155, that were coinduced in the brains of mice challenged with *Toxoplasma* in a strain-specific manner. These microRNAs define a characteristic fingerprint for infection by type II strains, which are the most prevalent cause of human toxoplasmosis in Europe and North America. Using forward genetics, we showed that strain-specific differences in miR-146a modulation were in part mediated by the rhoptry kinase, ROP16. Remarkably, we found that miR-146a deficiency led to better control of parasite burden in the gut and most likely of early parasite dissemination in the brain tissue, resulting in the long-term survival of mice.

INTRODUCTION

Toxoplasma gondii is a widespread obligate intracellular protozoan parasite causing toxoplasmosis, a potentially severe disease in immunocompromised or congenitally infected humans. Central to transmission and pathogenesis is the ability to traffic to the CNS following host infection and to undergo transformation from the rapidly dividing, disease-causing tachyzoite to a long-lived, slow-dividing bradyzoite contained within tissue cysts. This bradyzoite cyst sustains establishment of chronic infection. Bradyzoites can spontaneously reconvert back to tachyzoites, but their dissemination is effectively prevented by the immune system. Upon host cell invasion, *Toxoplasma* parasites grow inside a parasitophorous vacuole (PV), a dynamic niche shaped by the tachyzoite to support its growth. Host stromal and immune cells respond to infection with profound transcriptional changes, including genes involved in mounting Th1-type immune response to infection (Jensen et al., 2011; Hunter and Sibley, 2012). Several strain-specific parasite effectors are secreted into the host cells where they neutralize cell autonomous immune defenses or subvert gene expression (Howard et al., 2011; Hunter and Sibley, 2012). Many known secreted molecules are either released from rhoptry secretory organelles early in host cell invasion (e.g., ROP16, Saeij et al., 2007) or are released from dense granules (e.g., GRA24, Braun et al., 2013).

In addition, apicomplexan parasites may also interfere with host miRNA populations in a parasite-specific manner, suggesting this RNA silencing pathway as a way of reshaping cellular environment (Hakimi and Cannella, 2011). Previous work also suggests that miRNA-based regulatory pathways may contribute to the mounting of host cell responses/defenses (e.g., the inflammatory response) (Ding and Voinnet, 2007).

The present study confirms that *Toxoplasma* alters the host cell miRNA profile through a mechanism that requires live parasites that are able to invade and divide (Zeiner et al., 2010). Notably, we now describe significant and exclusive upregulation by cystogenic strains (type II) of miR-146a expression, a key inflammatory response regulator (Taganov et al., 2006). Using forward genetics, we show that strain-specific differences in miR-146a expression modulation are controlled by the parasite-secreted kinase ROP16. Furthermore, *miR-146a* ablation affects early parasite burden, leading to significant differences in interferon (IFN)-γ production and better survival in normally susceptible C57BL/6 mice. Last, mice challenged with type II strains had higher brain levels of miR-146a but also of miR-155, another immunoregulatory RNA (Lindsay, 2008). Collectively, these data uncover a microRNA signature that typifies *Toxoplasma* long-term persistence and latency in the host brain.

RESULTS

Specific Alteration of Host Cell miRNA Profile by *Toxoplasma* Infection

In this study, we profiled the steady-state levels of cellular mature miRNAs from primary human foreskin fibroblasts (HFF) infected with the virulent type I (RH) strain (Figure S1A; Table S1). Overall, our data mirror those published previously showing that *Toxoplasma* alters the levels of ~5%–10% of host miRNAs in tachyzoites-infected human fibroblasts (Zeiner et al., 2010). We compared host miRNA expression levels in cells infected with *Toxoplasma*, *Theileria*, or *Cryptosporidium* using our data set and previously published data sets, and this revealed that these apicomplexan parasites modulate the expression of a restricted panel of miRNAs in a specific manner, most likely in order to suit their respective biology. This can be illustrated by miR-155, a microRNA implicated in immune response (Lindsay, 2008) that was strongly induced by both *Toxoplasma* (Figures S1B) and *Theileria* (Marsolier et al., 2013) while being unaffected by *Crytosporidium* infection (Zhou et al., 2009).

Patterns of expression of host microRNA were linked to the time course of infection and multiplicity of infection (MOI) (Figure S1C; data not shown). Although the levels of miR-16, -26a, -29a, and -221 marginally decreased 6 hr postinvasion and showed a clear inhibition at 15 and 24 hr, miR-1246 increased rapidly at 6 hr and remained high thereafter (Figure S1C). Control cells exposed to heat-killed tachyzoites displayed a miRNA expression profile similar to that of uninfected control samples indicating that interference with the host RNA silencing machinery required live parasites, still able to invade and divide, rather than through signaling molecules diffusing in the cell-culture media (Figure S1C; data not shown). Overall, these changes are a specific response to *Toxoplasma* infection because the levels of the aforementioned miRNAs remain unchanged in cells infected with *Neospora caninum*, a close relative of *Toxoplasma* (Figure S1C).

Toxoplasma Infection Modulates Host miRNAs Biogenesis at Multiple Levels

Theoretically, interference with the miRNA pathway could occur at the levels of miRNA transcription, biogenesis, or activity (Hakimi and Cannella, 2011). In this respect, the inhibition of miR-16, -26a, and -29a may occur at the transcriptional level because the level of their precursors (pre-miRNA) decreased simultaneously postinvasion (Figure S1C). A common feature of these three microRNAs is that they are classified as repressed by the oncogenic transcription factor c-Myc (Chang et al., 2008). It appears that they belong to a larger panel of miRNAs defined as core Myc-regulated genes (~20 miRNAs) that are effectively repressed 6 hr postinvasion by *Toxoplasma* (Figure S2A). This is consistent with the induction of the Myc protein by *Toxoplasma* (Figure S2B). Although acting at the transcriptional level, *Toxoplasma* infection also potentiates the Dicer-mediated processing of host cell miRNAs. For instance, the mature miR-1246 drastically increased with the expected decrease of its precursor, suggesting that infection by live *Toxoplasma* tachyzoites affects dicer processing (Figure S1C). This response reminds us of the effect of the RNA binding protein Lin-28 on the processing of the microRNA Let-7 (Rybak et al., 2008).

Toxoplasma Upregulates miR-146a Levels in a Strain-Specific Manner

We next considered whether altered miRNA levels were relevant to differences in pathogenesis among strain types. We compared the levels of cellular microRNAs in human fibroblasts infected with type I (RH) strain, which normally does not make tissue cysts, to the cystogenic type II (ME49) strain (Figure 1A; Table S2). Among the miRNAs differentially expressed across strains, miR-146a was substantially and significantly induced by ME49 tachyzoites, whereas the RH parasites did not affect its expression (Figures 1A, 1B, and S2C). The miR-146a miRNA family has two evolutionary conserved genes: *miR-146a* and *miR-146b*, which are located on separate chromosomes, in quite unrelated sequence contexts, but differ in their mature sequence by only two nucleotides at the 3′ end (Figure S3A) (Taganov et al., 2006). We next assessed the kinetics of both miR-146a and miR-146b to discriminate between miR-146 family paralogs in human and murine cells infected with *Toxoplasma* strains of the three main clonal lineages (Figures 1C and 1D). Twenty-four hours postinvasion, miR-146a was dramatically induced (~30- to 60-fold) by type II (PRU and ME49) strains compared to human fibroblasts infected with type I (RH and GT1) or type III (CTG) strains (Figures 1C). miR-146a upregulation by ME49 started 6 hr postinvasion and was most dramatic after 24 hr (Figure 1D). This regulation is likely to be at the level of transcription because a similar upregulation was observed at the level of the primary miR-146a transcripts (pri-miR-146a) in cells infected with type II parasites (Figure 1E). Additionally, the levels of miR-146a increased slightly but exclusively in response to infection by a type II strain in human embryonic kidney 293 and astrocytoma U373 cells, reflecting the robustness of this phenotype using different host cell types (Figures S2D and S2E). Whereas the amounts of mature miR-146a were increased by *Toxoplasma* infection, miR-146b levels remained unchanged (Figure 1D), suggesting that this miRNA family has a rather specific mode of regulation of expression involving more than one point of control.

miR-146a is widely expressed throughout the hematopoietic system (Boldin et al., 2011). Its expression is initially low in precursor cells and increases with maturation and activation, typically in response to Toll-like receptor (TLR) agonists. In line with previous findings, we found that the levels of miR-146a in murine bone marrow-derived macrophages (BMDMs) and in human primary astrocytes were significantly higher when compared to fibroblasts (Figure S2F; data not shown). However, *Toxoplasma* did not alter miR-146a expression in BMDMs, nor in astrocytes, most likely because the maximum threshold limit was already reached in these cells. In summary, miR-146a expression was distinctively upregulated in a wide range of nonhematopoietic cells following infection by type II strains of *Toxoplasma*.

Toxoplasma Type II Strains Induce miR-146a, an NF-κB-Dependent Gene, in a GRA15-Independent Fashion

miR-146a was initially found to be a NF-κB-dependent gene (Figure S3A; Taganov et al., 2006). This response is consistent with the strain-specific activation of the host NF-κB pathway by *Toxoplasma* (Rosowski et al., 2011). Indeed, type II tachyzoites, unlike types I and III, activate the nuclear translocation of NF-κB p65

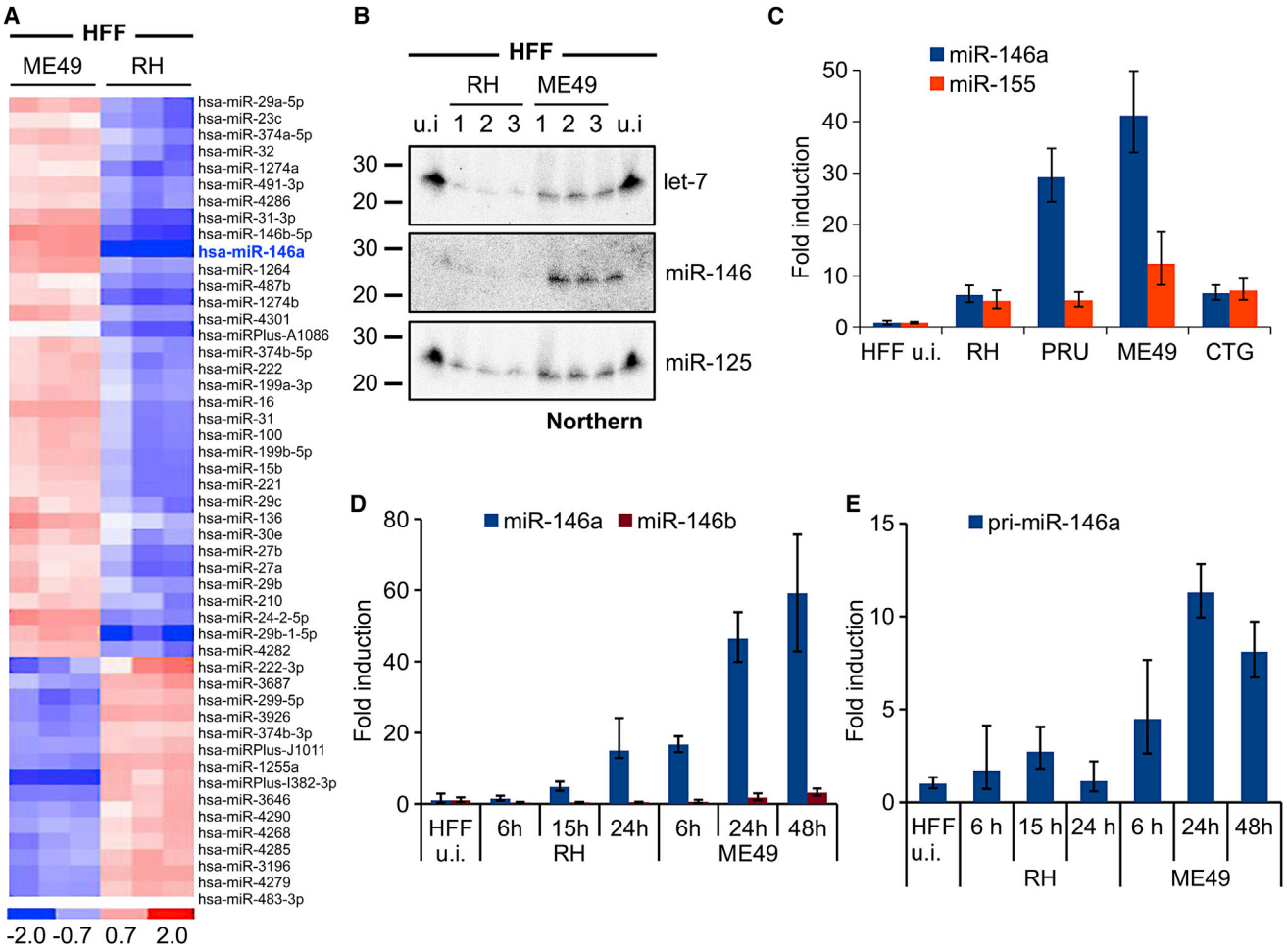


Figure 1. Expression Profiling of Mature miRNAs in Human Fibroblasts following Infection by Type I (RH) versus Type II (ME49) Strains

(A) Heatmap of quantitative miRNA profiling of RH- versus ME49-infected HFF cells. Hierarchical clustering was performed on the microRNAs that were differentially expressed between the two groups (n = 3 samples per strain). Gene expression values were mean log2 transformed and median centered for clustering. The scale of the heatmap is presented as fold change from the median value. The miRNAs list can be found in Table S2.

(B) Northern blot analysis of cellular miRNA levels in human fibroblast cells after 24 hr of mock treatment (u.i) or upon infection with type I (RH) and type II (ME49) strains. Each infection was performed in triplicate. To control for loading, blots were stripped and rehybridized with the indicated miRNA and control probe. RNA markers are on the left.

(C) Quantitative RT-PCR (qRT-PCR) analysis of both miR-146a and miR-155 levels in HFF cells left uninfected (u.i) or infected with type I (RH), type II (PRU and ME49), or type III (CTG) strains. microRNA levels were normalized by RNU24 levels. Mean values and SDs from three independent experiments are shown.

(D and E) Time-dependent expression of (D) miR-146a, miR-146b and (E) primary transcripts pri-miR-146a in HFF cells after *Toxoplasma* type I (RH) and type II (ME49) infections. microRNA levels were assessed by qRT-PCR and normalized by RNU24 levels.

Mean values and SDs from three independent experiments are shown. See also Figures S1 and S2 and Tables S1 and S2.

(RelA) promoting its transcriptional activity, which may account for the type II-dependent induction of miR-146a. The dense granule protein GRA15 was reported as sufficient to mediate the effect as it intersects the NF-κB pathway downstream of MyD88, interleukin (IL)-1R, and TRIF but upstream of TRAF6 and the IKK complex (Rosowski et al., 2011). We therefore asked whether GRA15 might be involved in miR-146a induction by *Toxoplasma*. However, *GRA15* deletion in the type II (Pru A7) did not significantly limit miR-146a induction in the infected fibroblast cells (Figure S3B). Similarly, ectopic expression of a type II allele of *GRA15* in a type I strain (Figure S3C), while activating NF-κB p65 (Rosowski et al., 2011; data not shown), did not alter miR-146a levels (Figure S3D). Therefore, miR-146a induction by *Toxoplasma* is independent of GRA15. However, the fact that $GRA15^{II}$ has an effect on some NF-κB family members (e.g., p65 subunit) but not on others (Rosowski et al., 2011) indicates that alternative parasite-derived factors other than GRA15 are likely involved in NF-κB-dependent (p65-independent) miR-146a regulation.

Genome-wide Scan of the miR-146a Phenotype Reveals a Single Major QTL

To further assess if the increase of miR-146a levels in cells infected with *Toxoplasma* takes place via an active mechanism

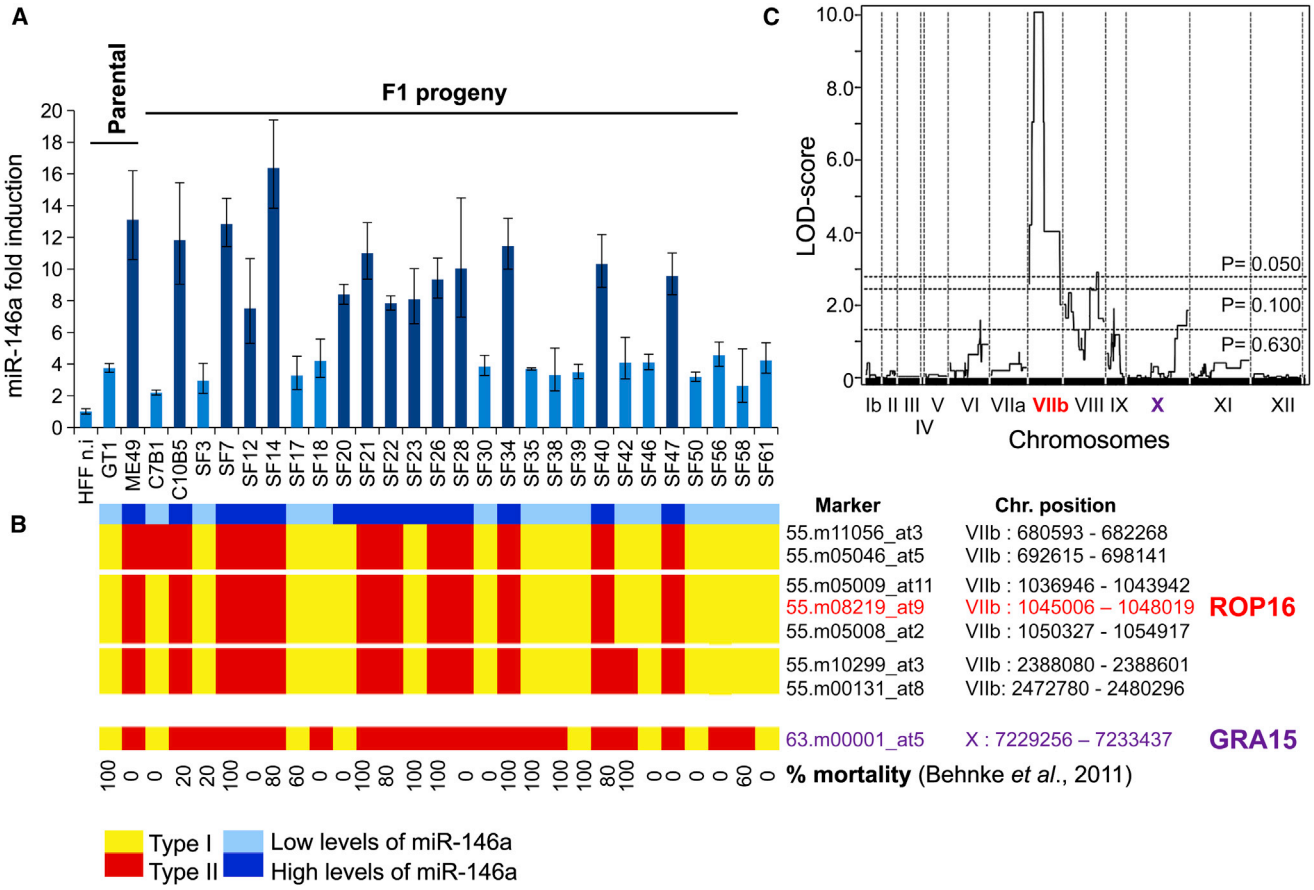


Figure 2. Genome-wide Scan for Association of miR-146a Expression with *Toxoplasma* Genetic Markers Reveals a Single Major QTL

(A) Expression levels of miR-146a in the parental lines (type I, GT1, and type II, ME49) and 27 F1 progeny were assessed by qRT-PCR. Data are displayed as fold difference relative to the uninfected cells. microRNA levels were normalized by RNU24 levels. Mean values and SDs from three independent experiments are shown.

(B) Plots indicate the log-likelihood association of expression of miR-146a (solid line) with markers aligned across the entire *Toxoplasma* genome in chromosome order. Significance levels determined by 3,000 permutations are indicated by horizontal lines (upper two lines are significant; lower line is suggestive).

(C) Fine mapping of the *Toxoplasma* chromosome VIIb locus involved in strain specific regulation of miR-146a. The *GRA15* locus on chromosome X is shown as a control. The QTL on VIIb is bound by genetic markers 55.m05046_at5 and 55.m010299_at3, which spans a region of 1.2 Mb including *ROP16*. The genotype for each of the F1 progeny at the locus is indicated by the boxes color (type I, yellow, and type II, red).

See also Figure S3.

controlled by a parasite-derived product, we took advantage of the aforementioned strain-specific difference to identify the responsible gene(s). If the strain-specific regulation of miR-146a has a genetic basis, it should accordingly segregate among F1 progeny derived from a cross between two strains (II versus I or III) that differ in its regulation. We therefore monitored miR-146a levels in fibroblasts infected separately with 27 F1 progeny derived from the I × II cross (Behnke et al., 2011). As expected, the parental type II (ME49-FUDR^{R}) strain induced significantly higher miR-146a levels compared to the type I (GT1-SNF^{R}) strain, and among the recombinant progeny a range of distinct phenotypes was observed (Figure 2A). Neither in vitro growth (data not shown) nor virulence reported previously (Behnke et al., 2011) were correlated with altered expression of miR-146a among the progeny (Figure 2B). A genome-wide scan for association of *Toxoplasma* genetic markers and the expression level of miR-146a identified a single high-probability quantitative trait loci (QTL) on chromosome VIIb with a high logarithm of odds score (LOD = 10, Figure 2C) that was associated with >92% of the inherited variation. Our data allowed us to restrict the locus involved to a region between the genetic markers *55.m05046_at5* and *55.m010299_at3*, representing a maximum size of 1.2 Mb spanning 200 predicted protein encoding genes (Figure 2B). Unexpectedly, we identified the *Toxoplasma* polymorphic ROP16 kinase as the most likely candidate (Figure 2B). ROP16 is a specialized phosphotyrosine kinase that is released from the rhoptries and injected into the host cell nucleus to activate STAT3/6 signaling pathways (Saeij et al., 2007). To strengthen our genetic analysis, we repeated the genome-wide scan using type II × III recombinant progeny (Khan et al., 2005) and validated the QTL containing ROP16 for the miR-146a phenotype (Figure S4). In addition, both genetic screens

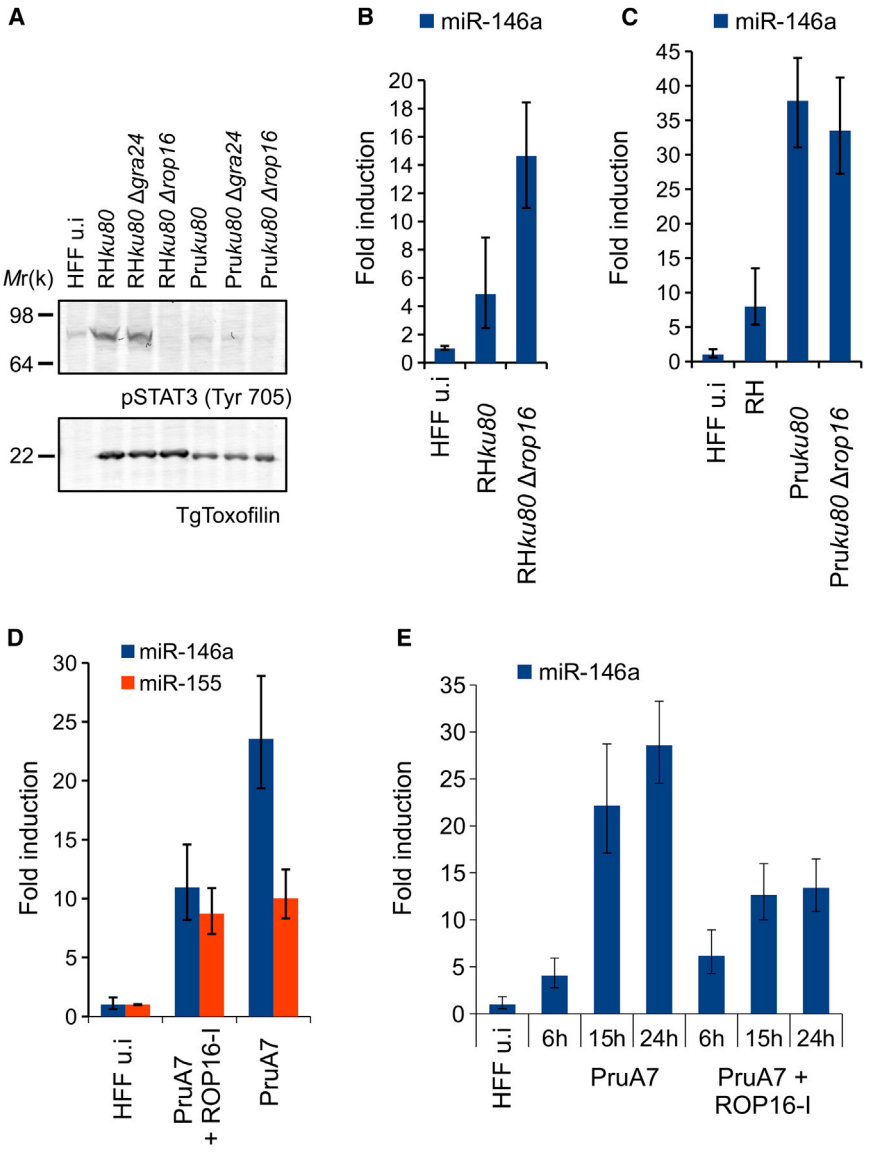


Figure 3. Suppression of miR-146a Expression Is Mediated by Type I ROP16

(A) Immunoblotting detection of phospho-STAT3(Y705) in nuclear cell lysates of HFF cells left uninfected (u.i.) or infected (24 hr) with the indicated strains. Toxofilin (parasite-specific) level is shown as loading control.

(B and C) qRT-PCR analysis of miR-146a levels in HFF cells left uninfected (u.i) or infected with (B) type I or (C) type II Δ*rop16* strains versus their parental strains. microRNA expression levels were normalized by RNU24 levels. Mean values and SDs from three independent experiments are shown.

(D) qRT-PCR analysis of miR-146a and miR-155 levels in HFF cells left uninfected (u.i) or infected with wild-type type II strain PruA7 or PruA7 ectopically expressing a type I allele of *ROP16*. microRNA expression levels were normalized by RNU24 levels. Mean values and SDs from six independent experiments are shown.

(E) qRT-PCR analysis of miR-146a, described in Figure 3D, was repeated during a 24 hr *Toxoplasma* infection time course. Mean values and SDs from two independent experiments are shown.

See also Figure S4.

confirmed that the type-II-dependent miR-146a induction was clearly GRA15 independent (Figures 2B and S4B).

ROP16 Suppresses miR-146a Expression in a Strain-Specific Fashion

To assess whether *ROP16* was directly involved in the strain-specific activation of miR-146a, we deleted the gene in both RHΔ*ku80* (type I) and PruΔ*ku80* (type II) strains (data not shown). In line with previous reports (Saeij et al., 2007), we showed that with parasites deleted for *ROP16* in type I (RHΔ*ku80*Δ*rop16*) the sustained STAT3/6 tyrosine phosphorylation was no longer observed (Figure 3A; data not shown). Deletion of *ROP16* also significantly enhanced the induction of miR-146a mediated by *Toxoplasma* (Figure 3B). In marked contrast, *ROP16* deletion in type II (PruΔ*ku80*) strain did not altered miR-146a levels (Figure 3C). Taken together, these data suggest that the kinase acts as a repressor of the expression of this specific miRNA. In this respect, ectopic expression of an additional type I allele of *ROP16* in the type II (Pru) strain (ROP16-I, Saeij et al., 2007) downregulated the level of miR-146a by 2-fold (Figure 3D). These data confirm that the type I allele of *ROP16* suppresses miR-146a expression, whereas the type II allele lacks this activity. Although ROP16 is released from the rhoptries in the early time of infection (1–6 hr postinfection, Saeij et al., 2007), it has a marked repressive effect on miR-146a beyond 15 hr of infection (Figure 3E), which is consistent with the ability of the kinase to sustain phosphorylation of STAT3/6 in the late phase of infection (Saeij et al., 2007).

miR-146a and miR-155 Are Highly Induced in Mice Brain Chronically Infected with Type II Strains

Because the induction of miR-146a in stromal cells was restricted to infection by type II strains, which exhibit low virulence and are cystogenic in mice, we further assessed whether this microRNA might play a critical role in chronic toxoplasmosis. Accordingly, we monitored the levels of eight microRNAs that were upregulated (miR-155 and miR-146a), downregulated (miR-150, miR-101, let-7b, miR-29a, and miR-125b), or unchanged (miR-146b and miR-132) following *Toxoplasma* infection of stromal cells (Figures 1A and S1A). We assessed miRNA levels in the brain of mice infected with a dose of parasites that allows survival through acute infection (<14 days) and establishment of chronic infection (Figure 4A). Surprisingly, miR-146a and miR-155 levels increased

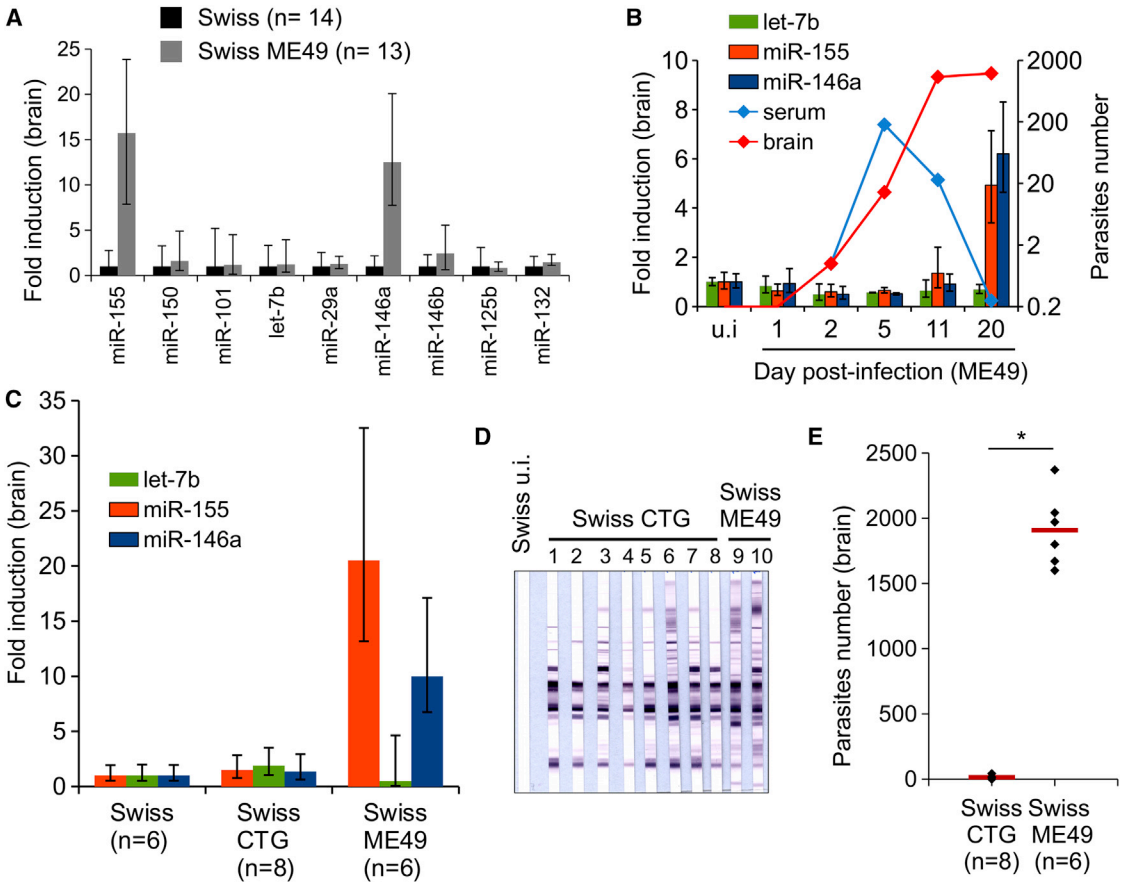


Figure 4. Evidence for a Typical microRNA Fingerprint in Cyst-Containing Brains of Mice Chronically Infected by Type II *Toxoplasma* Strains

(A) qRT-PCR analysis of selected microRNA levels in whole brains of Swiss mice left uninfected (n = 14 mice) or chronically infected (6–10 weeks postinfection) by 30–80 cysts of type II (ME49) strain (n = 14 mice) by intraperitoneal route. miRNA expression levels were expressed as normalized values using U6 snRNA as the endogenous control. Mean values and SDs from three independent experiments are shown.

(B) Parasite burden and miRNAs expression were determined in wild-type Swiss mice intraperitoneally infected with a dose of 10^6 ME49 tachyzoites. Parasites were enumerated by qPCR in the serum and from brain homogenates by qPCR at the indicated times after infection. The expression of the aforementioned microRNA was assessed in the brain using qRT-PCR. miRNA expression levels were expressed as normalized values using U6 snRNA as the endogenous control. Mean values and SDs from two animals in each group are shown.

(C) miRNA expression levels were assessed by qRT-PCR in brains of Swiss mice chronically infected by a type III (CTG) or a type II (ME49) *Toxoplasma* strains (10^5 tachyzoites by intraperitoneal route). miRNA expression levels were expressed as normalized values using U6 snRNA as the endogenous control. Mean values and SDs from six to eight animals in each group are shown.

(D) Serological status by western blot analysis of mice left uninfected or infected as mentioned in Figure 4C.

(E) Parasite burden in the brain of infected Swiss mice (six to eight animals/group) described above. *Toxoplasma* DNA PCR assay was performed with brain tissue sampled from mice at 7 weeks postinfection. *Significantly different ($p < 0.05$, Student's t test).

See also Figure S5.

in the brain of chronically infected mice in a sustained fashion (6–10 weeks postinfection [p.i.]), regardless of the genetic background of the mouse host (i.e., C57BL/6, BALB/c or Swiss) or the type II parasite used (i.e., Pru A7, PruΔ*ku80*, or ME49) (Figures 4A, S5A, and S5B). This miRNA signature is not a broad response to the mere presence of parasites in the brain (detected 11 days p.i.), but rather correlates well with the presence of fully developed cysts in the brain (>20 days p.i., Figure 4B) that persist throughout the life of the chronically infected mice (up to 10 weeks p.i.) (Figure 4A). In contrast, the relative levels of miR-155 and miR-146a are distinctly low in the brains of mice chronically infected with the mouse-avirulent type III (CTG) strain (Figure 4C); yet, this strain causes nonlethal chronic latent infection characterized by a proper positive serology (Figure 4D). The main difference with ME49 infection was that mice chronically infected with CTG had a low number of cysts in their brain (Figure 4E), suggesting a likely relationship between cyst burden and the alteration of miR-155 and miR-146a levels in the mice brain (Figures 4C and 4E). In this fashion, the high levels of miR-146a in mouse brains with high cyst burden are consistent with the type II strain-restricted regulation of the microRNA in stromal cells. Surprisingly, miR-155, while being induced in the brain only when infected by type II strains (Figures 4A, S5A, and S5B), has a strain-independent expression profile in stromal (Figure 1C)

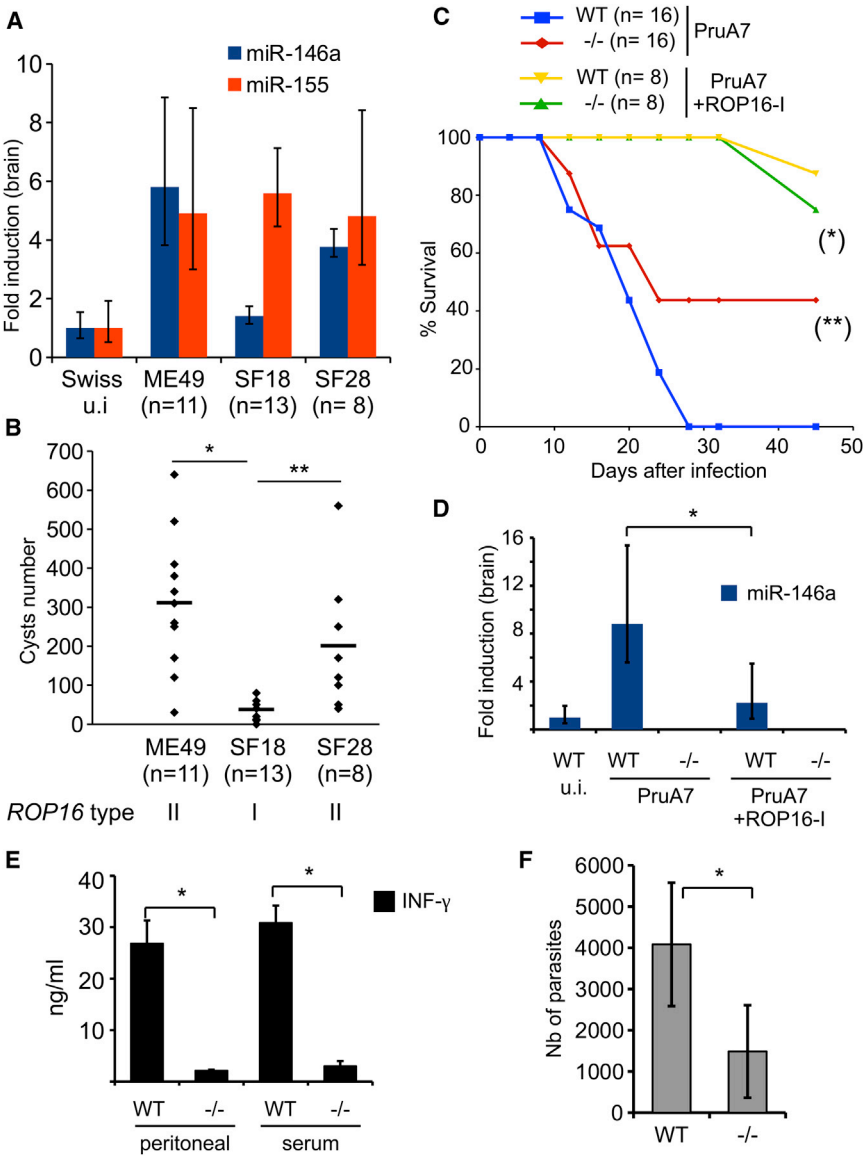


Figure 5. miR-146a Expression Affects the Control of *Toxoplasma* In Vivo

(A and B) Chronic cyst burden and microRNA expression were assessed in Swiss mice left uninfected or infected by the parental strain ME49 (n = 11) and F1 I × II progeny strains SF18 (n = 13) and SF28 (n = 8) using 10^5 tachyzoites and intraperitoneal route. (A) qRT-PCR analysis of miR-146a and miR-155 levels were assessed in mice brains. microRNA expression levels were normalized by U6 snRNA levels. Mean values and SDs from eight to 13 animals in each group are shown. (B) Cysts were enumerated by microscopy. *, **Significantly different ($p < 0.05$, Student's t test).

(C) C57BL/6 or C57BL/6 *miR-146a*$^{-/-}$ mice were intraperitoneally infected with a dose of 5×10^2 tachyzoites of PruA7 or PruA7+*ROP16*-I. Survival was monitored. Significance was tested using log-rank (Mantel-Cox) test and Peto & Peto modification of the Gehan-Wilcoxon test, *$p = 8.13 \times 10^{-6}$ and **$p = 4.31 \times 10^{-5}$ when compared to BL6 WT infected by PruA7 WT parasite strain.

(D) qRT-PCR analysis of miR-146a levels were assessed in the brain of C57BL/6 WT and *miR-146a*$^{-/-}$ mice infected with PruA7 or PruA7+*ROP16*-I and compared to those of uninfected C57BL/6 WT mice. miRNA expression levels were expressed as normalized values using miR-U6 as the endogenous control. Mean values and SDs from three independent experiments are shown.

(E) Peritoneal lavage fluid and serum were collected on day 4 after infection of C57BL/6 or C57BL/6 *miR-146a*$^{-/-}$ mice that had received an intraperitoneal (i.p.) dose of 10^3 PruA7 tachyzoites. Concentrations of IFN-γ were determined by ELISA. Data shown are means ± SD with n = 3 individual mice per mice genotype. Error bars represent SD from one experiment. *Significantly different from WT ($p < 0.05$, Student's t test).

(F) WT and *miR-146*$^{-/-}$ C57BL/6 mice were i.p. infected with 10^3 PruA7 tachyzoites. Parasites were enumerated using recovered i.p. contents at day 4 postinfection. Data shown are means ± SD (from three mice per mouse genotype). *Significantly different from WT ($p < 0.05$, Student's t test).

See also Figure S5.

and phagocytic cells infected in vitro (Figure S2F). This suggests that the miRNA fingerprint that marks cystogenesis in chronically infected mice has multiple strain-dependent and -independent determinants.

miR-146a belongs to a large family of circulating microRNA that cofractionates in plasma and serum with exosomes (Arroyo et al., 2011). We also provide evidence that miR-146a copurifies with exosome vesicles isolated from supernatant of *Toxoplasma*-infected cells (Figures S5C–S5E) or brain (data not shown), suggesting that the cell hosting the parasite might not be the only cell where miR-146a is operating as a microRNA.

Cyst Burden Promotes miR-155 and miR-146a Induction in the Brains of Chronically Infected Mice

Notwithstanding the ability of both type II and III strains of *T. gondii* to establish latent infections, our data strongly indicate that the induction of miR-146a or miR-155 was restricted to mice with high chronic cyst burdens (Figure 4). This potentially raises the question of whether ROP16 might be involved in this process, i.e., whether the kinase, by suppressing miR-146a induction (Figure 3) may contribute to lessen the cyst burden in the brain. We therefore monitored the course of chronic toxoplasmosis in mice infected with either ME49 or SF18 (*ROP16-I*) and SF28 (*ROP16-II*), two mouse-avirulent F1 progeny derived from the type I × II cross (Behnke et al., 2011) that were initially characterized as low and high inducers of miR-146a expression in stromal cells (Figure 2A). Our aim was to assess whether the ability of ROP16 to reduce miR-146a levels in the brain might impede or promote cystogenesis. We first confirmed that SF28 differs from SF18 by a strong induction of miR-146a in the brain (Figure 5A), thus mimicking the phenotype seen in stromal cells (Figure 2A) whereas miR-155 was equally induced by both strains

and at the same level as the parental ME49 strain (Figure 5A). Moreover, mice infected with the SF18 strain (*ROP16*-I allele) had markedly reduced cyst burden in the brain, compared with mice infected with ME49 (type II control) or SF28 (*ROP16*-II allele) strains (Figure 5B). These results mirror those using infection of mice with a type III strain that was typified by a low number of cysts and no induction of miRNAs whatsoever in the brain (Figures 4C and 4E).

miR-146a Ablation Promotes Host Resistance to *Toxoplasma* Infection in Susceptible C57BL/6 Mice

miR-146a expression, while being efficiently induced by *Toxoplasma*, was shown to be persistently suppressed by strains expressing type I or III *ROP16* alleles (Figures 4C and 5A). Given the established role of the ROP16 in generating innate resistance to oral challenge (Jensen et al., 2013), we decided to explore whether miR-146a influences host resistance during infection. As reported before, challenge of C57BL/6 mice with a transgenic type II strain expressing *ROP16*-I lead to better survival compared to a control infection with the parental type II Pru A7 strain (Figure 5C). To assess whether miR-146a was also required for host protection, we challenged *miR-146a*$^{-/-}$ knockout mice (described in Boldin et al., 2011) with 5×10^2 parasites of PruA7, and their survival was compared with that of C57BL/6 control mice. *miR-146a*$^{-/-}$ mice infected with the PruA7 WT strain were more resistant as evidenced by the 40% survival during the acute and chronic state of infection (50 days p.i., Figure 5C). However, this did not fully recapitulate the phenotype of the type II (PruA7) strain expressing *ROP16-I* (80% survival of challenged mice), thereby indicating that additional ROP16-regulated genes might contribute to promote host resistance to *Toxoplasma* infection in susceptible C57BL/6 mice. Histopathological analysis of brains showed that *miR-146a*$^{-/-}$ mice challenged with PruA7 developed a mild meningoencephalitis, whereas WT mice had a more severe toxoplasmic encephalitis (TE) characterized by the presence of significant numbers of parasites and areas of necrosis (data not shown).

Possibly, *miR-146a*$^{-/-}$ mice challenged with PruA7 survived longer to TE than WT C57BL/6 mice because of changes in the inflammatory response to the parasite. Indeed, amounts of IFN-γ were significantly lower in sera and peritoneal exudates of infected *miR-146a*$^{-/-}$ mice compared to their WT littermates (Figure 5E). However, levels of other proinflammatory cytokines like tumor necrosis factor (TNF)-α, IL-12, IL-1β, and IL-6 did not change significantly (data not shown). In vitro assay using tail-derived fibroblasts confirmed the above in vivo experiment and showed that *Toxoplasma* (PruA7) infection failed to induce *IFN-γ* gene transcription in *miR-146a*$^{-/-}$ tail-derived fibroblasts (Figure S5G). The early difference in IFN-γ production correlates well with high parasite burden in peritoneal exudates of WT animals (Figure 5F), which subsequently may affect parasite dissemination, load in the brain, and, ultimately, survival. It is noteworthy that infection by a type II strain expressing a *ROP16-I* allele led to a similar attenuation of *IFN-γ* mRNA expression to what observed in the absence of *miR-146a* (Figure S5G). Overall, these data indicate that miR-146a is required for full inflammatory response in mice during toxoplasmosis.

DISCUSSION

As obligate intracellular parasites, apicomplexans deploy sophisticated mechanisms to profoundly reorganize the host cell for their own needs. miRNAs were recently deemed as important components of gene regulatory networks that play critical roles in fine-tuning the host response to infection by several parasites of the phylum (Hakimi and Cannella, 2011). Here, we provide further evidence that the alteration of the microRNome mediated by apicomplexan infection, both in terms of the repertoire and the magnitude, is different from one parasite to another. Remarkably, we uncovered a reservoir of host genetic riboregulators whose expression was tightly regulated by *Toxoplasma* infection. We thus showed that *Toxoplasma* infection increased the levels of miR-146a and miR-155 in the brains of mice infected by type II strains, which are competent to form cysts in the neural tissue. In this regard, *miR-146a*$^{-/-}$ ablation did not alter miR-155 levels in the brain of mice challenged with type II strain, suggesting that miR-155 induction in this tissue is miR-146a independent (Figure S5F). It is worth mentioning here that the levels of miR-146b were neither influenced by *Toxoplasma* infection (Figure 1D) nor by the ablation of its paralog *miR-146a* (Figure S5F), which illustrates the uniqueness of this RNA signature that typifies type II parasite persistence in the host.

Both miR-155 and miR-146a are regulated by the transcription complex NF-κB, a component of a well-studied antipathogen pathway. The increase in the levels of miR-146a and miR-155 is not, however, a broad NF-κB-dependent inflammatory response to *Toxoplasma* infection. Several arguments support this assertion. First, the prominent upregulation of their activation was only evident at day 20 p.i. when the cysts were readily detectable and persisted in the chronic phase (Figures 4A and 4B). Second, other microRNAs whose expression was shown to be dependent on NF-κB pathway activation, e.g., miR-132 (Taganov et al., 2006) and miR-125b (Zhou et al., 2009), were not induced in the brain of chronically infected mice (Figure 4A). Finally, *Plasmodium berghei* ANKA strain, which causes cerebral malaria, selectively increases the levels of miR-150 but not those of miR-155 in the brain of CBA mice p.i. (El-Assaad et al., 2011), in quite the opposite fashion to infection by *Toxoplasma* (Figure 4A), suggesting that each apicomplexan infection is associated with a conserved, disease-specific signature of differentially expressed miRNAs.

We have clearly shown that the timing of miR-155 and miR-146a induction in the mice brains coincides with the presence of "mature" cysts and not with the mere occurrence of parasites (Figure 4B), suggesting that bradyzoite-specific effector(s) might operate in the host cell to regulate their levels. Meanwhile, we provided strong evidence that GRA15, while directly modulating the NF-κB pathway (Rosowski et al., 2011), did not alter the levels of miR-146a, nor of miR-155 (Figure S3). Another candidate dense granule protein, GRA24, which directly activates the MAPK p38α (Braun et al., 2013) pathway that interfaces with NF-κB, did not appear to be associated with the phenotype (data not shown).

Ablation of *miR-146a* expression in mice seems to play a role in the control of early proliferation of *Toxoplasma* in the gut, which was associated with a significant decrease in IFN-γ levels

in sera and peritoneal contents postinfection (Figures 5E and 5F). These early inflammatory events may lead to lower CNS colonization and cyst burdens in *miR-146a*$^{-/-}$ mice, which result in a less severe encephalitis and delayed time to death as shown in Figure 5C. Therefore, the higher survival rate of *miR-146a*-deficient mice might be more likely a consequence of early differences in parasite burden rather than a direct effect of miR-146a action in the brain.

Nevertheless, infection by type II strains, that are prone to cyst formation, systematically causes a significant increase in miR-146a levels in mice brain. However, our results do not reveal the primary mRNA target of the microRNA in the cyst-containing brain tissue. This could involve something akin to what has been reported in Treg cells, where miR-146a acts as a negative-feedback regulator to restrain IFN-γ-mediated pathogenic Th1 responses by targeting directly STAT1 (Lu et al., 2010). IFN-γ is the central cytokine in resistance against *Toxoplasma* during both the early and late stages of the infection. However, excessive or inappropriate activation of the IFN-γ pathway can be deleterious to the host. Thus, it appears that overproduction of IFN-γ in the genetically susceptible mice following *Toxoplasma* infection can lead to tissue destruction in small intestine and liver (Liesenfeld et al., 1999). *Toxoplasma* has developed means to prevent excessive host damage due to this cytokine. In this respect, SOCS-1 is induced during *Toxoplasma* infection and contributes to the inhibition of IFN-γ signaling by the parasite (Zimmermann et al., 2006). miR-146a may represent an alternative regulatory mechanism to fine-tune the IFN-γ-mediated inflammatory response throughout the chronic infection in mouse. In this model, miR-146a could directly target STAT1 protein translation or alternatively its upstream negative regulators like SOCS-1, the latter being previously identified as a downstream target of its coregulated counterpart, miR-155 (Cardoso et al., 2012).

In this study, we discovered a characteristic microRNA fingerprint typified by miR-146a and miR-155 that might be involved in the establishment of latent *Toxoplasma* infection through the buffering of the immune response in the brain of the infected host. Part of the variability of disease outcome in human infections may also be tied to this strain-specific microRNA signature. In North America and Europe, most human toxoplasmosis cases are mainly due to type II strains that exhibit a typical miR-146a/miR-155 signature in mice models. In contrast, type III strains that are more common in animals and rarely associated with human disease failed to induce these microRNAs in mice brain. Therefore, it will be important from a clinical point of view to establish if the aforementioned microRNA signature plays a role in determining severity of toxoplasmosis in humans.

EXPERIMENTAL PROCEDURES

Mouse and Experimental Infection

Six-week-old Swiss OF1 and BALB/cJRj or 9-week-old C57BL/6JRj mice were obtained from Charles River Laboratories. Nine-week-old female B6(FVB)-Mir146tm1.1Bal/J (http://jaxmice.jax.org/strain/016239.html) and their wild-type littermates (http://jaxmice.jax.org/strain/000664.html) were obtained from The Jackson Laboratory. Mouse care and experimental procedures were performed under pathogen-free conditions in accordance with established institutional guidance and approved protocols from the Institutional Animal Care and Use Committee. The infection protocols are detailed in Supplemental Experimental Procedures.

T. gondii Transfection

Parasites were transfected using electroporation parameters established previously (Braun et al., 2013).

Western Blot Analysis and Immunofluorescence Microscopy

Immunofluorescence assays and western blots were performed as described (Braun et al., 2013) using antibodies listed in Supplemental Experimental Procedures.

Cell Fractionation

Cell fractionation was detailed in Supplemental Experimental Procedures.

MicroRNA Microarray Analyses

HFF cells were left uninfected or infected (MOI = 3) for 6–18 hr with RH or ME49 tachyzoites. RNA was isolated using TRIzol reagent (Invitrogen), followed by phenol:chloroform:isoamyl extraction. Total RNAs were profiled by Exiqon (http://www.exiqon.com) using miRCURY LNA arrays. Further processing of RNAs, hybridization, and analysis of microarray data are in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.02.002.

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