MicroRNA 155 Contributes to Host Immunity Against *Toxoplasm Gondii*

Yu-Chao Zhu
Affiliated Hospital of Medical School Ningbo University and Ningbo City Third Hospital
Chou-Jian Qiu
Ningbo Yinzhou No 2 Hospital
Ya-Nan Xu
Ningbo Women and Children's Hospital
Chun-Xue Zhou
Shandong University
Jia Chen (chenjia@nbu.edu.cn)
Affiliated Hospital of Medical School Ningbo University and Ningbo City Third Hospital

Research

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Abstract

Background: Toxoplasma gondii is known to infect almost all the mammalian including human beings and avian species, with worldwide distribution, and cause serious toxoplasmosis, posing regards with public health problem. The role of microRNAs in the pathogenesis of T. gondii has not been well described. The objective of the present study was to investigate the role of microRNA-155 (miR-155) in mediating innate and adaptive immune responses during T. gondii infection.

Methods: The survival and parasite burden in T. gondii-infected miR-155-/- and WT C57BL6 mice were compared. In these two mouse models, ELISA were used for analysis of Th1-associated, Th-2 associated, and Th-17 associated cytokines, and flow cytometry were used for analysis of the subpopulations of NK, NKT, $CD8^+$T, $CD4^+$T cells and Tregs, as well as Ly6Chi inflammatory monocytes and DCs. Proinflammatory mediators and $CD8^+$ T cells responses were also analyzed by using qRT-PCR and flow cytometry, respectively. In the end, the expression of the direct target of miR-155, SHIP-1 and SOCS1 was analyzed by using qRT-PCR.

Results: The lack of miR-155 led to increased parasite burden and decreased survival of infected mice in contrast to WT mice. Innate and adaptive immune responses were reduced in the absence of miR-155, associated with diminished Proinflammatory mediators, Th1-associated and Th-2 associated cytokines and accumulation of lymphocyte subpopulations. Also, $CD8^+$ T cells exhaustion was also worsened in the absence of miR-155 via targeting to SHIP-1 and SOCS1, showing as up-regulated recruit of Tregs and expression of PD-1 and, and down-regulated expression of IFN-γ and TNF-α in $CD8^+$ T cells.

Conclusion: miR-155 is a critical immune regulator for the control of T. gondii infection, suggesting that miR155 can be explored as a potential molecular target for boosting immunity against T. gondii.

Background

As the causative agent of toxoplasmosis, *Toxoplasma gondii* can infect all warm-blooded animals, and has infected approximately 30% of the world's population throughout the world [1, 2]. Primary infection in immune-potent individuals is usually asymptomatic or presented as a mild, flu-like illness, concomitant with parasite conversion to dormant bradyzoite within a tissue cyst [3]. While severe toxoplasmosis in immunocompromised individuals could develop following reactivation of bradyzoites into disseminating tachyzoites, leading to toxoplasmic encephalitis (TE), eye disease, neurological problems and even death [2, 3]. Congenital infection can occur in the newborns resulting from the infection of pregnant women, associated with dysplasia, hydrocephaly and chorioretinitis [4]. *Toxoplasma gondii* infection can also cause large economic losses to the stock-raising industry [2]. Despite *T. gondii* acute infections could be controlled by medications, there are still no effective medications can be used for completely eliminate the chronic infection, and more effective chemotherapeutic agents are needed for this organism [5]. Additionally, there are no clinical licensed vaccines available for human infection [6]. Disclosing the immune response to *T. gondii* is vital for the design of effective vaccines and also drug targets.

It is well documented that the immune response in *T. gondii* infection is complex, including innate and adaptive immune response, which involves various immune cells, such as $CD4^+$ and $CD8^+$ T cells, natural killer (NK) cells, dendritic cells (DCs), macrophages and neutrophils [7]. $CD8^+$ T cells and their responses are essential for the control of infection by acting synergistically to $CD4^+$ T cells [8]. Primarily, T helper 1 (Th1) cell-mediated protective immunity to *T. gondii* drive the production of high levels of interleukin-12 (IL-12) and interferon-γ (IFN-γ) following by the migration of innate immune cells (e.g. DCs, macrophages and neutrophils) to the site of infection, which are indispensable for host resistance against *T. gondii*, and thus limit the parasite's proliferation and the progression of infection via multiple intracellular mechanisms with the production of various antiparasitic factors [9, 10]. The expansion of Th2 cells and those anti-inflammatory cytokines production of IL-4, IL-10, IL-13, IL-27, and transforming growth factor-β (TGF-β) are responsible for controlling the effects of excessive immune activation and preventing the immunogic pathology [10]. Collectively, these innate and adaptive immune responses, mainly Th1- and Th2-associated cytokines are contributed to protective immunity to *T. gondii* infection.

MicroRNAs (miRNAs), as class of non-coding RNAs, are involved in gene regulation at both transcriptional and post-transcriptional levels [11]. Through several mechanisms including translational repression and messenger RNA (mRNA) degradation, they have been shown to mediate and regulate some physiological processes and also to be related to human disease, such as carcino-genesis, and even extended to the immune system [12, 13]. It has been revealed that miRNAs can regulate the immune system biology, including both lymphocyte development and function and host immune responses, which involved in inflammation associated with $CD4^+$ T cell differentiation and $CD8^+$ T cell responses [13]. In particularly, miRNA-155 is a well-characterized miRNA, which is shown as a chief regulator of monocytes, macrophages, T-cells and B-cells by interference the expression of pro-inflammatory and anti-inflammatory cytokines [14, 15]. MiR-155 has a regulative role in visceral leishmaniasis by up-regulating of both Th1 and Th2 immune responses, which is contributed to the control of the infection [16]. MiR-155 has been demonstrated to be an important immune regulatory molecule critical for the control of *Trypanosoma cruzi* infection [17]. Likewise, miR-155 is ascertained to play a critical role in maintaining the survival of Mtb-infected macrophages and the function of Mtb-specific T-cells during Mycobacterium tuberculosis infection [18].

Despite the fact that miR-155 showed an elevated level in chronic *T. gondii* infection [19], but its specific role in involved in the innate and adaptive immune responses against this infection has not been uncovered. To determine if miRNA-155 influences the progression of *T. gondii* infection and immune responses in mice models, we have investigated the role of miRNA-155 during immunity to *T. gondii* infection in miR155 gene-deficient mice and those of their age- and sex-matched wild-type (WT) counterparts. Our results show that miR155 is required for control of *T. gondii* infection via mediating a wide spectrum of immune compartments.

Materials And Methods

Mice and parasite

Age-matched 6–8 week old female miR155KO C57BL/6 and WT C57BL/6 mice were purchased from purchased from Beijing Vital River Laboratory Animal Technology Co., China. All mice were maintained and bred in strict accordance according to the Animal Ethics Procedures and Guidelines of the People's Republic of China. Animal experiments were approved by the ethical committee of Ningbo University (permission: SYXK(ZHE)2019-0005).

The PRU strain (Type II) of *T. gondii* were used for the in vivo challenge of mice, which were propagated and harvested as described in our previous studies [20]. The tachyzoites-forming of PRU strain (Type II) of *T. gondii* were also used for preparation of soluble tachyzoite antigens (TLA) as previously described [20, 21].

Cytokine ELISA

According to our previously described method [20, 21], splenocytes were prepared by push the spleens through a wire mesh, and then purified by removing the red blood cells using RBC erythrocyte lysis buffer, and then re-suspended in DMEM medium supplemented with 10% FCS, 1% penicillin- streptomycin, and 1% HEPES. Following by plated at a concentration of 5×10^6 cells/ml and stimulated for 72 h with 15 μg /ml TLA, cell-free supernatants were collected and assayed for IL-2 and IL-4 at 24 h, for IL-22 activity at 48 h, for IL-13, IL-17A, IL-17F activity at 72 h, and for IFN-γ activity at 96 h using commercial ELISA kits according to the manufacturer's instructions (Biolegend, USA).

Flow cytometry

Single-cell suspensions were prepared according to the method mentioned above. Followed by blocking of FC receptors with addition of normal mouse serum, cells were stained with the following antibodies: CD3(BV421, clone: 145-2C11, BD Biosciences), CD4(BV510, clone: RM4-5, BD Biosciences), CD8(PE, clone: 53 – 6.7, BD Biosciences), NK1.1(PE-CY7, clone: PK136, BD Biosciences), CD11b (BV605, clone: M1/70, BD Biosciences), Ly6C (APC, clone: 560595, BD Biosciences). For intracellular cytokine staining (ICS) assays, prior to intracellularly stained with IFN- and (AF647, clone: XMG1.2, BD Biosciences) for 30 min at 4 ◦C, the cells were surface-stained CD8-PE (BD Biosciences), fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) for 20 min in dark, and then the data were acquired through performing these cells on a Beckman CytoFLEX S and were further analyzed by FlowJo software (Tree Star, Inc., Ashland, OR, USA).

qRT-PCR

The expression of IL-1α, IL-1 β, IL-6, SHIP-1 and SOCS1 were analyzed by qRT-PCR. Total RNA was isolated from three purified splenocytes of mice in each group by using Trizol reagent (Invitrogen, USA), as per the manufacturer's instructions. RNAs were dissolved in RNase-free ddH_2O (TaKaRa, China) and the cDNA was synthesized using a GoScript™ Reverse Transcription System (Promega, Madison, WI, USA), which were used as templates for quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR was performed using the Light Cycler 480 SYBR Green I Master (Roche, Switzerland). The primers

used for amplification are listed in Table 1. qRT-PCR analysis was performed on the Light Cycler 480 (Roche, Switzerland) and data were calculated using the comparative cycle threshold (CT) method ($2^{-\Delta CT}$).

Challenge and parasite burdens

A total of five mice in each group was challenged with 10 PRU tissue cysts of *T. gondii* PRU strain orally and the survival periods were recorded daily until all mice were dead. In the meanwhile, mean brain cyst loadings were counted at 4 weeks after the challenge, as described in our previous studies [20, 21]. All samples were counted in triplicate. The parasite reduction rate of brain cysts is relative to that of the control.

Statistical analysis.

Statistical analysis was conducted using GraphPad Prism 8. Student's unpaired *t* test was used to determine statistical significance of differences among the groups. P values < 0.05 were considered to statistically significant.

Results

miR155 deficiency enhanced the susceptibility to T. *gondii*

MiR-155 has recently been uncovered to be contributed to the resistance to the experimental parasitic diseases [16, 17]. To test if the lack of miR-155 would potentiate the chronic *T. gondii* in mice models, miR-155$^{-/-}$ mice were orally inoculated with 10 PRU tissue cysts. All of the miR-155$^{-/-}$ infected mice died after 17 days of infection, but WT mice infected with *T. gondii* showed a prolonged survival time (Fig. 1A). To further confirm the *T. gondii* infection in WT and miR-155$^{-/-}$ mice, the mean brain cyst loadings were counted. *T. gondii*-infected miR-155$^{-/-}$ mice showed more brain cyst (Fig. 1B). These data demonstrate that the absence of miR-155 enhance susceptibility to *T. gondii* in mice models.

miR155 deficiency impairs both Th1 and Th2 immune responses in the spleens of *T. gondii*-infected mice.

As miR-155 has been shown to be critical for regulating T cell responses as well as inflammatory responses and cytokine signals, which have the well-documented roles in the control of intracellular pathogens, including *T. cruzi* and *Leishmania donovani* [16, 17], we further analyzed the production of cytokines Th1-associated IFN-γ, IL-2, Th2-associated IL-4 and IL-13, Th17-associated IL-17A and IL-17F by splenic cells harvested from WT and miR155KO (miR-155$^{-/-}$) mice after infection with *T. gonii* PRU cysts.

After ELISA analysis of TLA-stimulated spleen cells supernatants, it has been shown that the production of IFN-γ and IL-2 was significantly reduced in *T. gondii*-infected miR-155$^{-/-}$ mice compared to WT

controls (Fig. 2). Likewise, the IL-13 production by splenic cells from *T. gondii*-infected miR-155$^{-/-}$ mice were dramatically decreased in contrast to WT controls (Fig. 2). However, there were no significant changes of the levels of IL-4, and IL-17A and IL-17F in these *T. gondii*-infected miR-155$^{-/-}$ mice and WT controls (Fig. 2). Taken together, these data suggest that this *T. gondii* infection in miR-155$^{-/-}$ is due to the lower protective cytokines production.

Lack of miR155 contributes to the impaired recruitments of CD4$^+$ T, CD8$^+$ T, NK and NK-T cells in the spleens of *T. gondii*-infected mice.

NK cells, NK-T, and T cells are usually recruited to kill *T. gondii* by hosts through the production of IFN-γ and the perforin-independent mechanism [10, 22]. To investigate whether the miR155 deficiency has an effect in these immune cells recruitment, the splenic cell population of CD4$^+$ T cells, CD8$^+$ T cells, NK cells and NK-T cells were analyzed from *T. gondii*-infected miR155$^{-/-}$ and WT mice by flow cytometry. We have found that these indicated splenic cell populations of *T. gondii*-infected miR-155$^{-/-}$ mice have been significantly decreased in contrast to WT counterparts (Fig. 3). These results suggest that miR155 plays a role in mediating recruitment of NK, NK-T cells, CD4$^+$ T cells and CD8$^+$ T cells, that control the *T. gondii* infection, while these decreased recruited cells in miR-155$^{-/-}$ mice could not activate the protective immunity effectively, leading to the subsequent aggravated parasitic infection and even death.

miR155 deficiency exacerbated CD8$^+$ T cell exhaustion

Since miR-155 have recently demonstrated to restrain CD8$^+$ T cell functional exhaustion in chronic virus infection and tumor, through targeting to several inhibitors of cytokine signaling, including SOCS1, SHIP1 [23, 24]. Thus, to better understand the role of miR-155 on CD8$^+$ T cell exhaustion during chronic infection with *T. gondii*, we analyzed the expression of IFN-γ, TFN-α and PD-1 in CD8$^+$ T cells and the number of regulatory cells (Tregs) by flow cytometry (Fig. 4). As we expected, miR155KO mice significantly decreased the numbers of IFN-γ CD8$^+$ and TNF-α CD8$^+$ T cells in contrast to WT mice, suggesting a decreased effector T cells in miR155KO mice (Fig. 4). In the meanwhile, the number of Tregs were also up-regulated by *T. gondii*-infected miR155KO mice, suggesting the enhanced immune-inhibitory effects followed by the deficient expression of miR155.

It is known that PD-1 expression by the myeloid cell population plays an important role in immune regulation in various infectious diseases as well as in several cancer models [25–27]. Since PD-1 has been implicated in suppressing T cell response in *T. gondii* [28], flow cytometric analysis of the expression PD-1 in CD8$^+$ T cell revealed that *T. gondii*-infected miR155KO mice up-regulated the expression PD-1 in CD8$^+$ T cell in contrast to *T. gondii*-infected WT mice. Furthermore, miR155KO mice showed increased expression of both SHIP-1 and SOCS1 by qRT-PCR (Fig. 4), which are known to be direct targets of miR155 [23, 24]. Together, these findings indicate that miR155 deficiency contribute to the increased T cell exhaustion by suppressing T cell responses via targeting to SHIP-1 and SOCS1.

miR155 deficiency decreased accumulation of splenic inflammatory monocytes and DCs and expression of pro-inflammatory mediators.

Recent studies have established the critical role of $CD11b^+Ly6C^+$ cells and DCs in *T. gondii* infection [29, 30]. Flow cytometric analysis of phagocyte populations has revealed that *T. gondii*-infected miR-$155^{-/-}$ mice contained significantly lower numbers of $CD11b^+Ly6C^+$ cells and DCs than their WT counterparts (Fig. 5). In the meanwhile, we further determined whether decreased numbers of $CD11b^+Ly6C^+$ cells and DCs in miR155KO mice consequently impaired the expression of pro-inflammatory mediators by Flow cytometric analysis. In consistent with decreased numbers of $CD11b^+Ly6C^+$ cells and DCs, the expression of IL-1α, IL-1β and IL-6 was significantly reduced in contrast to their *T. gondii*-infected WT counterparts (Fig. 5). These data suggest that miR155 deficiency leads to the decreased accumulation of DCs and $Ly6C^+$ inflammatory monocytes, in combination with reduced production of pro-inflammatory mediators could contribute to high parasitic burdens in miR155KO mice.

Discussion

It is well known that IFN-γ and IL-4-associated signaling pathways are desired immunological responses for defending against *T. gondii* infection [7]. In particually, releasing of Th1 type cytokines, IL-12, IL-2 and IFN-γ are essential for the development of T cell immunity against *T. gondii* infection [22]. Besides, T helper type 2 (Th2) cells-associated cytokines, such as IL-4, IL-10 and IL-13, which also play an important role in coordinating the immune response by dampening the systemic Th1 type cytokine production and thus prevents lethal immunopathology [8]. Th17-associated immune responses are also shown as significant protective immunity against *T. gondii* infection [31]. Despite miR155 has been shown to be able to regulate the development and activity of Th1, Th12, and $CD8^+$ T cells, its specific role in immunity against *T. gondii* infection has yet to be well studied. In our study, we have established that miR155 contributes to host immunity against *T. gondii* infection through the regulation of Th1 and Th2 immune responses, but it showed no any effect in Th17-associated immune responses.

Some recent studies have shown the important role of microRNAs during the infection [23, 24, 32]. In our study, it has been revealed that lack of miR155 led to a significant increase in parasitic brain cysts and decrease survival time. Since miR155 has been shown as the key regulator of IFN-γ production via the targeting of SOCS1 [33], it is not surprise that this regulative effect should be ascribed to the inability of miR-$155^{-/-}$ mice to mount an efficient Th1 immune response, with the reduced production of IFN-γ and IL-2. Besides, we have observed a decrease in Th2 immune responses in miR-$155^{-/-}$ mice, which is similar to a previous study in vitro and in *L. donovani*-infected miR-$155^{-/-}$ mice [16], indicating a potential role of miR155 that it is beneficial for the generation of optimal Th2 immune responses against *T. gondii* infection. These data demonstrate that miR155 plays a significant role in regulating both Th1 and Th2 immune responses during *T. gondii* infection.

MiR-155 regulates the activation of several immune subpopulations including $CD8^+$T cells, as well as NK and NKT cells [34, 35], which is critical for immunity against *T. gondii* infection [31]. Previous studies have found that miR-155 is important in control of the *Leishmania donovani* and *T. cruzi* infection by mediating the regulation of T-cell proliferation and thus the activation of $CD8^+$ T cells, NK and NKT cells [16, 17]. In support with these studies, our data indicate that those decreased recruitment of $CD8^+$ T cells, NK and NKT cells resulting from the absence of miR-155 expression, can lead to the lack of control of parasite infection in the miR-$155^{-/-}$ mice.

As the first line cell types in the initial stages of infection, inflammatory monocytes, especially $Ly6C^+$ monocytes are necessary to govern the control of chronic infection with *T. gondii* in mice [29], through the inductive production of proinflammatory mediators, such as IL-1α, IL-1β, IL-6, inducible NO synthase, TNF, and reactive oxygen intermediate. Additionally, DCs are essential for the expansion and subsequent T cell priming and activation of Ag-specific $CD8^+$ T cells during infection with *T. gnodii* [30]. Our studies have shown decreased accumulation of $Ly6C^+$ inflammatory monocytes and DCs in miR155KO mice, associated with a significantly increased parasite load and with the reduced expression of those proinflammatory mediators. However, it is contradictory that aberrant miR-155 expression has recently been shown to adversely affect $Ly6C^+$ inflammatory monocytes migration in *L. donovani* and *T. cruzi*, which is due to a fact that inflammatory monocytes facilitate the growth of the parasites in the spleen in visceral leishmaniasis and trypanosomosis [16, 17]. These results demonstrate that miR155 deficiency exacerbated *T. gondii* infection through the down-regulation of DCs and inflammatory monocyte infiltration.

Recent studies showed that chronic infection with *T. gondii* led to $CD8^+$ T cell exhaustion, concomitant with decreased $CD8^+$ T cell effector response, which is characterized as up-regulated expression of inhibitory receptor PD-1 on these $CD8^+$ T cells [28]. MiRNA-155 expression is essential for optimal $CD8^+$ T cell responses toward chronic infection with LCMV, and cancer, involved in regulation of $CD8^+$ T cell exhaustion [23, 24]. In this study, our analysis of the effects of miR155 on cellular immune responses showed that the deficiency of miR-155 ablated CD8 T cell responses during chronic *T. gondii* infection, with up-regulation of PD-1, down-regulation of IFN-γ and TNF-α in $CD8^+$ T cells. In the meanwhile, Tregs have also been augmented in miR-$155^{-/-}$ mice, which are shown as the roles of immune-counter in cancer [36], indicating a possible immune-inhibitor effect in *T. gondii*, but its specific role need further confirmation. As the direct target of miR-155, SHIP-1 and SOCS1 are indicated as negative regulators of IFN-γ production and T cell exhaustion [23, 24]. The higher SHIP-1 and SOCS1 levels in infected miR155KO mice, together with these perturbed responses of $CD8^+$ T cells suggest that T cell exhaustion during chronic *T. gondii* infection is mediated by miR155-dependent mechanisms.

Conclusions

We demonstrate the absence of miR-155 could enhance the susceptibility of mice to chronic *T. gondii* infection. It is clear that this effect of miR155 is ascribed to its contribution to host immunity via the

regulation of both Th1 and Th2 protective immune responses, as well as inflammatory monocyte and DCs infiltration. It should be noted that miR155 was essential to mediate the T cell exhaustion in this model. Therefore, miR155 can be exploited as a potential target for reinforcing hosts' immunity against *T. gondii*.

Declarations

Authors' contributions

YZ, CZ and JC designed the study. YZ, CQ and YX performed experiments. YZ, CQ and YX statistically analysed the data. LV, YZ, CQ and JC made major contributions to the writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article and its additional files. The raw datasets used and analyzed during the present study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

All individual authors declare that they have no competing interests.

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Figures

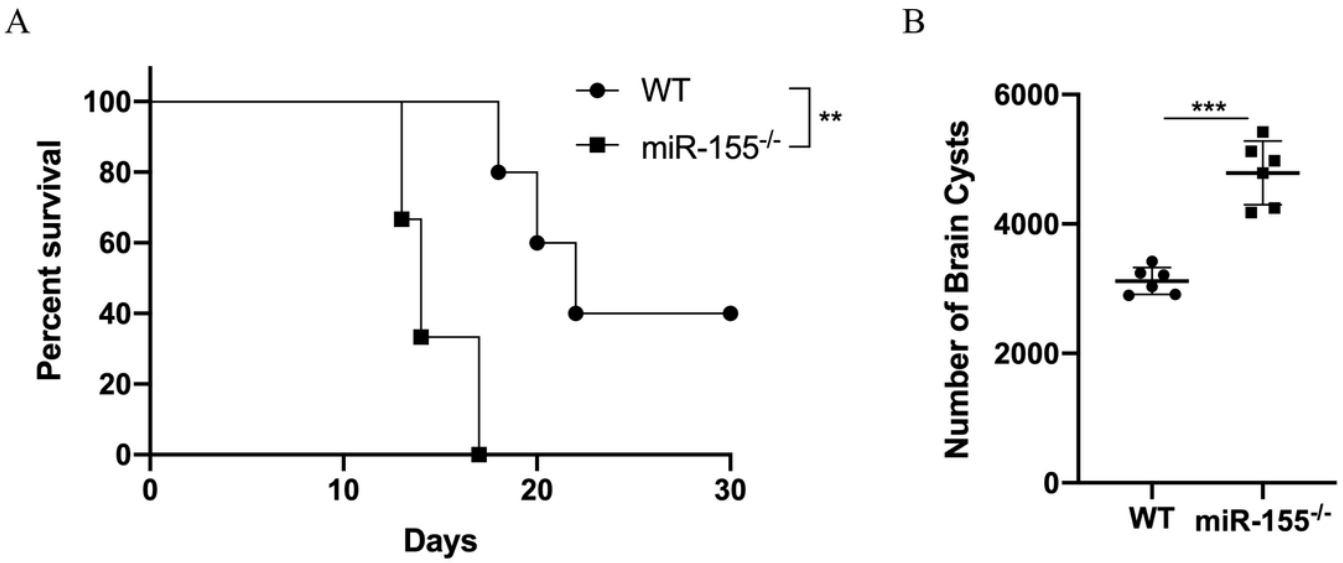

Figure 1

miR155 deficiency enhanced cyst burden and reduced survival time in mice models. (A) Survival curves of mice after challenge of cysts of the Pru strain. miR-155-/- mice had 0% survival at day 17. (B) Cyst loads were counted from whole brain homogenates of mice 4 weeks after challenge. The bars represented the mean cyst burden per mouse brain after challenge orally with a dose of 10 cysts of the Pru strain. Data are mean ± SD (representative of three experiments). ***$p < 0.001$, compared with the WT groups.

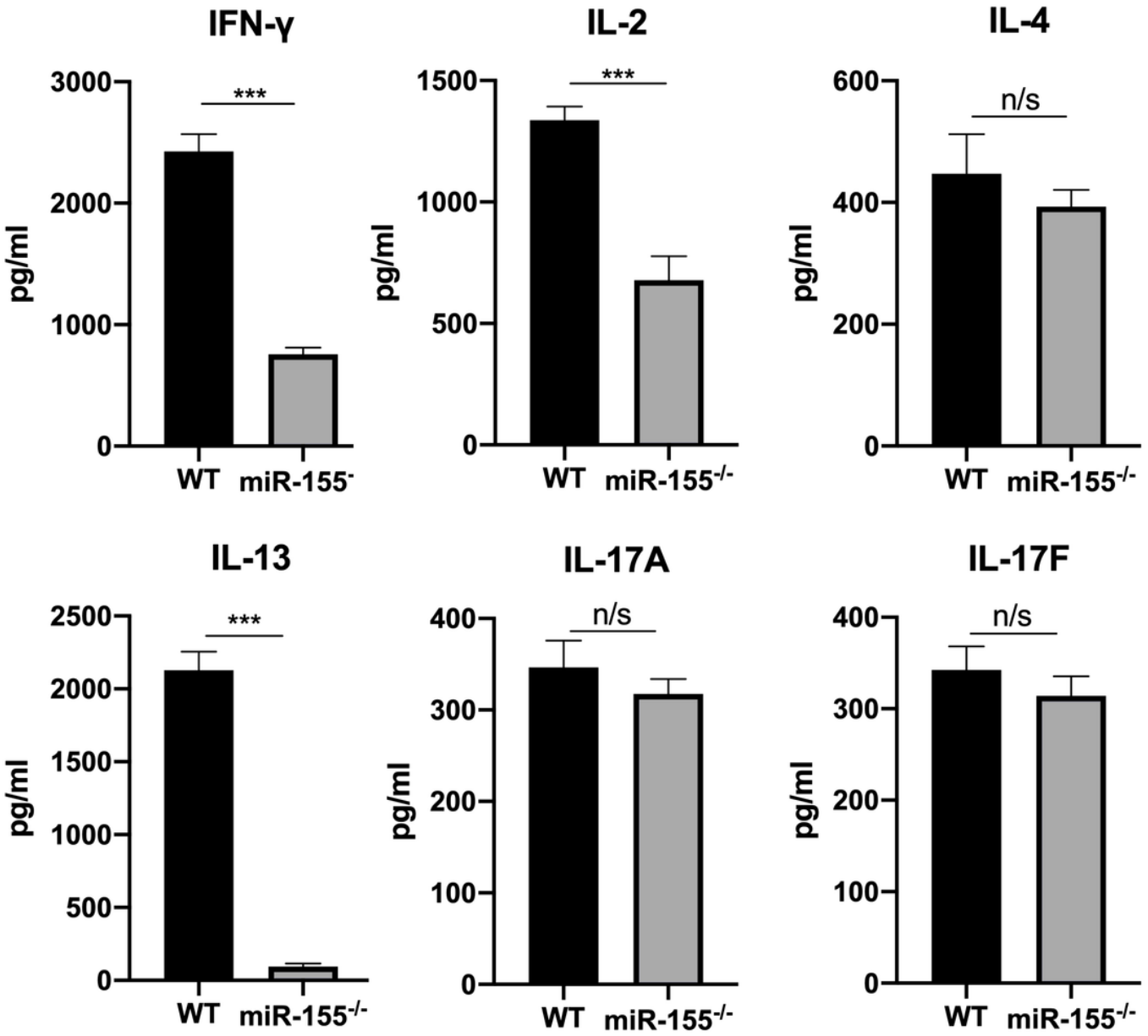

Figure 2

Evaluation of Th1\Th2\Th17 immune responses in WT and miR-155-/- mice. The levels of IFN-γ, IL-2, IL-13, IL-4, IL-17A and IL-17F were measured from splenic lymphocytes of WT- and miR155KO-infected mice. Significant decreases in IFN-γ, IL-2, IL-13 were observed in miR-155-/- mice. Data are shown from one representative experiment of three independent experiments and presented as a mean ± SD. ***, $P < 0.001$; N/S = not significant.

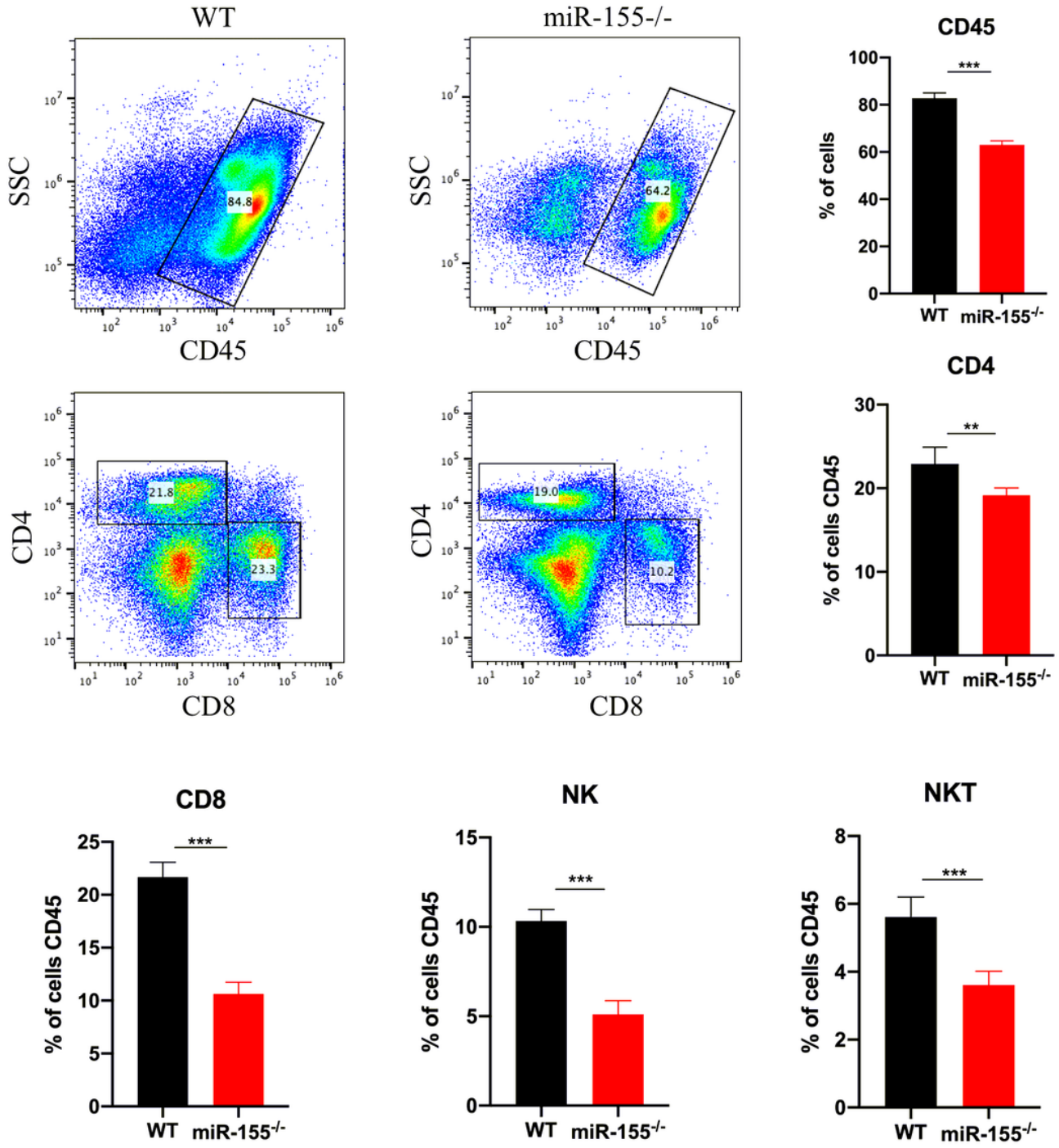

Figure 3

miR155 deficiency impairs the recruitments of CD4+ T cells, CD8+ T cells, NK and NKT cell. Spleens were collected from each mouse of WT and miR-155-/- groups. The splenocytes labeled with cell-specific fluorescent labeled antibodies were analyzed by flow cytometry. Results were calculated and expressed as a mean ±SD of three independent experiments. ***, P < 0.001; **, P <0.01.

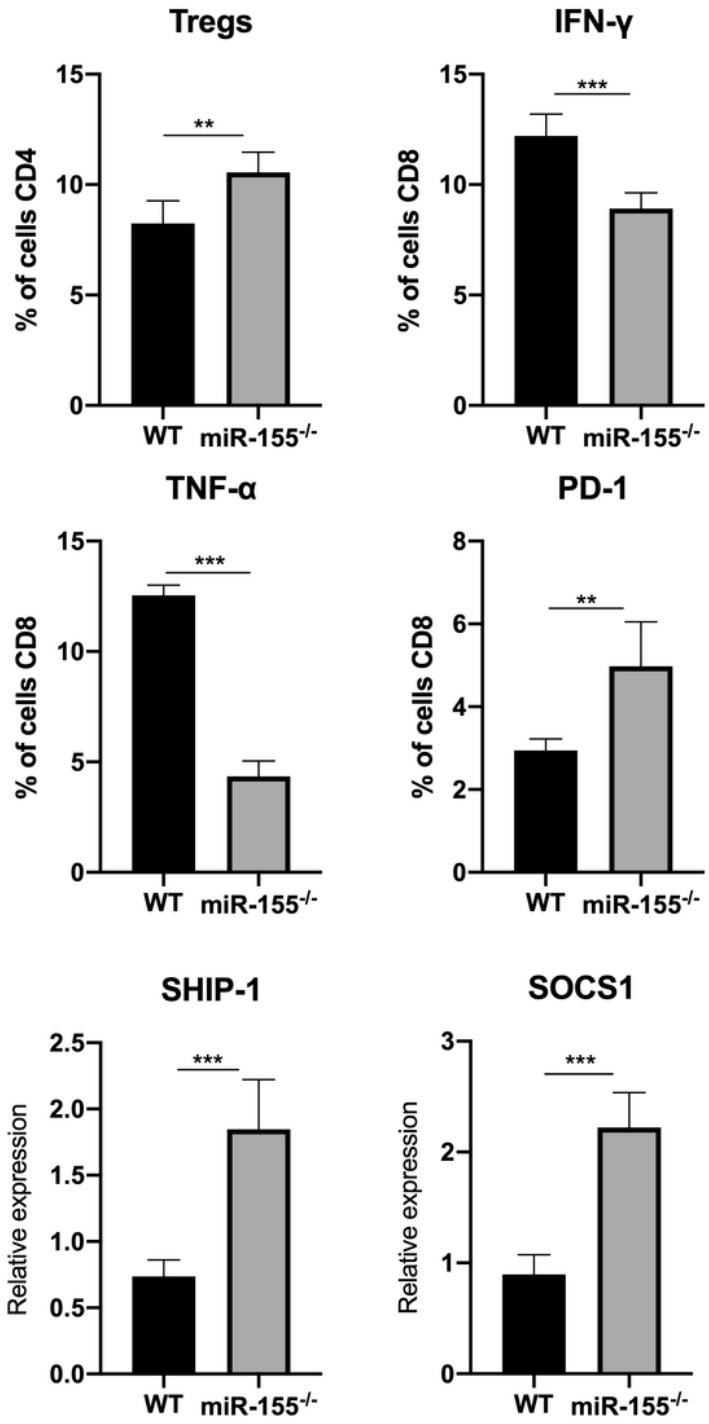

Figure 4

miR155 deficiency exacerbated CD8+ T cell exhaustion by targeting to SHIP-1 and SOCS1. Spleens were collected from each mouse of WT and miR-155-/-groups. The percentages of Tregs, IFN-γ CD8+ T, TNF-α CD8+ T and PD1 CD8+ T cells were analyzed by flow cytometric analysis. The expression of SHIP-1 and SOCS1 were estimated by qRT-PCR. Data are shown from one representative experiment of three independent experiments and presented as a mean ± SD. ***, $P < 0.001$; **, $P < 0.01$.

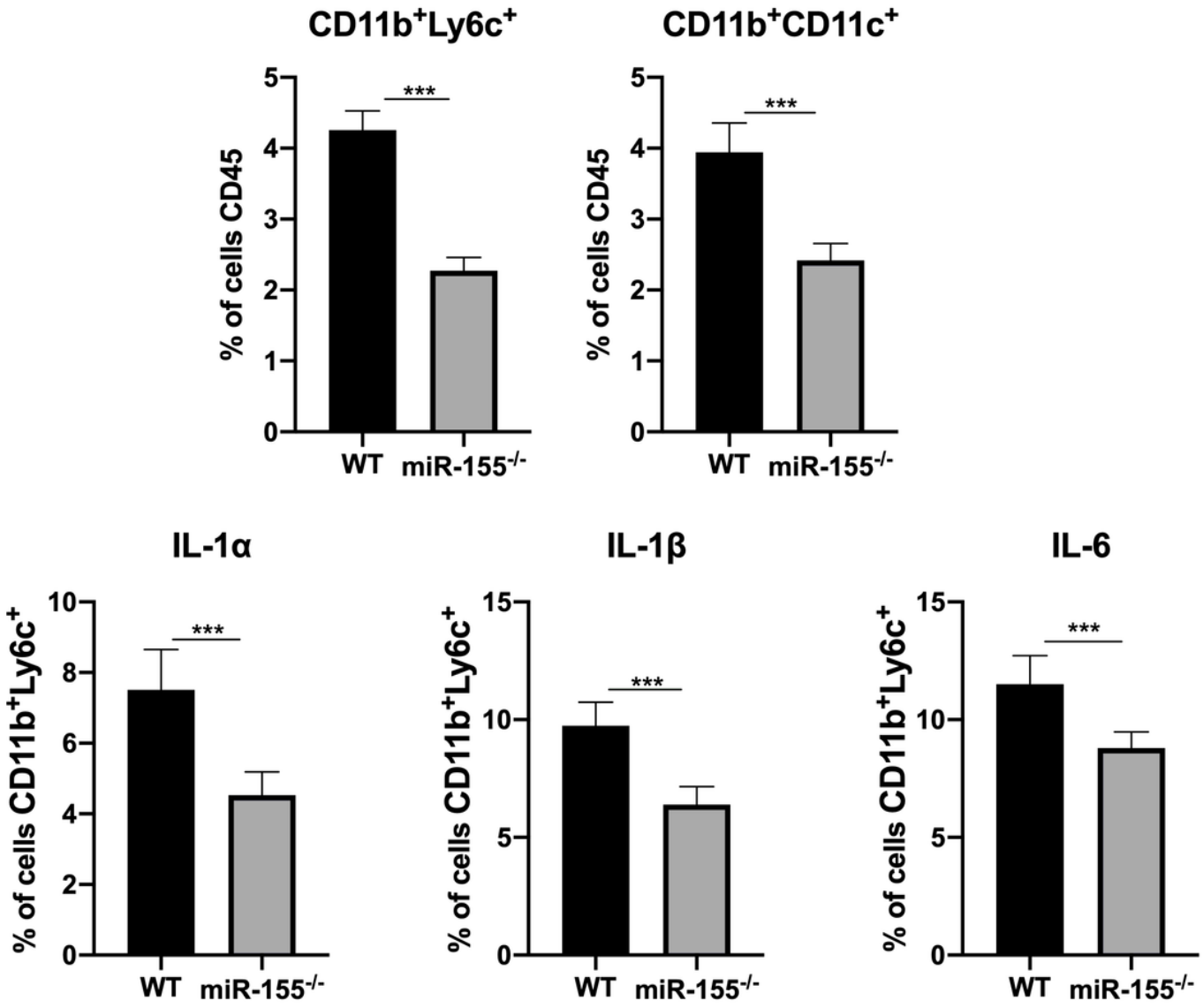

Figure 5

Decreased accumulation of splenic inflammatory monocytes and DCs and expression of pro-inflammatory mediators in miR155KO-infected mice. Flow cytometric analysis of splenic inflammatory monocytes and DCs of parasite infected-WT and miR-155-/- mice. The expression of pro-inflammatory mediators was analyzed by qRT-PCR. Data are presented as a mean ±SD of three independent experiments. The asterisks indicate the statistical significance of difference between WT and miR-155-/- mice. ***, P < 0.001; **, P <0.01.

Supplementary Files

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- TableS1.docx

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