International Journal for Parasitology 49 (2019) 993–997

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Succinctus

Host *miR-148* regulates a macrophage-mediated immune response during *Schistosoma japonicum* infection

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ARTICLE INFO

Article history:
Received 30 April 2019
Received in revised form 15 August 2019
Accepted 27 August 2019
Available online 11 November 2019

Keywords:
Extracellular vesicle
microRNA cargo
miR-148a
Schistosoma japonicum
Macrophage

ABSTRACT

Extracellular vesicles are critical regulators of host-parasite interactions. We previously demonstrated that *Schistosoma japonicum* EVs contain a remarkably high abundance of host *miR-148a*. Here, we characterised the abundance of *miR-148a* in circulation, in peripheral immune cells, and in plasma EVs of *S. japonicum*-infected mice. The results suggested the high abundance of *miR-148a* in macrophages to be likely linked to *S. japonicum* EVs. Additionally, *miR-148a* was found to target *PTEN* through the PI3K/AKT pathway to regulate cytokine production in macrophages. Consequently, our findings suggest that high abundance of *miR-148a* in macrophages may be associated with *S. japonicum* EVs, and regulate the host immune response during schistosome infection.

Schistosomiasis (bilharzia) is an intravascular disease caused by parasitic trematodes of the genus *Schistosoma*, affecting more than 200 million people in 78 tropical and subtropical countries (https://www.who.int/news-room/fact-sheets/detail/schistosomiasis). Morbidity is high and schistosomiasis contributes to approximately 200,000 deaths annually, with the majority of cases occurring in developing countries. Praziquantel is currently the only drug available to widely control and treat schistosomiasis; however, the frequent and continuous use of this drug has led to the development of drug resistance (Greenberg, 2013). Therefore, there is a great need to identify potential targets and develop novel therapeutic strategies against schistosomiasis.

Through long-term co-evolution, parasites have evolved to communicate, manipulate, and even utilise the host immune system to survive in the hostile host environment (Coakley et al., 2016). Therefore, understanding the mechanisms of host-parasite interactions could lead to the identification of potential targets for development of novel strategies against schistosomiasis. Several studies have focused on the secretion of parasitic signalling molecules such as proteins, lipids, glycans and nucleic acids, which are considered to play vital roles in host-parasite interactions (Wu et al., 2017). Recently, in-depth studies on extracellular vesicles (EVs) have demonstrated their importance in intercellular communication, tissue repair and immune surveillance through various mechanisms (Pitt et al., 2016; Silva et al., 2017). EVs have been characterised from different helminth parasites, and accumulated evidence has suggested the important role of EV cargo in mediating host-parasite interactions through host immune regulation (Coakley et al., 2016; Tritten and Geary, 2018). Previously, our preliminary study on *Schistosoma japonicum* exosome-like vesicles had suggested their potential regulatory role in host-parasite interactions (Zhu et al., 2016). EVs facilitate intercellular communication in both short and long ranges (van Niel et al., 2018), and EV-mediated intercellular communication is of fundamental importance for many immune system functions (Greening et al., 2015). MicroRNAs (miRNAs) carried by EVs can be transferred to recipient cells to modulate host immunity by directly regulating the expression of target genes (Buck et al., 2014). We had previously identified a small RNA population associated with *S. japonicum* EVs (SjEVs), which demonstrated a relatively high abundance of host miRNAs, particularly *miR-148a* (Zhu et al., 2016). In a separate study, we further demonstrated that SjEVs were primarily taken up by host macrophages both in vitro and in vivo, thereby indicating the important role played by the SjEV miRNA cargo in modulation of the host's immune response (Liu et al., 2019).

To test this hypothesis and elucidate the underlying mechanisms, we analysed an RNA-Seq dataset in two biological replicates from SjEVs, and found approximately 50% of reads belonged to *S. japonicum* rRNA/tRNA. Of the remaining reads, 10% specifically mapped to the rabbit genome. Out of those, 2% of reads belonged to tRNA and rRNA, while the remaining 3% were miRNA.

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https://doi.org/10.1016/j.ijpara.2019.08.002
0020-7519/

Interestingly, we observed certain host miRNAs, particularly *miR-148a*, to be associated with SjEVs in high abundance (Fig. 1A). To rule out the potential of contamination of *miR-148a* from the host during SjEV preparation, we investigated the abundance of *miR-148a* in circulation (plasma and serum) in mice or rabbits infected with *S. japonicum* cercariae where adult schistosomes reside. All the animal experiments were carried out following the guidelines for animal use in toxicology with approval from the Shanghai Laboratory Animal Management Committee and the Animal Care and Use Committee of the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China (Permit number: SYXK 2016-0010). Briefly, relative abundance of miRNA was quantified using quantitative reverse transcription-PCR by extracting total RNAs from plasma and serum, and cDNA was prepared using a miScript II RT Kit (Qiagen, Germany). Plasma and serum samples were normalised with spiked in *cel-miR-39* (UCACCGGGUGUAAAUCAGCUUG). All primer details are listed in Supplementary Table S1. Quantitative reverse transcription PCR (qRT-PCR) results indicated a low abundance of *miR-148a* in infected host serum and plasma (Supplementary Fig. S1A). Combined with the results of our previous study (Cheng et al., 2013), host *miR-10*, which is one of most abundant host miRNAs in circulation, was not found to be highly abundant in SjEVs. Consequently, these results suggested the high abundance of host *miR-148a,* associated with SjEVs, was not likely to be due to contamination from host circulation.

Next, we investigated the abundance of *miR-148a* in four different peripheral immune cell populations [T cell, B cells, natural killer (NK) cells, and monocytes] isolated from *S. japonicum*-infected and control mice, since adult schistosomes are in close contact with these host cells owing to their residence in the host mesenteric vein. The peripheral immune cells were then sorted using flow cytometry to distinguish between CD3e T cells, B220 B cells, CD11b+ Ly6C+ monocytes, and NK1.1 cells. Antibodies directed against the following markers were used to sort the peripheral blood immune cells for flow cytometry analysis using a BD FACSAria II system (BD Biosciences, CA, USA) (antibody information is provided in Supplementary Table S2). A significant reduction in the relative abundance of *miR-148a* was observed in isolated T cells of infected mice compared with that in T cells of uninfected control mice (Fig. 1B). However, there was no notable difference in *miR-148a* abundance in isolated NK or B cells between infected and uninfected mice (Fig. 1B). Notably, we found significantly increased abundance of *miR-148a* in monocytes (Fig. 1B) and in plasma EVs of hosts infected with schistosomes (Fig. 1C). In addition, we observed a significant increase in the abundance of *miR-148a* in cells treated with SjEVs, compared with that in control EV (NCTC clone 1469 cells) and PBS treatments (Fig. 1D). The method of EV treatment is provided in Supplementary Methods S1. Taken together, these results suggested the high abundance of *miR-148a* in monocytes to possibly be due to the uptake of SjEVs. However, the high abundance of *miR-148a* may also have resulted from host EVs and/or cells.

To reveal the effect of such high levels of *miR-148a* on the function of peripheral blood immune cells, particularly on macrophages, we predicted the target genes of *miR-148a* using different databases including miRWalk, TargetScan (v7.0; www.targetscan.org), and miRDB (Sticht et al., 2018; Wong and Wang, 2015). Among the several hundreds of candidate target genes, we selected eight targets that were co-identified from these databases, all of which were putatively involved in PI3K-AKT signalling, cell cycle, cell death, inflammatory response, and innate immunity. Network analysis of the selected target mRNAs of *miR-148a* is shown in Fig. 2A. Next, we determined the transcript levels of the selected target genes in cultured RAW264.7 cells (cells are macrophage-like; Abelson leukaemia virus transformed cell line derived from BALB/c mice) transfected with *miR-148a* mimic, in RAW264.7 cells treated with SjEVs and NCTC clone 1469 cells (cells are derived from mouse liver cell line NCTC 721) EVs, and in monocytes isolated from mice infected with schistosomes (Fig. 2B–D and Supplementary Fig. S2A–C). Total RNAs were extracted using TRIzol (Thermo Fisher Scientific, MA, USA) and cDNA was prepared using a PrimeScript RT reagent Kit (Takara, Dalian, China). qRT-PCR

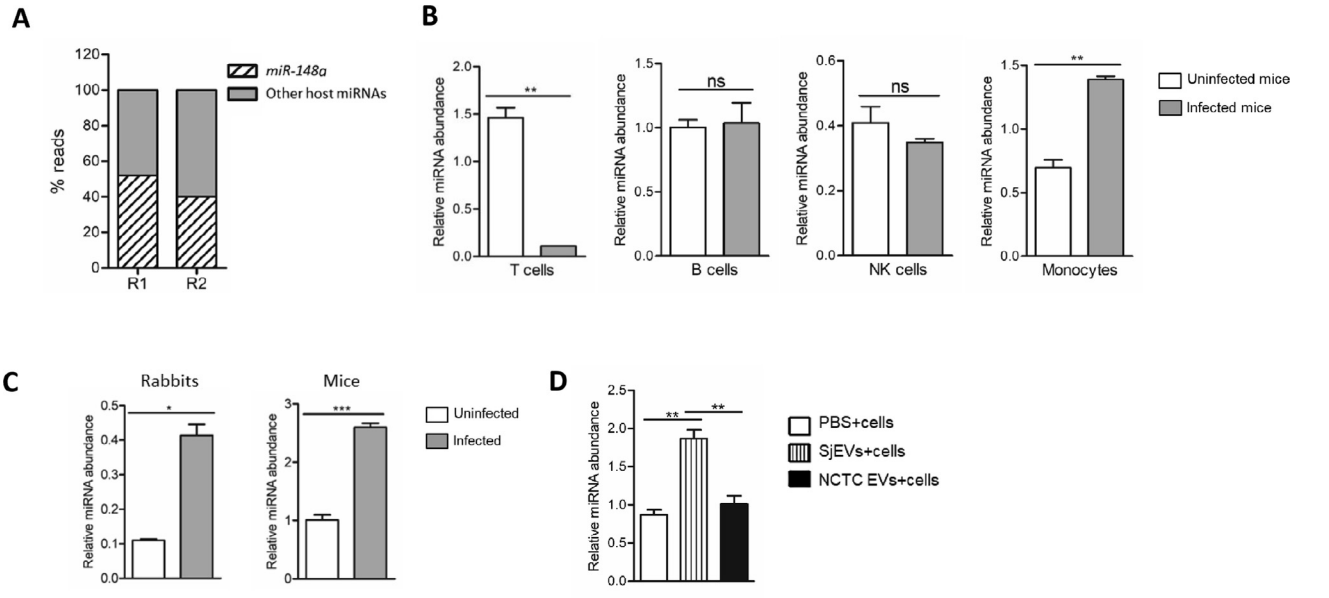

Fig. 1. Characterization of the abundance of *miR-148a* in *Schistosoma japonicum* extracellular vesicles, host plasma EVs, and host peripheral blood immune cells. (A) Percentage of host *miR-148a* in *S. japonicum* EV microRNAs (miRNAs) by RNA-Seq analysis. (B) Relative abundance of *miR-148a* in different peripheral blood immune cells sorted by fluorescence-activated cell sorting from mice infected with *S. japonicum* at day 28 p.i. and from control mice. (C) Relative abundance of *miR-148a* in plasma EVs isolated from mice and rabbits infected with *S. japonicum* at day 28 p.i. and control mice and rabbits. (D) Analysis of *miR-148a* abundance in RAW264.7 (cells are macrophage-like; Abelson leukaemia virus transformed cell line derived from BALB/c mice) cells treated with *S. japonicum* EVs, NCTC clone 1469 cells (cells are derived from mouse liver cell line NCTC 721) EVs and PBS (blank) by qRT-PCR quantitative reverse transcription PCR (qRT-PCR). Data are represented as means ± S.E.M. from three biological replicates. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ (Student's *t* tests). ns, not significant.

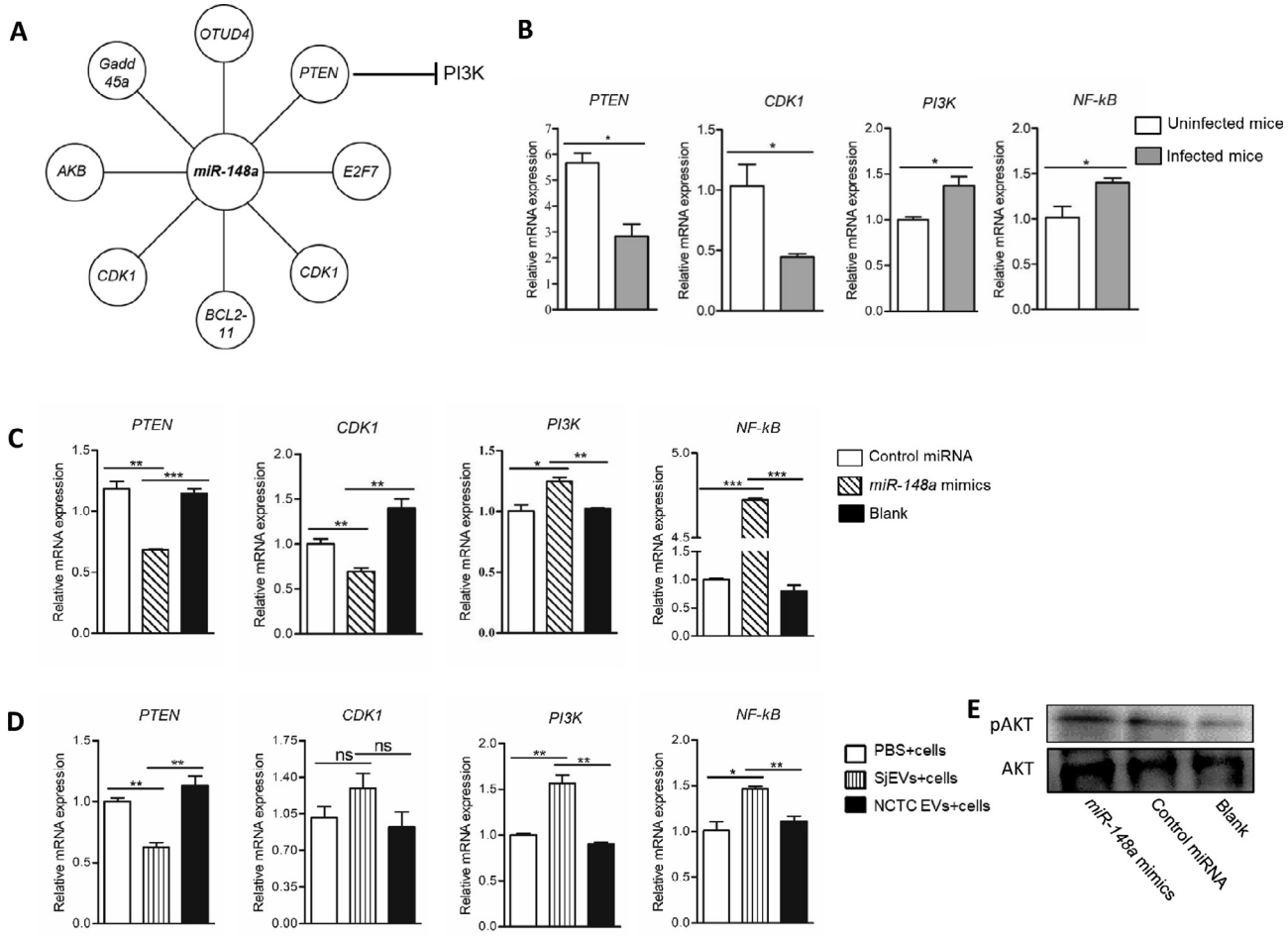

Fig. 2. Expression of *miR-148a* targets in monocytes isolated from mice infected with *Schistosoma japonicum*, RAW264.7 (cells are macrophage-like; Abelson leukaemia virus transformed cell line derived from BALB/c mice) cells transfected with a *miR-148a* mimic, and RAW264.7 cells treated with *S. japonicum* extracellular vesicles and NCTC clone 1469 cells (cells are derived from mouse liver cell line NCTC 721) EVs. (A) Diagrammatic view of the network of *miR-148a* and selected targets, and phosphatase and tensin homolog regulated PI3K/AKT. (B) Quantitative reverse transcription PCR (qRT-PCR) analysis of *miR-148a* targets *PTEN* and *CDK1* in isolated monocytes from mice infected with *S. japonicum* and controls. (C) qRT-PCR analysis of the expression of *miR-148a* targets in RAW264.7 cells transfected with *miR-148a* mimics and control miRNA. (D) qRT-PCR analysis of *miR-148a* targets in RAW264.7 cells treated with SjEVs, NCTC EVs and PBS (blank). (E) Western blot analysis of AKT (protein kinase B) and phosphorylated AKT from RAW264.7 cells transfected with a *miR-148a* mimic and control miRNA, and from normal cells without any treatment (Blank). For B and C, data are represented as means ± S.E.M. from three biological replicates. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ (Student's *t* tests).

analysis was performed using Hieff qPCR SYBR Green Master Mix (YeaSen Biotech, Shanghai, China). Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) was used as the internal control. The method of RAW264.7 cell transfection is provided in Supplementary Methods S1, all primer sequences are listed in Supplementary Table S1. qRT-PCR results indicated that the transcript levels of *PTEN* and *CDK1* genes were found to be significantly reduced in the transfected RAW264.7 cells, cells treated with SjEVs as well as in the isolated monocytes from infected mice, compared with those in controls, from among the eight targets investigated (Fig. 2B–D); the remaining six target genes showed no significance (Supplementary Fig. S2A–C). Cyclin-dependent kinases (CDKs) are vital for the regulation of cell proliferation and differentiation in eukaryotes (Wang et al., 2011), and overexpression of *miR-148a* had been shown to down-regulate the expression of CDKs and suppress the proliferation of cancer cells (Han et al., 2017). Previous studies demonstrated that phosphatase and tensin homolog) (PTEN) can negatively regulate the PI3K/AKT pathway, which in turn influences nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling that is critical for macrophage survival, migration, and proliferation (Asano et al., 2004; Song et al., 2005). Indeed, we found a significant upregulation of PI3K in monocytes isolated from *S. japonicum*-infected mice and in RAW264.7 cells treated with a *miR-148a* mimic (Fig. 2B and C), together with elevated expression of its downstream signalling molecules NF-κB (Fig. 2B and C) and AKT (protein kinase B) using western blots (Fig. 2E). Similar results were also observed in SjEV-treated cells (Fig. 2D). These results suggested that *miR-148a* may target the PI3K/AKT pathway via *PTEN* to regulate the functions of peripheral blood immune cells during schistosome infection.

Macrophages play a central role in innate immunity and initialization of adaptive immune response in vertebrates, and hence serve as a prime target for EVs derived from different helminth parasites such as *Heligmosomoides polygyrus*, *Litomosoides sigmodontis* and *Fasciola hepatica* (Coakley et al., 2017; de la Torre-Escudero et al., 2019). Macrophage polarisation and proliferation are associated with the secretion of different cytokines. We found higher expression levels of tumour necrosis factor-alpha (TNF-α) in *miR-148a* mimic-transfected RAW264.7 cells and in monocytes isolated from infected mice than the control, suggesting the regulation of macrophage polarisation by *miR-148a* (Fig. 3A and B). Interleukin-12 (IL-12) plays critical roles in host defence and in

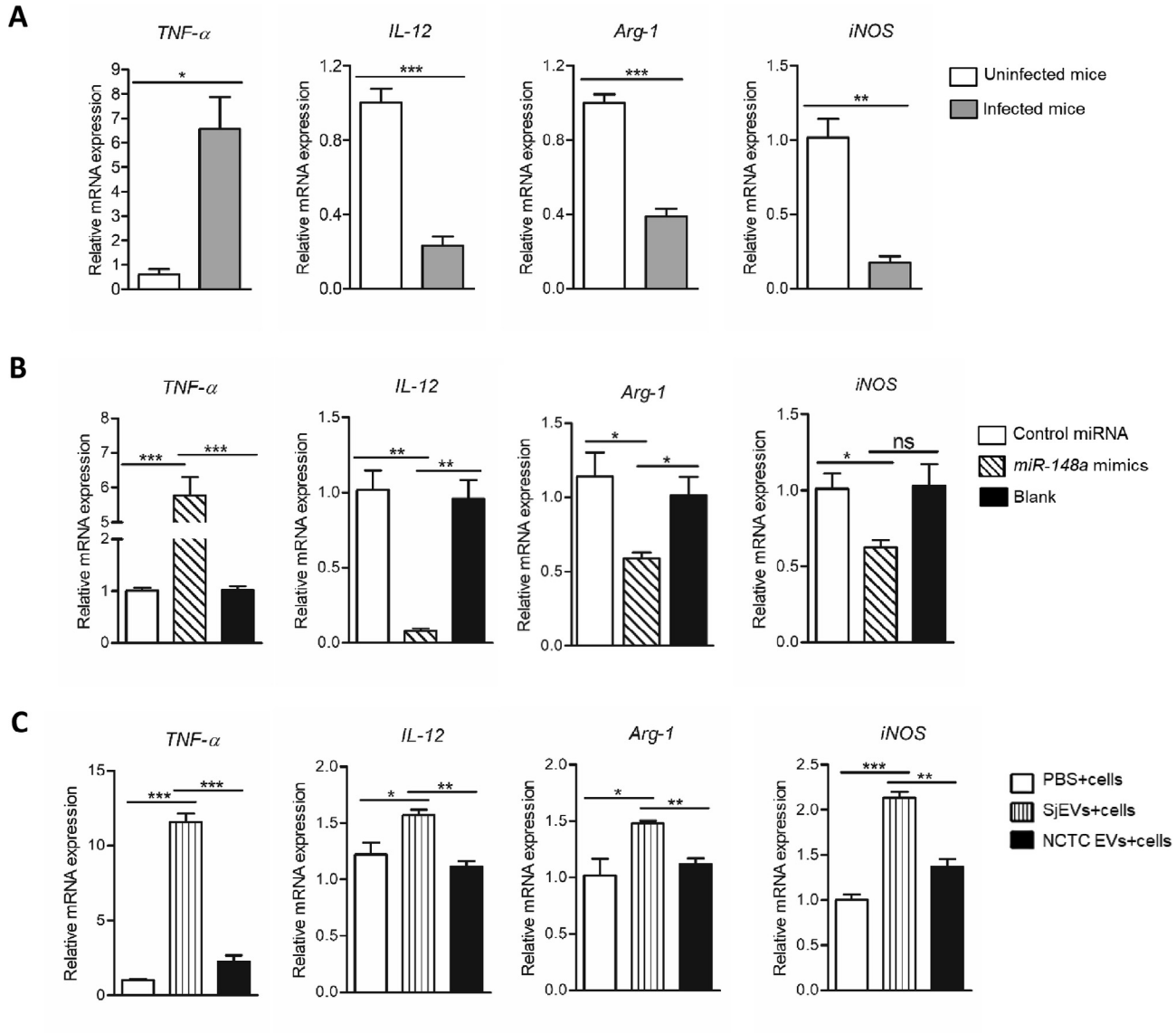

Fig. 3. Quantitative reverse transcription PCR (qRT-PCR) analysis of the expression of several M1/M2 markers in monocytes isolated from mice infected with *Schistosoma japonicum*, in RAW264.7 (cells are macrophage-like; Abelson leukaemia virus transformed cell line derived from BALB/c mice) cells treated with a *miR-148a* mimic, and in RAW264.7 cells treated with (*S. japonicum* extracellular vesicles and NCTC clone 1469 cells (cells are derived from mouse liver cell line NCTC 721) EVs. (A) qRT-PCR analysis of the expression of *TNF-α*, *IL-12*, *Arg-1* and *iNOS* in monocytes isolated from mice infected with *S. japonicum*. (B) qRT-PCR analysis of the expression of *TNF-α*, *IL-12*, *Arg-1* and *iNOS* in RAW264.7 cells transfected with a *miR-148a* mimic and control miRNA. (C) qRT-PCR analysis of the expression of *TNF-α*, *IL-12*, *Arg-1* and *iNOS* in RAW264.7 cells treated with SjEVs, NCTC EVs and PBS (blank). Data are represented as means ± S.E.M. from three biological replicates. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ (Student's *t* tests).

maintaining the immune homeostasis produced by antigen presenting cells, including macrophages and dendritic cells (Ma et al., 2015). However, we found a significant down-regulation of IL-12 expression in the monocytes of infected mice as well as in *miR-148* mimic-transfected RAW264.7 cells (Fig. 3A and B). Nitric oxide (NO) is a toxic defence molecule against worm infection and regulates the functions, growth and death of many immune cells including macrophages (MacMicking et al., 1997). Arginine (Arg-1) and inducible nitric oxide synthase (iNOS), enzymes involved in L-Arg-1 and NO metabolism, are closely involved in circulating inflammation and enhance macrophage-mediated immune responses to give rise to a killing response. However, in the present study, we observed a significant decline in the expression levels of *Arg-1* and *iNOS* in the monocytes of infected mice as well as in *miR-148a* mimic-transfected RAW264.7 cells (Fig. 3A and B). Increased expression of TNF-α was also observed in the cells treated with SjEVs while IL-12, *Arg-1* and *iNOS* showed significant upregulation (Fig. 3C). Collectively, these results suggest altered abundance of *miR-148a* in host macrophages may be involved in the regulation of macrophage polarisation. However, transfection of the *miR-148a* mimic did not influence cell proliferation (Supplementary Fig. S3).

Taken together, our study demonstrates that the abundance of *miR-148a* in host macrophages is associated with schistosome infection and that *miR-148a* can regulate the macrophage-mediated immune response by targeting *PTEN* through the PI3K/AKT pathway to alter the expression of several cytokines. Although we observed that the remarkable abundance of *miR-148a* is associated with SjEVs which, in turn, are primarily taken up by host macrophages, owing to technical limitations (i.e., lack of a tool for genetic manipulation to inhibit SjEVs), we could not directly demonstrate whether SjEVs release host *miR-148a* to modulate the immune response of macrophages. Nevertheless, our study revealed the important functions of *miR-148a* in the regulation of

macrophage functions during *S. japonicum* infection, suggesting a new role in host-parasite interaction.

Acknowledgements

This study was, in part or in whole, supported by the National Natural Science Foundation of China (31672550 and 31472187) and The Agricultural Science and Technology Innovation Program of the Chinese Academy of Agricultural Sciences.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.08.002.

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