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## miRNA regulation of macrophage fusion into multinucleated giant cells

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### Abstract

Cellular fusion of macrophages into multinucleated giant cells is a distinguishing feature of the granulomatous response to inflammation, infection and foreign bodies (1). We observed a marked increase in fusion of macrophages genetically deficient in Dicer, an enzyme required for canonical miRNA biogenesis. Gene expression profiling of miRNA deficient macrophages revealed an up-regulation of the IL4 responsive fusion protein Tm7sf4, analyses identify miR-7a-1 as a negative regulator of macrophage fusion, functioning by directly targeting Tm7sf4 mRNA. miR-7a-1 is itself an IL4 responsive gene in macrophages, suggesting feedback control of cellular fusion. Collectively these data indicate that miR-7a-1 functions to regulate IL4 directed multinucleated giant cell formation.

### Introduction

Circulating monocytes and tissue resident macrophages serve as sentinels of the immune system by sensing the presence of pathogens using a diverse array of membrane anchored and cytosolic detectors. Upon pathogen exposure, macrophages coordinate both intrinsic and extrinsic host defense mechanisms to eliminate the intruding organism and establish protective immunity against reinfection (1, 2). Macrophages are remarkably adaptable in their response to infection and activate distinct effector mechanisms commensurate with the pathogen detected. For example, detection of viruses through cytosolic sensors triggers a rapid type 1 interferon dominated response, whereas pro-inflammatory cytokine expression is induced following TLR2 recognition of peptidoglycan, a component of Gram-positive bacteria (1, 3).

Macrophage responses are further regulated by leukocyte derived soluble and cell surface molecules. Exposure of macrophages to TLR ligands in the presence of IFN $\gamma$  induces a classically activated state characterized by expression of TNF $\alpha$  and IL12, which together promote T<sub>H</sub>1 or T<sub>H</sub>17 dominated responses. In contrast, macrophages activated by the T<sub>H</sub>2 cytokines IL4 and IL13 participate in wound repair, immune suppression and defense against parasites (2, 4, 5). Additionally, IL4 and IL13 can induce macrophage fusion into multinucleated giant cells that serve to isolate and/or eliminate foreign bodies and parasites and are a hallmark of the granulomatous response (6, 7). Studies of molecular mechanisms that regulate mammalian cell fusion have identified several key fusogenic molecules (i.e. CD9, CD47, CD44, Tm7sf4/DC-STAMP, and SIRP $\alpha$ ) (8) (9). Whereas macrophage activation is generally considered a plastic and reversible response, the formation of multinucleated cells represents a terminally differentiated state and is thus likely subject to unique regulatory mechanisms.

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miRNAs are 21–23 nucleotide non-coding RNAs generated by sequential nuclear and cytosolic processing of precursor transcripts by the RNaseIII enzymes Drosha and Dicer, respectively (10). They negatively regulate gene expression by binding to conserved, partially complementary sequences most often found in the 3' non coding regions of target mRNAs. Several miRNAs are responsive to TLR agonists and function to dampen or reshape innate immune responses within macrophages (11). Here, we demonstrate a role for miR-7a-1 in regulating multinucleated giant cell formation by targeting the fusogenic cell surface protein Tm7sf4.

## Materials and Methods

### Mice

Mice in which myeloid cells were selectively deficient in Dicer were generated by crossing C57BL/6.129 Dicer<sup>fl/fl</sup> (12) with the C57BL/6.129 Lyz2<sup>tm1(cre)</sup> deleter strain (13) to generate C57BL/6 Dicer<sup>fl/fl</sup>Lyz2<sup>tm1(cre)/+</sup> mice. Throughout experiments, age and sex matched C57BL/6 Dicer<sup>fl/+</sup>Lyz2<sup>tm1(cre)/+</sup> mice were used as controls. Mice obtained from The Jackson Laboratory (Bar Harbor, ME) were housed under SPF conditions with approval of IACUC at the Institute for Systems Biology.

### Tissue culture

Bone marrow-derived macrophages (BMM) were cultured in complete DMEM (cDMEM; plus 10% FBS, 2 mM l-glutamine, penicillin and streptomycin) with recombinant human CSF-1 (50 ng/ml) for 6 d. To generate giant cells, bone-marrow cells were cultured as above for 4 d then mouse-IL-4 (50 ng/mL; Peprotech) was added for a further 6 d. Human embryonic kidney cells (HEK 293) for luciferase assays were grown in cDMEM. Macrophages derived from conditionally immortalized Hoxb8 progenitors were generated from C57BL/6 mice essentially as described (14) and maintained in their myeloid progenitor stage in RPMI, 10% FBS, 2 mM l-glutamine, 10ng/mL GM-CSF-1, 1 $\mu$ M  $\beta$ -estradiol. To generate Hoxb8 macrophages from wild-type and miRNA transduced lines, progenitors were harvested, washed 2 X in 1X PBS and resuspended in fresh BMM culture medium containing CSF-1 and cultured for 6 d as per the BMM protocol.

### Gene expression arrays

Total RNA was isolated using TRIzol (Invitrogen) and mRNA was labeled and hybridized to an Affymetrix GeneChip Mouse Exon ST 1.0 array. Expression data were acquired from six microarray hybridization experiments comprising three Dicer<sup>fl/fl</sup>Lyz2<sup>tm1(cre)/+</sup> mutant-derived macrophages, and three Dicer<sup>fl/+</sup>Lyz2<sup>tm1(cre)/+</sup> controls. Data were background-subtracted and normalized, then averaged across biological replicates using the log<sub>2</sub> intensities (15, 16). Significance testing was performed using log<sub>2</sub> intensities and expression cut-off value of 6 with a student's t-test; p-value < 0.01 regarded as significant. Gene array data are deposited in NCBI-GEO, accession number: GSE36585 (<http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE36585>)

### Retroviral transduction of miR-7a-1

Genomic sequences encoding pri-miR-7a-1 were cloned corresponding to genetic coordinates Chr13:58493908 and Chr13:58494429. The product was cloned into a MSCV-PIG vector (17) and a control construct in the same backbone was generated encoding a pri-miR-30 formatted shRNA against Tlr5, a gene not expressed by mouse BMM. Retroviral constructs were packaged using the Phoenix ecotropic 293 line. Macrophages were transduced and cells selected in puromycin for 21 days prior to analysis.

### Tm7sf4 3'UTR cloning, mutagenesis and luciferase reporter assays

The 3'UTR of Tm7sf4 was cloned from C57BL/6 mouse genomic DNA by PCR corresponding to Ensembl coordinates 15071 and 15621. The product was cloned 3' of the firefly luciferase ORF in a pViro-blasti (Invivogen) backbone carrying a control *Renilla* luciferase gene. Point mutations in the Tm7sf4 miR-7a target sequence were introduced by mutagenesis PCR. HEK 293 cells were co-transfected with luciferase and miRNA expressing vectors and assayed using a luminometer.

### DNA staining to determine giant cell ploidy

Ploidy staining was performed as described (18). Flow cytometry was performed using a Becton Dickinson FACSAria II and ploidy classes for giant cells determined.

### Gene expression analysis

To analyze recombination of *Dicer1* alleles, expression of genes and microRNAs, total RNA was isolated from macrophages using TRIzol (Invitrogen), treated with DNAase (Ambion), and used as template for reverse transcription (Superscript II, Invitrogen). Quantitative PCR (qPCR) reactions were performed using an ABI 2900 HT thermocycler and expression levels were calculated using the  $\Delta\Delta CT$  method relative to control *Efla* gene. MicroRNA-specific reverse transcription was performed for each miRNA using gene-specific primers (Applied Biosystems). MicroRNA levels were normalized to small-nucleolar-RNA sno202. The levels of Sno202 were unchanged by CSF-1, IL-4, or by a variety of TLR agonists in the primary bone-marrow derived macrophage or Hox macrophage systems.

### Western blotting analyses

Were performed using standard techniques, membranes were probed with relevant primary antibodies: rabbit anti-Stat6 (Cell signaling technologies, MA), rabbit anti-phospho-Stat6 (Abcam, MA), rabbit anti-mouse-Tm7sf4 (Santa Cruz, CA), rabbit anti-mouse beta-actin1-HRP (Jackson Immunoresearch labs).

### Statistical analysis

Significance was determined using an unpaired 2-tailed Student's t test.

## Results and Discussion

miRNAs have established roles in regulating classic innate immune responses. We took a genetic approach to globally assess a role for miRNAs in regulating macrophage fusion into multinucleated giant cells, a specialized  $T_H2$  driven response of macrophages to intrusion by parasites or synthetic foreign material. Mice carrying a floxed allele of Dicer were crossed to a myeloid restricted cre expressing strain (Lys2Cre). The resulting mice,  $Dicer^{fl/fl} Lys2Cre$ , were born at the expected Mendelian frequencies from the appropriate crosses and were overtly indistinguishable from littermate controls heterozygous for the floxed Dicer allele,  $Dicer^{fl/+} Lys2Cre$ . Dicer mRNA levels were reduced approximately 75 % in  $Dicer^{fl/fl} Lys2Cre$  bone marrow macrophages (BMM) relative to controls (Fig. 1). The extent of Dicer deletion could not be further increased by using mice homozygous for Lys2cre, prolonged culture or LPS stimulation (unpublished observations) and are in agreement with previous reports using the Lys2-cre deleter strain (19). A corresponding decrease in the expression of miRNAs abundantly expressed in BMMs was observed (Fig. 1A).

We used genome wide expression profiling to analyze the consequences of Dicer deficiency on macrophage responses to IL4. IL4 programs macrophages to dampen immune responses, repair damaged tissue and participate in defense against parasites (2, 4). The expression of

classic markers of IL4 macrophage activation including Arginase-1 (Arg1), Chitinase-3 like-3 (Chi3l3/Ym1) and Resistin-like molecule- $\alpha$  (Retnl1a/Fizz), is unaffected by Dicer depletion (Supplement 1B). IL4 additionally induces the expression of genes involved in macrophage fusion. Amongst this panel of genes, only Tm7sf4, a molecule required for macrophage fusion (20–22), was significantly altered in Dicer deficient cells (Supplement 1A) These data were confirmed by qRT-PCR and western blot analyses of resting and IL4 stimulated macrophages (Fig. 2A and 2B) and suggests a unique role for Dicer, and hence miRNAs, in the control of Tm7sf4 expression. Extended culture of BMMs in IL4 containing media leads to the generation of multinucleated giant cells. Under these conditions, we noticed a marked increase in the number of multinucleated cells in Dicer<sup>fl/fl</sup>, Lys2Cre relative to control cells (Fig. 2C and 2D). This suggests a direct link between the expression of the fusogenic molecule, Tm7sf4, and the fusion phenotype. Considering that IL-4 highly induces Tm7sf4 in Dicer<sup>fl/fl</sup>, Lys2Cre cultures relative to controls, we examined whether Dicer<sup>fl/fl</sup>, Lys2Cre macrophages were globally hyper-responsive to IL-4. This was not the case. For example, IL4-induced Stat6 phosphorylation and dephosphorylation were identical in Dicer<sup>fl/fl</sup>, Lys2Cre and control macrophages (Fig. 2E) Furthermore, another IL4-responsive fusogenic molecule, E-cadherin (Cdh1), was unaffected in Dicer<sup>fl/fl</sup>, Lys2Cre macrophages (Fig. 1B), demonstrating the specificity of regulation of Tm7sf4 by miRNA. Finally, the expression of a number of macrophage fusogenic molecules was unaffected in Dicer<sup>fl/fl</sup>, Lys2Cre macrophages (Supplement 1A).

Examination of the Tm7sf4 3'UTR using various target prediction algorithms (23) demonstrated a number of miRNA target sites. However, only the predicted miR-7a target sequence was conserved amongst mammalian species. There are three isoforms of miR-7, miR-7a-1, miR-7a-2 and miR-7b; miR-7a and 7b can be distinguished by PCR based on their mature form, but the miR-7a-1 and miR-7a-2 isoforms cannot. miR-7b is not expressed in macrophages whereas miR-7a-(1/2) are (data not shown). Moreover, miR-7a-(1/2) are strongly induced by IL-4 (Fig. 3A), suggesting a potential role in regulating Tm7sf4 (Fig. 3A). In order to distinguish between miR-7a-1 and miR-7a-2 we turned to epigenetic mapping (Supplementary Figure 2). These two miRNA's are found on chromosomes 13 and 7, respectively. Histone acetylation results in an open chromosome structure that facilitates transcription. ChIP-seq experiments in IL4-treated macrophages demonstrated substantial histone acetylation in the vicinity of miR-7a-1 on chromosome 13 whereas there was no detectable histone acetylation in the vicinity of miR-7a-2 on chromosome 7. This suggests that miR-7a-1 is the IL-4 responsive species (Supplement 2). We therefore considered miR-7a-1 to be a candidate miRNA regulating both Tm7sf4 expression and IL4 induced multinucleated giant cell formation.

To directly examine a role for miR-7a-1 in regulating Tm7sf4 expression, we transduced immortalized BMMs with retroviruses encoding either pri-miR-7a-1 or a control pri-miR-30. Decreased Tm7sf4 expression was specifically observed in miR-7a-1 over-expressing BMMs (Fig 3B). Moreover, IL4 induced multinucleated giant cell formation was attenuated in miR-7a-1 over-expressing BMMs (Fig. 3C). To determine if the ability of miR-7a-1 to regulate Tm7sf4 expression is through direct targeting of the 3'UTR, we fused these sequences downstream of a luciferase reporter. The resulting construct was tested in HEK 293 cells co-transfected with either miR-7a-1 or a control miRNA. As shown in Fig 3D, the Tm7sf4 3'UTR imparted miR-7a-1 specific suppression of luciferase expression. Transfection of the Tm7sf4 3'UTR containing a point mutation in the miR-7a-1 target sequence had no effect on luciferase activity (Fig. 3D). These experiments demonstrate that miR-7a-1 interacts directly and specifically with the Tm7sf4 3'UTR to regulate its expression.

Our study identifies miR-7a-1, an IL4 responsive miRNA, as critical negative regulator of IL4-mediated gene transcription of Tm7sf4. This novel mechanism allows for precise control of macrophage differentiation into multinucleated giant cells. In support of a role for miRNA in regulation of cellular differentiation, Dicer dependent pathways are required for the differentiation of Langerhans cells and T-cells (24–26). More specifically, miR-181 is required for human hematopoietic cell differentiation (27), while miR-150 controls B-cell differentiation (28), and miR-223 regulates neutrophils (29). By generating a myeloid specific deletion of Dicer, we have identified for the first time that miRNA can fine-tune the macrophage differentiation response to IL4. More specifically, miR-7a-1 acts as a negative regulator of Tm7sf4 and as a macrophage fusion rheostat.

An environment of chronic T<sub>H</sub>2 inflammation programs macrophages to differentiate to multinucleated giant cells, a hallmark of granulomatous disease. However, the agents driving giant cell formation in granulomatous diseases such as sarcoidosis remain elusive. It is possible that IL-4 regulated Tm7sf4 via miR-7a-1 may play a role in the etiology of chronic inflammatory diseases.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Non-standard condensed words

<b>miRNA</b>	microRNA
<b>miR-7a</b>	miRNA-7a

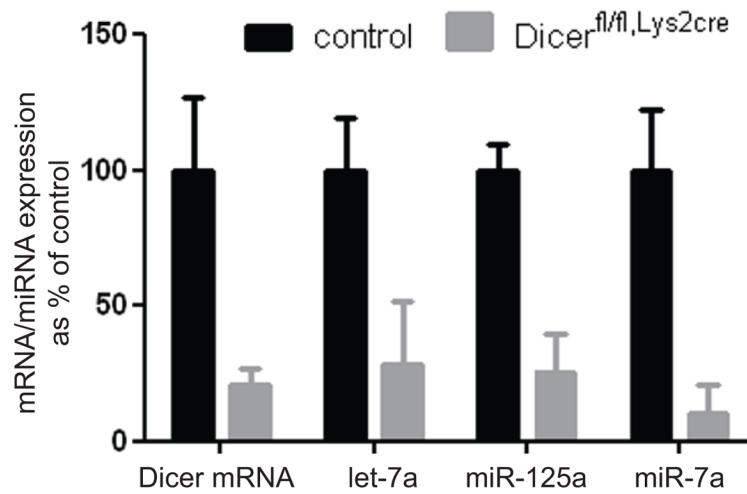
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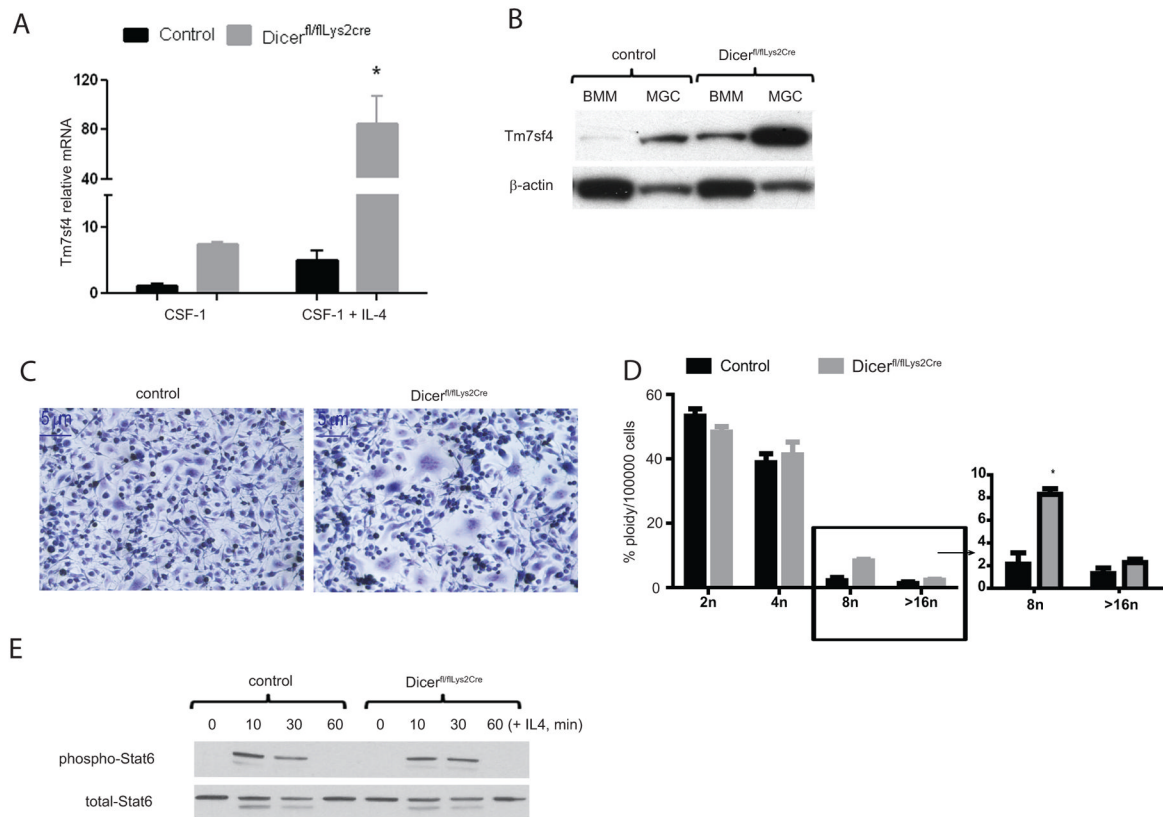
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**Figure 1. Deletion of Dicer mRNA in bone-marrow derived macrophages (BMM) reduces miRNA levels**

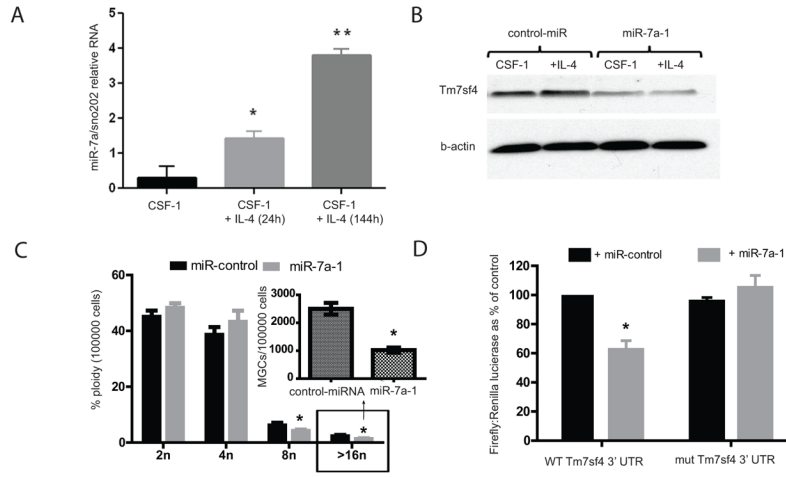
Quantification of Dicer mRNA levels in control (Dicer<sup>fl/+, Lys2cre</sup>) and Dicer deficient (Dicer<sup>fl/fl, Lys2cre</sup>) macrophages, and analysis of microRNAs let-7a, miR-125a-5p and miR-7a expression in control and Dicer<sup>fl/fl, Lys2cre</sup> macrophages by qPCR.





**Figure 2. IL-4 induces Tm7sf4 expression and excess giant cell formation in Dicer<sup>fl/fl</sup>Lys2cre macrophages**

**A)** qPCR of resting and IL4-induced (24 h) Tm7sf4 mRNA in control relative to Dicer<sup>fl/fl</sup>Lys2cre macrophages. Error bars denote SEM, asterisk represents significance; p value of < 0.05, representative of five independent experiments. **B)** Immunoblotting of Tm7sf4 protein expression in macrophages (BMM; CSF-1 derived) or IL-4 derived multinucleated giant cells (MGC; CSF-1/IL-4 derived) in control or Dicer<sup>fl/fl</sup>Lys2cre cells, representative of n = 3 experiments. **C)** Dicer deficiency increases cellular fusion and multinucleated giant cell formation. Hematoxylin staining of control or Dicer<sup>fl/fl</sup>Lys2cre macrophages stimulated with IL-4 for 144h; representative of five experiments. Images are 100X original magnification. **D)** Flow cytometric enumeration of the frequency of multinucleated cells in control or Dicer<sup>fl/fl</sup>Lys2cre multinucleated giant cells. Cells were stained with propidium iodide (PI). Bars indicate percent of cells against chromosomal copy number. **E)** Immunoblotting analysis of Dicer<sup>fl/fl</sup>Lys2cre cultures for total Stat6 protein and phospho-Stat6 response to IL-4, representative of n = 5 experiments.



**Figure 3. miR-7a is regulated by IL-4 and ectopic expression of miR-7a regulates Tm7sf4 mRNA and protein levels, leading to restricted giant cell formation**

**A)** qPCR analysis of miR-7a response to IL-4 in macrophages. Representative of three independent experiments. Asterisk denotes significance;  $p < 0.05$ . **B)** Immunoblotting of Tm7sf4 in macrophages expressing a control miRNA or miR-7a, cells were stimulated with IL-4 for 24 h and examined for Tm7sf4 expression relative to beta-actin. **C)** Flow cytometric analysis of multinucleated cells from control miRNA or miR-7a expressing cells. Asterisk denotes significance;  $p < 0.05$ . **D).** HEK 293 cells co-transfected with WT or mutated Tm7sf4 3' UTR and miR-7a, then assessed for luciferase activity. Asterisk denotes significance;  $p < 0.01$ .