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Cutting Edge: MicroRNA Regulation of Macrophage Fusion into Multinucleated Giant Cells

James R. Sissons, Jacques J. Peschon, Frank Schmitz, Rosa Suen, Mark Gilchrist, and Alan Aderem

Cellular fusion of macrophages into multinucleated giant cells is a distinguishing feature of the granulomatous response to inflammation, infection, and foreign bodies (Kawai and Akira. 2011. *Immunity* 34: 637–650). We observed a marked increase in fusion of macrophages genetically deficient in Dicer, an enzyme required for canonical microRNA (miRNA) biogenesis. Gene expression profiling of miRNA-deficient macrophages revealed an upregulation of the IL-4–responsive fusion protein Tm7sf4, and analyses identified miR-7a-1 as a negative regulator of macrophage fusion, functioning by directly targeting Tm7sf4 mRNA. miR-7a-1 is itself an IL-4–responsive gene in macrophages, suggesting feedback control of cellular fusion. Collectively, these data indicate that miR-7a-1 functions to regulate IL-4–feedback control of cellular fusion. The formation of 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 SIRPα) (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.

MicroRNAs (miRNAs) are 21- to 23-nt noncoding 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′-noncoding regions of target mRNAs. Several miRNAs are responsive to TLR agonists and function to dampen or reshape innate immune responses within macrophages (11). In this study, 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 Dicerfl/fl (12) with the C57BL/6.129 Lyz2tm1(cre) deleter strain (13) to generate C57BL/6 Dicerfl/flLyz2tm1(cre)/+ mice. Throughout experiments, age- and sex-matched C57BL/6 Dicerfl/fl and C57BL/6 Dicerfl/flLyz2tm1(cre)/+ mice were used as controls. Mice obtained from The Jackson Laboratory (Bar Harbor, ME) were housed under specific pathogen-free conditions with approval of the Institutional Animal Care and Use Committee at the Institute for Systems Biology.

Tissue culture

Bone marrow-derived macrophages (BMM) were cultured in complete DMEM (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, and then, mouse IL-4 (50 ng/ml; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE36585. Address correspondence and reprint requests to Dr. Alan Aderem, Seattle Biomedical Research Institute, 307 Westlake Avenue North, Suite 500, Seattle, WA 98109. E-mail address: alan.adерем@seattlebiomed.org

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PeproTech) was added for an additional 6 d. Human embryonic kidney cells (HEK 293) for luciferase assays were grown in complete DMEM. Macrophages derived from conditionally immortalized Hoxb8 progenitors were generated from C57BL/6 mice essentially as previously described (14) and maintained in their myeloid progenitor stage in RPMI 1640 medium, 10% FBS, 2 mM l-glutamine, 10 ng/ml GM-CSF-1, and 1 μM β-estradiol. To generate Hoxb8 macrophages from wild-type and miRNA-transduced lines, progenitors were harvested, washed twice in 1× FBS, 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 miRNA was labeled and hybridized to an Affymetrix GeneChip Mouse Exon ST 1.0 array. Expression arrays were acquired from six microarray hybridization experiments comprising three Dicer<sup>+/−</sup>Lyz2<sup>tm1(cre)/+</sup> mutant-derived macrophages and three Dicer<sup>+/−</sup>Lyz2<sup>tm1(cre)/+</sup> controls. Data were background-subtracted and normalized and then averaged across biological replicates using the log2 intensities (15, 16). Significance testing was performed using log2 intensities and expression cutoff value of 6 with a Student t test; p < 0.01 was regarded as significant. Gene array data are deposited in the National Center for Biotechnology Information-Gene Expression Omnibus accession number GSE36585 (http://www.ncbi.nlm.nih.gov/projects/gds/query/acc.cgi?acc=GSE36585).

Retroviral transduction of miR-7a-1

Genomic sequences encoding pri-miR-7a-1 were cloned corresponding to genetic coordinates Chr1:58493908 and Chr13:58494429. The product was cloned into a pmMSCV-Puro-IREs-GFP vector (17), and a control construct in the same backbone was generated encoding a pri-miR-30–formatted short hairpin RNA against Tfr5, a gene not expressed by mouse BMM. Retroviral constructs were packaged using the Phoenix ecotropic 293 line. Macrophages were transduced and selected in puromycin for 21 d prior to analysis.

Tm7sf4 3’-untranslated region cloning, mutagenesis, and luciferase reporter assays

The 3’-untranslated region (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 pViron-blasti (Invitrogen) 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 cotransfected with luciferase and miRNA expressing vectors and assayed using a luminometer.

DNA staining to determine giant cell ploidy

Ploidy staining was performed as described previously (18). Flow cytometry was performed using a BD FACSaria II, and ploidy classes for giant cells were determined.

Gene expression analysis

To analyze recombination of Dicer alleles, expression of genes and miRNAs, total RNA was isolated from macrophages using TRizol (Invitrogen), treated with DNase (Ambion), and used as template for reverse transcription (Superscript II; Invitrogen). Quantitative PCRs (qPCRs) were performed using an ABI 2900 HT thermocycler, and expression levels were calculated using the ΔCT method relative to control Ef1a gene. miRNA-specific reverse transcription was performed for each miRNA using gene-specific primers (Applied Biosystems). miRNA levels were normalized to small-nucleolar-RNA sno202. The levels of Sno202 were unchanged by CSF-1 and IL-4 or by a variety of TLR agonists in the primary BMM or Hox macrophage systems.

Western blotting analyses

Western blotting analyses were performed using standard techniques, and membranes were probed with relevant primary Abs: rabbit anti-Stat6 (Cell Signaling Technology), rabbit anti–phospho-Stat6 (Abcam), rabbit anti–mouse-Tm7sf4 (Santa Cruz Biotechnology), rabbit anti–mouse β-actin 1-HRP (Jackson ImmunoResearch Laboratories).

Statistical analysis

Significance was determined using an unpaired two-tailed Student 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 Th2-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<sup>fl/lt</sup>, 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<sup>+/−</sup>, Lys2Cre. Dicer mRNA levels were reduced ~75% in Dicer<sup>fl/lt</sup>, 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 (J.R. Sissons and J.J. Peschon, 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. 1).

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, chitinase-3-like-3 (Chi3l3/Ym1), and resistin-like molecule-α (Retnla/Fizz) is unaffected by Dicer deletion (Supplemental Fig. 1B). IL-4 additionally induces the expression of genes involved in macrophage fusion. Among this panel of genes, only Tm7sf4, a molecule required for macrophage fusion (20–22), was significantly altered in Dicer-deficient cells (Supplemental Fig. 1A). These data were confirmed by quantitative RT-PCR and Western blot analyses of resting and IL-4–stimulated macrophages (Fig. 2A, 2B) and suggests a unique role for Dicer, and hence miRNAs, in the control of Tm7sf4 expression. Extended culture of BMMs in IL-4 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/lt</sup>, Lys2Cre relative to control cells (Fig. 2C, 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/lt</sup>, Lys2Cre cultures relative to controls, we examined whether Dicer<sup>fl/lt</sup>, Lys2Cre macrophages were globally hyperresponsive to IL-4. This was not the case. For example, IL-4–induced Stat6 phosphorylation and dephosphorylation were identical in Dicer<sup>fl/lt</sup>, Lys2Cre and control macrophages.
Furthermore, another IL-4–responsive fusogenic molecule, E-cadherin (Cdh1), was unaffected in Dicerfl/fl, Lys2Cre macrophages (Fig. 1), demonstrating the specificity of regulation of Tm7sf4 by miRNA. Finally, the expression of a number of macrophage fusogenic molecules was unaffected in Dicerfl/fl, Lys2Cre macrophages (Supplemental Fig. 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 among 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). To distinguish between miR-7a-1 and miR-7a-2, we turned to epigenetic mapping (Supplemental Fig. 2). These two miRNAs are found on chromosomes 13 and 7, respectively. Histone acetylation results in an open chromosome structure that facilitates transcription. Chromatin immunoprecipitation-sequencing experiments in IL-4–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 (Supplemental Fig. 2). We therefore considered miR-7a-1 to be a candidate miRNA regulating both Tm7sf4 expression and IL-4–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–overexpressing BMMs (Fig. 3B). Moreover, IL-4–induced multinucleated giant cell formation was attenuated in miR-7a-1–overexpressing BMMs (Fig. 3B). To determine whether 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 firefly-luciferase reporter. The resulting construct was tested in HEK 293 cells cotransfected with either pri-miR-7a-1 or a control pri-miR-30. Decreased Tm7sf4 expression was specifically observed in miR-7a-1–overexpressing BMMs (Fig. 3B). Moreover, IL-4–induced multinucleated giant cell formation was attenuated in miR-7a-1–overexpressing BMMs (Fig. 3B).
and specifically with the Tm7sf4 3′-UTR to regulate its expression. Our study identifies miR-7a-1, an IL-4–responsive miRNA, as a critical negative regulator of IL-4–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), whereas miR-150 controls B cell differentiation (28), and miR-223 regulates hematopoietic cell differentiation (27), whereas 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, to our knowledge, that miRNA can fine-tune the macrophage differentiation response to IL-4. More specifically, miR-7a-1 acts as a negative regulator of Tm7sf4 and as a macrophage fusion rheostat. An environment of chronic Th2 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.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary figure 1

Expression level (log2)

A

B

Sirpa  Cd44  Cd81  Cd9  P2x7  Gja1  Lgals  Mrc1  Slc3a2  Anpep  Psen2  Sfpi1  Nfkb1

Ly6a  Cd11b  Itgax  Flrt2  Dhrs9  Atp6v0d2  Ccl8  Bex1  Il1rl2  Clec7a  Mmp12  Mmp13

Fabp4

control + IL4 control + IL4 control + IL4 Dicer

fl/flLys2Cre + IL4 Dicer fl/flLys2Cre + IL4 Dicer fl/flLys2Cre + IL4

7        10       13        7       10       14

7        10       14
Supplementary figure legends

Supplement 1. A) Transcriptional array analysis of select macrophage fusion molecules or related modulators. IL-4 responsive genes were analyzed from Dicer^{fl/fl,Lys2cre} macrophages and controls stimulated with IL-4 for 72 h (n = 3 biological replicates). Red, higher expression; green, lower expression in the Dicer^{fl/fl,Lys2cre} versus control macrophages. B) Transcriptional array analysis of Dicer^{fl/fl,Lys2cre} BMM stimulated with IL-4 for 72h. IL-4 responsive genes were analyzed from Dicer^{fl/fl,Lys2cre} macrophages and controls stimulated with IL-4 for 72 h (n = 3 biological replicates). Total RNA was used for a microarray analysis to determine mRNA expression differences between control and Dicer^{fl/fl,Lys2cre} macrophages. The top 20 differentially expressed IL-4 responsive genes from transcriptional arrays are shown and were expressed in equal amounts in Dicer^{fl/fl,Lys2cre} macrophages compared to controls; note expression of IL-4 responsive genes Retnla, Chi3l3, and Arg1, while Tm7sf4 was expressed at higher amounts. Red, higher expression; green, lower expression in the Dicer^{fl/fl,Lys2cre} versus control macrophages.

Supplement 2. Epigenetic mapping of miR-7ab isoforms A) Chip-Seq of bone marrow macrophages stimulated with IL-4 for 24h. Wild-type BMM were stimulated with IL-4 for 24h, followed by chromatin immuno-precipitation with anti-acetylated histone-4 antibody, purified chromatin was subject to RNA-sequencing. Note histone-4 acetylation corresponding to the coordinates of miR-7a-1, but not miR-7a-2 or miR-7b.