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

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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 *Journal of Immunology*, 2012, 189: 23–27.

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 IFN-dominated response, whereas proinflammatory 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-γ induces a classically activated state characterized by expression of TNF-α and IL-12, which together promote Th1- or Th17-dominated responses. In contrast, macrophages activated by the Th2 cytokines IL-4 and IL-13 participate in wound repair, immune suppression, and defense against parasites (2, 4, 5). In addition, IL-4 and IL-13 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 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 cytoplasmic 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) (13) mice. Throughout experiments, age- and sex-matched C57BL/6 Dicerfl/fl;Lyz2tm1(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 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;
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^{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^{+/f}, Lys2Cre. Dicer mRNA levels were reduced ∼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 (J.R. Sissons and J.J. Peschon, unpublished observations) and are in agreement with previous reports using the Lys2-cre deletor 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 IL-4. IL-4 programs macrophages to dampen immune responses, repair damaged tissue and participate in defense against parasites (2, 4). The expression of classic markers of IL-4 macrophage activation including arginase 1, chitinase-3 like-3 (Chi3l3/Ym1), and resistin-like molecule-α (Retnl1a/Fizz) is unaffected by Dicer depletion (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^{fl/fl}, 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^{fl/fl}, Lys2Cre cultures relative to controls, we examined whether Dicer^{fl/fl}, 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^{fl/fl}, Lys2Cre and control macrophages (Fig. 1B).
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. 3C). 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. 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 (Fig. 2E). 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).

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FIGURE 2. IL-4 induces Tm7sf4 expression and excess giant cell formation in Dicerfl/fl,Lys2Cre macrophages. (A) qPCR of resting and IL-4–induced (24 h) Tm7sf4 mRNA in control relative to Dicerfl/fl,Lys2Cre macrophages. Error bars denote SEM; *p < 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 Dicerfl/fl,Lys2Cre cells; representative of n = 3 experiments. (C) Dicer deficiency increases cellular fusion and multinucleated giant cell formation. Hematoxylin staining of control or Dicerfl/fl,Lys2Cre macrophages stimulated with IL-4 for 144 h; representative of five experiments. Original magnification ×100. (D) Flow cytometric enumeration of the frequency of multinucleated cells in control or Dicerfl/fl,Lys2Cre multinucleated giant cells. Cells were stained with propidium iodide. Bars indicate the percentage of cells against chromosomal copy number. *p < 0.05. (E) Immunoblotting analysis of Dicerfl/fl,Lys2Cre cultures for total Stat6 protein and phospho-Stat6 response to IL-4; representative of n = 5 experiments.
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

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