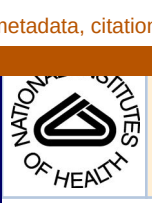

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Global microRNA expression is essential for murine mast cell development in vivo

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Abstract

microRNAs (miRNAs) are small, non-coding RNAs that have been shown to play a critical role in normal physiology and disease, such as hematopoietic development and cancer. However, their role in mast cell function and development is poorly understood. The major objective of this study was to determine how global miRNA expression affects mast cell physiology. The RNase III endonuclease, Dicer, is required for the processing of pre-miRNAs into mature miRNAs. To investigate the effect of global miRNA depletion on mast cells in vivo, we generated a mast cell-specific knock out of Dicer in mice. Transgenic mice (*Mcpt5*-Cre) that express Cre selectively in connective tissue mast cells were crossed with mice carrying the floxed conditional *Dicer* allele (*Dicer fl/fl*). *Mcpt5*-Cre x *Dicer fl/fl* mice with homozygous Dicer gene deletion in mast cells were found to have a profound mast cell deficiency with near complete loss of peritoneal, gastrointestinal, and skin mast cells. We examined the in vivo functional consequence of mast cell-specific Dicer deletion using an IgE-dependent passive systemic anaphylaxis (PSA) murine model. IgE sensitized wild type *Mcpt5*-Cre x *Dicer* +/+ and heterozygous *Mcpt5*-Cre x *Dicer fl/+* mice show marked hypothermia with antigen; however, homozygous *Mcpt5*-Cre x *Dicer fl/fl* mice were completely unresponsive to antigen challenge. These studies suggest a critical role for Dicer and miRNA expression for establishment of tissue compartments of functional mast cells in vivo.

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Introduction

Mast cells are critical effectors of allergic and inflammatory responses [1]. They also participate in normal innate immune responses to bacteria and parasites [2]. Dysregulated proliferation of mast cells manifest in diseases that range from benign cutaneous mastocytosis to mast cell leukemia [3]. They are derived from hematopoietic stem cells in the bone marrow, but they migrate and reside in the connective tissue of skin, lung, and gastrointestinal tract mucosa. Normal mast cell development is dependent on a network of transcription factors [4] which coordinate the expression of critical gene targets. The role of epigenetic regulators of gene expression in mast cells, such as miRNAs, has not been extensively studied.

miRNAs are small, non-coding RNA nucleotides, about 18 to 24 base pairs in size, and are expressed in a tissue-specific and developmentally-regulated fashion. They function primarily as negative regulators of protein expression. The RNase III endonuclease, Dicer, is necessary for mature, double-stranded miRNAs. Inhibition of Dicer by RNAi or gene targeting results in global depletion of miRNA expression [5,6]. miRNA profiling experiments have identified the expression pattern of miRNAs in bone marrow derived mast cells (BMMC) during development [7,8]. Our group previously identified the miR-381 and miR-539 cluster that regulates Mitf expression in response to c-Kit signaling [9]. Other investigators have shown roles for miRNAs in cell cycle regulation and proliferation, as well as apoptosis and degranulation [10–13]. Global depletion of miRNAs through deletion of Dicer function has been demonstrated to have critical roles in normal differentiation and function of myeloid cells such as neutrophils, macrophages, and dendritic cells [14–16]. The role of global depletion of miRNA in mast cells is yet unexplored. In order to address this question, we generated mice with a mast cell-selective deletion of Dicer by crossing the *Mcpt5*-Cre strain with Dicer fl/fl strain and found a profound loss of tissue mast cells. These studies demonstrate a critical role for miRNA expression in mast cell development.

Materials and Methods

Animals

Mcpt5-Cre mice were kindly provided by Dr. Axel Roers from Dresden, Germany [17]. These mice harbor a transgene in which the coding region of the first exon of the Mcpt5 gene is replaced with a Cre-encoding cDNA; Cre is expressed in the connective tissue mast cells of skin and peritoneum [17,18]. *Dicer fl/fl* mice were obtained from the Jackson Laboratory (Bar Harbor ME) and previously described [5]. These two strains are on the C57BL/6 background. Six to 12 week old *Mcpt5*-Cre x *Dicer fl/fl* mice were used to obtain splenocytes and bone marrow and were also used for the anaphylaxis experiments. Mice were maintained in the Johns Hopkins University Animal Facilities in strict accordance with institutional guidelines. All experiments were approved by the Johns Hopkins University Animal Care and Use Committee.

Passive systemic anaphylaxis (PSA) and active systemic anaphylaxis (ASA)

The passive and active systemic anaphylaxis experiments were previously described [19]. For passive systemic anaphylaxis, mice were given 10 ug of anti-DNP IgE and were challenged 24 hours later with 1 mg of DNP-HAS antigen (Sigma-Aldrich, St Louis) intravenously. For active systemic anaphylaxis, mice were immunized by intraperitoneal injection of 50 mcg OVA mixed with 1 mg Alum and challenged 2 weeks later by 1 mg OVA intravenously. For both passive and active anaphylaxis models, body temperature and clinical scores, including survival were recorded every 10 minutes up to 90 minutes after challenge. The t-test was used for analysis of body temperature change and clinical scores. The log-rank (Mantel-Cox) test (chi-square) was used for survival analysis. At the completion of the passive and active anaphylaxis experiments, mice were euthanized with a combination of Ketamine/Xylazine (400 mg/40 mg/kg) given intraperitoneally.

Quantification of tissue mast cells

Indicated tissues from mice of different genotypes were harvested and fixed in 10% buffered formalin, sectioned and stained with 0.5% toluidine blue (Sigma Aldrich, St. Louis, MO). For each sample, mast cells, as identified by the presence of metachromatic granules stained by toluidine blue. 5 to 10 fields were counted under 50X magnification. Average numbers of mast cells in a given field are represented. For identification of mast cells in the peritoneal cavity, peritoneal fluid was obtained by lavage, cytospined, and stained with Wright giemsa. Total cells and mast cell percentage were determined by counting cells from 5 to 10 fields under 50X magnification.

Results and Discussion

Mast cell-specific deletion of Dicer results in loss of tissue mast cells

Mice with homozygous mast cell deletion of Dicer *(Mcpt5*-Cre/*Dicer fl/*fl, herein referred to as MC-Dicer −/−) and heterozygous deletion of Dicer (MC-Dicer +/−) were viable and healthy and indistinguishable from wild type (*Mcpt5-Cre/Dicer* +/+) mice. However, there was a striking reduction of peritoneal mast cells in MC-Dicer −/− mice compared to wild type mice. In addition, mast cells in the skin and submucosa of stomach were profoundly reduced compared to tissues of wild type mice (Figure 1). Other tissues, including lung, small intestine, submucosa of stomach, and colon, were also examined. These tissues had almost no detectable mast cells by toluidine blue staining from both wild type and MC-Dicer −/− mice (data not shown). The expression of Cre in the *Mcpt5*-Cre mice is restricted to mast cells; other hematopoietic cell types, including basophils, are not affected (supplementary data).

MC-Dicer −/− mice are resistant to anaphylaxis

We examined the in vivo functional consequence of mast cell-specific Dicer deletion using an ovalbumin (OVA) allergen induced active systemic anaphylaxis (ASA) murine model [19]. As shown in Figures 2A and 2B, WT mice showed clear anaphylactic responses including significant drop in body temperature, severe clinical symptoms and death. However, MC-Dicer −/− mice showed only mild changes in body temperature, mild clinical symptoms, and no death. Five of 10 control WT mice did not survive to completion of the

experiment compared to 0 of 7 MC-Dicer −/− mice (p=0.034). There was also a statistically significance difference in clinical symptoms (score 3.7 in WT versus 1.0 in MC-Dicer −/−, p=0.0003).

We also examined mast cell-specific Dicer deletion in the passive systemic anaphylaxis (PSA) murine model which is dependent on mast cells [19,20]. As shown in Figure 2C, IgE sensitized WT mice showed symptoms of anaphylaxis as evidenced by marked hypothermia with antigen; however, MC-Dicer −/− mice were completely protected against systemic anaphylaxis. These results indicate that in the absence of Dicer function and mast cells do not mature normally; mast cell-dependent allergic responses require connective tissue mast cells rather than mucosal mast cells. The mild responses of MC-Dicer −/− mice in the ASA model may be mediated by basophils and macrophages, which would not be expected to be affected in the *Mcpt5*-Cre model [21].

Taken together, these studies suggest a critical role for Dicer and miRNA expression for establishment of tissue compartments of connective tissue mast cells in vivo. In our model, Dicer is deleted in committed mast cell precursors, with expression of the protease, Mcpt-5. The absence of connective tissue mast cells may be due to the requirement for Dicer in differentiation, migration, survival, or proliferation. However, this central question of how Dicer and microRNAs regulate the population of tissue mast cell compartments is yet to be addressed.

A challenge to further examining the mechanism of Dicer deletion on mast cell function and development is the lack of tissue mast cells to study in our in vivo model. BMMCs are a useful tool to examine mast cell differentiation and function ex vivo. However, because of low expression of Cre, BMMCs from these MC-Dicer −/− mice are not a suitable model system to examine Dicer function [17,22]. In BMMCs from MC-Dicer −/− mice, we found incomplete deletion of Dicer; and no discernable phenotypic differences in granule expression by toluidine blue stain and c-KIT/FcεRIα expression compared to WT BMMCs (supplementary data). We found that MC-Dicer −/+ mice have no significant differences in tissue mast cell populations and susceptibility to active or systemic anaphylaxis, suggesting that subphysiologic expression of Dicer is sufficient for normal mast cell development. Further studies of the mechanisms of Dicer function in mast cells will be aided by other approaches, such as inducible systems to delete Dicer in vivo and ex vivo.

In hematopoietic stem cells, Dicer has been shown to regulate the stem cell and progenitor cell pool; conditional deletion of Dicer results in increased apoptosis of this compartment [23]. In committed hematopoietic cells, loss of Dicer affects different physiologic functions. Dicer deletion in early myeloid progenitors (using a Cre driven by C/EBPA) results in severe reduction of macrophages and dysplastic neutrophils [14]. Using a different model with a Lyz2-Cre strain, Dicer deletion resulted in an increase in giant cell formation of macrophages [16]. In both T and B- lymphocytes, Dicer deletion can result in aberrations in differentiation or activation, depending the cell type and stage of development in which Dicer function is inhibited [24]. In our study, loss of Dicer function in committed mast cell progenitors results in profound loss of tissue mast cell compartments, suggesting a critical role for miRNAs in mast cell differentiation, growth or migration. Further studies are needed

to determine the essential miRNAs that mediate these functions and their potential impact on allergic and inflammatory diseases.

Supplementary Data. Incomplete deletion of Dicer in MC-Dicer −/− BMMC A) PCR from BMMC from wild type (*Mcpt5*-Cre x *Dicer* +/+) and MC-Dicer −/− (*Mcpt5*-Cre x *Dicer fl/fl*) is shown. Wild type allele denoted by * and floxed allele denoted by **. The deleted allele is shown with arrow. PCR from BMMC from two Dicer +/+ and two Dicer fl/fl mice shown. Incomplete deletion noted by the presence of floxed, and undeleted allele **. Cytokines for culture condition shown on left. B) BMMCs from MC-Dicer −/− mice show no difference in differentiation by co-expression of FcεRIα and c-Kit compared to wild type. C) Basophils cultured from MC-Dicer −/− mice show no difference in differentiation by co-expression of FcεRIα and CD49b compared to wild type. Two independent experiments shown for BMMC and basophil cultures.

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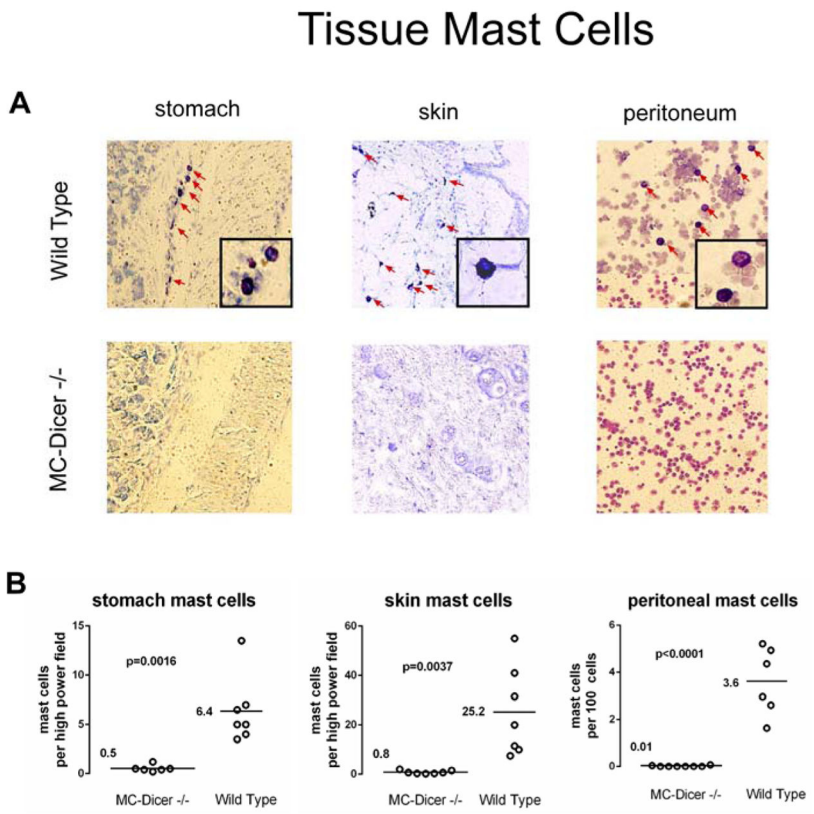

Figure 1.

Loss of tissue mast cells in mice with mast cell-specific deletion of Dicer. A) Tissue sections of stomach (submucosa), skin and peritoneum demonstrate near absence of mast cells in MC-Dicer −/− tissue. Mast cells denoted by red arrows. Stomach and skin mast cells stained with Toluidine blue and peritoneal mast cells stained with Wright Geimsa. Magnification 50x with insert 100X. B) Quantitation of mast cells in tissue sections demonstrating significant reduction in MC-Dicer −/− tissue compared to wild type tissue. Average numbers of mast cells per high power field are plotted for skin and stomach. Peritoneal mast cell numbers per total number of peritoneal cells are plotted.

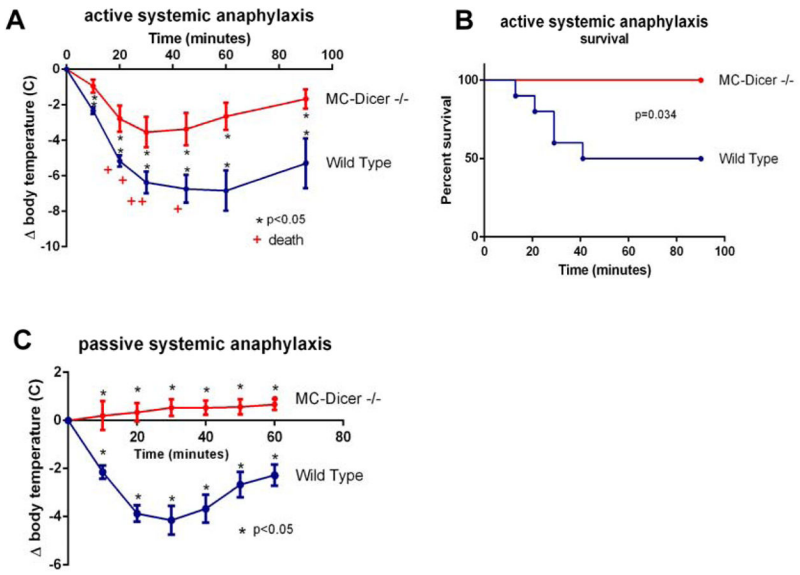

Figure 2.

Mast cell deletion of Dicer protects against anaphylaxis. A) MC-Dicer −/− mice are protected against active systemic anaphylaxis. MC-Dicer −/− show blunted drop in body temperature after challenge with antigen compared to wild type mice. WT (n=10); MC-Dicer −/− (n=7). B) Increased survival of MC-Dicer −/− mice in active systemic anaphylaxis model. Half of wild type mice do not survive after antigen challenge, while all MC-Dicer −/− mice survive. C) MC-Dicer −/− mice are resistant to passive systemic anaphylaxis. MC-Dicer −/− show no drop in body temperature with antigen challenge, while wild type mice show significant decrease in body temperature. WT (n=4); MC-Dicer −/− (n=3).