Essential Role of MicroRNA-155 in the Pathogenesis of Autoimmune Arthritis in Mice

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Objective. MicroRNAs (miRNA) are a new class of regulatory elements. Altered expression of miRNA has been demonstrated in the inflamed joints of patients with rheumatoid arthritis (RA). The aim of this study was to examine the role of miRNA in the pathogenesis of autoimmune arthritis, using 2 murine models.

Methods. Collagen-induced arthritis (CIA) and K/BxN serum-transfer arthritis were induced in wild-type (WT) and miR-155–deficient (miR-155−/−) mice. The severity of arthritis was determined clinically and histologically. Anticollagen antibodies and cytokines were measured by enzyme-linked immunosorbent assay. The cellular composition of the draining lymph nodes after induction of CIA was measured by flow cytometry.

Results. The miR-155−/− mice did not develop CIA. Deficiency in miR-155 prevented the generation of pathogenic autoreactive B and T cells, since anticol-lagen antibodies and the expression levels of antigenspecific T cells were strongly reduced in miR-155−/− mice. Moreover, Th17 polarization of miR-155−/− mouse T cells was impaired, as shown by a significant decrease in the levels of interleukin-17 (IL-17) and IL-22. In the K/BxN serum-transfer arthritis model, which only depends on innate effector mechanisms, miR-155−/− mice showed significantly reduced local bone destruction, attributed to reduced generation of osteoclasts, although the severity of joint inflammation was similar to that in WT mice.

Conclusion. These results demonstrate that miR-155 is essentially involved in the adaptive and innate immune reactions leading to autoimmune arthritis, and therefore miR-155 might provide a novel target for the treatment of patients with RA.

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease affecting ~1% of the population and is one of the most prevalent causes of disability worldwide (1). Its pathogenesis, however, although studied intensively, has not yet been clarified. Evidence from animal models and from therapeutic trials in patients with RA has suggested an involvement of T cells, B cells, osteoclasts, and several cytokines in the induction and/or perpetuation of the disease (2–8). However, because a substantial number of RA patients still do not respond sufficiently to current treatment options, the development of new therapeutic targets is needed (9). Evidence has emerged to indicate that microRNAs (miRNA) play a crucial role as posttranscriptional regulators of gene expression (10–12). MicroRNA-mediated gene regulation normally results in a reduction of the total amount of target protein that is produced. However, the fate of most messenger RNAs (mRNA) that are targeted by miRNA remains unclear.

The miRNA have been shown to repress expression of some of their target genes at the translational level, with mRNA levels remaining constant and the level of the encoded protein declining, whereas in other cases, miRNA repress target gene expression by triggering the degradation of target mRNA (13,14). In the first years after the discovery of miRNA, they were thought to be involved mostly in controlling proliferation, cell differentiation, or apoptosis (15–17). More recently, it has become clear that they also exert powerful regulatory functions in cancer development and the immune

1281
system, including regulation of T cells, B cells, and dendritic cells (DCs) (11,14,18–22).

The miRNA miR-155 has been detected in the joints and synovial fibroblasts of patients with RA and has been found to be involved in the regulation of matrix metalloproteinase 3 (23). RA is a disease characterized by chronic, destructive arthritis resulting from complex alterations of not only the adaptive immune system, but also the innate immune system. Whereas the adaptive immune system is thought to play an important role mainly in the initial phase of RA, disturbances of the innate immune system become more important at later stages of the disease (24). We therefore investigated the role of miR-155 in the pathogenesis of autoimmune-mediated arthritis, by analyzing 2 different animal models, each of them representing a main immune system pathway in RA. The collagen-induced arthritis (CIA) model depends on adaptive immune responses, whereas the K/BxN serum-transfer arthritis model depends on alterations of the innate immune response.

MATERIALS AND METHODS

Antibodies and reagents. The following antibodies were used: CD11b, F4/80, and Neu7/4 (rat anti-mouse) (Serotec), CD11c and CD80 (hamster anti-mouse) (BD Biosciences), Gr-1, CD25, CD40, CD86, I-A/I-E, CD44, and CD62L (all rat anti-mouse) (BD Biosciences), CD4 (rat anti-mouse) (Beckman Coulter), and CD69 hamster anti-mouse (PharMingen). The anticollagen enzyme-linked immunosorbent assay, Mouse Th1/Th2 10plex FlowCytomix Multiplex assay, and Flow Cytomix Simplex assay for interleukin-2 (IL-22) and IL-23 were obtained from Bender Medsystems, and all were used in accordance with the manufacturer’s protocols.

Mice. Breeding pairs of miR-155−/− mice were obtained from The Jackson Laboratory. Generated miR-155−/− mice and miR-155+/− littermates were used for this study. All experiments were approved by the local ethics committee.

Induction of CIA. Mice were immunized subcutaneously with 50 μg chicken type II collagen (Sigma-Aldrich) in 50 μl H2O, emulsified in 50 μl Freund’s complete adjuvant that was enriched with 10 μg/ml Mycobacterium tuberculosis (H37Rv; Difco/BD Biosciences), on day 1 and day 21. Mice in this model are expected to develop arthritis between week 3 and week 10, and were evaluated weekly for symptoms of arthritis using a semiquantitative scoring system that includes the degree of joint swelling and grip strength. Briefly, joint swelling was examined using a clinical score ranging from 0 to 3 (0 = no swelling, 1 = mild swelling of the toes and ankle, 2 = moderate swelling of the toes and ankle, and 3 = severe swelling of the toes and ankle). In addition, the grip strength of each paw was analyzed using a wire, 3 mm in diameter, to determine grip strength scores ranging from 0 to −3 (0 = normal grip strength, −1 = mildly reduced grip strength, −2 = moderately reduced grip strength, and −3 = severely reduced grip strength). Animals were killed between week 3 and week 10 after disease induction.

Induction of serum-transfer arthritis. After intraperitoneal application of 150 μl of K/BxN serum on day 1 and day 3, mice were scored clinically every other day for the development of arthritis using a semiquantitative scoring system, similar to that used for CIA, and were thereafter killed on day 12 to prepare tissue samples for histology.

Evaluation of inflammation and local bone erosions by histologic examination. Hind paws were fixed in formalin overnight and then decalcified in EDTA until the bones were pellable. Serial paraffin sections (2 μm) were stained with hematoxylin and eosin (H&E) or stained for tartrate-resistant acid phosphatase (TRAP) activity. TRAP staining was performed as previously described (8). For exact quantification of the areas of inflammation, H&E-stained sections were evaluated using an Axioskop 2 microscope (Carl Zeiss Micro-Imaging) and Osteomeasure Analysis System (OsteoMetrics), which allows absolute quantification of areas in histologic sections. The sum of the areas of inflammation for each single mouse was calculated by evaluating all tarsal joints. The same H&E-stained sections were analyzed similarly for the quantification of erosions. In addition, given the established role of osteoclasts in the pathogenesis of local bone erosions, the number of osteoclasts was counted in TRAP-stained serial sections.

Flow cytometric analysis of the draining lymph nodes (DLNs). DLNs were harvested and passed through a nylon mesh to obtain single-cell suspensions. Cells were then stained with the indicated antibodies and analyzed by flow cytometry (BD Biosciences Facscount II, with Facsdiva software).

DLN cell cultures. DLN cells were harvested, passed through a nylon mesh, and cultured at a density of 1 × 10⁶ cells/ml in RPMI 1640 with l-glutamine plus 10% fetal calf serum (FCS), penicillin/streptomycin, and β₂-mercaptoethanol and stimulated with plate-bound anti-CD3 monoclonal antibodies (145-2C11). Supernatants were collected for cytokine analysis after 48 hours. Cells were then incubated for a further 18 hours in the presence of 1 μCi/well of ³H-thymidine, to quantify proliferation.

In vitro restimulation of spleen cells with collagen. Spleen cells were harvested, passed through a nylon mesh, and cultured at a density of 2 × 10⁶ cells/ml in RPMI 1640 with l-glutamine plus 10% FCS, penicillin/streptomycin, and β₂-mercaptoethanol and stimulated with 100 μg/ml of type II collagen for 72 hours. Cells were then incubated during the last 18 hours of culture in the presence of 1 μCi/well of ³H-thymidine, to quantify proliferation.

In vitro osteoclast assay. Bone marrow cells were isolated and cultured for 3 days in 100 ng/ml macrophage colony-stimulating factor (M-CSF) to enrich for monocyte/macrophages, and were then cultured in 10% FCS/Dulbecco’s modified Eagle’s medium supplemented with 30 ng/ml M-CSF and 50 ng/ml RANKL (both from R&D Systems) for another 3–4 days. Osteoclasts were detected by the presence of TRAP+ multinucleated cells (≥3 nuclei).

Statistical analysis. The unpaired t-test was used to test statistically significant differences. A P value less than or equal to 0.05 was considered significant.
RESULTS

Lack of development of CIA in miR-155−/− mice.
We first evaluated the role of miR-155 in the CIA model (25). We therefore immunized miR-155−/− and wild-type (WT) mice, both having a C57BL/6 background, with collagen and evaluated clinical signs of arthritis weekly. WT animals developed clinical signs of arthritis, such as an increase in paw swelling and decrease in grip strength, starting 4 weeks after the induction of CIA, with a progressive course over time. In miR-155−/− mice, however, we did not observe any clinical signs of arthritis at any time point during the observation period (Figures 1A and B).

At the end of the experiment, 9 weeks after the induction of CIA, we analyzed histologic sections from the hind paws of both groups of animals. In WT mice, we observed severe joint inflammation, which was characterized by marked infiltration of the synovial membrane with inflammatory cells (Figures 1C and D). In contrast, we did not detect any inflammatory cell infiltration of the synovial membrane in miR-155−/− mice. In addition, we observed local bone destruction as well as osteoclasts attached to these bone erosions in the hind paws of WT mice, but there was no evidence of bone erosions or osteoclasts in the miR-155−/− animals (Figures 1C and D). These findings demonstrate that the presence of miR-155 is required for the development of clinical as well as histologic signs of CIA.

Prevention of autoreactive B and T cell generation and inhibition of Th17 polarization by miR-155 deficiency after induction of CIA. To elucidate the mechanisms of protection from CIA in miR-155−/− mice in further detail, we first measured anticolonagen antibody levels in the serum of WT and miR-155−/− animals. We found that the levels of IgG were significantly decreased, with a reduction of ~80% of total anticolonagen IgG antibodies, in miR-155−/− mice compared to that in WT mice (Figure 2A). This defect was apparent for all IgG subclasses analyzed (results not shown). Anticolonagen IgM levels were not significantly different between the 2 groups (Figure 2A).

We next analyzed whether the relative numbers of B cells or activation state of B cells were altered in miR-155−/− mice when compared to WT mice. We therefore harvested DLNs from the mice and analyzed the B cell population, defined as B220+ cells, by flow cytometry. However, we did not detect significant differences in the relative numbers of B220+ cells in the
DLNs between WT animals and miR-155−/− mice (Figure 2B, left panel). In addition, the expression of costimulatory molecules, such as CD80 (Figure 2B, middle panel) or the class II major histocompatibility complex (MHCII) (Figure 2B, right panel), on B220+ cells was not different between the groups. Taken together, these results demonstrate that the diminished production of IgG is not accompanied by a reduction of IgM and cannot be attributed to reduced numbers of B cells or altered expression of costimulatory molecules or MHCII on B cells.

Since the generation of IgG antibodies requires T cell help and the pathogenesis of CIA is also crucially dependent on the formation of autoreactive T cells, we next analyzed the effect of miR-155 deficiency on T cell polarization and activation. To this end, we harvested cells from the DLNs of WT and miR-155−/− mice after the induction of CIA, stimulated the cells with anti-CD3, and analyzed the supernatants for signature cytokine levels. When we assessed the cytokines considered prototypic of Th1 and Th2 polarization, we found no difference in the levels of interferon-γ (IFNγ) and only a trend toward increased levels of IL-4 in miR-155−/− mice. However, production levels of the Th17 signature cytokines IL-17 and IL-22 were found to be significantly reduced in miR-155−/− mice compared to WT animals (Figure 3A).

To investigate T cell activation after the induction of CIA, we restimulated mouse spleen cells in vitro with type II collagen, which resulted in a reduced proliferation of T cells in miR-155−/− mice, indicating that antigen-specific T cell activation is strongly impaired in miR-155−/− mice (Figure 3B). Consistent with this finding, when we further characterized the CD4+ T cells in the DLNs in vivo, we detected significantly elevated proportions of CD62L+CD44− cells, indicative of a higher prevalence of naive T cells, in miR-155−/− mice compared to WT mice (Figure 3C, left panel). Furthermore, we found a significantly higher number of CD62L−CD44+ memory T cells in the DLNs of WT mice compared to miR-155−/− mice (Figure 3C, middle panel). Similarly, the percentage of CD69+ activated T cells was increased in WT mice compared to miR-155−/− mice (Figure 3C, right panel). However, we did not observe a general defect in T cell proliferation, since DNL cells from immunized WT and miR-155−/− mice showed no difference in T cell proliferation when the cells were activated with CD3 (results not shown).

Since the generation of an antigen-specific T cell response requires priming of naive T cells with antigen-presenting cells (APCs), we also analyzed the relative numbers and activation status of APCs in the DLNs. We found that DCs and macrophages were present in comparable relative numbers (Figure 3D) and displayed a similar expression of costimulatory molecules (CD80, CD86, and MHCII) in the DLNs of both groups of mice (Figure 3E). These results demonstrate that the absence of miR-155 has no influence on the numbers and activation status of APCs in our model.
Decreased systemic levels of IL-6 and IL-17 in miR-155−/− mice after the induction of CIA. To evaluate whether the reduction in the levels of Th17-associated cytokines is present only locally in the DLNs of miR-155−/− mice or whether it is also found systemically in the serum of these animals, we measured the serum levels of IL-6 and IL-17 after the induction of CIA. Indeed, we found significantly lower levels of IL-6 and IL-17 in the serum of immunized miR-155−/− mice compared to WT mice (Figure 4, left and middle panel).

**Figure 3.** Deficiency in microRNA-155 (miR-155−/−) in mice inhibits Th17 polarization after the induction of collagen-induced arthritis (CIA). A, Draining lymph nodes (DLNs) of wild-type (WT) mice (n = 9) and miR-155−/− mice (n = 10) were harvested 2 weeks after the induction of CIA, and levels of the cytokines interferon-γ (IFNγ), interleukin-4 (IL-4), IL-17, and IL-22 were measured in the supernatant by enzyme-linked immunosorbent assay after in vitro stimulation with anti-CD3 antibody. B, Splenes were harvested 9 weeks after induction of CIA, and the proliferation of spleen cells from WT mice (n = 10) and miR-155−/− mice (n = 9), without or with restimulation with 100 μg/ml type II collagen (CII) in vitro, was quantified by 3H-thymidine incorporation. Bars show the mean ± SD. C–E, DLNs of WT and miR-155−/− mice were harvested 9 weeks after induction of CIA, followed by flow cytometric analysis to determine the relative numbers of CD4+CD25+CD44− naïve T cells, CD4+CD25−CD44+ memory T cells, and CD4+CD69+ T cells (as a percentage of total CD4+ cells) (C), the relative numbers of CD11c+ dendritic cells (as a percentage of total DLN cells) (D), and the expression levels of CD80, CD86, and class II major histocompatibility complex (MHCII) on CD11c+ dendritic cells (as the mean fluorescence intensity [MFI]) (E) in WT and miR-155−/− mice. Bars in A, C, D, and E show the mean ± SD. * = P ≤ 0.05 versus WT.

**Figure 4.** Serum cytokine levels after the induction of collagen-induced arthritis (CIA) in mice with deficiency in microRNA-155 (miR-155−/−). Serum samples were obtained from wild-type (WT) mice (n = 10) and miR-155−/− mice (n = 9) 4 weeks after the induction of CIA. Levels of interleukin-6 (IL-6), IL-17, and interferon-γ (IFNγ) were analyzed by enzyme-linked immunosorbent assay. Bars show the mean ± SD. * = P ≤ 0.05 versus WT.
Moreover, consistent with the pattern observed in the DLNs, the serum levels of IFNγ were not significantly different between the groups (Figure 4, right panel). Taken together, these results show that miR-155+/− mice have profound deficiencies in the generation of pathogenic autoreactive T and B cell function, leading to a complete abrogation of CIA, which represents a model of RA mainly triggered by the adaptive immune system.

**Impairment of local bone destruction via reductions in osteoclast differentiation in miR-155+/− mice.** We next wanted to determine whether miR-155 deficiency also affects erosive arthritis that is mainly triggered by the innate immune system. We therefore used the K/BxN serum-transfer arthritis model. In this model, mice carrying the KRN transgene, encoding a T cell receptor recognizing a peptide of glucose-6-phosphate isomerase (GPI), are crossed into an Aβ2-positive genetic background such as that in NOD mice. The autoreactive T cells promote the production of vast quantities of anti-GPI antibodies. These antibodies are then transferred into WT recipients, and these antibodies are sufficient to induce an inflammatory erosive arthritis in the mice, even in the absence of a functioning adaptive immune system (26,27). We injected the arthritogenic serum intraperitoneally in WT and miR-155+/− mice. Both groups of animals developed arthritis, and there were no significant differences in the scores for paw swelling between miR-155+/− mice and WT animals (Figure 5A). Similarly, histologic evaluation revealed no significant differences in the extent of synovial inflammation (Figure 5B).

To our surprise, however, the grip strength scores were significantly higher in miR-155+/− mice when compared to WT mice (Figure 5C). This was accompanied by a significant reduction in the extent of bone erosions in miR-155+/− mice (Figure 5D).

Furthermore, we observed reduced numbers of osteoclasts in the hind paws of miR-155+/− mice (Figures 5E and F). The reduction in osteoclastogenesis in the inflamed joints of miR-155+/− mice may be attributed either to a reduced potential of myeloid cells to...
differentiate into osteoclasts or to a reduced production of pro-osteoclastogenic factors, such as RANKL, by mesenchymal cells, such as synovial fibroblasts. To differentiate between these 2 mechanisms, we performed in vitro osteoclast differentiation assays in cultures without mesenchymal cells. We stimulated bone marrow macrophages with M-CSF and RANKL. However, in line with the results obtained in vivo, we found a reduced generation of mature osteoclasts from bone marrow cells of miR-155 \(^{-/}\) mice compared to that in WT mice (Figure 5G), suggesting that there is a defect in RANKL-dependent osteoclastogenesis in myeloid cells in these mice.

Because osteoclastogenesis is a multistep event, including M-CSF–dependent proliferation of preosteoclasts and RANKL–dependent differentiation into mature osteoclasts (28), we next addressed the question of whether impaired osteoclastogenesis is due primarily to early-phase monocyte proliferation or if it could be attributed to late-phase osteoclast differentiation. We therefore stimulated bone marrow cells with M-CSF and measured the number of osteoclast precursor cells, defined as CD11b and F4/80 double-positive cells. Our results showed that miR-155 deficiency did not affect the number of these osteoclast precursor cells (Figure 5H), and therefore we can conclude that miR-155 is an important factor in late-stage osteoclast differentiation. These findings demonstrate that miR-155 deficiency protects from local bone destruction by reducing the generation of bone-resorbing osteoclasts.

**DISCUSSION**

The contribution of miRNA to the pathogenesis of complex systemic autoimmune diseases such as RA is not known. This study is the first to demonstrate that the lack of a single miRNA, miR-155, is sufficient to interfere with 2 major pathways of destructive arthritis, namely, the adaptive and innate immune systems. It has been previously reported that miR-155 is involved in antibody formation after immunization with KLH or tetanus toxoid (20,29,30). Indeed, miR-155 deficiency completely prevents the development of CIA, which is dependent on adaptive immune–regulatory mechanisms, by reducing the levels of pathogenic anticollagen autoantibodies. This reduction is apparent in all classes of IgG analyzed, namely, IgG1, IgG2a, and IgG2c. However, anticollagen IgM levels, B cell numbers, and the expression of costimulatory molecules on these cells are not altered by the absence of miR-155.

In addition, proliferation of antigen-specific T cells after restimulation with collagen is reduced in miR-155 \(^{-/}\) mice compared to WT animals. These results demonstrate that miR-155 also plays an important role in regulating autoreactive T cell responses in our model. Furthermore, after stimulation with an anti-CD3 antibody, miR-155 \(^{-/}\) mouse T cells showed reduced production of the Th17 cytokines IL-17 and IL-22, both of which have been shown to be important in the development of CIA (31,32). Therefore, our experiments demonstrate that after immunization with collagen, miR-155 \(^{-/}\) mice show a selective defect in Th17 polarization.

In vitro assays have previously shown that miR-155 \(^{-/}\) mouse T cells preferentially differentiate into Th2 cells, and in vivo immunization experiments using tetanus immunization have revealed decreased production of IFN\(\gamma\) and IL-2 in miR-155 \(^{-/}\) mouse T cells, indicating that Th1 polarization is reduced (20,29). Interestingly, in our study, after immunization with collagen, the production of IFN\(\gamma\) and IL-4 by miR-155 \(^{-/}\) mouse T cells was not affected after nonspecific stimulation with an anti-CD3 antibody.

The miRNA miR-155 also plays an important role in regulating innate effector mechanisms of destructive arthritis. In the K/BxN serum-transfer arthritis model, we found similar amounts of synovial inflammation in WT and miR-155 \(^{-/}\) mice. This was somewhat surprising, since previous studies have shown an important involvement of miR-155 in innate inflammation pathways (18,33). However, we noticed an uncoupling of inflammation and bone destruction, since osteoclast-mediated bone erosions were significantly reduced in miR-155 \(^{-/}\) mice compared to WT mice. In line with our in vivo findings, miR-155 deficiency also decreased RANKL–induced osteoclastogenesis in vitro.

Taken together, these findings show that miR-155 has a dual effect on controlling autoimmune–triggered destructive arthritis. On the one hand, miR-155 deficiency inhibits the generation of pathogenic self-reactive T and B cell responses. On the other hand, miR-155 controls the development of local bone destruction. These data identify miR-155 as a possible novel target in the treatment of autoimmune arthritis.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Redlich had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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