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IL-23 Receptor Regulation by Let-7f in Human CD4⁺ Memory T Cells

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Abstract

CD4⁺ memory T cells include the Th17 cell population, which has been shown to be implicated in autoimmune and inflammatory diseases. These memory T cells express higher IL-23R and produce more IL-17 compared with their naive counterparts. However, the molecular mechanisms that regulate IL-23R expression in human T cells are not completely understood. MicroRNAs play important roles in a wide range of biological events through posttranscriptional suppression of target mRNAs. In this article, we provide evidence that a specific microRNA, Let-7f, inhibits IL-23R expression in human CD4⁺ memory T cells. Endogenous expression of Let-7f in memory T cells is significantly lower when compared with naive T cells, and Let-7f blocks IL-23R expression through its complementary target sequence within 3' untranslated region of target gene. Furthermore, exogenous transfection of a Let-7f mimic into memory T cells results in downregulation of IL-23R and its downstream cytokine, IL-17. Our findings reveal a novel mechanism in regulating the IL-23/IL-23R pathway and subsequent downstream IL-17 production, which may provide novel therapeutics for human inflammatory and autoimmune diseases.

Interleukin-17 and IL-17–producing effector cells have been increasingly implicated in the pathogenesis of autoimmunity (1–6). The proinflammatory roles of IL-17 have been identified in several human inflammatory diseases, including multiple sclerosis (7, 8), rheumatoid arthritis (9), Crohn's disease (10), and psoriasis (11). The differentiation and development of IL-17–producing T cells are governed by its cytokine microenvironment. TGF- β and IL-6 are sufficient to induce the differentiation of naive murine T cells into Th17 cells (12–14), and IL-23 can expand and maintain the effector function of Th17 cells (15, 16). However, IL-23 or IL-1 β alone appear sufficient to drive human Th17 development (17–19). Thus, there exists a strong link between IL-23 and the Th17 response, suggesting that IL-23 is important for human Th17 effector function.

The IL-23R is a heterodimeric receptor complex composed of the IL-12R β 1 and IL-23R subunits (20). The IL-23R is mainly expressed on memory T cells, NK cells, and to a lesser extent, by dendritic cells and monocytes (20, 21). IL-23, the primary cytokine ligand for

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IL-23R, is composed of an IL-23-specific p19 subunit that associates with the IL-12 (and IL-23) p40 subunit to exert its biological function (22). JAK2 and STAT3 are the predominant signaling molecules for the IL-23/IL-23R pathway (20, 22). IL-23R expression is regulated by cytokines such as IL-6, IL-21, and even IL-23 itself (23). However, our understanding of IL-23R regulation is still presently very limited.

MicroRNAs (miRNAs) are a group of noncoding, small (~22-nucleotide) RNAs that posttranscriptionally regulate the expression of protein-coding genes (24). miRNAs are believed to either block mRNA translation or reduce mRNA stability after imperfect binding of the guide strand to miRNA-recognition elements within the 3' untranslated region (UTR) of their target genes (25). More than 1000 miRNAs have been identified in mammals, and most have been implicated in wide range of biological functions, including organ development, cellular differentiation, and homeo-stasis. Of note, it is estimated that miRNAs may regulate as much as one third of all mRNAs (26, 27). Although the precise functions of most miRNAs have yet to be elucidated, miRNAs are being increasingly recognized in the regulation of the immune system (28), including the development of T and B cells (29), differentiation and survival of immune cells (30), and stimulation of cytokines.

In this study, we hypothesized that miRNAs may participate in regulating the expression of IL-23R gene in human T cells. We compared the expression of IL-23R and miRNAs with putative binding sites in the 3'UTR of the IL-23R in memory and naive CD4⁺ T cells from the peripheral blood of patients undergoing endoscopic procedures. We found that Let-7f, miR-17, and miR-936 inversely correlated with IL-23R expression. Furthermore, mutating the seed region of the Let-7f binding site in the 3'UTR of IL-23R resulted in altered IL-23R gene expression. Over-expression of Let-7f in memory T cells decreased IL-23R and associated IL-17 gene expression. Our finding suggests that miRNAs may play an important role in the regulation of IL-23R and downstream IL-17 gene expression.

Materials and Methods

Primary cell isolation and culture

Whole peripheral blood was obtained from healthy individuals undergoing endoscopic procedure at The Johns Hopkins University using an Institutional Review Board-approved protocol. Mononuclear cells were isolated after centrifugation using a BD Vacutainer CPT Cell Preparation Tube with Sodium Citrate (Becton Dickinson), following the manufacturer's instructions. Naive and memory T cells were then isolated via magnetic beads with the Naive or Memory Human T cell isolation kit (Miltenyi Biotec), following the manufacturer's instructions. Purity as measured by flow cytometry was >90%. CD4⁺ T cells were cultured in RPMI 1640 medium containing 10% FBS (Life Technologies) supplemented with 2 mM L-glutamine (Life Technologies), penicillin/streptomycin. Both naive and memory T cells were activated with beads coated with anti-CD2, anti-CD3, and anti-CD28 (Miltenyi Biotec) and then cultured for 5 d (23). In some experiments, IL-2, IL-1 β , or IL-23 (R&D Systems) was added in the culture system.

ELISA and intracellular cytokine staining

The cytokine-producing capacity of naive and memory T cells was assessed by stimulation of cells (1×10^6 /ml) for 72 h with beads coated with anti-CD2, anti-CD3, and anti-CD28, and cultured for 5 d. Cytokine levels in culture supernatants were measured by ELISA according to manufacturer's instructions (BioLegend). Intracellular staining for IL-17 and IFN- γ was performed on T cells stimulated for 5 h with PMA and ionomycin in the presence of Golgistop (BD Biosciences) for the final 3 h of culture. Cells were fixed and made permeable with BD Cytfix/Cytoperm Plus (BD Biosciences) according to the

manufacturer's instructions. Cells were incubated with FITC-labeled anti-IFN- γ (B27; BD Biosciences) and PE-labeled anti-IL-17 (eBio64CAP17; eBioscience); cells then were washed and data acquired on a FACSCalibur (BD Biosciences), and data were analyzed using Cell Quest software (BD Biosciences).

Western blot analysis for IL-23R

Because of the lack of commercial anti-IL-23R Ab available for the cell surface staining, we detected IL-23R using Western blot analysis. Cell extract (50 μ g protein/lane) from CD4⁺ memory T cells with Let-7f mimic transfection was separated on a 10% SDS-PAGE with Laemmli buffer system. Protein separated on gels was transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech) with an electrotransfer apparatus at 200 mA for 2 h. The membranes were blocked with PBS containing 5% nonfat milk. The blots were stained with anti-IL-23R Ab (catalog no. IMG-5092A; Imgenex) or anti- β -actin Ab (Sigma) for 1 h at room temperature, followed by a secondary staining with IRDye 700DX-labeled goat anti-rabbit IgG (H&L, Rockland, PA) for IL-23R and IRDye 800-labeled anti-mouse IgG (H&L) for β -actin. The protein bands on the filter were visualized via an Odyssey Infrared Imaging System (Li-Cor Biosystems).

Quantitative RT-PCR for miRNA and mRNA

RNA was obtained from naive and memory T cells using the TRIzol RNA isolation kit (Invitrogen). cDNA was generated from mRNA using the Two-Step quantitative RT-PCR (qRT-PCR) Kit (Invitrogen). cDNA was generated from miRNA using the Ncode Vilo miRNA cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. The Ncode SYBR Green miRNA qRT-PCR kit (Invitrogen) and the SYBR Green PCR Master Mix (Applied Biosystem) were used to detect the miRNA and mRNA expression, respectively. The expression of each target miRNA in T cells was calculated relative to U6B, a ubiquitously expressed small nuclear RNA. For miRNA quantitative PCR (qPCR), the reverse primer was the Ncode miRNA Universal qPCR Primer (Invitrogen). Forward miRNA primers and mRNA primers were obtained from Operon (Table I). A comparative threshold cycle method was used to compare each condition with control.

IL-23R 3' UTR construct and luciferase report assay

The IL-23R 3'UTR bearing binding sites for all putative miRNAs corresponding to nucleotides 1977–2801 (RefSeq NM_144701) was cloned into the SpeI and MuiI sites downstream of the firefly luciferase reporter vector, pMIR-Report (Ambion), according to the manufacturer's instructions. Mutant miRNA binding sites in the pMIR-3'UTR vectors were obtained from GenScript Corporation. Overall, five mutants were generated corresponding to the predicted binding sites on IL-23R 3'prime;UTR for Let-7e/f and miRs-17, -591, -378, and -19b-1 (Table II).

K562 cells were cultured in 12-well plates (0.8×10^6 /well). Each pMIR construct (800 ng/well), together with the Renilla luciferase control plas-mid, phRL-CMV (5 ng/well; Promega), was transfected into cells using Amaxa Nucleofector (Amaxa) according to the manufacturer's protocol for K562 cells (VCA-1003). Cells were harvested 24 h posttransfection, and luciferase activity was measured using the Dual Luciferase Report Assay System (Promega) according to the manufacturer's instructions. Experiments were performed at least in triplicate.

miRNA mimic transfection

Meridian miRNA mimics to Let-7f, Let-7e, and miR-17, and the cel-miR-67 negative control were obtained from Dharmacon. Human myeloid leukemia K562 cells were

transfected with synthetic Let-7f, Let-7e, miR-17 mimics via Amaxa Nucleofector (Amaxa) according to manufacturer's protocol for K562 cells (catalog no. VCA-1003). Typical transfection efficiency for K562 cells was ~80%. For CD4 memory T cells, a total of 5×10^6 CD4⁺ T cells were transfected with synthetic Let-7f, Let-7e, and miR-17 mimics via Amaxa Nucleofector (Amaxa), following the manufacturer's protocol for stimulated human T cells (VPA-1002) using program T-023. Typical efficiency in CD4 T cells using control GFP plasmid transfection was ~50%.

Statistics

Data for IL-23R expression, IL-17 production, and miRNA expression profile between naive and memory T cells were analyzed with Wilcoxon matched pair test (GraphPad Prism, version 5; GraphPad Software). Data for IL-23R and IL-17 expression after luciferase vectors and miRNA mimics transfection were analyzed with ANOVA. A *p* value <0.05 was considered significant (**p* < 0.05, ***p* < 0.01). Data are presented as mean ± SE unless indicated.

Results

CD4⁺CD45RO⁺ memory T cells are the principal IL-17–secreting population

To address the cellular source of IL-17 and its regulation in humans, we purified peripheral blood CD4⁺ T cells from 16 healthy individuals. The subjects were 63% male and 37% female, and ranged in age from 30 to 79 y old. CD4⁺ T cells were separated into CD45RA⁺ naive and CD45RO⁺ memory T cells, and both populations were stimulated with TCR and CD28 cross-linking (anti-CD2/anti-CD3/anti-CD28 beads) for 3 d and then cultured for 5 d. Their capacity to produce IL-17 was analyzed by ELISA and qRT-PCR. As anticipated, both IL-17 protein and mRNA were detected mainly in CD4⁺CD45RO⁺ memory T cells (Fig. 1A, 1B). Intracellular immunofluorescence data confirmed that the memory T cells were the main source of IL-17⁺ T cells after PMA/ionomycin stimulation (Fig. 1C). Consistent with a previous report, we also demonstrated that these memory T cells produce the same amount of IL-13 mRNA as naive T cells (31) (Supplemental Fig. 1).

To determine whether certain cytokines regulate IL-17 expression in T cells, we cultured purified CD4⁺ naive and memory T cells in the presence of IL-23, IL-1β, and their combination, and measured IL-17 levels in the culture supernatants. Addition of IL-23 alone did not amplify the IL-17 production in both memory and naive T cells (Fig. 1D, 1E). In contrast, IL-1β strongly drove IL-17 production in memory T cells (Fig. 1D). Furthermore, the combination of IL-1β and IL-23 led to 1.7-fold increase in IL-17 production in the culture supernatant of memory T cells compared with IL-1β alone (Fig. 1D). These results confirm previous reports of IL-17 production in CD4⁺CD45RO⁺ memory T cells and the enhanced expression of IL-17 in response to IL-1β and IL-23.

Let-7f is inversely correlated with IL-23R expression in memory and naive T cells

IL-23 is a heterodimeric cytokine that shares a ligand subunit (p40) with IL-12. IL-23 has been reported to be essential for the maintenance and survival of human and murine Th17 cells. IL-23R consists of unique IL-23R subunit and IL-12β1 subunit shared with IL-12R. Therefore, we assessed IL-23R mRNA expression by qRT-PCR in human memory and naive T cells. We found that IL-23R expression was 2.6-fold higher in memory T cells compared with naive T cells (Fig. 2A). High expression of IL-23R in memory T cells thus correlated with responsiveness to IL-23 and increased IL-17 production in this population (Fig. 1D).

RNA interference by miRNAs has been identified as a post-transcriptional mechanism regulating protein expression. An *in silico* analysis of the 3'UTR of the IL-23R using the MicroCosm targets registry (32) revealed 19 putative miRNA binding regions (data not shown). To test whether miRNAs regulate the IL-23R expression in naive and memory T cells, we screened the 19 miRNAs with putative binding sites in the 3'UTR of IL-23R gene by mature miRNA qRT-PCR. Of these predicted miRNAs, we found that only three miRNAs, *Let-7f*, miR-17 and miR-936, were downregulated in memory T cells (Fig. 2B, 2C, 2E). The expression of other remaining miRNAs with putative binding sites within the 3'UTR of the IL-23R was unchanged when comparing naive versus memory T cells (Supplemental Fig. 2). Some miRNAs such as miRs-875, -613, -19a, -19b-2*, and -583 were expressed at extremely low levels in both naive and memory T cells (data not shown). Furthermore, the expression of *Let-7e*, which shares the seed region with *Let-7f*, was not significantly changed in naive and memory T cells (Fig. 2D). The inverse correlation of IL-23R expression and *Let-7f*, miR-17, and miR-936 expression suggested that these three miRNAs may modulate target gene IL-23R expression.

Manipulation of *Let-7f* results in altered expression of IL-23R in K562 cells

To study potential miRNA regulation of IL-23R expression, we first assessed IL-23R mRNA expression in various human lymphoid and myeloid cell lines, using primers described in Table I. Specifically, IL-23R mRNA was found to be highly expressed in NKL and K562 cell lines. Low-level expression of IL-23R was found in Molt-4, Jurkat, THP-1, and U937 cell lines (Fig. 3A).

Next, we assessed the cell line expression of the same 19 miRNAs with putative binding sites in the 3'UTR of IL-23R (Fig. 3B), using primers described in Table I. Both *Let-7f* and *Let-7e* were found to be expressed at low levels in NKL and K562 cell lines but relatively higher levels in other cell lines (Fig. 4A, 4B). The inverse correlation of *Let-7e*, *Let-7f* with IL-23R in the NKL and K562 cell lines was consistent with the results from human primary naive and memory T cells.

We then tested whether alterations of cellular *Let-7f*, *Let-7e*, and miR-17, the three miRNAs downregulated in human memory T cells, affected IL-23R expression in cell lines normally expressing IL-23R. We transfected K562 cells with *Let-7f*, *Let-7e*, and miR-17 mimics for 24 h and measured IL-23R mRNA by qRT-PCR. When compared with the negative control mimics, we found that *Let-7f* mimic transfection decreased IL-23R mRNA expression by 56 and 70% using 25 and 100 nM, respectively (Fig. 4C). Similarly, miR-17 mimic transfection also decreased IL-23R mRNA expression by 58 and 70% using 25 and 100 nM, respectively (Fig. 4C). Interestingly, *Let-7e* did not significantly alter IL-23R expression at either the 25 or 100 nM concentrations (Fig. 4C). Furthermore, as a control for specificity, MIP-2 α expression, which is not a target gene for *Let-7f*, *Let-7e*, and miR-17, did not change in response to the transfection of these mimics (Fig. 4D). Taken together, these data revealed that *Let-7f* and miR-17 can mediate IL-23R expression in human K562 cells.

Let-7f affects IL-23R expression through its predicted 3'UTR binding site

miRNAs target mRNAs via base pair complementarities within the 3'UTR of a target gene, leading to either mRNA cleavage or translational suppression. By querying the MicroCosm targets registry, we identified considerable complementarities within the seed region of *Let-7f*, *Let-7e*, and miR-17, and the 3'UTR of IL-23R (Fig. 5A). Interestingly, *Let-7f* and *Let-7e* differ by only two nucleotides located outside the seed region (Fig. 5A).

To determine whether *Let-7f*, *Let-7e*, and miR-17 indeed interact with the corresponding IL-23R mRNA sequence, we designed a pMIR-REPORT luciferase construct containing the

wild type 3'UTR of IL-23R with the putative miRNA binding sites. Five additional pMIR-REPORT luciferase constructs containing mutations in each of the putative miRNA binding site seed regions for Let-7e/f and miRs-17, -378, -19, and -591 were generated by altering five nucleotides in the seed region of each corresponding miRNA (Table II).

K562 cells were transfected with each reporter construct, and luciferase activity was assessed 24 h after transfection. As shown in Fig. 5B, the Let-7e/f binding site mutant resulted in a significant increase in luciferase activity when compared with the wild type IL-23R 3'UTR. Mutants in the putative binding sites for miRs-378, -19, and -591 did not significantly influence reporter activity (data not shown). Moreover, the miR-17 mutant did not influence the luciferase activity despite the inverse correlation of miR-17 and IL-23R expression in human T cells (Fig. 5B). These results indicated that the Let-7e/f binding site, but not the miR-17 binding site, influences IL-23R expression in the human cell line.

Let-7f directly affects IL-23R and IL-17 transcripts in human memory T cells

To further determine whether exogenous Let-7f can directly repress IL-23R expression in human CD4⁺ memory T cells, we analyzed IL-23R mRNA in purified memory T cells transfected with either control mimic or Let-7f, Let-7e, and miR-17 mimics for 24 h. IL-23R mRNA expression in memory T cells transfected with the Let-7f mimic was decreased by 44% compared with control mimic (Fig. 6A). Consistent with the luciferase assay studies in K562 cells, neither the Let-7e mimic nor the miR-17 mimic had any effect on IL-23R expression in purified memory T cells (Fig. 6A). Western blot analysis of CD4⁺ memory T cells transfected with a Let-7f mimic demonstrated reduced expression of IL-23R protein, as compared with the control mimic (Fig. 6B).

In this study, we demonstrated that memory T cells are the principal IL-17-producing cells, and IL-1 β stimulation results in increased IL-17 production. An examination of miRNA expression in IL-1 β -stimulated memory T cells revealed reduced Let-7f expression but not Let-7e (Fig. 7). We next examined whether Let-7e, Let-7f, and miR-17 mimic transfection altered IL-17 production. Indeed, IL-17 mRNA expression was reduced by 35% in noncytokine-stimulated memory T cells transfected with the Let-7f mimic as compared with control mimic (Fig. 6C). Let-7f mimic transfection also resulted in reduced IL-17 protein expression in these CD4⁺ memory T cells, as compared with the control mimic (Fig. 6D). Furthermore, in cytokine-stimulated (IL-1 β and IL-23) CD4⁺ memory T cells, Let-7f mimic transfection also reduced intracellular IL-17 protein expression (Supplemental Fig. 3).

Of note, 3'UTR of IL-17 gene does not contain putative Let-7f, Let-7e, or miR-17 binding sites as assessed by querying miRBase targets registry. We further demonstrated that the Let-7f, Let-7e, and miR-17 mimic transfection into memory T cells did not inhibit expression of TNF- α (Fig. 6E), a gene without putative binding sites for any of these miRNAs. These findings suggested that Let-7f interference with IL-23R in memory T cells is indeed sufficient to influence both IL-23R and downstream IL-17 gene expression.

Discussion

In this study, we demonstrated an inverse correlation between the expression of IL-23R and one of the IL-23R putative binding miRNAs, Let-7f. Further luciferase and miRNA mimic experiments in the K562 cell line and human primary CD4⁺ memory T cells suggested that Let-7f can directly modulate IL-23R expression and the subsequent expression of its downstream target, IL-17. To our knowledge, this is the first study that provides a mechanistic insight into the role of miRNAs in controlling IL-23/IL-17 signaling pathway, which has been implicated in the pathogenesis of many inflammatory and autoimmune diseases.

Although the role of IL-23 in murine Th17 cell function is not yet fully defined, multiple studies support the concept that IL-23 is a critical cytokine for human Th17 cell development or survival (17). Like many cytokines, IL-23 engagement of the IL-23R complex results in activation of the JAK-STAT signaling pathway (20, 22). IL-23R is expressed on memory T cells, NK cells, NKT cells, monocytes, and dendritic cells but barely expressed on naive T cells (20, 21), which corresponds with the ability of those cells to respond to IL-23 to produce IL-17, as well as other pro-inflammatory cytokines. Our IL-23R expression profile and IL-17 production in memory T cells were consistent with these previous studies.

Let-7f was identified by Wu et al. (33) as one of six predominant miRNAs downregulated in CD8⁺ effector and memory T cells compared with naive T cells. The level of Let-7f expression was dynamically regulated during the differentiation of these T cells and inversely correlated with the T cell activation status. In our study, we focused on human CD4⁺ memory T cells, a different subtype of T cells from CD8⁺ T cells. Nevertheless, both naive CD4⁺ and CD8⁺ T cells are nonreplicating and functionally quiescent cell types relative to their effector or memory counterparts, respectively. In our study, Let-7f expression is significantly higher in naive CD4⁺ T cells than that in memory T cells, which is consistent with the study of Let-7f expression in CD8⁺ T cells.

Let-7f is not the only miRNA demonstrating different expression in CD4⁺ memory T cells. Our results also demonstrate reduced miR-17 expression in memory T cells as compared with naive T cells. These data support other recent reports of differential miRNA expression in human T cells during development. Neilson et al. (34) measured miRNA profiles from different stages of T lymphocyte development including double-negative stage 1, 3, 4, and CD4⁺CD8⁺ double-positive stage, as well as single-positive CD4 and CD8 T cells. They found that a few specific miRNAs are enriched at distinct stages. Specific enrichment of miRNAs can be correlated with the depletion of transcripts harboring seed matches to these miRNAs. For example, the CD4⁺CD8⁺ double-positive, stage-enriched miR-181a confers repression of CD69, Bcl-2, and TCR through the binding of miR-181 to their respective 3' UTR elements. Most recently, Weitzel et al. (35) reported that miR-184 inhibited NFAT1 protein expression and its associated inflammatory cytokines in umbilical cord blood-derived CD4⁺ T cells, which may contribute to one mechanism of peripheral tolerance exhibited by umbilical cord blood CD4⁺ T cells.

The true complexity of miRNA regulation of gene expression is reflected in our study. Of the 19 miRNAs with putative binding sites in the IL-23R 3'UTR, only 3 miRNAs were differently expressed when comparing naive and memory T cells. Of these miRNAs, only Let-7f was found to directly regulate IL-23R in K562 cells and CD4⁺ memory T cells. More importantly, Let-7e, which shares a common binding site with Let-7f and differs with Let-7f by only two nucleotides, was not differently expressed in memory T cells and naive T cells, and did not regulate IL-23R expression. The predicted conservation alignment scores, an indicator of complementarity between an miRNA and its putative binding sites, for Let-7e and Let-7f are 16.39 and 16.19, respectively. The two-nucleotide difference in complementarity to the IL-23R 3'UTR apparently is sufficient to account for a functional role of Let-7f but not Let-7e in regulating IL-23R expression. Like Let-7f, naive T cells also exhibited significantly higher miR-17 expression as compared with memory T cells, initially suggesting that miR-17 may regulate the expression of IL-23R. Previously, the miR-17-92 cluster expression was well characterized during the development of lymphocytes and myeloid lineages (36, 37). It is highly expressed in T and B cell precursor, and its expression diminished somewhat after maturation. Also, expression of miR-17, and its family miRNAs miR-20a and miR-106a, decreased during monocytopoiesis in vitro. Mice with ectopic overexpression of the miR-17-92 cluster in the lymphocyte compartment develop severe

lymphoproliferative disease and auto-immunity, and die prematurely (38). In our study, miR-17 mimic transfection downregulated the IL-23R expression in K562 cells. However, mutations within the miR-17 binding site in a luciferase vector containing 3'UTR of IL-23R did not result in altered luciferase activity, indicating that miR-17 most likely indirectly influences IL-23R expression.

In summary, we have observed that memory T cells endogenously express significantly less amounts of Let-7f as compared with naive T cells. Let-7f is capable of regulating IL-23R expression through its predicted complementary binding site within the IL-23R mRNA 3' UTR. This process in CD4⁺ T cells may comprise one mechanism underlying the relatively higher levels of IL-23R expression in memory T cells compared with naive T cells, resulting in their characteristically higher expression of the proinflammatory cytokine IL-17. Taken together, we have identified a novel mechanism in regulation of the IL-23/IL-17 signaling pathway with potential implications for our understanding of inflammation and autoimmune diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

miRNA	microRNA
qPCR	quantitative PCR
QRT-PCR	quantitative RT-PCR
3'UTR	3' untranslated region

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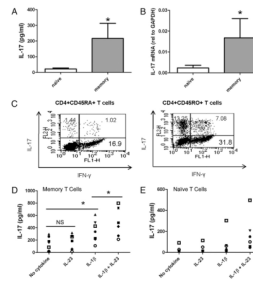


FIGURE 1.

Human memory T cells produce significantly more IL-17. Isolated naive and memory CD4⁺ T cells were cultured with anti-CD3, anti-CD2, and CD28 beads for 3 d and then culture for 5 d. RNA and culture supernatant were harvested. *A*, IL-17 secretion in cell culture supernatant was measured by ELISA. $n = 16$. $*p < 0.05$. *B*, IL-17 mRNA expression were assessed by qRT-PCR between naive and memory T cells. $n = 16$. $*p < 0.05$. *C*, Intracellular cy-tokine staining and flow cytometry were used to measure IL-17-producing cells after PMA and ionomycin stimulation for 5 h (representative of two experiments). *D* and *E*, IL-1 β , IL-23, or both were added into the memory or naive T cell culture system, and IL-17 production was detected by ELISA. $n = 7$. $*p < 0.05$.

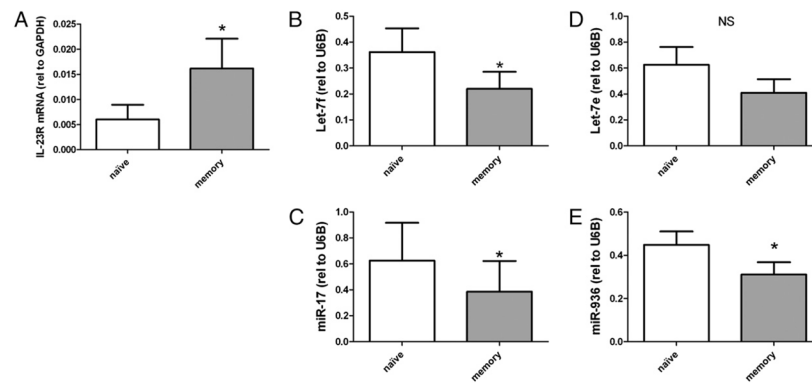


FIGURE 2. miRNA Let-7f, miR-17, and miR-936 inversely correlated with IL-23R expression. A, IL-23R mRNA expression in naive and memory T cells were assessed by qRT-PCR. $n = 16$. $*p < 0.05$. B–E, miRNAs with putative binding sites within 3'UTR of IL-23R gene were quantified in naive and memory T cells by qRT-PCR. $n = 16$. $*p < 0.05$.

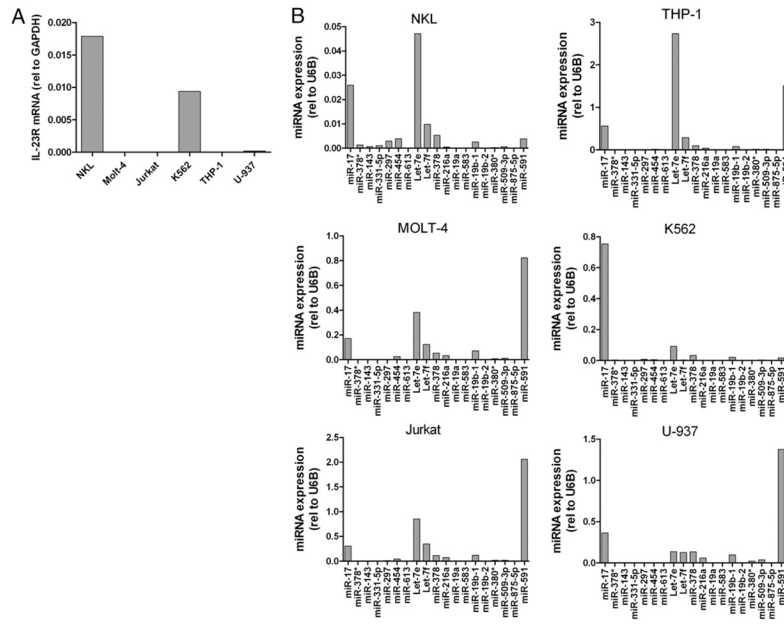
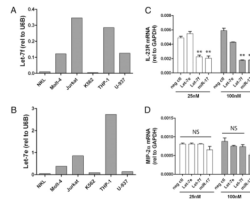


FIGURE 3. IL-23R and putative associated miRNA expression in human cell lines. *A*, IL-23R mRNA expression was assessed in human myeloid and lymphoid cell lines by qRT-PCR. *B*, miRNA expression was assessed in human and lymphoid cell lines by qRT-PCR.

**FIGURE 4.**

Exogenous transfection of Let-7f mimic inhibits IL-23R expression in human K562 cells. *A*, Let-7f expression in cell lines was measured by qRT-PCR. *B*, Let-7e expression in cell lines was measured by qRT-PCR. *C*, IL-23R mRNA level was qualified in K562 cells after Let-7e, Let-7f, and miR-17 mimics (25 and 100 nM) transfection by qRT-PCR. $**p < 0.01$. *D*, MIP-2 α mRNA level was measured in K562 cells after miRNA mimic transfection by qRT-PCR. $*p < 0.05$.

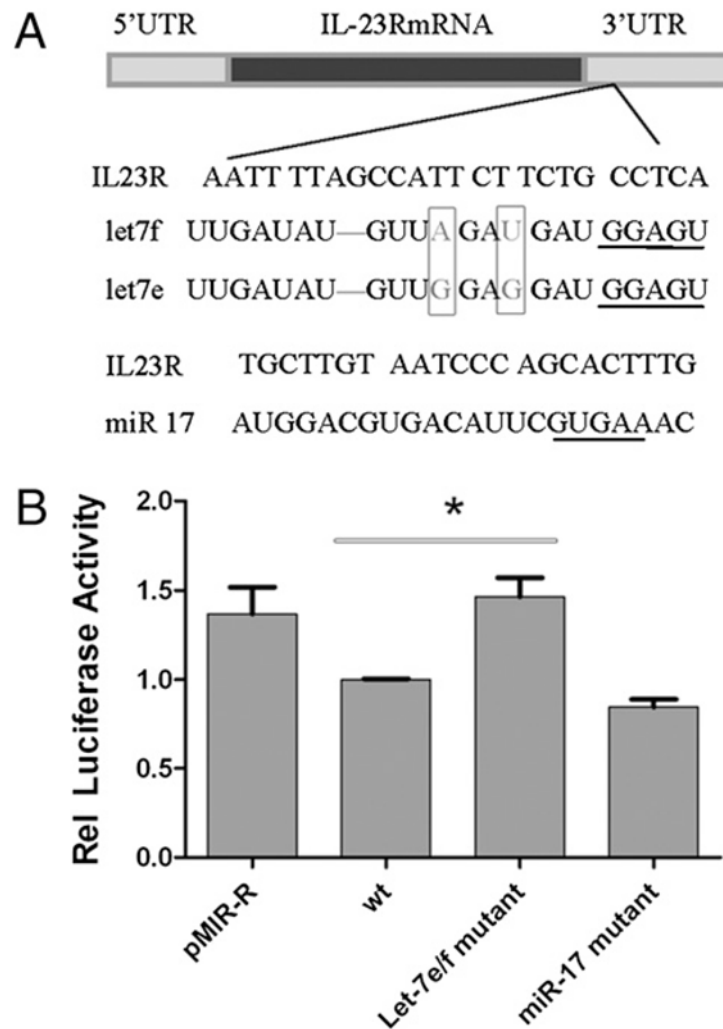
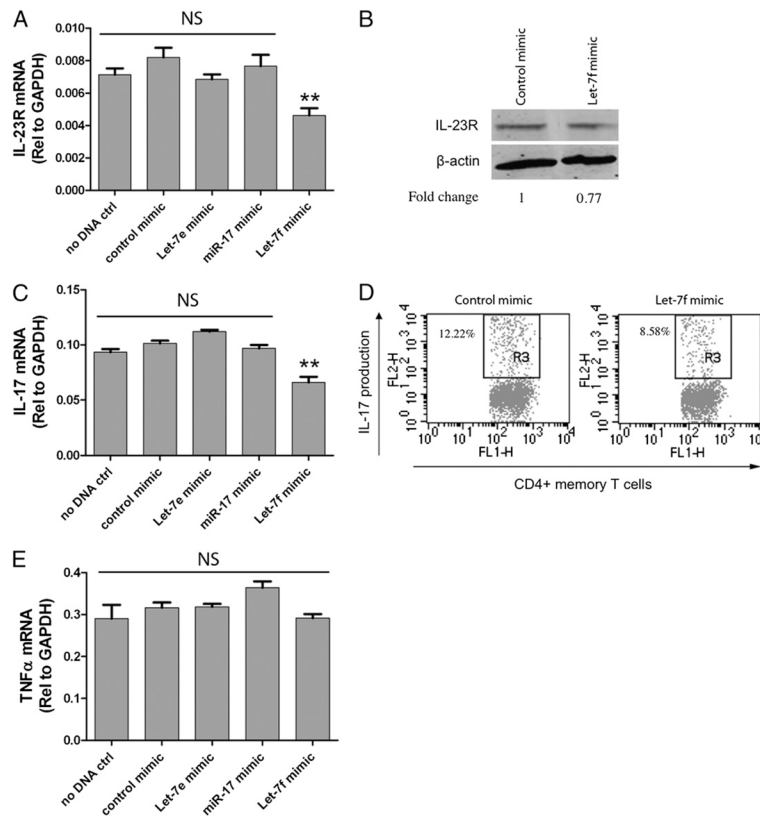
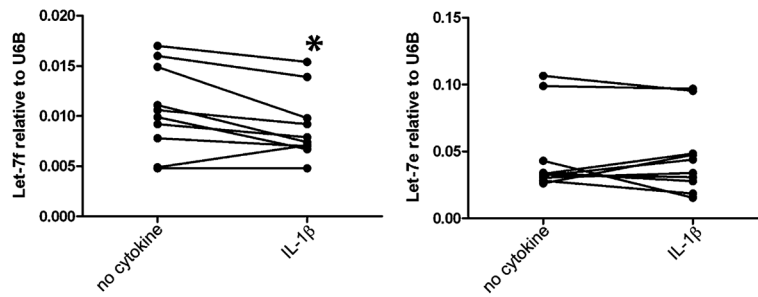


FIGURE 5.

IL-23R miRNA binding site mutations' effect on reporter gene expression. *A*, The IL-23R 3'UTR contains complementarities to miRNA Let-7f, Let-7e, and miR-17. Using in silico computational target prediction analysis, we identified that Let-7f, Let-7e, and miR-17 have five- to seven-nucleotide seed regions complementary to the 3'UTR of IL-23R mRNA. *B*, Luciferase reporter activity in the pMIR-IL-23R 3'prime;UTR reporter constructs and associated miRNA binding site mutants. Data shown are relative to the wild type pMIR-IL-23R 3'UTR report construct. $n = 9$. $*p < 0.05$.

**FIGURE 6.**

Let-7f inhibits IL-23R and IL-17 expression in human primary memory T cells. *A*, Memory T cells were transfected with control, Let-7f, Let-7e, and miR-17 mimics for 24 h. RNA was extracted and mRNA level of genes was detected by qRT-PCR. IL-23R mRNA expression was significantly reduced in memory T cells transfected with Let-7f mimic. $**p < 0.01$. Control mimic, Let-7e, and miR-17 had no effect. *B*, Memory T cells were transfected with control and Let-7f mimics for 24 h, and protein extracts were isolated. Western blot analysis demonstrated reduced IL-23R protein expression in Let-7f mimic transfected cells (representative of two individual donors). *C*, IL-17 mRNA expression was also significantly decreased in memory T cells after Let-7f mimic transfection. $**p < 0.01$. *D*, Decreased IL-17-producing cells were detected by intracellular staining after Let-7f mimic transfection. *E*, TNF- α mRNA expression was not inhibited by the Let-7f mimic and other mimics.

**FIGURE 7.**

Let-7f, but not Let-7e, is decreased in IL-1 β -stimulated human primary memory T cells. Primary memory T cells were treated in the presence or absence of IL-1 β for 48 h, RNA extracted, and miRNA expression assessed by qRT-PCR. Let-7f was significantly decreased in IL-1 β -treated memory T cells, whereas Let-7e was unchanged. * $p < 0.05$. $n = 10$.

Table I

Primers used for quantitative real-time PCR

Name	Primer (5'–3')
miRNA primers	
miR-17	Forward: CAAAGTGCTTACAGTGCAGGTAG
miR-378*	Forward: CTCCTGACTCCAGGTCCTGTGT
miR-331-5p	Forward: CTAGGTATGGTCCCAGGGATCC
miR-297	Forward: ATGTATGTGTGCATGTGCATG
miR-454	Forward: TAGTGCAATATTGCTTATAGGGT
miR-613	Forward: AGGAATGTTCTTCTTTGCC
miR-378	Forward: ACTGGACTTGGAGTCAGAAGG
miR-216a	Forward: TAATCTCAGCTGGCAACTGTGA
miR-19a*	Forward: AGTTTTGCATAGTTGCACTACA
miR-583	Forward: CAAAGAGGAAGGTCCCATTAC
miR-19b-1*	Forward: AGTTTTGCAGGTTTGCATCCAGC
miR-19b-2*	Forward: AGTTTTGCAGGTTTGCATTCA
miR-380*	Forward: TGGTTGACCATAGAACATGCGC
miR-509-3p	Forward: TGATTGGTACGTCTGTGGGTAG
miR-936	Forward: ACAGTAGAGGGAGGAATCGCAG
miR-875-5p	Forward: TATACCTCAGTTTATCAGGTG
miR-591	Forward: AGACCATGGGTTCTCATTGT
miR-21	Forward: TAGCTTATCAGACTGATGTTGA
Let-7f	Forward: TGAGGTAGTAGATTGTATAGTT
Let-7e	Forward: TGAGGTAGGAGGTTGTATAGT
mRNA primers	
GAPDH	Forward: GTCTCCTCTGACTTCAACA
	Reverse: CAGGAAATGAGCTTGACAAA
IL-23R	Forward: AACAGCAATGTTGTGAAAATGC
	Reverse: AGGCTTGTGTTCTGGGATGA
IL-17	Forward: ACCAATCCCAAAAGGTCCTC
	Reverse: TGGATGGGGACAGAGTTCAT
IL-13	Forward: GGAATCCCTGATCAACGTGT
	Reverse: CCACCTCGATTTTGGTGTCT
MIP-2 α	Forward: CTCAAGAATGGGCAGAAAGC
	Reverse: CTTCAGGAACAGCCACCAAT

Table II

Mutants of pMIR-IL-23R 3'UTR luciferase vectors

Binding Site	IL-23R 39UTR Sequence (5'-3') ^a	Construct
miR-17	TGCTTGTAATCCAGCACTTTG	WT
	TGCTTGTAATCCAGGTGAATG	Mutant
miR-378	GAGTTCGAGTCCAG	WT
	GAGTTCGACAGGTG	Mutant
miR-19	ATTGAAAGATGCAAAAC	WT
	ATTGAAAGATGGTTTTTC	Mutant
miR-591	GAACATGCTTCATGGTC	WT
	GAACATGCTTCTACCAC	Mutant
Let-7e/f	AATTTAGCCATTCTTCTGCCTCA	WT
	AATTTAGCCATTCTTCTGGGAGT	Mutant

^aUnderlined text indicates the seed regions for individual miRNAs.