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5-Lipoxygenase Is a Direct Target of miR-19a-3p and miR-125b-5p

Saskia Busch,* Eileen Auth,† Friederike Scholl,* Sabine Huenecke,† Ulrike Koehl,† Beatrix Suess,§ and Dieter Steinhilber*  

5-Lipoxygenase (5-LO) is the key enzyme in leukotriene biosynthesis. Leukotrienes are mediators of the innate immune system and inflammatory processes, and they might also be involved in cancer development. MicroRNAs (miRNAs) are important translational regulators and have been shown to be involved in development, differentiation, and cancer. Unraveling the miRNA network is important for understanding the cellular regulation processes. We identified two new miRNAs, miR-19a-3p and miR-125b-5p, regulating 5-LO and confirmed direct interaction by reporter gene assays. Furthermore, we investigated the regulation of 5-LO by these two miRNAs in several cell types. Inhibition of both miRNAs by antagonirs during differentiation of the myeloid cell line Mono Mac 6 led to a significant increase in 5-LO protein expression. Stimulation of human T lymphocytes with PHA resulted in a strong down-regulation of 5-LO mRNA expression and in the induction of miR-19a-3p. The inhibition of miR-19a-3p with an antagonir led to a significant increase in 5-LO mRNA expression in T lymphocytes. Taken together, our data reveal that miR-19a-3p and miR-125b-5p target 5-LO in a cell type– and stimulus-specific manner. The Journal of Immunology, 2015, 194: 1646–1653.

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eukotrienes are important mediators for inflammatory, allergic, and immunologic reactions. 5-Lipoxygenase (5-LO) is the key enzyme in leukotriene biosynthesis, catalyzing the conversion of arachidonic acid to leukotriene A₄ (1). Depending on the cell type, leukotriene A₄ is either metabolized to leukotriene B₄ or the cysteinyl leukotrienes (leukotrienes C₄, D₄, and E₄). Leukocytes are the major producers of leukotrienes, leading to induction and recruitment of macrophages and monocytes as well as dendritic cell maturation and migration from the peripheral site of action to the lymphoid nodes (2). As part of the adaptive immune response, leukotriene B₄ is a chemoattractant for T cells (3, 4). Moreover, 5-LO is involved in the regulation of Ig production in B cells (5). The expression of the 5-LO can mainly be found in the myeloid lineage of the hematopoietic cells (monocytes, macrophages, granulocytes, neutrophils, mast cells, and dendritic cells) as well as B lymphocytes (6, 7), where the expression and activity depend on stimulation and differentiation status of the cell (8).

MicroRNAs (miRNAs) are small, noncoding, regulatory RNAs. miRNAs can induce mRNA degradation or translational repression of their target genes (9). Their precursors, the pri-miRNAs, are transcribed by RNA polymerase II and metabolized in the nucleus by Drosha to pre-miRNA with a length of ∼70 nt. The pre-miRNAs, once in the cytoplasm, are further processed by Dicer to ∼21 nt double-stranded miRNA. Only one strand of the miRNA duplex, the guide strand, is then incorporated into the RNA-induced silencing complex and directs this complex to the 3′ untranslated region (3′UTR) of its target genes.

Even though regulation of gene expression by miRNAs has been studied intensively in recent years, not much is known about the regulation of the 5-LO pathway by miRNAs. In 2010, miR-135a and miR-19a-5p were the first two miRNAs identified to target the 5-LOactivating protein (10). miR-219-2 was the first and only miRNA identified so far as a regulator of 5-LO in macrophages (11).

Recently, it was shown that inhibition of the leukotriene receptors cysteinyl leukotriene 1 or leukotriene B₄ receptor-1 (BLT₁) receptor or a knockdown of the BLT₁ receptor led to a downregulation of several miRNAs (12). Among them were miR-19a-3p and miR-125b-5p. Overexpression of miR-125b-5p in the myeloid cell line HL-60 prevented their differentiation into macrophages or granulocytes by either DMSO or GM-CSF (13). Alternatively, it could be shown that high expression of 5-LO requires cell differentiation, for example, by DMSO, GM-CSF, or TGF-β (14–17), which provides a possible link between miR-125 and the 5-LO pathway. Mir-19a-3p is a member of the miR-17~92 cluster, which has been shown to be expressed in B cells (18). Within B cell development, the 5-LO is downregulated in germinal center cells (8, 19), whereas the miR-17~92 cluster as well as miR-125b-5p are upregulated (20–23). Furthermore, it has been shown that the miR-17~92 cluster regulates T cell proliferation (24, 25) and that miR-19a-3p plays a role in promoting Th1 responses (26).

All of these data suggest a possible role of miR-19a-3p and miR-125b-5p in the regulation of leukocyte functions and immune responses by regulation of the 5-LO pathway. In this study, we show that miR-19a-3p and miR-125b-5p directly regulate 5-LO expression in myeloid cells and in T lymphocytes.

Materials and Methods

Cell lines

Cos-7 cells were cultivated in DMEM medium (Invitrogen) supplemented with 10% FCS (Biochrom), 100 μg/ml streptomycin, and 100 U/ml

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Abbreviations used in this article: BLT₁, leukotriene B₄ receptor-1; 5-LO, 5-lipoxygenase; miRNA, microRNA; MM6, Mono Mac 6; SAC, Staphylococcus aureus Cowan strain protein A; 3′UTR, 3′ untranslated region.

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penicillin (Invitrogen). The cells were detached using trypsin-EDTA (Invitrogen) and split at a ratio of 5:1 twice a week. Mono Mac 6 (MM6) cells were cultivated in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (Biochrom), 100 μg/ml streptomycin, 100 U/ml penicillin (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 1× nonessential amino acids (Sigma-Aldrich), 1 mM oxaloacetate (AppliChem), and 10 μg/ml human insulin (Sanofi-Aventis). The cells were split twice a week to a density of 0.3 × 10^6/ml. For the time course experiment, the cells were seeded at a density of 0.3 × 10^6/ml and were cultivated for 4 d. MM6 cells were seeded at a density of 0.3 × 10^6/ml for untreated time courses and 0.2 × 10^6/ml for differentiation experiments. For differentiation, the cells were stimulated with 50 nM calcitriol (Sigma-Aldrich) and 1 ng/ml TGF-β1 (isolated and purified in-house) for up to 4 d. All used cell lines were kept at 37°C, 5% CO2, and 100% humidity and were purchased from the German Collection of Microorganisms and Cell Cultures.

miRNA target predictions

Three online bioinformatic target prediction programs were used: TargetScan (http://www.targetscan.org/), MicroRNA.org (http://www.microrna.org/microrna/getMiraForm.do), and MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl).

Plasmid construction

All plasmids used are based on the pMIR-Report plasmid (Invitrogen). The SacI and HindIII restriction sites within the multiple cloning site, which are located behind the firefly luciferase gene, were used to insert the 5-LO 3′UTR sequences. The upper construct in Fig. 1B carries the complete 5-LO 3′UTR cloned between SacI and HindIII. The so–named “triplicate 19α” or “triplicate 125β” plasmids carry a triplicate of the predicted miR binding site within the 3′UTR with a spacer of 15 nt in between and have the following sequences (the predicted miR binding site, as shown in Fig. 1A, is underlined): triplicate 125β, 5′-TTTATCTCTTGCTTCAGGG- GAATAAAGCTTTGGTGCTGAATAGCAGCTTGGGTCGAGATTTCTTCAATTCCTTGCACAATAAAGCTTTGGGTCTGCTTAGTC- GTTGAGATTATTTATCTTGGTCTACCGGAGG-3′; triplicate 19α, 5′-TGCAATGCACCTTGGCACAATAAGCTTTGGTGCTGCTTAGTFCTCAATGCACCTTGGCACAATAAGCTTTGGTGAATAGCAGCTTGGGTCGAGATTATTTATCTTGGTCTACCGGAGG-3′.

The plasmids with the perfect match contain the complementary sequences of the mature 19α and 125b miRNA behind the firefly luciferase gene. Mutagenesis of the seed regions, as shown in Fig. 1A, was performed by overlap PCR, as previously described (27). All sequences were confirmed by DNA sequencing.

Luciferase assay

The Cos-7 cells were seeded at a density of 40,000 cells/well into a 24-well plate in a volume of 1 ml 1 d prior to transfection. The pRL-SV40 plasmid from Promega, carrying the Renilla luciferase gene, was used as a transfection control. The pre-miRNAs for hsa-19a-3p and hsa-miR125b-5p as well as a negative control with a random sequence (Ambion Pre-miR control no. 2) were used to study miR overexpression. The mature miRNA for hsa-miR-29a-5p as well as a negative control were used (MISSION miRNA negative control 1 and MISSION microRNA mimic hsa-miR-29a*: Sigma-Aldrich). Every cotransfection was carried out with 150 ng pMIR reporter plasmid construct, 20 ng pRL-SV40, and 5 pmol precursor miRNA or mature miRNA. Cells were transfected with 1 μl Lipofectamine 2000 (Invitrogen) in 100 μl, according to the manufacturer’s protocol. The medium change from Opti-MEM to DMEM was performed 3 h after the transfection. Luciferase activity was determined 24 h after the medium change using the Dual-Glo luciferase assay system (Promega) and a Tecan Infinite M200 reader.

RNA isolation, cDNA, and quantitative PCR

RNA isolation of MM6 cells was performed with the phenol method. The harvested cell pellet of 1×10^6 200 μl PBS (150 mM NaCl, 25 mM sodium saccharose [Merck], 10 mM sodium acetate [AppliChem], adjusted to pH 4.8 using acetic acid [AppliChem]) in the presence of 1% SDS (AppliChem) and 200 μl 6 M guanidine thiocyanate (Sigma-Aldrich). The extraction with phenol and a 25:1 mixture of chloroform and isomyl alcohol was carried out twice. MaxTract high-density tubes (Qiagen) were used for phase separation. TurboDNase (Ambion) was used for DNase digest and was handled according to the manufacturer’s protocol. The RNA integrity was analyzed after the DNase digest by agarose gel electrophoresis. The RNA of the primary B and T cells was extracted with the miRNeasy kit (Qiagen) and eluted in 30 μl MilliQ water, according to the manufacturer’s protocol.

For cDNA synthesis the high-capacity RNA-to-cDNA kit (Invitrogen) was used according to the manufacturer’s protocol. All quantitative PCR experiments were carried out with a StepOne Plus device (Applied Biosystems). The endogenous control used for the MM6 cells was β-actin and for the primary cells RNU48.

For the specific cDNA synthesis of hsa-miR-19a-3p, 100 ng RNA was transcribed into specific cDNA using stem-loop primers, designed as previously described (28). Consequently, the quantitative PCR was carried out using a specific forward primer, a universal reverse primer, and Universal ProbeLibrary probe no. 21 (Roche Diagnostics). RNU48 served as the endogenous control. All primer sequences are available upon request. Hsa-miR-125b-5p was detected using the TaqMan miRNA assay kit according to the manufacturer’s protocol. RNU48 served as the endogenous control.

Western blot analysis

A minimum of 5 × 10^6 cells were lysed in 100 μl 0.1% Triton X-100 solution (Fluka BioChemika) in PBS (Invitrogen) supplemented with 1 mM PMSF (Fluka BioChemika) and frozen at −80°C. After thawing, the cells were centrifuged for 10 min at 10,000 × g (4°C) and the protein concentration of the supernatant was determined by the Bradford colorimetric assay (Bio-Rad Laboratories) (29). The samples (50–100 μg protein) were separated by SDS-PAGE on a 10% gel and then blotted onto a nitrocellulose membrane (Whatman reinforced nitrocellulose; GE Life Sciences). The membrane was blocked for 1 h at room temperature with Odyssey blocking buffer (LI-COR Biosciences) and afterward incubated with primary and secondary Abs. The Abs were diluted in PBS (Invitrogen) as follows: mouse anti–5-LO at 1:1,000 (6A12; Frankfurt University), donkey anti-mouse at 1:10,000 (IRDye; LI-COR Biosciences), goat β-actin at 1:5,000 (Santa Cruz Biotechnology), and donkey anti-goat at 1:10,000 (IRDye; LI-COR Biosciences). The visualization was carried out with an Odyssey Classic infrared imaging system (LI-COR Biosciences).

Isolation of B and T lymphocytes

The primary cells were isolated from buffy coats obtained from the German Red Cross Blood Donor Service Baden-Württemberg-Hessen in Frankfurt, Germany. A Ficoll separation was performed before enrichment of either B or T lymphocytes with the respective RosetteSep human enrichment kit (StemCell Technologies). The magnetic separation was carried out according to the manufacturer’s protocol. The purity of the cells was checked by staining with CD45-FITC, CD3-PE, CD19-ECD, viability dye 7-aminocinimidin D, and CD56-PC7 (Beckman Coulter). As an internal standard for quantification of the cell count accuracy, fluorescence beads (Flow-Count; Beckman Coulter) were added. The measurements were carried out with an FC500 by Beckman Coulter, and analysis of the data was performed with the CXP v2.2 software (Beckman Coulter).

The CD3+CD4+ T cells had a median purity of 97.1% with a SD of 1.8%; the remaining cells were <1% monocytes. The CD19+CD3− CD4+ B cells had a median purity of 97.3% with a SD of 7.5%; the remaining cells were <5% monocytes. The viability directly after the purification step was >99%.

Stimulation of primary B lymphocytes

B lymphocytes were taken into culture in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (Invitrogen), 2 mM glutamine (Invitrogen), 50 μg/ml streptomycin, 50 U/ml penicillin (Invitrogen), and 25 mM HEPES buffer (Invitrogen) at a density of 1 × 10^6/ml (culture volume 4 ml). The cells were stimulated with 10 ng/ml protein A from Staphylococcus aureus Cowan strain (SAC) (Sigma-Aldrich) and 20 U/ml IL-2 (PeproTech).

Stimulation of primary T lymphocytes

T lymphocytes were taken into culture in X-VIVO 10, without phenol red and gentamicin (Lonza), and supplemented with 10% human heat-inactivated plasma (Red Cross Baden-Württemberg-Hessen) at a density of 1 × 10^6/ml (culture volume 4 ml). The cells were stimulated with 5 ng/ml PHA (Sigma-Aldrich).

Antagonim experiment

The antagonims for miR-19a-3p and miR-125b-5p as well as a control, with a complementary sequence to GFP, were designed as previously described (30) and obtained from VBC Biotech (Vienna, Austria) with the following sequences: antagonim-19a, 5′-TCAGTTTTCATAGATTTCGACA-3′; antagonim-125b, 5′-AAGGCGCCTTGGATTGAACACT-3′; control antagonim-CO, 5′-AAGGCAAGCGUACCCUGAAGUU-3′.

The antagonims were added at a final concentration of 25 nM to the differentiating MM6 cells and at a final concentration of 150 nM to the
primary T cells. The efficiency of the antagomir treatment was measured by determination of mRNA levels of the verified hsa-miR-19a-3p target cyclin D1.

Statistical analysis
GraphPad Prism 5.03 (GraphPad Software) was used for statistical analysis. A p value <0.05 was considered significant.

Results
Target predictions
The search engines TargetScan, MicroRNA.org, and MicroCosm Targets were used to identify potential miRNA binding sites in the 5-LO 3’UTR. Seven putative miRNA binding sites were obtained that were further filtered by the quality of their predicted target site combined with their expression pattern in leukocytes. Taking these criteria into account, the number of miRNAs was further reduced from seven to three (i.e., miR-19a-3p, miR-125b-5p, and miR-29a-5p), which were further investigated.

The binding sites for these miRNAs within the context of the 3’UTR of 5-LO are illustrated in Fig. 1A and Supplemental Fig. 1. All binding sites have a good seed region and complementary base pairing in the 3’ end of the miRNAs.

MiR-19a-3p and miR-125b-5p can regulate 5-LO expression via two binding sites in the 5-LO 3’UTR
To validate the predicted binding sites, we performed luciferase reporter gene assays. The complete 5-LO 3’UTR was inserted into the luciferase reporter plasmid pMIR. Within the 3’UTR we mutated the seed regions to abolish miRNA binding (the mutated bases are underlined in Fig. 1A). Additionally, a plasmid containing only the predicted binding site in triplicate was created (31). As positive control, we constructed a reporter plasmid containing a sequence complementary to the respective miRNA (perfect match). All constructs are shown in Fig. 1B.

Cotransfection of the constructs containing the entire 5-LO 3’UTR with the pri-miRNAs 19a or 125b decreased luciferase activity by 23 and 35%, respectively. Mutation of the predicted binding sites abolished the decrease, confirming a direct interaction. Reporter gene activity was even further reduced (by 35 and 41%, respectively) when the miR-19a-3p and 125b-5p binding sites were inserted in triplicates without the 5-LO 3’UTR context. Cotransfection of the 3’UTR of the 5-LO with miR-29a-5p did not reduce the luciferase activity (Supplemental Fig. 1), hence miR-29a-5p did not interact with the 3’UTR and was therefore not further investigated.

Taken together, these results show that miR-19a-3p and miR-125b-5p target the 5-LO 3’UTR at the predicted binding sites.

5-LO is a target of miR-19a-3p and miR-125b-5p in MM6 cells
Next we investigated the expression pattern of 5-LO and both miRNAs in a native cellular context. As a model system we chose the human monocytic cell line MM6 to analyze a potential role of the miRNAs in monocytic cells. In this cell line, 5-LO mRNA and protein expression are induced after differentiation with TGF-β and calcitriol (17, 32, 33). In contrast, untreated cells show a low level of 5-LO mRNA expression but no detectable protein expression. We found that miR-19a-3p is prominently expressed in undifferentiated MM6 cells, but the obtained miR-19a-3p amounts were rather variable (Fig. 2A). Interestingly, when the 5-LO mRNA expression is plotted against miR-19a-3p expression in MM6 cell samples, there is a negative correlation between both
parameters ($r^2 = 0.6125$), suggesting that high 5-LO mRNA expression corresponds to a low expression of the miR-19a-3p and vice versa in undifferentiated MM6 cells (Fig. 2B). The expression of miR-125b-5p increased from day 0 to day 3 to ∼1.3-fold. The overall expression of hsa-miR-125b-5p in comparison with hsa-miR-19a-3p is much lower ($2^{-\Delta \Delta Ct}$ values were $65.8 \pm 46.6$ for miRNA-19a [$n = 9$] and $8.7 \pm 6.7$ for miRNA-125b [$n = 7$]).

To study the effect of miR-19a-3p and miR-125b-5p on the 5-LO protein expression, MM6 cells were incubated with a combination of 1 ng/ml TGF-β and 50 nM calcitriol for 4 d, which leads to a strong increase in 5-LO protein expression (17). The RNA expression was determined for 5-LO mRNA and both miRNAs. Treatment of the MM6 cells with the combination of TGF-β and calcitriol led, as already published, to a strong induction of 5-LO mRNA by ∼50-fold on day 2. In contrast, the expression pattern of both miRNAs did not change significantly (compare Fig. 2A with Fig. 2C).

During differentiation, the cells were treated with antagonirs against miR-19a-3p and miR-125b-5p at a final concentration of 25 nM. Each antagonir alone increased the protein only to a small extent, but the combination of both antagonirs increased the protein level significantly by ∼1.8-fold compared with the control antagonir (Fig. 2D). Because the antagonir treatment led to an increase in 5-LO protein expression, the effect of the antagonir treatment on 5-LO

![FIGURE 2. Analysis of 5-LO mRNA and miR-19a-3p/125b-5p expression in MM6 cells.](http://www.jimmunol.org/)

- (A) Time course of 5-LO mRNA, miR-19a-3p, and miR-125b-5p expression in MM6 cells. Values for 5-LO mRNA and miR-19a-3p/miR-125b-5p are normalized to β-actin or U48, respectively, and are expressed as means ± SEM ($n = 4–7$). $2^{-\Delta \Delta Ct}$ values for miR-19a-3p and miR-125b-5p expression were $65.8 \pm 46.6$ and $8.7 \pm 6.7$ ($n = 9$ and $n = 7$), respectively.

- (B) Correlation of miR-19a-3p and 5-LO expression in undifferentiated MM6 cells. Values were analyzed by linear regression ($r^2 = 0.6125$, $n = 35$). (C) Time course of 5-LO mRNA, miR-19a-3p, and miR-125b-5p expression in MM6 cells stimulated with 1 ng/ml TGF-β and 50 nM calcitriol. Values of 5-LO mRNA are normalized to β-actin and miR-19a-3p and miR-125b-5p expression to U48. Values are expressed as means ± SEM ($n = 3$). (D) Western blot analysis of 5-LO expression in MM6 cells. The cells were differentiated for 4 d with 1 ng/ml TGF-β and 50 nM calcitriol in the absence or presence of 25 nM antagonir as indicated and analyzed for 5-LO and β-actin expression by Western blot using anti-5-LO and anti–β-actin Abs and an Odyssey Classic infrared imaging system. Results are expressed as means ± SEM of four to six independent experiments. *$p < 0.05$ by one-way ANOVA and a Dunnett multiple comparison test. (E) 5-LO mRNA expression in antagonir-treated MM6 cells. The cells were differentiated for 4 d with 1 ng/ml TGF-β and 50 nM calcitriol in the presence of 25 nM antagonir. Results are given as means ± SEM of the relative 5-LO mRNA expression compared with the control antagonir. 5-LO values are normalized to actin expression and are the results of four independent experiments.
mRNA expression was also determined. Both antagonirs alone or in combination did not change 5-LO mRNA expression (Fig. 2E).

This, together with the reporter gene experiments, shows that miR-19a-3p and miR-125b-5p directly regulate 5-LO protein expression but do not interfere with 5-LO mRNA expression in MM6 cells.

5-LO, miR-19a-3p, and miR-125b-5p expression in B lymphocytes stimulated with SAC and IL-2

The 5-LO pathway plays an important role in T and B lymphocyte responses. Therefore, we investigated the expression of both 5-LO and the miRNAs miR-19a-3p and miR-125b-5p in peripheral B and T cells. B lymphocytes were either cultivated in the absence or presence 10 ng/ml SAC in combination with 20 U/ml IL-2 for the indicated times (Fig. 3). SAC leads to BCR-mediated activation and proliferation of naive B cells (CD27⁻, CD19⁺) (34, 35), and IL-2 was used as costimulus (36).

Fig. 3A illustrates the expression pattern of both 5-LO mRNA as well as miR-19a-3p and miR-125b-5p in untreated B cells after cultivation of the cells for up to 4 d. The expression of the 5-LO mRNA varied at ∼60–80% of the peak expression. The miRNAs showed a slight downregulation toward day 2 and an upregulation on day 4 to the level of the initial expression. When we stimulated the B cells with SAC and IL-2, there was a slight decrease in 5-LO mRNA as well as miR-19a-3p and miR-125b-5p expression (Fig. 3B). To analyze an influence on translation, 5-LO protein expression was determined by Western blot. As shown in Fig. 3C, 5-LO protein expression was detectable on all 4 d. Slightly stronger bands were observed in stimulated cells, but this coincided with increased β-actin expression, suggesting that the relative 5-LO protein expression is rather constant in B cells regardless of cell stimulation. Thus, taken together our results in B cells suggest that 5-LO expression seems not to be regulated by miR-19a-3p and miR-125b-5p under these cell culture conditions using these stimuli.

MiR-19a-3p regulates 5-LO in activated T lymphocytes

Next, we analyzed the influence of the miRNAs miR-19a-3p and miR-125b-5p on 5-LO expression in activated T cells. Freshly isolated T cells were stimulated with the lectin PHA, which leads to a strong polyclonal proliferation (37). T cell stimulation by PHA can be divided into three phases, that is, an increase in volume, followed by an onset of RNA synthesis, marking in G1b phase, and finally the DNA synthesis and cell proliferation leading to a 5-fold increase in cell counts after 96 h (38).

Fig. 4A shows the expression pattern of 5-LO mRNA and the two miRNAs in untreated cells during 4 d. Probably as a response to cell isolation and cultivation under cell culture conditions, 5-LO expression decreased on day 1 to ∼20% of its peak expression and remained constant around this level during the following days. The miRNAs did not significantly change their expression during the 4 d.
In contrast, stimulation with PHA decreased 5-LO expression dramatically on day 3 to ∼5% and reincreased to ∼20% on day 4 as shown in Fig. 4B. The two miRNAs showed different expression patterns compared with untreated T lymphocytes. MiR-125b-5p expression decreased to ∼40% on day 2 and remained on that level. In contrast, miR-19a-3p expression increased ∼5-fold on day 3 and slightly decreased again on day 4. Unfortunately, 5-LO protein expression in T cells varied between the different patients. Interestingly, low 5-LO expression could be detected by Western blot, which rapidly disappeared after 3 h in culture (data not shown). Taken together, there is an inverse regulation of 5-LO mRNA and miR-19a-3p expression in PHA-activated T lymphocytes, which suggested that 5-LO might be a target of miR-19a-3p in activated T cells.

To prove the regulation of 5-LO by miR-19a-3p, PHA-stimulated T cells were incubated with 150 nM of the antagomir against hsa-miR-19a-3 for 4 d. This treatment led to an increase in 5-LO mRNA expression on days 3 and 4 compared with the treatment with a control antagomir (Fig. 5). The efficiency of the antagomir treatment was verified by mRNA expression analysis of the proven miR-19a-3p target gene cyclin D1 (Supplemental Fig. 2) (39). Taken together, the inverse expression of 5-LO and miR-19a-3p as well as the antagonim results suggest that 5-LO is a target of miR-19a-3p also in PHA-stimulated T lymphocytes.

**Discussion**

Arachidonic acid metabolism by 5-LO plays a role in immune responses and inflammatory processes. 5-LO has also been shown to be involved in the development of prostate cancer as well as chronic myeloid leukemia in BCR-ABL mice (40, 41). Furthermore, 5-LO has been shown to be highly expressed in mantle cell lymphoma (42), B lymphocytes, as well as in differentiated myeloid cells such as granulocytes, monocytes, or dendritic cells.

Cell differentiation as well as leukocyte proliferation have been shown to be regulated by miRNAs (43). Within this study, our aim was to identify miRNAs that target 5-LO in immune-competent cells. Among several bioinformatically predicted miRNAs, we experimentally validated the interaction between 5-LO and the miR-19a-3p and miR-125b-5p. We used the monocytic cell line MM6 where 5-LO is expressed on a low basal mRNA level and treatment with the differentiation stimuli TGF-β and calcitriol leads to a strong increase in 5-LO mRNA, protein, and activity (17). In undifferentiated cells, we could detect a distinct negative correlation between the expression of 5-LO mRNA and miR-19a-3p. Interestingly, miR-19a-3p belongs to the miR-17∼92 cluster, which was reported to inhibit TGF-β signaling (44). Specifically, members of this cluster (miR-17 and miR-20) reduce the expression of the type II TGF-β receptor and miR-18, another member, limits the expression of Smad4, suggesting that regulation of 5-LO...
expression by the miR-17–92 cluster can occur at multiple steps either by modulation of TGF-β signaling or directly by targeting 5-LO (44).

Our study now revealed that miR-19a-3p and miR-125b-5p are involved in the direct regulation of 5-LO expression. We were able to show that knocking down both miRNAs with antagonors led to an increase in 5-LO protein expression in differentiated MM6 cells (Fig. 2D). The miRNA knockdown did not affect 5-LO mRNA expression. According to these data, the mechanism involved in the regulation of 5-LO in MM6 cells by the two investigated miRNAs is an inhibition of translation rather than mRNA decay. Interestingly, miR-19a-3p and miR-125b-5p have previously been discussed in conjunction with the leukotriene pathway. A recent study showed that inhibition of either the BLT1 receptor or the cysteinyl leukotriene 1 receptor decreases expression of both miRNAs in macrophages. Additionally, miR-125b-5p expression was decreased in BLT1−/− mice compared with wild-type mice (12) and is upregulated by resolvin D1 (45). These observations and our data suggest a possible negative feedback regulation of 5-LO via the 5-LO products by induction of these two miRNAs.

Another interesting aspect is the observation that overexpression of miR-125b-5p blocks the differentiation of the promyelocytic cell line HL-60 by DMSO (13) and induces myeloid leukemia in mice (13, 46). Alternatively, differentiation of HL-60 cells by DMSO strongly induces the 5-LO pathway in these cells (14). In B cell development miR-125b-5p seems to play an important role in the germline center reaction, and it has been shown that miR-125b-5p is upregulated in germinal center cells (20). In our study, expression of miR-125b-5p in myeloid cells and in isolated lymphocytes was much lower compared with miR-19a-3p, which might be due to the culture conditions.

Regarding miR-19a-3p, it has been reported that its expression in B cells increases during differentiation from naive to germinal center cells with an abrupt downregulation when the cells leave the germinal center (22). Alternatively, 5-LO is downregulated in germinal center cells (8) and this downregulation is stronger on protein than on the mRNA level, which supports a possible regulation by a miRNA. When human B cells were activated under cell culture conditions, we did not observe strong changes in either 5-LO or miR-19a-3p and miR-125b-5p expression, indicating that regulation of 5-LO expression in B lymphocytes by the respective miRNAs is restricted to certain physiological conditions and might not be apparent under the usual cell culture conditions.

The presence of the 5-LO in T cells has been controversially discussed over the years (6, 47). We were able to detect 5-LO in freshly isolated T lymphocytes on the mRNA level as well as on the protein level (data not shown). However, in contrast to Cook-Moreau et al. (47), who detected 5-LO protein by Western blot even after stimulation, we saw a strong decrease in the 5-LO mRNA and protein levels within 3 h of cell culture. Because we could not detect concomitant changes in the respective miRNAs and because the t1/2 of the 5-LO protein in PMNL and HL60 cells was shown to be 24 h, this 5-LO downregulation seems to be due to active degradation of the 5-LO protein when T cells are cultured. Interestingly, when we stimulated the T cells, we observed a further downregulation on the mRNA level, which, at least is in part, is due to the strong upregulation of miR-19a. In PHA-stimulated T cells, we observed a strong decrease in 5-LO mRNA expression down to 5% after 4 d. Concomitantly, we saw a 5-fold increase in miR-19a-3p expression after 3 d of stimulation (Fig. 4B). As expected, application of antagonists against miR-19a-3p upregulated 5-LO mRNA expression (Fig. 5). Importantly, cyclin D1, another target gene of miR-19a, was upregulated too (Supplemental Fig. 2). The strong induction of miR-19a-3p by PHA is in line with the observation that the miR-17–92 cluster regulates T cell proliferation (24, 25) and that miR-19a-3p plays a role in promoting Th1 responses (26).

Different mechanisms are discussed for the control of the gene expression by miRNAs. They can either switch off or only fine tune target gene expression (48). The latter case leads to a slight decrease in translation but still allows protein expression (49). From our data it can be concluded that the relationship between 5-LO and miR-19a-3p in MM6 cells as well as human peripheral T cells stimulated with PHA is an example for the target tuning mechanism, because a knockdown leads only to a small effect on 5-LO mRNA or protein expression.

Interestingly, the effects of both miRNAs seem to be cell type– and stimulus-specific. Because our reporter assay data suggest a direct binding of both miRNAs to the 3′UTR of 5-LO mRNA, one possible explanation for this phenomenon could be the cell-specific expression of miRNA or miRNA binding proteins that prevent interaction of the miRNAs with the 5-LO mRNA.

In conclusion, we were able to find two miRNAs regulating 5-LO. We determined the expression pattern of these miRNAs in stimulated B and T cells and were able to show that antagonizing miR-19a-3p in PHA-stimulated T cells leads to an increase in 5-LO mRNA expression. Furthermore, treatment of differentiated monocytic MM6 cells with antagonors for miR-19a-3p and miR-125b-5p increases 5-LO protein expression. The data suggest that both miRNAs modulate the leukotriene pathway via regulation of the key enzyme 5-LO. This observation unravels previously unrecognized roles for both miRNAs by linking 5-LO with B and T cell responses, and it indicates that 5-LO control by miRNAs is a key component in the complex cellular signaling network of innate immune responses.

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Disclosures

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References


