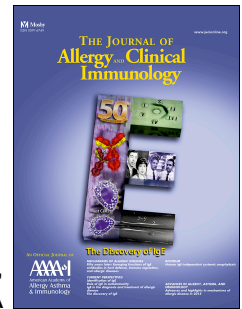


# Accepted Manuscript

miRNA-155 mediates down-regulation of the high-affinity receptor for IgE via Toll-like receptor signaling

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N. Leib *et al.*

**TITLE PAGE**

**miRNA-155 mediates down-regulation of the high-affinity receptor for IgE via Toll-like receptor signaling**

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**Capsule summary:**

miRNA-155 contributes to the inflammatory process in AD by regulating the expression of the high-affinity receptor for IgE (FcεRI) via its transcription factor PU.1. miRNA-155 may represent an interesting new target in AD therapy.

**Key words:**

Atopic dermatitis, FcεRI, dendritic cells, microRNA, PU.1, TLR

N. Leib *et al.*

**Abbreviations:**

AD – Atopic dermatitis

CD34LC – CD34<sup>+</sup> hematopoietic stem cell-derived LC

DC – Dendritic cell

FcεRI - high-affinity receptor for IgE

FSL-1 – Fibroblast-stimulating lipopeptide (Pam<sub>2</sub>Cys-GDPKHPKSF)

LC – Langerhans cell

LPS – Lipopolysaccharide

LTA – Lipoteichoic acid

miRNA – microRNA

P3C – Pam3Cys-SKKKK

rFI – relative fluorescence index

*S.a.* – *Staphylococcus aureus*

*S.p.* – *Streptococcus pyogenes*

SNP – single nucleotide polymorphism

**To the editor**

MicroRNA (miRNA) have been shown to be important fine tuners of posttranscriptional regulation of gene expression. In the immune system, two special miRNA have been extensively investigated: miRNA-146a is constitutively expressed in Langerhans cells (LC) and facilitates endotoxin-induced tolerance, while miRNA-155 is induced upon activation in macrophages and dendritic cells (DC) and can promote or suppress inflammation<sup>1, 2</sup>. miRNA-155 binds to PU.1, a key factor promoting the transcription of the high-affinity receptor for IgE (FcεRI). In atopic dermatitis (AD), a multifactorial severe skin disease, FcεRI is expressed on epidermal Langerhans cells (LC) and inflammatory dendritic epidermal cells (IDEC). AD skin is predominantly colonized with *Staphylococcus aureus* (S.a.) that can be recognized by Toll-like receptors (TLR). TLR activation on *in vitro* generated LC results in a down-regulation of FcεRI expression<sup>3</sup>. The aim of the present study was to explore regulatory processes of FcεRI expression after TLR signaling-related miRNA expression. We showed that TLR-induced and ectopic miRNA-155 decreased FcεRI on LC in a PU.1-dependent manner.

We used hematopoietic CD34<sup>+</sup> stem cell-derived LC (CD34LC) generated from human cord blood (provided by the Johanniter-Hospital Bonn and the St. Marien-Hospital Bonn, Germany, and with the approval of the local ethics committee of the University of Bonn in accordance with the declaration of the Helsinki principles) for stimulation or for transfection experiments (Online Repository Table EI and EII). CD1a<sup>+</sup> cells were obtained using CD1a-MircoBeads enrichment (Miltenyi Biotec GmbH, Germany). Mean purity of CD1a<sup>+</sup>-enriched cells was 83±12 %. CD1a<sup>+</sup> cells were subject to flow cytometry analysis, qPCR (antibodies and primers see Online Repository Table EIII and EIV) and TaqMan<sup>®</sup> MicroRNA Assays for hsa-miRNA-155-5p, hsa-miRNA-146a-5p and control RNU48 (Life Technologies GmbH, Germany). Significances (two-tailed Wilcoxon signed-rank tests), Pearson correlations and correlation significances were calculated using IBM SPSS Statistics 22 software (IBM Deutschland GmbH, Germany).

Our first experiments tested whether miRNA-155 and miRNA-146a were involved in TLR2-induced modulation of FcεRI expression in CD34LC. miRNA-155 was highly induced by TLR2 ligation (Fig. 1A). CD34LC were transfected with pre-miRNA-155 and pre-miRNA-146a. Transfection specificities were verified by Taqman<sup>®</sup> MicroRNA Assays (Fig. 1B-C). The levels of both PU.1 (Fig. 1D) and FcεRI (Fig. 1E-G) were reduced significantly in the presence of

ectopic miRNA-155 in comparison with the control. There was no effect in the presence of ectopic pre-miRNA-146a. Thus, transfection of CD34LC with ectopic miRNA-155 was sufficient for TLR-induced FcεRI regulation via its transcription factor PU.1.

The effect of different TLR engagements on the expression of miRNA, PU.1 and FcεRI were then investigated. miRNA-155 expression was shown to be induced strongly with P3C (8.5±4.8-fold) followed by FSL-1 (7.1±3.2-fold) and LTA (4.4±2.2-fold). *PU.1* and FcεRI expressions were reduced significantly and inversely proportional to miRNA-155 levels in all cases (Fig. 2A). Bacterial suspensions of *S.a.* (Pansorbin®) and of *S.p.* (serotype M49 strain 591) were then used to mimic the influence of AD-related skin bacteria and LPS to address TLR4. miRNA-155 was induced significantly by LPS, *S.a.*, *S.p.* and P3C (Fig. 2B). *PU.1* decreased significantly with all four stimuli, but showed the strongest reduction with *S.a.* (-57±14 %) and *S.p.* (-55±20 %) followed by P3C (-54±7 %) and LPS (-36±18 %) (Fig. 2B). FcεRI was decreased strongly at the mRNA and protein levels after each treatment. In addition, there was a significant negative correlation of *PU.1* and *FCER1A* mRNA with miRNA-155 on one hand but a significant positive correlation between *FCER1A* and *PU.1* mRNA (Fig. 2C) on the other hand. In contrast, *PU.1* and *FCER1A* did not correlate with miRNA-146a (Online Repository Fig. S1). Taken together, the results show that when miRNA-155 was induced upon TLR2 and TLR4 activation on LC there was a significant regulating effect on lowering the levels of *PU.1* and subsequently FcεRI.

Among inflammatory cells in AD, DC have been shown to express miRNA-155<sup>4</sup>. Therefore, since FcεRI on DC is assumed to be a key structure in the inflammatory process of AD, we investigated the influence of miRNA-155 and miRNA-146a on FcεRI expression. Upon TLR2 engagement miRNA-155 was highly induced, while miRNA-146a showed only little increase. Ectopic and different TRL-induced miRNA-155 sufficiently down-regulated *PU.1* and subsequently FcεRI. Negative correlations of miRNA-155 with *PU.1* and *FCER1A* expression and positive correlations for *FCER1A* and *PU.1* supported a relationship between miRNA-155 levels and FcεRI via PU.1.

A contradictory point is the high expression of FcεRI on cutaneous DC in the frequent presence of skin colonization with *S.a.* in AD. Our data would imply a down-regulation of the receptor

following the miRNA-155-PU.1-FcεRI pathway, but miRNA-155 as well as PU.1 themselves have been described to be elevated in AD, too<sup>5</sup>. In murine bone marrow derived mast cells (BMMC) *PU.1* mRNA and protein levels are increased upon IL-33 treatment<sup>6</sup>, an interleukin that is found to be released upon FcεRI cross-linking in mice and, controversially discussed, in human mast cells, but also in keratinocytes and endothelial cells of lesional AD skin<sup>7, 8</sup>. Additionally, IL-33 can be positively regulated by miRNA-155 as shown in chronic allergen challenged murine type 2 innate lymphoid cells (ILC2)<sup>9</sup>. Transferring the knowledge taken from the literature to our system, one could suggest that miRNA-155-induced PU.1 expression via IL-33 is more powerful than a concurrent down-regulation of PU.1 through direct miRNA-155/mRNA interaction. However, in AD, a high expression of PU.1 may overcome miRNA-155 levels and thus PU.1 down-regulation is reduced. Another point is the incidence of several single nucleotide polymorphisms (SNP) in the receptor's promotor region which go along with an increased FcεRI expression in AD. SNP can also be found in the 3' UTR of PU.1 (see: <https://www.ncbi.nlm.nih.gov/snp/?term=SPI1>). *In silico* analysis revealed that none of the known SNP in the PU.1 3' UTR affects the binding region of miRNA-155 (data not shown).

In summary, several factors may contribute to the regulation of FcεRI expression on cutaneous DC. We have shown that different TLR stimuli as well as whole bacterial lysates were able to significantly induce miRNA-155 and thereby decrease FcεRI levels in *in vitro* LC in a PU.1 dependent manner. These findings support the hypothesis for a functional TLR-miRNA-155-PU.1-FcεRI regulatory network in CD34LC. The sustained elevation of FcεRI in AD may therefore be owing to an interruption in the dialogue between miRNA and PU.1.

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N. Leib *et al.*

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**Figure Legends**

**Figure 1 Ectopic miRNA-155 decreases PU.1 and FcεRI expression.** CD34LC were left untreated (Ø), were treated with P3C or were transfected with indicated pre-miRNA for 24 h. Mean values ± SEM of stimulation experiments (n=7) are shown (A). miRNA expressions (B) and mean values ± SEM of miRNA levels (C), of mRNA (D, E) and rFI (F) of transfected cells (n=8-10) are depicted. Representative histograms of FcεRI protein expression are shown (G). (RQ=relative quantitation) \*p<0.05; \*\*p<0.01

**Figure 2 Different TLR stimuli were able to decrease PU.1 and FcεRI expression.** CD34LC were left untreated (Ø) or were treated as indicated and analyzed by Taqman<sup>®</sup> MicroRNA Assays and qPCR. Mean values ± SEM of miRNA-155-5p, mRNA levels of *PU.1* and *FCER1A* and rFI of FcεRI from n=5-8 experiments are shown (A, B). Pearson correlations and correlation significances of ΔCT values of *PU.1*, *FCER1A* and miRNA-155 of stimulation experiments are depicted. \*p<0.05

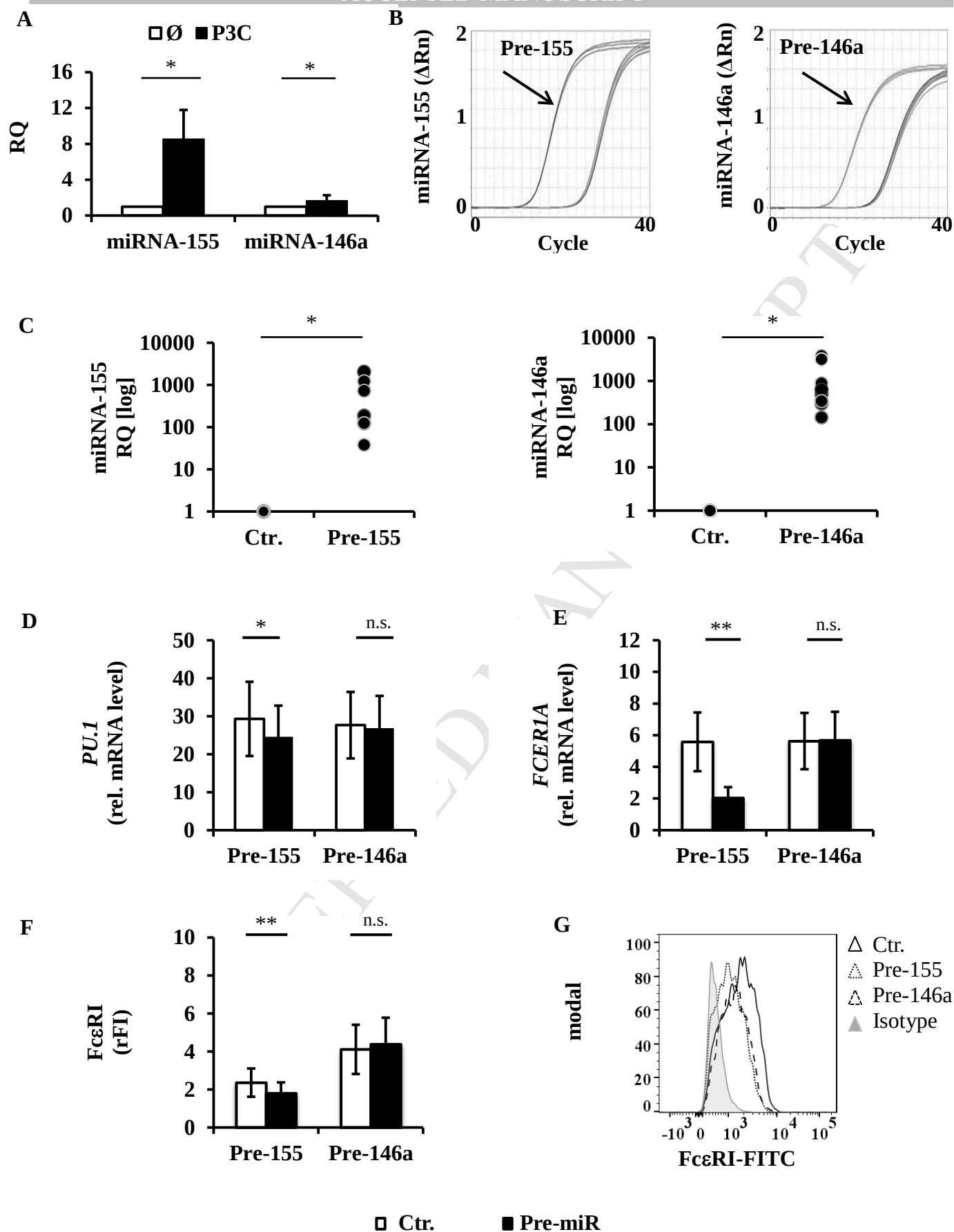


Figure 1

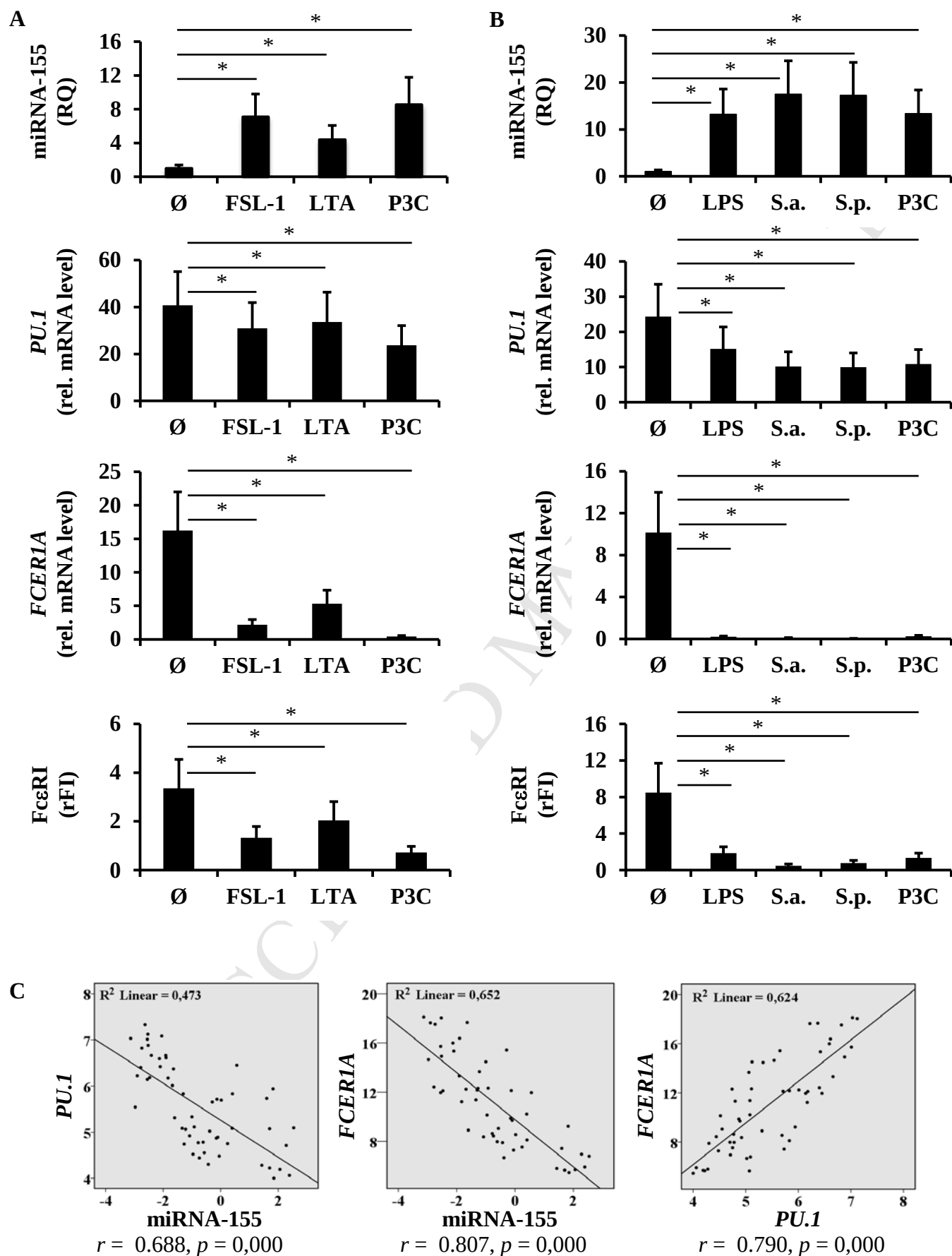


Figure 2

## Online Repository

**Table EI Ligands and concentration used for TLR mediated stimulation of CD34LC.**

| Stimulant / Treatment           | Manufacturer's specifications                                                                                                                                                                                                                              | Final concentration            |
|---------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|
| <b>FSL-1</b>                    | <b>Description:</b> Synthetic diacylated lipoprotein,<br><b>Derived from:</b> represents lipoprotein LP44 of <i>Mycoplasma salivarium</i><br><b>Ligand for:</b> TLR2 / TLR6<br><b>Manufacture:</b> EMC microcollections GmbH, Tübingen, Germany            | 16.6 x 10 <sup>-3</sup> µg/mL  |
| <b>LTA-SA</b>                   | <b>Description:</b> purified lipoteichoic acid, Enodotoxin <1.25 EU/mg<br><b>Derived from:</b> <i>Staphylococcus aureus</i><br><b>Ligand for:</b> range from 10 ng/mL to 10 µg/mL only activates TLR2<br><b>Manufacture:</b> InvivoGen, San Diego, CA, USA | 1.0 µg/mL                      |
| <b>LPS</b>                      | <b>Description:</b> ultra-pure lipopolysaccharide<br><b>Derived from:</b> <i>E. coli</i> 0111:B4<br><b>Ligand for:</b> TLR4<br><b>Manufacture:</b> InvivoGen, San Diego, CA, USA                                                                           | 0.1 µg/mL                      |
| <b>M 49 vaccine<br/>POD 591</b> | <b>Description:</b> Heat-killed bacteria<br><b>Derived from:</b> <i>Streptococcus pyogenes</i> serotype M49<br><b>Ligand for:</b> TLR2<br><b>Manufacture:</b> kind gift from R. Lütticken, University of Aachen, Germany                                   | 6.5 x 10 <sup>9</sup> cells/mL |
| <b>Pansorbin</b>                | <b>Description:</b> Heat-killed, formalin-fixed<br><b>Derived from:</b> <i>Staphylococcus aureus</i> Cowan 1<br><b>Ligand for:</b> TLR2<br><b>Manufacture:</b> ©Merck KGaA, Darmstadt, Germany                                                             | 10 µL suspension/mL            |
| <b>P3C</b>                      | <b>Description:</b> Synthetic triacylated lipopeptide<br><b>Derived from:</b> -<br><b>Ligand for:</b> TLR1 / TLR2<br><b>Manufacture:</b> EMC microcollections GmbH, Tübingen, Germany                                                                      | 1.0 µg/mL                      |
| <b>“untreated”</b>              | -                                                                                                                                                                                                                                                          | equal volume of medium         |

N. Leib *et al.*

## Online Repository

**Table EII miRNA molecules used for transfection experiments.** miRNA precursor and inhibitor molecules were purchased from Life Technologies GmbH, Darmstadt, Germany.

| miRNA Name                                                                                       | miRBase Accession #         | Species      |
|--------------------------------------------------------------------------------------------------|-----------------------------|--------------|
| Ambion <sup>®</sup> Pre-miR <sup>™</sup> Precursor<br>for hsa-miR-155-5p                         | MIMAT0000646                | Homo sapiens |
| Ambion <sup>®</sup> Pre-miR <sup>™</sup> Precursor<br>for hsa-miR-146a-5p                        | MIMAT0000449                | Homo sapiens |
| Ambion <sup>®</sup> Pre-miR <sup>™</sup> miRNA Precursor<br>Molecules - Negative Control # 1     | synthetically derived miRNA | -            |
| BLOCK-iT <sup>™</sup> Alexa Fluor <sup>®</sup> Red<br>Fluorescent Control (Ambion <sup>™</sup> ) | synthetically derived miRNA | -            |

## Online Repository

**Table EIII Antibodies used for flow cytometry analysis.**

| Antibody /<br>Conjugation                     | Immunogen /<br>Specificity | Clone         | Species | Isotype  | Manufacturer                                             |
|-----------------------------------------------|----------------------------|---------------|---------|----------|----------------------------------------------------------|
| <b>CD14-APC</b>                               | human CD14                 | TÜK4          | mouse   | IgG2a    | Miltenyi Biotec GmbH,<br>Bergisch Gladbach,<br>Germany   |
| <b>FcεRIa</b>                                 | human FcεRIa               | AER-37 (CRA1) | mouse   | IgG2b, κ | eBioscience, San<br>Diego, CA, USA                       |
| <b>Goat anti-<br/>mouse IgG,<br/>Fcγ-FITC</b> | mouse IgG Fcγ              | polyclonal    | goat    | IgG      | Jackson<br>ImmunoResearch<br>Europe Ltd., Suffolk,<br>UK |
| <b>CD1a<br/>(T6-RD1)</b>                      | human CD1a                 | SFCI19Thy1A8  | mouse   | IgG1     | Beckman Coulter Inc.,<br>Krefeld, Germany                |

## Online Repository

**Table EIV Oligonucleotides used for qPCR.** Oligonucleotides were purchased from Life Technologies GmbH, Darmstadt, Germany.

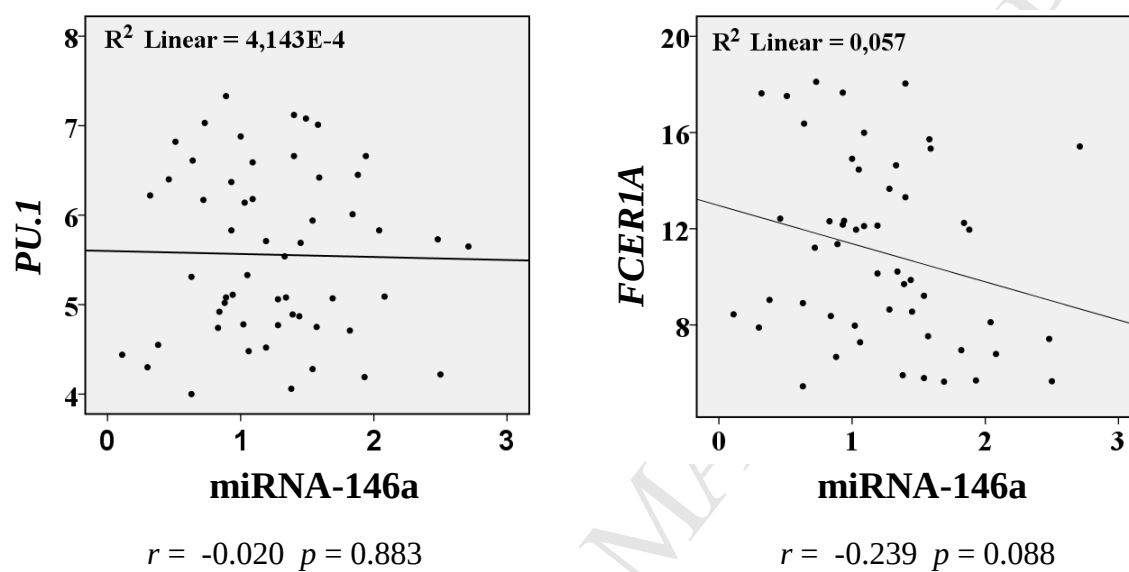
| Gene          | Forward sequence (5' → 3') | Reverse sequence (5' → 3') |
|---------------|----------------------------|----------------------------|
| <i>ACTB</i>   | AGCGCGGCTACAGCTTCA         | TCCTTAATGTCACGCACGATTT     |
| <i>FCER1A</i> | GGCAGCTGGACTATGAGTCTGA     | CTTCTCACGCGGAGCTTTTATT     |
| <i>FCER1G</i> | GATGCCATCCTGTTTCTGTATGG    | CACTTGGATCTTCAGTCGACAGTAG  |
| <i>PU.1</i>   | GGAGAGCCATAGCGACCATT       | GGAGCTCCGTGAAGTTGTTC       |

**Figure S1 *miRNA-146a* expression does not correlate with *PU.1* and *FCER1A* levels.** Pearson correlations of  $\Delta$ CT values of *PU.1*, *FCER1A* and *miRNA-146a* of stimulation experiments from Figure 2 are shown. ( $r$ =Pearson correlation)

**Figure S2 *miRNA-155* downregulates *MYD88*.** CD34LC were transfected with indicated pre-miRNA for 24 h. Mean values  $\pm$  SEM of *MYD88* mRNA levels of  $n=7-9$  experiments are shown. (RQ = relative quantitation),  $*p<0.05$

## Online Repository

## Supplemental Figure S1



# Online Repository

## Supplemental Figure S2

