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Carreras-Badosa, Gemma; Maslovskaja, Julia; Periyasamy, Kapilraj; Urgard, Egon; Padari, Kärt; Vaher, Helen; Tserel, Liina; Gestin, Maxime; Kisand, Kai; Arukuusk, Piret; Lou, Chenguang; Langel, Ülo; Wengel, Jesper; Pooga, Margus; Rebane, Ana

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Gemma Carreras-Badosa, Julia Maslovskaja, Kapilraj Periyasamy, Egon Urgard, Kärt Padari, Helen Vaher, Liina Tserel, Maxime Gestin, Kai Kisand, Piret Arukuusk, Chenguang Lou, Ülo Langel, Jesper Wengel, Margus Pooga, Ana Rebane

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CREDIT AUTHOR STATEMENT

Gemma Carreras-Badosa: Conceptualization, Investigation, Formal analysis, Visualization, Writing – Original Draft.
Julia Maslovskaja: Investigation, Formal analysis
Kapilraj Periyasamy: Investigation, Formal analysis
Egon Urgard: Investigation, Formal analysis
Kärt Padari: Investigation, Formal analysis
Helen Vahe: Investigation, Formal analysis
Liina Tserel: Formal analysis
Maxime Gestin: Conceptualization, Writing – Review & Editing
Kai Kisand: Formal analysis
Piret Arukuusk: Conceptualization, Writing – Review & Editing
Chenguang Lou: Investigation
Ülo Langel: Conceptualization, Writing – Review & Editing
Jesper Wengel: Conceptualization, Writing – Review & Editing
Margus Pooga: Conceptualization, Writing – Review & Editing
Ana Rebane: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – Review & Editing
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AUTHORSHIP AND AFFILIATIONS
Gemma Carreras-Badosa, Julia Maslovskaja, Kapilraj Periyasamy, Egon Urgard, Kärt Padari, Helen Vaher, Liina Tserel, Maxime Gestin, Kai Kisand, Piret Arukuusk, Chenguang Lou, Ülo Langel, Jesper Wengel, Margus Pooga, Ana Rebane

1 Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia
2 Institute of Molecular and Cell Biology, University of Tartu, Estonia
3 Department of Biomedicine, University of Tartu, Estonia
4 Department of Biochemistry and Biophysics, Stockholm University, Sweden
5 Institute of Technology, University of Tartu, Estonia
6 Nucleic Acid Center, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Denmark

CORRESPONDING AUTHOR
Ana Rebane, PhD
Institute of Biomedicine and Translational Medicine
University of Tartu
Ravila 14B
50411 Tartu, Estonia
Tel: +372 7 374 419
Email: ana.rebane@ut.ee

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CONFLICT OF INTEREST STATEMENT
None of the authors have any conflict of interest.

AUTHOR CONTRIBUTIONS
G.C.-B. contributed to the study design, carried out the experiments, analysed the data and wrote the manuscript. J.M., K.P., E.U., K.P., H.V. and C. L. carried out the experiments and analysed the data. L.T and K.K. analysed data. M.G., P.A., U.L., J.W. and M.P. contributed to the study design and review of the manuscript. A.R. designed and supervised the study and participated in the writing of the manuscript. All authors were involved in the interpretation of data and confirmed the final version of the manuscript.

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Abstract

MicroRNAs (miRNAs) are post-transcriptional gene expression regulators with potential therapeutic applications. miR-146a is a negative regulator of inflammatory processes in both tissue-resident and specialized immune cells and may therefore have therapeutic effect in inflammatory skin diseases. PepFect (PF) and NickFect (NF) type of cell-penetrating peptides (CPPs) have previously been shown to deliver miRNA mimics and/or siRNAs into cell cultures and in vivo. Here, we first demonstrate that selected PF- and NF-type of CPPs support delivery of fluorescent labelled miRNA mimics into keratinocytes (KCs) and dendritic cells (DCs). Second, we show that both PF- and NF-miR-146a nanocomplexes were equally effective in KCs, while NFs were more efficient in DCs as assessed by downregulation of miR-146a-influenced genes. None of miRNA nanocomplexes with the tested CPPs influenced the viability of KCs and DCs nor caused activation of DCs according to CD86 and CD83 markers. Transmission electron microscopy analysis with Nanogold-labelled miR-146a mimics and assessment of endocytic trafficking pathways revealed endocytosis as an active route of delivery in both KCs and DCs for all tested CPPs. However, consistent with the higher efficiency, NF-delivered miR-146a was detected more often outside endosomes in DCs. Finally, pre-injection of NF71:miR-146a nanocomplexes was confirmed to suppress inflammatory responses in a mouse model of irritant contact dermatitis as shown by reduced ear swelling response and downregulation of pro-inflammatory cytokines, including IL-6, IL-1β, IL-33 and TNF-α. In conclusion, NF71 efficiently delivers miRNA mimics into KCs as well as DCs, and therefore may have advantage in therapeutic delivery of miRNAs in case of inflammatory skin diseases.
INTRODUCTION

Therapeutic applications targeting the immune system have been of great interest in the last years, however, the gap in knowledge on delivery into primary immune cells is still a challenge. One of the central pathways activated during many immune system associated conditions is the NF-κB inflammatory signalling pathway. The controlled modulation of the NF-κB signalling could possibly have therapeutic effect in numerous immune system associated diseases and would be an alternative to antibody-based therapies targeting cells surface receptors or extracellular mediators.

Inflammatory skin diseases, such as atopic dermatitis or psoriasis, are lifelong conditions that severely impair life quality and affect up to 5% of the adult population in the developed countries. These diseases associate with sensitization of the immune system to environmental and/or intrinsic factors and result in a complex changes in interplay among keratinocytes (KCs), immune cells and inflammatory mediators in the skin. Current therapies are focused on neutralizing disease-related cytokines or chemokines using biologics, but targeting of immune cells instead, such as dendritic cells (DCs), could be an alternative approach to overcome safety issues and a great advantage allowing the modulation of adaptive immune responses involved in the development of these skin diseases. DCs as antigen-presenting cells could modulate adaptive immunity through interaction with T cells and production of cytokine and chemokine.

MicroRNAs (miRNAs), a family of small endogenous noncoding RNAs, are post-transcriptional gene expression regulators involved in many biological processes. Several miRNAs have been related to inflammatory diseases and have potential therapeutic applications. miR-146a plays an important role in the negative regulation of inflammatory responses: it acts as a feedback suppressor of the NF-κB pathway in KCs and has been shown to be upregulated in Langerhans cells (LCs) as compared to inflammatory DCs. In the skin, all these cell types, KCs, LCs and DCs can be considered the main cell types subjected to therapeutic targeting. DCs have been already used for different cancer therapies and siRNA delivery into DCs has been achieved as well. However, little is known about the delivery of miRNAs into DCs, as well as the influence of different delivery methods on the properties of DCs is not well studied. The delivery of miR-146a mimics, synthetic analogues of the endogenous miR-146a, to achieve the increase in miR-146a levels in these cells would be a potential therapeutic application to decrease skin inflammation.
The main challenge to surpass when delivering nucleic acids (NAs) is their limited ability to penetrate cell membranes due to their negative charge. To date, a vast number of substances have been used to develop NA delivery systems, including peptides\(^\text{17}\), but there is still great need for efficient, safe and specific methods for in vivo delivery of any type of NAs, including miRNA mimics\(^\text{18}\). Efficient internalization of therapeutic agents, without cytotoxicity or immunogenic effects, into target cells is very important to gain the desired therapeutic effect, especially when the purpose is to target unwanted inflammation.

Cell-penetrating peptides (CPPs) are a broad group of short peptides with membrane translocation activity, which have been successfully applied in vitro and in vivo to deliver potentially therapeutic molecules, including DNA, RNA, siRNA\(^\text{19}\) and recently also miRNA mimics\(^\text{20,21}\) into different cell types with minimal toxicity. CPPs condense the negatively charged NAs into self-forming non-covalent complexes through electrostatic interactions and thereby successfully deliver NAs into the cells. The uptake mechanism of CPPs is not fully understood, but various types of endocytosis have been suggested, depending on CPP, cargo, cell-type and treatment conditions\(^\text{22,23}\). During endocytosis, each vesicular trafficking event involves membrane budding characterized by the formation of spherical vesicles from flat membrane areas typically followed by regulated transport. Endocytosis is traditionally divided into macropinocytosis, clathrin- or caveolin-mediated endocytosis and clathrin- or caveolin-independent endocytosis that differ in the composition of the coat, the size of the detached vesicles, and the fate of internalized particles\(^\text{24}\). Despite the cell-penetrating ability of CPPs, low delivery efficiency is common and achieving effective delivery is still a challenge, especially into immune cells. It should also be noted that a very few studies have been performed to study endocytic or trafficking pathways in specialized immune cells\(^\text{25}\). Moreover, elucidating cellular delivery mechanisms in different cell types may contribute to the development of proper 3R (reduction, replacement and refinement) workflows that potentially enable to reduce the amount of animal experiments in future\(^\text{26}\).

In the last decade, several CPP modifications, such as addition of different fatty acids in N-terminus\(^\text{27}\) or changes in the sequence to enhance helicity of their structure\(^\text{28}\) have been used to enhance CPP activity. Examples are different PepFect (PF) and NickFect (NF) type peptides, which are active in siRNA delivery in multiple cell types and/or in vivo. PF-type and NF-type of CPPs are transportan (TP) analogues for in vitro application\(^\text{29,30,31}\). In addition, amphipatic Mgpe type peptides derived from phosphatase E, such as Mgpe9, have been previously described to be useful in delivery of NAs into the skin\(^\text{32}\). In the current study, these CPPs were chosen as carriers and miR-146a mimics were used as test miRNA to assess the efficiency,
safety and trafficking pathways in human primary KCs and in vitro differentiated DCs. In addition, the effect of NF71:miR-146a nanocomplexes, which had good characteristics both in KCs and DCs, were tested in vivo in a mouse model of irritant contact dermatitis.

EXPERIMENTAL PROCEDURES

Cell cultures

Pooled primary human epidermal KCs (Promocell, Germany) were seeded at 2×10⁴ KCs per well on 12-well plates in 1 mL of Keratinocyte-SFM medium with supplements (Life Technologies, Carlsbad, CA, USA) 24 h before transfection and incubated at 37 °C in 5% CO₂. Human monocyte-derived dendritic cells (mo-DCs) were prepared from blood as previously described³³. Blood samples were obtained from Blood Centre of Tartu University Hospital from healthy blood donors who all gave a written informed consent. The study was approved by Ethics Review Committee on Human Research of the University of Tartu (Approval 166/T-10). “Buffy coat” blood samples were centrifuged using a density gradient on Ficoll-PaqueTM Plus (GE Healthcare, UK), peripheral blood mononuclear cells (PBMCs) fraction was obtained and monocytes (MOs) were purified by positive sorting using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotech, Germany). MOs were differentiated into DCs at a concentration of 1 million cells per mL on 24-well plates in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FBS (all from PAA Laboratories, Canada) in the presence of 50 ng/mL GM-CSF and 25 ng/mL IL-4 (both from PeproTech, UK) for 6 days before transfection and incubated at 37 °C in 5% CO₂. GM-CSF/IL-4-dependent monocyte-derived DCs share many characteristics with inflammatory dendritic epithelial cells in vivo¹⁴.

Cell-penetrating peptides

A series of PepFect (PF: PF14 and C22PF14), NickFect (NF: NF55, NF70, NF71 and NF72) and MGPE (MGPE9 and stearyl-MGPE9 (s-MGPE9)) type of cell-penetrating peptides were used (Table 1). PF- and NF-type CPPs are chemically derived from TP-10²⁹ and MGPE-type is derived from human protein phosphatase 1E³⁴. PF14 and C22PF14 were ordered via Pepmic (Jiangsu, China). All other peptides were synthesized on automated peptide synthesizer (Biotage Initiator+ Alstra, Sweden) using fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis strategy as previously described²²,³⁵. Peptides were purified by reverse-phase liquid chromatography on C4 column (Phenomenex Jupiter C4, 5 μm, 300 A, 250 × 10 mm) using a
gradient of acetonitrile/water containing 0.1% TFA. The molecular weight of the peptides was determined by MALDI-TOF mass spectrometry (Brucker Microflex LT/SH, USA).

Transfection of miRNA mimics

Peptides were incubated with miRNA mimic at a 17:1 CPP:miRNA molar ratio (MR) in MQ-water in 1/10 of final treatment volume at room temperature (RT) for 1 h to form CPP:miRNA complexes; mixed with media and added to the cells after removing the old growth media as previously described. Transfections were performed at 30 nM or 100 nM (miRIDIAN microRNA Mimic Negative Control #1 and miRIDIAN microRNA hsa-miR-146a mimic; all from Thermo Fisher Scientific, USA) concentration of miRNA mimics in KCs and DCs, respectively, for 48 h or 24 h. Transfection using commercial Lipofectamine2000 (LF2000) reagent (Invitrogen, USA) was conducted as a positive control following manufacturer’s instructions. When indicated, KCs were stimulated with interferon-γ (IFN-γ) using 20 ng per mL concentration and DCs with lipopolysaccharide (LPS) using 1 μg per mL concentration. Cells were harvested with Qiazol (Qiagen, Germany) and kept at -80ºC until RNA extraction.

Transfection of fluorescently-labelled miRNA mimics for internalization assessments and endosome marker detection

Cells were seeded onto glass coverslips and incubated with CPPs:miRNA nanocomplexes as described in the previous paragraph. For confocal imaging, transfections were performed using DyLight547-labelled miRNA mimic at a concentration of 60 nM (Dy-547 labeled miRIDIAN microRNA Mimic Transfection Control #1 from Thermo Fisher Scientific, USA) for 24 h in KCs and DCs. Thereafter, cells were washed in PBS and fixed with 4% paraformaldehyde (PFA) for 10 minutes. Cell membrane was counterstained with wheat germ agglutinin (WGA; Thermo Fisher Scientific, USA) and nucleus with 4’,6-diamidino-2-phenylindol (DAPI; Roche, Switzerland). For endosome marker detection, after fixing, the cells were permeabilized with 0.2% Triton and blocked with 2%FBS in 1X PBS. The primary antibodies, anti-human-early endosome marker 1 (EEA1) and anti-human-lysosome marker (LAMP2), were used for cell staining followed by AF488-anti-mouse and AF488–anti-rabbit secondary antibodies. Finally, coverslips were reversely mounted using fluorescent mounting media (DAKO; Agilent Technologies, USA) onto slides. Images were taken at 60x/1.40 oil objective at 1024x1024 pixel resolution using a confocal laser scanning microscope (Olympus FV1200MPE; Olympus Corporation, Japan), analysed and processed with ImageJ software (http://imagej.nih.gov/ij/index.html; public domain, NIH, USA) and Adobe Photoshop (Adobe 652 Systems, Inc., San Jose, CA, USA). Lysosensor Green (acidic vesicle-marker; ThermoFisher...
Scientific, USA) was added into the media on the cells after transfection and live-cell images were taken using an inverted confocal microscope (Zeiss LSM710; Carl Zeiss AG, Germany). Relative colocalization was assessed with M2 coefficient (ImageJ software).

Synthesis of unlocked nucleic acid (UNA)-modified miRNAs

The UNA-modified miR-146 mimics (Table 2) were synthesized on an automated nucleic acid synthesizer, a PerSeptive Biosystems expedite 8909, using the phosphoramidite approach and following manufacturer’s standard protocols. The syntheses were performed in 1.0 µmol scale on polystyrene Glen Unysupport (Glen Research #26-5040-10) for the synthesis of miR-146a-uUNA (NAC8605), or 3’-thiol-modifier C3 S-S CPG support (Glen Research #20-2933-41) for the synthesis of miR-146a-Nanogold (NG) (NAC8882) and miR-146a-Cy5 (NAC9041), respectively. Standard RNA synthesis conditions of the synthesizer were used for the incorporation of RNA and UNA monomers using DCI (4,5-dicyanoimidazole) as an activator, and the stepwise coupling yield of unmodified RNA as well as UNA monomers were >99% based on the absorbance of the dimethoxytrityl cation released after each coupling step. Following standard RNA deprotection, purification and workup, the composition and purity (>80%) of the resulting oligonucleotides was confirmed by MALDI-MS analysis and ion exchange HPLC. Reactive thiol in miRNA-146a mimic was obtained by disulfide reduction using a buffered DTT (dithiothreitol) solution according to manufacturer’s standard protocols. Next, the miRNA-146a (SH) was coupled with sulfo-cyanine5-maleimide (Lumiprobe #13380) to give the miRNA-146a-Cy5 using the manufacturer’s recommended protocol.

Transfection of NG-labelled miRNA mimics and transmission electron microscopy (TEM)

MiRNA mimic containing thiol modifier C3 S-S (NAC8882, 49.8 nmoles) was associated with NG as described earlier. After removal of protection from the thiol group by reduction with DTT, NAC8882 was covalently labelled with monomaleimido NG (d1.4 nm; Nanoprobes Inc., NY). The miRNA mimic NG 1:1 conjugate was purified from unreacted free mimic and NG label by gel filtration on Superdex 30. The concentration of conjugate was calculated based on the absorption at 420 nm (NG) and its complexes with peptides were formed as described above. Cells were seeded onto glass coverslips and incubated with CPP:miRNA mimic complexes as described in the previous paragraphs. Transfections were performed at a NG-tagged mir-146a mimic concentration of 60 nM for 4 h in KCs and DCs. After incubation, coverslips with cells were washed with cacodylate buffer (pH 7.4) and fixed with 2.5% glutaraldehyde at RT for 1 h. To visualize the NG label in cells by TEM, it was magnified by silver enhancement (HQ Silver Kit, Nanoprobes Inc, NY) to about 10-12 nm size and stabilized with 0.05% gold chloride.
post-fixation with osmium tetroxide the cells were dehydrated with ethanol and embedded in epoxy resin (TAAB Laboratories Equipment Ltd., UK). The specimens were cut into ultrathin sections and contrasted with uranyl acetate and lead citrate. The sections were examined with Tecnai G2 Spirit transmission electron microscope (FEI, The Netherlands) and microphotos were analysed and processed with Adobe Photoshop CS4 software.

Transfection of siRNA mimics

DCs transfections were performed using siRNA mimics at a concentration of 60 nM [Silencer Select Negative Control Nº1 siRNA and Pre-designed siRNA hsa-IRF1 (all from Thermo Fisher Scientific, USA)] for 24 h following the same instructions as described for miRNA mimics transfection.

Inhibition of endocytosis

Peptides were incubated with miRNA mimic and transfections were performed as described above in KCs and DCs, respectively, for 24 h. When indicated, different inhibitors of endocytosis were used for 30 min to block the following endocytic pathways just before transfection as follows: 10 μM chlorpromazine to block clathrin-mediated endocytosis, 50 μM nystatin to block caveolin-mediated endocytosis and either 4 μM cytochalasin D or 50 μM 5-(N-ethyl-N-isopropyl)-Amiloride (EIPA) to block macropinocytosis. Cells were harvested with Qiazol (Qiagen, Germany) and kept at -80ºC until RNA extraction.

RNA purification and quantitative RT-PCR

RNA was purified using Qiazol (Qiagen, Germany) and Total RNA Zol-out kit (A&A Biotechnology, Poland) in case of cell cultures and Total RNA Zol-out D kit (A&A Biotechnology, Poland) in case of mouse tissue. To maintain small RNA fraction, a 1:1 ratio of isopropanol was added to the samples before loading them onto the kit columns. The concentration and quality of RNA was assessed with NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). cDNA from 300-500 ng of total RNA was synthesized using oligo(dT), RiboLock RNase Inhibitor, RevertAid and dNTP Mix according to the manufacturer's protocol (Thermo Scientific, USA). Real-time qPCR (RT-qPCR) was performed in triplicates with 5xHOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne, Estonia) and the primers from TAG Copenhagen (Supplementary Table S1) using ViiA™ 7 Real-Time PCR system (Applied Biosystems, USA). The relative gene expression levels were normalized according to the level of elongation factor 1 alpha (EF1A) in case of cell cultuers and beta-2 microglobulin (B2M) in case of mouse samples.
and calculated using the comparative Ct (ΔΔCt) method. The mean level of non-transfected cells or control experiments was equalized to 1.

Cell viability assay

KCs were seeded at density of $5 \times 10^3$ cells per well on 96-well plate and cultured for 24 h before transfection. DCs were cultured for 6 days before transfection starting from $1 \times 10^5$ cells per well of 96-well plates. All transfections were performed for 72 h, otherwise as described in the paragraph of miRNA mimics transfection description. Cell proliferation assay was performed according to manufacturer’s instructions using CellTiter- Glo Luminescent Cell Viability Assay (Promega, USA) and analysed with VICTOR X5 reader and Workout 2.5 software (Perkin Elmer, USA). Cell viability percentage was calculated as: 
\[
\frac{\text{transfected cell sample (luminescence units)}}{\text{non-transfected cell samples average (luminescence units)}} \times 100.
\]

Flow cytometry assay

DCs surface markers were analysed using flow cytometry. DCs were transfected for 24 h as described before. The conjugated antibodies phycoerythrin (PE)-labelled anti-CD83, fluorescein isothiocyanate (FITC)-labelled anti-DC-SIGN/CD209, FITC-labelled anti-CD86 and allophycocyanin (APC)-labelled anti-CD14 (all from Biologend, USA or Southern Biotech, USA) were used for cell staining. Cells were washed in ice-cold FACS buffer (PBS containing 0.5% BSA and 2 mM EDTA), pre-incubated with FcR Blocking Reagent (Milteny Biotec, Germany) for 5 minutes to block non-specific Ig binding and incubated with the panel of specific antibodies or respective fluorescent minus one (FMO) controls for 20 minutes at 4°C in the dark. Cells were then washed twice and suspended in FACS buffer for analysis. Data was acquired by BD LSRFortessa™ (BD Biosciences, USA) flow cytometer and analysed using BD FACSdiva software and FlowJo v10.6 (both from Becton Dickinson, USA).

Nanoparticle tracking analysis (NTA)

Peptides were incubated with miRNA mimic as described above and diluted 1:100 in MQ water. Analyses were performed with ZetaView PMX 110 v3.0 and ZetaView NTA software (Particle Metrix GmbH, Germany). Operating instructions of the manufacturer were followed before calibrating the instrument with a known concentration of 100 nm polystyrene nanoparticles (Applied Microspheres B.V., Netherlands). Particles were counted and size-distributed at three cycles under a sensitivity of 85 and shutter value of 70. Zeta potential measurements were performed at five cycles under a sensitivity of 85 and shutter value of 70. Temperature was maintained at 25°C during the measurement.
Mouse model of irritant contact dermatitis (ICD) and in vivo delivery of miRNA-146a nanocomplexes

All animal experiments were approved by the Animal Ethics Committee at the Ministry of Agriculture Estonian Government (01.03.2018, licence No. 117 and 21.01.2020, licence No. 158). Mice were maintained in the animal facility at the Institute of Biomedicine and Translational Medicine, University of Tartu in accordance with the institute’s regulations. Eight-to-ten-week-old wild type C57BL/6J female mice were used in all NF71 experiments and male mice were used for imaging analysis presented on supplementary materials. For in vivo delivery, 60 pmol of miRNA mimics (double stranded for miR-146a-Cy5 (NAC9041), Table 2; or unlabelled miRIDIAN microRNA Mimic Negative Control #1) at 28:1 CPP:miRNA MR in 20 μl of 5% glucose were injected subcutaneously per one ear (one ear control, one ear miR-146a). To induce ICD, 20 μl of 0.2% phorbol 12-myristate 13-acetate (PMA) in acetone was applied topically. Ear thickness was measured in a blinded manner with a Vernier digital calliper (Mitutoyo, Japan) in three different locations per one ear in each time-point. Mouse ears and draining lymph nodes were harvested in 4% paraformaldehyde for imaging procedures and/or in Qiazol using tissue homogenizing CK14 tubes (Precellys Lysing Kit, Bertin Technologies, France) for RNA isolation. For histology and immunofluorescence analyses, mouse ears and draining lymph node (LN) tissues were fixed in 4% paraformaldehyde for 24 h at 4°C, incubated in 30% sucrose for 24 h at 4°C and embedded in Neg-50 cryo-medium tissuetek (Thermo Fisher Scientific, USA). Ten-micrometer cryosections were obtained and stained with diff-quik for histology imaging. Histology slides were analysed with a Nikon Eclipse C1 light microscope (Nikon Instruments Europe BV, The Netherlands). Additionally, 10 μm cryosections of LN were permeabilized with 0.3% Triton-X-100 in PBS and stained with AF488-labelled anti-mouse-CD11c antibody overnight. Specimens were further counterstained with DAPI and slides were mounted with fluorescence mounting media (Dako Products, Agilent Technologies, USA). All slides were analysed with an Olympus FV1200 confocal laser scanning microscope (Olympus, Japan).

Statistical analyses

Results are expressed as mean ± SEM. Unpaired Student’s t-tests were used to study differences among treatments. In mouse experiments, two-way ANOVA analyses were performed to assess differences in mean values along different time-points and paired Student’s t-tests were used to study differences among treatments in the different ears of
same mice. Significance level was set at P<0.05. GraphPad Prism v.6 was used for preparing the graphs and the statistical analyses.

RESULTS

CPP:miRNA complexes are internalized by KCs and DCs

First, the selected CPPs (Table 1) were used to test their capacity to deliver fluorescently-labelled miRNA mimics into KCs and DCs. Confocal microscopy images show that internalization of the fluorescently-labelled miRNA was evident 24 h after transfection with PF14, NF55, NF70 and NF71 (Figure 1A) as well as C22PF14, NF72, MGPE9 and s-MGPE9 (Supplementary Figure S1A) in KCs, and to a lesser extent, in DCs (Figure 1A and Supplementary Figure S1A). Using an increased molar ratio (MR) of CPP:miRNA complexes, internalization of the labelled miRNA was apparently improved in DCs (Figure 1B and Supplementary Figure S1A). Interestingly, in case of both NF71 and NF70, the fluorescence signal was more evenly distributed in DCs and found to be present in every cell, while PF14, C22PF14, MGPE-9 and s-MGPE9 showed more particles that localized at the membrane both in KCs and DCs and some DCs lacked the signal (Figure 1, Supplementary Figure S1). Strong fluorescent signal and bigger vesicles were found in comparison to CPPs when LF2000 was used for transfection (Figure 1) in line with the mechanism used by liposomal reagents known to be a membrane fusion or engulfment of lipovesicles.

PF- and NF-type of CPP-delivered miR-146a mimic is efficient in the suppression of target genes in KCs

Next, all selected CPPs (Table 1) were used to deliver miR-146a and the control mimic into KCs and mRNA levels of target genes for miR-146a as well as related chemokines were measured to assess the effect. Liposomal reagent LF2000 (Figure 2) and naked mimic (Supplementary Figure S2) were used as controls, CPP:miRNA MR ratio was 17:1. As a result, the downregulation of the direct miR-146a target genes caspase recruitment domain family member 10 (CARD10) and interleukin 1 receptor associated kinase 1 (IRAK1), and miR-146a-influenced chemokines interleukin 8 (CXCL8) and chemokine C-X-C motif ligand 1 (CXCL1) was detected with LF2000, PF14, NF55, NF70 and NF71 (Figure 2A) as well as with C22PF14 and NF72, but not with MGPE-type of CPPs (Supplementary Figure S2A). When KCs where stimulated using interferon (IFN)-γ, the downregulation of direct targets IRAK1 and CARD10 persisted and a very strong downregulation of miR-146a-influenced chemokines was observed with all tested peptides, except for MGPE-type of CPPs (Figure 2B and supplementary Figure...
IFN-γ was used in the assay because it induces a strong inflammatory response in KCs and the skin. The transfection at CPP:miRNA MR 28:1 did not enhance the efficiency of MGPE-type of CPPs; while miR-146a mimic transfected with all other CPPs still efficiently suppressed the target genes (data not shown).

NF55-, NF70- and NF71-delivered miR-146a mimic is efficient in the suppression of target genes in DCs

Next, all tested CPPs (Table 1) were used to deliver miR-146a and the control mimic into DCs and mRNA levels of miR-146a-influenced genes IRAK1, interleukin (IL)-6 and CXCL8 were measured. As in KCs, the starting CPP:miRNA MR ratio was 17:1, liposomal reagent LF2000 (Figure 3A) and naked mimic (Supplementary Figure S3A) were used as controls. Consistent with immunofluorescence analysis (Figure 1), the downregulation of direct miR-146a target genes IRAK1 was detected using NF-type of CPPs, NF55, NF70 and NF71, however, only weak effect on miR-146a influenced cytokine IL-6 with LF2000 and NF55 was observed (Figure 3A).

Next, we used increased CPP:miRNA MR (28:1) and achieved the modest downregulation of IRAK1 also with PF14, NF55, NF70 and NF71 as well as IL-6 with NF70 (Figure 3B). The increasing of the CPP:miRNA MR to 28:1 was not beneficial for delivery with C22PF14, NF72 and MGPE-type CPPs (Supplementary Figure S3B). Stimulating DCs with LPS, which induces an inflammatory phenotype via toll-like receptor 4 (TLR4), did not enhance the effect of delivered miR-146a mimic on IL-6 and CXCL8 (data not shown).

The effect of CPP:miRNA transfection on cell viability and DC activation

To assess the viability of the cells during the delivery experiments using LF2000 and CPP:miRNA complexes, we used ATP dependent cell viability assay. Although LF2000 drastically decreased KCs cell viability as compared to non-transfected (NT) cells, neither DCs nor KCs were impaired after 72 h using the optimal 17:1 CPP:miRNA MR (Figure 4A and Supplementary Figure S4A). Next, as immunogenicity could be one of the drawbacks of using CPP-based delivery methods to target immune cells, we assessed the activation level of DCs during transfections with CPPs. We observed that none of the selected CPPs used in this study activated DCs, assessed by flow cytometry analysis of cell surface markers CD83 and CD86 (previously checked using LPS-stimulation, Supplementary Figure S4D). In addition, fluorescence minus one (FMO) controls were plotted in each histogram. The number of CD86+ cells using PF14, NF55, NF70 and NF71 (Figure 4B) and NF72, MGPE9 and s-MGPE9 (Supplementary Figure S4B) was significantly lower in comparison to LF2000 reagent indicating that transfection with used CPPs is less immunogenic as compared to LF2000. Using flow
cytometry for CD14+ and CD209+ markers, we also confirmed that differentiated DC population was well established (Supplementary Figure S4C).

**NF-type peptides efficiently deliver siRNAs in DCs**

As apparent differences between the efficiency of CPPs to deliver miRNA mimics into DCs as compared to KCs were observed, we analysed the relative miR-146a expression after transfection to see if it correlated with the observed effect of miR-146a. Indeed, the increase in the relative level of miR-146a after transfection in KCs was greater than in DCs (Supplementary Figure S5A). Interestingly, in correlation with the transfected miR-146a effect in DCs, we observed that the amount of miR-146a delivered by PF14 was lower than when using NF70 (Supplementary Figure S5A). Additionally, we found the endogenous expression level of miR-146a in cultured DCs was higher as compared to KCs (Supplementary Figure S5B), which is in concordance with previously observed very high miR-146a expression in human monocyte derived DCs and could be a plausible reason why the effect of miR-146a mimics in DCs is not so strong as in KCs. Therefore, we additionally assessed the efficiency of transfection of siRNA targeting interferon regulatory factor (IRF)1. We observed a strong downregulation of the *IRF1* gene expression after siRNA transfection in DCs achieved by the same CPPs, NF55, NF70 and NF71 (Supplementary Figure S5C) as in case of miRNA transfection (Figure 3) confirming that NF-type of CPPs are efficient in transfecting DCs.

**Cellular localization of the delivered miRNA mimics in KCs and DCs**

Next, we assessed whether the used CPPs condense miR-146a mimics to nanoparticle-type complexes and analysed the morphology (size and shape) of self-forming particles using TEM and NTA. All CPPs formed nanoparticles with NG-labelled miR-146a mimic (Supplementary Figure S6). The most regular nanoparticles formed with NF55 that packs miR-146a mostly into spherical particles with 50-70 nm in diameter (Ø). However, smaller particles of about Ø 15-25 nm were also observed, similar to previous results with CPP PepFect6 that efficiently delivers miRNA both *in vitro* and *in vivo*. NF70 and NF71 formed rather similar nanoparticles with mimic presenting less regular spherical shape and Ø 15-25 nm. These particles also showed tendency for association with each other forming clusters and branching structures of Ø 40-100 nm, which was also confirmed by the NTA (Supplementary Table S2). Additionally, we analysed the zeta potential of NF70:miRNA and NF71:miRNA nanoparticles with NTA and observed negative values of -21.7 or -27.9 mV, respectively (Supplementary Table S2). Interestingly, although PF14-miR-146a mimic nanoparticles are more spherical and have approximately the same size as the ones with NF70/71, they showed the highest tendency to
form long agglomerates of 50-200 nm in length. These results enable to conclude that NF-type of CPPs form more homogeneous particles than PF-type of CPPs when complexed with miR-146a mimics (Supplementary Figure S6).

To more precisely assess the cellular localization of the delivered miRNAs and visualize their internalization mechanism, we performed transfection with PF14, NF55, NF70 and NF71 nanoparticles with NG-labelled miR-146a mimic. Electron microscopy images showed that CPP:miRNA nanoparticles retain their shape upon association with the plasma membrane and inside cells both in KCs (Figure 5) and DCs (Figure 6).

In KCs, nanoparticles induced invagination of plasma membrane (Figure 5, white arrows) and cells uptake by endocytic vesicles (Figure 5, black arrows). NF70 and NF71 mostly triggered macropinocytosis (Figure 5E, 5G) whereas PF14 and NF55 were mostly taken up by cells in smaller vesicles, perhaps caveolar-coated vesicles (Figure 5A, 5C). Concordantly, when different endocytosis pathways were inhibited, both macropinocytosis and caveoae-mediated endocytosis processes were confirmed as main endocytic pathways for NF70:miRNA and NF71:miRNA complexes as downregulation of miR-146a target genes was not achieved in the presence of corresponding inhibitors (Supplementary Figure S5). Inside cells, CPP:miRNA complexes accumulated in multivesicular bodies (MVB) or late endosomes (LE) whose limiting membrane was in some cases destabilised enabling miRNA escape into cytosol (Figure 5, arrowheads).

In DCs, internalization substantially differed from that observed in KCs. The number of complexes on the surface was lower in DCs (Figure 6) as compared to KCs. In line with the fluorescence microscopy and transfection analysis data from previous sections, more nanoparticles were observed to be present in the cells when transfection was performed with NF70 or NF71 as compared to the transfections with PF14 and NF55. Furthermore, the miRNA complexes with PF14 and NF55 were trafficked in dissimilar manner from the ones with NF70 and NF71. PF14 and NF55 were mostly found in multilamellar vesicles (MLV) with low electron density, characteristic for DCs only, or outside vesicles (Figure 6D, 6G). NF70 and NF71 complexes were mostly found in MVB and endosomes with higher electron-density (Figure 6J, 6L). Moreover, using NF70 and NF71 the limiting membrane of these electron-dense vesicles was typically fragmented and higher amounts of miRNA complexes had escaped into cytosol (Figure 6J, 6L) as compared to PF14 and NF55. The differences observed in number and localization of NF70 and NF71 complexes in DCs is in concordance with the miRNA downstream effects found in these cells in vitro (Figure 3). Whether this is caused by
differences in the association of the CPP:miRNA complexes with the DC, by intracellular trafficking or by endosomal escape cannot be distinguished based on TEM data only.

**Cellular colocalization of endocytic pathway markers with the delivered miRNA mimics in KCs and DCs**

According to immunofluorescence and TEM analysis, the CPP:miRNA complexes were found mainly in endosome-like vesicles both in KCs and DCs. To better characterize the delivery processes, we next aimed to track the colocalization of CPP:miRNA complexes with different endocytic vesicle markers, such as EEA1 for early endosomes, Lysosensor for acidic vesicles, which are close to LE and LAMP2 for lysosomes. Colocalization between EEA1 marker, most used in clathrin-mediated endocytosis, and labelled miRNA was low in KCs and DCs within 30 min after transfection (Figure 7), indicating that clathrin-mediated endocytosis poorly contributes to uptake of CPP-based complexes in KCs and DCs. This finding is in line with TEM images showing more macrophagocytosis or caveolin-dependent endocytosis (Figures 5 and 6), as well as in line with inhibition of endocytic pathway experiments (Supplementary Figure S5).

Analysing the brightness, distribution and shape of the labelled acidic vesicles by microscopy, we observed a different organization in KCs and DCs: KCs showed small and highly acid (bright) vesicles that co-localized extensively with the miRNA mimic signal (Figure 7A), whereas DCs presented large and not-so acid (faint) vesicles and miRNA mimic signal was present close to the vesicles but not within them (Figure 7B) with all tested peptides. NF70 and NF71 presented significantly more colocalization with acidic tracker than PF-type CPPs (Figure 7). This slightly higher colocalization of acidic vesicles and labelled miRNA in case of NF70 and NF71 could indicate a different mechanism of escape, which is in line with TEM data (Figure 6), and could explain the observed enhanced efficiency of these types of CPPs in DCs.

**NF71-delivered miR-146a mimic is efficient in the suppression of inflammatory genes in vivo**

To assess the relevance of findings in cell cultures in *in vivo* context, we next tested whether NF71:miR-146a complexes, showing the best characteristics in KCs and DCs, were effective when pre-injected subcutaneously into mouse ears just before administering PMA reagent topically onto the whole ear to induce mouse model of ICD (Figure 8A). Results showed a reduction in ear swelling, assessed by ear thickness, when NF71:miR-146a nanocomplexes were injected (Figure 8B). Moreover, NF71:miR-146a complexes were able to downregulate pro-inflammatory cytokines such as IL-6, IL-1β, IL-33 and tumour necrosis factor alpha (TNF-α) at a 6 h or 24 h time-point after being injected (Figure 8C). In addition, Langerhans’s cell marker CD207 and intercellular adhesion molecule 1 (ICAM1) gene were downregulated by
NF71:miR-146a complexes at both 6 h and 24 h time point (Figure 8D). None of the following genes or cytokines presented expression changes: CARD10, IRAK1, chemokine C-X-C motif ligand 2 (CXCL2) and thymic stromal lymphopoietin (TSLP) (Supplementary Figure S7A). As safety feature, NF71:miR-146a complexes did not show any ear swelling or inflammatory signal when applied without PMA administration (Supplementary Figure S7B). Finally, labelled miR-146a was found next to dendritic cells (marked with CD11c antibody) in draining lymph nodes (Figure 8E). It is worth mentioning that NF71 was the best performer also when injected 24 h before PMA application (scheme in Supplementary Figure S7C) according to ear thickness assessments and miRNA internalization in ears (Supplementary Figure S7D and S7E) as compared to PF14, NF55 or NF70.

**DISCUSSION**

To develop CPP-based NA delivery methods for *in vivo* use, better description of internalization mechanisms and intracellular trafficking in human primary and immune cells as well as the effect on immune responses is needed. Listed drawbacks to solve in order to optimize the CPP-based delivery include ability to be taken up by the cells and efficient release of the cargo into the cytoplasm while being not harmful to the cells. In our study, we demonstrated that NF type of CPPs are efficient and safe in delivery of miRNA mimics into both KCs and DCs. Importantly, we demonstrated that NF-type of CPPs are the most efficient for delivery miRNA mimics into DCs while being not harmful or immunogenic. Moreover, we confirm that the NF71-delivered miR-146a is able to suppress the inflammation in a mouse model of ICD and to downregulate pro-inflammatory cytokines *in vivo* indicating the potential of NF71 for *in vivo* developments.

CPPs uptake is dependent on the peptide sequence, choice of cargo, cell type and experimental factors\(^{40}\). In the present study, we observed that all self-formed CPP:miRNA complexes with the selected CPPs were taken up by both KCs and DCs. However, some differences were seen between the different cell types, being the DCs the ones presenting a weaker and diffuse staining for the fluorescently-labelled miRNA mimic, especially when using NF-type of CPPs. It has already been described that most of CPPs when complexed with NAs enter cells via endocytosis and, after internalization, are mainly located in the endosomes. A punctate fluorescence pattern is often considered an indication of the tracer compound being entrapped in endosomes while a diffuse cytosolic staining implies cytosolic localization due to leakage from the endosomal vesicles\(^{41}\). Endosomal entrapment is a major bottleneck in cytosolic delivery of nanoparticles\(^{26,41}\). Self-formed complexes, once taken up via endocytic
pathways, often remain trapped inside endocytic organelles within cells and fail to reach the cytosolic space\textsuperscript{28,42}. Therefore, endosomal escape is a crucial factor to consider when optimizing nanoparticle delivery using CPPs. We used TEM to visualize whether the nanoparticles were located freely in the cytosol or sequestered in membranous vesicles. We observed that the labelled miRNA was localized in endosome-like vesicles as well as free in the cytoplasm, being NF-type of CPPs the ones presenting more cytoplasm-localization of NG-labelled miRNA mimics, hence more efficient endosomal escape. Later, studying the distribution of miRNA along endocytic pathway trafficking, we observed a slight difference in the colocalization of miRNA and acidic vesicles when delivered by NF-type of CPPs.

Multiple studies have shown the importance of both charge and amphipathicity on the peptide transfection efficiency\textsuperscript{34,43,44}. Stearylation of cell-penetrating peptides is a successful approach to increase the transfection efficiency of NAs due to forming more stable CPP:oligonucleotide complexes, and as several studies have shown, it also promotes endosomal escape\textsuperscript{45}. On the other hand, it is known that maturation of most types of EE to multivesicular LEs is coupled with a decrease in intravesicular pH and this acidification of endosomal compartments, in turn leads to the formation of LE and/or lysosome fusion known to decrease delivery efficiency\textsuperscript{46}. This endolysosomal sequestration and hydrolytic degradation of the nanoparticulate cargo implies that they should escape from the endosomes in a timely manner to exert or preserve their intended function\textsuperscript{41}. Because of that, more efficient CPPs have been designed aiming at creating CPP molecules that change their net charge under different pH conditions, and thereby reorganise the interactions between the CPP and the cargo, thus enhancing endosomal escape\textsuperscript{46}. Recent experiments found endosomal escape to occur specifically in moderately acidic vesicles\textsuperscript{47}. Some CPP retain their net charge at all physiologically relevant pH ranges, as for example PF14 stays always at +5, whereas other CPPs change their charge, like NF51 changes from +4 to +5.2 upon shifting pH from 7.5 to 5.5\textsuperscript{46}. Therefore, CPPs being able to change their net charge in different pH conditions are of great advantage in some cell types to enhance the efficiency of delivery. In our study, the main modification in the new NF-type series of CPPs, NF70 and NF71, was the inclusion of histidine residues\textsuperscript{48}, which have been described to be capable to enhance endosomal membrane disruption through proton sponge effect\textsuperscript{49,50} and therefore, these were added in order to make the peptides pH-responsive\textsuperscript{51}. Differences in cellular localization were indeed seen with these two modified peptides: NF70 and NF71 showed a more diffuse pattern, indicative of endosomal escape, when transfecting fluorescently-labelled miRNA mimics into DCs and fragmented MVB membrane leading to endosomal escape was observed with TEM assessments in DCs as well. Furthermore, NF70 and
NF71 were the ones consistently enabling efficient in transfection of DCs both with miRNA mimics and siRNAs. Finally, NF71 was able to deliver functional miR-146a when injected subcutaneously before initiation of inflammation in mouse ears.

Regarding our selected peptides, PF14 has been previously reported to deliver oligonucleotides and siRNAs into different cell lines\(^23\) as well as recently reported to efficiently deliver microRNAs\(^{20,21}\). In our study, we showed PF14 to be excellent vectors to target KCs as well. Likewise, NF55, designed with reduced net charge and distinct hydrophilic region on alpha-helical wheel projection, has been described to promote endosomal escape and transfect the majority of cells in large cell population transfections\(^28\), as well as the newly designed NF70 and NF71, with the introduced histidine residues in their sequence, showed improved efficiency of transfection\(^48\). Our results are in line with previous studies and confirm NF-type of CPPs having great advantage to transfect and deliver miRNAs, both in KCs and especially in immune cells as DCs. MGPE-type of CPPs, very efficient human protein phosphatase 1E (PPM1E) derived peptides that show serum stability and low cytotoxicity, have been suggested to be promising peptide-based NA delivery system\(^{34}\). MGPE9 used in our study was designed by adding a Cysteine residue both to N- and C- terminus of MGPE3 sequence in order to render stability to the nanoparticles through formation of disulphide linkages\(^52\). Although these peptides were specially designed to be used in skin, in our study they were not efficient in miRNA and siRNA delivery to KCs or DCs, suggesting that despite they have shown skin penetrating characteristics, they are not the best option for miRNA delivery.

Targeting of immune cells adds another challenge for CPP-based delivery as peptides could directly stimulate immunogenic responses, particularly when delivered in particle form. Although immunogenic responses might be of interest for enhancing immune system responses in immunotherapies for cancer or infectious diseases; they are totally undesirable in case of therapies aimed for attenuating inflammatory and autoimmune responses. It has been reported that some peptides with significant aggregation could affect immunogenicity as it is known that larger particles are more efficiently phagocytised and presented at the membrane level\(^53\). Activated DCs undergo morphological changes by developing extensions that increase cellular surface area to improve interaction with T cells, as well as upregulate major histocompatibility complex and costimulatory molecules such as CD86\(^{54}\). We did observe a significant upregulation of CD83 and CD86 when miRNA mimics were transfected using a lipid-based transfection agent but the selected CPPs did not show upregulation of these cell surface markers. In addition, the transfections using LF2000 reagent also upregulated the inflammatory response genes, CARD10, IRAK1, CXCL8 and CXCL1 in KCs, while the transfection
with miRNA nanocomplexes formed with CPPs did not affect the expression of these genes. These results enable to conclude that transfection with CPPs is less immunogenic than that of with LF2000.

One of the most effective CPP for delivery in cell cultures, NF71, was finally also tested for \textit{in vivo} application during skin inflammation. According to our results, NF71 was able to deliver efficiently miR-146a into the inflamed skin and thereby to suppress skin inflammation in a mouse model of ICD. The local subcutaneous pre-injection of NF71:miR-146a complexes into the mice ears subjected to ICD showed decreased ear swelling and downregulated pro-inflammatory cytokines IL-6, IL-1\(\beta\) IL-33 and TNF-\(\alpha\). Thus, although \textit{in vivo} studies are crucial to advance the field to be potentially translated in the future to the clinics, our study indicates that carefully planned cell culture studies in the initial phase of development may reduce the number of \textit{in vivo} experiments needed.

In conclusion, all selected PF- and NF-type of CPPs were efficient in delivering miRNA mimics into KCs, whereas only NF-type peptides were efficient delivering miRNA mimics into DCs. The most promising NF71 was chosen for \textit{in vivo} experiment and demonstrated to efficiently deliver miRNA \textit{in vivo} and evoke physiological response, suggesting its potential in the development of therapeutic delivery of miRNAs to suppress skin inflammation.

\textbf{DATA AVAILABILITY}

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.
REFERENCES


54. Ahmad S, Zamry AA, Tan H-TT, Wong KK, Lim J, Mohamud R. Targeting dendritic cells...

Table 1. Sequences of the selected cell-penetrating peptides used for the transfection of miRNA mimics.

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Cell-penetrating peptide sequence</th>
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*Synthesis continues from the sidechain amino group instead of α-amino group.

Table 2. Sequences of the synthesized oligonucleotides.

<table>
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<td>Cy5-labelled ss miR-146a-5p</td>
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<td>NAC8605</td>
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Unlocked nucleic acids (UNA) are marked as underlined. Ss: single stranded, NG: Nanogold, ds: double stranded; ON: oligonucleotides.

Figure 1: CPP:miRNA complexes are internalized by KCs and DCs.

KCs and DCs were transfected with Dy547-labelled miRNA (red) at 60 nM concentration using the indicated CPPs for 24 h at 17:1 CPP:miRNA molar ratio (MR) (A) or 28:1 MR (B). Cells were counterstained with DAPI (blue) and membrane marker WGA (green). White arrows indicate membrane localization of the complexes. Scale bar: 20 μm.

Figure 2: PF- and NF-delivered miR-146a mimic inhibits target genes in KCs.

(A) KCs were transfected with miR-146a or control miRNA at 30 nM concentration using 17:1 CPP:miRNA MR for 24 h or left non-transfected (NT). (B) KCs were transfected as in A and then stimulated with IFN-γ for additional 24 h or left non-stimulated and non-transfected (NS NT) or NT and stimulated with IFN-γ (NT IFN-γ). LF2000 was used as transfection control. Data are expressed as mean +/- SEM and relative to NT (A) or NT NS (B) cells. Unpaired student T test, *p<0.05, **p<0.01, ***p<0.001.

Figure 3: NF-delivered miR-146a mimic is efficient in the suppression of target genes in DCs.

DCs were transfected with miR-146a or control miRNA at 100 nM concentration using (A) 17:1 CPP:miRNA MR or (B) 28:1 MR. LF2000 was used as transfection control. Data is expressed as mean +/- SEM and relative to NT cells. Unpaired student T test, *p<0.05, **p<0.01.

Figure 4: CPP:miRNA transfection does not impair cell viability and does not activate DCs.

(A) Luminescent cell viability assay was performed 72 h after transfection with indicated CPP:miRNA or CPP:miR-146a complexes. Data are expressed as percentage relative to NT cells.

(B, C) Flow cytometry with anti-CD83 and anti-CD86 was performed 24 h after transfection. (B) Column graphs present % of positive cells; data is mean of two different experiments.

Unpaired student T test compared to LF2000 treatment. (C) Histograms are shown as
compared to fluorescent minus one (FMO) controls. (A, C) Unpaired student T test, *p<0.05, **p<0.01, ***p<0.001.

**Figure 5: Cellular localization of the delivered miRNA mimics in KCs.
Keratinocytes were incubated with CPP:miR-146a-Nanogold (NG) nanocomplexes for 4 h, cell specimens were fixed and processed for TEM analysis using standard methods. Localization of miR-146a-NG (small black dots ~10 nm) with PF14 (A, B, and magnified images b1, b2), NF55 (C, D, d), NF70 (E, F, f) or NF71 (G-J, I, j) on cell surface (white arrows), in endosomal vesicles (black arrows) and in cytosol (arrowheads) are shown. The boxed areas show zoomed in images. Scale bar: 500 nm.

**Figure 6: Cellular localization of the delivered miRNA mimics in DCs.
Dendritic cells were incubated with CPP:miR-146a-Nanogold (NG) nanocomplexes for 4 h, cell specimens were fixed and processed for TEM analysis using standard methods. Localization of miR-146a-NG (small black dots ~10 nm) with PF14 (A-D, and magnified image c), NF55 (E-G, g1, g2), NF70 (H-J, j) or NF71 (K, L, l) in endosomal vesicles (black arrows) and in cytosol (arrowheads) are shown. The boxed areas show zoomed in images. Scale bars: 200-500 nm.

**Figure 7: Cellular colocalization of endocytic pathway markers with the delivered miRNA mimics in KCs and DCs.
(A) KCs and (B) DCs were transfected with Dy547-labelled control miRNA (red) at 60 nM concentration for 30 min and stained with EEA1; or for 4 h and stained with lysosensor or LAMP2. Cells were counterstained with DAPI (blue). Scale bar: 20 μm. Relative colocalization was assessed with M2 coefficient using Image J software and is presented in column graphs. Unpaired student T test compared to PF14, *p<0.05, **p<0.01, ***p<0.001.

**Figure 8: Pre-injection of NF71:miR-146a nanocomplexes inhibits inflammation in a mouse model of irritant contact dermatitis.
(A) Irritant contact dermatitis (ICD) mouse model experimental scheme. One ear of each mouse was injected with control mimics and another with Cy5-labelled miR-146a mimics, both in complex with NF71. After one hour, the ears were treated with PMA. Ear thickness was measured at time-points 0 h, 6 h and 24 h. Tissue and RNA were harvested at time-point 24 h. (B) Ear swelling assessed by ear thickness (7 mice/group). Two-way ANOVA test, *p<0.05, ***p<0.001. RT-qPCR analysis of pro-inflammatory cytokines, IL-6, IL-1β, IL-33 and TNF-α (C) and markers CD207 and ICAM1 (D) 6 h and 24 h after the injections. Data is expressed as mean ± SEM and normalized to non-treated non-PMA group (=1). Paired student T test, *p<0.05, **p<0.01. (E) Localization of labelled Cy5-miR-146a (red) and dendritic cell marker CD11c (green) in draining lymph nodes at 24 h. Tissues were counterstained with DAPI (blue). Scale bar: 20 μm.
Figure 1
**Figure 2**

Graphs showing the relative expression of genes CARD10, IRAK1, CXCL8, and CXCL1 in nonstimulated and IFN-γ-stimulated KCs. The graphs compare the expression levels under control conditions and the effect of miR-146a.
Figure 3
Figure 4
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Figure 6
Figure 7
Figure 8
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: