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Research Article

Keywords: RNA, miRNA, Immunological

Posted Date: January 11th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-134973/v1>

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Immunological off-target effects of microRNA control sequences

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Abstract

Immunological off-target effects of RNA and RNAi therapy are a considerable challenge in research and the future of RNA-therapy. Here we investigated some of the hurdles we previously encountered when transfecting microRNA (miRNA) control sequences into chondrocytes in an inflammatory model simulating osteoarthritis (OA). We investigated different negative control sequences of different technologies; Pre-miR miRNA Precursor and mirVana from Thermo Fisher Scientific. We used RT-qPCR, western blot analysis and mass spectrometry to assess for the effects of the transfected control sequences.

The data did not show a global immunological off-target effect, however a specific off-target effect on IL6 and IL8 was observed. IL6 and IL8 were both upregulated by the negative control from the Pre-miR miRNA Precursor technology (Pre-neg #1), and downregulated by the negative control from the mirVana technology (mirVana-neg). Moreover, the results suggested that the effect on IL6 and IL8 was dependent on both sequence and type of chemical modifications in addition to donor variation. We conclude that negative controls should be selected wisely, and suggest that scientists need to test several controls to ensure correct interpretation of data before drawing any conclusions.

Introduction

miRNAs are also small regulatory RNAs of 21-22bp in length that mediate their regulatory effects, just like siRNAs, through the RNAi pathway. miRNAs are potent gene regulators as one miRNA can target up to 100 genes^{1,2}, and both the leading strand and the anti-sense strand exhibit regulatory effects. In a previous study we showed how miRNA-145 also had immunostimulatory off-target effects that were liposomal-delivery dependent and were mediated via RIG-1³. The expression of immune genes was not induced when miRNA-145 was electroporated into the cytosol. We have since taken into consideration the immunostimulatory off-target effects of using lipid based vehicles for transfection of regulatory RNAs and favored electroporation.

Immunological off-target effects to RNA has been previously reported in relation to siRNA and miRNA transfection into cells³⁻⁵. Undesirable off-target induction of interferon response, which is the classical anti-viral response involving many genes, was reported to be mediated by siRNAs⁶⁻⁸. However, synthetic siRNAs of 21 bp have been shown to bypass activation of the interferon pathway^{4,9}, and this has opened the door for the potential of using synthetic siRNAs to study gene silencing in vitro, and as therapeutic agents in vivo¹⁰. Nevertheless there are conflicting results on the degree of interferon pathway induction as a result of siRNA delivery that depends on factors like the particular siRNA sequence, chemical modification of the siRNA and cell type. In a study by Sioud et. al⁴ where 32 different siRNA sequences were investigated, the author concluded that the immunostimulatory capacity of the siRNAs, whether they are double-stranded or single-stranded, are sequence dependent and require endosomal intracellular signaling. About 50% of the siRNAs investigated induced the production of the inflammatory cytokines TNF α and IL6, and their ability to induce TNF α and IL6 varied depending on their sequence. Moreover the study showed that liposomal delivery of siRNAs was required for cytokine induction⁴.

siRNAs are highly specific and can be potent gene silencers with only one mRNA target and thus harbor great therapeutic potential, however such therapies will be limited to elimination of a specific target protein. While miRNAs having numerous targets simultaneously, some of which are transcription factors and regulatory molecules, are capable of altering complex networks¹¹. This however might make avoiding immunological off-target effects extremely difficult⁵. Here we report immunological off-target effects of miRNA negative control sequences delivered to the cells through electroporation in an inflammatory in vitro model. These negative control sequences should ideally not alter gene expression, as this can have a great impact on interpretation of the results.

In this study we investigated different control sequences made using different miRNA synthesis technologies; Pre-miR miRNA Precursor and mirVana from Thermo Fisher Scientific. The miRNA sequences studied were modified using two different chemical processes in order to protect against endonuclease degradation and to increase silencing performance¹²⁻¹⁴. Based on results from RT-qPCR analysis, mass spectrometry proteomics and western blot assays we conclude that there were few immunological off-target effects of transfection of the control sequences, but IL6 and IL8 were both upregulated by the negative control from the Pre-miR miRNA Precursor technology (Pre-neg #1). IL6 and IL8 were downregulated by the negative control from the mirVana technology (mirVana-neg). Further, the results suggested that the immunological off-target effects were dependent on both sequence and type of chemical modifications. We conclude that negative controls should be selected wisely.

Results

Pre-neg #1 and mirVana-neg are affecting IL6 and IL8 differently

Chondrocytes were electroporated with each RNA sequence separately in three parallel experiments using cells from three different donors. Four days later all the samples were stimulated with the inflammatory cytokines IL1 β and TNF α , used in other projects to simulate an osteoarthritic milieu in vitro. In addition to Pre-neg #1 and mirVana-neg, a mock control experiment (electroporated without the addition of RNA sequences) and a non-electroporated but cytokine stimulated experiment were also included in the analysis. All cells were harvested on day 5.

Fig.1 shows that IL1 β and TNF α upregulated all the cytokines investigated as expected. Mock upregulated IL6 and to a lesser extent IL8 protein levels, compared to the stimulated control, with notable donor variation. While Pre-neg #1 seemed to strongly upregulate IL6 and IL8 (donor 1) or bring them to similar levels of that of mock control (donor 2 and 3). mirVana-neg consistently downregulated IL6 and IL8 in all donors and exhibited a pattern of effect similar to that of the stimulated control (Fig. 1a). There were no consistent differences observed at *IL1B*, *IL6*, *IL8* and *TNF* mRNA levels, though an upregulatory tendency of *IL1B* in two of the donors by Pre-neg #1 was seen (Fig. 1b).

Immunological off-target effects of the controls are both sequence and chemical modification dependent

In order to understand whether the effects on IL6 and IL8 mediated by these two controls were restricted to their particular sequences, their chemical modifications or both we looked at additional negative control sequences produced by the same manufacturer. We included Pre-neg #2, which has the same chemical modifications as Pre-neg #1 but differs in sequence, and Pre-neg*, which shares sequence with Pre-neg #1 but has the same chemical modification

as mirVana-neg. The type of chemical modifications and the sequences are company properties and were not revealed to us. Fig.2 shows western blot analysis of IL6 and IL8 in response to transfection of the various control sequences in two donors. Again, electroporation performed in the mock experiment upregulated IL6 and IL8 protein levels. Further upregulation of IL6 and IL8 was seen after Pre-neg #1 transfection. Pre-neg #2 showed differential effects on IL6 in the two donors, and did not affect IL8 compared with mock. mirVana-neg again had a consistent downregulatory effect on both IL6 and IL8 as previously observed in Fig. 1a, and in a previous study (under publication). Finally, Pre-neg* showed downregulatory effects on IL6 and IL8 similar to mirVana-neg (Fig. 2).

In order to understand why those control sequences altered IL6 and IL8 proteins, we further checked for the expression of key genes involved in inflammatory signaling of the innate immune response in donor1; some pattern recognition receptors (PPRs) that respond to foreign RNA and further mediate the inflammatory signal; *TLR7,8*, and *3* and some of their downstream mediators *TRIF*, *MYD88*, *IRF3*, *OAS2*, two intracellular PRRs that also respond to RNA and elicit an immune response; *DDX58*, *PKR*, cytokines including *IL6* and *IL8* but also *IL1 β* , *TNF*, *CXCL10* and *CCL5*, and finally a pro-inflammatory (*IL1 β*) induced gene *PTGES*. *TLR7* and *8* were not detected and therefore not seen in Fig.3. Although there are not big differences in gene expression between the controls, mirVana-neg and Pre-neg* seem to affect the selected genes similarly (Fig. 3). Sharing the same chemical modifications could perhaps explain their similar effects on the mRNA expression of the selected genes and the protein expression of IL6 and IL8. Pre-neg #1 also showed a tendency to upregulate *IL1 β* and *CXCL10*, and downregulate *OAS2*. However none of these observations could explain why pre-neg #1 led to upregulation of IL6 and IL8, and why mirVana-neg led to the opposite.

Proteome alterations upon transfection of Pre-neg #1 control and miRvana-neg control

In the pursuit of possible explanation of the effects seen on IL6 and IL8 we performed proteomics analysis on cells from the first three donors used in Fig 1. Table 1 shows all altered proteins between the samples (Student t-test significance $p < 0.05$ and 2 fold change cut-off). To our surprise few proteins were altered between the samples, even without adjusting for multiple testing (FDR). When we corrected for multiple testing, no proteins were significantly altered between the control sequences. Notably the biggest difference between the samples in terms of the number of proteins altered, is in fact between the electroporated samples containing an RNA sequence (mirVana neg, Pre-neg#1 controls) and the unelectroporated control (stimulated), indicating the effect of transfection of RNA into the cell. Curiously ITGA5 was consistently downregulated by mirVana when compared to Pre-neg#1, mock, and stimulated control (Table 1a, 1b, and 1c respectively). IL6 and IL8 were not detected by the proteomics analysis.

Discussion

Immunological off-target effects as a result of introduction of RNA into the cell regardless of the transfection method make correct interpretation of data difficult, and may have unfortunate consequences for gene therapy. Indeed electroporation alone (mock) without any RNA sequence involved had notable immunological off-target effects as it upregulated IL6 and IL8 protein. However the fact that the consistent immunological off-target effects seen on IL6 and IL8 were generated by the negative control sequences (mirVana-neg) raises a concern.

The negative controls Pre-neg #1 and mirVana-neg had different sequences and different chemical modifications that seemed to give them opposing effects on our proteins of interest. The precursor technology of Pre-neg #1 seemed to have an upregulatory effects on IL6 and

IL8 protein levels that was also donor dependent.

The question of whether these effects are sequence-dependent or chemical modification-dependent was addressed by including additional controls. Pre-neg #2 sharing the same chemical modifications as Pre-neg #1, did not exhibit the same upregulatory effects on the interleukins which indicates that the upregulatory effects observed by Pre-neg #1 are partially sequence-dependent. Engineering Pre-neg#1 sequence with mirVana chemical modification (Pre-neg*) allowed us to conclude that the previously observed downregulatory effect mediated by mirVana-neg on IL6 and IL8 are due to their chemical modifications as evident by the effects Pre-neg* exhibited. In summary we conclude that the immunological off-target effects mediated by mirVana-neg and Pre-neg #1 are both sequence dependent and chemical modifications-dependent.

The specific effects on these interleukins made us wonder whether other cytokines, some upstream receptors and mediators of inflammatory signaling were altered and thus resulted in the effects observed on IL6 and IL8. However the additional cytokines we tested for showed no or small differences between the controls with no consistent pattern. We also tested for several TLRs; TLR7 and 8 were either not detected or barely detected in some of the samples and thus not included in the results. TLR3 was detected however no difference was observed between the samples. The downstream mediators of inflammatory signals tested did not show a considerable difference between the negative controls that could explain the effects seen on IL6 and IL8 protein levels. One possible explanation could be the timing of cell harvesting. Probably the inflammatory response to the RNA-sequences is quite immediate and thus many of these upstream mediators might have been restored to homeostatic levels after mediating the inflammatory signal. The cells were harvested 5 days after transfection and one day after stimulation with IL1 β and TNF α . Perhaps, it was long enough to see the effects on IL6 and IL8, but not to see the initial effects on upstream regulators and mediators. This is certainly a

limitation of the study and for future studies we would certainly check the expression of these molecules and perhaps several others at earlier time points. Moreover we only assessed for mRNA levels, perhaps on the protein level at different time points there could be more evident differences on these upstream mediators. Although we performed proteomics to our surprise the proteomics analysis did not show many altered proteins when we compared the various controls against each other. Applying multiple correction statistics, which are highly recommended in omics-studies, no proteins were actually differentially expressed between any of the samples. In this fashion it is a positive observation which implies that these controls do not have immunological off-target effects on a global scale. Although worth noting that proteomics analysis have its limitations and do not always detect all proteins and all differences in the cells. IL6 and IL8 for instance were not detected by the proteomic analysis. Cytokines having low molecular weight and existing in low abundance provide less peptides for mass spectrometry which makes them very difficult to detected¹⁵

Previously, we compared RNA-sequencing data of mock control and mirVana-neg control and found no genes to be differentially expressed (unpublished data). All this suggest that the effects seen on IL6 and IL8 mediated by these two controls are quite specific and yet to be explained. We and others have previously reported immunostimulatory effects of lipid based transfection of RNA³. In those studies the authors also showed that these immune responses were not observed when electroporation was used as method of RNA delivery. However in this study we report modulated immune response to RNA control sequences when delivered by electroporation. Moreover these modulated immune responses were specifically observed on IL6 and IL8 protein levels only. Certainly usually there is a discrepancy between RNA and protein expression in the cell^{16,17}. However this also suggests that the effect observed on IL6 and IL8 was regulated at the post-translational level.

Methods and Materials

Isolation and culture of human articular chondrocytes (ACs). ACs were isolated from discarded osteoarthritic cartilage tissue following total knee replacement surgery and cultured as previously described^{18–20}. Only apparently healthy tissue with no macroscopic signs of OA was used. Written informed consent from all donors was received. The study was approved by the Regional Committee for Medical Research Ethics, Southern Norway, and we confirm that all research was performed according to relevant guidelines and regulations. We also confirm that informed consent was obtained from all patients donating tissue after undergoing total knee replacement surgery. In brief, the cartilage was processed into tiny pieces then digested with Collagenase type XI (Sigma-Aldrich, St. Louis, MO) at 37°C for 90–120 min. The cells were washed three times and resuspended in culture medium consisting of Dulbecco's modified Eagle's medium/F12 (Gibco/ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% human plasma (Octaplasma AB, Oslo Blood Bank, Norway) with platelet lysate (corresponding to 10⁹ platelets/ml plasma) (PLP), 100 units/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B¹⁹. PLP was prepared as previously described²¹. Every 3–4 days the culture medium was changed and amphotericin B was removed after the first passage. Cells were trypsinized at 70–80% confluence with trypsin-EDTA (Sigma-Aldrich) and seeded into new culture flasks.

MicroRNA mimics, transfection and stimulation with IL1 β and TNF α . The Amaxa Human Chondrocyte Nucleofector Kit and the Amaxa nucleofector system were used for electroporation according to manufacturer's protocol (Lonza, Walkersville, MD). Shortly, each reaction contained 1.0 x 10⁶ cells, 5µM of miRvana mimics and Precursor mimics (Supplementary table S1) in a total volume of 100 µl nucleofection solution. The transfected

cells were then seeded in 20% PLP without antibiotics over night. The next day (day1) the medium was changed to 10% PLP with 1% penicillin/streptomycin. On day 4 chondrocytes were stimulated with 0.1 ng/ml recombinant IL1 β (IL1 β) and 10 ng/ml TNF α (R&D Systems, Minneapolis, MN) for 24 hours before harvesting for analysis.

Isolation of miRNA, cDNA synthesis, and qRT-PCR. Total RNA including miRNAs was isolated using the miRNeasy mini kit following manufacturer's protocol (Qiagen, Germantown, MD). cDNA synthesis and qRT-PCR were performed according to protocols from the manufacturer using the Taqman High capacity cDNA Reverse Transcription Kit for mRNA (Thermo Fisher Scientific, Waltham, MA, USA). 200 ng RNA in a total volume of 15 μ l was reverse transcribed into cDNA. All samples were run in technical triplicates. Each replicate contained 0.2 μ l cDNA in a total volume of 15 μ l for mRNAs. Thermo cycling parameters were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. GAPDH was used as housekeeping control gene for mRNAs. qRT-PCR results are shown as relative fold changes using mean values from technical triplicates with a 95% confidence interval.

Western blotting. Cell lysates corresponding to 200,000 cells were loaded onto a 4-20% gradient (Bio-Rad, Hercules, CA). Proteins were separated by gel electrophoresis, transferred to PVDF membranes and incubated with appropriate antibodies (Supplementary Table S1) prior to visualizing the bands using the myECL imager (Thermo Fisher Scientific).

Mass spectrometry. All experiments were performed on an Easy nLC1000 nano-LC system connected to a quadrupole – Orbitrap (QExactive Plus) mass spectrometer (ThermoElectron, Bremen, Germany) equipped with a nanoelectrospray ion source (EasySpray/Thermo). For

liquid chromatography separation we used an EasySpray column (C18, 2 μm beads, 100 \AA , 75 μm inner diameter) (Thermo) capillary of 50 cm bed length. The flow rate used was 0.3 $\mu\text{L}/\text{min}$, and the solvent gradient was 2% to 7 % in 5 minutes and then to 30% B in 120minutes. The column was finally washed in 90 % B wash for 20 minutes. Solvent A was aqueous 0.1 % formic acid, whereas solvent B was 100 % acetonitrile in 0.1 % formic acid. Column temperature was kept at 60°C.

The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 300 to 1,500) were acquired in the Orbitrap with resolution $R = 70,000$ at m/z 200 (after accumulation to a target of 3,000,000 ions in the quadrupole). The method used allowed sequential isolation of the most intense multiply-charged ions, up to ten, depending on signal intensity, for fragmentation on the HCD cell using high-energy collision dissociation at a target value of 100,000 charges or maximum acquisition time of 100 ms. MS/MS scans were collected at 17,500 resolution at the Orbitrap cell. Target ions already selected for MS/MS were dynamically excluded for 30 seconds. General mass spectrometry conditions were: electrospray voltage, 2.1 kV; no sheath and auxiliary gas flow, heated capillary temperature of 250°C, normalized HCD collision energy 25%.

Protein identification and label-free quantification. MS raw files were submitted to MaxQuant software version 1.6.1.2 (Cox J and Mann M, Nature Biotechnology 26, 1367-1372, 2008) for protein identification. Parameters were set as follow: carbamidomethylation as fixed modification and protein N-acetylation and methionine oxidation as variable modifications. First search error window of 20 ppm and mains search error of 4.5 ppm. Trypsin without proline restriction enzyme option was used, with two allowed miscleavages.

Minimal unique peptides were set to 1, and FDR allowed was 0.01 (1%) for peptide and protein identification. The Uniprot human database was used (download from uniprot Sept. 2018). Generation of reversed sequences was selected to assign FDR rates. This protocol was performed by the Proteomics Core Facility, Oslo University Hospital, Rikshospitalet.

Acknowledgment

Proteomics services were purchased from the Proteomics Core Facility, Oslo University Hospital, Rikshospitalet and performed by Tuula Anneli Nyman and Maria Stensland. We would like to acknowledge Dr. Bjørn Ødegaard, Department of Orthopedic Surgery, Lovisenberg Diakonale Hospital, Oslo, Norway, for providing cartilage tissue samples. This study was funded by a PhD grant from South-Eastern Norway Regional Health Authority, UiO Innovation funds, and The Olav Thon Foundation. None of the authors have any conflicts of interest to declare.

Author contributions

R.N.A, T.A.K and J.E.B conceptualized the idea. R.N.A wrote the main manuscript and prepared all figures. T.A.K. and J.E.B reviewed and edited the manuscript. J.E.B acquired funding and resources. T.A.K. and J.E.B. supervised the project. All authors reviewed the manuscript.

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Figure Legends

Figure 1 Pre-neg #1 and mirVana-neg affected OA mediators differently in response to IL1 β and TNF α

(a) Western blot analysis of IL6 and IL8 protein levels in unstimulated and IL1 β and TNF α -stimulated conditions in chondrocytes from 3 OA donors. β -actin (ACTB) was used as loading control. The unstimulated samples are cropped from another part of the same blot.

(b) RT-qPCR analysis of *IL1B*, *IL6*, *IL8*, and *TNF* mRNA levels in unstimulated and IL1 β and TNF α -stimulated conditions in the same donors. Error bars represent a 95% confidence interval from technical triplicates.

Figure 2 Comparison of additional negative controls and their effects on OA mediators in response to IL1 β and TNF α .

Western blot analysis of IL6 and IL8 protein levels in unstimulated IL1 β and TNF α -stimulated conditions in 2 donors. ACTB was used as loading control.

Figure 3 The effects mediated by the various negative controls on the expression of selected genes. (a) RT-qPCR analysis of, *IL6*, *IL8*, *IL1B*, *TNF*, *CXCL10*, *CCL5* mRNA levels in stimulated conditions. (b) RT-qPCR analysis of *TLR3*, *TRIF*, *MYDD88*, *IRF3*, *OAS2*, *PKR*, *DDX58*, *PTGES* mRNA levels under the same conditions. Error bars represent a 95% confidence interval from technical triplicates. Donor 1 was used for this experiment.

Table 1 Altered protein expression in response to the various controls

Table 1a

mirVana-neg vs. Pre-neg #1

Gene names	Protein names	Fold change
PDCD6	Programmed cell death protein 6	-5,8
ENO2	Gamma-enolase	-5,6
ITGA5	Integrin alpha-5	-3,1
THOP1	Thimet oligopeptidase	-2,8
PPL	Periplakin	-2,2
TMPO	Lamina-associated polypeptide 2, isoform alpha;Thymopoietin	-2,1
ENG	Endoglin	2,2
PMVK	Phosphomevalonate kinase	2,4
PFDN1	Prefoldin subunit 1	2,6

Negative fold change values indicate downregulation, and positive values indicate upregulation compared to mirVana-neg.

Table 1b

mirVana-neg vs. Mock

Gene names	Protein names	Fold change
TROVE2	60 kDa SS-A/Ro ribonucleoprotein	-5,0
TMX1	Thioredoxin-related transmembrane protein 1	-4,4
ITGA5	Integrin alpha-5	-3,8
TMTC3	Transmembrane and TPR repeat-containing protein 3	-2,4
PC	Pyruvate carboxylase, mitochondrial	-2,4
ASNS	Asparagine synthetase [glutamine-hydrolyzing]	-2,2
PTRHD1	Putative peptidyl-tRNA hydrolase PTRHD1	-2,1
METAP2	Methionine aminopeptidase 2	-2,1
GGH	Gamma-glutamyl hydrolase	-2,0
PFDN1	Prefoldin subunit 1	3,3
ABCD3	ATP-binding cassette sub-family D member 3	3,8

Negative fold change values indicate downregulation, and positive values indicate upregulation compared to mirVana-neg.

Table 1c

mirVana-neg vs. Stimulated

Gene names	Protein names	Fold change
KCTD12	BTB/POZ domain-containing protein KCTD12	-10,9
XIRP2	Xin actin-binding repeat-containing protein 2	-4,4
PPP1R9B	Neurabin-2	-3,5
ITGA5	Integrin alpha-5	-3,3
PDE12	2,5-phosphodiesterase 12	-2,8
PLOD3	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	-2,6

PC	Pyruvate carboxylase, mitochondrial	-2,2
XRN2	5-3 exoribonuclease 2	-2,1
PTRHD1	Putative peptidyl-tRNA hydrolase PTRHD1	-2,0
SAMHD1	Deoxynucleoside triphosphate triphosphohydrolase	2,0
CYCS	Cytochrome c	2,7
SLC25A20	Mitochondrial carnitine/acylcarnitine carrier protein	2,9
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	2,9
NUBP1	Cytosolic Fe-S cluster assembly factor NUBP1	3,1
MGLL	Monoglyceride lipase	3,5
ABCD3	ATP-binding cassette sub-family D member 3	4,2

Negative fold change values indicate downregulation, and positive values indicate upregulation compared to mirVana-neg.

Table 1d

Mock vs. Stimulated

Gene names	Protein names	Fold change
XIRP2	Xin actin-binding repeat-containing protein 2	-3,4
PLIN2	Perilipin-2	-3,1
GLIPR2	Golgi-associated plant pathogenesis-related protein 1	-2,0
SLC25A20	Mitochondrial carnitine/acylcarnitine carrier protein	2,5
MGLL	Monoglyceride lipase	4,4

Negative fold change values indicate downregulation, and positive values indicate upregulation compared to mock.

Table 1e

Mock vs. Pre-neg #1

Gene names	Protein names	Fold change
TMPO	Lamina-associated polypeptide 2, isoform alpha;Thymopoinetin	-3,6
CXCL6	C-X-C motif chemokine 6;Small-inducible cytokine B6	-2,4
STAT3	Signal transducer and activator of transcription 3	2,2
TMTC3	Transmembrane and TPR repeat-containing protein 3	2,3

Negative fold change values indicate downregulation, and positive values indicate upregulation compared to mock.

Table 1f

Stimulated vs. Pre-neg #1

Gene names	Protein names	Fold change
MRPL11	39S ribosomal protein L11, mitochondrial	-4,5
SMAP	Small acidic protein	-4,5
XIRP2	Xin actin-binding repeat-containing protein 2	-4,4
PMVK	Phosphomevalonate kinase	-2,6
GLIPR2	Golgi-associated plant pathogenesis-related protein 1	-2,2

XRN2	5-3 exoribonuclease 2	-2,2
ESYT2	Extended synaptotagmin-2	-2,1
PPP1R9B	Neurabin-2	-2,1
LRP1	Prolow-density lipoprotein receptor-related protein 1	-2,0
HSPB6	Heat shock protein beta-6	-2,0
GABARAPL1;GABARAP	Gamma-aminobutyric acid receptor-associated protein-like 1	2,4
TNPO2	Transportin-2	2,5
SLC25A20	Mitochondrial carnitine/acylcarnitine carrier protein	2,7
APOA1	Apolipoprotein A-I	3,4
MGLL	Monoglyceride lipase	4,5
IDI1	Isopentenyl-diphosphate Delta-isomerase 1	5,7

Negative fold change values indicate downregulation, and positive values indicate upregulation compared to stimulated.

Figures

Figure 1

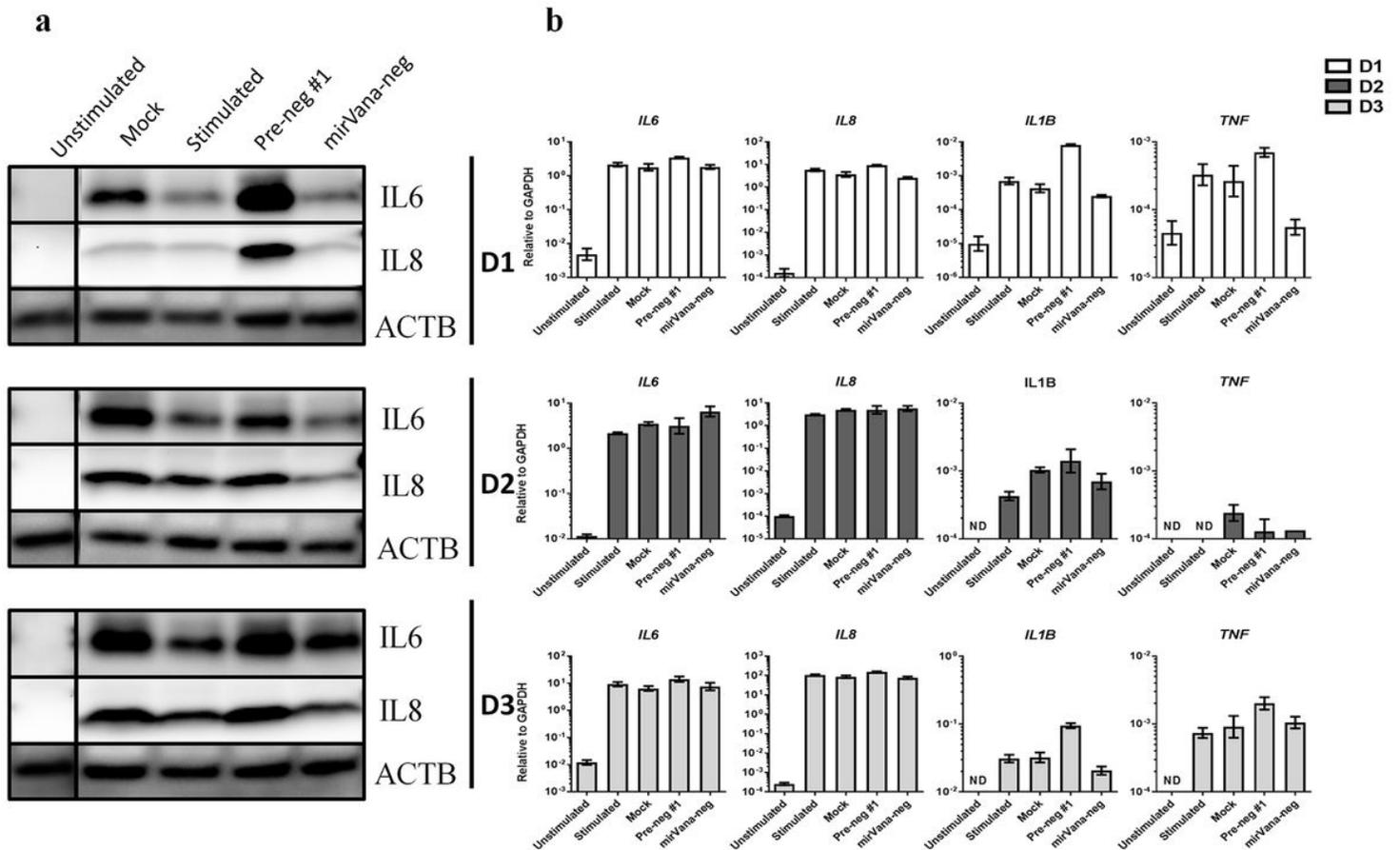


Figure 1

Pre-neg #1 and mirVana-neg affected OA mediators differently in response to IL1 β and TNF α (a) Western blot analysis of IL6 and IL8 protein levels in unstimulated and IL1 β and TNF α -stimulated conditions in chondrocytes from 3 OA donors. β -actin (ACTB) was used as loading control. The unstimulated samples are cropped from another part of the same blot. (b) RT-qPCR analysis of IL1B, IL6, IL8, and TNF mRNA levels in unstimulated and IL1 β and TNF α -stimulated conditions in the same donors. Error bars represent a 95% confidence interval from technical triplicates.

Figure 2

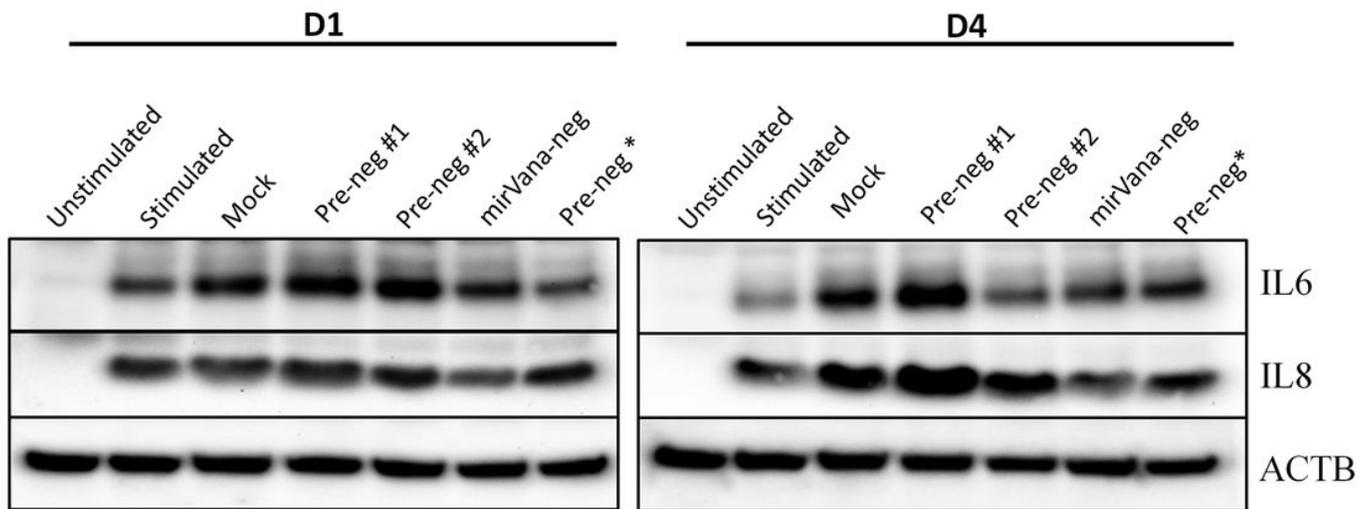
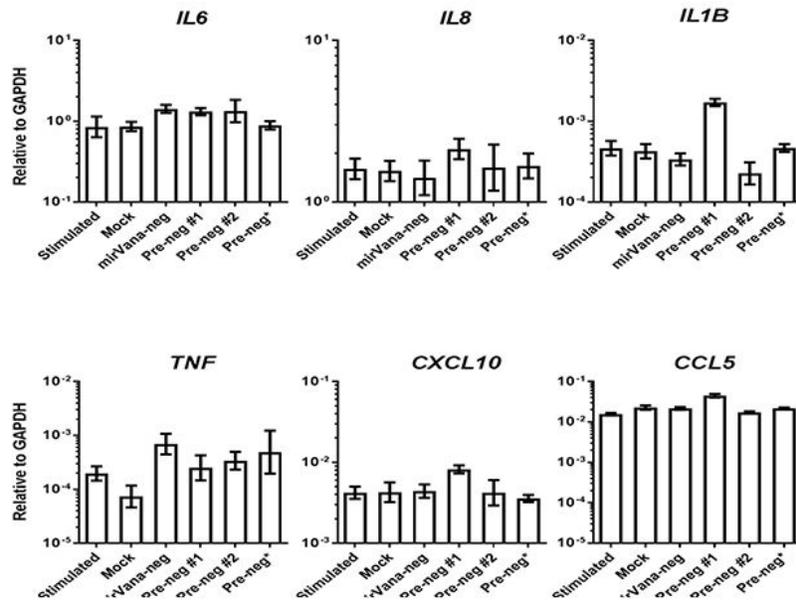


Figure 2

Comparison of additional negative controls and their effects on OA mediators in response to IL1 β and TNF α . Western blot analysis of IL6 and IL8 protein levels in unstimulated IL1 β and TNF α -stimulated conditions in 2 donors. ACTB was used as loading control.

Figure 3

a



b

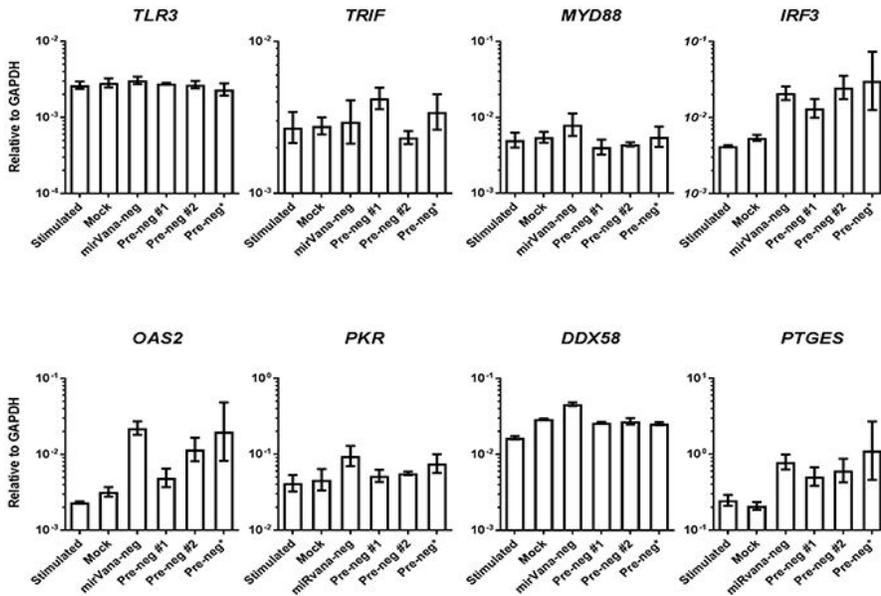


Figure 3

The effects mediated by the various negative controls on the expression of selected genes. (a) RT-qPCR analysis of, IL6, IL8, IL1B, TNF, CXCL10, CCL5 mRNA levels in stimulated conditions. (b) RT-qPCR analysis of TLR3, TRIF, MYDD88, IRF3, OAS2, PKR, DDX58, PTGES mRNA levels under the same conditions. Error bars represent a 95% confidence interval from technical triplicates. Donor 1 was used for this experiment.

Supplementary Files

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- [SupplementaryMaterial.pdf](#)