A distal enhancer is required for *TNF* gene transcription in human macrophages in response to TLR3 stimulation

Junfeng Gao[1], Yapeng Li[1], Dianzheng Zhao[1], Xiaoyu Guan[1], Hong Wei Chu[2], Hua Huang[1,3*]

[1]Department of Immunology and Genomic Medicine, National Jewish Health, Denver, CO 80206, USA.

[2]Department of Medicine, National Jewish Health, Denver, CO 80206, USA.

[3]Department of Immunology and Microbiology, University of Colorado Anschutz Medical Campus, Aurora, CO 80045, USA.

* Correspondence should be addressed to H.H. (email: huangh@njhealth.org).

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Abstract

Macrophages play a critical role in inflammatory responses during infections. Activated macrophages by infections through stimulation of TLRs expressed their cell surface produce pro-inflammatory cytokines, including TNF. However, distal enhancers that regulate *TNF* gene transcription in human macrophages have not been investigated. In this study, we identified the five putative *TNF* enhancers using H3K27ac ChIP-seq and ATAC-seq. We showed proximal enhancer (PE), E-16.0, and E-6.5 possessed enhancer activity in a reporter gene assay. Deletion of the distal *TNF* E-16.0 enhancer resulted in 73% reduction in *TNF* gene transcription in human macrophage cell line THP-1 in response to ploy(I:C) stimulation. Our study identifies a novel distal enhancer that regulates *TNF* gene transcription in human macrophages.

Introduction

Macrophages are among the first immune cells to encounter pathogens (1,2). These cells play a critical role in inflammatory responses during infections. Macrophages sense infections via pattern-recognition receptors, including Toll-like receptors (TLRs) (3,4). Toll-like receptors (TLRs) play a major role in the activations of macrophages (5). TLRs bind to viral and bacterial productions derived from many bacteria and viruses at some point of their replication cycle (6,7). TLR4 binds to bacterial product lipopolysaccharide (LPS) (8). TLR7 and TLR8 detect single stranded RNA, while TLR3 recognizes double-stranded RNA (dsRNA) (6,9,10) which can be mimicked by synthetic dsRNA poly(I:C). Activated macrophages by infections through stimulation of TLRs expressed their cell surface produce pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), chemokines (chemokine (CC motif) ligand 5 (CCL-5); chemokine (CXC motif) ligand 10 (CXCL10)) (11,12). Overproduction of pro-inflammatory cytokines and chemokines, such as IL-1β, IL-6, and IL-8, CCL2 and CXCL10, can lead to fatal outcomes during severe infections (13,14) and severe toxic side effects in cancer immune therapies (15). However, transcriptional codes, which consist of combinatorial transcription factor (TF) binding sites and interacting TFs and cofactors, detecting the numerous signal inputs triggered by TRL stimulations and infected epithelial cells is still poorly understood.

Enhancers play a critical role in regulating gene expression. Enhancers are segments of DNA located in the non-coding regions of genes (16). Decoding enhancers has been a longstanding goal in the field of gene transcription (17). *cis* regulatory elements, such as enhancers, work from a distance in animals.

Transcriptional codes hidden in the distal regions are often required for full transcription. Locating critical enhancers can be a significant challenge because critical enhancers can be located up to 100kb from the transcription start sites in non-coding regions that make up 99% of a genome (18). In this study, we focus on enhancer regulation of one of the most potent pro-inflammatory cytokine *TNF* gene in human lung macrophages.

The *TNF* gene locus lies in mouse chromosome 17 and human chromosome 6 and is comprised by the *TNF* gene and the genes encoding lymphotoxin-a and lymphotoxin-b (*LTA* and *LTB*) (19). However, how the *TNF* gene is regulated by distal enhancers in macrophages are not completely understood. A distal enhancer element 9 kb upstream of the mouse *Tnf* mRNA cap site (HHS-9) can bind NFATp and participate in intrachromosomal interactions with the *Tnf* promoter in mouse T cells upon activation (20). Additionally, A distal hypersensitive site ~8 kb upstream of the human *TNF* TSS (human hypersensitive site -8kb, hHS-8) is required for and mediates IFN-γ-stimulated augmentation of LPS-induced *TNF* gene expression via binding of IRF1 to a cognate hHS-8 site in human monocytes/macrophages (21). hHS-8 is also coordinately regulated with *TNF* and *LTA* gene expression in activated human T cells via a discrete and highly conserved NFAT binding site (22). However, distal enhancers that regulate *TNF* gene transcription in human macrophages is incompletely understood.

In this study, we identified the five putative *TNF* enhancers using H3K27ac ChIP-seq and ATAC-seq. We showed proximal enhancer (PE), E-16.0, and E-6.5 possessed enhancer activity in reporter gene assay. Deletion of the distal *TNF* E-16.0 enhancer resulted in 73% reduction in *TNF* gene transcription in human macrophage cell line THP-1 in response to ploy(I:C) stimulation. Deletion of the PE enhancer

resulted in 52% reduction in *TNF* gene transcription. Our study identifies a novel distal enhancer that regulates *TNF* gene transcription in human macrophages.

Results

Identification of putative *TNF* enhancers that respond to poly(I:C) stimulation

To determine the time course for *TNF* mRNA expression in human alveolar macrophages (AMs) in response to poly(I:C) stimulation, we treated primary human AMs from 7 donors without or with poly(I:C) for 4, 8, or 24 hours. We observed that *TNF* mRNA started to increase 4 hours after stimulation and reached the highest levels at 8 hours after stimulation (104.8-fold compared with the *TNF* mRNA in resting human primary AMs) and remained 15.6-fold higher than that in resting cells at 24 hours after stimulation (Fig. 1).

To identify potential enhancers, we treated AMs without or with poly(I:C) for four hours to detect early changes in permissive histone modification and chromatin accessibility. We performed H3K27ac ChIP-seq and Omni-ATAC-seq to identify non-coding DNA regions associated with increased H3K27ac modification and chromatin accessibility. We showed that there were four potential enhancer regions that were associated with increased chromatin accessibility and H3K27ac modification within 28.1 kb of the human *TNF* gene, which covers the intergenic regions between the *LTA* and *LTB* genes (Fig. 2). We named these potential enhancers proximal enhancer (PE), E-16.0, E-6.5, E+5.6 and E+6.3 based on the distances of the putative enhancers to the transcription start site (TSS) of the *TNF* gene.

The *TNF* E-16.0 and PE enhancers possess enhancer activity

Not all non-coding regions associated with H3K27ac modification and increased chromatin accessibility possess enhancer activity. To access the enhancer activity of the potential enhancers, we cloned the potential enhancers into the LentiMPRA vector containing a minimal promoter and barcoded *Gfp* reporter gene (23) (Fig. 3A). We

used a non-coding DNA fragment that was not associated with H3K27ac or chromatin accessibility as non-enhancer (NE) control (Fig. 2). We transduced human lung macrophage cell line THP-1 cells with the recombinant lentivirus containing enhancers. Because THP-1 cells expressed low levels of pro-inflammatory cytokine genes, we matured THP-1 cells with phorbol 12-myristate 13-acetate (PMA) for three days and found that matured THP-1 transcribed high levels of *TNF* mRNA in response to poly(I:C) stimulation (unmatured, 8.3-fold of induction after poly(I:C) treatment; matured, 91.6-fold of induction after poly(I:C) treatment, Supplemental Fig. 1), consistent with previous studies (24,25). Three days later, we treated the enhancers-transduced-THP-1 cells with poly(I:C) for four hours. We measured the number of RNA and DNA barcodes in RNA and DNA samples prepared from the enhancers-transduced-THP-1 cells. Barcodes in the RNA and DNA samples prepared from transduced cells were sequenced to determine the number of RNA barcode transcripts and DNA inserts. The log2 ratios of barcode RNA transcripts to barcode DNA inserts (as input controls) were used to determine enhancer activity. We found that the *TNF* PE, E-16.0, and E-6.5 showed significant enhancer activity (Fig. 3B). To determine which of *TNF* PE, E-16.0, and E-6.5 can respond to poly(I:C) stimulation with increased enhancer activity, we treated enhancers-transduced-THP-1 cells with poly(I:C) for four hours and measured barcodes in RNA and DNA barcodes by qPCR. Our results showed that *TNF* PE and *TNF* E-16.0 enhancers showed significant increase enhancer activity in response to poly(I:C) treatment (Fig. 3C).

The *TNF* E-16.0 enhancer is essential in *TNF* gene transcription in response to poly(I:C) stimulation

Not all enhancers identified using reporter gene assay are required for gene transcription in the context of naïve chromatin. To determine whether *TNF* E-16.0 and PE enhancers are required or contribute to *TNF* gene transcription in response to poly(I:C) stimulation, we deleted these enhancers and NE using CRISPR/Cas9. The current CRISPR deletion method using two sgRNA guides often resulted in deletions occurring in one chromosome, creating heterozygous deletion that does not have a phenotype. To overcome this technical challenge, we targeted each enhancer with four sgRNA guides, each contained within a bicistronic gene co-encoding for a different fluorescence protein GFP, RFP, BFP, or Thy1.1 molecule (Fig. 4A). We found that 9.4 % of THP-1 cells transduced with lentivirus containing the four sgRNA guides expressing BFP, GFP, RFP, and Thy1.1 (Fig. 4B). FACS-sorted cells positive for BFP, GFP, RFP, and Thy1.1 achieved complete homozygous deletion of WT *TNF* NE, E-16.0, and PE enhancers in bulk using this newly improved method (Fig. 4C). Deletion of the *TNF* E-16.0 enhancer resulted in 72.5 % reduction in *TNF* mRNA expression and deletion of the *TNF* PE enhancer led to 51.9 % reduction in *TNF* mRNA expression. In contrast, deletion of the *TNF* E-16.0 or the *TNF* PE did not affect *LTA* and *LTB* mRNA expression (Fig. 4 E and F). These data demonstrate that *TNF* E-16.0 is critical in *TNF* gene transcription in response to poly(I:C) stimulation.

Discussion

Distal enhancers regulating *TNF* gene transcription in human macrophages have not been determined. In this study, we demonstrated that a distal *TNF* E-16.0 is critical in *TNF* gene transcription in human macrophages in response to TLR ligand poly(I:C) stimulation.

Distal enhancers are critical in the assembly of TF-TF and TF-coF interactions with core promoters through a looping mechanism (26,27). Locating critical enhancers can be a great challenge because critical enhancers can be located up to 100kb from the transcription start sites in non-coding regions that make up 99% of a genome. The bioinformatics approach often assigns enhancers to the nearest genes (18). The bioinformatics approach thus is limited in finding distal enhancers. Thus, it is necessary to use CRISPR method to delete enhancer candidates. The CRISPR deletion method that uses two sgRNA guides often results in heterozygous deletion that does not have a phenotype. We targeted one enhancer with four sgRNA guides, each contained within a bicistronic gene encoding for a different fluorescence protein GFP, BFP, RFP, or Thy1.1 molecule. By coupling this with fluorescence activated cell sorting to select for cells that express all four marker genes, we achieved around complete enhancer deletion at two chromosomes without single-cell cloning. This technical improvement has allowed us to analyze *TNF* gene transcription in the context of naïve chromatin.

Previous studies reported that a distal hypersensitive site ~8 kb upstream of the human *TNF* TSS (human hypersensitive site -8kb, hHS-8) is required for LPS-induced *TNF* and *LTA* gene expression in macrophages and T cells (21,22). Although our results showed that deletion of the *TNF* E-16.0 did not affect *LTA* or *LTB* gene

transcription in human macrophages, we should take caution in interpreting this finding because human macrophages do not transcribe *LTA* and *LTB* genes at levels comparable to T cells (28,29). Our finding discovers a novel enhancer in human macrophages *TNF* E-16.0 and advance knowledge of *TNF* gene transcription in human macrophages.

Methods and Materials

Human alveolar macrophages

Human lung was obtained from de-identified organ donors whose lungs were not suitable for transplant and were donated for medical research. We obtained the donor lungs through the International Institute for the Advancement of Medicine (Edison, NJ) and the National Disease Research Interchange (Philadelphia, PA). Research on these human lungs has been deemed as nonhuman subject research and is given IRB exemption because the donors are deceased and de-identified. Alveolar macrophages (AMs) were isolated from lavage of the lung before the instillation of elastase, as described previously (30). The purity of the AMs was 92.6 ± 2.8% as measured by immunostaining of cytocentrifuge preparations. AMs were frozen. Previous studies have compared freshly isolated and frozen AMs and did not find noticeable differences (30). AMs were cultured in DMEM (CAT # SH3024301) plus 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B in the presence of 50 ng/ml GM-CSF (PeproTech, 300-03) in a humidified 37°C, 5% CO2 incubator.

Chromatin Immunoprecipitation and ChIP-seq

Human macrophages (5×10^6) were not treated or treated with 1 μg/mL Poly(I:C) for four hours were fixed with 1% formaldehyde (PI28908, Thermo Fisher Scientific), sonicated by using the Covaris S220 Focused-ultrasonicator in the SDS lysis buffer (1% SDS, 10 mM EDTA, 50mM Tris.HCl pH8) and precleared with Protein A Beads at 4 °C for 1h according to established protocols. The samples were incubated with 10 μg of following antibodies (1:100 dilution): anti-H3K27ac antibody (ab4729, Abcam,

Abcam, Cambridge, MA) at 4°C overnight and then with protein A agarose/salmon sperm DNA slurry (Millipore, Cat# 16-157) at 4°C for 1h. The beads were washed and eluted as described. The crosslinking of eluted immunocomplexes was reversed and the recovered DNA was recovered using a QIAGEN QIAquick PCR purification kit (Qiagen, Valencia, CA). ChIP-seq library was prepared using TruSeq ChIP Library Preparation Kit (IP-202-1024, Illumina, San Diego, CA) according to the manufacturer's instructions. Briefly, 10 ng of ChIPed DNA was converted into blunt-ended fragments. A single adenosine nucleotide was added to the 3' ends of the blunt-ended fragments before ligation of indexing adapters to the adenylated 3' ends. The ligated products were purified, size-selected and PCR amplified according to the manufacturer's instructions. The quality and quantity of the DNA library were assessed on 4150 TapeStation System (Agilent, CA). Paired-ended sequencing was performed on an Illumina NovaSEQ6000 platform.

Omni-ATAC-seq

Omni-ATAC-seq was performed according to the published method (31). Briefly, 50,000 AMs that were untreated, treated with Poly(I:C) for four hours were spun down and washed once with cold PBS. The cells were resuspended in 50 µl cold ATAC-RSB-lysis buffer and incubated for 3 minutes. The ATAC-RSB-lysis buffer was immediately washed out with 1 mL ATAC-RSB buffer. The cell pellet was resuspended in 50 µl transposition mix and incubated for 30 minutes at 37 °C. The reaction was stopped by adding 2.5 µl pH 8 0.5 M EDTA. The Qiagen MiniElute PCR purification kit (Qiagen) was used to purify the transposed DNA. Purified DNA was amplified using the following condition: 72°C for 5 min, 98 °C for 30 s, and 13 cycles:

98 °C for 10s, 63 °C for 30 s, 72 °C for 1min. The amplified libraries were purified, size-selected, and the quality and quantity of libraries were assessed on 4150 TapeStation System (Agilent, CA). The pair-ended sequencing of DNA libraries was performed on an Illumina NovaSEQ6000 platform.

ChIP-seq and Omni-ATAC-seq data analysis

Raw sequencing reads (average 40-80 million reads, 2 biological replicates for each treatment) were aligned to the hg38 reference genome using Bowtie2 with very-sensitive and -x 2000 parameters. The read alignments were filtered using SAMtools to remove mitochondrial genome and PCR duplicates. Peaks were identified by MACS2 with the q-value cut-off of 0.05 and the sequencing data was displayed using IGV.

Lentiviral MPRA barcoded enhancer and CRISPR plasmids constructions

The lentiviral MPRA vector pLS-SceI (Addgene, Plasmid #137725) containing a minimal promoter and barcoded *Gfp* reporter gene was used for reporter assay. The candidate enhancers were cloned into the lentiMPRA pLS-SceI vector through the AgeI and Sbf restriction sites. Polymerases, restriction and modification enzymes were obtained from New England Biolabs (Beverly, MA). All plasmids were verified by sequencing.

The bicistronic sgRNA guides targeting *TNF* enhancers were designed using the online CRISPick tool from Broad Institute (https://portals.broadinstitute.org/gppx/crispick/public). Each of four sgRNA

sequences targeting the same enhancer was cloned into LentiCRISPRv2GFP (Addgene, Plasmid # 82416), LentiCRISPRv2-mCherry (Addgene, Plasmid #99154), LentiCRISPRv2-BFP, or LentiCRISPRv2-THY1.1 vectors via the BsmBI cloning site. LentiCRISPRv2-THY1.1 vector was modified by replacing GFP gene in LentiCRISPRv2-GFP with the gene encoding THY1.1 using the SacII and BamHI restriction sites.

Lentivirus production and transduction

The 10cm dishes were coated with 4mL l0 μg/mL poly D lysine (Sigma, P0899) for 5 minutes at room temperature in H_2O. Plate cells at 2-3 x 10^6 HEK293T cells/dish in DMEM (10% FBS, but no antibiotics). Twenty-four hours later, HEK293T cells were transfected with 10 μg of pLS-SceI-BE plasmid or four color bicistronic sgRNA guides LentiCRISPRv2 plasmids, 9 μg PΔ8.9 and 1 μg VSV-G using $CaCl_2$. Seventy-two hours after transfection, the supernatants were collected and filtered with a 0.45 μm filter.

THP-1 cells were cultured in RPMI 1640 medium plus 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin and 2mM beta-mercaptoethanol in a humidified 37°C, 5% CO_2 incubator. The 1×10^6 THP-1 cells were seeded into one well of a 6-well plate with 10 mL lentivirus medium. The polybrene was added to each plate at the final concentration of 8 μg/mL and the HEPES was added to each plate at final concentration of 25 mM. Lentivirus supernatants were added to each plate. The plates were wrapped with parafilm and centrifuged at 2,500 rpm for 90 minutes at room temperature. The supernatant was removed by aspiration and 2 mL fresh medium per well was added. The spin infection step was repeated at the next

day and the day after for a total of three spin infections. Two days after the last spin infection, the cells for four color sgRNA guides deletion that expressed BFP, GFP, RFP, and Thy1.1 were FACS-sorted. For the reporter assay, the transduced THP-1 cells can be used for further analysis.

PMA maturation and Poly(I:C) stimulation

THP-1 cells express low levels of pro-inflammatory cytokines upon Poly(I:C) stimulation. These cells can be matured to become robust pro-inflammatory cytokine-producing cells by Phorbol 12-Myristate 13-Acetate (PMA, Sigma-Aldrich). Transduced-THP-1 cells were incubated with PMA (200 ng/mL) for three days. The matured THP-1 cells were not treated or treated with 20 μg/mL Poly(I:C) (4287, R&D Systems, Minneapolis, MN) for four additional hours before the cells were collected for analysis.

Sequencing RNA barcode transcripts and DNA barcode inserts and bioinformatics analysis

The untreated or Poly(I:C)-stimulated cells were washed with PBS three times, and genomic DNA and total RNA were extracted using a DNA/RNA mini kit (Qiagen) according to the manufacturer's instructions. LentiMPRA barcoded RNA-seq and DNA-seq libraries were constructed according to the published method (23). Barcodes were associated with enhancer sequences and the number of barcodes in the RNA and DNA samples was counted using software MPRAflow as described in the published bioinformatics workflows (23). Briefly, the genomic DNA was treated with RNase to remove contaminating RNA and the total RNA was treated with DNase to

remove contaminating DNA. For the enhancer–barcode association, a P5 flowcell sequence, the sample index sequence and a P7 flowcell sequence were added to the LentiMPRA barcoded libraries. For the RNA and DNA barcode counts, cDNA was synthesized by reverse transcription using construct-specific primers that contain P7 flowcell sequences and unique molecular identifiers (UMIs), to preserve the true counts of molecules through the amplification process. DNA or cDNA was amplified with the primers that contain the P5 flowcell sequence, sample index sequence, 16-bp UMI and P7 flowcell sequence. The pair-ended sequencing of DNA libraries was performed on an Illumina NovaSEQ6000 platform.

We analyzed the NGS sequencing data on Linux. The codes was downloaded from https://docs.conda.io/en/latest/miniconda.html and the MPRAflow was downloaded from https://github.com/shendurelab/MPRAflow.git. For the barcode association, the code is “nextflow run association.nf --fastq-insert "R1_001.fastq.gz" --fastq-insertPE "R3_001.fastq.gz" --design "ordered_candidate_sequences.fa" --fastq-bc "R2_001.fastq.gz"”. For the barcode counting, the code is “nextflow run count.nf --dir "bulk_FASTQ_directory" --e "experiment.csv" –design "ordered_candidate_sequences.fa" –association "dictionary_of_candidate_sequences_to_barcodes.p"”.

qPCR analysis

The untreated or Poly(I:C)-stimulated THP-1 cells were collected, genomic DNA and total RNA were extracted using a DNA/RNA mini kit (Qiagen) according to the manufacturer’s instructions. Quantitative PCR was performed in a QuantStudio 7 Flex Real-Time PCR System (ThermoFisher, MA). The sequences of qPCR primers are

listed in Supplemental Table □. Relative mRNA amounts were calculated as follows: Relative mRNA or DNA amount = $2^{[Ct(Sample)-Ct(\textit{HPRT})]}$. The barcode reporter activity was measured as the ratio of RNA and DNA.

Statistical analysis

The nonparametric Mann-Whitney U test or two-tailed student's *t*-test was used to determine significant differences between the two samples.

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Figure legends

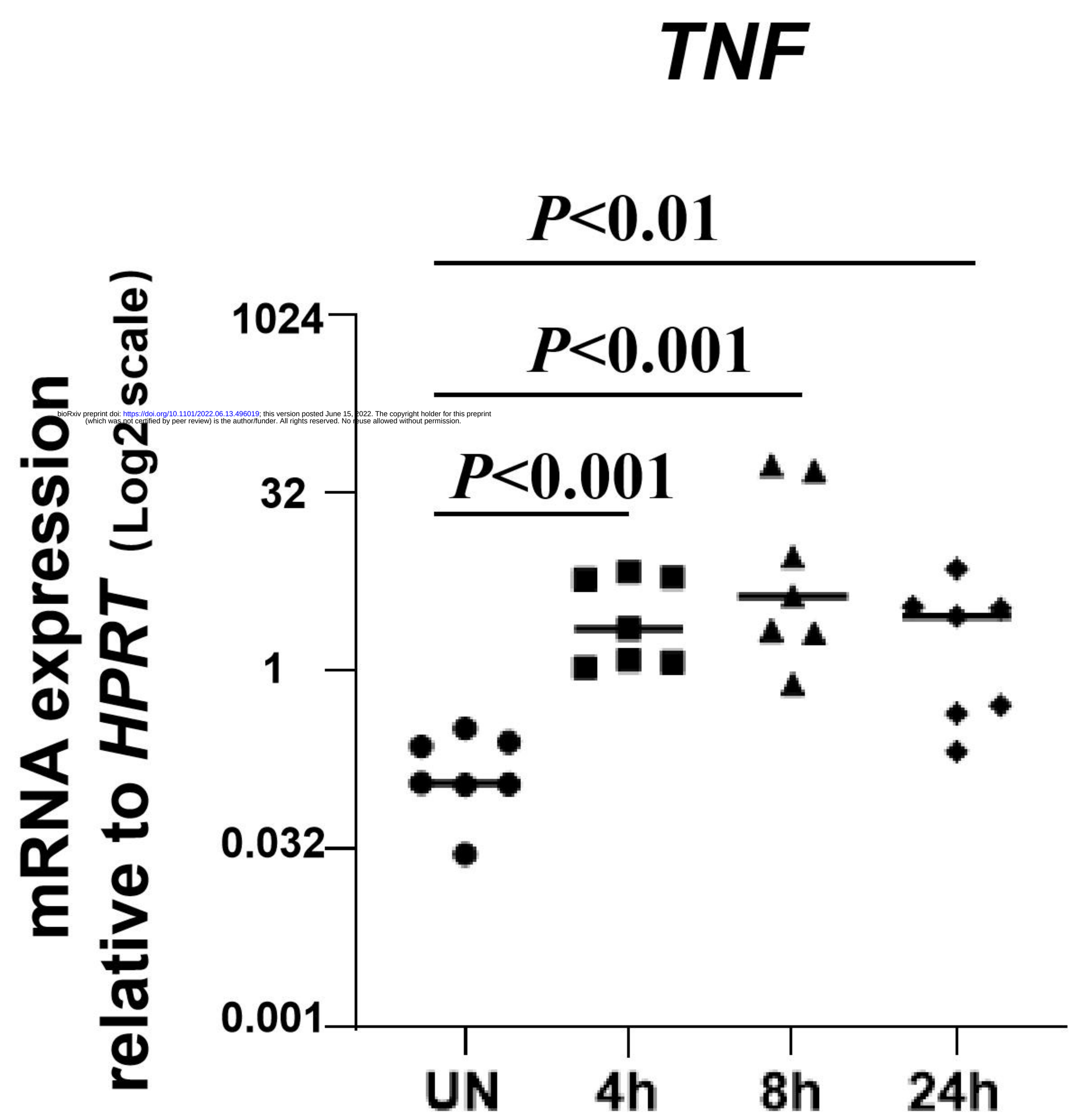
Figure 1. Primary human alveolar macrophages transcribe *TNF* genes to extremely high levels in responding to poly(I:C) stimulation. Human alveolar macrophages were untreated (UN), treated by poly(I:C) for four hours (4h), eight hours (8h) or twenty-four hours (24h). qPCR analysis of the mRNA expression of *TNF* genes. *P* values were calculated using the Mann-Whitney U test. Data represent mean ± SEM of seven biological samples.

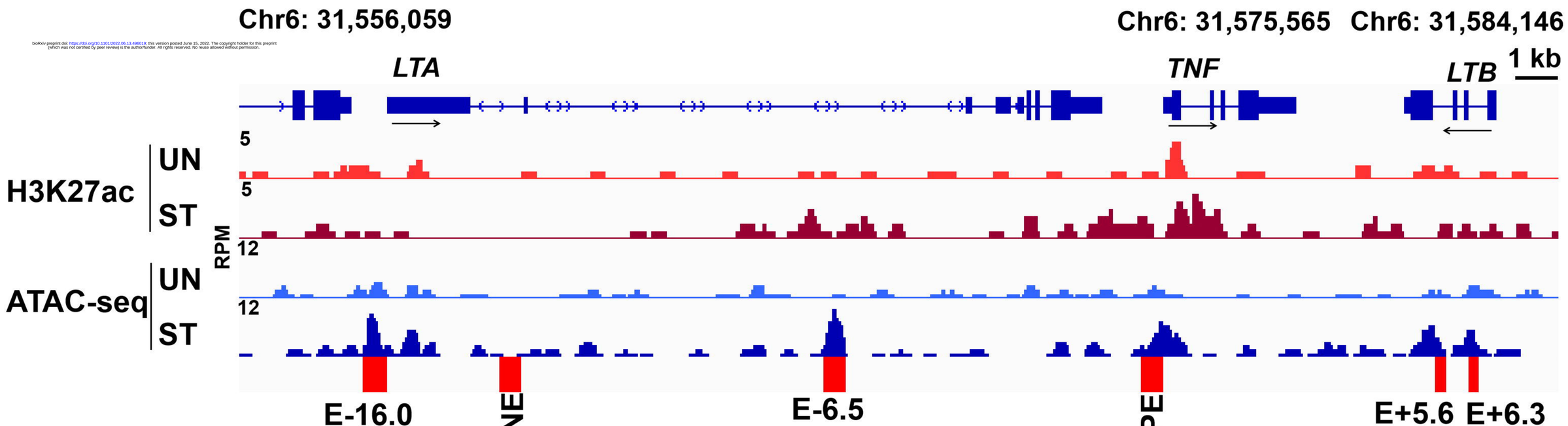
Figure 2. Identification of the putative *TNF* enhancers. Representative Integrated Genome Viewer (IGV) tracks from H3K27ac ChIP-seq and Omni-ATAC-seq. Human alveolar macrophages with unstimulation (UN) or poly(I:C) stimulation for four hours were used for H3K27ac ChIP-seq and Omni-ATAC-seq. Red bars indicate putative *TNF* enhancers that show increased H3K27ac modifications and chromatin accessibility. RPM: reads per million mapped reads; E: enhancer; PE: proximal enhancer; NE non-enhancer. The numbers following E indicate the distance (kb) of enhancer to the TSS of the *TNF* gene; + means the enhancers are located at the downstream of the TSS, and – means the enhancers are located at the upstream of the TSS. One IGV track was from one biological sample, representing two biological replicates with similar patterns.

Figure 3. *TNF* E-16.0 and PE possess enhancer activity in response to poly(I:C) stimulation. A. Barcoded GFP reporter gene constructs. **B.** Barcoded enhancer activity. The number RNA barcode transcripts and DNA barcode inserts in RNA and DNA samples prepared from the enhancers-transduced-THP-1 cells were determined

by NGS sequencing. The log2 ratios of barcode RNA transcripts to barcode DNA inserts (as input controls) were used to determine enhancer activity. Data represent mean ± SEM of two biological samples. **C.** qPCR analysis of RNA barcodes or DNA barcode inserts in samples prepared from the enhancers-transduced-THP-1 cells untreated or treated with poly(I:C) for four hours. Data represent mean ± SEM of three transduced samples. *P* values were calculated using two-tailed student's *t* test.

Figure 4. The *TNF* E-16.0 is required for *TNF* gene transcription in response to poly(I:C) stimulation. A. Targeting one enhancer with bicistronic sgRNA guides co-expressing BFP, GFP, RFP, and Thy1.1. **B.** FACS sorting gates. THP-1 cells were transduced with BFP, GFP, RFP, and Thy1.1 sgRNA guides (4c sgRNA guides). BFP^+, GFP^+, RFP^+, and $Thy1.1APC^+$ transduced cells were FACS-sorted using sorting gates indicated. **C.** DNA deletion efficiency analysis. Vector control (VC) and deleted DNA fragments in FACS-sorted BFP^+, GFP^+, RFP^+, and $Thy1.1APC^+$ cells were analyzed with PCR. **D.** *TNF* mRNA expression in the FACS-sorted transduced cells was measured by qPCR. The percentages indicate the percent reduction in *TNF* mRNA expression in enhancer-deleted (del) relative to non-transduced or vector-transduced THP-1 cells. **E.** *LTA* mRNA expression in the FACS-sorted cells was measured by qPCR. **F.** *LTB* mRNA expression in the FACS-sorted cells was measured by qPCR. *P* values were calculated using two-tailed student's *t* test. Data represent mean ± SEM of three transduced samples.





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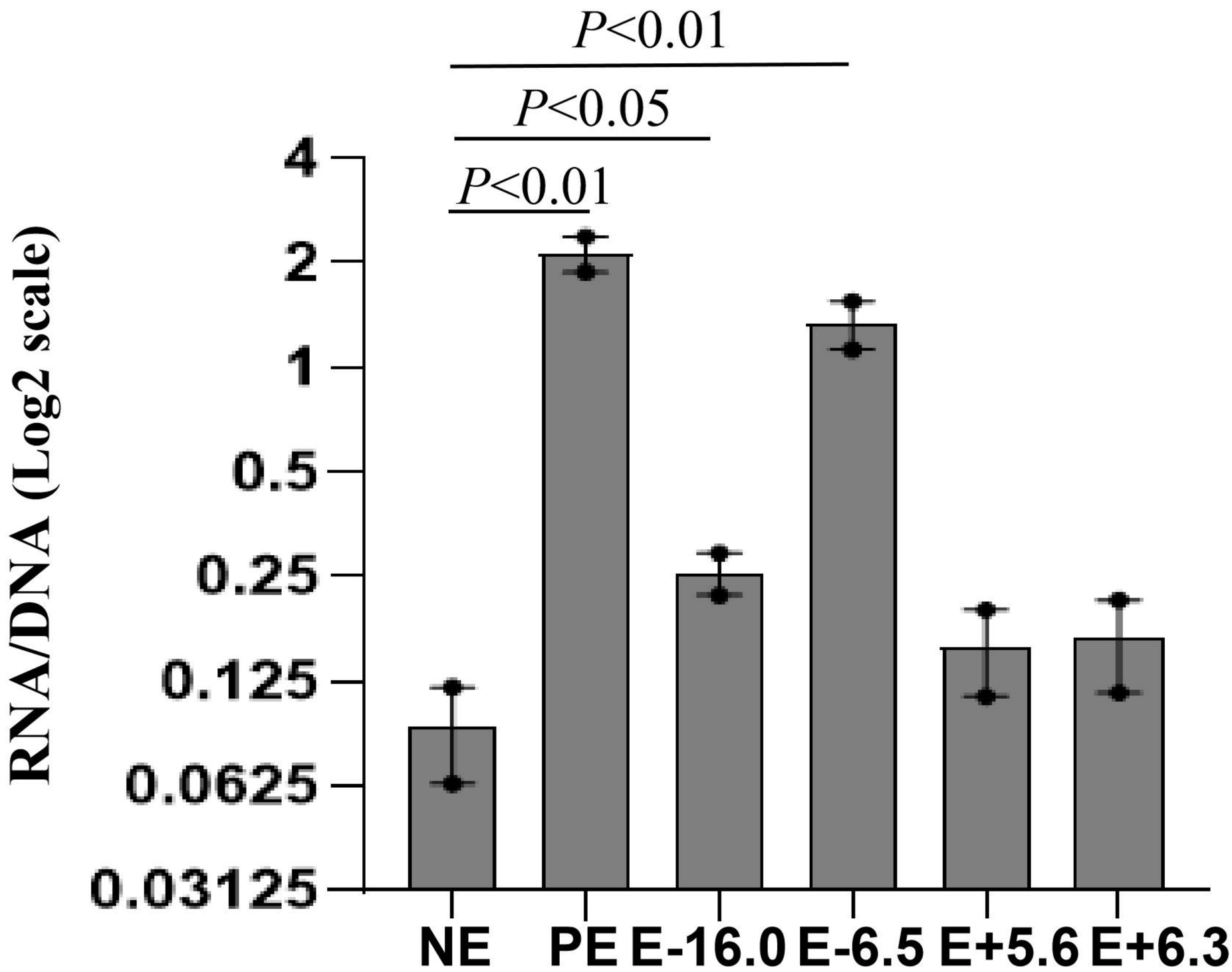

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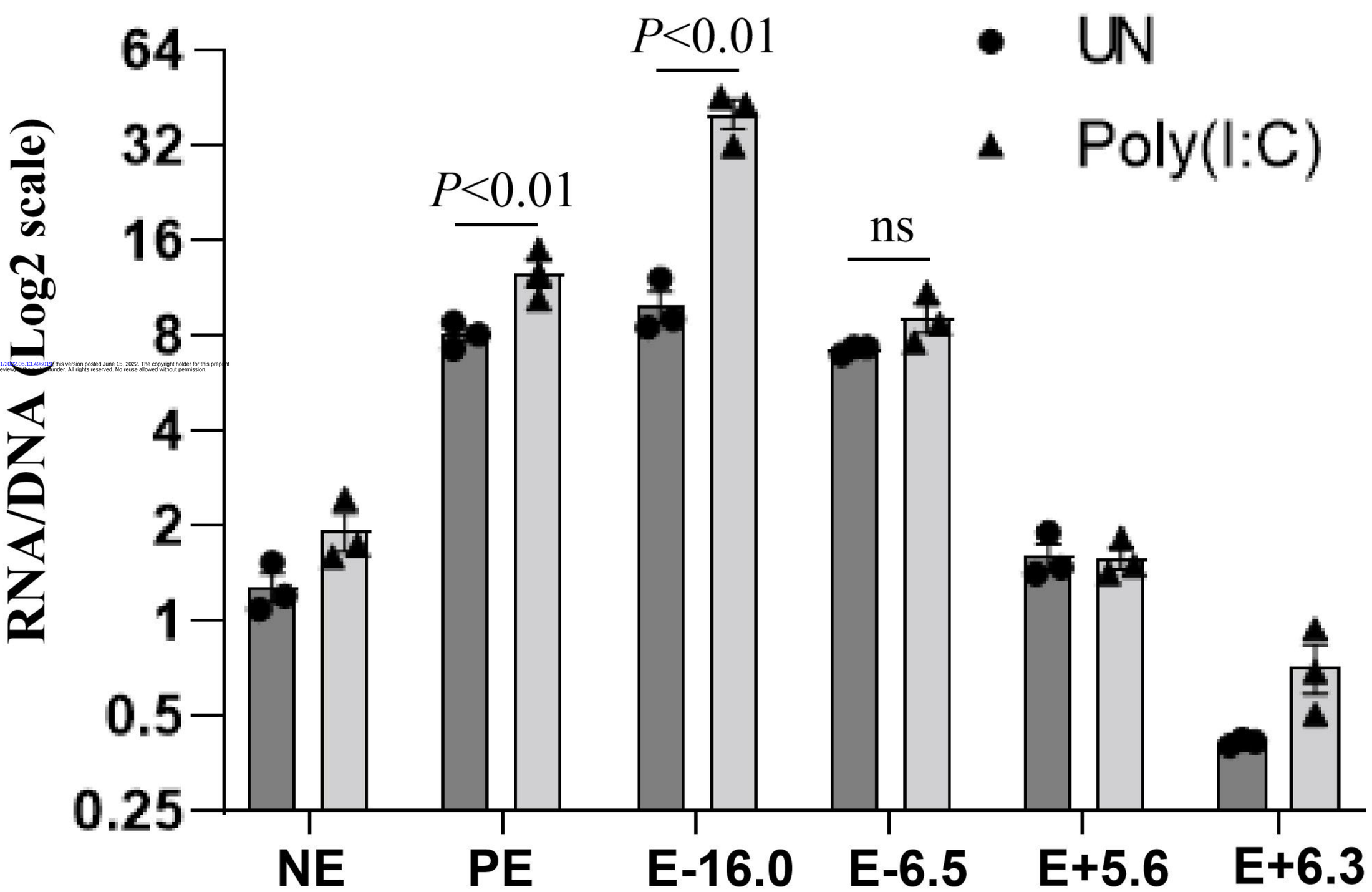

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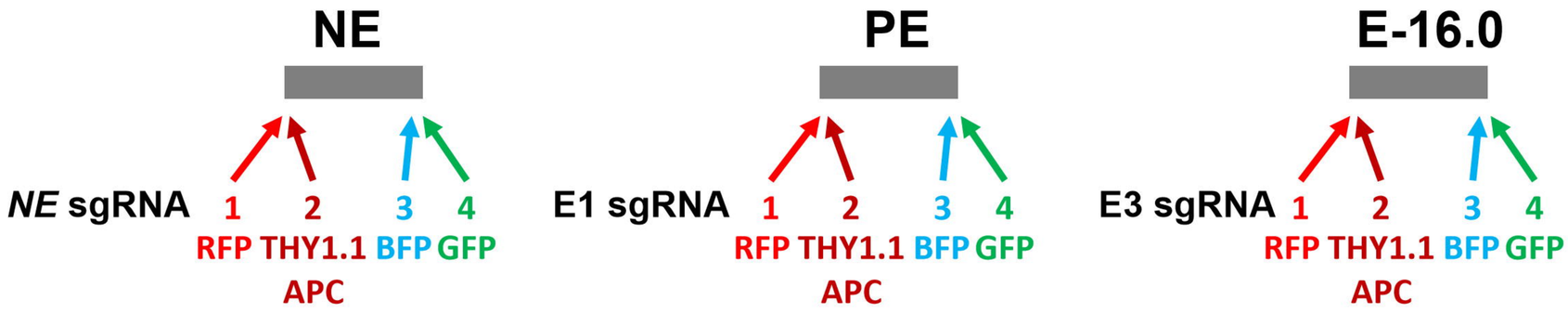

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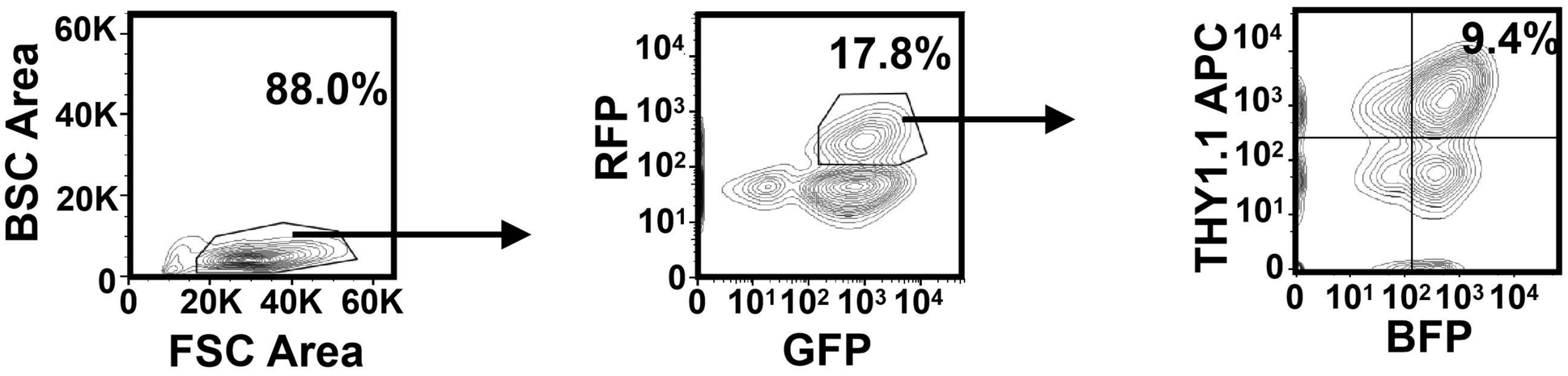

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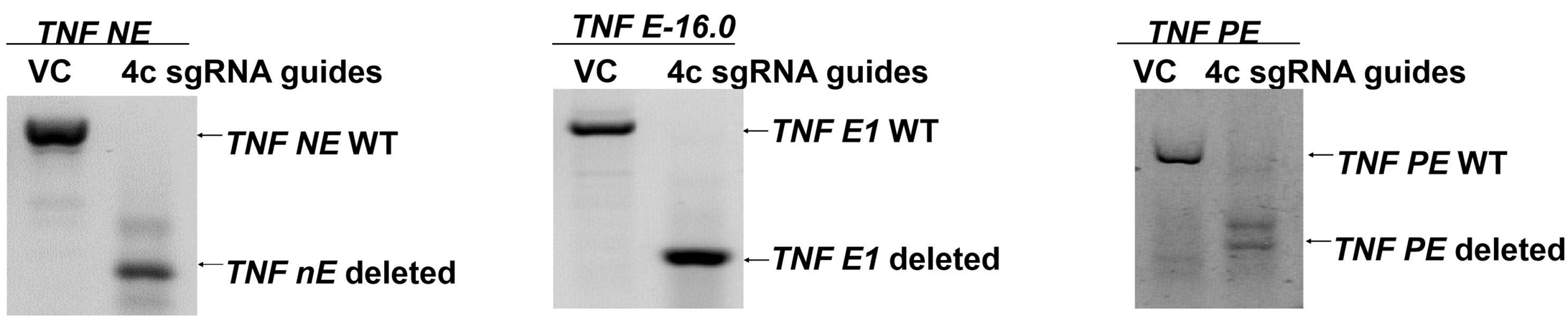

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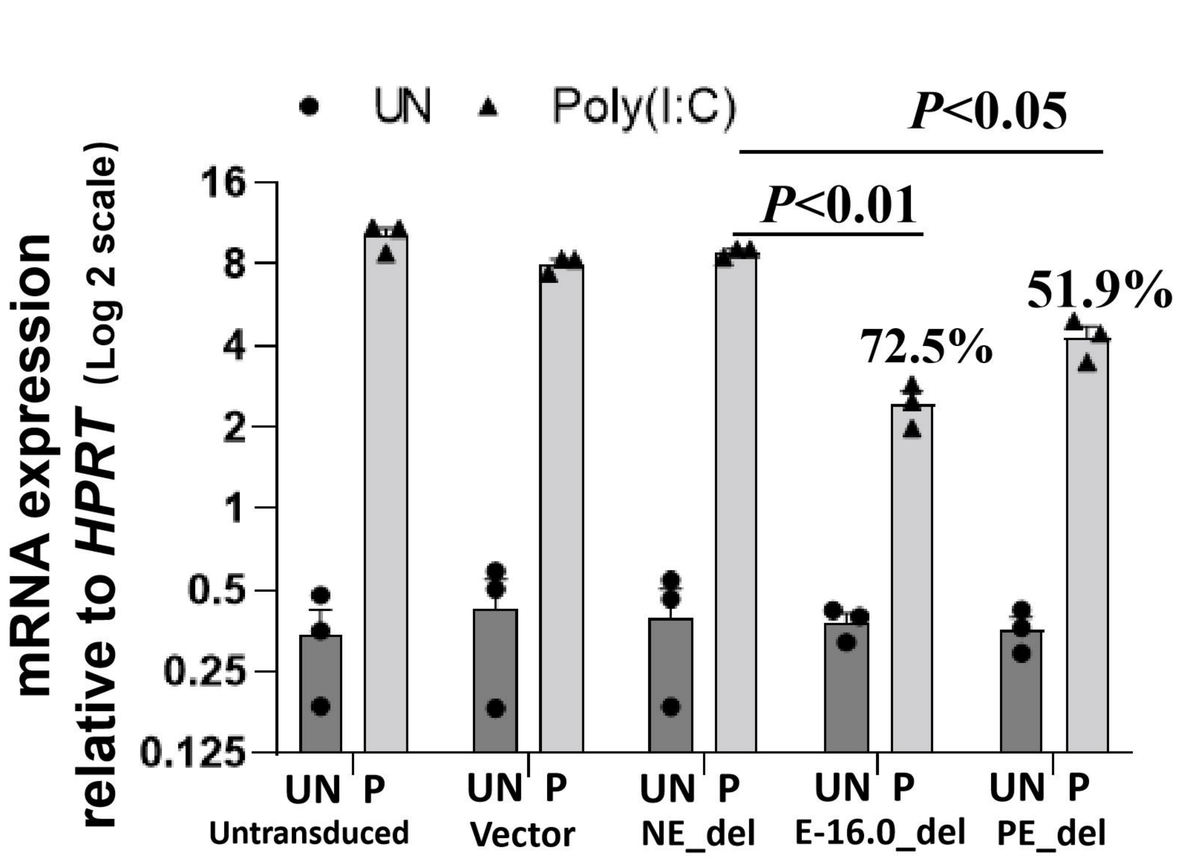

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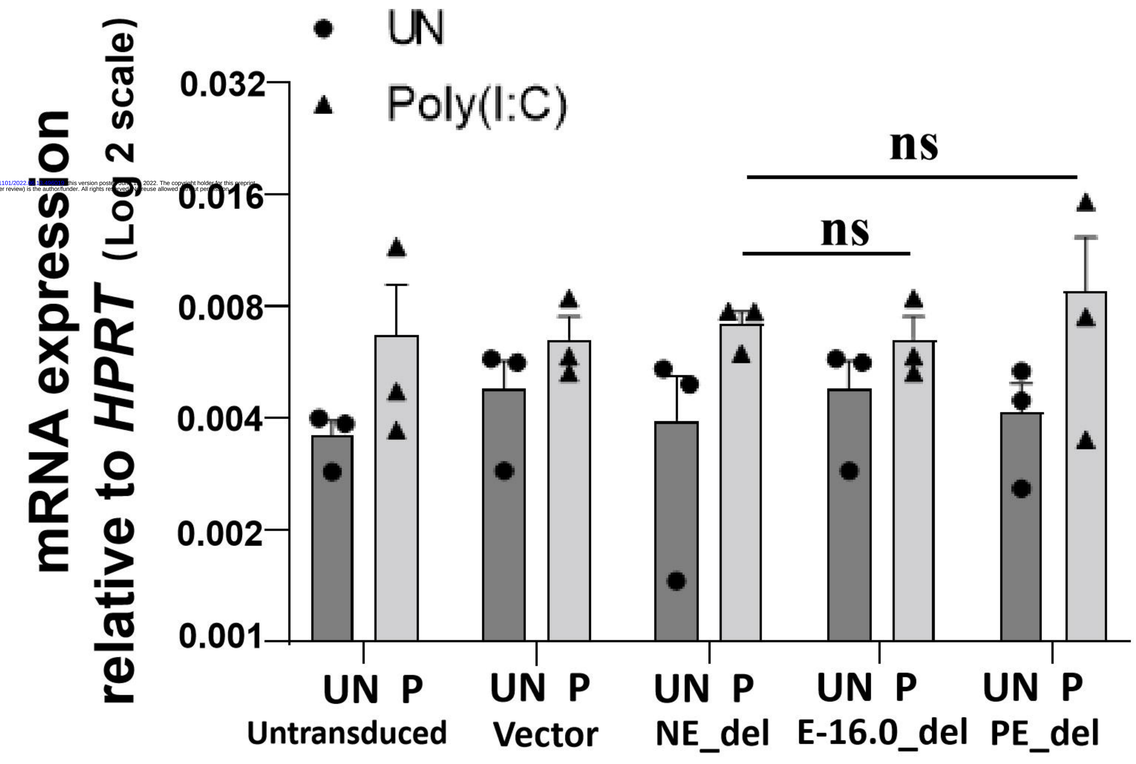

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