The role of enhancer RNA in IFN-γ priming effect on TNF induction by lipopolysaccharide

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Department of Medical Science

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Directed by Professor Jae Myun Lee

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Yonggeun Cho

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This certifies that the Doctoral Dissertation of Yonggeun Cho is approved.

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December 2017

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학위를 마치며 감사의 마음을 전하고자 합니다. 먼저 지난 5 년 동안 기초의학 교실에서 안정적으로 연구활동에 종사할 수 있도록 Physician-Scientist 제도를 만들어 주신 모교에 감사드립니다. 덕분에 늦은 나이에 시작한 실험실 생활에서도 시행착오를 겪으며 조금씩 더 성장해가는 기회를 누릴 수 있었습니다.

또한, 많은 분들의 도움과 가르침이 있었기에 여기까지 올 수 있었습니다. 무엇보다도 다양한 연구를 접하여 능동적으로 연구를 수행할 수 있게끔 토대를 마련해주시고, 과문한 가운데서 나온 설익은 아이디어에 대해서도 끊임없이 격려하고 아낌없이 지원해주신 지도교수님 이재면 교수님께 감사드립니다. 아울러 연구의 방향성이 명료해 지도록 갈피를 잡아주시고, 어수선한 글을 꼼꼼이 읽으며 논지와 문장을 가다듬어주신 박전한 교수님의 노고로 본 논업이 자그마한 책자로 만들어지는데 이를 수 있었습니다. 바쁘신 와중에도 본 연구를 위하여 각별한 조언을 해주신 김현숙 교수님, 서준영 교수님, 현영민 교수님께 깊은 감사를 드립니다. 오가며 마주칠 때 마다 조언과 격려의 말씀을 아끼지 않으신 최인홍 교수님, 신전수 교수님을 비롯한 미생물학교실 모든 교수님들께도 감사드립니다.

같은 과정에 참여한 동기들(김한상, 이승현, 한현호)과 선후배님들이 있었기에 낯선길이지만 물어 볼 데가 있어 든든하였습니다. 특히 승현이는 근처에 있다는 이유로 숱한 도움을 청하였는데 그때마다 다 들어 주어서 항상 고마웠습니다.

많은 시간을 함께하며 동고동락한 우리 파트의 여러분들께 감사를 표합니다. 실험실 생활에서 다방면으로 도움을 주며 또 랩을 이끌어 간 박사님들(이현규, 차혜란, 박광환, 심두희, 박필구, 김민정)의 노고가 있었고, 함께 박사과정을 진행한 양창모, 김혜미와 석사과정 학생들(김유진, 조수지, 황수진)의 고생이 있었으며, 권승현, 이지해, 문국영 선생님과 설지희씨의 수고가 있었습니다. 저의 여러 단점들에도 불구하고 이 모든 분들이 가진 많은 장점들 덕으로 앞으로 나아갈 수 있었습니다.

특히 동갑내기 창모를 만나 이런저런 고민을 나누며 외롭지 않게 길을 헤쳐 나갈 수 있었던 것은 행운이라고 생각합니다.

파트를 불문하고 아낌없이 도움을 주신 분들이 미생물학교실에 많이 계셨습니다. 저를 비롯한 실험실 내 모든 학생들이 고시환 선생님의 헌신적인 도움과 가르침을 받았습니다. 주 관심사였던 분자생물학적 기법에 대한 이해는 이강무 박사님께서 기꺼이 받아주신 토론과 격려 속에 무르익어갔고, 대식세포라는 저에게 생소한 세포주를 접하고 익히는 데에는 같은 분야의 연구를 먼저 천착해 나가며 터득한 바를 거리낌없이 공유해주고 또 연구수행에 물심양면으로 지지를 보내준 서진원 박사님의 도움이 절대적이었습니다. 김영훈, 무하마드, 신희욱, 황원태와 열성적으로 주고 받은 담소 내지는 토론들이 서로의 발전에 도움이 되었으리라 믿습니다. 그 밖에 지면에 싣지 못한 많은 분들의 도움이 있었습니다. 모두들 감사하고 뜻한 바 일들이 계속 잘 되시기를 기원합니다.

기초의학 교실에서 배우고 익힌 과학적 소양을 임상의사로 살아가는데 잘 접목시켜내는 것이 제게 남은 큰 숙제인 것 같습니다. 이에 대한 설계가 불투명한 때에 생각을 다잡아 주시고 또 힘들 때 위로의 손길을 내밀어 주신 김현숙 교수님께 다시 한 번 깊은 감사의 말씀을 올립니다. 석사학위 과정을 지도해주신 이래로 부족한 제가 학문의 길을 처음 내딛고 이후에도 계속 걸어갈 수 있도록 포기하지 않고 이끌어 주신 김정호 교수님께는 마음의 빚이 큽니다. 매사에 열일을 다 하시는 이상국 교수님은 의국 생활 때 처음 뵌 이래로 언제나 귀감이 되어 왔습니다.

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다시 한번 모두에게 감사의 마음을 전합니다.

2017 년 12 월
저자 조 용 근

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ABSTRACT

The role of enhancer RNA in IFN-γ priming effect on TNF induction by lipopolysaccharide

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(Directed by Professor Jae Myun Lee)

Tumor necrosis factor (TNF) is a pleiotrophic cytokine that conducts a potent proinflammatory function in inflamed tissue. Monocytes and macrophages are the major source of the cytokine. They produce TNF in response to lipopolysaccharide (LPS) stimulation, and interferon (IFN)-γ priming sensitizes the cells to augment expression of a set of genes including *TNF*. Despite major advances in understanding of the TNF induction by LPS:Toll-like receptor (TLR)4 signaling pathway, the mechanism of how IFN-γ potentiates its induction has been poorly understood. Recent study showed that recruitment of interferon regulatory factor (IRF)1 to an enhancer region 8 kb upstream of *TNF* transcription start site (hHS-8) is responsible for the priming effect, as well as the increased transcriptional activity at the enhancer region. However, characteristic of the eRNA transcribed in hHS-8 region and its possible role in mediating IFN-γ priming effect on TNF induction were not addressed.

In this study, I characterized enhancer RNA (eRNA)s transcribed on hHS-8 in IFN-γ primed monocyte upon LPS challenge. Balanced amount of bidirectional long non-coding RNA (lncRNA)s was produced by LPS, as the NF-κB signaling pathway being the major inducer, and which was augmented with pretreatment of IFN-γ. The eRNAs were transcribed without processing and only part of them were polyadenylated. They contained no ORF and localized mainly in the nucleus. I also found that they mediate enhancer function of augmenting TNF induction in IFN-γ primed monocyte upon LPS challenge, which was demonstrated by catabolically dead Cas9 mediated knock down experiment. These findings indicate that enhancer region of *TNF* gene generates lncRNAs with typical features of the eRNA, and these eRNAs play an important role in regulating TNF transcription in human monocyte.

Key words:, eRNA, hHS-8, TNF, IFN-γ, LPS, monocyte

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I. INTRODUCTION

Tumor necrosis factor (TNF) is the prototypic cytokine of the TNF superfamily, consisted of 19 member of ligands grouped on amino acid sequence homology to TNF, which interacts with 29 cognate receptors.[1] Versatile bioactivities are induced by TNF via binding to two distinct receptors; pathogenic effect of pro-inflammation is mediated through TNF receptor (TNFR)1 and homeostatic effect such as cell survival and tissue regeneration is promoted via TNFR2.[2] While membrane-bound TNF, which is the initially synthesized form, binds to both TNFRs, soluble TNF, which is released from membrane-bound form by proteolytic cleavage with TNFα-converting enzyme,[3] binds exclusively to TNFR1.[4] Thus, soluble TNF increasing early in the inflammatory cascade mainly promotes inflammatory activities of diverse cells, as TNFR1 is ubiquitously expressed,[5] in the implicated milieu. Those pro-inflammatory activities orchestrated by TNF encompass almost holistic aspects of the

process including induction of inflammatory mediators such as cytokines and lipid mediators, recruitment of inflammatory cells through induction of chemokines and activation of endothelial cells, augmentation of inflammatory cell survival, and promotion of tissue destruction by necroptosis.[5] Thereby, uncontrolled production of TNF found in various autoimmune diseases would serve important rate-limiting steps of the development and progression of the diseases.[6] Indeed, the concept of TNF as a therapeutic target has been well proven with the use of recently approved biologic agents targeting TNF that led a major progress in treating individuals with TNF-dependent chronic inflammatory diseases, such as rheumatoid arthritis, Crohn's disease, and psoriasis.[7] Meanwhile, current therapeutic paradigm of global TNF blockade also has side effects including increased risk of common and opportunistic infection.[5,8] Advances in the understanding of TNF biology in diverse aspects would render new approaches to regulate TNF in a more tailored manner.

Although TNF was first studied as the product of macrophages,[9] wide range of cell types including T cells, B cells, NK cells, mast cells, dendritic cells, and fibroblasts were demonstrated to express it.[10] And myriad of stimuli have been identified to induce TNF including pathogens, cytokines, mitogens, chemical stress, radiation, and ionophores.[10] Thus transcriptional regulation of TNF has been studied in the cell type and stimulus-specific manner. The i*n vitro* studies of TNF production in monocyte uses lipopolysaccharide (LPS), which is a common constitute of outer membrane of gram negative bacteria, as a stimulant modelling bacterial infection.[11] LPS binds to Toll-like receptor (TLR)4 on the surface of monocyte and initiates downstream signaling that leads to recruitment of distinct sets of transcription factor (TF)s on the proximal promoter region of *TNF* gene,[12] which reside in the ~20 kb *TNF/LT* locus within the class III major histocompatibility region on human chromosome 6. These TFs associate with coactivators to form more complex structure known as enhanceosomes to drive TNF transcription synergistically.[12,13]

In addition to LPS, the effect of interferon (IFN)-γ, the only cytokine of type II class of interferons, on monocyte has been studied *in vitro* as it was reported that IFN-γ sensitizes circulating monocytes and tissue resident macrophages to act against a diverse group of microbial targets.[14] While IFN-γ *per se* does not induce TNF expression, it activates cells of monocyte/macrophage lineage to produce more inflammatory cytokines including TNF, interleukin (IL)6, and IL12 upon LPS stimulation,[15] the phenomenon known as IFN-γ priming.[16] While the molecular events leading to TNF induction by LPS have been relatively well elucidated, as descried above, the mechanism how IFN-γ potentiates expression of TNF upon LPS challenge has been poorly understood; treatment of IFN-γ minimally affected TLR signaling[15] that directly drives TNF transcription, and the sole *TNF* promoter was insufficient to recapitulate IFN-γ priming effect in promoter assay.[17]

In a recent study, Yu et al. showed that IFN-γ induces sustained occupancy of transcription factors STAT1 and interferon regulatory factor (IRF)1 at promoter and enhancer region of *TNF*, *IL6*, and *IL12B* loci to epigenetically liberate those regions.[15] Nancy et al. further demonstrated that distal *TNF/LT* locus element 8 kb upstream of the *TNF* transcription start site (hHS-8) works as a distal enhancer element in IFN-γ priming through p300 recruitment to the element mediated by IRF1.[17] In the study, both genetic ablation of IRF1 and repression of the hHS-8 region with dead Cas9 linked to the Krüppel-associated box (KRAB) repressive domain successfully abolished IFN-γ priming effect on TNF expression.[17] Furthermore, they also showed that hHS-8 region has transcriptional activity generating a type of non-coding RNA (ncRNA), enhancer RNA (eRNA), of which level corresponded well to the epigenetic activation status of the region.[17] However, characteristic of the eRNA transcribed in hHS-8 region and its possible role in mediating IFN-γ priming effect on TNF induction were not addressed.

eRNA represents a relatively short ncRNA transcribed on enhancer element,

which could be presumably distinguished by histone modification marks such as high H3K4me^{1} and low H3K4me^{3}.[18] With recent advances in sequencing technologies, it has become apparent that those active enhancer elements conduct pervasive RNA transcription across diverse cell types.[18] Gross categorization of these eRNAs can be made based on the basic natures of their transcription; whether they undergo polyadenylation or not, and whether they are transcribed unidirectionally or bidirectionally.[18] More importantly, when its functionality is of concern, emerging evidences suggest that at least some eRNAs contribute to distinct steps in transcriptional process of target gene in diverse cell types.[19-21] In human breast cancer cells, eRNAs facilitate 17β-oestradiol dependent gene activation by stabilizing enhancer-promoter looping through an interaction with cohesion, which forms a complex with mediator to facilitate chromosomal looping.[19] In human neurons, eRNAs facilitate the transition of paused RNA polymerase II (RNAPII) into productive elongation by acting as a decoy for the negative elongation factor (NELF) complex upon induction of immediate early gene (IEG)s.[20] Notably, LPS stimulation of primary human monocytes caused widespread changes in the expression levels of tentative eRNAs, which corresponded well with the induction rate of nearby target genes, and IL1β expression was ameliorated by suppression of its eRNA, although the mechanism was not addressed.[21] Thus, eRNAs are important regulator of inflammatory genes in monocytes and functionality of each eRNA needs to be studied.

In this study, I characterized the eRNAs transcribed on *TNF* enhancer region, hHS-8, and found that these eRNAs have functional role in mediating IFN-γ priming effect on human monocytes to enhance TNF expression stimulated by LPS.

II. MATERIALS AND METHODS

1. Cell culture and treatment

The human monocyte leukemic cell line THP-1 (ATCC, Manassas, VA, USA) was cultured in RPMI-1640 (GE Healthcare Life Sciences, South Logan, UT, USA) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences) and 1% penicillin/streptomycin (Thermo Fischer Scientific, Waltham, MA, USA). Cells were maintained at concentration of 1-4 × 10^5 cells/mL. For differentiation into macrophage, 5 ng/mL phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) was treated for 48 hr.[22] In the study of TNF induction, THP-1 cells were primed with or without 100 ng/mL of recombinant human IFN-γ (Peprotech, Rocky Hill, NJ, USA) for 2 hr, followed by 100 ng/mL of LPS from *E. coli* O111:B4 (Sigma-Aldrich) challenge for additional 1 hr. In the study of pathway inhibition, following inhibitors in specified amount were pretreated with cells for 30 min at 37℃ before LPS challenge; U0126 at 10 μM, SB203580 at 10 μM, SP660125 at 10 μM, BAY11-7082 at 10 μM, Wortmanin at 1 μM, and Cyclosporin A at 1 μM. All inhibitors were purchased from Sigma-Aldrich.

2. Real time quantitative PCR (RT-qPCR)

Total RNA was extracted from THP-1 cells using Trizol (Thermo Fischer Scientific) reagent. One microgram of RNA was reverse transcribed by Superscriptase III (Thermo Fischer Scientific) using random hexamer. Fifty nanograms of synthesized cDNA served as a template for subsequent RT-qPCR. Reaction was performed in ABI Step One instrument sing a SYBR Green based detection kit (Kapa Biosystems, Boston, MA, USA) according to the manufacturer's instruction. The primer sequences for RT-qPCR were listed in **Table 1**. β-actin mRNA level in each sample was used as a control for internal normalization.

Table 1. The primer sequences for RT-qPCR

Names	Sequences
β-actin, F	CATGTACGTTGCTATCCAGGC
β-actin, R	CTCCTTAATGTCACGCACGAT
TNF, F	CCTCTCTCTAATCAGCCCTCTG
TNF, R	GAGGACCTGGGAGTAGATGAG
IRF1, F	GCCAGTCGACGAGGATGAGGAAGGG
IRF1, R	CCAGCGGCCGCCTGCTACGGTGCAC
IRF8, F	AGTAGCATGTATCCAGGACTGAT
IRF8, R	CACAGCGTAACCTCGTCTTC
IRF9, F	GCCCTACAAGGTGTATCAGTTG
IRF9, R	TGCTGTCGCTTTGATGGTACT
IL6, F	ACTCACCTCTTCAGAACGAATTG
IL6, R	CCATCTTTGGAAGGTTCAGGTTG
IL12B, F	ACCCTGACCATCCAAGTCAAA
IL12B, R	TTGGCCTCGCATCTTAGAAAG
(-)eRNA, F	TCCCAATTCAGGTTTTGAGGCT
(-)eRNA, R	AGTGGTCTTTGTGGAGAAACT
(+)eRNA, F	TCATGTCTCCCCATCACCCT
(+)eRNA, R	TGCAATTAACAGTGAGACACAGA
HIST1H2AG, F	AGTCCAAACCAACGGCTCTT
HIST1H2AG, R	AATCTGCGGTGTTCGCTACA
H2AFV, F	GGCCAAGGCTAAGGCAGTAT
H2AFV, R	CTCCAGAATCGCAGCACTGT
UBB, F	GGTCCTGCGTCTGAGAGGT
UBB, R	GGCCTTCACATTTTCGATGGT

RPPH1, F	CTAACAGGGCTCTCCCTGAG
RPPH1, R	CAGCCATTGAACTCACTTCG
CCNGL, F	ATGGATTGTTTCTGGGCGTA
CCNGL, R	GGTTGTGGAGAAAGGCTTCA
MALAT1, F	GACGGAGGTTGAGATGAAGC
MALAT1, R	ATTCGGGGCTCTGTAGTCCT
RPL30, F	GCTGGAGTCGATCAACTCTAGG
RPL30, R	CCAATTTCGCTTTGCCTTGTC
18S rRNA, F	AAACGGCTACCACATCCAAG
18S rRNA, R	CCTCCAATGGATCCTCGTTA

3. Determination of the subcellular localization of RNA

For determination of the subcellular localization of target transcripts, cellular fractionation was conducted as to the method described **(Fig. 1a)**.[23] Briefly, whole cells were lysed by incubation in hypotonic lysis buffer (HLB; 10 mM Tris (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 0.3% (vol/vol) NP-40 and 10% (v/v) glycerol) supplemented with RNase inhibitor (Thermo Fischer Scientific) for 10 min in ice. After centrifugation at 2000 g for 3 min, the supernatants were transferred into a new tube and cytoplasmic RNA was extracted using Trizol (1 mL of Trizol per 200 μL of supernatant). The nuclear pellet was washed once with ice cold HLB and nuclear RNA was extracted using Trizol. One hundred nanograms of RNAs from each compartment were reversed transcribed, and RT-qPCR was conducted as described above. Expression levels of the transcripts in each compartment were normalized to those of 18S rRNA.

4. Determination of the polyadenylation status of RNA

For determination of polyadenylation status of target transcripts, total RNAs were separated by an oligo dT tagged beads (oligotex direct mRNA mini kit; Qiagen, Hilden, Germany) according to the method recommended by the manufacturer. Additional separation step was conducted on both flow-through and eluate to increase the separation accuracy **(Fig. 1b)**. One hundred nanograms of RNAs from each compartment were reversed transcribed, and RT-qPCR was conducted as described above. Expression levels of the transcripts in each compartment were compared without normalization.

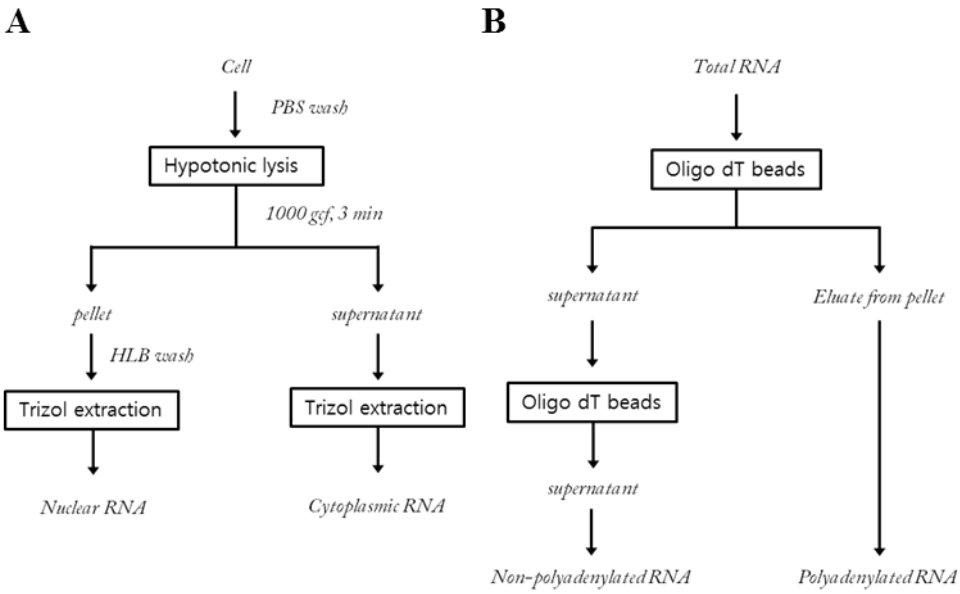

Figure 1. Flow charts of RNA separation for the characterization of eRNAs. (A) Flow chart of RNA localization study using cellular fractionation by hypotonic lysis (B) Flow chart of RNA polyadenylation status study using oligo dT bead based polyadenylated RNA separation.

5. Enzyme-linked immunosorbent assay (ELISA)

Soluble TNF levels in media was measured by a human TNF ELISA kit (BD Biosciences, San Diego, CA, USA) according to the method suggested by the manufacturer.

6. Lentiviral production and transduction

For generation of lentiviral particles, 1.2×10^6 HEK293T cells were seeded in a 6 well plate a day before transfection. A total of 3 μg plasmid DNAs composed of target construct, pMD2g, pMDL g/p RRE, and pRSV-rev in equimolar ratio were transfected into the HEK293T cells using Lipofectaime 3000 reagent (Thermo Fishcher Scientific) according to the method suggested by the manufacturer. After 6 hr of incubation, media was changed with lentivirus packaging media (Opti-MEM I reduced serum medium (Thermo Fischer Scientific) supplemented with 1 mM sodium pyruvate and 5% FBS) media and the virus containing media was harvested twice at 24 hr and 52 hr post transfection. Harvested media were mixed and filtered through 0.45 μm PVDF filter (Merck Millipore Ltd., Tullagreen, County Cork, Ireland). Then, the filtered viral media were aliquoted and kept frozen at -80℃ until use. Lentiviral transduction was conducted by adding 500 μL of the viral media to 500 μL of THP-1 cell (4×10^5 cells/mL) supplemented with 8 μg/mL hexadimethrine bromide (Sigma-Aldrich). Following warm spinoculation by centrifuging the samples at 800 g for 120 min at 32℃, cell pellets were resuspended in a complete RMPI media and cultured for 72 hr. Then, antibiotic selection was conducted against puromycin (InvivoGen, San Diego, CA, USA) at concentration of 0.5 μg/mL. The media was changed every 3 days for 1-2 weeks.

7. cDNA circularization and simultaneous inverse RACE PCR

To determine the whole sequence of eRNAs, cDNA circularization and 5' RACE PCR method[24] was adopted with minor modification to simultaneously determine both end sequences **(Fig. 2)**.[25] Firstly, total RNA was extracted from THP-1 cells primed with IFN-γ (100 ng/ml) for 2 hr followed by LPS (100 ng/ml) challenge for additional 1 hr. A total of 800 ng of the RNA was polyadenylated using *E. coli* poly A polymerase (NEB, Ipswich, MA, USA) according to the method suggested by the manufacturer. The polyadenylated RNA was purified with RNeasy MinElute Cleanup kit (Qiagen) and 160 ng of it was reverse transcribed with Superscriptase III using phosphorylated tagged oligo dT primer (Integrated DNA Technologies, Coralville, IA, USA) in 20 μL reaction volume. RNA bound to the first strand cDNA was removed by alkaline hydrolysis (adding 2 μL of 1 N NaOH and incubation at 70℃ for 10 min), and the solution was neutralized by adding 2 μL of 1 N HCl. About 100 ng of the cDNA were circularized with 50 U of T4 RNA ligase (NEB) in 100 μL reaction volume (1×T4 RNA ligase buffer supplemented with 25% PEG 8000 and 1 mM ATP) by incubation at 25℃ for 16 hr. The circularization was stopped by adding EDTA at final concentration of 2 mM. One microliter of the circularized cDNA was used for nested inverse PCRs and the amplicons obtained in the second round PCR were gel-excised, TOPO-ligated and sequenced. The primer sequences for nested inverse RACE PCR were listed in **Table 2**.

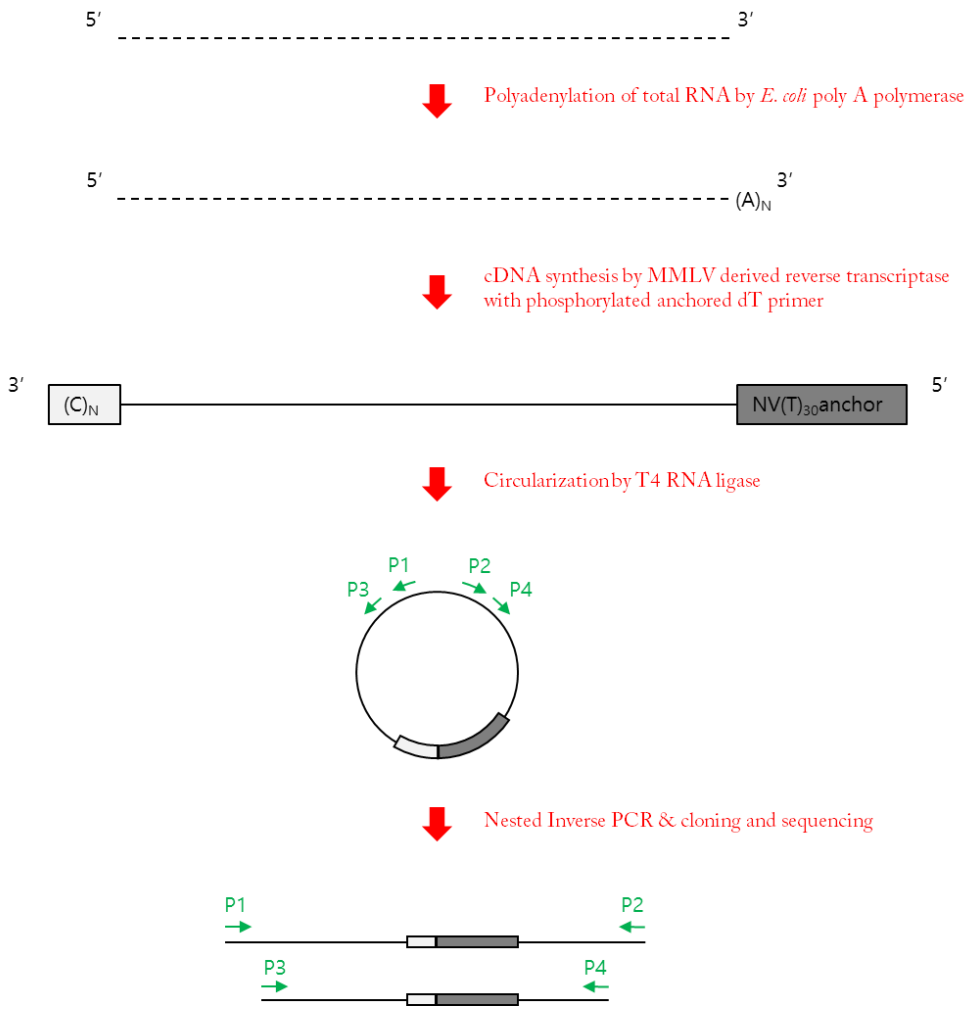

Figure 2. Flow charts of cDNA circularization and simultaneous RACE PCR using inverse primers. Reactions conducted and enzymes used in each step are described (in red). Primers used in inverse PCR are denoted and depicted as arrows (in green).

Table 2. The primer sequences for nested inverse RACE PCR

Names	Sequences
(-) strand primer 1	GCTTCAAACTTGAACTTAGCCTCA
(-) strand primer 2	TCTCATTTGGAGGCTTCAGAGA
(-) strand primer 3	AGCCTCAAAACCTGAATTGGGA
(-) strand primer 4	AGTTTCTCCACAAAGACCACT
(+) strand primer 1	GGAAGAATGGGACATTGAAAGACA
(+) strand primer 2	AATTTCTCTCTGTGTCTCACTGT
(+) strand primer 3	AGGGTGATGGGGAGACATGA
(+) strand primer 4	TCTGTGTCTCACTGTTAATTGCA

8. dCas9 mediated knock down of eRNAs on hHS-8

Catalically dead Cas9 (dCas9) was introduced on lentiCRISPR v2 vector[26] (Addgene plasmid #52961) by generating D10A and H840A amino acid changes[27] on Cas9 coding sequences on the vector, through site directed mutagenesis **(Fig. 3)**. To knock down eRNAs on hHS-8, sgRNA sequences targeting sense strand of each eRNA were designed with a web tool (http://crispr.mit.edu/). The designed sgRNAs and non-targeting sgRNA[28] sequences were inserted into the dCas9 containing lentiCRISPR v2 vector using golden gate assembly method.[29] The primer sequences for generation of dCas9 knock down of eRNAs on hHS-8 were listed in **Table 3** and **Table 4**.

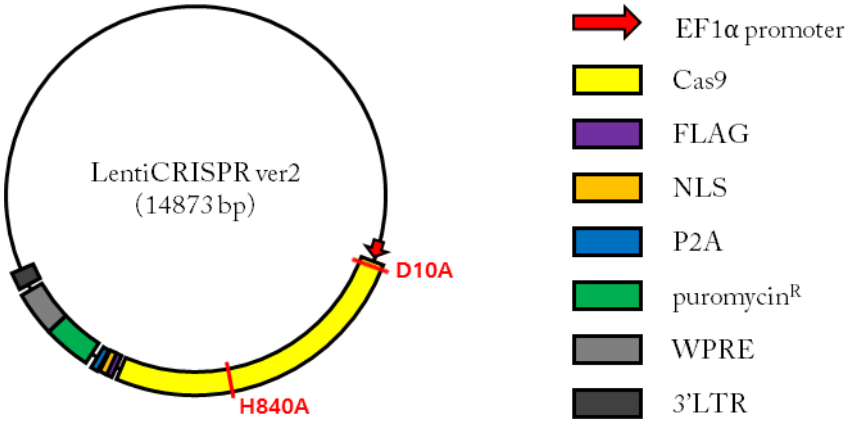

Figure 3. Construction of dCas9 expressing vector. Schematic illustration of Cas9 cassette on the Lenti CRISPR v2 vector (Addgene plasmid #52961) is presented. dCas9 was introduced on the vector by generating D10A and H840A amino acid changes (indicated by red bars and characters) with site directed mutagenesis.

Table 3. The primer sequences for generation of dCas9

Names	Sequences
D10A, F	GTACAGCATCGGCCTGGCCATCGGCACCAACTCTG
D10A, R	CAGAGTTGGTGCCGATGGCCAGGCCGATGCTGTAC
H840A, F	CGACTACGATGTGGACGCTATCGTGCCTCAGAGC
H840A, R	GCTCTGAGGCACGATAGCGTCCACATCGTAGTCG

Table 4. The primer sequences for construction of dCas9 mediated eRNA KD vectors

Names	Sequences
Non-targetting gRNA #1, F	CACCGTGAACTGCAATCTTATTAT
Non-targetting gRNA #1, R	AAACATAATAAGATTGCAGTTCAC
Non-targetting gRNA #2, F	CACCGGGCAGAAGTTGCTGTCCTG
Non-targetting gRNA #2, R	AAACCAGGACAGCAACTTCTGCCC
(-) strand eRNA gRNA #1, F	CACCGTCATTTCAGTCTCATTTGG
(-) strand eRNA gRNA #1, R	AAACCCAAATGAGACTGAAATGAC
(-) strand eRNA gRNA #2, F	CACCGAATGTCATTTCAGTCTCATT
(-) strand eRNA gRNA #2, R	AAACAATGAGACTGAAATGACATTC
(+) strand eRNA gRNA #1, F	CACCGGGACATTGAAAGACAAAAA
(+) strand eRNA gRNA #1, R	AAACTTTTTGTCTTTCAATGTCCC
(+) strand eRNA gRNA #2, F	CACCGAGAGAAATTTAAATTAAAG
(+) strand eRNA gRNA #2, R	AAACCTTTAATTTAAATTTCTCTC

9. Overexpression of eRNAs

For overexpression of the eRNAs under lentivral vector system, I introduced bovine growth hormone polyadenylation signal into EcoRI/BamHI sites of pCDH-CMV-MCS-EF1-Puro (CD510B-1; System Biosciences, Palo Alto, CA, USA) vector. Inserting polyA signal within LTR region renders expression of target RNA in designated length, meanwhile interfering with viral genome RNA replication and resulting in relatively lower amount of viral particles, though which was sufficient to transduce target cells.[30] Then, cDNA sequences of eRNAs or LacZα gene (control) were amplified from genomic DNA of THP-1 or pUC19 vector, respectively, and inserted into XbaI/EcoRI sites of the modified vector. The primer sequences for generation of the eRNA overexpression vectors were listed in **Table 5**. Production of viral particle in HEK293T and transduction of it into THP-1 cells were conducted as described above.

Table 5. The primer sequences for construction of eRNA overexpression vectors

Names	Sequences
bGH pA, F	GCTAGCGAATTCTTGTTTATTGCAGCTTATAATGGT
bGH pA, R	GGCCGCGGATCCATAAGATACATTGATGAGTTTGGACA
LacZα, F	GAAGATTCTAGATTCACTGGCCGTCGTTTTAC
LacZα, R	AAATTCGAATTCTCGCCATTCAGGCTGCGCAA
(-)eRNA, F	GAAGATTCTAGAATAGTGGTCTTTGTGGAGAAACT
(-)eRNA, R	AAATTCGAATTCCAAGATTAGCCCTAGAAACAGGGT
(+)eRNA, F	GAAGATTCTAGAAGTCTTACCCTTCCTTCCTCTGT
(+)eRNA, R	AAATTCGAATTCGCCCTCCAGCCTGGGCAA

Underline in the primer sequence highlights the restriction enzyme (EcoRI, BamHI, or XbaI) recognition site

10. Statistical analysis

Data were shown as the mean ± standard error of the mean (s.e.m.). I first performed the Shapiro-Wilk normality test for checking normal distributions of the groups. If normality tests passed, two-tailed, unpaired Student's t-test and if normality tests failed, Mann-Whitney tests were used for the comparisons between two groups. For the comparisons of three or four groups, I used one-way ANOVA if normality tests passed, followed by Tukey`s multiple comparison test for all pairs of groups. If normality tests failed, Kruskal-Wallis test was performed and followed by Dunn`s multiple comparison test. The GraphPad PRISM software (v6.0a, La Jolla, CA, USA) was used for statistical analysis. $P < 0.05$ was considered statistically significant. *P<0.05, **P<0.01, ***P<0.001.

III. RESULTS

1. IFN-γ poises the *TNF* gene for enhanced transcription in response to LPS in monocyte/macrophage.

For investigation of IFN-γ priming effect on TNF induction by LPS, a human monocytic leukemic cell line, THP-1, was used. THP-1 cells were primed with or without IFN-γ (100 ng/mL) for 2 hr, followed by LPS (100 ng/mL) challenge for additional 1 hr, then changes in expression levels of TNF transcripts or soluble TNF secreted into the supernatant were monitored. As a single stimulus, LPS alone significantly induced TNF transcription, whereas IFN-γ alone was not sufficient to induce *TNF* gene. However, priming of cells with IFN-γ significantly enhanced TNF mRNA levels compared with stimulation by LPS alone **(Fig. 4a)**. TNF protein levels were consistently elevated by exposure to LPS, but not by IFN-γ alone, while IFN-γ priming augmented the level significantly **(Fig. 4b)**.

IFN-γ priming effect was also observed in IL6, in which the expression level of the gene was synergistically augmented with treatment of both IFN-γ and LPS than the level induced by each stimulus alone. However, the priming effect was not yet observed in IL12B **(Fig. 4c)**, rather the expression level was decreased by pretreatment of IFN-γ than LPS alone with no statistical significance. Thus, I determined IL6 as a control gene, for the moment, to verify whether in terms of IFN-γ priming effect on TNF expression certain mechanism is used specifically or is used in general.

IFN-γ induces IRF1, IRF8, and IRF9 in monocyte,[31] and including these, all the nine-member IRF family of transcription factors share a cognate binding motif.[31] In the current study, when measured 3 hr post IFN-γ treatment, the levels of IRF1 and IRF9 were significantly elevated, while that of IRF8 yet remained comparable to none treated status **(Fig. 4d)**, resembling the report of more delayed expression of IRF8

than IRF1 observed in IFN-γ treated murine macrophage cells.[32] These imply IRF1 and IRF9, but not IRF8, could show some degree of functional redundancy at the moment, while the expression levels of IRF1 is dominant.

To verify whether IFN-γ priming effect on TNF induction by LPS in monocyte could be extended to macrophage, THP-1 cells were differentiated with minimal amount (5 ng/ml) of PMA for 48 hr.[22] In differentiated THP-1 cells, both TNF mRNA levels **(Fig. 5a)** and protein levels in supernatant **(Fig. 5b)**, in exposure to LPS with or without IFN-γ, were changed similarly as observed in undifferentiated THP-1. Thus I speculated that the mechanisms of IFN-γ priming that augments LPS induced TNF are similar in monocytes and macrophages, as a previous report demonstrated that the primary monocyte driven macrophages react similar to undifferentiated THP-1,[17] thus both conditions were used for the following experiments interchangeably.

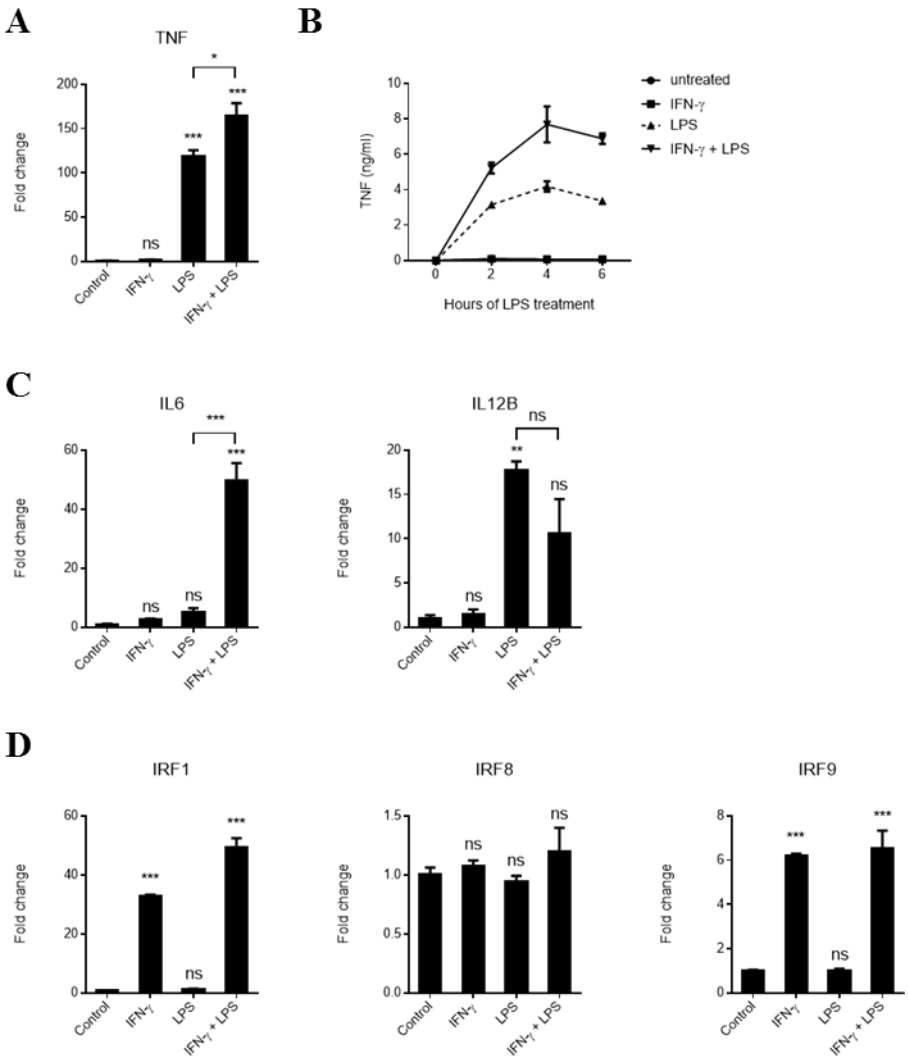

Figure 4. IFN-γ poises the *TNF* gene for enhanced transcription in response to LPS in monocyte. (A) THP-1 cells were primed with or without IFN-γ (100 ng/mL) for 2 hr, and stimulated by LPS (100 ng/mL) for additional 1 hr. TNF expression levels were measured by RT-qPCR. (B) Soluble TNF protein levels in supernatant

were measured at 2 hr, 4 hr, and 6 hr post LPS treatment with or without IFN-γ priming. (C, D) Transcript levels of other inflammatory cytokines (IL6 and IL12B in C) and IRFs downstream of IFN-γ (IRF1, IRF8, and IRF9 in D) were measured 1 hr post LPS treatment with or without IFN-γ priming.

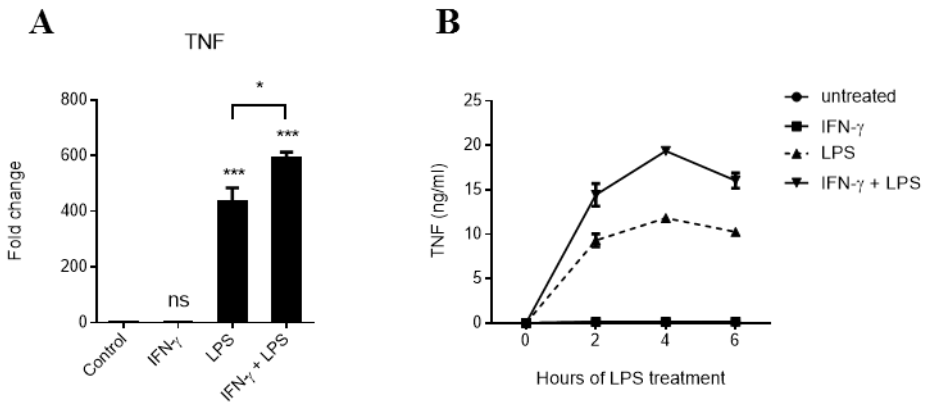

Figure 5. IFN-γ poises the *TNF* gene for enhanced transcription in response to LPS in macrophage. THP-1 cells were differentiated into macrophages with 5 ng/mL of PMA for 48 hr. (A) TNF transcription levels were measured after 1 hr of LPS treatement (100 ng/mL) with or without 2 hr of IFN-γ priming (100 ng/mL). (B) TNF protein levels in supernatant were measured 2 hr, 4 hr, and 6 hr post LPS treatment with or without IFN-γ priming.

2. LPS induces bidirectional unprocessed eRNAs from *TNF* enhancer region, hHS-8, which is augmented by IFN-γ priming

eRNA production at hHS-8 upon LPS stimulation has been reported, which was augmented by IFN-γ priming similarly to TNF expression.[17] In that study, primers targeting 3' flanking region of IRF1 binding sites at hHS-8 were used to assess changes in expression levels of the transcript spanning the region. To further characterize the RNA transcripts at hHS-8, I first retrieved cap analysis of gene expression (CAGE) peaks from FANTOM5 dataset viewed on UCSC genome browser **(Fig. 6)**. On visual inspection, bidirectional transcriptional activity from the flanking regions of IRF1 binding sites at hHS-8 was noticed **(Fig. 6)**. Thus I designed primer sets targeting 100 bp length segments on the both sides, 100 bp apart from the IRF1 binding sites, to confirm transcription at each side; (-)eRNA and (+)eRNA **(Fig. 6)**. RT-qPCR results showed induction of eRNAs on the both sides upon LPS and/or IFN-γ stimulus in the manner similar to TNF expression, although their changes were statistically not significant **(Fig. 7)**.

As much of eRNA operates within the nucleus to regulate the transcription of target genes,[21] I conducted cellular fractionation to determine subcellular localization of the eRNAs at hHS-8 **(Fig. 1a)**. The separation procedure was validated by amplification of a nuclear marker gene, *MALAT1*, and a cytoplasmic marker gene, *RPL30*, of which results were satisfactory **(Fig. 8a and 8b)**. While there were no significant changes in the expression levels of the marker genes, both eRNAs were augmented significantly upon stimulus of IFN-γ priming followed by LPS. eRNAs were predominantly localized in the nucleus of both unstimulated and stimulated cells **(Fig. 8c and 8d)**, indicating its subcellular location of act, if it had functionality, would be the nucleus.

Next, I assessed the polyadenylation status of the transcripts using an oligo dT based RNA separation method **(Fig. 1b)** to characterize transcriptional processing

occurring in the enhancer region and weigh the potential of the transcript to be translated. The separation procedure was validated by amplification of a polyadenylated (pA+) RNA marker gene, *UBB*, and a non-polyadenylated (pA-) RNA marker gene, *RPPH1*, of which results were satisfactory **(Fig. 9a and 9b)**. In both eRNAs, only part of the transcripts was polyadenylated, while the portion of polyadenylated transcripts were higher in (+)eRNA than in (-)eRNA **(Fig. 9c and 9d)**.

Furthermore, to map the exact sequence of both transcripts, I used a 5' RACE PCR method employing cDNA circularization[24] with minor modification[25] to simultaneously detect both ends sequences **(Fig. 2)**. As only a part of eRNAs from hHS-8 are polyadenylated, total RNAs were firstly polyadenylated by *E. coli* poly (A) polymerase to have binding site for tagged oligo dT primers for cDNA synthesis. Primers were designed by simply reversing the ones used for RT-qPCR and another primer sets residing inside the RT-qPCR target region were designed to conduct the inverse PCR in nested manner. After 2 rounds of PCRs, >200 bp single transcripts were identified at the both sides **(Fig. 10 and Table 6)**. On the sequence analysis, presence of tagging sequence at 3' end and the additional non-template cytosines in the 5' end, which are made during first strand synthesis using MMLV derived reverse transcriptase, confirmed that the directionality of the eRNA transcripts were outward the IRF1 binding sites as expected from CAGE peaks of FANTOM5 dataset **(Fig. 6)**. The transcripts contained no splicing region. And the 'AATAAA' termination signal, which serves also as a polyadenylation signal, was found only in (-)eRNA. This is quite opposite to the finding that the polyadenylated transcripts are more abundant in (+)eRNA than in (-)eRNA **(Fig. 9c and 9d)**. Furthermore, both eRNAs contained no open reading frame (ORF)s, and these transcripts localized mainly in the nucleus. Collectively, these data indicate bidirectional eRNAs, which have no translational potential, are transcribed at hHS-8.

Superimposing the PhastCons score from the PHAST dataset, which shows measurements of evolutionary conservation of the sequence among 100 vertebrate species, relatively higher conservation was observed in (-)eRNA coding region than in the region coding (+)eRNA **(Fig. 6)**. The central non-transcribed regions are highly conserved, outreaching the known IRF1 binding sites, possibly containing transcription factor binding sites that are responsible for the eRNA transcription with LPS stimulation alone **(Fig. 6 and Fig. 7)**.

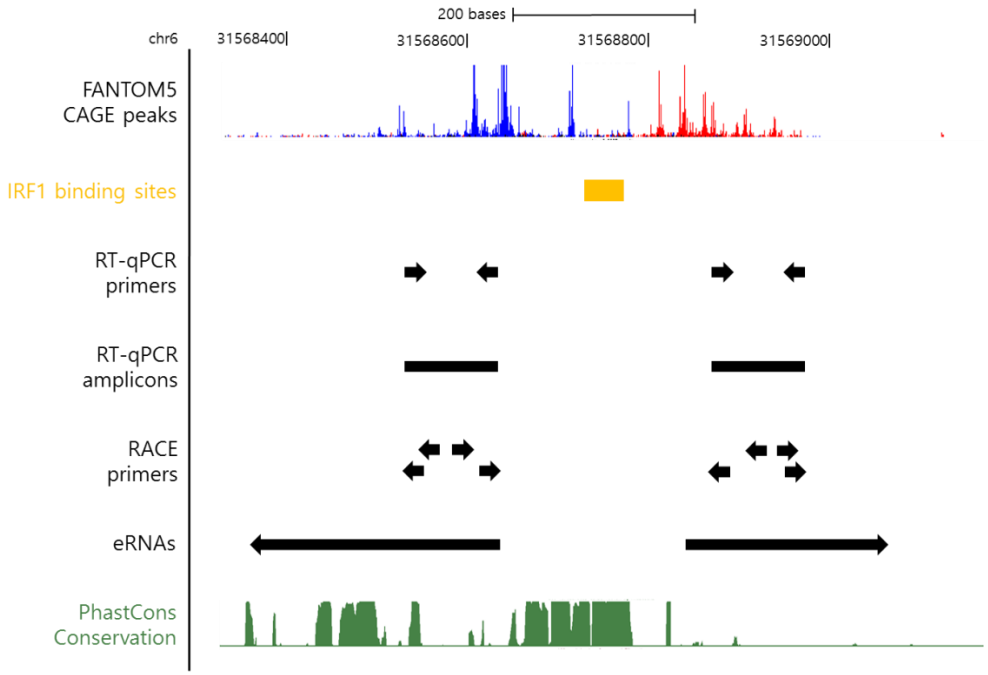

Figure 6. A part of genomic region (chr6:31568330-31569172) within hHS-8, with manual annotations and illustrates from UCSC browser view. On the top, blue and red peaks represent CAGE peaks retrieved from FANTOM5 dataset. IRF1 binding sites are denoted by yellow box. Arbitrary RT-qPCR primers used in current study and their target regions are presented in black arrows and bars. Below, inverse primer sets for RACE PCR and the annotation of the eRNAs sequenced are presented in black arrows. On the bottom, green peaks represent PhastCons scores retrieved from PHAST dataset.

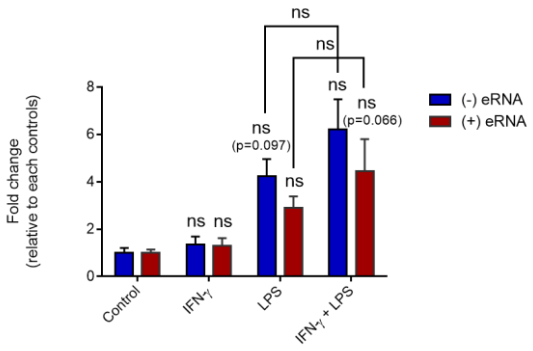

Figure 7. Bidirectional transcription at hHS-8 was observed upon LPS treatment, which was augmented by IFN-γ priming. THP-1 cells were primed with or without IFN-γ (100 ng/mL) for 2 hr, and stimulated by LPS (100 ng/mL) for additional 1 hr. Expression levels of the eRNAs were measured by RT-qPCR.

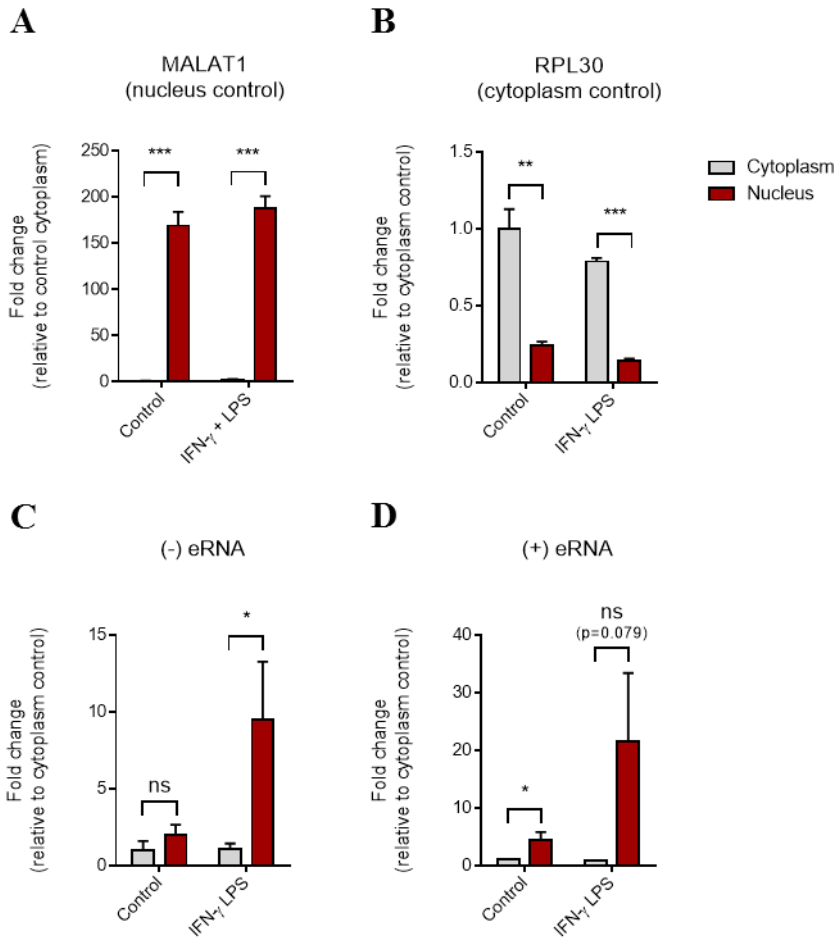

Figure 8. eRNAs are localized primarily in the nucleus. Expression levels of the both eRNA transcripts (C, D) in each subcellular compartment (nucleus or cytoplasm) were measured in THP-1 cells stimulated by LPS (100 ng/mL) for 1 hr with or without IFN-γ (100 ng/mL) priming for 2hr. RT-qPCR results were normalized against 18S rRNA. Expression levels of a nuclear marker gene, *MALAT1* (A), and a cytoplasmic marker gene, *RPL30* (B) were also measured to validate the separation procedure.

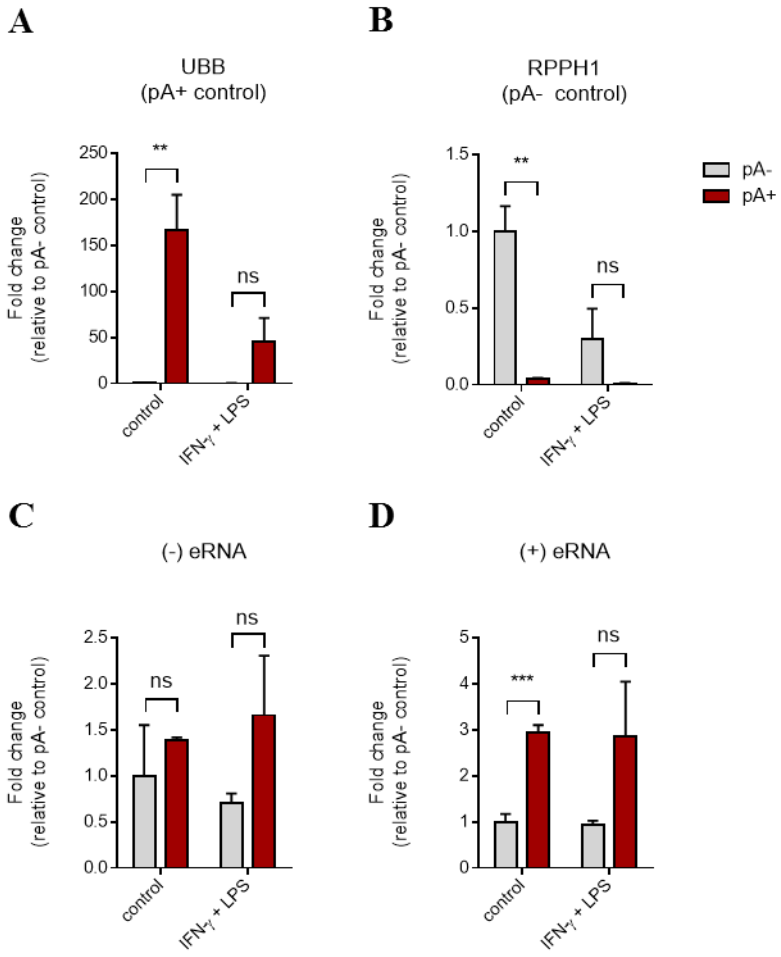

Figure 9. eRNAs are partially polyadenylated. Expression levels of the both eRNA transcripts (C, D) in each RNA fraction (pA+ or pA-; separated by oligo dT beads) were measured in THP-1 cells stimulated by LPS (100 ng/mL) for 1 hr with or without IFN-γ (100 ng/mL) priming for 2 hr. Expression levels of a pA+ marker gene, *UBB* (A), and a pA- markger gene, *RPPH1* (B), were also measured to validate the separation procedure.

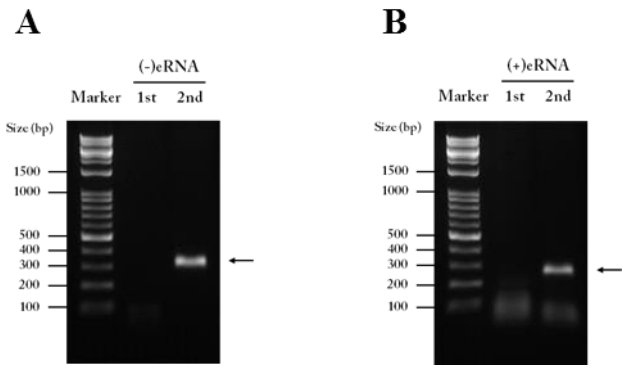

Figure 10. Nested inverse RACE PCR of circularized eRNAs. Total RNA from IFN-γ primed and LPS treated THP-1 cells were polyadenylated, reverse transcribed, and circularized **(Fig. 2)**. Inverse PCRs of the circularized cDNA were conducted in a nested manner, and the products of each round PCR were electrophoresed on 1.5% agarose gel. (-)eRNA (A) and (+)eRNA (B).

Table 6. The sequences of eRNAs on hHS-8

Names (lengh)	Sequences
(-)eRNA (275 bp)	ATAGTGGTCTTTGTGGAGAAACTAGTGAAATCTCTGAAGC CTCCAAATGAGACTGAAATGACATTAGCTTCAAACTTGAA CTTAGCCTCAAAACCTGAATTGGGATTTAATACCAACATC AACCCTAACCCAAATTTAACCTCAACCCAAATCACAACTC AAACTCAACCCCAACTGTAACCCTAACCTTAAATCTAAAC ACATCCCAATTAATAACCCCCTAAATAAAACTTCTCCTCTA CCCCAACCCAACCCTGTTTCTAGGGCTAATCTTG
(+)eRNA (223 bp)	AGTCTTACCCTTCCTTCCTCTGTGTCTCTCATGTCTCCCCA TCACCCTTCTTGCCTTCCCTTTTTTGTCTTTCAATGTCCCA TTCTTCCTCTTTAATTTAAATTTCTCTCTGTGTCTCACTGTT AATTGCAATACCTTTTTTTGTTTGCTTGTTTTGTTTTGTTTT GTTTTTTGGTTTGTTTGTTTGAAATGGAGTCTCACTTTGTT GCCCAGGCTGGAGGGC

Underline in the transcript sequence highlights the presence of ‘AATAAA’ sequence.

3. Transcription of eRNA on hHS-8 stimulated by LPS is mediated by NF-κB pathway

As the eRNAs on hHS-8 were transcribed upon LPS stimulation alone **(Fig. 7)**, I tried to determine which signaling cascade under LPS:TLR4 pathway is mainly driving the transcription. To this end, I treated THP-1 cells with the selected inhibitors of signaling pathways under TLR4 activation, 30 min prior to LPS challenge, then changes in the levels of eRNAs by LPS was measured by RT-qPCR. While the inhibition of ERK1/2, p38α, JNK, or calcineurin affected little on the level of (-)eRNA induction upon LPS challenge compared with that in control cells pretreated with same amount of DMSO, inhibition of NF-κB significantly suppressed (-)eRNA transcription to the level comparable to none challenged cells **(Fig. 11a)**. Meanwhile, inhibition of PI3K increased (-)eRNA expression, although it was not statistically significant ($p=0.0636$) **(Fig. 11a)**. Similar patterns were also found on the expression levels of (+)eRNA induced by LPS after treatment with the various inhibitors, which yielded in lesser differences **(Fig. 11b)**. The induction levels of TNF were increased with pretreatment of p38α or PI3K inhibitors, and decreased with ERK1/2, JNK, or NF-κB inhibitors, while inhibition of calcineurin made no statistically significant change **(Fig. 11c)**.

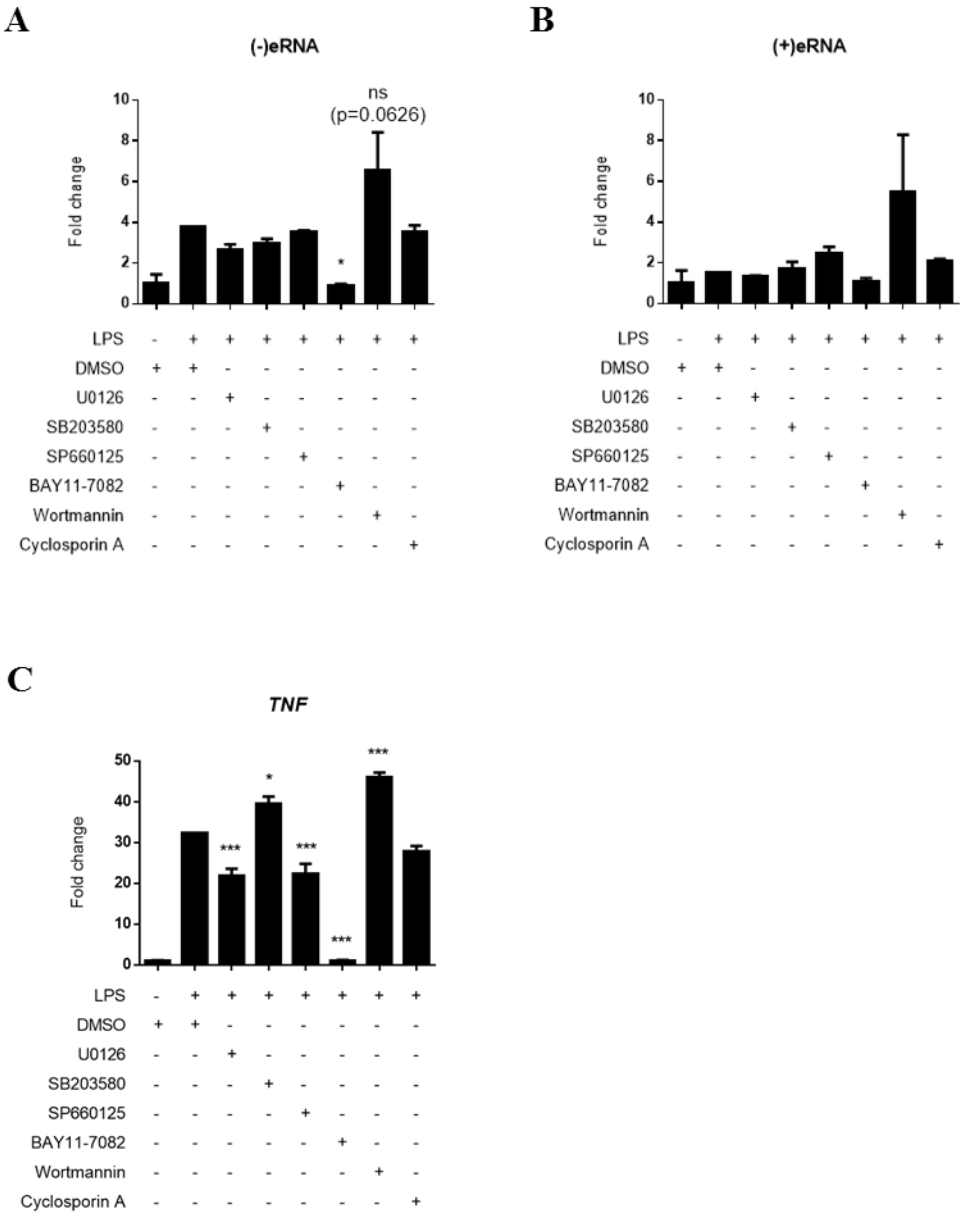

Figure 11. Transcription induction of eRNA on hHS-8 by LPS is mainly mediated by NF-κB pathway. THP-1 cells were pretreated with the specified amount of inhibitors for 30 min at 37℃ incubator; U0126 (ERK1/2 inhibitor) at 10 μM, SB203580 (p38α inhibitor) at 10 μM, SP660125 (JNK inhibitor) at 10 μM, BAY11-7082 (NF-κB inhibitor) at 10 μM, Wortmanin (PI3K inhibitor) at 1 μM, and Cyclosporin A (calcineurin inhibitor) at 1 μM prior to LPS (100 ng/mL) challenge for 1 hr. Cells were harvested and RNAs were extracted and reverse transcribed. The expression levels of (-)eRNA (A), (+)eRNA (B), and TNF (C) were measured by RT-qPCR and fold changes over LPS non-challenged control cells that was incubated with DMSO were obtained. Statistical comparison of mean fold changes among LPS challenged cells were conducted as DMSO pretreated cells being control.

4. dCas9 mediated knock down of each eRNA reduces TNF expression

To validate whether eRNAs transcribed from hHS-8 have functional role in mediating the IFN-γ priming effect on augmenting TNF expression induced by LPS, I tried to knock down these transcripts. RNA interference methods based on RISC mediated target RNA decay, siRNA transfection or transduction of shRNA vector containing viral particle, were initially attempted on the both strand eRNAs, but none of the siRNA sequences or shRNA constructs designed were effective (data not shown). Next I tried a dCas9 mediated knock down method,[27] which transposes catalytically dead Cas9 on the sense strand of target RNA coding regions to give steric hindrance to the transcriptional machinery passing by. For each eRNA, two sgRNA target sequences within coding regions were designed by a web tool (http://crispr.mit.edu/), and the relative position of the designed sequences to the gDNA are presented **(Fig. 12a)**. Transducing these dCas9 mediated knock down vectors into THP-1 cells, but not negative control vectors having non-targeting sgRNAs sequences,[28] reduced TNF expression upon stimuli **(Fig. 12b)**, indicating that both eRNAs have functional role in mediating IFN-γ priming effect on enhancing TNF expression.

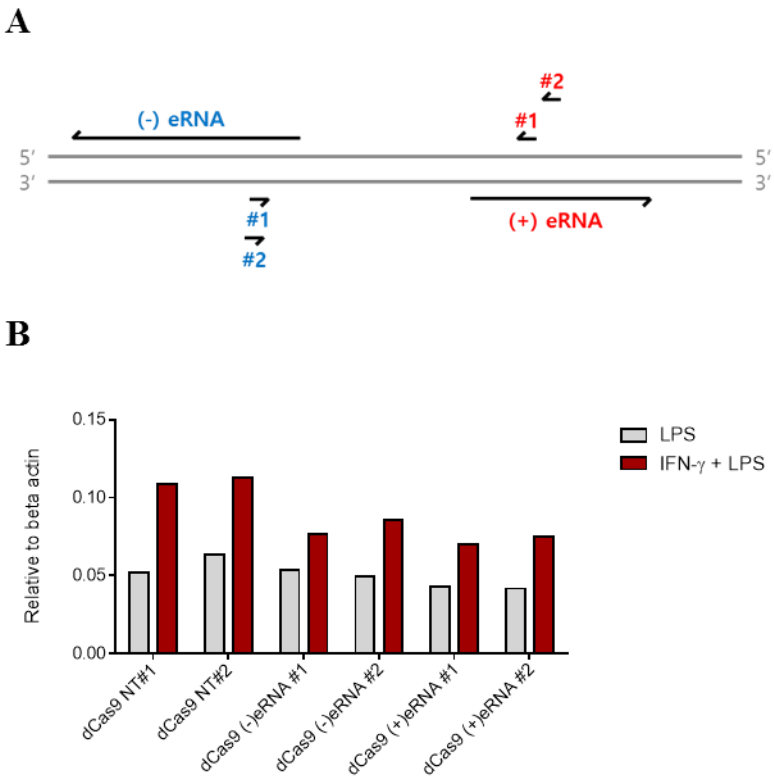

Figure 12. dCas9 mediated knock down of both eRNA reduces TNF expression. (A) Double stranded genomic DNAs within a part of hHS-8 region, chr6:31568330-31569172, are shown in grey lines. Black arrows above each line indicate coding region or designed sgRNA regions of (-)eRNA (with blue characters) or (+)eRNA (with red characters) (B) dCas9 mediated knock down cell lines were primed with or without IFN-γ (100 ng/mL) for 2 hr, followed by LPS (100 ng/mL) challenge. TNF expression levels were measured 1 hr post LPS challenge.

5. Overexpression of eRNAs does not alter TNF expression levels upon stimuli

Next, to test the effect of overexpressing these eRNAs on regulating TNF expression level, I utilized a commonly used lentiviral overexpression vector, pCDH-CMV-MCS-EF1-Puro **(Fig. 13a)**. Overexpressing cells lines were well constructed, as the basal expression levels of intended transcripts were detected in non-stimulated condition **(Fig. 13b)**. However, when TNF expression levels were measured by RT-qPCR post 1 hr LPS exposure, I could observe no statistically significant changes on the TNF induction level from the cell lines overexpressing each eRNA compared to that from the control cell lines overexpressing LacZα **(Fig. 14a)**. The effect of eRNA overexpression was tested on another time point of 6 hr post LPS treatment, when the endogenous eRNA expression level is anticipated to subside, yet the effect was not shown **(Fig. 14c)**. In those time points, IL-6 induction levels of IFN-γ primed cells were also remained unaffected **(Fig. 14b and 14d)**.

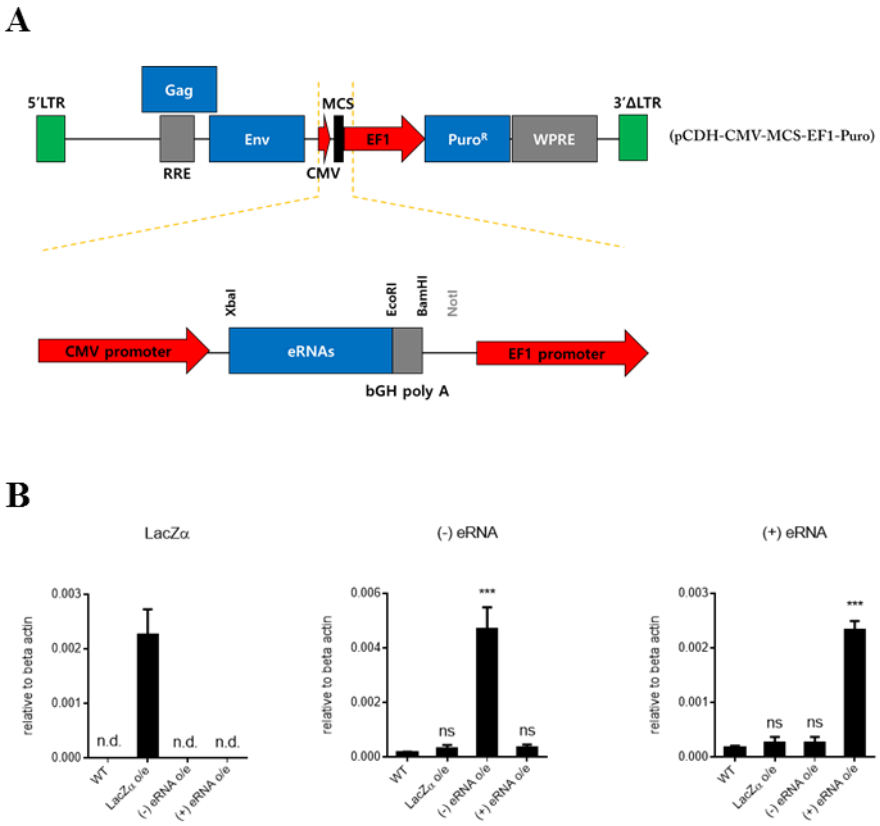

Figure 13. Construction of control (LacZα) or hHS-8 eRNAs overexpression lentiviral vectors and validation of THP-1 cell lines transduced with the vectors. (A) Schematic illustration of a LTR flanked regions in a commercial lentiviral vector (pCDH-CMV-MCS-EF1-Puro from System Biosciences) is presented with the magnification of MCS region to demonstrate restriction enzyme sites used for cloning bGH polyadenylation signal and target RNA sequence. (B) Expression levels of LacZα, (-)eRNA, or (+)eRNA transcripts were measured by RT-qPCR in THP-1 cells transduced with lentiviral overexpression vectors and wild type, in unstimulated condition. o/e : overexpression.

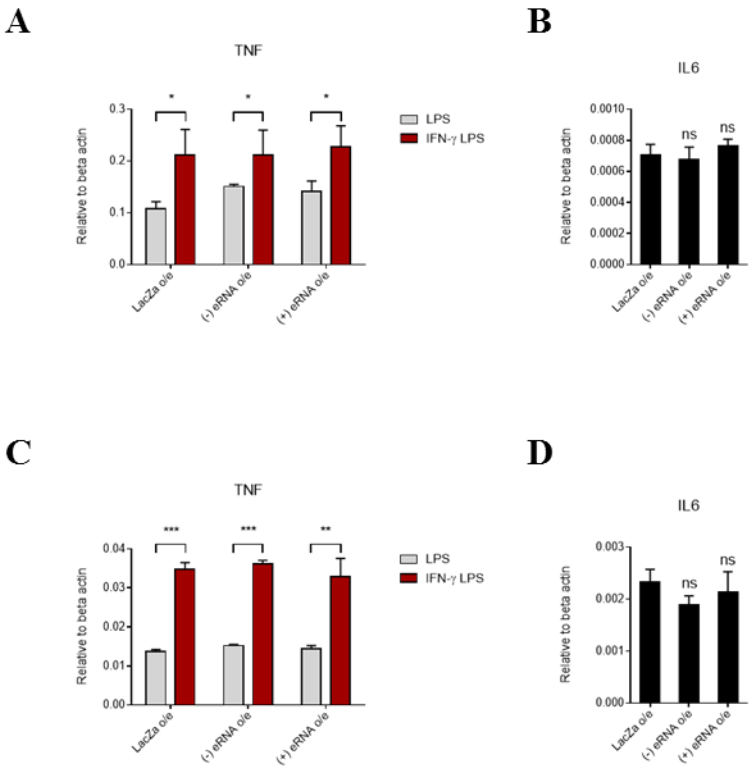

Figure 14. No effect of hHS-8 eRNAs overexpression during THP-1 cells challenged with LPS and/or IFN-γ. hHS-8 eRNAs overexpressing cell lines were primed with or without IFN-γ (100 ng/mL) for 2 hr, followed by LPS (100 ng/mL) challenge. TNF expression levels were measured 1 hr post LPS challenge (A) or 6 hr (C). IL6 expression levels were also measured 1 hr or 6 hr post LPS, (B) and (D) respectively. o/e: overexpression.

IV. DISCUSSION

In this study, I characterized eRNAs transcribed on the enhancer region of *TNF*, hHS-8, in IFN-γ primed monocyte upon LPS challenge. In a previous study, transcriptional activity at this region was observed,[17] but details of the transcript and its role in the enhancer function has not been addressed. When the region was viewed on UCSC genome browser, CAGE peaks from FANTOM5 dataset, which represent comprehensive TSSs observed in diverse human cell types,[33] indicated that bidirectional transcription occurs centering the previously found IRF1 binding sites[17] **(Fig. 6)**. To confirm the observed bidirectional transcriptional activity in THP-1 cells, arbitrary primers targeting gDNA sequences downstream of the TSS peaks on the both sides were designed **(Fig. 6)**, and the transcripts spanning those regions were detected by RT-qPCR **(Fig. 7)**. In the region, balanced amount of bidirectional transcripts was produced in exposure to LPS and which was augmented with IFN-γ priming **(Fig. 7)**, meanwhile the production of TNF also follows this incremental pattern **(Fig. 4a)**. Such bidirectional transcription has been observed in enhancer regions showing active transcriptional activity.[34]

In search of full sequence of the transcripts, two variant of RACE PCR methods[24,25] were used in combination. Inverse primers were designed within the sequences confirmed to be transcribed by RT-qPCR, to conduct a simultaneous RACE PCR in a nested manner,[25] which utilizes circularized cDNA as a template[24] **(Fig. 2 and Fig. 10)**. The circularization of cDNA enables simultaneous detection of sequences at both ends of transcript, and nested PCR endows enhanced sensitivity and specificity of template amplification, which is especially required for amplification of transcripts in low copy number[25] such as the eRNAs in this study. Furthermore, erroneous incorporation of fragmented gDNA into circularization is excluded by use of T4 RNA ligase, which ligate only single stranded nucleotide, and

the presence of both tag sequences at 3' end, which was added on the primers used for cDNA synthesis, and non-template addition of cytosine at 5' end guarantees that the amplicons used for sequencing is indeed originated from reverse transcribed native RNAs.[24]

Sequence analysis found that those transcripts are relatively short, although it belongs to 'long' ncRNA as it contains >200 bp of nucleotides **(Fig. 10)**, were produced without processing, and contain no ORF. RNA fractionation studies further revealed that these eRNAs are only partially polyadenylated **(Fig. 9)** and mainly localize in the nucleus **(Fig. 8)**. These features are consistent with the general features of eRNAs that they largely present in the nuclear and chromatin bound fractions[35] and rarely undergo splicing event.[34] As the recent reports indicate,[19-21] at least a subset of eRNAs have functional role in regulating cognate gene expression. When the functionality of eRNAs on hHS-8 are supposed, based on the characters of the transcripts, it is plausible to speculate that they might function in the nucleus as a RNA transcript *per se*.

For validation of the functional role of an eRNA, it is essential to knock down the transcript. There have been reports utilizing short hairpin RNA (shRNA), small interfering RNA (siRNA), or locked nucleic acid (LNA) that successfully addressed this issue.[36] In this study, I tested several kinds of shRNAs and siRNAs targeting each of eRNA on hHS-8, but all of them failed to influence the level of eRNA transcripts (data not shown). Next, I used dCas9 mediated knock down method, which transpose mutant Cas9, incapable of inducing DNA strand breakage, onto a target gDNA region to interfere with initiation/elongation step of the transcription encompassing the region.[27] To this end, dCas9 expressing vector was made by site directed mutagenesis of a widely used lentiviral Cas9 vector **(Fig. 3)**, and sgRNAs targeting non-template strand of each eRNA were designed and cloned into the vector **(Fig. 12a)**; targeting non-template strand is preferred over template strand for inducing steric hindrance on

RNAPII elongation step.[37] When THP-1 cells were transduced with dCas9-sgRNA complexes targeting each eRNA, the IFN-γ priming effect on augmenting TNF induction by LPS was attenuated, compared with that in control cells expressing dCas9 and non-targeting sgRNAs **(Fig. 12b)**. This indicates that both eRNAs have functional role in mediating IFN-γ priming effect on enhancing TNF expression by LPS.

In view of genomic sequence conservation of the eRNAs, while some parts of (-)eRNA are conserved well across the selected mammalians, as depicted by regions with high PhastCons score on a UCSC track, (+)eRNA contains no conserved element throughout the entire transcribed region **(Fig. 6)**. As there are various circumstances, other than the presence of functionality, that leads to the appearance of genomic sequence conservation,[38] mere presence of a conserved sequence within transcribed region should be carefully interpreted. Albeit, this prominent imbalance in sequence conservation among the eRNAs initially led me to speculate that if these eRNAs had sequence-dependent function as the major mechanism they utilize to augment TNF transcription, (-)eRNA would show dominant functionality over (+)eRNA. However, dCas9 mediated knock down experiment showed nearly equal contribution of each eRNA for augmenting TNF expression **(Fig. 12b)**, implying sequence-independent functions might be the major mechanism they utilize. Still, there remains a possibility that these conserved regions on (-)eRNA transcript conducts additional sequence-dependent function.

Another issue regarding the sequence conservation within hHS-8 lies on the central non-transcribed loci between each eRNA transcribed regions, where highly conserved elements are abundant **(Fig. 6)**. Currently accepted functional mechanism of enhancer transcription requires prerequisite binding of pioneer or lineage-determining transcription factors (LDTF) on the transcription regulatory region of an enhancer.[18] This event promotes preliminary chromatin remodeling of the enhancer

region to open nearby binding sites for the signal-dependent transcription factors (SDTF). When proper stimulation is given, SDTFs bind to the enhancer and recruit coactivator complex to initiate eRNA transcription.[18] Thus the highly conserved elements observed on the central non-transcribed loci might represent multiple binding sites for those pioneer TFs, LDTFs, or SDTFs. Indeed, the binding sites for IRF1 found in a previous study[17] constitute a part of those conserved elements **(Fig. 6)**. Still, most of the central conserved elements within hHS-8 needs to be functionally defined.

Since eRNAs on hHS-8 were transcribed by LPS treatment alone **(Fig. 7)**, in consistent with the previous study,[17] whether eRNA transcripts present in sole LPS challenge augment TNF induction is questionable. dCas9 mediated inhibition of eRNA transcription slightly decreased TNF induction when LPS was challenged alone **(Fig. 12)**. To further address this issue, enhanced knowledge on how hHS-8 function under sole LPS treatment is necessary. To this end, I tried to determine which signaling cascade under LPS:TLR4 pathway is responsible for the transcription of the eRNAs. Using selected inhibitors targeting specific signaling cascade, changes in expression levels of the both eRNAs were assessed and similar patterns were observed in both eRNAs under influence of each inhibitor, with lesser differences found in (+)eRNA than in (-)eRNA **(Fig. 11a and 11b)**. While treating with BAY 11-7082, which inhibits phosphorylation and degradation of IκBα to abrogate nuclear translocation of NF-κB,[39] decreased expression levels of eRNAs comparable to that found in unstimulated cells, inhibition of PI3K with Wortmannin increased eRNA expressions upon LPS treatment **(Fig. 11a and 11b)**. As the activation of the PI3K-Akt pathway in monocytes negatively regulates LPS-induced signaling cascades, including nuclear translocation of NF-κB,[40] it is plausible to conclude that these results from the experiments using two inhibitors coincide in that NF-κB is the major inducer of eRNAs on hHS-8 upon LPS challenge. Meanwhile, NF-κB also plays a

major role in expressing TNF upon LPS challenge **(Fig. 11c)**, in line with the previous report.[10] In this situation, it is difficult to determine the contribution of eRNA on TNF induction with the results from experiments using an NF-κB inhibitor, as the effect of NF-κB binding on the *TNF* promoter region, although it is controversial,[10] cannot be excluded. Thus, further study revealing the NF-κB binding site within hHS-8 region, which would probably locate among the highly conserved central non-transcribed elements, is necessary. The finding would render an experiment selectively inhibiting the NF-κB binding site on hHS-8 possible, and the result of which would demonstrate the contribution of eRNA on TNF transcription during sole LPS challenge.

To further validate the functionality of eRNAs on hHS-8, I overexpressed each eRNAs using a commonly used lentiviral vector with addition of a relatively weak polyadenylation signal **(Fig. 13a)** to ensure the transcript to be generated in size comparable to the native one. Although THP-1 cells transduced with these vectors overexpressing eRNAs sufficiently **(Fig. 13b)**, there were no differences in the induction level of TNF between cells overexpressing each eRNA and control cells that overexpress LacZα **(Fig. 14)**. This finding could be a matter of an unexpected deleterious effect by additional sequences that inevitably originate by use of the vector; sequences from the TSS in CMV promoter to the 5' end of eRNA, and sequences following 3' end of the eRNA to the bGH polyadenylation signal **(Fig. 13a)**. Or it could reflect that the mechanism eRNAs utilize is, by nature, not reproducible with conventional overexpression system; inserting gene cassettes on gDNA raises average concentration of a target transcript across the nucleus but it influences little on the concentration of overall transcripts.

Regarding the latter hypothesis, there is a report presenting a mechanistic model, which reveals that certain eRNAs function as an enhancer of target gene through local enrichment on the target promoter region.[20] In the study, Katie et al. found that IEGs of human neurons are poised to transcription as RNAPII is paused on the promoter

region by NELF. And during the induction of IEGs by proper stimuli, eRNAs facilitate the transition of the paused RNAPII into productive elongation by acting as a decoy for the NELF complex.[20] As the interaction between eRNA and NELF complex requires little or no apparent sequence or structural constraint,[41,42] this mechanism has a potential to be generally applied in genes with similar environment. In a murine macrophage cell line and bone marrow driven macrophage, *TNF* gene was also found to be poised for activation through RNAPII stalling via NELF.[43] Thus, extrapolation of this finding in human monocyte/macrophage lineage cells has a worth to be tested. And if it were found to be true, so is worth evaluating whether the regulatory mechanism of 'eRNA mediated NELF release' demonstrated in IEGs of human neuronal cells works in human monocyte/macrophage lineage or not. Including this, mechanistic models that can reconcile the seemingly contradictory findings presented in this study should be further searched for and tested to elucidate the mechanism how eRNAs on hHS-8 augment TNF induction.

V. Conclusion

In this study, I characterized eRNAs transcribed on hHS-8, enhancer region of the *TNF* promoter, in IFN-γ primed monocyte upon LPS challenge. Balanced amount of bidirectional lncRNAs were produced by LPS, as the NF-κB signaling pathway being the major inducer, and which was augmented with pretreatment of IFN-γ. The eRNAs were transcribed without processing and only a part of them underwent polyadenylation. They contained no ORF and localized mainly in the nucleus. I also found that they mediate enhancer function of augmenting TNF induction in IFN-γ primed monocyte upon LPS challenge, which was demonstrated by dCas9 mediated knock down experiment. These findings indicate that enhancer region of *TNF* gene generates lncRNAs with typical features of the eRNA, and these eRNAs play an important role in regulating TNF transcription in human monocyte.

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ABSTRACT (IN KOREAN)

지질다당류에 유도 발현되는 종양 괴사 인자의 양이 인터페론 감마에 의해 증가되는 과정에서 증폭자 리보핵산이 하는 역할

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조 용 근

종양 괴사 인자는 다양한 역할을 수행하는 사이토카인으로 염증 조직에서는 염증을 활성화 시키는 작용을 하며, 주로 단구 세포 및 대식 세포에서 분비된다. 이 세포들은 지질다당류에 반응하여 종양 괴사 인자를 발현하며, 인터페론 감마의 자극을 받으면 종양 괴사 인자를 포함한 다양한 염증성 사이토카인들의 발현을 증가시킨다. 이 과정에서 지질다당류:TLR4 신호 전달 체계가 종양 괴사 인자를 발현시키는 과정은 잘 연구되어 있는 반면, 인터페론 감마에 의해 발현이 증가되는 메커니즘은 잘 연구되어 있지 않았었다. 최근에 인터페론 감마에 의해 발현된 IRF1 이 종양 괴사 인자의 프로모터 부위 상류 8kb 지점에 위치한 증폭자 부위(hHS-8)에 결합하여 그 주변부의 전사를 일으키고, 또한 종양 괴사 인자의 발현도 증가 시킴이 밝혀졌다. 하지만 증폭자

부위에서 유래한 리보핵산의 특성과, 그것이 지질다당류에 의한 종양 괴사 인자 발현이 인터페론 감마에 의해 증가하는 데 어떠한 역할을 하는지는 아직 알려진 바가 없다. 나는 본 연구에서 단구 세포가 인터페론 감마에 감작된 후 지질다당류에 노출되었을 때 만들어내는 hHS-8 유래 증폭자 리보핵산의 특성을 밝혀냈다. 단구세포가 지질다당류에 자극 받았을 때, NF-κB 신호 전달 경로가 주요한 전사 인자로 작용하여, 비슷한 양의 긴 비암호화 리보핵산이 hHS-8 부위에서 양방향으로 전사 되었고 그 양은 인터페론 감마를 전처리 하였을 때 증가되었다. 전사된 증폭자 리보핵산은 편집과정을 거치지 않았으며 폴리아데닐화도 일부에서만 일어났다. 전사된 서열 내부에는 오픈 리딩 프레임이 존재하지 않았으며 증폭자 리보핵산은 전사된 후 주로 핵 내부에만 위치하였다. 또한, 나는 증폭자 리보핵산이 인터페론 감마로 인해 지질다당류 반응성 종양 괴사 인자 생산이 증가하는데 기여함을 dCas9 을 이용한 발현 조절 실험을 통해 발견했다. 이러한 결과들을 토대로 볼 때, 종양 괴사 인자의 증폭자 부위에서 생성되는 긴 비암호화 리보핵산은 전형적인 증폭자 리보핵산의 특징을 띠며, 이것은 인간 단구세포에서의 종양 괴사 인자 전사 조절 과정에 중요한 역할을 하고 있다.

핵심되는 말: 증폭자 리보핵산, hHS-8, 종양 괴사 인자, 인터페론 감마, 지질다당류, 단구 세포

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