THE ROLES OF LONG NONCODING RNAs IN INFLAMMATION AND IMMUNE RESPONSES

By

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Abstract

Inflammation is an immune response that protects the human body from infection, illness, or injury. The inflammatory response causes the activation of immune cells, such as leukocyte subsets (i.e., monocytes, macrophages, dendritic cells, neutrophils, eosinophils and others), T cells, and B cells, and induces production of inflammatory mediators such as cytokines, chemokines, and antibodies to fight against injury or infection. Uncontrolled and continued inflammation drives the development of many human diseases, including metabolic diseases, obesity, diabetes, autoimmune disorders, neurological disorder and cancer. Indeed, the signaling process associated with inflammation and immune response is very complex. Our understanding of inflammation and immune response are largely limited to genomic markers and protein-based factors. Even with the huge amount research and therapeutic drugs, many inflammatory diseases still cannot be treated. Therefore, understanding the detailed signaling mechanism associated inflammation and immune response is critical for developing effective therapies. Emerging evidence suggest that noncoding RNAs (ncRNA) play critical roles in inflammation and immune response. As a part of my thesis work, the roles of long noncoding RNAs (lncRNAs) in inflammation and immune response have been investigated.

Chapter 1 discusses the functions of known lncRNAs in the regulation of inflammation and immune response. Inflammation is the intrinsic immune response of the body towards invading pathogens. However, uncontrolled and continued inflammation drives the development of many human diseases from autoimmune disorders to cancer. The signaling process associated with inflammation and immune response is a part of immune system which is very complex. Indeed, human immune system constitutes of various immune cells such as neutrophils, eosinophils, monocytes, macrophage, dendritic cells (DCs), mast cells, natural killer (NK) cells, B cells, and T cells, which spread throughout the body and fight against foreign invaders. Emerging evidences suggest that noncoding RNAs (ncRNA) regulate the differentiation, development and function of those immune cells and control the immune response. For instances, lncRNAs (long

noncoding RNAs) such as LincRNA-Cox2, THRIL (TNFα and hnRNPL related immunoregulatory lncRNA), PACER (p50-associated COX2 extragenic RNA), and NEAT1 (Nuclear enriched abundant transcript 1) are involved in regulation of inflammatory response of myeloid cells such as macrophage, dendritic cells, and neutrophils. LncRNAs noncoding transcript in CD4+ T cells (NTT), growth-arrest-specific transcript 5 (Gas5), and noncoding repressor of NFAT (NRON) are associated with T cells subset differentiation and function. LncRNA FAS-AS1 plays a crucial role in the B cells development, differentiation, and function. However, understanding the roles of lncRNAs in the regulation of immune response is still limited. In the research, it has been aimed to discover novel lncRNA associated immune response and inflammation and investigate their mechanism of action. In the chapter, functions of various known lncRNAs associated with inflammation and immune response have been summarized.

Chapter 2 presents the studies on the functions of a well-known lncRNA HOTAIR in immune response and inflammation. LncRNAs are emerging as major regulators of a variety of cell signaling processes. Many lncRNAs are expressed in immune cells and appear to play critical roles in the regulation of immune response. My study demonstrates that lncRNA HOTAIR expression is induced in immune cells (macrophages) upon treatment with lipopolysaccharide (LPS). Knockdown of HOTAIR reduces NF-κB-mediated inflammatory gene and cytokine expression in macrophages. Inhibition of NF-κB resulted in down-regulation of LPS-induced expression of HOTAIR as well as IL-6 and iNOS expression. It has been further demonstrated that HOTAIR regulates activation of NF-κB and its target genes (IL-6 and iNOS) expression via facilitating the degradation of IκBα. HOTAIR knockdown reduces the expression of NF-κB target gene expression via inhibiting the recruitment of NF-κB and associated

cofactors at the target gene promoters. Taken together, my finding suggests that HOTAIR is a critical player in NF-κB activation in macrophages suggesting its potential functions in inflammatory and immune response.

Chapter 3 presents the studies on the role of lncRNA HOTAIR in the regulation of glucose metabolism in macrophage during inflammatory response. Inflammatory response plays a central role in immune response and the inflammatory response is closely linked with glucose metabolism. My recent study demonstrates that lncRNA HOTAIR plays key roles during inflammation. HOTAIR is upregulated upon inflammation in macrophage and regulates cytokines expression via regulation of NF-κB activation. Here it has been further investigated if HOTAIR plays any role in regulation of glucose metabolism in immune cells upon inflammation. HOTAIR in macrophages has been knocked down and measured the glucose uptake potential of macrophages in the absence and presence of LPS (lipopolysaccharide). Interestingly, my study demonstrated that LPS induces the expression of glucose transporter isoform 1 (Glut1) which controls the glucose uptake into macrophages and the lncRNA HOTAIR is required for LPS-induced Glut1 expression. My study demonstrated that HOTAIR induces NF-κB activation which in turn increases Glut1 expression in response to LPS. Importantly, it has been found that HOTAIR regulates glucose metabolism in macrophages during LPS-induced inflammation and knockdown of HOTAIR decreases LPS-induced increased glucose uptake. Overall, my study demonstrated that HOTAIR induces Glut1 expression and hence glucose uptake by activating NF-κB and regulates metabolic programming in immune cells potentially to meet the energy needs at the priming of immune response to pathogenic infection or other stressors.

Chapter 4 presents the studies on the discovery of novel lncRNAs associated with immune response and inflammation. As mentioned earlier that the ncRNAs, especially lncRNAs, are emerging as key players in a variety of cell signaling processes, in health and disease. A large number of lncRNAs have been discovered though most of the studies are linked to cancer. Information on lncRNAs linked to immune response and inflammation, is limited. My recent study has demonstrated that lncRNA HOTAIR plays critical roles in regulation of inflammation and immune response in macrophages. Here, to identify lncRNAs linked to inflammation in an unbiased manner, the whole transcriptome RNAseq analysis in primary BMDM (bone marrow derived macrophages) cells treated with LPS has been performed. A large number of lncRNAs that are potential regulators of inflammatory response have been identified. In particular, a novel lncRNA, termed as **LinfRNA1** (**L**ong noncoding **inf**lammation associated **RNA 1**) whose expression is significantly elevated upon treatment with LPS in macrophage, has been identified. My study demonstrated that LinfRNA1 regulates NF-κB activation and inflammatory response in macrophage. Knockdown of LinfRNA1 (using siRNA specific to LinfRNA1) reduces the level of LPS-induced degradation of IκBα and suppresses the level of LPS-induced accumulation of phospho-p56 (NF-κB), suggesting key roles of LinfRNA1 in NF-κB activation. Knockdown of LinfRNA1 also reduced the LPS-induced expression of NF-κB-regulated inflammatory gene and cytokine expression. Furthermore, inhibition of NF-κB resulted in down-regulation of LPS-induced expression of LinfRNA1 as well as IL-6 and iNOS expression. Taken together, my observation demonstrates that LinfRNA1 is a critical and novel regulator of NF-κB activation, inflammation, and immune response.

Overall, my research has discovered novel functions of lncRNAs in immune response and inflammation. In particular, my study demonstrated that HOTAIR, which is well-known as

repressor lncRNA, plays critical roles in NF-κB activation and inflammation. My study demonstrated that HOTAIR plays critical roles of regulation of glucose transporter and metabolism in macrophage cells especially under inflammatory condition. Finally, my study has discovered novel lncRNAs, called LinfRNA1 associated with inflammation and demonstrates that LinfRNA1 plays critical roles in regulation of NF-κB activation and cytokine expression. My research revealed novel cell signaling pathways involving lncRNA associated inflammation and immune response, and hence may provide novel platform for developing drugs for the treatment of immune and metabolic disorders.

Table of Contents

	Acknowledgements	3
	Abstract	4-8
	List of Illustrations	12
	List of Tables	13
	List of Abbreviations	14
	CHAPTER 1	
	Long noncoding RNAs (lncRNAs) and their roles in inflammation and immune response	16-36
1.1	Introduction	16
1.2	Inflammation and immune response signaling	17
1.3	Noncoding RNAs (ncRNAs), their classification, and functions	20
1.4	LncRNAs in Immune response	22
1.4.1	Roles of lncRNAs in innate immune response	23
1.4.1.1	LincRNA-Cox2.	26
1.4.1.2	THRIL (TNF-α and hnRNPL related immunoregulatory lncRNA)	26
1.4.1.3	PACER (p50-associated COX2 extragenic RNA)	26
1.4.1.4	HOTAIRM1 (HOXA transcript, antisense RNA myeloid-specific	27
1.4.1.5	Morrbid (myeloid RNA regulator of Bim-induced death)	27
1.4.1.6	Lnc-DC	28
1.4.1.7	Lethe	28
1.4.1.8	Nuclear enriched abundant transcript 1 (NEAT1)	28
1.4.1.9	LincRNA-EPS (erythroid pro-survival)	29
1.4.1.10	Lnc-13	29
1.4.2	Role of LncRNAs in adaptive immune responses	29
1.4.2.1	Role of LncRNAs in activation, development, and differentiation of T-cell	29
1.4.2.1.1	NRON (Noncoding repressor of NFAT)	30
1.4.2.1.2	LincRNA-MAF-4	30
1.4.2.1.3	Nettoie Salmonella pas Theilers's (NeST)	30
1.4.2.1.4	LincR-Ccr2-5' AS	31
1.4.2.1.5	RMRP	31
1.4.2.2	LncRNAs in B cells activation	32
1.5	Conclusions	32
1.6	Acknowledgements	33
1.7	References	33-36

CHAPTER 2

	LncRNA HOTAIR regulates lipopolysaccharide-induced cytokine expression and inflammatory response in macrophages	37-78
2.1	Introduction	37
2.2	Materials and methods	38
2.2.1	Cell culture and treatment with lipopolysaccharides (LPS)	38
2.2.2	RNA extraction, cDNA synthesis and real time PCR	39
2.2.3	Western blot analyses	39
2.2.4	Chromatin Immunoprecipitation (ChIP) assay	40
2.2.5	Antisense-mediated knockdown of HOTAIR	41
2.2.6	SiRNA-mediated knockdown of HOTAIR	41
2.2.7	NF-κB inhibition assay	42
2.2.8	Proteasomal inhibition assay	42
2.2.9	Enzyme linked immunosorbent assay (ELISA)	43
2.2.10	Immunofluorescence microscopy analysis	43
2.2.11	Isolation and culture of primary macrophage (BMDMs)	44
2.2.12	Statistical Analysis	44
2.3	Results	45
2.3.1	LncRNA HOTAIR expression is induced by Lipopolysaccharide in macrophages	45
2.3.2	Inhibition of NF-κB downregulates LPS-induced HOTAIR expression	48
2.3.3	HOTAIR knockdown abolishes LPS-induced IL-6 and iNOS expression	50
2.3.4	HOTAIR knockdown results in inactivation of NF-κB	53
2.3.5	HOTAIR promotes IκBα degradation and nuclear translocation of NF-κB	55
2.3.6	HOTAIR knockdown abolishes the recruitment of NF-κB at IL-6 and iNOS promoters	58
2.3.7	HOTAIR expression is induced by LPS in primary macrophages and is required for LPS-induced cytokine expression	61
2.4	Discussion	64
2.5	Acknowledgements	69
2.6	References	69-78

CHAPTER 3

	LncRNA HOTAIR regulates glucose transporters, glucose uptake and metabolism in macrophages during inflammation	79-105
3.1	Introduction	79
3.2	Materials and methods	81
3.2.1	Mouse macrophage cell culture	81
3.2.2	Cell treatment with Lipopolysaccharide (LPS)	82
3.2.3	RNA extraction and cDNA synthesis	82
3.2.4	Real time PCR	82
3.2.5	Western blot analyses	83
3.2.6	Chromatin Immunoprecipitation (ChIP) assay	83

3.2.7	SiRNA-mediated knockdown of HOTAIR	84
3.2.8	NF-κB inhibition assay	85
3.2.9	Glucose Uptake Assay	85
3.2.10	Statistical Analysis	85
3.3	Results	86
3.3.1	LPS-induces GLUT1 expression in macrophage	86
3.3.2	Glut1 expression is regulated by NF-κB during LPS stimulation	89
3.3.3	HOTAIR regulates LPS-induced Glut1 expression	91
3.3.4	HOTAIR regulates LPS induced NF-κB recruitment in the Glut1 promoter	93
3.3.5	HOTAIR regulates glucose uptake under LPS-simulation	96
3.4	Discussion	97
3.5	Acknowledgements	101
3.6	References	102-105

CHAPTER 4

	Discovery of lncRNA, LinfRNA1, a novel regulator of NF-κB activation and Inflammation	106-130
4.1	Introduction	106
4.2	Materials and methods	107
4.2.1	Isolation of BMDMs, treatment with LPS, and isolation of RNA	107
4.2.2	RNA isolation from BMDMs, RNA-Seq experiments and data analysis	108
4.2.3	Cell culture and treatment with Lipopolysaccharide (LPS)	109
4.2.4	RNA extraction and cDNA synthesis	110
4.2.5	Real time PCR	110
4.2.6	Western blot analyses	110
4.2.7	SiRNA-mediated knockdown of LinfRNA1	111
4.2.8	NF-κB inhibition assay	112
4.2.9	Enzyme linked immunosorbent assay (ELISA)	112
4.2.10	Statistical Analysis	112
4.3	Results	113
4.3.1	LinfRNA1 is a novel lncRNA associated with LPS-induced inflammation in macrophage.	113
4.3.2	LinfRNA1 expression is induced by LPS in primary macrophage (BMDMs) as well as in RAW264.7 macrophage cells	114
4.3.3	LinfRNA1 is regulated by NF-κB	118
4.3.4	LinfRNA1 knockdown down-regulates LPS-induced expression of cytokine and pro-inflammatory genes.	120
4.3.5	LinfRNA1 promotes IκBα degradation thereby activates NF-κB	122
4.4	Discussion	123
4.5	Acknowledgements	126
4.6	References	127-130

List of Illustrations

Figure 1.1	Inflammatory signaling pathways	19
Figure 1.2	Classification of the most widely found lncRNAs according to their genomic location	21
Figure 1.3.	Immune response and lncRNAs	23
Figure 2.1	LPS induces HOTAIR expression in macrophages.	47
Figure 2.2	Inhibition of NF-κB downregulates LPS-induced HOTAIR expression in macrophages	49
Figure 2.3	Knockdown of HOTAIR reduces LPS-induced IL-6 and iNOS expressions in macrophages	51-52
Figure 2.4	HOTAIR promotes IκBα degradation thereby activates NF-κB	54
Figure 2.5	HOTAIR promotes IκBα degradation and nuclear translocation of NF-κB	57
Figure 2.6	Knockdown of HOTAIR reduces the recruitment of transcription factors and coactivators at NF-κB binding sites on IL-6 and iNOS promoters	60
Figure 2.7	HOTAIR expression is induced by LPS in primary macrophages (Bone marrow derived macrophages, BMDM)	62-63
Figure 2.8	Proposed model for LPS-mediated HOTAIR induction in immune cells	68
Figure 3.1	LPS induces Glut1 expression in macrophages	88
Figure 3.2	Inhibition of NF-κB downregulates LPS-induced Glut1 expression in macrophages	90
Figure 3.3	Knockdown of HOTAIR reduces LPS-induced Glut1 expression in macrophages	92
Figure 3.4	Knockdown of HOTAIR reduces the LPS-dependent recruitment of NF-κB on Glut1 promoter	95
Figure 3.5	Knockdown of HOTAIR reduces the uptake of glucose in macrophages	97
Figure 3.6	Proposed model for HOTAIR mediated regulation of glucose metabolism during inflammation	101
Figure 4.1	RNA-Seq data showing the heat map for highly upregulated and downregulated genes and noncoding RNAs	114
Figure 4.2	LPS induces LinfRNA1 expression in BMDMs	116
Figure 4.3	LPS induces LinfRNA1 expression in macrophages	117
Figure 4.4	Inhibition of NF-κB downregulates LPS-induced LinfRNA1 expression in macrophages	119
Figure 4.5	Knockdown of LinfRNA1 reduces LPS-induced IL-6 and iNOS expressions in macrophages	121
Figure 4.6	Knockdown of LinfRNA1 reduces LPS-induced NF-κB signaling pathway	123
Figure 4.7	A model for LPS-stimulated LinfRNA1 induction in macrophage cells	126

List of Tables

Table 1.1	LncRNAs implicated in inflammation and immune response	24-25
Table 2.1	Sequences of primers and antisense oligonucleotides	45
Table 3.1	Sequences of primers	86
Table 4.1	Sequences of primers	113

List of Abbreviations

LncRNA	Long noncoding RNA
DC	Dendritic cell
NK cells	Natural killer cells
TNFα	Tumor necrosis factor α
IL-1β,6,8	Interlukin-1β,6,8
PPRs	Pattern recognition receptors
TLRs	Toll like receptors
NOD	Nucleotide-binding and oligomerization domain
NLRs	NOD-like receptors
RIG	retinoic acid-inducible gene
RLRs	RIG I-like receptors
PAMPs	Pathogen associated molecular patterns
LPS	Lipopolysaccharide
HMGB1	High mobility group box-1
DAMPs	Danger associated molecular patterns
iNOS	Inducible nitric oxide synthase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
AP1	Activator proteins
IRFs	Interferon Regulatory Factors
IFNs	Interferons
COX2	Cyclooxygenase II
ENCODE	Encyclopedia of DNA Elements
PRC2	Polycomb repressive complex 2
LSD1	Lysine specific demethylase 1
ChIP	Chromatin immunoprecipitation
DMEM	Dulbecco's modified eagle's medium
IMDM	Iscove's Modified Dulbecco's Medium
FBS	Fetal bovine serum
HOTAIR	HOX antisense intergenic RNA
LinfRNAs	Long noncoding inflammation associated RNAs
PBS	Phosphate buffer salime
RNAPII	RNA polymerase II
RT-PCR	Reverse transcriptase polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
EZH2	Enhancer of Zeste homolog 2
NTT cells	noncoding transcript in CD4+ T cells
Gas5	Growth-arrest-specific transcript 5
NFAT	Nuclear factor of activated T cells
NRON	Noncoding repressor of NFAT
PACER	p50-associated COX2 extragenic RNA
THRIL	TNF-α and hnRNPL related immunoregulatory lncRNA
NeST	Nettoie Salmonella pas Theilers's
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
BMDMs	Bone marrow derived macrophages
SPF	Specific pathogen free
IACUC	Institutional Animal Care and Use Committee
ELISA	Enzyme linked immunosorbent assay

siRNA	Small interfering RNA
NEAT1	Nuclear enriched abundant transcript 1

Chapter 1

Long noncoding RNAs (lncRNAs) and their roles in inflammation and immune response

1.1 Introduction

Inflammation is the intrinsic immune response of the body towards invading pathogens.[1] The inflammatory response causes activation of immune cells such as leukocytes (aka white blood cells), T cells, and B cells, and induces production of inflammatory mediators such as cytokines and chemokines to fight against injury or infection.[2] However, uncontrolled and continued inflammation drive the development of many human diseases, including metabolic diseases, obesity, diabetes, autoimmune disorders, neurological disorder, and cancer.[3-4] The signaling process associated with inflammation and immune response is very complex. Our understanding of inflammation and immune response are largely limited to genomic markers and proteins based factors that are involved in transcription, phosphorylation, ubiquitination, and protein–protein interactions.[2] Even with the huge amount research and options of many available therapeutics, many inflammatory diseases still cannot be treated effectively.[5] Therefore, understanding the detailed signaling mechanism associated inflammation and immune response is critical for developing effective therapies. Emerging evidences suggest that noncoding RNAs (ncRNAs), play critical roles in various cellular and physiological process including in gene expression, cell differentiation, development, and their dysregulation contributes to critical human disease.[6] NcRNAs are recently discovered highly heterogeneous group of transcripts that are coded by the genome, transcribed, but mostly remain untranslated.[7] Previously, ncRNAs were considered to be a consequence of transcriptional noise. Recent studies, however, suggest that ncRNAs have distinct cellular functions and are involved in different biological processes.[8]

NcRNAs are broadly classified based on their size, small ncRNA (< 50 nt long, such as microRNA), medium ncRNA (50- 200 nt, such as tRNA), and long noncoding RNAs (lncRNAs, > 200 nt long).[9] Based on genome-wide whole transcriptome sequencing analysis, a large number of ncRNAs, especially lncRNAs have been identified, though their detail structure and functions mostly remain elusive.[7, 9] LncRNAs, in particular, are highly abundant and widely expressed in various tissues. Many of them exhibit tissue specific expression, often identified in body fluids including in blood and urine and hence possess potential for novel biomarkers.[10] LncRNAs, being large in size, may develop large secondary structures which provides platform for interaction with various proteins and signaling molecules, which may influence enzymatic activation, assembly of multi-protein complexes, and hence regulate cell signaling process.[10] To achieve a better understanding of the regulatory pathways in inflammation and immune response, in my study, I would like to explore the potential functions of lncRNAs associated with inflammation and immune response. In this chapter, the functions of various known lncRNAs associated with inflammation and immune response have been summarized.

1.2 Inflammation and immune response signaling

Inflammation is an immune response that protects human body from infection, illness, or injury. The inflammatory response causes activation of immune cells such as leukocyte subsets (i.e., monocytes, macrophages, dendritic cells, neutrophils, eosinophils and others), T cells, B cells, and induces production of inflammatory mediators such as cytokines, chemokines, and antibodies to fight against injury or infection.[2] Inflammation can be acute that is induced for short period of time or chronic that is induced for long period of time. Acute inflammation is usually advantageous to health because of its purpose to remove the damaging agents and to restore tissue structure and function.[1] On the other hand, chronic inflammation occurs inside

human body without any noticeable symptoms. This type of inflammation can drive illnesses like diabetes, heart disease, fatty liver disease or even cancer. Importantly, chronic inflammation can occur when people are obese or under stressful situation.[1-2] Moreover, inflammation can also be provoked by an unwanted immune reaction towards body's own proteins and causes autoimmune disease. The inflammation can be detected by measuring inflammatory markers such as C-reactive protein, TNFα (Tumor necrosis factor α), or IL-6 (Interleukin-6) from blood.[8, 11]

Inflammation may be triggered by a variety of factors and may follow different signaling pathways.[2] Inflammatory responses may commence with activation of immune cells via priming their Pattern recognition receptors (PPRs) such as Toll like receptors (TLRs), the nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene (RIG) I-like receptors (RLRs), by pathogen associated molecular patterns (PAMPs) such as peptidoglycans, lipopolysaccharides (LPS), RNA, and DNA derived from pathogens, danger associated molecular patterns (DAMPs) such as high mobility group box-1 (HMGB1) derived from necrotic cells.[2, 11-13] A model showing the different receptors and associated signaling pathway is shown in Figure 1.1.

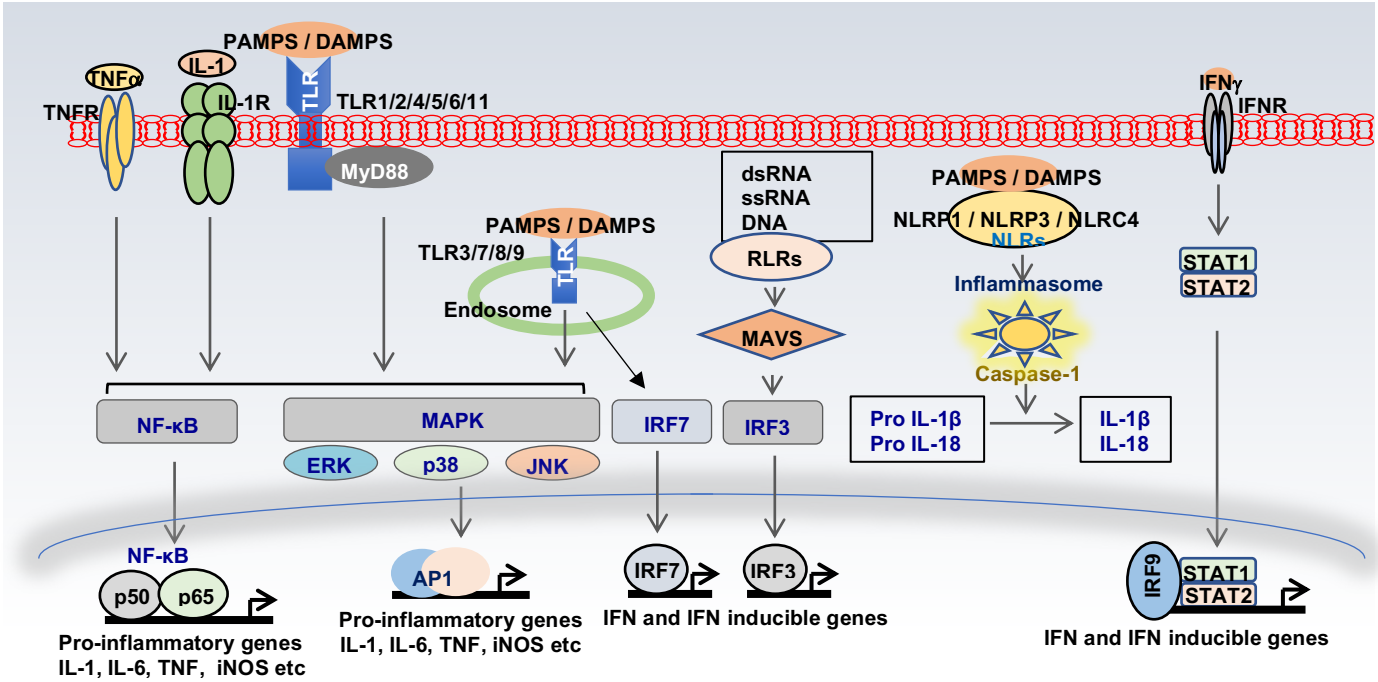

Figure 1.1. Inflammatory signaling pathways. Inflammatory immune response is associated with multiple pattern recognition receptors (PRRs) such as Toll Like Receptor (TLRs), NOD Like Receptors (NLRs), RIG Like Receptors (RLRs) which encounter pathogen associated molecular patterns (PAMPs) such such as peptidoglycans, lipopolysaccharides, RNA and DNA derived from pathogens, and danger associated molecular patterns (MAMPs) such as high mobility group box-1 (HMGB1) derived from necrotic cells. Once activated by their respective ligands (such as PAMPs, DAMPs, cytokines), PPRs recruit their specific repertoire of adaptor and effector proteins leading to activation and recruitment of transcription factors such as Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Activator protein 1 (AP1), Interferon Regulatory Factors (IRFs) to pro-inflammatory genes promoter, and induce their transcription. The resulting pro-inflammatory proteins such as cytokines, chemokines, interferons (IFNs) and other related proteins (i.e., inducible nitric oxide synthase (iNOS) and cyclooxygenase II (COX2)) mount inflammatory responses leading to inflammation.[2,12-14,16,21]

The immune cells can be also activated by IL-1β receptor, TNFα receptor or IFNγ receptor that encounter self-produced IL-1β, TNFα, or IFNγ respectively (Figure 1.1). All of these receptors and their respective ligands are involved in inflammatory signaling pathways that culminate in the activation of transcription factors such as NF-κB, activator protein 1 (AP1), Interferon regulatory factors (IRFs), STAT1/2 (Figure 1.1).[12-14] In turn, these activated transcription factors stimulate the expression of proinflammatory cytokines, chemokines,

interferons and other pro-inflammatory mediators, and thus propagating cellular inflammation.[2, 6, 13] Furthermore, several members of NLR family such as NLRP1, NLRP3 and NLRmC4 form a multi-protein complex called inflammasome with caspase-1 and ASC (apoptosis associated speck-like protein containing a CARD), and produce secretory activate form of IL-1β and IL-18 which also take part in inflammatory immune responses.[13, 15-16] Recent studies, including studies from our laboratory, demonstrate that noncoding RNAs are integral components of inflammation and immune signaling.[17]

1.3 Noncoding RNAs (ncRNAs), their classification, and functions

It was very big surprise when the first draft of the human genome revealed that exonic regions of protein-coding genes represents only < 2% of the whole genome.[9] Although some of the remaining DNA involves in the maintenance of DNA structure and regulation of mRNA expression (i.e., transcription binding sites, promoter and enhancer regions), subsequent studies revealed that a significant proportion is transcribed into noncoding RNAs (ncRNAs).[7, 9] According to the Encyclopedia of DNA Elements (ENCODE) project, about 80% of human genomic DNA is functional and the majority is transcribed into ncRNAs.[7, 9, 18] Indeed, ncRNAs are an emerging class of transcripts that are encoded by the genome but mostly are not translated into proteins. Although not translated, ncRNAs play crucial roles in regulating a variety of cellular and physiologic functions including chromatin dynamics, gene expression, cellular growth, differentiation, and development.[9, 11]

Over last decade, a large number ncRNA have been discovered and they may have many diverse functions. Effective classification of different ncRNAs into distinct groups is a major challenge.[19] However, ncRNAs are broadly classified based on their size, origin or mode of action. The most well-known classes of ncRNAs include tRNAs and rRNAs, which are abundant

and have well-defined structural and regulatory roles in translation.[20] Micro RNA (miRNAs), which belong to the family of small ncRNA are also well studied and play major roles in gene silencing.[9] LncRNA (> 200 nt long ncRNA), in particular, are very interesting and important because of their large size, diverse origins, structures, tissue specific expression patterns, and diverse modes of action. They are involved in regulating cell signaling pathways through their interaction with signaling proteins.[12, 20] Alternatively, they can enhance or inhibit gene transcription by interacting with transcription factors, acting as scaffolds for the recruitment of epigenetic regulators or other protein complexes, and as a 'sponge' to sequester miRNAs.[7] Based on the orgin, lncRNAs may be classified as intronic, intergenic, antisense, enhancer associated and others. For example, intronic lncRNAs (ilncRNAs) originate from the introns of protein-coding genes, intergenic lncRNAs (lincRNA) originate from the region between two protein coding genes, enhancer lncRNAs (elncRNA) originate from the promoter enhancer regions, and antisense transcripts originate from the antisense strands of the DNA (Figure 1.2).[7-8, 12] LncRNAs are abundantly expressed and widely associated with a variety of cancers, cardiovascular diseases, neurological disorder and others. Aberrant expression and mutations of lncRNAs are closely linked to tumorigenesis, and metastasis.[7] Although the functions of lncRNAs in tumorigenesis are most widely investigated and well documented, their involvement in inflammation, immune response

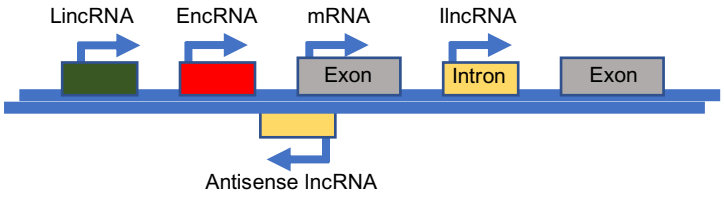

Figure 1.2. Classification of the most widely found lncRNAs according to their genomic location. LincRNAs (long intergenic ncRNAs) and encRNAs (enhancer ncRNAs) are stand-alone transcription units situated near protein coding gene. Intronic lncRNAs (IlncRNAs) are found within the introns of protein coding genes, while antisense lncRNAs are transcribed from the opposite strand from the exonic regions of protein coding genes.

and other metabolic diseases mostly remains elusive.[12] Here, the functions of various lncRNAs that are implicated in inflammation and immune response have been reviewed (Figure 1.3).

1.4 LncRNAs in Immune response

The immune system involves many types of immune cells such as neutrophils, eosinophils, monocytes, macrophage, dendritic cells (DCs), mast cells, NK cells, B cells, T cells, etc. and is spread throughout the body.[12] When immune system encounters a pathogen, such as a bacterium, virus, or parasite, it mounts a so-called innate immune response.[12] Human immune system elicits innate immune response as a first line of defense when senses invaders. This response is more general and non-specific. If the pathogen manages to dodge the innate immune system, adaptive or acquired immunity is activated.[6, 12, 21] Adaptive (also called acquired) immunity protects us from pathogens as we go through the life. As we are exposed to diseases or get vaccinated, we build up a library of antibodies to different pathogens and acquired the immunity called adaptive immunity.[6] Indeed, the innate and adaptive immune systems consist of diverse immune cells that work together to protect the host from pathogens. The innate immune system mainly relies on a surveillance system of neutrophils, monocytes, macrophages, and dendritic cells (DCs), which recognize and restrict pathogens and instruct the adaptive immune system. Adaptive immune cells (e.g., T and B cells) provide pathogen-specific immunity by detecting specific antigens to eliminate pathogens and pathogen-infected cells.[2, 12] The timing of these events is carefully coordinated and involves the differentiation and activation of immune cells in response to distinct external stimuli (microbial products, cytokines, or endogenous danger signals).[1, 22] Each type of immune cell expresses a specific repertoire of receptors (e.g., pattern-recognition receptors (PRRs), antigen receptors, and cytokine receptors) that detect these

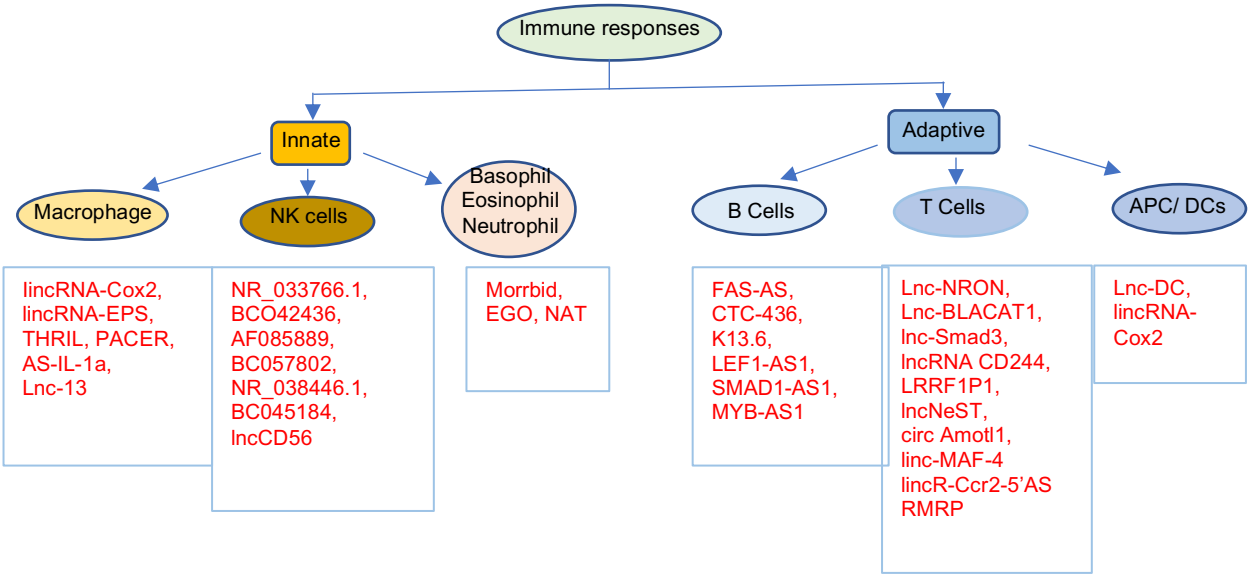

Figure 1.3. Immune response and lncRNAs. Immune system first mounts innate response and subsequently makes adaptive immune response. They involve various myeloid and lymphoid cells. Their development and function are associated with a huge range of lncRNAs.[6,11-13]

stimuli and activate downstream signaling pathways, chromatin modifying complexes, and transcription factors. These events lead to rapid and dynamic changes in gene expression that are hallmarks of activated immune cells.[1-2, 6, 21] Interestingly, various lncRNAs and miRNAs have been discovered to be associated with innate as well as adaptive immune system and they are summarized in Figure 1.3 and Table-1. Functions of selected lncRNAs linked to innate and adaptive immune response are discussed below.

1.4.1 Roles of lncRNAs in innate immune response

The first evidence of a potential role for lncRNAs in the inflammatory immune response was a report by Guttman *et al*. who used the intergenic deposition of epigenetic marks to identify lincRNAs induced in macrophages stimulated with lipopolysaccharide (LPS).[23] Now it is well established that lncRNAs regulate the development, differentiation and function of different immune cell lineages.[12, 20] Development of specific immune cell populations from precursor

hematopoietic stem cell is directed by the stepwise and combinatorial actions of lineage-determining transcription factors such as PU.1.

Table 1.1. LncRNAs implicated in inflammation and immune response

LncRNA	Origin (cell/tissue type)	Functions	Ref
LincRNA-Cox2 (Long intergenic noncoding RNA associated with cyclooxygenase II)	LPS- or Pam3CSK4-stimulated mouse bone marrow derived dendritic cells (BMDC) or macrophages (BMDM).	Regulates genes expression related to immune response.	23-24
THRIL (TNF-α and hnRNPL related immunoregulatory lncRNA)	Pam3CSK4-stimulated human monocytic THP-1 cells	THRIL regulates both basal and Pam3CSK4-stimulated gene expression through an interaction with hnRNPL	25
Lethe	TNFα-stimulated mouse embryonic fibroblasts	Lethe is induced upon exposure to IL1b and dexamethasone, and interacts and blocks the binding of the RelA (p65) subunit of NF-kB	26
NEAT1 (Nuclear enriched abundant transcript 1)	Poly (IC)- or influenza-stimulated HeLa and human epithelial A549 cells	NEAT1 expression induces the formation of paraspeckle. Redistribution of SFPQ from the CXCL8 promoter to the paraspeckles following NEAT1 binding leads to increased CXCL8 expression.	27
PTPRJ-AS1 (PTPRJ-Antisense RNA 1)	LPS-stimulated BMDM	Induced in response to LPS	28
PACER (p50-associated COX2 extragenic RNA)	PMA- and LPS-stimulated human U937 monocytic cell line	PACER is expressed upstream of the Cox2 promoter and positively regulates COX2 production. PACER binds to, and drives the release of, the repressive p50 dimer of NF-kB from the Cox2 promoter	29
Lnc-DC (Long noncoding RNA involved in differentiation and function of dendritic cells) (LOC645638)	Differentiation of human and mouse dendritic cells	Lnc-DC is required for monocyte differentiation into DCs. Lnc-DC promotes phosphorylation and activation of STAT3, a transcription involved in DC differentiation. blocking its dephosphorylation by SHP1	30
Lnc-IL7R (Long noncoding RNA interleukin-7 receptor)	LPS-stimulated monocytic THP1 cells	Lnc-IL7R is transcribed from the 30-UTR of IL7R in the sense orientation. Lnc-IL7R is induced following LPS stimulation and negatively regulates IL7R, IL8, IL-6,	31

		VCAM-1, and E-selectin expression, a process associated with diminished H3K27me3 levels	
NTT (noncoding transcript in CD4+ T cells)	Human T cell lines	NTT was identified in activated T cells	[32]
Gas5 (growth arrest specific transcript-5)	Human primary T cells and T cell lines (CEM-C7 and Jurkat)	- Gas5 levels increase upon growth arrest and inhibit cell-cycle progression and promote apoptosis - Inhibition of T cell proliferation through the mTOR antagonist rapamycin is mediated by upregulation of Gas5	[33-354]
NRON (noncoding repressor of NFAT)	Human Jurkat T cells and mouse T cells	NRON blocks the nucleocytoplasmic transport and therefore the transcriptional activity of NFAT through interaction with multiple proteins including members of the importin-b superfamily. NRON also attenuates NFAT dephosphorylation and thereby blocks NFAT nuclear translocation, activation, and induction of IL-2	[34-35]
NeST (Nettoie Salmonella pas Theilers's)	Transgenic mouse infected with Salmonella and Theiler's virus	Overexpression of NeST increases clearance of bacterial Salmonella infection but reduce resistance to the mouse Theiler's picornavirus. NeST induces the expression of IFN-g through an interaction with WD repeat domain 5 (WDR5), a core subunit of the MLL histone H3 lysine 4 (H3K4) methyltransferase complex	[22]
LincR-Ccr2-5′AS (Long intergenic noncoding RNA 5′-end of chemokine 2 antisense)	Mouse CD4+ T_H2 cells	LincR-Ccr2-5′AS was located at the 5′-end of Ccr2 in CD4+ T_H2 cells and regulates both the induction and suppression of gene expression during T_H2 differentiation. LincR-Ccr2-5′AS is also implicated in chemokine-mediated signaling including cell migration	[36]
PAN (polyadenylated nuclear)	KSHV-infected B- and T cell lines	PAN modulate host cell response including downregulation of IFNγ, IL-18, and interferon via polycomb repression complex 2 (PRC2)-mediated histone methylation.	[37-38]

Recent studies indicate that ncRNAs play important roles in these processes. Indeed, lncRNAs have been studied most extensively in the context of Toll-like receptor (TLR)

responses.[16] A large number of lncRNAs has been identified in macrophages and DCs upon exposure to microbial products, cytokines, and other immune stimuli. Those lncRNAs contribute to the regulation of gene expression in immune cells via TLRs and cytokine receptors signaling in both positive and negative ways.[16, 39-40]

1.4.1.1 LincRNA-Cox2

Induction of LincRNA-Cox2 has been observed in dendritic cells and macrophages when exposed to LPS, as well as other TLR ligands. LincRNA-Cox2, however, regulates the expression of both basal and TLR-induced genes in DCs and macrophages.[23] LincRNA-Cox2 is located 50 kb downstream from the Cox2 (Ptgs2) gene and regulates a huge list of genes including IL-6, CCL5 that are associated with immune response. The repressive action of lincRNA-Cox2 is mediated through interaction with hnRNP-A/B and hnRNP-A2/B1.[24] These hnRNPs are known to have a role in the processing of precursor mRNA, as well as in regulating gene expression. Recent studies, however, demonstrated that lincRNA-Cox2 is associated with the switch/sucrose nonfermentable (SWI/SNF) chromatin-remodeling complex and induces the expression of NF-κB regulated genes and implicated in immune responses.[23-24]

1.4.1.2 THRIL (TNF-α and hnRNPL related immunoregulatory lncRNA)

THRIL regulates basal and TLR2-inducible expression of TNFα in human monocytes. THRIL knockdown reduced expression of multiple inflammatory genes such as IL6, CXCL8, CXCL10, CCL1, and CSF1. As with lincRNA-Cox2, THRIL interacts with hnRNPL and the resultant complex binds to the TNFα promoter and induces its transcription.

1.4.1.3 PACER (p50-associated COX2 extragenic RNA)

PACER is located directly upstream of the Cox2 transcriptional start-site and expressed in the antisense direction. It promotes transcription of PTGS2 (COX2) by acting as a decoy

molecule in the NF-κB signaling pathway in human macrophages.[8]Increased PACER expression, following PMA-induced differentiation of the monocytes into macrophages and subsequent LPS-stimulation, is required for PMA/LPS-induced COX2 expression. Elevated level of PACER is required for an interaction between PACER and the inhibitory p50 homodimer of NF-κB. Indeed, PACER decreases p50–p50 occupancy at the Cox2 promoter and permits the binding of the active p50–p65 form of NF-kB and assembly of the RNA polymerase II pre-initiation complex.[29] This event supports recruitment of p300 histone acetyltransferase at COX2 promoter and increases histone acetylation and subsequent induction of COX2 expression.

1.4.1.4 HOTAIRM1 (HOXA transcript, antisense RNA myeloid-specific 1)

HOTAIRM1 is an antisense lncRNA expressed in the HOXA gene locus and implicated in myeloid cell development. HOTAIRM1 is selectively expressed in myeloid cells, controls the expression of CD11b and CD18 genes, which are crucial for myeloid cell differentiation. Thus, HOTAIRM1 is thought to regulate the development of myeloid immune cells.[8]

1.4.1.5 Morrbid (myeloid RNA regulator of Bim-induced death)

LncRNA Morrbid is involved in the development of short-lived myeloid cells (e.g., neutrophils, eosinophils, and classical monocytes) in response to pro-survival cytokines. Morrbid has been reported to transcriptionally represse Bcl2l11 (Bim) gene by interacting with the polycomb repressive complex 2 (PRC2) to promote H3K27me3 modification at the Bcl2l11 promoter.[20] Dysregulation of Morrbid levels are frequently found in patients with hyper-eosinophilic syndrome, a disease characterized by persistently elevated numbers of eosinophils and dysregulated immune response.[41]

1.4.1.6 Lnc-DC (Long noncoding RNA involved in differentiation and function of dendritic cells)

Lnc-DC regulates differentiation of human monocytes into dendritic cells (DCs). Expression of lnc-DC is 100 times greater in classical DCs (cDCs) compared to precursor monocytes.[42] Expression of lnc-DC is driven by PU.1 which is a critical driver of DC differentiation. Lnc-DC also contributes to the activation of DCs by regulating STAT3 (signal transducer and activator of transcription 3) and controlling the STAT3 transcriptional program in DCs.[30]

1.4.1.7 Lethe

Lethe blocks NF-κB-driven inflammatory responses. It acts as a negative-feedback regulator of the NF-κB signaling pathway to restrain inflammation. Expression of Lethe is induced upon exposure to IL-1β and the anti-inflammatory glucocorticoid receptor agonist, dexamethasone.[6] Lethe physically associates with the NF-κB p65 (RelA) subunit in the nucleus and blocks the binding of NF-κB p65 (RelA) with its target genes promoter and by this mechanism inhibit both the inflammatory response to TNFα and IL-1β, and promote the anti-inflammatory actions of dexamethasone.[26]

1.4.1.8 Nuclear enriched abundant transcript 1 (NEAT1)

NEAT1 has immune-related functions in humans. It is implicated in the expression of IL-8 in cells infected with herpes simplex virus (HSV-1) and influenza A virus (IAV), and in response to dsRNA. Indeed, NEAT1 promotes IL-8 transcription by relocating the transcriptional repressor SFPQ (splicing factor proline glutamine-rich) from the IL-8 promoter to the nuclear paraspeckle.[27]

1.4.1.9 LincRNA-EPS (erythroid pro-survival)

LincRNA-EPS regulates differentiation of erythrocytes. It also functions in TLR mediated signaling. Following TLR activation, lincRNA-EPS expression is reduced with concomitant induction of the expression of immune response genes. lincRNA-EPS is reported to abrogate tri-methylation of H3K4 at the promoters of immune response genes and inhibits their expression.[11, 42]

1.4.1.10 Lnc-13

Lnc-13 and lincRNA-EPS function in a similar manner. Human macrophages express lnc-13 which is rapidly degraded by the RNA decapping enzyme DCP2 upon TLR4 activation. lnc-13 interacts with hnRNPD and HDAC1 to suppress the transcription of immune genes in resting macrophages. Patients with celiac disease have a single-nucleotide polymorphism (SNP) in lnc-13 that disrupts its interaction with HDAC1. This inability to engage HDAC1 results in enhanced expression of immune genes that are a feature of celiac disease pathogenesis.[11, 42]

1.4.2 Roles of LncRNAs in adaptive immune responses

A wide range of studies have reported that lymphocytes also express lncRNAs that coordinate the development, differentiation, and activation of both T and B cells and regulate adaptive immune response.[1, 11] Here, we will discuss functions of lncRNAs in these cells focusing a few key examples to emphasize the importance of lncRNAs in T and B lymphocytes.

1.4.2.1 Roles of LncRNAs in activation, development, and differentiation of T cell

T cells has been reported to express may lncRNAs including the noncoding transcript in CD4+ T cells (NTT), growth-arrest-specific transcript 5 (Gas5), and noncoding repressor of NFAT (NRON).[32-35] The cytokine milieu coordinates the inducible expression and activation of lineage-defining transcription factors involved in the differentiation of naïve T helper cells into

specialized populations of effector cells, including Th1, Th2, and Th17 cells and regulatory T cells (Tregs).[33, 43] Distinct T cell subsets express unique profiles of lncRNAs at different stages of their development. Indeed, these lncRNA regulate the differentiation and function of T cells.[43]

1.4.2.1.1 NRON (Noncoding repressor of NFAT)

NRON, is a constitutively expressed intronic lncRNA, interacts with kayopherin importin-b1 (KPNB1) and blocks the nucleocytoplasmic transport and consequently reduces the transcriptional activity of Nuclear factor of activated T cells (NFAT). NFAT is a Ca2+-activated transcription factor that is an important mediator of T cell activation including IL-2 induction.[33, 43]

1.4.2.1.2 LincRNA-MAF-4

LincRNA-MAF-4 controls the development of Th1 cells through epigenetic silencing of the transcription factor c-MAF4. It is located ~140 kilobases upstream of c-MAF gene which encodes for a transcription factor involved in the differentiation of naive CD4+ T cells to Th2 and Th17 cells. The expression of lincRNA-MAF-4 and MAF has been reported to be inversely correlated in Th1 and Th2 cells.[33, 43] Therefore, depletion of lincRNA-MAF-4 in naive CD4+ T cells skews their differentiation toward Th2 cells by enhancing the expression of MAF. Furthermore, lincRNAMAF- 4 interacts with the transcriptional repressors LSD1 and EZH2 to deposit H3K27me3 marks at the promoter of MAF to silence its expression in Th1 cells.[33, 43]

1.4.2.1.3 Nettoie Salmonella pas Theilers's (NeST)

LincRNA NeST, originally identified as Tmevpg1, has been shown to regulate Th1 cell differentiation. NeST controls the persistence of Theiler virus in the central nervous system of mice. NeST is reported as a host factor in mice responsible for the persistence of Theiler virus. Depletion NeST has been reported to clear Theiler virus infection. Expression of NeST is

dependent on the transcription factors T-bet and STAT4, both of which control differentiation of Th1 cells.[22] NeST interacts with WDR5 (WD repeat–containing protein 5), a core subunit of the MLL H3K4methyltransferase com-plex that deposits H3K4me3 marks at the IFN-γ promoter, to turn on transcription in CD8+ T cells. Therefore, NeST is regarded as an enhancer RNA.[22] However, NeST expression alone is not sufficient to drive IFN-γ expression, since it requires the Th1-specific transcription factor T-bet for its function.

1.4.2.1.4 LincR-Ccr2-5′AS (Long intergenic noncoding RNA 5′-end of chemokine 2 antisense)

LincR-Ccr2-5′AS is located at the 5′ end of Ccr2 and transcribed in an antisense direction exclusively in T_H2 cells. Knockdown of this lncRNA does not affect the development of T_H2 cells in vitro but leads to impaired expression of chemokine genes Ccr1, Ccr2, Ccr3, and Ccr5, located in the same genomic loci that contain lincR-Ccr2-5_AS. Furthermore, lincR-Ccr2- 5′ AS knockdown in T_H2 cells has been reported to impair migration of T_H2 cells to the lung.[36]

1.4.2.1.5 RMRP (RNA component of mitochondrial RNA processing endoribonuclease)

RMRP is a 274–base pair lncRNA, unspliced transcript that is highly conserved, abundant, and ubiquitously expressed. RMRP controls the interaction of DDX5 with RORγt (retinoic acid receptor–related orphan nuclear receptor γt) to form a transcriptional activation complex in Th17 cells.[43] This complex is enriched at the promoters of RORγt-regulated genes to turn on their transcription in Th17 cells. RORγt controls expression of IL-17A, IL-17F, and IL-22, which play important roles in the development and functions of Th17 cells.[43] Genetic mutations in RMRP are associated with an early onset autosomal-recessive genetic disease: cartilage-hair hypoplasia (CHH). Patients with CHH display skeletal malformations and have some evidence of immune dysfunction. Mice expressing a mutant version of RMRP (270 G > T),

corresponding to a SNP in patients with CHH, had defective DDX5:RORγt-dependent effector functions in Th17 cells.[33]

1.4.2.2 LncRNAs in B cells activation

MicroRNAs such as miR-155 and miR-150 are well known to mediate crucial functions in the development and functions of B cells. However, understanding of lncRNAs in B cells is still limited. Recent studies demonstrate that transcription factor PAX5, which is crucial for B cell development, acts as an important driver for lncRNA expression in pro-B and mature B cells.[6] On the other hand, several transcriptome profiling studies revealed that a wide range of lncRNAs are expressed at different stages of B cell development. One such lncRNA with known functions in B cells is FAS-AS1 which regulates FAS receptor (CD95; TNFRSF6) signaling in B cell lymphomas.[20] In fact, soluble FAS (sFAS) ligand mediated activation of FAS receptor leads to cell death via apoptosis. Interestingly, FAS-AS1 binds RBM5 to block the alternative splicing of FAS pre-mRNA, which is required for sFAS production. Therefore, FAS-AS1 expression is inversely correlated with sFAS-induced apoptosis in B cell lymphomas.[12, 44] Furthermore, the expression of lncRNAs in B cell subpopulations have been reported to be strongly correlated with chromatin features such as histone modifications, chromatin architecture, and transcription factors.[13]

1.5 Conclusions

LncRNAs appear as emerging family of regulators of immune responses. Whole genome transcriptome profiling analysis revealed that a large number of lncRNAs are expressed by different immune cells. However, one of the great challenges is the identification of functionally relevant lncRNAs from the large lists that are commonly produced. In my study, I attempted to identify functionally active novel lncRNAs in immune cells and investigate their functions in

inflammation and immune response. In chapter 2, the function of a well-known lncRNA called HOTAIR (HOX antisense intergenic transcript) in inflammation and immune response has been investigated and my study indeed demonstrated that HOTAIR is a key player in inflammatory response. In chapter 3, the potential function of HOTAIR in glucose metabolism especially under inflammatory condition has been investigated. In chapter 4, based on RNAseq analysis, LinfRNA1 and other LinfRNAs as critical players in inflammation and immune signaling have been identified. Overall, my study has identified novel functions of lncRNAs in immune response and inflammation.

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CHAPTER 2

LncRNA HOTAIR regulates lipopolysaccharide-induced cytokine expression and inflammatory response in macrophages

2.1 Introduction

The mammalian immune system orchestrates innate and adaptive immune responses that are complex biochemical processes regulated by various protein and lipid mediators such as pattern recognition receptors, cytokines, chemokines, hormones, growth factors, and prostaglandins, etc.[1-4] Toll-like receptors (TLRs) play central roles in the innate reaction to bacterial infection and in immune responses against pathogens.[5-8] In particular, Toll-like receptor 4 (TLR4) is activated by endotoxin lipopolysaccharide (LPS) present in the outer membrane of Gram-negative bacteria.[9-13] TLR4 activation triggers a series of downstream signaling cascades including NF-κB activation[14-19] and induction of cytokines, chemokines, and pro-inflammatory genes.[6, 20-25] Increasing evidences suggest that noncoding RNAs (ncRNAs) are integral components of variety of cellular and physiological signaling processes including in immune response.[26-30] Notably, ncRNAs are recently discovered classes of transcripts, which are coded by the genome, but remains mostly untranslated into proteins.[31-35] Noncoding RNAs, which are longer than 200 nucleotides, are classified as long-noncoding RNAs (lncRNAs).[36-38] It is estimated that human genome encodes more than 28000 lncRNAs, though their functions and detailed characterization are yet to be revealed.[26, 37, 39] Examples of lncRNAs implicated in immune response include lincRNA-Cox2, linc1992/THRIL, nc-1L7R, NeST RNA, NEAT1 and others.[39-42] LincRNA-Cox2 regulates IL-6 expression, linc1992/THRIL regulates expression of

IL-8, CXCL10, CCL1, and CSF1,[40] nc-1L7R knockdown diminishes IL-6 and IL-8 mRNA levels,[41] NeST RNA induces secretion of IFNγ in $CD8^+$ T cells,[42] NEAT1 causes transcriptional activation of IL-8[40] and so on. Here we explored potential roles of a well-known lncRNA called HOTAIR (HOX transcript antisense intergenic RNA) in cytokine regulation and immune response. HOTAIR is a 2.2 kb long lncRNA, an antisense transcript and is associated with gene repression.[43-45] HOTAIR interacts with gene silencing and chromatin modifying factors such as polycomb repressive complex 2 (PRC2)[26] and lysine specific demethylase 1 (LSD1, a histone H3K4-demethylase) complexes.[43, 46] EZH2 (Enhancer of zeste homolog 2) is a H3K27-methylase which is an interacting component of PRC2.[47-49] H3K27-methylation by EZH2 and H3K4-demethylation by LSD1 are crucial to gene silencing.[43, 50-51] HOTAIR facilitates recruitment of PRC2 and LSD1 multi-protein complexes at the target genes promoters, which induce H3K27-methylation and H3K4-demethylaytion respectively and contribute to gene silencing.[46, 52] HOTAIR upregulation is associated with a variety of cancers.[38, 53] Beyond gene repression, HOTAIR is also found to be involved in protein degradation associated with neuronal function and diseases.[54-55] Previous studies from our laboratory showed that HOTAIR is required for the viability of breast cancer cells and its expression is regulated by estradiol and hypoxia.[56-58] Here, we demonstrate that HOTAIR is a critical player in LPS-induced cytokine expression, immune, and inflammatory response in macrophages.

2.2 Materials and methods

2.2.1 Cell culture and treatment with Lipopolysaccharide (LPS)

Mouse macrophage RAW264.7 cells were obtained from American Type Cell Culture Collection, and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10 % heat-inactivated FBS (Fetal bovine serum), 2 mM L-

glutamine, 100 units/mL penicillin and 0.1 mg/mL streptomycin in a humidified incubator with 5% CO2 and 95% air at 37°C.[59] 2 x10^6 cells were seeded in 60 mm cell culture plates and after overnight culture cells were treated with ultrapure *E.coli*-derived LPS (Invivogen) at 1.0 μg/mL concentration for different time periods.[59] Notably, this concentration of LPS has been widely used in various other laboratories to induce immune and pro-inflammatory response in macrophages.[59-60] Cells were harvested for the preparation of RNA and protein analysis.

2.2.2 RNA extraction, cDNA synthesis and real time PCR

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. Briefly RAW264.7 cells treated with various conditions were harvested using TRIzol, mixed with chloroform and centrifuged at 12000 rpm for 15 min. The aqueous layer was mixed with equal volume of 100 % ethanol and centrifuged at 12000 rpm for 10 min. The pellet was washed with 70 % ethanol, and RNA was finally dissolved in 30-50 μL of RNase-free water (Sigma) and quantified using a Nanodrop spectrophotometer.[59] 1 μg of the isolated RNA was reverse transcribed into cDNA using iScript RT-supermix (Bio-Rad). For semi quantitative PCR the cDNA was PCR-amplified by using Taq DNA Polymerase (Genscript) as described earlier[61] and primers are listed in Table 1. Real-time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad), with gene specific PCR primers as listed in Table 1, in CFX96 real-time detection system (Bio-Rad). Each experiment was repeated three times with three parallel replicates each time. Expression data were normalized to GAPDH and expressed as $2^{-\Delta Ct}$.[62-63]

2.2.3 Western blot analyses

The cells were washed in ice-cold PBS and then lysed in cell lysis buffer comprising 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 % Igepal CA-630, 0.5 % Na-deoxycholate, 2 mM Na_3VO_4, and complete protease inhibitor cocktail and phosphatase inhibitor cocktail

(Roche).[64] The resulting cell lysates were centrifuged for 10 min at 13,000 rpm at 4°C, and the protein concentrations in the supernatants were determined using a BCA protein assay kit (Pierce). 20μg proteins were loaded onto 10 % SDS-PAGE gels, followed by electro-transfer onto PVDF-membrane (Immobilon-P, Millipore). The membranes were blocked in 1 x TBST (0.1 % Tween-20, 20 mM Tris–Cl (pH 8.0), and 150 mM NaCl) containing 5 % skimmed milk and then incubated with the primary antibodies against IκBα (1:1000 dilution, 4814S, Cell Signaling), Phospho-IκBα (1:1000 dilution, 2859S, Cell Signaling), Phospho-p65 (NF-κB subunit, 1:1000 dilution, 3033S, Cell Signaling), IL-6 (1:1000 dilution, 12912S, Cell Signaling), iNOS (1:1000 dilution, 13120S, Cell Signaling), and β -actin (1:1000 dilution, A2066, Sigma) overnight at 4 °C. Membranes were washed 3 times (1xTBST), incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and then washed 3 times (1 x TBST). Finally, immunoreactive proteins were detected using ECL -super signal west femto substrate reagent (Thermo Scientific).[64] The amount has been quantified by ImageLab5.2.1software.

2.2.4 Chromatin Immunoprecipitation (ChIP) assay

The ChIP assay was performed as described earlier.[56, 61] Briefly, the cells were cross-linked with 1 % formaldehyde for 10 min at 37 °C, washed twice in ice-cold PBS and harvested using SDS lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris. HCl, pH 8.1) supplemented with complete protease inhibitor (Roche). Cells were subjected to sonication to shear the chromatin (∼200-300 bp range). The fragmented chromatin was pre-cleared with protein G agarose beads (16-266, EMD Millipore) and subjected to immunoprecipitation using antibodies specific to CBP (A22, Santa Cruz Biotechnology, Sc369), p300 (N15, Santa Cruz Biotechnology, Sc584), Phospho-p65 (3033, Cell Signaling), histone H3K4-trimethyl (07–473, EMD-Millipore), histone acetylation

(06–599, EMD-Millipore), RNA Pol II (8WG16, Abcam), and β-actin (A2066, Sigma). Immunoprecipitated chromatin was washed, de-crosslinked and deproteinized at 65 °C in presence of 5 M NaCl followed by incubation with proteinase K (Sigma) at 45 °C for 1 h. Purified ChIP DNA fragments were analyzed by semi-quantitative PCR and real-time PCR using primers spanning NF-κB binding sites present in the IL-6 and iNOS promoters (Table 1).[65-67]

2.2.5 Antisense-mediated knockdown of HOTAIR

For the antisense transfection, RAW 264.7 cells were grown up to 60 % confluency in 60 mm culture plates and transfected with HOTAIR-antisense (HOTAIR-AS) and scramble antisense (Scr-AS, no homology to HOTAIR) oligonucleotides[56-57] independently (Table 1.1) using GenMute siRNA and DNA transfection reagent (SL100568, SignaGen Laboratories) according to the manufacturer's protocol. Prior to transfection, a cocktail of transfection reagent and antisense oligonucleotides was made as follows. Initially, 12 μL (12 μg) of GenMute reagent was mixed with 300 μL DMEM (without FBS and antibiotics) in an eppendorf tube. In a separate eppendorf, antisense oligonucleotides were mixed with 100 μL DMEM (without supplements). Then the diluted antisense solution was mixed with diluted GenMute reagents and allowed to stand for 30 min in the dark. In the meantime, cells were washed twice with supplement-free DMEM and then 1.7 mL of supplement-free DMEM was added to each cell culture plate. Finally, antisense transfection reagents cocktail was applied to the cell plates, mixed gently and incubated for 48 h. Cells were then stimulated with LPS (1 μg/mL) for specified time period and then harvested for RNA/protein extraction or for ChIP assays.

2.2.6 SiRNA-mediated knockdown of HOTAIR

For the siRNA transfection, RAW 264.7 cells were grown up to 60 % confluency in 60 mm culture plates and transfected with HOTAIR-siRNA, a pool of 4 different siRNA constructs

(SI05685183, SI05685190, SI05685197, and SI05685204 Qiagen)[68] and scramble siRNA (1027310 Qiagen) independently using GenMute siRNA and DNA transfection reagent (SL100568, SignaGen Laboratories) according to the manufacturer's protocol. Prior to transfection, a cocktail of transfection reagent and antisense oligonucleotide was made as follows. Initially, 12 μL (12 μg) of GenMute reagent was mixed with 300 μL DMEM (without FBS and antibiotics) in an eppendorf tube. In a separate eppendorf, siRNA was mixed with 100 μL DMEM (without supplements). Then the diluted siRNA solution was mixed with diluted GenMute reagents and allowed to stand for 30 min in the dark. In the meantime, cells were washed twice with supplement-free DMEM and then 1.7 mL of supplement-free DMEM was added to each cell culture plate. Finally, siRNA transfection reagents cocktail was applied to the cell plates, mixed gently and incubated for 48 h. Cells were then stimulated with LPS (1 μg/mL) for specified time period and then harvested for RNA/protein extraction.

2.2.7 NF-κB inhibition assay

RAW264.7 macrophages (2 x 10^6) were seeded in 60 mm cell culture plates. After overnight incubation cells were initially treated with IKKβ inhibitor (25 μM, SC-514, Sigma)[69] for 1 h to inhibit NF-kB signaling pathway and then cells were treated with LPS (1 μg/mL) and incubated for additional period of time 4 h. Cells were harvested, total RNA was isolated using TRIzol reagent, reverse transcribed to cDNA and analyzed by qPCR for the expression of HOTAIR, IL-6 and iNOS. GAPDH was used as control. Protein was also extracted (SC-514 1 h and additional1 h LPS treatment) after cell harvesting for Western blot.

2.2.8 Proteasomal inhibition assay

RAW264.7 macrophages (2 x 10^6) were seeded in 60 mm cell culture plates. After overnight incubation cells were initially treated with or without HOTAIR-AS for 48 h . The cells were then

treated with MG132 (Sigma) (10μM) for 2 h followed by LPS (1 μg/mL) and incubated for additional 1 h and followed the procedure for immunofluorescence microscopy analysis.[70]

2.2.9 Enzyme linked immunosorbent assay (ELISA)

HOTAIR was silenced in RAW264.7 macrophages by using HOTAIR-AS and HOTAIR-siRNA, a pool of 4 different siRNA constructs (SI05685183, SI05685190, SI05685197, and SI05685204 Qiagen) by the use of GenMute siRNA transfection reagent (SL100568, SignaGen Laboratories) according to the manufacturer's protocol. 48h following transfection RAW264.7 macrophages were stimulated with LPS for 12h or kept untreated. Culture media were collected and amount of IL-6 secreted in culture media were measured using ELISA kits (DY406-05 R&D Systems) according to manufacturer's instruction.[59]

2.2.10 Immunofluorescence microscopy analysis

For immunofluorescence staining of macrophages, cells were seeded on cover slips and fixed in 4 % paraformaldehyde (PFA) for 15 min at room temperature, washed with 1X PBS (3 times for 5 min each) and blocked with 1X PBS containing 5 % goat normal serum and 0.3 % Triton-X100 for 1 h. The cells were then incubated with primary antibodies (rabbit anti-P-p65, 1:200, 3033, CST; and mouse anti-IκBα (L35A5, 1:400, 4814, CST) overnight at 4 °C. After that the cells were washed 3 times with PBS followed by incubation with anti-rabbit Alexa Fluor 564 (Invitrogen) and anti-mouse Alexa Fluor 488 (Invitrogen), conjugated secondary antibodies for 1 h at RT. Finally the cells were washed 3 times with PBS and mounted with mounting media containing DAPI. Images were taken by fluorescence microscope (Nikon ECLIPSE TE2000-U). The amount has been quantified by the National Institutes of Health (NIH) ImageJ software.

2.2.11 Isolation and culture of primary macrophages (BMDMs)

Bone marrow derived macrophages (BMDMs) were isolated from mice bones and cultured as described earlier.[59, 71] Wild-type (C57BL6/J) mice were purchased from Jackson Laboratory. All mice are maintained in a specific pathogen free (SPF) facility at UT Southwestern Medical center. All studies were approved by the Institutional Animal Care and Use Committee (IACUC) and were conducted in accordance with the IACUC guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For the isolation of BMDMs, femur and tibia were collected from mouse legs. Using 25G needle bone marrows were flushed out with Iscove's Modified Dulbecco's Medium (IMDM), (12440061, Life technologies) and processed for single cell suspension by passing through 22G needle two times. The suspension was centrifuged at 1000 RPM for 5 min. The pellet was re-suspended with BMDM culture media (L-cell-conditioned IMDM medium supplemented with 15% L929 supernatant, 10 % FBS, 1 % nonessential amino acid, and 1 % penicillin-streptomycin) followed by seeding in three 150 mm culture dishes and cultured for 6 days to differentiate into macrophages, while at day 3, 10 ml fresh BMDM culture media was added into each plate. After day 6, the culture plate was washed with ice cold PBS and using cell scrapper cells were gently scraped with ice cold PBS. The BMDM was centrifuged at 1000 rpm for 5 min and re-suspended into BMDM media. The BMDM was counted and seeded in 6-well ($2.5x10^6$/well) cell culture plates. After overnight incubation the BMDM was treated with LPS or HOTAIR antisense oligonucleotides and processed for further experiments.

2.2.12 Statistical Analysis

Each experiment was done in two or three replicates, and then cells were pooled (and treated as one sample), subjected to RNA extraction, RT-PCR, and ChIP analysis, and each experiment

was repeated at least three times (n = 3). The real-time PCR analysis of such samples were done in three replicate reactions and repeated in all three independent experiments (n=3). Data are presented as means ± SD (as stated in the figure legends). Statistical significance was determined by unpaired Student's t test (GraphPad Prism 6), and $P \leq 0.05$ was considered statistically significant.

Table 2.1: Sequences of primers and antisense oligonucleotides

Primers	Forward (5' – 3')	Reverse (5' – 3')
PCR Primers		
HOTAIR	TCCAGATGGAAGGAACTCCAGACA	ATAGATGTGCGTGGTCAGATCGCT
IL-6	CAAGAAAGACAAAGCCAGAGTC	GAAATTGGGGTAGGAAGGAC
iNOS	TGTGACACACAGCGCTACAACA	GAAACTATGGAGCACAGCCACAT
GAPDH	ACCCAGAAGACTGTGGATGG	CACATTGGGGGTAGGAACAC
ChIP PCR Primers		
IL-6 promoter [a]	GCACACTTTCCCCTTCCTAGTT	AGACTCATGGGAAAATCCCACATT
iNOS-promoter [a]	GTGTACCTCAGACAAGGGCA	AAGCATTCACACATGGCATGGA
Antisense Oligonucleotides		
HOTAIR Antisense	5'-C*C*T*T*C*C*T*T*C*C*G*C*T*C*T*T*A*C*T*C*T-3'	
Scramble Antisense	5'-C*C*T*C*T*T*C*T*G*T*C*T*C*T*T*C*C*C*G*C*T-3'	

[a]ChIP PCR primers are flanked around the NF-κB binding site.
*All phosphorothioate linkages instead of regular phosphodiester bonds.

2.3 Results

2.3.1 LncRNA HOTAIR expression is induced by Lipopolysaccharide in macrophages

To investigate the roles of lncRNA in immune response, initially we explored potential involvement of lncRNA HOTAIR. As HOTAIR is well-known as a repressor,[50-51, 72-73] initially

we hypothesized that HOTAIR may be involved in repression of cytokine and inflammatory gene expression. To test this hypothesis, we treated macrophage cells (RAW264.7) with LPS and analyzed its impacts on the expression of HOTAIR along with well-known cytokines (e.g. interleukin-6, IL-6) and pro-inflammatory genes (e.g. inducible nitric oxide synthase, iNOS).[74-75] RNA from the LPS-treated macrophages were analyzed by RT-qPCR. As expected, LPS-treatment induced the expression of IL-6 and iNOS in time-dependent manner (Figure 2.1A and B). The expression of IL-6 was induced by 522, 6722 and 17321 folds at 2, 4 and 6 h post LPS-treatment, respectively (Figure 2.1A). Similarly, the expression of iNOS was also induced by 31, 292 and 621 folds at 2, 4 and 6 h respectively upon treatment with LPS (Figures 2.1B). Interestingly, along with IL-6, iNOS and other cytokines and inflammatory genes, lncRNA HOTAIR expression was also induced upon treatment with LPS (5, 11 and 14 folds at 2, 4 and 6 h, respectively) (Figures 2.1C). Notably, LPS-dependent induction of IL-6 and iNOS is much higher compared to HOTAIR expression, though the LPS-induced HOTAIR expression is significant. The cDNA was also analyzed by semi-quantitative PCR and products were analyzed on agarose gel showing the LPS-dependent induction of IL-6, iNOS and HOTAIR (Figures 2.1D). Overall, these observations demonstrated that along with IL-6, iNOS, lncRNA HOTAIR expression is induced upon stimulation with LPS and it may be associated with inflammatory and immune response in macrophages.

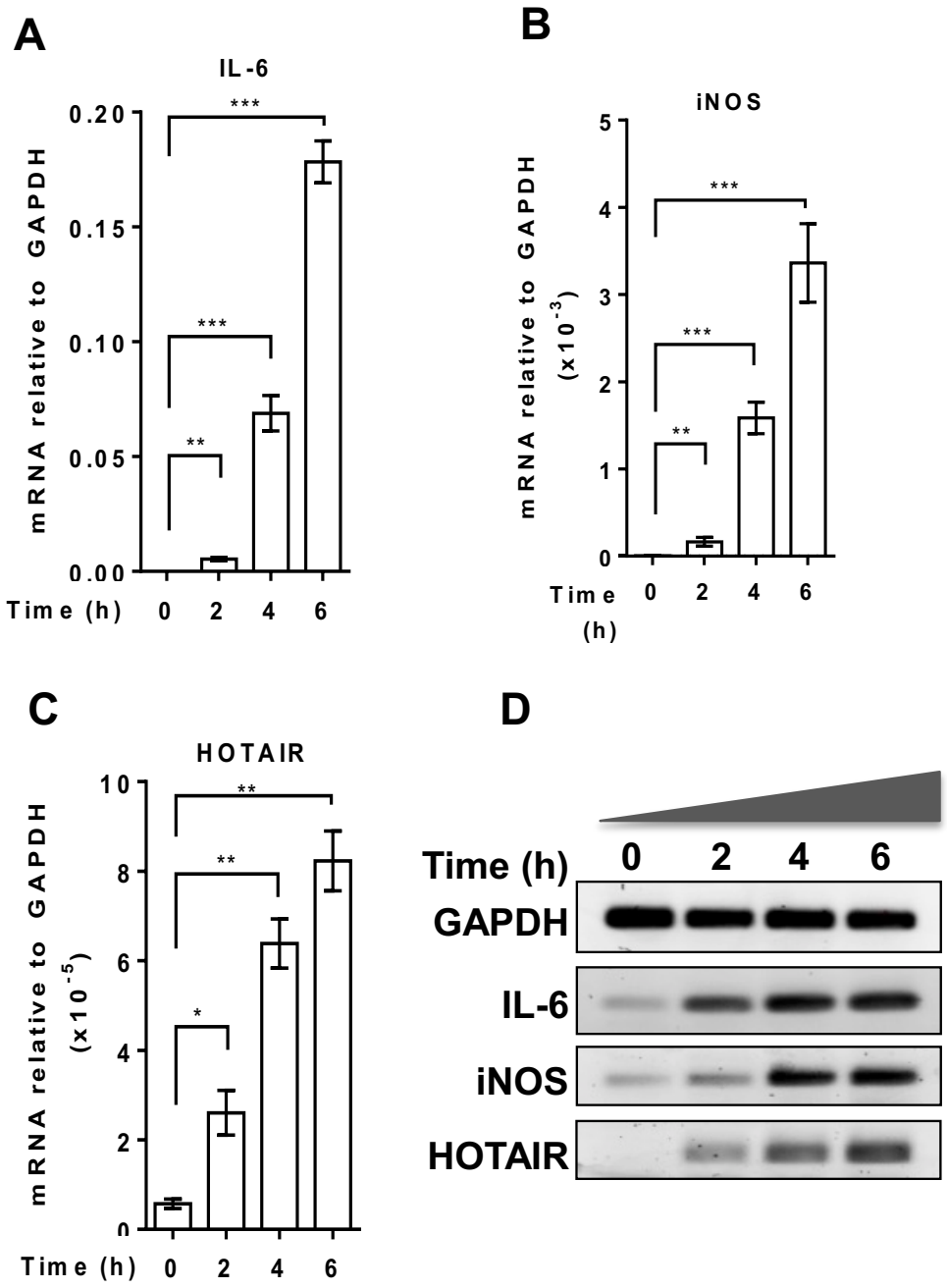


Figure 2.1. LPS induces HOTAIR expression in macrophages. RAW264.7 cells were treated with LPS (1 μg/mL) for varying period of time, total RNA was isolated, reverse transcribed to cDNA and analyzed by qPCR for expression of IL-6 (A), iNOS (B) and HOTAIR (C). cDNA was also analyzed by semi-quantitative PCR and agarose gel (D). Each experiment was repeated at least with three parallel replicates. GAPDH was used as loading control. Data represent mean ± SD (n=3); *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

2.3.2 Inhibition of NF-κB downregulates LPS-induced HOTAIR expression

The activation of transcription factor NF-κB is well-known to be associated with LPS-induced cytokine expression (including IL-6 and iNOS) and inflammatory response.[76-77] To investigate if NF-κB activation is associated with LPS-induced HOTAIR expression, we treated macrophages with an inhibitor of IKKβ (SC-514)[78] and analyzed its impacts on LPS-induced HOTAIR, IL-6 and iNOS expressions. Notably, IKKβ is a kinase which phosphorylates IκBα allowing its poly-ubiquitination, proteasomal degradation, and hence NF-κB activation.[6, 76, 79-80] Thus , inhibition of IKKβ (by SC-514) results in deactivation of NF-κB.[6, 76] Briefly, RAW264.7 cells were treated with IKKβ-inhibitor SC-514 (25 μM, 1 h) and stimulated with LPS. The concentration of the inhibitor (SC-514) used is chosen based on previous literature.[69] Proteins from the control and SC-514 treated cells were analyzed by Western blot to detect the levels of phospho-IκBα and phospho-p65.[79-80] As expected SC-514 treatment resulted in a decrease in phospho-IκBα and phospho-p65 (NF-κB submit) levels, indicating the effective inhibition of IKKβ kinase activity and consequent decrease in NF-κB activation (Figure 2.2A, quantifications in panel 2.2B). The RNA from the control and SC-514 treated cells were also then analyzed by RT-qPCR (Figures 2.2C-E) and semi-quantitative PCR (Figures 2.2F) for the expression of HOTAIR, IL-6 and iNOS. Interestingly, treatment with IKKβ inhibitor down-regulated LPS-induced expression of NF-κB target genes, IL-6 (5.4 folds) and iNOS (15.3 folds), along with a decrease in HOTAIR (2.4 folds) expression (Figures 2.2C-E, 2.2F). The treatment of SC-154 alone in the absence of LPS has no significant impact on IL-6, iNOS and HOTAIR expression (Figures 2.2C-F). These observations suggest that similar to IL-6 and iNOS, HOTAIR expression is also regulated by NF-κB upon LPS-stimulation.

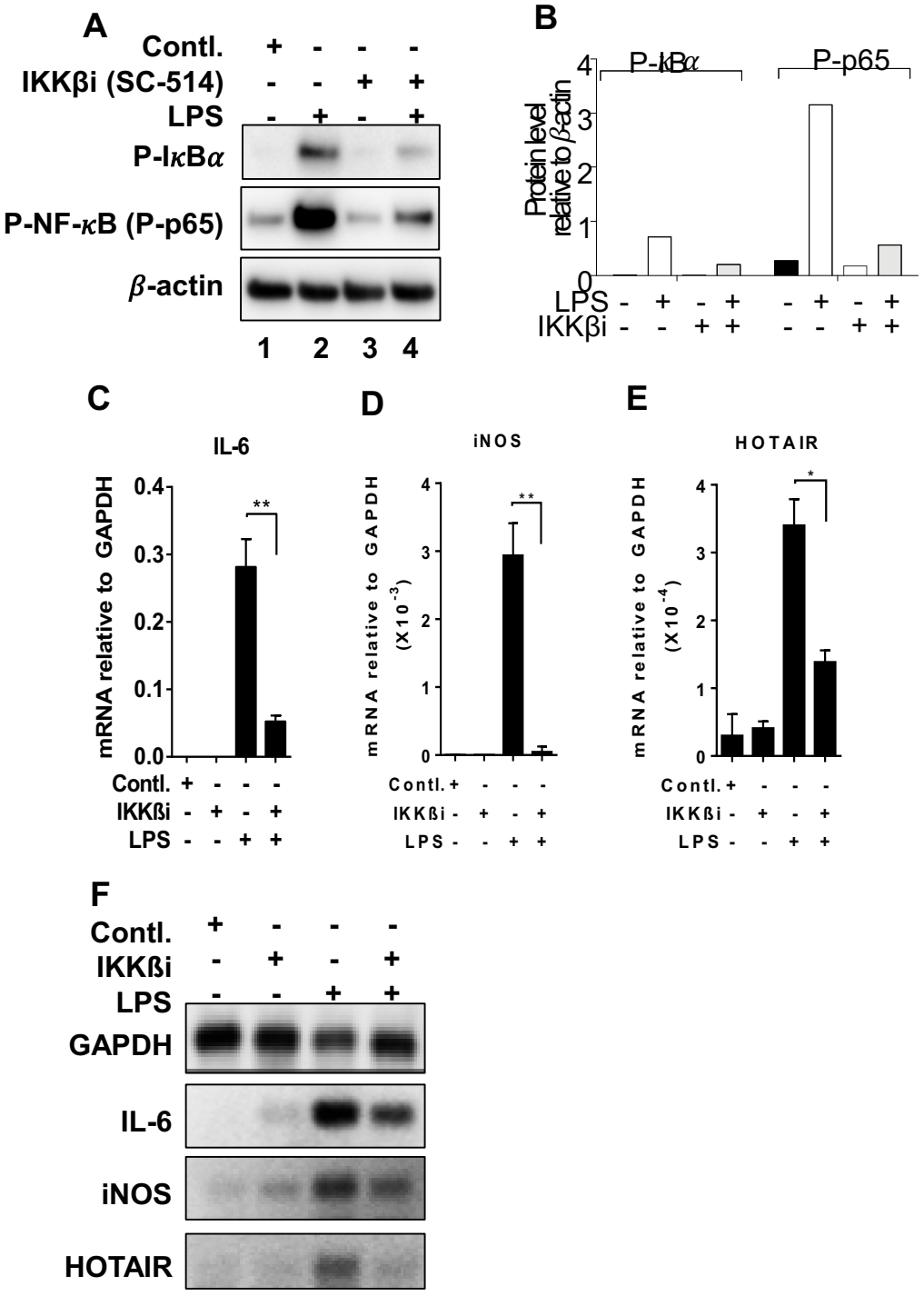


Figure 2.2. Inhibition of NF-κB downregulates LPS-induced HOTAIR expression in macrophages. RAW264.7 cells were initially treated with IKKβ-inhibitor SC-514 (for 1 h) and then treated with LPS for additional 1 h for protein and 4 h for RNA. Proteins were analyzed by Western blotting using antibody against phospho-IκBα, phospho-p65 (NF-κB subunit) and β-actin (loading control) (panel A, quantifications using ImageLab5.2.1software is shown in panel B). RNA was isolated and expressions of IL-6 (C), iNOS (D) and HOTAIR (E) were measured

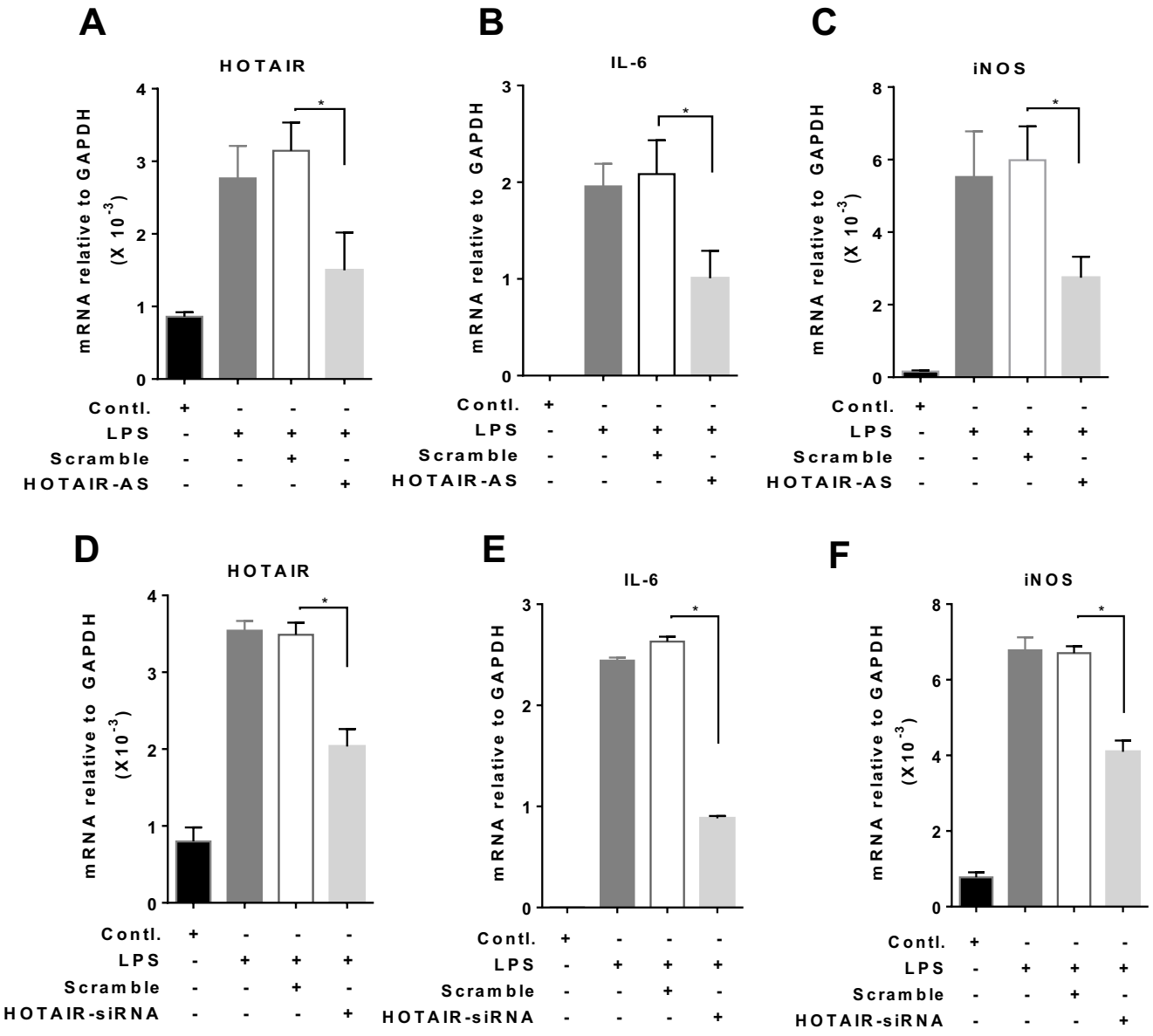
by RT-qPCR. cDNA was also analyzed by semi-quantitative PCR and agarose gel (F). GAPDH was used as control for PCR experiments. Data represent mean ± SD (n=3); *p < 0.05, **p < 0.001.

2.3.3 HOTAIR knockdown abolishes LPS-induced IL-6 and iNOS expression

To investigate any potential contribution of HOTAIR in cytokine regulation and inflammatory response, we knocked down HOTAIR in macrophages and analyzed its impacts on LPS-induced expression of IL-6 and iNOS. In brief, RAW264.7 cells were transfected with HOTAIR-antisense (HOTAIR-AS) and scramble-antisense (control) for 48 h, then treated with LPS (1 μg/mL, 4 h) and RNA was analyzed by RT-qPCR. As expected, LPS treatment induced the expression of HOTAIR as well as IL-6 and iNOS (Figures 2.3A-C). The application of HOTAIR-AS knocked down the levels of LPS-induced HOTAIR (Figure 2.3A). Scramble-AS did not down-regulate HOTAIR level (Figure 2.3A). Interestingly, the knockdown of HOTAIR resulted in down-regulation of the LPS-induced expression levels of IL-6 and iNOS significantly (Figures 2.3B and C). To further confirm the antisense-specificity of HOTAIR, we knocked down HOTAIR using HOTAIR-specific siRNA (a pool of 4 different siRNAs, targeting a different region 450-800 bp, 800-1050 bp, 1050-1550 bp and 1550-1900 bp of HOTAIR)[68] and then analyzed it impacts in LPS-induced expression of IL-6 and iNOS. Interestingly, HOTAIR-siRNA knocked down HOTAIR and also down-regulated LPS-induced IL-6 and iNOS expression levels (Figures 2.3D-F).

In addition to mRNA levels, we also analyzed the protein levels of IL-6 and iNOS in the absence and presence of LPS and under HOTAIR-conditions, using Western blots. As expected, IL-6 and iNOS were increased upon treatment with LPS (compare lanes 1 and 2) and their levels were decreased upon treatment HOTAIR-AS (compare lane 2 and 6) or HOTAIR-siRNA (compare lanes 2 and 10) (Figure 2.3G, quantification in H). Scramble antisense or siRNA has

no significant impacts on the LPS-induced expression of IL-6 and iNOS. Furthermore, we also measured the secretion of IL-6 in culture media using ELISA and found that secreted IL-6 level was increased upon treatment with LPS and that were decreased upon treatment HOTAIR-AS or HOTAIR-siRNA (Figure 2.3I). Taken together, our analysis demonstrated HOTAIR is required for LPS-induced expression of cytokines and inflammatory response genes in macrophages.



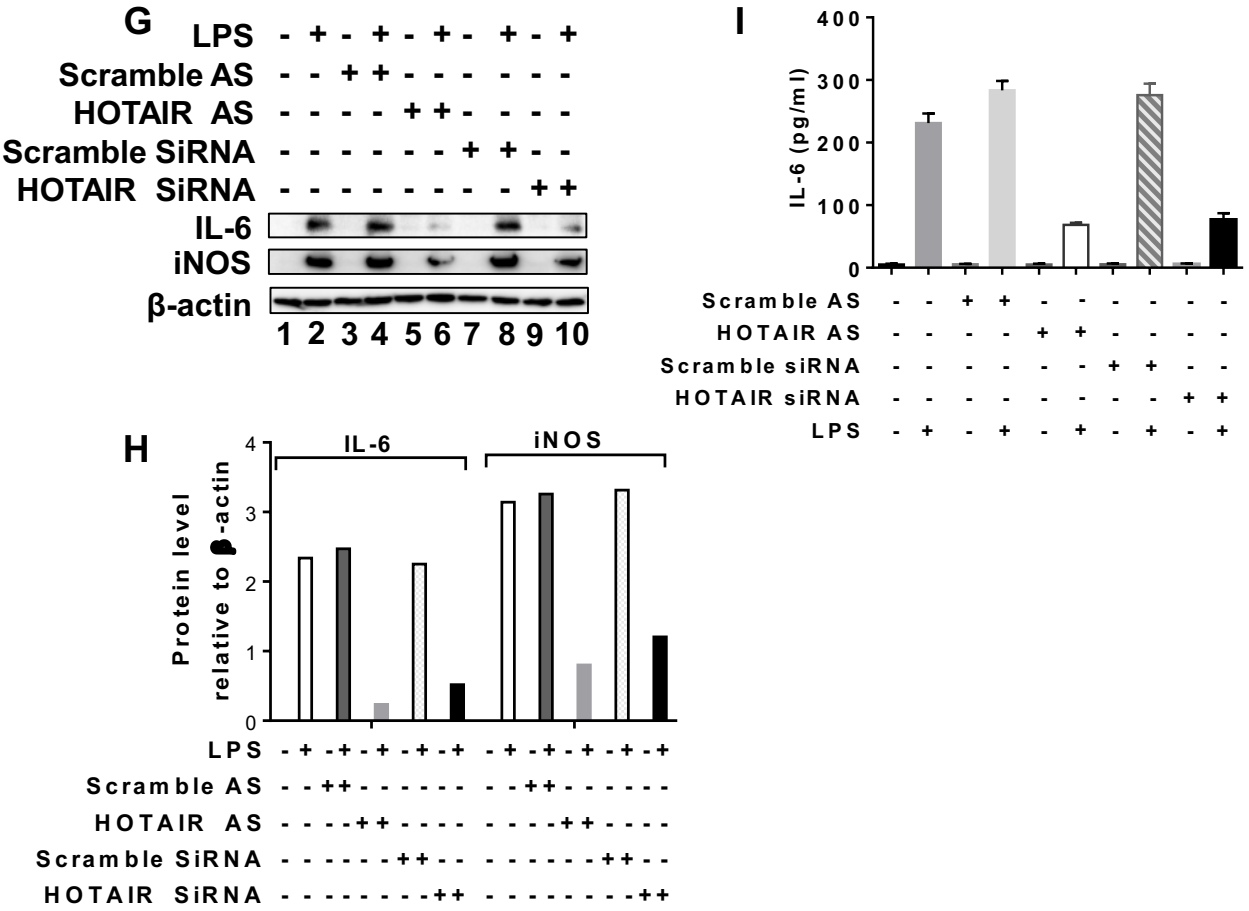


Figure 2.3. Knockdown of HOTAIR reduces LPS-induced IL-6 and iNOS expressions in macrophages. (A-C): Antisense-mediated knockdown of HOTAIR followed by treatment with LPS. RAW264.7 cells were transfected with HOTAIR-AS and scramble-AS, then treated with LPS for 4 h. RNA was analyzed by RT-qPCR for the expression of HOTAIR, IL-6, and iNOS. (D-F): siRNA-mediated knockdown of HOTAIR followed by treatment with LPS. RAW264.7 cells were transfected with HOTAIR-siRNA and scramble, RNA was analyzed RT-qPCR for the expression of HOTAIR, IL-6, and iNOS. GAPDH was used as control. Data represent mean ± SD (n=3); *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$. (G-H): Proteins from HOTAIR-antisense or HOTAIR-siRNA treatment followed by 6 h LPS-treated RAW264.7 cells were analyzed by Western blotting using antibodies against IL-6, iNOS and β-actin (loading control) (G). The changes in amounts of IκBα and NF-κB have been quantified by ImageLab5.2.1 software (H). (I): ELISA for IL-6 expression. HOTAIR was silenced in RAW264.7 macrophages by using HOTAIR-antisense and HOTAIR-siRNA separately, treated with LPS (12 h). Culture media were collected and amount of IL-6 secreted in culture media were measured using ELISA (n=4).

2.3.4 HOTAIR knockdown results in inactivation of NF-κB

As HOTAIR is required for LPS-induced expression of IL-6 and iNOS, we investigated its potential mechanism of action in this process. Notably, IL-6 and iNOS are regulated via activation of transcription factor NF-κB [81-83] and therefore, we explored if HOTAIR may be involved in NF-κB activation. We knocked down HOTAIR (using HOTAIR-antisense) in RAW264.7 macrophages and treated with LPS (varying time) and then analyzed the protein levels of IκBα and NF-κB (phospho-p65 subunit) (Figures 2.4A-C). Beta-actin was used a loading control. As expected, IκBα level was significantly decreased upon treatment with LPS (compare lane 1 with lanes 2 - 4, Figure 2.4A, quantification of IκBα levels relative to β-actin in Figure 2.4B). The degradation of IκBα was highest at 0.5 h of LPS-treatment and it was slightly increased over time (Figures 2.4A-B). As expected the level of phospho-p65 (NF-κB subunit) was also increased (NF-κB activation) upon LPS-treatment in comparison to the control (compare lane 1 with lanes 2 - 4, Figure 2.4A and quantification in 2.4C). The decrease in IκBα and concomitant increase in phospho-p65 upon LPS treatment suggest that LPS-stimulation has induced proteasomal degradation of IκBα resulting in activation of NF-κB. Interestingly, upon treatment with HOTAIR-AS (HOTAIR-knockdown), the level of LPS-induced IκBα degradation was decreased in comparison to LPS alone (compare lanes 2 with 6, 3 with 7, 4 with 8, Figures 2.4A and B). HOTAIR-knockdown also reduced phospho-p65 levels compared LPS-alone (compare lanes 2 and 6; 3 and 7; 4 and 8; Figures 2.4A and C). To cross verify the impact of HOTAIR-knockdown on deactivation of NF-κB, we also examined the IκBα and NF-κB (phospho-p65 subunit) levels under HOTAIR-siRNA treatment followed by LPS-stimulation (Figures 2.4D, E). Interestingly, siRNA-mediated knockdown of HOTAIR resulted in decrease in LPS-induced IκBα degradation and reduced phospho-p65 levels (compare lanes 2 with 4 in

Figure 2.4D, quantifications in 2.4E). Scramble-siRNA has no significant impacts on LPS-induced level of IκBα degradation and phospho-p65 (Figures 2.4D and E). These observations demonstrate that HOTAIR is required for LPS-induced proteasomal degradation of IκBα and NF-κB activation.

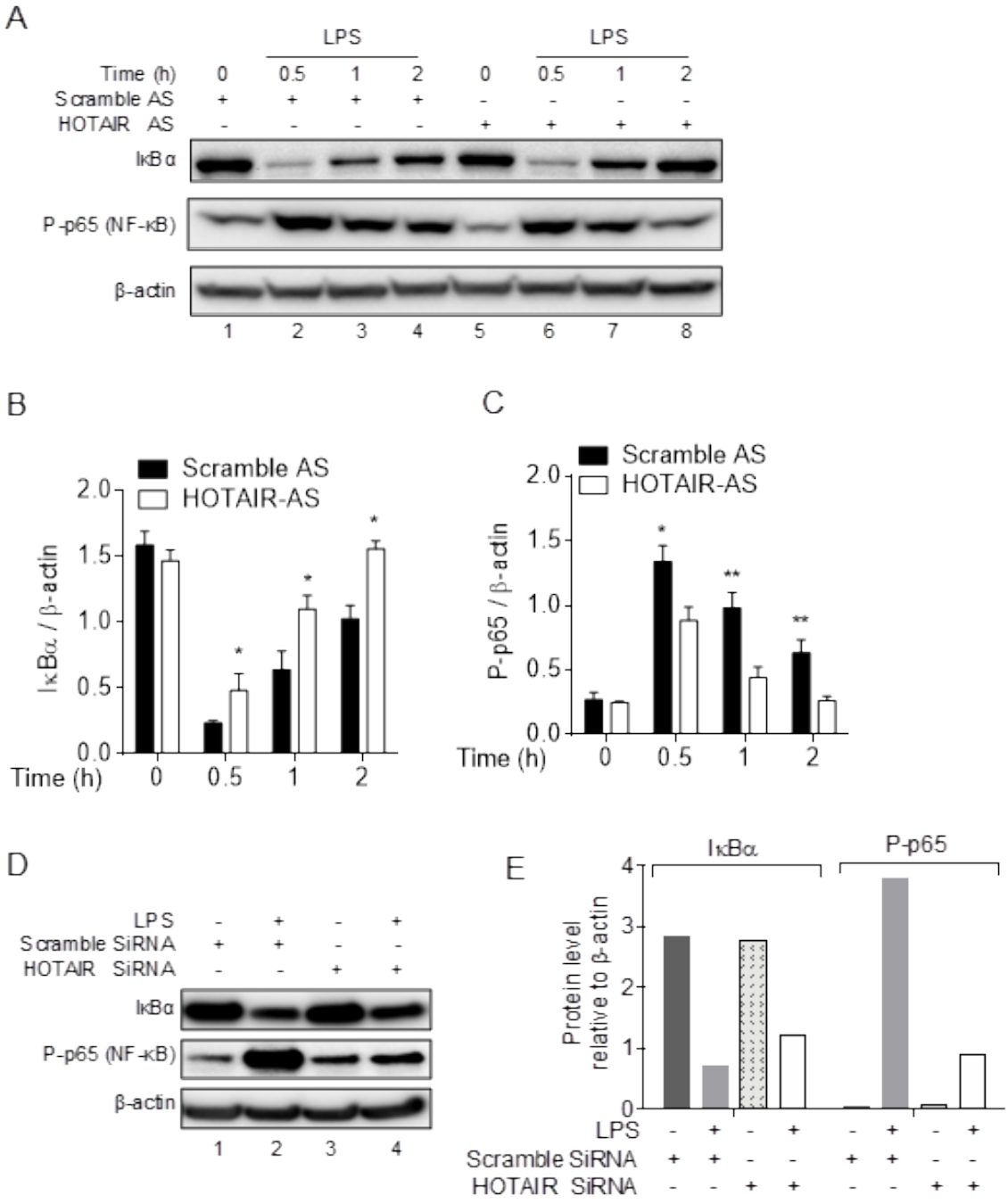


Figure 2.4. HOTAIR promotes IκBα degradation thereby activates NF-κB. (A-C): Antisense-mediated knockdown of HOTAIR followed by LPS-treatment. RAW264.7 macrophages were transfected with HOTAIR- or scramble antisense for 48 h, treated with LPS for different time periods (0.5 h, 1 h and 2 h) and protein was isolated. The protein was resolved on SDS-PAGE and immunoblotted with antibodies against IκBα, phospho p65 (NF-κB subunit) and β-actin (loading control). The changes in amounts of IκBα and NF-κB have been quantified by ImageLab5.2.1 software (B-C). (D-E): siRNA-mediated knockdown of HOTAIR followed by LPS-treatment. Proteins from HOTAIR- or scramble siRNA (48 h-treatment) followed by LPS (1 h) -treated RAW264.7 were analyzed by Western blot using antibody against IκBα, phospho p65 and β-actin. Quantifications (ImageLab5.2.1software) are shown in panel E. Data represent mean ± SD; *$p < 0.05$, **$p < 0.001$.

2.3.5 HOTAIR promotes IκBα degradation and nuclear translocation of NF-κB

To further understand the function of HOTAIR in NF-κB activation, we measured the co-expression of IκBα and phospho-p65 (NF-κB) in macrophages by using immunofluorescence assay. We knocked down HOTAIR in RAW264.7 cells by using HOTAIR-AS and scramble-AS (control) and then treated LPS. Control and antisense-treated cells were subjected to immunostaining with IκBα and phospho-p65 (NF-κB) antibodies. Cell nucleus was stained with DNA binding dye DAPI. We observed that IκBα protein levels were higher while phospho NF-κB protein levels were lower in unstimulated (in the absence of LPS) control cells (Figure 2.5A, top panel, quantification of the immunofluorescence staining images are shown in figure 2.5B). However, when stimulated with LPS we found a decrease in IκBα levels and increase in phospho NF-κB in macrophages compared to LPS-untreated control cells (compare top two panels, Figure 2.5A). Interestingly, upon HOTAIR-knockdown (HOTAIR-AS and LPS treatments), IκBα level was increased in comparison to LPS-treatment alone (compare panels 4 and 2, Figures 2.5A and B). The level of phospho-p65 protein (NF-κB) was decreased concomitantly upon knockdown of HOTAIR (HOTAIR-AS and LPS treatment) relative to LPS alone (compare panels 4 and 2, Figures 2.5A and B). Scramble antisense has no significant impact on LPS-induced expression of IκBα and phospho-p65 levels (Figures 2.5A and B). These results further indicate that HOTAIR

is required for LPS-induced proteasomal degradation of IκBα and activation of NF-κB in immune cells. Notably, to further understand the potential involvement of HOTAIR in proteasomal degradation of IκBα, we applied a well-known proteasomal inhibitor MG132 followed by treatment with LPS in the presence and absence of HOTAIR knockdowns. Interestingly, application of MG132 (followed by treatment with LPS) also inhibited the level of LPS-induced degradation of IκBα and lowered phospho-p65 level (compare panels 6 and 2, Figures 2.5A and B). The effects of independent treatment of MG132 or HOTAIR-AS on inhibition of LPS-induced degradation of IκBα and phospho-p65 levels are comparable (compare panels 6 and 4, Figures 2.5A and B). The application of MG132, in combination with HOTAIR-AS followed by LPS-treatment, showed no further significant impact in comparison to MG132 or HOTAIR-AS treatments alone (compare panels 7 with 6 and 4, respectively, Figures 2.5A and B). Overall, these studies demonstrate that HOTAIR plays potential roles in the regulation of proteasomal degradation of IκBα and subsequent activation of NF-κB in response to LPS in immune cells.

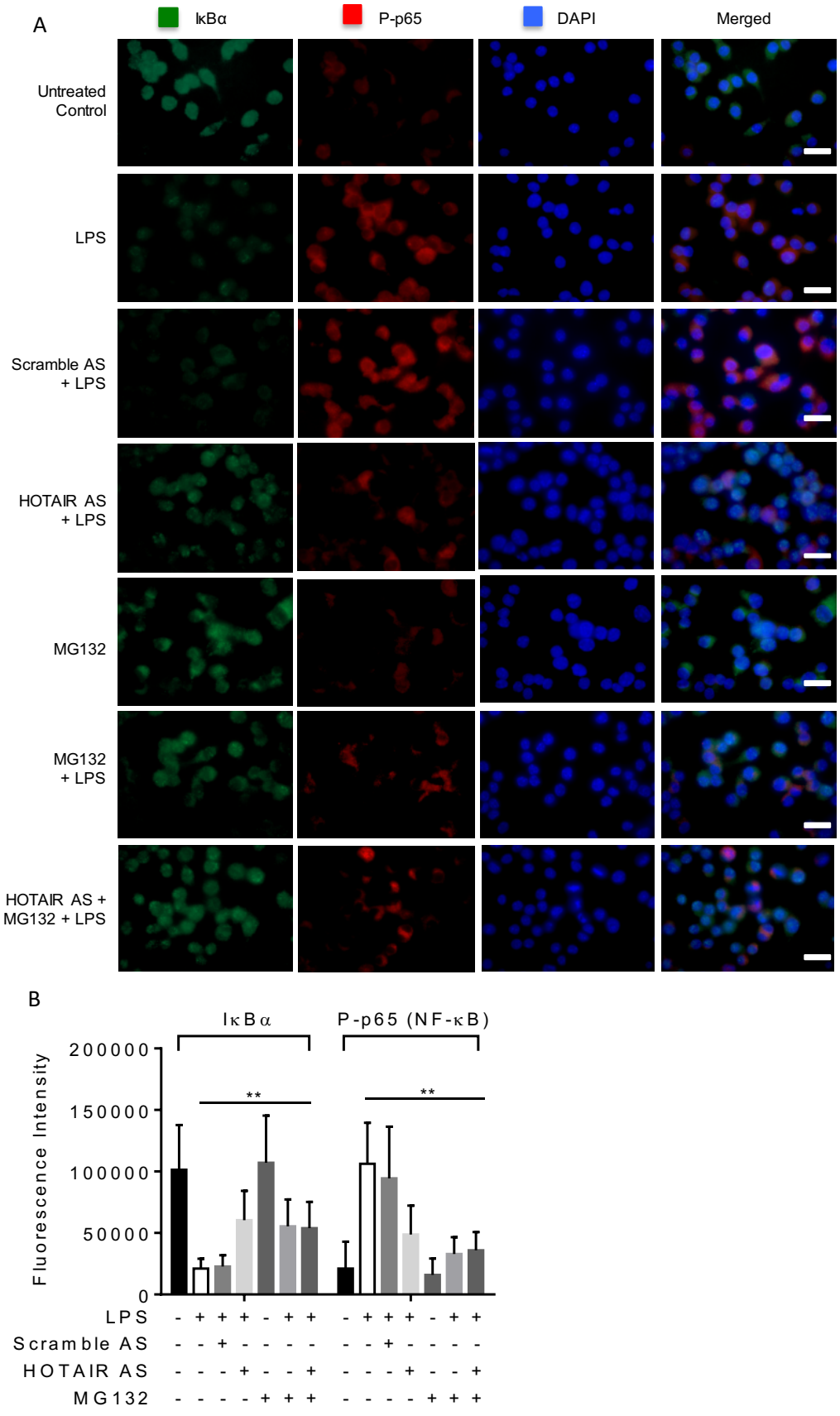

A
IκBα
P-p65
DAPI
Merged
Untreated Control
LPS
Scramble AS + LPS
HOTAIR AS + LPS
MG132
MG132 + LPS
HOTAIR AS + MG132 + LPS
B
IκBα
P-p65 (NF-κB)
Fluorescence Intensity
200000
150000
100000
50000
0
**
**
LPS - + + + - + + - + + + - + +
Scramble AS - - + - - - - - - + - - - -
HOTAIR AS - - - + - - + - - - + - - +
MG132 - - - - + + + - - - - + + +

Figure 2.5. HOTAIR promotes IκBα degradation and nuclear translocation of NF-κB. HOTAIR was silenced in RAW 264.7 cells by using HOTAIR antisense and scramble (control) for 48h. Additionally, cells were also treated with proteasomal inhibitor MG132 (2 h) alone or in combination with HOTAIR-knockdown and then treated with LPS (1 h). The cells were then fixed with paraformaldehyde and immunostained with antibody against IκBα and phospho NF-κB (P-p65), and counterstained with DAPI. Images were taken by fluorescence microscope (Nikon ECLIPSE TE2000-U) (A) and fluorescence intensity showing the expressions of IκBα and phospho-p65 was quantified and plotted by ImageJ software (B). Data represent mean ± SD; *$p < 0.05$, **$p < 0.001$.

2.3.6 HOTAIR knockdown abolishes the recruitment of NF-κB at IL-6 and iNOS promoters

The pro-inflammatory cytokine and inflammatory response genes such as IL-6 and iNOS are regulated via transcription factor NF-κB.[84-85] The promoters of IL-6 and iNOS contain NF-κB binding sites (NF-κB response elements) (Figures 2.6A and C). As HOTAIR is required for NF-κB activation and expression of NF-κB regulated genes IL-6 and iNOS expression, we analyzed HOTAIR-dependent recruitment of NF-κB at the NF-κB binding sites present in IL-6 and iNOS promoters as a function of LPS-treatment, using chromatin immunoprecipitation (ChIP) assay. Briefly, control and HOTAIR-knocked down RAW264.7 treated with LPS were subjected ChIP using phosphorylated p65 (NF-κB subunit) and β-actin (control) and ChIP DNA fragments were PCR-amplified using primers specific to NF-κB binding sites present in IL-6 and iNOS promoters.[61, 86] Interestingly, these analyses demonstrated that phospho-p65 (NF-κB) levels were enriched at the IL-6 and iNOS promoters (NF-κB response element regions) upon treatment with LPS and these LPS-induced NF-κB binding was reduced upon HOTAIR-knockdown in both IL-6 and iNOS promoters (see p-65 ChIP qPCR data in Figures 6A and C and also compare lanes 2 and 4, Figures 2.6B and D for regular PCR analysis). Notably, along with NF-κB, there are other coactivators which are associated with LPS-induced IL-6 and iNOS expression. For example, histone acetyltransferase CBP/p300 is known to interact with NF-κB to regulate NF-κB target genes.[87-89] Our ChIP analysis demonstrates that similar to NF-κB, the levels of CBP/p300 were

also enriched at IL-6 and iNOS promoters and these LPS-induced enrichments were alleviated upon knockdown of HOTAIR (Figures 2.6A and B for qPCR analysis and C-D for regular PCR analysis, compare lanes 2 and 4). Scramble antisense treatment has no significant impact on the LPS-induced enrichment of NF-κB and CBP/p300 at the IL-6 and iNOS promoters. As a control, we measured β-actin (antibody control), but no enrichment was observed irrespective of the LPS or HOTAIR-AS treatment (Figures 2.6A-D). These observations suggest that HOTAIR plays key roles in LPS-induced NF-κB activation and hence its enrichment at the promoters of NF-κB target genes such as IL-6 and iNOS to regulate their expression.

Histone H3K4-trimethylation and histone acetylation are also well known marks for gene activation.[61, 86, 90-92] ChIP analysis demonstrated that levels of H3K4-trimethylation and histone acetylation as well as the level of RNA polymerase II (RNA pol II) were enriched at IL-6 and iNOS promoters in the presence of LPS and this was decreased upon HOTAIR knockdown (Figures 2.6A-D). These observations suggested that HOTAIR is required for promoter activation (H3K4-methylation and histone acetylation) of IL-6 and iNOS and this is mediated via activation of NF-κB followed by recruitment of NF-κB and its coregulators including histone methyl-transferases and histone acetyl-transferases at the target gene promoters. Taken together our ChIP analysis demonstrated that LPS-induced expression of IL-6 and iNOS are regulated via transcription factors NF-κB, CBP/p300 and other coactivators and this is regulated by HOTAIR via regulation of NF-κB activation.

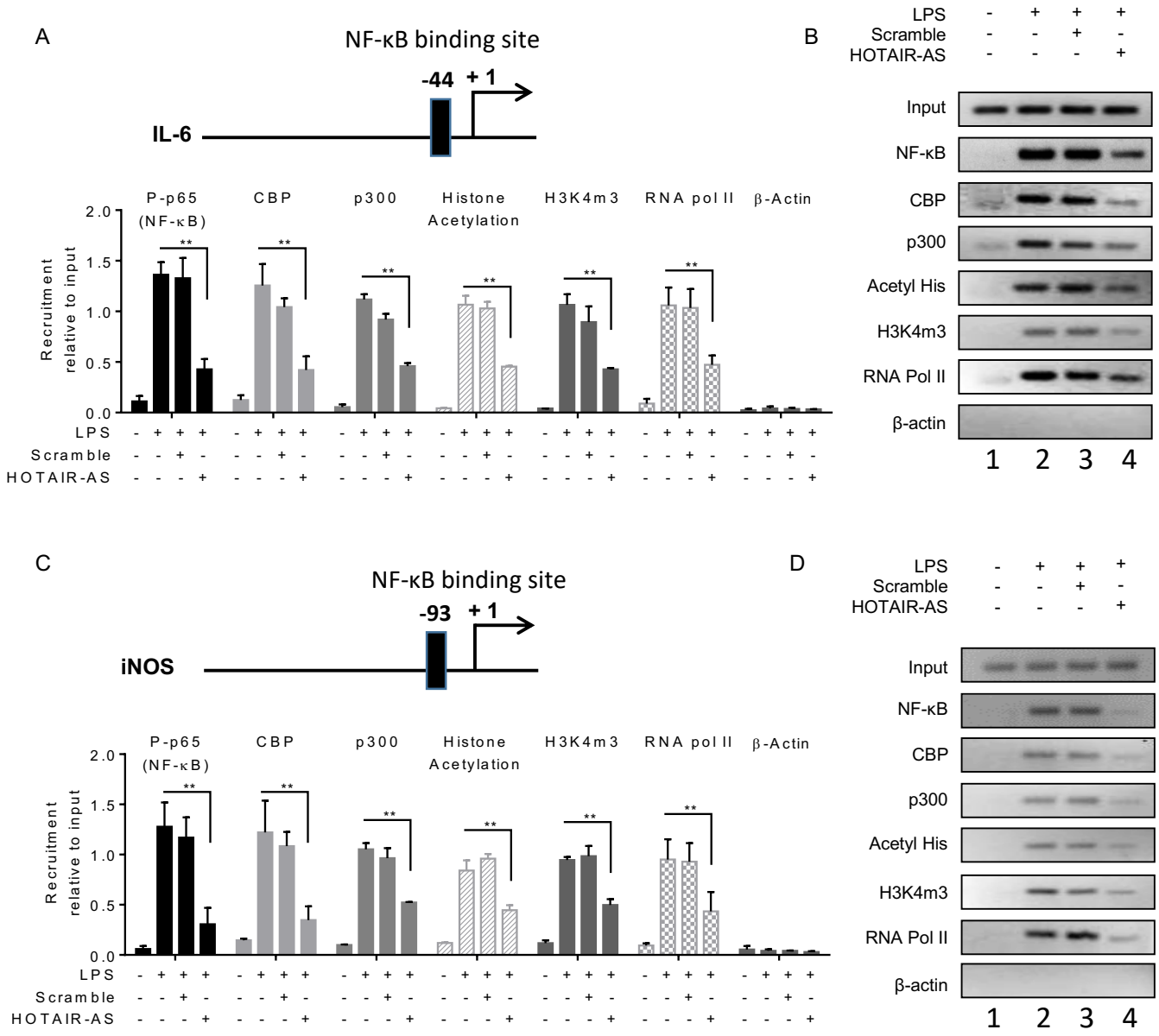


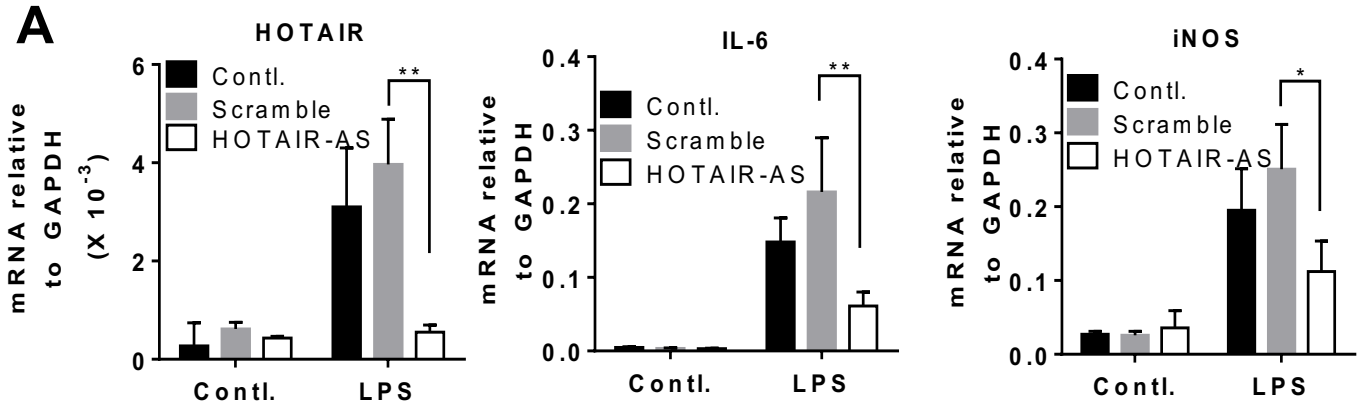
Figure 2.6. Knockdown of HOTAIR reduces the recruitment of transcription factors and coactivators at NF-κB binding sites on IL-6 and iNOS promoters. RAW264.7 macrophage cells were transfected with HOTAIR or scramble-antisense, then treated with LPS (1.5 h). Cells were then fixed with formaldehyde and subjected to ChIP assay using antibodies specific to phospho-p65, CBP, p300, histone acetylation, H3K4m3, RNA pol II and β-actin (control). The immunoprecipitated DNA fragments were analyzed by qPCR (panels A shows the ChIP analysis for IL-6, and C for iNOS) and semi-quantative PCR (panels B and D) using primers specific to the NF-κB binding regions on IL-6 and iNOS promoters. The location of NF-κB binding sites at the IL-6 and iNOS promoters are shown at the top of panels A and C respectively. Each experiment was repeated at least thrice (n = 3). Data represent mean ± SD; *p < 0.05, **p < 0.001.

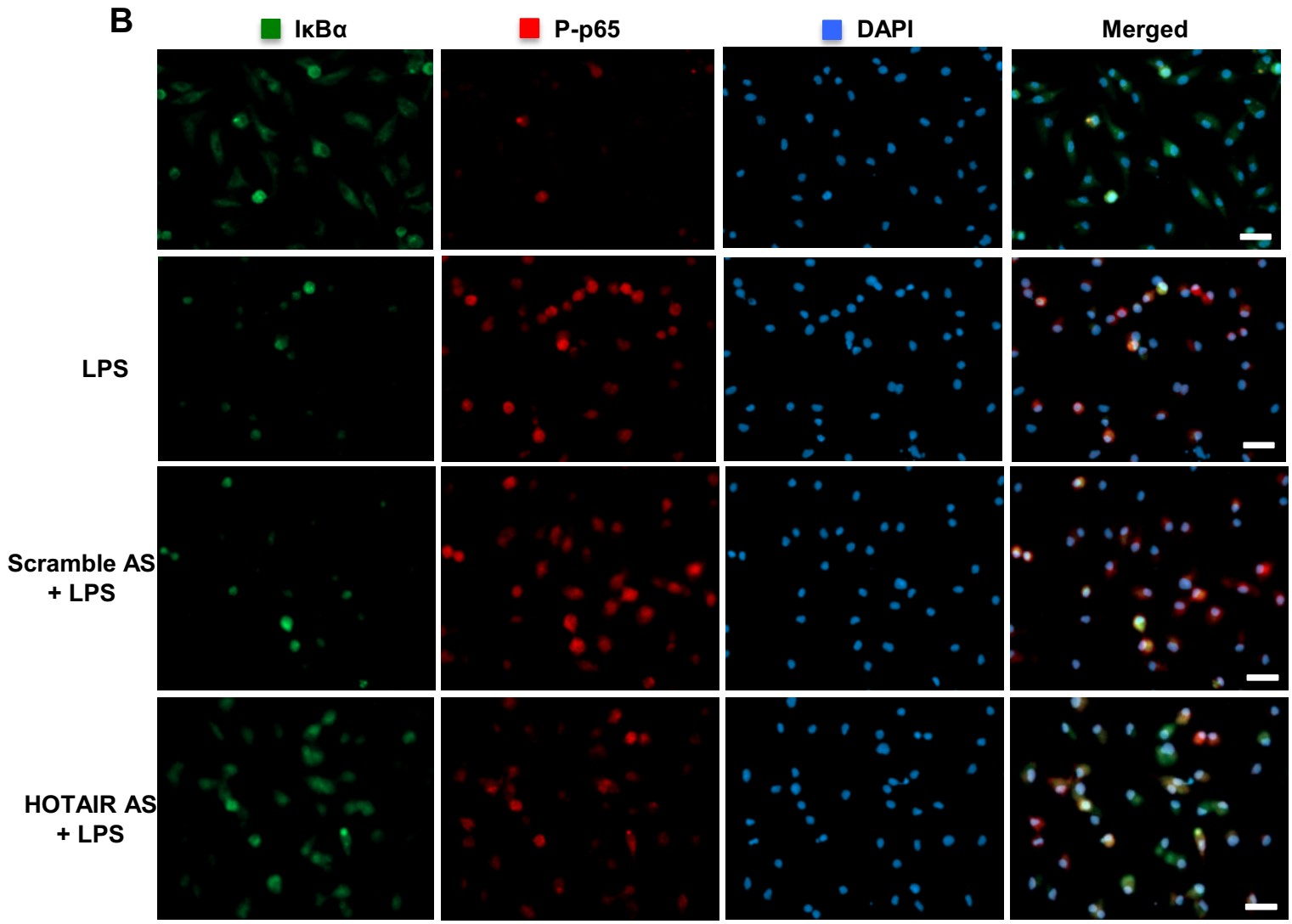
2.3.7 HOTAIR expression is induced by LPS in primary macrophages and is required for LPS-induced cytokine expression

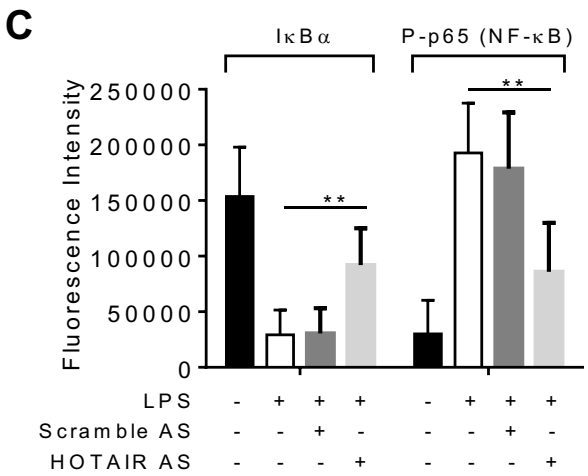
We investigated further the importance of HOTAIR in cytokine expression and immune response in primary macrophages, bone marrow derived macrophages. Briefly, BMDM cells were treated with LPS (1 μg/mL, for 4 h) in the presence and absence of HOTAIR and scramble (control) antisense, RNA was analyzed by RT-qPCR. Interestingly, the levels of IL-6 and iNOS as well as HOTAIR expression were induced upon treatment with LPS in BMDM (compare 1st and 4th bar graphs in each panel, Figure 2.7A). Application of HOTAIR-AS resulted in significant knockdown of LPS-induced HOTAIR expression level (compare 4th or 5th with 6th bar graphs, left panel, Figure 2.7A). Importantly, the levels of LPS- induced expression of IL-6 and iNOS were decreased upon HOTAIR knockdown (compare 4th or 5th with 6th bar graphs for IL-6 and iNOS panels, Figure 2.7A). HOTAIR-AS has no significant impact on IL-6 and iNOS expression in the absence of LPS and also scramble antisense has no significant impacts on LPS-induced IL-6 and iNOS expression (Figure 2.7A). These observations demonstrated that HOTAIR expression is induced upon LPS-stimulation in primary macrophages and it is required for the LPS-induced expression of IL-6 and iNOS.

HOTAIR-knocked down and LPS-treated BMDM cells were also analyzed by immunostaining to understand the function of HOTAIR in NF-κB activation using similar experiments outlined in figure 5. Interestingly, we found that IκBα protein levels were higher in the control BMDM (no LPS) and this was decreased upon treatment with LPS (Figure 2.7B). The level of NF-κB (phospo-p65) protein was increased upon treatment with LPS (Figure 2.7B). Interestingly, we also found that upon HOTAIR-knockdown, the level of LPS-induced decrease in IκBα protein level was rescued significantly while LPS-induced phospho-p65 NF-κB protein

level was decreased. Scramble antisense has no significant impacts on LPS-induced expression of IκBα and NF-κB (phospho-p65) protein levels. Quantification of immunofluorescence images showing the expression of IκBα and NF-κB (phospho-p65) protein levels under different treatments are shown in figure 2.7C. These observations based on primary macrophage analysis further demonstrate that HOTAIR plays a critical role in LPS-induced NF-κB activation and immune response.




B
IκBα
P-p65
DAPI
Merged
LPS
Scramble AS + LPS
HOTAIR AS + LPS


C
IκBα
P-p65 (NF-κB)
Fluorescence Intensity
250000
200000
150000
100000
50000
0
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LPS - + + + - + + +
Scramble AS - - + - - - + -
HOTAIR AS - - - + - - - +

Figure 2.7. HOTAIR expression is induced by LPS in primary macrophages (Bone marrow derived macrophages, BMDM). A) BMDM cells were treated by HOTAIR-AS and scramble-AS followed by LPS treatment and RNA was extracted. The expression of HOTAIR, IL-6, and iNOS was measured by real time PCR. B-C**)** BMDM cells were treated by HOTAIR-AS and scramble-AS, treated with LPS and were immunostained with antibodies against IκBα and phospho NF-κB (P-p65), and counterstained with DAPI to visualize the nucleus. Images were taken by fluorescence microscope (Nikon ECLIPSE TE2000-U) (B) and fluorescence intensity showing the expressions of IκBα and phospho NF-κB (P-p65) was quantified by ImageJ software and plotted in panel C. Each experiment was repeated at least thrice (n = 3). Data represent mean ± SD; *$p < 0.05$, **$p < 0.001$.

2.4 Discussion

The human genome contains about 3 billion base pairs of which only 1.5 % codes for proteins.[93-95] The ENCODE project has suggested that more than 80 % of the genome is functionally active and codes for ncRNA and regulatory sequences.[95-96] NcRNAs, even though are not translated into proteins, appear to play critical roles in a variety of cellular and physiological processes including gene regulation, cells signaling, differentiation and development.[38, 97] NcRNAs are misregulated in human diseases.[38 98] Increasing numbers of studies indicate that lncRNAs are associated with immune signaling and inflammatory response.[99] LncRNAs are expressed in immune cells including monocytes, macrophages, dendritic cells, neutrophils, T cells and B cells.[100] For example, lncRNA lincR-Ccr2-5′AS is associated with $CD4^+$ T cell differentiation.[97] LncRNAs are linked to pathogen-response pathways such as lincRNA-Cox2 expression is elevated upon activation of the toll-like receptors in bone-marrow-derived dendritic cells and macrophages.[32, 40, 97] LincRNA-Cox2 is required for the induction of other immune-related genes, such as IL-6, Tlr1, and IL-23a in bone marrow-derived macrophages by Pam3CSK4 treatment.[40, 101] NRON (non-protein coding RNA, repressor of NFAT) acts as a transcription regulator for immune regulation by inhibiting nucleocytoplasmic shuttling of NFAT (Nuclear Factor of Activated T cells).[102] Lethe acts as a decoy lncRNA and is a negative feedback inhibitor of NF-kB signaling in inflammation by being

increased with proinflammatory cytokines such as TNFα and IL-1β.[32, 103] THRIL (TNFα and heterogeneous nuclear ribonucleoprotein L related immunoregulatory lincRNA) plays immunoregulatory roles by binding with heterogeneous nuclear ribonucleoprotein L (hnRNPL) to induce the expression of TNFα, IL-6, IL-8, CXCL10, CCL1 and CSF1.[40, 104-105] Here, I investigated the importance of lncRNA HOTAIR in immune and inflammatory response in macrophages.

My study demonstrates that HOTAIR expression is induced in macrophage cells in response to LPS treatment. LPS, present on the Gram-negative bacteria cell wall, is one of the most potent pathogen-associated molecular patterns (PAMPs) known and responsible for the inflammatory response observed during endotoxic shock.[82, 106-108] LPS stimulation induces variety of cytokines, chemokines and inflammatory genes such as ILs, TNFs , interferons (IFNs), iNOS etc.[3, 84, 109-112] In my study, I observed that LPS-treatment (macrophage RAW264.7) induced the expression of various cytokines and inflammatory genes including IL-6, iNOS along with HOTAIR. Interestingly, I observed that HOTAIR is required for the LPS-induced cytokines and inflammatory genes expression. Antisense or siRNA-mediated knockdown of HOTAIR abolished the LPS-induced activation of IL-6 and iNOS. HOTAIR-knockdown down regulated the expression of IL-6 and iNOS, both at mRNA and protein levels. Independent knockdowns of HOTAIR with antisense or siRNAs targeting different regions of HOTAIR confirmed the HOTAIR-target specificity. These observations demonstrate that HOTAIR is a critical player in cytokine expression and inflammatory response in macrophages upon LPS-stimulation.

It is well known that LPS-induces activation of TLRs which activates down-stream signaling cascades and this ultimately results in degradation of IκBα and activation of NF-κB; the NF-κB activation triggers its target gene expression and induces immune and inflammatory

response.[113-117]To investigate if LPS-induced HOTAIR expression is regulated via NF-κB activation and if HOTAIR is required for LPS-induced NF-κB activation and its target gene expression, I blocked NF-κB activation via application of IKKβ-inhibitor (SC-514). I observed that upon treatment with IKKβ-inhibitor, the levels of phospho-IκBα as well as phospho-p65 were decreased. LPS-induced HOTAIR expression is also reduced upon IKKβ-inhibition. Notably, the kinase IKKβ phosphorylates IκBα at Ser 32/36, which triggers its polyubiquitination and proteasomal degradation, resulting in release and activation of NF-κB.[6, 76] Activation of NF-κB is accompanied by phosphorylation of its subunit p65 (at Ser 536).[79-80] Thus, the decrease in phospho-IκBα as well as phospho-p65 level indicated effective inhibition of NF-κB by SC-514. The decrease in HOTAIR expression upon IKKβ-inhibition indicated potential roles of NF-κB in LPS-induced HOTAIR expression. Furthermore, the biochemical and immunofluorescence studies demonstrated that HOTAIR is required for LPS-induced degradation of IκBα and activation of NF-κB and its nuclear translocation. HOTAIR-knockdown inhibited the LPS-induced degradation of IκBα and decrease on phosphorylation of p-65 (NF-κB), similar to the effects observed upon independent application of a proteasomal inhibitor MG132. This observation indicated that HOTAIR may be important for facilitating LPS-induced degradation of IκBα and activation of NF-κB. ChIP analysis demonstrated that HOTAIR is required for the recruitment of NF-κB and its coregulators at NF-κB target genes promoters regulating their expression. Experiments using primary macrophages isolated mouse bones further support that HOTAIR is induced by LPS and its expression is required for LPS-mediated NF-κB activation and hence IL-6 and iNOS expression. Thus, based on the biochemical studies in RAW264.7 macrophage cell lines and primary cells (BMDM) I demonstrated that HOTAIR

plays a central role in NF-κB activation and pro-inflammatory response in immune cells upon stimulation with LPS.

Notably, lncRNA HOTAIR is well known for its functions as repressor via interaction with chromatin modifying enzymes such as histone methylase EZH2 containing complex PRC2 and H3K4-demethylase LSD1.[46, 118-120] HOTAIR facilitates the recruitment of PRC2 and LSD1 at the target genes and induces gene silencing.[38, 121-122] HOTAIR is also implicated in DNA damage response,[123] proteasomal degradation via assembling E3-ubiquitin ligases as associated neuronal siganling[54, 124] HOTAIR induces ubiquitin-mediated proteolysis via interaction with E3 ubiquitin ligases Dzip3 and Mex3b, along with their respective ubiquitination substrates Ataxin-1 and Snurportin-1.[54] This leads to the degradation of Ataxin-1 and Snurportin-1.[38, 54, 125] A recent study also demonstrated that HOTAIR is involved in NF-κB activation and DNA damage response in ovarian cancer cells.[123] HOTAIR is overexpressed in variety of cancers.[126] Previous studies from our lab show that HOTAIR is required for the viability of breast cancer cells and its expression is transcriptionally regulated by estradiol via coordination of estrogen receptors (ERs) and ER-coregulators such as MLL (mixed lineage leukemia)-family of histone methyltransferases and CBP/p300 in breast cancer cells.[56-57, 61, 127] Here, I have demonstrated that HOTAIR expression is induced in immune cells and is required for pathogen-induced activation of cytokine expression and pro-inflammatory response. HOTAIR plays critical roles in IκBα degradation, which results in NF-κB activation followed by its target gene (cytokine, chemokines, and pro-inflammatory genes) expression. A model, showing the potential roles of HOTAIR in regulation of IκBα degradation and hence NF-κB activation, its translocation into the nucleus and binding to the target gene promoters inducing their expressions, is shown in figure 2.8.

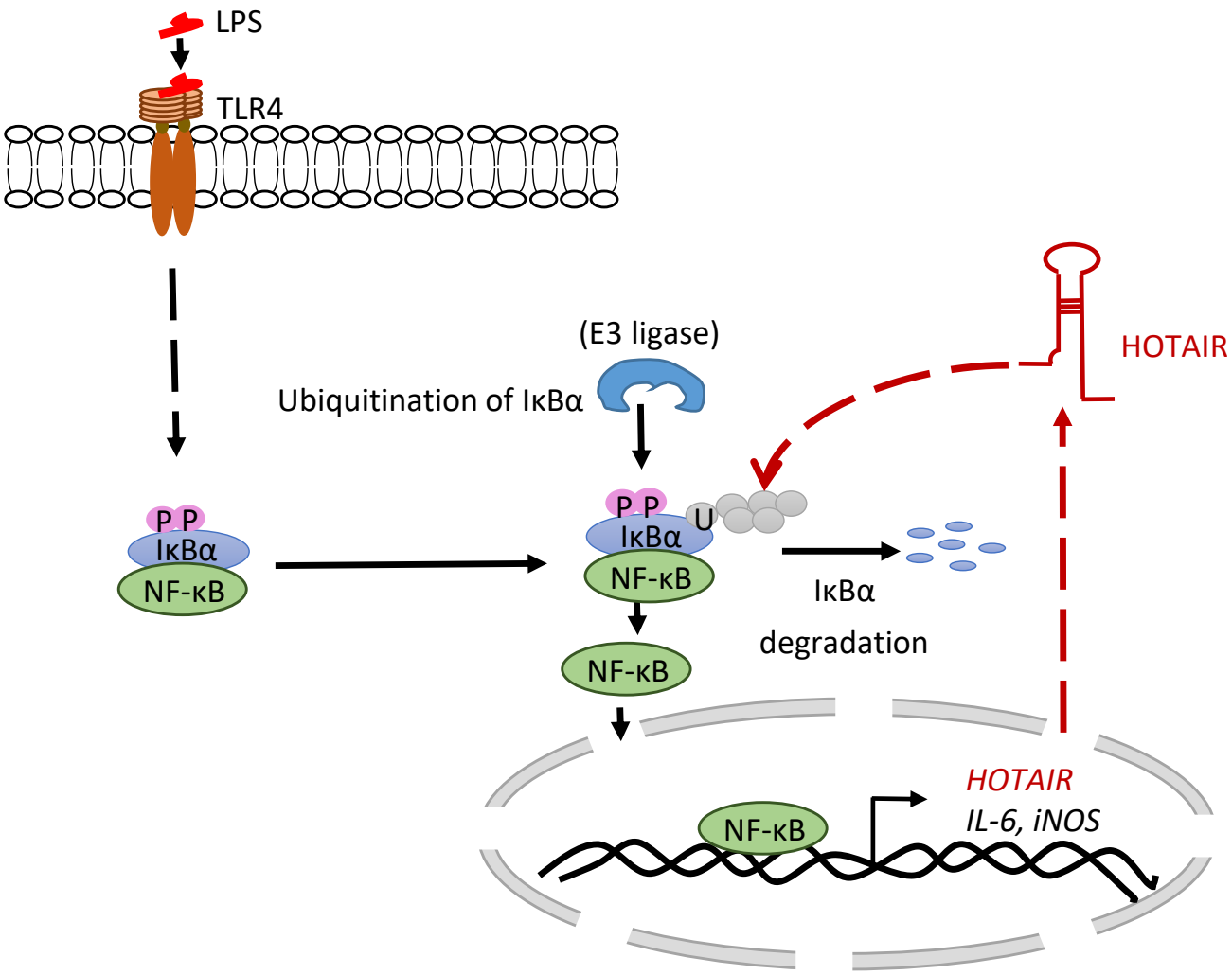

Figure 2.8: Proposed model for LPS-mediated HOTAIR induction in immune cells. When TLR4 senses LPS, NF-κB is activated that induces IL-6, iNOS and HOTAIR expression. In turn, HOTAIR facilitates IκBα degradation and enhances NF-κB activation, nuclear translocation and binding to NF-κB regulated genes (pro-inflammatory genes such as IL-6, iNOS) inducing their expressions.

The detailed mechanism by which HOTAIR regulates IκBα degradation remains elusive. Overall, our observations demonstrate for the first time that HOTAIR plays a central role in inflammation and immune signaling in immune cells and this may shed light into the novel immune signaling pathway that may aid development of novel therapeutics.

2.5 Acknowledgement

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CHAPTER 3

LncRNA HOTAIR regulates glucose transporters, glucose uptake and metabolism in macrophages during inflammation

3.1 Introduction

Inflammation is a biological response of the immune system. Inflammatory response is triggered by a variety of factors such as under pathogenic infection, and exposure to toxic compounds.[1]Activation of immune response removes injurious and toxic stimuli, helps healing and thus vital to health.[1-2] However, uncontrolled inflammation may results in severe inflammatory diseases.[3] Macrophages provide a first line of defense against pathogens and other invaders, and keep tissues healthy by eliminating foreign substances and apoptotic cells via phagocytosis.[4-5]To execute these functions, they migrate and constantly survey their immediate environment to sense the tissue damage or presence of invading organisms.[4-5]During surveillance, danger signals are detected through pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), nod-like receptors (NLRs) and others. Indeed, when tissue resident macrophages encounter stimuli like classical type 1 T-helper (T_H1) inflammatory cytokines such as interferon-gamma (IFNγ) and tumor necrosis factor alpha (TNFα) or foreign material such as lipopolysaccharide (LPS), the macrophages become activated to undergo a phenotypic change towards a classically activated M1 polarization state.[5-7] However, when they encounter prototypical type 2 T-helper (T_H2) cytokines such as IL-4 and IL-13, tissue-resident macrophages become activated to undergo a alternatively activated (suppressive) M2 polarization state.[1, 8]Upon activation, M1 macrophages secrete proinflammatory inflammatory cytokines and chemokines such as IL-1β, IL-6, TNFα, CXCL1, and induce inflammatory

immune responses.[1-2] In contrast, upon activation M2 macrophages secret anti-inflammatory cytokines such as IL-10, IL-18 and mitigate inflammation and promote tissue repair.[1, 8] Interestingly, M1 macrophages preferentially metabolize glucose as an energy substrate, while M2 macrophages primarily utilize fatty acids to fuel cellular behavior and activity.[9-10] Therefore, glucose metabolism is central to the function of classically activated M1 Macrophages and could be a potential target for modifying inflammatory responses.[1, 8-10] Emerging evidences suggest that proinflammatory immune response in M1 macrophages enhances expression of glucose transporter isoform 1 (Glut1), glucose-6-phosphate dehydrogenase, hexokinase, and increases glucose uptake.[9, 11] Glut1 is a major glucose transporter in macrophages and involved in glucose uptake into the cells.[9] Moreover, elevated Glut1-driven glucose metabolism drives inflammatory immune responses in macrophage.[12-13]

Emerging evidences suggest that, similar to proteins, noncoding RNAs (ncRNA) plays critical roles in variety of cell signaling process including in regulation of gene expression, cellular growth, differentiation and development.[14-15] NcRNAs are functional and integral components of signaling processes. An increasing number of publications report that ncRNAs play important roles in regulating aerobic glycolysis in cancer cells.[3] For instances, miR-199a, miR-138, miR-150 and miR-532-5p inhibit the expression of Glut1,[16-18] while miR-130b, miR-19a, miR-19b and miR-301a induce Glut1 expression in epithelial cancer cells.[15, 19] Recently, we discovered that long noncoding RNAs (lncRNAs) HOTAIR is involved in regulating inflammatory immune responses in macrophages.[14] HOTAIR is a 2.2 kb long lncRNA, an antisense transcript and is associated with gene repression.[20-21] HOTAIR interacts with gene silencing and chromatin modifying factors such as polycomb repressive complex 2 (PRC2) and lysine specific demethylase 1 (LSD1, a histone H3K4-demethylase) complexes.[22-23] HOTAIR

facilitates recruitment of PRC2 and LSD1 multi-protein complexes at the target genes promoters, which induce H3K27-methylation and H3K4-demethylaytion respectively and contribute to gene silencing.[24] HOTAIR is overexpressed in variety of cancer cells and suppresses expression of tumor suppressor. Studies from our laboratory demonstrated that HOTAIR is expressed in breast cancer cells and its expression is regulated via estradiol. HOTAIR expression is also misregulated upon exposure to estrogenic endocrine disrupting chemicals such as bisphenol-A (BPA) and diethylstilbestrol (DES), both *in vitro* and *in vivo*.[25] HOTAIR is an oncogenic lncRNA. Beyond gene repression, HOTAIR is also found to be involved in protein degradation associated with neuronal function and diseases.[26]

My recent study demonstrated that HOTAIR plays critical roles in regulation of NF-κB activation via degradation of IκBα and regulate expression of cytokines and pro-inflammatory genes regulating inflammation and immune response.[14] Importantly, activation of immune cells in response to infection or other stressors is a metabolically expensive event,[15] and immune cells preferentially meet their energy needs by metabolizing glucose.[27] As HOTAIR plays critical role in NF-κB activation and inflammatory response in macrophage, we hypothesized that HOTAIR play critical roles in inflammation induced metabolic reprogramming and therefore, we explored any roles of HOTAIR in glucose metabolism in macrophage during inflammation. My study demonstrated that HOTAIR regulates the expression of glucose transporter Glut1, glucose uptake, and hence metabolism during LPS-induced inflammation in macrophages.

3.2 Materials and methods

3.2.1 Mouse macrophage cell culture

RAW264.7 cells (mouse macrophages) were procured from American Type Cell Culture Collection. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-

Aldrich, St. Louis, MO) supplemented with 10 % heat-inactivated FBS (Fetal bovine serum), 2 mM L-glutamine, 100 units/mL penicillin and 0.1 mg/mL streptomycin in a humidified incubator with 5% CO2 and 95% air at 37°C. [28 28] Cells were counted to seed 2 x10^6 cells in 60 mm cell culture plates. After overnight incubation, macrophages were ready for treatment.[14, 28]

3.2.2 Cell treatment with Lipopolysaccharide (LPS)

Macrophage cells were treated with ultrapure *E.coli*-derived LPS (Invivogen), 1.0 μg/mL, for different time periods. The concentration of LPS has been broadly reported to be used by other laboratories to induce immune and pro-inflammatory response in macrophages.[14, 28] Cells were harvested for the preparation of RNA and protein analysis.

3.2.3 RNA extraction and cDNA synthesis

After treatment, total RNA was extracted from the cells using TRIzol (Invitrogen) according to the manufacturer's instructions.[28] Briefly RAW264.7 macrophages were harvested with TRIzol, mixed with chloroform and centrifuged at 12000 rpm for 15 min. The aqueous layer was mixed with equal volume of 100 % ethanol and centrifuged at 12000 rpm for 10 min. After washing the pellet with 70 % ethanol, purified RNA was dissolved in 30-50 μL of RNase-free water (Sigma) and quantified using a Nanodrop spectrophotometer. 1 μg of the isolated RNA was reverse transcribed into cDNA using iScript RT-supermix (Bio-Rad).[14, 28]

3.2.4 Real time PCR

Real-time PCR was done using iTaq Universal SYBR Green Supermix (Bio-Rad), with gene specific PCR primers as listed in Table 3.1. The CFX96 real-time detection system (Bio-Rad) was used for RT-qPCR. Each experiment was repeated three times with three parallel replicates each time. Expression data were normalized to GAPDH and expressed as $2^{-\Delta Ct}$. [29-30]

3.2.5 Western blot analyses

The treated macrophage cells were washed with ice-cold PBS and then lysed in cell lysis buffer comprising 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 % Igepal CA-630, 0.5 % Na-deoxycholate, 2 mM Na_3VO_4, and complete protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche). The resulting cell lysates were centrifuged for 10 min at 13,000 rpm at 4°C, and the protein concentrations in the supernatants were determined using a BCA protein assay kit (Pierce).[31] 20μg proteins were loaded onto 10 % SDS-PAGE gels, followed by electro-transfer onto PVDF-membrane (Immobilon-P, Millipore). The membranes were blocked in 1 x TBST (0.1 % Tween-20, 20 mM Tris–Cl (pH 8.0), and 150 mM NaCl) containing 3 % skimmed milk and then incubated with the primary antibodies against IκBα (1:1000 dilution, 4814S, Cell Signaling), Glut 1 (1:1000 dilution, 12939S, Cell Signaling), Phospho-p65 (NF-κB subunit, 1:1000 dilution, 3033S, Cell Signaling), and β -actin (1:1000 dilution, A2066, Sigma) overnight at 4 °C. Membranes were washed 3 times (1xTBST), incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and then washed 3 times (1 x TBST). Lastly, immunoreactive proteins were detected using ECL -super signal west femto substrate reagent (Thermo Scientific).[14, 31] The amount has been quantified by Image Lab 5.2.1 software.

3.2.6 Chromatin Immunoprecipitation (ChIP) assay

The ChIP assay was done as described earlier.[14, 25] The cells were cross-linked with 1 % formaldehyde for 10 min at 37 °C, washed twice with ice-cold PBS and harvested using SDS lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris. HCl, pH 8.1) supplemented with complete protease inhibitor (Roche). Cells were sonicated to shear the chromatin (~200-300 bp range). The fragmented chromatin was pre-cleared with protein G agarose beads (16-266, EMD

Millipore) and subjected to immunoprecipitation using antibodies specific to CBP (A22, Santa Cruz Biotechnology, Sc369), Phospho-p65 (3033, Cell Signaling), histone H3K4-trimethyl (07–473, EMD-Millipore), histone acetylation (06–599, EMD-Millipore), RNA Pol II (8WG16, Abcam), and β-actin (A2066, Sigma). Immunoprecipitated chromatin was washed, de-crosslinked and deproteinized at 65 °C in presence of 5 M NaCl followed by incubation with proteinase K (Sigma) at 45 °C for 1 h.[14, 25, 32] Purified ChIP DNA fragments were analyzed by semi-quantitative PCR and real-time PCR using primers spanning NF-κB binding sites present in the Glut1 promoter (Table 3.1).

3.2.7 SiRNA-mediated knockdown of HOTAIR

For the siRNA transfection, RAW 264.7 cells were grown up to 60 % confluency in 60 mm culture plates and transfected with HOTAIR-siRNA, a pool of 4 different siRNA constructs (SI05685183, SI05685190, SI05685197, and SI05685204 Qiagen)[26] and scramble siRNA (1027310 Qiagen) independently using GenMute siRNA and DNA transfection reagent (SL100568, SignaGen Laboratories) according to the manufacturer's protocol. Prior to transfection, a cocktail of transfection reagent and siRNA was made as follows. Initially, 12 μL (12 μg) of GenMute reagent was mixed with 300 μL DMEM (without FBS and antibiotics) in an eppendorf tube. In a separate eppendorf, siRNA was mixed with 100 μL DMEM (without supplements). Then the diluted siRNA solution was mixed with diluted GenMute reagents and allowed to stand for 30 min in the dark. In the meantime, cells were washed twice with supplement-free DMEM and then 1.7 mL of supplement-free DMEM was added to each cell culture plate. Finally, siRNA transfection reagents cocktail was applied to the cell plates, mixed gently and incubated for 48 h. Cells were then stimulated with LPS (1 μg/mL) for specified time period and then harvested for RNA/protein extraction or for ChIP assays.[14, 32]

3.2.8 NF-κB inhibition assay

RAW264.7 macrophages (2 x 10^6) were seeded in 60 mm cell culture plates. After overnight incubation cells were initially treated with IKKβ inhibitor (25 μM, SC-514, Sigma) for 1 h to inhibit NF-kB signaling pathway and then cells were treated with LPS (1 μg/mL) and incubated for additional period of time 4 h.[33] Cells were harvested, total RNA was isolated using TRIzol reagent, reverse transcribed to cDNA and analyzed by qPCR. Protein was also extracted (SC-514 1 h and additional 1 h LPS treatment) after cell harvesting for Western blot.[14]

3.2.9 Glucose Uptake Assay

Glucose uptake into the macrophages was determined using Glucose Uptake Assay Kit (Colorimetric, ab136955, Abcam) as per the manufacturer's Instructions.[12, 19] RAW264.7 macrophages were treated with HOTAIR siRNA for 48 hours and then seeded in 96 well plate in serum free medium. After overnight incubation cells were washed and incubated in 2% Bovine serum albumin (BSA) for 40 minutes. Then cells were stimulated with insulin (+/-), followed by 2-deoxyglucose addition for 20 minutes. 2-DG-6-phosphate (2-DG6P) standard curve was prepared. In this assay, the 2-DG6P was oxidized to generate NADPH, which was measured at 412 nm in a microplate reader.[12, 19]

3.2.10 Statistical Analysis

Each experiment was done in two or three replicates, and then cells were pooled (and treated as one sample), subjected to RNA extraction, RT-PCR, and ChIP analysis, and each experiment was repeated at least three times (n = 3). The real-time PCR analysis of such samples were done in three replicate reactions and repeated in all three independent experiments (n=3). Data are presented as means ± SD (as stated in the figure legends). Statistical significance was determined

by unpaired Student's t test (GraphPad Prism 6), and $P \leq 0.05$ was considered statistically significant.[14]

Table 3.1: Sequences of primers

Primers	Forward (5' – 3')	Reverse (5' – 3')
PCR Primers		
HOTAIR	TCCAGATGGAAGGAACTCCAGACA	ATAGATGTGCGTGGTCAGATCGCT
IL-6	CAAGAAAGACAAAGCCAGAGTC	GAAATTGGGGTAGGAAGGAC
iNOS	TGTGACACACAGCGCTACAACA	GAAACTATGGAGCACAGCCACAT
Glut1	GCTGTGCTTATGGGCTTCTC	CACATACATGGGCACAAAGC
Glut2	CCCTGGGTACTCTTCACCAA	GCCAAGTAGGATGTGCCAAT
Glut3	CTGGGGTCACAGGTTAAGGA	ACAGAAGCCGCTCTCAGAAG
Glut4	ACTCTTGCCACACAGGCTCT	CCTTGCCCTGTCAGGTATGT
Glut5	TGTACCCCACCTCTCACTCC	CTCGGGTAGCAATGGACAGT
GAPDH	ACCCAGAAGACTGTGGATGG	CACATTGGGGGTAGGAACAC
ChIP PCR Primer		
Glut1 promoter [a]	GCACACTTTCCCCTTCCTAGTT	AGACTCATGGGAAAATCCCACATT

[a]ChIP PCR primers are flanked around the NF-κB binding site.

3.3 Results

3.3.1 LPS-induces GLUT1 expression in macrophage

Our studies demonstrate that lncRNA HOTAIR plays critical roles in inflammation and immune signaling and regulates cytokine expression via regulation of NF-κB activation.[14] As glucose metabolism is well-known to be elevated during inflammation,[6, 9, 34] we investigated if HOTAIR plays any roles in glucose uptake and metabolism. To investigate roles of HOTAIR in glucose metabolism during inflammation, we examined if glucose transporters (Glut) expression are

affected upon LPS-stimulation in macrophage. We treated mouse macrophage cells (RAW264.7) with LPS and analyzed its impact on various Gluts expression at mRNA and protein levels. Briefly, RNA from the control and LPS-treated macrophages are reverse-transcribed into cDNA and analyzed by qPCR using primers specific to different glucose transporters (Glut1, Glut2, Glut3, Glut4 and Glut5). We also analyzed the expression of well-known inflammatory marker genes expression such as IL-6 and iNOS, and inflammatory noncoding RNA such as HOTAIR. As expected, our studies demonstrate that the expression of IL-6, iNOS and HOTAIR are stimulated by 9800, 272 and 6 folds, respectively, upon treatment with LPS for 4 h (Figure 3.1 A-C). Interestingly, expression of Glut1 is also significantly induced upon treatment with LPS in macrophage (Figure 3.1D and E). Additionally, Glut1 is the primary glucose transporter expressed in macrophage cells in the absence of LPS (Figure 3.1D). The basal expressions of other glucose transporters (Glut 2-5) and their LPS stimulation were relatively low (Figure 3.1D). Time course analysis demonstrates that LPS-induced stimulation of Glut1 is maximum at 4 h (Figure 3.1E). Western blot analysis also demonstrates that Glut1 expression is induced at the protein level as well upon treatment with LPS (Figure 3.1F, quantification in Figure 3.1G). These observations demonstrate that Glut1expression, which is the primary glucose transporter expressed in macrophages, is upregulated upon LPS-induced inflammation in macrophage and is potentially involved in glucose uptake and metabolism during inflammation.

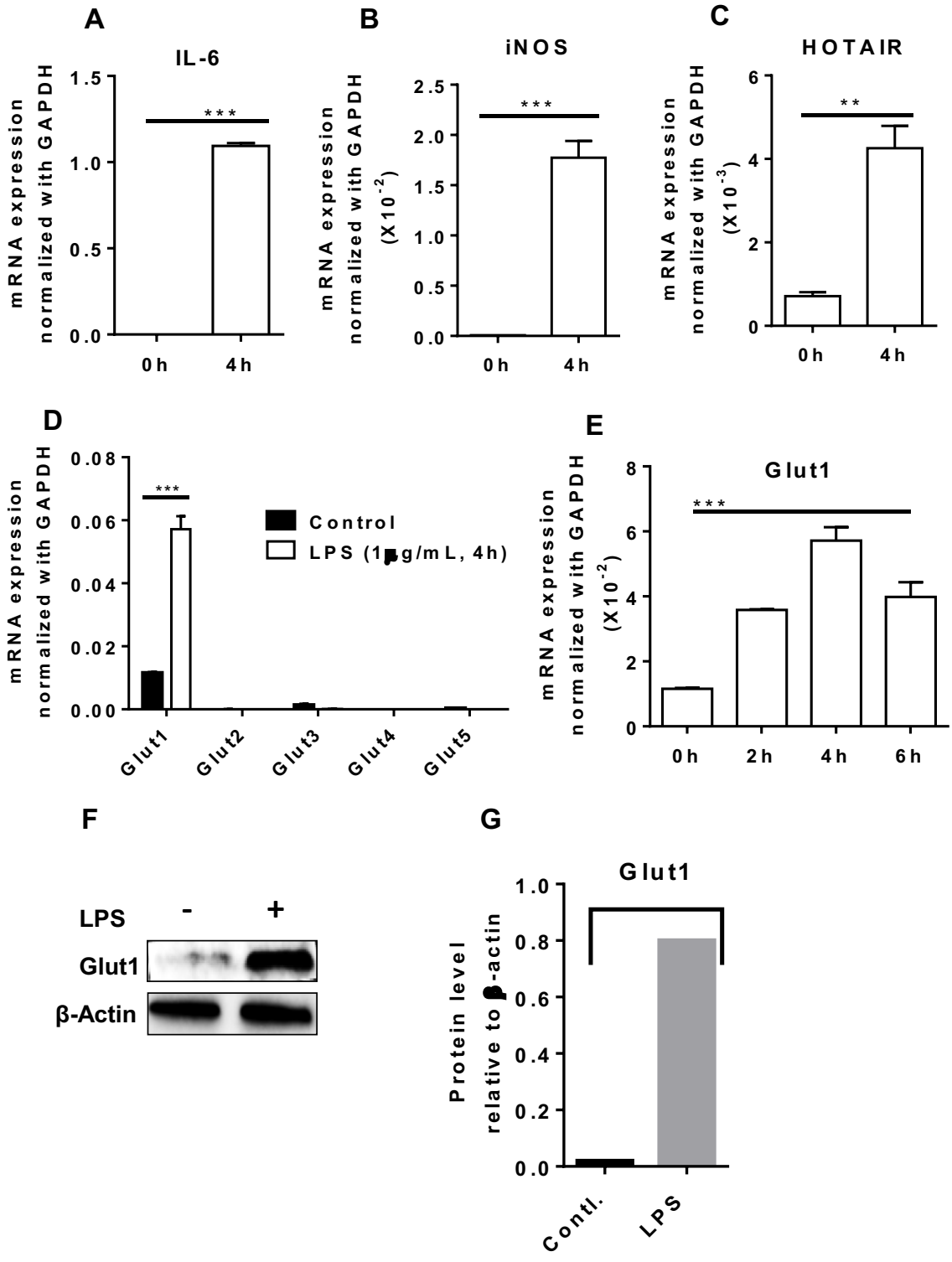

Figure 3.1. LPS induces Glut1 expression in macrophages. (A-E) RAW264.7 cells were treated with LPS (1 µg/mL) for 4 hours (or different time periods), total RNA was isolated, reverse transcribed to cDNA and the expressions of IL-6, iNOS, HOTAIR and different glucose transporters (Glut1, Glut2, Glut3, Glut4 and Glut5) were analyzed by RT-qPCR. GAPDH was

used as control. The expression (relative to GAPDH) of IL-6, iNOS, HOTAIR, Glut1, Glut2, Glut3, Glut4 and Glut5 are shown in panels A-D. The expression of Glut1 at different time periods of LPS-treatment is shown in panel E. (F-G) LPS stimulated protein (1 h LPS-treatment) was extracted from macrophages and analyzed by Western blot using antibodies against Glut1 and β-actin (loading control). Quantifications (using ImageLab5.2.1software) is shown in panel G. Each experiment was repeated at least with three parallel replicates. Data represent mean ± SD (n=3); *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

3.3.2 Glut1 expression is regulated by NF-κB during LPS stimulation

The transcription factor NF-κB activation plays central roles in inflammation and immune response. NF-κB activation is required for the expression of cytokine and pro-inflammatory genes.[1, 8] Notably, under basal condition (in the absence of inflammation), NF-κB is complexed with IκBα and remains inactive.[35] However, upon inflammation, IκBα gets phosphorylated that results in its poly-ubiquitination followed by proteasomal degradation, and hence release of NF-κB (NF-κB activation). IKKβ is a kinase which phosphorylates IκBα. Therefore, inhibition of IKKβ results in NF-κB deactivation.[7, 35-36] To investigate if NF-κB activation is associated with LPS-induced Glut1 expression, we treated macrophages with an inhibitor of IKKβ (SC-514) and analyzed its impacts on LPS-induced Glut1 expression. Western blot analysis demonstrate that, upon treatment with LPS, the level of IκBα is decreased, while the level phospho-p65 (NF-κB subunit) is increased (compare lanes 1 and 2, Figure 3.2A). Notably, the LPS-induced IκBα degradation as well as increased phospho-p65 level, are reversed upon treatment with IKKβ-inhibitor (SC-514) (compare lanes 1, 2 and 4, Figure 3.2A, quantification in panel B). These observations suggest that LPS treatment resulted in NF-κB activation and this is inhibited by SC-514 treatment. Interestingly, RT-qPCR analysis demonstrated that LPS-treatment induced the expression IL-6, iNOS and HOTAIR and upon IKKβ-inhibition (SC-514 treatment), suppressed the LPS-induced expression of IL-6, iNOS and HOTAIR (Figures 3.2C-E). Interestingly, the Glut1 expression is increased upon treatment with LPS and this LPS-induced Glut1 expression

level is decreased upon treatment with SC-514 (compare lanes 1, 2 and 4, Figure 3.2F). The LPS-induced increase in Glut1 protein level and its decrease upon SC-514-treatement is also evident in the Western blot (compare lanes 1, 2 and 4, Figure 3.2A, quantification in panel B). These observations demonstrated that Glut1 expression is augmented upon LPS-induced inflammation and this is regulated via NF-κB activation.

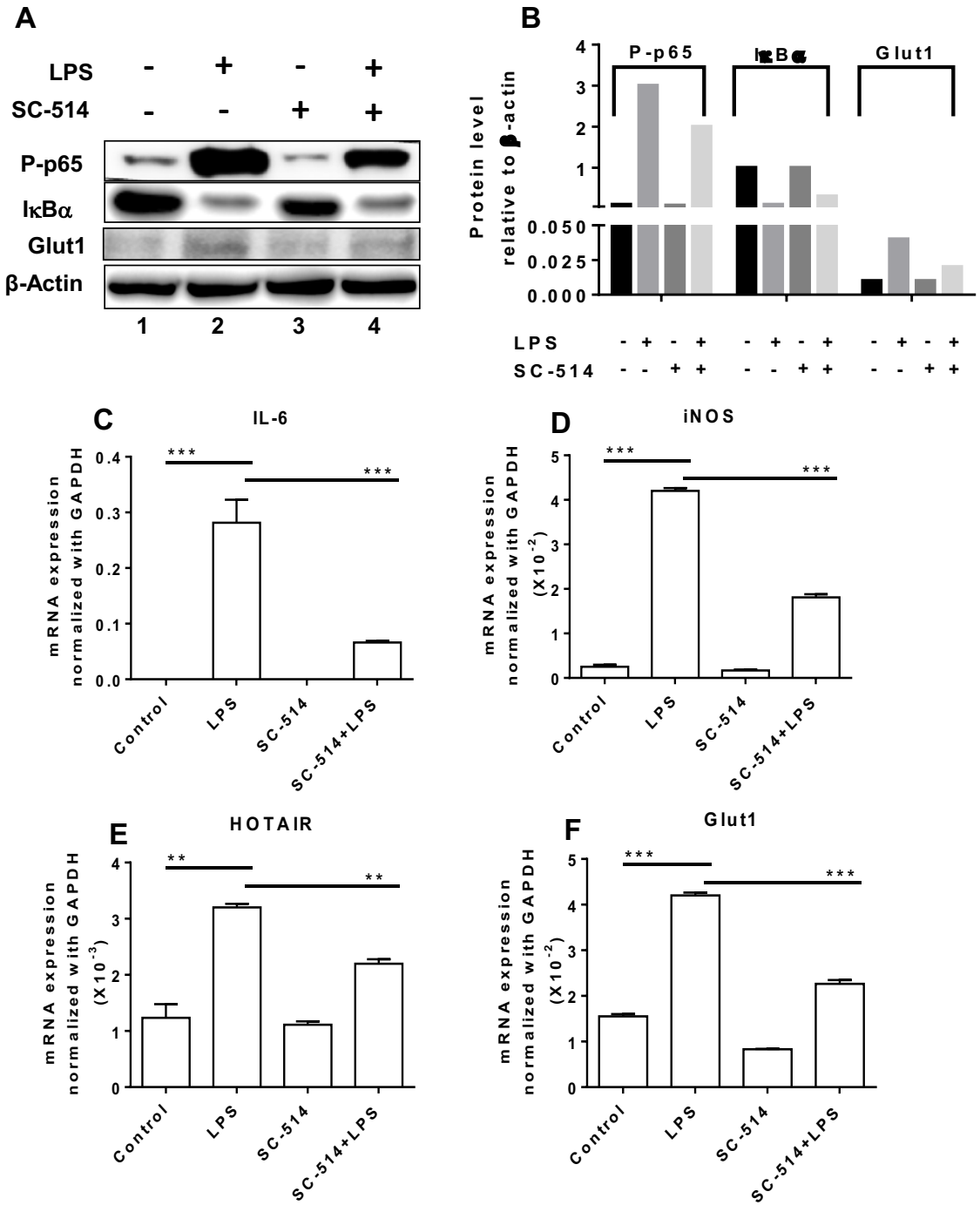

Figure 3.2. Inhibition of NF-κB downregulates LPS-induced Glut1 expression in macrophages. (A-B) RAW264.7 cells were initially treated with IKKβ-inhibitor SC-514 (for 1 h) and then treated with LPS for additional 1 h (for protein analysis) and 4 h (for RNA analysis). Proteins were analyzed by Western blotting using antibodies against phospho-p65 (NF-κB subunit), IκBα, Glut1, and β-actin (loading control) (panel A, quantifications in panel B). (C-F) RNA was isolated and expressions of IL-6, iNOS, HOTAIR, and Glut1 were measured by RT-qPCR. GAPDH was used as control. Data represent mean ± SD (n=3); *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

3.3.3 HOTAIR regulates LPS-induced Glut1 expression

Our recent studies demonstrated that HOTAIR plays key roles in NF-κB activation, cytokine regulation, and inflammation.[14] Here to investigate if HOTAIR plays any roles in regulation of Glut1 expression, glucose uptake, and metabolism, we knocked down HOTAIR (using siRNA) in macrophages, then treated with LPS, and analyzed its impacts on Glut1 expression (RNA and protein levels). Notably, RT-qPCR analysis demonstrated that the expression of HOTAIR is increased upon treatment with LPS and this LPS-induced expression of HOTAIR is significantly reduced (56%) upon application of HOTAIR siRNA (Figure 3.3A). Scramble siRNA has no significant impact on HOTAIR expression level (Figure 3.3A). Interestingly, RT-qPCR analysis demonstrated that Glut1 expression (mRNA) is increased upon LPS-treatment and the LPS-induced expression of Glut1 decreased upon HOTAIR-knockdown (Figure 3.3B). Western blot analysis demonstrated that Glut1 protein level is increased upon LPS-treatment and this LPS-induced Glut1 expression is also decreased upon HOTAIR-knockdown (HOTAIR-siRNA treatment (Figures 3.3C-D). These observations demonstrate that LPS-induced Glut1 expression is regulated by HOTAIR in macrophage cells.

Our previous mechanistic studies demonstrated that HOTAIR regulates LPS-induced degradation of IκBα and NF-κB activation.[14] Here, our WB analysis also showed that LPS-induced the degradation of IκBα and that is inhibited upon HOTAIR-knockdown (compare lanes

1, 2 and 4, Figure 3.3C-D). Concomitantly, the LPS-induced increase in phospho-p65 (NF-κB) level is also decreased upon HOTAIR-knockdown (compare lanes 1, 2 and 4, Figure 3.3C-D). These observations further demonstrate that HOTAIR regulates NF-κB activation via regulation of IκBα degradation and hence regulates Glut1 expression under LPS-induced inflammation.

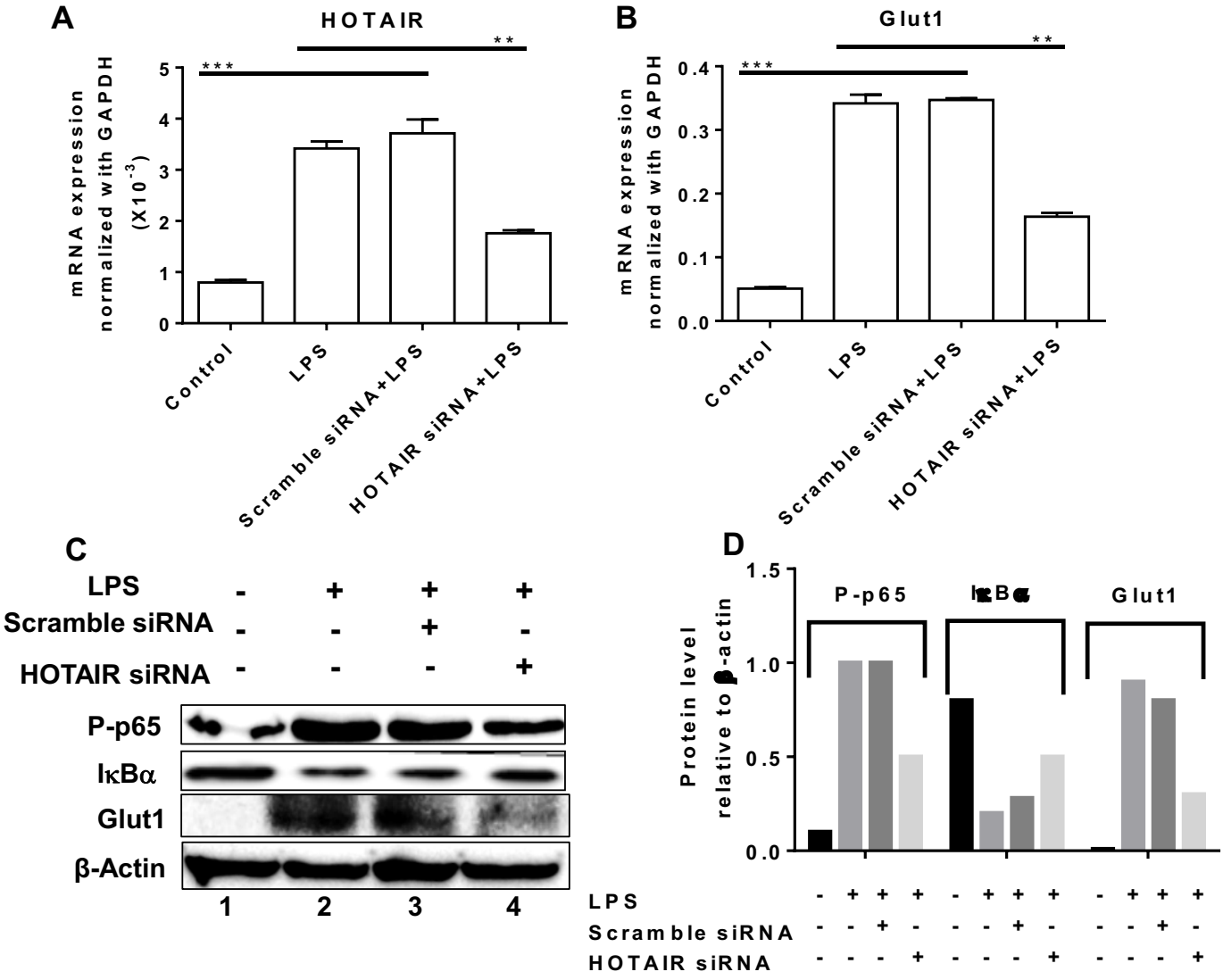

Figure 3.3. Knockdown of HOTAIR reduces LPS-induced Glut1 expression in macrophages. (A-B) RAW264.7 cells were transfected with HOTAIR-siRNA and scramble siRNA followed by treatment with LPS, RNA was analyzed by RT-qPCR for the expression of HOTAIR and Glut1. GAPDH was used as control. Data represent mean ± SD (n=3); *p < 0.05, **p < 0.001, ***p < 0.0001. (C) Proteins from HOTAIR-siRNA and scramble siRNA treatment

(48 h) followed by 1 h LPS-treated RAW264.7 cells were analyzed by Western blotting using antibodies against phospho-p65 (NF-κB subunit), IκBα, Glut1, and β-actin (loading control). (D) The changes in amounts of NF-κB, IκBα, and Glut1 have been quantified by ImageLab5.2.1 software.

3.3.4 HOTAIR regulates LPS induced NF-κB recruitment in the Glut1 promoter

Our studies demonstrate that HOTAIR is required for LPS-induced NF-κB activation.[14] NF-κB activation is critical for the expression of cytokines and pro-inflammatory genes.[1-2] Typically, upon inflammation, the activated NF-κB binds to the promoters of the NF-κB-regulated genes and cytokines and recruits other activators and coactivators resulting in NF-κB target gene activation.[37-38]As Glut1 expression is induced upon LPS-treatment in macrophage and is regulated by HOTAIR, and HOTAIR-regulates NF-κB activation upon LPS-stimulation, we investigated if NF-κB binds to the promoter of Glut1 in an LPS-dependent manner and if this is regulated via HOTAIR. Notably, we analyzed the Glut1 promoter for the presence of potential NF-κB binding sites as described by us previously[14, 32] and indeed, we found the presence of a typical NF-κB binding site(GGGGATGTCT) close to transcription start site (Figure 3.4, top panel). This is in agreement with previous studies.[39] The presence of NF-κB binding site close to the transcription start site of Glut1 suggests its potential regulation via NF-κB especially under inflammatory condition. Here, we examined the binding of NF-κB in the Glut1 promoter in the absence and presence of LPS and under HOTAIR-knockdown condition, using chromatin-immunoprecipitation assay (ChIP) as described by us previously.[14, 32] Briefly, control and HOTAIR-knocked down RAW264.7 treated with LPS were subjected ChIP using phosphorylated p65 (NF-κB subunit) and β-actin (control) and ChIP DNA fragments were PCR-amplified using primers specific to NF-κB binding sites present in Glut1 promoter. Interestingly, these analyses demonstrated that phospho-p65 (NF-κB) levels were enriched at the Glut1

promoter (NF-κB response element regions) upon treatment with LPS and this LPS-induced NF-κB binding was reduced upon HOTAIR-knockdown in Glut1 promoter (p-65 ChIP qPCR data in Figure 3.4 and also compare lanes 2 and 4).

Along with NF-κB, there are other coactivators which may be associated with LPS-induced Glut1 expression. For example, histone acetyltransferase CBP is known to interact with NF-κB to regulate NF-κB target genes.[37-38, 40] Our ChIP analysis demonstrates that similar to NF-κB, the level of CBP was also enriched at Glut1 promoter and this LPS-induced enrichment was alleviated upon knockdown of HOTAIR (Figure 3.4, compare lanes 2 and 4). Scramble siRNA treatment has no significant impact on the LPS-induced enrichment of NF-κB and CBP at the Glut1 promoter. As a control, we measured β-actin (antibody control), but no enrichment was observed irrespective of the LPS or HOTAIR siRNA treatment (Figures 3.4). These observations suggest that HOTAIR plays key roles in LPS-induced NF-κB activation and hence its enrichment at the Glut1 promoter to regulate its expression.

Histone H3K4-trimethylation and histone acetylation are also well known marks for gene activation.[3, 41-42] ChIP analysis demonstrated that levels of H3K4-trimethylation and histone acetylation as well as the level of RNA polymerase II (RNA pol II) were enriched at Glut1 promoter in the presence of LPS and this was decreased upon HOTAIR knockdown (Figure 3.4). This observation suggested that HOTAIR is required for promoter activation (H3K4-methylation and histone acetylation) of Glut1 and this is mediated via activation of NF-κB followed by recruitment of NF-κB and its coregulators including histone methyl-transferases and histone acetyl-transferases at the target gene promoters. The ChIP analysis demonstrated that LPS-induced expression of Glut1 is regulated via transcription factors NF-κB, CBP and other coactivators and this is regulated by HOTAIR via regulation of NF-κB activation.

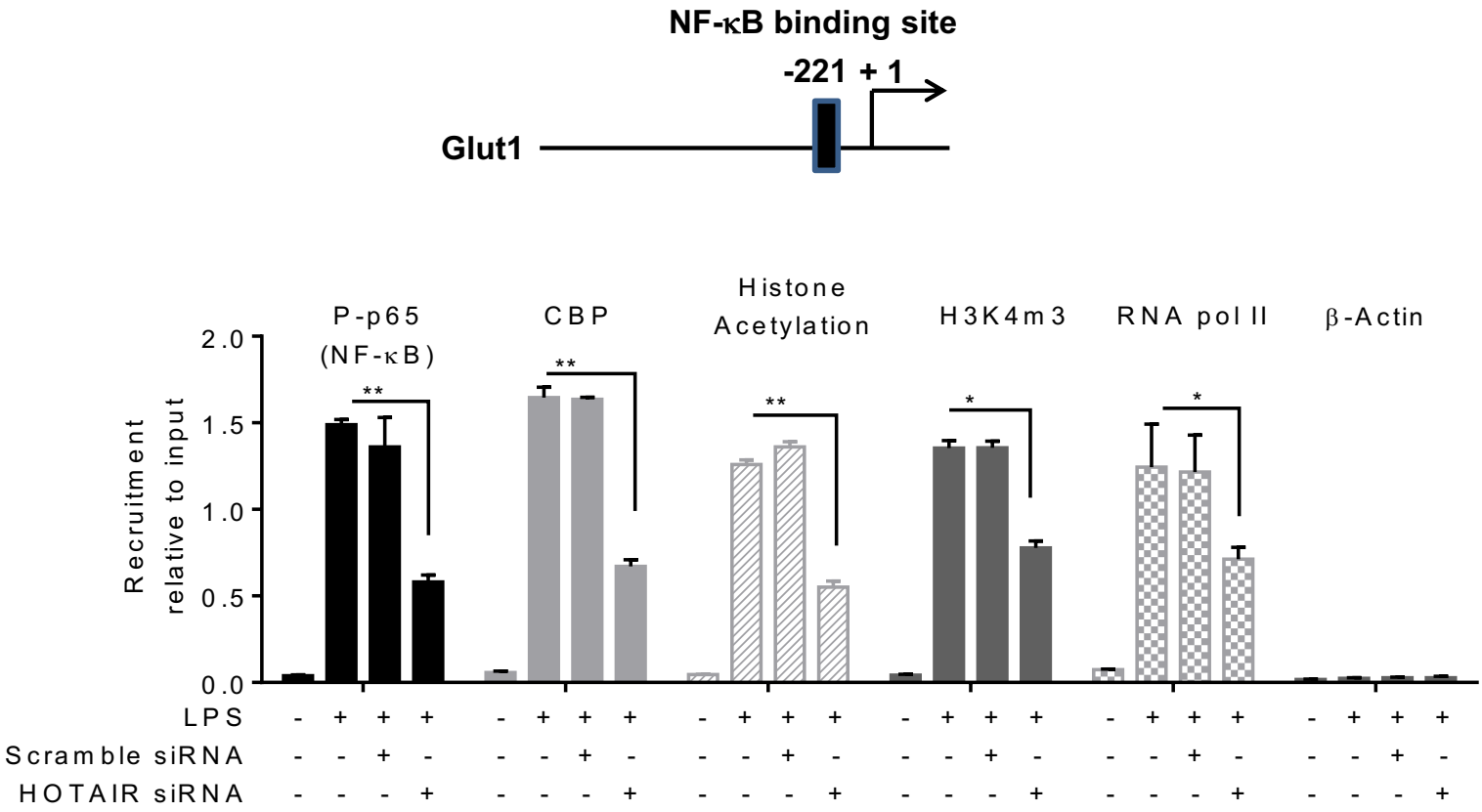

Figure 3.4. Knockdown of HOTAIR reduces the LPS-dependent recruitment of NF-κB on Glut1 promoter. RAW264.7 macrophage cells were transfected with HOTAIR and scramble-siRNA, then treated with LPS (1.5 h). Cells were then fixed with formaldehyde and subjected to ChIP assay using antibodies specific to phospho-p65, CBP, histone acetylation, H3K4m3, RNA pol II and β-actin (control). The immunoprecipitated DNA fragments were analyzed by qPCR using primers specific to the NF-κB binding regions on Glut1 promoter. The location of NF-κB binding site at the Glut1 promoter is shown. Each experiment was repeated at least thrice (n = 3). Data represent mean ± SD; *$p < 0.05$, **$p < 0.001$.

3.3.5 HOTAIR regulates glucose uptake under LPS-simulation

As HOTAIR regulated LPS-induced Glut1 expression in macrophage, we investigated if HOTAIR regulates level of glucose uptake in macrophage cells in presence of LPS. Notably, Glut1 is a cytosolic protein and this translocates to the cell membrane and thus allows glucose uptake during glucose metabolism.[43] It is well-recognized that the level of glucose uptake and metabolism is increased during inflammation and this helps cells to tackle critical inflammatory situation.[10, 13] Here, our studies demonstrate that Glut1 expression is increased upon LPS-stimulation in macrophage and this is regulated by HOTAIR lncRNA. Therefore, we hypothesize the under LPS-stimulation condition, the level of glucose uptake will be increased and this might be regulated via HOTAIR. To test this hypothesis, we measured the level of uptake of glucose (using a commercial kit) in macrophage cells under LPS treatment and in the presence of absence of HOTAIR-knockdowns. Briefly, macrophages were treated with HOTAIR siRNA (and scramble siRNA) for 48 hours and then stimulated with insulin followed by 2-deoxyglucose (2-DG) treatment. 2-DG is taken by the cells by glucose transporter (Glut1 in our case) and gets phosphorylated to 2-DG6P. Accumulation of 2-DG6P is proportional to glucose uptake by the cells. The accumulated 2-DG6P level was determined by colorimetric reactions.[12, 19] Our results demonstrated that the level of glucose uptake is increased (10 folds) in macrophages upon treatment with LPS and the LPS-induced increased glucose uptake level is significantly reduced upon HOTAIR knockdown (HOTAIR-siRNA treatment) (Figure 3.5). Scramble siRNA has no significant impacts on LPS-induced glucose uptake level. These observations demonstrate that HOTAIR regulates the LPS-induced glucose uptake during inflammation and this is likely mediated via induction of Glut1 expression.

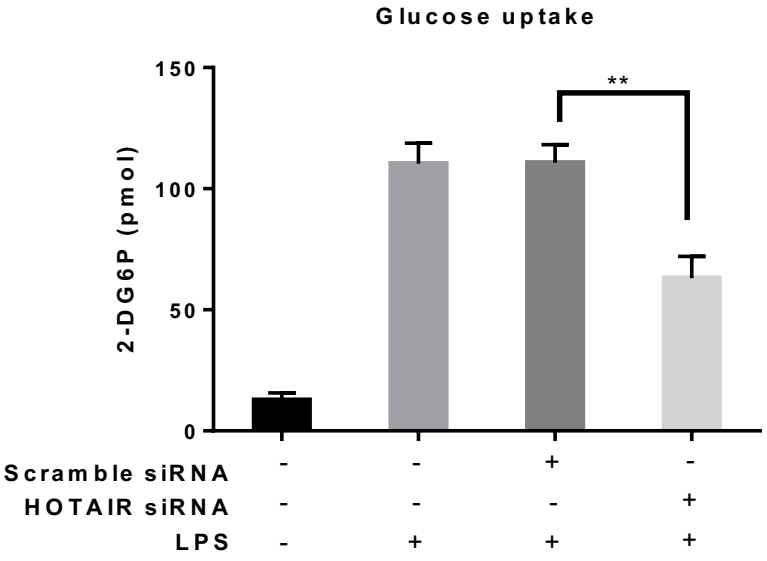

Figure 3.5. Knockdown of HOTAIR reduces the uptake of glucose in macrophages. RAW264.7 macrophages were treated with HOTAIR and scramble-siRNA for 48 hours and after overnight incubation, cells were stimulated with insulin (+/-), followed by 2-deoxyglucose addition for 20 minutes. 2-DG6P was oxidized and that generates NADPH, which was quantified calorimetrically (measured at 412 nm in a microplate reader). Each experiment was repeated at least thrice (n = 3). Data represent mean ± SD; *$p < 0.05$, **$p < 0.001$.

3.4 Discussion

LncRNA HOTAIR is involved in activation of immune cells (e.g. macrophages) in response to pathogen associated molecular patterns such as LPS.[14] Activation of immune cells in response to infection or other stressors is a metabolically expensive event.[15] The immune cells preferentially meet their energy needs by metabolizing glucose.[27] Furthermore, glucose metabolism is involved in mounting inflammatory immune responses.[11] Interestingly, the inflammatory response induces expression of glucose metabolizing enzymes and related factors such as Glut1, glucose-6-phosphate dehydrogenase, hexokinase, and increase glycolysis.[44-45]

Therefore, we investigated if HOTAIR is involved in inflammation induced metabolic reprogramming.

Glut1 is the primary glucose transporter in macrophages.[9] The proinflammatory activation of macrophages by LPS induces M1 polarization and increases Glut1 expression.[9] Consistent with previous findings, we found out that Glut1 is a major glucose transporter in macrophage and in response to LPS, macrophages induces higher expression of Glut1, as well as proinflammatory genes such as IL-6, iNOS (which are also indicators of macrophages M1 polarization) (Fig 3.1). Previously, we discovered that expression lncRNA HOTAIR is increased in macrophages upon exposure to TLR ligand (e.g. LPS).[14] We also discovered that HOTAIR induces IL-6 and iNOS expression by activating NF-κB via IκBα degradation.[14] Here, I aimed to understand whether HOTAIR controls the expression glucose transporter Glut1, glucose uptake, and metabolism in macrophages during inflammatory responses. Our studies indeed demonstrated that Glut1 is the primary glucose transporter in macrophage cells and its expression is induced upon treatment with LPS. Most importantly, our results also demonstrated that lncRNA HOTAIR is required for the LPS-induced expression of Glut1 in macrophage. siRNA-mediated knockdown of HOTAIR suppressed the LPS-induced expression of Glut1 level in macrophage, suggesting critical roles of HOTAIR in glucose metabolism during inflammatory reposne.

NF-κB activation plays a central role in immune response and inflammation. NF-κB activation induces expression of NF-κB regulated cytokines and pro-inflammatory genes.[46-47] Though it is well known that activation of immune cells in response to infection or other stressors is a metabolically expensive event and metabolizing glucose is a major source of energy to meet the energy requirement of inflammatory response, the detailed signaling mechanism by

which NF-κB activation is integrated to glucose metabolism remains elusive. Glut1 expression is known to be regulated by mTOR and cMyc in cancer cells.[9, 11, 13] Our mechanistic studies demonstrate that lncRNA HOTAIR play critical roles in regulation of NF-κB activation via degradation of IκBα.[14] Additionally, we observed that the inhibition (using SC-514 treatment) of NF-κB significantly reduces inflammation induced-Glut1 expression (Fig. 3.2). Furthermore, we found out that knockdown of HOTAIR by HOTAIR-specific siRNA leads to down regulation of LPS-induced Glut1 expression (Fig. 3.2). This likely because of reduced NF-κB activation under HOTAIR knockdown condition. Importantly, NF-κB activation regulates cytokines and pro-inflammatory genes expression via binding to its target gene promoters, followed by recruitment of activators, coactivators, chromatin modification and remodeling. Typical NF-κB regulated genes promoters contain binding sites for NF-κB and upon inflammation, activated NF-κB gets recruited to the target gene promoters resulting in their gene activations.[37] Our analysis demonstrated that similar to well-known NF-κB target genes (such as, IL-6, iNOS, etc), the Glut1 promoter has NF-κB binding site close to the transcription start site (Fig. 3.4 top pannel). Our ChIP analysis indeed demonstrated that upon LPS-stimulation, NF-κB activated and is enriched at the Glut1 promoter (NF-κB response element). Along with NF-κB, histone acetyl-transferase and NF-κB associated activators CBP/p300 is enriched at the Glut1 promoter in response to LPS-treatment.[37-38]Gene activation associated chromatin modifications such as histone H3K4-trimethylation and histone acetylation levels are also increased. Importantly, these LPS-induced enrichments in NF-κB, CBP, H3K4-trimethyl, histone acetylation and RNA polymerase II levels, were reduced upon knockdown of HOTAIR. HOTAIR promotes IκBα degradation which makes NF-κB free from its inhibitory effect and induces availability of activated NF-κB for binding to its target genes promoter such as Glut1 promoter. These

observations suggest further about the crucial roles of HOTAIR in LPS-induced activation of NF-κB, Glut1 expression, and potentially in glucose metabolism under inflammation.

Consistently, we found HOTAIR promotes glucose uptake and metabolism in macrophages during inflammatory responses. Indeed, Glut1-mediated glucose metabolism drives metabolic re-programming of macrophages by switching fuel metabolism from oxidative to glycolytic pathway. Furthermore, elevated Glut1-mediated glucose uptake and metabolism forced macrophages into a hyper-inflammatory state with increased production of multiple inflammatory mediators. Since we observed that Glut1 is a major glucose transporter in macrophages and HOTAIR induces Glut1 expression, we conclude that HOTAIR controls glucose metabolism as well as metabolic programming in macrophages and mounts inflammatory immune signaling in response to pathogenic infection. Notably, a recent study reported that HOTAIR promotes glycolysis in hepatocytes and this further supports our observation of about the potential roles of HOTAIR in regulation of glucose metabolsim.[48] A model, showing the potential roles of HOTAIR in regulation of glucose uptake via inducing

Glut1 expression through IκBα degradation and NF-κB activation, its translocation into the nucleus and binding to the Glut1 promoter is shown in figure 3.6.

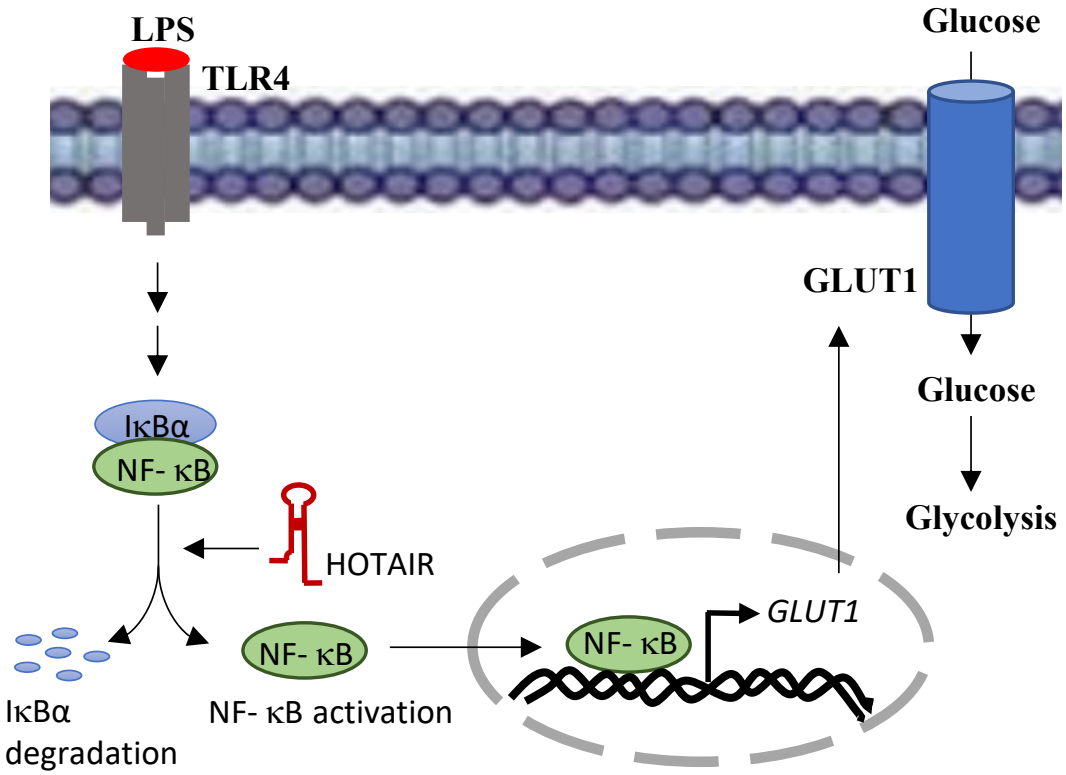

Figure 3.6: Proposed model for HOTAIR mediated regulation of glucose metabolism during inflammation. When TLR4 senses LPS, NF-κB is activated that induces HOTAIR expression. In turn, HOTAIR facilitates IκBα degradation and enhances NF-κB activation, its nuclear translocation, and binding to NF-κB regulated Glut1 gene promoter inducing its expressions. Overexpressed Glut1 is translocated to membrane, allows increased glucose uptake and glucose metabolism, during inflammation and immune response.

Importantly, glucose metabolism and inflammation in macrophages are closely associated with variety chronic metabolic disorder such as obesity and diabetes. Therefore, our studies showing the critical roles of lncRNA HOTAIR in inflammation and metabolic reprogramming in macrophages provides novel signaling pathways that may serve as a novel therapeutic avenue in the treatment of inflammatory, immune and metabolic disease.

3.5 Acknowledgement

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Chapter 4

Discovery of lncRNA, LinfRNA1, a novel regulator of NF-κB activation and Inflammation

4.1 Introduction

Inflammation is a vital biological process associated with immune response.[1] This is triggered by various harmful stimuli, such as pathogens, toxic compounds, or irradiation.[1-2] Immune response ultimately removes injurious stimuli and initiates the healing process.[3] However, uncontrolled inflammation may become chronic and contribute to a variety of inflammatory diseases including metabolic disorders, immune disorders, obesity, diabetes, and cancer.[4] Activation of inflammatory response triggers a cascade of signaling pathways, most commonly the NF-κB, MAPK, and JAK-STAT pathways.[5-6] Toll like receptors (TLRs) are cell surface receptors widely expressed on immune cells such as monocytes, macrophage, dendritic cells etc.[7] In particular, upon pathogenic infections, TLR4 is activated which triggers a series of signaling cascade including NF-κB activation, and that induces release of critical pro-inflammatory cytokines necessary for immune responses.[8-9]

Emerging evidence suggests that noncoding RNAs (ncRNAs) are crucial factors in regulation of variety signaling pathways including in immune response and inflammation.[10] For example, lincRNA-Cox2, linc1992/THRIL, nc-1L-7R, NeST RNA, NEAT1 and others have been reported as major players in the regulation of immune and inflammatory responses.[11-14] LincRNA-Cox2 controls IL-6 expression whereas linc1992/THRIL regulates expression of IL-8, CXCL10, CCL1, and CSF1,[12] nc-1L7R knockdown causes IL-6 and IL-8 mRNA levels to be reduced,[13] NeST RNA stimulates IFNγ secretion in $CD8^+$ T cells,[14] NEAT1 accelerates transcriptional activation of IL-8.[12]

In a recent preliminary study, we have made an exciting discovery for an essential role of a long noncoding RNA (lncRNA), HOTAIR (HOX transcript antisense intergenic RNA), in inflammatory response in macrophage.[15] HOTAIR is well known as a major player in gene silencing and cancer and this is coordinated via recruitment of gene silencing factors on the chromatins.[16-17] In our study, we found that HOTAIR expression is induced by lipopolysaccharide (LPS, a potent bacterial endotoxin) and is required for LPS-induced expression of cytokines, chemokines, and pro-inflammatory genes, in macrophage.[15] HOTAIR regulates NF-κB activation via degradation of IκBα, suggesting its potential roles in immune response and inflammation.[15] Importantly, though our initial studies are focused on evaluating functions of HOTAIR in inflammation, however, there may be other lncRNAs which are important to immune response and inflammation. To identify lncRNAs linked to inflammation in an unbiased manner, we performed whole transcriptome RNAseq analysis in primary BMDM (bone marrow derived macrophages) cells treated with LPS. These analyses resulted in discovery of several novel lncRNAs (**LinfRNAs** - **L**ong noncoding **inf**lammation associated **RNA**s) that are potential regulators of inflammatory response. In particular, we have demonstrated that LinfRNA1 is a novel lncRNA which regulates NF-κB activation and inflammatory response in macrophage.

4.2 Materials and methods

4.2.1 Isolation of BMDMs, treatment with LPS, and isolation of RNA

Mice bones were used to isolate and culture bone marrow derived macrophages (BMDMs) as described earlier.[15] After buying from Jackson Laboratory, wild type (C57BL6/J) mice were maintained in a specific pathogen free (SPF) facility at UT Southwestern Medical center. All studies were approved by the Institutional Animal Care and Use Committee (IACUC)

and were conducted in accordance with the IACUC guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Femur and tibia from mice legs were collected and bone marrows were flushed out with Iscove's Modified Dulbecco's Medium (IMDM), (12440061, Life technologies) by using 25G needle. The bone marrows were then processed for single cell suspension by passing through 22G needle two times. After centrifuging the suspension at 1000 RPM for 5 min, the pellet was re-suspended with BMDM culture media (L-cell-conditioned IMDM medium supplemented with 15% L929 supernatant, 10 % FBS, 1 % nonessential amino acid, and 1 % penicillin-streptomycin) followed by seeding in three 150 mm culture dishes and cultured for 6 days to differentiate into macrophages, while at day 3, 10 ml fresh BMDM culture media was added into each plate. The culture plate was washed with ice cold PBS after day 6 and cells were gently scraped with ice cold PBS using cell scrapper. BMDMs were centrifuged at 1000 rpm for 5 min and re-suspended into BMDM media, counted and seeded in 6-well ($2.5x10^6$/well) cell culture plates.

4.2.2 RNA isolation from BMDMs, RNA-Seq experiments and data analysis

BMDMs were incubated overnight and then treated with LPS (±). Total RNA from the treated BMDMs was extracted by RNeasy plus mini kit (Qiagen). Isolated RNA was quantified using a Qubit fluorometer (Invitrogen) and total RNA libraries were constructed using Illumina TruSeq Rib-zero Gold stranded kits. Each sample was uniquely indexed and all four libraries were mutliplexed into a single, pooled library along with 44 other libraries unrelated to this project. Libraries were sequenced on an Illumina NovaSeq S2 flowcell using 100bp paired-end sequencing. TruSeq adapter sequences were trimmed during the de-multiplexing via the sample sheet on Illumina BaseSpace cloud (basespace.illumina.com). Trimmed RNA-seq reads were mapped to the *Mus musculus* UCSC mm10 reference genome[18] using the STAR aligner[19] via the

RNA-seq Alignment v1.1.1 app in Illumina BaseSpace cloud. Differentially expressed reads between control and experimental groups were determined using the DEseq2 v1.1.0[20] app on Illumina BaseSpace cloud. Significantly differentially expressed genes were visualized across all samples as heat maps that were generated in R, with genes clustered by expression pattern similarity using the R-package vegan.[21] Significantly differential genes were grouped into 'up-regulated' and 'down-regulated' groups and were used separately to perform a GO enrichment analysis to determine if significantly differentially expressed gene sets were enriched for particular functional categories of genes.[22] We consider GO term categories as significantly enriched if the ratio test resulted in a Bonferonni-corrected p-value ≤ 0.05. For the sake of visualizing results, enriched GO terms were summarized by removing redundancies using REViGO with default settings.[23] To analyze non-coding RNA, reads pulled reads annotated as "ncRNA" from the GRCm38 mouse reference genome downloaded from Ensembl to obtain gene IDs that were not protein coding.[24] Genes of interest were visualized in heat maps using R. Genes were clustered by expression pattern based on an average linkage hierarchical clustering based on Bray Curtis dissimilarity matrix using an R-package, VEGAN.[21]

4.2.3 Cell culture and treatment with Lipopolysaccharide (LPS)

Mouse macrophage RAW264.7 cells were collected from American Type Cell Culture collection. The macrophages were then cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10 % heat-inactivated FBS (Fetal bovine serum), 2 mM L-glutamine, 100 units/mL penicillin and 0.1 mg/mL streptomycin in a humidified incubator with 5% CO2 and 95% air at 37°C.[15] After cell counting, 2 $x10^6$ cells were seeded in 60 mm cell culture plates. Macrophages were treated with ultrapure *E.coli*-derived LPS (Invivogen) at 1.0 μg/mL after overnight incubation for different time periods. The concentration

of LPS has been determined by literature review as it has been widely used in various other laboratories to induce immune and pro-inflammatory response in macrophages. Cells were harvested for the preparation of RNA and protein analysis.[15, 25]

4.2.4 RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. Briefly RAW264.7 cells treated with various conditions were harvested using TRIzol, mixed with chloroform and centrifuged at 12000 rpm for 15 min. The aqueous layer was mixed with equal volume of 100 % ethanol and centrifuged at 12000 rpm for 10 min. The pellet was washed with 70 % ethanol, and RNA was finally dissolved in 30-50 µL of RNase-free water (Sigma) and quantified using a Nanodrop spectrophotometer. 1 µg of the isolated RNA was reverse transcribed into cDNA using iScript RT-supermix (Bio-Rad).[15, 25]

4.2.5 Real time PCR

Real-time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad), with gene specific PCR primers as listed in Table 4.1, in CFX96 real-time detection system (Bio-Rad). Each experiment was repeated three times with three parallel replicates each time. Expression data were normalized to GAPDH and expressed as $2^{-\Delta Ct}$.[26-27]

4.2.6 Western blot analyses

RAW264.7 cells were washed in ice-cold PBS and then lysed in cell lysis buffer comprising 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 % Igepal CA-630, 0.5 % Na-deoxycholate, 2 mM Na_3VO_4, and complete protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche).[28] The resulting cell lysates were centrifuged for 10 min at 13,000 rpm at 4°C, and the protein concentrations in the supernatants were determined using a BCA protein assay kit (Pierce). 20µg proteins were loaded onto 10 % SDS-PAGE gels, followed by electro-

transfer onto PVDF-membrane (Immobilon-P, Millipore). The membranes were blocked in 1 x TBST (0.1 % Tween-20, 20 mM Tris–Cl (pH 8.0), and 150 mM NaCl) containing 3 % skimmed milk and then incubated with the primary antibodies against IκBα (1:1000 dilution, 4814S, Cell Signaling), Phospho-IκBα (1:1000 dilution, 2859S, Cell Signaling), Phospho-p65 (NF-κB subunit, 1:1000 dilution, 3033S, Cell Signaling) and β -actin (1:1000 dilution, A2066, Sigma) overnight at 4 °C. Membranes were washed 3 times (1xTBST), incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and then washed 3 times (1 x TBST). At last, immunoreactive proteins were detected using ECL -super signal west femto substrate reagent (Thermo Scientific).[28] The quantification was done by Image Lab 5.2.1 software.

4.2.7 SiRNA-mediated knockdown of LinfRNA1

For the siRNA transfection, RAW 264.7 cells were grown up to 60 % confluency in 60 mm culture plates and transfected with LinfRNA1-siRNA, a pool of 4 different siRNA constructs (SI02856490, SI02856497, SI02856504, and SI02856483, Qiagen) and scramble siRNA (1027310, Qiagen) independently using GenMute siRNA and DNA transfection reagent (SL100568, SignaGen Laboratories) according to the manufacturer's protocol.[29] Prior to transfection, a cocktail of transfection reagent and siRNA was made as follows. Initially, 12 µL (12 µg) of GenMute reagent was mixed with 300 µL DMEM (without FBS and antibiotics) in an eppendorf tube. In a separate eppendorf, siRNA was mixed with 100 µL DMEM (without supplements). Then the diluted siRNA solution was mixed with diluted GenMute reagents and allowed to stand for 30 min in the dark. In the meantime, cells were washed twice with supplement-free DMEM and then 1.7 mL of supplement-free DMEM was added to each cell culture plate. Finally, siRNA transfection reagents cocktail was applied to the cell plates, mixed

gently and incubated for 48 h. Cells were then stimulated with LPS (1 μg/mL) for specified time period and then harvested for RNA/protein extraction or for ChIP assay.[15]

4.2.8 NF-κB inhibition assay

RAW264.7 macrophages (2 x 10^6) were seeded in 60 mm cell culture plates. After overnight incubation cells were initially treated with IKKβ inhibitor (25 μM, SC-514, Sigma)[30] for 1 h to inhibit NF-kB signaling pathway and then cells were treated with LPS (1 μg/mL) and incubated for additional period of time 4 h. Cells were harvested, total RNA was isolated using TRIzol reagent, reverse transcribed to cDNA and analyzed by qPCR . GAPDH was used as control.[15] Protein was also extracted (SC-514 1 h and additional 1 h LPS treatment) after cell harvesting for Western blot.

4.2.9 Enzyme linked immunosorbent assay (ELISA)

LinfRNA1 was silenced in RAW264.7 macrophages by using LinfRNA1 (AW112010) siRNA, a pool of 4 different siRNA constructs (SI02856490, SI02856497, SI02856504, and SI02856483, Qiagen) by the use of GenMute siRNA and DNA transfection reagent (SL100568, SignaGen Laboratories) according to the manufacturer's protocol.[29] 48h following transfection RAW264.7 macrophages were stimulated with LPS for 12h or kept untreated. Culture media were collected and amount of IL-6 secreted in culture media were measured using ELISA kits (DY406-05, R&D Systems) according to manufacturer's instruction.[31]

4.2.10 Statistical Analysis

Each experiment was done in two or three replicates, and then cells were pooled (and treated as one sample), subjected to RNA extraction, RT-PCR, and each experiment was repeated at least three times (n = 3). The real-time PCR analysis of such samples were done in three replicate reactions and repeated in all three independent experiments (n=3). Data are

presented as means ± SD (as stated in the figure legends). Statistical significance was determined by unpaired Student's t test (GraphPad Prism 6), and $P \leq 0.05$ was considered statistically significant.

Table 4.1: Sequences of PCR primers

Primers	Forward (5' – 3')	Reverse (5' – 3')
LinfRNA1	TGGTGTGCTCATCATCTGCC	ATGACGACCTGGGTCTGGTA
IL-6	CAAGAAAGACAAAGCCAGAGTC	GAAATTGGGGTAGGAAGGAC
iNOS	TGTGACACACAGCGCTACAACA	GAAACTATGGAGCACAGCCACAT
Mir155	TAATGCTAATTGTGATAGGGGTTTT	TGTTAATGCTAACAGGTAGGAGTCAG
GAPDH	ACCCAGAAGACTGTGGATGG	CACATTGGGGGTAGGAACAC

4.3 Results

4.3.1 LinfRNA1 is a novel lncRNA associated with LPS-induced inflammation in macrophages.

In order to identify novel lncRNAs associated with inflammation and immune response in an unbiased manner, we performed RNAseq analysis in LPS-treated macrophages. Briefly, BMDM was isolated from mice bones and cultured as described earlier.[25, 32] All studies were approved by the Institutional Animal Care and Use Committee (IACUC). BMDM was treated with LPS (1 μg/mL) for 2 h. Total RNA from the control and LPS-treated BMDM were subjected to RNAseq analysis (UTA Genomics Core Facility). Significantly, differentially expressed genes were visualized across all samples as heat maps. Based on the RNAseq analysis, we found that upon LPS-treatment, 8,324 were significantly differentially expressed genes in comparison to the control **(Fig. 4.1)**. As anticipated, many well-known marker genes of inflammation such as IL6, NOS etc are significantly upregulated upon treatment with LPS (Figure 4.1). Interestingly, along with the well-known markers of inflammation, several noncoding RNAs (176 total lncRNA) were significantly differentially expressed upon LPS-

treatment in BMDMs. The most upregulated noncoding RNAs include Mir155, LinfRNA1 (AW112010), LinfRNA2 (G53001006RIK) and others are shown in the heatmap in Figure 4.1. We focused our studies in characterizing the functional roles of one lncRNA, LinfRNA1, in inflammatory signaling in macrophage.

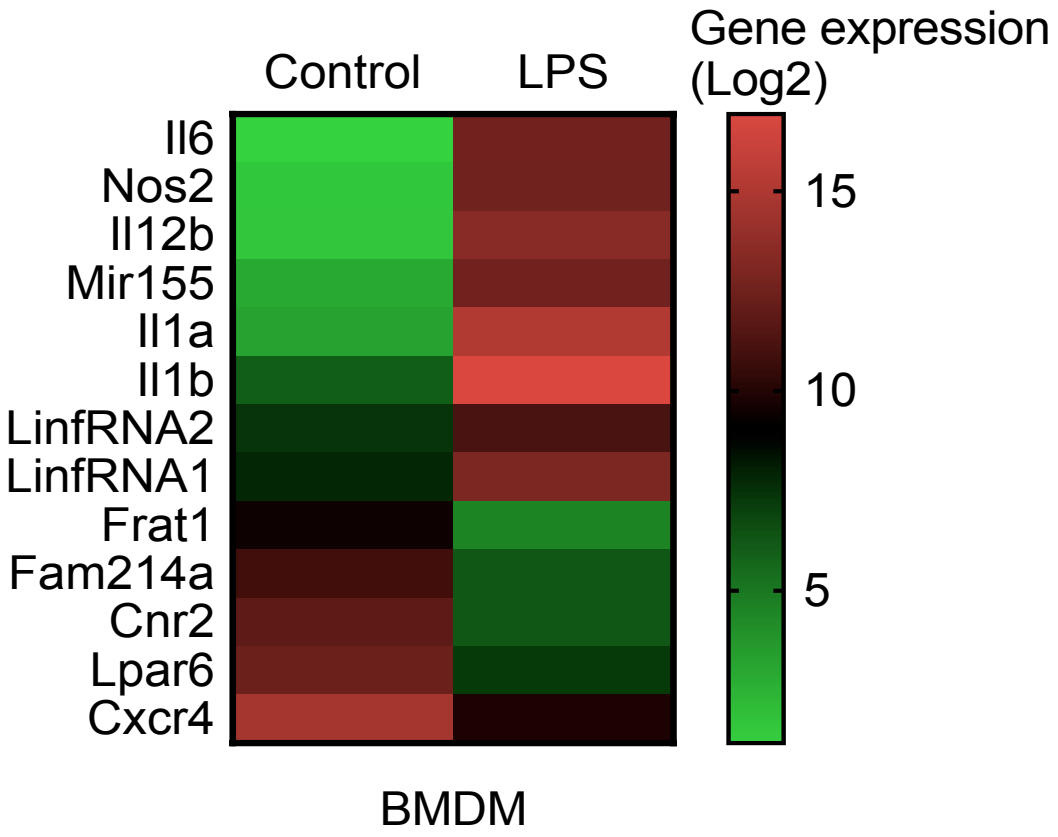

Figure 4.1. RNA-Seq data showing the heat map for highly upregulated and downregulated genes and noncoding RNAs. BMDMs (mouse) were treated with LPS (2 h). RNA was isolated from control and LPS-treated BMDMs. Total RNA libraries were constructed using Illumina TruSeq Rib-zero Gold stranded kits and sequenced on an Illumina NovaSeq S2 flowcell using 100bp paired-end sequencing. Significantly differential genes and noncoding RNAs were plotted as heat map. A new lncRNA, LinfRNA1 (AW112010), has been identified.

4.3.2 LinfRNA1 expression is induced by LPS in primary macrophage (BMDMs) as well as in RAW264.7 macrophage cells

To further confirm the LPS-induced expression of the noncoding RNAs and inflammatory genes, we performed RT-qPCR analysis with RNA samples obtained from the control and LPS-treated

BMDM cells. Briefly, BMDM cells were treated with LPS for 2 and 4 hours and then RNA was isolated, reverse transcribed into cDNA and analyzed by qPCR. The analysis of IL-6 and iNOS expression were used as positive control. These analyses demonstrated that, as anticipated, IL-6 and iNOS are significantly overexpressed in BMDM upon treatment with LPS. The expression of IL-6 was induced by 2300 and 4100 folds at 2 and 4 h post LPS-treatment, respectively (Figure 4.2A). Similarly, the expression of iNOS was also induced by 322 and 983 folds at 2 and 4 h, respectively, upon treatment with LPS (Figures 4.2B). Interestingly, RT-qPCR analysis also showed that expression of microRNA, miR155, and lncRNA, LinfRNA1, are also significantly induced upon treatment with LPS (Figure 4.2). The expression of miR155 increased up to 734 and 872 folds after 2 and 4 h LPS induction (Figures 4.2C) and the lncRNA linfRNA1 expression was also induced by 32 and 370 folds at 2 and 4 h, post LPS-treatment (Figures 4.3D). The induction of IL-6 and iNOS upon LPS stimulation demonstrated the validity of the experiment. The LPS-induced expression of miR155 and LinfRNA1 suggest their potential roles in immune response and inflammation. Notably, miR155 is a well known and well characterized micro RNA associated with inflammation and immune response and associated with a variety of human disease including in chronic lymphocytic leukemia and myeloproliferative disease.[33-35] Thus identification of miR155 demonstrates the validity of our RNAseq analysis data. Interestingly, however, very little is known about the structure and functions of LinfRNA1 and other LinfRNAs identified in our RNAseq studies. LinfRNA1 (AW112010) was originally detected in a genome wide expression screening and was expressed in inflammatory bowel disease and fibrosis.[36-37] It is 790 nt long lncRNA, located in chromosome 19. However, there is no functional studies done. Additionally, our studies also have identified a series of other novel LinfRNAs associated with inflammation, though their structural and functional significance in

immune response and inflammation remain mostly unknown. Here we have further investigated the function of LinfRNA1 in inflammation and immune signaling.

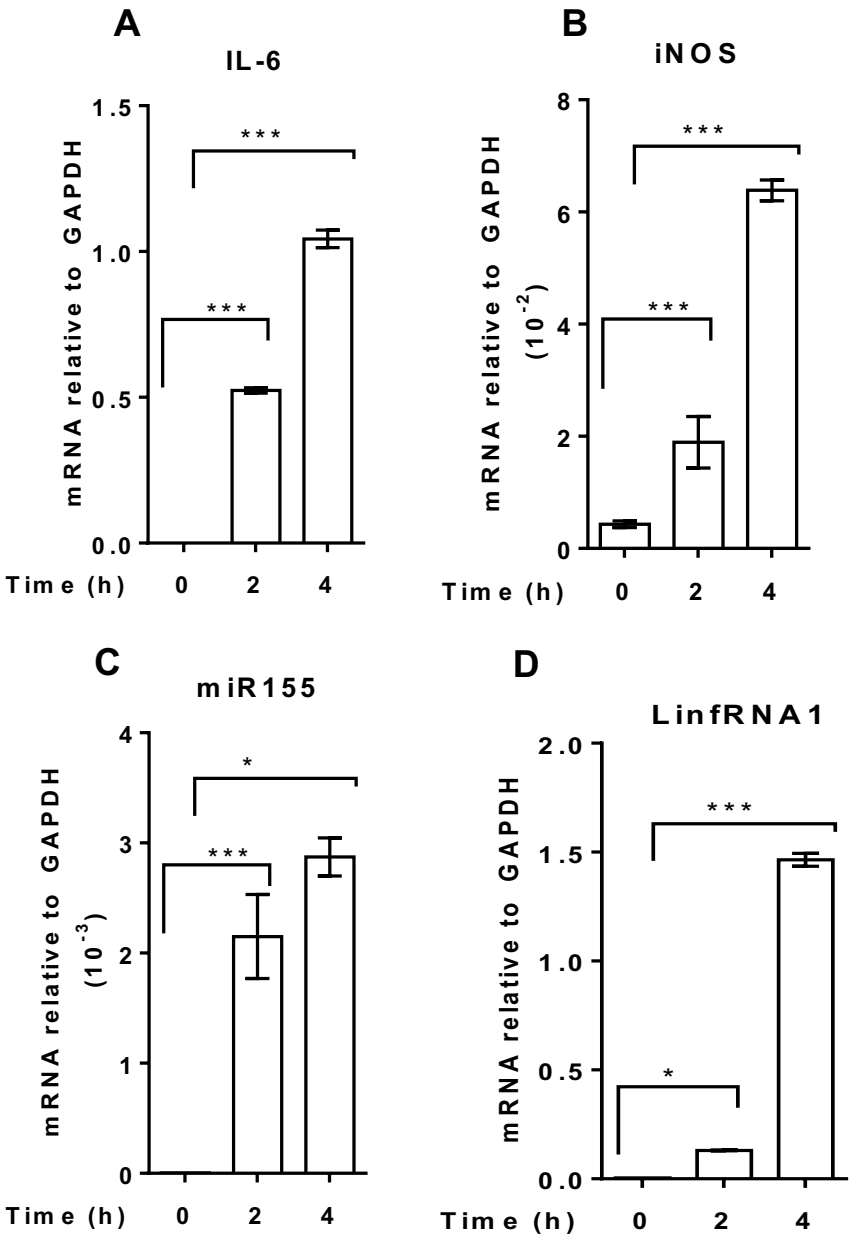

Figure 4.2. LPS induces LinfRNA1 expression in BMDMs. BMDMs were isolated from wild type (C57BL6/J) mice, treated with LPS (1 μg/mL) for different time periods, total RNA was isolated, reverse transcribed to cDNA and analyzed by qPCR for expression of IL-6, iNOS,

miR155 and LinfRNA1 (A-D). Each experiment was repeated at least with three parallel replicates. GAPDH was used as control. Data represent mean ± SD (n=3); *p < 0.05, **p < 0.001, ***p < 0.0001.

To further confirm our observations and also for the mechanistic studies, we examined the expression of LinfRNA1 and other inflammatory marker genes in cultured RAW264.7 mouse macrophage cells. We treated RAW264.7 with LPS and analyzed its impacts on the expression of LinfRNA1 along with IL-6 and iNOS. RNA from the LPS-treated macrophages were analyzed by RT-qPCR. As expected, LPS-treatment induced the expression of IL-6 and iNOS in time-dependent manner (Figure 4.3A and B). The expression of IL-6 was induced by 4700, 9013 and 16861 folds at 2, 4 and 6 h post LPS-treatment, respectively (Figure 4.3A). Similarly, the expression of iNOS was also induced by 756, 1683 and 2400 folds at 2, 4 and 6 h, respectively, upon treatment with LPS (Figures 4.3B). Interestingly, along with IL-6, iNOS, the lncRNA LinfRNA1 expression was also induced upon treatment with LPS (56, 291 and 299 folds at 2, 4 and 6 h, respectively) (Figures 4.3C). Overall, these observations further demonstrated that along with IL-6, iNOS, lncRNA LinfRNA1 expression is induced upon stimulation with LPS in macrophage and it may be associated with inflammatory and immune response.

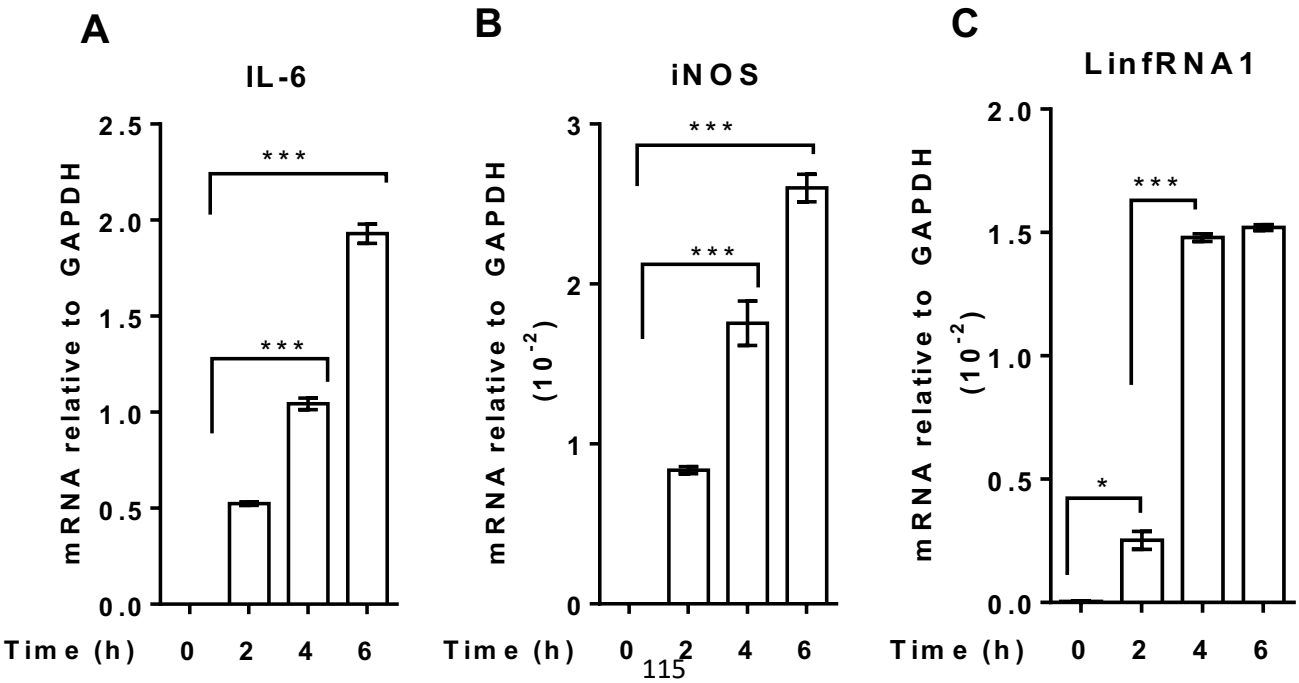

Figure 4.3. LPS induces LinfRNA1 expression in macrophages. RAW264.7 cells were treated with LPS (1 μg/mL) for varying periods of time, total RNA was isolated, reverse transcribed to cDNA and analyzed by qPCR for expression of IL-6, iNOS and LinfRNA1 (A-C). Each experiment was repeated at least with three parallel replicates. GAPDH was used as control. Data represent mean ± SD (n=3); *p < 0.05, **p < 0.001, ***p < 0.0001.

4.3.3 LinfRNA1 is regulated by NF-κB

As LinfRNA1 expression is induced by LPS in a similar manner as cytokines and pro-inflammatory genes, which are regulated via NF-κB activation, we investigated if our novel LinfRNA1 may be regulated via NF-κB activation as well. To investigate if NF-κB activation is associated with LPS-induced expression of LinfRNA, we treated macrophages with an inhibitor of IKKβ (SC-514)[3] and analyzed its impacts on LPS-induced IL-6, iNOS, and LinfRNA1 expressions. Notably, IKKβ is a kinase which phosphorylates IκBα allowing its poly-ubiquitination and proteasomal degradation, leading to NF-κB activation.[8-9, 38-39] Thus, inhibition of IKKβ results in deactivation of NF-κB.[8-9] Briefly, RAW264.7 cells were treated with IKKβ-inhibitor SC-514 (25 μM, 1 h) and stimulated with LPS.[30] Western blot analysis demonstrated that, as expected SC-514 treatment resulted in a decrease in phospho-IκBα and phospho-p65 levels, indicating the effective inhibition of IKKβ kinase activity and consequent decrease in NF-κB activation (Figure 4.4A, quantification in panel B). The RNA from the control and SC-514 treated cells were also analyzed by RT-qPCR (Figures 4.4C-E) for the expression of IL-6, iNOS, and LinfRNA1. Interestingly, treatment with IKKβ inhibitor down-regulated LPS-induced expression of NF-κB target genes IL-6 (4.8 folds) and iNOS (3.7 folds) along with a decrease in LinfRNA1 (1.5 folds) expression (Figures 4.4C-E). The treatment of SC-514 alone in the absence of LPS has no significant impact on IL-6, iNOS, and LinfRNA1 expression (Figures 4.4C-E). These observations suggest that similar to IL-6 and iNOS, LinfRNA1 expression is also regulated by NF-κB upon LPS-stimulation.

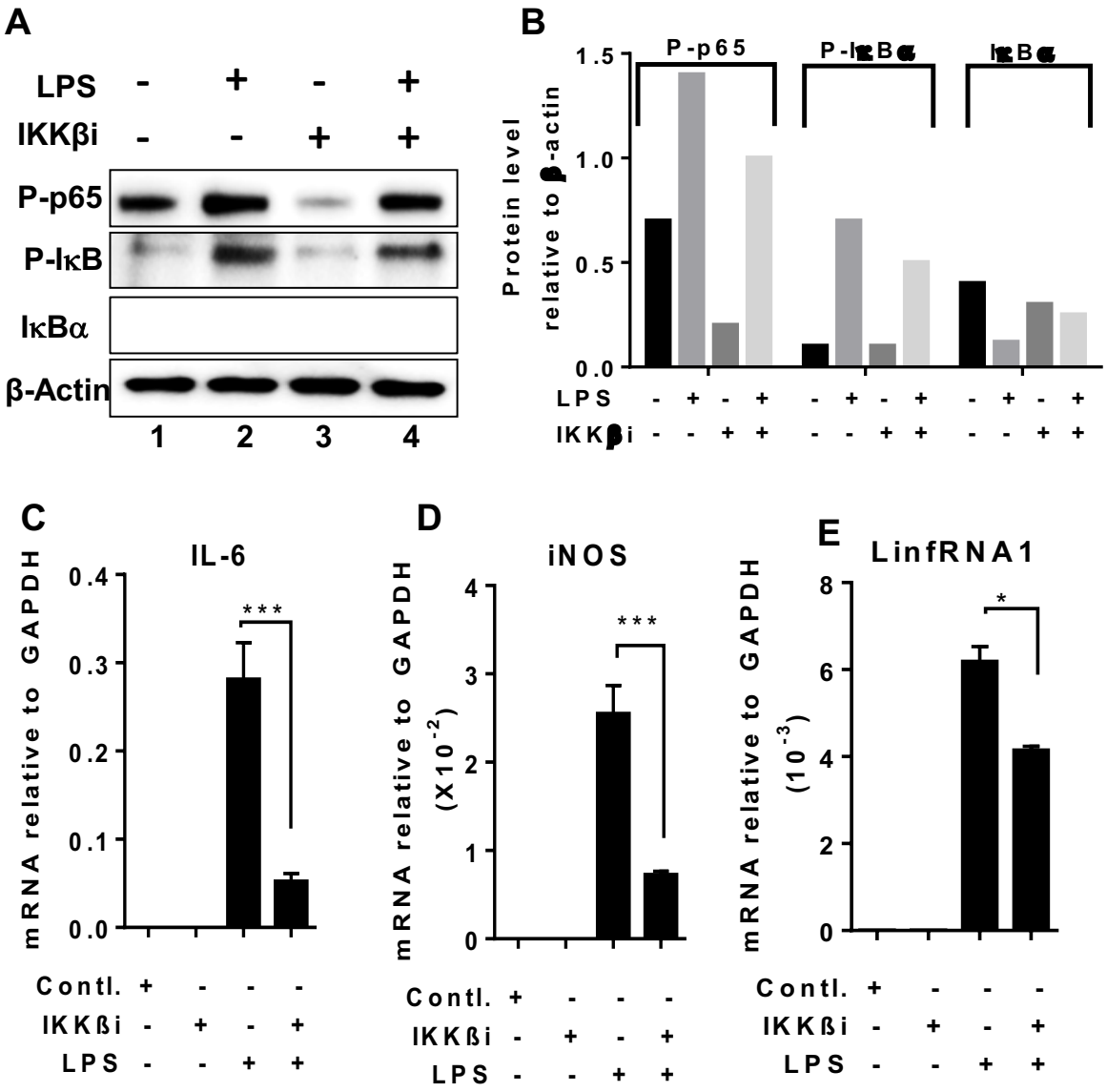

Figure 4.4. Inhibition of NF-κB downregulates LPS-induced LinfRNA1 expression in macrophages. (A) RAW264.7 cells were initially treated with IKKβ-inhibitor SC-514 (for 1 h) and then treated with LPS for additional 1 h (for protein analysis) and 4 h (for RNA analysis). Proteins were analyzed by Western blotting using antibodies against phospho-p65 (NF-κB subunit), phospho-IκBα, IκBα and β-actin (loading control) (Quantifications using ImageLab5.2.1software is shown in panel B). (C-E) RNA was isolated and the expressions of IL-6, iNOS and LinfRNA1 were measured by RT-qPCR. GAPDH was used as control for PCR experiments. Data represent mean ± SD (n=3); *p < 0.05, **p < 0.001.

4.3.4 LinfRNA1 knockdown down-regulates LPS-induced expression of cytokine and pro-inflammatory genes.

To investigate the roles of LinfRNAs in inflammation, we knocked down LinfRNA1 in macrophages and then analyzed its impacts on LPS-induced cytokine expression. Briefly, RAW264.7 cells were transfected with LinfRNA1-siRNA and scramble-siRNA (control) and then treated with LPS. RT-qPCR analysis of RNA showed that LinfRNA1 siRNA effectively knocked down (~50%) the level of LinfRNA1 (Figure 4.5). Interestingly, LinfRNA1 knockdown suppressed the LPS-induced expression of IL-6 and iNOS, suggesting critical roles of LinfRNA1 in regulation of cytokines expression and inflammation in macrophage (Figure 4.5A). Moreover, as IL-6 is a secretory protein, we also measured the IL-6 secretion in the culture media using ELISA and found that secreted IL-6 level was increased upon treatment with LPS and that were decreased upon treatment LinfRNA1 siRNA (Figure 4.5D). Taken together, our analysis demonstrated that LinfRNA1 is necessary for LPS stimulated expression of cytokines and inflammatory response genes in macrophages.

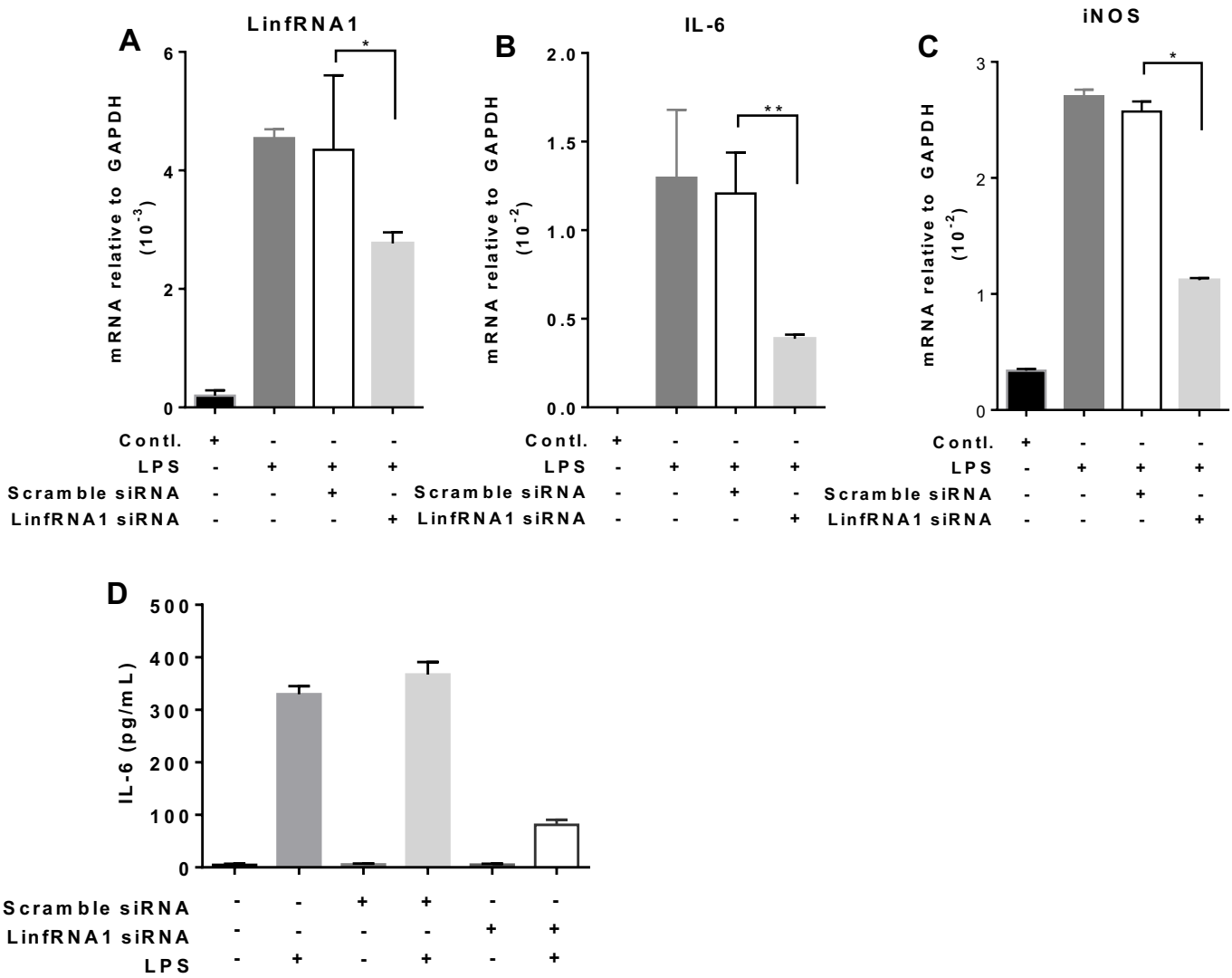

Figure 4.5. Knockdown of LinfRNA1 reduces LPS-induced IL-6 and iNOS expressions in macrophages. (A-C) RAW264.7 cells were transfected with LinfRNA1-siRNA and scramble siRNA (48 h), RNA was analyzed by RT-qPCR for the expression of LinfRNA1, IL-6, and iNOS. GAPDH was used as control. Data represent mean ± SD (n=3); *p < 0.05, **p < 0.001, ***p < 0.0001. (D) ELISA assay: LinfRNA1 was silenced in RAW264.7 macrophages by using LinfRNA1-siRNA, treated with LPS (12 h). Culture media were collected and amount of IL-6 secreted in culture media were measured using ELISA (n=4).

4.3.5 LinfRNA1 promotes IκBα degradation thereby activates NF-κB

Out studies demonstrate that LinfRNA1 is required for LPS-induced expression of cytokines and pro-inflammatory genes. It is also well-recognized that LPS-induced cytokines and pro-inflammatory genes expression are regulated via NF-κB activation.[8-9, 29] Here to understand the mechanism by which LinfRNA1 regulates inflammatory response and cytokine expression, we explored the role of LinfRNA1 in LPS stimulated NF-κB activation. Briefly, RAW264.7 cells were transfected with LinfRNA1-siRNA and scramble-siRNA, followed by stimulation with LPS. Proteins from the control, LinfRNA1-knocked down, and LPS-treated cells were analyzed by western blot to analyze the levels of phospho-p65 (NF-κB subunit), IκBα, and β actin (control). Interestingly, our studies demonstrate that, as expected upon treatment with LPS, the level of IκBα is decreased (due to degradation). Interestingly, however, the level of LPS-induced IκBα degradation was inhibited upon LinfRNA1-knocked down (compare lanes 1, 2 and 4, Figure 4.6). The scramble siRNA has no significant impacts on the LPS-induced IκBα degradation level. Along the same line, the level of phospho-p65 (NF-κB subunit) was increased upon LPS-treatment and this LPS-induced increased level of phospho-p65 was reduced upon LinfRNA1 knockdown. Notably, the LinfRNA1 knockdown also downregulated the LPS-induced phospho-IκBα level. The LPS-induced degradation of IκBα and associated elevation on phospho-p65 level, and its reversal upon LinfRNA1 knockdown suggest that LinfRNA1 plays critical roles in accelerating LPS-IκBα degradation and hence NF-κB activation. These observations demonstrate that LinfRNA1 is a functional lncRNA and is a critical player in inflammation and this is mediated via regulation of NF-κB activation.

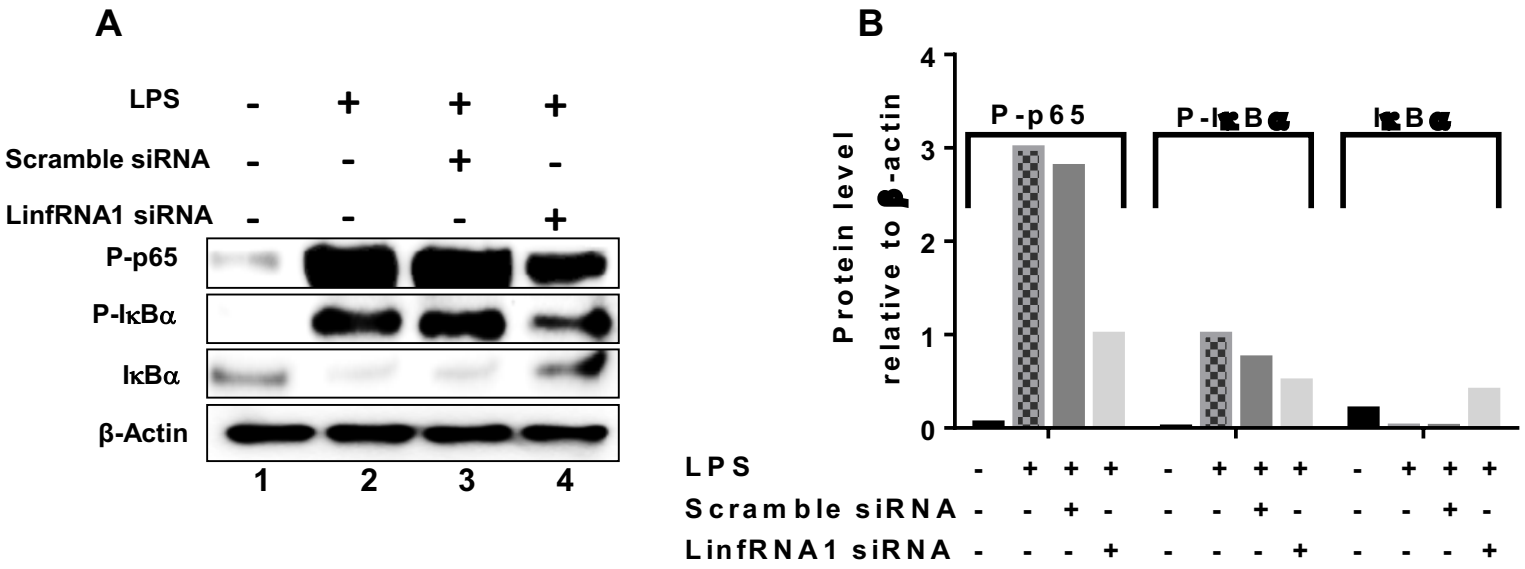

Figure 4.6. Knockdown of LinfRNA1 reduces LPS-induced NF-κB signaling pathway. (A) Proteins were extracted from LinfRNA1-siRNA and scramble siRNA (48 h) treatment followed by 1 h LPS treatment of RAW264.7 cells. These proteins were analyzed by Western blotting using antibodies against phospho-p65 (NF-κB subunit), phospho-IκBα, IκBα and β-actin (loading control). (B) The changes in amounts have been quantified by ImageLab5.2.1 software.

4.4 Discussion

The human genome database of GENECODE has reported 16,066 lncRNA genes and 29,566 lncRNA transcripts.[40] Despite the huge numbers of lncRNA in the genome, very little is known about the exact function, mechanism of action and types of lncRNAs.[41] LncRNAs have been reported to be involved in many biological processes. They have the potential to serve as biomarkers for various diseases.[42] Increasing numbers of studies indicate that lncRNAs are associated with immune signaling and inflammatory response.[43] LncRNAs are expressed in different types of immune cells and affect the signaling pathways.[44] For example, lncRNA lincR-Ccr2-5′AS causes $CD4^+$ T cell differentiation[45] whereas pathogen-response linked lincRNA-Cox2 expression is raised to induce other immune-related genes, such as IL-6, Tlr1,

and IL-23a in bone marrow-derived macrophages.[12, 46] NRON (non-protein coding RNA, repressor of NFAT) acts as a transcription regulator for immune regulation.[47] Lethe serves as a decoy lncRNA and negatively regulates NF-kB signaling in inflammation.[48-49] THRIL (TNFα and heterogeneous nuclear ribonucleoprotein L related immunoregulatory lincRNA) has roles in immunoregulation and causes the secretion of TNFα, IL-6, IL-8, CXCL10, CCL1, and CSF1.[12, 50-51] Here, we have discovered novel lncRNA associated with inflammation and immune response and investigated their potential mode of action.

RNA sequencing has provided an unbiased approach to study the unknown lncRNAs and their functions.[52] This approach provides a precise measurement of the levels of transcripts and their isoforms.[24] In our studies we have induced BMDMs with LPS to exert inflammation followed by RNA sequencing to investigate and discover the transcriptome, the total cellular content of mRNA, rRNA and tRNA. Along with expected inflammatory genes like IL-6, IL-12, iNOS, we discovered a novel group of lncRNAs, we termed as LinfRNAs. In particular, we have identified LinfRNA1 as a novel player in in immune and inflammatory response in macrophages.

My study demonstrates that LinfRNA1 expression is induced in both BMDMs and macrophage cells upon stimulation with LPS. LPS, a potent pathogen-associated molecular pattern (PAMP), origins inflammatory response via endotoxic shock.[53-56] Like our previous studies, we found that LPS-treatment prompts the expression of various cytokines and inflammatory genes including IL-6, iNOS, microRNA miR155, and unknown lncRNAs like LinfRNA1. Interestingly, it has also been revealed that LinfRNA1 is required for the LPS-induced cytokines and inflammatory genes expression. SiRNA-mediated LinfRNA1 knockdown downregulated the expression of IL-6 and iNOS, both at mRNA and protein levels. The siRNA mediated knockdown of LinfRNA1 targeting different regions of this lncRNA confirmed the

LinfRNA1-target specificity. These observations demonstrate that LinfRNA1 plays crucial roles in cytokine expression and inflammatory response in macrophages upon LPS-stimulation.

LPS is a well-known inducer for the activation of toll like receptors (TLRs) and stimulates down-stream signaling cascades resulting the degradation of IκBα and activation of NF-κB.[8-9] In turn, the NF-κB activation triggers its target gene expression and induces immune and inflammatory response.[57-61] To investigate the roles of LPS-induced LinfRNA1 expression via NF-κB activation and vice-versa, we inhibited NF-κB signaling pathway using IKKβ-inhibitor (SC-514). SC-514 treatment resulted in the decrease of the levels of both phospho-IκBα and phospho-p65. SC-514 also causes a reduction in the LPS stimulated LinfRNA1 expression. Remarkably, the kinase IKKβ phosphorylates IκBα at Ser 32/36, which triggers its polyubiquitination and proteasomal degradation, resulting in release and activation of NF-κB.[8-9] NF-κB activation is accompanied by the phosphorylation of its subunit p65 (at Ser 536).[38-39] So, reduction in the levels of phospho-IκBα and phospho-p65 indicates the inhibition of NF-κB by SC-514. SC-514-mediated down regulation of LPS-induced LinfRNA1 expression level suggest potential regulation of LinfRNA1 via NF-κB signaling pathway. Importantly, siRNA mediated knockdown of LinfRNA1 down regulated the LPS-induced increase in phospho-p56 and phospho-IκBα. Additionally, knockdown of LinfRNA1 also depressed the level of LPS-induced degradation of IκBα. Taken together, these observations demonstrate that lncRNA LinfRNA1 plays key roles in NF-κB activation via modulation of IκBα degradation and it is a novel and critical player in regulation of inflammation and immune response. A model, showing the potential roles of LinfRNAs, in regulation of IκBα degradation and hence NF-κB activation, its translocation into the nucleus and binding to the target gene promoters inducing their expressions, is shown in figure 4.7.

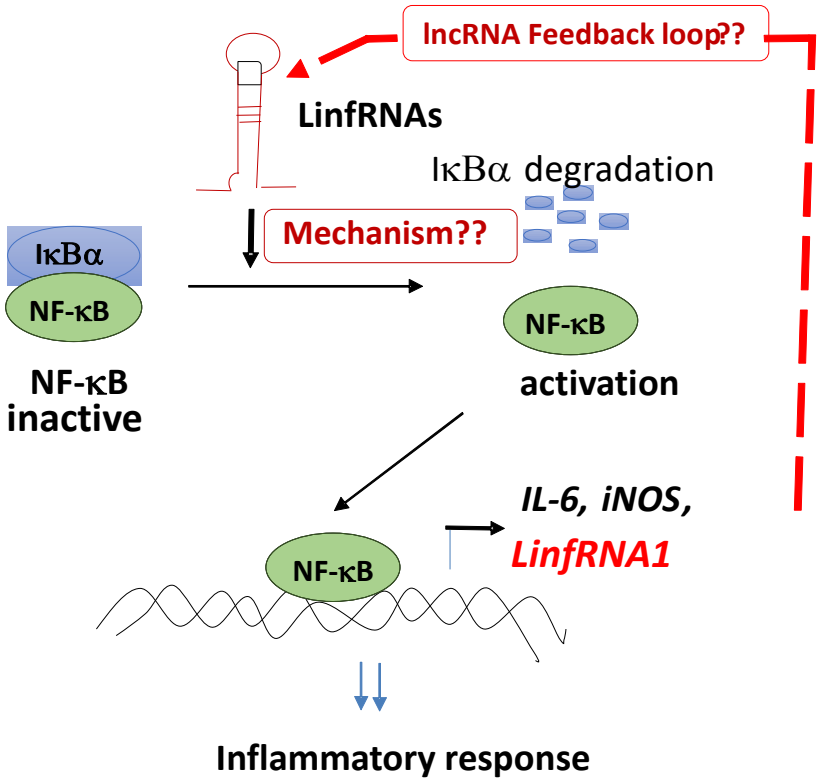

Figure 4.7. A model for LPS-stimulated LinfRNA1 induction in macrophage cells. NF-κB becomes inactive when bound to IκBα. Upon phosphorylation, IκBα is degraded, NF-κB is activated, and induces IL-6, iNOS and LinfRNA1 expression. In turn, LinfRNA1 facilitates IκBα degradation and enhances NF-κB activation, nuclear translocation and binding to NF-κB regulated genes (pro-inflammatory genes such as IL-6, iNOS) inducing their expressions.

Overall, my study leads to the discovery of novel lncRNA linked to inflammation and immune response. We also discovered novel inflammatory signaling pathways involving lncRNAs, LinfRNAs, which have advanced our understanding on the inflammation and immune signaling. My study provides novel LinfRNA-based therapeutic targets for the development of novel therapeutics for the treatment of inflammatory and immune diseases.

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