Functional Characterization Of A Putative Long Non-Coding Rna U90926: Role In Inflammation And Adipose Tissue Homeostasis

FUNCTIONAL CHARACTERIZATION OF A PUTATIVE LONG NON-CODING RNA *U90926*: ROLE IN INFLAMMATION AND ADIPOSE TISSUE HOMEOSTASIS

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ABSTRACT

Long non-coding RNAs (lncRNAs) are one of the most commonly transcribed functional non-coding RNAs in mammalian genomes, yet majority of them remain understudied. Recent advancement in high throughput sequencing and computational knowledge have revealed the identity of thousands of lncRNAs and their association with numerous diseases and cellular processes. In the last two decades, flurries of studies showed experimentally that lncRNAs regulate a myriad of essential biological processes including immune system. Although a large number of lncRNAs have been identified in a variety of immune cells, only a handful of them have been characterized for biological function and mechanisms. In this dissertation, we functionally characterized a mouse lncRNA named *U90926*, which was previously identified in our lab from transcriptomic data in activated macrophages.

First, we have investigated the role of *U90926* in macrophage function. We generated a *U90926*-deficient mice (U9-KO) to study *U90926* function. We showed that the expression of *U90926* is highly induced in macrophages and dendritic cells by toll-like receptor activation, in a p38 MAP kinase- and MyD88-dependent manner. Then, we have investigated the role of *U90926* in regulating macrophage function upon lipopolysaccharide (LPS) stimulation, such as cytokine secretion, expression of co-stimulatory molecules and gene expression. Surprisingly, we found minimal effects of *U90926* deficiency in cultured macrophages. The lack of macrophage-intrinsic effect by *U90926*, led us to explore protein-coding potential aspect of this putative lncRNA. Interestingly, we found that *U90926* RNA localizes to the cytosol, associates with ribosomes, and contains an open reading frame that encodes a novel glycosylated protein (termed U9-ORF), which is secreted from the cell. This led us to use an in vivo model of endotoxic shock where macrophages interact with many other cell types, for further characterization of *U90926*. Our data show that in comparison with WT mice, U9-KO mice exhibited increased sickness responses and mortality. Mechanistically, serum levels of IL-6 were elevated in U9-KO mice, and IL-6 neutralization improved endotoxemia outcomes in U9-KO mice. Taken together, these results suggest that *U90926* expression is protective during endotoxic shock, which is potentially mediated by the paracrine and/or endocrine actions of the novel U9-ORF protein secreted by activated myeloid cells.

We have also examined the role of *U90926* in adipose tissue homeostasis, using our U9-KO mouse model. *U90926* has been characterized as a repressor of adipogenesis in 3T3-L1 preadipocytes by another group. This led us to hypothesize that *U90926* deficiency in vivo will enhance adipogenesis or obesity-related phenotypes. However, we did not find any effect of *U90926* in weight gain or other obesity-related phenotypes like fat mass, adipose tissue morphology, metabolic parameters, and adipose tissue gene expression, both in the context of a normal diet or high-fat diet. Taken together, these data suggest that *U90926* may not have an essential role in adipocyte biology beyond its role in myeloid cell-mediated inflammation.

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DEDICATION

I dedicate this dissertation to my loving parents for their endless love, support, and inspiration in every phase of my life.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Much of the non-protein-coding portion of the genomes of humans and thousands of other organisms has historically been regarded as junk DNA. However, advancements in molecular biology and computational high throughput technologies over the last two decades have allowed in-depth experimentation of non-coding genomes with an unprecedented resolution and scale. Such studies revealed that this so-called junk DNA transcribes to non-coding RNAs (ncRNAs), which may be functionally and evolutionarily as important as protein-coding genes. Among the various ncRNAs, a class called long non-coding RNAs (lncRNAs), defined as transcripts of more than 200 nucleotides in size, has attracted attention for their unique roles in numerous cellular processes, including immune function. Nonetheless, the vast majority of lncRNAs remain uncharacterized. Since 2017, we have been working on the functional characterization of a putative mouse lncRNA called *U90926*. This dissertation will describe the following aims:

Aim 1: Initial characterization of lncRNA *U90926*, including its protein-coding potential

Aim 2: Investigating the role of *U90926* in myeloid cells and endotoxemia mouse model

Aim 3: Understanding the function of lncRNA *U90926* during adipogenesis *in vivo*

The journal article format is used here to describe the subsequent chapters. Chapter 2 describes aim 1 and 2 listed above (outlined in Fig.1-1) and chapter 3 describes the aim 3 (outlined in Fig. 1-2). Chapter 1 provides a comprehensive literature review summarizing the relevant research topics and the gap in knowledge in this field. Finally, chapter 4 contains thorough discussion and conclusions.

1.2. Long non-coding RNAs

The central dogma of biology states that genetic information encoded in the DNA is transcribed into RNA, followed by translation into protein, with the latter carrying out most of the cellular functions. This may essentially be true for prokaryotes and also presumed to be the fact for eukaryotes by regarding the non-protein coding genes as “junk” DNA. Recent advances in genomics revealed that the total number of classic protein-coding genes did not increase with the increasing complexity of the organisms. What did increase, however, was the amount of non-protein coding genes, the majority of which are, in fact, found to be transcribed(1–3). Finally, over the last 20-30 years, an increasing number of studies showed that these non-coding RNAs (ncRNAs) are functional and have crucial roles in regulating many major cellular processes. However, a vast majority of these ncRNAs still remain uncharacterized. Studies found that only 1% of the human genome encodes for proteins, whereas another ~ 4%-9% gets transcribed, yet whose functions are not known(4).

Non-coding RNAs can be broadly divided into two groups based on their functional attributes – housekeeping ncRNAs and regulatory ncRNAs. Housekeeping ncRNAs include ribosomal, transfer, small nuclear, and small nucleolar RNAs, which are usually expressed constitutively and predominantly involved with fundamental genetic phenomena(4, 5). Among the short Regulatory ncRNAs (<200 nucleotides) are microRNAs, small interfering RNAs, and Piwi-associated RNAs, which are primarily associated with transcriptional and post-transcriptional gene silencing(6). The most transcribed ncRNAs that are greater than 200 nucleotides (NTs) and have crucial regulatory roles in numerous cellular processes, including the cell cycle, differentiation, immune system, and metabolism, as well as in various diseases, are called long non-coding RNAs (lncRNAs)(2, 4, 7). However, this class has been largely understudied regardless of being the most commonly transcribed ncRNAs. Advancement in high throughput sequencing technologies has accelerated the discovery of new lncRNAs and their association with critical cellular processes and diseases. However, there still needs to be experimental tools to speed up the process of functional characterization of each lncRNA that is being identified.

1.2.1. Major mechanistic aspects of lncRNA

LncRNAs do not encode proteins like messenger RNAs (mRNAs); however, certain aspects of their biology are very similar to mRNAs. Like mRNAs, most lncRNAs are transcribed by RNA polymerase II (Pol II) and undergo capping and polyadenylation(8). Non-polyadenylated lncRNAs get stabilized by secondary structures like triple helical structures in their 3' ends(9). Extensive alternative splicing happens in the vast majority of the lncRNAs, to dramatically increase their potential number of isoforms(10). The extensive alternative splicing is a potential aspect of lncRNA functional diversity that gets overlooked often.

LncRNA expression levels are usually much lower than mRNA(11), but their expression is highly tissue-specific, which indicates a robust integral role in cell type-specific processes(8, 12–14). Unlike mRNA, lncRNAs generally lack sequence conservation among species, which makes its functional assessment more complicated(15, 16). At the same time, the lack of conservation also sheds light on the roles that lncRNA evolved to play in different species. Nevertheless, there are still subsets of lncRNAs that have sequence and/or genomic positional homology and may have similar functions across species(15–18). On the contrary, significant sets of lncRNAs with sequence homology and positional conservation between mice and humans may serve distinct functions because of different processing and subcellular localization(19). Additionally, structural conservation can also be found among lncRNAs based on their secondary or tertiary structures required for function, regardless of sequence homology(20). Thus, lncRNA conservation can be described in four dimensions – sequence, structure, function, and expression from syntenic loci or positional(21).

LncRNAs interact with DNA(22, 23), RNAs(24, 25), and/or proteins(26, 27) to regulate transcription, epigenetic modifications, RNA/protein stability, translation, and posttranslational modifications in the cells. Recently, it has been shown that lncRNAs can also directly interact with signaling receptors to carry out function(28). As the local molecular interactions dictate the function of lncRNAs, understanding the lncRNA subcellular localization is a key component to projecting their functions(29). Initially, all the lncRNAs that were functionally characterized were chromatin regulators, localized in the nucleus, such as *XIST*, *NEAT1*, and *MALAT1*, and these studies biased the notion that lncRNAs are generally nuclear(22, 30–32). However, recent studies indicate that cytoplasmic lncRNAs are much higher than previously estimated(33–35). In the nucleus, lncRNAs mainly regulate transcriptional programs by chromatin remodeling and interactions(36, 37), and orchestrate spatial organization of the nucleus compartment by scaffolding(38). However, in the cytoplasm, lncRNAs modulate translational programs, posttranscriptional control of gene expression, posttranslational modifications, mRNA stability, and scaffolding for tightly regulated signaling transduction programs(24, 39–42). Other than nucleus and cytosol, lncRNAs can also be located in other subcellular organelles like mitochondria, endoplasmic reticulum and ribosomes(29). Predictably, the same lncRNA can function distinctively in different subcellular compartments by interacting with different functional molecular partners and target(43).

Another essential aspect of lncRNA classes is knowing their genomic contexts, i.e., from where these RNAs get transcribed in the genome. It does not provide any crucial information about lncRNA evolutional origin or function; however, this genomic context-based classification helps tremendously in organizing these diverse RNA species. In this way, lncRNAs can be grouped into five broad categories which are mutually nonexclusive: i) sense, or ii) anti-sense, when transcribed from the same, or opposite strand, respectively, overlapping one or more exons of protein-coding genes, iii) bidirectional, its expression and a neighboring protein-coding gene expression on the opposite strand is initiated in close genomic proximity, iv) intronic, when it is transcribed entirely from introns of protein-coding genes, and v) intergenic, when it lies separately within the genomic interval between two genes(4, 44, 45).

For understanding lncRNA function, they can be classified into two major classes; *cis*-acting, which regulates expression and chromatin structure of the neighboring genes, and trans-acting, which leaves the site of transcription and executes an array of functions throughout the cell. For *cis*-acting lncRNA function, there are three mechanisms by which lncRNA can modulate local gene expression and chromatin state(7). First, sequence-dependent *cis*-regulation, where lncRNA itself recruits regulatory factors to the site to regulate the expression of neighboring genes and/or modulate their function, for example, the most famous and well-established *cis*-acting lncRNA, X-inactive specific transcript *Xist* works in this mechanism(22, 46, 47). Second, transcription- or splicing-dependent *cis*-regulation, where the process of transcription and/or splicing of a lncRNA confers a gene regulation functionality totally independent of the lncRNA sequence; for example, transcription and splicing of the *Blustr* RNA regulate the expression of the neighboring gene, *Sfmbt2*(48). Third, lncRNA Loci dependent *cis*-regulation, where functional DNA elements lie in the lncRNA promoter or gene loci to regulate *cis* action solely independent of the lncRNA transcript and its production, for example, *lincRNA-p21* locus modulates *Cdkn1a* gene expression(49).

Trans-acting lncRNAs can also be subdivided into three categories based on their mechanism of action(7). First, gene regulation by lncRNAs in *trans*, where lncRNAs regulate gene expression and chromatin state at regions distant from their transcription site. Examples of this class include mammalian lncRNAs *HOTAIR(50)* and *lincRNA-EPS(51)*, which work by recruiting chromatin-modifying complexes to regulate chromatin state in *trans*. Second, nuclear architecture and organization by lncRNAs, where lncRNAs influence nuclear structure to orchestrate transcription, RNA processing, and other steps and to provide spatial regulation upon these processes. For example, lncRNA *MALAT1* works as a linker that facilitates the nuclear speckles position at active gene loci(17, 32, 52). Third, interaction with other proteins and RNAs by lncRNAs, where lncRNAs can modulate activity and abundance of certain proteins and RNAs by directly binding to them.

For example, lncRNA *NORAD* binds protein PUM1/PUM2 to limit their availability(40), and *CDR1as* binds microRNAs to modulate their activity(53, 54).

Understanding the molecular mechanisms of regulating lncRNA gene expression genome-wide is crucial for appreciating the clinical and biological significance of lncRNAs. New tools, such as single-cell nascent RNA-seq analysis, can profile lncRNA transcription at a near single-cell level; however, it becomes invaluable to dissect different mechanisms of transcriptional regulation in the same cell(55). In the last decade, there was remarkable progress in the lncRNA research which gave us a clearer idea about their features and functional versatility. However, these studies are still just the tip of the iceberg, and knowledge remains incomplete in fine-tuning cell function and fate.

1.2.2. Protein-coding potential of lncRNAs

Classical annotation of protein-coding and non-coding RNAs is contingent upon some fundamental criteria. According to these criteria, any putative open reading frames (ORFs) encoded within the transcript are defined as a series of consecutively translated codons beginning with the canonical AUG start site(56, 57). Another primary criterion for defining protein-coding genes is the length of the novel ORFs. Traditionally, translation of ORFs smaller than 100 codons (or 300 NTs) is considered spurious and fails to produce functional proteins(58, 59). The same assumption is accepted widely for ORFs with poor phylogenetic conservation. A lack of ORF conservation in novel transcripts among species classifies those transcripts as non-coding(58, 60, 61). These properties may be used prominently in the field to predict the protein-coding potential of novel transcripts, but not indisputably.

Each of these assumptions discussed above is arbitrary, and the lack of empirical data supporting them renders the question of misannotation of many transcripts as non-coding. Over dozens of bona fide functional eukaryotic proteins encoded by ORFs shorter than 100 codons have been identified, and the numbers of these small functional ORFs are way higher than previously thought(58, 62). Additionally, several studies showed that ORFs with one nucleotide promiscuity at the start codon, as defined as non-AUG start site, encode stable and functional proteins(63–65). This discovery demonstrates that the definition of "putative ORF" must be reformed. There are also some technical issues associated with using phylogenetic conservation as a metric for distinguishing coding and non-coding transcripts. Limitation in comprehensiveness and accuracy of current protein annotation is one of them(66). Also, some lncRNAs evolve from protein-coding genes; therefore, there will be remnant signatures and homologies with their mRNA counterparts, which does not necessarily mean protein-coding, for example, *Xist* (67, 68). Finally, studies showed that many orphan ORFs (lack of conservation in other species) in yeasts can produce translated products that were annotated initially as spurious(69). Nevertheless, this evidence showed that even with the use of sophisticated algorithms, many understudied transcripts are wrongly assigned to protein-coding or non-coding group.

Another factor that complicates things further is the presence of bifunctional RNAs that function as both non-coding RNA and encode functional proteins, depending on the situation(58, 70). Additionally, high throughput transcriptome analyses have predicted that a significant fraction of protein-coding RNAs undergo alternative processing to generate nascent isoforms or are transcribed from an alternative promoter that may function as a non-coding RNA(71, 72). The discovery of these genes operating through a “bi-faceted” mechanism of action, suggests that the coding and non-coding categories are by no means mutually exclusive. Therefore, it is imperative to examine protein-coding potential of a lncRNA as an alternative function.

Multiple studies have shown that most cytoplasmic lncRNAs are recruited to ribosomes and associated with translational machinery(33, 35). However, there is much controversy on the functional significance of this association. Recently, a considerable number of annotated lncRNAs have been identified to encode bona fide small peptides(63, 64, 73–75). Some studies support that ribosome profiling in polysomal fractions showing lncRNA association with ribosomes is indicative of active translation(76–78). On the contrary, other studies showed that lncRNA association with ribosomes does not guarantee translation(79, 80). In fact, studies revealed that some lncRNAs are targeted to ribosomes for degradation by a process called nonsense-mediated decay (NMD)(35, 81). These reports suggest that ribosomal occupancy is insufficient to distinguish non-coding and protein-coding transcripts. Therefore, identifying lncRNA protein-coding potential has to be corroborated by specific ORF tagging and proteomics analysis(64, 81, 82).

Given the ambiguous classification of protein-coding and non-coding genes, misannotation of transcripts in either group has been easily attainable. Typically, like mRNAs, lncRNAs are transcribed by pol II, capped, spliced, polyadenylated, and accumulate in cytoplasm(33, 83). Additionally, lncRNAs are longer, and some contain one or more small ORFs, making them more likely to be translated among other ncRNA species(84). However, considering the current evidence of annotated lncRNA translation and disputes in the field, rigorous experimental interventions are needed to identify the protein-coding potential of both annotated and newly discovered lncRNAs(58, 61).

1.2.3. Role of LncRNAs in the immune system

Our mammalian immune system is broadly divided into two arms -the innate immune system and the adaptive immune system, consisting of diverse immune cells collaborating to protect the host against pathogenic microorganisms and other foreign materials. The innate immune system employs a surveillance system of immune cells such as neutrophils, monocytes, macrophages, and dendritic cells to recognize and control pathogens and relay the message to the adaptive immune system. Then, the adaptive immune cells - T and B cells undergo somatic hypermutation to detect specific antigens and ultimately eliminate pathogen or infected cells. The timing of these series of events is highly dynamic and carefully coordinated, and it involves rapid differentiation and activation of immune cells in response to different external stimuli, i.e., microbial products, cytokines, chemokines, and other endogenous mediators(85). All the immune cells express a specific repertoire of receptors to recognize specific stimuli and activate downstream signaling pathways to modulate chromatin complexes and transcription factors. These events lead to rapid changes in gene expression, which in turn causes phenotypic changes in activated immune cells. Recent evidence suggests that lncRNAs are involved in regulating and coordinating the development of immune cell lineages and controlling gene expression programs unleashed upon activation in these immune cells.

1.2.3.1 LncRNAs in the innate immune system

It is already established that lncRNAs are involved in coordinating different aspects of cell and tissue development, not surprisingly including immune cells(86). Given that lncRNA expression can be promoted utilizing the existing pools of transcription factors, it can rapidly affect the gene expression signatures of activated innate immune cells without requiring new protein synthesis(87). Therefore, it is not surprising that many of the initial discoveries of lncRNAs in the immune system focused on regulating innate immune cells. Because of the versatility of lncRNA mode of actions, biological functions of lncRNAs have to be interrogated on a one-by-one basis. Biological functions of some of the crucial lncRNAs are discussed below.

Among the lncRNAs that are involved in the development of innate immune cells, HOTAIRM1 (HOXA transcript, antisense RNA myeloid-specific 1) regulating myeloid cell development(88) and Morrbid (Myeloid RNA regulator of Bim-induced death) regulating the lifespan of short-lived myeloid cells for example, neutrophils, eosinophils and classical monocytes in response to prosurvival cytokines(89), are very popular. Another lncRNA, lnc-DC, plays a vital role in the differentiation and activation of classical dendritic cells (cDCs) by regulating STAT3 activity(90).

LncRNAs have also been investigated in functional aspects of innate immune cells. LincRNA-COX2 expressed upon toll-like receptors (TLRs)-activated macrophage modulates chromatin remodeling to activate or repress distinct sets of inflammatory genes(91, 92). Another lncRNA studied by the same group, lincRNA-EPS found to be highly expressed in resting macrophages, which interacts with hnRNPL to suppress target genes. However, upon stimulation, expression of lincRNA-EPS is reduced, and repression of these target genes is released, causing changes in gene expression signatures(51). A nuclear lncRNA PACER (p50-associated COX2 extragenic RNA) acts as a decoy molecule in the NF-κB signaling pathway to promote *PTGS2 (COX2)* gene expression in human macrophages(93). In human monocytes, TNFα and hnRNPL-related immunoregulatory lncRNA (THRIL) interacts with hnRNPL to control the transcription of TNFα during TLR activation(94). LncRNAs also contribute to regulating inflammation by modulating cytokine expression; for example, NEAT1 (nuclear enriched abundant transcript 1) coordinates IL-8 expression in herpes simplex virus (HSV-1)- and influenza A virus (IAV)-infected cells, and in response to dsRNA(95). A cytosolic lncRNA NKILA ((NF-κB interacting lncRNA) acts at the post-translational level to attenuate NF-κB dependent gene expression in human breast cancer cells(96).

All these lncRNA biological functions discussed above are their RNA-dependent mechanisms. In contrast, recent studies showed that some annotated lncRNAs could encode protein and function as protein-dependent mechanisms in immune cells. For example, the translated product of lncRNA Aw112010 is required for IL-12 production in macrophages upon lipopolysaccharide (LPS) stimulation(64). Another lncRNA annotated as 1810058I24Rik was found to encode a mitochondrial micropeptide-47 (Mm47), which is essential for activating the Nlrp3 inflammasome in macrophages(75). Taken together, these studies show that lncRNAs utilize diverse mechanisms to rapidly regulate inflammatory gene expression programs in innate immune cells in response to pathogenic cues, which adds a new and potent regulatory layer to the immune system.

1.2.3.2 LncRNAs in the adaptive immune system

The adaptive immune system, composed of T and B lymphocytes, is designed to generate an antigen-specific response and establish a long-lasting immunological memory avoiding autoimmunity and chronic inflammation. Several studies have shown that lncRNAs are differentially expressed in different subsets of T and B cells(97, 98). Functional characterization of lncRNAs in developing and mature B cells is still a nascent field of study. However, lncRNAs are emerging as critical regulators of defining steps in T cell immunity. Herein a few key examples of lncRNAs are discussed that are involved in the adaptive immune system.

In addition to the master transcriptional regulators, specific cytokines control the differentiation of naïve CD4+ T cells to their T helper cell lineages. For example, Th1 cells are characterized by the production of IFN-γ, Th2 by IL-4, and Th17 cells by the production of IL-17A and IL-17F. A well-characterized lncRNA, NeST has been shown to regulate Th1 cell differentiation by modulating *Ifng* expression in cis, in conjunction with Th1-specific transcription factor T-bet(99, 100). Another lncRNA, linc-MAF-4, regulates Th1 and Th2 differentiation inversely by repressing MAF gene, which is a transcription factor for Th2 differentiation(101). An anti-sense lncRNA, lincR-Ccr2–5' AS expressed in Th2 cells, is known to regulate the expression of chemokine genes *Ccr1, Ccr2, Ccr3,* and *Ccr5* by an unknown mechanism(102). LncRNAs have been found to restrict excessive activation of T cells as well. A nuclear lncRNA, NRON (noncoding RNA repressor of NFAT), restrains T cell activation by keeping the transcription factor NFAT (nuclear factor of activated T cells) in an inactive state by sequestering it during the resting stage(103). The lncRNA-CD244 functions similarly to that noted above in restricting the activation of

CD8+ T cells(104). LncRNAs are emerging to be modulating regulatory T cell functions as well. LncRNA Flicr, expressed in Foxp3+ regulatory T cells (Tregs), negatively regulates Foxp3 expression and Treg function by modulating chromatin accessibility within the *Foxp3* gene locus(105). Altogether, these studies demonstrate that lncRNAs control the development and effector function of T cells in conjunction with lineage-specific transcription factors.

The role of microRNAs, for example, miR-150 and miR-155, have been adequately established in B cell development and function(106). However, understanding the biological function of lncRNAs in B cells is still highly understudied. Recent transcriptomic studies revealed that hundreds of lncRNAs are differentially expressed at different stages of B cell development in mice and humans(107–109). One of the very few lncRNAs with known function in B cells is FAS-AS1, which regulates FAS receptor signaling in B cell lymphomas(110). LncRNA transcription has also been associated with regulating somatic hypermutation and class switch recombination processes in B cells(111).

Given the diverse mechanisms of interactions with target molecules like lncRNA-DNA, lncRNA-RNA, and lncRNA-protein, lncRNAs are the most likely to be the predominant RNA regulators in immune cells. There are flurries of studies that identify expression profiling of thousands of lncRNAs in immune cells; however, only a handful of lncRNAs have been further evaluated for their biological function in the immune system. There is a massive gap in our knowledge to unravel the function and molecular mechanisms of lncRNAs in a case-by-case manner. Developing new and refining existing experimental and computational techniques and unifying biological contexts from different areas will be paramount in filling this gap in understanding lncRNA biology.

1.2.4. LncRNA *U90926*

The main focus of this dissertation is to characterize a mouse lncRNA named *U90926* in terms of biological function and shed some light on the molecular mechanisms, such as protein-coding potential. In 2014, our lab had performed transcriptomic analysis in activated macrophages targeting one of the central pathways of the immune system, the p38-MAPK pathway and found that *U90926* gene is one of the most highly differentially regulated genes(112). At that period, there was nothing known about *U90926* function and molecular mechanisms, regardless of its differential expression pattern in different datasets. When we started characterizing *U90926* in 2017, there was only one paper that introduced a biological function to *U90926*, showing that it is a negative regulator of adipocyte differentiation in 3T3-L1 preadipocytes(113). Currently, several studies have enlightened some functional aspects of *U90926* in different cell types(114–116), described in more detail below. However, all these studies focused on the RNA-mediated function of *U90926*. Additionally, none of them elucidated *U90926* role in macrophages, which we have described in chapter 2 of this dissertation.

U90926 was first annotated as lncRNA by a large-scale genome exploration research in 2002 based on sequence features and expression patterns(117). *U90926* lncRNA is 522 bp in size, polyadenylated, and contains 5 exons and a single putative ORF (264 bp long) with a canonical ATG start codon and a valid stop codon. The *U90926* putative ORF could produce an 87-amino acids long protein. *U90926* is intergenic, located in mouse chromosome 5, residing between two protein-coding genes, *Uso1* and *Ppef2*. LncRNA *U90926* has been known to have very low sequence conservation across different species. However, a recent study showed that mouse *U90926* has a human ortholog, AC110615.1, which is positionally conserved (syntenic) between the two species, meaning that it is flanked by the same neighboring genes on human chromosome 4(114). They also showed that human ortholog has less than 50% sequence homology to the mouse *U90926*. Several studies have indicated that *U90926* is expressed in myeloid cells(116, 118, 119), preadipocytes(113), and retinal photoreceptor cells(115), which makes it not strictly cell type-specific, unlike many other lncRNAs. We and others showed that *U90926* is most typically a cytosolic lncRNA(113, 116, 118). However, one study showed that *U90926* is predominantly present in the nucleus in the mouse retinal photoreceptor cells(115). Nevertheless, mechanisms of action of *U90926* could vary in different cell types and based on the subcellular localization.

We have explored the protein-coding potential of *U90926* and its biological function in myeloid cells and in an endotoxemia mouse model, described in chapter 2. We have also further evaluated the role of *U90926* in weight gain and adiposity in vivo as a follow-up of in vitro study by Chen et al. in 2017 in 3T3-L1 preadipocytes(113). To elucidate this, we have used a diet-induced obesity model described in chapter 3. Besides our research on characterizing *U90926*, other studies elucidate some biological aspects of *U90926* from a different perspective. Firstly, *U90926* is characterized as a repressor of adipogenesis in vitro in 3T3-L1 preadipocytes(113). Then, another study showed that *U90926* works as a co-factor for herpes simplex virus type 1 (HSV-1) replication in mouse retinal cells(115). The same group identified the human ortholog of *U90926* and found that its expression correlates with HSV-1-induced acute retinal necrosis(114). Another recent study described that *U90926* in microglial cells could worsen ischemic brain injury by modulating neutrophil infiltration(116). Taken together, these studies confirm that annotated lncRNA *U90926* showing up as differentially expressed in transcriptomic data is not a coincidence; it has functional importance in different areas of biology.

LncRNA *U90926* has diverse roles in different cell types and in vivo models. Considering its versatility in terms of expression patterns, biological function, localization, conservation, and protein-coding potential, it is very likely that *U90926* has the potential to be one of the master regulators in many biological processes, including the immune system. Its protein-coding potential indicates a bifunctional role and adds an extra layer of complexity to its diversified mechanisms of action. Thus, distinct expression profiles of *U90926* observed in transcriptomic data is not a coincidence, and *U90926* function and molecular mechanisms need to be evaluated more meticulously in numerous biological processes.

1.3. Myeloid cells

Myeloid cells are a heterogeneous population of cells that make up a significant component of the innate immune system, playing critical roles in pathogen defense and inflammation, tissue homeostasis, and tissue repair. These cells originate from hematopoietic stem cells (HSCs) in the bone marrow and are supplied to all tissues constantly via circulation. They are recruited as first responders at the site of infection and tissue damage by various chemokine signaling. In the tissues, they are activated for phagocytosis and release inflammatory cytokines. They also work as antigen-presenting cells (APCs) to trigger the more specific adaptive immune response. Myeloid cells consist primarily of granulocytes, i.e., neutrophils, eosinophils and basophils, monocytes, macrophages and dendritic cells (DCs), and mast cells. Here, we have focused on macrophages as a classic paradigm of myeloid cells in the innate immune system.

1.3.1. Macrophages

Macrophages are prototypical innate immune cells strategically placed in nearly every tissue for their critical role in immune response, normal tissue development, tissue homeostasis, and repair of damaged tissue. Macrophages undergo functional phenotypic changes depending on their ontogeny, the local microenvironment in which they reside, and the type of pathogens and injuries to which they get exposed. The developmental origin of macrophages in various tissues has been a debatable topic in the field for a long time. In the 1960s, using labeling techniques after whole-body irradiation, van Furth and colleagues proposed that all the tissue macrophages originate from circulating bone marrow-derived monocytes(120). This notion prevailed for more than 40 years until, in the last decade, multiple independent studies have reversed it. They showed that macrophages derive from yolk sac or fetal liver precursors during embryonic development in many tissues and persist through adulthood independently of circulating blood monocytes(121–127). These tissue-resident macrophages, especially microglia in the brain, Langerhans cells in the skin, alveolar macrophages in the lung, and Kupffer cells in the liver, maintain their homeostatic pool without a contribution from circulating monocytes in the tissues through longevity or self-renewal(128–130). In other tissues, like the heart(127) and intestine(131), locally maintained embryonic macrophages and monocyte-derived macrophages coexist, playing distinct roles in tissue homeostasis and physiology. Currently, our conceptual understanding and advancements in genetic tools are sufficient to follow both embryonic and monocyte-derived macrophage subsets in health and disease. However, their specialized roles and how their function differs from the infiltrating monocytes during an injury are yet to be answered.

Generally, macrophages are characterized by their ability to engulf invading microbes or cell debris from the injured sites, secrete an array of immunomodulatory cytokines to recruit and activate other immune cells, present antigens to T cells, and act as an accessory to induce adaptive immune responses. Macrophages respond to an injury or infection by recognizing damage-associated molecular patterns (DAMPs; e.g., free heme, ATP) or pathogen-associated molecular patterns (PAMPs; e.g., LPS), which result in the initiation of a powerful pro-inflammatory cascade including recruitment of neutrophils and monocytes at the infected or wounded site(132–134). These PAMPs and DAMPs are detected by a collective set of pattern recognition receptors (PRRs) expressed in innate immune cells. In 1989, Charles Janeway Jr. first proposed the existence of PRRs, revolutionizing our understanding of the immune system(135). There are four prominent families of PRRs, the Toll-like receptors (TLRs), the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), the retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs), and the C-type lectin receptors (CLRs)(136, 137). TLRs are membrane-bound signal receptors, versatile and the most critical PRRs in the vertebrate immune system(138, 139). We will discuss more about macrophage response upon TLR-activation in the next section.

Functional polarization is another important avenue by which macrophages achieve distinct functional phenotypes depending on the environmental cues and under different pathophysiological conditions. When macrophages are recruited into tissues, they can have two different prototypical phenotypes, M1 (classically activated) and M2 (alternatively activated), depending on different stimuli. Microbial products or pro-inflammatory cytokines (for example, TLR ligands such as LPS, TNFα, IFNγ) stimulate M1 phenotypes, characterized by high production of pro-inflammatory cytokines such as IL-6, TNFα, IL-1, IL-12, IL-23, Type I IFN, CXCL1-3 and CXCL-5, high antigen presentation, and high production of nitric oxide (NO) and reactive oxygen species(140, 141). M1 macrophages are mainly involved in the removal of intracellular pathogens, resistance to tumors and can cause tissue damage during sterile conditions(142). On the other hand, M2 macrophages can play an immunoregulatory role, resist parasite infection, promote tumor growth, and orchestrate tissue repair(141). M2 macrophages get stimulated by different cytokines, such as IL-4, IL-13, IL-10, and specific TLR-ligands, and produce more anti-inflammatory cytokines that are important for regulatory mechanisms(142, 143). This classification of macrophages as M1 and M2 is oversimplified, which is very useful experimentally. In practical, macrophage polarization is transient and plastic, meaning M1 and M2 functions can be reversed rapidly by changing the stimulation(144, 145).

Taken together, macrophages are highly diverse and multifunctional innate immune cells that give protection against pathogens and tissue homeostasis. Their functional heterogeneity is needed for successful disease resolution and renewal of healthy tissue. For this dissertation, we have used a classic M1 macrophage *in vitro* model to characterize lncRNA *U90926* in chapter 2.

1.3.2. TLR signaling

In the last two decades, toll-like receptors (TLRs) have emerged as the center of attraction in biomedical research as this family of protein molecules is one of the earliest contributors to immune activation. TLRs are the best characterized among all the PRR families. These receptors can sense invading pathogens both extracellularly and intracellularly in endosomes or lysosomes(146). They are directly involved in regulating inflammatory responses and activation of innate and adaptive immune responses to eliminate foreign pathogens and cancer debris(147, 148). TLRs are highly expressed on myeloid cells of the innate immune system and have been widely studied in these cells. Major functions of TLR induction in innate immune cells include enhancement of antigen presentation, upregulation of costimulatory molecules such as CD40, CD80 and CD86, and promoting production of pro-inflammatory cytokines(147, 149). TLRs are also present in adaptive immune cells, T and B lymphocytes, and other non-immune cells like epithelial cells, endothelial cells, and fibroblasts, where they can play an innate immune-dependent or -independent role(150–153).

Toll gene product was first discovered in *Drosophila* and identified as a key molecule to antifungal immunity(154, 155). Shortly after that, a human homolog of *Drosophila* toll protein (presently known as TLR4) was identified as a receptor regulating antigen-presenting cells activity that promotes inflammation and adaptive immune response(156). Ever since, there have been a total of 10 TLRs identified in humans and 13 TLRs in mice, and a series of studies have been conducted to identify their respective ligands(139, 157). These TLRs have evolved to recognize a diverse range of PAMPs in microorganisms, including viruses, bacteria, fungi, and protozoa(146, 158, 159). At least one or more TLRs exist to bind any major biomolecule ligands, for example, protein, lipid, carbohydrate, or nucleic acid, that are being derived exogenously from pathogens(158–160). TLRs can also bind to endogenous host-derived DAMPs such as plasma membrane constituents, heat-shock proteins, and mitochondrial DNA(161). Specific ligands for each TLR have been reviewed elsewhere(138, 139, 158–160). In this dissertation, we have widely used a component of the gram-negative bacterial outer membrane, lipopolysaccharide (LPS), as a ligand that primarily binds to TLR4, to activate macrophages.

TLRs are type I transmembrane proteins, and ligand binding induces dimerization of the receptors to activate them(139, 158, 160). Like many other receptors in mammalian cells, the TLR signaling cascade depends on the ligand type, interacting TLR, and the recruited adaptor molecule involved in the signaling pathway. TLR signaling can be divided into two pathways based on the recruitment of two different adaptor molecules, either Myeloid differentiation primary response 88 (MyD88) or TIR domain-containing adaptor inducing IFN-β (TRIF) for downstream signaling cascades(139, 162, 163). Almost all the TLRs, except TLR3, utilize MyD88 as an adaptor protein, which leads to the increased expression of pro-inflammatory cytokine genes(164, 165). Nuclear factor (NF)-κB pathway and mitogen-activated protein kinase (MAPK) signaling pathway by activating JNK and p38-MAPK are mainly induced in MyD88-dependent TLR signaling(137, 138, 159). On the other hand, TLR3 and TLR4 use TRIF as an adaptor to trigger the enhanced expression of interferon type-1 genes by activating transcription factor IRF-3(137, 162). TLR4 is one of the most efficient PRRs that can transduce signal through both MyD88- and TRIF-dependent pathways to induce robust immune response upon activation(139, 166, 167).

Altogether, TLRs are an essential component of innate immune systems and play a crucial role in adaptive immunity by regulating APC function. Although TLRs are indispensable for host defense response against pathogens, aberrant TLR-activation by PAMPs, mutation in TLR signaling molecules, and DAMP-related TLR signaling can cause several other diseases like autoimmunity, chronic inflammation, and allergic diseases(168). Besides, TLRs have been studied extensively for the last two decades, mainly using loss-of-function genetic models. However, TLR signaling cascades and their crosstalk with other cell-intrinsic pathways in wild-type cells and organisms are still understudied, which is essential to understand the fundamental dynamics of TLRs. Moreover, TLR signaling provides plenty of targets to strategize novel therapeutics for the treatment of pathogen-associated diseases as well as chronic inflammation like cancer and autoimmune diseases.

1.4. Sepsis

Sepsis is a life-threatening medical condition that causes thousands of deaths and costs billions of dollars in healthcare annually in the USA alone(169). Sepsis arises when the body's dysregulated response to infection leads to its own tissue damage, organ dysfunction, and even death. Generally, sepsis develops when barrier tissues are infected with pathogenic bacterial strains; however, disruption of barrier tissue integrity by normal microbiota components can also cause sepsis(170, 171). The highest risk groups for sepsis or septic shock include older age (>65 years), infants, immunocompromised people, and patients with chronic disorders like autoimmune disease, tumors, kidney and lung diseases(172).

Innate immune activation is the critical factor in sepsis induction, and it occurs as different PRRs, such as TLRs, NOD, and RIG-like receptors and scavenger receptors, recognize PAMPs(173, 174). This receptor activation causes systemic production of pro-inflammatory cytokines and chemokines. PRRs can also recognize DAMPs released from damaged tissue, leading to excessive activation of immune cells and endothelial cells. This phenomenon causes the overproduction of cytokine, known as cytokine storm. It is followed by high cardiac output, lower systemic vascular resistance, subsequent blood pressure drops, increased coagulation, and decreased fibrinolysis, which eventually causes multiple organ failure and other pathophysiological alterations(175).

Despite its importance in the public health problem, developing a valid therapeutic agent for treating sepsis has yet to be successful. The use of antibiotics, fluid resuscitation, and primary supportive care for patients are still the best available treatment for sepsis(176). The primary reasons for this failure are the clinically complex and heterogeneous nature of sepsis and the lack of proper animal models that mimic human sepsis well. Many therapeutic agents have entered the clinical trials but universally failed to demonstrate efficacy in humans, in spite of showing tremendous promise in preclinical animal models(176–178). Nevertheless, the search for new therapeutic targets to treat sepsis continues. Despite being somewhat different in response to sepsis at the genomic level than humans, mouse models are widely used for sepsis research due to the ease of experimentation, the availability of genetically engineered species, and the relatively low cost(179).

1.4.1. Types of sepsis models

There are mainly three types of mouse models that are commonly used to induce experimental sepsis: injection of a toxic agent (e.g., LPS, CpG, zymosan or other PRR ligands), infusion or instillation of exogenous bacteria (e.g., induction of pneumonia, meningitis, urosepsis) and alteration of animal's endogenous barrier tissue integrity such as intestinal leakage and wound sepsis models.(176, 180–182). The first two groups are less invasive non-surgical models; however, the third group requires surgical interventions to be executed(176, 179).

LPS administration can successfully induce systemic inflammation that mimics the initial clinical features of sepsis, including increases in proinflammatory cytokines but without bacteremia(183, 184). Technically this model is easy, sterile, and the inflammatory response is reproducible. However, it induces a transient immune response and causes a variable hemodynamic response(185, 186). Additionally, there is an intrinsic dose response problem because sensitivity to toxins varies between species; for example, humans are more sensitive to LPS than mice(187). Bacterial infusion models mostly use a single pathogen to induce sepsis in a controlled and reproducible manner. Commonly, *Escherichia coli* is used to mimic gram-negative sepsis, whereas *Staphylococcus* and *Pseudomonas* are used to represent gram-positive human pathogens(182, 188). This model can approximate human sepsis caused by a single pathogen; however, it does not address the polymicrobial sepsis aspect. In this model, successful immune response varies depending on the dose, route of administration, and host's defense mechanism to clear the bacteria(189). In the most clinically relevant and widely used models, polymicrobial peritonitis is mimicked by disruption of gut integrity and allowing the microbial content to enter the peritoneal cavity(176). This can be achieved by two commonly used procedures, Cecal ligation and puncture (CLP)(190) and Colon ascendens stent peritonitis (CASP)(191). These models encompass many clinical features, including initial systemic inflammation to multiple organ failures and drug responses of human polymicrobial sepsis. However, the limitations of both of these procedures include these are technically challenging, difficult to reproduce, newborn mice cannot be used in these experiments, and it is not possible to use clinically relevant bacterial strains to induce sepsis(192).

Taken together, the available disease models have successfully imitated the initial stages of sepsis; however, the later stages of immunosuppression and secondary infection in these models have not been addressed completely. Considering the genetic differences between species and the complex nature of pathophysiological processes in sepsis, it seems unrealistic to expect great leaps in translational human trials using data from these animal models(193). However, this does not mean that animal sepsis models are not worth undertaking, but it calls for a change in our current approaches. In addition to their inherent problems, these models have extreme inter-laboratory and inter-operator variabilities(184). Therefore, scientists need to standardize the methods and protocols in the literature so that a uniform platform can be achieved to utilize these animal models for sepsis studies. Also, a systematic multi-model approach needs to be developed and standardized to test potential therapies for human sepsis. These animal models of sepsis are still very useful, depending on the interest and scale of the studies. In this dissertation, as a reductionist model to address a basic biological (rather than preclinical) question, we have used an LPS endotoxemia mouse model of sepsis in chapter 2, which is described below in more detail.

1.4.2. LPS endotoxemia model

Since 1930, when LPS was recognized as a critical effector molecule of human sepsis, murine endotoxemia models have become the staple model for studying experimental sepsis and have been utilized for almost 100 years to recapitulate the physiology of human sepsis(194). This model involves direct administration of LPS in the blood, peritoneum, or lung(179). LPS is an outer membrane component of gram-negative bacteria that act as PAMPs activating TLRs to trigger a heightened immediate inflammatory response that mimics the activation of the innate immune system in human sepsis(195). This model reproduces certain features of the acute phase of gram-negative sepsis and endotoxemia, such as lack of focal infection site, hypodynamic stage development without reaching a hyperdynamic stage, lactic acidosis, transient and abundant pro-inflammatory cytokine production such as TNF-α, IL-6, and IL-1, higher production of DAMPs, and strong innate immunity activation(176, 189, 196). Nevertheless, the murine response to LPS is dose-dependent, meaning that the dose can be titrated to recapitulate specific stages of sepsis; for example, a smaller dose can cause a hyperdynamic state, whereas a large dose can cause direct hypotension and hypothermia characteristics of septic shock(194, 195, 197).

The significant advantages of the LPS endotoxemia model are technical ease and creating reproducible responses using a quantifiable amount of LPS(176, 179). However, this model completely neglects the host response to a gram-positive bacterial infection, polymicrobial sepsis, and the host-pathogen interaction due to the host's defense mechanisms to clear the bacteria(182, 198). In this model, there is no microbial source for the continual release of LPS or other PAMPs, unlike human sepsis. In comparison with human sepsis, endotoxemia induces higher pro-inflammatory cytokine production, which peaks earlier in massive amounts but shows a faster resolution(171, 199). However, LPS in large doses has been shown to produce comparable cytokines in amounts and kinetics to a small subset of fulminant human sepsis, such as meningococcal sepsis(184, 200). Another caveat of comparing the LPS model to human sepsis is that humans are approximately 1000 times more sensitive to LPS than mice, owing to a greater dose of LD_{50} in mice(187, 201).

The endotoxemia model is tremendously helpful while studying specific biological pathways in sepsis, such as the immune response to particular PAMPs by its specific PRRs, for example, LPS and TLR4. While compared with other sepsis models believed to be more physiologically relevant to human sepsis, such as the CLP model, LPS dose can be manipulated to achieve a similar mortality rate and hemodynamic properties. However, cytokine profiling differs in these two models, where all the pro-inflammatory cytokines IL-6, TNF-α, keratinocyte chemoattractant (KC), and macrophage inflammatory protein-2 (MIP-2) peak earlier with a greater amount but for a short time in endotoxemia(184, 198).

Like other animal models, the LPS endotoxemia model also showed some promising results at the pre-clinical stage yet failed at the clinical stage for numerous reasons discussed in the earlier section. Based on the successful LPS model data, some clinical trials using anti-TNF-α and anti-IL-1 therapy were performed; however, they did not improve survival in septic patients(202, 203). Even so, for decades, the LPS endotoxemia model has remained a valuable tool to interrogate simplified subsets of the complex physiology of human sepsis. Thus, constant efforts to develop sophisticated and standardized methods will continue for the ideal pre-clinical therapeutic testing platform for sepsis.

1.4.3 Role of liver in sepsis

Multiple organ dysfunction is a hallmark of sepsis and septic shock. The organs more frequently affected during sepsis are the lung, kidney, liver, heart, and central nervous system(204); however, herein, we will focus more on liver function for the context of this dissertation. In normal conditions, the liver is involved in homeostasis, and its principal function includes the metabolism of carbohydrates, lipids, proteins, and hormones; biosynthesis of blood components and clotting factors; detoxification; bile production; metabolism of nitrogen compounds, synthesis of urea; storage of glycogen, cholesterol, vitamins, iron, and many more(205). Severe sepsis causes profound changes in normal liver function by rapidly shifting the balance of hepatic metabolic activity toward a response to inflammation with an acute phase reaction (APR)(206).

The liver plays a vital role in the systemic response during sepsis through the clearance of bacteria or toxins and through the APR, the release of liver-derived cytokines and other inflammatory mediators, and coagulation cascade components(206, 207). These functions are carried out by different types of cells in the liver, primarily by hepatocytes (HCs), liver macrophages known as Kupffer cells (KCs), and sinusoidal endothelial cells (SECs). In the case of sepsis or septic shock, HCs will shift their metabolic pathways towards the upregulation of inflammatory response. They increase the synthesis of acute phase proteins, predominantly mediated by IL-6(208). This leads to an increase in C-reactive proteins, α-1 antitrypsin, fibrinogen, prothrombin, and haptoglobin(209). The increase in acute phase proteins results in the inhibition of the protein C pathway, which is responsible for pro-coagulation activity in sepsis. Besides the passive role in immune modulation by HCs, recent studies revealed that they are involved in the direct inflammatory response to LPS exposure(210). In response to LPS, HCs are shown to activate TLR4 pathways to induce NF-κB activation and production of pro-inflammatory cytokines, TNF-α and IL-6, both in vitro and in vivo(211–213).

In contrast, KCs are mainly involved in scavenging bacteria and endotoxin. In response to LPS, KCs produce pro-inflammatory cytokines, TNF-α, IL-6, IL-1β, IL-12, and IL-18, reactive oxygen species (ROS), and nitric oxide (NO), which promote endothelial cell and hepatocyte injury(214–216). At the early stages of sepsis, KCs release TNF-α and leukotriene B4, which promote neutrophil recruitment to the liver to cause further damage(217). Similarly, SECs are also involved in producing pro-inflammatory cytokines in response to LPS, and they are the primary hepatic source of a potent vasoconstrictor, endothelin-1 (ET-1) production(218). ET-1 concentration is an early and sensitive indicator of mortality in septic patients(219).

Taken together, all the cell components of the liver have the capacity to respond to LPS and other microbial products and exhibit hyperresponsive immune activation, which in turn causes liver dysfunction. The pathophysiology of liver dysfunction has been better understood for the past few decades; however, a tool to recognize liver dysfunction early and accurately remains limited. Also, liver dysfunction treatment has only been in general therapeutic steps on sepsis syndrome management(220). Thus, an earlier and better diagnosis of liver dysfunction and novel therapeutic strategies for early treatment need to be developed to decrease septic patient mortality.

1.5. Adipogenesis and Obesity

Beyond the immune function of lncRNA *U90926*, we have also explored the effect of *U90926* on adiposity using an *in vivo* model in this dissertation's chapter 3. Therefore, some relevant topics are reviewed here from the vast literature of adipose tissue biology.

1.5.1. Adipose tissue

Adipose tissue has been regarded as an active endocrine organ that is at the center of energy homeostasis. It also plays a vital role in lipid storage and whole-body insulin resistance and protects delicate organs from mechanical stress(221). Adipose tissue is classified into two major groups, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT constitutes most of the fat depots in adult humans and mobilizes triglycerides and lipids systematically by lipolysis when required by other tissues(222). WAT is often subdivided into subcutaneous and visceral fat, where the latter is well associated with metabolic diseases, but the former is not(223). WAT is also crucial for energy homeostasis, insulin resistance, and endocrine actions. It is known to secrete a variety of substances including adipokines that help in regulating energy metabolisms, such as leptin, adiponectin, resistin, TNF-α, IL-6, monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), angiotensinogen, visfatin, retinol-binding protein-4, serum amyloid A (SAA), and others(222, 224). On the other hand, BAT is predominantly involved in nonshivering thermogenesis, mediated by uncoupling protein-1 (UCP1) in the mitochondria(225). BAT was initially well-established as an essential fat depot for postnatal babies in various mammals. However, several studies have shown that adult humans also contain BAT depots in the thoracic and supraclavicular regions and have suggested the metabolic role of BAT in human energy metabolism(226, 227). Brown adipocytes were also detected in subcutaneous WAT in mice(228), which has increased interest in their potential use in solving WAT pathologies in the field.

Adipose tissue comprises various cell types, including endothelial cells, blood cells, fibroblasts, pericytes, preadipocytes, macrophages, and other immune cells like T and B cells(229–231). However, mature adipocytes are the predominant cell types present in adipose tissue. WAT and BAT adipocytes are derived from different mesenchymal precursor cells and have distinct morphological characteristics. There is also evidence that adipocytes can be derived from bone marrow hematopoietic precursors via myeloid lineage(232). Morphologically, WAT adipocytes are generally spherical cells with a unilocular lipid droplet covering most of the cytosol, causing the other organelles to be in a confined arrangement. BAT adipocytes are characterized by a centered nucleus, multilocular smaller lipid droplets, and abundant distribution of cristae-rich mitochondria(233). Besides classic white and brown adipocytes, studies suggest an emerging presence of inducible brown-like beige adipocytes found in the WAT in response to various stimuli such as cold and β-adrenergic stimulation(234). Altogether, adipose tissue is a multifunctional endocrine organ that plays vital role in energy metabolism. Location and types of fat depots dictate their functional roles in different organs.

1.5.2. Adipogenesis

The process by which fibroblast-like progenitor cells differentiate into lipid-laden, insulin-responsive mature adipocytes is called adipogenesis(235). It can be described as two steps process to simplify the phenomenon. In the first step, the commitment step, a mesenchymal precursor cell (MSC) restricts itself to adipocyte precursor without any morphological changes to form a preadipocyte. This is followed by the differentiation step, during which preadipocytes undergo growth arrest, accumulate lipids, and form mature, functional, insulin-responsive adipocytes(235, 236). The differentiation process to develop mature adipocytes is an elegant progression of sequential activation of several transcription factors commencing with activating protein-1 (AP-1) family and continues with the induction and expression of peroxisome proliferator-activated receptor γ (PPARγ), a critical proadipogenic transcription factor. Other positive regulators of adipogenesis include CCAAT/enhancer-binding proteins (C/EBPs) family, single transducers and activators of transcription (STATs), sterol-regulatory-element-binding-protein-1

(SREBP1), insulin-like growth factor I (IGF-l), and insulin itself(235, 237). The transcriptional cascade of adipocyte differentiation was dissected mainly using experimental *in vitro* models such as 3T3-L1 preadipocyte and 3T3-F442A cell lines(238).

The most extensively characterized and used preadipocyte cell lines are 3T3-L1 and 3T3-F442A, derived from Swiss 3T3 mouse embryonic fibroblasts(239, 240). The main advantage of using these models is that they produce a homogeneous population of mature adipocytes morphologically and biochemically similar to adipocytes *in vivo*(241, 242). The downside is that molecular events constituting adipogenesis in a cell line may different significantly from human preadipocytes. Also, the ability of differentiation of a preadipocyte cell line decreases with the increasing passage number(243). On the other hand, the study of preadipocyte differentiation *in vivo* is even more difficult. Since human and animal adipose tissue is heterogeneous, it is hard to distinguish between preadipocytes and fibroblasts(244). Additionally, the inability to synchronize the preadipocytes at similar developmental stages is a confounding challenge to detailed *in vivo* studies(245). Preadipocyte primary culture has been used to overcome these issues(246). However, it also has shortcomings such as chances of contamination of other cells in the culture, low percentage of preadipocytes in total adipose tissue, and limited life span of preadipocytes(243). Therefore, the molecular control of adipogenesis has primarily been studied in these *in vitro* preadipocyte cell lines, and till now, they are the most widely used models to study adipogenesis.

Generally, adipose tissue expansion occurs in two ways; enhancing lipid accumulation in the existing adipocytes to increase adipocyte size (hypertrophy) and increasing differentiation of adipocyte precursor cells or adipogenesis to increase new adipocyte number (hyperplasia)(238, 247, 248). The balance between hypertrophy and hyperplasia is essential for individuals' metabolic health. Recent studies showed that adipogenesis is more beneficial in mitigating obesity-related metabolic diseases than hypertrophy(249, 250). Therefore, adipogenesis has emerged as a viable therapeutic target to treat metabolic consequences of high-fat exposure(251). Nonetheless, our understanding of adipogenesis and its key modulators are still limited, and new strategies and methods are required to unravel the significant functional determinants of adipogenesis and its pathophysiological relevance.

1.5.3. Obesity and metabolic changes

Obesity is defined by the excessive accumulation of WAT, which is clinically designated as a body mass index (BMI = kg/m^2) exceeding 30 (WHO, 2023). Obesity has become a significant public health concern worldwide, including in developing countries, as it is a major risk factor for many chronic conditions like type 2 diabetes, cardiovascular disease, and different types of cancer (WHO, 2023). The development of obesity has mainly been attributed to the overconsumption of food (increased energy intake) and lack of physical activity (decreased energy expenditure), which lead to a chronic positive energy balance resulting in the expansion of adipose tissue by storing excess energy as triglycerides within adipocytes(252). The expansion of adipose tissue during obesity is caused by both hypertrophy (increased adipocyte size) and hyperplasia (increased adipocyte number)(248, 250). Although, genetics and other obesogenic stimuli can affect the contribution of each mechanism in the accumulation of excess fat during obesity(248).

Some early studies in rodents and humans showed no increase in adipocyte number in obesity, indicating that hypertrophy is the sole reason for fat accumulation during obesity(253, 254). However, given the maximum range of adipocyte size(255), it is likely that the expansion of WAT only by hypertrophy would be limited. Some studies showed that hyperplasia occurs only on the early onset of obesity, prior to the developmental establishment of adipocyte number(256, 257). Others have shown that increased adipocyte number contributes to fat accumulation during the adult onset of obesity in both genetic and diet-induced obesity(253, 258–261). Interestingly, some of them also showed that hyperplasia-induced WAT expansion is not always restricted to severe obesity.

In general, adipocytes respond to a systemic signal to store lipids or mobilize lipids as the organism requires. During excess nutrition, blood glucose levels rise, and the pancreatic hormone insulin inhibits hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), promotes glucose uptake and activates de novo lipogenesis to package lipids to store the excess nutrients. During obesity-associated metabolic decline like type 2 diabetes, adipocyte fails to respond correctly to almost all these extracellular signals, especially insulin, leading to elevated plasma glucose and lipids(221, 262). As the adipocytes expand in size, they experience increased mechanical stress and hypoxia, which contribute to adipose tissue inflammation(263, 264). Other characteristics of adipocyte dysfunction in obesity include increased rates of adipocyte necrosis, aberrant adipokine (signaling proteins produced by adipocytes) release, such as increased production of pro-inflammatory cytokines TNF-α, IL-6, IL-1, and decreased production of adipokines, leptin and adiponectin, which play a vital role in systemic metabolism(265–267).

Obesity is characterized as a pro-inflammatory state where immune cells, especially macrophages, also play an essential role in adipose tissue inflammation. An increased amount of macrophage in adipose tissue is an essential hallmark of obesity(268, 269). Adipose tissue macrophages (ATMs) undergo a phenotype switch from M0 (resting) and M1(anti-inflammatory) in the lean state to M2 macrophages(pro-inflammatory) in obesity and release increased levels of TNF-α, IL-6, IL-1β, PAI-1, and MCP-1 to promote inflammation and modulate adipocyte function which leads to insulin resistance eventually(222, 270, 271).

In the past 100 years, adipose tissue biology has transitioned from the detailed observation of adipose tissue and molecular mechanisms of adipogenesis in vitro to the identification and characterization of in vivo adipocyte lineages. The use of genetic in vivo models of obesity and diet-induced obesity models has allowed the field to thoroughly investigate adipose tissue growth and remodeling in vivo. However, the lack of a direct methodology and genetic tool has limited our knowledge and understanding of the adipose tissue intrinsic mechanisms that regulate adipocyte size and number in vivo. Recent treatment approaches are targeting more on decreasing metabolic disorders and other co-morbidities associated with obesity. Some studies define obesity depending on the metabolic outcome, metabolically healthy obesity (increased adipogenesis and vascularization), and metabolically unhealthy obesity (increased adipocyte size and high levels of glucose and lipids in the circulation)(236, 250, 251). Thus, the cellular and molecular mechanisms of adipose tissue expansion significantly affect the pathogenesis of obesity. Therefore, studying adipose tissue accumulation in vivo can potentially identify novel therapeutic targets for obesity.

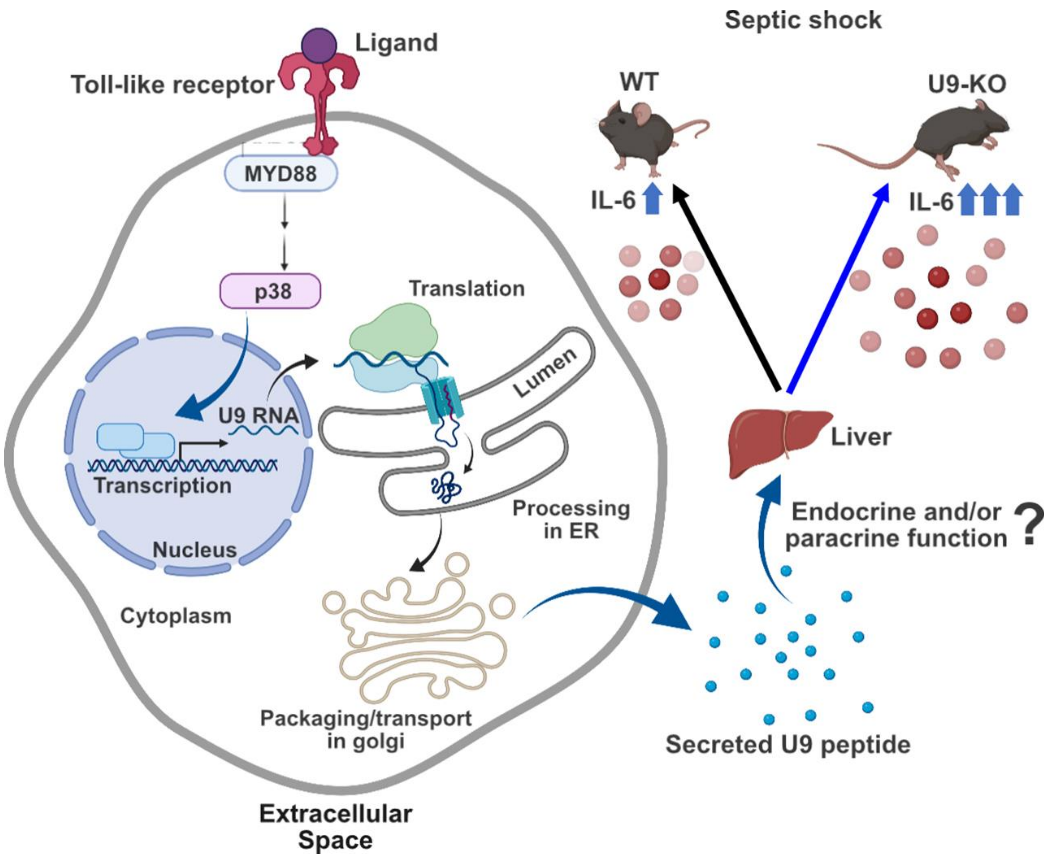

Figure 1-1. LncRNA *U90926* is highly expressed upon TLR-stimulation in macrophages, encodes a secreted protein and regulates sepsis outcome by modulating systemic IL-6 levels.

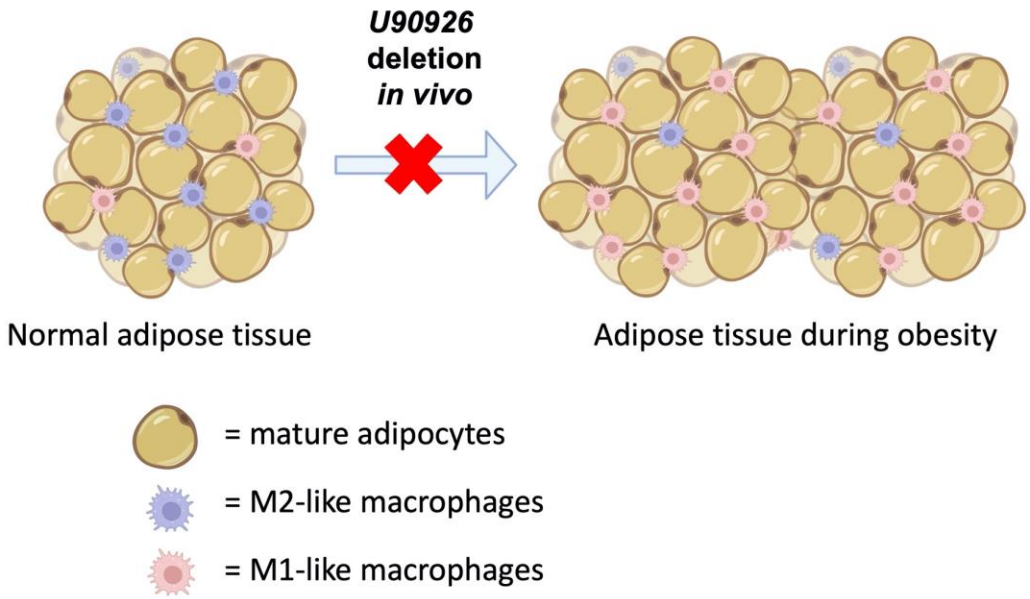

Figure 1-2. LncRNA *U90926* deficiency does not have major effect on adipose tissue homeostasis.

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CHAPTER 2: LNCRNA *U90926* IS INDUCED IN ACTIVATED MACROPHAGES, IS PROTECTIVE IN ENDOTOXIC SHOCK, AND ENCODES A NOVEL SECRETED PROTEIN

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Running Title: LncRNA *U90926* role in macrophages and systemic inflammation

Keywords: LncRNA, macrophages, toll-like receptors, endotoxic shock, IL-6

Keypoints:

LncRNA *U90926* is upregulated in TLR-stimulated macrophages

U90926 contains a translated ORF that encodes a secreted protein

U90926 knockout mice are more susceptible to endotoxic shock

ABSTRACT

Thousands of long non-coding RNAs (lncRNAs) are encoded in mammalian genomes, yet most remain uncharacterized. Here, we functionally characterized a mouse lncRNA named *U90926*. Analysis of *U90926* RNA levels revealed minimal expression across multiple tissues at steady state. However, the expression of this gene was highly induced in macrophages and dendritic cells by toll-like receptor activation, in a p38 MAP kinase- and MyD88-dependent manner. To study the function of *U90926*, we generated *U90926*-deficient (U9-KO) mice. Surprisingly, we found minimal effects of *U90926* deficiency in cultured macrophages. Given the lack of macrophage-intrinsic effect, we investigated the subcellular localization of *U90926* transcript and its protein-coding potential. We found that *U90926* RNA localizes to the cytosol, associates with ribosomes, and contains an open reading frame that encodes a novel glycosylated protein (termed U9-ORF), which is secreted from the cell. An in vivo model of endotoxic shock revealed that, in comparison with WT mice, U9-KO mice exhibited increased sickness responses and mortality. Mechanistically, serum levels of IL-6 were elevated in U9-KO mice, and IL-6 neutralization improved endotoxemia outcomes in U9-KO mice. Taken together, these results suggest that *U90926* expression is protective during endotoxic shock, potentially mediated by the paracrine and/or endocrine actions of the novel U9-ORF protein secreted by activated myeloid cells.

INTRODUCTION

Macrophages are the prototypical innate immune cell, strategically positioned to encounter infectious organisms, detect them via an array of microbial pattern recognition receptors (PRRs), and rapidly respond by producing pro-inflammatory mediators that alert the immune system. While this response is required for efficient clearance of infectious agents, an over exuberant response can drive immune-mediated pathology and fatal systemic shock(1). Therefore, regulatory mechanisms underlying these responses could be harnessed for treating conditions such a septic shock, which annually causes ~270,000 deaths in the U.S. alone(2).

Gene expression in mammals is a highly dynamic, complex, and tightly regulated process. This is particularly evident in innate immune cells, which can completely reprogram their entire gene expression repertoire within minutes of encountering an inflammatory stimulus. The central dogma of molecular biology dictates that information encoded in the genome is transcribed into RNA, followed by translation into protein, with the latter carrying out most cellular functions. However, recent advances in genomics have challenged this notion by demonstrating that protein-coding transcripts may in fact be outnumbered by non-canonical transcripts that do not encode proteins(3–5). Emerging evidence suggests that a large class of such transcripts, the so-called long non-coding RNAs (lncRNAs), defined as greater than 200 nucleotides in length and lacking a conserved open reading frame (ORF)(3), play unique cell type-specific roles in numerous cellular processes, including innate immune cell effector function(6–8).

Several early landmark studies have identified specific lncRNAs, such as lincRNA-Cox2, THRIL, and lincRNA-EPS, that function as positive or negative regulators of macrophage function and pro-inflammatory gene expression(9–11). These lncRNAs control gene expression and cellular functions in diverse ways, including interaction with proteins involved in gene expression regulation or signaling, interactions with chromatin, interactions with mRNAs or miRNAs, and others(6, 7). Each lncRNA appears to have its own unique mode of action, and novel mechanisms continue to emerge. Among these is the emerging evidence of pervasive translation of lncRNAs(12, 13), including two recent examples in which the protein products control macrophage inflammatory functions(14, 15).

Our previous studies(16–18) have focused on a central signaling pathway activated in macrophages downstream of PRRs, the p38 MAP kinase (MAPK) pathway, in which we identified an uncharacterized putative lncRNA named *U90926*, which was among the most highly downregulated genes in p38α MAPK-deficient murine macrophages(16, 18). Since the initial annotation of *U90926* lncRNA by a genome exploration research group(19), there was little known about the function of this gene. However, several studies have begun to shed some light on the biological function of *U90926*, including a possible role as a repressor of adipogenesis in vitro, a viral replication co-factor in retinal cells in vitro, and a pro-inflammatory factor in microglia during experimental stroke(20–22). Here, we focused on the function of *U90926* in macrophages. We demonstrate that the expression of this gene is highly inducible in myeloid cells by lipopolysaccharide (LPS) and other toll-like receptor (TLR) agonists in a MyD88- and p38α-dependent manner. To study the role of this gene in vivo, using CRISPR-Cas9, we generated the first (to our knowledge) mouse completely lacking the *U90926* locus (U9-KO). We demonstrate that, surprisingly, *U90926* deficiency has minimal intrinsic effects on macrophage pro-inflammatory gene expression in vitro. Unexpectedly, we found that *U90926* RNA localizes to the cytoplasm, associates with ribosomes, and contains an ORF that encodes a novel 87-amino acid-long protein that is secreted from the cell. In vivo, *U90926* deficiency exacerbates endotoxic shock through the upregulation IL-6 production. Taken together, our results suggest that TLR-mediated induction of *U90926* in macrophages may regulate inflammatory responses through paracrine production of the novel secreted protein encoded in this locus. These findings challenge the "non-coding" paradigm of lncRNA function, warranting a careful examination of the coding potential of ORFs contained in each of these genes.

MATERIALS AND METHODS

Animals

Wild type C57BL/6J (B6) mice and wild-derived PWD/PhJ (PWD) mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and were acclimated at the animal facility at UVM for at least 2 weeks prior to any experimentation, including breeding. p38αCKOLysM mice were generated in our laboratory previously(16), by crossing LysM-Cre mice (B6.129P2-*Lyz2tm1(cre)Ifo/J*)(23), and p38αf/f (floxed) mice (B6.129-*Mapk14*$^{tm1.2Otsu}$*OtsuRbrc*)(24). *U90926*-deficient mice (C57BL6/J-*U90926*^{em1Uvm}) were generated as described below. 8-12 weeks old male and/or female mice were used in all experiments. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Vermont.

Generation of *U90926*-deficient mice

U90926-deficient mice (U9-KO) were generated by CRISPR/Cas9 in B6 background. Using the CRISPOR tool(25), we designed two custom single guide RNAs (sgRNAs) targeting cut sites ~200 bp upstream of the first exon (site 1: 5'-ATACGGATGCAGCCTGTACA -3') and ~200 bp downstream of the last exon (site 2: 5'- GCGGCCCACGTACCATGCGT-3'), to delete the entire ~5.5 Kb *U90926* locus (Fig. 2a). The sgRNAs and recombinant Cas9 enzyme were prepared by Synthego, Inc (Redwood City, CA). Microinjection of single cell embryos was performed at the University of Rochester Mouse Genome Editing Resource, on a fee-for-service basis. Two primer sets were used for the initial PCR-based genotyping. One set of primers (forward primer: 5'-GCTGAAAGAGAACACATTGCCTT-3' and reverse primer: 5'-GGGCTAAGATGTTGCTTCCTGT-3') targets the 5' WT region and produces a 500 bp amplicon, which should be absent in KO. A second set of primers (forward primer: 5'-GCTGAAAGAGAACACATTGCCTT-3' and reverse primer: 5'-ACTGAGAGAAGCCAAAGCTCA -3') was designed flanking the entire region, which only yields product if the deletion is present (in between 200-500 bp, depending on size of deletion). A single male U9-KO mouse was chosen as the F0 founder to generate the U9-KO colony. To eliminate any potential off-target deletions made by CRISPR/Cas9 elsewhere in the genome, U9-KO mice were backcrossed with WT B6 mice. A single male KO founder was bred to two WT B6 females. Resulting heterozygous (HET) U9-KO were backcrossed to B6 for 2 more generations and subsequently bred as HET ^{x} HET to generate all subsequent KO animals and WT controls. An optimized PCR genotyping reaction was designed to identify U9-KO and WT genotypes. Three primers were used at in a single reaction: Forward primer (5'-GAGCCTTTGTGCAAGGCTTAT-3') was designed to bind upstream of the sgRNA site 1, KO reverse primer (5'-CAGCCCAAGTACACCTGCT-3') binds downstream of sgRNA site 2 (should yield a 152 bp product only if *U90926* whole locus is deleted), and a WT reverse primer (5'-ATGGATTCCCCACCTCATTCC-3') binds downstream of sgRNA site 1 and near the exon 1 (only yields a 346 bp product when *U90926* is not deleted). HET mice produce both the products. In all the PCR reactions, Accuris 2x Taq Red Dye Master Mix (Accuris instruments, Dublin, Ireland) was used. Annealing temperature was 550C for the primers and total 35 cycles were run in the Biometra Trio thermal cycler (Analytik Jena, Upland, CA). Backcrossed U9-KO mice were also validated by Sanger sequencing at the Vermont Integrative Genomics Resource core facility at UVM. A representative U9-KO genomic sequence (assembled contigs 5' to 3') showing the deletion is given below (SgRNA binding sites are underlined):

GGATGGGACCCCCCCAACACCTGAGGCAACATGACCATGCTGAGGTTGGACA
GGACAGGGGAGCCTTTGTGCAAGGCTTATACATCTATGCCAAGCTTCTGTCTC
CCGCCCAGTGAGACCCAGGGCAAAGCCCATACG……………………………………
…………………………TGGGTGATGCTATAGTGATATAGCAGGTGTACTTGGGC
TG

Cell culture experiments

Bone marrow-derived macrophages (BMDMs) and bone marrow-derived dendritic cells (BMDCs) were generated from mouse bone marrow using a standard 7-day M-CSF (10ng/ml, Peprotech) and GM-CSF (20ng/ml, Peprotech) differentiation protocol, respectively as previously described(17, 26). BMDMs were cultured in complete DMEM media containing 10% FBS, 2 mM L-glutamine, 100 Units/ml penicillin, and 100 µg/ml streptomycin. BMDCs were cultured in complete RPMI 1640 media containing 10% FBS, 2 mM L-glutamine, 100 Units/ml penicillin, 100 µg/ml streptomycin, and 55 µM beta-mercaptoethanol. At day 7, differentiated BMDM and BMDC were harvested and plated (1 million cells/well in a 12-well dish or 200K/well in a 96-well dish) for different in vitro experiments with indicated treatments. Cells from peritoneal cavity were isolated by lavage as described previously(16). Immortalized MyD88-KO macrophages(15) were generously provided by the Fitzgerald lab (UMass Medical School, MA) and cultured in complete DMEM with 10% FBS and penicillin and streptomycin. RAW 264.7 macrophages, HEK 293T cells, HeLa cells, 3T3 fibroblasts, and 3T3-L1 preadipocytes, originally obtained from ATCC and donated by various investigators, all were cultured in DMEM supplemented with 10% FBS and sodium pyruvate. AML12 cells were obtained from ATCC and cultured in DMEM F12: HEPES with 10%FBS, ITS-G 100X (Gibco) and 100nM dexamethasone (Gibco). For co-culture, AML12 and primary BMDMs were used at 4:1 ratio and cultured in AML12 media described above. For all the in vitro lipopolysaccharide (LPS) stimulation studies, we used 100 ng/ml of E. coli LPS O26:B6 (Sigma Aldrich, St. Louis, MO). Select agonists from the mouse TLR1-9 Agonist kit (InvivoGen, San Diego, CA) were used to activate TLR1/2, TLR3, TLR5, and TLR9 and cytokine receptors were activated using recombinant mouse TNFa and IL-1b (Peprotech). For the p38 MAPK inhibition experiment, SB203580 (Tocris Bioscience, Bristol, UK) was used at 10 □M. For all in vitro experiments, biological replicates were used, with each replicate representing independent BMDM cultures from individual mice.

RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

For most of the in vitro experiments, 12-well plates were seeded with 1x106 cells per well and stimulated at different time points. RNA was extracted according to the manufacturer’s instructions using the RNeasy plus Mini Kit by Qiagen (Hilden, Germany). For in vivo experiments, Direct-zol RNA Microprep Kits (Zymo Research, Irvine, CA) were used for RNA extraction as per manufacturer’s protocol. Whole liver tissue samples were collected into 2 ml screw cap tubes containing 1/3 full of 1.0mm diameter Silicon-Carbide particles (Biospec Products, Bartlesville, OK) and 1 ml of TRIzol reagent (Invitrogen, Waltham, MA) per tube. Then, the samples were homogenized using a Mini-Beadbeater instrument by Biospec Products (Bartlesville, OK) for total 2 min by 4 cycles, and placing tubes on ice-water bath in each interval to avoid over-heating. Then, tubes were centrifuged at 13,000 rpm for 2 min to pellet any debris, 200□□L of homogenate were collected and 100□L of fresh TRIzol was added, followed by extraction using the Direct-zol RNA Microprep Kit as above.

RNA concentration was determined by Nanodrop (Thermo Scientific NanoDrop 2000 Spectrophotometer) and cDNA synthesis reaction by reverse transcription was performed according to the manufacturer’s instructions using the qScript cDNA Super MIX kit (QuantBio, Beverly, MA). The qPCR reaction was performed according to the manufacturer’s instructions using the DyNAmo ColorFlash SYBR Green kit by Thermo Fisher Scientific (Waltham, MA), using target-specific primers. The qPCR was run on a

Quant Studio 3 Real-Time PCR System by Applied Biosystems (Thermo Fisher Scientific, Waltham, MA). Target gene expression was normalized by the expression of the housekeeping gene B2m (β-2-Microglobulin) and calculated by a comparative Ct method formula 2^-(deltaCt) and multiplied by a factor of 10,000 for ease of visualization, i.e. 2^-(Cttarget gene – CtB2m)*10,000. We did not normalize relative expression to a reference sample (i.e. delta-deltaCt).

Flow cytometry

For in vitro experiments, BMDMs and BMDCs differentiated for 7 days were seeded at 1x106 cells/well in a 12-well dish. Cells were treated with LPS, and 0 and 24 hours and after incubation cells were removed using non-enzymatic cell stripper liquid (Corning Inc., Corning, NY) and subjected to staining for flow cytometry. For in vivo experiments, spleen and lymph nodes were isolated from mice single cell suspensions were prepared followed by passing through a 70 μm strainer. Red blood cell (RBC) lysis was performed by incubating the cells in 0.8% ammonium chloride solution (StemCell Technologies, Cambridge, MA) and cells were subjected to staining for flow cytometry analysis. Cells were stained with the UV LIVE/DEAD fixable stain (Invitrogen) and then surface labeled for different combinations of following markers: CD11b, CD11c, CD80, CD86, CD40, CD19, TCRβ, Ly6G, MHCII, CD4, and CD8 (Biolegend, San Diego, CA) and fixed with 1% paraformaldehyde (Sigma Aldrich, St. Louis, MO). Samples were analyzed on an LSRII cytometer (BD Biosciences) or an Aurora (Cytek Biosciences). All flow cytometry data analysis was performed using FlowJo Software version 10.6.1 (BD Biosciences).

Cytokine quantification by ELISA

For the detection of cytokines in the cell culture supernatants or sera, ELISAs were performed as described previously(17), using the primary capture mAbs anti-TNFα, anti-IL-6, and anti-IL-1β and their corresponding biotinylated detection mAbs (Biolegend, San Diego, CA). Other ELISA reagents included: recombinant mouse TNFα, IL-6, and IL-1β as standards (Biolegend, San Diego, CA), HRP-conjugated avidin D (Vector Laboratories, Burlingame, CA), and TMB microwell peroxidase substrate and stop solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD). IL-10 cytokine was measured using Mouse IL-10 DuoSet ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. Biological replicates were used, representing independent BMDM cultures from individual mice.

RNAseq and bioinformatic analysis

Four biological replicates per genotype, representing independent BMDM cultures from individual mice, were used. BMDMs were seeded at 1x106 cells/well and treated with LPS for 4 and 24 hours. RNA was extracted as described above for in vitro experiments, followed by quantification, quality assessment, library preparation, and sequencing at the Vermont Integrative Genomics Resource core facility at UVM. cDNA libraries were synthesized using SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Takara, Japan), according to manufacturer protocol. Ribosomal RNA depletion was done to ensure recovery of a range of RNA species. Single-end 75 bp sequencing was performed on an Illumina HiSeq1500, generating around 30 million reads per sample.

For whole liver tissue RNAseq, RNA was extracted as described above, followed by quantification and quality assessment. Library preparation and sequencing were performed commercially by Novogene (Novogene Co, China), as follows. mRNA library preparation (poly A enrichment) was done by using NEB Next Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA) followed by paired end 150 bp sequencing using NovaSeq 6000 platform, to generate 20 million reads per sample.

Demultiplexed sequencing data was received from the sequencing facilities and quality control of the sequencing data was done using FastQC platform. Genomic alignment and transcript quantification from RNA-seq data was performed using Salmon tool(27). Differential gene-expression analyses were conducted using DESeq2(28) in R studio. Cutoffs for differential gene expression are described in the Results section. Gene ontology (GO) enrichment analysis on differentially expressed genes was performed using PANTHER(29). For upstream regulator analysis, Ingenuity Pathways Analysis (IPA) was used where we input list of DEGs and their fold change from cutoff logFC= |0.6| and Padj=<0.2.

RNA subcellular localization

WT BMDMs were stimulated with LPS for 4 and 24 hours to induce *U90926* expression, followed by lysis and nuclear and cytoplasmic fractionation. Nuclear and cytoplasmic fractions were separated by NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's protocol. After separation, TRIzol LS reagents (Thermo Fisher Scientific, Waltham, MA) were added to lysates according to manufacturer's suggested ratio to liquid sample. Then, we proceeded with the Direct-zol RNA micro prep kit for RNA extraction, followed by cDNA synthesis and RT-qPCR, as described above. Abundance of each transcript target in nuclear and cytoplasmic fractions was normalized to its respective abundance in an unfractionated input sample.

Plasmid constructs

U9-ORF cDNA was amplified by PCR from cDNA from LPS-stimulated BMDM. At the C-terminal end of the U9-ORF, before the stop codon, a hemagglutinin (HA) epitope tag (5'-TACCCATACGACGTGCCTGACTACGCC-3') was added by PCR. Amplified cDNAs were cloned into the pcDNA3.1 vector (ThermoFisher, USA) using EcoRI and NotI restriction sites. The correct sequence of the U9-ORF-HA construct was confirmed by Sanger sequencing. The pcDNA3.1 TYK2-HA construct was previously generated in our laboratory(30). The U9-full cDNA-HA construct consists of full *U90926* cDNA (522 bp) with HA tag sequence inserted just before the stop codon in the ORF, cloned into pBABE-puro vector (Addgene #1764). The U9-full cDNA-HA construct in pBABE-puro background was constructed commercially by insert synthesis and ligation at Agentide (Westfield, NJ).

Immunocytochemistry (ICC)

HeLa, 3T3, 3T3-L1, and RAW264.7 cells were cultured in 35 mm glass bottom dishes (MatTek, Ashland, MA). 80% confluent cells were transfected with different plasmids using Lipofectamine2000 (Invitrogen) according to manufacturer's protocol. 48 hours post transfection, cells were fixed with 1% PFA/PBS for 20 min at RT. Cells were washed 3x with PBS, blocked and permeabilized for 10 minutes with 1% BSA/PBS containing 0.1% Saponin, and incubated for 1 hour with 1:1000 diluted anti-HA.11 epitope tag mouse antibody (Biolegend, San Diego, CA) and 1:500 diluted anti-golgin-97 rabbit (Molecular Probes, Eugene, OR) primary antibodies in 1% BSA/PBS. Cells were washed 2x with PBS, blocked with 1% BSA/PBS for 1 hour, and incubated for an additional hour with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG secondary antibodies (Invitrogen). 2x PBS wash was preformed and DAPI (1:10,000) in PBS was added for 10 min. Cells were stored in PBS and imaged on a DeltaVision epifluorescence microscope (Cytiva/GE/Applied Precision, Issaquah, WA) with a Xenon illuminator, using an Olympus 60× PlanApo 1.42 NA oil immersion objective. Images were deconvolved using softWoRx software (Applied Precision). The same protocols were followed for experiments with protein transport inhibitors in which inhibitors were added 24 hours post transfection, meaning 24 hours before collection. Therein, GolgiPlug containing brefeldin A (BD Biosciences) at 1:2000 dilution or GolgiStop containing monensin (BD Biosciences) at 1:1000 dilution was used. Microscopy images were analyzed, and each panel were adjusted by Fiji software.

Immunoblot analysis

At 24 hours post-transfection, media was changed to serum free media and samples were collected at 48 hours post-transfection. Cell supernatants were collected and whole-cell lysates were prepared by lysing cells in RIPA buffer. For concentrating supernatants, StrataClean Resin (Agilent, Santa Clara, CA) was used according to manufacturer's protocol. Samples were diluted and boiled in Laemmli SDS sample buffer for 5 minutes and loaded on 4–15% Mini-PROTEAN TGX Stain-Free Protein Gels (Bio-Rad laboratories, Hercules, CA). After separation via SDS-PAGE, proteins were transferred to methanol activated polyvinylidene difluoride (PVDF) membrane. Mouse anti-HA.11 epitope tag antibody (BioLegend, San Diego, CA) was used as primary antibody and horseradish peroxidase (HRP)-conjugated rat anti-mouse Ig, κ light chain (BD Biosciences, New Jersey) was used as secondary antibody. Membranes were imaged using chemiluminescence detection upon addition of Clarity western ECL substrate (Bio-Rad Laboratories) on the Syngene PXi imager.

Immunoprecipitation and deglycosylation

Whole cell extracts were incubated with 1.5 ug of HA antibody overnight at 4°C with rotation. Washed protein G Dynabeads (Thermo Fisher Scientific, Waltham, MA) were added to the samples and incubated for 2 hours at 4°C with rotation. Immunoprecipitated protein was washed 5 times in NP40 lysis buffer followed by 3 washes in 1X TBS before resuspending in water. The deglycosylation reaction was performed under denaturing conditions overnight at 37°C using the Protein Deglycosylation Mix II from New England Biolabs and subsequently analyzed by immunoblot, as described above.

Peptide preparation and mass spectrometry

Gel regions indicated in Figure S4a were excised, diced to 1 mm cubes and transferred to separate microcentrifuge tubes. Gel pieces were washed with HPLC-grade H2O and then de-stained in 50 mM ammonium bicarbonate (NH4HCO3) and 50% acetonitrile (MeCN) at 37 °C for 30 minutes. De-stain was then removed, and gel pieces were dehydrated in 100% MeCN for 10 minutes. For reduction and alkylation of samples, dried gel pieces were incubated with 25 mM dithiothreitol solution (DTT) for 30 minutes at 55 °C, followed by 10 minutes at room temperature. Gel pieces were partially dehydrated with 100% MeCN. After a 5–10-minute incubation at room temperature, solvent was removed and 10 mM iodoacetamide in 50 mM NH4HCO3 (IAA) was added. Samples were incubated in the dark at room temperature for 45 minutes. The IAA solution was removed, and gel pieces were incubated in 50 mM NH4HCO3 for 5 minutes. Gel pieces were partially dehydrated in 100% MeCN for 5 minutes, then rehydrated in HPLC-grade H2O. De-staining, partial dehydration, and rehydration were repeated. Gel pieces were then completely dehydrated using 100% MeCN. On ice, dried gel pieces were rehydrated in 12 ng/μL sequencing grade modified trypsin in 50 mM NH4HCO3. After a 20-minute incubation at 4 °C, an additional equal volume of 50 mM NH4HCO3 was added to submerge gel pieces, followed by overnight incubation at 37 °C. After centrifugation, supernatants were transferred to new microcentrifuge tubes. Remaining peptides were extracted from gel pieces with the addition of 50% MeCN, 2.5% formic acid (FA). Supernatants were combined with the initial tryptic digest, and gel pieces were dehydrated in 100% MeCN. The final extraction was combined with the previous two extractions and peptides were dried in a speed-vac.

Peptides were resuspended in 2.5% MeCN/2.5% FA. Peptides were separated on a reverse-phase HPLC column (length=15 cm × 100 μm) packed in house with C18 resin (1.8 μm 120 Å, UChrom C18) prior to analysis via a linear ion trap-orbitrap (Orbitrap Eclipse; resolution=120,000) mass spectrometer fitted with an Easy-nLC 1200 (both instruments from Thermo Scientific; Waltham, MA, USA). Following loading (flow rate=300 nL/min) onto the C18 column in Solvent A (2.5% MeCN/0.1% FA), peptides were eluted using a 0–25% gradient of Solvent B (80% MeCN, 0.1% FA) over 60 min followed by an elution to 0-40% gradient of Solvent B over 10 minutes electrosprayed (1.9 kV) into the mass spectrometer. This gradient was followed by 9 minutes at 95% Solvent B before a 5 minute equilibration in 5% Solvent B.The precursor scan (375–1600 m/z) was followed by ten low energy collision-induced dissociation (CID) tandem mass spectra. Dynamic exclusion settings: repeat count=3, exclusion duration=15 sec, exclusion width= ±5 ppm).

SEQUEST searches were performed using a forward and reverse protein database which included known contaminants (such as human keratins and porcine trypsin) and the sequence for U9-ORF with its HA-tag as shown in Figure S4b. Tryptic peptides were required, 2 missed cleavages permitted, a precursor mass tolerance of ± 5 PPM and a fragment ion tolerance of ± 0.01 Da. The following differential modifications were permitted: oxidation of methionine (+15.9949 Da), carboxyamidomethylation of cysteine (+57.0215 Da), and acrylamidation of cysteine (+71.0371 Da). Peptides were filtered by cross-correlation (XCorr) scores dependent on charge state (charge states of 2 to 4 were considered): (XCorrz=+2 > or = 1.8; XCorrz=+3 > or = 2.0; XCorrz=+4 > or = 2.2) and only proteins with three or more identified peptides were further considered. These parameters resulted in a false discovery rate (FDR) <0.01%.

In vivo LPS challenge experiments

Age- and sex-matched male and female U9-KO or WT mice were injected intraperitoneally (i.p.) with E. coli LPS O26:B6 (Sigma Aldrich, St. Louis, MO) at 15 mg/kg body weight (low dose) or 60 mg/kg (high dose). LPS-challenged mice were monitored for survival and semi-quantitative evaluation of clinical signs induced by the sickness responses for 72 hours. A clinical scoring system was adapted from previous study(31) with minor modifications, as follows, 0 = no abnormal clinical signs, 1 = ruffled fur but lively, 2 = ruffled fur, moving slowly, hunched, and sick, 3 = ruffled fur, squeezed eyes, hardly moving, down, and very sick, 4 = same as 3 but with incontinence, 5 = moribund or dead. Blood and tissues were collected at different time points and analyzed. Blood was incubated for 30 min at room temperature to allow clotting, followed by storage on ice for 1-2 hours, centrifugation, and collection of serum. For the in vivo IL-6 neutralization experiments, immediately after LPS injection, 100 µg/mouse of mAb isotype control (anti-trinitrophenol) or anti-IL-6 antibodies (Bio X Cell, Lebanon, NH) were administered by i.p injection.

Statistical analyses

Statistical analyses not pertaining to RNA-seq data were carried out using GraphPad Prism software version 9.2.0 (GraphPad Software, San Diego, CA, USA). The specific tests used to assess the significance of the observed differences are detailed in the figure legends. All center values represent the mean, and error bars represent the standard error of the mean. A P-value of <0.05 was considered significant.

RESULTS

LncRNA U90926 is upregulated in macrophages and dendritic cells in response to TLR-ligands in a MyD88/p38α MAPK dependent manner

U90926 is a lncRNA located on mouse chromosome 5, between two protein-coding genes, Uso1 and Ppef2. The *U90926* gene is encoded in a ~5.5 Kbp locus, comprising 5 exons encoding a 522 bp polyadenylated transcript(32). Using microarray analysis, we originally discovered *U90926* as a highly downregulated gene in p38α MAP kinase (MAPK)-deficient peritoneal macrophages stimulated in vitro with heat-killed Mycobacterium tuberculosis(16), and more recently confirmed these data by RNAseq in LPS-activated bone marrow derived macrophages (BMDM)(18). To determine the cell/tissue type specificity of this transcript, mining transcriptomic data across >100 tissue and cell types, we identified the highest expression of this gene in LPS-stimulated BMDMs and thioglycolate-elicited peritoneal macrophages, with minimal expression in other cells/tissues at steady state (Fig. 2-1a). Leveraging published kinetic transcriptomic data from bone marrow derived dendritic cells (BMDCs) stimulated with an array of toll-like receptor (TLR) ligands(33), we found that *U90926* RNA expression was maximally induced ~4-6 hours after activation of TLR1/2, TLR4, TLR7, and TLR9, but not TLR3, and sustained for at least 24 hours (Fig. 2-1b). Using RT-qPCR, we confirmed that *U90926* RNA is upregulated with comparable kinetics in BMDMs and BMDCs (Fig. 2-1c) and determined that the expression of this gene in BMDM is induced by activation of TLR1/2, TLR4, TLR5, and TLR9, but not TLR3 (Fig. 2-1d). These results demonstrate that the expression of *U90926* is rapidly inducible in macrophages and dendritic cells by TLR signals. Given the documented heterogeneity of BMDC preparations(26), we focused the majority of our subsequent in vitro experiments on BMDM.

Next, we assessed the signals downstream of TLR activation required for the induction of *U90926* expression. Since myeloid differentiation factor 88 (MyD88) is a required adaptor protein for signaling downstream of all of the TLRs above with the exception of TLR3(34, 35), we investigated the necessity of MyD88 for *U90926* expression, using WT and MyD88-KO immortalized BMDM cell lines(15). *U90926* expression was induced by LPS in WT but not in MyD88-deficient cells, whereas TLR3 activation by polyI:C failed to induce the expression of *U90926* in either WT or MyD88-KO cells, as expected (Fig. 2-1e). Our previous transcriptomic findings implicated p38α MAPK, a major signaling pathway downstream of MyD88, in the expression of *U90926*(16, 18). We confirmed these findings using RTq-PCR, demonstrating that induction of *U90926* was significantly, though not completely, impaired in p38α-deficient BMDMs (Fig. 2-1f). Consistently, pretreatment of WT BMDM with SB203580, a pharmacological inhibitor of p38 MAPK, also highly reduced *U90926* expression after LPS stimulation (Fig. 2-1g). To investigate if p38 MAPK activation is sufficient to induce *U90926* expression independent of TLR-activation, we used NaCl-induced osmotic stress as a known activator of p38 MAPK in BMDMs and other cell types(36–38). NaCl-induced hyperosmotic stress through the addition of an extra 40 mM NaCl did not induce *U90926* expression (Fig. 2-1h), suggesting that p38 MAPK activation alone is not sufficient. We also investigated *U90926* expression in response to cytokine stimuli, including TNFa, which induces p38 MAPK activation(39), and IL-1b, which signals via MyD88 and p38

MAPK(40, 41). We found that TNFa did not stimulate *U90926* expression, but IL-1b stimulation induced significant *U90926* expression in WT BMDM, albeit very modest compared with LPS stimulation (Fig. 2-1i). Taken together, these findings demonstrate that the expression of *U90926* is robustly induced by TLR signals in a MyD88- and p38α-dependent manner.

Our original studies and the experiments above were performed utilizing the most widely used laboratory strain of mice, C57BL/6J (B6), which represents a single unique genotype, artificially selected in a captive setting for over a hundred years. However, we recently demonstrated that certain aspects of the LPS response in B6 myeloid cells may be idiosyncratic to classic inbred strains, and not conserved in the genetically divergent wild-derived inbred strain of mice, PWD/PhJ (PWD)(42). To investigate the conservation of *U90926* regulation across divergent genotypes, we used BMDM from PWD mice. BMDM from PWD and B6 mice demonstrated comparable induction of *U90926* in response to LPS stimulation (Fig. 2-1j), suggesting that regulation of *U90926* expression is conserved across divergent murine genotypes and not unique to the B6 strain. Given these findings, we continued our studies in the genetically tractable B6 model.

Generation of U90926-deficient mice (U9-KO)

To study the function of *U90926* in vivo and ex vivo, we generated *U90926*-deficient mice (U9-KO) by CRISPR-Cas9 on the C57BL/6J background. Two single guide RNAs (sgRNAs), targeted upstream of the 5’ and downstream of 3’ ends of the gene, were used to delete the entire ~5.5 Kb *U90926* locus (Fig. 2-2a) by microinjection together with Cas9 enzyme into single cell B6 embryos. We obtained multiple successful founders, all of which were confirmed by polymerase chain reaction (PCR) genotyping, showing deletion of all 5 *U90926* exons, with some variability around the cut sites, as expected from non-homologous end joining repair (Figure 2-S1a). We selected a single founder to generate the U9-KO mouse line (Pup ID# 11 in Figure 2-S1a). To eliminate any potential off-target deletions made by CRISPR/Cas9 elsewhere in the genome(43, 44), we backcrossed the U9-KO to WT B6 mice, starting with a single male U9-KO founder and followed by 2 more backcrosses. The resulting heterozygous (HET) mice were bred HET X HET to establish homozygous KO and WT control progeny in parallel, which were used in all subsequent experiments. Backcrossed U9-KO mice were validated by Sanger sequencing (see materials and methods) and PCR genotyping (Fig. 2-2b) to confirm deletion of the entire *U90926* genomic sequence.

To validate *U90926* deficiency at the RNA level, we generated BMDM and BMDC from WT B6 control mice and U9-KO mice, and stimulated them with LPS to induce expression of *U90926*. In contrast to WT cells, U9-KO BMDM and BMDC showed no detectable expression of *U90926* upon LPS stimulation (Fig. 2-2c and 2d). We next investigated whether *U90926* deletion alters BMDM and BMDC differentiation. WT and U9-KO BMDM and BMDC were assessed by flow cytometry to measure expression of the major myeloid differentiation markers CD11b and CD11c, which did not differ between WT and U9-KO mice, both at baseline and after 24h LPS stimulation (Fig. 2-2e and 2f). Next, we determined the effect of *U90926* deficiency on major leukocyte populations in spleen and lymph nodes at the basal level, using flow cytometry. No major immunophenotypic differences were found in U9-KO mice compared with WT mice with the exception of a decrease in Ly6G+ cell number in the spleen (Fig. 2-2g and 2h), which was not replicated in a repeat experiment (data not shown). Taken together, these data validate our U9-KO model for functional characterization of *U90926* gene function in vitro and in vivo, without any apparent confounding influences on leukocyte development.

Deletion of U90926 locus has minimal intrinsic effects on myeloid cell function in vitro

Given that *U90926* is highly expressed in macrophages and dendritic cells upon TLR-stimulation, we hypothesized that this gene may be involved in cell-intrinsic regulation of inflammatory gene expression, similar to other myeloid lncRNAs(9, 11, 45). Macrophages and myeloid DCs are professional antigen presenting cells, whose capacity for activation of naïve T cells is enhanced by TLR stimulation. Therefore, WT and U9-KO BMDMs were stimulated with LPS for 24 hours, followed by assessment of expression of cell surface co-stimulatory molecules involved in antigen presentation. Compared with WT, *U90926*-deficient BMDMs exhibited no significant differences in the surface expression of CD80, CD86 and CD40 (Fig. 2-3 a-c). Similar results were obtained in BMDCs (Figure S1b-d). These results suggest that *U90926* is not involved in the regulation of co-stimulatory molecule expression in macrophages and dendritic cells.

Next, we assessed cytokine production and secretion, as another essential myeloid cell effector function upon TLR-stimulation. We measured the secretion and mRNA expression of key pro-inflammatory cytokines, TNFα and IL-6, and the anti-inflammatory cytokine, IL-10. Additionally, we assessed IL-1β secretion in response to NLRP3 inflammasome activation by LPS and nigericin stimulation. No significant differences in cytokine secretion as measured by ELISA were detected in U9-KO BMDM when compared with WT (Fig. 2-3d-g). Similarly, we found no significant differences between WT and U9-KO BMDMs in Tnf, Il6, or Il10 cytokine mRNA expression (Fig. 2-3h and 3j). These data suggest that in spite of being TLR-inducible, *U90926* expression has no intrinsic effect on cytokine production and secretion by macrophages upon TLR-stimulation in vitro.

Next, we investigated the global effect of *U90926* deficiency on the myeloid cell transcriptome in an unbiased manner by RNA sequencing (RNAseq). Since *U90926* is expressed early after TLR-activation, we assessed gene expression at 4 hours, and 24 hours after LPS stimulation, to measure early and late effects in BMDMs generated from WT or U9-KO mice. Differential expression analyses using a standard cutoff for RNAseq, |Log2 Fold Change|>1 and Padj <0.05 revealed only two genes at each time point that passed filter, including *U90926* itself (both time points), Csnk2a1-ps3 (at 4 hours), and Gm12918 (at 24 hours) (Fig. 2-3k and 3l). Reanalysis using a relaxed cutoff of |Log2 Fold Change|>0.6, Padj <0.2 found only a total of 9 genes (at 4 hours) and 16 genes (at 24 hours) that were differentially expressed (Figure 2-S1e). Most of these differentially expressed genes were either functionally uncharacterized pseudogenes or predicted genes, and/or not TLR-inducible inflammatory genes, suggesting that *U90926* deficiency does not have a major impact on transcriptional regulation of macrophage inflammatory responses. Together with the results above, our findings suggest that *U90926* has minimal cell-intrinsic effects on macrophage function.

U90926 RNA localizes to the cytoplasm, associates with ribosomes, and contains an ORF that encodes a secreted peptide

Given the lack of cell-intrinsic effect of *U90926* on macrophage gene expression, we assessed the subcellular localization of *U90926* transcript using subcellular fractionation and RT-qPCR. We found that *U90926* RNA localized predominantly to the cytoplasm rather than the nucleus in BMDMs, similar to protein-coding mRNAs such as B2m, and unlike the small nucleolar RNA U6, which localized to the nucleus (Fig. 2-4a). Our in-silico analysis indicated that the *U90926* transcript contains a 264 bp open reading frame (ORF) that has the potential to generate a ~10 kDa protein of 87 amino acids (Fig. 2-4b), henceforth referred to as U9-ORF. BLAST analysis of U9-ORF amino acid sequence revealed no significant homology to any known proteins (data not shown). Surprisingly, we identified a predicted endoplasmic reticulum (ER) signal peptide sequence of 16 amino acids at the N-terminus (Fig. 2-4b), which could make U9-ORF a secreted or organelle lumen-resident protein. Seeking additional evidence for translation, we mined publicly available ribosome-profiling data from a study of LPS-activated BMDCs(46). We observed global ribosome footprints that mapped to all five of the exons of *U90926*, as well as initiating ribosome footprints that mapped to exon 2 (Fig. 2-4c), where the start codon for U9-ORF lies, suggesting that the *U90926* transcript associates with ribosomes and the U9-ORF may be translated.

To directly assess the protein coding potential of *U90926*, we cloned the 264 bp U9-ORF cDNA into a mammalian expression vector and added a hemagglutinin (HA) epitope tag at the C-terminus. The resulting U9-ORF-HA plasmid was transfected into HeLa cells, and the presence and localization of HA-tagged U9-ORF product was assessed by immunocytochemistry (ICC). We found that the U9-ORF-HA protein was translated and localized predominantly to the perinuclear Golgi apparatus in HeLa cells (Fig. 2-4d). Similar results were obtained by transfection of the U9-ORF-HA plasmid into RAW264.7 murine macrophages, 3T3 mouse embryonic fibroblasts, and 3T3-L1 mouse pre-adipocytes (Figure 2-S2a). To confirm that U9-ORF protein could be translated in the context of its native full-length mRNA, we also cloned the full length *U90926* cDNA (522bp) into an expression vector, inserted an HA tag at the C-terminus of the ORF, and transfected it into HeLa cells, followed by ICC. As before, we found that U9-ORF was translated and localized to Golgi apparatus (Fig. 2-4e), suggesting that this protein may be destined for exocytosis and secretion.

To determine whether U9-ORF can be secreted, we transfected HeLa cells with U9-ORF-HA or, as a negative control, an HA-tagged cytoplasmic kinase, TYK2(30), and analyzed cell lysates and supernatants by immunoblotting. While both proteins were present in the cell lysates, we found abundant U9-ORF-HA, but not TYK2-HA, protein in the supernatant, with or without concentration (Fig. 2-4f). We confirmed the correct identity of the U9-ORF protein in the U9-ORF-HA transfected cell supernatants by mass spectrometry (Figure 2-S3). To confirm that U9-ORF protein is secreted via the canonical exocytic pathway, we treated cells with vesicular transport inhibitors, monensin and brefeldin A, after transfection and analyzed by the cells by ICC, and cell lysates and supernatants by immunoblotting. Monensin and brefeldin A treatment resulted in a notable sub-cellular relocalization of U9-ORF protein (Fig. 2-4g and Figure 2-S2b). Similarly, by immunoblotting, we found that monensin and brefeldin A treatment almost completely inhibited U9-ORF secretion into the supernatant (Fig. 2-4h). Interestingly, immunoblot analysis indicated that both the predominant cell-associated and secreted forms of the U9-ORF-HA protein migrated at a molecular weight of 25 kDa (Fig. 2-4f and 4h), larger than its predicted mass of 12 kDa, which was visible as a minor band only in cell lysates (Fig. 2-4f). This suggested the presence of possible post-translational modifications (PTMs), such as glycosylation, which is a common on PTM on secreted proteins(47). In order to determine if the U9-ORF protein is glycosylated, we performed deglycosylation reactions on the U9-ORF-HA transfected cell lysates and supernatants, followed by immunoblotting. We found that deglycosylation caused a clear shift in the U9-ORF protein band to the predicted ~12kDa position (Fig. 2-4i), indicating that U9-ORF protein is indeed glycosylated. Taken together, our results suggest that *U90926* is a cytoplasmic RNA that encodes a novel U9-ORF protein, which is glycosylated, and secreted out from the cell. Together with the RNAseq data demonstrating minimal intrinsic effects of *U90926* deficiency on macrophage gene expression, these results suggest a model whereby in vivo U9-ORF secreted by macrophages may act on other cell types in a paracrine and/or endocrine manner.

U90926 expression protects mice from LPS-induced endotoxic shock by modulating IL-6 levels

Our findings so far had suggested that TLR-inducible expression of *U90926* in macrophages may exert non-macrophage intrinsic effects. To test this hypothesis in vivo, we employed the widely used LPS endotoxemia model, in which TLR4 signaling in macrophages promotes morbidity and mortality due to hypercytokinemia and systemic shock, by interacting with multiple other cell types(48),(49). We first determined whether

U90926 expression is inducible in vivo by LPS endotoxemia. WT B6 mice were challenged with a high dose (15 mg/kg) of E. coli O26:B6 LPS by intraperitoneal (i.p.) injection, followed by tissue collection at several time points. We found that *U90926* expression was induced within hours after systemic LPS administration, in peritoneal cells collected by lavage, liver tissue, and visceral adipose tissue, and its expression was sustained for up to 48 hours (Fig. 2-5a), similar to its kinetics in vitro in BMDM (Fig. 2-1d). To determine the contribution of *U90926* in severe endotoxemia, U9-KO and WT mice were challenged i.p. with high dose LPS (15 mg/kg), followed by monitoring of survival and quantitative evaluation of clinical signs induced by the sickness responses, as previously described(31, 50), at individual time points, and as a cumulative variable using area under the curve (AUC) analysis. In WT mice, this dose of LPS resulted in acute clinical signs that peaked at 12 hours, followed by recovery and survival in 86% of the mice (Fig. 2-5b-d). In contrast, U9-KO mice exhibited significantly sustained sickness responses and and reduced survival of 33% (Fig. 2-5b-d). Together, these results suggest that expression of *U90926* downstream of TLR4 activation in endotoxemia is protective in septic shock.

To determine the inflammatory mechanisms underlying exacerbated endotoxemia in U9-KO mice, we assessed the serum levels of the key pro-inflammatory cytokines TNFα and IL-6 by ELISA, after LPS challenge. TNFα levels peaked at 1 h post-challenge and did not differ significantly between WT and U9-KO mice at both 1 h and 4 h (Fig. 2-5e-f). IL-6 levels peaked at 4 hours post-challenge at comparable levels between WT and U9-KO mice (Fig. 2-5g). However, at 24 hours, IL-6 levels remained elevated at a significantly higher level in U9-KO mice compared with WT (Fig. 2-5h).

To determine whether elevated IL-6 in U9-KO mice was directly responsible for their increased susceptibility to endotoxic shock, we blocked IL-6 using a neutralizing antibody in the LPS endotoxemia model. U9-KO and WT mice were challenged with LPS (15 mg/kg) and simultaneously treated with isotype control or anti-IL-6 antibody, followed by monitoring of clinical signs and survival as described above. In WT mice, neutralization of IL-6 had no significant effect on clinical symptoms (Fig. 2-5i-j), and 100% of the mice in both treatment groups survived the challenge. In contrast, IL-6 neutralization in U9-KO mice diminished the sustained clinical signs (Fig. 2-5k-l), with 100% percent of the anti-IL-6 treated U9-KO mice surviving, compared with 50% of the isotype control treated U9-KO mice (P= 0.172 by Mantel-Cox analysis). Given the published evidence that IL-6 blockade has a protective effect in WT mice during sepsis(51, 52), we tested whether IL-6 neutralization will also protect WT mice from higher LPS dose challenge, since KO but not WT mice displayed high morbidity and mortality when challenged with the lower dose of LPS in our previous experiments (Fig. 2-5b-l). We found that neutralization of IL-6 was indeed protective in WT mice when challenged with a higher dose of LPS (Figure 2-S4a-c).

Taken together, the data above suggested that *U90926* could play a protective role in septic shock at least in part by negatively regulating systemic IL-6 levels during LPS endotoxemia, possibly via secreted U9-ORF from macrophages acting in a paracrine and/or endocrine manner. This would be consistent with our findings of unchanged IL-6 production by U9-KO BMDM in vitro (Fig. 2-3e,i) and the critical role of hepatocytes in IL-6 production in response to systemic LPS(53). To determine whether BMDM-derived

U9-ORF acts directly on hepatocytes to regulate IL-6 levels, we performed in vitro co-culture experiments between WT or U9-KO BMDMs and a mouse hepatocyte cell line, AML12 cells. We first tested whether hepatocytes express *U90926* RNA in response to LPS, and found that unlike BMDM, hepatocytes do not express *U90926* in response to LPS stimulation (Figure 2-S4d). While hepatocytes cultured alone also produced minimal IL-6 in response to LPS, their presence in co-culture with WT or KO BMDM resulted a significant boost in IL-6 production compared with BMDM alone. However, there was no significant difference in IL-6 production between WT and KO BMDM co-cultures, indicating that macrophage-derived U9-ORF does not directly regulate IL-6 production by hepatocytes, at least in vitro (Figure 2-S4e). Together with the findings that IL-6 neutralization is similarly protective in WT mice challenged with a higher dose of LPS (Figure 2-S4a-c) as in KO mice challenged with a lower dose of LPS (Fig. 2-5i-j), these data suggest that *U90926* deficiency may shift the threshold for endotoxic shock susceptibility, and subsequently causing elevated IL-6 levels downstream, rather than specifically and/or directly regulating IL-6 expression.

Given the critical involvement of both liver resident macrophages and hepatocytes in sepsis(54–58), to further examine the basis for the protective effect of *U90926* in LPS endotoxemia, we performed RNAseq analysis on whole liver tissue isolated from WT and U9-KO mice after 24 h of systemic LPS challenge. Our analysis revealed 71 genes that were differentially expressed in the liver of U9-KO vs WT mice, including key immune response genes such as Cxcl2, Ngp, Arrb2, S100a9 and Ptgds (Fig. 2-5m). Gene Ontology (GO) enrichment analysis of differentially expressed genes identified that distinct pathways were upregulated and downregulated in U9-KO mice compared to WT. The pathways associated with genes upregulated in U9-KO fell into three major categories: leukocyte chemotaxis, metabolic processes, and cell death (Fig. 2-5n), suggesting that *U90926* deficiency may exacerbate endotoxemia through augmented leukocyte recruitment, hepatocyte cell death, and/or dysregulated metabolism in the liver. In contrast, enrichment analysis of genes downregulated in U9-KO revealed much fewer significantly enriched pathways, but notably including defense response to Gram-positive and Gram-negative bacteria. To determine whether elevated IL-6 levels may have contributed to dysregulated gene expression in the liver of U9-KO mice, we performed upstream regulator analysis on genes differentially expressed in U9-KO liver tissue compared with WT (see Methods). We found significant enrichment of IL-6 as an upstream regulator (P=0.04) with a positive Z-score (0.161), with Cxcl3, Mmp7, S100a9, and Vldlr identified as target genes. Taken together, these data suggest that *U90926* deficiency exacerbates endotoxic shock in association with elevated systemic IL-6 levels, resulting in (or caused by) dysregulated liver function, and ultimately, death.

DISCUSSION

With recent advancement of high-throughput sequencing technologies and computational biology, the detection of novel lncRNA transcripts has become easier. However, functions of the most lncRNA remain largely unknown because in-depth experimental investigation of individual genes is needed to unravel their functions. Here, in this study we characterized a mouse putative lncRNA *U90926*. This gene lies between two conserved protein coding genes, Uso1 and Ppef2, on mouse chromosome 5. We became interested in *U90926* when we identified it as one of the most highly downregulated genes in TLR-stimulated p38α MAPK-deficient macrophages in our transcriptomic analyses(16, 18). Given this discovery, we focused on identifying possible roles for *U90926* in macrophages. We generated the first (to our knowledge) mouse model of *U90926* deficiency and discovered that the induction of this gene by inflammatory signals plays a protective role in endotoxic shock. We also found evidence that *U90926* encodes a novel secreted protein that could play paracrine roles in endotoxemia.

Prior to 2017, there had been no studies on the biological role of *U90926*. Since then, three independent groups have described possible functions for this gene. Using an in vitro model of adipocyte differentiation in 3T3-L1 pre-adipocyte cells, Chen et al discovered that 1) *U90926* expression was downregulated during adipocyte differentiation, and 2) overexpression of *U90926* inhibited adipocyte differentiation, while silencing had the opposite effect, thus implicating *U90926* as a possible repressor of adipogenesis(20). Another in vitro study found that *U90926* expression was highly upregulated in a murine retinal photoreceptor cell line 661W following herpes simplex virus 1 (HSV-1) infection, and silencing of this gene impaired HSV-1 replication, thus suggesting that this gene could serve as co-factor for DNA virus replication(21). Interestingly, the same group identified two human lncRNA transcripts syntenic with, and possibly homologous to, *U90926*, with the expression of one of these transcripts being elevated in the eye of individuals with acute retinal necrosis caused by HSV-1(59). Finally, a recent study demonstrated that *U90926* expression was elevated in microglia during ischemic stroke in vitro and in vivo, and that silencing microglial *U90926* reduced infarct volume and neutrophil recruitment in an experimental stroke model(22). These results are broadly consistent with our own findings, as discussed in more detail below, and suggest that expression of the *U90926* transcript is induced by a variety of inflammatory signals, and it plays diverse and context-dependent roles in regulating downstream events. Importantly, given our discovery that this gene may encode a protein, it would be of interest to determine the role of the U9-ORF in these other models.

We demonstrated that expression of *U90926*, while minimal across multiple tissues at steady state, is highly inducible in bone marrow-derived macrophages and DCs upon stimulation with agonists of TLR1/2, TLR4, TLR5, TLR7, and TLR9. The latter is consistent with the findings of Shirahama et al that HSV-1 infection, which also triggers TLR9(60, 61), induced expression of this gene(21), although we note that in our study, we used a mimic of bacterial CpG DNA as an agonist. This also suggests that expression of *U90926* may not be restricted to myeloid cells and may be present in other cell types that can respond to inflammatory signals such as TLRs, although we found no *U90926* expression in LPS-stimulated mouse hepatocyte cell line, AML12 cells (Figure 2-S4d). The findings of Xu and colleagues that ischemic stroke induced expression of *U90926* in microglia(22) suggest that damage associated molecular patterns released in damaged CNS tissue, which can induce signaling through several TLRs(62), could be driving this activation. Alternatively, inflammatory cytokines, such as TNFα or IL-1β, which are abundantly produced during stroke(63), could potentially serve to induce expression of *U90926*, in particularly since the receptors for these cytokines induce signaling through MyD88 and/or p38α MAPK, two signals that we showed are required for TLR4-dependent induction of *U90926*. Consistent with this, there is microarray evidence that TNFα can induce *U90926* expression in 3T3-L1 preadipocytes(64), although we did not detect any *U90926* expression upon TNFa stimulation in BMDMs (Fig. 2-1i). However, we did find moderate *U90926* expression in BMDMs upon IL-1b stimulation, which is consistent with the MyD88-dependence of IL-1R family(40). The findings of Chen et al that *U90926* may be expressed by pre-adipocytes in the absence of inflammatory cues(20) suggest that there may be additional signals that control its expression. We note that we did find detectable, albeit low, expression of *U90926* in whole adipose tissue, which was markedly increased upon systemic LPS challenge (Fig. 2-5a). Given the key role of tissue macrophages in adipose tissue homeostasis and inflammation(65–67), future studies could explore potential *U90926*-dependent cross-talk between adipocytes and adipose tissue macrophages.

Several previous studies have shown that expression of distinct lncRNAs either activated or repressed upon TLR-stimulation in macrophages plays an important regulatory role in controlling inflammation(9, 11, 45, 68). Surprisingly, we found that *U90926* deficiency did not have any major intrinsic effect on macrophage inflammatory gene regulation in vitro (Fig. 2-3). This suggests that *U90926* plays a role other than transcriptional regulation, unlike other macrophage lncRNA that are found in nucleus such as lincRNA-Cox2, lincRNA-EPS, Malat1 and MeXis(9, 11, 69, 70). Recent studies have shown that subcellular localization is critical in determining lncRNA function in the cell(71–73). Consistent with this, we found in our study that *U90926* is localized predominantly in the cytoplasm rather than the nucleus of BMDM (Fig. 2-4a), which validates the lack of major intrinsic effect of *U90926* deficiency on macrophage transcriptional regulation. Our findings on *U90926* localization in BMDM is consistent with other groups, who showed that it is localized to the cytoplasm of 3T3-L1 preadipocytes and primary microglia(20, 22). In contrast, another study showed the *U90926* RNA is localized primarily in nucleus in the HSV-1-infected 661W murine retinal photoreceptor cell line(21). It is possible that *U90926* localization differs across cell types or cell states, since it has been known that lncRNA localization can differ in different cell types depending on splicing and functional predisposition(71, 74). Another potential explanation for the apparent nuclear localization in 661W cells is that it was measured early before HSV-1 infection, at a time point when the expression of this gene is only at ~10% of its maximal expression(21), and thus it may represent an intermediate accumulation of unspliced *U90926* RNA prior to export out of the nucleus. Given our findings that the *U90926* transcript may be translated, cell type-dependent localization of this RNA could represent differential non-coding RNA vs. protein effects, and thus deserves further investigation.

Several recent studies showed that many genes currently annotated as lncRNAs, are pervasively translated to small proteins(75, 76). In general, ORFs size restrictions and the absolute requirement for AUG as the sole initiator of translation have constrained the identification of potentially important transcripts with non-canonical protein-coding potential(76, 77). The concept of functional translation of lncRNAs in macrophages is strongly supported by two recent studies. A lncRNA called AW112010 was recently identified to encode a peptide, which is important in controlling mucosal immunity during both bacterial infection and colitis(14). Another recent study showed that a classically annotated lncRNA 1810058I24Rik encodes a mitochondrial micropeptide named Mm47, which regulated Nlrp3 inflammasome function in macrophages(15). Given the emerging evidence of lncRNA being translated and cytoplasmic location of *U90926*, we were prompted to identify the protein-coding potential of *U90926*. Despite having an ORF with a canonical AUG start site that can potentially encode a predicted 87- amino acid long protein, no prior study has addressed the question if the ORF in *U90926* (U9-ORF) is functional. In this study, we found that not only did *U90926* RNA localize to the cytoplasm, but it associated with ribosomes. Using cDNA transfection, we further showed that epitope-tagged U9-ORF is translated into a stable glycosylated protein that traffics to the Golgi and is secreted from the cell (Fig. 2-4d-h), as predicted from the presence of a consensus ER signal peptide sequence at its N-terminus (Fig. 2-4b). Future studies will need to validate the expression and localization of endogenous U9-ORF in vivo.

The finding that U9-ORF is secreted potentially explains the lack of effect on macrophage-intrinsic function in vitro (Fig. 2-3), implicating paracrine cellular targets for this protein in vivo. In the LPS endotoxemia model, we found rapid induction of *U90926* expression in immune cells, and in liver and adipose tissue. Moreover, we found that U9-KO mice exhibit significantly heightened susceptibility and mortality compared to WT controls. We also demonstrated that U9-KO mice have sustained levels of IL-6 at 24 hours and neutralization of lL-6 can rescue the phenotype of U9-KO mice in the LPS endotoxemia model (Fig. 2-5), albeit WT mice were similarly protected if challenged with a higher dose of LPS (Figure 2-S4a-c). These data suggest that deletion of *U90926* lowers the threshold for endotoxic shock, which is results in elevated systemic IL-6 levels, which drive morbidity and mortality. These effects could be possibly mediated via secreted U9-ORF from macrophages acting in a paracrine and/or endocrine manner although our in vitro experiments do not support a direct regulation of hepatocyte IL-6 production by macrophage derived U9-ORF (Figure 2-S4e). An alternative possibility is that U9-ORF regulates IL-6 stability in vivo, which is an important way in which this cytokine is regulated(78, 79).

To further shed light on the basis of the protective role of *U90926*, we performed transcriptomic analysis on whole liver tissue, considering critical involvement of both the liver resident macrophages and hepatocytes during endotoxic shock(54, 55, 57, 58). We found that many immune genes and biological pathways are differentially regulated in U9-KO mice compared to WT, including pathways that are important in sepsis like cell chemotaxis, metabolic processes, and cell death(80–82), which were upregulated in U9-KO mice. This included an upregulation of neutrophil-specific genes Ngp and S100a9, and the chemokine Cxcl2, a neutrophil chemoattractant, suggesting enhanced neutrophil influx into the liver as a contributor to (or a result of) enhanced liver pathology in LPS-treated U9-KO mice, which is further consistent with the role of IL-6 in promoting neutrophilia in various inflammatory settings(83–85). Interestingly, silencing of *U90926* in microglia resulted in the downregulation of Cxcl2 expression and decreased neutrophil recruitment to the CNS(22), which is the inverse of our results, but nonetheless implicates myeloid cell mediated neutrophil recruitment as a possible common mechanism. Of note, the putative mechanism of action of *U90926* described in microglia involved its RNA form(22), and not the novel U9-ORF protein discovered in our study. Future studies will need to determine whether *U90926* function in regulating inflammatory processes in vivo require its RNA form, its protein form, or both, as has been reported for other lncRNAs(86). Notably, we found a significant enrichment for IL-6-regulated genes in the genes differentially expressed in liver tissue of U9-KO mice, suggesting that elevated systemic IL-6 may be upstream of the dysregulated gene expression in the liver.

Another important aspect of lncRNA biology is conservation across different species. Most lncRNA genes show low sequence conservation across species(7, 87), including many, if not most of the immune cell-specific lncRNAs identified to date that lack documented cross-species homologs(7). An emerging view of lncRNA conservation includes not only sequence, but structure, function and synteny(88). While we have so far been unable to find a human *U90926* homolog using sequence homology, using synteny, Shirahama et al identified a putative human homolog of *U90926*, AC110615.1, as a potential biomarker of acute retinal necrosis(59). We will characterize this potential human homolog of *U90926* in human macrophages in our future studies. Importantly, the lack of apparent conservation of the U9-ORF amino acid sequence in other species suggests that *U90926* could represent an example of a dual-function lncRNA that evolved to lack its protein coding potential in other species.

In conclusion, we have identified *U90926* as a key gene that is highly upregulated in activated macrophages and provided evidence for a protective role for this gene during endotoxic shock. Furthermore, we have discovered unexpected evidence for the translation of this putative lncRNA gene. These findings suggest manipulation of this gene and its products as potential experimental therapeutic approaches in sepsis, which remains a major unmet clinical need. More importantly, our findings yield provide important implications for the evolution of lncRNAs, their protein coding potential, and the physiological roles of this unique class of genes, which outnumbers canonically protein-coding genes.

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FOOTNOTES

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FIGURE LEGENDS

Figure 2-1. Expression of *U90926* is induced in mouse macrophage and dendritic cells by TLR signals in a MyD88- and p38α MAPK-dependent manner. (a) Microarray gene expression data for *U90926* were downloaded from BioGPS.org using the geneatlas_MOE430 dataset. A subset of representative tissues is shown in a heatmap. **(b)** *U90926* expression in BMDC stimulated with the indicated TLR ligands was extracted from a published Nanostring gene expression dataset(33). **(c)** BMDMs and BMDCs were isolated from B6 mice and stimulated with LPS (100 ng/ml) for 4h and 24h, followed by RT-qPCR for *U90926* expression measurement. (**d**) B6 WT BMDMs were stimulated with the TLR-ligands LPS (100 ng/ml), Pam3CSK4 (1 μg/ml), ODN1826 (1 μM), polyI:C (10 μg/ml), or ST-FLA (1 μg/ml) for the indicated times, followed by RT-qPCR to measure *U90926* expression. **(e)** Immortalized BMDMs from WT and MyD88-KO B6 mice were stimulated with LPS (100 ng/ml) or polyI:C (10 μg/ml) for 4 hours, and *U90926* expression was measured by RT-qPCR. **(f)** BMDMs were isolated from p38αCKO^{Lysm} (p38-CKO) and p38$\alpha^{flox/flox}$ Cre-negative littermate control (WT) B6 mice and stimulated with LPS *in vitro* for 4hrs, followed by RT-qPCR to measure *U90926* expression. **(g)** BMDMs were isolated from WT B6 mice and stimulated with LPS with or without pretreatment with the p38 MAPK inhibitor SB203580 (p38i, 10μM) for 4 hrs, followed by RT-qPCR. **(h)** BMDMs isolated from WT B6 mice were treated with LPS (100 ng/ml) or NaCl (40 mM) for 4 hours, and *U90926* expression was measured by RT-qPCR. **(i)** BMDMs isolated from WT B6 mice were treated with TNFa (10 ng/ml), IL-1b (10 ng/ml), and LPS (100 ng/ml) as positive control for 4 hours, followed by RT-qPCR. **(j)** BMDMs were isolated from B6 and PWD mice and stimulated with LPS (100 ng/ml) for 4 and 24 hours, followed by RT-qPCR. All RT-qPCR data are expressed relative to the housekeeping gene Beta-2-microglobulin (*B2m*) and calculated by a comparative Ct method formula 2^-(deltaCt), and multiplied by a factor of 10,000 for ease of visualization. Data are represented as the mean ± SEM. Significance was determined by two-way ANOVA for panel (**e**) and two-tailed Student's t test for panel (**f**). One-way ANOVA was performed for more than two groups comparison in panel (**g**) and (**h**). P values are represented as follows: ns>0.05, *=≤0.05, **=≤0.01, ***=≤0.001, ****=≤0.0001.

Figure 2-2. Generation and validation of U9-KO mice. (a) Schematic of CRISPR targeting of the *U90926* locus and the repaired double-strand break site (designated as "X") flanked by the indicated sequences, as identified by Sanger sequencing. (**b**) PCR-based genotyping of U9-KO mice. Three primers were used in a single reaction: Forward primer was upstream of the sgRNA site 1, KO reverse primer downstream of sgRNA site 2 (yields a product only if *U90926* whole locus is deleted), and a WT reverse primer downstream of sgRNA site 1 and near the exon 1 (only yields a product when *U90926* is not deleted). **(c, d)** BMDM and BMDC were isolated from WT and U9-KO mice (n=6 for each group) and stimulated with LPS (100 ng/ml) for 4h, followed by RT-qPCR to measure *U90926* expression. **(e, f)** BMDM and BMDC isolated from WT and U9-KO mice (n=8 for each group) were stimulated with LPS (100ng/ml) for 24h and analyzed by flow cytometry using CD11b and CD11c differentiation markers. **(g, h)** Cells were isolated from spleen and lymph nodes of naïve WT and U9-KO mice (n=8 for each group). Flow cytometry and cell counting was used to enumerate the following cell subsets: $CD19^+$ (B cells; $CD19^+$ $CD11b^-$ $TCR\beta^-$), $TCR\beta^+$(T cells; $CD19^-$ $CD11b^-$ $TCR\beta^+$), $CD4^+$ (CD4 T cells; $CD19^-$ $CD11b^-$ $TCR\beta^+$ $CD4^+$) and $CD8^+$ (CD8 T cells; $CD19^-$ $CD11b^-$ $TCR\beta^+$ $CD8^+$) and different myeloid cells markers, $CD11b^+$ ($CD19^-$ $CD11b^+$ $TCR\beta^-$ $CD11c^-$), $CD11c^+$ ($CD19^-$ $CD11b^-$ $TCR\beta^-$ $CD11c^+$) and $Ly6G^+$ ($CD19^-$ $CD11b^+$ $TCR\beta^-$ $CD11c^-$ $Ly6G^+$). Data are represented as the mean ± SEM. Significance was determined by Student's t test in panel (**c**) and (**d**). Two-way ANOVA was performed in panel (**e**) and (**f**) and Student's t test was performed for panel (**g**) and (**h**). P values are represented as follows: ns= >0.05, *= ≤0.05, **= ≤0.01, ***= ≤0.001.

Figure 2-3. *U90926*-deficiency has minimal intrinsic effects on macrophage function *in vitro*. (a-c) WT and U9-KO BMDMs were stimulated with LPS (100 ng/ml) for 24h. Cell surface expression of the co-stimulatory markers CD80, CD86, and CD40 was measured by flow cytometry, and expressed as mean fluorescence intensity (MFI). **(d-g)** WT and U9-KO BMDMs were stimulated with LPS (100 ng/ml) for the indicated times, and cytokine production in the supernatants was analyzed by ELISA. In **(f)**, BMDMs were stimulated with LPS (100 ng/ml) for 4h and followed by addition of nigericin (5 μM) for 1h. In **(g)**, IL-10 secretion was measured at 24h post-LPS only, since it was not detectable at 4h by ELISA. **(h-j)** RNA was isolated from WT and U9-KO BMDMs stimulated with LPS (100 ng/ml) at 0h, 2h, 4h and 24h, followed by RT-qPCR to measure mRNA expression of the indicated cytokine genes. RT-qPCR data are expressed relative to the housekeeping gene, Beta-2-microglobulin (*B2m*), and multiplied by a factor of 10,000 for ease of visualization. Data are represented as the mean ± SEM. Significance was determined by two-tailed Student's t test. **(k, l)** WT and U9-KO BMDMs (n=4 for each group) were stimulated with LPS (100 ng/ml) for 4h and 24h, and RNAseq was performed as indicated in Methods. DEseq2 analysis was used to identify differentially expressed genes. Volcano plots show differentially expressed genes passing a cutoff of |Log2 Fold Change|=2, Padj <0.05 (dotted lines on the plots), with up (red) = upregulated in U9-KO, down (blue) = downregulated in U9-KO.

Figure 2-4. *U90926* RNA localizes to the cytoplasm, associates with ribosomes, and contains an ORF that encodes a secreted protein. (a) WT BMDM were stimulated for 4h and 24h with LPS (100ng/ml), followed by isolation of nuclear and cytoplasmic fractions and RTq-PCR analysis of *U90926*, *B2m* (cytoplasmic control), and *U6* SnRNA (nuclear control) expression (each normalized to unfractionated input as equal to 1). **(b)** A schematic diagram of *U90926* cDNA sequence (522 bp) containing the 264 bp ORF spanning the exons is shown. Predicted amino acid sequences of the U9-ORF peptide (87 aa) and the signal peptide identified by SignalP software shown in red. **(c)** GWiPS was used to analyze of Ribo-seq data from LPS stimulated BMDC(46) at the *U90926* locus.

Global ribosome footprints or initiating ribosomes footprint data (in the presence of harringtonine) are shown, as indicated. **(d, e)** HeLa cells were transfected with U9-ORF-HA (**d**) and U9-full-lengthcDNA-HA (**e**) plasmids, followed by immunostaining for HA (yellow) and Golgin-97 (cyan; Golgi marker), and DAPI nuclear staining (magenta). **(f)** HeLa cells were transfected with U9-ORF-HA and TYK2-HA plasmids for 48 hours, followed by immunoblot analysis. Supernatants were loaded directly or concentrated using StrataClean resin, as indicated (Unconc. or Conc., respectively). **(g)** HeLa cells were transfected with U9-ORF-HA for total 48 hours, where protein transport inhibitor, monensin was added at 24 hours, and then cells were subjected to immunostaining at 48 hours of transfection, as above. **(h)** HeLa cells were transfected with U9-ORF-HA for a total of 48 hours, in which protein transport inhibitors, brefeldin A and monensin were added at 24 hours, and sample collection was done at 48 hours of transfection, followed by immunoblot analysis. Supernatants were analyzed as described above**. (i)** HeLa cells were transfected with empty vector control and U9-ORF-HA plasmid for 48 hours. Cell lysates were immunoprecipitated using an anti-HA antibody and supernatants were concentrated using StrataClean resin, followed by deglycosylation under denaturing conditions overnight at 37°C and analyzed by immunoblot.

Figure 2-5. *U90926* deficiency exacerbates LPS endotoxemia by elevation of IL-6 levels. (a) WT B6 mice were challenged with 15mg/kg LPS i.p. and tissues were collected at the indicated time points, followed by RNA extraction and measurement of *U90926* expression by RT-qPCR. *B2m* was used a normalizer for relative expression, as in **Fig. 1**. **(b-d)** WT (n=15) and U9-KO (n=12) mice were challenged with 15 mg/kg LPS i.p., followed by evaluation of sickness behavior (**b, c**) and survival (**d**). (**c**) Area under the curve (AUC) was calculated from kinetic data in (**b**), to calculate a cumulative measurement of the disease severity. Clinical score evaluation measurement is described in the Methods section. (**e-h**) Serum was collected from the LPS-challenged mice at indicated time points, and TNFα and IL-6 levels were analyzed by ELISA. **(i-l)** WT (n=7) and U9-KO (n=5) mice were challenged with LPS (15mg/kg) and simultaneously administerted isotype control (anti-trinitrophenol) or anti-IL-6 antibodies (100 μg/mouse) by i.p. injection, followed by evaluation of sickness behavior in (**i**) and (**k**). Area under the curve (AUC) calculated from data in (**i**) and (**k**) is shown in (**j**) and (**l**). Significance of differences was assessed using 2-way ANOVA (panels **b**, **i**, and **k**), Mantel-Cox test (panel **d**), and Welch's T test (panel **c, e-h, j, l**). P values are represented as follows: ns= >0.05, *= ≤0.05, **=≤0.01, ***=≤0.001. **(m)** WT (n=4) and U9-KO (n=4) mice were challenged with 15mg/kg LPS i.p. for 24 h and RNAseq was performed on whole liver tissue RNA as indicated in Methods. DEseq2 analysis was used to identify differentially expressed genes. Volcano plots showing differentially expressed genes at a cutoff of |Log2 Fold Change|>0.6, Padj <0.2 (dotted lines on the plots) with up (red) = upregulated in U9-KO, down (blue) = downregulated in U9-KO. Selected genes of interest are labelled. **(n)** Differentially expressed genes upregulated in U9-KO were analyzed by GO enrichment analysis by PANTHER. A subset of select pathways are shown, falling into three major categories, indicated in black (cell migration/chemotaxis), grey (metabolic processes) and red (cell death/apoptosis).

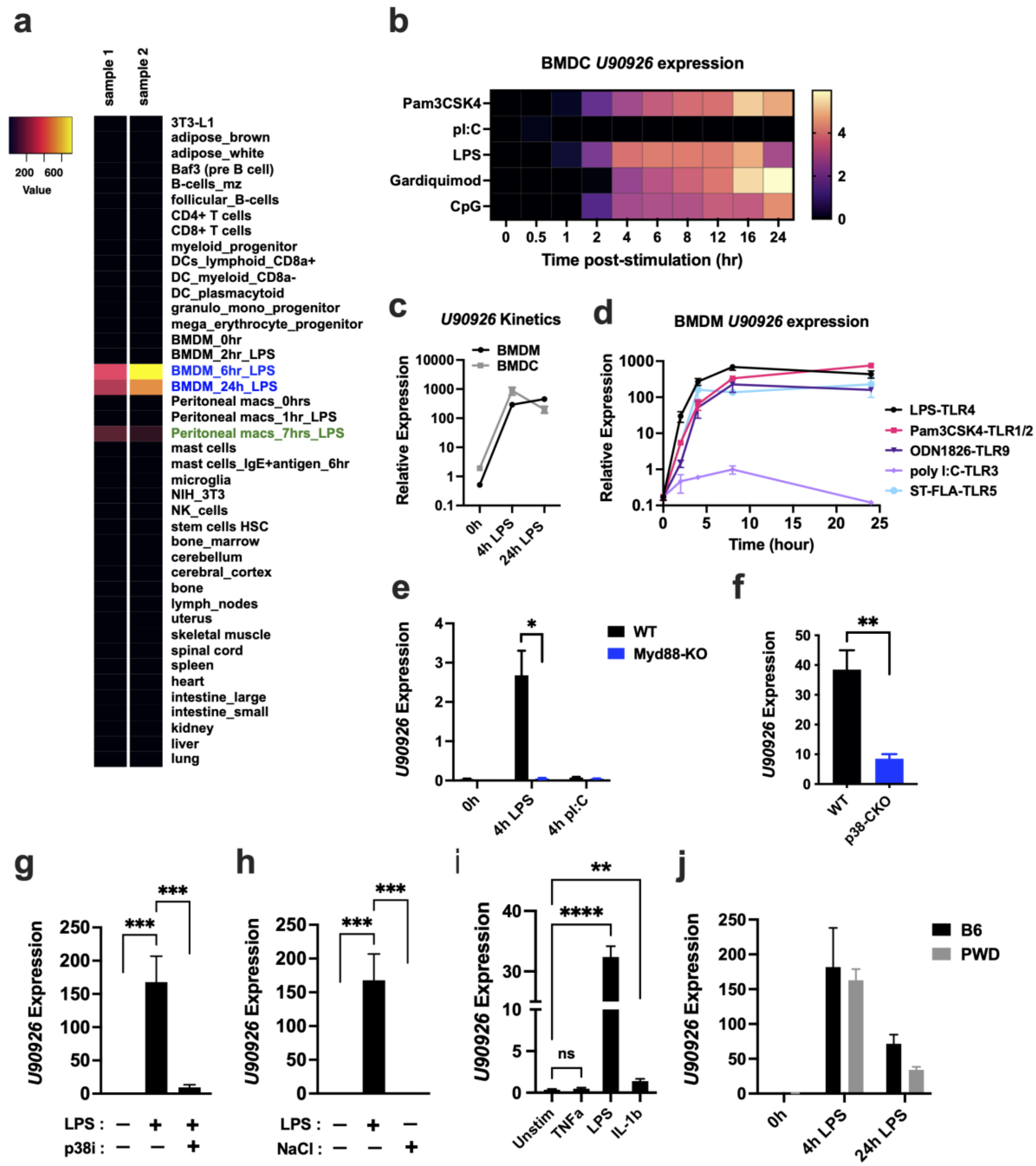

Figure 2-1. Expression of *U90926* is induced in mouse macrophage and dendritic cells by TLR signals in a MyD88- and p38α MAPK-dependent manner.

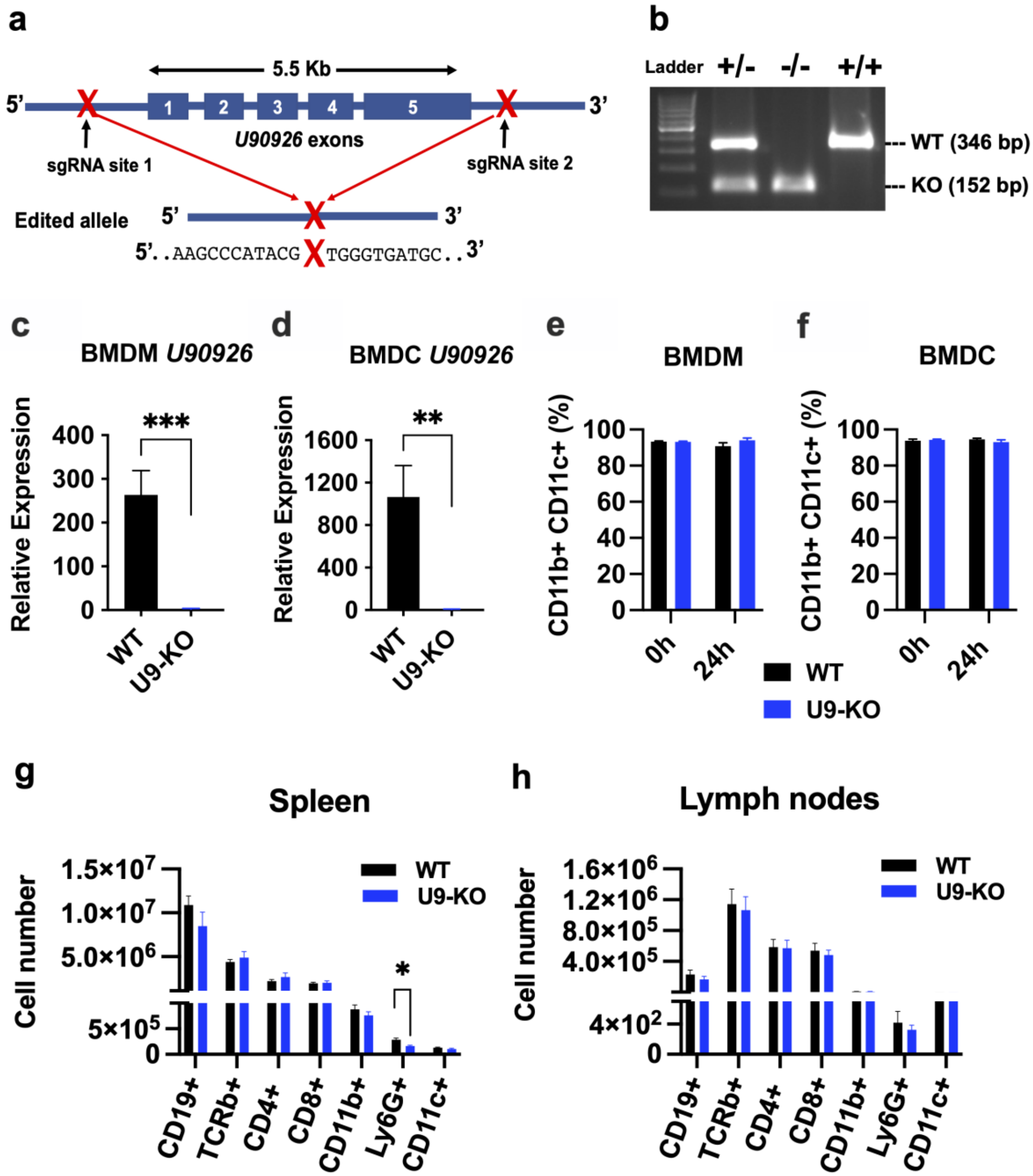

Figure 2-2. Generation and validation of U9-KO mice.

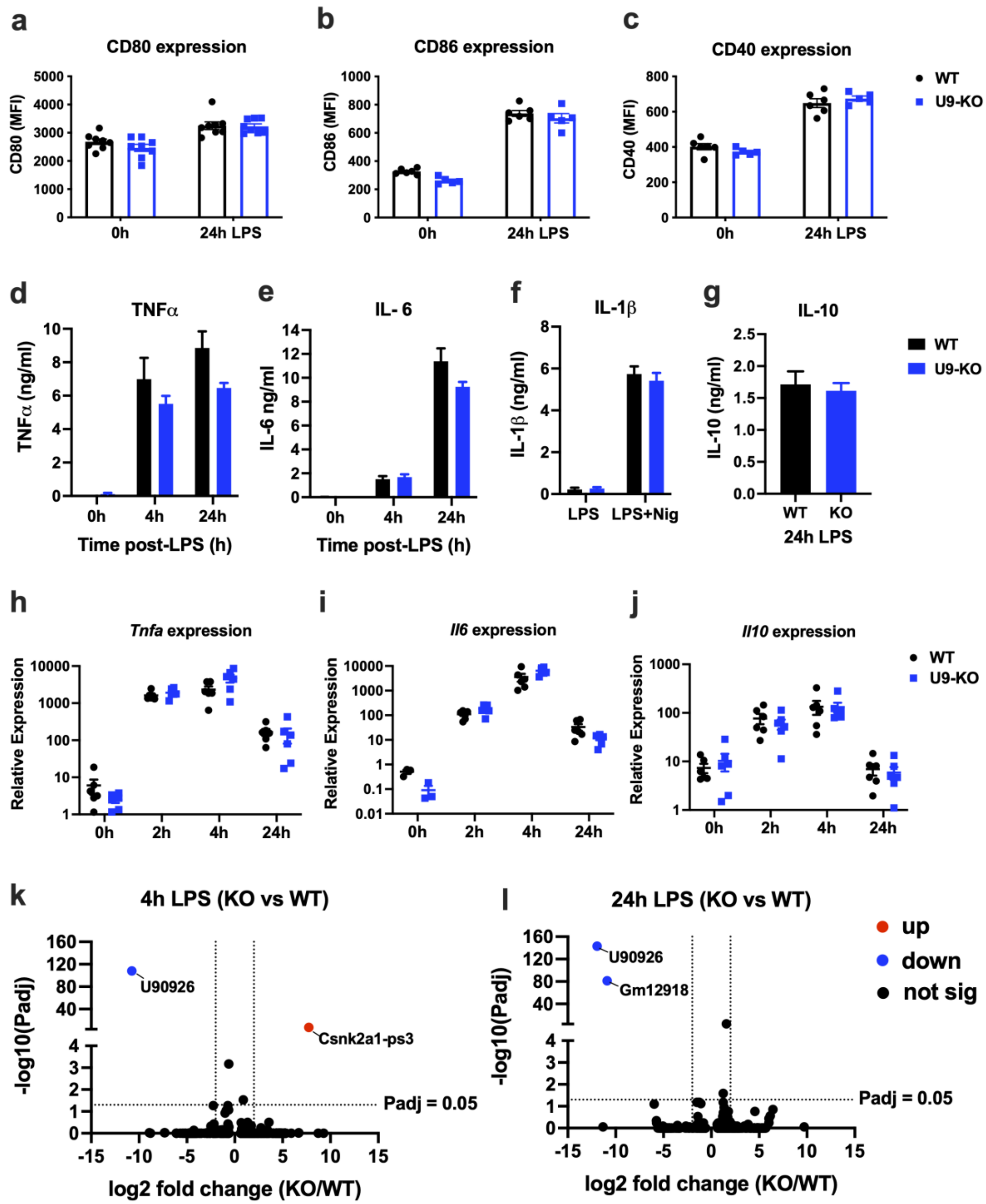

Figure 2-3. ***U90926*-deficiency has minimal intrinsic effects on macrophage function in vitro.**

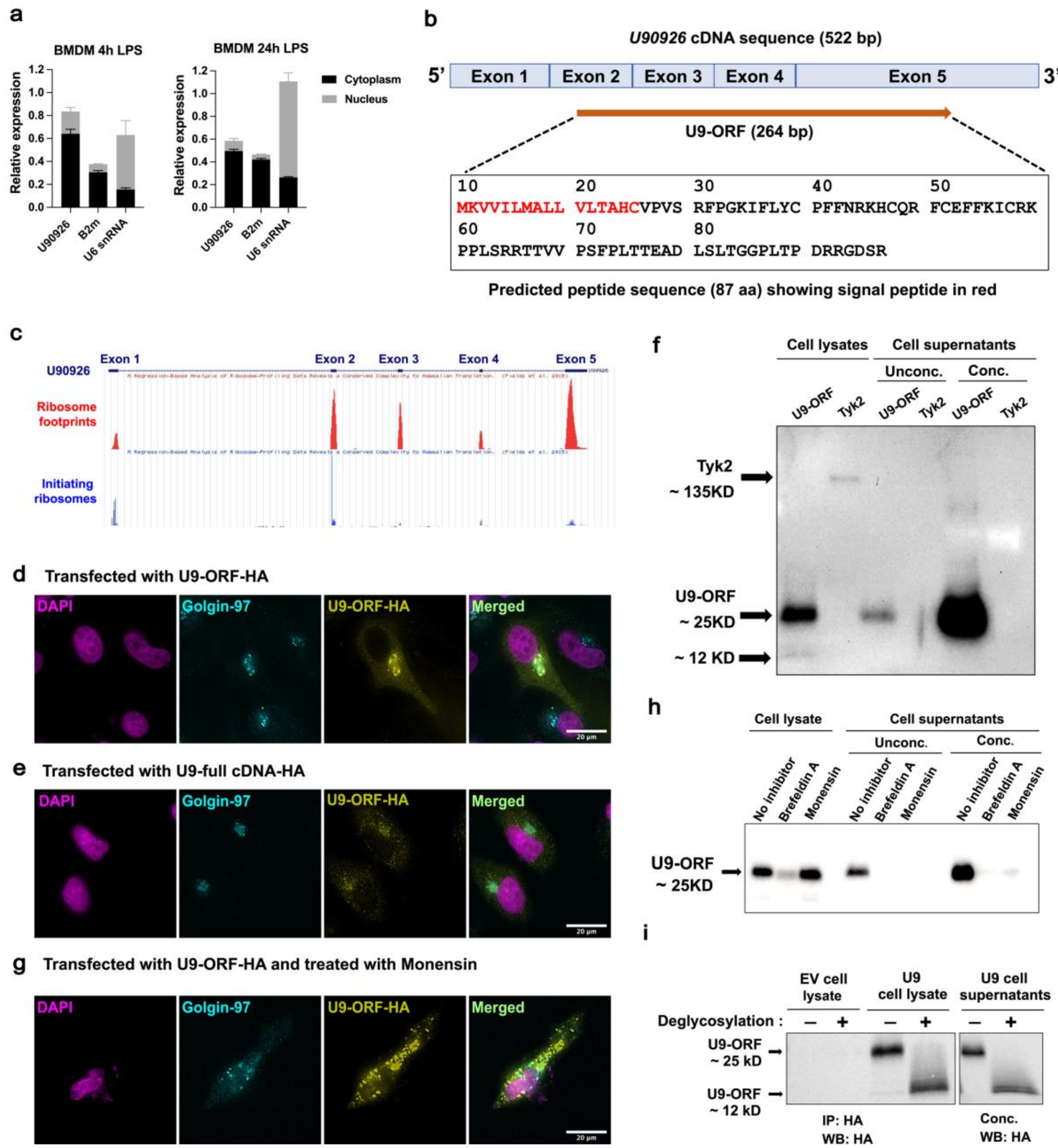

Figure 2-4. ***U90926*** **RNA localizes to the cytoplasm, associates with ribosomes, and contains an ORF that encodes a secreted protein.**

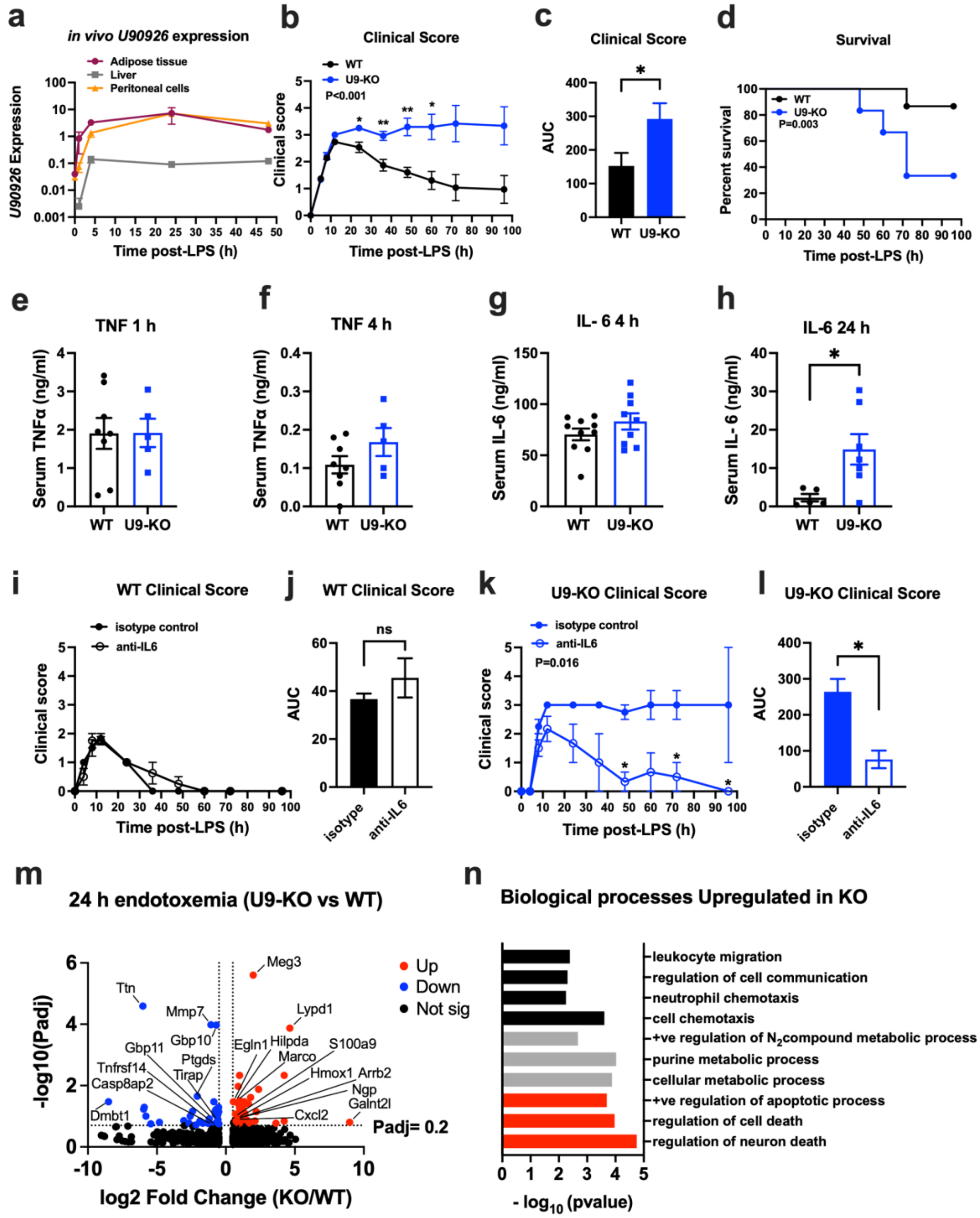

Figure 2-5. ***U90926*** **deficiency exacerbates LPS endotoxemia by elevation of IL-6 levels.**

SUPPLEMENTAL FIGURES

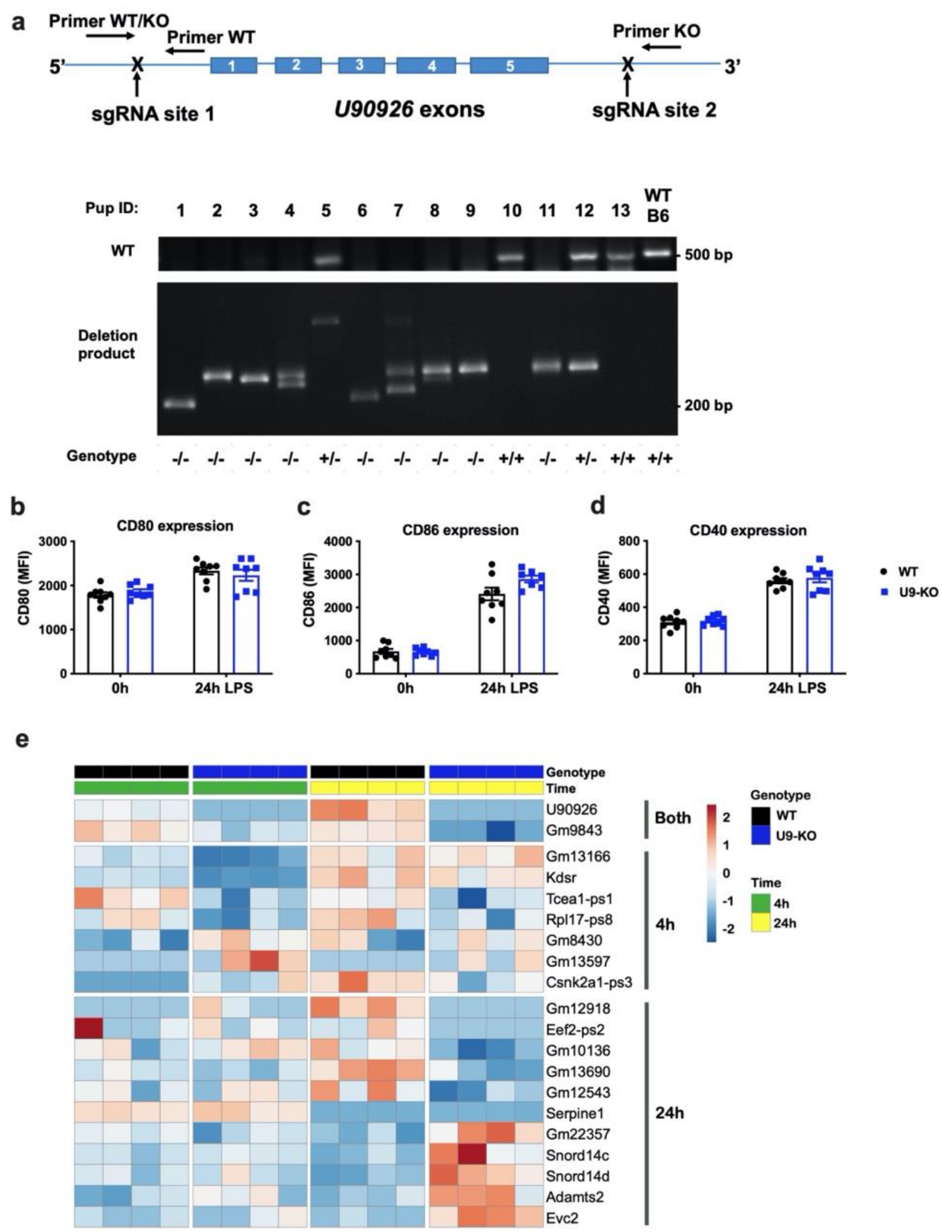

Figure 2-S1. Genotype screening of CRISPR/Cas9 edited pups and *U90926*-deficiency has none to minimal effect in BMDCs and BMDMs. (a) Two sgRNAs and recombinant Cas9 were microinjected into mouse single-cell embryos. PCR genotyping results from the resulting offspring are shown. Two primer sets were used: one set targeting the WT region, (absent in KO), and a second set flanking the entire region, which only yields product if the deletion is present (200-500 bp, depending on size of deletion). Here, U9-KO = -/- , WT B6 = +/+, and heterozygous = +/-. **(b-d)** BMDCs were generated from WT and U9-KO mice and stimulated with 100ng/ml LPS for 24h. Cell surface expression of co-stimulatory markers, CD80, CD86, and

CD40, was measured by flow cytometry and expressed as mean fluorescence intensity (MFI). Data are represented as the mean ± SEM. Significance was determined by student's t test. **(e)** RNAseq data shown in **Fig. 3k** and **3l** were reanalyzed using a relaxed cutoff of |Log2 Fold Change|=0.6, Padj <0.2. A heatmap demonstrating the row-normalized gene expression of the top 20 (by log2 fold change) differentially expressed genes at the indicated time points in WT and U9-KO BMDM.

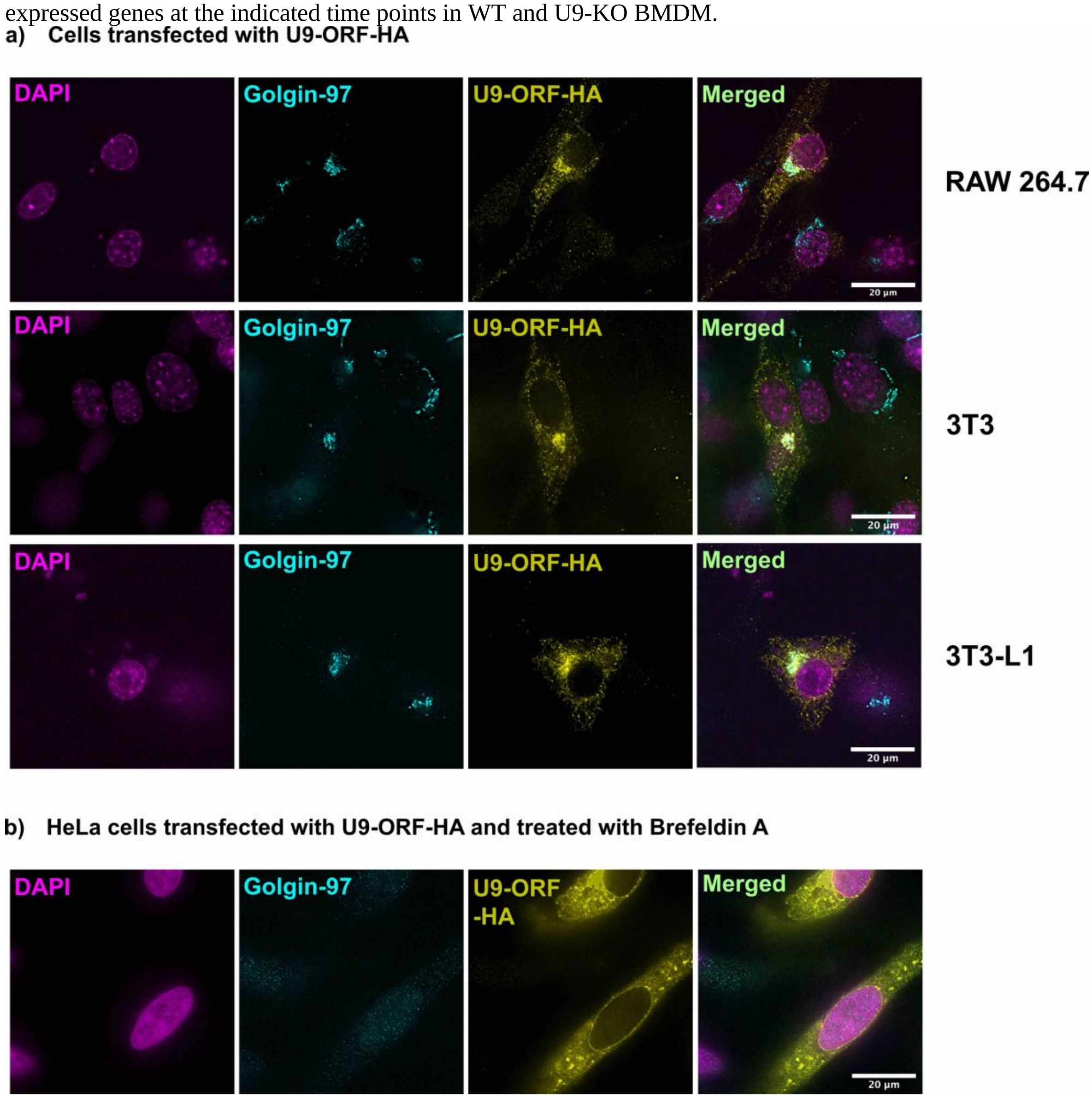

Figure 2-S2. U9-ORF protein localizes to the Golgi apparatus. (a) RAW 264.7 mouse macrophages, mouse 3T3 fibroblasts, and mouse 3T3-L1 preadipocytes were transfected with U9-ORF-HA plasmid, followed by immunostaining. **(b)** HeLa cells were transfected with U9-ORF-HA plasmid and treated with Brefeldin A, followed by immunostaining. Stained markers are as follows, Golgin-97 (cyan; Golgi marker), HA (yellow: U9-ORF peptide), and DAPI nuclear staining (magenta).

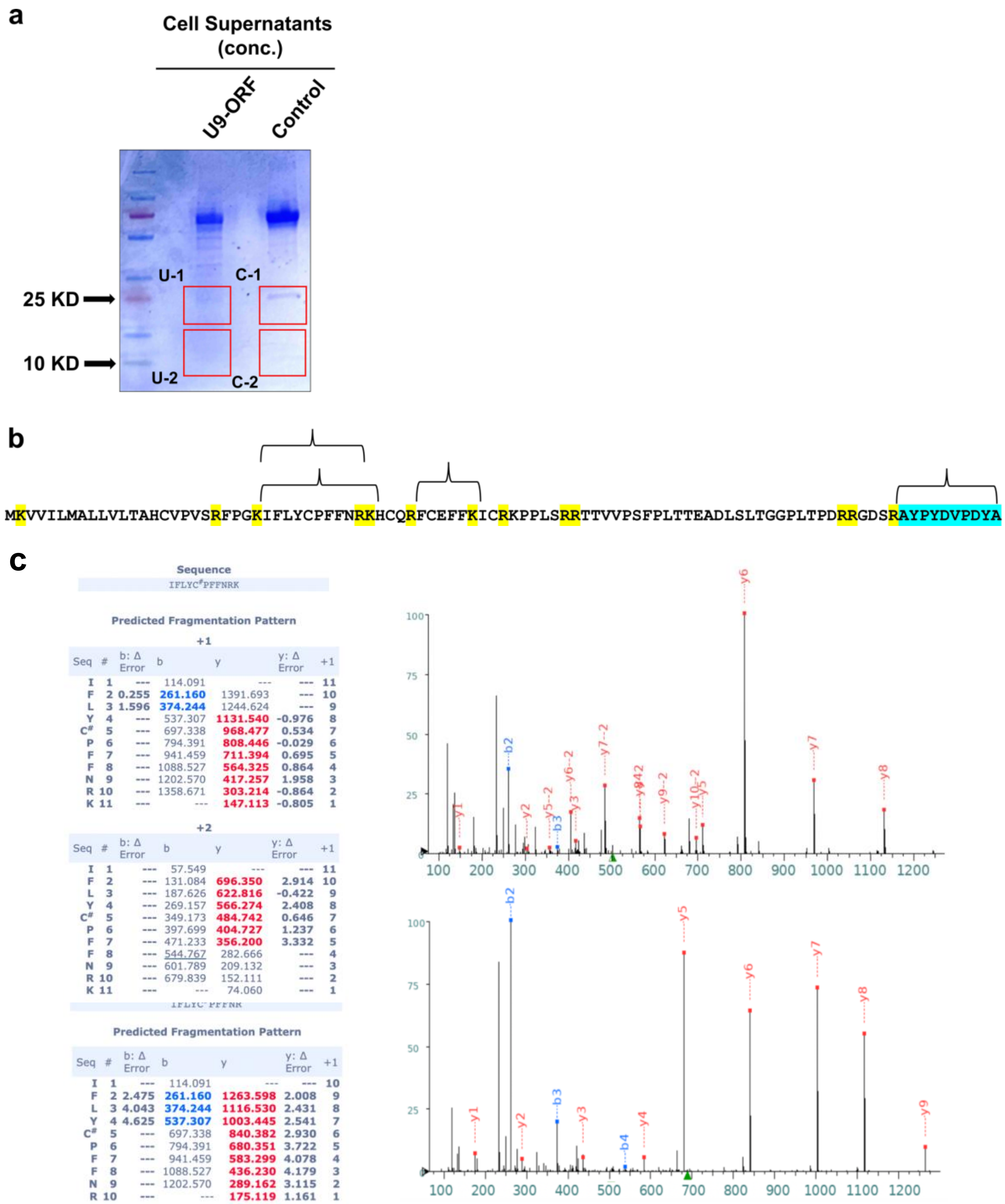

Figure 2-S3: LC-MS/MS identification of U9-ORF protein. HeLa cells were mock-transfected (control) or transfected with a plasmid encoding U9-ORF-HA. Supernatant was collected and concentrated as described earlier and subjected to SDS-PAGE. The gel was stained with Coomassie blue **(a).** Gel regions shown in **(a)** were excised and subjected to in-gel tryptic digestion. Tryptic peptides were extracted and subjected to LC-MS/MS. Tryptic peptides for U9-ORF were identified only in the transfected sample and the high confidence identified peptides are listed in Table 1 and are graphically depicted in **(b)**, with brackets indicating the identified tryptic fragments within the U9-ORF sequence. Note, yellow highlights indicate

potential tryptic cleavage sites. Blue highlight indicates the HA-tag with an N-terminal alanine linker. **(c)** MS/MS spectra for two of the identified tryptic peptides showing with b- and y-type ions labeled. Mass tables are included for each spectrum denoting expected fragment ion masses and mass accuracy (Δ Error, in ppm). Note upper and lower spectra overlap and display strong y-type ion series as expected.

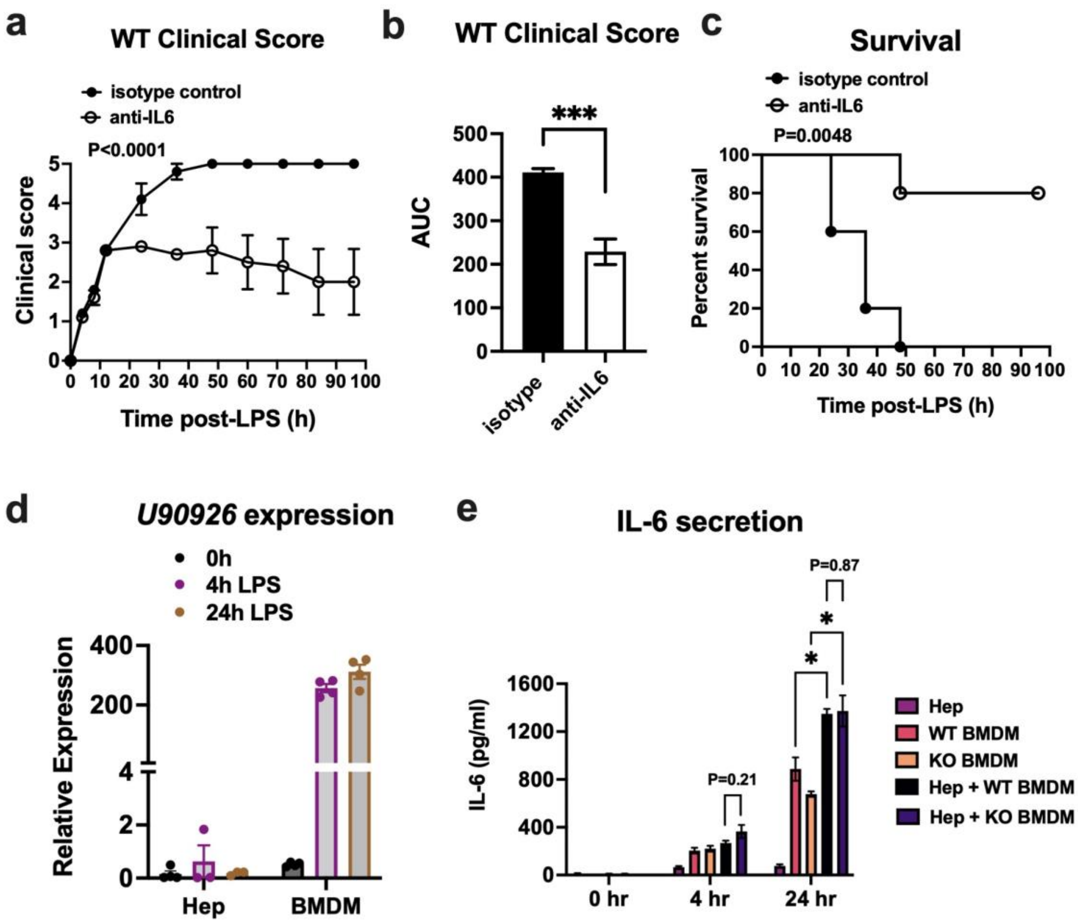

Figure 2-S4. IL-6 Neutralization is protective during LPS endotoxemia in WT mice challenged with a higher LPS dose, and U9-ORF regulation in heaptocytes. WT B6 mice (n=10) were challenged with high dose of LPS (60 mg/kg) and simultaneously administered isotype control (anti-trinitrophenol) or anti-IL-6 antibodies (100 µg/mouse) by i.p. injection, followed by evaluation of sickness behavior **(a** and **b)** and survival **(c).** Area under the curve (AUC) is shown in **(b),** which was calculated from kinetic data in (**a**), to calculate a cumulative measurement of the disease severity. Clinical score evaluation measurement is described in the Methods section. **(d)** A mouse hepatocyte cell line, AML12, or BMDMs generated from WT B6 mice (n=4) and were stimulated with LPS (100 ng/ml) at different time points indicated above. RNA was extracted, followed by RT-qPCR to measure *U90926* expression. RT-qPCR data are expressed relative to the housekeeping gene, Beta-2-microglobulin (*B2m*), and multiplied by a factor of 10,000 for ease of visualization. Data are represented as the mean ± SEM. **(e)** *In vitro* co-culture experiments were set up using mouse hepatocyte, AML12 and WT and U9-KO BMDMs at a 4:1 ratio and their control groups containing either AML12 or BMDM alone. Cells were stimulated with LPS (100ng/ml) for 4 and 24 hours and supernatants were analyzed by ELISA to measure IL-6 secretion. Significance of differences was assessed using 2-way ANOVA (panel a and e), Welch's T test (panel b) and Mantel-Cox test (panel c). P values are represented as follows: ns= >0.05, *= ≤0.05, **=≤0.01, ***=≤0.001.

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CHAPTER 3: DELETION OF LNCRNA *U90926* DOES NOT ALTER OBESITY-RELATED PHENOTYPES *IN VIVO*

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ABSTRACT

Obesity is a global health problem which is characterized by the excessive fat accumulation or adiposity in the body, which is associated with other chronic metabolic disorders such as type 2 diabetes, liver diseases, cardiovascular disease and certain types of cancer. Excess energy induces adipogenesis (hyperplasia) and lipid accumulation inside the existing adipocytes (hypertrophy) during obesity, which involves transcriptional changes and regulation. Recently, long non-coding RNAs (lncRNAs) have been implicated in adipogenesis regulation and adipose tissue function. Mouse lncRNA *U90926* was previously identified as a repressor of *in vitro* adipogenesis in 3T3-L1 preadipocytes. Based on this, we hypothesized that *U90926* may repress weight gain and adiposity *in vivo*. To test the hypothesis, we leveraged *U90926*-deficient (U9-KO) mice, which we recently generated. In mice fed a standard diet, we have found no differences in obesity-related phenotypes such as weight gain, fat mass accumulation, and plasma concentration of glucose, insulin, triglycerides, and free fatty acids in U9-KO mice in comparison with WT. Our data also show that *U90926* deficiency did not have a major effect on adipose tissue morphology and on gene expression signatures of adipogenesis or obesity. Furthermore, in high-fat diet-fed mice, we found no effect of *U90926* deficiency on weight gain, fat mass, adipogenesis marker expression and immune cell infiltration into the adipose tissue. Taken together, these data suggest that the lncRNA *U90926* lacks an essential role in obesity-related phenotypes as well as adipocyte biology *in vivo*.

INTRODUCTION

Obesity is characterized by the abnormal and excessive accumulation of adipose tissue, primarily white adipose tissue (WAT), that is harmful to human health. It is becoming increasingly prevalent worldwide. Obesity is a major risk factor for type 2 diabetes, non-alcoholic fatty liver disease, cardiovascular disease, and different types of cancers(1, 2). Expansion of adipose tissue and obesity occurs when adipocyte number or size increases(3, 4). Generally, preadipocytes differentiate into mature adipocytes by adipogenesis to increase adipocyte number, which has a profound effect on obesity(5). Understanding molecular mechanisms and modulation of adipogenesis is essential to combat obesity.

Obesity is often described as a chronic low-grade systemic inflammation characterized by the increased pro-inflammatory cytokines by adipocytes and increased infiltration of immune cells, mainly macrophages, into adipose tissue(6, 7). Adipose tissue macrophages (ATMs) play a vital role in obesity by regulating adipocyte function, decreasing efferocytosis, and secreting pro-inflammatory cytokines and chemokines that directly affect inflammation and metabolic imbalance, for example, insulin resistance(8, 9). The interplay between ATMs and adipocytes during obesity is regulated by many molecular factors, which is an ongoing area of research interest(10, 11).

Similar to the other areas of cell biology, long non-coding RNAs (lncRNAs) have emerged as essential regulators in adipocyte development(12). LncRNAs are unique transcripts that are 200 nucleotides in size, often polyadenylated, and usually do not encode for protein(13). They possess crucial regulatory effects in many biological processes, such as transcriptional repression, X chromosomal inactivation, chromatin remodeling, cell differentiation, and cancer metastasis(14–18). Recently, studies have shown that several lncRNAs, including SRA, lnc-RAPn, slincRAD, PU.1 AS, HOTAIR, ADINR, and NEAT1 regulate white preadipocyte differentiation(19–23).

We have recently characterized a murine lncRNA named *U90926*. We found that it is highly expressed in macrophages upon toll-like receptor (TLR) activation and is protective during endotoxic shock in mice(24, 25). Other groups have characterized *U90926* as a co-factor of HSV-1 virus replication and a modulator of neutrophil migration during ischemic brain injury(26, 27). Prior to these studies, in 2017, Chen et al. showed that lncRNA *U90926* is a repressor of adipogenesis in vitro, using 3T3-L1 preadipocytes(28). Based on this study, we hypothesized that deletion of *U90926* would promote weight gain and adiposity *in vivo*. To test our hypothesis, we leveraged a *U90926* knockout mouse model (U9-KO) that we recently generated, which lacks the whole *U90926* gene locus(24). Here, we have evaluated different parameters of obesity-related phenotypes using both a regular diet and a high-fat diet to determine the effect of *U90926* on adiposity.

MATERIALS AND METHODS

Animals

U90926-deficient mice (U9-KO) lacking the entire *U90926* locus on the C57BL/6J background were generated previously, and PCR confirmation of the U9-KO genotype was done as previously described(24). For German mouse clinic (GMC, München, Germany) phenotyping experiments, littermate WT C57BL/6J and homozygous U9-KO mice were generated from U9-KO heterozygous breeders (het x het crosses) to minimize any cage/parental effects. The animals were shipped to GMC at X weeks of age, and then subjected to the standard GMC phenotyping pipeline(29, 30). Mice at GMC received standard mouse chow. Diet-induced obesity experiments were performed at the UVM animal facility. Eight-week-old WT and U9-KO mice were switched from normal diet to high-fat diet (60%Kcal fat) purchased from Research Diets Inc. (catalog#D12492). WT control mice, C57BL/6J (B6) used for HFD experiment were bought from Jackson laboratory (Bar harbor, ME) and put on rest for two weeks to acclimate at UVM facility.

Cell culture

3T3-L1 preadipocytes were cultured in DMEM media with 10% FBS and sodium pyruvate. For adipogenesis, 3T3-L1 preadipocytes were grown to confluence in DMEM with 10% FBS (Gibco), after two days the culture medium was replaced with differentiation medium containing 10% FBS, 1 µM dexamethasone (Sigma), 0.5 mM 3-isobutyl-1-methylxanthie (IBMX) (Sigma), and 1 µg/ml insulin (Sigma). Two days later, the medium was changed to maintenance media (DMEM with 10% FBS + 1 µg/ml insulin). and maintained until day 8 with media change every two days. At each time point of the experiment, cells were collected in TRIzol reagent (Invitrogen, Waltham, MA) for RNA extraction. Bone-marrow derived macrophages (BMDMs) were isolated, cultured, and stimulated with LPS, as described previously(24).

RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

For both *in vitro* and *in vivo* experiments, Direct-zol RNA Microprep Kits (Zymo Research, Irvine, CA) were used for RNA extraction as per manufacturer's protocol. Fat tissue samples were collected into 2 ml screw cap tubes containing 1/3 full of 1.0mm diameter Silicon-Carbide particles (Biospec Products, Bartlesville, OK) and 1 ml of TRIzol reagent (Invitrogen, Waltham, MA) per tube. Then, the samples were homogenized using a Mini-Beadbeater instrument by Biospec Products (Bartlesville, OK) for total 2 min by 4 cycles, and placing tubes on ice-water bath in each interval to avoid over-heating. Then, tubes were centrifuged at 13,000 rpm for 2 min to pellet any debris, 200 μL of homogenate were collected and 100 μL of fresh TRIzol was added, followed by extraction using the Direct-zol RNA Microprep Kit as above.

RNA concentration was determined by Nanodrop (Thermo Scientific NanoDrop 2000 Spectrophotometer) and cDNA synthesis reaction by reverse transcription was performed according to the manufacturer's instructions using the qScript cDNA Super MIX kit (QuantBio, Beverly, MA). The qPCR reaction was performed according to the manufacturer's instructions using the DyNAmo ColorFlash SYBR Green kit by Thermo Fisher Scientific (Waltham, MA), using target-specific primers. The qPCR was run on a Quant Studio 3 Real-Time PCR System by Applied Biosystems (Thermo Fisher Scientific, Waltham, MA). Target gene expression was normalized by the expression of the housekeeping genes (HKG), either 18S rRNA or *B2m* (β-2-Microglobulin), as indicated, and calculated by a comparative Ct method formula 2^-(deltaCt) and multiplied by a factor of 10,000 for ease of visualization, i.e., $2^{-(Ct_{target} - Ct_{HKG})} * 10{,}000$. We did not normalize relative expression to a reference sample (i.e., delta-deltaCt).

Fat content analysis by DEXA and NMR

Fat content measurements at GMC were done by both Dual-energy X-ray absorptiometry (DEXA) and Time Domain Nuclear Magnetic Resonance (TD-NMR). DEXA was carried out consecutively using a Faxitron Ultrafocus equipped with 10 x 15 cm CMOS detector (Faxitron Bioptics, LLC, Tucson, AZ, USA). The mouse was anesthetized, and prior to imaging weight and length of the mouse were recorded. Subsequently, it was fixed on an X-ray-permeable plate and placed in the instrument. Image acquisition and analysis had performed using VisionDXA software (Faxitron Bioptics, LLC). Quantitative DEXA measures were computed automatically for the whole mouse. For TD-NMR, a whole-body composition analyzer (Bruker MiniSpec LF 50) was used as a robust method for the measurement of lean tissue and body fat in live mice without anesthesia, using TD-NMR signals from all protons in the entire sample volume and providing data on lean and fat mass.

Metabolic screening

At GMC, as part of metabolic screening, fasting glucose and other compounds were measured where mice were subjected to food withdrawal overnight and blood was collected. The screen was performed using a Beckman-Coulter AU 480 autoanalyzer and adapted reagents from Beckman-Coulter (Krefeld, Germany), except free fatty acids (NEFA) that were measured using a kit from Wako Chemicals GmbH (NEFA-HR2, Wako Chemicals, Neuss, Germany). In the primary screen, a broad set of parameters was measured including various enzyme activities, as well as plasma concentrations of specific substrates and electrolytes in *ad libitum* fed mice. Plasma insulin levels or insulin and leptin were determined using an electroluminescence-linked immunosorbent assay provided by Mesoscale Discovery (mouse/rat insulin kit K152BZC; mouse metabolic kit K15124C) and a MESO QuickPlex SQ 120 Sector imager (Mesoscale Discovery, Rockville, Maryland, USA).

Intraperitoneal Glucose Tolerance Test (IpGTT)

At GMC, mice were used for the glucose tolerance test after a 6-7 hour-long food withdrawal. Before food-withdrawal and in the beginning of the test, the body weight of mice was determined. For the determination of the fasting blood glucose level, the tip of the tail was scored using a sterilized scalpel blade and a small drop of blood was analyzed with the Accu-Chek Aviva glucose analyzer (Roche/Mannheim). Thereafter mice were injected intraperitoneally with 2 g of glucose/kg body weight using a 20 % glucose solution, a 25-gauge needle and a 1-ml syringe. 15, 30, 60 and 120 minutes after glucose injection, additional blood samples (one drop each) were collected and used to determine blood glucose levels as described before. Repeated bleeding was induced by removing the clot from the first incision and massaging the tail of the mouse.

White adipose tissue (WAT) analysis

At GMC, WAT was analyzed for morphology and molecular phenotyping. Mice were euthanized by CO_2 inhalation. For histology, a piece of inguinal WAT was frozen, cut and stained for hematoxylin and eosin (H&E) and analyzed under light microscope by a trained veterinary pathologist. For molecular phenotyping, perigonadal fat was collected from the mice and analyzed by RNA-seq. First, total RNA was isolated employing the RNeasy Mini kit (Qiagen) including Trizol treatment. The Agilent 2100 Bioanalyzer was used to assess RNA quality and RNA with RIN > 7 was used for RNA-seq analysis. RNA-seq was performed on an Illumina NovaSeq 6000 with a PE150 stranded protocol. Paired-end data was generated and analyzed by an RNA-seq pipeline consisting of quality control (FastQC, MultiQC), read trimming (trim_galore), genome alignment (STAR), and gene-level read counting (summarizeOverlaps, mode = 'Union'). Significantly differentially expressed genes were determined with DEseq2 tool(31) in R studio after filtering low expressed genes (<10 reads).

Flow cytometry

We have isolated visceral fat in the lab, and separated The stromal vascular cell (SVC) fraction and mature adipocyte fraction were isolated from visceral fat tissue by a collagenase based approach described previously(32). Red blood cell (RBC) lysis was performed on SVC by incubating the cells in 0.8% ammonium chloride solution (StemCell Technologies, Cambridge, MA) and then cells were subjected to staining for flow cytometry analysis. Cells were stained with the UV LIVE/DEAD fixable stain (Invitrogen) and then surface labeled for different combinations of following markers: CD45, CD11b, CD11c, CD206, CD301, CD19, TCRβ, Ly6G, F4/80, and Ly6C (Biolegend, San Diego, CA) and fixed with 1% paraformaldehyde (Sigma Aldrich, St. Louis, MO). Samples were analyzed on an LSRII cytometer (BD Biosciences) or an Aurora (Cytek Biosciences). All flow cytometry data analysis was performed using FlowJo Software version 10.6.1 (BD Biosciences).

Statistical analyses

Statistical analyses not pertaining to RNA-seq data were carried out using GraphPad Prism software version 9.2.0 (GraphPad Software, San Diego, CA, USA). The specific tests used to assess the significance of the observed differences are detailed in the figure legends. All center values represent the mean, and error bars represent the standard error of the mean. A P-value of <0.05 was considered significant.

RESULTS

LncRNA U90926 is expressed in adipose tissue

Previously, we have shown that lncRNA *U90926* is highly expressed upon TLR stimulation in myeloid cells(24). Additionally, Chen et al. demonstrated that *U90926* is constitutively expressed in 3T3-L1 preadipocytes, and its expression decreases as they differentiate into mature adipocytes(28). They have also shown a negative correlation of *U90926* expression in WAT with obesity using different obese mouse models such as *ob/ob*, *db/db*, and high-fat diet (HFD)-induced obesity. This led us to hypothesize that *U90926* deletion promotes weight gain and obesity-related phenotypes in mice. We have previously generated a U9-KO mouse model on C57BL/6J (B6) background, which we leveraged here to test this hypothesis. First, we validated *U90926* expression in our models. We found that *U90926* is expressed in visceral WAT depots from WT B6 mice but not U9-KO mice (Fig. 3-1A). We also compared the *U90926* expression level in WAT with LPS-stimulated bone marrow-derived macrophages (BMDMs), which we previously determined to exhibit very high levels of *U90926* expression, and with the 3T3-L1 model before and after differentiation. We found that *U90926* expression in WT WAT, while detectable, and is significantly lower compared with the LPS-stimulated BMDMs and 3T3-L1 preadipocytes (Fig. 3-1A).

We used a standard eight day-differentiation protocol for 3T3-L1 preadipocytes (see methods). Similar to Chen et al.(28), we found a significant reduction in *U90926* expression on day 8 (mature adipocyte) compared with baseline day 0 (preadipocyte), depicted in Fig. 3-1A. However, our parallel setup of control 3T3-L1 cultures, without addition of differentiation stimuli, showed a surprisingly similar decline in *U90926* expression during 3T3-L1 culture independent of adipocyte differentiation stimuli (Fig. 3-1B). Taken together, we have shown that *U90926* is expressed in fat tissue, and we were able to reproduce the data by Chen et al. that *U90926* expression declines as the cells approach toward differentiation.

Deletion of U90926 has no effect on weight gain and fat mass in vivo

We have performed a detailed phenotypic characterization of U9-KO mice on a normal diet in collaboration with German Mouse Clinic (GMC, München, Germany), using their standard mouse phenotyping pipeline (see Materials and Methods). We generated a cohort of control C57BL/6J WT (M=14, F=11) and homozygous U9-KO (M=11, F=14) mice, which were littermate offspring from U9-KO heterozygous breeders, thus minimizing any cage or littermate effects. Firstly, to determine if *U90926* grossly affects weight gain in mice fed a normal diet, body weight was measured weekly from 9-20 weeks of age. We found no difference in weight gain in female and male U9-KO mice compared to WT (Fig. 3-2A, B).

Since excessive fat accumulation is the major hallmark of obesity, we have determined whether *U90926* deficiency impacts the total fat mass. We have measured whole mouse fat mass by two different methods, DEXA (dual x-ray absorptiometry) and time domain nuclear magnetic resonance (TD-NMR), at different time points and found no differences in overall fat mass accumulation in U9-KO compared to WT (Fig. 3-2C-E). Adiposity index was calculated as fat mass (g) divided by lean mass (g), which showed no significant differences in either males or females U9-KO and WT (Fig. 3-2D, E). Taken together, these findings suggest that *U90926* does not regulate weight gain and total fat mass accumulation under a normal diet.

Obesity-related metabolic changes are unaltered in U9-KO mice

Obesity is a significant risk factor for numerous metabolic disorders, such as type 2 diabetes and nonalcoholic fatty liver disease which are accompanied by insulin resistance and elevated liver enzymes, respectively(33). In obesity, metabolic consequences leading to insulin resistance include an increased concentration of glucose, lipids, and free fatty acids in circulation(34). Here, we investigated plasma concentrations of glucose, triglycerides (TG), and non-esterified fatty acids (NEFA) in WT and U9-KO mice after overnight food withdrawal. We found that *U90926* deficiency does not affect fasting glucose, TG, and NEFA level (Fig. 3-3A-C). Next, to measure glucose uptake, thus the effect of obesity on metabolism, we have performed intraperitoneal glucose tolerance tests (IpGTT). We found no difference in glucose clearance between WT and U9-KO mice, indicating that U9-KO mice do not have impaired insulin signaling (Fig. 3-3D, E). Our metabolic screening also showed that *U90926* deficiency does not impact other major plasma biomarkers of obesity, such as fibroblast growth factor 21 (FGF21), leptin, and insulin (Table 3-1). Although there is a subtle decrease in leptin concentration in the male U9-KO mice compared with the WT, their food intake and other metabolic measurement do not suggest altered leptin signaling.

Obesity is associated with many liver diseases, such as nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), and cryptogenic cirrhosis(35). Like other liver injuries, these obesity-associated liver diseases are also accompanied by elevated liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the circulation(35). Given that, we have measured the plasma concentrations of AST, ALT, and lactate dehydrogenase (LDH) enzymes in our metabolic screening and found no unusual fluctuation of liver enzymes in U9-KO mice compared to WT (Fig. 3-3F-H). Altogether, these data suggest that *U90926* deletion does not affect glucose sensing and other obesity-related metabolic parameters *in vivo*.

U90926 deficiency does not impact the major WAT signatures in vivo

Since adipocyte size and the number increases in WAT during obesity(4), we have examined the effect of *U90926* deletion in WAT morphology *in vivo*. The microscopic examination followed by H & E staining was performed in inguinal WAT collected from male (data not shown) and female WT and U9-KO mice (Fig. 3-4A). We detected no apparent differences in adipocyte size and shape when comparing control WT mice with U9-KO (Fig. 3-4A). Both groups showed the typical morphology of WAT-adipocytes containing one large cytoplasmic droplet (also called unilocular) of lipid filling that takes up almost the entire cell and leads to the displacement of the nucleus to the periphery.

Adipogenesis is a well-established process that involves a sequential transcriptional cascade that controls adipocyte development at the molecular level(36). Chen et al. have shown that the knock-down of *U90926* in 3T3-L1 preadipocytes leads to the upregulation of adipogenesis markers, including peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα), fatty acid binding protein 4 (FABP4) and adiponectin (AdipoQ)(28). Based on that, we hypothesized that *U90926* deletion *in vivo* may affect molecular signatures related to adipogenesis and obesity. We performed RNA-seq analysis on perigonadal WAT depots collected from WT and U9-KO mice. Differentially expressed genes (DEGs) were identified using the DEseq2 package. Using a standard cutoff of Padj <0.05, 36 and 5 DEGs were found in males and females, respectively, with the relative expression of top 20 genes (based on p value) in both females and males shown as a heatmap in Fig. 3-4B and C. The DEGs showed relatively heterogenous expression across samples and contained no obvious adipogenesis markers or other obesity-related molecular markers, suggesting that *U90926* deficiency *in vivo* has minimal effects on obesity-related gene expression in WAT. Taken together with the histologic assessment and adiposity index measurements, these data suggest that *U90926* deficiency in vivo does not affect the major characteristics of WAT.

Diet-induced obesity phenotypes are no different in U9-KO mice

In the studies above, we had assessed if *U90926* deletion has any spontaneous role in weight gain or adiposity *in vivo* in mice fed a normal diet (ND). and found no major significant phenotypic changes in U9-KO mice. Next, we used a standard diet-induced obesity model (37), where we introduced a high-fat diet (HFD) containing 60% kcal fat to the control WT mice and U9-KO mice at the age of 8 weeks and continued for another six weeks. We found no difference in weight gain between WT and U9-KO mice fed a HFD (Fig. 3-5A, B). Perigonadal fat weights were also comparable between WT and U9-KO mice (Fig. 3-5C). These data indicate that *U90926* does not exert a major effect on weight gain and fat accumulation in diet-induced obesity.

A previous study showed that HFD-obese mice have decreased *U90926* expression in their subcutaneous and visceral WAT compared to ND, with expression ofadipogenesis markers PPARγ, FABP4, and adipoQ upregulated in WAT of HFD obese mice and in U9-knockdown 3T3-L1 preadipocytes(28). In constrast, we have found that WT HFD obese mice have significantly higher *U90926* expression in the visceral WAT than do ND-fed mice (Fig. 3-5D) with no differences in PPARγ and FABP4 expression in visceral WAT of HFD-fed WT and U9-KO mice (Fig. 3-5E, F).

Adipose tissue contains numerous immune cell populations in its stromal vascular cell (SVC) fraction, along with preadipocytes(38). During obesity, in addition to macrophages, other immune cells like neutrophils, monocytes, T cells, B cells, NK cells, etc., also infiltrate the adipose tissue, which plays an essential role in obesity-associated inflammation (9, 38). Therefore, to identify if the increased *U90926* expression in HFD-fed WT whole WAT tissue RNA (Fig. 3-5D) is associated with SVC fraction or adipocytes, we separated SVC and mature adipocytes from WAT collected from WT mice. We found that *U90926* expression is significantly higher in SVC fraction than in adipocytes (Fig. 3-5G). Since immune cell infiltration in WAT is a hallmark of obesity-related inflammation, we have examined if *U90926* has any effect on regulating immune cell infiltration in WAT, focusing on the myeloid cell population, given our published findings on the role of *U90926* in macrophages(24). We measured M1 macrophages (CD11c+), M2 macrophages (CD206+ or CD301+), adipose tissue-resident macrophages (F4/80+), and monocytes (Ly6C+) as a percentage of CD11b+ in perigonadal WAT collected from both ND-fed and HFD-fed WT and U9-KO mice by flow cytometry (Fig. 3-5 H, I). We found no differences in myeloid cell population in visceral WAT of U9-KO mice compared to WT regardless of diet. Altogether, these data suggest that *U90926* deletion *in vivo* lacks any effect on major phenotypes in diet-induced obesity.

DISCUSSION

Obesity has become a global pandemic that affects millions of people worldwide. It is a significant health risk being associated with different comorbidities such as type 2 diabetes, non-alcoholic fatty liver disease, cardiovascular disease like hypertension, myocardial infarction and stroke, musculoskeletal disease (osteoarthritis), and some types of cancer, for example, breast, ovarian, prostate, liver, kidney and colon(39). Adipogenesis or adipocyte differentiation is an essential aspect of fat expansion in obesity(5). Therefore, understanding molecular mechanisms and triggers for adipogenesis during obesity is helpful in combating obesity. Many research groups have implicated long non-coding RNAs (lncRNAs) as regulators of adipocyte differentiation(12, 40). Chen et al. studied this lncRNA in adipogenesis, showing that *U90926* is a negative regulator of adipogenesis using an in vitro 3T3-L1 preadipocyte differentiation system(28). Based on that, in this study, we have investigated the role of a mouse lncRNA called *U90926* in weight gain and other obesity-related phenotypes in vivo.

U90926 was first reported and classified as lncRNA by a genome exploration research group(41). Since then, many groups, including us, have characterized this lncRNA in distinct cell types, including 3T3-L1 preadipocytes, microglia, mouse retinal cells, and macrophages, with distinct functional roles in each cell type (24, 26–28). We have shown previously that *U90926* is highly expressed in myeloid cells in a TLR-Myd88-p38 MAPK-dependent manner and is protective during endotoxic shock(24). We are also the first to identify that *U90926* can encode a small protein in its 264 bp-long open reading frame (ORF)(24) suggesting that *U90926* may have a dual role in cellular processes, as lncRNA and a protein-coding gene. Here, we have evaluated the role of *U90926* in adiposity and weight gain using a knockout mouse model, U9-KO, where we deleted the whole genomic locus of *U90926*. In agreement with Chen et al.(28), we found that *U90926* is expressed at detectable levels in visceral WAT and is highly expressed in 3T3-L1 preadipocytes, with its expression decreasing as 3T3-L1 cells become mature adipocytes (Fig. 3-1A). However, we have found that the decrease in *U90926* expression in 3T3-L1 cells is not dependent on the presence of adipocyte differentiation stimuli; instead, it may be the effect of prolonged cell culture, as undifferentiated 3T3-L1 cells showed the same expression pattern. This could be due to contact inhibition or other intrinsic factors that change gene expression patterns when cells are in culture for a long time(42).

Chen et al. showed that *U90926* represses adipogenesis in vitro, as siRNA-mediated silencing of *U90926* causes increased expression of adipogenesis markers such as PPARg, CEBPα, FABP4, and AdipoQ and increased lipid accumulation during 3T3-L1 differentiation, while overexpression of *U90926* had the opposite effect(28). Based on this hypothesis, we expected that the U9-KO mouse model would show increased adipogenesis in vivo. On the contrary, we have not found any major effect of *U90926* deficiency in vivo in terms of weight gain and other obesity-related phenotypes such as fat accumulation, metabolic changes like plasma level of glucose, TG, NEFA, and insulin, WAT histology and adipogenesis molecular markers (Fig. 3-2,3,4). Leptin level was slightly decreased in male U9-KO mice (Table 3-1); however, their food intake (evaluated in GMC; data not shown) and other energy metabolism data did not correlate with this, indicating unaltered leptin signaling(43). Generally, the 3T3-L1 differentiation system is widely used for studying adipogenesis because of its uniform morphological and biochemical nature comparable with adipocytes in vivo(44). However, the drawback of this model is that molecular events of adipogenesis in vitro may not fully reflect adipogenesis in vivo, where different other cell types are also involved(45). Our data echo this and suggest that in vitro adipogenesis phenotypes may not be totally reflective of adipogenesis phenomena in vivo. Furthermore, the requirement of *U90926* in adipose tissue homeostasis may be redundant and may get compensated by other genes or pathways.

Since the deletion of *U90926* in vivo was insufficient to change any phenotypes related to obesity in mice fed standard chow, we stressed the system to robustly induce obesity using a HFD. We found that compared with ND, HFD increased *U90926* expression in whole WAT (Fig. 3-5), which contradicts Chen et al findings(28). Regardless, and as with ND, we have found no effect of *U90926* deletion in HFD-fed mice on weight gain, fat accumulation, adipogenesis markers in the WAT, and immune cell infiltration in the WAT (Fig. 3-5).

The role of adipogenesis in regulating obesity phenotypes is controversial in the field. Some studies showed that adipogenesis increases significantly during both genetic and diet-induced obesity(46–48). Others have shown that hypertrophy is the main contributor to obesity(49, 50). Recent studies revealed that increased adipogenesis has paradoxically beneficiary metabolic outcomes in obesity(51, 52). Thus, adipogenesis regulation in vitro may not be the indicator of obesity phenotypes in vivo. Regardless, the lack of changes in adipocyte number in WAT and adipogenesis marker expression in U9-KO compared to WT (Fig. 3-4) in our data suggests *U90926* deletion does not affect adipogenesis or other obesity phenotypes in vivo.

Besides adipocytes and preadipocytes, WAT tissue comprises many different cell types that influence adipogenesis and adipocyte activity in vivo(9, 38). Adipose tissue macrophages (ATMs) in particular, play a vital role in adipogenesis and metabolic changes during obesity(11), and we have shown previously that *U90926* is highly expressed in LPS-activated macrophages and even in whole WAT following systemic LPS administration in vivo(24). In addition, our data shows that *U90926* is highly expressed in stromal vascular cells (SVCs) instead in mature adipocytes (Fig. 3-5G), which indicates increased *U90926* expression signal in HFD-SVC may come from macrophages during obesity because of the chronic inflammation signal. Thus, there could be a macrophage-driven *U90926* role in obesity and/or sepsis-related adipose tissue responses that can be explored in future studies. A conditional knockout model specific to macrophage or adipocyte lineage could be used further to determine the role of *U90926* in obesity in vivo, with temporally inducible models to account for any developmental compensatory effects on loss of *U90926* expression. Nonetheless, our present results demonstrate that *U90926* does not affect obesity-related phenotypes in mice under a normal or high-fat diet condition, suggesting that unlike its role in myeloid cell-mediated inflammation, this gene may not have an essential role in adipocyte biology.

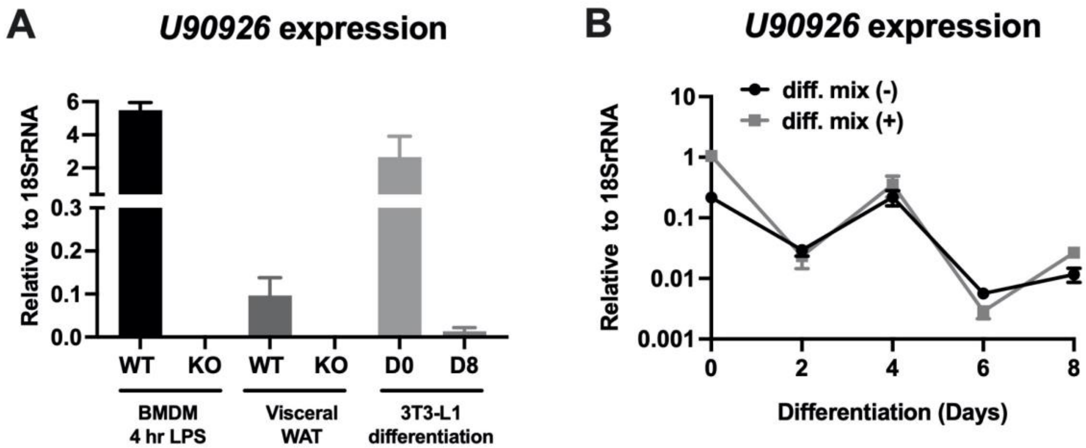

Fig. 3-1 LncRNA *U90926* expression in WAT and in the in vitro 3T3-L1 preadipocyte differentiation model. A) BMDMs and visceral fat tissue were isolated from WT B6 and U9-KO mice. BMDMs were stimulated with LPS (100 ng/ml) for 4h. 3T3-L1 cells collected at two time points of differentiation days D0 (day 0) and D8 (day 8). All the samples were subjected to RT-qPCR for *U90926* expression measurement. **B)** 3T3-L1 cells were plated for an eight-day differentiation protocol with or without differentiation mix (1uM dexamethasone + 0.5mM IBMX + 1ug/ml insulin). Samples were collected every 2 days, followed by RT-qPCR to measure *U90926* expression. All the data are expressed relative to the housekeeping gene 18SrRNA and calculated by a comparative Ct method formula 2^-(deltaCt) and multiplied by a factor of 10,000 for ease of visualization. Data are represented as the mean ± SEM. Student's t test was performed for each sample type in panel (A) and two-way ANOVA for panel (B) to assess the significance of differences between WT and U9-KO, and differentiated and undifferentiated samples, respectively.

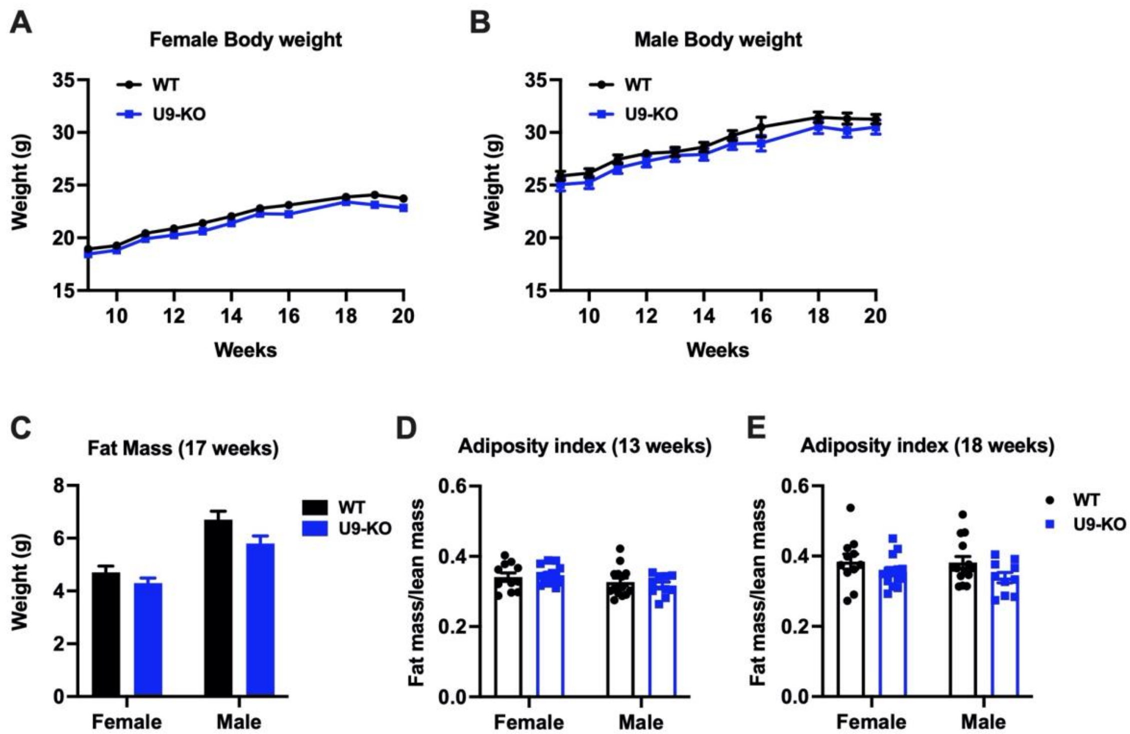

Fig. 3-2 WT and U9-KO mice show comparable total body weight and fat content. A) and **B)** are showing whole body weight of female (WT=11, U9-KO=14) and male (WT=14, U9-KO=11) mice respectively, to compare the difference between WT and U9-KO mice. **C)** At 17 weeks, same mouse groups as above were analyzed by DEXA for whole body fat content. **D)** and **E)** show two time points at 13 weeks and 18 weeks respectively, at which WT and U9-KO mice mentioned above were analyzed by NMR to compare their adiposity index. Adiposity index was calculated as fat mass/lean mass of a mouse, where the fat mass and lean mass were measured by NMR. Data are represented as the mean ± SEM. Two-way ANOVA was used to assess the significance of differences between WT and U9-KO mice.

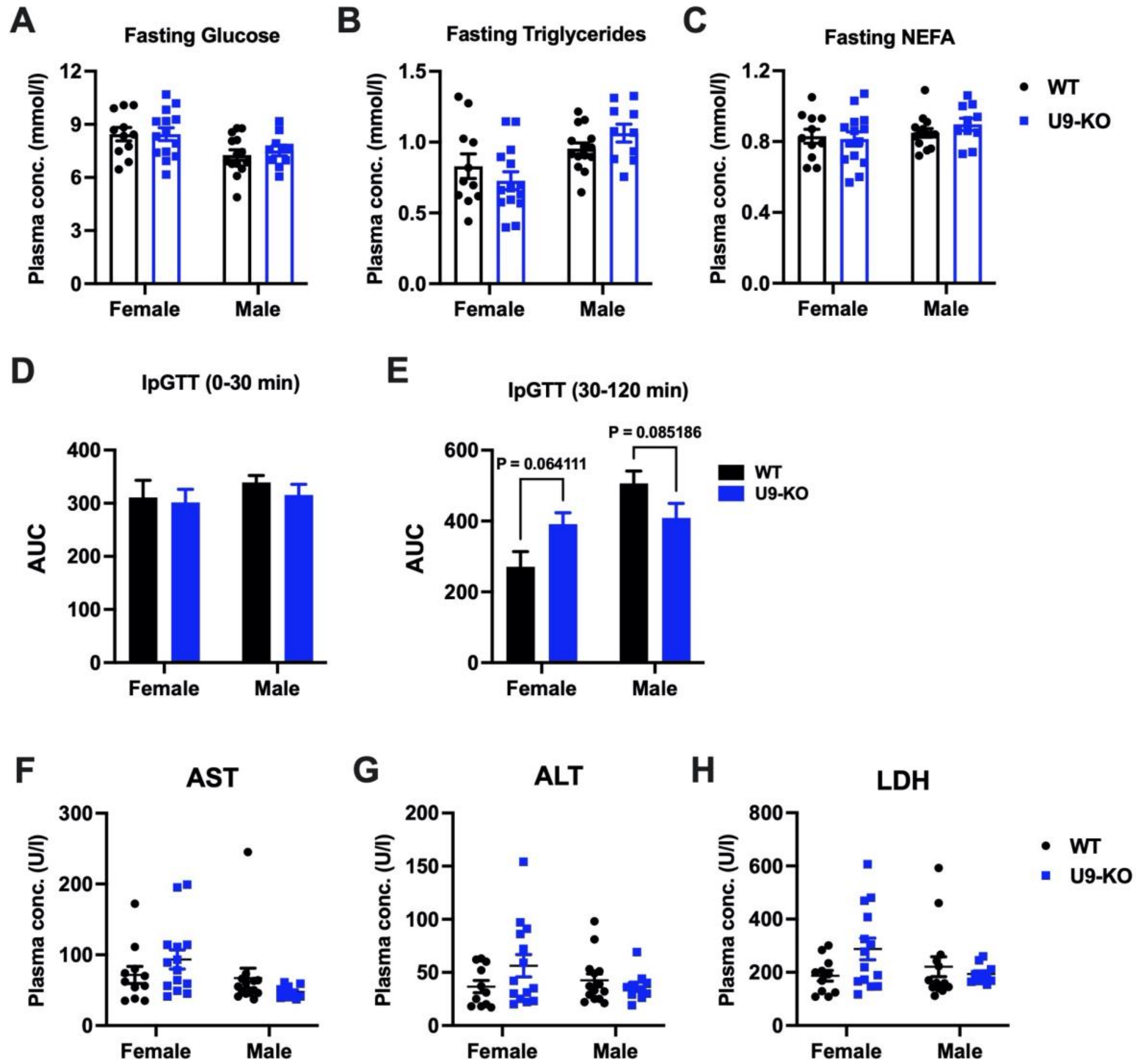

Fig. 3-3 *U90926* deficiency has no major metabolic effect *in vivo*. (A-C) Blood was collected from WT and U9-KO mice after overnight withdrawal of food. Then, plasma concentrations of glucose, triglycerides and non-esterified fatty acids (NEFA) were measured. **(D, E)** Intraperitoneal Glucose-Tolerance-Test (IpGTT) was performed on WT and U9-KO mice fasting for 6-7 hours, where glucose concentration was measured at the beginning, T=0 and at 15, 30, 60 and 120 min after glucose administration. Here, data shows area under the curve (AUC) analysis for 0-30 min in panel D, and for 30-120 min in panel E. **(F-H)** Blood were collected from *ad libitum* fed WT and U9-KO mice to measure the plasma concentration of liver enzymes, aspartate transaminase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH), depicted in panel F, G, and H respectively. Mice groups used for all of the above experiments are 13 weeks old, WT (M=14, F=11) and U9-KO (M=10, F=14) mice. Two-away ANOVA was performed for statistical analysis to assess the significance of differences between WT and U9-KO mice.

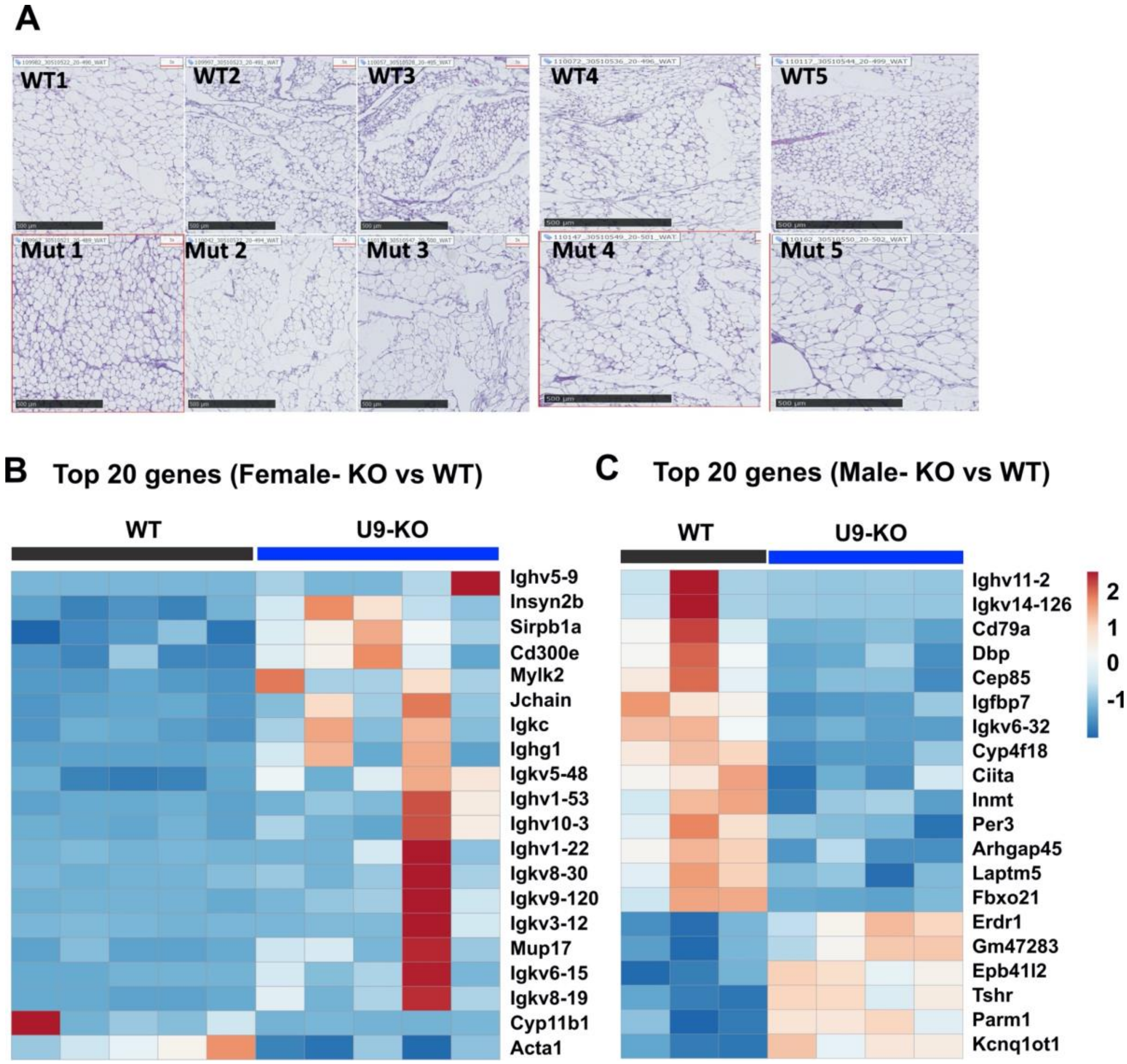

Fig. 3-4 Histological and transcriptional analysis shows no major obesity-related phenotypes in WAT from WT and U9-KO mice. (A) Histopathological analysis was performed using H&E as standard staining on inguinal white adipose tissue collected from female WT (n=5) and U9-KO (n=5) mice. Here, individual U9-KO mice are labeled as Mut 1, Mut 2, etc. **(B, C)** RNA-seq analysis was performed on RNA isolated from whole perigonadal adipose tissue collected from WT and U9-KO mice. DEseq2 analysis was done to identify the differentially expressed genes in U9-KO vs WT tissues. Heatmaps show the top 20 differentially expressed genes based on p value in female **(B)** and male **(C)** tissues.

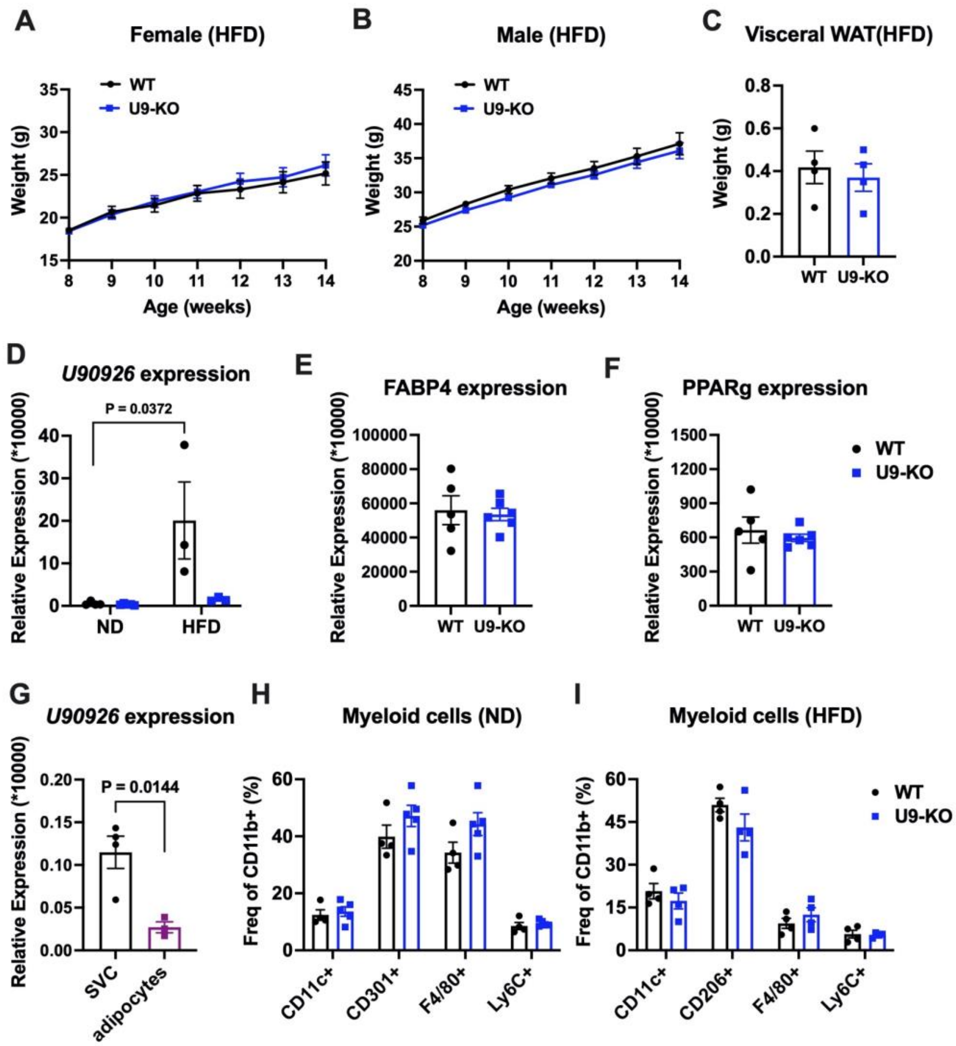

Fig. 3-5 *U90926* deletion does not alter WAT phenotypes during diet-induced obesity. (A, B) WT and U9-KO mice were exposed to high-fat diet (HFD) containing 60% fat at 8 weeks of their age and body weight measured every week for 6 weeks. Weight gain of female (WT=10, U9-KO=11) and male (WT=7, U9-KO=12) mice upon HFD are shown here in panel A and B, respectively. **(C)** Visceral/perigonadal fat depots were collected from female HFD-fed WT and U9-KO mice (n=4 in each group) for comparison in WAT weight. **(D)** *U90926* expression in perigonadal WAT was measured by RT-qPCR in tissues from normal diet (ND)- and HFD-fed WT and U9-KO mice. **(E, F)** Adipogenesis markers, such as PPARγ and FABP4 were measured by RT-qPCR in perigonadal WAT collected from HFD-fed WT and U9-KO mice. **(G)** WAT from HFD-fed WT mice were collected and stromal vascular cell (SVC) content and mature adipocytes were separated, followed by RT-qPCR to measure *U90926* expression in each cellular fraction. **(H, I)** SVCs were collected from ND-fed (panel H) and HFD-fed (panel I) WT and U9-KO WAT to measure different myeloid cell population by flow cytometry. Here, M1 macrophages (CD11c+), M2 macrophages (CD206+ or CD301+), adipose tissue resident macrophages (F4/80+) and monocytes (Ly6C+) expressed as percentage of the parent CD11b+ population are shown. All RT-qPCR data are expressed relative to the housekeeping gene Beta-2-microglobulin (B2m) and calculated by a comparative Ct method formula 2^-(deltaCt) and multiplied by a factor of 10,000 for ease of visualization. Data are represented as the mean ± SEM. Significance of differences between the indicated groups was determined by student t test and two-way ANOVA.

Table 3-1 Plasma biomarkers in WT and U9-KO mice measured by MSD at 13 weeks. Medians, first and third quartile and p-values calculated by a Wilcoxon rank-sum test.

	Female		**Male**		**Female**	**Male**	**Overall**
	WT N=11	U9-KO N=14	WT N=14	U9-KO N=10			
	median [25%, 75%]	median [25%, 75%]	median [25%, 75%]	median [25%, 75%]	P value	P value	P value
Leptin conc.	1339.92 [555.251, 2814.57]	1236.405 [949.114, 1813.168]	3580.02 [2574.76, 6398.07]	2177.775 [1388.622, 3486.51]	0.861	0.042	0.072
Insulin conc.	3360.145 [2621.298, 4282.233]	3484.87 [2315.84, 4558.015]	4142.68 [2538.037, 5016.63]	3834.645 [3063.49, 4312.927]	0.972	0.88	0.899
FGF21 conc.	572.787 [469.564, 1076.64]	643.901 [443.37, 858.939]	764.762 [526.418, 913.699]	608.813 [528.909, 688.819]	0.794	0.227	0.613

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CHAPTER 4: DISCUSSION

4.1. Background and Overview

For the last few decades, molecular biology has made revolutionary advancements that have allowed us to fully sequence and assemble complete genomes of humans and thousands of other organisms, revealing a wealth of information. One of the more surprising findings was that the total number of classic protein-coding genes did not increase with the increasing complexity of the organisms, at least for most multicellular organisms(1, 2). What did increase, however, was the amount of non-coding DNA(3). Recent studies, using the latest advances in sequencing technology and computation, have demonstrated that this DNA, while previously considered “junk,” is, in fact, comprised of essential genetic elements, which may be functionally and evolutionarily just as crucial as the protein-coding genes themselves(4). Among these genetic elements are numerous genes that encode so-called long non-coding RNAs (lncRNAs), defined by convention as RNAs larger than 200 bp and lacking a well-conserved open reading frame (ORF)(5). Current high throughput sequencing data has identified thousands of lncRNAs and their association with numerous pathophysiological processes and diseases(6). Over the last several years, a flurry of studies has begun to elucidate some of the functions of lncRNAs, but the majority of these genes still remain uncharacterized.

Here, in this dissertation, we characterized a mouse putative lncRNA *U90926*. This gene lies between two conserved protein-coding genes, *Uso1* and *Ppef2*, on mouse chromosome 5. A 540 bp transcript from this gene was initially annotated from a cDNA library in 1997 by an unpublished submission from investigators at the Memorial Sloan Kettering Cancer Center (Gure, A.O. and Old, L.J.), and named "TNF-resistance related gene" (GenBank: *U90926*.1). Subsequent genome-wide annotation of transcripts from the mouse genome resulted in an updated 522 bp transcript (NR_033483.1), which is currently annotated as a long intergenic non-coding RNA in the NCBI and Ensembl databases. We became interested in *U90926* when we identified it as one of the most highly downregulated genes in toll-like receptors (TLR)-stimulated p38α MAPK-deficient macrophages in our transcriptomic analyses(7). Given this discovery, we focused on identifying possible roles for *U90926* in macrophages. We generated the first mouse model of *U90926* deficiency (U9-KO), which lacks the whole *U90926* genomic locus. At the time when we started this project, there was only one study published on *U90926* characterization. In that study, using an in vitro model of adipocyte differentiation in 3T3-L1 preadipocyte cells, Chen et al. discovered that *U90926* expression was downregulated during adipocyte differentiation, and overexpression of *U90926* inhibited adipocyte differentiation, while silencing had the opposite effect, thus implicating *U90926* as a possible repressor of adipogenesis(8).

Given our discovery of *U90926* in TLR-stimulated p38α MAPK-deficient macrophages(7, 9) and the study on adipogenesis by Chen et al(8)., we continued to investigate the role of *U90926* using our global U9-KO mice in both inflammation and adipocyte biology, which are described here in this dissertation. Along the line, we have made an exciting discovery on the protein-coding potential of lncRNA *U90926*, which opens up a novel avenue for characterizing this gene.

4.2. *U90926* role in macrophages

Gene expression in mammals is a highly complex and tightly regulated yet dynamic process. This is particularly evident in innate immune cells, which can completely reprogram their entire gene expression repertoire within minutes of encountering an inflammatory stimulus. Macrophages are the prototypical innate immune cell strategically positioned to encounter infectious organisms, detect them via an array of microbial pattern recognition receptors (PRRs), and rapidly respond by producing pro-inflammatory mediators that alert the immune system. Many lncRNAs have been found to regulate TLR-mediated macrophage function, such as lincRNA-COX2, lincRNA-EPS, and Mirt2(10–12). These lncRNAs control gene expression and cellular functions in diverse ways, including interaction with proteins involved in gene expression regulation or signaling, interactions with chromatin, interactions with mRNAs or miRNAs, and others(13).

Our previous transcriptomic studies have focused on a central signaling pathway activated in macrophages downstream of PRRs, the p38 MAP kinase (MAPK) pathway(7, 9), where we have found that *U90926*, a putative lncRNA of unknown function is downregulated in p38α-KO macrophages(7). Since then, including Chen et al(8). research described above, a few independent groups have described possible functions for this gene, interestingly in different models. An in vitro study performed in a murine retinal photoreceptor cell line 661W showed that *U90926* expression is positively correlated with herpes simplex virus 1 (HSV-1) infection (14). Interestingly, the same group identified two human lncRNA transcripts positionally conserved with *U90926*, with the expression of one of these transcripts being elevated in the eye of individuals with acute retinal necrosis caused by HSV-16(15). Finally, a recent study demonstrated that *U90926* expression was elevated in microglia and it regulates neutrophil recruitment during ischemic stroke(16). These data suggest that *U90926* expressed in different cell types serves distinct roles.

In this dissertation, we demonstrated that *U90926* expression is minimal across multiple tissues at steady state, however, it is highly inducible in bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs) upon stimulation with agonists of all the TLRs except TLR3. TLR3 relays signal in a MyD88 (adaptor protein for most TLRs)-independent way(17), which suggests that *U90926* expression is MyD88-dependent. Our *U90926* expression data in MyD88-KO macrophages (Fig. 2-1e) and lack of *U90926* induction in TNFα-stimulated macrophages (Fig. 2-1i) validated that. Our expression profiling data of *U90926* in response to different TLRs activation agrees with Shirahama et al. who showed that HSV-1 infection, which also triggers TLR9, induced expression of this gene in murine retinal cells(14). This also suggests that expression of *U90926* may not be restricted to myeloid cells and may be present in other cell types that can respond to inflammatory signals such as TLRs. The findings of Xu and colleagues(16) that ischemic stroke induced expression of *U90926* in microglia suggest that damage-associated molecular patterns (DAMPs) released in damaged CNS tissue, which can induce signaling through several TLRs, could be driving this activation. Other than TLRs agonists, inflammatory cytokines could potentially induce expression of *U90926*, particularly since some cytokine receptors such as TNFα and IL-1β signaling through p38 MAPK and MyD88/p38 MAPK. Microarray evidence that TNFα can induce *U90926* expression in 3T3-L1 preadipocytes(18). Although we did not detect any *U90926* expression upon TNFα stimulation in BMDMs (Fig. 2-1i), however, we did find moderate *U90926* expression in BMDMs upon IL-1β stimulation, which is consistent with the MyD88-dependence of the IL-1R family(19). Chen et al. found that 3T3-L1 preadipocytes express *U90926*(8) in the absence of inflammatory cues suggesting that there may be additional signals that control its expression. Altogether, these data suggest the upstream signaling of *U90926* expression could be different depending on the cell types.

Here, we have investigated the effect of *U90926* in regulating major macrophage effector functions such as cytokine secretion, co-stimulatory molecules expression, and transcriptional change upon LPS stimulation. Surprisingly, we found that *U90926* deficiency had no significant intrinsic effect on macrophage function and inflammatory gene regulation in vitro (Fig. 2-3). This suggests that *U90926* plays a role other than transcriptional regulation, unlike many nuclear lncRNA in macrophages(10, 11). Many recent studies have shown that subcellular localization is critical in determining lncRNA function in the cell(20–22). Consistently, we found in our study that *U90926* is localized predominantly in the cytoplasm rather than the nucleus of BMDM (Fig. 2-4a), which validates the lack of a major intrinsic effect of *U90926* deficiency on macrophage transcriptional regulation. Our findings on *U90926* localization in BMDM are consistent with other groups, who showed that it is localized to the cytoplasm of 3T3-L1 preadipocytes(8) and primary microglia(16). However, another study found that *U90926* RNA is localized primarily in the nucleus in the HSV-1-infected 661W murine retinal photoreceptor cell line(14). *U90926* localization may differ across cell types or cell states since it has been known that lncRNA localization can differ in different cell types depending on splicing and functional predisposition(22, 23). Given the different biological role of *U90926* has been found in different cell types and models, it is not surprising that *U90926* localization differs among cell types.

Taken together, we have found that *U90926* is highly expressed in macrophages upon TLR stimulation in a Myd88-P38 MAPK-dependent manner. Despite its strongly increased expression in macrophages upon LPS stimulation, *U90926* has a minimal intrinsic effect on macrophage transcriptional regulation and other functions. However, we have not investigated the role of *U90926* in regulating phagocytosis which is another important effector function of macrophages and previously shown to be regulated by other lncRNAs(24). Nonetheless, the cytoplasmic location of *U90926* RNA and lack of total gene expression regulation in macrophages, suggest an alternative mode of action of lncRNA *U90926* other than the common nuclear lncRNA-mediated transcriptional regulation.

4.3. Protein-coding potential of *U90926*

Classical annotation of protein-coding genes and non-coding genes depends on some arbitrary criteria such as open reading frame (ORF) size, an absolute requirement of AUG as start codon, and lack of ORF conservation(25–27). Several recent studies have shown that many genes, currently annotated as lncRNAs, are pervasively translated to small proteins(28, 29). Two recent studies strengthen the concept of functional translation of non-canonical lncRNA ORFs in macrophages. A lncRNA called *AW112010* was recently identified to encode a peptide from a non-AUG start site, which is essential in controlling mucosal immunity during both bacterial infection and colitis(30). Another recent study showed that a classically annotated lncRNA *1810058I24Rik* encodes a mitochondrial micropeptide named Mm47, which regulated Nlrp3 inflammasome function in macrophages(31). Given the emerging evidence of lncRNA being translated and the cytoplasmic location of *U90926*, we were prompted to identify the protein-coding potential of *U90926*.

In general, when a lncRNA is cytoplasmic and contains predictive ORF, it is a common practice in the field to examine the protein-coding potential of that lncRNA. Nuclear lncRNAs are thought to be bona fide noncoding, however, a recent study has shown that a nucleolar lncRNA PAPAS can encode a protein named RIEP (Ribosomal IGS Encoded Protein) which plays a role in preventing stress-induced DNA damage(32). Therefore, regardless of subcellular localization, lncRNA ORFs should be tested for their protein-coding potential in order to meet their full characterization. Despite having an ORF with a canonical AUG start site that can potentially encode a predicted 87- amino acid long protein, no prior study has addressed the question if the ORF in *U90926* (U9-ORF) is functional. Notably, U9-ORF was also annotated as a "putative TNF-resistance related protein" (GenBank: AAB81542.1) from the original cDNA library transcript (GenBank: *U90926.1*). However, we note that this older transcript contains what appears to be a sequencing error in exon 5 that results in a longer predicted protein (data not shown). In this study, we found that not only did *U90926* RNA localize to the cytoplasm, but it is associated with ribosomes. We further showed that epitope-tagged U9-ORF is translated into a stable protein that traffics to the Golgi and is secreted from the cell (Fig. 2-4d-h), as predicted from the presence of a consensus ER signal peptide sequence at its N-terminus

(Fig. 2-4b). We have also shown that the U9-ORF protein is glycosylated, which explains the discrepancy in molecular weight (Fig. 2-4i). We also confirmed the peptide's correct identity using mass spectrophotometry (Supplemental fig. 2-S3). Recent data in our lab found the endogenous presence of U9-ORF protein in LPS-stimulated BMDM (data not shown) by western blot using U9-ORF specific antibody made in collaboration with Green Mountain Antibodies, Inc (Burlington, VT). Future studies must validate endogenous U9-ORF by mass spec and localization of endogenous U9-ORF in vivo by immunohistochemistry.

We demonstrate that lncRNA *U90926* encodes a small novel protein from a 264 bp long ORF, defying the classic annotation of protein-coding ORF size of more than 300 bp(26, 33). Another important aspect that is commonly perceived is that protein-coding genes are conserved across different species(25, 34) and most lncRNA genes show low sequence conservation across species(35, 36). An emerging view of lncRNA conservation includes not only sequence but structure, function, and synteny(37). While we have so far been unable to find a human *U90926* homolog using sequence homology, Shirahama et al. identified a putative human homolog of *U90926*, *AC110615.1,* using synteny, as a potential biomarker of acute retinal necrosis(15). Like *U90926*, another lncRNA in macrophages called *GAPLINC* has been identified as positionally conserved in human and mouse, which is found to play a role in controlling basally control the expression of inflammatory-response genes(38). Therefore, this potential human homolog of *U90926* in human macrophages must be characterized in future studies. Furthermore, the lack of apparent conservation of the U9-ORF amino acid sequence in other species suggests that *U90926* could represent an example of a dual-function lncRNA that evolved to lack its protein-coding potential in other species. In future studies, dissecting the function of both *U90926* as lncRNA and as a protein will be important. Plasmid constructs with mutated U9-ORF (by a point mutation which disrupts the protein) keeping the RNA expression intact can be transduced to characterize the prtoein in different types of cells such as macrophage, 3T3-L1 etc. in vitro.

4.4. *U90926* role in endotoxemia model

Innate immune cell such as macrophage activation is the critical factor in sepsis induction, and it occurs as different PRRs, for example, TLR4 (39, 40) in case of LPS endotoxemia, recognize pathogen-associated molecular patterns (PAMPs; e.g., LPS). This receptor activation causes systemic production of pro-inflammatory cytokines and chemokines leading to excessive activation of immune cells and endothelial cells. This “cytokine storm” eventually leads to organ dysfunction and even death(41). In our study, the finding that U9-ORF is secreted potentially explains the lack of effect on macrophage-intrinsic function in vitro (Fig. 2-3), indicating potential paracrine cellular targets for this protein in vivo. Therefore, we further characterized *U90926* role in an in vivo LPS endotoxemia model because macrophages play a vital role in septic shock.

In the LPS endotoxemia model, we found rapid induction of *U90926* RNA expression in immune cells, as well as in whole liver and adipose tissue. Moreover, we found that U9-KO mice exhibit significantly increased susceptibility and mortality compared to WT controls. We also demonstrated that U9-KO mice have sustained level of IL-6 at 24 hours and neutralization of lL-6 can rescue the phenotype of U9-KO mice in the

LPS endotoxemia model (Fig. 2-5), albeit WT mice were similarly protected if challenged with a higher dose of LPS (Supplemental fig. 2-S4a-c). These data suggest that deletion of *U90926* lowers the threshold for endotoxic shock, which results in elevated systemic IL-6 levels, which drive morbidity and mortality. These effects could be possibly mediated via secreted U9-ORF from macrophages acting in a paracrine and/or endocrine manner although our in vitro experiments do not support a direct regulation of hepatocyte IL-6 production by macrophage derived U9-ORF (supplemental fig. 2-S4e). Another possibility is that U9-ORF regulates IL-6 stability in vivo, which is an important way in which this cytokine is regulated(42, 43).

Considering critical involvement of the liver resident macrophages and hepatocytes during endotoxic shock(44–47), we performed transcriptomic analysis on the whole liver tissue. We found that many important immune genes and biological pathways that are important for sepsis, are differentially regulated in U9-KO mice compared to WT. This included an upregulation of neutrophil-specific genes *Ngp* and *S100a9*, and the chemokine *Cxcl2*, a neutrophil chemoattractant, suggesting enhanced neutrophil influx into the liver as a contributor to (or a result of) enhanced liver pathology in LPS-treated U9-KO mice, which is further consistent with the role of IL-6 in promoting neutrophil trafficking in various inflammatory settings(48–50). Interestingly, silencing of *U90926* in microglia in previous studies resulted in the downregulation of *Cxcl2* expression and decreased neutrophil recruitment to the CNS(16), which is the inverse of our results, but nonetheless implicates myeloid cell mediated neutrophil recruitment as a possible common mechanism. Of note, the putative mechanism of action of *U90926* described in microglia involved its

RNA form(16), and not the novel U9-ORF protein discovered in our study. Future studies will need to determine whether *U90926* function in regulating inflammatory processes *in vivo* require its RNA form, its protein form, or both, as has been reported for other lncRNAs(51).

Notably, we found a significant enrichment for IL-6-regulated genes including *STAT3*, as upstream regulators in the genes differentially expressed in liver tissue of U9-KO mice, suggesting that elevated systemic IL-6 may be upstream of the dysregulated gene expression in the liver. Interestingly, a recent ChIP-seq study of chromatin from isolated intestinal epithelial cells revealed that *U90926* promoter has binding sites for transcription factors, signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa beta (NF-κβ)(52). Given that STAT3 gets activated and induces transcription of genes downstream of IL-6(43) and STAT3 has a binding site for *U90926* promoter, it may be speculated that *U90926* may work in a negative feedback loop to inhibit IL-6 signaling, thus supports our data of sustained IL-6 in U9-KO mice (Fig. 2-5). Anti-inflammatory role of *U90926* is further supported by our data where we found anti-inflammatory cytokine, IL-10 is reduced in U9-KO BMDCs upon LPS stimulation (data not shown). Moreover, *U90926* may be induced by a pro-inflammatory signal, but works as an anti-inflammatory molecule to maintain the immune homeostasis like many other lncRNAs(53). Thus, in future studies, *U90926* role in anti-inflammatory mechanisms needs to be explored.

Altogether, our data has shown that deletion of *U90926* lowers the threshold for endotoxic shock, which results in elevated systemic IL-6 levels, which causes disease mortality and morbidity. Target cell or tissue of U9-ORF or the source of the heightened

IL-6 levels in our sepsis model are big questions remaining. In future, identifying the cell and tissue specificity of both the RNA and protein will be important to identify the *U90926* target. RNA-fluorescence in situ hybridization (FISH) in tissue sections and RT-qPCR on different population of cells sorted by FACS should be done in LPS-endotoxemia model, to identify the tissue and cellular expression of *U90926*. We already have the antibodies against endogenous U9-ORF, therefore, immunohistochemistry should be done to identify the protein. Our data in *ex vivo* BMDMs did not show an effect of *U90926* deficiency in IL-6 secretion. Furthermore, our co-culture data did not show a *U90926* effect on IL-6 production by hepatocyte cell line, AML12 (Supplemental fig. 2-S4), although hepatocytes are known to be an important contributor of IL-6 production during hepatic stimuli both in vitro and in vivo(54–56). That is why identifying the presence of both *U90926* RNA and protein in certain tissue and cells will help us to narrow down the target and find the possible source of IL-6.

For mechanistic aspects, in terms of physical interaction of *U90926* RNA or protein with other molecules could be analyzed by RNA pull down assays or co-immunoprecipitation, which will be informative to identify the specific pathway by which *U90926* is regulating IL-6 or other potential molecules. Furthermore, the phenotype of *U90926*-driven sepsis outcome that we have demonstrated here in this dissertation, is that effect of whole *U90926* gene deletion. In future, studies need to determine if the phenotype is U9-ORF-specific or RNA-specific of both. Synthetic U9-ORF can be administered to U9-KO mice along with LPS to determine if U9-ORF is sufficient to reverse the phenotype.

4.5. *U90926* effect on adipocyte biology

Obesity has become a significant public health concern worldwide, including in developing countries, as it is a major risk factor for many chronic conditions like type 2 diabetes, non-alcoholic fatty liver disease, cardiovascular disease, and different types of cancer (WHO, 2023). Obesity is defined by the excessive accumulation of white adipose tissue (WAT), which is clinically designated as a body mass index (BMI = kg/m2) exceeding 30 (WHO, 2023). In this dissertation, we have also examined the role of *U90926* in adipocyte biology in parallel to our work on immune function of *U90926*.

Obesity to WAT expansion is generally caused by two phenomena, hypertrophy (accumulation of lipids in the existing adipocytes) and hyperplasia (increase in adipocyte number by adipogenesis)(57, 58). Many studies have shown that lncRNAs are emerging as essential regulators of adipogenesis(59–61). In 2017, first functional characterization study on lncRNA *U90926* was published, and they showed that *U90926* repressed adipogenesis in 3T3-L1 preadipocytes(8). Based on that, in this study, we have investigated the role *U90926* in weight gain and other obesity-related phenotypes in vivo using our knockout mouse model, U9-KO. In agreement with Chen et al.(8), we found that *U90926* is expressed at detectable levels in visceral WAT and is highly expressed in 3T3-L1 preadipocytes, with its expression decreasing as 3T3-L1 cells become mature adipocytes (Fig. 3-1A). However, we have found that the decrease in *U90926* expression in 3T3-L1 cells is not dependent on the presence of adipocyte differentiation stimuli; instead, it may be the effect of prolonged cell culture, as undifferentiated 3T3-L1 cells showed the same expression pattern. This could be due to contact inhibition or other intrinsic factors that change gene expression patterns when cells are in culture for a long time(62). This makes the model skeptic to characterize this gene. However, Chen et al. showed compelling data on siRNA-mediated silencing of *U90926*, which causes increased expression of adipogenesis markers such as PPARγ, CEBPα, FABP4, and AdipoQ and increased lipid accumulation during 3T3-L1 differentiation, while overexpression of *U90926* had the opposite effect(8). Based on this, we expected that the U9-KO mouse model would show increased adipogenesis as well as obesity phenotypes in vivo. On the contrary, we have not found any major effect of *U90926* deficiency in vivo in terms of weight gain and other obesity-related phenotypes such as fat accumulation, metabolic changes like plasma level of glucose, TG, NEFA, and insulin, WAT histology and adipogenesis molecular markers (Fig. 3-2,3,4). Leptin level was slightly decreased in male U9-KO mice (Table 3-1); however, their food intake (according to German Mouse Clinic) and other energy metabolism data did not correlate with this, indicating unaltered leptin signaling(63).

In general, the 3T3-L1 differentiation system is widely used for studying adipogenesis because of its uniform morphological and biochemical nature comparable with adipocytes in vivo(64). However, the drawback of this model is that molecular events of adipogenesis in vitro may not fully reflect adipogenesis in vivo, where different other cell types are also involved(65). Our data agree with this, and it suggests that in vitro adipogenesis phenotypes may not be totally reflective of adipogenesis phenomena in vivo. Nonetheless, the study of preadipocyte differentiation *in vivo* is even more difficult. Since human and animal adipose tissue is heterogeneous, it is hard to distinguish between preadipocytes and fibroblasts(243). Additionally, the inability to synchronize the preadipocytes at similar developmental stages is a confounding challenge to detailed *in vivo* studies(244). In future, mesenchymal stem cells could be isolated from WT and U9-KO mice and in vitro adipogenesis could be performed in primary preadipocytes and examine the role of *U90926* in adipogenesis. Another possibility of our negative result is that, in vivo the role of *U90926* in adipogenesis may be redundant and its absence in U9-KO mice may compensated for by other genes.

Given that we did not observe any effect of *U90926* on adipose tissue homeostasis in mice fed a standard chow/normal diet (ND), we have used a robust model of obesity by introducing a high-fat diet (HFD; 60%Kcal fat). We found that compared with ND, HFD increased *U90926* expression in whole WAT (Fig. 3-5), which contradicts Chen et al findings(8). Regardless, and as with ND, we have found no effect of *U90926* deletion in HFD-fed mice on weight gain, fat accumulation, adipogenesis markers in the WAT, and immune cell infiltration in the WAT (Fig. 3-5).

The role of adipogenesis in obesity is debatable in the field, as some researchers think that hypertrophy is the main cause of obesity and find no evidence of increased adipocyte cell number during obesity, because adipocyte number is determined developmentally(66, 67). However, other groups of researchers showed that adipogenesis increases during obesity in both genetic- or diet-related onset(68–70). Some studies showed that increased number of smaller adipocytes that generated by adipogenesis during obesity may be even beneficial for obesity-related metabolic outcomes(71, 72). Thus, adipogenesis in vitro may be a not good indicator of obesity phenotypes in vivo.

Obesity is a slow chronic inflammation state where numerous immune cells, especially macrophages take part, besides preadipocytes and adipocytes(73). Adipose tissue macrophages (ATMs) play a vital role in regulating adipogenesis and metabolic changes during obesity(74, 75). Our previous data shows that *U90926* is upregulated in activated macrophages and in whole WAT following systemic LPS administration in vivo(76). We showed that *U90926* is significantly increased in stromal vascular cells (SVCs) compared to mature adipocytes in WAT of an obese mice (Fig. 3-5G), which is probably accounted for activated macrophages in chronic inflammation during obesity. Thus, there could be a macrophage-driven and/or sepsis-related *U90926* function in regulating adipose tissue homeostasis, which needs to be explored in the future studies. LPS endotoxemia model could be combined to HFD-obese mice to identify if there is a combinatorial effect of *U90926*, in future. The model we have used in this study is a global knockout model. In future, a conditional knockout model specific to macrophage or adipocyte lineage could be used further to determine the role of *U90926* in obesity in vivo, with temporally-inducible models to account for any developmental compensatory effects on loss of *U90926* expression. In conclusion, our present results demonstrate that *U90926* does not affect obesity-related phenotypes in mice under a normal or high-fat diet condition, suggesting that unlike its role in myeloid cell-mediated inflammation, this gene may not have an essential role in adipose tissue homeostasis.

4.6. Concluding Remarks

In this dissertation, we have identified an important gene *U90926* that is highly upregulated in macrophages upon TLR-activation in Myd88- and p38a MAPK-dependent manner. We have also shown that *U90926* is protective during endotoxic shock, possibly be regulating systemic IL-6 levels. Furthermore, we have discovered unexpected evidence for the translation of this putative lncRNA gene. Our discovery on protein-coding potential of *U90926* had shed light on the evolutionary aspect of lncRNAs and provoked concerns about the basis of current classification of protein-coding genes versus non-coding genes. In addition, these findings suggest manipulation of this gene and its products as potential model for experimental therapeutic approaches in sepsis, which remains a major clinical need. On the other hand, we have found that unlike its role in myeloid cell-mediated inflammation, this gene may not have an essential role in adipocyte biology. Future studies should focus on teasing apart the biphasic (both as RNA and as protein) mode of action of *U90926*, underlying mechanisms targeting sepsis outcome and source of IL-6 and investigating the crosstalk between macrophages and adipocytes in regulating adipose tissue homeostasis. Future study also should characterize the positionally conserved human *U90926* in order to determine if it has similar role in inflammation as mouse *U90926*. Overall, this study provides important implications for the evolution of lncRNAs, their protein coding potential, and biological function of this gene.

4.7. References

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