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Long Non-Coding RNAs Regulating Macrophage Functions in Homeostasis and Disease

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Abstract

Non-coding RNAs, once considered “genomic junk”, are now known to play central roles in the dynamic control of transcriptional and post-transcriptional gene expression. Long non-coding RNAs (lncRNAs) are an expansive class of transcripts broadly described as greater than 200 nucleotides in length. While most lncRNAs are species-specific, their lack of conservation does not imbue a lack of function. LncRNAs have been found to regulate numerous diverse biological functions, including those central to macrophage differentiation and activation. Through their ability to form RNA-DNA, RNA-protein and RNA-RNA interactions, lncRNAs have been implicated in the regulation of myeloid lineage determination, and innate and adaptive immune functions, among others. In this review, we discuss recent advances, current challenges and future opportunities in understanding the roles of lncRNAs in macrophage functions in homeostasis and disease.

Introduction

Cells of the myeloid lineage must adopt numerous roles in their quest to maintain homeostasis and defend against invading organisms. Macrophages in tissues, whether seeded during embryonic development (tissue-derived macrophages) or derived from recruited monocytes (monocyte-derived macrophages), respond to environmental cues to adopt a spectrum of activation states. These phagocytic cells play key roles in development, innate and adaptive immunity, as well as tissue homeostasis and repair, and in doing so, execute a myriad of key functions including efferocytosis of dying cells, pattern recognition of microbial or altered-self ligands, antigen processing and presentation, release of pro- and anti-inflammatory mediators, wound repair, and homeostasis of adipose tissue. Macrophages are, thus, remarkably plastic and their numerous functions are dynamically coordinated by transcriptional and post-transcriptional gene regulation¹. While research in the last several decades has helped to define key pathways and their means of regulation by proteins,

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advances in genomics have recently introduced a new variable to the regulation of gene expression and function: non-coding RNAs (ncRNAs).

Advances in the post-genomic era, particularly in next generation sequencing, have led to the appreciation that the majority of elements in the genome generate ncRNA. Greater than 70% of the human genome is now known to be transcribed into RNA, yet only ~2% codes for proteins^{2,3}. Although previously overlooked as junk DNA, the annotation and characterization of the ncRNA transcriptome have revealed numerous subclasses of highly active RNAs, including rRNAs and tRNAs required for protein synthesis, small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) required for mRNA splicing and nuclear organization, microRNAs (miRNAs) that repress mRNA translation, and a heterogeneous group of long non-coding RNAs (lncRNAs) that are over 200 nucleotides in length. This last group of ncRNA transcripts has proved to be enigmatic: although it is estimated that there are tens of thousands of lncRNAs coded for in the genome, and they share many of the same characteristics as mRNAs (RNA polymerase II-dependent, 5' methyl capped, multiexonic, and polyadenylated), the functions of only a few hundred have been uncovered to date. However, even from the small fraction of lncRNAs that have been characterized, it is clear that these ncRNAs play central roles in the organization and regulation of a diverse set of cellular processes, including genomic imprinting, X-chromosome inactivation, and 3-dimensional nuclear organization, among many others.

lncRNAs are able to bind to proteins, DNA, RNA, or a combination thereof, to exert their diverse actions. These actions, and their genomic or subcellular localization, can be used to classify lncRNAs as: intergenic lncRNAs, intronic lncRNAs, enhancer RNAs, antisense lncRNAs, pseudogenes, circular RNAs, competing endogenous RNAs, decoys, guides, or scaffolds. Most frequently, lncRNAs are characterized by their functions in interacting with proteins, other RNAs, and/or DNA. For example, 'decoy' lncRNAs are those which bind to proteins or other RNAs and, as a result of that interaction, block those proteins or RNAs from binding to their usual targets. Conversely, lncRNAs that interact with DNA as well as proteins that target DNA, such as chromatin modifiers, and thereby sustain that protein-DNA interaction are described as 'guides' or 'scaffolds' – in the case that lncRNAs that tether more than one protein to genomic loci. As will be discussed below, many lncRNAs have been shown to be transcriptionally induced or inhibited by inflammatory mediators such as cytokines, pathogen associated molecular patterns (PAMPs), and damage associated molecular patterns (DAMPs). Interestingly, certain classes of ncRNAs, such as antisense lncRNAs or intronic lncRNAs, can share regulatory elements with nearby genes, while intergenic lncRNAs (also called lincRNAs) often have their own unique promoters. Comparative analysis of the promoters of human lncRNA and protein-coding genes have begun to identify differences in genetic and epigenetic features that may help to explain the observed differential and highly tissue- or condition-specific transcriptional regulation of certain lncRNA genes compared to their protein-coding counterparts⁴, yet this remains an active area of investigation.

Accumulating evidence suggests that a single lncRNA may have more than one function, varying by cell type, stimuli, and/or cellular localization. While most lncRNAs are not conserved between species, it is important to note that the number of noncoding genomic

elements increases with organism complexity, suggesting a potential role for such RNAs in adaptation and evolution^{5,6}. Interestingly, the majority of human disease-associated sequence variants identified through genome-wide association studies are found in the non-coding landscape of the genome, suggesting important functions in human health. It has thus become clear that this lack of conservation does not imply a lack of function⁷. However, the low sequence conservation of most lncRNAs can limit the scope of *in vivo* studies possible for primate-specific lncRNAs, thereby hindering investigation of these most interesting lncRNAs. Nevertheless, the tools for studying lncRNAs and their -RNA, -DNA and -protein interactomes and higher-order structures are rapidly evolving, and these technological advances are likely to continue to drive important discoveries in lncRNA function and biology in general.

Recent studies have revealed several lncRNAs that play important roles in macrophage function by regulating inflammation and the initiation of the immune response. Insight into the mechanism of these macrophage-modulating lncRNAs not only provides insight into the inner workings of tightly controlled immunological processes, but also perhaps a means of fine tuning such systems. Here we review recent discoveries of lncRNAs that regulate macrophage function, their implications, and future challenges.

Immune-modulating long non-coding RNAs in macrophages

The actions of most immune-related lncRNAs are mediated through binding to proteins including signaling molecules, transcription factors, heterogeneous nuclear ribonucleoproteins, and components of chromatin-modifying complexes⁸. A number of lncRNAs have been identified to be upregulated in response to stimulation of innate immune signaling pathways by microbial or endogenous components that activate Toll-like receptors (TLR)⁹ and cytokine receptor signaling. These signaling pathways converge on NF- κ B, a master transcriptional regulator of immune response genes, and as described below, lncRNAs are emerging as potent regulators of the NF- κ B protein complex.

Lethe

Rapicavoli *et al.* performed an RNA sequencing (RNA-Seq) screen to identify lncRNAs regulated by tumor necrosis factor α (TNF α) in mouse embryonic fibroblasts, which revealed the changes in expression of numerous lncRNAs and pseudogene lncRNAs. Among the pseudogene lncRNAs identified, Lethe was found to be expressed upon stimulation with pro-inflammatory cytokines such as TNF α and interleukin (IL)-1 β , as well as the anti-inflammatory agent dexamethasone⁹. It was recently shown that Lethe controls the binding of the p65-NF- κ B complex to the NOX2 promoter in bone marrow derived macrophages (BMDMs). Overexpression of Lethe in primary macrophages reduced *Nox2* expression and subsequently the production of reactive oxygen species¹⁰. Interestingly, Lethe expression is downregulated in diabetic wounds, where healing is impaired¹⁰. As the balance of reactive oxygen species production is essential for effective wound healing¹¹, this suggests a potential role for Lethe in this metabolic disease.

LincRNA-Cox2

Whole-transcriptome profiling of murine BMDMs stimulated with the TLR2 ligand Pam3Csk4 revealed a significant upregulation of lincRNA-Cox2¹². LincRNA-Cox2 is an intergenic lncRNA (sometimes referred to as lncRNA-cox2), encoded on murine chromosome 1, and like many lncRNAs, is named for its location proximal to its neighboring protein coding gene (*Cox2*). LincRNA-Cox2 has three splice variants and is highly induced in mouse macrophages (RAW264.7), microglia (BV2), and primary bone marrow-derived dendritic cells after stimulation of TLR4 with lipopolysaccharide (LPS), but not in response to the TLR3 agonist poly(I:C) or the non-canonical NF- κ B pathway activator lymphotoxin B^{13,14}. Promoter studies using 3000 bp upstream of the transcription start site in a luciferase vector assay in combination with an NF- κ B inhibitors indicated regulation of lincRNA-Cox2 by NF- κ B. This was confirmed by ChIP analysis, which detected significant enrichment of NF- κ B in the promoter region of lincRNA-Cox2 upon LPS stimulation¹⁴. Cell fractionation followed by qPCR as well as RNA fluorescent *in situ* hybridization showed that lincRNA-Cox2 localizes to both the nucleus and the cytoplasm. Interestingly, lincRNA-Cox2 can repress as well as activate distinct subsets of genes related to the immune response, such as chemokines, chemokine receptors, and interferon (IFN)-stimulated genes. The repression of key immunoregulatory genes is dependent on its forming a complex with the RNA-binding proteins heterogeneous nuclear ribonucleoprotein A/B and A2/B1¹². In addition to this initial characterization, lincRNA-Cox2 has been shown to be required for the assembly of NF- κ B subunits, RelA and p50, into the SWI/SNF complex, which is in turn recruited to late-primary genes in macrophages in response to LPS stimulation¹⁴. More recently, yet another role for this lncRNA has been described in macrophage polarization¹⁵. Silencing of lincRNA-Cox2 corresponds with a decrease in production of M1 associated cytokines (including TNF α and IL-12), thereby reducing the ability of macrophages to inhibit hepatocellular carcinoma. Together, these studies suggest important immuno-regulatory roles for lincRNA-Cox2.

LincRNA-AK170409

Like lincRNA-Cox2, lincRNA-AK170409 was identified from a screen of macrophage lncRNAs upregulated by the TLR2/1 ligand Pam3CSK4¹², and it was independently identified by Lam *et al.* to be upregulated following LPS treatment of macrophages¹⁶. CRISPR/Cas9-mediated deletion of lincRNA-AK170409 in an immortalized murine BMDM reporter cell line expressing an NF- κ B-GFP showed that this lncRNA is a novel regulator of NF- κ B signaling in response to Pam3CSK4 and LPS¹⁷. Further studies will be required to understand how lincRNA-AK170409 regulates NF- κ B activation.

LincRNA-EPS

Using an approach similar to that described above for lincRNA-Cox2, the Fitzgerald group also identified lncRNAs in murine BMDMs that exhibited decreased expression upon TLR2 stimulation. Among those identified was an annotated long intergenic non-coding RNA, lincRNA-EPS (also called lncRNA-EPS), that was originally identified as a regulator of erythrocyte differentiation¹⁸. Mice lacking lincRNA-EPS were generated in order to test the role of lincRNA-EPS in regulating immune response genes. Subsequent RNA-Seq of wild

type and lincRNA-EPS^{-/-} BMDMs stimulated with LPS revealed changes in immune response genes in both resting and stimulated conditions. In resting macrophages, mass spectrometry and RNA antisense purification with qRT-PCR were used to determine that lincRNA-EPS associates with chromatin via hnRNPL through a CANACA motif located at the 3' end of the lincRNA. This interaction with hnRNPL promotes binding of lincRNA-EPS to the transcription start sites of many inflammatory chemokines (e.g. *Cxcl10*, *Cxcl9* and *Il27*) and IFN-stimulated genes. In non-activated macrophages, lincRNA-EPS maintains a repressed chromatin state, thereby restraining the basal expression of these inflammatory response genes. Upon activation, lincRNA-EPS is released from the chromatin, which leads to transcription factor recruitment and subsequent transcriptional activation¹⁹.

PACER

Like lincRNA-Cox2, the human-specific lincRNA PACER (p50-associated COX-2 extragenic RNA) is located upstream of the *COX2* gene locus. The *COX2* locus contains a conserved recognition site for the CCCTC-binding factor CTCF, a highly conserved 11-zinc finger DNA binding protein²⁰. Together with a multi-subunit protein, cohesin, CTCF forms a complex that regulates a variety of chromatin-related functions through chromosomal looping²¹. Although the mechanism of gene activation by CTCF/cohesin is not fully understood, CTCF binding to the *COX2* locus is required for expression of both PACER and *COX2* mRNA. PACER, like *COX2*, is highly upregulated after LPS stimulation of U937 monocyte derived macrophages. However, unlike lincRNA-Cox2, which does not directly affect *cox2* expression levels¹², PACER controls the transcription of the *COX2* mRNA and acts as 'decoy lincRNA' by physically interacting with excess levels of repressive p50 subunits and thus restricting their binding to the promoter of *COX2*. This interaction facilitates the recruitment of NF- κ B subunit p50/p65 heterodimers, allowing the subsequent recruitment of p300, which induces histone hyper-acetylation and subsequent assembly of RNA polymerase complexes leading to transcription activation²⁰. The well-defined role of cyclooxygenases and prostaglandins makes it attractive to speculate about the potential therapeutic benefits of controlling expression of lincRNAs such as PACER.

THRIL

THRIL (TNF α and hnRNPL related immunoregulatory lincRNA) was identified in a screen for lincRNAs differentially expressed following activation of the innate immune response using THP-1 monocyte-derived macrophages stimulated with the TLR1/2 agonist Pam3CSK4. THRIL expression was found to increase with macrophage activation. Using THRIL-targeting shRNAs, it was observed that repression of THRIL lead to a decrease in TNF α secretion. This appears to be transcriptional regulation, as shRNA-mediated repression of THRIL reduced *TNFA* mRNA, although additional post-transcriptional mechanisms have not been ruled out. THRIL was shown, by mass spectrometry, to interact with the protein hnRNPL. Together, THRIL and hnRNPL form a complex which binds to the *TNFA* promoter and regulates its expression, as well as the expression of many other immune-response genes. In support of this pro-inflammatory role, THRIL expression was correlated with Kawasaki disease severity, an inflammatory condition affecting medium-sized arteries²².

LncRNA-ACOD1

Unlike most described immuno-regulatory lncRNAs, which are induced by stimuli related to their respective branch of the immune response, the recently described lncRNA-ACOD1 is induced by multiple viruses, but not type I IFN. Interestingly, lncRNA-ACOD1 was found to be regulated by the NF- κ B pathway rather than IRF3. This cytoplasmic lncRNA was found to control viral replication in both mouse and human macrophages by directly binding the metabolic enzyme glutamic-oxaloacetic transaminase (GOT2) near the substrate niche, thereby enhancing its catalytic activity. As viruses use the metabolic products of GOT2 in replication, the activity of this lncRNA thus aids in viral replication and lethality. This represents a new example of how viruses can co-opt host metabolic networks²³ and illustrates how therapeutic targeting of lncRNA-ACOD1 may serve to reduce viral replication and infection.

MacORIS

Deep RNA-Seq of the human lncRNA transcriptome after macrophage differentiation to an M1 (LPS + IFN γ) or M2 (IL-4) phenotype revealed markedly altered lncRNA expression profiles in these macrophage subtypes when compared to each other, as well as to their undifferentiated 'M0' state. Furthermore, it revealed that a subset of lncRNAs overlapped with GWAS loci that were previously reported to be involved in cardiometabolic disorders. One of these, the human-specific lncRNA MacORIS (macrophage-enriched obesity-associated lncRNA serving as a repressor of IFN γ signaling), overlaps with a single nucleotide polymorphism (rs7081678) that is associated with central obesity. Although the potential functional role of this single nucleotide polymorphism on the expression of MacORIS needs to be investigated, it was shown that the cytoplasmic MacORIS represses IFN γ signaling through the JAK2/STAT1 phosphorylation pathway in macrophages²⁴.

LncRNA-CCL2

SIRT1 regulates macrophage immune responses during sepsis; however, the mechanisms for its effects have been unclear. Jia *et al.* found that SIRT1's interaction with lncRNA-CCL2 plays a role in this previously unknown mechanism. In a mouse model of sepsis, levels of SIRT1 were found to inversely correlate with levels of lncRNA-CCL2 in macrophages. Investigation of the underlying mechanism revealed that SIRT1 sustains a repressive chromatin state at the lncRNA-CCL2 locus, which in turn leads to a downregulation in the expression of inflammatory cytokines²⁵.

Mirt2

Mirt2 was first identified in a screen for lncRNAs unregulated in peritoneal macrophages after 24 hours of LPS stimulation. LPS activation of TLR4 at the plasma membrane stimulates inflammatory signaling pathways that depend on the E3 ubiquitin ligase TRAF6. Mirt2 was found to attenuate K63-linked ubiquitination of TRAF6, thus inhibiting activation of NF- κ B and MAPK pathways. Studies in mice using adenovirus gene transfer of Mirt2 showed increased protection from endotoxemia, further supporting a role for Mirt2 as a checkpoint to prevent excess inflammation²⁶.

FIRRE

Expressed in both humans and mice, the lncRNA FIRRE was first characterized as a lncRNA controlling nuclear architecture across chromosomes²⁷. While these initial characterizations were conducted using multiple human cell types including embryonic stem cells, HeLa, and MCF7 cell lines, additional roles for FIRRE have since been described, including a role in macrophages. Lu *et al.* reported that FIRRE expression in macrophages is regulated by NF- κ B signaling and that FIRRE positively regulates the expression of inflammatory genes in response to LPS. This function is dependent on FIRRE's previously described interaction with the protein hnRNPU, which regulates the stability of mRNAs²⁶.

GAS5

Macrophages are known to be highly plastic and able to change their functional phenotype in response to environmental stimuli. The lncRNA GAS5 (Growth Arrest Specific 5), previously described as a silencer of *CCL1* expression²⁸, was recently described to play a role in macrophage polarization by Ito *et al.* While the exact mechanism has yet to be elucidated, these studies revealed that the reduction of GAS5 by nonsense-mediated RNA decay contributes to M2b macrophage polarization, indicating that prolonged expression of GAS5 will not lead to this phenotypical switch²⁹.

SeT

The lncRNA SeT (named for LT/TNF locus lncRNA sense transcript³⁰), encoded in the murine *Tnfa* locus, was studied in genetically modified mice to investigate its effect on *Tnfa* gene expression. Mice with decreased expression of SeT in macrophages showed increased steady-state levels of *Tnfa*, as well as increased mortality. Although SeT is not a direct target of miR-155, SeT's stability was increased upon deletion of miR-155³¹, a miRNA known to regulate inflammatory signaling³². While the relationship between these RNAs needs to be further explored, this study indicates that various types of noncoding RNAs can cooperate to regulate *Tnfa* gene expression in mice.

LincRNA-Tnfaip3

Located proximal to the tumor necrosis factor α -induced protein 3 (*Tnfaip3*) gene on murine chromosome 10, lincRNA-Tnfaip3 is an early response gene controlled by NF- κ B in mouse macrophages. This lncRNA, like lincRNA-Cox2, mediates both activation and repression of groups of immune response genes. Ma *et al.* determined that lncRNA-Tnfaip3 interacts with the protein high-mobility group box 1 (Hmgb1), and assembles a NF- κ B/Hmgb1/lincRNA-Tnfaip3 complex. This complex mediates Hmgb1 histone modifications and, as a consequence, activation of inflammatory genes in mouse macrophages following microbial challenge³³.

MALAT1

The long noncoding RNA MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) also known as NEAT2³⁴ is a highly conserved nuclear lncRNA originally described as a predictive marker for metastasis development in lung cancer³⁵. Among other functions that have been noted since MALAT1's initial discovery, MALAT1 has been described to be

involved in attenuating the production of inflammatory mediators. LPS stimulation of the human THP-1 monocytic cell line leads to significantly enhanced expression of MALAT1. Upon this stimulation, MALAT1 binds the nuclear p65/p50 heterodimer of NF- κ B and prevents the binding of NF- κ B to the promoters of a subset of NF- κ B-regulated genes. Consequently, siRNA-mediated knock down of MALAT1 leads to increased expression of known inflammatory factors such as IL-6 and TNF α after LPS stimulation³⁶. Interestingly, MALAT1 exerts similar regulatory effects in cardiomyocytes and microvascular endothelial cells during sepsis^{26,37}. In addition to its described role interacting with p65/p50, MALAT1 also plays roles in atherosclerosis. Lipid uptake by THP-1 macrophages upregulates the expression of MALAT1 through the NF- κ B pathway. In response, MALAT1, through binding of β -catenin, induces the transcription of *CD36*, a major scavenger receptor required for the formation of foam cells³⁸. Unexpectedly, the expression of MALAT1 in atherosclerotic plaques is less expressed when compared to control internal mammary arteries³⁹. These inconsistencies underline that future research in MALAT1, as well as other lncRNAs in atherosclerotic development are of key importance to understand the role of lncRNAs and to develop potential therapeutic targets.

Macrophage long non-coding RNAs playing roles in atherosclerosis

Inflammation plays a fundamental role in all stages of atherosclerosis, from initiation to progression of plaques, as well as eventual thrombotic complications⁴⁰. Indeed levels of inflammatory cytokines within atherosclerotic plaques have long been known to correlate with disease severity⁴¹. Macrophages, which engulf accumulated lipoproteins in the intima, secrete inflammatory cytokines and chemokines, and play numerous roles in the progression of atherosclerosis. However, most lncRNAs implicated in atherosclerosis have been characterized using cardiac tissue or endothelial cells. These include: MALAT1^{42,43}; a natural antisense HIF-1 α transcript (aHIF)⁴⁴, Kcnq1ot1⁴⁵, and MIAT⁴⁶. While less well-described, lncRNAs in macrophages provide a unique opportunity to study the crossroads of inflammation and cardiovascular disease.

CARL

CARL (cardiac apoptosis-related lncRNA; also known as *Carlr*) was initially discovered in cardiomyocytes as a regulator of apoptosis during anoxia through its actions as competing endogenous RNA for miR-539⁴⁷. More recently, CARL was shown to have increased expression in human and mouse macrophages following NF- κ B activation. Upon NF- κ B activation, CARL is dynamically shuttled from the nucleus to the cytoplasm, where it regulates protein localization. Like PACER, Lethe, and MALAT1, CARL is able to directly interact with components in the NF- κ B protein complex. CARL preferentially binds the p65 complex, although it also shows affinity for I κ Ba and p50. Upon knock-down of CARL, expression levels of several inflammatory mediators, such as *COX2* and *IL1B*, are reduced. These effects suggest that CARL exerts a pro-inflammatory function, possibly by recognizing and facilitating transport of I κ Ba-free p65 back to the nucleus⁴⁸.

ANRIL and CircANRIL

Genome-wide association studies (GWAS) identified the locus on chromosome 9p21, containing the lncRNA ANRIL (Antisense noncoding RNA in the *INK4* locus), as the strongest genetic susceptibility locus for cardiovascular disease. Linear forms of *ANRIL* are positively associated with atherosclerosis risk⁴⁹ and mediate *trans*-regulation of gene networks leading to pro-atherogenic cellular properties, such as increased proliferation and adhesion⁵⁰. ANRIL appears to act as a scaffold to bind polycomb group proteins recruited to promoters of target genes. Interestingly, these functions depend on a primate-specific Alu motif present in *ANRIL* RNA and also interspersed throughout the genome. ANRIL can also circularize and in this form, contrary to its linear function, circANRIL confers atheroprotection in vascular smooth muscle cells and macrophages⁵¹. CircANRIL binds to the 60S-preribosomal assembly factor, pescadillo homologue 1, and induces cellular apoptosis in vascular smooth muscle cells and macrophages through impaired pre-rRNA processing. Although circRNAs are broadly expressed in eukaryotic cells, few have been linked to human disease. CircANRIL provides insight into how circularization of lncRNAs may alter their linear function in homeostasis and disease.

LincRNA-DYNLRB2-2

LncRNA microarray analysis of THP-1 macrophages loaded with oxidized or acetylated LDL revealed that the expression of lincRNA-DYNLRB2-2 was induced in a dose- and time-dependent manner upon cholesterol loading. Increased expression of lincRNA-DYNLRB2-2 activates the glucagon-like peptide 1 receptor signaling pathway and as a result the expression of *GPR119* and *ABCA1* is increased⁵². Both genes play atheroprotective roles in macrophages: *GRP119* inhibits the release of inflammatory cytokines such as TNF α and IL-1 β ⁵², and is thus anti-inflammatory, while the transmembrane transport protein *ABCA1* promotes the efflux of excess cellular cholesterol⁵³. The mechanisms by which lincRNA-DYNLRB2-2 upregulates the expression of *GRP119* and *ABCA1* are currently unknown, and remain to be explored.

RP5-833A20.1

RP5-833A20.1 was identified from a microarray analysis of macrophage foam cells, which showed that expression of lncRNA RP5-833A20.1 is upregulated with cholesterol loading. The enhanced expression of RP5-833A20.1 was accompanied by a down regulation of Nuclear Factor I A (*NFIA*), whose genomic location overlaps with RP5-833A20.1. *NFIA* regulates cholesterol transport across cellular membranes by stimulating the expression of *ABCA1* and *ABCG1*⁵⁴. Interestingly, expression of mature miR-382-5p, a miRNA targeting the 3'UTR of *NFIA*, was increased when RP5-833A20.1 was overexpressed. By contrast, inhibition of RP5-833A20.1 led to decreased levels of miR-382-5p. Together, these data suggest a relationship between RP5-833A20.1, *NFIA* and miR-382-5p in the regulation of cholesterol homeostasis⁵⁴, although the detailed mechanisms by which RP5-833A20.1 attenuates the expression levels of miR-382-5p remains to be unraveled.

NEAT1

NEAT1 was initially identified as one of three nuclear lncRNAs (Xist, NEAT1, NEAT2/MALAT1) with the potential to regulate gene expression or mRNA metabolism³⁴. Subsequent studies showed that NEAT1 has several macrophage-specific roles. The longer isoform of NEAT1, NEAT1_2, mediates the formation of paraspeckles, a process that, in THP-1 macrophages, can facilitate oxidized low-density lipoprotein uptake⁵⁵. NEAT1 is also induced by viral infection and TLR3 stimulation, resulting in paraspeckle formation⁵⁶. This contributes to NEAT1 regulation of antiviral genes such as IL-8. Under basal conditions, the splicing factor SFPQ is usually localized to the *IL8* promoter, inhibiting transcription. However, induction of NEAT1 relocates SFPQ from the *IL8* promoter to paraspeckles, and de-represses *IL8* transcription. These studies suggest roles for NEAT1 in regulating the innate immune response.

H19

A recent study from Han *et al.* showed that H19, a lncRNA previously studied in cancer and metastasis, plays additional roles in adipogenesis and inflammation. H19 was found to be elevated in the blood of atherosclerosis patients, as well as in RAW264.7 macrophages stimulated with oxidized LDL. To investigate H19's roles in atherosclerosis, H19 was silenced in macrophages and foam cell formation was analyzed. Oil red O staining revealed that H19 knockdown correlated with a decrease in lipid accumulation. ELISAs and western blotting were used to examine changes in cholesterol and lipogenic genes, revealing that shRNA knockdown of H19 decreased lipid accumulation in oxidized LDL treated macrophages in addition to decreasing pro-inflammatory factors such as TNF α and IL-1 β . Subsequent analyses revealed that silencing of H19 led to an upregulation of miR-130b. Interestingly, overexpression of H19 overcomes the effects of a miR-130b inhibitor on adipogenesis and inflammation⁵⁷. These findings suggest a role for H19 and miR-130b cooperation in regulating macrophage foam cell formation.

E330013P06

RNA-Seq analyses of BMDMs from diabetic db/db mice revealed that various lncRNAs showed altered expression levels when compared to non-diabetic control mice. One of the differentially expressed lncRNAs, E330013P06, was also significantly increased in macrophages derived from diet-induced insulin-resistant type 2 diabetic (T2D) mice. Furthermore, the human equivalent of E330013P06, MIR143HG, was increased in monocytes from T2D patients when compared to healthy controls. Of note, no changes in expression were found in type 1 diabetic mice, indicating that the expression of E330013P06 is associated with insulin resistance, hyperglycemia, and free fatty acids. Macrophages treated with high glucose and palmitic acid showed increased expression of E330013P06. Overexpression of E330013P06 in the murine macrophage cell line RAW264.7 using lentiviral vectors led to the upregulation of proinflammatory and proatherogenic genes such as *Nos2*, *Il6* and *Cox2*, while anti-inflammatory genes such as *Il10* were down-regulated. Furthermore, E330013P06 overexpression lead to increased lipid uptake through the upregulation of scavenger receptor *Cd36*, resulting in enhanced foam cell formation. Although siRNA-induced silencing of E330013P06 in db/db macrophages did not inhibit the

basal expression of inflammatory genes, these genes were significantly less responsive to treatment with high glucose and palmitic when compared to macrophages transfected with control siRNA⁵⁸. Although the exact mechanism by which E330013P06 regulates inflammatory genes remains to be elucidated, E330013P06 is of interest because of its conservation in humans, making it a potential therapeutic target.

Competing endogenous RNAs in macrophages

LncRNAs exported to the cytoplasm have been reported to function as competing endogenous RNAs (ceRNA) that modulate the concentration and biological functions of miRNAs^{59,60}. These ceRNAs contain miRNA response elements that act as sinks for active miRNAs, thereby sequestering them away from their mRNA target networks⁶⁰. This mechanism is thought to contribute to homeostatic maintenance by rapidly curtailing miRNA activity when perturbations are detected, liberating mRNA target genes from miRNA-induced silencing to return gene expression to homeostatic levels⁵⁹. This ceRNA hypothesis is somewhat controversial due to the notion that miRNA response elements in RNA sequences are generally in large excess over their lowly expressed post-transcriptional regulators (e.g. lncRNAs). Mathematical models suggest that significant regulatory effects may only be expected when there are extremely high expression levels of a ceRNA⁶¹. However, an increasing number of examples of lncRNAs being able to sponge and sequester miRNAs with regulatory effects are being described⁶². It is thus likely that the sequestering of miRNAs is supported by unknown factors (e.g. RNA binding proteins or RNA modifications) that might facilitate the interactions of ceRNAs with their targets in the cytoplasm. Nonetheless, a number of proposed ceRNAs have been shown to function in macrophages and to impact the immune response.

Lnc-MC

PU.1 is a critical effector of the differentiation of monocytes to macrophages, thereby regulating inflammation and the innate immune response⁶³. PU.1 directs the expression of a wide variety of genes during monocyte/macrophage differentiation, including the expression of a long non-coding monocytic RNA (lnc-MC) that is upregulated during the monocyte to macrophage differentiation of primary CD34⁺ hematopoietic stem cells and the THP-1 and HL-60 cell lines⁶⁴. Fluorescence *in situ* hybridization and quantitative PCR upon cellular fractionation showed that lnc-MC is cytoplasmic, raising the possibility that lnc-MC could interact with mature miRNAs, which accumulate in the cytoplasm. Indeed, it was shown that lnc-MC interacts with miR-199a-5p and can de-repress the translation and function of a known miR-199a-5p target ACVR1B⁶⁴. In turn ACVR1B promotes the activation of TGF- β facilitating monocyte/macrophage differentiation through Smad2/3 and C/EBP α ⁶⁵.

CHRF

In hypertrophic mouse cardiomyocytes, the lncRNA CHRF (Cardiac Hypertrophy Related Factor) has been shown to regulate *Myd88* expression, via targeting of miR-489⁶⁵. Interestingly, the CHRF/miR-489/*Myd88* axis has been identified as a contributing factor in silica-induced pulmonary fibrosis. Silica particles activate macrophages causing them to release oxidants and cytokines, which can ultimately lead to fibrosis. Notably, macrophages

exposed to silica show an upregulation of CHRF and reduced levels of miR-489. However, this discovery may have limited translational value, as full length of CHRF is poorly conserved, despite conservation homology of the miR-489 binding site across species⁶⁶.

TUG1

Taurine-upregulated gene (TUG1) has been reported to act as an oncogene in a variety of cancers⁶⁷. Within the vascular system, TUG1 expression in endothelial cells was found to be increased in *ApoE*^{-/-} mice fed a high fat diet. TUG1 regulation of the expression of miR-26a was shown to be associated with endothelial cell apoptosis⁶⁸. In addition, using several independent techniques, TUG1 was shown to function as a molecular sponge to repress miR-133a expression levels, and as a consequence to regulate the miR-133a target FGF1⁶⁹. TUG-1 expression is upregulated in mouse vascular smooth muscle cells and macrophages following oxidized LDL treatment. Furthermore, overexpression of TUG1 in these cell types lead to an increase in apoptosis and inflammatory gene expression. *In vivo*, TUG1 knockdown reduced levels of IL-6 and TNF α in the circulation of high fat diet-fed *ApoE*^{-/-} mice, suggesting that it has a proinflammatory, and thus, proatherosclerotic function.

Enhancer RNAs in macrophages

Enhancer RNAs (eRNAs) represent a novel and understudied class of lncRNAs that are transcribed from enhancer regions encoded within the DNA sequence. Enhancers are relatively short (50 – 1500bp) DNA sequences that can bind transcription factors and influence the transcription of distant genes in a cis-acting manner. Enhancers can be located either downstream or upstream of a gene, distal or proximal, making these powerful sequences enigmatic. They regulate gene expression by creating loops in chromatin, in which there is direct protein-protein contact between enhancer-associated transcription factors and components of the basal transcription machinery. These interactions likely contribute to the formation of large transcriptional complexes at promoter regions, from which transcription is initiated⁷⁰. Interestingly, genome-wide techniques such as RNA-Seq and ChIP-Seq have detected extensive RNA polymerase II binding to these enhancer sites, indicating putative non-coding RNA transcription of eRNAs^{71,72}. Currently, there is no consensus on the biological significance of eRNAs. eRNA synthesis may be required to generate and/or maintain certain chromatin landscapes⁷³ or, as some evidence suggests, eRNA transcripts themselves may be biologically relevant⁷⁴.

Only a few studies have described a functional role for eRNA transcripts during macrophage differentiation or activation. For instance, LPS stimulation of THP-1 macrophages leads to rapid induction of two nuclear eRNA transcripts from enhancer regions: *IL1 β -eRNA* and *IL1 β -RBT46*. In these cells, knockdown of *IL1 β -eRNA* and *IL1 β -RBT46* with antisense inhibitors in combination with LPS treatment decreased mRNA expression levels of *IL1B* and *CXCL8*, as well as the diminished secretion of IL-1 β and CXCL8, but not IL-6⁷⁴. Another study showed that TLR4 activation regulates macrophage gene expression primarily through a pre-existing enhancer landscape, but a large number of new enhancers were also activated and while others were inactivated. Compellingly, eRNA transcription preceded the recruitment of transcription-initiating proteins (e.g. histone methyltransferases) to these

enhancer regions; however, the presence of eRNA transcripts was not shown to be associated with these regulatory effects⁷³. These observations suggest a regulatory role for enhancer transcription independent of potential functions for the synthesized eRNA transcripts.

Long non-coding RNAs harboring putative micropeptides

The recent discovery that lncRNAs can harbor translatable small open reading frames that give rise to biologically active micropeptides has added yet another layer of complexity to their potential functions⁷⁵. Recent studies from the Olson laboratory have uncovered two micropeptides, myoregulin (MLN) and DWORF, which interact with SERCA to regulate calcium uptake in muscle tissue^{76,77}. These studies revealed that small peptides can have potent functions. No functional roles for micropeptides in macrophage and/or immunity have been reported to date. However, in HeLa cells, a TNF α responsive peptide (through eIF4E phosphorylation) named STORM (Stress- and TNF α -activated ORF Micropeptide) is encoded within *linc00689*⁷⁸. Given the recent discoveries of micropeptides in other fields of study and the emergence of bioinformatics techniques that are publicly accessible, it is likely that micropeptides affecting macrophage biology will soon be reported.

Concluding remarks

Collectively, the studies cited above show that lncRNAs (listed in Table 1) can coordinate diverse aspects of the inflammatory response, including the differentiation and activation of macrophages⁷⁹. To date, there is a large discrepancy between the number lncRNAs being discovered and the number of lncRNAs being functionally described in detail. This is, in part, due to the fact that lncRNAs act through diverse and complex mechanisms, which is exacerbated by a lack of prediction algorithms, which could accelerate discovery. Further complicating this, it appears that lncRNAs, like miRNAs, can display different biological activity in different cell types and in response to different stimuli or even subcellular localization. For example, the well-known lncRNA HOTAIR⁸⁰ has been described to play roles in a variety of cancers⁸¹, to be a circulating biomarker⁸² and to also function as a competing endogenous RNA when present in the cytoplasm^{68,83–86}. The scientific community is generating expansive RNA-Seq databases documenting lncRNA regulation in specific cell types, in response to various stimuli and in different disease conditions. Additionally, hundreds of lncRNAs have been identified to be dysregulated in disease using screens of RNA collected from patients with various conditions including cancer, cardiovascular disease, and diabetes, among others. While the mechanisms of lncRNA regulation in disease varies, many dysregulated lncRNAs have been found to be controlled by transcription factors already identified to be upregulated in those disease states, such as NF- κ B. The present challenge is to translate these changes in lncRNA expression to the discovery of lncRNA functions and contributions to disease and homeostasis.

In the last decade, non-coding RNAs have emerged as important new therapeutic targets and there has been a major effort to develop oligonucleotide-based therapies to enhance or antagonize miRNAs and lncRNAs. Over 100 antisense oligonucleotide (ASO)-based therapies have been tested in clinical trials, with fomivirsen and mipomersen receiving FDA approval to treat cytomegalovirus retinitis and familial hypercholesterolemia, respectively

(reviewed in⁸⁷). Programs targeting miRNAs are currently more advanced than those targeting lncRNAs, but similar techniques are being employed to inhibit these two forms of ncRNA. The current strategy to deliver miRNA mimics or inhibitors involves the use of synthetic modified oligoribonucleotides. In the case of miRNAs, their small size can be an advantage for targeting, while the longer size and highly structured nature of lncRNAs often confounds the design of effective inhibitors or mimics. Although clinical advances have yet to be made with lncRNAs, their versatile and specific biological functions make them attractive as an effective modality for therapeutic intervention in future studies.

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Table 1

List of lncRNA expressed in macrophages, localization and summarized functions.

lncRNA	Species	Localization	Stimuli	Function	Ref.
Lethe	Mouse	Nucleus	TNF α IL-1 β	Prevents binding of p65-NF- κ B complex to <i>Nox2</i> promoter.	9,10
LincRNA-Cox2	Mouse	Nucleus Cytoplasm	Pam3Csk4 LPS	Represses immuno-regulatory genes by forming complex with hnRNPs. Required for assembly of NF- κ B into SW1SNF complex.	12,14
LincRNA-EPS	Mouse	Nucleus	TLR2	Controls expression of inflammatory cytokines through association of chromatin through hnRNPL.	19
PACER	Human	Nucleus	LPS	Interacts with repressive p50-subunits to facilitate the expression of <i>COX-2</i> .	20
THRIL	Human	Nucleus	Pam3Csk4	Controls TNF α expression and secretion by interacting with the <i>TNFA</i> promoter through a complex with hnRNPL.	22
LncRNA-ACOD1	Human Mouse	Cytoplasm	Viruses	Binds directly to GOT2 enhancing its activity.	23
MacORIS	Human	Cytoplasm	LPS + IFN γ	Represses IFN γ signaling through JAK2/STAT1 phosphorylation.	24
LincRNA-AK170409	Mouse	Nucleus	Pam3Csk4	Regulates NF- κ B signaling.	17
LncRNA-CCL2	Mouse		SIRT1, sepsis	Repressed by SIRT1, dampening inflammatory cytokine expression.	25
Min2	Mouse	Cytoplasm	LPS	Acts as checkpoint to impede excess inflammation by ubiquitination of TRAF6.	26
FIRRE	Human Mouse	Nucleus	LPS	Stabilizes inflammatory genes through hnRNPU.	26
GAS5	Mouse	Cytoplasm	IFN γ IL-10	Participates in macrophage polarization.	29
SeT	Mouse		LPS	Deregulates steady-state expression of <i>Tnfa</i> .	31
LincRNA-Tnfrap3	Mouse	Nucleus	NF- κ B	Interacts with Hmgbl to modify histones and control inflammatory gene expression.	33
MALAT1	Human	Nucleus	LPS Lipid uptake	Binds p65-p50 complex to prevent NF- κ B binding to promoter regions of NF- κ B-responsive genes. Regulates transcription of CD36.	36,38
CARL/Carlr	Human Mouse	Nucleus Cytoplasm	NF- κ B	Facilitates transport of p65 from cytoplasm to nucleus.	48
CircANRIL	Human	Nucleus		Induces apoptosis through binding to PES1, impairing preRNA processing	51
LincRNA-DYNLRB2-2	Human		Cholesterol loading	Directs atheroprotection through the regulation of <i>GPR119</i> and <i>ABCA1</i> .	52
RP5-833A20.1	Human	Nucleus	Cholesterol loading	Controls cholesterol transport through <i>NFIA</i> and miR-382-5p.	54
NEAT1	Human	Nucleus	Cholesterol loading, Virus	Mediates the formation of paraspeckles – leading to multiple downstream effects.	55,56
H19	Human Mouse	Nucleus	oxLDL	Cooperates with miR-130b during foam cell formation.	57

lncRNA	Species	Localization	Stimuli	Function	Ref.
E330013P06	Mouse		High glucose Palmitic acid	Upregulates proinflammatory and proatherogenic genes upon stimulation. MIR143HG is human equivalent.	58
Lnc-MC	Human	Cytoplasm	Macrophage differentiation	Interacts with miR-199a-5p to derepress <i>ACVR1B</i> expression.	64
CHRF	Mouse	Cytoplasm	Silica particles	Binds to miR-489 to elevate expression levels of <i>Myd88</i> .	66
TUG1	Mouse	Cytoplasm	oxLDL	Acts as a molecular sponge for miR-133a to increase <i>FGF1</i> expression levels.	69
IL1β-eRNA, IL1β-RBT46	Human	Nucleus	LPS	Regulates the expression of IL-1β and CXCL8 as eRNA.	74
Linc00689	Human	Cytoplasm	TNFα	Encodes for TNFα-responsive micropeptide in HeLa cells.	78