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Control of immune cell homeostasis and function by lncRNAs

Walter K. Mowel^{1,2,†}, Jonathan J. Kotzin^{1,2,†}, Sam J. McCright^{1,2}, Vanessa D. Neal^{1,2}, and Jorge Henao-Mejia^{1,2,3}

¹Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

²Institute for Immunology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

³Division of Transplant Immunology, Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, Pennsylvania 19104

SUMMARY

The immune system is comprised of diverse cell types that coordinate responses to infection and maintain tissue homeostasis. In each of these cells, extracellular cues determine highly specific epigenetic landscapes and transcriptional profiles to promote immunity while maintaining homeostasis. New evidence indicates that long noncoding RNAs (lncRNAs) play critical roles in epigenetic and transcriptional regulation in mammals. Thus, lncRNAs have emerged as key regulatory molecules of immune cell gene expression programs in response to microbial and tissue-derived cues. Herein, we review how lncRNAs control the function and homeostasis of cell populations during immune responses, emphasizing the diverse molecular mechanisms by which lncRNAs tune highly contextualized transcriptional programs. Moreover, we discuss the new challenges faced in interrogating lncRNA mechanisms and function in the immune system.

The regulatory role of the non-coding genome

A longstanding observation in biology is that while the number of protein coding genes has remained relatively stable across multicellular organisms, the genomic content of non-coding DNA increases with the developmental complexity of organisms [1]. This seemingly paradoxical finding, termed the G-value paradox, suggests that the degree of regulatory control exerted by non-coding regions over protein-coding genes determines phenotypic complexity, which has increased throughout evolution [1]. In support of this inference, a number of high-throughput sequencing projects have recently shown that a great proportion of the mammalian non-coding genome comprises an immense network of *cis*-regulatory elements such as promoters, enhancers, and super-enhancers [2–4]. Strikingly, these

Corresponding author contact information: Jorge Henao-Mejia, jhena@mail.med.upenn.edu.

[†]These authors contributed equally to this work

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consortia also showed that nearly 70% of the human non-coding genome shows evidence of transcriptional activity. The function of the majority of these non-coding transcripts is unknown; nevertheless, several classes of non-coding RNAs have been found to contribute to the transcriptional and epigenetic regulation of gene expression programs. Among these functional non-coding RNAs, lncRNAs are the most abundant and have been extensively studied in recent years. As such, the role of lncRNAs in immune regulation has received a significant amount of attention and will be the focus of this review.

A diverse array of cells from the innate and adaptive immune system orchestrate protective immune responses that clear infections and promote the development of long term immunological memory while preventing the deleterious consequences of prolonged inflammation. The highly-specialized functions of each of these immune cell types are determined by their unique transcriptional programs. While much is known about how transcription factors and different extracellular cues regulate the development and functional specialization of innate and adaptive immune cells, our knowledge regarding the roles of lncRNAs in the immune system is in its infancy. A growing body of evidence indicates that lncRNAs are expressed in a more cell type-specific manner than protein-coding genes and are rapidly induced by unique tissue-derived and environmental signals [5, 6]. As such, these molecules have started to emerge as highly cell type-specific regulators of gene expression programs in the immune system. In the following sections, we will provide an overview of how, through diverse mechanisms of action, lncRNAs tightly control the function and homeostasis of specific cell populations during innate and adaptive immune responses.

MECHANISMS OF ACTION OF LNCRNA LOCI

lncRNAs accomplish a diverse range of biological functions through an equally diverse range of molecular mechanisms [7]. Important recent work has demonstrated that certain ribosome-associated lncRNAs which, by definition, were annotated as non-coding are now reported to code for short peptides with biological function [8–10]. Whether the cryptic translation events of other ribosome-associated lncRNAs in the immune system have meaningful biological function has yet to be determined. Therefore, this type of lncRNA is not discussed further in this review.

lncRNA molecules themselves can directly interact with proteins, DNA, and other RNAs to exert their functions. Nevertheless, not all lncRNA loci that are biologically active depend on the function of the RNA molecule itself. Herein, we distill the biological mechanisms of lncRNAs loci into 3 different groups: (i) lncRNA loci in which the RNA molecule itself is functional, (ii) lncRNA loci in which the act of transcription, but not the lncRNA molecule, has a functional role, and (iii) lncRNAs that represent proxy signals for active *cis*-regulatory elements. Throughout this section, we aim to exemplify each functional grouping with lncRNA loci described in the immune system, while in the following sections we will describe in detail the biological process they regulate in the context of immune responses.

Functional lncRNAs

The first lncRNAs were characterized by demonstrating direct biological activity of the lncRNA molecule itself [11–13], and this remains the gold standard for characterizing

lncRNA loci. Functional lncRNAs can localize to the nucleus or cytosol to specifically interact with proteins, DNA, or other RNAs to perform their molecular functions. The sequence constraints for RNA folding and structure are more lenient than those for protein folding, which has likely helped facilitate the evolution of such mechanistically versatile lncRNA molecules. The recent development of novel RNA-centric tools that directly test lncRNA structure, localization, and interactomes (Box 1) has paved the way for our understanding of the mechanisms of action of lncRNAs. Conceptually, we group functional lncRNAs within two classes: those that serve as inhibitors and those that serve as facilitators of binding partner interactions.

Class I of functional lncRNAs: Inhibitors – keeping things apart—Many protein-protein interactions conform to a classic molecular decoy archetype, wherein the decoy molecule binds to and inhibits one partner in an interaction; a classic example of this is the non-signaling interleukin 1 receptor type 2 (IL1R2), which binds the cytokine IL-1 β and prevents it from signaling through its normal receptor [14]. Here we expand this to include lncRNA-mediated processes in which factors are inhibited from interacting with one another (Figure 1A). This class most obviously applies to lncRNAs that act as sinks for microRNAs, such as the lncRNA Phosphatase And Tensin Homolog Pseudogene 1 (PTENP1), whose 3' UTR binds the same set of miRNAs sequences that normally target the tumor-suppressor gene PTEN [15]. In addition, this class also includes lncRNAs that specifically inhibit classical protein-protein interactions such as the lncRNA lnc-DC [16]. lnc-DC regulates the differentiation and activation profile of dendritic cells (DCs) in humans by binding STAT3 in the cytoplasm and preventing its dephosphorylation and inactivation by the tyrosine phosphatase SHP1 [16]. Additionally, this inhibitory class includes lncRNAs that block protein-DNA interactions, such as pseudogene-derived lncRNA Lethe [17]. Lethe physically associates with NF- κ B p65 (RelA) subunit and directly or indirectly blocks its recruitment to the promoters of certain immune genes such as *IL6* and *IL8* [17]. Of note, lncRNAs can directly act post-transcriptionally, as their function is not dependent on the process of translation. Thus, by blocking key DNA, RNA, or protein interactions, lncRNAs can exert rapid potent biological outcomes in the immune system.

Class II of functional lncRNAs: Facilitators – bringing things together—lncRNAs have been described to form complexes with protein, RNA, and/or DNA, and thus facilitate the interaction of specific binding partners. This class of RNA-dependent mechanism includes lncRNAs that serve as “scaffolds” or “guides” (Figure 1B). “Scaffold” lncRNAs can function as molecular platforms upon which other DNA, RNA, and protein molecules can assemble. For example, the lncRNA RMRP facilitates an interaction between the nuclear receptor RAR-related orphan receptor gamma (ROR γ t) and DEAD-box protein 5 (DDX5) [18]. This ROR γ t-DDX5 interaction is necessary for transcriptional co-activation of certain T helper 17 (Th17) genes [18]. On the other hand, lncRNAs that serve as “guides” can bind other DNA, RNA, and protein molecules and target their localization to induce a specific biological function. For example, X-inactive specific transcript (*Xist*) is, as its name implies, involved in the silencing of the second X chromosome in female placental mammals. *Xist* is expressed by and coats the inactive X chromosome from which it is transcribed by binding and promoting the recruitment of multiple repressive factors

including Polycomb, SHARP, and others [19–21]. Similarly, in the immune system, the lncRNA myeloid RNA regulator of Bim induced death (*Morrbid*) silences its neighboring pro-apoptotic gene *Bcl2l11* (*Bim*) in an allele specific manner by promoting the enrichment of polycomb repressive complex 2 (PRC2) within the *Bcl2l11* promoter [22].

The scaffold and guide classes are closely related. For example, a lncRNA can bind a protein and be required for that protein's localization to a specific genomic location; yet, this localization can occur through an independent biological effect of the lncRNA. The distinction of the scaffold and guide classes is typically determined by whether the lncRNA's function is either directly or indirectly dependent on localization. For instance, *Xist* has the ability to associate with many different protein complexes; nevertheless, its function is predominately dependent on the direct and indirect recruitment of these complexes to the inactive X chromosome. As such, for simplicity, *Xist* is typically categorized as a lncRNA guide. Of note, the general model of lncRNA direct protein recruitment is complicated by the lack of known protein-binding motifs within lncRNAs and little data on RNA binding specificity (reviewed in [23]). Future studies on lncRNA motifs and structure are needed to better understand how lncRNAs interact with specific proteins and how lncRNAs localize to specific sites.

Transcription across lncRNA has a functional role

As previously discussed, lncRNAs demonstrate exquisite cell type-specific expression and many are responsive to specific extracellular cues. Given the strict regulation of lncRNA biogenesis and that many lncRNAs impact the expression of neighboring genes, it has been suggested that the process of transcription and/or splicing of lncRNAs may impact the expression of spatially proximal genes through the recruitment of specific protein factors [24–26] (Figure 1C). This concept was recently dissected in further detail through the study of the protein coding gene *Sfmbt2* and its neighboring lncRNA *Blustr*. The authors found that *Sfmbt2* is upregulated by the splicing and transcription of *Blustr*, in a mechanism independent of the sequence of *Blustr* but likely to involve the recruitment of polymerases and chromatin-modifiers [27]. By elegantly inserting a synthetic polyadenylation sequence at different locations within this locus *in vitro*, these effects were found to be dependent on the length of transcription across the *Blustr* locus [27]. Additionally, promoter-proximal splice sites are known to enhance transcription, and deletion of this site of *Blustr* also impacted *Sfmbt2* transcription [27]. In parallel, Anderson and colleagues demonstrated *in vivo* that the heart development regulator *Hand2* is controlled *in cis* by the lncRNA *Uph*, but that this control was independent of the sequence of *Uph* [28]. Of note, the authors found that transcription of *Uph* was required to maintain the chromatin state of a cardiac superenhancer-like element within the *Uph* locus [28]. Although it is not clear mechanistically how transcription and/or splicing recruit critical regulatory factors to coordinate the expression of genes *in cis*, these studies are highly relevant to the lncRNA field as they clearly demonstrate that lncRNA biogenesis itself can impact proximal genes in a transcript and sequence independent manner. How transcription across lncRNA loci impact immune cells is still yet to be determined.

LncRNAs as proxy signals for cis-regulatory elements

The promoters of many lncRNA loci are conserved across species and have enhancer chromatin signatures, suggesting that certain lncRNA promoters may function as *cis*-acting enhancer elements that result in bystander noncoding transcripts [27, 29]. Given that lncRNA loci frequently contain or are in close proximity to important regulatory DNA elements, the production of lncRNAs from these loci can delineate critical *cis*-regulatory DNA elements within these loci (Figure 1D). For example, mapping of RNA polymerase II using Chromatin Interaction Analysis with Paired-End-Tag sequencing (ChIA-PET) revealed strong genome-wide promoter-centered intergenic interactions, suggesting that promoters of coding and non-coding genes may have dual functions as enhancers [30]. Therefore, certain lncRNA transcripts may arise as non-functional by-products, yet they may serve as proxy signals of the activity of important regulatory elements. For example, deletion of the lncRNA down-stream of *Cdkn1b* (*Lockd*) locus was found to impact the transcription of its neighboring gene *Cdkn1b*; however, pre-mature polyadenylation of *Lockd* had no impact on *Cdkn1b* transcription [31]. The *Lockd* promoter was found to have many enhancer-like features suggesting that this lncRNA locus may indeed function through DNA elements within its promoter and that the RNA transcript produced from this locus marks an important regulatory element [31]. Additionally, we recently showed that the lncRNA *Rroid* is specifically expressed in group 1 innate lymphoid cells (ILCs), and marks a critical *cis*-regulatory element important for the maturation, function, and lineage identity of these cells while being dispensable for the development and homeostasis of closely related ILC2s and ILC3s (Mowel et al. 2017), suggesting that other cell-type specific lncRNAs will demarcate critical *cis*-regulatory elements that control highly specialized processes in immune cells.

BIOLOGICAL FUNCTIONS OF LNCRNAS IN THE IMMUNE SYSTEM

LNCRNAS IN THE INNATE IMMUNE SYSTEM

In biological immune responses, the cells of the innate immune system – macrophages, dendritic cells, neutrophils, basophils, eosinophils, and ILCs – must undergo rapid changes in their gene expression programs to immediately respond to infection, tissue damage, or other changes in environmental conditions. As discussed above, one of the key advantages of lncRNA-mediated gene regulation is that existing pools of transcription factors can be utilized to promote expression of target lncRNAs, which can then rapidly affect expression of both individual genes or sets of genes within minutes without requiring new protein synthesis. As such, it is not surprising that many of the initial discoveries of lncRNAs in the immune system were focused on the regulation of inflammation in innate immune cells.

lncRNAs regulate inflammatory gene expression in innate immune cells—One of the first lncRNAs described in the immune system, lincRNA-Cox2, was discovered in bone marrow-derived dendritic cells stimulated with lipopolysaccharide (LPS), an agonist of TLR4 [32]. Strikingly, lincRNA-Cox2 is rapidly induced within 30 minutes in RAW264.6 macrophages in response to inflammatory cues [33]. Carpenter et al. showed that this lncRNA regulates a large set of inflammatory response genes, as knockdown of lincRNA-Cox2 resulted in increased expression of a gene set including *Ccl5* and *Cx3cl1* [34] (Figure 2A). lincRNA-Cox2 interacts with hnRNP-A/B and hnRNP-A2/B1 *in vitro*, and knockdown

of both hnRNP-A/B and hnRNP-A2/B1 results in de-repression of *Ccl5* [34]. In contrast, other work has shown that lincRNA-Cox2 in immortalized macrophage cell lines interacts with the SWI/SNF chromatin remodeling complex to promote its recruitment to the *Ccl5* promoter and activate transcription [33]. lincRNA-Cox2 has also been shown to interact with and recruit the Mi2/NuRD repressive complex to the *Il12b* promoter in response to TNF stimulation in intestinal epithelial cell lines to silence *Il12b* expression [35].

This type of mechanism, wherein lncRNAs act as guides or scaffolds to rapidly regulate the recruitment chromatin modifying complexes to their target genes, is likely widespread. Indeed, a recent study identified lnc13, which is downregulated in response to LPS, as a repressor of genes involved in inflammatory pathways such as *Myd88*, *Stat1*, *Stat3*, and *Il1ra*. This effect is mediated through the regulation of HDAC1 and hnRNPD recruitment to their target genes in an RNA-dependent mechanism [36]. Additionally, lincRNA-EPS suppresses genes such as *Cxcl10* and *Ccl5* by interacting with hnRNPL and regulating nucleosomal positioning at these target loci [37] (Figure 2B). *In vivo*, mice deficient for lincRNA-EPS exhibit significant increases in circulating proinflammatory cytokines and are extremely susceptible to LPS challenge.

Constitutively expressed transcription factors in innate immune cells transduce signals within minutes of TLR ligation, promoting inflammatory gene expression. lncRNAs regulate this process not only by directing the recruitment of chromatin modifying complexes to target genes, but can also directly control the ability of transcription factors to form complexes with other proteins or bind their target loci. As described above, one example of this activity is lnc-DC, which is highly expressed in human DCs and directly promotes STAT3 activity by preventing its dephosphorylation [16]. Functionally, this results in reduced expression of the costimulatory molecules CD80 and CD86, as well as MHC-II [16]. Thus, lnc-DC and upstream signals regulating its expression may play an important role in CD4⁺ T cell priming during *in vivo* immune responses. Similarly, the lncRNA Lethe interacts with the p65 subunit of NF- κ B to inhibit its binding to the TNF promoter, negatively regulating TNF production in mouse macrophages, while the lncRNA PACER blocks the inhibitory NF- κ B p50 subunit from binding the COX2 promoter, resulting in increased COX2 expression in human macrophages *in vitro* [17, 38]. Altogether, these studies show that lncRNAs utilize a variety of mechanisms to rapidly regulate inflammatory gene expression programs in innate immune cells in response to microbial-derived cues, representing a new and potent regulatory layer in inflammatory responses.

lncRNAs in the development and homeostasis of innate immune cells—While several lncRNAs regulating inflammatory processes have been described in the literature, very few are yet known to regulate the development or homeostasis of innate cells. HOTAIRM1 is one such example that is highly expressed in human granulocytes and induced by retinoic acid signaling and PU.1 during myeloid differentiation [39, 40]. Knockdown of HOTAIRM1 results in reduced expression of CD11b and CD18 as well as impaired granulocyte differentiation in cell lines [39]. However, the role of HOTAIRM1 in the differentiation of human granulocytes *in vivo* still unknown.

Recently, we showed that the lncRNA *Morrbid* controls the homeostasis of eosinophils, neutrophils, and Ly6C^{hi} monocytes *in vivo* by regulating the pro-apoptotic molecule *Bcl2l11*, also known as *Bim* [22]. *Morrbid* is induced in these short-lived myeloid cells in response to pro-survival cytokines and represses *Bcl2l11* transcription in an allele-specific manner by promoting the enrichment of the repressive PRC2 complex within the *Bcl2l11* promoter (Figure 2C). In the absence of *Morrbid*, *Bcl2l11* is de-repressed in these cells, resulting in their apoptosis [22]. This example illustrates a novel and critical pathway used to precisely regulate the lifespan of these highly inflammatory cell populations. Indeed, expression of *MORRBID* is highly upregulated in eosinophils collected from patients with hypereosinophilic syndrome (HES), a group of disorders characterized by an altered eosinophil lifespan [22]. Taken together, these data suggest that dysregulation of the *Morrbid-Bcl2l11* axis may be an important element in HES and other disorders of dysregulated myeloid lifespan such as autoinflammation and cancer.

Finally, the lncRNA biogenesis and proxy signal-based mechanisms of action are an emerging field, and as such have not yet been studied in detail in the context of the immune system. Interestingly, a recent study identified linc1405, a lncRNA expressed upstream of the *Eomes* gene in mouse embryonic stem cells [27]. Deletion of this locus or its promoter reduced *Eomes* expression in these cells. However, *Eomes*-dependent immune cells, such as natural killer (NK) cells were not examined. We have recently shown that a lncRNA, *Rroid*, marks a *cis*-regulatory element that is critical for promoting the homeostasis and function of group 1 ILCs, but not ILC2s or ILC3s (Mowel CITATION). The *Rroid* locus, but not the RNA itself, regulates ILC1 homeostasis by promoting open chromatin and STAT5 deposition at the promoter of *Id2*, a key gene required for ILC homeostasis. Although the *Rroid* locus forms a physical interaction with the *Id2* locus, the exact mechanisms of action are still unknown. It is intriguing to speculate that other lncRNAs expressed near key lineage-specific transcription factors or cytokines may mark *cis*-regulatory elements critical for the development, homeostasis, and function of varied immune cell subsets.

LNCRNAS IN THE ADAPTIVE IMMUNE SYSTEM

Composed of B and T lymphocytes, the adaptive immune system must generate an effective antigen-specific effector response and form long-term immunological memory while avoiding autoimmunity and chronic inflammation. Unbiased studies have shown that lncRNAs are differentially expressed by many, if not most subsets of B and T cells [41–43]. Mechanistic description of lncRNA function in developing lymphocytes and mature B cells remains a nascent area of inquiry, but lncRNAs are emerging as key regulators of the defining steps of helper T cell-mediated immunity. Herein we describe recent work on lncRNA function in the activation of naive CD4⁺ T cells, their differentiation into specific effector subsets, and production of subset-specific cytokines.

lncRNAs in T cell activation—Upon activation, naive T cells undergo transcriptional and epigenetic changes as they proliferate and differentiate into specific effector subsets. One canonical example of this process is the production of IL-2 and the switch from a low-affinity to a high-affinity IL-2 receptor, which promote naive T cell proliferation and survival. Production of IL-2 requires the transcription factors NFAT1 (nuclear factor of

activated T cells), AP-1, and NF- κ B. Of these, the function of NFAT1 in T cells is regulated by the lncRNA NRON (ncRNA repressor of NFAT) [44]. In resting T cells, inactive NFAT1 is sequestered in the cytoplasm. As T cells are activated, NFAT1 is dephosphorylated by the phosphatase calcineurin, and translocated to the nucleus [45]. NRON serves as a scaffold for the interaction of NFAT1 with IQGAP and the three inhibitory NFAT1 kinases CKE, GSKb, and DYRK, which leads to the retention of NFAT1 in the cytoplasm [44, 46] (Figure 3A). Importantly, CD8⁺ T cells from IQGAP knockout mice produce significantly increased levels of IFN- γ , a known NFAT1 target, suggesting that an NRON-IQGAP axis may be a key factor in regulating NFAT-dependent cytokine production in activated CD8⁺ T cells [46].

lncRNAs in CD4⁺ T cell polarization—Following their activation by cognate antigen, naive CD4⁺ T cells further differentiate into different lineages of helper and regulatory T cells with specific immunological functions. While environmental cues shape CD4⁺ T cell fate, the major lineage-specifying determinants are cytokines that activate networks of transcription factors leading to characteristic transcriptional profiles and functions. Th1 cells require the master transcription factor T-bet for their differentiation, Th2 cells require GATA3, and Th17 cells require ROR γ t. Recent work has shown that linc-MAF-4 plays a key role in regulating the expression of the transcription factors which control the Th1/Th2 fate decision [47, 48](Figure 3B). Ranzani et al. showed that linc-MAF-4 expression is high in Th1 cells but considerably reduced under Th2-polarizing conditions, and that knockdown of linc-MAF-4 in naive CD4⁺ cells results in expression of the Th2-specific genes MAF, GATA3, and IL-4 [47]. The authors went on to demonstrate physical interaction between linc-MAF-4 and the MAF promoter, increased H3K27 trimethylation at the MAF promoter, and association of linc-MAF-4 and the methyltransferases EZH2 and LSD1 [47]. Together, these observations suggest a model in which linc-MAF-4 associates with the MAF promoter via DNA looping, where it recruits or acts as a scaffold for a repressive chromatin-modifying complex.

A second lncRNA, LincR-Ccr2-5' AS, coordinates the expression of a Th2-specific gene program downstream of GATA3 signaling. Located in the cluster of genes encoding *Ccr1*, *Ccr3*, *Ccr2*, and *Ccr5*, LincR-Ccr2-5' AS regulates the expression of these genes and others by an unknown mechanism, independent of modulating H3K4 methylation or RNA pol II accessibility [49](Figure 3C). These studies and others suggest that lncRNAs influence T helper cell development by regulating the expression of a range of lineage-controlling transcription factors and other subset-specific genes.

lncRNAs control the function of adaptive immune cells—In addition to their master transcription factors, effector CD4⁺ T cell lineages are defined functionally by the cytokines they produce; Th1 cells are characterized by production of IFN- γ , Th2 by IL-4, and Th17 cells by the production of IL-17A and IL-17F. It has recently been shown that lineage-specific lncRNAs are preferentially located adjacent to or intergenic with cytokine coding genes, and act downstream of lineage-specific transcription factors to regulate their expression [49]. A well-characterized example of this is the lncRNA NeST, which is located in the cluster of genes encoding *Ifng* and *Ii22* and requires the Th1-specific transcription factors T-bet and STAT4 for its expression [50, 51]. NeST interacts with the

methyltransferase subunit WDR5, and expression of NeST is correlated with increased levels of transcriptionally permissive H3K4 trimethylation at the *Ifng* locus [52, 53] (Figure 3D). The lncRNA Th2-LCR appears to regulate cytokine production in Th2 cells by a similar mechanism. Th2-LCR is located in the IL-4, IL-5, and IL-13 gene cluster, co-precipitates with WDR5, and its knockdown *in vitro* reduced both H3K4 methylation and WDR5 binding of the IL-4 and IL-13 promoters [54, 55].

Th17 cells, a third lineage of CD4⁺ T cells, are characterized by production of the cytokines IL-17A, IL-17F, and IL-22 downstream of the transcription factor ROR γ t [56]. As mentioned above, Huang et al showed that the lncRNA RMRP is required for the association of ROR γ t and DDX5 [18]. Proper assembly of the DDX5-RMRP complex is not required for Th17 cell differentiation, but is required for ROR γ t-mediated expression of Th17-specific cytokines (Figure 3D). Significantly, a single G>T point mutation in the human RMRP gene is associated with cartilage-hair hypoplasia (CHH), a disorder characterized by immunodeficiency among a number of other symptoms. Mice bearing the CHH-associated RMRP allele harbor Th17 cells with a defective effector program, and this remains one of the only studies directly linking altered lncRNA function to a human immunological disease [18]. Another lncRNA, *Flicr*, is expressed in mouse and human Foxp3⁺ Tregs and functions to negatively regulate *Foxp3* expression and Treg function, as *in vivo* deletion of *Flicr* significantly protected mice from autoimmune diabetes [57]. The exact mechanisms through which *Flicr* regulates *Foxp3* expression are unknown, but appear to work in part through the control of chromatin accessibility at the CNS3/AR5 region within the *Foxp3* gene [57]. Together, these studies demonstrate that lncRNAs act in conjunction with lineage-specific transcription factors to control expression of cell type-specific effector molecules in the adaptive immune system.

LNCRNAS IN HUMAN INFLAMMATORY DISORDERS

A longstanding observation in human GWAS studies is that more than 90% of disease-related SNPs are associated with noncoding elements of the genome, and it is tempting to speculate that mutations in lncRNAs may explain some of these phenotypes [58]. Thus far, much of the lncRNA-related research that has been done in the immune system has focused on delineating the function of lncRNAs in mouse and human primary cells and cell lines. However, examples have begun to emerge that demonstrate the importance of lncRNAs in human inflammatory disorders. As we discuss above, the lncRNA *MORRBID* is highly upregulated in eosinophils collected from patients with HES, suggesting the *MORRBID*-*BCL2L11* axis may contribute to this disease [22]. lnc13, a highly expressed lncRNA in the healthy human gut, suppresses expression of a subset of inflammatory disease related genes and is significantly downregulated in patients with celiac disease, suggesting that dysregulated lnc13 expression may contribute to inflammation in this disease [36]. Indeed, celiac disease-associated SNPs have been found in lnc13 [36]. Additionally, linc-MAF-4 has been shown to be dysregulated in multiple sclerosis, and as discussed above, mutations in *RMRP* are associated with CHH [18, 48]. Together, the *in vivo* and *in vitro* data regarding lncRNAs in the immune system suggest that these molecules play key roles in mediating both protection and susceptibility to inflammatory diseases. In the future, it is critical that robust *in vivo* models are developed and utilized to test the physiological consequences of

lncRNAs in inflammatory disorders such as inflammatory bowel disease, diabetes, allergies, asthma, and cancer, among many others.

TECHNICAL CHALLENGES IN DISSECTING LNCRNA MECHANISM IN VIVO

Although high-throughput sequencing has allowed for great strides to be made in profiling immune cell lncRNA expression patterns, the functional characterization of these lncRNAs *in vivo* has lagged far behind (Figure 4A). Much of this delay can be attributed to the difficulty in separating the role of the genomic lncRNA loci from that of its RNA products. For example, many groups have deleted full lncRNA loci to test lncRNA functionality [22, 59, 60] (Figure 4B). While this deletion approach is a rapid way to initially screen functional lncRNA loci, especially with the efficiency of the CRISPR/Cas9 system, it does not separate any of the 3 lncRNA mechanism categories discussed above: functional RNAs, transcription-dependent, and proxy signals for *cis*-regulatory elements. As such, additional complementary experimental approaches need to be taken to disentangle the functionality of these lncRNAs [61].

RNA interference (RNAi)-based techniques, in particular short-hairpin RNAs (shRNAs), are an important addition to the experimental arsenal of lncRNA biologists as they allow for stable targeting of specific RNAs (Figure 4C). Of note, nuclear RNAi targeting is less well understood [62, 63]; thus, RNAi techniques are most well suited for testing the RNA-dependence of cytoplasmic lncRNAs. Antisense oligonucleotides (ASOs), provide an alternative post-transcriptional RNA targeting method [64] and their nuclear RNA targeting is significantly better characterized, yet they cannot be used to generate stable transgenic lines (Figure 4C). Moreover, if the lncRNA is suggestive to have functional roles in *trans*, complementation 'rescue' studies are a direct method to address their mechanism of action. To test transcription-dependent lncRNA function, additional genetic approaches can be taken to insert transcriptional terminator sequences [27, 28, 65] (Figure 4D). Targeted deletion of DNA elements within the lncRNA locus, such as the lncRNA promoter, may help to expose the lncRNA as a proxy for these important *cis*-regulatory elements. As with all genetic approaches, investigators must be vigilant and control for disruption of important DNA regulatory elements and inadvertent impact of inserted DNA elements. Importantly, it is not possible to predict lncRNA function from expression levels and sequence composition with current bioinformatics tools, especially with little known about conserved lncRNA motifs and secondary structures. Thus, a combination of the empiric approaches above must be taken and interpreted in the context of each lncRNA to accurately characterize its functionality.

CONCLUDING REMARKS

lncRNAs are critical epigenetic and transcriptional regulators that allow cells to specifically adapt their gene expression profiles to cues within their extracellular environment. It is becoming clear that the diverse cell types of the innate and adaptive immune system are no exception, and that lncRNAs are key regulatory molecules of immune cell gene expression programs in response to microbial and tissue-derived cues. Although much progress has been made in cataloging lncRNAs expressed in the immune system and in assessing their

functional impact, there still exists a large void in our understanding of how these lncRNAs function at the molecular level in the context of immune responses. The development of new experimental and bioinformatic technologies will be paramount in filling this gap in lncRNA biology. In particular, novel techniques directed towards predicting and interrogating the structure of lncRNAs will be essential as they will help to uncover lncRNA motifs and structures that ultimately dictate the localization and interactomes of these molecules. Additionally, novel techniques to address lncRNA impact on higher order 3D chromatin organization will help uncover parallels between certain lncRNAs and potentially new principles of lncRNA function. Ultimately, better understanding of lncRNA function in the immune system will expose new means to augment protective immunity, quell excess inflammation, and promote tissue homeostasis.

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GLOSSARY

lncRNAsLong

non-coding RNAs are defined as being RNA species that are greater than 200nt in length, capped, spliced, and polyadenylated. As their name implies, lncRNAs lack strong Kozak consensus sequences and therefore are not predicted to encode proteins.

Cis-regulatory element

A region of non-coding DNA which impacts the transcription of a neighboring or proximally located gene.

Cis-acting lncRNA

A lncRNA that directly impacts the transcription of genes located proximally to the lncRNA loci.

Trans-acting lncRNA

A lncRNA that directly impacts the transcription of distally located genes and is not confined to acting on the chromosome from which it is transcribed

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BOX 1**TOOLBOX FOR DETERMINING LNCRNA-INTERACTING MOLECULES AND SECONDARY STRUCTURE**

lncRNAs can associate with DNA, RNA, and proteins to impart an array of functions, many of which are still being discovered. Identifying specific lncRNA-binding partners, in addition to the structural motifs of lncRNAs that might govern these associations, can provide insight into the functional scope of lncRNAs. New RNA-centric tools use *in vivo* cross-linking methods and high-throughput technologies to discover the binding partners of a particular lncRNA of interest on a global scale. Three methods analogous to ChIP-seq have emerged in recent years to probe lncRNA-biomolecule interactions: ChIRP (chromatin immunoprecipitation by RNA purification), CHART (capture hybridization analysis of RNA targets), and RAP (RNA antisense purification) [66–68]. Although these techniques differ in specific details, they are similar in principle and take advantage of RNA hybridization to pull down a specific lncRNA along with its binding partners. By altering the cross-linking and purification conditions, DNA, RNA and protein partners can be co-purified with a lncRNA and subsequently identified through sequencing and mass spectrometry [69]. These RNA-centric methods for discovering binding partners will be an important component in untangling lncRNA functions.

In addition to identifying lncRNA binding partners, probing the secondary structures of lncRNAs will add to our understanding of lncRNA function. Given that many lncRNA sequences are not well conserved, it has been proposed that their secondary structures could form modular domains that bring an array of binding partners together in a context-specific manner. A new method called PARIS (psoralen analysis of RNA interactions and structures) identifies intra- and inter-molecular RNA-RNA interactions on a genome-wide scale at high resolution, in a manner analogous to 3C for chromatin-chromatin interactions [70]. Unlike ChIRP, CHART, and RAP, this new technology does not probe for a specific lncRNA, and instead analyzes all cross-linked RNAs on a global scale. In addition, PARIS can identify conserved RNA structures through cross-species comparisons, allowing to interrogate the possibility that lncRNA secondary structures are conserved in lieu of their sequences. By first identifying the secondary structures of lncRNAs, researchers can then uncover whether these conformations aid in function and impart binding specificity by introducing point mutations that disrupt these secondary structures. A combination of these approaches will be necessary to characterize lncRNA structure and interactomes in the immune system.

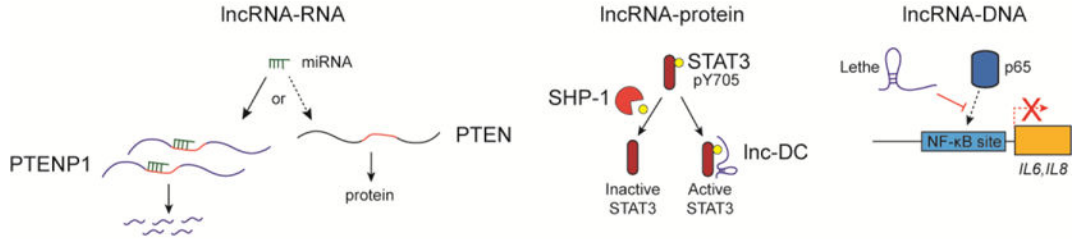
TRENDS

- Immune cells exhibit a wide variety of functions that are controlled by cell type-specific epigenetic and gene expression profiles. Long non-coding RNAs (lncRNAs) have recently emerged as key epigenetic and transcriptional regulators of immune cells.
- LncRNAs control the development and function of specific immune cell-types through a variety of mechanisms including interactions with other RNAs, DNA, and proteins. LncRNAs are frequently induced downstream of cytokine, innate and adaptive receptors, which allows immune cells to rapidly modulate their gene expression programs in response to stimuli.
- In innate immune cells, lncRNAs have been shown to directly regulate several biological functions, including cytokine production, development, and cell survival.
- In adaptive immune cells, lncRNAs have primarily been shown to regulate the polarization and effector function of CD4⁺ T cells.

OUTSTANDING QUESTIONS

- There is growing appreciation that transcription of lncRNA loci, rather than the lncRNA itself, is required to mediate the biological functions of lncRNAs. What is the role of transcription across lncRNA loci in protective immunity and immune cell development?
- Secondary structures are likely to be critical for lncRNA function. What lncRNA motifs and secondary structures are important for their function in the immune system?
- How do lncRNAs impact nuclear organization and 3D chromatin architecture of immune cells?
- The majority of known immune acting lncRNAs have been characterized *in vitro*. What are the *in vivo* functions of lncRNAs in the immune system?
- What are the roles of lncRNAs in human inflammatory disorders? What are the functional consequences of disease-associated SNPs that fall within lncRNA loci?

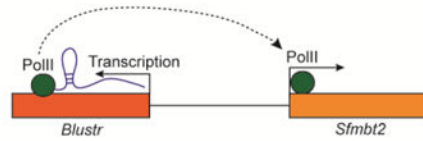
A Functional lncRNAs



B Scaffold Guide



C Transcriptional dependence



D Proxy signal

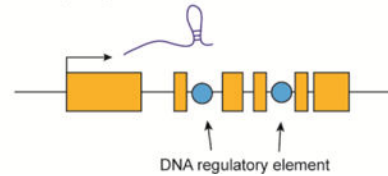


Figure 1. Mechanisms of lncRNA action

(A) lncRNA mechanisms that depend on the RNA molecule itself include lncRNA-RNA, lncRNA-protein, and lncRNA-DNA-based mechanisms. For example, *PTENP1* encodes miRNA target sequences shared by *PTEN* and acts as a “sponge” for miRNAs targeting *PTEN* (left). *lnc-DC* interacts directly with the protein STAT3 and blocks its dephosphorylation by the phosphatase SHP-1 (middle). lncRNA *Lethe* hinders the recruitment of NF-κB p65 subunit to its target DNA sequences (right).

(B) lncRNAs can activate gene expression by acting as scaffolds for interactions between other proteins, such as lncRNA *RMRP* (left), or as guides to direct the recruitment of chromatin modifying complexes to neighboring genes, such as *Morrbid* (right).

(C) The act of lncRNA transcription can intrinsically account for the activity of some lncRNA loci. Transcription across the lncRNA *Blustr* directly promotes transcription of its neighboring gene *Sfbmt2*.

(D) Some lncRNAs may not regulate transcription of their target genes, but instead act as a proxy signals for important DNA regulatory elements embedded within the lncRNA locus.

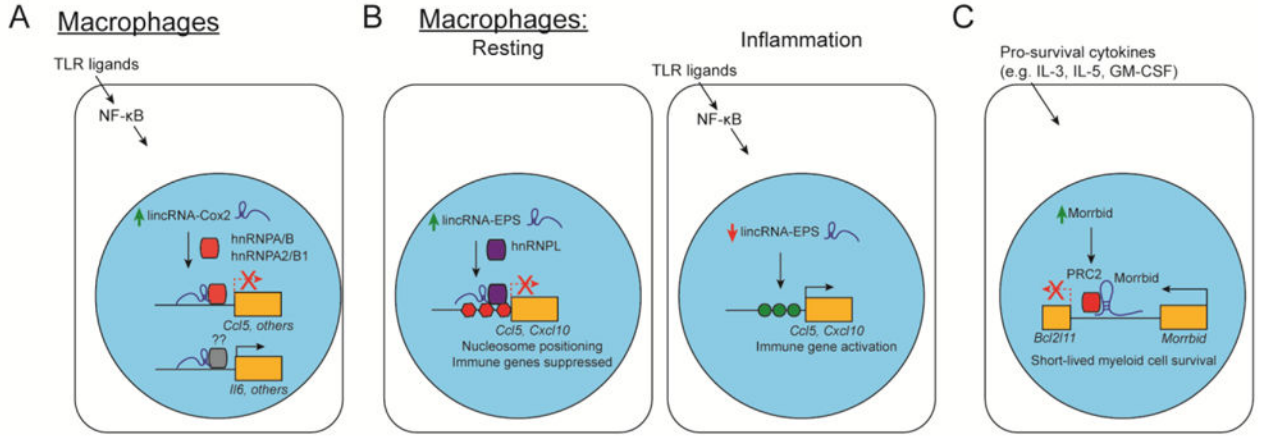


Figure 2. IncRNAs in the innate immune system

(A) LincRNA-Cox2 is induced by TLR ligands and associates with hnRNPA/B and hnRNPA2/B1 to suppress transcription of target genes, such as *Ccl5*, and promotes the expression of genes such as *Il6* through a yet unknown mechanism.

(B) Resting macrophages express high levels lincRNA-EPS, which interacts with hnRNPL to suppress target genes. Upon stimulation, expression of lincRNA-EPS is reduced, and repression of these targets is released.

(C) In short-lived myeloid cells, pro-survival cytokines induce expression of *Morrbid*, which acts as a guide to target the repressive PRC2 complex to the *Bcl2l11* locus (which encodes the pro-apoptotic molecule *Bim*), to promote the survival of these cells.

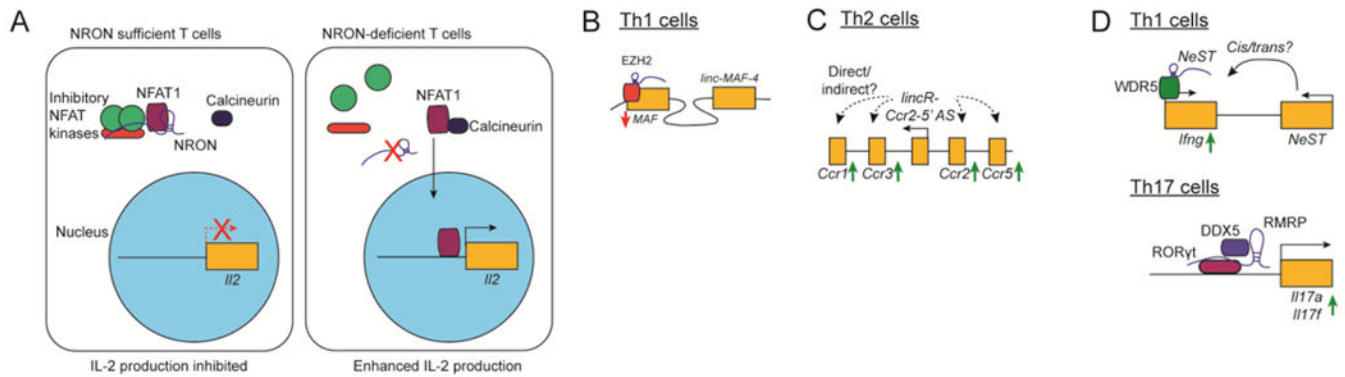


Figure 3. lncRNAs in the adaptive immune system

(A) The lncRNA *NRON* represses T cell NFAT1 activity by promoting its association with inhibitory factors in the cytoplasm. In the absence of *NRON*, the phosphatase Calcineurin is able to dephosphorylate NFAT1, resulting in its nuclear translocation and increased cytokine production.

(B) lncRNAs promote Th1 cell polarization. *linc-MAF-4* promotes the recruitment of EZH2 to the promoter of the Th2-specific transcription factor *c-MAF* to repress its expression.

(C) LincR-*Ccr2-5' AS* promotes expression of Th2 genes, including nearby chemokine receptors such as *Ccr1*, *Ccr2*, *Ccr3*, and *Ccr5*. Whether this regulation is direct or indirect, and the specific mechanisms through which LincR-*Ccr2-5' AS* regulate these genes is unknown.

(D) lncRNAs promote T cell effector function. *NeST* promotes WDR5 recruitment to the *Ifng* locus to activate its transcription (above), while *RMRP* promotes Th17 cytokine production by acting as a scaffold for the association of the Th17-specific transcription factor ROR γ t and the helicase DDX5 at the *Il17a* and *Il17f* loci.

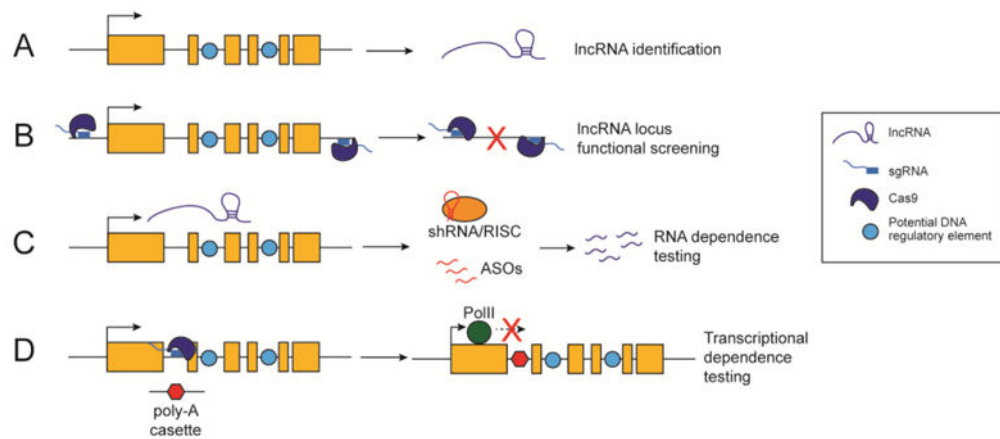


Figure 4. Testing lncRNA function *in vivo*

In vivo functional testing of lncRNAs remains challenging but is critical to understanding their relevance in the immune system.

(A) Cell-type specific lncRNA expression profiles can be generated from RNA-seq on purified cell populations or publicly available datasets.

(B) The functional relevance of these loci encoding these lncRNAs can be quickly and easily assessed by generating genomic deletions using the CRISPR/Cas9 system.

(C) RNA dependence of functional lncRNA loci can be assessed using shRNAs and ASOs.

(D) Transcriptional dependence can be determined by insertion of a premature poly-A cassette using CRISPR/Cas9.

Table 1

lncRNAs with identified immune functions

LncRNAs in innate immunity				
lncRNA	Species	Phenotype/function	Proposed mechanism	Reference
Lethe	Ms	Pseudogene lncRNA activated by TNF α . Negatively regulates NF- κ B.	Binds RelA to inhibit DNA binding	[17]
Linc-Cox2	Ms	Induced in BMDC by LPS (TLR4) and Pam3CSK (TLR2). Regulates inflammatory gene sets.	Interactions with hnRNP-A/B, hnRNP-A2/B1, and SWI/SNF	[32–34]
PACER	Hu	Antisense RNA upstream of COX-2 gene. Promotes COX2 by inducing epigenetic changes at COX2 promoter.	Interacts w/NF κ B p50 subunit, prevents it from binding & repressing COX2	[38]
THRIL	Hu	Decreased upon Pam3CSK stimulation and promotes TNF α . Associates with hnRNPL located at TNF α promoter	hnRNPL	[71]
AS-IL-1 β	Ms	Highly upregulated following LPS stimulation in RAW and peritoneal macrophages. Suppresses IL1 β expression.	Regulates RNAPolII recruitment to IL-1 β locus	[72]
IL1 β -eRNA & IL1 β -RBT46	Hu	eRNA and site of bidirectional transcription near IL-1 β locus. Knockdown attenuates IL-1 β and CXCL8 transcription/release.	Unknown	[73]
AS-IL-1 α	Ms	Induced by Pam3CSK, LPS, polyIC in BMDM to regulate levels of IL-1 α	Regulates recruitment of PolII to IL-1 α promoter	[74]
lnc-DC	Hu	Highly expressed in all DC populations. Promotes CD40, CD80, HLA-DR expression on DCs.	Blocks dephosphorylation of STAT3	[16]
Lnc13	Hu,Ms	Decreased in inflammation & active celiac. Overlaps 3' end of I18rap. Levels depend on levels of Dcp2 decapping enzyme. Shown to interact with Hdac1 and hnRNPD. Celiac risk alleles reduce association with hnRNPD	Localized to TSS of targets along with hnRNPD and Hdac1 ; likely a scaffold	[36]
lincRNA-EPS	Ms	Downregulated by TLR stimulation. Full deletion results in increased inflammatory genes basally and during inflammation.	Interacts w/hnRNPL through specific RNA motif; loss alters positioning of nucleosomes at target genes	[37]
Morrbid	Hu,Ms	Induced by prosurvival cytokines in short-lived myeloid cells (neutrophils, eosinophils, Ly6Chi monocytes). Inhibition results in increased short-lived myeloid cell death.	Interacts with repressive PRC2 complex to target it to Bcl2l11 promoter	[22]
lncHSC-1/2	Ms	Promote HSC function in vitro and in vivo; altered T/B/myeloid reconstitution upon knockdown.	At least partially by promoting recruitment of E2A to target genes	[75]
NRAV	Hu	Expressed basally & reduced by infection w/virus. Mouse phenotype is via transgenic overexpression. Negatively regulates ISG expression.	Increases H3K27me3 modifications	[76]
iNOS AS	Rat	Antisense RNA in 3' UTR of iNOS. Promotes expression of iNOS mRNA.	Interactions with 3' UTR of iNOS mRNA	[77]
HOTAIRM1	Hu	Knockdown results in reduced CD11B and CD18 expression;	Activated downstream of RA signaling; mechanism unknown	[39]

LncRNAs in innate immunity				
lncRNA	Species	Phenotype/function	Proposed mechanism	Reference
		reduced granulocytic differentiation of cell lines		
NKILA	Hu	Induced in response to TNF and IL-1b to regulate NF-kB signaling in breast cancer cell lines. Function in immune cells is unknown.	Blocks IκB phosphorylation	[78]
NEAT1	Hu,Ms	Regulates IL-8 synthesis following immune stimulation	Alters localization of repressor protein SFPQ	[79]
lncRHOXF1	Hu	Suppresses expression of viral response genes in trophoblast progenitors. Viral infection induces lncRHOXF1 expression.	Unknown	[80]
lncKdm2b	Ms	Regulates ILC3 homeostasis by promoting ILC3 proliferation	Interacts with Satb1	[81]
<i>Rroid</i>	Ms	Promotes <i>Id2</i> expression specifically in NK/ILC1 to control their homeostasis	Promotes STAT5 deposition and chromatin accessibility at <i>Id2</i> . RNA-independent	[MOWEL citation]
LncRNAs in adaptive immunity				
NeST / Tmevpg1	Ms,Hu	Promotes expression of <i>Ifng</i> in Th1 cells downstream of Stat4, Tbet, NFκB, Ets1	Recruits H3K4 methyltransferases at <i>Ifng</i> promoter	[51–53]
NRON	Ms,Hu	Regulates expression of IL-2 in Th1 cells	Maintains NFAT1 in cytoplasm by providing scaffold for inhibitory NFAT kinases	[44, 46]
linc-MAF-4	Hu	Represses Th2 differentiation by regulating levels of the master Th2 transcription factor c-MAF	Recruits LDS1 and EZH2 to the MAF locus	[47, 48]
Rmrp	Ms,Hu	Loss decreases production of IL-17A and IL-17F by Th17 cells. SNP associated with the genetic disease cartilage hair hypoplasia	Required for the interaction of RORγt with DDX5	[18]
Th2-LCR	Ms,Hu	Regulates expression of the IL-4, IL-5, and IL-13 gene cluster in Th2 cells	Associates with WDR5, promotes H3K4 methylation at the <i>Il4</i> and <i>Il13</i> promoters	[54, 55]
LincR-Ccr2-5' AS	Ms	Controls a Th2-specific gene expression program including <i>Ccr1</i> , <i>Ccr2</i> , <i>Ccr3</i> , and <i>Ccr5</i>	Unknown	[49]
Fas-AS1	Ms,Hu	Increases Fas/FasL-mediated apoptosis in B cells, T cells, and other cell types	Binds RBM5, reduces exon skipping in Fas	[82]
Gata3-AS	Hu	Coexpressed with GATA3 under Th2-polarizing conditions	Unknown	[49, 83]
Flicr	Ms	Partially overlaps FoxP3, specifically expressed in Tregs. Deletion reduces the number of FoxP3 ^{lo} Tregs and protects against diabetes in NOD mice. Negatively regulated by IL-2	May alter chromatin structure and accessibility at the CNS3/AR5 FoxP3 regulatory region	[57]