REVIEW

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Gene regulation in the immune system by long noncoding RNAs

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Long noncoding RNAs (lncRNAs) are emerging as critical regulators of gene expression in the immune system. Studies have shown that lncRNAs are expressed in a highly lineage-specific manner and control the differentiation and function of innate and adaptive cell types. In this Review, we focus on mechanisms used by lncRNAs to regulate genes encoding products involved in the immune response, including direct interactions with chromatin, RNA and proteins. In addition, we address new areas of lncRNA biology, such as the functions of enhancer RNAs, circular RNAs and chemical modifications to RNA in cellular processes. We emphasize critical gaps in knowledge and future prospects for the roles of lncRNAs in the immune system and autoimmune disease.

Precise regulation of the expression of genes of the immune system is critical to an organism's ability to generate robust immunity to pathogens while limiting autoimmunity to self antigens. The majority of studies so far have focused on the function of proteins in this process, particularly the roles of cell-surface receptors, secreted cytokines and transcription factors, but comparatively less is known about the functions of RNA. While RNA molecules have traditionally been viewed as merely passive carriers of information from DNA molecules to the proteins encoded, studies have now demonstrated that RNA is indeed an active participant in the regulation of gene expression at all levels, including transcription, translation and post-translational modification.

Advances in transcriptome sequencing over the past decade have demonstrated that greater than 70% of the genome is transcribed and that the vast majority of transcribed DNA encodes long noncoding RNAs (lncRNAs)[1–3]. As a result of such findings, the catalog of annotated and functionally analyzed lncRNAs has rapidly expanded, with some studies estimating the presence of over 58,000 lncRNAs in the human genome[4]. The vast majority of lncRNAs have not been tested for function, and many are possibly just transcriptional 'noise'. Nonetheless, many lncRNAs exhibit diverse functions in gene transcription and protein regulation.

The study of lncRNAs in the setting of the immune system has proven particularly advantageous. The immune system provides a highly organized biological context in which cellular phenotypes and functions are finely mapped, cellular components are easily accessible and manipulated, and perturbation at the molecular and cellular levels can be achieved through *in vitro* and *in vivo* models. Here we review recent advances in elucidation of the mechanisms of lncRNA function within the immune system, such as in hematopoietic development and cell-type-specific immunological pathways. In addition, we discuss several emerging fields in lncRNA biology, including the roles of circular RNAs (circRNAs), RNA editing and RNA modification in biological function. Although in some cases newer aspects of lncRNA biology have not yet been studied extensively in the context of the immune system, we envision and encourage the prospect that future studies exploit the immunological context to gain insight into the specific functions of, as well as the general principles that govern, lncRNA biology.

Gene regulation by lncRNAs

Noncoding RNAs are classified as short noncoding RNAs or lncRNAs on the basis of a sequence-length cutoff of 200 nucleotides. This distinction is used mainly to distinguish lncRNAs from classes of small RNA such as tRNA and microRNA (miRNA), which perform distinct functions. The lncRNAs are further classified as long intergenic noncoding RNAs (lincRNAs), intronic lncRNAs, antisense lncRNAs and enhancer RNAs (eRNAs) on the basis of their position relative to gene loci encoding protein-coding mRNA and enhancer regulatory elements of genes. Similar to mRNA, the majority of lncRNA classes are capped, spliced and polyadenylated. In contrast, eRNAs are generally transcribed bidirectionally from active enhancer elements and are capped but not spliced or polyadenylated[5,6]. However, these classifications can be blurred; for example, some RNAs transcribed from regulatory elements can be unidirectional and polyadenylated. Therefore, further classification of RNA as '1d-RNA' (unidirectional and polyadenylated) or '2d-RNA' (bidirectional, nonpolyadenylated and comparatively short in length) might be useful[7]. An emerging subset of lncRNAs has been classified as circRNAs on the basis of the self-ligation of 3′ and 5′ ends, generated through back splicing[8–12]. The circRNAs are spliced from precursor RNAs and are not capped or polyadenylated. Thousands of circRNAs have been identified in

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the human genome, and in many cases, circRNAs are the dominant isoform over the linear transcript[10–12].

The biological functions of lncRNAs are now starting to be understood, and critical roles for lncRNAs have been identified in nearly every biological system studied. For example, essential functions have been elucidated for the following lncRNAS: for Xist, in silencing of the X chromosome[13–15]; for H19, in genomic imprinting[16,17]; for lincRNA-RoR, in the differentiation of embryonic stem cells (ESCs)[18,19]; and for HOTAIR, in breast-cancer metastasis[20,21] (reviewed in refs. 22–24). Surprisingly, unlike other classes of RNA molecules, lncRNAs do not seem to have a predominant molecular archetype[25]. Instead, like protein scaffolds, lncRNAs function through modular domains that interact with DNA or RNA through nucleic-acid base pairing or with proteins through higher-order RNA structures[26,27] (**Fig. 1**). In many cases, the act of transcribing lncRNA itself can exert gene-regulatory effects through changes in chromatin accessibility or transcription-factor trapping at target-gene promoters[28–31].

lncRNA–DNA interactions

Most lncRNAs described so far function by modulating the transcription of target genomic loci in *cis* (neighboring genes) or in *trans* (distantly located genes) by binding to target DNA through the recognition of specific chromatin features or as an RNA–DNA heteroduplex or RNA–DNA–DNA triplex. RNA can form base pairs with single-stranded DNA through Watson-Crick interactions (duplex) or can interact with double-stranded DNA by inserting into the major groove of the duplex structure with sequence specificity (triplex) (reviewed in ref. 32). For example, lncRNAs can establish a stable duplex with promoter sequences[33] or can participate in triplex structures at ribosomal DNA promoters in fibroblasts[34]. The transcription of lncRNA itself can lead to local chromatin changes that are not mediated by the noncoding transcript[28–30]. In a study analyzing the mechanisms of local gene regulation mediated by twelve lncRNAs, five were found to regulate the expression of their neighboring genes in *cis*. However, surprisingly, none of those *cis* regulators required the lncRNA transcripts themselves but instead depended on processes associated with their transcription, including enhancer activity of lncRNA promoters, transcription, or splicing of the lncRNA[30].

lncRNA–RNA interactions

Several classes of lncRNAs function through RNA–RNA interactions. For example, the Epstein Barr virus (EBV) noncoding RNA EBER2 hybridizes with nascent transcripts from the terminal repeats (TRs) locus of the latent EBV genome and thereby recruits the transcription factor PAX5 to the TRs. That recruitment of PAX5 regulates the expression of genes near the TRs and thereby controls the lytic replication of EBV[35]. RNA binding is a major mechanism employed by circRNAs to modulate gene expression. So far, studies have described two functional outcomes of RNA–RNA interaction via circRNAs: a decrease in the availability of partner RNA transcripts through direct base pairing, in particular with miRNAs; and a decrease in the transcription of alternative RNA transcripts through competition for transcriptional machinery. As 'sponges' for miRNA, circRNAs can effectively 'titrate' miRNA from its functional gene target. For example, the circRNA ciRS-7 contains 70 binding sites for miR-7 and regulates mid-brain development by 'titrating' copies of miR-7 (refs. 8,9). ciRS-7 is completely resistant to miR-7-mediated target destabilization and therefore strongly suppresses the activity of miR-7 and results in increased abundance of miR-7's targets. Similarly, the circRNA Sry found in testis serves as a sponge for miR-138, in confirmation of published findings that circularization of Sry inhibits the translation of miR-138's targets into protein[8]. Overall, the lack of 5′ and 3′ ends in circRNA leads to greater stability than that of linear RNAs and suggests that lncRNAs can temporally affect gene expression through base-pairing interactions and modification of their secondary structure.

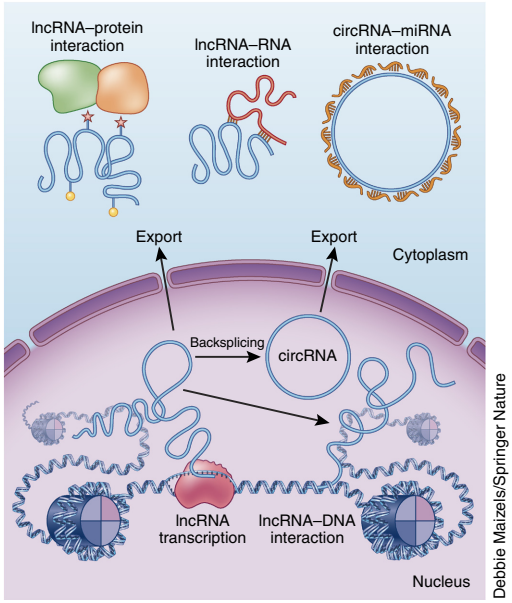

Figure 1 General lncRNA mechanism. lncRNAs function by interacting with DNA, RNA and proteins.

lncRNA–protein interactions

A single lncRNA can contain multiple modular domains that bind DNA, RNA and/or protein[27,36]. As a result, lncRNAs are able to coordinate the activities of various types of macromolecules. A chief mechanism of lncRNA function is the modular pairing of DNA binding and protein interaction to recruit chromatin-modifying proteins that regulate gene regulation via the chemical modification of histones[37]. For example, Xist binds the Polycomb repressive complex to silence the inactive X chromosome through the placement of repressive histone modifications[38,39]. Similarly, the lncRNA HOTTIP maintains gene transcription by binding to the adaptor WDR5, a core subunit of the MLL histone H3 Lys4 (H3K4)-methyltransferase complex. The HOTTIP–WDR5 complex recruits MLL factors to deposit activating histone marks on the *HOXA* locus (which encodes the HOXA family of transcription factors)[40]. lncRNAs have also been shown to partner with ribonucleoproteins to directly regulate chromatin accessibility and transcription and cytoplasmic proteins to control signaling pathways downstream of pathogen-response receptors[41–44].

eRNAs and circRNAs have also been demonstrated to control gene expression through interactions with proteins. eRNAs have been shown to bind the transcription factor YY1 at promoter-proximal elements to stabilize occupancy by transcription factors at target genomic loci[31]. Such functional protein 'trapping' at promoter sites can be diminished by the treatment of chromatin with RNase, and artificial tethering of eRNAs adjacent to YY1-binding sites through CRISPR-Cas9 technology leads to increased occupancy by YY1. Similarly, circRNAs have been found to interact directly with RNA polymerase II to augment efficient gene transcription[45]. Cross-linking and immunoprecipitation of RNA polymerase II, followed by RNA

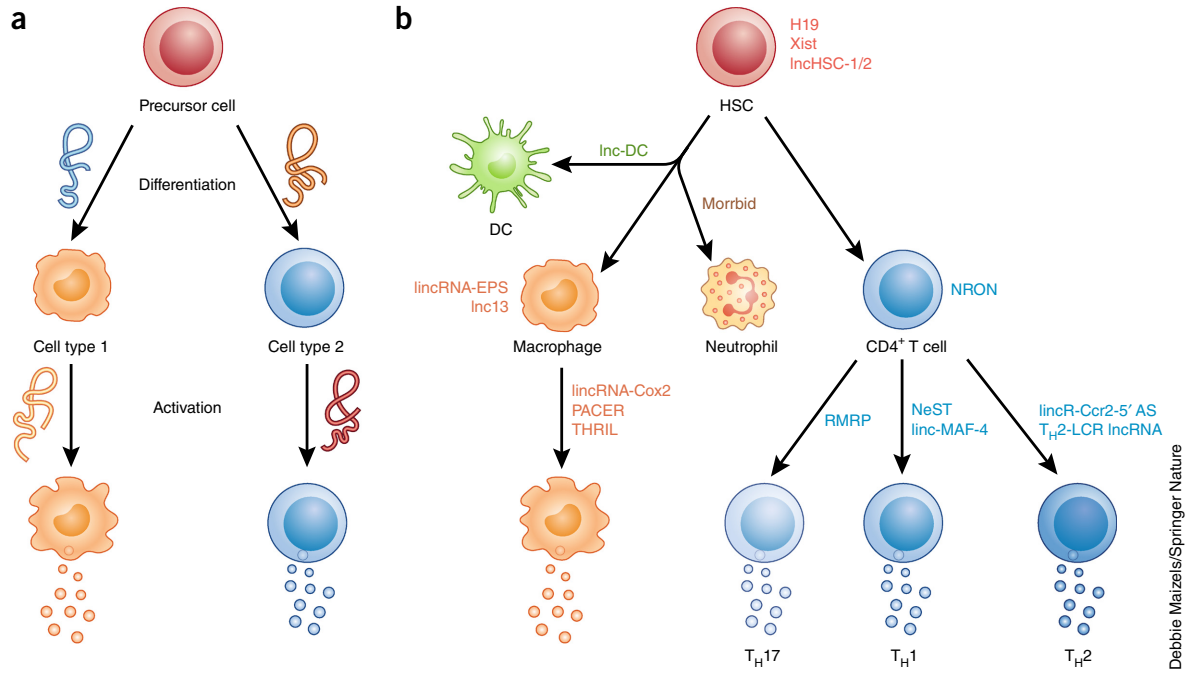

Figure 2 lncRNAs in the differentiation and function of immune cells. (**a**) lncRNAs can regulate the differentiation of precursor cells into mature cells, as well as the subsequent activation of those mature cells. (**b**) The selected lncRNAs presented here control the maturation of HSCs into mature immune-cell subtypes and control the subsequent activation of those cell types.

sequencing, has revealed over 100 bound circRNAs. The majority of these RNAs are exon–intron circRNAs, which contain full-length transcripts that have not been spliced. Many exon–intron circRNAs interact with the small nuclear ribonucleoprotein U1 and increase transcription of their parental spliced mRNA species[45]. The RNA-binding protein MBL ('Muscleblind') also directly binds nascent RNA and increases the expression of circRNAs[46]. Generation of the circRNA muscleblind competes with the generation of canonical mRNA, which suggests that circRNA–protein complexes can regulate the abundance of their linear counterparts.

Cell-type specificity of lncRNAs

The expression of lncRNAs is highly cell-type specific, and this cell-type specificity seems to have been conserved across evolutionary time[47–49]. Several studies have shown that the expression patterns of lncRNAs can be predictive of their tissue-specific functions. For example, the skin-specific lncRNA HOTAIR is expressed in posterior and distal skin fibroblasts and controls the expression of HOX-encoding genes required for the establishment of skin-cell positional identity[20,50]. Another lncRNA expressed in skin is TINCR, which is upregulated during keratinocyte differentiation and is needed to induce genes encoding products associated with barrier formation in the epidermis[51]. Analysis of the expression and function of lncRNAs in ESCs has identified 226 lncRNAs expressed specifically in ESCs[52]. Systematic knockdown of each lncRNA via short hairpin RNA has revealed that approximately 90% of these transcripts have a significant effect on gene expression in ESCs. On average, the expression of 175 protein-coding transcripts was affected by deletion of each lncRNA, which is similar in effect to the knockdown of well-studied regulatory proteins in ESCs. Notably, lncRNAs expressed specifically in ESCs physically interact with chromatin regulators to maintain pluripotency and repress differentiation toward terminal cell lineages. The specificity of lncRNAs is preserved for all classes of lncRNAs, including eRNAs and circRNAs. For example, circRNA expression is regulated at the cellular and developmental levels[10,53–55].

Such principles have also been demonstrated for lncRNAs expressed in immune cells. Gene-expression analysis of more than 40 mouse T cell populations has identified 1,500 lncRNAs, approximately half of which are expressed in a T cell subset–specific manner[56]. In contrast, only 6–8% of mRNAs show such specificity[56]. Similar results have been obtained in comparisons of more-diverse human lymphocyte populations[57]. In that data set, more than 70% of expressed lncRNAs were specific to one lymphocyte subset[57]. Together these findings suggest that the regulation of genes by lncRNAs might be critical for spatial and temporal aspects of the immune response.

lncRNAs in hematopoietic development

Productive immunity relies on the continuous self-renewal of hematopoietic stem cells (HSCs) and their differentiation into terminal functionally specialized types of immune cells. This process is tightly controlled through the coordinated expression of cell-type-specific genes, and evidence suggests that lncRNAs might also have an important role in this process, serving as an intermediate layer of control between cell-extrinsic signals and transcription-factor activity in the nucleus (**Fig. 2** and **Table 1**).

The lncRNA H19 has been shown to serve a critical role in sustaining long-term hematopoietic quiescence[58]. H19 had previously been demonstrated to regulate maternal imprinting during embryogenesis by recruiting complexes of the methyl-CpG-binding protein MBD1 and histone lysine methyltransferase to 'imprinted' target-gene loci[16,17]. Those complexes deposit repressive H3K9-methylation marks to target genes; the marks are then passed to progeny cells, which preserves their gene-expression state. Long-term HSCs have high expression of H19 that is immediately downregulated when long-term HSCs differentiate into short-term HSCs[58]. Notably, deletion of maternal H19 in the hematopoietic compartment results

Table 1 Summary of lncRNAs involved in the differentiation and function of immune cells

Immunity type	lncRNA	Type of cell	Function	References
Progenitor	lncHSC-1	HSC	Depletion results in increased myeloid differentiation	59
	lncHSC-2	HSC	Promotes HSC self-renewal by recruiting E2A to target genes	59
	H19	HSC	Regulates self-renewal of long-term HSCs	58
Innate	lnc-DC	DC	Controls dendritic cell differentiation by promoting the nuclear translocation and function of STAT3	61
	lincRNA-Cox2	DC and macrophage	Regulates expression of genes encoding inflammatory molecules through interaction with hnRNP-A/B and hnRNP-A2/B1	69
	THRIL	Macrophage	Regulates expression of genes encoding inflammatory molecules by recruiting hnRNPL to genomic loci	71
	PACER	Monocyte	Induces *PTGS2* expression by sequestering NF-κB p50 subunits away from the *PTGS2* promoter	72
	Lethe	Fibroblast	Binds nuclear RelA homodimers and prevents their accumulation at target gene loci to restrict excessive inflammatory response	44
	mcircRasGEF1B	Macrophage	Acts as a miRNA sponge that targets ICAM-1 to regulate expression of genes encoding inflammatory molecules	76
	Morrbid	Myeloid cell	Regulates lifespan of neutrophils, eosinophils, and monocytes by repressing *Bcl2l11* transcription	62
	lnc13	Macrophage	Binds to hnRNPD to suppress transcription of immune response genes	78
Adaptive	lincR-Ccr2-5′AS	T_H2 cell	Functions downstream of GATA-3 to regulate transcription of T_H2 cell chemokine-encoding genes	56
	NeST (Tmevpg1)	$CD8^+$ T cell and T_H1 cell	Regulates *Ifng* transcription by binding WDR5 and recruiting transcriptional activation complex to the *Ifng* promoter	80,83
	NRON	T cell	Binds phosphorylated NFAT to sequester it in cytoplasm of resting T cells	89
	Rmrp	T_H17 cell	Promotes assembly of the RORγt–DDX5 complex at genomic loci of genes encoding critical T_H17 cell effector molecules	65
	TH2-LCR	T_H2 cell	Regulates the transcription of the gene cluster encoding T_H2 cell cytokines, including IL-4, IL-5, and IL-13	64
	lncRNA-CD244	$CD8^+$ T cell	Inhibits expression of *IFNG* and *TNF* downstream of CD244	92
	Linc-MAF-4	T_H1 cell	Promotes T_H1 differentiation by repressing expression MAF	57

in a loss of long-term HSCs and a concomitant gain in short-term HSCs. H19-deficient long-term HSCs lose the ability to self-renew and instead enter the cell cycle and differentiate into downstream cell types. That phenotype is mediated at least in part by de-repression of the H19 target gene *Igf2* (which encodes the growth factor IGF2) and increased entry into the cell cycle that is dependent on the transcription factor Foxo3.

To determine the extent to which additional lncRNAs could be involved in early hematopoietic differentiation, a study used RNA-based next-generation sequencing to analyze purified hematopoietic progenitor cells and end-stage cell types to identify non-coding transcripts expressed specifically in HSCs[59]. In total, more than 2,500 lncRNAs were identified in all cell types, and HSCs showed enrichment for 159 lncRNAs, relative to the abundance of these lncRNAs in all cell types. Two HSC lncRNAs, lncHSC-1 and lncHSC-2, were functionally assessed by *in vitro* and *in vivo* knockdown studies. Depletion of lncHSC-1 resulted in altered myeloid differentiation, while depletion of lncHSC-2 resulted in impaired self-renewal of HSC and increased T cell differentiation. Furthermore, enrichment for lncHSC-2 and analysis of chromatin isolation by RNA purification and sequencing identified 264 genomic binding sites that were predominantly promoters and 5′ untranslated regions. lncHSC-2-binding sites show significant enrichment for the DNA-binding motif for the transcription factor E2A, which has critical regulatory roles in HSC and lymphoid-cell development; this suggests that lncHSC-2 might function to recruit E2A to target genes. Indeed, knockdown of lncHSC-2 impairs the recruitment of E2A to selected genomic targets, including *Nln* (which encodes neurolysin), *Slc35c2* (which encodes a solute carrier) and *Itgb2* (which encodes integrin β_2). Together these studies have established a role for lncRNAs in the regulation of HSC differentiation and indicate that lncRNA-mediated gene regulation, in concert with partner transcription-factor activity, might be a strategy frequently employed in hematopoiesis.

lncRNAs in myeloid differentiation

Short-lived myeloid cells, including macrophages and dendritic cells (DCs), are derived from common myeloid progenitor cells in the bone marrow (BM) and depend on cellular signals, cytokine stimulation and key transcription factors to develop along appropriate pathways[60]. Studies suggest that lncRNAs might also have critical roles in these pathways. In particular, two studies have performed in-depth analysis of the roles of the lncRNAs Morrbid and lnc-DC in the regulation of myeloid-cell survival and myeloid-cell differentiation, respectively[61,62].

Morrbid expression is induced as common myeloid progenitor cells differentiate into terminal cells and is highest in short-lived myeloid cell types, including neutrophils, eosinophils and monocytes[62]. Loss of Morrbid in hematopoietic cells leads to a decrease in the number of short-lived myeloid cells due to an increase in the death of terminal cells. Morrbid is found mainly in the nucleus, and RNA analysis in Morrbid-deficient cells has revealed that it acts in *cis* to repress the expression of its neighboring gene *Bcl2l11* (which encodes the pro-apoptotic molecule Bim). Morrbid is brought into close spatial proximity with *Bcl2l11* through chromosome looping and binds to and recruits the Polycomb repressive complex PRC2 to deposit silencing H3K27me3 histone marks on the *Bcl2l11* promoter[62]. Interestingly, expression of Morrbid can be induced *in vitro* by cytokines that signal through the common β-chain receptor, interleukin 3 (IL-3), IL-5 and the cytokine GM-CSF. Consistent with that, eosinophils from patients with hypereosinophilic syndrome, who have high concentrations of IL-5 in their plasma, also express significantly more MORRBID than do eosinophils from healthy control subjects, which suggests that this lncRNA might represent a therapeutic target in human disease.

The expression of lnc-DC is also upregulated during the differentiation of common myeloid progenitor cells or monocytes into DCs and is highest in lymphoid and non-lymphoid classical DC subsets[61]. Knockdown of lnc-DC during DC differentiation impairs the upregulation

of DC-specific genes encoding products involved in antigen presentation, T cell activation and cell migration, including *CD40*, *CD80*, *CD86* and *CCR7* (ref. 61). Accordingly, DCs depleted of lnc-DC are unable to efficiently prime $CD4^+$ T cells or secrete inflammatory cytokines after pathogen stimulation. Unlike Morrbid, lnc-DC seems to function mainly in the cytoplasm, by interacting with the key DC transcription factor STAT3. lnc-DC binds to the carboxyl terminus of STAT3 and promotes its phosphorylation and translocation into the nucleus. Analysis of additional proteins that bind to STAT3 in DCs has identified the tyrosine-phosphatase SHP1. Depletion of lnc-DC leads to more interactions between STAT3 and SHP1, less phosphorylation of STAT3 at Tyr705 and less resultant translocation of STAT3 to the nucleus. In contrast, overexpression of lnc-DC decreases interactions between STAT3 and SHP1 and results in more translocation of STAT3 to the nucleus. Therefore, lnc-DC regulates DC differentiation by controlling the post-translational modification of a critical DC transcription factor. Interestingly, the ortholog of lnc-DC in mice and non-human primates (Wdnm1-like) has been reported to encode a small secreted protein[63]. Whether lnc-DC exerts its function on DC differentiation in these organisms through conserved lncRNA–STAT3 binding or through unknown functions of the Wdnm1-like protein remains to be studied.

lncRNAs in $CD4^+$ T cell differentiation

The differentiation of $CD4^+$ T cells into helper T cell subsets is critical for the initiation of pathogen-specific adaptive immune responses, and several studies have indicated that lncRNAs might be critical molecular determinants of this process. Analysis of RNA expression in T cell subsets has identified the lncRNA lincR-Ccr2-5′AS as being specifically expressed in the T_H2 subset of helper T cells[56]. Expression of lincR-Ccr2-5′AS is regulated by the signature T_H2 cell transcription factor GATA-3, and knockdown of lincR-Ccr2-5′AS has revealed that it controls the expression of nearly 1,200 genes, a group that overlaps genes dependent on GATA-3. In particular, lincR-Ccr2-5′AS upregulates a cluster of genes encoding key T_H2 cell chemokines (*Ccr1*, *Ccr2*, *Ccr3* and *Ccr5*), and T_H2 cells from which lincR-Ccr2-5′AS is depleted display an impaired ability to migrate to the lungs after *in vivo* transfer, relative to that of control cells with sufficient lincR-Ccr2-5′AS. A study of human T_H2 cells has identified a similar regulatory function for lncRNA TH2-LCR, which is transcribed from the *RAD50* locus (which encodes a double-strand-break repair protein) and regulates the transcription of its neighboring gene cluster encoding T_H2 cell cytokines that contains *IL4*, *IL5*, and *IL13* (ref. 64).

The differentiation of T_H17 cells also depends on the transcriptional function of a lncRNA associated with a signature transcription factor. The DEAD-box RNA helicase DDX5 has been identified as a functional partner of the T_H17 cell transcription factor RORγt[65]. T_H17 cells generated *in vitro* from DDX5-deficient mice produce less IL-17A than do wild-type cells and have lower expression of RORγt's target genes, including *Il17a*, *Il17f*, *Il22* and *Il23r*. Accordingly, transfer of DDX5-deficient T cells *in vivo* fails to induce T_H17 cell–driven organ inflammation in a mouse model of autoimmune colitis and results in a lower abundance of intestinal $CD4^+RORγt^+$ T cells co-expressing IL-17A and interferon-γ (IFN-γ). Strikingly, the function of DDX5 relies on the RNA-helicase component of the protein, which suggests that critical RNAs mediate its function. Indeed, immunoprecipitation of DDX5 has demonstrated an association with the lncRNA Rmrp ('RNA component of the mitochondrial-RNA-processing endoRNase'). In T_H17 cells, Rmrp localizes to the nucleus and promotes assembly of the RORγt–DDX5 complex at genomic loci of genes encoding critical T_H17 cell effector molecules, including *Il17a* and *Il17f*. Furthermore, knockdown of Rmrp RNA in human T cells leads to compromised secretion of cytokines from T_H17 cells, and mutant forms of Rmrp cause cartilage-hair hypoplasia, a congenital disease associated with immunological dysfunction[66,67].

Finally, in T_H1 cells, the lncRNA linc-MAF-4 represses expression of the T_H2 cell transcription factor MAF to promote T cell differentiation toward the T_H1 cell lineage[57]. The genomic regions of linc-MAF-4 and *MAF* form long-distance chromosome contacts, and linc-MAF-4 recruits the chromatin remodelers EZH2-and LSD1 to place repressive chromatin marks on the *MAF* promoter and repress its transcription. As a result, knockdown of linc-MAF-4 skews T cell differentiation toward the T_H2 cell lineage. Together these studies of helper T cells have demonstrated that lncRNAs can serve as critical regulators of cell-type-specific effector programs, often in concert with critical lineage-specifying transcription factors.

lncRNAs in the activation of inflammation

The innate immune response is driven by several functionally distinct myeloid lineages, including DCs and macrophages, which recognize pathogens through pattern-recognition receptors and initiate the immune response. Several studies have identified critical functions for lncRNAs in this response at the level of gene transcription, post-transcriptional protein modification and chromatin accessibility. Globally, the expression patterns and functions of lncRNAs are highly specific in pathogen-response pathways[68,69] (**Fig. 2** and **Table 1**).

Among such signal-specific lncRNAs, the expression of lincRNA-Cox2 is induced more than 1,000-fold following activation of the Toll-like receptors TLR1 and TLR2 or activation of TLR7 and TLR8, but not after activation of TLR3, in bone-marrow-derived DCs and bone-marrow-derived macrophages[69]. Furthermore, transcription of lincRNA-Cox2 is dependent on signaling through the TLR adaptor MyD88 and the transcription factor NF-κB. Accordingly, depletion of lincRNA-Cox2 during inflammatory stimulation leads to altered expression of greater than 500 genes encoding inflammatory molecules, which shows that lncRNAs can coordinate the specific expression of large gene sets. In addition, lincRNA-Cox2 also has a substantial effect on gene expression in the absence of stimulation, repressing the activation of hundreds of genes encoding inflammatory molecules. Genes regulated by lincRNA-Cox2 include those encoding molecules involved in the inflammatory response (*Tlr1*, *Il6* and *Il23a*) and chemokines (*Ccl5* and *Cx3cl1*), as well as interferon-stimulated genes (*Irf7*, *Oas1a* and *Oas1l*). The proposed mechanism for the regulation of genes by lincRNA-Cox2 is through its interactions with the heterogeneous nuclear ribonucleoproteins hnRNP-A/B (encoded by *Hnrnpab*) and hnRNP-A2/B1 (encoded by *Hnrnpa2b1*)[69]. hnRNPs are large nuclear RNA-binding proteins that participate in the splicing, stabilization and transport of RNA and the transcription and translation of genes[70]. In association with lncRNAs, hnRNPs have been shown to repress gene expression in the context of lncRNAs induced by the tumor suppressor p53 (ref. 42). Depletion of *Hnrnpab* and *Hnrnpa2b1* in bone-marrow-derived macrophages results in the dysregulation of a set of genes encoding inflammatory molecules that overlaps the set of genes dysregulated by knockdown of lincRNA-Cox2. Furthermore, knockdown of *Hnrnpab* or *Hnrnpa2b1* in bone-marrow-derived macrophages overexpressing lincRNA-Cox2 reverses the repression of *Ccl5*, suggestive of a cooperative function for these molecules.

The lncRNA THRIL is another regulator of TLR signaling that also functions through RNA–protein interactions with an hnRNP[71]. THRIL was identified in an unbiased gene-expression screen of the THP-1 human macrophage cell line that identified 159 candidate lncRNAs

induced by activation of TLR2. Transcriptome-wide analysis of THP-1 cells depleted of THRIL identified more than 300 genes downstream of TLR2 that were expressed differentially in these cells relative to their expression in THP-1 cells with sufficient THRIL, including *TNF*, *IL8*, *CXCL10*, *CCL1* and *CSF1*. Similar to the function of lincRNA-Cox2, THRIL also regulates the expression of genes encoding inflammatory molecules at steady state. Mass-spectrometry studies have identified a specific interaction between THRIL and the RNA-binding protein hnRNPL, and knockdown of *Hnrnpl* results in a decrease in production of the cytokine TNF by macrophages, which suggests that these two molecules function as a complex. Furthermore, analysis of THRIL via chromatin isolation by RNA purification and sequencing has demonstrated an association between this lncRNA and the *TNF* genomic locus, and chromatin-immunoprecipitation analysis of hnRNPL resulted in enrichment for a region within the *TNF* promoter in a THRIL-dependent manner. Together these data demonstrate a specific action of THRIL in recruiting hnRNPL to a specific genomic locus where it can affect TLR-stimulated gene transcription.

The lncRNA PACER also acts as a decoy for the NF-κB signaling pathway[72]. PACER expression is induced by stimulation with lipopolysaccharide, and it specifically regulates its neighboring gene *PTGS2*, which encodes COX-2 ('cyclooxygenase 2'), a known mediator of the inflammatory response[73]. Mechanistically, PACER acts by binding the NF-κB subunit p50 and sequestering it away from the *PTGS2* promoter, where p50–p50 homodimers can repress transcription. The resulting decrease in the availability of free p50 subunits leads to the alternative generation of transcription-activating heterodimers of p50 and the NF-κB component p65 (RelA)and the recruitment of transcription-pre-initiation complexes to the *PTGS2* promoter. A similar 'decoy lncRNA archetype' has been observed in studies of the lncRNA Jpx during the process of X-chromosome inactivation. Jpx turns on Xist expression by binding to and diminishing the activity of the repressive transcription factor CTCF ('CCCTC-binding factor') at the onset of X-chromosome inactivation[74].

Potential roles for circRNAs in regulating genes encoding inflammatory molecules have also been identified. One study has shown that the delivery of purified circRNA stimulates a greater innate immune response than that stimulated by linear RNA with the same sequence[75]. Likewise, circRNA produced in the cell by foreign introns induces the expression of genes of the immune system. However, the same circRNA generated by endogenous introns is recognized as 'self' and is associated with a set of diverse RNA-binding proteins.

Another investigation has catalogued circRNA expression in macrophages and has found nearly 2,000 circRNAs that are induced following TLR4 stimulation[76]. One such circRNA, mcircRasGEF1B, is responsive to lipopolysaccharide, is stably expressed and is dependent on NF-κB[76]. Interestingly, upregulation of mcircRasGEF1B is cell type specific across 15 cell lines; for example, stimulation of mouse embryonic fibroblasts with lipopolysaccharide increases the expression of *Ccl5* but not that of mcircRasGEF1B. Knockdown of mcircRasGEF1B in lipopolysaccharide-stimulated macrophages has revealed a function for this circRNA in the expression of genes encoding inflammatory molecules: it results in a decrease in the abundance of mRNA and protein of the inflammatory adhesion molecule ICAM-1, which is required for the homing of leukocytes to inflammatory sites. mcircRasGEF1B localizes to the cytoplasm and regulates the stability of *ICAM1* mRNA rather than its transcription, and the authors speculate that mcircRasGEF1B might act as a 'sponge' for miRNAs targeting *ICAM1* (ref. 76). Notably, the sheer number of circRNAs induced in a cell-type-specific manner in macrophages and preliminary analysis of the functional consequences of the loss of one circRNA suggest that these RNAs might serve important functions in immunological regulation that remain to be explored.

lncRNAs restrict inflammatory responses

Several studies have identified protective functions for lncRNAs in restricting excessive inflammatory responses. The pseudogene lncRNA Lethe was identified as a gene substantially induced in mouse embryonic fibroblasts following stimulation with IL-1β and TNF[44]. Induction of Lethe expression is dependent on NF-κB activity; however, knockdown of Lethe results in the upregulation of NF-κB targets, which suggests a negative regulatory function. Consistent with that hypothesis, overexpression of Lethe leads to a decrease in the activity of an NF-κB reporter. RNA-immunoprecipitation analysis of the NF-κB component p65 (RelA) has shown that Lethe acts by specifically binding nuclear homodimers of RelA and preventing their accumulation at target-gene loci, including *Nfkbia*, *Il6* and *Il8*. Therefore, Lethe acts as a decoy receptor for NF-κB and provides negative feedback to limit inflammation in response to activation of the immune system.

Two studies have described functions for lncRNAs in restraining the expression of genes encoding inflammatory molecules through direct interactions with chromatin[77,78]. The lncRNA lincRNA-EPS is expressed in erythrocytes, macrophages and DCs, and its expression in macrophages and DCs is downregulated after activation of the innate immune system. While lincRNA-EPS-deficient mice do not display defects in erythroid development, these mice show profound 'hyper-activation' of immune responses *in vivo*. Bone-marrow-derived macrophages generated from lincRNA-EPS-deficient mice that are then left resting or are stimulated with TLR ligands show higher expression of genes encoding inflammatory molecules, including *Il6*, *Cxcl10*, *Ccl4* and *Irg1*, than that of their wild-type counterparts[77]. Consistent with that, lincRNA-EPS-deficient macrophages display a greater content of H3K4me3 (a mark associated with transcriptionally active or poised promoters), chromatin accessibility and nucleosome-free chromatin at the promoters of genes encoding immune-response molecules than that of wild-type cells. Such epigenomic changes are observed mainly in resting macrophages and are lost after stimulation of the immune system, consistent with the expression kinetics of the RNA. lincRNA-EPS is localized to the nucleus, and detailed biochemical studies have shown that it binds to hnRNPL to exert these regulatory effects. Together these findings indicate that lincRNA-EPS acts directly on chromatin to restrain the expression of response genes in myeloid cells by controlling chromatin accessibility and nucleosome positioning.

The lncRNA lnc13 also functions to repress the transcription of genes of the immune system in a manner similar to lincRNA-EPS[78]. lnc13 is expressed in macrophages and is downregulated after activation by TLR4. In resting cells, lnc13 localizes to the nucleus and functions in an RNA–protein complex with hnRNPD and the histone deacetylase HDAC1 to suppress the transcription of a distinct set of genes encoding immune-response molecules, including *Myd88*, *Stat1*, *Stat3* and *Tnf*. Moreover, lnc13 harbors single-nucleotide polymorphisms associated with celiac disease, and a form of lnc13 transcribed *in vitro* containing alleles linked to celiac disease shows an impaired ability to bind hnRNPD in cell lines. Accordingly, patients with celiac disease have lower expression of lnc13 and higher expression of lnc13-regulated genes, which suggests that noncoding single-nucleotide polymorphisms that affect the expression and function of lnc13 might underlie the development of inflammatory disease.

Finally, the lncRNA NKILA has been shown to negatively regulate NF-κB signaling, but at the level of post-transcriptional control[79].

NKILA is substantially induced in breast cancer cells following stimulation with IL-1β or TNF, and, similar to studies of Lethe, knockdown of NKILA enhances NF-κB activity, and its overexpression inhibits NF-κB. Biochemical studies have shown that NKILA forms an RNA–protein complex with NF-κB and its negative regulator IκB ('inhibitor of NF-κB') and directly masks phosphorylation motifs of IκB from the kinase IKK ('IκB kinase'). This association prevents the phosphorylation and degradation of IκB by IKK and results in the retention of NF-κB in the nucleus.

lncRNAs in T cell activation

One of the first examples of lncRNA function in the immune response *in vivo* was provided by studies analyzing the lncRNA NeST (Tmevpg1)[80]. NeST was identified as an intergenic lncRNA located in a region previously associated with persistence of neurotropic Theiler's virus infection[81–83]. B10.S mice clear infection with Theiler's virus, while SJL/J mice are susceptible to persistent infection, and the susceptibility phenotype has been narrowed to the genomic region containing the genes encoding NeST (*Tmevp3*), IFN-γ (*Ifng*) and IL-22 (*Il22*)[84–86]. Gene-expression analysis has revealed that NeST expression is much higher in T cells from mice that retain the locus derived from the SJL strain, and transgenic expression of that locus confers onto B10.S mice susceptibility to Theiler's virus[80]. Additional studies have shown that NeST is expressed in $CD8^+$ T cells, $CD4^+$ T_H1 cells and natural killer cells and that its expression depends on combinatorial activity of the transcription factors T-bet, STAT4 and NF-κB[56,87,88]. Those results, combined with the proximity of NeST to the *Ifng* locus, suggest that NeST might affect responses to pathogens through its regulation of *Ifng* transcription. Indeed, congenic or transgenic expression of NeST increases IFN-γ production in activated $CD8^+$ T cells[80]. Mechanistically, NeST induces *Ifng* transcription by binding to WDR5 and recruiting the transcription-activation complex to the *Ifng* promoter sequence in *trans*. Those studies identified a critical role for a lncRNA in T cell function and in the systems-level immune response to viral infection *in vivo*. Future studies of inbred mouse strains with deletion of NeST are needed to determine whether NeST acts specifically at a single locus or at many loci to regulate T cell function.

Finally, lncRNAs also have a role in restricting excessive activation of T cells (**Fig. 2** and **Table 1**). An *in vitro* screen using short hairpin RNA has identified the lncRNA NRON as a repressor of activation of the calcium-dependent transcription factor NFAT[89]. Co-immunoprecipitation studies have shown that NRON nucleates a large RNA–protein complex in resting T cells, which sequesters the phosphorylated form of NFAT in the cytoplasm[89,90]. The RNA–protein complex also includes the calmodulin-binding protein IQGAP1, the kinase LRRK2, and the nuclear-transport factor karyopherin β1. After stimulation of the T cell antigen receptor, NFAT is released from the NRON complex and is dephosphorylated by the Ca^{2+}-calmodulin–dependent phosphatase calcineurin and then is transported into the nucleus, where it can activate transcription. Accordingly, depletion of NRON results in increased dephosphorylation of NFAT and translocation of NFAT to the nucleus and production of cytokines following the activation of T cells[90]. NRON is proposed to function *in vivo*, on the basis of studies of mice deficient in LRRK2 (ref. 91). LRRK2 stabilizes the NRON–NFAT association, and loss of LRRK2 in mice leads to increased translocation of NFAT to the nucleus and the induction of downstream target genes. Notably, LRRK2 deficiency exacerbates experimental colitis in mice, which results in greater weight loss and clinical symptoms during disease, more inflammatory infiltration of the colon and higher expression of inflammatory cytokines.

In $CD8^+$ T cells, the lncRNA lncRNA-CD244 performs a function similar to that noted above, in restricting the activation of T cells[92]. Expression of lncRNA-CD244 is induced by signaling through the T cell inhibitory receptor CD244 (2B4) and mediates the repression of genes encoding the inflammatory molecules IFN-γ and TNF. Similar to linc-MAF-4, lncRNA-CD244 physically interacts with EZH2 and recruits it to the *IFNG* and *TNF* promoters for the deposition of repressive chromatin marks. Notably, knockdown of lncRNA-CD244 improves $CD8^+$ T cell function *in vivo.* In a model of infection with *Mycobacterium tuberculosis*, the transfer of $CD8^+$ T cells depleted of lncRNA-CD244 resulted in significantly lower organ bacterial burden in mice than did the transfer of lncRNA-CD244-sufficient (control) T cells[92].

Chemical modification of RNA

The developing field of epitranscriptomics (**Fig. 3**), which comprises RNA editing and RNA modification, has demonstrated the dynamic regulation of RNA activity on the basis of cellular changes and signals. The vast majority of RNA molecules experience some form of editing or modification through the course of their lifetime, either during transcription or after transcription[93–98]. The presence of specific modifications or base changes can greatly alter the function of the unmodified transcript, including the recruitment of proteins, a change to the 'read-out' identity of the base and adjustments to the higher-order structure of the RNA[99]. RNAs are also under immunosurveillance, and chemical modifications of RNA molecules affect both their immunogenicity and their function[100]. The precise placement of modifications can mark the RNA as nonpathogenic, which masks the nucleic acids from cellular sensors and suppresses immune responses to the RNA. Extensive work has been conducted on the motifs necessary for RNA editing or modification and the cell and tissue specificity of RNA editing[101–103], but much remains unknown about the functions of such modifications. The dysregulation of RNA editing and modification has greatly varying results, from no perceptible change in phenotype[93,104] to a dramatic block in development[105–107]. We will briefly highlight here a few studies that are relevant to lncRNAs and circRNAs.

The most common type of RNA editing in mammals is the deamination of adenosine to inosine in Alu repetitive elements found in introns or intergenic regions[108]. Adenosine and inosine have different base-pairing 'preferences', so the editing event changes RNA structure[109] by unwinding previously double-stranded regions *in vivo*[110]. This can effectively eliminate a recognition pattern that commonly triggers autoimmunity, since cytosolic RNA sensors such as MDA5 detect long double-stranded sections as pathogenic[111]. While the majority of editing occurs in protein-coding genes (65% of mRNAs), a substantial portion also occurs in ncRNAs (10%)[101,112]. The adenosine-deaminase (ADAR) family of enzymes catalyzes the adenosine-to-inosine deamination and consists of two active members: ADAR1, which is found in both nucleus[113–115] and cytoplasm[113]; and ADAR2, which is found only in the nucleus[112,116]. Dysregulation of these enzymes has been linked to several diseases, including schizophrenia, amyotrophic lateral sclerosis and cancer, and mice lacking these enzymes have severely deleterious phenotypes, which shows that editing is a necessary part of normal cellular processes[117–120].

Researchers are now working toward understanding the effects of editing on specific lncRNAs. Observations that ADAR affects disease phenotype[99,101,121–123] and controls tissue-specific expression of lncRNAs[103] suggest a wide range of cellular events in which ADAR participates. One mechanism by which RNA editing regulates gene expression is that the deamination reaction on lncRNAs changes the

ability of lncRNAs to bind miRNAs[99]. The lncRNA PCA3 is an example of this phenomenon: ADAR acts on the complex formed by PCA3 and its corresponding antisense gene, *PRUNE2*, to regulate *PRUNE2* expression[124]. The PCA3–*PRUNE2* complex is sensitive to activation by androgen receptors and affects cellular proliferation and transformation. Thus, the ADAR-mediated editing event controls the ability of PCA3 to form a complex with *PRUNE2* and regulates the progression of prostate cancer[124]. Editing by ADAR1 has also been shown to control development of the B cell lineage, regulate a cell's detection of foreign nucleic acids and modulate interferon responses[102,125–127].

ADAR has been linked to regulation of the abundance of circRNAs, since ADAR targets intronic regions that flank exons, which can splice to form circRNAs. The double-stranded regions in introns are hyperedited by ADAR[108], which results in decreased circRNA expression[128,129]. Likewise, Alu elements are also present in introns and are targeted by ADAR enzymes as well[109,130–132]. The frequency of adenosine-to-inosine editing is based on how much ADAR enzyme is present, so changes in ADAR expression lead to dynamic alterations in editing and circRNA production[101]. Additional observations suggest that the amount of circRNA changes in disease settings[133]; much is still unknown about the functions of specific circRNAs, as well as whether the overall abundance of circRNAs is relevant.

Over 100 such modifications have been identified, with the majority found on rRNA and tRNA[93,134]. N6-methyl-adenosine (m^6A) is the most abundant RNA modification on mammalian lncRNA and mRNA. The m^6A modification has been linked to a plethora of cellular functions, including translation, splicing, localization, stability, differentiation, regeneration and immunity, among others[107,135–139]. The placement of the m^6A modification has been shown to be sequence specific and shows enrichment near the 5′ untranslated region, stop codon and 3′ untranslated region of RNA[94,95]. Since the modification frequency is much lower than the appearance of the consensus sequence, this suggests that there are other levels of regulation for placement of the methyl group.

While m^6A was initially thought to be a static modification, the discovery of enzymes that read, write and erase m^6A suggests that m^6A is dynamic[105,140]. METTL3 and METTL14 are the core components of the complex that adds this modification in vertebrates[141–143], while proteins with the YTH domain[139,144], the ribonucleoprotein hnRNPC[140] and the translation-initiation factor eIF3 (ref. 145) recognize the modification or its corresponding secondary-structure change. The enzymes FTO[144] and ALKBH5 (ref. 146) remove the methylation. The combinatorial activity of these proteins provides finely tunable and precise control of the methyl-group placement. An important concept related to reading of the m^6A modification is that m^6A induces a structural switch in the secondary structure of RNA[140,147]. Each m^6A imposes a free-energy cost of ~1.5 kcal/mol on an RNA duplex, which favors the adoption of a single-stranded conformation by the RNA[148]. The switch from double-stranded RNA to single-stranded RNA opens binding sites for RNA-binding proteins and decreases the abundance of double-stranded RNA regions that triggers innate immunity.

One of the cellular functions of m^6A might be to signal that the RNA is nonpathogenic in mammalian systems, similar to other modifications, including inosine from ADAR activity[149]. The presence of m^6A on transcripts generated *in vitro* prevents recognition by TLR3, TLR7 and TLR8, whereas unmodified versions of such transcripts stimulate all three receptors[149]. When cytokine-derived DCs are stimulated with *in vitro*–synthesized transcripts, RNAs containing m^6A induce less secretion of TNF and IL-12 than do unmodified RNAs[149]. However, transcripts containing m^6A stimulate the secretion of TNF

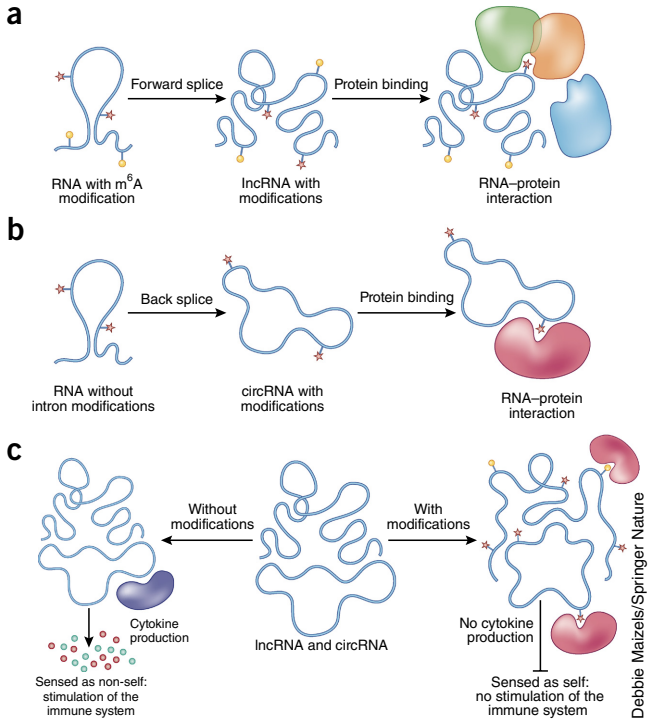

Figure 3 New mechanisms: RNA modification and circular RNA. (**a**) RNA with m^6A and intron modifications forms a secondary structure for forward splicing into linear RNA. (**b**) RNA with m^6A but no intron modification forms a secondary structure for back splicing into circRNA. (**c**) The presence or lack of modifications leads to differential recognition by the immune system.

from primary blood DCs, but the inclusion of pseudouridine (Ψ) on the same transcript ablates this response, which suggests that primary DCs have different recognition pathways for m^6A and Ψ. m^6A-modified RNAs diminish the ability of monocyte-derived DCs to induce cell-surface expression of CD80, the marker CD83, CD86 and major histocompatibility complex class II molecules. The ability of modified RNAs to induce cytokine secretion from DCs depends on the subclass of cell and the nature and level of modification, with a general trend toward the suppression of stimulatory ability by modified RNAs[149].

Nucleotide modifications represent one of the most ancient markers of the immune system; they allow the host to distinguish between its own nucleic acids and those from invading pathogens[149–151]. Studies investigating the scope and frequency of RNA modifications on circRNAs[152,153] suggest that the presence of m^6A or other modifications might be an additional level of regulation whereby mammalian cells distinguish endogenous circRNAs versus invading pathogens containing circRNAs.

Concluding remarks

The immune system is remarkably versatile, simultaneously mounting robust responses to pathogenic invaders while maintaining organ homeostasis and preventing autoimmunity. To distinguish between exogenous threats and endogenous normality, the immune system responds to changes in environment through macromolecules that detect, amplify and react to cellular stimuli. lncRNAs have now been demonstrated to be active participants in this process that participate in multiple stages of immune-cell development and in pathogen-response pathways. Notably, individual lncRNAs can act functionally through modular domains and often link protein activity to DNA or RNA targets through interactions with both. Moreover, dysregulation

of these functions has been demonstrated in the setting of human autoimmune disease.

A promising area of investigation includes elucidation of the roles, types and abundance of circRNAs in various immunological contexts. While there is currently limited understanding of circRNAs that are involved in the development and function of the immune system, we speculate that there are probably many circRNAs that act similarly to lncRNAs. It is possible that the current methods for finding and characterizing lncRNAs might have missed circRNAs, since the bioinformatics process for specifically detecting circRNAs is still nascent. Until recently, circRNAs were disregarded as products of low frequency of aberrant splicing or incorrect annotation of computational processing. Additional studies might provide further insight into the roles of circRNA in the immune system.

Another emerging area of investigation is the dynamic nature of the epitranscriptome, which will be greatly aided by the development of new technologies that will improve the functional delineation of RNAs *in vitro* and *in vivo*. Post-transcriptional modifications can affect RNA function and structure and how the cellular machinery recognizes RNA sequences. The ability to precisely place or remove RNA modifications in response to environmental signals allows rapid adjustments to modulate gene expression and affect other cellular changes. The relatively short half-lives of lncRNAs (on the order of hours)[154] means quick turnover of both the RNA and its modifications. While circRNAs persist for several orders of magnitude longer than the lifetime of typical lncRNAs, little is known about the existence or effects of modifications on circRNA. Continued investigation into circRNAs and lncRNAs will yield new discoveries and reveal insights into better therapeutics for treating human disease and infection.

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The authors declare competing financial interests: details are available in the online version of the paper.

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