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A Long Noncoding RNA Mediates Both Activation and Repression of Immune Response Genes

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An inducible program of inflammatory gene expression is central to antimicrobial defenses. This response is controlled by a collaboration involving signal-dependent activation of transcription factors, transcriptional co-regulators, and chromatin-modifying factors. We have identified a long noncoding RNA (lncRNA) that acts as a key regulator of this inflammatory response. Pattern recognition receptors such as the Toll-like receptors induce the expression of numerous lncRNAs. One of these, lincRNA-Cox2, mediates both the activation and repression of distinct classes of immune genes. Transcriptional repression of target genes is dependent on interactions of lincRNA-Cox2 with heterogeneous nuclear ribonucleoprotein A/B and A2/B1. Collectively, these studies unveil a central role of lincRNA-Cox2 as a broad-acting regulatory component of the circuit that controls the inflammatory response.

The innate immune system coordinates host defenses through germ line-pattern recognition receptors (e.g., Toll-like receptors (TLRs)), which recognize microbial products and induce the expression of hundreds of proteins involved in antimicrobial defense and adaptive immunity (1–3). Recent studies have identified thousands of long noncoding RNAs (lncRNAs) in mammalian genomes (4–9) that regulate gene expression in different biological processes [reviewed in (5)]. lncRNAs are differentially regulated in virus-infected cells (10) and in dendritic cells after lipopolysaccharide (LPS) stimulation (4). Recently, the lncRNA NeST was shown to control susceptibility to Thielers virus and Salmonella infection in mice through epigenetic regulation of the interferon-γ (IFN-γ) locus (11, 12). Although lncRNAs can be induced in innate immune cells (4, 10), whether lncRNAs act as regulators of gene expression in innate immunity is unknown.

To identify lncRNAs transcribed during the innate immune response, we conducted whole-transcriptome analysis (RNA-seq) (6) of mouse bone marrow–derived macrophages (BMDMs) stimulated with the synthetic bacterial lipopeptide Pam3CSK4, a Th2 ligand (Fig. 1A). Pam3CSK4 induced the transcription of numerous protein-coding genes involved in the immune response (Fig. 1A, inner track), as well as 62 lncRNAs (Fig. 1A, outer track, and table S1). The most significantly induced lncRNAs tended to occur in chromosomal regions where we also detected higher expression of immune genes, which suggests that these genes are co-regulated. lincRNA-Cox2 was among the most highly induced lncRNAs and is proximal to the prostaglandin-endoperoxide synthase 2 (Ptgs2) locus.

Fig. 1. lincRNA-Cox2 expression is induced by Th ligands in a MyD88- and NF-κB–dependent manner. (A) The Circos plot shows genome-wide differential expression (RNA-seq) between untreated and Pam3CSK4 (Th1/2)–stimulated (5 hours) BMDMs. The inner track shows log2 relative change for protein-coding genes that are classified into immune genes (red; see supplementary materials) and other genes (blue). The outer track shows log2 relative change for all lncRNAs. lincRNA-Cox2 is highlighted in red on chromosome 1 (arrow). (B) lincRNA-Cox2 encodes three splice variants. (C and D) RNA was extracted from primary BMDMs stimulated with LPS for 0, 2, 5, or 24 hours. Expression levels of lincRNA-Cox2 (C) and Ptgs2 (Cox2) (D) were examined by qRT-PCR and expressed relative to time 0 hours (which was set to 1, indicated by arrow). (E and F) Induction of lincRNA-Cox2 and Ptgs2 (Cox2) after piperine and MyD88+ expression relative to time 0 hours (which was set to 1, indicated by arrow). (G) lincRNA-Cox2 expression was examined by qRT-PCR and expressed relative to time 0 hours (which was set to 1, indicated by arrow). (H) lincRNA-Cox2 expression was examined by qRT-PCR and expressed relative to time 0 hours (which was set to 1, indicated by arrow).
of lincRNA-Cox2 (fig. S3, A and B). Induction of lincRNA-Cox2 and its neighboring gene Ptgs2 (Cox2) was dependent on the Tlr adaptor protein MyD88 (Fig. 1, E and F) and transcription factor NF-κB signaling (Fig. 1, G and H).

We next examined the protein-coding capacity of lincRNA-Cox2 by assessing its association with polysomes within cells. BMDMs were treated with cycloheximide to trap ribosomes on RNA molecules and either left untreated or pre-treated with EDTA (disrupting all RNA-protein interactions) or with harringtonine (specifically disrupting translation). Cell lysates were fractionated and RNA analyzed by qPCR (13–15). We compared Gapdh mRNA with lincRNA-Cox2 and another lincRNA, lncRNA-Eps, which had previously been shown to be noncoding (16). As expected, Gapdh mRNA sedimented with a high velocity through the sucrose gradient because it was associated with polysomes. In contrast, lincRNA-Cox2 and lncRNA-Eps remained in lighter fractions (fig. S4). Treatment with EDTA or harringtonine resulted in a shift of Gapdh mRNA, but not lincRNA-Cox2 or lncRNA-Eps, from the higher-velocity to the lower-velocity fractions. Furthermore, most of the open reading frames identified in lincRNA-Cox2 were found to have poor Kozak strength (fig. S5). Collectively, these studies indicate that lincRNA-Cox2 is unlikely to encode a protein product.

We next generated BMDM cell lines in which lincRNA-Cox2 expression was silenced by shRNA (Fig. 2A). Silencing of lincRNA-Cox2 did not alter expression of Ptgs2 (Cox2) (fig. S6). To identify potential targets of lincRNA-Cox2, we conducted RNA-seq in both control and lincRNA-Cox2–silenced cells before and after stimulation with Pam3CSK4. Silencing of lincRNA-Cox2 increased the expression of 787 genes by a factor of 3 or greater in unstimulated cells (table S2). A Gene Ontology (GO) enrichment analysis indicated that genes related to the immune response were significantly overrepresented in these up-regulated genes (Fig. 2B and table S3). This gene set included chemokines (Ccl5, Cx3cl1), chemokine receptors (Ccr1), and IFN-stimulated genes (ISGs) (Irf7, Oas1a, Oas1l, Oas2, Ifi204, and Ifi204) (Fig. 2B). In the same cells stimulated with Pam3CSK4, silencing of lincRNA-Cox2 resulted in attenuated expression of 713 genes by a factor of 3 or greater (table S4). Examples of these genes include Tlr1, Il6 (interleukin-6), and Il23a.

We confirmed these findings by means of an RNA profiling technology to simultaneously analyze mRNA expression levels of differentially regulated genes (nCounter; Nanostring, Seattle, WA). In unstimulated cells, silencing of lincRNA-Cox2 led to a marked increase in expression of Irf7, Ccl5 (Rantes), and ISGs relative to control cells (Fig. 2C, columns 1 and 2, and fig. S7), whereas the Pam3CSK4–induced expression of Tlr1 and I6 was attenuated (Fig. 2C, columns 3 and 4). We confirmed these findings in three independent shRNA lines by measuring protein levels for Ccl5 (Rantes) and I6 (Fig. 2, D and E). Heat map representation of differentially regulated genes performed on RNA extracted from control or lincRNA-Cox2 knockdown cells stimulated with Pam3CSK4 for 5 hours. (F and G) Cells were stimulated with Pam3CSK4 for 24 hours, and Ccl5 (F) and Il6 (G) levels were measured in lincRNA-Cox2 knockdown cells by enzyme-linked immunosorbent assay (ELISA) (n.d., not detected). (H to I) lincRNA-Cox2 was silenced in IFN α/β receptor (IFN α/β R) KO cells. Expression levels of lincRNA-Cox2 (H), Ccl5 (G), Irf7 (H), and Ifi204 (I) were measured by qRT-PCR. Data represent means ± SD from three independent experiments.

Fig. 2. lincRNA-Cox2 is a major regulator of immune genes. (A) qRT-PCR was carried out on BMDMs stably expressing lentiviral shRNA specific to lincRNA-Cox2 (shRNA) or a control shRNA. Expression of lincRNA-Cox2 was measured. (B) RNA-seq analysis on lincRNA-Cox2 knockdown or control shRNA BMDMs that were either stimulated with Pam3CSK4 or unstimulated. The heat map shows mRNA levels for genes annotated in GO as immune genes. These genes are among the top 50 up-regulated immune genes in unstimulated cells when lincRNA-Cox2 was silenced or the top 50 down-regulated immune genes in stimulated cells when lincRNA-Cox2 was silenced. The 100 genes were ranked according to absolute values of log2 relative change; the top 80 differentially expressed genes are displayed. (C) Heat map representation of differentially regulated genes performed on RNA extracted from control or lincRNA-Cox2 knockdown cells by enzyme-linked immunosorbent assay (ELISA) (n.d., not detected). (F to I) lincRNA-Cox2 was silenced in IFN α/β receptor (IFN α/β R) KO cells. Expression levels of lincRNA-Cox2 (F), Ccl5 (G), Irf7 (H), and Ifi204 (I) were measured by qRT-PCR. Data represent means ± SD from three independent experiments.
In contrast to these genes, the Thr2-dependent induction of Il1β was unaffected in cells deficient in lincRNA-Cox2 (fig. S8). We also observed reduced Il6 in macrophages stimulated with R848 and Pam3CSK4 (a ligand that activates a Thr2-Irf6 heterodimer) (fig. S10). We next silenced lincRNA-Cox2 in macrophages lacking the type I IFN α/β receptor (IFN α/βR KO) in order to distinguish between IFN-dependent and IFN-independent targets of lincRNA-Cox2 (fig. 2, F to I, and fig. S11, A and B). Ccl5 was regulated by lincRNA-Cox2 independently of type I IFN signaling, whereas regulation of Irf7 and Irf204 appeared to be secondary to type I IFN signaling. Despite the elevated expression of IFN pathway components, silencing of lincRNA-Cox2 did not render cells permissive to Thr2-induced TBK1 activation, a measure of Irf3 signaling (fig. S12). Finally, because shRNA silencing of lincRNA-Cox2 led to decreased expression of Thr1, we wanted to eliminate the possibility that we inadvertently impaired Pam3CSK4 signaling via the Thr1-Thr2 heterodimer. We restored expression of Thr1 in lincRNA-Cox2–silenced cells and confirmed that the differential regulation of Ccl5 and Il6 was not due to impaired expression of Thr1 (fig. S13). Taken together, these data indicate that lincRNA-Cox2 regulates distinct classes of immune genes both basally and after TLR stimulation.

We next conducted “gain-of-function” studies by generating macrophages that ectopically expressed lincRNA-Cox2 (Fig. 3A). Macrophages that ectopically expressed lincRNA-Cox2 had decreased levels of Ccl5, Irf7, and other ISGs (Fig. 3B). Il6 was not detected when lincRNA-Cox2 was overexpressed in unstimulated cells. However, Il6 levels were significantly enhanced when lincRNA-Cox2 was overexpressed in Pam3CSK4–stimulated cells (Fig. 3C). These results further demonstrate that lincRNA-Cox2 represses Ccl5 while simultaneously enhancing the expression of TLR-induced Il6.

lncRNAs can be found in the nucleus, cytoplasm, or both compartments (17–19). We examined the localization of lincRNA-Cox2 in macrophages by performing quantitative reverse
transcription PCR (qRT-PCR) on RNA isolated from nuclear or cytosolic fractions (fig. S14). Using RNA fluorescence in situ hybridization (FISH), substantial amounts of lincRNA-Cox2 were visible in both the nuclear and the cytosolic compartments of macrophages (Fig. 4A and fig. S15, A and B). Analysis of published RNA-seq data from nuclear and cytosolic fractions of macrophages (20) confirmed that lincRNA-Cox2 was present in both compartments, unlike Neat1, which was exclusively nuclear (fig. S16). RNA FISH analysis demonstrated efficient targeting of lincRNA-Cox2 in the nuclear fractions of our shRNA-silenced cells (fig. S15, C to F).

Many lncRNAs regulate transcription through their interactions with chromatin-modifying complexes or with heterogeneous nuclear ribonucleoproteins (hnRNPs) (5, 21–23). To identify binding partners for lincRNA-Cox2, we incubated in vitro–transcribed biotinylated lincRNA-Cox2 as well as an antisense lincRNA-Cox2 control RNA with nuclear or cytosolic extracts of macrophages and subjected RNA binding proteins to mass spectrometry for identification. hnRNPs A1 and hnRNPs A2/B1 were identified as specific binding partners for lincRNA-Cox2 in both the nuclear and cytosolic fractions (fig. S17). The ability of hnRNPs A1 and hnRNPs A2/B1 to bind lincRNA-Cox2 was confirmed by Western blot analysis (Fig. 4, B and C).

hnRNPs are multifunctional nuclear RNA binding proteins involved in various aspects of RNA biology (24, 25). hnRNPs also act as mediators of lncRNA-induced transcriptional repression (22, 23). hnRNPs A1 and hnRNPs A2/B1 have been linked to transcriptional repression of some genes (26, 27), and hnRNPs A2/B1 and hnRNPs A1/B have been shown to associate (28). Therefore, we hypothesized that lincRNA-Cox2 regulates the transcription of immune genes by associating with these hnRNPs. To test this directly, we generated macrophages lacking expression of hnRNPs A1 or hnRNPs A2/B1 by shRNA (fig. S18, A and B). Knockdown of hnRNPs A1 and hnRNPs A2/B1 did not modulate the levels of lincRNA-Cox2 itself (fig. S19) but did result in an enhancement of Ccl5 protein levels in both unstimulated and Pam3CSK4-stimulated cells (Fig. 4D). Multiplex RNA analysis also revealed elevated levels of Ccl5, Stat1, Tlr7, Icam1, and Ikb in hnRNPs-silenced macrophages (Fig. 4E). There was considerable overlap between genes that were regulated by lincRNA-Cox2 and these two hnRNPs proteins (fig. S20 and table S5). We also examined the occupancy of RNA polymerase II at the promoters of the Ccl5 and Ifr7 genes using chromatin immunoprecipitation (ChIP) and found increased levels of RNA polymerase II (Pol II) at the Ccl5 and Ifr7 promoters when lincRNA-Cox2 or either of the hnRNPs were silenced in unstimulated cells (Fig. 4, F and G, and fig. S21). The occupancy of Pol II on the promoter of Il6 was reduced when lincRNA-Cox2 but not hnRNPs A1 or A2/B1 was knocked down in Pam3CSK4-stimulated cells (fig. S22); this effect was consistent with hnRNPs having no effect on Pam3CSK4-induced Il6 mRNA levels (Fig. 4E).

Silencing of hnRNPs A2/B1 or hnRNPs A1/B in cells that overexpressed lincRNA-Cox2 also reversed the inhibitory effect of lincRNA-Cox2 on Ccl5 expression (Fig. 4H and fig. S23). Taken together, these experiments confirm that hnRNPs A1/B and hnRNPs A2/B1 form a complex with lincRNA-Cox2 to repress the transcription of immune genes. The identification of hnRNPs A1/B and hnRNPs A2/B1 further underscores the importance of hnRNPs in lincRNA function (21–23).

Innate immune responses have the capacity to both combat infectious microbes and drive pathological inflammation, which contributes to diseases such as atherosclerosis, autoimmunity, and cancer. A multitude of regulatory checkpoints control TLR signaling and inflammatory responses. We propose a model whereby TLR signaling induces lincRNAs, such as lincRNA-Cox2, that serve as repressors and activators of genes through their physical interactions with various regulatory complexes. As such, lincRNAs represent a component of the innate immune response that can both restrain and promote aspects of inflammatory signaling. Further characterization of these regulatory networks is likely to reveal novel drug targets and opportunities for therapeutic intervention in infectious and inflammatory diseases.

Cleavage of Fibrinogen by Proteinases Elicits Allergic Responses Through Toll-Like Receptor 4

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Proteinases and the innate immune receptor Toll-like receptor 4 (TLR4) are essential for expression of allergic inflammation and diseases such as asthma. A mechanism that links these inflammatory mediators is essential for explaining the fundamental basis of allergic disease but has been elusive. Here, we demonstrate that TLR4 is activated by airway proteinase activity to initiate both allergic airway disease and antifungal immunity. These outcomes were induced by proteinase cleavage of the clotting protein fibrinogen, yielding fibrinogen cleavage products that acted as TLR4 ligands on airway epithelial cells and macrophages. Thus, allergic airway inflammation represents an antifungal defensive strategy that is driven by fibrinogen cleavage and TLR4 activation. These findings clarify the molecular basis of allergic disease and suggest new therapeutic strategies.

Allergic asthma is a chronic inflammatory airway disease that is characterized by both airway obstruction and enhanced systemic and airway allergic inflammation marked by interleukin-4 (IL-4)–secreting Th helper 2 (Th2) cells, eosinophils, and serum immunoglobulin

References and Notes

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Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1240925/DC1 Materials and Methods Figs. S1 to S23 Tables S1 to S5 References (29–35)

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