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

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Science **341**, 789 (2013);
DOI: 10.1126/science.1240925

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.

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–encoded 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 Theiler's 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 Pam_3CSK_4, a Tlr2 ligand (Fig. 1A). Pam_3CSK_4 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*(*Cox2*)] gene (Fig. 1A); lncRNA-Ehd1 and lncRNA-Lyn were also induced after Tlr2 and Tlr4 stimulation (fig. S1, A and B).

A previous study demonstrated that lincRNA-Cox2 was induced in dendritic cells after stimulation with LPS (*4*). However, whether lincRNA-Cox2 regulates the inflammatory response that is associated with TLR signaling is unknown. We identified three splice variants of lincRNA-Cox2 (Fig. 1B; GenBank accession numbers JX682706, JX682707, and JX682708). Variant 1 was the most abundant transcript containing exons 1 and 4, common to all splice variants. Consequently, we designed primers for quantitative PCR (qPCR) and short hairpin RNA (shRNA) that targeted these regions. Using qPCR, we confirmed that LPS induced similar temporal patterns of expression of both lincRNA-Cox2 and its neighboring *Ptgs2* (*Cox2*) gene in bone marrow–derived dendritic cells (BMDCs, Fig. 1, C and D). R848, a synthetic antiviral compound that activates Tlr7/8, induced lincRNA-Cox2 and *Ptgs2* (*Cox2*) in BMDCs, whereas polyinosinic-polycytidylic acid, a synthetic double-stranded RNA that activates Tlr3, had no effect (fig. S2, A to D). Both *Listeria monocytogenes*–infected BMDMs and splenocytes from *Listeria*-infected mice also had elevated levels

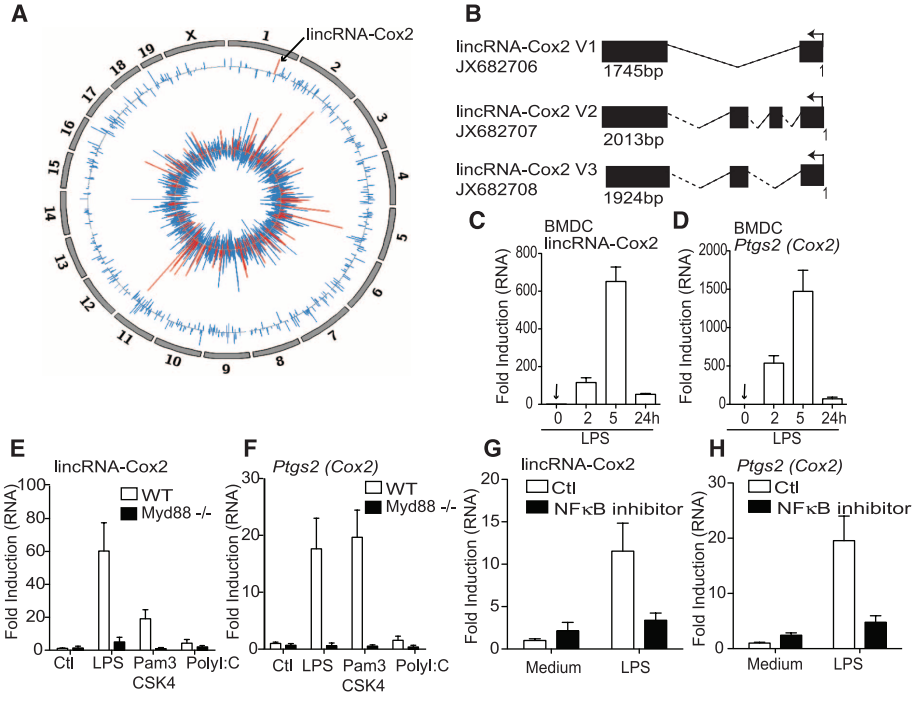

Fig. 1. lincRNA-Cox2 expression is induced by Tlr ligands in a MyD88- and NF-κB–dependent manner. (**A**) The Circos plot shows genome-wide differential expression (RNA-seq) between untreated and Pam_3CSK_4 (Tlr1/2)–stimulated (5 hours) BMDMs. The inner track shows $\log_2$ relative change for protein-coding genes that are classified into immune genes (red; see supplementary materials) and other genes (blue). The outer track shows $\log_2$ 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 BMDCs 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 qRT-PCR on BMDMs obtained from wild-type (WT) or *Myd88*$^{-/-}$ mice. (**G** and **H**) BMDMs were treated for 30 min with a NF-κB inhibitor (1 μg/ml), followed by stimulation with LPS (100 ng/ml); expression levels of lincRNA-Cox2 (G) and *Ptgs2* (*Cox2*) (H) were examined by qRT-PCR. Data represent means ± SD from three independent experiments.

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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 pretreated 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 Pam_3CSK_4. 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 (*Ccrl*), and IFN-stimulated genes (ISGs) (*Irf7*, *Oas1a*, *Oas1l*, *Oas2*, *Ifi204*, and *Isg15*) (Fig. 2B). In the same cells stimulated with Pam_3CSK_4, 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 Pam_3CSK_4-induced expression of *Tlr1* and *Il6* 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 Il6 (Fig. 2, D and

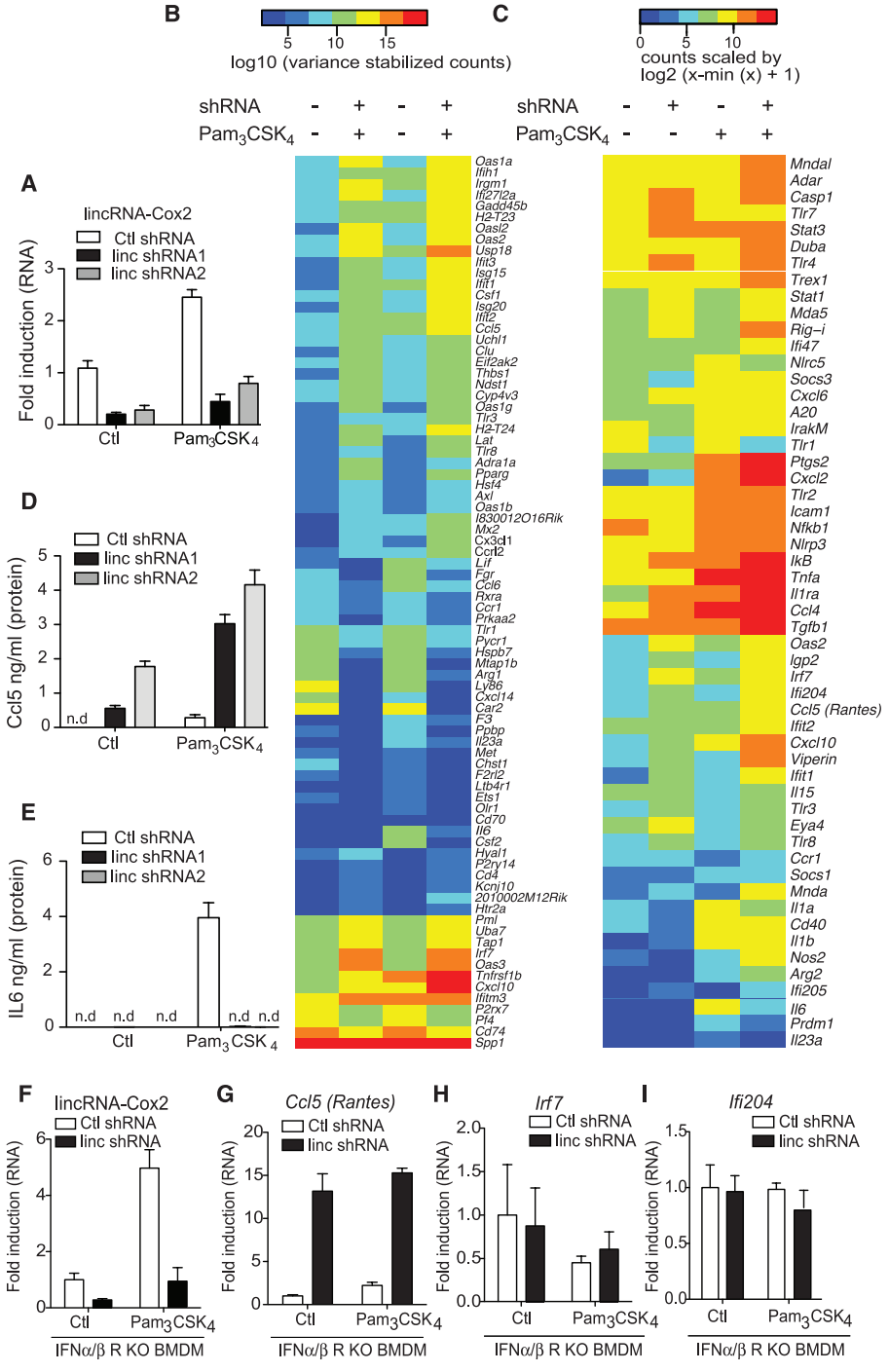

Fig. 2. lincRNA-Cox2 is a major regulator of immune genes. (**A**) qRT-PCR was carried out on BMDMs stably expressing lentiviral shRNA specific to lincRNA-Cox2 (shRNA) or a control shRNA. Expression of lincRNA-Cox2 was measured. (**B**) RNA-seq analysis on lincRNA-Cox2 knockdown or control shRNA BMDMs that were either stimulated with Pam_3CSK_4 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 $\log_2$ 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 stimulated with Pam_3CSK_4 for 5 hours. (**D** and **E**) Cells were stimulated with Pam_3CSK_4 for 24 hours, and Ccl5 (D) and Il6 (E) levels were measured in 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.

E, and fig. S8). In contrast to these genes, the Tlr2-dependent induction of Il1β was unaffected in cells deficient in lincRNA-Cox2 (fig. S9). We also observed reduced Il6 in macrophages stimulated with R848 and Pam_2CSK_4 (a ligand that activates a Tlr2-Tlr6 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 *Ifi204* 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 Tlr2-induced TBK1 activation, a measure of Irf3 signaling (fig. S12). Finally, because shRNA silencing of lincRNA-Cox2 led to decreased expression of Tlr1, we wanted to eliminate the possibility that we inadvertently impaired Pam_3CSK_4 signaling via the Tlr1-Tlr2 heterodimer. We restored expression of Tlr1 in lincRNA-Cox2–silenced cells and confirmed that the differential regulation of Ccl5 and Il6 was not due to impaired expression of Tlr1 (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 Pam_3CSK_4 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

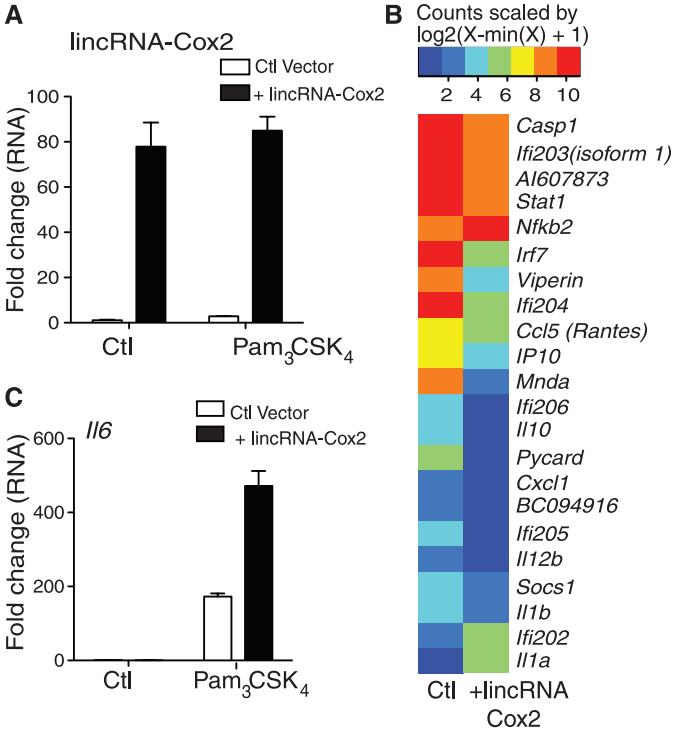

Fig. 3. Differential gene expression after overexpression of lincRNA-Cox2. (**A**) A BMDM cell line stably overexpressing lincRNA-Cox2 or a control vector was generated. qRT-PCR was carried out and overexpression of lincRNA-Cox2 was confirmed. (**B**) Heat map representation of differentially regulated genes from control or lincRNA-Cox2–overexpressing BMDMs. (**C**) qRT-PCR was carried out on control or lincRNA-Cox2–overexpressing cells stimulated with Pam_3CSK_4 for 5 hours. Data represent means ± SD from three independent experiments.

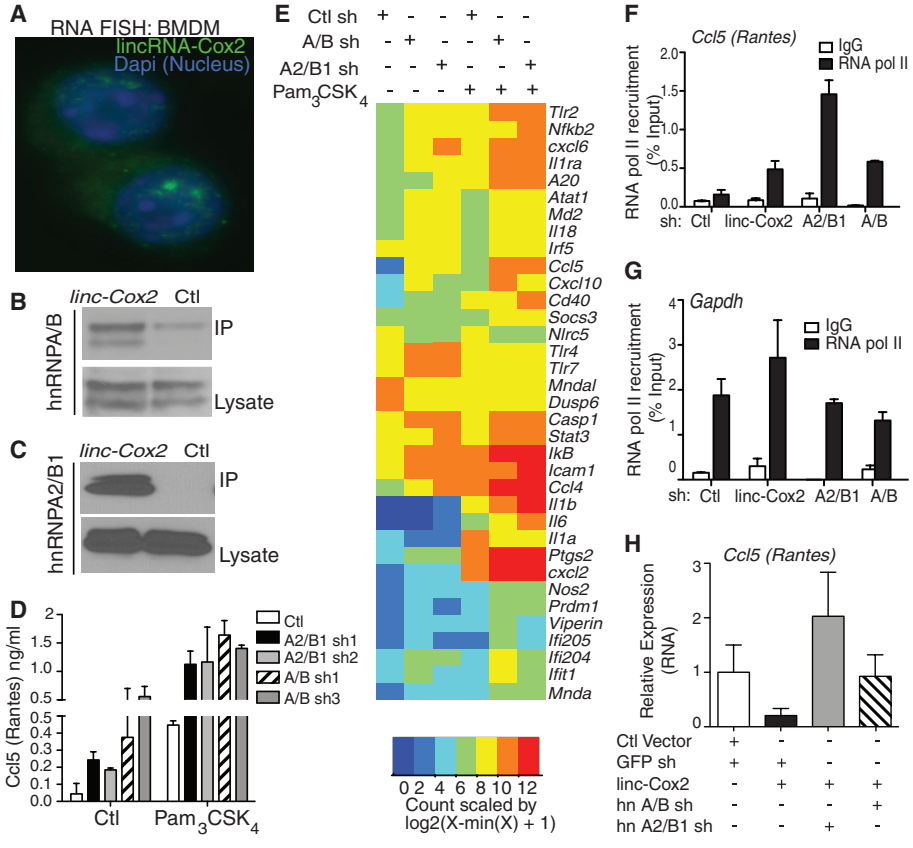

Fig. 4. lincRNA-Cox2 is localized to both the cytosolic and nuclear compartments and interacts with hnRNP-A/B and A2/B1 to regulate immune genes. (**A**) BMDMs were labeled with a lincRNA-Cox2 probe via RNA FISH and counterstained with 4′,6-diamidino-2-phenylindole (DNA to visualize the nucleus). (**B** and **C**) Biotinylated lincRNA-Cox2 or antisense RNA was incubated with nuclear extracts, and interaction with endogenous hnRNP-A/B (B) or hnRNP-A2/B1 (C) was assessed after immunoprecipitation and Western blot (upper panels). Expression levels of hnRNP-A/B (B) or hnRNP-A2/B1 (C) (lower panels) in input lysates were also examined. (**D**) Cell lines with shRNA targeting hnRNP-A/B or hnRNP-A2/B1 were stimulated with Pam_3CSK_4; Ccl5 production was measured in these cells by ELISA. (**E**) Heat map representation of differentially regulated genes of control shRNA–, hnRNP-A/B shRNA–, or hnRNP-A2/B1 shRNA–expressing BMDMs stimulated with Pam_3CSK_4 (100 nM) for 5 hours. (**F** and **G**) Recruitment of RNA Pol II to the Ccl5 promoter as determined by ChIP analysis in cells expressing shRNA targeting lincRNA-Cox2, hnRNP-A/B, or hnRNP-A2/B1. (**H**) hnRNP-A/B or hnRNP-A2/B1 was knocked down using lentiviral shRNA in lincRNA-Cox2–overexpressing BMDMs; *Ccl5* expression levels were measured using qRT-PCR. Data are representative of three separate experiments.

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. hnRNP-A/B and hnRNP-A2/B1 were identified as specific binding partners for lincRNA-Cox2 in both the nuclear and cytosolic fractions (fig. S17). The ability of hnRNP-A/B and hnRNP-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*). hnRNP-A/B has been linked to transcriptional repression of some genes (*26*, *27*), and hnRNP-A2/B1 and hnRNP-A/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 hnRNP-A/B or hnRNP-A2/B1 by shRNA (fig. S18, A and B). Knockdown of hnRNP-A/B and hnRNP-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 Pam_3CSK_4-stimulated cells (Fig. 4D). Multiplex RNA analysis also revealed elevated levels of *Ccl5*, *Stat1*, *Tlr7*, *Icam1*, and *IκB* in hnRNP-silenced macrophages (Fig. 4E). There was considerable overlap between genes that were regulated by lincRNA-Cox2 and these two hnRNP proteins (fig. S20 and table S5). We also examined the occupancy of RNA polymerase II at the promoters of the *Ccl5* and *Irf7* genes using chromatin immunoprecipitation (ChIP) and found increased levels of RNA polymerase II (Pol II) at the *Ccl5* and *Irf7* 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 RNA Pol II on the promoter of *Il6* was reduced when lincRNA-Cox2 but not hnRNP-A/B or A2/B1 was knocked down in Pam_3CSK_4-stimulated cells (fig. S22); this effect was consistent with hnRNPs having no effect on Pam_3CSK_4-induced *Il6* mRNA levels (Fig. 4E). Silencing of hnRNP-A2/B1 or hnRNP-A/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 hnRNP-A/B and hnRNP-A2/B1 form a complex with lincRNA-Cox2 to repress the transcription of immune genes. The identification of hnRNP-A/B and hnRNP-A2/B1 further underscores the importance of hnRNPs in lncRNA 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 lncRNAs, such as lincRNA-Cox2, that serve as repressors and activators of genes through their physical interactions with various regulatory complexes. As such, lncRNAs 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.

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Acknowledgments: We thank N. Singh for advice on RNA-seq, A. J. Crawford for help with quantification of RNA FISH, and K. Jeffrey, L. Stuart, N. Silverman, D. Golenbock, and E. Lien for critical reading of this manuscript. The data presented in this paper are tabulated in the main paper and in the supplementary materials. All RNA-seq data are available for download from NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under accession number GSE40978. Supported by Ireland Health Research Board/Marie Curie Fellowship MCPD/2010/04 (S.C.) and by NIH grants AI067497 (K.A.F.) and GM053234 (J.B.L.).

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1240925/DC1
Materials and Methods
Figs. S1 to S23
Tables S1 to S5
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23 May 2013; accepted 24 July 2013
Published online 1 August 2013;
10.1126/science.1240925

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 T helper 2 (T_H2) cells, eosinophils, and serum immunoglobulin