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## A long noncoding RNA induced by TLRs mediates both activation and repression of immune response genes

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### Abstract

An inducible program of inflammatory gene expression is central to anti-microbial defenses. Signal-dependent activation of transcription factors, transcriptional co-regulators and chromatin modifying factors collaborate to control this response. Here we identify a long noncoding RNA that acts as a key regulator of this inflammatory response. Germline-encoded 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 germline-encoded receptors, which recognize microbial products and activate signaling pathways that induce transcription of inflammatory genes (1). These surveillance receptors (e.g. Toll-like receptors) and their downstream pathways activate transcription factors (e.g. NFκB and Irf3) that act in concert with transcriptional co-regulators and chromatin modifying factors to regulate transcription (2–4). This complex transcriptional response leads to expression of

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Supplementary Materials

Materials and Methods, Figs S1–S15, Tables S1–5.

### Author contributions

S.C, D.R.C and K.A.F designed the research, S.C, M.A, P.G, E.R, L.H, M.B, B.M, M.H.B, J.L, M.M conducted experiments, analyzed data. D.A and D.R.C collected and processed all RNA-seq data. S.C, D.R.C and K.A.F wrote the manuscript. D.R.C, L.A.J.O’N and K.A.F supervised the project.

hundreds of proteins involved in antimicrobial defense, cell migration, tissue repair, adaptive immunity and resolution of inflammation (2, 5). Recent studies have identified thousands of long non-coding RNAs (lncRNAs) in mammalian genomes (6–11). lncRNAs have emerged as major regulators of gene expression and are involved in various biological processes that include genomic imprinting, embryonic development, cell differentiation, tumor metastasis and regulation of the cell cycle [reviewed in (7)]. lncRNAs are differentially regulated in virus-infected cells (12) and in dendritic cells following lipopolysaccharide (LPS) stimulation (6). Very recently, a lncRNA called NeST, (nettoie Salmonella pas Theiler's [cleanup Salmonella not Theiler's]) was shown to control susceptibility to Theiler's virus and Salmonella infection in mice through epigenetic regulation of the interferon- $\gamma$  locus (13, 14). Although lncRNAs can be induced in innate immune cells (6, 12), it is not yet known whether lncRNAs act as regulators of gene expression in the innate immune response.

To identify lncRNAs that are transcribed during the innate immune response, we conducted whole-transcriptome analysis (RNA-seq) (8) of macrophages stimulated with the synthetic bacterial lipopeptide Pam<sub>3</sub>CSK<sub>4</sub>, a TLR2 ligand (Fig. 1A). The TLR2 ligand induced the transcription of numerous protein-coding genes involved in the immune response (Fig. 1A inner track) as well as 62 lncRNAs (Table S1, Fig. 1A outer track). Consistent with previous studies, overall changes in lncRNA expression were less than that observed for protein coding genes. The most significantly induced lncRNAs tended to occur in chromosomal regions where there was also increased expression of immune genes, suggesting that these genes are co-regulated. *lincRNA-Cox2* was amongst the most highly induced lncRNAs and is proximal to the prostaglandin-endoperoxide synthase 2 (*Ptgs2/Cox2*) gene (Fig. 1A). In addition to *lincRNA-Cox2*, two additional lncRNAs, *lincRNA-Ehd1* and *lincRNA-Lyn* were also induced following TLR2 and TLR4 stimulation (Fig. S1A–B).

A previous study demonstrated that *lincRNA-Cox2* was induced in dendritic cells following stimulation with lipopolysaccharide (LPS) (6). However, it is not known whether *lincRNA-Cox2* regulates the inflammatory response that is associated with TLR signaling. Using PCR amplification, we identified three splice variants of *lincRNA-Cox2* (Fig. 1B, accession numbers JX682706, JX682707, JX682708). Variant 1 was the most abundant transcript and contains exons 1 and 4, which are common to all splice variants. Consequently, we designed primers for quantitative PCR (qPCR) and 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. 1C–D). Unlike Pam<sub>3</sub>CSK<sub>4</sub> (TLR2), Poly I:C, a synthetic double stranded RNA (TLR3) did not induce *lincRNA-Cox2* or *Ptgs2 (Cox2)* significantly in BMDCs (Fig. 1E–F). LPS, Pam<sub>3</sub>CSK<sub>4</sub> and R848 (which activates TLR7/8) also induced the expression of *lincRNA-Cox2* and *Ptgs2 (Cox2)* in macrophages (Fig. 1G–H). Both Listeria-infected macrophages (*in vitro*) and isolated splenocytes from Listeria-infected mice (24 hr post infection) had elevated levels of *lincRNA-Cox2* (Fig. S1C–D). Induction of *lincRNA-Cox2* and its neighboring gene *Ptgs2 (Cox2)* was dependent on the TLR signaling adaptor protein MyD88 (Fig. I–J) and on activation of the transcription factor NF $\kappa$ B (Fig. 1K–L).

We next examined the protein-coding capacity of *lincRNA-Cox2* by assessing its association with polysomes within cells. Macrophage cells were treated with cycloheximide to trap ribosomes on RNA molecules and either left untreated or pretreated with EDTA (which disrupts all RNA-protein interactions) or with harringtonine (which specifically disrupts translation). Cell lysates were then fractionated by sucrose density gradients and ultracentrifugation and RNA analysed in all fractions by qPCR (15–17). Using this approach we compared *Gapdh*, a well-translated mRNA with *lincRNA-Cox2* and another lincRNA, *lincRNA-Eps*, that has previously been shown to be non-coding (18). As expected *Gapdh* RNA sedimented with a high velocity through the sucrose gradient since it was associated with ribosomes. In contrast, *lincRNA-Cox2* and *lincRNA-Eps* both remained in lighter fractions (Fig. S2). Treatment with EDTA or specific disruption of translation by harringtonine resulted in a shift of *Gapdh* from the higher velocity to the lower velocity fractions indicating that this mRNA was actively translated. In contrast, the profile of *lincRNA-Cox2* or *lincRNA-Eps* were largely unaffected by harringtonine treatment.

We also examined *lincRNA-Cox2* for the presence of potential open reading frames (ORFs). Most of the ORFs identified were found to have poor Kozak strength suggesting that it is unlikely that this RNA sequence is translated, (Fig. S3A–B). Collectively, these studies indicate that *lincRNA-Cox2* is unlikely to encode a protein product.

To define the functional role of *lincRNA-Cox2* in inflammatory gene expression, we generated macrophage cell lines in which *lincRNA-Cox2* expression was silenced by shRNA (Fig. 2A). Silencing of *lincRNA-Cox2* did not alter expression of its neighboring gene *Ptgs2* (*Cox2*) (Fig. S4). 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<sub>3</sub>CSK<sub>4</sub>. Silencing of *lincRNA-Cox2* increased the expression of 787 genes by 3-fold or greater in unstimulated cells (Table S2). A GO enrichment analysis indicated that genes related to the immune response were significantly overrepresented in these up-regulated genes (Table S3, Fig. 2B). A number of chemokines (*Ccl5*, *Cx3cl1*), chemokine receptors (*Ccr1*) and interferon-stimulated genes (ISGs) (*Irf7*, *Oas1a*, *Oas1l*, *Oas2*, *Ifi204* and *Isg15*) were up-regulated when *lincRNA-Cox2* was silenced in unstimulated cells (Fig. 2B). In the same cells stimulated with Pam<sub>3</sub>CSK<sub>4</sub>, silencing of *lincRNA-Cox2* resulted in attenuated expression of 713 genes by 3-fold or greater (Table S4). Examples of these genes include *Tlr1*, *Il6* and *Il23a*.

We confirmed these findings using a non-enzymatic RNA profiling technology that employs bar-coded fluorescent probes to simultaneously analyze mRNA expression levels of differentially regulated genes (nCounter, Nanostring). In unstimulated cells, silencing of *lincRNA-Cox2* led to a marked increase in expression of *Irf7*, *Ccl5* (*Rantes*), and ISGs (*Ifi204*, *Ifi205* and *Viperin*) relative to control cells (Fig. 2C, columns 1–2), while the Pam<sub>3</sub>CSK<sub>4</sub>-induced expression of *Tlr1* and *Il6* was attenuated (Fig. 2C, columns 3–4). We confirmed these findings in three independent shRNA lines by measuring protein levels for *Ccl5* (*Rantes*) and *IL6* (Fig. 2D and E, Fig. S5). In contrast to these genes, the TLR2 dependent induction of *IL1β* was unaffected in cells lacking *lincRNA-Cox2* (Fig. S6). We also observed reduced *IL6* production in macrophages stimulated with Pam<sub>2</sub>CSK<sub>4</sub> and R848, ligands of TLR2/6 and TLR7, respectively (Fig. S7). We next silenced *lincRNA-Cox2*

in macrophages lacking the type I interferon  $\alpha/\beta$  receptor (IFN  $\alpha/\beta$ R KO) in order to distinguish between direct and indirect (IFN-dependent) targets of *lincRNA-Cox2* (Fig. 2F–I, Fig. S8). Non-enzymatic RNA profiling of gene expression in these cells indicated that while *Ccl5* was regulated by *lincRNA-Cox2* in a manner that was independent of type I IFN signaling, regulation of *Irf7* and *Ifi204* was secondary to type I IFN signaling. Despite the elevated expression of IFN pathway components, silencing of *lincRNA-Cox2* did not render these cells permissive to TLR2 induced TBK1 activation, a measure of the Irf3 signaling pathway (Fig. S9). Finally, since shRNA silencing of *lincRNA-Cox2* led to decreased expression of Tlr1, it was important to eliminate the possibility that we inadvertently impaired Pam<sub>3</sub>CSK<sub>4</sub> signaling via the TLR1/TLR2 heterodimer. To test this directly, we restored expression of mTLR1 in *lincRNA-Cox2* silenced cells and confirmed that the differential regulation of *Ccl5* and IL6 expression was not due to impaired expression of TLR1. Restoration of TLR1 did not alter the effects of *lincRNA-Cox2* silencing on *Ccl5* or IL6 protein levels (Fig. S10). Taken together, these data indicate that *lincRNA-Cox2* regulates distinct classes of immune genes both basally and following TLR-stimulation.

We next conducted ‘gain-of-function’ studies by generating macrophages that ectopically expressed *lincRNA-Cox2* (Fig. 3A). As expected, over-expression of *lincRNA-Cox2* mirrored the effects of silencing the lncRNA. Macrophages that ectopically expressed *lincRNA-Cox2* had decreased levels of *Ccl5*, *Irf5*, *Irf7*, *Stat1*, *Viperin*, and three members of the PYHIN protein family (*Ifi202*, *Ifi204*, *Mnda*) (Fig. 3B). Il6 was not detected when *lincRNA-Cox2* was over-expressed in unstimulated cells. However, *Il6* levels were significantly enhanced when *lincRNA-Cox2* was over-expressed in Pam<sub>3</sub>CSK<sub>4</sub> 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 in both compartments (19–21). We therefore examined the localization of *lincRNA-Cox2* in macrophages using RNA fluorescence in situ hybridization (FISH), a methodology that allows simultaneous detection and localization of RNA transcripts in relation to cellular substructures. Significant amounts of *lincRNA-Cox2* were clearly visible in both the nuclear and the cytosolic compartments of macrophages (Fig. 4A, Fig. S11). Analysis of RNA-seq data for different subcellular fractions of macrophages (22), confirmed that *lincRNA-Cox2* was present in both the cytosol and nucleus (Fig. S12). Importantly RNA-FISH analysis demonstrated efficient targeting of *lincRNA-Cox2* in the nuclear fractions of our shRNA silenced cells (Fig. S11C–F).

Many lncRNAs regulate transcription through their interactions with chromatin-modifying complexes or with heterogeneous nuclear ribonucleoproteins (hnRNP) (7, 23–25). 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 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 cytosolic and nuclear fractions (data not shown). The ability of hnRNP-A/B and hnRNP-A2/B1 to bind *lincRNA-Cox2* was confirmed by western blot analysis (Fig. 4B and C). hnRNPs are involved in several RNA-related biological processes such as transcription, pre-mRNA processing, mature mRNA transport to the cytoplasm, and

translation (26, 27). In addition, hnRNPs are known to form complexes with lncRNAs and are emerging as important mediators of lncRNA-induced transcriptional repression (24, 25). hnRNP-A/B has been linked to transcriptional repression of some genes (e.g. OPN and Sm-a-A) (28, 29) and hnRNPA2/B1 associates with hnRNP-A/B (30). Therefore, we hypothesized that lincRNA-Cox2 regulates transcription of immune genes by forming complexes with these hnRNP proteins. To test this hypothesis, we generated macrophages where expression of hnRNP-A/B and hnRNP-A2/B1 were silenced by shRNA (Fig. S13A–B). Knockdown of hnRNP-A/B and hnRNP-A2/B1 did not modulate the levels of *lincRNA-Cox2* expression (Fig. S14), indicating that these hnRNPs did not regulate *lincRNA-Cox2* levels. Similar to our *lincRNA-Cox2* silencing studies (Fig. 2D), we detected a significant enhancement of Ccl5 protein in TLR2-stimulated cells and unstimulated cells that lacked hnRNP-A/B and hnRNP-A2/B1 (Fig. 4D). Multiplex RNA analysis also revealed elevated levels of *Ccl5*, *Stat1*, *Tlr7*, *Icam1* and *IκB* RNA in hnRNP-silenced macrophages (Fig. 4E). There was considerable overlap between genes that were regulated by *lincRNA-Cox2* and these two hnRNP proteins (Fig. S15, Table. S5). We also examined RNA polymerase II recruitment to the promoter of the *Ccl5* and *Irf7* promoters using RNA polymerase II Chromatin immunoprecipitation (ChIP). As expected, recruitment of RNA Pol II to the promoters of *Ccl5* and *Irf7* was increased when *lincRNA-Cox2* or the hnRNPs were silenced (Fig. 4F–G, Fig. S16), indicating that *lincRNA-Cox2*, hnRNPA2/B1 and A/B negatively regulate transcription of these genes. In contrast to these genes, RNA Pol II displayed decreased recruitment to the promoter of *Il6* only when *lincRNA-Cox2* was knocked down (Fig. S16), an effect that was consistent with the hnRNPs having no effect on *Il6* mRNA levels (Fig. 4E). Silencing of hnRNP-A2/B1 or hnRNP-A/B in cells that over-expressed *lincRNA-Cox2* reversed the inhibitory effect of *lincRNA-Cox2* on *Ccl5* expression (Fig. 4H, Fig. S17). Taken together, these experiments confirm that hnRNP-A/B and A2/B1 form a complex with *lincRNA-Cox2* in order to repress the transcription of immune genes.

In summary, these studies identify *lincRNA-Cox2* as a critical component of the inflammatory response. The impact of this lncRNA on the TLR response was both dramatic and broad-acting with unprecedented effects not previously seen with other regulatory RNAs (e.g. microRNAs). The identification of hnRNP-A/B and A2/B1 as mediators of *lincRNA-Cox2*'s transcriptional repressive functions underscore the importance of hnRNPs in lncRNA function. Recently, *lincRNA-p21*, identified as a repressor in the p53-dependent transcriptional response, was shown to associate with hnRNP-K which facilitates the proper genomic localization of hnRNP-K at repressed genes to modulate p53 dependent apoptosis (23–25).

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 novel component of the innate immune response that can 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

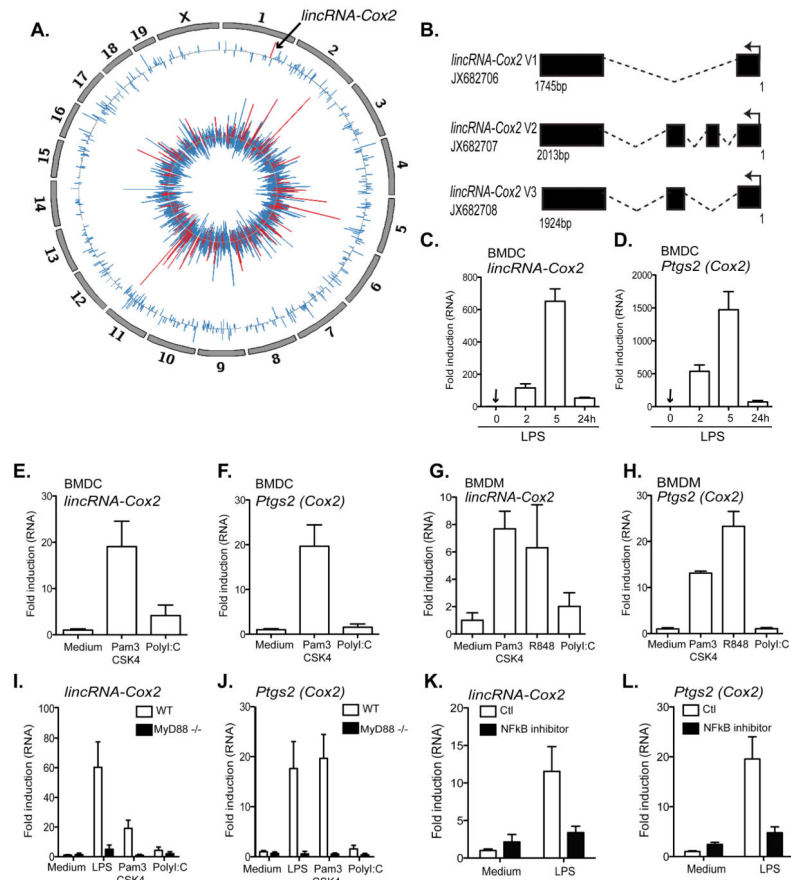
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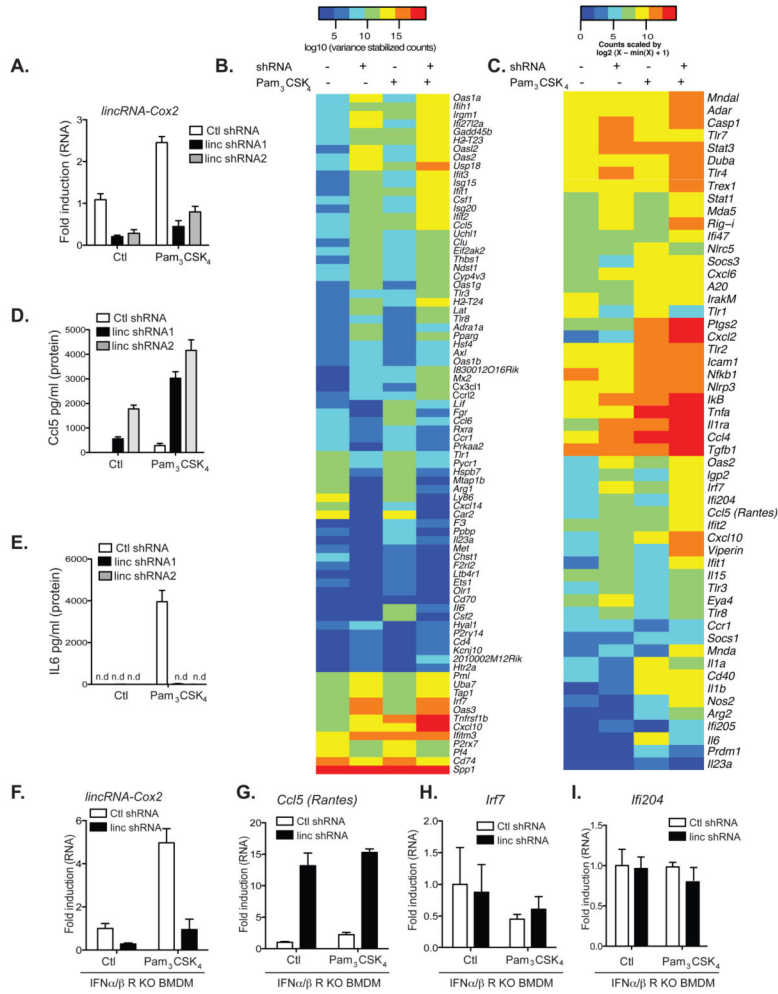
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**Figure 1. *lincRNA-Cox2* expression is induced by TLR ligands in a MyD88 and NF $\kappa$ B dependent manner**

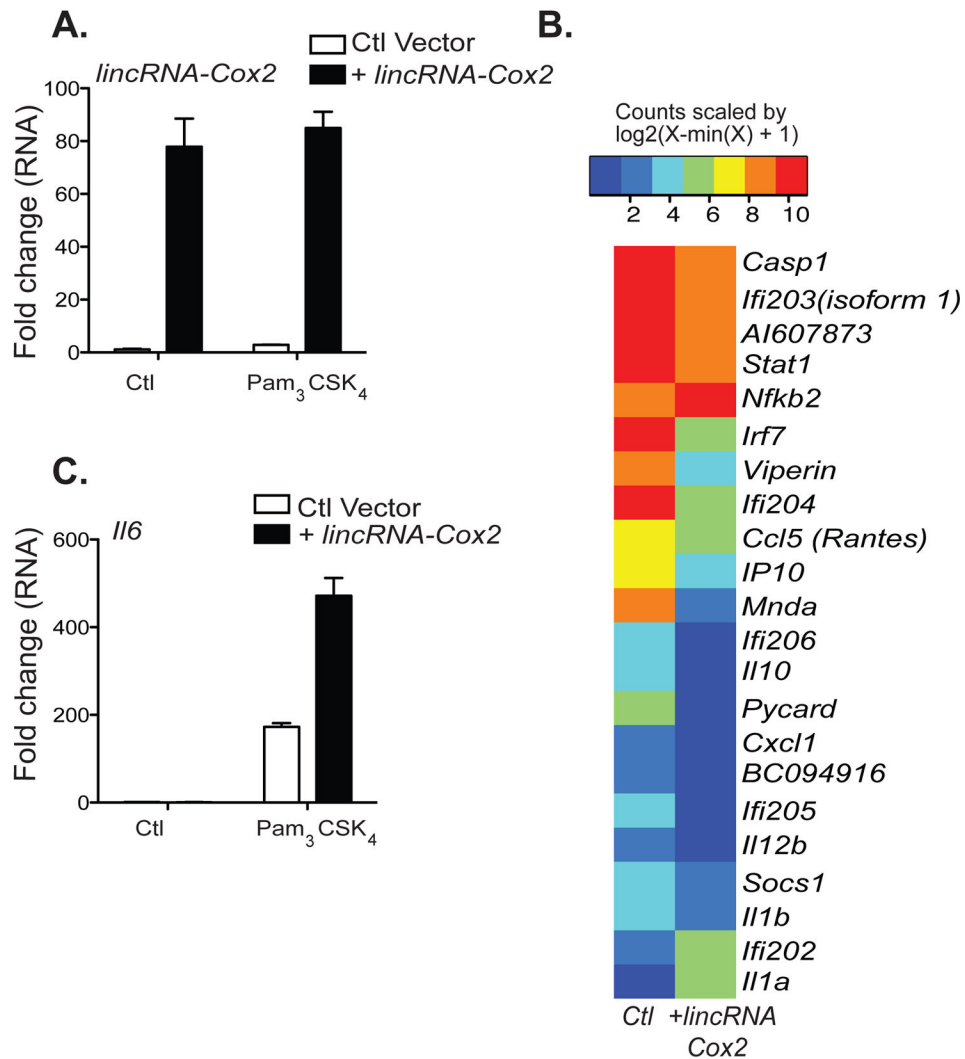
A, The Circos plot shows genome-wide differential expression (RNA-seq) between untreated bone marrow derived macrophages (BMDM) and BMDMs stimulated with Pam<sub>3</sub>CSK<sub>4</sub> (TLR1/2) (5 h). The inner track shows log<sub>2</sub> fold-change values for protein coding genes that are classified into immune genes (red, see methods) and other genes (blue). The outer track shows log<sub>2</sub> fold-change value for all lincRNAs. *lincRNA-Cox2* is highlighted in red on Chromosome 1 (arrow). B, *lincRNA-Cox2* encodes three splice variants. C–H, qRT-PCR was performed on bone marrow derived dendritic cells (BMDC) (C–F) or bone marrow derived macrophages (BMDM). Elevated levels of *lincRNA-Cox2* and *Ptgs2* were observed following LPS (TLR4) (C–D), Pam<sub>3</sub>CSK<sub>4</sub> (TLR2) (E–H), R848 (TLR7/8) (G–H) but not with Poly I:C (TLR3) (E–H) stimulation. I–J. Induction of *lincRNA-Cox2* and *Ptgs2* were found to be dependent on MyD88 following qRT-PCR on BMDMs obtained from wild type (WT) or MyD88 KO mice. K–L, BMDMs treated for 30min with an NF $\kappa$ B inhibitor (1  $\mu$ g/ml), followed by stimulation with LPS (100 ng/ml) resulted in reduced expression levels of *lincRNA-Cox2* (K) and *Ptgs2* (L) as examined by qRT-PCR. Data represents mean  $\pm$ SD from three independent experiments.



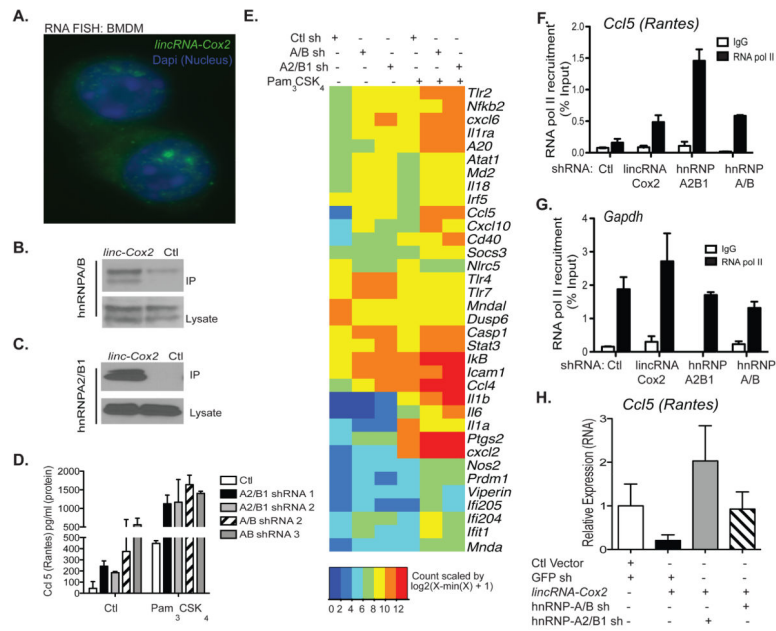


**Figure 2. *lincRNA-Cox2* is a major regulator of immune genes**

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 was performed on *lincRNA-Cox2* knockdown or control (ctl shRNA) BMDMs that were either stimulated with Pam<sub>3</sub>CSK<sub>4</sub> 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 by absolute log<sub>2</sub> fold-change values and the top 80 differentially expressed genes displayed. C, Heatmap representation of differentially regulated genes from a custom designed gene codeset performed on RNA extracted from Ctl or *lincRNA-Cox2* knockdown cells stimulated with Pam<sub>3</sub>CSK<sub>4</sub> for 5 h. D–E, Cells were stimulated with Pam<sub>3</sub>CSK<sub>4</sub> for 24 h, increased Ccl5 (Rantes) (D) and reduced IL6 (E) cytokine levels were identified in *lincRNA-Cox2* knockdown cells by elisa (n.d means not detected). F–I, *lincRNA-Cox2* was silenced in interferon  $\alpha/\beta$  receptor (IFN  $\alpha/\beta$  R) KO cells. Expression levels of *lincRNA-Cox2* (F), *Ccl5 (Rantes)* (G), *Irf7* (H) and *Ifi204* (I) were measured using qRT-PCR to define direct targets of *lincRNA-Cox2*.



**Figure 3. Differential gene expression following over expression of *lincRNA-Cox2***  
 A, BMDM stably over-expressing *lincRNA-Cox2* or a Ctl vector were generated. qRT-PCR was carried out and over-expression of *lincRNA-Cox2* was confirmed. B, Heatmap representation of differentially regulated genes from a custom designed gene codeset performed on RNA extracted from Ctl or *lincRNA-Cox2* over expressing BMDMs. C, qRT-PCR was carried out on Ctl or *lincRNA-Cox2* over expressing cells stimulated with Pam<sub>3</sub>CSK<sub>4</sub> for 5 h, increased *Il6* expression levels were identified using qRT-PCR. Data represents mean  $\pm$ SD from three independent experiments.



**Figure 4. *lincRNA-Cox2* is localized to both the cytosolic and nuclear compartments, interacts with hnRNP-A/B and A2/B1 to regulate immune genes**

A, BMDMs were labeled with a *lincRNA-Cox2* probe using RNA FISH, and counterstained with DAPI (DNA). B–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) assessed following IP/western (top 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<sub>3</sub>CSK<sub>4</sub>, elevated Ccl5 (Rantes) production was identified in these cells by elisa. E, Heatmap representation of differentially regulated genes of Ctl, hnRNP-A/B or hnRNP-A2/B1 expressing BMDMs stimulated with Pam<sub>3</sub>CSK<sub>4</sub> (100nM) for 5 h. F–G, Silencing of *lincRNA-Cox2*, hnRNP-A/B or hnRNP-A2/B1 promotes recruitment of RNA Pol II to the Ccl5 promoter as determined by CHIP analysis. H, hnRNP-A/B or hnRNP-A2/B1 was knocked down using lentiviral shRNA in *lincRNA-Cox2* over-expressing BMDMs, *Ccl5* expression levels were measured using qRT-PCR.