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The long noncoding RNA *THRIL* regulates TNF α expression through its interaction with hnRNPL

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Thousands of large intergenic noncoding RNAs (lincRNAs) have been identified in the mammalian genome, many of which have important roles in regulating a variety of biological processes. Here, we used a custom microarray to identify lincRNAs associated with activation of the innate immune response. A panel of 159 lincRNAs was found to be differentially expressed following innate activation of THP1 macrophages. Among them, linc1992 was shown to be expressed in many human tissues and was required for induction of TNF α expression. Linc1992 bound specifically to heterogenous nuclear ribonucleoprotein L (hnRNPL) and formed a functional linc1992-hnRNPL complex that regulated transcription of the TNF α gene by binding to its promoter. Transcriptome analysis revealed that linc1992 was required for expression of many immune-response genes, including other cytokines and transcriptional and posttranscriptional regulators of TNF α expression, and that knockdown of linc1992 caused dysregulation of these genes during innate activation of THP1 macrophages. Therefore, we named linc1992 *THRIL* (TNF α and hnRNPL related immunoregulatory LincRNA). Finally, *THRIL* expression was correlated with the severity of symptoms in patients with Kawasaki disease, an acute inflammatory disease of childhood. Collectively, our data provide evidence that lincRNAs and their binding proteins can regulate TNF α expression and may play important roles in the innate immune response and inflammatory diseases in humans.

innate immunity | inflammation | Toll-like receptors

Vertebrates are constantly exposed to microbial pathogens that can disrupt normal cellular processes and lead to diseases (1). The innate immune response has evolved as a rapidly mobilized first line of defense against such threats and is initiated by engagement of several classes of cell surface and intracellular pattern-recognition receptors (PRRs) that include the transmembrane Toll-like receptors (TLRs) (1, 2). TLRs recognize a variety of microbial molecules, including lipopeptides, lipopolysaccharides, and DNA, that trigger intracellular signaling cascades that activate transcription factors such as NF κ B and IFN regulatory factors (IRFs). NF κ B and IRFs regulate the expression of hundreds of genes involved in the immune response, including the proinflammatory cytokines TNF α , interleukin (IL)-1, and IL-6 (1, 2). The innate immune response must thus be tightly controlled to limit potential damage from excess inflammatory mediators and to allow tissue repair following infection.

In recent years, it has become clear that noncoding RNAs (ncRNAs) such as microRNAs play important regulatory roles in TLR signaling in response to microbial stimuli, acting at both the transcriptional and posttranscriptional levels (3, 4). However, microRNAs are only a small fraction of the noncoding regions of the mammalian genome, and additional ncRNAs, including large intergenic noncoding RNAs (lincRNAs), are expressed abundantly (5). LincRNAs are encoded similarly to coding genes but do not contain protein-coding sequences in the transcripts. LincRNAs are evolutionarily conserved, and emerging evidence suggests that they play key roles in a diverse array of cellular

processes such as X-chromosome inactivation (6), p53 pathway regulation (7), cell-cycle control (8), epigenetic regulation (9–11), self-renewal of embryonic stem cells (12), and embryonic development (13). Moreover, recent studies have indicated that mutation and/or dysregulated expression of lincRNAs could play a role in multiple human diseases, including cancer (9, 14), suggesting that they could be therapeutic targets. LincRNAs are thought to function primarily through specific interactions with cellular proteins, and a panel of these proteins have been identified (6, 10, 11, 15, 16). However, it is clear that other cellular lincRNA-binding factors and cell type-specific functions remain to be identified.

In this study, we identified a lincRNA that regulates the human macrophage response to an innate stimulus, suggesting that lincRNAs may play an unappreciated role in regulating cell-defense mechanisms and host–pathogen interactions. We designed a custom human lincRNA microarray to detect genome-wide changes in the expression of lincRNAs in a classical model of innate immune cell activation. The human THP1 monocyte cell line was differentiated to macrophage-like cells and stimulated with a synthetic lipopeptide ligand of TLR2. We identified a panel of 159 lincRNAs that were highly modulated in stimulated THP1 macrophages, one of which, linc1992, was essential for induction of TNF α , a critical cytokine released early in the innate immune response. Linc1992 functions through interactions with heterogenous nuclear ribonucleoprotein L (hnRNPL), which is highly expressed in THP1-derived macrophages but not known to be involved in regulating TNF α expression. HnRNPL is an RNA-binding protein found inside and outside the nucleolus. HnRNPL is one of the members of a family of proteins that associate with

Significance

Genome-wide identification of changes in the expression of large intergenic noncoding RNAs (lincRNAs) in a classical model of innate immune cell activation revealed a panel of 159 lincRNAs that were highly modulated in stimulated THP1 macrophages. One of the lincRNAs, named TNF α and heterogenous nuclear ribonucleoprotein L (hnRNPL) related immunoregulatory LincRNA (*THRIL*), was essential for induction of TNF α , functions through a ribonucleoprotein (RNP) complex with hnRNPL, and plays an important role as regulator of physiological and pathological inflammatory immune responses.

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hnRNAs (such as pre-mRNAs and mRNAs) and play major roles in the formation, packaging, and processing of mRNA (17). Recent studies indicate that hnRNPL is involved in the mammalian stress response and plays dual roles in the nucleus and cytoplasm (18, 19). In the nucleus, hnRNPL binds L1 retrotransposon RNAs and helps to silence endogenous retrotransposon expression (19) whereas, in the cytoplasm, hnRNPL is involved in a stress-responsive switch that controls VEGFA expression (18). However, the extent to which hnRNPL can bind other RNAs and regulate additional mammalian functions remains unclear. Given its close relationship with TNF α and hnRNPL, we named lincRNA *THRIL* (TNF α and hnRNPL related immunoregulatory lincRNA). Importantly, decreased *THRIL* expression was correlated with the acute phase of Kawasaki disease, an inflammatory disease of children, substantiating the importance of lincRNAs as regulators of physiological and pathological inflammatory immune responses.

Results

Many lincRNAs Are Highly Regulated During Innate Activation of Macrophages. We hypothesized that, if lincRNAs are involved in regulating innate immunity, their expression would likely be tightly controlled following cell stimulation with ligands of the innate immune system. To test this hypothesis, we designed a custom lincRNA microarray. cDNA sequences of all known human lincRNAs were extracted from two sources and used for probe design: 1,703 defined lincRNA transcripts were from the Ensembl database (release 61) and 2,915 transcripts were from the Havana database, as previously reported (20). Overall, 5–8 probes were designed per transcript, and ~26,000 commercially available mRNA probes were also included in the array for quality control. We chose the THP1 monocyte cell line as the model system because it has been used extensively to study the response of monocytes/macrophages to innate ligands, as well as the role of small ncRNAs in regulating the immune response (3,

4). THP1 cells differentiate into macrophage-like cells upon treatment with phorbol-12-myristate-13-acetate (PMA), allowing macrophages to be produced in culture in large quantities. To prepare cell samples for microarray analysis, THP1-derived macrophages were stimulated with Pam3CSK4 (Pam), a synthetic lipopeptide that induces proinflammatory gene expression (Fig. 1A). To identify changes in lincRNA expression early in the response, we harvested total RNA 8 h after treatment with Pam. These conditions have been used previously for microRNA studies in THP1 cells (4). We first validated the performance of our probes by comparing two independent samples of unstimulated or Pam-stimulated THP1 macrophages (Fig. S1A). Overall, the lincRNA probes performed as well as the established mRNA probes and exhibited good linear correlation between the two samples (Fig. S1A). From the microarray data, a lincRNA was classified as a hit if expression was suppressed or induced in both samples with at least 1.9-fold change and a P value < 0.05 . We identified 159 unique lincRNAs (240 probes) that were highly induced or suppressed in THP1 macrophages following Pam stimulation, of which 80% (127 lincRNAs, 191 probes) were suppressed and 20% (32 lincRNAs, 49 probes) were induced (Fig. 1B). These observations suggested that lincRNAs were largely suppressed during TLR2 activation of THP1 macrophages.

To validate the microarray data, 20 candidate lincRNAs were selected for validation by RT-qPCR based on the change in expression (1.9-fold, $P < 0.05$), the number of detected probes, and the context of genes within 1 Mb of the lincRNA locus. Of the 20 candidate lincRNAs analyzed, 15 were confirmed to be expressed in THP1 macrophages (Fig. S1B). RT-qPCR analysis detected the same pattern of expression upon Pam treatment as that determined by microarray analysis (Fig. 1C), but the change was statistically significant for only 9 of the 15 lincRNAs. There are at least two explanations for the discrepancy between the number of differentially expressed lincRNAs identified by

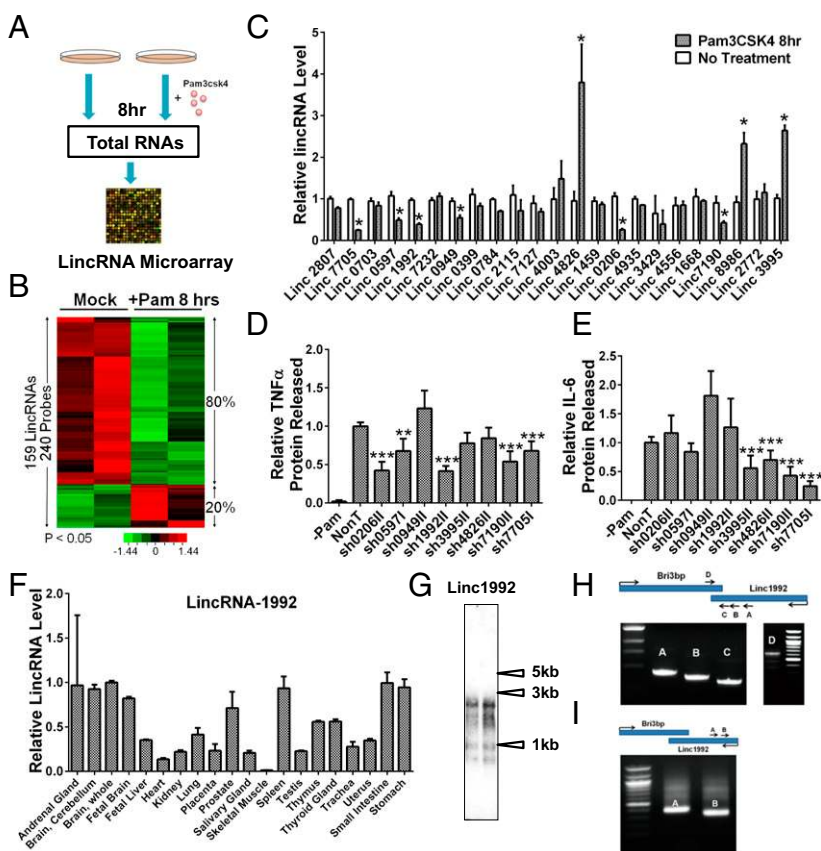


Fig. 1. Identification of lincRNAs associated with innate immunity. (A) Experimental design. THP1 macrophages were treated with 100 ng/mL innate activator Pam3CSK4 (Pam) for 8 h, and total RNA was then harvested for microarray analysis. (B) Heat map of 159 lincRNAs (240 probes) significantly changed upon Pam stimulation of THP1 macrophages. (C) RT-qPCR analysis of lincRNAs following treatment of THP1 macrophages with Pam for 8 h. Results are mean \pm SD of two independent experiments with duplicate wells. * $P < 0.05$. (D and E) TNF α (D) and IL-6 (E) release from THP1 macrophages expressing control shRNA (NonT) or shRNAs targeting the indicated lincRNAs. Cells were treated with Pam for 24 h, and culture supernatants were collected for ELISA. Results are expressed relative to secretion from NonT shRNA cells and are mean \pm SD of five (D) or four (E) independent experiments with duplicate wells. *** $P < 0.001$. (F) RT-qPCR analysis of linc1992 expression in human tissues. Results are mean \pm SD of duplicate wells. (G) Expression of linc1992 in THP1 macrophages by Northern blotting. (H) The 3' RACE of linc1992. (I) The 5' RACE of linc1992.

microarray and RT-qPCR. First, the lincRNA sequences were assembled from available cDNA libraries that have not been accurately mapped; thus, the qPCR primers may not detect the same transcripts as the array probes. Alternatively, some of the microarray probes may have given false-positive signals. Nevertheless, using a combination of microarray and RT-qPCR, we successfully identified a number of lincRNAs that were highly regulated during activation of THP1 macrophages by Pam.

Several lincRNAs Regulate Induction of Proinflammatory Cytokines.

We next determined whether the nine differentially expressed lincRNAs could regulate the THP1 macrophage response to Pam (Fig. 1C). THP1 cells were infected with two shRNAs for each lincRNA and treated with PMA to induce cell differentiation. Eight of the nine lincRNAs (the exception being linc8986) were confirmed to be efficiently knocked down by at least one of the shRNAs (Fig. S2A). To determine the effect of lincRNA knockdown on cytokine secretion, shRNA-infected THP1 macrophages were treated with Pam for 24 h, and the concentrations of proinflammatory cytokines TNF α and IL-6 in the supernatants were measured by ELISA. We found that knockdown of several of the lincRNAs modulated TNF α (Fig. 1D) and IL-6 (Fig. 1E) secretion. These results were confirmed with a second set of shRNAs to exclude the possibility of off-target effects (Fig. S2B and C). Among the tested lincRNAs, knockdown of linc1992 strongly suppressed TNF α induction, and two of the three shLinc1992s tested also strongly suppressed IL-6 induction (Figs. 1D and E and Fig. S2B and C). Analysis of lincRNA expression in 20 different human tissues revealed that linc1992 was widely distributed whereas most of the remaining eight lincRNAs examined showed more tissue-restricted expression patterns (Fig. 1F and Fig. S2D).

The size of linc1992 was examined by Northern blotting and shown to be ~2–2.5 kb in length (Fig. 1G). We used 5' and 3' RACE to map the exact sequence of linc1992, which is located in the reverse strand of the *in cis* gene *Bri3bp* with ~450 bp overlapping with the BRI3 binding protein (*Bri3bp*) mRNA 3' UTR (Fig. 1H and I). The RACE results identified two ~2-kb isoforms of linc1992, which differed only slightly in the first 40–50 nucleotides of the 5' end (SI Materials and Methods). Linc1992 was confirmed by *in vitro* translation analysis to be a noncoding RNA (Fig. S1C–E) and was present in THP1 macrophages at approximately eight copies per cell (Fig. S1F). Collectively, these results identified several lincRNAs that regulate proinflammatory cytokine induction during activation of THP1 macrophages. One of them, linc1992, is an ~2-kb RNA with a broad expression profile and regulates secretion of TNF α , a cytokine that plays a critical role in the innate immune response.

Linc1992 Regulates TNF α Expression Through a Negative Feedback Mechanism.

Because knockdown of linc1992 strongly reduced TNF α secretion following Pam stimulation of THP1 macrophages (Fig. 1D), we next asked whether linc1992 affects TNF α mRNA or protein expression. We transduced THP1 macrophages with four different shlinc1992s, each of which efficiently knocked down linc1992 expression (Fig. 2A), and analyzed TNF α induction in unstimulated or Pam-stimulated cells. Pam stimulation greatly increased TNF α mRNA production (Fig. 2B) and protein secretion (Fig. 2C) in cells transduced with nontargeting shRNA (shNonT) whereas production of both TNF α mRNA and protein were significantly lower in shLinc1992-expressing cells (Fig. 2B and C). TNF α and linc1992 expression following Pam treatment was also examined in the human lung adenocarcinoma epithelial cell line, A549, and the human monocyte-derived macrophages, MDMs, with similar results (Fig. S3A–C). Overexpression of linc1992 rescued both linc1992 and TNF α expression in linc1992-knockdown cells (Fig. S3D and E), which further supports that the effect of linc1992 shRNAs is specific. Thus, linc1992 appears to regulate TNF α transcriptionally, although we cannot rule out the possibility of additional posttranscriptional regulation. To

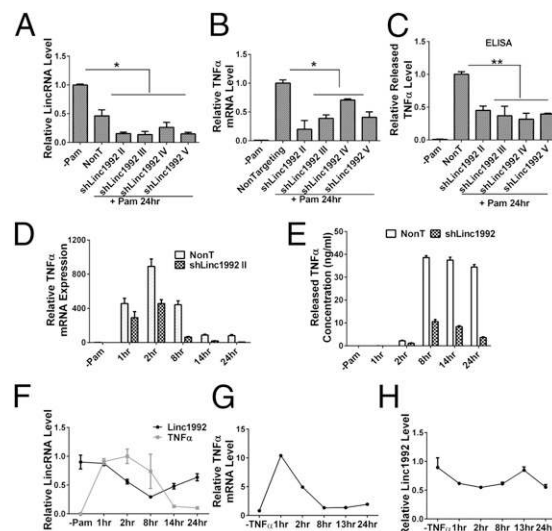


Fig. 2. Linc1992 regulates TNF α expression through a negative feedback mechanism. (A) Knockdown of linc1992 in THP1 macrophages with three distinct shRNAs. Results are mean \pm SD of two independent experiments with duplicate wells. * P < 0.05. (B and C) TNF α mRNA expression in THP1 macrophages. TNF α mRNA (B) was quantified by RT-qPCR analysis, and secreted TNF α (C) was quantified by ELISA. Results are mean \pm SD of two (B) and three (C) independent experiments with duplicate wells. * P < 0.05, ** P < 0.01. (D and E) Time course of TNF α mRNA (D) induction measured by RT-qPCR and secreted TNF α (E) measured by ELISA in Pam-stimulated THP1 cells expressing control or linc1992-specific shRNAs. Results are mean \pm SD of duplicate wells. (F) Kinetics of linc1992 and TNF α mRNA expression in Pam-stimulated THP1 macrophages. Results are mean \pm SD of duplicate wells. (G and H) Kinetics of TNF α mRNA (G) and linc1992 (H) expression in TNF α -treated THP1 macrophages. Signals were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. Results are mean \pm SD of duplicate wells.

determine whether linc1992 knockdown delayed TNF α expression, rather than decreased the magnitude of the response, we analyzed the time course of Pam-stimulated TNF α expression in THP1 macrophages expressing shNonT or shLinc1992. In control cells, TNF α mRNA was detectable within 1 h of Pam treatment and peaked at 2 h poststimulation (Fig. 2D) whereas protein secretion reached maximal levels at 8 h and remained at approximately the same level through 24 h, the last time point examined (Fig. 2E). Linc1992 knockdown cells showed significantly reduced levels of both TNF α mRNA and protein, as expected, but the kinetics of induction was unaffected (Fig. 2D and E). Thus, knockdown of linc1992 affected the magnitude but not the time course of TNF α induction by Pam. We further analyzed the relative expression of TNF α and linc1992 after Pam stimulation and discovered that linc1992 expression was down-regulated once TNF α production was detectable (Fig. 2F), suggesting that linc1992 may be part of a protective feedback loop to control TNF α levels, a mechanism that has also been proposed for microRNA function in innate immunity (4). To test this hypothesis, unstimulated THP1 macrophages were treated with purified recombinant human TNF α to avoid the possibility of contamination with other cytokines, and the time course of TNF α mRNA and linc1992 expression was analyzed over the following 24 h (Fig. 2G and H). We found that TNF α mRNA expression was elevated as early as 1 h after addition of exogenous TNF α and that this observation was mirrored by a decrease in Linc1992 expression, confirming the negative feedback regulation of linc1992 by TNF α . We also asked whether Pam-induced down-regulation of linc1992 was dependent on the expression of MyD88, a critical adapter protein for innate immune signaling (2, 21, 22). As expected, efficient shRNA-mediated knockdown of MyD88 (Fig. S4A) was accompanied by a substantial reduction in Pam-induced TNF α induction (Fig. S4B); however, linc1992

expression was unaffected (Fig. S4C). Notably, MyD88 mRNA was reduced by Pam stimulation (Fig. S4A), suggesting that MyD88 expression may also be subject to feedback regulation. Together, these data suggest that regulation of linc1992 upon innate activation occurs through MyD88-independent pathways.

Because linc1992 is located close to the coding gene Bri3bp and lincRNAs have been reported to have cis-regulatory effects on nearby genes (20), we investigated whether knockdown of linc1992 affected the expression of its *in cis* genes. Indeed, linc1992 knockdown resulted in ~50% reduction of Bri3bp mRNA (Fig. S4D) but did not affect expression of other nearby genes such as Aacs, Scarb1, Ubc, and Dhx37 (data not shown). To determine whether the reduction in TNF α expression in linc1992 knockdown cells was due to decreased expression of Bri3bp, we used two shRNAs to efficiently knockdown Bri3bp expression (Fig. S4E). Significantly, TNF α mRNA levels were reduced by 40–50% in Pam-stimulated shBri3bp-expressing cells compared with cells expressing shNonT (Fig. S4E). We reasoned that, if TNF α regulation by linc1992 was mediated solely through Bri3bp, Bri3bp overexpression should rescue the linc1992 knockdown phenotype. Although lentiviral-mediated overexpression of Bri3bp (Fig. S4F) increased Pam-induced TNF α induction in control THP1 cells, it could not rescue the reduction in TNF α caused by linc1992 knockdown (Fig. S4G). It is also worth noting that Bri3bp knockdown did not significantly affect hnRNPL expression (Fig. S4H). These data therefore suggest that Bri3bp is not the dominant mediator of linc1992 regulation of TNF α expression although decreased Bri3bp expression may contribute to the phenotype.

Linc1992 Interacts with hnRNPL to Regulate TNF α Induction. LincRNAs usually function by physically interacting with other cellular factors (9–11, 15, 16). To determine how linc1992 regulates TNF α expression, we sought to identify intracellular linc1992-binding factors using an unbiased approach. Full-length linc1992 was *in vitro* transcribed with biotinylated nucleotides (Fig. S5A), and biotin incorporation was confirmed by dot blotting (Fig. S5B). Biotinylated linc1992 (or partial lacZ mRNA without protein-coding potential as a negative control) was then incubated with total protein extracts from THP1 macrophages and pulled down with streptavidin. The associated proteins were analyzed by SDS/PAGE and silver staining (Fig. 3A). Two distinct bands specifically present in the linc1992 pull-down samples (Fig. 3B) were excised and analyzed by mass spectrometry, which identified four potential binding proteins: hnRNPL, DDX5, NONO, and vimentin. To confirm that the proteins specifically interact with linc1992, we repeated the pull-down assay with biotinylated linc1992 and probed for the four proteins by Western blot analysis. Only hnRNPL was confirmed to be bound specifically to linc1992 (Fig. 3C). To identify the hnRNPL-interacting region of linc1992, three fragments of linc1992 (1–699, 700–1405, and 1406–2012) were biotinylated and used in the pull-down assay with THP1 lysates. These experiments showed that the 5' fragment and, to a lesser extent, the 3' fragment of linc1992 mediated the interaction with hnRNPL (Fig. 3D). To substantiate these observations, anti-hnRNPL antibody was used to immunoprecipitate endogenous hnRNPL from nuclear extracts of THP1 macrophages, and RNAs bound to hnRNPL were extracted and analyzed. We detected ~sixfold enrichment of linc1992, but not control *GAPDH* RNA, in the anti-hnRNPL immunoprecipitates compared with the IgG control (Fig. 3E). These results confirmed that linc1992 and hnRNPL can form an RNP complex *in vivo*. We next asked whether hnRNPL was necessary for Pam-induced TNF α expression in these cells. HnRNPL expression was depleted using five different shRNAs, all of which were confirmed to efficiently knock down hnRNPL mRNA levels (Fig. S5C). Notably, knockdown of hnRNPL resulted in a dramatic decrease in Pam-stimulated TNF α induction (Fig. 3F) but did not affect expression of Bri3bp (Fig. S5C). Linc1992 expression was moderately decreased in shhnRNPL-expressing cells (Fig. S5D), possibly due to destabilization.

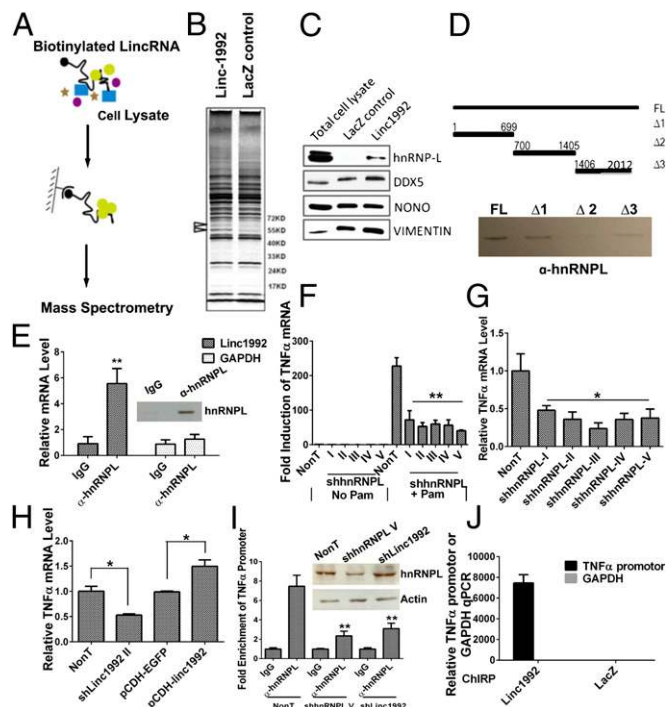


Fig. 3. Linc1992 functions by interacting with hnRNPL. (A) Experimental design for pull-down assays and identification of linc1992-associated cellular proteins. Linc1992 and lacZ RNA were biotinylated by *in vitro* transcription, refolded, and incubated with THP1 total cell lysates. (B) Silver staining of biotinylated linc1992-associated proteins. Two linc1992-specific bands (arrows) were excised and analyzed by mass spectrometry, which identified hnRNPL, DDX5, NONO, and vimentin. (C) Western blotting of proteins from lacZ and linc1992 pull-down assays. (D) Western blotting of hnRNPL in samples pulled down by full-length (FL) or truncated linc1992 ($\Delta 1$: 1–699, $\Delta 2$: 700–1405, and $\Delta 3$: 1406–2012). (E) Linc1992 association with hnRNPL. Nuclear lysates of THP1 macrophages were immunoprecipitated with control mouse IgG or anti-hnRNPL antibody, and the complexes were analyzed for the presence of linc1992 or *GAPDH* by RT-qPCR. Signals were normalized to actin mRNA. Results are mean \pm SD of two independent experiments with duplicate wells. $**P < 0.01$. Specific immunoprecipitation of hnRNPL was confirmed by Western blotting (inset). (F) TNF α mRNA levels in THP1 macrophages expressing NonT shRNA or five shRNAs targeting hnRNPL. TNF α mRNA was analyzed by RT-qPCR. Results are mean \pm SD of two independent experiments with duplicate wells. $**P < 0.01$. (G) Basal TNF α mRNA levels in hnRNPL-depleted THP1 macrophages. Experiment was performed as in F except cells were not treated with Pam. Results are mean \pm SD of two independent experiments with duplicate wells. $*P < 0.05$. (H) TNF α expression in linc1992-depleted or linc1992-overexpressing THP1 macrophages. Samples were analyzed by RT-qPCR 3 d after infection. Results are mean \pm SD of two independent experiments with duplicate wells. $*P < 0.05$. (I) ChIP analysis of hnRNPL association with the TNF α promoter region. ChIP with anti-hnRNPL or control IgG was performed as described in *Materials and Methods*. Results are expressed as the fold enrichment of TNF α promoter sequence in hnRNPL compared with IgG ChIP. Results are mean \pm SD of three independent experiments. $**P < 0.01$. Input samples were analyzed by Western blotting (inset) with anti-hnRNPL or anti-actin antibodies. (J) ChIP analysis of linc1992 binding to the TNF α promoter. ChIP was performed as described in ref. 23. Results are expressed as fold enrichment of TNF α or *GAPDH* promoter sequence in linc1992 compared with lacZ RNA ChIP.

We noted that hnRNPL knockdown not only affected Pam-stimulated TNF α expression in THP1 macrophages, but also significantly decreased basal TNF α mRNA expression (Fig. 3G), a phenomenon also observed in linc1992 knockdown cells (Fig. 3H). Overexpression of linc1992 (Fig. S5E) significantly up-regulated basal TNF α mRNA levels (Fig. 3H). Because pull-down assays showed that linc1992 was associated with hnRNPL in linc1992-overexpressing cells (Fig. S5F), we reasoned that linc1992 and

hnRNPL might regulate TNF α expression by forming a complex at the TNF α promoter. Indeed, chromatin immunoprecipitation (ChIP) assays revealed that hnRNPL bound to the TNF α promoter (Fig. 3I). Furthermore, knockdown of hnRNPL or linc1992 independently reduced binding of hnRNPL to the TNF α promoter (Fig. 3I), suggesting that linc1992–hnRNPL complex binding at the promoter is required to maintain basal transcription of TNF α . We also confirmed by ChIRP (chromatin isolation by RNA purification) assays (23) that linc1992 bound directly to the TNF α promoter (Fig. 3J). Overexpression of linc1992 didn't enhance TNF α expression in hnRNPL knocked down cells (Fig. S5 G and H), further confirming the collaboration of linc1992 and hnRNPL to regulate TNF α expression. Notably, knockdown of linc1992 did not affect hnRNPL expression (Fig. 3I), and Pam treatment didn't decrease the expression of hnRNPL (Fig. S5I).

Based on these findings, we propose a model in which hnRNPL and linc1992 form an RNP complex that regulates basal and stimulated transcription of TNF α by binding to the TNF α promoter. In activated cells, high levels of TNF α secretion initiate a negative feedback loop in which linc1992 and, in turn, TNF α expression is down-regulated (Fig. 4A).

Linc1992 Is Required to Maintain the Expression of Many Innate Immunity-Associated Genes. To identify additional linc1992-regulated target genes, we performed a transcriptome analysis by RNA-seq. Next, we analyzed samples of THP1 macrophages expressing shNonT or shLinc1992 and treated with or without Pam. We chose shLinc1992 II shRNA for these experiments as it most effectively reduced linc1992 levels (Fig. 2A). To ensure that changes in expression of a broad array of innate immunity-associated genes would be detected, we extended the Pam treatment time to 24 h. Samples from three independent cell incubations were analyzed for each condition by HiSeq2000, and between 10 and 27 million reads per sample were generated with a mapping rate of more than 80%. Read counts were then further processed to RPKM (reads per kilobase per million) and analyzed by ANOVA,

with a filter setting of at least a twofold expression change and a false discovery rate (FDR) = 0.05 (Fig. S6A).

We first compared the results of the control shRNA samples with or without Pam treatment, reasoning that a significant enrichment of innate immune genes should be seen in this dataset. Gene ontology analysis of 618 differentially expressed genes showed a significant representation of genes usually activated during an innate immune response (Fig. S6B and C). These data validated our overall experimental design and data processing.

We next compared cells expressing shLinc1992 and control shRNA in the absence of Pam stimulation. Surprisingly, among the 454 genes significantly modulated by shLinc1992, 444 were dramatically down-regulated and only 10 were up-regulated (Fig. S6D), strongly suggesting that linc1992 may be required to maintain basal transcription of a broad array of genes. Cells expressing shLinc1992 and shNonT showed no significant differences in the expression of cytokine or cytokine receptor genes, with the exception of a decrease in *Bmpr2* expression in shLinc1992 cells (Fig. S6E), confirming that innate signaling was not activated in the absence of Pam. Analysis of the dataset from Pam-stimulated cells indicated that 317 genes were differentially expressed in control and shLinc1992-expressing cells, of which 101 were up-regulated and 216 were down-regulated by linc1992 knockdown (Fig. 4B). We found 131 overlapping genes in the shLinc1992 – Pam and shLinc1992 + Pam groups, which was significant compared with random groups of genes ($P = 0.0004$, Fisher's exact test). Knockdown of linc1992 decreased Pam-induced expression of cytokines and chemokines in addition to TNF α (Fig. S6G), including IL-8, CXCL10, CCL1, and CSF1. Additional genes known to regulate cytokine induction and innate immunity were enriched in Pam-stimulated cells (Fig. S6F and Table S1), and most of these (24/32, 75%) were decreased upon knockdown of linc1992. A selection of 22 genes was further validated by RT-qPCR (Fig. 4C). Interestingly, several of these genes are involved in the transcriptional and posttranscriptional regulation of TNF α expression, including the transcription factor POU2F1 (23), and rhomboid family member 2 (RHBDF2), a protein that binds to TNF α -converting enzymes to facilitate their exit from the ER membrane (24). This finding suggests that linc1992 may modulate aspects of TNF α expression other than transcription. The expression of many other documented NF κ B target genes was similarly decreased upon knockdown of linc1992 (Fig. S6H). Collectively, these data demonstrate that reduced expression of linc1992 dysregulates the induction of cytokines during innate immune cell activation and strongly suggest that linc1992 may play a broad role in maintaining transcription of an array of genes. Based on the similar experiment, hnRNPL was knocked down, and 22 genes, which were selected as the potential targets of linc1992, were further validated by RT-qPCR. Four genes, protein kinase C alpha (PRKCA), RAGEF1B, MMP9, and RHBDF2, showed the same trend with the treatment of Pam as that shown in linc1992 knocked down cells (Fig. S7A). We further tested the binding of linc1992 to the promoters of PRKCA and RHBDF2 and found that linc1992 was indeed enriched at these loci, but not in MALT1 (Fig. S7B). Furthermore, linc7705 and 2807 were not associated with hnRNPL (Fig. S7C). These results further indicate that linc1992 and hnRNPL interact with each other specifically and may work together to regulate downstream genes. Given its importance in TNF α induction, hnRNPL binding, and the innate immune response, we named linc1992 *THRIL* (TNF α and hnRNPL Related Immunoregulatory LincRNA).

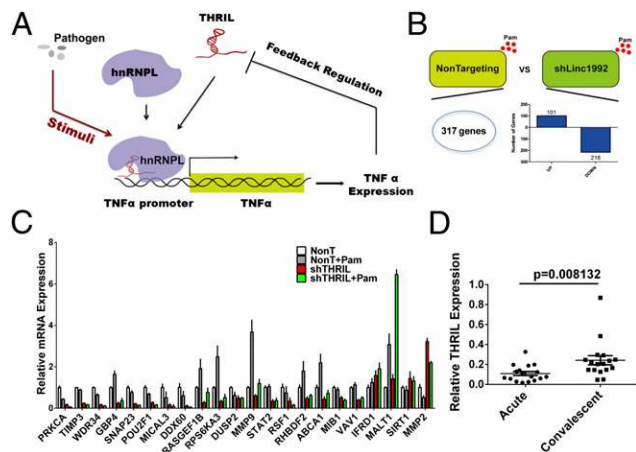


Fig. 4. Linc1992 knockdown dysregulates expression of innate immunity-associated genes. (A) Model for *THRIL*-mediated regulation of TNF α gene expression. (B) Identification of differentially expressed genes in Pam-stimulated THP1 macrophages expressing control or linc1992-targeting shRNA. Datasets were compared by ANOVA and filtered using a twofold change in expression and FDR of 0.05. (C) Validation of changes in gene expression by RT-qPCR. Of the 32 genes listed in Table S1, 22 with a single transcript were selected for validation. Results are mean \pm SD of two independent experiments with duplicate wells. (D) *THRIL* expression in the blood of Kawasaki disease patients. Whole blood samples were obtained from 17 patients during the acute phase of disease (before treatment) and 1–2 mo later during the convalescent phase. *THRIL* expression was measured by RT-PCR. Data were calculated using the $2^{-(\Delta\Delta Ct)}$ method and analyzed with Student *t* test.

***THRIL* Expression Is Associated with Kawasaki Disease.** To determine whether *THRIL* expression is modulated in human inflammatory diseases, we examined RNA samples from patients with Kawasaki disease, an acute self-limiting vasculitis of children that can lead to cardiovascular damage (24). The disease course is characterized by an acute phase, during which serum TNF α levels are elevated, followed by a convalescent phase. We compared the

expression of lincRNAs in paired samples of whole blood taken from 17 patients during the acute phase of disease (before treatment with i.v. Ig) and during the posttreatment convalescent phase when inflammation is resolving. The convalescent phase samples were collected within 1–2 mo of the acute phase, when the erythrocyte sedimentation rate and the platelet count had returned to normal. We observed that *THRIL* expression was significantly lower in the acute phase than in the convalescent phase (Fig. 4D). Expression levels did not correlate with the day of illness on which the sample was drawn (Fig. S6J), acute levels of C-reactive protein, white blood cell counts, or erythrocyte sedimentation rate, suggesting complex regulation of the innate immune response in these patients. *THRIL* expression was clearly lower during the acute phase of disease when TNF α levels were elevated. This effect mirrors the negative feedback loop of *THRIL* regulation demonstrated in our *in vitro* experiments and suggests that *THRIL* could be a novel biomarker for immune activation.

Discussion

The complete sequencing of the human genome revealed many surprises, particularly that a large portion of the genome was composed of noncoding regions. Many of these regions are actively transcribed into mRNAs and other RNA species. Among them, lincRNAs have been shown to be involved in regulating many important processes (6, 7, 9, 11, 25, 26) and exhibit cross-species conservation of their genomic loci but not of their sequences (13). LincRNA research is a rapidly expanding field and novel functions for lincRNAs will undoubtedly continue to be discovered.

In this study, we systematically analyzed the change in expression of lincRNAs upon activation of innate immune signaling in THP1 macrophages. Through a combination of genomic, biochemical, and cell biological approaches, we identified an unannotated lincRNA, termed *THRIL*, as a key player in regulating TNF α expression in these cells (Fig. 4A). Based on our result, *THRIL* was shown to function through forming an RNA–protein complex with hnRNPL, a protein involved in the stress response (18) but not previously documented to have a role in innate immunity. Our data does not rule out the possibility that the interaction of *THRIL* with hnRNPL may be mediated by other unidentified cellular factors within the protein complex. Meanwhile, transcriptome analysis of knockdown cells revealed that loss of *THRIL* dysregulated the expression of additional

immune-associated genes, many of which play well-documented roles in innate immunity. Therefore, *THRIL* may have a broad impact on many aspects of immunity that should be addressed further. In addition, our disease-association study indicated a positive association between decreased expression of *THRIL* and the acute phase of Kawasaki disease. The involvement of *THRIL* in TNF α regulation strongly suggests that it may also contribute to other common inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease, and thus further investigation of *THRIL* expression in human disease is warranted. This study reports a lincRNA functioning in the regulation of innate immune signaling, and our data provide the proof of concept that lincRNAs could be key players in regulating various host–pathogen interactions. Recently, two groups reported lincRNAs regulating innate immunity (27, 28). An enhancer-like lincRNA termed NeST [nettoie Salmonella pas Theiler's (cleanup Salmonella not Theiler's)] was reported to regulate epigenetic marking of IFN-gamma-encoding chromatin, expression of IFN-gamma, and susceptibility to a viral and a bacterial pathogen (27). Intriguingly, lincRNA-Cox2, a key regulator of inflammatory response, modulates both the activation and repression of immune response genes (28). Many lincRNAs and their mechanisms in regulating immunity and host–pathogen biology will emerge in future.

Materials and Methods

shRNA Design and Vector Construction. shRNA sequences were designed using the open-access tool from the Broad Institute. Probes containing both sense and antisense strands of shRNAs were cloned into the pLKO.1-puro lentiviral vector (Dataset S1).

ELISAs for Secreted TNF α and IL-6. Lentivirus-infected THP1 macrophages were treated with 100 ng/mL Pam3CSK4 for 24 h, and the culture supernatants were collected. TNF α and IL-6 levels were measured by ELISA (eBioscience) according to the manufacturer's instructions.

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