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IFNG-AS1 Enhances Interferon Gamma Production in Human Natural Killer Cells

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HIGHLIGHTS
Natural killer cells (NKs) express a previously undescribed transcript of IFNG-AS1
Upon activation, NKs upregulate IFNG-AS1 along with later IFNγ expression
Overexpression of IFNG-AS1 in an NK line augments IFNγ expression and secretion
IFNG-AS1 influences innate immunity, suggesting a general role in the IFNγ response

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Article

IFNG-AS1 Enhances Interferon Gamma Production in Human Natural Killer Cells

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SUMMARY

Long, non-coding RNAs (lncRNAs) are involved in the regulation of many cellular processes. The lncRNA IFNG-AS1 was found to strongly influence the responses to several pathogens in mice by increasing interferon gamma (IFNγ) secretion. Studies have looked at IFNG-AS1 in T cells, yet IFNG-AS1 function in natural killer cells (NKs), an important source of IFNγ, remains unknown. Here, we show a previously undescribed sequence of IFNG-AS1 and report that it may be more abundant in cells than previously thought. Using primary human NKs and an NK line with IFNG-AS1 overexpression, we show that IFNG-AS1 is quickly induced upon NK cell activation, and that IFNG-AS1 overexpression leads to increased IFNγ secretion. Taken together, our work expands IFNG-AS1’s activity to the innate arm of the type I immune response, helping to explain its notable effect in animal models of disease.

INTRODUCTION

The central dogma of molecular biology states that information travels from DNA to RNA to protein. Recently, a large body of work has shown that many species of RNA have roles other than shuttling information and are fully functional on their own. This includes the long, non-coding RNAs (lncRNAs).

One lncRNA that has been shown to be particularly important is IFNG-AS1, also known as TMEVPG1 or NeST. It was originally found as a candidate to explain differences in murine response to Theiler’s virus (Vigneau et al., 2001). Later works showed that IFNG-AS1 regulates interferon gamma (abbreviated IFN for the RNA transcript and IFNγ for the protein) (Vigneau et al., 2003) and proved that IFNG-AS1 controls the response to Theiler’s virus and Salmonella (Gomez et al., 2013). IFNG-AS1 dysregulation was found in a number of human diseases, and IFNG-AS1 levels correlate with clinical markers in some diseases (Li et al., 2016; Padua et al., 2016; Peng et al., 2015; Wang et al., 2016). This is consistent with the knowledge that IFNγ, which IFNG-AS1 regulates, is the pro-inflammatory end product of the type I immune response (Eberl, 2016) and with clinical findings that defects in the IFNγ pathway lead to increased susceptibility to a wide variety of diseases (Averbuch et al., 2011; Filipe-Santos et al., 2006).

IFNG-AS1 has also been studied on a molecular level. Significant work by Collier et al. and Spurlock et al. has characterized regulatory features of IFNG-AS1, including transcription factors important for IFNG-AS1 function and specificity to Th1 cells among the T cell lineages (Collier et al., 2012, 2014; Spurlock et al., 2015, 2017). Their study and that of others indeed focuses on Th1 cells, an important source of IFNγ. There is little data, however, on the function of IFNG-AS1 in another IFNγ producer, natural killer cells (NKs).

NKs are part of the innate immune system and are a subset of the type I innate lymphoid cells (Spits et al., 2016; Vivier et al., 2008). They were named for their ability to kill cancer cells without prior exposure to antigen, but have since been shown to recognize a wide variety of threats (Bar-On et al., 2017; Gur et al., 2015; Vitenstein et al., 2016), including Theiler’s virus itself (Paya et al., 1989). Accordingly, defects in human NKs cause serious immune deficiency (Orange, 2013).

NKs are also important secretors of various cytokines and chemokines. Several studies have suggested that NKs not only secrete IFNγ earlier than T cells but also play crucial regulatory roles, including activating the T cell phase of type I immunity (Heremans et al., 1994; Mocikat et al., 2003; Orange et al., 1995; Waggoner et al., 2016; Yang et al., 1997). Furthermore, NKs also express the transcription factors shown to be involved...
in IFNG-AS1 function (Miyagi et al., 2007; Paolini et al., 2015; Spits et al., 2016). Given the importance of NKs in the systemic IFNγ response, we set out to determine what, if any, role IFNG-AS1 plays in human NKs.

RESULTS

IFNG-AS1 Is Expressed in Activated Human Natural Killer Cells

We first determined the presence of IFNG-AS1 in NK cells by PCR. Using a sequence in exon 5 of IFNG-AS1, we found expression in cDNA prepared from bulk (activated) NKs, bulk CD4+ T cells, an NK cell line, and a T cell line (YTS and Jurkat, respectively, Figure 1A). To confirm that IFNG-AS1 expression in NKs is not donor specific, we analyzed NK cells derived from seven healthy donors and observed expression in all of them (Figure 1B).

To assess IFNG-AS1 abundance, we used quantitative real-time PCR, with the colon carcinoma line RKO and the mouse thymoma line BW as negative controls (Figure 1C). Consistent with the band strength in Figure 1A, NK and YTS cells both express IFNG-AS1 at higher levels than do CD4+ T cells, and the expression of IFNG-AS1 in Jurkat cells is minimal.

Intrinsic Regulation of IFNG-AS1 in NKs

An important form of lncRNA regulation involves cellular localization. Like proteins, IncRNAs can localize to a specific organelle, with a basic division between nuclear and cytoplasmic IncRNAs. Using RNA fluorescence in situ hybridization (FISH) we found IFNG-AS1 expression in the cytoplasm and nucleus of NKs.
In YTS cells, IFNG-AS1 was also expressed in both compartments; however, we observed clear nuclear enrichment (Figure 1D, middle row). To confirm specificity, we performed RNA FISH on YTS cells overexpressing IFNG-AS1 and found increased fluorescence (Figure 1D, bottom row; overexpression shown in Figure 4A).

IFNγ secretion is regulated at several stages, including at the post-transcriptional level. RNA-binding proteins actively downregulate IFNG transcripts by binding AU-rich elements (Ogilvie et al., 2009). Given that IFNG-AS1 is a globally AU-rich transcript (GC content = 38.45%), we wondered whether it shared this form of regulation with IFNG. Previous reports have used cycloheximide (CHX), a global translation inhibitor, to allow accumulation of IFNG transcripts (Lebendiker et al., 1987), so we applied the same treatment to NK cells. Using quantitative real-time PCR we indeed found that IFNG-AS1, like IFNG, accumulates in NKs after CHX treatment (Figures 1E and 1F).

Several cytokines can also stimulate NKs to secrete large amounts of IFNγ, notably interleukin (IL)-12 and the combination of IL-12 and IL-18 (Lusty et al., 2017; Miyake et al., 2010). We wished to check if such strong induction of IFNγ is associated with an increase in expression of IFNG-AS1. Treatment of these cytokines indeed upregulated IFNG-AS1 (Figure 1G), along with the expected induction of IFNγ at both RNA and protein levels (Figures 1H and 1I).

Sequence of IFNG-AS1 Expressed in NKs

As lncRNA function is influenced by its sequence and structure, we wished to ensure that we had the complete sequence of IFNG-AS1. Public databases provide two transcript variants for human IFNG-AS1. The first contains five exons, and the second lacks exon 4. This, however, contradicts previous reports, which refer to six exons (Vigneau et al., 2003) (Figure 2A). To determine the sequence expressed in NKs, we performed RNA FISH on YTS cells overexpressing IFNG-AS1, and observed increased fluorescence (Figure 1D, bottom row; overexpression shown in Figure 4A).

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The majority of our sequencing of IFNG-AS1 in NKs matched database sequences, whereas the 3’ end was previously unreported. We found no evidence of a sixth exon, although exon 5 is approximately 200 bp longer than database sequences (Figure 2A). We found this sequence in three healthy donors, as well as in YTS cells. Furthermore, we confirmed that the genomic region downstream to the end of our sequence is not A-rich, making off-target priming by oligo-(dT) in cDNA preparation highly unlikely. The sequence of IFNG-AS1 in NKs is shown in Figure 2B.

Triggering of the Natural Cytotoxicity Receptors Induces IFNG-AS1 and IFNG Expression

We then tested whether activation of various NK receptors affects IFNG-AS1 expression using a plate-bound antibody assay (PBA, please see the Transparent Methods for more information). We examined the three members of the natural cytotoxicity receptor (NCR) family—NKp30, NKp44, and NKp46—which are potent activators of NKs. Interestingly, we found that IFNG-AS1 is upregulated very quickly—1 h after activation (Figure 3A). By 5 h, IFNG-AS1 seems to be repressed (Figure 3A), and at 24 h it largely returns to baseline (data not shown). We noted that the kinetics were consistent with IFNG-AS1 being an enhancer, as its upregulation precedes that of IFNG (Figure 3B). In addition, stimulation of these receptors led to increased IFNy secretion (Figure 3C).

We next wanted to examine whether IFNG-AS1 and IFNG are similarly induced upon interaction of the NCRs with their cellular ligands (Figures 3D–3I). To assess NKp30 activation, we used the melanoma line 624mel overexpressing the NKp30 ligand B7H6 (Figures 3D, 3E, and S1A). For NKp44 and NKp46 we used the choriocarcinoma line Jeg3 preincubated with the PR8 strain of influenza, which expresses hemagglutinin—a ligand for both NKp44 and NKp46 (Arnon et al., 2001; Mandelboim et al., 2001) (Figures 3F, 3G, and S1B). We normalized IFNG-AS1 and IFNG expression levels using NKp44 and 2B4 (expressed only by...
the NKs) along with the housekeeping gene GAPDH, with the untreated NKs serving as a pure reference sample.

The kinetics was much faster in the cell-based models than in the PBA, and 15-, 30-, and 60-min time points are shown. Similar to the PBA, we saw quick induction and repression of IFNG-AS1 followed by induction of IFNG in the NCR-activated samples (Figures 3D–3G). With the PBA, the increase in RNA levels were accompanied by increased IFNγ secretion (Figures 3Ha and 3I).

IFNG-AS1 Overexpression Enhances IFNγ Production

To isolate IFNG-AS1’s effect, we overexpressed IFNG-AS1 in YTS cells using lentiviral transduction (Figure 4A). YTS cells do not express any of the NCRs in a functional manner, but instead are stimulated via the co-activating receptor 2B4 (Elias et al., 2014). As YTS cells also express 2B4’s ligand CD48 (Figure S1 C), the cells have a baseline level of activation and secrete IFNγ under normal growth conditions.

YTS cells can be further activated by a variety of methods. We activated the IFNG-AS1-overexpressing YTS cells using Toll-like receptor (TLR) ligation (lipopolysaccharide [LPS]), stimulatory cytokines (IL-2, IL-12, IL-12 + IL-18, and IL-15), non-specific lymphocyte activation (phorbol 12-myristate 13-acetate [PMA] and ionomycin), and target-mediated activation (721.221 cells, which express high levels of CD48 [Figure S1D]). As the treatments have different kinetics, we show peak IFNG RNA levels (Figure 4B) and cumulative IFNγ secretion after 24 h of incubation (Figure 4C). Representative time course graphs of IFNG expression are shown in Figure S2. For concentrations of treatments, please see the Transparent Methods.

As seen in Figures 4B and 4C, some forms of activation (721.221 cells, IL-2, and IL-15) activated all cells but caused significantly more IFNγ production in the cells overexpressing IFNG-AS1. IL-12 alone did not seem
to stimulate YTS cells, whereas the addition of IL-18 caused increases in all cells, but significantly more in the context of IFNG-AS1 overexpression. PMA/ionomycin increased production in all cells but showed no effect of IFNG-AS1 overexpression. Finally, LPS seemed to effectively activate only the cells overexpressing IFNG-AS1. These increases indicate that IFNG-AS1 is a functional regulator of IFNγ in NKs.

DISCUSSION

Here we have shown that the lncRNA IFNG-AS1 is not only relatively abundant in NKs but also enhances IFNγ secretion in these cells. Many of the facts about IFNG-AS1 in T cells hold true in NKs. In both cells, for example, IFNG-AS1 is upregulated upon cellular activation and contributes to increased IFNγ production. The models we used also add information not previously shown in T cells.

First and foremost, we provide a previously undescribed sequence of IFNG-AS1 in humans. The extra nucleotides described here may influence 3D structure, and therefore function. IFNG-AS1 in NKs is indeed spliced and polyadenylated, consistent with its function as an enhancer RNA (Gil and Ulitsky, 2018), and this sequence may alter processing. Second, whereas NKs express IFNG-AS1 at higher levels than do T cells (Figure 1C), RNA FISH shows even more expression than would be expected based on previous quantification. Almost all cells examined expressed IFNG-AS1, although we did find a few cells with no fluorescence. This may be consistent with higher copy numbers of IFNG-AS1 found by Gomez et al. when T cells were activated. We did, however, clearly see cytoplasmic expression, whereas previous work suggested nuclear restriction (Gomez et al., 2013). This, too, may be a by-product of basal levels of activation found in cultured NK cells, which could provide hints of IFNG-AS1’s mechanism of action.

Vigneau et al. originally observed the expression of IFNG-AS1 or IFNG, yet not both at any one time, and hypothesized that IFNG-AS1 inhibits IFNG. Subsequent works have shown that the opposite is true, and our use of time course assays may resolve the contradiction. We show that IFNG-AS1 and IFNG transcripts are simply upregulated at different time points following activation, and that expression largely does not overlap.

In addition, whereas other studies use PMA/ionomycin or anti-CD3 antibodies, we describe a variety of signals that activate IFNG via IFNG-AS1. This is similar to earlier works on IFNG itself (Billiau and Matthys, 2009). IL-2, IL-12, IL-15, and IL-18 are important activators of NKs, specifically with regard to IFNγ production (Carson et al., 1995; Handa et al., 1983; Schoenborn and Wilson, 2007). NKs also respond to TLR ligation (Vivier et al., 2008), and to specific receptors, especially the NCRs (Mandelboim et al., 1998). Broading our pool of signals may help in expanding IFNG-AS1 study to other cell types and experimental models.

IFNG-AS1 was originally found due to its role in the murine response to Theiler’s virus, and the initial focus was on the T cells in this process. Paya et al. show that NKs are crucial in the response to Theiler’s virus, and our findings may help link these works. Several studies have suggested that IFNγ produced by the innate immune system must prime the T cell response, with NKs playing a central part in this process (Guo et al., 2018; Heremans et al., 1994). NKs integrate a variety of signals received through their activating and inhibitory receptors (Kruse et al., 2014; Vivier et al., 2008). The first steps of this pathway are fairly well known, including, for example, the signaling molecules DAP12, ZAP70, and Syk (Jelencic et al., 2018; Pugh et al., 2018), but the later stages are less understood. IFNG-AS1 can now join the web of intracellular signaling at this phase of the immune response.

Although IFNG-AS1 specificity to T_h1 cells has been suggested, we look beyond the T cell populations and find an additional set of models in which IFNG-AS1 plays an important role. This can pave the way to study of IFNG-AS1’s significance in various disease models, and help us understand the dysregulation found in clinical works. Our findings also underscore the importance of IFNG-AS1 on a systemic level. The notable phenotypic differences that originally led to IFNG-AS1’s discovery imply a central role. Here we help explain that role by demonstrating IFNG-AS1’s activity in the innate immune system, suggesting that IFNG-AS1 is a general regulator of the type I immune response.

Limitations of the Study

As our work focuses on human cells, it is limited by the usual problems associated with ex vivo study, namely, the reductionism inherent to these methods. The human cells we use are themselves activated,
a state that reflects only our best approximation of the actual state of these cells in action. Finally, as genetic alteration in primary human NKs is an extremely inefficient process, we used an NK cell line, again an approximation of NK cells themselves.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

DATA AND SOFTWARE AVAILABILITY

The sequence for the isoform of IFNG-AS1 shown in Figure 2B is also available as GenBank: MK296539.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods and two figures and can be found with this article online at https://doi.org/10.1016/j.isci.2018.12.034.

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AUTHOR CONTRIBUTIONS

N.S. designed and performed experiments, analyzed results, and wrote the paper. O.B., D.S., A.D.-C., E.S., I.K., M.H., A.R., M.G., and R.Y. assisted in designing and performing experiments. P.T., A.O., and Y.C.-A. provided critical reagents. O.M. designed experiments, wrote the paper, and supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

IFNG-AS1 Enhances Interferon Gamma Production in Human Natural Killer Cells

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Figure S1
Figure S1. Cell-based assays, related to Figures 3 and 4.

(A-B) FACS staining of the target cells used in Figure 3C-D. Background staining is shown in the gray, filled histograms. (C-D) FACS staining of (C) YTS cells and (D) 721.221 cells for the 2B4 ligand CD48. Background staining is shown in the gray, filled histograms.
Figure S2
**Figure S2.** YTS Activation Assay Time Course Graphs, related to Figure 4.

Time course graphs of IFNG RNA levels in activation assays of YTS cells, the peaks of which are shown in Figure 4B. Each graph shows the untreated cells (black) and one other treatment (various colors). Cell type is indicated by marker shape (wild-type = circle, empty-vector transfected = square, and IFNG-AS1 overexpression = triangle). Expression is normalized to an independent sample of untreated parental YTS cells. Representative of three independent experiments. WT – wild-type, EV – empty-vector transfected, OE – IFNG-AS1 overexpression.
**Transparent Methods**

**Cell Culture and Transfection**

NK cells and CD4+ T cells were derived from healthy donors pursuant to approval of the Institutional Review Board of Hadassah Medical Center. The procedure has been described previously (Yamin et al. 2013). Briefly, PBMCs were purified on a ficoll gradient, and NKs were separated using the EasySep™ NK separation kit (STEMCELL). Cells were grown on irradiated feeder cells in the presence of PHA (Sigma-Aldrich) and IL-2 (PeproTech) and maintained with IL-2 containing medium.

YTS, Jurkat, BW, and 721.221 cells were maintained in RPMI (Sigma-Aldrich) supplemented with 10% fetal calf serum serum (Sigma-Aldrich), 2 mM glutamine (Biological Industries (BI)), 1 mM sodium pyruvate (BI), 1× nonessential amino acids (BI), 100 U/ml penicillin (BI), 0.1 mg/ml streptomycin (BI). RKO, 624mel, and Jeg3 cells were maintained in DMEM (Sigma-Aldrich) with the same supplements.

Lentiviral vectors were produced in 293T cells using TransIT-LT1 transfection reagent (Mirus) for a transient three-plasmid transfection protocol according to manufacturer instructions. The expression vector used was pHAGE-DsRED(-)eGFP(+).

**RNA and cDNA Preparation**

RNA was extracted from cells using the Quick-RNA™ MiniPrep Kit (ZYMO). Samples were quantified by spectrophotometry (DeNovix DS-11). Reverse transcription was performed using
anchored oligo-dT (ThermoFisher) and the M-MLV reverse transcriptase (Invitrogen), according to manufacturer instructions.

**PCR and qPCR**

PCR was performed using Phusion® high-fidelity DNA polymerase (ThermoFisher) according to manufacturer instructions. Primers used are listed in the following table. qPCR was performed using Platinum™ SYBR™ Green (Invitrogen) according to manufacturer instructions. qPCR data was analyzed using QuantStudio 12K software. Gene expression was normalized to GAPDH.

**RNA Fluorescence in-situ Hybridization**

IFNG-AS1 specific LNA™ was ordered from Exiqon using the probe generator. FISH was performed according to manufacturer instructions.

**3’ RACE**

RACE was performed using the ThermoFisher kit, according to manufacturer instructions.

**Oligo Sequences**

Sequences of oligos used in various experiments are shown in the following table. All oligos are shown in the 5’ to 3’ orientation.
Plate-Bound Antibody Assays

Antibodies were bound to 96F plates (DeGroot) overnight at 4° using binding buffer of 71.43mM NaHCO₃ and 28.59mM Na₂CO₃. 200μl of binding buffer containing 0.08μg of antibody were used per well. Plates were washed with 200μl DMEM. 75,000 NK cells per well were added in 200μl medium and incubated at 37° for the indicated times. Cells were then collected and RNA was extracted as described.

Activation Assays

For cell-based assays, target cells were plated in 96F plates in DMEM overnight at 37°. Approximately 40,000-70,000 cells were plated per well, depending on cell line and time until use. The following day, once cells had adhered and achieved 100% confluence, plates were
gently washed with medium, and 75,000 NKs in 200μl fresh medium were plated for the indicated times. For YTS assays, cells were plated in 96U plates (DeGroot). For influenza coating, 250,000 Jeg3 cells were plated in 6 well plates (DeGroot) and allowed to reach 70% confluence. PR8 was added to the cells at an approximate MOI of 5, and the cells were incubated overnight. The following day cells were collected, stained with the H17-L2 antibody to verify coating, and plated as described above. For YTS assays, 75,000 cells per well were plated in 96U plates (DeGroot). The final concentrations of treatments were: 721:221 cells – 150,000 cells per well, LPS – 5μg/ml, IL-2 – 500U/ml, IL-12 – 20ng/ml, IL-15 – 500ng/ml, IL-18 – 100ng/ml, PMA – 1ng/ml, ionomycin – 0.5 μM.

For protein measurements, supernatants were directly transferred to plates pre-bound with Biolegend clone NIB42. Detection antibody was biotin-conjugated Biolegend clone 4S.B3, followed by treatment with streptavidin-HRP (Jackson ImmunoResearch), and quantification with TMB one component substrate (Southern Biotech).

**Flow Cytometry**

FACS was performed using standard procedures with the following antibodies: R&D systems clone 875001, an anti-HA1 mAb (H17-L2, a kind gift from Dr. Jonathan Yewdell, National Institutes of Health), Alexa-Fluor® 647 F(ab’)2 goat anti-mouse (Jackson ImmunoResearch), PE-conjugated Biolegend clone BJ40, PE-conjugated Biolegend clone MOPC-21. Data were analyzed using FCSExpress 4.

**Statistical Analysis**
ANOVA was performed at statpages.info/anova1sm.html using Tukey’s \textit{post hoc} analysis.

Student’s T test was two-tailed with assumption of heteroscedasticity.

\textbf{Data Availability}

The sequence for the isoform of IFNG-AS1 shown in Figure 2B is also available as GenBank:MK296539.
Yamin, R. et al., 2013. The viral KSHV chemokine vMIP-II inhibits the migration of Naive and activated human NK cells by antagonizing two distinct chemokine receptors. *PLoS pathogens*, 9(8), p.e1003568.