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Interleukin-2 mediated NF-κB -dependent mRNA splicing induces interferon gamma protein production

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Abstract

Interleukin-2 mediated NF-κB -dependent mRNA splicing induces interferon gamma protein production

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Interferon-gamma (IFNγ) is a pleiotropic cytokine produced by natural killer (NK) cells during the early response to infection. IFNγ expression is tightly regulated to mount sterilizing immunity while preventing tissue pathology. Post-transcriptional effectors resolve expression of inflammatory cytokines, and several dampen IFNγ expression through *IFNG* mRNA degradation[1]. NK cells' acute induction of IFNγ defies transcription-translation kinetics, suggesting that cells may be poised for rapid IFNγ production. In this study, we identify mRNA splicing as a major regulator of IFNγ protein production. While treatment with the combination of IL-12 and IL-2 causes synergistic production of *IFNG* mRNA and protein, we observe that NK cells treated with IL-12 alone transcribe *IFNG* with its introns intact. When NK cells are treated with both IL-2 and IL-12, *IFNG* transcript is spliced to form mature mRNA with a

concomitant increase in IFNγ protein. We found that IL-2-dependent intron splicing occurs independently of nascent transcription and translation but is NF-κB signaling dependent. We propose that while IL-12 transcriptionally induces *IFNG* mRNA expression, IL-2 signaling stabilizes *IFNG* mRNA in an NF-κB dependent manner by inducing swift splicing of detained introns, ensuring that NK cells are poised for robust IFNγ protein expression. This study uncovers a novel role for splicing in regulating IFNγ protein production through a mechanism potentially applicable to immune control of other inflammatory mediators.

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DEDICATION

This dissertation is dedicated to the previous generations of women scientists who have fought to make it possible for me to complete this degree today.

Chapter 1. Introduction

Parts of this chapter were adapted from the following publication:

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1.1 Interferon gamma production in natural killer cells

At the onset of microbial infection, the immune system must quickly initiate both an immediate innate response to control infection, as well as longer-term adaptive processes to achieve sterilizing immunity and protect the host upon re-infection. Interferons (IFNs) were initially discovered in the 1950s as protein agents that quickly interfere with viral replication during the early stages of infection, allowing for the rapid ramp-up of the host immune response. IFNs are split into three distinct classes: type I interferon, which exists in 14 subtypes, of which IFNα and IFNβ are most prominent, ubiquitously expressed by nucleated cells; type II interferon, interferon gamma (IFNγ) its sole member, restricted in expression to specific immune cell subsets; and type III interferon, comprised of the interferon-lamba (IFNλ) family, expressed primarily by epithelial cells in mucosal barrier surfaces[2–5]. Interferon-γ (IFNγ, encoded by *IFNG*), the central focus of this dissertation, is a unique and crucial inflammatory cytokine that bridges the innate and adaptive immune responses to infection. Humans lacking IFNγ or its receptor IFNγR1/2 are highly susceptible to microbial infections, particularly those caused by mycobacteria like *Mycobacterium tuberculosis*, which remains a global human health threat[6,7]. However, an overabundance of IFNγ is also detrimental to human health, and is a known contributing cause of multiple chronic autoimmune disorders including rheumatoid arthritis and lupus disease[8]. Thus, precise regulation of IFNγ production is essential for maintaining immune

homeostasis both by providing protection from microbial disease and autoimmune tissue pathology.

IFNγ is expressed primarily by natural killer (NK) cells, innate effectors that fight pathogens during early stages of immune response, and by $CD4^+$ Th_1 cells, adaptive cells that quench infection and invoke long-lasting memory against foreign microbes[9]. Several secondary immune cell subsets produce IFNγ, including $CD8^+$ T-cells, NKT cells, type I innate-like lymphocytes (ILC1s), and in small amounts, phagocytes, and B cells; however, each of these populations expresses IFNγ to a significantly lesser extent than NK or $CD4^+$ T-cells[10–15]. The focus of this dissertation is the early response to infection by NK cell-derived IFNγ[16].

Human NK cells produce IFNγ protein when stimulated with a combination of cytokines: notably IL-12 + IL-2 or the combination of IL-12+IL-18[17–20]. Though IFNγ is produced in smaller quantities upon stimulation with any of these cytokines alone, their combination prompts rapid and robust production[17,20–23]. Other cytokines such as IL-15 and IFNα also stimulate IFNγ production, though induction via combination of these treatments is additive, not synergistic like IL-12 + IL-2[17,24,21,25]. While IL-12 and IL-18 are both cytokines produced by dendritic cells and other phagocytes when they encounter pathogens in early infection, IL-2 is produced primarily by $CD4^+$ T-cells upon engagement of the T-cell receptor with cognate antigen displayed by an antigen presenting cell[26]. Given that IL-12 and IL-2 come from distinct sources, an NK cell may receive signals to produce IFNγ in differing temporal order, depending on the activation state of surrounding cells in its immediate environment.

In vivo, the spatiotemporal interaction of NK cells with DCs producing IL-12 versus T-cells producing IL-2 is distinct. NK cells, primarily circulatory in homeostasis, home to lymph nodes during infection and there interact with mature DCs, which have also migrated to lymph node upon sensing pathogens via pathogen associated molecular patterns[27]. DC and NK cell homing to the lymph node occurs rapidly during an initial infection, outpacing the priming of naïve T-cells, which must encounter their specific cognate antigen before producing IL-2[28]. Therefore, NK cells may receive IL-12 stimulation from DCs prior to being stimulated with IL-2 derived from $CD4^+$ Th_1-cells. After initial interactions with DCs in the lymph node, populations of NK cells may also return to peripheral tissues, where they can encounter IL-2 producing resident T-cell populations, or IL-2 producing T-cells that have homed to the site of infection after exposure to antigen in the context of MHC-II in the lymph node[29]. The trafficking and migration of NK cells relative to DCs and T-cells again suggests NK cells will encounter IL-12 prior to IL-2 during primary infection. We propose that the initial transcriptional induction of *IFNG* during IL-12 exposure prepares NK cells for subsequent synergistic IFNγ protein production after encountering with IL-2.

The effects of NK cell-derived IFNγ are pleiotropic, activating macrophages, DCs, and T-cells during microbial infections (Figure 1.1). Upon IFNγ stimulation, macrophages and monocytes produce nitric oxide species and increase their ability to phagocytose pathogens[16]. When DCs are simulated by IFNγ, they mature and increase their IL-12 output, thus amplifying IFNγ-driven inflammation by providing stronger cytokine signals to enhance NK cell activation[16,30]. Mature DCs and IFNγ-activated macrophages also upregulate class II major histocompatibility complex molecules, which are required for presenting bacterial or viral antigens to naïve of effector T-

cells for T-cell priming and activation[9,16,31]. When naïve $CD4^+$ T-cells bind DC-presented antigen in the presence of DC-derived IL-12 and NK-derived IFNγ, they are primed as Th1 effectors. Mature $CD4^+$ Th1s enhance the feed-forward inflammation loop as IFNγ and IL-2 producers themselves[27,32,33].

IFNγ serves additional functions beyond aiding in activation, maturation and priming of immune cell subsets. The IFNγ receptor (IFNγR1/2) is expressed nearly ubiquitously in nucleated cells. In non-hematopoietic cells, IFNγR1/2 signaling induces expression of MHC-I molecules required for antigen presentation to $CD8^+$ T-cells, which are subsequently activated for sterilizing immunity during infection[4,31]. From activating innate phagocytic effectors to ensuring a robust T-cell response, the importance of NK-cell derived IFNγ in the immune response cannot be overstated.

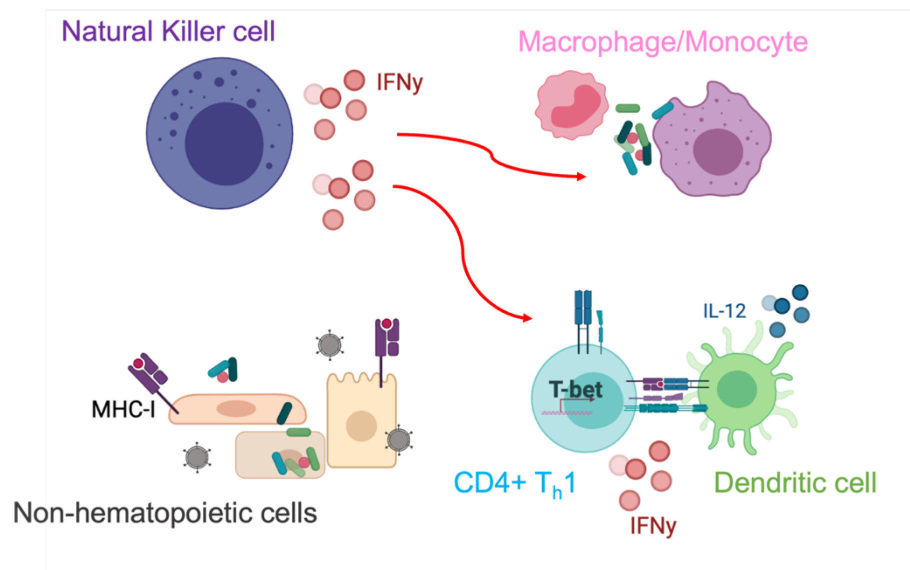

Figure 1.1 Pleiotropic effects of early NK-cell derived IFNγ. NK-derived IFNγ activates both hematopoietic and nonhematopoietic cell subsets, resulting in increased microbial phagocytosis, dendritic cell IL-12 production, $CD4^+$ Th_1 skewing, and MHC-I upregulation for enhanced T-cell activation in infected cell clearance.

1.2 IL-12R and IL-2R signaling in NK cells

This dissertation clarifies the mechanisms by which NK cells rapidly and synergistically express IFNγ upon stimulation with the combination of IL-12 and IL-2. This phenomenon has been robustly described in the literature and recapitulated in our hands (see Chapter 2)[21,23,25]. The IL-12 receptor in NK cells is comprised of two chains, beta subunit 1 and beta subunit 2, and transduces signals through tyrosine kinase 2 (TYK2) and Janus kinase 2 (JAK2)[34]. Upon ligation of IL-12 to the receptor, TYK2 and JAK2 auto-phosphorylate, then phosphorylate STAT4, which dimerizes and translocates into the nucleus where it binds gamma activation sequences (GAS) in the *IFNG* promoter to initiate gene transcription[35]. The IL-2 receptor (IL-2R) is more complex, with three central components: the alpha, beta, and gamma chains. When IL-2R contains the alpha chain, also known as CD25, it becomes the highest affinity version of IL-2R. CD25 is expressed primarily on T-lymphocytes and rarely by NK cells, though its expression has been shown to be inducible by combinations of stimulatory cytokines in human NK cells[36]. The beta and gamma chains in absence of the alpha chain are sufficient to transduce signal and are the subunits canonically expressed on human NK cells, but are lower affinity than the receptor containing the alpha chain.

Signaling downstream of IL-2R involves the activation of JAK1 and JAK3, which phosphorylate STAT1, STAT3, and STAT5[35]. Activation of STAT4 by IL-2 signaling has also been observed in both human primary NK cells and immortalized NK cell lines[37] . In addition to STAT activation, however, several other signal transduction pathways are activated downstream of IL2R ligation, including a MAP kinase cascade beginning with the G-protein coupled receptor

Ras/Raf and ending in the phosphorylation and nuclear translocation of ERK1/2. This MAP kinase signaling cascade has been shown to be critical for induction of *IFNG* in NK cells[38]. The last well-defined signaling pathway downstream of IL-2R is PI3K signaling through Akt, which culminates in mTOR activation and phosphorylation of S6, important for cell survival[39–41]. PI3K signaling branches into several other transduction pathways beyond the canonical mTOR pathway, including activation and phosphorylation of the NF-κB p65 subunit through Akt interaction with CARMA1[42,43]. Our study examines the effects of IL-2 signaling on the regulation of *IFNG* in determining how both the Ras/Raf and PI3K-Akt signaling pathways modulate post-transcriptional processes, impacting IFNγ production.

1.3 Post-transcriptional control of *IFNG*

Given its central role in immune modulation, and the damaging effects of aberrant expression, IFNγ must be tightly regulated to ensure a robust microbial response without causing damage to tissue. Mechanisms of *IFNG* transcriptional induction, including STAT, AP-1, and NF-κB transcription factor binding, as well as epigenetic modifications of the *IFNG* genetic locus, have been robustly characterized[12,18,44,45]. However, less-well understood but equally as important, post-transcriptional regulation of RNA is another key process that can control inflammation. The function and expression of RNA can be tuned at every stage of its life cycle, beginning with co-transcriptional control of capping, splicing, and polyadenylation; continuing with nuclear export and translation; and terminating with RNA degradation. RNA-binding proteins (RBPs) are inextricably involved in every facet of RNA metabolism. RNA molecules are decorated with RBPs from their inception and exist exclusively as ribonucleoproteins (RNPs). Several distinct RBPs bound to a single RNA molecule can act in concert with or against one another. Such

interactions between RBPs and RNA lead to transcript-specific molecular outcomes that ultimately shape the cellular transcriptome.

Non-coding RNAs also play key roles in RNA regulatory processes through RNA-protein as well as RNA-RNA interactions. Although only 2% of the human genome encodes proteins, more than 85% is transcribed into non-coding RNAs, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs)[46,47]. miRNAs are single-stranded RNAs that are 21-23 nucleotides in length. After extensive processing of primary miRNA transcripts, miRNAs are loaded into the RNA-induced silencing complex (RISC), where they bind to the 3′ UTRs of target mRNAs through their “seed sequence”, which spans nucleotides 2-8, causing degradation or translational suppression of target mRNAs[48–50]. At least 30% of human mRNAs have the potential for regulation via miRNA binding[48–50]. LncRNAs, thousands of which are encoded in the human genome, are defined as non-coding RNAs greater than 200 nucleotides in length and are often differentially expressed during viral infection and immune stimulation[51,52]. Until recently, lncRNAs were considered transcriptional noise due to their often-low levels of expression. However, lncRNAs are increasingly implicated as diverse regulators of transcription, protein modulation, and RNA activity, most often serving as molecular decoys, guides or scaffolds[53–57].

IFNG is post-transcriptionally regulated both directly and indirectly by several microRNAs and long noncoding RNAs including miRNA-29, miRNA-21, and *NeST,* which dampen IFNγ expression, and *Tmevpg1,* which promotes induction[58–63] (Fig 1.2). Both miRNA-29 and miRNA-21 indirectly dampen induction of *IFNG* and expression of IFNγ .While miRNA-29 is thought to target degradation of the transcription factors required for upregulation of *IFNG,* miRNA-21 may have effects on IL-12 receptor signaling in T-cells;, the mechanism of action in

NK cells is not yet elucidated[60,64]. The lncRNA *NeST* is thought to epigenetically silence the *IFNG* locus, contrasting with *Tmpevg1*, which amplifies *IFNG* transcription through STAT4 dependent mechanisms[61,62]. In other modes of post-transcriptional regulation, the 3´UTR of *IFNG* contains AU-rich elements that foster *IFNG* transcript degradation to avoid over-amplification of the immune response[59]. The RNA binding proteins (RBPs) TTP and ZFP36L2 destabilize *IFNG* transcript through interaction with these AREs[65,66]. Deletion of *IFNG* AREs causes chronic inflammatory disease in mice due to resulting overabundance of IFNγ protein[59]. Though several forms of *IFNG* post-transcriptional regulation have been studied, it is unknown whether these mechanisms account for the full repertoire of IFNγ regulation. Strikingly, apart from *Tmevpg1*, all known mechanisms are also negative regulators of the IFNγ response; few forms of positive induction via post-transcriptional regulation have been identified, despite the rapid speed at which IFNγ is produced during the early antimicrobial response[61]. Identifying other forms of *IFNG* post-transcriptional regulation will clarify our understanding of IFNγ-driven inflammation, with broad implications for disease treatment.

1.4 Alternative splicing as a mechanism of post-transcriptional control

Nearly 85% of all transcripts undergo alternative splicing[67]. Through alternative exon usage, intron retention, or splice site selection, alternative splicing serves to diversify the proteome and generate proteins with distinct properties. Further, alternative splicing can also contribute to post-transcriptional mRNA regulation through nonsense-mediated decay, nuclear retention of transcripts, or modulation of translation efficiency[68]. While post-transcriptional regulation of *IFNG* through microRNAs, lncRNAs and RBPs that bind the AU-rich elements of the 3´UTR

have been thoroughly investigated, alternative splicing of *IFNG* has received less attention, and is not widely understood to be a mechanism regulating IFNγ production.

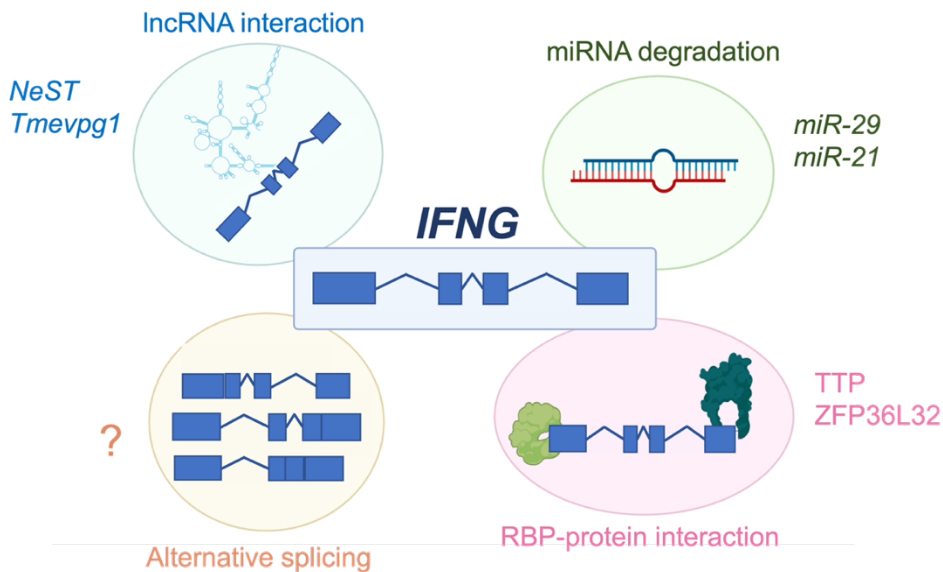

Figure 1.2 Post-transcriptional regulation of *IFNG* mRNA. LncRNAs and miRNAs indirectly regulate *IFNG* mRNA, while RNA-binding proteins interact directly with the 3´UTR to induce degradation of the transcript. Alternative splicing is a key post-transcriptional modulatory mechanism yet unexplored for regulation of *IFNG*.

In recent years, a particular form of alternative splicing known as intron detention (ID) has gained recognition as a modulator of essential cell processes, including host pathogen defense[69,70]. ID is a form of post-transcriptional regulation in which splicing of polyadenylated transcripts occurs on a delayed timescale, or not at all, causing introns to remain within mature transcripts. ID regulates protein expression through targeting mRNA transcripts for nonsense mediated decay or by causing transcript sequestration in the nucleus, delaying translation kinetics[69–71]. The latter allows for maintenance of a pool of pre-mature mRNA that may be rapidly translated in response to a triggering signal inducing transcript processing. For example, in *CXCL2*, a chemokine that attracts neutrophils to sites of inflammation early during infection,

detention of the third intron keeps transcript localized to the nucleus of unstimulated macrophages. Only upon additional inflammatory signaling via TLR4 ligation is *CXCL2* rapidly spliced and exported, allowing for its translation and initiating a robust innate response[72]. ID also affects inflammatory mediator expression through transcript interactions with RBPs. For *IRF7*, a transcription factor important in type I IFN induction, detention of the fourth intron has been shown to downregulate expression[73]. A splicing factor BUD13 is responsible for strengthening a weak splice site in the detained fourth intron, ensuring *IRF7* processing to allow type I IFN production, and demonstrating how the interaction between RBPs and unspliced introns may control innate immune activation. These examples illustrate a critical role for ID in regulating cytokine expression.

It is unclear whether alternative splicing, and specifically ID, modulates IFNγ expression. However, the mechanism by which NK cells produce and store *IFNG* transcript suggests a role for ID in the regulation of IFNγ expression[23,74]. In mice, *Ifng* transcript has been shown to be created and stored in NK cells from the time of cell development and differentiation[74]. The mechanism by which this transcript is stabilized is unknown but could be attributed to ID, or to mRNA interaction with RBPs that bind and sequester *Ifng* transcript until sufficient inflammatory stimulus invokes translation. In humans, the speed with which NK cells produce IFNγ upon stimulation with the combination of IL-12 and IL-2 suggests that factors beyond transcriptional control may be responsible for expression, as the quantities expressed defy the traditional timescale of transcription-translation. This thesis outlines a novel form of *IFNG* post-transcriptional control accounting for NK cell ability to acutely and robustly produce IFNγ to induce an immune response for rapid pathogen clearance and lasting immune memory.

Chapter 2. IL-2 mediates post-transcriptional *IFNG* mRNA stability and splicing

Parts of this chapter were adapted from the following publication:

Van Gelder, R.D., Gokhale, N.S., Genoyer E., Omelia D.S., Young, H.A., Savan R. (2024). Interleukin-2 mediated NF-κB -dependent mRNA splicing induces acute interferon gamma protein production. *Under Review* at *EMBO Reports*.

2.1 Introduction

Interferon-γ (IFNγ, encoded by *IFNG*) is a potent inflammatory cytokine central to the antimicrobial response through its activation of both innate and adaptive immune cell subsets[16,31,75,76]. During early stages of the immune response, natural killer (NK) cells are the first producers of IFNγ, inducing protein production in a matter of minutes[9]. Several cytokines can induce IFNγ in NK cells, including IL-12, IL-18, IL-15 and IL-2 (Barbulescu et al..; Chaix et al., 2008; Chan et al., 1992; Nguyen et al., 2002). Combinations of these cytokines, notably IL-12 with IL-2, promote rapid production of sizeable quantities of IFNγ protein [17,21–23,25,77]. While IL-12 is produced primarily by dendritic cells (DCs) upon sensing microbes in early infection, IL-2 is chiefly $CD4^+$ T-cell-derived, and expressed upon engagement of the T-cell receptor (Perera et al., 2012; Spolski et al., 2018). Infection prompts the migration of NK cells to draining lymph nodes and secondary lymphoid structures where DCs interact with NK cells to prime them for effector functions in vivo [25,29]. Early IFNγ produced by NK cells augments MHC Class II expression on APCs, allowing for subsequent T-cell receptor engagement and downstream IL-2 production [9,16,31]. Thus, NK cells *in vivo* may receive signals to produce IFNγ in a time-sequential manner, lending the possibility for multi-signal systems to govern the kinetics of *IFNG* expression.

IFNγ expression is stringently regulated to ensure its robust but transient expression, as chronic low-level IFNγ causes autoimmune disease [59,79]. Understanding the basis of inflammatory cytokine transcription has clarified many mechanisms of *IFNG* control, including characterization of epigenetic regulation, as well as transcriptional enhancers and their cognate transcription factors [12,80,81]. However, the acute induction of IFNγ by NK cells defies transcription-translation kinetics, indicating that cells may be poised to rapidly generate IFNγ by means beyond transcriptional control. In recent years, post-transcriptional regulation of mRNA has emerged as a key process mediating the fine-tuning of inflammation [82–85]. While most known post-transcriptional regulators of *IFNG* account for mRNA degradation and thus downregulation of IFNγ production, few mechanisms explain the swift and robust production of IFNγ in NK cells responsible for initiating antimicrobial responses. The post-transcriptional processes that positively regulate *IFNG* mRNA for rapid production of protein remain unknown and thus constitute the central focus of this study.

We have uncovered the molecular mechanisms that dictate synergistic induction of IFNγ in NK cells treated with the combination of IL-12 and IL-2. We show that *IFNG* mRNA splicing is mediated by IL-2. Signaling downstream of IL-2 receptor increases *IFNG* mRNA stability and prompts its splicing in the absence of nascent mRNA transcription, allowing for rapid translation of protein. This study lends key insights into previously unknown post-transcriptional control of *IFNG*, with implications for understanding the broader biology of inflammatory cytokines.

2.2 Results

2.2.1 Combination of IL-12 and IL-2 synergistically induces IFNγ

Previous studies have shown that NK cells rapidly and robustly produce IFNγ upon stimulation with the combination of the cytokines IL-12 and IL-2 [23,86]. In line with these observations, we showed that treatment of CD56+ NK cells from healthy human donors with either IL-12 or IL-2 alone induced low or undetectable levels of *IFNG* mRNA expression and IFNγ protein production; however, the combination of the two cytokines boosted production to levels far beyond additive effect of either cytokine alone (Fig 2.1A, 1B). We used a human immortal NK cell line, NK92, which phenocopied primary human NK cells in synergistic production of *IFNG* mRNA and IFNγ protein. In NK92 cells, at only 6 h post-stimulation, IL-2 and IL-12 in conjunction induced *IFNG* mRNA expression to levels almost three times greater in transcription and five times greater in protein than the additive quantity of either cytokine alone (Fig 2.1C, 1D, 2.3B).

For ease of IFNγ quantification, we developed a bioassay to measure IFNγ protein expression, which correlated well with our measurements of IFNγ protein through ELISA (Fig 2.1A, 1C, 2.3B). Briefly, a *Gaussia* luciferase reporter driven by GAS (Gamma activable sequence) elements from the IRF1 promoter was stably transduced into Huh7 cells. IFNγ induced STAT1 homodimers bind and activate the IRF-1 promoter reporter to induce luciferase activity (Fig 2.3A). Transfer of supernatants directly from cytokine treated NK cells onto IFNγ reporter cells accurately quantified IFNγ activity through *Gaussia* luciferase production (Fig 2.3A, 2.3B).

We first assessed whether synergistic IFNγ induction in NK cells was transcriptionally mediated. We postulated that the combination of IL-12 and IL-2 might open the *IFNG* transcriptional locus for increased transcription factor binding, quickly inducing transcription of large quantities of *IFNG* mRNA, and thus accounting for robust protein output [37,77]. To this end, we investigated nascent transcription levels of *IFNG* during IL-12 stimulation alone compared to the combination of IL-12 and IL-2. To measure nascent transcription, we used 4-thiouridine (4sU), which incorporates as a nucleoside analog into actively transcribed RNA [5,87]. After RNA isolation, 4sU-labelled RNA is biotinylated and precipitated with streptavidin beads to determine whether rates of nascent gene transcription vary upon differential stimulation conditions. Strikingly, we observed that the addition of IL-2 to IL-12 treated cells did not significantly induce nascent transcription of *IFNG* mRNA as compared to IL-12 treatment alone (Fig 2.1E, 2.3C), suggesting that factors beyond transcriptional regulation dictate acute synergistic production of IFNγ in NK cells.

2.2.2 IL-2 induces post-transcriptional stability of *IFNG* mRNA

Given that transcriptional variability was not sufficient to account for IL-12 + IL-2 synergism in inducing IFNγ, we wanted to determine whether active translation was necessary for the observed induction of *IFNG;* that is, whether rapid de-novo protein synthesis of another, distinct protein could account for synergistic *IFNG* induction. Using the translation inhibitor cycloheximide (CHX), whose function was validated with puromycin incorporation (Fig 2.3E), we analyzed differences in *IFNG* mRNA transcription in the absence of active translation during IL-12 versus IL-12 + IL-2 stimulation (Fig 2.3F). We found no significant difference in *IFNG* transcription between CHX- versus mock-treated cells, suggesting that nascent translation was not required for the synergistic effect of IL-12 + IL-2 stimulation. To probe whether IL-2 might

globally increase protein translation, resulting in higher IFNγ output, we assayed puromycin incorporation into total actively translated proteins upon IL-12 versus IL-12 + IL-2 treatment. We observed no notable differences in puromycin incorporation between stimulation conditions (Fig 2.3G), indicating that IL-2 did not globally enhance protein translation beyond levels induced by IL-12.

Due to the similarities in regulation of transcription and translation of *IFNG* between IL-12 versus IL-12 + IL-2 stimulated cells, we hypothesized that IL-2 may instead post-transcriptionally affect *IFNG* mRNA stability and synergistic induction. We observed that in the presence of IL-2, nearly 50% of *IFNG* mRNA induced upon 3 h IL-12 pre-stimulation was preserved 4 h after Actinomycin D (ActD) treatment, which blocks nascent transcription. Conversely, almost full degradation of the *IFNG* mRNA occurred during ActD treatment in absence of IL-2 (Fig 2.1F). These results are comparable to the previously reported increase in *IFNG* mRNA stability during treatment with IL-12 + IL-2 [23]. This finding established that IL-2 prompts post-transcriptional modulation of *IFNG* mRNA, which may be responsible for its rapid and robust induction.

2.2.3 IL-2 promotes *IFNG* mRNA splicing

We hypothesized that IL-2 dependent post-transcriptional stability of *IFNG* may be directly linked with splicing of the *IFNG* transcript. Splicing of mRNA, which often occurs co-transcriptionally, prevents transcript degradation and potentiates mRNA export and translation for subsequent protein expression [88,89]. Given the faster degradation of *IFNG* mRNA during IL-12 stimulation alone as compared with the combination of IL-12 and IL-2, we conjectured that IL-2 might enhance *IFNG* splicing, preventing transcriptional turnover of *IFNG* mRNA and thus

promoting increased translation. To test this, we first investigated intronic detention in *IFNG* mRNA via PCR primers that spanned the regions between each exon (Fig 2.4A, Fig 2.2A) in both the nuclear and cytosolic fractions of NK92 cells treated with IL-12 alone or with IL-2 + IL-12. We then visualized resulting amplicons by agarose electrophoresis (Fig 2.4B) or bioanalyzer (Fig 2.2B). Remarkably, data from the nuclear fraction of treated cells revealed that the addition of IL-2 to IL-12-treated cells significantly reduced intron-retaining *IFNG* mRNA transcripts, suggesting increased splicing occurred during IL-2 stimulation. We quantitatively validated this result by RT-qPCR using probes that spanned each of the three exon-intron regions and found the splicing phenotype particularly stark at the 6-hour stimulation timepoint (Fig 2.2C, D)**.** To ensure that the effect of IL-2 on *IFNG* mRNA splicing occurred post-transcriptionally, we tested the ability of IL-2 to enhance splicing out of *IFNG* introns in the absence of nascent transcription using the transcriptional inhibitor ActD. Even in the absence of active transcription, IL-2 induced splicing of *IFNG* in all three introns, confirming the phenomenon as a post-transcriptional regulatory mechanism (Fig 2.2E). We additionally examined the effect of CHX-mediated translational halt on IL-2 induced *IFNG* mRNA splicing and found that RNA processing occurred independently of translation (Fig 2.4D). Lastly, we analyzed the effects of IL-12 and IL-2 stimulation on splicing of the housekeeping gene *ACTB* to determine whether the enhanced splicing effect occurred globally (Fig 2.4E). Though there was some increased splicing in the second intron of the gene, overall IL-2 did not induce intron excision in *ACTB*, suggesting that the post-transcriptional effect of IL-2 is not ubiquitous. We conclude that the central role of IL-2 in inducing synergistic IFNg is post-transcriptional splicing of *IFNG*, conferring increased mRNA stability and heightened protein production on an abbreviated timescale.

2.3 Figures

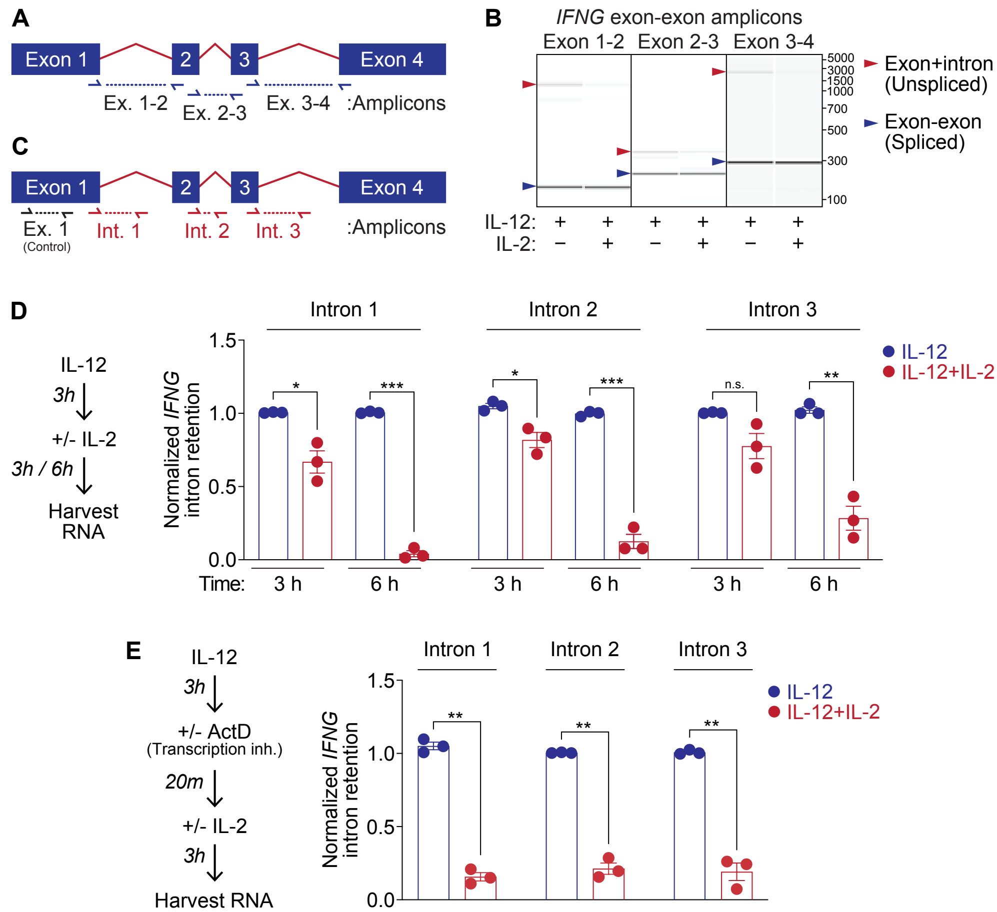

Figure 2.1 IL-2 induces post-transcriptional stability of *IFNG* mRNA Healthy human CD56+ NK cells were treated with IL-2 (100U/mL), IL-12 (10ng/mL) or both for 6h. Supernatants and cell lysates were collected for (**a**) IFNγ protein quantification via ELISA or (**b**) qPCR analysis of *IFNG* induction respectively. *IFNG* expression normalized to *HPRT1*. NK92 cells were stimulated 3 or 6h as in (**a**) and supernatants and cell lysates collected for (**c**) ELISA and (**d**) qPCR respectively, *IFNG* induction normalized to *HPRT1*. **e**) qPCR analysis of total versus 4SU labelled *IFNG* for IL-12 versus IL-12+IL-12 stimulated cells, normalized to *HPRT1* expression. **f**) Time course of *IFNG* mRNA stability in absence of nascent transcription during IL-2 stimulation, normalized to *IFNG* levels at 3h IL-12 treatment before addition of ActD. Data in **a-e** are mean ± SEM of 3 biological replicates, **a-d** analyzed with two-way ANOVA with Turkey's test for multiple comparisons test (**e**) with ratio paired T test with Holm-Šídák method for multiple corrections; **f** is mean ± SEM of 4 biological replicates analyzed with two-way ANOVA with Turkey's test for multiple comparisons * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, n.s. is not significant

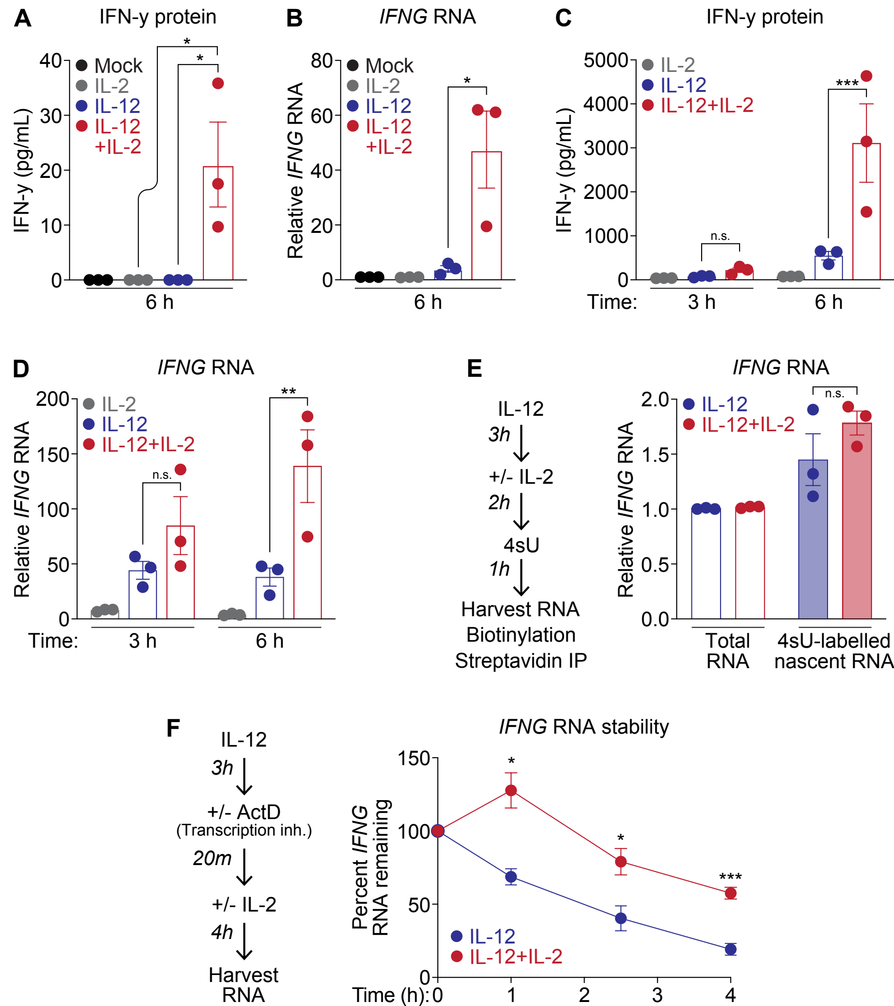

Figure 2.2 IL-2 promotes *IFNG* mRNA splicing (**a**) Primer design for PCR amplification of intra-exonic regions of *IFNG* mRNA; primers used in (**b**), (**FIG 2.4b**). **b**) Bioanalyzer analysis of PCR amplification of intra-exonal regions of *IFNG* isolated from fractionated nuclei from NK92 cells stimulated with IL-12 or IL-12 + IL-2 for 6h (**c**) Schematic of SYBR probe design for qPCR amplification of intronic regions of *IFNG* in (**d**) and (**e**) and all further experiments quantifying intron detention **d**) SYBR qPCR analysis of *IFNG* intron detention in nuclear lysates during stimulation with IL-12 or IL-12 + IL-2 for 3 or 6 h. Intron expression normalized to *IFNG* 5'UTR, representing total mature plus unspliced *IFNG* mRNA as quantified by amplification of the region spanning the 5'UTR into the coding region of Exon 1, depicted in (**c**) as control. **e**) SYBR qPCR *IFNG* intron detention analysis to determine the effect of IL-2 on splicing in the absence of nascent transcription. NK92 cells were stimulated with IL-12 for 3h to induce a pool of *IFNG* transcript, then treated with ActD to halt nascent transcription before 3h treatment with IL-2. Data from nuclear lysates shown. Data in (**b**) representative of 3 biological replicates. Data in **d-e** mean ± SEM of 3 biological replicates, paired T test with Holm-Šídák method for multiple corrections * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. is not significant.

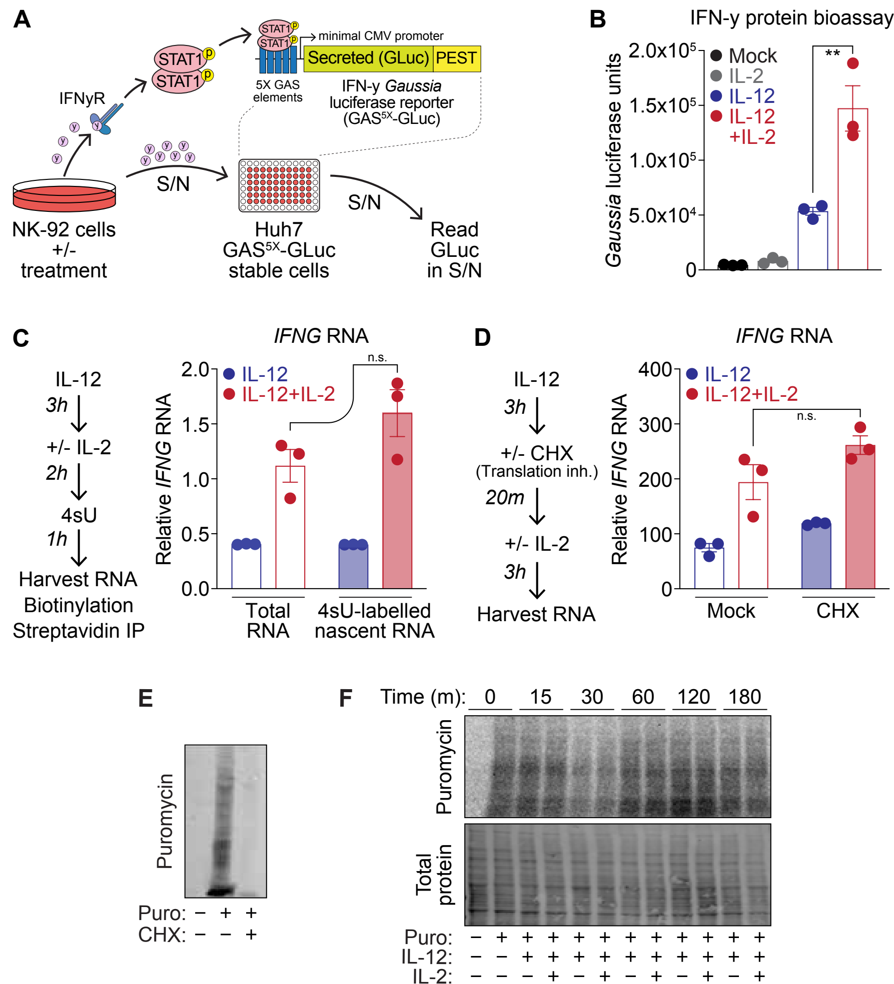

Figure 2.3 a) Schematic of IFNγ bioassay workflow for quantification of IFNγ in supernatants of treated NK92 cells via Huh7 *Gaussia* luciferase IRF1 GAS(5x) reporter cells **b**) Bioassay quantification of IFNγ protein production using supernatants from NK cells stimulated 24 h with IL-2 (100u/mL), IL-12 (10ng/mL) or IL-12+IL-2 **c**) qPCR analysis of 4SU labelled *IFNG* transcripts compared with total (4SU labelled + unlabeled) *IFNG* induction during IL-12 versus IL-12+ IL-2 treatment; normalized to *HPRT1*. **d**) qPCR analysis of *IFNG* mRNA induction during IL-2 stimulation in presence and absence of nascent translation, using cycloheximide (CHX) treatment (100µg/mL) to halt protein synthesis. Normalized to *HPRT1* expression **e**) Immunoblot depicting puromycin incorporation for cycloheximide protein synthesis halt control **f**) Immunoblot depicting puromycin incorporation in NK92 cells in NK92 cells stimulated over a time course of 0-3 h with IL-12 or IL-12 + IL-2. Total protein stain used for relative comparison of puromycin incorporation. Data in **b-d** are mean ± SEM of 3 biological replicates, **(b)** one way ANOVA with Turkey's comparison test for multiple comparisons, **c-d** ratio paired T test with Holm-Šídák method for multiple corrections (**e**) is representative of 3 biological replicates, (**f**) is representative of 2 biological replicates. $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. is not significant

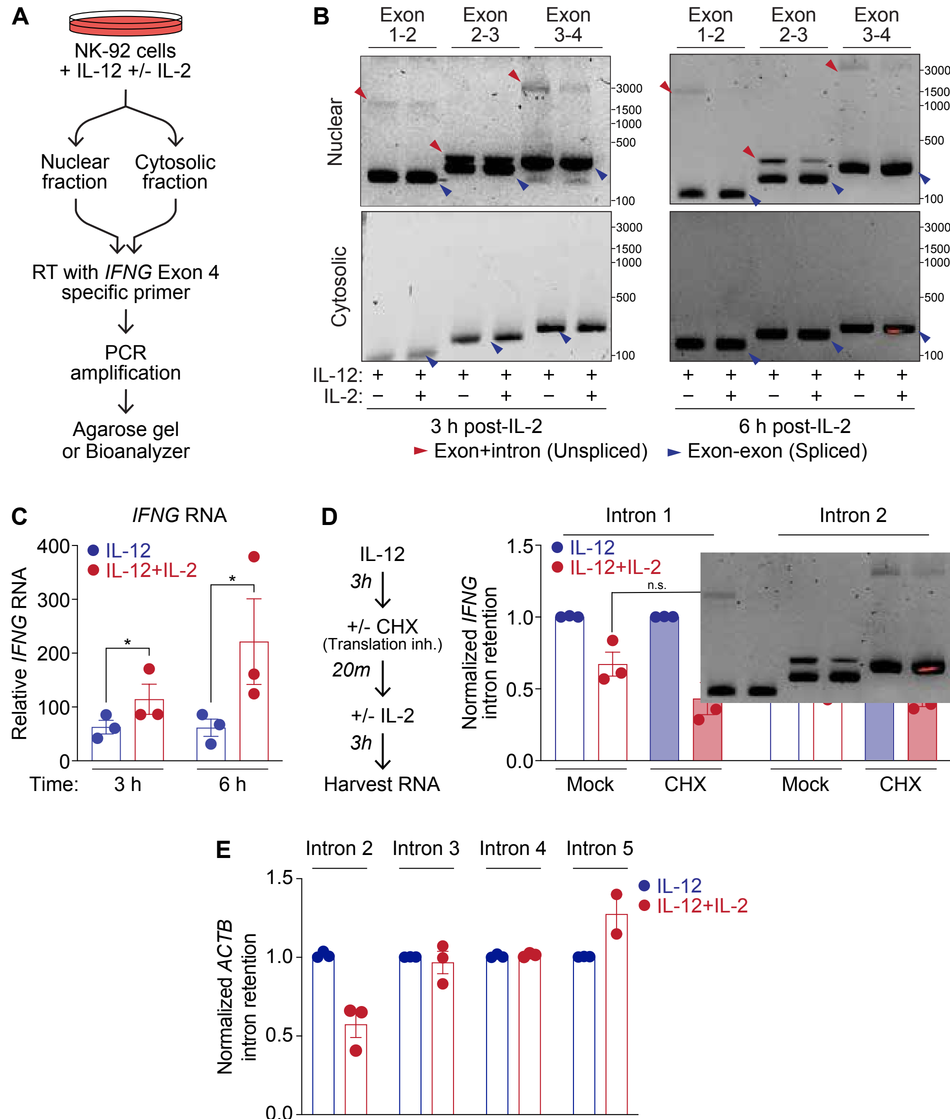

Fig 2.4 a) Schematic of NK92 stimulation and processing for determining *IFNG* intron detention via PCR as seen in **Fig 2.2b, Fig 2.4b. b**) Image of gels depicting PCR products from amplification of intra-exonal regions of *IFNG* from nuclear and cytosol fractionated NK92 cells stimulated with IL-12 or IL-12 + IL-2 for 3 or 6 h. Blue arrows depict fully spliced products while red arrows depict amplified intronic regions. **c**) total spliced *IFNG* induction normalized to *HPRT1* expression during stimulations outlined in Fig **2.2d. d**) SYBR qPCR analysis of *IFNG* intron detention upon IL-2 treatment with and without translation inhibition via CHX (100µg/mL) treatment. Normalized to total mature plus unspliced *IFNG* expression via 5'UTR-Exon 1 amplification as seen in Fig 2.2**c** schematic. **e**) Analysis of intron detention in *ACTB* during IL-12 or IL-12 + IL-2 stimulation for 6h, normalized to total expression of *ACTB* via amplification of Exon 6/3´UTR. Data in (**b**) representative of 3 biological replicates. Data in **c-e** depicts mean ± SEM of 3 biological replicates, where (**c**) is analyzed by ratio paired T test and (**d**) paired T test with Holm-Šídák method for multiple corrections * $p \leq 0.05$, n.s. is not significant.

2.4 Discussion

In this study, we uncover IL-2 mediated intron excision as a novel post-transcriptional mechanism inducing acute IFNγ production. Though it has been previously reported that the combination of the cytokines IL-12 and IL-2 stimulates rapid IFNγ production in NK cells, the mechanism by which these cytokines synergize to promote robust expression has not been elucidated [23,37,77,86]. We found that signaling downstream of IL-2 induces *IFNG* splicing, allowing for intron-containing *IFNG* transcripts generated during IL-12 stimulation to be rapidly spliced for rapid protein production (Figure 5.1).

Our study is unique in proposing one of few mechanisms of positive post-transcriptional regulatory control of *IFNG* mRNA. *IFNG* is post-transcriptionally regulated both directly and indirectly by several microRNAs and long noncoding RNAs, all of which besides a singular lncRNA dampen expression[58–62]. Additionally, the RNA binding proteins (RBPs) TTP and ZFP36L2 destabilize *IFNG* transcript through binding AU-rich elements in the 3´ UTR [65,66]. We have uncovered a novel pathway for induction rather than quenching of inflammatory mediator expression, which functions through alternative splicing of retained introns within the *IFNG* mRNA transcript.

Intron detention has been observed in the context of several drivers of innate inflammation, including *IRF7* and *CXCL2*, in which splicing upon a second signal promotes processing of mRNA for its eventual translation[72,73,90]. In conjunction with our findings, we propose that inflammatory modulation through intron splicing regulation could be a wide-spread post-transcriptional mechanism during an immune response. We postulate that other rapidly and

robustly produced cytokines such as TNFα could be governed by similar mechanisms, in which at homeostasis or in the presence of an initial signal, transcription of intron-containing mRNAs occurs, but a second signal is required to induce stability and splicing for robust protein production. There is evidence to suggest that *TNFa* in mouse bone-marrow derived macrophages is transcribed as a constitutive "pre-cursor" transcript in the unspliced form, wherein upon MyD88/TRIF dependent LPS stimulation, splicing occurs [91]. Additionally, in mouse T-cells, preformed TNFα appears to be stabilized upon antigen stimulation even in the absence of nascent transcription, which would allow for rapid protein production [92]. Such molecular systems drastically reduce the time required to induce expression of inflammatory mediators through bypassing the need for transcriptional induction: a ready-made pool of mRNAs is available for rapid translation when specific signals stimulate processing.

Table 2.1 Primers and gblocks used in this study

Primer/Construct	Sequence
IFNG Exon 1 Forward	5'-CAAGTTATATCTTGGCTTTTCAGCTCTGC-3'
IFNG Exon 2 Reverse	5'-CTCTTTCAATTCTTCAAAATGCCTAAG-3'
IFNG Exon 2 Forward	5'-AATGCAGGTCATTCAGATGTAGCG-3'
IFNG Exon 3 Reverse	5'-CGAATAATTAGTCAGCTTTTCGAAGTC -3'
IFNG Exon 3 Forward	5'-GAGAGTGACAGAAAAATAATGCAGAGCC-3'
IFNG Exon 4 Reverse	5'-CTGGGATGCTCTTCGACCTCG-3'
IFNG 5'UTR Forward SYBR	5'-GAAAGATCAGTTAAGTCCTTT -3'
IFNG Exon 1 Reverse SYBR	5'-GCTTCTTTTACATATGGGTCCTGG C-3'
IFNG Intron 2 Reverse	5'-GAAGGAAAGAGCACAAACAGAGGATG-3'
IFNG Intron 1 Reverse SYBR	5'-GCTACAGCAAGTCGATATTCAGTCAT-3'
IFNG Exon 3 Forward SYBR	5'-GTGGAGACCATCAAGGAAGACATG-3'
IFNG Exon 3 Reverse SYBR	5'CATAGCTTTAGCAACTGTTAAATAGCT-3'
qRT *ACTB* Reverse	5'-TCACCTTCACCGTTCCAGTTTT-3'
qRT *ACTB* Forward	5'-TGACAAAACCTAACTTGCGCAG-3'
ACTB Exon 2 Forward	5'-CTCACCATGGATGATGATATCGCCG-3'
ACTB Intron 2 Reverse	5'-CTGTGCAGAGAAAGCGCCCTTG-3'
ACTB Exon 3 Forward	5'-CTTCTACAATGAGCTGCGTGTGGC-3'
ACTB Intron 3 Reverse	5'-CAGAAGAGAGAACCAGTGAGAAAGGGC-3'
ACTB Exon 4 Forward	5'-TCCAGCTCCCTGGAGAAGA-3'
ACTB Intron 4 Reverse	5'-CAGGACTTAGCTTCCACAGCACAG-3'
ACTB Exon 5 Forward	5'-GCAAAGACCTGTACGCCAACACAG-3'
ACTB Intron 5 Reverse	5'-ACAGCTCCCCACACACCACA-3'
ACTB Exon 6 Forward	5'-ATCATTGCTCCTCCTGAGCGCA-3'
ACTB 3'UTR Reverse	5'-GGTGTAACGCAACTAAGTCATCCG -3'
IFNG full 3'UTR Reverse	5'-GGATTAAGTGAGACAGTCACAGGATATAGG-3'
IRF1 Gene Block Forward	5'-GAGTGTAGCCAGATCTCCCGGGATCTCGATATTTCCCGAAATTG-3'
IRF1 Gene Block Reverse	5'-CTCGAATTGGGCCCTACCCGGGATTTCGGGAAATGTAGTCTAC-3'
IRF1 GAS 5x Gene Block	CCGGGATCTCGATATTTCCCGAAATTGATCATCGCATTTCCCGAAATGCG

	AATCTGAATTTCCCGAAATCGCTTCGTAAATTTCCCGAAAT CGTAGACTACATTTCCCGAAATCCCGG
Colony PCR *IRF1* Forward	5'-CAGATCTCCCGGGATCTCGATATTTCCCGAAATTGATC=3'
Colony PCR *IRF1* Reverse	5'-AATTGGGCCCTACCCGGGATTTCGGG-3'

Chapter 3: NF-κB signaling is required for IL-2-mediated *IFNG* mRNA splicing

Parts of this chapter were adapted from the following publication:

Van Gelder, R.D., Gokhale, N.S., Genoyer E., Omelia D.S., Young, H.A., Savan R. (2024). Interleukin-2 mediated NF-κB -dependent mRNA splicing induces acute interferon gamma protein production. *In Revision at* EMBO Reports.

3.1 Introduction

While the initial portion of this dissertation focuses on characterization of the *IFNG* splicing phenotype driven by IL-2 stimulation, this chapter serves to elucidate the mechanisms underlying processing of *IFNG* mRNA. We strive both to clarify the signaling mechanisms downstream of IL-2R ligation required for splicing of the *IFNG* transcript, and separately, to identify the RNA binding proteins or splice factors mitigating mRNA processing.

In understanding the broader signaling mechanisms dictating post-transcriptional regulation of *IFNG,* we hope to widen the scope of intron detention and delayed splicing as potential modulators of immunity beyond *IFNG*; similar signaling pathways exist downstream of other cytokine receptors, so perhaps serve post-transcriptional functions for induction of other inflammatory mediators. As signaling downstream of IL-2R binding is complex and multifaceted (Chapter 1.2), we examine the effect of blocking multiple major transduction pathways, including Ras/Raf signaling, which culminates in the phosphorylation of MEK/ERK, as well as PI3K signaling, which activates Akt, a kinase that phosphorylates a plethora of targets including NF-κB[38,42,93]. We show that *IFNG* mRNA splicing induced by IL-2 is dependent upon NF-κB signaling, describing a non-canonical role for NF-κB downstream of IL-2 receptor signaling during inflammation, beyond its widely described function as a transcription factor. Additionally,

we validate this finding with another potent inducer of *IFNG* transcription, phorbol myristate acetate (PMA), which signals primarily through NF-κB, thus confirming the importance of this NF-κB signaling mechanisms in regulation of *IFNG* processing for rapid induction[1,94].

RBPs are integral and essential in splicing of pre-mature RNAs, including in cases of alternative splicing like intron detention[95]. Many structurally distinct RBPs have similar functions in mediating post-transcriptional regulation through blocking the ability of the spliceosome to bind for processing[96,97]. We postulated that specific RBPs are differentially activated downstream of NK cell IL-2R ligation, binding to or releasing from *IFNG* transcript to promote splicing of detained introns upon IL-2 stimulation. Through both narrow and wide-reaching hypotheses involving known post-translational modifications of RBPs downstream of IL-2 signaling, in addition to RBPs associated with NF-κB or NF-κB-related splicing, we probed nearly 20 putative RNA binding proteins for their effect on *IFNG* induction. None of the tested RBPs had significant effect on the upregulation of *IFNG* during IL-12 + IL-2 stimulation, leaving this investigation open-ended.

3.2 Results

3.2.1 IL-2-mediated splicing of *IFNG* mRNA is NF-κB dependent

Having characterized IL-2's effect on *IFNG* stabilization and splicing, we aimed to elucidate the mechanism downstream of IL-2 receptor (IL-2R) signaling responsible for IL-2's post-transcriptional effects. We hypothesized that post-transcriptional effects might be mediated by ERK1/2 phosphorylation downstream of Ras/Raf activation upon ligation of IL-2R (Fig 3.3A)[38,98,99]. Using the MEK1/2 phosphorylation inhibitor PD98059 we blocked ERK

phosphorylation and then evaluated *IFNG* transcription, protein production and splicing. PD98059 treatment did significantly curb induction of *IFNG* mRNA and protein, in keeping with past observations (Fig 3.3B-E) [38]. However, blocking the ERK signaling pathway did not affect splicing of *IFNG* (Fig 3.3F). While Ras/Raf signaling is necessary for transcriptional induction of *IFNG,* we conclude that this pathway does not control post-transcriptional processes responsible for robust induction of *IFNG* during the combination of IL-12 + IL-2 stimulation.

We therefore investigated whether alternative signaling downstream of the IL-2 receptor might instead control post-transcriptional regulation. PI3K signaling, thought to occur upon JAK3 phosphorylation, branches in multiple directions downstream of Akt beyond its canonical target, mTOR, which primarily controls cell proliferation and viability (Fig 3.4A) [98,100]. One of Akt's signaling targets is NF-κB, and intriguingly, we noted increased phosphorylation of p65 during the treatment of NK cells with the combination of IL-2 and IL-12 compared with IL-12 alone via immunoblot (Fig 3.4B). This contrasted with ERK1/2 phosphorylation, which was not notably altered during IL-12 versus IL-12 + IL-2 treatment (Fig 3.3B). We therefore investigated the importance of NF-κB p65 phosphorylation on IL-2 mediated *IFNG* induction. After stimulating *IFNG* transcription with IL-12, we blocked NF-κB signaling with the IκB dephosphorylation inhibitor BAY11-7802 (henceforth BAY-11) and measured *IFNG* transcription and translation (Fig 3.1B). Stimulation with IL-12 prior to BAY-11 treatment was essential for determining the effect of NF-κB inhibition on *IFNG* induction, as inhibiting NF-κB signaling before IL-12 stimulation resulted in complete loss of *IFNG* mRNA induction (Fig 3.4C). BAY-11 treatment resulted in a reversal of IL-2 mediated *IFNG* induction and processing, inhibiting the splicing phenotype afforded by IL-2 (Fig 3.1A-C) and revealing a previously uncharacterized role for

NF-κB in IL-2 signaling in NK cells. This phenomenon also held true in the absence of nascent transcription via treatment with ActD, confirming that increased induction and splicing are post-transcriptional effects reliant on NF-κB signaling (Fig 3.1D-E). To confirm that NF-κB dependent IL-2 mediated splicing was not a phenotype specific to the BAY-11 inhibitor, we also blocked NF-κB signaling with the NEDD8 ubiquitinoylation inhibitor MLN4924 [101,102]. MLN4924 inhibited induction of transcriptional *IFNG* and its splicing to a similar extent as BAY-11 (Figure 3.4E-F), lending validation to our studies.

Of note, we found that ActD treated cells stimulated with IL-12 plus IL-2 induce similar levels of mature *IFNG* mRNA as mock treated cells, when normalized to total degradation of mRNA within the condition, regardless of the complete lack of nascent transcription (Fig 3.1F, Fig 3.4D). We attribute the increased quantity of spliced *IFNG* mRNA in IL-12 + IL-2 stimulated, ActD treated cells to IL-2's post-transcriptional splicing and stabilization effect on *IFNG* mRNA. We postulate this splicing and stability prevents *IFNG* turnover and thus increases mature *IFNG* mRNA levels even in the absence of active transcription, a phenomenon reversed in the absence of NF-κB signaling. We believe this phenomenon also accounts for the slight increase in spliced RNA noted in Fig 2.1F after the addition of IL-2 to ActD treated cells.

We next sought to determine whether NF-κB regulation occurs through Akt signaling downstream of PI3K activation as is canonically described (Liu et al., 2020; Narayan et al., 2006). We blocked all Akt activity with the inhibitor Afuresertib after inducing a pool of *IFNG* mRNA with 3 h IL-12 treatment and observed that Akt inhibition restrained protein and mRNA induction to IL-12 levels even in the presence of added IL-2 (Fig 3.1G-H). Interestingly,

however, blocking Akt resulted in an intermediate phenotype for splicing of *IFNG* mRNA, wherein it did not appear to interrupt splicing to the same extent as BAY-11 or MLN4924 inhibition of NF-κB. We propose that while Akt signaling may partially account for the effect of NF-κB on post-transcriptional regulation of *IFNG*, another non-canonical signaling mechanism likely also prompts phosphorylation of NF-κB p65, inducing synergistic IFNγ expression through fostering *IFNG* mRNA stability and intron excision.

3.2.2 PMA promotes *IFNG* mRNA splicing in an NF-κB dependent manner

To determine whether we could replicate the effects of IL-2 in synergistic *IFNG* mRNA induction we used PMA, a mitogen that signals through PKC and induces NF-κB activation [1,94,101,104,105]. We hypothesized that PMA would recapitulate IL-2's effects on NF-κB signaling in synergistic induction and processing of *IFNG.* As a control, we used ionomycin, another well-characterized inducer of *IFNG* in NK and T-cells, but which signals through calcium resulting in NFAT translocation to the nucleus rather than NF-κB [106,107]. Upon treating cells with both IL-12 and PMA, we observed a synergistic increase in *IFNG* transcript and IFNγ protein expression that mirrored the effect of stimulation with combination of IL-12 + IL-2 (Fig 3.2A-C). The combination of ionomycin and IL-12 did not replicate this synergy (Fig 3.2B, 4C). Given the outsized effect of the combination of IL-12 and PMA in *IFNG* mRNA induction, we probed whether PMA could promote RNA processing in the same manner IL-2 mediated *IFNG* mRNA splicing. Treatment with PMA + IL-12 resulted in significant *IFNG* intron excision compared with IL-12 stimulation alone, to a similar extent of what we observed with IL-2 + IL-12 treatment (Fig 3.2D). However, addition of ionomycin to IL-12 did not promote *IFNG* splicing, again suggesting a specific role for NF-κB in *IFNG* regulation and processing.

To validate that PMA-mediated synergistic induction and processing of IFNγ functions primarily through NF-κB signaling, we used BAY-11 to halt NF-κB signaling after inducing a pool of *IFNG* mRNA via IL-12 treatment, but before further stimulation with PMA (Fig 3.2E). We hypothesized that blocking NF-κB signaling would abrogate the synergy between IL-12 and PMA at both the transcriptional and protein levels, as well as any splicing effects. Indeed, we found that BAY-11 + PMA treated cells were unable to induce IFNγ protein or RNA to levels beyond those observed with IL-12 alone (Fig 3.2E-F). Additionally, BAY-11 abrogated intron splicing to levels even lower than those seen with IL-12 treatment alone (Fig 3.2G). We therefore concluded that in the case of synergistic IFNγ protein production and mRNA expression induced the combination of IL-12 and PMA or IL-12 and IL-2, NF-κB signaling was indispensable.

3.2.3 Putative RNA binding proteins do not affect *IFNG* expression upon knockdown

To further clarify the mechanisms driving IL-2 mediated splicing of *IFNG,* we investigated numerous RNA binding proteins we postulated may be involved in *IFNG* processing. Seminal papers have described the role of RNA binding proteins in controlling the export and translation of pre-mature mRNAs, including transcripts modulating innate immunity[73,107,108]. Using POSTAR3, which employs several algorithms to predict putative RNA binding protein interaction with mRNAs, we found several high probability interactors with *IFNG* mRNA[109]. *TARDBP* arose as top hit in several algorithms. TDP-43, the protein for which *TARDBP* codes, is a well-characterized splice factor implicated in alternative splicing, principally exon skipping, in several disease models including cystic fibrosis and amyotrophic lateral sclerosis[110–112]. However, knockdown of *TARDBP* via siRNA (Fig 3.6A) had no effects on *IFNG* mRNA processing or total *IFNG* mRNA induction (Fig 3.5A, B)[109,113].

We attempted to cast a wider net in determining RBP interactors using a previously published data set of RBPs differentially phosphorylated upon IL-2 stimulation in T-cells[114]. Given the short time frame in which we observe *IFNG* mRNA to be spliced, between 3 and 6 h after treatment with IL-12 + IL-2, we postulated there would be little time for novel translation of an RBP involved in *IFNG* processing, so hypothesized that post-translational modifications like phosphorylation could modulate the activation state of an involved RBP, readying it for its function hindering or promoting mRNA processing. We designed a mini-screen of 16 proteins differentially phosphorylated during IL-2 stimulation, associated with RNA binding, splicing, and transport[114]. We knocked down these proteins for 36 h in NK cells via dsiRNA electroporation before stimulating silenced cells with IL-12 + IL-2 for 6h. We then processed mRNA for qPCR to determine whether knockdown affected total induction of *IFNG* mRNA. Most proteins appeared to have no effect on *IFNG* expression as compared with the control. However, both *THOC5* and *HNRNPU* appeared to slightly increase *IFNG* transcription upon their knockdown (Figure 3.5C). We therefore validated these hits with a separate set of dsiRNAs, whose function we confirmed via qPCR (Figure 3.5D, 3.5E; 3.6B, 3.6C). Upon validation, we did not recapitulate the effect observed with the original screen, leading us to conclude that the initial result may have been an artifact of natural variance during the knockdown process.

Interestingly, the RBP DDX17, which functions in conjunction with NF-κB to splice HTLV, arose as a hit in the proteins differentially phosphorylated during IL-2 stimulation in T-cells, from which we derived our mini-screen[114,115]. Its knockdown did not yield differential *IFNG* induction (Fig 3.5C) but given that it was shown to be associated with NF-κB and affected post-translationally downstream of IL-2 signaling, we decided to validate our screen results with a

separate set of dsiRNAs. Upon DDX17 knockdown with a combination of two distinct siRNAs, we observed no difference in *IFNG* mRNA levels during IL-12 + IL-2 stimulation versus the dsiRNA non-targeting control, and therefore conclude that DDX17 is not involved in the regulation of *IFNG* downstream of IL-12 and IL-2 stimulation in NK cells (Fig 3.5E).

Lastly, we hypothesized that Sam68, another NF-κB associated RBP, might regulate *IFNG* induction. Sam68 (encoded by *KHDBRS1*) has been shown to regulate alternative splicing of immune genes such as CD44, and is also a modulator of CD25, the alpha chain of the IL-2 receptor, in T-cells[116,117]. Additionally, Sam68 is thought to promote $CD4^+$ T-cell differentiation into a Th_1 lineage, which is strongly associated with *IFNG* in both promoting Th_1 skewing and also potentiating IFNγ protein production[118]. Of further relevance, Sam68 has several ties to NF-κB family members, including p65, in the context of CD25 regulation, and is thought to be activated downstream of PI3K signaling, which we demonstrated may be partially responsible for the inhibition of *IFNG* splicing in NK cells downstream of IL-2 stimulation (Fig 3.1F, 3.1G)[117,119]. We therefore tested Sam68's relevance in controlling *IFNG* induction in NK cells during IL-12 plus IL-2 stimulation. Knockdown of Sam68, validated through qPCR (Fig 3.6D) had no effect on either *IFNG* induction or IFNγ protein expression (Fig 3.5F, G). The RNA binding protein or proteins responsible for IL-2 mediated RNA processing of *IFNG* therefore remain unelucidated.

3.3 Figures

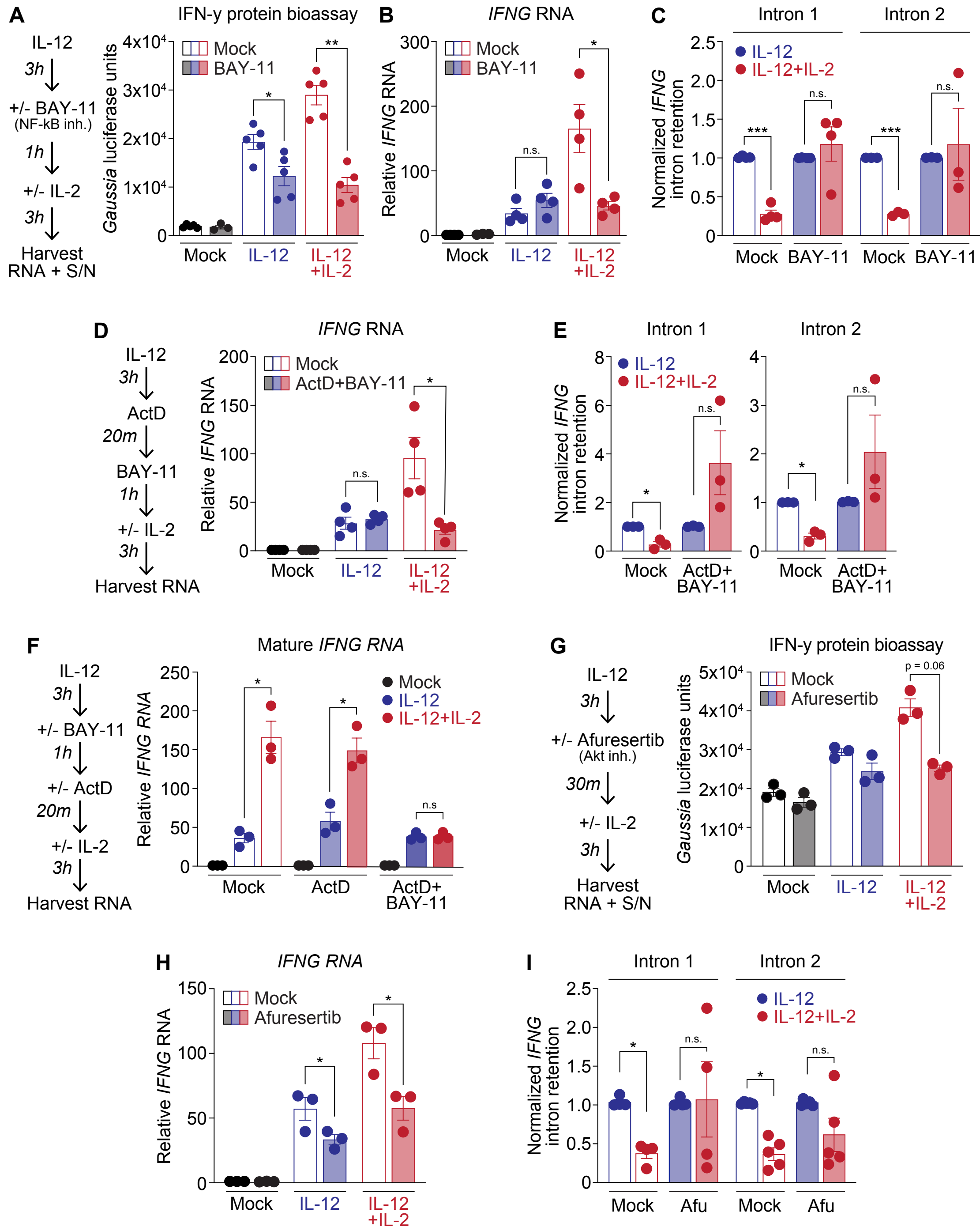

Figure 3.1: Post-transcriptional IL-2 mediated splicing of IFNG is NF-κB-dependent. a) Schematic for inhibition of NF-κB signaling with BAY-11 during IL-2 stimulation and *Gaussia* luciferase bioassay for IFNγ protein quantification (**b**) qPCR analysis of total *IFNG* mRNA induction normalized to *HPRT1* and (**c**) SYBR qPCR analysis of *IFNG* mRNA intron detention upon inhibiting NF-κB signaling with BAY-11 (10μM) treatment prior to IL-2 treatment. **d**)

mature *IFNG* mRNA induction and (**e**) *IFNG* intron detention upon inhibition of both nascent transcription and NF-κB signaling prior to IL-2 treatment; normalized to *HPRT1* and to 5'UTR of *IFNG* mRNA respectively **f**) *IFNG* mRNA induction normalized to *HPRT1* and to the no stimulation condition within each treatment (no treatment no stim, ActD no stim, and ActD + BAY-11 no stim). **g**) *Gaussia* luciferase bioassay for IFNγ protein and (**h**) qPCR analysis of *IFNG* mRNA normalized to *HPRT1* during inhibition of Akt activity with Afuresertib (10μM) for 30min prior to IL-2 stimulation **i)** SYBR qPCR analysis of *IFNG* mRNA intron detention upon inhibiting Akt, normalized to 5'UTR of *IFNG* mRNA. Data in **a-i** is mean ± SEM for 3 to 5 biological replicates with (**a**)**,** (**b**), (**d**)**, f-h** analyzed by ratio paired T test and (**c**)**,** (**e**), (**i**) by paired T test with Holm-Šídák method for multiple corrections. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n.s. is not significant.

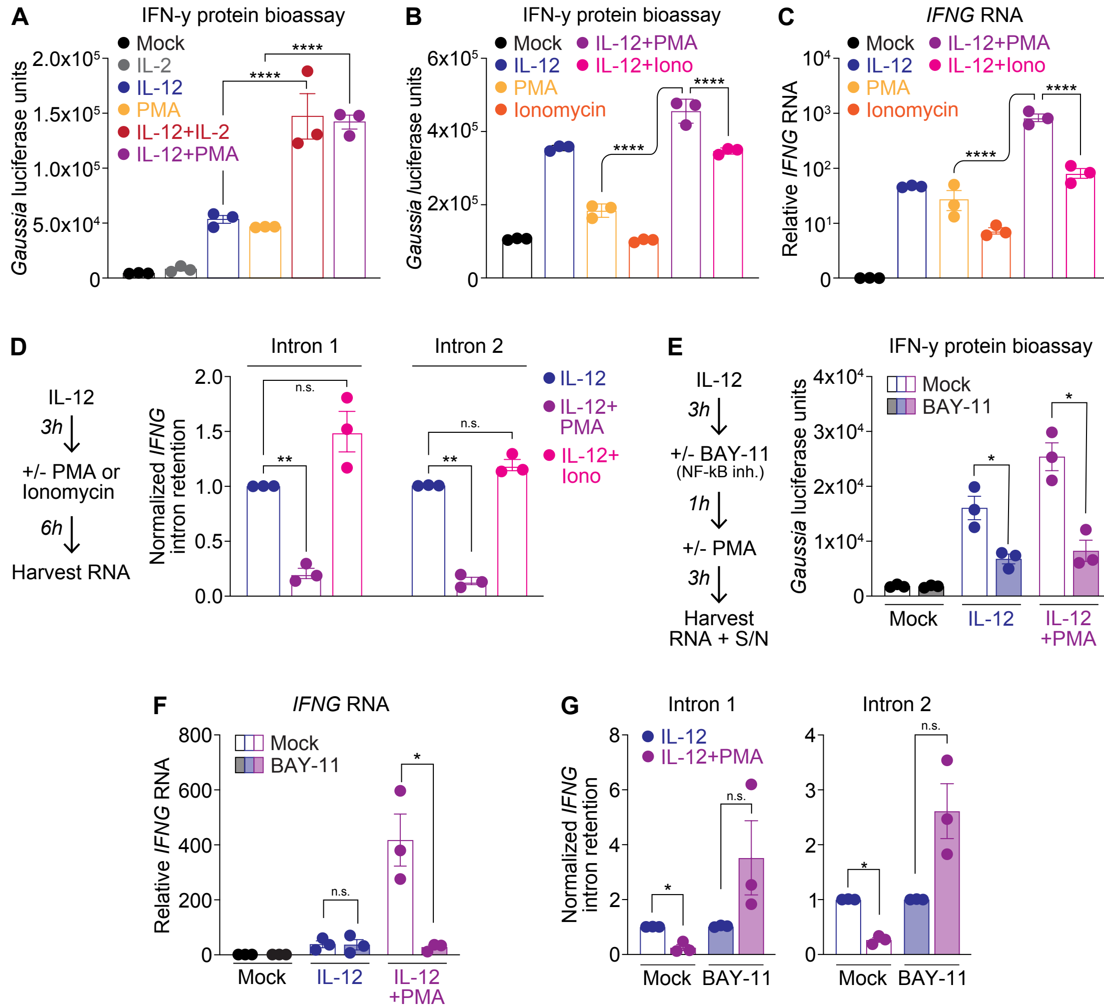

Figure 3.2 NF-κB signaling is required for PMA-mediated splicing of *IFNG*
a) Bioassay quantification of IFNγ protein production using Huh7 *Gaussia* luciferase IRF1 GAS(5x) reporter cells treated for 24 h with supernatants collected after 24h NK92 stimulation with IL-2 (100U/mL), IL-12 (10ng/mL), PMA (10nM), or a combination. (**b**) Bioassay quantification of IFNγ protein production and (**c**) qPCR analysis of *IFNG* induction during stimulation with PMA (10nM), Ionomycin (1μg/mL), IL-12 (10ng/mL) or a combination for 24 h (**b**) or 6 h (**c**). **d**) SYBR qPCR analysis of *IFNG* mRNA intron detention of total lysates from cells treated with IL-12 (10ng/mL), IL-12 + PMA (10nM), or IL-12 + Ionomycin(1μg/mL) for 6h, normalized to total mature plus unspliced *IFNG* (**e**) *Gaussia* luciferase bioassay for IFNγ protein quantification (**f**) *IFNG* mRNA transcriptional induction normalized to *HPRT1* and (**g**) *IFNG* intron detention as amplification normalized to 5'UTR-Exon 1 amplification in the presence versus absence of 1 h NF-κB inhibitor BAY-11 (10μM) treatment before 3h PMA stimulation. Data in **a-c** is mean ± SEM for 3 biological replicates, one way ANOVA with Turkey's comparison test for multiple comparisons. Data in **d-g** is mean ± SEM for 3 biological replicates**, d, g** paired T test, **e, f** ratio paired T test with Holm-Šídák method for multiple corrections * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, n.s. is not signifi

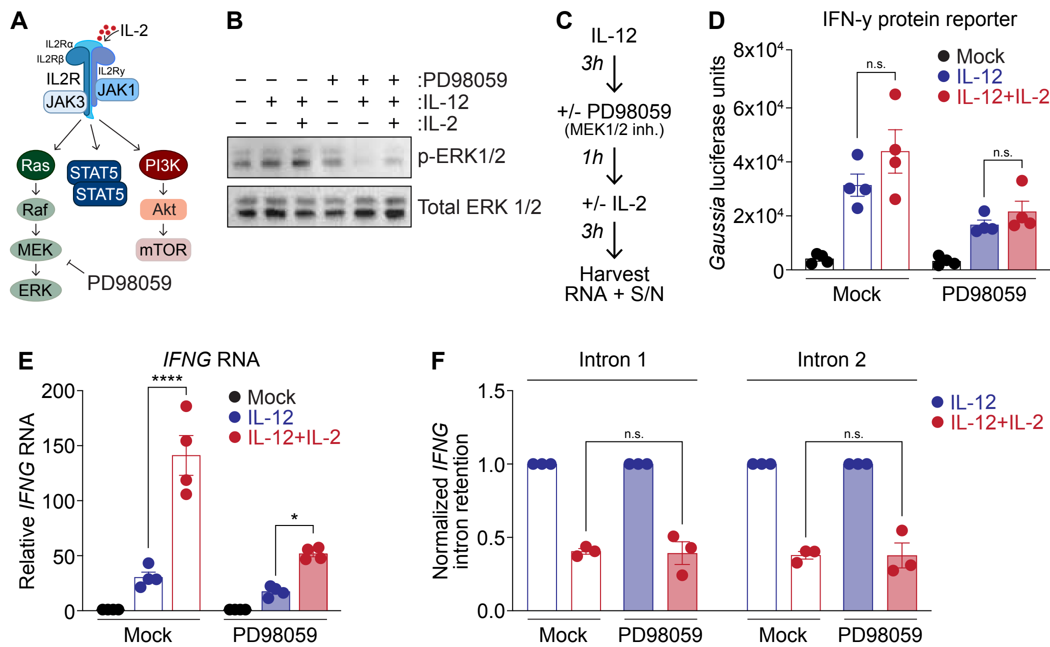

Figure 3.3 a) Schematic of canonical IL-2R signaling **b**) Immunoblot confirming effect of inhibitor PD98059 (10µM) on preventing phosphorylation ERK1/2 downstream of MEK1/2 inhibition during IL-12 and IL-2 treatment in NK92 cells **c**) schematic of PD98059 treatment in determining effect on IFNγ induction and splicing **d)** *Gaussia* luciferase bioassay for IFNγ protein quantification (**e**) total *IFNG* transcript induction and (**f**) *IFNG* intron detention upon treating cells with PD98059 (25µM) prior to IL-2 treatment, normalized to total spliced plus unspliced *IFNG*. Data in **d-f** is mean ± SEM for 3 or 4 biological replicates. (**b**) is representative of 3 biological replicates. **d-e** analyzed two-way ANOVA with Turkey's test for multiple comparisons and (**f**) by paired T test with Holm-Šídák method for multiple corrections. $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, n.s. is not significant.

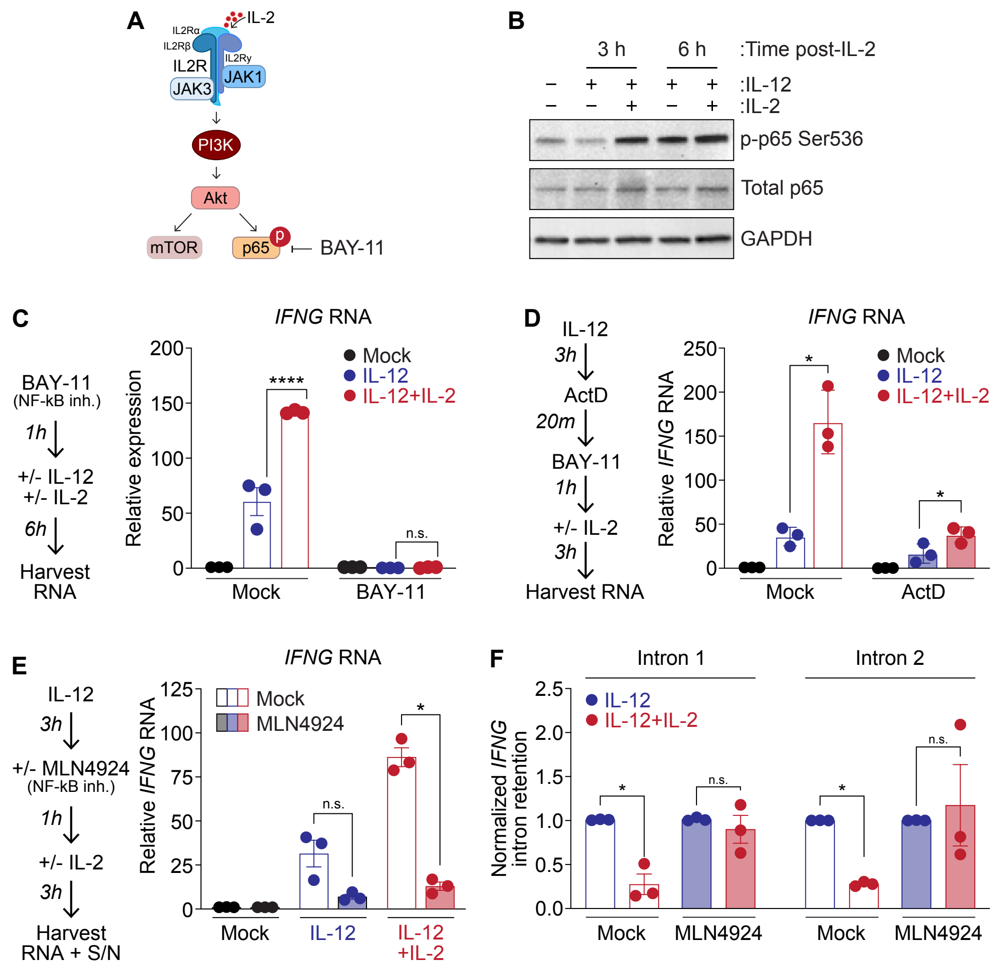

Figure 3.4 a) Schematic depicting alternative signaling through Akt downstream of the IL-2 receptor in NK cells **b**) immunoblot for total and phosphorylated NF-κB p65 NK cells treated with IL-12 or IL-12 + IL-2 for 3 or 6 h with GAPDH as loading control **c**) qPCR analysis of *IFNG* induction upon pre-treatment with the NF-κB inhibitor BAY-11, prior to any stimulation **d**) *IFNG* mRNA induction normalized to *HPRT1* with all samples normalized to the no stimulation condition within the mock treatment to confirm functioning ActD halt of nascent transcription **e**) qPCR analysis of total *IFNG* mRNA induction and (**f**) SYBR qPCR analysis of *IFNG* mRNA intron detention upon inhibiting NF-κB signaling with MLN4924 (10μM) (**b**) is representative of 5 biological replicates where 4 of 5 showed the depicted result. Data in **c-f** is mean ± SEM for 3 biological replicates with **c-d** analyzed by one-way ANOVA with Turkey's test for multiple comparisons, **e** analyzed by ratio paired T test and **f** by paired T test with Holm-Šídák method for multiple corrections * p ≤ 0.05, ****p ≤0.0001, n.s. is not significant

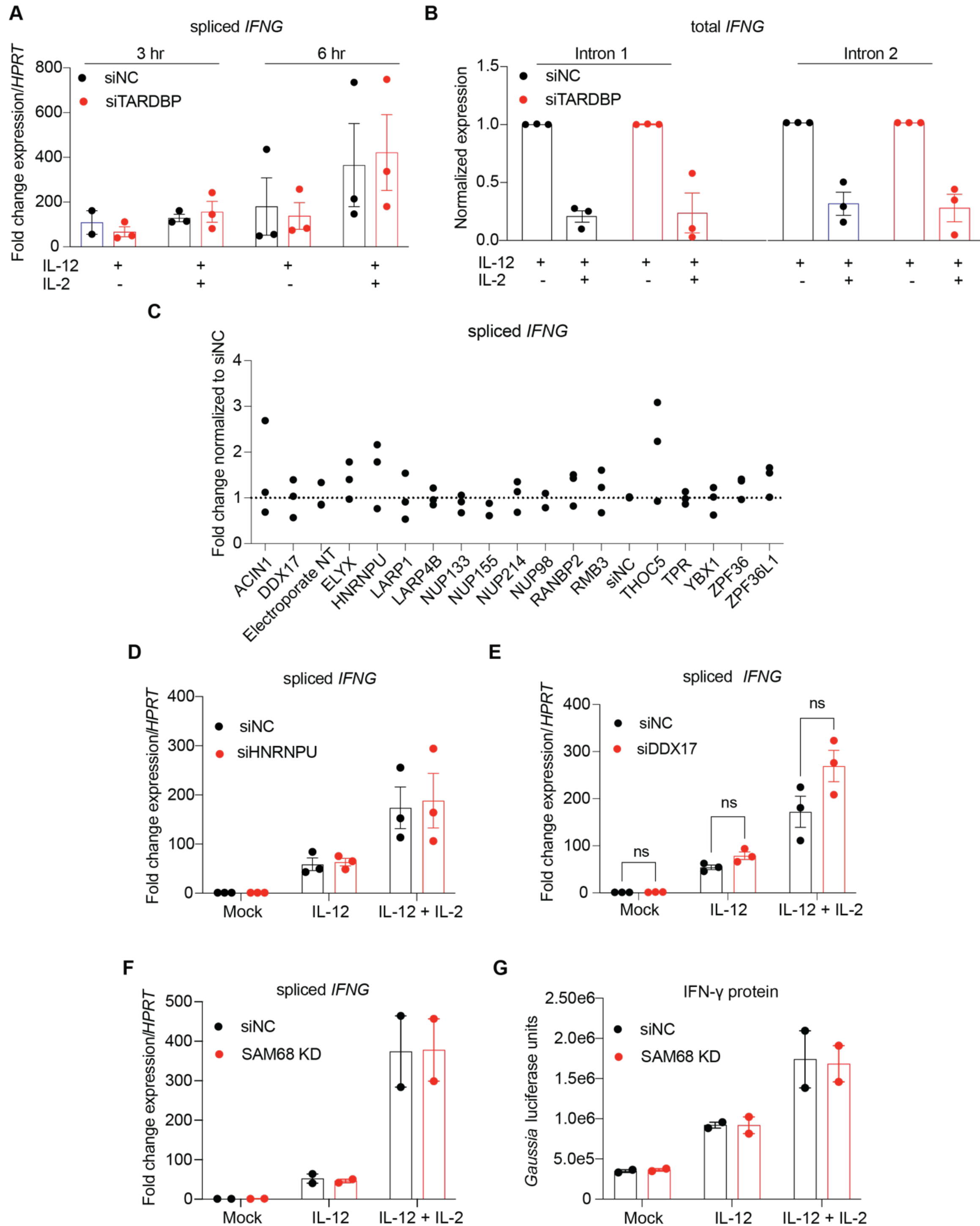

Figure 3.5 Putative RNA binding proteins do not affect *IFNG* induction. **a)** qPCR analysis of *IFNG* induction after 36h knockdown of *TARDBP* followed by 3 or 6 h treatment with IL-12 and IL-12 + IL-2; normalized to *HPRT1*. **b)** SYBR qPCR analysis of *IFNG* mRNA intron detention after 36 h *TARDBP* knockdown, during stimulation with IL-12 or IL-12 + IL-2 normalized to mature plus unspliced *IFNG* mRNA. **c)** qPCR analysis of *IFNG* induction,

normalized to *HPRT1*, after 36 h knockdown of 16 RNA-binding proteins. NK92 cells were treated with or IL-12 + IL-2 for 6h, and induction of *IFNG* mRNA normalized to the non-targeting control siNC. qPCR analysis of *IFNG* induction normalized to *HPRT1* and to mock within each condition after dsiRNA knockdown of **(d)** *HNRNPU* **(e)** *DDX17* **(f)** *SAM68*, stimulated for 6 h with either IL-12 or the combination of IL-12 + IL-2. **(h)** *Gaussia* luciferase bioassay for IFNγ protein quantification after knockdown of *SAM68* during 6h stimulation with IL-12 or IL-12 + 2. For each experiment, data is mean ± SEM for n=2 or 3 biological replicates. Data in **(e)** analyzed by ratio paired T test where n.s. = not significant.

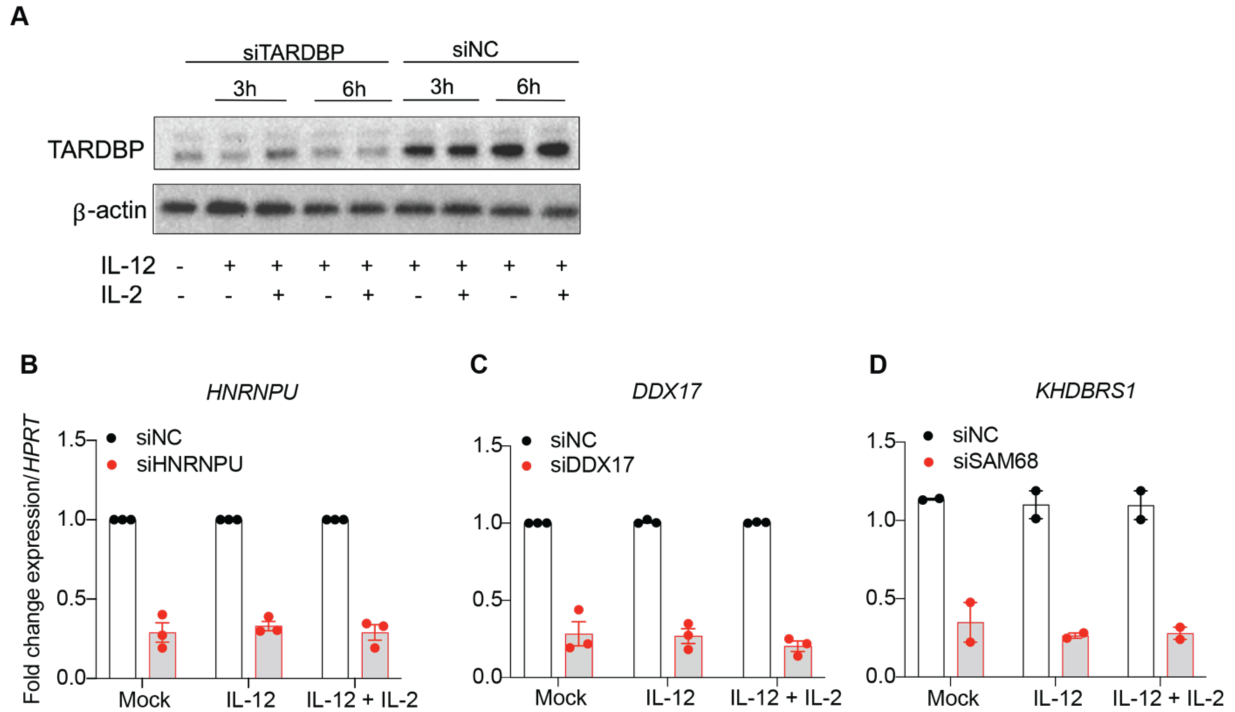

Figure 3.6 (a) Representative immunoblot confirming siRNA knockdown of TDP-43 during the stimulation conditions depicted in Fig 3.5a, Fig 3.5b. qPCR analysis of siRNA knockdown of (b) *HRNRNPU*, (c) *DDX17* and (d) *KHDBRS1* (codes for Sam68) for the experiments shown in Fig 3.5d-g, normalized within each stimulation condition to the siNC control and *HPRT1* expression. Data in a is representative of 4 biological replicates. Data in b-d is mean ± SEM for n=2 or 3 biological replicates.

3.4 Discussion

Our findings identify NF-κB as a novel regulator of post-transcriptional processes downstream of IL-2 receptor signaling. Though NF-κB p65 is activated for cytokine production in NK cells, this is thought to occur primarily downstream of activating receptors like NKG2D rather than IL-2R, which is instead canonically associated with cell survival and proliferation[93,98,120]. We postulate that NF-κB is likely controlling processing of *IFNG* mRNA through interactions with other RNA-binding proteins or splice factors, perhaps through acting as a scaffold to recruit proteins to intronic regions where they can facilitate mRNA processing. This seems especially pertinent given that each intron within the *IFNG* gene contains at least one NF-κB binding sequence (Howard Young, unpublished data). Indeed, p65 has been shown to serve in conjunction with DDX17 as a splice factor for HTLV[121].

We tested the effect of knockdown on *IFNG* mRNA transcription for 16 RNA binding proteins identified by Ross et. al to be differentially phosphorylated during IL-2 stimulation in T-cells, but found none to have significant impact on *IFNG* levels (Figure 3.5C-F) [114]. Additionally, silencing of the RNA binding protein and splice modulator Sam68, associated with NF-κB, had no effect on *IFNG* induction (Fig 3.5F-G). While our understanding that NF-κB contributes to the splicing of *IFNG* is clear, the effectors or mechanism downstream of NF-κB remain to be elucidated. Nevertheless, our observations provide new insights into how IL-2 induces robust IFNγ production and introduce a new role for IL-2 signaling in NF-κB activation in NK cells.

Overall, this study sheds light on the mechanism by which crucial immune signals are stringently regulated to ensure the appropriate balance of inflammation and resolution during the immune

response. The availability of pre-mRNA transcripts poised for immediate processing could serve as model for many cytokines in allowing rapid and robust production, but also sufficient resolution of inflammation upon termination of the signals required for post-transcriptional processing. The understanding of the post-transcriptional modulation of *IFNG* mRNA can serve as a framework for investigation of the regulation of other inflammatory cytokines.

AUTHOR CONTRIBUTIONS

Conceptualization: R.D.V.G., N.S.G. and R.S.; *Investigation*: R.D.V.G., D.S.O., and E.G. *Methodology*: R.D.V.G., N.S.G, and R.S., *Formal Analysis*: R.D.V.G.; *Visualization*: R.D.V.G. and N.S.G., *Resources*: R.S., H.A.Y.; *Writing—original draft*: R.D.V.G.; *Writing-- review and editing*: R.D.V.G., N.S.G., E.G., H.A.Y., R.S. *Funding acquisition*: R.D.V.G., H.A.Y., R.S.

Table 3.1 Reagents used in this study

Reagent	Company	Catalog Number
4-thiouridine	Cayman Chemical Company	13957-31-8
Afuresertib	Selleck Chemicals	S7521
Applied Biosystems Power SYBR Green PCR Master Mix	Thermo Scientific	4367659
Applied Biosystems Power SYBR Green PCR Master Mix	Thermo Scientific	4367659
BAY-11	Selleck Chemicals	S2913
Bio-Rad Protein Assay Kit II	Bio-Rad	5000002
CD56+ NK Cells Negatively Selected	Bloodworks	4570-66
Cyclohexamide	Sigma Aldrich	239763-M
Direct-zol 96 RNA isolation kit	Zymo Research	R2067
Dulbecco's modified Eagle medium	Thermofisher	11960069
Dynabeads M-280 Streptavidin	Thermo Fisher Scientific	11205D
DYNAL MyOne Dynabeads Streptavidin C1	Thermo Fisher Scientific	65001
EZ-Link HDPD-Biotin	Thermo Fisher Scientific	A35390
FastDigest SmaI	Thermo Fisher Scientific	FD0664
Fetal Bovine Serum	Atlas Biologicals	F-0500-A
Halt Protease and Phoshphatase Inhibitor	Thermo Scientific	78444
HEK293T cells	ATCC	CRL-3216
HPRT1 probe	Taqman	Hs.PT.58v.45621572
Huh7 cells	ATCC	
Ifn gamma Human ELISA kit	Thermo Scientific	KHC4021
IFNG probe	Taqman	Hs.PT.58.3781960
Immobilon-FL PVDF Membrane	Sigma Aldrich	IPFL00010
Invitrogen Superscript IV Reverse Transcription Kit	Thermo Fisher Scientific	18-090-050
Lenti-X	Clontech	631231
Midi-Prep Kit	Macherey Nagel	740420
Mini-Prep Kit	Macherey Nagel	740588

Mirus Bio TransIT-X2 Dynamic Delivery	Fisher Scientific	MIR6000
MLN4924	Cell Signaling Technologies	85923
NK92 cells	Howard Young	
Nucleospin RNA isolation	Macherey Nagel	740955.25
NuPAGE 10% Bis-Tris Protein Gels	Thermo Fisher Scientific	NP0301BOX
NuPAGE 4-12% Bis Tris Protein Gels	Thermo Fisher Scientific	NP0336BOX
NuPAGE MOPS SDS Running Buffer	Thermo Fisher Scientific	NP0001
P98059	Selleck Chemicals	S1177
PCR and Gel Clean Up	Macherey Nagel	740609
Pen-Strep-L-Glutamine	Corning Fisher-Scientific	MT30009CL
Peroxidase HRP Anti-Rabbit IgG Goat	Cell Siganling Technologies	7074S
Phase Lock Gel	VWR	10847-802
Pierce Bradford Plus Protein Assay Kits	Thermo Fisher Scientific	23236
Pierce Gaussia Luciferase Glow Assay	Thermo Scientific	16161
Precision Plus Protein Dual Color Standards	Bio-Rad	1610374
Primescript Reverse Transcription Kit	Takara	RR037B
Recombinant human IL-12	PeptroTech	200-12
Recombinant human IL-15	Shenandoah Biotechnology	10086100UG
Recombinant human IL-2		
RPMI 1640	Corning	15-040-CV
SYBR Safe	Invitrogen	S33102
Syringes with BD Luer-Lok TIP	BD	302995
Taqman Universal Master Mix II no UNG	Thermo Fisher Scientific	4440048
Trizol LS	Thermo Fisher Scientific	10296028
UltraPure Phenol:Chloroform:Isoamyl	Sigma Aldrich	15593031

Chapter 4: Materials and Methods

Cells and cell culture conditions

All cells were grown at 37°C in 5% CO2. Human NK92 cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), L-glutamate at 2mM, penicillin at 100U, streptomycin at 100µg, IL-15 at 10ng/mL and IL-2 at 200U/mL growth media. Before any treatment or stimulation, NK92 cells were resuspended at a concentration of $5*10^5$ cells per mL in resting media (RPMI 1640, 10% FBS, L-glutamate at 2mM penicillin at 100U, streptomycin at 100µg/mL with no added cytokines) overnight. During stimulation, rhIL-12 was used at 10ng/mL and rhIL-2 was used at 100U/mL. HEK293T-cells for generation of lentivirus and Huh7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% FBS, L-glutamate at 2mM, penicillin at 100U, streptomycin at 100µg/mL. Human CD56+ primary NK cells were obtained from Bloodworks (CAT#4570-66) and rested for 1h at a concentration of $5*10^5$ cells/mL in low serum (1% FBS in RPMI) media for one hour after thawing, prior to cytokine treatments.

Cytokine treatments and inhibitors

Cells were treated with 10ng/mL rhIL-12, 100U/mL rhIL-2, 10nM PMA, and 1µg/mL ionomycin for stimulations. The MEK1/2 phosphorylation inhibitor PD98059 (Selleck Chemicals CAT#S1177) was used at 10µM. NF-κB inhibitors were used at the following concentrations: BAY11-7802 (Selleck Chemicals CAT#S2913) at 10µM, MLN4924 (CST CAT#85923) at 10µM for 1 h prior to cytokine or PMA/ionomycin treatments. The pan-Akt inhibitor Afuresertib (Selleck Chemicals CAT# S7521) was used at 10µM for 30 min prior to stimulation. Actinomycin D (ActD) for inhibition of nascent transcription was used at 5µg/mL

for 20-30 min prior to cytokine treatments, and cycloheximide (CHX) for inhibition of translation was used at 100µg/mL for 15 min prior to cytokine or PMA/ionomycin treatments.

Gene expression analysis

RNA was isolated from NK92 cells using the Macherey Nagel NucleoSpin RNA isolation kit as dictated by manufacture guidelines (CAT #740955). A cDNA library was then prepared using PrimeScript RT (Takara Bio CAT#RR037B) with random hexamers and polyAdt per manufacturer guidelines. cDNA was amplified via qPCR using the ViiA7 qPCR system with Taqman probes and relative amplification normalized to the housekeeping gene, *HPRT1*. Primers and probes are described in Expanded View Tables 1 and 2.

Quantification of IFNγ by ELISA

Supernatants from treated NK92 samples were collected at the time of RNA and/or lysate harvesting and stored at -80°C until processing. Samples were then diluted 1:5 and processed with the IFN gamma Human ELISA kit (Thermo Scientific Cat#KHC4021) as per manufacturer instructions.

***Gaussia* luciferase assay to detect IFNγ activity**

All primer sequences used for cloning are provided in Table 1. A gblock containing 5x repeat of the human IRF1 promoter was amplified via PCR with the Adv2 system, then cloned via InFusion into the lentiviral post-transcriptional regulation IP ISGF3 5x hGLUC-MODC-PEST plasmid, previously described in Schwerk et al, cut with SmaI Fast Digest enzyme. Lentivirus was generated using PPAX2 and VSVG with Mirus Transit X2 transfection in 293FTs. Filtered

lentivirus was placed directly on Huh7 cells for 24h. Cells were selected with puromycin, then diluted for single cell cloning. Several single-cell clones were screened for robust range of *Gaussia* luciferase production via rhIFNγ and supernatants from NK92 cells treated with rhIL-2 and rhIL-12. For bioassay quantification of IFNγ protein via *Gaussia* luciferase readout in Huh7 IRF1 GAS(5x) cells, supernatants from stimulated NK92 cells were taken at the time RNA and lysates were collected, then frozen down at -80°C until use. Huh7 IRF1 GAS(5x) reporters were plated at a concentration of $1.25*10^5$ cells/well in a 24 well plate, then allowed to rest O/N. Media was refreshed immediately prior to adding supernatants to each well. Cells were allowed to incubate with supernatant at 37°C, 5% CO_2 for 24 h. 100µL of the supernatant from each well was harvested, and either frozen or prepared immediately for *Gaussia* luciferase quantification with the Pierce *Gaussia* Luciferase Glow Assay Kit (Thermo Scientific CAT#16161). Luminescence was quantified using a 96 well plate with the Synergy HT microplate reader and Biotek Gen5 analysis software.

4SU-labelling to determine nascent mRNA transcription

Briefly, cells under varying stimulation conditions were pulsed in the final hour of treatment with 500µM concentration 4SU. Cells were then lysed with RA1 from the NucleoSpin RNA Kit by Macherey Nagel (CAT#) and RNA was isolated per manufacturer's instructions. Total RNA (minimum 25µgper condition) was biotinylated with 1mg/mL biotin-HPDP (Thermo Fisher Scientific CAT#A35390) in DMF. Preparation per 1µg of RNA was as follows: 1µL10x biotinylation buffer (100mM Tris, pH7.5, 10mM EDTA in H_2O), 7µL RNA (1µg diluted in RNAse free H_2O), 2µL biotin-HPDP. RNA was incubated in the biotinylation solution for 2 h at RT with rotation, then phenol:chloroform:isoamyl alcohol (Sigma Aldrich CAT#15593031)

extracted twice in phase lock tubes (VWR CAT#10847-802). RNA was precipitated with sodium acetate (12.5 µL per 100µL aqueous phase) and pellet washed in 70% ethanol. RNA was resuspended at a concentration of 1 µg/µL. Labeled RNA was then separated using streptavidin beads. Beads were washed 3x in 0.1M NaCl, then once in streptavidin bead binding buffer (10mM Tris-HCL, pH 7.5, 1mM EDTA, 2M NaCl). RNA was denatured at 65°C for 10 min, then added to an equal volume of washed/primed streptavidin beads (Thermo Fisher Scientific CAT#12205D) and topped to a final volume of 200µL in binding buffer. Streptavidin beads and RNA were rotated for 15 min at RT, then placed on a magnet for separation. Beads were washed 4x with washing buffer (100mM Tris pH 7.5, 10mM EDTA, 1M NaCl, 0.1% Tween 20 in RNAse free), then eluted 2x in 10mM DTT with 5 min incubation. RNA was then cleaned up with the NucleoSpin RNA Clean-up Kit (Macherey Nagel CAT#740948) as per manufacturer instructions. RNA was reverse transcribed and gene expression quantified as per "Gene expression analysis".

mRNA stability assay

NK92 cells were stimulated with IL-12 for 3 h. Subsequently, transcription was halted with Actinomycin D (5µg/mL) for 20-30 mins. Cells were treated with or without IL-2 for 4 h, and cells and supernatants were harvested at 1 h, 2.5 h and 4 h after stimulation. RNA was isolated with the Macherey Nagel kit and analyzed via qRT PCR.

mRNA splicing assay

For quantification of intron detention via gel electrophoresis or Bioanalyzer, RNA was isolated from NK92 cells using the Macherey Nagel NucleoSpin RNA isolation kit as dictated by

manufacture guidelines (CAT#740955.250). An *IFNG* specific cDNA library was created from isolated RNA with the Invitrogen Super Script IV kit (ThermoFisher Scientific CAT #18-09-050) using a reverse primer specific to Exon 4 of *IFNG* (Table S1). cDNA was amplified using primers spanning the regions between each exon of the *IFNG* gene, provided in Expanded View Table 1. PCR products were run on 1% agarose gel with SYBR safe or Bioanalyzer Chip. For quantification of intron detention via qPCR, RNA was isolated from NK92 cells using the Macherey Nagel NucleoSpin RNA isolation kit as dictated by manufacture guidelines (CAT#740955.250). An *IFNG* specific cDNA library was then created with Invitrogen Super Script IV kit (ThermoFisher Scientific CAT#18-09-050) using a reverse primer specific to Exon 4 of the *IFNG* gene. cDNA was then amplified via qPCR using the ViiA7 qPCR system with SYBR Green Power Mix (Thermo Scientific CAT#4367659), using primers for specific targets to regions spanning the exon-intron gap of each intron of the *IFNG* gene. Amplification of each intron was normalized to a region spanning the 5'UTR and Exon 1 of *IFNG.* Primer sequences can be found in Expanded View Table 1.

Immunoblotting

Whole cell lysates were generated using RIPA buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH 7.4) supplemented with a protease phosphatase inhibitor cocktail (Thermo Scientific Cat#78444). Protein concentration was quantified with Bradford reagent (Bio-Rad Protein Assay Kit II CAT#50000002). 3-10μg of protein was resolved by SDS-PAGE and transferred onto PVDF. Primary antibody incubation occurred overnight in with antibodies diluted in 3% BSA in TBS-T, and species specific HRP conjugated secondary antibodies for 1h after 3x washes in TBS-T. Chemiluminescence was detected with a

ChemiDoc touch. The following antibodies were used in Western Blot analysis: anti-phospho ERK1/2 (CST CAT#4695S), anti-ERK1/2 (CST CAT#4370S), anti-puromycin (Sigma Aldrich Cat#MABE343), anti-phosphorylated NF-κB p65 (CST CAT#3033), anti-NF-κB p65 (CST CAT#8242), GAPDH (CST CAT#51774S).

siRNA-mediated gene knockdown by nucleofection in NK92

Cells were rested overnight in cytokine free media. Prior to electroporation with the Lonza 4D Nucleofector, 1x10^6 cells were resuspended in 25ul of Lonza 4D Nucleofection Cell SF serum with supplement plus the siRNA for a final concentration of 20nM. Cells were then pulsed with the CM-130 setting, resuspended in 1-2mL cytokine free media per million cells and allowed to rest for 36-48 hours. Knockdown efficiency was determined by either immunoblotting or qPCR.

Chapter 5. Conclusions

5.1 Summary and impact

The work presented in this dissertation has yielded advances in understanding the regulatory factors contributing to post-transcriptional control of a crucial inflammatory cytokine, IFNγ. Though the transcriptional mechanisms of *IFNG* induction are largely elucidated, they do not account completely for the rapid and robust production of IFNγ observed in NK cells during stimulation with the combination of cytokines IL-12 and IL-2. We have discovered that alternative splicing, specifically intron detention, plays a role in the positive regulation of IFNγ. This finding is novel: mRNA splicing has not been previously implicated in control of IFNγ production. In congruence with previously published studies, we found that NK cell stimulation with the combination of IL-12 and IL-2 induces rapid synergic IFNγ expression, and that IL-2 has a stabilizing effect on *IFNG* mRNA even in the absence of concurrent transcription (Figure 2.1, 2.2)[22,23]. We took these findings a step further in discovering that beyond its stabilizing effect, IL-2 also mediates post-transcriptional splicing in all three introns of *IFNG* mRNA.

In attempting to deconvolute the signaling pathways required for *IFNG* post-transcriptional mRNA processing, we uncovered a phenomenon parallel to the synergistic induction of IFNγ observed during stimulation the combination of IL-12 and IL-2: treatment with the combination IL-12 and PMA yielded remarkably similar increases *IFNG* mRNA and IFNγ protein expression. Upon further investigation, we found that PMA also mediates *IFNG* splicing, like IL-2. However, the known *IFNG* inducer ionomycin does not recapitulate either robust induction or splicing of *IFNG* mRNA when combined with IL-12 (Figure 3.2). PMA and ionomycin signal transduction pathways are distinct: while ionomycin signals through calcium to induce cleavage

of IP3 and DAG for NFAT phosphorylation, PMA signals through PKC resulting in NF-κB p65 phosphorylation and translocation to the nucleus[106,107]. We found that inhibiting NF-κB p65 phosphorylation with the IκB phosphorylation inhibitor BAY-11 reversed the effect of PMA in mediating splicing and synergistic upregulation of *IFNG* (Figure 3.2).

Given the similarities of PMA and IL-2 in regulating synergistic *IFNG* induction and splicing, we questioned whether NF-κB may have a non-canonical role in post-transcriptional regulation of *IFNG* downstream of the IL-2 receptor. When we blocked NF-κB signaling in transcriptionally halted cells, we reversed the splicing and upregulation phenotype afforded by IL-12 in combination with IL-2 stimulation (Figure 3.1). Though NF-κB has been implicated in transcriptional upregulation of *IFNG*, its post-transcriptional effects, and effects downstream of IL-2R, are not well elucidated. Thus, the post-transcriptional involvement of NF-κB in IFNγ control constitutes a novel role for this transcription factor, previously unappreciated. To better determine the extent of the IL-2 receptor signaling governing NF-κB involvement in synergistic IFNγ expression, we determined whether the PI3K-Akt pathway mediated the observed effect of IL-2 on post-transcriptional regulation of *IFNG*, and determined it was partially, but not completely responsible for resulting mRNA processing and IFNγ production (Figure 3.1). Intriguingly, PMA and IL-2R signaling also converge downstream of Ras/Raf; however, blocking phosphorylation of ERK1/2, which is the end result of this transduction pathway, did not affect the post-transcriptional modulation of *IFNG* mRNA (Figure 3.3). Overall, this dissertation's principal impact is uncovering novel regulation of *IFNG* mRNA processing, via understanding the signaling pathways responsible for governing post-transcriptional processes mediated by IL-2 (Figure 5.1).

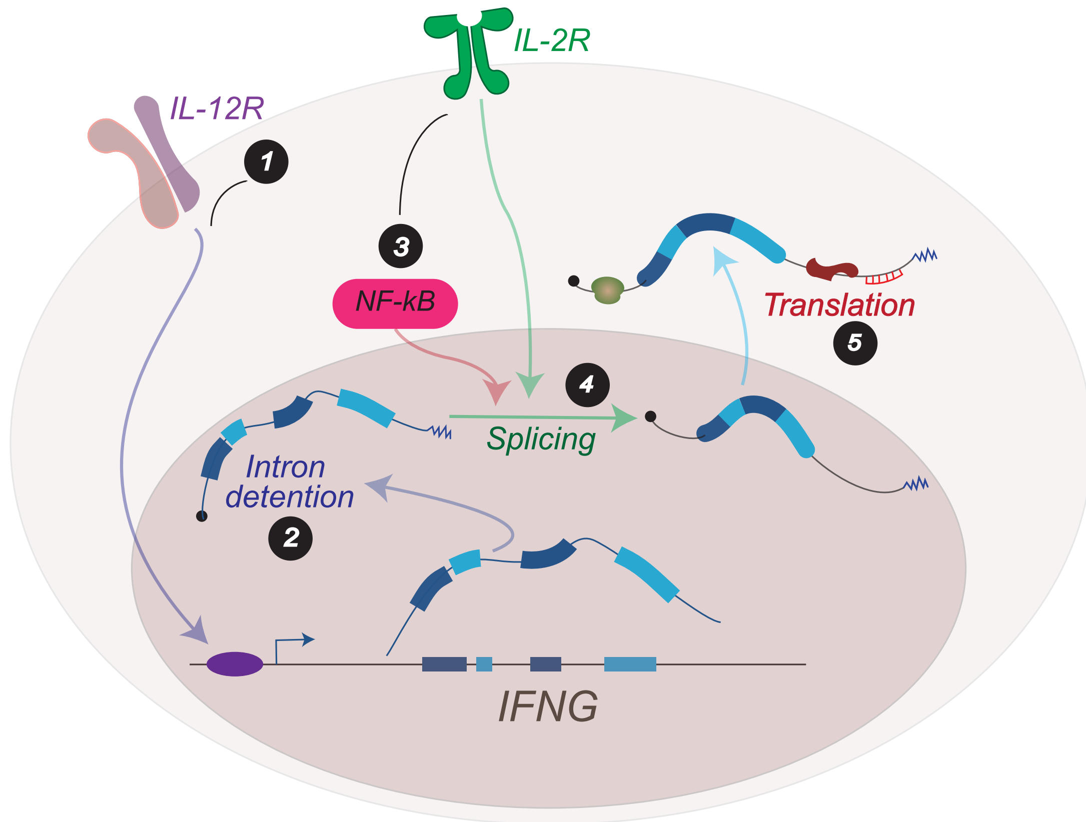

Figure 5.1. Working model of IL-2 post-transcriptional modulation of *IFNG*. We postulate that while IL-12 signaling induces transcription of *IFNG* mRNA, (1-2) IL-2 is required for stabilization and splicing (3) through an NF-κB-mediated mechanism (4). This allows for rapid translation of robust quantities of *IFNG* mRNA (5) allowing for a swift innate response to antimicrobial pathogens.

5.2 Future directions

This dissertation lays out novel findings in the fields of cytokine biology and RNA regulation of innate inflammation. However, many questions arise from our observations. The central gap in our understanding of post-transcriptional regulation of *IFNG* downstream of IL-2 is the mechanistic connection between NF-κB signaling and splicing—how is it that this transcription factor induces mRNA processing? We hypothesized that NF-κB interacted with an RNA-binding protein, allowing for *IFNG* mRNA binding and thus promoting splicing. However, as laid out in Chapter 3, we attempted both larger scale mini-screens from existing data sets, as well as targeted hypotheses of RBPs that associate with NF-κB without yielding any significant hits. A future approach to identify RBPs would likely need to be completely unbiased, such as comprehensive identification by Mass-Spectrometry (ChiRP), which involves crosslinking RNA to RBPs and using biotinylated oligos along the length of the desired RNA to immunoprecipitate all associated proteins[122].

Another avenue for future investigation is elucidating whether intron detention in pre-mRNA of inflammatory mediators extends beyond *IFNG* mRNA in NK cells. Other rapidly induced cytokines may be regulated by similar mechanisms, in which an unstable, un-spliced form of a given gene is transcribed upon one signal and then processed upon a second. For instance, even other inflammatory mediators produced by NK cells, such as granzyme and perforin, are rapidly produced in robust quantities during the initial stages of the immune response[22,23,123]. As outlined in Chapter 2 discussion, TNFα would also be a primary target for investigation, as in memory T-cells, its protein levels are not drastically reduced even in the absence of nascent transcription, much like *IFNG*, suggesting a role for post-transcriptional regulation[79].

Lastly, the question remains of whether *IFNG* mRNA is post-transcriptionally regulated in cell types beyond NK cells, namely $CD4^+$ T-cells, which are the other major producer of this cytokine. Because IL-12 is not a primary stimulator of *IFNG* mRNA transcription in $CD4^+$ T-cells, the signals governing induction and processing may differ between NK cells and $CD4^+$ T-cells. Logically, TCR signaling, and co-stimulation via CD28 may occupy the functions of IL-12 and IL-2 signaling for NK cells in activating $CD4^+$ T-cells; perhaps TCR ligation acts as a catalyst for transcription of *IFNG* while co-stimulation foments processing for rapid translation. We attempted to investigate this possibility in Jurkat cells, a human $CD4^+$ T-cell line, but found that these cells transcribed very little *IFNG* RNA, even when stimulated by strong mitogens like PMA and ionomycin. Primary mouse T-cells isolated from naïve or pathogen challenged mice could serve as a more tenable model for elucidating the role of intron detention in $CD4^+$ produced *IFNG.*

This study opens many doors for future investigation of cytokine regulation with the potential for high impact in clarifying the how the immune system fine-tunes inflammation. We hope these findings will inspire further elucidation of post-transcriptional regulation of immunity.

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VITA

Rachel was born to Suzanne Dintzis and Russell Van Gelder in Des Peres, Missouri. Her younger brother is Maxwell Van Gelder, also a scientist, who uses computation for ecological conservation efforts. Her family relocated to Mercer Island, Washington in 2008 and she graduated from Mercer Island High School in 2014. In 2018 she completed her degree in biology modified with mathematics at Dartmouth College. She was heavily involved in undergraduate research at Geisel School of Medicine in the Hogan lab, completing an Honors Thesis studying the phenotypic and genotypic heterogeneity of *Pseudomonas aeruginosa* isolates collected from the lungs of individuals with cystic fibrosis. Rachel continued directly into the Immunology PhD program at the University of Washington following her undergraduate degree and joined the Ram lab in summer of 2019. She completed her dissertation in 2024. She would like some chickens and dogs in the future and to live on a big farm with all her friends.