Synthetic RIG-I-agonist RNA induces death of hepatocellular carcinoma cells

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Abstract

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Retinoic acid-inducible gene I (RIG-I) is a critical sensor of viral RNA and is activated in response to binding to RNA containing exposed 5'-triphosphate (5′ppp) and poly-uridine to trigger innate immune activation and response including induction of type I and III interferons (IFNs). RIG-I signaling plays a key role in not only restricting RNA virus infection but also suppressing tumor progression via oncolytic signaling. We evaluated the actions of a specific RIG-I agonist RNA (RAR) as a potential therapeutic against model tumor cell lines representing hepatocellular carcinoma (HCC). RAR constitutes a synthetic-modified RNA motif derived from the hepatitis C virus genome that is specifically recognized by RIG-I and induces innate immune activation when delivered to cells. We found that RAR directs RIG-I-dependent signaling to drive HCC cell death. Analysis of knockout cell lines lacking RIG-I, MAVS, or IRF3 confirmed that RAR-induced cell death signaling propagates through the RIG-I-like receptor (RLR) pathway to mediate caspase activation and HCC cell death. RAR-induced cell death is potentiated by type I IFN. Thus, RAR

actions trigger HCC cell death through RIG-I linkage of RLR, caspase, and IFN signaling programs. RAR offers a potent application in antitumor therapeutic strategies leveraging innate immunity against liver cancer.

TABLE OF CONTENTS

LIST OF FIGURES

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DEDICATION

I dedicate this work to my parents Lynette Ulloa and Julio Ulloa Jr., and to my siblings Julia Celina Ulloa and Julio Rey Ulloa.

Mom and Dad, thank you for your unwavering support of all my dreams, big and small. Your love and encouragement gave me the strength to pursue all my scientific and creative endeavors. From academic activities, art lessons, and sports - thank you for always being there for me and giving me the freedom to pursue everything that inspired me. I am so grateful to you both and love you so much.

Julia and Rey, the first two loves of my life, thank you for making me a big sister. The joy that you two bring to my life is immeasurable. I am so grateful that despite our age gaps and years of long distance that we remain close. Watching you two grow up to be the wonderful people that you are, has been an honor and great source of inspiration. I hope that you pursue all your dreams without fear and know that I am always in your corner. I love you always.

Chapter 1: Introduction

Host cells possess multiple mechanisms to respond to virus infection[1–3]. Such mechanisms include the activation of innate immune responses and cell intrinsic responses, such as autophagy and cell death[2–5]. PRR in the cytosol, such as RLRs, detect viral infections and initiate transcription of a cohort of antiviral genes including IFN and ISGs which ultimately block viral replication[1,2]. RIG-I is a cytoplasmic RLR helicase which acts as an immunoreceptor ubiquitously expressed in human tissues, and its presence across cancer cells is also well documented[3,6–9]. A number of studies suggest that RIG-I acts as a tumor suppressor in hepatocellular carcinoma (HCC), acute myeloid leukemia (AML), and cervical cancer[6,10,11]. The two most important outcomes of the RIG-I signaling pathway in cancer are production of type I IFNs and preferential activation of programmed tumor apoptosis[6,7]. Tumor cells develop various strategies to evade host immunosurveillance[8,12]. Specifically, they can modulate intrinsic (e.g. immunosuppressive cytokines) and extrinsic pathways (e.g. T-cell exhaustion) to evade endogenous antitumor immunity and establish an immunosuppressive tumor microenvironment (TME) that fosters cancer progression[7,13–15]. Tumors can be classified as immune desert, excluded, or inflamed, depending on the density and diversity of tumor-infiltrating immune cells, with increased inflammation being correlated to improved immunogenicity[16–18]. Hence, the TME and the associated inflammatory processes play a significant role in cancer progression. A relevant feature of T-cell inflamed tumors is the expression of type I IFNs and ISGs[12]. The importance of IFN signaling contributions to spontaneous activation of antitumor T-cell response highlights the correlation between innate and adaptive responses[13]. Thus, engaging innate immunity by targeting the RIG-I pathway is a promising strategy to promote inflammation and tumor immunogenicity through the induction of

IFN and ISGs as a result of RIG-I antiviral signaling, skewing non-immunogenic tumors into immunogenic tumors[7,9,17].

1.1 RLR Innate Immune Signaling

The innate immune response is our first line of defense against viral infection[3,4,19]. During a viral infection, innate immunity is triggered in an infected cell when pathogen recognition receptors recognize and bind to pathogen-associated molecular patterns (PAMPs)[2]. Retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) are a family of cytoplasmic helicases that are one of the main groups of PRRs that drive innate immunity and inflammation[1,3,20]. The RLR family of proteins includes RIG-I, melanoma differentiation antigen 5 (MDA5), and Laboratory of Genetics and Physiology 2 (LGP2)[1,2]. HCV is a hepatotropic, positive-sense, single-stranded RNA virus that unless interrupted by antiviral therapy, establishes a chronic infection in most people after acute exposure[10,21]. RLR signaling plays a crucial role in sensing HCV through the actions of RIG-I[1,3,20]. RIG-I recognizes the 5′ppp of the viral genome RNA in combination with the 3′ untranslated region (UTR) poly(U)-rich motif[2]. Upon recognition of viral PAMP, RIG-I utilizes its caspase activation and recruitment domains (CARDs) that facilitate signaling through CARD interactions with the adaptor protein mitochondrial antiviral-signaling protein (MAVS)[2]. Downstream of this, IRF-3 induces the expression of many target genes including antiviral and immune modulatory genes[1,2,22]. NF-κB also directs the expression of a variety of inflammatory mediators including interleukin 1β[2]. Together, IRF-3 and NF-κB participate in the induction of type I and III IFN expression. Interferon stimulated gene products have antiviral, inflammatory, and immune-modulatory functions that restrict virus replication and spread[2,23]. This also polarizes the adaptive immune response for effector actions against infection[1,23]. During acute HCV infection, RIG-I

recognizes viral RNA as non-self and then triggers downstream signaling through MAVS leading to innate immune activation and viral clearance[2,20]. RIG-I signaling is summarized in Figure 1.1.

1.2 RLR mediated cell death pathways

Cellular mechanisms by which RIG-I triggers programmed cell death include activation of the intrinsic apoptotic pathway, the extrinsic apoptotic pathway, and pyroptosis[9,24–27]. Intrinsic apoptosis is regulated by the proapoptotic BCL2 family members (e.g., NOXA and PUMA) and results in permeabilization of the mitochondrial outer membrane[28–30]. Subsequent leakage of apoptosis-inducing proteins, such as cytochrome c, out of the mitochondria activates the initiator caspase-9 resulting in a caspase cascade that concludes with cellular apoptosis[27,31]. Extrinsic apoptosis involves engagement of a death receptor by its cognate death ligand, resulting in the activation of initiator caspase-8 or -10 which then activates effector caspases-3, -6, and -7, effectively executing cellular apoptosis[24]. RIG-I signaling can also induce pyroptosis, where RIG-I engagement activates the inflammasome by forming a protein complex containing apoptosis-associated speck like proteins (ASC) and caspase-1[32,33]. Activated caspase-1 promotes gasdermin D translocation to the plasma membrane to form membrane pores, causing cellular swelling and lysis[27]. In addition to apoptosis and pyroptosis, an alternate form of caspase-independent programmed cell death is necroptosis[34–38]. Necroptosis can be initiated by TNF or by TLR3/TLR4 ligands, DNA damaging agents and T-cell receptor ligation[34]. Signaling in cells deficient of the death receptor adapter protein FADD or caspase-8 leads to deubiquitination of the serine/threonine kinase, RIP1, and its recruitment to family member, RIP3, to form a ‘necrosome’ complex[27,35]. Some cells can also be sensitized to necroptosis following treatment of a pan-caspase inhibitor zVAD-fmk (zVAD) which blocks apoptosis and pyroptosis[27,39]. Another known pathway of RIG-I

mediated cell death is the transcription-independent pathway RLR-induced IRF3-mediated pathway of apoptosis (RIPA)[40,41]. RIPA activation of IRF3 is independent of and distinct from the known pathway of transcriptional activation of IRF3[40,41]. It requires linear polyubiquitination of IRF3 which triggers a caspase cascade that ultimately results in apoptosis, without the expression of ISGs[41,42]. RIG-I mediated cell death pathways are summarized in Figure 1.2.

1.3 Current RLR oncolytic agonists

Cancer virotherapy has attracted attention following the observation that some cancer patients undergo remission in response to viral infection[6]. These oncolytic viruses selectively replicated in cancer cells and led to intrinsic cancer cell death[14]. Replication, however, is not required as several replication-incompetent RLR-agonists, such as Sendai virus envelope and fragments (HJV-E) have been found to selectively induce apoptosis of human cancer cell lines but not of transformed cells or primary human fibroblasts[43,44]. RIG-I ligands are thus attractive targets for chemotherapeutic development because RLR agonists can trigger tumor-resident innate immune receptors to initiate apoptotic programs in tumor cells[7]. Virally derived double-stranded (ds) RNA or synthetic analogs have been shown to activate several innate immune receptors, and once stimulated, these factors initiate signaling cascades that result in the production of proinflammatory cytokines and type I interferon, apoptosis and translational inhibition[7,13]. In melanoma, breast, prostate, and HCC cells, dsRNA has been shown to trigger a caspase-dependent apoptotic response[23,45–47]. Clinical trials for breast and gastric cancer that incorporate dsRNA in combination with standard care demonstrated improved overall survival[6,17,45]. All together, the outcome of RIG-I agonists in the context of cancer have been shown to release tumor antigens,

produce type I IFNs, initiate T-cell activation and ultimately result in cancer cell death (summarized in Figure 1.3).

For our studies, we utilize RAR which is generated from the 3' untranslated region (UTR) region of the HCV genome. Previous studies showed that RIG-I recognition of HCV is dependent on the 100 nucleotide (nt) poly-uridine/cytosine-rich motif found in the 3′ UTR partially encoded by RAR[3,20,21]. Though several other RIG-I agonists have been studied for their activation of caspase-mediated tumor cell death[25,45,48], the research discussed here positions RAR as a promising agonist to study for oncolytic therapy driven by RLR-activated caspase dependent cell death.

1.4 Central Hypothesis: RAR induces RIG-I-mediated caspase-dependent apoptotic cell death in hepatocellular carcinoma cells.

We have found that RAR, a 100 nucleotide RIG-I agonist derived from HCV, containing an exposed triphosphate motif, can promote in vitro cell death in hepatocellular carcinoma cell lines. Preliminary data show that addition of the pan-caspase-inhibitor zVAD ablates cell death, suggesting that RAR directs a form of caspase-mediated apoptosis. We aimed to characterize the cell death pathway mediated by RAR, identify the key effector genes that regulate induction of cell death, and also understand the contribution of interferon on RAR induced cell death. The impact of this work will provide a more complete understanding of the RIG-I-mediated cell death pathway that is induced by RAR. Moreover, the outcome of these findings could position RAR for potential use in tumor immunotherapy beyond hepatocellular carcinomas.

1.4.1 Hypothesis #1: RAR induced cell death is regulated by RIG-I signaling members.

Preliminary work reveals that RIG-I is required for RAR induced cell death. It is therefore highly likely that downstream RIG-I signaling players MAVS and IRF3 also play a crucial role in RAR induced cell death. We hypothesize that the complete RIG-I signaling cascade, including MAVS and IRF3 is required for RAR induced cell death, and in the absence of these key members cell death will be abrogated. To investigate this, we utilized knockout cell lines to study RAR induced cell death in the absence of RIG-I signaling.

1.4.2 Hypothesis #2: Interferon regulates or potentiates RAR induced cell death.

RIG-I and RLR signaling direct IFN expression and mediate an IFN-feedback loop in which IRF3-target genes and interferon-stimulated genes (ISGs) together function to enhance and diversify the innate immune response. We hypothesize that in RAR-induced cell death, this IFN feedback loop also plays a role in regulating or enhancing RAR-induced cell death in our hepatocellular carcinoma cells of interest. We therefore assessed the impact of IFNβ treatment on RAR-induced cell death signaling.

1.4.3 Hypothesis #3: RAR induces caspase-dependent apoptotic cell death.

Preliminary data shows that the addition of the pan caspase inhibitor zVAD restricts cell death when RIG-I is intact in our hepatocellular carcinoma cells of interest. This suggests that RAR induced cell death is caspase dependent. We therefore hypothesize that RAR induces caspase dependent cell death such that key hallmarks of apoptosis, Caspase 3 and PARP, are activated and cleaved following RAR transfection. To investigate this, we performed caspase inhibitor experiments and also monitored the expression of key apoptotic hallmarks via WB.

1. 5 Figures

Figure 1.1

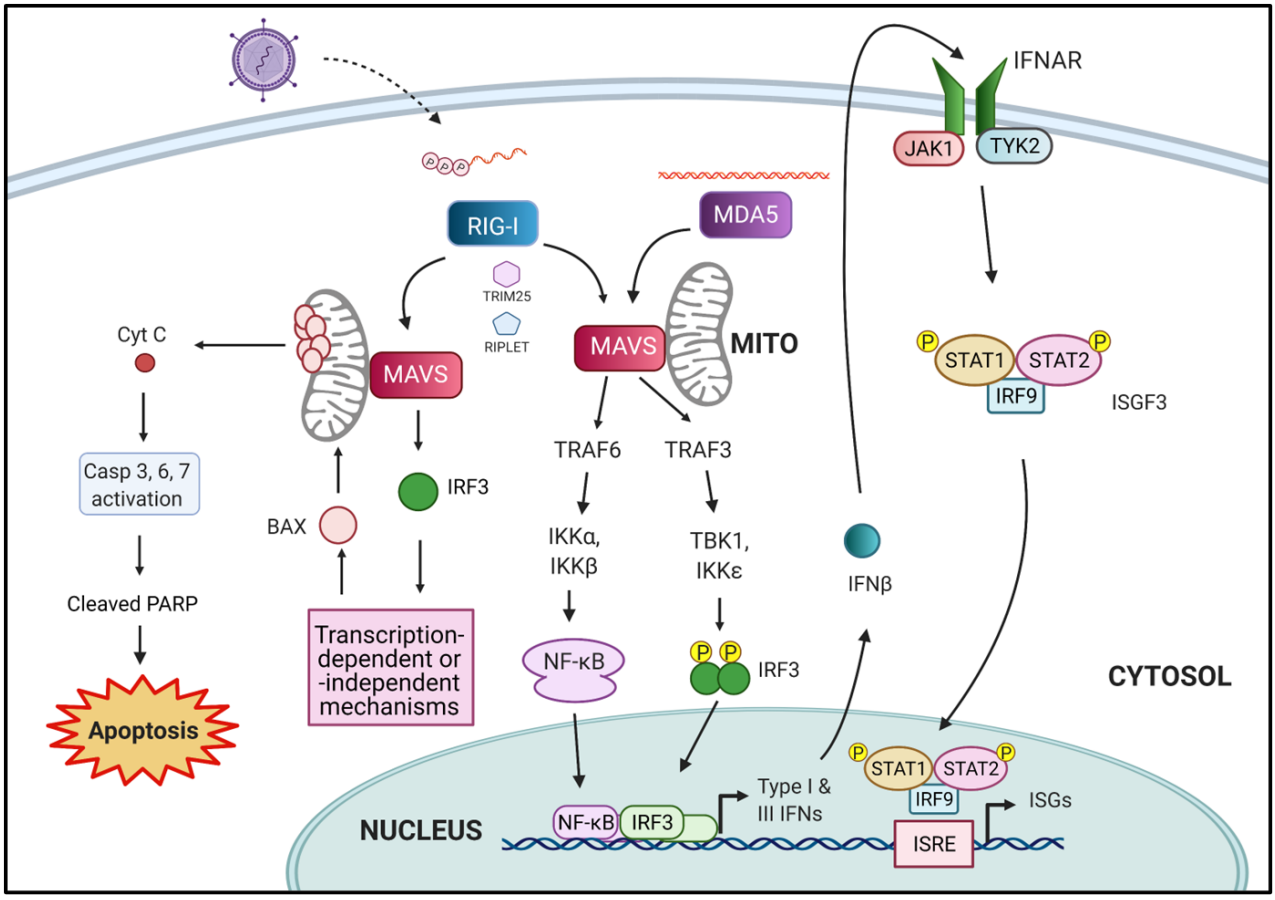

Figure 1.1 RIG-I signaling cascade activation. RIG-I preferentially recognizes and binds single stranded RNA PAMP species containing an exposed 5' triphosphate motif. Once RIG-I recognizes a PAMP species, RIG-I undergoes signaling activation through MAVS to induce IRF3 phosphorylation and activation leading to concomitant innate immune induction. Downstream production of Type I and III interferons results in activation of the ISRE and ISG production, ultimately resulting in viral restriction and/or clearance. RIG-I and certain RLR pathway components are themselves interferon stimulated genes, thus RIG-I signaling is amplified and enhanced by interferon signaling. RIG-I signaling can also result in the activation of caspase-mediated apoptosis marked by the activation of effector caspase-3 and cleavage of PARP.

Figure 1.2

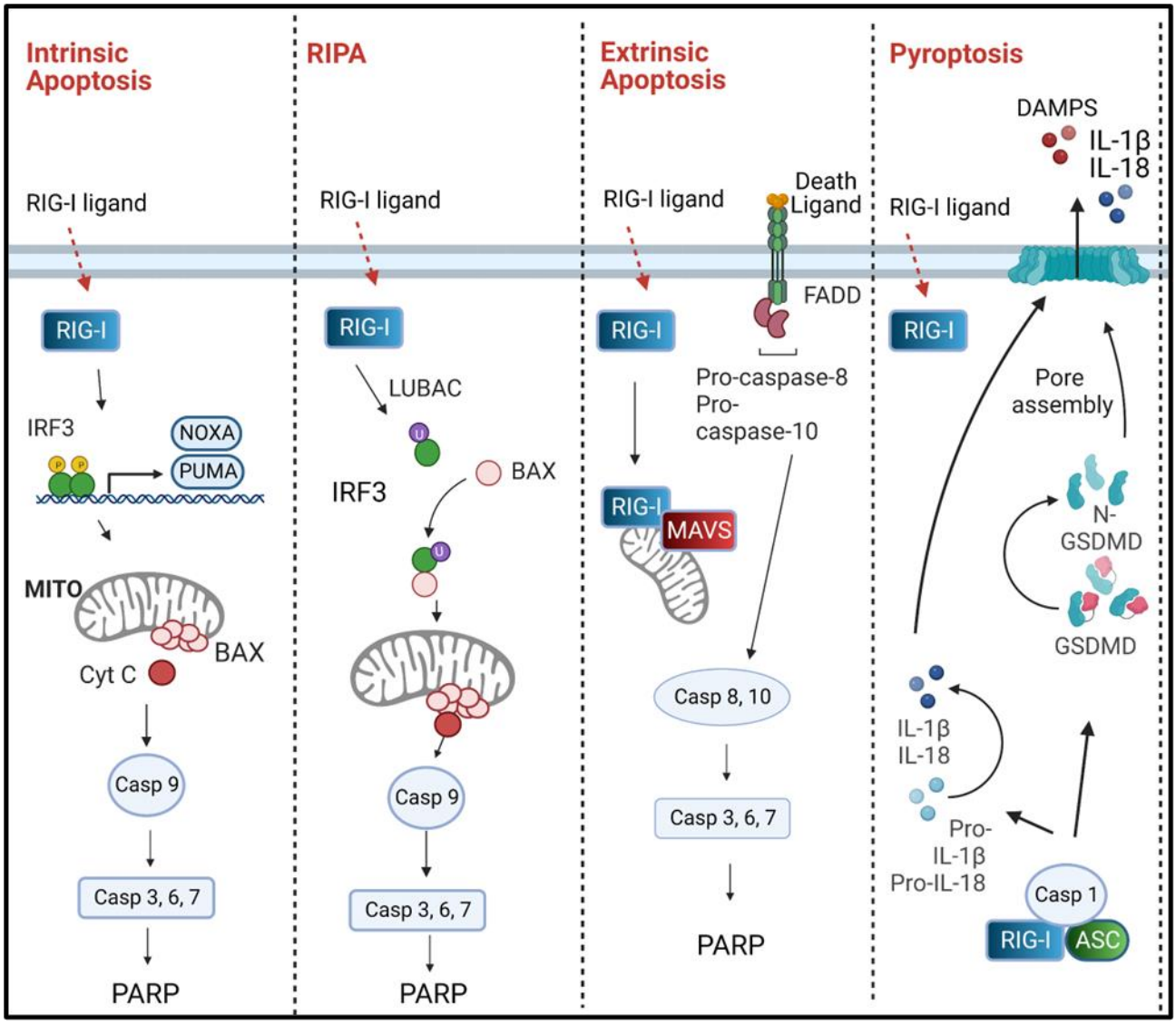
Intrinsic Apoptosis
RIPA
Extrinsic Apoptosis
Pyroptosis
RIG-I ligand
RIG-I
IRF3
NOXA
PUMA
MITO
BAX
Cyt C
Casp 9
Casp 3, 6, 7
PARP
LUBAC
Death Ligand
FADD
Pro-caspase-8
Pro-caspase-10
MAVS
Casp 8, 10
DAMPS
IL-1β
IL-18
Pore assembly
N-GSDMD
GSDMD
Pro-IL-1β
Pro-IL-18
Casp 1
ASC

Figure 1.2 RIG-I mediated cell death pathways. Intrinsic apoptosis is regulated by the proapoptotic BCL2 family members (e.g., NOXA and PUMA) and results in permeabilization of the mitochondrial outer membrane. Subsequent leakage of apoptosis-inducing proteins, such as cytochrome c, out of the mitochondria activates the initiator caspase-9 resulting in a caspase cascade that concludes with cellular apoptosis. The transcription-independent pathway RLR-induced IRF3-mediated pathway of apoptosis (RIPA) is independent of and distinct from the known pathway of canonical transcriptional activation of IRF3. It requires linear polyubiquitination of IRF3 which triggers a caspase cascade that ultimately results in apoptosis, without the expression of ISGs. Extrinsic apoptosis involves engagement of a death receptor by its cognate death ligand, resulting in the activation of initiator caspase-8 or -10 which then activates effector caspases-3, -6, and -7, effectively executing cellular apoptosis. RIG-I signaling can also induce pyroptosis, where RIG-I engagement activates the inflammasome by forming a protein complex containing apoptosis-associated speck like proteins (ASC) and caspase-1. Activated caspase-1 promotes gasdermin D translocation to the plasma membrane to form membrane pores, causing cellular swelling and lysis.

Figure 1.3

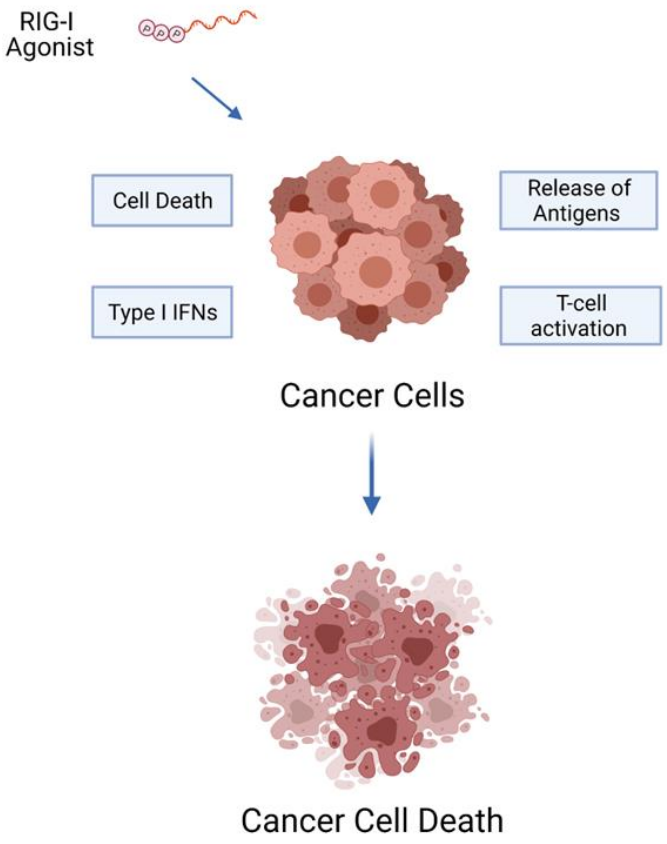
RIG-I
Agonist
Cell Death
Release of
Antigens
Type I IFNs
T-cell
activation
Cancer Cells
Cancer Cell Death

Figure 1.3 RIG-I agonists can induce cancer cell death. RIG-I ligands are attractive targets for therapeutic developments against cancer because RLR agonists have been shown to trigger a variety of outcomes that ultimately culminate in cancer cell death. RIG-I ligands can release tumor antigens, making them visible to immune cells. RIG-I ligands also result in the production of IFNs and polarizes the adaptive immune response for effector actions against cancer. Together these outcomes can culminate in cancer cell death.

Chapter 2: Synthetic RIG-I-agonist RNA induces death of hepatocellular carcinoma cells

This chapter is adapted from the following manuscript:

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2.1 Introduction

In vertebrates the intracellular innate immune response to virus infection is triggered by pathogen recognition receptors (PRRs). Retinoic acid inducible gene I (RIG-I) is the charter member of the RIG-I-like receptor (RLR) family of cytosolic PRRs that recognize RNA virus infection to trigger the innate immune response[1–5]. RLRs signal through the MAVS adaptor protein to direct downstream activation of interferon regulator factor 3 (IRF3), NF-KB, and other transcription factors and their target genes, including types I and III interferons (IFN), to induce an antiviral state that can limit virus replication and spread through direct antiviral actions and cell death signaling [3,5,6,49]. IFN and other cytokine products of RLR signaling also serve to program innate immune cells for activating and polarizing the adaptive immune response[1–3,5]. RIG-I is expressed in most cell types, including cancer cells, at a low level and is induced to higher levels in response to IFN[2,3,7]. While RIG-I plays an essential role to program immunity against most RNA viruses[1–3,5], an increasing number of studies show that it can also direct anti-tumor actions triggered through RLR signaling to suppress different cancer types, including melanoma, acute myeloid leukemia, and cervical cancer[6–9,14,16,17,45,47,48,50–53]. In cancer cells, the two major outcomes of RIG-I signaling are type I IFN production and programmed cell death[7,9,50]. Problematically, tumor cells impose various strategies to evade immunosurveillance[12] and establish an immunosuppressive tumor microenvironment (TME) that fosters cancer progression[7,19–22]. Type I IFN can contribute to spontaneous activation of anti-tumor T-cell responses, highlighting the innate immune actions as a strategy for controlling cancer progression[7,54]. Thus, engaging innate immunity by targeting RIG-I and the RLR pathway to mediate innate immune activation offers a promising approach in immuno-therapeutic strategies against cancer that can overcome the immunosuppressive TME.

Hepatocellular carcinoma (HCC) is among the most common malignancies and causes of cancer-related deaths[55]. Increasingly, the incidence of HCC is linked with obesity and related pathology including fatty liver disease and liver cirrhosis[56]. Moreover, persistent infection by hepatitis C virus (HCV) or hepatitis B virus continues to be a major etiology of HCC worldwide[10,57]. Treatments for HCC historically have focused on systemic therapies that slow cancer progression wherein just a fraction of patients obtain long-term benefit. Targeted therapeutics, against HCC show promise for clinical benefit[58]. Targeting HCC solid tumors and tumor cells for suppression and cell death presents an attractive strategy to limit tumor progression and enhance anti-tumor immunity[58]. For example, RIG-I expression level[59] and activity[60] have been linked with death of HCC cells and M1 immune polarization, implying that strategies to target and activate RIG-I in the HCC tumor cell can offer an immunotherapeutic approach, alone or in combination, to treat HCC.

Several RIG-I or RLR-agonists have been shown to selectively induce apoptosis of human cancer cell lines and model tumors without triggering cell death in immortalized or primary cells[25,43,44,53,61–63]. Fragments of genomic viral RNA can selectively promote apoptosis in cancer cells via the induction of pro-apoptotic genes including tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and NOXA, downstream of the RIG-I/MAVS pathway[6,9,48,52]. Mechanisms by which RIG-I triggers programmed cell death include activation of the intrinsic apoptotic pathway, the extrinsic apoptotic pathway, and pyroptosis[8,24,48,64,65]. Intrinsic apoptosis is regulated by the pro-apoptotic BCL2 family members (e.g., NOXA and PUMA) and results in permeabilization of the mitochondrial outer membrane[28,54,66]. Subsequent leakage of apoptosis-inducing proteins, such as cytochrome c, out of the mitochondria then activates the initiator caspase-9 resulting in a caspase cascade that concludes with cellular apoptosis[9,67]. Extrinsic

apoptosis involves engagement of a death receptor by its cognate death ligand, resulting in the activation of initiator caspase-8 or -10 which then activates effector caspases-3, -6, and -7, effectively executing cellular apoptosis[24,64,65]. RIG-I signaling can also induce pyroptosis, where RIG-I engagement activates the inflammasome by forming a protein complex containing apoptosis-associated speck like proteins (ASC) and caspase-1[27,32,33]. Activated caspase-1 promotes gasdermin D translocation to the plasma membrane to form membrane pores, causing cellular swelling and lysis. In addition to apoptosis and pyroptosis, an alternate form of caspase-independent programmed cell death is necroptosis[34–37,68]. Necroptosis can be initiated by multiple stimuli including but not limited to TNF or by TLR3/TLR4 ligands, DNA damaging agents and T-cell receptor ligation[27,34,35]. Signaling in cells deficient of the death receptor adapter protein FADD or caspase-8 leads to deubiquitination of the serine/threonine kinase, RIP1, and its recruitment to family member, RIP3, to form a 'necrosome' complex[26,34]. Cell death programs can be blocked by treatment of cells with the pan-caspase inhibitor zVAD-FMK (zVAD)[39].

Here we show that synthetic RIG-I agonist RNA (RAR), representing a modified 100 nucleotides (nt) RIG-I PAMP motif of the HCV RNA genome[3,20] containing an exposed triphosphate motif, promotes in-vitro death of Huh7 cells and HepG2 hepatocellular carcinoma (HCC) cells. Addition of the pan-caspase-inhibitor, zVAD, ablates cell death, revealing that RAR directs a form of caspase-mediated cell death in parallel with RIG-I mediated innate immune activation, with resulting IFN production and signaling potentiating RAR-mediated cell death. These findings inform specific applications for further consideration of RAR as a possible component of HCC tumor therapy.

2.2 Results

2.2.1 RAR induces parallel innate immune activation and cell death signaling in HCC cell lines.

We manufactured RAR and control XRNA. The XRNA is a 100 nt RNA encoding the HCV genome 3' non-translated region harboring 3 stem loops but lacking the poly-uridine (p-U) motif. Both RAR and XRNA are single stranded (ss)RNA and contain 5'ppp. XRNA does not activate RIG-I and therefore serves as an HCV genome-derived and length-matched non-agonist ssRNA control to RAR[3,20,21]. Both ssRNAs were produced through a large scale T7 RNA polymerase production system programmed by template DNA and purified as previously described[3,20,21]. To confirm that the manufactured lot of RAR has biological function as a RIG-I agonist in the different HCC lines, we transfected each line with RAR or XRNA control and analyzed via immunoblot assay for innate immune gene/protein expression[1,3]. Huh7 and HepG2 cells were transfected with RAR or XRNA each at a concentration of 250 ng/mL or were mock-transfected. Protein extracts were collected at 8, 16, and 24 hours post-transfection and were subject to immunoblot assay to measure the abundance of RIG-I, MAVS, IRF3, phosphoserine (p)-IRF3, IFIT1, and Actin (internal gel-loading control). As expected, RAR induced innate immune activation and response as marked by the expression of RIG-I, MAVS, P-IRF3, and IFITI over the time course but protein expression was not induced by either XRNA or mock-transfection (Fig. 2.1 A, 2.1B).

A fluorescence microscopy real-time cell death assay measuring cell uptake of Sytox green, a cell-permeable dye that penetrates compromised membranes characteristic of dead cells[69], was utilized to assess cell death signaling potential of RAR. Staurosporine treatment of cells was used as a positive control inducer of cell death for treatment of Huh7 and HepG2 cells[31,70]. Both Huh7 and HepG2 cells were sensitive to increasing concentrations of staurosporine over a 48-hour

monitoring period to establish our cell death assay. Cultures of each cell line were transfected in parallel with RAR or XRNA and monitored for Sytox green uptake. While XRNA control neither induced innate immune activation nor cell death, high dose (400 ng/mL) RAR was found to be a strong inducer of cell death when compared directly to staurosporine treatment (Fig. 2.1C, 2.1D), and each cell line displayed a RAR-induced cell death dose-response (Fig. 2.1E, 2.1F). Thus, RAR is a potent agonist that drives innate immune activation in Huh7 and HepG2 cells and directs parallel cell death signaling in these HCC lines.

2.2.2 Innate immune and cell death signaling by RAR is RIG-I-dependent and propagated through the RLR pathway.

To assess RAR signaling specificity in cell death we utilized a series of CRISPR knockout (KO) cell lines lacking expression of RIG-I, MDA5, MAVS, IRF3, or type I IFN receptor (IFNAR), as well as HepG2 cells lacking RIG-I, or MAVS, while cells expressing a nontargeting guide RNA (NTC) were used as controls (Fig. 2.2A, 2.2B). Compared to parental (wild type) or NTC Huh7 cells, HCC KO cells lacking RIG-I, MAVS, or IRF3 fail to induce p-IRF3 or increased expression of RIG-I or IFIT1 in response to RAR treatment. Huh7-IFNAR KO cells displayed reduced p-IRF3 accumulation following RAR treatment (Fig. 2.2A). Real-time reverse transcription quantitative PCR (RT-qPCR) assay demonstrated that RAR, but not XRNA, treatment of Huh7-NTC cells induces mRNA expression of a range of RIG-I responsive genes including IFITM1, IFIT1, MX1, IFNβ, IL-6, and OAS1 (Fig. 2.2C). In contrast, Huh7 KO cells lacking RIG-I, MAVS, or IRF3 were recalcitrant to RAR-mediated gene upregulation (Fig. 2.2C). Importantly, RAR treatment (400 ng/mL) of Huh7 parental/wild type cells robustly induced cell death but cells lacking RLR pathway components RIG-I, MAVS, or IRF3 were refractory to RAR-

induced cell death and remained viable through the 50-hour observation period (Fig. 2.2D). Similarly, RAR treatment resulted in cell death of HepG2-NTC cells but minimal death in HepG2 KO cells lacking RIG-I or MAVS (Fig. 2.2E). Thus, RIG-I mediated RLR signaling is essential for RAR-induced death of Huh7 and HepG2 HCC cells, and cell death signaling occurs in parallel with RIG-I-dependent RLR signaling following RAR treatment.

2.2.3 Interferon potentiates RAR-induced cell death signaling.

RIG-I and RLR signaling direct IFN expression and mediate an IFN-feedback loop in which IRF3-target genes and interferon-stimulated genes (ISGs) together function to enhance and diversify the innate immune response[19,71]. We therefore assessed the impact of IFNβ treatment on RAR-induced cell death signaling. Huh7 cells induce ISG expression (MX1) in response to IFNβ treatment (Fig. 2.3A). When treated with IFNβ alone, parental/wild type Huh7 (Fig. 2.3B) or HepG2 (Fig. 2.3C) cells do not undergo cell death even at high treatment concentration but parallel treatment with RAR results in cell death of each within the observation period. We note that RAR treatment induces a level of p-IRF3 and IRF3-target gene expression independent of IFN signaling[4,72,73] as observed in Huh7-IFNAR KO cells (see Fig. 2.2A). We therefore interrogated if IFN signaling amplifies RAR-induced cell death. We conducted a cell death assay comparing Huh7 parental/wild type cells to Huh7-IFNAR1 KO cells that were mock-treated or treated with RAR. Compared to Huh7 parental/wild type cells, IFNAR KO cells display an approximate 50% reduction in cell death over the observation period, suggesting that IFN signaling potentiates RAR-induced cell death (Fig. 2.3D). To determine if IFNβ treatment can indeed potentiate RAR-induced cell death, Huh7 parental/wild type cells were pre-treated with 100 units (U) of IFNβ or culture media alone for 24 hours followed by RAR treatment, and cell death was monitored over a

subsequent 36-hour observation period. IFNβ pre-treatment followed by RAR resulted in response with enhanced kinetics and frequency of cell death compared to RAR treatment alone (Fig. 2.3E). We also confirmed that IFNβ alone was not sufficient to induce cell death, showing that cell death signaling is RAR-dependent and is potentiated by IFN signaling.

2.2.4 RAR induces caspase dependent death in hepatocellular carcinoma cells.

RIG-I signaling and IRF3 are linked to caspase activation and apoptosis induced by virus infection and other stimuli[74]. To determine if RAR signaling of cell death links with signaling of caspase activation we conducted immunoblot assay to evaluate the abundance of caspase-3 and substrate poly (ADP-ribose) polymerase (PARP), which are major markers of caspase-mediated cell death signaling[75]. Parental/wild type Huh7 (Fig. 2.4C) and HepG2 (Fig. 2.4D) cells that were treated with RAR in the presence or absence of the pan-caspase inhibitor zVAD-FMK (zVAD)[76] displayed RAR-induced p-IRF3 and IFIT1 expression. In the absence of zVAD treatment cleaved caspase-3 and cleaved PARP accumulated in RAR-treated cells concomitant with p-IRF3 and IFIT1, but zVAD treatment prevented the accumulation of cleaved caspase-3 and PARP (Fig. 2.4C, 2.4D). We also found that zVAD treatment of cells suppressed RAR-induced cell death (see Fig. 2.4A, 2.4B). These observations indicate that RAR directs caspase-dependent cell death signaling in HCC cells. Together, our observations demonstrate that RAR can direct apoptotic cell death in HCC cell lines concomitant with innate immune activation, and that IFN signaling serves to potentiate cell death signaling.

2.3 Discussion

Our study shows that RAR induces caspase-dependent cell death in hepatic tumor HCC cell lines, that RIG-I and RLR signaling are required for caspase activation of this cell death program, and that IFN potentiates RAR-mediated cell death signaling. Based on previously published work[1,3,19–21,41,67,71–73] and the current study, we propose a model for RAR-induced cell death (Fig. 2.5) as follows: (i) RAR triggers RIG-I activation and the downstream RLR signaling pathway following its binding by RIG-I[20]. RIG-I then signals downstream through MAVS and IRF3 to induce gene expression including IRF3 target genes. (ii) IFN production from RLR signaling facilitates an IFN-signaling feedback-loop that drives ISG expression including RIG-I. Because RIG-I is an interferon stimulated gene, cell death signaling is potentiated by IFN resulting in increased kinetics and frequency of RAR-induced cell death. Importantly, interferon treatment alone will not induce cell death without RAR treatment to mediate activation of RIG-I and RLR signaling. (iii) Caspase-mediated cell death activation: RAR-induced RIG-I signaling culminates in the activation of caspase-mediated apoptosis characterized by the activation of effector caspases-3 and PARP cleavage in a zVAD-sensitive manner.

RIG-I is a cytoplasmic PRR ubiquitously expressed in human tissues, and its role extends beyond that of a pattern recognition receptor[3,6,19]. In cancer cells, the two major outcomes of RIG-I signaling are the production of interferons and the activation of programmed tumor apoptosis[6,8,9,23,25,65]. Host defense responses against virus-infected cells and against tumors share intrinsic molecular features, it is therefore theorized that RIG-I activation is selective in cancer cells as a way to mimic a viral infection and either induce immunogenic cell death or direct cancer cell apoptosis[6,9,16,45,65]. Interferons are pleiotropic immune-modulatory cytokines that are well known for their essential role in host defense responses against viruses, bacteria, and other

pathogenic microorganisms[13,19,23,71,77–79]. Interferon signaling results in the induction of IFN-stimulated genes influencing different cellular pathways including direct anti-viral responses, immune-modulation or cell death[2,20,78]. Interferons can induce or modulate gene expression of essential signaling players of cell death pathways including apoptotic, pyroptotic and necroptotic cell death. In our study, we show that RAR, an RLR agonist, induces caspase-dependent cell death and that this cell death program can be modulated by interferon. While interferon alone does not induce cell death in our HCC model, interferon enhances the RAR-induced RLR-mediated cell death program. IRF3 was found to be another key regulator of RAR-induced RLR-mediated cell death. IRF3 also plays a key role in several other forms of cell death, including transcription-dependent and transcription-independent cell death pathways[40,41,67]. For example, the RIG-I induced pathway of apoptosis, known as RIPA, utilizes transcription-incompetent IRF3 mutants in a manner to direct transcription-independent apoptotic cell death[40,41]. Ongoing studies will define the signaling processes by which RAR promotes caspase-dependent cell death.

RLR agonists can trigger tumor-resident innate immune receptors to initiate apoptotic programs in tumor cells, making RAR and other RIG-I ligands attractive targets for chemotherapeutic development[80]. Double stranded (ds)RNA species have been studied for their ability to induced various forms of cell death in different cancer cell models. Moreover, virally derived double-stranded RNA or synthetic analogs, have been shown to activate several innate immune receptors, including RIG-I, TLR3, MDA5, and PKR[80]. Once stimulated, these factors initiate signaling pathways that lead to the production of proinflammatory cytokines and type I interferon, apoptosis and translational inhibition[80]. Clinical trials for breast and gastric cancer that included dsRNA in combination with standard care showed improved overall survival and progression-free survival[45,78,81]. In melanoma, breast, prostate, and HCC cells, dsRNA has been

shown to trigger a caspase-dependent apoptotic response[46,52,53,59,81–83]. RAR is generated from the 3' UTR region of the HCV genome. HCV is a hepatotropic, positive-sense, single-stranded RNA virus that unless interrupted by antiviral therapy, establishes a chronic infection in most people after acute exposure[1]. Previous studies showed that RIG-I recognition of HCV is dependent on the 100 nt poly-uridine/cytosine-rich motif found in the 3′ untranslated region (3′ UTR) partially encoded by RAR[3,20]. Though several other RIG-I agonists have been studied for their activation of caspase-mediated tumor cell death, our current study represents the first examination of RAR in this context. Our observations show that RAR drives caspase-dependent cell death, and further reveal that it induces apoptosis via PARP cleavage. These findings pose RAR as a promising agonist to study for oncolytic therapy driven by RLR-activated caspase dependent cell death.

In summary, our study demonstrates that RAR induces RIG-I and RLR signaling-dependent innate immune activation, caspase activation, and cell death signaling in HCC cells, and that RAR induction of cell death is potentiated by interferon. RIG-I and RLR signaling are increasingly recognized as important components of tumor control[7,13,25,53,62,83]. We conclude that RAR treatment could contribute to strategies for the control of liver cancer and other cancer types.

2.4 Materials and Methods

Cells and culture methods

Huh7 and HepG2 cells were obtained from the American Type Culture Collection (ATCC). All cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 1x nonessential amino acids and antibiotic-antimycotic solution (Fisher). Huh7 IRF3-KO[22], Huh7 IFNAR1-KO[84], and Huh7 MAVS-KO[85] cells were generously provided by RS and the Horner Lab.

RAR and XRNA

RAR is in preclinical development for applications as a therapeutic agent. RAR and XRNA have been extensively described and the sequence of each is reported[3,20]. RAR and XRNA were generously supplied by HDT Bio Corp. Both were generated from template DNA using T7 polymerase. All RNA synthesis reagents were kept on ice but assembled at room temperature. DNA template was produced from oligonucleotides as described[3,20]. Oligonucleotides were annealed using a heat block incubation in normal saline starting at 95 °C for 5 min, dropping 1 °C every 30 seconds until reaching 4 °C. RNA was purified by phenol-chloroform extraction, ethanol precipitation, and size-exclusion chromatography. RNA products were verified by gel electrophoresis and imaging.

RAR transfection

RAR and XRNA (negative control) were transfected using the Mirus TransIT-mRNA Transfection Kit. TransIT-mRNA and mRNA-Boost reagents were warmed to room temperature and gently vortexed. 100 uL of Opti-MEM Reduced-Serum Medium was placed in sterile tubes. 1 ug (1uL of a 1ug/ul stock) RNA was added and gently pipetted to mix. 1 uL mRNA Boost Reagent and 1 uL

of TransIT-mRNA were also added and pipetted gently to mix. Samples were gently flicked 15 times and subjected to a quick 2 second spin. Samples were then incubated at room temp for 2-5 minutes to allow for complexes to form. MOCK samples were prepared with transfection reagents only (no RAR or XRNA). The complexes were then distributed to the cells in complete growth medium. The vessel was rocked back and forth gently to evenly distribute the transfection complexes. Samples were incubated for 4-48 hours depending on the nature of the goal of each experiment. Cells were harvested and assayed as required.

Staurosporine treatment

Staurosporine (Millipore) was used as a cell death control. Staurosporine was prepared at concentrations of 0.5, 1, 5, 10 μM in supplemented DMEM. Cells were seeded the prior day and staurosporine was administered by replacing the media with prepared staurosporine media. Cell death was then monitored via incucyte.

Immunoblot

Cells were lysed in RIPA buffer (Sigma) with freshly added protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail (Millipore) and Okadaic acid (Thermo). Lysates were separated on 8% Bis-Acrylamide SDS gels and transferred onto PVDF membranes (Millipore), then blocked/probed overnight in 4 °C incubation with primary antibody diluted in 4% BSA blocking buffer. After washing, membranes were incubated for 1 hour at RT with HRP-conjugated secondary antibodies diluted in TBS-tween. Membranes were incubated in ECL (Fisher Scientific) and detected using a ChemiDoc XRS+ System (Bio-Rad).

Cell death assays

Hepatocytes were seeded at concentration of 1 x10^5 cells per well in 24 well dishes (Corning). 16 hours later, cells were transfected with MOCK, XRNA, or RAR 400 ng/mL. 4 hours post transfection, cells were then stained with the cell permeable dye Sytox green 1:1000. PAMP induced cell death was assessed in real time via quantification of Sytox dye uptake through live cell imaging using an incucyte instrument over a 24–50-hour period. Percent cell death was calculated as a ratio of dead cells (Sytox) over live cells (Syto) and monitored over time.

qRT-PCR

Total RNA was isolated from cell lysates using the RNeasy Kit (QIAGEN) and digested with DNase I (QIAGEN) on column. 200 ng total RNA was subjected to cDNA synthesis using the iScript cDNA Synthesis Kit (BioRad). cDNA was diluted 1:4 in H_2O and qPCR was performed using SYBR Select Master Mix (Thermo) and gene specific primers on the ABI 7500 Real-Time PCR System.

Interferon treatment

Human IFNβ (Toray) was prepared in complete supplemented DMEM at concentrations of either 10 U, 100 U, or 1000 U per mL. This was then used to replace the media on Huh7 or HepG2 cells that had been seeded the day before (about 16 hours before). 4 hours post treatment, cells were stained with Syto and Sytox and cell death was monitored over time via incucyte. For Interferon pre-treatments, Interferon was administered as stated above, followed by RAR transfection 24 hours later.

zVAD-FMK treatment

To interrogate cell death signaling, zVAD-FMK (Sigma) was used as a pan-caspase inhibitor. zVAD-FMK was prepared at a concentration of 100 μM in supplemented DMEM. Cells that had been seeded previously the day before, received fresh caspase inhibitor media as treatment. PAMP transfection was performed 2 hours post zVAD treatment. Cells were harvested or assayed as appropriate either by immunoblot or incucyte.

2.5 Figures

Figure 2.1

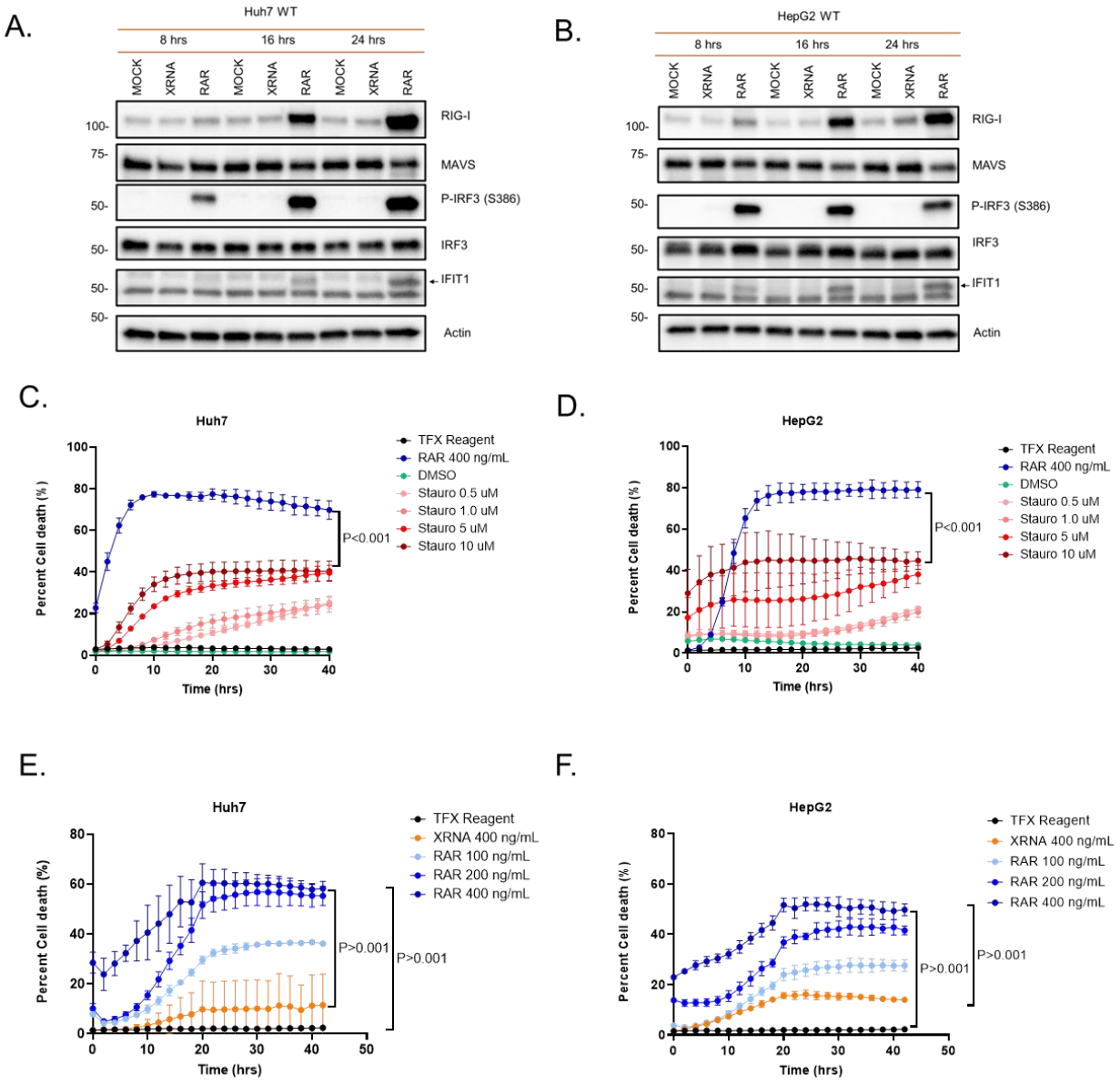


Figure 2.1. **RAR induces cell death in hepatocellular carcinoma cells**. Immunoblot analyses showing that RAR is a RIG-I agonist that induces innate immune gene expression in Huh7 (A) and HepG2 (B) HCC cells. RAR was transfected at a dose of 250 ng/mL and protein lysates were harvested at 8, 16, and 24 hours post-RAR treatment. Staurosporine treatment (0.5 μM, 1 μM, 5 μM, 10 μM) was included as a cell death-inducer control for Huh7 (C) and HepG2 (D) HCC cells. RAR dose response cell death analysis of Huh7 (E) and HepG2 (F) HCC cells compared to XRNA treatment and transfection reagent control treatment. 2way ANOVA and Sidaks multiple comparison tests were performed to calculate the p values shown. Data are representative of three independent experiments.

Figure 2.2

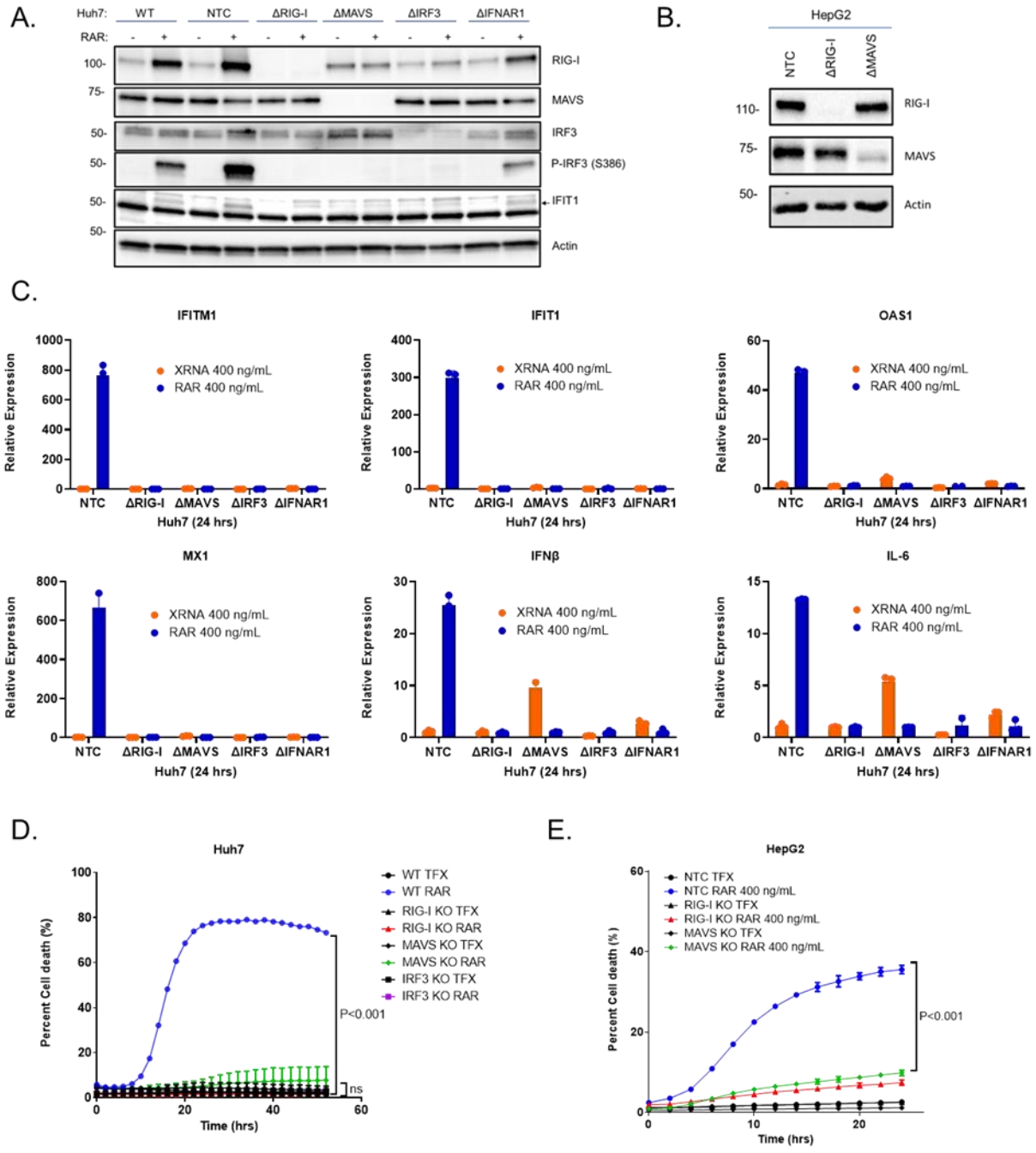

A.
Huh7:
WT
NTC
ΔRIG-I
ΔMAVS
ΔIRF3
ΔIFNAR1
RAR:
RIG-I
MAVS
IRF3
P-IRF3 (S386)
IFIT1
Actin
B.
HepG2
NTC
ΔRIG-I
ΔMAVS
RIG-I
MAVS
Actin
C.
IFITM1
IFIT1
OAS1
MX1
IFNβ
IL-6
Relative Expression
XRNA 400 ng/mL
RAR 400 ng/mL
NTC
ΔRIG-I
ΔMAVS
ΔIRF3
ΔIFNAR1
Huh7 (24 hrs)
D.
Huh7
Percent Cell death (%)
Time (hrs)
WT TFX
WT RAR
RIG-I KO TFX
RIG-I KO RAR
MAVS KO TFX
MAVS KO RAR
IRF3 KO TFX
IRF3 KO RAR
P<0.001
ns
E.
HepG2
NTC TFX
NTC RAR 400 ng/mL
RIG-I KO TFX
RIG-I KO RAR 400 ng/mL
MAVS KO TFX
MAVS KO RAR 400 ng/mL
P<0.001

Figure 2.2. **RAR-induced cell death is dependent on RIG-I and RLR pathway signaling**. Huh7 (A) or HepG2 (B) HCC cells were treated with 250 ng/mL of RAR. Cells were harvested 24 hours (hrs) later and analyzed by immunoblot for specific protein expression. (C) Huh7 HCC NTC and gene-specific KO cells were treated with RAR or XRNA (400 ng/mL) and subjected to RT-qPCR analysis for specific gene expression 24 hrs later. (D) Huh7 control or specific gene KO cells were treated with 400 ng/mL RAR or transfection reagent only (internal control) and monitored for cell death. (E) HepG2 HCC NTC cells (control) or gene KO cells were treated with 400 ng/mL RAR or transfection reagent only (internal control) and monitored for cell death. 2way ANOVA and Sidaks multiple comparison tests performed to calculate p-values as shown. Data are representative of three independent experiments.

Figure 2.3

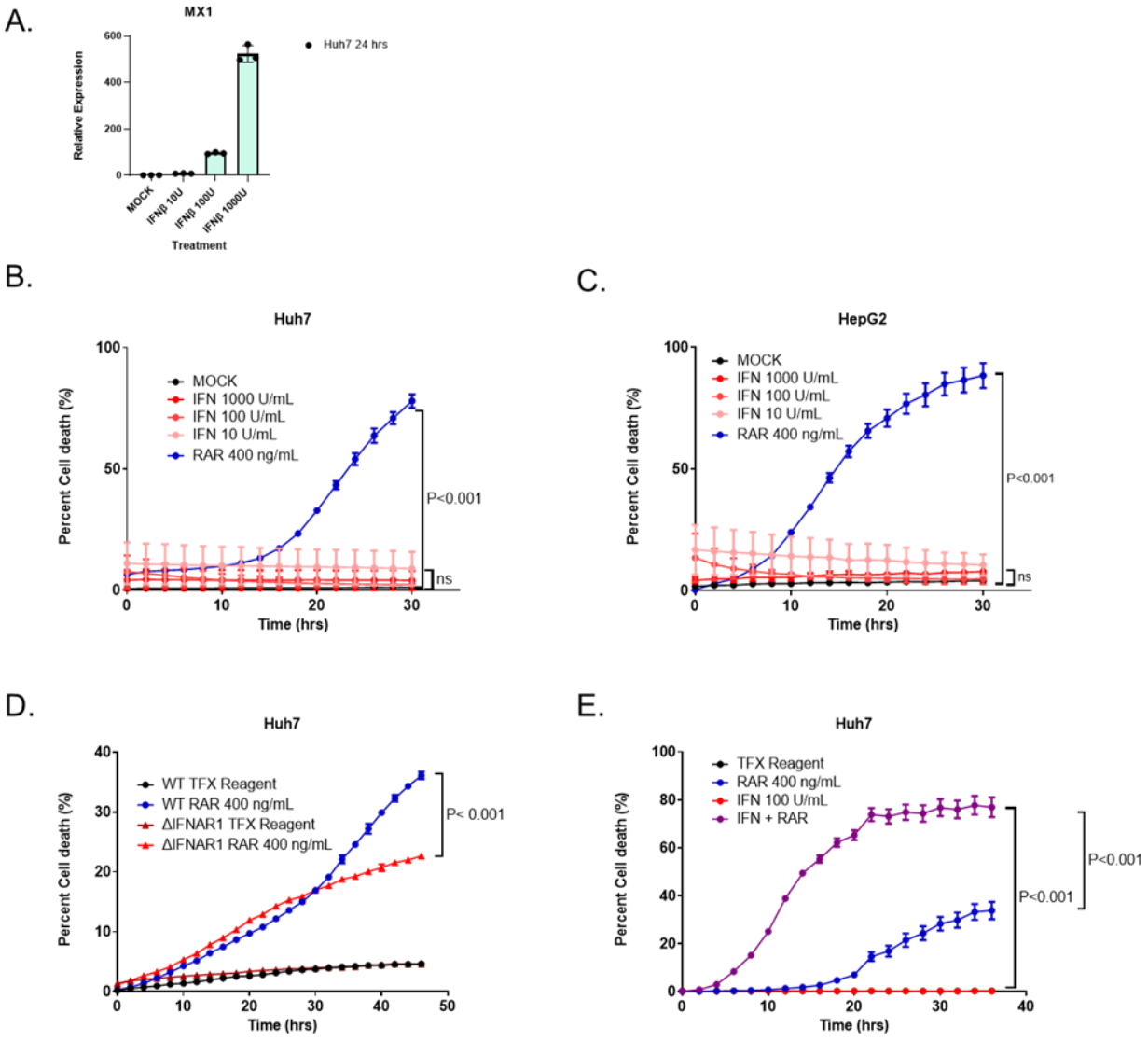

A.
MX1
Relative Expression
600
400
200
0
Huh7 24 hrs
MOCK
IFNβ 10U
IFNβ 100U
IFNβ 1000U
Treatment
B.
Huh7
Percent Cell death (%)
100
50
0
MOCK
IFN 1000 U/mL
IFN 100 U/mL
IFN 10 U/mL
RAR 400 ng/mL
P<0.001
ns
0
10
20
30
Time (hrs)
C.
HepG2
Percent Cell death (%)
100
50
0
MOCK
IFN 1000 U/mL
IFN 100 U/mL
IFN 10 U/mL
RAR 400 ng/mL
P<0.001
ns
0
10
20
30
Time (hrs)
D.
Huh7
Percent Cell death (%)
40
30
20
10
0
WT TFX Reagent
WT RAR 400 ng/mL
ΔIFNAR1 TFX Reagent
ΔIFNAR1 RAR 400 ng/mL
P< 0.001
0
10
20
30
40
50
Time (hrs)
E.
Huh7
Percent Cell death (%)
100
80
60
40
20
0
TFX Reagent
RAR 400 ng/mL
IFN 100 U/mL
IFN + RAR
P<0.001
P<0.001
0
10
20
30
40
Time (hrs)

Figure 2.3. **Type I interferon potentiates RAR induced cell death in HCC cells**. (A) RT-qPCR analysis of Mx1 mRNA expression following a 24 hr dose-response treatment of Huh7 cells with interferon-β (10, 100, 1000 units U/mL). (B) Huh7 and (C) HepG2 HCC cells, were respectively mock-treated, treated with increasing doses of interferon-β, or were treated with 400 ng/mL RAR and monitored for cell death. (D) Huh7 (control) or Huh7-IFNAR KO cells were treated with transfection reagent only (internal control) or 400 ng/mL RAR and monitored for cell death. (E) Huh7 HCC parental cells were pre-treated with 100 U/mL interferon-β or media alone for 24 hrs. followed by treatment with transfection reagent alone (internal control) or with 400 ng/mL RAR, and cultures were monitored for cell death. 2way ANOVA and Sidaks multiple comparison tests were performed to calculate the p-values shown. Data are representative of three independent experiments.

Figure 2.4

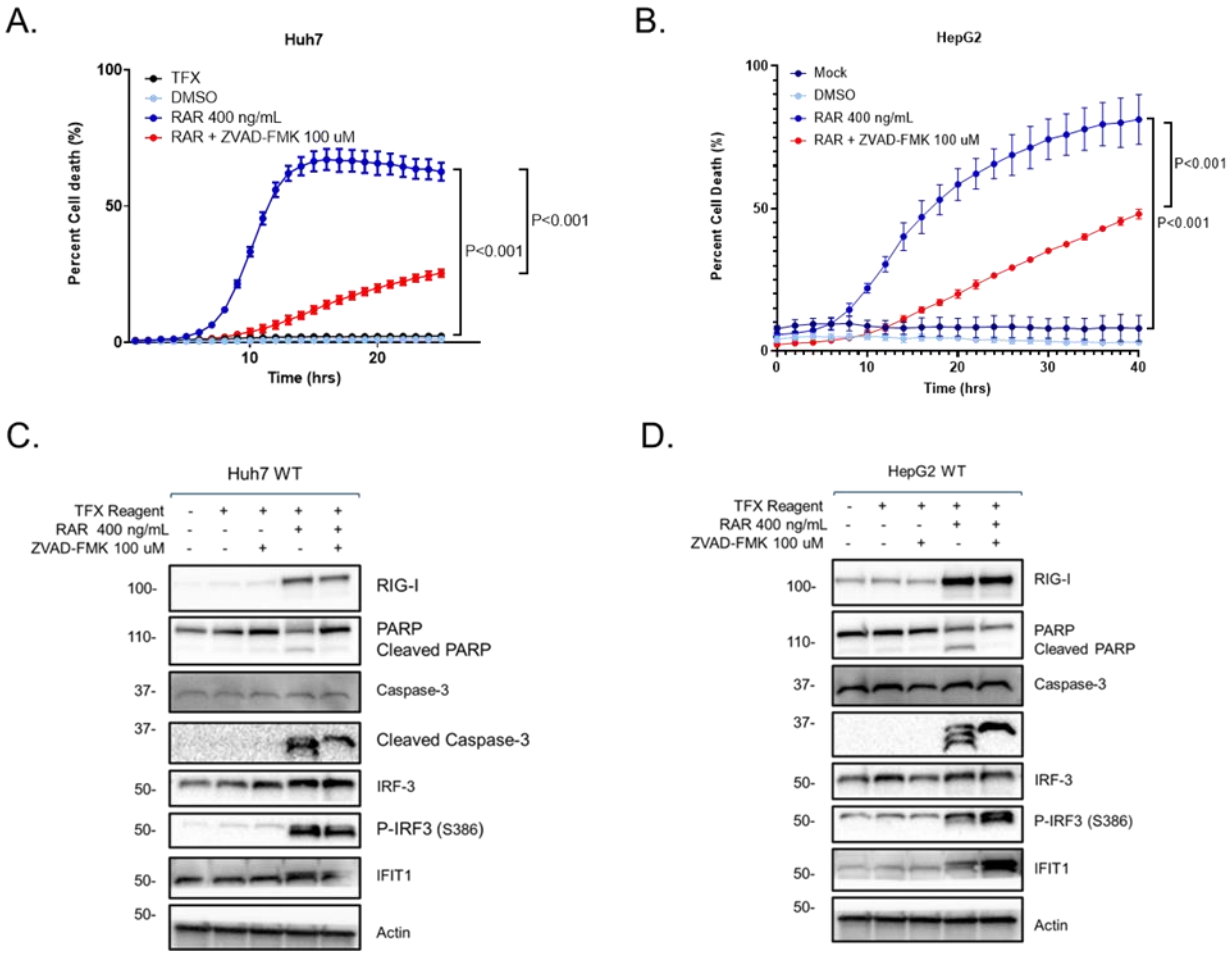
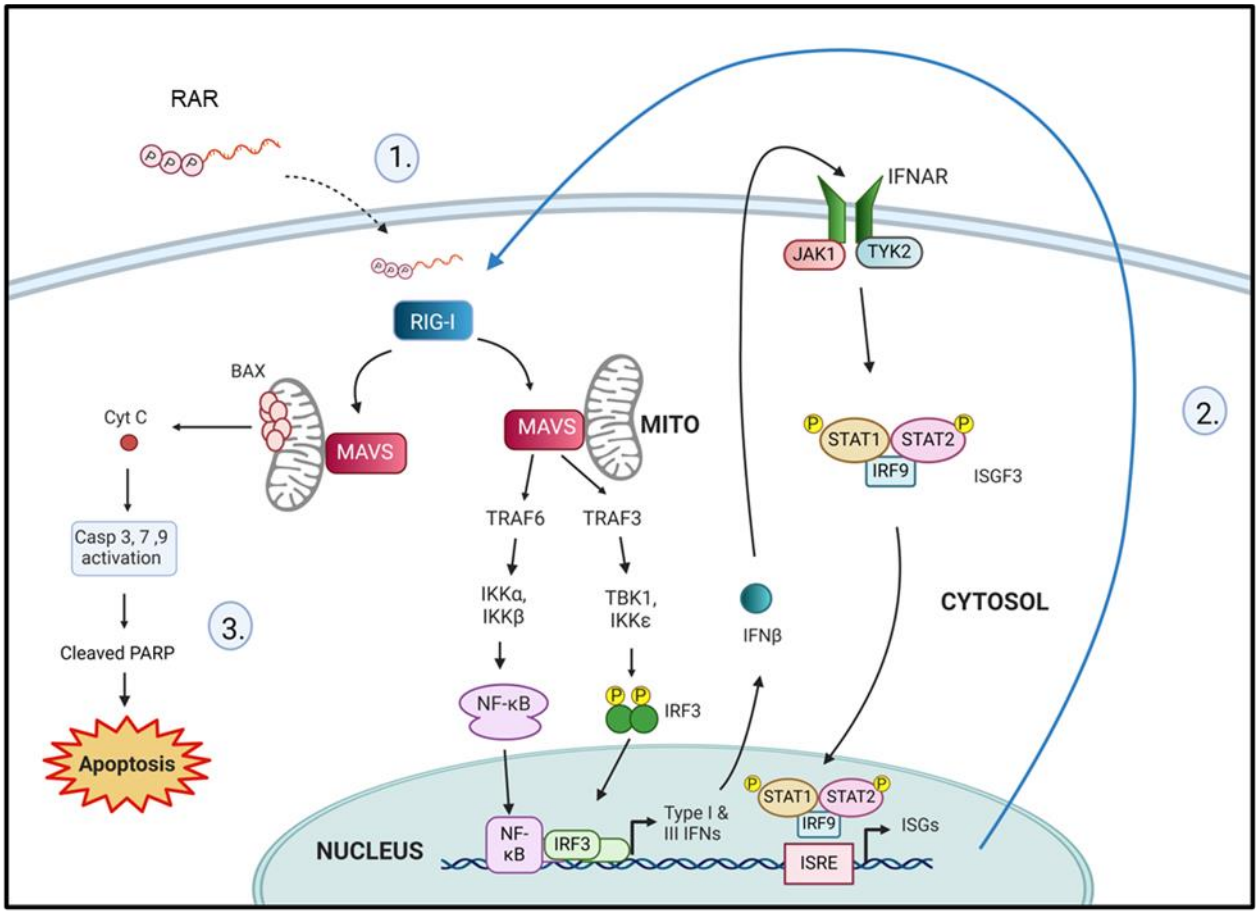

A.
Huh7
TFX
DMSO
RAR 400 ng/mL
RAR + ZVAD-FMK 100 uM
Percent Cell death (%)
Time (hrs)
P<0.001
P<0.001
B.
HepG2
Mock
DMSO
RAR 400 ng/mL
RAR + ZVAD-FMK 100 uM
Percent Cell Death (%)
Time (hrs)
P<0.001
P<0.001
C.
Huh7 WT
TFX Reagent
RAR 400 ng/mL
ZVAD-FMK 100 uM
RIG-I
PARP
Cleaved PARP
Caspase-3
Cleaved Caspase-3
IRF-3
P-IRF3 (S386)
IFIT1
Actin
D.
HepG2 WT
TFX Reagent
RAR 400 ng/mL
ZVAD-FMK 100 uM
RIG-I
PARP
Cleaved PARP
Caspase-3
IRF-3
P-IRF3 (S386)
IFIT1
Actin

Figure 2.4. RAR induces caspase-dependent death of HCC cells. Huh7 (A) or HepG2 (B) HCC cells were treated with transfection reagent or DMSO (internal controls), 400 ng/mL RAR, or a combination of 400 ng/mL RAR with 100 μM ZVAD-FMK and analyzed for cell death. Western blots of Huh7 cells (C) and HepG2 cells (D) treated with transfection reagent with or without 400 ng/mL RAR or 100 μM ZVAD-FMK for 12 hrs. Cells were harvested and subjected to immunoblot analysis of the indicated proteins. (A) and (B): Data sets were evaluated by 2way ANOVA and Sidaks multiple comparison tests to generate the p-values shown. Data are representative of three independent experiments.

Figure 2.5



Created with BioRender.com

Figure 2.5: Model of RAR induced RIG-I-mediated caspase-dependent death of HCC cells. (i) RIG-I signaling cascade activation: following RAR binding by RIG-I, RIG-I undergoes signaling activation through MAVS to induce IRF3 phosphorylation/activation leading to concomitant innate immune induction and RAR-induced cell death. (ii) Interferon feedback-loop activation: because RIG-I and certain RLR pathway components are themselves interferon stimulated genes, RIG-I/RLR-mediated cell death signaling is amplified and enhanced by RAR/RIG-I/RLR signaling-induced interferon production or interferon-β treatment. Note that interferon-β treatment alone will not induce cell death without RAR and an active RLR signaling cascade. (iii) Caspase-mediated cell death activation: RAR-induced RIG-I signaling culminates in the activation of caspase-mediated apoptosis marked by the activation of effector caspase-3 and cleavage of PARP.

3. Summary and Concluding Remarks

3.1 Discussion

The results of our study show that RAR induces caspase-dependent cell death in hepatocellular carcinoma cell lines, that RIG-I and RLR signaling are required for caspase activation of this cell death program, and that IFN potentiates RAR-mediated cell death signaling. RAR triggers RIG-I activation and the downstream RLR signaling pathway following its binding by RIG-I. RIG-I then signals downstream through MAVS and IRF3 to induce gene expression including IRF3 target genes. IFN production from RLR signaling facilitates an IFN-signaling feedback-loop that drives ISG expression including RIG-I. Because RIG-I is an interferon stimulated gene, cell death signaling is potentiated by IFN resulting in increased kinetics and frequency of RAR-induced cell death. Importantly, interferon treatment alone will not induce cell death without RAR treatment to mediate activation of RIG-I and RLR signaling. RAR-induced RIG-I signaling then culminates in the activation of caspase-mediated apoptosis characterized by the activation of effector caspases-3 and PARP cleavage in a zVAD-sensitive manner.

Though several other RIG-I agonists have been studied for their activation of caspase-mediated tumor cell death[5,8,43,48,50,52,53], our current study represents the first examination of RAR in this context. Our observations show that RAR drives caspase-dependent cell death, and further reveal that it induces apoptosis via PARP cleavage. These findings pose RAR as a promising agonist to study for oncolytic therapy driven by RLR-activated caspase dependent cell death.

Our study serves as a good starting point for studying the potential of RAR in cancer cell death applications, however the results of our study lead us to many more questions. There are several things that would enhance and further inform the capabilities of RAR to serve as a

promising oncolytic agent. The first important study would be to compare RAR induced cell death in a non-cancerous cell model. This would reveal if RAR induced cell death is cancer cell selective. If RAR is found to be cancer cell selective, that would be monumental in positioning it as a promising oncolytic agent. In addition, our studies primarily focused on studying RAR by interrogating caspase dependent apoptosis. It would be important to study RAR by interrogating other cell death pathways, and perhaps more interestingly, interrogating RAR in a translation-independent cell death pathway such as RIPA[40,41]. Furthermore, to better inform what could prime cancer cells to be more preferable targets of RAR induced cell death, it would be necessary to identify differentially expressed genes in cancerous vs non-cancerous cells following RAR treatment. To address these items, key future studies are outlined below. Together, the results of these key future experiments could enhance the findings of our study and better inform us of RAR's potential for oncolytic therapy.

3.2 Key Future Studies

Impact of RAR in non-cancerous cells

Our preliminary studies focus on hepatocellular carcinoma cell lines, however, it would be valuable to determine if RAR induces cell death in a cancer selective manner by performing parallel studies in non-cancerous hepatocytes. To investigate whether PAMP induced RIG-I-mediated cell death is specific to cancer cells, RAR can be transfected into a panel of non-malignant cells and a panel of malignant cells. Incucyte analysis will be used to measure real-time cell death rates following RAR transfection via quantification of Sytox dye by Incucyte. RAR will be transfected into a panel of non-malignant immortalized or primary cell samples from liver, lung,

breast, and skin tissues. RAR will also be transfected into a panel of corresponding cancer cell lines, including liver cancer (Huh7 and HepG2), lung cancer (A549), breast cancer (MCF7), and skin cancer (A-375). Incucyte analysis will be used to measure real-time cell death rates of malignant vs. non-malignant cells following RAR transfection by quantifying the uptake of Sytox via incucyte. Transfection efficiency between different tissue types or between cancer vs primary cells can greatly vary. To overcome this, the transcription efficiency can be monitored by incorporating a GFP-tagged vector that can be observed by microscopy. Cells will be transfected with RAR along with the GFP expressing vector. Any differences in transfection rates can be identified by monitoring the presence of GFP in each cell type. Transfection conditions can be adjusted to optimize transfection efficiency for each cell type and to ensure similar transfection efficiencies across each cell type.

Next, it would be important to investigate differences in protein expression following RAR treatment via WB analysis of cell death/RIG-I activation in cancer vs. non-cancer cell lines. Markers of cell death and RIG-I activation will be monitored following RAR or Poly(I:C) transfection. Protein lysates will be harvested at 4, 8, 16, and 24 hours post-transfection. We can compare protein expression readouts of cell death and RIG-I activation across cell types at each timepoint as previously outlined above. Together, these experiments will identify tissue specific cell death differences in response to RAR. These experiments will also identify cell death responses that are specific to cancer or non-cancerous cells.

Impact of RAR on transcription independent cell death

We have shown that IRF3 is required for PAMP induced cell death, but it has dual functions in antiviral defense. In transcriptional pathways IRF3 induces antiviral genes like IFNβ and ISGS. In a non-transcriptional pathway (RIPA) IRF3 is recruited to BAX to cause mitochondrial activation and apoptotic cell death. It would be interesting to investigate how RAR cell death varies in different transcriptional scenarios, and one way to do this is to study RAR in a RIPA skewed system. Previous studies identified RIPA as a transcriptional-independent IRF3 mediated pathway, which utilizes phosphorylation-incompetent IRF3 mutants that lead to the findings that IRF3 can be ubiquitinated and interact with the pro-apoptotic gene BAX and ultimately induce apoptosis in a transcription-independent manner. We can target RIPA by inhibiting IRF3 recruitment to LUBAC- knockout or by blocking TRAF2/TRAF6. This would prevent LUBAC-mediated ubiquitination of IRF3 and downstream recruitment to BAX. RAR can be transfected to determine if RAR induces cell death in RIPA skewed conditions, and we can use the above methods to determine if restricting RIPA abrogates RAR induced cell death. Furthermore, another experiment that can be utilized is a dose response of exogenous ubiquitin to determine if RAR induced cell death is amplified via RIPA skewing. It would be interesting to see if we can force RIPA conditions by introducing exogenous ubiquitin, and if ubiquitin has a dose-dependent effect on RAR induced cell death. This would provide a novel connection between RIPA, ubiquitin, and RAR. The results of these experiments could inform novel ways of utilizing RAR induced cell death in a non-canonical fashion.

Impact of differentially expressed genes on RAR-induced cell death in cancer vs. non-cancer cells

If we can determine that RAR is cancer-selective through additional studies, it would be important to expand on those findings and identify what the gene differences are between cancer and non-cancerous cells that prime cancer cells for RAR induced cell death. To first identify differentially expressed genes (DEGs) in cancer vs non-cancer cells induced by RAR signaling, we can compare hepatoma cell lines Huh7 and HepG2 to the immortalized non-neoplastic hepatocytes PH5CH8. These cell lines will be seeded for treatment with RAR, Poly(I:C), or the TLR4 agonist LPS, to identify gene signatures specific to RAR. RNA will be harvested at 8-, 16, and 24-hours post-treatment. RNA sequencing analysis of these samples can be utilized to discern which genes are upregulated or downregulated following treatment of each agonist to obtain a DEG list. These gene lists can then be used to identify co-expression modules through low parameter gene correlations. From these modules we can then identify associated functions of these correlated genes by conducting an over-representation analysis (ORA), which will identify cellular processes associated with co-expressed genes. Downstream analyses utilizing curated databases, such as Ingenuity Pathway Analysis, and manual construction of networks will aid in predicting potential key regulators or "gene hubs" specific to RAR cell death functional signaling.

Based on the results of the RNA sequencing and downstream bioinformatic analysis, we test the regulatory functions of the genes of interest by knockdown of these genes and determine if this enhances or restricts cell death. First, we can generate knockout cell lines using CRISPR/Cas9 for each gene of interest, and also confirm knockout by qPCR and WB. Following knockout via CRISPR, cells will be treated with RAR and cell death will be quantified over a 24-hour period using incucyte analysis. Genes of interest will either restrict or enhance RAR cell death

following their knockout. As a result of these experiments, we will demonstrate the influential role of hub genes located downstream of RIG-I/RAR interactions, while also gaining an improved understanding of RAR cell death signaling.

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