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Determining the effect of double-stranded RNA treatment in ovarian cancer

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DETERMINING THE EFFECT OF DOUBLE-STRANDED RNA TREATMENT IN OVARIAN CANCER

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Biochemistry at Virginia Commonwealth University.

by

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Abstract

DETERMINING THE EFFECT OF DOUBLE-STRANDED RNA TREATMENT IN OVARIAN CANCER

By Charlotte Faye Roberts, B.A.

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Biochemistry at Virginia Commonwealth University.

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Epithelial ovarian cancer is a lethal gynecological malignancy. Due to its asymptomatic nature it is typically detected in the latter metastatic stages. Standard treatment protocol involves surgical cytoreduction, followed by a combination of taxane and platinum-based chemotherapeutics. Initially this treatment is successful however, most patients face recurring tumors that over time become resistant to current drug regimens. Thus, novel chemotherapeutic development is necessary. Cancer cells express receptors of the innate immune system, pattern recognition receptors (PRRs) that function to alert the host of invading pathogens. PRRs such as toll-like receptor 3 (TLR3), retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and

dsRNA-dependent protein kinase receptor (PKR) recognize double-stranded RNA (dsRNA), a viral replication intermediate, and trigger apoptosis. Numerous studies have been conducted on the four dsRNA receptors in cancer. The findings have shown that stimulation of individual or a group of these receptors have led to a multitude of responses such as activation of apoptosis, inhibition of tumorigenic growth, and inhibition of metastasis in several cancer types (prostate, breast, nasopharyngeal, and melanoma cancer). Previous work in the Bell lab has shown that within a panel of ovarian cancer cell lines, one subset upregulates dsRNA receptors upon stimulation with polyinosinic-polyuridylic acid (pI:pC) and leads to apoptosis. A second subset of ovarian cancer cells do not upregulate dsRNA receptors and their survival is not affected by dsRNA treatment. We hypothesize that all or a subset of dsRNA receptors are required to elicit a dsRNA-induced apoptotic response. To test this hypothesis we examined the dsRNA-induced apoptotic response the responding cell lines (CAOV3 and OVCAR3) via three methods: selective ligand assays, transient knockdowns with siRNA, and stable lentiviral knockdowns with shRNA. Then we examined the dsRNA-induced apoptotic response in the non-responders (DOV13 and SKOV3). The first objective was to determine if all or a subset of these four dsRNA receptors were required for the dsRNA-induced apoptotic response. The second objective of this thesis was to examine if dsRNA receptor expression in cell lines resistant to dsRNA-induced apoptosis could restore dsRNA responsiveness. To execute the first objective, we first examined receptor contribution to the dsRNA-induced apoptotic response via a selective antagonist (2-aminopurine) to PKR and a selective agonist (polyadenylic-polyuridylic acid, pA:pU) to

TLR3. Inhibition of PKR did not blunt the apoptosis levels in the responders and was determined to be inessential for the dsRNA-induced apoptosis. Selective ligation of TLR3 with pA:pU showed an increase in apoptosis, but not to levels seen with pI:pC. Objective one was also carried out via transient knockdown using siRNA. Knockdowns via this method were less than 70% and the lipid vehicle of one of the transfection reagents was found to be sensitizing to the cells. Stable lentiviral knockdowns with shRNA were utilized to conduct the knockdown assays. By qPCR, lentiviral knockdown of TLR3 showed an 85% decrease and showed a great decrease in the dsRNA-induced apoptotic response in the cell death assay. The lentiviral knockdown of RIG-I showed a 54% decrease via qPCR and did not alter dsRNA-induced apoptotic responses. The lentiviral knockdown of MDA5 could only be assessed via the TLR3/MDA5 double knockdown, and it showed a 53% decrease via qPCR analysis. The cell death assay of the TLR3/MDA5 double knockdown showed a great decrease in the dsRNA-induced response. The work presented in this thesis is the first to address the contribution of all four dsRNA receptors to the dsRNA-induced apoptotic response in one study. In this work, we have found that PKR is not needed for the dsRNA-induced apoptosis. Loss of TLR3 in the responders reduces death, but not back to basal levels. This may be due to the delivery method of pI:pC such that it goes directly to the endosome. Forced expression of the dsRNA receptors (TLR3, MDA5, and RIG-I) can all induce apoptosis to similar levels indicating redundancy. The importance of this work reveals that any of the three dsRNA receptors, TLR3, MDA5, and RIG-I, could be possible targets for

individualized chemotherapeutic regimens for women with ovarian cancer expressing these receptors.

Chapter 1: Introduction

1.1 Ovarian Cancer

Ovarian cancer occurs when malignant cells develop in the ovaries, the female reproductive glands that are responsible for ova (egg) production. This malignancy can be detected in different tissues of the ovary including germ, stromal, and epithelial tissue. Epithelial tumors account for about 90% of the ovarian cancer forms[1].

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy due to an insidious onset and absence of distinct, early, physical symptoms[2]. Although more than 80% of women with ovarian cancer have symptoms (even when still limited to the ovaries), these symptoms are present in much more common conditions affecting the gastrointestinal and genitourinary areas[3]. Thus, when finally detected, the cancer has usually progressed to the late stages (>III) when metastasis has occurred.

Genetically heterogeneous and plagued by persistence or frequent recurrence, ovarian cancer patients typically receive a poor prognosis[4]. The 5-year survival rate of women with ovarian cancer (across all stages) is 46%. Only 20% of patients are diagnosed in stage I where a 90% cure rate can be achieved[5]. Instead, 60% of patients are diagnosed at stage III and later, with a 5-year survival rate of 25%[4]. Late stage diagnosis arises from a lack of effective screening strategies, such as well-defined prognostic

markers. Cancer antigen 125 (CA125) is a commonly used prognostic factor in ovarian cancer[6]. CA125 is consistently elevated in epithelial ovarian cancer and the rate of its decline during primary chemotherapy has been an important prognostic indicator. The issue with CA125 is that it is elevated in many other conditions such as endometriosis, breast cancer, colon cancer, and lung cancer[6]. Although prevention and early diagnosis are areas of active research, the prevalence of late stage diagnosis necessitates aggressive treatment.

Typical first-line treatment involves surgical cytoreduction to remove or reduce the size of the tumor followed by combination drug therapy consisting of platinum- and taxane-based drugs. Initially, the drug regimen is effective, but over time most patients relapse with drug-resistant tumors[7]. These drug resistant tumors are thought to arise from a small population of cells that remain dormant, but progressively grow. To attack these recurring, drug resistant tumors, development of novel chemotherapeutics or new combination therapies with established chemotherapeutics is necessary. The focus of this study is to examine how an innate immune agonist may be used as a chemotherapeutic to directly alter the survival of primary or drug resistant tumors.

1.2 Innate Immunity

Due to the heterogeneous nature of ovarian cancer, development of more efficient treatments involves creating strategies to treat the individual rather than a generic regimen used to treat all patients presenting with ovarian cancer. Currently, this generic regimen, of platinum- and taxane-based drug combination therapy is the standard protocol but the field is moving towards individualized therapies to address disease

heterogeneity. These strategies focus upon identifying molecular targets that can alter the survival of the cancer. In the 1890s, Dr. William B. Coley, a bone sarcoma surgeon, noticed that a small fraction of his patients who developed post-operative bacterial infection went into remission and were cured of their cancer[8]. In 1891, he injected patients that had inoperable bone cancer with streptococcal organisms and noticed a regression in the cancer[9]. He therefore linked bacterial infection to cancer regression. Although Dr. Coley didn't know it at the time, this was the first recorded usage of an innate immune agonist as a cancer chemotherapeutic. However, innate immunity had yet to be identified.

Around the same time, 1908 Nobel Laureate and Russian biologist, Dr. Ilya Mechnikov was working on starfish larva, in which he stuck thorns from a tangerine tree into the starfish and observed the process that essentially became known as phagocytosis[10]. Previous scientists such as Edward Jenner and Louis Pasteur had determined that immunization led to resistance against infection reliant upon the adaptive immune response, but Mechnikov's work led to the discovery of the innate immune system[11]. This prompted scientists to seek the components that initiated this phagocytic response.

1.3 Pattern Recognition Receptors

The next step in elucidation of the innate immune system was discovery of the host's internal components that recognized foreign material such as the bacterial toxins Dr. Coley injected in his patients and the tangerine tree thorns Dr. Mechnikov observed

being phagocytosed in starfish larvae. The discovery of Toll receptor helped to answer this question.

The Toll receptor was first discovered as a component for dorsal/ventral patterning in embryonic development in *Drosophila melanogaster*[12]. Mutations to the Toll receptor in the adult flies led to an inability to fight fungal infections[12]. Human Toll homologues were discovered via computational searches and in situ hybridization assays[13]. Through antibody ligation to a Toll-like receptor (TLR), Medzhitov and Janeway discovered that TLRs activated pathways initiating immune responses[14]. Due to the ability of the TLRs to recognize foreign material and alert the host to activate an immune response, they belong to a large collection of receptors referred to as pattern recognition receptors (PRRs).

Pattern recognition receptors, located both intra- and extracellularly, surveil the cell's internal and external environments for danger signals. Currently, there are three defined categories of pattern recognition receptors based upon function. The first class includes proteins that initiate phagocytosis to sequester and eliminate the host of pathogen via neutrophils and macrophages[15]. The second class is comprised of components secreted from cells to act in the cells surrounding environment. Receptors of this class facilitate the complement pathway as well as targeted phagocytosis by macrophages[15]. The third class of PRRs contains the receptors that signal to alert the host of infection[15]. These receptors are found in cells that populate host surfaces that may encounter foreign material like immune, epithelial, fibroblast, keratin, and endothelial cell types. These receptors recognize evolutionarily conserved pathogen-associated

molecular patterns (PAMPs) that activate transcription factors, which lead to the production of pro-inflammatory cytokines, type I interferons (IFNs), and chemokines[16].

Innate immune receptor agonists and antagonists have become targets for drug development to modulate the robust, sometimes detrimental, inflammatory response mediated by the PRRs. For example, TLR7 and 8 agonists have been used to treat viral warts caused by the Human Papillomavirus (HPV)[17]. TLR4 is being studied as a therapeutic target for treatment of gram-negative sepsis[18]. Synthetic dsRNA, polyinosinic-polycytdylic acid, is recognized by TLR3 and stimulates an IFN-mediated anticancer immune response as well as apoptosis in human breast cancer cells[19]. A dsRNA mimic, IPH-3102, is being developed as a vaccine adjuvant in the treatment of breast cancer cells[19].

DsRNA can engage four pattern recognition receptors. These receptors are toll-like receptor 3 (TLR3), melanoma differentiation-associated gene 5 (MDA5), retinoic acid-inducible gene I (RIG-I), and double-stranded RNA-dependent protein kinase receptor (PKR). They all are capable of triggering pro-inflammatory cytokines, type I interferons, and an apoptotic response via different signaling pathways that at some points converge (**Figure 1**).

1.3.1 Toll-like receptor 3

Located primarily in the endosome, toll-like receptor 3 (TLR3) was first identified as a dsRNA receptor that leads to the production of type I interferons (IFN)[20]. TLR3 is a type I transmembrane glycoprotein that contains several leucine-rich repeats in the ectodomain and a cytoplasmic tail containing the toll/interleukin-1 receptor (TIR) domain

for cytosolic signaling[21]. Upon ligation of TLR3 with dsRNA, it undergoes ligand-promoted multimerization to signal[22]. TLR3 activation initiates signaling pathways that phosphorylate the transcription factors, interferon regulatory factor 3 or 7 (IRF3 or IRF7), which homodimerize and translocate to the nucleus to initiate production of interferon-beta (IFN- ß). Once interferon-beta is secreted, it acts in an autocrine/paracrine manner to activate the heterodimeric, extracellular receptor interferon alpha receptor 1/2 (IFNAR1/2). This initiates the JAK/STAT pathways leading to production of interferon stimulatory genes resulting in abrogation of protein synthesis and initiation of apoptosis. Activated TLR3 also leads to the activation of the transcription factors, activator protein 1 (AP-1) and nuclear factor-κB (NFκB), and also activates caspase 8.

1.3.2 The helicases: Melanoma differentiation-associated gene 5 and Retinoic acid-inducible gene I

Melanoma differentiation-associated gene 5 and retinoic acid-inducible gene I are cytosolic helicases that recognize long dsRNA (more than 200 base pairs in length) and short dsRNA (less than 200 base pairs in length with a 5' 3-triphosphate group), respectively[23]. These helicases belong to the DExD/H box helicase family and contain central helicase domains and two N-terminal caspase recruitment domains (CARDs)[23]. The CARDs interact with the CARD-containing adapter, mitochondrial antiviral signaling adapter (MAV) in the outer mitochondrial membrane[23]. Activation of MDA5 and RIG-I leads to the activation of the transcription factors IRF3, IRF7, activator protein 1 (AP-1), and NFκB. This results in pro-inflammatory cytokine and type I interferon production. The helicase activation can also lead to cleavage of caspase 8 resulting in

apoptosis. TLR3 and the helicases differ in the adaptor molecule usage, but converge downstream to activate similar transcription factors and caspase.

1.3.3 Double-stranded RNA-dependent protein kinase receptor

Double-stranded RNA-dependent protein kinase receptor (PKR) is a cytosolic receptor whose expression is induced by the production of type I interferons[24]. PKR contains two N-terminal dsRNA-binding domains and a C-terminal serine/threonine kinase domain [25]. Double-stranded RNA with precise secondary stem loop structures is a potent activator of PKR[10]. Once PKR interacts with activators such as dsRNA, its conformation becomes modified, resulting in homodimerization and autophosphorylation[25]. Activated, PKR phosphorylates several targets, most notably eukaryotic initiation factor-2α (eIF-2α) on serine residue 51, which leads to inhibition of protein translation, suppression of cell growth, and apoptosis[24].

Figure 1. Double-stranded RNA signaling pathways

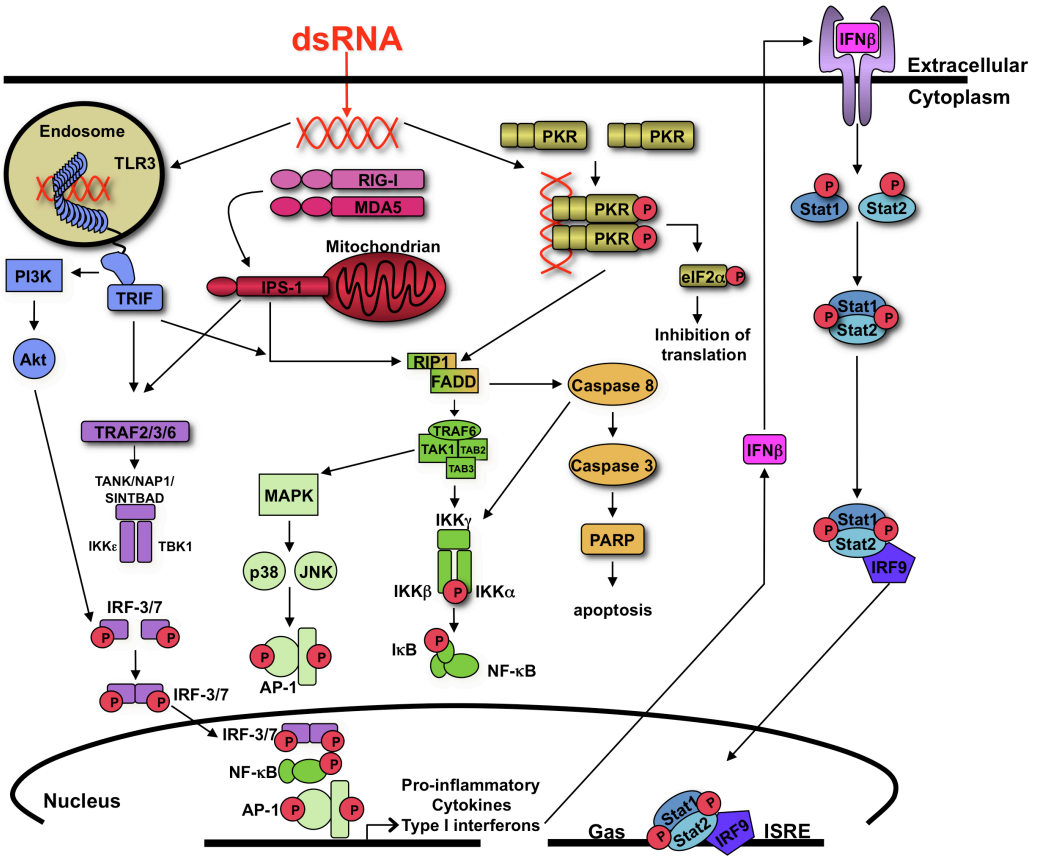
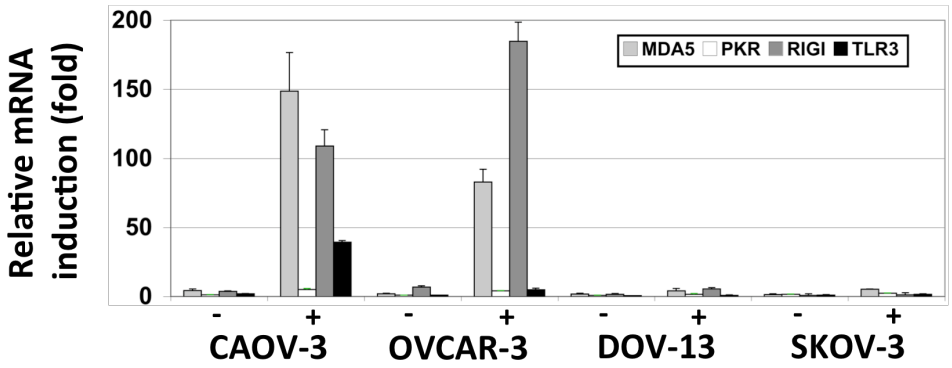


Figure 1. Double-stranded RNA signaling pathways. Cartoon diagram of the four receptors that recognize double-stranded RNA and their downstream signaling pathways. TLR3 is located in the endosome. RIG-I, MDA5, and PKR are cytosolic. Receptor ligation leads to a series of interrelated signaling pathways that lead to the production of type I interferon (purple), proinflammatory cytokines (green), and activation of caspases (yellow).

Figure 2. mRNA and protein expression levels of double-stranded RNA receptors (Danielle N. Van)

A.



B.

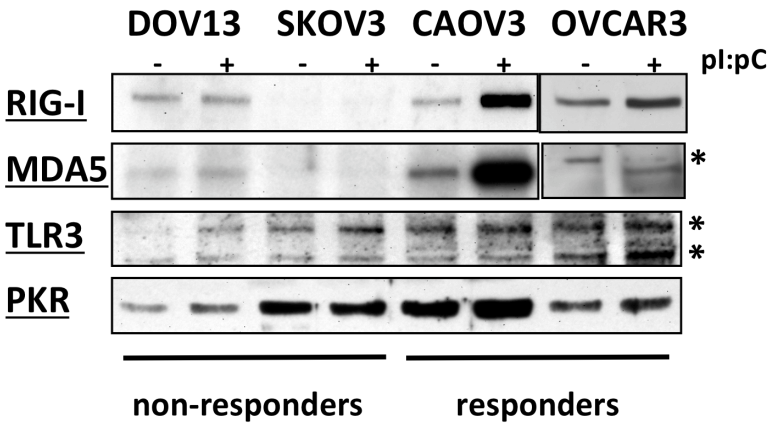


Figure 2. mRNA and protein expression levels of double-stranded RNA receptors (Danielle N. Van). Cells were (un)treated with 50 µg/mL pI:pC for 24h and RNA (A) or protein (B) was collected for analysis. **A.** Extracted RNA was made into cDNA and levels of receptor mRNA were quantified via TaqMan qPCR. Expression is reported as relative induction normalized to GAPDH. **B.** Protein lysates were analyzed via immunoblot using primary antibodies specific to receptors. * denotes non-specific bands.

In our lab, previous work had discovered a panel of ovarian cancer lines (CAOV3, OVCAR3, DOV13, and SKOV3) with a dichotomy of responses to dsRNA and associated treatment pathways, apoptosis or unaltered survival (Figure 2). These assays revealed that before stimulation, all four ovarian cancer cell lines had similar levels of mRNA and protein. However, upon stimulation with pI:pC, two of the cell lines (CAOV3 and OVCAR3) upregulated their mRNA and protein expression for the dsRNA receptors. However, the other two cell lines (DOV13 and SKOV3) did not upregulate their expression levels of mRNA and protein upon stimulation with pI:pC. The cell lines that upregulated their expression of dsRNA receptors were found to undergo apoptosis upon treatment with pI:pC. The cell lines that did not upregulate dsRNA receptor expression did not respond to pI:pC at all. Thus, the four cell lines were divided into two groups responders (CAOV3 and OVCAR3) and non-responders (DOV13 and SKOV3) to pI:pC stimulation.

Ovarian cancer cells with unaltered survival upon dsRNA stimulation were examined for restored responsiveness upon forced dsRNA receptor expression. These experiments would determine if pathways retain function in the non-responsive cell lines and can be targeted for activation in cancers that repress receptor expression. The cell lines that did not survive upon dsRNA stimulation were examined for reduced or lost responsiveness upon knockdown of receptor via siRNA and shRNA. The responsive cell lines were also treated with selective agonists to determine the essentiality of individual dsRNA receptors. These experiments were conducted to determine if all or a subset of the receptors are essential for the dsRNA-induced apoptotic response.

Double-stranded RNA has been used as a chemotherapeutic since the 1970's. The first therapeutic use of dsRNA was as its synthetic analog, pI:pC, in leukemia patients in 1976[26]. The study was stopped due to severe side effects in the patients, such as shock and renal failure[26]. A derivative of pI:pC, Ampligen®, was made containing unpaired uracil and guanine bases (polyI:C_{12}U) to reduce the toxicity while retaining the bioactivity of pI:pC[27]. This redeveloped dug was initially used to treat chronic fatigue syndrome [27]. It is now being developed as a vaccine adjuvant for therapy in advanced ovarian cancer [28].

Aside from clinical trials, more work is being conducted in the laboratory setting to examine the effectiveness of dsRNA as a potential chemotherapeutic. TLR3 has been found to be important for pI:pC-induced apoptosis in colon, pharyngeal, breast, prostate, squamous cell carcinomas of the head and neck, and melanoma cancers[29,30,31,32,33]. TLR3 has also been implicated in growth inhibition upon ligation with pI:pC in renal cell carcinoma and prostate cancer[34,35]. Upon pI:pC stimulation, TLR3 has also been shown to decrease metastasis in nasopharyngeal carcinoma, alter tumor microenvironments in ovarian cancer cells to increase the immune response, and cause autodigestion in melanoma cells[36,37,38].

The helicases RIG-I and MDA5 have also been implicated in the anti-cancer effects upon pI:pC stimulation. RIG-I has been found to cause death in ovarian, liver, and melanoma cancers[39,40,41]. MDA5 has been shown to cause melanoma cells to undergo autodigestion in the presence of pI:pC[38]. TLR3 was assessed in this same study, but was not found to be as efficient as MDA5 in the activation of autodigestion[38]. This may be due to the delivery method of pI:pC used in this assay. Polyethyleneimine (PEI) was used to

deposit pI:pC directly into the cytoplasm, completely by-passing the endosome where TLR3 is located[38]. MDA5 has also been shown to be important for cell death in liver an melanoma cancer[40,41].

In previous studies like the aforementioned ones, only a subset of the dsRNA receptors were examined for their effect on altering cancer cell survival. In all of these studies, no one has examined all four dsRNA receptors and their contribution to the dsRNA-induced response. The work presented here examines all four dsRNA receptors and their individual contribution to the dsRNA-induced apoptotic response.

The overall findings in this work revealed that PKR was not essential for the dsRNA-induced apoptotic response as determined by selective inhibition with 2-aminopurine. Also, a reduction in dsRNA-induced apoptosis was only noted when TLR3 or TLR3 with MDA5 were knocked down in the responders, implicating TLR3 as necessary for the dsRNA-induced apoptotic response. As for the non-responders, all of the receptors were capable of activating the dsRNA-induced apoptotic response when overexpressed in the non-responders. This revealed two things. One, there are functional death pathways in the non-responders. Two, there appears to be redundancy in the dsRNA signaling pathways.

The purpose of this thesis was to evaluate and determine the contribution of each of the four dsRNA receptors to the dsRNA-induced apoptotic response to identify which receptor(s) is/are absolutely essential for response. If a subset of receptors could be determined as essential, then key signaling components common to the essential receptors may be identified and used as possible biomarkers for dsRNA-based chemotherapy.

Chapter 2: Materials and Methods

2.1 Cell culture

Ovarian cancer cell line DOV13 was received from the Fang laboratory at Virginia Commonwealth University, Richmond, Virginia. CAOV3, OVCAR3, and SKOV3 ovarian cancer cell lines along with the breast cancer cell line CAMA1 were purchased from American Type Culture Collection (ATCC). The HEK293 cells were a gift from Dr. David M. Segal (National Institutes of Health - NIH). All ovarian, breast, and HEK293 cell lines were cultivated in complete media (RPMI 1640) supplemented with 10% low endotoxin fetal bovine serum, 200mM L-glutamine, 1X non-essential amino acids (Sigma), 5 mM sodium pyruvate, 5 mM HEPES (all purchased from Invitrogen Gibco) at 37°C and 5% CO_2. Experiments were completed with cells that had undergone less than 20 passages in culture.

2.2 Pattern Recognition Receptor ligand preparation

2.2.1 Polyinosinic-polycytidylic acid preparation

The dsRNA synthetic analog, polyinosinic-polycytidylic acid (pI:pC – Sigma P0913), prepared as per manufacturer's protocol to a final concentration of 5 mg/mL. Aliquots were stored at -20 ºC in sterile micro-centrifuge tubes.

2.2.2 Polyadenylic-polyuridylic acid preparation

The dsRNA synthetic analog (TLR3 specific agonist), polyadenylic-polyuridylic acid (pA:pU- Sigma P1537), was prepared as per manufacturer's protocol to a final concentration of 5 mg/mL. Aliquots were stored at -20 ºC in sterile micro-centrifuge tubes.

2.2.3 2-Aminopurine preparation

The PKR inhibitor, 2-Aminopurine, was dissolved in 1X PBS:glacial acetic acid (200:1) to a concentration of 150 mM. Powder 2-aminopurine was dissolved for 1 minute in a hot water bath at 60 ºC. The solution was then mixed and sterilely aliquoted in hood at 200 µL and stored at -20 ºC in sterile micro-centrifuge tubes.

2.3 Transfections

2.3.1 Receptor overexpression in SKOV3 and DOV13 cells

SKOV3 or DOV13 cells ($0.2x10^5$ cells/well) were seeded into 24-well plates. After 24 hours, the cells received fresh complete media and were then transfected with 500 ng of receptor DNA (MDA5, RIG-I, or TLR3), 500 ng of empty vector DNA (pCDNA3.1), or a combination of receptor and empty vector at a combined total of 500 ng using *Trans*IT LT1 (Mirus Bio Corp.) as per manufacturer's protocol. Twenty-four hours post-transfection, the cells received complete media alone or complete media plus polyinosinic-polycytidylic acid (pI:pC – Sigma P0913) at 50 µg/mL.

2.3.2 Receptor overexpression

SKOV3 or DOV13 cells ($3x10^5$ cells/well) and HEK293 cells ($4.5x10^5$ cells/well) were seeded into 6-well plates. After 24 hours, the cells received fresh complete media and were then transfected with 2.5 µg of receptor DNA (MDA5, RIG-I, or TLR3), 2.5 µg of empty vector DNA (pCDNA3.1), or a combination of the receptor and empty vector DNA

maintaining a total of 2.5 µg using either *Trans*IT LT1 (Mirus Bio Corp.) or Lipofectamine 2000 (Invitrogen) as per manufacturer's protocol. Twenty-four hours post-transfection, the cells received complete media alone or complete media plus polyinosinic-polycytidylic acid (pI:pC – Sigma P0913) at 50 µg/mL. After 24 hours, the media was removed; the cells were rinsed with ice cold, sterile 1X PBS. Cells were lysed with 250 µL of cold lysis buffer comprised of 20 mM Hepes, pH 7.4, 150 mM NaCl, 135 mg ß-glycerophosphate, 1.5 mM $MgCl_2$, 10 mM NaF, 1M DTT, 1 mM $NaVO_4$, 2 mM EGTA, 1 mM PMSF, 1X protease inhibitor (Complete-EDTA free Protease inhibitor tablet, Roche), 0.5% TritonX-100, and 1 mg/mL N-ethylmaleimide (lysis buffer). The cells were scraped from the plate using a sterile cell scraper and pipetted into microcentrifuge tubes. The lysates were kept on ice and vortexed on medium speed for 1 second every 10 minutes for 40 minutes. Cell debris was separated from soluble sample via centrifugation at 14,000 rpm for 10 minutes at 4 ºC. The supernatant was pipetted into new microcentrifuge tubes and stored at -20 ºC for short-term storage or long-term at -80 ºC.

2.3.3 GFP transfection

SKOV3 cells ($3x10^5$ cells/well) were seeded into 24-well plates. After 24 hours, the cells received fresh complete media and were then transfected with 5, 50, or 500 ng of GFP plasmid DNA (Lonza). For cells receiving less than 500 ng of GFP plasmid, empty vector DNA was added at various amounts to bring total transfected DNA to 500 ng. Control cells were transfected with 500 ng of empty vector DNA (pCDNA3.1). All transfections were conducted using *Trans*IT LT1 (Mirus Bio Corp.) as per manufacturer's protocol. Twenty-four hours post-transfection, the cells received fresh complete media

with 5 µL of 1 mg/mL Hoechst 33342 (Sigma-Aldrich). The stained cells incubated in the dark for 5 minutes at room temperature. Nuclei were counted under UV light on a Nikon TE300 Eclipse microscope equipped with an Hg-lamp and red/blue excitation fluorescence filters to determine total cells. In the same field, GFP fluorescent cells were counted. Transfection efficiency was determined by GFP positive cells over total, Hoechst stained, cells.

2.4 siRNA knockdowns

OVCAR3 or CAOV3 cells ($3x10^5$ cells/well) were seeded into 6-well plates. Cells were grown to a 50% confluence and were then transfected with siRNA (25 nM final concentration) specific to MDA5 (Qiagen, siRNA ID: SI03648981), RIG-I (Qiagen, siRNA ID: SI03019646), TLR3 (Qiagen, siRNA ID: SI02655156), or scrambled (Qiagen, siRNA ID: 1027285) using either DharmaFECT®1 (Thermo Scientific), Lipofectamine® 2000 (Invitrogen), or *Trans*IT TKO (Mirus BioCorp.) according to manufacturer's recommendations. Cells were washed at 18 and 24h post-transfection with sterile 1X PBS and given fresh complete media. Polyinosinic-polycytidylic acid was delivered to the cells in fresh complete media at 50 µg/mL 60 hours post-transfection. Twenty-four hours post-stimulation, media was removed and cells were washed with 1X PBS. Cells were harvested by scraping and their RNA was extracted using an RNeasy Mini kit (Qiagen) as per manufacturer's protocol. RNA was stored at -20°C. Levels of dsRNA receptor mRNA were quantified via Taqman quantitative Real Time-PCR by Bell laboratory colleague, Danielle Van.

2.5 Lentiviral shRNA knockdowns

2.5.1 Puromycin Assay

CAOV3 and OVCAR3 cells were seeded into 96-well plates at ($3.5x10^4$ or $4.5\ x10^4$ cells/well, respectively) and allowed to adhere for 24 hours. The following day, the cells received either fresh complete media alone or in conjunction with puromycin (Santa Cruz Bio.) at 2, 4, 6, 8, or 10 µg/mL. Twenty-four hours later, the puromycin media was removed and the cells were rinsed with 20 µL sterile 1X PBS. Cell viability was determined by trypan blue assay.

2.5.2 Polybrene assay

CAOV3 and OVCAR3 cells were seeded into a 24-well plate at ($5x10^4$ or $6\ x10^4$ cells/well, respectively) and allowed to adhere for 24 hours. The following day, the cells received media containing increasing concentrations of polybrene (Santa Cruz Bio.) at 2, 4, 6, 8, or 10 µg/mL. Twenty-four hours later, the plates were stained with Hoechst stain and propidium iodide. Nuclei were assessed under UV light on a Nikon TE300 Eclipse microscope equipped with an Hg-lamp and red/blue excitation fluorescence filters. The percentage of cell death was quantified by counting apoptotic cells versus total cells. The concentration of polybrene with the lowest cell death after a 24-hour exposure was used for the lentiviral transfections.

2.5.3 Lentiviral infections

CAOV3 and OVCAR3 cells were seeded to a 24-well plate at ($5x10^4$ or $6\ x10^4$ cells/well, respectively) and allowed to adhere for 24 hours. The following day, the cells were at 50% confluency. One well per cell line was reserved for counting to know how

many cells were in each well. The media was removed from each of these wells and then the cells were rinsed with 100 µL sterile 1X PBS. The cells received 40 µL of trypsin and a 10 µL aliquot was mixed with 10 µL Trypan Blue stain and were counted using a hemocytometer. The volume of lentivirus to use to infect the cells was determined using the cell count. For each receptor knockdown and the control, a multiplicity of infection of 1:1 and 1:2 were calculated. The cells then received media containing polybrene at 4 µg/mL while the lentivirus was freeze-thawing. The virus was added drop-wise, the plates were swirled and then allowed to incubate for 24 hours. The following day, the cells received fresh complete media and were given a 24 hour rest period. The cells were then split 1:3 from one 24-well plate into three 24-well plates. The cells were given a 48 hour rest period.

2.5.4 Colony selection

The responding cell lines that successfully integrated the genomic material to make receptor-specific shRNA into their genome also incorporated puromycin resistance. Puromycin was added to the plated cells at 4 µg/mL directly into the media every three days. Every three days the cells were checked until stable colonies could be identified. Once stable colonies were identified, they were selected via two methods. The first method involved selecting single-cell derived colonies. Four hundred and fifty microliters of media were removed from the wells, leaving fifty microliters of media to cover the cells. The plates were held such that the bottom of the wells could be visualized. Large colonies were circled on the bottom of the plate and then analyzed under microscope to insure encircled colonies were isolated from other cells. The plates were taken back into the hood and the

colonies were selected using sterile swabs. The swabs were then swished into new 24-well plates containing media with puromycin at 4 μg/mL. The second method of selecting colonies involved selecting all of the colonies in the well. The media was removed from the 24-well plates and the cells were rinsed with 100 μL of sterile 1X PBS. Then the colonies received 100 μL of trypsin, and once detached from the plate, the cells were mixed in 1.9 mL of complete media containing puromycin at 4 μg/mL. Then the cells were pipetted into 6-well plates. This method was also used to collect colonies in some wells to freeze for later use.

2.6 Hoechst/Propidium Iodide cell staining

After stimulating cells with 50 μg/mL pI:pC, the cells received 5 μL of 1 mg/mL Hoechst 33342 (Sigma-Aldrich) and 1 mg/mL propidium iodide (Fluka BioChemika) added directly to the media. The stained cells incubated in the dark for 5 minutes at room temperature. Then the nuclei were assessed under UV light on a Nikon TE300 Eclipse microscope equipped with an Hg-lamp and red/blue excitation fluorescence filters. The percentage of cell death was quantified by counting apoptotic cells versus total cells.

2.7 Lysate harvest

At the time of harvest, media was removed from cells in 6-well plates. The cells were then washed with 1 mL cold 1X PBS. Cells were lysed with 250 μL of cold lysis buffer (lysis buffer). The lysates were stored on ice and vortexed for 1 second every 10 minutes over a 40-minute time span. Cell debris was separated from soluble sample via centrifugation at 4ºC and 14,000 rpm for 10 minutes. The supernatant was removed and stored at -20ºC until further use.

2.8 RNA extraction

Qiagen RNeasy Mini Kit was used to perform total RNA isolation according to manufacturer's instructions. Total RNA (40 μL) isolated from OVCAR3 and CAOV3 cells were stored at -20°C.

2.9 Immunoblotting

Protein concentrations of the lysates were determined via Bio-Rad protein assay (BioRAD). Equal amounts of protein (50-100 μg) were prepared for SDS-PAGE separation in NUPAGE® LDS sample buffer (Invitrogen) containing 5 mM 2-mercaptoethanol (Sigma-Aldrich). Samples were separated by SDS-PAGE (8 or 10% Tris Glycine) and then transferred to Hybond ECL nitrocellulose membrane (Amhersham Biosciences). The blots were blocked in either 5% milk/ 1X TBS + 0.1% Tween-20 or 5% bovine serum albumin (BSA)/ 1X TBS + 0.1% Tween-20 for 1 hour at room temperature with agitation. Primary antibodies were diluted as follows:

1. α-hMDA5 receptor Ab (Fisher Lab), 1:10,000 in 5% BSA/ 1X TBS + 0.1% Tween-20
2. α-hTLR3 receptor Ab (Imgenex), 1:1,000 in 5% milk/ 1X TBS + 0.1% Tween-20
3. α-hRIG-I receptor Ab (Cell Signaling), 1:1,000 in 5% BSA/ 1X TBS + 0.1% Tween-20
4. α-Actin Ab (Santa Cruz), 1:1,000 in 5% milk/ 1X TBS + 0.1% Tween-20
5. α-phospho-eIF2α Ab (Cell Signaling), 1:1,000 in 5% BSA/ 1X TBS + 0.1% Tween-20
6. α-eIF2α Ab (Cell Signaling), 1:1,000 in 5% BSA/ 1X TBS + 0.1% Tween-20
7. α-Tubulin Ab (Cell Signaling), 1:1,000 in 5% BSA/ 1X TBS + 0.1% Tween-20

The blots were incubated with the primary antibodies overnight at 4ºC with agitation. The following day the blots were washed either three times with 1X TBS + 0.1% Tween-20

buffer for 5 minutes per wash or four times with 1X TBS + 0.1% Tween-20 buffer for 15 minutes each (specifically for MDA5) at room temperature with agitation. HRP-conjugated secondaries were diluted as follows:

1.Goat- Anti-mouse (Santa Cruz), 1:10,000 in 5% milk or 5% BSA with 1X TBS + 0.1% Tween-20

2. Goat-Anti-rabbit (Santa Cruz), 1:10,000 in 5% milk or 5% BSA with 1X TBS + 0.1% Tween-20

The blots were incubated with secondary antibody for 1 hour at room temperature with agitation. The blots were then washed 3 times for 5 minutes each in 1X TBS + 0.1% Tween-20 buffer. Immunoblots were developed using ECL or ECL-PLUS reagents (GE Healthcare).

2.10 Protein concentration

Protein concentrations were determined using the Bio-Rad protein assay protocol in accordance with the manufacturer's recommendations.

2.11 Data analysis and statistics

All results are representative of three replications. The results were reported as the mean plus or minus the standard deviation. Differences between groups were analyzed for statistical significance using One-Way ANOVA, JMP 8.0. Software. A p-value ≤ 0.05 was considered significant.

Chapter 3. Results

Four immortalized epithelial ovarian cancer cell lines (CAOV3, OVCAR3, SKOV3, and DOV13) were previously shown to have varied responses to pI:pC stimulation. Two of the cell lines, CAOV3 and OVCAR3, responded to pI:pC stimulation by undergoing programmed cell death (apoptosis). The other cell lines, DOV13 and SKOV3, did not undergo apoptosis or show any response to the pI:pC stimulation. As a result, the cell lines were divided into two groups, responders (OVCAR3 and CAOV3) and non-responders (SKOV3 and DOV13).

Western blot analysis of the four dsRNA receptors revealed that the responding cell lines had higher levels of these receptors, and also up-regulated them 24 hours following pI:pC stimulation (Figure 2). The non-responding cell lines had very low or undetectable endogenous levels of the receptors and did not up-regulate their expression levels after pI:pC stimulation. We hypothesize that all or a subset of dsRNA receptors are required to elicit a dsRNA-induced apoptotic response. To test this hypothesis the following experiments were designed to explore: one, if all or a subset of the dsRNA receptors were required for the dsRNA-induced apoptotic response, and two, if transient dsRNA receptor expression can restore the dsRNA-induced apoptotic response.

3.1 Ligand assays

A simple way to analyze individual receptor contribution to the pI:pC-induced apoptotic response was to utilize receptor-specific ligands. An inhibitor of PKR, 2-aminopurine, and a ligand recognized by TLR3, polyadenylic-polyuridylic acid (pA:pU), were used to assess the importance of these two receptors to the apoptotic response induced by pI:pC stimulation.

3.1.1 pI:pC assay

In order to conduct the selective ligand assays, an optimal range of pI:pC that would allow a visible increase in the apoptotic response above basal, but not overwhelm the cells had to be determined. This would allow for accurate examination of the receptor's contribution to the dsRNA-induced apoptotic response. This concentration was optimal for the assays at 5 μg/mL for both responsive cell lines (Figure 3).

3.1.2 2-Aminopurine assay

The ability of PKR to phosphorylate the alpha subunit of eukaryotic translation initiation factor 2 is inhibited by 2-aminopurine. Analysis of 2-aminopurine inhibition on PKR signaling in the presence of pI:pC first required optimizing the concentration of pI:pC.

The Li group reported that 5 mM 2-aminopurine was able to inhibit PKR's ability to phosphorylate eukaryotic translation initiation factor 2 alpha (eIF-2α)[42]. A titration of 2-aminopurine was conducted to find the optimal concentration for the PKR inhibition assay. OVCAR3 cells were seeded to 6-well plates and treated with various concentrations of 2-aminopurine in the presence of pI:pC at 5 μg/mL. A decrease in phosphorylation of eIF-2α was seen following treatment with 5, 10, and 50 mM 2-aminopurine (Figure 4). Cell death

was seen upon treatment of the cells with 50 mM 2-aminopurine. The vehicle of 2-aminopurine contains acetic acid and this increase in the media's acetic acid concentration may have caused this death. A change in the color of the media was noted after adding in the vehicle at the volume necessary to deliver 2-aminopurine at a concentration of 50 mM. When collecting the protein lysates, the media was spun down to collect the dead cells and add to the protein lysate. A long exposure of the immunoblot of tubulin revealed unequal loading of protein for vehicle and 50 mM 2-AP in the presence of pI:pC. A 5 mM concentration of 2-aminopurine was chosen to complete the assay.

Figure 3. pI:pC titration for selective ligand assays

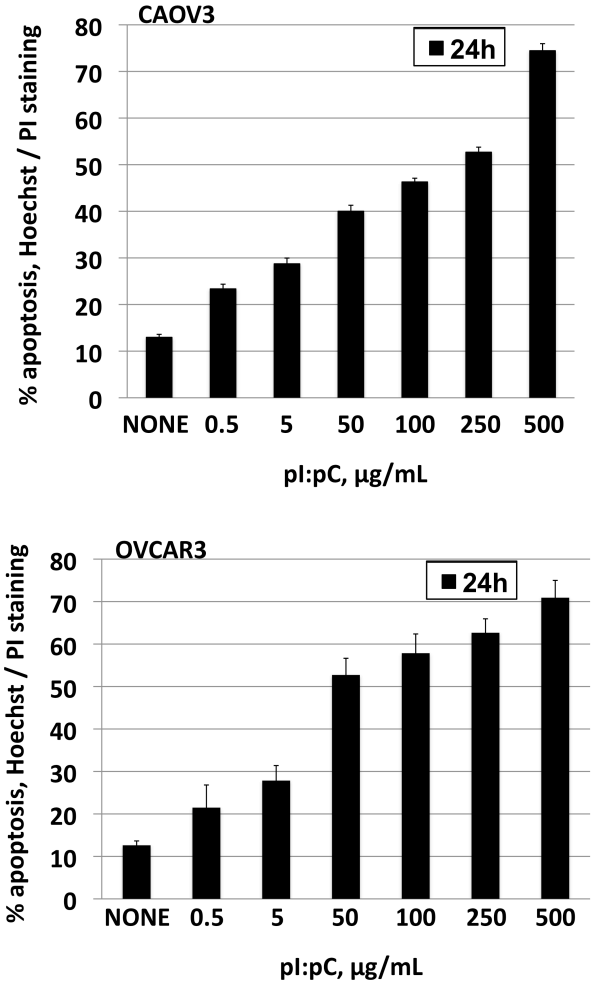


Figure 3. pI:pC titration for selective ligand assays. CAOV3 and OVCAR3 cells were seeded to 24-well plates and were treated with pI:pC at various concentrations for 24 hours. Cells were stained with Hoechst and propidium iodide to assess apoptotic nuclei.

Figure 4. 2-Aminopurine at 5 mM inhibits phosphorylation of eIF-2α phosphorylation

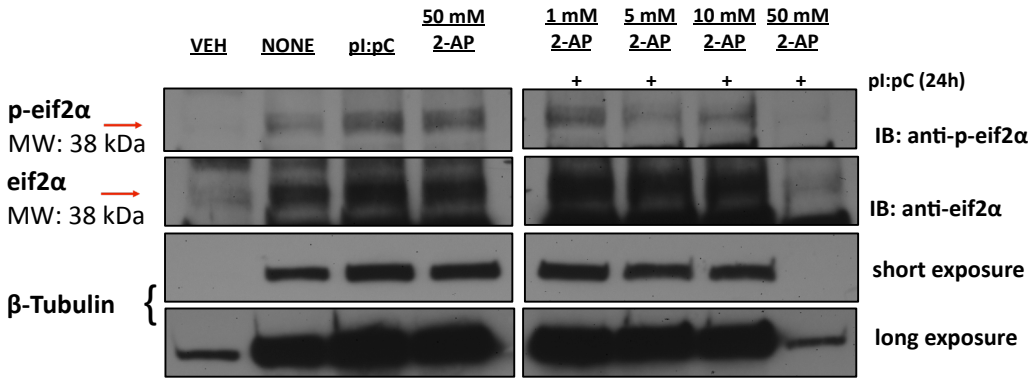


Figure 4. 2-Aminopurine at 5 mM inhibits phosphorylation of eIF-2α phosphorylation. OVCAR3 cells were seeded to 6-well plates and were (un)treated with 2-aminopurine vehicle (PBS:acetic acid at 200:1 ratio), 5 µg/mL pI:pC, and either 1,5,10, or 50 mM 2-aminopurine (2-AP) for 24 hours. Cells were lysed and phosphorylation of eukaryotic translation initiation factor 2 alpha on Ser51 was analyzed via immunoblot.

Figure 5. Inhibition of PKR with 2-aminopurine does not affect dsRNA-induced apoptosis

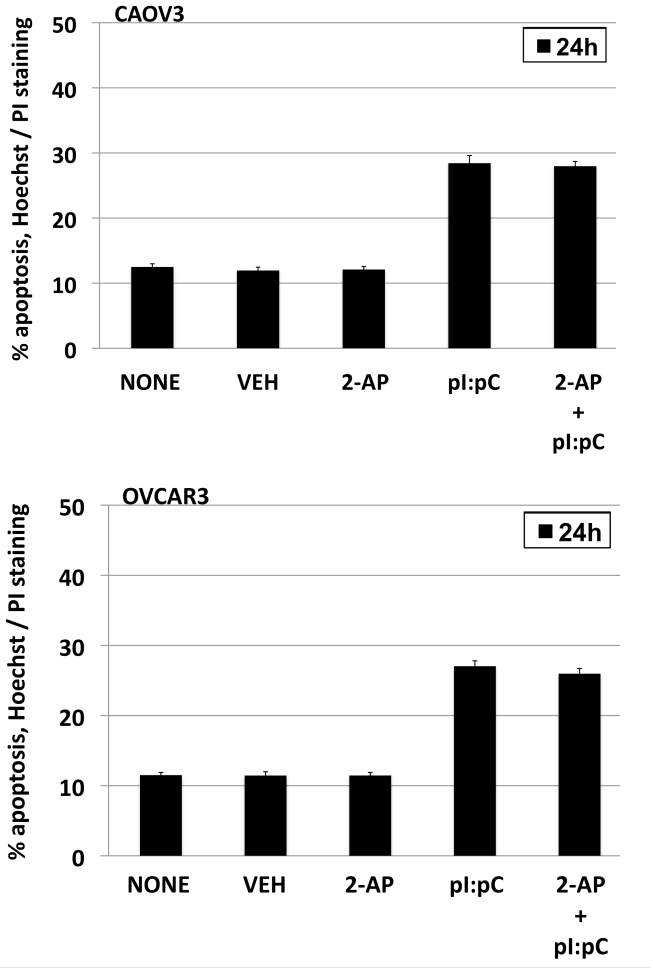


Figure 5. Inhibition of PKR with 2-aminopurine does not affect dsRNA-induced apoptosis. CAOV3 and OVCAR3 cells were seeded to 24-well plates and were treated with 5 µg/mL pI:pC, 5 mM 2-aminopurine (2-AP), or both for 24 hours. Cells were stained with Hoechst and propidium iodide to assess apoptotic nuclei.

To study the effect of PKR signal inhibition, both responsive cell lines, CAOV3 and OVCAR3, were seeded to 24-well plates and treated with 5 mM 2-aminopurine and pI:pC at 5 μg/mL for 24 hours (Figure 5). The cells were then stained with Hoechst and propidium idodide stain to assay for apoptosis. Inhibition of PKR's ability to signal did not dampen the apoptotic response induced by pI:pC. Therefore, PKR was determined to be inessential for the pI:pC-induced apoptotic response. Consequently, this receptor was not analyzed any further.

3.1.3 pA:pU assay

The Caux group in 2010 conducted a luciferase assay in which they transfected HEK293T cells with the promoter for interferon stimulatory response elements (ISRE) attached to luciferase[43]. They also transfected in TLR3, RIG-I, or MDA5 plasmid and then treated the cells with either polyadenylic-polyuridylic acid (pA:pU) or polyinosinic-polycytidylic acid (pI:pC) at increasing concentrations from 0.01-100 μg/mL. They found that at any concentration, TLR3 was able to recognize both dsRNA ligands[43]. RIG-I and MDA5, however, only recognized pI:pC at any concentration and required pA:pU at concentrations of 10 and 100 μg/mL**,** respectively to be recognized[43]. Due to these findings, TLR3 receptor contribution to dsRNA-induced apoptosis could be assessed using pA:pU stimulation at concentrations lower than 10 μg/ml.

To assess if TLR3 responded to pA:pU below 10 μg/ml in our cells, pA:pU was titrated in a cell death assay to find an optimal treatment concentration (Figure 6). At a concentration of 5 μg/mL, the cell death was detected above basal levels for both responsive cell lines, therefore that concentration was chosen to conduct the assay.

Figure 6. pA:pU titration

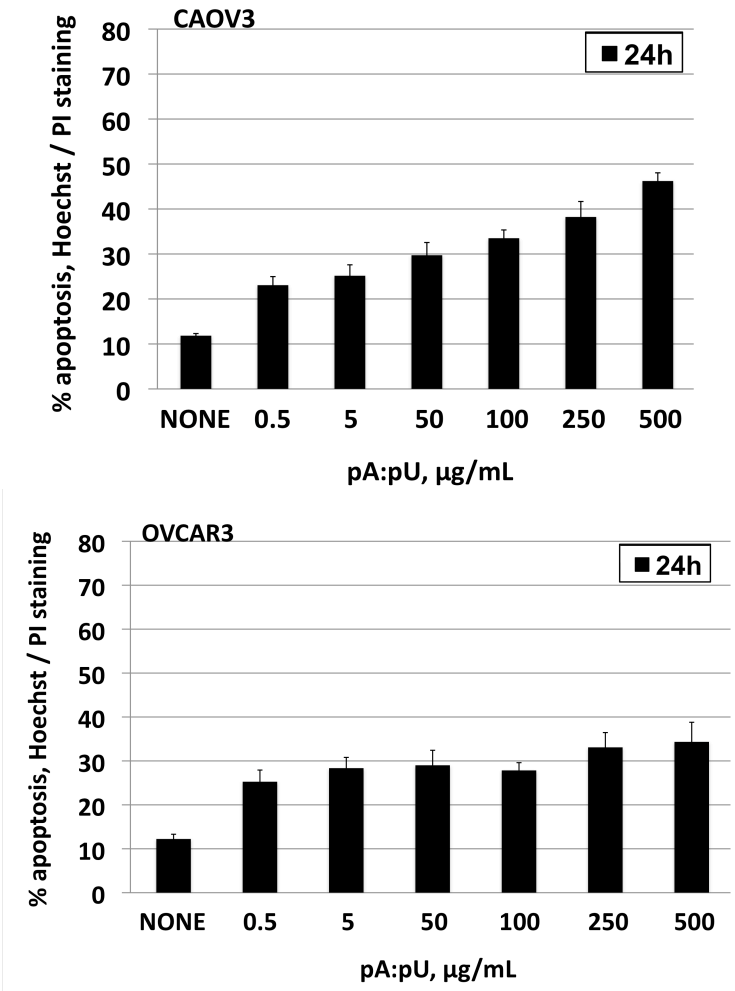


Figure 6. pA:pU titration. CAOV3 and OVCAR3 cells were seeded to 24-well plates and were treated with pA:pU at various concentrations for 24 hours. Cells were stained with Hoechst and propidium iodide to assess apoptotic nuclei.

With this optimal pA:pU concentration for selective activation of TLR3, OVCAR3 and CAOV3 cells were treated with either pA:pU or pI:pC at 5 μg/mL for 24, 48, and 72 hours and then assessed for their pA:pU induced apoptotic response. Some wells were left untreated as a control. The findings revealed that pI:pC induced increasing levels of apoptosis over the 72 hour period (Figure 7). However, pA:pU could induce cell death comparable to pI:pC at 24 hours, but this cell death level remained unchanged at 48 and 72 hours (Figure 7).

Figure 7. TLR3 does not induce increasing levels of apoptosis

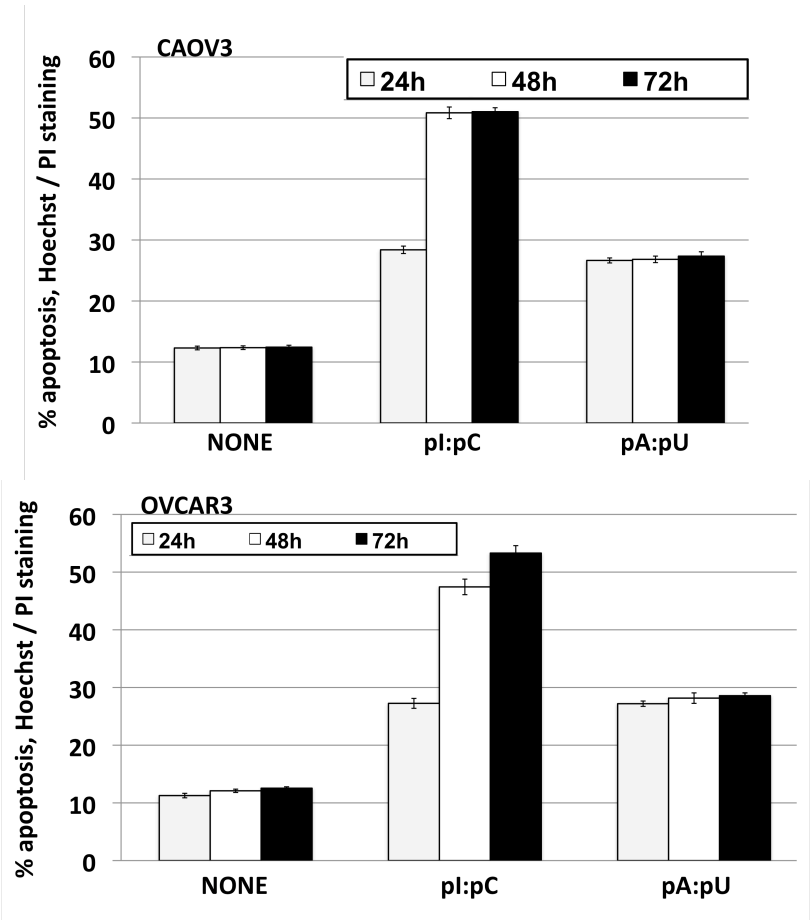


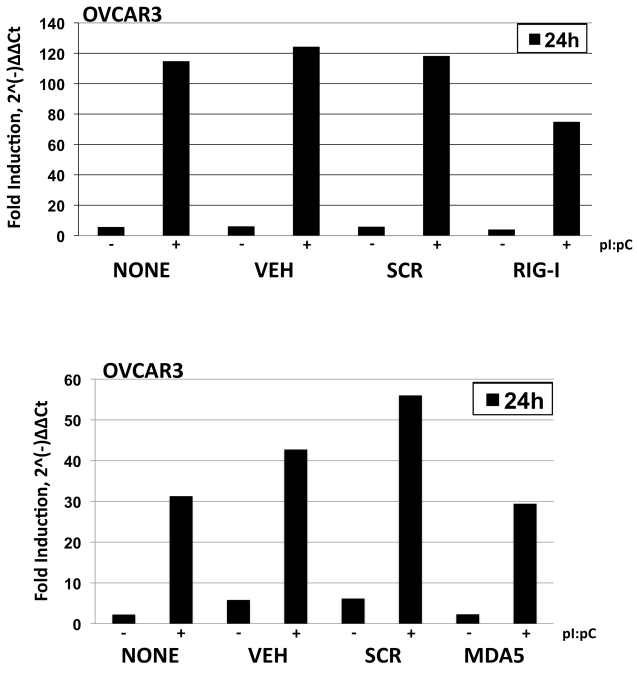
Figure 7. TLR3 does not induce increasing levels of apoptosis. CAOV3 and OVCAR3 cells were seeded to 24-well plates and were treated with 5 μg/mL pI:pC or pA:pU for 24, 48, and 72 hours. Cells were stained with Hoechst and propidium iodide to assess apoptotic nuclei.

3.2 Transient knockdown of receptors

Another way to analyze the receptor importance in the dsRNA-induced apoptotic response is to use siRNA to knockdown individual receptors and then treat cells with pI:pC and measure a change in the dsRNA-induced apoptotic response. We analyzed the importance of TLR3, MDA5, and RIG-I to the dsRNA-induced apoptotic response via this method. It was previously determined in the inhibition assay with 2-aminopurine that PKR was not essential to the dsRNA-induced apoptotic response; therefore it was not further analyzed. For these experiments, the Bell lab had previously identified commercially available, single, validated siRNA sequences (Qiagen) for these experiments. The first experiments were conducted in OVCAR3 using Lipofectamine 2000 to transfect in siRNA specific to RIG-I and MDA5. The mRNA levels for cells +/- siRNA transfection were analyzed via qPCR and a percentage knockdown determined. The percentage knockdown was determined as follows: (100-(fold induction in receptor mRNA for siRNA treated + pI:pC/fold induction in receptor mRNA (native) + pI:pC)=% knockdown. Using this analysis, RIG-I mRNA levels could be knocked down 34%, whereas MDA5 mRNA levels were knocked down 32% in OVCAR3 cells (Figure 8A). When DharmaFECT was used as the siRNA vehicle delivery reagent, RIG-I mRNA levels in CAOV3 cells was knocked down 16%, however when comparing receptor siRNA treated to scrambled siRNA treated both plus pI:pC, the knockdown was 34%, Figure 8B. To significantly reduce the level of receptor in the cell, a knockdown of at ~70% was targeted. With only a reduced level targeted receptor, its role in the dsRNA-induced apoptotic response could not be assessed. This level of knockdown, however, was not achieved using these siRNA transfection

Figure 8. Transient knockdown of RIG-I and MDA5 in responders

A.



B.

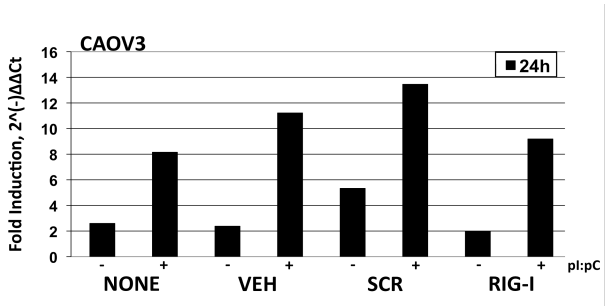


Figure 8. Transient knockdown of RIG-I and MDA5 in responders. OVCAR3 and CAOV3 cells were seeded to 6-well plates and transfected with siRNA specific to either RIG-I or MDA5 (25 nM final concentration). OVCAR3 cells were transfected using Lipofectamine 2000. CAOV3 cells were transfected using DharmaFECT1. A non-specific, scrambled (SCR, 25 nM final concentration) was used as a control. Forty-eight hours post-transfection the cells received pI:pC at 50 μg/ml. The RNA was extracted 24 hours post-stimulation and analyzed via q-PCR.

reagents (Lipofectamine 2000 or DharmaFECT). Therefore, a third transfection reagent (Transit TKO) was examined for improved knockdown efficiency.

A control experiment was conducted in CAOV3 cells to test if this lipid-based vehicle would sensitize the cells to dsRNA stimulation. An analysis using qPCR looking for RIG-I mRNA revealed that only cells receiving pI:pC upregulated their RIG-I mRNA levels (Figure 9). However, in the untransfected control, the RIG-I mRNA levels do not increase as previously observed for this cell line (see Figure 2). Upon stimulation of cells with pI:pC, it was noted that the untransfected cells and the cells receiving only vehicle were much more confluent than the cells that received siRNA, suggesting that the addition of siRNA affected cell proliferation. A cell plating assay was conducted to insure that cells across treatment wells remained the same confluency at the time of stimulation with pI:pC such that the cell number:ligand concentration ratio would be similar in all groups. This required plating 2/3 fewer cells for the untransfected and vehicle alone treatment wells.

With these additional controls in place, the knockdown efficiencies with Transit TKO for TLR3 and MDA5 receptors were assessed. The receptors were individually knocked down and efficiency of knockdown was analyzed via qPCR. Again, no upregulation of receptor mRNA was observed in the untransfected, but pI:pC stimulated cells. Ligand integrity was excluded as a potential reason for the failed upregulation as its ability to upregulate dsRNA receptor mRNA levels and induce an apoptotic response in a control cell line (CAMA-1[30]) compared to previous lots of ligand was unaltered (data not shown, Danielle Van). In addition, new vials of CAOV3 and OVCAR3 were purchased from ATCC, propagated and assessed for pI:pC induced upregulation of

Figure 9. Control Experiment using Transit TKO

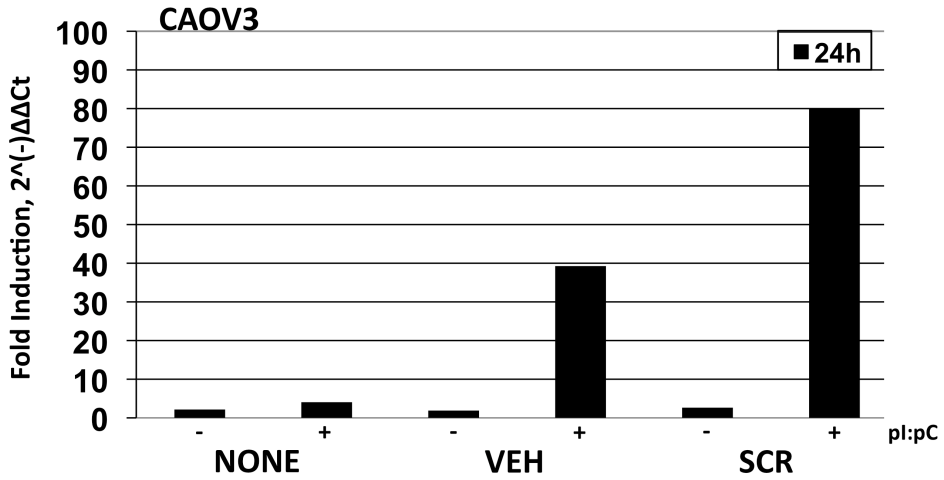


Figure 9. Control experiment using Transit TKO. CAOV3 cells were seeded to 6-well plates and transfected with scrambled siRNA (25 nM final concentration) using Transit TKO. Eighteen and twenty-four hours post-transfection, cells received fresh complete media. Forty-eight hours post-transfection the cells received pI:pC at 50 μg/ml. The RNA was extracted 24 hours post-stimulation and analyzed via q-PCR.

Figure 10. Transient knockdowns using Transit TKO

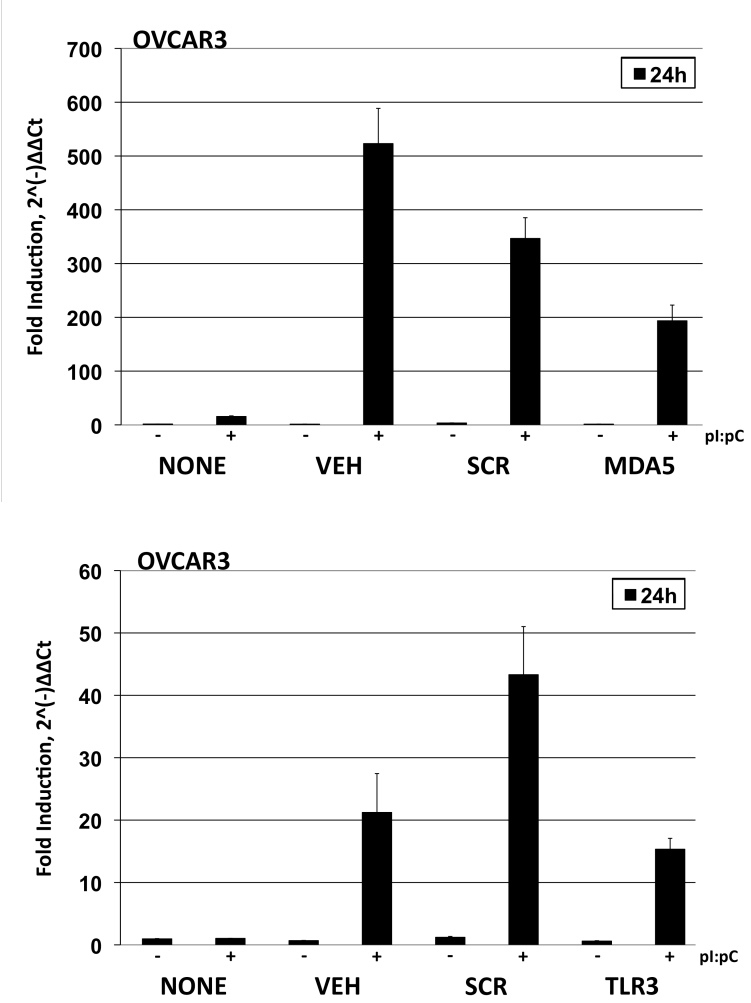


Figure 10. Transient knockdowns using Transit TKO. OVCAR3 cells were seeded to 6-well plates and transfected with receptor siRNA (MDA5 or TLR3, 25 nM final concentration) or scrambled siRNA (25 nM final concentration) using Transit TKO. Eighteen and twenty-four hours post-transfection, cells received fresh complete media. Forty-eight hours post-transfection the cells received pI:pC at 50 μg/ml. The RNA was extracted 24 hours post-stimulation and analyzed via q-PCR.

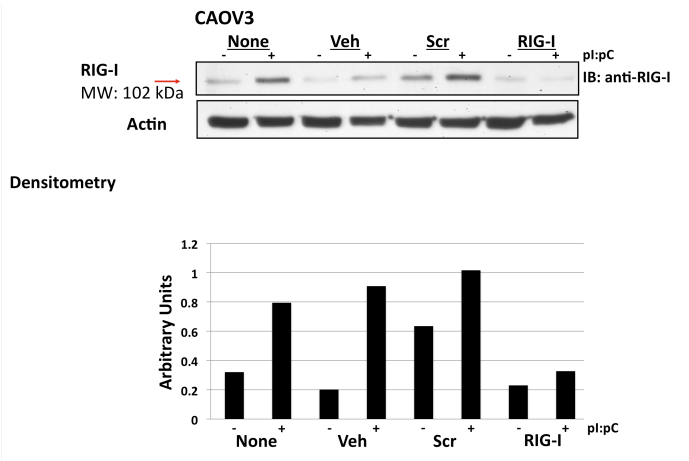
dsRNA receptor mRNA. Both the older version and newly propagated CAOV3 and OVCAR3 samples showed dsRNA-induced increases in dsRNA receptor mRNA (data not shown, Danielle Van). At this time, the loss of receptor mRNA upregulation for non-transfected, but pI:pC stimulated samples in these experiments cannot be explained. As the non-transfected control receptor mRNA levels are not consistent with our initial data and subsequent control experiments to isolate the source of this difference, for the Transit TKO experiments, the level of mRNA knockdown was calculated as follows: (100-(fold induction in receptor mRNA for siRNA treated + pI:pC/fold induction in receptor mRNA for siRNA scrambled control + pI:pC)=% knockdown. The knockdown efficiencies using this approach were found to be 44.2% for MDA5 and 64.7% for TLR3 in the OVCAR3 cell line (Figure 10).

As our assessment comparing mRNA levels revealed either low level knockdown (Lipofectamine 2000, Dharmafect) or suffered for inconsistent results for control experiments (Transit TKO), the protein level of targeted dsRNA receptor for transient knockdowns was directly examined. RIG-I was targeted for siRNA knockdown in CAOV3 using Transit TKO and the knockdown efficiency was analyzed via immunoblot. Figure 11A shows that the RIG-I siRNA treated sample has lower receptor levels than the non-transfected, but stimulated control sample. Using densitometry, each receptor band was normalized to its corresponding actin (loading control) band and plotted, Figure 11A. Using these values, the percentage of protein expression knockdown was calculated as follows: (100-(normalized, +pIpC RIG-I siRNA receptor densitometry/normalized, +pI:pC non-transfected receptor densitometry))=% protein knockdown. The knockdown of RIG-I

was found to be 55% when compared to stimulated untransfected cells. In comparison to stimulated scrambled control, a knockdown of 67% was achieved. A cell death assay was conducted. At 24 hours, the cell death was not blunted by reduced expression of RIG-I (Figure 11B). At 24 h post-dsRNA stimulation, the level of apoptosis was equivalent for all treatments. For all time-points after 24 hours, all cells receiving lipid vehicle and pI:pC had undergone cell death (Figure 11B). Therefore, it was concluded that the lipid vehicle sensitized the cells to pI:pC possibly by aiding pI:pC entry into the cells. This possibility had been addressed by the experimental design in which cells are washed multiple times (3) following siRNA transfection to remove lipid vehicle. Even with these precautions, the lipid vehicle may have remained at the cell surface or altered the cell surface altering the cells response to pI:pC stimulation.

Figure 11. Protein expression and apoptosis following transient knockdown

A.



B.

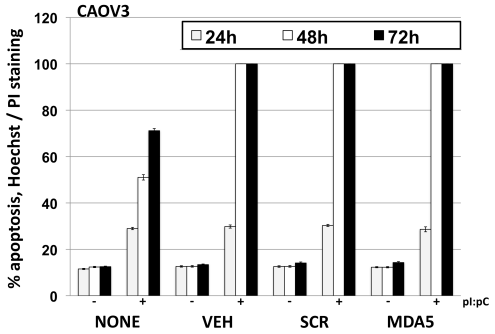


Figure 11. Protein expression and apoptosis following transient knockdown. A. CAOV3 cells were seeded to 6-well plates and transfected with RIG-I siRNA (25 nM final concentration) using Transit LT1. Fresh complete media was given to the cells 18 and 24 hours post-transfection. Forty-eight hours post-transfection the cells received pI:pC at 50 μg/ml. Twenty-four hours post-transfection, the cell lysates were collected and analyzed via immunoblot. Densitomtery values are reported below the blot. **B.** CAOV3 cells were plated in 24-well plates and treated the same as in A up to stimulation with pI:pC. Twenty-four, forty-eight, and seventy-two hours post-transfection, the cells were stained with Hoechst / propidium iodide to measure apoptosis.

3.3 Stable knockdown of receptors using lentivirus

Due to the difficulties discussed in the previous section with regards to using siRNA and lipid vehicle to knockdown individual dsRNA receptors, stable knockdowns using a lentiviral delivery system were undertaken to examine individual dsRNA receptor contribution to the dsRNA-induced apoptotic response. The lentiviral system chosen imparted puromycin resistance when successfully integrated into the target cell allowing selection of stable clones in puromycin-containing media. Prior to lentiviral infection, puromycin kill curves for each cell line were completed, data not shown, and from these curves, a concentration of >2 mg/ml puromycin was shown to be sufficient for selection. Cells were also tested for polybrene sensitivity following a 24 h exposure, Figure 12. Polybrene is a cationic polymer used to increase the efficiency of infection with a retrovirus in cell culture but at too high of a concentration can cause cell death. Polybrene at <6 μg/ml was well tolerated by both cell lines. For lentiviral infections, 4 μg/ml polybrene was used. Lentiviral particles containing three expression constructs each encoding target-specific shRNA designed to knockdown an individual receptor (RIG-I, MDA5 or TLR3) were used to infect each of the responder cell lines. As a control, the responder cells lines were also infected with lentiviral particles encoding an expression construct for scrambled shRNA (no target). Two multiplicity of infections (MOI), MOI 1:1 and 1:2 cells:viral plaque forming units, were tested to examine if the potential for multiple integrants would increase knockdown efficiency. A summary of lentiviral constructs undertaken is given in Table 1.

Figure 12. Non-sensitizing concentration of Polybrene is less than 6 µg/mL

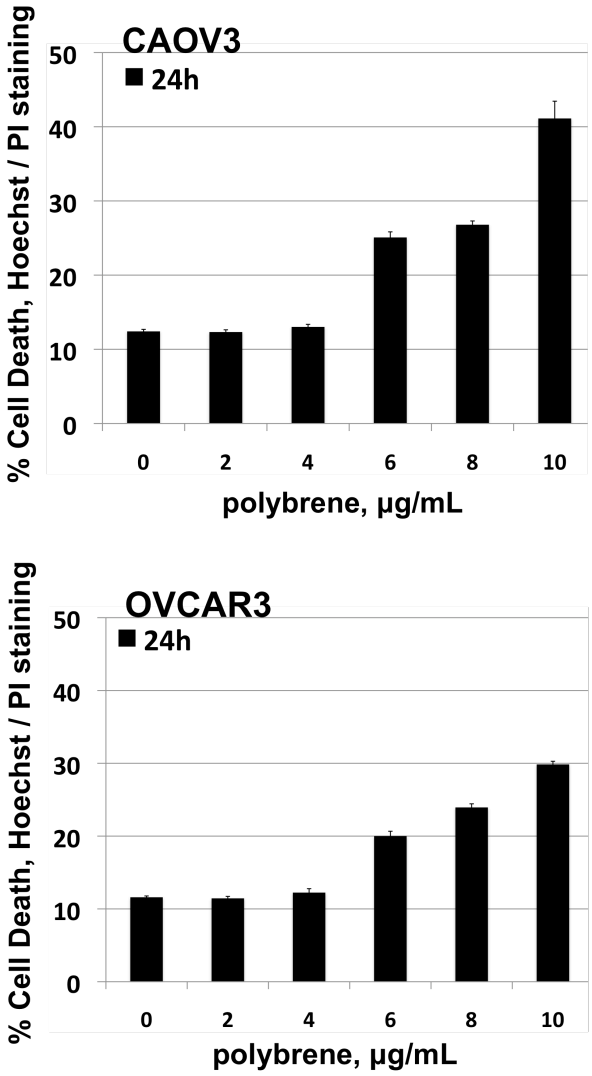


Figure 19. Transfection reagents sensitize cells. CAOV3 and OVCAR3 cells received 0, 2, 4, 6, 8, or 10 µg/mL polybrene. Twenty-four hours later, cells were stained with Hoechst and propidium iodide to measure cell death.

Table 1. Summary of Lentiviral Stable Knockdown Cell Lines.

CAOV3 & OVCAR3 Virus	CAOV3 qPCR	CAOV3 Immunoblot	CAOV3 Apoptosis	
Scr 1:1	X	X	X	Scrambled shRNA did not alter dsRNA-induced responses.
Scr 1:2	X	X	X	
TLR3 1:1	X	X	X	TLR3 shRNA showed 85% KD and descreased dsRNA-induced responses.
TLR3 1:2	X		X	
RIG-I 1:1	X	X	X	RIG-I shRNA showed 54% KD and did not alter dsRNA-induced responses.
RIG-I 1:2	X		X	
TLR3/MDA5	X		X	TLR3/MDA shRNA showed 85% (TLR3), 53% (MDA5) KD and descreased dsRNA-induced responses. This experiment is currently in progress.
RIG-I/TLR3		X		
MDA5/RIG-I				This experiment is currently in progress.

First column, All cell lines constructed. Scr, scrambled. Ratio refers to Cell/viral PFU. Columns 2-5 are results pertaining to CAOV3-derived cell lines.

Following infection and selection in puromycin-containing media, resistant colonies were first selected. Because OVCAR3 cells grew in patches and CAOV3 cells spread evenly within the wells, the single-cell derived stable colonies were only readily selected from OVCAR3 wells. However, at this low cell density, the selected OVCAR3 colonies did not survive either because of dilute conditions or inefficient viral infection. Whole-well stable colony selections were obtained from both OVCAR3 and CAOV3 cell lines. Far fewer OVCAR3 cells survived the puromycin selection, which may indicate that CAOV3 are more readily infected by lentiviral systems. OVCAR3 also have a longer doubling time than CAOV3 and are particularly sensitive to cell density, further slowing their growth. As a result, data are only shown for the CAOV3-derived stable cell lines.

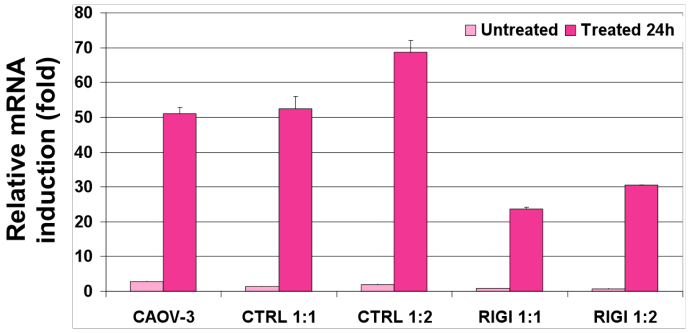
In the CAOV3 cell line, receptors RIG-I, TLR3, and MDA5 were targeted for knockdown. Each receptor knockdown was evaluated three ways. First, the change in targeted receptor mRNA levels +/- pI:pC stimulation was analyzed via qPCR. Second, the change in targeted receptor protein levels +/- pI:pC stimulation was analyzed via immunoblot. For mRNA and protein levels, the knockdown percentage is calculated as follows: (100-(targeted knockdown + pI:pC/uninfected control + pI:pC))=% knockdown. Third, the dsRNA-induced apoptotic response was evaluated via cell death assay using Hoechst and propidium iodide stain following pI:pC stimulation 24, 48 and 72 h after treatment. In our control experiment, the scrambled shRNA at an MOI of 1:1 or 1:2 did not alter dsRNA receptor mRNA or protein levels for any receptor. Also, scrambled shRNA did not alter the level of dsRNA-induced cell death.

Evaluation of RIG-I knockdown at a multiplicity of infection (MOI) of 1:1 and 1:2 in CAOV3 examining mRNA receptor levels via qPCR revealed a 55% and 41% knockdown as compared to stimulated uninfected control, respectively (Figure 13A). The immunoblot of RIG-I 1:1 knockdown revealed that RIG-I receptor protein was still expressed at levels comparable to the uninfected, unstimulated parent cell line, but receptor protein levels could no longer be upregulated in the presence of pI:pC (Figure 13B). The cell death assay revealed that knockdown of RIG-I did not reduce the dsRNA-induced apoptotic response at any time-point (Figure 13C).

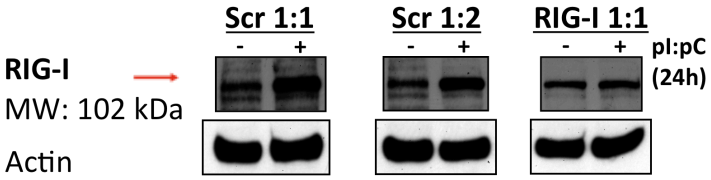
Analysis of TLR3 knockdowns examining TLR3 mRNA levels via qPCR revealed an 85% knockdown when uninfected, stimulated cells are compared to the stimulated TLR3 1:1, 1:2, and TLR3/MDA5 double knockdowns (Figure 14A). In the immunoblot the expression of TLR3 in the stimulated TLR3/MDA5 knockdown sample was reduced and TLR3 was not visible for the 1:1 knockdown pre- and post-stimulation (Figure 14B). The cell death was blunted by knockdown of TLR3 (Figure 14C). For the TLR3 knockdown at an MOI of 1:1 and 1:2, the cell death was reduced from 21% to 15% when compared to stimulated control 1:1 at 24h. At 48h, the cell death for TLR3 1:1 and 1:2 was reduced from 50% (control 1:1 stimulated) to 28 and 21%, respectively. At 72h, TLR3 1:1 and 1:2 revealed a cell death decrease from 58% (control 1:1, stimulated) to 30 and 31%, respectively. The cell death assay for the TLR3/MDA5 double knockdown revealed a decrease from 21% to 16% at 24h, 50% to 29% at 48h, and 58% to 32% at 72 hours when comparing stimulated control 1:1 to TLR3/MDA5 stimulated (Figure 14C). Overall, at 48 and 72 hours, the loss of TLR3 signaling reduces cell death by ~50%.

Figure 13. Stable knockdown of RIG-I does not affect the dsRNA-induced apoptotic response

A.



B.



C.

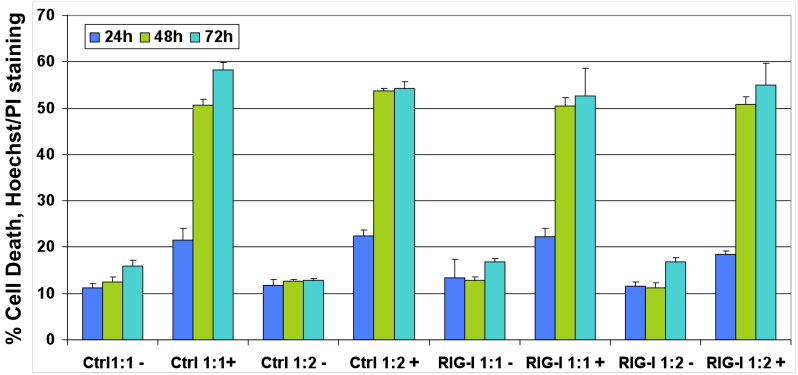
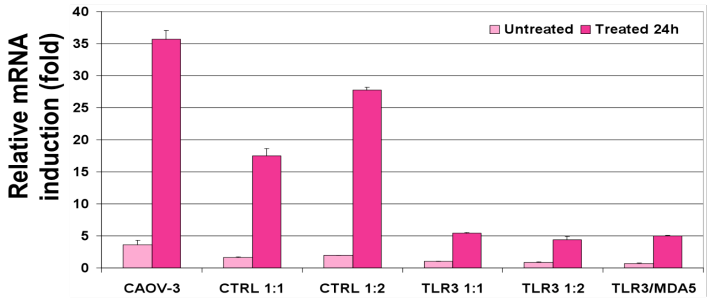


Figure 13. Stable knockdown of RIG-I does not affect the dsRNA-induced apoptotic response. Cells were (un)treated with 50 µg/mL pI:pC for 24h and RNA (A) or protein (B) was collected for analysis. **A.** Extracted RNA was made into cDNA and levels of receptor mRNA were quantified via TaqMan qPCR.

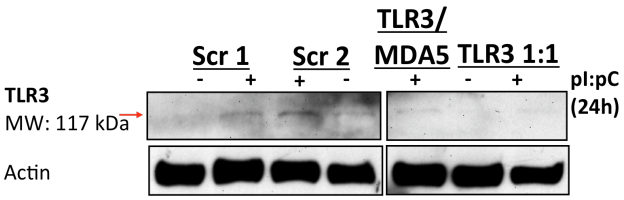
Expression is reported as relative induction normalized to GAPDH. **B.** Protein lysates were analyzed via immunoblot using primary antibodies specific to RIG-I receptor. **C.** CAOV3 cells stimulated with 50 μg/mL pI:pC. Twenty-four, forty-eight, and seventy-two hours post-transfection, the cells were stained with Hoechst / propidium iodide to measure apoptosis.

Figure 14. Stable knockdown of TLR3 reduces the effect of the dsRNA-induced apoptotic response

A.



B.



C.

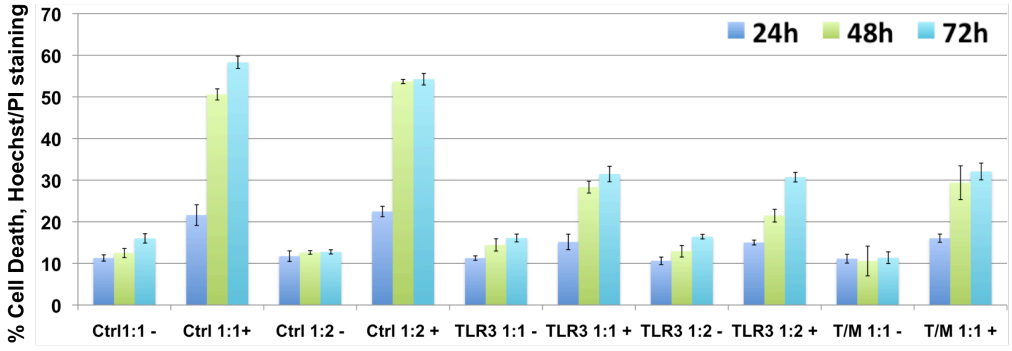


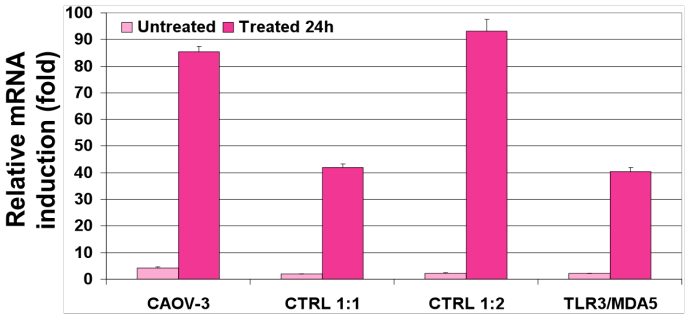
Figure 14. Stable knockdown of TLR3 reduces the effect of the dsRNA-induced apoptotic response. Cells were (un)treated with 50 µg/mL pI:pC for 24h and RNA (A) or protein (B) was collected for analysis. **A.** Extracted RNA was made into cDNA and levels of receptor mRNA were quantified via TaqMan qPCR. Expression is reported as relative induction normalized to GAPDH. **B.** Protein lysates were analyzed via

immunoblot using primary antibodies specific to TLR3 receptor. **C.** CAOV3 cells stimulated with 50 µg/mL pI:pC. Twenty-four, forty-eight, and seventy-two hours post-transfection, the cells were stained with Hoechst / propidium iodide to measure apoptosis.

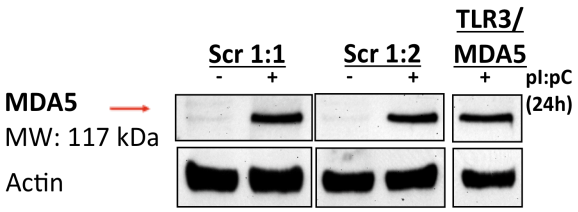
MDA5 knockdown was only evaluated via the double knockdown of MDA5/TLR3. The single knockdowns of MDA5 in both responding cell lines were unsuccessful. The knockdown of MDA5 in comparison to uninfected stimulated cells revealed a 54% knockdown of receptor mRNA levels when evaluated via qPCR (Figure 15A). The immunoblot of MDA5 for the double knockdown of MDA5 and TLR3 showed no decrease in the receptor expression post-stimulation (Figure 15B). Cell death assays revealed a decrease in cell death as previously noted in the TLR3 data above (Figure 15C). This cell death, however, cannot be attributed solely to any alterations in MDA5 expression because of the concomitant loss of TLR3 expression from these cells as described in the TLR3 data above.

Figure 15. Stable knockdown of TLR3/MDA5 reduces the effect of the dsRNA-induced apoptotic response

A.



B.



C.

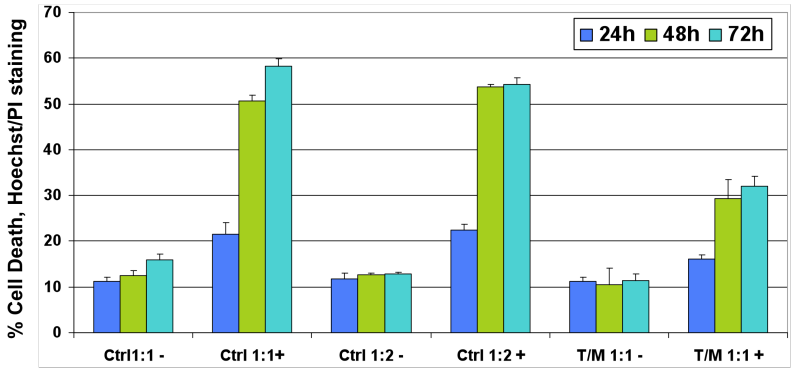


Figure 15. Stable knockdown of TLR3/MDA5 reduces the effect the dsRNA-induced apoptotic response. Cells were (un)treated with 50 µg/mL pI:pC for 24h and RNA (A) or protein (B) was collected for analysis. **A.** Extracted RNA was made into cDNA and levels of receptor mRNA were quantified via TaqMan qPCR. Expression is reported as relative induction normalized to GAPDH. **B.** Protein lysates were analyzed

via immunoblot using primary antibodies specific to MDA5 receptor. **C.** CAOV3 cells stimulated with 50 µg/mL pI:pC. Twenty-four, forty-eight, and seventy-two hours post-transfection, the cells were stained with Hoechst / propidium iodide to measure apoptosis.

3.4 Overexpression of Pattern Recognition Receptors in non-responding cell lines

To determine if restoration of the pattern recognition receptors would permit an apoptotic response upon pI:pC stimulation, the receptors were overexpressed in SKOV3 and DOV13 cells. The success of the transfections was confirmed via immunoblot (Figure 16).

The next step was to conduct an apoptosis assay to see if overexpression restored the ability of the cells to respond to pI:pC. SKOV3 cells were seeded into 24-well plates and transfected 24 hours later with 0.5 μg receptor DNA (MDA5, RIG-I, or TLR3) or 0.5 μg pCDNA3.1, empty vector control. Twenty-four hours post-transfection, the cells were stimulated with pI:pC at 50 μg/mL for 24, 48, and 72 hours. The apoptosis was assayed via Hoechst and propidium iodide staining. Although apoptosis was seen in the receptor transfected SKOV3 cells when stimulated with pI:pC, the level of death in the pre-stimulated empty vector control and dsRNA receptor transfected groups was much higher than basal (no transfection, no stimulation) apoptosis (Figure 17).

To address the issue of sensitization, the level of receptor over-expression was first examined. The amount of dsRNA receptor was titrated to optimize the amount of over-expression while attempting to lower the amount of pre-stimulated cell death to basal levels (Figure 18A). The concentration of total DNA transfected into each group was held constant whilst the ratio of dsRNA receptor DNA to control (pCDNA3.1 empty vector) was varied. The 1:3 ratio of dsRNA receptor DNA to control DNA reduced the unstimulated apoptosis to that of the basal empty vector control and was subsequently used in the apoptosis assay (Figure 18B).

Figure 16. Overexpression of Pattern Recognition Receptors in non-responding cell lines

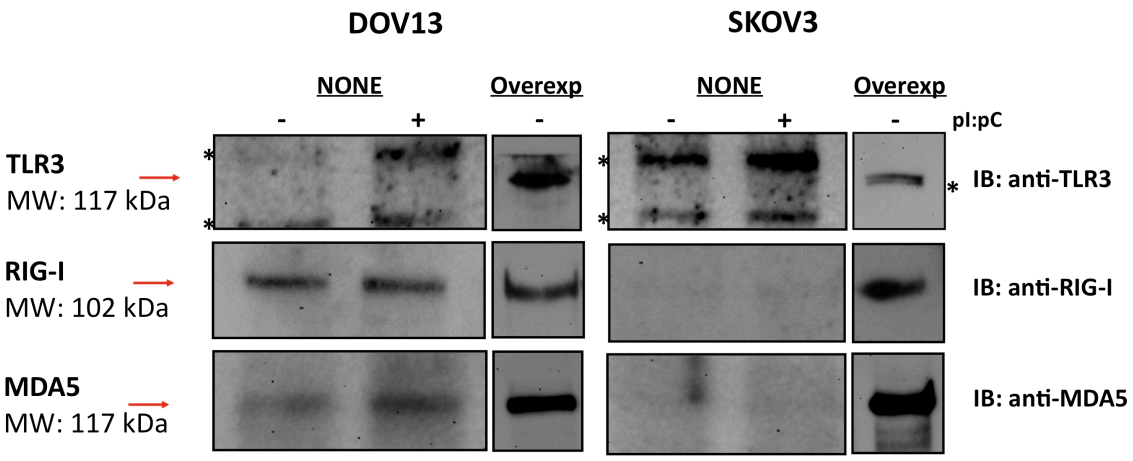


Figure 16. Overexpression of Pattern Recognition Receptors in non-responding cell lines. Ovarian cancer cell lines DOV13 and SKOV3 were (un)transfected with 2.5 μg receptor DNA. Twenty-four hours post-transfection, cells were (un)stimulated with 50 μg/ml pI:pC for 24 hours and whole cell lysates were collected. Receptor protein expression levels were analyzed via immunoblot, * designates non-specific bands.

Figure 17. Overexpression sensitizes cells

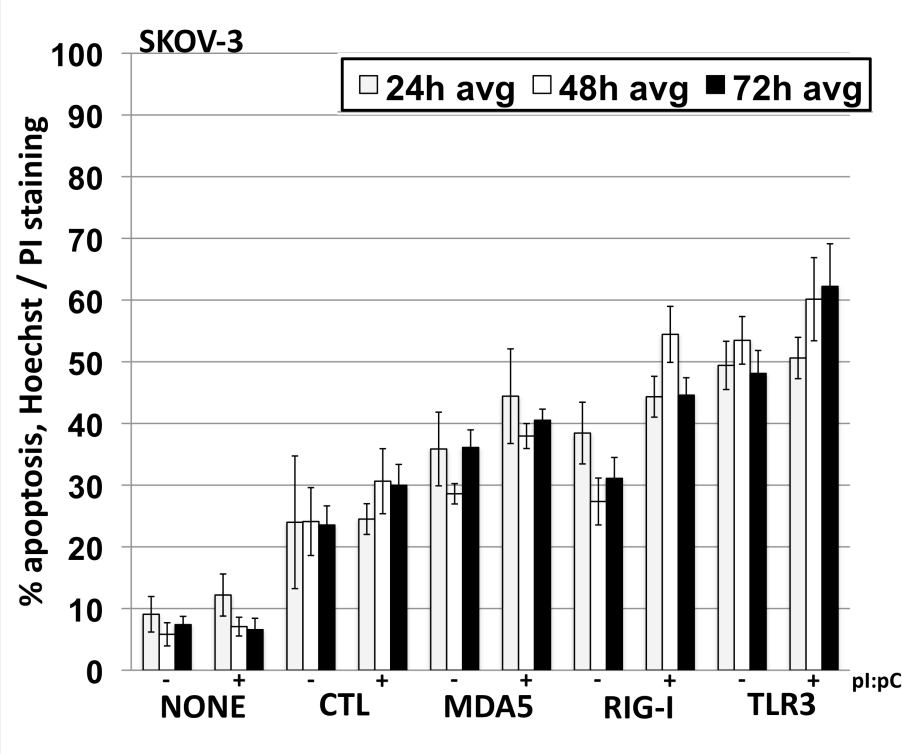
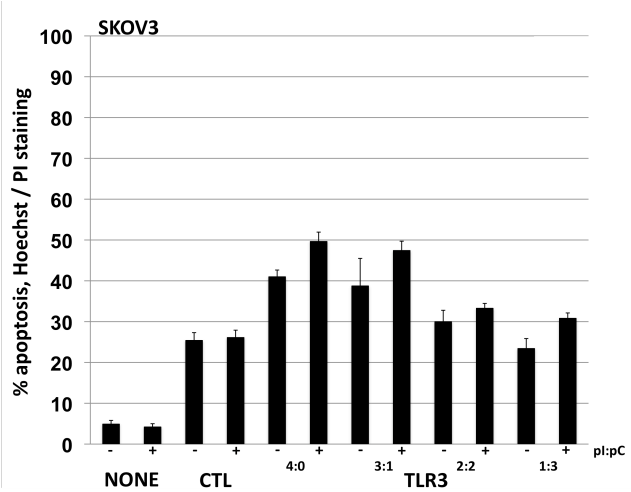


Figure 17. Overexpression sensitizes cells. Ovarian cancer cell line SKOV3 was (un)transfected with 0.5 μg receptor DNA or control DNA with Lipofectamine LTX. Twenty-four hours post-transfection, cells were (un)stimulated with 50 μg/ml pI:pC for 24, 48, and 72 hours. Hoechst / propidium iodide staining was used to quantitate apoptosis.

Figure 18. Titration of receptor DNA lessens sensitivity

A.



B.

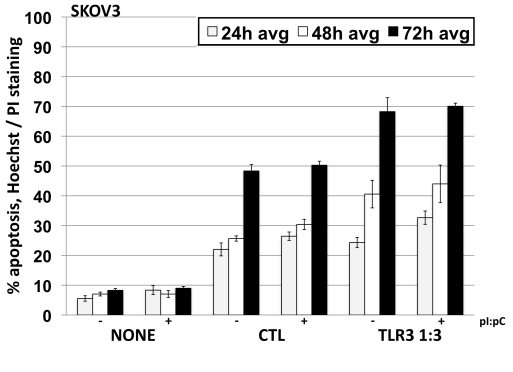


Figure 18. Titration of receptor DNA lessens sensitivity. SKOV3 cells were (un)transfected with 0.5 μg TLR3 DNA, empty vector (control) DNA, or a ratio of the two using Lipofectamine LTX. **A.** TLR3 DNA was titrated (4:1, 3:3, 2:1, 1:3) with control DNA at a total of 0.5 μg. Twenty-four hours post-transfection, cells were (un)stimulated with 50 μg/ml pI:pC for 24 hours. Hoechst / propidium iodide staining was used to measure apoptosis. **B.** TLR3 DNA was titrated with control DNA at a total of 0.5 μg. Twenty-four hours post-transfection, cells were (un)stimulated with 50 μg/ml pI:pC for 24, 48, 72 hours. Hoechst / propidium iodide staining was used to measure apoptosis.

The pre-stimulated cell death in the overexpressed cells decreased, but was still twice that of basal cell death in non-transfected controls at 24 hours. This amount of cell death continued to increase over the next 48 hours. Moreover, the empty vector control had higher pre-stimulated cell death than the non-transfected control suggesting that although we may have optimized the level of dsRNA receptor DNA additional parameters such as the vehicle delivery reagent must be assessed.

To determine if the transfection reagent was also contributing to the high amount of death seen in the cells, an apoptosis assay was conducted on several transfection reagents. Transit LT1, Fugene 6, and calcium phosphate were all shown to be less sensitizing to the cells (Figure 19A). The receptor MDA5 was overexpressed in DOV13 cells using Transit LT1 following identification of this non-sensitizing reagent (Figure 20). A slight increase above basal apoptosis in MDA5 overexpressed cells was detected 24 hours post-stimulation with 50 μg/mL pI:pC. Also, both the empty vector control and pre-stimulated MDA5 overexpressed cells had similar apoptosis levels as compared to non-transfected cells. Therefore, the effect of overexpressing the receptor could be directly interpreted. These preliminary results warranted further investigation. Thus, in both non-responsive cell lines, DOV13 and SKOV3, MDA5, RIG-I, and TLR3 were overexpressed with using Transit LT1 (Figure 21). Apoptosis increased in the stimulated, dsRNA receptor transfected cells. The pre-stimulated, overexpressed cells exhibited similar levels of apoptosis as compared to non-transfected controls. However, at 48 and 72 hours, the pre-stimulated, dsRNA receptor transfected cells increased their apoptosis levels compared to non-transfected controls.

Figure 19. Transfection reagents sensitize cells

A.

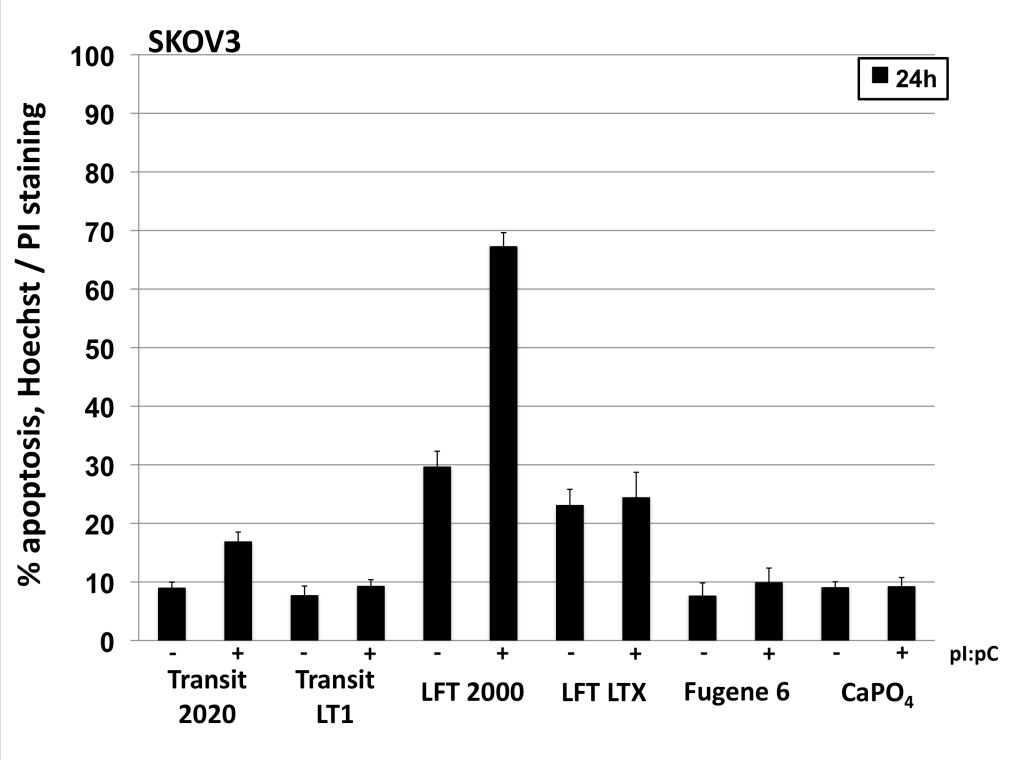


Figure 19. Transfection reagents sensitize cells. SKOV3 cells were seeded to 24-well plates and then (un)transfected with 0.5, 0.8, or 1 μg control DNA. Twenty-four hours post-transfection, cells were (un)stimulated with 50 μg/ml pI:pC for 24 hours. Hoechst / propidium iodide staining was used to measure apoptosis.

Figure 20. Transit LT1 does not sensitize cells

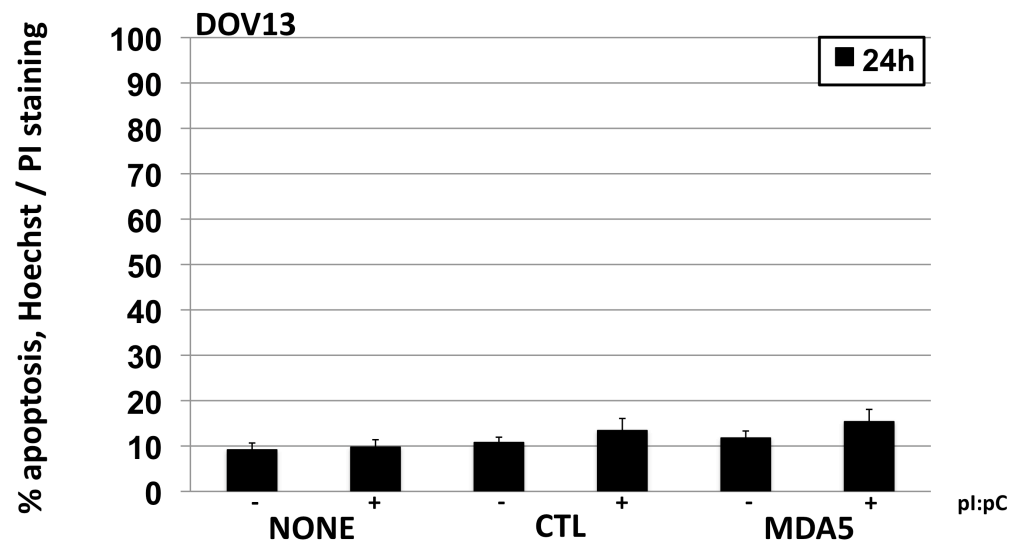


Figure 20. Transit LT1 does not sensitize cells. DOV13 cells were (un)transfected with 0.5 μg MDA5 or empty vector (control) DNA using Transit LT1. Twenty-four hours post-transfection, cells were (un)stimulated with 50 μg/ml pI:pC for 24 hours. Hoechst / propidium iodide staining was used to measure apoptosis.

Figure 21. Overexpression of receptors restores sensitivity to dsRNA stimulation

A.

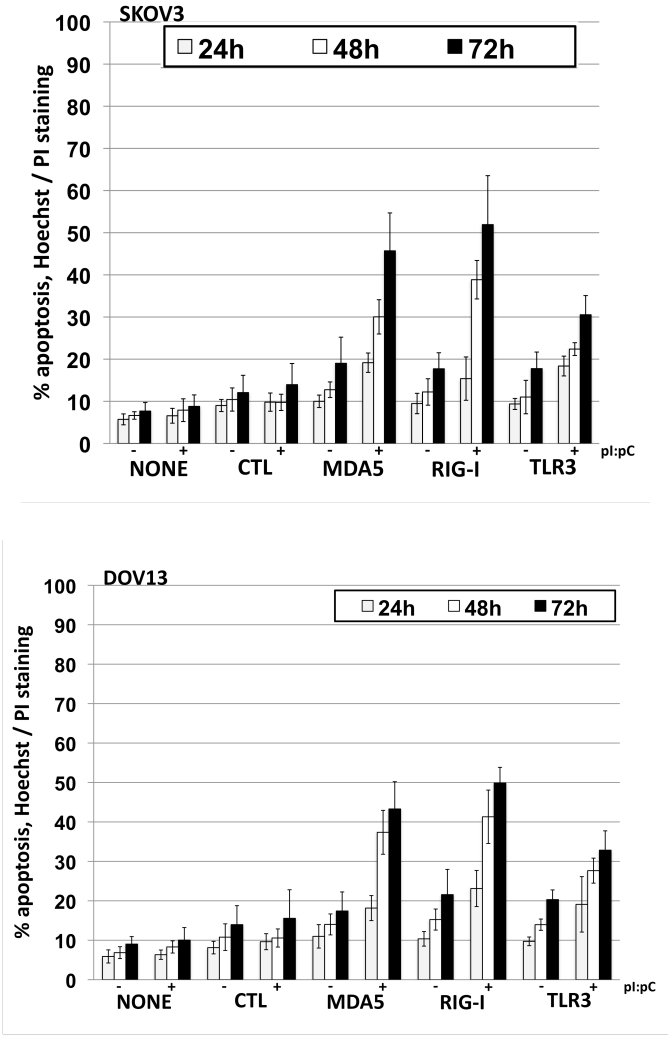


Figure 21. Overexpression of receptors restores sensitivity to dsRNA stimulation. SKOV3 or DOV13 cells were seeded to 24-well plates and then (un)transfected with 0.5 μg receptor DNA or control DNA using Transit LT1. Twenty-four hours post-transfection, cells were (un)stimulated with 50 μg/ml pI:pC for 24, 48, and 72 hours. Hoechst / propidium iodide staining was used to measure apoptosis.

This increase in apoptosis may have been due to the level of receptor protein being great enough to allow receptor multimerization that could initiate the receptor's signal pathways in the absence of a ligand. In a study conducted by the Brennan group, they showed that overexpression of the HER2/neu receptor led to both unregulated oligomerization and signaling[44].

To address this issue, a receptor DNA titration assay was conducted in SKOV3 cells (Figure 22A). Cells receiving 5 ng of receptor DNA had pre-stimulated apoptotic levels comparable to non-transfected controls. However, apoptosis did not increase post-stimulation until 72 hours in these cells. At 50 ng of receptor DNA, the pre-stimulated cell death was equivalent to non-transfected controls but also these cells had higher levels of apoptosis following stimulation with pI:pC. The 500 ng of receptor DNA allowed pre-stimulation apoptosis in a time-dependent manner above non-transfected control apoptotic levels. Thus, 50 ng of receptor DNA was the optimal amount of DNA with which to conduct the apoptosis assays. As our cell death assay assumes that all cells are equally expressing the transfected receptor, the transfection efficiency of our reagent, Transit LT1, and DNA concentrations were tested (Figure 22B). Results revealed that <2% of the cells were transfected with 5 ng of receptor DNA. The 50 and 500 ng receptor DNA titrations had similar transfection efficiencies at 33%. At our chosen DNA concentration for cell death assays, on average, only one in three cells will be overexpressing the target dsRNA receptor. Given this limitations, the cell death seen in Figure 8A is much more significant. If one assumes that transfected and untransfected cells are randomly distributed within the

well and during any given count of cell death the random distribution is also true, the largest percentage of cell death that could be observed in this assay would be 33%.

Figure 22. Titration of receptors lessens sensitivity

A.

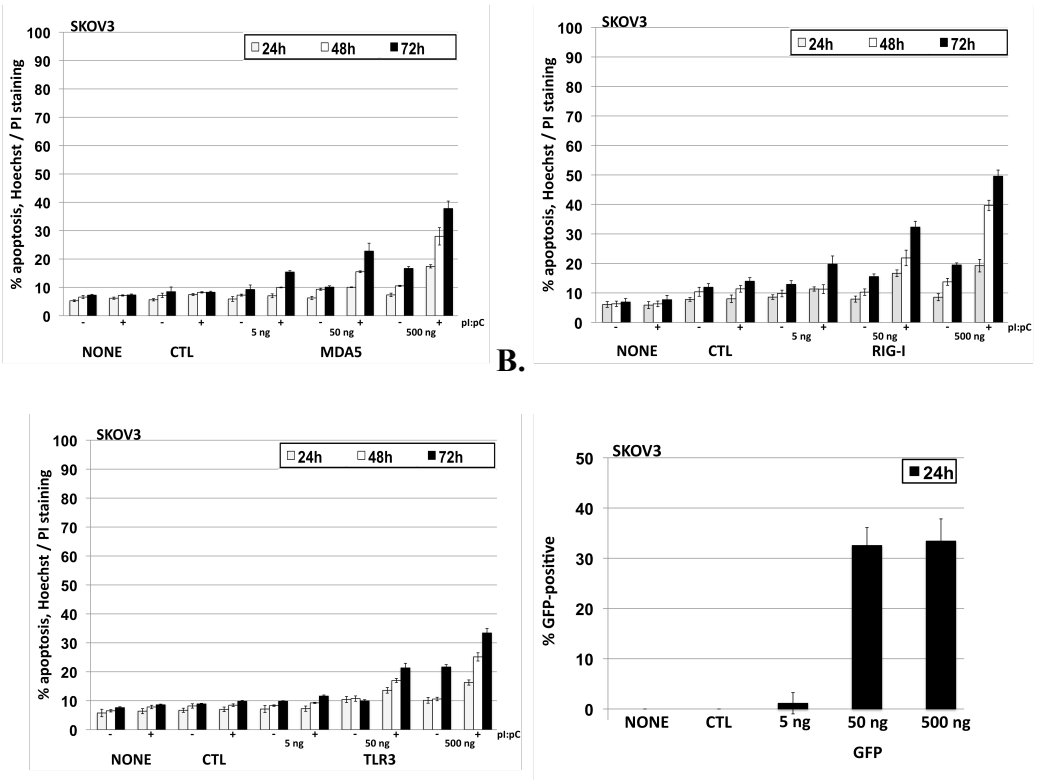


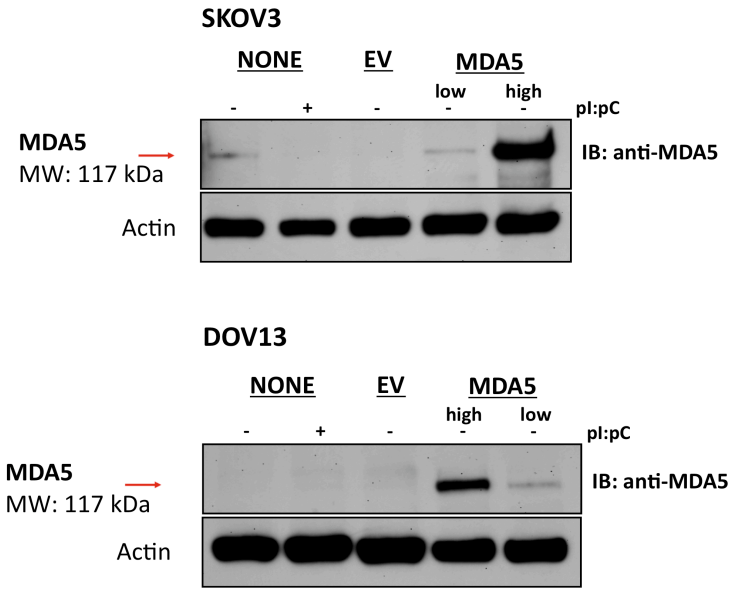
Figure 22. Titration of receptors lessens sensitivity. A. SKOV3 cells were seeded to 24-well plates and (un)transfected with 5, 50, or 500 ng of receptor DNA (MDA5, RIG-I, or TLR3) or 500 ng of control DNA using Transit LT1. Twenty-four hours post-transfection, cells were (un)stimulated with 50 μg/ml pI:pC for 24, 48, and 72 hours. Hoechst / propidium iodide staining was used to measure apoptosis. **B.** SKOV3 cells were seeded to 24-well plates and (un)tranfected with 5, 50, or 500 ng of GFP plasmid, or 500 ng of control DNA using Transit LT1. Twenty-four hours post-transfection, transfection efficiency was assessed via quantification of GFP fluorescence with a fluorescence microscope.

In our assays, RIG-I approaches this limit (31%) while MDA5 and TLR3 are slightly lower, 23% and 21%, respectively, suggesting that, when present, dsRNA receptors very efficiently induce cell death upon stimulation.

In responsive cell lines (CAOV3 and OVCAR3) a much higher level pI:pC-induced apoptosis is observed than in the non-responders with transfected dsRNA receptor. As mentioned above, this may be due to only one in three non-responsive cells receiving the dsRNA receptor DNA via transfection leading to a lower expression level of receptor in these cells as compared to responder cell lines. Immunoblots of MDA5 receptor overexpression in both SKOV3 and DOV13 cell lines were analyzed at 50 ng (low) and 500 ng (high) receptor DNA/transfection (Figure 23A) for receptor expression levels. The slight band in the pre-stimulated SKOV3 negative control is from a spillover of the adjacent positive control (not shown). Densitometry was used to compare the overexpressed receptor levels in the non-responsive cells to the pre- and post-stimulated endogenous receptor levels in the responsive cell lines (immunoblot not shown). The densitometry reveals that the low (50 ng receptor DNA) receptor expression level is equivalent to the pre-stimulation receptor expression level in responder cells lines and that the high (500 ng receptor DNA) mirrors the increased expression observed in the responder cell lines post-dsRNA stimulation. Figure 23B.

Figure 23. Overexpression of MDA5 in non-responders are comparable to upregulated endogenous levels in responders

A.



B.

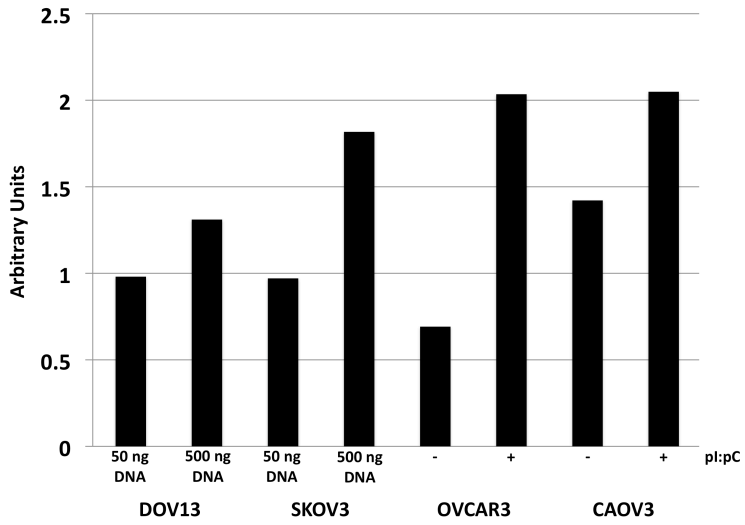


Figure 23. Overexpression of MDA5 in non-responders are comparable to upregulated endogenous levels in responders. A. SKOV3 and DOV13 were (un)transfected with .05 μg (low) or .5 μg (high) receptor

DNA. Twenty-four hours post-transfection, cells were (un)stimulated with 50 μg/ml pI:pC for 24 hours and whole cell lysates were collected. Receptor protein expression levels were analyzed via immunoblot. Note: the faint band in SKOV3 none (-) lane is from a spillover of the adjacent positive control (not shown). **B.** Densitometry was used to compare the overexpression levels in non-responders (unstimulated) to the endogenous levels in responders.

Chapter 4. Discussion

Double-stranded RNA has been shown to induce apoptosis in several cancer cell types via type I interferon production and caspase cleavage[45]. Previously, the four dsRNA receptors had not been analyzed in one study. Therefore, all of the studies to date have been incomplete analyses of the full complement of the dsRNA receptors in the dsRNA-induced responses. In addition, of the various cancer cell types, the responses of ovarian cancer lines have had limited evaluation in the presence of dsRNA. The significance of this study was that all four dsRNA receptors were analyzed for their role in dsRNA-induced apoptotic response and report these findings for a panel of ovarian cancer cell lines.

A previous experiment in the Bell laboratory revealed that two ovarian cancer cell lines (OVCAR3 and CAOV3) underwent apoptosis in response to pI:pC and two additional cell lines (SKOV3 and DOV13) did not. Analysis of the dsRNA receptor mRNA and protein expression levels revealed that the non-responsive cell lines had very low or undetectable receptor levels, whereas the responsive cell lines did have detectable receptor levels that were upregulated in the presence of pI:pC as analyzed via qPCR and immunoblot.

Based upon the results of the previous experiments, we hypothesize that all or a subset of dsRNA receptors are required to elicit a dsRNA-induced apoptotic response. To test this hypothesis the focus of this study was to alter the dsRNA receptor expression or

signaling ability of the receptors to examine if and what changes occurred in the dsRNA-induced apoptotic response. If essential receptors could be identified, then shared signaling components downstream of the receptors may be identified as essential for the dsRNA-induced apoptotic response as biomarkers for chemotherapy. Several approaches were taken to identify if all or a subset of dsRNA receptors are essential.

Two approaches were taken in the responsive cell lines to identify necessary dsRNA receptors for dsRNA-induced cell death. First, the responsive cell lines were treated with selective ligands to examine individual receptor contribution. Next, the dsRNA receptors of the responsive cell lines were individually knocked down with either siRNA or shRNA and cells were then treated with pI:pC to analyze the dsRNA-induced apoptotic response. In the non-responsive cell lines, we examined if any of the dsRNA receptors could restore sensitivity to dsRNA-induced cell death. In the non-responsive cell lines, individual dsRNA receptors were overexpressed, treated with pI:pC, and then assessed for the dsRNA-induced apoptotic response.

The selective ligand assays in the responsive cell lines were used to determine receptor contribution to dsRNA-induced apoptosis. Perrot et al. in 2008 conducted an assay to analyze type I interferon response in immune cells overexpressing either TLR3, MDA5, or RIG-I upon stimulation with pI:pC or pA:pU[43]. They found that Polyadenylic-polyuridylic acid to be a selective agonist to TLR3[43]. It was therefore used to determine TLR3's contribution to dsRNA-induced apoptosis. At 24 hours, the amount of cell death caused by pA:pU was comparable to that of pI:pC at the same concentration, 27% death. After 24 hours however, the cell death induced by pI:pC continued to increase to 50% at

48 and 72 hours, whereas the death induced by pA:pU reached a plateau at 24h. This seems to indicate that TLR3 is not solely responsible for the dsRNA-induced cell death in the responsive cells. When TLR3 binds ligand, this leads to signaling that up-regulates the cytosolic dsRNA receptors, MDA5, RIG-I, and PKR. When pI:pC was the ligand, it was delivered directly into the cells' media and taken into the endosome via the scavenger A receptor. Once inside the endosome it was recognized by TLR3 but also escaped from the leaky endosome into the cytosol where it was recognized by the cytosolic dsRNA receptors and led to increased signaling and therefore increased cell death. However, when pA:pU was the ligand, the cytosolic receptors were most likely still upregulated, but were unable to recognize pA:pU and were therefore unable to increase signal, and consequently, cell death reached a plateau. In the selective ligand assay for PKR, an inhibitor 2-Aminopurine, was used. A titration to examine the optimal concentration of inhibitor to use in the ovarian cancer cell lines revealed that 5 mM 2-aminopurine reduced phosphorylation of eIF-2α to basal levels. A concentration of 50 mM 2-aminopurine was cytotoxic. Inhibition of PKR did not lessen or inhibit the dsRNA-induced cell death in the responsive cells. We concluded that PKR is not essential for the dsRNA-induced apoptotic response.

Transient receptor knockdown via siRNA technology was used also to determine individual receptor contribution to the dsRNA-induced apoptotic response. This technology was employed because it was a widely used, effective approach to study individual components of a complex system. RIG-I mRNA levels were knocked down 34%, whereas MDA5 mRNA levels were knocked down 32% in OVCAR3 cells using Lipofectamine 2000. When DharmaFECT was used as the delivery vehicle for the siRNA,

RIG-I mRNA levels in CAOV3 cells was knocked down 16%. However, comparison of receptor siRNA treated to scrambled siRNA treated both plus pI:pC, the knockdown was 34%. Knockdown of ~70% was targeted to significantly reduce receptor level and conduct the cell death assay. Using Lipofectamine 2000 and DharmaFECT did not achieve this level of knockdown so the receptor's role in the dsRNA-induced apoptotic response could not be assessed. Thus, a third transfection reagent, Transit TKO, was examined for increased knockdown efficiency. A control experiment revealed that adding in siRNA reduced cell proliferation. A plating control was conducted to keep cell confluency uniform across all treatments. Due to numerous inconsistencies in the mRNA analysis of the receptor knockdown, protein knockdown levels were assessed. The knockdown of RIG-I was found to be 55% when compared to stimulated untransfected cells, but was 67% when compared to stimulated scrambled control. This was an acceptable level of knockdown, so a cell death assay was conducted. Cell death was not blunted by reduced expression of RIG-I 24 hours post-stimulation with pI:pC. At 48 h and 72h post-dsRNA stimulation, all cells receiving lipid vehicle and pI:pC had undergone cell death. Therefore, it was concluded that the lipid vehicle may have adhered to cells after multiple washes and aided in the entry of pI:pC into the cytoplasm leading to the increased cell death. Alternative methods were considered such as nucleofection and stable knockdown using lentivirus. Stable knockdown assays were undertaken to address individual receptor contributions to dsRNA-induced cell death.

Stable receptor knockdown analysis was used after transient knockdowns were found to be ineffective in the cell lines. Lentiviral delivery was chosen because it did not

utilize sensitizing lipid vehicles. If successful, new cell lines absent of receptor would be created and could be used for future assays. In addition, the virus used delivers three shRNA sequences to the cells and the cells were infected at two different multiplicities of infection, making efficiency of knockdown more likely. The findings revealed knockdowns of 85%, 54%, and ~48% for TLR3, MDA5, and RIG-I, respectively. Double-stranded RNA-induced cell death was decreased to about 50% at 48h and 72h following pI:pC stimulation for the TLR3 and TLR3/MDA5 knockdowns. Knockdown of RIG-I did reach high enough levels to accurately assess its contribution to the dsRNA-induced apoptotic response and it did not reduce the dsRNA-induced cell death. From these findings, TLR3 appears to be important for the dsRNA-induced apoptotic response. Reduction of MDA5 expression was not as high as TLR3 and receptor protein was still present when analyzed via immunoblot. Therefore, MDA5's contribution to the reduction in cell death seen in the TLR3/MDA5 double knockdown cannot be adequately assessed. One way to further analyze dsRNA-induced cell death with our current cell lines would be to take advantage of receptor localization. We normally deliver pI:pC directly into the cells' media so that it can be taken into the endosome by the scavenger A receptor. Thus, TLR3 is the first to recognize pI:pC. The cytosolic dsRNA receptors are also able to recognize pI:pC when it leaks from the endosome. Polyethyleneimine (not lipid based) could be used to deliver pI:pC directly into the cytoplasm, by-passing TLR3. Using the TLR3 knockdown cell line, we could then assess if direct stimulation of RIG-I and MDA5 could restore the high levels of apoptosis seen in the wild type cells.

Overxepression of the receptors in the non-responsive cell lines was able to restore sensitivity to pI:pC. The increase in the amount of apoptosis was 32% for RIG-I, 22% for MDA5 and 21% for TLR3 72 hours post-stimulation when the overexpression was titrated to 50 ng of DNA, which can be partially explained by the transfection efficiency assay using plasmid-GFP. It revealed that only one in three cells were transfected. In addition, an alternative method that could be used in the future is transfection of a reporter gene under a tet promoter and a stable selection marker. This would allow a cell population with homogenous reporter gene to be selected and then expression induced simultaneously with dsRNA stimulation to mimic responder cell lines' dsRNA response. Our current results reveal that there are functional pathways in the non-responsive cell lines that are now activated and causing apoptosis. While it is unclear by what mechanism these cells are dying, future experiments will be carried out to elucidate the pathways these cells are now activating. Previous experiments in the Bell laboratory revealed that the non-responsive cell lines did not cleave caspase 3 and did not produce detectable interferon beta. Future experiments such as caspase 3 cleavage immunoblots in cells overexpressing dsRNA receptors may be conducted. Also, an ELISA may be conducted to determine if the cells with overexpressed receptor can now produce detectable interferon beta. The cells were able to respond to pI:pC when any of the receptors (TLR3, RIG-I, or MDA5) were overexpressed, which is indicative of a redundant system. This redundancy is not surprising as viruses continually attack these pathways to subvert our innate immune response[46].

Overall, the experiments in the responders revealed that knockdown of TLR3 greatly reduced the amount of dsRNA-induced cell death when pI:pC was delivered directly into the media. Death was not brought back down to basal, however. This finding was consistent with the pA:pU selective ligand assay. Both results show that TLR3 is important for eliciting a dsRNA-induced apoptotic response, but is incapable of controlling the entire response. From these experiments, it appears that TLR3 is responsible for initiating the dsRNA-triggered signaling cascade, and the amount of cell death observed at that point is high, but not as high as it could be with the other receptors. The TLR3 signal leads to the up-regulation of MDA5, RIG-I, PKR, and itself to increase signaling. This is where the largest amount of dsRNA-induced cell death is observed. To insure that the importance of TLR3 is not an artifact of pI:pC delivery method, the experiments should be conducted using a vehicle, like polyethyleneimine, to transfect pI:pC directly into the cytosol. If there would be observations similar to the results presented here, the importance of TLR3 would be strengthened. From this study, it was shown that TLR3, RIG-I, and MDA5 could induce cell death in all of the ovarian cancer cells upon dsRNA synthetic analog stimulation. The significance of this work is that TLR3, MDA5, and RIG-I, could be possible targets for individualized chemotherapeutic regimens for women with ovarian cancer expressing these receptors.

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APPENDIX A

"ANALYSIS OF POST-TRANSLATIONAL MODIFICATIONS OF SIKE"

Abstract

Innate immunity is the first line of defense following host infection with pathogen. One important signaling pathway in the innate immune response is the Toll-like receptor 3 signaling pathway. Toll-like receptor 3 (TLR3) recognizes double-stranded RNA (dsRNA), a replication intermediate of certain viruses, and initiates a signaling cascade leading to production of type-I interferon. The TLR3: interferon signaling pathway contains a kinase complex that consists of two kinases, I-Kappa-B Kinase Epsilon (IκBε) and TANK-binding kinase 1 (TBK1), and a scaffold, NAK-associated protein 1 (NAP-1). An inhibitory protein, silencer of inhibitor of κBα (SIKE), has been identified in the signaling pathway in association with the kinase complex. SIKE's mechanism of inhibition has yet to be defined. Understanding of the post-translational modifications of SIKE will offer new insight into its possible mechanism of inhibition. The goal of this work was to determine the post-translational modifications of SIKE upon stimulation with polyinosinic-polycytidylic acid (pI:pC, a synthetic dsRNA analog). In execution of the objective, HEK 293 cells were transiently transfected with hemagglutinin-tagged SIKE and stimulated with

pI:pC. The lysates were immunoprecipitated with anti-hemagluttinin beads and immunoblotted with anti-phosphoserine to look for phosphorylation. SIKE was found to be phosphorylated after 24h of pI:pC stimulation. Multiple bands were detected above the phosphorylated SIKE band, possibly indicating ubiquitination. If the presence of dsRNA in the host leads to a signal for SIKE's inhibition to be released, phosphorylation may be that signal. Future assays will determine the phosphorylation kinetics of SIKE to determine the earliest time at which SIKE becomes phosphorylated when in the presence of dsRNA. The work presented here details a post-translational modification of SIKE that may reveal further details of SIKE's function.

Chapter 1: Introduction

The protein silencer of inhibitor of κBα (SIKE) negatively regulates the Toll-like receptor 3/Interferon regulatory pathway exclusively. It's mechanism of inhibition is yet undefined. What is known, however, is that under physiological conditions SIKE and TANK-binding kinase 1 (TBK1) interact with each other, but in the presence of dsRNA this interaction is lost[1]. The possible signal for SIKE to dissociate from the kinase complex and allow it to signal is unknown. It is the goal of this project to help determine the signal via post-translational modification analysis.

Chapter 2: Materials and Methods

2.1 Cell culture

The HEK293 cells were a gift from Dr. Donald M. Segal (National Institutes of Health - NIH). HEK293 cells were cultivated in complete media (RPMI 1640) supplemented with 10% low endotoxin fetal bovine serum, 5% L-glutamine, 5% non-essential amino acids (Sigma), 5% sodium pyruvate, 5% HEPES (all purchased from Invitrogen Gibco) at 37°C and 5% CO_2 under strictly endotoxin-free conditions. Cells were used for less than 20 passages in continuous culture.

2.2 Transfection

HEK293 cells ($4x10^5$ cells/well) were seeded to 6-well plates in 2 mLs of complete media. After 24 hours, the cells received fresh complete media and were then transfected with 4 µg of hemagglutinin-tagged SIKE DNA or 4 µg of empty vector DNA (hemagglutinin-tagged pCDNA3.1) using Lipofectamine 2000 (Invitrogen) as per manufacturer's protocol. Twenty-four hours post-transfection, the cells received complete media alone or complete media plus naked polyinosinic-polycytidylic acid (pI:pC – Sigma P0913) at 50 µg/mL. After 24 hours, the media was removed from the cells and then they were rinsed in ice cold, sterile 1X PBS. Then the cells were lysed with 100 µL of cold lysis buffer comprised of 20 mM Hepes, pH 7.4, 150 mM NaCl, 135 mg ß-glycerophosphate, 1.5 mM $MgCl_2$, 10 mM NaF, 1M DTT, 1 mM $NaVO_4$, 2 mM EGTA, 1 mM PMSF, 1X

protease inhibitor (Complete-EDTA free Protease inhibitor tablet, Roche), 0.5% TritonX-100, and 1 mg/mL N-ethylmaleimide (Buffer 1). The cells were scraped from the plate using a sterile cell scraper and pipetted into microcentrifuge tubes. The lysates were kept on ice and vortexed on medium speed for 1 second every 10 minutes for 40 minutes. Cell debris was separated from soluble sample via centrifugation at 14,000 rpm for 10 minutes at 4 ºC. The supernatant was pipetted into new microcentrifuge tubes and stored at -20 ºC for short-term storage or at -80 ºC for long-term storage.

2.3 Immunoprecipitation

HEK293 cells ($4.5x10^5$ cells/well) were seeded in 6-well tissue culture plates and transfected 24 hours later with 4 µg of target DNA using Lipofectamine 2000 as per manufacturer's protocol. Cells labeled for stimulation received 50 µg/mL pI:pC 24 hours post-transfection. Unstimulated cells received complete RPMI 1640 media 24 hours post-transfection. The cells were lysed 24 hours post-stimulation in IP buffer (Buffer 1). Protein concentrations were determined via Bio-Rad protein assay protocol. Equal amounts of protein were added to 40 µLs of resuspended anti-HA agarose beads (Sigma). To obtain the resuspended resin, the anti-HA beads were spun in centrifuge at 2,000 rpm, 4ºC for 2 minutes. The ethanol was removed and 1 mL of IP buffer (Buffer 1) was used to resuspend the beads. They were spun twice at the same conditions again and the IP buffer was removed. The beads were then resuspended in 40 µLs of IP buffer. Lysates and resuspended resin were brought up to 1.25 mLs in IP buffer and incubated overnight at 4ºC on an orbital rotator. The following day, the resin was recovered via centrifugation at 3,000 rpm at 4ºC for 2 minutes in 500 µL of IP buffer. The supernatant was removed and the

resin was resuspended in 75 µL of NUPAGE® LDS sample buffer 2X (Invitrogen) loading dye. The samples were boiled at 95ºC for 3 minutes. Then the samples were spun twice at 10,000 rpm for 5 minutes at 4ºC. The supernatant was pipetted into a new tube with 1.5 µL of 2-mercaptoethanol added.

2.4 Immunoblotting

The samples were separated on 8% SDS-polyacrylamide gels and then transferred to Hybond ECL nitrocellulose membrane (Amhersham Biosciences) or PVDF membrane (Bio-Rad). The blots were blocked in 3% milk/ 1X TBS + 0.1% Tween-20 for 1 hour at room temperature with agitation. Primary antibodies were diluted as follows overnight at 4ºC with agitation:

1. anti-phosphoserine 4A4 Ab (Upstate), 4 µg/mL in 3% BSA/ 1X TBS + 0.1% Tween-20
2. anti-Hemagglutinin Ab (Covance), 1:1,000 in 3% milk/ 1X TBS + 0.1% Tween-20

The following day the blots were washed three times with 1X TBS + 0.1% Tween-20 buffer for 5 minutes per wash at room temperature with agitation. HRP-conjugated secondaries were diluted as follows:

1. Anti-mouse (Santa Cruz), 1:5,000 in 3% milk/ 1X TBS + 0.1% Tween-20

The blots were washed 3 times for 5 minutes each in 1X TBS + 0.1% Tween-20 buffer. Immunoblots were developed using ECL-PLUS reagents (GE Healthcare).

Chapter 3: Results

To analyze the post-translational modifications of SIKE an immunoprecipitation assay was conducted. HEK 293 cells were transiently transfected with hemagglutinin-tagged SIKE and stimulated with pI:pC for 24h. The lysates were collected and then immunoprecipitated with anti-hemagglutinin beads. The success of the immunoprecipitation was confirmed via immunblot for anti-hemagglutinin. Another immunoblot of the immunoprecipitated SIKE protein looked for phosphorylation using anti-phosphoserine. This blot revealed that SIKE becomes phosphorylated following 24h stimulation with pI:pC (Figure 1).

Figure 1. SIKE is phosphorylated in the presence of pI:pC

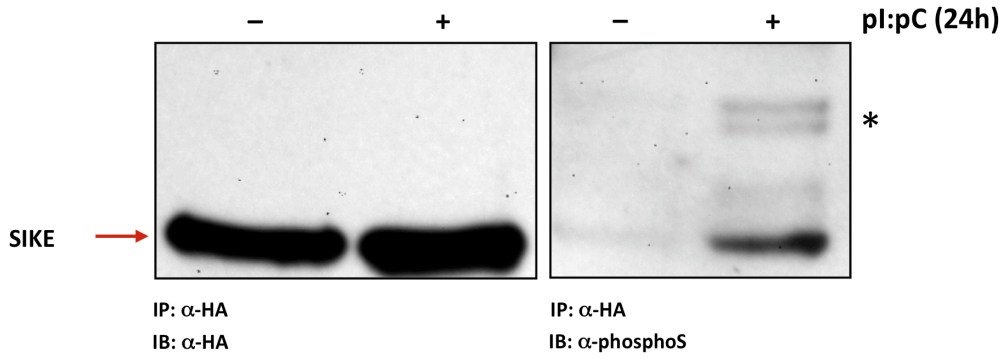

Figure 1. SIKE is phosphorylated in the presence of dsRNA. HEK 293's were seeded to 6-well plates and then transfected with 4.0 μg of HA-tagged SIKE DNA. Twenty-four hours post-transfection, cells were (un)stimulated with 50 μg/ml pI:pC for various hours and whole cell lysates were collected. The SIKE protein was then immunoprecipitated with anti-HA resin. Protein expression levels were analyzed via immunoblot, * designates possible ubiquitinated bands.

Chapter 4: Discussion

SIKE is an inhibitory protein known to associate with the TBK1/IKKε kinase complex of the TLR3 interferon pathway under physiological conditions and upon stimulation with dsRNA this association is lost[1]. It was determined that in this project the possible signal for SIKE's release of the kinase complex may be phosphorylation of SIKE. The immunoblot of phosphorylated SIKE revealed what appeared to be a laddering effect. This may be indicative of ubiquitination of SIKE once phosphorylated. The long-term goal of this project is to determine the time at which SIKE is first phosphorylated and then to conduct an ubiquitination assay to determine if SIKE becomes ubiquitinated once phosphorylated that would then signal for SIKE degradation. Further understanding of SIKE's post-translational modifications may help elucidate how the host controls this essential anti-viral response pathway.

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VITA

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