The Adjuvant Properties of RNA Origami for Immunotherapy in a CT26 Cancer Model

by

Ryan Rodriguez del Villar

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Graduate Supervisory Committee:

Yung Chang, Chair
Xiaowei Liu
Xiaodong Qi

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ABSTRACT

The properties of adjuvants to stimulate an immune response to treat cancer has sparked a major area of research in the field of immunotherapy. Given the presence of multiple RNA sensors in mammalian host cells for eliciting innate immunity, synthetic RNA nanostructures present a unique opportunity for adjuvant exploration. While RNA nanostructures are organic and biocompatible in nature than other adjuvants, they could be tailored to have desired structural stability and functional diversity for *in vivo* application. In this study, a rectangular RNA origami nanostructure was designed to contain double-stranded RNA motifs and possess high structural stability. Using *in vitro* assays, RNA origami was shown to stimulate the toll-like receptor 3 (TLR3) signaling pathway, which has been reported to activate antigen presenting cells (APCs), natural killer (NK) cells, cluster of differentiation 8 (CD8) T-cells, and the secretion of proinflammatory cytokines. To explore RNA origami as an adjuvant for cancer immunotherapy, intraperitoneal administration of a murine colon cancer cell line (CT26) was used as a model system to mimic peritoneal metastasis (PM), in which RNA origami was investigated for its activities in mitigating PM tumor microenvironment and improving anti-tumor immunity. Given the poor outcome of the patients with PM and urgent need for new interventions, this study aims to translate the adjuvant activities of RNA origami demonstrated *in vitro* into potent anti-cancer immunotherapeutics. Here, it was shown that multiple intraperitoneal injections of RNA origami could inhibit tumor growth, leading to a significant delay and/or regression of metastatic tumor growth in the peritoneum. Furthermore, tumor-free mice, after being treated with RNA origami, were also resistant to a second challenge of tumor cells, indicating the development of the adaptive anti-tumor immunity. This immunity is

dependent on T-cells since nude mice succumbed to tumor growth with or without RNA origami treatment. Thus, RNA-origami can function as an adjuvant to activate the innate immunity and subsequently the adaptive anti-tumor immunity, leading to tumor regression. Conceivably, RNA origami could be explored as an immunotherapeutic agent to improve the disease outcome of patients with peritoneal metastasis and peritoneal carcinogenesis.

DEDICATION

This thesis is dedicated to my friends and family that helped me through this journey:

To my parents who raised me, helped me grow, guided me, and supported me in all my endeavors. It is their unconditional love and faith that has helped me reach where I am at today.

To my brother who is always there for me and has helped me grow as both a person and in faith.

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PREFACE

The scope of this thesis is to present a novel RNA-based adjuvant that could activate the host immune system to fight against cancer. Specifically, one objective is to demonstrate that a self-assembled RNA origami structure can be used as an immuno-adjuvant to elicit an immune response that can combat cancer. The second objective is to test the feasibility of peritoneally injected CT26 colon cancer cells as a model for peritoneal metastasis to examine the adjuvant's activities in the context of a highly immunosuppressive tumor microenvironment. Third objective is to evaluate the use of intraperitoneal treatments as a regiment to treat peritoneal metastasis that has very poor prognosis and shows unmet therapeutic needs. Overall, this thesis is intended to explore the adjuvant activity of self-assembled RNA origami for the application of cancer immunotherapy against a CT26 metastasized colon cancer model.

CHAPTER 1

Nucleic acid origami. Nucleic acids (NA) have been defined as one of the key building blocks of life and one of the cornerstones of the central dogma. They are present in both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). In turn, DNA and RNA are universally found as genetic material in all organism. This takes the shape in several different forms depending if it is DNA or RNA. DNA is needed for coding genes and being a genetic template for transcribing all the components of a cell and an orgasm (Lodish, 2016). RNA is more diverse in function where it is needed for the translation of proteins, has the potential to regulate genes, and can be converted back into DNA in certain cases (Lodish, 2016). It is these abilities and applications for nucleic acids that have made them a key area of interest in how they can be used both naturally in the body and even synthetically as a point of medicine. The advancements in technology and the collection of knowledge in fields such as biology, chemistry, and physics have led to ways of analyzing and manipulating DNA and RNA, both in terms of their natural functions and also their structural abilities. One of the most prominent areas that this has led to is the formation of nanoparticles and nanostructures, specifically in this case, origami structures designed either as two-dimensional or three-dimensional shapes through complementary base pairing (Lodish, 2016). The unique component of nucleic acid origami is that it normally does not use DNA or RNAs abilities to code, but instead how they can be assembled structurally. The notion of this idea came about in the 1950's by Richard Feynam who challenged scientist to manipulate and control things on a nano and atomic scale (Lee, 2016, p. 4199). This would not come to fruition until almost the 90's by Dr. Nadrian Seeman. Dr. Seeman proposed ideas that would incorporate the complementary properties

of Watson-Crick base pairing (adenine paring to thymine and cytosine pairing to guanine) of DNA to create specifically designed structures, as shown in *Figure 1* (Seeman, 1982, p.

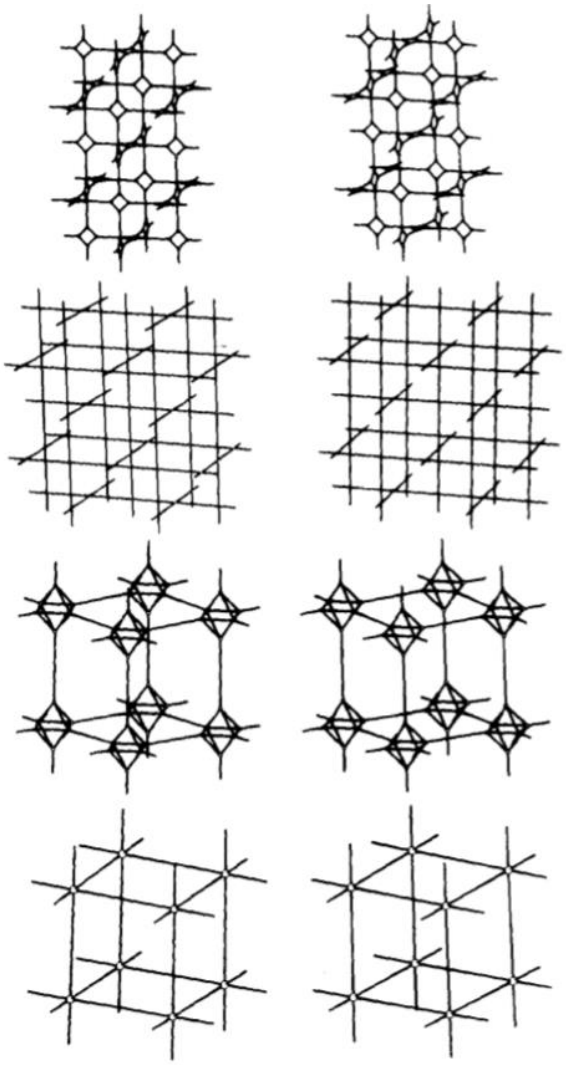

Figure 1. Initial DNA origami designs. Dr. Nadrian Seeman's concept for structures where the lines represent the double-stranded DNA (dsDNA) segments (Seeman, 1982, p. 239).

237). The ideas proposed by Dr. Seeman would then foster the creation of a wide-range of other structures. His initial intention was to use the DNA structures to target various types of bio-molecules, but as the complexity and intricacy of these origami shapes became more complex, as shown in *Figure 2*, so did their possible applications. The potential of DNA nanoparticles has not only led to designing uniquely sized and shaped origami structures,

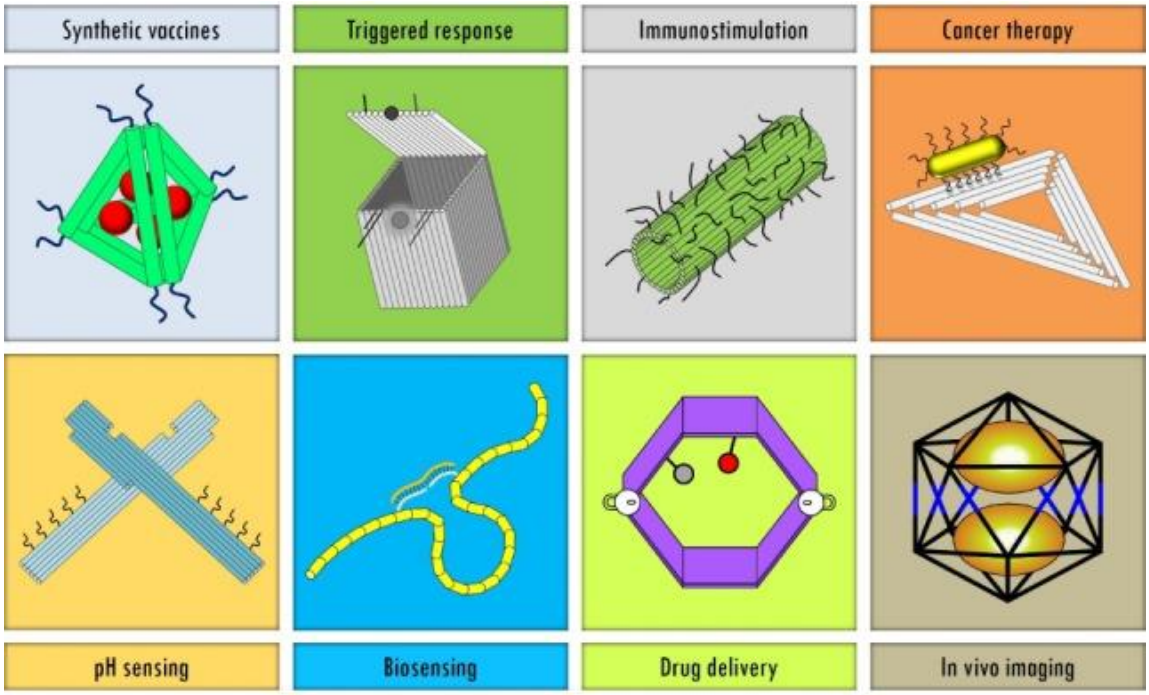

Figure 2. Applications of DNA origami. Various structures and how they can be designed for various biological and medicinal purposes (Chandrasekaran, 2016).

but origami that have certain modifications and abilities that can be utilized in a vast number of ways. These include the medical applications, such as being a vehicle to deliver drugs for different types of diseases and cancer, the development of synthetic vaccines, and as an adjuvant for immunotherapy (Chandrasekaran, 2016). Additionally, DNA origami has been implemented in disease diagnosis as well: being a sensor for certain biomarkers, detectors in the variation in pH, and tools that can aid in imaging and quantifying situations that are happening *in vivo* (Chandrasekaran, 2016). As seen above, the applications for DNA origami structures are immense and the potential for them continues to grow. Although the use of DNA in nanoparticles is a major focus of research currently, it is not the only nucleic acid structure that is found in cells. In comparison to DNA, the functions and structures of RNA have been relatively unknown and even misunderstood (initial only viewed as a transitional unit from DNA to protein and was more challenging to

detect/measured since it is not contained to one region and are short in length) (Lodish, 2016). More recent discoveries though have started to show that RNA based structures are much more dynamic and involved in more functions than DNA. Various types of RNA found naturally in a cell continue to be discovered as well as their purposes, ranging from the more common types such as ribosomal RNA, messenger RNA, and transfer RNA, which play key roles in translation, to lesser known kinds such as small interference RNA, non-coding RNA, and microRNA, which are utilized in gene regulation, splicing, and RNA interference, and viral RNA which can be reverse transcribed into DNA and elicit immune responses in foreign hosts (Lodish, 2016). These applications for RNA are just a small part of a much larger picture. In turn, RNA as a nanoparticle is much more appealing than DNA, not just because it could possibly take advantage of some of these naturally occurring features of RNA, but also can be structurally manipulated, like DNA, into origami. The problem with the use of RNA origami compared to DNA origami is that, in general, DNA is more stable than RNA. This is due to DNA lacking the hydroxyl group on the second carbon on the pentose ring on the ribose sugar. Since RNA has the hydroxyl group, it is more susceptible to degradation by hydrolysis. Despite this, the current technology has allowed for the design and synthesizing of strands, permitting for certain origami shapes to be created to counter this and make the RNA origami as stable as DNA origami if so desired (Han, 2017).

Immunotherapy as cancer treatment platform. The term immunotherapy has been described as modulating the host's immune system to treat a disease either by amplifying the response or suppressing it (Owen, 2009). The applications and developments of immunotherapy have been around for centuries where early forms of

medicine harnessed the immune system to fight infectious diseases. The actual intensive purpose of specifically targeting the immune system as a form of treatment though has not been explored until the last few decades (Owen, 2009). The potential of using the host's immune system to fight off disease has really become a key point in research in several different fields. This includes battling various types of ailments: immunotherapy to modulate allergies (extreme food allergies, asthma, or rhinitis), reduce autoimmunity (autoimmune disease or organ transplants), and now most prominently as a tool to fight cancer (Jones, 2014, p. 318) (Rotrosen, 2002, p. 17). The origins of using immunotherapy against cancer goes back to the late 1800s under the scientist William Coley who tried to treat cancer with bacteria by having them elicit an immune response (Yang, 2015, p. 3335). While the thought of immunotherapy as a therapeutic treatment for cancer has existed for years, the results have been limited. The reason for this is that many instances that try to elicit the host's immune cells to destroy the cancer have difficulty due to the tumor's ability to avoid recognition by appearing as cells that are a part of the host's system and develop various strategies to actively suppress immune responses (Yang, 2015, p. 3335). To counteract this issue, many different approaches have been taken which involve applying antibodies that block immunosuppressive cells, adoptive transfer of engineered T-cell (chimeric antigen receptor (CAR) T-cells) that can recognize target specific cancer antigens with high affinity, and the use of adjuvants: a substance that can provoke and activate the host immune responses (Yang, 2015, p. 3336). The latter of those mentioned, the adjuvant, provides a unique form of immunotherapy where it could elicit a strong immune response such that the host immune system's tolerance may be alleviated to some extent. In addition, adjuvants can be combined with other forms of treatments, such as

chemotherapy, radiation therapy, cancer vaccines, checkpoint inhibitors, and other treatments to synergize the therapeutic efficacy. For a broader comparison, a general and widely featured adjuvant is alum ("Vaccine adjuvants", 2011). Although it has been clinically approved and capable of eliciting an immune response, it elicits the humoral immune system (stimulates B-cells and antibodies) and has low T-cell activity. The problem with this is that the humoral response cannot effectively fight tumor cells, a cellular immune response (T-cells) is necessary for tumor mitigation. This can be a common issue with other types of immunostimulatory adjuvants, hence not making them ideal candidates for anti-cancer immunotherapy. On the other hand, cellular immunity could be induced by a variety of adjuvants that are derived from pathogen associated molecular patterns (PAMP), which are often procured from microorganisms and even part of host cellular components that are known as "danger signals", e.g., uric acids (Schlee, 2016, p. 566). These components bind and engage pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), retinoic acid inducible genes (RIG), retinoic acid-inducible gene-I-like receptors (RIG-I), nucleotide-binding oligomerization domain-like receptors (NOD), and others, to activate the innate immunity (Parham, 2015). The PAMP-PRR interactions in antigen presenting cells (APCs) promotes APC maturation and activation, and secretion of pro-inflammatory cytokines, which in turn helps activate the adaptive immune response, as illustrated in *Figure 3* ("Vaccine adjuvants", 2011).

Nucleic acids can act on many of PPRs, including TLR3 (for endosomal dsRNA), TLR7/8 (for endosomal single stranded RNA (ssRNA)), TLR9 (for endosomal CpG-containing DNA), RIG-I (for small cytoplasmic double stranded RNA (dsRNA)) and melanoma differentiation associated (MDA) genes (for binding to large cytoplasmic

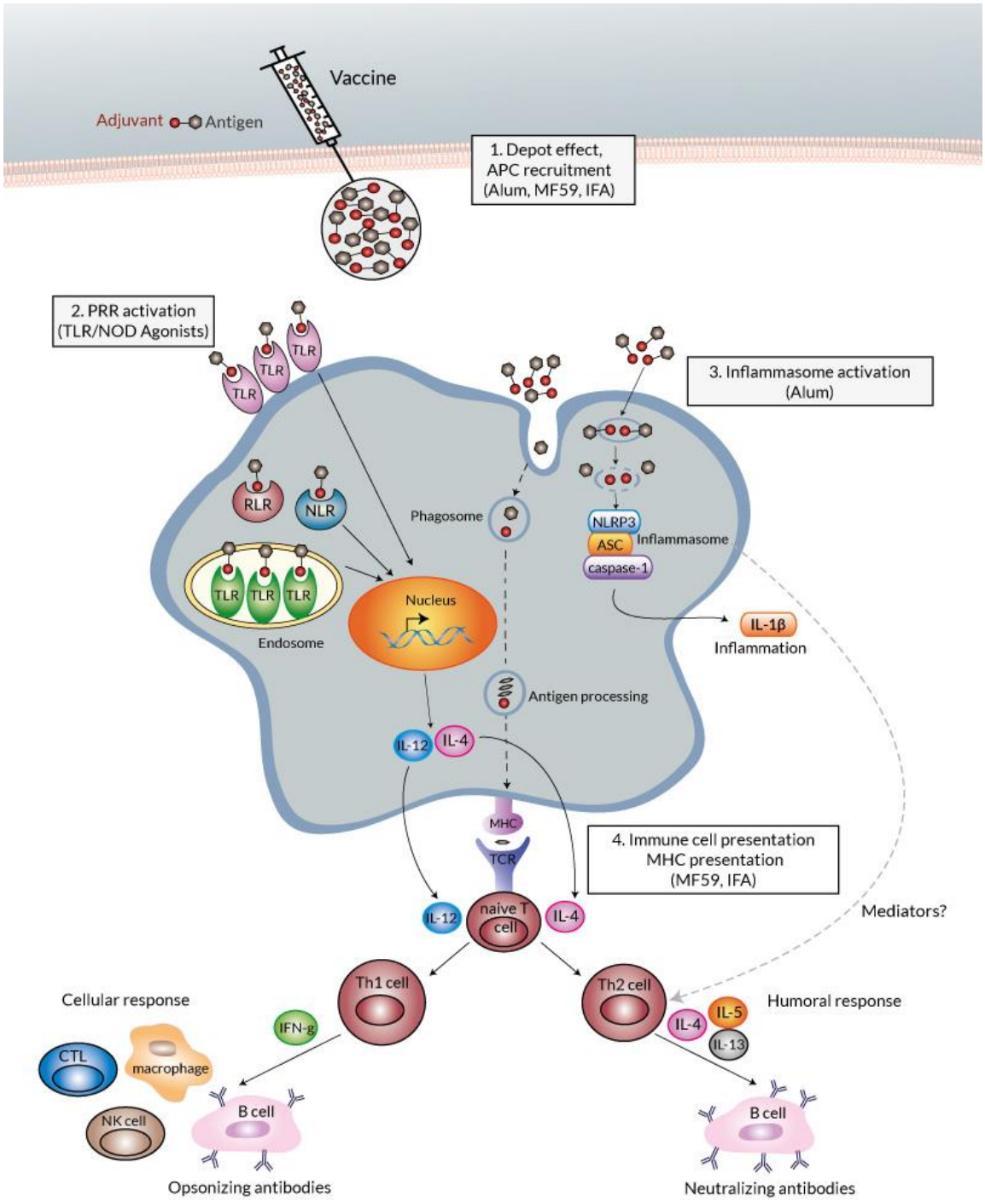

Figure 3. Adjuvant immunotherapy. Shows how an adjuvant can be incorporated and its effects on the immune system. The adjuvant (alum) is able to elicit an innate response causing inflammation and promotion of the adaptive immunity in conjunction with an antigen. The adaptive immunity generates antibodies (humoral response) leading to immunity (“Vaccine adjuvants”, 2011).

dsRNA), or stimulators of interferon (IFN) genes ((STING) for cytosolic DNA) (see *Figure 4*) (Schlee, 2016, p. 569). Although NAs can engage these PRRs to stimulate both innate and adaptive immune responses to eliminate pathogens and remove danger signals, they could also inflict autoimmunity. Thus, to avoid this dire consequence, the host cells, especially immune cells, are wired to maintain homeostasis by regulating various processes, such as degradation of NAs, activation pathways, and negative feedback (also seen in *Figure 4*) (Schlee, 2016, p. 568). Looking at these pathways that can be used in

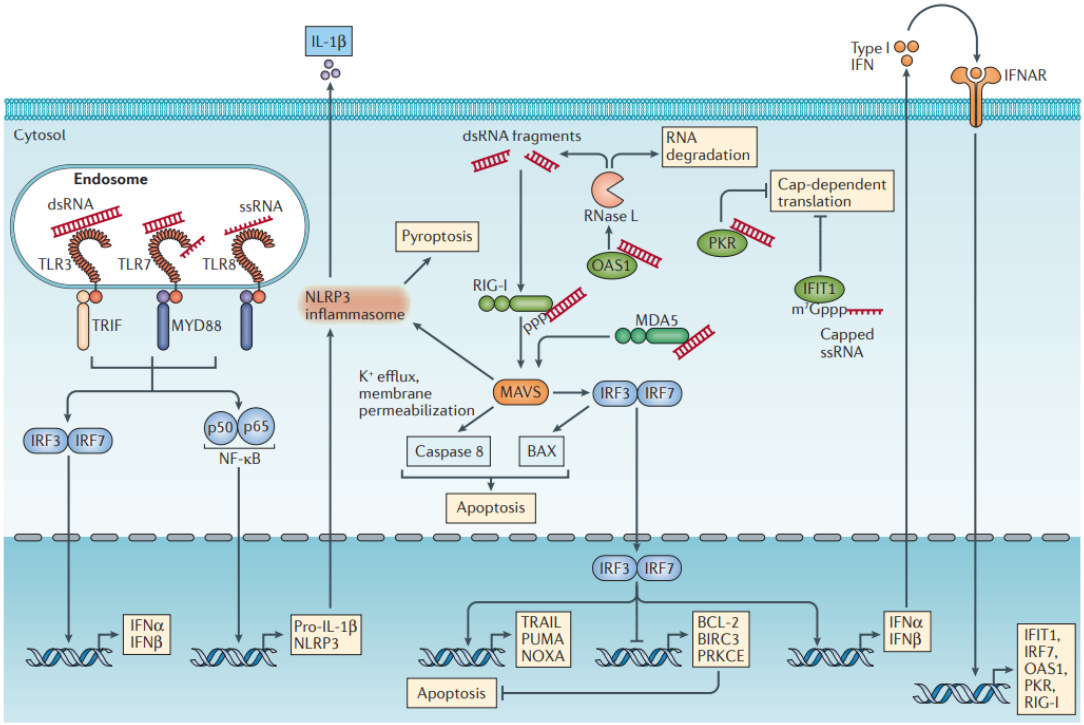

Figure 4. Viral RNA PRR pathways. It illustrates the various receptors that RNA can initiate and the resulting responses. Shows how TLR3 can lead to the upregulation of interferons, which can help elicit an immune response (Schlee, 2016, p. 568).

immunotherapeutic approaches, it begs the question of what is the current state of immunostimulatory adjuvants. These revelations from studying other adjuvants have driven the research into studying nucleic acids since they can initiate signaling pathways that elicit immune responses, seen in response to viral and bacterial nucleic acid strands in

a host. This leads to the question as to why choose RNA over DNA as the immunostimulatory therapeutic. Both of these use base-pairing principles and can be designed as nanoparticles, where DNA has substantially influenced the applications of RNA as a nanoparticle (Jasinski, 2017, p. 1142). Like RNA, DNA can also interact with PRRs to initiate an immune response. The most studied one is CpG DNA that is enriched in bacteria genome. However, one of the drawbacks to using CpG DNA as an adjuvant is that it does not induce a substantial response in humans (Heidenreich, 2014, p. 372). This is due to the expression of TLR9, which is a sensor for CpG DNA and is limited in its cellular distribution in humans (Heidenreich, 2014, p. 372). On the other hand, although DNA can be sensed via the STING pathway to activate innate immunity, its activation in the absence of CpG motif is not very robust (unpublished observation), therefore, making DNA nanostructures less feasible for being a functional anti-cancer adjuvant. Instead, DNA-nanostructures have been exploited as a vehicle for drug delivery or targeted immune modulators (molecules/peptides that target other molecules, such as T-cell, B-cell, or immune cell markers) (Schuller, 2011, p. 9696) (Li, 2018, p. 258).

RNA nanostructures have recently emerged as another robust platform for biomedical application, such as delivery of siRNA, tumor-targeting, and carriers of CpG-motif to function as adjuvants (Jansinski 2017). Another key components that helps differentiate RNA from DNA is that general structure of dsRNA has increased thermostability compared to dsDNA due to the sugar conformations in the RNA and the folding of the secondary structures (dsDNA form usually B-helix structures while dsRNA forms A-helix structures, which results in a longer structure with different groove sizes and sugar conformations) (Jasinski, 2017, p. 1142). This stability gives it another upper hand

over DNA since it has the chance of remaining in the host organism's system for longer periods of time, allowing for greater exposure to the immune system. Besides DNA as a nucleic acid adjuvant, this primarily leaves RNA structures to be tested as an adjuvant. In addition to having to test various other structures similar to RNA origami to see if they too could be utilized as adjuvants, there are two other prominent RNA nanostructure that has been tested for its immunostimulatory properties: polyinosinic-poycytidylic acid (Poly(I:C)) and a dsRNA mimic structure named ARNAX. Poly(I:C) has been looked at as a potential adjuvant for several years. It is a synthetic construct that is similar to dsRNA. The difference is that Poly(I:C) integrates inosinic acid bound to cytidylic acid instead of some of the traditional base pairs for RNA (Feng, 2018). These changes result in a mismatched dsRNA structure and have been shown to elicit immunostimulatory effects (Feng, 2018). The only major concern with Poly(I:C) is that it has been shown to have toxic side effects when used in high doses (Ammi, 2015, p. 120) (Gralnick, 1983, p. 146). Some clinical trials that have been conducted using Poly(I:C) have revealed that high levels of toxicity are present in the host, which led to symptoms such as fever, hypertension, flu-like symptoms and high pain/inflammation in certain parts of the body (liver, nervous system, bone marrow) (Ammi, 2015, p. 121). The research that has been conducted with Poly(I:C) and its ability to lead to toxic side effects led to the development of ARNAX. ARNAX is still in early stages of research, but initial reports show that it can stimulate the immune system through the TLR3 pathway (Seya, 2016). It was intentionally designed to target the TLR3 pathway because with Poly(I:C) it can interact with RIG-I and MDA5 and lead to cytokinemia, which can contribute to the elevated toxicity (Seya, 2016). In order to do this, they modified dsRNA to have a 5'-cap of phosphorothioated GpC DNA and kept the length

of the structure relatively short (140 base-pairs), where both conditions would help prevent it from activating the other two pathways (Seya, 2016). Overall, results have illustrated that it can elicit an immune response and activate NK cells without provoking the cytokinemia, but the findings are still preliminary, and more analysis needs to be conducted (Seya, 2016).

Development of cancer testing model. The efforts to combat cancer have been around since the dawn of modern medicine, and yet the struggle is still very real. There have been great improvements regarding cancer survivors and the options for treatment, but despite this, the main problems with cancer are how diverse and how broadly the disease is defined (DeSantis, 2014, p. 252). Since cancer development results from accumulation of mutations and changes in a cell, which lead to rapid and uncontrolled division of the cell, the ways it can arise are almost endless (Lodish, 2016). This means many types of cancers can exist with the most prominent in humans being prostate (43%), colorectal (9%), and melanoma (8%) in men and breast (43%), uterine (8%), and colorectal (8%) in women (DeSantis, 2014, p. 252). In addition to the breadth of cancer types, the disease can tolerate the organism's immune system due to the cancerous cells not being viewed as foreign since it is usually derived from the host's native cells (Parham, 2015). In turn, this and, more prominently, the broadness of the term cancer itself makes it challenging to generate a cancer model that is both applicable to various types of cancer, but clinically relevant when translating the models to clinical studies. Thusly, many cancer models have been developed to focus on a specific type or component of a cancer and how it can be treated. Many models include cancer cell lines from known types that can be grown, modified, and applied in modeling systems, both *in vitro* and *in vivo*. This includes methods such as injecting the cancer cells into a specific region of a model organism such

as inoculating it into the peritoneal cavity or subcutaneously for more of an external growth (Groza, 2018) (Frampas, 2011, p. 147). This is relatively easy to perform and quantify, but the drawback is that in many cases they do not completely illustrate how that cancer interacts when it naturally occurs, which can lead to clinical irrelevance. To counteract this, other strategies involve orthotopic models, i.e. the cancer is grown then either injected or engrafted onto the site that the cancer line arises (Tseng, 2007, p. 484). The problem with this is that these methods are technically challenging and can be hard to replicate successfully. In addition to these, other models have tried to replicate cancer be inducing it into the model organism either by a carcinogen or a viral compound (Connor, 2018) (Zhang, 2014, p. 695). This method can lead to a plethora of other complications namely, low mutations rates, off-target effects, difficulties in monitoring, and the development of other types of cancers in the host. Due to the development of various ways to model cancer, the path to choosing one really depends on the system of the treatment and the type of cancer that is the primary target for the therapeutic. Based off this, a cancer model can be developed that can illustrate whether the treatment works but also simplifies the process of studying it with reduced variables and, ideally, clinical applications.

CHAPTER 2

RNA ORIGAMI PROPERTIES AND FUNCTION

Structural components of RNA origami. RNA origami nanoparticles started to be designed in the early 2000's. The potential for designing them was influenced by the ability to assemble various structures of DNA and seeing how that could be similarly applied to RNA (Jasinski, 2017, p. 1142). As opposed to DNA, most naturally occurring RNA in a cell are single-stranded RNA (ssRNA). Since this is the case, the single-stranded RNA typically folds onto itself at various areas due to the complementary base pairs interacting, in turn, resulting in unique structures (motifs) to form, as shown in *Figure 5*.

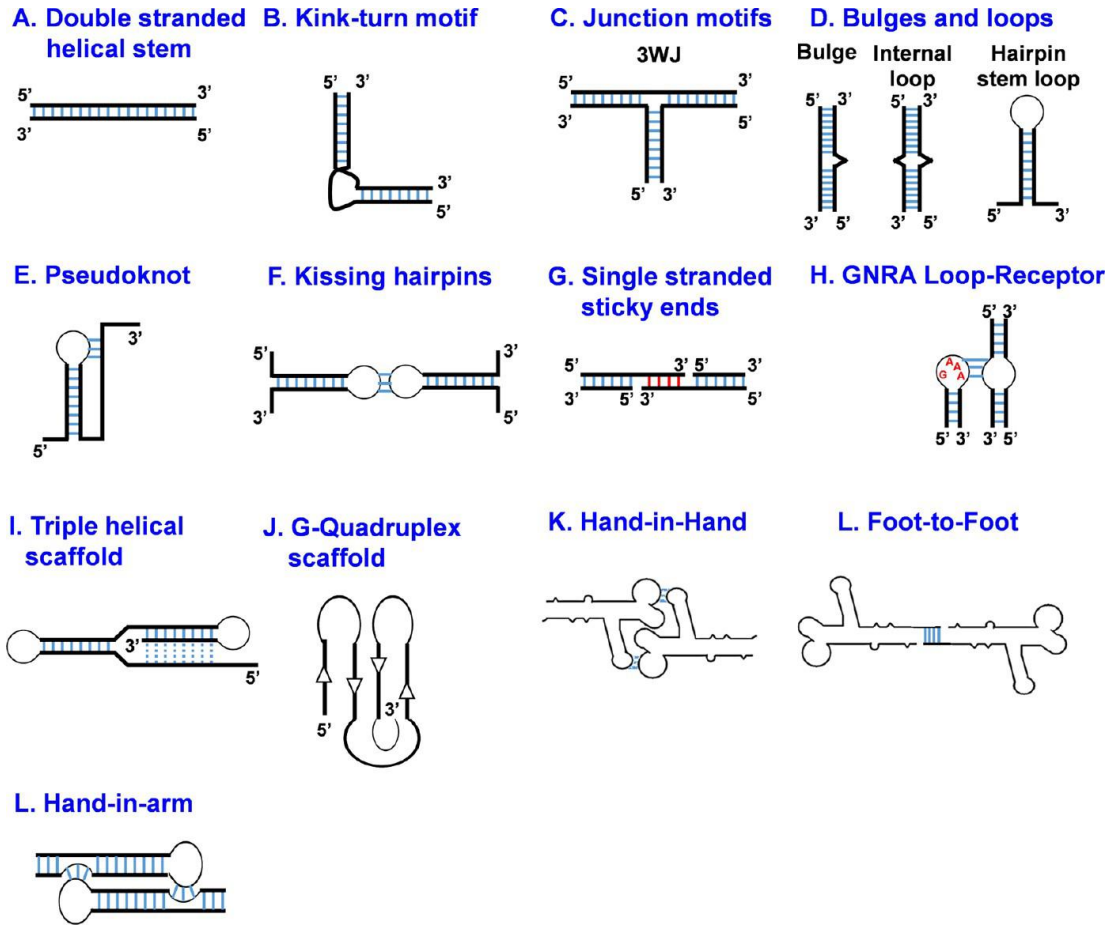

Figure 5. Various structural motifs for RNA. These motifs can be used and analyzed to find ways to assemble RNA nanoparticles (Jasinski, 2017, p. 1142).

These various motifs can impact how the RNA is utilized in the cell as well as its ability to function (Parham, 2015). Due to this phenomenon, the ability to assemble RNA as nanoparticles was first looked at by how RNA strands could be designed to form different structural motifs. These various motifs have shown how RNA strands can be stabilized by folding on itself in various ways and how the RNA functions naturally due to its variations in structure (Jasinski, 2017, p. 1142). Based off the analysis of how RNA naturally forms these motifs, these structures helped lay the groundwork for how more complex shapes could be assembled for RNA origami. This led to the development of various techniques to generate RNA origami involving RNA that self-assembles based-off structural motif interactions (uses packaging RNA to interact with itself to form the shape), and by intentionally analyzing specific motifs to find ways for them to intermingle to derive a specific structure (Jasinski, 2017, p. 1142). From these methods on how to design RNA origami structures and the influence from DNA nanostructures, many different RNA origamis have been developed, as shown in *Figure 6* (Shu, 2013, p. 767). Beyond using structural motifs to make RNA origami, strategies for developing it from the ground up have been developed by computer-generated sequences. These can integrate single-stranded strains, double-stranded strains, short fragments, long strands, or a combination of these. This adds another layer to the structural development of the origami allowing for even more complex shapes and nanoparticles that can vary greatly in terms of size, as seen in *Figure 7* (Han, 2017). Based off the shape, components, and the size of the RNA nanoparticles, RNA origami has been explored for many different applications. These include designing carriers for drug delivery (Agudelo, 2013), incorporating siRNA for interference to regulate gene expression (Qiu, 2013**,** p. 12952), using it as a marker for

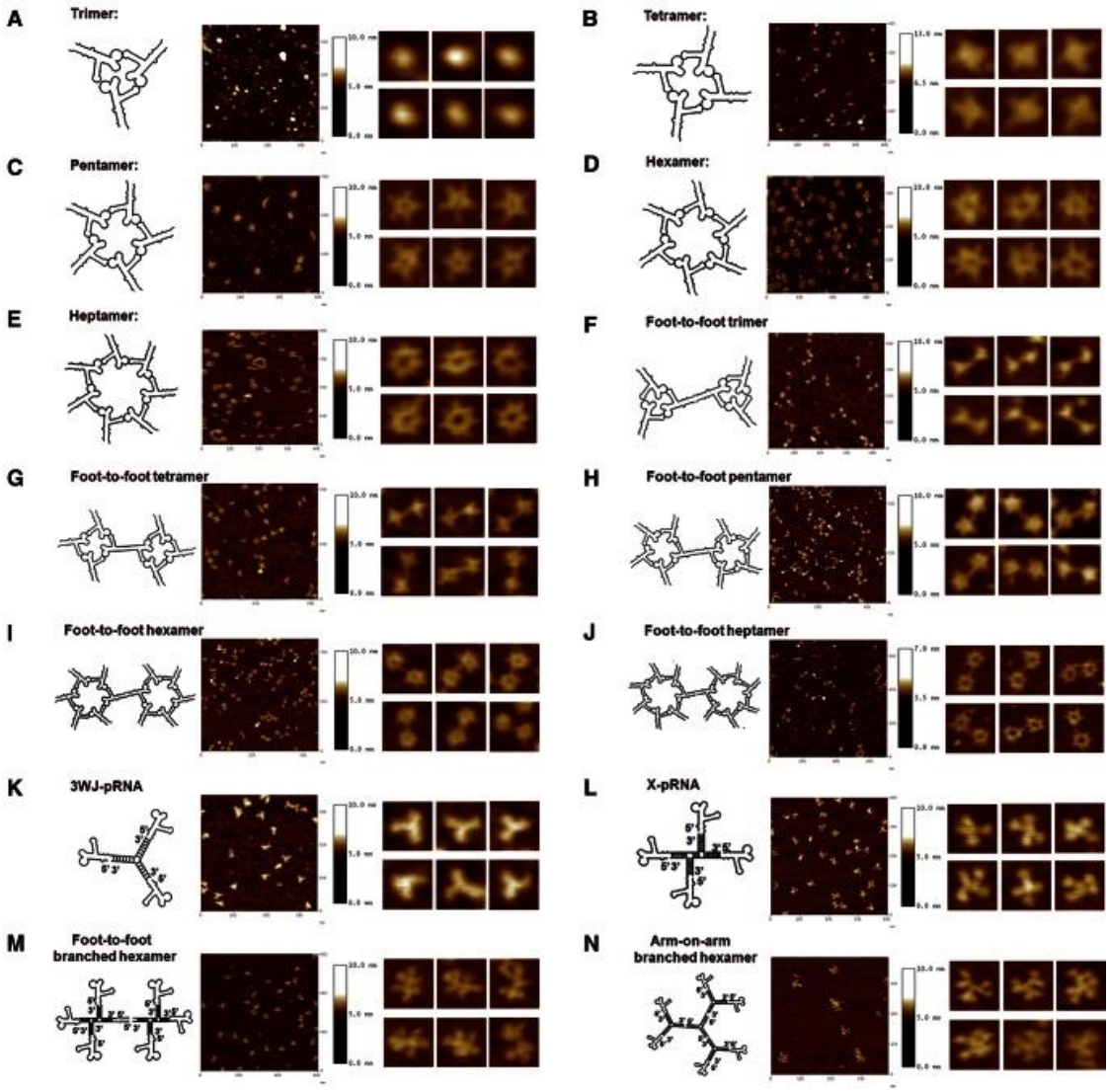

Figure 6. Motif-based RNA origami. These used packaging RNA with hand-in-hand motifs to self-assemble in bacteriophages. The structured were imaged using atomic force microscopy (AFM) (Shu, 2013, p. 771).

monitoring biological applications and diagnostics (Cui, 2015), and testing for targeted delivery of RNA origami to tumor cells (Rychahou, 2015, p. 1108). These examples illustrate that RNA origami has the potential for numerous applications. For focus of the study in this thesis, RNA origami nanostructures were examined for its potential to enhance immune reactions. The RNA origami used here was developed by the Hao Yan lab, courtesy of Dr. Xiaodong Qi. The structure provided and experimented with was a

rectangle that was comprised of linearized dsRNA folded onto itself. The process for the design and assembly was conducted by Dr. Xiaodong Qi, which is roughly illustrated in *Figure 8* as well as the resulting structure.

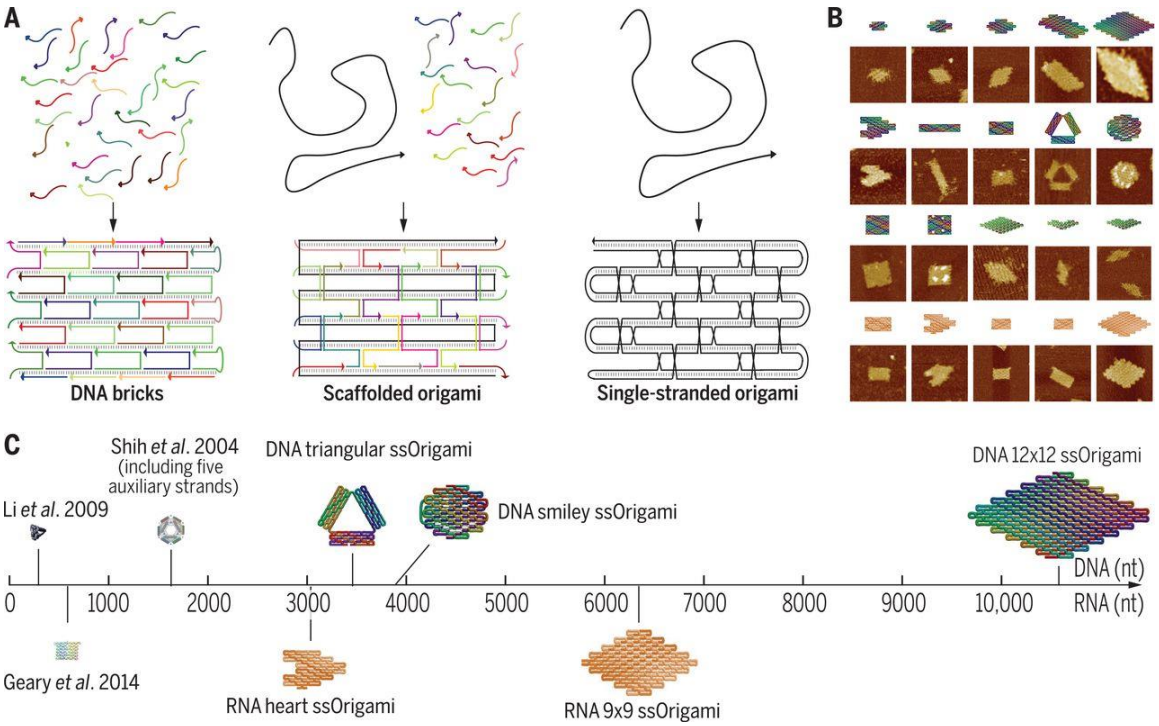

Figure 7. DNA origami and RNA origami assembly and structures. (A) illustrates how different size strands can be used together to start to assemble a structure. (B) shows the many types of shapes that can be created (top three rows are DNA and last row is RNA). (C) plots how size can vary from one type of origami to another and can be considered for a desired purpose (Han, 2017).

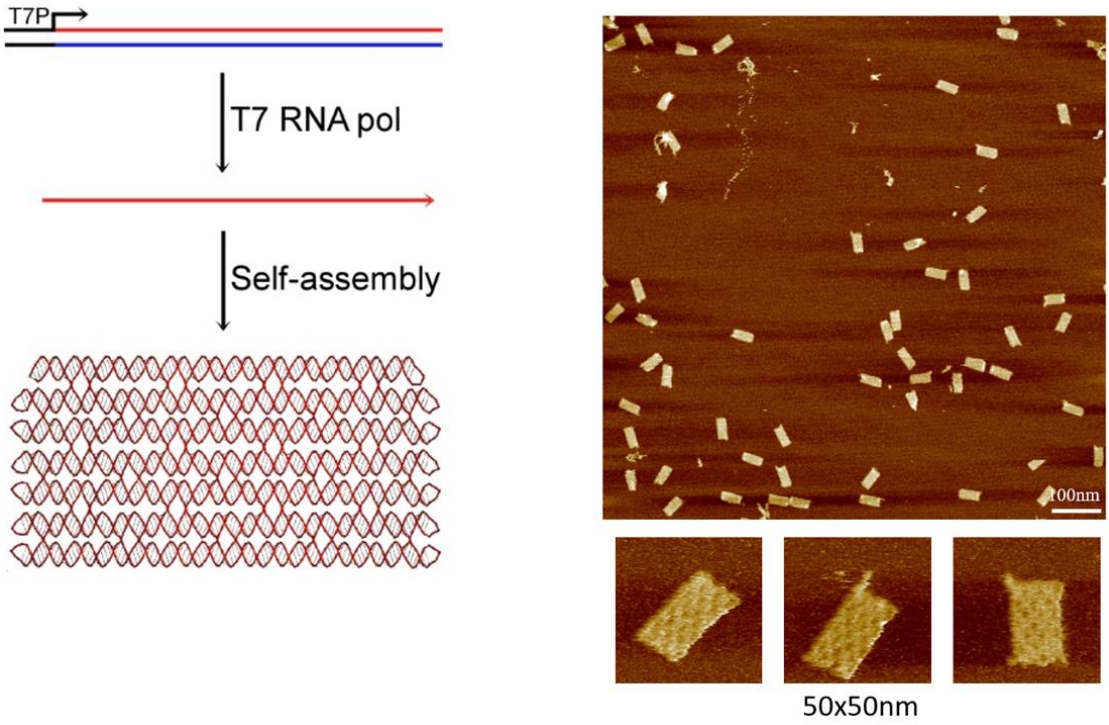

Figure 8. RNA origami adjuvant structure. The illustration shows the basic design principles for the rectangular RNA origami structure and the final structure, which was imaged using atomic force microscopy (AFM), kindly provided by Dr. Xiaodong Qi.

RNA origami functions as an adjuvant. Here, the goal was to focus on the exploration of RNA origami as an immunological adjuvant for anti-cancer treatment. The prospect of RNA as an adjuvant arises from the realization that some viruses carry dsRNA or ssRNA, these can be sensed by host cells to trigger a series of host defense reactions, including stimulation of both innate and adaptive immunity. Several mechanisms are implemented in a cell to differentiate self from non-self RNA, which are controlled by features intrinsic to the endogenous RNA: 1) RNA concentrations; 2) physical location of the RNA extracellularly and intracellularly, in particular, within cellular compartments, and 3) RNA sequences and structural motifs (Schlee, 2016, p. 566). These characteristics allow the RNA to be detected by the PRRs. Depending on whether the RNA structure is double-stranded or single stranded, certain receptor pathways can be initiated. As

mentioned earlier, the most common types of PRRs that sense RNA include TLR3, TLR7, TLR8, RIG-I, and MDA5 (for this instance, TLR3 will be the focus since this was the pathway shown to recognized RNA origami) (Schlee, 2016, p. 567). TLR3 is a transmembrane protein found in endosomes (cellular compartments that are derived from the Golgi in eukaryotic cells) and, on some cell types the cellular surface (fibroblasts and tumor cells) (Owen, 2009). It is expressed in myeloid/conventional dendritic cells (cDCs), macrophages, epithelial cells, neurons, and fibroblasts (Tatematsu, 2014, p. 195). These cell types are the ones that are commonly agreed upon to express TLR3, but there are studies that show other types are used as well: natural killer (NK) cells, T-cells, and some types of tumor cells (Ammi, 2015, p. 120). The key cell types when looking at RNA origami as an adjuvant are the immune cells: cDCs, macrophages, NK cells, and T-cells, and the tumor cells. Many of these cell lines are part of the innate immune system (cDCs, macrophages, and sometimes NK cells) which can lead to inflammation and the release of cytokines (small signaling proteins that can activate or repress immune cells) (Parham, 2015). Cytokines, such as interferon (IFN), have been reported to be secreted in the presence of TLR3 recognized RNA, which helps prime the intensification in the immune response, both innate and adaptive, by recruiting immune cells to the site of infection (Ammi, 2015, p. 121). The increased presence of these cytokines leads to the stimulation of innate immune cells and antigen presenting cells (APCs), which link the innate immunity to the adaptive immunity. Furthermore, the cytokines cause the upregulation in the expression of major histocompatibility complexes (MHCs) on cell surfaces. The MHC's can then present antigens, in this case tumor-associated antigens (TAAs), that can then activate T-cells (Schlee, 2016, p. 566). Depending on the MHC, whether class I or

class II, different T-cell types can be triggered. The antigens on MHC II would recruit cluster of differentiation 4 (CD4) positive helper T-cells that could lead to the activation of cytotoxic T-cells, macrophages, and B-cells (Parham, 2015). In the instance of MHC I presentation, cytotoxic T-cells would be recruited directly due to expressing cluster of differentiation 8 (CD8), a transmembrane protein that binds to it, which would lead to the tumor cells death by apoptosis (Parham, 2015). Additionally, due to the upregulation of cDCs there is a chance of MHC I cross-presentation from exogenous TAAs, thus allowing for another way to induce cytotoxic T-cells (Parham, 2015). Based off what is known from the research with Poly(I:C) and dsRNA, RNA origami has the potential to activate these pathways. Additionally, one could speculate the potential of RNA origami and what else it could lead too if it turns out to be comparable to the other RNA-based adjuvants. One of these is the possibility of developing memory T-cells (T-cells that can recognize a specific antigen and lead to the direct attack and elimination of the infected cell), which when viewed in the context of cancer, could result in immunity (Parham, 2015).

Current adjuvants and their influence on RNA origami. In immunotherapy, the use of adjuvants is not a novel idea and has led to many different types. The novelty of RNA origami is that it contains dsRNA, which can allow it to function similarly to Poly(I:C), a known adjuvant. Additionally, by analyzing the NA-based adjuvants against the concept of this RNA origami structure, two key areas were highlighted that RNA origami had to perform well in besides being able to elicit a strong immune response. The first of these was ensuring RNA origami was stable and did not degrade easily. For this, studies performed by Dr. Xiaodong Qi tested the length of time it could stay intact in the presence of RNase (enzymes that breakdown RNA) and how long RNA origami could

retain its structure without degrading (Lodish, 2016). In the presence of RNase, the structure did not degrade after 10 or even 30 minutes, shown in *Figure 9*. Additionally, the

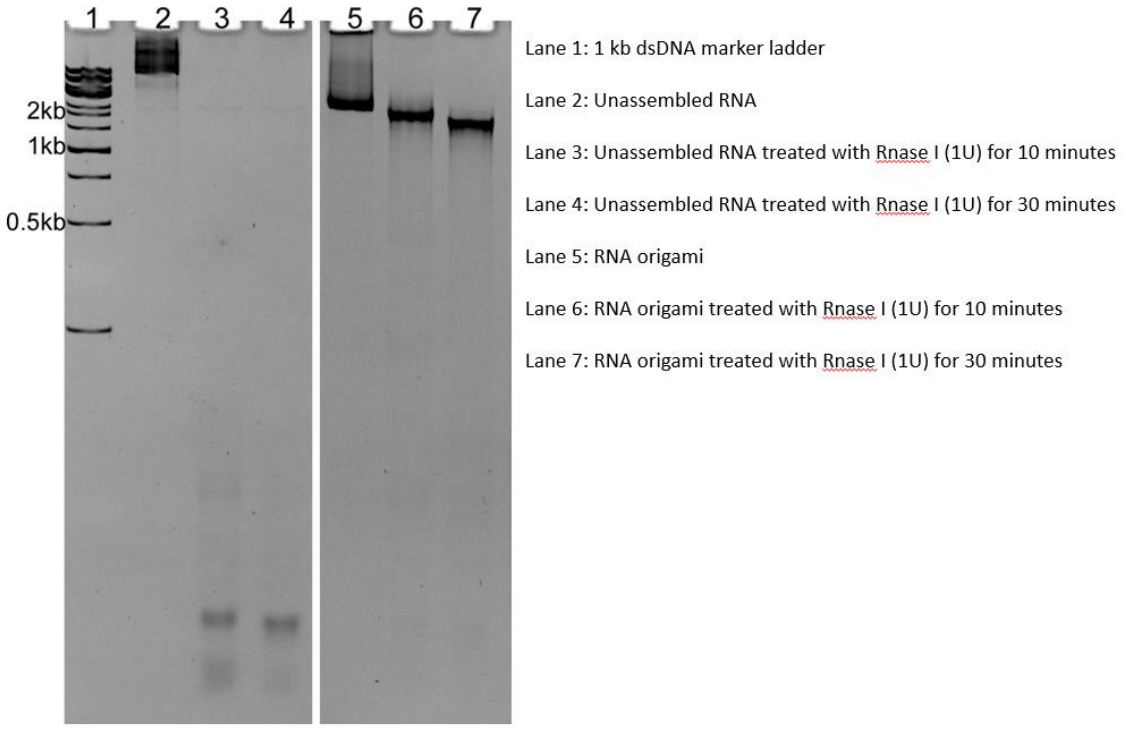

Figure 9. RNA origami degradation resistance. Agarose gel with both unassembled RNA and RNA origami in both the presence with RNase 1 and without. Used in permission by Dr. Xiaodong Qi.

importance of the RNA origami structure was shown by comparing it to unassembled RNA origami strands, where the unassembled RNA origami degraded right away in the presence of RNase. Supplementary studies were performed by Dr. Xiaodong Qi, where the RNA origami was incubated with mouse serum overnight and there was still no degradation of the structure (image not shown). In analyzing whether the RNA origami could keep its shape over time, the RNA origami was stored for four months and then evaluated. Even under these conditions it was able to keep its structure, as seen in the *Figure 10* courtesy of Dr. Xiaodong Qi. Thusly, these various tests demonstrated the stability potential of the RNA origami. The second component that RNA origami had to perform well in was not

causing high levels of toxicity, which has been shown with Poly(I:C). In order to do this, several assays were conducted comparing RNA origami and Poly(I:C), which is discussed in Chapter 4.

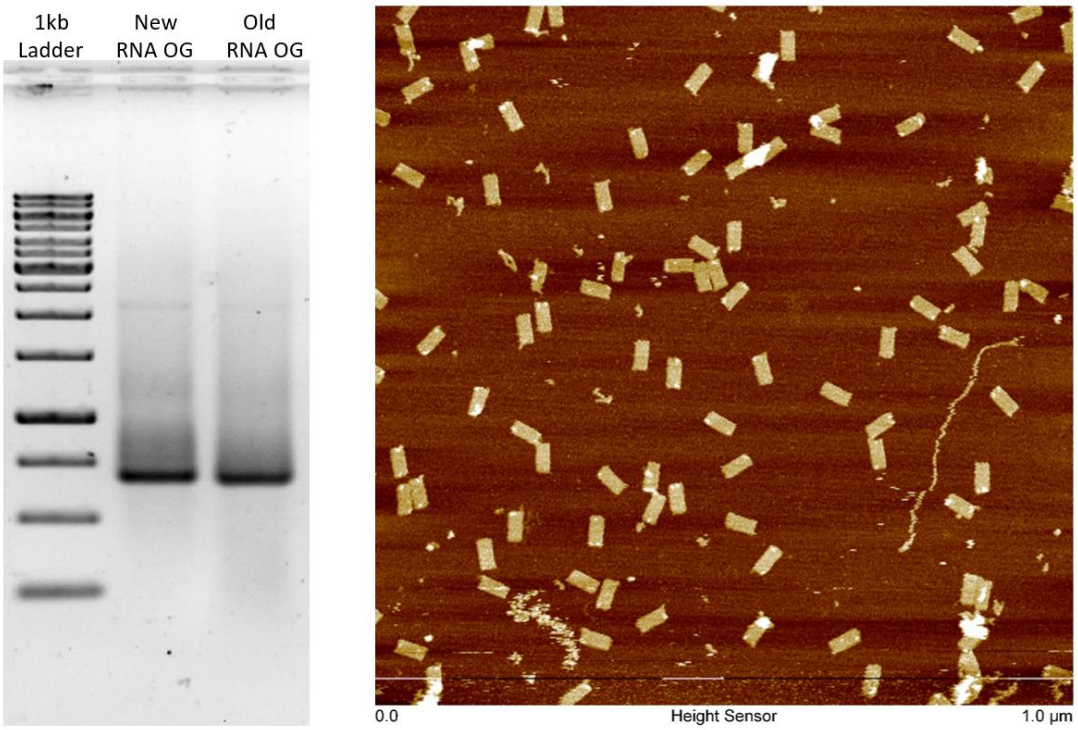

Figure 10. RNA origami time-based integrity. (Left) Freshly assembled RNA origami (New) and RNA origami that had been stored for 4 months (Old). (Right) Atomic force microscopy performed on the old RNA origami (same as left) where the structure was shown to be still intact (compare to *Figure 7*). Both images were kindly provided by Dr. Xiaodong Qi.

CHAPTER 3

METHODS FOR DESIGNING TUMOR MODEL

Introduction to colon cancer. Colon cancer or more frequently grouped as colorectal cancer, is one of the most common types of cancer. Overall, it is the fourth most common cancer among men and women where there is a chance that for every 20 people, one person will develop colorectal cancer in their life ("Colorectal cancer information", 2018). Most cases occur in people over the age of 50, but it can develop/come about sooner. Both colon and rectal cancer are grouped because in most cases, one leads to the other. This is due to the large intestine being connected to the rectum and the type of cancer is named based off the proximity to that region ("Colorectal cancer information", 2018). Colorectal cancer can cause sever irritable bowls, weight-loss and fatigue, and death. It develops regularly since that is where waste collects and the colon lining is a prime area for mutations to occur and tumors to form ("Colorectal cancer information", 2018). One of the key benefits of colorectal cancer over other forms of cancer is that there have been many developments in ways to screen for the cancer including colonoscopy, stool samples, as well as some types of imaging like MRI or CT scans (Souza, 2018). The problem is that the treatments for colorectal cancer are the same as many other types of cancer: surgery to remove parts of the colon, radiation therapy, and chemotherapy ("Colorectal cancer information", 2018). All of these treatments are extremely hard on the body and can lead to death. Additionally, none of these guarantee that the cancer will not grow back again, will metastasize to another region, or will be completely eliminated, making this an ideal cancer candidate to test immunotherapy so that the immune system can destroy the cancer if it returns. Another concern with colorectal cancer is its ability to metastasize to the

peritoneal cavity. The peritoneal metastasis (PM) of colorectal cancer advances into the disease known as peritoneal carcinomatosis. While the frequency of peritoneal carcinomatosis happening is low (appears in roughly 15 percent of patients), when it occurs the consequences are more dire. The disease is more urgent and less treatable than that of non-metastasized colorectal cancer where it has poor prognosis and can lead to death in less than two years (Strohlein, 2016).

Peritoneal tumor model. Colorectal cancer is a wide-spread form of cancer, making it a prime candidate to test RNA origami adjuvant immunotherapy. For modeling purposes, the colon carcinoma cell line for mice that can be injected is CT26. Despite having an existing cell line, it can still be challenging to model in a way that portrays colorectal cancer in its native state. As mentioned earlier, there have been various methods developed including subcutaneous injection of the cancer cells, orthotopic modeling, and mutagenesis and oncogene-mediated transgenic models that allow spontaneous development of cancer cells. For this model, the logic behind rejecting these other methods was that the orthotopic and spontaneous developed models would be impractical due to their technical difficulties, especially for preliminary studies, and the subcutaneous model could lead to clinical irrelevance for this type of cancer. Additionally, the way that the treatments are performed in these could be problematic as well because most of the time the treatments are done intratumorally (injecting the therapeutic directly in to the tumor) (Aznar, 2017, p. 31). This would be an issue when it comes to treating colorectal cancer since it would be challenging to deliver the therapeutic directly into the tumor in this region. Since colorectal cancer can metastasize into the peritoneal cavity, causing peritoneal carcinomatosis, this can help design another model that can remove the technical

difficulties, but also mimic clinically relevant situations. The model would consist of injecting the cancer cells into the peritoneal cavity, replicating PM (Strohlein, 2016). However, measurements of tumor growth in this model would primarily be based on an enlargement of abdomen and/or histological examination of the peritoneum (the barrier that covers the peritoneal cavity) for tumor nodules, which requires surgical procedures or euthanization of the animal. To allow better and sensitive assessment of tumor growth, the CT26 line was engineered to express near infrared fluorescence protein (iRFP), which was generated by Drs. Sriram Sokalingam and Xiaowei Liu. The presence of iRFP in the cells makes it possible to monitor tumor growth in real time *in vivo*, especially for detecting and quantifying small number of tumor clusters on the peritoneum. This is critical to the study of cancer immunotherapy in this compartment since the balance between anti-tumor immunity and immunosuppression in the peritoneal tumor microenvironment is very dynamic and sensitive to tumor loads, therefore demanding a highly sensitive detection method for monitoring the process, not just end results of tumor growth. Information gained from these analyses could have important clinical implications, since peritoneal carcinomatosis has poor prognosis and can occur by other types of cancers that can metastasize into the peritoneal cavity, i.e. gastric, cervical, and ovarian cancer (Strohlein, 2016). Thus, new discoveries made from these studies could also benefit the management of these malignancies as well. For example, due to technical ease to gain access to the peritoneal cavity, this model can be used to test and screen a variety of anti-cancer agents, including chemical compounds, cytokines, adjuvants, and checkpoint inhibitors.

Tumor microenvironment in peritoneal cavity for immune interrogation. The peritoneal cavity offers a unique microenvironment for immune cells. Since the peritoneal

cavity can house and acquire many different types of bacteria that are taken into bowls through ingestion, it has to have a fast-acting immune response to prevent pathogens from disseminating into other systemic regions (Strohlein, 2016). In turn, this provides a unique region that could take advantage of an adjuvant, such as RNA origami, and could elicit a strong immune response against the cancer. The immune cells that comprise the peritoneal cavity include roughly 45 percent macrophages, 45 percent T-cells (both CD8+ and CD4+), 8 percent NK (natural killer) cells, and 2 percent DCs (dendritic cells) (Strohlein, 2016). All of these cell types are crucial in the adaptive immunity and could be stimulated by an adjuvant. Additionally, mesenchymal cells are found here which are a cell type that can produce pro-inflammatory and anti-inflammatory factors (cytokines and chemokines), depending on the context (Strohlein, 2016). Despite the peritoneal cavity offering a dynamic and sensitive immunological environment, it is also pro-cancer growth as well. In addition to various nutrients circulating through to promote tumor growth, the lining layer of the peritoneum serves as an anchor for cancer cells to seed, adhere, and spread (Strohlein, 2016). Moreover, just as how fast the immune system is to respond in the peritoneal cavity, it is also quick to stop it. In the tumor environment, myeloid cells could be driven into tumor associated macrophages (TAMs), and myeloid derived suppressor cells (MDSCs) by factors made by tumor cells, fibroblasts, mesenchymal cells, as well as other immune cells (such as regulatory T-cells), which in turn creates an immunosuppressive milieu to counteract anti-tumor immunity (Strohlein, 2016). This is shown in *Figure 11* in ovarian cancer and how it can manipulate macrophages in their favor (Worzfeld, 2017). However, the ability of an adjuvants to enhance both the innate and

adaptive immune response highlights their potential in reprograming a pro-tumor microenvironment into a tumor-hostile battle ground for elimination of tumor cells.

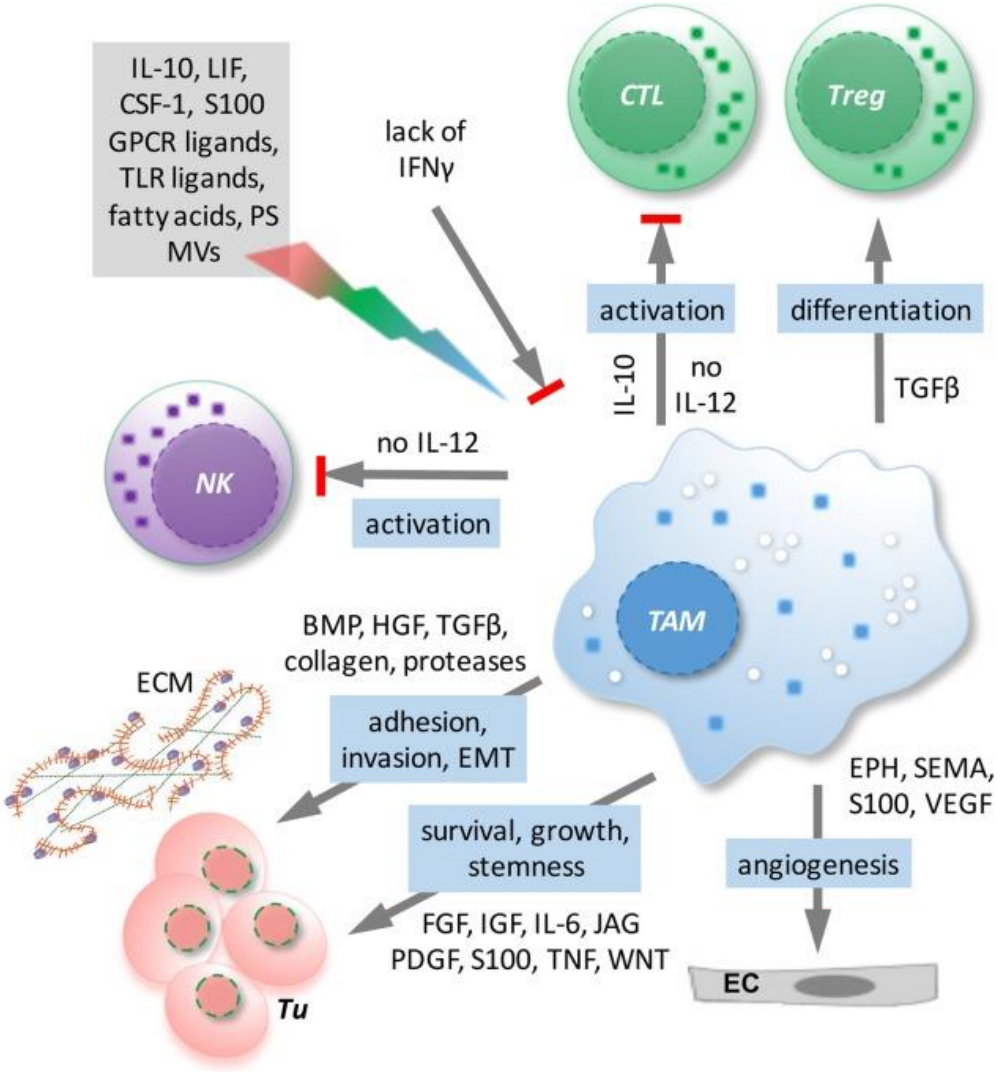

Figure 11. Tumor-associated macrophages (TAM). Illustrates how ovarian cancer can interact with TAMs leading to secretion of various cytokine and chemokines that can suppress the immune cells (Worzfeld, 2017).

CHAPTER 4

RESULTS OF RNA ORIGAMI IN CANCER TREATMENT

In vitro results of RNA origami as an adjuvant. Before RNA origami could be looked at with the primary results generated for this thesis, many *in vitro* studies had to be conducted to show the plausibility of RNA origami as an immuno-adjuvant, which were performed by Dr. Yung Chang and other members of her lab. As a comparison, many of the assays looked at Poly(I:C) (both a high molecular weight (HMW or H) and a low molecular weight (LMW or L), where the HMW contained a longer Poly(I:C) chain and had an increased chance for toxicity) since it has been established as a potential adjuvant that could be integrated in anti-cancer immunotherapy (Ammi, 2015, p. 120) (Gralnick, 1983, p. 146). Studies were conducted to investigate whether the RNA origami could function as an adjuvant that could elicit an anti-tumor immune response, what receptors it utilizes to enter the cells, and what signaling pathways it relies to trigger the innate reactions.

RNA origami macrophage internalization and stimulation. To verify that RNA origami was internalized by APC's, the origami structured was modified to have a fluorescent tag (Alexa 488) that could be excited when stimulated with the corresponding laser using flow cytometry (a device that passes cells through a stream of fluid that uses various lasers that can excite corresponding dyes, allowing for sorting and cell counting) (Lodish, 2016). The APC that this was performed on was a macrophage line (RAW 264.7 cells). In addition to testing for internalization, a different dye that would bind to cluster of differentiation 40 (CD40) was used to test for APC stimulation, since CD40 is considered

as a co-stimulator of APC, is upregulated upon APC stimulation, and is required for initiating an adaptive immune response. As shown in *Figure 12* (provided by Dr. Yung

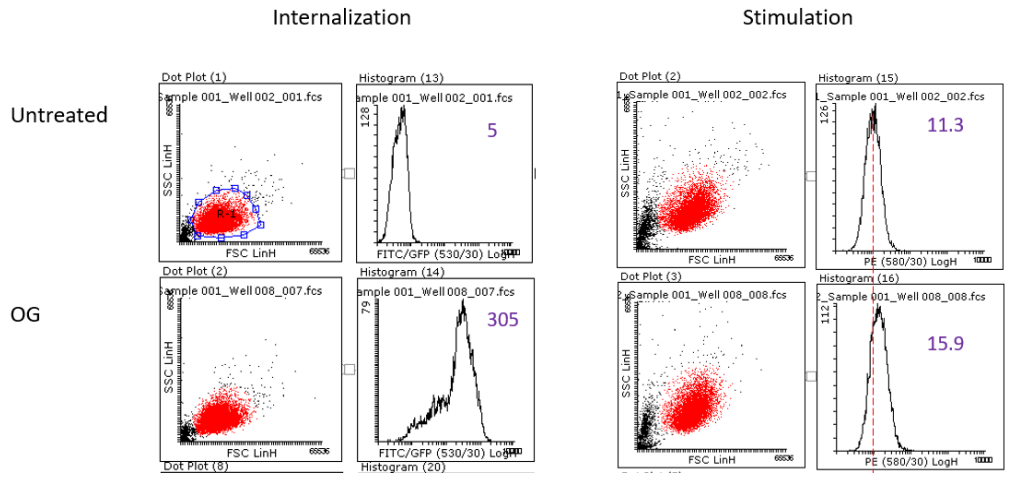

Figure 12. RAW 264.7 internalization and stimulation. Flow cytometry results tested for both RNA origami (OG) internalization and stimulation of the macrophages. It was conducted on RAW 264.7 cells that had been treated with the RNA origami and ones that received no treatment. Courtesy of Lawrence Matiski and Dr. Yung Chang.

Chang), two-hour incubation of the macrophage line with Alexa 488-RNA origami results in drastic increase in mean fluorescence intensity (MFI) of the cells (*Figure 12*, left panel), implicating its binding and possible internalization (since unbound probes were removed by trypsinization). Such binding led to upregulation of CD40, revealed by an increase in MFI (*Figure 12*, right panel). Furthermore, as demonstrated by Dr. Yung Chang in *Figure 13*, RNA origami was a much more potent stimulator than HMW Poly(I:C) for macrophages. This was revealed not only by the greater magnitude of CD40 macrophages present due to the RNA origami based off the MFI (*Figure 13*, left panel), but also by being able to stimulate macrophages at a faster rate in shorter incubation times. This is distinctly shown by the increased stimulation of the RAW 264.7 cells by the RNA origami in all three time points in comparison to phosphate-buffered saline (PBS) treated macrophages, where Poly(I:C) does not show an increase in stimulation until the 24-hour incubation.

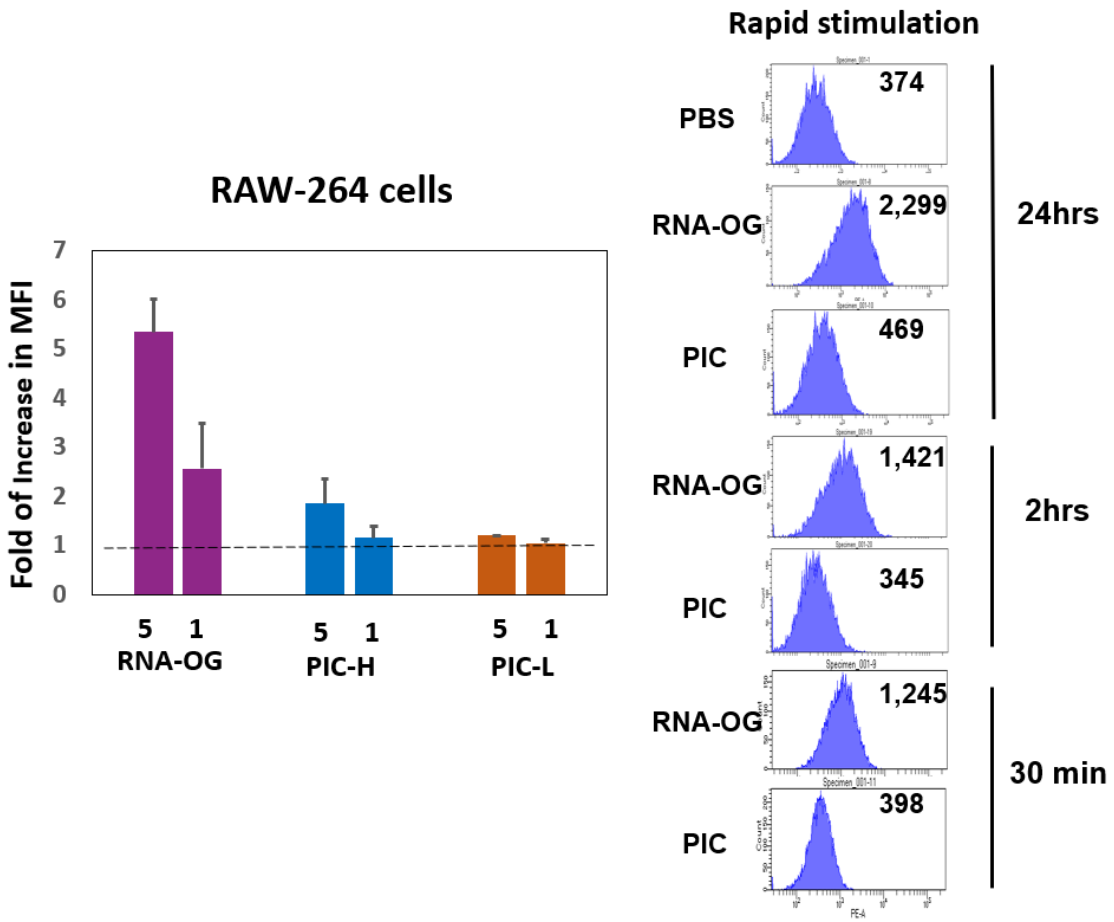

Figure 13. RAW 264.7 time-dependent stimulation. (Left) The MFI quantifications for all three treatments. There were two dosages of the treatments: a 5 ug/ml and a 1 ug/ml. (Right) Stimulation of the RAW 264.7 cells at various incubation times with RNA origami, HMW Poly(I:C), and PBS. Contributed by Dr. Yung Chang.

RNA origami PRR pathway. In order to examine which PPRs RNA origami reacted with for innate activation, two types of cell lines were utilized. The first one is a TLR3-reporter line, engineered from Human Embryotic Kidney cells (HEK293) that express the TLR3 and contains nuclear factor-kappa B (NF-kB) dependent reporter substrates for revelation of TLR3-mediated activation. As displayed in *Figure 14* (provided by Dr. Yung Chang), both HMW Poly(I:C) and RNA origami could activate this cell line, indicating RNA origami functions as a TLR3 ligand. Although RNA origami's activity is slightly lower than the one induced by HMW Poly(I:C), Poly(I:C) is a known TLR3 agonist, hence

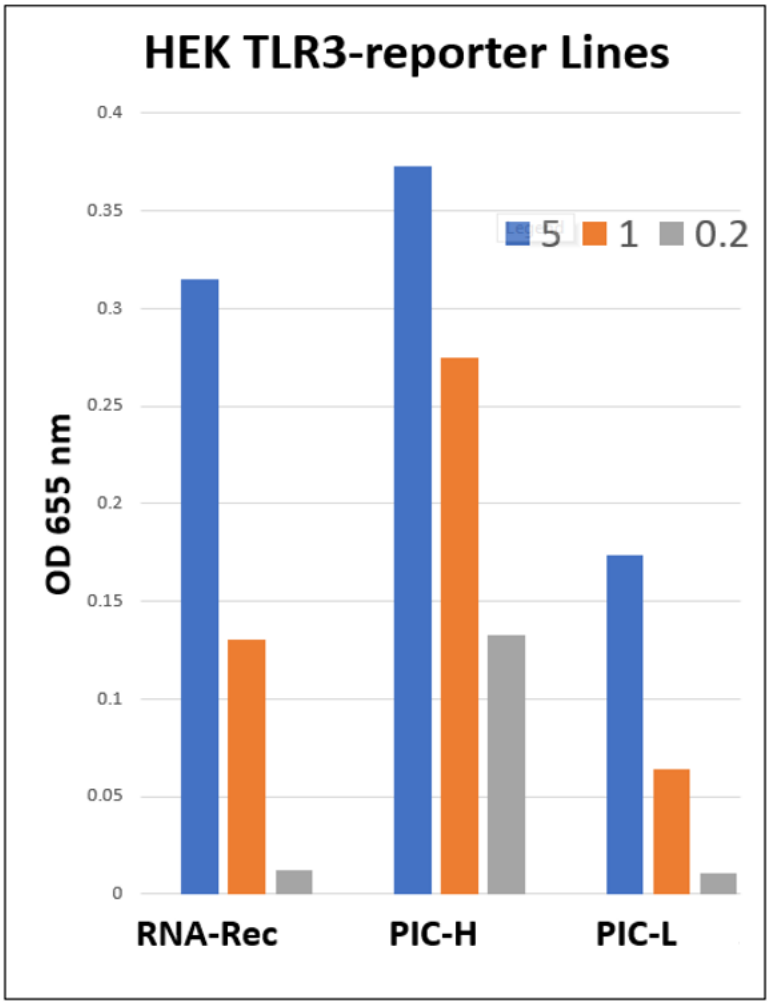

Figure 14. TLR3 RNA origami expression. HEK-Blue cells were exposed to three treatments: RNA origami (RNA-Rec), HMW Poly(I:C) (PIC-H), and LMW Poly(I:C) (PIC-L) for 16 hours. There were three different dosages for treatments: 5 ug/ml, 1 ug/ml, and 0.2 ug/ml. The optical density (OD) described by the kit's protocol was used in the plate reader. Kindly provided by Lawrence Matiski and Dr. Yung Chang.

illustrating RNA origami is as well. However, it is known HMW Poly(I:C) can also interact with cytoplasmic dsRNA receptors, such as RIG-I and MDA5 signaling pathways, which have been implicated for high levels of type I interferon produced *in vivo*. To determine whether RNA origami also goes through these pathways, two reporter lines were obtained from *Invivogen*, a wild type A549 reporter line (A549-Dual^{TM}WT-MAVS) that are positive for both RIG-1 and MDA5 dsRNA receptors and contain dual reporter genes, and a mutant

A549 reporter line that is defective in mitochondrial antiviral stimulatory protein (MAVS), an adaptor downstream of both RIG-1 and MDA5 signaling pathway. As expected, HMW Poly(I:C) upon transfection was found to activate both NF-kB and interferon regulatory factor (IRF) signaling in wild type A549 reporter line, but not the mutant line (seen in *Figure 15*, generated by Lawrence Matiski). However, RNA origami and unstructured RNA (Poly(A:U)) failed to activate either of these cell lines, therefore, they are not likely ligands of RIG-1 and MDA5 dsRNA sensors. This finding suggests that RNA origami functions as a TLR3-restricted ligand, which has been reported to have better therapeutic activity, and lower *in vivo* toxicity.

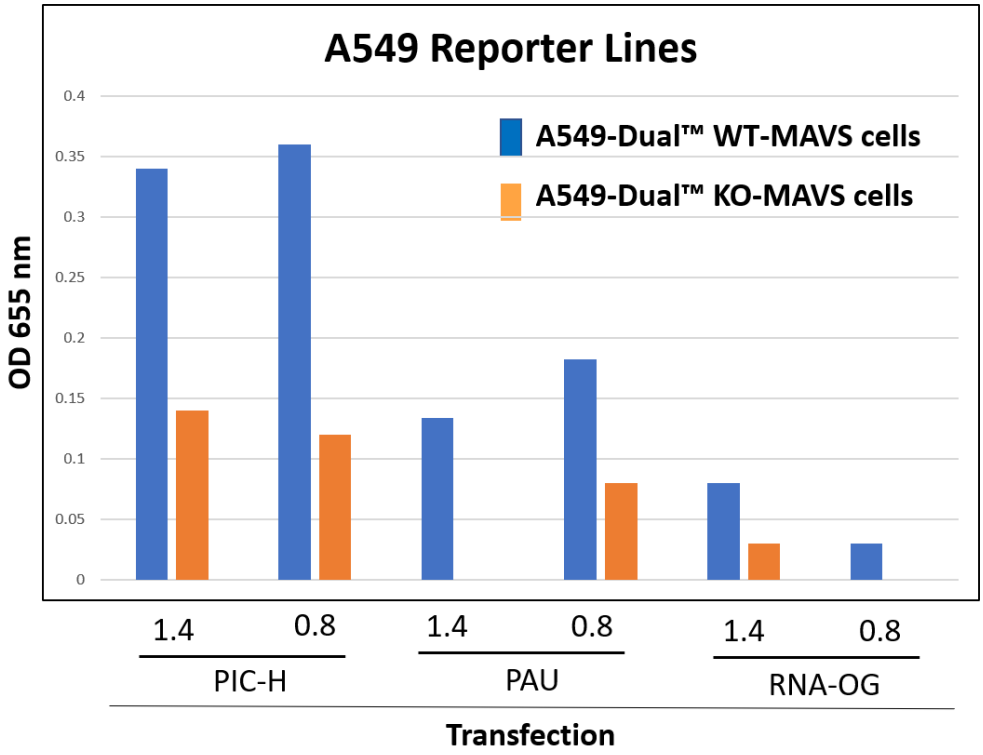

Figure 15. RNA origami MAVS excitation. Two types of A549-Dual cells (wildtype (WT) containing MAVS and a knockout (KO) without MAVS) were exposed to three treatments: RNA origami (RNA-OG), HMW Poly(I:C) (PIC-H), and PAU (Poly(A:U)). There were two different dosages for the treatments: 1.4 ug/ml and 0.8 ug/ml. The optical density (OD) described by the kit's protocol was used in the plate reader. Kindly provided by Lawrence Matiski and Dr. Yung Chang.

RNA origami in vitro cytokine profile. To examine the cytokine profile produced by immune cells in response to RNA origami, splenocytes (spleen cells which are major contributors to generating immune responses) were incubated with different treatments: PBS, Poly(I:C), and RNA origami. The culture supernatant from these incubations were collected 24 hours later for cytokine analysis. The flow cytometry-based multiplex cytokine array kit (Biolegend) was used to profile pro-inflammatory cytokines, as shown in *Figure 16* (kindly provided by Dr. Yung Chang). Two chemokines were found to

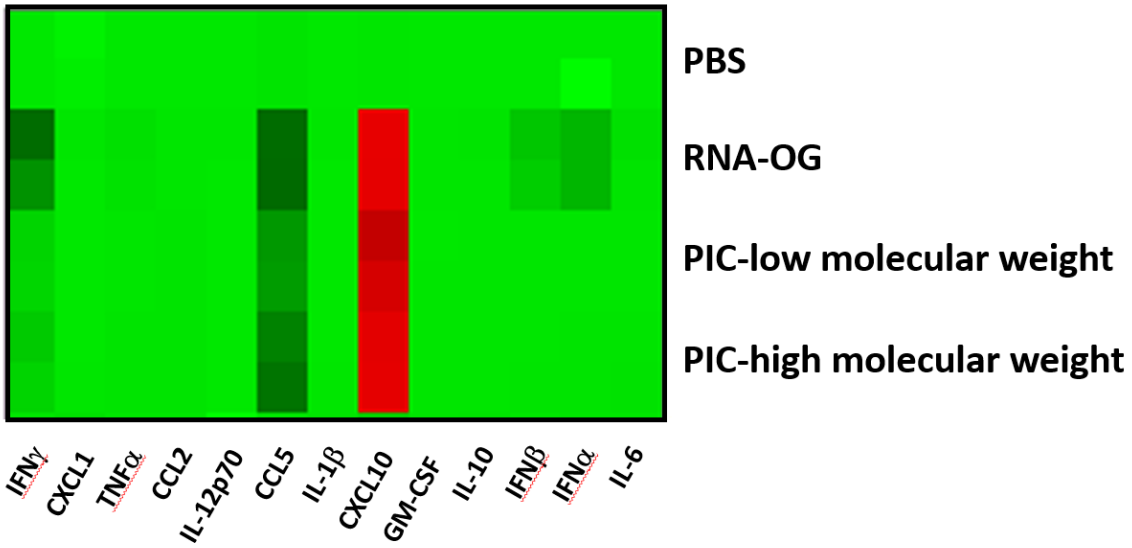

Figure 16. Splenocyte cytokine profile. Various proinflammatory cytokines were tested under four treatments: PBS, RNA origami, LMW Poly(I:C), and HMW Poly(I:C). Permission to use by Dr. Yung Chang.

significantly upregulated by both RNA origami and the Poly(I:C): CCL5 (reported to recruit T-cells, eosinophils, and basophils) and CXCL10 (known to recruit various APCs, T-cells, and NK cells to tumor sites for generating anti-tumor activity) (Owen, 2009). Interestingly, as compared to Poly(I:C) (both high and low molecular species), RNA origami induced slightly elevated levels of IFN alpha and beta (type I interferons that can stimulate immune responses typically against viruses and can stop DNA and RNA

replication) and IFN- gamma (a type II interferon which stimulates cytotoxic T-cells and helper T-cells) (Parham, 2015), highlighting its potent stimulatory activities exerted on splenocytes. An additional cytokine analysis was performed with the same process, except the cytokine profile was derived from serum withdrawn from naïve mice that received treatments three hours prior. These treatments included PBS, RNA origami, and Poly(I:C) and the results are shown in *Figure 17* kindly provided by Dr. Yung Chang. The assay

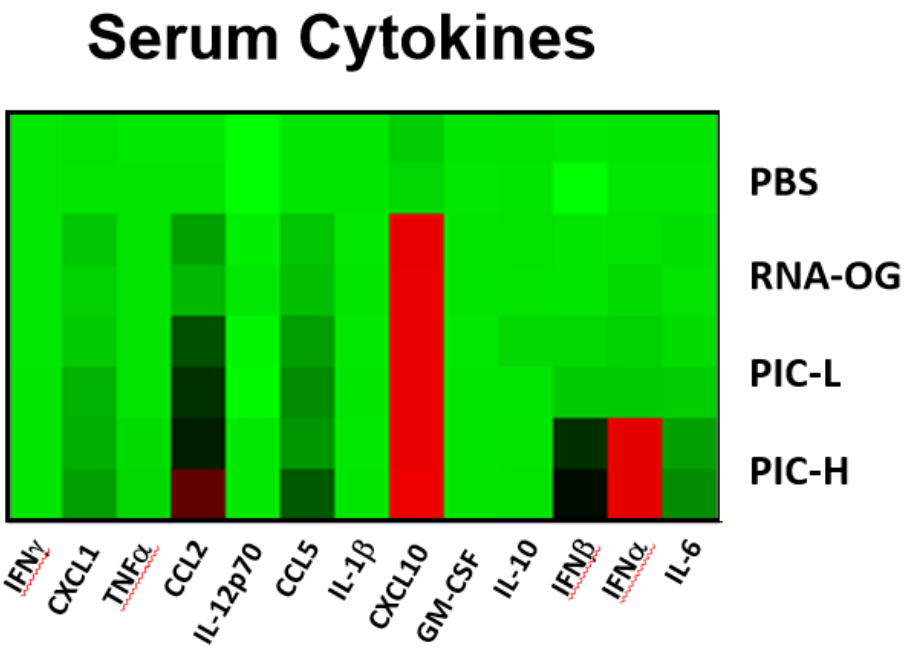

Figure 17. Serum cytokine profile. Treatments were done using 16 ug in 100 ul with IP injections in the mouse and the serum was extracted three hours later. Permission to use by Dr. Yung Chang.

displayed similar outcomes to the splenocyte analysis. RNA origami and the Poly(I:C) upregulated CCL5 and CXCL10 again, but they also expressed elevated levels of CXCL1 (attracts neutrophils and increases proinflammatory activity) and CCL2 (recruits monocytes, memory T-cells, and DCs) (Parham, 2015). The major difference was that HMW Poly(I:C) upregulated IFN alpha and beta in the serum, while RNA origami did not in this case.

RNA origami adaptive immune cell stimulation. The final *in vitro* assays looked at what immune cells were present, or more specifically, whether T-cells and B-cells were upregulated by the presence of RNA origami. This also used flow cytometry on cultured splenocytes that had been gated into B-cells and T-cells. Both of cell types were stained with an antibody against cluster of differentiation 69 (CD69 which is found on activated immune cells such as these). Both the B-cells and the T-cells were incubated for 24 hours with the treatments and the outcome is seen in *Figure 18*, contributed by Dr. Yung Chang.

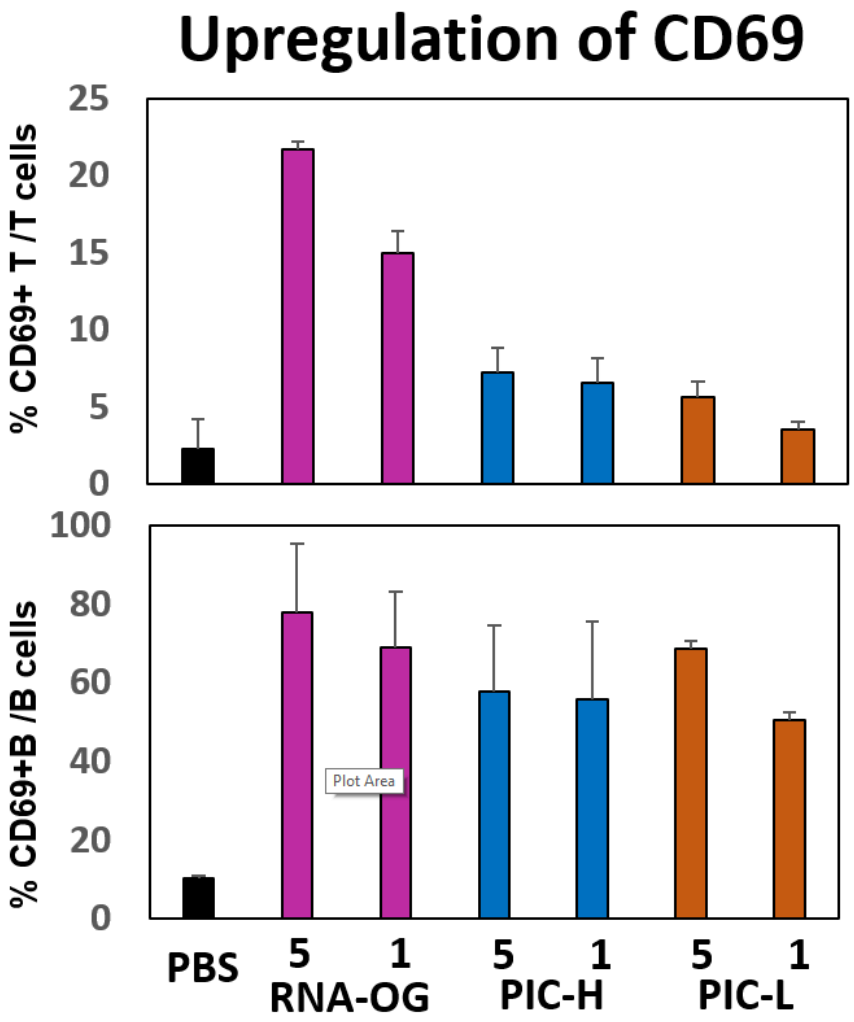

Figure 18. RNA origami B-cell and T-cell upregulation. There were three treatments and a control (PBS). The two dosages were 5 ug/ml and 1 ug/ml. (Top) T-cell upregulation. (Bottom) B-cell upregulation. Done by Dr. Yung Chang.

Upregulation was shown for both T-cells and B-cells that were treated with RNA origami. The results for the T-cells showed that RNA origami stimulated CD69 the most, even in comparison to Poly(I:C). As for the CD69 B-cells, the stimulation was almost identical between RNA origami and Poly(I:C). Additionally, a similar experiment was conducted, but on APCs: dendritic cells, B-cells, and macrophages. These were gated to display the expression of cluster of differentiation 86 (CD86 which is on activated antigen presenting cells when trying to stimulate T-cells) on several types of antigen presenting cells (APCs), including conventional dendritic cells (cDC), plasmacytoid dendritic cells (pDC) (producers of INF alpha), B-cells (B), and macrophages (Mac), which are all critical to activation of the adaptive immune system. As shown in Table 1 that was compiled by Drs. Xiaowei and Yung Chang, the mean fluorescence intensity (MFI) of CD86 co-stimulator was significantly increased in these APCs after being treated with RNA origami, as compared to Poly(I:C).

Table 1

Average Mean Fluorescence Intensity of CD86 APCs

Treatment	CD86-MFI			
	cDC (Cd11c+/B220-)	pDC (CD11c+/B220+)	B (CD11c-/B220+)	Mac (CD11b+/CD11c-)
PBS	1,054 (1)	2,610 (1)	660 (1)	251 (1)
RNA-OG (5ug/ml)	2,382 (2.3)	12,350 (4.7)	4,866 (7.4)	693 (2.8)
PIC-H (5 ug/ml)	1,734 (1.6)	12,107 (4.6)	2,624 (4.0)	275 (1.1)

In vivo results of RNA origami as an adjuvant. To really demonstrate the application of RNA as an anti-cancer adjuvant, testing was conducted on several murine models (initial testing and repeats of RNA origami, time of treatment variations, and re-challenge tumor models). In addition to these models, different aspects of the effects of RNA origami immunostimulatory properties were looked at, including tumor growth, the

importance of treatment timing, immunity assessment, and cellular stimulator components from *in vivo* studies. For these instances, the RNA origami was tested in the designed CT26-iRFP metastasized colon cancer model. The mouse strain in the models was BALB/c mice (common albino laboratory mouse strain). BALB/c mice were chosen for this research due to them being synergetic with the CT26 cell line, i.e. having identical MHC alleles between the host recipient and tumor cells to recapitulate tumor recognition in human, in which tumor cells are derived from self-tissues, therefore bearing identical MHC. Additionally, the tumor cells were injected intraperitoneally (IP) where each mouse received usually 500,000 cells; the design of the experiments is shown in *Figure 19*. Since the cells fluoresced iRFP, the tumor size and growth could be monitored in the injected mice. This was done using a LI-COR Bioscience Pearl Imager: a small animal imager that contained a 700-channel laser (excited at the wavelength of 685 nm) and a white channel (imaged the entire animal for background regardless of fluorescence). The mice were monitored for one month (~30 days) or until

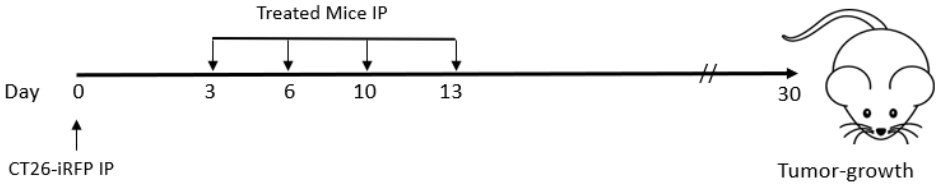

Figure 19. General murine model layout. The days listed show possible treatment days over the course of the 30-day monitoring period. The number of treatments varied between groups, but the least amount performed was four.

they reached endpoint and imaged two to three times a week. The imaging was performed while the mice were anesthetized using isoflurane so that the images could be taken without them moving. As a control to ensure that the uptake of the cancer cells occurred, there was always one group of mice that received PBS at the same time as the RNA origami treated.

CT26 peritoneal tumor growth in murine models. The initial trial group of mice had five PBS treated mice and five RNA origami treated mice. Since this was the first group and the exact number of CT26-iRFP cells that would appear and be lethal within a month timespan was still being determined, 800,000 cells were injected IP. For this group, there as a total of six treatments which were on days 1, 5, 8, 12, 19, and 25. Each mouse was treated IP with 100 ul of the corresponding treatment (the RNA origami groups concentration was 16 ug of RNA origami per mouse per treatment). The images of this group are shown in *Figure 20*. As seen, all of the PBS mice had died by day 19 due to

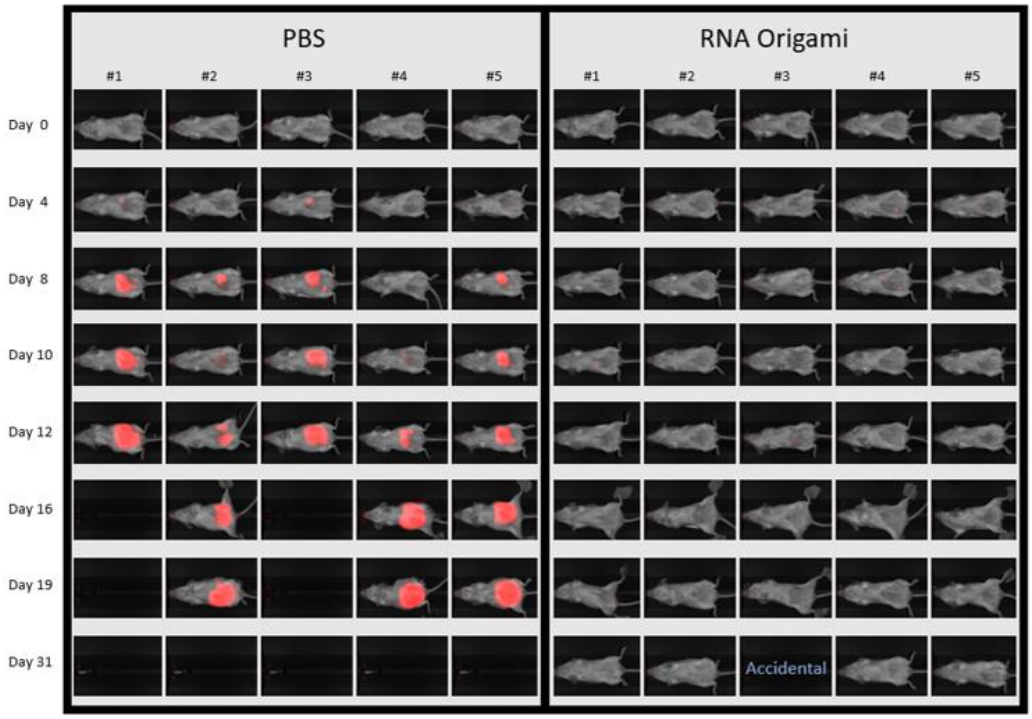

Figure 20. Initial murine treatment group. Ten BALB/c mice injected IP with 800,00 CT26-iRFP. The left group was treated with PBS (100 ul) IP six times over a month and the right group was treated with RNA origami (100 ul at 16 ug) IP six times over a month. Treatments started one day after cancer cell injections. Images taken with a LI-COR Bioscience Pearl Imager

tumor growth, while all of the RNA origami mice survived. Based off these results, further testing was done under similar conditions. A repeat experiment was done, but only 500,000

CT26-iRFP were injected in order to see if a lesser number of cells could be used to conserve and save time from culturing. Also, the amount of treatments and dosages stayed the same, but the exact days for treatment varied (treatment days were 1, 5, 8, 12, 15, and 19). Additionally, the imaging setup for these pictures was adjusted to show heat mapping so that the magnitude of the tumor size could be better visualized. The results for the repeat group are revealed in the *Figure 21*. The outcome for this experiment concluded very

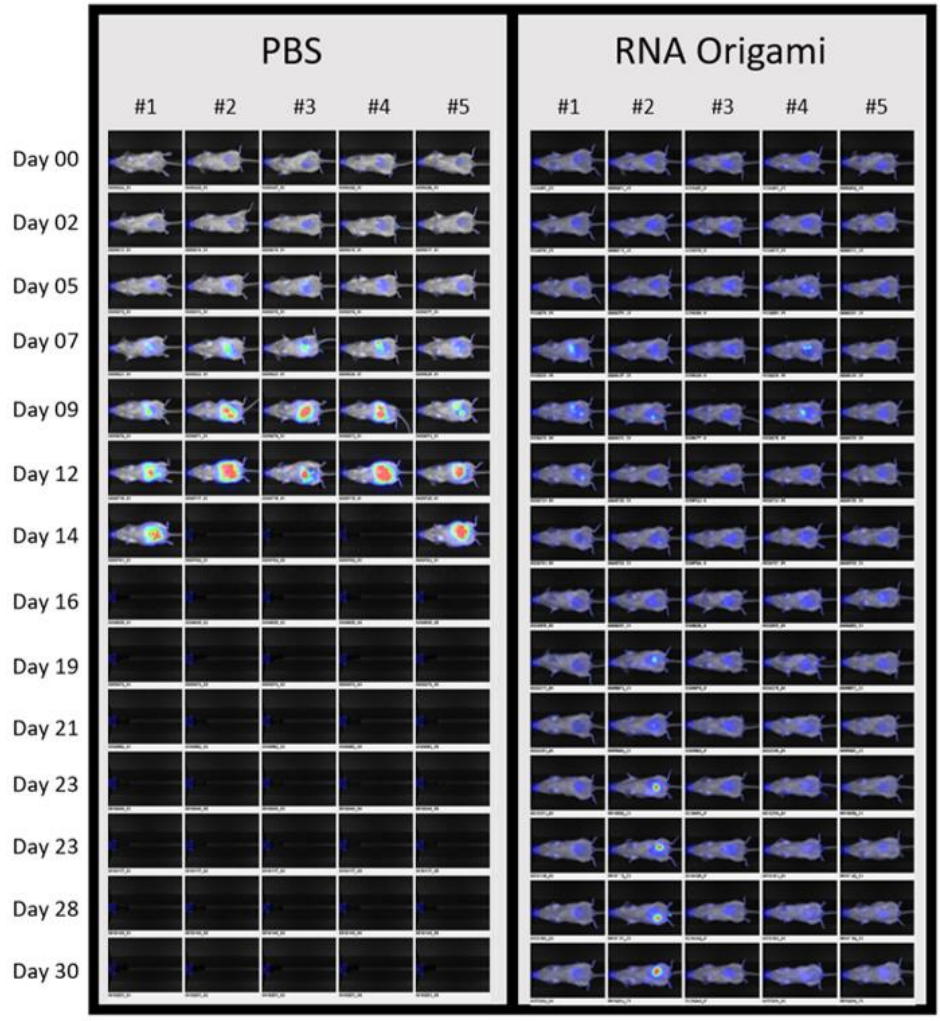

Figure 21. Repeat murine treatment group. Ten BALB/c mice injected IP with 500,00 CT26-iRFP. The left group was treated with PBS (100 ul) IP six times over a month and the right group was treated with RNA origami (100 ul at 16 ug) IP six times over a month. Treatments started one day after cancer cell injections. Images taken with a LI-COR Bioscience Pearl Imager.

similarly to the first one. All of the PBS mice had died by day 14 and all but one RNA origami mice were tumor free. The one that did die due to tumor development lasted all 30 days of monitoring and still was not at endpoint. Something to note in this study over the previous one was that there was an RNA origami mouse that displayed tumor growth but showed regression after further treatments. Some of the drawbacks of these initial tests were the treatments started on day 1, the presence of the tumor growth was usually undetectable by the imager for the RNA origami group, and the number of necessary treatments could not be concluded.

RNA origami treatment delay and effects on tumor growth. These initial trials then led to varying the day that the mice received the treatments. This next group contained fifteen mice where there would be a PBS group (still would receive the treatments on day 1), a day 1 treated RNA origami group, and a day 5 treated RNA origami group. Six treatments were still administered and were done on days 1, 5, 8, 12, 15, and 19 (the final treatment for the day 5 group was done on day 22), the results are pictured in *Figure 22*. Over the month-long span for this group, slightly different results appeared, especially for the day 5 treated. The day 1 RNA origami mice still did well with three mice being tumor-free and two mice that had delayed tumor growth. The PBS group still did poorly, as expected, with all mice dead by day 17. The day 5 treated RNA origami mice ended up almost being comparable to the PBS group except for one mouse that showed tumor regression and eventually elimination. This trial led to another, but this time the mice were treated starting on day 3. One of the noticed trends from all of the previous groups was that the number of treatments needed might be off, where it seemed treatments after the fourth

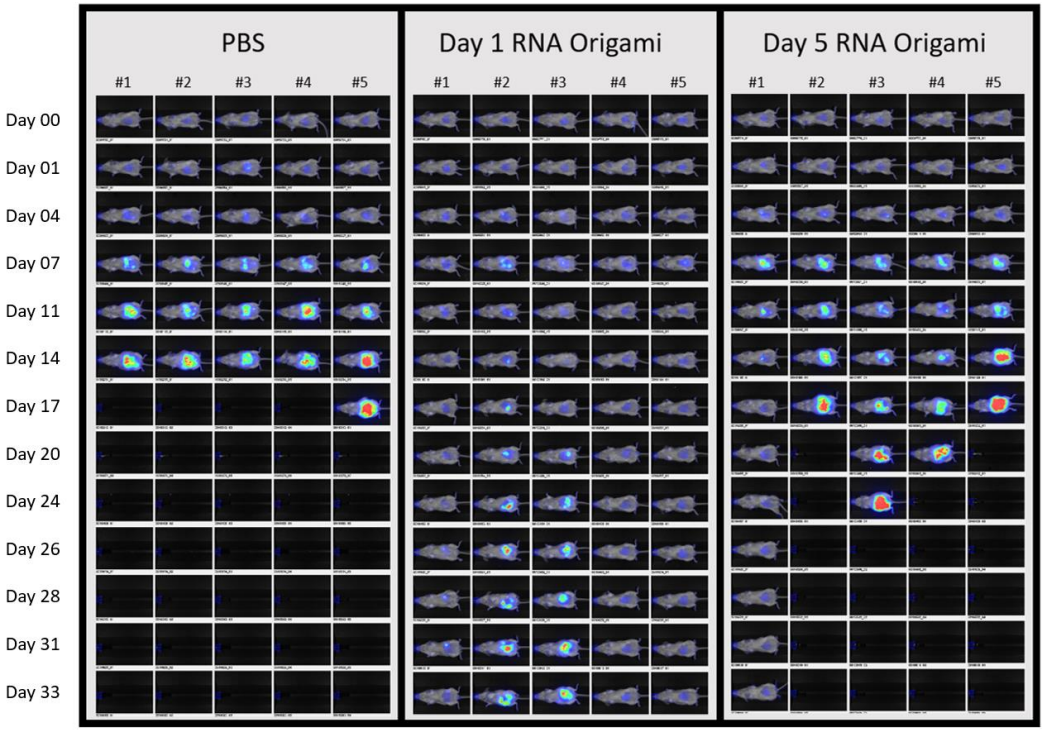

Figure 22. Five-day delayed treatment murine group. Fifteen BALB/c mice injected IP with 500,00 CT26-iRFP. The left group was treated with PBS (100 ul) IP six times over a month, middle group was treated with RNA origami (100 ul at 16 ug) IP six times over a month and the right group was treated with RNA origami (100 ul at 16 ug) IP six times over a month. Treatments started one day after cancer cell injections for the left and middle group, while the right group received first treatment five days post cancer injection. Images taken with a LI-COR Bioscience Pearl Imager.

round were unnecessary/redundant. The results for this group led to the subsequent group that still had a PBS group, but just one RNA origami treated group, treated on day 3. The treatment days for this group landed on days 3, 6, 10, and 13, which are shown in *Figure 23*. This group illustrated that day 3 treatments still worked for RNA origami and really highlighted the regression of the tumor growth. Almost all of the PBS mice were dead by day 18 and only one RNA origami mouse died due to tumor growth. Since most of the mouse in all of these groups were tested and treated under comparable conditions, the overall survival could be looked at and how the start of treatment could impact RNA origami's ability to be a potential adjuvant. *Figure 24* shows the survival curve for all of

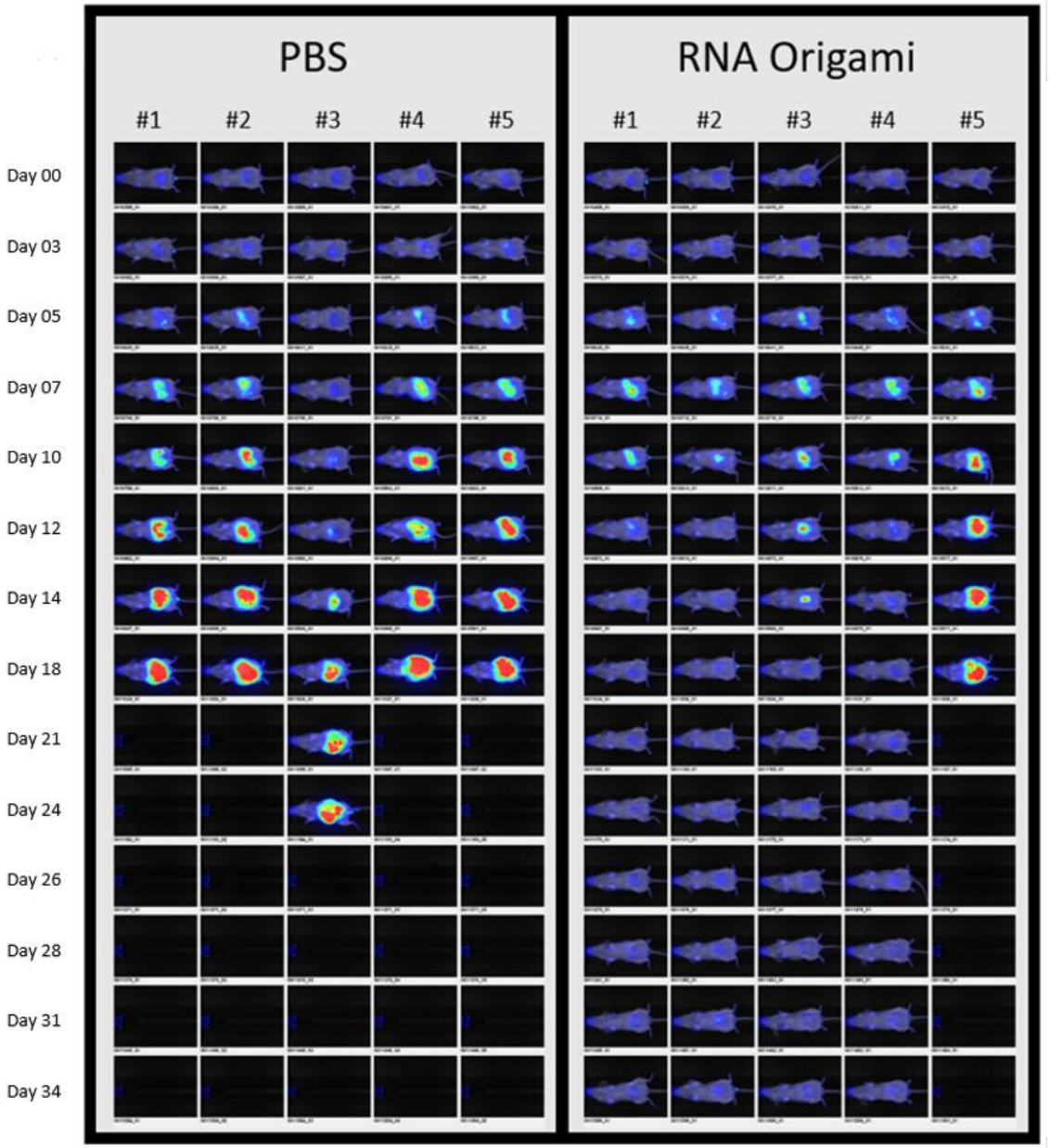

Figure 23. Three-day delayed treatment murine group. Ten BALB/c mice injected IP with 500,00 CT26-iRFP. The left group was treated with PBS (100 ul) IP four times over a month and the right group was treated with RNA origami (100 ul at 16 ug) IP four times over a month. Treatments started three days after cancer cell injections. Images taken with a LI-COR Bioscience Pearl Imager.

the previously mentioned mice, including a group of Poly(I:C) treated mice, images in the supplemental material. The Poly(I:C) group was only done in conjunction with the initial testing group of mice. Since Poly(I:C) has been shown to be an adjuvant against cancer, it

was used more as a control. Due to the concentration of Poly(I:C) inoculated into the mice, none of the other three trials received it because it was too low to show the toxic side-effects and toxicity is not as prevalent in mice as compared to humans (Ammi, 2015, p. 121). Also noticeable was that the more time had passed, the more progressive the cancer had become in the mice, leading to a worst outcome for the RNA origami as a therapeutic. Day 3 treated mice still showed promising results, but by day five many of the RNA origami mice were not able to survive for all 30 days. Although time does appear to be a factor in the efficacy of RNA origami as an immuno-adjuvant, all the group faired better than the negative control that was PBS, which had the largest sample size displayed in the graph, yet had no mice reach the end (day 30). Despite this, the RNA origami day 1 treated were comparable to the Poly(I:C) day 1 treated.

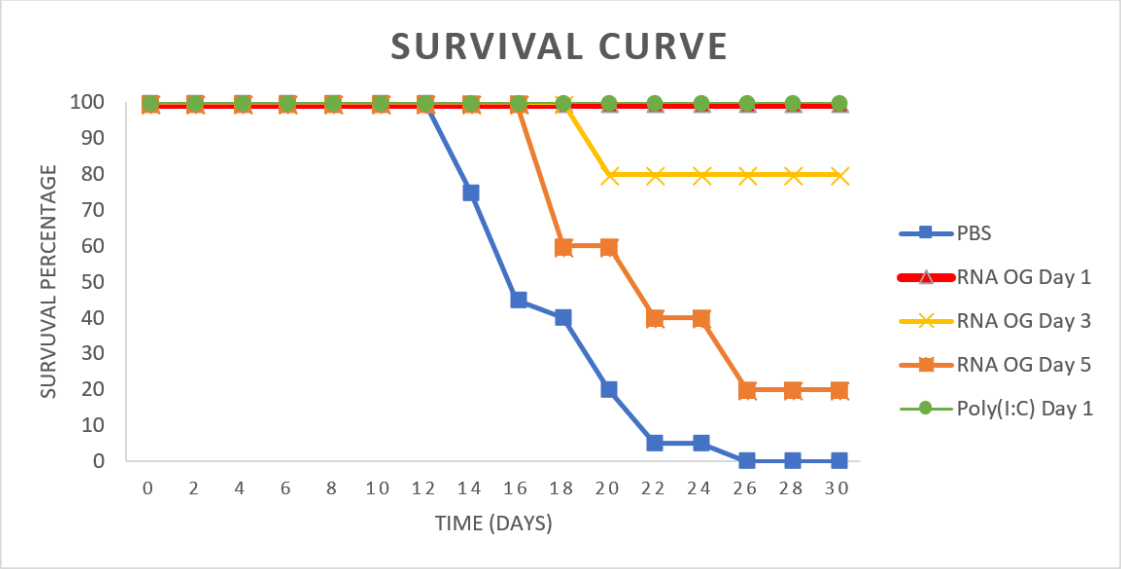

Figure 24. Survival curve for murine models. Survival percentage was used for the y-axis instead of the number of mice. There were 20 PBS treated, 15 RNA origami day 1 treated, 5 RNA origami day 3 treated, 5 RNA origami day 5 treated, and 5 Poly(I:C) treated.

CT26 immunity assessment in murine models. The results from these monitoring groups led to two other paths of testing. The first of these was to check for the presence of

anti-tumor immunity by re-challenging RNA treated mice that had shown regression. Four mice were used for this, two mice from the second experiment (49 days post last RNA origami treatment) and two mice from the third (36 days post last RNA origami treatment, day treated). These mice were injected a second time with 500,000 CT26-iRFP cells, but this time it was done subcutaneously on the abdomen of the mice. No treatments were given here, and the results are shown in *Figure 25*. Out of the four mice, only one ended

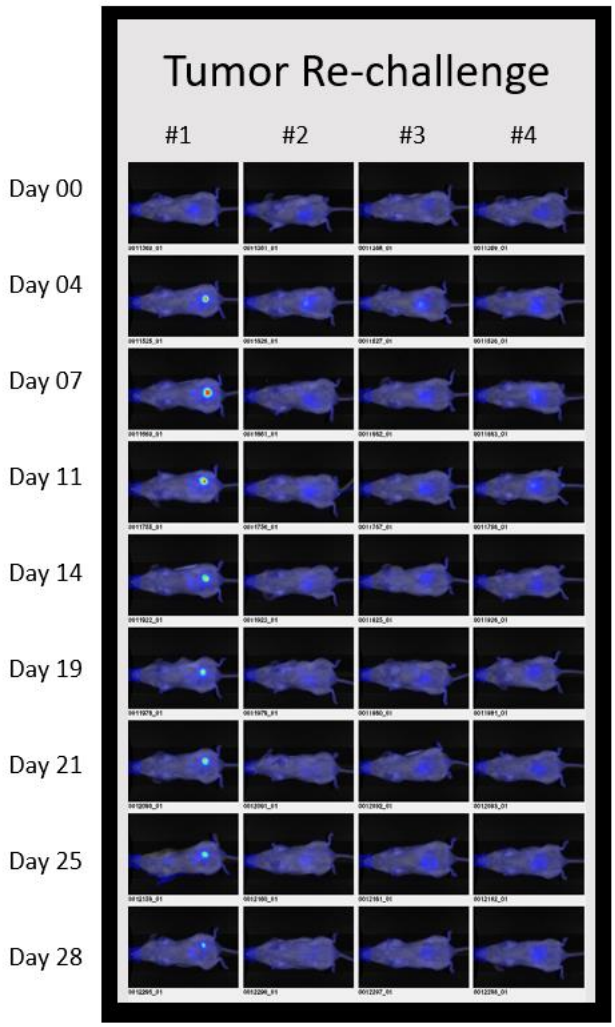

Figure 25. Re-challenge cancer murine model. Four BALB/c mice that had been injected with CT26 and treated with 16 ug of RNA origami six times starting on day 1 over the course of a month and had regression or no tumor growth were re-injected with 500,000 CT26-iRFP cells (mice #1 and #2 received re-challenge 49 days after final treatment and mice #3 and #4 received re-challenge 36 days after final treatment).

up growing a tumor and even this mouse's tumor regressed, illustrating the possibility of a recall response from the T- cells. The second test to further the finding of the immunological effects of RNA origami, was to test what would happen to mice that were immunocompromised (nude BALB/c mice that have no thymus, thus lacking T-cells). For this group, there were four groups of mice each one having 5 mice and they received 500,000 CT26-iRFP cells IP. Two of the groups were like the initial experiments where one was treated with PBS and the other treated with RNA origami. These two groups were treated on days 1, 5, 8, and 12. The other two groups received no form of treatment. One group was only injected with the cancer cells, while the other group was injected the day before the cancer cells with 20 million splenocytes IP that were harvested from one of the RNA origami treated mice from the second group that showed tumor regression. This adoptive transfer group of mice would display whether memory T-cells had formed against CT26 cancer cells and that T-cells are crucial responders to the RNA origami's adjuvant properties. The outcome for the group is in *Figure 26* The results were promising in regard to RNA origami's anti-tumor attributes. Since the RNA origami treated group failed, this means that T-cells are required for the RNA origami to combat cancer, illustrating that this is a form of immunotherapy and it is not the RNA origami that is actually doing the treatment.

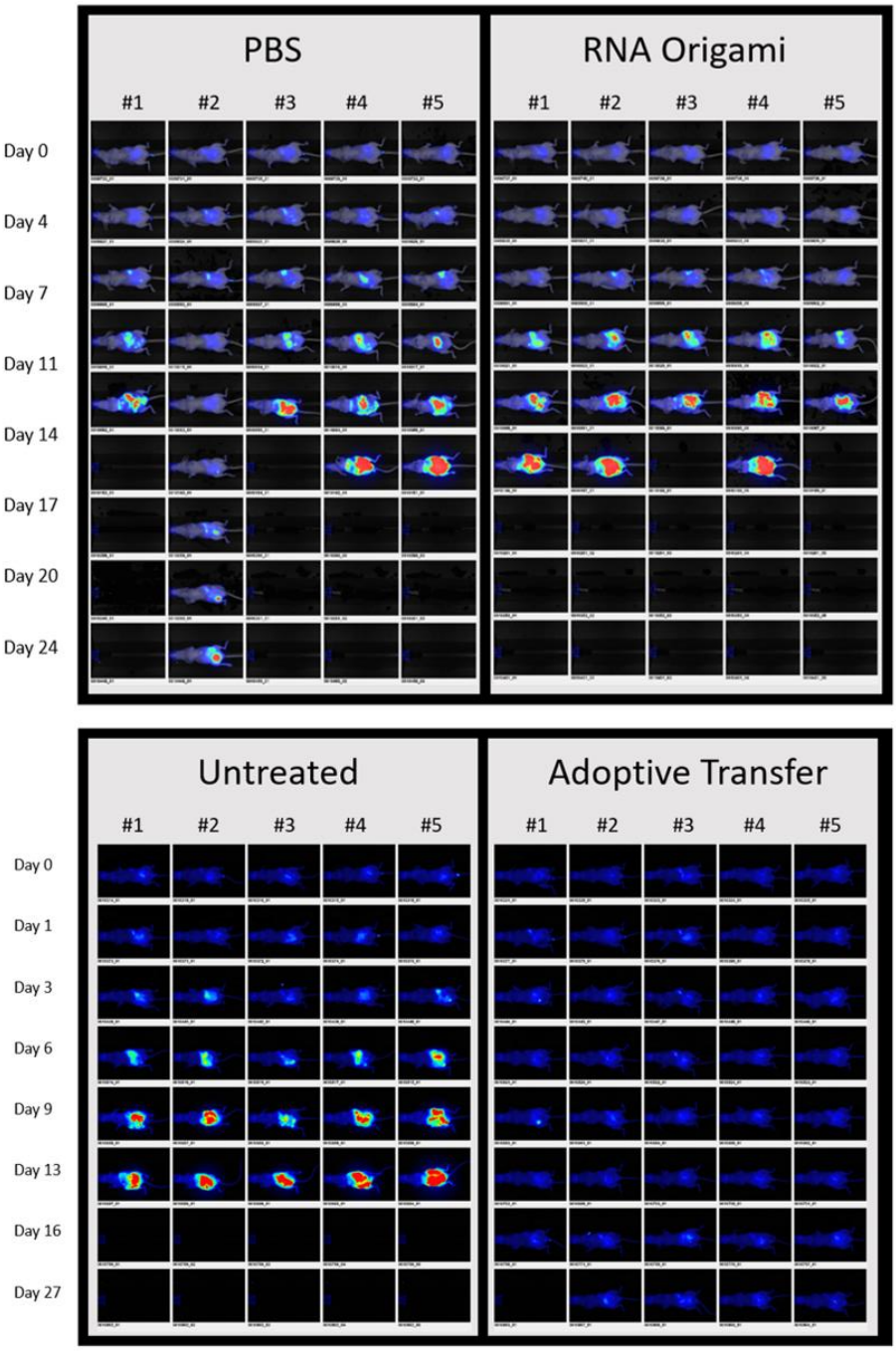

Figure 26. Immunocompromised treated and transfer group. Twenty nude BALB/c mice injected IP with 500,00 CT26-iRFP. The upper left group was treated with PBS (100 ul) IP four times over a month and the upper right group was treated with RNA origami (100 ul at 16 ug) IP four times over a month. Treatments started one day after cancer cell injections. The bottom left group received no treatments and the bottom right group received splenocytes from an RNA origami treated mouse IP the day before the cancer cell injection. Mouse #1 from adoptive transfer group was removed experimentally, not succumbed to tumor growth. Images taken with a LI-COR Bioscience Pearl Imager.

Evaluation of IFN gamma-producing T cells in tumor-bearing mice. In addition to the monitoring of tumor progression in the mice, some of the mice that were part of these experimental groups were sacrificed at the end of studies to look into more detail the immune-stimulant properties. This involved harvesting splenocytes from treated mice, in this case RNA origami and HMW Poly(I:C), to see if that during the effector phase (point in the adaptive immunity when T-cells and B-cells are active and releasing cytokines to stimulate an immune response (Parham, 2015)) IFN gamma was present. In order to do this, an ELISPOT assay (enzyme-linked immunospot where specifically coated plates can use antibodies to bind to corresponding antigens that are then bound with a corresponding antibody with a substrate that can be visually detected, formation of spots, when activated, (Lodish, 2016)) was performed on the harvested splenocytes to see if IFN gamma was released in the presence of CT26 cells, results shown in *Figure 27*. The outcome presented that both RNA origami and Poly(I:C) treated mice release IFN gamma. In comparison to the PBS control, the RNA origami and Poly(I:C) IFN gamma levels were almost four-fold greater, with RNA origami having the most abundant presence. Furthermore, unassembled RNA origami showed that it could stimulate the secretion of IFN gamma, but at a much lower level than RNA origami and Poly(I:C), unassembled RNA origami murine images were part of the five-day delay treatment group shown in the supplemental materials. Therefore, ELISPOT showed the inverse relationship between the presence of IFN-gamma and the growth of the tumor, where the greater number of IFN-gamma producing cells found in a region, the lesser prevalence of tumor growth occurred.

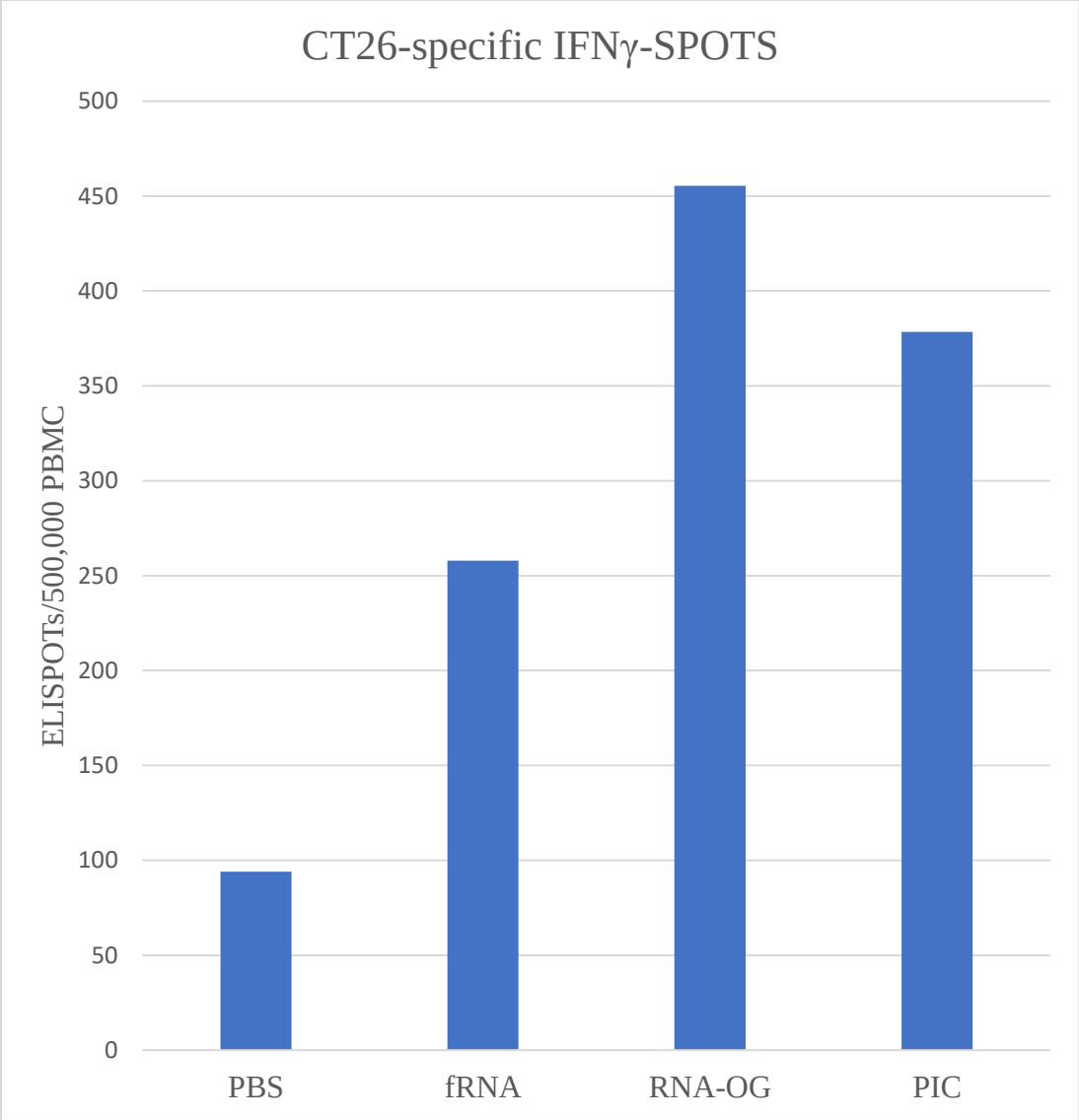

Figure 27. IFN-gamma ELISPOT results. Standard ELIPOT protocol was used, triplicates were done for each group and averaged, control backgrounds were done for all of the cells and the controls average was subtracted out from each group. The presence of spots indicated the presence of IFN-gamma. There were four groups: PBS, fRNA (unassembled RNA origami), RNA origami, and Poly(I:C). Peripheral blood mononuclear cells (PBMC) were acquired by bleeding for mouse serum.

Analysis of cytokine profiles from in vivo murine models. In addition to ELISPOT, cytokine analysis was performed on ascites (fluid that can collect in the peritoneal cavity) extracted from treated mice (PBS and RNA origami) and a naïve mouse for comparison. This cytokine profile analysis was conducted by Dr. Chang and looked at specific

proinflammatory cytokines (IFN-gamma and tumor necrosis factor alpha (TNF)) and anti-inflammatory cytokines (transforming growth factor (TGF) b-beta 1, TGF-beta 2, interleukin (IL) 10 and IL-4). TGFb-beta1, TGF-beta2, IL-10, and IL-4 have been known to contribute to the suppression of various immune cells and immunological responses (Owen 2009). The analysis demonstrated that the RNA origami was able to upregulate proinflammatory cytokines and down regulate anti-inflammatory ones clearly showing RNA origami's ability to induce an immune response, as seen in *Figure 28*. The tumor

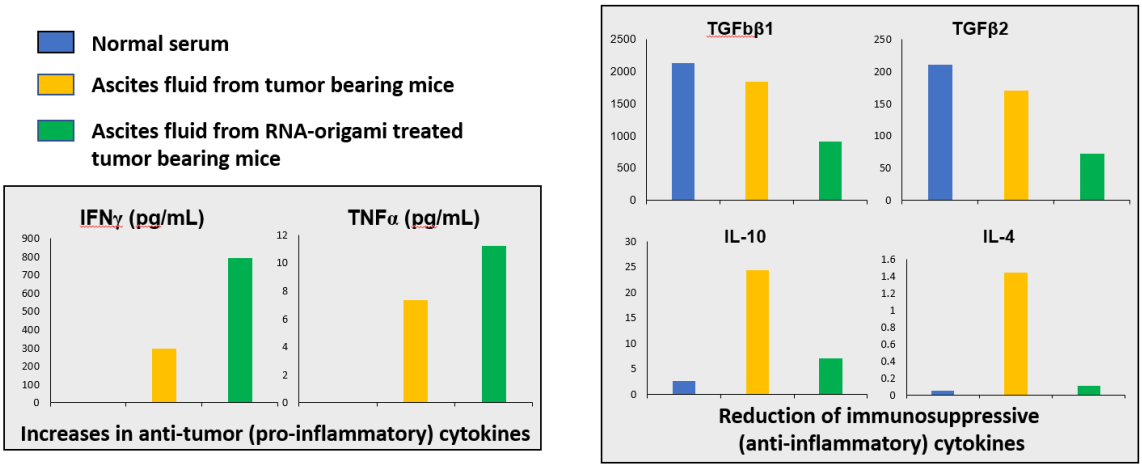

Figure 28. Proinflammatory and anti-inflammatory cytokine profile. There were three groups: naïve mice serum, PBS tumor-bearing mice ascites, and RNA origami treated ascites. Proinflammatory cytokines tested: IFNγ and TNFα. Anti-inflammatory cytokines tested TGFbβ1, TGFβ2, IL-10, and IL-4.

bearing PBS treated mice also demonstrated that in the presence of tumors the anti-inflammatory cytokines were the stimulated (the TGFb-beta1 and TGF-beta2 levels were high in the naïve mice due to these being necessary to prevent the immune system from being constantly stimulated). This highlights the ability of cancer cells to induce tolerance and immunosuppression to interfere with the generation of anti-cancer immunity. On the other hand, the RNA origami could clearly reverse this trend and attenuate the immunosuppression of the tumor environment in peritoneal metastasis. This finding led to

further examination of immunosuppressive cells in this compartment, i.e. myeloid-derived suppressor cells (MDSC) (Parham, 2015). There were two types of MDSCs, monocytic, M-MDSCs and granulocytic, i.e., G-MDSC, which were defined by the staining of two sets of surface markers, Ly6G and Ly6C. Thus, murine M-MDSCs were defined as $CD11b^{+}Ly6G^{-}LyC^{hi}$ and G-MDSC as $CD11b+Ly6G+Ly6C^{lo}$ (Parham, 2015). This was done by using corresponding dyes that check for the expression of lymphocyte antigen complex G (Ly6G: blocks recruitment of neutrophils) and lymphocyte antigen complex C (Ly6c: regulates IL-2 production with T-cells, which can suppress T-cells) on the extracted cells (Parham, 2015). The appearance of both of these on a cell would demonstrate the presence of MDSCs being upregulated in that region. As seen in *Figure 29*, this was the case for untreated tumor-bearing mice in both the spleen and the peritoneal cavity, but not for the mice that had received the RNA origami treatment in some form. The RNA origami mice showed some differences between it and the tumor-bearing mice in the spleen, but the difference was more noticeable in the peritoneal cavity. The nude-immunocompromised mice that had RNA origami treated splenocytes exhibited the greatest variance of all, especially in the peritoneal cavity. Thus, this indicated that the RNA origami is able to elicit an immune response and not have it suppressed by MDSCs, allowing for continued stimulation of the adaptive immune response that could attack the cancer.

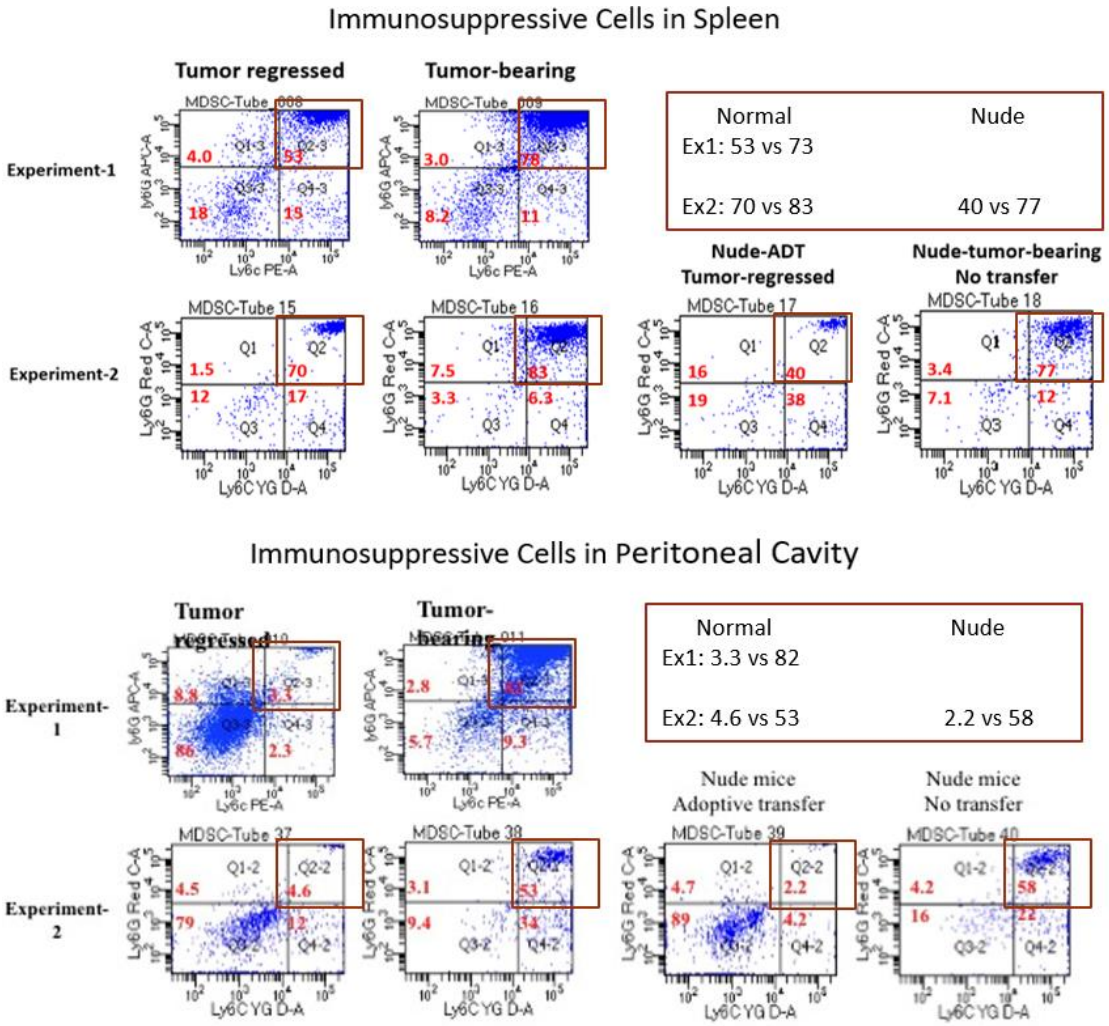

Figure 29. MDSC detection in murine models. There were four mice types: RNA origami treated mouse that had tumor regression, tumor-bearing mouse (PBS treated), nude mice that received adoptive transfer of splenocytes from an RNA origami treated tumor regressed mouse, and a nude tumor-bearing mouse with no treatments. On the plots, the top left showed cells with ly6G, the top right showed cells with both ly6G and ly6C (MDSC), the bottom left showed that neither one was detected, and the bottom right showed cells with ly6C. The top left value indicated whether immunosuppressive cells were present. Values in the plot were the percent of cells in that quadrant. Result done in assistance by Dr. Yung Chang.

Discussion on RNA origami as a therapeutic adjuvant. The results from both the *in vitro* and the *in vivo* experiments showed promising outcomes for RNA origami as a therapeutic anti-cancer adjuvant. The *in vitro* studies highlighted that RNA origami was able to elicit various immune cells to promote the innate and adaptive immune response

and provoke the release of immuno-active cytokines, all of which can mostly be contributed to RNA origami's initiation of the TLR3 pathway. All of these components were evident in the comparisons done with Poly(I:C), which has already been established as an immuno-adjuvant (Ammi, 2015, p. 120). As seen in Table 2, the comparison between

Table 2

Summary of the In Vitro Effects of RNA Origami Compared to Poly(I:C)

	Control (PBS)	**HMW Poly(I:C)**	**RNA Origami**
TLR3 Pathway (HEK-blue)			
	-	++	++
RIG-I and MDA5 Pathway (A549 WT-MAVS and KO-MAVS)			
	-	++	-
Macrophage Line (RAW-264.7) Activation			
	-	+	++
Ex Vivo Lymphocyte Stimulation			
T-Cells (CD69)	-	+	++
B-Cells (CD69)	-	++	++
cDC	-	+	++
pDC	-	++	++
B-cells (APC)	-	++	++
Macrophages	-	+	++
Ex Vivo Cytokine Production			
CCL5	-	+	+
CXLC10	-	++	++
IFNα	-	-	+
IFNβ	-	-	+
IFNγ	-	-	+
Serum Cytokines			
CCL2	-	++	-
CCL5	-	+	-
CXLC10	-	++	++
IFNα	-	++	-
IFNβ	-	++	-

Note. (+) indicated at least a one-fold difference than the control. (++) indicated two-fold or greater difference than the control. (–) indicated little to no difference to the control.

Poly(I:C) and RNA origami was promising. In many instances, RNA origami performed better than Poly(I:C). The main difference between Poly(I:C) and RNA origami, *in vitro*, was the PRRs that they activated (Poly(I:C) can interact with RIG-I and MDA5 and RNA origami could not). RNA origami's inability to activate these might play in its favor due to these features of Poly(I:C) possibly contributing to the toxicity levels that are reported with it when used at high concentrations. This could be due to the overexpression of certain immune responses which end up being toxic to the host, thus giving RNA origami a more favorable appeal over Poly(I:C). These positive outcomes from the *in vitro* studies helped led to the *in vivo* studies. The *in vivo* studies with RNA origami showed that it could be used as an effective therapeutic in treating cancer in the designed PM model. As illustrated in the previous images and summarized in Table 3, RNA origami effected the growth of the cancer, especially if the treatments were performed earlier on (primarily day one treated). In several cases, it proved that the RNA origami elicited an immune response that prevented the growth of the CT26 cell, where they were eliminated before detection could be achieved from the imager. In other cases, clear tumor regression was observable. The tumor development was initially seen both visually by the enlarging of the abdominal region and by the fluorescent detection from the imager, but as time went on and the mice were treated, the tumor eventually was destroyed by the immune system. Additionally, it showed that RNA origami could lead to tumor growth delay, usually in the delayed treatment studies (day three and day five treated). This was seen in comparison to the PBS group which had the mice reach their endpoint much faster that the mice treated with RNA origami, thus demonstrating the potential effects the stimulation of the immune system had on tumor progression. This was only further supported by the results from tests observing

Table 3

Summary of the In Vivo Effects of RNA Origami

	Control (PBS)	HMW Poly(I:C)	RNA Origami
CT26 Tumor Growth			
Day 1	++	-	-
Day 3	++	N/A	-
Day 5	++	N/A	+
IFNγ in Murine Model Serum (ELISPOT)			
	+	++	++
In Vivo Murine Model Ascites Cytokine Profile			
IFNγ	+	N/A	++
TNFα	+	N/A	++
TGFbβ1	++	N/A	+
TGFβ2	++	N/A	+
IL-10	++	N/A	-
IL-4	++	N/A	-
MDSC Presence in Murine Model			
Splenocytes	++	N/A	+
Peritoneal Cells	++	N/A	-

Note. (+) indicated at least a one-fold difference than the control. (++) indicated two-fold or greater difference than the control. (–) indicated little to no difference to the control. N/A indicated that the category was not apart to the study.

immune-suppressive cells and anti-inflammatory cytokines. RNA origami treated mice showed that MDSC were present at reduced levels as well as cytokines such as TGFbβ1, TGFβ2, IL-10, and IL-4, which are known to regulate and suppress immune-stimulation. Even more validation for RNA origami's aptitude to interact with the immune system was seen with both the tumor re-challenge group and the active transfer group. Both cases presented how it is the adaptive immune system that is attacking the cancer by either preventing the CT26 cancer cells from growing when reinjected into a previously RNA origami treated mouse, or the ability of RNA origami treated splenocytes to recognize the cancer and prevent it from growing in a new host that is also immunocompromised.

Furthermore, this nude group of mice also showed the development of immunity to the CT26 cancer cells, meaning that the RNA origami could lead to the development of memory T-cells against the cancer line. Finally, the *in vivo* trials also supported some of the findings in the initial *in vitro* studies by illustrating that RNA origami could stimulate T-cells, promote the secretion of the similar cytokines found in the *in vitro* studies, and cause the production of IFN-gamma, a vital cytokine that helps upregulate the both the innate and adaptive immune system.

CHAPTER 5

CONCLUSION

Perspective of RNA origami as a viable therapeutic against cancer. From the initial studies using RNA origami as a therapeutic against CT26 cancer in the PM model, there does appear to be validity in the findings. The work done towards this thesis, specifically the *in vivo* testing in the murine models only help confirm the findings from the *in vitro* studies. The murine models that were looked at illustrated that the RNA origami could elicit an immune response substantial enough to lead to complete remission of the metastasized cancer. Furthermore, it highlighted that RNA origami could halt growth of the tumor, leading to regression, causing a delay in the progression of the cancer, and/or even possibly leading to anti-tumor immunity. Moreover, the findings helped verify some of the results from the previous *in vitro* tests such as the possible cytokine presence and the immune cells that are stimulated by RNA origami. Lastly, the research done here established the potential of the peritoneal metastasized colon cancer model. It exhibited that the model was valid in demonstrating tumor growth and that it could be comparable to how cancer can develop in certain situations; metastasis leading to peritoneal carcinomatosis. Additionally, it illustrated how IP treatments provide a unique approach that could take advantage of the distinct immune microenvironment in the peritoneal cavity. Overall, the results shown here, while preliminary, displayed RNA origami as a functional adjuvant in immunotherapy against, at least, metastasized cancer in the peritoneal cavity and offered a possible therapeutic means against peritoneal carcinomatosis, something that desperately needs other techniques for treatment to manage these malignancies.

Future of RNA origami as an immunotherapy against cancer. The research that has been done so far with RNA origami shows great promise. Further studies need to be conducted to continue to demonstrate its ability as an anti-cancer adjuvant. Many of the findings need to be repeated and re-challenged with increased sample sizes to validate the conclusions. Additionally, some to the parameters require refinement such as testing the effects of increased concentrations of RNA origami administered as a therapeutic, comparisons to other adjuvants, and further developments in finding what the optimal time to start treatment before the RNA origami loses its efficacy. Another area of focus for future studies is applying it to other forms of cancer. In this cancer model, other cancers that could be valid include gastric, cervical, liver, ovarian, and renal cancer to name a few. Beyond the current model, there are other types of cancers that RNA origami can be tested against, such as breast, skin, and so on. With this though, the tumor model and the method of deliver would have to change with the RNA origami. Additionally, the RNA origami itself could be further refined and optimized. This might mean that the structure could be altered, such as how DNA origami continues to be modified, so that the origami could elicit a greater immune response, stay longer in the host's system, or have certain modifications that can improve its ability to target/fight cancer. Other options would include complexing the RNA origami with various peptides that make it target the cancer or ones that improve the stimulation of the immune response. Furthermore, the RNA origami could be applied as a vessel to deliver or used in conjunction with a drug that could either help eliminate the tumor or suppress regulation of the host's immune system. In addition to trying to improve the function of RNA origami as an active treatment against cancer, the RNA origami could also be tested as a preventive therapeutic. This includes trying to integrate the RNA

structure as a platform for a preventative vaccine. As shown with the active transfer murine model, the RNA origami exhibited that it could result in the development of memory cells against the cancer, which means that various methods could be developed to allow the RNA structure to be incorporated prophylactically so that the host's system would be immune to that type of cancer. The combination of all these possibilities demonstrates how RNA origami can continue to be manipulated to combat various types of cancer.

Possible applications of RNA origami in biology. As mentioned earlier, the use of RNA origami as an anti-cancer therapeutic is promising and it also brings up the possibilities of other ways it can be developed. Although it is currently being looked at as an immunotherapeutic against cancer in this case, this does not limit it from other applications. Regarding immunotherapy, it could be applied as an adjuvant that could fight other types of ailments besides cancer with similar results. Beyond this, RNA origami could end up taking a similar approach to DNA origami. Since RNA nanostructures can be designed and modified, so too, can their applications. The multipurpose of RNA in biology allows for a wide-range of uses for these designed structures. This would include other medicinal purposes such as drug delivery or other forms of vaccines. Additionally, like DNA nanoparticles, they could be created for biological diagnostic purposes as well. In conclusion, the versatility and potential for RNA origami is great, and as shown with its ability to be used as an immunostimulatory adjuvant, it could prove to be a valuable tool in both the fields of medicine and biology.

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APPENDIX A

SUPPLEMENTAL FIGURES FOR CHAPTER 4

BIOLOGICAL APPLICATION OF RNA ORIGAMI IN CANCER TREATMENT