INVESTIGATION OF CAVEOLAE-MEDIATED ENDOCYTOSIS IN RESPONSE TO INTERFERON

BY

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THESIS

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ABSTRACT

Cancer contributes substantially to overall mortality and morbidity worldwide, resulting in considerable research focused on treatments. Yet many types of cancers continue to have a poor prognosis[1]. Cancer is an incredibly diverse affliction, resulting from numerous changes in normal cell physiology. Treating such a diverse disease will take highly interdisciplinary work from clinicians and scientists to discover novel methods to fight this condition and improve existing treatments. The broad objective of this work is to obtain a better understanding of the actions of type I interferons (IFN), cytokines that display potent antiviral as well as anti-cancer activity. IFN has a variety of cellular effects, some of which lead to its anti-tumor nature, but many of which are not fully understood. Some cellular effects may contribute to the detrimental side effects that may occur during IFN treatment and significantly affect a patient's quality of life. Thus, additional research is needed to improve the use of IFN as a cancer treatment.

This work sheds light on type I interferon's (IFN) effects on cellular endocytosis. Endocytosis, a vital process in healthy cells used to internalize material, is a likely IFN target. The anti-tumor nature of IFN may be an unintentional byproduct of its antiviral effects, which are designed to halt pathogenic assault, including pathogen entry into the cell via endocytosis[2,3]. IFN-induced effects on endocytosis may also contribute substantially to its anti-tumor nature, as abnormal endocytosis has also been found in multiple cancers[4,5]. To examine the effects of IFN on endocytosis, several fluorescent techniques were used to quantify the internalization of cholera toxin in IFN-treated and untreated cells. Flow cytometry was used to quantify the internalization of fluorescent pathway-specific cholera toxin with established concentrations[6], and the differences between the IFN-treated and untreated cell samples were assessed with statistical methods. The commercially available fluorescent reagent, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate (DiD) was also used to stain the plasma membrane and fluorescent-labeled cholera toxin to image IFN-treated and untreated

cells with confocal microscopy. This work showed that IFN induced a statistically significant increase in the endocytosis of cholera toxin in NIH-3T3 cells.

This work not only expands the basic knowledge of IFN and its cellular effects, but also further characterizes a potential IFN target, namely endocytosis. This knowledge may lead to its improved use as a cancer treatment, and may also benefit gene/drug delivery research. Elucidating these IFN-induced changes in endocytosis may also lead to improved therapies which mimic IFN's effects while bypassing adverse side effects.

ACKNOWLEDGEMENTS

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CHAPTER 1: INTRODUCTION

1.1 Interferon

Interferon (IFN) is comprised of a family of signaling molecules secreted by cells that initiate immune responses against invading pathogens. While there are multiple types of IFN, this work focuses on Type I IFN due to their importance in medicine, specifically in cancer treatment, and the extent of research available. Type I IFN is comprised of IFN-α and IFN-β, which display potent antiviral activity. IFN has diverse effects, including manipulation of cellular metabolism, activation of immune counterparts, and antiproliferative effects[7,8]. IFN stimulates over 300 genes that detect viral molecules and modulate signaling pathways to block viral replication[7].

Type I IFN gene transcription is induced when cells detect foreign pathogens. This detection occurs through numerous pathways, however, only the most commonly reported methods will be discussed due to the complexity of the IFN signaling pathway. In the classical pathway, retinoic acid-inducible gene 1 (RIG-1), an intracellular receptor, recognizes viral RNA[9–11]. This recognition causes a conformation change of RIG-1 that leads to a signaling cascade responsible for the transcription of IFN genes[9,10]. Pathogens can also be recognized by toll-like receptors (TLRs). TLRs are members of a family of receptors that are located within numerous areas of the cell such as the plasma membranes, organelles, and cytosol[9]. TLRs recognize a variety of pathogen-associated molecules derived from viruses, bacteria, fungi, and protozoa[12–14]. TLR pathogen recognition also triggers a signaling cascade which leads to the transcription of IFN genes[9]. Following the production of IFN from cells which detect a pathogen, IFN can signal other neighboring cells in a paracrine-type fashion. Type I IFN interacts with the IFN-α/β receptor (IFNAR) located at the plasma membrane. Binding of IFN to IFNAR activates the heavily researched JAK-STAT signaling pathway, leading to a signaling cascade which activates hundreds of target genes[7,9].

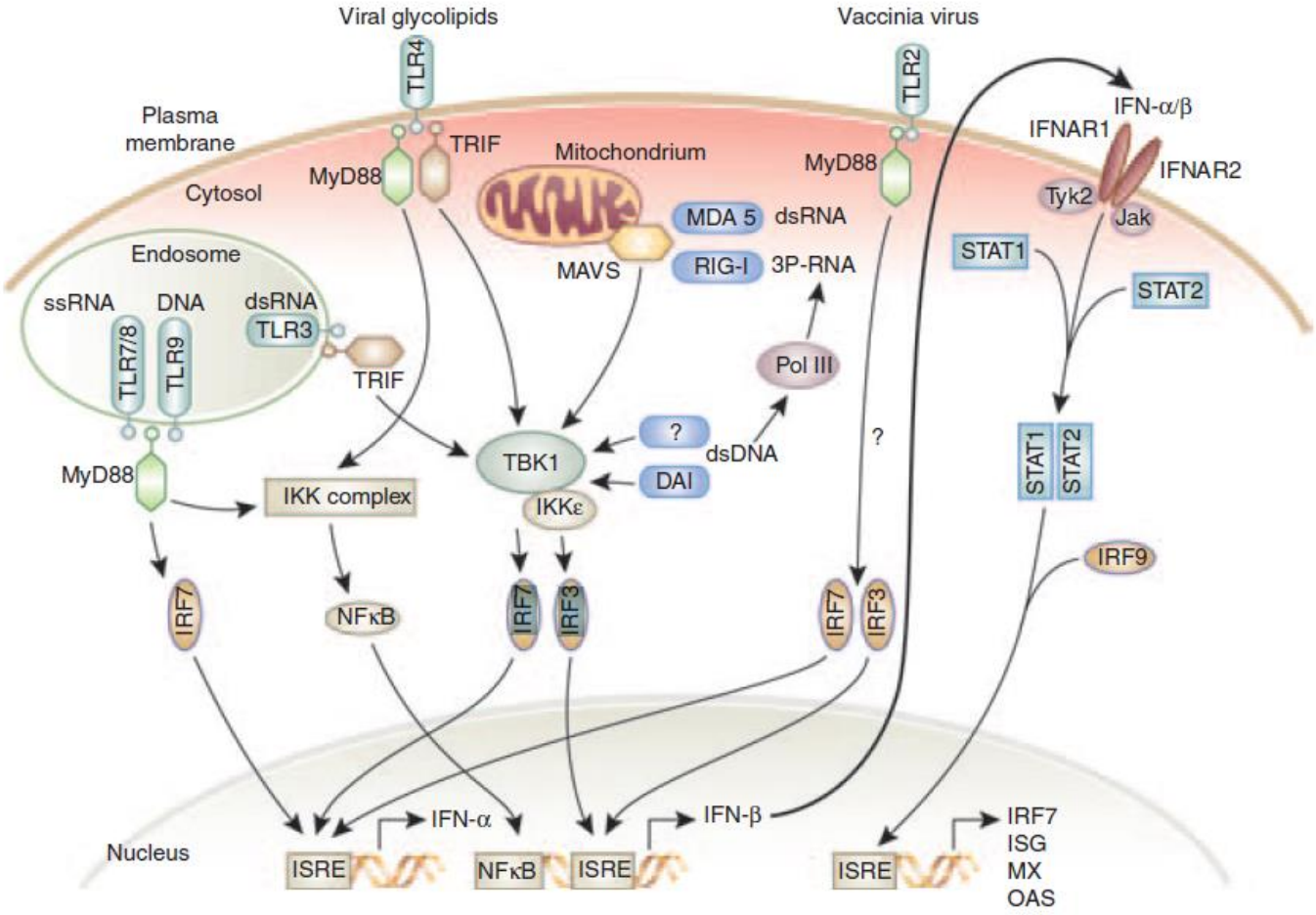

Figure 1.1: IFN production and signaling. Induction of type I IFN (IFN-α and IFN-β) is regulated by pathogen recognition receptors. TLRs are located in various regions on and within cells and are activated by unique pathogen-associated molecular patterns (PAMPs). RIG-I is a helicase and cytosolic sensor for viral RNA. RIG-I interacts with ssRNA and dsRNA. IFN-α and IFN-β are recognized by the IFNAR1/2 receptor, activating the JAK/STAT1 pathway. Reprinted by permission from Nature Publishing Group: [Kidney International] (Ref.11), copyright (2010)).

IFN induces a multitude of effects within a cell which serve to protect it from perceived pathogens. IFN is not only an important aspect of our immunity against pathogens, but holds promise as a therapeutic agent. Not surprisingly, clinicians have leveraged the powerful effects of IFN against a variety of disorders, most notably cancer. Continued research will strengthen our current understanding of IFN and help discern its diagnostic potential.

1.2 Interferon as a Cancer Treatment

Cancer is the second leading cause of death in the United States with costs estimated near $125 billion in 2010 and a projected increase of nearly 27% by 2020[1,15]. Type I IFN are not only crucial cytokines that mediate a host's innate immune defense against pathogens, but are clinically important in the fight against many solid tumors and hematological malignancies such as melanoma, renal cell carcinoma, hairy cell leukemia, follicular lymphoma and AIDS-related Kaposi's sarcoma[16]. Unfortunately, cancer treatment with IFN also carries adverse side effects which can substantially affect a patient's quality of life. These can range from various constitutional effects, such as fatigue[17], headache, and malaise[16], to more severe symptoms indicative of neurological-[18], hepatic- and hematologic-toxicity[19] and autoimmune-mediated thyroid dysfunction[20]. These unwanted reactions show the great need for continued IFN research as a viable cancer treatment.

Type I IFN are obvious candidates for anti-cancer therapy due to their regulation of innate and adaptive immunity which could enhance a patient's natural abilities to fight cancerous cells, and its antiangiogenic and proapoptotic effects[21]. IFN has also been linked to cell growth, a key process for tumorigenesis, through p27, a tumor suppressor that plays an important role in regulating progression through the G1–S phases of the cell cycle[22].

While IFN holds promise as a cancer treatment, both clinical and basic research demonstrate the enormous complexity surrounding IFN and highlight our incomplete understanding of these cytokines. IFN has been used to treat melanoma, a form of skin cancer responsible for the vast majority of skin cancer deaths. An IFN subtype was found to significantly improve disease-free survival among

melanoma patients, however, significant side effects and conflicting data regarding the overall survival lead many clinicians to question its use as a treatment[23]. IFN has also been used for nearly three decades in the treatment of multiple myeloma[24], a cancer of the plasma cells[25]. While its long-standing use as treatment certainly underscores the importance of IFN in myeloma treatment, many conflicting reports have surfaced detailing the inconsistencies reported among clinical studies and the efficacy of IFN treatment[26–31]. The disparities evident in the literature only serve to highlight the need for continued IFN research.

1.3 Linking Interferon and Endocytosis

While the clinical applications of IFN are heavily investigated, the direct mechanisms conveying the wanted, as well as the unwanted, effects of IFN treatment remain unclear. Endocytosis, a vital cellular process to internalize various materials including nutrients, membrane components, metabolites, and extracellular ligands[32,33] is suspected to be a process targeted by IFN[34,35]. Endocytosis is important to many cellular functions aside from its obvious function of internalization, such as the regulation of cell-surface receptor expression, maintenance of cell polarity, and cellular metabolite homeostasis[36]. Aberrant endocytosis has been found to be a hallmark of multiple cancers[4,5]. Dysfunction of important endocytosis machinery such as caveolin-1 (CAV-1), the characteristic caveolae-mediated endocytosis membrane protein, and Huntingtin interacting protein-1, HIP1, a known clathrin-binding protein that serves as a cofactor for clathrin-mediated endocytosis, has been shown in some cancers[37]. Upregulation of CAV-1 has been found to correlate with aggressive, metastatic tumors[38]. This CAV-1 dysfunction has also been linked with poor prognosis in prostate cancers[38,39]. Overexpression of HIP1 has been linked to lymphomas[40], prostate and colon cancer[41], primary breast tumors[42], and gliomas[43].

More and more research points to complex functions of endocytosis that contribute far more to cell biology than merely the process of internalization. Among other things, endocytosis serves to regulate signaling, sort vital biomolecules to their proper places within the cell, and control morphogenetic

aspects. Aberrant control of such processes could account for the metastatic nature and abnormal signaling displayed by some tumor cells[4,5,44].

Research has shown IFN treatment markedly reduces the penetration of vesicular stomatitis virus into cells without affecting the adsorption of virus to the cell surface, an observation consistent with IFN-specific regulation of endocytosis[35]. IFN has the potential to block entry of liposome-DNA complexes[45] and is implicated in the reduced efficiency of adenovirus-aided gene delivery[46]. The antiviral state naturally induced by IFN could limit the endocytosis of material in order to protect the cell from perceived invading pathogens, a manipulation likely induced in healthy and cancerous cells upon IFN treatment. Limiting internalization could slow cell growth due to the decrease in nutrients and signaling factors necessary to proceed through the cell cycle. A decrease in cell growth rate could directly account for the antitumor nature of IFN as an increased rate of cell growth is required to support the rapid proliferation central to tumor growth[47].

Caveolae are 50-100 nm flask-like invaginations located in lipid-specific plasma membrane microdomains and are heavily implicated as an important endocytosis route[48–51]. This endocytosis pathway is known to be a key entry point for virus, including influenza, Simian virus 40, and ebola virus making it a promising IFN target[52,53]. Caveolae are found within plasma membrane microdomain regions that are believed to be enriched in sphingolipids and cholesterol[54]. CAV-1 is a protein found in caveolae that is responsible for the de novo formation of caveolae[55]. This integral membrane protein also binds cholesterol[56]. These findings have led many researchers to speculate that CAV-1 is central to the structure and function of caveolae[56–58]. The cholesterol-binding properties of CAV-1 highlight the intimate relationship between plasma membrane cholesterol levels and caveolae endocytosis. In fact, cholesterol depletion causes disassembly of caveolae leading to disruption of the normal caveolae shape[58]. The control of cholesterol and sphingolipids could provide a means to control caveolae endocytosis.

Recent research suggests a link between IFN-induced proteins and cellular membrane components that are important to the shape and function of caveolae, such as cholesterol and lipids, as well as their

biosynthesis pathways[59,60]. IFN has also been found to decrease plasma membrane fluidity[61], implying modulation of plasma membrane molecular components. Furthermore, viperin, an IFN-inducible protein known to inhibit influenza infection, reduces the activity of an enzyme involved in cholesterol biosynthesis[62,63]. These findings suggest a link between IFN and plasma membrane components vital to caveolae. Though the precise mechanisms for the antiviral effects of down regulated sterol biosynthesis have not been established, the abundance of cellular cholesterol is known to affect caveolae which is also essential for virus infection[64,65]. Distinct membrane microdomains that are enriched with cholesterol have been associated with caveolae, and cholesterol-depletion induced disruption of these microdomains may underlie these changes in endocytosis[66]. Reorganization of membrane components and the alteration of cholesterol and lipid cellular concentrations could provide a means to control caveolae, bolstering our theory that IFN is modulating endocytosis.

1.4 Flow Cytometry

Flow cytometry enables the rapid study of single-cell characteristics using the optical properties of cells and fluorescence. In this technique, cells are labeled either with a fluorescent dye that binds specific cell regions, such as cellular DNA, or antibodies which have been fluorescently labeled. Once prepared cells are suspended, they are drawn into the flow cytometer which uses a sheath isotonic fluid stream, known as simply “sheath fluid”, to surround the cell suspension and create a laminar flow (Figure 1.2). This laminar flow enables single cells to pass through the laser beam to detect single-cell fluorescence. Cells emit light in all directions and a series of filters and mirrors are used to isolate specific wavelength bands. Photomultiplier tubes detect these light signals and convert them for digital analysis[67].

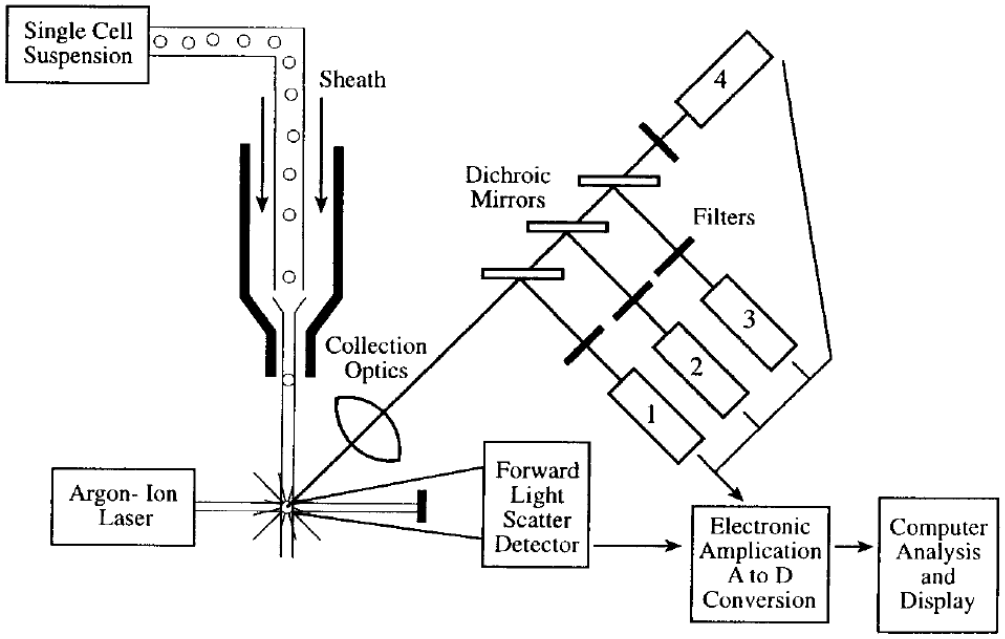

Figure 1.2: Schematic of a flow cytometer. Sheath fluid is used to focus the single cell suspension to intersect a laser. Signal collection occurs via a forward angle light scatter detector, a sidescatter detector (1), and fluorescence emission detectors (2–4)[67]. Reprinted by permission from P.B. HOEBER: [Clinical Chemistry] (Ref. 67), copyright (2000)).

Information from the detected light signals are typically converted and viewed as a histogram or two-dimensional dot plot. The way light is scattered enables not only the fluorescent molecules bound to the cell to be quantified, but also the size of the cell. Flow cytometry is a versatile tool used to analyze a cell's size, DNA and RNA content, and the expression levels of a host of plasma membrane-bound or cytosolic proteins[67].

1.5 Laser Scanning Confocal Microscopy

Fluorescence microscopy is a commonly used technique which provides real-time visualization of fluorescently labeled molecules. Today, researchers are afforded a wide spectral range of commercially available fluorophores[68]. This allows simultaneous imaging of several different fluorescent molecules within cells. Laser scanning confocal microscopy (LSCM) allows optical sectioning of specimen where a

spatial filter, such as a pinhole, is used to eliminate undesired fluorescence above and below the focal plane of interest (Figure 1.3). This allows only fluorescence from the focal point of interest to reach the photodetector[69].

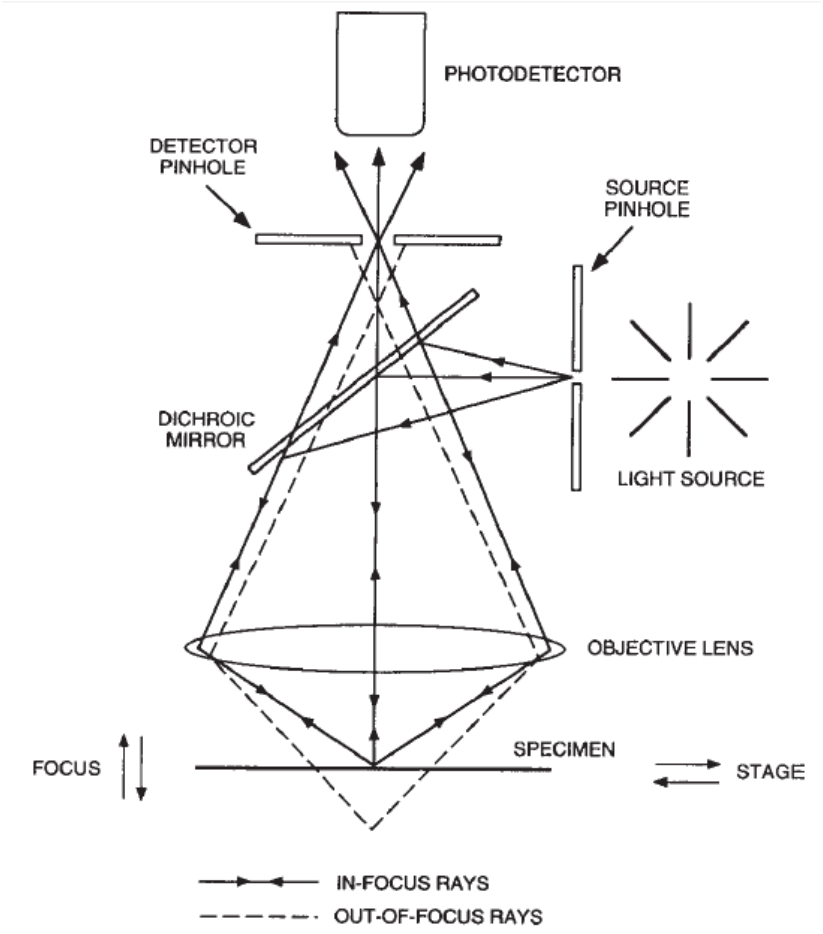

Figure 1.3: The light path in a typical LSCM showing the pinholes placements to produce optical sectioning. Note the pinhole at the light source and the pinhole in front of the detector are all confocal with one another[70]. Reprinted by permission from Springer: [Molecular Biotechnology] (Ref. 70), copyright (2000)).

LCSM allows imaging of fixed intervals within a specimen with both lateral and vertical axis, creating what are known as z-series or z-stacks. These z-stack images can be digitally combined to

produce three-dimensional images of the specimen[70]. LCSM is a powerful tool to visualize key structural components.

1.6 Objectives of this Work

To improve IFN as a cancer treatment we must better understand the intricacy involved in IFN-induced cellular changes, such as its effects on endocytosis, and how these changes disrupt normal cell physiology. Understanding these complex relationships will lead to better knowledge of the tumor-fighting capabilities of IFN, as well as how these IFN-induced changes contribute to adverse side effects. This thesis aims to characterize the key changes in important endocytosis pathways induced by IFN. This study will broaden the current knowledge of IFN and the regulation of endocytosis. Knowledge of these mechanisms could provide the rationale for the design of new cancer treatments that exploit similar pathways while minimizing adverse side effects. Understanding the cellular effects of IFN will not only improve clinical applications, but further basic immunology research. Advances in immunology could help in the fight against common immune disorders as well as provide insights into pathogen clearance that could pave the way for new drug treatments and therapies. Knowledge of the mechanisms by which the innate immune system thwarts viral entry can provide the rationale for the design of not only new antiviral treatments that exploit similar pathways, but also shed light on the IFN-induced cellular responses that must be suppressed to achieve more efficient gene and drug delivery.

The overall goal of this work is to elucidate type I IFN effects on cellular endocytosis. The specific aim focuses on understanding the effects of type I IFN on caveolae-mediated endocytosis. This work characterizes the extent of IFN control on endocytosis, namely through caveolae-mediated pathways. Chapters 2 and 3 provide a detailed methodology and discussion of the experimental results. Briefly, commercially available IFN-αA/D (Sigma), a recombinant universal type I interferon hybrid which is able to cross the species barrier, was used to stimulate the IFN response using established literature to estimate the desired IFN concentration and time of treatment[60,71]. Flow cytometry was used to quantify the internalization of fluorescent pathway-specific cholera toxin with established concentrations,[6] and

the differences between the IFN-treated and untreated cell samples were assessed with statistical methods. The results of the flow cytometry were confirmed by imaging the plasma membrane, which was stained with the commercially available fluorescent reagent, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate (DiD), with respect to the fluorescent-labeled cholera toxin using confocal microscopy. This work showed that IFN induced a statistically significant increase in the endocytosis of cholera toxin in NIH 3T3 cells. This finding refutes the original hypothesis that IFN treatment decreases caveolae-dependent endocytosis. Lastly, in Chapter 4 we discuss our conclusions and provide future directions.

CHAPTER 2: INTERFERON UP-REGULATES THE INTERNALIZATION OF CHOLERA-TOXIN IN MURINE NIH 3T3 FIBROBLAST CELLS

2.1 Introduction

The objective of this work was to determine whether interferon (IFN) limits caveolae -dependent endocytosis, potentially explaining the anti-cancer effects IFN displays. Limiting caveolae-dependent endocytosis may primarily serve to limit virus' entry into cells, helping to slow infection and prevent the spread of the virus into healthy cells; however, this manipulation of an important uptake pathway could result in other consequences. Fluorescence Activated Cell Sorting (FACS) techniques were used to investigate the internalization of caveolae-specific cholera toxin-conjugated Alexa Fluor 488 (CTx-488) in IFN treated and untreated cells. Cholera toxin was chosen as the uptake marker as it is known to enter cells through caveolae[72,73]. Both live and fixed cells were chosen for study and numerous FACS experiments were undertaken to ensure errors did not contribute to the observed trend.

2.2 Materials and Methods

2.2.1 Reagents

Interferon-αA/D (IFN-αA/D) and sterile trypan blue solution were purchased from Sigma-Aldrich. Alexa Fluor 488-conjugated cholera toxin subunit B (CTx-488) was purchased from Invitrogen. Paraformaldehyde at 16% solution was purchased from Electron Microscopy Sciences. Dulbecco's Modified Eagle Medium (DMEM) without phenol red from Gibco was used during flow cytometry analysis.

2.2.2 Cell Culture

NIH 3T3 cells, a mouse fibroblast cell line, were obtained from the American Type Culture Collection (ATCC). Cells were grown at 37 °C in a humidified incubator with 5% CO_2 in DMEM supplemented with 10% calf serum and 1% penicillin-streptomycin. Cells were cultured in 150x20 mm sterile cell culture dishes from Sarstedt.

2.2.3 Cell Preparation for Flow Cytometry and Flow Cytometry Analysis

To investigate the caveolae-mediated endocytosis pathway and its response to IFN treatment, commercially available IFN-αA/D (Sigma) was used to treat NIH 3T3 murine fibroblast cells over several days. IFN-αA/D is a recombinant universal type I IFN hybrid and was used due to its ability to cross the species barrier. IFN treatment concentrations were chosen based on literature[60,71]. One day prior to IFN treatment, cells were seeded in 60x15 mm sterile culture dishes from BD Falcon. Following IFN treatment of 1000 U/mL in cell growth media for 0, 24, or 48 h at 37 °C, 2 µg/mL CTx-488 in cell growth media was placed in the cell dishes for 30 min at 37 °C[74]. Following CTx-488 treatment, cell samples were washed several times with Phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 10 min at room temperature, and analyzed in PBS with a BD FACS Aria II enclosed in a biosafety level 2 cabinet (10,000 events). Some fixed cell samples were also analyzed in trypan blue solution (data indicated below). Live cell samples were washed several times in PBS and analyzed in 1:1 trypan blue:DMEM without phenol red supplemented with 10% calf serum (data indicated below). Live cell samples were kept on ice following preparation until actual analysis. Flow cytometry data was analyzed using FCS Express 3 flow cytometry software.

2.2.4 Flow Cytometry Quantification and Statistics

One-way ANOVA with repeated measures analysis was performed using GraphPad Prism Statistical Software. A Tukey's Multiple Comparison Test was also performed using GraphPad Prism Software to assign p-values to individual groups compared to the 0 h IFN sample. P-values are indicated in all figures by asterisks: *$p<0.01$ and **$p<0.001$.

2.3 Results and Discussion

As shown in Figure 2.1, CTx-488 uptake increased with increasing duration of IFN exposure. One-way ANOVA with repeated measures analysis performed using GraphPad Prism Statistical Software

indicated the observed trend of increasing CTx-488 uptake was statistically significant (p-value<0.0001).

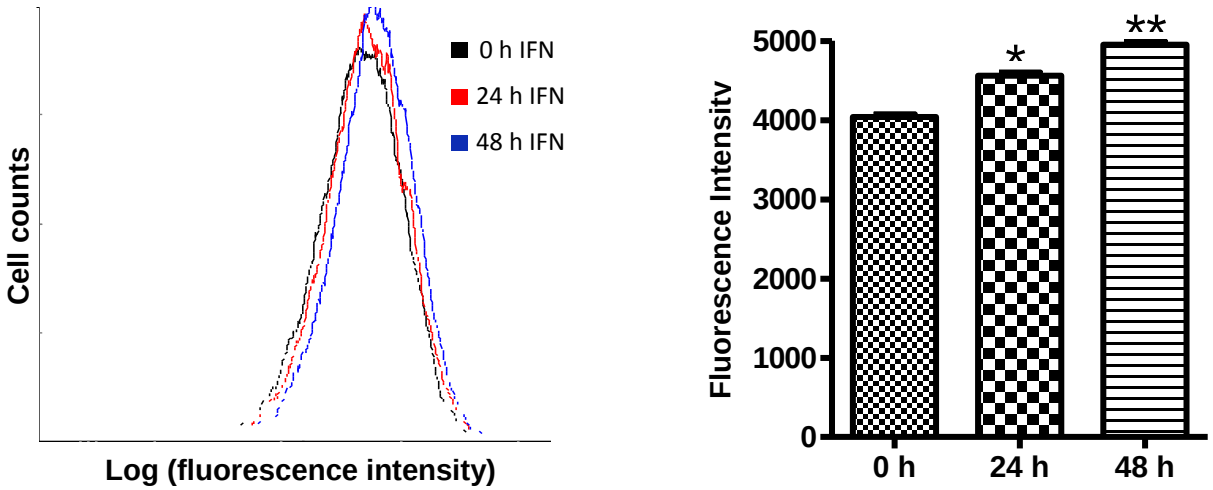

Figure 2.1: IFN up regulates endocytosis of CTx-488 in 3T3 fixed murine fibroblast cells. (A) Histograms show the mean value of each measurement (B) Values are the mean and standard error of the mean (SEM) from two separate experiments with duplicate samples. Differences from 0 h were found to be statistically significant at *p<0.01 and **p<0.001.

A Tukey's Multiple Comparison Test was also performed using GraphPad Prism Software to assign p-values to individual groups compared to the 0 h IFN sample. This finding is the opposite of the original hypothesis that IFN treatment would decrease caveolae-dependent endocytosis.

This unexpected trend calls into question the methods employed. A major question that had to be addressed was whether the increasing trend was merely due to errors associated with the FACS fluorescence detection, such as whether bound excess CTx-488 on the outside of the cell, but not internalized, was responsible for the trend. To evaluate the contribution of bound CTx-488, fixed cells were analyzed in trypan blue. Because trypan blue is a florescence quencher that cannot cross intact plasma membranes[75,76], the fluorescence from surface-bound CTx-488 would be quenched by trypan blue, and only endocytosed CTx-488 would be detected. As shown in Figure 2.2, CTx-488 uptake again

increased with increasing duration of IFN exposure. One-way ANOVA performed using GraphPad Prism Statistical Software indicated the observed trend of increasing CTx-488 uptake was statistically significant (p-value<0.0001). A Tukey's Multiple Comparison Test was also performed using GraphPad Prism Software to assign p-values to individual groups compared to the 0 h IFN sample. This finding supports the original findings shown in Figure 2.1.

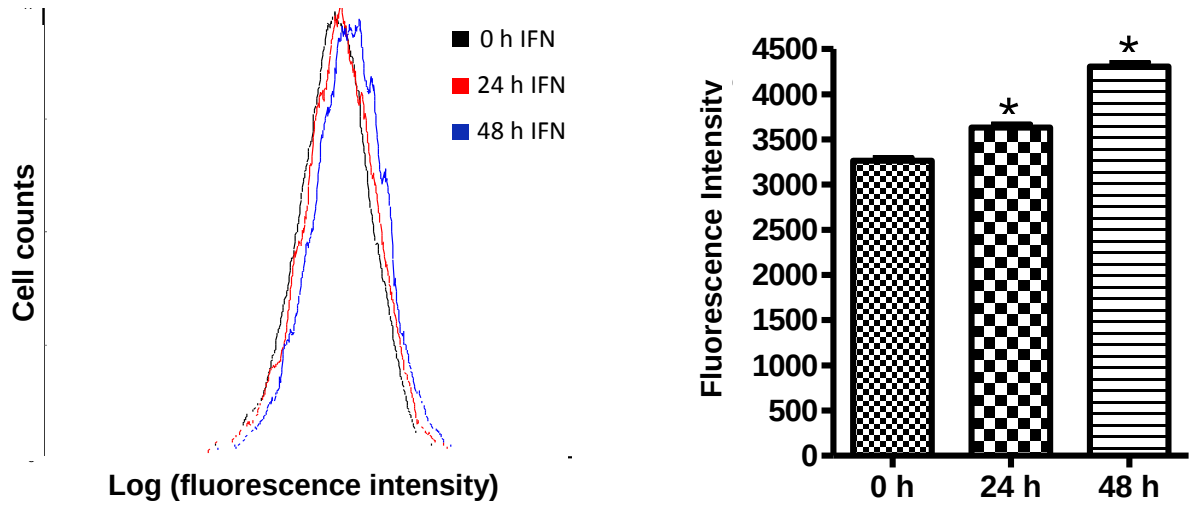

Figure 2.2: IFN up regulates endocytosis of CTx-488 in 3T3 fixed murine fibroblast cells analyzed in trypan blue. (A) Histograms show the mean value of each measurement (B) Values are the mean and standard error of the mean (SEM) from one experiment. Differences from 0 h were found to be statistically significant at *p<0.01.

Although the importance of the trends shown in Figures 2.1 and 2.2 should not be minimized, it should be noted that fixed cells were used to gather this data. The question of whether this trend could be shown in living cells is important. Some fixation techniques can lead to the detection of background fluorescence which could impact the data gathered from FACS. Fixation with formaldehyde, specifically, has also been shown to distort cells[77]. Paraformaldehyde is merely a higher polymer weight of formaldehyde[78]. Formaldehyde is a highly reactive species, interacting with a multitude of different

functional groups of biological molecules such as glycoproteins[77], a major constituent of a cell's plasma membrane[79]. Research has demonstrated that formaldehyde can form plasma membrane vesiculation, the accumulation of fluid-filled vesicles at the plasma membrane[77,80]. These artifacts and drastic distortions evident from fixation call into question the actual similarities between fixed and live cells. If fixation causes major changes within the cells plasma membrane, the entry of excess CTx-488 through means such as diffusion, instead of genuine endocytosis, is a real concern.

To investigate the effects of fixation on CTx-488 internalization, live cells were treated as described above, excluding fixation, and trypan blue was added prior to FACS analysis. Again, trypan blue was used as a florescence quencher to mitigate the fluorescence from surface-bound CTx-488 as it would be quenched, and only endocytosed CTx-488 would be detected. As shown in Figure 2.3, the same increase in CTx-488 uptake with increasing duration of exposure to IFN was observed. One-way ANOVA with repeated measures analysis performed using GraphPad Prism Statistical Software indicated the observed trend was statistically significant (p-value<0.0001). This data supports the earlier finding that caveolae-dependent endocytosis increases as the duration of time that the cells were exposed to IFN increases. A Tukey's Multiple Comparison Test was also performed using GraphPad Prism Software to assign p-values to individual groups compared to the 0 h IFN sample. This finding supports the original findings found in fixed samples.

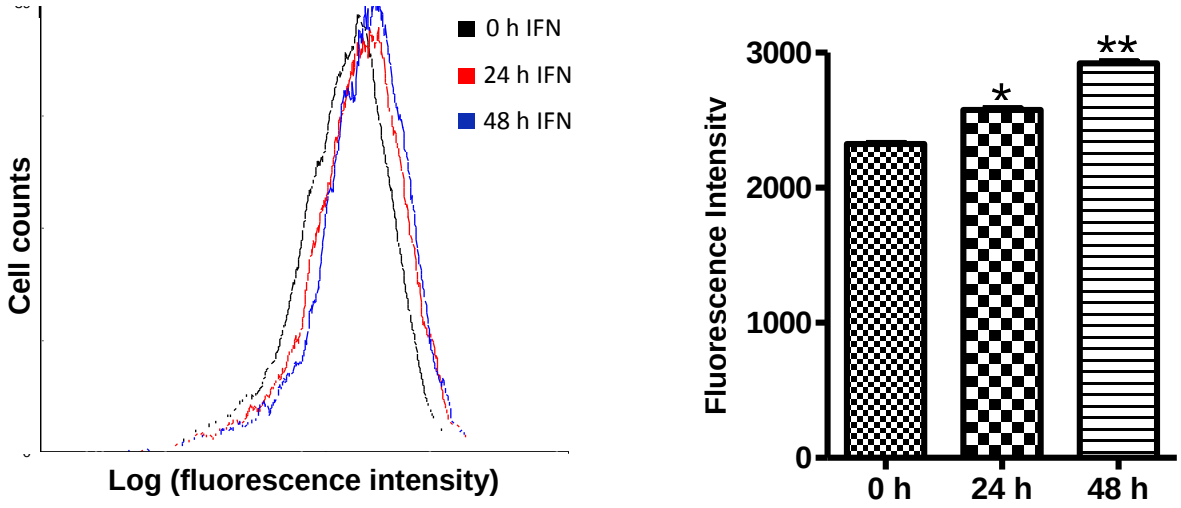

Figure 2.3: IFN up regulates endocytosis of CTx-488 in 3T3 live murine fibroblast cells. (A) Histograms show the mean value of each measurement (B) Values are the mean and standard error of the mean (SEM) from three separate experiments with duplicate samples. Differences from 0 h were found to be statistically significant at *p<0.01 and **p<0.001.

The data presented in Figures 2.1-2.3 provide evidence in disagreement with the hypothesized role of IFN in the regulation of endocytosis. The increasing endocytosis of CTx-488 after treatment with IFN goes against the hypothesis that IFN would decrease caveolae in order to prevent pathogen entry. Several errors that may have contributed to the unexpected trend, such as the detection of fluorescence material on the outside of the cell, have been invalidated. These findings suggest a true trend was found.

CHAPTER 3: CONFOCAL MICROSCOPY CONFIRMS THE TRENDS FOUND USING FLOW CYTOMETRY

3.1 Introduction

While the data discussed in Chapter 2 provide evidence in support of a real trend, it contradicts expected predictions. Caveolae is a major entry pathway of numerous pathogens including influenza, Simian virus 40, and ebola virus[52,53]. Because IFN is theorized primarily to be an anti-virus tool a cell uses to prevent or limit infection[7,8], the up-regulation of caveolae-dependent endocytosis seems counterintuitive. This makes the use of a second technique to verify the trend essential. Confocal microscopy serves as a powerful tool, enabling researchers to directly observe internal objects with excellent resolving capabilities. Although trypan blue was used to verify the internalization of CTx-488 during FACS, confocal microscopy enables the ability to clearly distinguish internal and external compartments of a cell, provided an appropriate dye is available, by eye.

To verify the internal location of the fluorescent CTx-488, DiD membrane stain was used to visualize the plasma membrane of the cells. Using a confocal scanning laser microscope, it was shown that CTx-488 was, in fact, located within the cell. This technique also enabled the visualization of the entire plasma membrane to ensure its integrity, providing evidence that the cell plasma membranes were intact and CTx-488 had entered cells through active endocytosis as opposed to pores or membrane breaks.

3.2 Materials and Methods

3.2.1 Reagents

Interferon-αA/D (IFN-αA/D) was purchased from Sigma-Aldrich. Alexa Fluor 488-conjugated cholera toxin subunit B (CTx-488) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate (DiD) plasma membrane stain were purchased from Invitrogen. Paraformaldehyde at 16% solution was purchased from Electron Microscopy Sciences.

3.2.2 Cell Culture

NIH 3T3 cells, a mouse fibroblast cell line, were obtained from the American Type Culture Collection (ATCC). Cells were grown at 37 °C in a humidified incubator with 5% CO_2 in DMEM supplemented with 10% calf serum and 1% penicillin-streptomycin. Cells were cultured in 150x20 mm sterile cell culture dishes from Sarstedt.

3.2.3 Cell Preparation for Laser Confocal Microscopy and Imaging

NIH 3T3 cells were treated for 0, 24, or 48 h IFN at 1000 U/mL in cell growth media. One day prior to imaging, cells were seeded in 35 mm high glass bottom µ-Dishes™ from Ibidi. On the day of imaging, cells were washed with PBS and then incubated with 2 µg/mL CTx-488 in growth media for 30 min at 37 °C. Next, cells were washed three times with PBS and fixed with 4% paraformaldehyde solution for 25 min. The staining solution was made using 5 µL DiD dye diluted in 1 mL of growth media and was placed on the fixed subconfluent cell layers. The dye was incubated with the fixed cells for 10-15 min at 37 °C. Following incubation, the dye solution was drained and cells layers were washed with PBS and incubated with warmed growth media for 10 min at 37 °C. This cycle of washing with PBS and incubation with growth media was repeated three times. Cells were imaged in PBS on a Carl Zeiss LSM700 confocal microscope with 63X1.4 NA oil immersion objective lens. A 488 nm laser and a 633 nm laser were used for excitation of CTx-488 and DiD, respectively.

3.3 Results and Discussion

Following 30 min of incubation at 37 °C, CTx-488 is internalized by NIH 3T3 cells. Using a confocal scanning laser microscope, it was shown that CTx-488 was located within the cell cytosol. DiD plasma membrane stain is a lipophilic dye which binds cell plasma membrane[81]. As shown below in Figure 3.1, the DiD membrane dye clearly delineates the plasma membrane, showing the CTx-488 located in the cell cytosol. The integrity of the cell plasma membrane is also upheld, as we see minimal red DiD stain located in the cell cytosol. This was important to show that cells were actively internalizing CTx-488, as

opposed to CTx-488 diffusing through pores or breaks within the cell plasma membrane. This data supports the FACS data shown in Chapter 2, proving CTx-488 is clearly entering the cells through active endocytosis means.

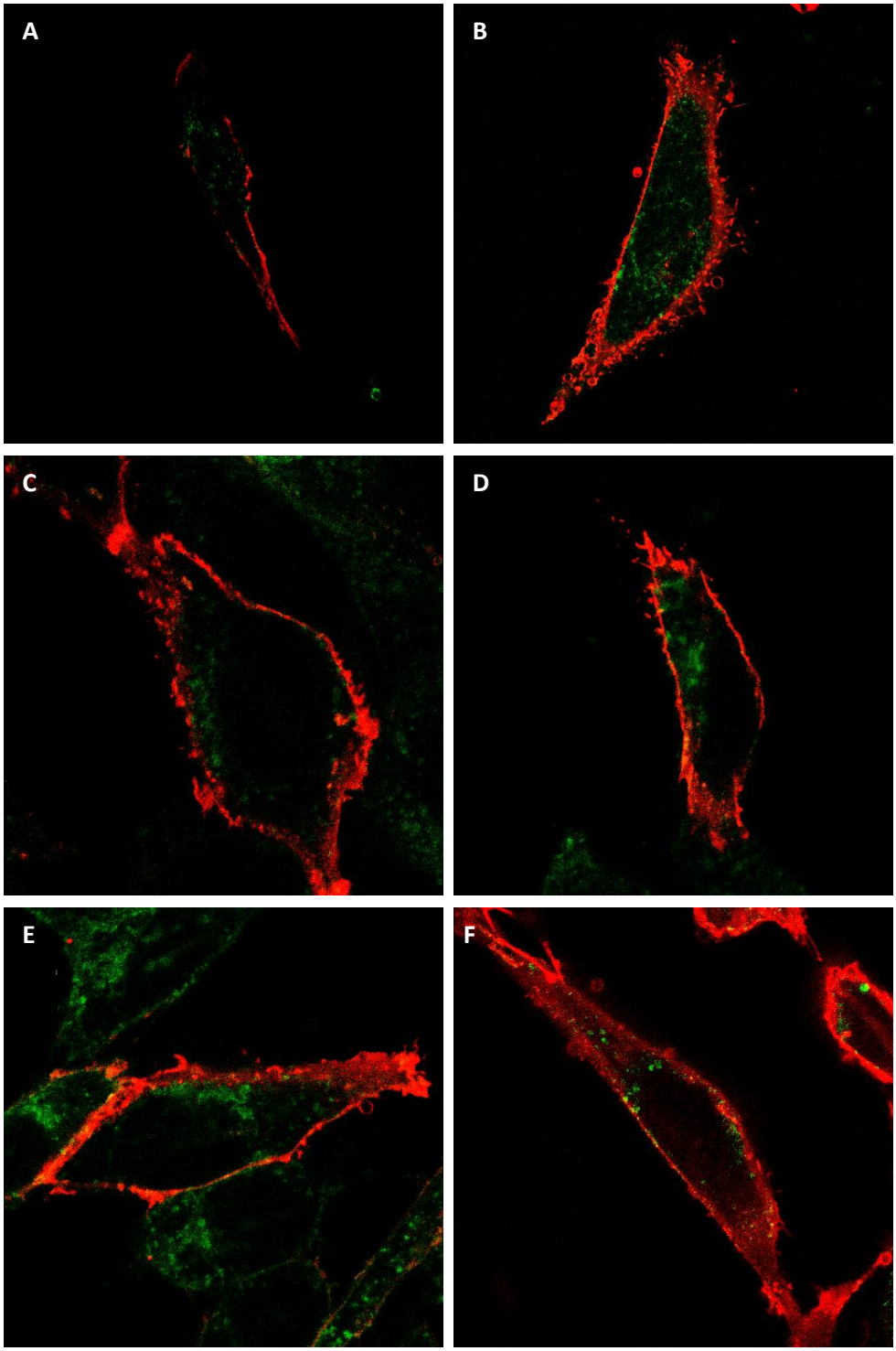

Figure 3.1: Confocal scanning laser microscopy images showing CTx-488 (green) and DiD (red) staining in fixed 3T3 murine fibroblast cells. The plasma membrane is clearly stained red and the green CTx-488 is located in the cytosol. (A-B) Control cells. (C-D) Cells treated with IFN for 24 h. (E-F) Cells treated with IFN for 48 h.

The data presented thus far suggest an increase in caveolae-mediated endocytosis following treatment with IFN. As previously explained this trend is unexpected and must be rigorously researched to ensure the trend is real and not merely a symptom of errors or inherent limitations of the methods used to gather the data. To verify the trend shown using FACS, confocal microscopy was also used to visually inspect the internalized CTx-488. The images shown below are taken from a mid-level section, clearly discerning the cell nuclear membrane. This ensures the plane at which each image was taken did not vary between the different IFN treatment lengths and the images clearly show cells with intact nuclear membranes.

Shown below, the differences visible from the 0 h IFN treated and 24 h IFN treated samples (Figure 3.2 and 3.3) are not quite as apparent; however, the differences from 0 h IFN treated and 48 h IFN treated samples (Figure 3.2 and 3.4) are stark. Although the differences shown in Figures 2.1-2.3 may seem small, as evident in the histograms, the confocal microscopy images prove these differences are detectable by the naked eye. The data shown in Figures 3.2-3.4 support the data gathered using FACS.

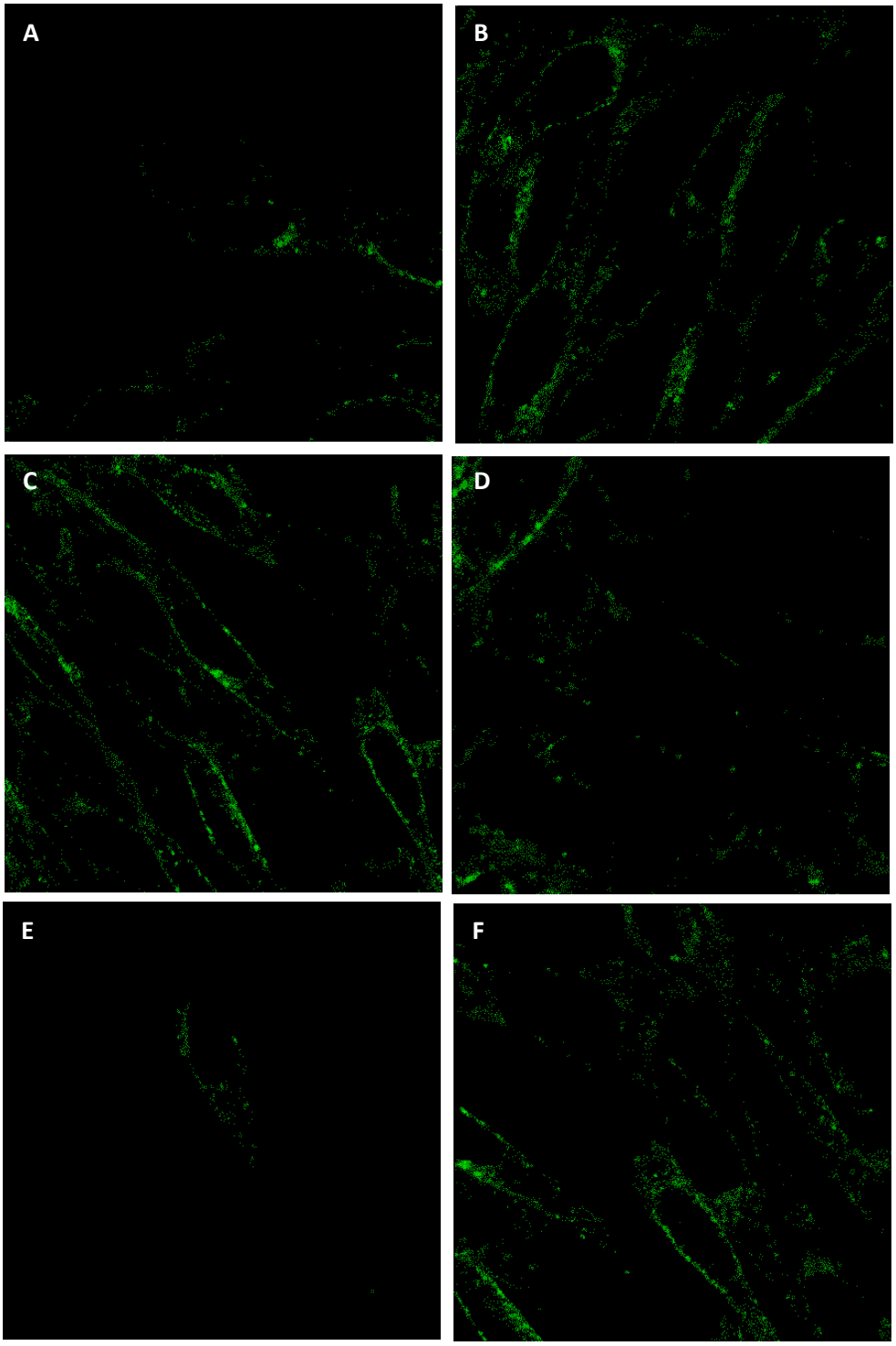

Figure 3.2: (A-F) Confocal scanning laser microscopy images showing CTx-488 (green) staining in fixed 3T3 murine fibroblast cells with no IFN treatment. The red DiD plasma membrane stain was not shown to display the green CTx-488 more easily.

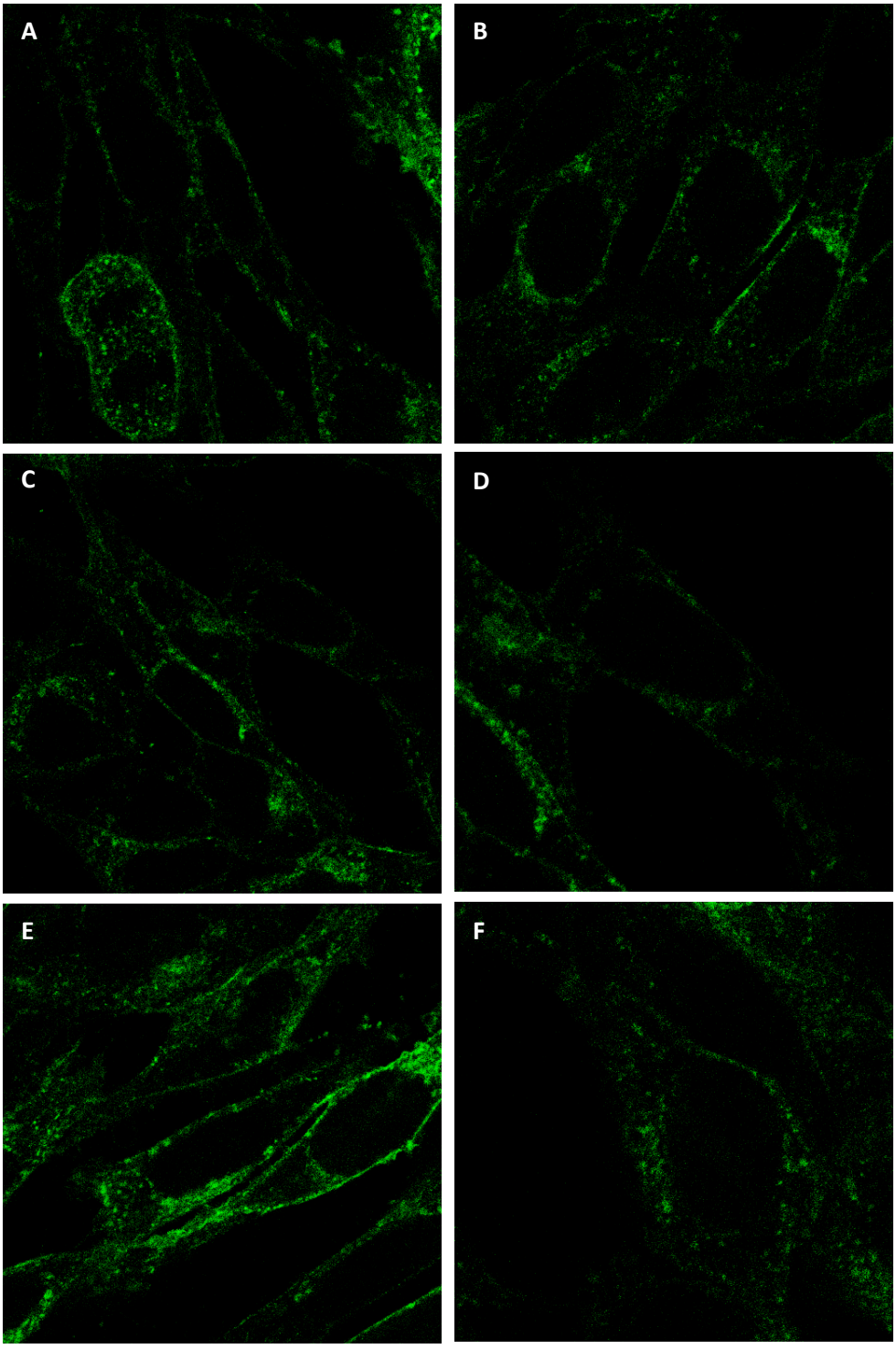

Figure 3.3: (A-F) Confocal scanning laser microscopy images showing CTx-488 (green) staining in fixed 3T3 murine fibroblast cells after 24 h of IFN treatment. The red DiD plasma membrane stain was not shown to display the green CTx-488 more easily.

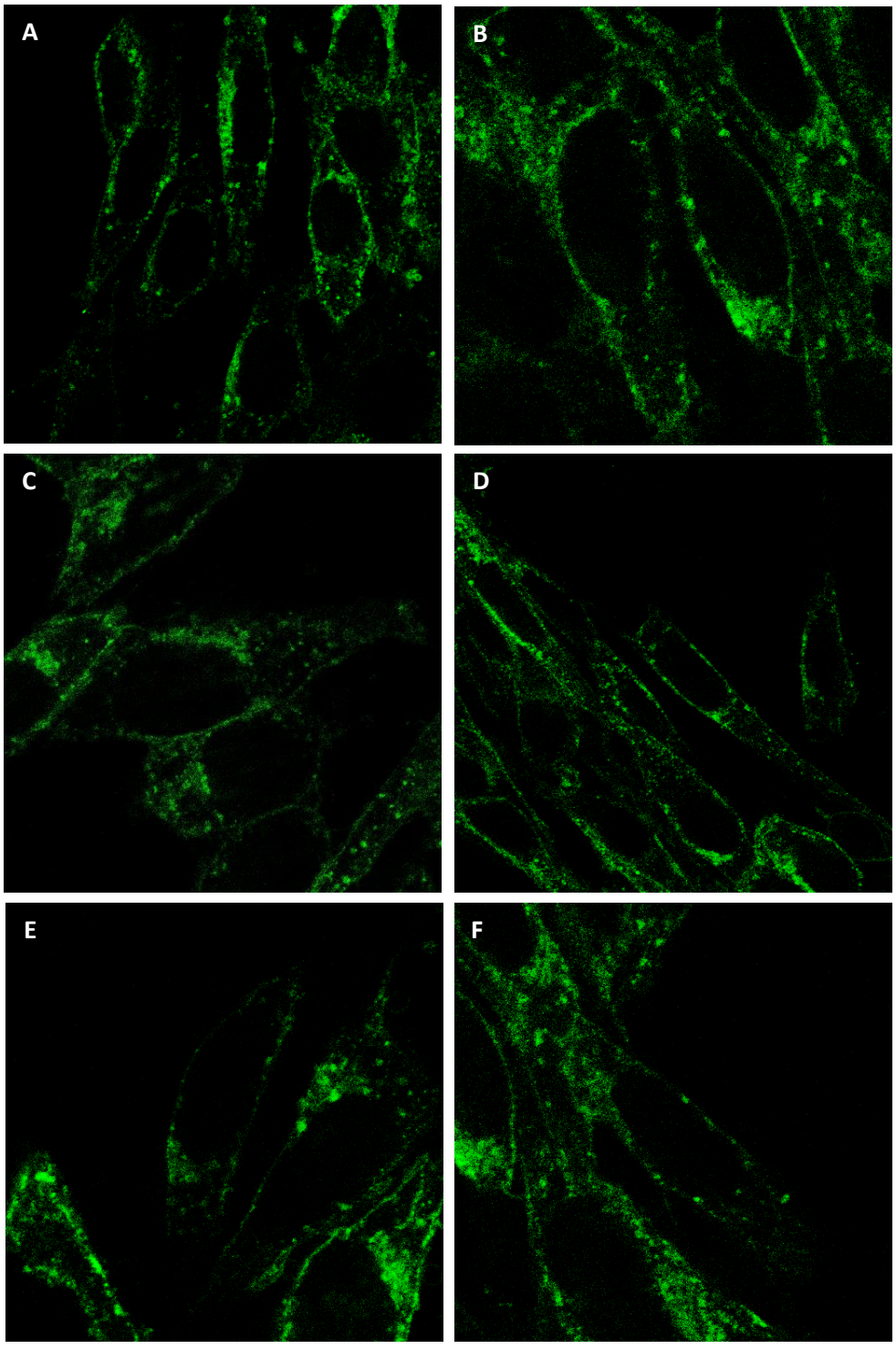

Figure 3.4: (A-F) Confocal scanning laser microscopy images showing CTx-488 (green) staining in fixed 3T3 murine fibroblast cells after 48 h of IFN treatment. The red DiD plasma membrane stain was not shown to display the green CTx-488 more easily.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

In summary, the data shown suggest an increasing caveolae-dependent endocytosis trend following interferon treatment. Verifying that CTx-488 was actually internalized was vital to determine whether endocytosis, as opposed to other means of plasma membrane penetration, was being measured. Two methods were used to prove the internal location of CTx-488. In agreement with the flow cytometry data, confocal microscopy imaging confirmed CTx endocytosis was up-regulated in IFN-treated cells. Both FACS with trypan blue and confocal microscopy using a plasma membrane stain were also used to validate an intact cell membrane.

These results challenge the simplistic notion that endocytosis would be down-regulated following IFN treatment so as to prevent perceived pathogens from gaining access into the cell. These findings point to a much more complex regulation of caveolae. Caveolae-dependent endocytosis may exist as two distinct pathways, one to the endoplasmic reticulum (ER) and one to the Golgi Apparatus[82]. With two separate pathways linked to one plasma membrane marker, namely caveolin, each separate pathway may include multiple levels of regulation not only at the plasma membrane where caveolae are located, but also internally along each endocytosis pathway. This knowledge suggests caveolae also serve as an important signaling pathway, with the abilities to direct cargo to specific locations within the cell. This signaling role of caveolae may be the true target of IFN, rather than their endocytosis-ability.

It is important to note that CTx may also be internalized into cells via non-caveolae-dependent pathways. CTx has been shown to enter cells through the clathrin-mediated endocytosis pathway, however, the portion is minor and caveolae is thought to be the major entry pathway in NIH 3T3 cells[82]. Future experiments will employ chlorpromazine to block clathrin-mediated endocytosis and ensure caveolae-specific internalization of the aforementioned fluorescent-conjugate[83,84].

As mentioned in Chapter 1, IFN manipulates plasma membrane components, such as cholesterol, that are important to caveolae. Due to the interesting trend outlined in this work, the future direction of this project will explore IFN manipulation of cell plasma membrane components. Identification of IFN-

induced alteration of the abundance of cholesterol and/or sphingolipids at caveolae requires the ability to image cholesterol, sphingolipids, and caveolae- or clathrin-specific membrane proteins without using bulky labels that may alter component organization (i.e., fluorophores)[85–88]. For this purpose, we will use high-resolution secondary ion mass spectrometry (SIMS) to chemically image the distributions of metabolically incorporated, isotope-labeled cholesterol and sphingolipids, and protein-specific immunolabels in the plasma membranes of IFN-treated and untreated cells. Statistical analysis of the resulting compositional maps will allow quantitative assessment of differences in the local composition of caveolae. This technique combines high sensitivity with a lateral resolution as high as 90 nm to allow mapping the elemental and isotopic composition at the surface (top 5 nm) of a biological sample. In the analyses a primary cesium ion beam is scanned over the surface of the sample (i.e., a cell). Monoatomic and diatomic secondary ions, which are charged fragments of the molecules that were located on the sample surface, with up to five different mass-to-charge ratios (*m*/*z*) are collected in parallel at every pixel (Figure 4.1).

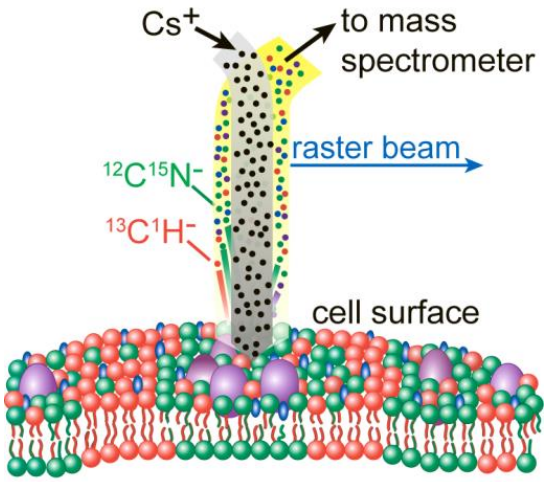

Figure 4.1: Depiction of NanoSIMS

The intensities of these secondary ions are then used to map the element and isotope composition at the sample surface. Because this instrument detects monoatomic and diatomic secondary ions, each species of interest must contain a distinct stable isotope so that the secondary ions generated during NanoSIMS analysis can be linked to the parent molecule. The distinct stable isotopes that encode for component identity are selectively incorporated into the lipid species of interest with established metabolic labeling techniques[89–91]. NanoSIMS analysis is performed under ultra-high vacuum, so sample preparation methods that preserve the intricate organization of the cellular membrane are required. Established fixation methods are used to preserve the spatial arrangement of the membrane biomolecules[92].

Though these SIMS experiments will not directly link IFN-induced cell membrane changes to caveolae-mediated endocytosis, it will investigate a possible mechanism of endocytosis control that may help to explain the interesting trends found in Chapters 2 and 3. Successful completion of the future research will shed new light onto the IFN response and its ability to modulate the plasma membrane and caveolae-mediated endocytosis.

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