Spring 2022

The Effect of Poly(I:C) and IFN-α on the Immune System as Observed Through IL-12 Production by Dendritic Cells

Sarah Kleb

The effect of poly(I:C) and IFN-α on the immune system as observed through IL-12 production by dendritic cells

By

Sarah S. Kleb

This thesis is submitted in partial fulfillment of the requirements for Honors in the Discipline in the Department of Biological and Environmental Sciences and the Elizabethtown College Honors Program

Due Date: 05/02/2022

Thesis Advisor (signature required) ____Jodi L. Lancaster_____

Second Reader __Debra LaWall_____

Third Reader _____Diane Bridge_____

THE HIGH LIBRARY
ELIZABETHTOWN COLLEGE

Honors Senior Thesis Release Agreement Form

The High Library supports the preservation and dissemination of all papers and projects completed as part of the requirements for the Elizabethtown College Honors Program (Honors Senior Thesis). Your signature on the following form confirms your authorship of this work and your permission for the High Library to make this work available. By agreeing to make it available, you are also agreeing to have this work included in the institutional repository, JayScholar. If you partnered with others in the creation of this work, your signature also confirms that you have obtained their permission to make this work available.
Should any concerns arise regarding making this work available, faculty advisors may contact the Director of the High Library to discuss the available options.

Release Agreement

I, as the author of this work, do hereby grant to Elizabethtown College and the High Library a non-exclusive worldwide license to reproduce and distribute my project, in whole or in part, in all forms of media, including but not limited to electronic media, now or hereafter known, subject to the following terms and conditions:

Copyright

No copyrights are transferred by this agreement, so I, as the author, retain all rights to the work, including but not limited to the right to use in future works (such as articles or books). With this submission, I represent that any third-party content included in the project has been used with permission from the copyright holder(s) or falls within fair use under United States copyright law (http://www.copyright.gov/title17/92chap1.html#107).

Access and Use

The work will be preserved and made available for educational purposes only. Signing this document does not endorse or authorize the commercial use of the content. I do not, however, hold Elizabethtown College or the High Library responsible for third party use of this content.

Term

This agreement will remain in effect unless permission is withdrawn by the author via written request to the High Library.

Signature: Sarah Kleb **Date:** 5/2/2022

Abstract

Activation of dendritic cells (DCs) through toll-like receptor (TLR) agonists causes release of cytokines. DC2.4 cells are immature murine DCs derived from the bone marrow that express TLRs, including TLR3. The response of DC2.4 cells to varying doses and times of the TLR3 agonist, poly(I:C), was evaluated. In contrast to previously studied MuTu DCs, DC2.4 cells produced little to no interleukin-12 (IL-12) after 12-hour exposure to doses from 0.1-100 μg/mL poly(I:C). This was surprising because literature suggests that the expression of TLR3 dramatically increases when DCs are maturing, which should occur upon poly(I:C) exposure. Once fully mature, DCs receive inflammatory signals to reduce TLR3 expression. Treatment of DCs with interferon-a (IFN-α) has been found to enhance IL-12 production by increasing TLR3 expression. DC2.4s exposed to IFN-α prior to poly(I:C) treatment also failed to produce IL-12. Experiments using lipopolysaccharide (LPS) confirmed that the cells can make IL-12 through activation of the TLR4 pathway. Ongoing studies are investigating the functionality of the TLR3 pathway in the cells. Luciferase assays with IFN-β plasmids are being conducted to confirm the presence of a functional TLR3. Western blotting and immunofluorescence are also being explored to qualitatively determine whether the DC2.4 cell line expresses TLR3, as reported in literature. Additionally, the maturation status of the lab's DC2.4 cells is being considered. Analysis of TLR3 levels and production of IL-12 by DC2.4s allows for greater understanding of how different lab-derived lines of DCs exhibit responses to TLR agonists.

Introduction

Dendritic cells (DCs) are antigen presenting cells that connect the innate and adaptive immune system in the body. DCs start in an immature state, and progress to a mature state

throughout their development. These two phases in the cell's life show different functional and morphological characteristics. Immature DCs are circular and have lower levels of costimulatory molecules, such as CD80, CD86, MHC I, and MHC II. Mature DCs have longer dendrites and express higher levels of costimulatory molecules that aid in T cell activation. In this way, DCs are the link between the innate and adaptive immune responses. Additionally, immature DCs have a phagocytic ability that mature DCs do not (Kim & Kim, 2019). The DC2.4 cell line being used were generated from the bone marrow of mice and transitioned to an immortal cell line with endless growth potential (He et al., 2007). Knowing about the maturity of the DC2.4 cell line in the lab has demonstrated to be important in understanding results. Due to previous work with MuTu DCs, it was hypothesized that an increase in the dose of poly(I:C) and IFN-α would cause increased production of IL-12 from DC2.4. In this experiment, HeLa cells were used as a positive control because literature suggests that HeLa cells express all 10 human TLRs (Nishimura & Naito, 2005).

Poly(I:C) is a synthetic analog of viral dsRNA that acts an immunostimulant. It promotes the release of cytokines from innate immune cells, such as DCs, as part of the inflammatory response (Lever et al., 2015). From the literature, poly(I:C) is generally known as one of the most appropriate maturation agents for DCs. Poly(I:C) acts through TLR3, which is a receptor expressed by DCs (Rouas et al., 2004). However, TLR3 is exclusively expressed by DCs and absent in precursor monocytes. The expression of TLR3 dramatically increases when DCs are differentiating. After reaching a certain point in their maturity, however, DCs receiving inflammatory signals to fully mature reduce TLR3 expression. For example, *in vitro* experiments demonstrated that TLR3 expression was inhibited upon exposure to LPS or cytokines that are used to induce functional maturation (Muzio et al., 2000). In one study, the decrease in

production of IL-12 was confirmed by stimulating human DCs with poly(I:C) with 80% of samples showing no detectable IL-12. However, IFN-α as a treatment for DCs has been found to enhance IL-12 production (Spelmink et al., 2016). Finding the balance between maturing the cells enough for increased TLR3 expression, and subsequent loss of this expression as cells become fully mature, seems to be a challenge.

Assuming TLR3 is prevalent, functional, and has been activated, one of the outcomes of receptor-ligand interaction of interest to this research is production of the inflammatory cytokine IL-12. A functional TLR3 is required to induce complete IL-12 secretion because IL-12 depends on this dsRNA receptor (Spelmink et al., 2016). Mature DCs are characterized by secretion of IL-12, a key cytokine for priming the response of CD8+ cytotoxic T lymphocytes, CD4+ T helper 1 cells, and antitumor responses (Rouas et al., 2004). DCs are the main producers of IL-12, ultimately driving differentiation of these cells and inducing other innate immune cells to produce cytokines such as IFN- γ (Spelmink et al., 2016).

In previous experiments, the cytokine IL-12 was not produced by the DC2.4 cell line when cells were treated with poly(I:C). According to the literature, DC2.4s express TLR3, making it so these cells should produce IL-12 when properly stimulated (Rouas et al., 2004). The main goal of this work is to characterize production of IL-12 by induction of TLR3 in the DC2.4 cell line. We chose to attempt stimulation of DC2.4 by IFN-α in addition to poly(I:C), as this has been the most common treatment reported. IFN-α serves to increase TLR3 expression while poly(I:C) is used to stimulate these receptors. IFN-α seems to be a consistent contributor in the production of IL-12 in DCs. One mode of treatment proposed by Spelmink et al. (2016) was treatment of DCs with IFN-α (500 U/mL) for eight hours to slightly increase expression of TLR3, improving the overall level of IL-12 production. This project used this dose of IFN-α at

varying timepoints to further expression of IL-12 in the DC2.4 cell line. It was hypothesized that any increase in chemical application of poly(I:C), IFN-α, and LPS would lead to an increase in production of IL-12 by the DC2.4 through TLR3 and TL4 respectively.

Materials and Methods

Cell Culture:

The DC2.4 were grown in RPMI media (He et al., 2007). This media consisted of 10% fetal calf serum (FCS), 1% Hepes buffer, 1.6% sodium bicarbonate ($NaCO_3$), 1% beta-mercaptoethanol, 1% Penn/Strep, and 1% glutamine. The cells were placed in loose capped or filtered capped flasks and incubated at 37°C in 6% CO_2. Cells were routinely split at a 1:10 ratio approximately every three days. When ready to harvest, a confluent T75 flask was aspirated and 2mL trypsin/versine was added. The flask was left in the incubator for two minutes, then agitated by smacking the side of the flask. Approximately 4 mL of complete RPMI media was added back. When counting cells, a 1:20 dilution of cells to trypan blue was used. Of this diluted cell solution, 12 uL was inserted into each side of the hemocytometer. The top three and bottom three quadrants were counted and averaged to obtain an overall average of total cells using the following equation:

$$\frac{\#\ cells\ counted}{6\ quadrants} * \frac{dilution\ factor}{1} * \frac{10000}{1} * \frac{6mL\ volume\ the\ cells\ are\ in}{1} = Total\ \#\ cells$$

The WT19 cells were grown in DMEM media which consisted of 5% FCS, 2% $NaCO_3$, 2% Hepes buffer, 1% Penn/Strep, and 1% glutamine. The cells were placed in tight capped flasks to cut off the flow of oxygen and incubated at 37°C in 6% CO_2. Cells were routinely split at a 1:10 ratio approximately every three days.

The HeLa cells were grown in DMEM media which consisted of 10% FBS, 3% $NaCO_3$, and 1% Penn/Strep. The base DMEM media already contained L-glutamine. The cells were placed in loose capped or filter capped flasks in the 37°C incubator in 6% CO_2. Cells were routinely split at a 1:8 ratio every three days.

Microscopy

Cell growth was monitored using inverted light microscopes. Cells were examined under the 10X lens to determine confluency and appropriate split ratios. Additionally, when counted, cells were looked at on the hemocytometer on the compound light microscope under the 10X lens. The fluorescent microscope was used for immunofluorescence protocols. Cells were examined under the 10X lens for focusing, then magnification was increased to 100X to look at the location of the protein in the cell based on its fluorescence.

ELISA Experiments

All ELISA assays were conducted using the BD OptEIA Mouse IL-12 ELISA. Manufacturer instructions were followed. Average absorbances at each known concentration of IL-12 were detected using the TECAN infinite F50 ELISA plate reader.

Analysis of IL-12 production by DC2.4 exposed to increased poly(I:C) concentrations:

The first ELISA assay was conducted with increased poly (I:C) doses and exposure time, as compared to work previously done in the lab. In this experiment, $1x10^6$ DC2.4 cells were added to each well of a 12-well culture plate. All treatments were done in triplicate and incubated for a total of 42 hours before the supernatant was removed to conduct the ELISA. Table 1 delineates the setup for the ELISA performed. Columns 1-3 contain the IL-12 standards

in triplicate, while columns 4-12 contain the treated cell groups. Group 1 had no treatment, group 2 had poly(I:C) at 100 ug/mL, group 3 had poly(I:C) at 100 ug/mL with an inhibitor, group 4 had no treatment, group 5 had poly(I:C) at 50 ug/mL, and group 6 had poly(I:C) at 100 ug/mL. With each ELISA conducted, a standard curve was generated. Using the standard curve equation in the form y=mx+b, IL-12 in ng/mL was calculated. To do this, the b-value from the equation was subtracted from the absorbance value for the given sample, then divided by the m-value of the equation.

Table 1: Set-up of ELISA #1, analyzing IL-12 production by DC2.4 exposed to increased poly(I:C) concentrations and exposure times.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1000 pg/mL	1000 pg/mL	1000 pg/mL	Group 1 #1	Group 1 #1	Group 1 #1	Group 3 #3	Group 3 #4	Group 3 #5	Group 6 #2	Group 6 #2	Group 6 #2
B	500 pg/mL	500 pg/mL	500 pg/mL	Group 1 #2	Group 1 #2	Group 1 #2	Group 4 #1	Group 4 #1	Group 4 #1	Group 6 #3	Group 6 #3	Group 6 #3
C	250 pg/mL	250 pg/mL	250 pg/mL	Group 1 #3	Group 1 #3	Group 1 #3	Group 4 #2	Group 4 #2	Group 4 #2	/	/	/
D	125 pg/mL	125 pg/mL	125 pg/mL	Group 2 #1	Group 2 #1	Group 2 #1	Group 4 #3	Group 4 #3	Group 4 #3	/	/	/
E	62.5 pg/mL	62.5 pg/mL	62.5 pg/mL	Group 2 #2	Group 2 #2	Group 2 #2	Group 5 #1	Group 5 #1	Group 5 #1	/	/	/
F	31.25 pg/mL	31.25 pg/mL	31.25 pg/mL	Group 2 #3	Group 2 #3	Group 2 #3	Group 5 #2	Group 5 #2	Group 5 #2	/	/	/
G	16.8 pg/mL	16.8 pg/mL	16.8 pg/mL	Group 3 #1	Group 3 #1	Group 3 #1	Group 5 #3	Group 5 #3	Group 5 #3	/	/	/
H	Water	Water	Water	Group 3 #2	Group 3 #2	Group 3 #2	Group 6 #1	Group 6 #2	Group 6 #3	/	/	/

Analysis of IL-12 production by DC2.4 exposed to IFN-α then poly(I:C):

The second ELISA assay was conducted with IFN-α and poly(I:C). In this experiment, $2x10^6$ cells were added to each well of a 12-well culture plate. All treatments were done in triplicate and incubated for a total of 24 hours before the supernatant was removed to conduct the ELISA unless otherwise indicated. Group 1 had no treatment, group 2 had IFN-α at 1500 U/mL, group 3 had IFN-α at 500 U/mL for 12 hours then poly(I:C) at 5 ug/mL for an additional 12 hours, group 4 had IFN-α at 1000 U/mL for 12 hours then poly(I:C) at 5 ug/mL for an additional 12 hours, and group 5 had IFN-α at 1500 U/mL for 12 hours then poly(I:C) at 5 ug/mL for an additional 12 hours.

Analysis of IL-12 production by DC2.4 exposed to LPS and IFN-α:

The third ELISA assay was conducted with LPS and IFN-α. In this experiment, $1.5x10^6$ cells were added to each well of a 12-well culture plate. All treatments were done in triplicate and incubated for a total of 20 hours before the supernatant was removed to conduct the ELISA. Group 1 cells received no treatment, group 2 LPS at 5 ug/mL, group 3 had LPS at 5 ug/mL with IFN-α at 1000 U/mL, and group 4 had IFN-α at 1000 U/mL.

Immunofluorescence Experiments

For immunofluorescence experiments, appropriate cell lines were seeded into 60 mm dishes with three coverslips per dish. Coverslips were pushed to the bottom of the dishes to ensure cell attachment. After cells attached, media was drained from the dishes, and the coverslips were washed three times with PBS. After the final wash, 5 mL of a 1:1 mixture of methanol and acetone was added as a fixative, dishes were incubated for five minutes at room temperature, the fixative was aspirated off, and the coverslips air dried. The slips were then washed with PBS for two minutes and coverslips were transferred to a new 60 mm dish.

The primary antibody used in various experiments was prepped at a 1:100 dilution in sterile PBS. For applicable groups, 100 uL of the primary antibody was added on top of the slip. For treatment groups not receiving the primary antibody, 100 uL PBS was added directly to the slip instead. These coverslips were incubated for 30 minutes at 37°C, washed three times with PBS, and again transferred to a new 60mm dish. The secondary antibody used in experiments was diluted 1:60 in sterile PBS. For applicable groups, 100 uL of the secondary antibody were added, or 100 uL of PBS for control groups. These coverslips were incubated at 37°C for 30 minutes. Slips were then washed two times with PBS and then washed again with water, and

aspirated. The coverslips air dried and were mounted onto slides with ~20 uL Cytoseal, with the cell side facing down. Slides were then examined under the fluorescent microscope.

Transfection of DC2.4 and HeLa, measured by luciferase assay and immunofluorescence

Using PEI as the transfection method, a 3:1 ratio was arbitrarily chosen, as common ratios include 1:2, 1:3, and 1:6. Because this ratio worked with the first transfection, a 1:3 ratio of PEI:DNA was used for all subsequent experiments. After the transfection mix was made, it was incubated at room temperature for 15 minutes before being added to each well. In the 12 well plate used for transfection, each well was treated with a combination of 100 uL serum free media, 1 ug of IFN-beta pGL3 DNA plasmid, and 3 uL of PEI at 1 ug/uL for the 3:1 ratio. These mixtures were added dropwise to the cells in the 12 well plate. The plates were put back into the incubator for 24 hours when media on the cells was changed. Appropriate treatments were added, if applicable, for the timeframe before conducting the luciferase assay.

Using the ONE-Glo Luciferase Assay System design from Promega, light units were measured for each well in the 12 well plate. Each well was aspirated, washed with PBS, and received 100 uL 1X lysis buffer. The plate was gently rocked, then 100 uL from each well was placed into a microcentrifuge tube on ice. With each of these tubes, 5 uL was added into new tubes at room temperature, analyzing each well of the 12 well plate in triplicate. Immediately before being placed into the luminometer, 25 uL of luciferase substrate was added to each tube, and light units were recorded. When using the microscope to assess for DAPI for nuclear staining, and EGFP for cells that had taken up the plasmid, samples were prepared by aspirating media from each well, washing with PBS, and removing one cover slip to be mounted onto a slide with mounting agent.

DC2.4 and HeLa transfection with pGL3 plasmid:

Confluent DC2.4 T25 and HeLa T25 flasks were harvested and counted. In each well of a 12-well cell culture plate, $1x10^5$ cells were added. All treatments were done in triplicate and incubated for ~24 hours before the plasmid was added. Cells were tested for transfection efficiency using a pGL3 plasmid at 1 ug/well, using PEI as the transfection method at a 3:1 ratio. After transfection had occurred, samples were analyzed through the luminometer to measure light units associated with each sample for quantitative data. Additionally, immunofluorescence was done using EGFP to qualitatively measure transfection efficiency. DC2.4 and HeLa were transfected with an IFN-β plasmid and treated with poly(I:C) and LPS, following this protocol.

Results

ELISA

The overall goal of this research was to observe immune system activity, through the production of IL-12, after the treatment of DC2.4 cells with varying doses of poly (I:C), IFN-α, or LPS. In all cases, a standard curve of known concentrations of IL-12 was used as depicted in Figure 1. As seen in Figure 1, the R^2 value is 0.9795, indicative of a strong correlation between increased IL-12 and increased absorption with the slope of the line being $y=3x10^{-5}+0.0307$. For every ELISA, a new standard curve was used to calculate the amount of IL-12 produced by DC2.4 under varying conditions

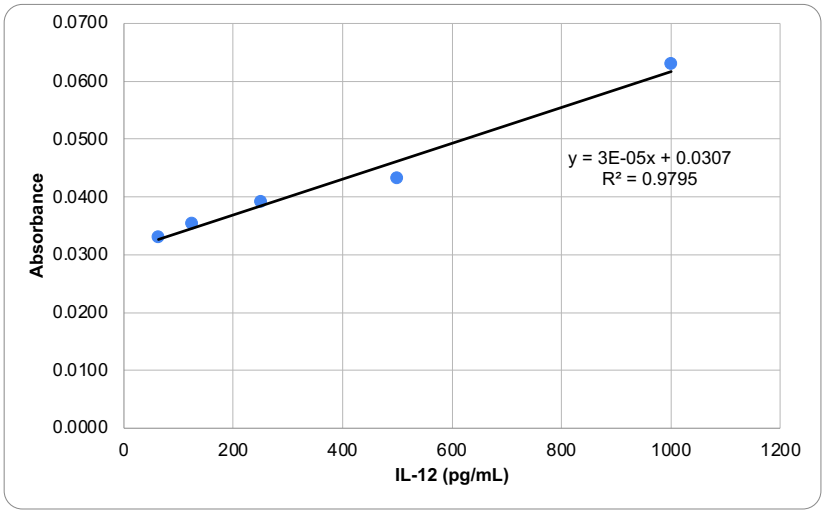


Figure 1: Representative IL-12 standard curve showing relationship between known concentrations of IL-12 (pg/mL) and the subsequent absorbance (nm). The slope of the line is used to determine the amount of IL-12 produced by the different treatment groups of DC2.4 cells.

ELISA #1, shown below as Figure 2, had all experimental samples with values below zero as compared to the standard curve. DC2.4 in group 1 had no treatment, group 2 had

poly(I:C) at 100 ug/mL, group 3 had poly(I:C) at 100 ug/mL with an inhibitor, group 4 had no treatment, group 5 had poly(I:C) at 50 ug/mL, and group 6 had poly(I:C) at 100 ug/mL. As expected, DC2.4 cells receiving no treatment did not produce IL-12. However, DC2.4 exposed to varying doses of poly(I:C) also did not produce IL-12. This did not support the hypothesis that cells treated with poly(I:C) would have TLR3 stimulated, ultimately producing more IL-12.

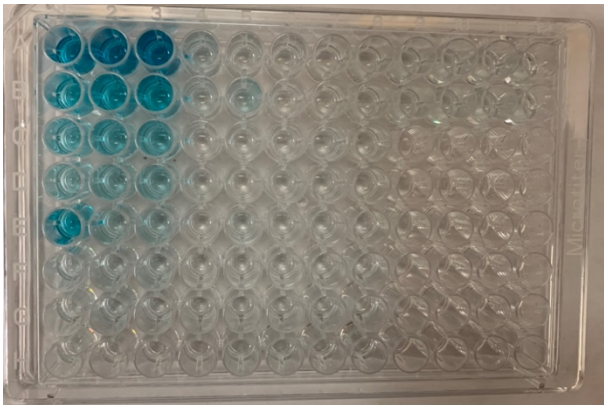


Figure 2: Completed plate of ELISA assay #1 with blue visualization of IL-12 production. The wells that turned blue are the known concentrations of IL-12. The DC2.4 not treated, treated with higher poly (I:C) concentrations, and treated with poly (I:C) and the inhibitor, failed to produce any IL-12 as evidenced by the lack of blue color in columns 4 through 12.

The results for ELISA #2, shown in Figure 3, demonstrated all experimental samples with values below zero as compared to the standard curve, again illustrating a complete lack of IL-12 production even in the presence of IFN-α. DC2.4 cells in group 1 had no treatment, group 2 had IFN-α at 1500 U/mL, group 3 had IFN-α at 500 U/mL for 12 hours then poly(I:C) at 5 ug/mL for an additional 12 hours, group 4 had IFN-α at 1000 U/mL for 12 hours then poly(I:C) at 5 ug/mL for an additional 12 hours, and group 5 had IFN-α at 1500 U/mL for 12 hours then poly(I:C) at 5 ug/mL for an additional 12 hours. This did not support the hypothesis that cells treated with poly(I:C) and/or IFN-α would have TLR3 stimulated, ultimately producing more IL-

12. Additionally, IFN-α according to literature, should have helped DC2.4s through the maturation process, increasing the number of TLR3 receptors on the membrane, producing more IL-12.

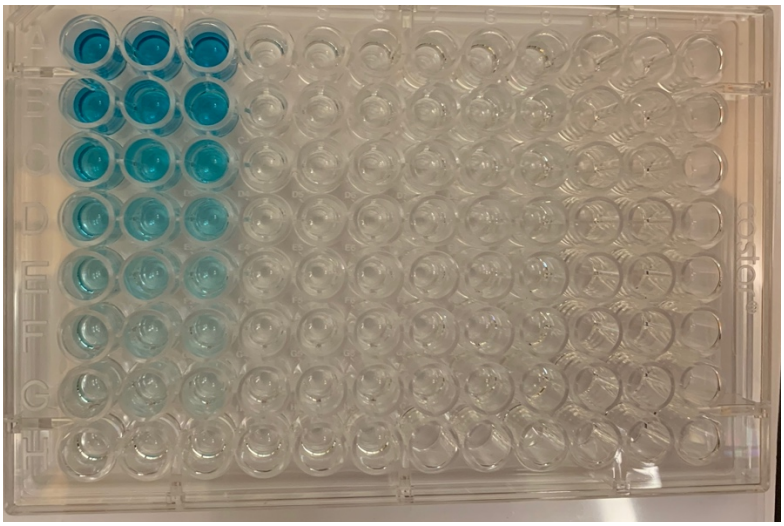


Figure 3: Completed plate of ELISA assay #2 with blue visualization of IL-12 protein. The wells that turned blue are the known concentrations of IL-12. The DC2.4 cell experimental groups consisted of no treatment, treatment with IFN-α, and a combination of IFN-α and poly(I:C). All experimental groups failed to produce any IL-12.

To demonstrate whether the DC2.4 cells could make IL-12 through a different pathway, DC2.4 cells were treated with LPS, which activates the TLR4 receptor resulting in IL-12 production. Finally, in ELISA #3, shown in Figure 4, experimental treatments did result in IL-12 production as compared to the standard curve. DC2.4 cells in group 1 received no treatment and produced no IL-12 as expected. DC2.4 in group 2 had LPS at 5 ug/mL, group 3 had LPS at 5 ug/mL with IFN-α at 1000 U/mL, and group 4 had only IFN-α at 1000 U/mL. All LPS containing groups successfully produced IL-12 protein. This did support our hypothesis that cells treated with LPS would have TLR4 stimulated, ultimately producing more IL-12. Group 2 with

LPS at 5 ug/mL had the most significant results, showing IL-12 production. This protein level, as compared to the standard curve, was at 9.37 ng/mL of IL-12. Group 3 had LPS at 5 ug/mL with IFN-α at 1000 U/mL, showing slightly less IL-12 production then the LPS treatment alone. The data related to IFN-α treatment does not support the hypothesis that IFN-α treatment would increase DC maturation and TLR production in DCs.

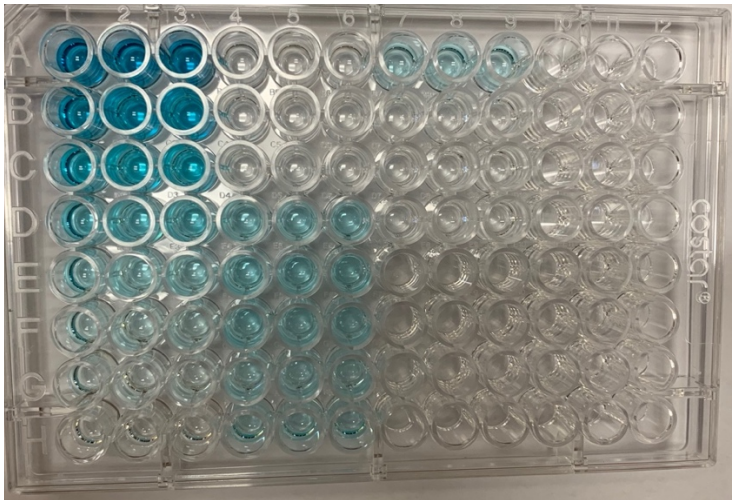


Figure 4: Completed plate of ELISA assay #3 with blue visualization of IL-12 protein. The wells that turned blue are the known concentrations of IL-12. The DC2.4 not treated, treated with LPS, and a combination of LPS and IFN-α showed that cells treated with LPS were able to produce IL-12.

After DC2.4 cells demonstrated, by ELISA, that they could produce IL-12 through TLR4 stimulation, the expression of the receptors of interest were measured in the cells. Immunofluorescence was performed for both TLR3 and TLR4 in DC2.4 cells to confirm their presence on the endosomal and outer cell membranes, respectively. With the confirmation that these receptors are present, this would allow for their stimulation by poly(I:C) and LPS respectively, to produce IL-12.

Immunofluorescence

Using immunofluorescence, WT19 cells were used as a positive control. WT19 cells express the SV40 T antigen protein, predominately in the nucleus. The primary antibody used was specific for T antigen and came from a mouse host. The secondary goat anti mouse antibody used was labeled with FITC which fluoresces green on the fluorescent microscope. This was used as a positive control for the procedure used for the DC2.4s in Figure 5. As expected, T antigen protein is present in the nucleus of the cells.

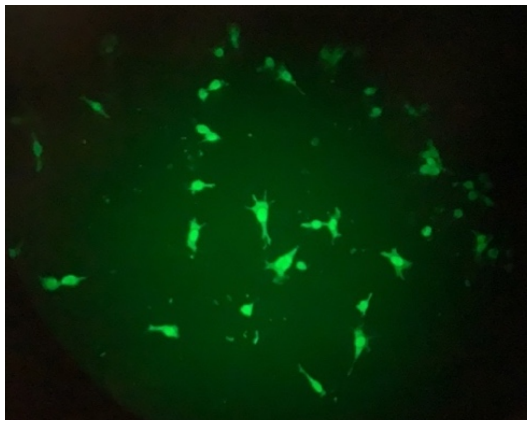


Figure 5: Immunofluorescence results of WT19 cells and T-antigen. Cells were viewed under 100X magnification to visualize T antigen in the nucleus of the cells.

Using immunofluorescence, DC2.4 cells were treated with primary and secondary antibodies to analyze TLR4 expression on the cell surface. The primary antibody was a monoclonal antibody for TLR4, from Invitrogen. The secondary was a goat anti-mouse antibody labeled with FITC. Figure 6 shows the results for DAPI, simply showing that cells are present. DAPI causes the nucleus to appear blue on the fluorescent microscope. Unfortunately, as shown in Figure 6, there was no cell-specific green light for TLR4. The experiment was repeated for

TLR3 in DC2.4 and the same results occurred. The primary for that experiment was a TLR3 monoclonal antibody from Invitrogen. The secondary antibody was labeled with FITC which fluoresces green but did not fluoresce in this experiment. Neither TLR3 or TLR4 were detected by immunofluorescence.

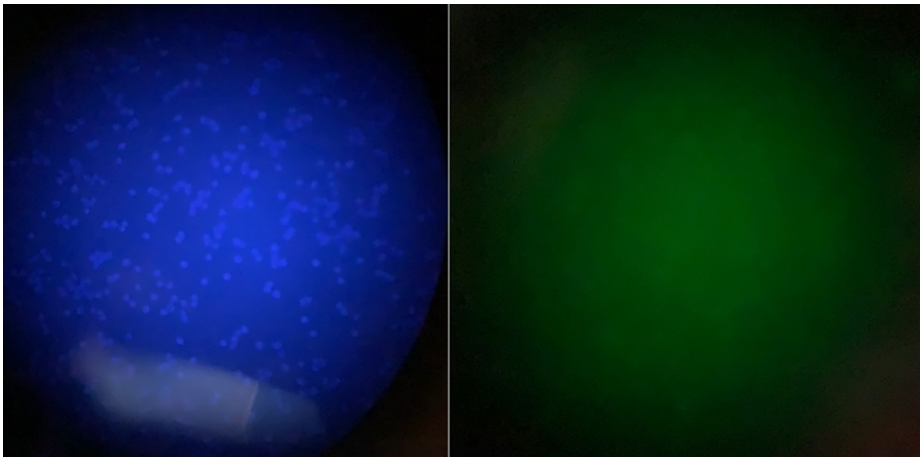


Figure 6: Immunofluorescence results of DC2.4 looking for TLR4. The image on the left is stained with DAPI, showing that cells were present on the coverslips after treatments. The image on the right shows the cells under FITC, showing that the combination of TLR4 antibodies did not cause any fluorescence in the DC2.4 cells. The same results occurred for TLR3 in DC2.4 cells.

Luciferase

The lack of IL-12 production by DC2.4 in response to poly(I:C) led to speculation about whether the TLR3 intracellular signaling pathway was functional. To examine the function of the intracellular signaling pathway, luciferase assays were conducted. The TRIF pathway that TLR3 uses to ultimately produce IL-12 is also required for TLR4-mediated production of IL-12 (Fitzgerald et al., 2003). When TLR3 stimulation was not working, TLR4 was tested. When

treating DC2.4 with LPS, the TLR4 agonist, the DC2.4s did produce IL-12, showing that this function in the cells was not compromised (Figure 4). Luciferase assays examining intracellular signaling pathways downstream of TLR3 and TLR4 were conducted. Literature has shown that DCs make IFN-β and IFN-α through the TRIF pathway from both TLR3 and TLR4 (Figure 7) (McCarthy et al., 2017). With IFN-β being one of the end products of this pathway, a luciferase assay using an IFN-β plasmid was conducted. DCs were transfected with the plasmid and treated with poly(I:C) or LPS to determine the functionality of the TLR3 and TLR4 pathways, respectively. In this experiment, HeLa cells were used as a positive control because HeLa cells express all 10 human TLRs (Nishimura & Naito, 2005).

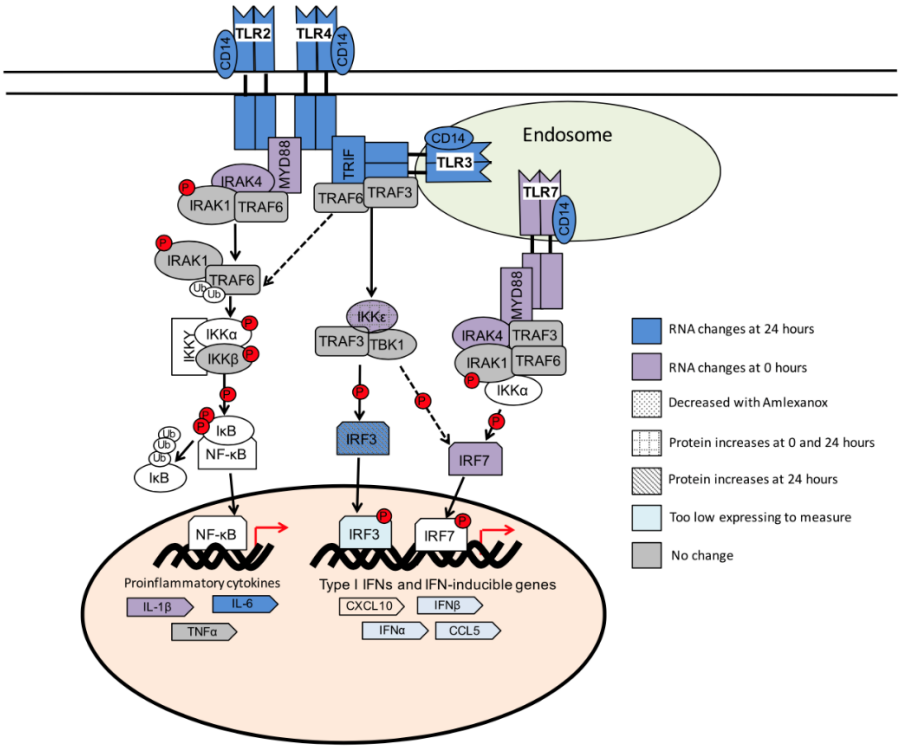
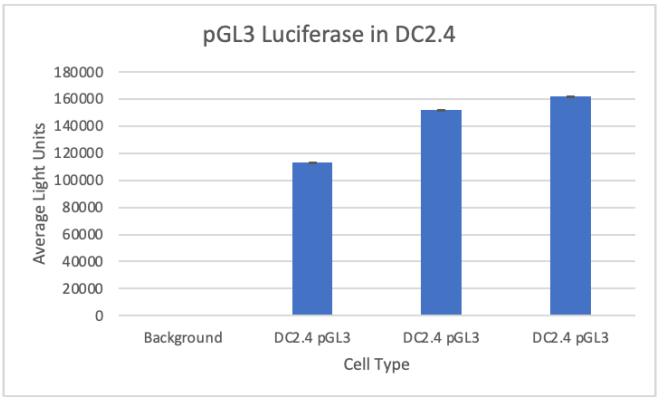


Figure 7: Schematic of the TLR signaling pathways, including TLR3 and TRL4 using the TRIF pathway to produce proinflammatory cytokines and Type I IFNs (McCarthy et al., 2017).

Verification of transfection before luciferase and immunofluorescence

Before checking the desired IFN-β plasmid, DC2.4s and HeLa cells were transfected with pGL3, which has been shown to have a very good transfection rate in many cell types. Both cell lines showed significant transfection efficiencies, giving large values for light units in the luminometer for both DC2.4 and HeLa cells. Although the transfection of HeLa cells was significantly higher than the DC2.4s, the DC2.4s still had a value significantly above the background level, which demonstrates that they can be transfected.

A.)



B.)

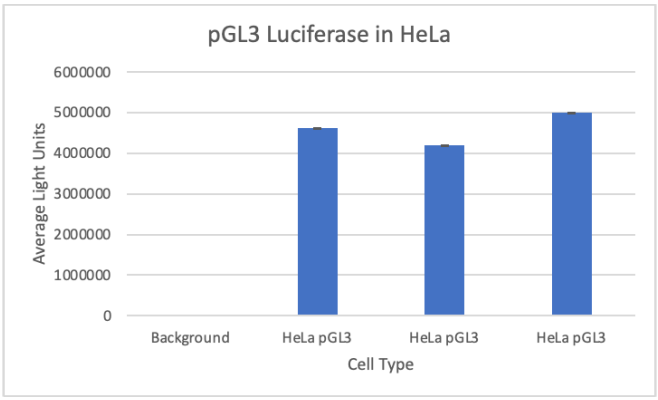


Figure 8: Replicate luciferase assay of DC2.4 and HeLa using pGL3 control plasmid. **A.)** Shows high promoter activity in DC2.4 with pGL3 plasmid, showing high transfection levels of the plasmid. **B.)** Shows higher promoter activity in HeLa with pGL3 plasmid, showing about a 30 times greater transfection level than in DC2.4.

In the same pGL3 experiment in Figure 8 above, some cells were transfected with an EGFP plasmid. To qualitatively test transfection efficiency, cells were observed for DAPI and EGFP staining. In the DC2.4 cell line, as shown by DAPI versus EGFP in Figure 9, this transfection efficiency was nearly 100%, so it was not tested again in further experiments. The amount of cells with stained nuclei is approximately equal to those that took up the EGFP plasmid during the transfection protocol. This again demonstrates that the DC2.4 can be readily transfected.

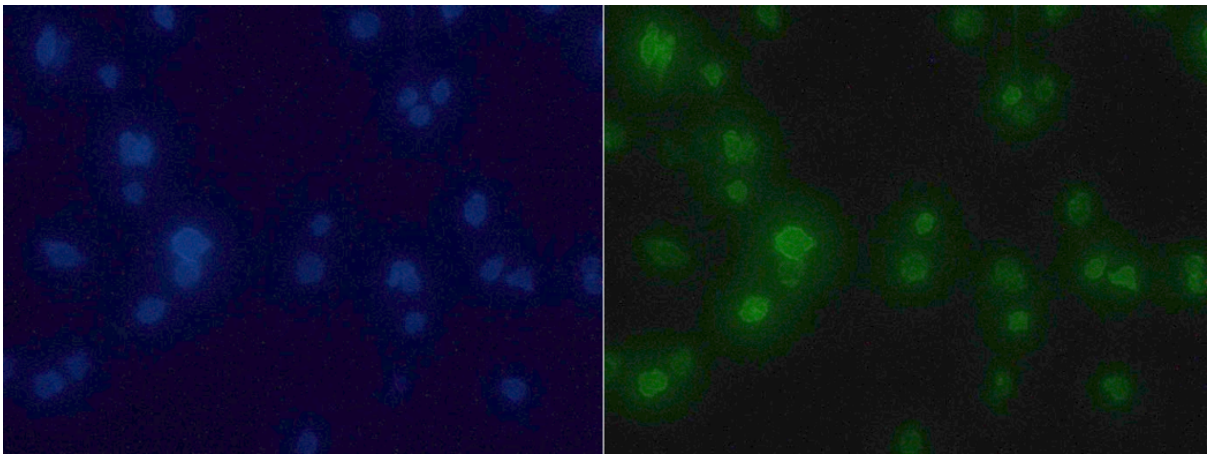


Figure 9: DC2.4 cells under immunofluorescence, looking at DAPI and EGFP, showing nearly a 100% transfection efficiency.

DC2.4 and HeLa cells were transfected with an IFN-β plasmid and later treated with 5 ug/uL poly(I:C) 24 hours prior to being assessed on the luminometer. According to literature, poly(I:C) treatment stimulates TLR3, ultimately producing more cytokines and chemokines. Among these should be IFN-β at the end of the TRIF pathway. However, the results shown in Figure 9 do not support the hypothesis that poly(I:C) treatment would increase IFN-β promoter activity in the cells as shown by lower promoter activity in the treated groups for both cell lines.

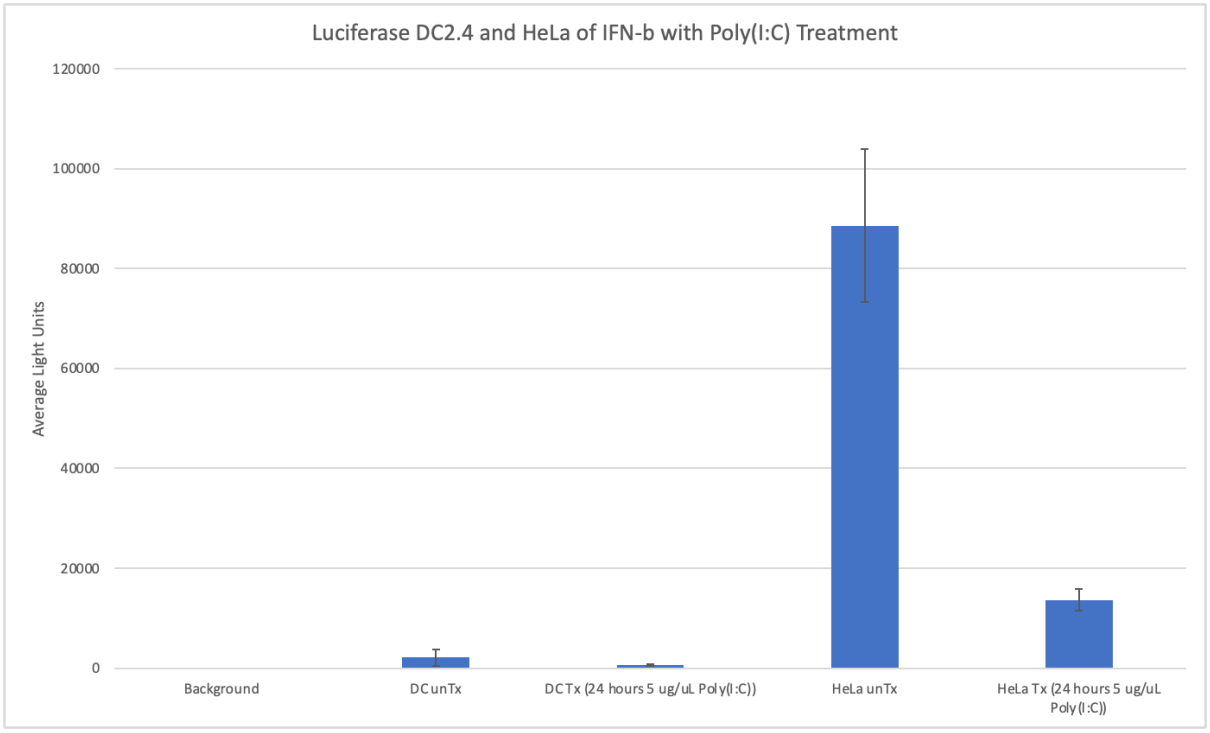


Figure 10: Luciferase assay of DC2.4 and HeLa using an IFN-β plasmid with poly(I:C) (5 ug/uL) treatment 24 hours before reading on the luminometer.

DC2.4 and HeLa cells were transfected with an IFN-β plasmid and later treated with 5 ug/mL LPS for 12 and 24 hours prior to being read on the luminometer. According to literature, LPS treatment stimulates TLR4, ultimately producing more cytokines and chemokines (Fitzgerald et al., 2003). Among these should be IFN-β at the end of the TRIF pathway. However, the results shown in Figure 11 do not support the hypothesis that LPS treatment would increase IFN-β in the cells as shown by insignificant differences in the untreated and treated groups for both cell lines. These results were surprising because based on ELISA #3 (Figure 4), LPS stimulation did increase IL-12 production in cells, which is a branch off from the same TRIF pathway used in IFN-β production.

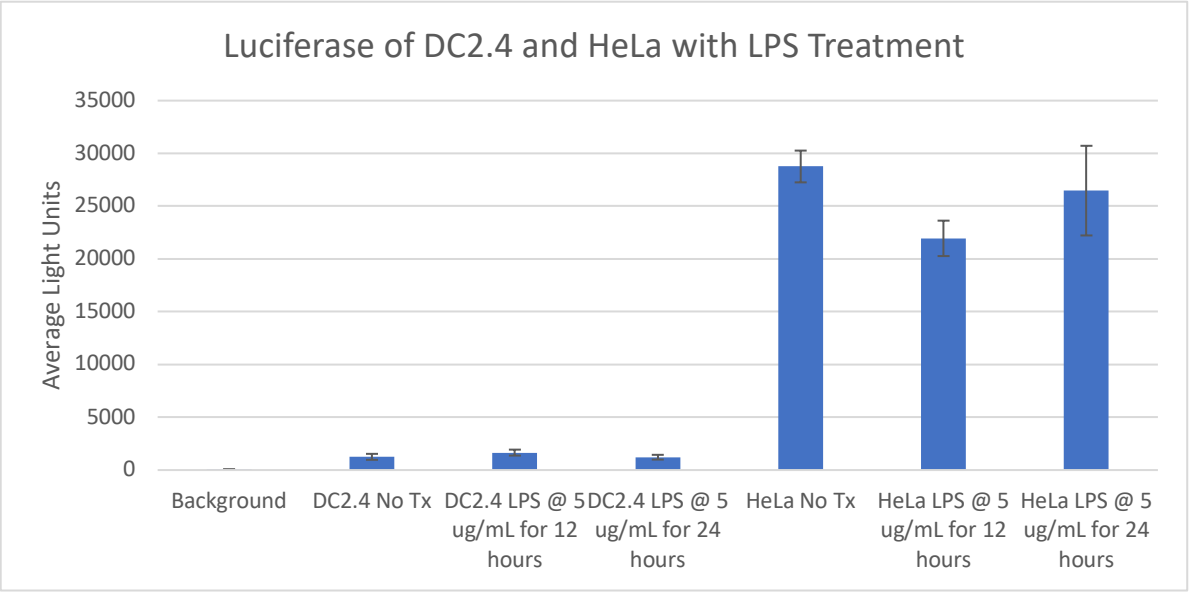


Figure 11: Luciferase assay of DC2.4 and HeLa using an IFN-β plasmid with LPS (5 ug/mL) treatment 12 and 24 hours before reading on the luminometer. There was no statistical significance across the same cell lines.

Discussion

Throughout this experimentation, the overall goal has been to observe the effect of chemicals on the immune response in lab derived DCs. Poly(I:C), IFN-α, and LPS were the primary chemicals utilized and their effect on DC2.4 was observed through IFN-β promoter activity, and more importantly the production of IL-12, a prolific immune system cytokine. The direction of this study came from literature which supported that DC2.4 release IL-12 through the TLR3 pathway (Spelmink et al., 2016). Prior to experimentation, it was hypothesized that any increase in chemical application of poly(I:C), IFN-α, and LPS would lead to an increase in production of IL-12 by the DC2.4 through TLR3 and TL4 respectively.

Because poly(I:C) is generally known to be the ideal stimulating agent for DCs, previous studies in the lab, used poly(I:C) as the pathogen-associated molecular pattern (PAMP) to stimulate the DC2.4 dendritic cells (Rouas et al., 2004). However, exposure of DC2.4 to varying doses of poly(I:C) at varying time points resulted in no IL-12 production, which was not expected based on past reports that TLR3 is expressed by DC2.4 cells. Different maturation agents and environmental factors affect cells differently in the way they secrete cytokines. Other ways of DC maturation that have been proposed are CD40L combined with IFN- γ and poly(I:C), as well as a cocktail of prostaglandin E_2 and the proinflammatory cytokines IL-1β, IL-6, and TNF-α (Rouas et al., 2004). The expression of TLR3 is also downregulated by the presence of these inflammatory cytokines, so there needs to be a balance of stimulating the maturation of DCs, while not decreasing the expression of TLR3 (Muzio et al., 2000). TLR3 is needed to lead to production of IL-12; the cytokine of interest in current studies.

A possible explanation for these unexpected outcomes was observed in the media. During the first and second ELISA, the media had yellowed, but the cells were not dead when harvested.

This acidification of the media could have caused IL-12 to denature (Loscher et al., 2005). A conformational epitope is the target of the antibodies used in the ELISA, so denaturation could have led to the failed detection of the IL-12. Another potential reason could have to do with the particular cell line used. These DC2.4 were derived from the bone marrow of mice. Because this is where this cell type both originates and matures, it is possible that this cell line is not yet matured to the point of releasing cytokines. This contrasts with published literature, but there are differences in cell lines cultured in different labs. This is an ongoing area of investigation.

Another possible reason for why the DC2.4 produced no IL-12 in response to poly(I:C) stimulation is because TLR3 is located inside of the cell. With the receptor being located inside of the cell membrane, the DC must phagocytose the poly(I:C) so that it can reach TLR3 to cause an intracellular signaling cascade. If the DC2.4 cell line is too mature, the level of phagocytosis may have decreased to the point of no poly(I:C) being engulfed into the cell. To test the maturity of the cell line, fluorescent bacteria can be fed to the cells, watching under the fluorescent microscope to see if cells take up the bacteria through phagocytosis or not. If cells have fluorescent bacteria inside of their cell membrane, they are still at an immature point in their cell cycle and can continue being used in current studies. However, if DC2.4 cells do not engulf the fluorescent bacteria, they may be too mature, explaining the lack of TLR3 stimulation thus far, since TLR3 expression decreases as cells fully mature (Kim & Kim, 2019).

From studies failing to produce IL-12 through TLR3 in the endosomal membrane, the functionality was questioned. DCs also produce IL-12 through TLR4 from the outer cell membrane, so this receptor was used to stimulate the cells to produce IL-12. Both TLR3 and TLR4 use the TRIF pathway to activate different cytokines and chemokines (Fitzgerald et al., 2003; Jiang et al., 2004). However, only TLR4 uses the MyD88 pathway. Specifically relating to

this lab's area of study, TLR3 and TLR4 both use an activated toll-IL-1-resistance domain containing adaptor-inducing IFN-β (TRIF) pathway to produce IFN-α and IFN-β. However, even though TRIF functions downstream of both TLR3 and TLR4 pathways, the function of the activating component, toll-receptor-associated molecule (TRAM), is only present in the TLR4 pathway. TRAM functions in activating interferon regulatory factor 3 (IRF3) and NF-κβ. This literature suggests that TRIF and TRAM both function in TLR4 signaling, and consequently LPS stimulation, but not with TLR3 and poly(I:C) stimulation (Figure 12) (Fitzgerald et al., 2003). Knowing this, there could be something wrong with the functionality of TRIF pathway in the DC2.4 line that TLR3 is unable to avoid without TRAM activation, making TLR3 unable to produce type I IFNs and additionally secrete IL-12. Since the TLR3 pathway, like TLR4, uses IRF3 to produce IFN-β, if IRF3 is not being activated in a way that does not use TRAM, the cytokines can never be produced.

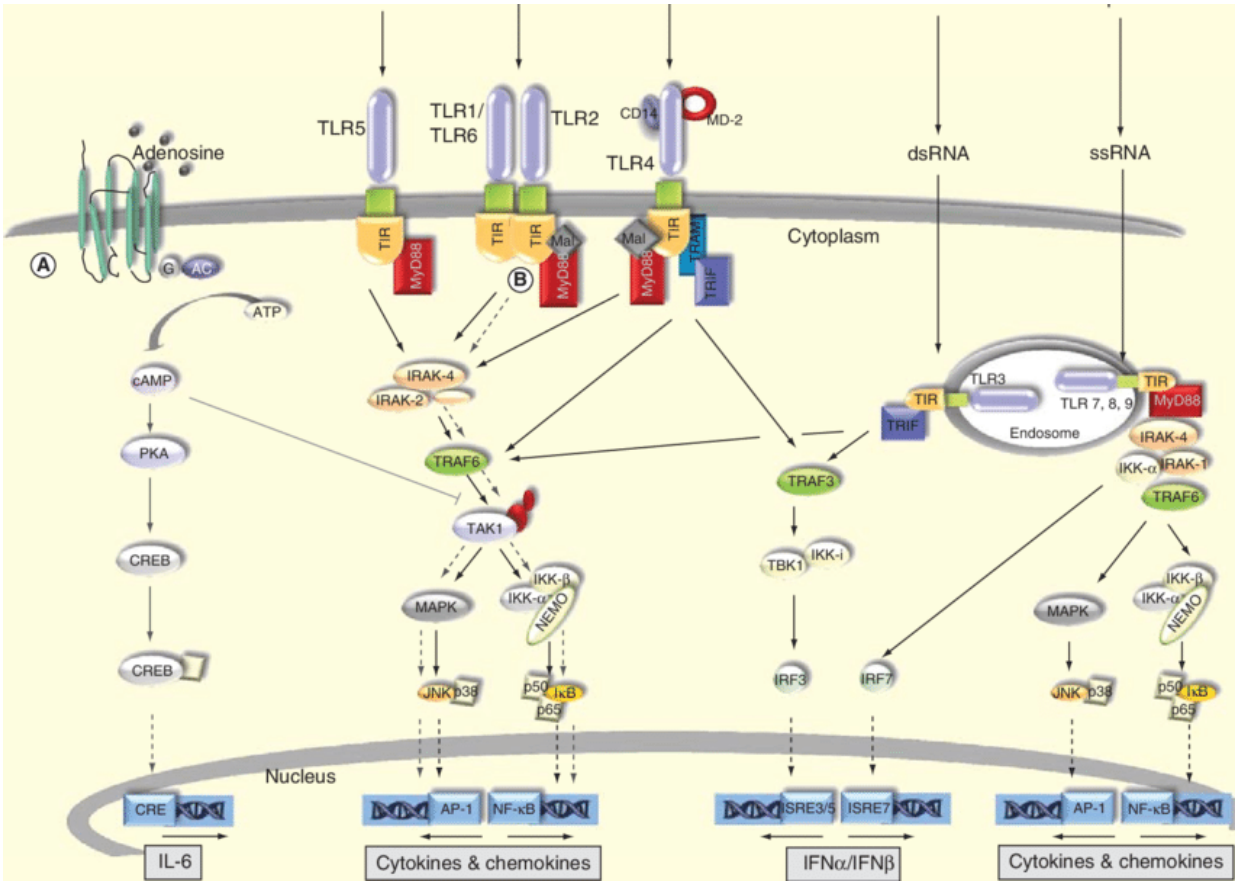


Figure 12. MyD88-dependent and TRIF-dependent TLR signaling pathways characterized by the TIR-domain-containing adaptors MyD88, MAL, TRIF and TRAM. MyD88, which is common to all TLR signaling pathways except TLR3, leads to the production of pro-inflammatory cytokines and chemokines, whereas TLR3- and TLR4-mediated TRIF-dependent signaling activates NF-kB and IRF3 and results in the induction of pro-inflammatory genes and Type 1 IFNs (Glaser & Speer, 2013).

Studies were conducted using an IFN-β plasmid during transfection to look at the functionality of the TLR3 TRIF pathway and subsequent IRF3 activation. In the DC2.4 cells, the resulting luciferase assay did not yield significant results in poly(I:C) or LPS treatment as compared to untreated groups with the plasmid. Induction of the plasmid allows us to measure

the functionality of the TLR3 and TLR4 receptors in and on the cell. Questions about the maturity of the cells have been brought up. According to literature, murine studies have shown that only maturation of cells with TLR ligands generates mature DCs that produce IL-12 to promote T cell help in the body (Schreibelt et al., 2010). Knowing this, our cell line should express the proper TLRs given the treatments they have been given. Additionally, immature DCs have a circular shape, while mature ones have longer dendrites (Kim & Kim, 2019). In our cell line, most of the cells take on this circular shape, showing that they are still immature, which is what is wanted during treatments. However, given that some cells do not take on this circular shape, the current cell line may be too mature to alter the expressed receptors and produce the corresponding cytokines and chemokines. The maturity of the cells can be analyzed based on phagocytosis levels of fluorescent bacteria. By introducing the fluorescent bacteria to the DC2.4 cells, activity of phagocytosis can be monitored in real time under the fluorescent microscope. With higher fluorescence inside of the cell membranes, this would be an indication of a more immature cell line, as more immature DCs have higher phagocytosis levels than mature DCs (Kim & Kim, 2019).

These experiments are relevant due to their focus on immune system activation. Due to impacts on public health and infectious disease, understanding the mechanisms behind these pathways is crucial to human medical development. This study investigates the release of IL-12, a prolific cytokine, which plays a role in inflammation and communication between immune cells. It was hypothesized that any increase in chemical application of poly(I:C), IFN-α, and LPS would lead to an increase in production of IL-12 by the DC2.4 through TLR3 and TL4 respectively Both inflammation and communication are important in maintaining health. As research continues, the functionality of the TLR3 pathway will be further explored. For example,

Western Blot analysis can confirm the presence of TLR3 in the cells. Similarly, Western Blots can be done on resulting cytokines such as IFN-α and IFN-β to see if the cells are able to correctly make products at the end of the pathway. Additionally, qPCR could be used to determine whether different genes in the TRIF pathway downstream of TLR3 are expressed as expected, including NAP1, TBK1, IRF3, and IRF7. This would allow investigation of whether a key component of the TRIF pathway for TLR3 is not being transcribed. With proper IL-12 stimulation through TLR4, there should not be something wrong with these proteins. Looking at sections of the pathway that are unique to TLR3, a missing link may be identified.

References

Fitzgerald, K. A., Rowe, D. C., Barnes, B. J., Caffrey, D. R., Visintin, A., Latz, E., Monks, B., Pitha, P. M., & Golenbock, D. T. (2003). LPS-TLR4 signaling to IRF-3/7 and NF-KB involves the toll adapters tram and trif. *Journal of Experimental Medicine*, *198*(7), 1043–1055. https://doi.org/10.1084/jem.20031023

Glaser, K., & Speer, C. P. (2013). Toll-like receptor signaling in neonatal sepsis and inflammation: A matter of orchestration and conditioning. *Expert Review of Clinical Immunology*, *9*(12), 1239–1252. https://doi.org/10.1586/1744666x.2013.857275

He, T., Tang, C., Xu, S., Moyana, T., & Xiang, J. (2007). Interferon γ Stimulates Cellular Maturation of Dendritic Cell Line DC2.4 Leading to Induction of Efficient Cytotoxic T Cell Responses and Antitumor Immunity. *Cellular & Molecular Immunology*, *4*(2), 105–111.

Jiang, Z., Mak, T. W., Sen, G., & Li, X. (2004). Toll-like receptor 3-mediated activation of NF- B and IRF3 diverges at Toll-il-1 receptor domain-containing adapter inducing IFN- . *Proceedings of the National Academy of Sciences*, *101*(10), 3533–3538. https://doi.org/10.1073/pnas.0308496101

Kim, M. K., & Kim, J. (2019). Properties of immature and mature dendritic cells: Phenotype, morphology, phagocytosis, and migration. *RSC Advances*, *9*(20), 11230–11238. https://doi.org/10.1039/c9ra00818g

Lever, A. R., Park, H., Mulhern, T. J., Jackson, G. R., Comolli, J. C., Borenstein, J. T., … Prantil-Baun, R. (2015). Comprehensive evaluation of poly(I:C) induced inflammatory response in an airway epithelial model. *Physiological Reports*, *3*(4), 1–11. https://doi.org/10.14814/phy2.12334

Loscher, C. E., Draper, E., Leavy, O., Kelleher, D., Mills, K. H., & Roche, H. M. (2005). Conjugated linoleic acid suppresses NF-KB activation and IL-12 production in dendritic cells through Erk-mediated IL-10 induction. *The Journal of Immunology*, *175*(8), 4990–4998. https://doi.org/10.4049/jimmunol.175.8.4990

McCarthy, G. M., Warden, A. S., Bridges, C. R., Blednov, Y. A., & Harris, R. A. (2017). Chronic ethanol consumption: Role of TLR3/TRIF-dependent signaling. *Addiction Biology*, *23*(3), 889–903. https://doi.org/10.1111/adb.12539

Muzio, M., Bosisio, D., Polentarutti, N., D'amico, G., Stoppacciaro, A., Mancinelli, R., … Mantovani, A. (2000). Differential Expression and Regulation of Toll-Like Receptors (TLR) in Human Leukocytes: Selective Expression of TLR3 in Dendritic Cells. *The Journal of Immunology*, *164*(11), 5998–6004. https://doi.org/10.4049/jimmunol.164.11.5998

Nishimura, M., & Naito, S. (2005). Tissue-specific mrna expression profiles of human toll-like receptors and related genes. *Biological and Pharmaceutical Bulletin*, *28*(5), 886–892. https://doi.org/10.1248/bpb.28.886

Rouas, R. (2004). Poly(I:c) used for human dendritic cell maturation preserves their ability to secondarily secrete bioactive IL-12. *International Immunology*, *16*(5), 767–773. https://doi.org/10.1093/intimm/dxh077

Schreibelt, G., Benitez-Ribas, D., Schuurhuis, D., Lambeck, A. J., van Hout-Kuijer, M., Schaft, N., Punt, C. J., Figdor, C. G., Adema, G. J., & de Vries, I. J. (2010). Commonly used prophylactic vaccines as an alternative for synthetically produced TLR ligands to mature monocyte-derived dendritic cells. *Blood*, *116*(4), 564–574. https://doi.org/10.1182/blood-2009-11-251884

Spelmink, L., Sender, V., Hentrich, K., Kuri, T., Plant, L., & Henriques-Normark, B. (2016). Toll-Like Receptor 3/TRIF-Dependent IL-12p70 Secretion Mediated byStreptococcus pneumoniaeRNA and Its Priming by Influenza A Virus Coinfection in Human Dendritic Cells. *MBio*, *7*(2), 767–773. https://doi.org/10.1128/mbio.00168-16