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

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By

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Abstract

Activation of dendritic cells (DCs) through chemical treatment causes release of cytokines. Original studies demonstrated that MuTu DCs, derived from the spleen of mice, responded to stimulation of Toll-like receptor 3 [TLR3] with poly(I:C) by producing IL-12. Challenges in culture of MuTu DCs led to the transition to culture and use of the DC2.4 cell line. DC2.4 cells are also murine dendritic cells but derived from the bone marrow. DC2.4 also express TLR3. To compare the response of DC2.4 cells to MuTu DCs, studies using varying doses and times of poly(I:C) exposure were conducted. In contrast to MuTu DCs, DC2.4 cells produced little to no IL-12 after 12-hour exposure to doses less than 5 µg/mL poly(I:C). Additional experiments determined what dose of poly(I:C) and exposure time was required to stimulate DC2.4 to produce larger amounts of IL-12. Other ongoing studies are investigating whether corticosterone (CORT) alters IL-12 production by DC2.4. CORT is a stress hormone known to suppress IL-12 production by the MuTu DCs. Following activation, DCs often undergo programmed cell death, or apoptosis. This occurred when MuTu DCs were stimulated with poly(I:C). Bim, a member of the Bcl-2 family of proteins, promotes apoptosis, which can be triggered through administration of chemicals such as poly (I:C). Bim was successfully detected in Human Diploid Fibroblasts (HDF), which will serve as a positive control to optimize Bim detection in DCs. Bim was also successfully detected in the DC2.4 cells. Analysis of the production of IL-12 by DCs and their subsequent apoptosis allows for greater understanding of how stress-induced chemicals affect our ability to fight pathogens.

Introduction

Dendritic cells [DCs] are of particular interest because they are activated early in an infection and they direct later stages of the immune response (Murphy, 2017). DCs have pattern recognition receptors [PRRs] on their surface that are capable of interacting with unique pathogen associated molecular patterns [PAMPs] found on pathogens (Murphy, 2017). This allows the recognition of potentially harmful, pathogenic material by our immune system (Murphy, 2017). The interaction between the DC's PRR and the pathogen's PAMP allows the innate immune response to begin which consists of proteins and cells in the extracellular space either directly killing the cell, causing inflammation, or marking the cell for destruction by other immune system cells (Murphy, 2017). The link between this innate response and the subsequent adaptive immune response lies in DCs (Murphy, 2017). DCs are innate immune response cells that play a significant role in the activation of T cells (adaptive immune response lymphocytes) through the release of activation signals (Murphy, 2017).

MuTu DCs, a cell line derived from the spleen of mice, contain TLR3 which is activated through the addition of the chemical poly (I:C), resulting in the release of the cytokine IL-12 (Fuertes et al., 2011). IL-12 is an activating signal that communicates with the adaptive immune response and T cells (Murphy, 2017). This general mechanism led to DC2.4s becoming the focus of this research, because they are a cell line that would respond to treatment similarly to MuTu DCs. DC2.4s are murine dendritic cells derived from the bone marrow of mice (Montes-Casado et al., 2020). DC2.4s are similar to MuTu DCs in their presentation of engulfed antigens, ability to phagocytose pathogens, and in their expression of characteristic dendritic cell features (Van Elsas et al., 1999) which are, for the most part, common among dendritic cell lines. Quantitative analysis has been conducted via ELISA to measure IL-12 production by DC2.4s. Because both

MuTu DC and DC2.4 express TLR-3, DC2.4s are a logical choice to be used for furthering this research following the difficulty in culture of the MuTu DCs in the Fall semester.

When a chemical stressor, such as poly(I:C), is introduced to DC2.4s, they mature and release cytokines as part of the subsequent immune response (Montes-Casado et al., 2020). Cytokines are proteins secreted by cells of the immune system which act as communicators in the immune response (Murphy, 2017). One such cytokine is IL-12, which is released as part of the immune system in response to poly(I:C) through a mechanism involving toll-like receptors (Rhule et al, 2008). Toll-like receptors [TLRs] are membrane bound pattern recognition receptors that interact with unique pattern-associated molecular patterns to activate dendritic cells (Rhule et al., 2008). TLR3 is the specific toll-like receptor that, upon the exposure of DC2.4s to poly(I:C), leads to the ultimate release of IL-12 (Ullrich et al., 2020). This secretion occurs through the upregulation of IL-12's two subunits which are linked by disulfide bonds (Ullrich et al., 2020). The subunits, p35 and p40, are encoded on chromosome 3 and 5, respectively, and their simultaneous expression leads to the formation of the p70 heterodimer which is equivalent to biologically active IL-12 (Ullrich et al., 2020). IL-12 plays pro-inflammatory roles in the immune response making it a good measurement of immune system activity (Ullrich et al., 2020). IL-12 influences the differentiation of CD4+ T cells into one of their helper T cell subtypes (Murphy, 2017), specifically T helper 1 [Th1] subtype, which is relevant because these are proinflammatory compared to other CD4+ T cell subtypes (Murphy, 2017).

The communication between DCs and T cells subsequently leads to the activation, proliferation, and aforementioned differentiation of those T cells (Murphy, 2017). Following this, DCs undergo programmed cell death which is referred to as apoptosis (Murphy, 2017). This

is a necessary process in terms of avoiding extensive inflammatory conditions which can lead to tissue damage (Murphy, 2017).

Bim is a protein that is a member of the Bcl-2 family, which promotes this apoptosis (O'Connor, 1998). The Bcl-2 family of proteins regulates caspases which are the executioners of apoptosis. Apoptosis can be triggered by developmental cues or stress, such as from chemicals, including poly(I:C) or corticosterone [CORT], or by other types of damage and occurs in multiple cell types including human dermal fibroblast cells ([HDF] cells found in the skin that produce the extracellular matrix of skin connective tissue (Kálman et al., 2016)) and MuTu DCs (Fuertes et al., 2012). Poly(I:C) is an immunostimulant that acts to promote the release of cytokines from innate immune cells such as dendritic cells [DC] and CORT is a stress hormone that suppresses immune responses (Lever et al., 2015). The exposure of MuTu DCs to poly(I:C) induces apoptosis and literature suggests that Bim plays a key role in this process (Fuertes et al., 2011).

In this research, the overarching goal is to observe the effect of chemicals on the immune response. Assessing for the connection between the response to the chemicals, poly (I:C) and CORT and the apoptotic protein, Bim, helps display a connection between the immune response and apoptosis which is a crucial process in avoiding harmful inflammation. Verifying Bim expression in DC2.4 will further this connection. Using the dendritic cells outlined above and quantifying the expression of immune system cytokine, IL-12, allows a relationship between chemical stimulation and the immune system to be observed. Overall, the effect poly(I:C) and CORT have on different cell lines is being. Due to previous work with MuTu DC, it was hypothesized that an increase in the dose of poly (I:C) would cause increased production of IL-12 from DC2.4 as well as increased detection of Bim.

Materials and Methods

Cell Culture:

The DC2.4 were grown in RPMI media which was supplemented with 10% fetal calf serum [FCS], 1% glutamine, 1% Penn/Strep, and 2% beta-mercaptoethanol (Steinman and Cohn, 1973). Cells were incubated at 37 degrees C in 6% CO2 conditions. Cells were routinely split at a 1:5 ratio for maintenance approximately every three days. Cell harvest consisted of aspirating a confluent, T75 flask and adding 2 mL of trypsin/versine solution. This was left to incubate for 2 minutes prior to agitation of the flask and adding back approximately 4 mL of cell culture media. Cell counting consisted of a 1:20 dilution of the harvested cells into trypan blue and use of a hemocytometer. The four outer quadrants of each side were averaged to obtain an overall average of total cells using the following equation:

Total # cells / 4 quadrants * dilution factor * ($1x10^4$) = # of cells/mL

Analysis of IL-12 production by DC2.4 exposed to varying doses of poly (I:C) or CORT:

ELISA assay #1

Confluent T75 flasks of DC2.4s were harvested and counted. $5x10^5$ cells were added to each well of a 12-well cell culture plate with nine total wells being utilized. The treatments were done in triplicate with three wells remaining untreated (control), three wells being treated with CORT ($1x10^6$ M), and three wells being treated with poly(I:C) (5 µg/mL). Treatments were applied for 12 hours prior to the supernatants being removed to conduct an ELISA. The kit used was a mouse IL-12 (p40) ELISA set from BD Biosciences (BD OptEIA). Table 1 delineates the

set up of this ELISA. Columns 1-3 contain the IL-12 standards in triplicate and columns 4-9 contain the treated samples in triplicate. Samples in J1-J3 were not treated, samples in J4-J6 were supernatant treated only with CORT, and samples J7-J9 was supernatant treated only with poly(I:C).

Table 1: Set-up of ELISA #1, analyzing IL-12 production by DC2.4 exposed to poly (I:C) or CORT.

	1	2	3	4	5	6	7	8	9
A	1000pg/µL	1000pg/µL	1000pg/µL	J1	J1	J1	J9	J9	J9
B	500 pg/µL	500 pg/µL	500 pg/µL	J2	J2	J2	/	/	/
C	250 pg/µL	250 pg/µL	250 pg/µL	J3	J3	J3	/	/	/
D	125 pg/µL	125 pg/µL	125 pg/µL	J4	J4	J4	/	/	/
E	62.5 pg/µL	62.5 pg/µL	62.5 pg/µL	J5	J5	J5	/	/	/
F	31.25pg/µL	31.25pg/µL	31.25pg/µL	J6	J6	J6	/	/	/
G	16.8 pg/µL	16.8 pg/µL	16.8 pg/µL	J7	J7	J7	/	/	/
H	H2O	H2O	H2O	J8	J8	J8	/	/	/

Analysis of IL-12 production by DC2.4 exposed to varying doses of poly (I:C) and/or CORT: ELISA assay #2

Exposure of DC2..4 to varying doses of poly (I:C) was conducted using the same ELISA. The additional groups involved cells treated with 5 µg/mL poly (I:C), 1 µg/mL poly (I:C), and 0.1 µg/mL poly (I:C). Table 2 delineates the set up of the ELISA for this experiment. Columns 1-3 contain the IL-12 standards in triplicate and columns 4-12 contain the treated supernatant

samples in triplicate. J1-J3 was not treated, J4-J6 was supernatant from DC2.4s only treated with CORT, J7-J9 supernatant that was only treated with poly (I:C). For samples in S1-S3 DC2.4 cells were not treated, S4-S6 was treated with 5 µg/mL poly (I:C), S7-S9 was treated with 1 µg/mL poly (I:C), and S10-S12 was treated with 0.1 µg/mL poly (I:C).

Table 2: Set-up of ELISA #2, analyzing IL-12 production by DC2.4 exposed to varying doses of poly (I:C) and/or CORT.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1000pg/µL	1000pg/µL	1000pg/µL	J1	J1	J1	J9	J9	J9	S8	S8	S8
B	500 pg/µL	500 pg/µL	500 pg/µL	J2	J2	J2	S1	S1	S1	S9	S9	S9
C	250 pg/µL	250 pg/µL	250 pg/µL	J3	J3	J3	S2	S2	S2	S10	S10	S20
D	125 pg/µL	125 pg/µL	125 pg/µL	J4	J4	J4	S3	S3	S3	S11	S11	S11
E	62.5 pg/µL	62.5 pg/µL	62.5 pg/µL	J5	J5	J5	S4	S4	S4	S12	S12	S12
F	31.25 pg/µL	31.25 pg/µL	31.25 pg/µL	J6	J6	J6	S5	S5	S5	/	/	/
G	16.8 pg/µL	16.8 pg/µL	16.8 pg/µL	J7	J7	J7	S6	S6	S6	/	/	/
H	H2O	H2O	H2O	J8	J8	J8	S7	S7	S7	/	/	/

ELISA assay #3:

The third ELISA assay was conducted with increased poly (I:C) doses and exposure time. In this experiment $1.5x10^6$ was initially added per well of a 12-well culture plate. One culture contained the following treatments in triplicate: untreated cells, cells treated with 100 µg/mL poly (I:C), and cells treated with 100 µg/mL poly (I:C) and $1x10^{-6}$ M CORT. The other culture contained the following treatments in triplicate: untreated cells, cells treated with 50 µg/mL poly

(I:C), and cells treated with 100 µg/mL poly (I:C). These treatments were all applied for 46 hours prior to harvest of cell culture supernatants and the subsequent ELISA. Table 3 illustrates the set-up of this ELISA. Columns 1-3 contain the IL-12 standards in triplicate and columns 4-12 contain the treated samples in triplicate. J1-J3 was not treated, J4-J6 was treated with 100 µg/mL poly (I:C), J7-J9 was treated with 100 µg/mL poly (I:C) + 100 µg/mL $1x10^{-6}$ M CORT, S1-S3 was not treated, S4-S6 was treated with 50 µg/mL poly (I:C), and S7-S9 was treated with 100 µg/mL poly (I:C)

Table 3: Well treatments of ELISA #3, analyzing IL-12 production by DC2.4 exposed to varying doses of poly (I:C) and/or CORT.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1000pg/µL	1000pg/µL	1000pg/µL	J1	J1	J1	J9	J9	J9	S8	S8	S8
B	500 pg/µL	500 pg/µL	500 pg/µL	J2	J2	J2	S1	S1	S1	S9	S9	S9
C	250 pg/µL	250 pg/µL	250 pg/µL	J3	J3	J3	S2	S2	S2	/	/	/
D	125 pg/µL	125 pg/µL	125 pg/µL	J4	J4	J4	S3	S3	S3	/	/	/
E	62.5 pg/µL	62.5 pg/µL	62.5 pg/µL	J5	J5	J5	S4	S4	S4	/	/	/
F	31.25 pg/µL	31.25 pg/µL	31.25 pg/µL	J6	J6	J6	S5	S5	S5	/	/	/
G	16.8 pg/µL	16.8 pg/µL	16.8 pg/µL	J7	J7	J7	S6	S6	S6	/	/	/
H	H2O	H2O	H2O	J8	J8	J8	S7	S7	S7	/	/	/

Analysis of Bim production by HDF cells exposed to 5-fluorouracil:

Western Blot #1:

A western blot was conducted which contained the positive control for Bim, HDF cells treated with 5µM 5-flurouracil for two hours, and untreated HDF cells. The cells for this western blot were obtained through harvest and subsequent lysis. The harvesting procedure consisted of aspirating confluent T75 flasks, adding 1 mL of trypsin/versine solution, incubating for 2 minutes, agitating the flask, and checking for adequate cell movement. Next 5 mL of DMEM media was added and this was transferred to a conical vial for subsequent centrifugation at 1200 RPM for 3 minutes. This was then aspirated and 5 mL of sterile 1X PBS was added before agitating the pellet. The centrifugation was repeated, followed by aspiration of the PBS.

Following brief freezing, this pellet was resuspended in 1 mL of cold PBS to carry out the lysis procedure. The cells were transferred to chilled microfuge tubes to be centrifuged at 13000 RPM for 3 minutes at 4 degrees C. The PBS was then aspirated, and the pellet left was placed on ice. The cell pellet was then lysed with protease inhibitor, which is diluted in EBC lysis buffer, resuspended, and placed on a rotator in a refrigerator for 30 minutes. This is then centrifuged again at 13000 RPM for 3 minutes at 4 degrees C. The supernatant was then transferred to clean/chilled tubes and frozen (several months prior to the conduction of this western blot).

A Bradford assay was carried out to quantify the amount of protein that each of these lysates contained. 225 µg of protein was added to each well from these samples. They were run next to a protein ladder during the gel electrophoresis to ensure detection of the correct protein. Based on its weight, Bim is typically found at approximately 23 kDa. The primary antibody was Bim (C34C5) rabbit mAB catalog #2933S from Cell Signaling Technology. The secondary

antibody was anti-rabbit IgG, HRP linked antibody catalog #7074S from Cell Signaling Technology. The primary antibody utilized was applied at a 1:800 dilution and the secondary antibody was applied at a 1:2000 dilution.

Analysis of Bim production by DC2.4 cells exposed to poly (I:C) and/or CORT:

Western Blot #2

The treatments applied to the DC2.4 line consisted of one that was untreated (control), one treated with 100 µg/mL poly (I:C), and one treated with 100 µg/mL poly (I:C) + $1x10^{-6}$ M CORT. All treatments were applied for 24 hours. The harvest and lysis procedures were carried out in the same way as previously described with 2 confluent T75 flasks. A standard curve was obtained from another Bradford assay to determine the amount of protein to be added per well. Each well was given the maximum amount of protein possible that could be mixed with 9 µL of 6X loading dye (this was 41 µL of lysis supernatant for the 50 µL wells utilized). The total amount of protein added for each sample was: 160.97µg for the control, 194.29 µg for the poly (I:C) treated, and 120.54 µg of the poly (I:C) + CORT treated cells. This was calculated utilizing the accompanying Bradford assay results which quantified the amount of desired protein present. The antibodies used (as well as their dilutions) were the same as in the previous western blot.

Results

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) and/or

corticosterone. In all cases, a standard curve of known concentrations of IL-12 was used as depicted in Figure 1 below. 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=3E-05+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.

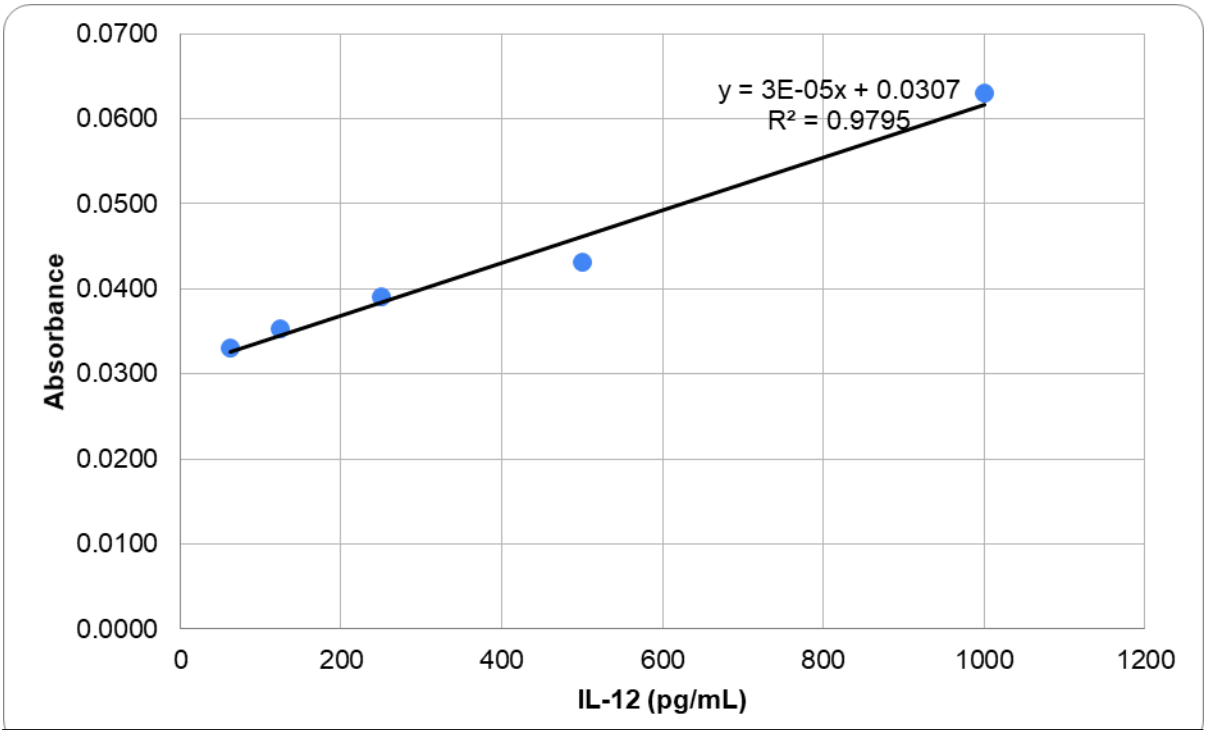

Figure 1: IL-12 standard curve depicting relationship between known concentrations of IL-12 and the subsequent absorbance. The slope of the line is used to determine the amount of IL-12 produced by varying treatment groups of DC2.4 cells. Values represent the average absorbance at each known concentration of IL-12 and was detected using the TECAN infinite F50 ELISA plate reader.

ELISA #1 yielded absorbance values for experimental samples (as compared to their standard curve) which all fell below zero. This did not support our hypothesis as little to no IL-12 was produced in response to poly (I:C) treatment. It did, however, support our hypothesis about CORT because IL-12 production was not seen when only CORT was applied to the DC2.4

cells. ELISA #2 compared varying doses of poly (I:C) and yielded the results observed in Figure 2 below:

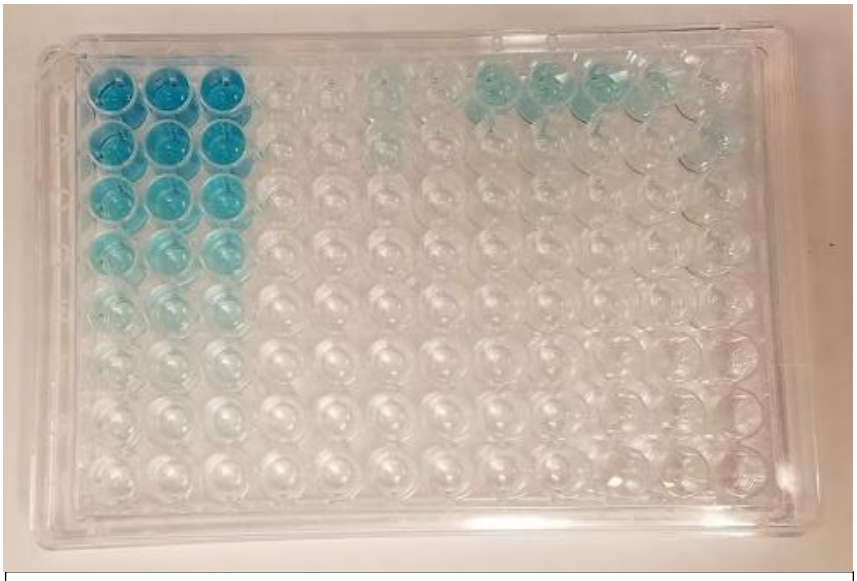

Figure 2: Completed plate of ELISA assay #2 with blue visualization of IL-12 production from DC2.4 not treated, treated with poly (I:C), and treated with poly (I:C) and CORT (based on experiment set-up outlined in Table 2).

As seen in Figure 2, the samples for ELISA #2 display very little blue coloration which is indicative of very little IL-12 production. The absorbance values of this plate were compared to its standard curve and once again, values below zero were found. This once again did not support our hypothesis that poly (I:C) application would increase IL-12 production. ELISA #3 was conducted with an increased number of cells, increased doses of poly (I:C), and an increased treatment time. It's results also, when compared to its standard curve, yielded measurements of IL-12 less than zero. This did not support our hypothesis that poly (I:C) would increase the amount of IL-12 produced by these cells.

Western blot detection of Bim was conducted to determine if the application of poly (I:C), which is known to increase inflammation, would also lead to subsequent apoptosis, which is a necessary process to avoid chronic inflammation. Western blot #1 was conducted with HDF cells treated with 5µM 5-flurouracil for two hours and untreated HDF cells as an optimization procedure for detecting Bim through western blot. This was successful as Bim was detected which is visible in Figure 3a below. To the right in Figure 3b, the expected weight of proteins is shown for comparison. Bim is expected to fall at approximately 23 kDa.

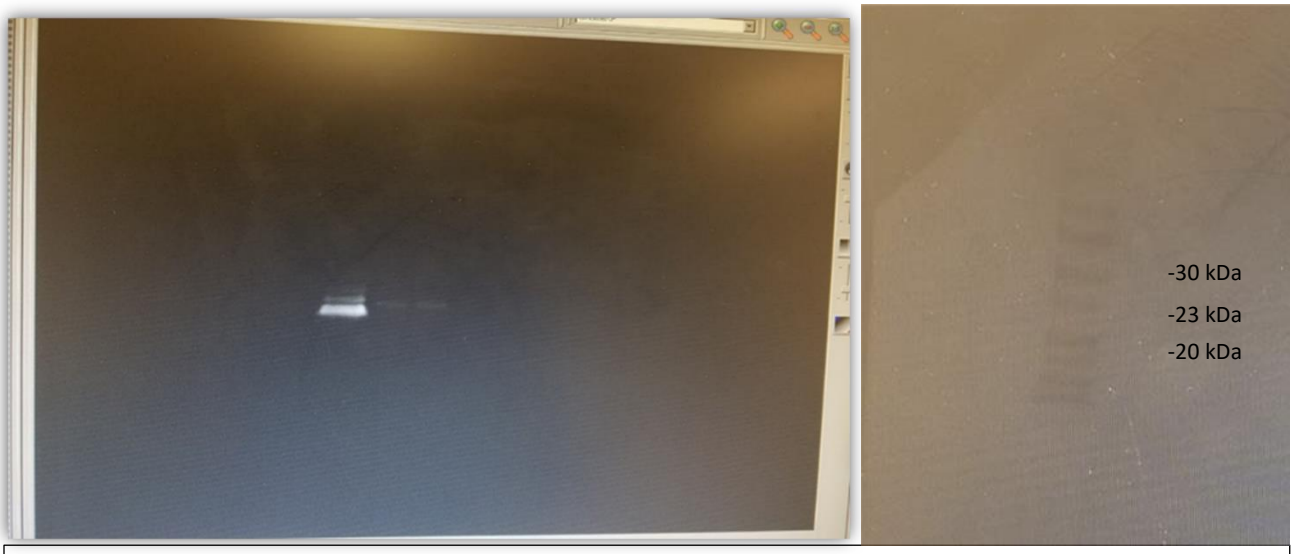

Figure 3a: (Right) Western blot results with the positive control for Bim in the left most lane, Bim detection in the 5µM 5-flurouracil treated HDF cells in the middle lane, and Bim detection in the untreated HDF cells in the rightmost lanes.

Figure 3b: (Left) Labelled protein ladder depicting weights of varying protein sizes with Bim's typical range (23 kDa) highlighted.

As evident in Figure 4 below, Bim was successfully detected in the DC2.4 cells as well. The bands observed in Figure 4 fall at approximately 23 kDa as expected for Bim. This supported our hypothesis that Bim production would be increased following treatment of poly (I:C).

Figure 4: Western blot results with positive control for Bim in lane #1 (left most lane), lane #2 showing Bim detection after no treatment of DC2.4 cells, lane #3 showing Bim detection in poly (I:C) treated DC2.4 cells, and lane #4 showing Bim detection in poly (I:C) and CORT treated DC2.4 cells.

Discussion

Throughout this experimentation, the overall goal has been to observe the effect of chemicals on the immune response. Poly (I:C) and CORT were the primary chemicals utilized and their effect on DC2.4 was observed through the production of IL-12, a prolific immune system cytokine. The direction of this study came from previous literature which supported that

DC2.4 release IL-12 through the TLR3 pathway (Hu et al., 2021) similar to the mechanism within MuTu DC. Observation of Bim in DC2.4 in response to these chemicals was also measured to find the connection to the apoptotic mechanism. Prior to experimentation, it was hypothesized that any increase in chemical application would lead to an increase in observation of IL-12 or Bim in their respective analyses.

As evident by the lack of substantial absorbance value and blue coloration seen in the ELISA depicted in Figure 1, CORT alone does not trigger IL-12 in DC2.4s. This was as expected because CORT typically acts as an immune response suppressor (Lever et al., 2015) so the subsequent lack of IL-12 production supported the literature. The absorbance values for the first ELISA were minimal suggesting no IL-12 production. This finding led to the second ELISA with increased poly (I:C) dose and time of exposure of DC2.4. Again, these absorbances were low and IL-12 production was not detected. DC2.4 have been found to express TLR3 and it has also been found that IL-12 is produced by these cells. It is possible that this was the case due to the handling of the poly (I:C) as it was kept at room temperature overnight instead of being refrigerated. Another possible explanation for these outcomes was observed in the media. During the second ELISA, it was observed that while the cells were not actively dying when the supernatant was harvested, the media had yellowed. This implies an acidification of the media which could have caused IL-12 to denature. Because it is likely that a conformational epitope is the target of the capture and detecting antibodies used in the ELISA, denaturation could have led to the failed detection of the IL-12. Another potential reason why IL-12 production was not observed could have to do with the particular cell line used. These DC2.4 were derived from the bone marrow. 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.

Bim is known to play a role in inducing apoptosis as it comes from the Bcl-2 family of proteins which are known to be pro-apoptotic. As previously noted, different amounts of protein were loaded into each well so a qualitative conclusion about whether more Bim was present in the poly (I:C) treated DC2.4 cannot be made. Moving forward, now that it is known that Bim can be detected in these cells following poly (I:C) application, a repeat Western Blot will be conducted that contains equal amounts of protein loaded into each well.

This study is highly relevant due to its focus on the immune system. Understanding the innerworkings of these mechanisms leads to findings that can have a huge impact on public health as infectious diseases and anything that can be passed between humans, somehow comes into contact with the immune system. This study in particular, is investigating the release of a prolific cytokine, IL-12, which plays a role in inflammation and communication between immune cells and is important to control to maintain a person's health. As the research on this topic continues, the focus will shift towards understanding why DC2.4 cells treated with poly (I:C) are not producing IL-12. For example, the next step could be to ensure that this particular DC2.4 line expresses TLR3 which is crucial for the subsequent release of IL-12. Another direction could be to expose the DC2.4 to different PAMPs, such as lipopolysaccharide [LPS], to see if this can stimulate the release of IL-12. Steps may also be taken to address the potential that the DC2.4 are not mature enough, such as adding the application of a PAMP that would first mature the cells prior to poly (I:C) exposure for subsequent experimentation.

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