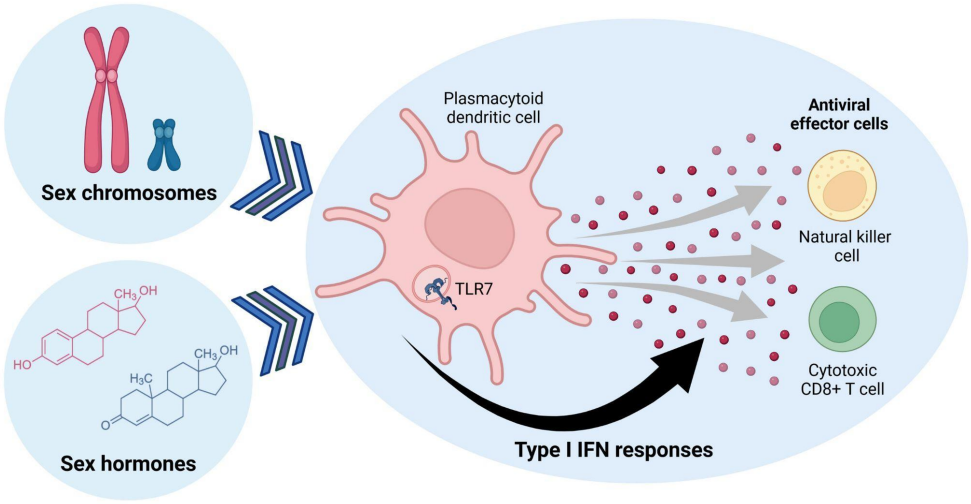
UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

ROLE OF ANDROGEN RECEPTOR SIGNALING IN THE ACTIVATION AND DIFFERENTIATION OF PLASMACYTOID DENDRITIC CELLS



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Degree project for Master of Science (120 hec) with a major in Biology
BIO727, Degree project in Physiology and Cell Biology and 60 hec
Second cycle
Semester/year: Autumn 2022, Spring 2023
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https://doi.org/10.3389/fimmu.2022.986840

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Abstract

Androgens are steroid hormones essential for the development and maintenance of male reproductive organs and secondary sexual characteristics. Plasmacytoid dendritic cells (pDCs) are immune cells that act as the first line of defense against viral infections. These cells have specialized receptors such as the toll-like receptors (TLR7) that recognize viral nucleic acids and initiate the production of antiviral cytokines such as Type I interferons (IFN-α1 and IFN-α2). Androgen receptor signaling might affect pDC functions throughout their activation and maturation in response to viral infections via the androgen receptor (AR), a nuclear hormone receptor that binds to hormones such as testosterone, an androgen hormone. Androgens have also been shown to promote the replication and intensity of some viruses, most notably SARS-CoV-2, an RNA virus. Understanding the mechanism of androgen signaling is critical for developing effective antiviral therapies. Considering this, we stimulated human peripheral mononuclear cells (PBMCs) with a TLR7 agonist such as Gardiquimod, an AR agonist R1881, and the synthetic androgen named Testosterone propionate. An antagonist namely G15 for the G-protein coupled estrogen receptor was also used. These compounds are used to activate the AR in order to assess its role in interferon production. Furthermore, qPCR was used to examine the gene expression of *IFNα1, IFNα2, IRF7, and MX1* using *GAPDH* as the endogenous control. Flow cytometry was also performed after the cells were stained with BDCA2, CD3, CD19, CD123, and IFNα2 to evaluate the pDC population and interferon production. The findings showed an increase in IFN response when the PBMCs were stimulated with Gardiquimod and the agonists, with individual differences. To date, relatively few studies have been conducted on the role of androgens in immune system regulation. Further research can generate effective treatment targets and lessen the impact that certain infections have on human health.

1. Introduction

1.1 Sex Hormones and the Immune System

The immune system is comprised of two types: the innate immune system which is made up of a variety of physical, chemical, and cellular mechanisms that act as the first line of defense against pathogens, and the adaptive immune system which responds to specific antigens (Chaplin 2010). Sex plays a significant role in the innate immune system, and gender disparities in immunity have been thoroughly documented in years of scientific research. In general, women have stronger immunological responses than men. This is assumed to be owing to the effects of sex hormones on the immune system, such as estrogen and testosterone. Estrogen has been demonstrated to stimulate the immune system, whilst testosterone can suppress it. Moreover, the ability to respond to infection and vaccination is sex-biased (Ruggieri et al. 2016). Furthermore, many immune-related genes such as *TLR7, TLR8, IRAK1,* etc. are expressed on the X chromosome. Because females have two X chromosomes, they have a greater number of immune-related genes than men who have only one X chromosome. However, this increased immunological response in women may contribute to a higher occurrence of autoimmune diseases, which occur when the immune system mistakenly attacks the body's own tissues (Rojas-Villarraga et al. 2010). For example, Systemic lupus erythematosus (SLE), Rheumatoid Arthritis (RA), and Multiple Sclerosis prevail more in women than men. On the contrary, men are more susceptible to infectious diseases. An analysis of death among 17 million adults during COVID-19 showed that being female was a robust protective factor (Williamson et al. 2020). Despite both genders exhibiting similar vulnerability, men faced a greater likelihood of being infected and experiencing a higher mortality risk (Ramírez-Soto, Ortega-Cáceres, and Arroyo-Hernández 2021). The involvement of sex differences in viral infections is evident although the mechanisms have quite not been explored extensively. An example of how testosterone affects a variety of cell types in response to infections and vaccinations is shown in Fig 1 (Trigunaite, Dimo, and Jørgensen 2015).

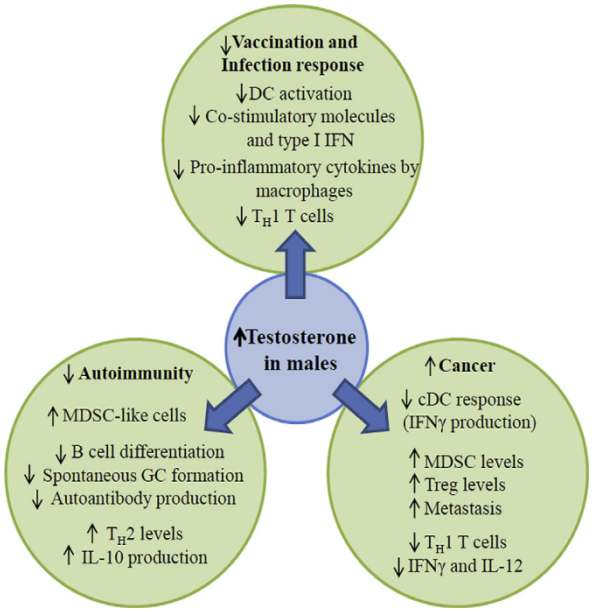

Fig 1: Testosterone regulation in males is responsible for immune responses.

1.2 Androgen Receptor Signaling

Androgens are a group of steroid hormones, and they are primarily produced in the testes of males and in lower amounts in the adrenal glands of both sexes. There are five types of androgens where testosterone is produced in Leydig cells and adrenal glands, and a more potent form of testosterone named dihydrotestosterone (DHT) is the one responsible for male sexual characteristics and male reproductive organs. The effects of androgens are mediated through androgen receptor (AR). The AR is a ligand-dependent nuclear receptor, meaning it is activated by binding to steroid hormones such as androgens (Sever and Glass 2013). The classical pathway by which AR acts is illustrated in Fig 2 below.

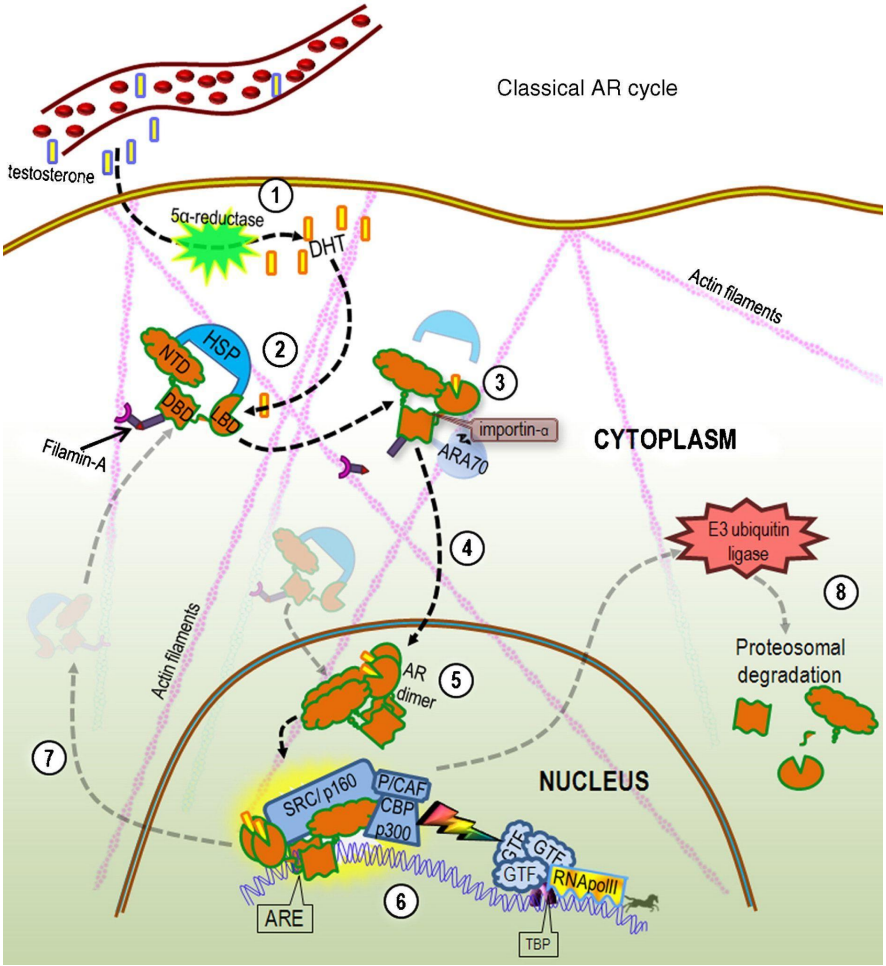

Fig 2: (1) The enzyme 5-reductase in the basal epithelial cells of the prostate converts testosterone to DHT. (2) DHT enters epithelial cells' cytoplasm and binds to AR. (3) Ligand interaction causes a conformational change in AR, causing heat shock proteins (HSPs) to dissociate to enable nuclear translocation. (4) In the nucleus, AR dimerizes (6) Various co-activators attach to AR within the nucleus, and the AR-DBD enhances nucleic acid binding at androgen response elements (ARE). This stimulates the results in chromatin remodeling. This permits TATA-binding protein (TBP), general transcription factors (GTF), and RNApolII to bind and activate transcription. (7) AR that is not bound to ligands is recycled back to the cytoplasm in preparation for future ligand binding ("Molecular Cell Biology of Androgen Receptor Signalling" 2010).

1.3 Complete Androgen Insensitivity Syndrome

The modulation of AR function is dependent on mutations that arise in various loci on the AR gene located on the X chromosome. These mutations might result in a reduction or complete loss of gene expression. Due to the presence of only one X chromosome in males, these mutations are expressed in all cells, whereas in females, the second copy of the X chromosome counteracts their effects leading them to be silent carriers (Quigley et al. 1995). Androgen Insensitivity Syndrome (AIS) is a functional consequence of the AR mutation where individuals are insensitive to androgens including the variation in the absence of phenotypic characteristics (Hughes et al. 2012). The severity of this condition varies in different degrees from partial, mild to complete.

Complete Androgen Insensitivity Syndrome (CAIS), an X-linked disease is a severe form of AIS where individuals are completely resistant to androgens. This is an extremely rare genetic disorder that occurs in 1 in 20,000 to 64,000 male births (Ahmed et al. 2000). Individuals with CAIS carry the 46, XY karyotype and testes. However, they exhibit the female phenotype with normal female external genitalia and breast development (Quigley et al. 1995). It is common for these individuals to go undiagnosed until they reach puberty and when they are examined to have an inguinal hernia. Individuals with CAIS are considered for this project because they are unable to respond to androgens completely and to see how they respond to similar experimental conditions besides healthy male individuals. Hence, they can be assigned as the control group.

1.4 Plasmacytoid Dendritic Cells

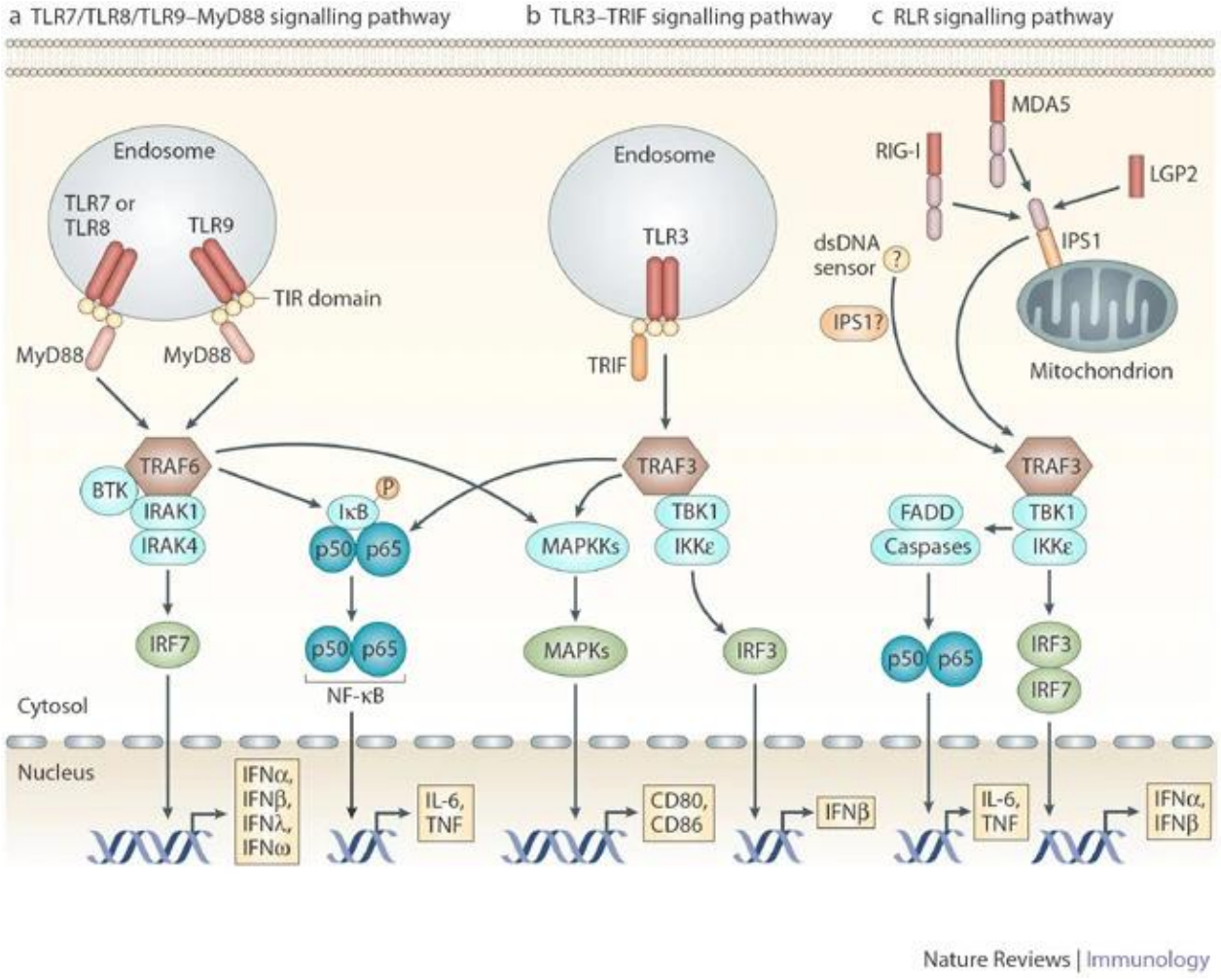

Fig 3: There are three signaling pathways of pDC activation and differentiation. Following viral or nucleic acid activation, TLR7, and TLR9 move from the ER to the endosomes to interact with their RNA or DNA agonists. TLR conformational alterations activate MyD88 (myeloid differentiation primary-response gene 88), which subsequently connects with TRAF6 (tumor necrosis factor (TNF) receptor-associated factor 6), BTK (Bruton's tyrosine kinase), and IRAK4 (interleukin-1-receptor-associated kinase 4). To spread downstream signals, the "MyD88-TRAF6-IRAK4" complex activates IRF7 (interferon-regulatory factor 7), TAK1 (transforming-growth-factor-activated kinase 1), nuclear factor-kB (NF-kB), and IRF5. TRAF3, IRAK1, IKK (NF-B kinase inhibitor), osteopontin (OPN), and phosphoinositide 3-kinase (PI3K) are the primary activators of IRF7. Following ubiquitylation and phosphorylation, IRF7 translocate to the nucleus and initiates type I interferon transcription (Lund et al. 2003; Gilliet, Cao, and Liu 2008).

Plasmacytoid dendritic cells (pDCs), also known as "interferon-producing cells," are a type of specialized subset of dendritic cells that are antigen-presenting cells found in lymphoid organs and consist of 0.4% of peripheral blood mononuclear cells (Blom et al. 2000). These cells can express low levels of MHC II molecules which can be upregulated when activated and present to CD4+ T cells. pDCs recognize single-stranded viral RNA through pattern recognition receptors such as the toll-like receptor 7 (TLR7) as shown in the case of HIV and Influenza Virus (Beignon et al. 2005; Cella et al. 2000). Moreover, they can also recognize double-stranded DNA viruses through Toll-like receptor 9 such as Herpes Simplex Virus-2 (HPV) (Lund et al. 2003). When encountering viral nucleic acids, this specialized subgroup of dendritic cells is the initial responder in the innate immune system against invaders, secreting higher levels of Type 1 Interferons, primarily IFN alpha as an anti-viral response (Coccia et al. 2004) as shown in Fig (c). Studies have suggested that females have higher expression of TLR7 as well as higher production of IFN alpha (Pisitkun et al. 2006). Moreover, these cells can also secrete cytokines that can activate Natural Killer (NK) cells to mediate the lysis of target cells (McKenna, Beignon, and Bhardwaj 2005; Swiecki et al. 2010). An impairment of pDC function can lead to chronic conditions of certain diseases such as HIV (McKenna, Beignon, and Bhardwaj 2005). In humans, male mononuclear cells generate lower type I IFNs in response to TLR7 ligands and more IL-10 in response to TLR9 ligands than females (Meier et al. 2009). Recent studies have shown that pDC impairment is a critical factor in causing severe medical conditions in patients infected by the Sars-Cov2 virus (Zhou et al. 2020). Looking at how pDCs have contributed to the innate immune system, it is important to study these cells.

2. Aim

To assess the role of androgen receptor signaling on Type I Interferon by pDCs. Evaluation of Interferon production by pDCs will be done after PBMC stimulation of healthy male individuals and CAIS patients and then compared to see if the gene and protein expression levels varied among the two groups.

3. Materials and methods

Table: Materials required

Reagents	Company	Catalog number
Gibco ™ RPMI 1640 media	Thermo Fisher Scientific	61870-010
TheraPEAK ™ XVIVO ™ - 15 media	Lonza	BE02-060F
Gardiquimod	Invivogen	tlr-gdq-5
R1881	Sigma Aldrich	965-93-5
Testosterone Propionate	Sigma Aldrich	86541-5G
G15	TOCRIS	2B/277935
2-Mercaptaethanol	gibco	21985-023
Benzonase Endonuclease	Sigma Aldrich	1.01695.0001
IC Fixation Buffer	Invitrogen	00-8222-49
Permeabilization Buffer	Invitrogen	00-8333-56
CD3	BioLegend	300430
CD19	BioLegend	302230
BDCA2	BD Biosciences	566427
CD123	BioLegend	306010
IFN-α2	BD Pharmingen ™	560097
Fc block	BD Biosciences	564220
Ficoll	cytiva	17144003
DMSO	SIGMA	D2438

3.1 Isolation of PBMCs from the blood by Ficoll

Fresh blood was taken from healthy volunteers at Sahlsgrenska University Hospital in Heparin tubes, which were inverted three times and kept at room temperature for 30 minutes before being put into a single 50ml falcon tube rinsed with PBS. Blood was diluted in PBS RT at a 1:1 ratio and divided into two 50 ml falcon tubes. 15 ml Ficoll was introduced to a fresh 50 ml falcon tube. We added the diluted blood at a 45-degree angle into the Ficoll at a maximum of 20 mL of blood into the tube and centrifuged at 400g for 40 minutes at room temperature, acceleration of 5, and brake of 0. The lymphocyte-containing top layer was collected and placed in 10 ml PBS. After that, 50 ml PBS was added and centrifuged for 20 minutes at 4℃, acceleration: 7, and deceleration: 7. After centrifugation, the supernatant was discarded by pouring and 50 ml cold PBS was added and centrifuged again. The supernatant was poured out again and 1ml cold PBS was added, pipetted up and down to mix, and set for counting.

3.2 Freezing of PBMCs

First, we centrifuge and resuspend cells of 500 µl in FBS and put them on ice. 500 µl with 20% Dimethyl sulfoxide (DMSO) was prepared where 100 µl is DMSO with 400 µl of FBS. 500 µl of cell suspension was added to each cryovial. Then we added the 20% DMSO+FBS solution to the vials so that there is 10% DMSO in each vial. The vials were then put immediately in Mr. Frosty Freezing Container under the -80℃ freezer for two days and then transferred to the -150℃ freezer.

3.3 Thawing of PBMCs

A total of four individuals with buffy coats (Male 1: B488; Male 2: B044, Male 3: B479; Male 4: 11.22) with the highest cell count were included in this project. 20 ml of RPMI+10% charcoal Fetal Bovine Serum (FBS)+1%PenStrip+50 µl 2-Mercaptaethanol (BME) was taken and 0.5 µl of Benzonase Endonuclease (BE) was added to it and 4 ml was set aside in another falcon tube. The reason for using charcoal FBS is to deplete the media of hormones. 4 vials each containing the same buffy coat were thawed in a hot water bath for 30 seconds and then with a pipette, 1 ml of it was added slowly to the 16 ml RPMI media that we made earlier and rested for 10 minutes. Centrifugation was done at 250g, 9 acceleration (acc), 7 deceleration (dec), for 10 minutes at 22℃. After discarding the supernatant, 3 ml of the mix was added to it and centrifuged again at 7 acc, 5 dec, and for 10 minutes. The supernatant was discarded and 1ml of RPMI media was added to it. Then we calculated the amount of media for each well (500 µl) and cell suspension and add it to a 48-well plate. For example, 500 µl multiplied by 12 wells, we need 6ml in total. Therefore, 5 ml media was added to the 1 ml of cell suspension.

3.4 Stimulation of PBMCs

The initial concentration of R1881 was 35 mM; to make it 350 µM, 1 µl was added to 99 µl of complete media, the step was repeated, and 1.7 µl was put into the wells, with each well having 1.19 µM. The second R1881 concentration would be 3.5 µM, thus we added 1 µl of the 350 µM concentration to 99 µl of complete media. The wells were then filled with 1.7 µl of 11.9 nM solution. For two different time points such as 0 and 3 hours, we calculated different concentrations of Gardiquimod, a TLR7 agonist, R1881, an AR agonist, Testosterone Propionate, and an antagonist namely G15. The starting concentration of testosterone propionate was 20 millimolar. 1 µl of it was added to 1 ml of media, resulting in a concentration of 20 µM, the step was repeated to obtain a concentration of 20 nM, and the concentration was 100 pM after we added 2.5 µl to the wells. For the second concentration, 10 pM, we took 1 µl of 20 nM and mixed it with 99 µl media before adding 2.5 µl to the well. In the instance of G15, an antagonist for GPER, we use a concentration of 0.5 µg, so we add 1 µl to 1 ml of media and 5 µl to the wells. All of this in their final concentrations were added to the wells and incubated at 37 ℃ for two hours. For the Gardiquimod, we add 9 µl of it to 21 µl media, then add 15 µl of the mix to 15 µl media, and finally add 5 µl to the wells. The final concentration of 1.5 µg per ml was added after two hours of incubation and incubated for 3 hours. RL buffer was prepared with 5 ml of media and 50 µl of BME. 500 µl from the wells were taken into Eppendorf tubes and centrifuged at 200 g, 22 ℃, for 10 minutes, and 350 µl of RL buffer was added to the wells. The supernatant was discarded while we pipetted the RL buffer up and down the wells and collect it into the tubes with the pellet. The tubes were then vortexed for 10 seconds at -80 ℃.

3.5 Purification of RNA

Total RNA purification Plus Micro Kit with product code no. 48400 from Norgen Biotek Corp was used for RNA extraction and purification according to the manufacturer's protocol.

3.6 cDNA synthesis

The iScript cDNA Synthesis Kit (BioRad) Cat#1708891 was used to generate cDNA according to the manufacturer's instructions. Nanodrop was used to determine the concentration of RNA.

3.7 qPCR

To perform quantitative PCR, The SsoAdvanced Universal SYBR Green Supermix from BioRad #L001894 B was used following the recommended protocols provided by the manufacturer. Pre-designed qPCR primers were obtained from Sigma Aldrich. The qPCR was done in duplicates.

3.8 Statistical data

Microsoft Excel was used to analyze the data from the qPCR which typically contained cycle threshold (Ct) values that were organized with each column representing a sample and each row representing the target gene and its specific Ct value. We calculated the ΔCt (delta Ct) value for each sample by subtracting the Ct value of the reference gene from the Ct value of the target gene. Next, we calculated the ΔΔCt (delta-delta Ct) value for each sample by subtracting the average ΔCt value of the control group from the ΔCt value of the experimental group. Then we calculated the fold change in gene expression known as the Relative Quantification (RQ) value for each sample by using the formula 2^-ΔΔCt. The data was further analyzed on the software GraphPad Prism 9.

3.9 Flow cytometry

X-Vivo ™ 15 media which is a serum-free media designed for hematopoietic cells was used beside the RPMI media following the same protocol of cell stimulation. Brefeldin A (1000x) which is a Golgi apparatus blocker was added taking 5 µl of it for all the wells and resting for 3 hours. Afterward, the Fc block solution was prepared by calculating first. 1 µl of Fc block was needed for 100 µl of FACS buffer and 50 µl of Fc block for each sample. The next step was surface staining for which 2 µl of CD3, 2 µl of CD19, 2 µl of BDCA2, and 0.5 µl of CD123 were taken. For 25 samples, 2 x 25=50 µl for the first three antibodies, and for the last one, 12.5 µl was taken. The cocktail in a falcon tube contained 2500-165=2335 µl of FACS buffer and the antibodies that we just calculated but we took 165 µl with 3 µl of viability dye. The cells were collected by pipetting up and down and then 300 µl of PBS+EDTA was added to the cells, pipetted up and down, and collected. Centrifugation of the tubes was done at 300 g, for 5 minutes. The supernatant was sucked out and then 50 µl of Fc block solution was added to it, pipetted up and down, and added to the flow plate. The cocktail mix of 100 µl was added to the plate and rested for 30 minutes. A buffer mix with IC fixation buffer which is used to fix cells before permeabilization and 100 µl FACS buffer were prepared. We needed 200 µl x 25 = 5 ml so we added 2500 µl of the IC fixation buffer and 2500 µl of FACS solution. After 30 minutes, we added 200 µl of FACS to wash and centrifuge the well plate at 1500 rpm, 3 mins, 4℃. We repeated the steps two more times. We added the fixation buffer to the wells, sealed the plate, and put it in the fridge overnight. The next day, we need to permeabilize the cells for intracellular staining i.e., making holes in the membrane. So, we added a permeabilization buffer 100 µl to the wells to dilute and centrifuge at 1500 rpm, for 3 minutes. Meanwhile, we prepared 12.5 µl Fc block and 1250 µl of Permeabilization buffer to permeabilize the cells for intracellular staining. The solution was discarded by inverting and centrifugation twice. The IFN-α2 was added for intracellular staining. 5 µl of antibody in 95 µl of permeabilization buffer was taken. For 25 samples, 125 µl of antibody and 2375 µl of permeabilization buffer was calculated. 50 µl of Fc block and 95 µl of IFN-α2 were added to the plate, sealed, and put in room temperature for one hour. FACS buffer was added taking 100 µl, washed, centrifuged twice at 1500 rpm for 3 minutes. 200 µl of FACS buffer was added to the wells, pipetted up and down, and with the plate sealed, it was run in FACS.

3.10 Gating strategy for flow cytometry

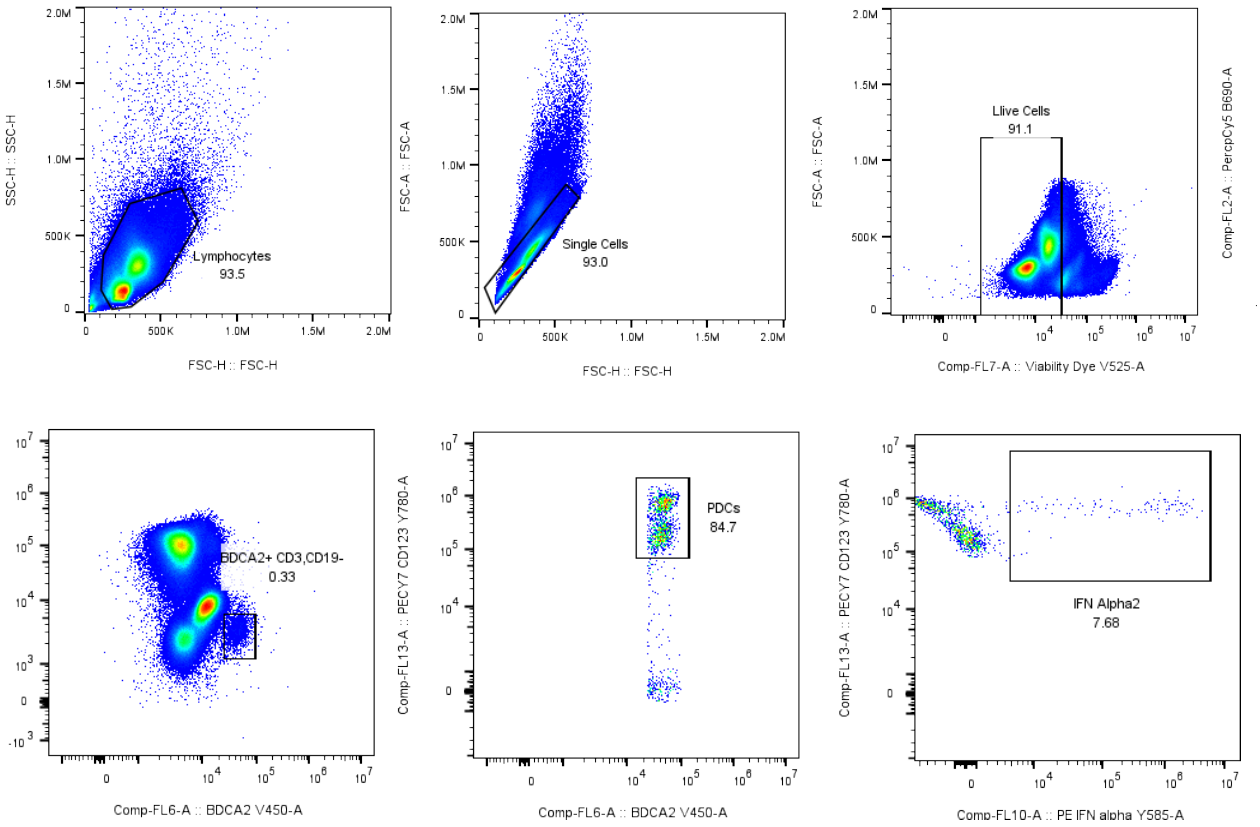

Fig 4: The gating strategy for flow analysis is to target leukocytes, followed by single cells, the viability of cells, the exclusion of T and B cells, and lastly, the pDCs. BDCA2 (Flurochrome: BV421) and CD123 (Fluorochrome: PE) pDC markers distinguish the pDC population (Dzionek et al. 2000). Finally, we can see the amount of IFN alpha 2.

The data is further analyzed in the software FlowJo and GraphPad Prism 9.

3.11 Ethical declaration

Any human blood cells used in my research were collected in accordance with ethical and regulatory standards. Necessary precautions were taken to ensure the safe handling, storage, and disposal of these cells, including adhering to biosafety regulations and guidelines.

Ethical permit number: 2019-00205/2022-02545-2.

4. Results

At first, we decided to test different concentrations of Gardiquimod which is a TLR7 agonist meaning that it activates the TLR7 receptor. In order to observe its effects on the expression of the targeted genes, we tried three different concentrations such as 0.75 µg/ml, 1.5 µg/ml, and 3 µg/ml.

4.1 Gene expression by qPCR after stimulation with Gardiquimod

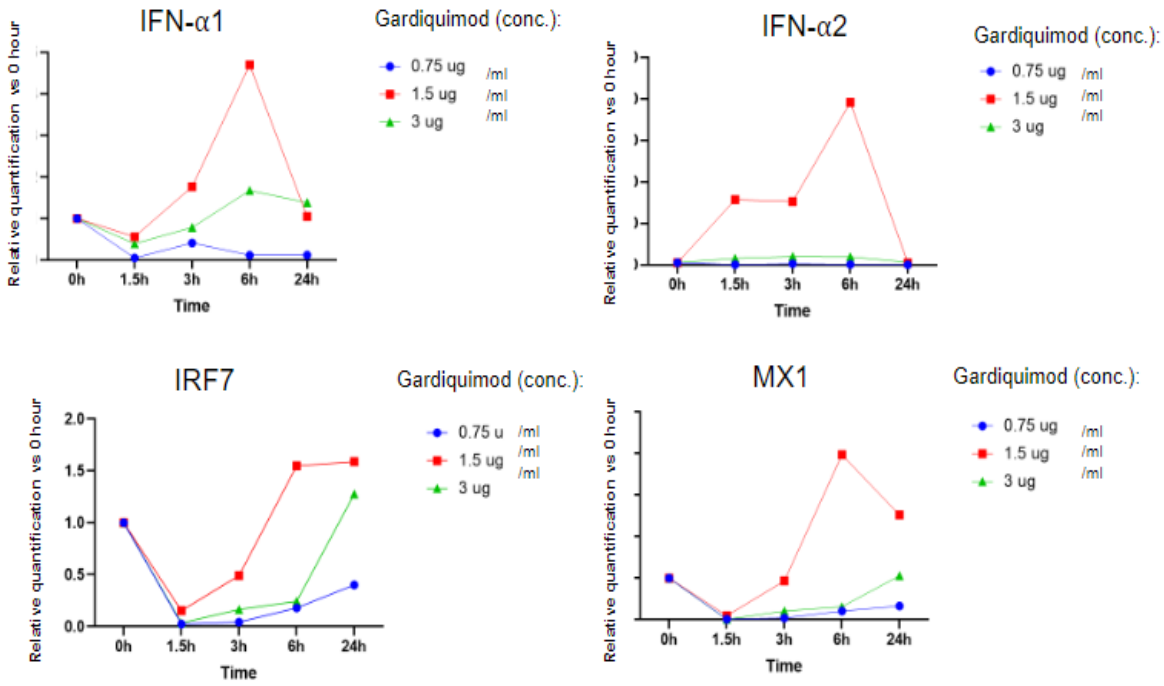

Figure 5: Different concentrations (0.75 µg/ml, 1.5 µg/ml, and 3 µg/ml) of Gardiquimod, a TLR7 agonist show different effects of gene regulation on one male individual at different time points.

From Figure 5, we saw an upregulated gene expression of the targeted genes such as *IFN-α1, IFN-α2, IRF7,* and *MX1* at the concentration of 1.5 µg/ml at 3 hours. IFN-α1 and IFN-α2 are the common type of IFN alpha that are produced as an antiviral response and IRF7 and MX1 are IFN responsive genes with IRF7 responsible as a transcription factor for IFN production. At 6 hours, there is a peak with a downregulation reaching the lowest levels at 24 hour-mark. At 0.75 µg/ml, the gene expression levels were not significant. Moreover, at concentration of 3 µg/ml, there was not significant gene expression levels either.

4.2 Gene expression by qPCR after stimulation with Gardiquimod and R1881

After testing the Gardiquimod, we proceeded further with the R1881, an AR agonist which activates the AR receptor. We added the R1881 in two different concentrations with the Gardiquimod. We are looking to see what kind of effect could result when the TLR7 receptor and the AR are activated.

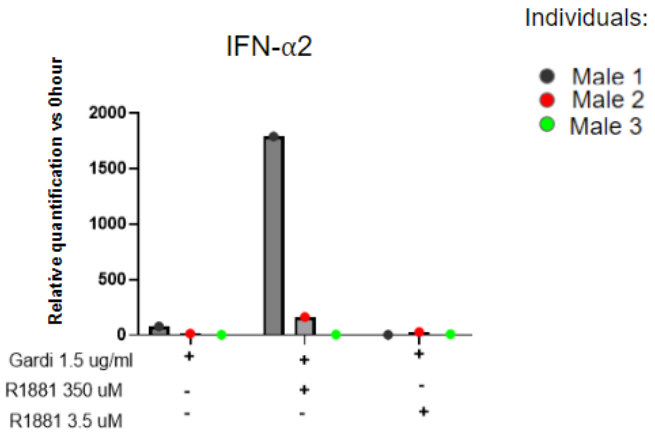

Figure 6: The IFN-α2 gene expression varied when PBMCs from three male individuals were thawed in RPMI and stimulated with Gardiquimod, a TLR7 agonist, and two concentrations of R1881 which is an AR receptor agonist (350 µM, 3.5 µM). The symbols (+) and (-) indicates the presence and absence of the stimulants.

Figure 6 indicates the differences in the gene expression levels of IFN-α2 among three male individuals. Male 1 had the highest upregulation when stimulated with Gardiquimod and the higher concentration of R1881. However, with the lower concentration of R1881 and Gardiquimod, there was a significant downregulation in the gene expression levels. In the case of Male

4.3 Gene expression by qPCR after stimulation with Gardiquimod and Testosterone Propionate

We stimulated PBMCs with Testosterone Propionate which is a synthetic androgen in two different concentrations and Gardiquimod. We expected the synthetic androgen to suppress the gene expression of IFN-α2 given the theory that androgens are responsible for having immunosuppressive effects against viral infections.

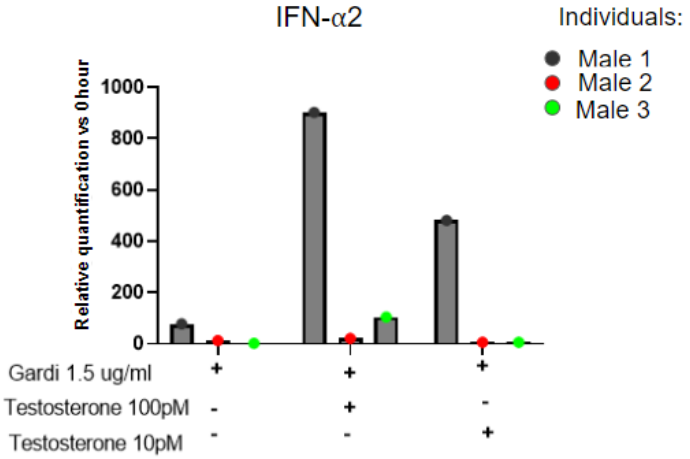

Figure 7: After thawing PBMCs from male people in RPMI media and stimulating them with Gardiquimod, a TLR7 agonist, and two doses (100 pM, 10 pM) of Testosterone Propionate, a synthetic androgen, IFN-α2 gene expression levels differed.

As we see from Figure 7 that when stimulated with Gardiquimod and Testosterone Propionate, Male 1 had the highest upregulation of the gene at both concentrations, whereas Male 3 had a minor upregulation with the higher dose. On the contrary, Male 2 did not show any effects.

4.4 Gene expression by qPCR after stimulation with Gardiquimod and G15

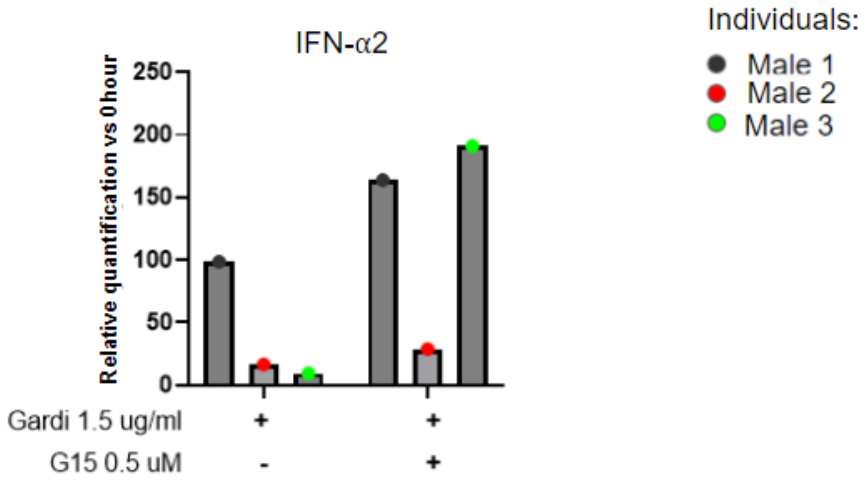

Figure 8: IFN-α2 productions varied after stimulating PBMCs from male subjects in RPMI medium with Gardiquimod, a TLR7 agonist, and G15, an antagonist for the G-protein coupled estrogen receptor. The (+) and (-) symbols indicate whether stimulants are present.

It can be seen in the above figure that all three male individuals had a higher gene expression of IFN-α2 when stimulated with the Gardiquimod and G15, an antagonist in contrast to the previous stimulants. The expectation was that G15 would block the expression of IFN alpha, but it did have any effect after stimulation, and we saw a higher expression.

4.5 Effect of R1881 on IFN-α2 protein expression by flow cytometry

After obtaining data from the qPCR, we evaluated the protein expression of IFN-α2 by flow cytometry.

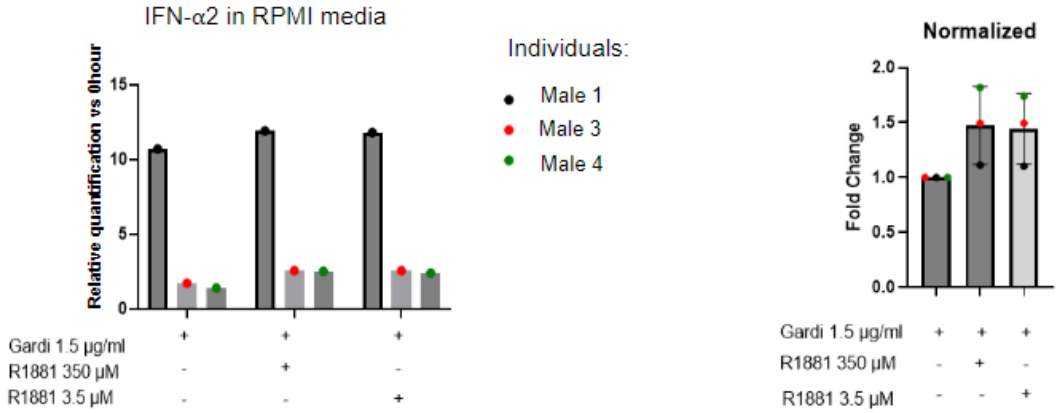

Figure 9: IFN-α2 protein expression in RPMI media analyzed by flow cytometry showed a slight if not significant upregulation in all three male individuals in the higher concentration of the AR agonist, R1881, and Gardiquimod.

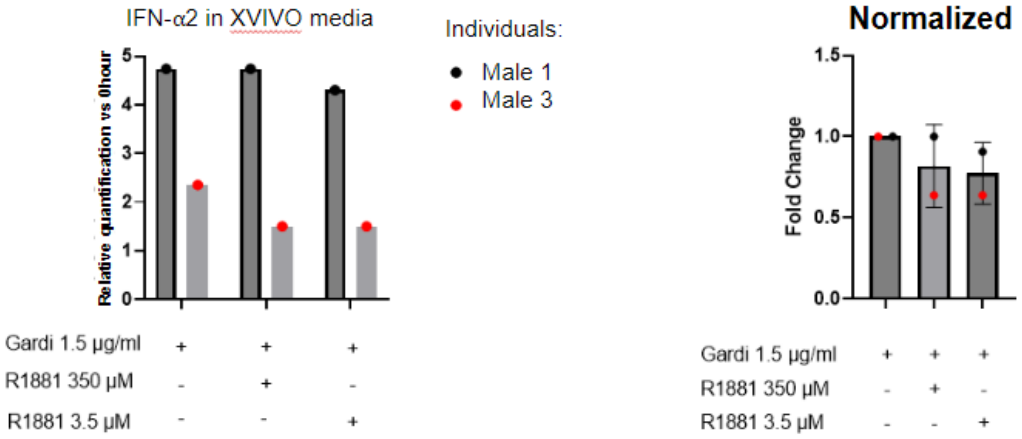

Figure 10: When stimulated in the X-VIVO ™ 15 media, there seems to be a downregulation of the IFN-α2 protein expression in two male individuals following stimulation with R1881 and Gardiquimod.

Here, we observe different effects of the stimulants in different media such as the RPMI and XVIVO ™ 15. We decided to go for serum-free media to confirm the effects of the stimulants since serum can contain hormone-binding proteins. In the XVIVO ™ 15 media, we see consistency in the downregulation of the protein expression in two male individuals that showed a bit of upregulation in the RPMI media which was interesting.

4.6 Effect of Testosterone Propionate on IFN-α2 protein expression by flow cytometry

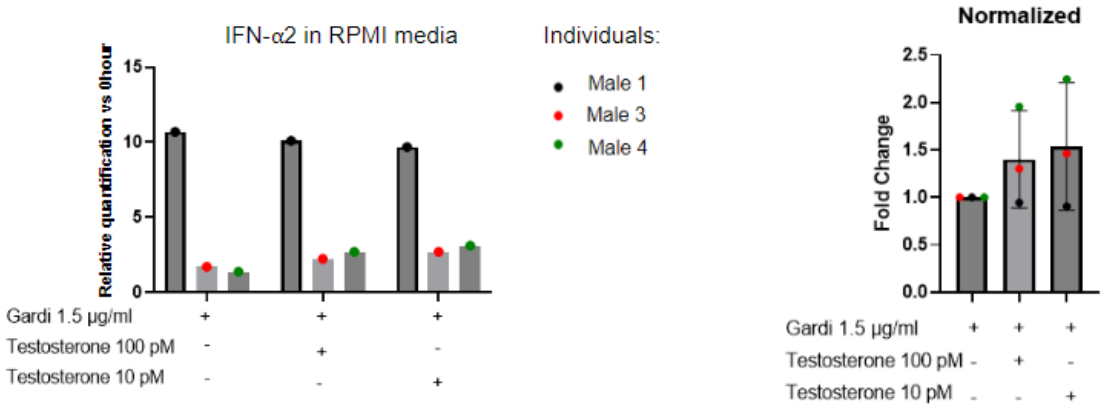

Figure 11: Flow cytometry analysis of IFN-α2 protein expression in RPMI medium revealed a slight, albeit not significant, decrease in one male individual while little upregulation can be seen in the other two males when stimulation was performed with the synthetic androgen, Testosterone Propionate, and Gardiquimod.

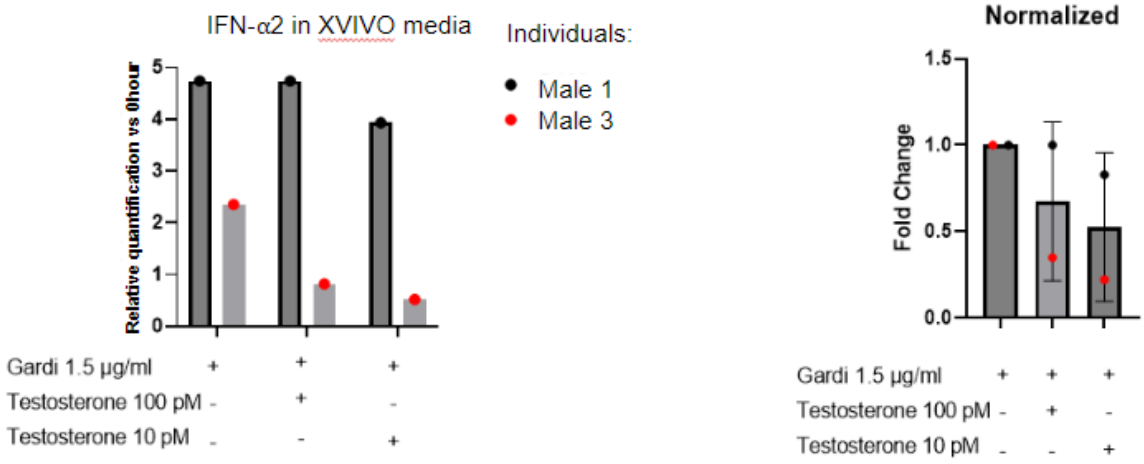

Figure 12: In two male individuals, stimulation of PBMCs with Testosterone Propionate and Gardiquimod showed downregulation in Male 1 with the lower concentration and Male 2 in both concentrations of the synthetic androgen compound.

Figures 11 and 12 show the effects of the Testosterone Propionate on both RPMI and XVIVO ™ 15 media. There was a slight upregulation of protein expression when it came to Male 3 in RPMI media while in XVIVO, it had a rather downregulation which from theory shows that androgens have suppressive effects. The difference could be due to the presence of some hormone-binding proteins in serum such as estrogen-binding proteins that could trigger the upregulation of the stimulant used in this scenario.

4.7 Effect of G15 on IFN-α2 protein expression by flow cytometry

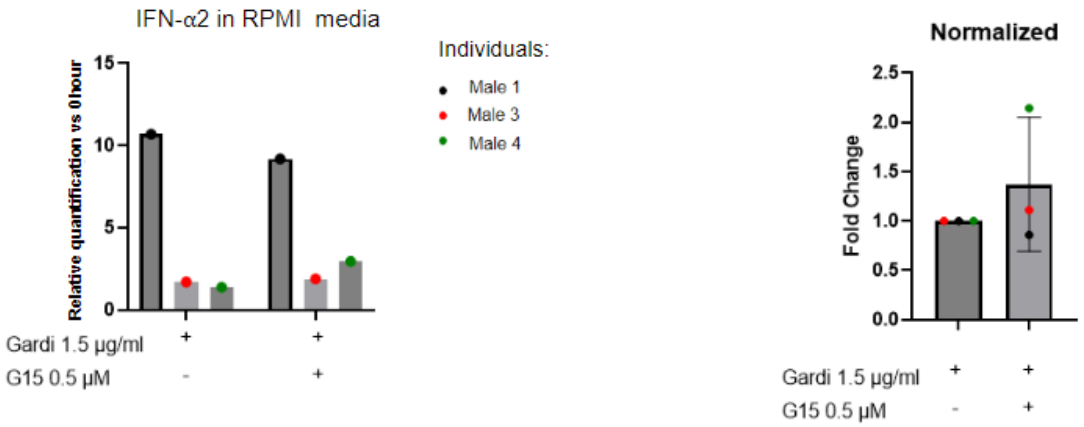

Figure 13: PBMCs of three male individuals when stimulated with G15, an antagonist for GPER along with Gardiquimod, a TLR7 agonist in RPMI media show a slight upregulation in two male individuals.

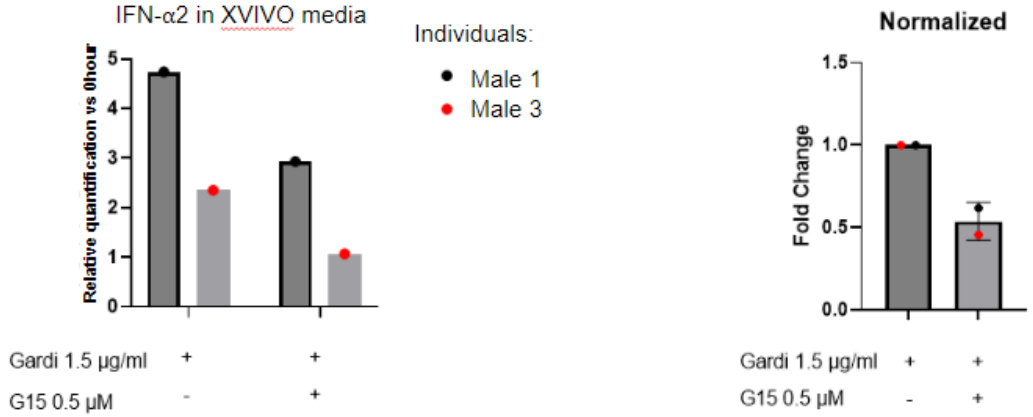

Figure 14: PBMCs after 3 hours of stimulation with Gardiquimod and G15 showed downregulation in protein expression of IFN-α2 in two male individuals.

To ensure the effect of testosterone, we tested G-15 as estrogen is known to promote IFN production. Here in Figure 13 and 14, similar results are observed with enhanced interferon levels after stimulation was done with Gardiquimod and G15 in RPMI media. In the X VIVO ™ 15 media, we could see downregulation which means that the G15 is able to block the protein expression even though it is not significant. These results are in line with previous studies done on estrogen signaling.

5. Discussion

The purpose of this project was to evaluate the production of IFN-α2 secreted by plasmacytoid dendritic cells as a means of fighting off infectious agents such as viruses. In the event of COVID-19, failure in pDC response was a critical factor for inducing the severity of the patient's conditions (Venet et al. 2023). The buffy coats that were selected for this study were based on the donors having a higher number of cell counts ranging from 10 to 20 million. The findings of this project suggest that the IFN-α2 production which is the most common cytokine out of the 13 subtypes of human IFN alpha secreted after encountering viruses varied between individuals (Yang et al. 2020; Zhang et al. 2020). Among the four individuals, Male 1 had the highest level of the targeted gene and protein expression which could be due to the presence of an early infection before donating blood since their pDCs will have already been primed. The upregulation of IFN-α2 when stimulated with R1881, an AR agonist which activates the AR, in RPMI media, contrasted with the theory that androgens possess immunosuppressive effects which was rather surprising. The effect of testosterone propionate which is a synthetic androgen diluted to the lowest concentrations for their potency showed similar results with upregulation in the targeted gene expression in Males 3 and 4. The effect of the antagonist G15 was shown to have upregulation when used with the RPMI media.

This led us to seek an alternative for RPMI media since it could be a possibility that the FBS present in the media following charcoal depletion which was done in order to remove sex hormones from the serum that could contain hormone-binding proteins such as estrogen-binding proteins might be responsible. FBS is not only variable, but it also differs from the fluid to which cells are naturally exposed (Baker 2016). Given that the stimulants are potent in nature, only a little amount of hormones in FBS can induce a response. Over the last few decades, there has been research to find alternatives to FBS with no potential outcome. One of the top suggestions for using alternatives was to use depletion or serum-free media. Animal-free media could be a suggestion, but in that case, it would still need to be chemically defined in a way that promoted certain cell types. The X-VIVO ™ 15 media which is a serum-free media and completely devoid of hormones or any other factors of undetermined composition was used to further evaluate the effects of the stimulants and how they influence the production of IFN-α2. The results gave different expression levels of IFN-α2 protein that was quite the opposite of what we obtained from RPMI media. The protein expression levels were downregulated in all the individuals. Although the differences in the downregulation were not immensely significant, it, however, points towards androgens exerting inhibitory effects. This indicates that FBS in the RPMI media could have had a role in the upregulation.

All these results were surprising based on previous observations regarding the role of estrogens and testosterone on pDCs. It is to be noted that the presence of sex hormones is not the sole factor for modulating innate or adaptive immune responses against infectious diseases. Other epidemiological factors include increased age, being male, genetic factors, and underlying medical conditions (Pijls et al. 2021). This brings us to the sample size which was a limitation in this study due to time constraints. Another limitation was a delay in collecting samples from CAIS patients which would have otherwise been taken as a control group thus the data could not be compared. The studies are still ongoing with the next step would be to perform the same experiments on the CAIS patients repeatedly so the data could be compared. So, there could be more clear insights into the androgen receptor and its role in the innate immune system.

6. Conclusion

The data obtained from this project concludes that androgens can play a major role in the innate immune system. The theory that this hormone has immunosuppressive effects on the innate immune system through impaired pDC activation and differentiation leading to a reduced amount of IFN-α2 generations and that being male is a demographic factor against viral diseases can be realized from the final sets of experiments that were done in the X-VIVO ™ media. Since autoimmune diseases prevail in women, emphasis has been given to studying the role of estrogens. With the research still ongoing and after the limitations are overcome, we can expect that this could lead to an in-depth understanding of how the androgen receptor signaling works and contributes to disease severity. While there are androgen deprivation therapies that are being used to treat prostate cancers, further research in this area can lead to the development of potential therapeutic strategies such as anti-viral therapies to treat male patients to fight off infectious diseases and provide optimal disease management for both genders.

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Acknowledgments

First and foremost, I would like to express my heartfelt gratitude to Mattias Svensson, my thesis supervisor, for giving me the opportunity to work at his laboratory and for his insightful feedback, and constructive criticism during the whole research process. I am truly grateful for it.

I want to extend my deepest appreciation to Negar Ayoubzadeh, my lab supervisor for her invaluable guidance, expertise, and consistent motivation which have played an important role in defining the direction and quality of my work.

I would like to address the contributions of my friends and classmates, who provided me with valuable insights, worthwhile discussions, and moral support during moments of doubt. Their friendship and camaraderie have added to the fulfillment and enjoyment of this academic quest.

Lastly, I would like to offer my heartfelt gratitude and appreciation to everyone else who has helped me along the way to completing my master's thesis. This accomplishment would not have been achieved without their direction, encouragement, and constant support.

Thank you all for being a part of this important milestone in my life.

Popular Science Summary

Do androgens put men at higher risk of infection against viral diseases?

Our bodies are complex machines consisting of countless interconnected processes, and the immune system is our body's natural defense mechanism that protects us from foreign invaders such as bacteria, viruses, and fungi. Sex hormones are one of the key soldiers to building that defense. These hormones do so through secreting different pro-inflammatory cytokines that relay signals that help our body's immune system to fight off invaders. Because of their unique sex hormones such as androgens and estrogens, men and women may fall ill in slightly different ways. Men may be more likely to get sick from viruses, whereas women may be more susceptible to autoimmune illnesses. It's as though each gender has its very own kryptonite. For a long time, scientists have been researching how the female sex hormone, estrogen impacts our bodies, but they have not done studies on the male sex hormone, androgen as thoroughly. Based on this fact, I have done my project to discover more about how androgens aid our immune systems to combat viral diseases.

At first, we collected blood samples from male donors and isolated peripheral blood mononuclear cells (PBMCs) which are made up of different types of cells such as monocytes, lymphocytes, natural killer cells, and most importantly, dendritic cells which are the first responders to viral pathogens. We begin stimulating the PBMCs with different concentrations of TLR7 agonists which are substances that bind to and activate certain receptors in the body which in this case is the TLR7 receptor and begin the production of interferons such as IFN-α1 and IFN-α2. They can disrupt viral protein synthesis and viral replication, limit viral assembly, and promote the destruction of infected cells. Interferons also increase the activity of natural killer cells and cytotoxic T cells, both of which are required for eliminating virus-infected cells. We then analyzed the data by molecular techniques such as qPCR and flow cytometry. Now, what we expected to see is that there would not be a tremendous amount of IFN production since different studies supported androgens having suppressive effects on the immune system. However, in some cases, there seemed to be the opposite of our expectations. We believe that it had something to do with the media that we used earlier that could have possibly contained some sort of hormonal residues to have triggered a higher production of the desired cytokine.

To ensure effective research methods, it is essential for all researchers to adopt innovative approaches. Individuals with complete androgen insensitivity syndrome (CAIS) are significant in this perspective. CAIS patients have this disorder because of mutations in the androgen receptor on the X chromosome otherwise known as X-linked recessive disorder, which results in a complete lack of androgens. Given that testosterone has no effects on these patients, they provide an appropriate basis for comparison with healthy individuals in order to investigate the differences in interferon production. After comparison, it would be possible to conclude the importance of androgen signaling in fighting off viral infections. Understanding androgen receptor signaling is necessary in order to develop therapies in the future.