Bachelor Degree Project

UNIVERSITY OF SKÖVDE

# SEXUAL DIMORPHIC ANTIVIRAL RESPONSES OF PLASMACYTOID DENDRITIC CELLS: ROLE OF TLR7 AND TYPE 1 INTERFERON SIGNALLING

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# Abstract

Sexual dimorphism is shown to affect the immune response, with females presenting an increased immune reactivity in comparison to males. The difference in immune response is impacted by an intricate interplay of sex chromosomes and sex hormones. Plasmacytoid dendritic cells (pDCs), recognised for their vital role in antiviral immunity, are influenced by these factors. However, the exact mechanism beneath this influence is still unknown. This study explores the sexual dimorphic antiviral responses of pDCs, specifically within the role of Toll-Like Receptor 7 and type 1 interferon signalling. Peripheral blood mononuclear cells (PBMCs) and pDCs were isolated from the blood of healthy human donors obtained from Sahlgrenska University Hospital and stimulated with gardiquimod (a Toll-like receptor 7 agonist) and lipopolysaccharide (a Toll-like receptor 4 agonist). Quantitative polymerase chain reaction (qPCR) and fluorescence activated cell sorting (FACS) were used to analyse the gene expression and protein levels. The findings in this study illustrate sex-specific differences in the immune-related genes. Gene expression analysis displayed higher *IFNα2* levels in male pDCs than females. Conversely, female pDCs exhibited increased IFNα2 protein levels than males. *IRF7* and *TLR7* expression levels were increased for female pDCs but higher levels of *TNF* expression were displayed for male pDCs. These findings allow us to understand the complex interaction between sex hormones, sex chromosomes and the immune response, specifically in the antiviral functions of pDCs. This also provides insight into the possible sex-specific differences in disease susceptibilities and therapeutic approaches.

# Popular Scientific Summary

This study dives into the captivating field of discovering how differences between males and females impact the immune response. Past research illustrated notable differences in how the immune system responds in females and males. For example, males are more susceptible to infections (viral, bacterial or parasitic), whereas females are more prone to autoimmune diseases, in which the immune system mistakenly attacks the own body. Such differences suggest that females have a higher immune response to threats that are external as well as internal.

One element involved in causing these differences is sex chromosomes (structures in our cells that carry genetic information). X chromosomes contain many important genes (instructions in our body causing certain functions) relating to the immune system. Females carry two X chromosomes, meanwhile, males carry one. In females, one of the two X chromosomes gets inactivated randomly. This is so that similar to males, only one of the X chromosomes will be active in females. However, sometimes the inactivation is only partial leading to changes in the immune response.

Another factor that can play a role in the differences in the immune response is sex hormones. Females usually have a higher level of the sex hormone oestrogen, while males have a higher level of testosterone. Previous research shows that testosterone can suppress the immune response. On the other hand, oestrogen has more of a complex role since it can both inhibit and stimulate the immune response depending on the cell environment.

This research focuses on a particular immune cell called plasmacytoid dendritic cell (pDC), which produces elevated levels of a virus-fighting component called interferon alpha 2 (IFNα2). In this study, it was found that although male pDCs produced higher levels of the instructions for making *IFNα2*, female pDCs produced higher levels of the actual IFNα2 protein. These differences could be because of the different factors such as sex hormones and chromosomes playing a role in the production of IFNα2 by pDCs.

Within the pathway of IFNα2 production, other genes are involved to help make it. These genes, for example, *TLR7* and *IRF7*, were shown to be higher in female pDCs than males. This could mean that female's immune responses are faster than males, which may be why they get less infections. However, how the sex hormones or chromosomes influence these immune responses is not known exactly. It is also important to mention that not all results in this study, such as the difference in *TLR7* between the sexes showed a meaningful difference. Since a small sample size was used in this experiment, some results could be due to chance.

Altogether, this study offers insight into the complex relationship between sex chromosomes, sex hormones and the immune response. Understanding this is important as it can help figure out why more males get infections and females get autoimmune diseases. It can also bring about new treatments for diseases and shed light on creating personalised therapies that are tailored to each patient's needs.

# Abbreviation list

| | |
|---|---|
| AR | Androgen Receptor |
| ARE | Androgen Response Element |
| cDNA | Complementary Deoxyribonucleic Acid |
| DHT | Dihydrotestosterone |
| ER | Oestrogen Receptor |
| ERE | Oestrogen Response Element |
| FACS | Fluorescence Activated Cell Sorting |
| IFNa2 | Interferon alpha 2 |
| IFN-I | Type 1 Interferon |
| IL1B | Interleukin 1B |
| IL6 | Interleukin 6 |
| IRF7 | Interferon Regulatory Factor 7 |
| ISG | Interferon Stimulated Gene |
| mRNA | Messenger Ribonucleic Acid |
| Mx1 | Myxovirus Resistance 1 |
| PBMC | Peripheral Blood Mononuclear Cell |
| pDC | Plasmacytoid Dendritic Cell |
| qPCR | Quantitative Polymerase Chain Reaction |
| TLR7 | Toll-Like Receptor 7 |
| TLR4 | Toll-Like Receptor 4 |
| TNF | Tumour Necrosis Factor |

# Table of Contents

# Introduction

The phenomenon of sexual dimorphism insinuates a distinction amongst biological sexes, transcending beyond their reproductive features. Sexual dimorphism is greatly presented within humans regarding the immune response, with males showcasing a higher rate of infections than females for a wide range of parasitic, bacterial and viral pathogens (Shepherd et al., 2021). Whereas a predilection for autoimmune diseases is exhibited in females than males, with an estimation of 80% autoimmune diseases occurring in females (Marquez 2020). These observations display females possessing enhanced immune responses to both internal and foreign molecules, hence the higher immunity in females than males (Shepherd et al., 2021).

One factor contributing to this difference is the influence of sex hormones on the immune system (Taneja, 2018). Generally, oestrogen is thought to stimulate the immune response, which can contribute to the progression of autoimmune diseases (Fan et al., 2014). Nevertheless, the function of oestrogen is complex as it is immune stimulating in some diseases but immune inhibitory in others. For example, in systemic lupus erythematosus (SLE), during pregnancy, the disease symptoms tend to worsen. Whereas in other autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA), the disease symptoms improve (Da Silva & Spector, 1992; Confavreux et al., 1998).

Oestrogen receptor (ER) has two main isoforms expressed extensively throughout immune cells: the alpha receptor (ER-α) and the beta receptor (ER-β). ER binding to a ligand contributes to its binding to the oestrogen response elements (EREs) in the gene promoter region. Therefore, it can act as a transcription factor to inhibit or promote transcription (Laffont et al., 2014). Moreover, ER bound to a ligand may also cooperate with additional transcription factors, co-repressors and co-regulators to obliquely impact downstream transcription. This relates to many immune pathways as numerous genes encoding for cell surface immune markers, cytokines and chemokines involve regulation by oestrogen signalling (Shepherd et al., 2021). For instance, oestrogen influences the Toll-Like receptor (TLR) signalling pathway, which is involved in modulating antiviral immune functions (Laffont et al., 2014).

On the other hand, androgen and testosterone display an immunosuppressive effect (Trigunaite et al., 2015). Testosterone mainly exerts its effects through androgen receptor (AR) signalling, while a more potent agonist of this receptor is its derivative, dihydrotestosterone (DHT). The AR gene on the X chromosome encodes AR, which is expressed in various immune cells. When unbound, heat shock proteins (HSPs) and chaperone proteins bind to the AR in the cytoplasm. However, when AR binds to an androgen ligand e.g. testosterone or DHT, the AR translocates to the nucleus. This allows the binding of AR to androgen response elements (AREs) to regulate the target gene expression with aid from co-repressors and co-activators (Shepherd et al., 2021).

Previous research indicates that androgens suppress inflammatory immune cells such as dendritic cells as well as suppress the immune response to infections and vaccination (Trigunaite et al., 2015). Additionally, testosterone displays immunosuppressive effects too, so it leads to weakened immune responses to infections and vaccinations in males (Kanda et al., 1996). Autoimmune diseases such as SLE or RA worsening in males are suggested to occur due to low testosterone levels combined with

abnormal oestrogen levels (Kanda et al., 1996). Therefore, highlighting the impact sex hormones have on the immune response.

Another factor contributing to sex bias in immune response and female preponderance of autoimmune disorders is gene diversity and dosage. Generally, males carry one X chromosome whereas females carry two X chromosomes, in which one is arbitrarily transcriptionally inactivated. A variety of genes on the X chromosome are linked to immune function regulations. For instance, genes encoding immune response regulations such as *TLR7* are located on the X chromosome (Taneja, 2018). Past research implied that a female's immune system has increased responsiveness due to the biallelic X chromosome-related immune genes that escape X chromosome inactivation (XCI). Double gene dosage can occur since the genes that escape XCI, can be expressed from both the active and inactive X chromosome. Approximately 15% X-chromosome related genes are expressed biallelically in females (Laffont et al., 2014).

Moreover, although oestrogen has been reported to influence autoimmune diseases such as SLE which occur more frequently in females, X chromosome-related gene dosage could also have an impact on the disease pathogenesis (Laffont et al., 2014). Males with Klinefelter syndrome with an XXY karyotype are reported to have a similar risk as females to developing SLE. However, females with Turner syndrome, possessing only one X chromosome, are less frequently reported to have SLE (Laskowski et al., 2019). This illustrates the influence of sex chromosomes on immunity.

The impact of sexual dimorphism can be further explored by examining the innate immune system, which serves as the initial defence mechanism against viral infections. This mechanism swiftly identifies pathogen-associated molecular patterns (PAMPs), such as viral nucleic acids, through pattern recognition receptors (PRRs) such as TLR7 (Meier et al., 2007). This leads to an antiviral response through the production of type 1 interferon (IFN-I), IFN-III and pro-inflammatory cytokines. Also, it contributes to the activation of IFN-stimulated genes (ISGs) such as myxovirus resistance 1 (*Mx1*) to prevent viral replication and interferon regulatory factors 7 (*IRF7*) to regulate *IFNα2* expression (Horisberger et al., 1983; Venet et al., 2023).

A key player in the defence mechanism against viral infections is plasmacytoid dendritic cells (pDCs), known for their exceptional capabilities to swiftly produce high levels of IFNα2, a form of IFN-I, despite comprising only 0.1-0.5% of human peripheral blood mononuclear cells (PBMCs; Hagen et al., 2020; Ye et al., 2020). pDCs recognise pathogens via nucleic acid-detecting TLR7 and TLR9, which sense single-stranded RNA and unmethylated CpG motif involving DNA, correspondingly (Webster et al., 2018). This detection allows the expression of *IRF7* to occur via signalling pathways such as the adaptor myeloid differentiation primary response protein 88 (MYD88; Honda et al., 2005). Therefore, promoting the transcription of *IFNα2* as well as ISGs such as *IRF7* (Arroyo Hornero & Idoyaga, 2023).

However, although most pDCs showcase high *IRF7* levels, most do not secrete IFN-I after CpG or viral stimulation. These have been verified for both murine and human pDCs at single-cell level. Additionally, it is shown within individually stimulated human pDCs that only a subpopulation secretes IFN-I, implying that within the pDC response, functional heterogeneity may occur. Therefore, indicating that certain pDCs can have functions other than IFN-I secretion, such as antigen presentation to CD4+ T cells, which can also contribute to the antiviral functions of pDCs (Ye et al., 2020; Arroyo Hornero & Idoyaga, 2023).

Interestingly, the production of IFNα2 through pDCs show sex bias in humans (Hagen et al., 2020). In autoimmune diseases such as SLE, female pDCs produce higher levels of IFNα than males. Furthermore, in infectious diseases such as human immunodeficiency virus (HIV), female pDCs produced higher levels of IFN-I than males (Meier et al., 2009). Synergetic effects of oestrogen combined with the X chromosome complement in females can cause the differences perceived in the immune response (Laffont et al., 2014; Ye et al., 2020). This illustrates the influence sex hormones and sex chromosomes have on the sexual dimorphism observed in immunity and within the antiviral functions of pDCs.

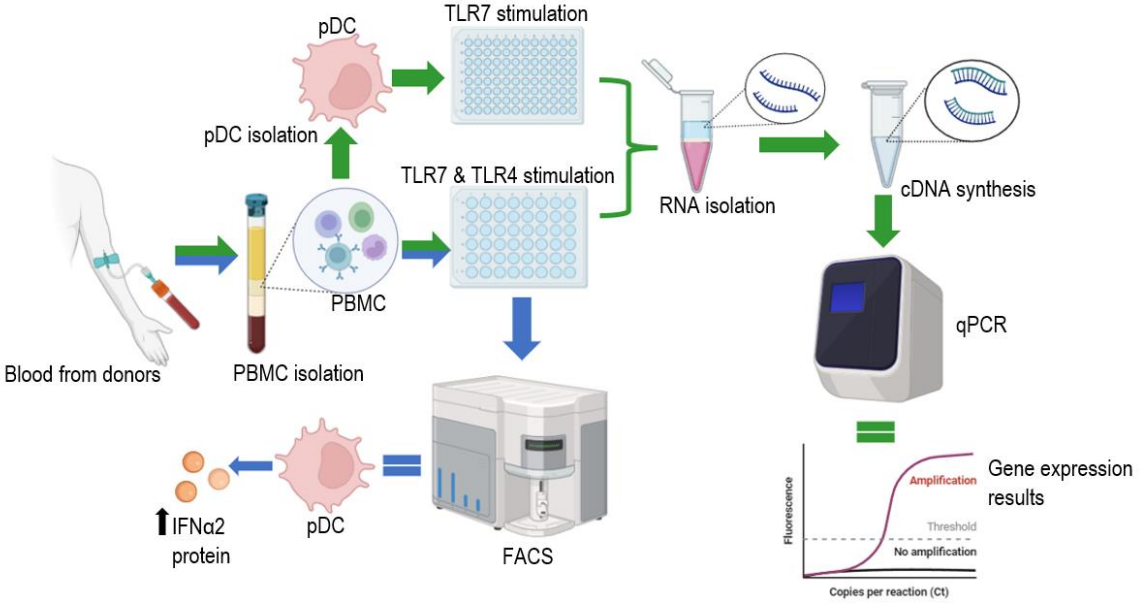
Since the mechanism by which sex hormones or chromosomes influence the role of pDCs remains largely unknown, this study aimed to determine the impact such factors have on the antiviral functions of pDCs, specifically focusing on TLR7 and type 1 interferon signalling. PBMCs and pDCs were isolated from the blood of healthy human donors. The isolated cells were stimulated with gardiquimod and lipopolysaccharide. RNA was isolated, followed by cDNA synthesis to perform qPCR for gene expression analysis. Additionally, FACS was performed post-stimulation to assess protein expression. This experiment aims to contribute to a greater understanding of sexual dimorphism in immunity and potentially aid in the development of personalised therapies for individuals.

# Material and Methods

## Project workflow

To initiate the experimental procedures, PBMCs and pDCs were isolated from healthy blood donors, serving as the foundational step in the project workflow as depicted in Figure 1. Subsequent to isolation, the cells were stimulated with a TLR7 and TLR4 agonists. This enabled the assessment of gene and protein expression through quantitative polymerase chain reaction (qPCR) and fluorescence activated cell sorting (FACS), respectively.

Figure 1. Overview of the project workflow. Blue arrows indicate the use of FACS on stimulated PBMCs, resulting




in pDCs as the main IFNα2 producing cell. The green arrows indicate the use of qPCR on stimulated PBMCs and pDCs, resulting in the gene expression of the immune-related genes involved in the pDC functions. Figure created in BioRender.

## PBMC isolation from blood

Whole blood samples were obtained from two healthy human volunteers at Sahlgrenska University Hospital. All participants gave informed consent. The blood was collected in heparin tubes, inverted three times and kept at room temperature for 30 minutes. The blood was diluted in a 1:1 ratio with recommended media containing phosphate-buffered saline (PBS; Corning) supplemented with 2% charcoal-stripped fetal bovine serum (FBS; Cytiva). First Ficoll (Cytiva) was added to SepMate tubes, then the diluted blood to ensure a total volume of 22 ml in each tube. The tubes were centrifuged at 1200 xg for 10 minutes at room temperature (Eppendorf Centrifuge 5810R). The separated top layer containing the plasma and mononuclear cells was collected into new tubes, filled up to 50 ml with recommended media and centrifuged at 300 xg for 8 minutes at room temperature. The supernatant was discarded and the pellet was resuspended with recommended media. A haematology analyser (Sysmex) was used for cell counting.

## PBMC thawing

Previously collected PBMCs from 12 healthy volunteers in buffy coats were obtained from the blood bank at Sahlgrenska University Hospital. The PBMCs were already isolated from the buffy coat samples and cryopreserved at -150°C. The samples were thawed using a thawing media containing 0.2 µl of benzonase endonuclease (Sigma-Aldrich) combined with complete media involving RPMI 1640 medium (Gibco) supplemented with 10% charcoal-stripped FBS, 1% penicillin and streptomycin (pen-strep; Gibco) and 1% 2-β-mercaptoethanol (Gibco). Centrifugation was done at room temperature for 10 minutes at 300 g with acceleration of 7 and deceleration of 9. The supernatant was discarded and the pellet was resuspended with thawing media and centrifuged again. Before the final centrifugation, only the pellet was resuspended in complete media and then counted using a cell counter. 500 µl of the cell suspension was transferred to a 48-well plate and rested for 2 hours in the incubator maintained at 37°C and 5% $CO_2$. Approximately 10% of the total PBMC cell population was used for stimulating PBMCs whilst the rest were used for pDC isolation. For example, if the total number of PBMCs was 100 million cells, 10 million were used for PBMC stimulation and 90 million for pDC isolation. Approximately 800,000 cells were used per well for PBMC stimulation. It is important to note that the number of cells could vary from sample to sample as the initial PBMC cell count was different for each sample.

## pDC isolation

pDC isolation was done using the EasySep Human Plasmacytoid DC Isolation Kit (#17977, Stemcell technologies). Cell suspension combined with trypan blue (Invitrogen) was used for cell counting (Thermo Fisher Scientific Countess 3). 250 µl of cell suspension was transferred to a 96-well plate and rested in the incubator for 30 minutes (37°C and 5% $CO_2$). As previously mentioned, the number of cells per well was dependent on the initial number of PBMCs, which varied from sample to sample. The total cell population of pDCs were equally divided to ensure that there were 20,000 to 75,000 cells per well.

## PBMC and pDC stimulation

Once the resting period was done, PBMCs were stimulated with 5 µl of a final concentration of 1.5 µg/ml of gardiquimod (Invivogen) and 5 µl of a final concentration of 10 ng/ml of lipopolysaccharide

(LPS; Sigma-Aldrich) which were added to the wells with time points: 1.5, 3 and 6 hours in the incubator (for each stimulant). pDCs were stimulated with 2.5 µl of a final concentration of 1.5 µg/ml of gardiquimod at the stimulation time points: 1.5 and 3 hours in the incubator. All incubation was kept at 37°C and 5% $CO_2$. A control of 0 hours was also included without stimulation for both PBMCs and pDCs. Gardiquimod, a TLR7 agonist was chosen for its capability to trigger the immune response through TLR7, a receptor predominantly encoded on the X chromosome. LPS was also selected as it is a TLR4 agonist and it is not expressed on sex chromosomes, hence serving as a control to diminish any possible sex bias in the PBMCs response.

Once the stimulation period was completed, both PBMCs and pDCs were transferred to 1.5 ml Eppendorf tubes. 750 µl TRIzol LS (#10296028, Invitrogen) reagent was added to the pDC cell suspension. PBMCs were centrifuged for 10 minutes at room temperature at 300 g (Fisher Scientific accuSpin Micro 17R). The supernatant was discarded, and the pellet was resuspended in 500 µl TRIzol reagent (#15596018, Invitrogen). The addition of TRIzol and TRIzol LS reagent was to instantly halt the ribonuclease activity and preserve RNA integrity. For pDCs, TRIzol LS was used instead of TRIzol due to a lower number of cell population obtained compared to PBMCs. Also, TRIzol is used with cell pellets whereas TRIzol LS can be added directly to cell suspension, which is beneficial for smaller cell populations. Both PBMCs and pDCs were stored at -22°C.

## RNA isolation

For PBMCs, RNA isolation was done using 500µl of TRIzol reagent (#15596018, Invitrogen) and 100 µl of chloroform (Fisher Bioreagents). The samples were mixed for 30 seconds and then placed on ice for 15 minutes. Centrifugation was done at 12,000 xg at 4 °C for 15 minutes and the aqueous phase was moved into new tubes. 300 µl of ice-cold isopropanol (Fisher Bioreagents) was added to the aqueous phase. Samples were incubated for 20 minutes on ice, then centrifugation was performed at 17,000 xg at 4 °C for 40 minutes. The supernatant was discarded and the pellet was washed in 1 mL of 75% ethanol (Fisher Bioreagents). Samples were centrifuged again but for 10 minutes at 17,000 xg at 4°C. Ethanol was discarded and samples were air dried for 20-30 minutes. Finally, RNA was eluted in 15µl of RNase-free water. Samples were stored at -80°C.

For pDCs, RNA isolation protocol using TRIzol LS reagent was followed as per the manufacturer's description (#10296028, Invitrogen). RNA clean-up was also performed using the "RNA clean-up and concentration kit" (#43200, Norgen Biotek Corp.) as described by the manufacturer. Samples were stored at -80°C.

The purity and concentration of RNA were established by absorbance at 260 nm (A260) and 280 nm (A280), and A260 and 230 nm (A230) using NanoDrop one Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific).

## Complementary DNA (cDNA) synthesis

RNA was reverse transcribed into cDNA on a T100 thermal cycler (Bio-Rad) using the iScript cDNA synthesis kit (#1708891, Bio-Rad) according to the manufacturer's protocol. cDNA equivalent to 400 ng of RNA from PBMCs and variable RNA amounts from pDCs was used for each qPCR reaction. pDCs had a smaller cell population which resulted in variable RNA amounts, but it was ensured that no more than 400 ng of RNA was utilised.

## qPCR analysis

SsoAdvanced Universal SYBR Green Supermix (#1725274, Bio-Rad) following the supplier's instructions. The following pre-designed primers (Sigma-Aldrich) were used: GAPDH, IFNα2, Mx1, TLR7, IRF7, TNF, IL1B and IL6. qPCR was conducted in duplicates on QuantStudio 6 pro Real-Time PCR system (Thermo Fisher Scientific). The expression levels of the reference gene GAPDH were used for normalising the gene expression data. $2^{-\Delta\Delta Ct}$ method was utilised to calculate the relative fold change of the gene expression levels.

## Flow cytometric analysis

The same process as PBMC thawing and stimulation (only with gardiquimod) was done as mentioned above, with the addition of 5µl of 5 mg/ml Brefeldin A (diluted 1000-fold; BioLegend) after 3 hours to halt vesicle creation and transport between the Golgi apparatus and endoplasmic reticulum.

FACS analysis was conducted on CytoFlex S instrument and CytExpert software (Beckman coulter). Fc block (BD Biosciences) diluted in a ratio of 1:100 with FACS buffer containing PBS, 2% FBS and 1mM EDTA (Invitrogen) was added to the cells. After centrifugation for 2 minutes at 1500 RPM at 4°C, the supernatant was discarded, and cells were resuspended in FACS buffer. The washing step was repeated twice with FACS buffer. Surface staining performed for 30 minutes on ice included the following anti-human antibodies: CD19-APC (BioLegend), CD3-Percpcy5.5 (BioLegend), CD8A-AF700 (BioLegend), CD14-BV786(BD BioSciences), CD16-APCH7 (BD BioSciences), CD56-FITC (BioLegend), CD123-PECY7 (BioLegend), BDCA2-BV421 (BD BioSciences) as well as the viability dye eFluor 506 (Invitrogen). The samples were washed twice again. Intracellular Fixation buffer (Invitrogen) was added to the cells and stored overnight at 4°C in the fridge. The following day, centrifugation was done at 1500 RPM for 2 minutes at 4°C to discard the supernatant and resuspended cells in permeabilisation buffer (Invitrogen) diluted from 10x stock in di$H_2O$. Cells were washed twice with permeabilisation buffer. Fc block diluted 1:100 with permeabilization buffer was added to the cells along with IFNα2-PE mouse anti-human (BD Pharmingen) and kept at room temperature for 1 hour in the dark to allow intracellular staining to occur. FlowJo software version 10.10.0 (Treestar) was used to analyse all data.

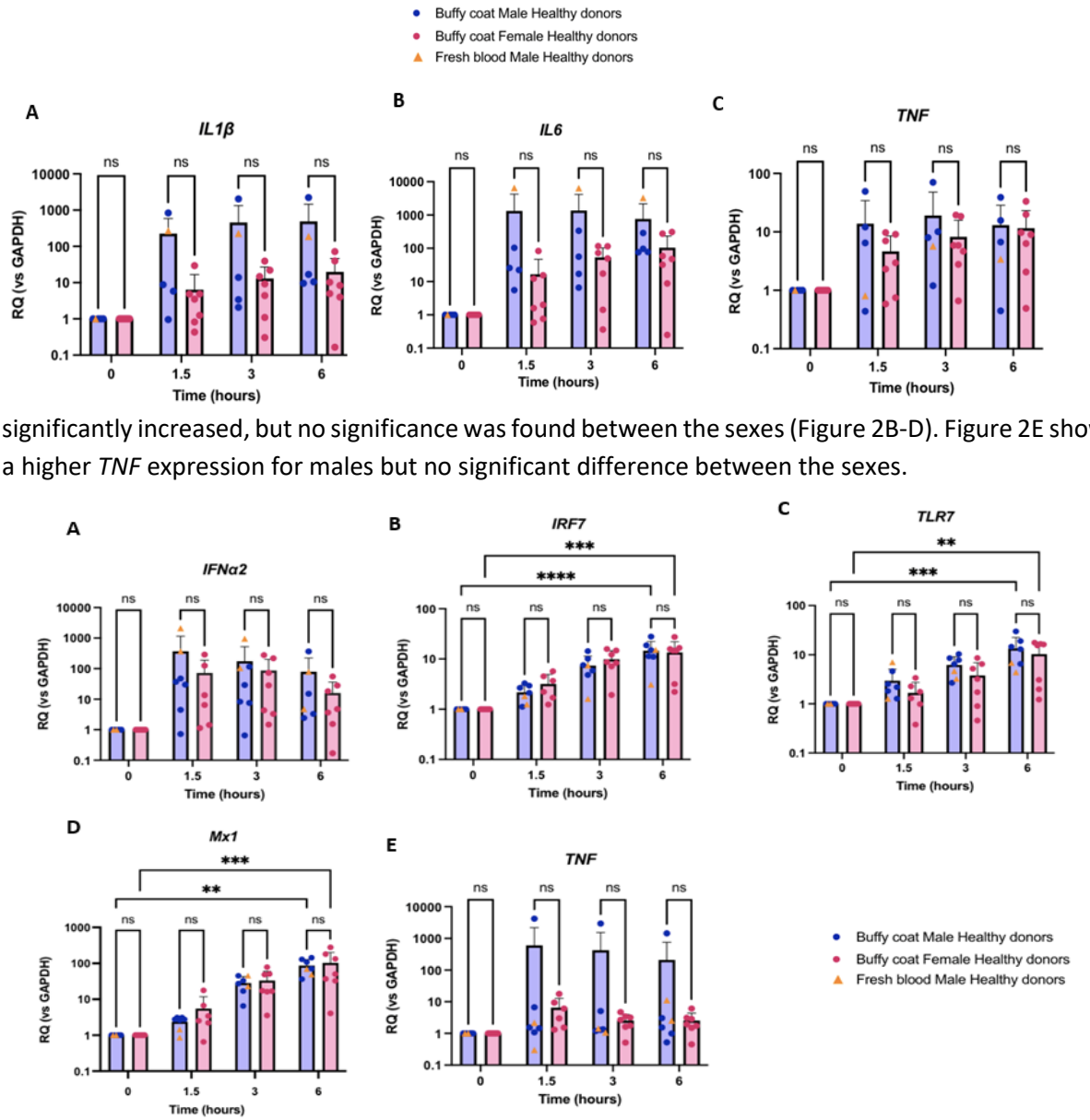
## Statistical analysis

GraphPad Prism version 9.5.1 was used to perform all the statistical analysis. Two-way analysis of variance (ANOVA) was used to compare the expression between males and females as well as comparing the different stimulation time points within the same sex. A comparison was deemed significant if the p-value was less than 0.05.

# Results

## TLR7-stimulated PBMC

Female and male PBMCs were stimulated by gardiquimod (TLR7 agonist; Figure 2). A trend was observed when comparing the PBMCs of males and females for *IFNα2*, in which as the stimulation time increased, the level of *IFNα2* declined but the expression of *IFNα2* was overall higher in males than females, although no significant difference was observed (Figure 2A). *IRF7*, *TLR7* and *Mx1* expression show that as the stimulation hours increased from 0 to 6 hours, the expression of the gene also




significantly increased, but no significance was found between the sexes (Figure 2B-D). Figure 2E shows a higher *TNF* expression for males but no significant difference between the sexes.

Figure 2. Female (n= 6-7 buffy coats) and male (n= 7, 2 fresh blood, 5 buffy coats) PBMC stimulated with gardiquimod. The data is shown as the relative quantification (RQ; relative to GAPDH) in Log10 against the time points 0 (control), 1.5, 3 and 6 hours after stimulation. The gene expressions are displayed for **(A)** *IFNα2* **(B)** *IRF7* **(C)** *TLR7* **(D)** *Mx1* **(E)** *TNF*. Data represents mean ± SD. P-values were obtained using 2-way ANOVA, **p <0.01, ***p < 0.001, ****p < 0.0001, ns, not significant.

## TLR4-stimulated PBMC

Female and male PBMCs were stimulated by LPS (TLR4 agonist; Figure 3). The gene expression of *IL1B*, *IL6* and *TNF* was higher for males than females, though no significant difference was found between the sexes (Figure 3A-C).

Figure 3. Female (n= 7 buffy coats) and male (n= 5, 1 fresh blood, 4 buffy coats) PBMC stimulated with LPS. The data is shown as the relative quantification (RQ; relative to GAPDH) in Log10 against the time points 0 (control), 1.5, 3 and 6 hours after stimulation. The gene expressions are displayed for **(A)** *IL1B* **(B)** *IL6* **(C)** *TNF*. Data represents mean ± SD. P-values were obtained using 2-way ANOVA, ns, not significant.

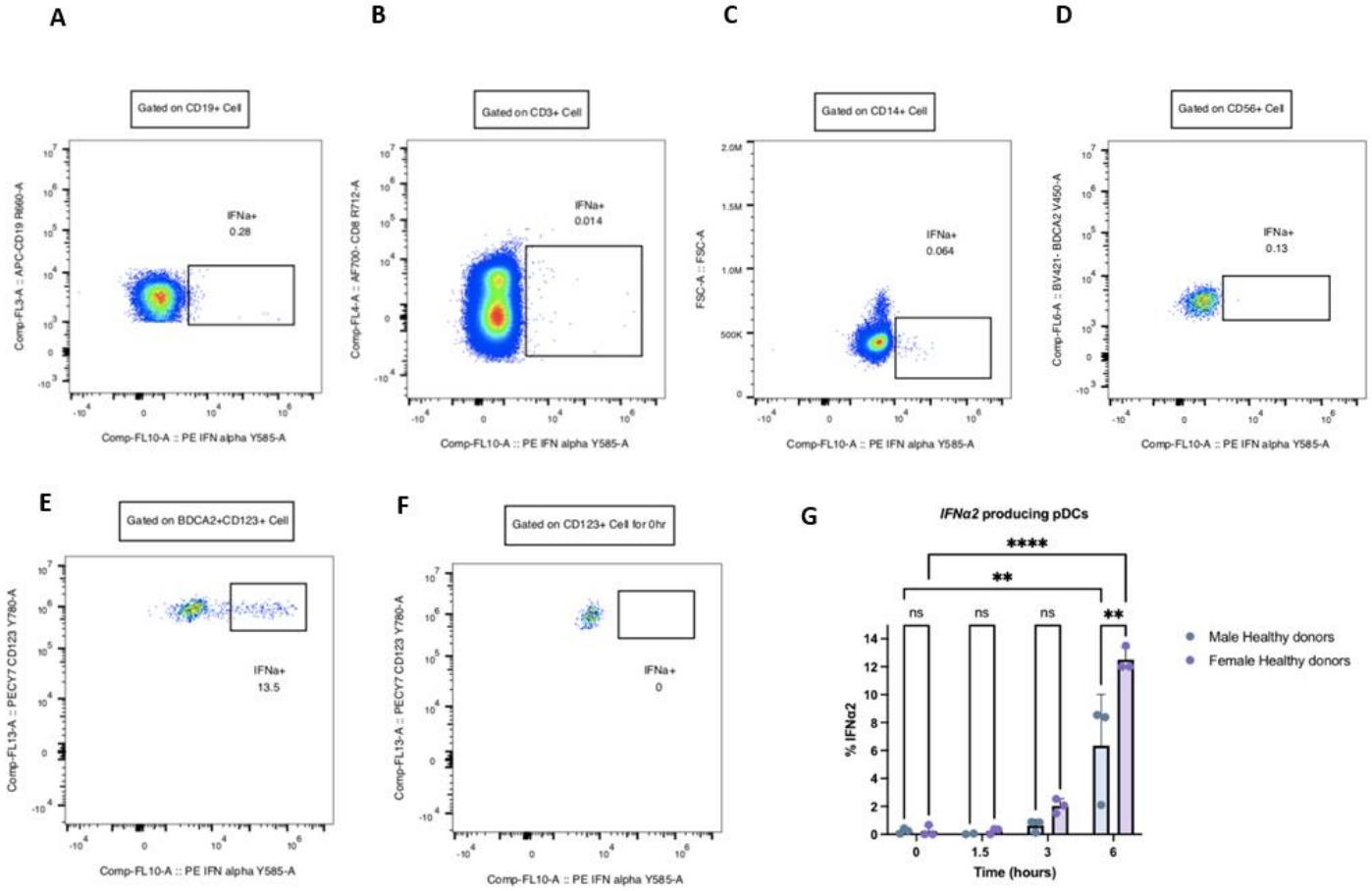
## FACS:

FACS was conducted on the PBMCs stimulated with gardiquimod to determine the protein expression of IFNα2 (Figure 4). Figure 4E illustrates pDCs as producing the highest number of IFNα2 after 6 hours of stimulation compared to the other immune cells in PBMCs such as B-cells, T-cells, monocytes and NK-cells (Figure 4A-D). Figure 4F displays IFNα2 expression at 0 hour with no stimulation. Figure 4G depicts the IFNα2 producing pDCs with a significant difference between the male and female percentage (%) of IFNα2 protein levels after 6 hours of stimulation, with higher IFNα2 protein production in female pDCs. Also, a significant difference was seen in the IFNα2 protein expression from 0 to 6 hours after stimulation for both male and female IFNα2 producing pDCs (Figure 4G).

Figure 4. FACS results of IFNa2 protein production of PBMC stimulated with gardiquimod. The IFNa2 secretion after 6 hours of stimulation is shown for **(A)** CD19+ B-cells **(B)** CD3+ T-cells **(C)** CD14+ Monocytes **(D)** CD56+ NK-cells **(E)** BDCA2+ and CD123+ pDCs. **(F)** The IFNa2 production of pDCs at 0 hours serves as a control. The results of one representative buffy coat PBMC are presented from **(A)** to **(F)**. **(G)** The percentage (%) of IFNa2 protein secretion in the pDC population of PBMCs of females (n= 3 buffy coats) and males (n= 3 buffy coats) at 0, 1.5, 3 and 6 hours after stimulation. Data represents mean ± SD. P-values were obtained using 2-way ANOVA, **p <0.01, ****p < 0.0001, ns, not significant.

## TLR7-stimulated pDCs

Female and male pDCs were stimulated with gardiquimod (Figure 5). Interestingly, Figure 5A displays the *IFNα2* mRNA expression being higher for males (specifically fresh blood samples) than females, though no significance was found between the sexes. After 3 hours of stimulation, a significant difference was found between males and females for *IRF7* expression, with females producing higher levels of *IRF7* (Figure 5B). Also, as seen in Figure 5B, 3 hours after stimulation, significance was found in female *IRF7* gene expression compared to males. Higher *TLR7* expression was seen in females and a trend of a slight increase in *TLR7* expression was also observed as stimulation time increased but no

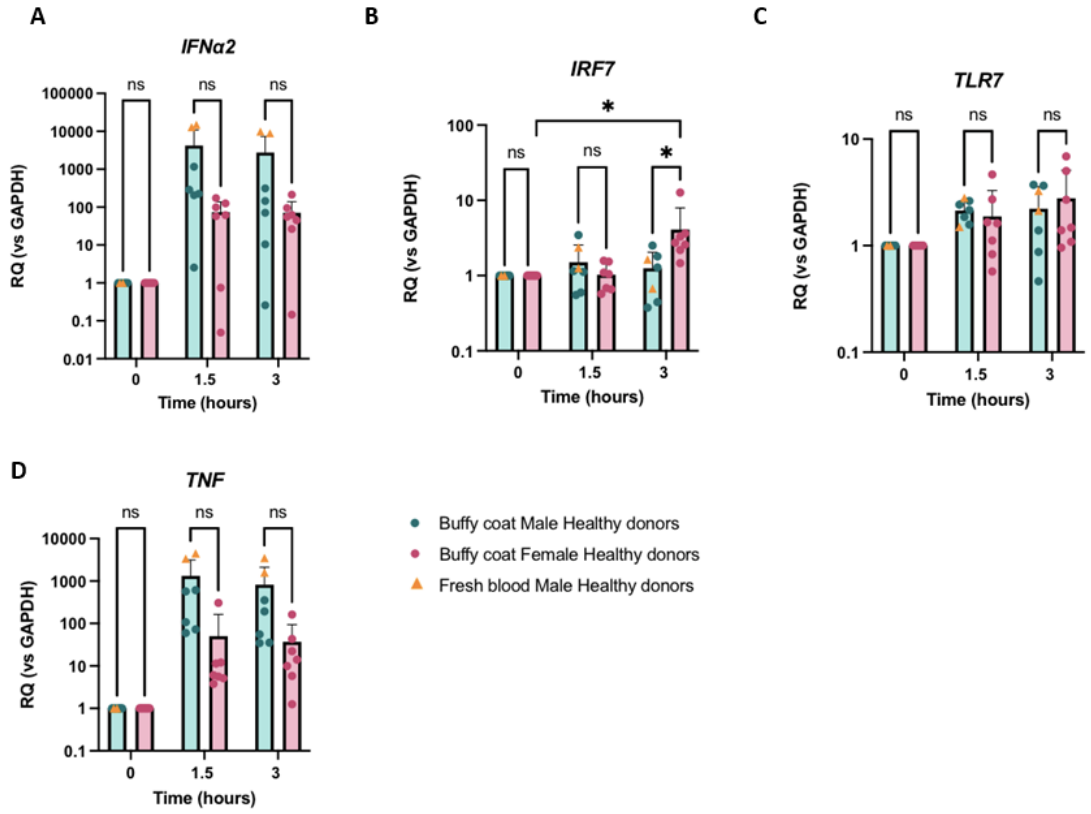


significance was seen (Figure 5C). Whereas Figure 5D shows higher *TNF* expression levels for males (particularly fresh blood samples) but no significant difference was seen between the sexes.

Figure 5. Female (n= 7 buffy coats) and male (n= 7, 2 fresh blood, 5 buffy coats) pDCs stimulated with gardiquimod. The data is shown as the relative quantification (RQ; relative to GAPDH) in Log10 against the time points 0 (control), 1.5 and 3 hours after stimulation. The gene expressions are displayed for **(A)** *IFNα2* **(B)** *IRF7* **(C)** *TLR7* **(D)** *TNF*. Data represents mean ± SD. P-values were obtained using 2-way ANOVA, *p <0.05, ns, not significant.

## Discussion

The study attempts to comprehend the sexual dimorphism observed in the antiviral functions of pDCs. pDCs were presented as the major IFNa2-producing cells in PBMCs, hence its critical anti-viral functions (Hagen et al., 2020). The IFNa2 protein expression in female pDCs were significantly higher than in male pDCs. Whereas TLR7-induced stimulation showed higher *IFNα2* mRNA levels in male pDCs although no significant difference was seen between the sexes. Other genes involved in the pathway of IFNa2 productions such as *IRF7*, displayed higher levels in female pDCs than male pDCs. Whereas TLR4-induced stimulation exhibits a trend of higher *IL1B*, *IL6* and *TNF* levels in male PBMCs. Taken




together, these genes can contribute to the antiviral functions of pDCs but are expressed differently in males and females. The differences could be due to the involvement of sex hormones since sex hormone receptors can be found on immune genes and impact expression levels (Foo et al., 2016). Nevertheless, it can also be due to sex chromosomes playing a role, for instance, X-linked immune genes escaping XCI (Souyris et al., 2018).

Souyris et al. (2018) observed some female pDCs demonstrating nuclear foci with synthesised *TLR7* at certain transcription sites. One of these foci overlapped with the inactive X chromosome. Furthermore, increased *TLR7* expression was also found in female pDCs. These findings show that *TLR7* escaping XCI in females and females possessing two X chromosomes, increases *TLR7* dosage in females. In agreement with Souyris et al. (2018), the current study also portrayed higher *TLR7* expression in female pDCs. One can presume that the escape of *TLR7* from XCI can lead to the enhanced *TLR7* levels seen in female pDCs. This contributes to a higher antiviral mechanism of pDCs in females since *TLR7* leads to the production of *IFNα2*, the key component in the antiviral functions of pDCs, making females less susceptible to viral infections than males (Hannah et al., 2008).

Initially, oestrogen was suspected as the probable cause of heightened IFN levels in females (Laffont et al., 2014). Laffont et al. (2014) conducted an experiment blocking oestrogen receptors and discovered TLR7-associated IFN-I production decreased in pDCs. However, additional experiments were also conducted, in which female pDCs were transplanted into male mice. This resulted in high IFN-I production by the transplanted female pDCs in the male mice. This insinuates that both female sex hormones and x chromosomes contribute to the increased IFN-I production in pDCs.

Despite Laffont et al. (2014) results, the gene expression of *IFNα2* for male pDCs was slightly higher than for female pDCs in the current experiment. However, the difference in the results can be due to using cryopreserved buffy coat samples compared to using fresh blood samples. Also, one should note that fresh blood samples were collected and used for males but not for females in this experiment due to time constraints. Ida et al. (2006) highlighted that *IFNα2* response declined when cryopreserved PBMCs were stimulated with TLR agonists compared to freshly isolated PBMCs. Furthermore, it was shown that *IFNα2* secretion by pDCs became profoundly weaker after thawing and freezing. Whereas fresh blood had a more accurate representation of cytokine and cellular environment (Ida et al., 2006). Since fresh blood samples were used only for males in this study, higher *IFNα2* levels were observed for males than cryopreserved buffy coats used for all females, so the effect of sex hormones could have weakened.

A link between increased *TLR7* and TLR7-induced IFN-I levels is suggested (Hagen et al., 2020). Hagen et al. (2020) demonstrated increased levels of IFN-I and *TLR7* in female pDCs through stimulation with a TLR7 agonist. Contrary to this, the results in the current experiment displayed slightly higher higher *IFNα2* in male pDCs but higher *IRF7* mRNA levels for female pDCs. Furthermore, female pDCs produced significantly higher IFNα2 protein levels than males after 6 hours of TLR7 stimulation. Differences in the self-priming and post-transcriptional mechanisms of each gender's pDC along with sex hormones or chromosomes impacting these differences could potentially play a part in the results obtained in this study (Ivashkiv & Donlin, 2013; Kim et al., 2014).

pDCs have a self-priming mechanism as they produce low levels of IFN-I mRNA continuously when undergoing TLR stimulation. Even if unstimulated, pDCs produce low amounts of IFN-I (Kim et al., 2014). This signifies that pDCs maintain a baseline level of IFN-I. Additionally, post-transcriptional modifications also have a role in regulating the secretion of IFN-I, hence playing a role in the antiviral activities of pDCs. The existence of lots of different RNA regulatory elements in the mRNA transcripts of IFNs can influence their instability or stability. Post-transcriptional regulatory factors such as RNA-binding proteins and specific motifs in the mRNA can impact the mRNA stability and efficiency of translation (Ivashkiv & Donlin, 2013). Therefore, the difference between the mRNA levels of *IFNα2*

between male and female pDCs, along with the differences seen in IFNα2 protein levels, could derive from these post-transcriptional regulatory factors. This underlines the complexity of gene expression regulations whilst emphasising the importance of taking post-transcriptional mechanisms into consideration. Overall, such mechanisms along with factors such as sex hormones could affect the functions of pDCs, hence the difference in the antiviral activities of each sex (Hannah et al., 2008).

The adaptor MYD88 interacts and activates the transcription factor *IRF7*. This interaction is crucial for enhanced production of IFNs and causing the antiviral functions seen in pDCs (Honda et al., 2005). Transcription factors showcase sex-biased regulatory targeting tendencies (Lopes-Ramos et al., 2020). Hannah et al. (2008) inoculated rats with Seoul virus. Female rats were found to have increased expression of IRF7 and Mx protein. The current results exhibited a significant difference in the *IRF7* expression of pDCs, with females producing higher levels than males. Also, with PBMCs it was displayed that the expression of *Mx1* was similar but slightly higher for females than males. This can explain why females are less susceptible to infections since ISGs such as *Mx1* which aid pDCs in preventing viral replication were found to be slightly higher in females in this study (Verhelst et al., 2012). Therefore, allowing females to have a higher immune response against viral infections than males (Hannah et al., 2008).

Hannah et al. (2008) also induced that since oestrogen and androgen response elements are found in the promoter region of immune genes, this played a role in female rats producing a higher immune response. This validates sex hormone's influence on immune genes. Therefore, this suggests that sex hormones could impact the functions of pDCs involving *IRF7* and *Mx1*. Although generalisation of Hannah et al.'s (2008) finding can be questioned as the study was done on rats whereas this study involved human samples.

Asai et al. (2001) discovered that male PBMCs had increased LPS-induced *TNF* levels than females. Asai et al. (2001) implied that this may be due to differences in the TLR, NF-KB and cytokine mRNA transcription between the sexes. Furthermore, the study demonstrated that oestrogen combined with LPS decreased the pro-inflammatory *TNF* levels in male PBMCs. This could relate to females producing lower levels of *TNF* in this study as the anti-inflammatory effects of oestrogen could have an impact on the functions of pDCs by decreasing the production of *TNF*. However, Aomatsu et al. (2013) found that males have increased TLR4 levels which causes higher responsiveness to LPS stimulation (TLR4 agonist). This can also explain the high *TNF* levels by LPS stimulation in male PBMCs, but high *TNF* levels were also seen in male pDCs and PBMCs stimulated by gardiquimod (TLR7 agonist). Also, Aomatsu et al. (2013) used neutrophils in the experiment, but the current study utilises PBMC which does not include neutrophils (Kleiveland, 2015).

Additionally, TLR-stimulated pDCs have shown that *TNF* lowers *IFNα* by downregulating *IRF7* pathways but endorses pDC maturation instead. The effect of *TNF* led to an increase in antigen presentation and T cell activation pathways. Therefore, *TNF* promoted pDC maturation by adopting a conventional dendritic cell (cDC) phenotype instead of the primary functions as IFN*α*-producing cells (Psarras et al., 2021). The increased IFNα2 protein levels and *IRF7* mRNA levels in female pDCs along with higher *TNF* levels in male pDCs could suggest sex differences in *TNF* regulations and their impact on pDCs. This could imply that females show a more robust innate immune response, marked by a faster secretion of IFNα2, which is essential for antiviral functions (Meier et al., 2009). Whereas males could have a more prominent adaptive immune response, marked by increased antigen presentation and T-cell

activation, possibly resulting in robust antibody production (Rantala et al., 2012). Hence, the function of pDCs might be affected by both their inflammatory milieu and sex hormones.

T cells secrete *IL2* and express CD40L to signal pDCs to release *IL6* thus activating B cells. *IFNα2* produced by pDCs promotes the differentiation of B cells into plasma blasts. Whereas *IL6* produced by pDCs allows these plasma blasts to turn into antibody-releasing plasma cells (Jego et al., 2003). *IL6* can be both a pro-inflammatory and anti-inflammatory cytokine (Chomarat et al., 2000; Márquez et al., 2020). Furthermore, males possess higher testosterone and androgen levels, which are said to be immunosuppressive and therefore weaken the immune response (Rantala et al., 2012). The current study illustrates higher levels of *IL6* in male PBMCs than females. This could further support the idea of pDCs functioning differently in the sexes. Since testosterone suppresses the immune, perhaps the increase in *IL6* in pDCs could attempt to counteract this by increasing antibody production and boosting the immune response against viral infections in males (Jego et al., 2003; Rantala et al., 2012).

Márquez et al. (2020) discovered that as age increases, certain cytokines get activated, with more noticeable activation seen in males. Cytokines such as *IL1* and *IL6* were increased in elderly males than elderly females. It is also known that with age, the concentration of sex hormones also changes (Bjørnerem et al., 2004). Moreover, pDC functions have been reported to decline as age increases, with a decline also seen in *IFNα2* levels leading to weaker antiviral function of pDCs in the elderly (Shodell & Siegal, 2002). The current experiment demonstrated higher levels of *IL1B* and *IL6* in male PBMCs than females. Thus, one can postulate that perhaps this may be due to the age group of males being higher than females. However, higher *IFNα2* levels were also seen in male pDCs than females, which may suggest otherwise. As most samples were buffy coats obtained from a biobank, it cannot be certain what age group the participants were due to the anonymity of the samples.

Nevertheless, this opens a paradox in the connection between sex hormones, functions of pDCs and the immune response. Testosterone and androgen have immunosuppressive effects and as males age, their concentration of these sex hormones also decreases leading to increased vulnerability to infections and a weakened immune response (Kanda et al., 1996; Bjørnerem et al., 2004). Whereas increased levels of *IL6* and *IL1B* can act as a compensatory mechanism for the susceptibility to infections and a weakened immune response (McElvaney et al., 2021). However, excessive levels of these pro-inflammatory cytokines can also have a detrimental impact on health and increase inflammatory responses (Márquez et al., 2020). The higher *IL1B* and *IL6* levels along with increased *IFNα2* levels seen in males in the current experiment could perhaps mean a balance between immune suppression and compensatory functions of pDCs in males. Nonetheless, additional research is required to understand this. One should also note that no significant differences were observed between the sexes for the mRNA levels of *IFNα2*, *IL1B* and *IL6*.

Gómez-Carballa et al. (2022) displayed an upregulation of *TLR7* in female whole blood COVID-19 samples than males. However, in the current study, male PBMCs showed a slightly higher *TLR7* expression than females. A possible reason for this, other than the difference in whole blood and PBMC sample usage, can be due to most samples in this study being buffy coats for all females and the majority of males. Moreover, the buffy coat samples were cryopreserved in dimethyl sulfoxide (DMSO) and stored in the freezer for approximately 3 years.

DMSO has cytotoxic abilities, and the storage time of cryopreserved cells can have an impact on cell viability (Hønge et al., 2017). Therefore, this could have affected the expression levels of the buffy coat

samples used in this study. Additionally, no fresh blood samples were used for females due to time constraints in sample collection, but two fresh blood samples were used for males. This may also play a role in the differences in the gene expression shown in this experiment, with males showing higher expression for certain genes such as *IFNα2* than females. Although no significant difference was observed between the genders.

Prior research has also disclosed that a variety of factors can impact the concentration of sex hormones, which can further affect the immune response. For example, total oestradiol level is impacted by age, body mass index (BMI) and menopause (Bjørnerem et al., 2004). Since sex hormones have an impact on immunity, the PBMC and pDC results displayed in the current experiment could be due to such factors impacting the sex hormones, which further influence the immune responses exhibited (Foo et al., 2016). This might suggest how different factors, biological or environmental could influence sex hormones and lead to an effect on pDCs antiviral mechanisms.

Nevertheless, factors such as age, diet, state of the menstrual cycle or menopause can affect sex hormones and cause further influences on the immune response (Rehman et al., 2021). However, such biological and environmental factors are unknown in this study due to the buffy coat samples being anonymous because of ethical concerns. Therefore, this is a limitation of this experiment as one can only make possible assumptions regarding such factors in the experimental results.

Further limitations of this study along with the use of cryopreserved buffy coats and the lack of information regarding the buffy coat samples include the small sample size used due to time constraints. This could be the reason behind the lack of statistical significance in the results. Also, the lack of fresh blood for females compared to male samples can play a part in the differences observed in the expression levels in this experiment.

## Conclusion

To conclude, this study highlights sexual dimorphism in the expression of immune genes related to the antiviral mechanisms of pDCs, specifically in the role of TLR7 and type 1 interferon signalling. Male pDCs expressed higher *IFNα2* mRNA whereas female pDCs displayed higher IFNα2 protein levels. Through TLR4 stimulation, males showcased a trend of higher *TNF*, *IL6* and *IL1B* levels in PBMCs. Whereas with TLR7 stimulation females exhibited higher *IRF7* levels in pDCs. This suggests potentially influences from both sex hormones and sex chromosomes on the antiviral functions of pDCs. Additional research needs to be conducted to clearly elucidate the specific contributions of sex-related factors to pDCs functionality, thereby increasing the understanding of sexual dimorphism in the antiviral immune responses.

For future research, it would be beneficial to use more fresh blood samples than cryopreserved buffy coat samples to ensure an accurate representation of the cell environment. A greater number of samples should also be utilised to achieve results with statistical significance. Also, if under ethical regulations, certain information about the participants such as environmental or biological factors that can impact the sex hormones could be obtained, it would help comprehend the results better.

## Ethical aspects and impact of the research on society

Within this study, blood from healthy human participants was used for the experimental procedures. All participants were given informed consent regarding the project. Additionally, all participant's information and privacy were kept confidential. Participants were also protected from any physical or psychological harm. Furthermore, blood was collected under ethical and regulatory principles. Buffy coat samples from human participants were also used. Safe handling, disposal and storage of the human cells was done with the required precautions and adherence to the biosafety guidelines. The buffy coat samples used were obtained from the Biobank located at the Sahlgrenska University Hospital, thus all participant information was kept anonymous to protect the sample donor's information. The ethical permit number obtained from the Swedish Ethical Review Authority is 2023-07050-02.

The project aimed to look at how sex hormones and sex chromosomes influence the antiviral functions of pDCs. Human males and females previously showed a difference in their immune response. Therefore, it was necessary to use human samples to showcase an accurate representation of the mechanisms involved in the immune responses that occur in humans.

The societal impact this project has lies in increasing the understanding of the sexual dimorphism observed in the immune responses, specifically in the antiviral functions of pDCs. By elucidating the influence sex hormones and chromosomes have on the mechanisms of pDCs, this research can contribute to an increased understanding of autoimmune diseases that are more prone in females as well as infections that are more susceptible to males. Moreover, knowledge gained through this project could contribute to the development of additional targeted treatments along with therapies personalised for each patient's need.

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