Influence of Maternal Inflammation on Immune Modulation

by

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Declaration

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Abstract

Background

Perturbations within intrauterine microenvironment, due to maternal stressors such as chronic inflammation, metabolic dysfunction and psychological stress, can result in lifelong detrimental consequences in affected offspring. Given the substantial contribution of non-communicable diseases to the global morbidity and mortality rate, the potential impact of maternal prenatal chronic disease on foetal development is of increasing concern. Additionally, inheritance of these developmental defects, such as behavioural, neurological, glucocorticoid and immune maladaptations, can persist for multiple generations in offspring. Although inflammation that forms part of the aetiology of chronic diseases, very few studies have reported on the immunological and glucocorticoid maladaptations in offspring exposed to maternal inflammation. Moreover, even less is known about the extent of this generational transfer, the sex-specific inheritance or the transgenerational plasticity of these maladaptations in the F1 and F2 generations. Thus, we aimed to delineate the immune functionality and glucocorticoid sensitivity in leukocytes in two generations of offspring in a mouse model of chronic induced maternal inflammation.

Methodology

Pregnant C57/BL/6 (F0) dams were exposed to either 10μg/kg lipopolysaccharide (LPS) or 0.9% saline (control) every seven days for the duration of gestation, with no further intervention after gestation. At 8 weeks of age, the F1 offspring were crossed with either a wild-type untreated C57/BL/6 mate or F1 LPS-affected non-sibling mate to create the second generation of offspring, F2. For experimental analyses, F0, F1 and F2 mice were profiled to assess the changes in differential splenic and circulatory leukocyte populations, corticosterone concentrations, splenic leukocyte glucocorticoid sensitivity and *ex vivo* cytokine responses.

Results

LPS-treated F0 dams displayed augmented glucocorticoid receptor expression in splenic leukocytes, which transferred to both F1 and F2 offspring, as well as elevated inflammatory cytokine responses in LPS-stimulated splenocytes. Both male and female F1 offspring displayed glucocorticoid hypersensitivity, indicated by elevated corticosterone and

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leukocyte glucocorticoid receptor levels, as well as a heightened inflammatory phenotype, which was transferred to F2. However, no sex-specific traits were observed. Interestingly, F1 LPS-affected offspring displayed sex-specific transfer of inflammatory effects to F2. The glucocorticoid dysregulation and the resultant pro-inflammatory phenotype appeared to be transferred in the F1 maternal lineage to her F2 offspring, affecting the male offspring to a greater extent. In contrast, F1 LPS males only transferred a pro-inflammatory phenotype to their offspring. The cumulative inheritance of maladaptation in F2 offspring, from F1 maternal and paternal lineages being LPS-affected, displayed blunted functional immune responses, and dampened glucocorticoid levels, but unchanged leukocyte numbers and may be suggestive of immune senescence.

Conclusion

This study contributes to the knowledge of inheritance of susceptibility to non-communicable chronic diseases. Our findings illustrate that parental chronic inflammation may cause lifelong reprogramming to a maladapted pro-inflammatory phenotype, persisting to at least two subsequent generations of offspring. Moreover, divergent adverse outcomes are seen in F2 offspring, in terms of sex-differences and cumulative inheritance of inflammatory effects. Given the potential impact of these findings, more focussed research in this context is required to confirm and further characterise the mechanisms underlying this paradigm, to facilitate the development of future interventions.

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Opsomming

Agtergrond

Dit is bekend dat versteurings binne die mikro-omgewing in die uterus, as gevolg van moederlike stressors soos chroniese inflammasie, metaboliese disfunksie en sielkundige stres, tot nadelige gevolge vir die nageslag selfs tot in volwassenheid lei. Gegewe die aansienlike bydrae van nie-oordraagbare siektes tot wêreldwye morbiditeit en mortaliteit, is die potensiële impak van chroniese inflammasie in die moeder, op fetale programmering 'n groeiende kommer. Daarbenewens kan die oorerflikheid van hierdie ontwikkelingsdefekte, soos gedrags-, neurologiese, glukokortikoïede en immuun-wanaanpassings, voortduur vir verskeie geslagte in die nageslag. Alhoewel inflammasie deel uitmaak van die etiologie van chroniese siektes, is baie min studies gerapporteer oor die immunologiese en glukokortikoïede wanaanpassings by nageslagte wat *in utero* aan swangerskap inflammasie blootgestel is. Daar is selfs minder bekend oor die omvang van generasie-oordrag van hierdie aspekte, sowel as die geslagspesifieke oorerwing en transgenerasie plastisiteit van hierdie wanaanpassings in die F1- en F2-generasies. In hierdie studie het ons dus ten doel gehad om die immuunfunksionaliteit en leukosiet glukokortikoïedsensitiwiteit by twee generasies nageslagte in 'n muismodel van chroniese geïnduseerde swangerskap inflammasie te omskryf.

Metodes

Dragtige C57/BL/6 (F0) wyfies is elke 7 dae blootgestel aan óf 10 μg/kg lipopolisakkaried (LPS) óf slegs 0,9% soutoplossing (kontrole) tot aan die einde van die swangerskap. Geen verdere ingryping is na dragtigheid gegee nie. Op die ouderdom van 8 weke is die nageslag (F1) gekruisteel met wilde-tipe onbehandelde C57/BL/6-muise of F1-LPS-geaffekteerde muise, wat nie verwant is nie, om die tweede generasie nageslag, F2, te skep. Vir eksperimentele ontledings is F0, F1 en F2 generasies omskryf om die effek van chroniese swangerskap inflammasie in differensiële milt- en bloedsomloop leukosietpopulasies, kortikosteroon, milt leukosiet glukokortikoïed sensitiwiteit en ex vivo sitokienrespons te bepaal.

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Resultate

Die LPS-behandeling van F0-wyfies het gelei tot 'n verhoogde uitdrukking van glukokortikoïed reseptore in die meeste van die miltleukosiet-subpopulasies wat in beide F1- en F2-nageslagte ge-evalueer is, asook verhoogde inflammatoriese sitokienrespons in splenosiete wat in vitro met LPS gestimuleer is. F1-nakomelinge (beide mannetjies en wyfies) het oor die algemeen verhoogde kortikosteroonvlakke en leukosiet-glukokortikoïed reseptor-uitdrukking getoon, wat 'n aanduiding is van hipersensitiwiteit vir glukokortikoïede, sowel as 'n verhoogde inflammatoriese fenotipe wat oorgedra is na F2, ondanks die insluiting van 'n onaangetaste ouer. Hierdie fenotipe het geslagspesifieke oorerwing getoon van F1 tot F2. Glukokortikoïed wanregulering en 'n gepaardgaande pro-inflammatoriese fenotipe is klaarblyklik in die F1-moederlyn oorgedra na haar F2-nageslag, wat die manlike nageslag tot 'n groter mate beïnvloed het, terwyl F1 LPS-mannetjies daarenteen slegs 'n meer pro-inflammatoriese fenotipe oordra, maar geen glukokortikoïed wanregulering nie. Interessant genoeg vertoon die kumulatiewe oorerwing van wanaanpassing by F2-nakomelinge, vanaf die F1-moederlike en vaderlike geslagslyne wat deur die LPS aangetas is, funksionele immuunrespons, en gedempte glukokortikoïedvlakke, maar onveranderde leukosiet getalle, wat 'n aanduiding is van immuun gerontisme.

Gevolgtrekkings

Die huidige studie dra by tot ons kennis van ouerbydrae tot die vatbaarheid vir die ontwikkeling van nie-oordraagbare chroniese siektes in nageslagte. Ons bevindinge illustreer dat chroniese ontsteking deur ouers lewenslange herprogrammering tot 'n wanaanpassende pro-inflammatoriese fenotipe in die nageslag kan veroorsaak, wat voortduur tot ten minste twee daaropvolgende generasies. Verder word uiteenlopende nadelige uitkomste in F2 generasies gesien, in terme van geslagsverskille en kumulatiewe oorerwing van inflammatoriese effekte. Gegewe die potensiële impak van hierdie bevindings, is meer gefokusde navorsing in hierdie konteks nodig om die meganismes onderliggend aan hierdie paradigma te bevestig en verder te karakteriseer, ten einde die ontwikkeling van toekomstige intervensies te vergemaklik.

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Research Outputs

Manuscripts

1. Adams, RCM and Smith C. Chronic Gestational Inflammation: Transfer of Maternal Adaptation over Two Generations of Progeny, Mediators of Inflammation, vol. 2019, Article ID 9160941, 16 pages, 2019. https://doi.org/10.1155/2019/9160941.

2. Adams, RCM, Smith, C. *In utero* exposure to maternal chronic inflammation transfers a pro-inflammatory profile to generation F2 via sex-specific mechanism. Frontiers Immunology (manuscript accepted for publication, January 2020).

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List of Abbreviations

11β-HSD1	Type 1 11β-hydroxysteroid dehydrogenase
11β-HSD2	Type 2 11β-hydroxysteroid dehydrogenase
A	Area
ACTH	Adrenocorticotropic hormone
APC	Antigen presentation cell
ASC	Apoptosis-associated speck-like protein containing a CARD domain
BD	Becton Dickinson
CARD	Caspase activation and recruitment domain
COR	Cortisol
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
CRS	Chronic restraint stress
CSD	Chronic social disruption
CSS	Chronic social stress
CTL	Cytotoxic T-lymphocytes
CVS	Chronic variable stress
CYP17	17-α hydroxylase enzyme
DAMP	Damage-associated molecular pattern
DBD	DNA-binding domain
DC	Dendritic cell
DPBS	Dulbecco’s Phosphate Buffered Saline F0
DSS	Dextran-sulfate-sodium
ELISA	Enzyme-linked immunosorbent assay
F0 LPS	Generation 0 LPS mothers
F0	Parental generation affected
F1 LPS	Generation 1 offspring from LPS-injected mothers
F1	Generation of offspring derived from F0, i.e. first generation of offspring
F2 LPS	Generation 2 offspring from LPS-injected mothers

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F2	Generation of offspring derived from F1, i.e. second generation of offspring
F3	Generation of offspring derived from F2, i.e. third generation of offspring
F4	Generation of offspring derived from F3, i.e. fourth generation of offspring
FOXP3	Forkhead box P3
FSC	Forward scatter
GATA-3	GATA binding protein 3
GC	Glucocorticoid
G-CSF	Granulocyte colony-stimulating factor
GR	Glucocorticoid receptor
GRE	Glucocorticoid-responsive elements
H	Height
HFD	High fat diet
HPA	Hypothalamic-pituitary-adrenal
HSP70	Heat shock protein 70
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin
iNKT	Invariant natural killer T-lymphocyte
IP_3	Inositol 1, 4, 5-triphosphate
IRAK	Interleukin-1 receptor (IL-1R) associated kinase
IRF7	Interferon regulatory factor
JNK	Jun kinase N-terminal
LBD	Ligand-binding domain
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
m-CSF	Macrophage colony-stimulating factor
MFI	Median fluorescent intensity
MHC I	Major histocompatibility complex I
MHC II	Major histocompatibility complex II
MIA	Maternal immune activation

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MPSI	Maternal periconception systemic inflammation
MR	Mineralocorticoid receptor
NF-κB	Nuclear Factor Kappa B
NK	Natural killer
NKT	Natural killer T-lymphocytes ()
NLR	Nod-like receptor
NLRP3	Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3
NTD	N-terminal transactivation domain
PAMP	Pathogen-associated molecular pattern
PHA	Phytohaemagglutinin
PKB	Protein kinase B
PKC	Protein kinase C
PMN	Polymorphonuclear leukocyte
PRR	Pattern recognition receptor
PTSD	Post-traumatic stress disorder
PYD	Pyrin domain
RPMI 1640	Roswell Park Memorial Institute 1640
RSD	Repeated social defeat
SCC	Side scatter
sGC	Synthetic glucocorticoid
TAB2	TAK1-binding protein 2
TAK1	TAB2-associated TGF-β- activated kinase 1
T-bet	T-box–containing protein expressed in T cells
T-bet	T-box–containing protein expressed in T cells
TCR	T-cell receptor
TGF-β	Transforming growth factor
TGF-β	Transforming growth factor beta
T_H0	Naïve helper T-lymphocytes
T_H1	Type I helper T-lymphocyte
T_H2	Type II helper T-lymphocyte
T_H3	Type III helper T-lymphocyte
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha

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TRAF6	TNF receptor associated factor
T-reg	Regulatory T-lymphocytes
TRIF	Toll/interleukin-1 receptor domain TIR-domain-containing adapter-inducing interferon-β
VEGF	Vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2
W	Width
WBC	White blood cell count
WHO	World Health Organisation

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Chapter 1

Introduction

Inflammation, as an acute and transient immune response, is essential for host protection against pathogens and foreign bodies. Acute inflammation is characterised by leukocyte infiltration and the production of mediators to facilitate pathogen destruction and elimination, as well as tissue regeneration and repair (Kumar, Kawai & Akira, 2011). The acute inflammatory response and the subsequent production of inflammatory cytokines can activate the hypothalamic-pituitary-adrenal (HPA) axis and stimulate the production of the adrenocorticotropic hormone and glucocorticoids (Straub & Cutolo, 2016). Glucocorticoids are one of the key role-players in the acute inflammatory response. On the one hand, this hormone is responsible for releasing energy (from sources such as glucose, ketone bodies, amino acids and free-fatty acids) to sustain leukocytes and allow leukocyte redistribution (Straub et al., 2010; Straub & Cutolo, 2016), while on the other hand, glucocorticoids represent the body's main endogenous anti-inflammatory feedback loop to ensure the resolution of inflammation. In contrast to acute inflammation, which is self-limiting, chronic inflammation is a persistent, low level inflammatory response over an extended period. This pathological condition is often due to lack of resolution of inflammation or continuing exposure to the triggering agent, typically leading to tissue damage and degeneration (Franceschi & Campisi, 2014). The chronic elevation of the inflammatory response and HPA axis can result in detrimental consequences in the host.

A widely recognised hallmark of most chronic diseases is the onset of a chronic inflammatory state, especially in non-communicable diseases, such as metabolic syndrome, chronic pulmonary diseases, diabetes mellitus type 2 and cardiovascular diseases (Lumeng & Saltiel, 2011; Yu, Li, et al., 2019). Of particular relevance, these abovementioned conditions are also reported as the some of the leading causes of morbidity and mortality globally (World Health Organization, 2018).

A known aetiological factor in chronic diseases is the combination of chronic inflammation and HPA axis dysregulation. For example, chronic psychological stress

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is characterised by high serum cortisol (excluding situations of adrenal burnout) and low lymphocyte GR expression (Gotovac et al., 2003; Vidović et al., 2011), as well as increased leukocyte presence in circulation (Vidović et al., 2011; Lindqvist, Mellon, et al., 2017) and a relative pro-inflammatory cytokine profile in circulation (Song et al., 1999; Carpenter et al., 2010; Murphy et al., 2014; Lindqvist, Dhabhar, et al., 2017; Niraula et al., 2019). Similarly, the aetiology in metabolic diseases also includes glucocorticoid dysregulation, increased inflammatory cytokine production and macrophage infiltration into adipose tissue (Hotamisligil, 2006; Lumeng & Saltiel, 2011; Saltiel & Olefsky, 2017; Yu, Li, et al., 2019). This phenomenon can lead to resistance to glucocorticoids at a cellular level.

Given the adverse effects caused by chronic stress, Barker postulated, in what is known as the "foetal programming hypothesis", that abnormalities occurring during foetal development influences the physiological outcome in affected offspring and their susceptibility to disease later in life (Barker, 1998, 2007). It has been widely described that exposure to unfavourable environments during prenatal development, is associated with a higher risk of disease in later life. Emerging evidence suggests that this early life programming is influenced by several factors, including aspects of poor nutrition (Heijmans et al., 2008; Ling et al., 2018), psychological stress (O'Connor et al., 2013; Lehrner et al., 2014; Kubo et al., 2018), pollutants (Capra et al., 2013) and innate immune activation (Spann et al., 2018). These adverse events may contribute to the onset of immune and glucocorticoid dysfunction and predispose the affected individuals to developing chronic diseases. Indeed, human and animal studies have demonstrated that maternal and intrauterine perturbations affect not only maternal, but also foetal endocrine and immunological processes during gestation. For example, maternal prenatal stress or inflammation is associated with increased concentrations of IL-6 and TNF-α in circulation and in placental tissue (Coussons-Read, Okun & Nettles, 2007; Christian et al., 2009; Ma et al., 2019). Furthermore, it has been reported that maternal exposure to infectious or inflammatory agents result in maladaptation in offspring. Studies have shown that cytokines or inflammatory agents (e.g. lipopolysaccharide) does not directly cross the placenta (Zaretsky et al., 2004; Ashdown et al., 2006), thus foetal inflammation seen in offspring is probably due to placental damage resulting from maternal inflammation (Babu et al., 2018; Ginzel et al., 2016; Fricke et al., 2018; Yan et al., 2019). This indirect *in utero* exposure to

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inflammation results in adverse consequences in glucocorticoid (Kirsten et al., 2013; Sasaki et al., 2013), neurological (Straley et al., 2014) metabolic (Dudele et al., 2017; Xie et al., 2018) and immunological (Hodyl et al., 2007; Mandal et al., 2011; Williams, Teeling, Perry, Fleming, et al., 2011) function in offspring born to the affected mothers. However, while literature has described the effect of maternal inflammation on the offspring born to the mother, little is known about the transgenerational inheritance of the inflammatory effects across more than one generation, or inheritance through the paternal lineage.

Ancestral exposure to maternal stressors is known to impact the health outcomes of the offspring, without any further stress exposure. Several studies have described transgenerational effects of maternal stress in the first generation - even up to the fourth generation – of offspring, with sex-specific heritability of adaptations also being reported (Buchwald et al., 2012; Sominsky et al., 2012; Cheong et al., 2016; Moisiadis et al., 2017). In terms of maternal inflammatory stress, many studies report on the transgenerational disturbances in psychological behavioural traits and susceptibility to neurobiological disorders in the second and third generation of offspring (Penteado et al., 2014; Reis-Silva et al., 2016; Weber-Stadlbauer et al., 2017; Berger et al., 2018; Boulanger-Bertolus, Pancaro & Mashour, 2018). Yet, very little is known about the immunological outcome following *in utero* exposure to the maternal acute immune activation or even chronic maternal immune activation beyond the first generation of offspring. Given that the transgenerational heritability of neurobiological adaptations caused by maternal inflammation is evident in generations of offspring, we postulate that a similar phenomenon exists in terms of the immunological and glucocorticoid response.

The delineation of cross-generational susceptibility of inherited immunological dysfunction in offspring is imperative in the epidemiology of health and disease. With this in mind, the main aim of this thesis was to investigate the longevity of transgenerational inheritance of immunological and stress hormone maladaptation in F1 and F2 offspring born to F0 C57/BL/6 female mice exposed to chronic low grade inflammation (through repeated low dose LPS exposure) during gestation. A second aim was to determine the sex-specificity of the postulated inherited maladaptation and

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the potential cumulative effects on the immunological phenotype of F2 offspring born from **two** *in utero* affected F1 offspring.

In this dissertation, I will review the current knowledge on the topic in the following chapter, in terms of the control of the HPA axis on the immune response as well as the generational inheritance of maternal stress and immune activation beyond the first generation. At the end of the review, I will present my hypothesis and aims for the dissertation. Chapter 3 will investigate the immunological and glucocorticoid response outcome of chronic maternal inflammation in two generations of offspring. Chapter 4 will describe the sex-specificity of this inherited phenotype and effects of cumulative inheritance in offspring from two affected F1 parents. This will be followed by Chapter 5, which will provide a general discussion and final conclusions of findings, as well as recommendations for future research.

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Chapter 2

Literature Review

The purpose of this chapter is to provide insight into the impact of the hypothalamic-adrenal-pituitary axis on the immune system and vice versa. The review will cover the glucocorticoid regulation in immune cells and also discuss the concept of generational inheritance of maladaptation, the impact of maternal stress on offspring and the plasticity of inheritance of the altered offspring phenotype.

2.1. Overview of the innate and adaptive immune response

The primary role of the host immune system is defence from - and elimination of - pathogenic microbes, toxins and allergens. The immune system is broadly categorised into innate and adaptive components. The innate immune arm mediates the early host response to pathogens and plays a key role in the activation of the adaptive immune response (Iwasaki & Medzhitov, 2004; Chaplin, 2010; Kumar, Kawai & Akira, 2011). In contrast, the adaptive arm of the immune system, while slower in response than the innate arm, has high specificity and the capability of immune memory to allow faster pathogen clearance in the event of re-exposure to the same pathogen (Chaplin, 2010).

2.1.1. Innate immunity

Apart from the anatomical and physiological barriers (e.g. the skin and mucosal lining of the respiratory, gastrointestinal, and genitourinary tracts), the innate immune system employs a range of cell types to respond to invading pathogens and enact immunological functions. Monocytes, macrophages, neutrophils, eosinophils and basophils form part of the granular cell lineage and are derived from common myeloid progenitor stem cells in the bone marrow. NK cells and NKT-lymphocytes are considered part of the lymphoid lineage and differentiate from the common lymphoid progenitor. These cells have specific functions in the innate immune response (Chaplin, 2010) (Chaplin, 2010; Stone, Prussin & Metcalfe, 2010; Turvey & Broide, 2010). Below, brief descriptions are provided for the different innate effector cells and

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their major roles, followed by a brief review of the processes by which these cells recognise pathogens and become activated to mount the appropriate response.

2.1.1.1. Monocytes and macrophages

Monocytes and macrophages play a key role in the innate immune system by recognising and sequestering microbes, thereby degrading them by phagocytosis, and as such, process the microbial antigens internally (Chaplin, 2010; Turvey & Broide, 2010). Macrophages also play an important role in the immune response as antigen presentation cells (APCs). APCs recognise pathogen-associated or host-associated moieties, e.g. recognition of lipopolysaccharide (LPS) or heat shock protein 70 (HSP70), via conserved germline-encoded host receptors, termed pathogen recognition receptors (PRRs), and primarily secrete pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin (IL)-1, IL-6, IL-8, and IL-12 upon recognition of these inflammatory stimuli (Kumar, Kawai & Akira, 2011; Duque & Descoteaux, 2014; Escoter-Torres et al., 2019).

Macrophages can be categorised into M1 and M2 subtypes. M1 macrophages (classically activated macrophages) secrete pro-inflammatory cytokines and mediate pro-inflammatory actions in response to inflammatory stimuli or antigen exposure. Classically activated (M1) macrophages are primary producers of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-12 and interferon gamma (IFN-γ) allowing regulation of the adaptive immune responses. The M2 macrophages are characterised by the anti-inflammatory actions and are activated by cytokines, such as IL-4, IL-10, and IL-13 (Chaplin, 2010; Turvey & Broide, 2010; Martinez & Gordon, 2014). Macrophage pathogen recognition and signalling subsequently activates adaptive immune cells (Duque & Descoteaux, 2014).

2.1.1.2. Granulocytes

Neutrophils, eosinophils, and basophils form the granulocyte population of leukocytes, (also known as polymorphonuclear leukocytes (PMNs)). PMNs are present in the blood circulatory system and migrate into tissues in response to a chemoattractant, such as IL-8 (attracting neutrophils) or eotaxin (attracting eosinophils and basophils) (Geering et al., 2013).

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Neutrophils also have a phagocytic role in the immune response. They primarily engulf bacteria, viruses and damaged cells by phagocytosis and mediates intracellular killing. Neutrophils possess PRRs that, when activated, produce cytokines and chemokines to recruit immune cells, such as macrophages, dendritic cells (DCs), NK cells, and B- and T-lymphocytes to the inflamed site (Mantovani et al., 2011; Geering et al., 2013). Neutrophils produce large quantities of reactive oxygen species and release lytic enzymes from their internal granules to enact antimicrobial cytotoxicity to bacterial pathogens (Chaplin, 2010; Mantovani et al., 2011).

Basophils are best known for their activation of inflammation and allergic effector responses. Basophil activation is triggered by immunoglobulins (immunoglobulin (Ig) E, IgG, IgD, etc), cytokines and chemokines as well as PRRs (Steiner et al., 2016). Upon activation, basophils release various vasoactive mediators, including histamine, cysteinyl leukotrienes, prostaglandins, anti-microbial peptides and cytokines, mediating their immune responses (Steiner et al., 2016). Basophils act against protozoa, parasites and helminthic infections, and induce T_H2 function by their synthesis of copious amounts of IL-4 and IL-13 in response to IgE crosslinking, which can be protective against these pathogens (Karasuyama et al., 2011; Cromheecke, Nguyen & Huston, 2014; Steiner et al., 2016). Basophils are responsible for eliciting hypersensitivity reactions, and contribute to autoimmunity development (Steiner et al., 2016; Chirumbolo et al., 2018).

Eosinophils play a major role in allergy (along with basophils) (Martin et al., 1996; Cromheecke, Nguyen & Huston, 2014), asthma and parasitic infections (O'Connell & Nutman, 2015). Eotaxins chemotactically recruit eosinophils to inflamed tissue, where they are involved in clearance of larger microorganisms by releasing cytotoxins and mediate tissue repair and remodelling in the affected site. Eosinophils encourage the recruitment of T_H1 T-lymphocytes through chemoattraction by expression of chemokines. CXCL10 and CCR3 are known to bind to CXCR3 on T-lymphocytes, possibly as a mechanism for the production of antigen-specific memory cells (Jacobsen et al., 2007). Eosinophils can modulate adaptive immunity through the production of T_H2-inducing cytokines, e.g. IL-4, IL-8, IL-10, and IL-13, polarizing the T-lymphocytes to a T_H2-mediated inflammatory response (Jacobsen et al., 2007; O'Connell & Nutman, 2015; Diny, Rose & Čiháková, 2017).

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2.1.1.3. Natural killer cells and natural killer T-lymphocytes

Natural killer cells (NKs) and natural killer T-lymphocytes (NKTs) are considered part of the innate immune system as they do not require antigenic exposure for activation (Chaplin, 2010; Wu & Kaer, 2013). However, NKTs are considered an intermediary between NKs and T-lymphocytes due to their expression of the T-lymphocyte receptor (TCR) as well as conventional NK cell receptors. (Chaplin, 2010; Wu & Kaer, 2013). Both NKs and NKTs are producers of IFN-γ and TNF-α and can promote the maturation of DCs, which in turn produce type I interferons and IL-12 to activate NK cells. NK cells are able to kill infected or neoplastic cells within the host by inducing apoptosis of the affected cell (Cerwenka & Lanier, 2016). NK cells also affect adaptive immunity by acting directly on B- and T-lymphocytes, either promoting their activity or killing activated cells (Vivier et al., 2008).

2.1.1.4. Pathogen recognition in the innate immune system

Antigen presentation cells (APCs) (e.g. monocytes, macrophages and dendritic cells) in the innate immune system recognise a broad range of microbes by relying on PRRs. PRRs bind conserved specific moieties, either pathogen-associated molecular patterns (PAMPs) present on large group of pathogens, or host “danger” signals present as result of cellular damage, named damage-associated molecular patterns (DAMPs). DAMPs are host molecules released during the cell lysis and tissue damage in infectious and sterile inflammation, and include moieties such as ATP, heat shock proteins, and uric acid (Turvey & Broide, 2010; Kumar, Kawai & Akira, 2011).

Several PRR classes have been characterised, e.g. toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG)-I-like receptors (RLRs) and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) that can identify various molecules, such as proteins, lipids, carbohydrates and nucleic acids (Iwasaki & Medzhitov, 2004; Kumar, Kawai & Akira, 2011). The recognition of PAMPs by plasma membrane- and endosomal TLRs activate TLR signalling pathways. All TLRs activate the MyD88-dependent signalling pathway, with the exception of TLR3. TLR3 activates the toll/interleukin-1 receptor domain (TIR)-domain-containing adapter-inducing interferon-β (TRIF)-dependent signalling. MyD88-dependent signalling is initiated by the activation of interleukin-1 receptor (IL-1R) associated kinase (IRAK) family proteins

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and subsequently TNF receptor associated factor (TRAF)6. TRAF6 then recruits TAK1-binding protein 2 (TAB2) and activates TAB2-associated TGF-β- activated kinase 1 (TAK1), which in turn activates the IκB kinase (IKK)-complex, thereby inducing NF-κB subunits to initiate the transcription of inflammatory cytokine gene expression. The TLR7- and TLR9-mediated activation pathways trigger NF-κB signalling, and in addition, activate type I interferon production via MyD88/interferon regulatory factor (IRF) 7 pathway. Recognition of PAMPs in RLR signalling occurs through IPS-1, a mitochondria-localized adaptor protein, and results in NF-κB and IRF3/IRF7 activation, and thus the production of inflammatory cytokines and type I interferons. PAMP recognition by NOD1 and NOD2 in NLR signalling initiates the NF-κB signalling via the IKK complex.

Another member of the NLR family is the inflammasome, which is a multiprotein complex controlling the release of the potent inflammatory cytokine IL-1β and IL-18 (part of the IL- family, T_H1 inducing cytokine, stimulates IFN-γ production). The release of these inflammatory mediators is tightly regulated by these cytosolic multiprotein complexes. The inflammasome is responsible for the cleavage and activation of caspase-1, which in turn, cleaves the biologically inactive pro-IL-1β and pro-IL-18 into functional IL-1β and IL-18 (Menu & Vince, 2011; Strowig et al., 2012).

The nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) complex is the best characterised inflammasome complex, especially in the context of inflammatory conditions. This inflammasome complex consists of several subunits, namely, the NLRP3 scaffold, the apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) adaptor and caspase-1. The ASC adaptor molecule, which is made up of two domains of the death-fold superfamily, a pyrin domain (PYD) and a caspase recruitment domain (CARD), is central to the inflammasome function. NLRP3 inflammasome activation in macrophages require two signals: (1) an initial priming signal via PAMP activation (e.g. TLR4 activation by lipopolysaccharide (LPS)), resulting in the transcription and the synthesis of pro-IL-1β and (2) endogenous products released during cellular damage, disease or stress, such as ATP (Ling & Groop, 2009; Schroder & Tschopp, 2010; Paugh et al., 2015). Upon initiation of inflammasome activation via signal 1 and 2, the ASC adaptor initiates the process by recruiting pro-caspase-1 via CARD-CARD

interactions. The biologically inactive pro-caspase-1 then undergoes cleavage to become the bioactive caspase-1. The caspase-1, in turn, cleaves the pro-IL-1β and pro-IL-18 into IL-1β and IL-18 (Schroder & Tschopp, 2010; Sester et al., 2015; Jha et al., 2017).

The innate activation of the adaptive immune system is illustrated in Figure 2.1. Following recognition by PRRs, APCs process foreign bodies and present these processed antigens on their cell surface via major histocompatibility (MHC) complexes. This antigen-presentation signal (signal 1), as well as the subsequent expression of co-stimulatory molecules (signal 2) and specific cytokine production (signal 3) directs and controls the type and extent of the adaptive immune response in the host (Chaplin, 2010; Goenka & Kollmann, 2015).

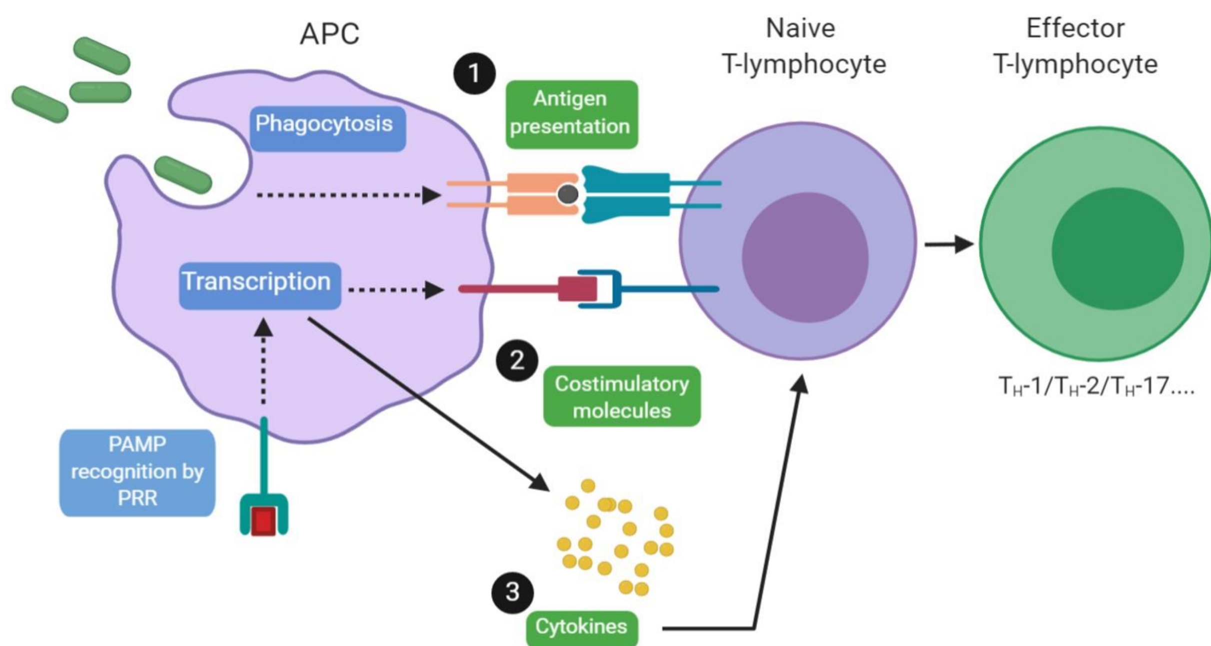

Figure 2.1 Innate control of adaptive immune responses (adapted from Goenka & Kollmann, 2015). Signal 1, signal 2 and signal 3 all determine the adaptive immune responses.

2.1.2. Adaptive immunity

The adaptive immunity allows for highly specific and variable immune responses to pathogens. It can also confer lifelong immunological memory against a pathogen, and form a rapid immunological response with re-exposure to the same pathogen. B- and

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T-lymphocytes are the effector cells of the adaptive immune system and differentiate from common lymphoid progenitor cells (Chaplin, 2010). These lymphocyte subsets tend to traffic from the spleen and other lymphoid tissues to circulation and tissue to wield their effector function when and where necessary (Bonilla & Oettgen, 2010).

2.1.2.1. B-lymphocytes

B-lymphocytes mature within the bone marrow are primarily characterised by their immunoglobulin (Ig) or antibody production, e.g. IgM, IgD, IgA, IgE and IgG subclasses. B-lymphocytes mediates humoral immunity by means of antibody production from mature plasma cells after stimulation from T-lymphocytes and APCs. During maturation, B-lymphocytes undergo several developmental stages for antigenic specificity, in the absence of foreign antigen exposure, before entering circulation in a naïve state. The second phase of B-lymphocyte maturation is antigen-dependent (either T-lymphocyte dependent or independent) and, depending on the secondary stimuli received, the B-lymphocyte will become either a memory cell, responsible for antigenic memory, or a plasma cell, responsible for producing large amounts of antibodies directed against a specific antigen (Bonilla & Oettgen, 2010).

2.1.2.2. T-lymphocytes

T-lymphocytes mature in the thymus and are characterised by the expression of the αβ TCR on their cell surface. The TCR interacts with the major histocompatibility complexes (MHC) class I or II on APCs during antigen presentation. MHC class I proteins are expressed on all nucleated cells, and this complex presents processed peptides from endogenous host antigens (either antigens from the host genome or virally infected host cells) or from intracellular antigens. However, the MHC class II complex is only expressed on APCs and present antigens identified by PRRs. T-lymphocytes are divided into two major subsets, namely the CD4+ T-lymphocytes and CD8+ T-lymphocytes, to effect the necessary immune function (Bonilla & Oettgen, 2010; Chaplin, 2010).

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2.1.2.2.1. Helper T-lymphocytes

CD4+ T-lymphocytes are known as T-helper (T_H) lymphocytes and are the largest T-lymphocyte subpopulation. T_H subsets are derived from naïve T_H0 precursor lymphocytes and their resultant activated subpopulations are influenced by specific antigen and cytokine exposure and their resulting capacity to mediate their effector functions (Bonilla & Oettgen, 2010; Chaplin, 2010).

T_H1 type CD4+ lymphocytes differentiate from T_H0 with IL-12 and IFN-γ stimulation, which activate T-box–containing protein expressed in T cells (T-bet) and are characterized by the production of IFN-γ and IL-2. These T_H1 cytokines drive the pro-inflammatory and cell-mediated responses, such as activation of cytotoxic T-lymphocytes and M1 macrophages and stimulate killing of infected host cells by NK cells (Bonilla & Oettgen, 2010). T_H2 CD4+ lymphocytes characteristically produce IL-4, IL-5, IL-10, and IL-13, and their differentiation is directed by IL-4 and GATA-3 transcription factor activation. The T_H2 cytokine profile stimulates antibody production from B-lymphocytes, immune responses against parasitic infections, as well as hypersensitivity reactions (Bonilla & Oettgen, 2010).

The T_H17 lymphocyte lineage is induced by transforming growth factor (TGF)-β and IL-6, and the resulting activation of the RAR-related orphan receptor gamma transcription factor (RORγt) transcription factor. T_H17 cells produce IL-17A and IL-17F, which promote IL-6 and TNF-α production and enhanced neutrophil recruitment into tissue (Bonilla & Oettgen, 2010). T_H9 lymphocytes are induced by stimulation of T_H2 lymphocytes by IL-4 and TGF-β, and they characteristically produce IL-9. IL-9 is a mast cell growth factor and mediates anti-helminthic immune responses. The regulation of the inflammatory immune responses is a function of regulatory CD4+ T-lymphocytes (T-regs), also known as T_H3 CD4+T-lymphocytes. This subset expresses the forkhead box P3 transcription factor (FOXP3) and is induced by TGF-β production and produces immunosuppressive cytokines such as IL-10 and TGF-β (Bonilla & Oettgen, 2010).

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2.1.2.2.2. Cytotoxic T-lymphocytes

The CD8+ cytotoxic T-lymphocytes (CTLs) primarily target and kill transformed or infected host cells in a contact-dependent mechanism via MHC I. The recognition of foreign peptides on the MHC I of the target cell leads to the formation of an immunological synapse. CTLs then release serine protease granules (e.g. granzymes and perforin) into the synapse that target proteins in the host cell, initiating apoptosis in the target cell. The TCR/MHC activation also upregulates Fas ligand expression on the CTLs, which bind Fas on the host cell membrane, triggering apoptosis.

The complementary activity between the innate and adaptive immune system allows for efficiency in the immune response. The ability of the innate system to sense and discriminate “danger” - being the first responders to infection - and subsequently activating the adaptive immune system, is beneficial for immune homeostasis (Bonilla & Oettgen, 2010; Chaplin, 2010; Turvey & Broide, 2010).

The crosstalk between the innate and adaptive immune system determines the type and duration of the inflammatory response. The initial acute inflammation is tightly controlled and essential for an effective host immune response. This process mobilises leukocyte crossing the endothelial cell barrier, moving from circulation to affected site (Hopkin et al., 2019). However, in chronic inflammation, the immune response is disproportionate and more long-term, increasing the risk of extensive tissue damage. In order to maintain homeostasis, activation of the hypothalamic-pituitary-adrenal axis and the resulting glucocorticoids have been known to exert immunomodulatory effects to curb excessive inflammation (Busillo & Cidlowski, 2013).

2.2. The hypothalamic-pituitary-adrenal axis in immune regulation

This section is aimed to provide a brief overview of the steroidogenesis of glucocorticoids and glucocorticoid signalling. This will be followed by a discussion of the impact of the HPA axis on the glucocorticoid and HPA-axis regulation of inflammation and the immune response.

2.2.1. The hypothalamic-pituitary-adrenal-axis

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The HPA axis plays an important role in homeostasis and regulation of many physiological processes. Endogenous glucocorticoids (GCs), mostly cortisol in humans and other mammals and corticosterone in rodents (although humans do produce corticosterone, but to a significantly lower extent than rodents, and this also accounts for rodents and cortisol), are stress hormones that regulate various transcriptional pathways in cellular development, regulating carbohydrate, fat, and protein metabolism as well as the cardiovascular, immune, reproductive, and central nervous systems. GCs are synthesized from cholesterol through steroidogenesis in a circadian rhythm- and stress-dependent manner and are able to exert their function in almost every part of the body (Oakley & Cidlowski, 2011; Biddie, Conway-campbell & Lightman, 2012; Kadmiel & Cidlowski, 2013).

2.2.2. The hypothalamic-pituitary-adrenal axis pathway and glucocorticoid synthesis

Endogenously GCs are synthesised in - and released from - the zona fasciculata of the adrenal glands, a process tightly controlled by the dynamic circadian and ultradian rhythms of the hypothalamus. Cortisol (COR) is the primary endogenous adrenal steroid in most mammals, including humans. Rodents, however, lack the 17-α hydroxylase enzyme (CYP17) present in the adrenocortical zona fasciculata, thus produce corticosterone (CORT) in their adrenals (Gallo-Payet & Battista, 2014). Corticotropin-releasing hormone (CRH) is secreted by the hypothalamus and subsequently induces the production and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. The release of ACTH results in the synthesis and secretion of COR/CORT from the adrenal glands into the bloodstream. In peripheral blood, COR/CORT is inactive as it is bound to corticosteroid-binding globulins. Type 1 11β-hydroxysteroid dehydrogenase (11β-HSD1) catalyses the conversion of inactive cortisone to COR/CORT, while the type 2 11β-hydroxysteroid dehydrogenase (11β-HSD2) enzyme catalyses the conversion of active COR/CORT to the inactive form. The glucocorticoid homeostatic balance is maintained by negative-feedback mechanisms that suppress ACTH production in the anterior pituitary and CRH levels in the hypothalamus (Biddie, Conway-campbell & Lightman, 2012; Kadmiel & Cidlowski, 2013; Quax et al., 2013). The adrenal GC production

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induced upon activation of the HPA axis results in GC release into the circulatory system to exert its functions.

While stressors result in the synthesis of GCs, their subsequent physiological functions are mediated through the activation of their glucocorticoid receptors (GRs). Its noteworthy to mention that endogenous GCs also have a high affinity to the mineralocorticoid receptor (MR), a receptor specific for aldosterone, which in turn regulates sodium reabsorption. While MRs are highly expressed in the kidneys, colon, heart and hippocampus, it is expressed at low levels on leukocytes (Cain & Cidlowski, 2017).

2.2.3. The glucocorticoid receptor structure and function

GCs are lipophilic and diffuse through cell plasma membranes and bind to cytosolic GR to enact their physiological functions. The GR is part of the nuclear receptor family and encoded by *NR3C1* gene on chromosome 5 in the human genome (Baschant & Tuckermann, 2010; Oakley & Cidlowski, 2011; Quax et al., 2013). *NR3C1* has nine exons and undergoes alternative splicing, which results in various GR isoforms: GRα, GRβ, GRγ, glucocorticoid receptor A and glucocorticoid receptor P (Quax et al., 2013). GRα is generally accepted to be the biologically active isoform, whereas GRβ acts as its transcriptional inhibitor (Oakley & Cidlowski, 2011; Quax et al., 2013). Due to its ubiquitous expression on almost all mammalian cell types, GR is considered a necessity for sustaining life.

GR signalling has been extensively reviewed in the literature (Baschant & Tuckermann, 2010; Biddie, Conway-campbell & Lightman, 2012; Kadmiel & Cidlowski, 2013). The GR is distributed primarily within the cytoplasm of cells as part of a large protein complex and is composed of several subunits. This receptor has a N-terminal transactivation domain (NTD), which binds co-regulatory molecules and transcription components; a DNA-binding domain (DBD); which consists of two zinc finger motifs that recognise and bind target DNA sequences, namely glucocorticoid-responsive elements (GREs); and a ligand-binding domain (LBD) on its C-terminal, which consists of 12 α-helices and four β-sheets, and forms a hydrophobic pocket for binding GCs. Upon GC-GR interaction, a conformational change is triggered and the GR dissociates from its multiprotein complex, hyperphosphorylation occurs and the receptor then

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translocates into the nucleus. In the nucleus, the GR dimerises and binds directly to GREs to either activate or suppress the expression of target genes. GR binding to the GRE results in another conformational change and the recruitment of co-regulators and chromatin-remodelling complexes, ultimately modifying gene transcription by directing RNA polymerase II activity. The receptor interacts only briefly with target genes, as it rapidly cycles on and off the GRE, possibly allowing the GR to bind to many binding sites and interacting proteins (Baschant & Tuckermann, 2010; Biddie, Conway-campbell & Lightman, 2012; Kadmiel & Cidlowski, 2013). Although the HPA-axis has many roles in homeostasis, this review will focus on the role of the HPA-axis in modulating immune function.

2.2.4. Glucocorticoid activity in host immune homeostasis

GCs play an essential role in the resolution of the inflammatory immune responses, thereby shielding against detrimental hyperinflammatory outcomes (Silverman & Sternberg, 2012). GCs affect the functions on the innate and adaptive immune cells (Escoter-Torres et al., 2019). Due to their immunomodulatory nature, glucocorticoids exert influence on various leukocytes in the innate and adaptive effector cells by manipulating their survival and functional response. This will be reviewed in more detail below.

2.2.4.1. Innate immune cells

Beginning the discussion with innate immune cells, GCs suppress the macrophage M1 type inflammatory responses via TLRs, inhibiting NF-κB and AP-1 transcription activity of pro-inflammatory cytokines and chemokines and shift towards an M2 phenotype (Franchimont et al., 1999; Bhattacharyya et al., 2007; Martinez & Gordon, 2014; Dong et al., 2018). This GC action mediates resolution of inflammation, avoiding undue hyper-inflammatory responses. GCs affect the recruitment of mononuclear phagocytes (e.g. macrophages, myeloid dendritic cells) by reducing the expression of adhesion molecules endothelium (Cronstein et al., 1992), thereby limiting their tissue infiltration and localised inflammation. The GR has been shown to play a protective role in the host by limiting LPS-mediated inflammation in macrophages. For example, mice with a conditional GR deletion in their macrophages were more susceptible to LPS-induced systemic inflammation (Bhattacharyya et al., 2007) and demise. GCs

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suppress inflammatory cytokine and chemokine (TNF-α; IL-1β; MCP-1, MIP2 and IP-10) production associated with contact allergy (Tuckermann et al., 2007).

Similar to monocytic cells, granulocyte populations, i.e. neutrophils, eosinophils, and basophils, are also under GC regulation. GCs inhibit neutrophil apoptosis (Saffar, Ashdown & Gounni, 2011) and at the same time GCs limit neutrophil migration into tissue by downregulation of L-selectin and CD18 adhesion molecules on the neutrophils and endothelium (Filep et al., 1997). GCs reduce superoxide and myeloperoxidase release (Liu et al., 2014) and downregulate pro-inflammatory cytokine production induced by LPS, such as IL-1β, TNF-α and IL-8 (Hirsch et al., 2012). GC treatment has been shown to induce apoptosis in basophils (Yoshimura et al., 2001), indicating mediation in allergy. Similar to basophils, GCs induce apoptosis in activated eosinophils, which has clinical implications in asthmatic patients. Improvement in these patients was associated with increased apoptotic eosinophils in bronchial biopsies and in sputum samples (Meagher et al., 1996; Druilhe, Létuvé & Pretolani, 2003).

2.2.4.2. The NLRP3 inflammasome complex

A complex central to the inflammatory response – and the topic of this thesis – which is similarly assembled in most innate immune cells, is the NLRP3 inflammasome complex. Glucocorticoids play a role in the NLRP3-mediated response to DAMPs in the acute inflammatory response to enhance tissue repair. LPS-stimulated macrophages rapidly upregulate NLRP3 production, in the presence of GCs. Also, in combination with ATP, GC-treated macrophages enhance NLRP3 processing and the release of IL-β, along with enhanced production of TNF-α and IL-6 (Busillo, Azzams & Cidlowski, 2011). However, in chronic inflammatory conditions such as acute lymphoblastic leukaemia, high expression of caspase-1 and NLRP3 is reported. This overexpression of caspase 1 results in cleavage of the GR, thereby diminishing the GR-mediated translocation and transcriptional response, inducing glucocorticoid resistance (Paugh et al., 2015).

Inflammasome activation is known to activate NK cells, e.g. via their release of IL-18. In contrast, murine NK cells treated with dexamethasone (a synthetic GC) show less proliferation and reduced cell activation, pushing them to a more immature state by

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favouring CD11b-CD17- expression. NKT-lymphocytes however, were not significantly affected by GC treatment (Chen, Jondal & Yakimchuk, 2018). GCs interaction results in the downregulation of TNF-α and IFN-γ production in splenic and liver NK cells, and inhibits their activation mediated by either IL-2/IL-15 (Morgan & Davis, 2017) or IL-12/IL-18 (Quatrini et al., 2017) , although not via driving them to apoptosis.

2.2.4.3. Adaptive immune cells

Glucocorticoid receptor activation hinders T-lymphocyte signalling via the TCR-mediated pathway by inhibiting protein kinase (PK) Scr, leading to the suppression of the phosphorylation downstream of the pathway, including mitogen-activated protein kinase (MAPK), Jun kinase N-terminal (JNK), protein kinase B (PKB), protein kinase C (PKC), and p38. At low doses, GCs inhibit the calcium signalling (required as a secondary messenger in TCR activation) in the TCR transduction pathway downstream by acting on inositol 1, 4, 5-triphosphate (IP_3). GCs act on mitochondria present in immature double positive T-lymphocytes, by altering transmembrane potential and ATP availability, inducing apoptosis (Zen et al., 2011). Thymocytes with suboptimal or TCR signalling or with a high affinity to host proteins are particularly susceptible to GC-induced apoptosis (Wiegers et al., 2001; Zen et al., 2011).

GCs inhibit the transcription of many T-lymphocyte derived cytokines. In differentiated mature T-lymphocytes, GC administration reduces T-lymphocyte numbers (CD3+ CD4+ and CD8+) (Chen, Jondal & Yakimchuk, 2018), suppresses T-bet (T_H-1 response) transcription factor activity and subsequently the production and release of T_H1-specific pro-inflammatory cytokines such as IL-12, IFN-γ and TNF-α (Elenkov & Chrousos, 1999; Baschant & Tuckermann, 2010). The activity of the T_H2 defining GATA binding protein 3 (GATA-3) transcription activity is suppressed by the GR activity, suppressing cytokines such as IL-4 and IL-5 (Baschant & Tuckermann, 2010).

In contrast, T-reg lymphocyte proliferation is enhanced with GC treatment (Baschant & Tuckermann, 2010; Zen et al., 2011; Chen, Jondal & Yakimchuk, 2018). GC treatment encourages the expansion of both thymic and splenic T-regs, and upregulates FOXP3 expression and the production of immunosuppressive cytokines

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IL-10 and TGF-β (Pap et al., 2019). This lymphocyte subset is crucial in the resolution of inflammatory responses.

In terms of B-lymphocytes, a study revealed that murine B-lymphocytes express GR in all developmental stages both in the spleen and bone marrow (Gruver-Yates, Quinn & Cidlowski, 2014). While dexamethasone is able to induce B-lymphocyte apoptosis on all developmental stages, *in vivo* results show that, similar to T-lymphocytes, immature B-lymphocytes are particularly susceptible to this, while multiple GC doses are necessary to reduce B-lymphocyte counts (Gruver-Yates, Quinn & Cidlowski, 2014). This is seen with chronic GC treatment, the predominant effects of GCs on B-lymphocyte proliferation and IgG production (Cupps et al., 1985).

In addition, it is necessary to consider other role players in the context of HPA-axis and immune function. One such factor, which has recently come under the spotlight, and which has relevance given the dissertation topic, is sex differences.

2.3. Sex differences in immune and HPA regulation

The sexual dimorphism in immune function and glucocorticoid regulation has been recognised, with differences in X and Y chromosomes and hormones being given as the reason (Klein, 2012; Goel et al., 2014). In terms of immunity, this topic has been well reviewed by several articles in the general context as well as in disease (Robinson & Klein, 2012; Klein & Flanagan, 2016; Rainville, Tsyglakova & Hodes, 2018). Generally, females seem to show greater immune reactivity than males. More specifically, females typically have a higher count of innate and adaptive immune cells, as well as immunoglobulin levels. Thus, they can theoretically mount a more robust immune responses than males. In terms of adaptive immunity, females have higher CD4+ T-lymphocytes counts and a higher CD4+/CD8+ T-lymphocyte ratio, suggesting a more T-helper lymphocyte presence. However, males show increased CD8+ T-lymphocytes, which, along with their known higher NK cell count, indicates a more cytotoxic phenotype. This is supported by the T_H1 bias in cytokine production in males. In contrast, females show a relative T_H2 cell bias (Klein, 2012; Klein & Flanagan, 2016; Rainville, Tsyglakova & Hodes, 2018). In terms of innate pathogen recognition, females tend to have higher expression of PRRs on APCs (monocytes, macrophages, and DCs), and they respond more readily to *in vitro* stimulation (Klein, 2012; Klein &

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Flanagan, 2016). The phagocytic activity of neutrophils and macrophages is also higher in females (Klein, 2012; Klein & Flanagan, 2016; Rainville, Tsyglakova & Hodes, 2018).

When considering GCs, human studies show less consistency in their findings. Reasons for this may be due to the lack of standardisation in type of sample collected (saliva vs plasma or serum), time of day or type and timing of stressor (Goel et al., 2014). Nevertheless, a meta-analysis of the COR responses reported a sex-specific trend for higher COR in female children and adolescents than males in response to stress (Hollanders et al., 2017). Animal studies show a more consistent picture and confirmed that females indeed produce more GCs than males, especially in response to stress. According to a review on the topic, the increased CRH levels, may result in increased GC synthesis in the paraventricular nucleus of the hypothalamus, along with heightened sensitivity of the pituitary and adrenals explains the increased HPA axis responsiveness in females (Goel et al., 2014).

In terms of investigations on leukocyte GR expression, few studies look at the sexual dimorphism of GR expression, given the evidence for sex-specific dimorphism in the HPA-axis and immune responses. From the limited literature, it is reported that GR expression is varied among the leukocyte subsets overall, with eosinophils exhibiting highest GR expression (Lu, Cooper, et al., 2017; Lu, Radom-Aizik, et al., 2017). When looking specifically at sex differences, females had an overall lower GR expression on monocytes, T-lymphocytes and NK cells, NKT cells, neutrophils and eosinophils in circulation. Furthermore, testosterone production was positively associated with increased GR expression in T lymphocytes and NK cells, while oestradiol production was negatively associated by GR expression in NKTs and T-lymphocytes (Lu, Radom-Aizik, et al., 2017). Taken together, GCs play a significant role in the immune modulation, and may contribute to the sex-specific regulation of immune cell number and function.

Of particular relevance, males seem more able to mount T_H1 inflammatory responses and cytotoxic activity than females (Robinson & Klein, 2012; Klein & Flanagan, 2016; Rainville, Tsyglakova & Hodes, 2018). This sex-specific differences may point to specific requirements in the female body, such as a less inflammatory phenotype, to support fertilization and pregnancy.

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2.4. Maternal Immune and endocrine regulation during the gestational period

Mammalian gestation is an immunological phenomenon that relies on tolerance of a semi-allogeneic foetus (Sykes et al., 2012; Morelli et al., 2015). Full term pregnancy is essential for species survival; thus, a fine balance exists in endocrine–immune functions that determine the outcome of pregnancy. During pregnancy, the process of decidualisation occurs, which involves remodelling of various compartments of the uterine mucosal cellular layer (endometrium) for implantation and establishment and support of pregnancy. The maternal decidua comprises of different parenchymal cellular compartments, namely the glandular epithelial and luminal epithelial compartments, as well as the endothelium and stromal cells, all of which are transformed and remodelled in preparation for gestation (King, 2000; Morelli et al., 2015).

The maternal–foetal interface is identified as the interface between the uterine mucosa and the extraembryonic tissues. Leukocytes are abundant in the maternal decidua during normal pregnancy. In early pregnancy, NK cells, macrophages and T-lymphocytes make up the majority of the decidual leukocytes present (King, 2000; Sanguansermsri & Pongcharoen, 2008; Mor & Cardenas, 2010; Bonney, 2016; Solano, 2019), although other B-lymphocytes, dendritic cells and neutrophils are present, but rare.

NK cells (70%) and macrophages (20%) are the most abundant leukocyte cell type in the maternal decidua thought to be primarily responsible for decidual remodelling during gestation (King, 2000; Mor & Cardenas, 2010; Morelli et al., 2015). In human decidua, subsets of NK cells produce IFN-γ, TNF-α, IL-17 and IL-22, which are thought be important in tissue remodelling as well as in response to infection (Bonney, 2016; Solano, 2019). NK cell cytotoxic cell function is limited (Morelli et al., 2015) at this interface and is found to be regulated by inhibitory receptors, such as killer Ig-like receptors (KIRs), and activating receptors, inhibiting the placental trophoblast killing, which lack MHC-I expression (Bonney, 2016). Macrophages are 20% of decidual leukocytes (Morelli et al., 2015). While both M1 and M2 activation states are present during gestation, its generally considered that M2 activation predominates in gestation (Morelli et al., 2015; Solano, 2019). When considering uterine cytokine production at the maternal interface, IL- 4, IL-6, IL-10, IL-13, and macrophage colony-stimulating

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factor (M-CSF) production by T-lymphocytes and trophoblasts (outer layer of the blastocyst, which will develop into a large part of the placenta) favour a T_H-2 predominant environment. This is associated with a successful pregnancy outcome (Sykes et al., 2012; Morelli et al., 2015).

When considering foetal programming, the *in utero* milieu is finely regulated and dependent on various endocrine and immune factors to sustain a favourable environment for development and maturation. In this context, the mechanisms of glucocorticoid action have been extensively studied as a critical endocrine mediator of foetal programming. The GR is present in most foetal tissue and placenta, even in the embryonic phase, and is essential for foetal development and survival, e.g. maturation of liver, lungs, gut, skeletal muscle and adipose tissue (Seckl & Holmes, 2007; Harris & Seckl, 2011; Korgun et al., 2012).

As GCs are lipophilic, they can readily cross the placental barrier. However, foetal glucocorticoid concentrations remain much lower than those in pregnant females due to their functional inactivation of biologically active COR and CORT into inactive cortisone and 11-dehydrocorticosterone by the 11β-HSD2 enzyme produced by the placenta. Thus, the placenta modulates the availability of endogenous glucocorticoids in foetal tissue in response to maternal circulating GC levels (Seckl & Holmes, 2007; Harris & Seckl, 2011; Korgun et al., 2012). High 11β-HSD2 levels are also found in the placenta. Here, this enzyme plays an important role in protecting the foetus from the relatively high maternal glucocorticoid levels (Korgun et al., 2012; Moisiadis & Matthews, 2014).

In the context of inflammation, maternal prenatal inflammation is known to be a cause of preterm birth or foetal loss (Weinstock, 2005; Szarka et al., 2010; Sykes et al., 2012; Li et al., 2015; Ander, Diamond & Coyne, 2019), stressing the importance of a balanced homeostatic environment for sustaining gestation.

Interestingly, the interplay between the glucocorticoid system and immunoregulation is best observed in immune challenges that could result in adverse birth outcomes. In cases of preterm birth, typically caused by infection, inflammatory mediators TLR4, TNF-α, IL-1 and IL-6 are elevated in the amniotic fluid, increasing the risk of preterm birth and mortality (Romero et al., 2006; Pandey, Chauhan & Awasthi, 2017). As

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inflammatory cytokines are known to induce COR production (Dunn, 2006), the resulting elevated HPA axis which may be caused by glucocorticoid-induced suppression of inflammatory responses (Entringer et al., 2012).

2.5. Maternal transgenerational inheritance of disease

2.5.1. The development of chronic disease

World Health Organisation (WHO)-defined non-communicable diseases (NCDs), in particular cardiovascular diseases, chronic respiratory diseases (e.g. chronic obstructive pulmonary disease and asthma) and type 2 diabetes have become an important public health concern. NCDs, (referred to as chronic diseases) account for 41 million deaths worldwide, of which 36% are classified as premature deaths (age 30-69) (World Health Organization, 2018). Recently, psychological disorders (e.g. depression and anxiety) were classified as chronic disease due to their comorbidity with other NCDs (Stein et al., 2019). The risks of NCDs are exacerbated by a Western lifestyle, including poor diet and a sedentary habits (Gluckman, Hanson & Pinal, 2005; World Health Organization, 2018; Stein et al., 2019).

Considering this, a known aetiology in chronic diseases is chronic inflammation and HPA axis upregulation. In PTSD sufferers, patients have been reported to have altered serum COR and lymphocyte GR expression (Gotovac et al., 2003; Vidović et al., 2011), high blood neutrophil and monocyte counts (Lindqvist, Mellon, et al., 2017) and higher proinflammatory cytokine profile (Song et al., 1999; Carpenter et al., 2010; Murphy et al., 2014; Lindqvist, Dhabhar, et al., 2017; Niraula et al., 2019). The mechanism of this immune activation is better demonstrated in rodent models of chronic psychological stress. Chronic stress in mice was shown to induce haematopoiesis and trafficking of myeloid derived cells from spleen and bone marrow to tissue, resulting in increased inflammatory cytokine production and subsequent tissue-specific inflammation and injury (Wohleb et al., 2014; Ramirez, Fornaguera-Trìas & Sheridan, 2017; Li et al., 2018; McKim et al., 2018; Yin et al., 2019). Overall, this strong bias towards increased GC and T_H1 and T_H17 pro-inflammatory phenotype in psychological stress (Harpaz et al., 2013; Bergamini et al., 2018) can lead to GC resistance and increase the risk of exaggerated inflammation and development of autoimmune diseases (Harpaz et al., 2013).

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Similarly in obesity models, there is a clear link between inflammation, obesity and chronic disease in literature (Hotamisligil, 2006; Lumeng & Saltiel, 2011; Saltiel & Olefsky, 2017). HPA dysfunction is part of the disease aetiology, with high levels of COR and its tetrahydrometabolites being associated with metabolic syndrome, loss of β-cell function and insulin sensitivity. Obesity is widely characterized by the infiltration of M1 macrophage into adipose tissue and upregulation of PRRs such as TLR4 on inflammatory macrophages (Yu, Li, et al., 2019). TLR4 is widely implicated in obesity-induced chronic inflammation and metabolic disorders, as the expression of this PRR is correlated with the severity of insulin resistance, and elevated serum concentrations of IL-1β, IL-6, IL-8, and TNF-α in obese or diabetic patients, implicating TLR4-mediated inflammatory responses (Yu, Li, et al., 2019). In addition, obesity and metabolic disease is associated with increased the production of IL-1β, as well as the increased expression of *IL1B* and *NLRP3* mRNA, in adipose tissue macrophage and decreased regulatory T-lymphocyte counts (Esser et al., 2013).

Given the long-term effects of chronic disease on immune modulation, it stands to reason that maternal stress, as such, may not only affect the mother, but the developing foetus as well. The foetal programming hypothesis popularised by Barker proposes that aberrations occurring during the critical foetal development determine the physiological and metabolic phenotype established in the offspring and, thus, their susceptibility to disease later in life (Barker, 1998, 2007). Barker conceptualised that foetal undernutrition *in utero* resulted changes the offspring body structure, hormones (e.g. COR, insulin) and metabolism, increasing the risk of developing insulin resistance and hypertension, stroke (Barker, 1998) and coronary heart disease (Barker, 2007) in later life. While foetal plasticity is a necessary and advantageous feature to develop a physiological phenotype able to adapt to changes in the environment, some insults during the foetal developmental stage may result in deleterious consequences.

2.5.2. Inheritance of maternal chronic inflammation

Considerable evidence supports the developmental programming of chronic diseases. In human cohorts, maternal transmission of maladaptation has been seen in aspects of malnutrition (Lumey et al., 2007; Heijmans et al., 2008), obesity (Entringer et al., 2012; Wilson et al., 2015; Sureshchandra et al., 2017; Ling et al., 2018), psychological stress (Brand et al., 2006; Yehuda et al., 2008; Franklin et al., 2010; O'Connor et al.,

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2013; Lehrner et al., 2014; Kubo et al., 2018), and immune activation (Spann et al., 2018). These adverse events could contribute to, not only the onset of immune and HPA dysfunction in childhood but could predispose the affected individuals to developing chronic diseases in adulthood.

Human and animal studies have confirmed that maternal and intrauterine perturbations affect the endocrine and immunological processes during gestation. For example, maternal psychological stress is associated with an inflammatory profile in gestational mothers, with increased concentrations of IL-6, TNF-α and C-reactive protein (CRP) and low IL-10 concentrations in circulation, (Coussons-Read, Okun & Nettles, 2007; Christian et al., 2009). Similarly, pregnancy-induced hypertension and pre-eclampsia are considered inflammatory conditions, with elevated serum pro-inflammatory cytokines and chemokines, and M1 bias in macrophages in peripheral blood, which was associated with M1 macrophage infiltration into placenta (Ma et al., 2019). Maternal obesity and the subsequent peripheral and placental inflammation have been described, with increased plasma IL6, IL-8 and CRP, along with increased *TLR4, IL-8 and IL-6* mRNA expression in the placenta of obese compared to lean women. TLR4 expression in obese mothers was associated with increased placental cytokine expression and insulin resistance, and thus implicated in the propagation of placental inflammation (Yang et al., 2016). While the concept of maternal stress *in utero* has been described, information on the inheritance of the adaptive aspects of HPA control and immune regulation is lacking. Without knowledge of maternal transfer, we cannot decipher pregnancy-related exposures and sex-specific parental effects.

2.5.3. The nature of generational inheritance

Ancestral (parent, grandparent, great-grandparent, etc) environmental exposure is increasingly recognised as a determinant in offspring development and wellbeing. This resulting altered offspring phenotype can persist in later life (Painter et al., 2008; Manor & Koupil, 2010; Smith et al., 2010; Spann et al., 2018). In particular, maternal exposure to prenatal adversity can have a direct effect on offspring foetal development via placental transfer of nutrients, hormones or other metabolites (Devoto et al., 2017; Lindsay et al., 2017; Sureshchandra et al., 2017; Lye et al., 2018). For this reason, as shown by the abovementioned studies, developmental programming can be

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transmitted across generations, even without exposure of the grandchild/great-grandchild to the triggering adversity. Thus, the investigation into the cross-generational susceptibility of inherited maladaptation in offspring phenotype is imperative in the epidemiology of health and disease.

Generational transfer of environmental exposures has been summarised in Table 2.1 and is defined as either intergenerational or transgenerational. Briefly, intergenerational inheritance occurs when maternal (F0, first generation affected) exposure to the triggering stressor during gestation has direct influence on the phenotype of the developing foetus (F1, second generation affected), with the potential to persist to adulthood. This *in utero* stress could potentially also affect the primordial germline of F1 offspring, and subsequently continuing the altered phenotype of the F1 offspring to the grand-offspring (F2, third generation affected). Transgenerational inheritance of the stressor occurs when the altered maternal F0 adaptation still persists to the great-grand-offspring (F3, fourth generation) generation and further, with no further exposure to the triggering agent (Skinner, 2008; Dunn, Morgan & Bale, 2011; Portha, Grandjean & Movassat, 2019). If the F3 generation still retain the altered phenotype, this is likely due to epigenetic modification of the germline by either DNA methylation, histone modification or microRNA alterations (Taouk & Schulkin, 2016). Interestingly, paternal F0 stressors persisting into F2 and beyond is considered a transgenerational epigenetic inheritance of the altered phenotype, as there is no *in utero* exposure to the triggering stress past the F1 generation (Portha, Grandjean & Movassat, 2019).

Table 2.1 Definitions commonly used when describing transfer of information across generation.

Type of transmission	Definition
Intergenerational inheritance	Maternal exposure *in utero* (F0) results in altered phenotype in offspring, and potentially on the germ line of the F1 resulting in altered phenotype in F2.
Transgenerational inheritance	Maternal F0 exposure persists in F1, F2 and F3 on the maternal line or F2 and F3 in the paternal line.
Epigenetic multigenerational inheritance	Transmission of altered phenotype is passed on across generations via epigenetic modifications of the germline in the absence of any direct exposure or genetic manipulation.

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Evidence for generational inheritance has been observed both in animal models and in human studies and can be classified in into three main categories, depending on the generation affected (Drake & Liu, 2010; Dunn, Morgan & Bale, 2011; Szyf, 2015). The first category pertains to the phenotype developing presenting in F1 offspring only. For example, a previous study reported in a mouse model of *in utero* nutrient insufficiency, that low birth mass F1 and bone density deficiencies did not persist past F1 to F2 and F3, once malnutrition was corrected (Anevska et al., 2019). The second category is a modification of the first category and describes the resultant exposure in F1 offspring *in utero* affecting their embryonic somatic cells and gametes (to which will eventually form the F2 offspring). This is a more common inheritance category and has been described in human and rodent models of nutritional deficiency (Heijmans et al., 2008; Joaquim et al., 2017), obesity (Jimenez-Chillaron et al., 2009), maternal immune activation (Reis-Silva et al., 2016; Ronovsky et al., 2017) and GC administration (Iqbal et al., 2012), as well as psychological stress (Yehuda, Halligan & Grossman, 2001; Murgatroyd et al., 2016; Yehuda et al., 2016; Hicks-Nelson et al., 2017a; Nephew et al., 2017). Within this category, sex-specific heritability can be determined, if present. However, due to the exposure of the F1 primordial cells (that will form the F2 generation) to the triggering stressor, it is unclear whether the altered phenotype will persist to F3 or further. The last category is the transgenerational transmission of ancestral adversity to the F3 offspring and further. This is considered to be stable inheritance of the phenotype and has been shown in previous studies investigating behavioural differences in response to sGCs (Moisiadis et al., 2017), maternal immune activation (Weber-Stadlbauer et al., 2017) and obesity (Chamorro-Garcia et al., 2017). This phenotype occurs due to enduring epigenetic reprogramming of the F2 primordial germline and is potentially a permanent transformation. If the F3 offspring still carry an altered phenotype, this is likely due to epigenetic modification of the germline by either DNA methylation, histone modification or microRNA alterations (Taouk & Schulkin, 2016).

2.5.4. Epidemiology of maternal generational inheritance: evidence from other chronic conditions

In utero transmission of maternal adaptation is well described in historical human models and the mechanisms have been investigated in animal models. The first

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evidence of maladaptation resulting from grandmaternal and maternal exposure was reported from the Dutch Famine cohort studies. Exposure to famine in this period increased adiposity and *IGF DMR* hypomethylation in F1 males (Ravelli, Stein & Susser, 1976; Lumey et al., 1993, 2007), and linked to increased adiposity and poor health consequences in F2 offspring born to F1 mothers exposed to famine *in utero* (Painter et al., 2008). Transgenerational effects of psychological stress persisting into adulthood have been reported in the offspring of Holocaust survivors. Specifically, maternal PTSD was shown to increase glucocorticoid receptor sensitivity and induce hypomethylation of PTSD GR-1_F promoter hypermethylation in offspring with both parents suffering from PTSD, and to induce increased FKPB5 promotor methylation in leukocytes of adult offspring born to Holocaust survivors (Lehrner et al., 2014; Yehuda et al., 2014, 2016). In the adult children of Holocaust survivors, the changes in GC sensitivity and HPA signalling in leukocytes could result in an altered inflammatory immune profile, similar to what is observed in PTSD patients (Lindqvist, Dhabhar, et al., 2017; Lindqvist, Mellon, et al., 2017). These abovementioned observations highlight that susceptibility to disease can be transferred to subsequent generations, in the absence of direct exposure of offspring to the triggering agent. This has brought light to the topic of transgenerational inheritance in other models.

2.5.4.1. Human models

Evidence for maternal transgenerational inheritance of immune dysregulation has been demonstrated in chronic stress conditions, such as malnutrition/obesity, diabetes and psychological stress during gestation in humans and rodents. Diet-induced obesity is considered to result in a chronic inflammatory process, due to the increased inflammatory mediators associated with increased body fat (Strandberg et al., 2009; Zhou & Pan, 2015), and it presents with innate immune dysfunction in mice, can result in impaired pathogen clearance (Strandberg et al., 2009). In humans, delayed inflammatory responses has been reported in neonatal umbilical cord blood monocytes and myeloid dendritic cells from F1 offspring from obese mothers in response to TLR2/1 stimulation (Wilson et al., 2015) or TLR4 stimulation (Sureshchandra et al., 2017), with a pronounced skewing to T_H17-driven adaptive immune responses (Wilson et al., 2015). These abnormalities were linked to DNA hypomethylation of promoter regions of genes involved in defensive immune

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responses (*HLA-DRA, HLA- DRB5, IFNAR1, IFNGR2, IRF2, CD180, and CXCL1*), haematopoiesis (*KLF4, CD28, CEBPA, VEGFA, and EPHA2*) and apoptosis (*BCL6, KDM2B, PSMA6, FLT4, DAD1, and ERCC3*), ultimately driving an M2 phenotype in these offspring (Sureshchandra et al., 2017). However, discreet changes in DNA methylation in human studies should be carefully interpreted, as its extrapolation may highly specific to specific cell types and conditions.

In terms of psychological stress, a definite trend to inflammatory responses was observed. In humans, prenatal stress is associated with increased basal levels of IL-1β, IL-4, IL-5, IL-6 and IL-8 in neonatal cord blood (Andersson et al., 2016). Prenatal stress also resulted in increased innate immune activation (high TNF-α and IL-8 production after TLR3, TLR2/6 and TLR9 stimulation), greater magnitude T_H2 adaptive responses to allergy (high IL-13 with dust mite exposure) and T_H1 adaptive responses (diminished phytohaemagglutinin (PHA)-induced IFN-γ production) in F1 offspring (Wright et al., 2010). This points towards immune hyperresponsiveness and risk of allergy and atopy. Studies have shown that this dysfunction persists to later life. Adolescents and adults born to prenatally stressed mothers exhibited lower T-lymphocyte counts and increased innate TLR4-mediated inflammatory cytokine production (TNF-α, IL-1β, and IL-6) *in vitro* as well as enhanced production of T_H2 cytokines (IL-4 and IL-13) in response to *in vitro* PHA stimulation, which suggest a bias towards T_H2 increased polarization of CD4+ T-lymphocytes (Entringer et al., 2008; Veru et al., 2015). Their findings show that neonatal immune dysregulation induced by maternal prenatal stress indeed does persist to later life.

2.5.4.2. Animal models

Animal models have been employed in transgenerational studies to investigate the mechanisms of inheritance across several generations in a controlled setting. In rodent models, maternal hypercaloric diet consequently lead to increased immune activation in adult offspring, with increased TNF-α at basal level and in response to acute LPS challenge (Joaquim et al., 2017) as well as increased blood leukocytes (Dudele et al., 2017). Dudele and colleagues (2017) also described sexual dimorphism in the inheritance of metabolic maladaptation: F1 females demonstrated an altered metabolic phenotype, while F1 males showed pro-inflammatory phenotype and increased immune activation with exposure to prenatal high fat diet (HFD)

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programming *in utero.* Maternal HFD resulted in offspring intestinal inflammation (increased IL-1β, IL- 6, IFN-γ, and TNF-α mRNA) and microbial dysbiosis, enhancing the offspring susceptibility to dextran-sulfate-sodium (DSS)-induced colitis (Xie et al., 2018). Interestingly, the differences in rodent and human findings may be due to the age of F1 neonates. The reasoning for this could be due to the vast and rapid changes in blood composition of foetal/neonates and during early life during development (Olin et al., 2018), thus the permanency of the maladaptation may only present in later life where the phenotype is more stable.

In terms of psychological stress, chronic social stress (CSS) in early life (F0 dams were exposed to stress during lactation of F1 offspring), F1 CSS dams displayed persistently elevated plasma CORT levels in puberty, adulthood and during lactation of their F2 offspring, without any stress exposure (Carini & Nephew, 2013). The same research group also reported that F2 lactating CSS-affected dams (without any CSS intervention), had reduced milk and plasma CORT levels, but still demonstrated similar maternal behaviour in comparison to F1 CSS-affected dams (Nephew et al., 2017). In similar CSS paradigms, sex-specific transmission was reported with maternal CSS. While F2 CSS-affected females and males displayed dampened basal HPA activity (CORT) as juveniles (Babb et al., 2014), no difference in basal CORT levels were reported in adulthood. Instead, F2 CSS exhibited sex differences in their HPA-axis response to stressors. F2 CSS-affected females displayed enhanced HPA activity (ACTH and CORT) in response to immune stimulation (IL-1β administration) while F2 CSS-affected males increased HPA activation with psychological stress (restraint) (Grundwald & Brunton, 2015).

Immune functionality is also affected in rodent models of psychological stress. *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) vaccinated F2 CSS-affected offspring showed inflammatory cytokine responses (increased IL-6 production) in response to Concanavalin A, but they did not display protective T_H1 immunity (diminished IFN-γ) to BCG upon immune challenge (Hicks-Nelson et al., 2017a). Transgenerational CSS may thus have consequences for adaptive and innate immune functionality, by maintaining innate immune activation involving IL-6, while simultaneously reducing the IFN-γ-mediated development of effector and memory adaptive immune responses (Hicks-Nelson et al., 2017a).

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From the abovementioned literature, we can acknowledge the role of inflammation in the development of chronic disease and as a factor in the immunological outcome of offspring from mothers exposed to chronic disease during pregnancy. However, it is apparent that few studies focus on the transgenerational inheritance of immune maladaptation in F1 offspring, with even fewer assessing to the F2 generation and further. To delineate the role of inflammation, we will now consider maternal transgenerational models that directly induce inflammation or immune activation in order to assess the outcome in offspring.

2.5.5. Maternal immune activation

Maternal infection and/or inflammation is known to alter early foetal development and increase the risk for maladaptation during pregnancy. Epidemiological studies on maternal immune activation (MIA) reveal a unique relationship between maternal gestational inflammation and altered foetal brain and behaviour development, as offspring born to mothers exposed to infection or inflammation during pregnancy have a higher risk of developing neurobiological disorders (Brown et al., 2014; Canetta et al., 2014; Zerbo et al., 2015; Gilman et al., 2016; Jiang et al., 2016; Solek et al., 2018). While MIA cohorts provide invaluable insight into the aetiology of maternal stress studies, there are significant limitations on the topic of inheritance. Firstly, the extensive research in MIA is mainly focused on the aetiology of neurological disorders resulting from exposure to maternal inflammation *in utero* (reviewed by Solek et al., 2018) and do not investigate immune dysfunction in offspring in response to *in utero* MIA exposure. Secondly, MIA studies of offspring outcome is limited to the F1 generation, and no human data is available on transgenerational inheritance of MIA to F2 and beyond. Lastly, it is difficult to conclusively identify specific biological mechanisms for maladaptation due to the potential confounding factors present in human studies (e.g. maternal age, other stressors present, etc).

The effect MIA on offspring has been studied in experimental animal models, thereby limiting confounding factors present in human studies. Maternal immune activation is induced in animal models by activating the innate immune system. TLR3 activation with polyinositic: polycitidylic acid (poly(I:C)), a double stranded RNA moiety simulates viral infection; or TLR4 activation with LPS, a bacterial cell wall component on gram-negative bacteria, are popular choices for animal MIA models. Both LPS and poly(I:C)

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mimics host infection and induces an innate immune response, stimulating the production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α via TLR signalling and NF-κB activation (Meyer, 2014). Other inflammatory agents have also been used to induce MIA models, e.g. TLR7/8 (mimicking single stranded DNA viruses) (Missig et al., 2019), influenza virus (Shi, Tu & Patterson, 2005) or IL-6 (Smith et al., 2007). Considering the context of this thesis, which involves chronic maternal immune activation by LPS administration, the review of these models will focus on LPS-induced mouse models.

As expected in MIA, exposure to infection or immune stimulant typically results in elevated pro-inflammatory mediators (e.g. CRP, TNF-α, IL-6, IL-1β) in pregnant women (Coussons-Read, Okun & Nettles, 2007; Brown et al., 2014; Canetta et al., 2014; Spann et al., 2018) and animals (Gayle et al., 2004; Ashdown et al., 2006; Mandal et al., 2011; Fricke et al., 2018; Missig et al., 2019). Similarly, *in utero* MIA also upregulates inflammatory mediators in F1 offspring (Gayle et al., 2004; Ashdown et al., 2006; Ginsberg et al., 2012; Kirsten et al., 2013). However, these inflammatory effects have been demonstrated to be mediated by placental injury rather than exposure to the inflammatory agent. In a model of LPS-induced MIA (100*μ*g/kg LPS, once mid-gestation), it has been shown that LPS does not cross the placental barrier (Gayle et al., 2004; Ashdown et al., 2006; Fricke et al., 2018; Yan et al., 2019), but maternal LPS and subsequent inflammation was mirrored in placenta, triggering necrosis of the placental tissue. The resulting placental inflammation and necrosis lead to microbiome dysbiosis and intestinal (ileum) damage in MIA foetal offspring, which resembled neonatal necrotizing enterocolitis, and these effects persisted well into offspring adulthood (56 days old in mice) (Fricke et al., 2018). To further delineate the mechanism of necrotising enterocolitis, another MIA study stated that LPS-induced (50*μ*g/kg LPS, once, mid-gestation) inflammation reduced intestinal microvasculature and microvilli formation and downregulated vascular endothelial growth factor (VEGF) and its receptor (VEGFR2) (responsible for angiogenesis), thus resulting in necrotising enterocolitis and neonatal death. This disruption of vascular development was associated neonatal MIA-induced TNF-α production (Yan et al., 2019). While inflammatory cytokines do not cross the placenta under normal conditions (Aaltonen et al., 2005), it suggested that the offspring inflammation and intestinal damage could be due to exposure to placental by-products or cytokines in its compromised state.

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2.5.5.1. MIA: outcome in offspring

Not many studies have reported on the extent and longevity of MIA-induced inflammatory phenotype in F1 offspring. Some variability has been shown in acute versus chronic MIA models with differing adaptations in the affected offspring. In terms of data on offspring outcome, the effect of prenatal stress on the HPA-axis has been previously reviewed (Weinstock, 2005). In summary, gestational stress has been shown to result in increased production of maternal and foetal plasma CORT and thus downregulate foetal glucocorticoid receptors and impairing the negative feedback loop of the HPA axis. Considering the substantial pro-inflammatory modulation caused by *in utero* LPS exposure, as well as the link to MIA and behavioural adaptation, MIA studies have also investigated the CORT regulation with contrasting findings on their effect on F1 offspring in adulthood. A single administration of LPS in early gestation (Kirsten et al., 2013) or late gestation (Straley et al., 2014) induced a strong CORT spike in F0 rat dams after LPS administration, and is diminished over 24 hours. The resulting maternal LPS-induced IL-1β production can also influence foetal exposure to maternal corticosterone. IL-1β is able to downregulate placental 11β-HSD2 production, resulting in increased corticosterone exposure in foetal offspring via the placenta (Straley et al., 2014). This supports evidence suggesting that this maladapted endocrine state is linked to dysfunctional immunological responses in neonatal offspring. However, CORT findings in LPS-affected F1 adult offspring are somewhat different. While F1 LPS-affected offspring show no change in their basal CORT in comparison to controls (Williams, Teeling, Perry, Fleming, et al., 2011; Kirsten et al., 2013), they strongly upregulate plasma CORT in response to *in vivo* LPS challenge (Kirsten et al., 2013). This difference may be attributed to sensitization of the GC response to LPS but could also be an effect of timing of the maternal intervention (periconception versus early/mid gestation) as well as the dose of LPS received both during maternal LPS exposure and offspring immune challenge.

In terms of immune outcome, in an acute MIA model induced by LPS administration 0.5 days post conception, Williams et al (2011) demonstrated a blunted innate immune response with *in vivo* LPS (50 μg/kg) challenge in F1 LPS-affected offspring. This was dependant on the LPS dose received by the mother (10 -150 μg/kg), as offspring affected by higher doses of LPS *in utero* displayed a more pronounced blunted

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cytokine responses to LPS exposure than offspring from mothers that received lower doses. Furthermore, a sex-dependence was observed in their cytokine response to the LPS challenge. Males from 10 *μ*g/kg LPS-treated dams only blunted levels of IL-1α and granulocyte colony-stimulating factor (G-CSF), while males from 50 *μ*g/kg LPS-treated dams had blunted levels of increasingly more inflammatory mediators (MCP-1, IL-1α, IL-9, IL-10, G-CSF, MIP-1β and RANTES) (Williams, Teeling, Perry, Fleming, et al., 2011). Males from 150 *μ*g/kg LPS-treated dams also had blunted levels of IL-1α, IL-9, IL-1β, IL-10, MCP-1, MIP-1β and RANTES, but to a greater extent than the group exposed to 50 *μ*g/kg (Williams, Teeling, Perry, Fleming, et al., 2011). Female offspring show a different trend in response to the dose-dependent MIA. Low-dose MIA (10 *μ*g/kg) did not alter cytokine responses to LPS challenge in female offspring compared to saline controls (Williams, Teeling, Perry, Fleming, et al., 2011). However, moderate (50 *μ*g/kg) and high (150 *μ*g/kg) LPS-treated dams induced severe blunting of cytokine responses than that of controls (offspring from 50 *μ*g/kg LPS-treated dams. This treatment resulted in blunted levels of IL-1β, IL-12p40, G-CSF, CXCL1, MCP-1, MIP-1β, MIP-1β, RANTES and TNF-α in offspring. Offspring from 150 μg/kg LPS-treated dams had blunted levels of IL-1β, IL-12 p40, G-CSF, MCP-1, MIP-1α, IL-1a, IL-2, RANTES and TNF-α) (Williams, Teeling, Perry, Fleming, et al., 2011). This data suggests that MIA male offspring exerts a dose-dependent modulation of immune responses from moderate to severe blunting, while female offspring are not affected by low dose MIA but are prone to immune dysregulation at moderate and high dose MIA exposure.

Variations in dosage and timing of MIA is also an important consideration when looking at offspring immune responses. While Williams and colleagues (2011) induced MIA at low LPS doses early in pregnancy, a moderately high LPS-induced MIA (100 *μ*g/kg LPS) model by Kirsten and colleagues administered in mid-gestation (day 9.5) induced elevated inflammatory responses (higher TNF-α and IL-1β) in LPS-affected adult mice in response to LPS challenge (100 *μ*g/kg) (Kirsten et al., 2013). In contrast to Kirsten and colleagues, high LPS dose MIA in late gestation (500 *μ*g/kg, day 18) induced significantly lower IL-1β, IL-6 and TNFα in neonatal offspring in response to LPS challenge (Beloosesky et al., 2010). Thus, differences in timing, age and LPS dose are key factors that influence immunological outcome.

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In acute MIA models, single- and high LPS doses mimic prenatal infection or chorioamnionitis, and thus cannot be extrapolated to chronic disease experienced in human or mammalian pregnancy. As chronic inflammation is an ongoing immunological event, better insight might be gained when considering chronic or repeated dose MIA models. In a rodent MIA model, repeated LPS administration (200 *μ*g/kg) in pregnant dams during late gestation (day 16, 18 and 20) resulted in delayed innate immune development in their F1 offspring. Pups born to LPS-exposed dams produced lower TNF-α and IL-1β than pups born to saline-administered controls postnatally, but this was not maintained into pubescent age of offspring. The effects of postnatal immune activation continued to adulthood, resulting in immune senescence, as F1 offspring demonstrated less circulating monocytes than control offspring (Hodyl et al., 2007). Hodyl and colleagues also observed sex-specific differences in LPS-induced MIA in adult and aging rodents (2007). The LPS-affected female adult offspring had significantly lower basal leukocytes but were still able to mount comparable cytokine responses and leukocyte responses to *in vivo* LPS challenge compared to males. In aged LPS-affected mice, females maintained a stronger TNF-α response to *in vivo* LPS immune challenge than males (Hodyl et al., 2007). A more recent study looking at chronic LPS-induced MIA (6.45 *μ*g/day) during gestation and lactation reported higher blood leukocyte counts with chronic LPS exposure, with a particularly higher leukocyte count in F1 males. Furthermore, while no significantly elevated cytokine responses were reported at basal level, female F1 offspring produced somewhat higher cytokine responses than F1 males in response to LPS. However, F1 males exposed to LPS *in utero* seemed to exhibit higher hypothalamic inflammatory cytokines (Dudele et al., 2017).

When looking at a different acute MIA model at mid-gestation (20 mg/kg poly I:C, day 12.5), single dose MIA resulted in a more pronounced M1 macrophage phenotype in adult offspring, and may result in immune dysregulation by inducing T_H1 skewing, promoting a relatively more pro-inflammatory state (Onore et al., 2014). Immunological outcomes were assessed in a more chronic MIA paradigm (250 μg/kg poly I:C, 3 times) in a primate model in either mid- or late gestation. Offspring was followed up in juvenile (year 1) and adult (year 4) stages. MIA juvenile offspring exhibited higher plasma cytokine levels at both time points. Their peripheral mononuclear cells also displayed higher *ex vivo* cytokine responses under basal conditions as well as in response to

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poly I:C and LPS stimulation. In adulthood (year 4), the analysis of plasma revealed the maintained presence of an inflammatory milieu, and *ex vivo* peripheral blood mononuclear cell culture revealed high IL-1β production and a more T_H2/T_H17 bias with TLR4 and TLR3 stimulation in MIA offspring compared to controls; the latter switched to a more directed immunomodulatory or allergy phenotype in older offspring (Rose et al., 2017).

As mentioned previously, the variations in immune functionality in different acute MIA models may have been as a result of the timing chosen for the induction of prenatal MIA , and as such, could account for the differences in immune function. Chronic or repeated dose MIA seems to result in a hyper-inflammatory profile in F1 offspring, especially in the innate immune system. Nevertheless, the inheritance of inflammatory dysregulation in the F1 adult generation is mostly observed in their innate immune functionality and less dysregulation of the GC production, which can lead to "inflammaging". Previous studies have indicated that moderate inflammation and immune senescence observed in chronic disease, in the absence of infection (i.e. sterile inflammation) is associated with poor health outcomes seen in the elderly (Franceschi & Campisi, 2014; Bauer et al., 2015).

As shown in other models mentioned, maternal environmental exposure, nutritional adaptations or trauma/adversity induces phenotypic changes that persists to at least the F1 generation, it is worth investigating whether similar inheritance of MIA immune and/or CORT phenotype changes seen in F0 mothers and F1 offspring do indeed persist to subsequent F2 generations and further.

2.5.5.2. Evidence of maternal transgenerational transfer in MIA

Data on the inheritance of behavioural adaptations have been reported in the rodent LPS-induced MIA paradigm in both F2 (Penteado et al., 2014; Reis-Silva et al., 2016; Ronovsky et al., 2017) and F3 generations (Weber-Stadlbauer et al., 2017). However, very little is known about the effect of ancestral MIA exposure on the CORT functionality and immune function in the F2 and/or F3 generations. From previous findings in transgenerational models of social stress, chronic HPA activation results in a pro-inflammatory phenotype in F2 offspring, but the offspring display reduced functional capacity (immune memory and adaptive immune responses) (Hicks-Nelson

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et al., 2017a). As chronic psychological stress results in upregulation of maternal inflammatory profile, as well as result in immune dysregulation in F1 and F2 offspring, we speculate that a similar pattern of maladaptation may be present in the F2 generations exposed to MIA, however, this needs to be confirmed.

Considering the relative scarcity of data on the transgenerational effects of MIA, clearly more research is required in this context. However, in terms of the exact protocol used, several factors should be considered in order to accurately simulate the physiological condition to be investigated. These considerations will be discussed in the next section.

2.6. Methodological considerations in animal MIA models

2.6.1. Nature of intervention model

Several different approaches and administration time points have been utilised in maternal immune activation studies, and thus, produce diverse results depending the method used to induce inflammation, time of administration, routes of administration, dosage of immune activating agent as well as whether an acute or chronic model will best depict the outcome of the study (Rana, Aavani & Pittman, 2012; Solek et al., 2018). This is important for several reasons and will be addressed in this section.

2.6.1.1. Timing of administration (acute versus chronic)

In terms of timing, maternal cytokine production subsequent to MIA may alter normal developmental processes. It's been shown that acute LPS administration in early gestation versus. Late gestation has different offspring effects, with early administration resulting in blunted responses whereas mid/late gestation seems to result in hyper-inflammatory responses. It should be noted that dosage alters responses in offspring (Beloosesky et al., 2010; Williams, Teeling, Perry, Fleming, et al., 2011; Kirsten et al., 2013).

In MIA models, sub-chronic (2x repeated exposure) or chronic (3 or more exposures) maternal immune activation is achieved by repeated administration of the immunogen to pregnant animals during gestation, which may be a better model for chronic maternal gestational inflammation. Specifically, while repeated administrations are

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thought to dampen the immune response to the inflammatory agent (Meyer, Feldon & Fatemi, 2009), in some studies, at low to moderate MIA doses, its shown to heighten offspring immunological responses, especially to immune challenge (Hodyl et al., 2007; Dudele et al., 2017; Rose et al., 2017). Conversely, at high LPS doses (250mg and higher), foetal immune activation and leukocyte infiltration into lungs and tissue is seen, even increased risk of foetal death (Hudalla et al., 2018). Consequently, dosage and timing of chronic exposure is crucial considerations.

2.6.1.2. Routes of administration

In terms of routes of administration, most immune-activating agents are administered via the intraperitoneal (i.p.), intravenous (i.v.) or subcutaneous (s.c.) routes. In MIA studies, i.p. is the preferred route of administration as it is relatively fast and easy to perform. Intraperitoneal administration also result in relatively fast absorption, minimises handling stress and the risk of requiring to re-puncture the dam. However, care is required with i.p. administration in pregnant rodents during late gestation to introduce injuries to the foetus or placental tissue (Meyer, Feldon & Fatemi, 2009).

2.6.1.3. Age of offspring

In terms of F1 and F2 generation analyses, analysis can either take place in the foetal or neonatal age until weaning (postnatal day 1 to day 21) (Ashdown et al., 2006; Carini & Nephew, 2013; Kirsten et al., 2013), sexual maturity (6 to 8 weeks of age) or at adulthood and senescence (Hodyl et al., 2007; Dudele et al., 2017; Hicks-Nelson et al., 2017a). As discussed, the age of the offspring determines the outcome observed. It has been demonstrated that postnatal responses may not be a true reflection of the offspring outcome due to rapid change in cell populations during the first few weeks of life (Hodyl et al., 2007; Olin et al., 2018). Sexual maturity or adulthood may be an appropriate time for analysis for the heritability of the immune and GC phenotype due to stability of the phenotype. Immune senescence can introduce confounding factors, such as “inflammaging”, which may exacerbate the present inflammatory phenotype (Franceschi & Campisi, 2014).

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2.6.2. Whole blood versus splenocytes for immunological analysis

Apart from the intervention model itself, sample collection is another crucial consideration, especially in small rodents, where sample volume is very limited. In order to assess physiological changes, and in particular, immune parameters, in mothers and offspring from MIA models, blood samples and/or spleen may be collected for analyses. While many studies have analysed the effect of stress and MIA on serum cytokine production and the blood leukocytes, few have discussed the changes in splenic leukocytes to inflammation. Considering that the spleen is a key haematopoietic organ (Bronte & Pittet, 2013), little is known about the impact of MIA on these leukocytes in these organs. The spleen is commonly used in immune studies to determine the vaccine response or *ex vivo* immune stimulation. Previous research has shown that the spleen contributes to redistribution of myeloid derived cells and lymphocytes in disease and stress (McKim et al., 2016, 2018; Li et al., 2018; Yin et al., 2019). Furthermore, a recent study has highlighted the functional splenic leukocytes distribution in non-infectious chronic inflammation (Li et al., 2017). It was also demonstrated that splenic leukocytes are altered in response to GR and GC upregulation (Li et al., 2018). Thus, an assessment of the distribution of leukocytes in blood and spleen may provide insight into immune cell trafficking in models of chronic stress, as the spleen, at least in part, may contribute to the variation in peripheral circulation induced by stress.

2.6.3. Methodological analysis: justification for use of flow cytometry

Lastly, sample analysis is an important factor for consideration in MIA. Multiparameter flow cytometry is one of the most popular technologies for cell or particle analysis and is routinely used in immunological studies, but not limited to this field. Multiparameter flow cytometry (defined as five or more fluorescent markers) is employed to identify and characterise multiple markers within one sample, allowing for a comprehensive depiction of cell responses, subset characterisation or expression of markers of interest, in their frequencies of expression (as a percentage) and/or relative intensity of marker expression (expressed as median fluorescent intensity (MFI)). This method has been applied to several different applications (O'Donnell, Ernst & Hingorani, 2013). I will limit my discussion to the parameters most pertinent to my thesis topic.

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2.6.3.1. GR expression on leukocytes

GRs are ubiquitously expressed in almost all nucleated cells (Quax et al., 2013), with differing levels of expression on cell types. One of the earliest investigations of GR protein expression, analysed by western blot, demonstrated that GR is differentially expressed in the spleen, thymus and lymph nodes of rats. Furthermore, differences in GR expression was also reported in peripheral monononuclear cells as well as neutrophils. This gave insight into the effect of GR on immune cells and tissues at single-cell level (Miller et al., 1998), but gave little information on cell-specific expression of GR, which may be clinically relevant in treatment of inflammatory diseases with sGCs. Flow cytometric assessment of GR allowed for sensitive and accurate analysis of GR in various leukocyte populations by flow cytometry and had potential for monitoring the GR in clinical diagnostics (Berki et al., 1998). This method has been employed in several studies looking at asthma (Lu, Radom-Aizik, et al., 2017), systemic lupus erythematous (SLE) (Du et al., 2009), chronic obstructive pulmonary disease (Hodge et al., 2015), and psychological stress in humans (Gotovac et al., 2003; Vidović et al., 2007, 2011) and rodents (Gruver-Yates, Quinn & Cidlowski, 2014; Gurfein et al., 2017). This multiparameter flow cytometric methodology enables the sensitive and accurate assessment of GR expression and its role in immune modulation. This was our preferred technique for monitoring immunoregulatory action of glucocorticoids, especially when small sample volume is a consideration.

2.6.3.2. Inflammasome activation

Previous literature has assessed NLRP3 inflammasome activation by the modification of specific proteins with either western blots or enzyme-linked immunosorbent assay (ELISA). Using western blots, cell lysates were used to assess the upregulation of NLRP3 protein complex. NLRP3 activation was confirmed by the decline in pro-IL-1β and pro-caspase1 p35, the upregulation of caspase 1 p20, and importantly, the presence of the bioactive 17kDa IL-1β (Busillo, Azzams & Cidlowski, 2011; Paugh et al., 2015; Zasłona et al., 2017) in the cell lysate. Using the ELISA method, IL-1β concentrations were quantified in either cell culture supernatant or serum. While these methods allow of quantification of the NLRP3 activation, a limitation of these methods is that the inflammasome response at a single cell level cannot be assessed.

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In recent literature, flow cytometry has again been successfully employed to also assess inflammasome activation. The detection of cell-specific IL-1β production using intracellular flow cytometry is challenging, as this cytokine does not employ the same mechanism of packaging and release via the endoplasmic reticulum and Golgi apparatus as other cytokines (Lopez-Castejon & Brough, 2011). For this reason, studies have assessed either its precursor, pro-IL-1β or the ASC adaptor speck formation in flow cytometric assessment of the inflammasome (Sester et al., in press, 2015; Ramirez et al., 2012; Tzeng et al., 2016). Inflammasome activation has been evaluated in murine bone marrow macrophages via the presence of its precursor (pro-IL-1β) . The study assessed the variation of intracellular pro-IL-1β production in young versus aged mice and reported that TLR stimulation alone (with LPS) resulted in an increase of pro-IL-1β expression, while NLRP3 inflammasome activation (via LPS with ATP stimulation) resulted in a decline in pro-IL-1β production. This result correlated with the increase in IL-1β expression in culture supernatant with LPS and ATP stimulation (Ramirez et al., 2012).

Inflammasome activation can also be assessed by ASC speck formation in cells. The mobilisation of ASC adaptors from a disseminated state into a single localised speck allows for analysis by flow cytometry in ASC-labelled cells. As the labelled cell passes by the laser interrogation point in a flow cytometer, the electronic component stores in formation on the pulse of emitted fluorescence from the fluorescently labelled cell, namely the area (A) of the pulse (total emitted fluorescence), the height (H) and the width (W) of the peak. This assay utilizes the H and W of flow cytometry measurements, as cells of the same size should have equivalent H:A or W:A ratios while aggregates have a higher H:A or W:A ratio. In stimulated macrophages and monocytes exposed to media only (control) or to media with LPS, the ASC is diffuse in the cytosol and nucleus of the cell. In this resting or an LPS activated state, the ASC+ cells are expressed as a broad peak with a low H:A ratio. Upon activation of the inflammasome complex with LPS and ATP or nigericin, the ASC adaptor mobilizes from its diffuse state and aggregates into a speck inside the activated cell, resulting in a high ASC H:A or W:A ratio. Thus, the H and W parameters can reveal information regarding the oligomerisation ASC complex and thus the activation of the inflammasome. This data correlated with confirmation of speck formation by

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microscopy and data was reported as a percentage of cells with ASC speck formation (Sester et al., in press, 2015).

These two flow cytometric assays allow rapid and direct assessment of inflammasome activation in stimulated cells and can be paired to simultaneously assess both pro-IL-1β production and ASC formation in the same cell, in combination with either lineage or activation markers for multiparameter analysis.

2.7. Summary

Animal models of generational programming have been established to study several human diseases and have contributed greatly in deciphering the potential mechanisms behind the maladaptation underlying the findings in epidemiological studies. In addition, due to the nature and timing of breeding, animal studies allow for investigation of transgenerational inheritance up to F3. Further considerations such as maternal and offspring age, severity, type and dosage of inflammatory antigen, gestational period and frequency of exposure are important in conducting animal studies, as this can dictate the phenotype driven. While the findings in animal studies do require confirmation by human models, animal models of gestational programming provide the first step in understanding the mechanisms behind the inheritance of phenotype and risk of susceptibility on offspring.

From my review of the literature, a recurrent theme is the maternal transfer of inheritance of either a similar or altered phenotype in F1 offspring. There is a distinct relationship between maternal prenatal stress, obesity/metabolic disease psychological stress, obesity and the inflammatory dysfunction, as these conditions tend to exist in comorbidity. In studies investigating the sex-specificity of inheritance, female offspring are shown to be more immunomodulatory. However, while there is an abundance of literature discussing the impact of maternal transgenerational stress in the abovementioned disorders, very little literature is available on the effects of maternal inflammation during pregnancy on subsequent generations. Moreover, in the literature reviewed, the susceptibility of MIA offspring to neurodevelopmental dysfunction is commonly reported, with little emphasis on immunological adaptations. Maternal immune activation results in increased inflammatory responses, subsequently affecting placental development and functionality. This resulting effect is

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passed on to the offspring. Despite this, prenatal MIA studies employed to examine the consequences of prenatal exposure to psychological stressors or obesity have demonstrated definitive sex-specific differences in brain development, behaviour and obesity. A major limitation in previous MIA transgenerational studies is the lack of information on the F0 MIA chronic inflammation on GC response and leukocyte functionality on F2 offspring and the sex-specific heritability of F1 to F2 without further exposure. In this study, we aimed to address the gap in literature on the effect of chronic low-dose LPS administration on F1 and F2 generations. For our study we investigated the persistent immune dysfunction and GC alterations F1 and F2 generations. Although sex-specific heritability is still a factor to be determined, to our knowledge, this will be the first study to demonstrate the effect of chronic maternal inflammation on two generations of offspring.

2.8. Hypothesis and Aims

2.8.1. Hypothesis

For this study, we hypothesise that chronic low-grade LPS-induced maternal immune activation will lead to dysfunction in the GC production, inflammatory immune responses and leukocyte GR levels in F1 offspring. Furthermore, we postulate that these effects will persist to the 2nd generation of offspring (F2), with no further intervention, with sex-specific heritability of the inflammatory phenotypes from F1 to F2. Lastly, we propose that cumulative inheritance in F2 offspring born from two *in utero* LPS-affected parents will result in more severe outcome for these offspring.

2.8.2. Aims

The current study was aimed to delineate the transgenerational inheritance of immunological and HPA functionality in F1 offspring born to LPS-induced maternal immune activation in C57/BL/6 female mice. Furthermore, the study aimed to investigate the longevity and sex-specific heritability to F2 offspring the chronic maternal low-dose LPS exposure in the F1 and F2 offspring and to elucidate potential sex-specificity in the heritability of the F0 inflammatory phenotype.

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2.8.3. Objectives

In order to achieve the set aims, the following specific objectives were formulated:

- Establish a chronic low-grade maternal inflammation model using LPS. This entails inducing gestational inflammation by LPS administration during gestation to produce the F1 offspring and crossing F1 sexually mature offspring with wild type unaffected mice to produce the F2 offspring.
- Determine the basal HPA function by assessing plasma corticosterone concentrations in blood plasma in F0, F2 and F2 generations.
- Assess the basal leukocyte profile and leukocyte subset distribution in peripheral blood and spleen in F0, F2 and F2 generations.
- Determine a basal HPA sensitivity in leukocyte subpopulation by assessing GR expression by flow cytometry in F0, F2 and F2 generations.
- Determine immune responsiveness in F0, F2 and F2 generations at basal level and in response to acute immune stimulation (IL-1β, IL-6, IL-10, IFN-γ and TNF-α cultured splenocyte supernatant).
- Determine the effect of maternal chronic LPS exposure on inflammasome activation pathway for IL-1β by assessing pro-IL-1β expression and ASC speck formation in F0, F2 and F2 generations.
- Determine sex-specificity of maladaptations observed in F1 and F2 generations.

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Chapter 3

Chronic gestational inflammation: transfer of maternal adaptation over two generations of progeny

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3.1. Abstract

Fluctuations in the *in utero* environment can result in generational transfer of maladapted physiology in the context of conditions such as stress, obesity and anxiety. Given the significant contribution of non-communicable diseases – which are characterised by chronic inflammation – to population mortality, the potential for chronic maternal inflammation mediating foetal programming is a growing concern.

The extent of generational transfer in terms of immune functionality and leukocyte glucocorticoid sensitivity was investigated over two generations of offspring (F1 and F2) in a model of chronic LPS-induced maternal inflammation in C57/BL/6 mice. Maternal inflammation resulted in glucocorticoid hypersensitivity (increased glucocorticoid receptor expression levels) in the majority of leukocyte subpopulations in both F1 and F2 offspring. Furthermore, splenocytes stimulated with LPS in vitro, exhibited exacerbated inflammatory cytokine responses which were even more prominent in F2 than F1 – this effect could be ascribed to NLRP3 inflammasome hyperactivity in F1, but not F2.

Current data illustrates that parental chronic inflammation may mediate inflammatory profile in offspring, potentially propagating a maladapted, pro-inflammatory phenotype in subsequent generations.

3.2. Introduction

The high mortality resulting from non-communicable diseases – currently accounting for over 70% of global death rates, with cardiovascular disease, cancers, diabetes and chronic pulmonary diseases taking the forefront (World Health Organization, 2017, 2018) – increases the potential risk for generational transfer of maladapted physiology. This is a significant concern of modern societies, with published literature showing a precedent for transgenerational inheritance in offspring: the impact of maternal stress exposure, whether acute or chronic, is reportedly passed on to her offspring and to an extent, her grand offspring (Weaver et al., 2004; Gluckman, Hanson, & Pinal, 2005; Naumova et al., 2016).

The plasticity of foetal development is notoriously sensitive to environmental fluctuations, which is mediated by a variety of stressors. The foetal programming

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hypothesis suggests that adaptations occurring during the critical embryonic and foetal developmental stages, determines the established point of physiological and metabolic responses and susceptibility to disease in later life (Barker, 1998). This dysfunction is evident in transgenerational studies involving obesity (Dunn, Morgan, & Bale, 2011; Entringer et al., 2012; Dudele et al., 2017; Joaquim et al., 2017), social stressors (Carini & Nephew, 2013; Hicks-Nelson et al., 2017a), anxiety (Moisiadis et al., 2017) and LPS exposure (Williams, Teeling, Perry & Fleming, 2011; Reis-Silva & Bernardi, 2012; Dudele et al., 2017), which is, at least in part, due to changes in the *in utero* microenvironment.

The *in utero* milieu is subject to a variety of endocrine and immune adaptations, which is induced to sustain a favourable microenvironment for growth and maturation at the maternal-foetal interface. In terms of immunity, the primary alteration is the predominant type II helper T-lymphocyte (T_H2) bias that exists during pregnancy to facilitate maternal tolerance at the maternal-foetal interface (Morelli et al., 2015). The increased progesterone, estradiol, and prostaglandin D2 (PGD2) levels during gestation seems to further encourage this T_H2 profile, in so doing maintaining a relatively more immunosuppressive state in mothers (Robinson & Klein, 2012; Sykes et al., 2012). Maternal inflammation during gestation seems to disrupt this T_H2 balance, resulting in a more proinflammatory T_H1 phenotype, which adversely affects offspring. Both maternal pre- or perinatal inflammation is known to be causal in preterm birth and foetal loss (Weinstock, 2005; Sykes et al., 2012; Li et al., 2015). Furthermore, it has far reaching effects on offspring behaviour (Lin, Lin & Wang, 2012; Depino, 2015), metabolic function (Dudele et al., 2017; Ling et al., 2018) and immune functionality (Williams, Teeling, Perry & Fleming, 2011; Dudele et al., 2017; Parent et al., 2017; Ling et al., 2018). Glucocorticoid and hypothalamic-pituitary-adrenal (HPA) homeostasis (Lin, Lin & Wang, 2012; Zager et al., 2013; Parent et al., 2017) is also affected by gestational inflammation. The impact of maternal stress on the foetal HPA-axis in the perinatal state has been comprehensively reviewed in by Weinstock et al. (2005), who concluded that gestational stress and higher levels of maternal and foetal plasma corticosterone can result in downregulation of foetal glucocorticoid receptors, impairing the feedback loop of the HPA-axis into adulthood. Emerging evidence is suggesting that this maladaptive endocrine state may also be linked to sustained maladapted immunological functionality. For example, in rodents, stressed generation

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zero (F0) dams displayed higher circulating pro-inflammatory cytokine concentrations (Williams, Teeling, Perry & Fleming, 2011), higher leukocyte counts (Dudele et al., 2017) and increased circulating cortisol levels (Jafari et al., 2017). This dysregulation was reported to persist to the F1 and F2 generations of stressed groups (Murgatroyd et al., 2016; Hicks-Nelson et al., 2017a; Nephew et al., 2017).

Based on previous literature in transgenerational rodent models of social stress, as well as human models of post-traumatic stress disorder (PTSD) and trauma (Yehuda, Halligan & Grossman, 2001; Yehuda et al., 2009, 2016; Devoto et al., 2017), chronic HPA-axis activation promotes glucocorticoid insensitivity, resulting a pro-inflammatory phenotype, predisposing subsequent generations to increased risk of morbidity from non-communicable disease in adulthood. Although the effects of psychological stressors during the gestational period on maladaptation of the HPA-axis have been comprehensively reported on, little data is available on the effects of chronic stressors on functional capacity of the immune response in F1 and F2 generations, or their glucocorticoid sensitivity in response to chronic maternal inflammation. Thus, the purpose of our study was to delineate the plasticity of generational transfer in immune functionality and leukocyte glucocorticoid sensitivity, in a model of chronic LPS-induced maternal inflammation. Furthermore, the role of the NLRP3 inflammasome was investigated in this context.

3.3. Materials and methods

3.3.1. Animal experiments

Ethical clearance was obtained from the Stellenbosch University Animal Research Ethics Committee (SU-ACUM14-00004). C57/BL/6 mice were housed under temperature-controlled conditions under a 12-hour dark-light cycle, with *ad libitum* access to standard rodent chow. After one-week acclimatization, 6-week-old dams (generation F0) were naturally mated with age-matched males. The breeding and propagation of the mice is illustrated in Figure 3.1. Females were placed with males overnight and removed the following morning. Successful mating was confirmed by the presence of a vaginal plug. The plug-positive dams were moved to separate cages and randomised to receive either LPS (from *Escherichia coli*; Sigma, USA; serotype

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0127: B8) at 10 μg/kg bodyweight, prepared in 0.9% saline solution, or 0.9% saline solution (control) at a final volume of 50 μl.

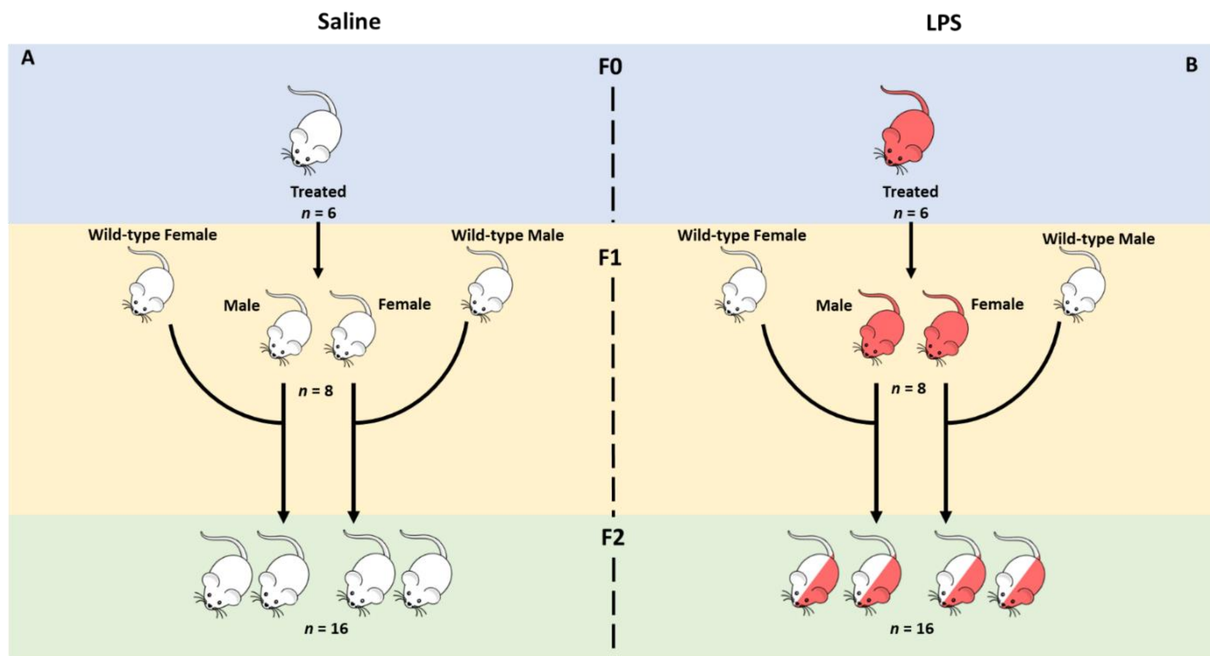

Figure 3.1 Breeding schematic for (A) Saline and (B) LPS-exposed groups. The n represents the number of mice for each subsequent generation and gender group.

The LPS or Saline treatment were administered via intraperitoneal injection and repeated every seven days for the duration of gestation. The dose was based on body mass prior to the first injection (~200 ng LPS per 20g body weight) and was kept constant throughout the duration of the gestation. A total of 3 injections were administered to each dam for the duration of gestation. For the duration of the intervention protocol, females were monitored daily for any signs of morbidity, such as lethargy, weight loss or vaginal bleeding - none were observed. F0 was terminated 4 weeks after weaning of offspring (i.e. at 14 weeks of age and 5 weeks after the last LPS injection).

The first generation of offspring (F1), resulting from the F0 mating, was weaned at 3 weeks of age. Offspring siblings were grouped together, but sexes were separated into two cages until 8 weeks of age, after which 4 males and 4 females per treatment group were terminated for further experimental analysis. The remaining animals were bred with wild-type animals for the second generation of offspring (F2).

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For F2 generation, the F1 mice were mated with wild-type C57/BL/6 mice and the same procedure was followed for weaning, but with no further intervention. Data on breeding, offspring litter size and gestational duration did not seem to be differ across generations or as result of LPS exposure (Supplementary material I). As for F1, F2 mice were killed by cervical dislocation at 8 weeks of age.

3.3.2. Sample collection

Whole blood was collected by cardiac puncture and transferred to K_2EDTA microtubes. An aliquot was assigned for full blood and differential leukocyte counts on the CellDyne 3700CS haemocytometer (Abbott Diagnostics), while the remaining blood sample was used to collect plasma for assessment of corticosterone concentration.

Corticosterone concentrations was determined by quantitative ELISA (DEMEDITEC Corticosterone rat/mouse ELISA, Demeditec Diagnostics, Germany), as per manufacturer's instructions. The concentrations were calculated in Microsoft Excel using a 6-point standard curve with a logistic regression algorithm. The detection range of the kit was 6.1 - 2250 ng/ml respectively. The kit has an intra-assay variation of 8.9% and inter-assay variation of 7.2%.

Mouse spleens were dissected under sterile conditions and collected into ice-cold complete RPMI 1640 medium (supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin, 1% gentamicin). It was noted that LPS-treated dams or LPS-affected offspring visually displayed larger spleen sizes in comparison to saline-treated dams or saline-affected offspring. This, however, was not added as a parameter for analysis, due to the concern for sterility.

3.3.3. Cell preparation

A single cell suspension of murine splenocytes was generated by mechanical dissociation, by passing dissected tissue through a sterile 70 μm cell strainer (BD Biosciences, USA). The cell strainer was rinsed with complete RPMI 1640 medium to remove any attached cells. Red blood cells were lysed with 1X ACK lysis buffer (150 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM NA_2EDTA in ddH_2O) for 5 minutes at room

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temperature and splenocytes were washed with 1X Dulbecco's phosphate buffered saline (DPBS, Gibco, USA). The cells were pelleted at 300xg for 5 minutes at room temperature, the supernatant was aspirated, and the pellet was resuspended in complete RPMI 1640. The cells were counted and adjusted to $1x10^7$/ml viable cells and used immediately for cell counting assays or frozen and stored in liquid nitrogen for subsequent batch analysis of the inflammasome and splenocyte functional capacity.

3.3.4. Basal leukocyte glucocorticoid receptor assessment

All reagents were prepared as per manufacturer's instructions prior to use. For permeabilisation of samples, the BD Cytofix/CytoPerm kit was used.

The staining buffer was prepared as 1X DPBS with 5% bovine serum albumin (Invitrogen, USA) and 1% NaN_3 and stored at 4ºC until use. The antibodies were titrated to determine optimal dilution for experiments. The antibodies and dyes and their respective dilutions is as follows: CD16/32 FC Block, (BD Biosciences); Zombie Aqua Fixable Viability dye (Biolegend); NK1.1 BV421, clone PK136 (Biolegend); TCRβ FITC, clone H57-597 (BD Biosciences); F4/80 PE-CF594, clone T45-2342 (BD Biosciences); CD11b PerCP-Cy5.5, clone M1/70, (BD Biosciences); NR3C1 Ax647, clone BugR2 (Novus Biologicals); Ly6G APC-Cy7, clone 1A8 (BD Biosciences).

Briefly, $1x10^6$ splenocytes were incubated with Zombie Aqua dye in DPBS for 30 minutes at room temperature. After incubation, the cells were washed twice with DPBS and the supernatant aspirated. The cells were then incubated with CD16/32 Mouse FC block antibody for 5 minutes in staining buffer, where after a master mix of the appropriate cell surface marker antibodies are added. The cells were mixed thoroughly and incubated for 30 minutes at 4˚C. The cells were then washed twice with staining buffer and permeabilised for 20 minutes at 4˚C. After incubation, the cells were washed in 1X Perm Buffer and pelleted at 600xg for 5 minutes. Splenocytes were then resuspended with the appropriate dilution of the intracellular NR3C1 antibody. The samples were incubated at 4˚C for 30 minutes in the dark. After incubation, the cells were washed twice with 1X Perm Buffer and as a last step, resuspended in 300µl staining buffer after centrifugation. The samples were stored at 4˚C for a maximum of 6 hours before acquisition using flow cytometry.

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3.3.5. Assessment of inflammasome activation

Splenocytes were thawed at 37°C washed twice (300xg, 5 minutes) with pre-warmed complete RPMI 1640 media. Cells were seeded at a density 2 × 10^6/ml in 10cm bacteriological plates in 20ml complete RPMI 1640 media supplemented with 10% L929 media and incubated at 37°C at 5% CO_2. On day 3, the plate was washed with pre-warmed DPBS, to remove unattached cells and the media was replaced. On day 6 the cells were harvested using 5 ml Accutase and resuspended at a concentration of 2x10^5 cells per well in poly-HEMA-coated 48-well plates in 490 μl RPMI 1640.

Splenocytes were resuspended in RPMI 1640 medium and plated into three wells per sample. Two wells were treated with LPS (100 ng/ml) and one well was treated with RPMI 1640 only and the wells were incubated for 6 hours at 37°C. After incubation, w, nigericin (10 μM, Sigma Aldrich, USA) was added to one of the LPS wells and the wells were incubated for another 30 minutes. Thus, for each sample, this assay generated (a) a control (RPMI only) sample, (b) LPS treated sample and (c) LPS+nigericin treated sample. Following incubation, the cells from each well was transferred into 1.5 ml labelled microcentrifuge tubes and centrifuged at 300xg for 5 minutes to pellet cells, after which they were fixed with 4% paraformaldehyde.

Antibodies used for labelling were titrated to determine optimal dilution for experiments. The antibodies and dyes and their respective dilutions used for the study is as follows: Mouse FC Block (BD Biosciences); CD11b BV421, clone M1/70 (BD Biosciences); F4/80 PE, clone T45-2342 (BD Biosciences); Pro-IL-1β PE-Cy7, clone NJTEN3 (eBiosciences); ASC/TMS1 Ax647 (Novus Biologicals).

The cells were permeabilised with BD CytoFix/CytoPerm Buffer for 20 minutes at 4°C, and subsequently washed twice. Prior to staining, CD16/32 Mouse FC Block was added to the samples for 5 minutes at 4˚C to block non-specific binding. Thereafter, a master mix of the appropriate antibodies for intracellular and extracellular markers was added and the samples incubated for 30 minutes at 4˚C in the dark. After incubation, the cells were resuspended in 1X BD Perm Buffer and centrifuged at 600xg for 5 minutes, at room temperature. After washing the supernatant was discarded and the cells resuspended in staining buffer before acquisition on the flow cytometer.

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3.3.6. Flow cytometric acquisition and analysis

Acquisition was performed on the BD FACSAria IIu flow cytometer (BD Biosciences), with BD FACSDiva™ version 8.1 software for data acquisition and analysis. Applications settings in BD FACSDiva software were used to standardize experimental data. As an experimental control, lot-specific 8-peak bead control was included as daily standardisation validation to ensure that all settings were valid and reproducible on any flow cytometer employed for this purpose. All data files were exported as FCS 3.1 files and further analysed in FlowJo™ v10.4.2.

The samples were resuspended by vortexing for 5 seconds prior to data acquisition. For the assessment of glucocorticoid receptor expression level on specific leukocyte subpopulations, a minimum of 200 000 and a maximum of 500 000 live, gated, singlet events were collected for each sample. The gating strategies are defined in Figure 3.2. Splenocytes were identified using FSC vs. SSC, thereafter dead cells were excluded. Doublet discrimination was performed by applying a gate around the linear population in the SSC-H vs. SSC-A plot. Cells of interest were then identified from the single cell population as follows: T-lymphocytes (TCRβ+ NK1.1-), NKT lymphocytes (TCRβ+ NK1.1+) NK cells (TCRβ- CD11b+ NK1.1+), neutrophils (TCRβ- CD11b+ Ly6G+), monocytes (TCRβ- CD11b+ F4/80-) and macrophages (TCRβ- CD11b+ F4/80+). Relative glucocorticoid receptor (NR3C1) expression for each cell populations was quantified as relative median fluorescence intensity (MFI). Bulk gating was used to apply these gate coordinates to each generation and all the gates were inspected and adjusted manually for each sample, if needed. All data for the experimental design was exported to Microsoft Excel.

For the inflammasome assay, a minimum of 5000 CD11b+F4/80+ macrophages were collected per sample. All samples were run on application settings and compensation was performed with every run. The gating strategies are defined in Figure 3.2. Macrophages were gated on the FSC vs SSC dot plot and doublets were excluded using FSC-H vs FSC-A. Macrophages were further identified by CD11b+F4/80+ expression and within this population, pro-IL-1β expression was quantified as relative median fluorescent intensity (MFI). Inflammasome Adaptor Protein Apoptosis-Associated Speck-Like Protein Containing CARD (ASC) speck formation was assessed by plotting ASC-A vs ASC-H. The ASC speck-containing cells were gated

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for quantification in the doublet gate as defined by accepted methodology (Sester et al., in press). As a brief introduction, the inflammasome complex is a multiprotein protein structure, responsible for the tightly controlled secretion of both IL-1β and IL-18 that recognises pathogens via Toll-Like receptor binding in combination with NOD-like receptor binding. Inflammasome assembly, and thereby the release of biologically active IL-1β is a two-step process: firstly, by the production of inactive pro-IL-1β, stimulated by TLR ligand binding and secondly, the formation of the inflammasome complex (ASC formation) which cleaves inactive pro IL-1β into active IL- 1β (Jha, Brickey, Pan, & Ting, 2017; Strowig, Henao-Mejia, Elinav, & Flavell, 2012). The NLRP3 is the best-studied inflammasome complex, and has been implicated in obesity, heart disease, neuroinflammation, and other systemic inflammatory dysregulation (Jha et al., 2017; Menu, Vince, Vince, & Menu, 2011; Strowig et al., 2012).

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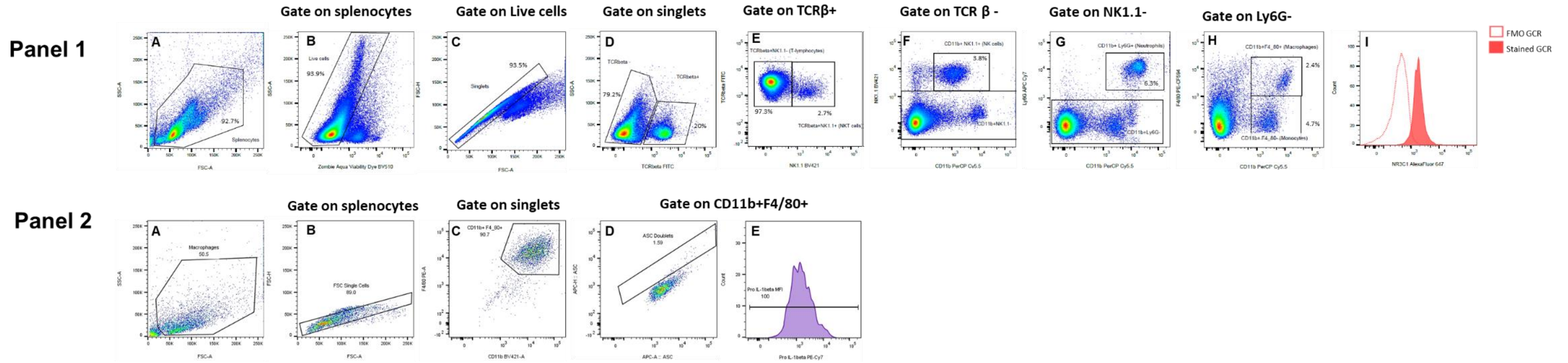

Figure 3.2 Representative images illustrating the gating strategy for basal glucocorticoid receptor expression on splenocytes (Panel 1) and assessment of inflammasome function (Panel 2).

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3.3.7. Splenocyte *ex vivo* functional capacity

Functional capacity of splenocytes were for all three generations of mice, in terms of their basal and LPS-induced cytokine secretion profile. Splenocytes were resuspended in RPMI1640 at a cell concentration of $1x10^6$ cell/ml and plated in 24-well plates at 1ml per well. The splenocytes were treated with either LPS (from Escherichia coli; Sigma, USA; serotype 0127: B8) at 1 μg/ml in RPMI 1640 (LPS-induced/stimulated) or complete RPMI 1640 only (basal/unstimulated) and incubated for 18 hours at 37°C, 5% CO_2. After stimulation, culture supernatants were collected and stored at -80°C for batch analysis.

The MAP Mouse Cytokine/Chemokine Magnetic Bead panel kit (Millipore, USA) was employed to assess the cytokine profile (IL-1β, IL-6, IL-10, TNF-α and IFN-γ) in stimulated and unstimulated supernatant samples, using the Bioplex 200 system (BioRad, USA) equipped with Bio-Plex Manager™ software. Cytokine concentrations were automatically calculated based on a 6-point standard curve (in duplicate) fitted with a five-parameter logistic regression algorithm. The lowest limit for detection for IL-1β, IL-6, TNF-α, IL-10, and IFN-γ, 1,1pg/ml, 2,3pg/ml, 2,0pg/ml, and 1,1pg/ml respectively. The highest limit of detection is 10 000pg/ml for all cytokines assessed.

3.3.8. Data reduction and statistical analysis

For flow cytometric data, percentage of cells for each leukocyte population identified and median fluorescent intensity (MFI) was used in statistical analyses. All data was exported to Microsoft Excel from respective analysis programs and consolidated. Data was analysed in Statistica version 13.2 (Statsoft Software, USA) and graphs were generated in GraphPad Prism 7.04 (GraphPad Software Incorporated, USA). After confirming normalcy of data distribution, one-way analysis of variance (ANOVA) was performed for the F0 LPS and Saline comparison, and a two-way analysis of variance (ANOVA) was employed for the F1 and F2 comparison. A Fisher's LSD post hoc tests were employed to analyse the statistical significance of differences between control and LPS-affected groups within the same generation and across generations F0, F1 and F2, respectively. Data is presented as means and standard errors of the mean (SEM) and $p<0.05$ or less was regarded as significant. Symbols were used to denote

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significance as follows: follows: * p<0.05, ** p<0.01, *** p<0.001 and follows: *** p<0.0001.

3.3.9. Supplementary material

Supplementary material I illustrates comparative data on breeding, mating partner and offspring size across generations.

Supplementary material II depicts the basal corticosterone levels for F0 mothers, their F1 offspring and subsequent F2 offspring in plasma.

Supplementary Material III illustrates *in vitro* basal and acute LPS-induced cytokine release by splenocytes of mothers (F0) at the time of sample collection, which took place 4 weeks after the last LPS injection.

Supplementary material IV illustrates *in vitro* basal cytokine release by splenocytes isolated from F1 and F2 offspring.

3.4. Results

3.4.1. Gestational chronic LPS-induced inflammation affects maternal physiology even after recovery period

After 3 weeks of recovery, plasma corticosterone levels were similar between control vs LPS-exposed groups. Insufficient numbers per group exclude firm conclusions on this, but individual data (Supplementary Material II) seems to suggest that at this time point, the majority – but not all – mothers have fully recovered in terms of circulating glucocorticoid levels.

The spleen size in LPS-treated dams and their offspring, which is reported by other models of stress (McKim et al., 2018; Yin et al., 2019), although we did not consider this as a parameter. In the blood and splenocyte absolute counts and relative distributions are summarized in Table 1. In line with the glucocorticoid (GC) data, in circulation, neither total nor differential leukocyte counts differed between groups. When considering splenocyte counts, the same representation is seen, with the exception of splenic eosinophil counts, which were significantly higher (p<0.05) in the LPS-exposed group. It is possible that activated neutrophils were counted as

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eosinophils by the automated cell counter. Since it was not logistically possible to exclude this possibility through manual assessment of blood smears, this result was excluded from interpretation. (Given the relatively low eosinophil counts, it is unlikely that neutrophil counts would have been significantly affected).

Table 3.1 Peripheral blood and splenic differential leukocyte counts in the F0 Control and F0 LPS-exposed groups. One-way ANOVA was used to compare F0 LPS and F0 Saline groups. Data is represented as means ± SD.

	Compartment	**F0 Control** *1x10⁹ cells/L*	**F0 LPS** *1x10⁹ cells/L*
Total leukocytes	Circulation	2.82 ± 0.41	3.15 ± 0.70
	Spleen	3.80 ± 0.54	4.04 ± 0.55
Lymphocytes	Circulation	1.29 ± 0.48	1.37 ± 0.33
	Spleen	3.11 ± 0.48	3.24 ± 0.69
Monocytes	Circulation	0.147 ± 0.061	0.094 ± 0.048
	Spleen	0.231 ± 0.037	0.279 ± 0.074
Neutrophils	Circulation	1.21 ± 0.78	1.57 ± 1.05
	Spleen	0.127 ± 0.041	0.256 ± 0.129
Eosinophils	Circulation	0.024 ± 0.013	0.020 ± 0.007
	Spleen	0.016 ± 0.004[a]	0.032 ± 0.016[b]
Basophils	Circulation	0.152 ± 0.092	0.090 ± 0.059
	Spleen	0.319 ± 0.063	0.382 ± 0.115

LPS; Lipopolysaccharide. Results are depicted as mean ± SEM. F0 Control, n=5; F0 LPS, n=6.Significance: [ab], p<0.05

When analysing relative distribution of splenocytes using flow cytometry (Figure 3.3), again most cell types appeared to have returned to control levels, except for NKT-lymphocytes, which remained relatively lower in the LPS-exposed group even after 5 weeks of recovery.

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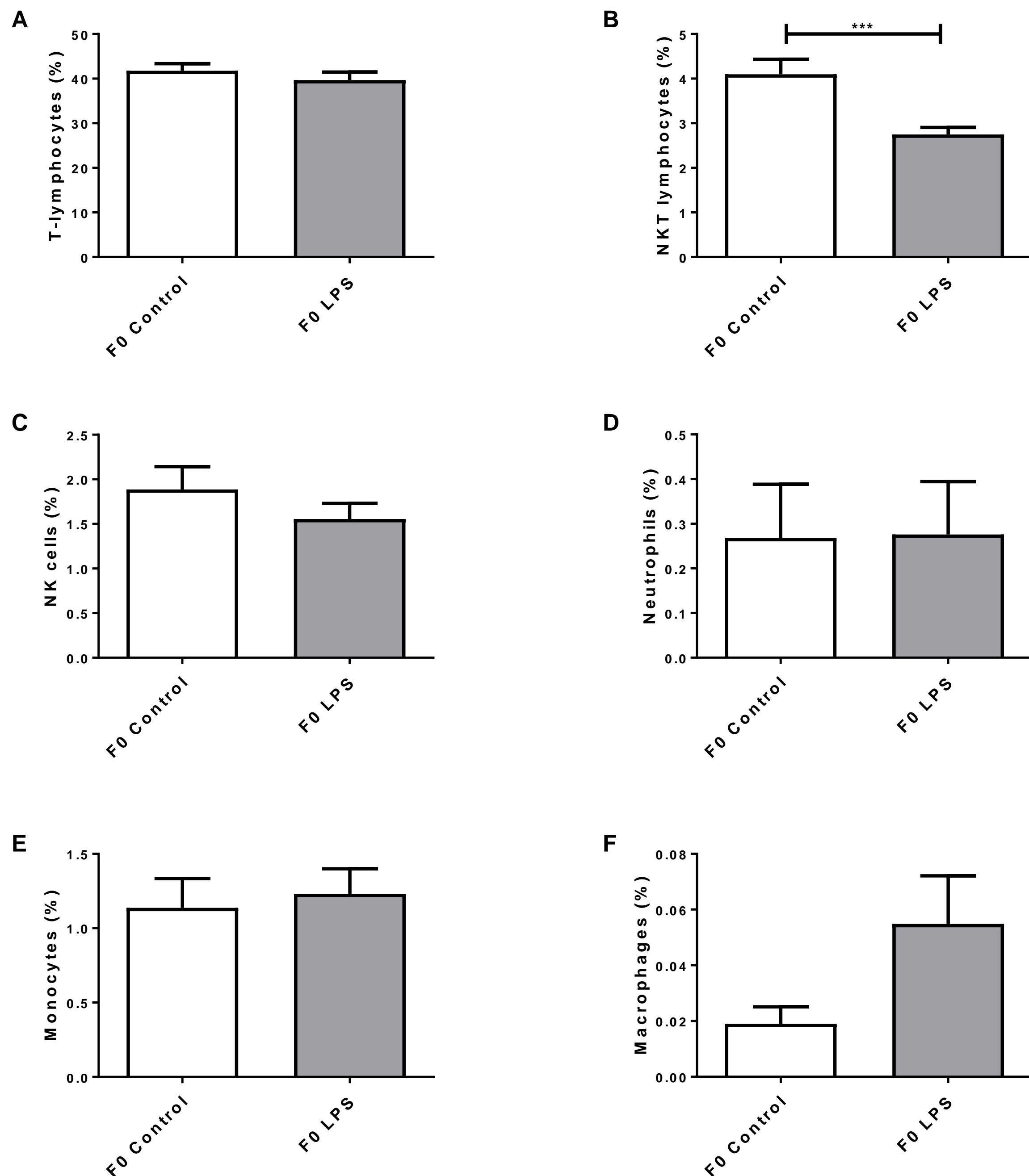

Figure 3.3 Frequency distribution of splenocyte populations between control and LPS-exposed female mice (F0), 3 weeks after the final LPS challenge: (a) T-lymphocytes, (b) NKT-lymphocytes, (c) NK cells, (d) neutrophils, (e), monocytes and (f) macrophages. One-way ANOVA was used to compare F0 LPS and F0 Saline groups. Data is represented mean ±SEM, F0 Control, n=5; F0 LPS, n=6. Significance is depicted as follows: *** p<0.001

Relative basal GR expression in response to repeated LPS exposure in F0 mice differed between different types of splenocytes at a time point 3 weeks after the last LPS challenge (Figure 3.4). Previous LPS-exposure resulted in maintained relatively higher GR expression on T-lymphocytes, NK cells and monocytes, but lower GR

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expression on macrophages and seemingly no lasting effect on neutrophils and NKT-lymphocytes.

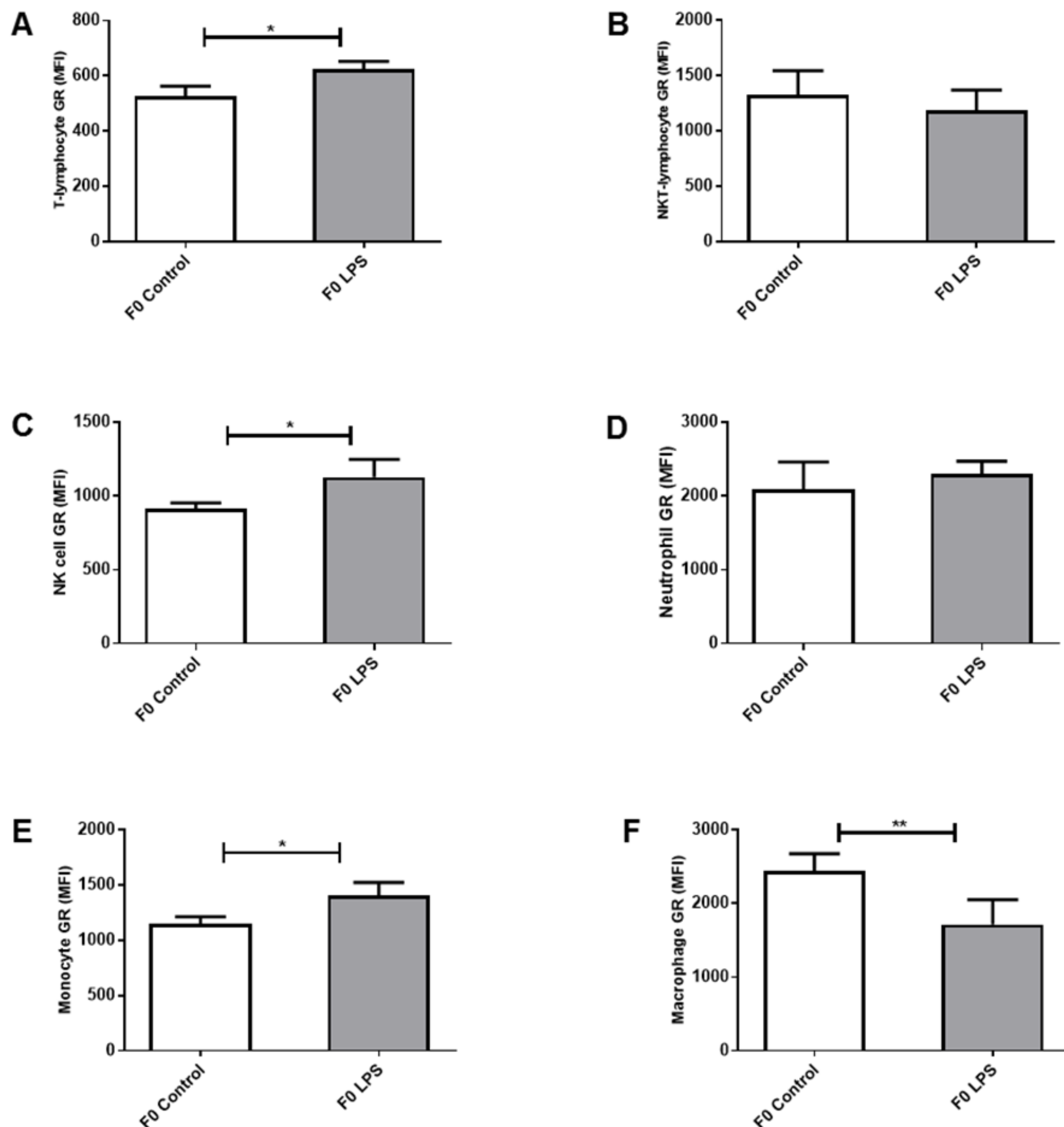

Figure 3.4 Basal cytoplasmic glucocorticoid receptor protein expression levels on splenocyte populations collected from F0 control mice vs. mice 3 weeks after repeated LPS treatment: (a) T-lymphocytes, (b) NKT-lymphocytes, (c) NK cells, (d) neutrophils, (e) monocytes and (f) and macrophages. One-way ANOVA was used to compare F0 LPS and F0 Saline groups. Data is represented mean ±SEM, F0 Control, n=5; F0 LPS, n=6. Significance is depicted as follows: * p<0.05; ** p<0.01

Both basal and LPS-induced capacity of splenocytes to secrete pro-inflammatory cytokines were not significantly affected by previous repeated LPS exposure

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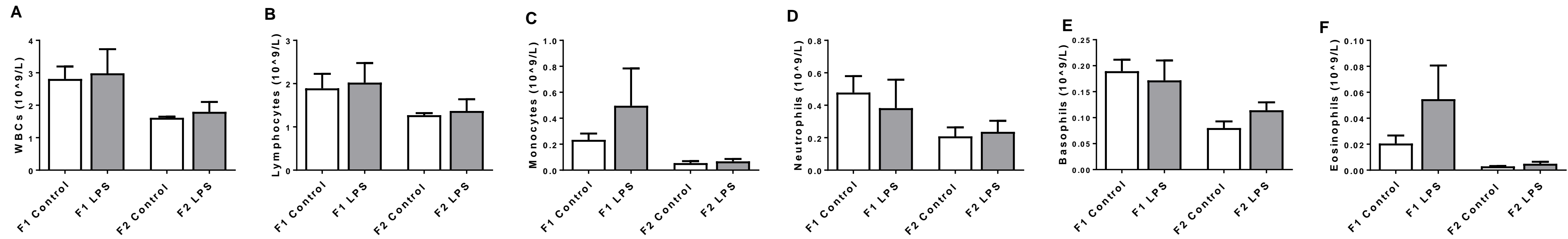
(Supplementary material III). However, a general pattern seems to exist averages for both pro- and anti-inflammatory cytokines were on average slightly higher in previously LPS-exposed groups. This of course remains to be substantiated in a larger group of animals.

3.4.2. Transgenerational inheritance of chronic LPS exposure

Plasma corticosterone levels for the F1 and F2 generations showed no significant response to the F0 maternal intervention, when comparing LPS groups to their respective generational controls, albeit somewhat inconclusive due to limited sample size (Supplementary material II).

In terms of leukocyte counts (Figure 3.5), total and differential leukocyte count was unaffected in circulation (Figure 5, A-F). In contrast, total count was significantly increased in spleens of generation 1 LPS-affected ($F1_{LPS}$) mice, but significantly decreased in F2 LPS-affected ($F2_{LPS}$) animals (Figure 5, G-L). Total lymphocyte counts showed significantly higher in association with inherited LPS exposure in both generations. Interestingly, counts for both neutrophils and basophils – the early phase pro-inflammatory role players – were significantly lower in spleens of LPS-affected animals, an effect that was even more pronounced in F2 than F1. A similar effect was also seen for splenic monocytes, with statistical significance only reached in F2.

Circulation



Spleen

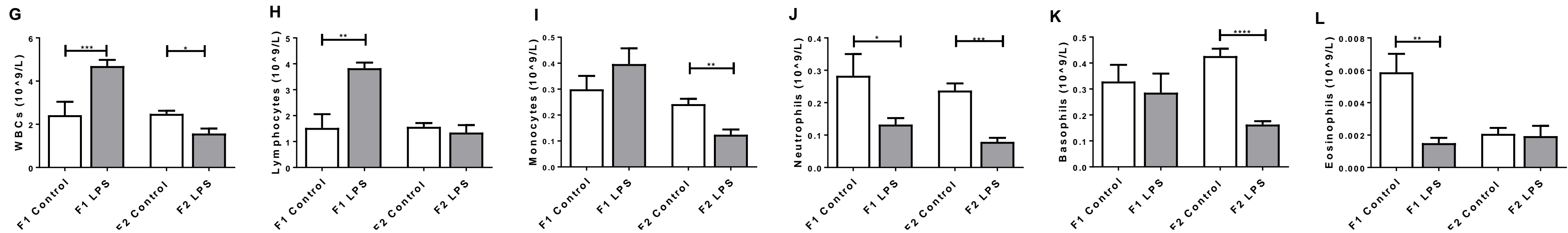


Figure 3.5 Total and differential leukocyte counts in peripheral blood circulation (A-F), and spleen (G-L) from LPS-affected vs control mice across two generations of offspring from LPS-treated F0 mothers. A two-way ANOVA was used to compare F0 LPS and F0 Saline groups. Data is represented mean ±SEM, *n*=8 per group. Significance is depicted as follows: * p<0.05; ** p<0.01; *** p<0.001, **** p<0.001.

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The relative frequencies of specific splenocytes mirrored absolute count data, also showing lower relative counts for neutrophils and monocytes in response to ancestral LPS exposure (basophils were not assessed) (Figure 3.6). Although relative counts for macrophages again reflects a decrease in association with LPS for F2, the opposite is seen in F1 – this may indicate a phenotype switch to favour F4/80^{+} macrophages. In addition, this analysis revealed that the increased splenic lymphocyte count can be ascribed to T-lymphocytes rather than NKT-lymphocytes or NK cells, which appeared unaffected by the LPS intervention.

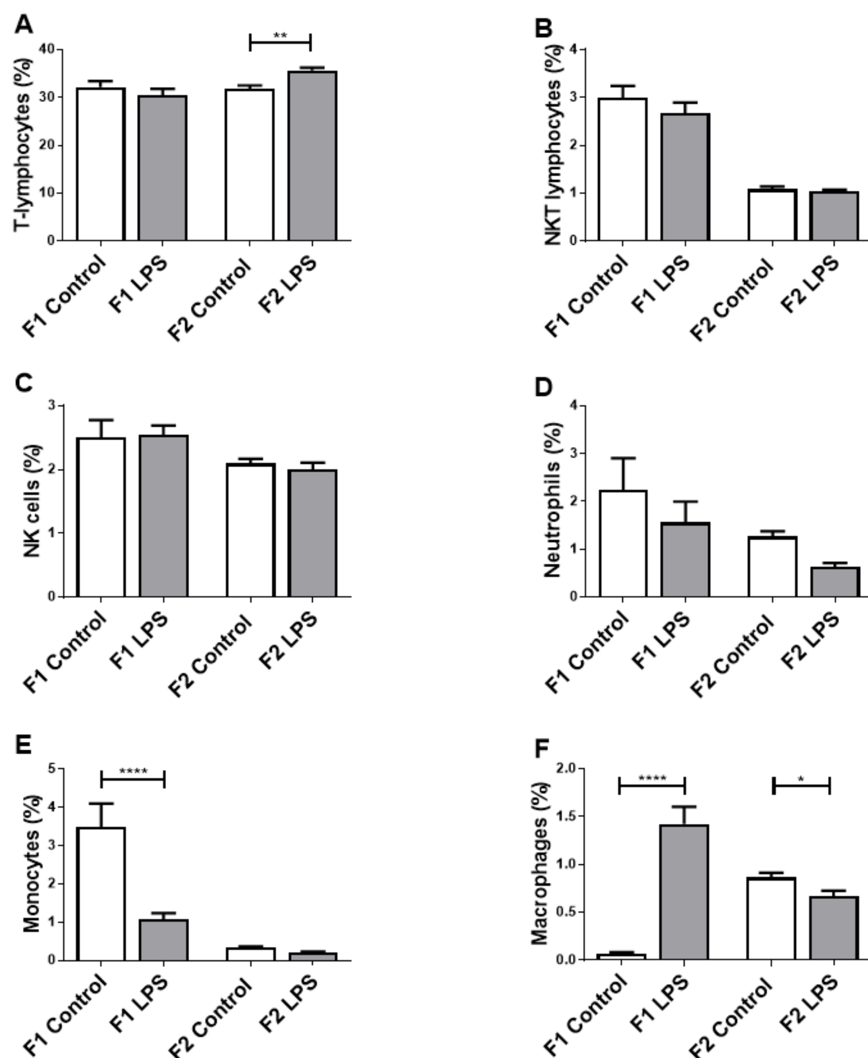

Figure 3.6 Frequency of splenocyte populations between LPS-affected and control F1 and F2 generations. (a) T-lymphocytes, (b) NKT-lymphocytes, (c) NK cells, (d) neutrophils, (e), monocytes and (f) macrophages. . A two-way ANOVA was used to compare F0 LPS and F0 Saline groups. Data is represented mean ±SEM, *n*=8 per group. Significance is depicted as follows: * p<0.05; ** p<0.01; *** p<0.001, **** p<0.001.

Turning attention to GR expression levels in offspring, the majority of leukocyte types exhibited increased GR expression levels in response to the LPS intervention (Figure

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3.7). Of interest, two exceptions were evident – for F1, both neutrophil GR and macrophage GR seemed unaffected. However, when considering F0, F1 and F2 responses to LPS together, this generation seems to reflect a transitional phase to effects only statistically significantly evident in F2.

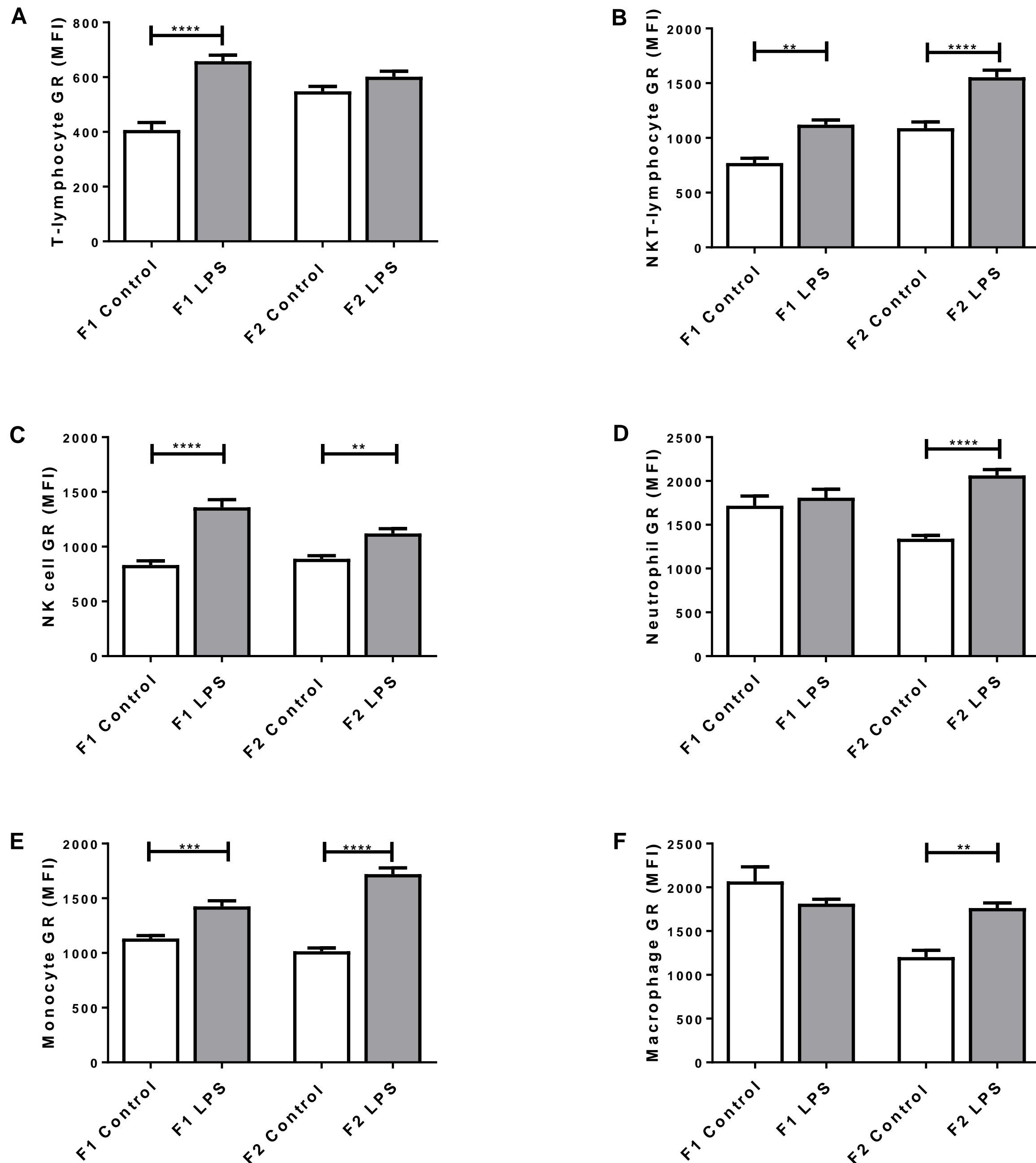

Figure 3.7 Glucocorticoid receptor levels in spleen T-lymphocytes (a), NKT-lymphocytes (b), NK cells (c), neutrophils (d) monocytes (e) and macrophages (f) of F1 and F2 untreated and LPS-treated generations. . A two-way ANOVA was used to compare F0 LPS and F0 Saline groups. Data is represented mean ±SEM, *n*=8 per group. Significance is depicted as follows: ** p<0.01; *** p<0.001, **** p<0.001.

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In terms of functional capacity, basal cytokine secretion was not affected by ancestral LPS exposure in either F1 or F2, with levels mostly below kit detection thresholds (Supplementary Material III).

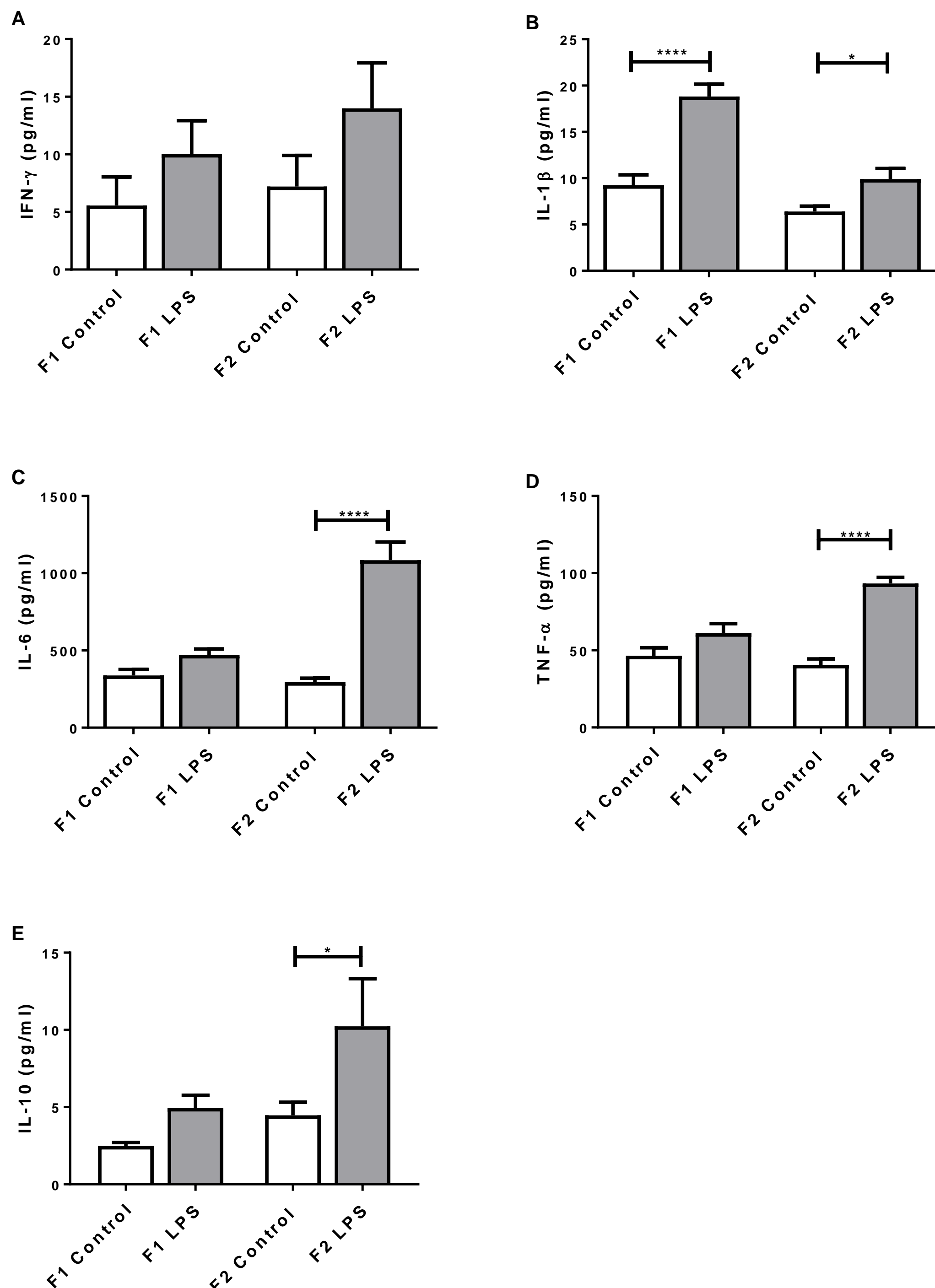

Figure 3.8 LPS-stimulated *ex vivo* cytokine responses of splenocytes from control vs LPS-affected mice across two generations of offspring. IFN-γ (a), IL-1β (b), IL-6 (c), TNF-α (d) IL-10 (e) levels were analysed after 18-hour incubation with 1ug/ml LPS. A two-way ANOVA was used to compare F0 LPS and F0 Saline groups. Data is represented mean ±SEM, *n*=8 per group. Significance is depicted as follows: * p<0.05; ** p<0.01; *** p<0.001, **** p<0.001

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However, in response to *in vitro* LPS challenge, the non-significant increase in cytokine production seen in mothers even after a period of recovery (Supplementary Material III) and which suggests a transient cytokine response to the LPS stimuli, were propagated and seemed to become more significant with each subsequent generation for the majority of cytokines assessed (Figure 3.8).

3.4.3. Contribution of NLRP3 inflammasome

In the current study, mouse splenocytes were frozen for an extended period of time (approximately 6 months) to facilitate batch analysis of all generations. In these previously frozen cells, the addition of nigericin proved to be unnecessary to facilitate conversion to IL-1β. Thus, only basal and LPS-induced inflammasome activation are presented (Figure 3.9). The control F0 splenic F4/80^{+} macrophages exhibited the expected LPS-induced increase in pro-IL-1β production, as well as conversion to IL-1β (as indicated by ASC complex formation). In the LPS-exposed group, basal intracellular pro-IL-1β levels were significantly higher ($p<0.05$) when compared to controls. However, relatively less efficient conversion occurred in response to acute LPS challenge. In generation F1, cells seem to maintain constant pro-IL-1β expression levels, but *in utero* LPS exposure was associated with more efficient conversion to IL-1β both basally and in response to acute LPS challenge. In F2, no significant differences are evident between control and LPS-exposed offspring.

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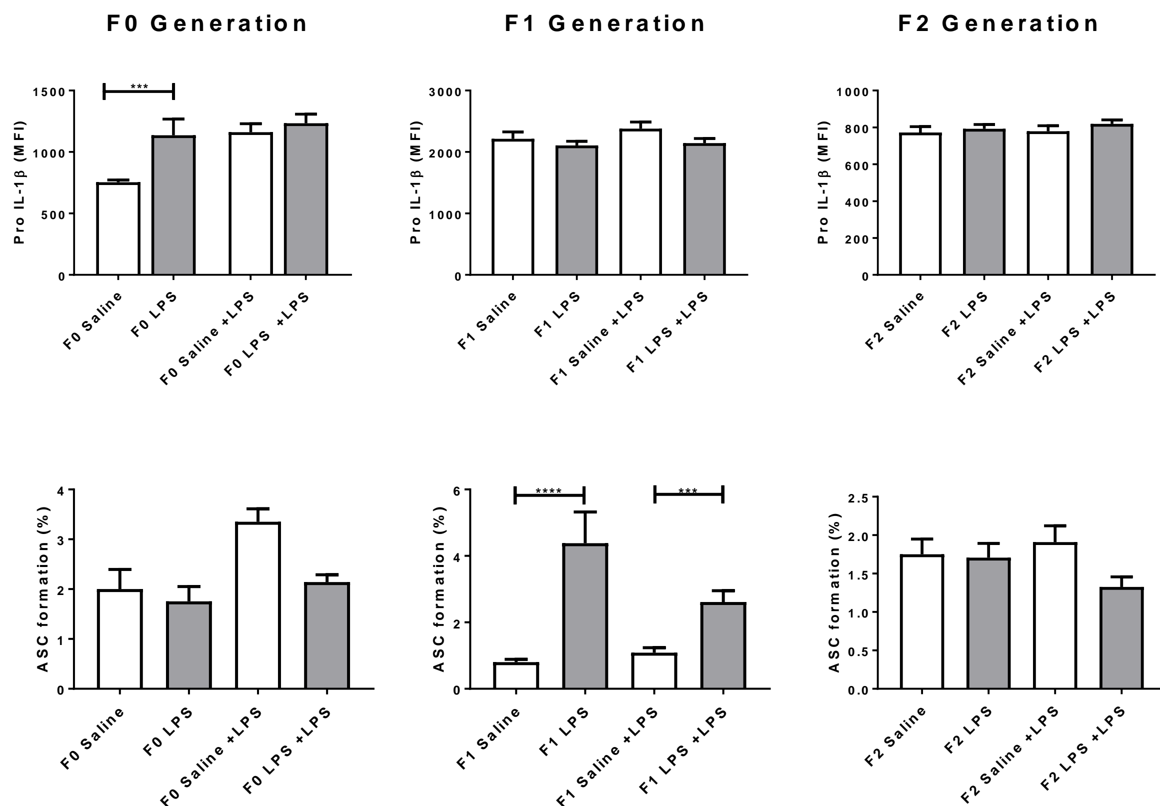

Figure 3.9 Relative expressions levels of Pro-IL-1β and ASC formation in CD11b+F4/80+ splenic macrophages. Basal and LPS responses were assessed in F0, F1 and F2 for both LPS and Control groups. . A two-way ANOVA was used to compare F0 LPS and F0 Saline groups. Data is represented mean ±SEM, *n*=8 per group. Significance is depicted as follows: *** p<0.001, **** p<0.001.

3.5. Discussion

The current study has successfully established an *in vivo* mouse model of chronic maternal inflammation, by expansion of the maternal periconception systemic inflammation (MPSI) protocol established by Williams *et al.* (2011). For the current model, the low-dose (10 μg/kg, every 7 days) LPS intraperitoneal administration in pregnant dams was continued until the end of the gestation period. Significant alterations in the immunological and HPA functionality are reported for all generations.

Chronic MPSI induced postnatal changes in the HPA-axis, as well as in leukocyte profile and functional capacity in mothers, some of which remained evident even after a recovery period. Furthermore, significant lasting effects of the LPS-affected *in utero* microenvironment are evident in the F1 and F2 generations, with regards to both circulating and reservoir immune cells, as well as glucocorticoid responsiveness and cytokine responses to *ex vivo* LPS challenge.

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The current data contributes to the knowledge regarding transgenerational inheritance of physiological adaptations to gestational chronic inflammation. The detailed leukocyte subpopulation-specific analyses in particular, provide new insight into the role of an altered *in utero* environment on the immunological phenotypes of both the F1 and F2 generations of offspring.

3.5.1. Maternal adaptation to gestational chronic LPS administration

In terms of gestation and litter size, neither was significantly affected by chronic MPSI, although offspring number was lower than that reported after either single dose LPS (Williams, Teeling, Perry & Fleming, 2011) or immobilisation stress during gestation (Amugongo & Hlusko, 2014), suggesting that the current model could represent a comparatively more severe stressor.

An acute single low dose (10 μg/kg) LPS challenge is known to induce an inflammatory cytokine response in dams, which return to control levels by 72 hours post-administration (Williams *et al.*, 2011). Similarly, in a mild model of chronic systemic administration (6.45 μg/kg LPS per day, administered by osmotic pump during pregnancy and lactation) (Dudele et al., 2017), LPS-administered dams displayed no significant impact on the postnatal systemic inflammatory profile at one week after cessation of LPS challenge. However, in the current study, a relatively more severe LPS challenge (weekly bolus injection of 10μg/kg) resulted in a long-term reduction in splenic NKT lymphocyte counts. Also, in line with published data on GR expression generated in total leukocytes or total lymphocytes in circulation (Bhattacharyya et al., 2007), macrophage GR was indeed lower in response to the chronic inflammatory stimulus. However, in contrast, subpopulation-specific analysis indicated an increased GR expression on T-lymphocytes, NK cells and monocytes – minority cell types which may not necessarily reflect in total T or total leukocyte GR analyses – suggesting a more differential leukocyte response to this particular chronic stress than previously thought.

The gestational LPS challenge indicated more significant effects on NKT cell numbers in the spleen, which is in line with and expand on available relevant literature. The low splenic NKT-lymphocyte frequencies reported here are likely due to NKT cell migration from the spleen into the maternal decidua (Boyson *et al.*, 2008; Li *et al.*, 2012). Alternatively, a decreased availability of NKT cells for storage in the spleen may result

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from increased recruitment of NKT cells from circulation into the maternal decidua. Previously, LPS exposure and risk of foetal loss was attributed to the increased presence of invariant NKT lymphocytes (iNKTs) in the maternal decidua (Ito et al., 2000). iNKTs contribute to the majority of NKT numbers and typically co-express T-lymphocyte receptors as well as NK cell receptors. iNKTs are significantly implicated in LPS-induced pregnancy loss (Li et al., 2013) and preterm delivery (Li et al., 2012, 2013, 2015) through inflammatory cell activation as well as T_H-1 and T_H-17 responses. This interpretation is in line with findings in a pilot study to the current study, where initiation of LPS-induced inflammation prior to conception prevented pregnancy in the majority of dams (data not shown). Furthermore, the NKT adaptation in the current study were sustained for several weeks after the end of administration of the LPS stimulus, suggesting an inability of pregnant dams to readily recover, which may have predisposed their offspring to related (mal)adaptations.

Assessment of GR expression levels in a leukocyte subpopulation-specific manner provided more information. Clinical evidence has substantiated the hypothesis that chronic stress directly influences leukocyte GR expression and functionality, although some contrasting data has been reported. For example, in PTSD veterans, lower total leukocyte GR density was reported (de Kloet et al., 2007) and specifically in T-lymphocytes, B-lymphocytes and NK cells (Gotovac et al., 2003). In contrast, PTSD and anxiety disorders has also been associated with elevated lymphocyte GR expression (Yehuda et al., 1993) as well as specifically in neutrophils (Gurfein et al., 2017). This is possibly the result of glucocorticoid adaptation occurring across a continuum where GR levels initially increase in response to acutely increased glucocorticoid levels, followed by GR downregulation when glucocorticoid hypersecretion becomes chronic. In terms of inflammation, this results firstly in an anti-inflammatory effect, which changes to a more pro-inflammatory outcome with onset of glucocorticoid resistance. In the current study, significantly higher GR expression was evident in T-lymphocytes, and NK cells in response to maternal chronic gestational LPS exposure. The higher GR levels in T-lymphocytes may, at least in part, be due to the presence of T-regulatory cells, which promote autoimmune protection during pregnancy (Engler et al., 2017). Future detailed analysis could shed more light on the validity of this interpretation. Moreover, even under normal conditions, NK cells are particularly sensitive to glucocorticoids (Eddy et al., 2014). It is thus not unexpected that this cell type in particular would respond by further increasing GR and thus GC

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sensitivity under conditions of chronic inflammatory activation. This may further indicate that the current protocol was not long enough in duration to result in chronic down-regulation of GC sensitivity, i.e. the dams did not have a sustained pro-inflammatory phenotype. However, given the continuum of GR adaptation, if offspring were to inherit a hyper-responsiveness to glucocorticoids – as seen here in NK cells and T-lymphocytes – they may be at risk of reaching the threshold for glucocorticoid insensitivity relatively earlier in life. This is in line with the earlier incidence of non-communicable diseases in the modern era (Smith & Essop, 2009; Okafor, 2011; Aguilar et al., 2015).

Monocyte and macrophage populations and their response to LPS are well characterised. In the current study, monocytes indeed exhibited an increased GR expression in dams exposed to LPS, which is in line with the relevant literature (Bhattacharyya et al., 2007; Miller et al., 2014; Bennett & Smith, 2018) and an interpretation of a predominating inflammatory profile existing in the LPS-treated dams. In contrast, macrophages had decreased GR expression levels in response to LPS. Similar to current data, splenic macrophages previously displayed insensitivity to glucocorticoids and enhanced IL-6 production in a model of chronic social stress (Stark et al., 2001). Although the corticosterone levels were not indicative of glucocorticoid insensitivity in the current study, the modulation of splenic cell composition and GR levels, which was also reported in another model of chronic mild stress (Gurfein et al., 2017), suggests a selective GR insensitivity in the splenic macrophages, priming the immune system to a relatively more pro-inflammatory phenotype.

Elucidation of splenic macrophage NLRP3 activation in the chronic LPS MPSI model, through the expression of pro IL-1β and ASC, further supports this interpretation. In the current study, when compared to saline-exposed controls, chronic LPS administration resulted in higher unstimulated pro-IL-1β production by F4/80+ CD11b+ splenic macrophages of F0 dams. This finding corresponds to the increased basal pro IL-1β production reported macrophages of aged mice, (Ramirez et al., 2012), as well as in chronic LPS exposure in rats, where significantly higher IL-1β secretion is reported at basal level (Guan, Lin & Tang, 2015). Interestingly, when an acute added stressor is applied, IL-1β secretion is unaltered from basal (Guan, Lin & Tang, 2015), which is again in line with current data. This suggests that the NLRP3 complex formation has a rate-limiting step in its response to chronic stress, possibly to prevent

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continuous activation. This is supported by the literature, which names caspase-1 as rate-limiting enzyme in the cleavage of pro-IL-1β to release IL-1β, in the context of neuroinflammation (Frank et al., 2016).

More knowledge is undoubtedly required to fully elucidate all mechanisms and role players involved, particularly within a human model. Nevertheless, gestational exposure to chronic inflammation clearly results in significant effects on the mother which is not readily resolved and may thus be transferred to her progeny as a relatively pro-inflammatory phenotype already at birth.

3.5.2. Transgenerational inheritance of chronic LPS administration in F1 and F2 generations

There is a growing body of evidence in support of generational transfer of increased susceptibility to disease and dysregulation. In example, in diabetes and obesity there is a significant association between parental history of obesity and diabetes and levels of serum fatty acid binding protein 4, retinol binding protein 4 and adiponectin, favouring obesity the risk developing of these disorders in offspring (Zachariah et al., 2017). Furthermore, that the foetal-maternal uterine environment is reported to have direct effects on offspring weight and metabolic outcomes (Tanvig, 2014). Diet-induced obesity has also been associated altered splenic CD4+ cells, macrophages and dendritic cells in mice (Boi et al., 2016) and elevated inflammatory cytokines and risk of mortality (Strandberg et al., 2009), with this metabolic dysfunction is also seen to be transferable to offspring in murine studies (Babu et al., in 2018). In the current study, both F1 and F2 generations of offspring to the LPS-dams exhibited significantly compromised physiology. Our data is in line with the abovementioned findings, and also expands on the current knowledge, illustrating that this inherited “proinflammatory proneness” may not limited to specific diseases, but could be more generally applicable to any parental chronic exposure involving substantial inflammation.

In line with previous reports (Williams, Teeling, Perry & Fleming, 2011; Kirsten et al., 2013), maternal LPS exposure did not affect offspring basal corticosterone levels when compared to controls. This is in support of our interpretation of inherited inflammatory proneness, as $F1_{LPS}$ exhibited HPA-axis hyperactivation, while $F2_{LPS}$ displayed an HPA-axis response may be attributed to relative adrenal burnout (Lynn et al., 2018). This may also explain the rise in GR in the majority of splenocytes assessed for F1.

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However, $F2_{LPS}$ splenocytes maintained the increased GR levels when compared to F2 Control mice, perhaps as a countermeasure to the relative adrenal hypo-responsiveness. Thus, in response to chronic LPS exposure, maladaptation in offspring may be indicated by changes in HPA functionality rather than GR levels specifically.

In our opinion, the GC and GR hyper-response in $F1_{LPS}$ splenocytes reported is due to an inflammatory phenotype resulting from maternal inflammatory response and subsequent placental inflammatory responses, that was shown to induce indirect foetal damage, specifically intestinal damage, that persists way beyond the postnatal period (Fricke et al., 2018).

This placental inflammatory response, subsequent offspring inflammatory response may be the reason for the explain the changes in leukocyte numbers in $F1_{LPS}$. While circulating leukocyte number and distribution remained unaffected, a clear picture of inherited altered immune system is evident in splenocytes. *In utero* inflammatory response to maternal LPS exposure was associated with increased splenocyte counts in, primarily due to a rise in lymphocyte populations. In contrast, neutrophil numbers were relatively decreased – which we interpret as tissue sequestration of neutrophils participating in affected areas of inflammation. This is further supported by flow cytometry results indicating an increased conversion of splenic CD11b+ monocytes to CD11b+F4/80+ macrophages that primarily produce IL-1β. Although the proinflammatory phenotype observed in $F1_{LPS}$ had no increased capacity for NLRP3 activation or increased proinflammatory cytokine secretion basally, during acute LPS challenge, both NLRP3 conversion of pro-IL-1β to active IL-1β and pro-inflammatory cytokine secretion was significantly exacerbated in $F1_{LPS}$ in comparison to controls. Chronic preconditioning with inflammatory mediators, such as IL-1β, have been shown to induce immunotolerance, by downregulation of TLR4 and through stimulation of corticosterone production (Alves-Rosa et al., 2002), which may be the case in $F1_{LPS}$.

When considering $F2_{LPS}$, a picture of relative immune tolerance seems to emerge, suggesting that effects of chronically elevated IL-1β on TLR4 in F1 may have been inherited by $F2_{LPS}$. For example, in contrast to the increase in splenic WBCs in $F1_{LPS}$, in $F2_{LPS}$ splenic WBCs were decreased. Most notably, macrophage frequency decreased in $F2_{LPS}$. In addition, the available macrophages failed to activate NLRP3

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in response to stimulus, effectively resulting in a much smaller net IL-1β response upon acute *in vitro* challenge, in line with TLR4 downregulation. This picture of relative immune tolerance was associated with an approximate (but statistically insignificant) 20% decrease in GC levels.

Interestingly, the sustained upregulation of leukocyte GR into the second generation of offspring may suggest epigenetic adaptation or primordial germline inheritance adaption, This is also in line with a previous report with upregulated GR in response to chronic mild psychological stress (Gurfein et al., 2017). In the study by Nephew and colleagues, the hypomethylation of GR gene promotor regions was lost in the second generation offspring. However, their model was milder than the one employed in the current study (Nephew et al., 2017). A review by Dunn, Morgan and Bale, (2011) hypothesised that for a phenotype to be inherited into one subsequent generation, *in utero* modification (epigenetic adaptation) is the only requirement. However, for a phenotype to be inherited into two or more subsequent generations, the stimulus has to be robust for resulting adaptations to stably alter germ cells. Taken together, the current study and that of Nephew et al. (2017) suggests that the plasticity of generational transfer may be severity-dependent. Importantly, it also indicates that this adaptation is not limited to infectious stimuli.

Despite the increased leukocyte GR expression, showed increased secretion IFN-γ, TNF-α, IL-6 and IL-10 in $F2_{LPS}$, a response not significantly present in $F1_{LPS}$. Previously, increased induced TNF-α (Hicks-Nelson et al., 2017a) and IL-6 (Carpenter et al., 2010; Murphy et al., 2014) was also reported in a model of chronic social stress, again suggesting a stimulus-independent adaptive effect. Furthermore, data suggest independence of this adaptation from the NLRP3 inflammasome. Nevertheless, the increased levels of cytokines other than IL-1β, suggest an alternative source, possibly lymphoid cells or neutrophils.

Though basal cytokine levels do not allude to a systemic pro-inflammatory phenotype, which have been reported in other stress models (Cheng, Jope & Beurel, 2015; Hicks-Nelson et al., 2017a; Nephew et al., 2017), there are two potential mechanisms that we propose to this outcome. Firstly, in $F1_{LPS}$, the upregulation of glucocorticoid sensitivity possibly reduced cytolytic activity by reduction of histone promoter acetylation for perforin and granzyme B, and TNF-α, IL-6 and IFN-γ production, as

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previously reported for NK cells (Krukowski et al., 2011; Eddy et al., 2014). However, in $F2_{LPS}$, where a picture of relative GC hyposecretion seemed present, this downregulation may have been abolished, resulting in increased cytokine release from NK – and potentially also other – cells. Secondly, neutrophils specifically are negatively implicated in the primordial generational programming in the F2 generation, as acute high dose LPS have been shown to increase neutrophil counts as well as GR receptor level on neutrophils (Bergquist et al., 2014) and GR expression on macrophages (Southworth et al., 2012). Furthermore, impaired translocation of activated GR was also demonstrated in at least neutrophils and T-lymphocytes after LPS exposure (Bergquist et al., 2014). Thus, in a chronic stimulus setting, sustained impaired GR function on specific leukocytes is probable and in line with current results suggesting a relative proinflammatory outcome despite down-regulated macrophage NLRP3 and increased GR expression levels. Future purpose designed studies could shed further light on this probability.

When specifically considering the leukocyte GR data, the response seen in the F0 model is mirrored in F1 and F2, with exacerbation of the response with each successive generation. This expands on the generally accepted report ascribing reduced hippocampal GR promotor methylation in offspring from low grooming arch back nursing (LG-ABN) mothers to diminished GR sensitivity, that persisted to adulthood (Weaver et al., 2004). Our current data is also in line with data from a transgenerational chronic social stress (CSS) mouse model, where lower GR methylation was reported in F1 CSS offspring, but not in F2 CSS (Nephew et al., 2017). This may allude to epigenetic inheritance or primordial germline inheritance, due to the chronic grandparent and *in utero* parental exposure to LPS, with even higher responsiveness in the F2 generation despite the addition of an unaffected parent.

3.6. Conclusion

In the current study, we show that in a mouse model of chronic low-grade maternal inflammation induced by LPS administration may facilitate longstanding reprogramming of the offspring phenotype, and that this effect is perpetuated in the next generation without any further stimulus. The maternal inflammatory state is mirrored and exacerbated in two subsequent generations of offspring, indicating transgenerational inheritance of the inflammatory phenotype and perhaps underlying

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epigenetic adaptation. The current results also suggest that similar primordial germline programming may occur in response to LPS and/or psychological stressors. Thus, current study contributes to our understanding of parental contribution to predisposition for development of non-communicable chronic diseases.

Given these insights, it is imperative to confirm and further characterise the mechanisms underlying this “proinflammatory programming”, particularly in a human model, in order to facilitate development of interventions to ameliorate these effects.

3.7. Author contributions

CS and RCMA conceptualized and designed the study. RCMA performed the experiments, data reduction and statistical analysis under the supervision of CS. Both CS and RCMA contributed to data interpretation and manuscript preparation.

3.8. Acknowledgements

The authors declare no competing financial interests. The South African National Research Foundation and Stellenbosch University is acknowledged for financial assistance and a bursary to RCMA. Mr LD Africa and Miss AC Bennett are acknowledged for technical assistance in sample preparation.

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3.9. Supplemental Material

3.9.1. Supplementary Material I: Breeding data, offspring litter size, gestational length

Table 3.2 Breeding data, offspring litter size, gestational length for F0 dams

Breeding	Treatment	**N**	Mating Partner	Gestation days	Litter size Mean ± SEM
F0	Saline	5	Wild-type	20	6,5 ± 1,26
F0	LPS	6	Wild-type	20	6,0 ± 0,95
F1	Saline	8	Wild-type	20	5,25 ± 0,48
F1	LPS	8	Wild-type	20	5,83 ± 0,88

LPS; Lipopolysaccharide

3.9.2. Supplementary material II: Plasma corticosterone levels for F0, F1 and F2 generations

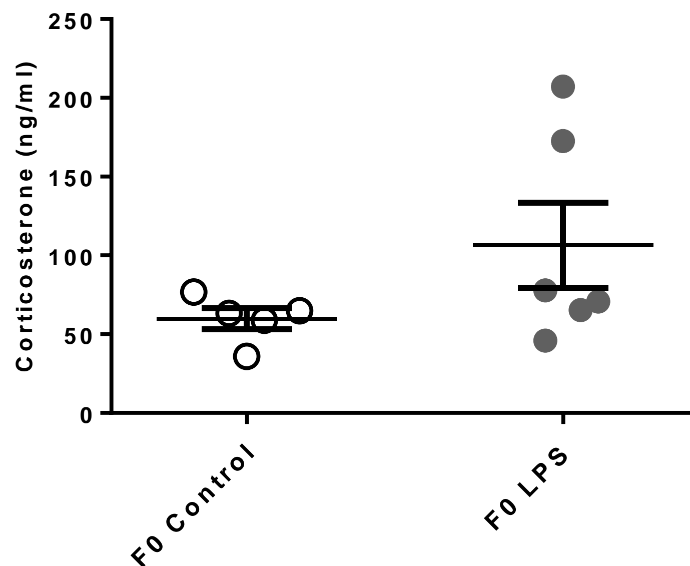

Figure 3.10 Basal plasma corticosterone for LPS-exposed and control groups for F0 generation 4 weeks after weaning of offspring. Data is represented mean ±SEM, F0 Control, n=5; F0 LPS, *n*=6.

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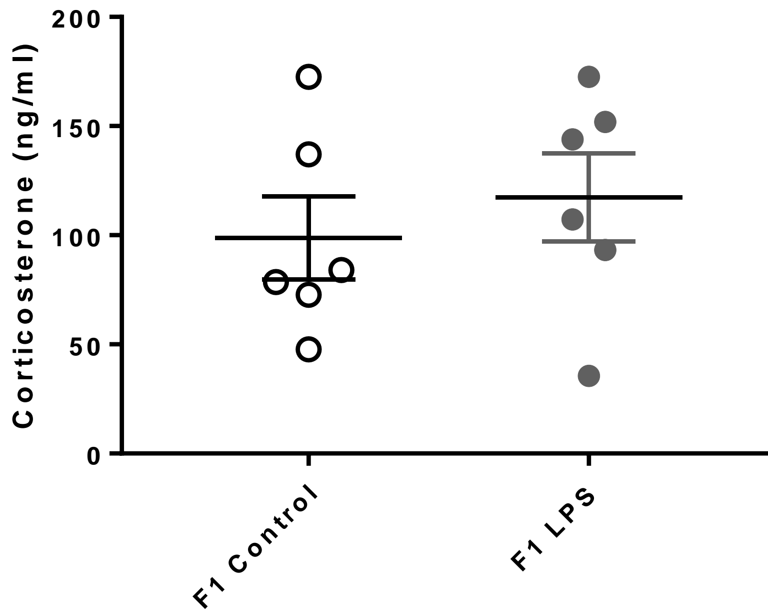

Figure 3.11 Basal plasma corticosterone for LPS-exposed and control groups for F1 generation. Data is represented mean ±SEM, F1 Control, *n*=8; F1 LPS, *n*=8.

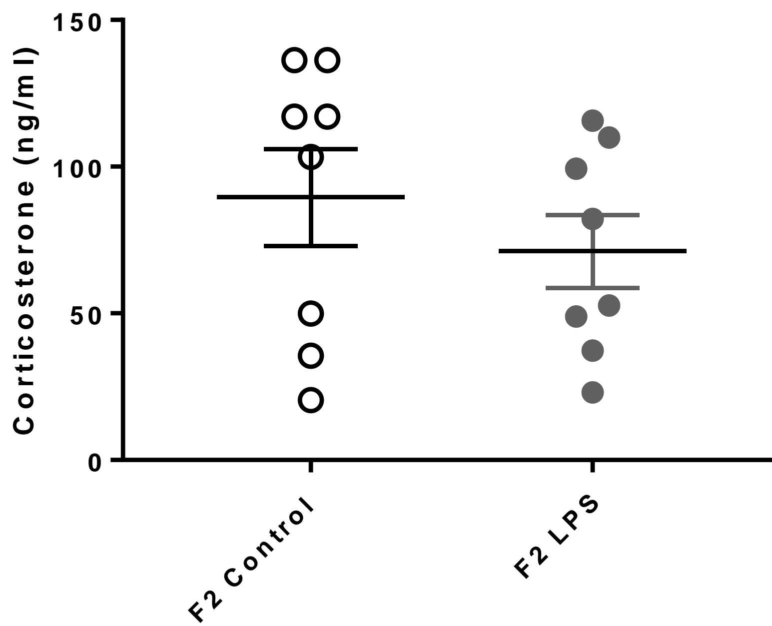

Figure 3.12 Basal plasma corticosterone for LPS-exposed and control groups for F2 generation. Data is represented mean ±SEM, F2 Control, *n*=8; F2 LPS, *n*=8.

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Supplementary Material III: *Ex Vivo* Cytokine Response

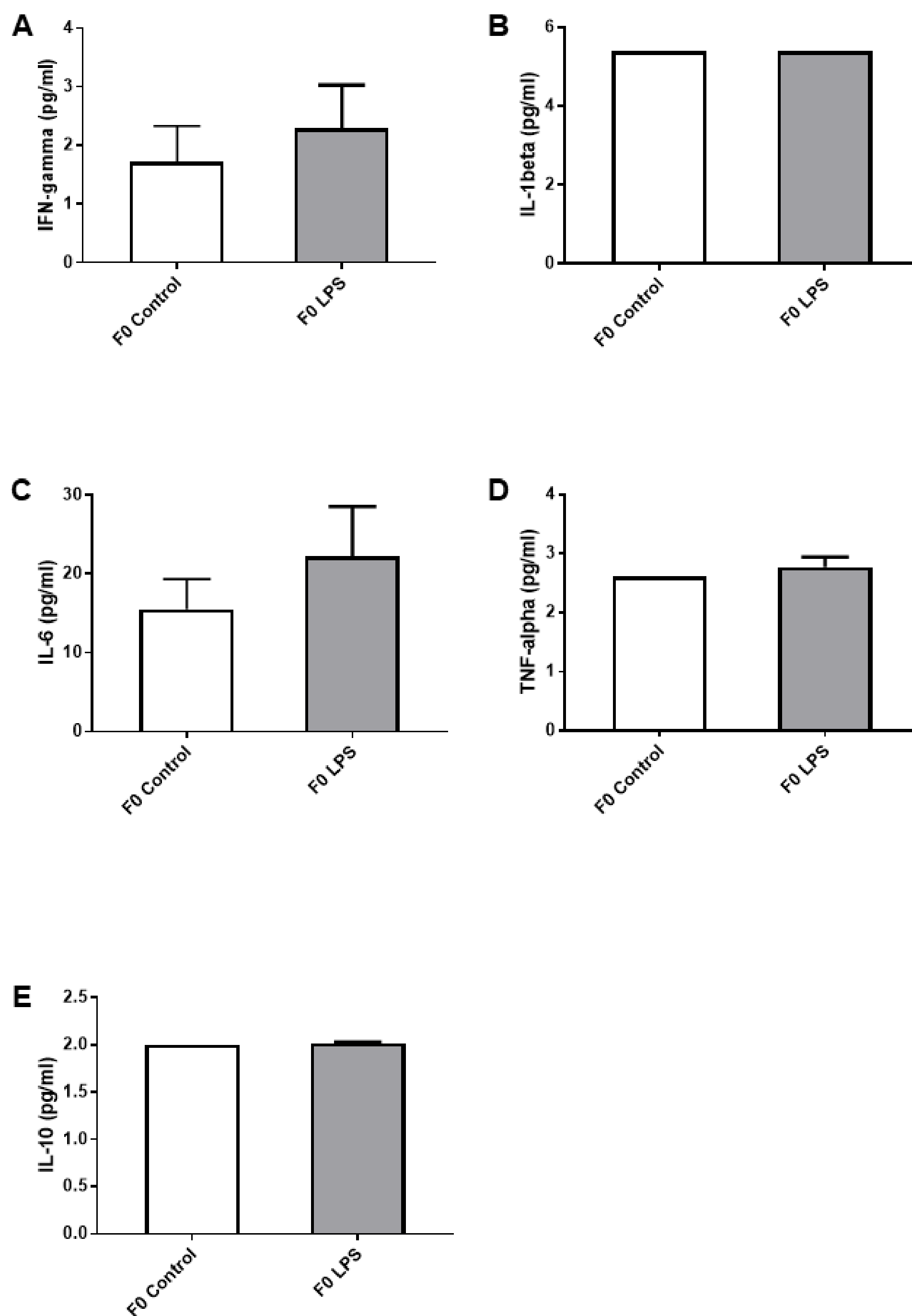

Figure 3.13 Basal *ex vivo* cytokine responses of splenocytes from control vs LPS-affected mice for the F0 generation. IFN-γ (a), IL-1β (b), IL-6 (c), TNF-α (d) IL-10 (e) levels were analysed after 18-hour incubation with RPMI 1640. Data is depicted as mean ± SEM, n = 5 and n = 6 for F0 Control and F0 LPS respectively.

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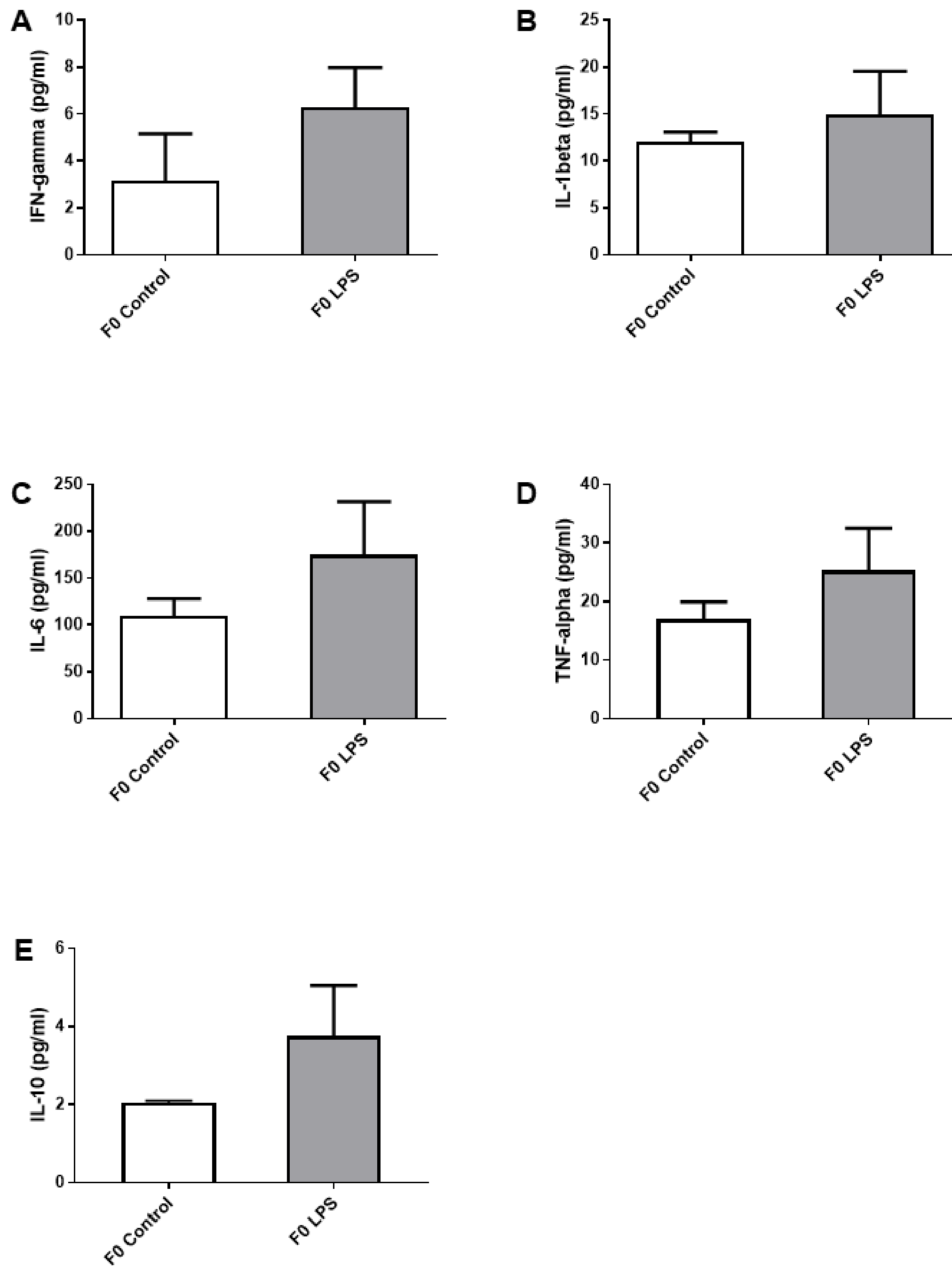

Figure 3.14 LPS-stimulated ex vivo cytokine responses of splenocytes from control vs LPS-affected mice for the F0 generation. IFN-γ (a), IL-1β (b), IL-6 (c), TNF-α (d) IL-10 (e) levels were analysed after 18-hour incubation with 1ug/ml LPS. Data is depicted as mean ± SEM, n = 5 and n = 6 for F0 Control and F0 LPS respectively.

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Supplementary Material IV: F1 and F2 *Ex vivo* Cytokine Response

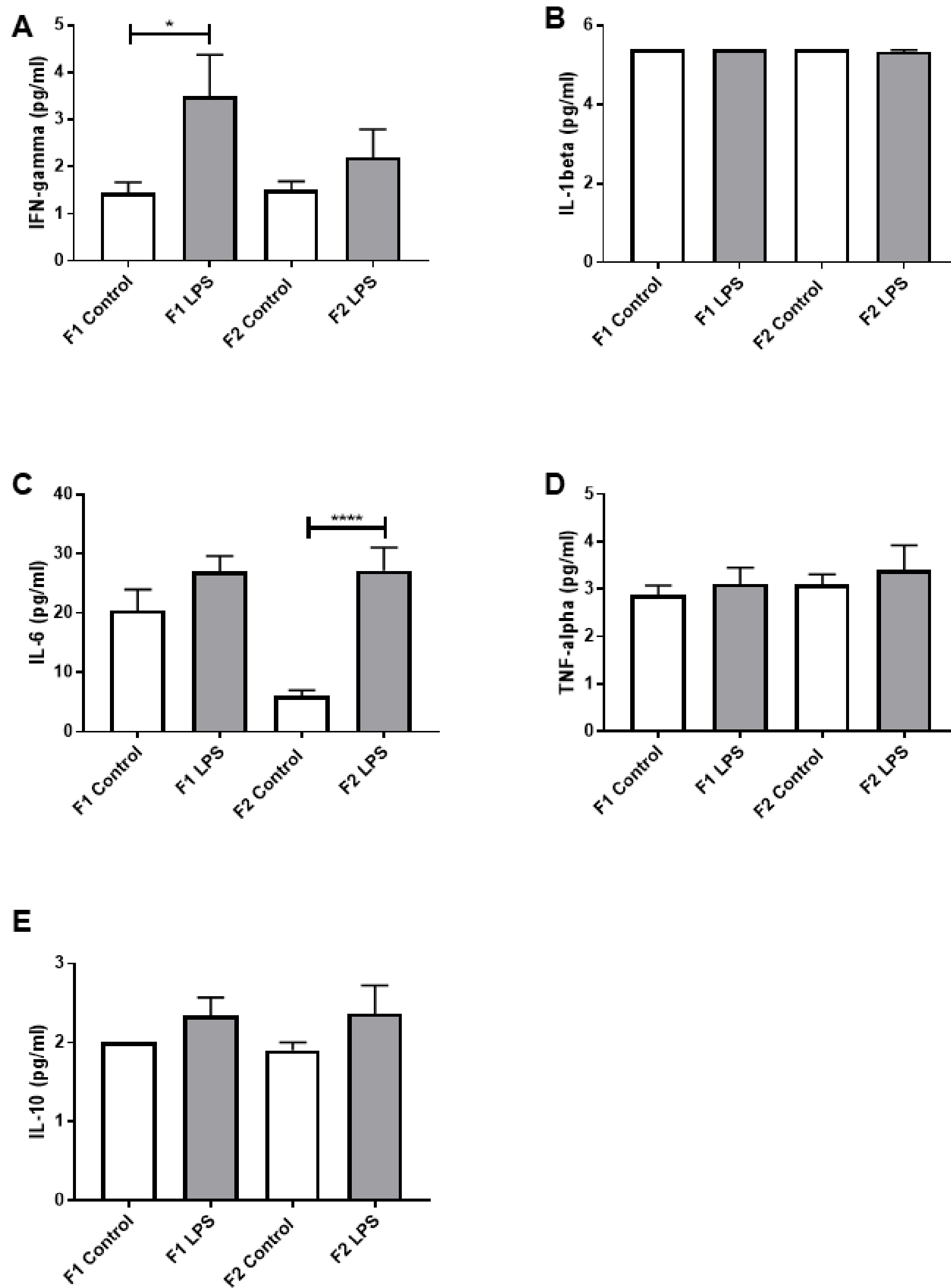

Figure 3.15 Basal ex vivo cytokine responses of splenocytes from control vs LPS-affected mice for the F1 and F2 generation. IFN-γ (a), IL-1β (b), IL-6 (c), TNF-α (d) IL-10 (e) levels were analysed after 18-hour incubation with RPMI 1640. Data is depicted as mean ± SEM, *n* = 8 per group."

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Chapter 4

In utero exposure to maternal chronic inflammation transfers a pro-inflammatory profile to generation F2 via sex-specific mechanisms

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4.1. Abstract

Generational transfer of maladaptations in offspring have been reported to persist for multiple generations in conditions of chronic inflammation, metabolic and psychological stress. Thus, the current study aimed to expand our understanding of the nature, potential sex-specificity, and transgenerational plasticity of inflammatory maladaptations resulting from maternal chronic inflammation. Briefly, F1 and F2 generations of offspring from C57/BL/6 dams exposed to a modified maternal periconception systemic inflammation (MSPI) protocol were profiled in terms of leukocyte and splenocyte counts and cytokine responses, as well as glucocorticoid sensitivity. Overall, F1 male and female LPS groups presented with glucocorticoid hypersensitivity (with elevated corticosterone and increased leukocyte glucocorticoid receptor levels) along with a pro-inflammatory phenotype, which carried over to the F2 generation. The transfer of inflammatory and glucocorticoid responsiveness from F1 - F2 is evident, with heritability of this phenotype in F2. The findings suggest that maternal (F0) perinatal chronic inflammation resulted in glucocorticoid dysregulation and a resultant pro-inflammatory phenotype which is transferred in the maternal lineage but seems to affect male offspring to a greater extent. Of further interest, upregulation of IL-1β cytokine responses is reported in female offspring only. The cumulative maladaptation reported in F2 offspring when both F1 parents were affected by maternal LPS exposure is suggestive of immune senescence. Given the potential impact of current results and the lack of sex-specific investigations, more research in this context is urgently required.

4.2. Introduction

It is proposed by a large body of literature that adversities (i.e. infectious, metabolic or psychological stress) during gestational development has a major impact on the long-term health outcomes of offspring into adulthood. Extensive research on human and animal studies have shown that, in the context of maternal stress or physiological disturbances, adversity during gestation can result in long term pathological outcomes in offspring. For example, in models such as poor nutrition and chronic social stress, in humans, altered serum concentrations of fatty acid binding protein 4 and adiponectin (Zachariah et al., 2017) were reported. In rodent models, altered corticosterone (CORT), prolactin, and oxytocin (Babb et al., 2014), intercellular adhesion molecule-1

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(ICAM-1), granulocyte-macrophage colony stimulating factor (GM-CSF), IL-18, progesterone and vascular endothelial growth factor (VEGF) (Murgatroyd et al., 2016) has been illustrated. Furthermore, these outcomes were demonstrated to exceed the first generation and to persist up to three generations downstream (Murgatroyd et al., 2015; Moisiadis et al., 2017). Moreover, the immune response in offspring (F1 to F3) is also altered by maternal exposure to psychological stress (Falcone et al., 2017; Hicks-Nelson et al., 2017b), soluble glucocorticoid challenge (Iqbal et al., 2012; Moisiadis et al., 2017) or immune challenge (Hodyl et al., 2007; Williams, Teeling, Perry, Fleming, et al., 2011; Dudele et al., 2017; Weber-Stadlbauer et al., 2017) during gestation.

Although some of these studies have investigated transgenerational inheritance of the altered phenotype by investigations up to F3, little information was gathered on sex-specificity of the changes maintained up to F3. Nevertheless, the fact that one group repeated reported a paternally transferred increase of in glucocorticoid sensitivity in response to maternal gestational exposure to exogenous glucocorticoids (Iqbal et al., 2012; Moisiadis et al., 2017, 2018), suggests that sex-specificity indeed does exist. However, although many of the adaptive/maladaptive changes reported in offspring relate back to inflammation, a known aetiological factor in most modern chronic disease states, very little data exists on the role of inflammation in this context.

From the limited data available, decreased capacity to mount an immune response to acute experimental infection was reported in F1 offspring to mothers acutely challenged with LPS during periconception (12). More in line with our context of low grade inflammation in chronic disease aetiology, chronic low dose maternal LPS exposure during pregnancy and lactation resulted in a male specific upregulation of blood leukocyte count and hypothalamic inflammatory cytokines in F1 offspring, in the absence of further specific stimulus (i.e. basally) (Dudele et al., 2017). F1 offspring is not indicative of generational inheritance, due to the potential confounding influence of the *in utero* microenvironment (for e.g. LPS-associated damage to the placenta (Kirsten et al., 2013; Fricke et al., 2018)). Employing a model capable of reflecting maternal generational transfer, we have recently reported in mice that experimentally induced chronic maternal gestational inflammation resulted in altered inflammatory profiles in at least two generations of offspring (Adams & Smith, 2019), which is consistent with an interpretation of multigenerational inheritance. Importantly, in terms

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of preventative treatment, in line with the “foetal origins of adult disease” hypothesis by Barker (2007), recent publications have described successful *in utero* treatment of inherited diseases, such as brittle bone disease (Sagar et al., 2018), haemophilia (Gupta et al., 2015) and Gaucher’s disease (Massaro et al., 2018). Thus, given the phenomenon of generational transfer of maladapted physiology already demonstrated in the context of stress and obesity, both conditions with an inflammatory component, the option for corrective intervention in utero may be more widely applicable than just congenital disorders. Indeed, the possibility of decreasing risk for development of especially non-communicable diseases – which are currently the number one cause of mortality world-wide (World Health Organization, 2018) – by addressing foetal inflammatory phenotype, will have huge impact on the health sector globally. However, more detailed information on the nature and generational plasticity of specific mechanisms by which maladaptive inflammatory responses may occur, is required.

The current gap in terms of the sex-dependence on these inherited inflammatory maladaptations, is a major obstacle in the development of strategies such as the *in utero* corrective intervention just mentioned, but also in terms of preventative medicine practices. Our previously published study reflected the transgenerational adaptations that occur in chronic gestational LPS administration and its effect on two subsequent generations of offspring (Adams & Smith, 2019). In an attempt to bridge this gap in literature, we here expand on our previous study in two ways to determine potential sex differences in the pro-inflammatory maladaptation previously reported and potentially delineate sex-specific heritability. Firstly, we present a reanalysis of a portion of the data previously published (Adams & Smith, 2019) to reflect sex-specificity of maladaptive inheritance. Secondly, we report novel data of cumulative inheritance in F2 offspring born from two LPS-affected (F1) parents.

4.3. Materials and Methods

4.3.1. Experimental animals

Ethical clearance for the study was obtained from the Stellenbosch University Animal Research Ethics Committee (Ref # SU-ACUM14-00004). The C57/BL/6 mouse strain was used for the current study. Mouse siblings were housed in groups of five, separated by sex, with the exception of late stage pregnant and lactating mothers who

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were individually housed. All animals were subjected to temperature-controlled and humidity controlled conditions (22 °C and 40% humidity) with a 12-hour dark-light cycle, for the duration of the study, with ad libitum access to standard rodent chow and water, as well as cage enrichment in the form of nesting material and tubing.

The breeding protocol followed is visually presented in Figure 4.1. For the initial breeding of the F0 generation, seven-week-old dams were naturally crossed with age-matched studs. The following day plug-positive dams were randomised to receive either LPS (from E. coli (Sigma-Aldrich, USA)) at 10 μg/kg body mass by intraperitoneal injection (LPS group), prepared in 0.9% saline, or 0.9% saline only (CTRL group), at a final volume of 50 μl. The F0 mothers were administered their respective injections every 7 days until the end of the gestation period (on average 20 days) and after this time point no further interventions were given to either mothers or resulting F1 and F2 offspring for the duration of the study. As a safety precaution, the F0 dams were monitored for adverse reactions post-administration of intervention.

The first generation of offspring, F1, was bred to 7 weeks of age and were then either terminated via cervical dislocation for sample collection, or naturally crossed with an age-matched wild-type (i.e. unaffected) C57/BL/6 mouse to produce the second generation of offspring, F2. In other words, a F1 LPS-affected or F1 CTRL female was crossed with a wild-type male C57/BL/6 mouse that had received no form of intervention. Similarly, a F1 LPS-affected or F1 CTRL male was crossed with a wild-type female C57/BL/6 mouse that had received no form of intervention. Additionally, a group of F1 LPS-affected males were crossed with (non-sibling) F1 LPS-affected females to produce a F2 generation with both parents affected by LPS. Grouping of animals for either termination or further breeding were performed randomly by an independent person. Information on litter size is presented in Table 4.1. All F2 offspring were again bred to 7 weeks of age and then terminated by cervical dislocation, for sample collection and analysis. Thus, for both F1 and F2, all animals were terminated at the same age.

For analysis, the designated groups were then separated into male and female mice. In the F1 generation, mice were grouped as F1 Female (F1 F) and F1 Male (F1 M) group, for the CTRL and LPS intervention groups. In the F2 generation, offspring were classified as follows for the CTRL groups: F2M Male CTRL - male offspring resulting

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from F1 Male CTRL x wild-type C57/BL/6 female; F2M Female CTRL – female offspring resulting from F1 Male CTRL x wild-type C57/BL/6 female; F2F Male CTRL - male offspring resulting from F1 Female CTRL x wild-type C57/BL/6 male; F2F Female CTRL – female offspring resulting from F1 Female CTRL x wild-type C57/BL/6 male.

The classification for the F2 LPS group is as follows: F2M Male LPS1 - male offspring resulting from F1 Male LPS x wild-type C57/BL/6 female; F2M Female LPS1 – female offspring resulting from F1 Male LPS x wild-type C57/BL/6 female; F2F Male LPS1 - male offspring resulting from F1 Female LPS x wild-type C57/BL/6 male; F2F Female LPS1– female offspring resulting from F1 Female LPS x wild-type C57/BL/6 male.

The classification for the F2 LPS offspring from F1 LPS male and female parents is as follows: F2 Male LPS2 - male offspring resulting from F1 Male LPS x F1 Female LPS; F2 Female LPS2 – female offspring resulting from F1 Male LPS group x F1 Female LPS.

Table 4.1. Gestation data for LPS-exposed and LPS-affected mice. Values are depicted as mean ± SEM.

	No. of mice	Mating Partner	Gestation length (days)	Total no. of offspring	Mean Litter Size	Male:Female Ratio
F0 F CTRL	5	-	20	33	6.6 ± 0.98	1.3 ± 0.35
F0 F LPS	5	-	20	30	6.0 ± 0.94	1.5 ± 0.47
F1 M CTRL	4	WT Female	20	19	4.8 ± 0.47	1.4 ± 0.53
F1 M LPS	4	WT Female	20	22	5.5 ± 0.50	1.0 ± 0.38
F1 F CTRL	4	WT Male	20	22	5.5 ± 0.23	1.3 ± 0.29
F1 F LPS	4	WT Male	20	24	6.0 ± 1.30	1.5 ± 0.55
F1 F LPS	3	F1 LPS Male	20	20	10.0 ± 1.10 [1]	1.3 ± 0.03

[1] *Significantly different from all other groups (at least p<0.05)*

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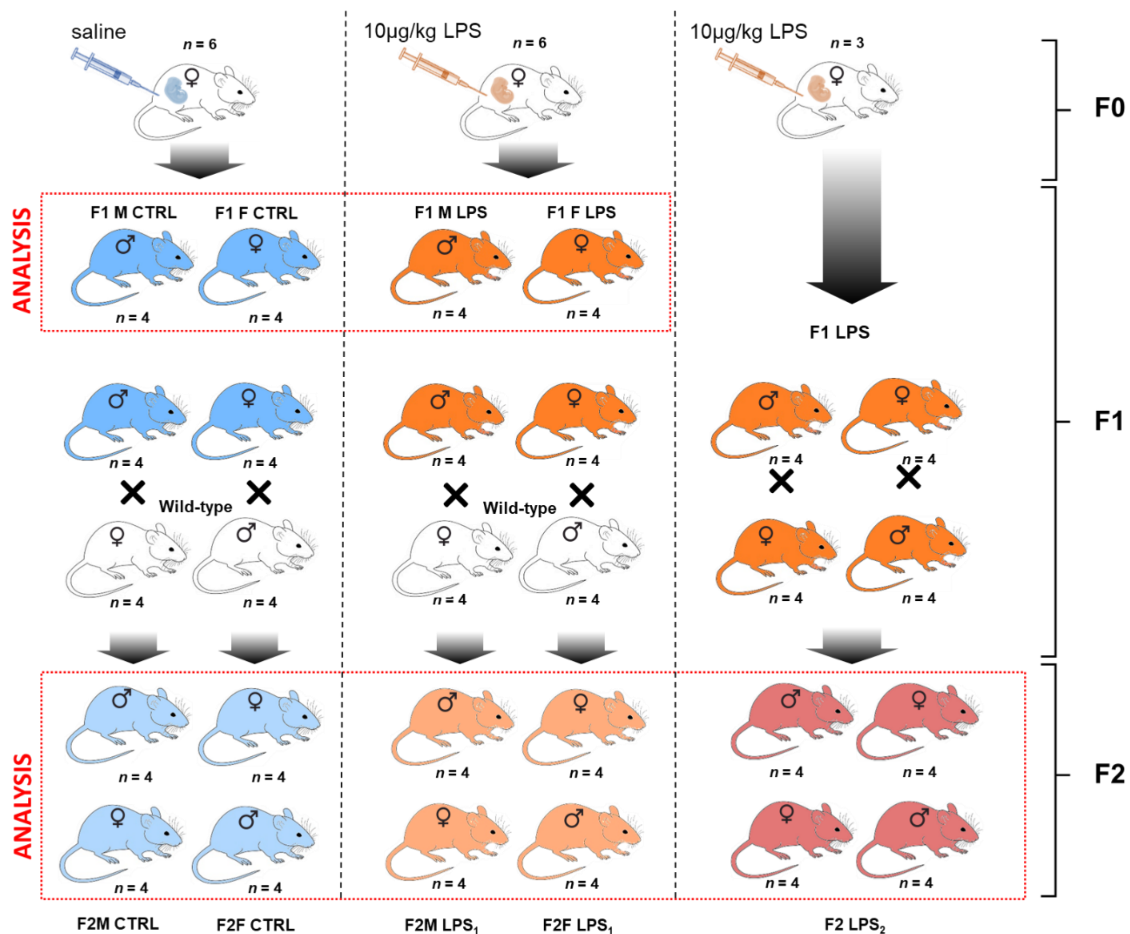

Figure 4.1 Breeding paradigm for control and LPS-affected mice. The *n* represents the number of mice for each subsequent intervention group and sex per F1 and F2 generations used for analysis (demarcated in the red boxes).

4.3.2. Sample collection

Whole blood was collected via cardiac puncture and transferred into K_2EDTA microtubes for full and differential blood counts on the CellDyne 3700CS haemocytometer (Abbott Diagnostics, USA). Plasma was collected from the whole blood samples and analysed for basal corticosterone (CORT) concentrations by quantitative ELISA (DEMEDITEC Corticosterone rat/mouse ELISA, Demeditec Diagnostics, Germany). Concentrations were calculated on a 6-point standard curve with a logistic regression algorithm. The assay detection range was 6.1 – 2250 ng/ml.

Spleens were collected in ice-cold complete RPMI 1640 (cRPMI) medium, consisting of 10% foetal bovine serum, 1% penicillin-streptomycin and 1% gentamicin, for isolation of splenocytes.

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4.3.3. Cell preparation

Spleens were mechanically dissociated and strained through a 70 µm cell strainer to acquire single cell suspensions. Red blood cells were lysed for 5 minutes at room temperature using ammonium–chloride–potassium (ACK) lysis buffer and the splenocytes counted on the Countess Cell Counter (Thermofisher Scientific, USA). Splenocyte counts were then adjusted to 1.0×10^7 cells per ml in cRPMI.

4.3.4. *Ex vivo* cytokine stimulation

For *ex vivo* stimulation, splenocytes were cultured at a concentration of 1×10^6 cells/ml either in the absence or presence of LPS (1µg/ml) for 18 hours, at 37 ºC, 5% CO_2. Supernatant was collected to determine cytokine profile. The MAP Mouse Cytokine/Chemokine Magnetic Bead panel (Millipore, USA) was used to assess the levels of IL-1β, IL-6, IL-10, TNF-α and IFN-γ. The samples were prepared in duplicate, as per manufacturer's instructions, and run on the Bioplex 200 system (Biorad, USA) equipped with Bio-Plex Manager™ software. Cytokine concentrations were automatically calculated using a 6-point standard curve fitted with a five-parameter logistic regression algorithm. The kit lowest detection thresholds were as follows: IL-1β, 5.4 pg/ml; IL-6, 1.1 pg/ml; TNF-α, 2.3 pg/ml and IL-10, 2.0 pg/ml.

4.3.5. Immunocytochemistry

Glucocorticoid receptor expression was assessed in splenic leukocyte subsets by flow cytometry. After isolation, the splenocytes were stained with Zombie Aqua Fixable Viability Dye (Biolegend) for 30 minutes at room temperature and washed with 1X DPBS (Gibco, USA). Mouse FC Block (BD Biosciences) was then added for 5 minutes before cell surface proteins were labelled with an antibody cocktail containing the following antibodies: Brilliant Violent 421 (BV421) conjugated anti-NK1.1 (Biolegend); Fluorescein isothiocyanate (FITC) conjugated anti-TCRβ (BD Biosciences); PE-CF594 conjugated anti-F4/80 (BD Biosciences); Peridinin chlorophyll (PerCP)-Cy5.5 conjugated anti-CD11b (BD Biosciences); allophycocyanin-Cy7 (APC-Cy7)-conjugated anti-Ly6G, (BD Biosciences). After a 30-minute incubation, cells were washed twice with staining buffer (1X DPBS containing 5% bovine serum albumin (Invitrogen, USA) and 1% sodium azide (Sigma Aldrich, USA)). The cells were fixed and permeabilised using the Cytofix/Cytoperm kit (BD Biosciences, USA) as per

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manufacturer's instruction. For intracytoplasmic staining of the GR receptor, cells were incubated with the anti-NR3C1 antibody (Alexafluor 647 conjugated anti-NR3C1, (Novus Biologicals)) for 30 minutes. After incubation, cells were washed twice and resuspended in staining buffer before acquisition on the flow cytometer.

4.3.6. Flow cytometry

The flow cytometric analysis was performed at the Central Analytical Facilities' Fluorescence Imaging Unit at Stellenbosch University. The gating strategy employed is illustrated in Supplemental Figure 1. All prepared samples were analysed on the BD FACSAria IIu flow cytometer (BD Biosciences, USA), with BD FACSDiva™ ver. 8.01 software for data acquisition and analysis.

For the leukocyte glucocorticoid receptor expression analysis, a minimum of 200 000 and a maximum of 500 000 live, gated, singlet events were acquired from each sample tube. Relative glucocorticoid receptor (NR3C1) expression was assessed in T-lymphocytes (TCRβ+ NK1.1-), NKT lymphocytes (TCRβ+ NK1.1+) NK cells (TCRβ- CD11b+ NK1.1+), neutrophils (TCRβ- CD11b+ Ly6G+), monocytes (TCRβ- CD11b+ F4/80-) and macrophages (TCRβ- CD11b+ F4/80+), as identified by their specific marker expression. The gating strategy was previously described in more detail (21).

For each respective assay, samples were run using application settings to verify MFI targets and compensation was performed with every run. All data files were further analysed in FlowJo™ v10.4.1 and reported as percentage positive (frequency) and median fluorescent intensity (MFI).

4.3.7. Statistical analysis

Data are reported as means and standard errors of the mean. Data were analysed using Statistica software 13.4 (Statsoft, USA) and graphs were generated in GraphPad Prism 7 (GraphPad Software Incorporated, USA). The F1 and F2 datasets were analysed separately using a two-way ANOVA to assess the main effects of LPS exposure *in utero* (F1) or ancestral LPS exposure (F2) between sexes for each generation. To avoid interpretation of seasonal immune differences between F1 and F2 generations (Paynter et al., 2015; Bisig, Petri-Fink & Rothen-Rutishauser, 2018) as resulting from prior LPS exposure or generational differences, the two generations was

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not directly compared with each other. A Fisher's post hoc analysis was performed to compare individual group means for statistical differences. All data are presented as mean ± SEM and $p<0.05$ was regarded as significant.

4.4. Results

4.4.1. Body mass changes in responses to generational LPS exposure

In terms of body mass, in F1 a sex-specific difference was seen, with males generally heavier than females, which was not unexpected. However, no effect of *in utero* LPS was evident (Table 4.2).

Table 4.2 Body mass (g) for the F1 generation for LPS-affected and CTRL groups. Data is depicted mean ±SEM (*n*=4-6 per group).

	F1 Males	F1 Females
CTRL	29.6 ± 0.86	23.7 ± 1.93 [1]
LPS	28.5 ± 0.34	21.7 ± 0.65 [2]

[1] Significantly different from F1 Male CTRL (p<0.05)

[2] Significantly different from F1 Male LPS (p<0.05)

In the F2 generation, males still maintained a higher body mass than females (Table 4.3). In addition, F2 females generally maintained similar body mass irrespective of parental LPS exposure. In contrast, F2 male offspring from LPS-affected mothers exhibited higher body mass when compared to their controls. In addition, F2 males from two LPS-affected parents had even higher body mass.

Table 4.3 Body mass (g) of generation F2. Data is depicted mean ±SEM (*n*=4 per group).

	F2M Males	F2M Females	F2F Males	F2F Females	F2 LPS_2 Males	F2 LPS_2 Females
CTRL	22.9 ± 0.70	21.0 ± 0.41	20.80 ± 0.89	19.3 ± 0.28	-	-
LPS	24.4 ± 1.69	22.2 ± 1.91	26.4 ± 1.90 [1]	21.4 ± 0.33	28.5 ± 0.34 [2]	21.9 ± 0.23 [3]

[1] Body mass significantly higher than F2F Male CTRL (p=0.05)

[2] Body mass significantly higher than all other male groups (p<0.05 at least)

[3] Body mass significantly lower than F2 LPS_2 Males (p<0.05)

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4.4.2. Changes in leukocyte distribution in circulation versus spleen

4.4.2.1. Peripheral leukocytes

Generally, in generation F1, females displayed higher total peripheral blood leukocyte count than males (Figure 4.2), independent of maternal chronic inflammation. In addition, in the LPS-affected group, F1 males displayed further significant decreases in leukocyte counts in comparison to their female counterparts.

When looking closer at specific leukocyte subsets, a similar trend emerges more noticeably with the lymphocyte-, monocytes-, and neutrophil counts, where a male-specific reduction in peripheral cell count is associated with exposure, contributing to relatively higher peripheral leukocyte counts in LPS-affected females.

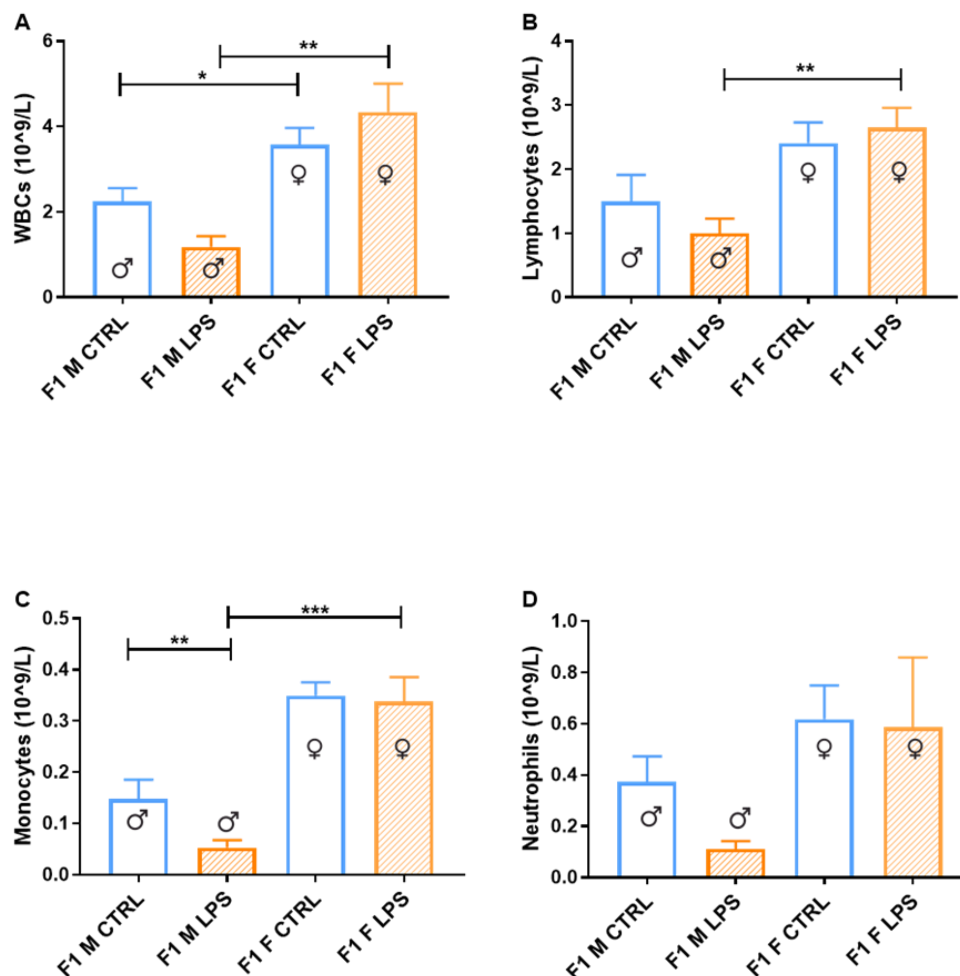

Figure 4.2 Peripheral blood leukocytes between the saline Control and LPS-affected groups for male and female F1 groups. The data is depicted as mean ± SEM. The cell subset comparisons for $F1_{Pat}$ and $F1_{Mat}$ Control and LPS-affected are depicted as follows: total WBCs (A), lymphocytes (B), monocytes (C), neutrophils (D) (*n*=4 per group).

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In the F2 generation, differences were again observed between the sexes (Figure 4.3). The F2M LPS males displayed significantly higher total WBCs in peripheral blood in comparison to F2M controls, as well as in comparison to the F2M Female LPS and F2F Male LPS group. This effect may be due specifically higher lymphocyte and neutrophil counts, which are the two subpopulations that primarily showed this pattern as well. Interestingly, F2F Male LPS group also showed significantly higher neutrophil counts in comparison to their F2F Male controls, while females did not – this is in line with an interpretation of a sex-specificity in the response to LPS exposure.

When comparing the F2 LPS_2 male and female groups, both groups had substantially higher total WBCs in both sexes compared to both the offspring from a single affected parent (LPS_1) and those from unaffected parents (CTRL). In addition, in this group, while both sexes exhibited higher lymphocyte numbers, the F2 LPS_2 males also displayed sex-specific increase in monocyte and neutrophil counts, while the F2 LPS_2 females had significantly higher monocyte counts in peripheral blood.

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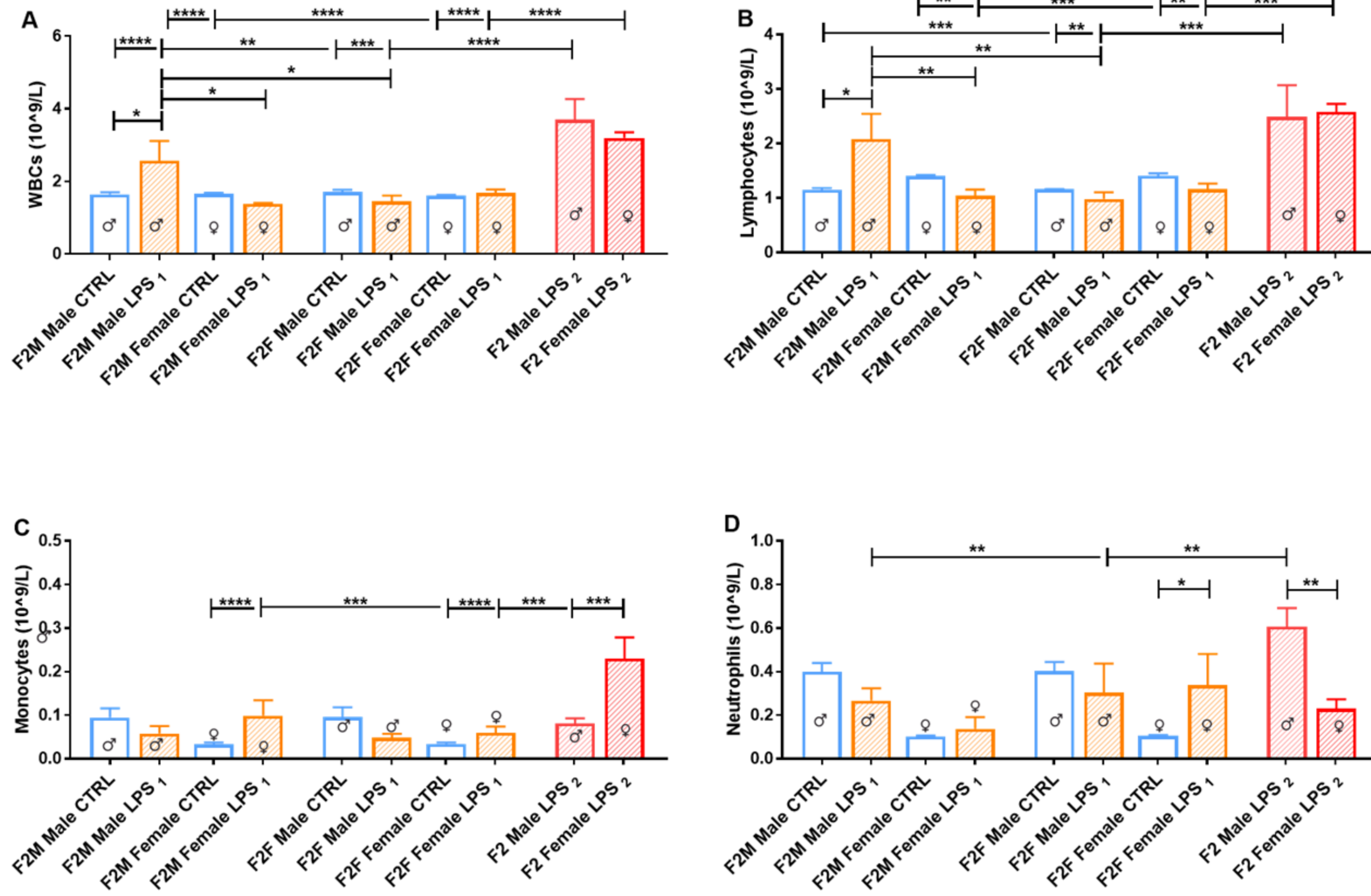

Figure 4.3 Peripheral blood leukocytes for male and female F2 Control, LPS1 and LPS2 groups. The data is depicted as mean ± SEM. The cell subset comparisons are depicted as follows: total WBCs (A), lymphocytes (B), monocytes (C), neutrophils (D) (n=4 per group). Significance is as follows: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

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4.4.2.2. Splenic leukocytes

Comparable to leukocyte counts in peripheral blood, females exhibited much higher splenic leukocyte counts than males, for the majority of leukocyte subpopulations assessed (Table 4.4). Maternal LPS exposure resulted in increased total leukocyte counts for both sexes when compared to their CTRL groups, but only reached statistical significance in the F1 males. In both sexes, the maternal LPS exposure also drastically increased lymphocyte counts, with males showing the largest incremental response. This suggests that the change in total leukocyte count is mainly due to the increase in lymphocyte numbers in the spleen.

Table 4.4 Differential leukocyte counts in spleen between the different sexes for the F1 generation for LPS-affected and CTRL groups. Data is depicted mean ±SEM (n=4 per group). For each group, cell count is depicted at 10^9/L.

	WBCs (10^9/L)	Lymphocytes (10^9/L)	Monocytes (10^9/L)	Neutrophils (10^9/L)
F1 M CTRL	1.20 ± 0.07	0.43 ± 0.03	0.25 ± 0.02	0.3 ± 0.06
F1 M LPS	1.57 ± 0.42 [(1)]	3.6 ± 0.23 [(1)]	0.43 ± 0.12	0.15 ± 0.04
F1 F CTRL	3.65 ± 0.88 [(1,3)]	2.63 ± 0.70 [(1)]	0.35 ± 0.10	0.27 ± 0.12
F1 F LPS	4.82 ± 0.44	4.07 ± 0.35 [(2)]	0.36 ± 0.04	0.12 ± 0.01

[(1)] *Significantly different from F1 M Control (at least p≤0.05)*
[(2)] *Significantly different from F1 F Control (p<0.001)*
[(3)] *Significantly different from F1M Control (P<0.01)*

In the F2 generation, LPS-affected animals again had significantly affected splenic leukocyte counts (Table 4.5). In the F2 offspring, leukocyte counts for the majority of subsets in LPS-affected groups were consistently reduced, with no specific sex-specificity. Only one exception was observed: total leukocyte count in LPS-affected F2F males did not show a decrease – this can potentially be ascribed to somewhat higher lymphocyte and significantly higher neutrophil counts in this group only. The significance of this remains to be validated.

F2 LPS2 splenic total WBCs did not show much response to LPS after exposure in both parental lines. F2 LPS2 males showed somewhat higher WBC counts than F2M

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and F2F LPS and CTRL males, as well as F2 LPS2 females. This was attributed to the significantly increased lymphocyte count seen in this group.

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Table 4.5 Differential leukocyte counts in spleen between the different sexes for the F2 generation for the Control, LPS_1 and LPS_2 groups. The affected parental lineage is described as offspring of F1 Males or F1 Females, which describes whether the affected (LPS-affected or saline CNTRL) parental group is male or female respectively. The groups are further grouped into male and females for their respective parental group. Data is depicted mean ±SEM (*n*=4 per group). For each group, cell count is depicted at 10^9/L.

			WBCs (10^9/L)	Lymphocytes (10^9/L)	Monocytes (10^9/L)	Neutrophils (10^9/L)
Offspring to F1 Males	**F2M Males**	F2M Male CNTRL	2.28 ± 0.33	1.43 ± 0.06	0.19 ± 0.01	0.3 ± 0.04
		F2M Male LPS_1	1.76 ± 0.02 [6]	1.32 ± 0.08 [6]	0.15 ± 0.03 [6]	0.07 ± 0.007 [1]
	F2M Females	F2M Female CNTRL	3.32 ± 0.22 [1,7]	2.41 ± 0.14 [1]	0.27 ± 0.05	0.3 ± 0.05 [7]
		F2M Female LPS_1	1.24 ± 0.08 [2]	0.81 ± 0.10 [2]	0.14 ± 0.006 [2]	0.08 ± 0.004 [2,7]
Offspring to F1 Females	**F2F Males**	F2F Male CNTRL	2.00 ± 0.11	1.05 ± 0.06	0.27 ± 0.07	0.26 ± 0.02
		F2F Male LPS_1	2.30 ± 0.67	1.78 ± 0.74	0.12 ± 0.01 [3,6]	0.11 ± 0.03 [3]
	F2F Females	F2F Female CNTRL	2.38 ± 0.06	1.42 ± 0.07	0.26 ± 0.03	0.2 ± 0.01 [2,7]
		F2F Female LPS_1	1.19 ± 0.16[4,5,7]	0.88 ± 0.23	0.13 ± 0.07[4]	0.05 ± 0.01[4,7]
Offspring of F1 LPS Male x F1 LPS Female	**F2 LPS_2**	F2 Male LPS_2	2.38 ± 0.17	1.70 ± 0.167	0.26 ± 0.03	0.17 ± 0.02
		F2 Female LPS_2	2.14 ± 0.09	1.13 ± 0.08 [6]	0.18 ± 0.03	0.85 ± 0.28

[1] *Significantly different from F2M Male CTRL (at least p≤0.05)*

[2] *Significantly different from F2M Female CTRL (at least p≤0.05)*

[3] *Significantly different from F2M Male LPS_1 (at least p≤0.05)*

[4] *Significantly different from F2F Male Control (at least p≤0.05)*

[5] *Significantly different from F2F Female Control (at least p≤0.05)*

[6] *Significantly different from F2F Male LPS_1 (at least p≤0.05)*

[7] *Significantly different from F2 Male LPS_2 (at least p≤0.05)*

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4.4.3. Adaptations in plasma corticosterone concentrations

The majority of F1 females showed higher CORT levels in plasma when compared to males, regardless of LPS exposure, pointing towards a physiological difference between sexes (Figure 4.4). Furthermore, LPS-affected F1 males exhibited significantly higher CORT levels when compared to male controls, while the F1 females did not exhibit a CORT response to maternal periconception chronic inflammation.

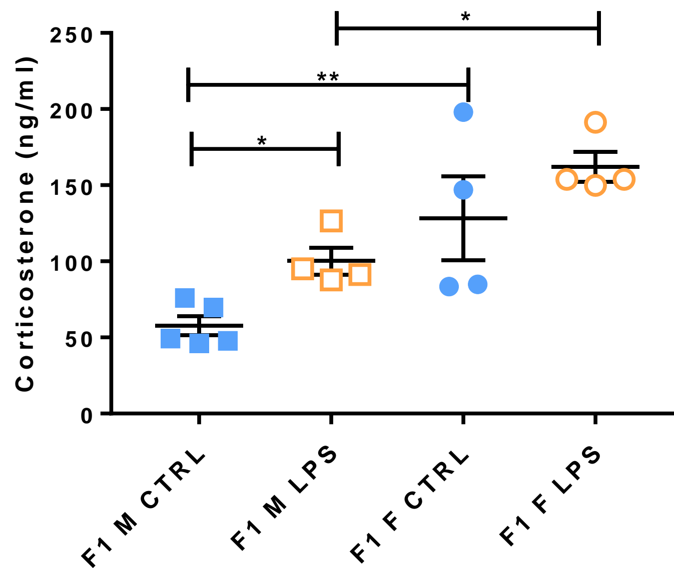

Figure 4.4 Basal plasma corticosterone concentrations in LPS-affected and saline controls in the F1 generation. All data is depicted as mean ± SEM (*n*=4 per group). Significance is as follows: * p<0.05; ** p<0.01.

This male-specific effect was perpetuated to the offspring of F1 Males, where basal plasma CORT concentrations in F2M females was again higher than that of F2M males, regardless of LPS (Figure 5). However, unlike in F1 males, F2M LPS-affected males did not exhibit upregulated CORT levels. Of interest, the F2F Male LPS group seemed to have somewhat higher CORT levels than their controls (although not statistically significant), resulting in statistically significantly higher CORT when compared to F2F Female LPS (Figure 4.5). In addition, while the F2F Male LPS group had higher plasma CORT levels when compared to the F2M Male LPS group, CORT levels in F2F Female LPS were significantly lower than that of F2M Female LPS.

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F2 LPS_2 males and females seemed to have blunted CORT levels, as their values were comparable to their respective F2F and F2M CTRL groups. Furthermore, F2 LPS_2 male CORT levels were significantly lower than the F2F LPS males. F2 LPS_2 females had CORT levels with significantly lower than the F2F and F2M LPS females as well as F2M CTRL females.

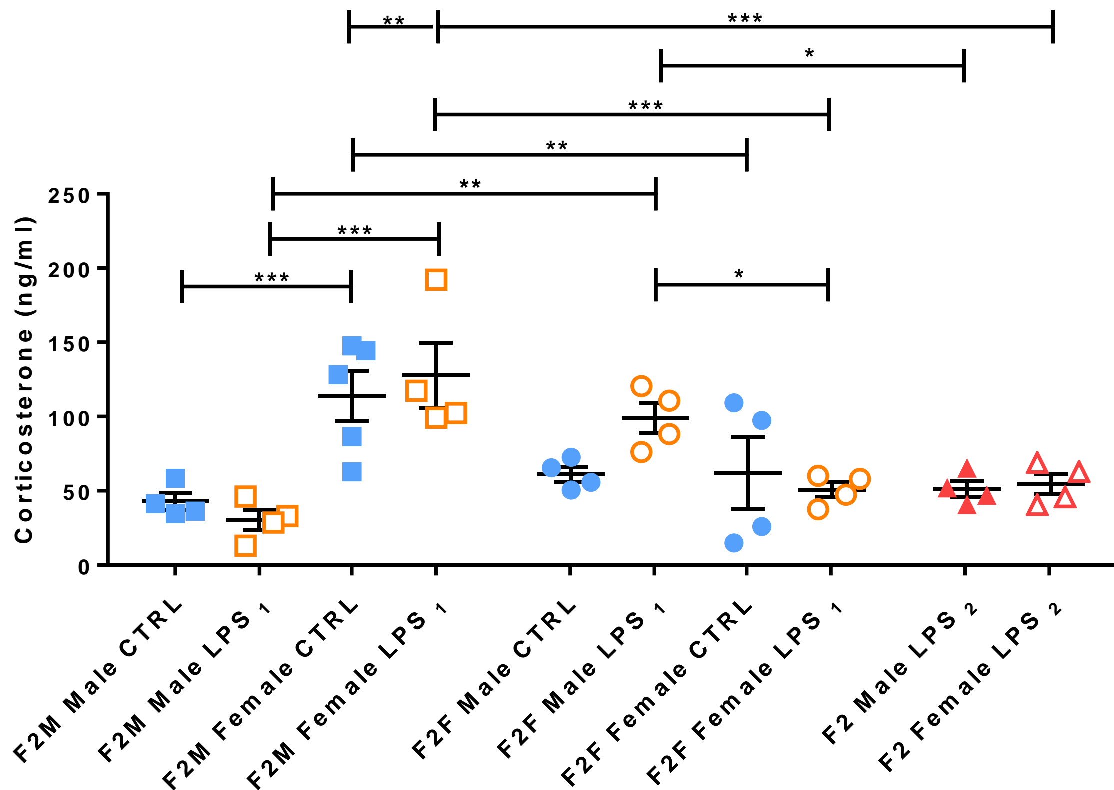

Figure 4.5 Basal plasma corticosterone concentrations in the Control, LPS_1 and LPS_2 groups in the F2 generation. All data is depicted as mean ± SEM (*n*=4 per group). Significance is as follows: * p<0.05; ** p<0.01, *** p<0.001.

4.4.4. Basal glucocorticoid receptor expression in splenic leukocytes

Both sexes in the F1 generation exhibited significant upregulation of GR expression in response to LPS for most of the cell subtypes analysed (T-lymphocytes, innate-like lymphocytes (NKT-lymphocytes and NK cells) and monocytes (Figure 4.6)). In contrast, both macrophage and neutrophil GR seemed unaffected by LPS, with neutrophils being the only cell subtype to display a sex-specific effect, with a higher GR expression in F1 females, independent of LPS exposure.

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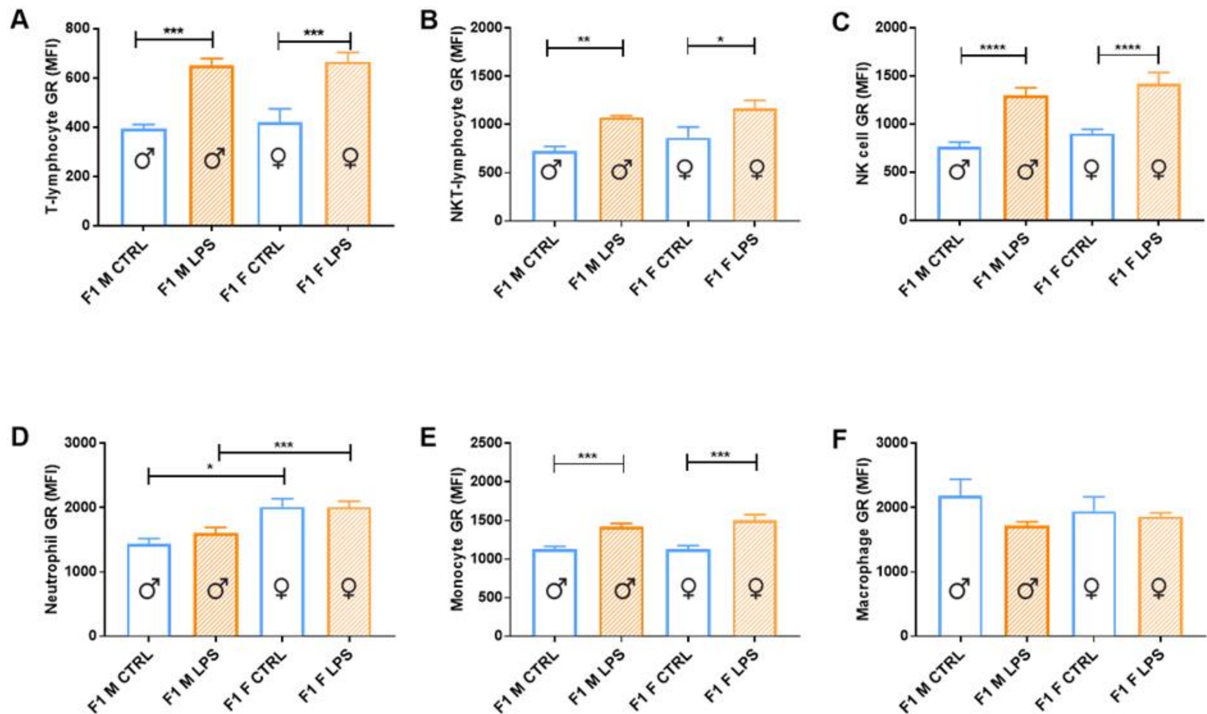

Figure 4.6 Splenocyte leukocyte subset expression of glucocorticoid receptor for male and female CTRL and LPS groups within the F1 generation. The glucocorticoid receptor expression comparisons are depicted as follows: T-lymphocytes (A), NKT-lymphocytes (B), NK cells (C), neutrophils (D) monocytes (E) and macrophages (F) (n=4 per group). All data is depicted as mean ± SEM (n=4 per group). Significance is as follows: * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

GR expression levels in F2 offspring differed with parental LPS exposure and was dependant on the affected lineage (F1 male or female) (Figure 4.7). In the F2M group, only males from LPS-affected fathers had significantly higher GR expression in T-lymphocytes, NKT-lymphocytes, NK cells, monocytes and macrophages, compared to the F2M CTRL, while females from LPS-affected fathers, GR expression was only upregulated in neutrophils. In the F2F group, offspring from affected F1 females, were both sexes seemed to increase GR expression in most of the cell subtypes, with LPS-associated changes in GR expression levels seeming to mirror that illustrated for F1. This indicates that GR adaptation was maintained into F2. When comparing F2F vs F2M, GR expression levels for all leukocyte subsets except neutrophils, were generally significantly higher in F2 LPS female group than F2 M LPS group.

Looking at the F2 LPS_2 group, this group showed no specific sex differences between their male and female counterparts for all the parameters assessed. Furthermore, in the second generation (F2), both parents' exposure to LPS only affected GR expression for monocytes, NK cells and NKT cells, when compared to single parent

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exposure (male or female). Of significance, NKT-lymphocytes and monocytes downregulated GR expression, and NK cell GR expression was upregulated with dual LPS-affected in both F2 LPS_2 males and females, which was especially lower the F2 LPS_1 females.

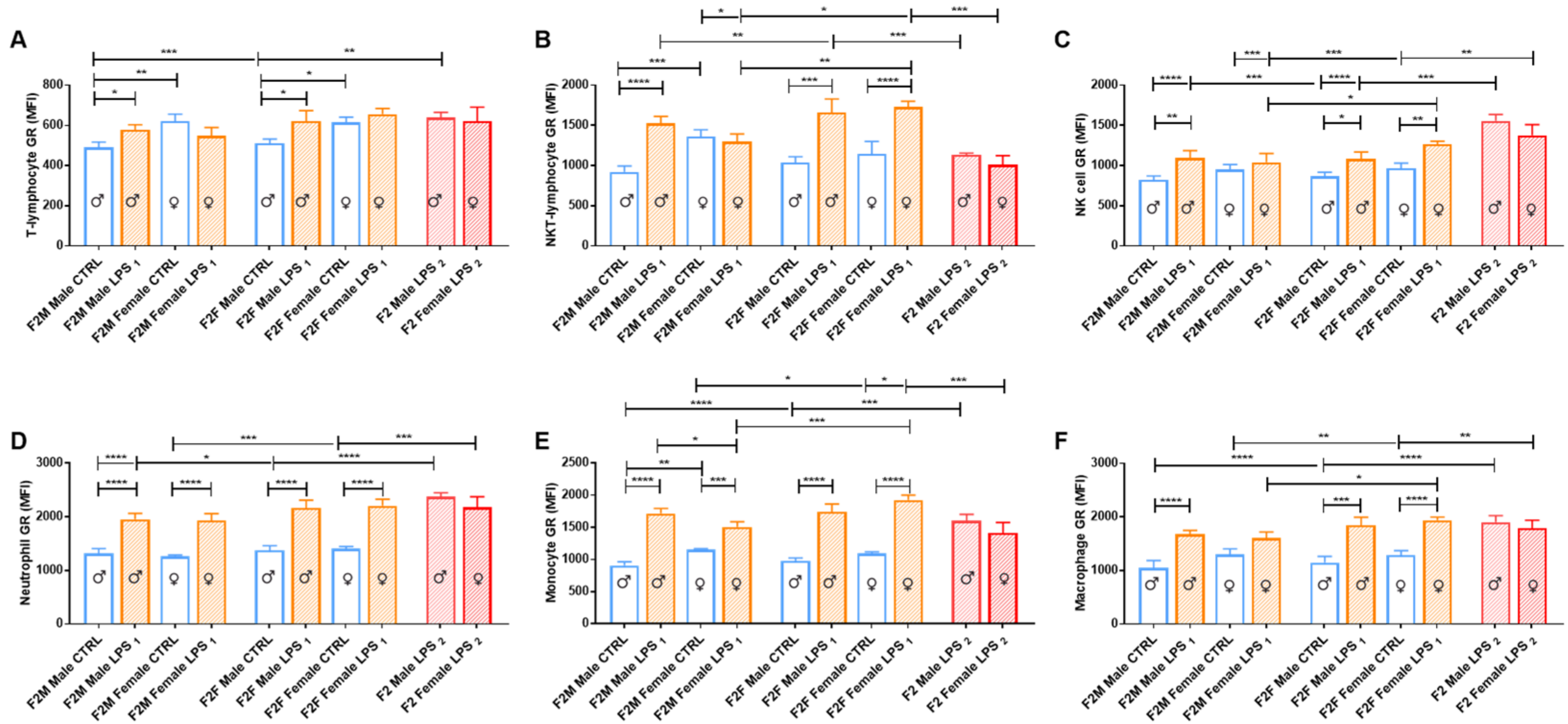

Figure 4.7 Splenocyte leukocyte subset expression of the glucocorticoid receptor for male and female CTRL, LPS1 and LPS2 groups within the F2 generation. The glucocorticoid receptor expression comparisons are depicted for the following leukocyte subsets: T-lymphocytes (A), NKT-lymphocytes (B), NK cells (C), neutrophils (D) monocytes (E) and macrophages (F) (*n*=4 per group). All data is depicted as mean ± SEM. Significance is as follows: * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001

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4.4.5. Splenocyte cytokine response

When considering the cytokine responses to acute *in vitro* LPS challenge, in F1 offspring (to primary affected *mothers)*, acute LPS challenge elicited a significant IL-1β response (Figure 4.8A) in both sexes. This response was maintained in F2, but only for offspring of F1 LPS-affected mothers (F2F LPS), while in F2M this hyper-response was not evident (Figure 4.9A).

IL-6 showed a sex-specific increase in F1 LPS females compared to controls (Figure 4.8B), but TNF-α production was similar to F1 CTRL group for both sexes. However, in F2, LPS-affected individuals all exhibited an exacerbated IL-6 and TNF-α response when compared to controls (Figure 4.9B-C).

In terms of anti-inflammatory cytokine response, F1 LPS-affected males seemed to mount relatively normal IL-10 responses to acute LPS challenge, while the females exhibited exacerbated responses to acute stimulation (Figure 4.8D). In the F2 group, the F2F LPS females had a normal IL-10 response. However, all other LPS-affected F2 offspring showed significantly increased IL-10 responses relative to controls, although this was only significant for the F2M male LPS group. (Figure 4.9D).

Splenocytes from F2 LPS_2 offspring seemed unable to elicit proper responses with LPS stimulation for all the cytokines assessed, when comparing to the offspring of the single LPS-affected parents. TNF-α and IL-6 concentrations from F2 LPS_2 males and females were significantly lower than the F2 LPS_1 sex-specific counterparts. No difference was observed when considering other cytokines assessed.

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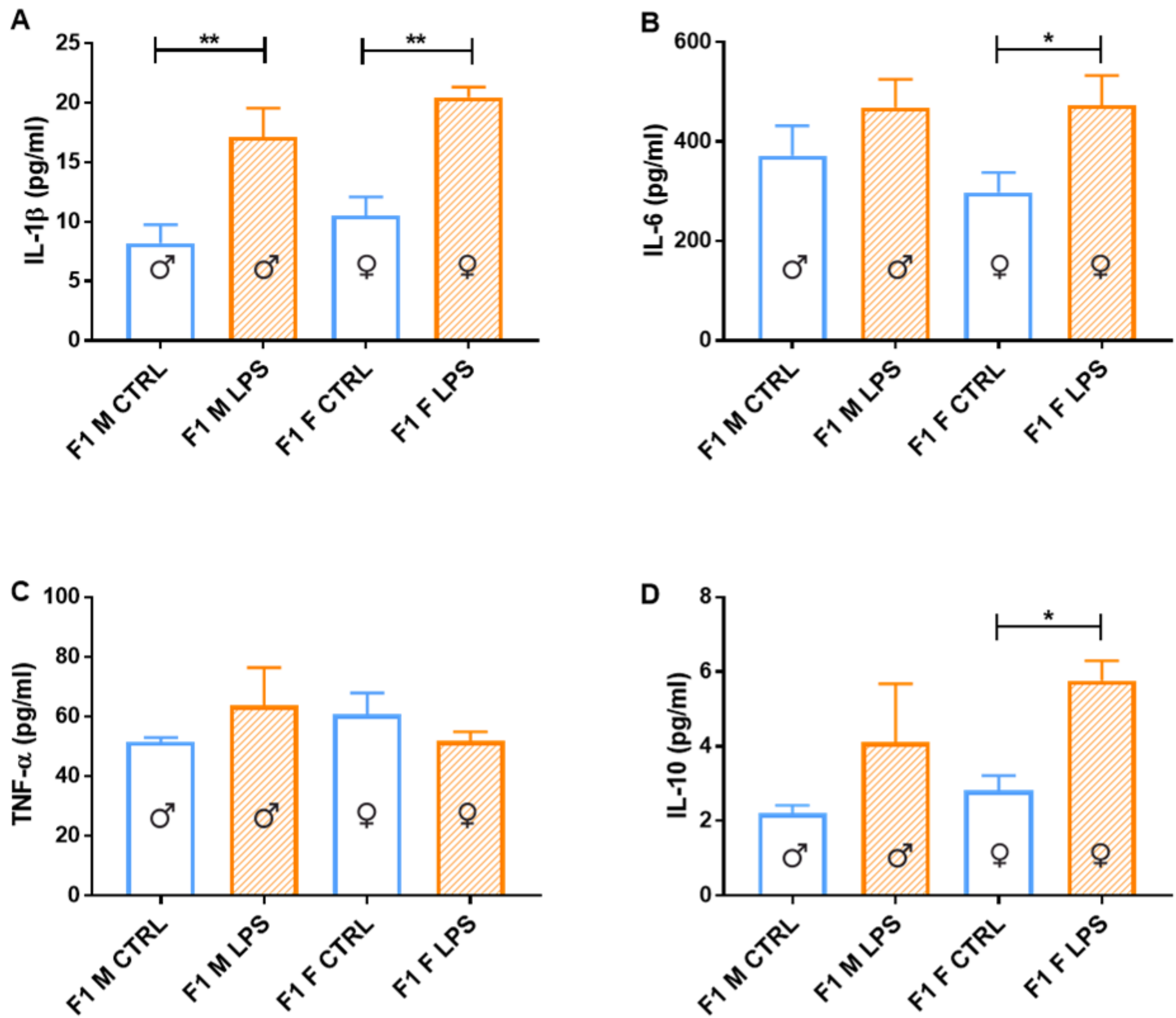

Figure 4.8 Splenocyte LPS-induced *ex vivo* cytokine responses between the male and female CTRL and LPS groups for the F1 generation. Concentrations of IL-1β (A), IL-6 (B), TNF-α (C) and IL-10 (D) were analysed after 18-hour incubation with 1ug/ml LPS. Significance is as follows: *p<0.05; ** p<0.01.

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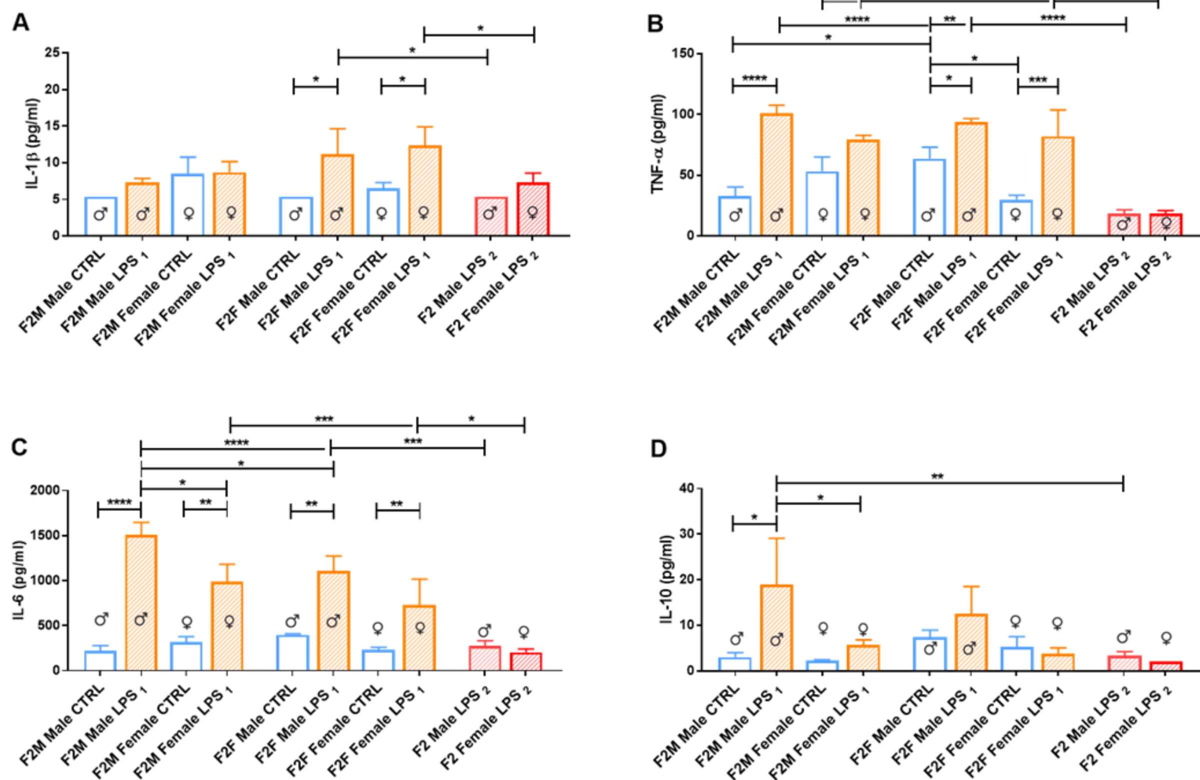

Figure 4.9 Splenocyte LPS-induced *ex vivo* cytokine responses between the male and female CTRL and LPS groups for the F1 generation. Concentrations of IL-1β (A), IL-6 (B), TNF-α (C) and IL-10 (D) were analysed after 18-hour incubation with 1ug/ml LPS. Significance is as follows: * p<0.05; ** p<0.01.

4.5. Discussion

The current study explored the generational transfer of HPA-axis- and immune adaptations across two generations of offspring, with the aim of delineating their possible sex-specific inheritance into subsequent generations. We expand on a previous report from our group (Adams & Smith, 2019) with the aim of delineating sex differences in the risk of disease vulnerability, as well as the potential of these effects to be cumulative. Our current *in vivo* mouse model of chronic maternal inflammation, described in Adams and Smith (2019), is a modification of the maternal periconception systemic inflammation (MPSI) protocol (a single LPS injection at the start of gestation) established by Williams et al. (2011) – our protocol entails weekly (every 7 days, starting immediately following plug-positive confirmation) LPS injections throughout the gestational period, to achieve consistent maternal exposure to the affecting agent during gestation. LPS administered intraperitoneally during gestation is known to not

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cross the placental barrier. However, the direct placental damage and subsequent foetal damage due to maternal LPS intervention was previously shown to persist into adulthood (Fricke et al., 2018). Furthermore, the primordial germ cells present in the F1 generation, from which the F2 generation will arise, are affected by the F1 *in utero* exposure, and result in maladaptive programming that may affect subsequent generations (Skinner, 2008; Portha, Grandjean & Movassat, 2019). The extent of this plasticity remains to be determined in later generations. The current data corroborates the findings reported by Fricke and colleagues (Fricke et al., 2018) and expands on it to include the F2 generation.

We report several sex-specific maladaptations resulting from gestational chronic maternal inflammation, some of which are maintained or even exacerbated in the second generation of offspring. We further add to the data by reporting on the cumulative maladaptations resulting from combining F1 LPS-affected male and female lineages, which severely impacted HPA-axis- and immunological functionality.

4.5.1. Immune adaptation to LPS exposure in the F1 vs F2 generation

Generally, chronic stress and inflammation results in the characteristic upregulation of haematopoiesis and increased circulating leukocytes (Wohleb et al., 2014; McKim et al., 2016; Dudele et al., 2017), which we similarly reported in the F0 female generation in our previous study (Adams & Smith, 2019). We have shown that F0 dams exposed to LPS throughout gestation maintained raised leukocyte counts several weeks after the last administration of the low dose (10ug/kg) LPS, showing persistence in the inflammatory haematopoiesis. Here we demonstrate that this effect was modulated in the F1 offspring and exhibited sex-dependence. Most notably, the leukocyte hypo-response was evident in F1 males and not females. In contrast, Dudele and colleagues reported a considerably higher WBC count in their model of chronic MSPI (Dudele et al., 2017). This may be attributed, in part, to their model, which was designed to sustain LPS-induced low grade inflammation throughout gestation and lactation, thus predisposing offspring to longer exposure to inflammatory mediators. Interestingly, similar to our findings, repeated social stress also resulted in a male-specific reduction in peripheral blood leukocytes (Engler et al., 2004). This is in line with the glucocorticoid hypersensitivity reported in males in the literature (Iqbal et al., 2012)

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and reflected in our own data. In terms of cytokine responses, and similar to current results, Dudele and colleagues showed that their F1 male offspring displayed chronic immune activation (pro-inflammatory cytokine mRNA expression in liver tissue; increased hypothalamic inflammatory cytokines) that persisted at least up to 25 weeks of age (mature adulthood in mice). Of specific relevance in the context of generational transfer of inflammation, the demonstrated maladaptation to LPS exposure was similar to that seen in a peer group fed a high fat diet in the same study (Dudele et al., 2017). The current study further expands by also reporting a picture of immune activation in affected F1 males. In our model, circulating peripheral blood neutrophil counts were relatively depleted in F1 LPS-affected offspring, suggesting their sequestration into tissue. This interpretation was supported by relatively higher leukocyte counts in the spleen, compared to saline controls. This is in line with results reported in mouse models of chronic psychological stress (e.g. repeated social defeat) (McKim et al., 2018; Yin et al., 2019), although the current study is the first to illustrate this in response to MSPI and offspring carryover.

We further expand on the current existing literature by identifying subsequent changes in the resulting F2 generation. Surprisingly, all four single parent (LPS_1) LPS-affected groups exhibited a general depletion of leukocytes from the spleen along with a raised leukocyte profile in the peripheral circulation, suggesting their trafficking from spleen to blood and possibly into tissue, an effect that seemed sex-independent. This result, given the sex-dependent maladaptation evident in F1 males, cannot be explained from immune data alone. Rather, other factors, such as known changes in endogenous anti-inflammatory feedback capacity, may come into play.

In terms of potential for cumulative maladaptive changes, the F2 LPS_2 offspring exhibited sex-dependent compartmental redistribution of cells, with male neutrophils depleting in the periphery in favour of increased sequestration in the spleen, while in contrast, female monocytes seemed to remain in large numbers in the periphery to result in relative depletion in the spleen.

In terms of functional immune capacity, both LPS_1-affected sexes in F1 exhibited increased IL-1β responses to LPS stimulation in *ex vivo* culture, which is in line with available literature available on circulating plasma levels (Kirsten et al., 2013; Fricke et al., 2018) as well as data reported for a similar *ex vivo* stimulation model in F1

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primates born to TLR3 agonist exposed mothers (Rose et al., 2017). In addition, when considering F2, the increased IL-1β response was only maintained in F2F LPS_1 male and female groups (offspring from F1 LPS-affected females). Current data may suggest mitochondrial dysfunction from the F2 maternal lineage - mitochondrial stress has been shown to activate the NLRP3 complex in aged haematopoietic stem cells, inducing IL-1β production (Luo et al., 2019). This interpretation is also in line with findings where maternal LPS exposure was associated with mitochondrial DNA abnormalities and metabolic dysregulation in offspring (Yu et al., 2018). Furthermore, current data specifically suggests that this mechanism may have sex dependency, pointing toward a female-linked intergenerational dysregulation of IL-1β signalling resulting in hyperresponsiveness in subsequent generations.

The previously reported hyperresponsiveness of TNF-α and IL-6 in both F1 and F2 (Adams & Smith, 2019), did not show sex-specificity, in line with related literature (Kirsten et al., 2013; Voorhees et al., 2013; Heidt et al., 2014; McKim et al., 2016; Li et al., 2018). Of further interest, F2 LPS_2 offspring to two affected parents, did not show this hyper-response. However, in our opinion, it unlikely that the hyper-responsive maladaptation was abolished; rather, we believe that these data suggests a relatively blunted or delayed reactivity to immune challenge which may again relate to altered HPA-axis functionality.

4.5.2. Distinct HPA status and functionality in F1 vs F2 generation

In terms of sex differences, our current results are in line with literature showing that female mice produce more glucocorticoids than males under basal conditions (Chmielarz, Kreiner & Nalepa, 2015; Yu, Hwa, et al., 2019). In the F1 generation, *in utero* LPS exposure elevated CORT in plasma, although this finding was only significant in F1 M LPS, and also upregulated GR expression in most of the cell subsets analysed. The basally elevated CORT and leukocyte GR expression in F1 animals, in combination with slightly elevated cytokine responsiveness, is in line with an interpretation of long-lasting inflammatory phenotype resulting from *in utero* exposure to maternal inflammation, which at this point did not seem to have sex specificity in terms of offspring affected. In further support of our interpretation, intraperitoneal administration of LPS was previously reported not to result in any

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change in blood CORT levels in F1 offspring (Williams, Teeling, Perry & Fleming, 2011), suggesting that the upregulated CORT in F1 resulted from maternal inflammation-associated reprogramming.

In F2, male offspring from LPS-affected males (F2M male LPS) showed no generational carryover of CORT hypersecretion, but F2F male LPS did not show the same plasticity, as evidenced bytheir higher CORT levels. Notably, crossing of the LPS-affected male phenotype with an unaffected female was able to amend the CORT maladaptation, while the LPS-affected female crossed with an unaffected male could not. This implies a maternal-linked inheritance of maladaptation, which further exacerbated the relatively greater GC sensitivity already known to exist in males (Lu, Radom-Aizik, et al., 2017; Moisiadis et al., 2017).

The F2 LPS_2 generation displays an entirely different phenotype with dual parental exposure, when compared to F2 LPS_1. Specifically of interest is the selective reduction in GR expression in NKT lymphocytes and monocytes in both sexes. Previous studies demonstrated increase $CD28_{null}$ NKT-lymphocytes in chronic obstructive pulmonary disease, with reduced GR expression. The lack of CD28 co-stimulatory molecule on lymphocytes is linked to lymphocyte senescence, and associated with a further increase in their pro-inflammatory/cytotoxic (TNF-α, IFN-γ, granzyme and perforin production) phenotype and glucocorticoid resistance (Hodge et al., 2015; Hodge & Hodge, 2019). In systemic lupus erythematosus, similar results are observed in monocytes, with loss of GR on monocytes being the hallmark of steroid resistance, which is also associated with an increased pro-inflammatory cytokine profile (Du et al., 2009). The current result may therefore be indicative of a predisposition to developing inflammatory disorders in later life, although we acknowledge that small sample size in the current study precludes firm conclusions at this point.

4.5.3. Perspectives and Significance

As mentioned, only F2 exhibits a clear depiction of inheritance, as the profile seen in F1 may also, at least in part, have resulted from *in utero* influences (maternal inflammation). In order to extrapolate from our data on F2 in particular, it is important to be reminded of the sequence of events known to occur in chronic stress. Chronic stress is known to result in changes across a continuum, where CORT and GR

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increases at first, followed by decreased GR. The resulting continued increase in CORT production finally results in adrenal burnout – the point where inflammatory processes escalate due to insufficient GC feedback control.

The current findings suggest that maternal (F0) gestational chronic (LPS-induced) inflammation resulted in transmission of altered HPA programming and maladapted functional immune response, in a sex-dependent manner, in their succeeding lineages. The male offspring (F1) seems to have inherited a maternal-linked maladapted HPA programming that rendered them CORT hyper-responsive, which along with the general increase in GR levels seen in both sexes, may lead to GC resistance. In addition, the F1 male offspring exhibit a relatively exacerbated pro-inflammatory cytokine response. Thus, in our opinion, at the point of adrenal burnout – which for both sexes seem to be brought forward by MPSI, but more pronounced in the males – the males seem to be relatively more vulnerable to inflammation-associated chronic disease. Our finding of higher body mass is LPS-affected F2 males further supports this interpretation. Although this data is in accordance with other studies linking specifically the male sex to maladapted generational transfer, we do acknowledge that translation of these results into a human model is required for validation. For example, this data, cannot account for sex differences in terms of behavioural or neurological adaptations to chronic stress. However, given that females in general are known to be more likely to make healthier lifestyle choices than men (Hardin-Fanning & Gokun, 2014; Patrão et al., 2017), the current data suggests that, in terms of inflammatory disease, the male population may be more susceptible than females.

Although current data and published literature paint a relatively bleak picture for males in particular in terms of clinical health outcome, in our opinion, females are not immune to inheriting maldaptations. Although LPS-affected F1 females did not exhibit a more compromised phenotype than their male counterparts, their offspring displayed relatively more maladapted endocrine and inflammatory (IL-1β) immune function, than F2 offspring from F1 LPS-affected males.

On a more technical note, baseline sex differences observed in control animals also provide valuable information. For example, in F1, females displayed higher peripheral leukocyte counts than males in all subpopulation assessed. However, in F2, females

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had consistently lower counts for peripheral monocytes and neutrophils than males (although not significant in this small sample number). This confirms that seasonal differences – which has been reported for humans (Paynter et al., 2015; Bisig, Petri-Fink & Rothen-Rutishauser, 2018) – may also be present in experimentally housed mice and that this variation is sex-dependent. In contrast, in both F1 and F2, control females exhibited higher splenic counts for total leukocytes and lymphocytes than control males, suggesting a sex difference that was not influenced by season. This may indicate that indeed, as utilised in the current study, splenic cells may be a better model than peripheral cells, with which to investigate sex differences in the context of immunity. Given the significance of this information, more studies is warranted to confirm this data statistically, as the relatively low n in the current study does not allow firm conclusions in this regard.

4.6. Limitations

In terms of limitations, we were not able to determine more conclusively the contribution of heritability to changes observed, as changes in the epigenome were not assessed. Breeding to the F3 generation in future studies would also further confirm the plasticity and sex-specificity of transgenerational epigenetic inheritance. Despite this, we are satisfied that consistent trends and patterns are emerging which clearly indicates that in investigations of this nature, both sexes should be considered, as mechanisms for maladaptation may differ substantially between the two sexes.

We acknowledge that a significant limitation of this study is the relatively small sample size. However, as mentioned in the introduction section, the data presented here resulted from the re-analysis of data not originally intended to be distinguish between sexes. However, we are confident that we were significantly conservative in our statistical analysis and approach to interpretation of data. Furthermore, the data is in line with relevant published data.

Furthermore, we did not compare F1 to F2 directly, due to the unavoidable seasonal variation in terms of parameters assessed, following our study design. In order to facilitate such a comparison, staggered entry into the protocol (and using higher n) may be a feasible approach.

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In terms of translation to human models, we acknowledge the requirement to further validate these results in longitudinal human studies.

4.7. Conclusion

In conclusion, it is clearly acknowledged that exposure to an adverse metabolic milieu before or during gestation accounts for some of the adverse consequences in resulting affected offspring. Current data suggest that in response to MPSI, male offspring exhibit a relatively more pro-inflammatory phenotype than female offspring, as well as a relative glucocorticoid hyperresponsiveness, which is likely due to maternal transgenerational inheritance. In addition, our data on offspring to two affected parents provides preliminary data suggesting cumulative inheritance of maladaptations, which may result in increased chronic disease risk, e.g. due to the immune senescence our data implies. These findings may have substantial implications for the development of disease and require further, more focused investigations.

4.8. Declarations

4.8.1. Ethical approval

The study was ethically cleared by the Stellenbosch University Animal Research Ethics Committee, as detailed in the methods section.

4.8.2. Consent for publication

Not applicable

4.8.3. Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

4.8.4. Competing interests

The authors declare that they have no competing interests.

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4.8.5. Funding

The South African NRF is acknowledged for financial assistance. The funder had no influence on the design of the study, collection, analysis, and interpretation of data or in writing the manuscript.

4.8.6. Author contributions

The study was jointly designed by CS and RCMA. RCMA conducted experimental work and data analysis under supervision of CS. Data interpretation and manuscript preparation was jointly performed by CS and RCMA.

4.8.7. Acknowledgements

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Chapter 5

Conclusion

In this study we have shown that in models of maternal gestational inflammation, repeated inflammatory challenge results in chronically inflamed F0 phenotype that does not fully recover even several weeks after final LPS challenge. We add to the existing literature by showing that in response to maternal inflammation, the affected offspring exhibits glucocorticoid hypersensitivity and exacerbated pro-inflammatory cytokine signalling. We have summarised the main findings of the current thesis in Figure 5.1.

In terms of our contribution to the knowledge base in the context of transgenerational inheritance of maladapted inflammatory phenotype, current data illustrates that *in utero* exposure to the inflammatory microenvironment results in distinctly different foetal programming when compared to that resulting from intergenerational inheritance. For example, F1 exhibited a pro-inflammatory phenotype which can be ascribed to NLRP3 inflammasome hyper-activation. However, in F2, an even more exacerbated pro-inflammatory maladaptation seems to be independent of NLRP3 inflammasome activation and to rather result from a relative adrenal burnout. Furthermore, from re-analysis of data to elucidate sex-specific adaptations, we conclude that the glucocorticoid hypersensitivity seems to result from HPA-dysregulation transferred via the maternal lineage, in a manner affecting male offspring to a greater extent. In contrast, NLRP3 inflammasome hyper-responsiveness seemed to occur only in offspring to LPS-affected F1 females. Alarmingly, these seemingly different maladaptive pathways had a cumulative effect on the offspring born to two LPS-affected parents. Moreover, these F2 offspring exhibited a profile of immune senescence.

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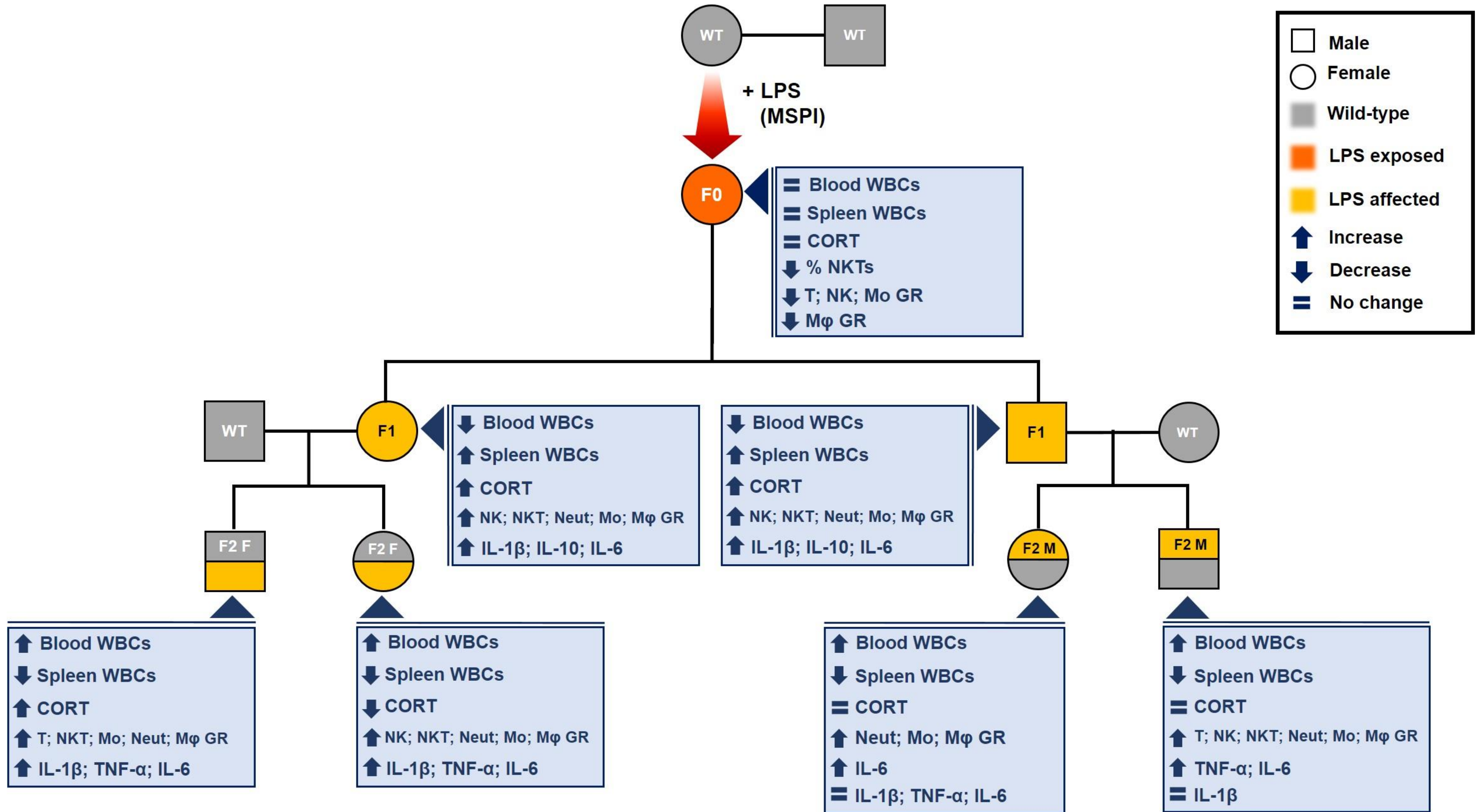

Figure 5.1 Inheritance of inflammatory maladaptations from F0 to F1 and F2 generations. Graphical summary depicting the inheritance pattern for the HPA-axis - and immune functionality dysregulation. WBCs, White blood cells (leukocytes); T, T-lymphocytes; NKT, NKT-lymphocytes; NK, NK cells; Neut, Neutrophils; Mo, Monocytes; Mφ, Macrophages.

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In terms of practical implications of the findings, this data suggests that the relatively pro-inflammatory phenotype seem in modern life stressors may have a significant impact on the health outcome of future generations to come. Taking this into consideration, we do acknowledge some limitations within the study that may preclude firm conclusions in this context. Firstly, the current study design was focussed on maternal gestational inflammation only (in the F0 generation). The multigenerational maladaptations due to primary paternal inflammation remains to be elucidated. The fact that paternal inheritance of maladaptation has been implicated in the context of PTSD and poor nutrition, indicates the importance of sex-specific inclusive research. Secondly, largely due to logistic factors, sex-specific data is limited by small numbers. Given the potential far-reaching impact of findings reported, larger scale studies are recommended to confirm the current preliminary interpretation. Thirdly, on a more technical note, a large portion of the current data was generated from investigations using spleen leukocytes. While this choice was necessary, given the sample volume required for the assessments performed, we acknowledge that leukocytes isolated from spleen may not fully represent the exact nature of adaption/maladaptation in all body compartments.

These abovementioned limitations, as well as the potential criticism of poor translation of rodent models in general, would possibly only be clearly elucidated in longitudinal human cohort studies. Although studies of this nature require extensive resources, in our opinion, such studies are warranted given the already high prevalence of chronic disease with an inflammatory component. Furthermore, future animal research on chronic maternal inflammation is still needed to identify the potential mechanism(s) implicated in this foetal programming as well as determining whether the effects persist to F3 and beyond. Both these studies will be beneficial as it could facilitate the development of targeted intervention strategies aimed at the gestational period pregnancies to diminish or ameliorate the development of chronic inflammatory diseases in future offspring.

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Appendix I: Ethical Approval for the animal study

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Protocol Approval

Date: 04-Apr-2016

PI Name: Adams, Rozanne RCM Protocol

#: SU-ACUM14-00004

Title: Influence of inflammation on transgenerational epigenetic modulation.

Dear Rozanne Adams, the Progress Report, was reviewed on 04-Apr-2016 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is only valid for a period of twelve months. Ethics clearance of protocols spanning more than one year must be renewed annually through submission of a progress report, up to a maximum of three years.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are ac ng within the scope of practice for their profession.

Please remember to use your protocol number, SU-ACUM14-00004 on any documents or correspondence with the REC: ACU concerning your research protocol.

Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research.

Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

We wish you the best as you conduct your research.

If you have any questions or need further help, please contact the REC: ACU secretariat at WABEUKES@SUN.AC.ZA or 0218089003.

Sincerely,

Winston Beukes

REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use

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Appendix II: Detailed overview of methodology

Preparation of reagents

Spleen isolation media

For the media, RPMI 1640 (Gibco, USA) with 1% penicillin-streptomycin and 10% heat inactivated, sterile filtered, foetal bovine serum was prepared and thoroughly mixed. Thereafter sterile filtered and stored at 4 ^{o}C and used within one week.

Red blood cell lysis buffer (ACK lysis buffer)

To lyse the red blood cells, without compromising a viability of the splenic leukocytes, an ACK lysis buffer was used. In one litre of sterile distilled water, 8.26g ammonium chloride (NH4Cl), 1g potassium bicarbonate (KHCO3) and 0.037g EDTA was added, mixed thoroughly. The pH was adjusted to pH7.4 and the lysis buffer was sterile filtered and stored at 4°C for up to 6 months.

Complete RPMI-1640 media

RPMI-1640 medium supplemented with 1× GlutaMAX (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA), 25 mM HEPES (Gibco, USA), 10% (v/v) heat-inactivated foetal calf serum. The media was sterile filtered and store at 4°C for up to 3 months

Flow BLOCK buffer

The blocking buffer was prepared with 1× DPBS supplemented with 3% (v/v) heat-inactivated foetal calf serum, 0.1% (w/v) bovine serum albumin (Sigma Aldrich, USA) A9647), 0.1% (w/v) sodium azide (Sigma Aldrich, USA). The buffer was sterile filtered and stored at 4°C for up to 18 months

Flow PERM buffer

The permeabilization buffer was prepared with 1× DPBS supplemented with 1% (v/v) heat-inactivated foetal calf serum, 0.1% (w/v) bovine serum albumin (Sigma Aldrich, USA) A9647), 0.1% (w/v) sodium azide (Sigma Aldrich, USA) and 0,1% (w/v) saponin (Sigma Aldrich, USA). The buffer was sterile filtered and stored at 4°C for up to 18 months

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LPS for ex vivo stimulation and Inflammasome Priming Stimulus

A 1mg/ml stock of LPS from Salmonella Minnesota Re595 (Sigma Aldrich, USA) in DPBS and sonicated before use. For the inflammasome priming stimulus, the stock was diluted to a working solution of 10µg/ml in complete RPMI 1640 media. The LPS was stored at −20°C for up to 12 months.

Inflammasome activation stimulus

Nigericin sodium salt (Sigma Aldrich, USA) was to a concentration of 5mM in 100% high grade ethanol and mixed by inversion until dissolved. The stock was stored at 4°C, well-sealed with parafilm for up to 12months. To prepare the working solution of nigericin (100µM), a 1:50 dilution was prepared in complete RPMI1640 and mix thoroughly. This was prepared prior to use and any unused solution was discarded.

Poly-HEMA solution

Poly-HEMA prevents cell adherence and allows easy harvesting without loss of cells. The poly-HEMA solution (Sigma Aldrich, USA) was prepared as previously published by Kuroda et al, 2013. Briefly, the coating solution poly-HEMA was prepared by adding 38 ml of 99.5% (vol/vol) ethanol and 2 ml of double distilled water into a 50-ml conical tube and mixing thoroughly. Thereafter, 1.2 g of poly-HEMA into the conical tube and dissolved using a plate rotator for 5–6 h at 37 °C. The poly-HEMA stock solution was stored at 4 °C for up to 2 months. To each well in the 48-well plate, 70µl of the poly-HEMA solution was added under sterile conditions and distributed over the entire well. The plates were dried overnight at room temperature and stored for up to 3 months after coating.

Study Methodology

Animal Study Design and Ethical approval

Ethical approval for the *in vivo* was obtained from the Stellenbosch University Research Ethics Committee (ethics no. SU-ACUM14-00004). For the initial breeding study, 10 six-week old female and 10 male sibling pairs of C57BL/6 mice were obtained from the University of Cape Town animal breeding facility and were housed at the Stellenbosch University animal facility under temperature-controlled conditions and a reverse dark-light cycle. The mice were acclimatized for one week before any

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treatment was commenced and were routinely monitored. The generational breeding experiments were initiated when mice reached 7 weeks.

Establishment of Chronic Inflammatory mouse model

Preparation of inflammatory stimulus

Lipopolysaccharide (LPS) was used in our model for chronic inflammation. To prepare the LPS, the LPS was diluted in 0.9% saline to a stock solution of 1mg/ml and sonicated for 10 minutes to ensure the lipids are were suspended. The stock was stored at -80˚C until use. To prepare a working the stock was diluted to 5ug/ml LPS in 0.9% saline and stored in single use aliquots of 50ul at -80˚C until use. Before use, all aliquots were sonicated and vortexed thoroughly. Any unused aliquots were discarded appropriately.

Breeding for F-0, F-1 and F-2 mouse generations

In order to obtain a chronic maternal inflammatory model, first generation of female mice, referred to as F0, were mated with sibling males and within 12 hours after mating the females were administered 10ug/kg LPS (from Salmonella), prepared in 0,9% saline solution, intraperitonially. The control group received 40µl saline solution interperitoneally to account for the effect of the vehicle. The administration of LPS and the vehicle was repeated every seven days for the duration of gestation. The dose was based on their initial weight prior to the first injection and was kept constant throughout the duration of the gestation. Pregnancy was confirmed by the animal technician and the female mice were monitored daily for any adverse effects and weighed weekly for the duration of gestation. For the subsequent generations, no further injections were administered.

The second-generation offspring (F1) were weaned at 3 weeks of age and separated into different cages accounting for the maternal study number and gender. At 8 weeks of age, four male and four female offspring from each group were terminated by cervical dislocation and blood, spleen and brain was collected. The remaining animals were bred for generation three (F2).

For F2 generation, the offspring was mated with a respective unaffected wild-type C57/BL/6 mouse and the same procedure was followed for weaning, with no further injections administered. As a separate group, a small number of LPS affected F1 mice

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were mated with an LPS affected mouse of a different parent. The mice were weaned at 3 weeks and separated into different cages based on maternal or paternal study number and gender.

The F-0, F-1 and F-2 generations were killed by cervical dislocation, either at 16 weeks after weaning offspring, (F-0 generation) or at 7-8 weeks of age (F-1 and F-2 generations). Blood was collected by cardiac puncture and the spleen was dissected and cells were isolated for further immune analysis.

Sample collection and cell isolation

Blood collection

For our study, samples were collected after cervical dislocation. For full and differential blood counts as well as plasma, whole blood was collected via cardiac exsanguination after cervical dislocation with a 25-gage needle and 2ml syringe and transferred into K2EDTA microtubes. The tubes were inverted 5 times and kept at room temperature until analysis.

Isolation of leukocytes from mouse spleen

Mouse spleens were harvested under sterile conditions and collected into 1.5ml microcentrifuge tubes, with 1ml spleen isolation media aliquoted into each tube, and kept on ice until cell isolation. Eppendorf, with FBS-Complete media solution (1mL) on ice. Keep on ice until use.

Under sterile laboratory settings, mechanical isolation technique was used to isolate single cells. Seventy micron (70µm) cell strainers were placed on 50ml conical tubes and the spleens were placed in the strainers. Using the end barrel of a 10ml syringe, splenic tissue was broken apart and gently pushed through the strainer. Afterwards, about the cell strainer was rinsed three times with 10ml spleen isolation media to remove any remnants of the cells that may be attached to the cell strainer. After rinsing, the tubes were centrifuged at 1200rpm for 10 minutes at 4ºC, the supernatant was discarded, being careful not to disturb the pellet.

To lyse the red blood cells, the pellet was resuspended in 3ml ACK lysis buffer at room temperature. The cells were lysed for 5 minutes, shaking the tube every minute. After 5 minutes, 45ml Dulbecco's phosphate buffered saline (1X) was added to each tube

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and the samples were centrifuged at 1200rpm for 10 minutes at 4ºC. The supernatant was carefully decanted, and the pellet was resuspended in 1ml RPMI 1640.

To assess viability, 10µl Trypan Blue was resuspended with 10µl of the splenic leukocyte suspension and counted on the haemocytometer. The cell number was then adjusted to 1×10^7 viable cells per ml for further experiments.

Experimental analyses

Full and differential cell count

To determine the leukocyte count in both peripheral blood and isolated splenocytes, an aliquot was run on the haemocytometer, to analyse the counts of lymphocytes, monocytes, neutrophils, eosinophils and basophils in both cell suspensions.

Plasma corticosterone concentrations

The plasma corticosterone levels were measured using a quantitative competitive ELISA kit, DEMEDITEC Corticosterone rat/mouse ELISA (Demetec Diagnostics, Germany). Plasma samples were thawed and diluted 1:2 in the kit diluent and plates were prepared as per manufacter's instruction. The plates were run on the Biochrom EZ Read 400 microplate reader (Biochrom, UK), using the Galapagos Expert v1.3 software platform, at a wavelength of 450nm. The optical density readings were exported to Microsoft Excel. The corticosterone concentrations were calculated using a 6-point standard curve (prepared in duplicate), with a logistic regression algorithm in Microsoft Excel. The R^2 value was > 0,97. The lowest detection limit was 6,1ng/ml and the highest detection limit was 2250ng/ml.

***Ex vivo* cytokine analysis**

Ex vivo cytokine profile for all the generations was assessed using the MAP Mouse Cytokine/Chemokine Magnetic Bead panel (Millipore, USA). In our experiment, we assessed IL-1β, IL-6, IL-10, TNF-α and IFN-γ. The cytokine profiles were assessed as per manufacturer's instructions. Before analysis, the supernatant collected from the ex vivo LPS stimulation was thawed and prepared plates were run on the Bioplex 200 system (Biorad, USA) equipped with Bio-Plex Manager™ software. The sample cytokine concentrations were automatically calculated by the software based on a 6-point standard curve (in duplicate) fitted with a five-parameter logistic regression algorithm. The lowest limit for detection for IL-1β, IL-6, TNF-α, IL-10 and IFN-γ are

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5,4pg/ml, 1,1pg/ml, 2,3pg/ml, 2,0pg/ml and 1,1pg/ml respectively. The highest limit of detection is 10 000pg/ml for all cytokines of interest.

Methodology for flow cytometry

Description of instrumentation

The BD FACSAria IIu flow cytometer is a high-speed fixed-alignment benchtop cell sorter, with the acquisition speeds up to 100,000 events per second. The instrument is equipped with blue (488-nm), red (633-nm) and violet (405-nm) solid state lasers for excitation of fluorochromes, to enable analysis of a maximum of 9 fluorescent markers and two scatter parameters, forward scatter (FSC) and side scatter (SSC) simultaneously.

The cell sorter employs detector arrays with photomultiplier tubes (PMTs) and a photodiode detector to detect and amplify photons emitted by the fluorescent markers. An octagon detector array contains six PMTs that detect SSC and up to five fluorescence signals excited by the blue laser. The trigon arrays contain two PMTs to detect fluorescence signals excited by the red and violet lasers, respectively. The FSC signal is detected by the photodiode detector. The PMTs convert photons into electrical pulses, which are subsequently processed by the electronics system and converted into visual data. Acquisition and BD FACSAria II cytometer functions are performed and controlled by BD FACSDiva v8.1 software.

The flow cytometric training and subsequent analysis of all samples was performed by the investigator at the Central Analytical Facilities Fluorescent Imaging Unit, Stellenbosch University. Prepared samples were analysed on the BD FACSAria IIu flow cytometer (BD Biosciences). As we have two separate flow cytometry experiments, we have illustrated the light path, filters, detectors for all the markers employed in our study in Table I.

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Table I: Detectors, Parameters and Filters on BD FACSAria IIu

Laser	Detector	Parameter	Long Pass Filter	Band Pass Filter	Amplification type
Blue 488nm	FSC	FSC	n/a	n/a	Linear
	SSC	SSC	n/a	488/10	Linear
	488-E	FITC	502	530/30	Log
	488-D	PE	556	585/20	Log
	488-C	PE-Texas Red	595	610/20	Log
	488-B	PerCP-Cy5.5	655	695/40	Log
	488-A	PE-Cy7	735	780/60	Log
Red 633nm	633-B	APC-Cy7	755	780/60	Log
	633-A	APC	n/a	660/20	Log
Violet 407nm	405-B	Brilliant Violet 421	502	450/40	Log
	405-A	Alexa Fluor 430	n/a	530/30	Log

Assessment of basal glucocorticoid receptor expression in splenic leukocyte

Cell Staining

All the reagents were prepared prior to use, as per manufacturer's instructions. For permeabilisation of samples, the BD Cytofix/ CytoPerm kit was employed. The staining buffer for washing and staining was prepared with 5% bovine serum albumin (Invitrogen, USA) (5g/100ml in deionised water), 10% sodium azide (10g/100ml in deionised water), and 1X Dulbecco's phosphate-buffered saline (DPBS) (Gibco, USA). The solution was stored at 4ºC until use. The paraformaldehyde solution (Sigma Aldrich) was prepared as a 4% (4g/100ml) solution in DPBS. The antibodies used are listed in Table 3.2 and were titrated to determine optimal dilution for experiments.

To determine the optimal antibody dilution, the antibody conjugates were titrated. Antibody titration allows one to identify the best antibody concentration to use for a given assay/ marker of interest to be detected, whilst minimizing the signal to noise (S/N) ratio, which represents background noise. Although the vendor will provide a specific concentration to use, this may not be appropriate for your assay.

For the staining procedure, $1x10^6$ splenocytes was transferred into 1.5ml microcentrifuge tubes and resuspended in 100ul DPBS. Thereafter 1µl Zombie Aqua

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viability dye (Biolegend, USA) was aliquoted and the sample tubes were incubated at room temperature for 30 minutes in the dark, as the fluorochromes are light-sensitive. After incubation, 1ml staining buffer was aliquoted into each tube and centrifuged at 300*xg* for 5minutes at room termperature. This wash was done to remove any residual dye. The supernatant was aspirated and the pellets were resuspended in 50µl staining buffer containing Mouse FC block. The samples were incubated for 5 minutes at 4 ˚C. For the antibody staining of cell surface markers, a mastermix was prepared in the dark. The volume for each antibody was added as described in Table 3.2. A total of 40µl staining buffer was added per sample and the mastermix was thoroughly mixed before added to each tube, and the samples were incubated for 30minutes at 4 ˚C in the dark. After incubation cells were washed two times by aliquoting 1ml staining buffer into the tubes and centrifuging at 300xg for 5 minutes.

The remaining pellets in each tube were resuspended in 250µl of the CytoFix/CytoPerm solution and incubated in the dark for 20 minutes at 4 ˚C. After incubation, the cells were resuspended in 1ml 1X PermWash solution and centrifuged at 600xg for 5 minutes at room temperature. The supernatant was discarded and the pellets were resuspended in 100ul PermWash solution with the appropriate dilution of the intracellular glucocorticoid receptor antibody. The samples were incubated at 4˚C for 30 minutes in the dark. After incubation, 1ml 1X PermWash was added to each sample tube and the samples were centrifuged for 600xg for 5 minutes at room temperature washed in PBSAN and centrifuged at 600xg for 5minutes. This process was repeated once more and the pellets were resuspended in 300µl PBSAN after centrifugation. The samples were stored at 4˚C for up to 6 hours until acquisition.

The antibody conjugates, their clones as well as their respective titrations used in this study are illustrated in Table II.

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Table II: Antibody panel for Basal Glucocorticoid Receptor Analysis

Fluorochrome	Marker	Clone	Supplier	Dilution	Volume/sample
FITC	TCRbeta	H57-597	BD Biosciences	1:320	0,3125µl
PE-CF594	F4/80	T45-2342	BD Biosciences	1:160	0,625µl
PerCP Cy5.5	CD11b	M1/70	BD Biosciences	1:160	0,625µl
AlexaFluor 647	NR3C1	BuGR2	Novus Biologicals	1:40	2,5µl
APC-Cy7	Ly6G	1A8	BD Biosciences	1:80	1,25µl
Brilliant Violet 421	NK1.1	PK136	Biolegend	1:160	0,625µl

Fluorescence minus one controls

Fluorescence minus one (FMO) controls for each antibody conjugate included in the staining panel. FMO controls were performed to delineate correct positive and negative populations for the marker of interest. This is required to determine accurate and unbiased gating methods for the experiment. For the FMO staining, the cell controls were treated similarly to the samples by aliquoting the volumes of the antibodies as described in Table 3.2, with the exception of the tested antibody. In example, the FMO sample for TCRbeta FITC would have all the other antibodies in the tube, with the exception of TCRbeta FITC, so as to determine the spillover of all the other markers into the FITC channel. This is done for each antibody in the panel. The cell controls were then acquired on the BD FACSAria IIu and analysed for the spillover for each tested antibody. The gates were then adjusted, to account for this spillover.

Instrument setup and Compensation

To determine optimal voltage settings for the experiment, single stain cell controls were employed for each antibody, using the optimal dilutions in Table II as well as an unstained cell control. To ensure reproducibility of our data, we used Applications settings in BD FACSDiva software, in order to standardize our experimental data. For each of our channels, we adjusted the voltages to ensure that the negative population was more than 2.5 times and less than 10 times the value of the electronic noise (rSD). We then checked that the positive populations were still within the log range and not exceeding the maximum channels on the log, and we adjusted accordingly. The settings were saved as Application Settings, which is linked to the daily quality control Cytometer Setup and Tracking (CST) check. These Application Settings accounted

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for voltage changes, using the CST as its measure and automatically adjusts the voltages accordingly, ensuring the reproducibility of the data. As a secondary measure, we also included a lot specific 8-peak bead control as our daily standardisation check to ensure that our settings are valid and also to be able to reproduce the experiment on a different cytometer, if necessary.

For the compensation, Anti-Mouse and Anti-Rat/Hamster Ig CompBeads were employed (BD Biosciences, USA). For the live/dead sample. A methanol fixed sample was used. Single-stained compensation controls were prepared for each antibody in the staining panel. The positive and negative anti-Mouse Ig CompBeads were vortexed at high speed for a minimum of 15 seconds. One drop of the positive and negative anti-Mouse Ig CompBeads was added to each well and the appropriate antibody/dye was added to the single stained beads or cells. The controls were vortexed thoroughly and incubated in the dark for 30 minutes at room temperature. After incubation, 1ml PBSAN was added to the wells and the plate was centrifuged at 600xg for 5 minutes at room temperature and the supernatant was discarded. The beads were resuspended in 300µl of 1:10 dilution paraformaldehyde and stored at 4˚C and protected from light.

The samples were acquired, and the compensation matrix (Table III) was then applied to the experiment for the acquisition and analysis of the samples.

Table III: Representative Compensation Matrix for Basal Glucocorticoid Receptor Analysis

	FITC	PE-CF594	PerCP-Cy5.5	AxF647	APC-Cy7	BV421	BV510
FITC	100	8,81	1,31	0	0	0,09	2,464
PE-CF594	0,14	100	25,30	0,75	0,12	0,04	0,022
PerCP-Cy5-5	0	0,03	100	6,63	17,33	0,02	0,049
AxFl 647	0	0,02	0,30	100	15,32	0	0,000
APC-Cy7	0,02	0,01	0,05	5,05	100	0,06	0,043
BV421	0,48	0,23	0,09	0,09	0,07	100	7,194
BV510	0,56	0,27	0,10	0,10	0,12	13,20	100

Acquisition and analysis

Sample tubes were resuspended by vortexing for 5 seconds before acquisition. All samples were analysed on the BD FACSAria II flow cytometer, which employed BD

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FACSDiva™ version 8.1 software for data acquisition and analysis. For data acquisition, a minimum of 200 000 and a maximum of 500 000 live, gated, singlet events were collected for each sample tube. All samples were run on compensated application settings, as previously described in the optimisation and compensation was performed with every run. For further analysis, all files were exported as FCS 3.1 files and further analysed in FlowJo™ v10.4.1

Identification of subsets and basal glucocorticoid receptor expression

Dot plots employed for the identification of splenic leukocytes of interest (Figure I). Back gating was performed on each leukocyte population to verify their position in relation to the initial FSC versus SSC plot (Figure 3.2 to 3.4). The primary gating strategy (FSC vs. SSC) was used to yield the Splenocyte gate to separate the cells of interest from debris. Within the splenocyte gate, dead cells were removed by only gating on Zombie Aqua negative cells. Doublet discrimination was performed by applying a gate around the linear population in the SSC-H vs. SSC-A plot. Within this population, TCRβ+ and TCRβ- cells were gated to distinguish lymphoid cells from non-lymphoid cell types. T-lymphocytes (TCRβ+ NK1.1-) and NKT lymphocytes (TCRβ+ NK1.1+) were derived from the TCRβ+ gate. In the TCRβ- population, NK cells (CD11b+ NK1.1+) were separated from non-NK cells, and in this latter population neutrophils (CD11b+ Ly6G+) were also isolated from other CD11b+ cell types. Lastly monocytes and macrophages sub populations were identified by the expression of CD11b+F4/80- and CD11b+ F4/80+ markers respectively. Bulk gating was used to apply these gate coordinates to each generation and all the gates were checked and adjusted manually for each sample.

To determine the glucocorticoid expression for each leukocyte population of interest, a histogram plot was employed and the entire population was gated using an interval gate. This was performed as the glucocorticoid receptor is expressed uniformly in all cells. All data for the experimental design was exported to Microsoft Excel for further analysis.

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Assessment of inflammasome Activation

Cell culture

Splenocyte culture

Splenocytes from each animal were thawed at 37° C in a water bath and washed twice (300xg, 5 minutes) with prewarmed complete RPMI 1640 media to remove any freezing media. Thereafter the cells were counted and seeded at 2×10^6 viable splenocytes per ml in complete RPMI 1640 media, and 450µl of the cells suspension was added to each well in a 48-well plate and incubated at 37°C at 5% CO_2 for 24 hours to allow resting.

Inflammasome Priming and Activation

The experiment consisted of three treatments per subject; control, LPS and LPS + Nigericin. To the control wells, 50µl of complete RPMI 1640 media was added, to the LPS and LPS + Nigericin wells, 50µl of 100 ng/ml LPS was added. All the wells were mixed thoroughly before placing the plate back into the incubator for 6 hours at 37°C and 5% CO_2.

After incubation, 55µl of 100µM Nigericin working solution was added to the LPS + Nigericin well and the plate was placed back in the incubator for 60 minutes at 37°C and 5% CO_2. Following incubation, the cells were transferred from the culture plate wells to 1.5-ml microcentrifuge tubes and centrifuged at 974xg for 3 minutes, and the pellet was retained for flow cytometric staining.

Cell staining

Fixation, permeabilization and staining

The samples were fixed in 400µl of 4% paraformaldehyde for 5 minutes on ice, after which 1ml Flow BLOCK was added to each tube and centrifuged for 5 minutes at 600 × g, room temperature. The supernatant was discarded, and the pellets were resuspended in 250µl Flow PERM buffer and incubated for 20 min at 4°C. after permeabilization, the cells were resuspended in 1ml Flow WASH and for 5 minutes at 600 × g and the supernatant for each sample was discarded.

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Similar to the glucocorticoid experiment, the antibodies were titrated to determine the optimal antibody dilution (Table IV). An antibody mastermix was prepared to a final volume of 100ul per sample in Flow PERM buffer and added into the sample tubes and before resuspending. The tubes were then incubated at 4°C for 30 minutes. After incubation, the cells were resuspended in 1ml Flow WASH and centrifuge for 5 min at 600×g, room temperature. After washing the supernatant was decanted, being careful not to disturb the pellet and the cells were resuspended in 500µl Flow WASH buffer before acquisition on the flow cytometer.

Table IV Antibody panel for Inflammasome Assay

Fluorochrome	Marker	Clone	Supplier	Dilution	Volume/sample
PE-Cy7	Pro-IL-1β	NJTEN3	eBiosciences	1:160	0,625µl
PE	F4/80	T45-2342	BD Biosciences	1:80	1,25µl
Brilliant Violet 421	CD11b	M1/70	BD Biosciences	1:160	0,625µl
AlexaFluor 647	ASC/TMS1	polyclonal	Novus Biologicals	1:40	1µl

FMO controls

Fluorescence spillover was analysed by use of fluorescence minus one (FMO) controls for each antibody conjugate included in the staining panel. The FMO experiment was done using unstimulated mouse splenocytes at a concentration of $1x10^6$ splenocytes per sample. The cell controls were then acquired on the BD FACSAria IIu and analysed for the spillover for each tested antibody. The analysis gates were then adjusted, to account for this spillover.

Instrument setup and Compensation

To determine optimal voltage settings for the experiment, single stain cell controls were employed for each antibody, using the optimal dilutions in Table IV as well as an unstained cell control. To ensure reproducibility of our data, we used Application settings in BD FACSDiva software platform, in order to standardize our experimental data. The settings were saved as Application Settings and as a secondary measure, we also included a lot specific 8-peak bead control as our daily standardisation check to ensure that our settings are valid and also to be able to reproduce the experiment on a different cytometer, if necessary. For the compensation, Total Comp beads were

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employed (Thermofisher Scientific, USA) and prepared according to manufacturer's instructions. The samples were acquired, and the compensation matrix (Table V) was then applied to the experiment for the acquisition and analysis of the samples.

Table V Representative Compensation Matrix for Inflammasome panel

	PE	PE-Cy7	AxF647	BV421
PE	100	2.088	0.085	7.043
PE-Cy7	2.289	100	0.2184	0
Ax647	0	0	100	0
BV421	0	0	0	100

Acquisition and analysis

Sample tubes were resuspended by vortexing for 5 minutes before acquisition. All samples were analysed on the BD FACSAria II flow cytometer, which employed BD FACSDiva™ version 8.1 software for data acquisition and analysis. For data acquisition, a minimum of 200 000 and a maximum of 500 000 gated, singlet events were collected for each sample tube. All samples were run on compensated application settings, as previously described in the optimisation and compensation was performed with every run. For further analysis, all files were exported as FCS 3.1 files and further analysed in FlowJo™ v10.4.1

Identification of macrophages and gating for inflammasome activation analysis

Dot plots employed for the identification of cells of interest (Figure 3.2). To separate cells from debris, a FSC vs. SSC plot was created and the cell population was gated to exclude debris. To exclude doublets, the cell population was plotted in a FSC-W vs. FSC-A plot. The resulting singlets were plotted as a histogram of ASC-A and the high ASC-A expressing cells were gated, as they are monocytes/macrophages. In this population, a ASC-H vs. ASC-A dot plot was created to quantify cells with an ASC speck. The ASC speck containing cells were gated for quantification. All data for the experimental design was exported to Microsoft Excel for further analysis.