Differential effects of activin A on macrophage responses: Implications for testis immunology

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Differential effects of activin A on macrophage responses: Implications for testis immunology

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Abstract

The immune privilege of the testis facilitates the protection of the germ cells from autoimmune reactions and inflammatory damage in case of an infection. The macrophages, which comprise the majority of immune cells present in the testis, help facilitate immune privilege with their immunosuppressive phenotype. How exactly this phenotype is obtained, however, remains to be elucidated. The immunoregulatory Sertoli cells potentially express a factor driving the macrophage polarisation towards an anti-inflammatory phenotype. One factor secreted by the Sertoli cells, which could possibly be involved in the immunoregulatory phenotype of testicular macrophages is activin A. Activin A has previously been shown to be involved in many biological processes including immunoregulatory functions and can elicit pro- as well as anti-inflammatory responses. To assess the influence of activin A on macrophage phenotype, several different macrophage models were investigated *in vitro*. The phenotype and the possible influence on the immune responses was assessed in activin A-treated macrophages in a resting, as well as lipopolysaccharide-activated state with several different markers at the gene expression and protein level, as well as regarding possible metabolic implications. This study revealed, that activin A elicits differential effects on commonly used murine macrophage models, depending on their origin or maturity, the context and time points of the treatments, as well as the factors which were investigated. Generally, activin A appeared to induce a more pro-inflammatory phenotype in macrophage colony-stimulating factor-differentiated primary bone marrow-derived macrophages, while it could reduce pro-inflammatory responses in two murine cell lines tested. Additionally, it was demonstrated that activin A can alleviate the pro-inflammatory metabolic switch in activated macrophages in an immortalised bone marrow-derived macrophage cell line. Given the opposing findings of the effects of activin A in different macrophage in vitro cultures, results obtained with these commonly utilised models need to be assessed and evaluated with caution.

This study demonstrates the complex actions of activin A in immune responses and suggests that activin A may also influence the microenvironment in the testis facilitating the immunoregulatory phenotype of testicular macrophages, supporting homeostasis and protection from inflammatory damage.

Declaration

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature:

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Date: 26.11.21

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List of Abbreviations

Actb	Beta-actin
ACVR2 (A, B)	Activin-specific type II receptor (A, B)
ADP	Adenosine diphosphate
AKT	Protein kinase B
ALK4	Activin receptor-like kinase 4
AMPK	5’ adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen-presenting cell
Arg1	Arginase 1
Atf3	Activating transcription factor 3
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BMDM	Bone marrow-derived macrophages
BMDM A	Bone marrow-derived macrophages cultured in Australia at Monash University
BMDM G	Bone marrow-derived macrophages cultured in Germany at Justus-Liebig-University
bp	Base pairs
BSA	Bovine serum albumin
BTB	Blood-testis barrier
°C	Degree Celsius
cAMP	Cyclic adenosine monophosphate
Casp1	Caspase 1
Ccl2	CC-chemokine ligand 2
cDNA	Complementary DNA
CD (4, 8, 11b, 14, 32, 45, 86, 206)	Cluster of differentiation (4, 8, 11b, 32, 45, 86, 206)
Chil3/Ym1	Chitinase-like protein 3
Clec7a	C-type lectin domain containing 7a/dectin-1
CLR	C-type lectin receptor
CO_2	Carbon dioxide
CREB	CAMP response element-binding protein

CT	Cycle threshold
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DPBS	Dulbecco’s phosphate buffered saline
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK (1/2)	Extracellular signal-regulated kinase (1/2)
ETC	Electron transport chain
EU	Endotoxin units
$FADH_2$	Flavin adenine dinucleotide
FasL	Fas ligand
FAO	Fatty acid oxidation
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone
Folr2	Folate receptor beta
Fpr2	Formyl peptide receptor 2
FSC	Forward scatter
FSH	Follicle-stimulating hormone
g	Gram
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Gpr18	G protein-coupled receptor 18
h	Hours
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF1α	Hypoxia inducible factor 1 alpha
HLA-DR	human leukocyte antigen-DR
IDO	Indoleamine 2,3 dioxygenase
IFNγ	Interferon gamma
IκB (α)	Nuclear factor of kappa-light-chain-enhancer of activated B cells inhibitor (alpha)

IKK (α, β)	Inhibitor of nuclear factor of kappa-light-chain-enhancer of activated B cells kinase (alpha/beta)
IL-1β	Interleukin 1 beta
IL (-4, -5, -6, -10, -12, -13, -17, -23)	Interleukin (4, 5, 6, 10, 12, 13, 17, 23)
iMACs	Immortalised bone marrow-derived macrophages
IRF (3, 7)	Interferon regulatory factor (3, 7)
JLU	Justus-Liebig-University Giessen
JNK	C-Jun N-terminal kinase
kD	Kilodalton
Klf4	Krüppel-like factor 4
L	Litre
LBP	LPS-binding protein
LC	Leydig cell
LPS	Lipopolysaccharide
M	Molar
MAPK	Mitogen-activated protein kinase
MARP	Monash Animal Research Platform
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Macrophage colony-stimulating factor
MD-2	Myeloid differentiation factor 2
MEK	Mitogen-activated protein kinase kinase
MEM	Minimum essential medium
MHC (class I, II)	Major histocompatibility complex class I, II
min	Minutes
ml	Millilitre
mM	Millimolar
Mn	Manganese
Monash	Monash University
Mrc1	Mannose receptor C-type 1
mRNA	Messenger ribonucleic acid
mTORC (1, 2)	Mammalian target of rapamycin complex 1, 2
MyD88	Myeloid differentiation factor 88
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

NAGly	N-arachidonoyl glycine
ND	Not detectable
NED	*N*-1-naphtylethylenediamine
NF-κB	Nuclear factor of kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NK	Natural killer
NLR	NOD-like receptor
nm	Nanometre
NO	Nitric oxide
NOD	Nucleotide-binding oligomerisation domain
NOS2/iNOS	Nitric oxide synthase 2/ inducible nitric oxide synthase
NOX2	Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2
OCR	Oxygen consumption rate
OM	Oligomycin
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGC-1β	Peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1β
PI3K	Phosphatidylinsositol-3-kinase
Pik3cd	Phosphatidylinsositol-3-kinase catalytic subunit delta
PIP_2	Phosphatidylinositol-4,5-biphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
pmol	Picomole
PPARγ	Peroxisome proliferator-activated receptor gamma
PTC	Peritubular cell
qRT-PCR	Quantitative real-time PCR
RAW	RAW264.7
RBC	Red blood cell
RIG	Retinoic acid-inducible gene
RIPK1	Receptor-interacting serine/threonine-protein kinase 1

RLRs	RIG-1-like receptors
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
Rot/AA	Rotenone/antimycin A
Rplp0	Ribosomal protein P0
rpm	Revolutions per minute
RPMI 1640	Roswell Park Memorial Institute 1640
PRR	Pattern recognition receptor
RT	Room temperature
s	Seconds
SC	Sertoli cell
SD	Standard deviation
Seahorse analysis	Seahorse real-time cell metabolic analysis
SEM	Standard error of the mean
Slamf1	Signalling lymphocytic activation molecule family member 1
SMAD	Small body size Mothers Against Decapentaplegic
SSC	Side scatter
STAT (3, 6)	Signal transducers and activators of transcription (3, 6)
TAE	Tris-acetate-EDTA
TCA	Tricarboxylic acid
TGFβ	Transforming growth factor beta
Th (1, 2, 17)	T helper (1, 2, 17)
TIR	Toll-IL-1 receptor
TLR (3, 4)	Toll-like receptor (3, 4)
TMB	3,3’,5,5’-tetramethylbenzidine
TM(I)	Interstitial testicular macrophage
TM(P)	Peritubular testicular macrophage
TNF	Tumour necrosis factor
TNFR (1, 2)	Tumour necrosis factor receptor (1, 2)
Tr	Regulatory T cell
TRAF (3, 6)	TNF receptor associated factor (3, 6)
Tregs	Regulatory T cells
TRIF	Toll-receptor-associated activator of interferon

U	Units
UV	Ultraviolet
µg	Microgram
µl	Microlitre
µM	Micromolar

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1 Introduction

The testis, as an immune privileged site, does not show strong immune responses to inflammatory stimuli, probably in order to protect the germ cells from damaging inflammatory reactions. Macrophages are the most prominent immune cell type in the testis and help sustain the immunoregulatory environment. How macrophages acquire their immunosuppressive functions, however, is not fully understood. The cytokine activin A, which reportedly can be both pro-, as well as anti-inflammatory, and is expressed by the Sertoli cells in the testis, was investigated regarding its effect on macrophage responses and therefore its potential implications in testis immunology.

1.1 The immune system

The immune system acts as a defence mechanism to protect the host against invading pathogens and aims to sustain the integrity of tissues. It is a complex system comprised of a vast variety of interacting cells, physical barriers such as the skin and mucous membranes, as well as soluble substances like the complement system, all collaborating to protect its host from pathogens, and detect and eliminate infectious agents (Delves et al., 2017; Parham, 2015; Parkin & Cohen, 2001).

Once pathogenic microorganisms enter the body, they are detected by the immune system's pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), via pathogen-associated molecular patterns (PAMPs) expressed by the invading pathogen, which ultimately elicits an immune response. These reactions of the immune system are generally divided into two responses: the more rapid innate and the more specialised adaptive immune response. The first line of defence against infectious agents is the innate immune response, which can act rapidly upon detecting pathogens. The innate immune system is comprised of cells with phagocytic and antigen-presenting capacities and consists of granulocytes (neutrophils, basophils, eosinophils, mast cells), dendritic cells, macrophages and their monocyte precursors. Cells of the innate immune system are derived from a myeloid progenitor, which originates from hematopoietic stem cells of the bone marrow (Janeway et al. 2001; Delves et al. 2017). Granulocytes and monocytes circulate in the bloodstream and can be recruited to sites of infection by tissue-resident myeloid cells like macrophages, dendritic cells, and mast cells. Macrophages and dendritic cells serve as sentinels and are phagocytes as well as antigen-presenting cells (APCs), engulfing invading pathogens and displaying their specific antigens to activate immune cells and elicit the immune response. Upon activation, they secrete cytokines and chemokines to

activate and recruit other immune cells, such as neutrophils and monocytes, to the site of infection. Mast cells increase the permeability of blood capillaries for the blood circulating immune cells and plasma proteins to invade the compromised tissue, whereas the neutrophils, specialised in rapid phagocytosis of pathogens, aid in attempting to eliminate the infectious agents (Delves et al., 2017).
The presentation of antigens as well as the secretion of cytokines and chemokines by the cells of the innate immune system activates and informs the cells of the specialised adaptive immune response. Antigens are presented via the major histocompatibility complex (MHC) proteins class I and class II. While MHC I molecules are expressed on all nucleated cells of the body, MHC class II molecules are expressed on APCs. Predominantly MHC class I molecules display patterns of endogenous proteins which the immune cells can recognise as being 'self' and determine the cells as part of the host. If a cell is compromised by pathogens or otherwise damaged (MHC class I), or the APCs engulf pathogens and process their proteins (MHC class II), the MHC molecules present antigens which then can be detected as foreign (Delves et al., 2017; Roche & Furuta, 2015; Rock et al., 2016). Similar to the cells of the innate immune system, the cells of the adaptive response (lymphocytes) also originate from a common stem cell progenitor in the bone marrow, which further develops into the lymphoid progenitor, finally differentiating into different types of lymphocytes. These lymphocytes are generally categorised into B cells, T cells and natural killer (NK) cells (also part of the innate immune response). T cells comprise cytotoxic T cells, regulatory T cells (Tregs), and T helper (Th) cells. The cells of the adaptive immune response are activated by the innate immune cells, such as the dendritic cells, which upon engulfing the pathogen, enter the lymph nodes to present the specific antigen to lymphocytes like naïve T cells, activate them through binding to their antigen-specific T cell receptor, and elicit the clonal expansion of the matured effector T cell. Additionally, presented antigens also bind to the B cell receptor of B lymphocytes, which upon activation proliferate and differentiate into plasma cells that produce specific antibodies against the antigen. This response is memorised by the B cells upon a second encounter with the antigen (Delves et al., 2017).
The subtypes of T cells can be characterised by different cell-surface molecules called cluster of differentiation (CD), primarily as CD8+ T cells, which recognise MHC class I, or CD4+ T cells, recognising MHC class II. Cytotoxic T cells are CD8+ and, similarly to NK cells, directly eliminate infected cells and tumour cells through induction of apoptosis. The subtype of CD4+ cells comprises Th cells as well as Tregs. Due to their differing responses upon activation, Th cells can be further subdivided into Th1, Th2, and Th17 cells. The Th1 cells induce immune responses against intracellular pathogens by inducing the production of opsonising antibodies by B cells and the secretion of cytokines such as interferon gamma (IFNγ) to promote the bactericidal actions of

macrophages. Responses elicited by the Th2 cells involve mostly the defence against extracellular parasites, inducing the secretion of cytokines such as interleukin (IL)-4, IL-5, and IL-13 to promote the recruitment of eosinophils and mast cells, as well as the maturation of B cells producing immunoglobulin E. The responses of Th17 have been implicated in chronic inflammatory reactions, as well as the resolution of fungal infections and induce the secretion of cytokines of the IL-17 family. Finally, Tregs are involved in regulating and resolving immune responses to limit tissue damage and alleviate inflammation (Chaplin, 2010; Iwasaki & Medzhitov, 2010; Marshall et al., 2018; Parham, 2015; Rock et al., 2016). NK cells can already be active during the innate immune response and are able to detect intracellular pathogens or cancerous cells by screening for the composition of MHC. In the absence of appropriate MHC presentation, the cytotoxic NK cells of the immune system can induce programmed cell death through apoptosis or cell lysis (Delves et al., 2017).

In addition to clearing pathogens, the immune system is also important for tissue homeostasis and normal physiology. For this, immune cells such as macrophages adapt to their surroundings as tissue-resident macrophages and serve tissue-specific purposes. Among these specialised adaptations of the immune system is the immune privilege in certain tissues, such as the anterior chamber of the eye, the brain, the lungs, and the testes, or under specific circumstances like the pregnant uterus. This immune privilege allows for a certain amount of tolerance to protect the tissue from inflammatory damage (Rink et al., 2015).

1.1.1 Macrophages and their phenotypes

Macrophages, as APCs, are located in most vertebrate tissues and are predominantly recognised for their essential role in host defence and removal of apoptotic cells. The phagocytic properties of macrophages were first described in the nineteenth century by Eli Metchnikoff, suggesting their role in immunity (Okabe & Medzhitov, 2016; Tauber, 2003). In case of infection, macrophages provide the first line of defence against microbial invaders, including bacteria, viruses and parasites, initiating adaptive and innate immune responses (Bhushan & Meinhardt, 2017; Okabe & Medzhitov, 2016). However, various studies have elucidated a broad range of fundamental biological functions where macrophages are involved, such as systemic metabolism, cold adaptation, tissue homeostasis, development of organs like the brain, and tissue-repair responses (Okabe & Medzhitov, 2016; Wynn et al., 2013).

Macrophages can be differentiated and activated into two broad phenotypes, the classical, inflammatory M1-phenotype and the alternatively activated, immunosuppressive M2-phenotype

(Fig. 1). Given the complex environment inhabited by macrophages with various cytokines and growth factors, this distinct classification into two extreme phenotypes does not entirely reflect the plasticity and continuum of macrophage states. However, they provide a tool for discrimination between apparently different populations (Murray, 2017; Sica & Mantovani, 2012; Wynn et al., 2013).

Generally, the function of M1 macrophages is to mediate the inflammatory response, with secretion of pro-inflammatory factors as a reaction to invading pathogens. In contrast, M2 macrophages exert immunoregulatory properties, including resolution of inflammation and tissue homeostasis (Italiani & Boraschi, 2014; Wynn et al., 2013). Multiple factors have been found to induce the polarisation of macrophage precursor cells towards the M1 or M2 phenotype. Among factors promoting a differentiation towards the classical M1 phenotype are IFNγ, granulocyte-macrophage colony-stimulating factor (GM-CSF), TLR ligands such as lipopolysaccharides (LPS) or tumour necrosis factor (TNF). In response to these factors, M1 macrophages secrete high amounts of pro-inflammatory cytokines, such as IL-1, IL-6, IL-12, IL-23, TNF, as well as reactive nitrogen and oxygen species (ROS), while also promoting pro-inflammatory Th1 responses (Italiani & Boraschi, 2014; Sica & Mantovani, 2012).

Conversely, polarisation towards the alternative, immunosuppressive M2 phenotype can be mediated via macrophage colony-stimulating factor (M-CSF), IL-10, IL-4, IL-13, transforming growth factor β (TGFβ), prostaglandins, and glucocorticoids (Martinez & Gordon, 2014; Murray, 2017). As mentioned previously, macrophages, especially those considered to belong to the immunosuppressive M2 phenotype, consist of a variety of subsets with differing characteristics, which is possibly accounted for by their assorted functions in immunity, such as their involvement in allergic inflammation, but also wound healing (Rőszer, 2015). The M2 subsets are proposed to be categorised by their various differential stimuli into M2a (IL-4, IL-13), M2b (immune complexes and bacterial LPS), M2c (glucocorticoids, TGF-β) and possess different functions in immune reactions such as repair and wound healing, defence against parasites or viral pathogens. However, these categorisations were based on *in vitro* models and do not take various other signalling factors into account, or the range of activation states of macrophages (Rőszer, 2015). For the purpose of the present study and due to their plasticity, M2 macrophages will not be further subdivided into specific subsets and will be referred to as M2. Generally, M2 macrophages express anti-inflammatory factors, including large amounts of IL-10 and TGFβ, promoting immunoregulation and resolution of inflammation, while only secreting low amounts of pro-inflammatory substances, such as TNF (Bhushan et al., 2015; Bhushan & Meinhardt, 2017; Italiani & Boraschi, 2014). Alternatively

activated M2 macrophages are commonly characterised, among others, by the secretion of scavenging molecules and the expression of galactose and mannose receptors, specifically CD206, also known as mannose receptor C-type 1 (Mrc1) (Rőszer, 2015; Sica & Mantovani, 2012). Due to the various subtypes of M2 macrophages and their activation, however, the specificity of a M2 characterisation via the CD206 marker alone, especially *in vitro*, could possibly be inconclusive (Jablonski et al., 2015; Rey-Giraud et al., 2012; Rőszer, 2015). Therefore, additional markers to distinguish the phenotypes are frequently investigated. While CD206 alone is not a definitive marker for the M2 phenotype, for the purpose of the present study CD206+ macrophages will be referred to as M2.

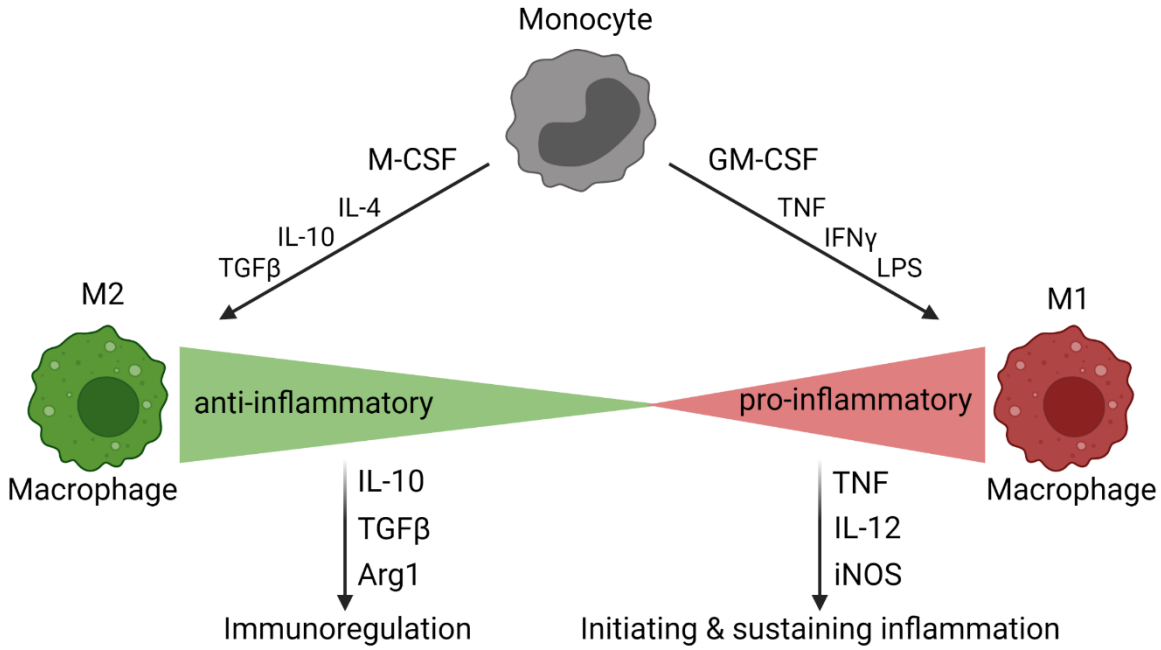


Figure 1: Simplified illustration of macrophage polarisation into anti- or pro-inflammatory phenotypes. Monocyte precursors can be polarised towards pro-inflammatory M1 macrophages (by factors such as LPS and TNF) or anti-inflammatory M2 macrophages (by factors such as IL-10 and TGFβ), with a wide spectrum of M2 polarised macrophage subtypes. Generally, M2 macrophages have immunoregulatory properties and secrete factors such as Arg1 and IL-10, while M1 macrophages initiate and sustain inflammation by secreting factors such as TNF and iNOS. Arg1: arginase 1, GM-CSF: granulocyte-macrophage colony-stimulating factor, IFNγ: interferon gamma, IL-4, -10, -12: interleukin 4, 10, 12, iNOS: inducible nitric oxide synthase, LPS: lipopolysaccharide, M-CSF: macrophage colony-stimulating factor, TGFβ: transforming growth factor beta, TNF: tumour necrosis factor (created with BioRender.com, license number: JN237GPISJ; information described in Italiani & Boraschi, 2014; Sica & Mantovani, 2012)**.**

1.1.1.1 Responses of macrophages in inflammation

The innate immune response initiates inflammation as a protective response against any injurious agents, such as invading pathogens or tissue injury. The inflammatory response aims at the

elimination of the threat, the removal of compromised tissue and finally the regeneration or repair of the inflamed site to regain tissue homeostasis where possible. An acute inflammation is induced when cells of the immune system, such as neutrophils and macrophages, detect injurious stimuli via their PRRs, including the TLRs, initiate signalling cascades, secrete pro-inflammatory cytokines and chemokines to attract immune cells to the site of infection, and activate the adaptive immune response. Infiltrating neutrophils and macrophages phagocytose pathogens and cell debris to eradicate the injurious agents before the inflammatory response can be resolved. If the resolution of the inflammatory response fails, for example due to incomplete removal of the injurious agents, chronic inflammation can develop, which can promote the development of diseases through excessive fibrosis or tissue dysfunction (Oishi & Manabe, 2018; Ospelt & Gay, 2010).

Macrophages appear to be important mediators in the inflammatory response and its resolution, and in the coordination of entire immune responses with their diverse and flexible phenotypes ranging from pro- to anti-inflammatory, as well as their ability to promote tissue regeneration and homeostasis. Macrophages orchestrate the mechanisms from inflammation to new tissue formation as the signalling pathways and cellular processes controlling the inflammatory response have been found to be connected to the processes of healing; however, their specific response highly depends on the microenvironment of the affected tissue (Oishi & Manabe, 2018).

Broadly, as an initial reaction to an infection, macrophages are activated towards a pro-inflammatory phenotype, which in turn produces pro-inflammatory signals, cytokines and chemokines, and initiates an appropriate adaptive immune response with either Th1 and Th17 cells or a Th2 response against extracellular invaders. Once the infection has been cleared, the phenotype of the macrophages changes towards an anti-inflammatory response to resolve inflammation, as well as to restore tissue integrity (L. Zhang & Wang, 2014).

1.1.1.1.1 Inflammatory signalling pathways

Immune responses can be initiated and modulated through various interacting signalling pathways, some of which will be introduced in the following section. One of the most prominently studied of the signalling pathways which induce and trigger immune responses is TLR4 signalling, elicited through the recognition of the gram-negative bacterial cell-wall endotoxin, LPS, and appears especially relevant in *in vitro* macrophage experiments where the cells are routinely activated with LPS to study their phenotypes. TLR4 signalling induces, among other responses, the signalling cascades of the nuclear factor of kappa-light-chain-enhancer of activated B cells (NF-κB) and the

phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) signalling pathways, which have been shown to play important roles in the regulation of immune responses (Ciesielska et al., 2021; Vergadi et al., 2017).

Injurious agents can be detected by various PRRs, such as the TLRs already mentioned, as well as C-type lectin receptors (CLRs), nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene (RIG)-1-like receptors (RLRs), whose activation leads to the initiation of signalling cascades ultimately inducing the transcription of the appropriate genes (Ahmed, 2011).

TLRs are among the best characterised PRRs and are type 1 transmembrane glycoprotein receptors located either in the outer cell membrane or intracellularly in the endosome. Once a ligand binds to the TLRs, the receptors homodimerize or heterodimerize and a signalling cascade is induced via binding of an adaptor to their cytoplasmic domain, Toll-IL-1 receptor (TIR). Depending on the stimulus, different combinations of downstream molecules can be activated by the adaptor molecule leading to specific responses. Generally, two main pathways of adaptor molecules have been studied: the myeloid differentiation factor 88 (MyD88) and the adaptor molecule Toll-receptor-associated activator of interferon (TRIF) (Kawasaki & Kawai, 2014; Ospelt & Gay, 2010).

The endotoxin LPS is detected by TLR4 and initiates both the MyD88- and TRIF-dependent pathways, which can induce pro- and anti-inflammatory responses, as well as metabolic changes and the adaptive immune response. The activation of the TLR4 signalling pathways by LPS induces the dimerization of TLR4/myeloid differentiation factor 2 (MD-2) molecules in the cell membrane. Subsequently, the adaptor protein MyD88 can bind to the TIR region of the receptor and initiate signalling cascades such as the type I PI3K/AKT pathway via phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PIP_3). Additionally, MyD88 signalling recruits TNF receptor associated factor (TRAF) 6, which finally leads to the initiation of the phosphorylation of nuclear factor of kappa-light-chain-enhancer of activated B cells inhibitor (IκB) kinases α/β (IKKα/β), as well as mitogen-activated protein kinases (MAPK). While the phosphorylation of IKKα/β leads to the translocation of NF-κB into the nucleus, the MAPK induce activating protein (AP)-1 and the cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB). The activation of the MyD88-dependent signalling pathway induces the expression of pro-inflammatory genes like TNF and IL-6, as well as the anti-inflammatory cytokine IL-10, while modulating the metabolism through activation of AKT and subsequent increase of glycolysis. When TLR4 is internalised into an endosome, the TRIF-dependent pathway is induced. Following this, the induction of TRAF3 leads to the phosphorylation of IRF3, a

transcription factor which initiates gene expression of type I IFNs, chemokines, as well as IL-10. In addition to IRF3, TRIF also activates TRAF6 and receptor-interacting serine/threonine-protein kinase 1 (RIPK1), leading to a late activation of NF-κB, as well as activation of extracellular signal-regulated kinase 1/2 (ERK1/2), inducing the production of cytokines. Studies also indicate that the activation of TLR4 by LPS is involved in the activation of the inflammasome (Ciesielska et al., 2021; Everts et al., 2014; Kawasaki & Kawai, 2014; Kuzmich et al., 2017).

The NF-κB signalling pathway is a main mediator of inflammatory responses and, aside from the induction through TLR4 by LPS, can be activated via other PRRs, as well as various stimuli, such as cytokine receptors, TNF receptors and B and T cell receptors. While two different NF-κB pathway activations can be distinguished, the canonical and non-canonical pathways, LPS recognition by TLR4 induces the canonical signalling cascade. When inactive, NF-κB dimers remain inhibited in the cytoplasm through binding of IκB family proteins, such as IκBα, preventing the transcription factor from entering the nucleus. The upstream kinase complex IKK is phosphorylated and activated during TLR4 signalling, and in turn phosphorylates IκBα. Following ubiquitination, IκBα is degraded and releases NF-κB, allowing for its translocation into the nucleus. As a transcription factor, NF-κB regulates the expression of multiple inflammatory genes such as cytokines like TNF, chemokines, interferons, transcription factors and is involved in the activation and differentiation of both the cells of the innate, as well as those of the adaptive immune system. The regulation of a multitude of immune responses mediated through NF-κB signalling is additionally influenced by crosstalk between other signalling pathways and feedback loops, which can be elicited by autocrine signalling function of cytokines originally induced by NF-κB (Dorrington & Fraser, 2019; T. Liu et al., 2017).

Aside from activation through TLR4, the PI3K/AKT signalling pathway can also be induced through insulin receptors, other PPRs, cytokine and chemokine receptors, as well as adipokine receptors. The PI3K/AKT signalling pathway is involved in the regulation of inflammatory responses, proliferation and viability, as well as metabolic changes and the polarisation of macrophages into different phenotypes. The AKT signalling molecules consist of a family of three serine-threonine kinases, namely AKT1, AKT2 and AKT3, while class I PI3K proteins include p110α, β, δ and γ with the isoforms PI3Kδ (encoded by *Pik3cd*) and PI3Kγ being the most predominantly expressed isoforms in hematopoietic cells. Once a heterodimer of a catalytic and regulatory subunit of PI3K has been activated by TLR4 signalling and facilitated the phosphorylation of PIP_2 to PIP_3, AKT is recruited and phosphorylated under involvement of mammalian target of rapamycin complex (mTORC) 2. The phosphorylated AKT leads to the activation of mTORC1 controlling cytokines, as well as metabolic changes (Linton et al., 2019; Vergadi et al., 2017).

1.1.1.1.2 Immunological markers and mediators

A plethora of factors are involved in the orchestration of immune responses, some of which are commonly utilised to differentiate between different phenotypes of macrophages or their expression or secretion has been attributed to a more pro-inflammatory or anti-inflammatory response, while many cannot be assigned unambiguously to either response and their effects depend on the environmental context.

Factors associated with a pro-inflammatory response or the M1 macrophage phenotype are TNF, IL-1β, IL-6, nitric oxide synthase 2 (NOS2; also called inducible nitric oxide synthase: iNOS) and caspase 1 (CASP1), while the G protein-coupled receptor 18 (GPR18) and the formyl peptide receptor 2 (FPR2) have recently been implicated (Jablonski et al., 2015; Rőszer, 2015; Sica & Mantovani, 2012). These factors are often utilised as markers to describe the classically activated macrophage phenotype, while commonly employed markers associated with the alternatively activated M2 macrophage or a more anti-inflammatory response comprise IL-10, arginase 1 (ARG1), chitinase-like protein 3 (CHIL3/YM1), krüppel-like factor 4 (KLF4), MRC1/CD206 and activating transcription factor 3 (ATF3) (Jablonski et al., 2015; Sica & Mantovani, 2012).

The classic pro-inflammatory cytokine, TNF, is secreted by many different cell types, but most prominently by macrophages. Its secretion is induced by inflammatory signals such as LPS and can be elicited via the majority of PRRs, promoting and sustaining the inflammatory response of the immune system through stimulation of inflammatory genes, influencing metabolism, proliferation and differentiation, as well as apoptosis and necroptosis. Two different transmembrane receptors can be activated by TNF: the TNF receptor 1 (TNFR1) and TNFR2, with TNFR1 being expressed in most tissues, while TNFR2 is expressed primarily by immune cells. The activation of the TNFR1 pathway by a TNF trimer induces several mediators, such as NF-κB, c-Jun N-terminal kinases (JNKs), p38 MAPKs and mitogen-activated protein kinase kinase (MEK)-ERK. These mediators facilitate the expression of cytokines and promote cytotoxicity and proliferation. Moreover, the activation of TNFR2 has been shown to also signal through NF-κB and promote cytotoxicity, and is additionally involved in the proliferation of lymphoid cells (Parameswaran & Patial, 2010; Varfolomeev & Vucic, 2018; Wajant & Siegmund, 2019).

Aside from TNF, IL-1β and IL-6 also are prominent pro-inflammatory factors. The endogenous pyrogen IL-1β binds to the IL-1 receptor and is produced predominantly by macrophages early in the immune response, but can also be induced in B cells, dendritic cells, NK cells, fibroblasts, and epithelial cells. In order to become active, pro-IL-1β has to be cleaved first by CASP1, which in turn

is mediated by the inflammasome. The activated IL-1β then is involved in many immunological responses, including the induction of prostaglandins, acute phase proteins and fever, acts as a chemoattractant, and can promote the differentiation of T cells (Duque & Descoteaux, 2014).

Although IL-6 is often employed as a pro-inflammatory marker, it has additional functions as an anti-inflammatory cytokine, depending on the signalling pathway it induces. As a pro-inflammatory pyrogen, it induces fever and acute phase proteins and exerts its actions through trans-signalling with soluble IL-6 receptors that bind to glycol protein 130, which is expressed ubiquitously. Further pro-inflammatory actions of IL-6 entail the promotion of B cells and cytotoxic T cells, while Tregs are inhibited, beside the attraction of immune cells to the compromised tissue. As an anti-inflammatory mediator, IL-6 can promote tissue repair and inhibit apoptosis through the classical pathway where it binds to an IL-6 receptor (Duque & Descoteaux, 2014).

Gpr18 and *Fpr2* have been shown to be significantly upregulated in classically activated M1 macrophages and have therefore been postulated as markers for the M1 phenotype (Jablonski et al., 2015). FPR2, however, appears to act in a context- and ligand-dependent manner, and seems to be involved in the switch from pro- to anti-inflammatory responses elicited by annexin 1 through 5' adenosine monophosphate-activated protein kinase (AMPK). The overexpression of FPR2, however, has also been reported to enhance the pro-inflammatory response of LPS-activated macrophages and its suppression has been shown to alleviate the inflammatory response in acute lung injury (H. Liu et al., 2020; McArthur et al., 2020; Ye et al., 2009). Although it is highly upregulated in M1 stimulated macrophages, GPR18 also has been suggested to be involved in the resolution of inflammation by induction of apoptosis elicited by N-arachidonoyl glycine (NAGly) and mediating neuroprotective actions together with NAGly (Grabiec et al., 2019; Takenouchi et al., 2012).

C-type lectin domain containing 7a (CLEC7A) or dectin-1, which has been suggested as a M2 subtype macrophage marker due to its increased expression in alternatively activated macrophages, is a receptor for beta-glucans and has been shown to be involved in many immunological processes, including antifungal responses (Rőszer, 2015; Willment et al., 2003). Conversely, it has been reported to increase the induction of inflammatory cytokines together with TLR2 and TLR4 receptors, as well as to mediate the polarisation of M1 macrophages through the NF-κB pathway involving autophagy and, consequently, has also been cited as a M1 marker (Ferwerda et al., 2008; Gantner et al., 2003; X. Li et al., 2019).

Prominent markers for M1 and M2 macrophages are NOS2 as a classic pro-inflammatory factor and ARG1 as a major anti-inflammatory marker. NOS2 (or iNOS) oxidises L-arginine which results in the production of the microbicidal nitric oxide (NO) (Amici et al., 2017; Banete et al., 2015). Its

counterpart, ARG1, is induced in M2 macrophages and competes for the same substrate, L-arginine, which it hydrolyses into urea and the polyamine precursor L-ornithine, ultimately promoting cell growth and proliferation. Regulatory effects of ARG1 are also elicited through the limitation of the substrate for NOS2 (or iNOS) (Amici et al., 2017; Banete et al., 2015; Z. Li et al., 2012).

An additional prominent marker for anti-inflammatory responses is the cytokine IL-10. Its anti-inflammatory actions have been reported to be mediated through signal transducers and activators of transcription (STAT)3 signalling after binding to the IL-10 receptor dimer, and lead to the induction of other immunoregulatory genes, such as *Mrc1* and *Tgfβ*, while also inhibiting the expression of pro-inflammatory cytokines (Martinez & Gordon, 2014; Sica & Mantovani, 2012).

Also a commonly employed marker for anti-inflammatory responses or M2 macrophages is CHIL3 (also known as YM1), which promotes repair and resolution of inflammatory responses; although the exact mechanisms remain to be elucidated, an involvement of STAT6 has been implicated (Sutherland et al., 2018; Welch et al., 2002; Zhu et al., 2020).

The previously mentioned M2 marker, CD206 or MRC1, is a carbohydrate receptor and has been shown to be upregulated in microglia and in macrophages of an immunoregulatory phenotype, while classical activation caused a suppression of MRC1 activity. In addition, MRC1 appears to be involved in phagocytosis as a scavenger receptor (Chroneos & Shepherd, 1995; Stein et al., 1992; Tarique et al., 2015; von Ehr et al., 2020).

Furthermore, the transcription factors ATF3 and KLF4 are both markers for anti-inflammatory phenotypes in macrophages. ATF3 can be induced by LPS and its anti-inflammatory properties have been shown to be elicited through the inhibition of TLR4 mediated expression of inflammatory mediators such as TNF (Sha et al., 2017). Similarly, KLF4 has also been reported to inhibit inflammatory cytokines, such as IL-1β through NF-κB signalling, as well as promote an anti-inflammatory phenotype through cooperation with STAT6 (Ghaleb & Yang, 2017; Liao et al., 2011).

Additional mediators of the inflammatory response are chemoattractants, such as CC-chemokine-ligand 2 (CCL2; also known as monocyte chemoattractant protein 1, MCP-1), which regulates the recruitment of macrophages to the site of infection and has been reported to induce a subtype of M2 macrophages. The co-stimulatory type I membrane protein, CD86, regulates the activation of T cells via the T cell receptor, and has been suggested as both a marker for the M1 phenotype, as well as for a subtype of the M2 macrophages (Sica & Mantovani, 2012; L. Wang et al., 2019).

The signalling lymphocytic activation molecule family member 1 (SLAMF1) or CD150, has been reported to be upregulated in both alternatively-, as well as classically activated murine macrophages (Gensel et al., 2017; Makita et al., 2015). The members of the SLAM family are co-stimulatory

molecules interacting with T cells, NK cells, as well as APCs, and SLAMF1 has been reported to be involved in the regulation of the phagosome as well as induction of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 NOX2, which promotes reactive oxygen species production (Berger et al., 2010; Ma et al., 2012). An induction of pro-inflammatory cytokines TNF and IL-1 could additionally be observed when overexpressing *Slamf1* (Song et al., 2015). However, in human macrophages, SLAMF1 has been postulated as a marker for M2 macrophages (Spencer et al., 2010). As indicated, many factors are involved in regulating immune responses and while they can present with specific pro- and/or anti-inflammatory actions, none of them act as definitive markers of the M1 or M2 phenotype on their own.

1.1.1.2 Immunometabolic changes of macrophages

The increasingly relevant field of immunometabolism focuses on the connection of metabolic changes with the regulation of immune responses. Depending on immunological stimuli, metabolic pathways can be reprogrammed and consequently influence immune responses by changes in energy generation, as well as by providing certain metabolites which modify immune cells on both transcriptional and post-transcriptional levels. The most important metabolic pathways involved are the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), glycolysis, fatty acid oxidation (FAO), amino acid metabolism, and the pentose phosphate pathway (O'Neill et al., 2016; Viola et al., 2019).

Specifically in macrophages, various metabolic changes are associated with the classically and alternatively activated phenotypes. Prominently, pro-inflammatory activation of the cells induces a metabolic switch from OXPHOS to glycolysis, accompanied by the disruption of the TCA cycle, ultimately facilitating a rapid production of energy and metabolites which help maintain the pro-inflammatory, classically activated macrophage phenotype. While OXPHOS and FAO are downregulated, the utilisation of the pentose phosphate pathway is increased. In contrast, alternatively activated macrophages rely on an intact TCA cycle and utilise enhanced OXPHOS, as well as FAO to accommodate high energy demand (Diskin & Pålsson-McDermott, 2018; Kelly & O'Neill, 2015; O'Neill et al., 2016; Viola et al., 2019).

Glycolysis takes place in the cytosol of the cell. Glucose is taken up into the cell and metabolised into pyruvate, along with several other metabolites, including two molecules of adenosine triphosphate (ATP) per unit of glucose. Although glycolysis is not a highly effective way for ATP production, it provides a rapid boost in pro-inflammatory macrophages and leads to the generation of other

intermediates, such as the important co-factor nicotinamide adenine dinucleotide (NADH), needed for many enzymatic reactions. Especially in proliferating cells, pathways that promote cell growth, such as the PI3K and MAPK pathways, enhance glycolysis needed to provide essential metabolites for synthesising ribose, fatty acids, and amino acids. The glycolytic pathway can only generate part of the energy comprised in glucose and delivers with pyruvate a substrate for the oxidation to carbon dioxide within the TCA cycle in the mitochondrial matrix (O'Neill et al., 2016).

In most quiescent cells, the predominant form of energy generation is the production of ATP through OXPHOS, for which the TCA cycle provides the necessary intermediates. Aside from fuelling OXPHOS, the metabolisation of pyruvate in the TCA cycle generates various other important precursors, which are needed for amino acid production or are used in other metabolic pathways. Pyruvate, generated by glycolysis or fatty acids enter the TCA cycle by conversion to acetyl coenzyme A, which further condenses with oxaloacetate and is oxidised in a cyclic series of reactions into carbon dioxide and water. Ultimately, the TCA cycle generates NADH and flavin adenine dinucleotide ($FADH_2$), which provide high energy electrons for the electron transport chain (ETC) and OXPHOS in the mitochondrial membrane. In the ETC, the electrons are passed through a series of membrane protein complexes to oxygen as the acceptor molecule, while generating a proton gradient between the mitochondrial matrix space and the intermembrane space. The proton gradient is utilised to fuel ATP synthase through which protons flow back into the matrix, while generating ATP from adenosine diphosphate (ADP) and phosphate (Burgot 2002; Diskin and Pålsson-McDermott 2018; O'Neill et al. 2016).

Various intermediates of the TCA cycle can influence immune responses and accumulate, especially in the disrupted TCA found in pro-inflammatory macrophages, which leads to an increase of citrate, itaconate and succinate. Citrate enhances pro-inflammatory responses by increasing NO and ROS in macrophages, while itaconate can decrease the expression levels of the pro-inflammatory cytokines, *Nos2*, *Il6* and *Il1β*. Succinate can increase the expression of *Il1β* and glycolytic genes by stabilising the transcription factor, hypoxia inducible factor 1 alpha (HIF1α). Additionally, fumarate enhances inflammatory responses, while α-ketoglutarate acts to alleviate inflammatory reactions (Diskin & Pålsson-McDermott, 2018; Liang et al., 2020). In alternatively activated macrophages, the high energy demand resulting from glycosylation of the highly expressed lectin and mannose receptors is facilitated by OXPHOS (Diskin & Pålsson-McDermott, 2018; Viola et al., 2019). It has been shown that factors associated with alternative stimulation of macrophages, such as IL-10 and IL-4, stimulated OXPHOS. Specifically, IL-4 affected the OXPHOS through STAT6 and peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1β (PGC-1β) signalling. In addition,

IL-10 exerted an inhibitory effect on the production of mitochondrial ROS, while also increasing the clearance of reprogrammed mitochondria through mitophagy (Diskin & Pålsson-McDermott, 2018). Aside from its effects on immune responses, studies have shown an influence of metabolic changes on the specific functions of tissue-resident macrophages that maintain the integrity of tissue, such as the ability of alveolar macrophages to clear surfactant facilitated by mediation of their lipid metabolism (Liang et al., 2020; Svedberg et al., 2019).

1.2 The Testis

1.2.1 Testis immunology and immune privilege

The mammalian testes are one of a small number of organs with a unique immunological environment, providing so-called "immune privilege". This privilege was initially characterised by the ability to tolerate allografts and xenografts for prolonged periods without provoking inflammatory immune responses and resulting rejection (Forrester et al., 2008; Meinhardt & Hedger, 2011). More specifically, immune privilege is created by multiple mechanisms, actively providing suppression of immune responses against antigens (Meinhardt & Hedger, 2011). Immune privilege was first discovered by John Hunter in 1767, when a cock testis was transplanted into the belly of a hen and could subsequently be retrieved intact (Setchell, 1990; Zhao et al., 2014). Over the following centuries, numerous transplantation studies were performed, identifying a number of immune privileged organs, including the eye and brain by tumour transplants, the pregnant uterus by skin allografts and the testis by transplantation of parathyroid grafts (Simpson, 2006).

In the reproductive system, the testes exert the crucial function of producing the male germ cells, whose protection against the immunological environment needs to be facilitated. The challenges faced by the immune system in this regard are, on the one hand, ensuring the defence against invading pathogens, but, on the other hand, preventing responses against the autoantigen-expressing germ cells (Zhao et al., 2014). As the immune system is already established prior to the onset of spermatogenesis during puberty, the produced germ cells display autoantigens and appear as 'foreign' to the immune system, potentially evoking immune responses. It is assumed, that immune privilege in the testis is present in order to prevent disruption of spermatogenesis due to autoimmunity against the newly developing cells (Meinhardt & Hedger, 2011).

Multiple mechanisms appear to be involved in facilitating the immune privilege of the testis, including physical barrier structures, an immunosuppressive milieu and systemic immune tolerance (Zhao et al., 2014). If the immunosuppressive environment of the testis is disturbed due to infections, trauma, or other non-infectious processes, orchitis can arise. This means infiltration of leukocytes into the testis and induction of inflammatory processes, potentially resulting in damage to the seminiferous epithelium, which could finally result in infertility (Schuppe et al., 2008; Zhao et al., 2014). Orchitis can be differentiated into acute, symptomatic, and chronically asymptomatic. The symptoms of acute orchitis are testis swelling and pain. Among acute orchitis, isolated orchitis is rare and usually due to viral infections, while in 90 % of bacterial epididymitis, orchitis arises as a result of an ascending infection. The incidence for acute epididymitis has been reported at 290 cases per 100.000 men per year, while isolated orchitis is estimated at 14 cases per 100.000 men per year. Furthermore, in 25 % of testicular biopsies of azoospermia, a medical condition with a lack of sperm in the ejaculate, chronic asymptomatic orchitis is found, meaning infiltrates of immune cells. Without an early diagnosis and therapy with antibiotics, depending on the cause of the infection, serious complications can arise, such as abscess formation and finally infertility (Pilatz et al., 2019; Street et al., 2017). Therefore, it is vital that the processes involved in maintaining immune privilege and testis homeostasis should be understood in order to treat or potentially ameliorate inflammatory damage of the testis.

The principal structure of the testis is comprised of the seminiferous tubules, which is the site of spermatogenesis, and the interstitial spaces between these tubules, containing several cell types such as Leydig cells, which ensure steroidogenesis (Fig. 2). In the seminiferous tubules, the developing germ cells are embedded between the columnar Sertoli cells. Sertoli cells and myoid peritubular cells are located at the outer rim of the seminiferous tubules as part of the tubular wall. Here, they secrete factors that assemble a basal lamina around the seminiferous tubules, which contributes to the tubular wall (Zhao et al., 2014). A physical structure, which was believed to be crucial for maintaining immune privilege, is the blood-testis barrier (BTB), seemingly providing protection of autoimmunity through antigen sequestration. The BTB is comprised of tight junctions, basal ectoplasmic specialisations, desmosome-like junctions and gap junctions between adjacent Sertoli cells, dividing the seminiferous epithelium in a basal and an adluminal compartment (Hedger, 2012; N. Li et al., 2012; Zhao et al., 2014). Even though the BTB sequestrates the majority of autoantigens of the developing germ cells in the adluminal compartment from systemic immunity, it is not sufficient for maintaining immune privilege of the testis. This has been implied by several studies, such as the activation of the testicular immune system against its autoantigens via transfer of activated

lymphocytes from an immunised to an untreated animal (Mahi-Brown et al., 1987; Meinhardt & Hedger, 2011).

A pivotal role in the immunoregulatory environment of the testis is played by the somatic cells, specifically the Sertoli cells, which have been reported to express or secrete immunosuppressive molecules such as activins, TGFβ, Fas ligand (FasL) and indoleamine 2,3 dioxygenase (IDO) (De Cesaris et al., 1992; Meinhardt & Hedger, 2011; Suarez-Pinzon et al., 2000; Wyatt et al., 1988). Co-transplantation studies additionally emphasise the immunosuppressive properties of Sertoli cells. In these transplantation studies, xenografts were transferred together with Sertoli cells, where they were able to protect the grafts from immune responses for a prolonged time (Sanberg et al., 1996; Suarez-Pinzon et al., 2000). Furthermore, it has been shown that Sertoli cells can prevent autoimmune reactions by phagocytosis of the debris of apoptotic germ cells, as well as the cytoplasm shed by maturing spermatids, therefore eliminating the autoantigens, which could potentially provoke an immune response (Nakanishi & Shiratsuchi, 2004; Tanaka et al., 2010).

The interstitial tissue between the seminiferous tubules is inhabited by various cell types contributing to the immune privilege of the testis (Fig. 2). Besides Leydig cells as the largest cell population in the interstitium, most types of immune cells can be found, macrophages being the most frequent (Zhao et al., 2014). A major function of the steroidogenic Leydig cells is the synthesis of androgens necessary for spermatogenesis, but also for secretion into the peripheral circulation targeting other androgen responsive organs (Diemer et al., 2003; Zhao et al., 2014). Leydig cells are also able to influence the immunosuppressive milieu of the testis via several mechanisms, such as innate antiviral responses and the ability to regulate macrophage and lymphocyte numbers (Dejucq et al., 1998; Melaine et al., 2003; Raburn et al., 1993; Zhao et al., 2014). Furthermore, the secreted androgens possess the ability to suppress autoimmune responses and thus contribute to immune privilege (Fijak et al., 2011; Melaine et al., 2003; Meng et al., 2011).

Among the immune cells in the interstitial tissue are mast cells, dendritic cells and lymphocytes, such as CD8+ T cells, CD4+ T cells, NK cells and CD4+CD25+ Tregs. However, B cells are not found in the testicular interstitium. In particular, Tregs appear to be involved in immunoregulation in the testis, suggested by the observation that Tregs are increasingly mobilised in allograft studies in mouse testis, while memory T cells are destroyed (Dai et al., 2005; Nasr et al., 2005; Zhao et al., 2014).

At 20 % of the total, macrophages make up one of the largest populations of cells in the interstitium (Hedger, 2002; Zhao et al., 2014). Macrophages are antigen presenting cells, and in the testis they display an immunoregulatory phenotype with diminished pro-inflammatory action, but high immunosuppressive properties. These properties include the secretion of relatively high levels of

anti-inflammatory cytokines, such as IL-10 and TGFβ (Bhushan et al., 2011; Kern et al., 1995; Winnall et al., 2011). Additionally, testicular macrophages contribute to the testicular microenvironment by influencing the development and steroidogenic activity of Leydig cells (Hutson, 2006; Zhao et al., 2014). Moreover, the finding that the infiltration of circulating macrophages into the testis during orchitis leads to disruption of spermatogenesis, further endorses the importance of the immunosuppressive phenotype of the testicular macrophages in immune privilege, making them an important candidate for investigation (Rival et al., 2008; Theas et al., 2008; Zhao et al., 2014).

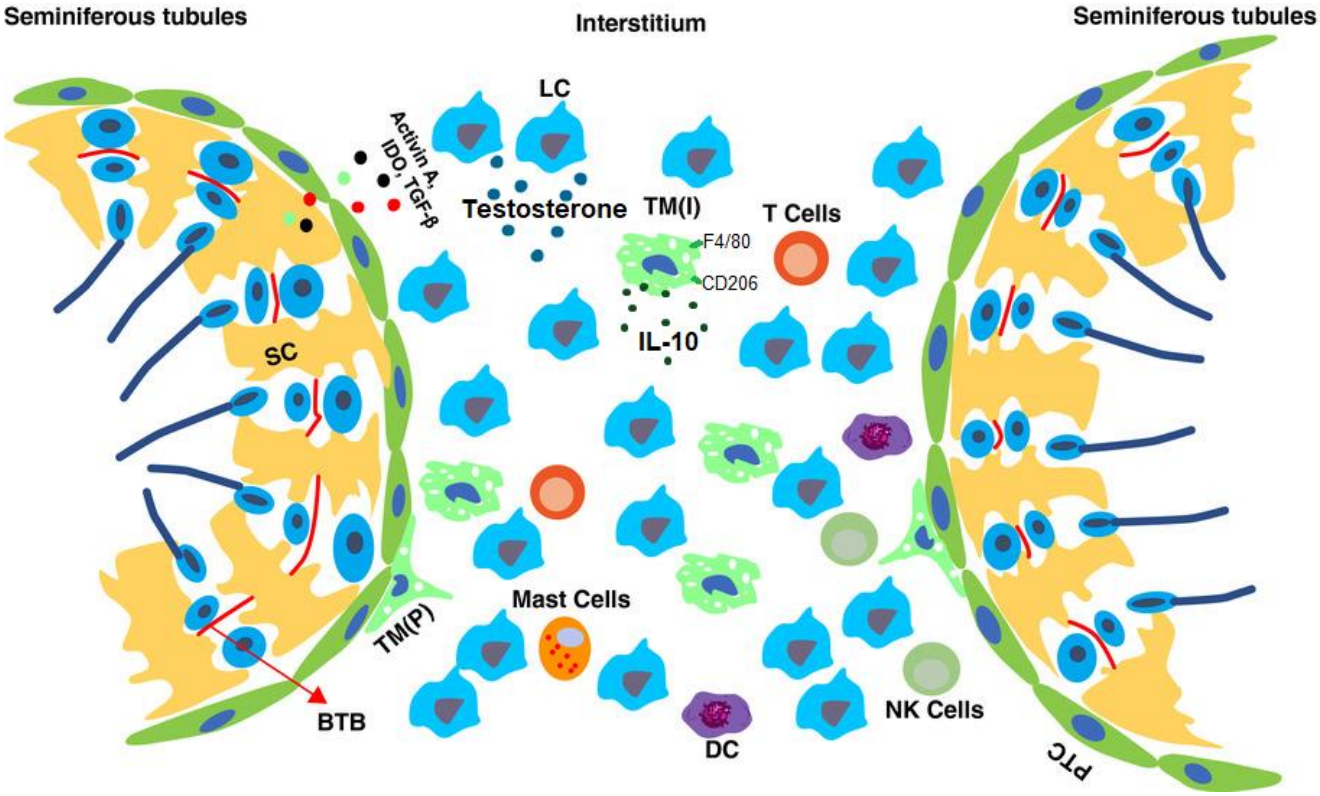


Figure 2: Simplified illustration of seminiferous tubule with interstitial cells of the testis. The testis is comprised of the seminiferous tubules and the interstitium between these tubules, containing several cell types such as Leydig cells and immune cells including testicular macrophages, T cells, NK cells, dendritic cells, and mast cells. The peritubular cells are located at the outer rim of the seminiferous tubules and germ cells are embedded between Sertoli cells. Sertoli cells secrete regulatory factors such as activin A, while testicular macrophages secrete the anti-inflammatory IL-10. BTB: blood-testis-barrier, CD206: cluster of differentiation 206, DC: dendritic cell, IDO: , IL-10: interleukin 10, LC: Leydig cell, NK cell: natural killer T cell, PTC: peritubular cell, SC: Sertoli cell, TM(I): interstitial testicular macrophages, TM(P): peritubular testicular macrophages (adapted from Meinhardt et al., 2018; with the permission of the publisher, license number: 5184370217543).

1.2.2 Testicular macrophages

Concomitant with the immunosuppressive environment of the testis, the resident macrophages in the interstitial tissue are predominantly activated towards the alternative M2 phenotype, exhibiting anti-inflammatory properties. This is indicated by several *in vitro* studies, demonstrating that inflammatory challenge of testicular macrophages results in high secretion of the anti-inflammatory IL-10, while low amounts of the pro-inflammatory TNF are expressed, as opposed to other tissue-resident macrophages of the M2 phenotype (Bhushan et al., 2011, 2015; Winnall et al., 2011). The promotion of an immunosuppressive phenotype appears to be facilitated through the local microenvironment of tissue-resident macrophages (Amit et al., 2016). In other organs, such as the brain, the anti-inflammatory cytokine, TGFβ, is involved in the generation of the alternative M2 phenotype of macrophages via down regulation of the transcription factor IRF7 (M. Cohen et al., 2014). Correspondingly, the polarisation of peritoneal macrophages is influenced by retinoic acid through the GATA6 transcription factor (Okabe & Medzhitov, 2014). Evidence is suggesting the possibility that there are also essential factors in the interstitial fluid of the testis, inducing the alternatively activated testicular macrophages.

1.2.2.1 Factors contributing to the immunosuppressive phenotype of testicular macrophages

The testicular interstitial fluid has recently been shown to induce an immunoregulatory phenotype in macrophages *in vitro* by increasing IL-10 secretion, while decreasing TNF, similar to the characteristics of testicular macrophages. In the interstitial fluid of the rat testis, corticosterone was recently found to be a seemingly crucial factor for immunoregulation by facilitating the skewing of testicular macrophages towards the immunosuppressive M2 phenotype. Treating macrophages with corticosterone before their activation with LPS led to an increase in IL-10 while pro-inflammatory mediators were reduced. Further, the glucocorticoid receptor was necessary to facilitate a skewing of the macrophages toward the M2 phenotype (M. Wang et al., 2017). Corticosterone also affected the immunometabolism by increasing the oxygen consumption rate in bone marrow-derived macrophages (BMDM) to a similar profile as seen in testicular macrophages, indicating a polarisation toward the M2 phenotype (Z. Zhang et al., 2020). In addition, testosterone and prostaglandins also appear to promote an anti-inflammatory phenotype and, more specifically, have been shown to induce immunosuppressive properties in BMDM by shifting M1 skewed macrophages towards the M2 phenotype (Meinhardt et al., 2018; M. Wang et al., 2017). Nevertheless, in the testes, the polarisation

of macrophages towards the alternatively activated phenotype appears to be a multifactorial event, with its specific processes and interdependencies remaining to be elucidated (Bhushan et al., 2020; Meinhardt et al., 2018).

As a possible mechanism by which testicular macrophages could regulate the immune microenvironment of the testis, a low expression of genes associated with TLR signalling has been suggested. In addition, the ubiquitination and degradation of IκBα, which serves as an inhibitor for NF-κB, was dimished in testicular macrophages, which could also be observed in macrophages treated with testosterone. Without the degradation of IκBα the NF-κB signalling pathway cannot be activated, which led to low levels of TNF and NO but high levels of IL-10 and activation of the CREB signalling pathway (Bhushan et al., 2015, 2020; M. Wang et al., 2017). Both, testosterone and corticosterone have been shown to activate CREB signalling, while corticosterone appeared to mediate its effects on macrophages through AMPK (M. Wang et al., 2017; Z. Zhang et al., 2020).

The cytokine, activin A, which is produced by the Sertoli cells in the testis, has previously been investigated for its properties regarding the polarisation of macrophages. The results were diverse, showing both, pro- and anti-inflammatory properties of activin A, making it an interesting target of further examination in the context of the testicular macrophages (Ogawa et al., 2006; Sierra-Filardi et al., 2011).

1.3 Activins

Activins are cytokines belonging to the TGFβ superfamily. They were first identified in ovarian extracts as antagonists of inhibin, which suppresses the production of follicle-stimulating hormone (FSH) by the anterior pituitary and acts by blocking the induction of FSH by activins (Hedger & Winnall, 2012; Ling et al., 1986).

Aside from regulating FSH production, activins have been found to be involved in a number of biological processes, such as cell proliferation, differentiation, inflammation, stress and immunoregulation (Phillips et al., 2009). Structurally, activins and inhibins are composed of disulfide-linked dimers of α- and/or β-subunits, with activin A being composed of two β_A-subunits (Hedger & Winnall, 2012; Vale et al., 1986). While inhibins appear to be predominantly produced in the gonads, activins are broadly expressed by most cells and tissues. This has been suggested by studies showing that ovariectomy and orchidectomy diminishes blood levels of inhibin drastically,

whereas it marginally affects activin A (Hedger & Winnall, 2012; McFarlane et al., 1996; Robertson et al., 1988).

In the testis, studies have demonstrated that activin A is predominantly produced by pubertal and mature Sertoli cells, and to a lesser extent by immature peritubular cells (Hedger & Winnall, 2012; Okuma, O'Connor, et al., 2005; Okuma, Saito, et al., 2005; Winnall et al., 2009). Additionally, immune cells, particularly activated monocytes and macrophages produce activins (Ebert et al., 2007; S.-Y. Wang et al., 2008).

In its wide functional spectrum, activin A establishes a relationship between reproduction and immunity, due to its involvement in spermatogenesis and inflammatory responses. Activin A has been shown to regulate spermatogenic and Sertoli cell proliferation, as well as differentiation during foetal and neonatal stages of the testis (Hedger & Winnall, 2012; Itman et al., 2006; Loveland et al., 2007). Additionally, several studies indicate regulatory properties of activin A on adult spermatogenesis, such as its ability to induce deoxyribonucleic acid (DNA) synthesis in developing spermatogonia (Hakovirta et al., 1993; Hedger & Winnall, 2012).

1.3.1 Signalling pathway

Activins exert their effects through the Small body size Mothers Against Decapentaplegic (SMAD) signalling pathway, among others.

In case of activin A, upon binding to a cell surface activin-specific type II receptor (ACVR2A or ACVR2B), the type II receptor dimerises with an activin-specific type I receptor serine/threonine kinase (activin receptor-like kinase, ALK), ALK4. The dimerisation of the receptor complex leads to phosphorylation of SMAD proteins 2 and 3, which in turn combine with SMAD4 to form a transcription factor, regulating genes implicated in proliferation and differentiation of cells (Hedger et al., 2011; Hedger & Winnall, 2012).

Aside from the SMAD signalling pathway, activins are able to exert their actions through inflammatory and stress signalling pathways, which involve TRAF6, the MAPKs, the JNKs and the ERKs (Hedger & Winnall, 2012; Heldin et al., 2009).

1.3.2 The influence of activin A on immune responses

Activin A has been reported to exert various effects in immune responses in different contexts, affecting both pro- as well as anti-inflammatory actions (Chen & ten Dijke, 2016; Hedger et al., 2011; Sierra-Filardi et al., 2011; S.-Y. Wang et al., 2008).

During inflammatory processes, the activity of Sertoli cells as well as their communication with spermatogenic cells appear to be altered, contributing to disruption of spermatogenesis, possibly due to inflammatory signals within the seminiferous epithelium including increased production of activin A (Hedger, 2011a; Hedger et al., 2011b; Hedger & Winnall, 2012). Moreover, it has recently been shown that activin A is upregulated in experimental autoimmune orchitis in mice, together with fibrotic proteins (Kauerhof et al., 2019; Nicolas, Michel, et al., 2017). Along with this, increased activin A production in response to inflammatory stimuli can be blocked by follistatin, an endogenous activin-binding protein, dampening inflammatory responses (Hedger et al., 2011; Nicolas, Muir, et al., 2017). Further pro-inflammatory properties of activin A are indicated by its ability to directly influence macrophages by increasing the expression of pro-inflammatory factors, such as IL-1β and TNF (Hedger & Winnall, 2012; Nüsing & Barsig, 1999; Yamashita et al., 1993). Macrophage polarisation has also been reported to be skewed by activin A towards the M1 phenotype for example by suppressing the secretion of the anti-inflammatory IL-10 in LPS-activated M-CSF-derived BMDM. In addition, its expression is increased in GM-CSF-derived BMDM compared to BMDM differentiated with M-CSF (Sierra-Filardi et al., 2011).

Interestingly, while activin A increases production of pro-inflammatory factors, such as IL-1β and IL-6 in resting macrophages, it showed a suppressive effect on IL-1β and NO in LPS-activated cells, suggesting context-dependent actions of activin A potentially related to the activation status of the macrophages (Fig. 3) (Ge et al., 2009; Hedger et al., 2011; S.-Y. Wang et al., 2008). In addition, activin A inhibits LPS-induced inflammation and promotes tolerogenic responses in lymphocytes as shown for example by an induction of IL-10 in NK cells and has been described to exert suppressive effects on the maturation of dendritic cells and their ability to stimulate T cells (Hedger et al., 2011; Huber et al., 2009; Kim et al., 2015; Segerer et al., 2008). Moreover, prolonged exposure to activin A evidently skews macrophages towards an immunoregulatory and anti-inflammatory phenotype (Famulski et al., 2008; Hedger & Winnall, 2012; Ogawa et al., 2006), and suppresses the activation of T and B cells (Hedger et al., 1989; Hedger & Winnall, 2012; Zipori & Barda-Saad, 2001). In the context of the testis microenvironment it was recently reported that macrophages appear skewed

towards an M2 phenotype in a transgenic mouse model with elevated activin A levels (Indumathy et al., 2020).

These reports indicate an involvement of activin A in spermatogenesis, as well as in pro- and anti-inflammatory processes, pointing towards a context-dependent role for activin A in testicular immunoregulation and immune privilege to facilitate fertility. In this regard, with activin A in the interstitial fluid at constitutively high levels, it could be a potential mediator of testicular macrophage polarisation towards the M2 phenotype (Fig. 3) (Hedger & Winnall, 2012; O'Bryan et al., 2005).

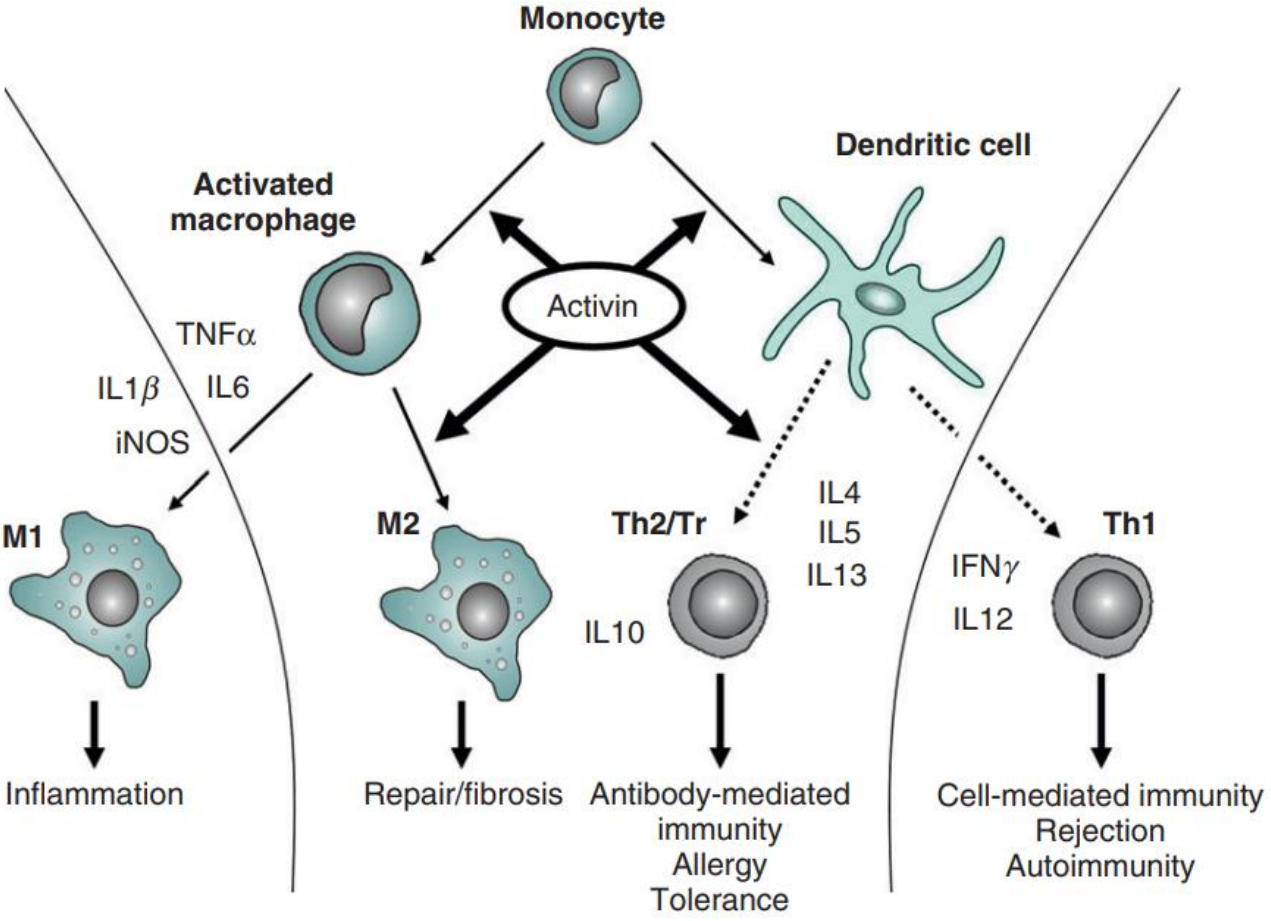


Figure 3: Schematic of the effects of activin A on immune responses. Activin A appears to promote immune responses by modulating the differentiation of monocytes towards dendritic cells or activated macrophages. In activated macrophages, activin A seems to regulate the immune response towards the M2 phenotype while inducing a Th2/Tr response in T cells. IFNγ: interferon gamma; IL1β, 4, 5, 6, 10, 12, 13: interleukin 1 beta, 4, 5, 6, 10, 12, 13; iNOS: inducible nitric oxide synthase; TNFα: tumour necrosis factor alpha; Th1, 2: T helper cell 1, 2; Tr: regulatory T cell (Hedger et al., 2011; with the permission of the publisher, license number: 5184551193188).

2 Research aims

The testicular macrophages contribute to the immune privilege of the testis, a mechanism required to prevent autoimmune reactions against the antigen expressing spermatogenic cells. The anti-inflammatory properties of testicular macrophages, based on their alternatively activated M2 phenotype contribute to the immunosuppressive microenvironment of the testis. Immune privilege can be disturbed by inflammatory processes, such as urogenital bacterial infections, sexually transmitted diseases or other causes, which can elicit disruption of spermatogenesis and androgen deficiency, potentially causing permanent damage (Bhushan et al., 2009; Pilatz et al., 2015). Therefore, it is vital to understand how the unique environment in the testis is created and preserved. The function of macrophages appears to be influenced by the Sertoli cells, which have also been shown to protect tissue grafts in co-transplantation studies, by their immunoregulatory properties (Suarez-Pinzon et al., 2000; J. Wang et al., 1994).

As TGFβ superfamily members, activins are involved in various essential biological processes, such as development, reproductive function, and inflammation (Hedger & De Kretser, 2013; Phillips et al., 2009). Specifically, it was shown that activin A is implicated in induction of pro-inflammatory cytokines by inert macrophages, but also inhibits LPS-induced inflammation and promotes tolerogenic responses in lymphocytes (Kim et al. 2015; Huber et al. 2009). These and other observations indicate a context-dependent function of activin A in inflammatory processes, rendering it an interesting target for further investigation. Additionally, the highly immunoregulatory Sertoli cells are the main producer of activin A in the testis under normal conditions, reinforcing a possible role of activin A in the regulation of the immunological microenvironment of the testis, potentially altering inflammatory responses (Hedger & Winnall, 2012).

Hypothetically, the Sertoli cell-derived activin A could influence the phenotype of testicular macrophages, contributing to immune privilege in the testis. Additionally, activin A could be involved in alleviating inflammation and pathogenesis due to infections and potentially reducing inflammatory damage.

This study focuses specifically on investigating the influence of activin A on the immunological responses of macrophage models from different origin *in vitro*. To assess the context-dependent effects of activin A in resting and in LPS-activated macrophages, their responses were subsequently evaluated for gene expression of M1 and M2 markers, additional immunologically relevant genes, as well as for other functional processes, specifically related to protein secretion and immunometabolic

changes. For culturing BMDM the effects of the growth factors GM-CSF and M-CSF as well as the effect of activin on the phenotype of BMDM were assessed with flow cytometry, hypothesising that M-CSF will skew cells more towards a M2 phenotype than GM-CSF and that activin may induce the M2 phenotype. The potential transcriptional changes induced by activin A influencing the phenotype of macrophages were further analysed by quantitative real-time polymerase chain reaction (qRT-PCR) comparing potentially differential effects on expression of genes related to immune responses in cell lines versus BMDM. To identify functional changes elicited by activin A in the different macrophage models, protein secretion was investigated by enzyme-linked immunosorbent assay (ELISA) and immunometabolic changes were assessed with Seahorse analysis. These studies aimed to shed light on the possible context-dependent effects of activin A, how macrophage culture models compare, and provide indications for the involvement of activin A in the immunosuppressive microenvironment of the testis, specifically the immunoregulatory phenotype of the testicular macrophages.

3 Materials and Methods

3.1 Materials

All materials, including equipment, solutions and reagents are specified in the appendices.

3.2 Methods

3.2.1 Animals

To investigate the effect of activin A on the immune responses in primary cells, male, adult wild-type C57BL/6J mice aged between eight to ten weeks were purchased either from Charles River Laboratories (Sulzfeld, Germany) for studies at Justus-Liebig-University Giessen (JLU; laboratory of Prof. Dr. Andreas Meinhardt), or from the Monash Animal Research Platform (MARP) of Monash University, Clayton campus, for studies at Monash University (Monash; laboratory of Prof. Dr. Mark Hedger). The mouse strain is commonly employed to harvest bones for primary bone marrow culture or tissue for further biological analysis.

The animals were housed under standard conditions with a light/dark cycle of 12 hours and temperatures of 20~22°C with *ad libitum* access to water and standard food pellets. All animals were euthanised by either inhalation of CO_2 or isoflurane and subsequent cervical dislocation prior to the extraction of femur and tibia.

All experiments were performed in strict accordance with the guidelines for Care and Use of Animals for Experimental Purposes under the German Law of Welfare or under the Australian Code for the Care and Use of Animals for Scientific Purposes. The experiments were either approved by the local ethics committee on animal care in Germany (Regierungspraesidium Giessen, Germany; M_684) or by the Monash University Animal Ethics committee in Australia (MMCB2017/40).

3.2.2 Cell culture

To investigate the differing effects of activin A on immune responses, macrophages of different origin were cultured *in vitro* and compared. The employed murine macrophages which are commonly used in immunological studies are the primary BMDM, as well as the cell lines RAW264.7 (RAW) macrophages and immortalised bone marrow macrophages, iMACs. All macrophages were cultured under normoxic conditions at 37°C and 5 % CO_2 unless otherwise specified. All cell culture work was conducted in a tissue culture hood under sterile conditions, to prevent possible contamination.

3.2.3 Isolation and culture of bone marrow-derived macrophages

Bone marrow cells were isolated from male 8-10 week-old C57BL/6J mice to further differentiate them into primary macrophage cultures. After euthanizing the animal, femur and tibia were extracted using sterile instruments, cleaned from muscle and placed into ice-cold 1X Dulbecco's phosphate buffered saline (DPBS) on ice. The bones were then washed with ice-cold DPBS to remove hair and blood, before gently cleaning off the remaining tissue on the bones with sterilized gauze in a tissue culture hood. Femur and tibia were then carefully separated at the knee joint, the knee was removed, and the bones placed into ice-cold DPBS containing gentamicin (50 µg/ml) as an antimicrobial treatment. Subsequently, the femoral head was removed with sterile forceps, and the bone marrow was flushed out with DPBS containing gentamicin by inserting a 24 G needle (femur) or 30 G needle (tibia) attached to a 10 ml syringe into the soft middle of the bone on the sides where the knee was attached. The cells were either directly filtered through a sterile 70 µm cell strainer to remove cell clumps and the filter washed with additional DPBS + gentamicin (JLU), or the cell clumps were first gently brought into suspension via pipetting and filtered after lysing the red blood cells (Monash). In both cases, the cells were next centrifuged at 1500 rpm for 6 min and resuspended in sterile Red Blood Cell (RBC) Lysis buffer (at JLU: Qiagen, at Monash: Invitrogen, sterile filtered prior to use) and incubated for either 2-3 min (JLU), or 10 min (Monash) with occasional shaking. The lysis buffer was inhibited either by fetal bovine serum (FBS)-containing Roswell Park Memorial Institute (RPMI) 1640 BMDM culture medium (RPMI 1640 supplemented with 10 % heat-inactivated FBS, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM Sodium Pyruvate, 1X minimum essential medium (MEM) non-essential amino acids, 50 µg/ml penicillin-streptomycin and freshly added 0.05 mM 2-mercaptoethanol) (JLU), or by DPBS (Monash). The bone marrow cells

isolated at Monash were then filtered through a 70 µm cell strainer, and in both cases the cells were subsequently centrifuged again at 1300 rpm for 6 min. Following this procedure, the cell pellet was resuspended in the BMDM culture medium and counted with 1:2 (JLU) or 1:10 (Monash) trypan blue staining to exclude dead cells from the cell count. At JLU, a hemocytometer was used while at Monash an automated cell counter was utilised. The isolated cells were seeded in 12- or 6-well plates at 2 x 10^6 or 3.3 x 10^6 cells per well, respectively, in RPMI 1640 BMDM medium. The bone marrow precursor cells were then differentiated into macrophages for 7 days with M-CSF (25 ng/ml) or GM-CSF (25 ng/ml), with medium changes including replenishment of the growth factors and treatments on day 3 and 6 after seeding. The cells were either treated with activin A (50 ng/ml, endotoxin levels < 0.1 ng/µg of protein; < 1 EU/g) from the day of isolation for 7 days, or on day 6 for 18 h prior to the activation of the differentiated macrophages on day 7 with *Escherichia coli* LPS (10 ng/ml). To activate the macrophages on day 7, the medium including the growth factors was changed again, the cells were treated with activin A and after 30 min, the macrophages were activated with LPS for 3 h, 6 h or 24 h (see appendix 7.2.5, Fig. 44A, B for treatment regimen). The supernatant was collected for ELISA and the cells were lysed for either protein analysis or gene expression. The cell culture data collected at JLU and Monash were analysed separately and final results were compared, taking into account the unavoidable variations between experiments in different laboratories, while the employed protocols were kept as consistent as possible.

3.2.4 Culture of cell lines

The macrophage like cell line RAW (cell line derived from male BALB/c mice (Guo et al., 2015)) and the immortalised bone marrow cell line iMAC (derived from male C57BL/6 mice; kindly gifted by Assoc. Prof. Dr. Ashley Mansell and immortalised after De Nardo et al., 2018) were both cultured in 75 cm^2 flasks until confluency was reached. The cells were then passaged for further culture in flasks at least once before harvesting for seeding in experiments. To detach the cells from the culture flask, the RAW macrophages were gently lifted with a cell scraper, while the iMACs were detached with TrypLE Express for 6 min at 37°C. After centrifuging and resuspending the cells in fresh culture medium they were counted with a hemocytometer (RAW) or an automated cell counter (iMACs) while employing trypan blue staining in both cases for exclusion of dead cells from the cell count. The cells were seeded at 5 x 10^5 cells per well for RAW macrophages or 3 x 10^5 cells per well for iMACs, in 6-well tissue-culture treated plates. After allowing the cells to settle and attach for 4 h,

they were treated with activin A (50 ng/ml) for 18h prior to a media change and subsequently activation with LPS (10 ng/ml or 100 ng/ml) for 3 h, 6 h, or 24 h (see appendix 7.2.5, Fig. 44C for treatment regimen). After the experiment, the supernatant was collected for ELISA and the cells were lysed for either protein analysis or gene expression. RAW macrophages were cultured in RPMI 1640 with 10 % heat-inactivated FBS and 50 µg/ml penicillin/streptomycin, while iMACs were cultured in 1X Dulbecco's Modified Eagle's Medium (DMEM) containing glucose and supplemented with 10 % heat-inactivated FBS and 2 mM L-glutamine.

3.2.5 Complementary DNA (cDNA) synthesis and qRT-PCR

To investigate the influence of activin A on immune responses through modulation of gene expression of immunological markers, the total messenger ribonucleic acid (mRNA) was harvested after experimental treatments, extracted, reverse transcribed into cDNA and subsequently the gene expression levels were determined by qRT-PCR.

3.2.5.1 Extraction of total mRNA

The cells were first washed twice with cold DPBS and then lysed in the well with RLT lysis buffer from the RNeasy Mini or Micro Kit with freshly added 2-mercaptoethanol (10 µl/ml), after which they were homogenised by aspiration with a 22-26 G needle (JLU) or with a tissue lyser using steel beads (Monash). The total mRNA was extracted according to the manufacturer's instructions using the RNeasy Mini or Micro Kit. Briefly, the lysed and homogenised cells were mixed with 70 % Ethanol and loaded into the provided spin columns. After centrifuging, the mRNA was washed with RW1 buffer, and any contaminating DNA was digested on the columns with deoxyribonuclease (DNase) I in RDD buffer for 20-30 min at room temperature (RT) (Monash) or in an additional step after the extraction of the mRNA (JLU). Following this procedure, the mRNA was washed again with RW1 buffer and then RPE Buffer. When using the RNeasy Micro Kit, the mRNA was additionally treated with 80 % Ethanol. After the described steps, the columns were dried by centrifugation and finally the mRNA was eluted with 14 µl (Micro Kit) or 30 µl (Mini Kit) ribonuclease (RNase)-free water. After the extraction, the concentration of the total mRNA was measured with a NanoDrop and 100 ng-2.5 µg mRNA was reverse transcribed into cDNA.

For DNase treatment following mRNA extraction (JLU), 2.5 µg of mRNA was digested with 0.9 µl DNase I (10 U/µl) and 2 µl 10x DNase I buffer/buffer RDD mixture with RNase free water for a volume of 20 µl. The mixture was then incubated at 37°C for 40 min, followed by 72°C for 10 min to inactivate the enzyme and subsequently cooled down on ice.
The extracted mRNA was either used directly or stored for further processing at -80°C.

3.2.5.2 cDNA synthesis

In the next step, the extracted mRNA was reverse transcribed into cDNA, to be able to investigate the expressed genes in the samples. The reagents used for cDNA synthesis differed between samples obtained at JLU versus the samples collected at Monash, however the general principle of the method remained the same.
For the samples at JLU, 2.5 µg of the isolated mRNA was incubated with 2 µl oligo-dT primer (10 pmol/µl) and denatured for 10 min at 70°C, after which the samples were cooled down on ice for 2 min. Subsequently, the reverse transcription mix was added as described in the table below (Table 1), and the samples were heated at 42°C for 1 min. Following this procedure, the reverse transcriptase was added, and the reaction further incubated at 42°C for 60 min. After the cDNA synthesis, the reverse transcriptase was inactivated at 72°C for 15 min and following cooled down on ice. Until further analysis, the samples were stored at -80°C.

Table 1: Mixture for cDNA synthesis at JLU.

Volume (µl)	Reagent
8	5X M-MLV RT buffer
2	dNTP mix (10 mM)
1	RNAsin (RNase inhibitor, 40 U/µl)
6	RNase-free water
1	Reverse transcriptase M-MLV RNase H-minus 200 U/µl

For cDNA synthesis at Monash, 100-400 ng of extracted mRNA was incubated with 1 µl Random Hexamers (50 ng/µl) and 1 µl dNTPs (10 mM) at 65°C for 5 min for denaturation, after which the samples were cooled on ice.

For the reverse transcription reaction, the samples were incubated with the reverse transcription mixture, as detailed in the table below (Table 2), for 10 min at 25°C, 50 min at 50°C, and finally for 5 min at 85°C. After cooling the mixture on ice, the remaining RNA in the synthesised cDNA sample was removed with the addition of 1 µl RNase H (2 U/µl) and incubation of 37°C for 20 min. The cDNA was stored for at -20°C until further analysis.

Table 2: Mixture for cDNA synthesis at Monash.

Volume (µl)	Reagent
2	10X RT buffer
4	$MgCl_2$ (25 mM)
2	DTT (0.1 M)
1	RNase out (40 U/µl)
1	Superscript III reverse transcriptase (200 U/µl)

3.2.5.3 Polymerase chain reaction (PCR) and Agarose gel electrophoresis

To verify successful digestion of contaminating DNA in the extracted mRNA samples, as well as synthesis of cDNA, a PCR for the abundantly expressed protein β-actin, encoded by *Actb*, (Table 3) using the thermal protocol listed in Table 4, with a subsequent agarose gel electrophoresis was performed.

Table 3: PCR mixture for amplification of *Actb*.

Volume (µl)	Reagent
1	Template (mRNA or cDNA)
5	5X Flexi Buffer
2	$MgCl_2$ (25 mM)
0.5	dNTPs (10 mM)
0.5	*Actb* primer mix (10 pM each forward and reverse primer)
0.25	Go Taq G2 DNA Polymerase (5 U/µl)
15.75	RNase/DNase-free water

Table 4: Program for *Actb* PCR.

Temperature (°C)	Time	
94	4 min	
94	40 s	25 cycles
57	40 s	
72	40 s	
72	10 min	
8	∞	

After the amplification of *Actb* by PCR, the resulting product was examined by agarose gel electrophoresis. This method separates the amplified DNA fragments by its molecular weight in a gel, revealing bands at the appropriate size of the target gene. For the gel, 1.5 % agarose was dissolved in 1X Tris-acetate-EDTA (TAE) buffer (see appendix 7.1.9) via boiling in a microwave oven with occasional shaking. Afterwards, ethidium bromide was added (0.5 µg/ml) to later visualise the DNA bands with UV light. The solution was poured into a vertical gel casting chamber, a comb was added to mold loading pockets for the PCR product and left for solidification of the gel. The comb was removed, and the gel transferred into an electrophoresis chamber containing 1X TAE buffer. After mixing the PCR product samples as well as positive and negative controls with 6X loading buffer to a final concentration of 1X, they were introduced into the gel pockets alongside a DNA ladder marking different band sizes. To separate the DNA fragments in the gel, they were run at 110 V until the visible dye front reached the end of the gel. Finally, the bands were visualised with a UV transilluminator and documented by photographing (Fig. 4).

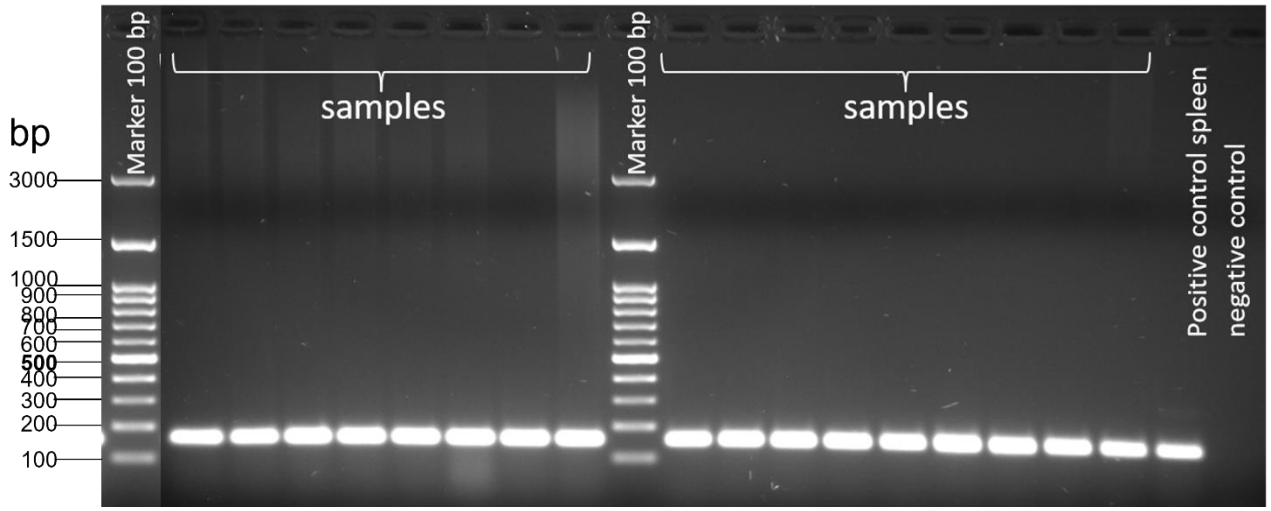


Figure 4: Representative photograph of verification of cDNA synthesis with agarose gel electrophoresis of PCR products showing the *Actb* gene product at the expected size of 156 bp. The DNA ladder has been duplicated and is provided as the first lane to depict the bp of the individual lanes.

3.2.5.4 Quantitative real-time PCR

To finally determine the gene expression patterns of selected immunological markers potentially influenced by activin A, a qRT-PCR was performed employing specifically designed primer pairs (see appendix 7.1.8, designed with NCBI Primer-BLAST) to detect the genes of interest. With the fluorescent dye SYBR Green intercalating in double-stranded DNA, the amplification of the target genes could be quantified. While the primers were designed for 60°C, the ideal annealing temperatures for the primer pairs were verified using a gradient PCR.

The same principle and primers were used for samples obtained at JLU and Monash, however the reagents and Thermal Cyclers used for the reaction differed.

For samples synthesised at JLU, the cDNA was first diluted in RNase/DNase-free water (1:10), added into a 96-well plate in duplicate or triplicate, and then mixed with the qRT-PCR cocktail described in the table below (Table 5), containing a specific primer and amplified using the thermal protocol detailed in Table 6 in a CFX96 thermal cycler. In each run, the reference gene *Actb* (see appendix 7.1.8) was included, as well as positive and negative controls and a melt curve analysis (Fig. 5). For the reaction and analysis, the CFX96 Touch Real-Time PCR Detection System and CFX Manager Software 3.1 was utilised.

Table 5: qRT-PCR mixture used at JLU.

Volume (µl)	Reagent
3	cDNA template (diluted 1:10)
12.5	iQ SYBR green Supermix
1	Primer mix (10 µM each reverse and forward)
8.5	DNase/RNase-free water

Table 6: qRT-PCR program for CFX96 Touch Real-Time PCR Detection System.

Temperature (°C)	Time	
95	30 s	
95	5 s	40 cycles
60	30 s	
65	5 s	
95		
4	∞	

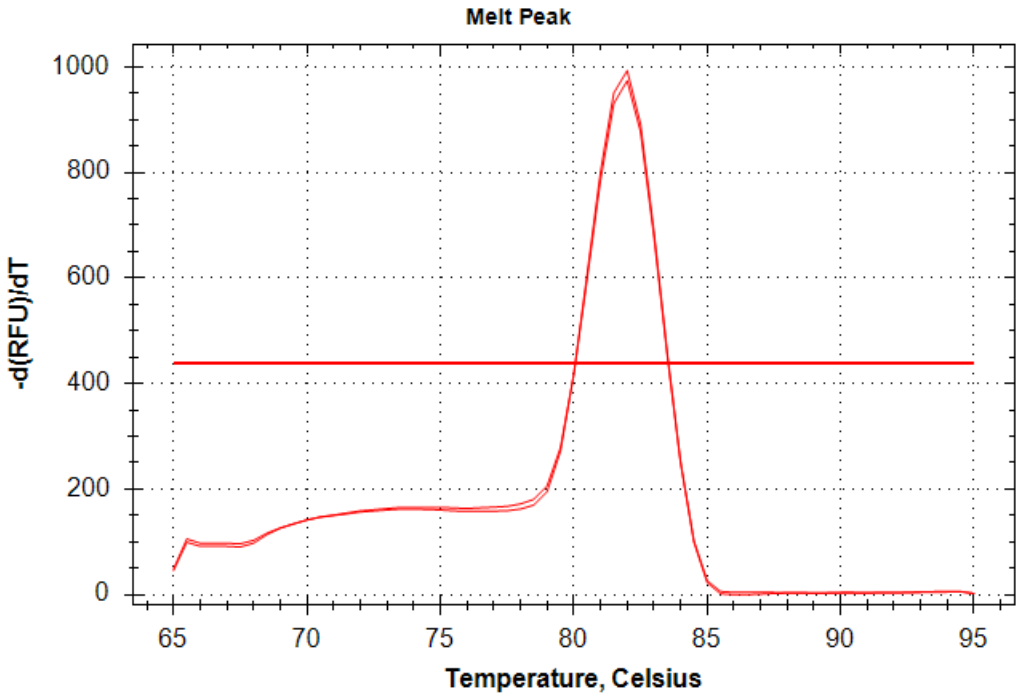


Figure 5: Representative melt curve of *Actb* amplification in qRT-PCR, showing single peak in each reaction of a duplicate sample.

For the analysis of gene expression in the samples obtained at Monash, 1 µl of the synthesised cDNA was used in 10 µl reaction volume and added into 384-well plates. Subsequently, the qRT-PCR mixture detailed in the table below (Table 7) was added to the cDNA and the plate was analysed employing the QuantStudio 6 Flex Real-Time PCR System following the thermal protocol set out in Table 8. With each plate, the reference genes *Actb* and *Rplp0* (see appendix 7.1.8), encoding the ribosomal protein P0, were included, as well as positive and negative controls and a melt curve analysis.

Table 7: qRT-PCR mixture used at Monash.

Volume (µl)	Reagent
1	cDNA template
5	Power SYBR green PCR master mix
0.5	Primer mix (10 µM each forward and reverse)
3.5	DNase/RNase-free water

Table 8: qRT-PCR program for QuantStudio 6 Flex Real-Time PCR System.

Temperature (°C)	Time	
50	2 min	
95	10 min	
95	15 s	40 cycles
60	60 s	
95	15 s	
60	60 s	
95	15 s	

All data was subsequently analysed using the $2^{(-ddCT)}$ method (Schmittgen & Livak, 2008), calculating the expression of genes relative to the amplification of the reference gene, as well as relative changes in expression values compared to the untreated control samples.

3.2.6 Enzyme-linked immunosorbent assay

For a more functional insight into the effects of activin A on immune responses, in addition to the effects on gene expression, the resulting levels of some secreted cytokine proteins were measured in resting and LPS-activated macrophage cultures.

After experimental treatments of the cells, the medium of the macrophage cultures was collected after 3 h, 6 h and 24 h LPS activation, along with control samples. The medium was centrifuged at 2500 rpm and 4°C for 7 min to remove insoluble material and collect the supernatant, which was either used directly or stored at -80°C until further analysis. The ELISAs were performed with the collected supernatant for the pro-inflammatory cytokine TNF and the anti-inflammatory cytokine IL-10, according to manufacturer's protocol. Briefly, 96-well plates were coated with the capture antibody diluted in Coating buffer (see appendix 7.1.9) and incubated at 4°C over night. The plates were then washed with 1X PBS containing 0.05 % Tween-20 (wash buffer) and blocked for 1 h with 10 % heat-inactivated FBS in 1X PBS (assay diluent). Before the incubation with the collected culture supernatant, the LPS-treated samples had to be diluted 1:5, 1:10 or 1:20 with assay diluent for the TNF ELISA to be able to detect the secreted cytokine within the detection range of the assay. For the IL-10 ELISA, the samples could be used undiluted. After washing, 100 µl of the sample supernatants were dispensed in duplicate into the plate coated with the capture-antibody. In each assay a standard curve consisting of a serial dilution of the provided standard with known concentrations was also included. The samples and standard dilutions were incubated for 2 h at room temperature. Following, the plate was washed with the wash buffer and incubated for 1 h with the Working Detector, consisting of the detection antibody linked to horseradish peroxidase. The signal was then developed for 30 min using 3,3',5,5'-tetramethylbenzidine (TMB), which detects the horseradish peroxidase. After stopping the reaction with 2 N H_2SO_4 the signal could be read at 430 nm with a wavelength correction of 570 nm with a plate reader. The concentrations of the cytokines could be determined based on the standard curve.

To normalise the ELISA results to the amount of cells in each experimental well (approximated by total protein concentration), after the supernatant was harvested for ELISA, the macrophages were washed with cold DPBS and lysed in a protein lysis buffer (see appendix 7.1.9) on ice for 30 min with shaking of the plates every 5 min. The lysate was harvested with cell scrapers and lysed further on ice for 30 min while repeatedly vortexing. Any insoluble material was then removed by centrifugation at 14000 rpm for 30 min at 4°C and the supernatant was stored at -80°C until further usage. The concentration of total protein was then measured by employing the colorimetric

bicinchoninic acid (BCA) Protein Assay Kit according to the manufacturer's protocol. Briefly, the protein lysates were diluted 1:3 in the lysis buffer and added into a 96-well plate alongside a dilution series of the provided bovine serum albumin (BSA) protein standard. The Working Reagent of the Kit was added and briefly mixed with the samples on a plate shaker before being incubated for 30 min at 37°C. Following this, developed signal was analysed with a plate reader at 570 nm. The protein concentrations could then be determined based on the BSA standard curve and used to normalise ELISA results.

3.2.7 Griess reagent assay

To further elucidate functional influences of activin A on immune responses in macrophages, nitrite was measured as an indicator of the production of pro-inflammatory NO. Since NO is quickly metabolised, one of its breakdown products, nitrite, is measured in the Griess Reagent System as an estimate for total NO production. To measure nitrite, sulfanilamide is first added to a sample, leading to the formation of a diazonium salt, after which an azo coupling reaction with *N*-1-naphtylethylenediamine (NED) produces a pink-red azo dye.

The assay was performed according to the manufacturer's protocol. For this, the cell culture medium was collected after the experimental treatments, cells and debris were centrifuged down for 7 min at 2500 rpm and 4°C and the supernatant was either used directly or frozen at -80°C. Briefly, after letting the reagents equilibrate to room temperature, 50 µl of experimental samples were dispensed in duplicates or triplicates into a 96-well plate, 50 µl of the Sulfanilamide Solution was added to each sample and to the dilution series of a nitrite standard, which was included in each assay. After incubation at room temperature in the dark for 5-10 min, 50 µl of NED Solution was added to all wells and incubated for another 5-10 min at room temperature in the dark. The developing colour was read with a plate reader at 570 nm within 30 min. The concentration of nitrite in the experimental samples was afterwards determined based on the absorbance of the known concentrations of the standard curve.

3.2.8 Arginase activity assay

The activity of arginase was additionally measured as a functional counterpart to the measurement of nitrite, as arginase and nitric oxide synthase compete for the same substrate, L-arginine. Several studies indicate inhibitory activities of arginase on NO production via several different mechanisms (Durante et al., 2007). Additionally, the anti-inflammatory arginase may regulate pro-inflammatory responses induced by NO, making it especially interesting to look at in macrophages immune responses. Arginase catalyses the conversion of arginine to urea and ornithine. The arginase activity assay utilises a colour development reaction with the resulting urea, which produces a colour signal proportional to the arginase activity in the sample. The arginase activity was measured in lysed protein samples of the macrophages after the respective treatments. The assay was performed according to manufacturer's protocol.

Briefly, after experimental treatment, the cells were washed with DPBS and lysed on ice for 10 min in 100 µl of protein lysis buffer (10 mM Tris-HCL, 0.05X protease inhibitor cocktail, 0.4 % Triton X-100; see appendix 7.1.9). After lysis and harvest of the cells with a cell scraper, the insoluble material was removed by centrifugation for 10 min at 14000 rpm at 4°C.The samples were either used directly or stored at -80°C until further use.

For the assay, 40 µl of samples were added in duplicates into a 96-well plate providing a test well and a blank well. Additionally, the provided urea standard is added to the plate. Following, 10 µl of the Substrate Buffer, consisting of Arginine Buffer and Manganese (Mn) Solution, was added to each of the test wells, except the blank wells, and incubated for 2 h at 37°C in the dark. Afterwards, 200 µl of the Urea Reagent was added to all wells to stop the arginase reaction. Finally, 10 µl of the Substrate Buffer was dispensed into the blank wells and incubated for 60 min at RT. Subsequently, the absorbance could be measured at 430 nm with a plate reader. The activity of the enzyme arginase could then be calculated based on the absorbance values of the urea standard.

3.2.9 Flow cytometry

Flow cytometry is a technique commonly used to measure the characteristics of cells in a population based on specific markers. In fluorescently labelled single cell suspensions, the flow cytometer can detect the cells positive for several markers with lasers, ultimately showing the distribution of cells with the investigated characteristics within populations. In these studies, flow cytometry was utilised to investigate the abundance of cells positive for the commonly used marker for M2 macrophages, CD206, within macrophage and immune cell populations, to gain insight into the effect of the growth factors M-CSF and GM-CSF on bone marrow-derived macrophage differentiation, as well as the effect of activin A on these cells.

To prepare the BMDMs after their respective treatments, the cells were washed twice with DPBS and detached with macrophage detachment solution for 40 min at 4°C and if necessary, longer at room temperature. For collection, 1 % BSA in DPBS (flow buffer) was added, the plate was gently flushed several times and the cell suspension was transferred into a 50 ml falcon tube. After centrifuging the cells at 1500 rpm for 7 min, the pellet was resuspended in DPBS containing 1 % BSA and counted with a hemocytometer using trypan blue staining to exclude dead cells. The cells were then allotted at 5 x 10^5 cells into flow cytometry tubes and centrifuged at 1500 rpm for 7 min at 4°C. After washing the cells again in 1 % BSA in DPBS and repeating the centrifugation step, they were resuspended in 50 µl of the flow buffer containing the Fc-blocker CD32/16 (1:50) to block unspecific binding sites for 10 min at 4°C. In the following step, fluorescently conjugated antibodies chosen for characterising the macrophage population were added to the cell suspension and incubated for 30 min at 4°C in the dark. Specifically, CD45 as a marker for immune cells, F4/80 and CD11b to determine macrophages, and a vital stain, efluor, to exclude dead cells (all 1:50). After the labelling, the cells were washed once with flow buffer and centrifuged at 1500 rpm for 7 min at 4°C. Since CD206 is an intracellular marker, the cells were fixed and permeabilised with a Fixation/Permeabilisation working solution for 30 min at 4°C in the dark. After centrifuging at 1500 rpm for 7 min at 4°C, the cells were washed twice with 1x Permeabilisation buffer and centrifuged at 1500 rpm for 7 min at 4°C. Subsequently, the BMDMs could be stained with CD206 (1:50) in flow buffer and incubated for 30 min at 4°C in the dark. Before resuspension in flow buffer for analysis, the cells were again washed in 1X Permeabilisation buffer and centrifuged at 1500 rpm for 7 min at 4°C. For the flow cytometric analysis of the labelled cells, a MACSQuant Analyzer 10 was utilised, and the subsequent analysis of the obtained data was performed with FlowJo software version 10.

3.2.10 Seahorse real-time cell metabolic analysis (Seahorse analysis)

To investigate whether activin A treatment could potentially lead to changes in the metabolic profile of macrophages, a Seahorse XFp Analyzer was utilised to determine whether the mitochondrial respiration is a target for activin A action.

Seahorse analysis is a tool to measure the metabolic changes in cells, specifically mitochondrial respiration. The oxygen consumption rate (OCR) indicates the usage of the ETC in the mitochondria and, therefore, can inform about the metabolic status of the cells. To analyse the metabolic changes in cells, Seahorse analysis is used to measure the consumption of oxygen and production of protons, which can be used to calculate the OCR, as well as the extracellular acidification rate. These measurements can indicate the metabolic status of the macrophages. Depending on the activation status of macrophages, the mitochondrial activity is reprogrammed (see chapter 1.1.1.2). Classically activated macrophages switch from OXPHOS in the resting state, to glycolysis in the activated state and therefore utilise less oxygen than resting macrophages. Alternatively activated macrophages rely mostly on OXPHOS and even show increased consumption of oxygen compared to resting macrophages (Fig. 6C). The mitochondrial respiration can be determined by measuring the OCR in a three-step protocol, the mitochondrial stress test, which is based on the ETC in the mitochondrial membrane (Fig. 6A, B). The ETC produces ATP via the transport of electrons along several complexes. During this process, oxygen is consumed and reduced to water and H+ protons are pumped into the intermembrane space. The Seahorse XFp Analyzer measures both the flux of oxygen and protons in the medium of cells. Firstly, it measures the basal oxygen consumption of the cells. Several compounds are added via an automated port system during the course of the measurement to test the respiratory capacity of the macrophages. The first compound is oligomycin, which inhibits the ATP synthase in the ETC, so that no new oxygen is needed for ATP production and the OCR indicates how much oxygen is used for basal ATP production. Next, the uncoupling agent carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) is added to the cells, which uncouples the ETC, leading to an increased flux of protons through the membrane showing the maximal oxygen consumption possible as well as the spare capacity of the cells in case of a high energy demand. Lastly, the cells are treated with rotenone (Rot)/antimycin A (AA), which blocks the electron transport via the proton pumping complex I and III. As a result, the OCR drops to a minimum and the oxygen consumption via the ETC in the mitochondria stops. The remaining oxygen consumption that can be observed is due to non-mitochondrial respiration. Furthermore, the difference between the baseline of the curve when treated with Rot compared to the treatment with oligomycin, which

inhibits the ATP synthase, shows the oxygen consumption due to the H+ proton leak across the membrane (Van den Bossche et al., 2015, 2016).

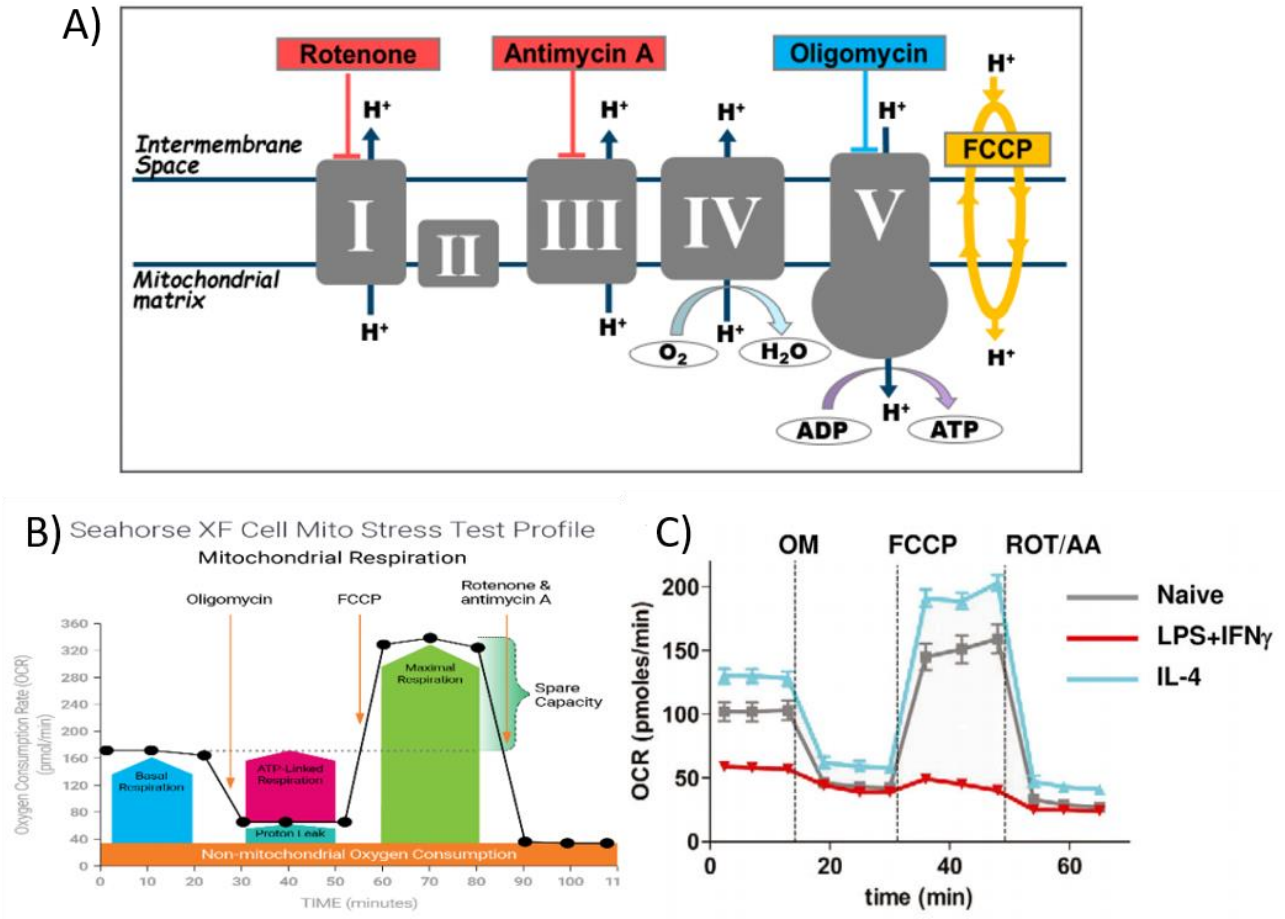


Figure 6: Representation of modulation of Seahorse XFp Cell Mitochondrial Stress Test of (A) the ETC, (B) the typical OCR curve, and (C) OCR changes in unstimulated, LPS- or IL-4-stimulated macrophages. (A) Depiction of oligomycin blocking the ATP synthase, Rotenone blocking complex I and Antimycin A blocking complex III of the ETC and FCCP as uncoupling agent. (B) Depiction of determination of the parameters basal respiration, ATP-linked respiration, proton leak, maximal respiration, spare capacity, and non-mitochondrial oxygen consumption with a mitochondrial stress test employing oligomycin, FCCP and Rot/AA. (C) Representative mitochondrial stress test with classically activated macrophages (red) displaying lower OCR than naïve macrophages (grey), while alternatively activated cells show an increased OCR (blue). ADP: adenosine diphosphate, ATP: adenosine triphosphate, FCCP: carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone, INFγ: interferon gamma, IL-4: interleukin 4, LPS: Lipopolysaccharide, OCR: oxygen consumption rate, OM: oligomycin, ROT/AA: rotenone/antimycin A. (A, B: © Agilent Technologies, Inc. 2019, reproduced with permission, courtesy of Agilent Technologies, Inc., Seahorse XF Cell Mito Stress Test Kit User Guide Kit 103015-100, 2019; C: Van den Bossche et al. 2016; published under Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND 4.0) https://creativecommons.org/licenses/by-nc-nd/4.0/)

To differentiate BMDM for Seahorse analysis, the cells were cultured in non-tissue culture treated square plates with 25 ng/ml M-CSF, with or without 50 ng/ml activin A. On day 3 of the culture, two-thirds of the medium was replaced with fresh medium including the M-CSF and activin A. To

harvest the cells on day 6 for seeding, the plate was rinsed once with cold DPBS and the cells were gently lifted with a cell scraper in DPBS. For the collection of BMDM, the plate was rinsed three times with DPBS and centrifuged at 1600 rpm for 5 min before counting.

The iMAC cell line was cultured and harvested as indicated in 3.2.4. Both cell suspensions were counted with an automated cell counter and a previously determined number of cells was seeded in 6 wells of an 8-well mini-culture plate. The mounts around the wells of the minichamber were filled with 400 µl warm 1 x DPBS and the cells were grown in 130 µl cell culture medium. The cells were incubated at 37°C with 5 % CO_2. After 4h of incubation the cells were treated with either activin A (50 ng/ml) or its solvent control (PBS/FBS/water) and incubated for 18 h. On the next day, the culture medium was replaced with fresh pre-warmed medium, and the cells were treated with activin A or its solvent control again for 30 min prior to the LPS treatment (10 ng/ml for BMDM or 100 ng/ml for iMACs) for 24 h. One day prior to the Seahorse analysis, the sensor cartridges were calibrated by dispensing calibration solution into the wells and placing the cartridges into a non-CO_2 incubator.

On the day of the assay, the cells were washed twice with the assay medium (Seahorse XF base medium containing glucose (10 mM), sodium pyruvate (2 mM) and L-glutamine (2 mM), which then replaced the culture medium. For calibration, the cells were also incubated in a non-CO_2 incubator for 1 h prior to the assay.

Possible bubbles were removed from the sensor cartridge by lifting the lid to ensure ideal measurements. The compounds from either the Mitochondrial Stress Test Kit were dissolved and diluted in assay medium, or individually purchased reagents (kindly gifted by Dr. Daniel Gough) were solubilized in dimethyl sulfoxide (DMSO) and were further diluted in the assay medium. Oligomycin (1-1.5 µM), FCCP (0.5-3.5 µM), and Rot/AA (1 µM) were dispensed into the sensor cartridge, respectively. The loaded cartridge was placed into the Seahorse XFp Analyzer to warm up and calibrate for the assay. After the cell culture plate had been incubated in the absence of CO_2 for 1 h, the plate was transferred to the Seahorse XFp Analyzer, where it was matched with the sensor cartridge. Following this procedure, the mitochondrial stress test assay was started as explained above with automated addition of oligomycin, FCCP and Rot/AA and three measurements per compound producing the typical curve. After the assay, the cells were fixed with ice-cold methanol for 20 min and subsequently stained with 0.05 % crystal violet for 30 min to determine the relative number of cells in each well, washed with water and then dried. For measurement and normalisation of the Seahorse analysis data to cell numbers, the dried staining was solubilised with 10 % acetic acid, transferred into a 96-well plate, and measured with a plate reader at 570 nm.

3.2.11 Statistical analysis

The statistical analysis of the results was performed using GraphPad Prism version 9.1.0 for Windows (GraphPad Software, Inc., San Diego, California USA). For analysing the differences between each treatment group in the qRT-PCR, arginase activity and Seahorse experiments, an ordinary multiple comparisons one-way analysis of variance (ANOVA) comparing means of each column with the mean of every other column and *post-hoc* Tukey's test was conducted. With each ordinary ANOVA, a Brown-Forsythe test for clustered or heteroscedastic residuals was performed to determine equal standard deviations (SDs). Data without equality of means determined by the Brown-Forsythe test was analysed with Brown-Forsythe and Welch ANOVA test with *post-hoc* Dunnett T3 test. The analysis of the ELISA data was performed using the unpaired two-tailed t-test, while the Griess assay was analysed with the one sample t- and Wilcoxon test, comparing the mean of the samples to a hypothetical mean. For all analyses, the statistical significance was defined as $P<0.05$. All data are presented as mean ± standard error of the mean (SEM) unless specified otherwise.

4 Results

4.1 Effect of GM-CSF, M-CSF and activin A on the phenotype of bone marrow-derived macrophages

To assess the role of activin A in modulating immune responses and its potential influence on the immune privilege in the testis, specifically the regulation of the testicular macrophages, BMDM were utilised as a surrogate model to study specific treatment effects.

Growth factors GM-CSF and M-CSF are used to differentiate bone marrow precursor cells into macrophages (Sierra-Filardi et al., 2011). Differentiation with GM-CSF typically skews the macrophage more towards a more heterogeneous pro-inflammatory phenotype, while M-CSF induces anti-inflammatory attributes and leads to a more homogeneous culture (Fleetwood et al., 2007; Hamilton et al., 2014; Na et al., 2016; Sierra-Filardi et al., 2011). The phenotype of GM-CSF- as well as M-CSF-derived BMDM was analysed by flow cytometry. After differentiation with either growth factor for 7 days, the cells were harvested and labelled with antibodies against CD45 as a marker for immune cells, F4/80 and CD11b for detection of macrophages, and CD206 as a marker for the M2 phenotype. Living cells were determined by addition of the viability marker, efluor450.

The gating strategy to distinguish the amount of CD206+ cells within the viable macrophage population is described in Fig. 7. For the demonstration of the gating strategy, GM-CSF derived BMDM were chosen.

After the removal of the debris with the forward- and side-scatter, single cells and subsequently efluor450-negative viable cells were chosen, showing a percentage of 87.2 % living cells. Within the viable population, 99.6 % of the cells were CD45+ immune cells. To further extract the macrophage population within the immune cells, F4/80+CD11b+ cells were gated with 86.1 % positive cells, showing a successful differentiation of the bone marrow precursor cells into macrophages. Lastly, within the macrophage population, the CD206+ cells were gated, which constitute the M2 macrophages and make up 64.2 % in this population.

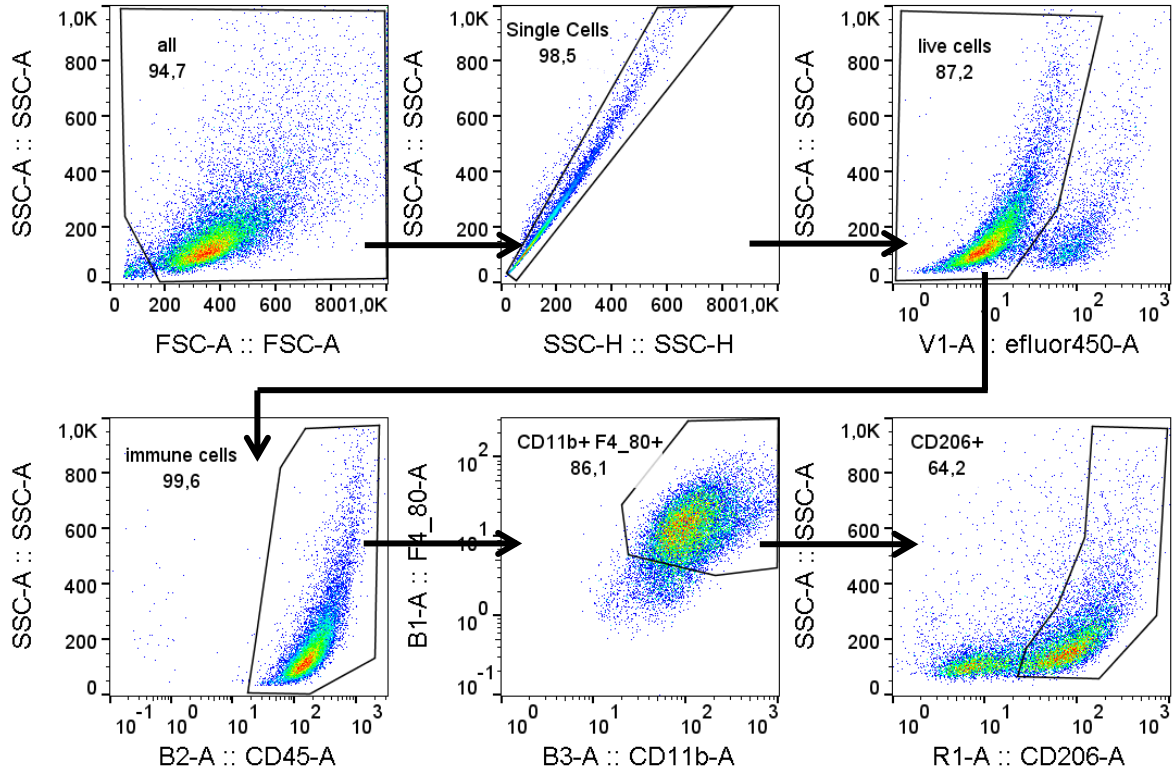


Figure 7: Gating strategy of GM-CSF-differentiated BMDM after flow cytometry with markers for macrophages. BMDM were differentiated with GM-CSF (25 ng/ml) for 7 days and then analysed by flow cytometry. After removal of the debris via gating of all cells of interest, single cells and live cells were chosen. Following this procedure, CD45+ immune cells were gated, including CD11b+F4/80+ macrophages. Within the macrophages 64.2 % of CD206+ macrophages were gated. Representative data of one experiment.

To compare the effects of the different growth factors on the phenotype of macrophages, BMDM differentiated with either GM-CSF or M-CSF were analysed according to the gating strategy illustrated in Fig. 7 and the F4/80+CD11b+ macrophage populations as well as the prevalence of the M2 phenotype determined by CD206+, were investigated (Fig. 8).

When comparing the F4/80+CD11b+ populations, indicating the macrophages within the immune cells, GM-CSF-derived BMDM were comprised of 58.3 % in this culture, while M-CSF-derived BMDM showed a much higher abundance of F4/80+CD11b+ macrophages at 97.8 %. As previously indicated, differentiation with GM-CSF can lead to a more heterogeneous population of immune cells than with M-CSF (Na et al., 2016), however, other GM-CSF-derived BMDM cultures showed a much higher percentage of F4/80+CD11b+ macrophages, for example 86.1 % in Fig. 7. As expected, the differentiation with M-CSF led to a much higher population of CD206+ macrophages at 89.2 % compared with 44.6 % in GM-CSF-derived BMDM, indicating a population skewed more towards the M2 phenotype.

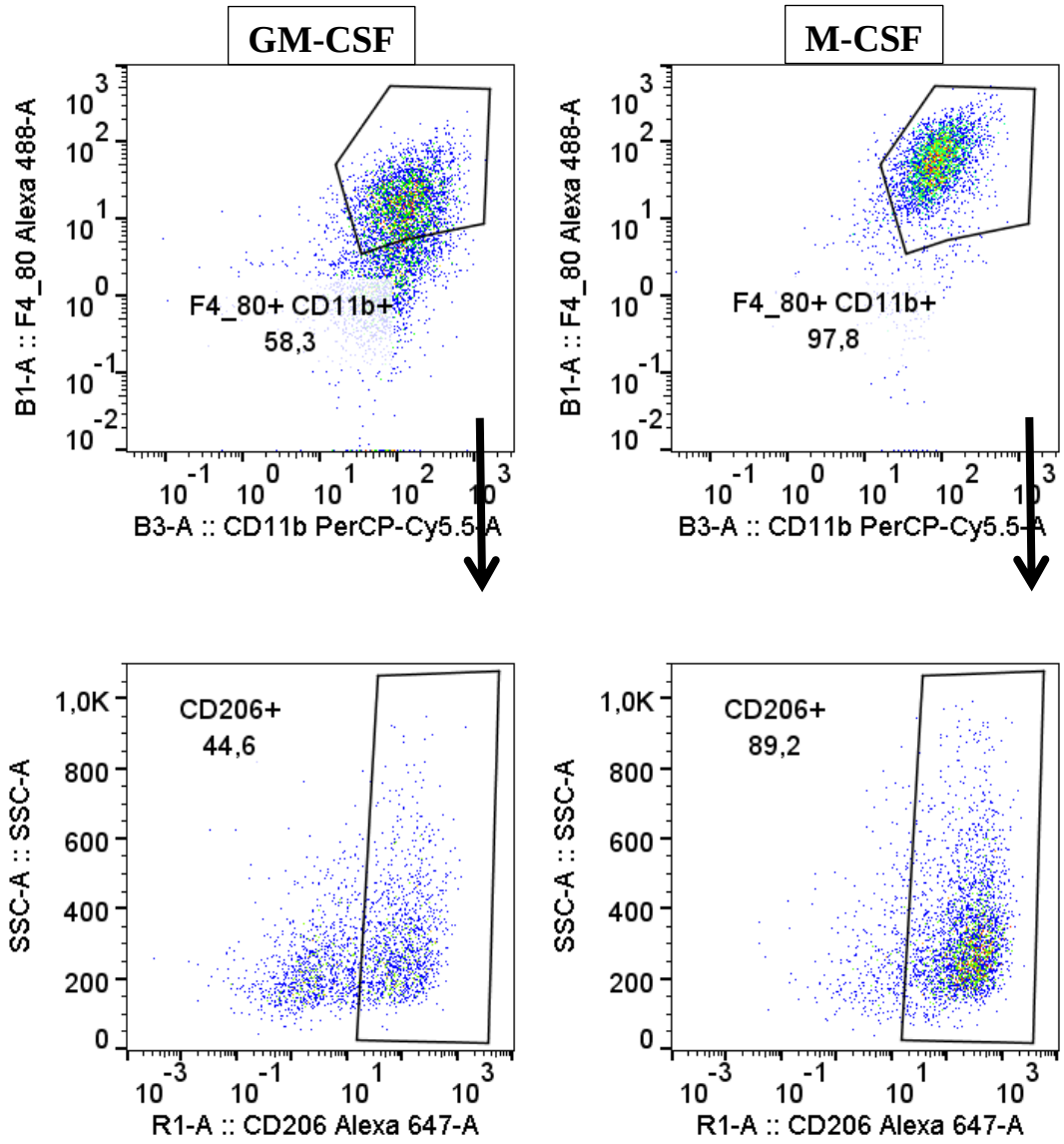


Figure 8: Flow cytometric analysis of the effects of GM-CSF and M-CSF on the phenotype of BMDM. Cells were cultured with either GM-CSF (25 ng/ml) or M-CSF (25 ng/ml) for 7 days and subsequently analysed by flow cytometry utilising the following antibodies: CD45, F4/80, CD11b, CD206 and a viability dye. The cell populations were analysed according to the gating strategy outlined in Fig. 7. Representative data of one experiment.

The effects of activin A on the anti-inflammatory phenotype of the macrophages was investigated by flow cytometry in GM-CSF-derived BMDM (Fig. 9). This approach was chosen based on previously unpublished experiments within our laboratory indicating an increase of CD206+ M2 macrophages following activin A treatment, within the population of GM-CSF derived BMDM, which typically display more characteristics of M1 skewed macrophages.

The cells were treated with or without activin A during the differentiation with GM-CSF from bone marrow precursor cells into BMDM. After 7 days, the influence of activin A on the cell populations

was investigated by flow cytometry and the cells were analysed (Fig. 9) according to the gating strategy outlined in Fig. 7. When comparing the F4/80+CD11b+ cell populations in untreated and activin A-treated BMDM, there appeared to be an increase in the population from 83 % in untreated cells to 92.8 % in activin A stimulated macrophages (Fig. 9). The proportion of F4/80+CD11b+ macrophages following activin A treatment also appeared denser and therefore, potentially more homogeneous than in the untreated cells.

As described in the previous experiments (Figs. 7, 8), the GM-CSF derived BMDM typically display characteristics of M1 skewed macrophages, with relatively low M2 CD206+ cells within the F4/80+CD11b+ macrophage population of 62.4 % in the untreated cultures (Fig. 9). With the activin A treatment of the GM-CSF-derived BMDM, however, the M2 CD206+ population could be increased to 86.2 %, indicating that the macrophages were skewed from the M1 phenotype towards an M2 phenotype by activin A treatment (Fig. 9).

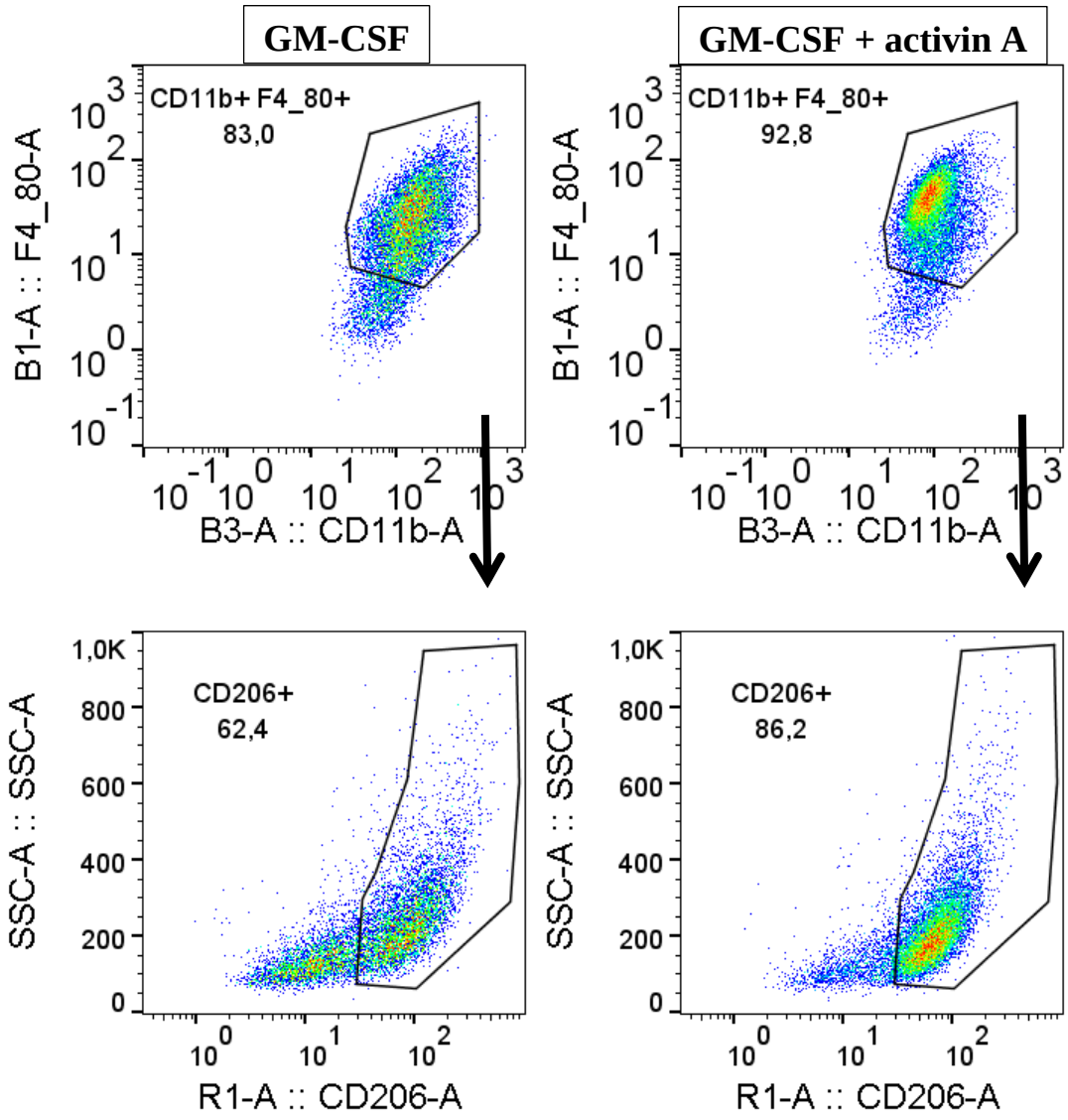


Figure 9: Influence of recombinant activin A on GM-CSF-derived BMDM. Analysis of CD206+ cells within the CD11b+F4/80+ macrophage population after 7-day culture with either GM-CSF (25 ng/ml) (left) or GM-CSF with activin A (50 ng/ml) (right) and subsequent analysis by flow cytometry. Representative data of one experiment.

When repeating the experiment and statistically analysing the influence of activin A on GM-CSF-derived BMDM, a slight relative increase in CD206+ cells was observed compared to the control, although the difference was not statistically significant (Fig. 10).

The flow cytometry results indicate that M-CSF-derived BMDM display a more M2-phenotype with higher levels of CD206+ cells compared to GM-CSF-derived macrophages, as has previously been reported, while the treatment with activin A may potentially show a tendency of increasing the proportion of CD206+ cells in GM-CSF-derived BMDM, which are typically associated with more

M1 characteristics. Further investigation would be needed to resolve this possibility requiring increased replicate numbers due to high variation.

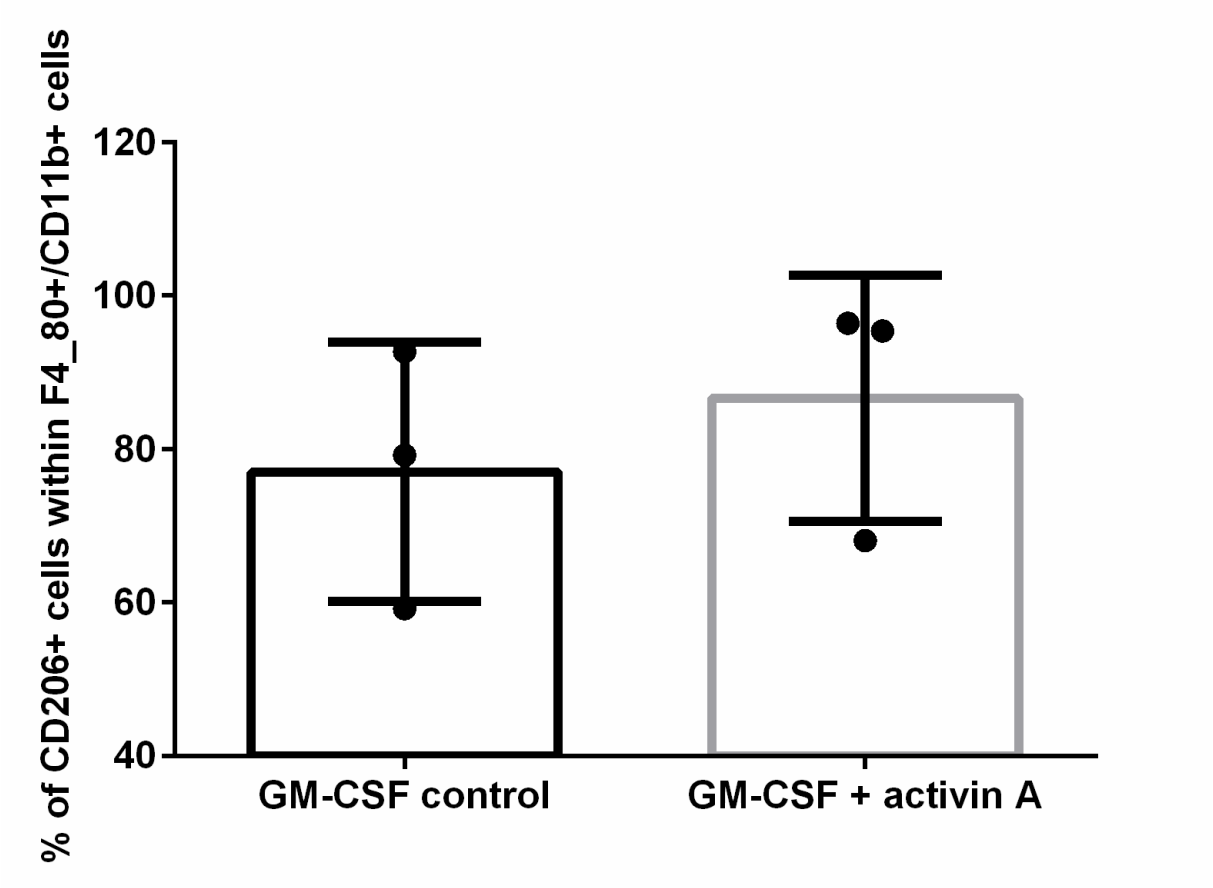


Figure 10: Influence of recombinant activin A on the CD206+ cell population in GM-CSF-derived BMDM. Quantification of CD206+ cells within the CD11b+F4/80+ macrophages after BMDM were treated for 7 days with GM-CSF (25 ng/ml) or GM-CSF with activin A (50 ng/ml) and subsequently analysed by flow cytometry. Analysed by unpaired two-tailed t-test. Results are expressed as mean ± SD. N=3 separate experiments, individual data points represent biologically independent replicates.

4.2 Effects of activin A on macrophages: comparison of murine cell lines and primary bone marrow-derived macrophages

Previous reports have shown differential influences of activin A on immune responses in different cell types, including primary cells or cell lines. Activin A seemingly has pro- and anti-inflammatory properties, depending on the context.

To investigate how activin A influences the immune responses in commonly used *in vitro* models, effects on murine macrophages derived from primary cells and two cell lines were compared. Primary BMDM, RAW macrophages and iMACs were treated with activin A in a resting state or following activation by LPS. Subsequently, the effect of activin A on gene expression, protein secretion and on changes to immunometabolism was analysed to gain insight on possible modulatory effects of activin A on macrophage function.

4.2.1 Comparison of gene expression patterns by primary macrophages and cell lines in response to activin A measured by qRT-PCR

To determine the different effects activin A may have on the immune response of macrophages of different origin, the gene expression of immunologically relevant genes was analysed after BMDM, RAW cells or iMACs were treated with activin A in the resting state or prior to activation with LPS. After 3 h, 6 h or 24 h LPS treatment, total RNA was harvested and the expression of *Tnf, Il10, Arg1, Fpr2, Gpr18, Il6, Il1β, Mrc1, Chil3/Ym1, Slamf1, Clec7a, Casp1, Klf4, Atf3, Akt1, Akt3, Pik3cd, Cd86,* and *Ccl2* was determined by qRT-PCR, in relation to the reference genes *Actb* (JLU) or both *Actb* and *Rplp0* (Monash) (primers: see appendix 7.1.8). The stability of the employed reference genes was ensured by comparing their expression in different treatments (appendix, Fig. 41) and the primers were validated by standard curves (appendix, Fig. 42). The results are shown as expression relative to untreated control samples. Several genes were investigated in the laboratory at JLU, but not at Monash, and therefore expression levels between BMDM G (BMDM cultured in Germany at JLU) and RAW macrophages (JLU), but not BMDM A (BMDM cultured in Australia at Monash) and iMACs (Monash), were compared for *Gpr18*, *Mrc1*, *Chil3/Ym1*, *Atf3*, *Akt1*, and *Pik3cd*.

The influence of activin A on the immune response of primary BMDM was investigated at different maturation states of the macrophages. The cells were either treated with activin A (50 ng/ml) from the time of isolation for 7 days (BMDM G), or the 7 day-treatment was compared to an activin A

treatment after the differentiation with M-CSF (25 ng/ml) for 18 h prior to the LPS (10 ng/ml) activation (BMDM A), similarly to the protocol used in the cell line macrophages. Both RAW macrophages and iMACs were treated with activin A for 18 h and then activated with LPS for 3 h, 6 h and 24 h. Different concentrations of activin A were tested in resting as well as activated iMACs, indicating a dose-dependent effect on the expression of the investigated genes, revealing 50 ng/ml as an appropriate effective concentration within the tested range (appendix Figs. 39, 40).

4.2.1.1 Effects of activin A on the expression of genes associated with pro-inflammatory responses, T cell activation and proliferation

To elucidate which parts of the immune response are influenced by activin A, the expression of different immunomodulatory genes was compared between the cell types in quiescent and activated macrophages at different time points.

In order to determine the effect of activin A on genes that are associated with pro-inflammatory responses, T cell activation and proliferation the relative expression of *Tnf*, *Il1β*, *Fpr2*, *Nos2*, *Il6* and *Gpr18* was analysed (Figs. 11-16).

The stimulation with LPS to activate the macrophages significantly increased the expression of the pro-inflammatory gene *Tnf*, inducing the activated phenotype of the macrophages at all time points in all cell types tested (Fig. 11A-D). A significant influence of activin A on the expression of *Tnf* compared with the basal levels was not observed in BMDM G, BMDM A or iMACs (Fig. 11A, B, D); however, in RAW macrophages, activin A significantly suppressed the expression of *Tnf* after 24h (Fig. 11C). The 7-day treatment of activin A significantly increased the expression of the pro-inflammatory gene *Tnf* in LPS-activated cells in both BMDM G (Fig. 11A) as well as BMDM A, whereas the 18h treatment prior to LPS activation did not significantly change the relative expression (Fig. 11B). Conversely, in both cell lines tested, activin A treatment led to a decrease in relative gene expression of *Tnf* in LPS-activated macrophages after 6 h and 24 h (Fig. 11C, D). Notably, the expression of *Tnf* in the LPS-activated BMDM appeared to increase over the time course from 3 h to 6 h (Fig. 11A, B), whereas in the cell line macrophages, the expression appears to be declining over time from 3 h, 6 h to 24 h (Fig. 11C, D).

The observations indicated that activin A treatment had opposing effects on *Tnf* expression in LPS-activated primary BMDM compared to cell lines and did not seem to show an effect when given 18 h prior to the activation with LPS in BMDM.

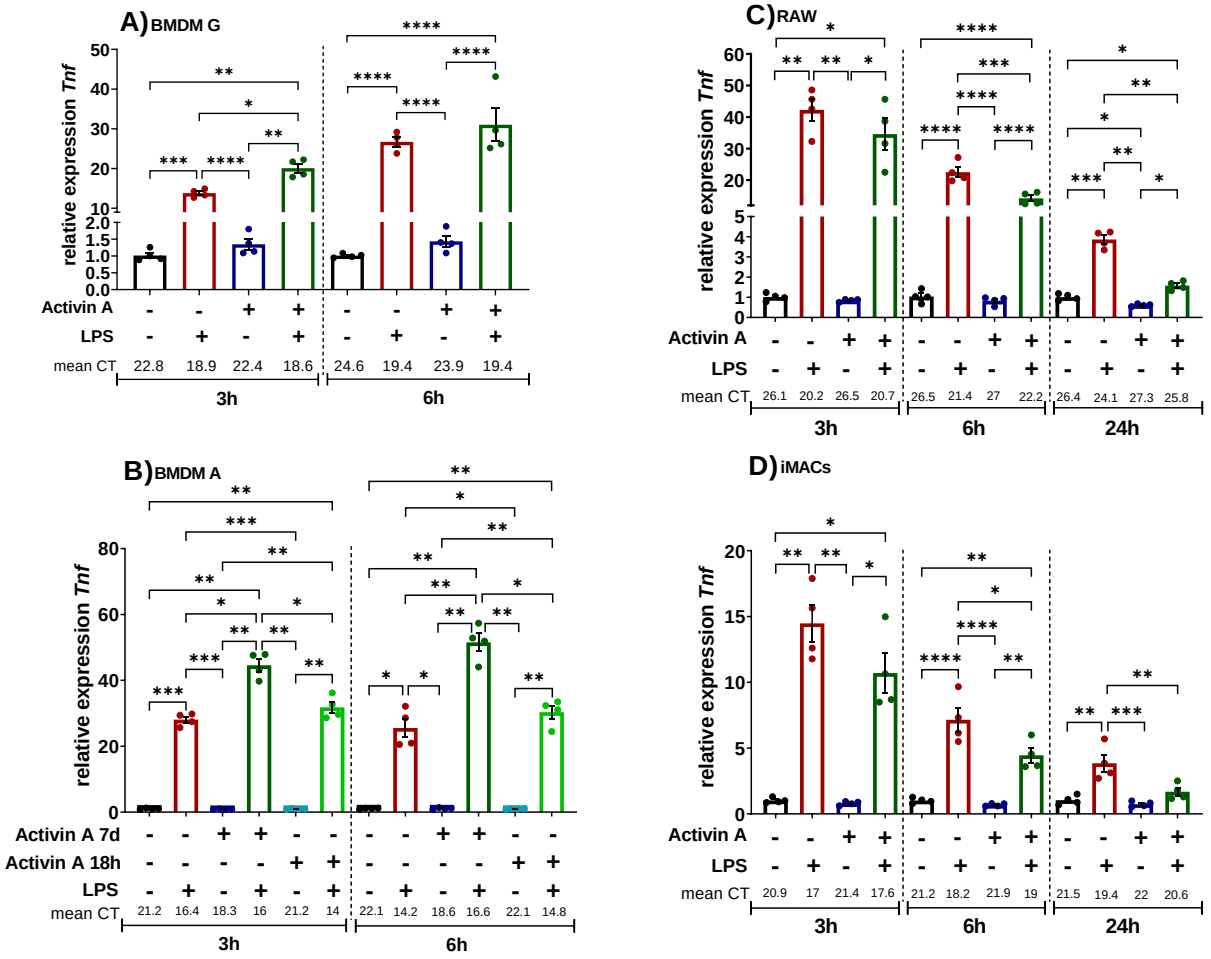


Figure 11: Regulation of mRNA expression of *Tnf* by activin A in macrophages. Relative mRNA expression in M-CSF-induced BMDM after activin A (50 ng/ml) and subsequent 3 h or 6 h activin A plus LPS (10 ng/ml) treatment, collected at (A) JLU and (B) Monash, compared to (C) RAW macrophages after activin A (50 ng/ml) and subsequent 3 h, 6 h or 24 h activin A plus LPS (10 ng/ml) treatment and (D) iMACs after activin A (50 ng/ml) and subsequent 3 h, 6 h or 24 h activin A plus LPS (10 ng/ml) treatment relative to untreated controls. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. Results are expressed as mean ± SEM. N=3-5 separate cultures denoted by individual data points.

The relative expression of the pro-inflammatory cytokine *Il1β* was upregulated by LPS activation of the macrophages in all cell lines tested and at all time points investigated (Fig. 12A-D).

Similar to the trend observed for the regulation of *Tnf* expression (Fig. 11), *Il1β* expression in LPS-activated BMDM was increasing over the time course of 3 h and 6 h (Fig. 12A, B), whereas it declined in RAW macrophages (Fig. 12C). However, in the iMACs, the expression increased from 3 h to 6 h (Fig. 12D), similarly to BMDM, and was then reduced at 24 h.

When investigating basal expression of *Il1β*, the 7-day treatment of activin A significantly increased the expression compared to the untreated control after 3 h in BMDM G (Fig. 12A). When repeating

the experiment with BMDM A, in order to compare the 7-day activin A treatment with a 18 h treatment prior to LPS activation, the same effect of the long-term activin A treatment was not observed (Fig. 12B). However, even though not significantly increased, there appeared to be a tendency for the long-term activin A treatment to increase the expression in LPS-activated BMDM. Similar to the expression of *Tnf* (Fig. 11), the 18 h treatment did not appear to change the expression of *Il1β* compared with the LPS-activated cells (Fig. 12B). Conversely, in both cell line macrophages, the activin A treatment led to a suppressed expression of *Il1β* in the LPS-activated cells, after 6 h and 24 h in RAW macrophages (Fig. 12C), and after 3 h in iMACs (Fig. 12D). The overall pattern of the *Il1β* transcript regulated by activin A appeared to be similar to the responses observed for *Tnf* (Fig. 11), although no significant changes of *Il1β* expression in the LPS-activated BMDM were found.

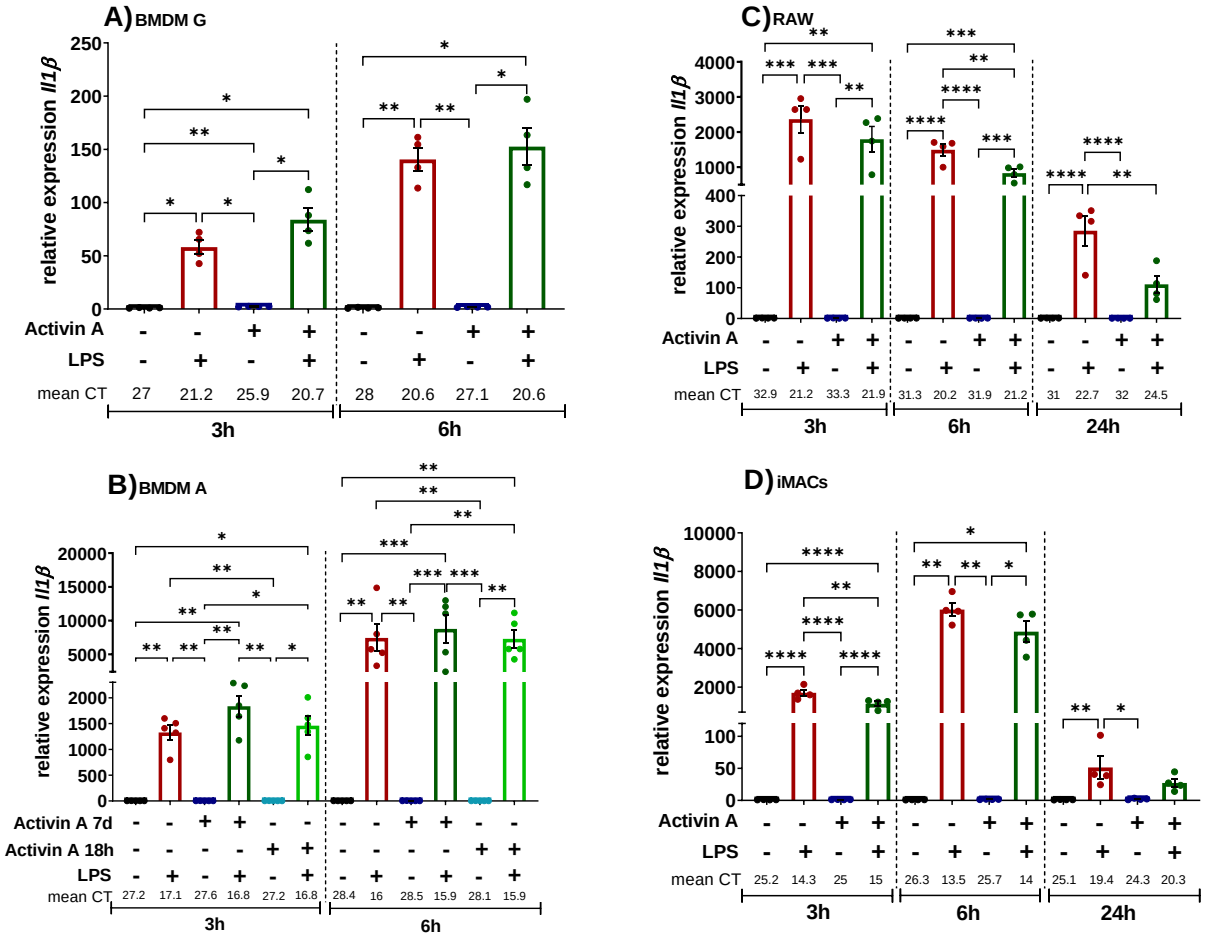


Figure 12: Regulation of mRNA expression of *Il1β* by activin A in macrophages. Refer to legend of Figure 11 for details of experiment. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=3-5 separate cultures denoted by individual data points.

The gene expression of the pro-inflammatory marker *Fpr2* was elevated by the long-term treatment with activin A compared to basal expression, after a 6 h treatment in BMDM G (Fig. 13A), however, this effect was not reproduced in BMDM A, where no significant difference was observed (Fig. 13B). The activation of BMDM with LPS successfully increased the relative expression of *Fpr2*, but no significant changes were found with additional long-term or 18 h activin A treatment in BMDM (Fig. 13A, B). In both cell lines tested, the treatment with activin A in LPS-activated macrophages led to a significant reduction of the relative expression of *Fpr2* after 3h (Fig. 13C, D). Additionally, in the iMACs, activin A treatment significantly decreased the expression of *Fpr2* compared to basal expression in untreated cells after 3 h and 6 h (Fig. 13D). These results appeared similar to the previously shown effects of activin A on the other pro-inflammatory genes (Figs. 11, 12), where a reduction of the gene expression was detected in the cell line macrophages.

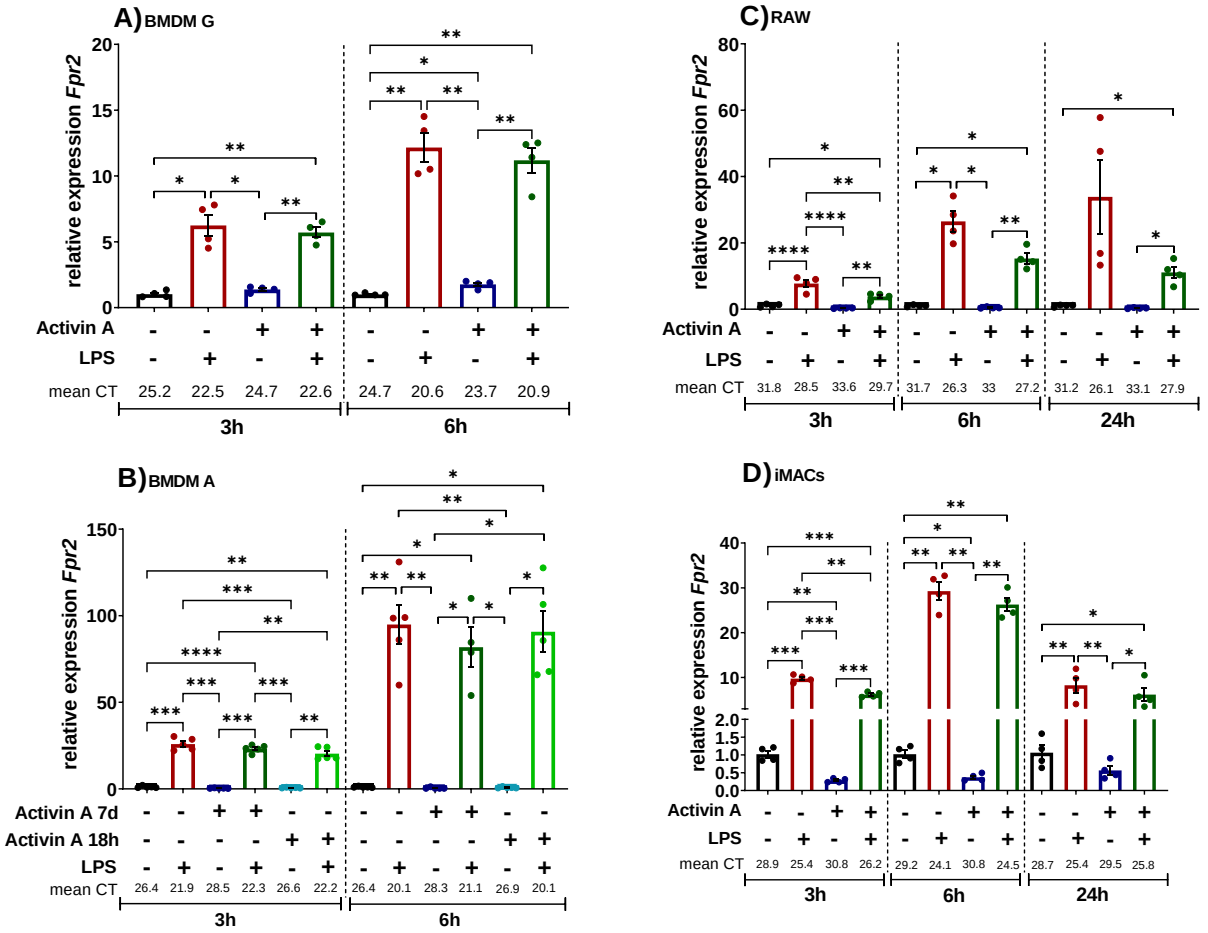


Figure 13: Regulation of mRNA expression of *Fpr2* by activin A in macrophages. Refer to legend of Figure 11 for details of experiment. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=3-5 separate cultures denoted by individual data points.

Similar to the previously analysed markers, LPS was able to increase the gene expression of *Il6* in both BMDM as well as RAW macrophages (Fig. 14A, B). The long-term treatment with activin A did not significantly change the expression of *Il6* in LPS-activated BMDM or basal expression levels (Fig. 14A). In RAW macrophages, however, activin A treatment did significantly suppress the expression of *Il6* in LPS-activated macrophages after 24 h treatment (Fig. 14B). Although not significant, there was an apparent reduction in relative *Il6* expression levels by activin A treatment after 3 h and 6 h in LPS-activated RAW macrophages (Fig. 14B). These observations appeared similar to the inhibiting effect of activin A in cell lines on the previously investigated pro-inflammatory genes (Figs. 11-13).

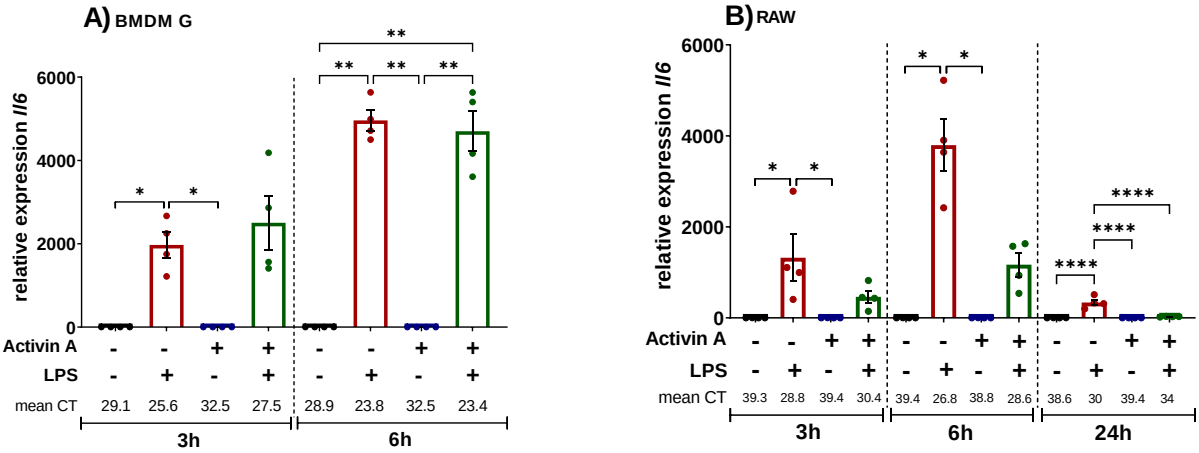


Figure 14: Regulation of mRNA expression of *Il6* by activin A in macrophages. Relative mRNA expression in (A) M-CSF-induced BMDM after activin A (50 ng/ml) and subsequent 3 h or 6 h activin A plus LPS (10 ng/ml) treatment, compared to (B) RAW macrophages after activin A (50 ng/ml), and subsequent 3 h, 6 h or 24 h activin A plus LPS (10 ng/ml) treatment compared to untreated controls. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. Results are expressed as mean ± SEM. N=4 separate cultures denoted by individual data points.

In BMDM, activin A treatment did not change the expression of *Nos2* compared with basal levels; however, treatment with activin A for 7 days prior to the LPS activation significantly increased the expression of *Nos2* after 6 h (Fig. 15A). The opposite effect was detectable in RAW macrophages, where activin A treatment of LPS-activated macrophages led to a decrease in the expression of *Nos2* after 24 h (Fig. 15B). This effect was similar to the observed influence of activin A on increasing the gene expression of markers associated with pro-inflammatory responses, T cell activation and proliferation in BMDM, but decreasing their relative expression in cell line macrophages.

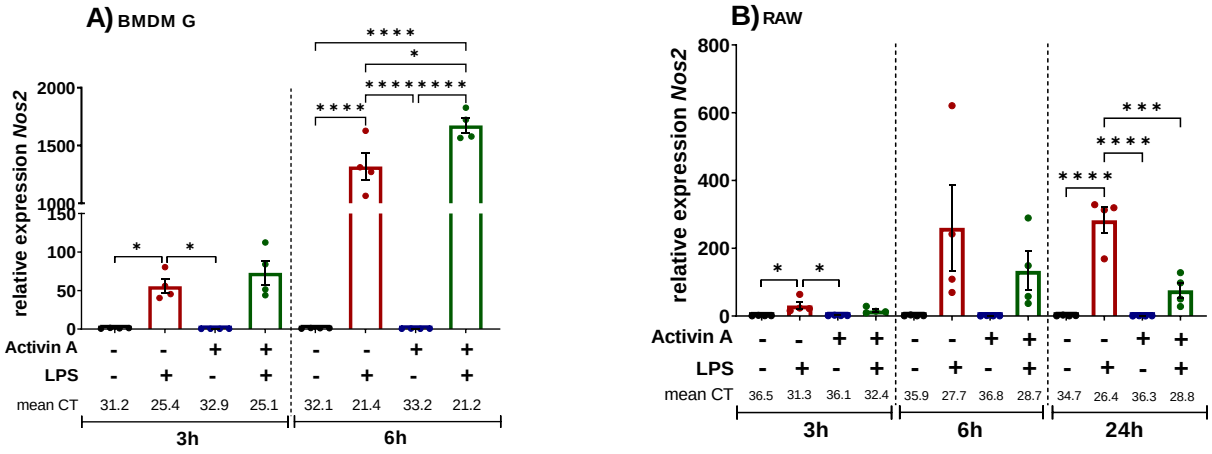


Figure 15: Regulation of mRNA expression of *Nos2* by activin A in macrophages. Refer to legend of Figure 14 for details of experiment. ****P<0.0001, ***P<0.001, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=4 separate cultures denoted by individual data points.

The relative expression of the pro-inflammatory marker *Gpr18* was significantly induced by LPS activation in both BMDM G and RAW macrophages (Fig. 16A, B). The long-term treatment with activin A significantly increased the relative expression of *Gpr18*, both compared with basal expression and in LPS-activated BMDM after 3 h (Fig. 16A), similar to the regulation seen for the previously studied pro-inflammatory markers. Correspondingly, activin A showed the opposite effect in RAW macrophages and significantly decreased the gene expression of *Gpr18* in LPS-activated macrophages after 24 h (Fig. 16B). In RAW macrophages activin A treatment did not significantly affect the gene expression compared to basal levels.

Taken together, when investigating the effect of activin A on the expression of genes associated with pro-inflammatory responses, T cell activation, and proliferation, activin A appears to regulate primary BMDM in a different manner to the cell line macrophages. Specifically, in BMDM the investigated genes were upregulated by activin A treatment, increasing the inflammatory response, whereas in cell line macrophages, activin A decreased the expression levels. Furthermore, the influences observed in BMDM were only detectable in the long-term activin A treatment, but not in the 18 h activin A treatment; however, the effects were not reversed by the duration of the treatment.

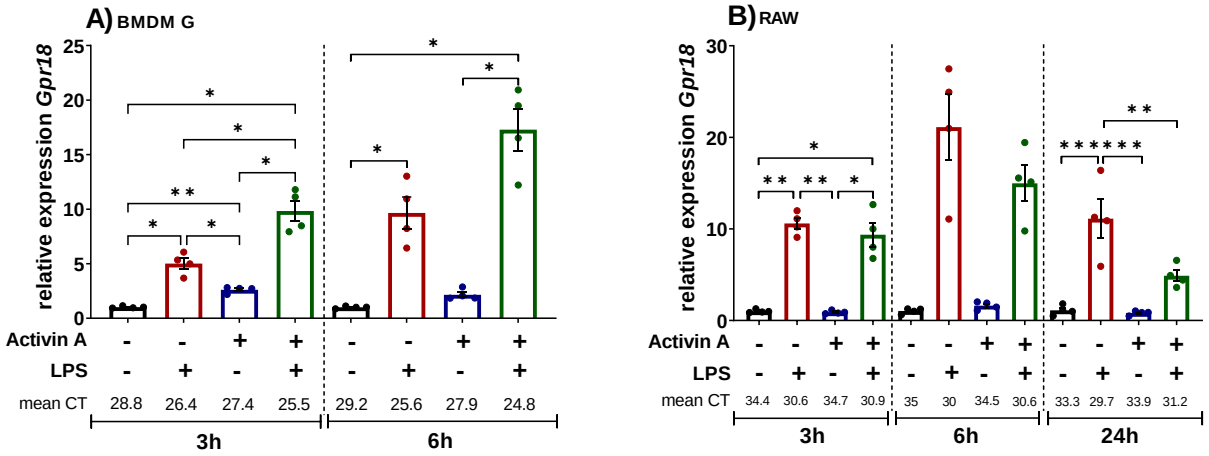


Figure 16: Regulation of mRNA expression of *Gpr18* by activin A in macrophages. Refer to legend of Figure 14 for details of experiment. ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=4 separate cultures denoted by individual data points.

4.2.1.2 Effects of activin A on the expression of genes associated with anti-inflammatory responses and immunoregulation

To gain more insight into the roles of activin A in regulating specific immune responses, a set of genes associated with anti-inflammatory and immunoregulatory responses was investigated (*Arg1*, *Il10*, *Klf4*, *Atf3*) in BMDM and cell line macrophages.

The treatment with activin A significantly increased the expression of the major anti-inflammatory marker *Arg1* in all cell types tested and in both the long-term as well as the 18h activin A treatment in BMDM (Fig. 17). In BMDM G, the significant increase of *Arg1* expression was detectable after both 3 h and 6 h (Fig. 17A). In RAW macrophages the increase was observed after 3 h (Fig. 17C) and in iMACs significantly elevated *Arg1* expression could be shown after all time points, 3 h, 6 h and 24 h (Fig. 17D). In the BMDM A data set, the significant induction of *Arg1* seen in long-term treatment with activin A in BMDM G could not be confirmed (Fig. 17B), however, a tendency towards an increase could be observed. The shorter 18 h activin A treatment significantly induced the expression of *Arg1* after 3 h and also showed a similar tendency after 6 h.

Following activation of the primary BMDM G with LPS, the expression of *Arg1* was significantly reduced after 6 h. However, this effect was not reproduced in the BMDM A study, although there appeared to be a trend towards a reduction of the gene expression after 3 h. Compared with a slight reduction of *Arg1* expression by LPS stimulation in BMDM, in the cell line macrophages, an increase of *Arg1* expression following LPS treatment was observed (Fig. 17C, D). In RAW macrophages, this

tendency appeared to be most prominent after 24 h; however, due to high variance, the response did not reach significance. In iMACs, similar to RAW macrophages, a significantly increased expression of *Arg1* was observed after 24 h LPS treatment. Activin A treatment of the LPS-stimulated macrophages led to an increase of the *Arg1* transcript in all cell types tested. In BMDM A, however, no significant increase was detected for the long-term activin A treatment, but was for the 18 h activin A treatment after 6 h of LPS stimulation (Fig. 17B). Nonetheless, in BMDM G the long-term treatment with activin A led to a significant elevation of *Arg1* expression after both 3 h and 6 h (Fig. 17A). In RAW macrophages, significantly induced *Arg1* expression was observed after 3 h (Fig. 17C) and in iMACs after 3 h and after 6 h (Fig. 17D).

In contrast to the observation of the opposing effect of activin A in BMDM and cell line macrophages within the pro-inflammatory gene set (Figs. 11-16), the anti-inflammatory marker *Arg1* appeared to be similarly upregulated by activin A treatment among all the cell types investigated. The response to LPS activation seems to be different between cell types, with LPS leading to *Arg1* reduction in BMDM G but to an increase in the cell lines after 24 h.

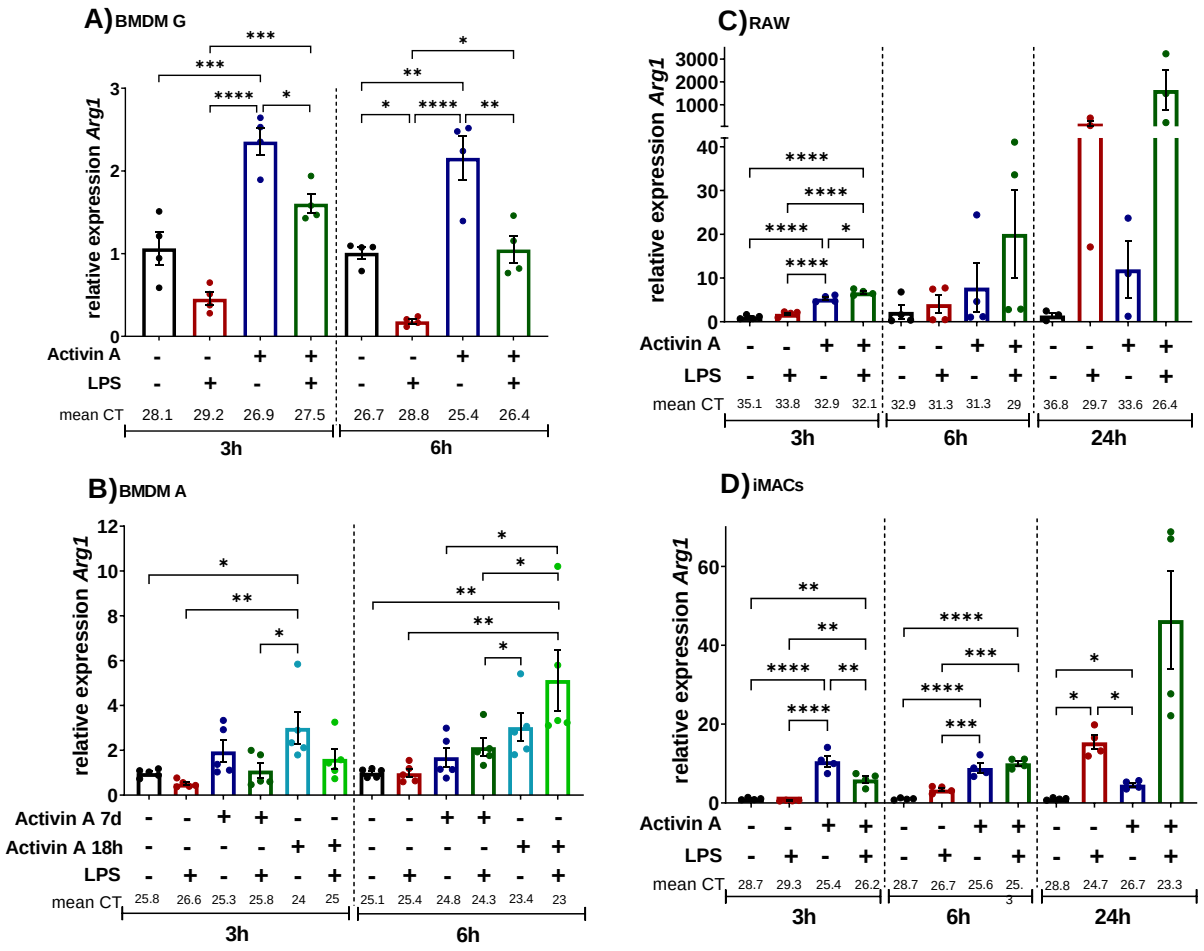


Figure 17: Regulation of mRNA expression of *Arg1* by activin A in macrophages. Refer to legend of Figure 11 for details of experiment. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=3-5 separate cultures denoted by individual data points.

As another classic anti-inflammatory marker, the expression of *Il10* was investigated in resting as well as activated macrophages (Fig. 18). In contrast to *Arg1*, the expression of *Il10* was significantly upregulated by stimulation with LPS in BMDM G and BMDM A after both 3 h and 6 h (Fig. 18A, B). A similar result was observed in iMACs, where LPS stimulation led to elevated *Il10* levels at every time point, most prominently after 3 h stimulation (Fig. 18D). In contrast, only a slight increase was observed in RAW macrophages after 6 h and 24 h, with no change after 3 h (Fig. 18C). Notably, in RAW macrophages the mean detected cycle threshold (CT) values were above 31.9 in each treatment group, indicating very low transcript numbers of *Il10*. In comparison, the CT values for iMACs range between 24.1 (LPS) and 27.7 (activin A) and in BMDM between 19.8 (LPS) and 32.5 (activin A).

The long-term treatment with activin A significantly reduced the expression of *Il10* in BMDM G after 6 h compared with basal expression levels (Fig. 18A). This effect was replicated in BMDM A and

was observed after both, 3 h and 6 h (Fig. 18B). Similar to the effects of long-term activin A treatment, the 18 h treatment reduced the expression of activin A in BMDM compared with basal levels. Likewise, the activin A treatment in iMACs significantly decreased the expression of *Il10* compared with the controls at all time points, 3 h, 6 h, and 24 h (Fig. 18D). In RAW macrophages, no significant reduction of *Il10* transcript after activin A treatment was observed.

When combining LPS stimulation with the activin A treatment, a significant reduction in the expression of *Il10* was detected following long-term treatment in BMDM G after 3 h, and in BMDM A after both 3 h and 6 h (Fig. 18A, B). Although there appeared to be a tendency of the 18 h activin A treatment to reduce the expression of *Il10* in LPS-stimulated BMDM A, it was not statistically significant (Fig. 18B). Remarkably, at the 6 h time point in BMDM G, the long-term activin A treatment appeared to slightly increase the expression of *Il10* in LPS-activated macrophages, although this was not significant (Fig. 18A). Activin A treatment showed a similar effect on the LPS-stimulated iMACs, where a significant reduction of *Il10* expression could be observed after all time points, 3 h, 6 h and 24 h (Fig. 18D). Despite the low expression values in RAW macrophages, a significant reduction of *Il10* levels was detected in activin A-treated, LPS-stimulated macrophages after 24 h (Fig. 18C).

In contrast to the anti-inflammatory marker *Arg1*, the transcript levels of the immunoregulatory gene *Il10* appeared to be inhibited by activin A in both resting, as well as LPS-activated macrophages.

While the markers investigated in the pro-inflammatory gene set appear to be oppositely regulated by activin A between primary BMDM and cell line macrophages, the modulation of both *Arg1* and *Il10* by activin A seems to be towards a similar direction among the cell types tested.

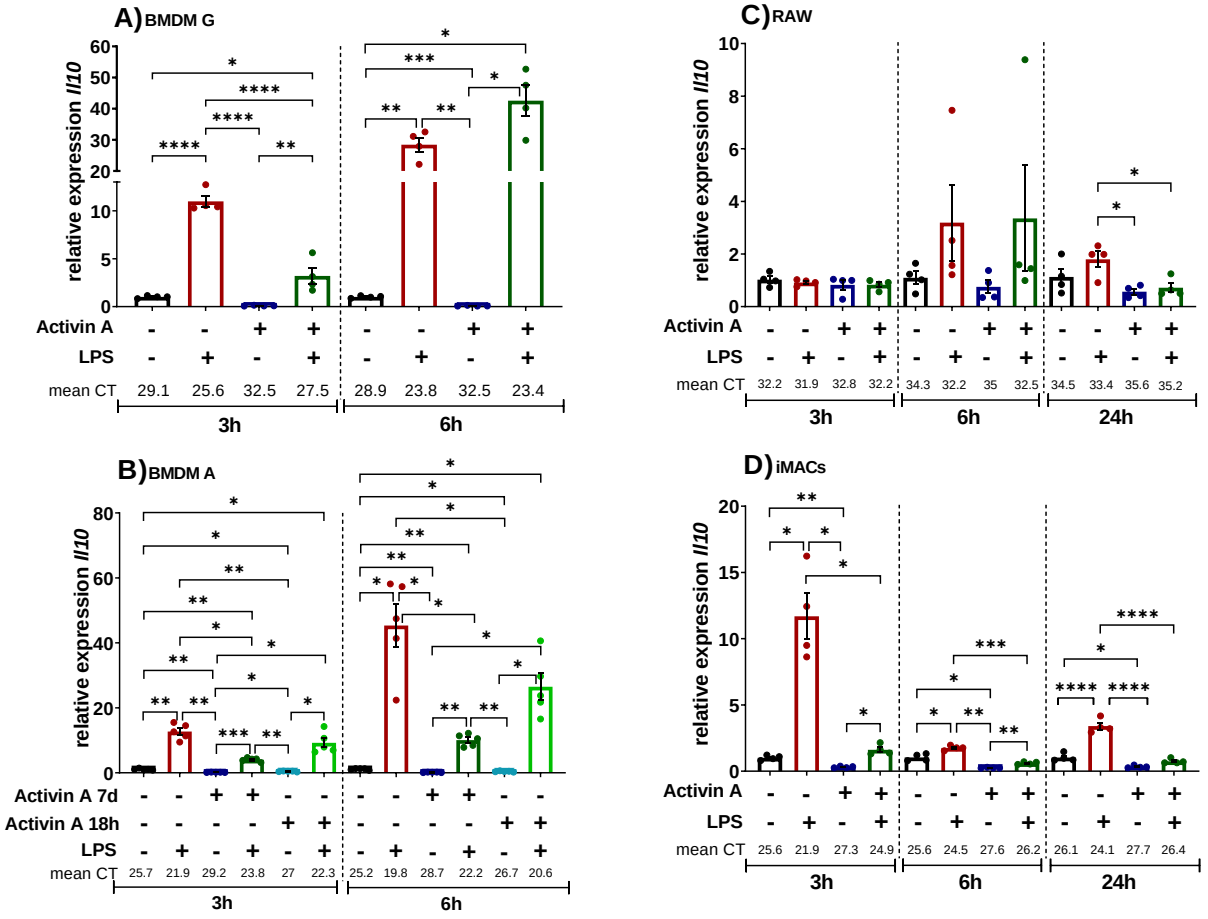


Figure 18: Regulation of mRNA expression of *Il10* by activin A in macrophages. Refer to legend of Figure 11 for details of experiment. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=3-5 separate cultures denoted by individual data points.

The expression of transcription factor *Klf4*, which is involved in the polarisation of macrophages towards an M2 phenotype, was significantly reduced by long-term activin A treatment compared with basal levels in BMDM G at both 3 h and 6 h (Fig. 19A). This result was not confirmed in BMDM A, although a tendency towards reduction was observed following the long-term treatment and, to a lesser extent, following the 18 h activin A treatment. The absence of significance, despite similar tendencies, may be explained by a higher variance among the samples.

In both cell lines, treatment with activin A led to no changes in the gene expression compared with control (Fig. 19C, D).

The activation of the macrophages with LPS showed no difference in expression after 3 h in BMDM G but led to a significant increase after the 6 h treatment, although these observations could not be confirmed in the BMDM A studies (Fig. 19A, B). Conversely, in both cell lines the stimulation with

LPS led to a significant reduction of the *Klf4* transcript after 3 h and 6 h, with the tendency still evident after 24 h of LPS activation (Fig. 19C, D).

When investigating the influence of activin A on LPS-activated macrophages, a significant reduction in the expression of *Klf4* was observed with the 7-day activin A treatment after LPS-activation for 6 h in BMDM G, as well as after 3 h in BMDM A (Fig. 19A). In contrast, the 18 h treatment seemingly showed no effects compared to the LPS-activated macrophages, although a diminishing tendency could be observed (Fig. 19B). In both cell lines, no significant effect of activin A treatment on LPS-activated macrophages was observed (Fig. 19C, D).

Similar to *Il10*, but unlike the anti-inflammatory marker *Arg1*, treatment with activin A led to a reduction of the transcription factor *Klf4* in BMDM. In the cell line macrophages, no significant effect of activin A was observed. This could suggest a differential regulation of the transcription factor *Klf4* between the investigated BMDM and the cell line macrophages, similar to the response of the pro-inflammatory markers. Additionally, opposing effects were observed in response to the stimulation with LPS, with a reduction of *Klf4* expression seen in the cell line macrophages compared to an increased expression in BMDM G.

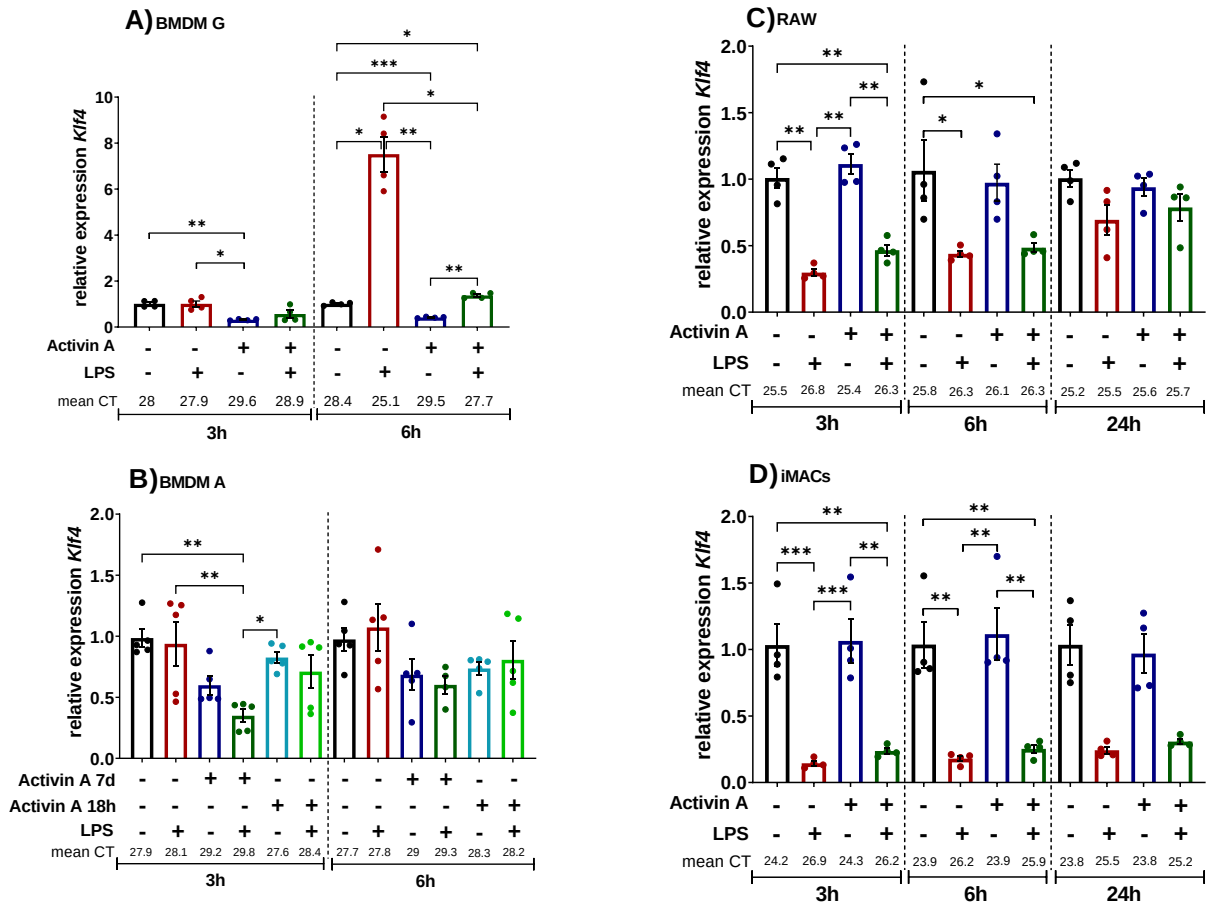


Figure 19: Regulation of mRNA expression of *Klf4* by activin A in macrophages. Refer to legend of Figure 11 for details of experiment. ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=3-5 separate cultures denoted by individual data points.

The effect on the expression of the transcription factor *Atf3*, which promotes the expression of M2 phenotype markers (Sha et al., 2017), was investigated at JLU only, where BMDM G and RAW macrophages were compared (Fig. 20).

Treatment of BMDM G with activin A for 7 days led to a significant reduction in the expression of *Atf3* at the 3 h time point and showed a similar tendency at the 6 h time point (Fig. 20A). In the RAW macrophages, treatment with activin A led to no difference compared with the basal expression of *Atf3* (Fig. 20B).

After activating the cells with LPS, an apparent increase of *Atf3* levels could be observed after 6 h in BMDM G, though this was not statistically significant (Fig. 20A). Similarly, in RAW macrophages LPS stimulation led to a tendency of increased expression of *Atf3* compared with the control after 3 h and 6 h, while a slight reduction after 24 h was observed, but neither trend was statistically significant (Fig. 20B).

The treatment with activin A prior to LPS activation did not show a significant change in *Atf3* expression either in BMDM G or RAW macrophages (Fig. 20A, B).

Similar to *Il10* and *Klf4*, the anti-inflammatory transcription factor *Atf3* appeared to be suppressed by a 7-day activin A treatment in BMDM G. At the same time, no changes could be observed in the RAW macrophages, which could indicate a different regulation between the cell types.

The observation that activin A is reducing the mentioned anti-inflammatory and immunoregulatory genes, aside from *Arg1*, was in accordance with the previous finding that activin A appeared to increase the expression of the pro-inflammatory gene set in BMDM. In contrast, the immunoregulatory genes appeared to be more similarly regulated among the cell types investigated. Specifically, either similar changes in expression or no changes were found. However, no opposing effects, as was observed for the pro-inflammatory genes, where activin A appeared to regulate the gene expressions in opposite directions between BMDM and cell line macrophages, were found.

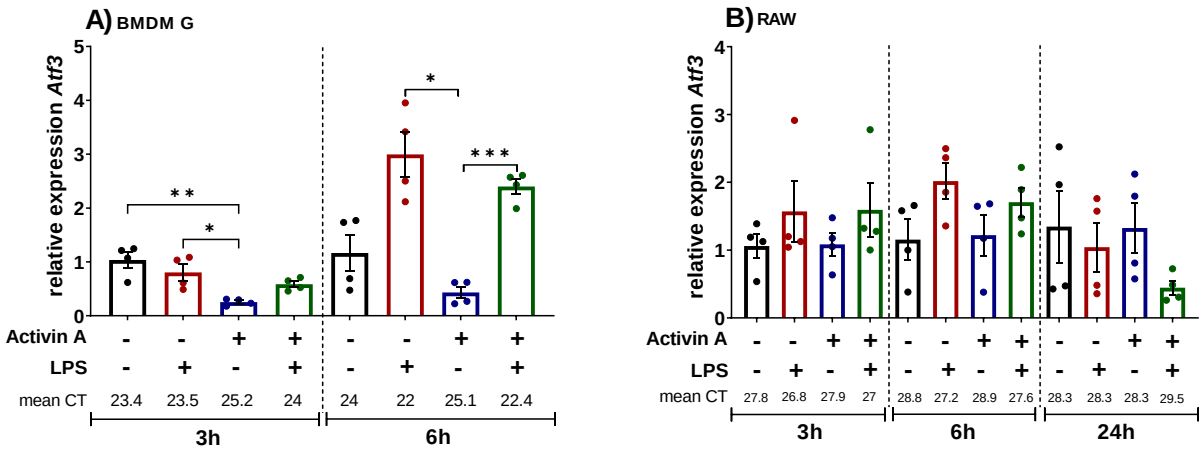


Figure 20: Regulation of mRNA expression of *Atf3* by activin A in macrophages. Refer to legend of Figure 14 for details of experiment. ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=4 separate cultures denoted by individual data points.

4.2.1.3 Effects of activin A on the expression of genes associated with antigen-presentation, co-stimulation and tolerance

To further elucidate the role activin A plays in regulating immune responses in macrophages, genes associated with antigen-presentation, co-stimulation, and tolerance were investigated, namely *Slamf1* and *Cd86*.

Long-term treatment with activin A was able to significantly decrease the expression of *Slamf1* compared to control in BMDM G at the 3 h time point (Fig. 21A). This effect was not confirmed in BMDM A (Fig. 21B). However, *Slamf1* showed low expression with CT values up to 35.1, and relatively high variation between the samples (Fig. 21B). In iMACs, the activin A treatment did not cause any changes compared to the basal expression levels (Fig. 21D). Expression of *Slamf1* was below detectable limits in RAW macrophages and therefore could not be analysed (Fig. 21C).

After activating the macrophages with LPS, a significant increase in the expression of *Slamf1* was detectable in BMDM G after 3 h and 6 h, in BMDM A after 6 h and in iMACs at all time points (Fig. 21A, B, D).

Long-term treatment with activin A led to a significant reduction of the *Slamf1* transcript in LPS-activated BMDM G after 3 h and 6 h (Fig. 21A), whereas in BMDM A the apparent decrease was not significant, possibly due to high variation between the samples (Fig. 21B). The shorter 18 h treatment with activin A did not change the expression of *Slamf1* after 6 h, but showed a tendency towards an increase after 3 h, suggesting a difference in regulation by activin A depending on duration of the treatment.

The effect of activin A observed in BMDM G was also seen in iMACs, where activin A significantly reduced the expression of *Slamf1* in LPS-activated cells after 3 h and 6 h (Fig. 21D).

The results suggest that *Slamf1* may be similarly regulated among the cell types investigated.

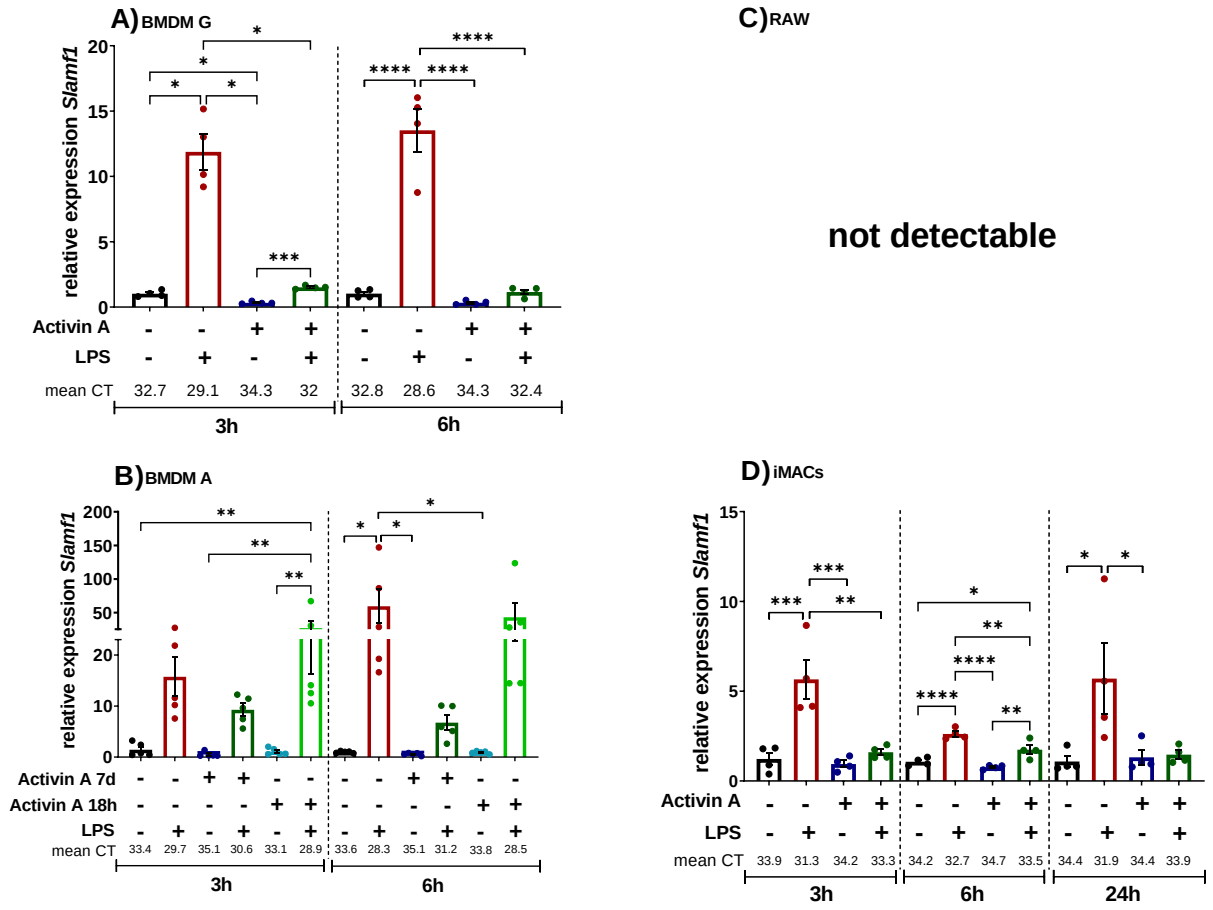


Figure 21: Regulation of mRNA expression of *Slamf1* by activin A in macrophages. Refer to legend of Figure 11 for details of experiment. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=3-5 separate cultures denoted by individual data points.

Unpublished findings in the Monash laboratory in transgenic mouse models with altered activin A levels indicated an effect of activin A on the co-stimulatory gene, *Cd86.* This could only be tested in BMDM A (Fig. 22).

The activation of BMDM with LPS significantly increased the expression of *Cd86* after 3 h and further after 6 h, compared with the control. Neither the long-term, nor the 18 h activin A treatment affected the expression of *Cd86* compared to basal levels, and no difference was detectable in activin A-treated cells after stimulation with LPS for 3 h versus LPS alone. Although not significant, a reduction of the *Cd86* transcript was apparent in the long-term treatment with activin A when stimulated with LPS for 6 h compared to LPS alone.

Taken together, it appears that activin A may inhibit expression of the investigated genes associated with antigen-presentation, co-stimulation, and tolerance in LPS-activated macrophages. The observed effects seemed to show similar tendencies between the cell types investigated. Of note, only the

long-term activin A treatment caused significant changes in BMDM to basal and LPS-activated levels of *Cd86* expression.

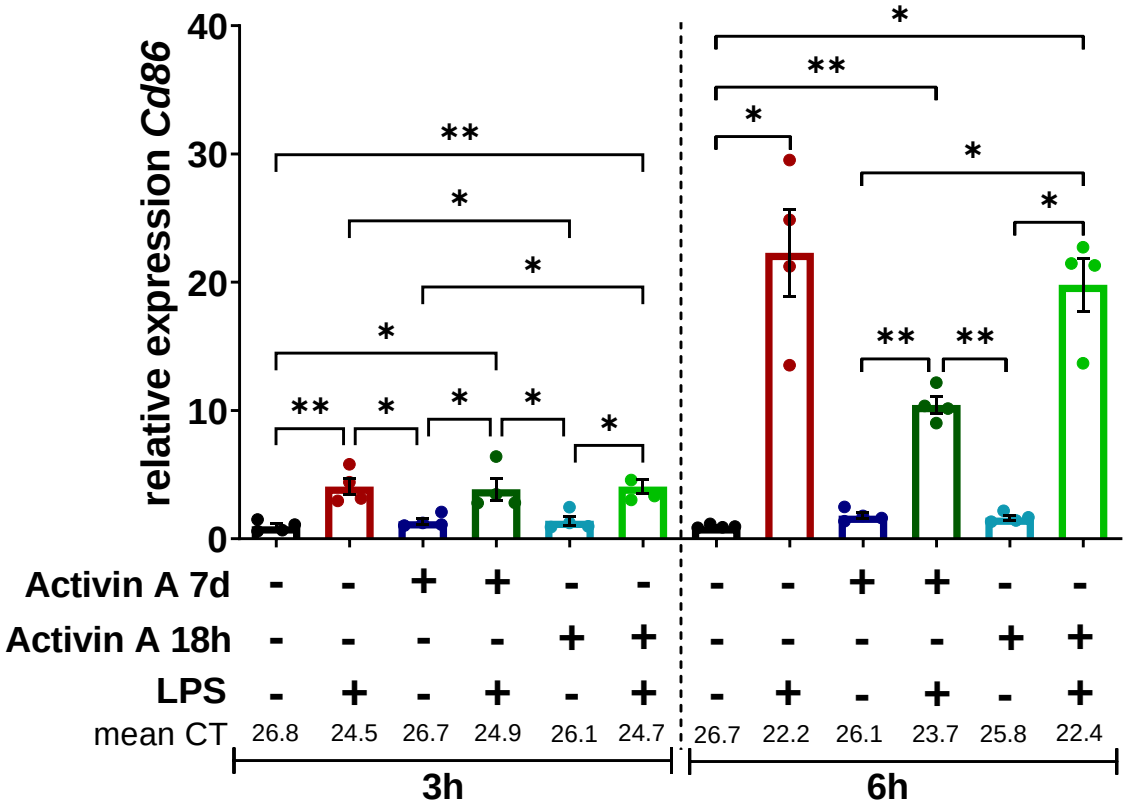


Figure 22: Regulation of mRNA expression of *Cd86* by activin A in BMDM. Relative mRNA expression of M-CSF-induced BMDM after activin A (50 ng/ml) and subsequent 3 h or 6 h activin A plus LPS (10 ng/ml) treatment, compared with untreated controls. ***P*<0.01, **P*<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. Results are expressed as mean ± SEM. N=4 separate cultures denoted by individual data points.

4.2.1.4 Effect of activin A on the expression of genes associated with pathogen recognition

Since one of the major functions of macrophages is the recognition of pathogens, the effect of activin A on the regulation of genes involved in detection of pathogen-associated molecules was investigated. The expression of the PRR dectin-1, encoded by the gene *Clec7a*, was studied in response to activin A treatment under resting and LPS-activated conditions (Fig. 23).

When activated with LPS a significant reduction in the expression of *Clec7a* in iMACs was observed after 24 h (Fig. 23D). The LPS activation of BMDM G also led to an apparent decrease in *Clec7a* transcripts after 3 h and 6 h, though not statistically significant (Fig. 23A). In LPS-treated RAW

macrophages, a non-significant decrease in *Clec7a* expression was observed after 3 h and 6 h (Fig. 23C).

In BMDM G and BMDM A, long-term treatment with activin A significantly reduced the basal *Clec7a* transcript levels compared to control at the 3 h time point (Fig. 23A, B). After 6 h, the reduction was no longer significant. A shorter 18 h treatment with activin A caused no significant changes in BMDM A compared to control levels (Fig. 23B). However, an elevation of *Clec7a* expression in the 18 h activin A treatment could be observed after 3 h, which was significantly different to the long-term treatment with activin A (Fig. 23B). Similarly, in RAW macrophages, a tendency towards an increase of the expression of *Clec7a* was observed after 3 h and 6 h, but these changes were not significant (Fig. 23C). In the iMACs, activin A did not significantly affect the expression of *Clec7a,* compared to basal levels (Fig. 23D).

A significant effect of activin A on the expression of *Clec7a* in LPS-stimulated macrophages could not be detected in BMDM (Fig. 23A, B). Similarly, in both cell lines tested, activin A treatment in LPS-stimulated macrophages did not lead to significant changes (Fig. 23C, D).

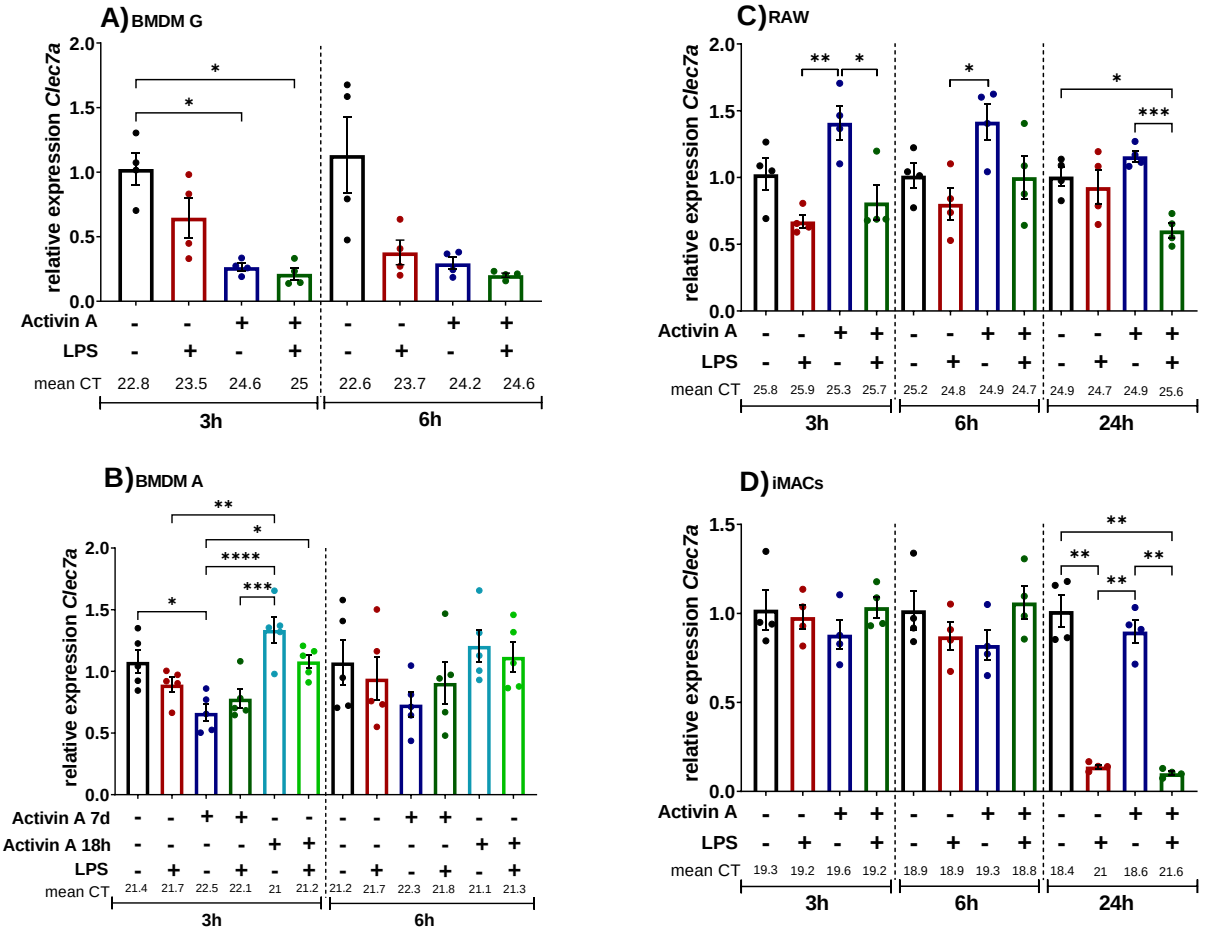


Figure 23: Regulation of mRNA expression of *Clec7a* by activin A in macrophages. Refer to legend of Figure 11 for details of experiment. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=3-5 separate cultures denoted by individual data points.

An additional PRR, the mannose receptor *Mrc1* (also known as CD206), which is commonly utilised as an M2 phenotype marker, was studied in BMDM G and RAW macrophages (Fig. 24).

In BMDM G, LPS stimulation of the cells led to a significant decrease in the expression of *Mrc1* after 6 h (Fig. 24A). Although a similar tendency was observed in RAW macrophages, especially after 24 h, the changes were not statistically significant (Fig. 24B). Notably, variation between the samples in RAW macrophages were relatively high.

Treatment with activin A was able to significantly decrease the expression of *Mrc1* at the 3 h and 6 h time points in BMDM G (Fig. 24A), and at the 3 h in RAW macrophages (Fig. 24B), compared with basal levels. The same trend could still be observed after 6 h and 24 h in the RAW cells, though this was not statistically significant.

In addition, activin A also significantly suppressed *Mrc1* levels in LPS-activated macrophages: after 6 h in BMDM G (Fig. 24A) and after 3 h in the RAW macrophages (Fig. 24B). In BMDM G, similar

tendencies towards a reduction were observed after 3 h (Fig. 24A) and in RAW macrophages after 6 h (Fig. 24B), although not significant.

In summary, activin A appears to have an inhibitory effect on the investigated genes associated with pathogen recognition, thus augmenting the effect of LPS activation. The regulation of *Mrc1* seemed to be similar between the cell types tested, while *Clec7a* may be differently regulated in RAW macrophages, and the duration of activin A treatment may influence its effects in BMDM.

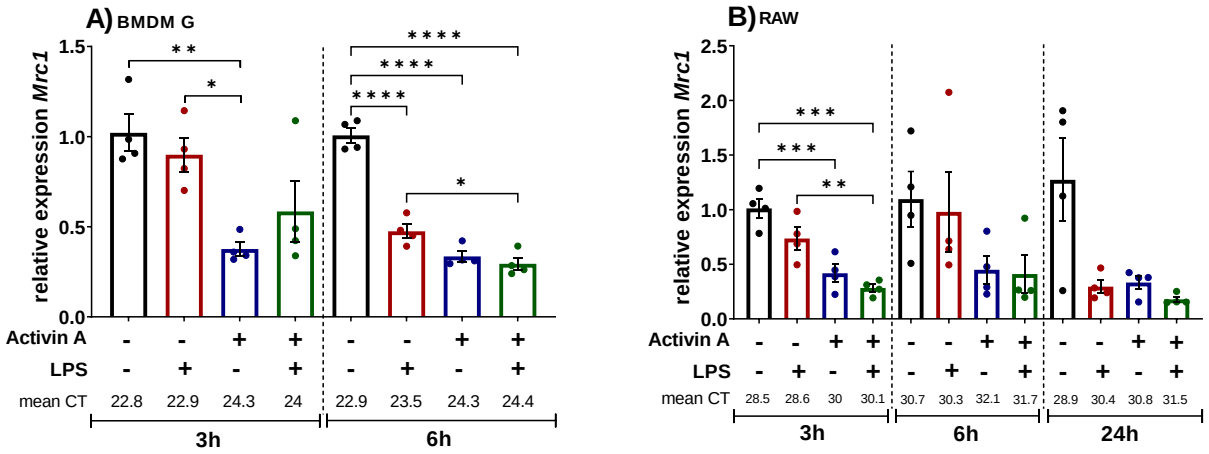


Figure 24: Regulation of mRNA expression of *Mrc1* by activin A in macrophages. Refer to legend of Figure 14 for details of experiment. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=4 separate cultures denoted by individual data points.

4.2.1.5 Effect of activin A on the expression of genes of the PI3K/AKT signalling pathway

Given that the PI3K/AKT signalling pathway is directly involved in modulating immune responses as well as metabolic changes, the possible regulation of this pathway by activin A was investigated by analysing the expression levels of *Akt1*, *Akt3* and *Pik3cd*.

The genes *Akt3* and *Akt1* encode for family members of the AKT kinases, which are involved in regulating many aspects of cell signalling, differentiation, and proliferation. The AKT kinases are activated by PI3K, which consists of several subunits. Among them, the subunit *Pik3cd* has been reported to be expressed in leukocytes and to be involved in regulation of the immune response (Lucas et al., 2016).

Activation with LPS did not change the relative expression of *Akt3* after 3 h in both BMDM G and BMDM A, but an apparent, non-significant increase was observed after 6 h of LPS treatment

(Fig. 25A, B). This could be due to relatively high variation among the replicates. In contrast with the effect of LPS in BMDM, in both cell lines tested LPS led to a tendency of reduced *Akt3* expression after 3 h, and to a significant decrease after 6 h in RAW macrophages (Fig. 25C) and after 24 h in iMACs (Fig. 25D). Notably, in RAW macrophages, no difference in *Akt3* expression was observed between LPS-treated cells and control cells after 24 h (Fig. 25C).

In BMDM G and BMDM A, neither the long-term nor the 18 h activin A treatment affected the expression levels of *Akt3* at any time point (Fig. 25A, B). In the cell line macrophages, a slight increase of *Akt3* expression could be observed, which became significant at the 6 h time point in RAW macrophages (Fig. 25C), but not in iMACs (Fig. 25D).

In the LPS-stimulated macrophages, neither the 7-day activin A treatment, nor the 18 h treatment changed the expression levels of *Akt3* compared to LPS-induced levels in BMDM G or BMDM A (Fig. 25A, B). Similarly, in the macrophage cell lines, no significant changes were found with activin A treatment of the LPS-stimulated macrophages in either RAW cells or iMACs (Fig. 25C, D).

Overall, in response to LPS stimulation BMDM and cell lines showed opposing tendencies with BMDM displaying an increase of *Akt3* expression, whilst in the investigated cell lines a decrease was observed. Activin A only had significant effects on the RAW macrophages.

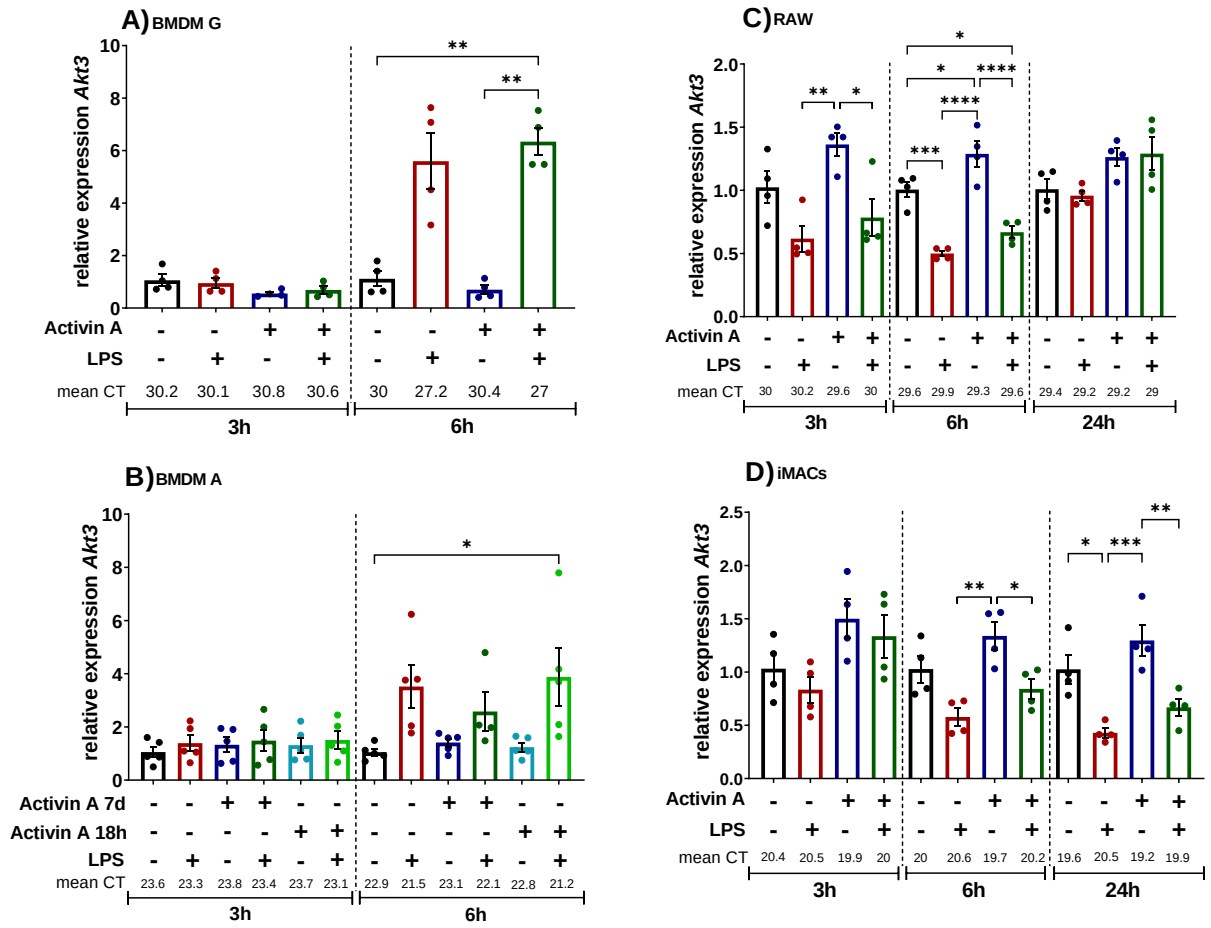


Figure 25: Regulation of mRNA expression of *Akt3* by activin A in macrophages. Refer to legend of Figure 11 for details of experiment. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=3-5 separate cultures denoted by individual data points.

In contrast to the expression of *Akt3*, the expression of family member *Akt1* was significantly reduced in both BMDM G and RAW macrophages by LPS stimulation at all investigated time points (3 h, 6 h, 24 h) (Fig. 26A, B). In BMDM G, the treatment with activin A significantly reduced the expression of *Akt1* at 3 h and 6 h (Fig. 26A). In RAW macrophages, activin A did not change the expression of *Akt1* compared with basal levels (Fig. 26B).

In both cell types, no significant influence of activin A treatment on *Akt1* expression was found in LPS-stimulated macrophages compared with the activated cells (Fig. 26A, B). However, in RAW macrophages the significant reduction of *Akt1* induced by LPS after 24 h was abolished by activin A treatment (Fig. 26B).

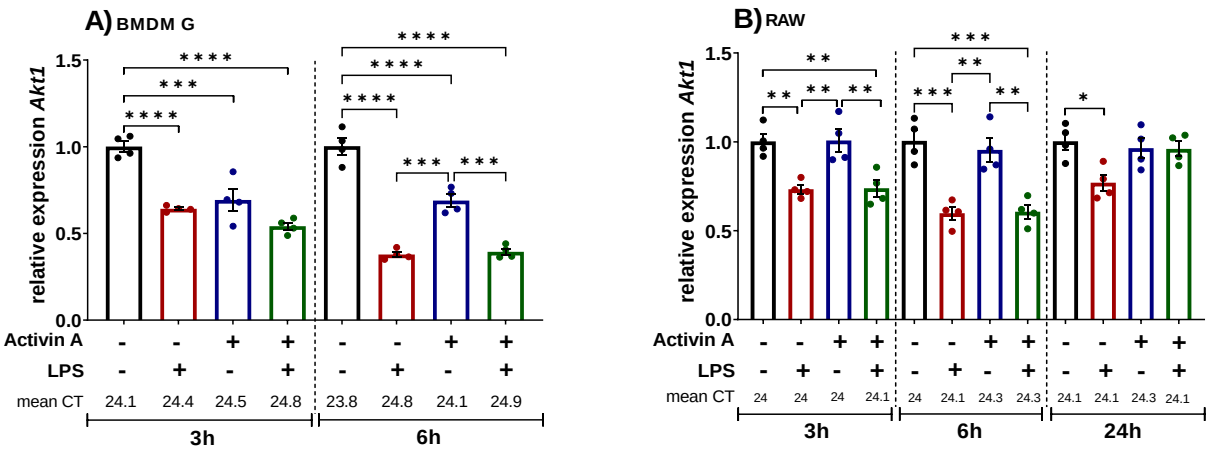


Figure 26: Regulation of mRNA expression of *Akt1* by activin A in macrophages. Refer to legend of Figure 14 for details of experiment. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=4 separate cultures denoted by individual data points.

The activation of BMDM with LPS initially led to a slight reduction in the *Pik3cd* transcript at 3 h, but then significantly increased its expression after 6 h (Fig. 27A). A similar tendency was observed in LPS-activated RAW macrophages, with a significant reduction in *Pik3cd* expression after 3 h, a slight increase after 6 h, followed by a non-significant reduction after 24 h LPS treatment (Fig. 27B). Activin A significantly reduced *Pik3cd* expression compared with control levels at 3 h in BMDM G, with a similar tendency at the 6 h time point (Fig. 27A). In RAW macrophages, no significant changes were observed with activin A treatment.

Treatment with activin A significantly reduced the expression of *Pik3cd* in LPS-stimulated cells in both BMDM G and RAW macrophages after 3 h and caused no effect at the other time points (Fig. 27A, B).

The response of the BMDM and RAW macrophages to LPS stimulation appears similar in tendency, with activin A able to significantly reduce the expression of *Pik3cd* in activated cells in both cell types. However, activin A significantly reduced the expression of *Pik3cd* in resting BMDM, but had little effect in resting RAW macrophages when compared with control levels.

Taken together, activin A seemingly affects the PI3K/AKT signalling pathway in specific subunits and cell types. *Akt3* appeared to be influenced by activin A in cell line macrophages, but not in BMDM, while *Akt1* levels were changed by activin A in BMDM, but not in RAW macrophages. *Pik3cd* could be suppressed by activin A in both LPS-activated cell types. Interestingly, LPS stimulation appeared to induce opposite responses in *Akt3* expression in BMDM and cell line macrophages.

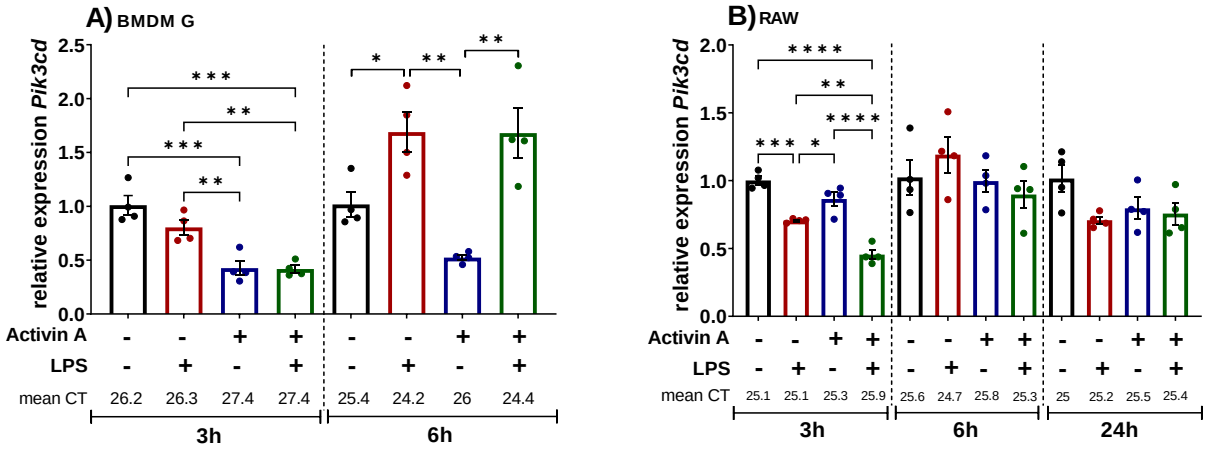


Figure 27: Regulation of mRNA expression of *Pik3cd* by activin A in macrophages. Refer to legend of Figure 14 for details of experiment. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=4 separate cultures denoted by individual data points.

4.2.1.6 Effect of activin A on the expression of genes associated with the inflammasome

To investigate a possible involvement of the inflammasome in activin A influenced immune responses in macrophages, the gene expression of the inflammasome protease *Casp1*, which activates the pro-inflammatory cytokine *Il1β*, was investigated (Sollberger et al., 2014).

The activation of the macrophages with LPS significantly increased the expression of *Casp1* in BMDM G after 6 h, and in BMDM A after both 3 h and 6 h (Fig. 28A, B). In contrast, limited change of *Casp1* expression with LPS treatment was observed in both RAW macrophages and iMACs, after 3 h and 6 h, with a slight progressive increase between 6 h and 24 h in RAW macrophages (Fig. 28C). The increase of the *Casp1* transcript was significant after 24 h LPS treatment in the iMACs (Fig. 28D).

The long-term treatment with activin A significantly suppressed *Casp1* expression compared with control levels after 3 h in BMDM G (Fig. 28A). This was not confirmed in BMDM A, although a similar tendency was visible, which seemed less apparent in the 18 h activin A treatment (Fig. 28B). A minor decrease of *Casp1* was observed at 3 h and 24 h in activin A treated RAW macrophages, compared with control levels (Fig. 28C), while a slight increase was apparent in the iMACs (Fig. 28D).

Although no significant changes were observed in BMDM G, despite a slight suppression of the *Casp1* transcript by activin A in LPS-stimulated macrophages (Fig. 28A), in BMDM A the observed reduction in the long-term activin A-treated cells was significant after the 3 h and 6 h LPS treatment

(Fig. 28B). The shorter treatment with 18 h activin A prior to activation with LPS also showed a slight, though not significant reduction. In RAW macrophages, activin A appeared to have no effect on LPS-stimulated cells (Fig. 28C). However, *Casp1* levels were significantly reduced in the iMACs by activin A treatment in LPS-activated macrophages at 24 h, while a minor increase was apparent at 3 h (Fig. 28D).

It appears that activin A exerts similar effects on *Casp1* expression in both primary macrophages and cell lines. However, the cell lines appear to be far less responsive to either activin A or LPS at the earlier time points of 3 h and 6 h, with RAW macrophages not showing any significant changes of the *Casp1* transcript.

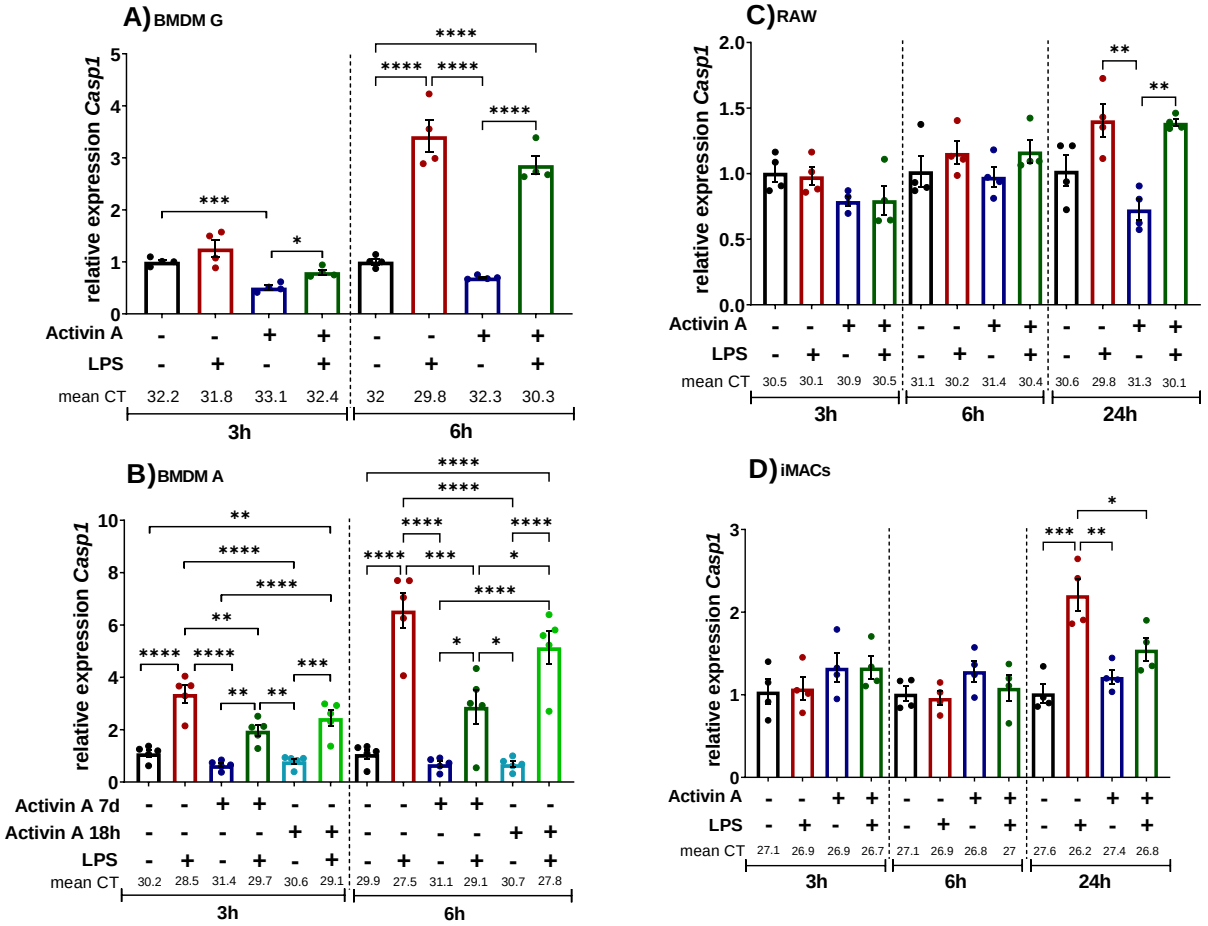


Figure 28: Regulation of mRNA expression of *Casp1* by activin A in macrophages. Refer to legend of Figure 11 for details of experiment. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=3-5 separate cultures denoted by individual data points.

4.2.1.7 Effect of activin A on the gene expression of chemokines

As an important function of macrophages is the attraction of immune cells to a site of inflammation, the chemokine *Ccl2* was investigated in LPS-stimulated and activin A treated BMDM A. Unpublished data from the Monash laboratory observed significant changes in expression levels of *Ccl2* in transgenic mouse models with altered activin A levels.

Due to large variation between the sample replicates, no significant changes in *Ccl2* expression were detectable between the treatment groups (Fig. 29). However, an increase of the *Ccl2* transcript was observed when the macrophages were activated with LPS after both 3 h and 6 h. The long-term treatment with activin A led to evidently lower expression of *Ccl2* than the shorter 18 h activin A treatment. Especially in the LPS-activated macrophages, the long-term treatment with activin A reduced the expression of *Ccl2* at both 3 h and 6 h, almost reaching basal levels after 3 h LPS treatment. This effect was not observed following the 18 h activin A treatment, where only a minor reduction was visible after 3 h and a minute elevation after 6 h.

There may be significant effects of activin A on suppressing the expression of *Ccl2* in both resting and LPS-activated macrophages, which could not be detected in this dataset due to high variations between samples.

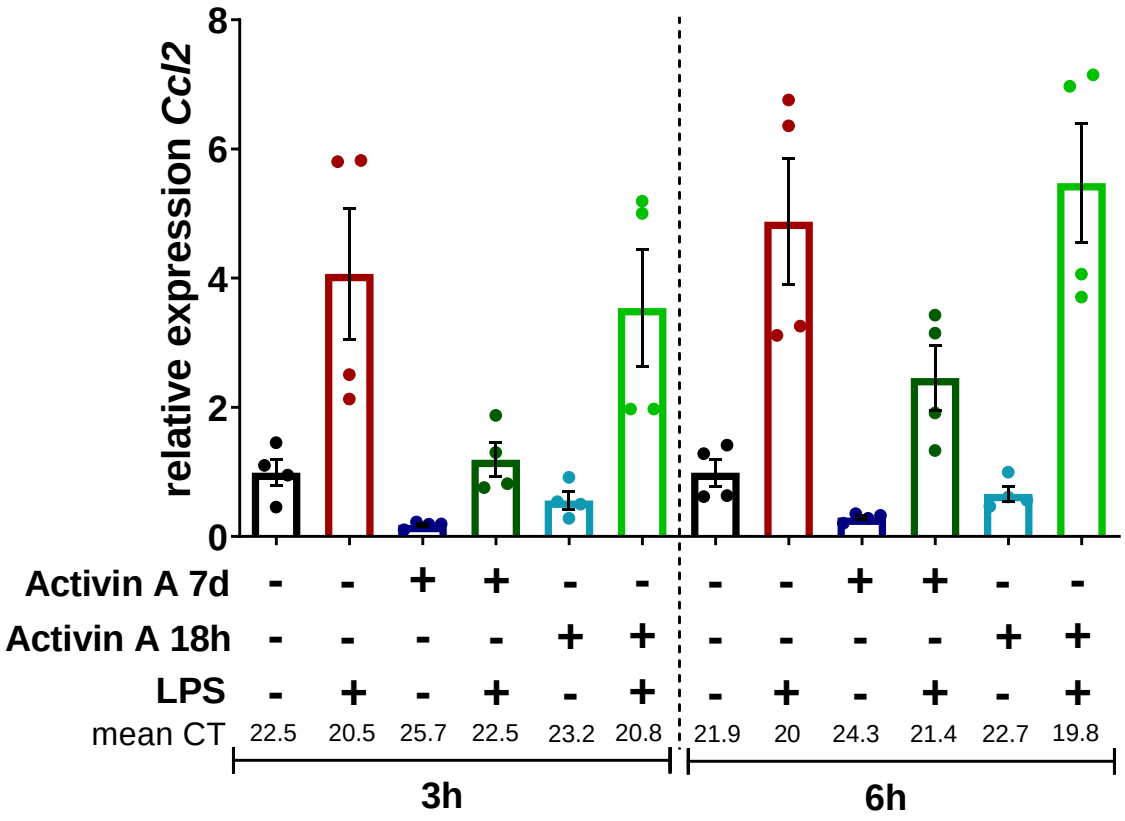


Figure 29: Regulation of mRNA expression of *Ccl2* by activin A in BMDM. Relative mRNA expression of M-CSF-induced BMDM after activin A (50 ng/ml) and subsequent 3 h or 6 h activin A plus LPS (10 ng/ml) treatment, compared to untreated controls. Statistics determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. Results are expressed as mean ± SEM. N=4 separate cultures denoted by individual data points.

4.2.1.8 Effect of activin A on expression of macrophage attachment genes

The commonly used M2 marker, *Chil3/Ym1*, was additionally investigated to gain insight into the effect of activin A on macrophage attachment genes.

The expression of the marker gene *Chil3/Ym1* could be detected in BMDM G (Fig. 30A), but was not detectable in the RAW macrophages (Fig. 30B).

In BMDM G, stimulation with LPS only led to a small increase in *Chil3/Ym1* expression, while the long-term treatment with activin A in the absence of LPS significantly reduced the gene levels at the 3 h time point, and non-significantly suppressed *Chil3/Ym1* at the 6 h time point, compared to control levels (Fig. 30A). In the macrophages treated with LPS, expression of *Chil3/Ym1* was significantly reduced by activin A compared with cells activated with LPS after both 3 h and 6 h, with similar levels observed in macrophages treated with activin A alone.

Activin A appeared to strongly reduce the expression of the M2 marker *Chil3/Ym1* in both resting and activated macrophages, consistent with the regulation of other genes associated with the M2 phenotype, such as and *Il10* and *Mrc1* (Figs. 18, 24).

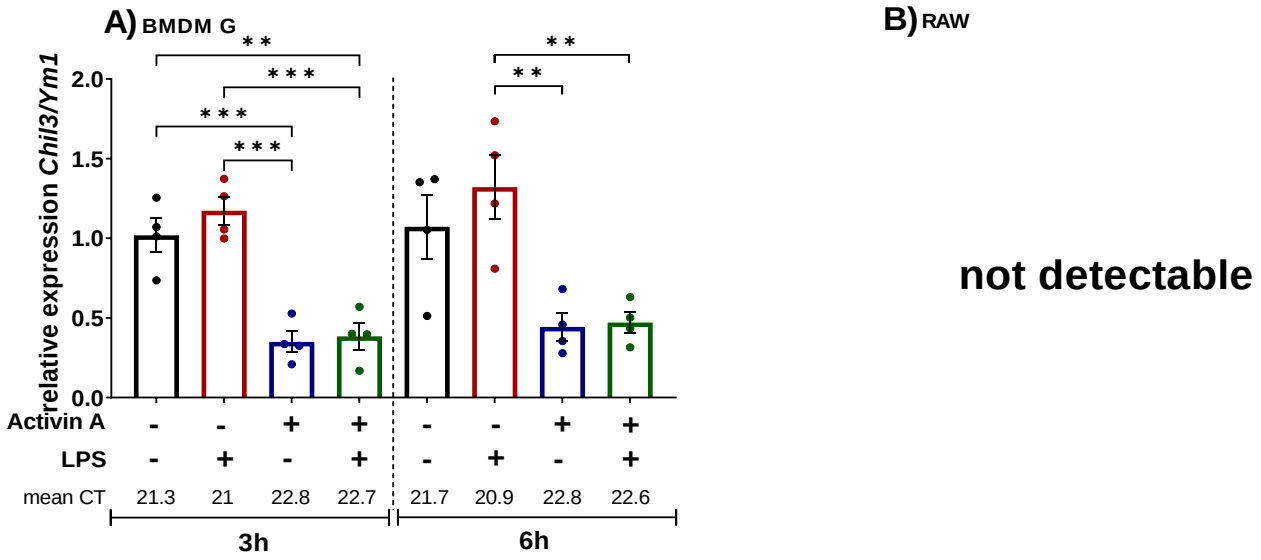


Figure 30: Regulation of mRNA expression of *Chil3/Ym1* by activin A in macrophages. Refer to legend of Figure 14 for details of experiment. ***P<0.001, **P<0.01 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=4 separate cultures denoted by individual data points.

The findings of the gene expression study, investigating the influence of activin A on immune response genes in macrophages of different origin *in vitro*, are summarised in Table 1. This table highlights the consistently observed responses of the macrophages to LPS and activin A.

One of the most prominent results of the gene expression studies was that activin A appeared to exert its effects on immune responses depending on the context of the treatment, as well as the type of macrophage investigated.

The group of genes associated with pro-inflammatory responses, which included *Tnf*, *Nos2*, *Gpr18*, *Fpr2*, *Il1β*, as well as the PRR *Clec7a*, were differently or oppositely regulated in primary BMDM compared with the cell line macrophages RAW and iMACs. While the pro-inflammatory genes were upregulated by activin A in primary macrophages, potentially increasing their pro-inflammatory activity, these genes were downregulated by activin A in the cell line macrophages. In addition, the typically pro-inflammatory marker *Il6* was also significantly suppressed by activin A in the cell line macrophages but was not significantly changed in BMDM. The PRR *Clec7a* was found to be

suppressed by activin A in primary BMDM but was significantly increased by activin A in RAW macrophages.

Genes involved in regulatory or anti-inflammatory immune responses, or genes typically associated with the M2 phenotype of macrophages, appeared to be similarly regulated by activin A in all the investigated macrophage cell types. Specifically, *Il10*, *Mrc1*, and *Chil3/Ym1* were consistently suppressed by activin A in all macrophages tested, while *Arg1* was the only gene consistently stimulated by activin A treatment.

The co-stimulatory molecule *Slamf1*, as well as *Pik3cd*, which is part of the PI3K/AKT signalling pathway, were also consistently suppressed by activin A in all the cell types tested, while *Casp1* was similarly reduced in BMDM and iMACs, but not in in RAW macrophages.

Several genes were not affected by activin A treatment or were only changed in one cell type. For example, expression levels of *Atf3* and *Akt1* were suppressed in primary BMDM, but not in the cell lines. Another gene in the PI3K/AKT-signalling pathway, namely *Akt3*, was found to be stimulated by activin A in the RAW macrophages but did not appear to be affected in the primary BMDM cultures.

In addition to the cell types, the duration of activin A treatment impacted the effect of activin A on immune responses in macrophages. Specifically, *Clec7a* was only suppressed by the 7-day activin A treatment, whereas 18 h treatment had no effect. Similarly, the inflammasome gene *Casp1*, and the anti-inflammatory marker *Klf4* were suppressed by the 7-day activin A treatment, while the 18 h treatment did not significantly change their expression.

Interestingly, it was observed that the LPS stimulation of the macrophages could lead to opposing gene expression regulation between primary BMDM and cell line macrophages in some cases. Namely, *Arg1* was suppressed in BMDM, but induced in both macrophage cell lines, while *Pik3cd*, *Akt3* and *Klf4* were found to be induced by LPS in BMDM but inhibited in the cell lines investigated.

Taken together, activin A appears to act in a context-dependent manner and the effect was influenced by the origin or maturation status of the macrophages investigated, in addition to the time point or duration of the activin A treatment. Moreover, the overall effects of activin A on immune responses could not be clearly segregated into promoting either a pro- or anti-inflammatory phenotype. However, it appears that the effects of activin A tend more towards promotion of a pro-inflammatory status in the primary BMDM cultures. This is opposed to cell line macrophages, where the expression of pro-inflammatory markers appears to be inhibited. In addition, macrophages from different origins responded differently to LPS stimulation for some genes, indicating that the different macrophage

types are at different states of activation prior to culture, which could have been responsible for the differing responses when subsequently stimulated with activin A.

Table 9: Summary of gene expression changes influenced by activin A in resting and LPS-activated macrophages, comparing primary BMDM with cell line macrophages RAW and iMAC. Arrows indicate upregulation (↑, ↑↑), downregulation (↓), or no change (↔), arrows in parentheses in the table indicate a consistent tendency without statistical significance being established. Genes highlighted in blue are differentially affected in different cell types, genes highlighted in beige are suppressed by activin A treatment, and genes highlighted in green are upregulated by activin A. Genes highlighted in red are affected by the 7-day activin A treatment and genes in without highlight are not significantly changed. ND: expression not detectable.

Gene	BMDM LPS	BMDM Activin (18h)	BMDM Activin (7d)	RAW LPS	RAW Activin	iMac LPS	iMac Activin	Cumulative effect of activin	Baseline expression
Tnfα	↑↑	no effect	↑	↑↑	↓	↑↑	↓	context-dependent	Ct 20-23 (high)
Nos2	↑↑		↑	↑↑	↓			context dependent	Ct 31-33 (low)
Gpr18	↑		↑	↑	↓			context-dependent	Ct 28-29
Fpr2	↑↑	no effect	↑	↑↑	↓	↑↑	↓	context-dependent	Ct 25-27
Il1ß	↑↑	no effect	↑	↑↑	↓	↑↑	↓	context-dependent	Ct 26-28
Il6	↑↑		no effect	↑↑	↓			inhibits expression	Ct 29-32
Il10	↑↑	(↓)	↓	(↑)	↓	↑↑	↓	inhibits expression	Ct 25-30
Slamf1	↑↑	no effect	↓↓	ND	ND	↑	↓	inhibits expression	Ct 33-34 (low)
Mrc1	↓		↓	(↓)	↓			inhibits expression	Ct 22-23 (high)
Chil3	↔		↓	ND	ND			inhibits expression	Ct 29-30
Pik3cd	↑		↓	↓	↓			inhibits expression	Ct 25-26
Atf3	(↑)		↓	(↑)	no effect			inhibits expression	Ct 23-25
Akt1	↓		↓	↓	no effect			inhibits expression	Ct 24-25
Casp1	↑	no effect	↓	(↑)	no effect	↑	↓	inhibits expression	Ct 32-33 (low)
Klf4	↑	no effect	↓	↓	no effect	↓	no effect	inhibits expression (7d treatment)	Ct 28-29
Clec7a	↓	no effect	↓	↓	(↑)	↓	no effect	context-dependent	Ct 19-25 (high)
Arg1	↓	↑	↑	↑	↑	↑	↑	stimulates expression	Ct 28-29
Akt3	↑	no effect	no effect	↓	↑	↓	no effect	stimulates expression	Ct 23-27
CD86	↑	no effect	(↓)					no effect	Ct 21-26 (high)
Ccl2	↑	no effect	(↓)					no effect	Ct 19-26 (high)

Gene	Observation
Tnf, Clec7a, Nos2, Gpr18, Il1ß, Fpr2	Responses to activin are different between cell types
Arg1	Only gene consistently stimulated by activin
Il-10, Slamf1, Mrc1, Chil3, Pik3cd, Casp1	Consistently suppressed by activin
Il6	Only suppressed by activin in cell lines
Atf3, Akt1	Only suppressed by activin in primary cells
Akt3	Stimulated by activin in cell line
Klf4, Clec7a	Only suppressed in long-term activin treatment

4.2.2 Effect of activin A on cytokine protein secretion by macrophages

Supplementary to the investigation of the effects of activin A at the transcriptional level, the changes in secretion of the key cytokines TNF and IL-10 were investigated in BMDM and RAW macrophages.

4.2.2.1 Cytokine protein secretion in BMDM

Bone marrow macrophages were differentiated with M-CSF (25 ng/ml) as previously described, with and without activin A (50 ng/ml) treatment. On day 7 they were activated with LPS (10 ng/ml), and the culture supernatant was collected after 6 h and 24 h, and subsequently analysed by ELISA.
The activation of BMDM with LPS increased the protein secretion of the pro-inflammatory cytokine TNF (Fig. 31A). Similar to the observations for transcript levels in BMDM, long-term treatment with activin A significantly increased the secretion of TNF protein at both 6 h and 24 h time points.
In contrast, the LPS-induced elevation of secreted IL-10 protein appeared to be reduced by activin A, although no significant change was detected at either individual time point (Fig. 31B). This tendency is similar to the observed inhibitory effects of activin A on the *Il10* transcript in LPS-activated BMDM.

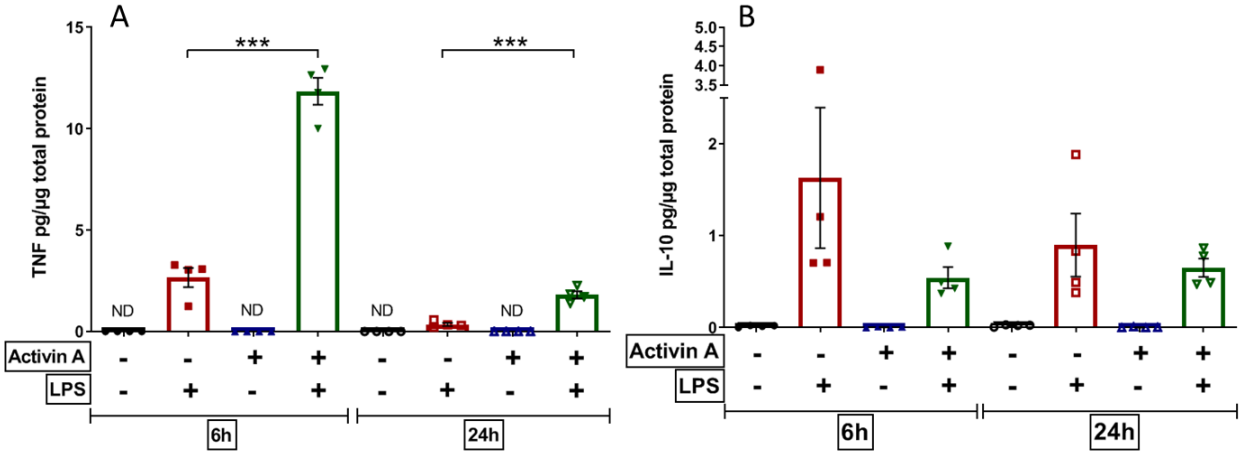
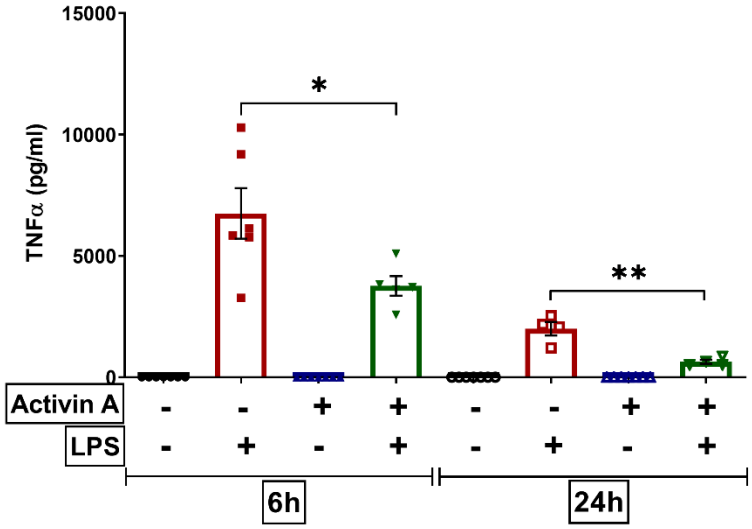


Figure 31: Effect of activin A on the protein secretion of TNF and IL-10 from BMDM. Relative concentration of (A) TNF and (B) IL-10 measured in the medium of M-CSF-induced BMDM after activin A (50 ng/ml) and subsequent 6 h or 24 h activin A plus LPS (10 ng/ml) treatment, compared to untreated controls. ***P<0.001 determined by unpaired two-tailed t-test. Results are expressed as mean ± SEM. N=4 separate cultures denoted by individual data points.

4.2.2.2 Protein secretion in RAW macrophages

The treatment with activin A was able to significantly inhibit the LPS-induced secretion of TNF by RAW macrophages after both 6 h and 24 h (Fig. 32). This observation was similar to the effect of activin A on the transcript level, where activin A treatment led to a reduction of *Tnf* gene expression in LPS-stimulated RAW macrophages, confirming a functional reduction of the pro-inflammatory cytokine.

Consistent with the low expression of *Il10* at the transcript level, IL-10 protein secretion in RAW macrophages was below the detection limit of the ELISA.



IL-10 below detection limit

Figure 32: Effect of activin A on the protein secretion of TNF from RAW macrophages. Relative concentration of TNF in the medium of RAW macrophages after activin A (50 ng/ml) and subsequent 6 h or 24 h activin A plus LPS (10 ng/ml) treatment, compared to untreated controls. Protein secretion of IL-10 was below the detection limit of the assay. $^{**}P<0.01$, $^{*}P<0.05$ determined by unpaired two-tailed t-test. Results are expressed as mean ± SEM. N=4-7 separate cultures denoted by individual data points.

4.3 Production of NO and changes in arginase activity in response to activin A

Further functional studies of the effects of activin A on immune responses were performed by investigating the concentration of nitrite as a means to indicate the production of NO, a pro-inflammatory product of macrophages. In addition, the enzyme activity of arginase was measured, as arginase competes with NOS2 for the same substrate, L-arginine.

RAW macrophages showed an increase of nitrite levels following LPS stimulation, suggesting a pro-inflammatory response (Fig. 33A). When treated with activin A, the LPS-activated RAW macrophages displayed a reduction of nitrite concentration, indicating reduction of the pro-inflammatory response. These observations were consistent with the previous results on transcript and protein secretion levels, where activin A inhibited the LPS-stimulated induction of pro-inflammatory protein and genes, including *Nos2*, in RAW macrophages. In BMDM, the levels of nitrite were below detection limits of the assay.

In the BMDM (Fig. 33B), a preliminary assessment (n=1 with triplicate culture wells) indicated an increased arginase activity by long-term activin A treatment compared to control, which was similar to the finding for transcript levels, where activin A treatment induced the expression of *Arg1*. Stimulation with LPS did not change the activity levels of arginase, but the treatment with activin A increased the activity of the enzyme in LPS-activated macrophages to the same levels as seen with activin A in resting cells. This observation was also similar to the effects of activin A on transcript levels, where it increased *Arg1* expression in LPS-activated cells.

Taken together, investigation of arginase activity and NO shows results consistent with the effects of activin A on the transcript levels of *Arg1* and *Nos2*. In RAW macrophages, activin A appeared to alleviate the LPS-induced pro-inflammatory response of NO, while the activity of arginase was enhanced by activin A in both resting and LPS-activated BMDM.

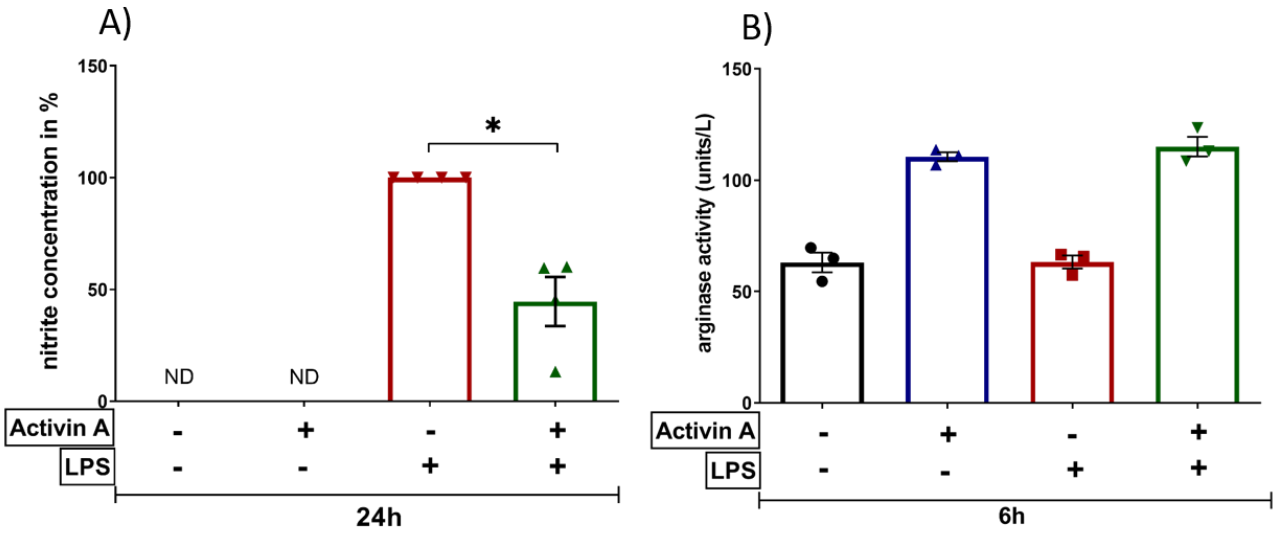


Figure 33: Effect of activin A on nitrite concentration in the medium of RAW macrophages and arginase activity in BMDM. (A) Relative concentration of nitrite in the medium of RAW macrophages after activin A (50 ng/ml) and subsequent 24 h activin A plus LPS (100 ng/ml) treatment, compared to untreated controls. N= 4 separate cultures denoted by individual data points. (B) Representative data of arginase activity in BMDM after activin A (50 ng/ml) and subsequent 6 h activin A plus LPS (10 ng/ml) treatment, compared to untreated controls. N= 1 culture with triplicate culture wells. **P*<0.05 determined by one sample t-test. Results are expressed as mean ± SEM.

4.4 The effect of activin A on the immunometabolic changes of resting and LPS-activated macrophages

Since activated macrophages change their metabolic profile from the usage of the ETC and OXPHOS to the predominant use of glycolysis in order to ensure rapid production of ATP, the mitochondrial respiration of macrophages in response to activin A treatment was analysed via the OCR measured during a mitochondrial stress test with the Seahorse XFp Analyzer.

BMDM and iMACs were treated with or without activin A (50 ng/ml) and activated with LPS for 24 h, in order to investigate the influence of activin A on their metabolic state. The conditions for Seahorse analysis first had to be optimised for each individual cell type, specifically the seeded cell numbers needed to be adjusted to be able to measure the oxygen consumption in an appropriate range for the machine. Moreover, the FCCP concentration needed to be titrated to allow for the measurement of the maximum respiratory capacity of the cells in the mitochondrial stress test. The experimental data were normalised to cell content ratio where appropriate, as determined by crystal violet staining, which had been tested for accuracy with a standard curve (appendix, Fig. 43).

4.4.1 Optimisation of Seahorse analysis for BMDM

In order to ensure collection of reliable data utilising Seahorse analysis, the conditions were first optimised for each cell type investigated. Specifically, it was tested which cell numbers produced OCR levels in the ideal detection range (60-150 pmoles/min), how much FCCP was necessary to reach maximum respiratory capacity, which LPS concentrations should be used to ensure reduction of OXPHOS in pro-inflammatory macrophages, and the functionality of different compounds were compared (Fig. 34).

In initial tests, different cell numbers of BMDM were seeded into Seahorse miniplates after differentiation for 6 days with M-CSF and subsequently analysed on day 2 after seeding, with different concentrations of FCCP (Fig. 34A, B). The seeded cell numbers tested were 2×10^4, 2.5×10^4, 3×10^4, 3.5×10^4, 4×10^4, and 4.5×10^4. While all seeded cell numbers led to basal OCR readings in the ideal detection range of the assay, the preferred readings of ~130 pmoles/min, which ensure the most reliable and reproducible results, could be detected around 2.5×10^4 seeded cells, while 2×10^4 seeded cells showed basal levels around 100 pmoles/min. An increase of the cell numbers to $3.0 - 4.5 \times 10^4$ increased the basal readings to around 150 pmoles/min. Based on these observations, a seeding number of 2.5×10^4 cells per well was chosen for further experiments.

To investigate the metabolic profile of the cells and their mitochondrial activity, the mitochondrial stress test was employed. After initial basal readings of the OCR, oligomycin (1.5 µM), FCCP and Rot/AA (1µM) were added to the cells, respectively. However, the ideal concentration of FCCP first had to be determined. Using different cell numbers, concentrations of 1 µM, 1.5 µM and 3 µM of FCCP were tested to elicit maximum respiratory capacity in the cells. An increase in the OCR was detectable from 1 µM to 1.5 µM, while 3 µM of FCCP led to a decrease again, showing 1.5 µM as the ideal FCCP concentration used for further experiments (Fig. 34A, B).

After determining ideal cell numbers and FCCP concentration in untreated BMDM, different LPS concentrations were tested to ensure the metabolic switch from OXPHOS in resting macrophages to glycolysis in activated cells. This is indicated by the absence of a FCCP-induced maximum respiratory capacity, where the stimulation could no longer increase the respiratory rate above basal levels (Fig. 34C). The tested concentrations of 10 ng/ml and 100 ng/ml of LPS, provided 24 h prior to analysis, revealed that both concentrations appear to efficiently block an increase of the respiratory rate by FCCP compared with the control cells. Given that 10 ng/ml LPS was sufficient for the metabolic switch and, additionally, was the concentration used in previous experiments investigating gene expression and protein secretion, this concentration of LPS was chosen to activate the

macrophages in all further experiments with BMDM. Both, the FCCP and the LPS concentrations determined for the mitochondrial stress test in BMDM were consistent with the literature (Van den Bossche et al., 2015).

After observing inconsistencies in the performance of the compounds of the Mitochondrial Stress Test Kit (oligomycin, FCCP, Rot/AA) between separate experiments, the compounds from the Kit (referred to as "Kit") were compared to compounds bought as individual components (referred to as "Compounds") (Fig. 34D). The Kit compounds were directly resolubilised in assay medium, a step that could potentially impact their effects, as for example oligomycin is not very soluble in aqueous solutions (Masamune et al., 1958). In contrast, the individually bought compounds were first dissolved in DMSO at high concentrations, before further diluting the cytotoxic DMSO to low concentrations with assay medium prior to testing. When comparing the compounds from different sources in a mitochondrial stress test using the same concentrations, it was observed that in the control macrophages, the effects of the different compounds at the same concentrations appeared to be similar in reducing (oligomycin, Rot/AA) or increasing (FCCP) the respiratory capacity to comparable levels (Fig. 34D). Notably, oligomycin from the Kit led to a slope-like decrease of the OCR, while the individually bought oligomycin was able to induce a stable decrease of the OCR, as would have been expected in the Seahorse analysis. Similarly, in the LPS-activated macrophages, the individually bought oligomycin led to a more consistent reduction of the OCR, while the oligomycin from the Kit could not induce a decrease of the OCR at all in this experiment. The effect of FCCP from either source, however, appeared to be comparable in both control and in LPS-activated macrophages. Due to the similar effects between the tested compounds, but the higher reliability of the individually bought compounds compared to the Kit substances, the individually bought compounds were selected for further experiments at the same concentrations as the Kit reagents.

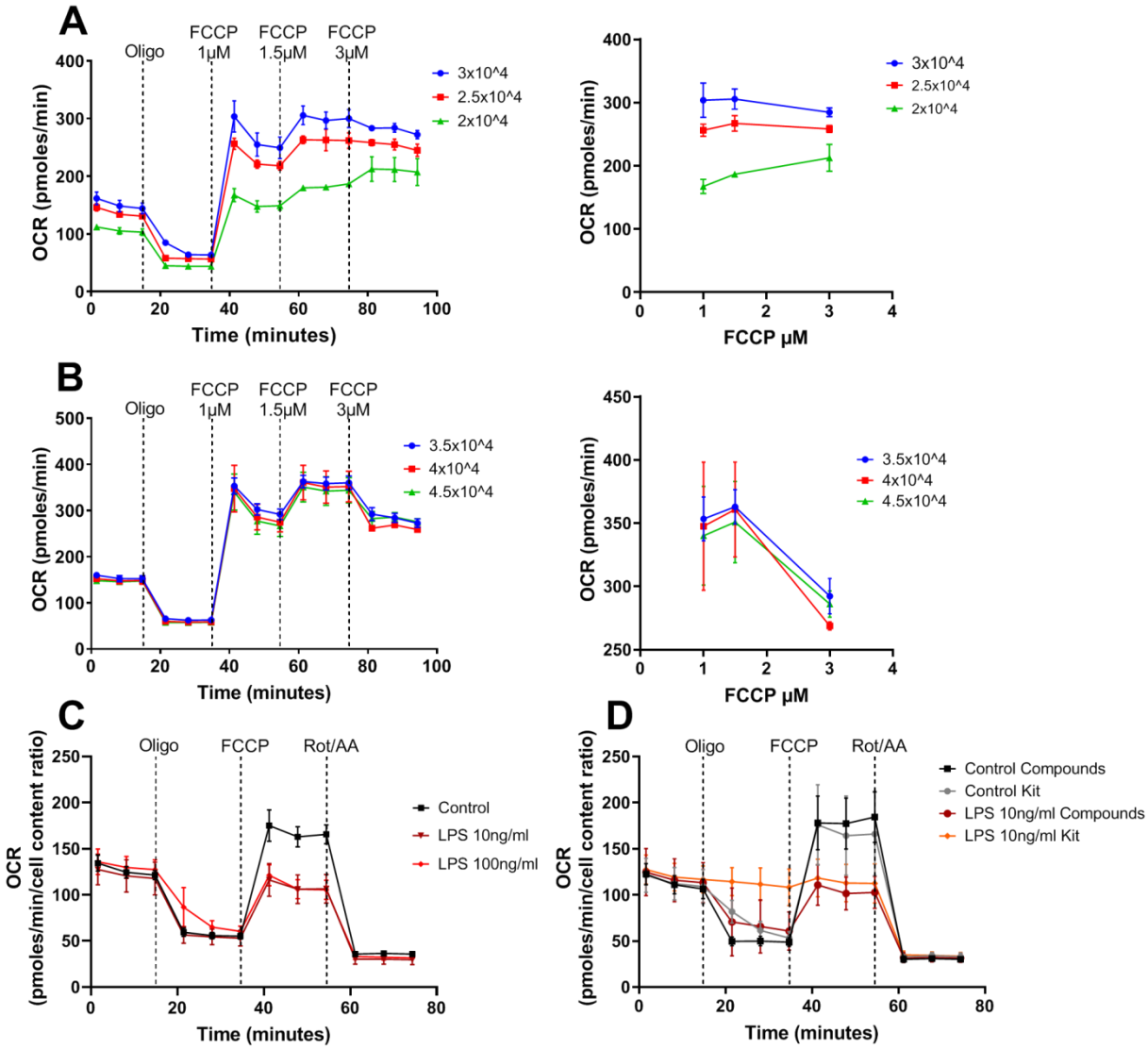


Figure 34: Optimisation of cell numbers and FCCP concentration for Seahorse analysis in BMDM.
(A, B) OCR measured in the medium of BMDM treated with oligomycin (1.5 µM) with different cell seeding numbers showing 2.5 x 10^4 seeded cells per well in ideal basal OCR range. FCCP titration was performed as depicted as a dose response curve to the right of the corresponding graphs, to confirm 1.5 µM as ideal concentration. (C) The LPS concentration of 10 ng/ml was chosen over 100 g/ml and (D) separately purchased compounds were preferred over ready to use compounds from a Kit (D). Results are expressed as mean ± SD. (A, B) N=2 replicate wells in one experiment, or (C, D) n= 2-3 separate experiments.

4.4.2 Effect of activin A on the immunometabolism of BMDM

After establishing the ideal conditions for Seahorse analysis using BMDM, the effect of activin A given at different time points was investigated with respect to metabolic changes in resting cells. In addition, the impact of activin A on the metabolic switch elicited by activating macrophages with LPS was examined (Fig. 35).

The mitochondrial stress test assay evidently was successful as the assay-typical OCR could be observed over the time measured (Fig. 35A). Specifically, oligomycin injection to block the ATP synthase led to a reduction in measured OCR, followed by an increase to maximum respiration with addition of the uncoupler FCCP. Subsequently, the mitochondrial oxygen consumption was blocked with Rot/AA, which reduced the OCR (Fig. 35A).

When comparing the graphs with different treatments, no obvious changes could be observed between control and activin A treatments or between LPS-activated cells with or without activin A treatment. However, the activation with LPS led to an inhibition of the FCCP-induced increase of respiratory capacity in all activated cells.

Based on the curve measurements, different parameters involved in mitochondrial metabolic activity could be determined (Fig. 35B-G). The basal respiration measured without additional injection of compounds was not found to be significantly different among the investigated groups (Fig. 35B). Similarly, no significant differences were found in the ATP-linked respiration, the proton leak, or the non-mitochondrial respiration (Fig. 35E-G). A slight increase in the proton leak could be observed in LPS-activated macrophages, which appeared to be reduced by the 18 h activin A treatment, but not by the 7-day treatment (Fig. 35F). When comparing the spare respiratory capacity, LPS was able to significantly decrease the capacity compared with non-activated macrophages (Fig. 35C). While LPS activation by itself appeared to lead to a slightly lower respiratory capacity compared with the baseline, a slight increase was observed with the 18 h activin A treatment, whereas the 7-day activin A treatment only marginally increased the capacity. Similarly, the maximum respiratory capacity was also significantly suppressed by activation with LPS compared with non-activated macrophages and no significant differences were found with additional activin A treatment (Fig. 35D).

Despite a slight elevation in the OCR graph when comparing FCCP-induced maximum respiratory capacity, activin A treatment did not significantly change the metabolic activity of the mitochondria in either non-activated or LPS-activated cells, when calculating the parameters based on the measurements of the assay.

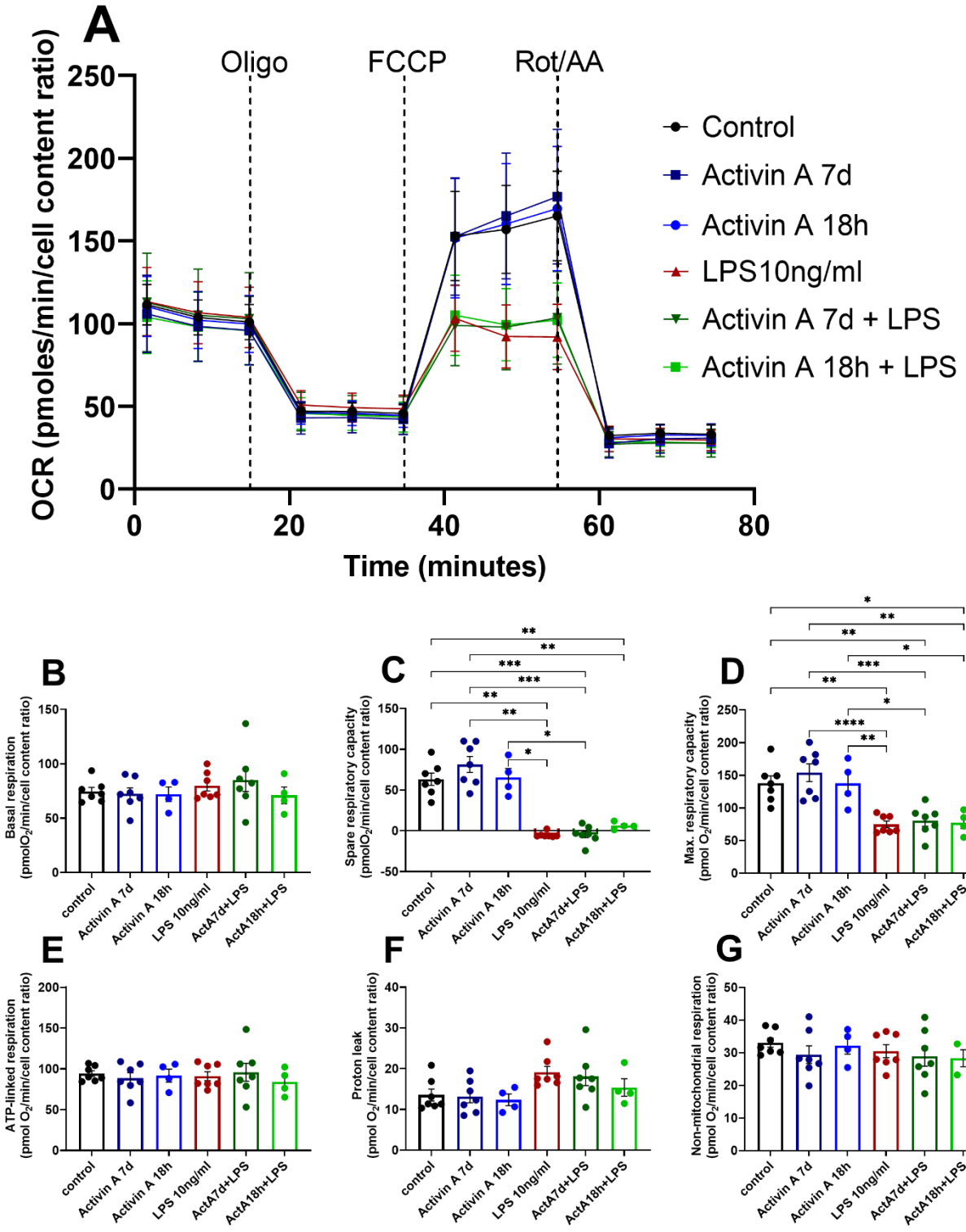


Figure 35: Effect of different duration of activin A treatment on immunometabolism in BMDM. A) OCR measured in the medium of BMDM comparing 18 h and 7-day activin A (50 ng/ml) treatment in resting and LPS-activated (10 ng/ml) cells. The oxygen consumption curve is measured during a mitochondrial stress test using oligomycin (1.5 µM), FCCP (1.5 µM) and Rotenone/Antimycin A (1 µM). The OCR values are normalised to the cell content ratio measured by crystal violet staining. Based on the OCR measured during the mitochondrial stress test, (B) basal respiration, (C) spare respiratory capacity, (D) maximum respiratory

capacity, (E) ATP-linked respiration, (F) proton leak, and (G) non-mitochondrial respiration were calculated. Results are expressed as mean ± SD. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=4-7 separate experiments.

4.4.3 Optimisation of Seahorse analysis for iMACs

After harvesting confluent iMACs, different cell numbers were seeded in Seahorse miniplates and analysed two days later for ideal basal OCR levels and the FCCP concentration eliciting maximum respiratory capacity (Fig. 36A-C). Additionally, it was tested whether different oligomycin concentrations (1 µM or 1.5 µM) would influence the OCR curve or the effect of FCCP, as various protocols recommended one of either concentration. Lastly, the appropriate concentration of LPS to successfully induce a metabolic switch, and therefore inhibit the increase of the maximum respiratory capacity by FCCP, was determined (Fig. 36D).

According to the confluency seen after initial testing, first 3 x 10^3 and 4 x 10^3 cells per well were seeded, where the basal OCR readings for both cell numbers appeared outside the upper range of the ideal levels, with 3 x 10^3 cells at around 190 pmoles/min, and 4 x 10^3 cells per well at around 300 pmoles/min (Fig. 36A). While there appeared to be no difference between using 1 µM or 1.5 µM oligomycin, none of the tested concentrations of FCCP with 2 µM, 3 µM and 3.5 µM could induce an increase in respiratory capacity, indicating that the concentrations were too high (Fig. 36A).

In further tests, low and high range FCCP concentrations were titrated with 3 x 10^3 seeded cells, in order to establish a range of FCCP (Fig. 36B), after which exact concentrations could be determined more accurately with different cell numbers in an additional assay (Fig. 36D).

Even though the basal OCR readings with 3 x 10^3 seeded cells were below the desired 130 pmoles/min, the levels were still well in the detection range of the assay (Fig. 36C). The titration of FCCP in low (0.5, 1, 2 µM) and high ranges (2, 3, 3.5 µM) successfully revealed that the respiratory capacity could be increased from 0.5 µM to 1 µM FCCP, while 2 µM FCCP led to a decline in induced respiratory capacity compared to 1 µM, which decreased further using 3 µM and 3.5 µM FCCP.

Based on these findings, a maximum FCCP concentration of 1 µM was confirmed by applying the low range FCCP titration to different seeded cell numbers, namely 2 x 10^3, 3.5 x 10^3, and 4 x 10^3 cells per well (Fig. 36D). While the basal OCR levels for 2 x 10^3 seeded cells per well were much lower than desired with around 70 pmoles/min, both 3.5 x 10^3 and 4 x 10^3 seeded cells showed basal OCR levels around 110-120 pmoles/min and were close to the 130 pmoles/min basal OCR levels

desired. As an immortalised cell line, each new vial of thawed cells appeared to grow slightly differently. This was seen when comparing two separate experiments with basal OCR levels of almost 300 pmoles/min in Fig. 36A and only 120 pmoles/min in Fig. 36C, despite using the same number of seeded cells (4×10^3). An additional reason for the lower basal OCR readings could have been that cells were accidentally washed off during medium changes. Since the seeded cell numbers of 4×10^3 cells per well resulted in too high basal OCR readings in previous experiments, it was decided to perform further experiments with 3.5×10^3 cells seeded per well. For the other parameters tested, oligomycin was used at 1.5 µM to ensure effectiveness and 1 µM FCCP proved to elicit maximum respiratory capacity.

In iMACs, different concentrations of LPS were tested to determine a successful metabolic switch, and inhibition of the FCCP-induced spare respiratory capacity in the Seahorse analysis (Fig. 36D). Similar to BMDM, LPS activation of the iMACs was tested at 10 ng/ml and 100 ng/ml in a mitochondrial stress test. Notably, these tests were performed prior to determining that the compounds bought individually were more reliable than the compounds from the Kit, therefore the oligomycin used in this trial lead to a slope-like decline of the OCR levels instead of a stable drop. Nevertheless, the comparison of 10 ng/ml or 100 ng/ml LPS still appeared valid and showed that both concentrations reduced the basal OCR readings and seemingly inhibited an increase of spare respiratory capacity by FCCP. Given that 100 ng/ml LPS led to slightly lower OCR levels than 10 ng/ml LPS, it was decided that 100 ng/ml LPS would be more reliable in iMACs to ensure the metabolic switch in LPS-activated macrophages.

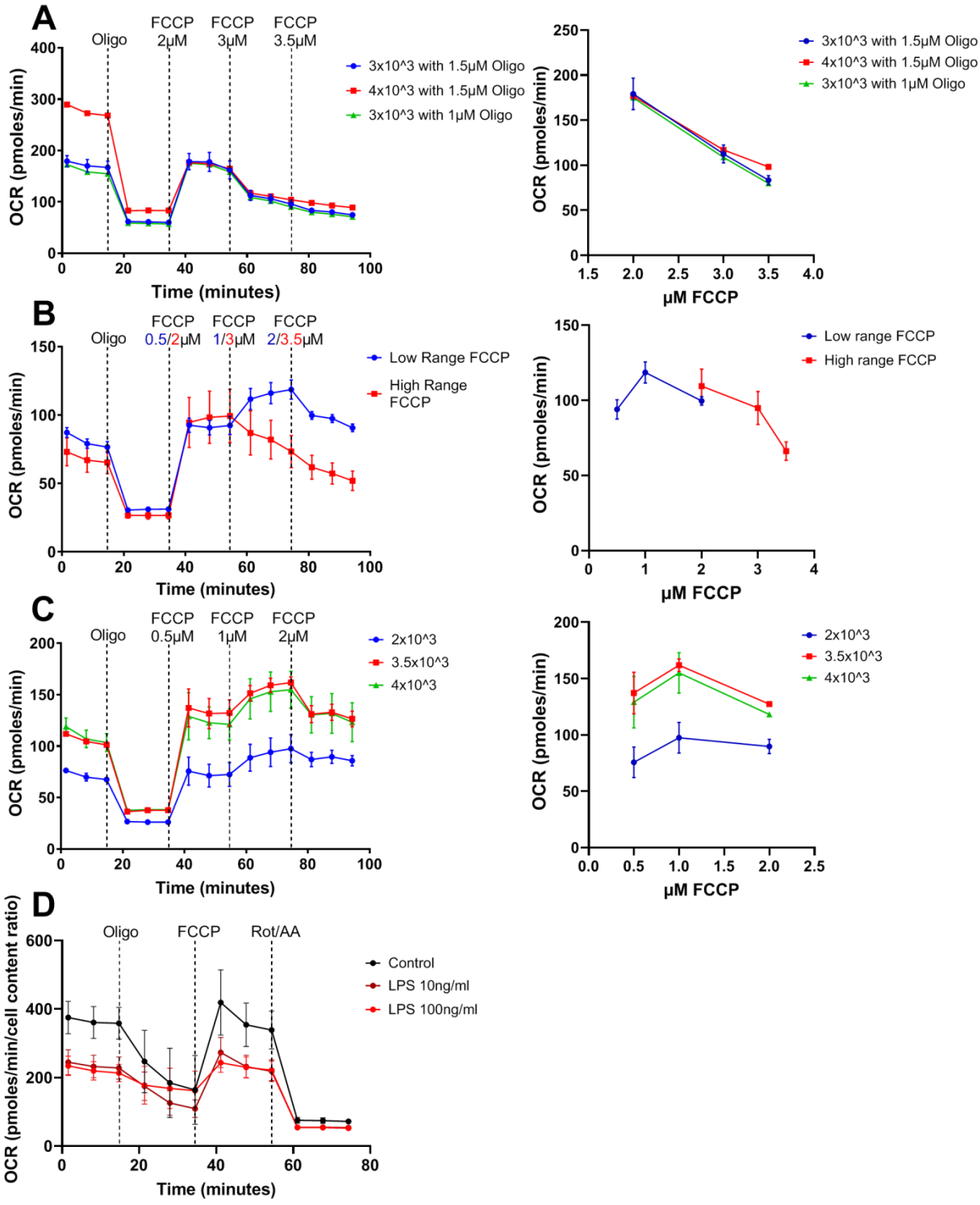


Figure 36: Optimisation of cell numbers and concentrations of oligomycin and FCCP in Seahorse analysis with iMACs. OCR measured in the medium of iMACs. (A, B) Oxygen consumption curve of iMACs seeded at different cell numbers, treated with oligomycin (1 or 1.5 µM) and FCCP, showing 1 µM FCCP as maximum concentration, as depicted in a dose response curve to the right of the corresponding graphs (C) Further testing of ideal FCCP concentration (0.5, 1, 2 µM) at different cell numbers seeded (2, 3.5, 4 x 10^3 cells/well) confirming 1 µM FCCP as ideal for 3.5 x 10^3 cells/well seeding density, as depicted in a dose

response curve to the right of the corresponding graphs. (D) The LPS concentration of 100 ng/ml was chosen over 10 ng/ml. Results are expressed as mean ± SD. (A-C) N=2-3 replicate wells in one experiment, or (D) n= 2 separate experiments (D).

4.4.4 Effect of activin A on the immunometabolism of iMACs

Based on optimised assay conditions for the iMAC cell line, it was further investigated, whether activin A treatment could influence mitochondrial oxygen consumption and affect metabolic immune responses of the macrophages (Fig. 37).

Similar to BMDM, the effect of activin A treatment was determined in resting and LPS-activated iMACs, assayed after 24 h of LPS treatment with the Seahorse XFp Analyzer.

The mitochondrial stress test assay performed with oligomycin, FCCP and Rot/AA was successful, and the assay showed the typical curve progressions after each compound injection (Fig. 37A).

When observing the curves of the graph, it could already be detected that the LPS activation led to a reduced basal respiration, beside the absence of a FCCP-induced increase of the spare respiratory capacity, when compared with the untreated control. While activin A appeared to lead to a slight reduction in OCR levels in resting macrophages, a distinct increase of OCR levels was observed when activin A-treated iMACs were activated with LPS.

These initial observations were confirmed after calculating the parameters based on the OCR measured with the Seahorse XFp Analyzer. Stimulation with LPS led to a significant reduction of the basal respiration compared with the control (Fig. 37B). This effect could be reversed almost back to control levels by activin A treatment in activated macrophages. Activin A treatment in resting cells, however, only led to a marginal reduction of basal respiration that was not significant.

Both the spare and the maximum respiratory capacity were significantly reduced by LPS activation of the iMACs compared with the control (Fig. 37C, D). While in both cases an increase with activin A treatment of LPS-activated iMACs was observed, the effect of activin A was only significant for the maximum respiratory capacity (Fig. 37D). The treatment with activin A in resting macrophages did not significantly change either the spare or maximum respiratory capacity (Fig. 37C, D).

Similar effects could be observed when calculating the ATP-linked respiration, as well as the proton leak (Fig. 37E, F). In both parameters, stimulation of the cells with LPS led to a reduction in respiration, which could be reversed almost to control levels by activin A treatment of LPS-activated macrophages. Again, the treatment with activin A hardly changed the investigated parameters compared with the untreated control. Lastly, the non-mitochondrial respiration was calculated, showing a reduction in LPS-activated iMACs compared with control levels (Fig. 37G). Activin A

treatment did not significantly change the non-mitochondrial respiration in LPS-activated iMACs or in resting cells.

Taken together, activin A treatment of LPS-activated iMACs was able to alleviate the LPS-induced metabolic switch, where macrophages preferably utilise glycolysis over OXPHOS in pro-inflammatory conditions. Contrary to the findings in BMDM, where activin A did not show any significant effects on metabolic immune responses, in the cell line iMACs, activin A led to a significant relief of the pro-inflammatory phenotype of the macrophages. This indicates a regulatory or anti-inflammatory role in cell lines. These observations were in accordance with the previous findings in cell lines on gene expression and protein secretion levels, emphasising the context-dependent effects of activin A in macrophages from different origins and maturation status.

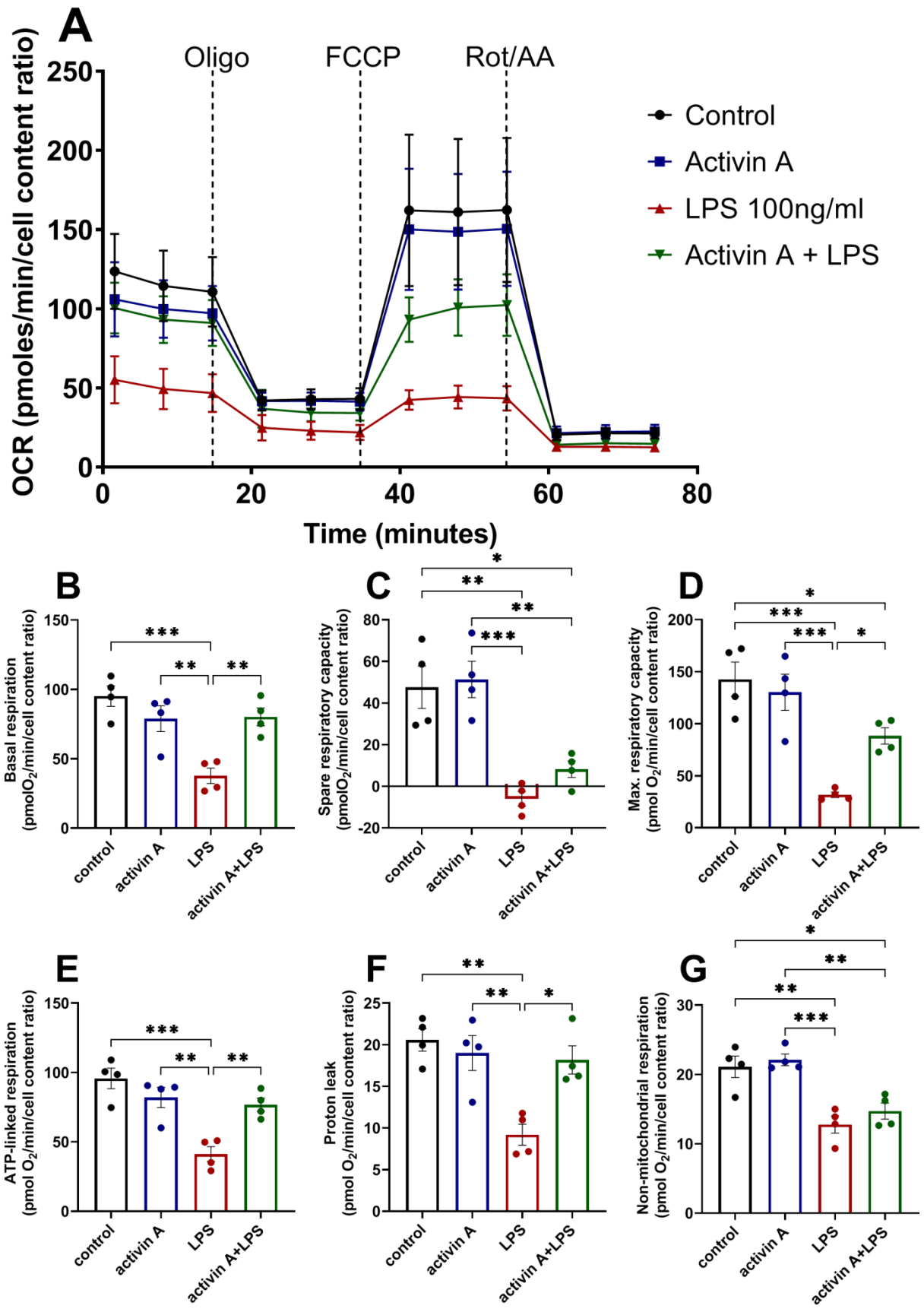

A
Oligo
FCCP
Rot/AA
OCR (pmoles/min/cell content ratio)
Time (minutes)
Control
Activin A
LPS 100ng/ml
Activin A + LPS
B
Basal respiration (pmolO2/min/cell content ratio)
C
Spare respiratory capacity (pmolO2/min/cell content ratio)
D
Max. respiratory capacity (pmol O2/min/cell content ratio)
E
ATP-linked respiration (pmol O2/min/cell content ratio)
F
Proton leak (pmol O2/min/cell content ratio)
G
Non-mitochondrial respiration (pmol O2/min/cell content ratio)
control
activin A
LPS
activin A+LPS

Figure 37: Influence of activin A treatment on immunometabolism in iMACs. OCR measured in the medium of iMACs treated with activin A (50 ng/ml) for 18 h in resting and LPS-activated (100 ng/ml) cells. The oxygen consumption curve is measured during a mitochondrial stress test using oligomycin (1 µM), FCCP (1 µM) and Rot/AA (1 µM). The OCR values are normalised to the cell content ratio measured by crystal violet staining. Based on the oxygen consumption rate measured during the mitochondrial stress test, (B) basal respiration, (C) spare respiratory capacity, (D) maximum respiratory capacity, (E) ATP-linked respiration, (F) proton leak, and (G) the non-mitochondrial respiration were calculated. Results are expressed as mean ± SD. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 determined by one-way ANOVA and *post-hoc* Tukey's test or Brown-Forsythe and Welch ANOVA and *post-hoc* Dunnett's T3 test where applicable. N=4 separate experiments.

5 Discussion

The regulatory phenotype of the testicular macrophages contributes to the immune privilege of the testis and therefore plays an important role in inflammatory damage and infertility (Hedger, 2012). How exactly the testicular macrophages obtain their immunoregulatory phenotype is, however, not well understood. It appears that the polarisation of testicular macrophages is multifactorial and potentially determined by the microenvironment of the testis (Bhushan et al., 2020). Multiple locally produced immunoregulatory factors such as testosterone, prostaglandins, and corticosterone, have been described as possible candidates influencing the phenotype of testicular macrophages and have been shown to induce immunosuppressive properties in BMDM (Bhushan et al., 2020; M. Wang et al., 2017; Z. Zhang et al., 2020).

The cytokine activin A, which is produced in the testis, has previously been implicated in various aspects of immune responses, promoting both pro- as well as anti-inflammatory actions in macrophages (Chen & ten Dijke, 2016; Sierra-Filardi et al., 2011; S.-Y. Wang et al., 2008). Additionally, activin A is thought to elicit anti-inflammatory effects on microglia, the macrophage-like cells of the immune privileged brain, suggesting activin A as an viable candidate for immunosuppressive actions in the testis (Sugama et al., 2007).

The Sertoli cells are among other cell types in the testis, which express activin A. Upon an inflammatory stimulus the Sertoli cells increase their production of activin A, suggesting a role in the immune response in the testis (Hedger & Winnall, 2012; Kazutaka et al., 2011). Additionally, Sertoli cells have been shown to possess regulatory and anti-inflammatory properties in transplantation studies and have been reported to secrete immunoregulatory factors, which could contribute to immune privilege (Meinhardt & Hedger, 2011; Sanberg et al., 1996; Suarez-Pinzon et al., 2000).

To investigate the potentially context-dependent actions of activin A and its influence on macrophage phenotypes, *in vitro* cultures of macrophages of different origin, specifically cell line macrophages and primary BMDM, were studied for their responses to activin A treatment in resting and activated conditions. These *in vitro* cultures were utilised as surrogate models for testicular macrophages, as the numbers of these cells which can be isolated from mouse testes are very limited and thus not sufficient for extensive cultures and experiments. Moreover, testicular macrophages are already primed by the testis environment, complicating, or even preventing analysis of the effects of activin A on naïve macrophages. The responses of the cultured macrophages were assessed by flow

cytometry, qRT-PCR, ELISA, Griess assay, arginase activity assay, as well as metabolic analysis with the Seahorse XFp Analyzer to gain insight on actions on gene expression and functional activity.

5.1 Differential effects of GM-CSF, M-CSF and activin A on the phenotype of BMDM

To investigate the influence of activin A on macrophage phenotypes in a resting and activated state, *in vitro* cell culture of primary BMDM was employed. The bone marrow precursor cells needed to be differentiated towards macrophages first to study their responses. Commonly utilised growth factors for *in vitro* differentiation of bone marrow precursor cells into BMDM are GM-CSF and M-CSF, whereby GM-CSF appears to skew the cells towards a more M1 phenotype, while M-CSF primes the cells towards M2 macrophages (Fleetwood et al., 2007; Hamilton et al., 2014). In order to determine the most suitable culture system for the present study, the cells were analysed with flow cytometry after being differentiated into BMDM with either growth factor. Sufficient viability and successful differentiation of the precursor cells into macrophages was verified in most experiments by using the appropriate markers for immune cells (CD45) and macrophages (CD11b and F4/80). This demonstrates a generally functioning experimental setup. However, GM-CSF did not seem to consistently induce a uniform CD11b+F4/80+ macrophage population. While in two separate experiments the CD45+ immune cell population contained between 83 % and 86 % CD11b+F4/80+ macrophages, only 58.3 % of the cells were CD11b+F4/80+ in another experiment. The observed inconsistencies could be due to technical or experimental issues but could also be a result of the differentiation of the cells with GM-CSF, since M-CSF induced a macrophage population of more than 97 % CD11b+F4/80+ cells. While the growth factor GM-CSF has been utilised for BMDM differentiation in the literature, it is additionally employed for differentiation of bone marrow precursor cells towards dendritic cells, and it has been shown that GM-CSF elicits a heterogeneous population of macrophages, granulocytes, and dendritic cells from bone marrow cultures (Helft et al., 2015; Na et al., 2016). Differences in the detected macrophage populations could be due to slight variations in the handling of the cells when changing medium, as well as the proportion of the detachment of the adherent macrophages from the cell culture dish, as dendritic cells seemingly are loosely adherent, while macrophages would be more strongly adherent to the culture dish (Helft et al., 2015). Therefore, slightly different populations could have been harvested prior to analysis.

Nevertheless, when further analysing the CD11b+F4/80+ macrophages for expression of the M2 macrophage marker CD206 after differentiation with either growth factor, as expected M-CSF differentiation led to a highly induced M2-phenotype, while GM-CSF showed less than half of the macrophage population to be CD206+, indicating a more M1-like phenotype. Based on the literature, it was expected that M-CSF would skew the macrophages towards an M2 phenotype, while GM-CSF would induce an M1 phenotype (Fleetwood et al., 2007; Hamilton et al., 2014).

After establishing a lower percentage of CD206+ and therefore fewer M2 macrophages in the GM-CSF-differentiated BMDM, compared to M-CSF-derived BMDM, the effect of activin A was examined. Specifically, it was investigated whether activin A could potentially skew the GM-CSF-derived M1 macrophages towards a M2 phenotype by increasing the CD206+ population, which would help elucidate the context-dependent effects of activin A on macrophage responses. Preliminary unpublished experiments from the JLU laboratory indicated an increase of CD206+ cells by activin A treatment in GM-CSF-derived BMDM. However, in the present study these results could not be significantly reproduced, despite individual experiments showing an increase in the CD206+ population after activin A treatment. A lack of a significant effect of activin A treatment in GM-CSF-derived BMDM could potentially be due to the inconsistency and heterogeneity of both the differentiated CD11b+F4/80+ macrophages, as well as the CD206+ population. The CD206+ population varied considerably between around 60 % and 90 % across repeated experiments. Therefore, a further increase of CD206+ cells by activin A, from already around 90 % in the untreated control, could not be detected, although an increase was visible when the control population showed fewer CD206+ cells. An increase of experimental replicates could potentially differentiate between outliers or an actual effect of activin A on inducing CD206+ M2 macrophages from a M1-skewed population.

In the literature it has been shown that activin A exerts both pro- and anti-inflammatory effects (Hedger et al., 2011; Indumathy et al., 2020; Sierra-Filardi et al., 2011). More specifically, on the one hand activin A has been reported to promote M1 polarisation by GM-CSF in BMDM and shows elevated excretion levels in GM-CSF-derived macrophages compared with BMDM differentiated with M-CSF (Sierra-Filardi et al., 2011). However, in this study by Sierra-Filardi et al. (2011), the M2 marker CD206 was not investigated, potentially pointing towards factor-specific differences in the actions of activin A on immune responses. On the other hand, in the context of testicular macrophages it was reported that elevated activin A levels in a transgenic mouse model skew macrophages towards the M2 phenotype (Indumathy et al., 2020).

The potential tendency of activin A to slightly increase the CD206+ M2 population in GM-CSF-derived BMDM enhances the impression of a context-dependent, or factor-dependent, mechanism of the reported dual role of activin A actions in immune responses.

For the following experiments in the present study, M-CSF was chosen as the macrophage differentiation factor due to higher reproducibility and homogeneity of macrophage cultures compared to GM-CSF-derived cells, but also due to its relevance in the testis immune microenvironment and testicular macrophage development, where M-CSF has been shown to be essential for sustaining testicular macrophages as well as fertility (P. E. Cohen et al., 1996, 1997; Meinhardt et al., 2018; Pollard et al., 1997).

5.2 Effects of activin A on macrophages: comparison of cell lines and primary bone marrow-derived macrophages

Activin A has been shown to exert both pro- as well as anti-inflammatory actions in immune responses in various contexts (Hedger et al., 2011). In the testis microenvironment, activin A is upregulated in experimental autoimmune orchitis in mice (Nicolas, Michel, et al., 2017), while also skewing testicular macrophages towards a M2 phenotype in transgenic mouse models with elevated activin A levels (Indumathy et al., 2020). Additionally, many studies have investigated the effects of activin A on immune responses *in vitro*, utilising a variety of cell types and models. Notably, activin A reduced phagocytosis in LPS-activated murine peritoneal macrophages, while it increased phagocytosis in activated murine microglia (Diesselberg et al., 2018; Zhou et al., 2009), highlighting differential effects on immune cells from different tissues. Additionally, studies with the immortalised murine macrophage cell line RAW showed a suppressive effect of activin A on LPS-induced pro-inflammatory cytokine production, while an increase of pro-inflammatory factors elicited by activin A was found in resting macrophages (Ge et al., 2009; S.-Y. Wang et al., 2008). This again highlights the context-dependent actions of activin A, which may be related to the origin, maturation, or activation status of the macrophage.

To further elucidate the role of activin A in immune responses in different contexts, especially in *in vitro* culture systems and the potential implication of activin A in the testis microenvironment, its effects on macrophage cultures was examined. Specifically, resting and activated primary BMDM, as well as two murine cell lines, RAW macrophages and immortalised bone marrow-derived iMACs,

were investigated with regard to their gene expression and secretion profiles of pro- and anti-inflammatory markers, as well as their changes in immunometabolic responses.

5.2.1 Regulation of pro- and anti-inflammatory gene expression patterns by activin A differs between primary and cell line macrophages

To assess the influence of activin A on immune responses of macrophages from different origins, the gene expression profiles of immunologically relevant genes were investigated in resting and activated BMDM, RAW macrophages and iMACs. Different concentrations of activin A indicated a dose-dependent effect on the expression of the genes investigated in both resting and activated iMACs. The activation of the cells by LPS proved to be successful, as shown by an upregulation of the pro-inflammatory genes compared to the untreated control cells, which indicated a functioning culture system to study the effects of activin A on stimulated macrophages. As consistent with the literature, the results showed differential effects of activin A treatment on the different macrophage models in resting and activated cells. Most strikingly, it was found that between primary BMDM and cell lines, activin A exerted opposing effects on cytokines associated with a pro-inflammatory or M1 phenotype, such as *Tnf*, *Nos2*, *Gpr18*, *Fpr2*, *Il1β*, and *Clec7a*. In primary BMDM cultures, the gene expression of these pro-inflammatory markers, with the exception of *Clec7a*, was promoted by activin A treatment, while in both cell lines, RAW macrophages and iMACs, the expression of these genes was suppressed, potentially reducing the inflammatory response. In contrast, *Clec7a* expression was suppressed by activin A treatment in primary BMDM, while a slight elevation was observed in RAW macrophages. This regulation of *Clec7a* may be explained by its suggested role as M2 subtype marker based on its increased expression in alternatively activated macrophages, despite its known capacity to promote pro-inflammatory cytokines (Ferwerda et al., 2008; X. Li et al., 2019; Rőszer, 2015; Willment et al., 2003). These findings compare with previous reports of the effects of activin A, showing inhibition of the LPS-induced pro-inflammatory phenotype in the RAW macrophages (S.-Y. Wang et al., 2008). In contrast, studies with BMDM showed a promotion of the M1 phenotype by activin A (Sierra-Filardi et al., 2011).

Although the investigated macrophage models showed consistent tendencies in the expression patterns among the pro-inflammatory genes investigated, not all of them were significantly changed by activin A in LPS-stimulated cells or when compared with untreated cells. While *Tnf*, *Nos2*, and *Gpr18* expression was significantly increased by activin A in LPS-activated BMDM, the expression

of *Il1β* and *Fpr2* was significantly induced by activin A compared with basal expression levels. Notably, a similar tendency towards an increased expression of *Il1β* by activin A treatment in LPS-stimulated BMDM was observed, while the expression of *Fpr2* appeared unchanged or even slightly reduced by activin A in LPS-activated BMDM, though neither of these effects were statistically significant. In addition, when repeating the activin A treatments of BMDM in a different laboratory (Monash), the increase of *Fpr2* expression by activin A treatment was not observed. Indeed, the basal expression levels appeared to show a tendency towards a reduction, which could be attributed to different conditions in the two laboratories (for example, mice, reagents, or equipment), despite keeping to the same conditions and culture protocol as far as possible. A potential reason could be that *Fpr2* has been implicated in both pro- and anti-inflammatory responses. On the one hand, *Fpr2* has been postulated as a marker for the M1 phenotype due to its significant upregulation in classically activated macrophages (Jablonski et al., 2015). On the other hand, it also appears to be involved in the switch from pro- to anti-inflammatory responses through annexin 1 mediated AMPK signalling (McArthur et al., 2020). However, the actions of *Fpr2* appear context- and ligand-dependent, as its suppression in endotoxin-induced acute lung injury alleviated the inflammatory response, and its over-expression enhanced the pro-inflammatory response in LPS-activated macrophages *in vitro* (H. Liu et al., 2020; Ye et al., 2009). In both macrophage cell lines, activin A treatment of LPS-stimulated macrophages led to a significant reduction in the expression of *Tnf, Nos2, Gpr18,* as well as *Il1β* and *Fpr2*. In addition to the effect of activin A on LPS-activated cell line macrophages, a decrease in basal expression induced by activin A could also be observed for *Tnf* in RAW macrophages and *Fpr2* in iMACs.

In contrast to previous reports, no opposing regulation of pro-inflammatory genes was observed when comparing the effect of activin A on resting and LPS-activated macrophages in either primary BMDM or cell line macrophages. Activin A either enhanced or inhibited the gene expression in both LPS-stimulated and resting macrophages within the same cell type studied. It did not promote opposite reactions of the cells towards a more pro-inflammatory phenotype in resting cells and anti-inflammatory in activated macrophages as described for RAW macrophages by Ge et al. and Wang et al., who showed an increase of IL-1β and IL-6 secretion in resting cells (Ge et al., 2009) but a reduction of IL-1β and NO in LPS-activated cells (S.-Y. Wang et al., 2008), whereby the latter finding could be reproduced in the present study.

Similar to the previously discussed pro-inflammatory genes, the pro-inflammatory marker *Il6* was found to be significantly reduced by activin A in LPS-activated RAW macrophages, however no significant changes were detected in BMDM cultures. A pro-inflammatory effect of activin A on

resting RAW macrophages, measured by increased IL-6 secretions as proposed by Ge et al. (2009), could not be found in the present study. However, the findings of Ge et al. (2009), were based on secreted protein, while the present study examined mRNA expression. Therefore post-transcriptional influences of activin A could potentially explain the increased secretion levels. In addition, in activated lymphocytes, activin A treatment resulted in inhibited secretion of IL-6, again demonstrating the context- and cell type-specific actions of activin A (Hedger et al., 2000).

In contrast to the opposing effects on pro-inflammatory markers elicited by activin A in primary BMDM and cell line macrophages, the regulation of genes involved in the M2 phenotype or regulatory immune responses, appeared consistent among the investigated macrophage cell types. Most strikingly, the expression of the major anti-inflammatory marker *Arg1* was the only gene consistently stimulated by activin A in all macrophage types investigated in the resting, as well as LPS-activated state, while other typical M2 markers, *Il10*, *Mrc1*, and *Chil3/Ym1*, were suppressed by activin A in all tested cells and both in resting and LPS-activated macrophages. Notably, *Chil3/Ym1* could not successfully be detected in RAW macrophages due to low expression. Similarly, *Il10*, although detectable, showed very low expression in RAW macrophages with high variance and delayed induction by LPS. In the BMDM experiments at the Monash laboratory, the 7-day treatment of activin A did not significantly increase *Arg1* expression in resting cells as opposed to the previous findings at the JLU laboratory. However, the tendency of an increased expression was still visible and potentially was not significant due to a somewhat higher variance between the samples. The shorter 18 h activin A treatment significantly increased the expression of *Arg1* to a seemingly greater extent than the long-term treatment.

With the exception of *Arg1*, these findings would support a more pro-inflammatory action of activin A by suppressing the expression of anti-inflammatory mediators. This assumption has also been postulated by Sierra-Filardi et al., who described an increased *Il10* mRNA expression following blocking of activin A and observed a promotion of the M1 phenotype by activin A through suppression of anti-inflammatory mediators in BMDM (Sierra-Filardi et al., 2011). The suppression of anti-inflammatory markers could have been expected in BMDM, since activin A treatment increased the expression of pro-inflammatory markers and promoted the M1 phenotype. However, in the cell line macrophages, an alleviation of the pro-inflammatory response was observed. Therefore, an additional suppression of anti-inflammatory genes by activin A appeared counterintuitive, further highlighting context- and target-dependent actions of activin A in immune responses. Moreover, the consistent upregulation of *Arg1* as the only induced anti-inflammatory gene is interesting, especially in the BMDM where activin A seemingly promotes the pro-inflammatory phenotype. Therefore, the

induction of *Arg1* appears potentially conserved among the macrophage cell types tested and may be an effect independent of the otherwise context-dependent actions of activin A. The increased expression of *Arg1* and, fittingly, a decreased expression of *iNos* (or *Nos2*), had also previously been shown in an additional macrophage type, the peritoneal macrophages (Ogawa et al., 2006). This led to the assumption that activin A promotes an anti-inflammatory phenotype in macrophages (Ogawa et al., 2006).

The treatment with activin A suppressed the expression of the co-stimulatory molecule *Slamf1* compared to basal levels in BMDM, but not in iMACs. However, in LPS-activated cells activin A reduced the expression of *Slamf1* in both BMDM and iMACs. A similar regulation was found for the p110δ subunit of PI3K encoded by *Pik3cd*. The expression of *Pik3cd* was suppressed by activin A in LPS-activated BMDM and RAW macrophages, while in BMDM alone, activin A also reduced basal expression. Similar to *Chil3/Ym1*, *Slamf1* also could not be detected in RAW macrophages due to its low expression. While *Slamf1* was also minimally expressed in resting macrophages, LPS stimulation induced its expression in BMDM and iMACs. This stimulation could be almost abolished by activin A in BMDM G, as well as iMACs. Notably, when repeating the experiment, the significant effect elicited by activin A could not be replicated in BMDM A. However, the same tendency was clearly observable. The literature yielded no indication that a direct inhibitory effect of activin A on *Slamf1* expression has been found previously, indicating that *Slamf1* has been identified as a novel target of activin A in the present studies. Furthermore, *Casp1*, as part of the inflammasome, was suppressed in BMDM and iMACs, but was unchanged in RAW macrophages. Despite comparable tendencies of the effects of activin A on the expression of *Casp1* in BMDM G and BMDM A, a significant decrease in basal levels was only found in BMDM G, while a significant reduction in LPS-activated macrophages was detected in BMDM A. Casp1 is part of the inflammasome and cleaves pro-IL-1β into mature IL-1β (Afonina et al., 2015). Based on the regulation of other pro-inflammatory genes, including *Il1β,* it could have been assumed that *Casp1* would also be induced by activin A in BMDM. However, *Casp1* appeared to be differently regulated than the other investigated genes associated with pro-inflammatory responses.

Activin A treatment could not change the expression of all investigated immune related genes and several of them were only affected in one of the cell types investigated. The transcription factor, *Atf3*, which is associated with the promotion of the M2 phenotype (Sha et al., 2017), as well as the anti-inflammatory transcription factor *Klf4* (Liao et al., 2011), were suppressed by activin A in resting primary BMDM G, similar to the M2 markers previously discussed, while cell line macrophages were unaffected. Due to high variances between the samples, however, the effects of activin A on basal

expression levels of *Klf4* could not be replicated in BMDM A, despite similar tendencies. In LPS-activated macrophages, the expression of *Klf4* was suppressed by activin A in both BMDM G and BMDM A. A comparable regulation could also be observed for an additional member of the PI3K/AKT pathway, namely *Akt1*, which was suppressed by activin A in resting BMDM, but unchanged in RAW macrophages. Conversely, *Akt3*, also part of the PI3K/AKT-signalling pathway was not affected by activin A treatment in primary BMDM, but was stimulated by activin A in the cell line RAW macrophages. Several members of the PI3K/AKT signalling pathway appeared to be differently influenced by activin A in the macrophages investigated. This is indicating a possible involvement of the signalling pathway in the effect of activin A on differing immune responses. Activin A had previously been shown to activate the PI3K pathway in colon cancers, while PI3K/AKT signalling is also known to regulate immune responses through both induction of inflammatory signalling, as well as its resolution (Amici et al., 2017; Bauer et al., 2015).

In addition to the described cell type specific actions of activin A, its effect on immune responses also appeared to be impacted by the duration of the activin A treatment. The previously observed changes of gene expression in BMDM elicited by activin A were mostly seen in the long-term 7-day activin A treatment, while the shorter 18 h treatment (administered after 6 days of MCSF-induced differentiation into macrophages) showed less effects. Only the induction of *Arg1* by the 18 h activin A treatment appeared even more pronounced than with the long-term treatment, while *Il10* was suppressed by 18 h activin A compared to basal levels. Other previously described effects of the long-term treatment with activin A in BMDM were not observed when activin A was given 18 h prior to the LPS stimulation. For example, the 7-day treatment increased the expression of *Tnf* in LPS-activated cells, but no changes were detected with the 18 h treatment. Similarly, the expression of *Clec7a* was significantly suppressed by the 7-day activin A treatment compared to basal levels, whereas the 18 h treatment showed no significant effects and even appeared to slightly increase the expression. Additionally, *Casp1* and the anti-inflammatory marker *Klf4* were suppressed by the 7-day activin A treatment, but the 18 h treatment did not significantly change the expression of these genes. The observation that activin A administered after differentiation of BMDM (18 h treatment) did not change the expression patterns of most of the genes investigated, while activin A provided during differentiation (7-day treatment) changed basal expression patterns of several genes, could indicate that activin A has a stronger impact on the basal polarisation state of the macrophages during their differentiation and maturation. This could indicate that activin A may play an important role in the microenvironment of the testis, especially during the differentiation or maturation of the testicular macrophages towards an immunoregulatory phenotype. In addition, all investigated macrophages

were replenished with medium containing activin A prior to the activation with LPS. This means the already differentiated macrophages that received the shorter 18 h activin A treatment were exposed a second time to activin A prior to LPS stimulation. In contrast, the long-term activin A-treated BMDM had been exposed to activin A for the duration of their differentiation from the day of their isolation. It could be hypothesised that the second stimulation of the matured BMDM with activin A in the 18 h treatment led to a tolerance towards the stimulus in a similar manner as seen for repeated LPS activation, therefore not eliciting a response (Butcher et al., 2018). In contrast, as mentioned above, the exposure to activin A during the 7-day differentiation process of BMDM could have led to a polarisation of the basal state of the mature macrophages, as indicated by changes in their basal expression pattern. An effect of activin A on the differentiation and maturation of cells has been suggested in previous studies. In similar experiments, it was shown that activin A treatment while differentiating M-CSF-derived macrophages reduced the expression of the M2 marker folate receptor beta (*Folr2*) continuously when the gene expression was investigated during the differentiation at 3 days as well as after the differentiation at 7 days (Sierra-Filardi et al., 2011). Further studies showed an inhibitory effect of activin A treatment on the maturation of monocyte-derived dendritic cells with a suppression of human leukocyte antigen-DR (HLA-DR), immature morphology and diminished ability to activate T cells (Segerer et al., 2008). For a more extensive insight on the context-dependent effects of activin A, different combinations of time points of treatment as well as different combinations of activin A and LPS concentrations could be investigated.

Interestingly, the expression of some genes was also oppositely regulated by the LPS stimulation of the primary BMDM compared to the cell line macrophages. This observation suggests possibly differing maturation or even activation states in the putatively resting cells prior to activation with LPS, or alternatively, differences in the inflammatory signalling mechanism of the activated cells. These potential differences between the investigated macrophages models could indicate that previously found opposing regulations may not be solely activin A-dependent differences. Specifically, LPS suppressed the expression of the anti-inflammatory marker *Arg1* in BMDM, but induced it in both cell line macrophages, while the expression of the anti-inflammatory transcription factor *Klf4*, and the PI3K/AKT signalling members *Akt3* and *Pik3cd*, was increased by LPS stimulation of BMDM and suppressed in the cell lines. This could indicate a different regulation of the induction of the PI3K/AKT signalling pathway in BMDM as opposed to the cell line macrophages, as well as differences in the regulation of the transcription of anti-inflammatory mediators. Differences in the responses of primary isolated cells compared with cell line macrophages have been observed before (Berghaus et al., 2010; Chamberlain et al., 2009; Guo et al., 2015).

Although RAW macrophages were reported to respond most similarly to BMDM when compared to splenic macrophages and bone marrow-derived dendritic cells, differences could still be found (Berghaus et al., 2010). Specifically, RAW macrophages and BMDM both expressed high levels of CD11b and F4/80, and showed a high production of TNF in response to LPS compared to the other primary cells (Berghaus et al., 2010). However, RAW macrophages exhibited higher levels of CD14 compared to BMDM (Berghaus et al., 2010). Moreover, proteomic analysis of the phagosomes of RAW macrophages and BMDM revealed, among others, higher levels of Chil3/Ym1 and Mrc1 in BMDM, while RAW macrophages showed higher levels of Clec7a and the IFNγ receptor (Guo et al., 2015). Therefore, caution is required when extrapolating results obtained by cell line macrophages, especially as it has also been reported that their responses can change depending on the duration of a continuous culture (Berghaus et al., 2010; Chamberlain et al., 2009; Guo et al., 2015). A general difference in the regulation of inflammatory responses by the macrophages investigated was also indicated by expression patterns of genes over time. Specifically, both *Tnf* and *Il1β* showed an increase of their expression over the time course measured in BMDM, while in the cell line macrophages, their expression pattern declined over time. Of note, the cell models investigated were grown in different culture media with different additives, as appropriate, which potentially could have an impact on their responses. It could be investigated whether the growth factor M-CSF, utilised to differentiate BMDM, could change the effects of activin A in cell line macrophages towards the effects seen in M-CSF-derived BMDM.

In summary, the findings support the hypothesis that activin A acts in a very context-dependent manner and can influence the transcription profiles of genes associated with immune responses differently in macrophages from different origin. The actions of activin A seemed to be influenced by the origin or maturation status of the macrophage type investigated, as well as by the duration of the activin A treatment, which could give an insight into the diverse roles of activin A described in the literature. Due to its context-dependent actions, a clear distinction of activin A as a factor promoting either pro- or anti-inflammatory responses cannot be made. The data in the present study establish that effects of activin A on macrophage development are complex and may be pro- or anti-inflammatory under different biological or experimental conditions. Nevertheless, it can be concluded that the actions of activin A on primary BMDM are predominantly of a pro-inflammatory nature, while its effect on the cell line macrophages leads to a reduction of the pro-inflammatory response.

The comparison of primary BMDM with commonly employed cell line macrophages revealed additional opposing responses to LPS on the gene expression level. This specifically affected the expression of anti-inflammatory genes, as well as members of the PI3K/AKT signalling pathway.

These opposing effects seen by LPS stimulation further support the observation of differing immune responses elicited by activin A in BMDM compared to cell line macrophages.

5.2.2 The effects of activin A on protein secretion in macrophages

In parallel with the investigation of the effect of activin A on immune gene expression responses in macrophages from different origin, the secretion of pro-inflammatory TNF and anti-inflammatory IL-10 was investigated with ELISA in BMDM and RAW macrophages. Similar to the changes found on the mRNA level, the protein secretion of TNF was oppositely influenced by activin A treatment in LPS-activated BMDM compared with RAW macrophages. Specifically, activin A elicited an increase of TNF secretion in BMDM after 6 h and 24 h LPS activation, whereas it suppressed TNF in LPS-activated RAW macrophages at the same time points. While IL-10 secretion in BMDM could not be significantly reduced by activin A treatment, a tendency towards a reduction was still apparent. In RAW macrophages, the secreted levels of IL-10 were below the detection limit of the assay and could therefore not be analysed, which is in line with the observed low mRNA expression levels of *Il10* in the present study. It could be suspected, however, based on the significant reduction of *Il10* mRNA in activin A treated RAW macrophages, that the secretion of IL-10 may also be reduced by activin A. The observed trend of a reduced IL-10 secretion in LPS-activated BMDM elicited by activin A, could potentially become significant with an increase in replicate numbers. This would be in line with previous studies, where activin A treatment led to a significant reduction of IL-10 secretion (Sierra-Filardi et al., 2011). In the same study, however, activin A showed no effect on TNF secretion in LPS-activated, M-CSF-differentiated macrophages (Sierra-Filardi et al., 2011), in contrast to the prominent increase in BMDM detected in the present study. In non-activated BMDM from rat, however, activin A significantly increased the release of TNF (Nüsing & Barsig, 1999), similar to the effect seen on activated murine BMDM in the present study.

5.2.3 Production of NO and changes in arginase activity in response to activin A

Consistent with the observed effect of activin A in dampening the pro-inflammatory response in RAW macrophages, it was found that activin A significantly reduced the concentration of nitrite, used as a marker for NO, in LPS-activated RAW macrophages. Similarly, in the literature it has been shown that activin A reduced NO levels in both activated RAW macrophages and in murine peritoneal macrophages (Ogawa et al., 2006; S.-Y. Wang et al., 2008; X. J. Zhang et al., 2005).

It could not be determined whether the production of nitrite was upregulated by activin A in LPS-activated BMDM, because the levels were below the detection limit of the assay. Given the pro-inflammatory effect of activin A on BMDM gene expression and protein secretion, an increase in nitrite production would have been expected, in contrast to the observed reduction of pro-inflammatory mediators, including nitrite, in RAW macrophages. In agreement with these assumptions, it has been shown in prior literature that activin A treatment increases the levels of nitrite in quiescent rat BMDM (Nüsing & Barsig, 1999).

The activity of the enzyme arginase was investigated in BMDM, as it is associated with anti-inflammatory properties of macrophages through its competition with iNOS (or NOS2) for the same substrate, L-arginine, and its promotion of tissue repair (Rath et al., 2014). Following activin A treatment of BMDM, a potential increase of arginase activity was observed, which was in line with the increased mRNA expression levels of *Arg1* elicited by activin A in the present study. A similar induction of *Arg1* expression by activin A treatment has previously been reported for murine peritoneal macrophages (Ogawa et al., 2006).

5.2.4 The effect of activin A on the immunometabolic changes of resting and LPS-activated macrophages

Inflammatory processes and different phenotypes of macrophages have been linked to changes in the metabolism of the cells. According to the stimulus, a reprogramming of metabolic pathways can influence immune responses by providing metabolites and generating energy (O'Neill et al., 2016). To gain further insight into the trajectory of the effects of activin A on the immune responses of macrophages, the influence on changes in macrophage immunometabolism were investigated.

This study shows, for the first time, the influence of activin A on the immunometabolic changes of activated macrophages: specifically, the reprogramming of the mitochondria, where a metabolic

switch takes place in classically activated macrophages. Among other changes, pro-inflammatory macrophages predominantly utilise glycolysis for rapid energy production instead of OXPHOS. This is opposed to resting and anti-inflammatory macrophages, which are relying on OXPHOS. The utilisation of OXPHOS can be measured via the oxygen consumption of the cells, which is reduced in M1 macrophages, but increased in M2 macrophages compared with resting controls (Diskin & Pålsson-McDermott, 2018; Van den Bossche et al., 2015).

This study found that activin A was able to increase the OCR in the LPS-stimulated cell line iMACs, which displayed reduced OXPHOS utilisation and switch to glycolysis due to the activation with LPS. Activin A treatment of primary BMDM, however, showed no significant effects on the reduced oxygen consumption in response to LPS. The observed increase of oxygen consumption elicited by activin A treatment of LPS-activated iMACs indicates an alleviation of the pro-inflammatory response of the cells, similar to the observed effects on mRNA and protein secretion in the cell line macrophages, all pointing towards a suppression of the pro-inflammatory response. Specifically, activin A treatment of LPS-activated iMACs appeared to rescue the reduced basal respiration rate of the cells. Moreover, activin A could increase the maximum respiratory capacity displayed by the iMACs, which had been abolished by the LPS activation of the cells, indicating a switch to glycolysis upon activation. Further parameters determined by the assay that were positively influenced by activin A in the LPS-activated iMACs were ATP-linked respiration and proton leak. Non-mitochondrial respiration was not affected. These findings indicate that activin A can increase the LPS-induced inhibition of OXPHOS in iMACs by increasing the ATP-linked respiration and the proton leak. The specific mechanism by which activin A may affect the mitochondrial respiration in iMACs, however, remains to be further investigated.

In BMDM a similar alleviation of the pro-inflammatory metabolic switch by activin A treatment was not observed and would not have been suspected due to its increase of the pro-inflammatory phenotype as seen on gene expression and protein levels.

Similar effects on increasing the inhibited OXPHOS as seen in iMACs, had been found by Van den Bossche et al. in M1-activated murine BMDM, through inhibition of iNOS (or NOS2) prior to macrophage activation with LPS and IFNγ (Van den Bossche et al., 2016). The inhibition of iNOS significantly increased the basal respiration, as well as ATP production and maximum respiratory capacity, similar to the effect of activin A on LPS-activated iMACs in the present study (Van den Bossche et al., 2016). Notably, in the present study, activin A was able to significantly reduce both the expression of *Nos2* and the production of nitrite in the LPS-activated cell line RAW macrophages. In contrast, in LPS-activated BMDM G, *Nos2* expression was significantly increased by activin A.

However, the present study did not investigate *Nos2* in iMACs. Van den Bossche et al. did not find any changes in the production of pro-inflammatory cytokines such as TNF upon inhibition of iNOS in LPS and IFNγ-activated macrophages (Van den Bossche et al., 2016). In contrast, in the present study, pro-inflammatory markers were inhibited by activin A in cell line macrophages and increased in BMDM, indicating a more complex effect of activin A on the immune responses in the investigated cells, possibly involving the inhibition of iNOS, or a different mechanism altogether.

5.3 Implications for testis immunology

Recent studies have reported on roles of activin A in the testicular environment under physiological and pathological conditions. Indumathy et al. demonstrated in a transgenic mouse model with increased activin A levels that macrophages became skewed towards an M2 phenotype (Indumathy et al., 2020), while studies with experimental autoimmune orchitis showed an upregulation of activin A along with the induction of fibrotic proteins contributing to inflammation associated fibrosis (Kauerhof et al., 2019). Although fibrotic remodelling is often pathological, it is also part of the regulatory immune response of tissue repair and regeneration, if controlled adequately (Gieseck et al., 2018). This could point to a putative damage resolving action of activin A in the testis environment.

In addition, other factors that are relevant in the testis such as testosterone, prostaglandins, and corticosterone have been shown to induce a M2 macrophage phenotype in studies of GM-CSF-differentiated rat and murine macrophages *in vitro* (Meinhardt et al., 2018; M. Wang et al., 2017; Z. Zhang et al., 2020). Although in the present study, activin A did not significantly increase the CD206+ population in the GM-CSF-derived BMDM, possibly due to high variability, a tendency was still visible. This trend could indicate an effect of activin A in skewing GM-CF-derived BMDM towards a more M2 phenotype, as has been reported for testosterone, prostaglandins, and corticosterone (Meinhardt et al., 2018; M. Wang et al., 2017; Z. Zhang et al., 2020). Specifically, corticosterone treatment of murine GM-CSF-derived BMDM could induce the M2 marker CD206, while also increasing *Il10* expression and decreasing the expression of pro-inflammatory *Tnf* (Z. Zhang et al., 2020).

Studies on the immunometabolic phenotype of murine testicular macrophages state that they show a similar metabolic profile as the corticosterone treated BMDM, which are displaying a M2 phenotype with increased OXPHOS (Z. Zhang et al., 2020). The observed M2 phenotype was decreased after

the inhibition of AMPK in both testicular macrophages as well as BMDM, indicating its relevance in inducing an anti-inflammatory phenotype via corticosterone in both cell types (Z. Zhang et al., 2020). Compared to the present study, activin A could not induce an anti-inflammatory phenotype in the investigated BMDM. However, the BMDM utilised by Zhang et al. were differentiated with GM-CSF, skewing macrophages towards the M1 phenotype, as opposed to the M-CSF-derived macrophages in the present study, rendering the cells to a M2-like phenotype (Hamilton et al., 2014; Z. Zhang et al., 2020). The flow cytometric analyses in the present study indicated that activin A could potentially skew GM-CSF-derived BMDM towards a more anti-inflammatory population by slightly increasing the number of CD206+ cells. Therefore, it can be speculated that activin A could have similar influences on the metabolic profile of GM-CSF-derived BMDM as corticosterone (Z. Zhang et al., 2020). In the present study, activin A elicited an anti-inflammatory metabolic phenotype in the cell line iMACs, albeit the effect was only significant in LPS-activated macrophages. Although Zhang et al. did not investigate the metabolic activity of activated testicular macrophages, in the resting state a regulatory phenotype was described (Z. Zhang et al., 2020). When activated, testicular macrophages have previously been reported to show subdued inflammatory responses by an increase in IL-10 (Bhushan et al., 2015; Winnall et al., 2011). These observations could lead to the assumption that testicular macrophages potentially also display anti-inflammatory metabolic properties when activated. Therefore, testicular macrophages could hypothetically resemble a similar metabolic phenotype as the activin A treated iMACs in this study, when activated with LPS. While direct links between activin A action and AMPK are not known, it has been reported that the TGFβ1/SMAD3 pathway leads to a suppression of AMPK signalling in hepatocytes (Yadav et al., 2017). In turn, AMPK negatively regulates TGFβ mediated gene expression (Gao et al., 2018), suggesting a potential implication of activin A in AMPK signalling. Future studies could investigate the metabolic profile of testicular macrophages compared with BMDM and iMACs in their response to activin A treatment in a resting and LPS-activated state.

Taken together, studies suggest a predominantly regulatory role of activin A on the testicular macrophage phenotype and show no promotion of pro-inflammatory cytokines (Indumathy et al., 2020). In contrast, an increase of pro-inflammatory properties was seen in the present studies in BMDM. These observations could suggest that BMDM differentiated with M-CSF while being exposed to activin A do not represent an ideal surrogate model to investigate the immune responses of testicular macrophages. Conversely, the macrophage cell lines investigated in the present study, show an alleviation of the pro-inflammatory phenotype following treatment with activin A, although this is not mediated through changes to the anti-inflammatory markers. Similar to a diminished

induction of TNF seen in activated testicular macrophages of rat compared to peritoneal macrophages (Bhushan et al., 2015), the activin A treatment of activated cell lines showed a reduction of TNF in the present study. Due to the regulatory role of activin A found in the cell line macrophages, similarly to reports on testicular macrophages (Indumathy et al., 2020), the cell lines could be an interesting surrogate model to study the effects of activin A on testicular macrophages if placed under similar conditions found within the testis. However, caution must be applied when extrapolating findings, especially regarding the regulation of IL-10. Increased IL-10 production is a particular characteristic of the regulatory phenotype in testicular macrophages (Bhushan et al., 2015; Winnall et al., 2011). In both cell lines, however, activin A treatment suppressed the expression of *Il10* and in RAW macrophages, the secretion of IL-10 was below detection limits of the assay. This could suggest that either activin A may potentially not be a crucial factor for inducing the immunoregulatory phenotype in testicular macrophages via IL-10 regulation, or that the regulatory effects of activin A seen in the cell lines, are mediated through a different mechanism than in testicular macrophages. In addition, it has been suggested that RAW macrophages may display more pro-inflammatory characteristics in a putatively resting state, based on higher expression of the IFNγ receptor compared to BMDM (Guo et al., 2015). This would be in contrast to the immunoregulatory phenotype of testicular macrophages (Bhushan et al., 2015; Winnall et al., 2011).

Although *in vitro* cultures can hardly replicate the complex microenvironment of the testis *in vivo*, they can help to provide specific insights and facilitate experimental investigations of multiple treatment combinations. Future studies could aim to replicate the testis microenvironment *in vitro* more closely by for example co-culturing Sertoli cells with the macrophage models or treating the macrophages with Sertoli cell supernatant or extracted testicular interstitial fluid. In these experiments, activin A could be either blocked or the cells treated with exogenous recombinant activin A to further elucidate its role in the testis environment in resting and activated macrophages. *In vivo* studies could look at mouse models with altered activin A levels and how the testicular macrophages respond when activated.

5.4 Conclusion

The investigation of the potential influences of activin A on immune responses and the phenotype of macrophages revealed distinct outcomes in different commonly utilised macrophage *in vitro* models, indicating differences between primary isolated BMDM and cell line macrophages. This should mandate caution when extrapolating *in vitro* findings to *in vivo* environments, and potential effects in diseases. This study further highlighted findings showing both pro- as well as anti-inflammatory actions of activin A in immune responses and could potentially help to differentiate between context-dependent effects on these cells. Activin A showed opposing effects in primary compared to cell line macrophages, especially regarding the induction of pro-inflammatory markers (Fig. 38). Specifically, it appeared that activin A skews primary BMDM towards a more pro-inflammatory phenotype when administered continuously during the differentiation of the cells into macrophages with M-CSF, increasing pro-inflammatory markers and decreasing anti-inflammatory markers at both the gene and protein level. In contrast, hardly any effect was detectable when activin A was provided after differentiation. In cell line macrophages, however, activin A appeared to alleviate the pro-inflammatory phenotype of macrophages by suppressing pro-inflammatory markers both on gene expression and protein level. Interestingly, activin A also appeared to mitigate pro-inflammatory changes in immunometabolism in iMACs. In contrast, anti-inflammatory markers were generally regulated in a similar manner by activin A in all cell types investigated. Specifically, activin A treatment predominantly suppressed anti-inflammatory markers on gene and protein levels, with the exception of arginase, as its expression and activity were consistently upregulated by activin A in all cell types and at all time points investigated. These observations indicate that the differences in the actions of activin A may be modulated through changes in the pro-inflammatory mediators. Aside from differences between the tested macrophage types, the duration and frequency of activin A treatment, as well as the time point of analysis after LPS activation, could influence the observed effects of activin A. In addition to the effects of activin A on immune mediators observed on levels of transcription and secretion, it also influenced the immune response via immunometabolic changes. Specifically, activin A led to the alleviation of the pro-inflammatory metabolic switch and increased OXPHOS in iMACs, while no changes were observed in BMDM.

The opposing effects found between primary and cell line macrophages could indicate differences in the upstream signalling pathways leading to the expression of the investigated pro-inflammatory markers. Potential reasons include the M-CSF-dependent differentiation of the utilised BMDM, which could skew the macrophages towards a more M2 phenotype (Hamilton et al., 2014), while

RAW macrophages have been suggested to show a more pro-inflammatory putatively resting state compared to BMDM by expressing higher levels of the IFNγ receptor (Guo et al., 2015). It has also been reported that RAW cells display a more monocyte/macrophage intermediate state compared with BMDM based on lower levels of F4/80 expression as a marker for highly differentiated macrophages, while also expressing higher levels of CD14, attributed to monocytes. In addition, the RAW cells were described to be morphologically smaller, more rounded and showed fewer cytoskeletal features and extensions than the larger and more spreading BMDM. The same study found that RAW cells exert diminished responses to LPS-activation compared with BMDM (Chamberlain et al., 2009). Furthermore, the cell lines were originally obtained from different tissues of origin, a factor known to influence the immune responses of macrophages (Stevens et al., 2021).

Although sample sizes were limited and some conclusions need to be considered in light of this and would benefit from increased replicate numbers, the overall results presented in this study showed consistent changes or tendencies over different cell models and laboratories, reinforcing the observations made.

Taken together, this study revealed new targets of activin A, such as *Slamf1*, and showed for the first time the effect of activin A on immunometabolic changes. The findings support the seemingly differential effects of activin A on immune responses and showed that activin A acts in a context-, cell type-, time point-, and factor-specific manner on gene expression, protein secretion, and immunometabolism. Activin A potentially elicits its differential effects via the distinct regulation of pro-inflammatory mediators, while it showed consistent regulation of anti-inflammatory mediators.

As an advantage over a number of other studies, the present investigations determined changes in multiple markers involved in immune responses, which allowed for a more extensive insight as opposed to studies examining for example solely arginase as a M2 marker and NOS2 as a M1 marker. In the present study, the upregulation of *Arg1* by activin A, if investigated alone, could have led to the misleading assumption that activin A induces anti-inflammatory responses, while other additional M2 markers were suppressed.

The present study could inform and give directions for future investigations to determine the mechanisms involved in the differential effects of activin A on immune responses, as it points to distinct differential actions on the regulation of pro-inflammatory genes and changes in immunometabolic processes.

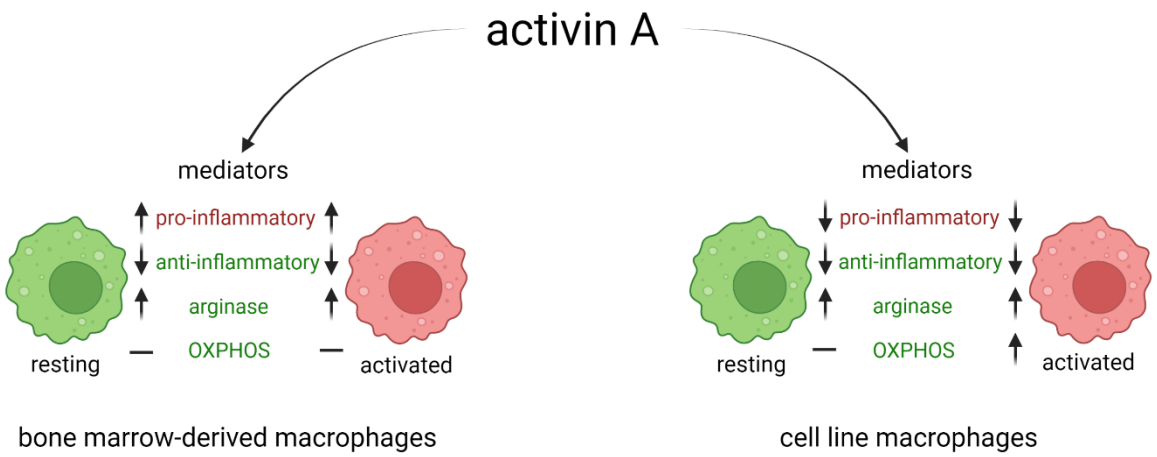


Figure 38: Simplified illustration of differential effects of activin A on primary BMDM and cell line macrophages. Activin A increases pro-inflammatory mediators in BMDM and inhibits them in cell line macrophages. Anti-inflammatory mediators are suppressed by activin A in all cells. As an exception, arginase is increased by activin A in all cells. In the cell line iMACs, OXPHOS is increased by activin A treatment in activated macrophages but is unchanged in resting cells and in BMDM. OXPHOS: oxidative phosphorylation (created with BioRender.com, license number: IW23753OE4).

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7 Appendices

7.1 Materials

7.1.1 Equipment

Biofuge Fresco	Heraeus, Hanau, Germany
Buerker Counting Chamber	Superior Paul Marienfeld, Lauda Koenigshofen, Germany
Cell culture CO_2 incubator	Binder, Tuttlingen, Germany
Cell culture CO_2 incubator HeraCell, Heraeus	Kendro Laboratory Products, Hanau, Germany
CFX96 Real-time C1000 Touch Thermal Cycler	Biorad, Munich, Germany
Clean bench	BDK, Sonnenbuehl-Genkingen, Germany
Consort Mini Electrophoresis Power Supply E143	Sigma-Aldrich, Steinheim, Germany
Countess II	Invitrogen, Waltham, MA, USA
DNA Engine Gradient Thermal Cycler PTC-200	MJ Research, Watertown, MA, USA
Eppendorf E3 electronic dispenser	Eppendorf AG, Hamburg, Germany
FLUOstar OPTIMA	BMG LABTECH, Ortenberg, Germany
Gel iX Imager	Intas, Goettingen, Germany
Haake shaking water bath SWB25	ThermoFisher Scientific, Waltham, MA, USA
Hera Safe safety cabinet	Heraeus, Hanau, Germany
Horizontal mini electrophoresis system	PEQLAB, Erlangen, Germany
Hot plate magnetic stirrer Rct Basic	IKA-Werke, Staufen im Breisgau, Germany
Inverted microscope CKX53	Olympus, Hamburg, Germany
Labofuge 400 function line centrifuge	Heraeus, Hanau, Germany

Labofuge 400 R function line centrifuge	Heraeus, Hanau, Germany
MACSQuant Analyzer 10 flow cytometer	Miltenyi Biotec, Bergisch Gladbach, Germany
Microplate reader Tristar LB941	Berthold, Bad Wildbad, Germany
Mini centrifuge Galaxy	VWR, Darmstadt, Germany
NanoDrop ND 2000	ThermoFisher Scientific, Waltham, MA, USA
NanoDrop ND-1000	ThermoFisher Scientific, Waltham, MA, USA
Orbital Mixer Incubator	Ratek Instruments, Boronia, VIC, AUS
PCMT Thermoshaker	Grant Instruments, Shepreth, UK
PCR thermocycler GeneAmp PCR system 9700	Applied Biosystems, Waltham, MA, USA
pH-meter 766	Knick, Berlin, Germany
PHM210 Standard pH meter	Radiometer Analytical SAS., Lyon, France
Pico 21 microcentrifuge	Heraeus, Hanau, Germany
Primo Vert inverted microscope	Zeiss, Oberkochen, Germany
QuantStudio 6 Flex Real-Time PCR Systems	ThermoFisher Scientific, Waltham, MA, USA
Seahorse Bioscience XFp Analyzer	Agilent, Santa Clara, CA, USA
Sigma 1-14 centrifuge	Sigma, Osterode am Harz, Germany
Sigma 2-5 centrifuge	Sigma, Osterode am Harz, Germany
Tissue Lyser LT	Qiagen, Hilden, Germany
Top Safe 1.2 ABC microbiological safety cabinet	EuroClone, Pero, MI, Italy

7.1.2 Software

Bio Rad CFX Manager 3.1	Biorad, Munich, Germany
Graphpad Prism 9.1.0	San Diego, CA, USA
FlowJo software version 10	Tree Star, Ashland, USA
QuantStudio Real-Time PCR Software v1.3	ThermoFisher Scientific, Waltham, MA, USA

7.1.3 Chemicals

2-Mercaptoethanol	Sigma-Aldrich, Steinheim, Germany
Acetic acid glacial	Merck, Darmstadt, Germany
Acetic acid glacial	Chem-supply, Gillman, SA, AUS
Agarose	Invitrogen, Carlsbad, USA
Crystal violet	Sigma-Aldrich, Steinheim, Germany
D-Glucose	Sigma-Aldrich, St. Louis, MO, USA
Dimethyl sulfoxide	Carl Roth, Karlsruhe, Germany
Ethanol	Sigma-Aldrich, Steinheim, Germany
Ethanol absolute for analysis	Merck, Darmstadt, Germany
Ethidium bromide	Carl Roth, Karlsruhe, Germany
FCCP (sc-203578)	Santa Cruz Biotechnology, Dallas, TX, USA
H_2SO_4	Carl Roth, Karlsruhe, Germany
Igepal CA-630 (NP-40)	Sigma-Aldrich, Steinheim, Germany
KCl	Carl Roth, Karlsruhe, Germany
KH_2PO_4	Carl Roth, Karlsruhe, Germany
Methanol	Sigma-Aldrich, Steinheim, Germany
NaCl	Carl Roth, Karlsruhe, Germany
Na_2HPO_4	Carl Roth, Karlsruhe, Germany
NaH_2PO_4	Carl Roth, Karlsruhe, Germany
N, N, N', N'-Tetramethylethylenediamin (TEMED)	Roth, Karlsruhe, Germany
Oligomycin A (75351)	Sigma-Aldrich, St. Louis, MO, USA
Phenylmethylsulfonyl fluoride (PMSF)	Carl Roth, Karlsruhe, Germany
Protease inhibitor cocktail (100X)	Sigma-Aldrich, Steinheim, Germany
Sodium chloride	Carl Roth, Karlsruhe, Germany
Tris	Carl Roth, Karlsruhe, Germany
Tris/HCl	Carl Roth, Karlsruhe, Germany
Triton X-100	Sigma-Aldrich, Steinheim, Germany
Tween 20	Sigma-Aldrich, Steinheim, Germany

7.1.4 Kits

Arginase Activity Assay Kit (MAK112)	Sigma-Aldrich, Steinheim, Germany
Fixation/Permeabilization	Invitrogen, Waltham, MA, USA
Griess Reagent System (G2930)	Promega, Madison, WI, USA
Mouse TNF (mono/mono) ELISA Set (555268)	BD Biosciences, Heidelberg, Germany
Mouse IL-10 ELISA Set (555252)	BD Biosciences, Heidelberg, Germany
Pierce BCA Protein Assay (23227)	Thermo Scientific, Rockford, IL, USA
RNeasy Mini Kit	Qiagen, Hilden, Germany
RNeasy Micro Kit	Qiagen, Hilden, Germany
RNase-Free DNase Set (79254)	Qiagen, Hilden, Germany
Seahorse XFp Cell Mito Stress Test Kit (103010100)	Agilent, Santa Clara, CA, USA
Seahorse XFp FluxPak (103022100)	Agilent, Santa Clara, CA, USA
SuperScript III First-Strand Synthesis System for RT-PCR	Invitrogen, Carlsbad, USA
TMB Substrate Reagent Set (555214)	BD Biosciences, Heidelberg, Germany

7.1.5 Reagents

2-Mercaptoethanol 50 mM (31350-010)	Gibco, Grand Island, NY, USA
2-Mercaptoethanol 1000X 55 mM in DPBS (21985023)	Gibco, Grand Island, NY, USA
5X M-MLV RT buffer	Promega, Mannheim, Germany
6X Blue/orange DNA loading dye	Promega, Mannheim, Germany
Bovine Serum Albumin	Sigma-Aldrich, Steinheim, Germany
Deoxynucleotide triphosphates (dNTPs) 10 mM	Promega, Mannheim, Germany
DMEM Medium, high glucose (11965092)	Gibco, Grand Island, NY, USA
DNA ladder 1 Kb plus	Invitrogen, Carlsbad, USA
DPBS, no calcium, no magnesium (14190144)	Gibco, Grand Island, NY, USA
eBioscience 1X RBC Lysis Buffer	Invitrogen, Carlsbad, USA
Fetal Bovine Serum (10270-106)	Gibco, Grand Island, NY, USA
Foetal Bovine Serum (SFBS)	Bovogen Biologicals, Keilor East, VIC, Australia
Gentamicin Reagent Solution (50 mg/ml) (15750060)	Sigma-Aldrich, Steinheim, Germany

Go Taq G2 Flexi DNA polymerase (M7805)	Promega, Mannheim, Germany
HEPES Buffer Solution 1 M	Gibco, Grand Island, NY, USA
iTaq Universal SYBR Green Supermix (172-5124)	Biorad, Munich, Germany
L-glutamine (200 mM) (25030-081)	Gibco, Grand Island, NY, USA
LPS-EB Ultrapure from E.coli 0111:B4 strain-TLR4 ligand (tlr-3pelps)	InvivoGen, San Diego, CA, USA
Macrophage Detachment solution DXF	PromoCell, Heidelberg, Germany
MEM Non-Essential Amino Acid Solution 100X (M745)	Sigma-Aldrich, St. Louis, MO, USA
Oligo-dT primer	Promega, Mannheim, Germany
Penicillin/Streptomycin (15070-063)	Gibco, Grand Island, NY, USA
Penicillin/Streptomycin (15140-122)	Gibco, Grand Island, NY, USA
Power SYBR green PCR master mix	Applied Biosystems, Waltham, MA, USA
Recombinant RNasine Ribonuclease inhibitor	Promega, Mannheim, Germany
Reverse Transcriptase (M-MLV RT)	Promega, Mannheim, Germany
RPMI Medium 1640 1X (21875-034)	Gibco, Grand Island, NY, USA
Red Blood Cell Lysis Solution	Qiagen, Hilden, Germany
Seahorse XF Base Medium (103334-100)	Agilent, Santa Clara, CA, USA
Sodium Pyruvate 100 mM	Gibco, Grand Island, NY, USA
Trypan blue solution 0.4 %	Sigma-Aldrich, Steinheim, Germany
TrypLE™ Express (1X) (12604013)	Gibco, Grand Island, NY, USA

7.1.6 Antibodies

Antigen Flow Cytometry	**Conjugated Fluorochrome**	**Manufacturer**	**Clone**	**Reference**
CD11b	Per/Cy5.5	Biolegend, San Diego, CA, USA	M1/70	101228
CD206	Alexa 647	Biolegend, San Diego, CA, USA	C068C2	141711

CD45	PE	eBioscience, Santa Clara, CA, USA	30-F11	12-0451-82
efluor 450	efluor 450	eBioscience, Santa Clara, CA, USA	-	42997348
F4/80	Alexa 488	Biolegend, San Diego, CA, USA	BM8	123120
CD32/16	-	BD Pharmingen, San Diego, CA, USA	D34-485	550271

7.1.7 Cytokines

GM-CSF	PeproTech, Hamburg, Germany
Mouse M-CSF, research grade (130-094-129)	Miltenyi Biotec, Bergisch Gladbach, Germany
Recombinant Human/Murine/Rat Activin A (CHO derived) (120-14P)	PeproTech, Hamburg, Germany

7.1.8 Primers

Primers used at JLU purchased from Eurofins Genomics, Ebersberg, Germany. Primers used at Monash purchased from Merck, Darmstadt, Germany.

Primer	**Forward 5'-3'**	**Reverse 5'-3'**	**Amplicon size (bp)**	**Accession number**
Actb	TGACAGGATGCAGAAGGAGAT	TACTCCTGCTTGCTGATCCAC	156	NM_007393.5
Rplp0	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG	85	NM_007475.5

Tnf	CAAATTCGAGTGACAAGCCTG	GAGATCCATGCCGTTGGC	113	NM_013693.3
Nos2	TCCTGGACATTACGACCCCT	AGGCCTCCAATCTCTGCCTA	93	NM_001313922.1
Gpr18	ACCACCTACCTAACACAGGC	TCAGGGTGGCCATCTTACAG	144	NM_182806.2
Fpr2	CCACAGGAACCGAAGAGTGTAA	CCACAGAACTCTGGAGATGGT	131	NM_008039.2
Arg1	ATGGGCAACCTGTGTCCTTT	TCTACGTCTCGCAAGCCAAT	127	NM_007482.3
Il1β	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT	138	NM_008361.4
Il6	AGCCAGAGTCCTTCAGAGAGAT	GAGAGCATTGGAAATTGGGGT	106	NM_031168.2
Il10	GGTTGCCAAGCCTTATCGGA	TCAGCTTCTCACCCAGGGAA	115	NM_010548.2
Klf4	GCCACCCACACTTGTGACTA	CTGTGTGTTTGCGGTAGTGC	175	NM_010637.3
Clec7a	CTAGGGCCCTGTGAAGCAAT	AATGGGCCTCCAAGGTGAAG	156	NM_001309637.2
Slamf1	GAGCCTCTTATGCTTCAAACAACA	CAGCAGCATTGCCAAACAGT	101	NM_013730.4
Casp1	AAGAAACATGCGCACACAGC	CCCTCAGGATCTTGTCAGCC	141	NM_009807.2
Atf3	CCAGCCACAGTCTCACTCAG	GACCTGGCCTGGATGTTGAA	197	NM_007498.3
Chil3/Ym1	GGGCCCTTATTGAGAGGAGC	CCAGCTGGTACAGCAGACAA	194	NM_009892.3
Mrc1	ATGGATTGCCCTGAACAGCA	TGTACCGCACCCTCCATCTA	71	NM_008625.2
Akt3	TGGGTTCAGAAGAGGGGAGAA	TTGGCTTTGGTCGTTCTGTT	178	NM_011785.4
Akt1	GGGACCTGAAGCTGGAGAAC	GTCCTTGATCCCCTCCTTGC	89	NM_001331107.1

Pik3cd	CTCTCCTGTGCTGGCTACTGT	GCTCTCGGTTGATTCCAAACT	157	NM_001164052.1

7.1.9 Solutions

Protein lysis buffer (BCA)

Stock Buffer	**Working Buffer concentration**	**Volume for 10 ml**
1 M Tris/HCL pH 7.4	10 mM	100 µl
5 M NaCl	150 mM	300 µl
Triton X-100	1 %	100 µl
Igepal CA-630 (NP-40)	0.25 %	25 µl
0.5 M EDTA	2 mM	40 µl
100 mM PMSF	1 mM	100 µl
Protease inhibitor cocktail (100X)	1X	100 µl
water	-	9235 µl

Protein lysis buffer (arginase activity assay)

Stock Buffer	**Working Buffer concentration**	**Volume for 5 ml**
1 M Tris/HCl, pH 7.4	10 mM	50 µl
Protease inhibitor cocktail (100X)	0.05X	2.5 µl
Triton X-100	0.4 %	20 µl
water	-	4927.5 µl

10X Phosphate buffered saline (PBS)

4 g KCl

4 g KH_2PO_4

160 g NaCl

23 g Na_2HPO_4

Fill up to 1 L with water

Coating buffer (0.2 M Sodium phosphate, pH 6.5)

12.49 g Na_2HPO_4

15.47 g NaH_2PO_4

Fill up to 1 L with water

Assay diluent	Wash buffer
100 ml 10X PBS	999.5 ml 1X PBS
100 ml FBS	0.5 ml Tween-20
800 ml water	

50X TAE electrophoresis buffer	10 % acetic acid
242 g Tris base	10 ml glacial acetic acid
57.1 ml Glacial acetic acid	90 ml water
14.6 g EDTA	
934 ml water	
adjust pH to 8.0 with HCL	

7.1.10 Consumables

Cell culture Plate, 6 well, Tissue culture treated	Corning Incorporated, Kennebunk, ME, USA
Centrifuge tubes (50 ml)	Corning Incorporated, Corning, NY, USA
Combitips advanced®, 0.1 ml	Eppendorf AG, Hamburg, Germany
Combitips advanced®, Biopur®, 0.2 ml	Eppendorf AG, Hamburg, Germany
Corning 75 cm2 Flask	Corning Incorporated, Kennebunk, ME, USA
Costar Assay Plate, 96 well, high binding non-pyrogenic, polystyrene	Corning Incorporated, Corning, NY, USA
Disposable Serological Pipette, 1 ml, 5 ml, 10 ml, 25 ml	Corning Incorporated, Corning, NY, USA
EASYstrainer 70 µm-sterile (542070)	Greiner Bio-One, Frickenhausen, Germany
Eppendorf Tubes, 0.5 ml, 1.5 ml, 2 ml	Eppendorf SE, Hamburg, Germany
F96 Maxisopr NUNC-Immuno Plate	Thermo Fisher Scientific, Roskild, Denmark

Falcon® cell strainer 70 µm, sterile	Corning Incorporated, Corning, NY, USA
Filter tips	Axygen Inc., Union City, CA, USA
Filter tips	Nerbe plus, Winsen/Luhe, Germany
Filtropur S 0.2	Sarstedt, Nuembrecht, Germany
Flow cytometry tubes	Sarstedt, Nuembrecht, Germany
Individual PCR tubes	Biorad, Munich, Germany
MaxyClear Microtubes	Axygen Inc., Union City, CA, USA
Minisart Syringe Filter	Sartorius, Goettingen, Germany
Microseal B adhesive seals	Biorad, Munich, Germany
Needle 24 G, 30 G	BD, Franklin Lakes, NJ, USA
PCR plates 96 and 364	Biorad, Munich, Germany
Petri dish 100x100x20 mm	Sarstedt, Nuembrecht, Germany
Pipette, graduated and sterile	Greiner Bio-One, Frickenhausen, Germany
Pipette tips	Sarstedt, Nuembrecht, Germany
Safe-Lock Tubes 2 ml	Eppendorf AG, Hamburg, Germany
Cellstar tubes, graduated and sterile	Greiner Bio-One, Frickenhausen, Germany
Stainless steel beads, 5mm (69989)	Qiagen, Hilden, Germany
Syringe, 1 ml, 10 ml, 20 ml	BD, Franklin Lakes, NJ, USA
Syringe Injekt	B Braun SE, Melsungen, Germany
TC Cell scraper	Sarstedt, Nuembrecht, Germany
Tissue culture flasks (T75)	Sarstedt, Nuembrecht, Germany
Tissue culture plates	Sarstedt, Nuembrecht, Germany

7.2 Supplemental data

7.2.1 Activin A dose response in iMACs

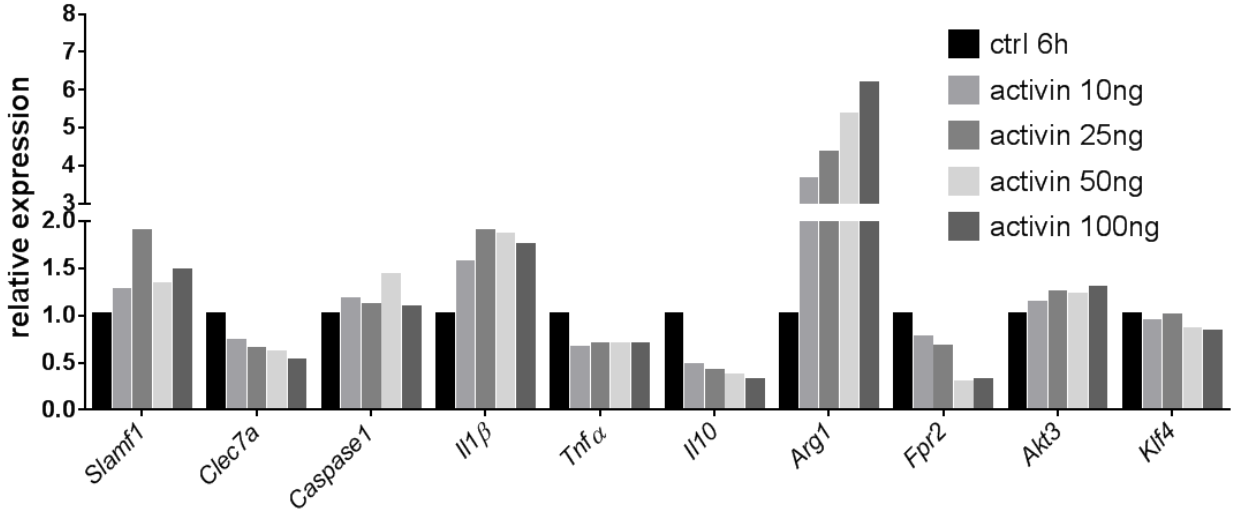


Figure 39: Regulation of mRNA expression by activin A in iMACs. Relative mRNA expression in iMACs after treatment with different concentrations of activin A (10 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml) for 6 h compared to untreated controls. N=1 experiment

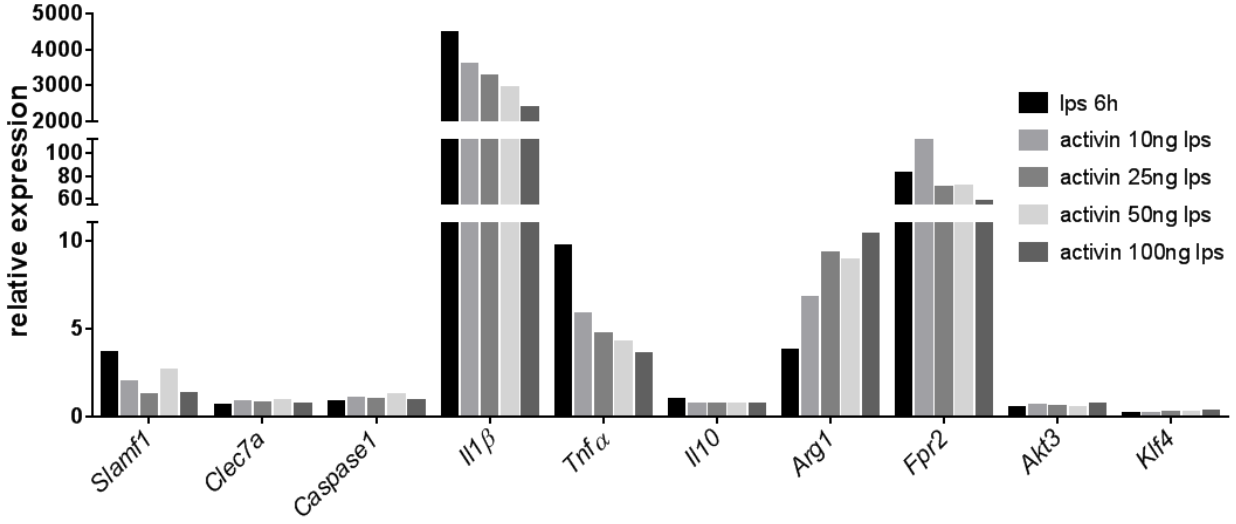


Figure 40: Regulation of mRNA expression by activin A in LPS-activated iMACs. Relative mRNA expression in iMACs after treatment with different concentrations of activin A (10 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml) and subsequent treatment with LPS (10 ng/ml) for 6 h. N=1 experiment

7.2.2 Stability of reference genes

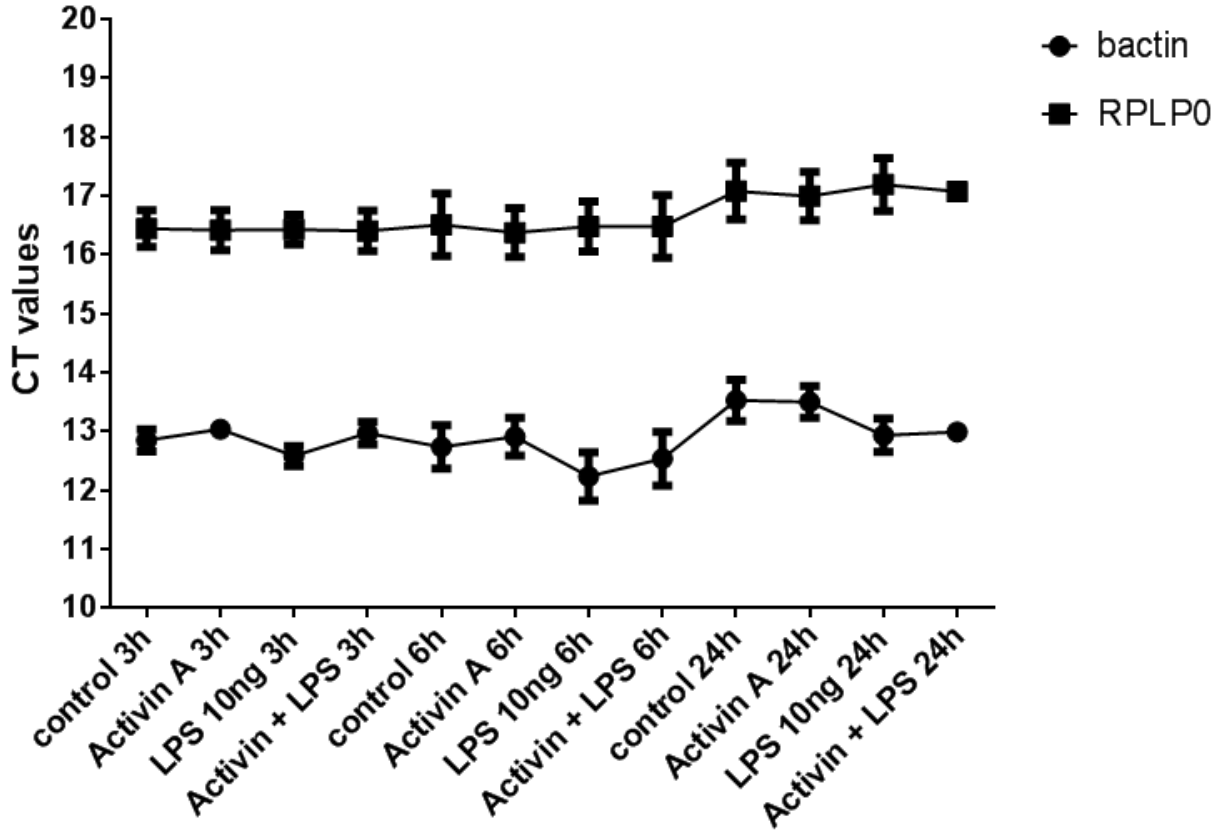
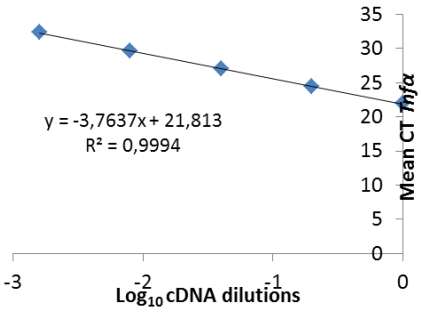
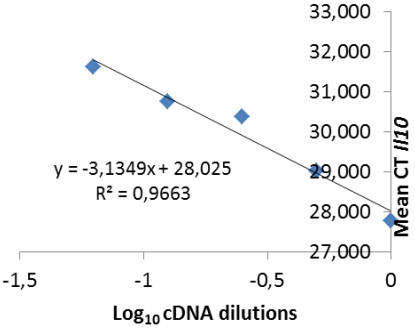
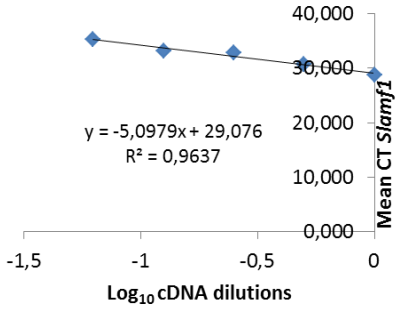


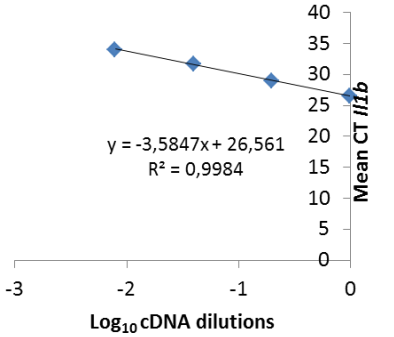
Figure 41: Stability of the expression of the reference genes *Actb* and *Rplp0* in iMACs. CT values of qRT-PCR analysis of *Actb* and *Rplp0* as reference genes in iMACs after treatment with activin A (50 ng/ml) and subsequently with LPS (10 ng/ml) for 3 h, 6 h, 24 h. N=4 separate experiments.

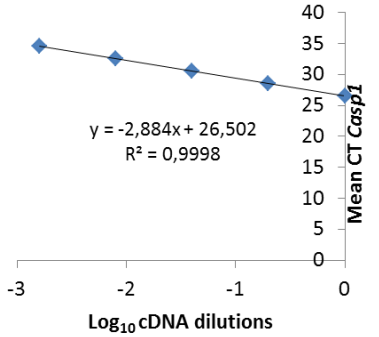
7.2.3 Primer validations

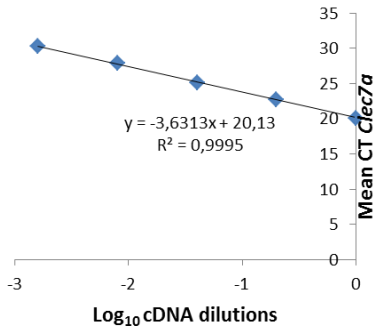

y = -3,7637x + 21,813
R² = 0,9994
Mean CT Tnfa
Log10 cDNA dilutions

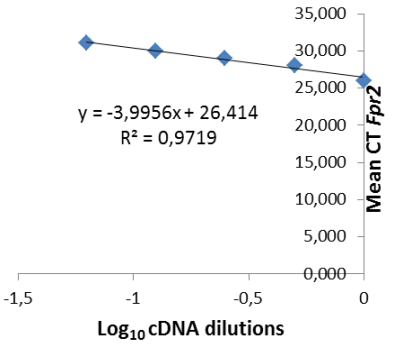

y = -3,1349x + 28,025
R² = 0,9663
Mean CT Il10
Log10 cDNA dilutions

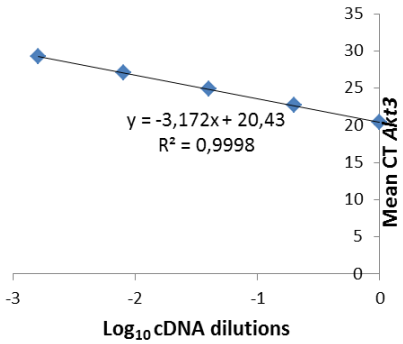

y = -5,0979x + 29,076
R² = 0,9637
Mean CT Slamf1
Log10 cDNA dilutions

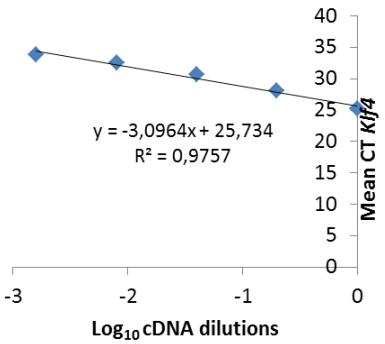

y = -3,5847x + 26,561
R² = 0,9984
Mean CT Il1b
Log10 cDNA dilutions

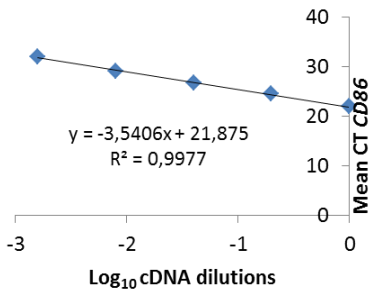

y = -2,884x + 26,502
R² = 0,9998
Mean CT Casp1
Log10 cDNA dilutions

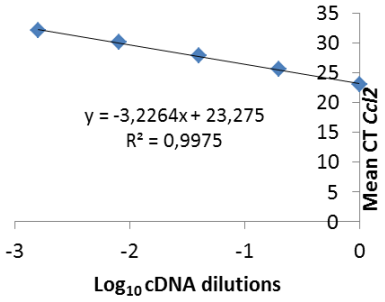

y = -3,6313x + 20,13
R² = 0,9995
Mean CT Clec7a
Log10 cDNA dilutions

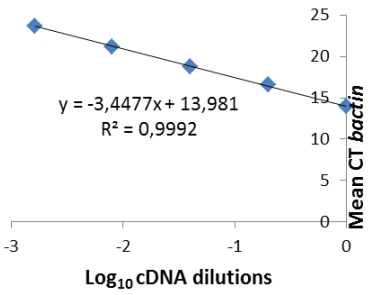

y = -3,9956x + 26,414
R² = 0,9719
Mean CT Fpr2
Log10 cDNA dilutions


y = -3,172x + 20,43
R² = 0,9998
Mean CT Akt3
Log10 cDNA dilutions


y = -3,0964x + 25,734
R² = 0,9757
Mean CT Klf4
Log10 cDNA dilutions


y = -3,5406x + 21,875
R² = 0,9977
Mean CT CD86
Log10 cDNA dilutions


y = -3,2264x + 23,275
R² = 0,9975
Mean CT Ccl2
Log10 cDNA dilutions


y = -3,4477x + 13,981
R² = 0,9992
Mean CT bactin
Log10 cDNA dilutions

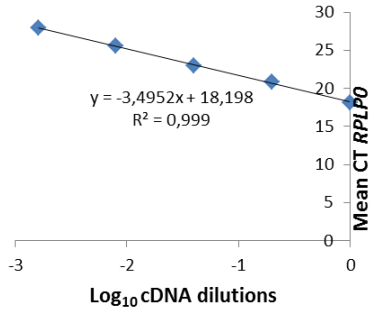

y = -3,4952x + 18,198
R² = 0,999
Mean CT RPLP0
Log10 cDNA dilutions

Figure 42: Primer validation. Standard curve showing mean CT values of primers using a serial dilution (1:5 or 1:2) of iMACs or spleen cDNA. Primer efficiencies: *Tnf*: 84,3 %, *Il10*: 108,4 %, *Slamf1*: 57,1 %, *Il1β*: 90 %, *Casp1*: 122,2 %, *Clec7a*: 88,5 %, *Fpr2*: 77,9 %, *Akt3*: 106,6 %, *Klf4*: 110,4 %, *Cd86*: 91,6 %, *Ccl2*: 104,1 %, *Actb*: 95 %, *Rplp0*: 93,2 %.

7.2.4 Crystal violet staining for normalisation of Seahorse analysis data

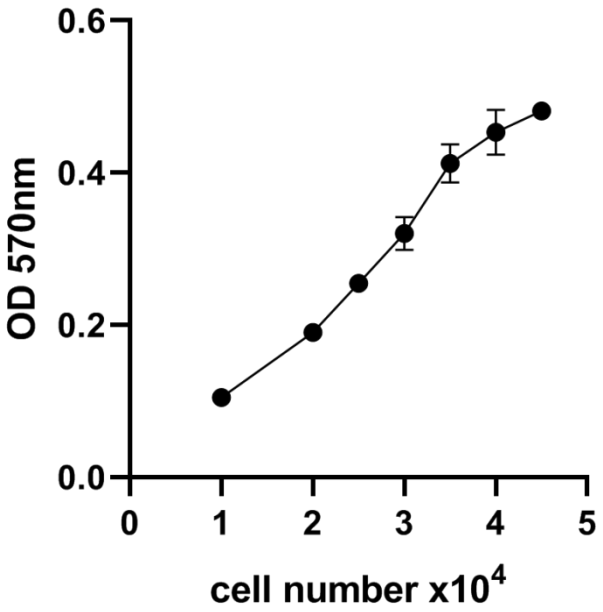


Figure 43: Titration curve of cell numbers for crystal violet staining used for normalisation of Seahorse analysis data to cell content. Optical Density of solubilised crystal violet staining measured at 570 nm to measure different cell numbers.

7.2.5 Cell culture treatment regimen

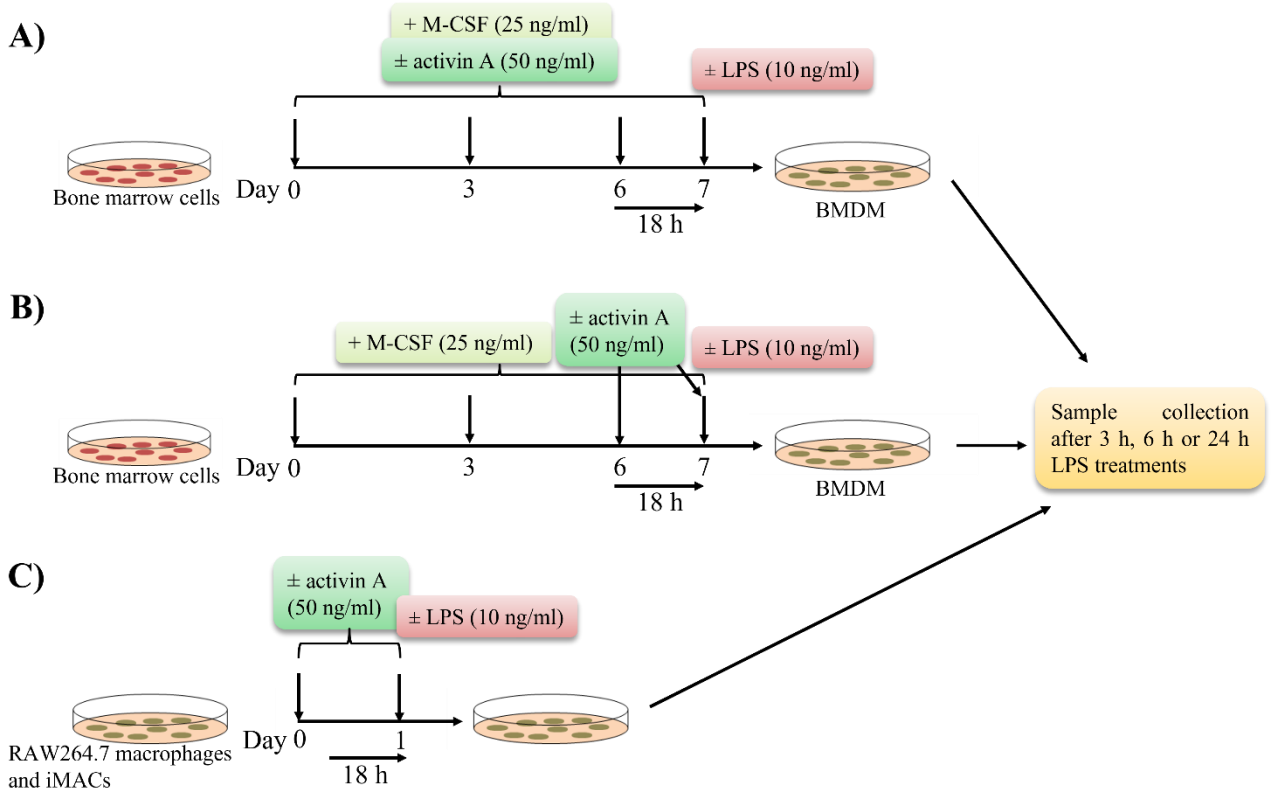


Figure 44: Cell culture treatment regimen. A) Long-term activin A treatment of BMDM from day of isolation, day 6 treatment 18 h prior to the treatment with LPS. B) 18 h activin A treatment of BMDM starting on day 6 after isolation, 18 h before the treatment with LPS. C) Treatment of cell lines RAW and iMACs after seeding for 18 h prior to the treatment with LPS.

Zusammenfassung

Das Immunprivileg der Hoden ermöglicht im Falle einer Infektion den Schutz der Keimzellen vor Autoimmunreaktionen und inflammatorischen Schäden. Die Makrophagen machen einen Großteil der im Hoden vorhandenen Immunzellen aus und tragen mit ihrem immunsuppressiven Phänotyp dazu bei, das Immunprivileg aufrecht zu erhalten. Wie genau dieser Phänotyp zustande kommt, ist jedoch nicht gut verstanden. Die immunregulatorischen Sertoli-Zellen exprimieren möglicherweise Faktoren, die die Makrophagen in Richtung eines antiinflammatorischen Phänotyps polarisieren. Ein Zytokin, welches von den Sertoli-Zellen sezerniert wird und am immunregulatorischen Phänotyp der Makrophagen in den Hoden beteiligt sein könnte, ist Aktivin A. Frühere Studien haben gezeigt, dass Aktivin A an vielen biologischen Prozessen beteiligt ist. Dies schließt immunregulatorische Funktionen, mit sowohl proinflammatorischen als auch antiinflammatorischen Wirkungen ein.

Um den Einfluss von Aktivin A auf den Phänotyp von Makrophagen zu untersuchen, wurden verschiedene Makrophagen-Zellkulturmodelle analysiert. Ein möglicher Effekt von Aktivin A auf Immunantworten und Veränderungen des Phänotyps wurde in unstimulierten und Lipopolysaccharid-aktivierten Makrophagen mit Hilfe von verschiedenen Markern auf Genexpressions- und Proteinebene, sowie hinsichtlich metabolischer Veränderungen untersucht. In dieser Studie wurde gezeigt, dass Aktivin A unterschiedliche Effekte auf häufig verwendete murine Makrophagen-Zellkulturmodelle ausübt. Diese Effekte scheinen abhängig von der Herkunft oder Reife der Makrophagen, dem Kontext und dem Zeitpunkt der Behandlungen, sowie den untersuchten Faktoren. Im Allgemeinen wiesen die Ergebnisse darauf hin, dass Aktivin A bei primären Knochenmarkzellen, die mit Hilfe des Makrophagen-Kolonien-stimulierenden Faktor zu Makrophagen differenziert wurden, einen proinflammatorischen Phänotyp induziert, während proinflammatorische Reaktionen in zwei getesteten murinen Makrophagen-Zelllinien, inhibiert werden konnten. Darüber hinaus konnte gezeigt werden, dass Aktivin A die proinflammatorischen Veränderungen im Metabolismus einer Makrophagen-Zelllinie abschwächen kann. Angesichts der gegensätzlichen Effekte von Aktivin A in den verschiedenen Makrophagen-Zellkulturmodellen, müssen die Ergebnisse, die mit diesen häufig verwendeten Modellen erzielt werden, mit Vorsicht und im Kontext bewertet werden.

Die vorgelegte Studie zeigt jedoch, dass Aktivin A das Mikromilieu in den Hoden beeinflussen könnte, indem es den immunregulatorischen Phänotyp von testikulären Makrophagen moduliert.

Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus-Liebig-University of Giessen in carrying out the investigations described in the dissertation.

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

Signature:

Print Name: Julia Bender

Date: 26.11.21

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Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.
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