EFFECT OF ATORVASTATIN ON MACROPHAGE ACTIVATION BY INTERFERON-γ

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Abbreviations

-/-	Double knockout
7,8-NP	7,8-dihydroneopterin
acLDL	Acetylated low density lipoprotein
ACN	Acetonitrile
ANOVA	Analysis of variance
AP	Activator protein
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ATS	Atorvastatin
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CD	Cluster of differentiation
CHD	Coronary heart disease
CNS	Central nervous system
COOH	Carboxylic acid
CVD	Cardiovascular disease
DAMP	Damage associated molecular pattern
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FBS	Foetal bovine serum
FFPP	Farnesyl farnesyl pyrophosphate
FRB	Free radical biochemistry
GGPP	Geranyl geranyl pyrophosphate
GTP	Guanosine triphosphate
GTPCH1	Guanosine triphosphate cyclohydrolase 1
HCL	Hydrochloric acid
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMGR	HMG-CoA reductase
HPLC	High performance liquid chromatography

hs-CRP	Human serum – C-reactive protein
HUVEC	Human umbilical vein endothelial cell
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN-γ	Interferon gamma
IFNGR	Interferon gamma receptor
IL-1β	Interleukin 1-beta
JAK	Janus kinase
KLF	Krüppel like factor
KYN	Kynurenine
LDL	Low density lipoprotein
LDLc	Low density lipoprotein cholesterol
LDLr	Low density lipoprotein receptor
M-CSF	Macrophage – colony stimulating factor
MCP-1	Monocyte chemoattractant protein-1
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKT	Natural Killer T-cell
NO	Nitric oxide
NOX	NADPH oxidase
NP	Neopterin
oxLDL	Oxidised low-density lipoprotein
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PMA	Phorbol 12-myristate 12-acetate
PP	Pyrophosphate
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SMC	Smooth muscle cell

SR	Scavenger receptor
STAT	Signal transducer and activator of transcription
TCR-γδ	Gamma delta T-cell
TH-1	T-helper type 1
TNF	Tumour necrosis factor
TNP	Total neopterin
TRP	Tryptophan
UV	Ultra-violet
VSMC	Vascular smooth muscle cells

Units

%	Percentage	**M**	Molar
(aq)	Aqueous	**mM**	Millimolar
^{o}C	Degrees Celsius	**µM**	Micromolar
Å	Angstrom	**min**	Minute
g	Grams	**mm**	Millimetre
mg	Milligram	**µm**	Micrometre
µg	Microgram	**nm**	Nanometre
g	Relative centrifugal force	**p**	P-value
gmol-1	Grams per mol	**psi**	Pounds per square inch
L	Litre	**U**	Units
µL	Microlitre	**$^{v}/_{v}$**	Volume/volume

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Abstract

Heart attacks and strokes are predominantly caused by atherosclerosis, a chronic inflammatory condition, where immune cells (monocytes, macrophages, and T-cells) are continuously recruited into the arterial wall where they accumulate cholesterol. The resulting arterial plaques may rupture, triggering blood clot formation and the blockage of blood supply to the heart (heart attack) or brain (stroke).

Statins are lipid-lowering drugs which inhibit cholesterol synthesis, decreasing plasma cholesterol levels, thereby the growth of cholesterol filled inflammatory cells within the arterial wall. Statins appear to possess additional anti-inflammatory properties which may reduce arterial inflammation. This research investigates the anti-inflammatory effects of atorvastatin, to determine how it may alter the levels of inflammatory markers within plaques. This was achieved using the model mixed cell culture system of peripheral blood mononuclear cells (PBMCs), consisting predominately of T-cells and monocytes. PBMCs isolated and prepared from whole human blood were cultured with varying levels of atorvastatin treatment in combinations with the inflammatory activator interferon-γ (IFN-γ). PBMC activation was quantified by measuring the production of inflammatory markers 7,8-dihydroneopterin, neopterin and kynurenine by HPLC.

Experimental results demonstrated that atorvastatin treatment of PBMCs in various combinations including IFN- γ activation, consistently produced an anti-inflammatory effect by inhibiting the production of respective inflammatory markers. In contrast, treatment conditions including IFN-γ only and DMSO produced statistically significant increases in inflammatory marker production. The TNP/NP ratios produced for atorvastatin treatments indicate a greater level of oxidative stress compared to inflammation; however, the mechanism by which this occurs remains unclear. The experimental results provide evidence for atorvastatin inhibiting IFN-γ activated production of 7,8-dihydroneopterin, neopterin and kynurenine in PBMCs. These results suggest atorvastatin may possess anti-inflammatory effects within atherosclerotic plaques.

Chapter 1

Introduction

1.1 Overview

Cardiovascular diseases (CVDs) have been the leading cause of death in the industrialised world (Braunwald, 1997). Atherosclerosis is the most common pathological process leading to CVD, a disease which affects large and medium sized arteries, which is characterised by the formation of atherosclerotic plaques. Plaque development is initiated by low density lipoprotein particles (LDL) exiting circulation into the arterial intima (Ross, 1999). Hypercholesterolemia is considered a risk factor for fatal and non-fatal CVDs, therefore clinical approaches typically target lowering plasma cholesterol (Taylor et al., 2013). This is predominantly achieved through statins, drugs which limit cholesterol synthesis by competitively inhibiting HMG-CoA reductase (HMGR) which catalyses the committed step in cholesterol biosynthesis, converting HMG-CoA to CoA and mevalonate (Corsini, Maggi, & Catapano, 1995).

Views regarding the pathophysiology of atherosclerosis have substantially evolved over time. Initially, the thinking was governed by the link between lipids and atherosclerosis. In recent years, the scientific community began to appreciate a prominent role for inflammation in atherosclerosis (Libby, Ridker, & Maseri, 2002; Russell Ross & Laurence Harker, 1976). Experimental investigations have implicated a role for inflammation in various phases of atherosclerotic development. Evidence from various experimental and clinical studies support statin drugs producing additional atheroprotective effects via anti-inflammatory mechanisms, as well as their lipid lowering effects resulting in a greater reduction in CVD-risk than other cholesterol lowering modalities (Paul M Ridker et al., 2008). Given the amassing evidence for statins possessing anti-inflammatory effects, and the prevalence and success of statins as CVD preventative treatment strategies, it is of interest whether these anti-inflammatory properties affect the production of macrophage generated inflammatory biomarkers. This research characterises the effects of atorvastatin on peripheral blood mononuclear cells, by measuring the generation and oxidation of the inflammatory biomarkers: 7,8-dihydroneopterin, produced by GTP cyclohydrolase-1, and kynurenine, a product of tryptophan degradation by indolamine-2,3-dioxygenase.

1.2 Atherosclerosis

1.2.1 Atherosclerosis and cardiovascular disease

For the past century, cardiovascular diseases (CVD) have been the leading cause of death in the industrialised world, accounting for approximately 30% of all deaths globally. Moreover, CVDs are the leading cause of death in New Zealand, accounting for approximately 35% of deaths annually, with one in twenty adult New Zealanders diagnosed with CVD (Braunwald, 1997; Health-Research-Council, 2010). These include coronary heart disease, blood vessel disease and stroke, all of which involve the underlying process of atherosclerosis, a complex disease which features many components of the vascular, metabolic, and immune system. Atherosclerosis is the most common pathological process which leads to CVD, a disease which affects large and medium sized arteries which is characterised by the formation of atherosclerotic plaques. Advanced atherosclerotic plaques often consist of a necrotic core, calcified regions, accumulated modified lipids, inflamed smooth muscle cells, endothelial cells, leukocytes and foam cells (Ross, 1999). Rupture of an advanced plaque produces a thrombus, which can cause an acute occlusion leading to myocardial infarction (heart attack) or cerebral ischemia (stroke). Epidemiological studies have identified a number of risk factors associated with the development of atherosclerosis, showing that there is a complex interplay between lifestyle-based factors, including smoking, inactivity, and alcohol consumption, and fixed factors, including age, gender, familial history and menopausal status (Frohlich & Al-Sarraf, 2013).

1.2.2 Disease initiation and progression

Initial atherosclerotic plaque development begins when low density lipoprotein (LDL) particles exit circulation into the arterial intima, where they accumulate. These LDL particles are subsequently oxidised into oxLDL, proinflammatory particles which elicit a response from the innate immune system. Initiation of inflammation occurs when endothelial cells are activated by oxLDL and excrete adhesion molecules and smooth muscle cells (SMC) secrete chemokines and chemoattractants which together recruit immune cells to the intimal space (Insull, 2009). Once located within the intima, monocytes acquire the characteristics of the tissue-macrophage. This occurs through the triggering of endothelial SMC, via oxLDL, to secrete monocytic maturation factors, such as monocyte colony stimulating factor (M-CSF) (Hazen, 2008). Subsequently, differentiated tissue macrophages located within the intima proceed to uptake modified LDL particles in an unregulated manner to form foam cells. Plaque growth

continues over time due to the recruitment of further monocytic cells, fibroblasts, and smooth muscle cells from the arterial wall. The development of an advanced plaque can be attributed to cell death occurring within the centre of the plaque mass, with a characteristic necrotic core of cell debris and lipid deposits rich in cholesterol and cholesterol esters. The rupture of these plaques results in thrombus formation, which may occlude blood flow (**Figure 1.2**). In the case of blood flow of the coronary arteries supplying the heart, this constitutes a heart attack and which arteries supplying the brain, stroke (Gieseg, Crone, & Amit, 2010; Rader & Daugherty, 2008; Silvestre-Roig et al., 2014).

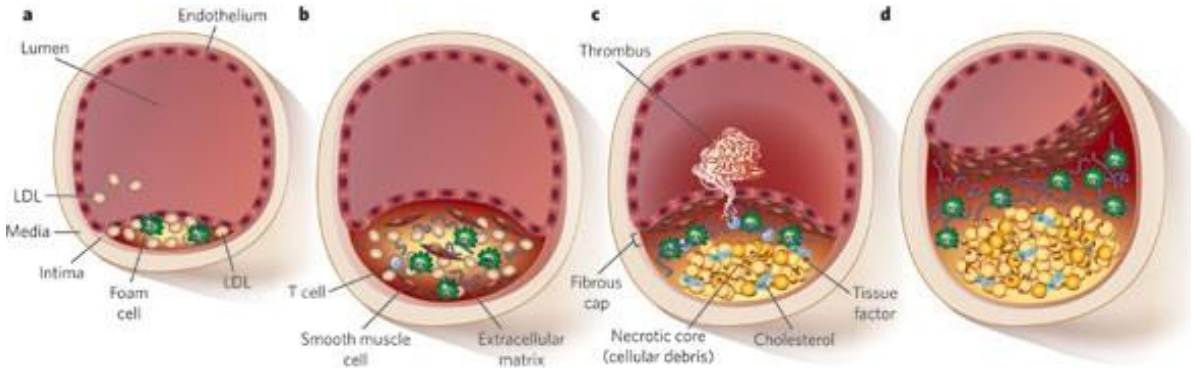


Figure 1.2 Atherosclerotic plaque: Stages of development Depicting: a.) LDL particles exit circulation, entering the arterial intima where it accumulates. Within the intima, the LDL particles are modified to the pro-inflammatory oxLDL which elicit a response from the immune system. Unregulated uptake of modified LDL particles forms foam cells. b.) Endothelial and smooth muscle cells contribute to inflammatory progression via the secretion of chemokines and chemoattractants which together recruit immune cells to the intimal space. As the plaque increases in size, arterial remodelling occurs resulting in a narrowing of lumen diameter. c.) Foam cell death results in the release of cellular contents contributing to necrotic core of cell debris and lipid deposits rich in cholesterol and cholesterol esters. The rupture of a plaque may result in thrombus formation which may occlude blood flow resulting in the clinically observable events such as heart attack or stroke. d.) Alternatively, the plaque continues to grow without rupture, further impeding blood flow which results in clinically obstructive disease. Adapted from (Rader & Daugherty, 2008).

1.2.3 Lipoproteins and oxidised LDL

Water-soluble lipoprotein particles mediate the transport of triacyl-glycerides, phospholipids, and cholesterol esters in circulation around the body. The oxidation of the cholesterol carrying lipoprotein particle, low density lipoprotein (LDL), in the walls of arteries is a significant step in the development of development of heart disease, stroke and often high blood pressure, collectively referred to as CVD (Gieseg, Crone, & Amit, 2009). The oxidation of LDL is a complex process, during which both the protein and lipids undergo oxidative changes forming complex products. These changes include non-enzymatic oxidative changes in amino acids, as well as proteolysis and cross-links of apoprotein B (apo-B) occur, which result in extensive alteration of lipoproteins composition and structure (Fong, Parthasarathy, Witztum, & Steinberg, 1987).

LDL has a high content of poly-unsaturated fatty acids (PUFAs). These fatty acids react readily and rapidly with oxygen to form lipid peroxides via peroxyl radical chain reaction. This appears to occur through a number of redox active transition metal ion binding sites present on LDL which initiate radical generation (Gieseg, Crone, et al., 2009). Initially, production of oxLDL shows an initial lag phase, where antioxidants, predominantly α-tocopherol (vitamin-E) remove and neutralise lipid peroxyl radicals inhibiting the peroxidation chain reaction (Puhl, Waeg, & Esterbauer, 1994). Additionally, naturally occurring antioxidants such as 7-8-dihydrogeneopterin and ascorbate (vitamin-C) can extend the lag phase by scavenging lipid peroxyl radicals (Esterbauer, Dieber-Rotheneder, Striegl, & Waeg, 1991; Gieseg, Reibnegger, Wachter, & Esterbauer, 1995).

The LDL receptor (LDLr) pathway is a negative feedback-controlled system which plays important roles in maintaining plasma and intracellular cholesterol homeostasis. To maintain cholesterol homeostasis, LDLr expression is tightly regulated therefore it should not be possible for regular LDL to be taken-up in sufficient quantities to be responsible for foam cell formation (Y. Zhang, Ma, Ruan, & Liu, 2016). To investigate this, LDL particles were modified by the addition of an acetyl-group (acLDL), so they would not be recognised by LDLr, however uptake still occurred. AcLDL is not synthesised in-vivo, however, oxLDL is formed in vivo due to radical formation, and interacts with receptors similarly to acLDL.

1.2.4 Scavenger Receptors

The unregulated uptake of oxLDL was found to be mediated by scavenger receptors (SR) first described by Brown and Goldstein in 1979. The uptake of modified LDL-particles such as oxLDL, by scavenger receptors which are not inhibited by the cell's cholesterol content (Brown, Goldstein, Krieger, Ho, & Anderson, 1979). Although the uptake of excess LDL particles from the intima may appear to be beneficial as it removes excess cholesterol, the unregulated manner by which uptake occurs leads to build-up of cholesterol-laden macrophages and the establishment of chronic inflammation. These cholesterol-laden macrophages are termed foam-cells, due to them being so packed with cholesterol they appear visibly foamy under a microscope. The ability to target endocytosed oxLDL for degradation differs between individual scavenger receptors (SR). Both cluster of differentiation 36 (CD36) and SR-B1 bind and internalise oxLDL very efficiently however, CD36 mediates efficient degradation of oxLDL, but with cholesterol ester accumulation SR-B1 does not. The inability of SR's like SR-B1 to provide efficient targeting of modified lipoproteins such as oxLDL for degradation in macrophages is one of the critical processes which result in foam cell formation (Kzhyshkowska, Neyen, & Gordon, 2012).

1.3 Inflammation and immune cell activation in CVD

1.3.1 Atherosclerosis and inflammation

Over time, the views on the pathophysiology of atherosclerosis have substantially evolved. At first, the thinking was dominated by the link between lipids and atherosclerosis based on strong experimental and clinical relationships between hypercholesterolemia and plaque (R. Ross & L. Harker, 1976). However, 20 years ago, the scientific community began to appreciate a prominent role for inflammation in atherosclerosis (Libby et al., 2002). Evidence from various trials (JUPITER, LIPID and PRINCE) over time demonstrated that statin treatment reduced CVD risk in primary prevention. The trials focussed on individuals with varying levels of cholesterol and the inflammatory marker C-reactive protein, and indicated statins possessed anti-inflammatory effects which may contribute to their success as preventative strategies. However, they were unable to verify that the improved CVD risk was independent of the statin's lipid-lowering effects (S Antonopoulos, Margaritis, Lee, Channon, & Antoniades, 2012). More recently, evidence from the CANTOS and CIRT studies, which finished in 2017 and 2018 respectively, showed a significant reduction in the primary end point in patients who

received a 150 mg dose of the drug canakinumab, 4 times a year over a 4-year period compared to the placebo (Ridker et al., 2017). This drug was selected as it possessed potent anti-inflammatory properties and no lipid-lowering ability. This provided clear evidence that targeting inflammation independent of lipid-lowering produced positive outcomes by reducing CVD risk, confirming the relationship between CVD risk and inflammation. This also indicates the value of markers of inflammation when it comes to the diagnosis of CVDs.

1.3.1 Cells mediating inflammation

1.3.1.2 T-cell activation

Both innate and adaptive immune components play crucial roles in atherosclerotic pathogenesis. As atherosclerotic lesions mature, T-cells mediate the dominant driving forces of inflammatory progression. All subsets of T-cells ($CD4^+$, CD8+, $TCR\gamma\delta^+$ and NKT cells) have been identified as present in human and mouse atherosclerotic plaques at all stages of atherosclerotic development (Andersson, Libby, & Hansson, 2010; Daughtery, 2000; Hansson, Robertson, & Söderberg-Nauclér, 2006). Analysis of expression markers revealed that plaques are highly enriched with activated T-cells, also confirming that CD8 (cytotoxic) T-cells are proportionately more activated in patient plaques than in patient blood (Grivel et al., 2011). Contact mediated signalling on monocytes by stimulated T-cells is a potent proinflammatory mechanism which triggers massive upregulation of proinflammatory cytokines, and tumour necrosis factors (TNF) (Burger & Dayer, 2002). Subsequently, the activated T-cells initiate production of pro-inflammatory mediators, by which the inflammatory response intensifies and the pathology progresses and worsens (Ait-Oufella, Taleb, Mallat, & Tedgui, 2011). Additionally, T-cells involved in atherosclerosis have the characteristics of TH-1 (T-helper type1 cells) as a result of their production of proinflammatory cytokines, such as Interferon-γ (IFN- γ), a prominent cytokine secreted by these activated T-cells in atherosclerosis.

1.3.1.3 Macrophage activation

Monocyte derived macrophages have been well documented as playing integral roles in both the early development and advanced progression of atherosclerotic plaques. The early inflammatory response to retained apo-B lipoprotein particles facilitated by endothelial cells results in the secretion of chemokines, recruiting predominantly circulating blood monocytes, to the intimal space. These monocytes become stimulated by local SMC secreted M-CSF and differentiate into mature tissue macrophage (Hazen, 2008). These macrophages then proceed

to uptake LDL particles in an unregulated manner through scavenger receptors as previously mentioned (section 1.2).

Proliferation of these resident macrophages contributes predominantly to the local macrophage population, more so than recruited monocytes. This was demonstrated in murine atherosclerotic lesions, where macrophages were shown to rapidly turnover after 4-weeks, with their replenishment depending predominantly on local proliferation, rather than monocyte influx (Moore, Sheedy, & Fisher, 2013). The inhibition of macrophage proliferation by the administration of Simvastatin to ApoE deficient mice resulted in rapid reduction in plaque inflammation and favourable phenotype remodelling (Tang et al., 2015). This self-sustaining macrophage population is activated by multiple stimuli associated with atherosclerotic risk factors including oxLDL, advances glycosylation endpoints of diabetes, angiotensin-II, and endothelin. Alternatively, activation may occur through Th-1 and Th-2 secreted cytokines such as IFN-γ (Boyle, 2005). Activation depending on macrophage type leads to the expression further pro-inflammatory particles such as TNF-α and IL-1β, metalloproteinases, production of ROS and induce apoptosis (Bobryshev, Ivanova, Chistiakov, Nikiforov, & Orekhov, 2016; Boyle, 2005). All of these associated effector functions worsen the progression of atherosclerosis by some means and contributes to disease advancement.

1.3.2 Interferon-γ

Interferon-γ (IFN-γ) is a prominent inflammatory cytokine secreted by activated T-cells, critical for innate and adaptive immune system functionality (Murphy, 2012). IFN-γ is the central stimulus for the activation of the enzyme GTP cyclohydrolase-1, whereby in 6-pyruvoyl-tetrahropterin synthase-deficient human monocytes/macrophages IFN-γ facilitates the accumulation of 7,8-dihydroneopterin and subsequently its oxidation product neopterin. 7,8-dihydroneopterin is a marker of inflammation and immune cell activation, as such measuring the 7,8-dihydropneopterin produced by a population of cells serves as an indication of immune cell activation.

IFN-γ has been shown to be pro-atherogenic in murine models through the activation of the JAK/STAT pathway (**Figure 1.3.2**). The JAK/STAT signalling cascade consisting of Janus kinases 1 and 2 (JAK) and signal transducer and activator of transcription (STAT) proteins, has been implicated in a variety of inflammatory pathologies. Binding of the ligand IFN-γ to the extracellular portion of the IFN-γ receptor complex (IFNGR) triggers the juxtaposition and phosphorylation of various proteins in the cascade, eventuating in STAT1 translocation to the

nucleus. Once located in the nucleus, STAT1 subsequently binds the promoter of IFN-γ inducible genes. Findings show incubating human monocyte-like THP-1 cells with IFN-γ, produces maximal STAT1 activity (phosphorylation) (N. Li et al., 2010).

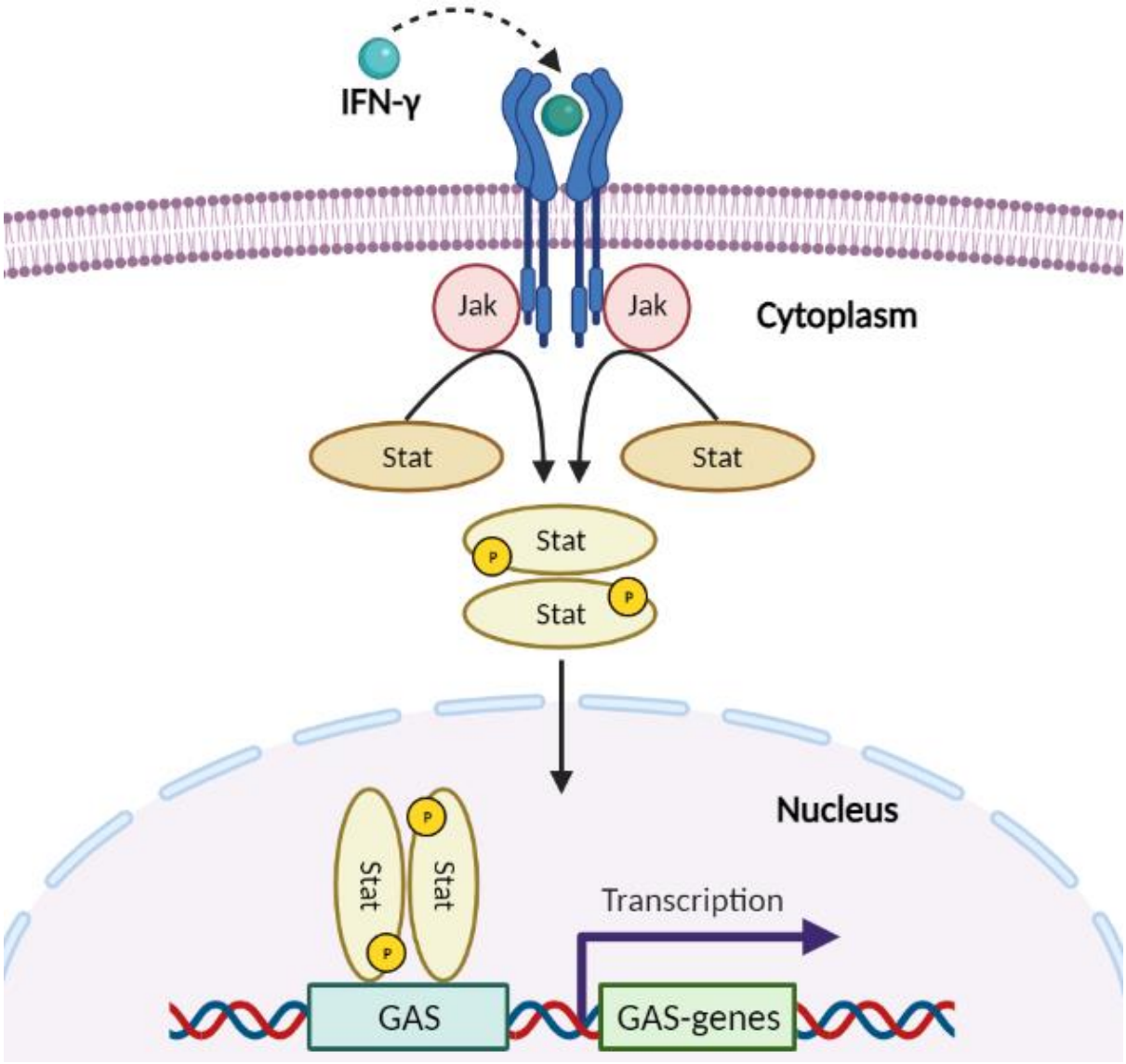


Figure 1.3.2 IFNGR–JAK/STAT signalling pathway. Upon binding the ligand, IFN-γ, cytokine associated JAKs are activated. The activated JAK proteins facilitate phosphorylation of specific receptor residues and recruited STAT proteins. The activated STAT proteins are then liberated from the receptor, proceed to dimerize, and translocate to the nucleus. Once located in the nucleus, the active STAT complex binds to members of the Gamma Activated Site (GAS) family of enhancers and subsequent transcription of GAS-genes occurs. Adapted from (Kisseleva, Bhattacharya, Braunstein, & Schindler, 2002).

1.3.3 Neopterin and 7,8-dihydroneopterin as inflammatory biomarkers

Clinically, levels of inflammation occurring as a result of physical trauma CVD, cancer, bacterial, parasitic, and viral infections can be assessed by measuring the concentration of plasma and urinary neopterin (Baydar et al., 2009; Eisenhut, 2013; Melichar et al., 2017; Pedersen et al., 2011; Signorelli et al., 2013). Neopterin is produced in response to IFN-γ activation of monocytes and macrophages, therefore it provides a direct measure of immune activation. Additionally, neopterin is highly fluorescent, making it easily detectable at low concentrations by methods such as HPLC. Neopterin may also be measured by ELISA-based assays, which are available for use clinically. This research group has made extensive use of neopterin analysis to assess oxidative stress *in vitro* cell culture and exercise-induced injury (A. Lindsay et al., 2015; A. Lindsay, Janmale, Draper, & Gieseg, 2014; Angus Lindsay, Lewis, Scarrott, Draper, & Gieseg, 2014).

The convenience of measuring neopterin alone disregards the basic biology that macrophages do not directly produce neopterin enzymatically. Elevations in tissue and fluid neopterin concentrations are the product of immune-activated macrophages and oxidants reacting with 7,8-dihydroneopterin to produce neopterin. Consequently, the ratio of neopterin to 7,8-dihydroneopterin may vary depending on the extent of immune activation and oxidative conditions at the site of inflammation. Therefore, this research group has recommended that clinically, both neopterin and 7,8-dihydroneopterin be measured in conjunction to achieve a more accurate measurement of macrophage activity during inflammation (Fuchs et al., 1989; Gieseg, Baxter-Parker, & Lindsay, 2018). The biochemical effects of neopterin and 7,8-dihydroneopterin will be described in **Section 1.5**.

1.3.4 Interferon-γ drives inflammatory progression

The chronic inflammation which is characteristic of atherosclerosis is hypothesised to be as a result of the continual recruitment of immune cells, such as monocytes, and T-cells to the site of atherosclerotic lesions. IFN-γ acts as a mediator of chemokine release and shapes the adaptive TH-1 immune response. IFN-γ is responsible for inducing a number of chemokines, such as the CC chemokine monocyte chemoattractant protein 1 (MCP-1), which attracts/directs both monocytes and T-cells to the site of the atherosclerotic lesion. The role of MCP-1 in atherosclerosis is significant with regard to the progression of atherosclerotic plaques. This was demonstrated in MCP-1-knockout mice, which displayed a reduction in the size of atherosclerotic lesions (Harvey EJ, 2007). Additionally, the binding of IFN-γ to other sensitive

chemokines stimulates further accumulation of activated CD4-TH1 T-cells in atherosclerotic regions. In contrast to its pro-atherogenic activities, IFN-γ has also been shown to display some anti-atherogenic functionality. These include inhibiting macrophage lipoprotein-lipase (LPL) expression, an enzyme which functions as a triglyceride hydrolase, in receptor-mediated uptake of lipoprotein, and the oxidation of LDL (Hughes TR, 2002). Although IFN-γ displays some anti-atherogenic function, atherosclerosis is an incredibly complex pathology and the actions of IFN-γ have been shown to be predominantly pro-atherogenic and worsen the disease by driving inflammatory progression.

1.4 Statins

1.4.1 HMG-CoA reductase inhibition

The accumulation and oxidation of the cholesterol carrying lipoprotein particle, low density lipoprotein (LDL) within the arterial wall is a significant step in the development of CVD. High blood cholesterol (hypercholesterolaemia) is a risk factor for both fatal and non-fatal CVD in people with and without a past CVD (Taylor et al., 2013). Virtually all cells synthesize cholesterol through the coordinated action of several enzymes. This process is mainly controlled by a feedback mechanism mediated by mevalonate-derived products which act on key enzymes such as 3-hydoxy-3methylglutaryl-co-enzyme-A (HMG-CoA) reductase (HMGR) (**Figure 1.4**). HMGR catalyses the committed step in cholesterol biosynthesis, involving the four electron diacylation of HMG-CoA to CoA and mevalonate. The main clinically used inhibitors of HMGR are the statin drugs, which limit cholesterol synthesis by inhibiting this committed step in the biosynthesis of isoprenoids and sterols (Corsini et al., 1995).

1.4.2 Statin treatment strategies

In 1994, the landmark Scandinavian Simvastatin Survival Study established clear benefits of HMGR inhibition on patient mortality (Scandinavian Simvastatin Survival Study, 1994). Since then, evidence has proven statins to be effective as both a disease onset preventative and disease impact reduction (primary and secondary preventative) strategies. Both high cholesterol and non-high cholesterol groups showed improved outcomes as a primary strategy, and secondary by lowering cholesterol by an average of 1.8 mmol/L, reducing coronary heart disease event risk by 60% and risk of stroke by 17% (Law, Wald, & Rudnicka, 2003; Taylor et al., 2013).

Statin treatment is recommended as both primary and secondary preventative strategies at an intensity dependent on an individual's determined cardiovascular risk. Statin treatment strategy is determined by associated risk factors including age, LDL-c level (mg/dL), hs-CRP level (mg/L) familial history, evidence of genetic hyperlipidaemias, determined calcium levels, sex and ethnicity (Stone et al., 2014).

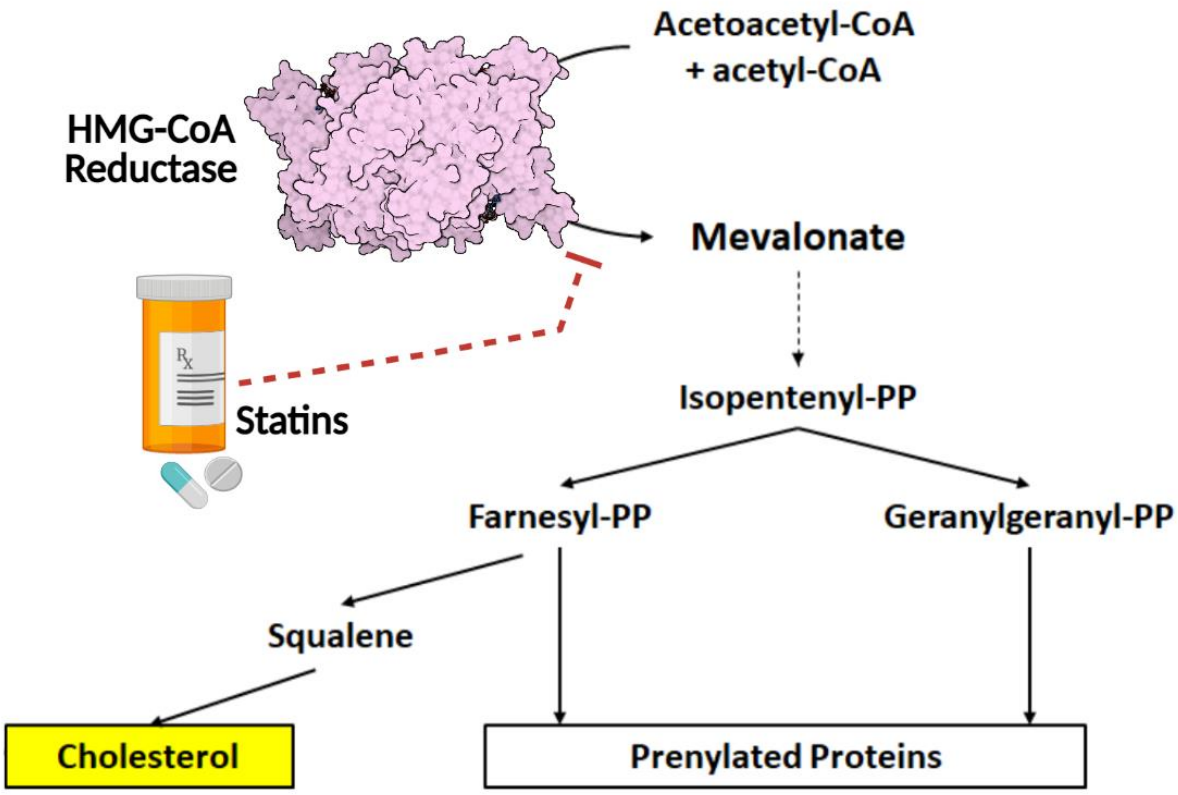


Figure 1.4.1 3-hydroxyl-3-methyl coenzyme-A reductase pathway. Depicts HMGR and its products/effects downstream. Acetoacetyl-CoA and acetyl-CoA are converted by HMGR to mevalonate. Statin drugs competitively inhibit HMGR activity. If HMGR is uninhibited, mevalonate is converted through to isopentyl-PP which is then converted to either of the isoprenoids: farnesyl-PP or geranylgeranyl-PP. The isoprenoids are subsequently either utilised in protein prenylation or are converted to squalene; the precursor molecule of all sterols, namely cholesterol which is essential for life. Adapted from (Schönbeck & Libby, 2004).

1.4.3 Statin structure and type

Structurally, statins all possess an HMG-like moiety, which may be present in an inactive prodrug lactone form, which is subsequently hydrolysed in vivo to the active hydroxy-acid form. Statins all share rigid, hydrophobic groups which are covalently linked to an HMG-CoA like moiety. Statins act as competitive inhibitors of HMGR with respect to the binding of the substrate: HMG-CoA, but not with respect to the binding of NADPH. Statin binding occurs through several polar interactions formed between their HMG-CoA like moieties and residues located in the cis-loop of HMGR, as well as hydrogen-bonding and salt-bridge formation. Hydrophobic residues of HMGR also participate in binding via Van der Waals contacts with statin molecules. It appears the innate flexibility of the COOH-terminal region of HMGR is utilised by statins to procure a binding site for the inhibitor molecules. The structurally diverse, rigid hydrophobic regions of statins are accommodated in a shallow non-polar groove that is present only when these COOH-terminal residues are disordered. Subsequently, the bulky hydrophobic components of statin molecules occupy the HMG-binding pocket and part of the binding surface for CoA, consequently the access of the substrate HMG-CoA to HMGR's active site is blocked (E. S. Istvan & Deisenhofer, 2001).

Statins differ in structure apart from their shared HMG-like moiety (**Figure 1.4**), with these structural differences resulting in comparatively different binding characteristics excluding the possessed HMG-like moiety. Statins may be divided into two classes of inhibitor based on their structure. Type 1 inhibitors (simvastatin and pravastatin) feature a decalin ring as well as the shared HMG-like moiety. In contrast, type 2 inhibitors (atorvastatin) feature a fluorophenyl group and a methylethyl group, not present in the type 1 inhibitors in addition to the shared HMG-like moiety. The methylethyl structure of type 2 inhibitors functionally replaces the decalin ring of type 1 inhibitors, additionally type 2 statins exhibit additional binding interactions between their fluorophenyl groups HMGR Arg^{590} residue. Type 2 inhibitors differ with regard to the central ring structure of the molecule, whereas type 1 do not (E. Istvan, 2003).

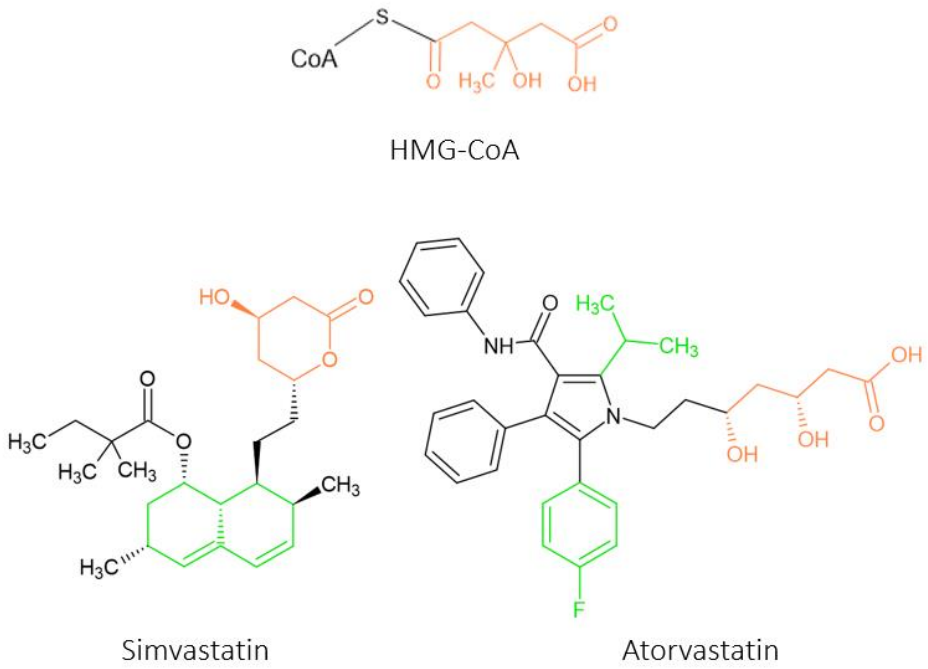


Figure 1.4.3 Structural formulas of substrate HMG-CoA, type 1 and 2 statins. Depicted: HMG-CoA, the substrate of HMG-CoA reductase, atorvastatin an example of a type 2 statin and simvastatin, an example of a type 1 statin. The HMG-like moiety through which the molecules bind to the enzyme is indicated in orange. The decalin ring of simvastatin, and the fluorophenyl group and methylethyl of atorvastatin are indicated in green. Chemical structures were obtained from RCSB-PDB and resolved using ACD-ChemSketch .

1.5 7,8-dihydroneopterin

1.5.1 GTP, 7,8-dihydroneopterin and products

Neopterin is the oxidation product of 7,8-dihydroneopterin (Gregory Baxter-Parker et al., 2020), a pteridine biosynthetically derived from guanosine triphosphate (GTP). 7,8-dihydroneopterin is the product of the IFN-γ-mediated upregulation of GTP cyclohydrolase-1 (GTPCH1) and is primary pterin generated in immune activated monocyte-derived macrophages. Subsequently, oxidants and immune-activated macrophages reacting with 7,8-dihydroneopterin generate neopterin, resulting in an observable elevation in tissue and fluid neopterin concentrations (Gieseg et al., 2018). The ratio of neopterin to 7,8-dihydroneopterin may differ depending on the level of immune activation and oxidative environment at sites of inflammation.

IFN-γ induces the activity of GTPCH-1, depicted below (**Figure 1.5**), which converts GTP into 7,8-dihydroneopterin-triphosphate (E.R. Werner et al., 1990). Subsequently, phosphatase enzymes hydrolyse 7,8-dihydroneopterin-triphosphate to generate 7,8-dihydroneopterin (Wachter et al., 1992). Co-stimulation with tumour necrosis factor-α (TNF-α), dexamethasone or LPS has also been demonstrated to enhance synthesis, however not as strongly as IFN-γ (Werner-Felmayer et al., 1995; Werner-Felmayer et al., 1990). Breakdown occurs by reactive oxidant species and/or immune-activated macrophages reacting with molecules of 7,8-dihydroneopterin, producing neopterin, 7,8-dihydroxanthoperin and xanthopterin. 7,8-dihydroneopterin has been shown to be oxidised to neopterin by radical scavenging of superoxide and hypochlorite (HOCl) in cellular and cell free systems (Gregory Baxter-Parker et al., 2020; Yang, Whiteman, & Gieseg, 2012) The measurement of neopterin is therefore a measurement of both oxidative stress due to superoxide and HOCl generation and inflammatory activation generating 7,8-dihydroneopterin.

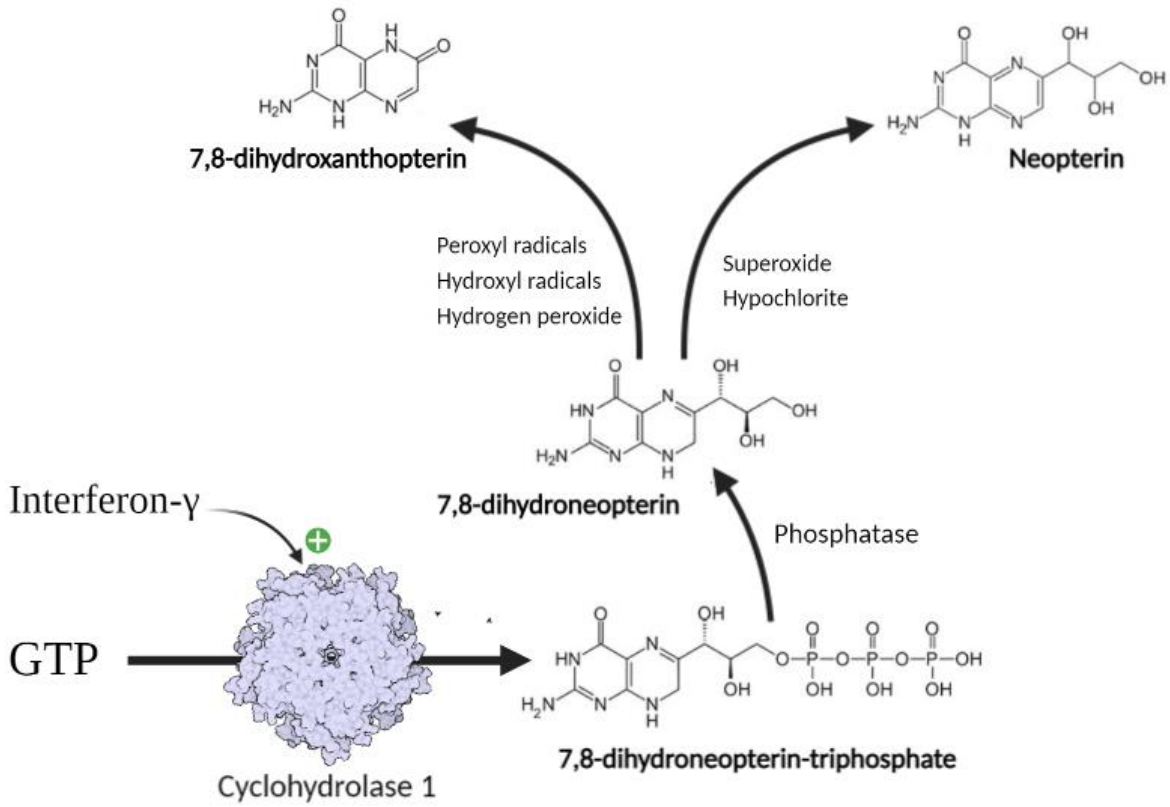


Figure 1.5.1 Formation and oxidation of 7,8-dihydroneopterin. In macrophages, interferon-γ upregulates the cytosolic enzyme GTP cyclohydrolase-1, which catalyses the conversion of Guanosine triphosphate (GTP) to 7,8-dihydroneopterin-triphosphate. Subsequently, the activity of non-specific phosphatases produces 7,8-dihydroneopterin. The oxidation of 7,8-dihydroneopterin produces neopterin or 7,8-dihydroxanthopterin, determined by the oxidant. Adapted from (Gieseg et al., 2018).

In non-primate macrophage cells and other non-monocytic derived cells located in the body, 7,8-dihydroneopterin is converted to tetrahydrobiopterin, which serves as a key cofactor for multiple synthetic enzymes. This is facilitated by the combined enzymatic action of 6-pyruvoyltetrahydropterin synthase followed by sepiapterin synthase, (E.R. Werner et al., 1990). In primate macrophages, 7,8-dihydroneopterin synthesis occurs predominantly due to IFN-γ not increasing the expression of 6-pyruvoyltetrahydropterin synthase. Consequently, the main product of GTP metabolism via the GTPH1 pathways is cytosolic 7,8-dihydroneopterin during macrophage activation in humans (Gieseg et al., 2018; Schoedon et al., 1987).

1.5.2 7,8-dihydroneopterin in atherosclerosis

Atherosclerotic plaques are sites of chronic inflammation as indicated by the high number of immune cells like macrophages, and the presence of various inflammatory markers. Macrophage cells are capable of secreting an array of oxidising agents including superoxide, hydrogen peroxide, lipid peroxides, lipoxygenase and possibly hypochlorite which are all compounds known to contribute to the oxidation of LDL (Gieseg, Leake, et al., 2009). Research has previously demonstrated atherosclerotic plaques to be a source of 7,8-dihydroneopterin and neopterin in symptomatic atherosclerosis patients (Janmale et al., 2015). In addition, direct stimulation of macrophages in live excised carotid-plaque samples generated a sustained increase in neopterin and 7,8-dihydroneopterin (Hannah Prebble et al., 2018).

Reduced pterins, such as 7,8-dihydroneopterin have been demonstrated to act as antioxidants and are produced by the macrophage cells present in atherosclerotic plaques. 7,8-dihydroneopterin has been demonstrated to have an athero-protective effect, functioning as a potent radical scavenging and chain-breaking antioxidant, which may outcompete α-tocopherol for the lipid peroxyl radical during LDL oxidation, (Gieseg et al., 1995). Furthermore, 7,8-dihydroneopterin has been shown to interfere with reactions mediated by reactive oxygen species (ROS), also preventing free radical damage from both hydroxyl and peroxyl radicals (Gieseg, Duggan, Rait, & Platt, 2002; Oettl, Greilberger, Dikalov, & Reibnegger, 2004).

In addition to 7,8-dihydroneopterin's antioxidant properties, it is hypothesised a second mode of athero-protection exists via the down regulation of CD36, a transmembrane plasma protein, which along with class A scavenger receptors was demonstrated to be responsible for approximately 75-90% of acetylated and oxidised LDL uptake (Kunjathoor et al., 2002). Previous work has shown 7,8-dihydroneopterin has a modulating effect on CD36 expression. 7,8-dihydroneopterin downregulated CD36 in U937 cells and human monocyte derived

macrophages, inhibiting foam cell formation, however, no reduction in oxLDL mediated cell death was observed. These results suggest that 7,8-dihydroneopterin may modulate foam cell formation in atherosclerotic plaques (Ghodsian, Yeandle, & Gieseg, 2021; Gieseg, Amit, Yang, Shchepetkina, & Katouah, 2010; Shchepetkina, Hock, Miller, Kennedy, & Gieseg, 2017). The importance of monocyte/macrophage-related CD36 activity during the initiation and growth of atherosclerotic plaque has been supported by a reduction in this size of plaque by inactivation of CD36 in ApoE-deficient mice (Febbraio et al., 2000; Handberg et al., 2008). Previous research shows various statins upregulate the expression of CD36 surface protein and mRNA. These effects were reversed with the addition of mevalonate and geranylgeraniol, suggesting that geranylgeranylation of proteins is required for regulation of CD36 (Ruiz-Velasco, Domínguez, & Vega, 2004)

1.6 Kynurenine and tryptophan

Activation of the immune system results in accelerated tryptophan degradation through the kynurenine pathway. Human dendritic cells can metabolise L-tryptophan into kynurenine through the enzymatic action of indoleamine 2,3-dioxydenase (IDO). This enzyme possesses minimal activity in non-pathogenic conditions; however, it is highly inducible by proinflammatory cytokines, especially IFN-γ (**Figure 1.6**) (Byrne et al., 1986; Thomas & Stocker, 1999; Ernst R. Werner et al., 1987). IDO's catabolic activity may also be induced by TNF-α, or via activation through recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Heyes et al., 1992; Pemberton, Kerr, Smythe, & Brew, 1997). Tryptophan is an essential amino acid essential for protein synthesis, and the IDO-induced degradation of tryptophan appears to function as a defence mechanism limiting the growth of intracellular pathogens or malignant cells by diminishing the availability of tryptophan. It is believed this occurs to limit T-cell and bacterial proliferation.

With regard to coronary heart disease (CHD) decreased tryptophan concentrations were found in a significant proportion of CHD patients which coincided with an increased kynurenine to tryptophan ratio ($kyn.trp^{-1}$) alongside an increased neopterin ratio indicating an activated cellular immune response (Wirleitner et al., 2003). PBMCs containing monocytes and Th-1 helper cells isolated from human blood and cultured *in vitro*, have been shown to degrade tryptophan and produce high levels of kynurenine upon direct stimulation with

phytohaemagglutinin or concanavalin A (Jenny et al., 2011). These factors make kynurenine an excellent candidate to be utilised as a biomarker in detecting immune system activation and inflammation. Kynurenine is the product of enzymatic catabolism, it is not the product of oxidation like neopterin, thus it serves as a direct measure of immune cell activation and stimulation. This is particularly relevant as with cell culturing the concentration of tryptophan may already be quantified before experimentation begins.

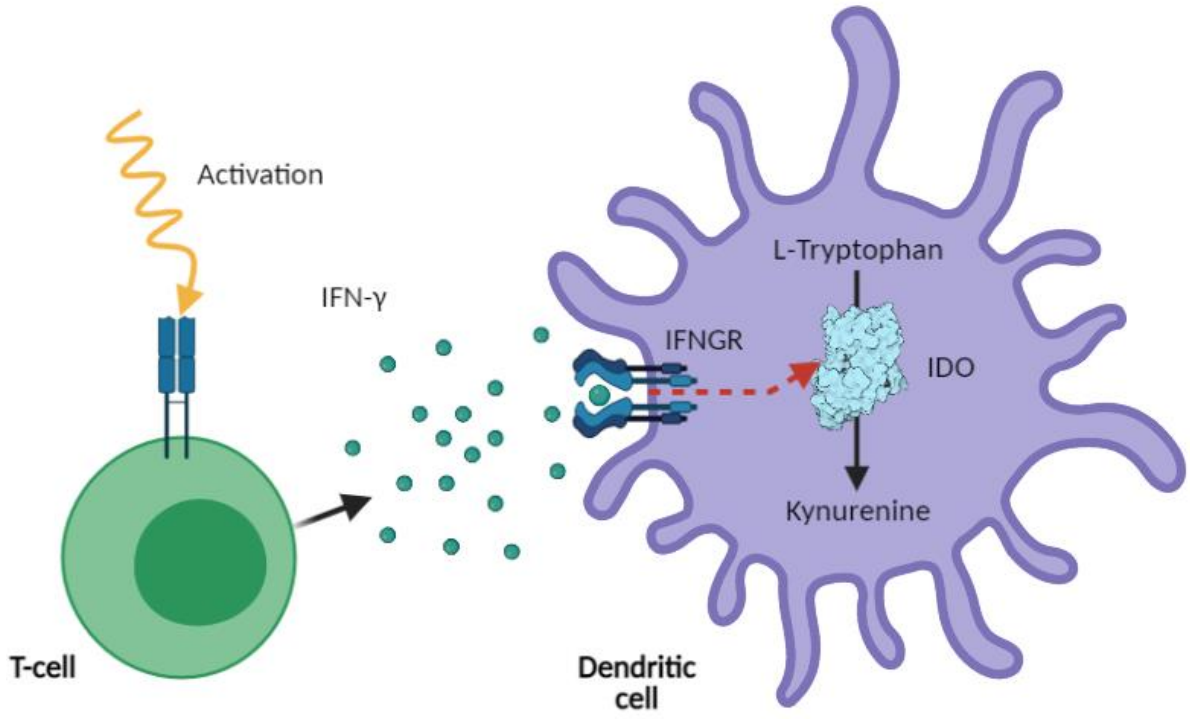


Figure 1.6 IFN-γ-tryptophan-kynurenine pathway. T-cell is stimulated/activated. T-cell secretes potent pro-inflammatory cytokine: IFN-γ, which binds to extracellular portion of the IFN-γ receptor (IFNGR). IFN-γ signal is transduced through plasma-membrane via IFNGR complex and subsequently induces IDO activity. IDO activity enzymatically converts cellular L-tryptophan though to kynurenine via its activity, increasing the ratio of kynurenine to tryptophan ($kyn.trp^{-1}$).

1.7 Statins as anti-inflammatory agents

1.7.1 Pleiotropic effects of statins

Experimental investigations implicate a role for inflammation in various phases of atherogenesis, from disease initiation and progression to potentially fatal plaque rupture, occlusion, and abrupt reduction in blood flow to vital organs. Evidence from both experimental and clinical studies support statins producing effects beyond lipid-lowering, termed "pleiotropic effects". The pleiotropic effects of statins further lower CVD-risk through various additional benefits, resulting in a greater reduction in CVD-risk than other LDL lowering modalities. As well as cholesterol-lowering, the anti-inflammatory properties of statins have been considered responsible for their protective effects in patients with coronary heart disease. For example, the JUPITER study demonstrated that treatment with statins reduces CV risk as a primary preventative measure, even in healthy individuals presenting with regular cholesterol, but elevated C-reactive protein levels (CRP) (Paul M. Ridker et al., 2008). Inhibitors of HMGR have also been associated with decreased serum neopterin levels, previously described as a biomarker of inflammation (**section 1.3.3**), in stable coronary artery disease. Neopterin was shown to be lower in subjects using statins compared to those were not to a statistically significant extent. This statistically significant association was also established as extending to a patient subgroup presenting with coronary artery disease (R. B. Walter, D. Fuchs, G. Weiss, T. R. Walter, & W. H. Reinhart, 2003).

1.7.2 Molecular mechanisms underlying anti-inflammatory effects

1.7.2.1 Mevalonate pathway

Drug therapy involving statins is implemented to lower LDL cholesterol by inhibiting HMG-CoA reductase, the committed step in the mevalonate pathway, responsible for cholesterol biosynthesis. Co-incubation of human vessels with statins and mevalonate demonstrated a reversal in the beneficial effects of statins on vascular redox state, underlining the importance of the mevalonate pathway in mediating the pleiotropic effects of statins (Antoniades et al., 2011). L-Mevalonate, the product of HMG-CoA reductase, is the precursor in the formation of the important isoprenoid metabolic intermediates: farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) (Schönbeck & Libby, 2004). These isoprenoids are utilised in prenylation, a form of post-translational modification involving the covalent addition of either farnesyl (15-carbon) or geranylgeranyl (20-carbon) to a protein via conserved cysteine

residues at or close to a proteins C-terminus. Isoprenylation provides proteins with a hydrophobic C-terminus, greatly increasing their capacity to interact with cellular membranes which contain a high concentration of signalling molecules. Effectively isoprenylation facilitates membrane association, subcellular localisation, and intracellular trafficking of proteins, in addition to important protein-protein interaction (Wang & Casey, 2016; F. L. Zhang & Casey, 1996).

Protein prenylation affects many signal transduction molecules in vascular and myocardial signalling pathways. The small guanosine triphosphate (GTP) binding proteins, Rho Rac and Ras, are significant intracellular signalling pathways modulated by statins; consequently, the diverse effects of such small GTP-binding proteins are diminished. These small GTP-binding proteins modulate pro-atherogenic and pro-inflammatory pathways and are dependent on isoprenylation for their activation. Rho proteins affect the expression of proinflammatory cytokines and are involved in the formation and maintenance of the actin cytoskeletal system. Ras proteins regulate cellular proliferation and hypertrophy, while Rac signal transduction affects the generation of ROS (Wolfrum, Jensen Kristin, & Liao James, 2003).

1.7.2.2 Nuclear factors

Statin modulation of cell-surface and intracellular signalling eventuates in nuclear events regulating gene expression, which subsequently effects cellular function. A diverse range of pro-inflammatory stimuli intersect a few key-transcriptional pathways, including nuclear factor-κB (NF-κB) and activator protein-1 (AP1) which induce the expression of pro-adhesive and pro-thrombotic genes in vascular cells. Statins have been shown to limit NF-κB nuclear accumulation, thought to occur by increasing the expression of Inhibitor of NF-κB (I-κB). NF-κB typically exists in the cytosol bound to I-κB. I-κB phosphorylated in response to inflammatory stimuli, resulting in degradation, which in turn liberates NF-κB to translocate to the nucleus (Hayden & Ghosh, 2008; Jain & Ridker, 2005). With regard to AP-1, statins have demonstrated a reduction in c-Jun expression, one of the two proteins comprising the AP-1 complex (Dichtl et al., 2003).

Another mechanism involving nuclear factors by which statins exhibit anti-inflammatory benefits is through Kruppel-like factor-2 (KLF-2) and peroxisome proliferator activated receptors (PPARs) (Feinberg, Lin, Fisch, & Jain, 2004; Plutzky, 2003). Sustained expression of KLF-2 was shown to induce key factors such as eNOS and thrombomodulin, and inhibit cytokine-mediated activation of pro-adhesive, pro-thrombotic factors (SenBanerjee et al.,

2004). Inhibition of NF-κB transcriptional activity was also shown to impart KLF-2 the ability to inhibit pro-inflammatory activity (SenBanerjee et al., 2004). The PPAR family of transcriptional factors has also demonstrated activation and interaction with statins. PPARs are lipid-activated receptors which regulate components of lipid and lipoprotein metabolism, glucose homeostasis and haematosis (Duval, Chinetti, Trottein, Fruchart, & Staels, 2002). Studies have focussed predominantly on PPAR-γ and PPAR-α, which have been identified in vascular cells and shown to possess potent anti-inflammatory properties (Marx, Libby, & Plutzky, 2001; Ricote, Li, Willson, Kelly, & Glass, 1998). This occurs partly via inhibition of NF-κB and AP-1's transcriptional activity, with both PPAR-γ and PPAR-α specific agonists demonstrating strong inhibition of experimental atherosclerosis and macrophage foam cell formation (A. C. Li et al., 2004).

1.7.3 Anti-inflammatory effects

1.7.3.1 Endothelial regulation

Among the key functions of the endothelium with respect to CVD is to maintain an anti-adhesive, anti-thrombotic surface and to regulate blood vessel tone (Gimbrone Jr, 1999). Statins favourably induce endothelial nitric oxide synthase (eNOS) expression, which in turn generates Nitric oxide (NO) in the vessel wall. This process is modulated by the inhibition of intracellular isoprenoid formation, thereby reducing the activation of the GTP-binding protein: Rho. Reduced Rho activation facilitates increased eNOS gene expression through nuclear factor KLF-2 and eNOS mRNA stabilisation via polyadenylation. Statins increase eNOS gene expression via stimulation of the PI3-Akt protein kinase pathway (S Antonopoulos et al., 2012). NO confers anti-inflammatory benefits such as vascular relaxation and inhibition of smooth-muscle-cell proliferation, leukocyte-endothelial interaction, platelet aggregation and endothelial platelet exocytosis (Ignarro, Buga, Wood, Byrns, & Chaudhuri, 1987). Thus, by inducing eNOS accumulation within endothelial cells, statins mitigate many of the negative consequences of reduced NO availability in the inflammatory setting. Inflammatory stimuli such as cytokines also induce expression of immune-recruiting leukocyte adhesion molecules such as VCAM-1 and ICAM-1 upon endothelial surfaces. Expression of leukocyte adhesion molecules promotes monocyte accumulation within the vessel wall by binding and mediating the transport of monocytes between endothelial cells (Cybulsky et al., 2001). Statins have been shown to inhibit the expression of these adhesion molecules, consequently providing the anti-

inflammatory effect of reducing immune/inflammatory cell investment within the vessel wall (Jain & Ridker, 2005).

1.7.3.2 Redox regulation

The vascular redox state is crucial in maintaining global vascular homeostasis and in atherosclerotic development. The redox state is the summation of ROS formation by pro-oxidant sources and elimination by antioxidant production and radical scavenging. Statins have been shown to up-regulate the gene expression and activity of several cellular antioxidant enzymes, including catalase, superoxide dismutase and thioredoxin (Haendeler, Hoffmann, Zeiher, & Dimmeler, 2004; Umeji et al., 2006; Wassmann, Wassmann, & Nickenig, 2004). In addition, the critical antioxidant defence system: heme oxygenase-1, which degrades heme, is up-regulated in vitro after atorvastatin treatment (Ali et al., 2007). NADPH oxidase (NOX) primarily functions to produce superoxide-anion radicals from the reduction of NADPH and molecular oxygen in the presence of pathogens. Statins have demonstrated the ability to reduce NOX activity, a major contributor to ROS production in the vascular wall. The reduction is facilitated by a mevalonate reversible inhibition of isoprenoid formation and membrane translocation of the small GTP-binding protein Rac1. Consequently, NOX modulated ROS production is reduced, and oxidative stress eased (Antoniades et al., 2010).

1.7.3.3 Immune cell activation

Atherosclerotic progression is propelled by the production and secretion of pro-inflammatory stimuli which recruit immune cells into the developing plaque and activate them. Simvastatin treatment of PBMCs and hypercholesterolemic patients demonstrated in both cases a decrease in the production of CVD associated pro-inflammatory cytokines: IL-6, IL-8 and MCP-1. This was demonstrated *in vivo* after six weeks of treatment and ELISA serum analysis and *in vitro* through mRNA extraction and Northern blot analysis. Similar results were obtained in HUVECs and analysed by flow cytometry (Rezaie-Majd et al., 2002). *In vitro* treatment of macrophages and resident CNS antigen-presenting cells with lovastatin prevented the expression of the pro-inflammatory modulators: TNF-α and IL-1β (Greenwood, Steinman, & Zamvil, 2006). To explore the potential of statin drug's anti-inflammatory ability on affecting organ transplant outcomes, animal (murine) models of allograft atherosclerosis were treated with relevant doses of cerivastatin. Statin treatment reduced expression of the chemokines: Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES) and MCP-1. Consequently, T-lymphocyte and macrophage accumulation in graft samples was significantly

decreased in the statin-treated recipients. Chemokine related mRNA expression was diminished also for both RANTES and MCP-1 in statin-treated recipient graft samples, further showing statins effectiveness at lowering immune cell activation (Shimizu, Aikawa, Takayama, Libby, & Mitchell Richard, 2003).

Previous research demonstrated that atorvastatin suppresses IFN-γ induced neopterin formation and tryptophan degradation to kynurenine in human peripheral blood mononuclear cell (PBMC) lines. The research showed that atorvastatin was capable of suppressing neopterin production after the addition of potent proinflammatory stimulants including IFN-γ, concanavalin A, and phytohemagglutinin. Kynurenine production was also similarly affected for each of the respective stimulants, with the ratio of kynurenine to tryptophan significantly reducing with the addition of atorvastatin (Neurauter et al., 2003). In addition, previous unpublished findings suggest that atorvastatin inhibits 7,8-dihydroneopterin generation.

1.7.3.4 Platelet activation

Hypercholesterolaemia is associated with platelet hyper-reactivity and both participate in thrombotic complications of advanced atherosclerosis. (Notarbartolo et al., 1995). Activated platelets intensify the chemotaxis of inflammatory cells and inflammation of the vascular wall through the production of pro-inflammatory particles. Platelets possess the CD40/CD40 ligand, belonging to the TNF superfamily, possessing dual prothrombotic and proinflammatory properties. (Antoniades, Bakogiannis, Tousoulis, Antonopoulos, & Stefanadis, 2009). Atorvastatin and simvastatin treatment *in vitro* of HUVECS in contact with activated platelets lead to a reduction in CD40 surface expression, as well as the expression of the aforementioned cellular adhesion molecules: VCAM-1 and ICAM-1. This decrease in the ability of platelets to participate in pro-inflammatory and thrombotic events may attribute to some of the pleiotropic ability of statins to reduce atherosclerotic progression exclusive to their effect on LCL-c (Stach et al., 2012).

1.7.3.5 Vascular smooth muscle cell proliferation

Statins have also demonstrated effect on vascular smooth muscle cell (VSMC) proliferation. The proliferation of SMC in the arterial wall is a prominent feature of atherosclerosis. During a response to injury, activated VSMCs efficiently proliferate and chemotaxis, contributing to repair. However, in a chronic inflammatory setting aberrant regulation leads to increased VSMC dedifferentiation and extracellular matrix formation in plaque (Chistiakov, Orekhov, &

Bobryshev, 2015). Pravastatin was found to inhibit the DNA synthesis of VSMC in a dose-dependent manner, inducing cell cycle arrest in the G1 phase. The ability of mevalonate to restore cycle progression suggested mevalonate or its metabolites are required for VSMC proliferation. Further research showed only the metabolite GGPP restored activity and that small GTP-ase Rho translocation was also inhibited by pravastatin (Terano et al., 1998). Evidence in rat models suggests that statin-mediated cholesterol depletion may coordinate VSMC migration and adhesion to different ECM proteins; regulating cellular thickness, cytoskeletal orientation, consequently the biomechanics of cellular and aortic functionality (Sanyour et al., 2020).

1.8 Objectives of research

The aim of this research project was to study and characterise the effect of statin treatment on immune cell activation by IFN-γ, by measuring 7,8-dihydroneopterin generation and oxidation. Previous unpublished findings by this research group suggest that atorvastatin inhibits 7,8-dihydroneopterin generation. Given the amassing evidence for statins anti-inflammatory effects and statins prevalence and success as preventative strategies by managing LDLc concentration, this research aims to elucidate how statins effect inflammatory markers related to CVD.

This research was carried out on the mixed model cell culture: peripheral blood mononuclear cells (PBMCs), to establish the nature of the statin treatment effect. PBMCs consist of any peripheral blood cell having a round nucleus, predominantly monocytes and lymphocytes, and are often isolated from anticoagulated whole blood for use in preclinical and clinical studies. The relative distribution of the PBMC populations may be influenced by age, ethnicity or gender (Tollerud et al., 1989). Previous research conducted has demonstrated production of the inflammatory markers 7,8-dihydroneopterin and neopterin in isolated PBMCs using various stimulants, including IFN- γ, PHA and PMA (Prebble, 2018). Other studies have also demonstrated that freshly isolated PBMC can be utilised as a screening method for anti-inflammatory properties of compounds by measuring tryptophan degradation (kynurenine) and neopterin formation (Baxter-Parker, 2019). Therefore, PBMCs are very capable of serving as candidates for monitoring immune cell inflammatory production (Jenny et al., 2011; Weiss et al., 1999).

Neopterin can be measured directly by HPLC, and 7,8-dihydroneopterin indirectly through pre-columnal oxidation using tri-iodide treatment and subsequently measured by HPLC. In samples which have undergone pre-columnal oxidation, the measured value is referred to as 'total neopterin'. The concentration of 7,8-dihydroneopterin can then be inferred by subtracting the measured neopterin concentration from total neopterin. Typically, in a clinical setting only serum neopterin levels are analysed. Measuring both neopterin and 7,8-dihydroneopterin takes this a step further. Additionally, kynurenine will be used as a measure of activation of monocytes and macrophage as a measure of inflammation. Kynurenine serves as a suitable addition as it is a marker of inflammation also modulated by IFN-γ. Furthermore, it is the product of catabolism, not oxidation and is affected by independent enzymes.

This research hypothesises that immune cell activation and the subsequent release of 7,8-dihydroneopterin is inhibited by statin treatment. The effect of the statin drug atorvastatin on immune cell activation will be investigated. This statin was selected as it is one of the three statin medications currently listed on the Pharmaceutical Schedule of New Zealand (K. Wilson, 2021). Preliminary investigations and published studies suggest atorvastatin suppresses neopterin formation in PBMCs and plaque derived cells (Neurauter et al., 2003). It is unclear whether atorvastatin inhibits 7,8-dihydroneopterin oxidation to neopterin by suppressing oxidant release or whether it is a suppression of overall immune cell activation resulting in decreased synthesis of 7,8-dihydroneopterin. Furthermore, it is not clear whether simvastatin or pravastatin, which are similar structurally, have the same effect as structurally different atorvastatin.

The focus of this research project is to investigate how atorvastatin affects 7,8-dihydroneopterin synthesis, and whether this affect occurs through inhibiting IFN-γ stimulation, or through blocking the oxidation through to neopterin. This will be achieved by measuring levels of total neopterin (7,8-dihydroneopterin), neopterin and kynurenine produced by activated and non-activated PBMCs. Activation will be achieved by treatment with the pro-inflammatory cytokine interferon-γ (IFN-γ), which is the predominant means by which T-cells activate macrophages and has been well-documented in literature. Investigating the effect of statin treatment on PBMC activation and subsequent pterin production in PBMCs will assist in elucidating how statins effect identified markers of plaque inflammation. Results from this research may aid in the development of total neopterin as a potential clinical diagnostic marker for monitoring a patient's cardiovascular health.

Chapter 2

Materials & Methods

2.1 Chemicals

All the chemicals and reagents used in this research were of analytical grade or higher.

7,8-Dihydroneopterin (7,8-NP)	Schricks Laboratory
Acetonitrile (ACN)	J. T. Baker, NJ, USA
Ammonium acetate	Sigma-Aldrich Co. LCC, New Zealand
Ascorbic Acid	Sigma-Aldrich Co. LCC, New Zealand
Atorvastatin (Calcium Salt)	Sigma-Aldrich Co. LCC, New Zealand
Dimethyl sulphoxide (DMSO)	BDH Lab Supplies Ltd, Poole, England
Ethanol	BDH Lab Supplies Ltd, Poole, England
Formic acid	BDH Lab Supplies Ltd, Poole, England
Hydrochloric acid	BDH Lab Supplies Ltd, Poole, England
Interferon-γ (IFN-γ)	Sigma Chemical Co., MO, USA
Iodine	BDH Lab Supplies Ltd, Poole, England
Isopropanol	BDH Lab Supplies Ltd, Poole, England
Iodine	Sigma-Aldrich Co. LCC, New Zealand
Kynurenine	Schricks Laboratory
Lymphoprep™	STEMCELL technologies, Germany
Methanol	Merck, Darmstadt, Germany
Neopterin	Schricks Laboratory
Orthophosphoric acid	BDH Lab Supplies, Poole, England
Penicillin/Streptomycin liquid	Invitrogen, Life Technologies, New Zealand

Phosphate buffered saline (PBS)	Sigma-Aldrich Co. LCC, New Zealand
Potassium Iodide	Sigma-Aldrich Co. LCC, New Zealand
RPMI-1640 media, with phenol red	Sigma-Aldrich Co. LCC, New Zealand
Sodium Hydroxide	Merck, Darmstadt, Germany

2.2 General solutions, buffers, and media

All solutions were prepared with de-ionised water which has been filtered and purified using a Milli-Q ultrafiltration system (Milli-Q, Massachusetts, USA). Purified water referred to as nano-pure water. When required, solutions were pH corrected and measured using an EDT Instruments BA350 pH meter (EDT directION, Dover, United Kingdom).

2.2.1 Acidic iodide

Acidic iodide solution was used for the oxidation of 7,8-dihydroneopterin within a sample to neopterin for detection via fluorescence. The solution contains 5.4% I_2 and 10.8% KI in 1 M hydrochloric acid (HCL) thus forming trioide in equilibrium with aqueous iodide and iodine: $I_{3\ (aq)}^{-} \rightleftharpoons I_{(aq)}^{-} + I_{2\ (aq)}$. The acidic iodide solution was prepared by the addition of powdered iodine (2.7 g) and potassium iodide (5.4 g) into 35 mL of nano-pure water. 4.37 mL of concentrated HCL was then added, followed by the addition of nano-pure water to give a final volume of 50 mL.

2.2.2 Ammonium Acetate (0.3% formic acid)

10 mM solution of ammonium acetate and 0.3% formic acid was prepared to be used in conjunction with the amine column method for cell pteridine, kynurenine and tryptophan determination. Typically, 1L was made by dissolving 0.77 g of solid ammonium acetate into 1000 mL of nano-pure water and adding 3 mL of concentrated formic acid. The solution was filtered through a 45 µm membrane filter, into a clean 1 L bottle for storage and use.

2.2.3 Ascorbic acid

A 0.6 M solution of ascorbic acid was prepared fresh when required by dissolving powdered (0.106 g) ascorbic acid into 1 mL of nano-pure water. To ensure the ascorbic acid is completely dissolved, the mixture was vortexed thoroughly and subsequently sonicated for 10 minutes.

Due to the instability of ascorbic acid, the solution was stored in the dark on ice when not being used and as much as possible.

2.2.4 Cell media

A mixture of penicillin and streptomycin solution was added to RPMI-1640 with phenol red (Life Technologies) each time a new bottle of media was prepared. This was done at a concentration of; 1000 U penicillin G and 1000 µg of streptomycin /mL. Depending on the type of cells being cultured, the RPMI-1640 media was supplemented with either 10% human serum for PBMCs or 5% foetal bovine serum (FBS) for U937 cells.

2.2.5 Phosphate buffered saline

Sterile, endotoxin-free phosphate buffered saline (PBS) was often used in the preparation of cell solutions. This was supplied ready-made (Sigma-Aldrich Co.).

2.2.6 HPLC mobile phases, standards

The mobile phase used during HPLC analysis is dependent on the method and column employed for a sample separation. Unless otherwise stated, the standard was diluted into the mobile phase buffer of the corresponding method.

2.2.6.1 Kynurenine standard

A stock solution of Kynurenine (100 µM) was prepared by dissolving Kynurenine in ammonium acetate with no adjustment to pH. To ensure that all the kynurenine has dissolved the solution was vortexed thoroughly and subsequently sonicated for 5 minutes. The standard solution was then stored at -20^{o}C in microtubes as 500 µL aliquots. When required, standards were thawed and diluted into the mobile phase buffer at the appropriate concentration for analysis. Kynurenine standards were very stable and are therefore able to be frozen and refrozen multiple times.

2.2.6.2 Neopterin standard

A stock solution of D-neopterin (100 µM) was prepared by dissolving D-neopterin in $AmPO_4$ at pH 6.0. To ensure that all the D-neopterin has dissolved the solution was vortexed thoroughly and subsequently sonicated for 10 minutes. The standard solution was then stored at -20^{o}C in microtubes tubes as 500 µL aliquots. When required, standards were thawed and diluted into the mobile phase buffer at an appropriate concentration for analysis. D-neopterin standards as

such are very stable and therefore are able to be frozen and thawed multiple times as needed (Baxter-Parker, 2019) .

2.2.6.3 7,8-Dihydroneopterin standard

7,8-Dihydroneopterin has been shown to be unstable in solution or when frozen in solution at -80^{o}C with significant amounts of the compound breaking down after time periods as short as 6 hours (G. Baxter-Parker, Chu, Petocz, Samman, & Gieseg, 2019). Consequently, 7,8-dihydroneopterin was prepared immediately prior to when it was required experimentally. Approximately 1 mg of 7,8-dihydroneopterin would be weighed out using a highly sensitive microbalance (Mettler Toledo UMX2 micro) and transferred into a new 15 mL centrifuge tube wrapped in foil to minimise light exposure. 10 mL of solvent (appropriate buffer, such as RPMI) was added to the tube followed by vortexing the solution thoroughly and subsequently sonicating for 10 minutes to ensure all the 7,8-dihydroneopterin powder is dissolved. The concentration of 7,8-dihydroneopterin was calculated from the actual mass weighed out by microbalance, molecular-weight (255.2 gmol^{-1}) and solution volume

2.3 Methods

2.3.1 Cell culturing conditions

All cellular experiments were performed under aseptic conditions inside a class II biological safety cabinet (Clyde-apex BH 200). All instruments and plasticwares were either purchased pre-sterilised (Greiner, Greiner Bio-one, Neuburg, Germany), or otherwise were sterilized by autoclaving (15 min, 121°C, 15 psi). All media and solutions were sterilised by autoclaving or by filtration through a 0.22 µm membrane filter (Membrane solutions USA). All equipment and items intended for cell culture work were sprayed extensively with 70% ($^{v}/_{v}$) ethanol diluted in distilled water prior to transfer inside the class II biological safety cabinet.

Cell cultures were incubated at 37^{o}C in a humidified incubator, with the atmosphere calibrated to 5% carbon dioxide: 95% air (Sanyo Electric Co. Ltd, Japan). Viable cells were counted using a haemocytometer (Marienfield, Germany) with a light microscope after staining with a trypan blue solution at a ratio dependent on the cell type. Images of cells were captured prior to taking samples and/or changing media using a LEICA DMIL microscope with a LEICA DFC290 camera.

2.3.2 Blood collection

Approval for the use of human blood in this research was granted by the Canterbury Ethics Committee (98/07/069). Blood was obtained from consenting patients, deemed healthy be a registered nurse, of the haemochromatosis clinic of the New Zealand Blood Bank (15 Lester lane, Addington, Christchurch). The blood collected was stored in Compoflex® autologous bags containing the anticoagulant: citrate-phosphate-dextrose-adenine (CPDA-1) for the preparation of cells. When blood was intended for human serum preparation, alternatively it was collected into dry bags (Fresenius Kabi; Homburg, Germany). Blood obtained for cell preparation was either used the same night as collection or alternatively stored overnight refrigerated at 4 ^{o}C for use the next morning.

2.3.3 Isolation of peripheral blood mononuclear cells

Depending on whether obtained blood is used the same night or the next morning, centrifugation times vary; the next morning times will be denoted in brackets after the same night time. If blood was stored overnight at 4 ^{o}C, it was placed on the bench for 30 minutes to gradually bring the blood bags to room temperature. Blood from autologous bags was mixed gently by inversion 10 times before being transferred equally into 50 mL centrifuge tubes (x10) and centrifuged for 20 minutes (30 minutes) at 1000 g (Multifuge 1 S-R, Heraeus) with unbraked deceleration, allowing for the separation of red blood cells and plasma with the buffy coat layered in between. The buffy coat, approximately 5 mL per tube, was then transferred to fresh 50 mL centrifuge tubes containing room temperature phosphate buffered saline (PBS) making the volume up to 35 mL. Following this, the addition of 15 mL of the Ficoll-Isopaque density gradient, Lymphoprep™ facilitated sedimentation of red blood cells upon centrifugation. During this step, care was taken to underlay the Lymphoprep™ solution to the bottom of each tube. Alternatively, the Lymphoprep™ was pre-aliquoted to the bottom of the intended centrifuge tubes, and the PBS-buffy coat mixture was then gently overlayed. This was achieved by holding the tube at an angle (~45^{o}) using a syringe and canula to slowly layer the PBS buffy coat on top. Subsequently, the tubes were centrifuged for 20 minutes (30") at 1000g, with a soft start and no breaking as to not cause undesired mixing.

Following centrifugation, the PBMC layer was visible approximately half-way up the centrifuge tube, between the Red blood cell/Lymphoprep™ and PBS layers. The PBS solution was removed by suction from the top until just above the desired layer using a clean cannula. The PBMC layer was then removed by fresh syringe and transferred to fresh 50 mL centrifuge

tubes. The centrifuge tubes containing the PBMC layer were topped up to 50 mL with PBS and subsequently centrifuged for 15 minutes (20") at 500 g with a fast start and the break on to wash/pellet the cells. Following this the supernatant was removed leaving only the cell pellets, which were then resuspended in 10 mL of Milli-Q filtered water, which was filtered through a 0.22 µM syringe prior, for 10 to 15 seconds and drawn up and down using a fresh cannula and syringe in order to lyse any remaining red blood cells remaining within the cell pellet. The tubes were then immediately topped up to 50 mL with PBS and again spun for 15 minutes (20") at 500g with a fast start and the break on. This step was repeated two more times with just PBS to wash the cells thoroughly. After the final wash, the supernatant was removed, and the cells were combined and resuspended in 30 mL of a RPMI-1640 + penicillin/streptomycin solution with 10% human serum for PBMC preparation.

The cells were then counted on a haemocytometer by combining 20 µL of the RPMI-cell solution and 180 µL of Trypan-blue together in an microtube. Following the cell count, the RPMI-cell solution was diluted appropriately to a concentration of $5e^6$ in RPMI with 10% human serum for the desired volume. Lastly, the cells were plated in 12 well adherent plates (Cellstar^R, Greiner Bio-one; North Carolina, USA) and placed into the incubator. The cell-populated plates were then left overnight (or for a period of approximately 24 hours) to settle down and adhere to the wells before experimentation.

2.3.3.1 Human serum preparation

Blood intended for human serum preparation was collected in dry bags, not containing an anticoagulant. After being left standing upright overnight at 4^{o}C, dry bags were cut open the next morning, and had the serum and un-clotted blood transferred evenly amongst 50mL centrifuge tubes. These tubes were then spun for 20 minutes at 2000 g to pellet any remaining clotted material and red blood cells. The resulting supernatant serum was subsequently transferred to new 15 mL centrifuge tubes in aliquots of 10 mL and stored at -80^{o}C until required for cell culturing media.

2.3.4 Solution preparation for cell culture work

All solutions for cell work were prepared under aseptic conditions inside a class II biological safety cabinet (Clyde-apex BH 200). When possible, compounds of interest were made directly into RPMI-1640. To ensure sterility, cell treatment solutions were filtered through 0.22 µM filters using a syringe.

2.3.4.1 Interferon gamma

Interferon Gamma (Sigma-Aldrich) was dissolved in PBS in order to produce a 10,000 U/mL stock solution. 50 µL of which produced a 500 U/mL concentration

2.3.4.2 Atorvastatin

Atorvastatin calcium salt (Sigma-Aldrich) was dissolved in 100% DMSO in order to produce a 2000mM/mL stock solution. 5 µL of which produced a 10 µM concentration

2.3.5 Cell culturing

2.3.5.1 Imaging cells

Each well was examined by microscopy every 24 hours to ensure no contamination had occurred. A candidate well was selected from each row (experimental condition) of a 12-well plate. Prior to each sample collection and changing over of media every 24 hours, the candidate well was photographed using a LEICA DMIL microscope with a LEICA DFC290 camera.

2.3.5.2 Sample collection and preparation

Each plate was gently titled (~45^{o}) and 1mL of cell media was removed from the corner of each well and transferred to its own microtube. A fresh pipette tip was used for each row/condition. Following this, the media in each well was replenished and the wells treated according to experimental conditions. The microtubes containing media were then spun down in a centrifuge at 20,000 g for 20 minutes. Following this step, any cells should appear as a small white coloured pellet at the bottom of the tube. 500 µL of the resulting supernatant was then collected, with care not to disturb the pellet from each tube, before combining with 500 µL of ice-cold ACN (appropriate buffer, stored in -20^{o}C degree freezer) in a new microtube. The 50:50 mixtures of supernatant and ACN were then vortexed for 10 seconds to ensure complete protein precipitation. Subsequently, the samples were spun down at 20,000 g at 4^{o}C degrees for 20 minutes to pellet the precipitated protein. Following this step, any excess protein in the sample should appear as a small brown coloured pellet at the bottom of the microtube. The supernatant (900 µL) was then collected taking care not to disturb the pellet and transferred to new microtubes. Samples are then labelled appropriately and stored frozen at -80^{o}C degrees until required for analysis by amine-HPLC. This process was repeated every 24 hours for an experimental period of 96 hours (4 times).

2.3.5.3 Experimental treatment

Each row of three wells on an adherent plate served as a triplicate for each experimental condition. Experimental treatment occurred when replacing a wells media. The media (firstly) and treatment solution (secondly) volume added up to 1000 µL total in each condition. After each well received its appropriate media and treatment, the lid was placed back on the plate and plate was gently gyrated for 5 seconds in each direction to ensure that the treatment/media was evenly distributed throughout the well.

2.3.5.4 Sample preparation for total neopterin determination

In contrast to neopterin, due to its low absorbance properties 7,8-dihydroneopterin is not easily detectable through HPLC. This makes the detection of biologically relevant concentrations of 7,8-dihydroneopterin not possible with the techniques and equipment available in our laboratory. A common method for the detection of 7,8-dihydroneopterin is to oxidise it to neopterin. This can be achieved using a solution of acidic iodide and the reporting the value as total neopterin (neopterin + oxidised 7,8-dihydroneopterin).

For acidic iodide oxidation, samples were diluted in the appropriate buffer (**Section 2.3.5.2**). Following this, 40 µL of acidic iodide solution was added (**Section 2.2.1**). Subsequently, the samples were vortexed and then left in the dark at room temperature for 15 minutes, allowing the acidic iodide to oxidise any 7,8-dihydroneopterin to neopterin. In order to quench the reaction by reducing the remaining acidic iodide, 20 µL of ascorbic acid (**Section 2.2.3**) is added to the samples before vortexing again. Samples were then spun down at 20,000 g for 10 minutes at 4^{o}C to pellet any potential remaining cellular debris. 100 µL of the samples were then analysed (**Section 2.5.3.3**) for total neopterin concentration from which 7,8-dihydroneopterin could be inferred.

2.3.6 HPLC analysis

2.3.6.1 Detection of 7,8-dihydroneopterin

7,8-dihydroneopterin is an inherently unstable compound, being both heat and UV light labile, thus it requires special care to be taken for sample preparation (Fuchs et al., 1989). All specimens must be kept cool and have exposure to light limited. If samples are not immediately analysed, they must be stored in a -80^{o}C freezer. The detection and quantification of 7,8-

dihydroneopterin can be made using absorbance spectroscopy at 254 nm following separation by chromatography (A. Lindsay et al., 2016). Most biological samples contain concentrations of 7,8-dihydroneopterin which absorbance spectroscopy is not a sensitive enough method of detection to be viable. For example, cell cultures where the concentration 7,8-dihydroneopterin are far below the limit of detection, or urinary analysis where chromatograms experience interference from other compounds possessing similar retention times. In order to circumvent these issues, 7,8-dihydroneopterin is oxidised to neopterin, which is highly fluorescent character, and so is easily detectable at low concentrations.

There are two predominant methods utilised; one implements an acidic iodide incubation followed by ascorbic acid to quench the reaction (Flavall, Crone, Moore, & Gieseg, 2008; Fukushima & Nixon, 1980; A. Lindsay et al., 2014), and the other uses manganese dioxide (Fuchs et al., 1989; Krämer et al., 1989). However, this introduces issues with reproducibility and reliability between methods (Tomšíková, Tomšík, Solich, & Nováková, 2013). The free radical biochemistry (FRB) laboratory utilises the acidic iodide method (**Section 2.3.5.4**). Therefore, the measurement of 7,8-dihydroneopterin requires two injections of the sample: one untreated to measure neopterin concentration and a second treated with acidic iodide and quenched with ascorbic acid to measure "total neopterin". 7,8-dihydroneopterin concentration may be calculated by subtracting neopterin concentration from "total neopterin" concentration or expressed as a ratio (Fuchs et al., 1989; A. Lindsay et al., 2014).

2.3.6.1 Kynurenine and tryptophan detection

Kynurenine and tryptophan may be separated and measured simultaneously using HPLC coupled with fluorescence and absorption detection. Kynurenine exhibits a strong absorbance characteristic at 360 nm, making it easily detectable. Tryptophan is able to be measured using fluorescence detection with excitation and emission wavelengths of 285 and 365 nm respectively (Hensler et al., 1997; Laich, Neurauter, Widner, & Fuchs, 2002). Many previous studies have implemented a C-18 reverse phase method for detection of kynurenine and tryptophan in biological samples, such as serum (Hensler et al., 1997), or urine (Shibata & Onodera, 1991). A well characterised technique developed and employed by this research group utilising amine-HPLC, enables the simultaneous detection of neopterin, kynurenine and tryptophan within a single sample injection.

2.3.6.3 Cell media analysis: Amine HPLC

Preparation of cell samples required protein precipitation. This was achieved by taking 500 µL of cell sample and vortexing it with 500 µL of ice cold ACN in a microtube (**Section 2.3.5.1**). If frozen at -80^{o}C degrees, the samples were thawed by leaving them in the refrigerator at 4^{o}C degrees for 20 minutes. Once thawed, the samples were then spun at 20,000 g for 10 minutes at 4^{o}C to pellet any potential remaining cellular debris. 100 µL of the sample was then pipetted into an HPLC insert and vial for analysis of neopterin. To assess total neopterin, acidic iodide treatment (**Section 2.3.5.2**) was conducted to oxidise present 7,8-dihydroneopterin.

Unless otherwise stated, samples were always centrifuged prior to injection. In this research, all cell samples were run through a Phenomenex Luna™ 5 µM NH_2 100 Å LC column 250 x 4.6mm. Analysis was conducted using a Shimadzu 20A HPLC with a Sil 20A autosampler, RF-20Axls fluorescence detector and M20A SPD absorbance detector. The autosampler was set to 4^{o}C to reduce any heat liability of analytes. This was run isocrastically with on line mixing. Line A consisted of 10 mM Ammonium acetate 0.3 % formic acid (**Section 2.2.2**.); line B was 100% ACN. These were mixed as 28 %-line A and 72 %-line B to achieve desirable separation of the compounds of interest. Simultaneous detection of neopterin, kynurenine and tryptophan were possible using this method. Neopterin eluted at approximately 9 minutes and was detected using fluorescence with excitation and emission wave lengths of 353 and 438 nm, respectively. 7,8-dihydroneopterin is weakly absorbent at 254 nm and was detected by PDA when possible. Kynurenine was measured using absorbance detection at 360 nm and eluted at approximately 6.5 minutes. Unless otherwise stated, 10 µL of sample was loaded onto the column by the autosampler.

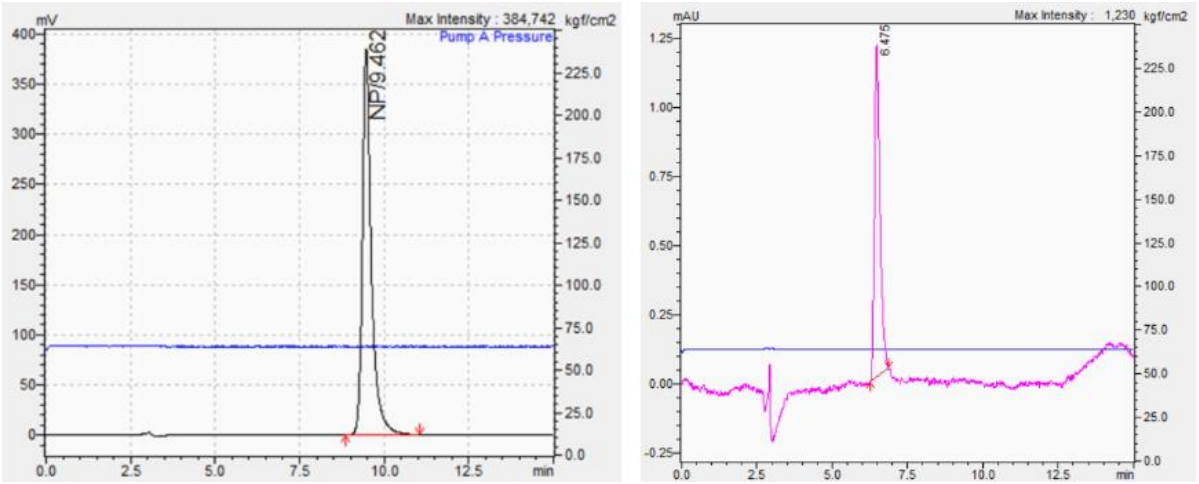


Figure 2.3.6.3 Chromatograms of neopterin and kynurenine standards. [5 µM] neopterin pictured left and kynurenine right dissolved in 10 mM AmAc (without acid), analysed using the amine method. neopterin detected at excitation/emission 353 and 438 nm, kynurenine absorbance detected at 360 nm

2.3.7 Cell assay methods

2.3.7.1 Preparation of cellular lysate

By washing and lifting all of the protein adhered to each well, it is possible to determine the protein concentration present, which may in turn serve as an estimate of cellular concentration. After the final sample (96 hour) has been taken from each well, this is achieved by "scraping" the plate. Plates should be washed with 1mL of PBS to ensure the removal of extracellular protein. To each well was added mL of a 0.1 M sodium hydroxide solution (NaOH). Each well is then scraped using a 1 mL pipette tip. To ensure the protein is fully lifted, the tip is scraped back and forth across the well 10 times. Following this the plate is rotated 45 degrees and scraped again, then rotated and repeated a further two times for a total of four scraping angles which encompass the majority of the well. following this, the cellular lysate containing NaOH is sucked up and down using a pipette 3 times, The cellular lysate of each well is then collected and stored in a microtubule prior to conducting the BCA assay and stored in the –80^{o}C freezer. To ensure that all protein has been lifted/collected, the well is briefly examined under a microscope. For each row/condition, a fresh pipette tip was used.

2.3.7.2 Determination of protein concentration – BCA assay

Protein concentration of cell lysate was quantified using bicinchoninic acid (BCA) protein determination kit assay (Pierce TM , Rockford, USA) in which the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium can be measured via a BCA reagent. The product of this assay reaction exhibits a strong absorbance at 562 nm with increasing protein concentration in the range of 25-250 µg/mL and was measured by spectrophotometry

Fresh working reagent solution was prepared prior to each use by combining reagent A ($NaCO_3$, $NaHCO_3$, BCA and sodium tartrate in 0.1 M sodium hydroxide) and reagent B (4% $CuSO_4.5H_2O$) at a ratio of 50:1 (20 µL of reagent A to 1000 µL of reagent B). The assay was run by mixing 50 µL of cell lysate diluted with nano-pure water as required (typically 10:1) with 1 mL of working reagent immediately prior to a 30-minute incubation on a shaking heating block at 60^{o}C to initiate the reaction. When complete, the reaction was halted by placing the

samples in a shallow water bath until cooled. Following this, the absorbance was determined using a spectrophotometer (Spectramax, Molecular Devices, USA) at 562 nm, using nano-pure water with the working reagent as the reference absorption (blank). Protein concentration was determined utilising a standard curve by measuring the absorbance of known concentrations of bovine serum albumin (BSA) (0-250 µg/mL) under the same conditions.

2.3.8 Statistical Analysis

High performance liquid chromatography data analysis was performed using Shimadzu LabSolutions software (version 5.96). With regard to analysis of results, the analysis method is written in the method section for the technique. All statistical analysis and graphing were conducted using GraphPad Prism version 9 (GraphPad Software Inc, USA). Results shown are obtained from single experiments, representing a minimum of three repetitions of the same experimental condition.

Chapter 3

Results

3.1 Characterisation of HPLC methods

HPLC analysis of neopterin and kynurenine generation was implemented as a measure of monocyte activation in this research. In order to determine that the devised amine HPLC method was capable of producing accurate measurements of such inflammatory markers, research began with confirming the linearity of responses by the HPLC instrument to known concentrations of neopterin and kynurenine standards.

3.1.1 Neopterin standard curve preparation

In this quantitative assay, neopterin standards were fitted to a regression model in order to determine the unknown quantitative concentrations of neopterin within experimental samples using the amine HPLC method. The neopterin linear regression (standard) curve, consisting of serially diluted concentrations between 1 and 50 µM of a 100 µM stock solution with ammonium acetate (without formic acid). 10 µL of these standards were injected into the HPLC instrument (**Section 2.3.5.3**) and data output was expressed computationally as chromatograms, from which the peak area is proportional to the concentration of neopterin present in the experimental solutions. A standard curve was generated by plotting peak area on the Y-axis and prepared neopterin standard concentration on the X-axis (**Figure 3.1.1**).

Simple linear regression analysis of the data gave an equation of Y = 589688*X + 362935, with an R-squared value of 0.9996. The R-squared value indicates a very close fit to the linear regression between neopterin concentration and area under the peak. The R-value produced was 0.9998, further indicating a strong linear association between neopterin concentration and area under the peak. These results demonstrated that from a known concentration of a neopterin, unknown concentrations of neopterin may be confidently derived.

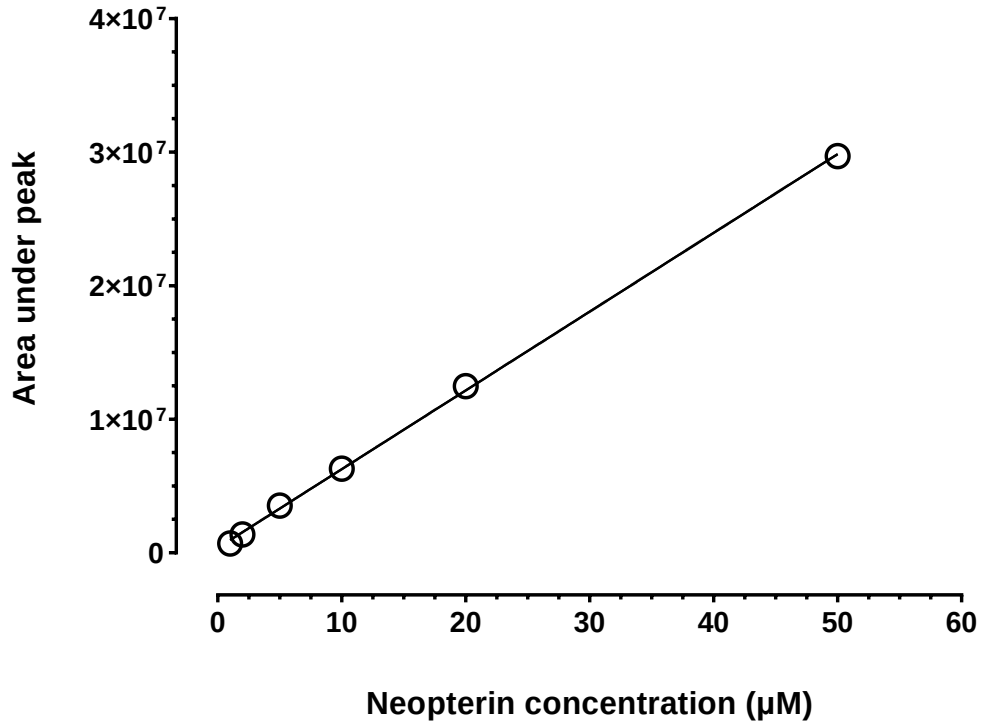


Figure 3.1.1. Neopterin standard curve. Linear regression analysis of 1 – 50 µM standards

3.1.2 Kynurenine standard curve preparation

In this quantitative assay, kynurenine standards were fitted to a regression model in order to determine the unknown quantitative concentrations of kynurenine in experimental samples using the amine HPLC method. The kynurenine linear regression (standard) curve consisted of serially diluted concentrations between 5 and 50 µM of a 100 µM stock solution with ammonium acetate (without formic acid). 10 µL of these standards were injected into the HPLC instrument (**Section 2.3.5.3**). The data output was expressed computationally as a chromatogram, from which the peak area is proportional to the concentration of kynurenine present in the experimental solutions. A standard curve was generated by plotting the peak area on the Y-axis and prepared kynurenine standard concentration on the X-axis (**Figure 3.1.2**).

Simple linear regression analysis of the data gave an equation of Y = 13466*X + 6523, with an R-squared value of 0.9998. The R-squared value indicates a very close fit to the linear regression between kynurenine concentration and peak-area. The R-value produced was 0.9999, further indicating a very strong linear relationship between the kynurenine concentration and area under the peak. As with neopterin, These results demonstrated known concentration of kynurenine standard, unknown concentrations may be confidently derived.

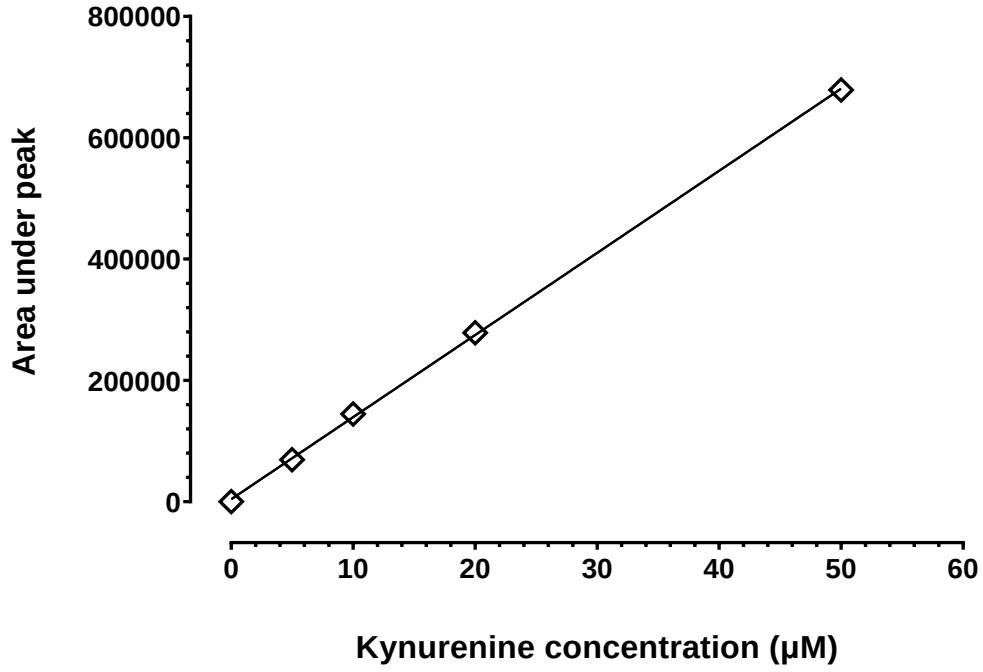


Figure 3.1.2 Kynurenine standard curve. Linear regression analysis of 5 – 50 µM standards

3.1.3 Acidic iodide oxidising potential testing

7,8-dihydroneopterin's low fluorescent character and nM concentration in plasma and cell media make it relatively difficult to detect within a sample. Therefore, the 7,8-dihydroneopterin within a sample is oxidised to neopterin, which in contrast possesses highly fluorescent character and is easily detectable at low concentrations. This is typically achieved by using an acidic iodide solution (5.4% I_2 and 10.8% KI in 1 M HCL), which oxidises 7,8-dihydroneopterin ≥98% through to neopterin (Flavall et al., 2008; A. Lindsay et al., 2014; Ziegler, 1985). Although routinely utilised by this research group, the oxidising potential of the acidic iodide solution has not been thoroughly assessed for some time. Therefore, before any PBMC sample analysis could take place, appropriate experiments were undertaken in order to determine the ability of the acidic iodide solution (**Section 2.2.1**) to oxidise 7,8-dihydroneopterin through to its oxidant product neopterin.

Using a highly sensitive, 5 decimal place microbalance, between 1 and 1.5 mg of pure 7,8-dihydroneopterin (Schick's Laboratory, Switzerland) was weighed out into a foil cup. The contents of the foil cup once weighed was transferred to a 15 mL, aluminium foil covered tube. Following this the 7,8-dihydroneopterin was dissolved by the addition of 10 mL of RPMI solution and sonicated until dissolved. Appropriate calculations and dilutions were made to

give a range of 7,8-dihydroneopterin concentrations: 5, 10, 25, 50 and 100 µM, to oxidise to neopterin. The acidic iodide solutions ability was tested over both 15- and 30-minute incubation periods by the addition of 40 µL of acidic iodide solution to 100 µL of these 7,8-dihydroneopterin solutions which were promptly vortexed to mix. The test concentrations were then stored in the dark for their respective incubation periods. Following the incubation, the samples were immediately quenched by the addition of 20 µL of 0.6 M ascorbic acid solution (**Section 2.2.3**) and promptly vortexed to mix thoroughly. The samples were then analysed by amine HPLC (**Section 2.3.5)**.

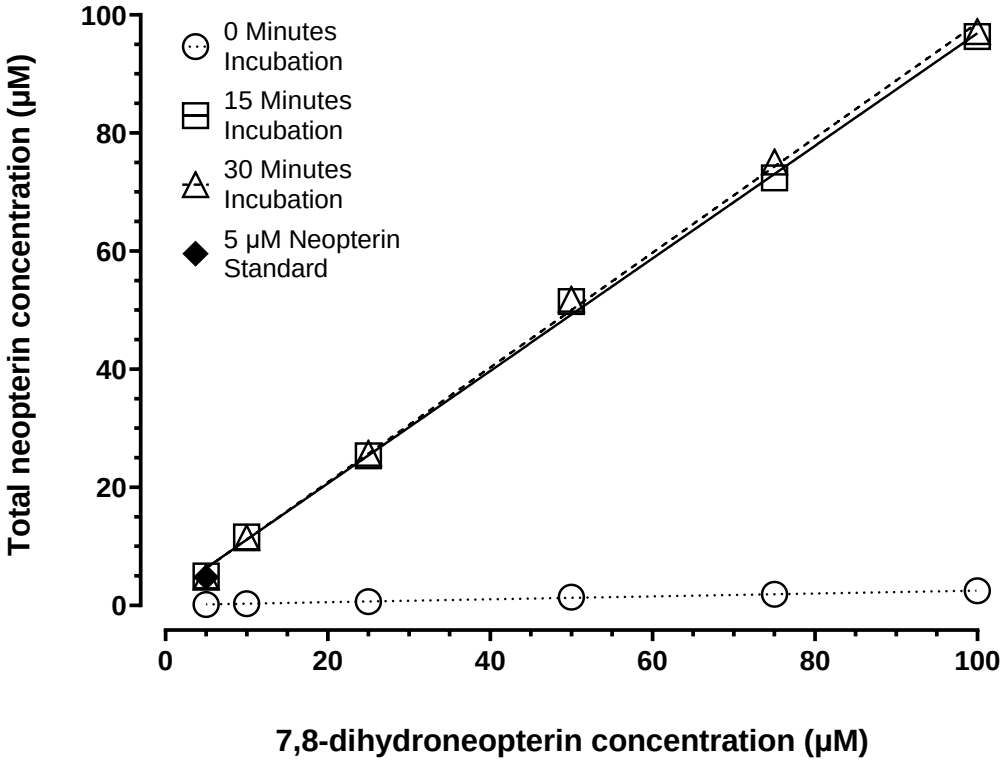


Figure 3.1.3 Acidic iodide incubation of 7,8-dihydroneopterin. 7,8-dihydroneopterin concentrations incubated with acidic iodide solution, then quenched by ascorbic acid solution. Concentrations analysed by Amine HPLC. Oxidation measured as total neopterin. Data analysed by simple linear regression.

Linear regression analysis of the produced data shows the slope of the 0 minutes incubation to be 0.02454, showing a poor relationship between the rate of change of 7,8-dihydroneopterin concentration (X) to total neopterin concentration (Y). In contrast the slopes for 15 minutes and 30 minutes incubation both showed strong linear relationships between X and Y, having

equations of Y = 0.9529*X + 1.597 and Y = 0.9717*X + 1.431, respectively. The slopes produced by the linear regression indicate an oxidation rate of 95.3% and 97.2% respectively for the 5 to 100 µM range of values. The equations produced by linear regression analysis of the 15- and 30-minute incubation periods show that both incubation periods are capable of oxidising 98% of a sample lesser than or equal to 50 µM in concentration. Therefore, the results of the incubation clearly demonstrate the reliability of acidic iodide solution to sufficiently oxidise samples for the purpose of TNP analysis.

3.2 Peripheral blood mononuclear cell experimental results

The peripheral blood mononuclear cell (PBMC) experimental results are presented a series of clinical investigations. Each PBMC isolation was prepared from individual donors on different dates over time. Experiments carried out on each primary culture of human PBMCs, isolated from respective blood donations, are presented as groups of experimental results. The ethics approval granted (**Section 2.3.2**) allowed for the blood collection from hemochromatosis patients and subsequent experimentation, however, it did not allow access to donor information. Therefore, it is not possible to speculate on a donor's overall health, medical history, or characteristics. As it is not possible to ascertain any information on respective donations, the assumption is made throughout that each PBMC preparation comes from a different respective donor. However, the blood selected for research, donated by hemochromatosis patients, was assessed, and decided by registered nurses, who deemed the health of each respective donor to be of an acceptable standard to be given to the laboratory group for research purposes.

3.2.1 Methods Overview

Primary cultures of human PBMCs were isolated from fresh whole blood donated under ethics approval granted by the Canterbury Ethics Committee (98/07/069) and cultured as described in the methods section (**Chapter 2**).

PBMCs were diluted to a concentration of 5 x 10^6 in RPMI (+ pen/strep) with 10% human serum and plated in 12-well adherent plates, The cell-populated plates were then left for a period of approximately 24 hours to settle down and adhere to the well-bottoms. Each row of three wells served as triplicate for an experimental condition. Each well had its media removed and replaced every 24 hours for a period of 96 hours, with the appropriate treatment and RPMI

+ serum mixture totalling 1000 µL replacing it. Prior to media removal, candidate wells of each experimental condition were visualised and photographed under microscope to demonstrate viability and confirm no contamination had taken place.

Following the 96-hour period and final media removal, cellular lysate was removed and collected by scaping each well of the plate after the addition of 1 mL of 1 M NaOH solution. Samples were analysed by amine HPLC described in chapter 2. Protein concentration of the cellular lysate was determined by BCA assay and spectrophotometry.

All Statistical analysis was conducted using GraphPad Prism version 9 (GraphPad Software Inc, USA). A multiple comparisons two-way analysis of variance (ANOVA) was performed on all HPLC experimental data for total neopterin, neopterin and kynurenine, comparing each PBMC experimental condition with the control condition. Protein determination data was assessed by An ordinary one-way multiple comparison's ANOVA, comparing as above. Significance is indicated as *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

3.2.3 Experimental concentrations

Previous work has shown that in IFN-γ stimulated PBMC, 100 µM atorvastatin, dissolved in dimethyl sulfoxide (DMSO), inhibited neopterin formation and tryptophan degradation to kynurenine completely, and 10 µM atorvastatin produced partial inhibition (Neurauter et al., 2003). Based on the previous findings establishing the effect on neopterin production, the decision was made to begin experimental work with the base experimental concentration of 10 µM, working upwards to 100 µM. The atorvastatin calcium salt (Sigma-Aldrich) is sparingly soluble in aqueous buffers and required an organic solvent. DMSO was utilised as the solvent as implemented in previous studies was readily available.

With regards to IFN-γ concentration, previous work by this laboratory has established 500 U/mL produces a statistically significant rise in total neopterin and neopterin production in PBMCs (Prebble, 2018). IFN-γ has also been demonstrated by this laboratory to produce statistically significant rises in kynurenine production, with 100 U/mL in PBMCs (Baxter-Parker, 2019) Therefore, the concentration of 500 U/mL (50 µL of 10,000 U/mL stock solution) was determined suitable for IFN-γ inflammatory stimulation and utilised throughout the experimental conditions.

3.2 Initial Statin Experiments

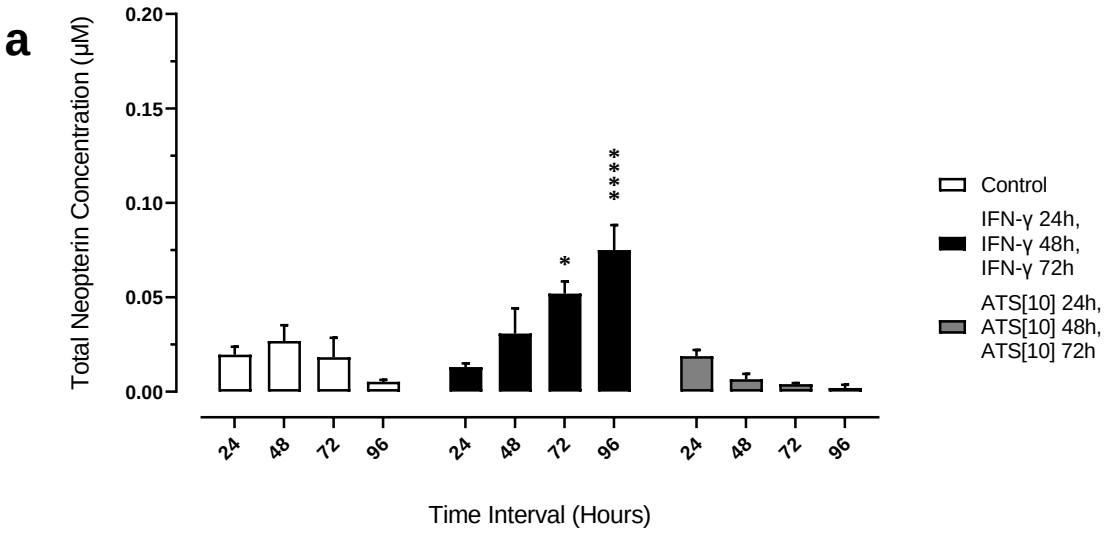
3.2.1 Characterising effect of IFN-γ and atorvastatin treatment

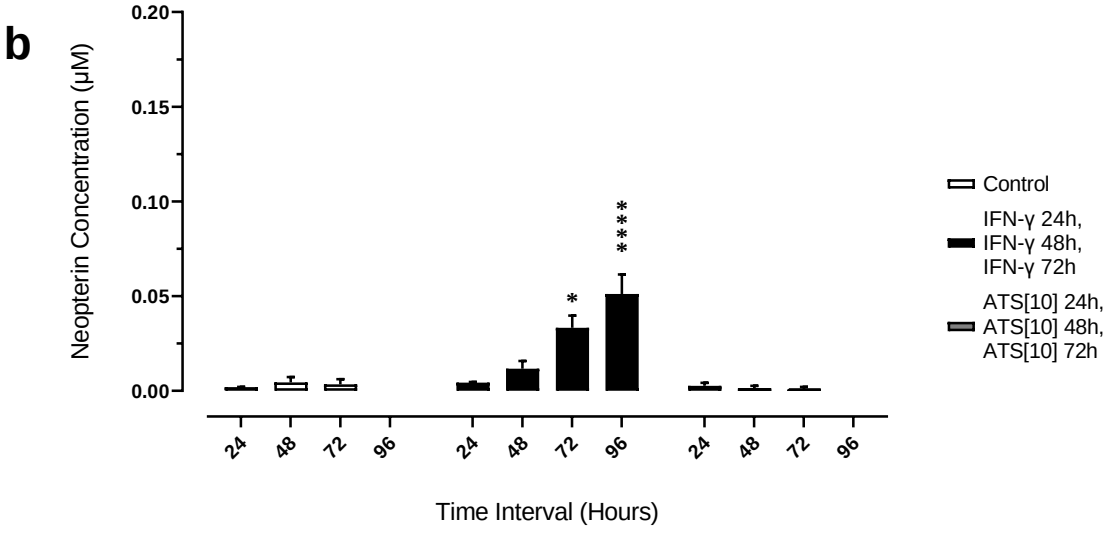
To firstly characterise the effect of IFN-γ and atorvastatin treatment, PBMCs were treated consistently with IFN-γ for 3 days, and 10 μM of atorvastatin (ATS) for 3 days following an initial media-only day (**Figure 3.2.1**).

The IFN-γ stimulation produced consistent increasing level of total neopterin over the 96-hour period with a significant rise after 72 hours (0.052 μM, $p = 0.0133$) and 96 hours increasing further (0.075 μM, $p<0.0001$). Neopterin increased significantly after 72 hours (0.033 μM, $p = 0.0146$) and 96 hours (0.051 μM, $p<0.0001$). Kynurenine increased significantly after 48 hours (13.78 μM, $p<0.0001$), although showing a decreasing trend with sustained significance after 72 hours (12.26 μM, $p<0.0001$) and 96 hours (10.70 μM, $p<0.0001$).

In contrast, the consistent 10 μM atorvastatin treatment alone produced no increase with regard to total neopterin, neopterin or kynurenine concentration. In all three instances the concentration produced by the PBMCs consistently decreased over time from the initial media-only day.

These initial results show that treatment with IFN-γ is capable of stimulating PBMCs, by the significant rise in the production of each inflammatory marker. In contrast, the treatment with atorvastatin alone produced a notable decrease in the production of all measured inflammatory markers, in this instance demonstrating that atorvastatin does not stimulate an inflammatory response from PBMCs.


a
Total Neopterin Concentration (µM)
0.20
0.15
0.10
0.05
0.00
*
****
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 24h,
IFN-γ 48h,
IFN-γ 72h
ATS[10] 24h,
ATS[10] 48h,
ATS[10] 72h


b
Neopterin Concentration (µM)
0.20
0.15
0.10
0.05
0.00
*
****
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 24h,
IFN-γ 48h,
IFN-γ 72h
ATS[10] 24h,
ATS[10] 48h,
ATS[10] 72h

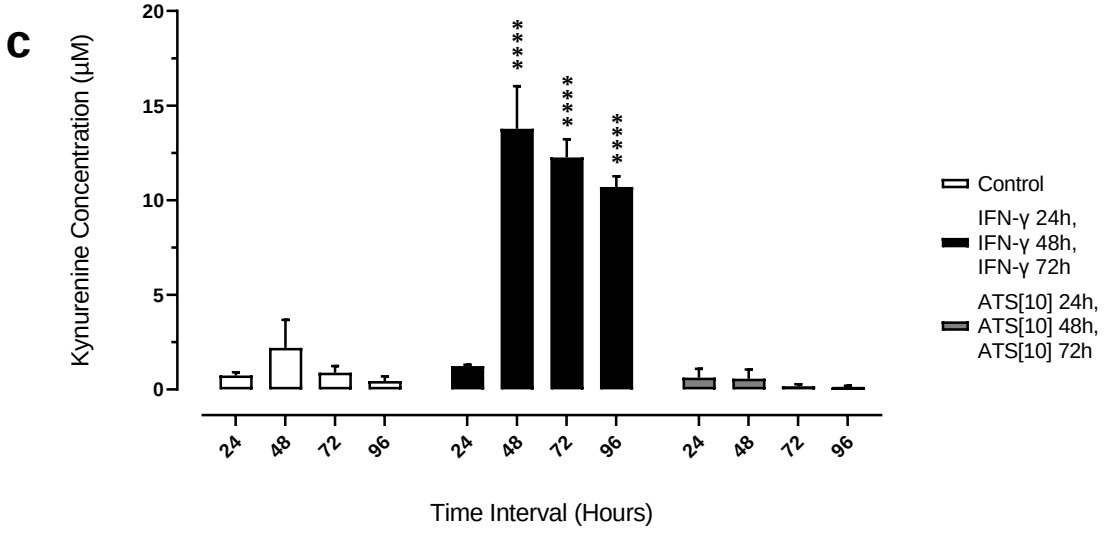
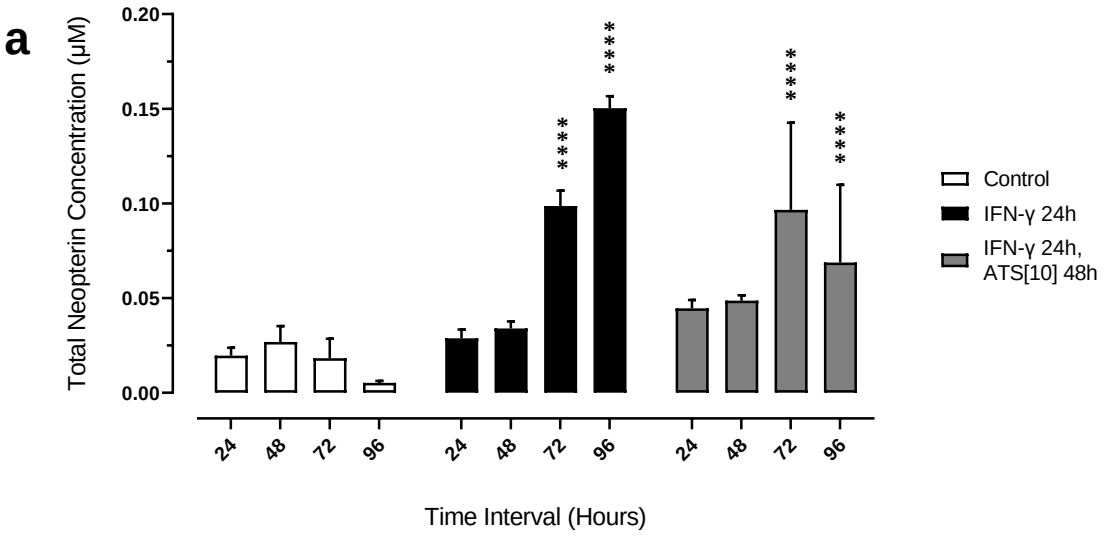

c
Kynurenine Concentration (µM)
20
15
10
5
0
****
****
****
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 24h,
IFN-γ 48h,
IFN-γ 72h
ATS[10] 24h,
ATS[10] 48h,
ATS[10] 72h

Figure 3.2.1 TNP, NP and KYN production with Consistent IFN-γ and ATS treatment. following an initial media-only day, PBMCs were treated consistently with IFN-γ (500 U/mL) for 3 days, and 10μM ATS for 3 days. (**a**) Shows the total neopterin, (**b**) shows the neopterin, and (**c**) shows the kynurenine concentration for the same time period as measured by amine HPLC (μM/L). A multiple comparisons two-way ANOVA was conducted on each. Significance is indicated as compared to the control condition at the same time point (24-hour period): *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

3.2.2 Characterising IFN-γ treatment followed by atorvastatin treatment

In order to characterise the possible inhibitory effect of atorvastatin on IFN-γ activation, PBMCs were treated with after 24 hours to establish a positive control, and with IFN-γ after 24 hours followed by 10 μM atorvastatin after 48 hours (**Figure 3.2.2**). The IFN-γ only control established an increase in total neopterin with a significant increase after 76 hours (0.099 μM, $p<0.0001$), increasing from there at 96 hours (0.15 μM, $p<0.0001$). Neopterin increased after 24 hours but not to a statistically significant extent and remained at a consistent level. Kynurenine increased significantly after 48 hours (13.66 μM, $p<0.0001$) decreasing but remaining significant after 72 hours (5.82 μM, $p = 0.0009$) and 96 hours (4.67 μM, $p = 0.0068$).

The IFN-γ followed by 10 μM atorvastatin treatment showed a similar total neopterin results with a significant increase after 76 hours (0.097 μM, $p<0.0001$), remaining significant but decreasing at 96 hours (0.069 μM, $p<0.0001$) unlike the IFN-γ only control. Neopterin results showed a significant increase at 72 hours (0.037 uM, $p = 0.0046$) decreasing to at 96 hours. Kynurenine increased significantly after 48 hours (6.04 μM, $p=0.0191$), increasing further and in significant at 72 hours (7.78 μM, $p<0.0001$) and decreasing after 96 hours (4.15 μM, $p = 0.0262$) however remaining significantly increased.


a
Total Neopterin Concentration (µM)
0.20
0.15
0.10
0.05
0.00
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24
48
72
96
24
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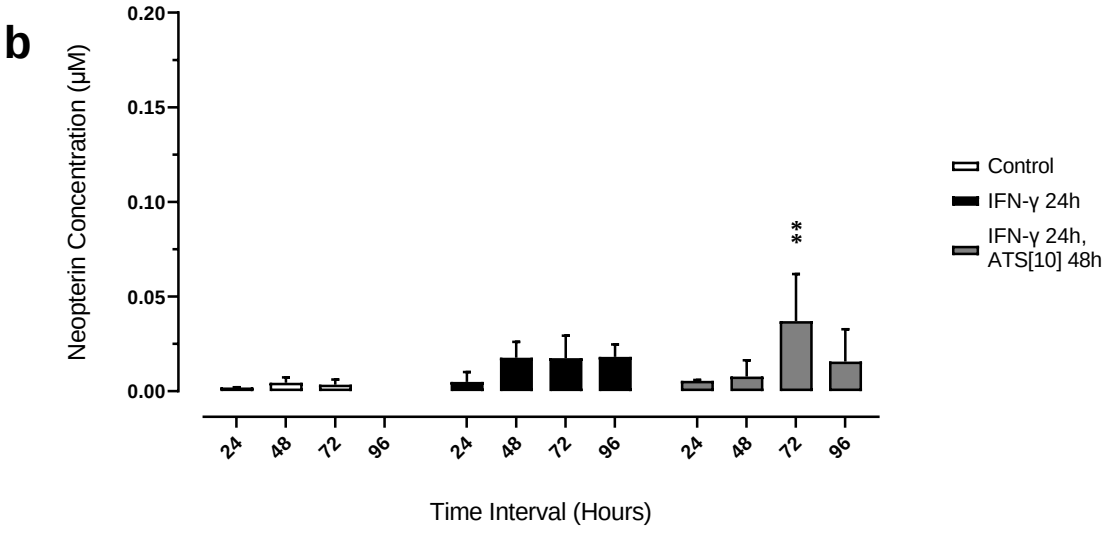
72
96
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 24h
IFN-γ 24h, ATS[10] 48h


b
Neopterin Concentration (µM)
0.20
0.15
0.10
0.05
0.00
**
24
48
72
96
24
48
72
96
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 24h
IFN-γ 24h, ATS[10] 48h

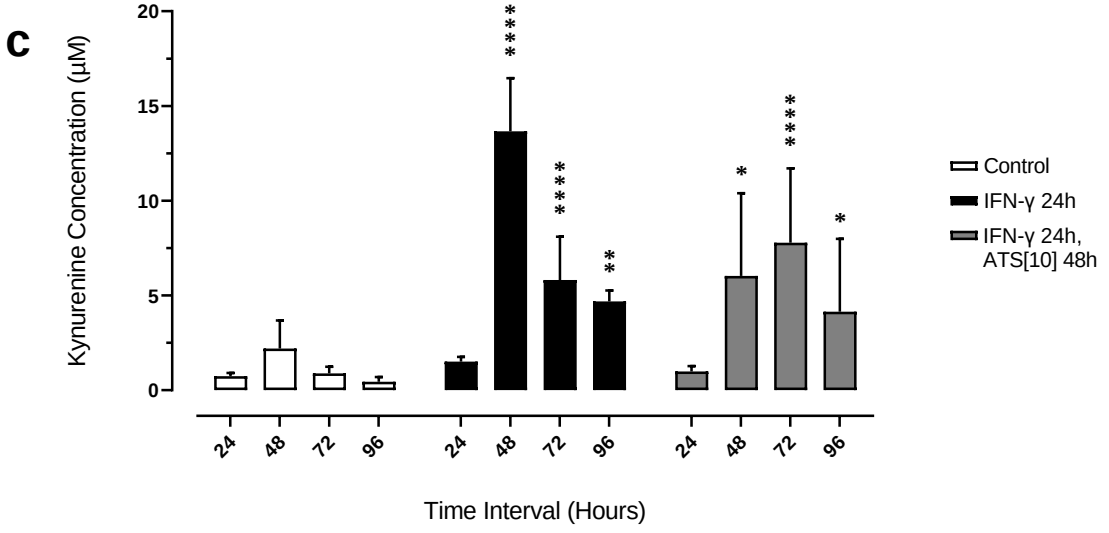

c
Kynurenine Concentration (µM)
20
15
10
5
0
****
****
**
*
****
*
24
48
72
96
24
48
72
96
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 24h
IFN-γ 24h, ATS[10] 48h

Figure 3.2.2 TNP, NP and KYN production with IFN-γ, followed by ATS treatment. Following an initial media-only day, PBMCs were treated with IFN-γ 500 U/mL after 24 hours alone, and with IFN-γ 500 U/mL after 24 hours followed by 10μM of ATS after 48 hours. (**a**) Shows the total neopterin, (**b**) shows the neopterin, and (**c**) shows the kynurenine concentration for the same time period as measured by amine HPLC (μM/L). A multiple comparisons two-way ANOVA was conducted. Significance is indicated as compared to the control condition at the same time point (24-hour period): *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

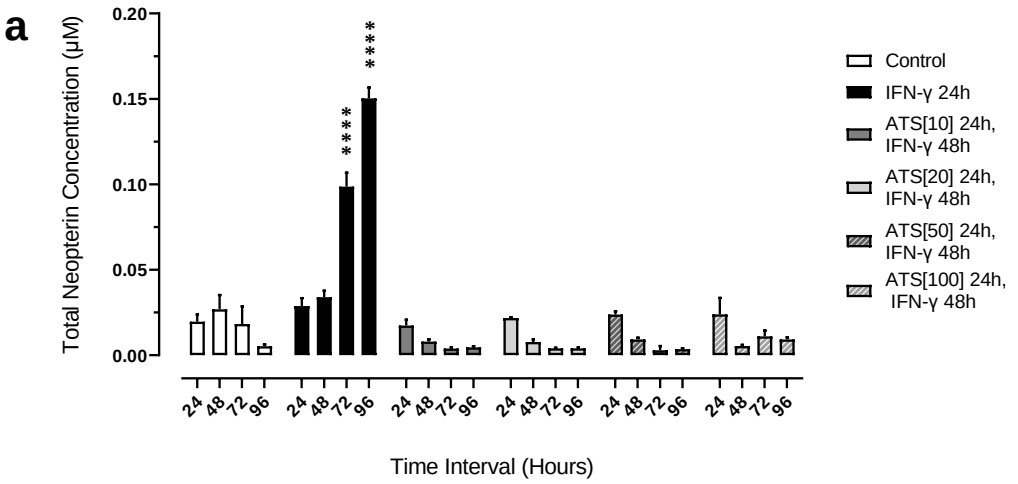
3.2.3 Characterising atorvastatin pre-treatment followed by IFN-γ treatment

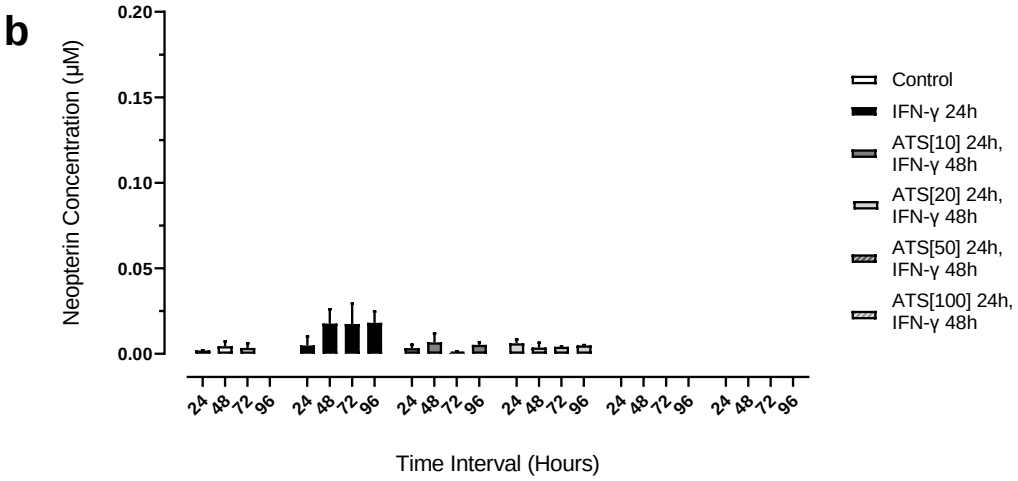
In this section, the possible effect of atorvastatin pre-treatment on IFN-γ activation of PBMCs was investigated. This was achieved by treating PBMCs with a range of statin concentrations, between the 10 and 100 μM concentrations used in previous work. PBMCs were treated after 24 hours with 10, 20, 50 and 100 μM of atorvastatin, followed by IFN-γ after 48 hours. These results were displayed alongside the 24-hour IFN-γ positive control condition previously discussed above in the beginning of **Section 3.2.2**.

The atorvastatin pre-treatment after 24 hours, at all concentrations showed a decrease in total neopterin at 48 hours unlike the control and positive control treatment which increased, but not in a significant manner. After the addition of IFN-γ at 48 hours again for the 10, 20 and 50 μM concentrations at 72 hours displayed a decrease in the production of total neopterin. In contrast to this the 100 μM atorvastatin treatment showed a slight rise compared to the others, but not in a significant manner. The pre-treated groups all showed not much difference after 96 hours all remaining at similarly low levels. This is remarkably different to the IFN-γ positive control, which after IFN-γ treatment alone at 24 hours produced significant rises in total neopterin after 72 and 96 hours.

With regard to neopterin production, there was no significant production for each of the pre-treated atorvastatin concentrations. Neopterin production for 10 μM atorvastatin was consistently lower than the positive control, which itself did not produce significant neopterin levels. The production decreased at 72 hours and then climbed back to its previous production at 96 hours. The production for 20 μM atorvastatin again was consistently lower than the positive control, however, it did not decrease and increase between 76 and 96 hours. Interestingly, the 50 μM and 100 μM atorvastatin treatments did not produce detectable levels of neopterin.

Kynurenine production also did not show significant production for each of the pre-treated atorvastatin concentrations. The production for the 10 µM pre-treatment remained low, not increasing after 48 hours as the control and IFN-γ positive control conditions did. It remained comparable after 48 hours, lowering slightly after 72 and rising up after 96 hours, but not to a significant concentration. The 20 µM pre-treatment produced similarly to 10 µM, but the 96-hour rise was not as large. for 50 µM and 100 µM pre-treatment the kynurenine production was similar to the others for the initial 24-hour result but produced very low kynurenine for 50 µM and not detectable levels of kynurenine for 100 µM after 48 hours. Subsequently, after 72 and 96 hours both the 50 and 100 µM produced non-detectable levels of kynurenine.


a
Total Neopterin Concentration (µM)
0.20
0.15
0.10
0.05
0.00
****
****
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)
Control
IFN-γ 24h
ATS[10] 24h, IFN-γ 48h
ATS[20] 24h, IFN-γ 48h
ATS[50] 24h, IFN-γ 48h
ATS[100] 24h, IFN-γ 48h


b
Neopterin Concentration (µM)
0.20
0.15
0.10
0.05
0.00
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)
Control
IFN-γ 24h
ATS[10] 24h, IFN-γ 48h
ATS[20] 24h, IFN-γ 48h
ATS[50] 24h, IFN-γ 48h
ATS[100] 24h, IFN-γ 48h

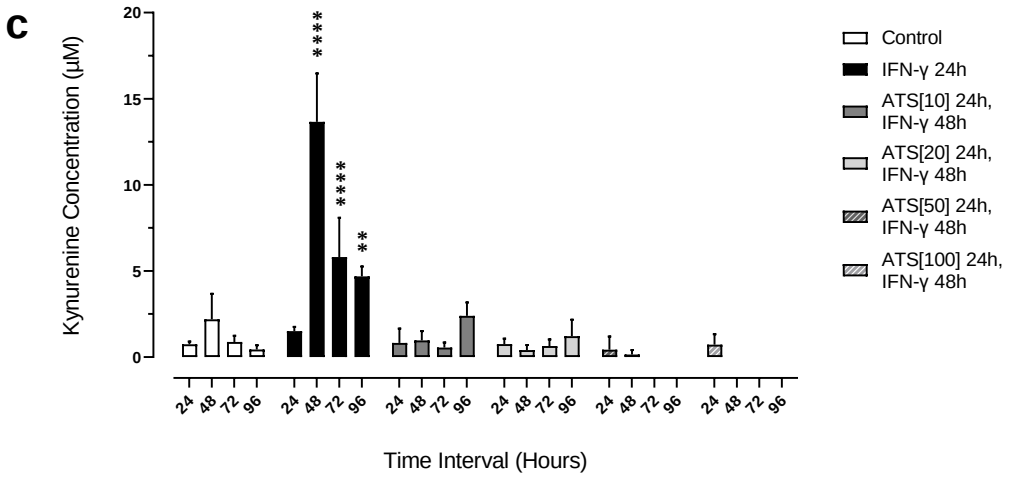

c
Kynurenine Concentration (µM)
20
15
10
5
0
****
****
**
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)
Control
IFN-γ 24h
ATS[10] 24h, IFN-γ 48h
ATS[20] 24h, IFN-γ 48h
ATS[50] 24h, IFN-γ 48h
ATS[100] 24h, IFN-γ 48h

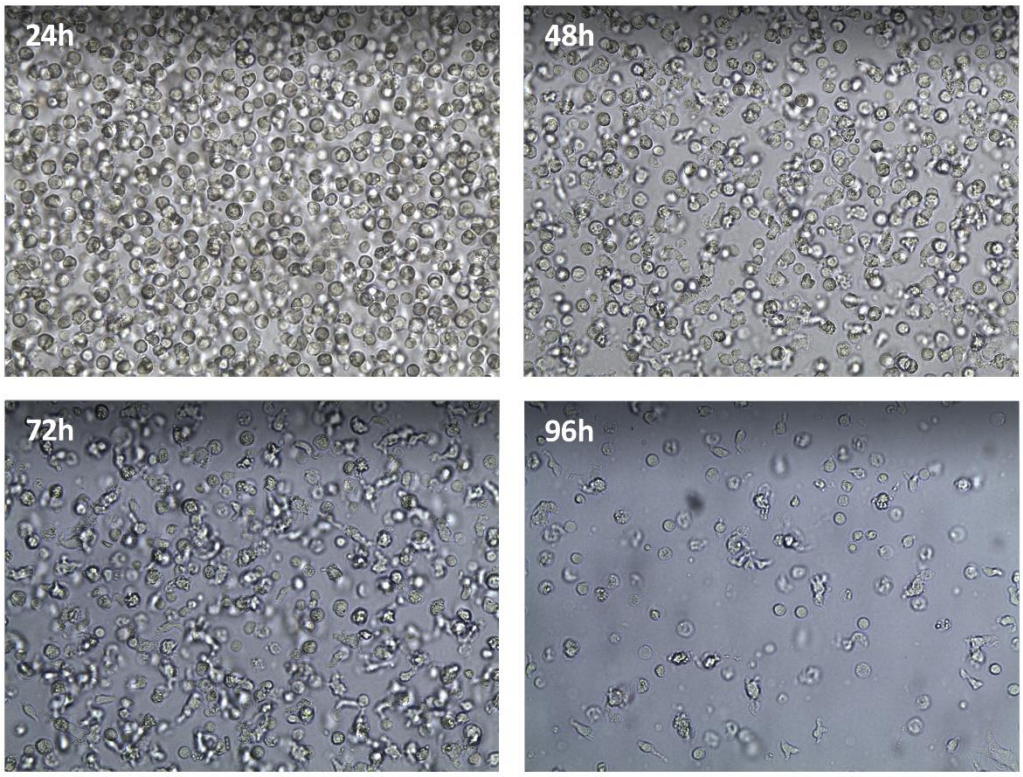
Figure 3.2.3 TNP, NP and KYN production with ATS pre-treatment, followed by IFN-γ. Following an initial media-only day, PBMCs were treated with IFN-γ 500 U/mL after 24 hours alone, and with ATS at varying concentrations: 10, 20, 50, 100 µM after 24 hours, followed by IFN-γ 500 U/mL after 48 hours. (**a**) Shows the total neopterin, (**b**) shows the neopterin, and (**c**) shows the kynurenine concentration for the same time period as measured by amine HPLC (µM/L). A multiple comparisons two-way ANOVA was conducted. Significance is indicated as compared to the control condition at the same time point (24-hours): *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, $p<0.0001$; ****

3.2.4 Cell Imagery and viability

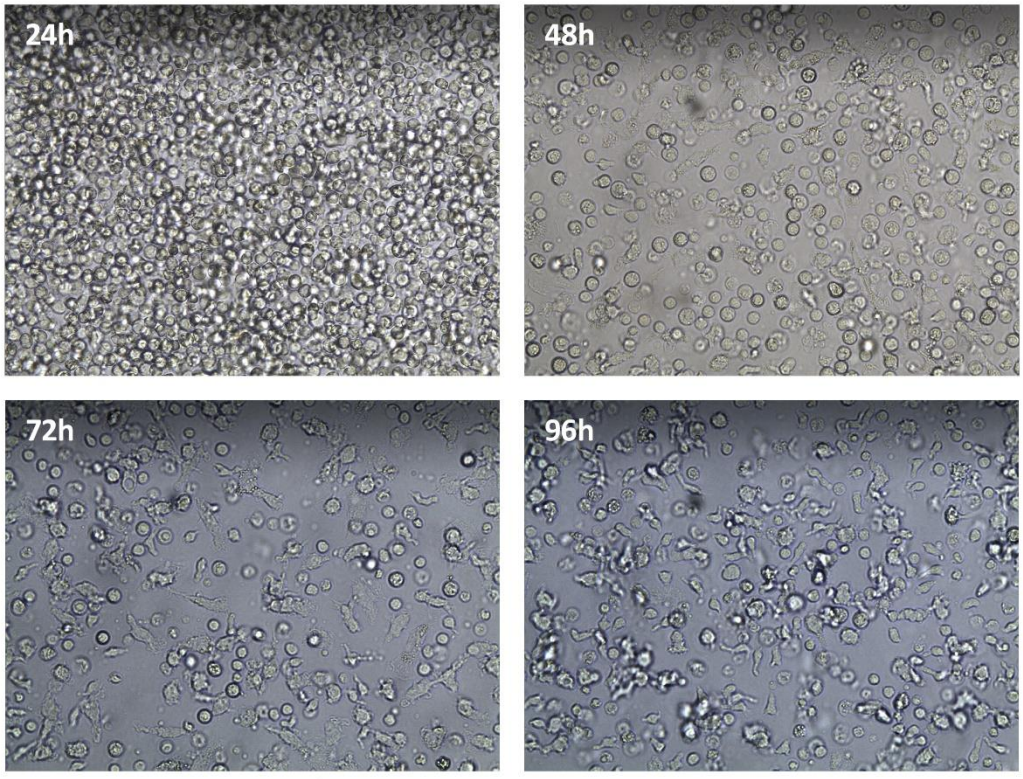
Images taken (**Figure 3.2.4.1**) indicate that the cells were less tolerant of the higher atorvastatin concentrations. There is visible some slight swelling of the cells which have been treated with higher concentrations of atorvastatin (panels **c** and **d**), particularly 100 µM atorvastatin. This is not the case with the IFN-γ treated and control cells (panels **b** and **a**). It appears the proportion of swelling increases in a concentration dependent manner, with the higher atorvastatin concentration displaying a greater amount. This may be due to the DMSO solvent required for atorvastatin, which is discussed later. This swelling indicates cell death may be occurring in an unregulated manner through necrosis, characterised by loss of membrane integrity and intracellular swelling at the higher atorvastatin concentration, resulting in less 7,8-dihydroneopterin release.

3.2.5 Protein determination

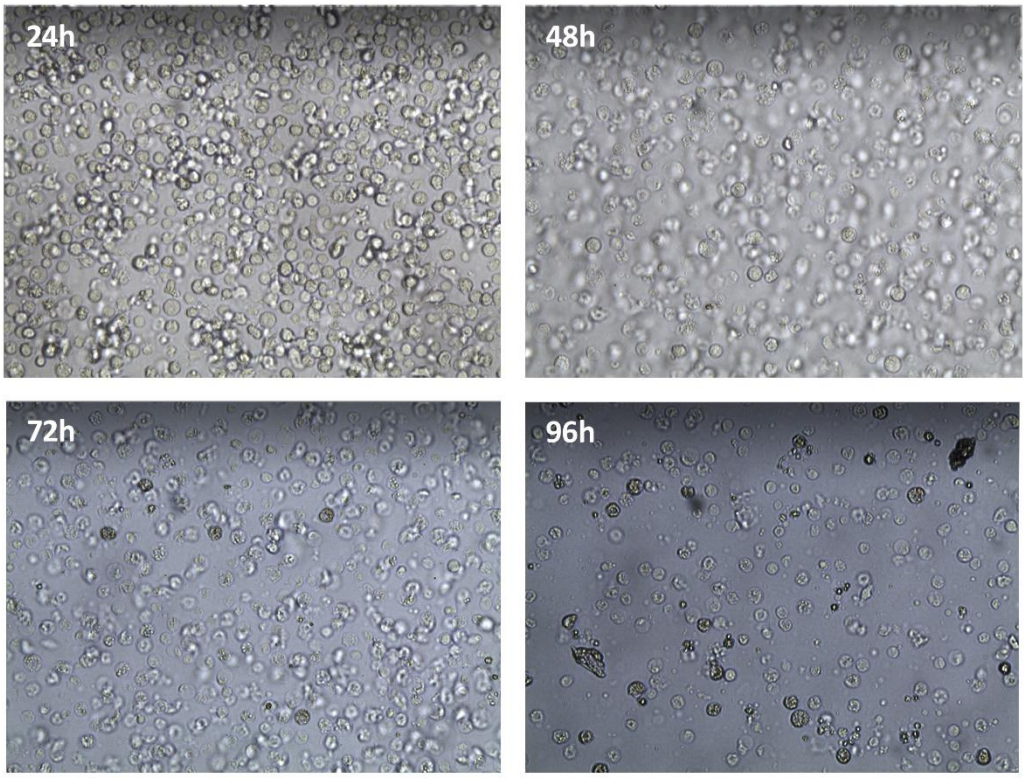
In this instance, an ordinary one-way ANOVA was conducted, utilising multiple comparisons between the control and treatment conditions individually (**Figure 3.2.4**). The treatment conditions all show similar, but varying levels of cellular protein concentration. Results of the multiple comparisons analysis showed that while different, there was no statistical significance to the difference in cellular protein concentrations between experimental conditions and the control (230 µg/mL). This data indicates that the more treatment received, the higher overall level of protein production, with the greatest concentration coming from the consistent IFN-γ treatment (426.2 µg/mL). The only treatment lower than the control in this instance was the 20 µM atorvastatin pre-treatment, however, 10, 50 and 100 µM were all higher.



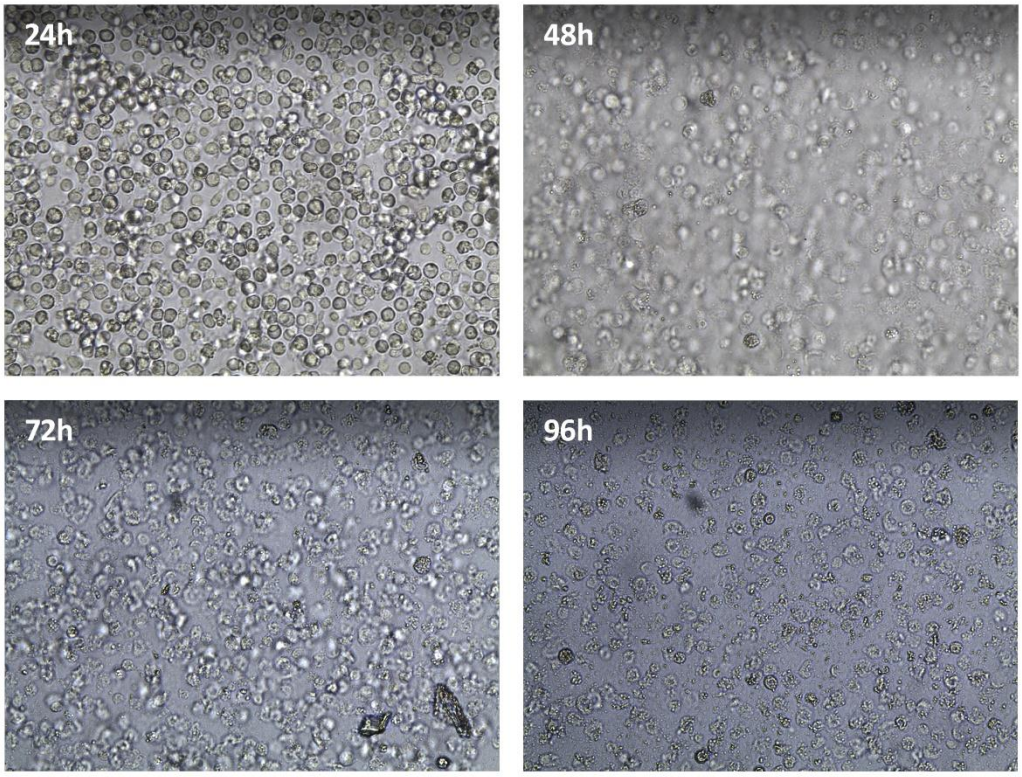
a.) Control - no treatment



b.) IFN-γ [500 U] at 24h



c.) ATS – [50 μM] at 24h, IFN-γ [500U] at 48h



d.) ATS – [100 μM] at 24h, IFN-γ [500U] at 48h

Figure 3.2.4 Images depicting cells with initial experimental conditions. PBMC images taken every 24-hours before media removal over 96-hour incubation with initial conditions. Images taken with LEICA DMIL microscope and LEICA DFC290 camera.

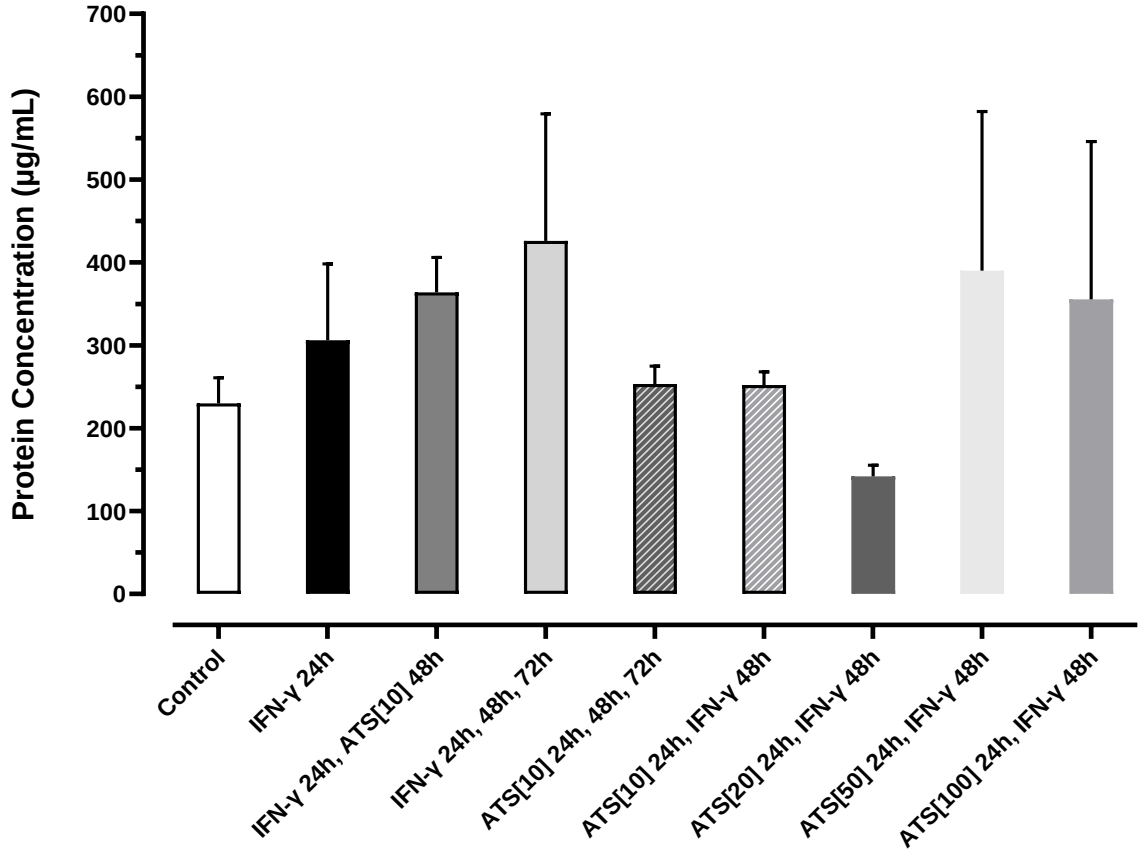


Figure 3.2.5 Protein concentration of cellular lysate from initial experimental conditions. Post 96-hour plate scraping sample of 50 µL, diluted 10:1, was added to 1 mL of BCA working solution, placed on a heat block at 60ºC and shaken for 30 minutes. Absorbance at 562 nm was determined by spectrophotometry and concentration derived from absorbance. An ordinary one-way multiple comparisons ANOVA was conducted, no significance was produced compared to the control.

3.3 Initial statin experiments repeat

After subjecting PBMCs to the initial experimental conditions, it was decided to repeat the conditions to ensure the results produced were representative of the PBMCs response to the IFN-γ and atorvastatin treatment. The following section describes results from experiments repeating the initial experimental conditions with a different donation of whole blood and subsequently isolated and culture of PBMCs.

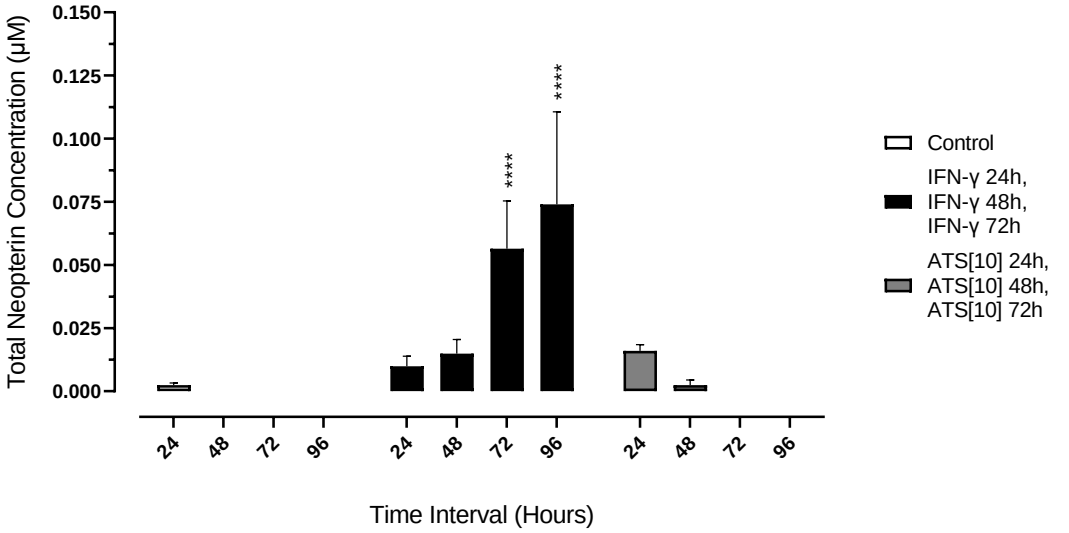
3.3.1 Characterising effect of IFN-γ and atorvastatin treatment

Characterising the effect of consistent IFN-γ and atorvastatin treatment was repeated. PBMCs were treated consistently with IFN-γ for 3 days, and 10 μM atorvastatin for 3 days following an initial media-only day (**Figure 3.3.1**).

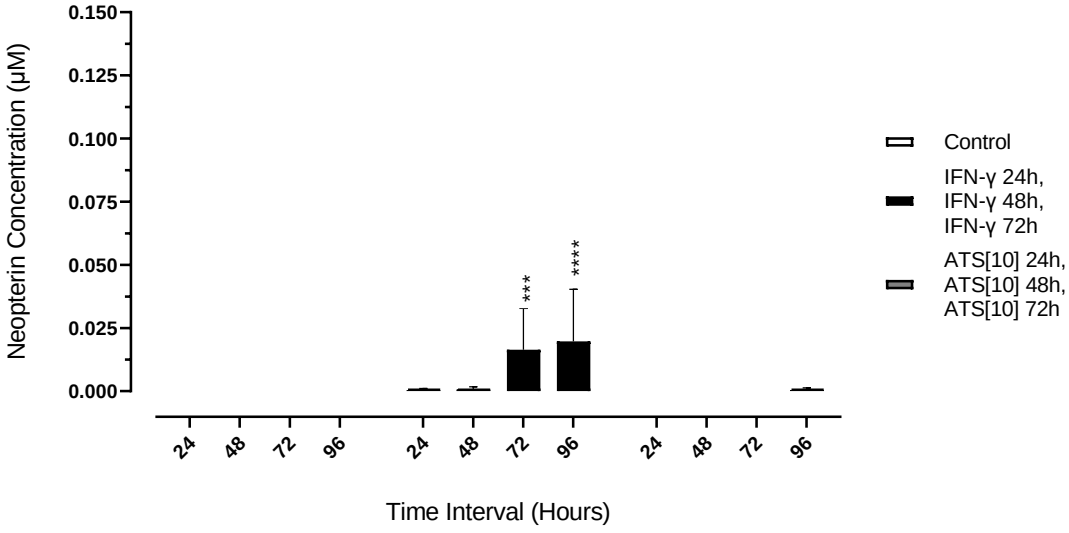
The consistent IFN-γ treatment over 3 days produced an increasing trend for total neopterin over the 96-hour period, with a significant rises after 72 hours (0.056 μM, $p<0.0001$) and 96 hours (0.074 μM, $p<0.0001$). Neopterin showed similar significant increases after 76 (0.016 μM, $p<0.001$) and 96 hours (0.020 μM, $p<0.0001$). Kynurenine increased significantly after 48 hours (3.71 μM, $p<0.001$), more so at 72 hours (4.70 μM, $p<0.0001$) and decreased at 96 hours (1.51 μM, $p<0.1$) while remaining significant.

Conversely, the consistent 10 μM atorvastatin treatment over 3 days did not produce any significant rises in total neopterin, neopterin or kynurenine over time. In fact, in this instance, given the control condition was not producing detectable levels of neopterin or kynurenine and minimal total neopterin initially, there were no notable rises from a non-detectable level of each measured inflammatory marker from the initial media-only day onward for this condition.

a


Total Neopterin Concentration (µM)
0.150
0.125
0.100
0.075
0.050
0.025
0.000
****
****
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 24h,
IFN-γ 48h,
IFN-γ 72h
ATS[10] 24h,
ATS[10] 48h,
ATS[10] 72h

b


Neopterin Concentration (µM)
0.150
0.125
0.100
0.075
0.050
0.025
0.000
***
****
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 24h,
IFN-γ 48h,
IFN-γ 72h
ATS[10] 24h,
ATS[10] 48h,
ATS[10] 72h

c

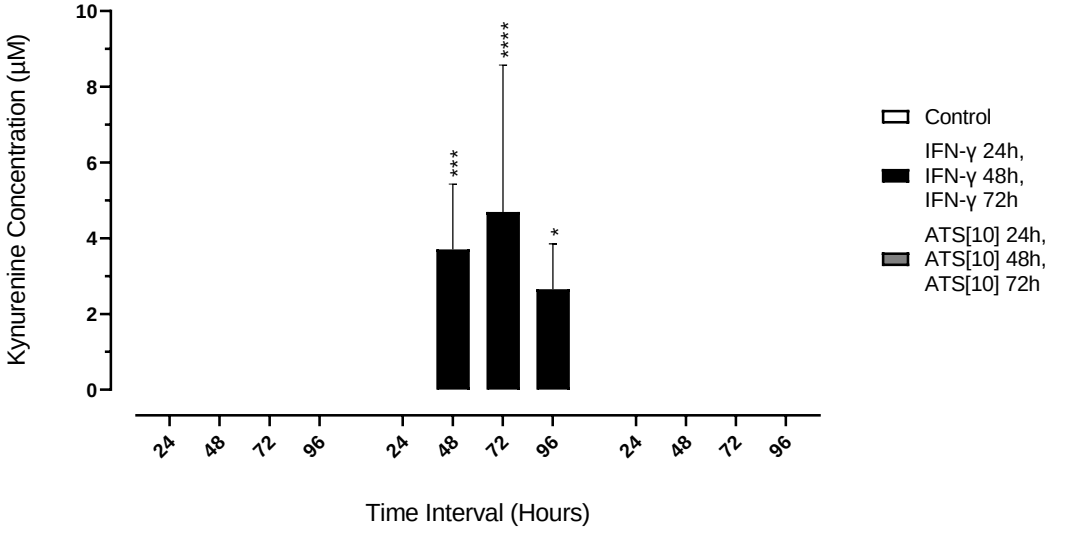

Kynurenine Concentration (µM)
10
8
6
4
2
0
***
****
*
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 24h,
IFN-γ 48h,
IFN-γ 72h
ATS[10] 24h,
ATS[10] 48h,
ATS[10] 72h

Figure 3.3.1 TNP, NP and KYN production with Consistent IFN-γ and ATS treatment. Repeated experimental condition: following an initial media-only day, PBMCs were treated consistently with IFN-γ 500 U/mL for 3 days, and 10 μM ATS for 3 days. (**a**) Shows the total neopterin, (**b**) shows the neopterin, and (**c**) shows the kynurenine concentration for the same time period as measured by amine HPLC (μM/L). A multiple comparisons two-way ANOVA was conducted on each. Significance is indicated as compared to the control condition at the same time point (24-hours): *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

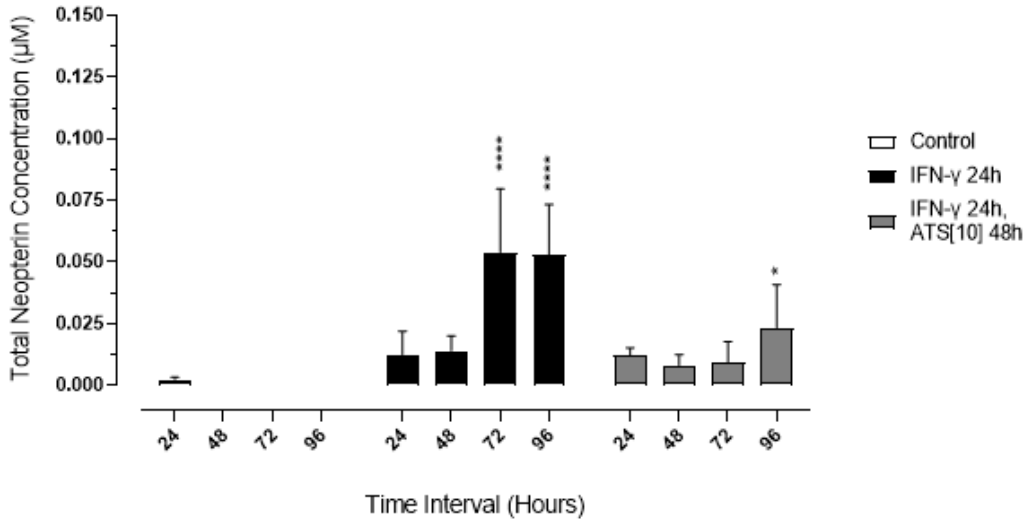
3.3.2 Characterising IFN-γ treatment followed by atorvastatin treatment

Characterising the effect of IFN-γ stimulation followed by atorvastatin treatment was repeated. PBMCs were treated with IFN-γ only after the initial media only 24 hours to establish a positive control for stimulation. In order to characterise how PBMCs are affected by IFN-γ followed by atorvastatin, PBMCs were treated after 24 hours with IFN-γ followed by 10 μM atorvastatin after 48 hours (**Figure 3.3.2**).

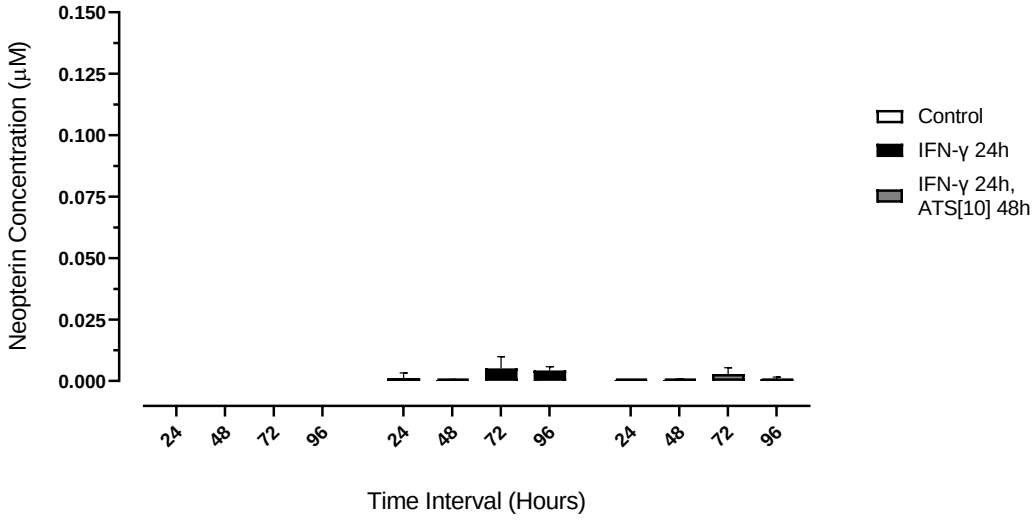
The IFN-γ only control established an increase in total neopterin, producing a significant increase after 72 hours (0.054 μM, $p<0.0001$), decreasing very slightly at 96 hours (0.053 μM, $p<0.0001$) remaining significant. Neopterin production was minimal in comparison, with no significant production, reaching its highest point at 72 hours (0.05 μM). Kynurenine production increased significantly after 48 hours (3.1 μM, $p<0.01$), rising slightly higher after 72 hours (3.4 μM, $p<0.01$), following that decreasing to a non-significant concentration after 96 hours.

The IFN-γ followed by 10 μM atorvastatin treatment in comparison showed a diminished rise in total neopterin, not rising noticeably at 76 hours like the positive control, producing a less significant concentration after 96 hours (0.023 μM, $p<0.05$). Similarly, to the positive control, minimal neopterin production occurred, with the highest point again being reached after 72 hours (0.03 μM). Kynurenine increased significantly prior to atorvastatin treatment at 48 hours (4.0 μM, $p<0.001$), then decreased to non-significant concentrations after 72 and 96 hours (2.0 μM and 0.5 μM) indicating the treatment had attenuated IFN-γ stimulation, which agrees with results from the previous experiment.

a


Total Neopterin Concentration (µM)
0.150
0.125
0.100
0.075
0.050
0.025
0.000
****
****
*
24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)
Control
IFN-γ 24h
IFN-γ 24h, ATS[10] 48h

b


Neopterin Concentration (µM)
0.150
0.125
0.100
0.075
0.050
0.025
0.000
24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)
Control
IFN-γ 24h
IFN-γ 24h, ATS[10] 48h

c

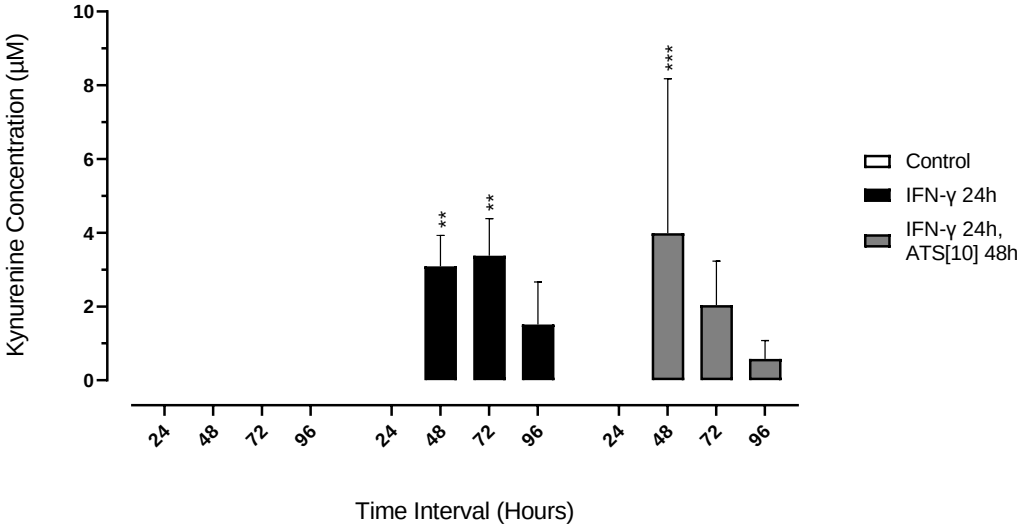

Kynurenine Concentration (µM)
10
8
6
4
2
0
**
**
***
24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)
Control
IFN-γ 24h
IFN-γ 24h, ATS[10] 48h

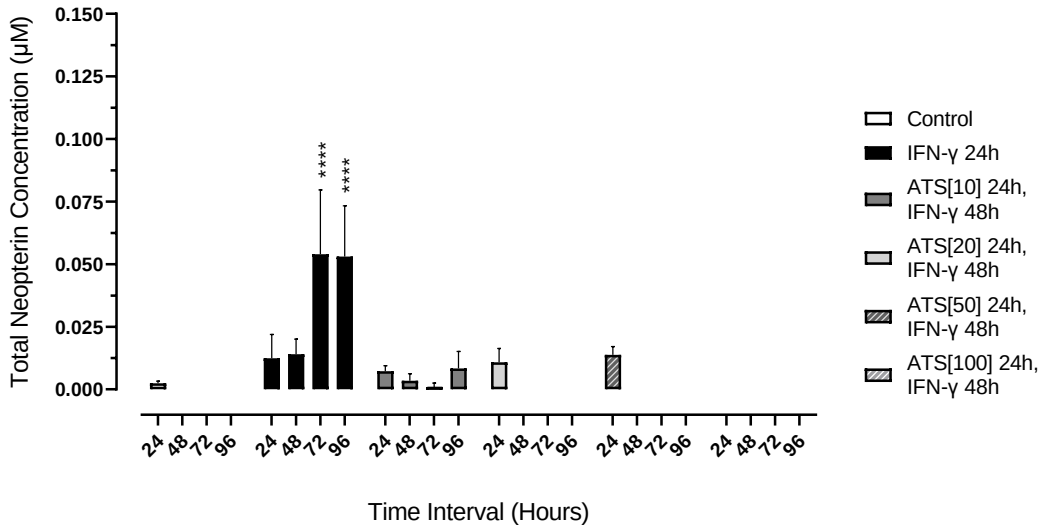
Figure 3.3.2 TNP, NP and KYN production with IFN-γ, followed by ATS treatment. Repeated experimental condition: following an initial media-only day, PBMCs were treated with IFN-γ 500 U/mL after 24 hours alone, additionally with IFN-γ 500 U/mL after 24 hours followed by 10 µM ATS after 48 hours. (**a**) Shows the total neopterin, (**b**) shows the neopterin, and (**c**) shows the kynurenine concentration for the same time period as measured by amine HPLC (µM/L). A multiple comparisons two-way ANOVA was conducted on each. Significance is indicated as compared to the control condition at the same time point (24-hours): *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

3.3.3 Characterising atorvastatin pre-treatment followed by IFN-γ treatment

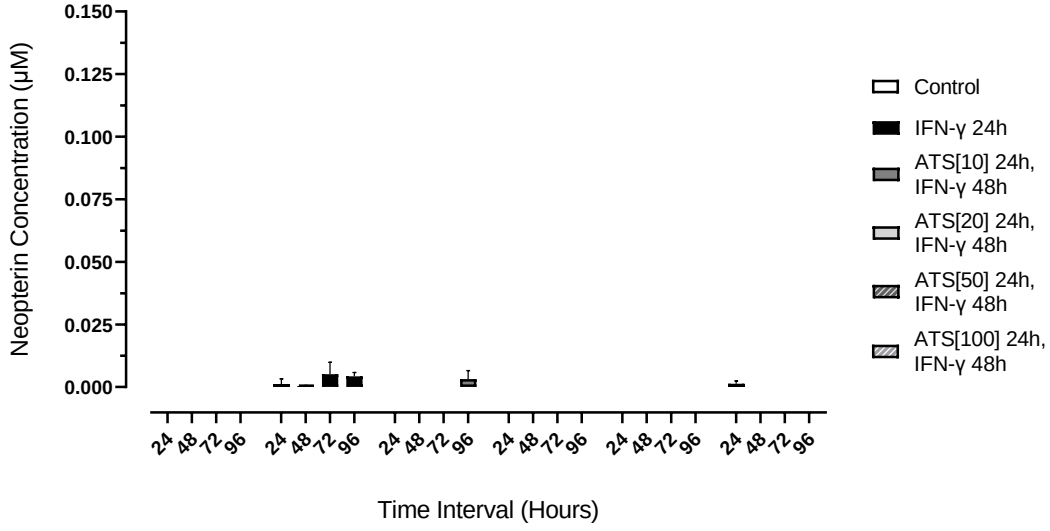
The next experimental conditions were to repeat the to characterise the possible effect of atorvastatin pre-treatment on IFN-γ activation of PBMCs (**Figure 3.3.3**). This was achieved by again treating PBMCs with a range of statin concentrations between the 10 and 100 µM used in previous work. PBMCs were pre-treated after 24 hours with 10, 20, 50 and 100 µM atorvastatin, followed by IFN-γ after 48 hours. The atorvastatin pre-treatment results were displayed alongside the 24-hour IFN-γ positive control condition previously discussed above (**Section 3.3.2**).

With regard to total neopterin, the pre-treatment with 10 µM atorvastatin after the initial 24 hours, showed a decreasing trend. Following the addition of IFN-γ after 48 hours, there was a decrease after 72 hours and an increase upwards at 96 hours, however the levels reached were not comparatively high or significant. The 20 and 50 µM atorvastatin pre-treatments produced non-detectable total neopterin after the initial 24 hours, and the 100 µM produced no detectable levels of total neopterin at all. Neopterin production (b) was similarly low, with only a small amount of production after 96 hours for the 10 µM pre-treatment, and a minute amount in the initial 24 hours for 100 µM. Kynurenine production for was non-detectable for each pre-treatment concentrations for the first 48 hours, following this production was only achieved by 10 µM after 76 and 96 hours, however not reaching a significant concentration. This indicates an complete inhibitory effect for atorvastatin concentrations ≥20 µM .

a


Total Neopterin Concentration (µM)
0.150
0.125
0.100
0.075
0.050
0.025
0.000
****
****
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)
Control
IFN-γ 24h
ATS[10] 24h, IFN-γ 48h
ATS[20] 24h, IFN-γ 48h
ATS[50] 24h, IFN-γ 48h
ATS[100] 24h, IFN-γ 48h

b


Neopterin Concentration (µM)
0.150
0.125
0.100
0.075
0.050
0.025
0.000
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)
Control
IFN-γ 24h
ATS[10] 24h, IFN-γ 48h
ATS[20] 24h, IFN-γ 48h
ATS[50] 24h, IFN-γ 48h
ATS[100] 24h, IFN-γ 48h

c

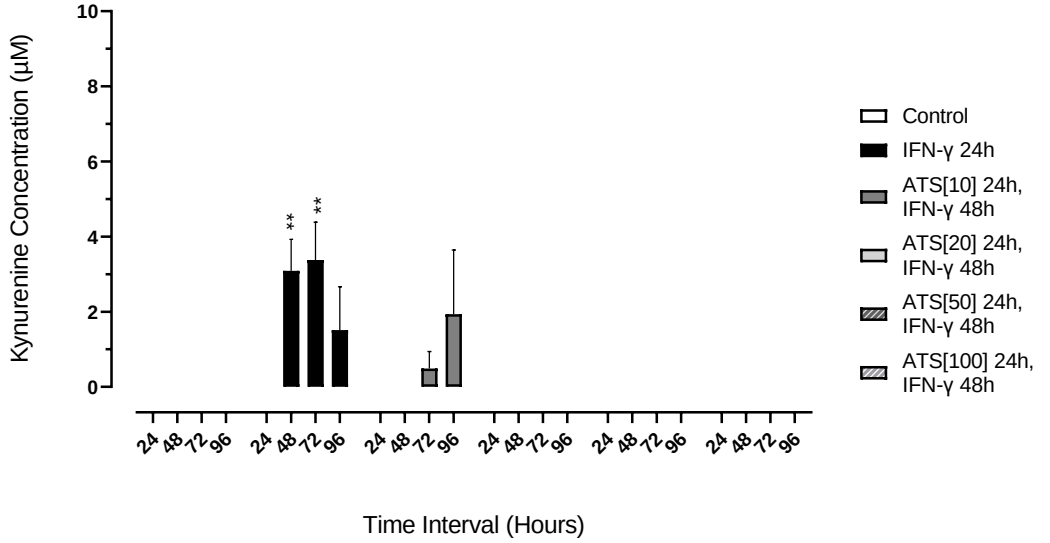

Kynurenine Concentration (µM)
10
8
6
4
2
0
**
**
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)
Control
IFN-γ 24h
ATS[10] 24h, IFN-γ 48h
ATS[20] 24h, IFN-γ 48h
ATS[50] 24h, IFN-γ 48h
ATS[100] 24h, IFN-γ 48h

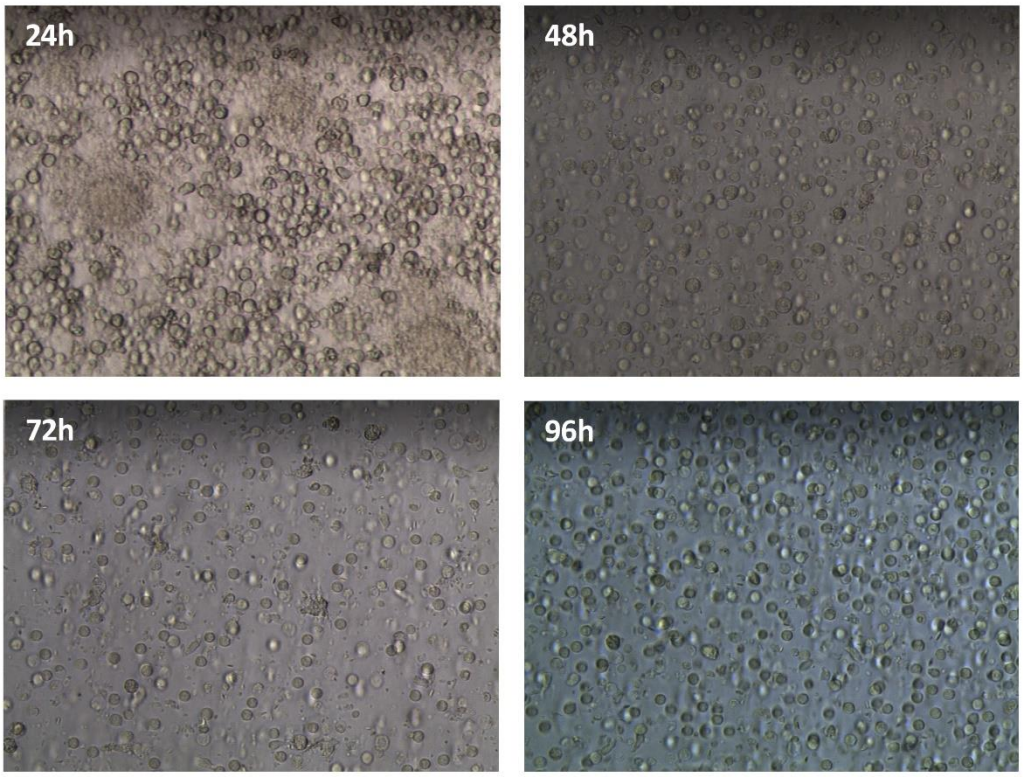
Figure 3.3.3 TNP, NP and KYN production with ATS pre-treatment, followed by IFN-γ. Repeated experimental condition: following an initial media-only day, PBMCs were treated with IFN-γ 500 U/mL after 24 hours, and with ATS at varying concentrations: 10, 20, 50, & 100 µM after 24 hours, followed by IFN-γ 500 U/mL after 48 hours. (**a**) Shows the total neopterin, (**b**) shows the neopterin, and (**c**) shows the kynurenine concentration for the same time period as measured by amine HPLC (µM/L). A multiple comparisons two-way ANOVA was conducted on each. Significance is indicated compared to the control condition at the same time point (24h): *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, $p<0.0001$; ****

3.3.4 Cell imagery and viability

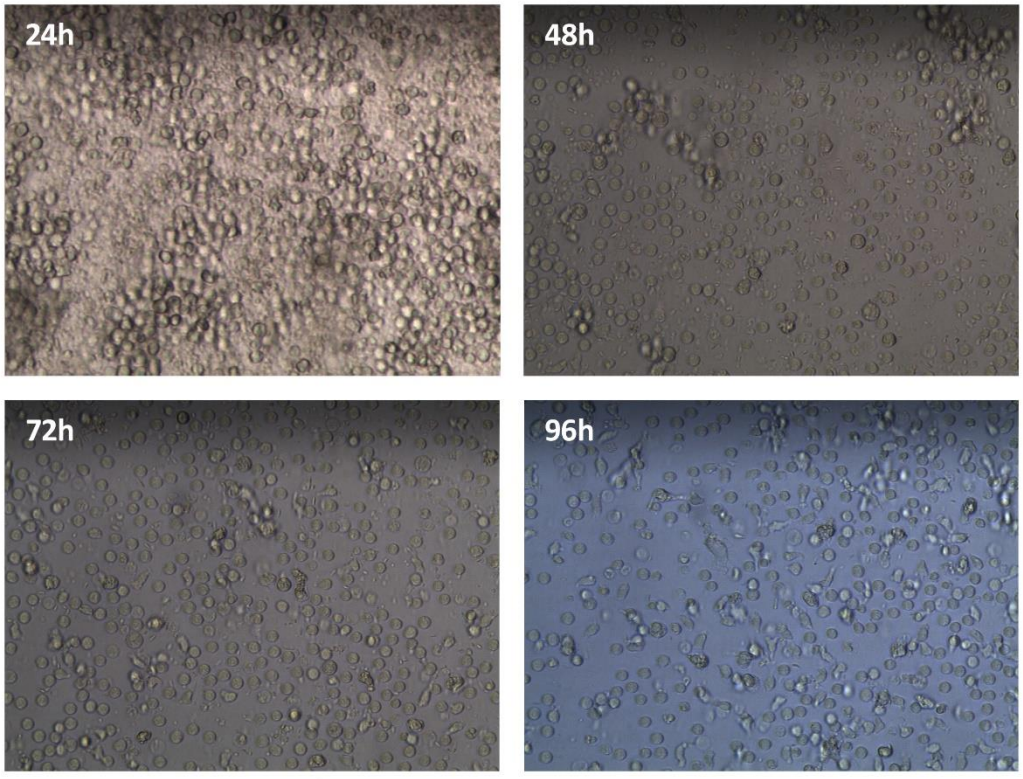
In the repeated experimental conditions, the images taken of the cells (**Figure 3.3.4.1**) again indicate that PBMCs were less tolerant of the higher atorvastatin concentrations, 50 and 100 µM. There is especially notable swelling of the PBMCs treated with atorvastatin. Again (**c.** and **d.**), with less cells visible over time. This is again not the case with the IFN-γ treated and control cells (**b.** and **a.**). The extent of the swelling is greater this experiment than previously. The swelling present in the 50 and 100 µM atorvastatin treated cells is extensive, although not as in focus for 100 µM, its clearly visible that there are swollen cells. This dramatic swelling (oncosis) indicates cell death is occurring in an uncontrolled manner by necrosis, characterised by intracellular swelling and loss of plasma membrane integrity (Zong & Thompson, 2006).

3.3.5 Protein determination

The BCA assay was conducted to determine cellular protein content and to serve as a means of estimating cellular concentration. (**Figure 3.3.4.2).** The treatment conditions show similar, but varying levels of protein concentration. Results of an ordinary one-way ANOVA utilising multiple comparisons analysis showed that there was not any statistical significance to the difference in cellular protein concentrations between experimental conditions and the control (294.2 µg/mL). The data indicates that PBMCs treated with IFN-γ alone have higher protein concentration than those treated with atorvastatin (374.2 and 353.4 µg/mL). the 10 µM pre-treated atorvastatin concentration produced higher protein concentration (333.7 µg/mL) than the control. However, each of the other atorvastatin pre-treated experimental conditions 20, 50 and 100 µM of atorvastatin produced lower concentrations of protein than the control condition (between 187.4 and 241.7 µg/mL). Again, in this experiment, the 20 µM pre-treatment concentration produced the lowest protein concentration (187.4 µg/mL).

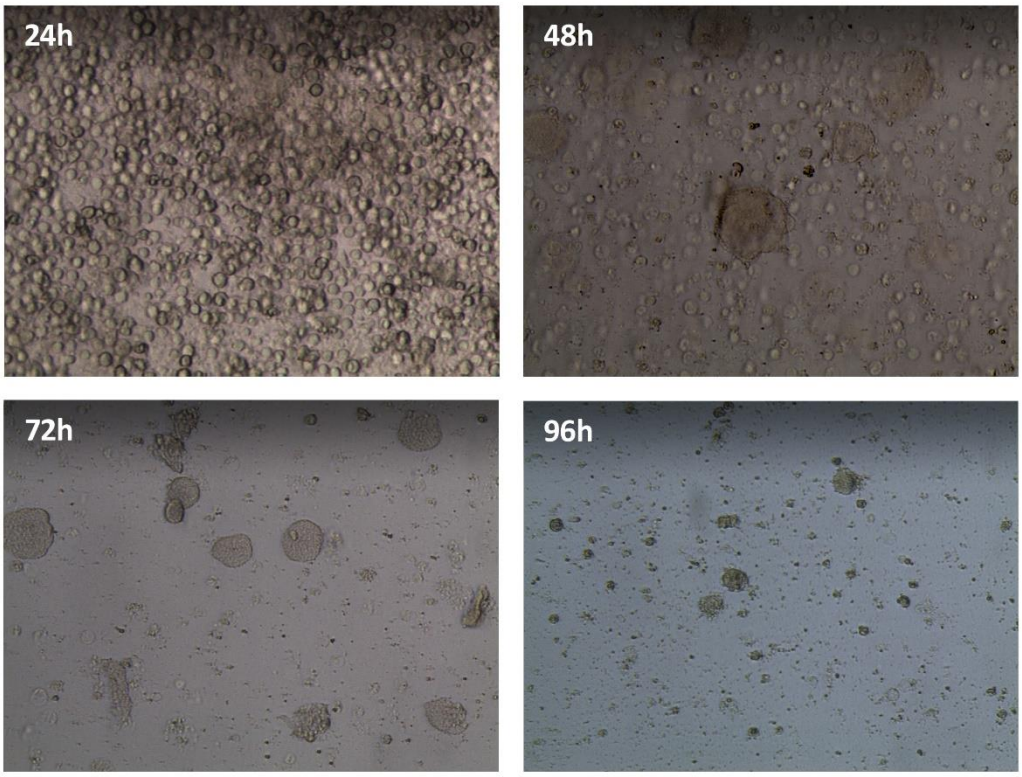


a.) Control - no treatment

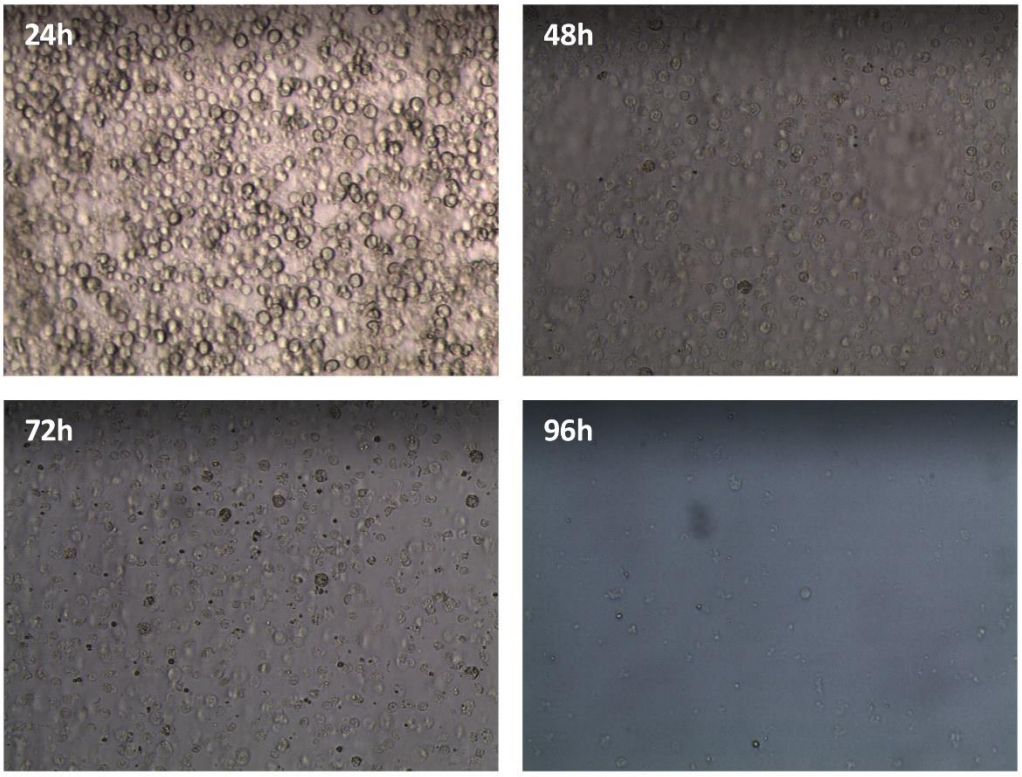


b.) IFN-γ [500 U] at 24h

Note: For these images, there was a fault with the camera colour balance which was later resolved.



c.) ATS – [50 μM] at 24h, IFN-γ [500U] at 48h



d.) ATS – [100 μM] at 24h, IFN-γ [500U] at 48h

Figure 3.3.4 Images depicting cells with repeated initial experimental conditions. PBMC images taken every 24-hours before media removal over 96-hour incubation with repeated initial conditions. Images taken with LEICA DMIL microscope and LEICA DFC290 camera.

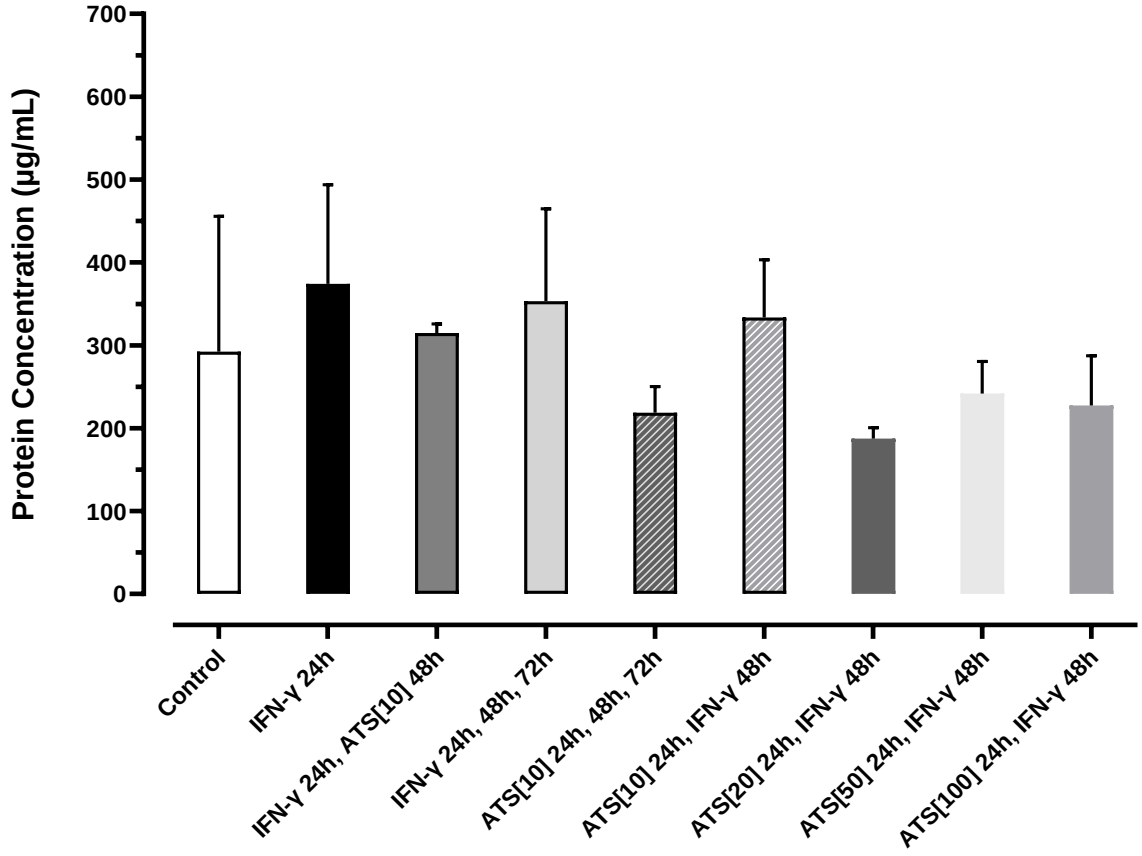


Figure 3.3.5 Protein concentration of cellular lysate from repeated initial conditions. Post 96-hour plate scraping sample of 50 µL, diluted 10:1, was added to 1 mL of BCA working solution, placed on a heat block at 60ºC and shaken for 30 minutes. Absorbance at 562 nm was determined by spectrophotometry and concentration derived from absorbance. An ordinary one-way multiple comparisons ANOVA was conducted, no significance was produced compared to the control.

3.4 Further investigation of statin inhibition of activity

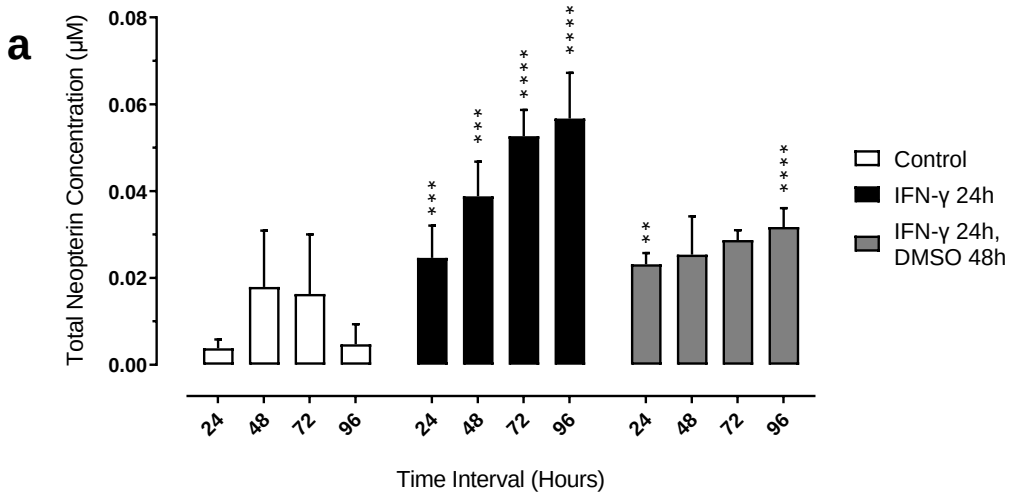
Results from the initial experimental conditions indicated that the lower concentrations 10 and 20 µM of atorvastatin were capable of producing comparable effects to the higher concentrations 50 and 100 µM on the PBMCs production of total neopterin, neopterin and kynurenine. Images taken of the higher atorvastatin concentration experimental conditions showed the cells were swelling, indicating that the PBMCs were not tolerating the higher conditions as well as the lower concentrations. Based on the HPLC data produced and images taken of the cells, the lower concentrations 10 and 20 µM were determined to be the optimal concentrations for further experimental work and the higher concentrations 50 and 100 µM were not utilised in the further statin experiments on subsequent PBMC preparations.

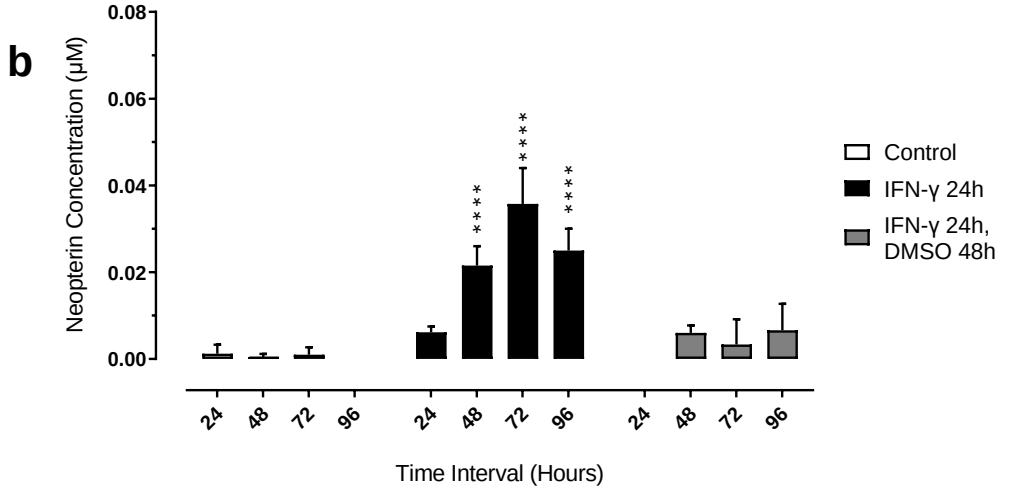
3.4.1 Characterising IFN-γ treatment followed by DMSO treatment

The atorvastatin calcium salt required an organic solvent, for which DMSO was required. Therefore, it was necessary to test that DMSO was not responsible for the anti-inflammatory effects shown in the initial experiments. PBMCs were treated with IFN-γ only after the initial media only 24-hours to establish a positive control for stimulation. In order to characterise how DMSO may possibly effect IFN-γ activation followed by, simulating the previous condition in **Section 3.2.2** without atorvastatin, PBMCs were treated after 24 hours with IFN-γ, followed by 5 µL of DMSO after 48 hours (**Figure 3.4.1**). The 5 µL of DMSO is equivalent to the volume containing 10 µM of atorvastatin, and 0.5% of the overall volume.

The IFN-γ control produced significant increases in total neopterin, rising after 48 hours (0.039 µM, $p<0.0006$), rising further after 72 hours (0.053 µM, $p<0.0001$) and 96 hours (0.057 µM, $p<0.0001$). Neopterin produced increased with significance after 48 hours (0.021 µM, $p<0.0001$), increasing at 72 hours (0.036 µM, $p<0.0001$) and decreasing slightly but remaining significant after 96 hours (0.025 µM, $p<0.0001$). Kynurenine production increased after 48 hours (14.5 µM, $p<0.0001$), decreasing but remaining at significant concentrations after 72 hours(12.5 µM, $p<0.0001$) and 96 hours (9.4 µM, $p<0.0001$).

IFN-γ followed by DMSO treatment produced a significant rise in total neopterin after 96 hours (0.032 µM, $p<0.0001$). Neopterin did not rise in to a significant concentration over time, rising after the IFN-γ treatment after 48 hours from a non-detectable level, decreasing slightly after 72 hours and rising slightly after 96 hours. Kynurenine also did not rise to a significant concentration over time, decreasing after 72 hours and rising slightly at 96 hours.


a
Total Neopterin Concentration (µM)
0.08
0.06
0.04
0.02
0.00
Control
IFN-γ 24h
IFN-γ 24h, DMSO 48h
24
48
72
96
Time Interval (Hours)


b
Neopterin Concentration (µM)
0.08
0.06
0.04
0.02
0.00
Control
IFN-γ 24h
IFN-γ 24h, DMSO 48h
24
48
72
96
Time Interval (Hours)

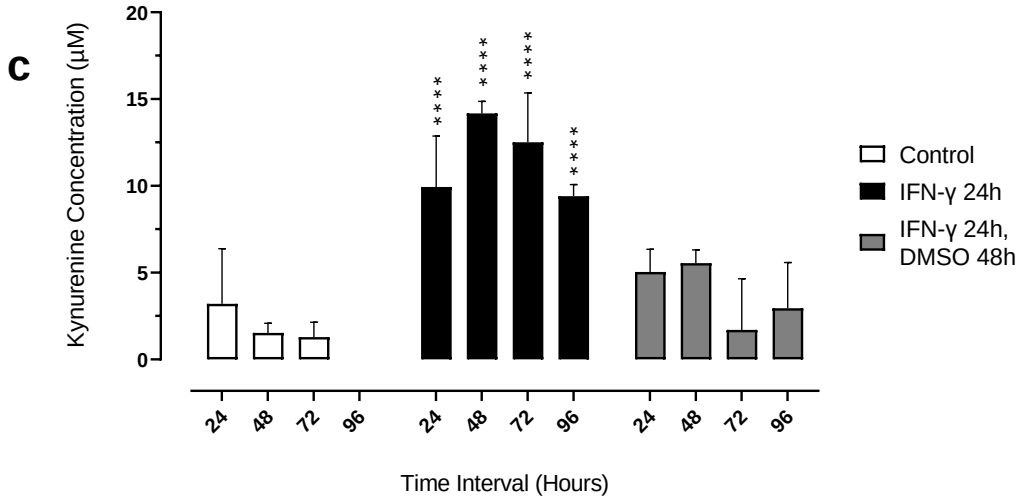

c
Kynurenine Concentration (µM)
20
15
10
5
0
Control
IFN-γ 24h
IFN-γ 24h, DMSO 48h
24
48
72
96
Time Interval (Hours)

Figure 3.4.1 TNP, NP and KYN Production with IFN-γ, followed by DMSO treatment. Following an initial media-only day, cells were treated with IFN-γ 500 U/mL after 24 hours alone, and with IFN-γ 500 U/mL after 24 hours followed by 5 µL of DMSO after 48 hours. (**a**) Shows the total neopterin, (**b**) shows the neopterin, and (**c**) shows the kynurenine concentration for the same time period as measured by amine HPLC (µM/L). A multiple comparisons two-way ANOVA was conducted. Significance is indicated as compared to the control condition at the same time point (24-hours): *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

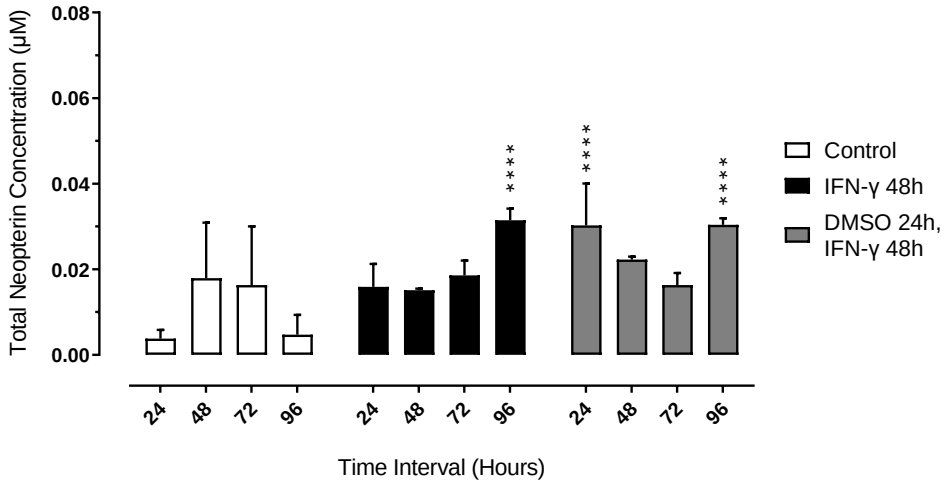
3.4.2 Characterising DMSO pre-treatment followed by IFN-γ treatment

The possible anti-inflammatory effect of atorvastatin pre-treatment on IFN-γ activation was further examined by pre-treatment with the solvent DMSO followed by IFN-γ, simulating the previous condition in **Section 3.2.3** without atorvastatin. PBMCs were treated after 24 hours with 5 µL of DMSO followed by IFN-γ, after 48 hours. A positive control for this was implemented by treating PBMCs with IFN-γ only after 48 hours and displayed alongside the DMSO treatment (**Figure 3.4.2**)

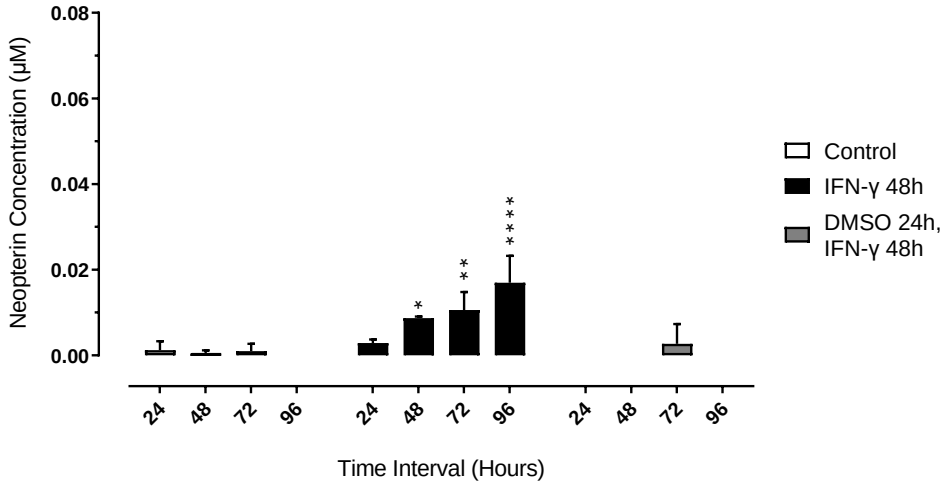
The positive control established a slight rise after 72 hours and significant total neopterin production after 96 hours (0.032 µM, $p<0.0001$). Significant concentrations of neopterin were produced after 48 (0.009 µM, $p<0.0313$), increasing further after both 72 (0.011 µM, $p<0.0083$) and 96 hours (0.017 µM, $p<0.0001$). Kynurenine began significant (10.1 µM, $p<0.0001$) but decreased after IFN-γ treatment to non-significant levels.

In the DMSO treatments, total neopterin production was elevated and significant after the initial media-only 24 hours (0.030 µM, $p<0.0001$), dropping after 48 and 72 hours to non-significant levels, and increasing again to a significant concentration after 96 hours (0.030 µM, $p<0.0001$) very similar to the positive control. Neopterin production for the DMSO treatment was non-detectable after 24, 48 and 96 hours, only producing a small amount of neopterin after 72 hours. Kynurenine production for this condition peaked after the initial media only day, dropped after 48 hours, rose slightly after IFN-γ treatment after 72 hours and was non-detectable after 96 hours.

a


Total Neopterin Concentration (µM)
0.08
0.06
0.04
0.02
0.00
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 48h
DMSO 24h,
IFN-γ 48h

b


Neopterin Concentration (µM)
0.08
0.06
0.04
0.02
0.00
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 48h
DMSO 24h,
IFN-γ 48h

c

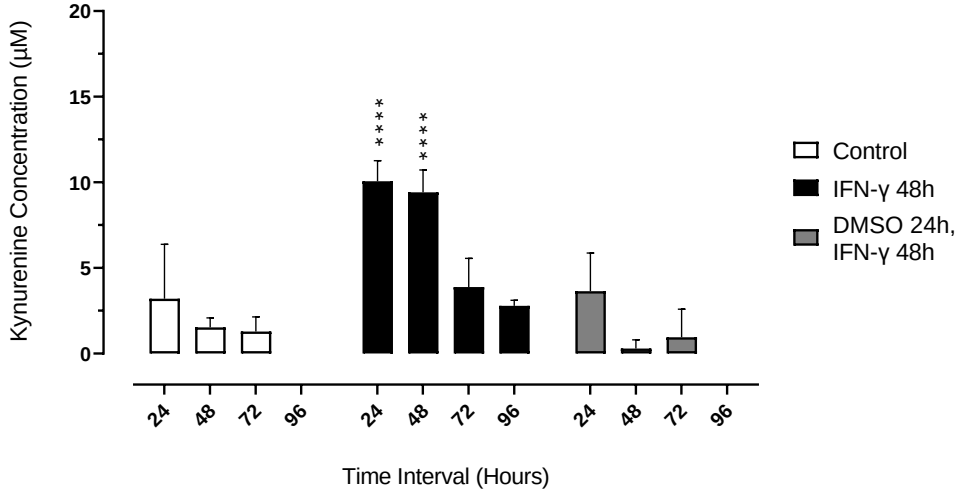

Kynurenine Concentration (µM)
20
15
10
5
0
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 48h
DMSO 24h,
IFN-γ 48h

Figure 3.4.2 TNP, NP and KYN production pre-treated with DMSO, followed by IFN-γ. Following an initial media-only day, cells were treated with IFN-γ 500 U/mL after 48 hours alone, and with 5 µL of DMSO after 24 hours followed by IFN-γ 500 U/mL after 48 hours. (**a**) Shows the total neopterin, (**b**) shows the neopterin, and (**c**) shows the kynurenine concentration for the same time period as measured by amine HPLC (µM/L). A multiple comparisons two-way ANOVA was conducted. Significance is indicated as compared to the control condition at the same time point (24-hours): *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

3.4.3 Characterising atorvastatin pre-treatment followed by two IFN-γ treatments

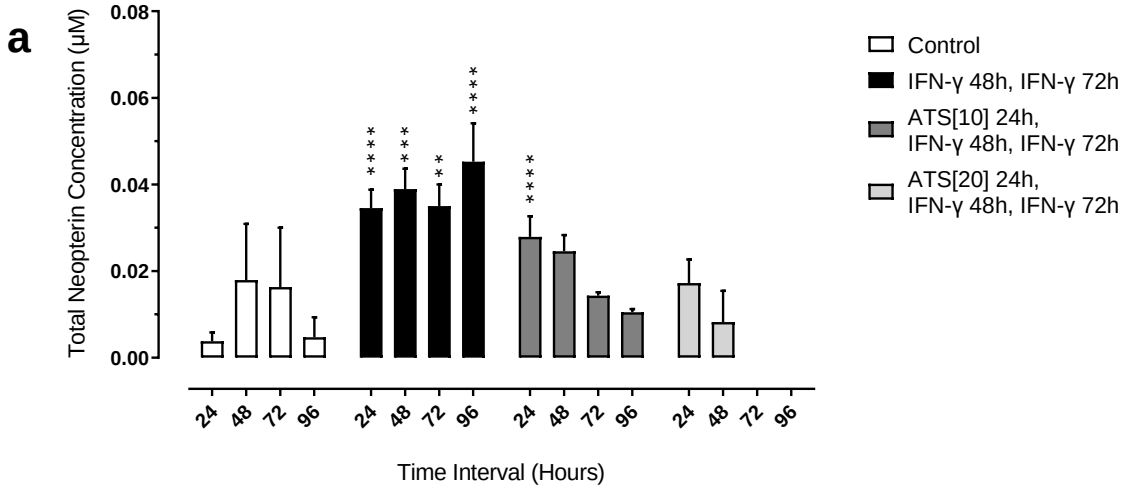
After the initial experimental results showing a single IFN-γ treatment after atorvastatin pre-treatment was not producing an inflammatory response, the logical next step was to increase stimulation to determine if it would produce a more measurable response. To achieve this, PBMCs were pre-treated with 10 and 20 µM atorvastatin and treated with two subsequent treatments of IFN-γ, to see if more stimulation would produce a stronger inflammatory response. This was achieved by treating PBMCs after 24 hours with 10 and 20 µM atorvastatin, followed by two treatments of IFN-γ, after 48 and 72 hours, respectively. These results were displayed alongside a positive control treatment, in which treated PBMCs with IFN-γ only after 48 and 72 hours respectively (**Figure 3.4.3**).

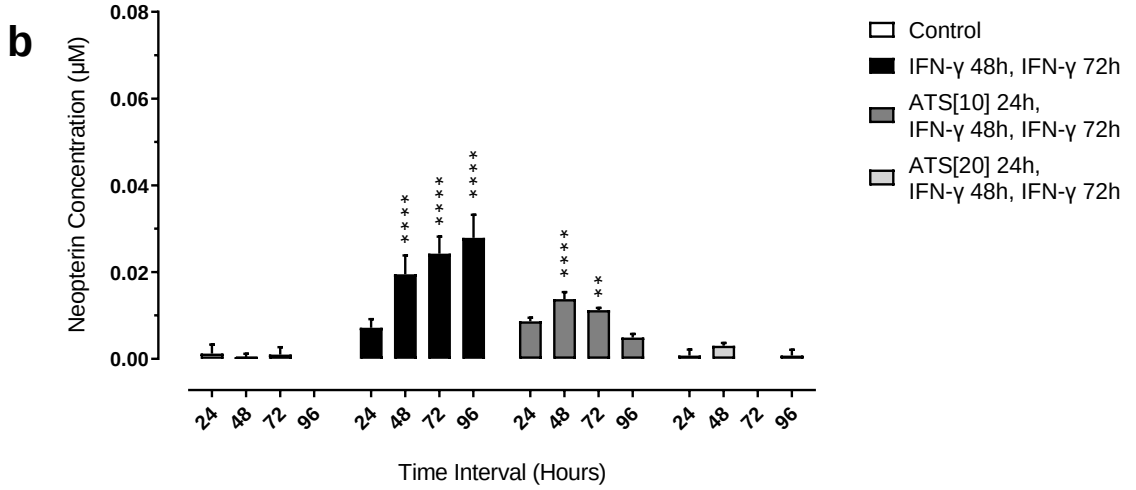
The positive control shows from 24 hours (0.035 µM, $p<0.0001$) onward significantly increased total neopterin production. after 48 hours (0.039 µM, $p<0.0001$), decreasing slightly after 72 hours (0.035 µM, $p<0.001$) and increasing to reach its highest point after 96 hours (0.045 µM, $p<0.0001$). Neopterin production began low after 24 hours, increasing significantly after 48 hours (0.0072 µM, $p<0.0001$), rising further after 72 hours (0.020 µM, $p<0.0001$) and 96 hours (0.024 µM, $p<0.0001$) respectively. Kynurenine production began elevated after 24 hours (9.3 µM, $p<0.0001$), rising further after 48 hours (11.2 µM, $p<0.0001$), then remaining significantly high, but decreasing after 72 (7.4 µM, $p<0.001$) and 96 hours (6.9 µM, $p<0.0001$).

Total neopterin concentration for the 10 µM of atorvastatin pre-treatment followed by two IFN-γ treatments began significantly high after 24 hours (0.028 µM, $p<0.0001$) similarly to the positive control. Post atorvastatin treatment, the concentration decreased slightly to a non-significant concentration, decreasing further after subsequent IFN-γ treatments at 72 and 96 hours, not showing any indication of increasing. Neopterin production was significant after 48 hours (0.014 µM, $p<0.0001$), decreasing and remaining significant after 72 hours (0.011 µM,

$p<0.004$) and decreasing further to non-significance after 96 hours. Kynurenine production began significantly elevated similarly to the positive control after 24 hours (12.2 µM, $p<0.0001$), however after atorvastatin treatment decreased at 48 hours (8.2 µM, $p<0.0001$), further still after 72 and 96 hours to non-significant concentrations with subsequent IFN-γ treatments received.

Total neopterin for the 20 µM atorvastatin pre-treatment followed by two IFN-γ treatments began non-significant, lower than the other two experimental conditions, decreasing at 48 hours after atorvastatin treatment, decreasing further to non-detectable levels after 72 and 96 hours in contrast to the other conditions. Neopterin production was negligible, producing its peak at 48 hours, non-detectable after 72 hours and very low production after 96 hours. In similarity to the other treatment conditions, the kynurenine production began significantly elevated after 24 hours (8.2 µM, $p<0.0001$), then after atorvastatin treatment decreased to non-significant concentrations after 48 hours, further still after 72 hours and down to non-detectable concentrations after 96 hours.


a
Total Neopterin Concentration (µM)
0.08
0.06
0.04
0.02
0.00
Control
IFN-γ 48h, IFN-γ 72h
ATS[10] 24h, IFN-γ 48h, IFN-γ 72h
ATS[20] 24h, IFN-γ 48h, IFN-γ 72h
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)


b
Neopterin Concentration (µM)
0.08
0.06
0.04
0.02
0.00
Control
IFN-γ 48h, IFN-γ 72h
ATS[10] 24h, IFN-γ 48h, IFN-γ 72h
ATS[20] 24h, IFN-γ 48h, IFN-γ 72h
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)

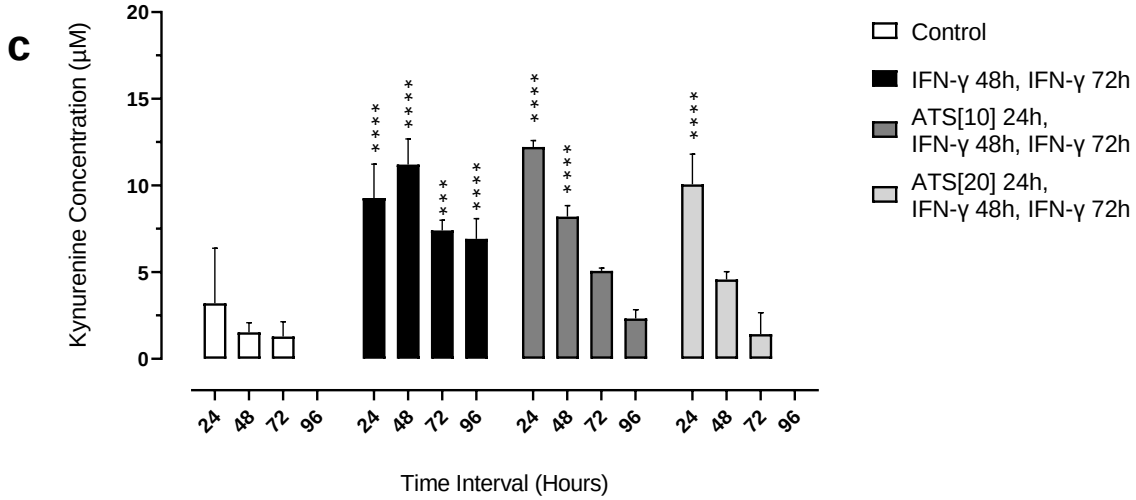

c
Kynurenine Concentration (µM)
20
15
10
5
0
Control
IFN-γ 48h, IFN-γ 72h
ATS[10] 24h, IFN-γ 48h, IFN-γ 72h
ATS[20] 24h, IFN-γ 48h, IFN-γ 72h
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)

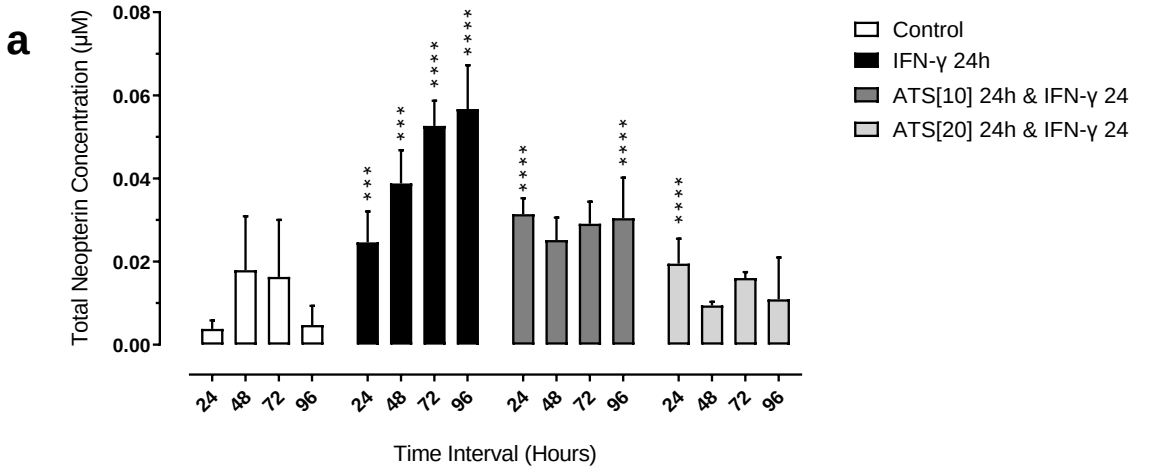
Figure 3.4.3 TNP, NP and KYN production with ATS pre-treatment, followed by subsequent IFN-γ treatments. Following an initial media-only day, PBMCs were treated with IFN-γ 500 U/mL after 48 hours and 72 hours alone and pre-treated with 10 and 20 µM ATS after 24 hours followed by IFN-γ 500 U/mL after 48 hours and 72 hours. (**a**) Shows the total neopterin (**b**) shows the neopterin, and (**c**) shows the kynurenine concentration for the same time period as measured by amine HPLC (µM/L). A multiple comparisons two-way ANOVA was conducted. Significance is indicated as compared to the control condition at the same time point (24-hour period): *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

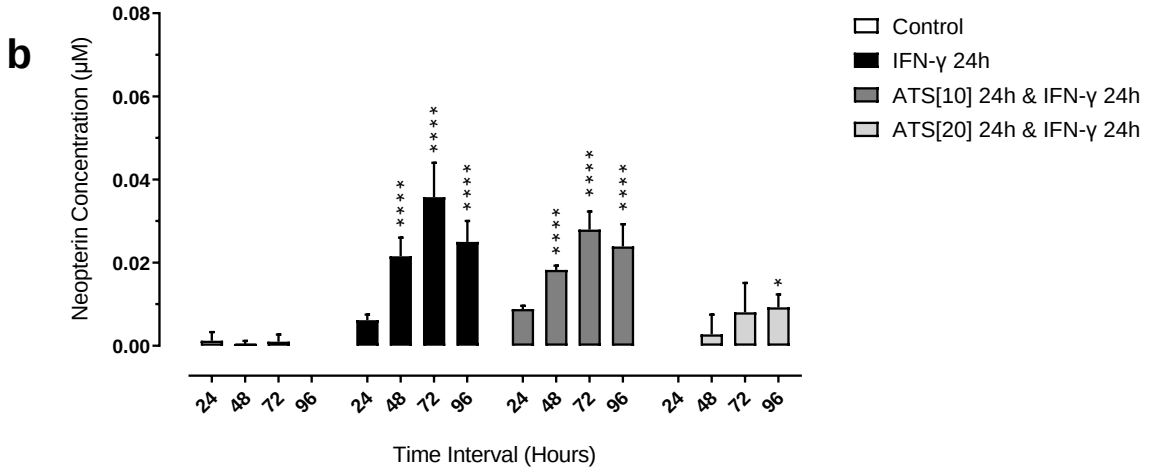
3.4.4 Characterising simultaneous atorvastatin and IFN-γ treatment

PBMCs were treated simultaneously with 10 and 20 µM of atorvastatin and IFN-γ after the initial media only day. This was displayed alongside a positive control treatment, in which PBMCs were treated with only IFN-γ after 24 hours (**Figure 3.4.4**). The results for the positive control may be viewed above in **Section 3.4.1**.

The 10 µM atorvastatin and IFN-γ condition began significantly high after 24 hours (0.031 µM, $p<0.0001$), decreasing slightly to a non-significant concentration after the simultaneous treatment at 48 hours, rising after 72 hours, then continuing to rise at 96 hours to a significant concentration (0.030 µM, $p<0.0001$). Neopterin reached significant levels after simultaneous treatment at 48 hours (0.018 µM, $p<0.0001$), reaching its highest point at 72 hours (0.028 µM, $p<0.0001$), followed by a slight decrease after 96 hours (0.024 µM, $p<0.0001$). Kynurenine began elevated significantly after 24 hours (13.5 µM, $p<0.0001$), following simultaneous treatment the concentration steadily decreased over time while remaining significantly elevated after 48 (12.0 µM, $p<0.0001$), 72 (11.7 µM, $p<0.0001$) and 96 hours (9.5 µM, $p<0.0001$).

The 20 µM atorvastatin and IFN-γ condition began significantly elevated after 24 hours (0.020 µM, $p<0.0001$), then after simultaneous treatment decreased at 48 hours to a non-significant concentration, rising after 72 hours but not to a significant extent, and decreasing at 96 hours. Neopterin production began non detectable, rising gradually over time, reaching its highest concentration after 96 hours (0.009 µM, $p=0.0120$.which showed slight significance. Kynurenine began low, increasing over time and reaching a significantly high concentration after 72 hours (9.7 µM, $p<0.0001$), decreasing after 96 hours (6.3 µM, $p<0.0002$) but remaining significant.


a
Total Neopterin Concentration (µM)
0.08
0.06
0.04
0.02
0.00
Control
IFN-γ 24h
ATS[10] 24h & IFN-γ 24
ATS[20] 24h & IFN-γ 24
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)


b
Neopterin Concentration (µM)
0.08
0.06
0.04
0.02
0.00
Control
IFN-γ 24h
ATS[10] 24h & IFN-γ 24h
ATS[20] 24h & IFN-γ 24h
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)

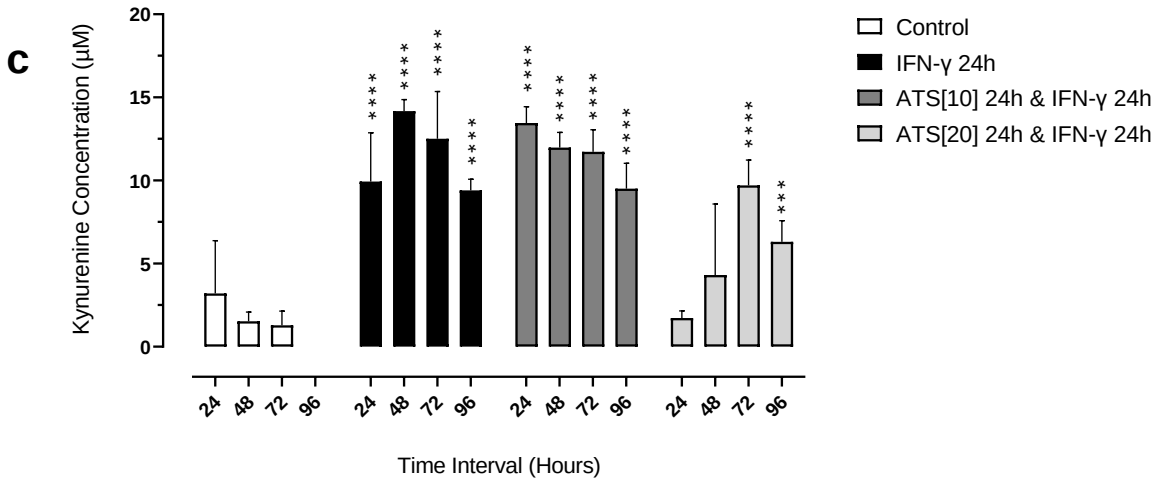

c
Kynurenine Concentration (µM)
20
15
10
5
0
Control
IFN-γ 24h
ATS[10] 24h & IFN-γ 24h
ATS[20] 24h & IFN-γ 24h
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)

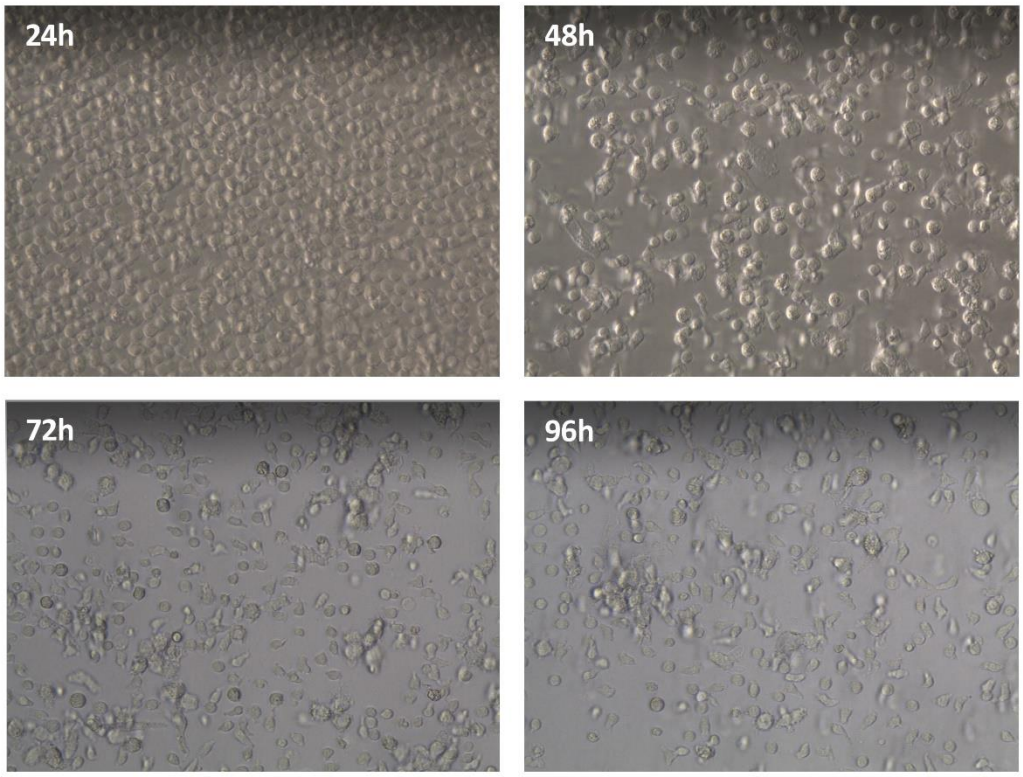
Figure 3.4.4 TNP, NP and KYN production with simultaneous ATS and IFN-γ treatment. Following an initial media-only day, PBMCs were treated with IFN-γ 500 U/mL alone after 24 hours and treated with 10 and 20 µM ATS and IFN-γ 500 U/mL simultaneously after 24 hours simultaneously. (**a**) Shows total neopterin (**b**) shows neopterin, and (**c**) shows kynurenine concentration for the same time period as measured by amine HPLC (µM/L). A multiple comparisons two-way ANOVA was conducted. Significance is indicated as compared to the control condition at the same time interval: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

3.4.5 Cell imagery and viability

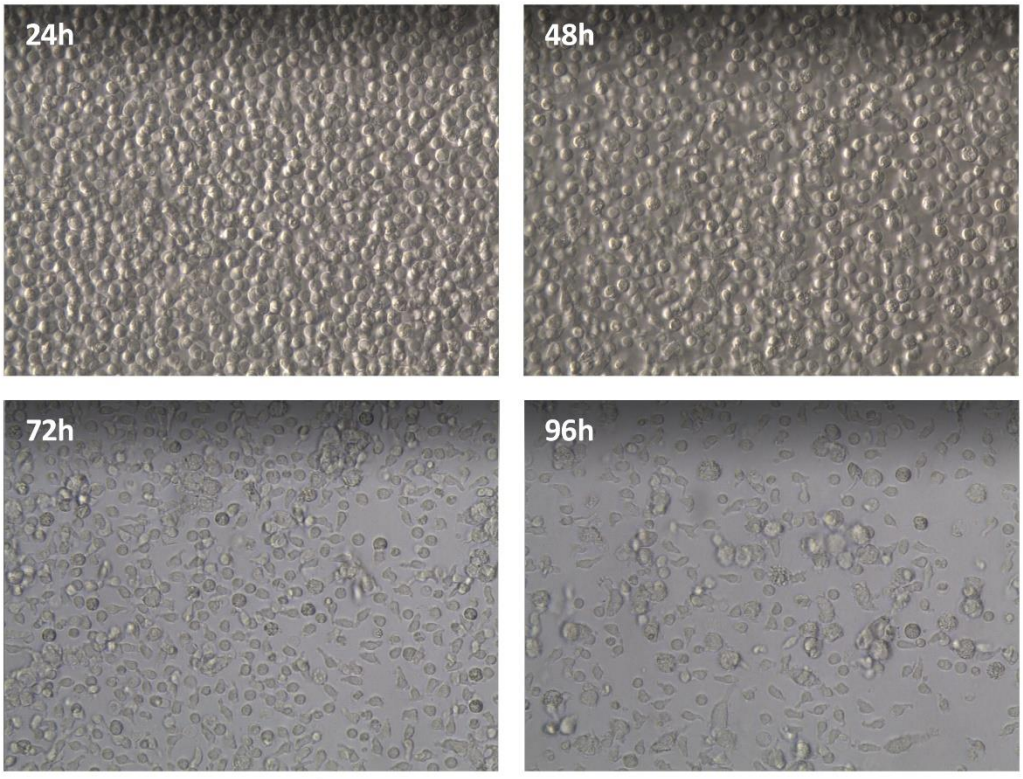
Depicted below (**Figure 3.4.5.1**), the 20 µM atorvastatin pre-treatment followed by two subsequent IFN-γ treatments (**d.**) shows no cellular swelling, indicating that the PBMCs tolerated the lower concentrations better than the higher concentrations 50 and 100 µM previously utilised. DMSO treatment followed by IFN-γ (**c.**) also appears tolerable over time, indicating that the solvent utilised at such a concentration is not having negative effects. The aforementioned conditions both appear similar to the control non-treatment (**a.**) and the single dose IFN-γ after 24 hours (**b.**) treatment. The similarity between different treatment images and lack of swelling, indicates that the PBMCs were tolerant of experimental conditions and of reasonable health. Overall, this preparation of PBMCs showed a lesser response to IFN-γ compared to the previous preparations

3.4.6 Protein determination

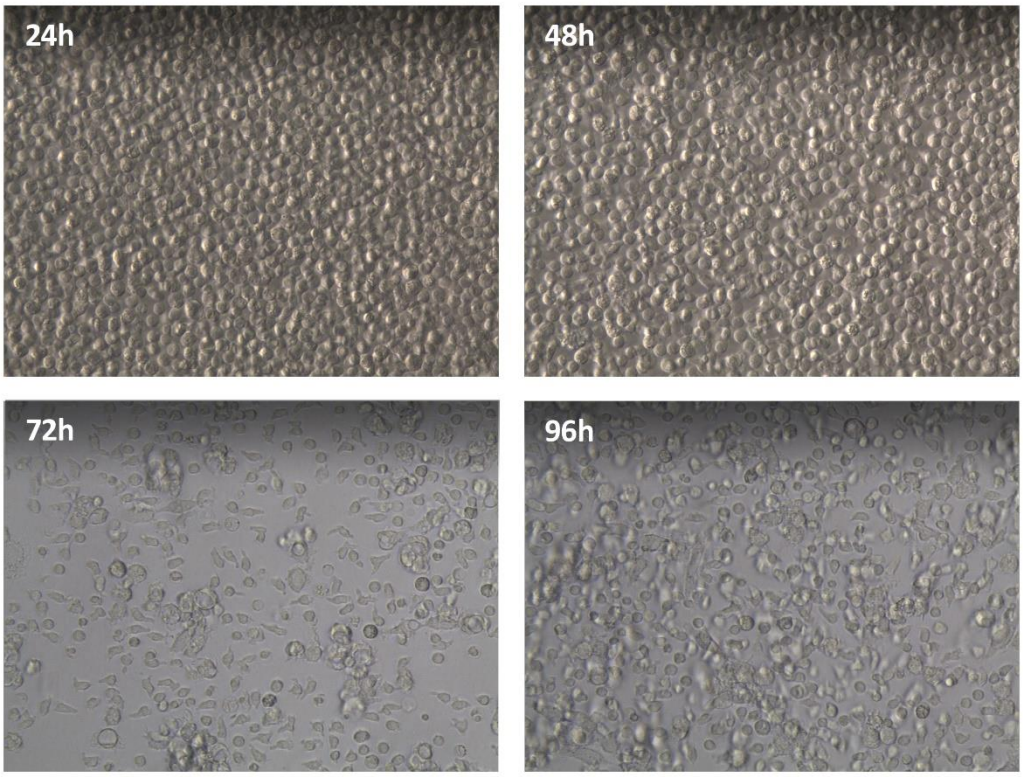
The BCA assay was conducted to examine cellular protein content and to serve as a means of estimating cellular concentration. An ordinary one-way ANOVA was conducted utilising multiple comparisons between the control and treatment conditions (**Figure 3.4.5.1**). The results of the analysis compared to the control (305.1 µg/mL), showed that there was one instance of statistically significant increase: DMSO pre-treatment followed by IFN- γ (532.6 µg/mL, $p = 0.036$). Experimental conditions showed varying levels of protein concentration. Each of the atorvastatin treatments produced a lower level of protein than the control (between 181.2 and 253.3 µg/mL), the lowest concentration being produced by the simultaneous 20 µM ATS and IFN-γ treatment. In contrast, each of the other treatment conditions which included a variation of DMSO and/or IFN-γ treatments produced a greater protein concentration than the control. These results agree with the results of the previous experiments.



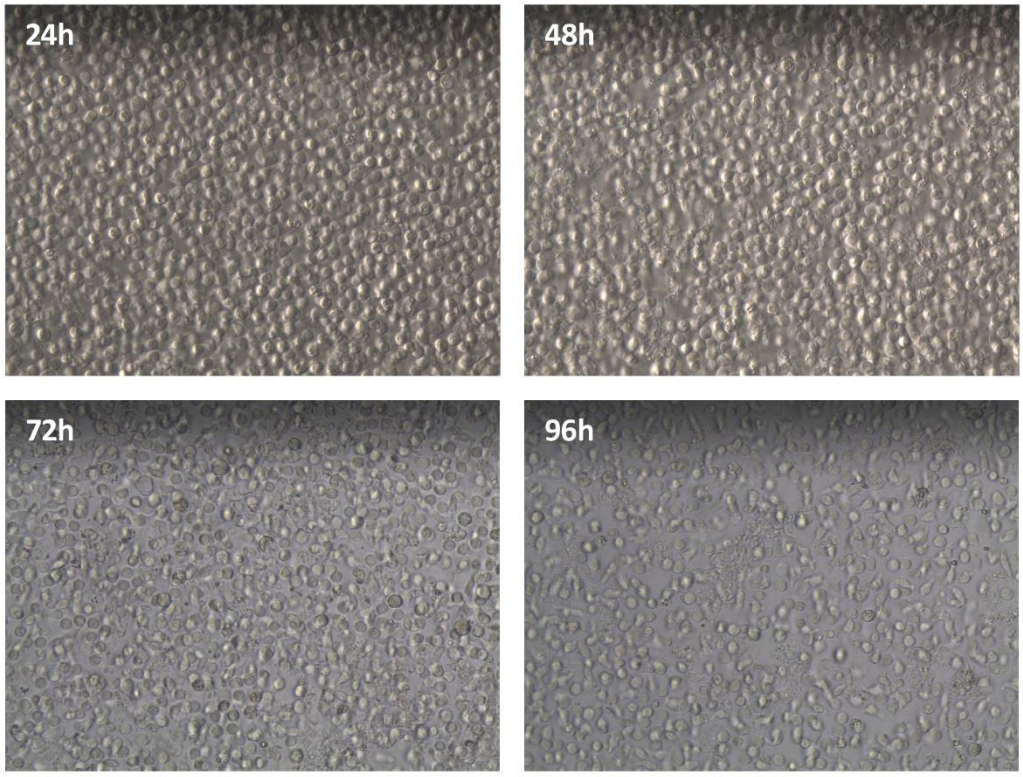
a.) Control - no treatment



b.) IFN-γ [500 U] at 24h



c.) DMSO 5 µL at 24h, IFN-γ [500U] at 48h



d.) ATS [20 µM] at 24h, IFN-γ [500 U] at 48h and IFN-γ [500 U] at 72h

Figure 3.4.5 Images depicting cells with further experimental conditions. PBMC images taken every 24-hours before media removal over 96-hour incubation with further treatment conditions. Images taken with LEICA DMIL microscope and LEICA DFC290.

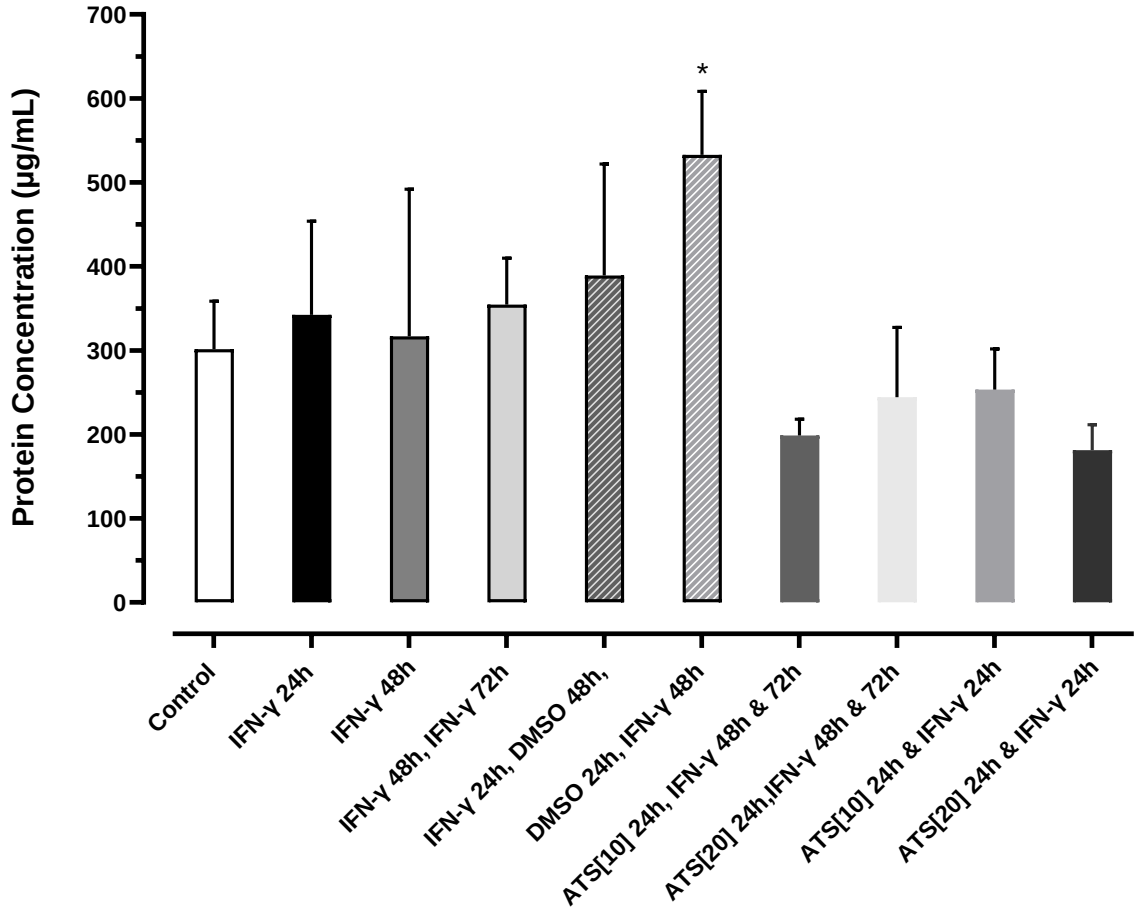


Figure 3.4.6 Protein concentration of cellular lysate from further conditions. Post 96-hour plate scraping sample of 50 µL, diluted 10:1, was added to 1 mL of BCA working solution, placed on a heat block at 60^{o}C and shaken for 30 minutes. Absorbance at 562 nm was determined by spectrophotometry and concentration derived from absorbance. An ordinary one-way multiple comparisons ANOVA was conducted. significance was indicated when comparing experimental conditions to the control: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

3.5 Further experimental conditions repeat

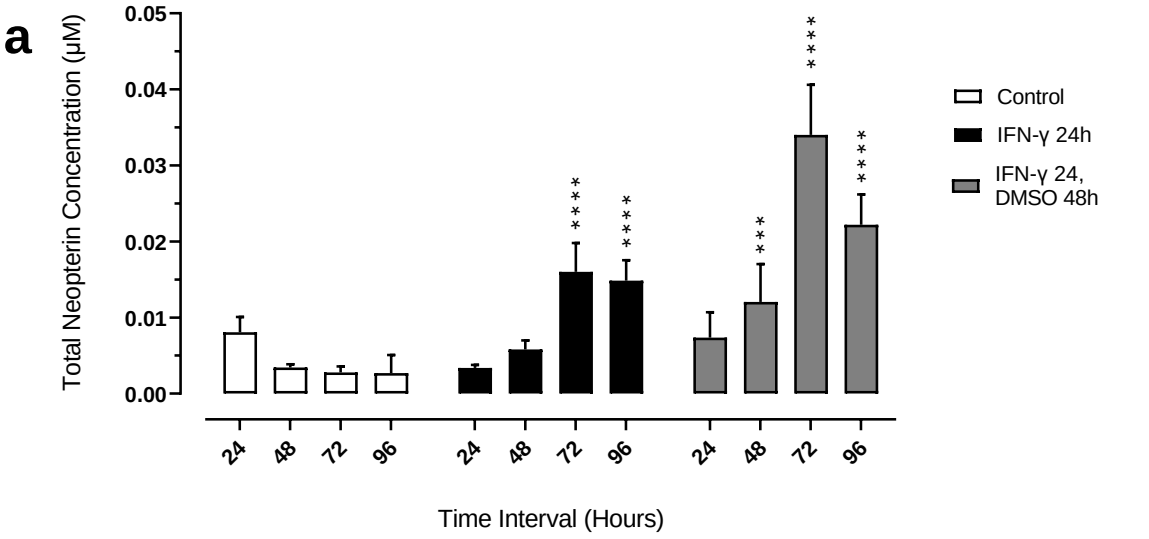
As with the initial conditions, it was decided to repeat the further conditions them to ensure that the results produced were representative of the PBMCs response to the experimental conditions. The following section describes results from experiments repeating the further experimental conditions with a different donation of whole blood and subsequently isolated and cultured batch of PBMCs.

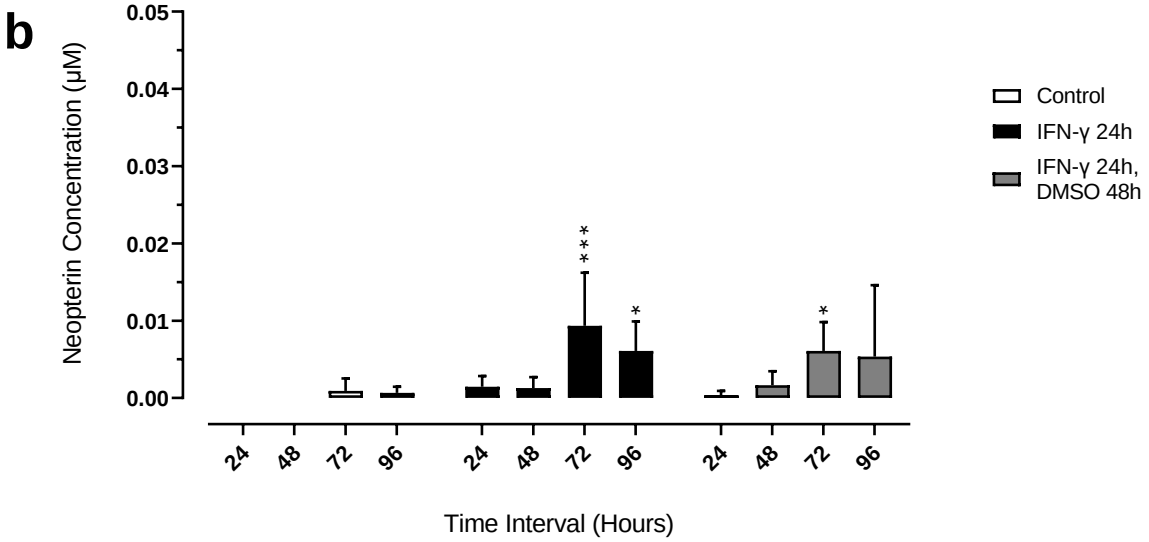
3.5.1 Characterising IFN-γ treatment followed by DMSO treatment

Repeating previous experimental conditions, PBMCs were treated with IFN-γ only after the initial media only 24-hours to establish a positive control for stimulation. In order to characterise the effect of IFN-γ followed by DMSO treatment, PBMCs were treated after 24 hours with IFN-γ, followed by 5 µL of DMSO after 48 hours (**Figure 3.5.1**).

The IFN-γ only control showed total neopterin rose over time reaching a significant concentration after 72 hours (0.016 µM, $p<0.0001$), decreasing slightly after 96 hours remaining significant (0.015 µM, $p<0.0001$). Neopterin production showed similar results, reaching a significant concentration after 72 hours (0.009 µM, $p<0.0002$), then decreased, however more so after 96 hours (0.006 µM, $p =0.0357$) to a less significant concentration. Kynurenine production started non-detectable, increasing to a significant level after treatment after 48 hours (5.4 µM, $p =0.0027$) increasing further after 72 hours (8.8 µM, $p<0.0001$) and decreasing to a non-significant concentration after 96 hours.

The IFN-γ followed by DMSO treatment produced a significant rise in total neopterin concentration after 48 hours (0.012 µM, $p<0.0001$), increasing further reaching its highest after 72 hours (0.032 µM, $p<0.0001$), followed by a decrease but remaining significant after 96 hours (0.022 µM, $p<0.0001$). Neopterin production rose overtime, reaching slight significance after 72 hours (0.006 µM, $p =0.05$) then decreasing to a non-significant concentration after 96 hours. Kynurenine production started non-detectable and rose significantly after 48 hours (8.7 µM, $p<0.0001$), decreasing but remaining significant after 72 hours (6.0 µM, $p =0.0006$) and decreasing to a non-significant concentration at 96 hours.


a
Total Neopterin Concentration (µM)
0.05
0.04
0.03
0.02
0.01
0.00
Control
IFN-γ 24h
IFN-γ 24, DMSO 48h
24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)


b
Neopterin Concentration (µM)
0.05
0.04
0.03
0.02
0.01
0.00
Control
IFN-γ 24h
IFN-γ 24h, DMSO 48h
24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)

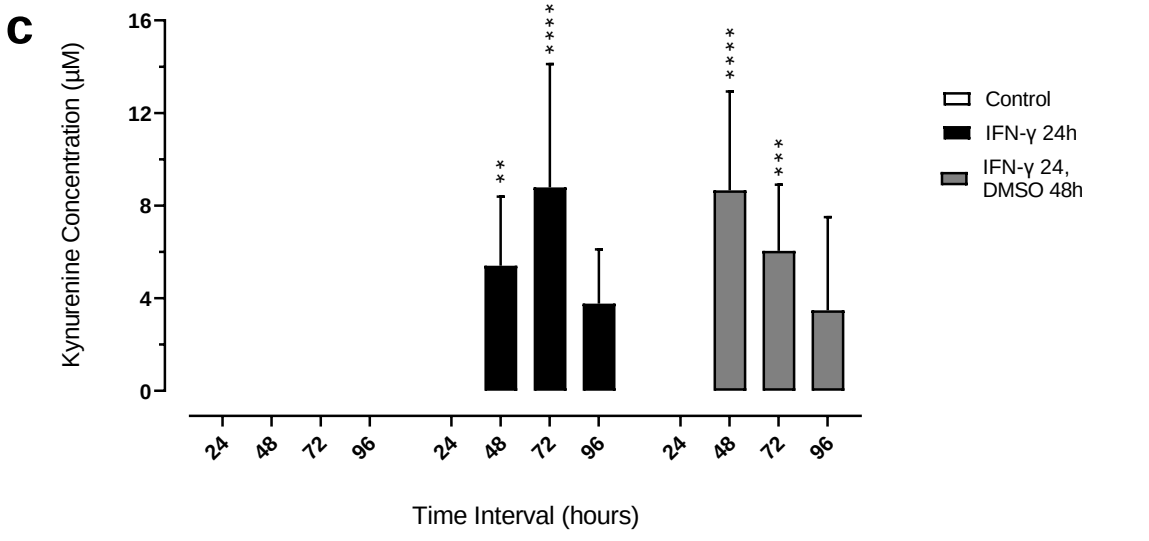

c
Kynurenine Concentration (µM)
16
12
8
4
0
Control
IFN-γ 24h
IFN-γ 24, DMSO 48h
24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (hours)

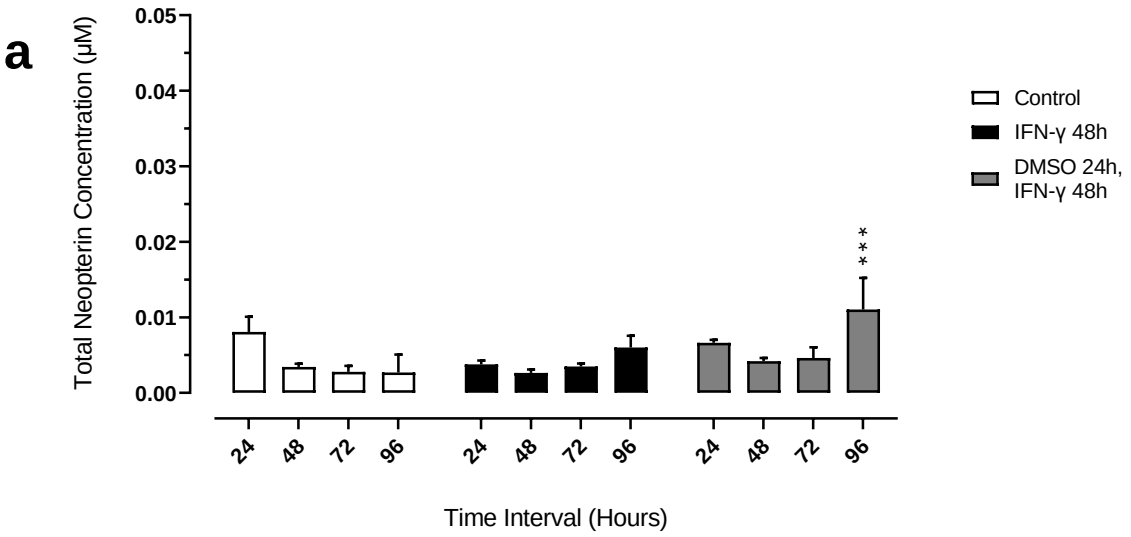
Figure 3.5.1 TNP, NP and KYN Production with IFN-γ, followed by DMSO treatment. Repeated experimental condition: Following an initial media-only day, cells were treated with IFN-γ 500 U/mL after 24 hours alone, and with IFN-γ 500 U/mL after 24 hours followed by 5 µL of DMSO after 48 hours. (**a**) Shows total neopterin, (**b**) shows neopterin, and (**c**) shows kynurenine concentration for the same time period, as measured by amine HPLC (µM/L). A multiple comparisons two-way ANOVA was conducted. Significance is indicated as compared to the control condition at the same time point (24-hour period): *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

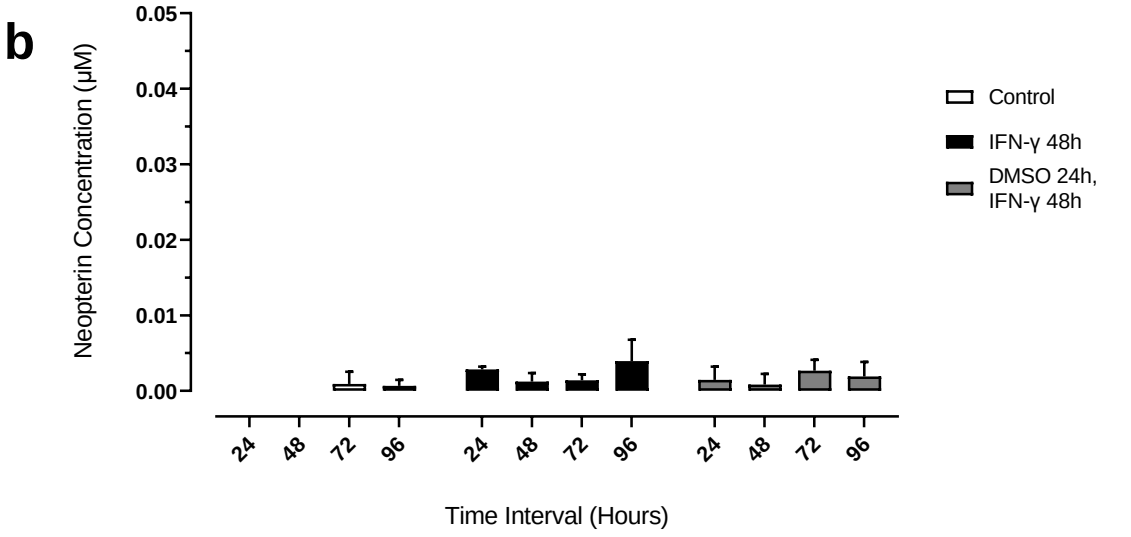
3.5.2 Characterising DMSO pre-treatment followed by IFN-γ treatment

Repeating previous experimental conditions, to test that DMSO was not responsible for the anti-inflammatory effects shown in the initial experiments. PBMCs were treated with IFN-γ only after the initial media only 24-hours to establish a positive control for stimulation. In order to characterise how DMSO may possibly effect IFN-γ activation followed by, simulating the previous conditions without atorvastatin. PBMCs were treated after 24 hours with 5 µL of DMSO followed by IFN-γ after 48 hours (**Figure 3.5.2**).

The positive control showed a rise over time for total neopterin, but not producing any significance, peaking after 96 hours (0.006 µM, $p =0.38$). Neopterin production remained low, not producing any significance over time, reaching its highest point after 96 hours. Kynurenine began low after 24 hours, decreasing to non-detectable after 48 hours then following IFN-γ treatment produced elevated levels of kynurenine after 72 and 96 hours, however no significance was produced. This is the only instance that IFN-γ has not produced a significant rise throughout my experimental results. These results show the difficulties of primary cell culture experiments and the patient, by extension PBMC preparation specificity of the results.

In contrast, the DMSO followed by IFN-γ produced an increased significant concentration of total neopterin after 96 hours (0.011 µM, $p =0.0002$). Neopterin production remained low, reaching its highest point after 72 hours, but no significance was produced. Kynurenine began non-detectable for the first 48 hours of treatment then following IFN-γ treatment at 48 hours, produced an elevated significant concentration after 72 hours (5.0 µM, $p =0.007$) , decreasing after 96 hours to a non-significant concentration.


a
Total Neopterin Concentration (µM)
0.05
0.04
0.03
0.02
0.01
0.00
***
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 48h
DMSO 24h,
IFN-γ 48h


b
Neopterin Concentration (µM)
0.05
0.04
0.03
0.02
0.01
0.00
24
48
72
96
Time Interval (Hours)
Control
IFN-γ 48h
DMSO 24h,
IFN-γ 48h

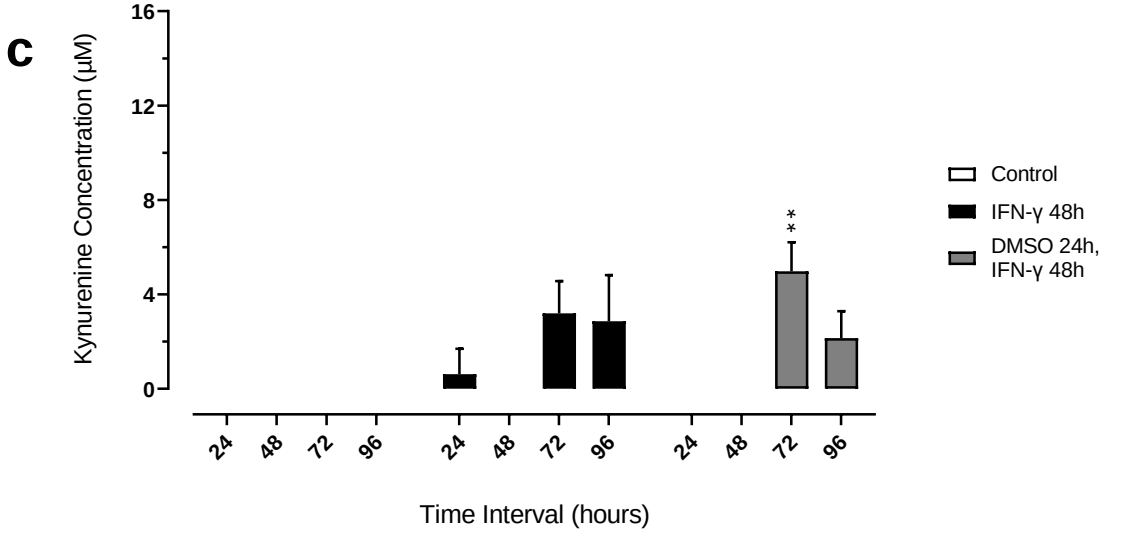

c
Kynurenine Concentration (µM)
16
12
8
4
0
**
24
48
72
96
Time Interval (hours)
Control
IFN-γ 48h
DMSO 24h,
IFN-γ 48h

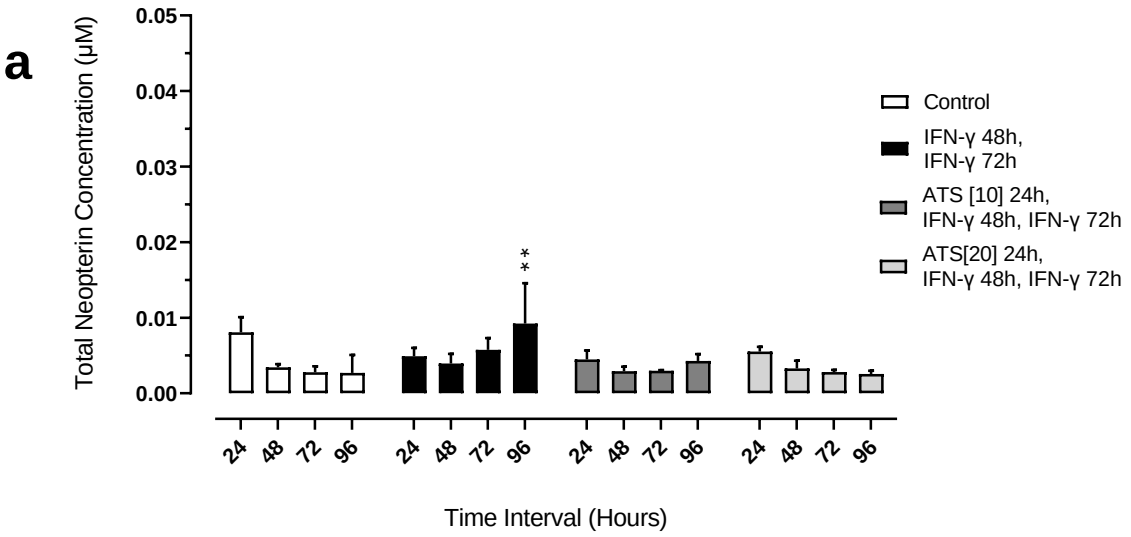
Figure 3.5.2 TNP, NP and KYN production pre-treated with DMSO, followed by IFN-γ. Repeated experimental condition: Following an initial media-only day, cells were treated with IFN-γ 500 U/mL after 48 hours alone, and with 5 µL of DMSO after 24 hours followed by IFN-γ 500 U/mL after 48 hours. (**a**) Shows total neopterin, (**b**) shows neopterin, and (**c**) shows kynurenine concentration for the same time period, as measured by amine HPLC (µM/L). A multiple comparisons two-way ANOVA was conducted. Significance is indicated as compared to the control condition at the same time point (24-hour period): *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

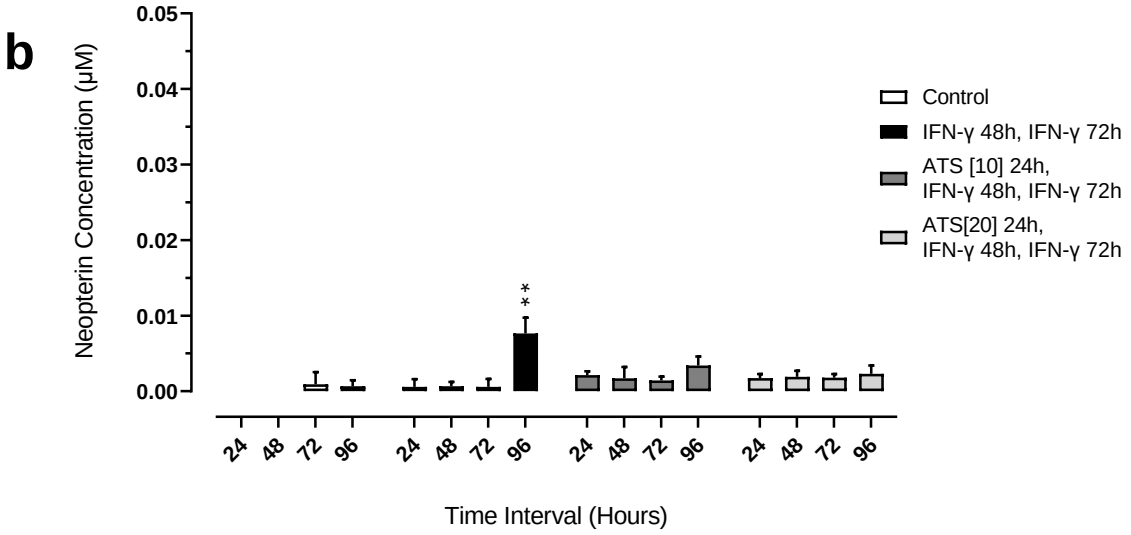
3.5.3 Characterising atorvastatin pre-treatment followed by two IFN-γ treatments

Repeating previous experimental conditions, PBMCs were pre-treated with 10 and 20 µM atorvastatin and treated with two subsequent doses of IFN-γ. This was achieved by treating PBMCs after 24 hours with 10 and 20 µM atorvastatin, followed by two subsequent treatments of IFN-γ after 48 and 72 hours. These results were displayed alongside a positive control treatment which treated PBMCs with IFN-γ after 48 and 72 hours.

The positive control showed low levels of total neopterin over the first 72 hours, then increased to a significant concentration after 96 hours (0.009 µM, $p =0.005$). Neopterin production behaved similarly, remaining low for the first 72 hours, increasing to a significant concentration after 72 hours (0.008 µM, $p =0.003$). Kynurenine production began low after 24 hours, decreasing to non-detectable levels after 48 hours. then following IFN-γ treatment at 48 hours, produced an elevated concentration after 72 hours, increasing further to a significant concentration after 96 hours (7.7 µM, $p<0.0001$).

The 10 and 20 µM pre-treatments with atorvastatin followed by IFN-γ treatments did not produce any significance with regard to total neopterin. Both concentrations remained low over time, with the 10 µM atorvastatin condition rising slightly after 96 hours in comparison to the 20 µM of, but not to a significant extent. Neopterin production behaved similarly, remaining low over time with 10 µM atorvastatin condition, rising slightly after 96 hours, but not to a significant extent. Kynurenine production began for 10 and 20 µ atorvastatin conditions non-detectable for the first 48 hours, then following IFN-γ treatment at 48 hours, produced detectable concentrations after 72 and 96 hours, 10 µM slightly higher than 20 µM atorvastatin condition, but not to a significant concentration.


a
Total Neopterin Concentration (µM)
0.05
0.04
0.03
0.02
0.01
0.00
**
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)
Control
IFN-γ 48h,
IFN-γ 72h
ATS [10] 24h,
IFN-γ 48h, IFN-γ 72h
ATS[20] 24h,
IFN-γ 48h, IFN-γ 72h


b
Neopterin Concentration (µM)
0.05
0.04
0.03
0.02
0.01
0.00
**
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)
Control
IFN-γ 48h, IFN-γ 72h
ATS [10] 24h,
IFN-γ 48h, IFN-γ 72h
ATS[20] 24h,
IFN-γ 48h, IFN-γ 72h

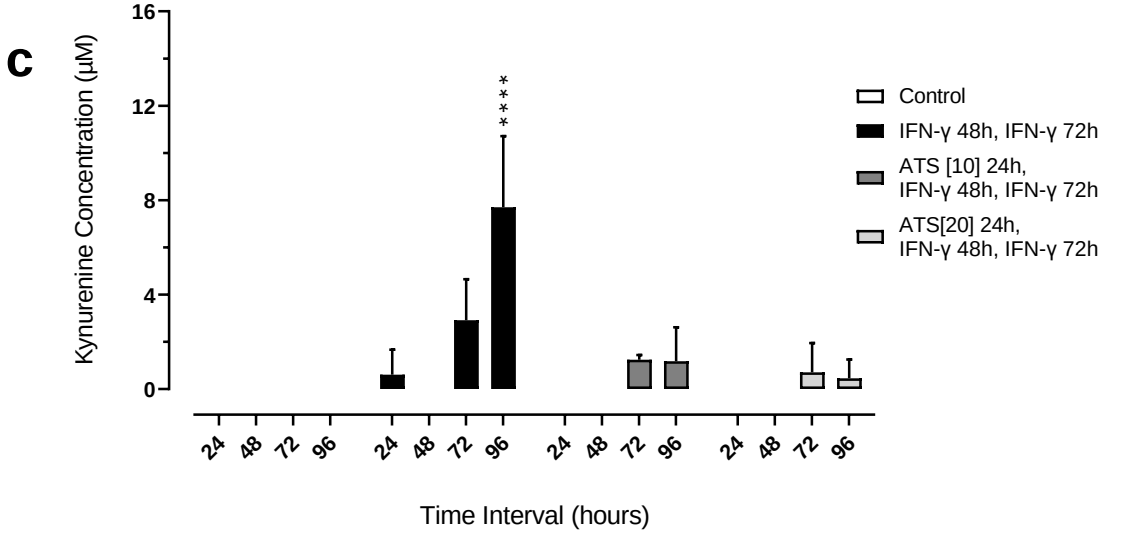

c
Kynurenine Concentration (µM)
16
12
8
4
0
****
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (hours)
Control
IFN-γ 48h, IFN-γ 72h
ATS [10] 24h,
IFN-γ 48h, IFN-γ 72h
ATS[20] 24h,
IFN-γ 48h, IFN-γ 72h

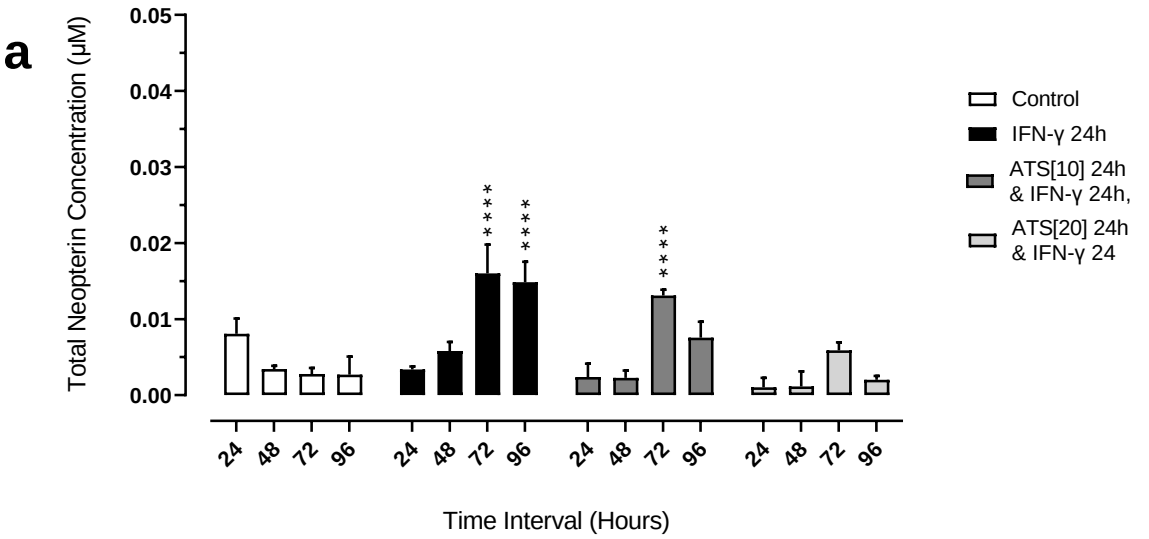
Figure 3.5.3 TNP, NP and KYN production with ATS pre-treatment, followed by subsequent IFN-γ treatments. Repeated experimental condition: Following an initial media-only day, PBMCs were treated with IFN-γ 500 U/mL after 48 hours and 72 hours alone and pre-treated with 10 and 20 μM ATS after 24 hours followed by IFN-γ 500 U/mL after 48 hours and 72 hours. (**a**) Shows total neopterin (**b**) shows neopterin, and (**c**) shows kynurenine concentration for the same time period as measured by amine HPLC (μM/L). A multiple comparisons two-way ANOVA was conducted. Significance is indicated as compared to the control condition at the same time point: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

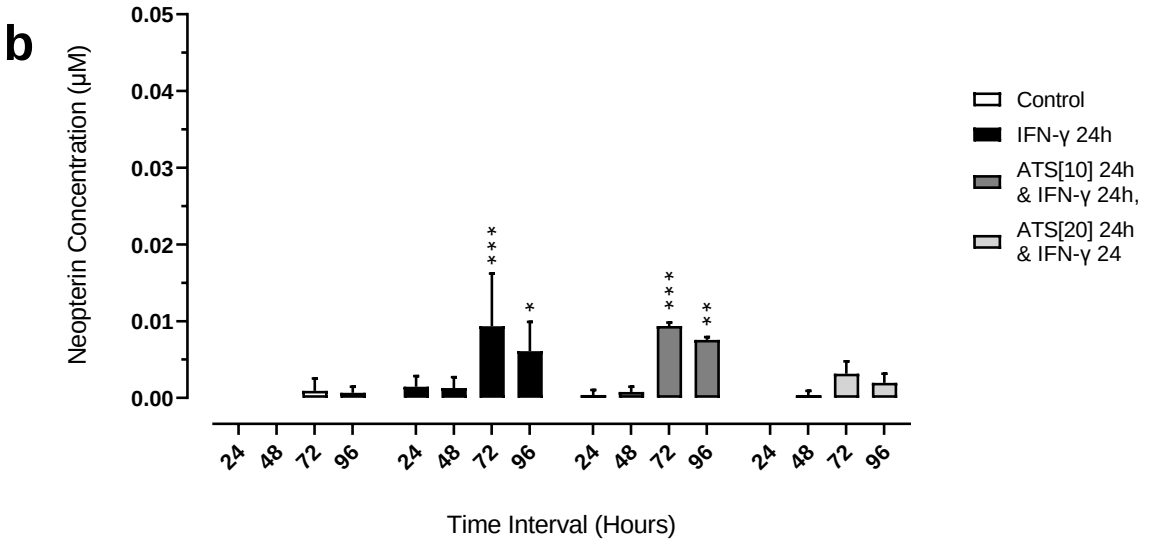
3.5.4 Characterising simultaneous atorvastatin and IFN-γ treatments

Repeating previous experimental conditions, PBMCs were treated simultaneously with 10 and 20 μM of atorvastatin and IFN-γ after the initial media-only day. This was displayed alongside the IFN-γ only after 24 hours positive control (**Figure 3.5.4**). The results for the positive control may be viewed above in **Section 3.5.1**.

The simultaneous 10 μM atorvastatin and IFN-γ condition's total neopterin production began low for the first 48 hours, rising reaching a significant concentration after 72 hours (0.013 μM, $p<0.0001$), dropping down to a non-significant concentration after 96 hours. Neopterin production began low over the first 48 hours, then increased to a significant concentration after 72 hours (0.009 μM, $p =0.0002$), decreasing after 96 hours (0.008 μM, $p =0.004$), remaining significant. Kynurenine production began non-detectable, increasing after treatment to a significant concentration at 48 hours (10.9 μM, $p<0.0001$), increasing slightly more after 72 hours (11.7 μM, $p<0.0001$), decreasing to slight significance to after 96 hours (4.1 μM, $p =0.042$).

The simultaneous 20 μM atorvastatin and IFN-γ condition's total neopterin production, similarly to 10 μM, began low for the first 48 hours, increasing after 72 hours, but in this case not to a significant concentration, then decreasing after 96 hours. Neopterin production began non-detectable, producing a low concentration after 48 hours, reaching its highest point after 72 hours but not significant, and decreasing after 96 hours. Kynurenine production began non-detectable, increasing after treatment at 48 hours, the to a significant concentration after 72 hours (6.2 μM, $p =0.0005$) , decreasing to a non-significant concentration after 96 hours.


a
Total Neopterin Concentration (µM)
0.05
0.04
0.03
0.02
0.01
0.00
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)
Control
IFN-γ 24h
ATS[10] 24h & IFN-γ 24h,
ATS[20] 24h & IFN-γ 24


b
Neopterin Concentration (µM)
0.05
0.04
0.03
0.02
0.01
0.00
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (Hours)
Control
IFN-γ 24h
ATS[10] 24h & IFN-γ 24h,
ATS[20] 24h & IFN-γ 24

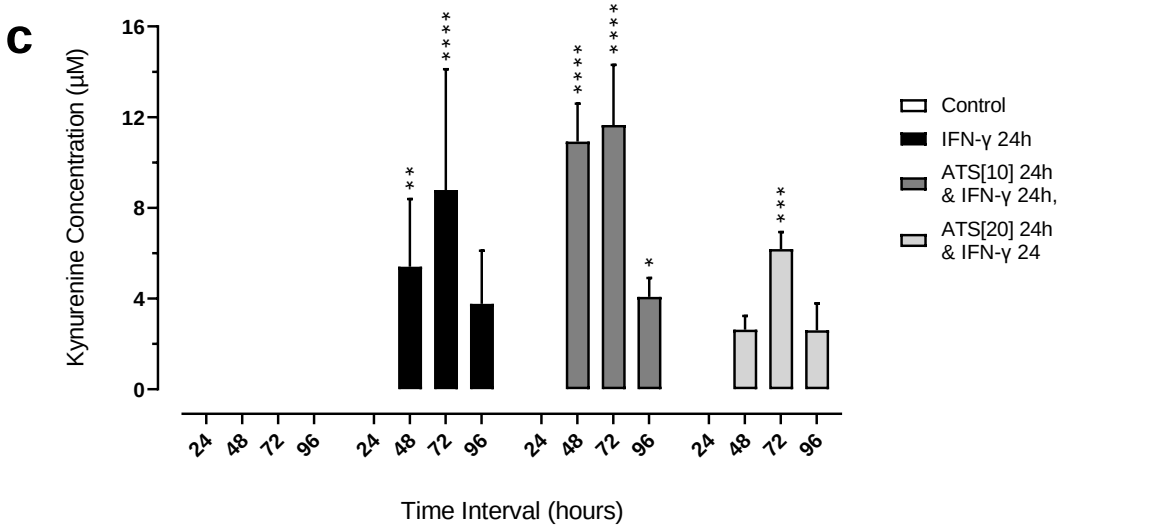

c
Kynurenine Concentration (µM)
16
12
8
4
0
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Time Interval (hours)
Control
IFN-γ 24h
ATS[10] 24h & IFN-γ 24h,
ATS[20] 24h & IFN-γ 24

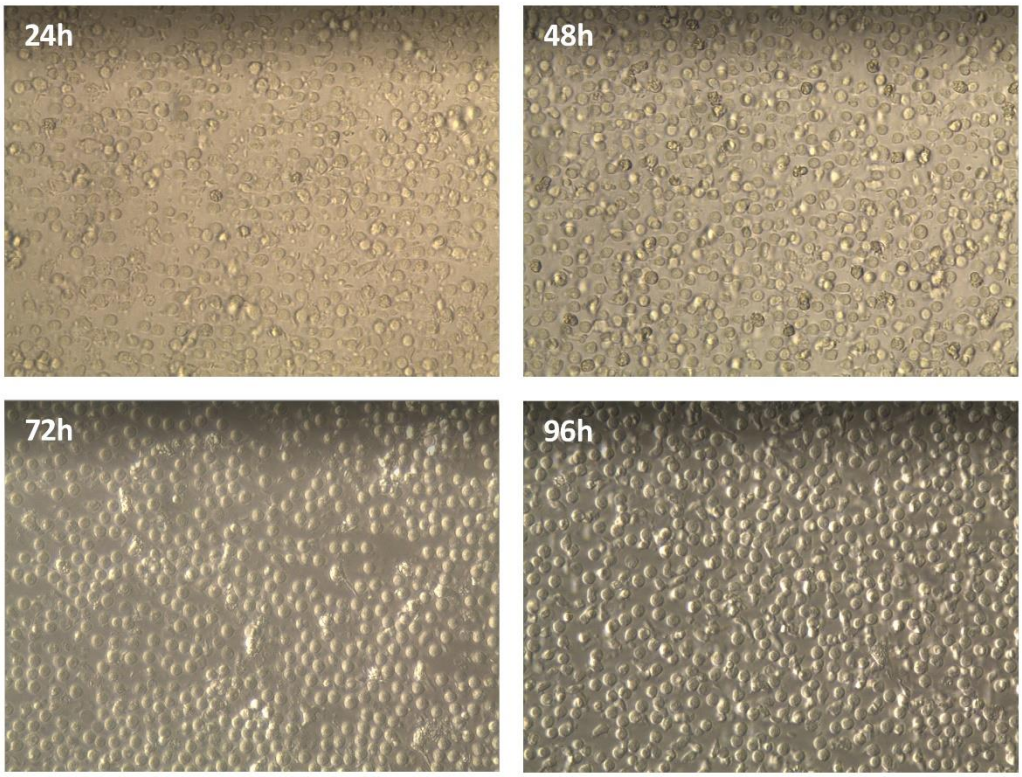
Figure 3.5.4 TNP, NP and KYN production with simultaneous ATS and IFN-γ treatment. Repeated experimental condition: Following an initial media-only day, PBMCs were treated with IFN-γ 500 U/mL alone after 24 hours and treated with 10 and 20 µM ATS and IFN-γ 500 U/mL simultaneously after 24 hours simultaneously. (**a**) Shows total neopterin (**b**) shows neopterin, and (**c**) shows kynurenine concentration for the same time period as measured by amine HPLC (µM/L). A multiple comparisons two-way ANOVA was conducted. Significance is indicated as compared to the control condition at the same time interval: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

3.5.5 Cell Imagery and viability

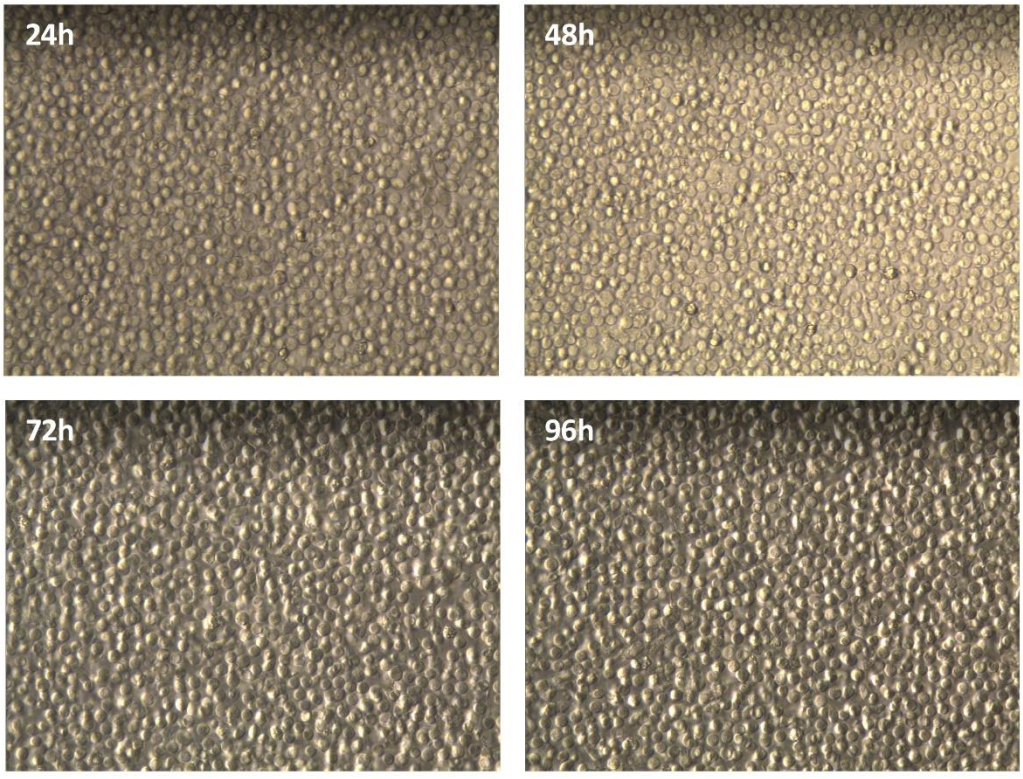
Similarly (**Figure 3.5.5.1**), to the previous experiment, the repeated 20 µM atorvastatin pre-treatment followed by subsequent IFN-γ treatments (**d.**) shows the cells were not swelling, as they did in the initial experimental conditions which utilised higher concentrations 50 and 100 µM of atorvastatin. The DMSO followed by IFN-γ treatment (**c.**) also appears tolerable over time. This agrees with the previous experiment's images. The aforementioned conditions also appear very similar to the control non-treatment (**a.**) and IFN-γ (**b.**) treatment. Again, no swelling visible, indicating that the PBMCs were tolerant of the experimental conditions and of a showed reasonable level of health

3.5.6 Protein Determination

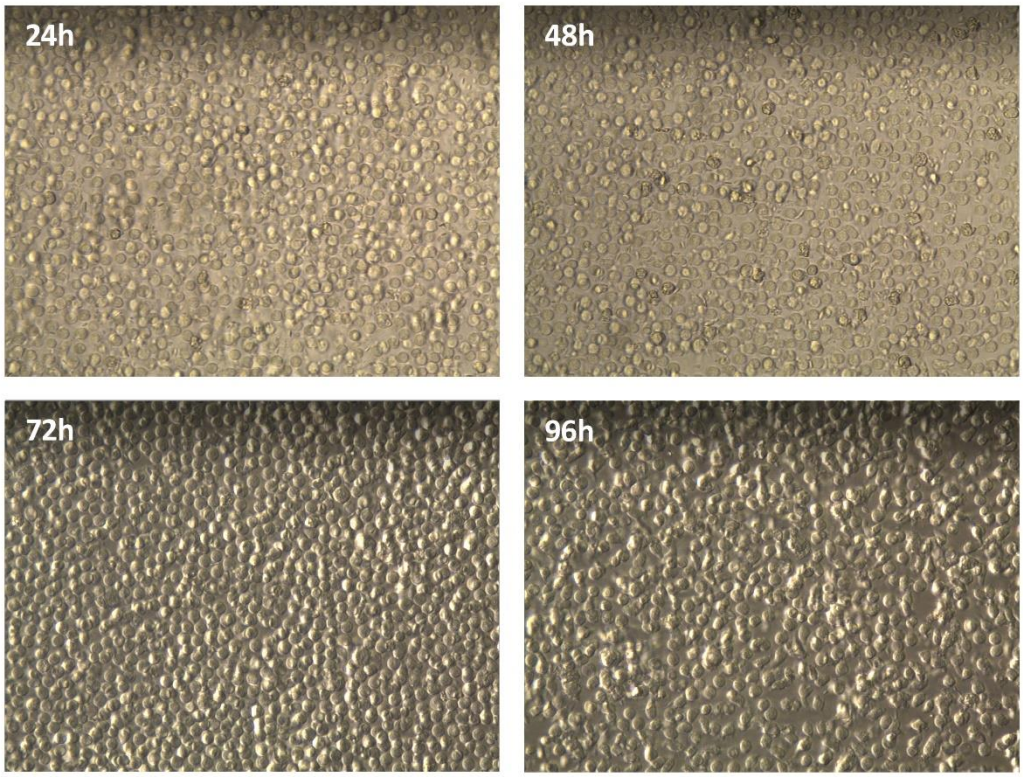
The BCA assay was conducted to determine cellular protein content and to serve as a means of estimating cellular concentration. An ordinary one-way ANOVA was conducted utilising multiple comparisons between the control and treatment conditions individually (**Figure 3.5.5.1**). Treatment conditions showed varying levels of protein concentration, however the results of the multiple comparisons analysis showed that there was not any statistically significant difference in concentrations produced compared to the control (155.7 µg/mL). The data for this batch of PBMCs shows the lowest protein concentration produced, with a mean of 225.4 µg/mL, compared to the others which ranged between 298.1 and 316.0 µg/mL. The protein concentration varied more so in this instance displaying a less clear distinction between those treated with atorvastatin and those not. The IFN-γ after 24h alone produced the greatest protein concentration (297.6 µg/mL), and the lowest was produced by the 20 µM atorvastatin pre-treatment (120.5 µg/mL). These results agree with all previous experiments.



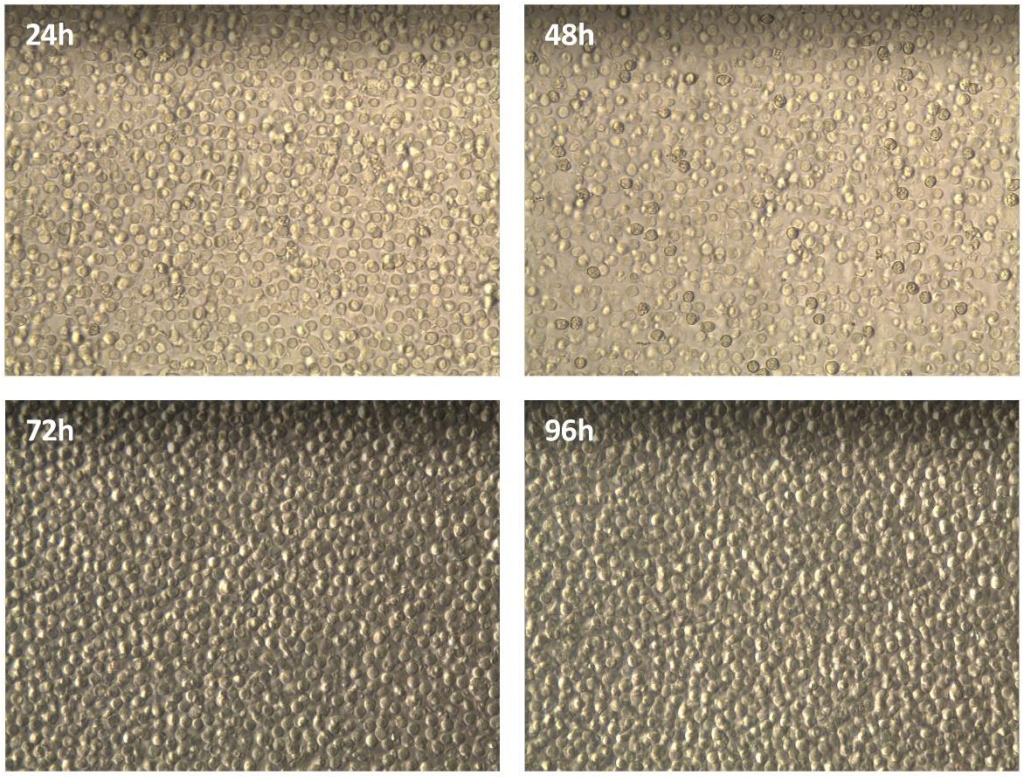
a.) Control - no treatment



b.) IFN-γ [500 U] at 24h



c.) DMSO 5 µL at 24h, IFN-γ [500U] at 48h



d.) ATS [20 µM] at 24h, IFN-γ [500 U] at 48h and IFN-γ [500 U] at 72h

Figure 3.5.5 Images depicting cells with repeated further experimental conditions. Images of PBMCs taken every 24 hours prior to media removal over 96-hour incubation for repeated further experimental conditions. Images taken with LEICA DMIL microscope using a LEICA DFC290 camera.

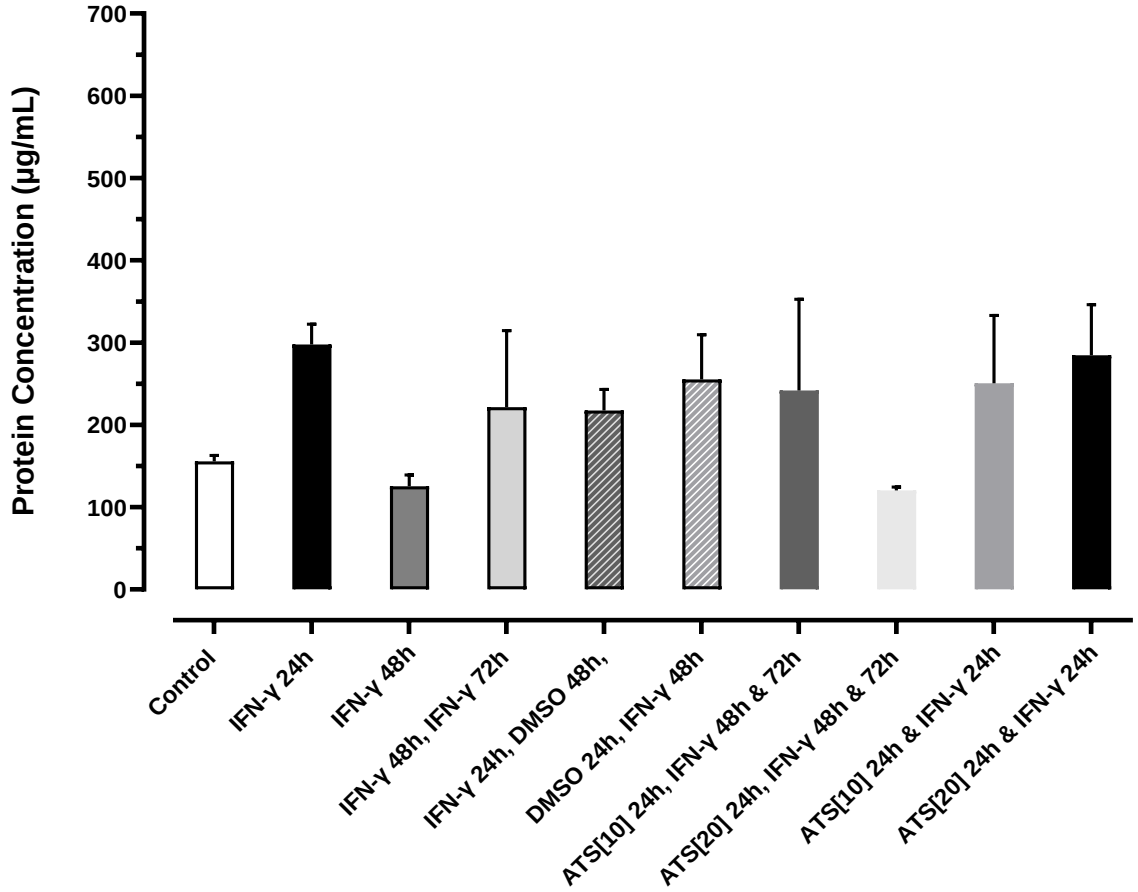


Figure 3.5.6 Protein concentration of cellular lysate from repeated further conditions. Repeated experimental condition: Post 96-hour plate scraping sample of 50 µL, diluted 10:1, was added to 1 mL of BCA working solution, placed on a heat block at 60ºC and shaken for 30 minutes. Absorbance at 562 nm was determined by spectrophotometry and concentration derived from absorbance. An ordinary one-way multiple comparisons ANOVA was conducted. No significance was indicated when comparing experimental conditions to the control.

3.6 Assessment of inflammation versus oxidation

The use of the total neopterin (7,8-dihydroneopterin + neopterin) over neopterin (TNP/NP) ratio for investigation into the dynamics between oxidative stress and immune system activation has been previously demonstrated by this research group (G. Baxter-Parker et al., 2019; Gregory Baxter-Parker et al., 2019). The ratio of total neopterin to neopterin may differ depending on the levels of immune activation and oxidation, i.e., a ratio closer to one indicates that there is a high level of immune system activation, generating ROS such as superoxide through the activity of NOX (Gieseg et al., 2018). In order to assess this for experimental work, where possible average total neopterin production was divided by the average neopterin production by PBMCs.

The results displayed show the TNP/NP ratio, graphed as individual lines for the 72-hour period after the initial media only day. This time period was determined due to in some instances non-detectable concentrations of neopterin prior to stimulation/treatment.

3.6.1 Initial experimental conditions

The ratio for the initial experimental conditions varied over time (**Figure 3.6.1**), ranging between 8.30 and 0.82 TNP/NP. After 96 hours at the end of the experimental treatment, There is a noticeable trend that the treatment conditions including atorvastatin were closer to one than the control or IFN-γ conditions. The lowest ratio, was produced by the 20 μM atorvastatin pre-treatment (0.82 TNP/NP), closely followed by the 10 μM atorvastatin pre-treatment (0.86 TNP/NP). This indicates the possibility of greater oxidative stress in these instances. The highest ratio indicating the least potential oxidative stress was produced by the IFN-γ after 24-hour treatment (8.3 TNP/NP). However, the three subsequent IFN-γ treatments condition (1.47 TNP/NP) produced a ratio much closer to one than the single treatment.

The subsequent repeat of the initial experimental conditions produced a lower overall level of neopterin production compared to total neopterin, with multiple instances of non-detectable neopterin levels. Consequently, ratios were unable to be produced for this PBMC preparation.

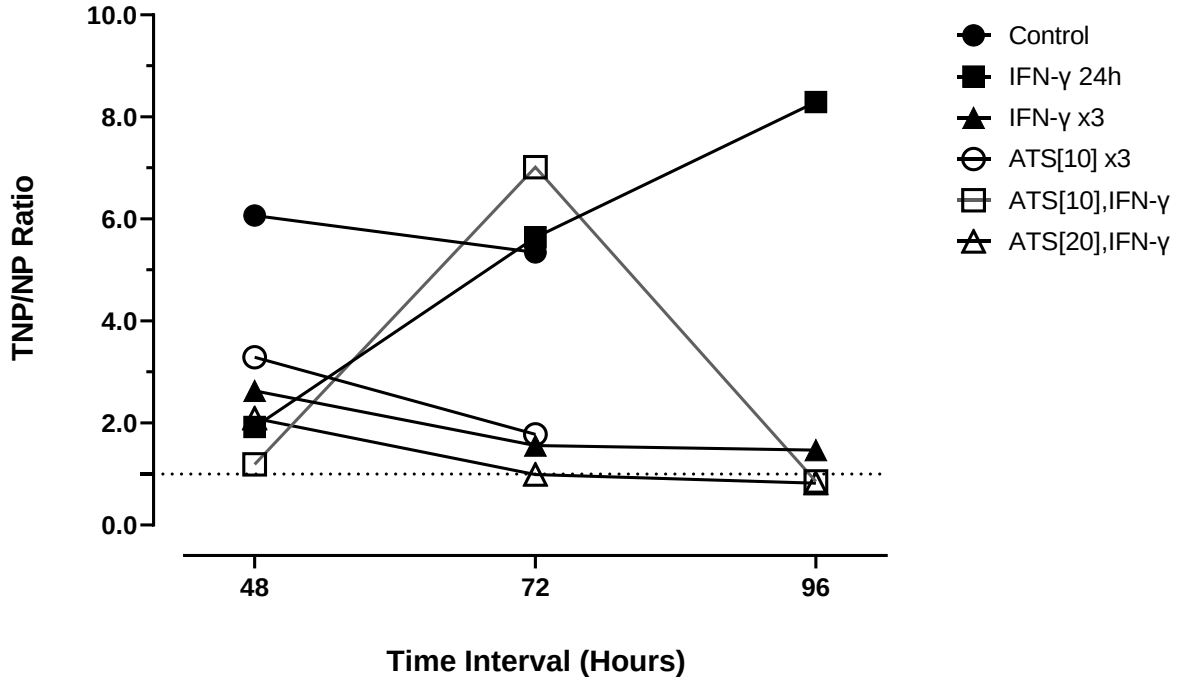


Figure 3.6.1 Initial treatment conditions TNP/NP ratio. Total neopterin and neopterin were measured in PBMCs over time with initial treatment conditions. TNP/NP ratios calculated by division of averages. Treatments involving ATS depicted in white, others depicted in black.

3.6.2 Further experimental conditions

The ratios produced by the further conditions varied less so (**Figure 3.6.2**); however, followed a similar treatment dependent trend to the initial conditions, ranging between 8.45 and 1.04 TNP/NP. After 96 hours at the end of the experimental treatment, the conditions produced a trend, in contrast less noticeable than the initial, in which the atorvastatin conditions produced a ratio closer to one than the IFN-γ and DMSO treatment conditions. In this instance, the control did not produce detectable concentrations of NP to utilise with TNP to produce a 96-hour ratio, indicating low oxidative stress. The lowest ratio was produced by the 10 µM atorvastatin pre-treatment (1.27 TNP/NP) after 96 hours, closely followed by the 20 µM atorvastatin pre-treatment (1.77 TNP/NP), again indicating the possibility of greater oxidative stress for the atorvastatin treatments. In this instance, the IFN-γ after 24 hours treatment (2.14 TNP/NP) produced a ratio lower than the initial, but still greater than the atorvastatin treatments. The highest ratio indicating the least potential oxidative stress was produced by the IFN-γ followed by DMSO treatment (4.79 TNP/NP). This result suggests that the DMSO solvent, is not causative regarding the lower ratio produced by the treatment conditions involving atorvastatin.

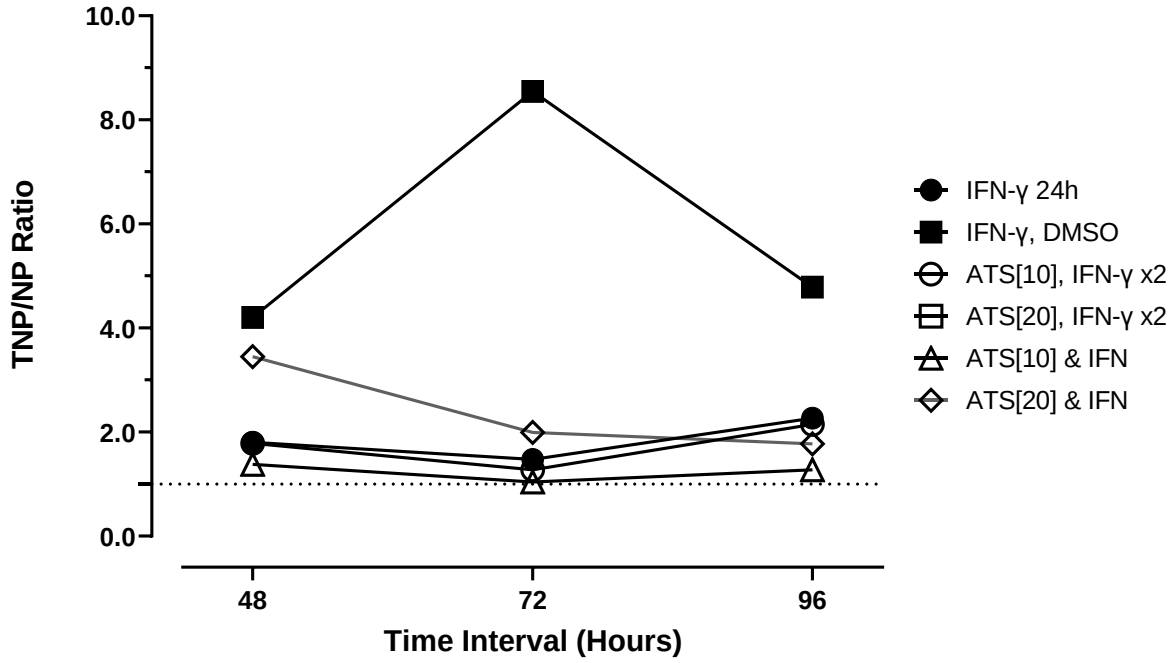


Figure 3.6.2 Further treatment conditions TNP/NP ratio. Total neopterin and neopterin were measured in PBMCs over time with further treatment conditions. TNP/NP ratios were calculated by division of averages. Treatments involving ATS depicted in white, others depicted in black.

3.6.3 Further experimental condition repeated

The ratios produced by the repeat were more varied than the previous batch (**Figure 3.6.3**), however, again followed to same treatment dependent trend as the initial and further conditions, ranging between 7.31 and 1.00 TNP/NP. In agreement with all previous experiments, after 96 hours at the end of experimental treatment, the conditions produced a trend in which the atorvastatin conditions each produced ratios closer to one, than the IFN-γ and DMSO treatment conditions. In this instance, the control produced a ratio of 3.99 TNP/NP after 96 hours, indicating comparatively low oxidative stress for no treatment received. The lowest ratio was produced by the simultaneous 10 µM atorvastatin and IFN-γ condition (1.00 TNP/NP) closely followed by the simultaneous 20 µM atorvastatin and IFN-γ condition (1.02 TNP/NP). These results again indicate the possibility of greater oxidative stress produced by the atorvastatin treatments. The highest ratio indicating the least potential oxidative stress was produced again by the IFN-γ followed by DMSO treatment (4.15 TNP/NP). This result again suggests that the DMSO solvent is not implicated in the lower ratio produced by the treatment conditions involving atorvastatin.

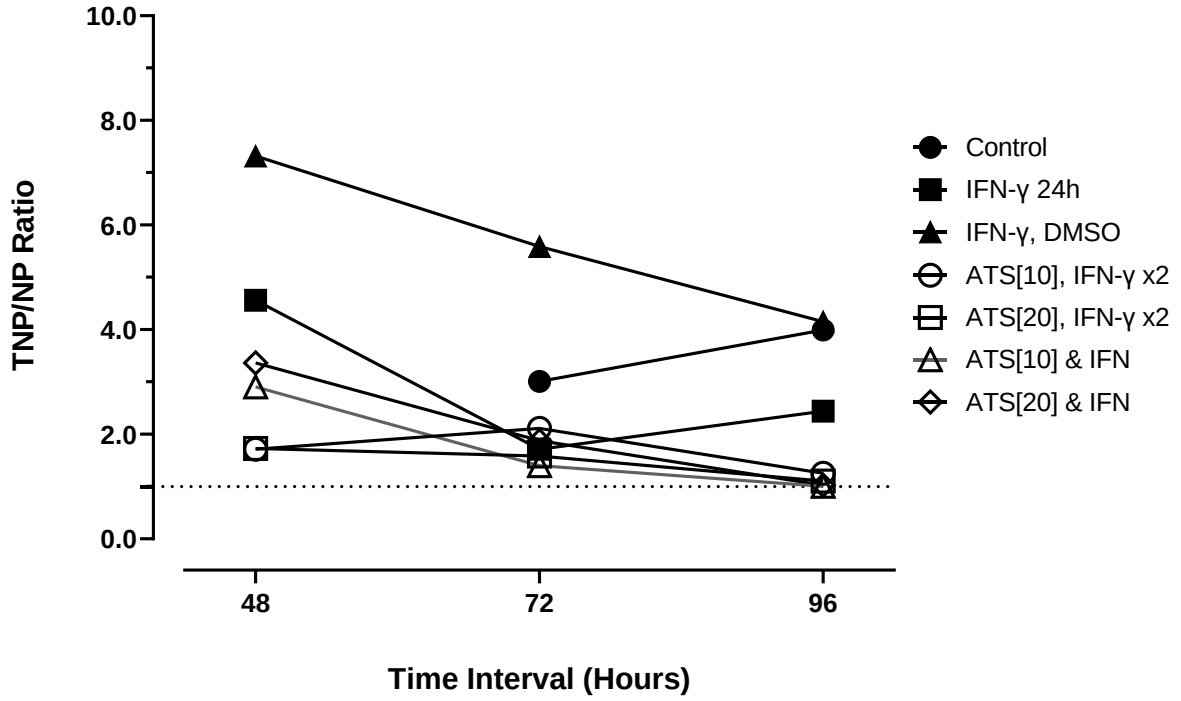


Figure 3.6.3 Repeated further treatment conditions TNP/NP ratio. Total neopterin and neopterin were measured in PBMCs over time with further treatment conditions repeated. TNP/NP ratios were calculated by division of averages. Treatments involving ATS depicted in white, others depicted in black.

Chapter 4

Discussion

4.1 Anti-inflammatory effects of atorvastatin on IFN-γ activation of PBMCs

The key aim of this research was to investigate the potential anti-inflammatory effect of atorvastatin treatment on immune cell activation by measuring 7,8-dihydroneopterin generation and oxidation. Peripheral blood mononuclear cells (PBMCs) were determined appropriate for this research, as this mixed model primary cell culture has been well characterised and previously demonstrated production of the inflammatory markers: 7,8-dihydroneopterin, neopterin, and kynurenine. Throughout this research, the 7,8-dihydroneopterin within a sample was oxidised and measured as total neopterin (TNP), in order to circumvent absorbance difficulties. HPLC analysis of experimental samples to determine the concentrations of respective inflammatory markers was utilised to measure macrophage activation in response to IFN-γ activation and atorvastatin treatments. This methodology allowed for simultaneous detection of neopterin and kynurenine within the same sample to high accuracy. The effect of experimental conditions on inflammation in this research were assessed by comparing the concentrations produced of respective biomarkers by each treatment condition to the no-treatment condition (control) within their respective PBMC preparations. This comparison was achieved by utilising a multiple comparisons two-way ANOVA.

Experiments conducted in this research clearly and consistently demonstrated that treatment of PBMCs with atorvastatin in varying concentrations and different combinations inhibited PBMC activation by IFN-γ with respect to the production of each of the inflammatory markers. This was shown by the absence of statistically significant increases in production compared to the no-treatment control conditions. In contrast, the IFN-γ only treatment conditions produced statistically significant increases in production of each of the inflammatory markers. These results are consistent with the amassing evidence throughout literature and research for statins possessing pleiotropic anti-inflammatory effects beyond their lipid lowering capacity.

Throughout experimental work TNP production was consistently inhibited by atorvastatin treatment. Pre-treatment with atorvastatin at varying concentrations, as low as 10 μM of atorvastatin, resulting in notable decreases in TNP concentration. This inhibitory effect was shown to extend over multiple IFN-γ treatments following pre-treatment. Simultaneous

treatment of PBMCs with atorvastatin and IFN-γ produced a less dramatic, but still notable inhibition of TNP production, the extent of which increased with atorvastatin concentration. Treatment with IFN-γ initially followed by atorvastatin produced similar inhibitory results, however also not as dramatic as the pre-treatment conditions. Atorvastatin treatment only of PBMCs consistently over time also showed no rises in TNP production. The results all showing of inhibition of TNP and by extension 7,8-dihydroneopterin production to various extents are in agreement with previous unpublished findings from this research group that atorvastatin inhibited 7,8-dihydroneopterin production in PBMCs. These results also support this research's hypothesis that immune cell activation and the subsequent release of 7,8-dihydroneopterin is inhibited by statin treatment. Presently, there exists a gap in literature and research published regarding the effect of statins on the production of 7,8-dihydroneopterin, therefore there is no comparison, however these findings will help to fill this gap in literature.

Neopterin production was inhibited similarly to TNP over each respective atorvastatin containing experimental treatment condition. Once more, concentrations as low as 10 µM of atorvastatin resulted in decreased neopterin production for each of the experimental conditions. The production of neopterin was entirely inhibited by conditions containing the higher statin concentrations, including 50 and 100 µM. The results from these experiments regarding neopterin production are in direct concurrence with previously published findings by Neurauter et al., showing partial inhibition of IFN-γ (100 U/mL) activated neopterin production in PBMCs with 10 µM of atorvastatin treatment and complete inhibition with 100 µM atorvastatin treatment. Findings from this research also demonstrated that 50 µM of atorvastatin treatment is capable of producing a comparable inhibitory effect previously shown by 100 µM. Further, this research employed a concentration of IFN-γ 5-times greater (500 U/mL) than previously by Neurauter et al., demonstrating a comparable inhibitory effect on a considerably higher level of IFN-γ activation (Neurauter et al., 2003). The results from this research are also in agreement with previous findings which associate inhibitors of HMG-CoA reductase (statins) with decreased serum neopterin levels in stable coronary artery patients (Roland B. Walter, Dietmar Fuchs, Günter Weiss, Thomas R. Walter, & Walter H. Reinhart, 2003).

Each respective atorvastatin containing treatment condition inhibited kynurenine production in addition to TNP and neopterin. The results regarding kynurenine were less consistent throughout the PBMC preparations, in some instances beginning elevated prior to treatment and some comparatively not so. However, decreases in kynurenine production were observed consistently with atorvastatin treatment, regardless of the measured initial concentration. The

response to IFN-γ activation by kynurenine appeared more rapid than TNP and neopterin, typically rising in the 24-hours following treatment. An instance where kynurenine production differed to the other markers was the simultaneous atorvastatin and IFN-γ treatment, where kynurenine production appeared to be less inhibited than TNP and neopterin, particularly with 10 μM. They are produced by different enzymes and processes so this is not unexpected; however, it was unique within these experimental results. Overall, results regarding kynurenine production are aligned with previous findings, showing diminished production by demonstrating a decrease in the ratio of kynurenine to tryptophan after 10 μM treatment with atorvastatin and IFN-γ, decreasing further with 100 μM, but not in a concentration dependent manner (Neurauter et al., 2003). These results also coincide with findings showing both serum kynurenine and the Kyn/Trp ratio were significantly decreased after cholesterol lowering treatment (simvastatin), to values comparable with healthy control in chronic kidney disease patients (Zinellu et al., 2015).

It remains unclear mechanistically how atorvastatin inhibits IFN-γ activation of PBMCs with respect to 7,8-dihydroneopterin production. It is well documented that protein prenylation affects many significant intracellular signalling pathways, including those which are pro-inflammatory and pro-atherogenic (Wolfrum, Jensen Kristin, & Liao James, 2003). By inhibiting HMGR, it the reduces the production and availability of isoprenoid intermediates, resulting in less potential for signal transduction. Previous *in vitro* studies demonstrate that cytokine-mediated upregulation of GTP-cyclohydrolase-1 (GTPCH-1) requires the coordinated activation of NF-κB and the Jak2/Stat pathway. The cytokine IFN-γ and TNF-α have a synergistic impact on the induction of GTPCH-1. This synergistic effect also extends to the kynurenine producing enzyme, indolamine 2,3-dioxygenase (IDO), which is optimally upregulated in the presence of both IFN-γ and TNF-α (Huang, Zhang, Chen, Hatakeyama, & Keaney John, 2005; Robinson, Shirey, & Carlin, 2003). Statins have been shown to limit the nuclear accumulation of NF-κB. This is thought to occur by increased expression of its inhibitor, I-κB, which is phosphorylated in response to inflammatory stimuli, releasing NF-κB to translocate to the nucleus (Hayden & Ghosh, 2008; Jain & Ridker, 2005). Findings have also shown that *in vitro* treatment of macrophage and resident CNS antigen presenting cells with lovastatin prevented the production of TNF-α and IL-1β (Greenwood et al., 2006). It is plausible that atorvastatin treatment resulted a reduction in various nuclear factors, such as NF-κB and the availability of isoprenoids in the PBMCs. As a result, the combination of decreases

in various signalling and activating factors results in a significant downregulatory effect for GTPCH-1, thus inhibiting 7,8-dihydroneopterin's production.

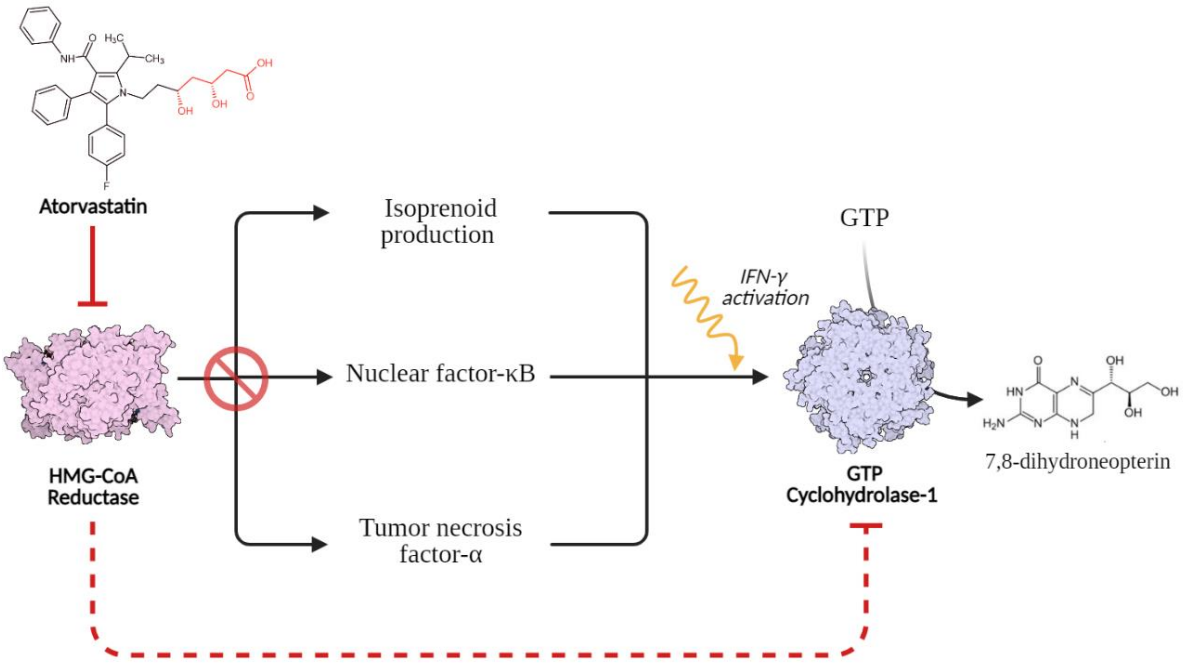


Figure 4.1 Possible mechanism of GTPCH-1 inhibition by atorvastatin. Combinatory effect of atorvastatin on various nuclear factors and isoprenoid availability, known to modulate GTPCH-1, may contribute its indirect inhibition, resulting in decreased production of the inflammatory marker 7,8-dihydroneopterin.

In this research, the results produced from atorvastatin pre-treatment experimental conditions are most similar to simulating what would be occurring *in vivo*. The majority of scenarios in which statins may affect measured clinical biomarkers, such as TNP or neopterin, an individual would already be prescribed and taking statins as primary or secondary preventative strategies to manage CV-risk (Stone et al., 2014). Therefore, the statin would already be in systemic circulation, having also distributed into the body's various tissues and built up to biologically relevant concentrations. In regard to developing TNP as a potential clinical diagnostic marker for monitoring patient's cardiovascular health, the pre-treatment conditions offer the most pragmatic insights. In this research the pre-treatment conditions have provided valuable insights into how the production of inflammatory markers may be affected by atorvastatin

treatment. Therefore, they would be suitable and appropriate and for future studies of statin treatment effect on PBMCs.

In previously published cell culture experiments, 10 µM was shown as the lowest atorvastatin concentration capable of producing significant pleiotropic effects (Youssef et al., 2002). This directly aligns with results from this research, showing decreased inflammatory marker production with 10 µM of atorvastatin. In human serum, the mean concentration of atorvastatin has been demonstrated to be 15 nM for doses of 20 mg (Stern et al., 2000). These results display a large difference, of 667-fold, between cell culture and measured serum concentrations of atorvastatin. This difference also extends to liver tissue, where statins typically accumulate, in which the concentration was only two-fold greater than in serum (Thelen et al., 2006). This presents a notable limitation for cell culturing with atorvastatin. Presently the concentrations of statins in endothelial, blood and immunologically active cells remain unknown (Björkhem-Bergman, Lindh, & Bergman, 2011). During the development of atherosclerosis, macrophages accumulate within the intimal space. In contrast to primary cell culture, *in vivo* mature macrophages have a very long lifespans, ranging from months to years (van Furth & Cohn, 1968). Statin drugs all possess hydrophobic character, providing a potential mechanism by which the molecules in circulation may pass through the membranes of such cells and accumulate producing a concentrating effect. As a result, the 20 mg of atorvastatin as a daily dose may produce a similar effect to 10 µM in cell culture, with sustained treatment over a longer time period. Previous findings from this research group observed a depression in TNP production in live excised carotid plaque samples from patients prescribed statins (Prebble, 2018). While there exists a disconnect between cell culture and physiological statin concentration, the depressive effect on TNP production exists and was demonstrated in both live excised plaque samples and PBMCs. As the concentrations of statins in cells which plaque tissue is proportionally rich in (endothelial, blood and immunological cells) remain unclear it is not possible to ascertain the extent of difference within plaque tissue and serum. The disconnect could be partially resolved, by establishing the effect of a range of atorvastatin concentrations on TNP production in both PBMCs and live excised plaque tissue and comparing the two.

4.2 DMSO solvent effect on IFN-γ activation of PBMCs

Experiments conducted in this research demonstrated that dimethyl-sulphoxide (DMSO) does not produce an anti-inflammatory effect on IFN-γ activation of PBMCs. Treatment conditions involving DMSO and IFN-γ in each instance produced a statistically significant increases in total neopterin concentration. Further, in some instances DMSO containing treatments actually produced more TNP than the IFN-γ only conditions, however this is likely due to variation among PBMCs within and between preparations. Based on these results showing DMSO did not greatly affect total neopterin production, it can be concluded that any anti-inflammatory effect regarding TNP, and by extension 7,8-dihydroneopterin, was produced by the atorvastatin suspended in DMSO, can be attributed to atorvastatin and not the required DMSO solvent.

Results showed that in treatment conditions involving DMSO and IFN-γ treatment neopterin production was diminished compared to the IFN-γ only control. DMSO [$(CH_3)_2SO$] is a amphipathic molecule with a highly polar domain and two apolar groups making it soluble in both aqueous and organic media, making it a very effective solvent for water insoluble compounds (Santos, Figueira-Coelho, Martins-Silva, & Saldanha, 2003). DMSO is known to be an effective hydroxyl radical scavenger (Ashwood-Smith, 1967; Del Maestro, Thaw, Björk, Planker, & Arfors, 1980; Jacob & Herschler, 1986). As such, DMSO's anti-inflammatory and reactive oxygen species scavenging abilities have resulted in its approval for and utilisation as a human therapeutic agent, such as in the management of several gastrointestinal diseases (Salim, 1991, 1992). The results of DMSO and INF-γ treatment producing a comparative reduction in neopterin concentrations are therefore likely a consequence of the documented effectiveness of DMSO at scavenging radicals. Consequently, a greater proportion of the pterins produced by PBMCs remained as 7,8-dihydroneopterin, as seen in the statistically significant production of TNP in these instances.

Kynurenine release was less consistent, with one PBMC preparation producing statistically significant increases in kynurenine, greater than INF-γ only treatments in each instance. In contrast, the other PBMC preparation did not produce statistically significant increases in kynurenine, appearing somewhat diminished. These inconsistencies may be attributed to variation among the PBMC preparations. Each respective preparation is assumed to come from a different donor, which may possess varying levels of IDO expression and or bioavailable tryptophan affecting its activity. The statistically significant increases in production of kynurenine achieved suggest that DMSO is not affecting kynurenine production.

In future experiments involving drugs such as atorvastatin, requiring organic solvents like DMSO, it would be advantageous to add equal concentrations of DMSO into each experimental condition. For example, if at least 0.5% of the overall cell media volume was DMSO in each experimental condition, it would have the equivalent of solvent required for the 10 µM atorvastatin concentration factored in. By including DMSO in every experimental condition in some manner, it would remove the need for a DMSO-control experiment as every experimental condition has it built in. This would also prevent potential inconsistencies between PBMC preparations and allow for more accurate characterisation of DMSO's effect in future statin experiments.

4.3 Assessment of inflammation versus oxidation using TNP/NP Ratio

Previous studies investigating the effect of statins on the production of neopterin, in both serum and cells, have neglected to examine 7,8-dihydroneopterin release in tandem or separately. This disregards the basic biology that activated macrophages do not produce neopterin in response to IFN-γ activation. Rather the activated immune cells produce 7,8-dihydroneopterin, which is then converted to neopterin upon reacting with oxidants and activated immune cells (Gieseg et al., 2018). By implementing the ratio of total neopterin over neopterin (TNP/NP) with regard to atorvastatin treatment, it was possible to assess the level of immune cell activation and oxidative stress. The TNP/NP ratio may vary depending on the respective levels of immune activation and oxidative stress. A ratio close to one indicates that the majority of 7,8-dihydroneopterin is being converted to neopterin, indicating comparable levels of immune activation and oxidative stress, whereas a greater ratio indicates a high level of immune activation compared to oxidative stress (Gieseg et al., 2018).

Within their respective PBMC preparations, the TNP/NP ratios produced by treatment conditions including atorvastatin produced the lowest ratios, closest to one. This suggests that atorvastatin treatment resulted in a greater amount of oxidative stress. In contrast, the TNP/NP ratios produced by other conditions, including IFN-γ and DMSO (**Figure 3.6.2-3)**, each produced higher ratios than those including atorvastatin. Besides one instance, only treatment conditions not including atorvastatin produced statistically significant concentrations of neopterin compared to the control. However, in these instances the ratio produced was greater as the production of TNP was also higher, reaching statistical significance, likely due to the absence of atorvastatin's anti-inflammatory effects. DMSO is known to act as an effective

radical scavenger (Ashwood-Smith, 1967; Del Maestro et al., 1980; Jacob & Herschler, 1986), and produced lower concentrations of neopterin in the experimental results. In addition, the INF-γ only treatments produced statistically significant quantities of TNP, consequently producing comparatively higher TNP/NP ratios. Based on the results, It remains unclear how the atorvastatin treatments produced lower TNP/NP ratios, as results past and present have demonstrated an anti-inflammatory effect on biomarker production (Neurauter et al., 2003).

While it is possible that atorvastatin generates a greater amount of oxidative stress in PBMCs, it may also be the case that the overall production of oxidative stress remains relatively unchanged. Rather, the production of 7,8-dihydroneopterin is inhibited, with a similar level of neopterin being produced due to oxidative stress, resulting in a lower TNP/NP ratio. HMGR catalyses the committed step in the mevalonate pathway, which in addition to controlling cholesterol production also controls the production of isoprenoid intermediates necessary for prenylation. Small GTP binding proteins regulating pro-inflammatory pathways are reliant on prenylation to facilitate their signal transduction. NADPH-oxidase (NOX) is a key contributor of ROS in in various pathological states of the cardiovascular system (V Goncharov, V Avdonin, D Nadeev, L Zharkikh, & O Jenkins, 2015). Important components of this enzyme are its cytosolic subunits which come together to form active NOX complexes and the small GTP binding protein Rac. The small GTP-binding protein Rac, elaborated upon in the introduction (**Section 1.7.2**), is directly affected by the reduction in isoprenoid intermediates and itself modulates NOX activity. As a result of atorvastatin potentially decreasing NOX activity through affecting prenylation, it is unlikely that atorvastatin is producing an increased extent of oxidative stress, rather the opposite. NOX is capable of maintaining physiological levels of O^{2-} and H_2O_2 with low levels of expression and activity (W. Zhang, Bai, Tian, Jia, & Zhou, 2016). It is possible that some NOX complexes had already formed in the PBMCs and the low levels of expression were responsible for maintaining a similar level of ROS production. It may also be the case that other sources of oxidative stress such as mitochondrial ROS production (Corral-Debrinski, Shoffner, Lott, & Wallace, 1992) could contribute to neopterin production.

4.4 Atorvastatin treatment effect on protein concentration

In tandem with imaging PBMCs using microscopy, this research implemented the BCA assay to measure the protein concentration of cellular lysate to demonstrate PBMCs were viable, with sufficient protein to be responsible for producing or not producing measured inflammatory markers. The mean protein concentrations varied between respective PBMC preparations, from 226.7 to 316.0 µg/mL. It was shown consistently between PBMC preparations, that the lowest protein concentrations measured was produced by a condition implementing 20 µM of atorvastatin treatment. This is interesting, as 50 and 100 µM concentrations were also implemented as experimental conditions and both produced higher protein concentrations than 20 µM. It was noticed that the higher concentrations of atorvastatin including 50 and 100 µM were less tolerated by PBMCs, in one preparation causing dramatic swelling indicating cell death occurrence. It is possible in these scenarios that the higher concentrations caused cell death in an unregulated and/or regulated manner, prior to the cells having a chance to be affected with respect to protein concentration. In contrast, the 20 µM concentrations, which appeared more tolerable throughout each respective PBMC preparation, produced notable decreases in protein concentrations.

The activation of the small GTPases like Rho, Rac and Ras are dependent on their prenylation. These GTPases function as major facilitators of signal transduction, leading to the transcription and translation of many proteins associated with inflammatory responses (Wolfrum et al., 2003). Any disruption to the synthesis of isoprenoid intermediates required for prenylation may result in a lower magnitude of signal transduction, leading a reduction in protein production, accounting for the experimental observations. Previous studies have shown simvastatin administration to mice decreases the prenylation of small g-proteins, including Ras, but not Rho, as measured by decreases in membrane association (Spindler et al., 2012). With respect to K-Ras protein translocation in pancreatic cancer cells, the IC_{50} was shown to be 27 µM for atorvastatin (Gbelcová et al., 2008). It may be the case that the 20 µM concentrations in this research, somewhat lesser than the previously stated IC_{50} for K-Ras protein translocation, are decreasing the amount of isoprenoid intermediates available leading to decreased protein prenylation. The protein concentration produced by 20 µM atorvastatin concentrations were also lesser than their respective controls. It is possible that the various mechanisms affected by decreased prenylation are acting in a combinatory manner subsequently decreasing protein production. This likely explains the control conditions producing a greater protein concentration via a baseline level of protein prenylation occurring uninhibited. These lesser

protein concentrations suggest that the expression of GTPCH-1 and IDO may be lessened, thus contributing to the decrease in 7,8-dihydroneopterin and kynurenine production. However, from the overall protein concentrations alone it is not possible to ascertain GTPCH-1 and IDO concentrations. To determine if the expression of inflammatory biomarker producing enzymes is diminished as the protein concentrations imply, it would be necessary to directly measure their respective protein expressions. Implementing a western blot procedure for GTPCH-1 and IDO would accurately measure their expression, from which a more confident conclusion could be made.

4.5 Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs), including monocytes are often isolated from anti-coagulated blood for use in preclinical and clinical research (Betsou, Gaignaux, Ammerlaan, Norris, & Stone, 2019). In this research PBMCs served as merited candidates for monitoring immune cell inflammatory marker production An advantage of using a primary cell line such as PBMCs over immortal monocyte-like cell lines like U937 and THP-1, is they provide a better representation of the *in vivo* tissue environment. As the PBMCs are derived directly from native body tissues and not modified, they simulate the *in vivo* state and physiology, therefore providing an excellent model system for studying inflammatory responses. A distinct disadvantage of using PBMCs is they only remain viable for a limited time period, unlike an immortal cell line. Therefore, for research purposes it is typically necessary to produce multiple preparations of cells for experimental work. This introduces the potential for variation between preparations, as cells taken from different donors may respond differently to treatment resulting in varying inflammatory responses.

During this research there was notable variation with regard to inflammatory biomarker production between respective PBMC preparations. In some instances, there was significant inflammatory production prior to treatment during the initial media only day, and in some instances none at all. Previous studies have shown gene transcription in PBMCs prepared from blood samples over weekly intervals to be remarkably consistent within samples from a single donor, conversely marked differences were observed in expression between donors (Eady et al., 2005). The relative distribution of the PBMC populations may also be influenced by age, ethnicity or gender (Tollerud et al., 1989). As these preparations are assumed to come from different donors, this likely is responsible for the variation observed. It is also possible that

some of the variation, particularly decreases between the respective PBMC preparations inflammatory marker production may be due to the donors medicating with anti-inflammatory medicines, such as statins. The ethics approval made it possible to collect and experiment on the donor's blood. However, this is the extent of the approval, and it was not possible to learn anything further about donor's health and history, such as any medications they may be taking. The blood donations were sourced from hemochromatosis patients who routinely undergo therapeutic phlebotomy as a part of their treatment regime. As such, these donations are not intended for clinical use, therefore the screening process for this blood is not as stringent regarding medications taken. It is possible that one or more of the donors was prescribed and taking statins to manage their own CVD-risk, thus producing diminished or non-significant rises in biomarker concentration in response the IFN-γ activation. This issue could potentially be resolved by slightly expanding upon the ethics approval to include donor's prescription history. Conversely, this could be achieved by implementing a short survey of relevant questions with donor consent, like whether or not they are routinely taking any medication, which is already routinely asked prior to regular blood donations.

4.6 Peripheral blood mononuclear cell tolerance to experimental conditions

Images from experiments with higher concentrations, 50 and 100 µM, of atorvastatin showed dramatic swelling of cells, indicative of cell death occurring through necrosis (**Figure 3.3.4.1**). The fundamental feature distinguishing necrosis is rapid loss of cellular membrane potentials, resulting in cytoplasmic swelling, rupture of the plasma membrane and cellular disintegration (Zong & Thompson, 2006). Previous studies on human PBMCs have demonstrated via staining and flow cytometry, that DMSO concentrations of 10% $^{v}/_{v}$ increased PBMC death after 24-hours and 5% $^{v}/_{v}$ after 120 hours (de Abreu Costa et al., 2017). The observed swelling in this research implemented treatment conditions with 50 and 100 µM of atorvastatin, for which the concentrations of DMSO in solution were 2.5 and 5% $^{v}/_{v}$, respectively. While not reaching 10% $^{v}/_{v}$, the volume percentage of DMSO was still at and close to a concentration shown to increase PBMC death. The PBMCs of that particular preparation may have been particularly sensitive in comparison to other preparations to deviations from normal, resulting in PBMC death. During this particular experimental work, the microscope was experiencing technical difficulties requiring servicing to fix, resulting in somewhat inconsistent images. However, the protein concentrations produced by the experimental conditions with swollen cells were

comparatively normal, producing a greater level of protein than the control. HPLC analysis of the initial experimental conditions demonstrated that the lower concentrations, 10 and 20 µM, were producing comparable anti-inflammatory and inhibitory effects to 50 and 100 µM. Based on these experimental results and concerns regarding viability, the lower concentrations were determined to be most suitable for further experimental work.

4.7 Acidic iodide incubation

This study implemented an acidic iodide solution (5.4% I_2 and 10.8% KI in 1 M HCL) in order to oxidise 7,8-dihydroneopterin within a sample to neopterin, so that it may be more easily and reliably detected (Flavall et al., 2008; A. Lindsay et al., 2014; Ziegler, 1985). The protocol involved a 15-minute incubation in the absence of light, after the addition of the acidic iodide solution to samples. Following this, the addition of ascorbic acid (0.6 M) was used to quench the solution. Prior to sample analysis the acidic iodide solution was assessed for its ability to oxidise samples over the incubatory period, which according to previous works should be ≥98%. Using the equation produced by linear regression analysis, calculations showed the acidic iodide solution to be capable of oxidising ≥98% of samples up to 50 µM of 7,8-dihydroneopterin in solution.

Previous work by Flavall et al. implementing the same acidic iodide oxidation protocol showed that in healthy versus septicaemia (blood infection) patients the highest concentration of serum TNP produced was less than 0.2 µM (Flavall et al., 2008). Further, work examining IFN-γ activated live excised carotid plaque tissue sections showed the greatest concentration of TNP produced in media retrieved was equal to 9 µM (H Prebble et al., 2018). The 7,8-dihydroneopterin standard concentration of 50 µM oxidised to 98%, is significantly higher than biologically relevant concentrations of TNP produced in these instances of inflammation. Therefore, the acidic iodide solution was determined to be effective and sufficient for the oxidation of 7,8-dihydroneopterin within experimental samples.

4.8 Further Research

Presently, there are three statin medications listed on the New Zealand Pharmaceutical Schedule: atorvastatin, simvastatin, and pravastatin (K. Wilson, 2021). In order to further elucidate the potential anti-inflammatory effect of statin treatment on immune cell activation by measuring 7,8-dihydroneopterin generation and oxidation, it is necessary to characterise at least one of either simvastatin or pravastatin. Structurally, statins all share an HMG-like moiety enabling them to function as competitive inhibitors of HMG-CoA reductase. Otherwise, statins differ in their respective structures, in particular the hydrophobic groups covalently linked to the HMG-like moiety. Based on their hydrophobic groups, statins are sorted into two types, simvastatin and pravastatin are classed as type-1 inhibitors, and atorvastatin a type-2 inhibitor. These differences in structure result in subtle differences in binding characteristics, therefore it is necessary to examine the effect of a type-1 inhibitor to supplement the results produced by the type-2 atorvastatin. Simvastatin is the next most potent statin available behind atorvastatin, and the most prescribed type-1 statin (New Zealand Formulary, Assessed April, 2021; NZ, 2017). Therefore, simvastatin will serve as an appropriate candidate for future experimental work. A consideration that will need to be made for other statins, is the relative concentrations at which they are required to achieve comparable LDL-c reductions due to varying potencies. For example, to achieve 30% to <50% (moderate intensity), 10 to 20 mg of atorvastatin is required in comparison to 20 to 40 mg simvastatin (Chou, Dana, Blazina, Daeges, & Jeanne, 2016). Considering this, potential future experimental conditions may require reviewing on a statin by stain basis in order to produce comparable results.

One such way this experimental work could be taken further is by examining the anti-inflammatory effect of statins on immune cell activation in excised live plaque tissue. Previous work published by this research group has demonstrated IFN-γ induced macrophage activation in live carotid atherosclerotic plaque tissue. While utilising PBMCs has value as they represent the *in vivo* tissue environment, this still does not allow for investigation of the more complex interactions between an array of cell types (smooth muscle cells, macrophages, monocytes, and T-cells) and cytokines present in the inflammatory setting that is plaque tissue (Hannah Prebble et al., 2018). Continuing the experimental work on excised plaque tissue would enable further elucidation of the potential anti-inflammatory effects of statins *in vivo*, by generation of 7,8-dihydroneopterin, neopterin and kynurenine.

To further elucidate how 7,8-dihydroneopterin production is affected by statin treatment, Interleukin-1β (IL-1β) expression could be investigated. The interleukin-1 family of ligands and receptors is the main cytokine family associated with acute and chronic inflammation (Dinarello, 2011). IL-1β has been shown in patients presenting with CVD to be increased in a manner which is directly proportional to the disease's severity (Galea et al., 1996). The enzyme responsible for the production of the inflammatory marker 7,8-dihydroneopterin: GTP-cyclohydrolase-1, has been demonstrated to be upregulated by IL-1β (PlÜSs, Werner, Blau, Wachter, & Pfeilschifter, 1996). Based on previously unpublished findings, this research group hypothesises that 7,8-dihydroneopterin and IL-1β function together in a feedback mechanism, whereby the presence of 7,8-dihydroneopterin decreases the release of IL-1β. Therefore, by measuring the expression of IL-1β, it would provide another modality of determining how the production of 7,8-dihydroneopterin is affected by statin treatment. An advantage of this would be that IL-1β may be measured in culture media by ELISA, thus it would not require any further experimental work with regard to tissue culture, as the media collected from this experimental work may be utilised for this purpose.

4.9 Summary

This study has characterised the anti-inflammatory effect of atorvastatin on interferon-γ (IFN-γ) activation of PBMCs by measuring the production of 7,8-dihydroneopterin, neopterin and kynurenine. It has also examined the extent of inflammation versus oxidation as a result of atorvastatin treatment in PBMCs by assessing the TNP/NP ratios produced. Treatment of PBMCs with atorvastatin in various combinations including IFN- γ activation each produced an anti-inflammatory effect by inhibiting the production of the respective inflammatory markers. This supports the research hypothesis that immune cell activation and the subsequent release of 7,8-dihydroneopterin is inhibited by atorvastatin. In contrast, the IFN-γ only and DMSO treatment conditions produced statistically significant increases in respective inflammatory markers. The TNP/NP ratios for atorvastatin treatments indicate a greater level of oxidative stress compared to inflammation, but the mechanism by which this occurs remains unclear. These findings provide evidence for atorvastatin inhibiting the production of 7,8-dihydroneopterin, neopterin and kynurenine in PBMCs. This information will assist in developing TNP as a potential clinical diagnostic marker for cardiovascular health. Further research is necessary to confirm the anti-inflammatory effects of statins, including other statins.

Chapter 5

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