



The Potential Immunomodulatory Effects of the Statins in Uveitis

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The Potential Immunomodulatory Effects of the Statins in Uveitis

Table of Contents

Table of Contents	2
Table of Figures.....	10
List of Tables.....	15
Abstract.....	16
Statement of Disclosure.....	18
Acknowledgements	19
Abbreviations.....	20
Chapter1.....	26
General Introduction.....	27
1.1 The Immune System.....	28
1.2 Th1/Th2 Balance.....	30
1.3 Immunologic Tolerance and Autoimmunity.....	40
1.3.1 Mechanisms of Tolerance.....	40
1.3.1.1 Clonal Deletion and Anergy.....	41
1.3.1.2 Idiotype Regulation.....	41
1.3.1.3 Suppressor or Regulatory T (Tr) cells.....	42
1.3.1.4 Inhibitory Cytokines.....	43
1.3.1.5 Failed Tolerance.....	43
1.3.2 Autoimmune Disease.....	43
1.4 The Ocular Immune System.....	44
1.4.1 Immune Privilege.....	44

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.4.1.1 The Blood-Retinal Barrier (BRB).....	47
1.4.1.1.1 Anterior BRB.....	47
1.4.1.1.2 Posterior BRB.....	48
1.4.1.2 The Blood-Aqueous Barrier (BAB).....	50
1.4.1.3 Anterior Chamber Associated Immune Deviation (ACAID).....	50
1.5 Intraocular Immune Disease.....	52
1.5.1 Experimental Autoimmune Uveoretinitis (EAU).....	53
1.5.1.1 Retinal Autoantigens.....	53
1.5.1.2 Induction of EAU.....	54
1.5.2 Leukocyte Trafficking in Immune-mediated Diseases of the Posterior Segment.....	55
1.5.3 Cytokines in Immune-mediated Diseases of the Posterior Segment.....	58
1.5.3.1 IL-1 β	59
1.5.3.2 IL-2.....	59
1.5.3.3 IL-4.....	60
1.5.3.4 IL-5.....	61
1.5.3.5 IL-6.....	62
1.5.3.6 IL-10.....	62
1.5.3.7 IFN- γ	64
1.5.3.8 TNF- α	64
1.5.4 Chemokines.....	65
1.5.4.1 IL-8.....	66
1.5.5 Clinical Uveitis.....	66

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.5.5.1 Idiopathic Uveitis.....	69
1.5.5.2 Behçet's disease.....	70
1.5.5.3 Sarcoidosis.....	71
1.5.5.4 Vogt-Koyanagi-Harada disease.....	72
1.5.5.5 Reiter's Syndrome.....	73
1.5.5.6 Ankylosing Spondylitis.....	74
1.5.5.7 Fuch's Heterochromic Cyclitis.....	75
1.6 Therapies for Intraocular Autoimmune Disease.....	77
1.6.1 Established Therapies for Intraocular Autoimmune Disease.....	77
1.6.1.1 Glucocorticoids.....	77
1.6.1.2 Cyclosporin A (CsA).....	79
1.6.1.3 Rapamycin.....	80
1.6.1.4 Mycophenolate mofetil.....	81
1.6.2 New Therapies for Intraocular Immune Disease.....	83
1.6.2.1 Anti-TNF- α Therapies.....	83
1.6.2.2 Other Monoclonal Antibody Therapies.....	83
1.6.2.3 Other Novel Treatments.....	84
1.6.3 Potential Therapies in Uveitis.....	85
1.6.3.1 Rheumatoid Arthritis-based Therapies.....	85
1.6.3.2 Antibody Fragment Therapies.....	85
1.6.3.3 Statin Therapies.....	86
1.6.3.3.1 Statins in Experimental Inflammatory Models of Disease.....	89
1.7 Aims.....	92

The Potential Immunomodulatory Effects of the Statins in Uveitis

Chapter 2.....	93
Materials and Methods.....	94
2.1 Materials.....	95
2.2 Antibodies.....	97
2.3 Donors.....	99
2.3.1 Multiplex Cytokine Detection with Cytometric Bead Array.....	99
2.3.2 T Cell Viability and Proliferation Inhibition.....	99
2.3.3 Intracellular Cytokine Staining.....	100
2.3.4 Multiplex Cytokine Detection with Fluorescent Bead Immunoassay.....	100
2.4 Stock Solutions.....	101
2.4.1 2-Mercaptoethanol.....	101
2.4.2 Atorvastatin.....	101
2.4.3 CFSE Stop Buffer.....	101
2.4.4 Cyclosporin A.....	101
2.4.5 Dexamethasone.....	102
2.4.6 FACS Lysing Solution.....	102
2.4.7 Lovastatin.....	102
2.4.8 Mevalonate.....	102
2.4.9 Mycophenolate mofetil.....	103
2.4.10 Permash Buffer.....	103
2.4.11 Phosphate Buffered Saline.....	103
2.4.12 Propidium Iodide.....	103
2.4.13 Rapamycin.....	104

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.4.14 Simvastatin.....	104
2.4.15 T Cell Medium.....	104
2.5 Multiplex Cytokine Detection with Cytometric Bead Array.....	105
2.5.1 Sampling Procedure.....	105
2.5.2 Cytometric Bead Array (CBA).....	105
2.5.2.1 Preparation of Th1/Th2 Cytokine Beads.....	105
2.5.2.2 Preparation of Th1/Th2 Cytokine Standards.....	106
2.5.2.3 Th1/Th2 Cytokine CBA Assay Procedure.....	106
2.5.2.4 Flow Cytometry.....	107
2.5.3 Enzyme-linked Immunosorbent Assay (ELISA).....	107
2.5.4 Analysis.....	108
2.6 T Cell Viability and Proliferation Inhibition.....	109
2.6.1 Sampling Procedure.....	109
2.6.2 MNC Counts.....	109
2.6.3 T Cell Culture.....	111
2.6.4 Flow Cytometry.....	112
2.6.5 Analysis.....	112
2.7 Intracellular Cytokine Staining.....	114
2.7.1 Sampling Procedure.....	114
2.7.2 Cell Culture.....	114
2.7.3 Staining for Intracellular Cytokines.....	114
2.7.4 Analysis.....	115

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.8 Multiplex Cytokine Detection with Fluorescent Bead Immunoassay.....	117
2.8.1 Sampling procedure.....	117
2.8.2 Cell Culture.....	117
2.8.3 Multiplex Fluorescent Bead Immunoassay (FBI).....	117
2.8.3.1 Preparation of Assay Buffer.....	118
2.8.3.2 Preparation of Biotin-Conjugate.....	118
2.8.3.3 Preparation of Beads.....	118
2.8.3.4 Preparation of Th1/Th2 Standards.....	119
2.8.3.5 Protocol.....	119
2.8.3.6 Flow Cytometry.....	120
2.8.3.7 Analysis.....	120
 Chapter 3.....	 122
Cytometric Bead Array.....	123
3.1 Introduction.....	124
3.2 Aims.....	125
3.3 Results.....	126
3.3.1 Optimising Cytokine Detection in AH by CBA and ELISA.....	126
3.3.2 CBA Cytokine Detection in Uveitis and Controls.....	129
3.3.3 Effect of Steroid Usage.....	132
3.3.4 Effect of Systemic Disease.....	134
3.3.5 Cytokine Correlation in AH.....	135

The Potential Immunomodulatory Effects of the Statins in Uveitis

3.4 Discussion.....	137
3.4.1 IL-2.....	137
3.4.2 TNF- α	138
3.4.3 IFN- γ	139
3.4.4 IL-4.....	139
3.4.5 IL-5.....	140
3.4.6 IL-10.....	142
 Chapter 4.....	 144
Viability and Proliferation Assays.....	145
4.1 Introduction.....	146
4.2 Aims.....	148
4.3 Results.....	149
4.3.1 T Cell Viability.....	149
4.3.2 T Cell Proliferation Inhibition.....	157
4.4 Discussion.....	168
 Chapter 5.....	 172
Intracellular Cytokine Staining.....	174
5.1 Introduction.....	175
5.2 Aims.....	176
5.3 Results.....	176
5.4 Discussion.....	178

The Potential Immunomodulatory Effects of the Statins in Uveitis

Chapter 6.....	187
Fluorescent Bead Immunoassay.....	188
6.1 Introduction.....	189
6.2 Aims.....	190
6.3 Results.....	191
6.4 Discussion.....	217
 Chapter 7.....	 231
General Discussion.....	232
 Chapter 8.....	 243
Bibliography.....	244

The Potential Immunomodulatory Effects of the Statins in Uveitis

Table of Figures

Figure 1.1 The balance between Th1 and Th2 cytokines.....	32
Figure 1.2 Cytokines influencing the development of antigen-activated naïve CD4 ⁺ T cells into Th1 and Th2 cells.....	35
Figure 1.3 Tight junction proteins of the blood-retinal barrier.....	49
Figure 1.4 Leukocyte transendothelial migration.....	57
Figure 1.5 Signs of anterior uveitis.....	67
Figure 1.6 Fundus fluorescein angiogram of posterior uveitis.....	67
Figure 1.7 Structures of statins.....	87
Figure 1.8 Pleiotropic effects of HMG-Co A reductase inhibitors.....	88
Figure 1.9 Intracellular effects of HMG-CoA reductase (inhibition by statins).....	90
Figure 2.1 The principles of density gradient centrifugation.....	110
Figure 2.2 WinList histogram plot series showing region definition of cell populations to analyze live stimulated cells in order to track their proliferation with CFSE labeling.....	113
Figure 2.3 WinList histogram plot series showing quadrant analysis resulting in 4 region percentages enabling identification of cells double positive for CD3 and IFN-γ.....	116
Figure 3.1 Cytokine levels (pg/ml) in AH of patients with uveitis as determined by CBA versus ELISA.....	127
Figure 3.2 Cytokine levels (pg/ml) in AH of patients with AU and panuveitis (PAN) versus controls.....	130

The Potential Immunomodulatory Effects of the Statins in Uveitis

Figure 3.3 Cytokine levels (pg/ml) in AH of controls compared with AU either on or off steroids, and PAN taking steroids.....	133
Figure 3.4 Cytokine levels (pg/ml) in AH of controls compared to AU patients with or without systemic disease, and PAN with or without systemic disease.....	136
Figure 4.1 CD3/CD28 stimulated viable T cells (%) with increasing Atorvastatin concentrations.....	150
Figure 4.2 CD3/CD28 stimulated viable T cells (%) with different Lovastatin concentrations.....	151
Figure 4.3 CD3/CD28 stimulated viable T cells (%) with different Simvastatin concentrations.....	152
Figure 4.4 Unstimulated and CD3/CD28 stimulated viable T cells (%) with different Rapamycin and Mycophenolate mofetil concentrations.....	154
Figure 4.5 Unstimulated and CD3 stimulated viable T cells (%) with different CsA concentrations.....	155
Figure 4.6 Unstimulated and CD3/CD28 stimulated viable T cells (%) with different Dexamethasone concentrations.....	156
Figure 4.7 Inhibition of CD3/CD28 stimulated proliferation of T cells with increasing Atorvastatin concentrations with and without Mevalonate.....	158
Figure 4.8 Inhibition of CD3/CD28 stimulated proliferation of T cells with increasing lovastatin concentrations with and without Mevalonate.....	159
Figure 4.9 Inhibition of CD3 and CD28 stimulated proliferation of T cells with different Simvastatin concentrations.....	160

The Potential Immunomodulatory Effects of the Statins in Uveitis

Figure 4.10 Inhibition of CD3 and CD28 stimulated proliferation of T cells with different Rapamycin concentrations.....	163
Figure 4.11 Inhibition of CD3 and CD28 stimulated proliferation of T cells with different Mycophenolate concentrations.....	164
Figure 4.12 Inhibition of PHA, CD3/CD28 and CD3 stimulated proliferation of T cells with different CD3 and CsA concentrations.....	165
Figure 4.13 Inhibition of CD3 stimulated proliferation of T cells with increasing CsA concentrations administered at time 0 or 2 hours prior to stimulation.....	166
Figure 4.14 Inhibition of CD3 and CD28 stimulated proliferation of T cells with increasing Dexamethasone concentrations.....	167
Figure 5.1 Percentage of IFN- γ , IL-10, IL-4 and IL-2 expression in PMA/Ionomycin stimulated whole blood with or without drug treatment.....	177
Figure 6.1 Percentage change in cytokine expression following Atorvastatin addition to PMA/Ionomycin stimulated whole blood.....	193
Figure 6.2 Percentage change in cytokine expression following Lovastatin addition to PMA/Ionomycin stimulated whole blood.....	194
Figure 6.3 Percentage change in cytokine expression following Simvastatin addition to PMA/Ionomycin stimulated whole blood.....	195
Figure 6.4 Comparison of percentage change in cytokine expression between Atorvastatin, Lovastatin and Simvastatin following their addition to PMA/Ionomycin stimulated whole blood.....	196
Figure 6.5 Percentage change in cytokine expression following Rapamycin addition to PMA/Ionomycin stimulated whole blood.....	198

The Potential Immunomodulatory Effects of the Statins in Uveitis

Figure 6.6 Percentage change in cytokine expression following Mycophenolate addition to PMA/Ionomycin stimulated whole blood.....	199
Figure 6.7 Percentage change in cytokine expression following CsA addition to PMA/Ionomycin stimulated whole blood.....	200
Figure 6.8 Percentage change in cytokine expression following Dexamethasone addition to PMA/Ionomycin stimulated whole blood.....	202
Figure 6.9 Percentage change in cytokine expression following Atorvastatin and Rapamycin addition to PMA/Ionomycin stimulated whole blood.....	203
Figure 6.10 Percentage change in cytokine expression following Atorvastatin and Mycophenolate addition to PMA/Ionomycin stimulated whole blood.....	204
Figure 6.11 Percentage change in cytokine expression following Atorvastatin and CsA addition to PMA/Ionomycin stimulated whole blood.....	206
Figure 6.12 Percentage change in cytokine expression following Atorvastatin and Dexamethasone addition to PMA/Ionomycin stimulated whole blood.....	207
Figure 6.13 Comparison of percentage change in cytokine expression between Atorvastatin, Dexamethasone, Atorvastatin and Dexamethasone following their addition to PMA/Ionomycin stimulated whole blood.....	209
Figure 6.14 Percentage change in cytokine expression following Atorvastatin and Dexamaethasone and CsA addition to PMA/Ionomycin stimulated whole blood.....	210
Figure 6.15 Comparison of percentage change in cytokine expression between Atorvastatin and Dexamethasone, Atorvastatin, Dexamethasone and CSA following their addition to PMA/Ionomycin stimulated whole blood.....	212

The Potential Immunomodulatory Effects of the Statins in Uveitis

Figure 6.16 Percentage change in IL-4 expression in whole blood stimulated with PMA/Ionomycin and treated with various drug combinations.....	213
Figure 6.17 Comparison of percentage change of cytokine expression between whole blood treated with CsA at time of stimulation with PMA/Ionomycin (time 0) and whole blood pretreated with CsA for 2 hours prior to stimulation with PMA/Ionomycin.....	216
Figure 7.1 A simplified model of the possible interactions between polarised Th1 and Th2 responses.....	238

The Potential Immunomodulatory Effects of the Statins in Uveitis

List of Tables

Table 2.1	Capture antibodies on microspheres and detector antibodies used in Cytometric Bead Array.....	97
Table 2.2	Unconjugated primary antibodies used in T cell viability and proliferation assay studies.....	97
Table 2.3	Directly conjugated primary antibodies used in cell surface and intracellular cytokine staining studies.....	98
Table 2.4	Capture antibodies on microspheres and detector antibodies used in Fluorescent Bead Immunoassay.....	98
Table 2.5	Minimum and maximum levels ($\mu\text{g/ml}$) of cytokines detected by FBI.....	121
Table 6.1	Summary of P value significances in the evaluation of various drug combinations on cytokine expression as compared to control, within whole blood stimulated with PMA/Ionomycin.....	214

The Potential Immunomodulatory Effects of the Statins in Uveitis

Abstract

Uveitis is the umbrella term used to designate intraocular inflammation which manifests as various clinical phenotypes. Much remains to be elucidated with regards to the aetiologies of the immune-mediated uveitides but they are largely T cell-mediated. T cell cytokine profiling has enabled a greater understanding of the immunopathogenic mechanisms involved with resultant development of cytokine-specific immunotherapies. There is, however, always room for inexpensive and relatively side-effect free treatments which offer themselves as either adjunctive therapy to or ideally replacement therapy for the more powerful immunosuppressants such as the corticosteroids.

The first aim of the study was to delineate further the patterns of cytokine expression which occur in various forms of immune-mediated uveitis in order to better understand their origins. Here the cytometric bead array (CBA) system to detect multiple cytokines within aqueous humor (AH) volumes from uveitis patients was utilized and evaluated against the more established enzyme-linked immunosorbent assay (ELISA).

The main body of the study investigated the potential immunomodulatory role of the group of compounds known as the statins as adjunctive or steroid-sparing therapy in uveitis. Viability studies and proliferation assays using carboxyfluorescein succinimidyl ester (CFSE) were conducted to assess their effect on normal human whole blood-derived T cells as compared to more well known immunosuppressants. Further comparisons were made by intracellular cytokine staining and another multiplexed cytokine detection assay-fluorescent bead immunoassay (FBI) on drug-treated normal human whole blood samples.

The Potential Immunomodulatory Effects of the Statins in Uveitis

In the first part of the study we demonstrated the usage of the cytometric bead array system to detect multiple cytokines simultaneously in a small AH volume with greater efficacy than ELISA. This has enabled us to more accurately define the interplay of cytokines between anterior uveitis and panuveitis groups and suggest a more marked Th1 cytokine polarization in panuveitis as compared to anterior uveitis. In addition, Th1 cytokines appear to be reduced in those panuveitis patients on steroids. Cytometric bead array appears to be a useful tool for the understanding of the pathogenesis of uveitis in order to tailor novel, disease-specific, cytokine-directed therapies in the future.

In the second part of the study atorvastatin, lovastatin and simvastatin were all demonstrated to reversibly inhibit lymphocyte proliferation without significantly affecting viability. Intracellular cytokine staining revealed significant reductions in the Th1 cytokine, IFN- γ , below control expression for each of the statins. Rapamycin, mycophenolate, cyclosporin A and dexamethasone showed a slightly greater inhibition of IFN- γ production as compared to the statins. With the fluorescent bead immunoassay, atorvastatin appeared to be the most effective statin at decreasing the pro-inflammatory and Th1 cytokines and increasing the Th2 cytokines. The combination of atorvastatin and dexamethasone synergistically reduced the pro-inflammatory and Th1 cytokines, as well as IL-1 β , while increasing all of the Th2 cytokines.

This data provides evidence for the potential of statins in the treatment of uveitis as a steroid-sparing monotherapy or as part of combination therapy. Further *in vitro* work with the statins on peripheral blood and AH samples of uveitis patients appears warranted.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Disclosure

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

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

The Potential Immunomodulatory Effects of the Statins in Uveitis

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The Potential Immunomodulatory Effects of the Statins in Uveitis

Abbreviations

AAU	Acute anterior uveitis
Ab	Antibody
ACAID	Anterior chamber associated immune deviation
AC	Anterior chamber
Ag	Antigen
AH	Aqueous humor
AP	Activator protein
APC	Antigen presenting cell
AS	Ankylosing spondylitis
AU	Anterior uveitis
BAB	Blood-aqueous barrier
BBB	Blood-brain barrier
BD	Behçet's disease
BFA	Brefeldin A
BRB	Blood-retinal barrier
CAD	Coronary artery disease
CBA	Cytometric bead array
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CFSE	Carboxyfluorescein succinimidyl ester
CIITA	Class II transactivator
ConA	Concanavalin A

The Potential Immunomodulatory Effects of the Statins in Uveitis

CoQ10	Co-enzyme Q10
CRP	C reactive protein
CsA	Cyclosporin A
DC	Dendritic cell
DTH	Delayed-type hypersensitivity
EAE	Experimental autoimmune encephalitis
EAU	Experimental autoimmune uveoretinitis
EC	Endothelial cell
EIU	Endotoxin-induced uveitis
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	ELISA-based assay for detecting cells secreting analyte
ERM	Ets transcription factor protein
FBI	Fluorescent bead immunoassay
Fc	Fragment constant
FHC	Fuch's Heterochromic Cyclitis
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GATA	Transcription factor which binds to the consensus DNA sequence Guanine-Adenosine-Taurine-Adenosine
GC	Glucocorticoid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTP	Guanosine-5-triphosphate
h	Human

The Potential Immunomodulatory Effects of the Statins in Uveitis

HLA	Human leukocyte antigen
HMG-CoA	3-hydroxy-3-methyl-glutaryl coenzyme A
I	Inserted
IAU	Idiopathic anterior uveitis
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IKK	I- κ B kinase
IL	Interleukin
IMPDH	Inosine monophosphate dehydrogenase
iNOS	inducible nitric oxide
IP3	Inositol phosphate 3
IRBP	Interphotoreceptor retinoid-binding protein
IVIg	Intravenous immunoglobulin
JAK	Janus kinase
L-site	Lovastatin-site
LDA	Limiting dilution analysis
LDL	Low-density lipoprotein
LFA	Leukocyte function antigen
LPS	Lipopolysaccharide
LT	Lymphotoxin
MAPK	Mitogen activation kinases
MCP	Monocyte chemoattractant protein

The Potential Immunomodulatory Effects of the Statins in Uveitis

ME	Mercaptoethanol
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MLR	Mixed lymphocyte reaction
MMF	Mycophenolate mofetil
MNC	Mononuclear cell
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
NaOH	Sodium hydroxide
NFAT	Nuclear factor of activated T cells
NF	Nuclear factor
NK	Natural killer
NSAIDS	Non-steroidal anti-inflammatory drugs
OX-LDL	Oxidized low-density lipoprotein
PAN	Panuveitis
PB	Peripheral blood
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin chlorophyll
PHA	Phytohemagglutinin

The Potential Immunomodulatory Effects of the Statins in Uveitis

PI	Propidium iodide
PKC	Protein kinase(s) C
PLC	Protein lipase C
PMA	Phorbol 12-myristate 13-acetate
PP	Pyrophosphate
R	Receptor
RA	Rheumatoid arthritis
RIA	Radioimmunoassay
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RPMI	Roswell Park Memorial Institute media 1640 Dutch modification without L-glutamine
RS	Reiter's syndrome
RT	Room temperature
RT-PCR	Reverse-transcription followed by polymerase chain reaction
SAg	Retinal soluble antigen
scFV	single-chain variable-domain fragment
SD	Standard deviation
SEM	Standard error of the mean
SRL	Sirolimus
SSC	Side scatter
STAT	Signal transducer and activator of transcription
Tc	Cytotoxic T cell

The Potential Immunomodulatory Effects of the Statins in Uveitis

TEM	Transendothelial migration
Th	Helper T cell
TCR	T cell receptor
TCM	T cell medium
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Tyk	Tyrosine kinase
VC	Vehicle control
VKH	Vogt-Koyanagi-Harada disease
VSMC	Vascular smooth muscle cell

The Potential Immunomodulatory Effects of the Statins in Uveitis

Chapter 1

The Potential Immunomodulatory Effects of the Statins in Uveitis

General Introduction

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.1 The Immune System

The human immune system comprises a myriad of responses generated through the complex interplay of a constellation of cells and their cytokines in order to protect the body against disease. Immunity, the condition which confers resistance, is composed of a series of dualities. It can also be thought of as either natural or artificial, either active or passive but most importantly it can be subdivided into innate and adaptive (acquired) immunity. The innate or 'nonspecific' immune system is what we are born with and is genetically based. All antigens are defended against equally by this first line of protection. It comprises of surface barriers or mucosal immunity such as the skin and mucous membranes but also has a cellular component including macrophages, natural killer cells, neutrophils, eosinophils and dendritic cells (DCs). Each of these cells have germ line encoded pattern-recognition receptors which bind to broad classes of infectious organism antigens.

Adaptive or acquired immunity on the other hand is 'specific' in nature as it responds to antigens in a highly regulated manner. The adaptive immune system is concerned firstly with cell-mediated immunity defined by the non-antibody producing T lymphocytes produced in the bone marrow and sensitized in the thymus. Secondly it also comprises humoral immunity mediated by bone marrow developed, antibody (Ab) producing mature B lymphocytes called plasma cells. These antibodies, also called immunoglobulins (Igs) are soluble proteins which inactivate antigens by complement fixation, neutralization, agglutination and precipitation.

The Potential Immunomodulatory Effects of the Statins in Uveitis

T lymphocytes, or T cells as they are also known, recognize antigen (Ag) through the T cell receptor (TCR). Recognition involves presentation of the Ag by appropriate Major Histocompatibility Complex (MHC) molecules. MHC class I molecules are found on virtually every cell in the human body while MHC class II molecules are found only on B cells, macrophages and other antigen presenting cells (APCs). MHC class I molecules present Ag to cluster of differentiation (CD)8⁺ T cells, including cytotoxic T cells (Tcs) which lead to lymphotoxin release and cell lysis. MHC class II present Ag to CD4⁺ T cells otherwise known as helper T (Th) cells which amplify the immune response through the secretion of lymphokines which stimulate cytotoxic T cells and the clonal expansion of B cells into plasma cells and memory B cells as well as attract neutrophils and enhance the phagocytic ability of macrophages. Tc production is inhibited by suppressor T cells while memory T cells are generated to recognize and respond to a pathogen once it has been cleared.

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.2 Th1/Th2 Balance

Cytokines are paracrine-signaling molecules composed of proteins or peptides, some of which are glycosylated such as the interferons (IFNs, Roitt *et al* 1998, Chadha *et al* 2004). They are the effector elements released by cells during innate and adaptive immune responses and include several groups of molecules such as the interleukins, growth factors, colony stimulating factors, transforming growth factors, tumor necrosis factors, chemokines and monokines (Gupta 1998). When activated, CD4⁺ T cells differentiate into two lines differing in the types of cytokines secreted. Type 1, or Th1 cells secrete interleukin (IL)-2, IFN- γ and lymphotoxin (LT, tumor necrosis factor (TNF)- β) and generate a cell-mediated immune response through the activation of Tcs and macrophages to produce reactive oxygen intermediates and nitric oxide (NO) as well as stimulate their phagocytic function and enhance their ability for antigen presentation by upregulation of MHC class II molecules. In addition Th1 cells promote the induction of complement-fixing, opsonizing antibodies and of antibodies involved in antibody-dependent cell cytotoxicity, for example IgG1 in humans. Type 2, or Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 potentiating the maturation of B cells and immunoglobulin class switching to IgE and subtypes of IgG that do not fix complement as well as deactivation of macrophages and degranulation of mastocytes thereby instigating a humoral response (Abbas *et al* 1996).

Th1 cells are thought to confer protection against viruses, intracellular parasites, raise the delayed-type hypersensitivity (DTH) skin response to viral and bacterial antigens and fight tumor cells. Th2 cells are believed to respond to extracellular

The Potential Immunomodulatory Effects of the Statins in Uveitis

pathogens such as multicellular parasites (Santana and Rosenstein 2003). The Th1 or type-1 pathway dominance is thought to generate organ-specific autoimmune disease e.g., arthritis and multiple sclerosis (Singh *et al* 1999). The Th2 or type-2 pathway is regarded as responsible for allergy and related IgE-based disease and promoting systemic autoimmune disease (Dent 2002). Either pathway can antagonize the other with one or the other pathway thought to be dominant in a response to a particular antigen in any one circumstance. These stereotypes however have been demonstrated to be arbitrary resulting in criticism of this paradigm (Zhai *et al* 1999).

The Potential Immunomodulatory Effects of the Statins in Uveitis

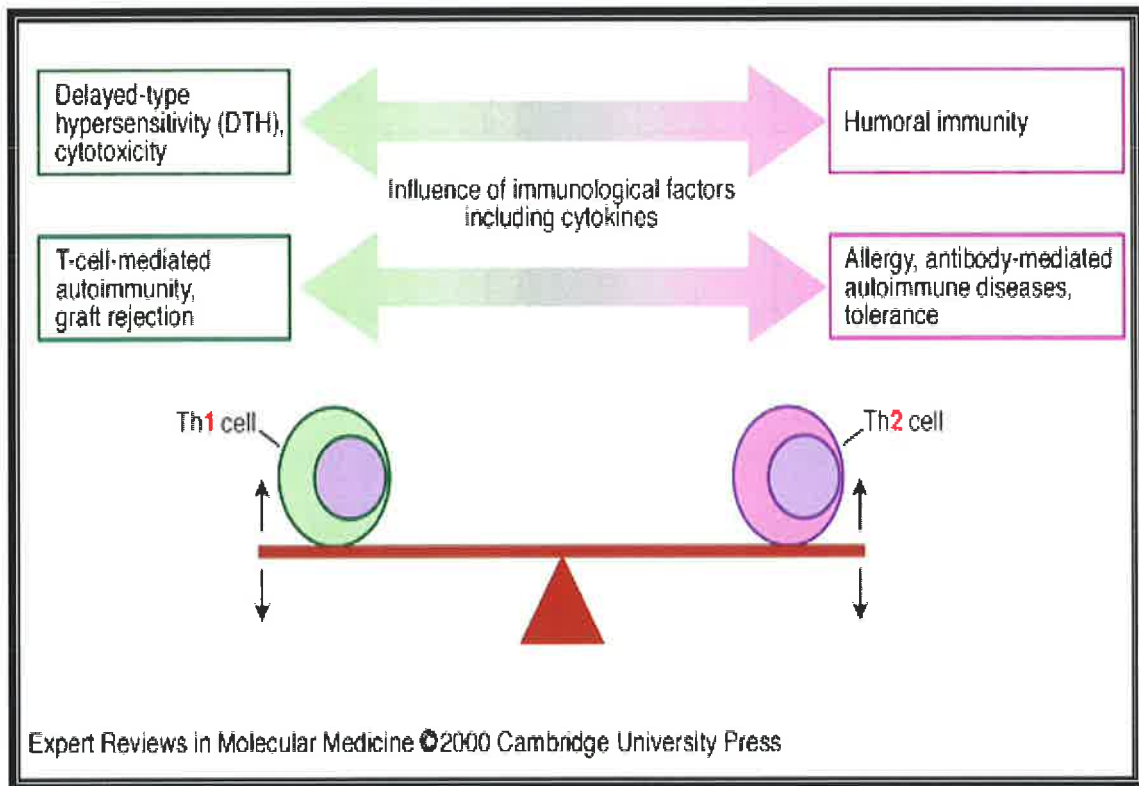


Figure 1.1. A model to illustrate the complex balance between T helper 1 (Th1) and Th2 cells.

The Th1/Th2 paradigm hypothesises that, under the influence of a variety of factors including the cytokines interleukin 4 (IL-4) and IL-12, naive T cells can mature into one of two phenotypes, Th1 or Th2, that counter-regulate each other and are involved in different aspects of immunological response and disease.

Taken and adapted from: Harber *et al* (2000).

The Potential Immunomodulatory Effects of the Statins in Uveitis

The Th1/Th2 balance hypothesis originated from murine models which proposed that Th1 and Th2 cells were important regulators of the class of immune response (Mosmann *et al* 1986). This concept was then applied to human immunity (Mosmann *et al* 1989) with the dichotomy varying greatly and influenced by a large number of experimental variables including whether the study is done *in vivo*, *ex vivo* or *in vitro* as with increasing delay before assay, cells typically undergo profound modification (Balkwill 2000, Romagnani 2000). Other Th1/Th2 maturation factors include nature and dose of the antigen, direct cell-to-cell interaction with APCs and the cytokine receptors available on the naïve cell (Kidd 2003).

Certain sets of interleukins are involved in the differentiation of immune cells into type 1 or type 2 phenotypes (O'Garra 1998). The initial DC or related APC, depending on the type of pathogen exposed to, will become type 1 or type 2 biased. Type 1 pathway bound APCs migrate to a nearby lymph node and secrete IL-12 and type I IFNs. Natural killer (NK) cells respond to the IL-12 by producing IFN- γ . IL-12 and type I IFNs induce naïve T cells to differentiate into Th1 cells which also produce IFN- γ . IFN- γ is an inhibitor of the Th2 pathway and thus promotes Th1 differentiation indirectly by stimulating APCs to produce more IL-12 in a self-fulfilling autocrine loop. The generation of Th2 cells is likely to result from IL-6 secretion by type 2 pathway committed APCs. IL-4 from NK cells, mast cells and eosinophils is also thought to play a role. A Th2 autocrine loop is also effected as Th2 cells produce IL-4 (Figure 1.2, Santana and Rosenstein 2003). IL-11 is also thought to be a strong inducer of Th2 differentiation

The Potential Immunomodulatory Effects of the Statins in Uveitis

as it acts directly on T cells to stimulate IL-4 and IL-5 synthesis and inhibits IL-12 expression by macrophages (Curti *et al* 2001).

Membrane-associated accessory molecules important for priming of T cells are upregulated by activated APCs (Santana and Rosenstein 2003). The most important co-receptor in T cells is CD28. CD28 signaling increases the level and duration of an activated state of intermediate molecules of the TCR signaling cascade (Michel *et al* 2001). CD28 signals directly propagate the Th2 pathway through the upregulation of the specific Th2 transcription factor which binds to the consensus DNA sequence Guanine-Adenosine-Taurine-Adenosine (GATA)-3 both *in vivo* and *in vitro* (Rodriguez-Palmero *et al* 1999).

Leukocyte function antigen (LFA)-1 (CD11a/CD18) is the most important integrin for the adhesion between APCs and T cells (Bachmann *et al* 1997). If cells are stimulated through CD11a/CD18 alone, lymphocytes produce the type 1 cytokine IFN- γ and granulocyte-macrophage colony-stimulating factor (GM-CSF); whereas if the cells are co-stimulated with the LFA-1 ligand (Intercellular adhesion molecule (ICAM)-1), and by ligation of the TCR, cells express IL-10, a regulatory cytokine that inhibits the expression of type 1 cytokines (Bleijs *et al* 1999).

The Potential Immunomodulatory Effects of the Statins in Uveitis

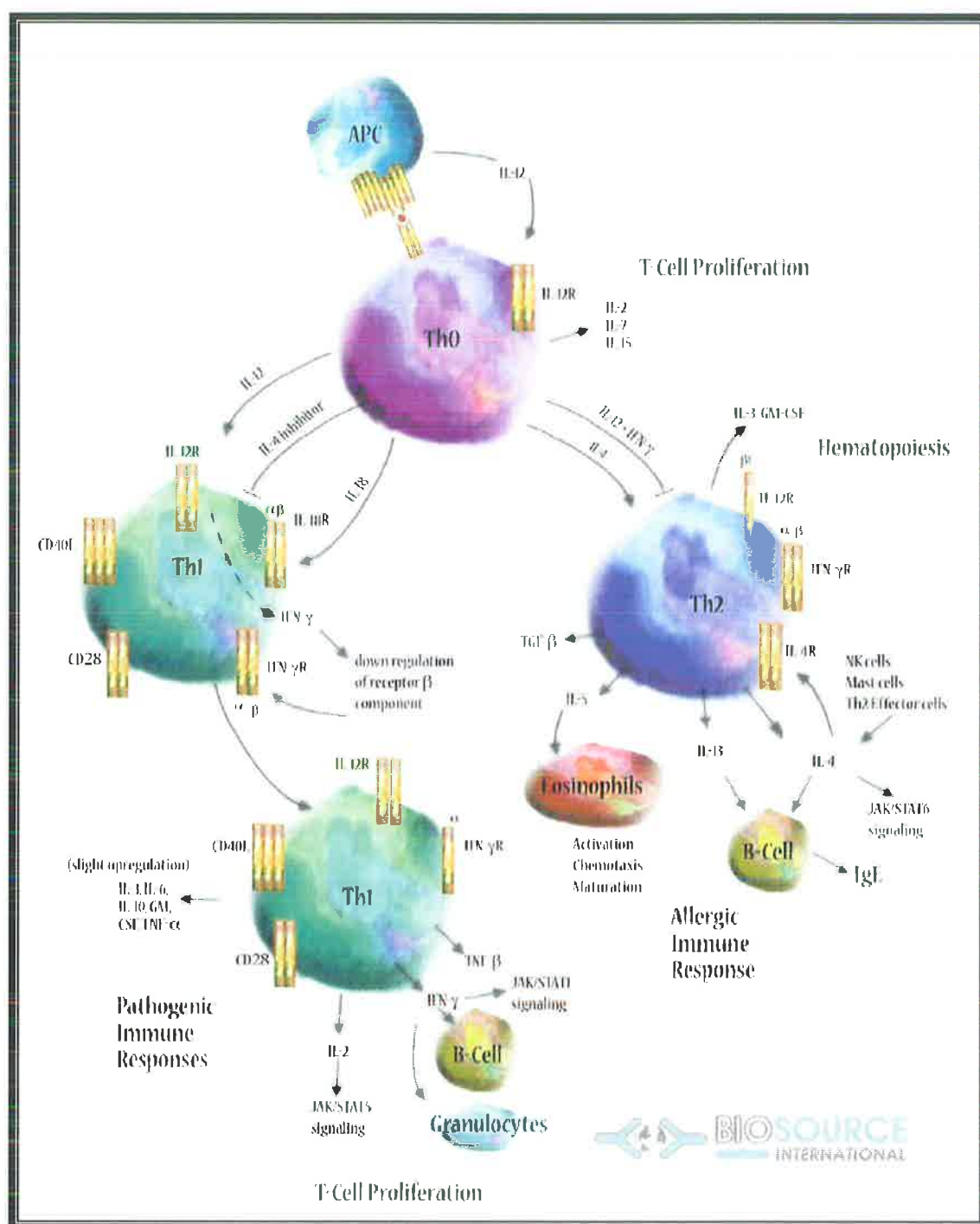


Figure 1.2 Schematic representation of cytokines influencing the development of antigen-activated naïve CD4⁺ T cells into Th1 and Th2 cells.

Taken from: <http://www.biosource.com/content/techCornerContent/immunPathways.asp>

The Potential Immunomodulatory Effects of the Statins in Uveitis

CD2, which has been shown to participate in adhesion and activation of T cells, has also been demonstrated to influence the Th1/Th2 balance. CD2 signals decrease IFN- γ and GM-CSF levels induced in human cells stimulated via the TCR thereby polarizing cells towards the Th2 pathway (Semnani *et al* 1998)

Four developmental stages need to be completed in order for a naïve T cell to be fully differentiated into an effector T cell. They are: activation of cytokine genes; commitment of the cells; silencing of opposing cytokine genes; and physical stabilization (Grogan *et al* 2001). Several signaling cascades are involved in the initiation of the first stage.

Firstly antigen specific T cell activation is initiated by tyrosine phosphorylation of specific domains named immunoreceptor tyrosine-based activation motifs (ITAM)s. ITAMs are present on CD3 and associated with the TCR. A chain of tyrosine kinase activation events leads to the recruitment of adaptor molecules to the cell membrane, and to the activation of small guanosine-5-triphosphate (GTP)ases. The activation of a cascade of serine and threonine kinases, generically named mitogen activation kinases or MAPK is then facilitated; these induce cell proliferation and the activation of activator protein (AP)-1 transcription factors (Santana and Rosenstein 2003).

Membrane lipids are also important for the generation of other signaling cascades. For example, the phosphatidil inositol kinases (PI3K and PI4K) and protein lipase C (PLC) mediate the production of second messengers, like inositol 3 phosphate (IP3), that

The Potential Immunomodulatory Effects of the Statins in Uveitis

generate internal release of calcium which results in the activation of the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin, inducing the translocation to the nucleus of nuclear factor of activated T cells (NFAT) transcription factors important for the expression of a number of cytokine genes (Santana and Rosenstein 2003). The rise of intracellular calcium after TCR/CD3 cross-linking has also been shown to activate calcineurin (Clipstone and Crabtree 1992).

Protein kinases C (PKC) are activated by diacylglycerol, short calcium fluxes, or other lipid second messengers. PKC are serine threonine kinases that are important mediators of T cell activation through the activation of MAP and of I κ B kinase; these lead to the activation of AP-1 and nuclear factor (NF) κ B transcription factors, respectively (Santana and Rosenstein 2003). Cytokines such as TNF- α and IL-1 β activate members of the MAP kinase family, resulting in increased binding AP-1 and NF κ B to DNA (Roitt *et al* 1998)

Many cytokine receptors are associated with molecules called Janus kinases (JAKs). Stimulation of these cytokine receptors involves rapid tyrosine phosphorylation of intracellular proteins such as the signal transducers and activators of transcription (STATs). These transcription factors then undergo dimerization and translocate to the nucleus where they regulate gene expression. For example, IL-4 activates JAK1 and JAK3, leading to STAT6 activation while IL-12 activates JAK1 and tyrosine kinase (Tyk)2 with STAT4 activation (Santana and Rosenstein 2003). IL-2, on the other hand, activates JAK3 with STAT5 induction (Miyasaki *et al* 1994).

The Potential Immunomodulatory Effects of the Statins in Uveitis

The first gene to be expressed is IL-2. IL-2 is a survival and growth factor necessary for expansion of the cell clones (Bird *et al* 1998). The IL-2 promoter has DNA sequences recognized by AP-1, NFκB and NFAT transcription factors induced by all the signaling cascades mediated by MAP kinases, PKC and calcium fluxes. Conversely expression of IFN-γ and IL-4 genes, which are the designated genes for Th1 and Th2 cells, is differentially controlled by transcription factors that become activated by the predominance of one signaling cascade over the other. For example if lymphocytes are activated mainly through calcium mediated cascades they differentiate into Th1 cells. The dominance of PKC signals however, lead to the maturation of cells into the Th2 type (Noble *et al* 2000).

Cytokine gene expression is regulated by a number of transcription factors, some of which are specific for one or other subset of T cells. STAT6, GATA3 and the proto-oncogene, c-Maf are induced in Th2 cells; while STAT4, Ets transcription factor protein (ERM), homeobox protein, Hlx and a member of the brachiury family of T-box transcription factors, T-bet are synthesized in Th1 cells (Murphy *et al* 2000). GATA3 and T-bet can induce and stabilize Th1 and Th2 phenotypes (Szabo *et al* 2000; 2003, Zheng and Flavell 1997). The NFAT family of transcription factors has a differential effect on cytokine gene expression arising from the specificity of binding of the different members of this family on the promoters of the particular genes. For example, NFATp and NFAT4 promote the expression of cytokine genes of the Th1 phenotype and repress Th2 associated genes, whereas NFATc has the opposite effects (Hodge *et al* 1996, Rengarajan *et al* 2002, Xanthoudakis *et al* 1996). NFκB is widely recognized as a critical mediator of

The Potential Immunomodulatory Effects of the Statins in Uveitis

immune and inflammatory reactions being a key player in the regulation of both innate and adaptive immunity. It is found in the cytoplasm of most cell types where it is associated with the inhibitory protein I- κ B. A wide array of stimuli such as TNF- α , IL-1, T cell activation signals, bacterial endotoxins, viral transforming proteins, certain growth factors and reactive oxygen intermediates lead to the rapid nuclear accumulation of NF κ B following the phosphorylation of I- κ B kinases (IKKs) (Verma 2004). NF κ B is one of the pivotal regulators of pro-inflammatory gene expression and induces transcription of pro-inflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteinases, cyclo-oxygenase 2 and inducible nitric oxide (iNOS) (Baeuerle and Baichwal 1997, Tak and Firestein 2001). These specific factors are induced by cytokines by the fourth day of differentiation (Grogan *et al* 2001) and mark the commitment stage of effector T cells.

The genesis of an effector T cell involves the activation and repression of genes as well as a full differentiation program where chromatin is remodelled and the accessibility of the selected genes to transcription factors is increased. Simultaneously the silencing of cytokine genes of the opposite phenotype occurs thereby constituting the third stage of development. TCR and cytokines mediate chromatin rearrangement in T cells (Agarwal and Rao 1998, Weinman *et al* 2001, Zhao *et al* 1998)

At a certain point, the process of chromatin rearrangement becomes irreversible. At this stage of stabilization or fourth stage of differentiation, T cells are not readily able to convert from one phenotype to another (Grogan *et al*)

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.3 Immunologic Tolerance and Autoimmunity

Immune system disorders may occur in the absence of clear evidence for a direct causation by a foreign infective organism. In many of these diseases, antibodies and T cell-mediated immune responses to 'self antigens' are in evidence and these disorders can be considered to be 'autoimmune diseases'. In the normal state, however, there is an immunologic non-responsiveness to self-antigens, otherwise known as tolerance. Tolerance may be defined more fully as antigen-induced inhibition of the development, growth or differentiation of antigen-specific lymphocytes (Abbas *et al* 1994). Tolerance has the following properties:

- 1) It is antigen specific – individuals who are tolerant to one antigen are not necessarily tolerant to all or even a second antigen.
- 2) Tolerance to autoantigen is acquired during development – immature lymphocytes develop tolerance more easily than adult ones
- 3) Maintenance of tolerance requires persistence of (auto)antigen throughout the life of the individual.
- 4) Tolerance to foreign antigens can be induced if the conditions are right.

1.3.1 Mechanisms of Tolerance

Tolerance is always acquired but differences arise when the acquisition of tolerance occurs in the thymus during development (central) or in the peripheral lymphoid tissues during adulthood (peripheral). Several mechanisms of tolerance have been suggested.

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.3.1.1 Clonal Deletion and Anergy

Clonal deletion refers to the process by which the immune system prevents autoimmunity by deleting lymphocytes bearing receptors specific for ubiquitous self molecules. They are deleted in the thymus at an early stage in lymphoid cell development and are therefore absent from the repertoire of mature lymphocytes.

Anergy is the state of inactivation that a T cell undergoes when it recognizes an antigen in the absence of co-stimulation by an APC. This allows self antigens expressed on tissue cells to induce tolerance in the peripheral T cell population and is considered to be the main mechanism of induction of tolerance in the adult. The inability of anergic T cells to produce IL-2 is the most important factor in their prevention of proliferation and differentiation into effector T cells when they encounter antigen even if the antigen is subsequently presented by antigen-presenting cells that express co-stimulatory cells. (Janeway *et al* 2005)

1.3.1.2 Idiotypic Regulation

Antibodies and TCRs, being proteins in their own right can act as antigens when injected into allogeneic individuals as well as in syngeneic individuals with the induction of an immune response in the same way that tumor cells induce immune responses by virtue of being new mutations. This is termed an idiotypic response with the site on the antibody or TCR that causes the response called an idioypic. Each idiotypic response in turn induces its own response which may be inhibitory or stimulatory to the overall

The Potential Immunomodulatory Effects of the Statins in Uveitis

immune reaction. This network theory may be a way of diluting out the original immune response thereby switching it off (Jerne *et al* 2004).

1.3.1.3 Suppressor or Regulatory T (Tr) cells

Powrie *et al* (1994) was the first to describe a subset of CD4⁺ that were able to prevent inflammatory bowel disease, in a murine model, through the inhibition of Th1 responses. These regulatory T (Tr) cells were first described as belonging to the subset of murine peripheral lymphocytes designated CD25⁺ CD4⁺ by Takahashi *et al* (1998). A similar subset has since been identified in human peripheral blood and was shown to strongly inhibit the proliferative responses of both naïve and memory CD4⁺ T cells to alloantigens. They were also found to produce IL-10, TGF-β along with low levels of IFN-γ and no IL-2 or IL-4 (Levings *et al* 2001). More recently the differentiation and expansion of T cells with regulatory function from human peripheral lymphocytes by stimulation in the presence of TGF-β has been demonstrated (Rao *et al* 2004). Furthermore it has just been shown that tolerance by co-stimulation blockade preferentially works through CD25⁺ CD4⁺ regulatory T cell-mediated suppression thereby contributing to the functional dominance of Tr cells in their prevention of alloreactive effector T cell expansion (Coenen *et al* 2005).

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.3.1.4 Inhibitory Cytokines

Immune responses may be 'switched off' or downregulated by inhibitory cytokines. One such cytokine is TGF- β which has been demonstrated to be a potent immunosuppressive molecule. IL-4, IL-6 and IL-10 have been demonstrated to inhibit DTH T-cell mediated responses, while IFN- γ inhibits B cell responses.

1.3.1.5 Failed Tolerance

Autoimmune disease arises when there is a failure to develop or maintain tolerance to autoantigens. Tolerance is incomplete to an extent and 'natural autoimmunity' to autoantigens is the norm. This has been shown for most autoantigens including retinal soluble antigen (SAg) in the eye. There are immunological mechanisms in place, however, that inhibit excessive expression of natural autoimmunity (Forrester *et al* 1996).

1.3.2 Autoimmune Disease

Autoimmune disease is the dysfunction or damage of tissue caused by immune responses to autoantigens. It can take many forms, from organ and even cell-specific antibody-mediated diseases to widespread systemic diseases such as systemic lupus erythematosus where the antigen, deoxyribonucleic acid (DNA) is distributed in all tissues. Autoimmune disease can be antibody-mediated or initiated by CD4⁺ T cells with damage to tissues caused by any of the well documented forms of immunopathologic DTH mechanisms (types I-IV).

The Potential Immunomodulatory Effects of the Statins in Uveitis

Several mechanisms have been postulated to explain this phenomenon:

- 1) Molecular mimicry between foreign and autoantigen – with the large variety of antigenic peptides occurring in infective organisms, sequence homologies occur with predictable frequency. Foreign antigenic peptide processing might therefore lead to activation of autoreactive T cells if the foreign antigen is ‘mistaken’ for self.
- 2) Idiotypic dysregulation – an idiotypic response to a foreign antigen may reveal an idio type on an antibody or TCR that has sequence similarity to an autoantigen and thus lead to activation of the autoreactive T cells.
- 3) Polyclonal B cell activation – certain compounds such as endotoxin can activate B cells directly, either to produce cytotoxic antibody or to act as APCs and therefore present autoantigen to receptive T cells
- 4) Failure of suppressor cell activity – with the failure of their ability to homeostatically inhibit autoreactive T cells, possibly through direct cell contact or release of cytokine, an autoimmune response might be induced.
- 5) Superantigen – simultaneous activation of many subsets of T cell by superantigens, which do not require processing since they link the T cell and MHC antigen directly, may also lead to activation of autoreactive T cells (Forrester *et al* 2002).

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.4 The Ocular Immune System

The eye generates all aspects of immune response like any other tissue. Both the innate and acquired immune systems function in ocular defense mechanisms but the immune response is modulated by the cells and tissues of the eye. In this respect the eye (and the brain) are regarded as immunologically privileged.

1.4.1 Immune Privilege

The concept of immune privilege was first described in 1948 by Peter Medawar as the “protection against rejection conferred upon foreign tissue when grafted to a particular site”. From observations upon the longevity of survival of allogeneic or xenogeneic transplants to sites such as the eye, brain and testis he surmised that their evasion from the immune system, or immune privilege, was a result of physical isolation of the organ by means of a cellular barrier. In the eye such barriers exist as the blood-retinal barrier (BRB) and the blood-aqueous barrier (BAB) which are collectively known as the blood-ocular barrier. In addition the absence of intraocular lymphatic drainage channels allows the putative sequestration of antigens in the eye (Medawar 1948). The ability of immune privileged sites or tissues to accept foreign grafts is no longer thought to be simply due to the presence of isolating cellular barriers and lack of efferent lymphatics. Various other factors have also been identified including the absence of blood vessels in the cornea and presence of specialized vascular endothelium within the iris stroma; direct drainage of AH into the venous circulation; soluble immunomodulatory factors in AH that are released from the cells and tissues surrounding the anterior chamber (AC) and secreted by the ciliary body; immunomodulatory ligands

The Potential Immunomodulatory Effects of the Statins in Uveitis

on the surface of ocular parenchymal cells and indigenous, tolerance-producing APCs in iris stroma and (perhaps) in the trabecular meshwork and outflow pathways (Streilein 2003).

The generation of an immunosuppressive microenvironment is contributed to by the local production and secretion of cytokines in AH such as transforming growth factor (TGF)- β (Cousins *et al* 1991, Willbanks and Streilein 1991). TGF- β_2 has been found to downregulate the production of the Th1-inducing cytokine IL-12 while inducing the synthesis of IL-10 (D'Orazio and Niederkorn 1998). Moreover TGF- β_2 also suppresses NK cells, the activation of T cells and induces APCs to present antigen in a manner that demotes the generation of a typical Th1 response (Niederkorn 2002). There are also the presence of neuropeptides such as α -melanocyte stimulating hormone, vasoactive intestinal peptide and calcitonin gene-related peptide which suppress Th1 DTH responses in the eye (Taylor *et al* 1994, Taylor 1999). Macrophage migration inhibitory factor (MIF) is also present which provides additional suppression of the potentially injurious NK cells, while AH soluble and corneal endothelial cell membrane-bound inhibitors of complement protect the eye against the intraocular activation of the complement cascade (Bora *et al* 1993, Shimada 1970). In addition, endothelial cells also have reduced or absent MHC class Ia and II expression thereby protecting the corneal endothelium from injury inflicted by effector T cells that have been primed elsewhere. The presence of intratissue barriers i.e. tight junctions in retinal pigment epithelial (RPE) cells also contributes to a static ocular immune environment. RPE cells as well as other ocular tissues and APCs also have Toll-like receptors (TLR) which recognize microbial pathogens on their surface.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Their resultant signaling plays an important role in innate and adaptive immune responses within the retina (Chang *et al* 2004, Kumar MV *et al* 2004). Also important is the secretion of corticosteroids and the presence of the apoptosis-inducing cell membrane molecule, Fas ligand (FasL). FasL (CD95L) is widely expressed on ocular cells and is effective in deleting inflammatory cells that enter the eye in response to various insults (Bechmann *et al* 1999, Griffith *et al* 1995).

Current research suggests that the CNS and retina are constantly surveyed for foreign antigen by activated T cells (Hickey *et al* 1991). Although these cells are able to enter the CNS, several mechanisms are thought to prevent T cell-initiated immune responses. Two known mechanisms of T cell downregulation within the CNS are the presence of downregulatory cytokines and the induction of apoptosis (Pender *et al* 1993). Evidence suggests the induction of apoptosis, as a mechanism of immune privilege, is crucial for the protection of the delicate microenvironment of the eye and brain for the preservation of organ function (Griffiths *et al* 1995).

1.4.1.1 The Blood-Retinal Barrier (BRB)

1.4.1.1.1 Anterior BRB

The tight junctions that exist between the retinal endothelial cells (RECs) of the retinal vascular endothelium constitute the BRB (Figure 1.3). The retinal vessels are impermeable to the passage of molecules greater than 20-30000 Da, and small molecules such as glucose and ascorbate are transported by facilitated diffusion (Forrester *et al* 1996). RECs which are thought to share numerous properties with brain endothelial cells

The Potential Immunomodulatory Effects of the Statins in Uveitis

of the blood-brain barrier (BBB) are known to be in direct contact with circulating leukocytes and are therefore thought to mediate control of leukocyte extravasation. RECs have been characterized as poor at binding lymphocytes (Hughes *et al* 1988, Male *et al* 1990, Wang *et al* 1993) and under normal conditions are thought to be low expressors of MHC class II. An enhanced expression of MHC class II has been demonstrated with experimental autoimmune uveoretinitis (EAU) however (Liversidge *et al* 1988) and ICAM-1 can be induced upon REC in response to IFN- γ (Liversidge *et al* 1990).

1.4.1.1.2 Posterior BRB

The role of the RPE in the posterior BRB is based also on the presence of tight junctions. In contrast to the vascular endothelium, the RPE lies beyond the vasculature within the cellular architecture of the eye. It is therefore thought the leukocyte recruitment at the posterior BRB is in part regulated by the choroidal endothelium as a result of cytokine influence from the RPE. During the course of EAU the RPE is thought to be activated and is induced to express MHC class II antigens (Chan *et al* 1986) and has been shown to be capable of antigen presentation as a result of trypsin treatment of cells (Liversidge *et al* 1990).

The Potential Immunomodulatory Effects of the Statins in Uveitis

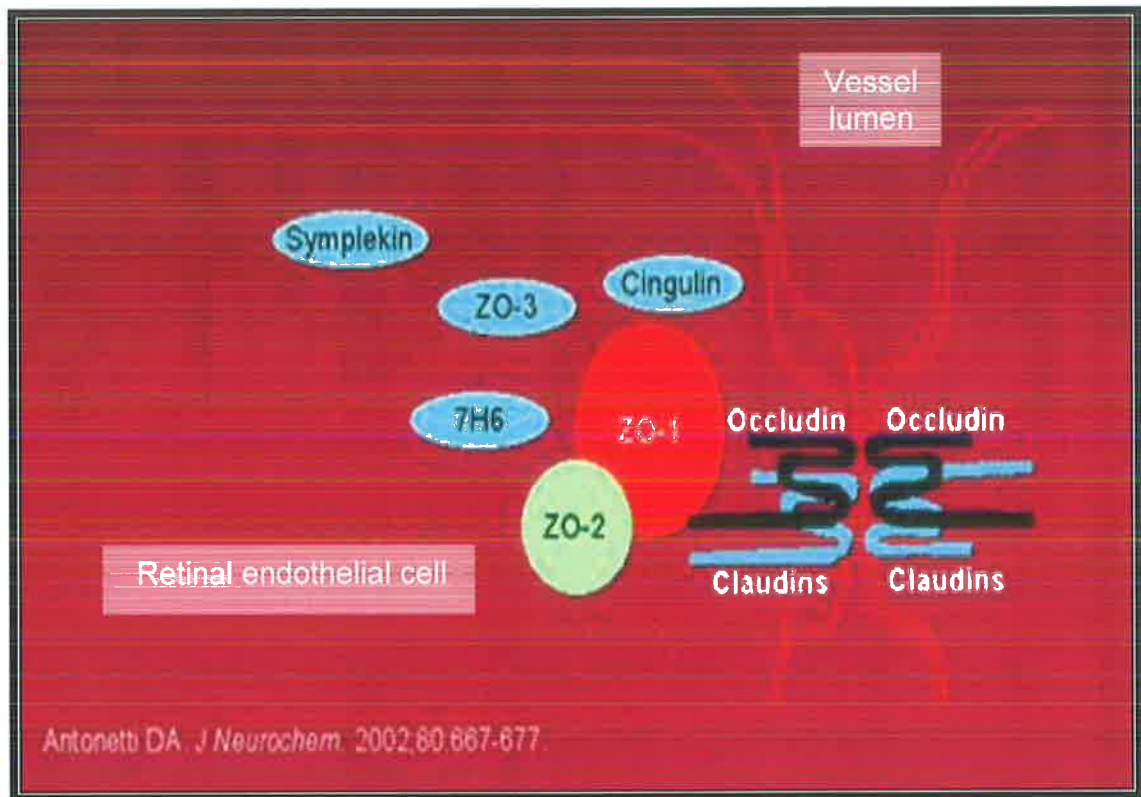


Figure 1.3 An illustration of the tight junction proteins of the blood-retinal barrier.

ZO-1, ZO-2 and ZO-3 = Zonula Occludens 1, 2 and 3

Tight junctions create a selective barrier to lipids, proteins, water and solutes.

Symplekin is located in the cytoplasm and is recruited in cells forming tight junctions.

Cingulin is located at the junctional region and is composed of two coiled peptides.

ZO proteins have a molecular structure which indicates a functional capacity for multiple protein/protein interactions possibly enabling organization of tight junctions.

7H6 may function to regulate paracellular permeability.

Occludins and claudins are transmembrane proteins that form the seal between cells containing tight junctions.

Taken and adapted from:

<http://images.medscape.com/pi/editorial/cmecircle/2003/2636/images/gardner/slide07.gif>

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.4.1.2 The Blood Aqueous Barrier (BAB)

Aqueous humor (AH) is produced by nonpigmented ciliary epithelial cells and is derived from plasma. It is composed however of different concentrations of electrolytes and other small molecules, and a lower concentration of specific proteins. Thus a 'barrier' exists between the plasma transudate in the ciliary body stroma and the AH in the posterior chamber of the eye. Tight junctions between nonpigmented ciliary epithelial cells form this barrier to the free diffusion of molecules. In the iris, where tight junctions between the epithelial cells do not exist, the barrier is formed by tight junctions between the vascular endothelial cells. These endothelial tight junctions contain the same set of proteins as epithelial tight junctions (Forrester *et al* 1996).

1.4.1.3 Anterior Chamber Associated Immune Deviation (ACAID)

Immune privilege within the eye is also an active immunoregulatory process that is thought to be achieved partly through the initiation of a deviant form of systemic immunity in response to antigens present in the eye (Streilein 1987). ACAID is a stereotypic antigen-specific systemic immune response to eye-derived antigens that is typified by the selective deficiency of Th1 type antigen-specific delayed hypersensitivity and B cell-derived complement-fixing antibodies. ACAID is observed when a variety of antigens including viruses, corneal allografts, tumor cells and hapten-derived spleen cells are introduced into the anterior chamber (Streilein *et al* 1997) and relies upon the eye spleen axis to remain anatomically intact for 4-5 days post inoculation (Streilein and Niederkorn 1981, Niederkorn and Streilein 1982). Eyes of EAU animals are unable to support the induction of ACAID, which is believed to be due to the disruption of

The Potential Immunomodulatory Effects of the Statins in Uveitis

important components of immune privilege, which may encompass breakdown of the blood-ocular barrier and loss of an immunosuppressive environment (Ohta *et al* 1999). Tr cells have also been demonstrated to influence the induction of ACAID (Sonoda *et al* 2001). It is of note that ACAID has now been shown to be inducible in mice, rats, rabbits and monkeys and there is also circumstantial evidence that ACAID exists within man (Kezuka T *et al* 2001, Kezuka T *et al* 2002).

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.5 Intraocular Autoimmune Disease

Within developed countries, intraocular inflammatory eye disease remains a significant cause of visual handicap in the working age population (Rothova *et al* 1996, Suttorp-Schulten *et al* 1996). Its aetiology is deemed multifactorial and includes genetic susceptibility, nutritional status, innate stimuli such as injury and concomitant ocular and systemic infection, and the presence of autoreactive T cells. When no obvious infectious agent is isolated in patients with uveitis, it is presumed that autoimmunity is the underlying pathogenesis (Forrester 1992). Despite retinal antigen-specific Th1 CD4⁺ mediating experimental models of posterior uveitis, what triggers uveitis in man remains unknown (De Smet *et al* 1998, Forrester *et al* 1989, Froebel *et al* 1989, Hirose *et al* 1988). The animal models which have significantly helped delineate the molecular mechanisms underlying ocular autoimmune disease and ocular inflammation are divided into 3 groups:

- 1) Uveitis mediated through nonocular-derived antigens
- 2) EAU
- 3) Ocular, but nonretinal, antigen-derived autoimmune disease

Uveitis mediated through nonocular-derived antigen models includes endotoxin-induced uveitis (EIU), cytokine-induced inflammation and adjuvant-induced uveitis. The effects of EIU are likely the result of the lipid moiety within the lipopolysaccharide (LPS) molecule (de Smet and Chan 2001). Shortly after injection, an acute inflammatory response emerges in the anterior segment of the eye (Rosenbaum *et al* 1980). EIU is thought to represent a number of sight-threatening human inflammatory eye diseases such as Behçet's disease and Reiter's disease. A variety of inflammatory factors have been suspected to contribute to the development of EIU. Upregulation of TNF- α IL-1 β

The Potential Immunomodulatory Effects of the Statins in Uveitis

and IL-6 messenger ribonucleic acids (mRNAs) within ocular tissues during EIU have been reported (Planck *et al* 1994, Yoshida *et al* 1994). In addition these cytokines have been reported directly in AH of rat eyes exhibiting EIU (De Vos *et al* 1994, Hoekzema *et al* 1991).

1.5.1 Experimental Autoimmune Uveoretinitis (EAU)

EAU enables the study of posterior uveitis through it being a prototypic CD4⁺ T-cell mediated disease which has a very polymorphic phenotype, manifesting itself as fulminant retinitis, granulomatous choroiditis or segmental vasculitis depending on the immunizing antigen, its dose, and the chosen animal model. Thus it can mimic a wide variety of human inflammatory conditions and is therefore considered a good experimental model for investigating how T cells cross the BRB, induce retinal inflammation and uveitis (Pras *et al* 2004). A number of experimental models of posterior uveitis have been established using a variety of retinal antigens in a number of animal species with models described in the rat, mouse, guinea pig, rabbit and monkey (Caspi *et al* 1997). Of note is that species-specific and strain-specific differences exist between different models of EAU. Because EAU can be induced in so many different animal species, including the primate, it is tempting to consider that retinal antigens are responsible for a variety of human uveitis conditions of unknown origin (Pras *et al* 2004).

1.5.1.1 Retinal Autoantigens

EAU can be induced by SAg, interphotoreceptor retinoid-binding protein (IRBP), rhodopsin, recoverin and other retinal proteins (Bieganowska *et al* 1997, de Smet *et al*

The Potential Immunomodulatory Effects of the Statins in Uveitis

1990, de Smet and Chan 2001, Yamamoto *et al* 1993). SAg is a 48kD protein of the retina. Located within the rod outer segments, SAg is involved in the phototransduction cascade and responsible for binding photoactivated-phosphorylated rhodopsin. SAg from retinal rod photoreceptor cells (PRCs) induces a CD4⁺ T cell-mediated autoimmune disease of the retina and uveal tract of the eye and the pineal gland (Chan *et al* 1985). IRBP is a 148 kD protein found within the photoreceptor matrix and is thought to transport vitamin A between the PRc layer and the RPE. IRBP immunization of Lewis rats induces severe autoimmune uveoretinitis and pinealitis (Dua *et al* 1992, Gery *et al* 1986). Rhodopsin and its illuminated form, opsin, is the rod cell visual pigment protein. The pathogenicity of this 40kD protein has been shown to be conformation-dependent (Schalken *et al* 1988). Recoverin is a 23 kD calcium-binding protein present in certain retinal neurons: rods, cones, a subset of bipolar cells and a minor subpopulation of cells in the ganglion cell layer (Dizhoor *et al* 1991, Milam *et al* 1993, Wiechmann and Hammarback *et al* 1993). It may regulate visual transduction in rods and cones and recently it has been inferred that it potentiates light-triggered phosphodiesterase activity in retinal rods (Makino *et al* 2004). Synthetic peptides containing the recoverin sequence have been shown to induce severe EAU (Ohkoshi *et al* 2001)

1.5.1.2 Induction of EAU

SAg-induced uveitis in Lewis rats is a short acute disease (De Kozak *et al* 1981, Mochizuki *et al* 1985). A similar disease can be induced by immunization of synthetic peptides of retinal autoantigens such as SAg or IRBP in an antigenic adjuvant, such as Freund's adjuvant (Fling *et al* 1991, Hankey *et al* 2001). The amino acid sequences of

The Potential Immunomodulatory Effects of the Statins in Uveitis

SAg from bovine, human, murine and rat retinas were deduced from cDNA sequencing and showed high homology (Abe and Shinohara 1990, Shinohara *et al* 1987, Tsudu M *et al* 1988, Yamaki *et al* 1988). The cleavage fragments of SAg and synthetic peptides were used to identify domains involved in lymphocyte recognition, immunogenicity and pathogenicity. In the Lewis rat, specific short amino acid sequences were able to induce EAU and proliferation of sensitized lymphocytes (de Smet *et al* 1993, Gregerson *et al* 1990). De Smet *et al* (2001) documented cellular responsiveness to ocular antigens in man to be augmented during active disease rather than remission, in diffuse forms of uveitis (panuveitis) rather than purely posterior ones, in subacute or chronic disease states rather than in acute self-limited disease, and after the disease has evolved for some time.

As yet, no definitive link has been made between retinal autoantigens and human retinal inflammation, although retinal autoantigens are thought to be implicated at some time in the pathogenesis of a number of uveitic conditions. Over the years, a number of researchers have autoimmunized themselves with SAg or IRBP. None, with the possible exception of one person, incurred ocular sequelae (Nussenblatt *et al* 1981).

1.5.2 Leukocyte Trafficking in Immune-mediated Diseases of the Posterior Segment

Leukocyte recruitment from the circulation to peripheral tissues is a key event in the pathogenesis of inflammatory disease including inflammatory diseases of the retina. It is postulated that it could be a sequelae following exposure to an inflammatory stimulus which induces clonal expansion and release of activated T cells from secondary lymphoid tissue. Such cells circulate in the blood stream producing cytokines and induce short-term

The Potential Immunomodulatory Effects of the Statins in Uveitis

chemokine-mediated up-regulation of adhesion molecules on endothelial cells (ECs) in various tissues permitting transendothelial migration (TEM) of T cells into the tissues. If some of these activated T cells meet cross-reactive cognate autoantigen, autoimmune tissue damage might ensue (Xu *et al* 2003).

In the eye the process of TEM, which is also termed extravasation or diapedesis, is dependent upon the interaction of activated leukocytes with ECs that form the BRB. The 'multistep paradigm' culminating in diapedesis was first described by Springer in 1994 and consisted of four sequential stages; these are, leukocyte tethering and rolling of leukocytes along the endothelium (Springer 1994), EC signaling to leukocytes, firm adhesion and then diapedesis (Figure 1.4). However, it has since been shown that leukocytes are able to signal to the endothelium and therefore there are five main stages involved in leukocyte TEM. T cells are able, at least in experimental models, to upregulate ICAM-1 and induce transient breakdown of the BRB (Xu *et al* 2003) possibly through a paracellular route in the initial stages of the disease (Lightman and Greenwood 1992). From the work of Hickey (1991) on the migration of haematogenous cells through the BBB and the initiation of CNS inflammation, it would appear that T cells of both CD4⁺ and CD8⁺ groups require activation on crossing blood-endothelial barriers in order to initiate inflammation during autoimmune reactions (Hickey 1991).

The Potential Immunomodulatory Effects of the Statins in Uveitis

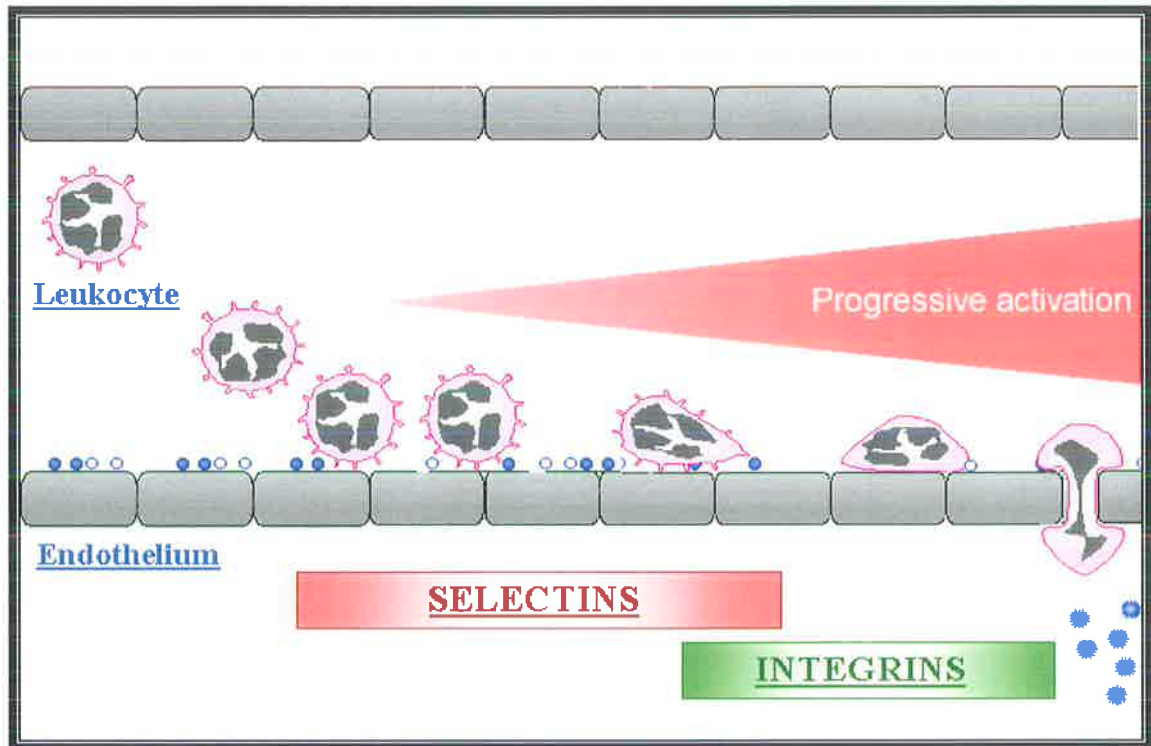


Figure 1.4 Leukocyte transendothelial migration.

The leukocyte adhesion cascade is a sequence of adhesion and activation events that ends with extravasation of the leukocyte, whereby the cell exerts its effects on the inflamed site. At least four steps of the adhesion cascade are involved in leukocyte adhesion to and migration out of the vascular endothelium: (1) Tethering and rolling, in which selectins and glycol-conjugated selection-ligands are involved; (2) Slow rolling and activation, mediated by soluble and/or membrane-bound chemokines; (3) Firm adhesion and (4) Transmigration, both mediated by antigens and their ligands, eg intercellular adhesion molecules. Each of these four steps appears to be necessary for effective leukocyte recruitment, because blocking any step can severely reduce leukocyte accumulation in the tissue.

Taken from: www.helsinki.fi/ml/biotiet/biokemia/lag/fig9.html

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.5.3 Cytokines in Immune-mediated Diseases of the Posterior Segment

The continuous, dynamic control of immune responses is conducted by cytokines which control leukocyte activation and function including APCs, antigen-specific T cells and non-specific leukocytes such as macrophages and granulocytes. Furthermore, tissues such as the eye are imbued with powerful immunomodulatory mechanisms in which cytokines play a critical role in controlling immune responses, limiting tissue damage and restoring homeostasis (Dick 1999, Dick and Carter 2003). Experimental models of anterior and posterior uveitis demonstrate a pivotal role for antigen-specific CD4⁺ T cells and pro-inflammatory cytokines IFN- γ , IL-2 and TNF- α (Forrester 1992). Studies in man have confirmed, for the most part, that immune responses in uveitic patients are pro-inflammatory, corresponding to the Th1 CD4⁺ T cell responses seen in animal models (Kilmartin *et al* 2001). Increased CD4⁺ T cell IL-2 receptor (R) and IFN- γ expression (Sugi-Ikai *et al* 1998) and circulating IL-2R (Arock-Mettinger *et al* 1990, Scheinberg *et al* 1992) and ICAM-1 (Whitcup *et al* 1999) have all been documented. On the premise therefore that during uveitis ocular antigens are presented via DC, CD4⁺ T cells become activated and polarized depending upon cytokine stimulation corresponding to the Th1/Th2 paradigm described in mice (Mosmann and Coffman 1989). Within this framework it appears that not only do the principle cytokines drive either a Th1 (IFN- γ , IL-2 and IL-12) or Th2 (IL-4, IL-5 and IL-10) response but also the same cytokines are capable of mutually regulating cytokine activity and resultant T cell and non-T cell activation. In addition, IL-2 and IL-15 regulate generation of antigen-specific regulatory cells (Dieckmann *et al* 2001), including TGF- β secreting Th3 cells (Weiner 2001) and IL-10-secreting Tr cells (Read and Powrie 2001).

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.5.3.1 IL-1 β

IL-1 β along with IL-1 α make up IL-1. Each has its own receptor but receptor usage is not highly restricted thus IL-1 α and IL-1 β have broadly similar effects on cells (Forrester *et al* 2002). IL-1 β is a pro-inflammatory cytokine derived predominantly from activated macrophages but also from B cells and ECs. It has been postulated as an important factor in the pathogenesis of human retinal inflammation. Experimental animal models have demonstrated that intravitreal injected IL-1 β is associated with a breakdown in the BRB and induces an inflammatory response characterized by an infiltrate of polymorphonuclear and mononuclear cells (Bamforth *et al* 1997, Claudio *et al* 1994). The majority of cells have been shown to enter the retina by migrating through retinal ECs with a smaller contribution from the ciliary body (Bamforth *et al* 1997). It has also been found in the aqueous and vitreous aspirates of patients with uveitis and may act as a local amplification signal in pathological processes associated with chronic eye inflammation (Franks *et al* 1992).

1.5.3.2 IL-2

IL-2 is produced by activated T lymphocytes and can activate T cells and NK cells in cytotoxic responses. Activation of T cells through the TCR is followed by a cascade of intracellular signaling events, culminating in the transition from the G₀ to the G₁ phase of the cell cycle and the induction of cytokine gene expression, leading to secretion of IL-2 and surface expression of the IL-2R. The interaction of IL-2 with IL-2R initiates a second phosphorylation cascade and sequential wave of gene expression that drives the cells to S phase, followed by clonal expansion (Crabtree 1989, Smith 1988). In

The Potential Immunomodulatory Effects of the Statins in Uveitis

addition to activating T cell proliferative responses genes, IL-2 can also induce expression of the cytokines IL-3, GM-CSF (Oster *et al* 1989), IFN- γ (Kasahara *et al* 1983), TNF- α , TNF- β (Nedwin *et al* 1985), IL-4 (Bello-Fernandez *et al* 1991) and IL-5 (Enokihara *et al* 1989). IL-2 has been shown to induce inflammation in experimental models after intraocular injection (Samples *et al* 1993). In humans, IL-2 has been demonstrated in ocular tissues (Hooks *et al* 1988) and in the AH of uveitis patients (Lacomba *et al* 2000). It has also been demonstrated to be produced by vitreous humor (VH) derived T cell lines of uveitis patients (Muhaya *et al* 1999).

1.5.3.3 IL-4

IL-4 is a multifunctional cytokine which can be secreted by several cell types, such as basophils and mast cells but the most important producers of IL-4 are CD4⁺ Th2 cells activated by Ag recognition through TCR. Among the effector functions of IL-4 are induction of IgE production by B cells (Gascan *et al* 1991) and direct differentiation of naïve CD4 T cells into a Th2 population thereby favoring the growth of cells which produce IL-4 and IL-5 while inhibiting the production of IFN- γ (Seder and Paul 1994). IL-4 is also involved in the inhibition of TNF- α and IL-1 production by activated monocytes (te Velde 1990) and in macrophages it has been demonstrated to suppress IFN- γ through the inhibition of NF κ B activity (Takeshita *et al* 1996). It also acts as a growth factor for both B and Th2 cells (Howard *et al* 1982, Romagnani 1993). Since it elicits responses which are associated with allergy, asthma and inhibition of autoimmunity, the regulation of its expression is critical in determining the overall character of immune responses (Dorado *et al* 2002). TCR signaling leads to activation of

The Potential Immunomodulatory Effects of the Statins in Uveitis

NFAT proteins by dephosphorylation mediated by calcineurin (Loh *et al* 1996). Once dephosphorylation has taken place, conformational changes occur, leading to promotion of transcriptional activity (Okamura *et al* 2000). Stat6, which binds to the P1 NFAT site, also seems to be essential in the initial IL-4 production during development (Kaplan *et al* 1996). In man, IL-4 has only been detected in low levels in the AH (Lacomba *et al* 2000, Ongkosuwito *et al* 1998) and VH of patients with uveitis (Ongkosuwito *et al* 1998). It has also been found to be produced at low levels by T cells derived from VH of uveitis patients (Muhaya *et al* 1999).

1.5.3.4 IL-5

IL-5 is a cytokine expressed by activated Th2 cells and mast cells, which in humans selectively stimulates differentiation, proliferation and functional activation of the eosinophil lineage. It also acts on mature eosinophils to prolong survival and activation (Takatsu 2004). IL-5 plays a major role in the induction of eosinophilia associated with helminth infection and allergic disease including asthma (Coffman *et al* 1989, Foster *et al* 1996, Lopez *et al* 1988, Sanderson 1992). IL-5 gene transcription in human T cells can be induced by TCR- or cytokine-mediated activation for example with IL-2 and IL-4. Stimulation of either pathway induces similar IL-5 mRNA expression kinetics and requires *de novo* synthesis of regulatory proteins (Valentine and Sewell 1997). High IL-2:IL-5 and IFN- γ :IL-5 ratios have been found in the peripheral blood lymphocyte culture supernatants of patients with presumed sarcoid intermediate uveitis thereby further illustrating the polarization of the systemic immune response towards Th1 in ocular sarcoidosis intermediate uveitis at least (Murphy *et al* 2004).

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.5.3.5 IL-6

IL-6 is a macrophage- and monophage-derived cytokine also thought to be produced by ECs and ocular parenchymal cells (Castell *et al* 1988, Ohta *et al* 2000). It is a pleiotropic cytokine which is able to function in a pro- and anti-inflammatory manner. Readily induced by TNF- α , IFN- γ and IL-1, IL-6 has been shown to stimulate production of acute phase proteins such as fibrinogen and C-reactive protein (Castell *et al* 1988). Evidence exists to suggest that IL-6 is involved in the induction of IL-2 receptor expression, in differentiation, and in proliferation of T cells after stimulation through the TCR for antigen (La Flamme and Pearce 1999). It is even thought to be more active in this respect than both IL-1 and TNF- α (Joseph *et al* 1998). Macrophage activation and the differentiation of B cells to immunoglobulin-secreting plasma cells are other known key roles played by IL-6. In EIU the presence of IL-6 in inflamed AH is thought to antagonize the immunosuppressive properties of TGF- β and also prevent the ocular environment from promoting ACAID. IL-6 has been detected in AH and VH aspirates of patients with uveitis (Franks *et al* 1992). Furthermore, in patients with recent-onset uveitis apoptotic lymphocytes have been found to be absent in their AH. It is therefore proposed that combinations of IL-6 and IL-6R are highly effective inhibitors of T cell apoptosis mediated by uveitis AH (Curnow *et al* 2004)

1.5.3.6 IL-10

IL-10 is an essential anti-inflammatory multifunctional cytokine produced primarily from T cells and activated macrophages. It was first recognized for its ability to inhibit activation and effector function of T cells, monocytes and macrophages. In

The Potential Immunomodulatory Effects of the Statins in Uveitis

addition to its limiting activity on inflammation, IL-10 regulates growth and/or differentiation of B cells, NK cells, Tc and Th cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells. IL-10 plays a key role in differentiation and function of T regulatory (Tr) cells which figure prominently in control of immune responses and tolerance *in vivo* (Moore *et al* 2001). Tr cells themselves produce IL-10 as well as TGF- β (Levings *et al* 2002). IL-10 in this scenario is thought to act as a positive autocrine factor for the development of Tr cells (O'Garra and Barrat 2003). IL-10 also strongly inhibits cytokine production and proliferation of CD4⁺ T cells and T cell clones via its downregulatory effects on APC function (de Waal Malefyt *et al* 1991, Fiorentino *et al* 1991). In addition to depressing APC function, IL-10 also affects DC maturation thereby providing a potential bimodal feedback inhibition of Th1 and Th2 responses (Akdis *et al* 1998, Grunig *et al* 1997, Moore *et al* 2001). IL-10 also directly affects the function of T cells and inhibits IL-2, TNF and IL-5 production depending on activation conditions as well as expression of CXCR4 and chemotaxis in response to the CXCR4 ligand SDF1 (Jinquan *et al* 2000). It is not clear how IL-10 acts as a suppressor for IFN- γ production in T cells, but in macrophages it has been demonstrated that IL-10 reduces the stability of mRNA (Takeshita *et al* 1996). In contrast, IL-10 has stimulatory effects on CD8⁺ T cells and induces their recruitment, cytotoxic activity and proliferation (Groux *et al* 1998, Jinquan *et al* 1993, Santin *et al* 2000, Schwarz *et al* 1994). In patients with uveitis, levels of AH IL-10 have been found to be low by several groups (Calder *et al* 1999, Lacomba *et al* 2000, Murray *et al* 1999).

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.5.3.7 IFN- γ

IFN- γ is produced by CD4⁺, CD8⁺ and NK cells. Specifically CD4⁺ cells of the Th1 type secrete IFN- γ which helps to mediate DTH responses via upregulation of HLA-DR activation of macrophages. IFN- γ has been shown to induce inflammation in rat models after intraocular injection (Lee and Pepose 1990). This pro-inflammatory cytokine has also been identified in ocular tissue (Hooks *et al* 1988), in AH (Abi-Hanna *et al* 1989), in VH-derived T cell lines (Muhaya *et al* 1999) and in the peripheral blood obtained from patients with uveitis (Lacomba *et al* 2000) thereby implicating it in the pathogenesis of the uveitides.

1.5.3.8 TNF- α

TNF- α is an important mediator in metabolic and immune responses and may be regarded as one of the earliest and most critical mediators in inflammation. This pleiotropic cytokine is synthesized by monocytes, macrophages, neutrophils, mast cells, NK and T cells (Tracey 1997). During the inflammatory process it orchestrates the initiation of further leukocytic infiltration via adhesion molecule upregulation, dendritic cell maturation and survival, macrophage activation and driving Th1 T cell responses within tissues (Dick *et al* 2004). Intravitreal injection of TNF- α in rats has been shown to induce acute uveitis with an increase in AH protein and an infiltration of polymorphonuclear granulocytes in the AC (de Vos *et al* 1995) through an increase in BRB permeability (Claudio *et al* 1994). Moreover in EAU, increased tissue concentrations of TNF facilitate the on-going T cell effector responses and macrophage activation (Dick *et al* 2004). TNF- α has also shown to be elevated in the AH of uveitis

The Potential Immunomodulatory Effects of the Statins in Uveitis

patients and is therefore thought to play a role in the pathogenesis of clinical uveitis (Lacomba *et al* 2001)

1.5.4 Chemokines

Chemokines are a family of small, secreted polypeptides, which function to recruit leukocytes to sites of infection and inflammation. They are classified into two main groups based on a particular amino acid sequence involving two cysteine residues: -C-C-chemokines and -C-X-C-chemokines in which the latter contains an intervening noncysteine residue. Many chemokines are known to be produced during an inflammatory reaction and can be produced by EC in response to cytokines such as TNF- α and IL-1. Chemokines are known to play a major role in the control of leukocyte adhesion, chemotaxis and activation (Eugenin and Berman 2003). These chemotactic cytokines include the 'inflammatory' chemokines (CC and CXC) as well as the 'immune' chemokines (C and CXC3) and are therefore thought to play a major role in inducing/regulating inflammation and various immune responses. A number of ocular chemokines have now been discovered which contribute to the recruitment of inflammatory cells into the eye in uveitis. These include monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α and MIP-1 β (Kawashima 2003). More recently, the dual-adhesion molecule-chemokine, fractalkine (FKN, CXXXC), has been found to be expressed in a variety of ocular tissues and cells. It is thought to play an important role in regulating leukocyte efflux in inflammatory eye diseases such as anterior uveitis and retinochoroiditis (Silverman *et al* 2003)

The Potential Immunomodulatory Effects of the Statins in Uveitis

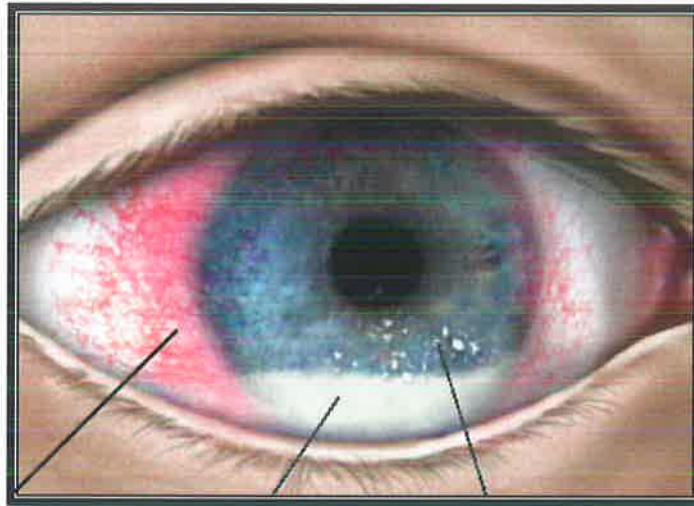
1.5.4.1 IL-8

IL-8 was the first chemokine discovered and belongs to the CXC chemokine family (Yoshimura *et al* 1987). It activates and attracts neutrophils after secretion by lipopolysaccharide-stimulated monocytes (Zeilhofer and Schorr 2000). Following activation of IL-8 receptors there is an increase in the intracellular concentration of free calcium which is responsible for many of the subsequent steps in the neutrophil activation process. IL-8 has been found to mediate neutrophil infiltration in EIU and its presence in the eye has been shown to be regulated by IL-1 and TNF- α (Kawashima 2003, Mo *et al* 1999). IL-8 and its homologues are potent signals for leukocyte migration but not for rolling or arrest (Becker *et al* 2000). Raised IL-8 levels in patients with uveitis have been significantly associated with active disease (Bardak and Aridogan 2004, Klok *et al* 1998, Sakaguchi *et al* 1998).

1.5.5 Clinical Uveitis

Inflammation of the eye can be caused by a variety of diseases other than bacterial infection, many of which threaten vision. These include the group of intraocular immune-mediated diseases, known as uveitis, which is responsible for 10% of all registered blind patients under the age of 65 years. Uveitis is the term given to any inflammatory condition affecting the uvea which is composed of the iris, ciliary body and choroid. Causes of uveitis can include autoimmune disorders, infection or exposure to toxins.

The Potential Immunomodulatory Effects of the Statins in Uveitis



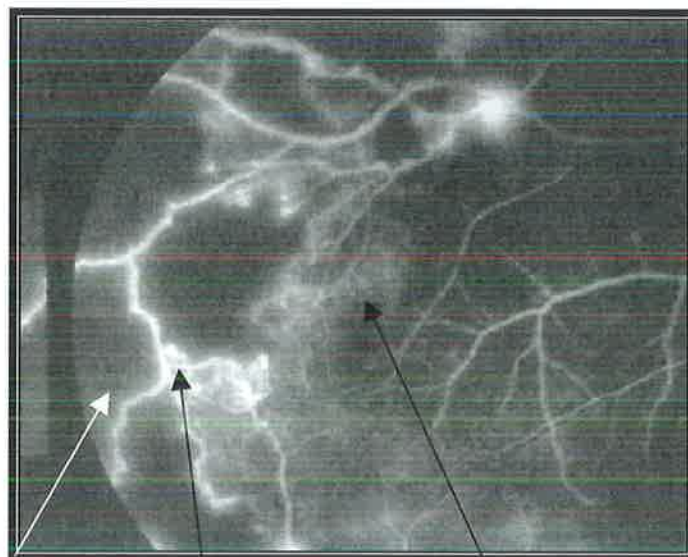
Circumciliary injection

Hypopyon

Keratic Precipitates

Figure 1.5 Illustration of anterior segment of the right eye showing some of the signs of anterior uveitis.

Taken and adapted from: <http://www.vos.dk/vos22003/VOS0203-internet04.html>



Ischaemic retina

Arteriolar vasculitis

Diffuse microvascular leakage (ferning)

Figure 1.6 Fundus Fluorescein Angiogram photo of left retina in posterior uveitis

Taken and adapted from: <http://www.uveitis.org/medical/articles/case/vasculitis.html>

The Potential Immunomodulatory Effects of the Statins in Uveitis

Uveitis is most commonly classified anatomically as anterior, intermediate, posterior or diffuse (panuveitis) depending on which segment of the eye is affected. The most common form of uveitis in Western countries is anterior uveitis (AU, Chang and McCluskey 2002), which involves inflammation in the front part of the eye, usually isolated to the iris or ciliary body (Figure 1.5). This condition is often called iritis and iridocyclitis and is generally the most symptomatic form of uveitis, generally presenting with pain and redness, photophobia and decreased vision. The inflammation may be associated with autoimmune diseases but in most cases occurs in healthy people and does not indicate an underlying disease. Most common in young and middle-aged people, AU does not generally lead to visual loss. Intermediate uveitis (IU) affects the area immediately behind the iris and lens in the region of the ciliary body and pars plana and may also be referred to as ‘cyclitis’ and ‘pars planitis’. IU is classically painless and presents with floaters and decreased vision. This form of disease predominantly affects teenagers or sometimes young children. Symptoms present as visual blurring without pain or redness and usually affect both eyes.

Posterior uveitis is the second most common type of uveitis in Western countries (Chang and McCluskey 2002) and signifies a number of forms of uveitis affecting the posterior segment of the eye. Inflammation presents in the choroid, retina or the blood vessels as choroiditis, retinitis, vasculitis or optic neuritis (Figure 1.6). Posterior uveitis is usually painless but is far more likely to cause visual impairment and is often associated with numerous other systemic diseases including Behçet’s disease and sarcoidosis. Drops and injections fail to reach the back of the eye and treatment is therefore generally

The Potential Immunomodulatory Effects of the Statins in Uveitis

administered systemically, which means the whole body is affected and may suffer side effects. Panuveitis is the third most common type of uveitis in Western countries (Chang and McCluskey 2002) and is the term given to inflammation involving all parts of the eye, including anterior, intermediate and posterior structures. It may produce any or all of the aforementioned symptoms and signs. Complications of uveitis may result in profound and irreversible visual loss, especially when unrecognized or treated improperly with the most frequent complications including cataract, glaucoma, retinal detachment, neovascularization of the retina, optic nerve or iris and cystoid macular oedema.

1.5.5.1 Idiopathic Uveitis

Generally the most common form of AU, intermediate, posterior and panuveitis in Western countries is idiopathic (Chang and McCluskey 2002). In children however, only intermediate and diffuse uveitis cases are commonly idiopathic with juvenile idiopathic arthritis most prevalent in AU and toxoplasmic retinochoroiditis in PU (Cunningham 2000). It is unknown as to whether the immune processes in idiopathic uveitis are the same as those in which a systemic immune disease is also occurring because where there is an associated systemic disease, the ocular inflammatory activity does not necessarily parallel systemic inflammation (Calder *et al* 1999). In addition it has been suggested that in some cases of idiopathic uveitis there is concurrent abnormal systemic immune activation (Deschênes *et al* 1988).

In one study there was a significant increase in IL-2R α chain expression specifically on CD4⁺ T cells in the blood in idiopathic posterior uveitis groups (Feron *et*

The Potential Immunomodulatory Effects of the Statins in Uveitis

al 1995). Similarly a selective increase in AH CD4⁺ T cells, but not CD8⁺ T cells, has since been reported, supporting the hypothesis that CD4⁺ T cells are involved in more aggressive forms of disease because inflammation in idiopathic uveitis is often clinically more severe than in uveitis associated with a systemic disease (Calder *et al* 1999). Idiopathic adult uveitis patients have been shown to have elevated AH and serum levels of TNF- α (Lacomba *et al* 2001), IFN- γ and IL-2 (Lacomba *et al* 2000). A decrease in AH IL-10 has also been demonstrated (Calder *et al* 1999)

1.5.5.2 Behçet's disease

Behçet's disease (BD) is a systemic vasculitis of unknown origin that affects veins and arteries of all sizes producing recurrent mucocutaneous lesions and frequent ocular involvement. Musculoskeletal, neurological and gastrointestinal manifestations are also seen. Prevalence of the disease is highest in Turkey, the Middle East, Japan and Korea. Age of onset is most usually 20-30 years in men, and young patients have a more severe disease progression. It has been associated with the HLA-B51 phenotype but the strength of this association varies worldwide and so the diagnosis is currently clinically based (McCluskey and Powell 2004). Raised IFN- γ and IL-2 levels have been found in the AH of BD patients (Lacomba *et al* 2000). Elevated serum IL-6, IL-8, TNF- α (Bardak and Aridogan 2004) and IFN- γ (Hamzaoui *et al* 2002) levels have been documented in BD patients.

Some patients develop isolated mild anterior uveitis that can be treated adequately with local corticosteroids. Typically ocular involvement leads to pronounced morbidity

The Potential Immunomodulatory Effects of the Statins in Uveitis

due to episodes of severe panuveitis which are associated with occlusive vasculitis affecting both the retinal arteries and veins which results in progressive, irreversible, ischaemic damage to the retina and optic nerve (Chavis and Tabbara 1995) despite aggressive immunosuppressive therapy (Muhaya *et al* 2000). High-dose oral and intravenous corticosteroids are usually used to control acute exacerbations of uveitis and continuous systemic immunosuppression (eg. cyclosporin and azathioprine) to delay onset of severe visual loss and minimize long-term corticosteroid use (Diaz-Llopis *et al* 1990, Hamuryudan *et al* 1997, Yazici *et al* 1997)

1.5.5.3 Sarcoidosis

Sarcoidosis is a common granulomatous inflammatory disorder of unknown cause that typically affects the respiratory system but can also damage the skin, eyes, neurological system and liver. Only histological examination of affected tissue can confirm clinical diagnosis (Jones 2002).

Intraocular sarcoidosis might present as acute or chronic uveitis and typical signs include acute anterior uveitis, chronic anterior uveitis, intermediate uveitis, multifocal choroiditis, retinal vasculitis and optic disc swelling (Rothova 2000). In those patients who present with classic ocular signs without evidence of disease in other organ systems, there are neither predictors for which patients will go on to develop systemic sarcoidosis nor pathognomonic ocular signs to diagnose sarcoidosis. Up to 45% lose vision from the consequences of ocular sarcoidosis (Edelsten *et al* 1999). T cells in AH of sarcoidosis patients have been shown to produce large amounts of IL-1 α , IL-6 and IL-8 (Sakaguchi

The Potential Immunomodulatory Effects of the Statins in Uveitis

et al 1998). Within the peripheral blood of sarcoidosis patients cytokine profiling has revealed increases in IL-2, IFN- γ and TNF- α (Belli *et al* 2000).

Sarcoid uveitis should be managed according to its merits and treatment depends on visual threat. About 50% of patients need systemic steroids or other immunosuppressive medication, such as methotrexate, cyclosporine and mycophenolate mofetil to control ocular inflammation (Edelsten *et al* 1999, Jones 2002).

1.5.5.4 Vogt-Koyanagi-Harada disease

Vogt-Koyanagi-Harada disease (VKH) is a chronic, bilateral, granulomatous panuveitis associated with aseptic meningitis, vitiligo, alopecia and poliosis (Moorthy *et al* 1995). The exact cause of this condition remains unknown, but cell-mediated autoimmunity directed against melanocytes appears to be the common mechanism. VKH disease is one of the most common causes of uveitis in Japan and women are more affected than men. It typically has a prodromal neurological and auditory phase, characterized by headache, fever, hyperacusis, dysacusis and tinnitus followed by severe panuveitis several days later (McCluskey and Powell 2004).

Uveitis is a feature of VKH and is an acute onset panuveitis characterized by multifocal choroiditis, serous retinal detachments and pink, swollen optic discs (Read *et al* 2001). The presence of uveitis usually leads to the diagnosis being considered and is a serious threat to vision, representing the major morbidity of the disease (McCluskey and Powell 2004). T cells from AH of VKH patients have been shown to produce

The Potential Immunomodulatory Effects of the Statins in Uveitis

significantly larger amounts of IL-6, IL-8 and IFN- γ (Sakaguchi *et al* 1998). Serum cytokine profiling of VKH patients has also shown a parallel expression of IFN- γ (Hirayama *et al* 1990).

Treatment is high-dose corticosteroid therapy and as VKH is very sensitive to steroids, rapid improvement of uveitis and other clinical features ensues. A slow tapering course of corticosteroids over 12 months or more is needed to control the disease and some might need steroid-sparing drugs.

1.5.5.5 Reiter's Syndrome

Reiter's syndrome (RS) was classically defined by the triad of arthritis, nongonococcal urethritis and conjunctivitis (Reiter 1916). Although a relatively rare syndrome, RS, also termed reactive arthritis, develops in 1% to 3% of men after a nonspecific urethritis (Keat *et al* 1978) and a variety of enteric infections (Sievers *et al* 1972, Yli-Kerttula 1984). The systemic manifestations of RS usually appear within 1 month after an acute nonspecific urethritis or acute diarrhea. These manifestations combine four syndromes: a peripheral arthritis syndrome, an enthesopathic syndrome, a pelvic and axial syndrome and an extramusculoskeletal syndrome (Amor 1998). RS usually also occurs more frequently in men and usually between the ages of 15 and 40 years. It is associated with the human leukocyte antigen (HLA)-B27 histocompatibility antigen in approximately 75% to 90% of patients (Keat 1988).

The Potential Immunomodulatory Effects of the Statins in Uveitis

The second most common ocular symptom of RS after conjunctivitis is anterior uveitis, occurring in up to 12% of patients (Lee *et al* 1986). Patients present with an acute onset, unilateral and frequently recurrent nongranulomatous anterior uveitis (Rosenbaum 1989). Signs include fine-sized to medium-sized white keratic precipitates, mild cells and flare. Anterior uveitis is more frequent in patients who are HLA-B27 positive and in those patients who have sacroiliitis (Ostler *et al* 1971). Disc oedema, retinal oedema and retinal vasculitis have also been reported intraocularly with RS (Lee *et al* 1986, Mattson 1955, Ostler *et al* 1971). In one study HLA-B27 positive patients were shown to have increased TNF- α levels in AH and peripheral blood but none of the sample population included RS (Pérez-Guijo *et al* 2004). There has been one RS-specific study which documented elevated IL-6 levels in the blood serum of patients with Reiter's disease but low levels of IL-4 (Drozdova 2004).

Acute episodes of ocular manifestations of RS typically are treated with topical or periocular steroids. A more significant inflammatory reaction may require a course of systemic steroids (Kiss *et al* 2002). Recurrent intraocular inflammation may be controlled by a maintenance dose of oral non-steroidal anti-inflammatories (Rosenbaum 1989).

1.5.5.6 Ankylosing Spondylitis

Ankylosing spondylitis (AS) is a chronic inflammatory disease of the axial skeleton with variable involvement of peripheral joints and non-articular structures. It is the most common among a group of diseases known as the seronegative spondyloarthropathies. Other extra-articular manifestations of AS besides uveitis that

The Potential Immunomodulatory Effects of the Statins in Uveitis

contribute to disease-associated morbidity include enthesopathy, aortitis, heart block and fibrocystic pulmonary disease (Scalapino and Davis Jr 2003).

Uveitis usually presents as an acute unilateral anterior phenomenon (Wakefield *et al* 1991). Corresponding serum and AH elevations of TNF- α have been noted in AS patients with AU (Pérez-Guijo *et al* 2004). A more marked elevation of IL-6 in AH as compared to serum has however been noted. Serum cytokine assays have revealed AS to be associated with increased TNF- α as well as IL-6 but not IL-1 β or IFN- γ (Gratacos *et al* 1994). A close correlation between serum IL-6 and disease activity and severity has also been demonstrated (Gratacos *et al* 1994). IL-4 has also been noted in serum samples of AS patients to be decreased (Drozdova *et al* 2004)

Current therapies for AS include physical therapy, non-steroidal anti-inflammatory drugs (NSAIDS), corticosteroids, pamidronate, thalidomide, sulfasalazine and biological agents such as etanercept and infliximab (Scalapino and Davis Jr 2003). Infliximab and etanercept have been evaluated for usage in uveitis but despite almost universal beneficial effects on joint disease, etanercept has, however, been documented to be associated with a poorer uveitis response rate and in some cases a flare of uveitis only after etanercept therapy had commenced (Smith *et al* 2001).

1.5.5.7 Fuch's Heterochromic Cyclitis

Fuch's Heterochromic Cyclitis (FHC) is a chronic, usually unilateral anterior segment uveitis syndrome which is relatively benign and spontaneously remits and

The Potential Immunomodulatory Effects of the Statins in Uveitis

relapses (Jones 1993). It is usually painless with limited inflammation characterized by diffuse, scattered, small and medium-sized keratic precipitates with AC flare, minimal AC cells and iris atrophy that leads to heterochromia. The clonal nature and predominance of CD8⁺ T cells in the AH of FHC patients is suggestive of an antigen-driven process with a possible viral initiating factor (Labalette *et al* 2002). Recently the discovery of AH rubella Abs and genome in FHC patients has led to the suggestion that it is a rubella virus-driven disease with persistence of the virus preferentially detected in younger patients (Quentin and Reiber 2004). VH-derived T cell cytokine studies of FHC patients have shown increased IFN- γ and IL-10 with low levels of IL-2 but no IL-4 (Muhaya *et al* 1999).

Unlike other uveitic syndromes, the disease does not respond to corticosteroid therapy. It is suggested that since the beneficial effect of steroids in the control of disease activity may be due to their capacity to increase the spontaneous expression of IL-10, the raised IL-10 in FHC precludes further beneficial steroid effect (Muhaya *et al* 1999). The discovery that FHC could be caused by persistent rubella virus offers another explanation to clinicians as to the ineffectiveness of steroids in their empirical experience and allows for the development of FHC-specific treatment modalities (Quentin and Reiber 2004).

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.6 Therapies for Intraocular Autoimmune Disease

The main aims of uveitis therapy are to relieve pain and discomfort, prevent sight loss and other possible complications and to treat the causes of the disease wherever possible. However, the cause of uveitis in the majority of cases remains undiagnosed. As a result of this, the immediate aim of treatment is to decrease inflammation and so alleviate symptoms of disease. This is achieved through steroids, immunosuppressives and mydriatics with the latter to alleviate symptoms of pain and discomfort associated with AU.

1.6.1 Established Therapies for Intraocular Autoimmune Disease

1.6.1.1 Glucocorticoids

Glucocorticoids (GCs) including dexamethasone, are amongst the most potent immunosuppressive and anti-inflammatory drugs currently available and are efficacious in the treatment of both Th1 and Th2 mediated inflammatory diseases, including rheumatoid arthritis and asthma (Karin 1998, Wilckens and De Rijk 1997). GCs inhibit both T cells and APCs at the level of proliferation and cytokine production (Karin 1998, Wilckens and De Rijk 1997). Most importantly dexamethasone is known to inhibit the activation and action of important transcription factors involved in cytokine gene regulation, such as AP-1 and NF- κ B via protein-protein interactions as well as NFAT which leads to the inhibition of IL-4 and possibly IL-2 gene expression (Chen *et al* 2000, De Bosscher *et al* 1997, Karin 1998). More recently a combination of the immunosuppressive drugs, vitamin D3 and dexamethasone has been shown to induce human and mouse naïve CD4⁺ T cells to differentiate *in vitro* into regulatory T cells

The Potential Immunomodulatory Effects of the Statins in Uveitis

which exhibited the ability to prevent experimental autoimmune encephalitis (EAE; Barrat *et al* 2002).

GCs also form the first line of defence for the treatment of non-infectious uveitis (Jabs *et al* 2000). This is due to their rapid ability to suppress inflammation through mimicking the function of cortisol (hydrocortisone). For the treatment of uveitis, steroids can be administered topically, systemically, intraocularly or in a periocular manner while in sight-threatening cases it is possible to administer intravenous steroids if so required. Prednisone is one such steroid used for the treatment of uveitis. There are however several side effects associated with steroid therapy including nausea, weight gain, fluid retention, osteoporosis, hypertension and diabetes. In addition long term therapy has been shown to give rise to severe ocular complications such as cataract and secondary glaucoma. The different techniques of steroid administration have associated complications themselves, for example, periocular complications include blepharoptosis, orbital fat herniation, globe perforation and even retinal and choroidal vascular occlusion. Intraocular injections and depot devices, meanwhile, have a risk of vitreous detachment, retinal detachment and endophthalmitis (Becker *et al* 2005).

Dexamethasone has been shown to significantly inhibit the secretion of monokines (IL-1 β , IL-6, IL-8 and TNF- α) and lymphokines (IL-2, IL-4, IL-10 and IFN- γ). Both IL-4 and IL-10 reductions were not as pronounced as that of IFN- γ thereby favoring a shift from the Th1 to Th2 cytokine profile of secretion (Franchimont *et al* 1998). Dexamethasone inhibits IL-2 induced IL-5 expression mediated by the TCR (Rolfie *et al* 1992). It has, however, been shown to inhibit IFN- γ and CXCR3 expression

The Potential Immunomodulatory Effects of the Statins in Uveitis

to a greater level than that of IL-5 and CCR4 thereby favoring a Th1 response suppression (Moed *et al* 2004). A more complete Th1/Th2 switch has also been seen with decreased IFN- γ production seen in conjunction with raised IL-4 and IL-10 in both human and rat experimental work (Agarwal and Marshall 2001, Ramirez 1998). GCs have also been shown to induce the production of TGF- β , which is known to be a potent inhibitor of T-cell functions, by various cell types including T cells (Batuman *et al* 1995).

1.6.1.2 Cyclosporin A (CsA)

CsA is an inhibitor of T cell activation which functions by blockade of gene transcription and hence proliferation and cytokine production. This is mediated by its ability to inhibit calcineurin and hence its substrates, the transcription factors NFAT and NF- κ B (Ho *et al* 1996, Martinez-Martinez and Redondo 2004). CsA binds to cyclophilin, an intracellular isomerase, also known as rotamase, which is a member of the immunophilin family, and which plays an important role in stabilizing and trafficking proline-containing proteins. The CsA-cyclophilin complex binds to calcineurin and blocks its phosphatase activity (Liu *et al* 1991). It has previously been found to be highly effective in the treatment of a number of different forms of uveitis (Nussenblatt *et al* 1983) with success comparable to that of steroid therapy (Nussenblatt *et al* 1991). CsA does have side effects which include muscle cramps, nephrotoxicity and elevated blood pressure with a major side effect of suppression of the immune system leading to an increased rate of infection. Long term cyclosporine therapy has also been shown to be highly detrimental for development (Ellis *et al* 1985).

The Potential Immunomodulatory Effects of the Statins in Uveitis

IL-2 production is reduced by CsA (van den Berg 1998) and IL-5 mRNA expression induced by TCR signaling is also blocked by CsA (Mori *et al* 1995). Likewise IFN- γ and TNF- α production has also been shown to be reduced but to a greater extent than the reduction of IL-4 and IL-5 (Härtel *et al* 2003). The decreased production of the chemokines CXCR3, CCR10 and CCR4 is also documented (Moed *et al* 2004).

1.6.1.3 Rapamycin

Rapamycin (sirolimus, rapamune) is a potent anti-fungal agent which has antitumor/antiproliferative and immunosuppressive activities. This antibiotic, which belongs to the macrolide family, inhibits CD28-mediated sustained upregulation of IL-2 transcription in T cells (Ikeda *et al* 1997, Sehgal 2003). Rapamycin forms an immunosuppressive complex with intracellular protein, FKBP12. This complex binds to the mammalian target of Rapamycin (mTOR) and blocks its function. By interfering with the function of mTOR, Rapamycin inhibits the mTOR-mediated signal-transduction pathways, resulting in the arrest of the cell cycle in the G1 phase (Sehgal 2003).

Rapamycin has been found to be highly effective in the inhibition of EAU (Roberge *et al* 1993, Ikeda *et al* 1997). The effects of Rapamycin are limited not only to IL-2- or IL-4- mediated proliferation of T cells but also include inhibition of IL-12 (Bertagnolli *et al* 1994) and IL-15 driven proliferation of activated T cells (Strehlau *et al* 1998). Furthermore it has been demonstrated to be an effective and potent immunosuppressive treatment in the majority of patients with non-infectious uveitis and can reduce the need for long term supplementary corticosteroid therapy

The Potential Immunomodulatory Effects of the Statins in Uveitis

(Shanmuganathan *et al* 2005). However drawbacks of rapamycin include occasional intolerable gastrointestinal and dermatological side effects and hyperlipidemia, including both hypercholesterolemia and hypertriglyceridemia (Groth *et al* 1999, Shanmuganathan *et al* 2005).

1.6.1.4 Mycophenolate mofetil

Mycophenolate mofetil (MMF) is a xenobiotic immunosuppressive agent that inhibits the proliferation of both T and B cells. T and B cell DNA synthesis requires both the purine salvage pathway and the *de novo* purine biosynthetic pathways. Since inosine monophosphate dehydrogenase (IMPDH) is the rate-limiting enzyme for the *de novo* pathway and since MMF inhibits IMPDH, it decreases the intracellular guanine nucleotide pools including GTP (Qiu *et al* 2000). This depletion leads to a reduction in both RNA and DNA synthesis thereby leading to inhibition of proliferation (Smak Gregoor *et al* 2000). Lymphocyte apoptosis has also been noted to be increased after MMF treatment in mitogen-stimulated cells (Barten *et al* 1999).

This deficiency of GTP in monocytes as well as lymphocytes inhibits glycosylation of membrane glycoproteins. This leads to less effective adhesion molecules (LFA-1 and ICAM-1) which are required for the recruitment of leukocytes at sites of inflammation (Allison *et al* 1993, Dambrin *et al* 2000). Concordantly mycophenolate has been found to block T cell infiltration *in vitro* with regard to (a) adhesion to endothelial cells, (b) horizontal migration along these cells and (c) penetration through the endothelial cells. Specifically the binding activity of the lymphocytic ligand LFA-1 and

The Potential Immunomodulatory Effects of the Statins in Uveitis

the endothelial ICAM-1 have been shown to be decreased by mycophenolate. In the same study, the regulatory activity of LFA-1 was seen to be decreased after incubation of T cells with high ($>10\mu\text{M}$) concentrations of mycophenolate (Blaheta *et al* 1998).

The inhibition of expression of other T cell surface proteins includes the receptors for IL-2, transferrins and TNF- α ; and secondly, the costimulatory ligand CD40 ligand (CD154) and CD28 (Dambrin *et al* 2000). Possibly through the blockage of IMPDH with a concomitant reduction in ATP pools, MMF has also been shown to block the induction of CDK6 and the IL-2-induced downregulation of the cyclin-dependent kinase inhibitor p27^{Kip1} thereby blocking activated T cells in the mid-G₁ phase of the cell cycle (Quéméneur *et al* 2002). Favorable effects on Th1/Th2 cytokine profiles have also been documented (Huang *et al* 2004, Izeradjene 2001) as has its short- and long-term efficacy in the management of acute and refractory uveitis (Larkin and Lightman 1999, Lau *et al* 2003). Side effects, however, include gastrointestinal complaints, immunosuppression sequelae and occasionally skin malignancy (Wang *et al* 2004).

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.6.2 New Therapies for Intraocular Autoimmune Disease

More recently treatment of uveitis has focused upon more specific targets including the use of biological therapies such as:

1.6.2.1 Anti-TNF- α Therapies

Etanercept, a fusion protein comprised of the extracellular ligand binding portion of the human TNF-receptor and the Fc portion of human IgG1, is capable of inhibiting the function of TNF- α . Conflicting evidence exists for the efficacy of etanercept in the treatment of uveitis at present (Reddy and Backhouse 2003, Reiff *et al* 2001). A second anti-TNF- α therapy is infliximab, a chimeric monoclonal antibody which binds to both membrane and soluble TNF- α (Scallan *et al* 1995) with exceptional efficacy as documented for rheumatoid arthritis (Maini *et al* 1998). Infliximab therapy has been shown to be successful in a number of forms of uveitis (El-Shabrawi and Herman 2002, Joseph *et al* 2003, Sfrikakis *et al* 2001). Lenercept has also now been used to treat patients with active chronic relapsing posterior segment intraocular inflammation, not improved by prednisolone or at least one immunosuppressive agent. Effect has also been in part thought to be through an increase in the expression of IL-10. A fourth TNF therapy investigating the ability of the p55 TNF receptor fusion protein (TNFr-Ig) has shown clinical efficacy in the treatment of posterior uveitis (Murphy *et al* 2004).

1.6.2.2 Other Monoclonal Antibody Therapies

Campath-1 or Alemtuzumab is a monoclonal antibody directed against the CD52 antigen expressed by human lymphocytes. Antibody recognition of CD52⁺ leukocytes

The Potential Immunomodulatory Effects of the Statins in Uveitis

results in their death and therefore leukocyte depletion. The antibody is fully humanized and has previously been found to have clinical efficacy in refractory ocular inflammatory diseases (Isaacs *et al* 1996, Dick *et al* 2000). Daclizumab (Zenapax) is a humanized monoclonal antibody, which recognizes the IL-2R and inhibits binding of IL-2 to its receptor. It functions by the suppression of IL-2 mediated activation of lymphocytes and has since been deemed safe for long term use in intermediate and posterior uveitis (Nussenblatt *et al* 1999; 2003). Anti-MIF monoclonal antibody has also been found to decrease histological disease severity in a rat model of EAU (Kotake *et al* 2002).

1.6.2.3 Other Novel Treatment Options

Intravenous immunoglobulin (IVIg) has anti-inflammatory effects that may be mediated by the inhibitory Fc receptor pathway (Samuelsson *et al* 2001). There have been reports of its efficacy in BD (Seider *et al* 2001). Several studies have shown that IFN- β treatment shifts the balance of cytokines in favour of a net anti-inflammatory response, by either inhibiting Th1 or by upregulating Th2 cytokine production or both (Dhib-Jalbut 1997, Yong *et al* 1998). It has also been used to treat uveitis associated with multiple sclerosis which was refractory to GCs (Becker *et al* in press). Oral tolerance induction is a natural immune mechanism based on the ingestion of soluble autoantigen which leads to inhibition of DTH reactions that has been demonstrated to be effective in autoimmune uveitis (Nussenblatt *et al* 1990, Thureau *et al* 1999).

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.6.3 Potential Therapies in Uveitis

1.6.3.1 Rheumatoid Arthritis and other Autoimmune disease-based Therapies

Most of the immunosuppressive treatments used in uveitis have been extensively explored in patients with rheumatoid arthritis (RA) and other rheumatic diseases and so it useful to monitor the new developments being used in rheumatology with a view to potential use in uveitis. Leflunomide, adalimumab, anakinra, rituximab, alefacept and cytotoxic T-lymphocyte-associated antigen 4-Ig and tocilizumab and have all been earmarked for further follow-up (Becker *et al* 2005). Anakinra is a recombinant human IL-1 receptor antagonist that has been shown to ameliorate signs and symptoms of active disease in RA patients (Furst 2004). Tocilizumab is a novel neutralizing antibody to suppress IL-6 signaling mediated by both membranous and soluble IL-6R. It has recently been shown to improve disease activity in RA as well as Crohn's disease (Mihara *et al* 2005). Another Crohn's therapy being trialed is fontolizumab, a humanized anti-IFN- γ monoclonal antibody (Bourne *et al* 2004, Dumont 2005). Th1-mediated experimental colitis has also been ameliorated with the administration of TGF- β 1 (Kitani *et al* 2000).

1.6.3.2 Antibody Fragment Therapies

Recent engineering developments have resulted in the generation of monovalent single-chain variable-domain fragment (scFV) and divalent miniantibodies have been shown via topical administration to pass through intact pig and human corneas into the AC and into the vitreous of the rabbit, with retained antigen-binding capacity (Thiel *et al* 2002, Williams *et al* 2004). Such fragment constructs may have therapeutic potential for immunopathological diseases affecting the anterior and posterior segments of the eye.

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.6.3.3 Statin Therapies

The 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins are a group of compounds which were originally isolated from *Penicillium* species (Endo *et al* 1976). They are a well-established class of drugs that are the most powerful in lowering serum cholesterol levels. They can be subdivided into natural and fully synthetic compounds. Examples of the former include lovastatin, simvastatin and pravastatin while drugs included in the latter are atorvastatin and fluvastatin (Figure 1.7).

Research has shown that their beneficial effects in the reduction of cardiovascular mortality relate also to their cholesterol-lowering independent properties. Other modalities proposed include a reduction of endothelial dysfunction, decreased blood coagulation, anti-inflammatory and immunomodulatory mechanisms of action (Blanco-Colio *et al* 2003, Corsini *et al* 1995). Furthermore, *in vitro* studies have shown that the statins can modulate the adhesion cascade at several points through their effects on endothelial cells and leukocytes. This is achieved through the blocking of expression and activation of integrins such as LFA-1 in leukocytes and by decreasing ICAM-1 and MCP-1 expression in activated monocytes and the endothelium (Weitz-Schmidt 2002, Niwa *et al* 1996, Andrews *et al* 1999, Lewis *et al* 1999). As a result statins are able to decrease T cell activation, inhibit the recruitment of T cells and monocytes into the arterial wall, and stabilize atherosclerotic plaques (Figure 1.8).

The Potential Immunomodulatory Effects of the Statins in Uveitis

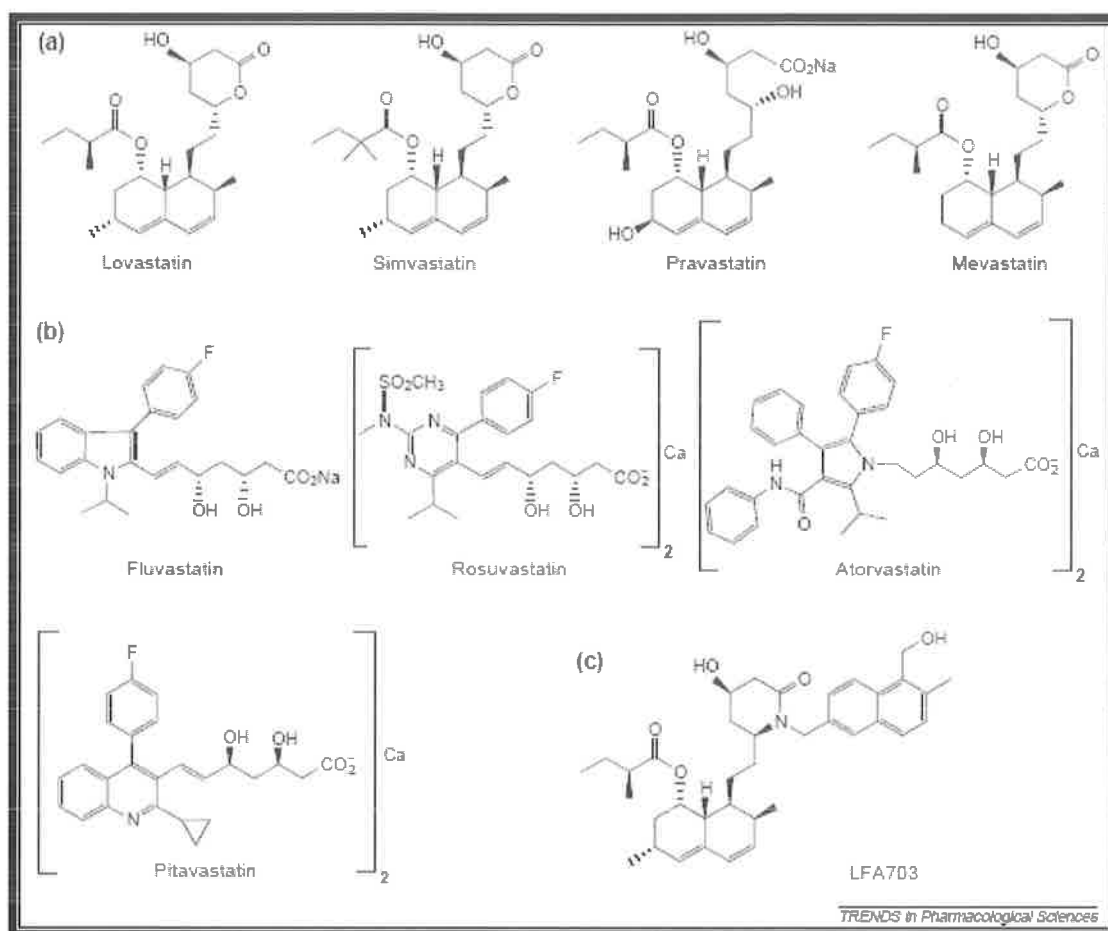


Figure 1.7 Structures of statins and the statin-derived lymphocyte-function-associated antigen 1 (LFA-1) inhibitor LFA703.

- (a) Lovastatin, simvastatin, pravastatin and mevastatin are natural statins.
- (b) Fluvastatin, atorvastatin, rosuvastatin and pitavastatin are fully synthetic statins
- (c) LFA703 is a statin-derived compound designed to potently inhibit the $\beta 2$ integrin LFA-1

Taken from: Weitz-Schmidt (2002)

The Potential Immunomodulatory Effects of the Statins in Uveitis

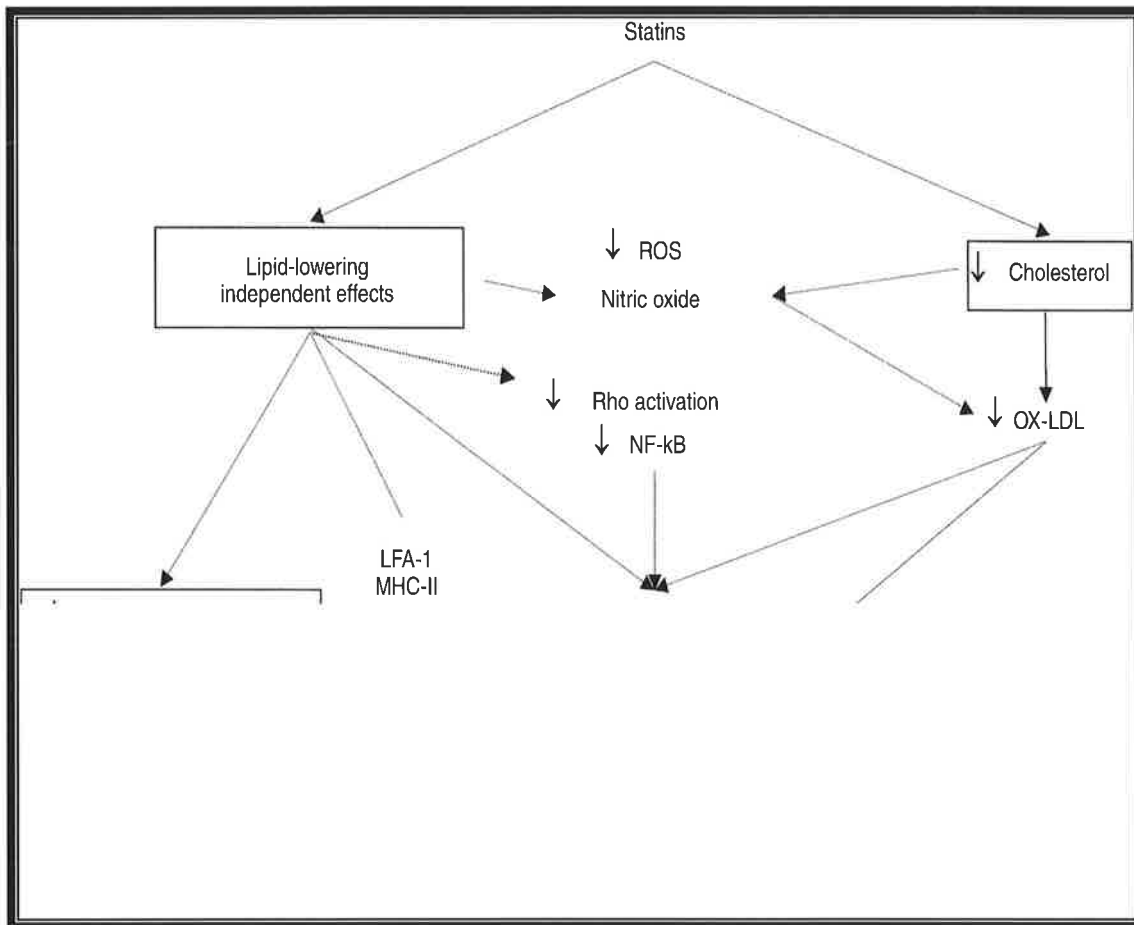


Figure 1.8 Pleiotropic effects of HMG-Co A reductase inhibitors

LFA-1 = Leukocyte function antigen-1

MHC-II = Major histocompatibility complex class-II antigen

OX-LDL = Oxidized low-density lipoprotein

ROS = Reactive oxygen species

VSMC = Vascular smooth muscle cell

Taken from: Blanco-Colio *et al* (2003)

The Potential Immunomodulatory Effects of the Statins in Uveitis

Many of these effects are related to the inhibition of isoprenoid synthesis, which serve as a lipid attachment for a variety of proteins involved in intracellular signaling. The Rho family of GTPases in particular, whose proper membrane localization and function are dependent on isoprenylation, may play an important role in the lipid-lowering independent effects of the statins (Blanco-Colio 2003). They interfere with NF- κ B which is involved in the transcriptional regulation of cytokines, chemokines, adhesion molecules and acute phase proteins CRP, which is a marker of inflammation (Pahl 1999). This HMG-CoA reductase led inhibition has been shown to be reversible by the addition of mevalonate or isoprenoids (Figure 1.9, Diomedea *et al* 2001, Niwa *et al* 1996, Liu *et al* 1999). Certainly in the clinical setting high-dose atorvastatin has been shown to reduce CRP levels as well as the Th1/Th2 ratio of the cytokines IFN- γ and IL-4 in patients with acute coronary syndrome after 16 weeks of treatment (Shimada *et al* 2004, Kinlay *et al* 2003).

1.6.3.3.1 Statins in Experimental Inflammatory Models of Disease

Recent studies have revealed the immunomodulatory effects of statins in mouse models of EAE, a Th1 mediated autoimmune disorder and model for multiple sclerosis (Aktas *et al* 2003, Youssef *et al* 2002, Greenwood *et al* 2003). Atorvastatin has been found to inhibit and reverse chronic and relapsing encephalomyelitis (Youssef *et al* 2002). In this study, in addition to upregulating Th2 cytokines (IL-4, IL-5 and IL-10), atorvastatin induced STAT6 tyrosine phosphorylation, which is associated with IL-4 upregulation. Conversely, STAT4 phosphorylation, associated with IL-12 induction, was inhibited and transcription of Th1 cytokines (IL-2, IL-12, IFN- γ and TNF- α) suppressed.

The Potential Immunomodulatory Effects of the Statins in Uveitis

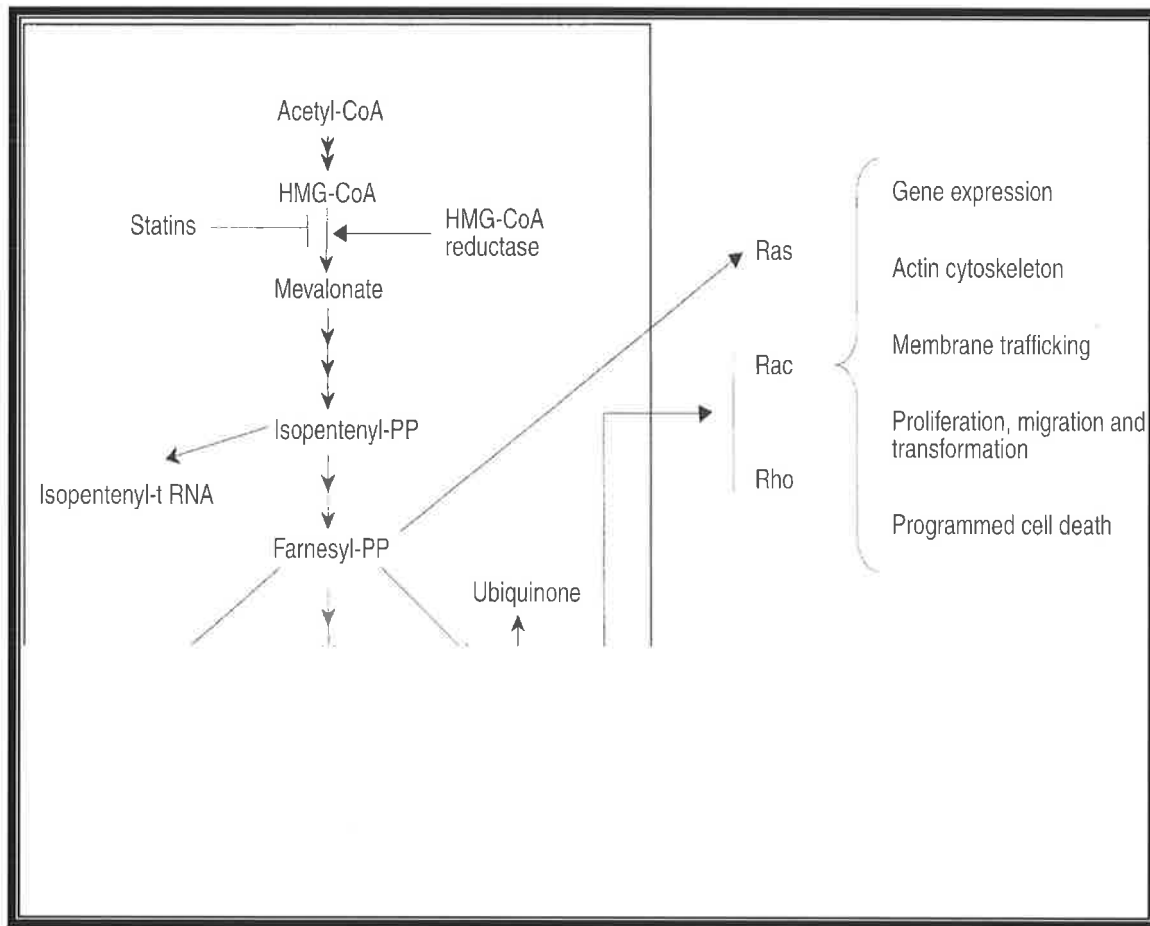


Figure 1.9 Intracellular effects of HMG-CoA reductase (inhibition by statins)

Inhibition of HMG-CoA reductase by statins decreases isoprenylation of small G proteins such as Ras, Rac and Rho. This effect leads to alteration of different functions in the cell.

PP = Pyrophosphate

Taken from: Blanco-Colio *et al* (2003)

The Potential Immunomodulatory Effects of the Statins in Uveitis

Surprisingly in a very recent study investigating the effects of atorvastatin in IRBP-induced EAU, atorvastatin was found not to modulate the immune response. The pro-inflammatory cytokines, IL-1 α and IL-1 β and Th1 cytokines, TNF- α and IL-2 were upregulated equally in control and atorvastatin treated groups. IL-12 and Th2 cytokines were not upregulated in all three groups. Histological grading suggested mildly decreased inflammation in the high dose treated group but mean differences between the treated groups and the control group were not statistically significant (Thomas *et al* 2005).

Lovastatin has also been shown *in vivo* by Nath *et al* (2004) to reduce the duration and clinical severity of active and passive EAE with reduced pro-inflammatory cytokine production and increased Th2 and Tr cytokine induction. Very recently, however, lovastatin was found parenterally attenuate a mouse model of EAU but without a Th2 cytokine induction. IFN- γ was shown to be significantly reduced but IL-2 production was not changed and TNF- α was increased. In addition IL-4 and IL-5 levels were below the assay detection limit and IL-10 was significantly decreased. The administration of mevalonolactone was able to reverse the lovastatin treatment effects (Gegg *et al* 2005).

Simvastatin was found by Leung *et al* (2003) to reduce pro-inflammatory cytokines as well as IL-10 in a murine model of collagen-induced arthritis but the presence of any Th2 cytokines was not detected. A reduction in expression of both Th1 and Th2 cytokines however has been documented in a murine model of allergic asthma which demonstrated the therapeutic potential of statins in allergic airways disease (McKay *et al* 2004).

The Potential Immunomodulatory Effects of the Statins in Uveitis

1.7 Aims

A number of studies have now looked at cytokine profiles within the AH of patients with uveitis using conventional enzyme-linked immunosorbent assay (ELISA) techniques. As a result only small numbers of cytokines are able to be studied at a time due to limited AH sample volumes. With the advent of flow cytometric bead-based technology, we therefore set about to try and more clearly define the complex interplay of cytokines within the AH of uveitis patients as the first aim of the thesis, as it allows for multiple cytokine analytes to be more rapidly quantified in single samples with better reproducibility and sensitivity than ELISA (Morgan *et al* 2004). The Cytometric Bead Array (CBA) system was applied in comparison with ELISA for the quantitative and qualitative cytokine profiling of six cytokines in AH of uveitis patients.

The second and major aim of my project was to evaluate the effects of the statins, and their reversibility, on T cell populations from the peripheral blood of normal human donors in comparison with those of known immunomodulatory drugs. In doing so we first of all investigated their effect on isolated lymphocyte viability and then proliferation through the use of carboxyfluorescein succinimidyl ester (CFSE). We then assessed their effect on cytokine profiles within whole blood through the use of intracellular staining techniques for the detection of four cytokines and through a flow cytometric multiplex bead-based array system in order to assess their possible usage in uveitis patients, having seen their potential in several experimental models of inflammatory disease. The bead-based system used here was the Fluorescent Bead Immunoassay (FBI) system employed for the quantitative and qualitative profiling of ten cytokines.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Chapter 2

The Potential Immunomodulatory Effects of the Statins in Uveitis

Materials and Methods

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.1 Materials

Product	Product Code	Supplier
2-Mercaptoethanol	M6250	Sigma-Aldrich UK
Atorvastatin	N/A	Parke-Davis US
Brefeldin A	B7651	Sigma-Aldrich UK
CFSE (CFDA tracker kit)	V12833	Molecular Probes UK
CFSE stop buffer	N/A	In-lab produced
Cyclosporin A	N/A	Sandoz UK
Cytofix/Cytoperm Kit	554772	BD Biosciences UK
Cytometric Bead Array Kit	550749	BD Biosciences US
Dexamethasone (MINIMS)	N/A	Chauvin UK
Dimethyl sulfoxide	D5879-100ML	Sigma-Aldrich UK
DL-Mevalonic acid lactone	M4667	Sigma-Aldrich UK
Ethanol 100%	E702-3	Aldrich US
FACS Lysing Solution	349202	BD Biosciences US
Fetal calf serum (heat inactivated)	F9665	Sigma-Aldrich UK
Fluorescent Bead Immunoassay	BMS710FF	Bender Medsystems US
Gentamicin	G1397	Sigma-Aldrich UK
Glutamine	25030-024	Invitrogen UK
Histopaque	1077-1	Sigma-Aldrich UK
Human AB+ serum	H4522	Sigma-Aldrich UK
Ionomycin	I0634	Sigma-Aldrich UK
Lovastatin	438185	Calbiochem UK

The Potential Immunomodulatory Effects of the Statins in Uveitis

Mycophenolate mofetil	N/A	Roche US
Non-essential amino acids	11140-035	Invitrogen UK
OptEIA Human IFN- γ set	2613KI	Pharmingen BD UK
OptEIA Human IL-5 set	2629KI	Pharmingen BD UK
OptEIA Human IL-10 set	2617KI	Pharmingen BD UK
OptEIA Human TNF- α set	2637KI	Pharmingen BD UK
Perm/Wash Buffer	554723	Pharmingen BD UK
Phosphate Buffered Saline	P4417	Sigma-Aldrich UK
Phorbol 12-myristate 13-acetate	P8139	Sigma-Aldrich UK
Phytohemagglutinin	L9017	Sigma-Aldrich UK
Propidium iodide	28,707-5	Aldrich UK
RPMI 1640 Dutch modification without L-glutamine	22409-015	Invitrogen UK
Simvastatin	N/A	MSD UK
Sirolimus (Rapamycin)	N/A	Wyeth UK
Sodium hydroxide	72068	Sigma-Aldrich UK
Sodium pyruvate	11360-039	Invitrogen UK
Trypan Blue Solution (0.4%)	T8154	Sigma-Aldrich UK
Tween 80	P4780	Sigma-Aldrich UK

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.2 Antibodies

Table 2.1 Capture antibodies on microspheres and detector antibodies used in Cytometric Bead Array

Capture Antibody	Detector Antibody
Anti-human IL-2	PE-conjugated anti-hIL-2
Anti-human IL-4	PE-conjugated anti-hIL-4
Anti-human IL-5	PE-conjugated anti-hIL-5
Anti-human IL-10	PE-conjugated anti-hIL-10
Anti-human IFN- γ	PE-conjugated anti-hIFN- γ
Anti-human TNF- α	PE-conjugated anti-hTNF- α

Table 2.2 Unconjugated primary antibodies used in T cell viability and proliferation assay studies

Antibody	Clone	Host	Isotype	Source
Anti-human CD28	CD28.2	Mouse	IgG _{1,k}	BD Biosciences
Anti-human CD3	HIT3a	Mouse	IgG _{2a}	BD Biosciences

The Potential Immunomodulatory Effects of the Statins in Uveitis

Table 2.3 Directly conjugated primary antibodies used in cell surface and intracellular cytokine staining studies

Antibody	Clone	Host	Isotype	Source
Anti-human CD3-PerCP	SK7	Mouse	IgG _{1,κ}	BD Biosciences
Anti-human IFN-γ-FITC	4S.B3	Mouse	IgG ₁	BD Biosciences
Anti-human IL-2-PE	5344.111	Mouse	IgG _{1,κ}	BD Biosciences
Anti-human IL-4-FITC	MP4-25D2	Rat	IgG ₁	BD Biosciences
Anti-human IL-10-PE	JES3-9D7	Rat	IgG ₁	BD Biosciences

Table 2.4 Capture antibodies on microspheres and detector antibodies used in Fluorescent Bead Immunoassay

Capture Antibody	Detector Antibody	Fluorescent Detector
Anti-human IL-1β	Biotin-conjugated anti-hIL-1β	Streptavidin-PE
Anti-human IL-2	Biotin-conjugated anti-hIL-2	Streptavidin-PE
Anti-human IL-4	Biotin-conjugated anti-hIL-4	Streptavidin-PE
Anti-human IL-5	Biotin-conjugated anti-hIL-5	Streptavidin-PE
Anti-human IL-6	Biotin-conjugated anti-hIL-6	Streptavidin-PE
Anti-human IL-8	Biotin-conjugated anti-hIL-8	Streptavidin-PE
Anti-human IL-10	Biotin-conjugated anti-hIL-10	Streptavidin-PE
Anti-human IFN-γ	Biotin-conjugated anti-hIFN-γ	Streptavidin-PE
Anti-human TNF-α	Biotin-conjugated anti-hTNF-α	Streptavidin-PE
Anti-human TNF-β	Biotin-conjugated anti-hTNF-β	Streptavidin-PE

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.3 Donors

2.3.1 Multiplex Cytokine Detection with Cytometric Bead Array

Control AH was collected from 10 patients undergoing routine phacoemulsification none of whom were receiving ocular medication or previously had an ocular problem. Samples of AH were taken from 36 patients with active uveitis who had at least 2+ cells in the AC. The protocol had Ethics committee (Moorfields Eye Hospital) approval and informed consent was given by all patients. 22 patients with idiopathic uveitis (mean age, 54.4 years) were diagnosed as either having panuveitis ($n = 4$) or anterior uveitis (AU, $n = 18$) after detailed questioning and appropriate investigations were all negative. Of these 22 patients, 10 were on topical steroids while the remainder were receiving no treatment. In addition, a further 14 uveitis patients with an associated systemic disease (mean age, 39.2 years) comprised of 8 AU (5 ankylosing spondylitis, 2 sarcoidosis and 1 Reiter's disease) and 6 panuveitis patients (4 Behçet's disease, 1 sarcoidosis and 1 Vogt-Koyanagi-Harada syndrome). Two of the Behçet's disease patients were on systemic steroids with one of them additionally on CsA. The remaining 12 active uveitis patients with an associated systemic disease were all on topical steroids except for one who was receiving no treatment. All the studies involving human subjects were conducted according to the tenets of the Declaration of Helsinki.

2.3.2 T Cell Viability and Proliferation Inhibition

Peripheral venous blood was obtained from 10 healthy donors (mean age, 34.5 years, male:female ratio, 3:2). The protocol had Ethics committee (Moorfields Eye Hospital) approval and informed consent was given by all donors. All the studies

The Potential Immunomodulatory Effects of the Statins in Uveitis

involving human subjects were conducted according to the tenets of the Declaration of Helsinki.

2.3.3 Intracellular Cytokine Staining

Peripheral venous blood for final analysis was obtained from 6 normal human donors (mean age, 33.2 years and male:female ratio, 1:1). The protocol had Ethics committee (Moorfields Eye Hospital) approval and informed consent was given by all donors. All the studies involving human subjects were conducted according to the tenets of the Declaration of Helsinki.

2.3.4 Multiplex Cytokine Detection with Fluorescent Bead Immunoassay

Peripheral venous blood for final analysis was obtained from the same 6 normal human donors as in 2.8.1 (mean age, 33.2 years and male:female ratio, 1:1) in order to test cytokine production for the same drug combinations as in 2.8.3. Peripheral venous blood from 4 normal human donors (mean age, 35.3 years and male:female ratio, 1:1) was obtained to compare the effect on cytokine production by pretreatment of whole blood with CsA 2 hours prior to PMA/ionomycin stimulation versus simultaneous addition of CsA and PMA/ionomycin stimulation to whole blood at time 0. The protocol had ethics committee approval and informed consent was given by all donors. All the studies involving human subjects were conducted according to the tenets of the Declaration of Helsinki.

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.4 Stock Solutions

2.4.1 2-Mercaptoethanol

2-Mercaptoethanol (2-ME) was purchased as a 14.3M stock solution. 14.3M 2-ME was diluted in phosphate buffered saline [1/14.3 v/v] (PBS). In aseptic conditions this was then sterilized by passing through a 0.22µm filter, aliquoted and stored at -20°C until use.

2.4.2 Atorvastatin

10mg of atorvastatin was dissolved in 8.655ml Roswell Park Memorial Institute media 1640 Dutch modification without L-glutamine (RPMI) and filtered to give a stock concentration of 1mM. 200µl aliquots were stored at -20°C.

2.4.3 CFSE Stop Buffer

10% fetal calf serum dissolved in RPMI stored at 4°C

2.4.4 Cyclosporin A

100mg cyclosporin A supplied as a 0.3ml liquid/oil preparation was dissolved in 0.2ml Tween 80 along with 3.5ml RPMI and 1ml 100% ethanol. A final stock concentration of 1mg/ml was achieved by diluting to 20ml with RPMI. Storage was in a Universal tube at 4°C. A vehicle control stock solution was also made up using TCM dissolved in Tween 80 and 100% ethanol as above.

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.4.5 Dexamethasone

MINIMS preparation of 0.1% weight per volume dexamethasone was diluted 1:10 with RPMI to use at a final concentration of 400µg/ml and stored at room temperature (RT).

2.4.6 FACS Lysing Solution

Concentrated FACS lysing solution was diluted 1 in 10 in deionized water and stored at RT.

2.4.7 Lovastatin

25mg of lovastatin was dissolved in 1.545ml dimethyl sulfoxide (DMSO) and filtered to make a 40mM stock. Storage was at -20°C. A vehicle control stock solution was also made up by dissolving T cell medium (TCM) in DMSO as above.

2.4.8 Mevalonate

1g DL-mevalonic acid lactone was converted to sodium mevalonic acid by solubilization in 3ml of 1 M sodium hydroxide. Further sodium hydroxide was added until a pH of 7.36. The resulting solution was then sterilized by filtration and made up to a volume of 15.36ml in RPMI at a stock concentration of 0.5M. Storage was at -20°C. A vehicle control stock solution was also made up by dissolving TCM in sodium hydroxide as above.

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.4.9 Mycophenolate mofetil

500mg mycophenolate was dissolved in 500 μ l DMSO and 99.5ml RPMI added to give a 10mM stock concentration. The resulting solution was sterilized by filtration. 1ml aliquots were stored at -20°C. A vehicle control stock solution was also made up by dissolving TCM in DMSO and RPMI as above.

2.4.10 Permash Buffer

Concentrated permash buffer was diluted 1 in 10 in deionized water. This solution was stored at 4°C.

2.4.11 Phosphate Buffered Saline

Phosphate buffered saline (PBS) was prepared according to the manufacturer's instructions. Briefly one PBS tablet was dissolved in 200ml deionized water. PBS was sterilized using a 0.22 μ m filter.

2.4.12 Propidium Iodide

Propidium iodide (PI) was dissolved at 1mg/ml in deionized water, aliquoted, stored at -20°C and thawed as required. Once thawed, PI solution was stored at 4°C.

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.4.13 Rapamycin

1mg rapamycin was dissolved in 100% ethanol and made up to a 0.1mg/ml stock solution with RPMI. Following filter sterilization, storage was in 100µl aliquots in 200µl Eppendorfs at -20°C. A vehicle control stock solution was also made up by dissolving TCM in 100% ethanol and RPMI as above.

2.4.14 Simvastatin

10mg of simvastatin was dissolved in 0.6ml DMSO and sterilized. Total volume was corrected to 2.4ml by the addition of 1.8ml of T cell medium (TCM) giving a 10mM stock concentration. Storage was at -20°C in 10µl aliquots in 200µl Eppendorf tubes. A vehicle control stock solution was also made up by dissolving PBS in DMSO and TCM as above.

2.4.15 T Cell Medium

44ml RPMI was supplemented by 50µl gentamicin, 5ml human AB+ serum, 2- ME, 0.5 ml L-glutamine, 0.5ml sodium pyruvate and 0.5ml non-essential amino acids.

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.5 Multiplex Cytokine Detection with Cytometric Bead Array

2.5.1 Sampling Procedure

A maximum of 50 – 150µl AH from active uveitis patients with at least 2+ cells in the AC was collected by anterior chamber paracentesis using a 30-gauge needle on a 1ml insulin syringe, via the temporal limbal approach and a rolling technique. It was immediately aliquoted into EDTA coated Eppendorf tubes to prevent cell clumping. On receipt, the samples were centrifuged 400 rpm for 5 minutes to precipitate any cells present and the AH stored frozen (-70°C) until testing. AH from cataract patients was taken at the beginning of the procedure, spun, aliquoted and frozen (-70°C).

2.5.2 Cytometric Bead Array (CBA)

The CBA kit was used as per manufacturer's instructions. Briefly, 7.5µm beads dyed to six different fluorescent intensities are coupled to antibodies against one of six capture cytokine antibodies, IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ, forming six discrete populations. These bind to their respective cytokines in the samples and the cytokines are then visualized using a second, detection, anti-cytokine antibody, coupled to phycoerythrin (PE). The standards (20 – 5000pg/ml) comprise a mixture of all six cytokines which are incubated with the capture beads and detector antibodies. A single sample, standard or test, therefore allows enumeration of all six cytokines.

2.5.2.1 Preparation of Th1/Th2 Cytokine Capture Beads

The number of assay tubes was determined (samples, 9 cytokine standard dilutions and 1 negative control). Each of the 6 capture bead suspensions, stored at +4 °C,

The Potential Immunomodulatory Effects of the Statins in Uveitis

were vigorously vortexed for a few seconds before mixing. A 10 μ l aliquot of each of the 6 capture bead suspensions, for each assay tube to be analyzed, was added to a single tube to form a capture bead mixture which was then vortexed thoroughly.

2.5.2.2 Preparation of Th1/Th2 Cytokine Standards

1 vial of lyophilized Th1/Th2 cytokine standards was reconstituted with 0.2ml of kit assay diluent (A buffered serum protein solution). The reconstituted standard was allowed to equilibrate for at least 15 minutes before dilutions were made. The vial was agitated to mix thoroughly. 9 tubes were labelled "Standard 1 to 9". 900 μ l of assay diluent was added to Standard 1 and 300 μ l of assay diluent was added to each of the remaining tubes. 100 μ l of the reconstituted standard was transferred to the Standard 1 tube and mixed thoroughly. A serial dilution was then made by transferring 300 μ l from Standard 1 to the Standard 2 tube and mixing thoroughly. Continued serial dilutions were then made by transferring 300 μ l from Standard 2 to the Standard 3 tube and so on to the Standard 9 tube, mixing thoroughly each time. The 9 standard tubes corresponded to the concentrations of 5000, 2500, 1250, 625, 312.5, 156, 80, 40 and 20pg/ml. The assay diluent served as the negative control.

2.5.2.3 Th1/Th2 Cytokine CBA Assay Procedure

50 μ l of the capture bead mixture were vortexed and then added to the appropriate tubes. 50 μ l of the kit Th1/Th2 PE detection reagent was added to the assay tubes. 10 μ l of the cytokine standard dilutions were then added to the control assay tubes. 10 μ l of each test sample was in turn added to the test assay tubes. The assay tubes were then incubated

The Potential Immunomodulatory Effects of the Statins in Uveitis

for 18 hours in the dark at RT (Cook *et al* 2001). The beads were washed and the pellets resuspended in 300µl of wash buffer.

2.5.2.4 Flow Cytometry

Two colour flow cytometric analysis was performed using a FACScan (BD). 1800 events were acquired using CellQuest software (BD) with a monomeric microsphere population gated on forward and side scatters following the protocol supplied. Analysis was performed using CBA dedicated analysis software (BD). The minimum levels of detection for each cytokine were: IL-2 and IL-4, 2.6pg/ml, IL-5, 2.4pg/ml, IL-10 and TNFα 2.8pg/ml and IFN-γ, 7.1pg/ml. In all samples where the calculated concentration was below the given sensitivity, it was treated as undetectable.

2.5.3 Enzyme-linked Immunosorbent Assay (ELISA)

Where there was sufficient AH remaining (100µl) the levels of IL-4, IL-10, TNF-α and IFN-γ were determined using commercially ELISA kits as per the manufacturer's instructions. Briefly, all reagents were brought to RT before starting the experiment. The assays employed a quantitative sandwich enzyme immunoassay, in which a monoclonal capture antibody specific for one of IL-4, IL-10, TNF-α or IFN-γ had been pre-coated onto a 96-well plate. Standard curves were generated by 7-fold serial dilutions of the supplied cytokine stock standard ranging from 0-500pg/ml for IL-4, 0-300pg/ml for IL-10, 0-500pg/ml for TNF-α and 0-300pg/ml for IFN-γ. Samples and standards were incubated with the pre-coated plates for 2 hours at RT followed by washing 5 times with wash buffer (supplied). 100µl of the cytokine conjugate (detection antibody + avidin-

The Potential Immunomodulatory Effects of the Statins in Uveitis

horseradish peroxidase reagent) was added to each well and incubated for 1 hour. The plate was then washed 7 times and then 100µl of substrate solution was added to each well followed by incubation for 30 minutes at RT. 50µl of stop solution containing 2N H₂SO₄ was then added to each well and the optical densities measured after 30 minutes using a SpectraCount microplate reader (Beckman). Triplicate readings of each well were taken at 450nm and 570nm to correct for optical imperfections in the plate.

Cytokine concentrations were normalized to the 0pg/ml standard and calculated using the regression formula of the linearized standard curve. All values were corrected for background readings. The minimum detection level of cytokines was 11pg/ml. In all samples where the calculated concentration was below the given sensitivity it was treated as undetectable.

2.5.4 Analysis

Comparisons of cytokine levels determined by CBA or ELISA were analysed using a paired t-test with significance where $p < 0.01$. Non-parametric statistical analysis of results from different patient groups was performed using the Mann-Whitney test without a Bonferroni correction as there were sufficient differences in average rank positions between groups. Correlation of cytokines within AH groups was carried out using Spearmann's rank coefficient test. Results were significant where $p < 0.05$.

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.6 T Cell Viability and Proliferation Inhibition

2.6.1 Sampling procedure

Peripheral blood was collected through venepuncture of the cubital fossa veins and then heparinized. T lymphocytes were isolated from whole blood by Histopaque density gradient (1.077) centrifugation using an MSE Harrier 18/80 centrifuge (Sanyo, Gallenkamp PLC, U.K.).

Heparinized whole blood was diluted 1:1 with RPMI and layered onto an equal volume of Histopaque. The tubes were then centrifuged at 400 x g at 20°C and no brake. In this process red blood cells which have a greater density precipitate to the bottom of the tube; while plasma and platelets remain at the top and the live mononuclear cells (MNC)s form a layer at the top of the Histopaque (Figure 2.1). A soft tip pipette was used to aspirate the MNCs which were washed with RPMI by centrifugation at 300 x g for 10 minutes at 20°C and no brake. The cell pellets were resuspended, pooled and washed with RPMI. The resulting pellet was resuspended in 4ml of RPMI for cell counting.

2.6.2 MNC counts

Cell counts were performed by diluting 10µl of RPMI resuspended T cells 1 in 1 with 10µl Trypan Blue Solution 0.4% and utilizing a haemocytometer (Weber Scientific International, West Sussex, UK).

The Potential Immunomodulatory Effects of the Statins in Uveitis

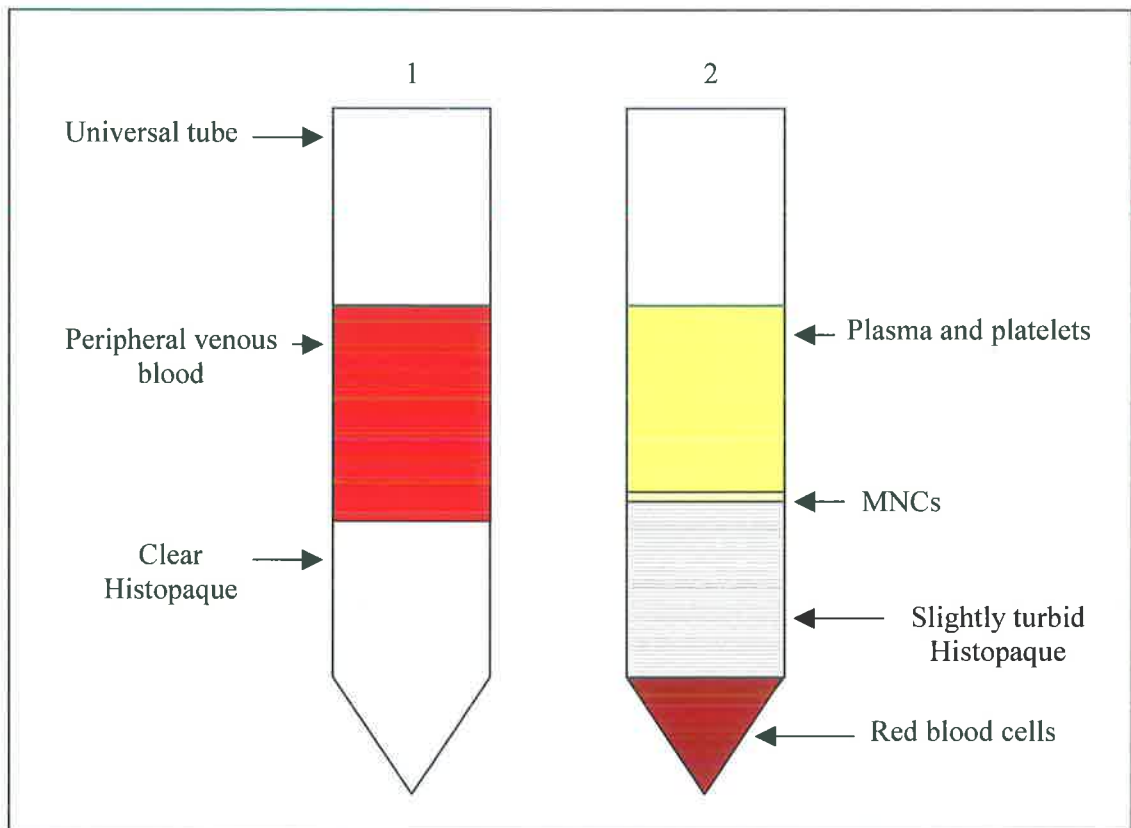


Figure 2.1 The principles of density gradient centrifugation.

Tube 1 shows the layered peripheral venous blood on to the Histopaque.

Tube 2 shows the distribution of the cells post centrifugation. The mononuclear cells (MNCs) remain at the interface of the Histopaque on the basis of a density less than 1.077 (the density of the Histopaque). Red blood cells, granulocytes and debris pass freely through the Histopaque density gradient and sediment at the bottom of the tube. The interface layer containing live mononuclear cells is then aspirated and the cells washed to remove residual Histopaque.

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.6.3 T cell culture

T cell viability and proliferation assays were performed in triplicate. Cells were incubated for 10 minutes in a 37°C water bath with CFSE at 7.5µM final concentration in serum free RPMI. 1ml cold CFSE stop buffer was then added before incubation at RT for 30 minutes. Cells were washed once with RPMI before being resuspended at 2×10^6 /ml in TCM.

Cells were then cultured at 2×10^5 cells/ml in 96-well plates for 5 days (previously optimized) in the absence or presence of one of the following drugs: simvastatin, lovastatin, atorvastatin, rapamycin, mycophenolate and CsA (total volume per well is 200µl). The reversibility of statin function was tested by addition of mevalonate. Parallel experiments with and without stimulation were conducted. Stimulation was performed with all drugs, except CsA, by adding anti-CD3 [10ng/ml] and anti-CD28 [5ng/ml] antibodies at start of culture. CsA pre-treatment of lymphocytes occurred for 2 hours prior to the addition of 5µg/ml phytohemagglutinin (PHA) and increasing concentrations of anti-CD3 antibodies [10-5000ng/ml]. Identical experiments were also conducted using vehicle controls (VCs) for all the drugs tested to assess effect of drug solvent on T cell viability and proliferation.

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.6.4 Flow cytometry

Propidium iodide (PI) was added to cells harvested from each well. Two-colour flow cytometry was performed on a FACScan flow cytometer with 488nm argon LASER (Becton Dickinson, Oxford, U.K.). Analysis gates were set on lymphocytes according to forward (FSCC) and side scatter (SSC) characteristics. Listmode data on 15,000 to 25,000 events were collected using CellQuest acquisition software (Becton Dickinson, Oxford, U.K.). Dead cells were excluded from analysis based on staining with PI.

2.6.5 Analysis

Data was analysed using WinList (Verity Software House, Topsham, ME). The total numbers of stimulated (blast) T cells were determined by using FSC and SSC characteristics and region gating to define the cell populations. Histograms were used to track the divisions of CFSE-labelled cells enabling identification of the percentage of divided (proliferated) cells. All data presented are from analyses of live (PI-negative) cells only (Figure 2.2). Statistical analysis was performed using paired t-tests.

The Potential Immunomodulatory Effects of the Statins in Uveitis

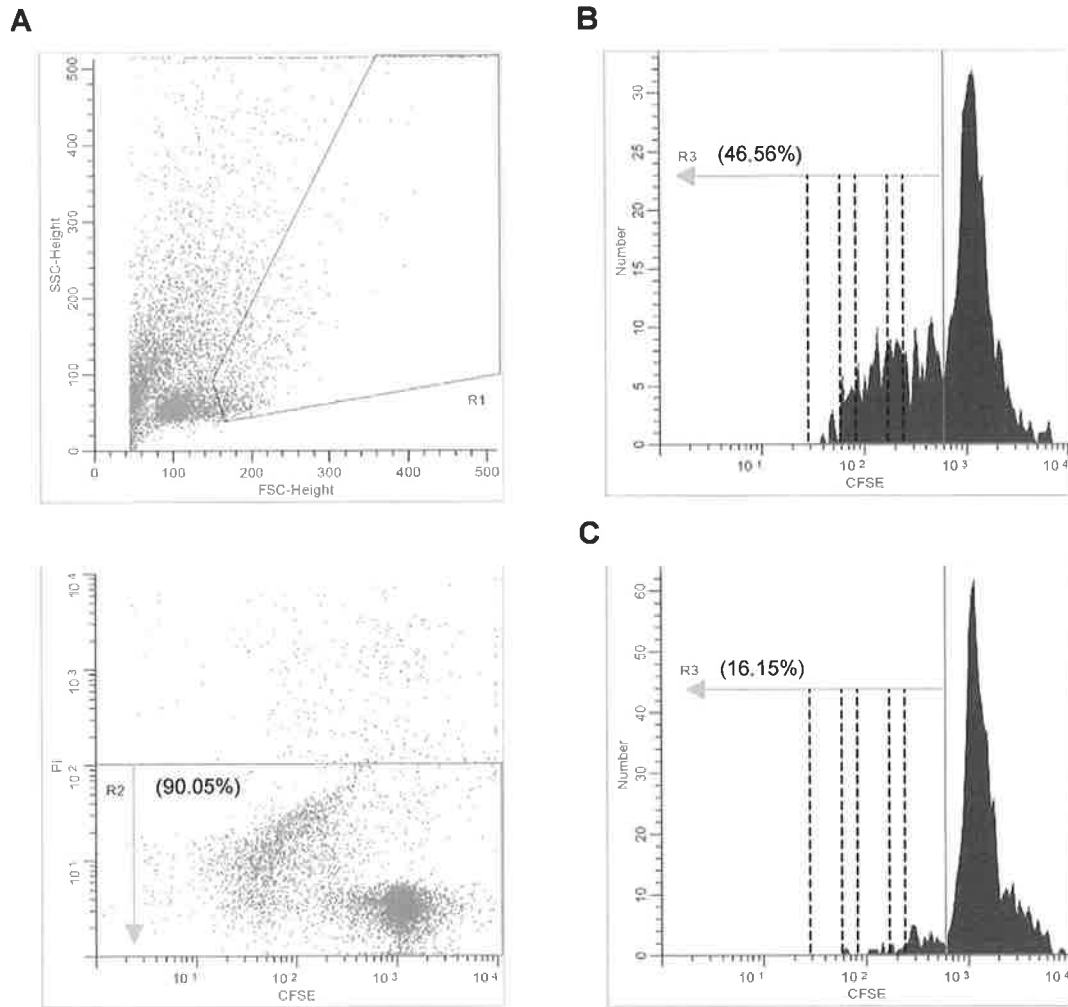


Figure 2.2 WinList histogram plot series showing region definition of cell

populations to track the proliferation of live stimulated cells with CFSE labelling

A, Stimulated (blast) T cells were gated through R1 (*upper panel*) into a dot plot where live PI-negative cells were then gated through R2 (*lower panel*) with both parameters (R1+R2) then gated into a histogram for fluorescence so division profiles could be analyzed where each cycle of cell division is shown by a dashed line. **B**, Control histogram for CFSE fluorescence showing percentage divided T cells in R3 without the addition of any drug. **C**, CFSE fluorescence histogram showing a decreased percentage of divided T cells in R3 with the addition of atorvastatin [50 μ M] as compared to control.

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.7 Intracellular Cytokine Staining

2.7.1 Sampling Procedure

Aseptic technique was used with blood drawn through venepuncture from the cubital fossa veins and then heparinized.

2.7.2 Cell Culture

100 μ l of heparinized whole human peripheral blood was added per well of a 96 well plate in the absence or presence of one of the following drugs at the appropriate final concentration: simvastatin, lovastatin, atorvastatin, rapamycin, mycophenolate, CsA and dexamethasone. Drug combinations of atorvastatin with rapamycin, atorvastatin with mycophenolate, atorvastatin with CsA, atorvastatin with dexamethasone and atorvastatin with CsA and dexamethasone were also used. The total volume was made up to 200 μ l through the addition of RPMI, 5 μ l of phorbol 12-myristate 13-acetate [PMA; 1 μ g/ml] and 4 μ l of ionomycin [50 μ g/ml] with 4 μ l of brefeldin [BFA; 0.1mg/ml] added to each well. BFA is a fungal metabolite which interferes with vesicular transport from the rough endoplasmic reticulum to the Golgi complex by disrupting the structure and function of the latter, which leads to the accumulation of intracellular cytokines. The plate was then incubated for 18 hours (previously optimized by my supervisors through timecourse experiments) at 37°C to maximize cytokine detection.

2.7.3 Staining for Intracellular Cytokines

Cytokine production was then analysed by intracellular immunofluorescence staining. The contents of each cell culture well were transferred to 1.5ml Eppendorf tubes.

The Potential Immunomodulatory Effects of the Statins in Uveitis

1ml 1x FACS Lysing Solution was added for 10 minutes and the tubes centrifuged at 4000 rpm for 3 minutes using a Microcentaur centrifuge (MSE, U.K.). 100µl CytoFix/Perm was then added for 20 minutes at 4°C. CytoFix/Perm enables cell fixation with paraformaldehyde to maintain their structural integrity and permeabilization with saponin thereby enabling intracellular cytokine staining. Cells were washed in 1ml 1x Perm/Wash Buffer and the tubes centrifuged at 4000 rpm for 3 minutes. Finally the intracellular cytokines were added. They were diluted in Perm/Wash Buffer to maintain the cells in a permeabilized state, according to the manufacturers' specifications. 5µl neat of CD3 PerCP (Peridinin chlorophyll), 5µl neat of phycoerythrin (PE)-conjugated anti-IL-2 and 50µl of PE-conjugated IL-10 (diluted 1:100 in Perm/Wash Buffer) were added as well as 50µl each of fluorescein isothiocyanate (FITC)-conjugated anti-IL-4 (diluted 1:200 in Perm/Wash Buffer) and IFN- γ (diluted 1:50 in Perm/Wash Buffer), were added for 30 minutes at 4°C. The cells were washed as before, resuspended in 250µl of PBS and stored at 4°C prior to flow cytometry with the FACScan. Analysis regions were set on lymphocytes according to FSC and SSC characteristics. CD3 positive T lymphocytes were defined and density plots of CD3 PerCP versus IFN- γ /IL-2 or IL-4/IL-10 were generated. Listmode data of 5000 events were collected using CellQuest software.

2.7.4 Analysis

Data was analysed using WinList. The percentage of cytokine positive cells within the CD3 positive population was determined through quadrant analysis of histogram plots (Figure 2.3). Statistical analysis was performed using Mann-Whitney *U* tests as there were sufficient differences in average rank positions between groups.

The Potential Immunomodulatory Effects of the Statins in Uveitis

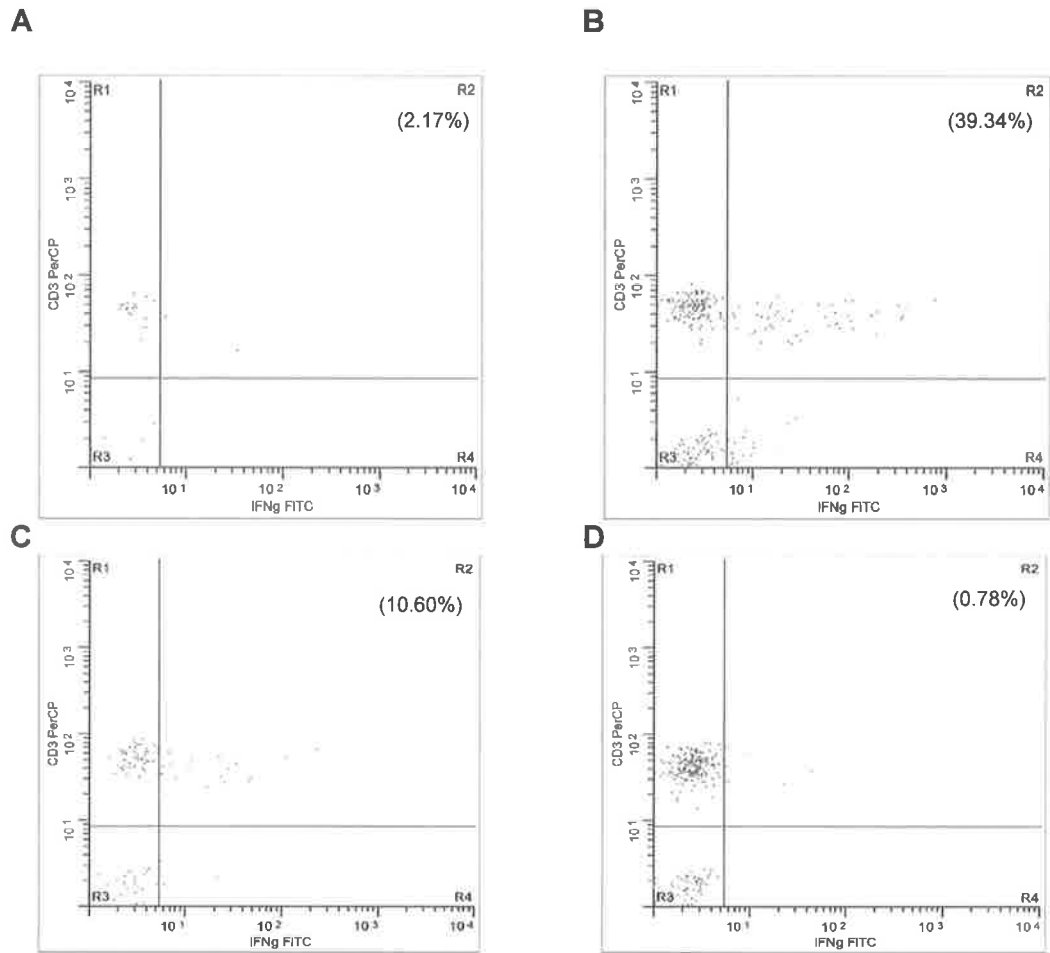


Figure 2.3 WinList histogram plot series showing quadrant analysis to identify live cells double positive for CD3 and IFN- γ .

A, Quadrant analysis of unstimulated and untreated CD3-positive cells to determine their percentage of background IFN- γ expression at baseline. **B**, Quadrant analysis of stimulated and untreated CD3-positive cells to determine their control percentage of IFN- γ expression. **C**, Quadrant analysis of stimulated and atorvastatin [50 μ M] treated CD3-positive cells showing decreased IFN- γ expression as compared to control. **D**, Quadrant analysis of stimulated and dexamethasone [400 μ g/ml] treated CD3-positive cells showing decreased IFN- γ expression as compared to control. Region (R)=CD3 positive, R2=CD3 positive and IFN- γ positive, R3=CD3 negative and IFN- γ negative, R4=IFN- γ positive.

The Potential Immunomodulatory Effects of the Statins in Uveitis

2.8 Multiplex Cytokine Detection with Fluorescent Bead Immunoassay

2.8.1 Sampling Procedure

Aseptic technique was used with blood drawn through venepuncture from the cubital fossa veins and then heparinized as in 2.8.2.

2.8.2 Cell Culture

100µl of heparinized whole human peripheral blood was added to each well of a 96 well plate then 5µl of PMA and 4µl of ionomycin in the absence or presence of one of the following drugs at the appropriate final concentration: simvastatin, lovastatin, atorvastatin, rapamycin, MMF, CsA and dexamethasone. Combinations of T cells and atorvastatin with rapamycin, T cells and atorvastatin with MMF, T cells and atorvastatin with CsA, T cells and atorvastatin with dexamethasone and T cells and atorvastatin with CsA and dexamethasone were also used as in 2.8.3. Additionally CsA was also added for 2 hours prior to PMA/ionomycin stimulation. No BFA was added however. The total volume was then made up to 200µl through the addition of RPMI. The plate was then incubated for 18 hours at 37°C and then centrifuged for at 2300 rpm for 10 minutes with temperature at 20°C and the brake at 0. Supernatants were collected via soft tip pipette and stored at -70°C until assayed.

2.8.3 Multiplex Fluorescent Bead Immunoassay (FBI)

FBI was conducted with the BMS710FF kit as per the manufacturer's instructions. The principles are that two sets of microspheres of two differing sizes, 4.4 µm and 5.5 µm, are coupled to antibodies against one of ten capture cytokine antibodies, IFN-γ, IL-

The Potential Immunomodulatory Effects of the Statins in Uveitis

IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF- α and TNF- β . Each of the two sets consist of five bead populations internally dyed with varying intensities of a fluorescent dye. This allows for simultaneous quantification of ten analytes by incubating a mixture of the coated beads for each analyte with the samples or Th1/Th2 cytokine standard mixture composed of ten cytokine standards (14 – 10000pg/ml). The cytokines present in the sample bind to the antibodies adsorbed to the beads. A biotin-conjugated second antibody mixture is added which binds to the cytokines captured by the first antibodies. Streptavidin-PE is then added which binds to the biotin-conjugate and emits fluorescent signals which are able to be resolved in a single fluorescence channel of the FACScan.

2.8.3.1 Preparation of Assay Buffer

Assay buffer, at RT, was diluted 1:10 with deionized water and mixed gently to avoid foaming.

2.8.3.2 Preparation of Biotin-Conjugate

Biotin-conjugates were stored at +4°C until usage whereupon they were brought to RT and vortexed well before use. 600 μ l of each of the 10 biotin-conjugates was pipetted into 1 tube, resulting in a total volume of 6ml i.e. a 1:10 dilution of each biotin-conjugate stock solution.

2.8.3.3 Preparation of Beads

Beads were stored at +4°C until usage whereupon they were brought to RT and vortexed well before use. 300 μ l of each of the 10 bead sets was pipetted into 1 tube

The Potential Immunomodulatory Effects of the Statins in Uveitis

resulting in a total volume of 3ml bead mixture i.e. a 1:10 dilution of each bead stock solution.

2.8.3.4 Preparation of Th1/Th2 Standards

Lyophilized standards were stored at +4°C until usage whereupon they were brought to RT and vortexed well before reconstitution with sterile water for injection according to the manufacturer's protocol. The vial was swirled thoroughly to ensure quantitative solubilization of contents.

A 1:20 dilution of each reconstituted standard was made as follows:

100µl assay buffer was added to the tube labelled Standard 1. 10µl of each reconstituted standard was then added to the tube and the contents mixed (Standard 1 = 10000pg/ml).

A serial dilution of the standard mixture was then made:

100µl assay buffer was added to 6 tubes labeled Standard 2 to 7. 50µl of Standard 1 was transferred to tube 2 and then mixed. The 50µl contents of tube 2 were then transferred to tube 3 and the procedure repeated creating 7 standard solutions corresponding to 10000, 3333, 1111, 370, 123, 41 and 14pg/ml.

2.8.3.5 Protocol

25µl of assay buffer was added to a blank tube. 25µl of each sample or standard was added to designated tubes. 25µl of diluted bead mixture was added to all tubes (standards, samples and the blank tube). 50µl of diluted biotin-conjugate was added to all

The Potential Immunomodulatory Effects of the Statins in Uveitis

tubes. The resultant mixture was incubated at RT for 2 hours in the dark. The beads were washed in 1ml of assay buffer and spun at 4000 rpm for 5 minutes. The supernatants were discarded. 50µl of Streptavidin-PE (made up according to the manufacturer's instructions) was added to all tubes which were incubated at RT for 1 hour. Beads were washed in 1ml of assay buffer as before and resuspended in 300µl of assay buffer for flow cytometer analysis.

2.8.3.6 Flow Cytometry

Two-colour flow cytometric analysis was performed using a FACSan. 3000 events (300 events per analyte) were acquired using CellQuest software with dimeric microsphere populations gated on forward and side scatters following the protocol supplied.

2.8.3.7 Analysis

Analysis was performed using BMS FlowCytomix Software included in the kit. . The lower limits of detection of each analyte defined as the analyte concentrations resulting in fluorescent intensity significantly higher than that of the dilution medium (mean plus three standard deviations) are shown in Table 2.5. Results were initially expressed as picograms per millilitre but due to the variation between peripheral venous blood samples, data was normalized and represented as percentage increase or decrease in comparison with the positive control of stimulated cells alone. Statistical analysis was performed using one-way ANOVA tests without a Bonferroni correction as there were sufficient differences in average rank positions between groups to draw significant results.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Cytokine	Minimum level of detection (pg/ml)
IL-1 β	6.8
IL-2	13.3
IL-4	9.6
IL-5	7.9
IL-6	7.1
IL-8	9.6
IL-10	10.3
IFN- γ	11.9
TNF- α	4.8
TNF- β	10.5

Table 2.5 Minimum concentration (pg/ml) of cytokines detected by FBI as determined by the manufacturer.

Chapter 3

The Potential Immunomodulatory Effects of the Statins in Uveitis

Cytometric Bead Array

The Potential Immunomodulatory Effects of the Statins in Uveitis

3.1 Introduction

Immunohistological studies of uveitis have demonstrated infiltration by mainly neutrophils but also lymphocytes, plasma cells and fewer numbers of eosinophils and mast cells. Despite these similarities in cellular infiltration, the response to treatment varies with disease. The phenotype of the infiltrating cells and the cytokines they secrete have been studied using various methodologies including enzyme-linked immunosorbent assay (ELISA) and intracellular cytokine staining (Calder *et al* 1999, Hill *et al* 2004, Muhaya *et al* 1998; 1999). AH from patients with idiopathic anterior uveitis (IAU) as compared to non-inflamed controls showed increased CD4⁺ T cell expression and decreased IL-10, with or without an associated systemic disease (Calder *et al* 1999). AH cytokine profiling has also shown balanced IFN- γ and IL-10 with decreased IL-12 in FHC as compared to IAU (Muhaya *et al* 1998). T cell-derived cytokines in the vitreous humor of intermediate uveitis patients have been investigated via T cell line expansion and have been shown to behave similarly to those in the AH of IAU thus validating the sampling of AH in studies of panuveitis (Muhaya *et al* 1999). In addition, AH-derived T cell expression of IFN- γ and IL-10 in acute anterior uveitis (AAU) and panuveitis has been detected by intracellular cytokine staining and flow cytometry (Hill *et al* 2004). However, these techniques require the T cells to be stimulated *in vitro* to express cytokines.

One of the limitations of testing AH is that only a small sample volume can be obtained (typically 50-150 μ l), thus only one to two cytokines can be assayed using traditional ELISA techniques. Cytokines found in AH from uveitis patients include

The Potential Immunomodulatory Effects of the Statins in Uveitis

interleukins -2, -6, -10 and -12, IFN- γ , macrophage migration inhibitory factor (MIF), TGF- β 2 and TNF- α (Abe *et al* 2003, Calder *et al* 1999, El-Shabrawi *et al* 1998, Lacomba *et al* 2001, Santos Lacomba *et al* 2001, Muhaya *et al* 1998, Murray *et al* 1990, Ongkosuwito *et al* 1998, Taguchi *et al* 2001). Even using sequential ELISA, levels of only two or three cytokines per sample can be measured. The alternative is to use pooled samples, which can mask individual patient differences. With the advent of flow cytometric bead-based technology, multiple cytokine analytes can now be more rapidly quantified in single samples, with better reproducibility and sensitivity than ELISA (Morgan *et al* 2004). Up to 15 cytokines in a 100 μ l volume have been accurately measured (Carson and Vignali 1999). The cytometric bead array (CBA) system has been tested using cell culture supernatant, human serum, tears and nasal lavage (Chen *et al* 1999, Morgan *et al* 2004).

3.2 Aims

In this first study we evaluated the use of CBA as compared to ELISA for cytokine determinations (IL-5, IL-10, TNF- α and IFN- γ) in fresh AH of normal controls. CBA was then used to compare subgroups of uveitis AH with controls for their cytokine levels and profiles.

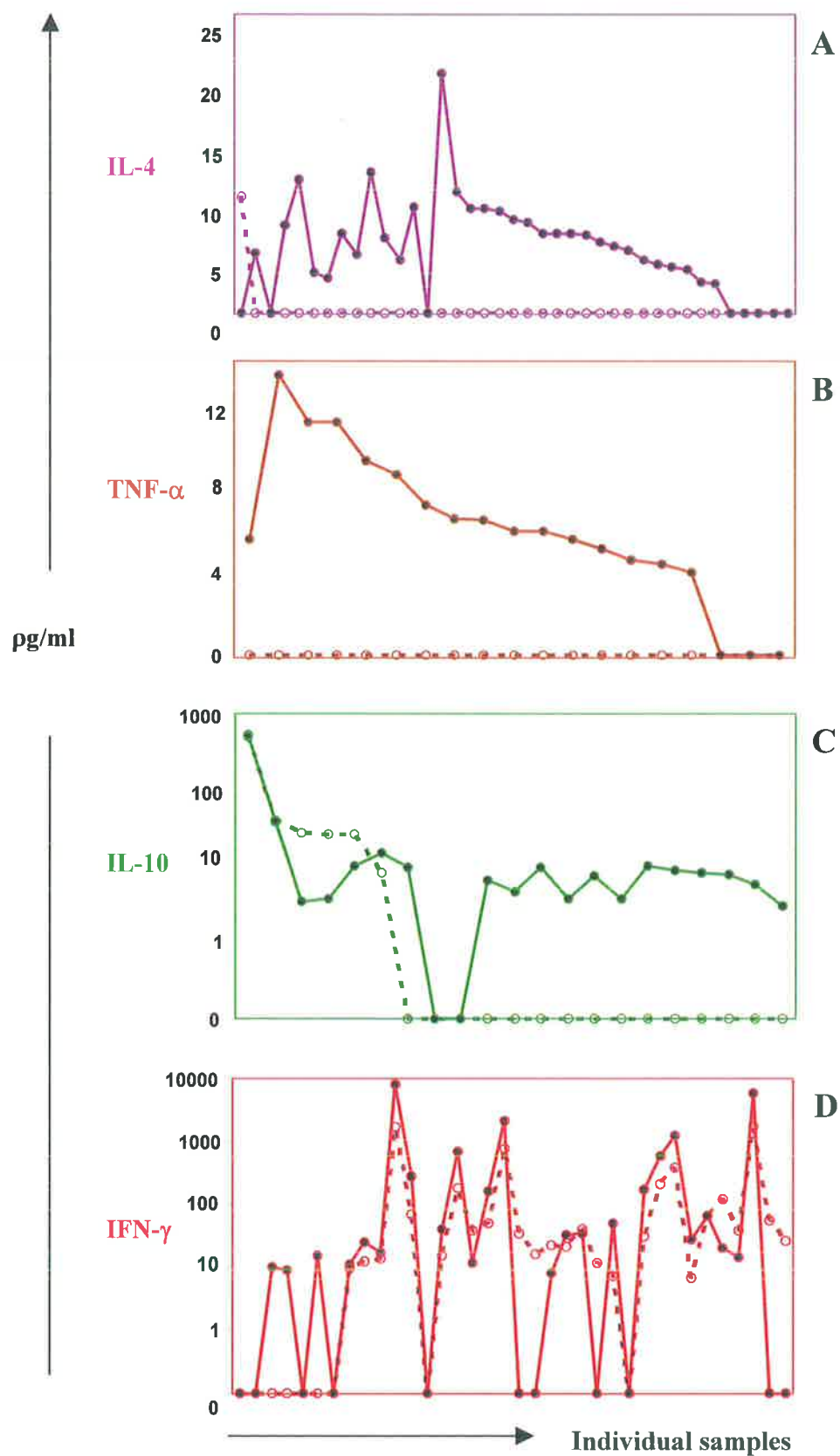
3.3 Results

3.3.1 Optimising Cytokine Detection in AH by CBA and ELISA

Due to the high albumin concentration in AH which may affect binding of antibodies, we initially determined the ability of CBA to detect cytokines in AH was initially determined by spiking cytokine standards in pooled samples of control AH ($n = 7$) and comparing the levels with those in the kit diluent. To the pooled 50-100 μ l AH samples were added the cytokine standards, serially diluted to concentrations of 625, 156, 80, 40, 20 and 0pg/ml. These concentrations were chosen to correspond to the concentrations of cytokines previously detected in AH by ELISA. This resulted in a final concentration of 98.75% AH in the highest cytokine standard and no significant differences in levels of cytokines detected in AH or diluent were seen.

Six cytokines were detected for each AH sample by CBA compared to one with ELISA. The CBA technique was more sensitive than ELISA in detecting IL-4 ($p < 0.01$; Figure 3.1A) and TNF- α ($p < 0.01$; Figure 3.1B) since the majority of samples were below the level of detection for ELISA. There were no statistically significant differences between CBA and ELISA in the detection of IL-10 (Figure 3.1C) and IFN- γ using paired t-tests (Figure 3.1D). Comparing CBA and ELISA, detection of IFN- γ showed good correlation in the levels detected (Figure 3.1D).

The Potential Immunomodulatory Effects of the Statins in Uveitis



The Potential Immunomodulatory Effects of the Statins in Uveitis

Figure 3.1 Cytokine levels (pg/ml) in AH of patients with uveitis as determined by CBA (*filled circles*) versus ELISA (*open circles*).

CBA technique was more sensitive in detecting IL-4 ($p<0.01$) [Figure 3.1A] and TNF- α ($p<0.01$) [Figure 3.1B] at the lower end of the concentration range. There were no statistically significant differences between CBA and ELISA in the detection of IL-10 [Figure 3.1C] and IFN- γ [Figure 3.1D]. CBA and ELISA detection of IFN- γ showed good correlation in the levels detected between the two techniques [Figure 3.1D].

The Potential Immunomodulatory Effects of the Statins in Uveitis

3.3.2 CBA Cytokine Detection in Uveitis and Controls

CBA was then used to detect IL-2, IL-4, IL-5, IL-10, TNF- α and IFN- γ in AH of uveitis patients as compared to controls. There was a highly significant increase in levels of IFN- γ in both the AU and panuveitis groups as compared to controls ($p < 0.01$; Figure 3.2A). Interestingly there was no IL-5 detected in the panuveitis group as compared to controls ($p < 0.01$), and the AU group ($p < 0.05$; Figure 3.2B). For IL-2, IL-4 (Figure 3.2C) and IL-10 (Figure 3.2D), no differences in levels between uveitis groups and controls were found with IL-2 not detectable in any of the samples. There were also no differences in TNF- α levels, comparing controls with uveitis (Figure 3.2E).

The Potential Immunomodulatory Effects of the Statins in Uveitis

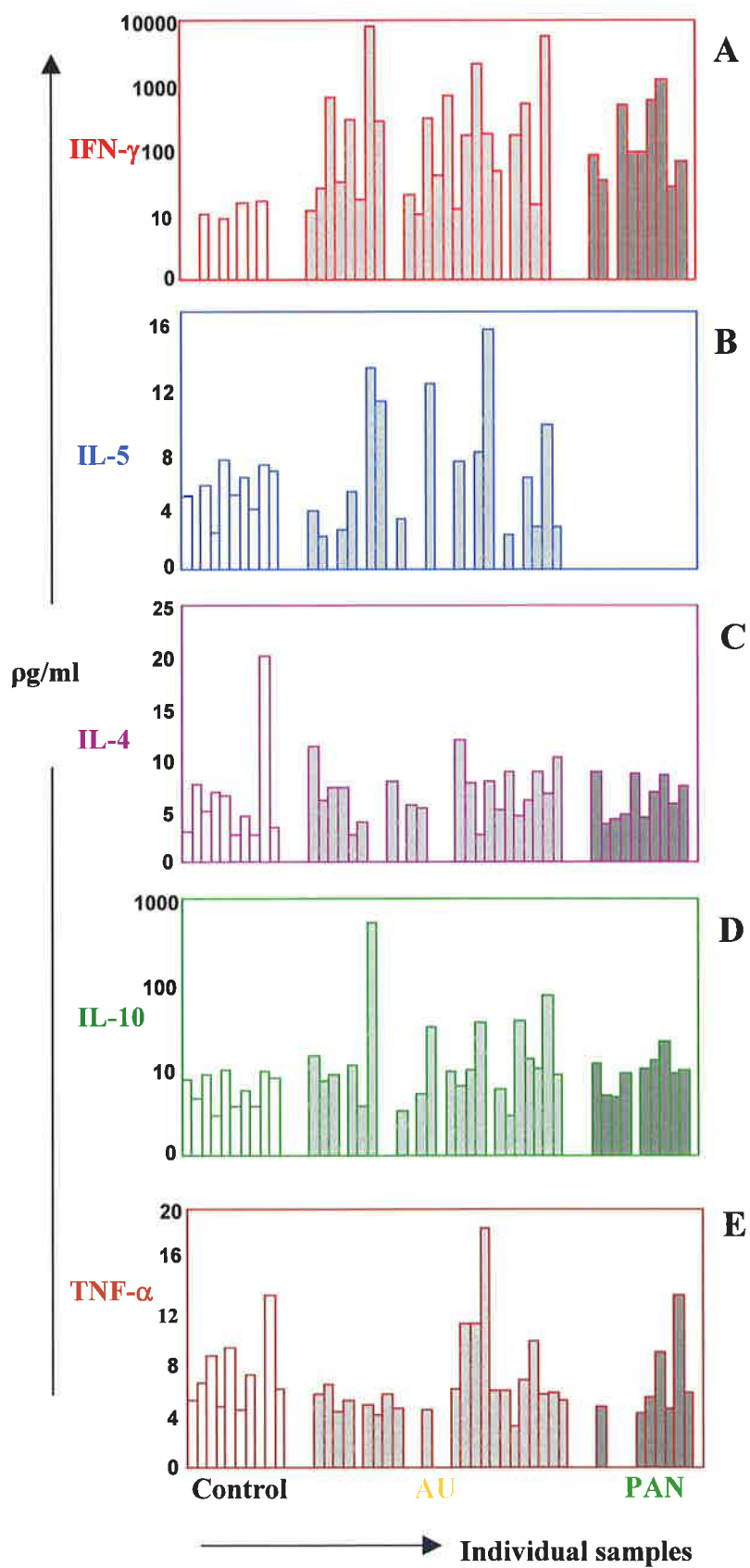


Figure 3.2

The Potential Immunomodulatory Effects of the Statins in Uveitis

Figure 3.2 Cytokine levels (pg/ml) in AH of patients with AU and panuveitis (PAN) versus controls.

There were raised levels of IFN- γ in both the AU and PAN groups as compared to controls ($p < 0.01$; Figure 3.2A). There was no IL-5 in the PAN group as compared to controls ($p < 0.01$), and the AU group ($p < 0.05$; Figure 3.2B). For IL-4 (Figure 3.2C), IL-10 (Figure 3.2D) and TNF- α (Figure 3.2E) no differences in levels between uveitis groups and controls were found.

The Potential Immunomodulatory Effects of the Statins in Uveitis

3.3.3 Effect of Steroid Usage

Cytokine levels in the control group were compared with the panuveitis group who were taking steroids and those AU patients either taking or not taking steroids. Since there were only 2 AH samples in the panuveitis group not receiving steroids, this data was not included.

No significant differences were noted for IL-2, IL-4 and TNF- α between any of the groups. The panuveitis group on steroids had no detectable levels of IL-5 compared with controls ($p<0.01$) and those AU patients not taking steroids ($p<0.01$; Figure 3.3A). There was no difference in IL-5 levels comparing the panuveitis group on steroids and those AU patients on steroids as the levels of IL-5 were relatively low and variable (Figure 3.3A).

There were no significant differences in IL-10 production between the control group and those with AU regardless of their steroid usage (Figure 3.3B). Those patients with panuveitis and steroid use, however, showed a significant increase in IL-10 levels as compared with controls ($p<0.01$; Figure 3.3B).

There was a significant increase in IFN- γ production in both AU patient groups with and without steroid usage compared to controls ($p<0.01$; Figure 3.3C). This was also true for the panuveitis patients taking steroids, with significant increase in IFN- γ as compared to controls ($p<0.01$; Figure 3.3C).

The Potential Immunomodulatory Effects of the Statins in Uveitis

3.3.4 Effect of Systemic Disease

The correlation of cytokines between AU patients with and without systemic disease and controls was investigated. Panuveitis patients with and without systemic disease were also compared to controls. There were no significant differences in production of IL-2, IL-4 or IL-10 in any of the uveitis groups as compared to controls.

Panuveitis patients whether with or without systemic disease, by virtue of a total lack of production, had significantly less IL-5 as compared to controls ($p < 0.01$ and $p < 0.05$; Figure 3.4A). IL-5 production in panuveitis groups with or without systemic disease was less than their corresponding AU groups ($p < 0.05$ and $p < 0.01$; Figure 3.4A). There were no significant differences in IL-5 production in both AU groups when compared with the control group (Figure 3.4A). Interestingly, those few AH samples giving the highest IL-5 values in the AU group with a systemic disease, were associated with AS ($n = 5$), although this failed to reach statistical significance. TNF- α production was also higher in those patients AU patients with AS compared with all uveitis patients with systemic disease ($p < 0.05$; data not shown). TNF- α production generally in all the uveitis groups was not significantly increased as compared to controls.

When IL-10 production was analysed, there were no significant differences in any of the uveitis groups when compared with controls. The increase in IFN- γ production in the AU group was statistically higher than in the control group whether with or without an associated systemic disease ($p < 0.01$; Figure 3.4B). Similarly the increase in IFN- γ production in the panuveitis group as a whole was significantly higher than the control

The Potential Immunomodulatory Effects of the Statins in Uveitis

group whether with ($p<0.05$) or without an associated systemic disease ($p<0.01$; Figure 3.4B). There were no significant differences in IFN- γ production between any of the uveitis groups whether with or without a systemic disease (Figure 3.4B).

3.3.5 Cytokine Correlation in AH

Positive correlations were seen between between TNF- α and IL-4 ($p<0.01$), TNF- α and IL-10 ($p<0.05$), TNF- α and IFN- γ ($p<0.01$) as well as IL-10 and IL-5 ($p<0.05$) in control AH (data not shown). Positive correlations were also seen between IFN- γ and IL-10 for all uveitis groups ($p<0.01$) but not in the control group. Only within FHC samples was a positive correlation seen between IL-5 and IL-4 ($p<0.01$), however there were only 5 FHC samples, so it is not possible to be confident of this data

The Potential Immunomodulatory Effects of the Statins in Uveitis

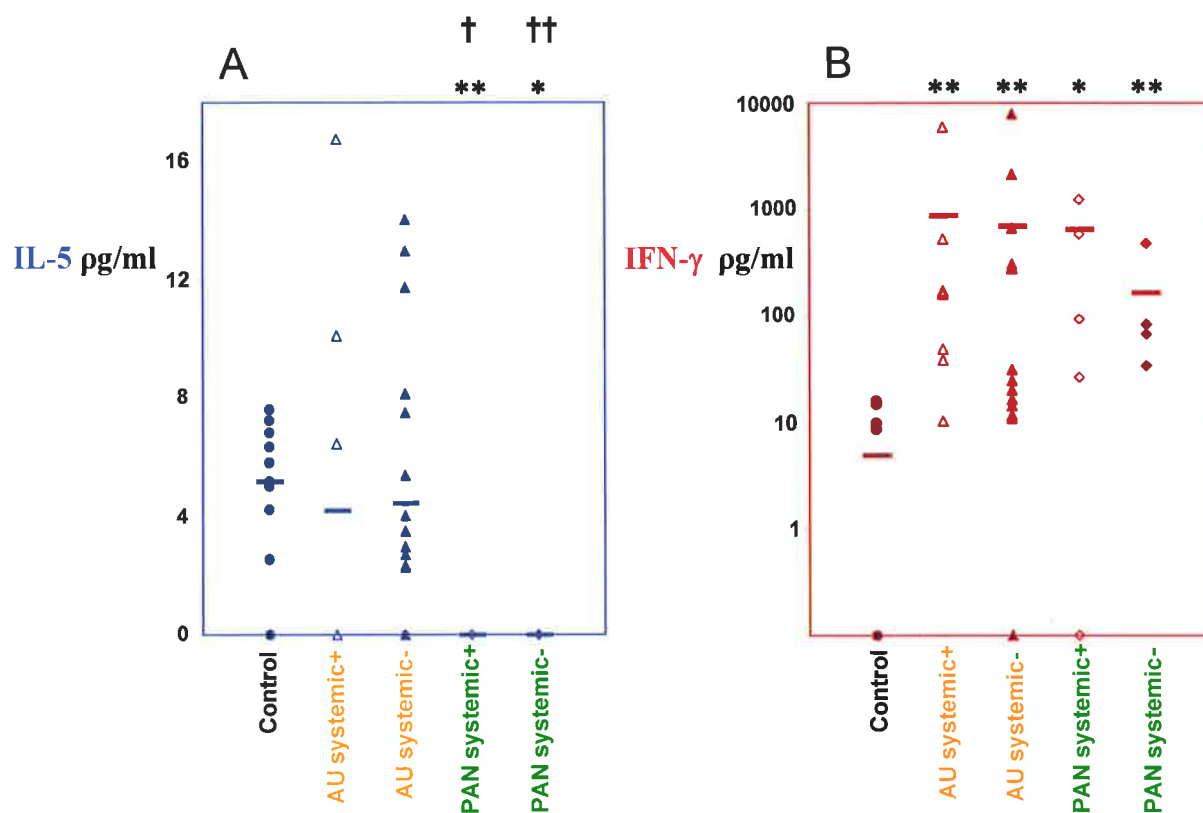


Figure 3.4 Cytokine levels (pg/ml) in AH of controls compared to AU patients with or without systemic disease, and PAN with or without systemic disease.

*p<0.05 compared with control. **p<0.01 compared with control. †p<0.05 compared with AU systemic+. ††p <0.01 compared with AU systemic-.

3.4 Discussion

In this study we firstly compared the efficacy of CBA versus ELISA in the detection and quantification of 4 cytokines in the AH of patients with uveitis. ELISA was comparable to CBA in the detection of IL-10 and IFN- γ but CBA proved to be more sensitive than ELISA in the detection of IL-4 and TNF- α . In agreement with others, CBA was a rapid means of measuring multiple cytokine analytes from a single, small volume, which, in this study, was usually 75 μ l of AH.

Since uveitis describes a variety of clinical entities with differing clinical phenotypes, it is possible that different mechanisms are involved in each clinical subtype. However T lymphocytes have been shown to dominate the cellular immune response in the flow cytometric analysis of lymphocytes from ocular fluids during uveitis (Deschênes *et al* 1986; 1988). The magnitude and pattern of the cytokine response in different types of uveitis, however, remains unclear. Using CBA we therefore focussed on a panel of T cell-related cytokines, some of which have previously been detected in AH in uveitis.

3.4.1 IL-2

Levels of IL-2 in AH of uveitis patients and controls were below the level of detection. This is in contrast with Hooks *et al* (1988) who demonstrated the presence of increased amounts of IL-2 and IFN- γ in the ocular tissue of 1 patient with idiopathic uveitis and 3 patients with sympathetic ophthalmia but not in 3 normal individuals. Since these cytokines were detected using an immunoperoxidase procedure, direct comparisons

The Potential Immunomodulatory Effects of the Statins in Uveitis

cannot be made. Lacomba *et al* (2000) also detected IL-2 by ELISA in AH from uveitis patients, which reached statistical significance in all of their idiopathic uveitis subset unlike the present study in which only 2 showed any increase. Our results, however, agree with those of Ongkosuwito *et al* (1998) who demonstrated that IL-2 levels remained below the ELISA detection limit in their cohort of 'autoimmune' uveitis patients with Behçet's and sarcoidosis. It is possible that although the patients were clinically phenotyped as having active uveitis, the production of early activation cytokines such as IL-2 might have peaked prior to sampling or that the activated T cells were using any available IL-2 for ongoing activation.

3.4.2 TNF- α

The literature concerning the prevalence of TNF- α in uveitis patients has been conflicting. We did not detect TNF- α in AH to any level of statistical significance but Lacomba *et al* (2001) demonstrated significantly higher levels of TNF- α in the AH and sera in autoimmune uveitis. Our results however are consistent with others who found no appreciable elevation of TNF- α in either AH or sera of their uveitis group (Feys *et al* 1994). These contrasting results may be related to differing assay techniques and uveitis populations as well as the absence or presence of immunomodulators at time of sampling. Contrary to our findings, it was shown that the concentration of TNF- α in AH in patients who are HLA-B27 positive is significantly greater than in those who are HLA-B27 negative (Perez-Guijo *et al* 2004). This may be due to their larger sample size and the fact that their HLA-B27 population comprised of other entities besides AS. Our cohort of HLA-B27 positive AS patients however, did have a significantly greater production of

The Potential Immunomodulatory Effects of the Statins in Uveitis

TNF- α than the uveitis group as a whole, supporting the hypothesis that anti-TNF α therapies may be of therapeutic benefit (Smith *et al* 2001, Greiner *et al* 2004).

3.4.3 IFN- γ

We found consistently higher levels of IFN- γ produced in both the uveitis groups than in the control group. This was true whether they were associated with a systemic disorder or not and regardless of steroid use. The increased levels of IFN- γ correlated with decreased levels of IL-4 and IL-5. Within the FHC group only, there was a correlation between IL-4 and IL-5. Raised IL-4 has not previously been detected and may relate to the sensitivity of detection methods employed (Muhaya *et al* 1998, Murray *et al* 1999). We also observed an equal ratio of IFN- γ :IL-10 within the FHC group as compared to the idiopathic uveitis group and a negative correlation coefficient confirmed decreased IFN- γ balanced by IL-10 within FHC samples. Recently rubella antibodies and the rubella virus have been detected in FHC (Quentin and Reiber 2004). Cytokine profiling after rubella vaccine inoculation has demonstrated marked plasma elevations in IL-4 and IL-10 with a significant reduction in IFN- γ (Pukhalsky *et al* 2003). This parallels the cytokine profiles in AH in this study and further supports a role for rubella in FHC pathogenesis.

3.4.4 IL-4

IL-4 was significantly detected by CBA at the lower end of the concentration range but not detected at all by ELISA. Perhaps CBA proved more sensitive than ELISA as the latter is an inherently slower technique thereby allowing for greater cytokine

The Potential Immunomodulatory Effects of the Statins in Uveitis

degradation during assay. IL-4 levels did not differ between groups and controls. It is likely therefore that this cytokine does not play an important role in uveitis affecting man possibly because its production is down-regulated by IFN- γ , which is detectable in AH in uveitis (Lacomba *et al* 2000, Muhaya *et al* 1998).

3.4.5 IL-5

In contrast to AU and controls, IL-5 production in AH was not detectable in the panuveitis group. No significant differences in IL-5 production were found between the AU groups with or without an associated systemic disease and controls. Although no such direct comparisons have been made in the literature this differential IL-5 production might suggest that Th1 polarity is more marked with greater uveal tract involvement in disease. Moreover a greater decreased production of IL-5 was evident in the population of panuveitis with an associated systemic disease, suggesting that an associated systemic disease leads to a greater polarisation of the AH immune response towards Th1. Although such studies have not been done with ocular lymphocytes, a similar parallel has been reported in the peripheral blood lymphocyte culture supernatants of presumed sarcoid uveitis patients where the demonstration of high IL-2:IL-5 and IFN- γ :IL-5 ratios suggests a Th1 polarisation of the systemic immune response (Murphy *et al* 2003). The significantly decreased production of IL-5 in the panuveitis group taking steroids suggests that glucocorticoids have no upregulatory effect on IL-5 in the AH. Overall the levels of IL-5 detected in the AH were considerably lower than those of IFN- γ , suggesting that IFN- γ is the predominant cytokine in the AC.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Interestingly production of IL-5 was relatively increased in those patients with AS and HLA-B27 positivity as compared to other systemic disorders. Statistical significance may not have been reached because of the relatively small sample size. Various autoimmune disorders including AS have been linked with HLA-B27 positivity and concurrent infection (Ooi *et al* 2004). Reports of linkages between IL-5 and HLA-B27 are scarce but Lahesmaa *et al* (1992) did identify *Yersinia enterocolitica* antigen in 2 HLA-B27 positive patients in whom it was thought the antigen activated a Th1-like T cell subset in reactive arthritis. These T cells produced IL-4, IL-5 or both cytokines upon activation suggesting that these cells were pathogenic in this disease. In airway eosinophilic inflammation IL-5 production by Th2 cells has been shown to be increased in the presence of *Candida albicans* (Mori *et al* 2001). More recently it has been reported that IL-4, at least in murine studies, if selectively neutralized, will improve survival from airway eosinophilic inflammation in susceptible strains (Lilic *et al* 2003, Romani *et al* 1992). Indeed it has been previously reported that the presence of *Candida albicans* may be a trigger for Churg-Strauss syndrome with its eosinophilic pneumonia and ocular involvement in a patient with AS and HLA-B27 positivity (Ooi *et al* 2004). This concurs with earlier *in vitro* work which showed an increased lymphocyte proliferative response to yeast histone H3 peptide in 2 patients with idiopathic human uveitis (Rajasingh *et al* 1996). Closer work with a larger population of AH samples in patients with uveitis and HLA-B27 positivity may reveal an infectious cause with concomitant IL-4 and IL-5 rise.

The Potential Immunomodulatory Effects of the Statins in Uveitis

3.4.6 IL-10

There were no statistical differences in IL-10 levels detected in any of the uveitis groups compared with the controls regardless of whether a systemic disease was present. This is concordant with el-Shabrawi (1998) who found increased IL-10 in only 3 of their 22 patients with uveitis. This lack of IL-10 production is also consistent with our previous findings by ELISA (Calder *et al* 1999). In contrast, in 5 FHC patients thus far investigated, we detected significant increases in IL-10 ($p < 0.05$; data not shown) which is also in keeping with our previous findings (Hill *et al* 2004, Muhaya *et al* 1998). IL-10, along with TGF- β , is thought to participate in immunoregulation of disease processes due to their effect on pro-inflammatory cytokines such as TNF- α and their inhibitory effects on Th1 and Th2 cell function (Horwitz *et al* 2003). It is also thought to act as a negative regulator for IL-12-induced inflammation, with IL-10 levels upregulated to counteract high levels of IL-12 (Borish and Rosenwasse 1996, Meyaard *et al* 1996). IL-10 is produced by a range of cell types including Th0, Th1, Th2 and Tr cell subsets, B cells, eosinophils, mast cells and monocytes/macrophages, with the latter being the most important source in humans (Del Prete *et al* 1993, Groux *et al* 1997, Horwitz *et al* 2003, Lamkhieued *et al* 1996, Moore *et al* 2001). Interestingly the increased level of IL-10 production in the panuveitis group taking steroids reached statistical significance over the control group. This correlates with the work of Barrat *et al* (2002) who showed that a combination of vitamin D3 and dexamethasone induced human naïve CD4⁺ T cells to differentiate *in vitro* into Tr cells which produced only IL-10. Mozo *et al* (2004) demonstrated that production of IL-10 by monocytes could also be up-regulated *ex vivo*

The Potential Immunomodulatory Effects of the Statins in Uveitis

by dexamethasone. A similar effect could be occurring *in vivo* for immunomodulation of Th1 effector function.

One advantage of CBA is the ability to compare relative cytokine levels within single samples. Positive correlations were seen between TNF α and IL-4, TNF α and IL-10, TNF α and IFN- γ as well as IL-10 and IL-5 within the control AH suggesting a balance of Th1 and Th2 cytokines. Positive correlations were also seen within AH between IFN- γ and IL-10 for all other uveitis groups, suggesting an immunoregulatory response with the upregulation of IL-10 in an attempt to counter the pro-inflammatory effects of IFN- γ .

In summary, we have demonstrated the usage of CBA to detect multiple cytokines simultaneously in a small AH volume with greater efficacy than that of ELISA. This has enabled us to more accurately define the interplay of cytokines between AU and panuveitis groups and suggest a more marked Th1 cytokine polarization in panuveitis as compared to AU. In addition, Th1 cytokines appear to be reduced in those panuveitis patients on steroids. Larger numbers of each disease category in further studies are ideally required to confirm these findings. CBA would, however, appear to be an important tool for the understanding of the pathogenesis of uveitis in order to tailor novel, disease-specific, cytokine-directed therapies in the future.

Chapter 4

The Potential Immunomodulatory Effects of the Statins in Uveitis

Viability and Proliferation Assays

The Potential Immunomodulatory Effects of the Statins in Uveitis

4.1 Introduction

Statins have been found clinically and in experimental models to exert immunosuppressive effects in addition to their effect on lowering cholesterol. The effects of statins *in vitro* on T cell responses are less well understood. The inhibition of T cell proliferation by HMG-CoA reductase inhibitors through blockage of LFA-1 binding to ICAM-1 (lovastatin and simvastatin) and the up-regulation of p27^{kip1}, the cyclin-dependent kinase 4 (CDK4) inhibitor involved in negative regulation of the T cell cycle (atorvastatin), has been documented (Aktas *et al* 2003, Weitz-Schmidt *et al* 2001).

Lovastatin has been identified as an extracellular inhibitor of LFA-1 (Kallen *et al* 1999, Weitz-Schmidt *et al* 2001) and has been shown to decrease LFA-1 mediated leukocyte adhesion to ICAM-1 and T cell co-stimulation. In fact lovastatin was shown to completely block the proliferative response of T cells induced by ICAM-1 in combination with a monoclonal antibody against the TCR (Weitz-Schmidt *et al* 2001). However, this inhibition was partially reversed by the addition of mevalonate, the immediate downstream metabolite of HMG-CoA reductase. This reversal indicates that the inactive lactone form of lovastatin is hydrolyzed to the active hydroxyl acid form, which seems to interfere with the T cell response by inhibiting HMG-CoA reductase in lymphocytes. It has been reported that HMG-CoA reductase inhibition in lymphocytes leads to impaired proliferative responses to IL-2 or phytohemagglutinin (PHA; Kurakata *et al* 1996). Lovastatin has also been shown to bind to a hitherto unknown site in the LFA-1 inserted (I) domain. This site termed the 'lovastatin site' (L-site) is distant from the ICAM-1 binding site, which indicates that lovastatin inhibits LFA-1 via an allosteric mechanism

The Potential Immunomodulatory Effects of the Statins in Uveitis

(Lu *et al* 2001). Simvastatin has also been shown to inhibit LFA-1 by binding to the L-site (Weitz-Schmidt *et al* 2001).

In *in vitro* assays, atorvastatin has not only inhibited antigen-specific responses, but also decreased T cell proliferation mediated by direct TCR engagement independently of MHC class II and LFA-1. In one study, inhibition of proliferation has been demonstrated to be not due to apoptosis induction, but linked to a negative regulation on cell cycle progression. Early T cell activation, however, was unaffected as reflected by unaltered calcium fluxes. Growth inhibition by atorvastatin was associated with a down-regulation of CDK4. The expression of p27^{kip1}, a CDK inhibitor involved in negative regulation of the T cell cycle, was also found to be up-regulated on atorvastatin incubation, which was reversed by mevalonate (Aktas *et al* 2003).

Historically the assessment of T cell function has involved assays measuring various aspects of T cell physiology, typically their ability to undergo mitosis or produce cytokines in response to mitogen or antigen. The classical means of measuring T cell proliferative response to such stimuli has been through their uptake of [³H]-thymidine during the final hours of a 3 to 5 day culture. This assay is labour-intensive, requiring handling of radioisotopes, scintillation fluid and expensive counting equipment. Most importantly it does not give information as to the phenotype of the proliferating T cell in mixed T cell proliferations. Interpretation of results is also problematic; a low level of [³H]-thymidine uptake could be explained either by a depressed T cell count or impaired cell function or (Fulcher and Wong 1999).

The Potential Immunomodulatory Effects of the Statins in Uveitis

CFSE is a newer, simpler technique which enables the tracking of cells *in vivo* and also tracing of their mitotic activity (Lyons and Parish 1994). This fluorescent dye stains intracellular proteins and produces a fluorescent signal that sequentially halves with each mitosis, producing peaks which each represent a division. This reduction in fluorescence intensity can be measured via flow cytometry. In addition, cultured cells can be stained for expression of other cell surface markers to define lineage or activation state. In addition to being cheaper and less labour-intensive than [³H]-thymidine, it has the advantage of allowing for specific gating on particular T cell subsets and simultaneous assessment of activation marker expression directly and in terms of cell cycle (Fulcher and Wong 1999).

4.2 Aims

In this study we looked at both the effect on T cell viability and the inhibition of T cell proliferation by atorvastatin, lovastatin and simvastatin. These were compared with three known immunosuppressants currently used in uveitis therapy – cyclosporin A (CsA), rapamycin (rapamune, sirolimus, SRL) and mycophenolate mofetil (MMF; Masri 2003). The specific T cell inhibitory effects of the statins were controlled for by testing for mevalonate reversibility. Viability was assessed through the use of PI while proliferation was tracked with CFSE labeling. Proliferation stimulation was achieved with unconjugated primary anti-human CD3 and CD28 antibodies in order to achieve a T cell-specific response by engaging the TCR-associated signaling pathways.

The Potential Immunomodulatory Effects of the Statins in Uveitis

4.3 Results

4.3.1 T Cell Viability

T cell viability was assessed through the use of PI over a range of drug concentrations for each of the drugs tested as well as their vehicle controls. T cell viability was not significantly affected by drug vehicle controls. Mevalonate alone did not have any significant adverse effect on viability (Figures 4.1, 4.2 and 4.3).

T cell viability at maximum concentration of atorvastatin tested without the addition of mevalonate was $88.7 \pm 1.9\%$ (Figure 4.1). The addition of mevalonate to atorvastatin appeared to increase T cell viability as compared to atorvastatin alone at the two highest atorvastatin concentrations tested although significance was only reached with the maximum concentration (Figure 4.1). T cell viability at maximum concentration of lovastatin tested was $84.2 \pm 2.0\%$ which was significantly decreased from the control group (Figure 4.2). The addition of mevalonate slightly increased T cell viability at all lovastatin concentrations tested above $0.5\mu\text{M}$ (Figure 4.2). Simvastatin $50\mu\text{M}$ significantly decreased T cell viability to $88.08 \pm 0.16\%$ but at maximum concentration viability was $93.01 \pm 0.86\%$ (Figure 4.3). The addition of mevalonate slightly increased T cell viability at all simvastatin concentrations tested (Figure 4.3).

The Potential Immunomodulatory Effects of the Statins in Uveitis

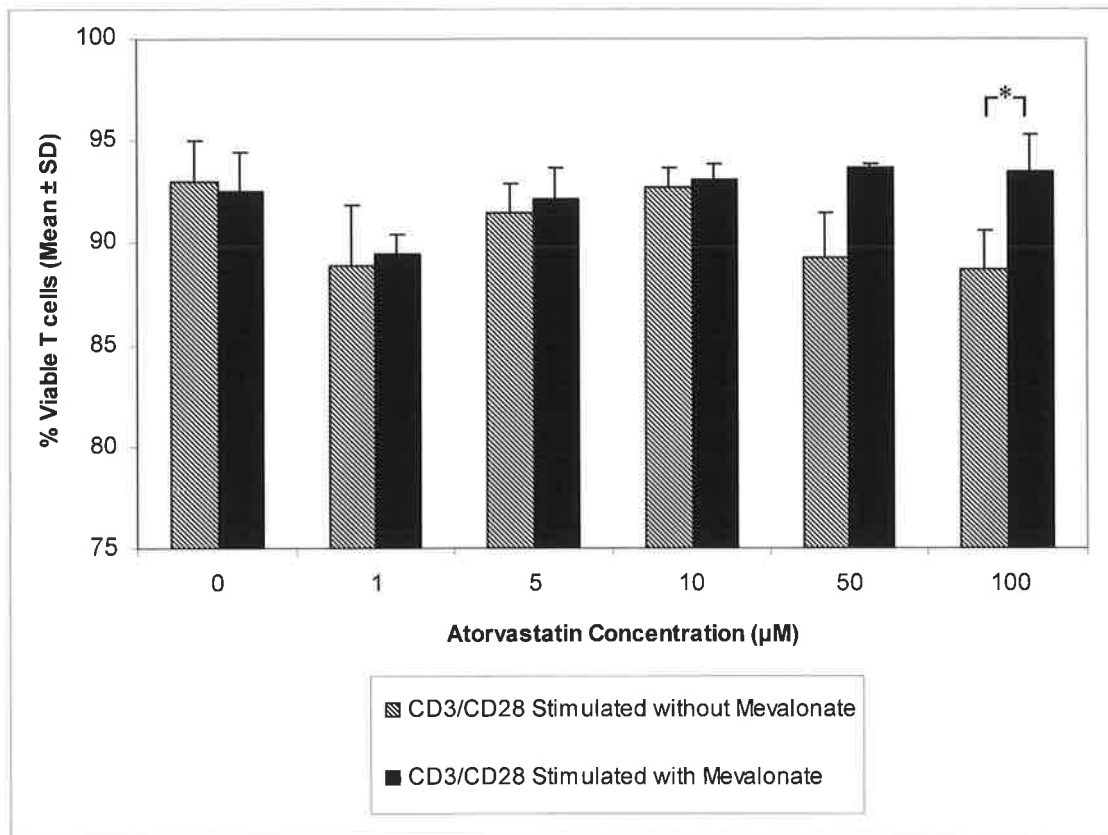


Figure 4.1 CD3/CD28 stimulated viable T cells (%) with increasing Atorvastatin concentrations.

CFSE-labelled T cells were cultured for 5 days in the presence of increasing concentrations of atorvastatin and unconjugated primary anti-human CD3 (10ng/ml) and CD28 (5µg/ml) antibodies for proliferation stimulation.

Viability was assessed through staining with PI.

Data is shown as the means of 3 triplicate assays ± SD.

Statistical analysis was performed using the paired t-test. *p<0.01

The Potential Immunomodulatory Effects of the Statins in Uveitis

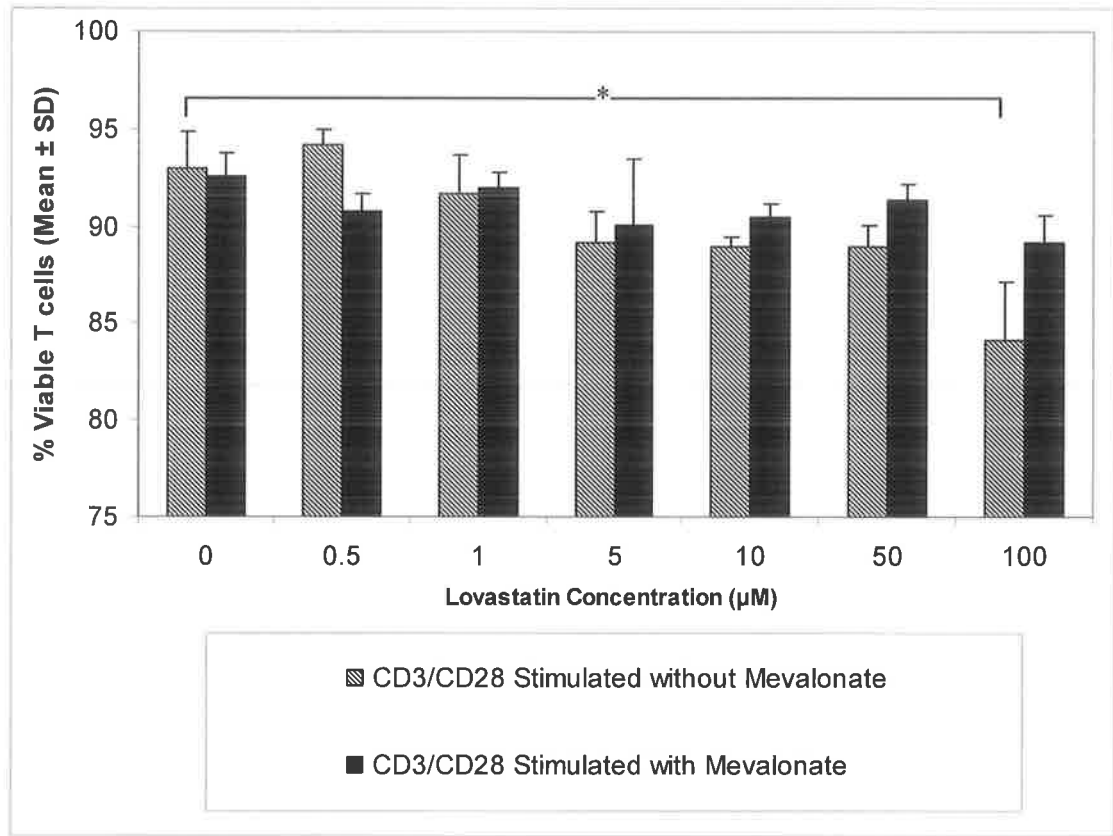


Figure 4.2 CD3/CD28 stimulated viable T cells (%) with increasing Lovastatin concentrations.

CFSE-labelled T cells were cultured for 5 days in the presence of increasing concentrations of lovastatin and unconjugated primary anti-human CD3 (10ng/ml) and CD28 (5µg/ml) antibodies for proliferation stimulation.

Viability was assessed through staining with PI.

Data is shown as the means of 3 triplicate assays $\pm$ SD.

Statistical analysis was performed using the paired t-test. * $p < 0.05$

The Potential Immunomodulatory Effects of the Statins in Uveitis

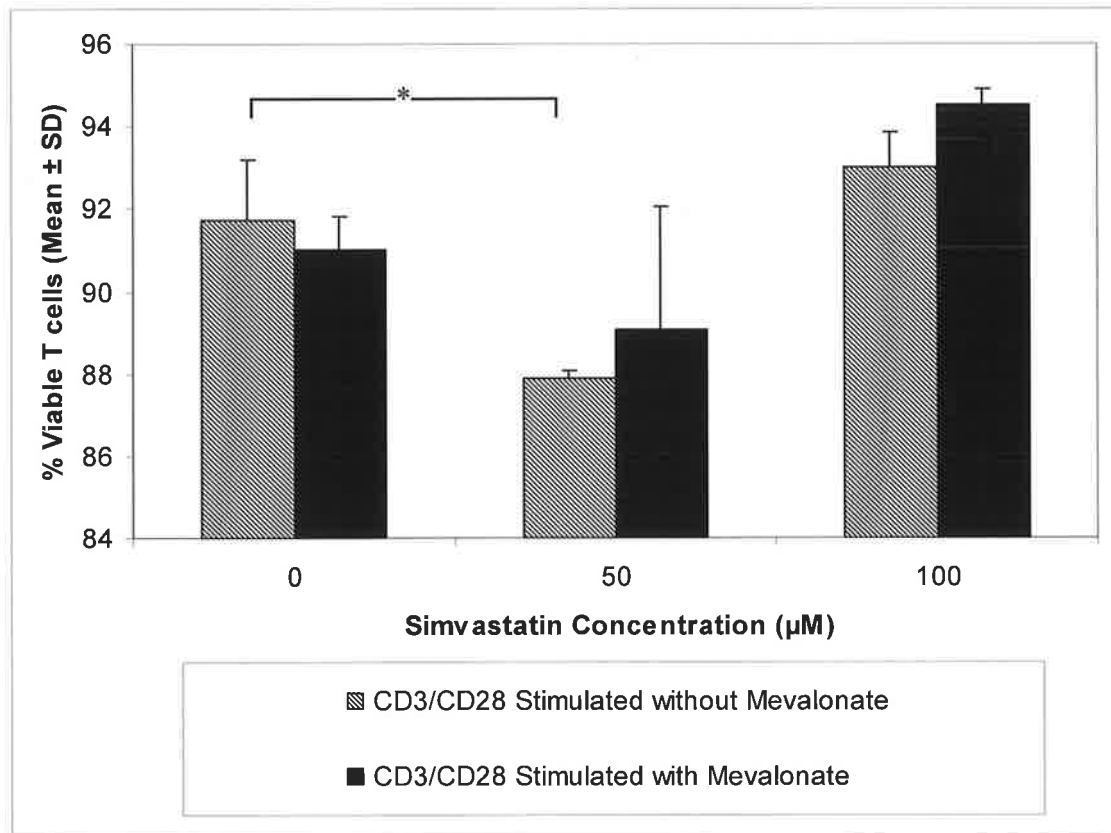


Figure 4.3 CD3/CD28 stimulated viable T cells (%) with increasing Simvastatin concentrations.

CFSE-labelled T cells were cultured for 5 days in the presence of increasing concentrations of simvastatin and unconjugated primary anti-human CD3 (10ng/ml) and CD28 (5μg/ml) antibodies for proliferation stimulation.

Viability was assessed through staining with PI.

Data is shown as the means of 3 triplicate assays ± SD.

Statistical analysis was performed using the paired t-test. *p<0.05

The Potential Immunomodulatory Effects of the Statins in Uveitis

T cell viability at maximum concentrations of rapamycin and mycophenolate tested were $90.72 \pm 1.90\%$ and $88.09 \pm 3.86\%$ respectively (Figure 4.4). Unstimulated T cell viability at maximum concentration of CsA (500ng/ml) was $91.33 \pm 0.22\%$ and significantly reduced as compared to the viability with 250ng/ml CsA ($p < 0.05$; Figure 4.5). Stimulated T cell viability at maximum concentration of CsA (500ng/ml) was $91.49 \pm 0.74\%$ and significantly reduced as compared to the viability with 250ng/ml CsA ($p < 0.05$; Figure 4.5). T cell viability at maximum concentration of dexamethasone tested was $90.85 \pm 1.85\%$ (Figure 4.6).

The Potential Immunomodulatory Effects of the Statins in Uveitis

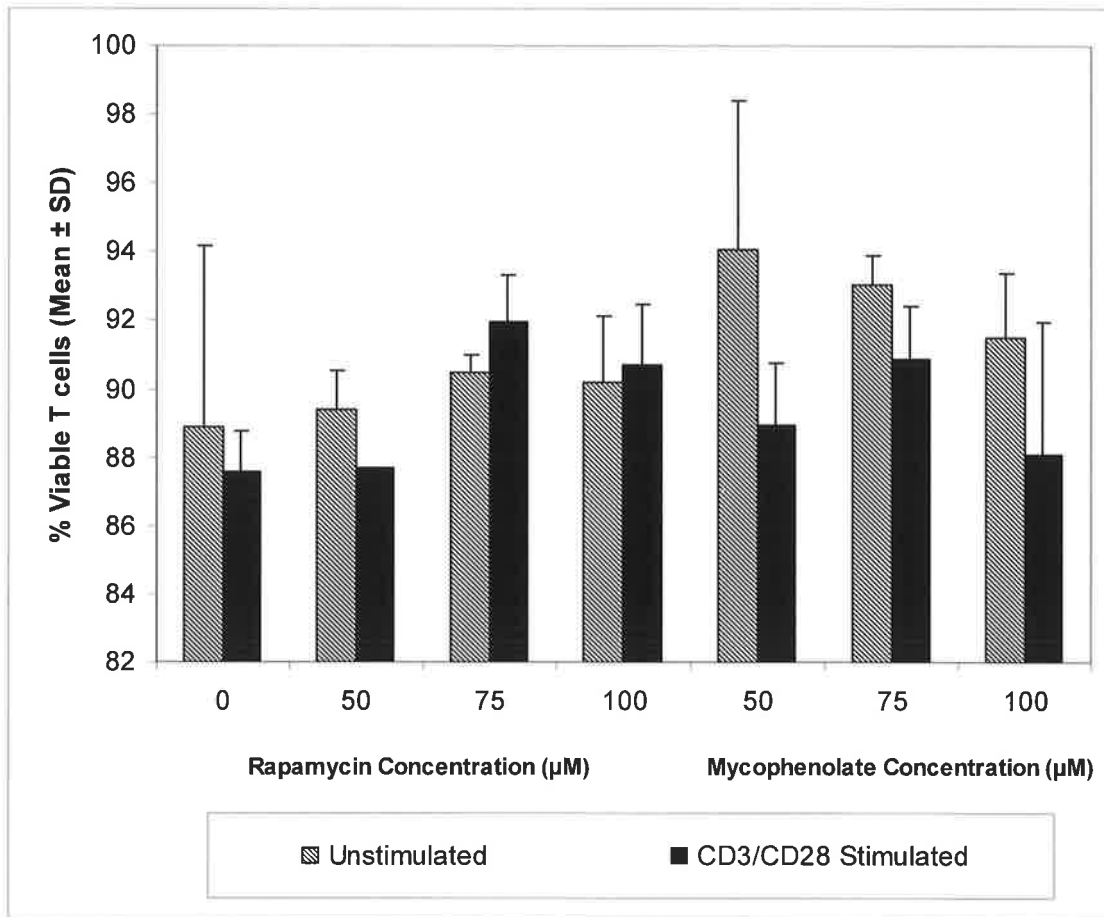


Figure 4.4 Unstimulated and CD3/CD28 stimulated viable T cells (%) with increasing Rapamycin and Mycophenolate mofetil concentrations.

CFSE-labelled T cells were cultured for 5 days in the presence of increasing concentrations of rapamycin and mycophenolate mofetil and unconjugated primary anti-human CD3 (10ng/ml) and CD28 (5μg/ml) antibodies for proliferation stimulation.

Viability was assessed through staining with PI.

Data is shown as the means of 3 triplicate assays ± SD.

Statistical analysis was performed using the paired t-test.

The Potential Immunomodulatory Effects of the Statins in Uveitis

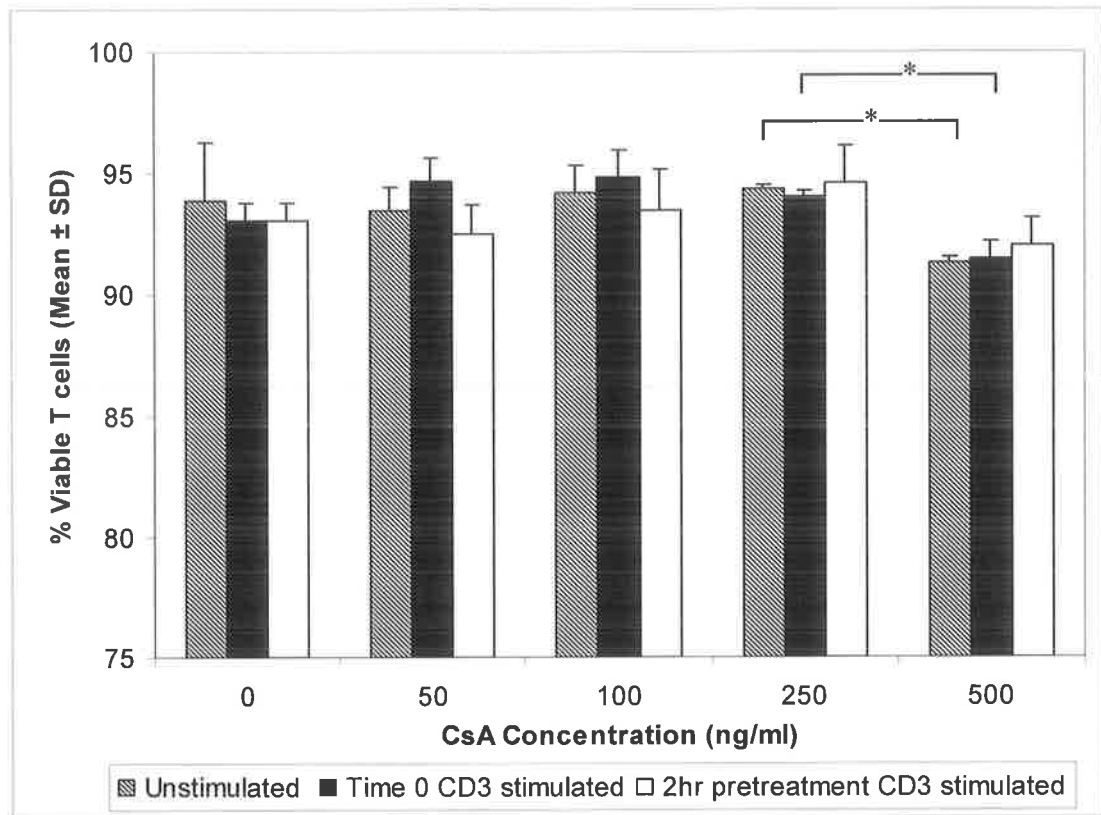


Figure 4.5 Unstimulated and CD3/CD28 stimulated viable T cells (%) with increasing CsA concentrations.

CFSE-labelled T cells were cultured for 5 days in the presence of increasing concentrations of CsA either administered 2 hours prior to or at the same time (time 0) as the addition of unconjugated primary anti-human CD3 (10ng/ml) alone for proliferation stimulation.

Viability was assessed through staining with PI.

Data is shown as the means of 3 triplicate assays $\pm$ SD.

Statistical analysis was performed using the paired t-test. * $p < 0.05$

The Potential Immunomodulatory Effects of the Statins in Uveitis

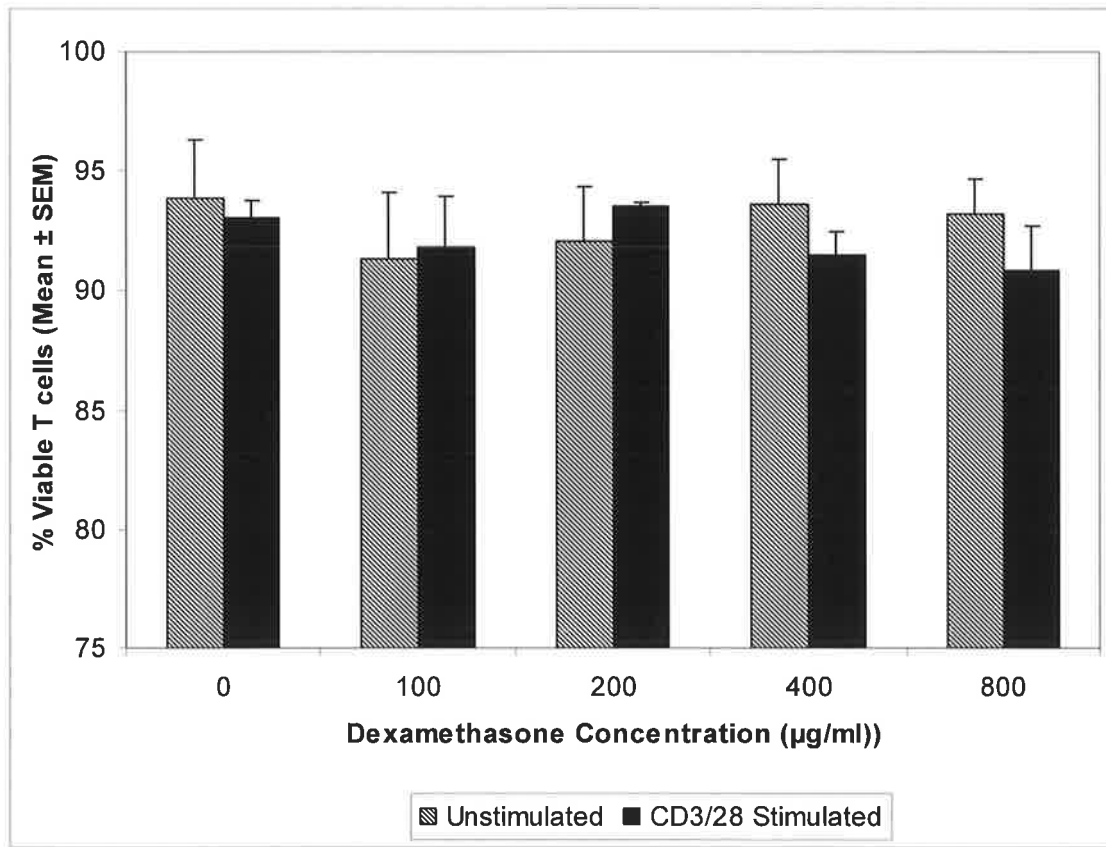


Figure 4.6 Unstimulated and CD3 stimulated viable T cells (%) with increasing dexamethasone concentrations.

CFSE-labelled T cells were cultured for 5 days in the presence of increasing concentrations of dexamethasone and unconjugated primary anti-human CD3 (10ng/ml) and CD28 (5µg/ml) antibodies for proliferation stimulation.

Viability was assessed through staining with PI.

Data is shown as the means of 3 triplicate assays ± SD.

Statistical analysis was performed using the paired t-test.

The Potential Immunomodulatory Effects of the Statins in Uveitis

4.3.2 T Cell Proliferation Inhibition

Atorvastatin was shown to inhibit CD3 and CD28 stimulated T cell proliferation in a dose-dependent fashion plateauing at 50 μ M (Figure 4.7). Significant reductions in proliferation as compared to untreated stimulated T cells were observed at concentrations of 10 μ M, 50 μ M and 100 μ M (Figure 4.7). The addition of mevalonate reversed the inhibition of proliferation to baseline untreated stimulated T cell levels (Figure 4.7).

Lovastatin was shown to inhibit CD3 and CD28 stimulated T cell proliferation in a dose-dependent fashion which plateaued at 50 μ M (Figure 4.8). Significant reductions in proliferation as compared to untreated stimulated T cells were observed at concentrations of 10 μ M, 50 μ M and 100 μ M (Figure 4.8). The addition of mevalonate reversed the inhibition of proliferation to baseline untreated stimulated T cell levels (Figure 4.8).

Simvastatin was shown to significantly inhibit CD3 and CD28 stimulated T cell proliferation at the higher end of the concentration range tested [50-100 μ M] as compared to untreated stimulated T cells (Figure 4.9). 100 μ M was found to induce maximum inhibition of T cell proliferation (Figure 4.9).

The Potential Immunomodulatory Effects of the Statins in Uveitis

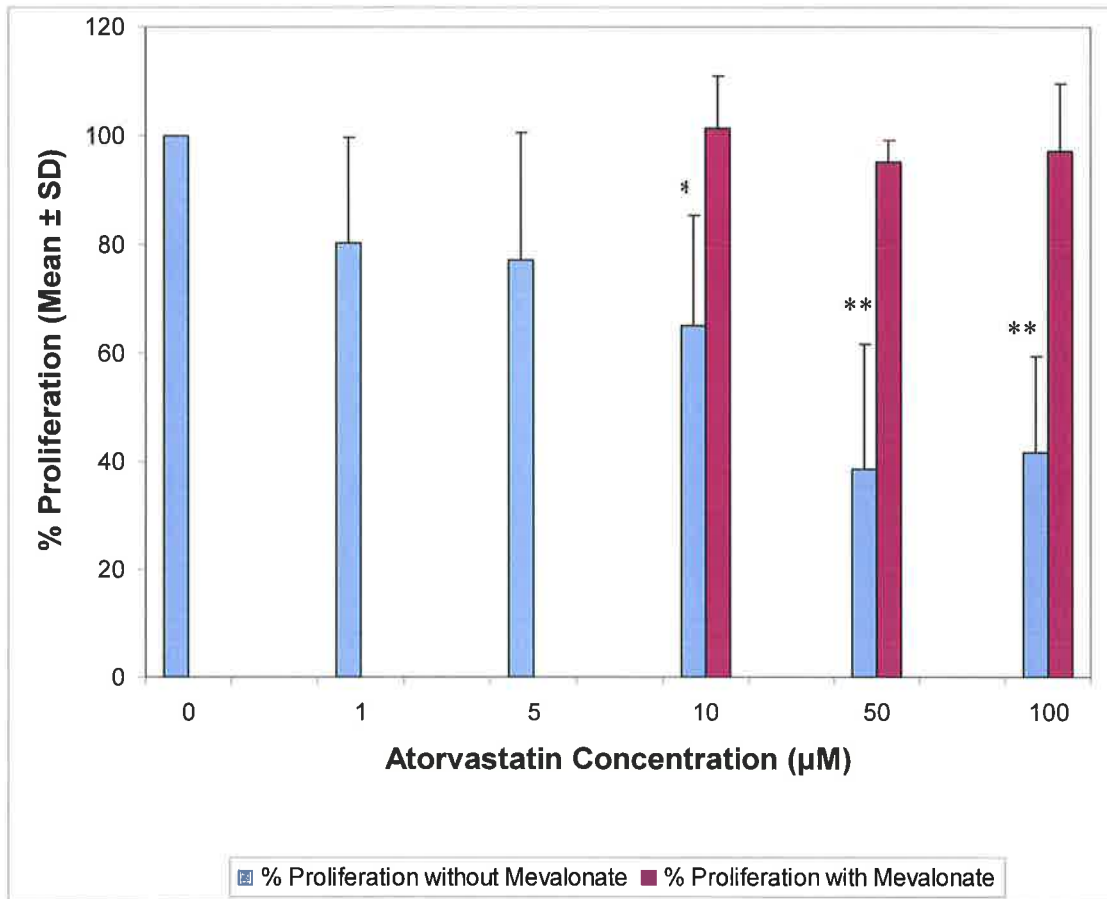


Figure 4.7 Inhibition of CD3/CD28 stimulated proliferation of T cells with increasing Atorvastatin concentrations with and without Mevalonate (200μM).

T cells were cultured for 5 days in the presence of increasing concentrations of atorvastatin and anti-human CD3/CD28 antibodies to stimulate T cell proliferation.

Proliferation was tracked through CFSE labeling of PI defined viable cells.

Each data point represents the mean percentage T cell proliferation $\pm$ SD.

Due to the variation between normal peripheral blood lymphocyte (PBL) samples, data points from each experiment ($n \geq 3$) were normalized against the positive control of percentage T cell proliferation without drug, allowing experiments to be pooled ($n \geq 3$).

Statistical analysis was performed using the paired t-test. * $p < 0.05$ and ** $p < 0.01$.

The Potential Immunomodulatory Effects of the Statins in Uveitis

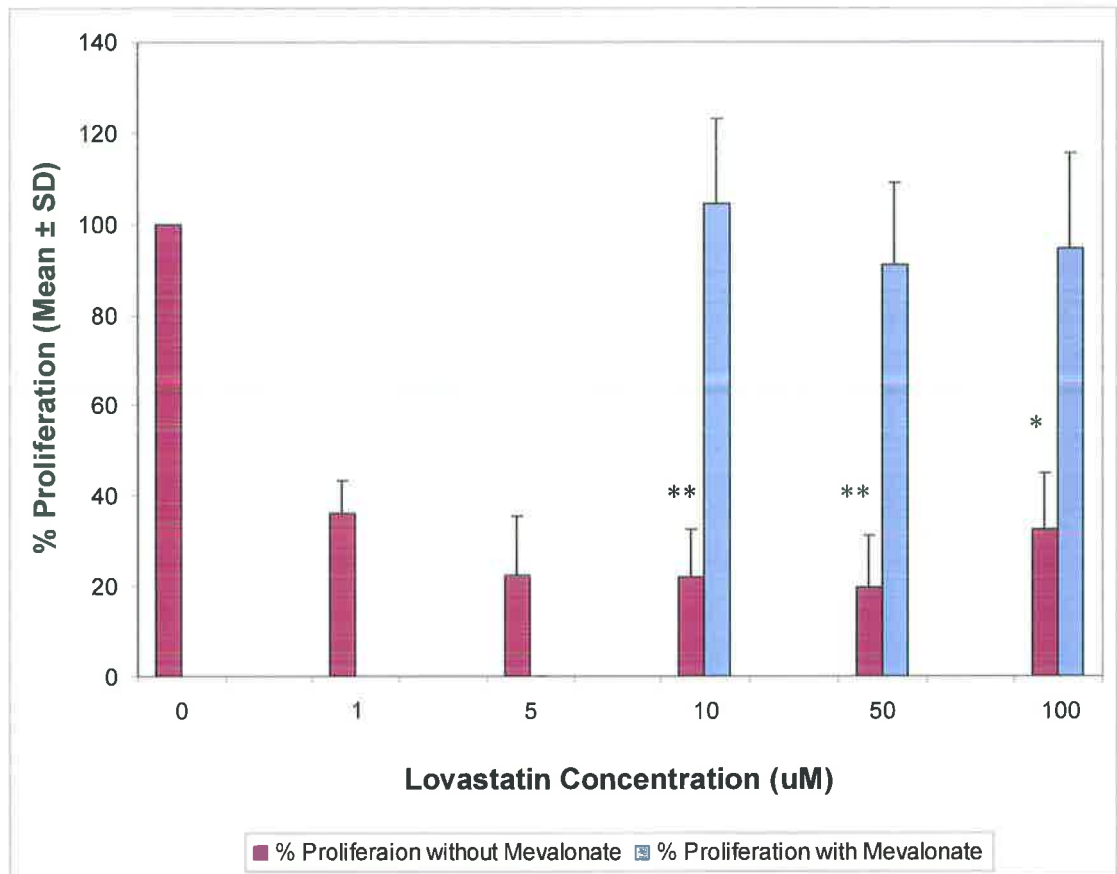


Figure 4.8 Inhibition of CD3/CD28 stimulated proliferation of T cells with increasing Lovastatin concentrations with and without Mevalonate (200uM).

T cells were cultured for 5 days in the presence of increasing concentrations of lovastatin and anti-human CD3/CD28 antibodies to stimulate T cell proliferation.

Proliferation was tracked through CFSE labeling of PI defined viable cells.

Each data point represents the mean percentage T cell proliferation $\pm$ SD.

Due to the variation between normal PBL samples, data points from each experiment ($n \geq 3$) were normalized against the positive control of percentage T cell proliferation without drug, allowing the experiments to be pooled ($n \geq 3$).

Statistical analysis was performed using the paired t-test. * $p < 0.05$ and ** $p < 0.01$.

The Potential Immunomodulatory Effects of the Statins in Uveitis

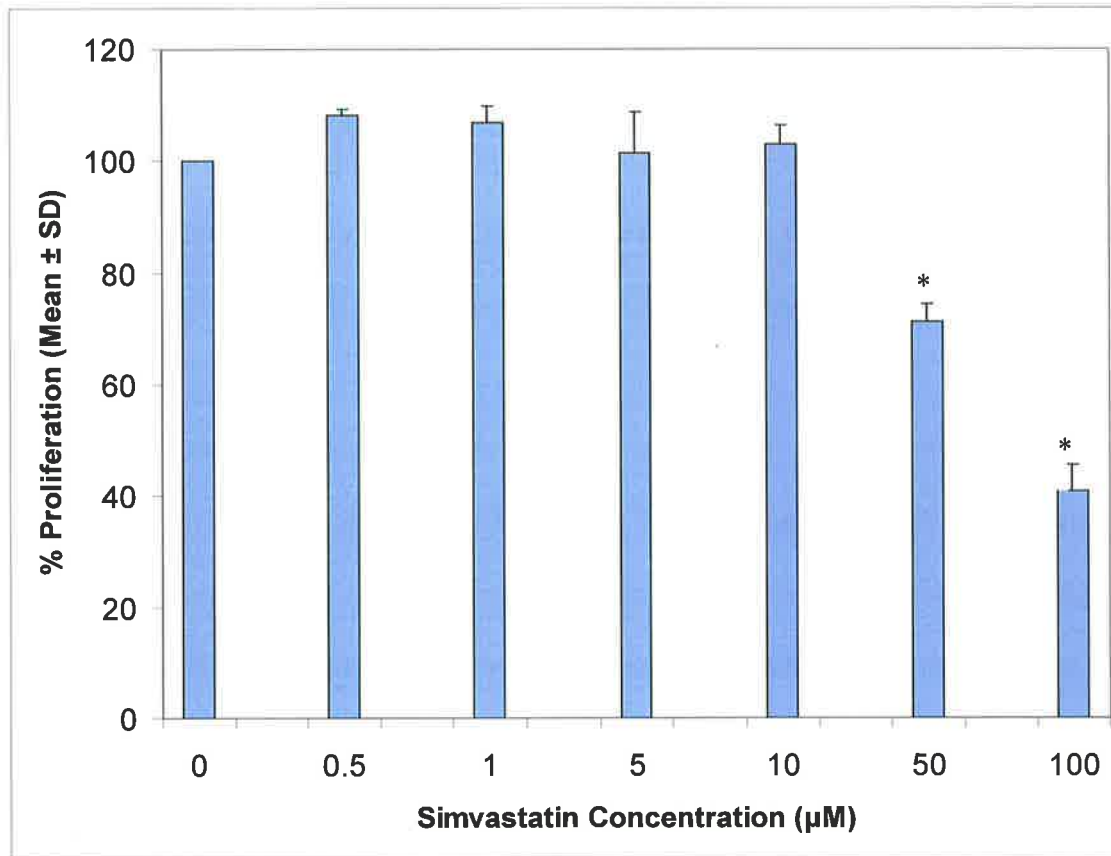


Figure 4.9 Inhibition of CD3 and CD28 stimulated proliferation of T cells with increasing Simvastatin concentrations.

T cells were cultured for 5 days in the presence of increasing concentrations of simvastatin in the presence of anti-human CD3/CD28 antibodies to stimulate T cell proliferation.

Proliferation was tracked through CFSE labeling of PI defined viable cells.

Each data point represents the mean percentage T cell proliferation $\pm$ SD.

Due to the variation between normal PBL samples, data points from each experiment ($n \geq 3$) were normalized against the positive control of percentage T cell proliferation without drug, allowing the experiments to be pooled ($n \geq 3$).

Statistical analysis was performed using the paired t-test. * $p < 0.01$

The Potential Immunomodulatory Effects of the Statins in Uveitis

Rapamycin significantly inhibited CD3 and CD28 stimulated T cell proliferation at 50-100 μ M (Figure 4.10). 100 μ M was found to be the maximum inhibitory concentration of rapamycin (Figure 4.10). Mycophenolate significantly inhibited CD3 and CD28 stimulated T cell proliferation at 10-100 μ M (Figure 4.11). 100 μ M was found to be the maximum inhibitory concentration of mycophenolate (Figure 4.11).

With CsA, the level of T cell proliferation observed using CD3 stimulation alone was as good as PHA or combined CD3 and CD28 stimulation (Figure 4.12). There was no significant reduction of PHA stimulated T cell proliferation with all concentrations of CsA tested (Figure 4.12). The reduction of CD3 and CD28 stimulated T cell proliferation with increasing concentrations of CsA was not as marked as the reduction with CD3 T cell stimulated proliferation alone (Figure 4.12). When comparing CD3/CD28 stimulated cells to 10ng/ml CD3 stimulated cells alone, significant differences were noted with paired T tests for 250ng/ml and 500ng/ml CsA concentrations ($p < 0.05$). CsA reduction of CD3 stimulated T cell proliferation decreased with increasing concentrations of CD3 employed (Figure 4.12). 500ng/ml concentration of CsA with CD3 (10ng/ml) stimulated T cell proliferation appeared to produce the greatest inhibition with statistical significance reached as compared to 1000ng/ml CD3 ($p < 0.05$) and 5000ng/ml CD3 ($p < 0.01$; Figure 4.12). 100ng/ml CsA with CD3 (10ng/ml) stimulated T cell proliferation did also produce a significantly greater inhibition as compared to 1000ng/ml CD3 ($p < 0.05$; Figure 4.12). There were, however, no significant differences in proliferation inhibition between any of the tested concentrations of CsA (100, 250 and 500ng/ml) in conjunction with 10ng/ml CD3 stimulation (Figures 4.12 and 4.13).

The Potential Immunomodulatory Effects of the Statins in Uveitis

When comparing T cells cultured for 5 days in the presence of increasing concentrations of CsA either administered 2 hours prior to or at the same time (time 0) as the addition of 10ng/ml CD3 stimulation, all concentrations tested significantly inhibited proliferation as compared to control ($p<0.01$; Figure 4.13). No significant differences were noted between T cells stimulated at time 0 as compared to those stimulated following 2 hours of CsA pretreatment except at 500ng/ml CsA ($p<0.05$; Figure 4.13).

Dexamethasone significantly inhibited CD3/28 stimulated proliferation of T cells at all concentrations tested ($p<0.01$; Figure 4.14). No significant differences were found, however, between any of the concentrations tested (Figure 4.14).

The Potential Immunomodulatory Effects of the Statins in Uveitis

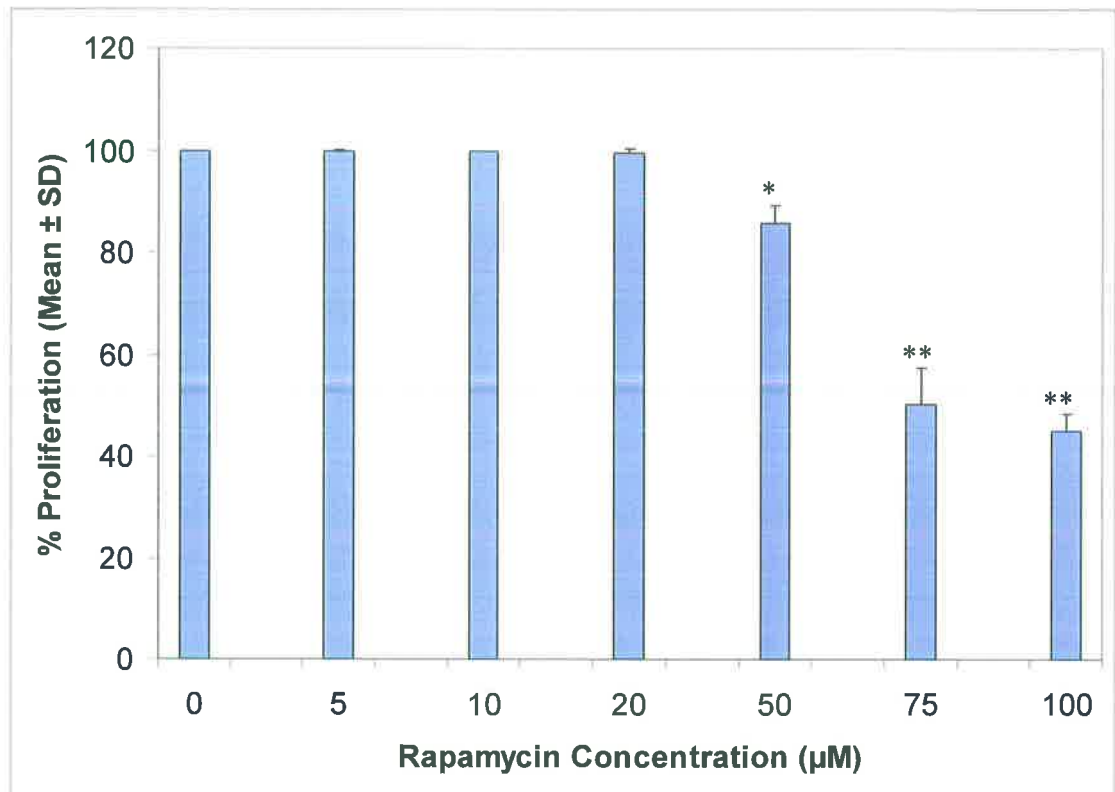


Figure 4.10 Inhibition of CD3 and CD28 stimulated proliferation of T cells with increasing Rapamycin concentrations.

T cells were cultured for 5 days in the presence of increasing concentrations of rapamycin and anti-human CD3/CD28 antibodies to stimulate T cell proliferation.

Proliferation was tracked through CFSE labeling of PI defined viable cells.

Each data point represents the mean percentage T cell proliferation $\pm$ SD.

Due to the variation between normal PBL samples, data points from triplicate wells were normalized against the positive control of percentage T cell proliferation without drug, allowing the experiments to be pooled ($n \geq 3$).

Statistical analysis was performed using the paired t-test. * $p < 0.05$ and ** $p < 0.01$

The Potential Immunomodulatory Effects of the Statins in Uveitis

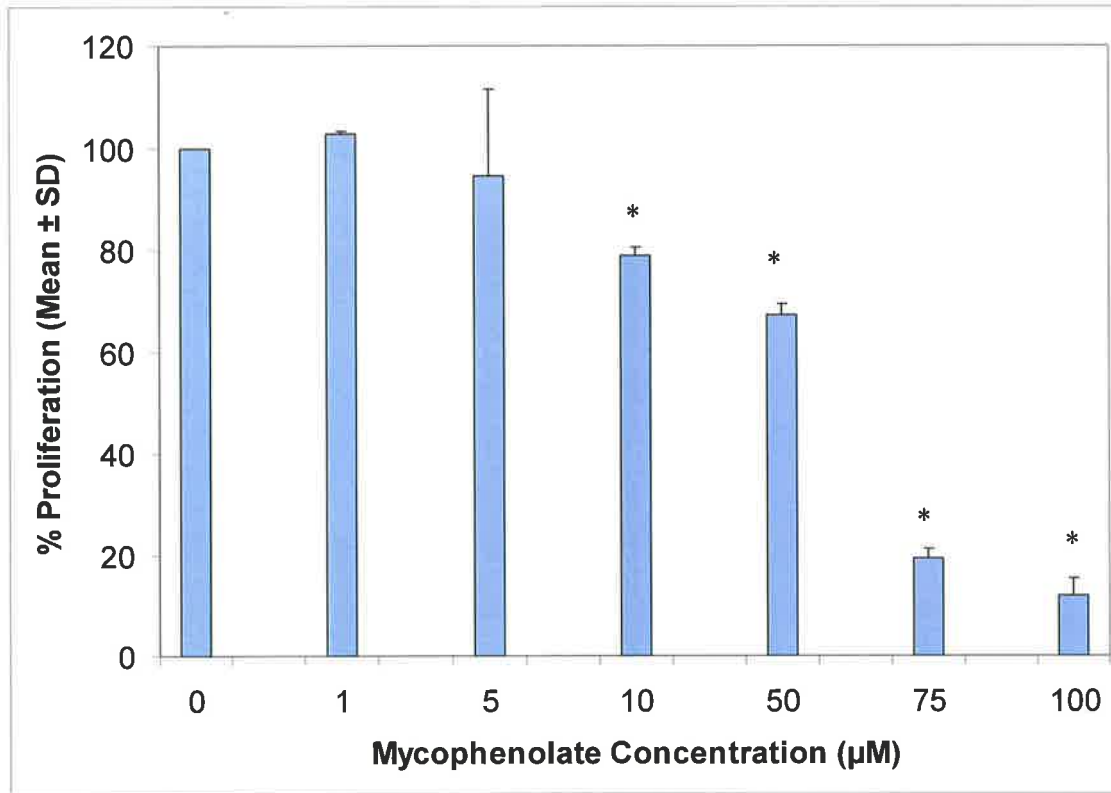


Figure 4.11 Inhibition of CD3 and CD28 stimulated proliferation of T cells with increasing Mycophenolate concentrations.

T cells were cultured for 5 days in the presence of increasing concentrations of mycophenolate and anti-human CD3/CD28 antibodies to stimulate T cell proliferation.

Proliferation was tracked through CFSE labeling of PI defined viable cells.

Each data point represents the mean percentage T cell proliferation $\pm$ SD.

Due to the variation between normal PBL samples, data points from triplicate wells were normalized against the positive control of percentage T cell proliferation without drug, allowing the experiments to be pooled ($n \geq 3$).

Statistical analysis was performed using the paired t-test. * $p < 0.01$.

The Potential Immunomodulatory Effects of the Statins in Uveitis

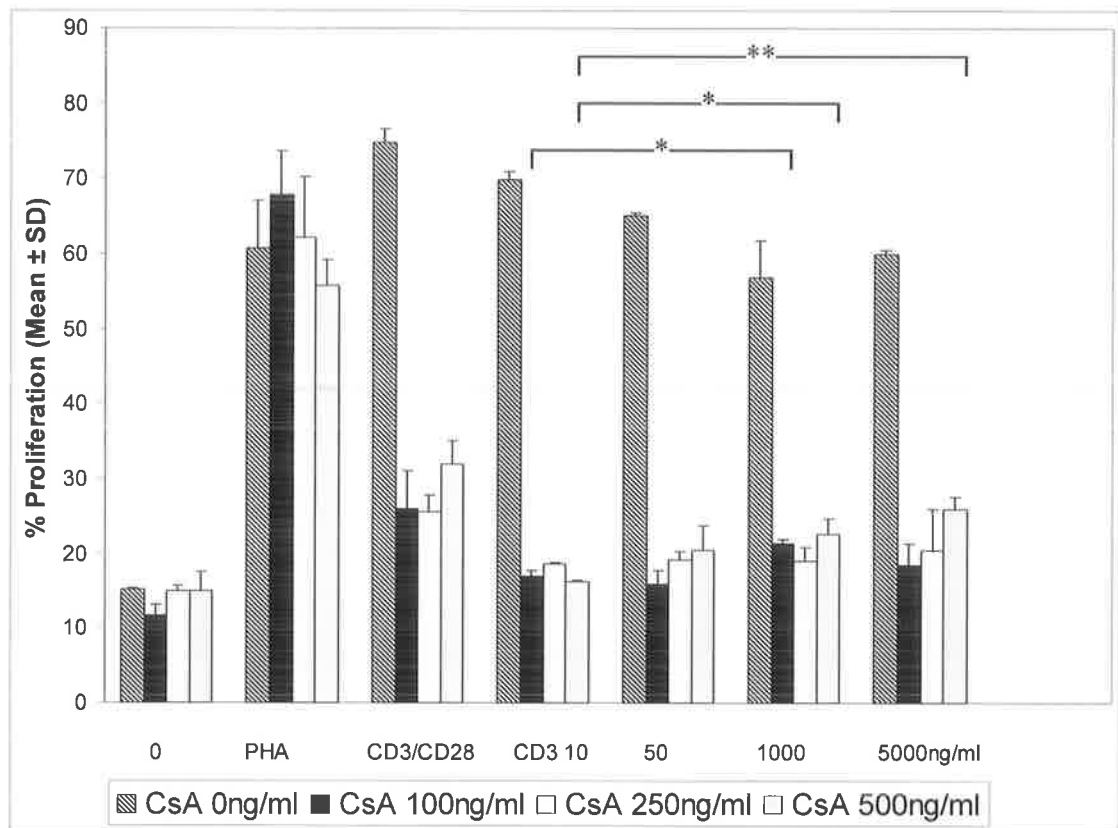


Figure 4.12 Inhibition of PHA, CD3/CD28 and CD3 stimulated proliferation of T cells with increasing CD3 and CsA concentrations.

T cells were cultured for 5 days in the presence of either PHA (5 μ g/ml) or unconjugated primary anti-human CD3 (10ng/ml) and CD28 (5 μ g/ml) antibodies or increasing concentrations of anti-human CD3 antibodies (10-5000ng/ml) alone to stimulate T cell proliferation, and with increasing concentrations of CsA.

Proliferation was tracked through CFSE labeling of PI defined viable cells.

Each data point represents the mean percentage T cell proliferation $\pm$ SD.

For each concentration, a single experiment was performed in triplicate wells giving a minimum of 3 data points.

Statistical analysis was performed using the paired t-test. *p<0.05 and **p<0.01.

The Potential Immunomodulatory Effects of the Statins in Uveitis

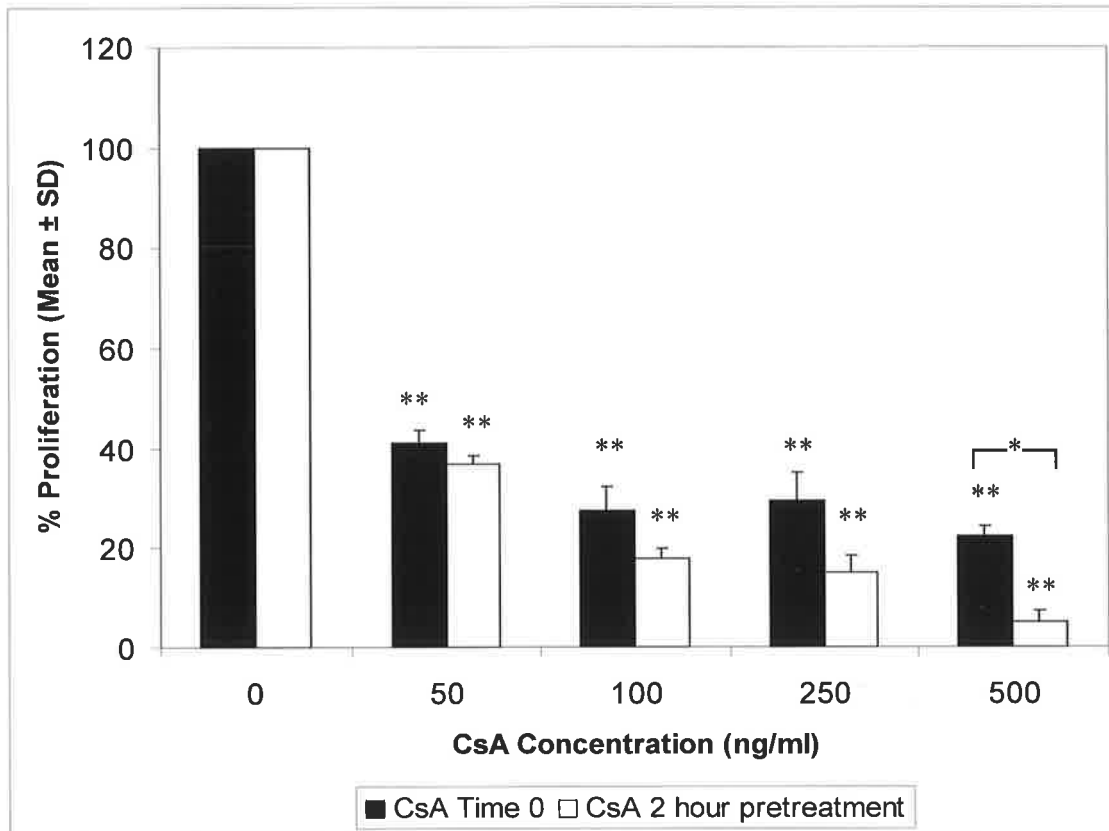


Figure 4.13 Inhibition of CD3 stimulated proliferation of T cells with increasing CsA concentrations administered at time 0 or 2 hours prior to stimulation

T cells were cultured for 5 days in the presence of increasing concentrations of CsA either administered 2 hours prior to or at the same time (time 0) as the addition of unconjugated primary anti-human CD3 (10ng/ml) alone for proliferation stimulation.

Proliferation was tracked through CFSE labeling of PI defined viable cells.

Each data point represents the mean percentage T cell proliferation $\pm$ SD.

Due to the variation between normal PBL samples, data points from triplicate wells were normalized against the positive control of percentage T cell proliferation without drug, allowing the experiments to be pooled ($n \geq 3$).

Statistical analysis was performed using the paired t-test. * $p < 0.01$.

The Potential Immunomodulatory Effects of the Statins in Uveitis

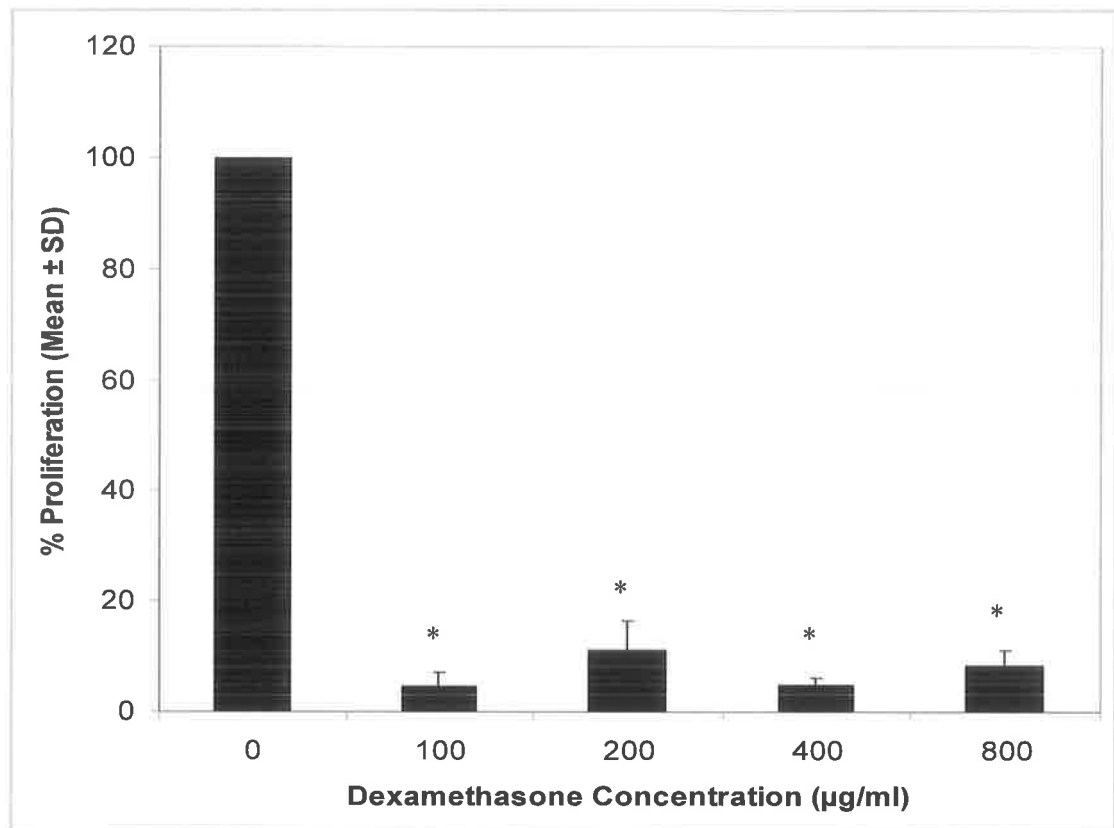


Figure 4.14 Inhibition of CD3 and CD28 stimulated proliferation of T cells with increasing Dexamethasone concentrations.

T cells were cultured for 5 days in the presence of increasing concentrations of dexamethasone and anti-human CD3/CD28 antibodies to stimulate T cell proliferation. Proliferation was tracked through CFSE labeling of PI defined viable cells.

Each data point represents the mean percentage T cell proliferation $\pm$ SD.

Due to the variation between normal PBL samples, data points from triplicate wells were normalized against the positive control of percentage T cell proliferation without drug, allowing the experiments to be pooled ($n \geq 3$).

Statistical analysis was performed using the paired t-test. * $p < 0.01$.

The Potential Immunomodulatory Effects of the Statins in Uveitis

4.4 Discussion

3 triplicate assays were conducted for all viability assays. Not all the same donors were used but baseline viability percentage i.e. without drug, was between at least 90-95% in all drug assays except for Rapamycin. In the Rapamycin assay, although there was a greater range of percentages at Rapa [0], there was no statistically significant difference in viability from 0 concentration of drug to maximum concentration of drug tested. Valid viability comparisons are thus able to be made.

Although a variable number of proliferation assays were used here with different donors data points from each experiment ($n \geq 3$) were normalized against the positive control of percentage T cell proliferation without drug, allowing experiments to be pooled ($n \geq 3$) and therefore able to be compared.

Atorvastatin reduction of CD3 and CD28 stimulated T cell proliferation was significantly greater at the concentration of 100 μ M than at 50 μ M. This corroborates with previous literature in which the anti-CD3 and anti-CD28 proliferation of human T cells was inhibited by atorvastatin in a dose-dependent manner (Aktas *et al* 2003). Mevalonate reversed this inhibition to a level of proliferation at baseline levels, in agreement with others (Diomedes *et al* 2001, Aktas *et al* 2003). This provides evidence that the immunomodulatory effects of atorvastatin are mediated by inhibition of HMG-CoA reductase (Aktas *et al* 2003). T cell viability showed little variation except at 100 μ M where mevalonate appeared to confer a viability advantage.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Lovastatin reduction of CD3 and CD28 stimulated T cell proliferation was statistically maximal at 50 μ M in the absence of a significant change in T cell viability. The addition of mevalonate to lovastatin-treated T cells also reversed the level of inhibition to a level of proliferation of controls.

Although simvastatin at the maximum concentration of 100 μ M inhibited proliferation by 40%, T cell viability was maintained above 90%. A similar proliferation study was conducted in which normal human peripheral blood (PB)-derived mononuclear cells stimulated with anti-CD3/anti-CD28 Abs were shown to have proliferation suppressed in a dose-dependent manner by simvastatin (Leung *et al* 2003). As with atorvastatin and lovastatin, the addition of mevalonate to simvastatin-treated cells conferred a viability advantage over statin-treated cells alone.

Similarly rapamycin reduced T cell proliferation in response to CD3 and CD28 stimulation to 40% of baseline at the maximum concentration tested of 100 μ M. T cell viability was maintained at above 90% under this condition of stimulation. This is consistent with the literature which documents rapamycin to inhibit proliferation to a range of stimuli, which induce either calcium-dependent or -independent signal transduction pathways (Sehgal 2003). Rapamycin has been shown to be able to inhibit T cell proliferation even when added up to 12 hours after the initiation of the stimulation (Dumont *et al* 1990, Sehgal and Bansbach 1993). Upon rapamycin conjugation with FKBP12, the resultant complex binds to mTOR to effect two biochemical alterations which underlie its ability to suppress proliferation. The first is the inhibition of the p70 S6 kinase (p70^{S6k}), whose major substrate is the 40S ribosomal subunit S protein, thereby

The Potential Immunomodulatory Effects of the Statins in Uveitis

inhibiting the translation of certain mRNA encoding for ribosomal proteins and elongation factors, resulting in decreased protein synthesis. The second is a suppression of the enzymatic activity of the CDK2-cyclin E complex, which functions as a crucial regulator of G1/S transition. This inhibition results from a prevention of the decline of the p27 CDK inhibitor that normally follows IL-2 stimulation (Dumont and Su 1996). IL-2 activation of JAK1, which is coupled to the induction of the *bcl-2* gene, which regulates apoptosis, is also thought to be mediated by mTOR. Finally, rapamycin has also been shown to inhibit CD3/CD28- as well as IL-2-mediated proliferation of T cells without affecting sustained upregulation of IL-2 transcription in T cells, thereby leading to inhibition of proliferation (Sehgal 2003).

Mycophenolate inhibited proliferation of CD3 and CD28 stimulated T cells to 10% of baseline at the maximum concentration tested of 100 μ M. Mycophenolate acts through its metabolite, mycophenolic acid, to inhibit IMPDH, an enzyme essential for GTP synthesis in lymphocytes (Franklin and Cook 1969, Tran *et al* 2001). Two isoforms of human IMPDH have been identified and sequenced (Franchetti and Grifantini 1999). Type I is constitutively expressed and is the predominant isoform in normal resting cells, whilst type II is selectively upregulated in neoplastic and replicating cells. Griesmacher *et al* (1997) showed inhibition of both isoforms *in vitro* by mycophenolate. The addition of exogenous guanine, guanosine or deoxyguanosine has been demonstrated to restore intracellular GTP and dGTP pools and reverse the antiproliferative effect of mycophenolate (Allison *et al* 1991). Our experimental work mirrors that of Quéméneur *et*

The Potential Immunomodulatory Effects of the Statins in Uveitis

al (2002) who also demonstrated mycophenolate-induced inhibition of anti-CD3 and anti-CD28 monoclonal antibody-activated PBL proliferation as tracked by CFSE staining. PBL activation, however, was also carried out with consecutive PHA and IL-2 stimulation. Cells were then incubated with medium alone or with IL-2, IL-15, IL-4 or IL-7, in the presence or absence of mycophenolic acid. Specifically, IL-2- and IL-15-dependent T cell proliferation were also blocked by mycophenolate, maximal at 10 μ M. As with our findings, 10 μ M mycophenolate inhibited proliferation without a major decrease of activated T cell viability as determined by trypan blue exclusion and annexin V labeling of apoptotic cells (Quéméneur *et al* 2002).

CsA inhibition of CD3 (10ng/ml) and CD28 (5 μ g/ml) co-stimulated T cell proliferation was not as effective as CsA inhibition of CD3 (10ng/ml) stimulated T cells alone. This is in keeping with a recent study whereby it was shown that costimulation of the B7-CD28 pathway led to a strong and universal CsA-resistant proliferation of TCR-activated IL-2 producing T cells in all tested healthy individuals (Geginat *et al* 2000). This confirms an earlier study in which it was proposed that an IL-2-independent regulation of T cell proliferation existed on CD28 costimulation (Appleman *et al* 2000). Full activation of T cells requires the activation of the TCR/CD3 complex and, in addition, a second signal induced by stimulation of CD28 (Robey and Allison 1995). Increasing CD3 stimulation with incremental increases in CD3 concentration led to decreasing CsA effectiveness in inhibition of proliferation. 500 μ M concentration of CsA

The Potential Immunomodulatory Effects of the Statins in Uveitis

marginally produced a greater reduction of proliferation inhibition of CD3 (10ng/ml) stimulated T cell proliferation than a 100 μ M concentration but this was not statistically significant. T cell viability was however greater at CsA 100 μ M under both CD3 stimulated and unstimulated conditions as compared to CsA 500 μ M. 2 hour pretreatment of T cells with CsA was not associated with a significantly greater inhibition of T cell proliferation than CsA added at time 0 except at 500ng/ml CsA.

The significant inhibition of T cell proliferation by dexamethasone is also consistent with the literature (Wilckens and De Rijk 1997). Taking into consideration T cell viability, optimum drug concentrations for inhibition of T cell proliferation were therefore deemed to be atorvastatin 50 μ M, lovastatin 50 μ M, simvastatin 100 μ M, rapamycin 100 μ M, mycophenolate 100 μ M, CsA 100ng/ml and dexamethasone 400 μ g/ml.

Chapter 5

The Potential Immunomodulatory Effects of the Statins in Uveitis

Intracellular Cytokine Staining

The Potential Immunomodulatory Effects of the Statins in Uveitis

5.1 Introduction

The characterization of functional subsets of CD4⁺ T cells with differential cytokine production was based originally on the characterization of mouse T cell clones using cytokine ELISA of culture supernatants (Mosman *et al* 1996). This method allows for the measurement of integrated amounts of secreted protein but is impractical when faced with large numbers of heterogeneous cells obtained *ex vivo*. Several other methods of measuring cytokine expression have been developed including limiting dilution analysis (LDA), ELISA-based assay for detecting cells secreting analyte (ELISPOT) and intracellular cytokine staining which are the most appropriate ways of estimating the frequency of specific cytokine producing cells. Reverse-transcription followed by polymerase chain reaction (RT-PCR) measures semi-quantitative levels of inducible mRNA while *in situ* hybridisation (ISH) and immunohistochemistry are useful for localization of cytokine producing cells in tissues (Pala *et al* 2000).

Intracellular cytokine staining was pioneered by the Andersons in Stockholm in the 1980s, initially to immunostain tissue sections (Sander *et al* 1991). After the introduction of methods to fix and permeabilise lymphocytes, the next development was the use of secretion inhibitors to accumulate cytokines intracellularly, allowing improvement of the signal/noise ratio (Jung *et al* 1993, Schmitz *et al* 1993). Finally, screening of large panels of monoclonal antibodies to select those that bind cytokines in their fixed form allowed practical methods to be developed (Openshaw *et al* 1995). Flow cytometric analysis on intracellular cytokine stained cells allows individual characterization of large numbers of cells and can fully display the heterogeneity of cell

The Potential Immunomodulatory Effects of the Statins in Uveitis

populations. It is dependent on the gating strategy used in the selection of blasts but experimental variability was minimized by defining and maintaining quadrant settings for each acquisition. Compared to ELISA, a great advantage of intracellular cytokine staining is that multicolour staining can demonstrate exclusive or mutual co-expression of different cytokines in individual cells, thus allowing the characterization of T cell subsets via cytokine production rather than just surface markers (Pala *et al* 2000).

There have been conflicting reports as to the effects of the statins on T cell cytokine production. In one study simvastatin was shown to significantly increase the production of the potentially harmful cytokines IFN- γ and IL-12. Although IL-4 was also strongly upregulated, IL-10 was downregulated and overall there seemed to be increased Th1 cytokine release from peripheral blood mononuclear cells from their healthy donors and multiple sclerosis (MS) patients. This was despite a dose dependent reduction in concanavalin A (conA)-, PHA- and anti-CD3-induced T cell proliferation by lovastatin as well as simvastatin (Neuhaus *et al* 2002). In chronic and relapsing experimental autoimmune encephalomyelitis, a CD4⁺ Th1-mediated central nervous system (CNS) demyelinating disease model, it was demonstrated that atorvastatin inhibited STAT4 phosphorylation and secretion of Th1 cytokines (IL-2, IL-12, IFN- γ and TNF- α). Atorvastatin also induced STAT6 phosphorylation and secretion of Th2 cytokines (IL-4, IL-5, IL-10 and TGF- β) thereby promoting Th2 differentiation and inhibition of proliferation and Th1 cytokine secretion (Youssef *et al* 2002). Simvastatin has also been shown to reduce the plasma concentrations of IL-2 and TNF- α in patients with hypercholesterolemia (Zubelewicz-Szkodińska *et al* 2003)

The Potential Immunomodulatory Effects of the Statins in Uveitis

5.2 Aims

Having optimized the concentrations of the drugs to be tested in the previous experiment, their effect on Th1 and Th2 cytokine production in whole blood through intracellular cytokine staining was determined. Specific combinations of atorvastatin and conventional immunomodulatory drug(s) were also used to assess the possibility of statin-potentiated systemic steroid and steroid sparing therapies in the management of uveitis. PMA/Ionomycin proliferation stimulation was used on this occasion. This was to physiologically stimulate all cell types in order to generate a whole blood response thereby enabling a more accurate assessment of the overall effects of the statins as systemic medications. Atorvastatin combinations were chosen as previous intracellular cytokine staining of mice T cells has shown this drug to downregulate IFN- γ and increase IL-4 to a greater degree than lovastatin and simvastatin (Hakamada-Taguchi *et al* 2003).

5.3 Results

There were significant reductions in intracellular cytokine staining for IFN- γ in whole blood with the addition of atorvastatin, lovastatin, simvastatin, rapamycin, mycophenolate, CsA, dexamethasone, atorvastatin and dexamethasone and the combination of atorvastatin, dexamethasone and CsA (Figure 5.1). The decrease in IFN- γ was most marked with dexamethasone while simvastatin was the most effective statin in inducing a decrease in IFN- γ expression (Figure 5.1). Atorvastatin /rapamycin, atorvastatin/mycophenolate and atorvastatin/ CsA combinations did not significantly reduce IFN- γ (Figure 5.1). There were no significant differences from baseline cytokine expression of IL-10, IL-4 and IL-2 with the addition of all drug combinations.

The Potential Immunomodulatory Effects of the Statins in Uveitis

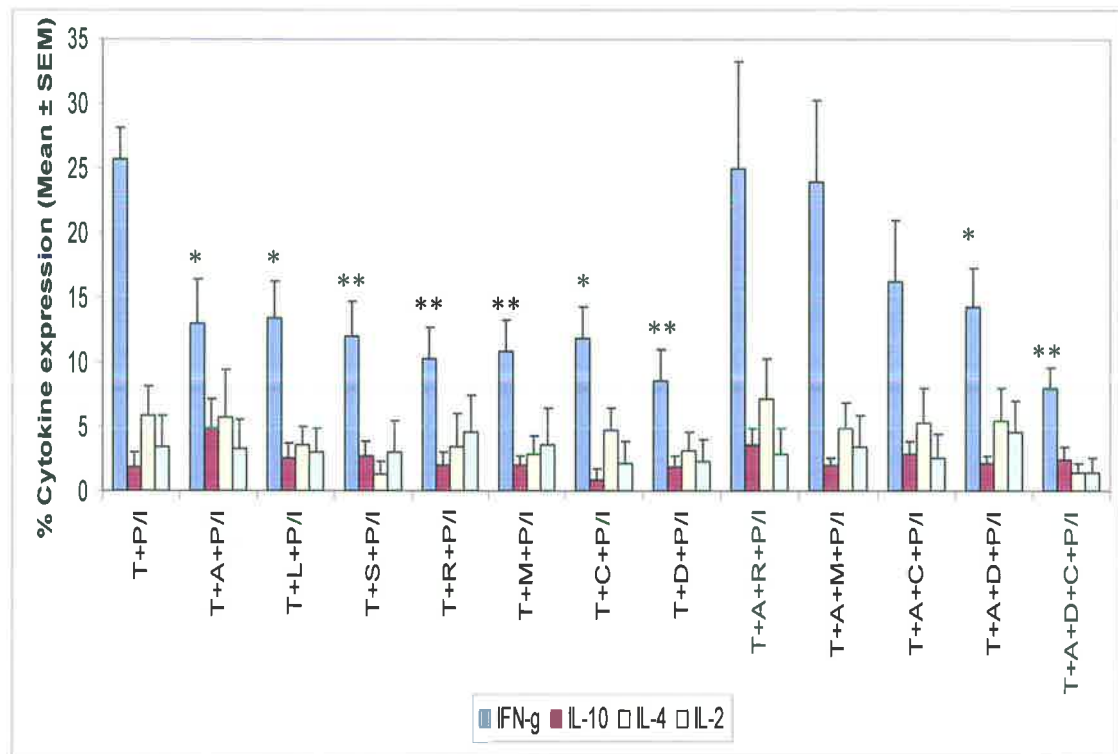


Figure 5.1 Percentage of IFN- γ , IL-10, IL-4 and IL-2 expression from live-gated CD3⁺ cells in PMA/Ionomycin stimulated whole blood with or without drug treatment. P/I=PMA/Ionomycin, A=Atorvastatin [50 μ M], L=Lovastatin [50 μ M], S=Simvastatin [100 μ M], R=Rapamycin [100 μ M], M=Mycophenolate [100 μ M], C=CsA [100ng/ml] and D=Dexamethasone [400 μ g/ml].

Whole blood cells were stimulated with PMA/Ionomycin and BFA was added prior to culture for 18hrs $\pm$ drugs at previously optimized concentrations.

Cells were stained intracellularly for IFN- γ /IL-10 and IL-2/IL-4.

Cytokine expressions are from live-gated CD3⁺ cells.

Each bar represents the means $\pm$ SEM from 6 experiments, each performed in triplicate and each with unstimulated background control cytokine expression subtracted.

Statistical analysis was performed using the Mann-Whitney *U* test. * p <0.05, ** p <0.01

The Potential Immunomodulatory Effects of the Statins in Uveitis

5.4 Discussion

All the immunomodulatory drugs tested inhibited the intracellular cytokine production of IFN- γ . No significant differences were seen, however, in the expression of IL-2, IL-4 and IL-10 from baseline with any of the drugs suggesting possibly that these cytokines underwent degradation either before or during the fixation process or were just poorly bound. Production of these particular cytokines as well may have already peaked or may not have even begun to rise. On the other hand the drugs tested may indeed have had no upregulatory effect on these cytokines.

Atorvastatin was found to significantly decrease the percentage of intracellular IFN- γ accumulation but had no significant effects on the levels of IL-10, IL-4 and IL-2 by human T cells in whole blood. A similar effect has been previously reported in an EAE model in which atorvastatin decreased the Th1 response in cells from mice spleens through a reduction in IFN- γ secretion as well as that of IL-12. That study also demonstrated an atorvastatin-associated down-regulation of CDK4, known to be involved in the positive regulation of the T cell cycle (Aktas *et al* 2003). This mechanism appeared to decrease cytokine expression independent of the reduction of MHC class II upregulation by inhibition of the inducible promoter IV of the transactivator CIITA (Kwak *et al* 2000) and the blockade of LFA-1/ICAM-1 interactions (Weitz-Schmidt *et al* 2001). The addition of mevalonate was also found to reverse IFN- γ secretion thereby providing further direct evidence that the immunomodulatory effects of atorvastatin are mediated by inhibition of HMG-CoA reductase (Aktas *et al* 2003). An atorvastatin-induced IFN- γ reduction was also reported in a chronic and relapsing EAE model which

The Potential Immunomodulatory Effects of the Statins in Uveitis

also documented the secretion suppression of IL-2 with an associated increase in IL-4 and IL-10 secretion (Youssef *et al* 2002). It would thus appear that in my particular analysis, atorvastatin exhibits Th1 inhibitory properties, consistent with the literature, as evidenced by the reduction in IFN- γ . A Th2 shift as mentioned by Youssef *et al* (2002), however, was not seen but this may have been due to a fundamental difference in the types of experimental models used. To our knowledge the atorvastatin-induced decrease in IFN- γ expression in human T cells has not previously been reported.

Lovastatin was also found to significantly reduce the amount of intracellular cytokine staining for IFN- γ but had no significant effects on the levels of IL-10, IL-4 and IL-2. There is little in the literature concerning the effects of lovastatin on intracellular cytokine staining profiles. One study did show lovastatin inhibition of IFN- γ and IL-2 in peripheral blood mononuclear cells (PBMCs) as demonstrated by supernatant ELISPOT studies (Li *et al* 2001). Another study using intracellular cytokine staining failed to detect the presence of IL-10 in LPS stimulated DCs but surprisingly noted an upregulation of pro-inflammatory cytokine production although IFN- γ and IL-2 were not specifically investigated (Sun and Fernandes 2003). It can therefore be surmised that in intracellular cytokine staining of whole blood, at least, that lovastatin also exhibits anti-inflammatory properties consistent with the literature, as evidenced by the reduction of the Th1 cytokine IFN- γ . Once again no Th2 shift was observed as evidenced by the lack of IL-4 expression. In addition the paucity of IL-10 noted by Sun and Fernandes (2003) paralleled to some extent our findings of no significant change in IL-10 expression.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Again, simvastatin was found to significantly decrease the percentage of intracellular IFN- γ accumulation but had no significant effects on the levels of IL-10, IL-4 and IL-2. This was however not in keeping with the *in vitro* findings in one study on healthy human donors where pretreatment with simvastatin induced a dose-dependent increase of the pro-inflammatory cytokines IFN- γ and IL-12 in the supernatant of anti-CD3-stimulated T cells derived from peripheral blood. Intracellular staining for IL-4 was also found to be significantly increased but TNF- α and IL-10 were significantly decreased (Neuhaus *et al* 2002). A more recent study on human PB-derived mononuclear cells stimulated with anti-CD3/anti-CD28 Abs, however, showed that IFN- γ release as detected by ELISA was reduced in a dose-dependent manner by simvastatin (Leung *et al* 2003). Also very much in keeping with our study was the fact that Leung *et al* (2003) found no IL-4 and no significant change in IL-10 levels in culture supernatants.

Rapamycin was found to produce a highly significant decrease in the percentage of intracellular IFN- γ staining but did not significantly affect levels of IL-10, IL-4 and IL-2. The reports on the effect of rapamycin on cytokine production have been conflicting. Contrary somewhat to our findings, rapamycin has been found to have limited effects on cytokine production including no effect on the production of IFN- γ , IL-4 and IL-2 in mitogen and antigen stimulated T cells (Sehgal *et al* 1995, Sehgal 2003). A flow cytometry study for the detection of intracytoplasmic cytokines after rapamycin intake in normal human controls also revealed minimal effects on cytokine production from PBMCs. No effects were seen for IL-10, IL-4 and IL-2 while the frequency of IFN- γ -expressing cells increased, although this was not statistically significant. Stimulation

The Potential Immunomodulatory Effects of the Statins in Uveitis

was with PMA and ionomycin for 18 hours as with our study but in contrast to our experiment on whole blood, PBMCs were separated on Ficoll gradient density before and then 2 hours and then 4 hours after drug intake (Tkaczuk *et al* 1998). The cell cycle progression inhibited by rapamycin has however been demonstrated to induce T cell clonal anergy and interestingly, upon rechallenge, the same anergic clones were found to display a decrease in IL-3 and a >10-fold decrease in IFN- γ production under optimal activation conditions. It is not clear whether this is due to more complete anergy, as a result of the ability of rapamycin to fully prevent the G1 to S phase transition, or its ability to effect an additional pathway (Powell *et al* 1999).

Mycophenolate was also found to produce a highly significant decrease in the percentage of intracellular IFN- γ staining but did not significantly affect levels of IL-10, IL-4 and IL-2. In one study of EAE mycophenolate treated brainstems of rats had lower levels of mRNA for Th1 (IL-2 and IFN- γ) and Th2 (IL-4) and Tr (IL-10) cytokines (Tran *et al* 2001). In another study allogeneic T cells stimulated by mycophenolate treated DCs expressed lower levels of cytokines such as IL-2 and IFN- γ but higher levels of IL-4 and IL-10 as compared to controls. Here it was concluded that in this mixed lymphocyte reaction (MLR), mycophenolate drove a shift of Th1 to Th2 cytokines (Huang *et al* 2004). Hence it can be seen our study that, despite not being directly comparable due to the differing experimental models employed, mycophenolate produces a downward shift in the expression of the Th1 cytokine IFN- γ consistent with the literature. A Th2 shift was not observed as per Huang *et al* (2004) but once again a direct comparison is not possible due to their differing stimulus for T cell proliferation.

The Potential Immunomodulatory Effects of the Statins in Uveitis

CsA was found to significantly decrease the percentage of intracellular IFN- γ staining but did not significantly affect levels of IL-10, IL-4 and IL-2. A previous study using whole blood has shown, by RT-PCR, variable cytokine mRNA profiles following CsA preincubation for 2 hours and costimulation with anti-CD3/CD28 antibodies for 24 hours. 3 of 11 subjects showed a marked (>50%) suppression of IL-2 mRNA and a partial inhibition of IL-4 and IFN- γ mRNA expression which parallels our results to some extent at least with IFN- γ . In the remaining 8 individuals cytokine expression levels were unaffected or even increased. A decrease in IL-10 mRNA expression corresponded with CsA resistance for IL-2 mRNA expression (Härtel 2003). Another study also described CsA inhibition of IL-2 and IFN- γ cytokine expression as measured by radioimmunoassay (RIA) and ELISPOT in stimulated human PBMCs cultured *in vitro* (Li *et al* 2001). In a nickel dermatitis model with nickel-induced T cell proliferation, IFN- γ production was also shown to be decreased by CsA (Moed *et al* 2004). Other studies have shown evidence for a decrease in IL-2 (van den Berg *et al* 1998) and IFN- γ (van den Berg *et al* 1998) cytokine production in transplant patients as determined by intracellular cytokine staining.

Dexamethasone was the most effective of all the drugs in its ability to significantly decrease the percentage of intracellular IFN- γ staining but did not significantly affect levels of IL-10, IL-4 and IL-2. A number of studies have looked at the effects of the glucocorticoids on cytokine secretion. Franchimont *et al* (1998) showed a significant inhibition by dexamethasone on the secretion of the lymphokines, IL-2, IL-4, IL-10 and IFN- γ as detected by ELISA in PHA stimulated whole blood of healthy

The Potential Immunomodulatory Effects of the Statins in Uveitis

volunteers. IL-4 and IL-10 were less inhibited than IFN- γ . This supports the idea that glucocorticoids may induce a shift from the Th1 to Th2 profile of cytokine secretion (Franchimont *et al* 1998). In a nickel dermatitis model with nickel-induced T cell proliferation, IFN- γ production was also shown to be decreased by dexamethasone (Moed *et al* 2004). Further evidence for type 2 cytokine promotion has been shown in a study on human PBMCs stimulated *in vitro* with tetanus toxoid where dexamethasone decreased IFN- γ production and increased IL-4 and IL-10 (Agarwal and Marshall 2001). Indeed work done by Richards *et al* (2000) has demonstrated the ability of dexamethasone, in conjunction with anti-CD3 and IL-2 human T cell stimulation, to induce CD4⁺ and CD8⁺ T cell populations which synthesize high levels of IL-10. It can therefore be inferred that the Th1 cytokine reduction in our study is in keeping with the literature. Few comparative studies have been undertaken, but in our study, at least, dexamethasone appeared to achieve the highest level of IFN- γ inhibition. Contrary to fairly concordant findings in the literature we did not observe any increases in IL-10 thereby giving weight to its possible degradation.

Atorvastatin in combination with rapamycin showed no significant decrease in intracellular cytokine staining from control for IL-2, IL-4, IL-10 and IFN- γ . IFN- γ levels, in addition, showed large variability. The literature concerning the combination of atorvastatin and rapamycin on cytokine profiles is scarce. Atorvastatin through its cholesterol lowering effects could be used to offset the adverse effects of hypercholesterolemia in rapamycin. Both drugs are metabolized by the P-450 3A4 system however and one case report showed that the clinical combination of atorvastatin and

The Potential Immunomodulatory Effects of the Statins in Uveitis

rapamycin resulted in elevated serum rapamycin trough concentrations requiring reduction of rapamycin dosing (Barshes *et al* 2004). Thus it would appear that the combination of atorvastatin and rapamycin would require careful monitoring of rapamycin dosage with the potential lipid lowering offering little offset through the absence of any additive effect in IFN- γ reduction. IFN- γ expression actually appeared to be increased by this particular combination.

Atorvastatin in combination with mycophenolate showed no significant decrease in intracellular cytokine staining from control for IL-2, IL-4, IL-10 and IFN- γ . IFN- γ levels, in addition, showed variability. There is little in the literature investigating the combined effect of the statins and mycophenolate on cytokine production. It would appear, at least from a cytokine perspective, that this particular statin combination also confers little beneficial effect and may in fact be counterproductive.

Atorvastatin in combination with CsA showed no significant decrease in intracellular cytokine staining from control for any of the four cytokines. IFN- γ levels, in addition, showed variability. Atorvastatin has been increasingly used as a cholesterol-lowering agent in solid organ transplant recipients receiving CsA. Both are metabolized by cytochrome P4503A4 which is why a bilateral interaction between the two is likely. Adding atorvastatin to CsA-treated transplant patients has been shown to result in a sixfold higher atorvastatin plasma HMG-CoA reductase inhibitory activity with resultant decrease in total and low density lipoprotein cholesterol (Asberg *et al* 2001). CsA however has also been documented to cause neuropathies and myopathies even when

The Potential Immunomodulatory Effects of the Statins in Uveitis

given at therapeutic doses (Guennoc *et al* 2005). Neuropathies and myopathies are also known side effects of the statins and the combination of CsA and statins has been identified as potentially leading to a harmful CsA-statin interaction such as the potentiation of rhabdomyolysis (Ratz Bravo *et al* 2005). Based on our *in vitro* results which showed a variable effect on IFN- γ production and on what is known in the literature with regards to potential side effects, any *in vivo* trials with this combination would have to proceed with caution.

Atorvastatin in combination with dexamethasone showed a significant decrease in intracellular cytokine staining from control for IFN- γ but not for the other cytokines. IFN- γ levels, in addition, showed variability. The decrease in IFN- γ did not reach the same level of significance as dexamethasone alone suggesting that the anti-inflammatory effects of atorvastatin did not augment those of dexamethasone. To date there have been no reports on the effect of atorvastatin and dexamethasone on cytokine profiles *in vitro*.

Atorvastatin in combination with CsA and dexamethasone showed a significant decrease in intracellular cytokine staining from control for IFN- γ but not for the other cytokines. The decrease in IFN- γ did not reach the same level of significance as dexamethasone alone. This poorer than expected IFN- γ reduction, as with the other atorvastatin-combinations, might be accounted for by experimental variation or possibly an interference of each other's immunosuppressive potential through as yet undefined mechanisms. There is little in the literature concerning the combination of atorvastatin, CsA and dexamethasone on cytokine profiles. The combination of CsA and prednisolone,

The Potential Immunomodulatory Effects of the Statins in Uveitis

however, has been studied. Therapy with CsA and prednisolone greatly reduced both IFN- γ^+ /CD3 $^+$ lymphocytes and serum IL-12 levels associated with a general clinical improvement. Furthermore, in the same study, *in vitro* work demonstrated that CsA/prednisolone therapy impairs the capacity of mature DCs to secrete IL-12 and inhibits their MLR activity (Frassanito *et al* 2003).

It has been documented that Th2 cytokines may be difficult to detect and are only present transiently after stimulation in a minority of cells. In contrast, IFN- γ and TNF- α may be present in a very high proportion of cells for a long time after stimulation and stain very brightly. This does not necessarily mean, however, that the biological outcome is dominated by the effects of IFN- γ . It is thought that IFN- γ has potentially relatively weak effects locally and that this weakness is corrected for by its abundance. On the other hand IL-4 and IL-5 are very potent and only need to be produced in very small amounts to dominate the immunopathological process (Pala *et al* 2000). The lack of effect on cytokine expression by the drugs could be due to the use of the intracellular staining technique, which relies on the ability of the cells to respond to PMA in the presence of brefeldin. The untreated cells in this study showed disappointingly low levels and so an alternative technique with greater sensitivity was applied.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Chapter 6

The Potential Immunomodulatory Effects of the Statins in Uveitis

Fluorescent Bead Immunoassay

The Potential Immunomodulatory Effects of the Statins in Uveitis

6.1 Introduction

Intracellular cytokine staining, for all its T cell characterization specificity, does have important limitations. The permeabilization procedure frequently causes high autofluorescence and many antibodies that bind specifically in other conditions do not work well on permeabilized, fixed cells (Pala *et al* 2000). In addition cytokine synthesis is generally not constitutive, and only a small proportion of cells obtained *ex vivo* stain for intracellular cytokines. The kinetics of activation and asynchronous cytokine production by individual cells may account for this. It is accepted that even clonal populations of lymphocytes produce different cytokines asynchronously (Openshaw *et al* 1995). Cytokines each have different kinetics of expression, so that optimal times for detection vary. These variables hinder investigation of co-expression of different cytokines in single cells. It may be possible to determine how many cells co-express two or more cytokines at a single point in time, but the sequential expression of cytokines by the same cells may be impossible to discover by this technique (Pala *et al* 2000).

In comparison to some methods, intracellular staining can be relatively insensitive, even after using monensin or brefeldin A to cause intracellular accumulation. It must also be remembered that the presence of intracellular cytokine does not equate to secretion of that cytokine *in vivo*, or to its biological effects. A final major drawback of intracellular cytokine staining is that cells have to be killed in order to visualize cytokines. Further functional studies are therefore precluded, allowing only a snapshot, static view of cytokine production to be obtained (Pala *et al* 2000).

The Potential Immunomodulatory Effects of the Statins in Uveitis

Because of the extreme interdependence of cytokine functions, a simultaneous measurement of multiple cytokines in a single fluid sample is desirable. FBI, like CBA, is a multiplexed fluorescent bead-based immunoassay for detecting and quantifying multiple cytokines. Its degree of multiplexing (i.e. the number of sequences that can be detected in a single run), however, is greater than that of CBA as it employs two sets of microspheres of two differing sizes, 4.4 μ m and 5.5 μ m. This makes it possible to detect ten capture cytokine antibodies by FBI as to the six through CBA. In addition good correlation has been demonstrated between intracellular detection of cytokines and extracellular cytokine secretion as detected by bead arrays (Schuerwegh *et al* 2003).

6.2 Aims

To address the hypothesis that other cytokines might be inhibited by statins, which were not seen by intracellular staining. To this end we used FBI to detect the presence of the cytokines IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF- α and TNF- β within the supernatants collected from parallel drug treated whole blood from the same normal human donors used in the intracellular cytokine staining experiments in the absence of brefeldin. Cells were stimulated for 18 hour as before with PMA/Ionomycin, with drug treatment initiated at time of stimulation (See Chapter 5). In addition we compared the effects of CsA added to the cells at time of PMA/Ionomycin stimulation versus CsA pretreatment of the cells for 2 hours prior to stimulation. This was an attempt to obtain a more complete picture of the efficacy of the statins as immunomodulators through their effects on extracellular as well as intracellular cytokine profiles, in comparison with the more conventional immunosuppressives.

The Potential Immunomodulatory Effects of the Statins in Uveitis

6.3 Results

All cytokines investigated in the first FBI experiment showed good recovery except TNF- β , which exhibited none. TNF- β was however recovered in the second FBI experiment comparing the effects of CsA pretreatment. Data for all cytokines are represented as the mean percentage change in cytokine expression $\pm$ SEM except for IL-4. Data for IL-4 are expressed in raw form as pg/ml as relatively small amounts and large inter-individual variability precluded statistically significant comparisons. All assigned p values in the first experiment are shown collectively after the graphical representations in Table 6.1.

Atorvastatin decreased the non-T cell specific costimulatory cytokine for T cell activation, IL-1 β by $50.9 \pm 56.9\%$ and the pro-inflammatory cytokines IL-2 by $46.6 \pm 61.8\%$, TNF- α by $59.9 \pm 47.2\%$ ($p < 0.05$), IFN- γ by $59.1 \pm 44.3\%$ ($p < 0.05$), IL-6 by $46.7 \pm 39.2\%$ by and IL-8 by $46.7 \pm 42.0\%$ (Figure 6.1). The Th2 cytokine, IL-4, was increased (Figure 6.16) but IL-5 was significantly decreased by $50.5 \pm 30.7\%$ ($p < 0.05$, Figure 6.1). IL-10 was also significantly decreased by $49.0 \pm 33.5\%$ ($p < 0.05$, Figure 6.1).

With lovastatin, IL-1 β was increased by $52.9 \pm 58.4\%$ (Figure 6.2). Lovastatin, however, decreased IL-2 by $36.8 \pm 35.8\%$, TNF- α by $41.1 \pm 21.5\%$ ($p < 0.01$), IFN- γ by $34.4 \pm 31.5\%$ and IL-6 significantly by $12.7 \pm 3.0\%$ ($p < 0.01$, Figure 6.2). IL-8, however, was increased by $12.2 \pm 12.6\%$ (Figure 6.2). IL-4 was increased (Figure 6.16) while IL-5 expression was not significantly changed from control (Figure 6.2). IL-10 was increased by $136.5 \pm 158.6\%$ (Figure 6.2).

The Potential Immunomodulatory Effects of the Statins in Uveitis

Simvastatin significantly increased IL-1 β by $110.4 \pm 35.1\%$ ($p < 0.05$, Figure 6.3). Simvastatin did decrease IL-2 by $36.0 \pm 50.7\%$ and IFN- γ by $11.5 \pm 54.6\%$ in whole blood supernatants. TNF- α , however, was slightly increased by $9.5 \pm 68.5\%$. IL-6 expression was not significantly changed from control but IL-8 was increased significantly by $23.3 \pm 3.8\%$ ($p < 0.01$, Figure 6.3). The Th2 cytokine, IL-4, was increased by $188.4 \pm 186.0\%$ (Figure 6.13) while IL-5 showed no significant change from baseline at $5.9 \pm 83.2\%$ above control. IL-10 was decreased by $39.6 \pm 28.1\%$ ($p < 0.05$; Figure 6.3).

Upon comparisons between the statins (Figure 6.4), atorvastatin inhibited a greater number of pro-inflammatory cytokines and also to a greater degree than lovastatin and simvastatin. Both lovastatin and simvastatin were associated with an increase in IL-1 β and IL-8 (Figure 6.4). There were significant differences between the reduced levels of IL-1 β and IL-8 with atorvastatin as compared to simvastatin ($p < 0.05$; Figure 6.4). Simvastatin alone was associated with a comparatively slight increase in IL-6 as compared to atorvastatin but this did not reach significance (Figure 6.4). All statins were associated with an increase in IL-4 (Figure 6.16) with a trend towards atorvastatin showing the greatest increase (Figure 6.16). Atorvastatin alone was associated with a decrease in IL-5 while lovastatin alone was associated with an increase in IL-10 (Figure 6.4). No significant differences in cytokine expression were noted between lovastatin and simvastatin.

The Potential Immunomodulatory Effects of the Statins in Uveitis

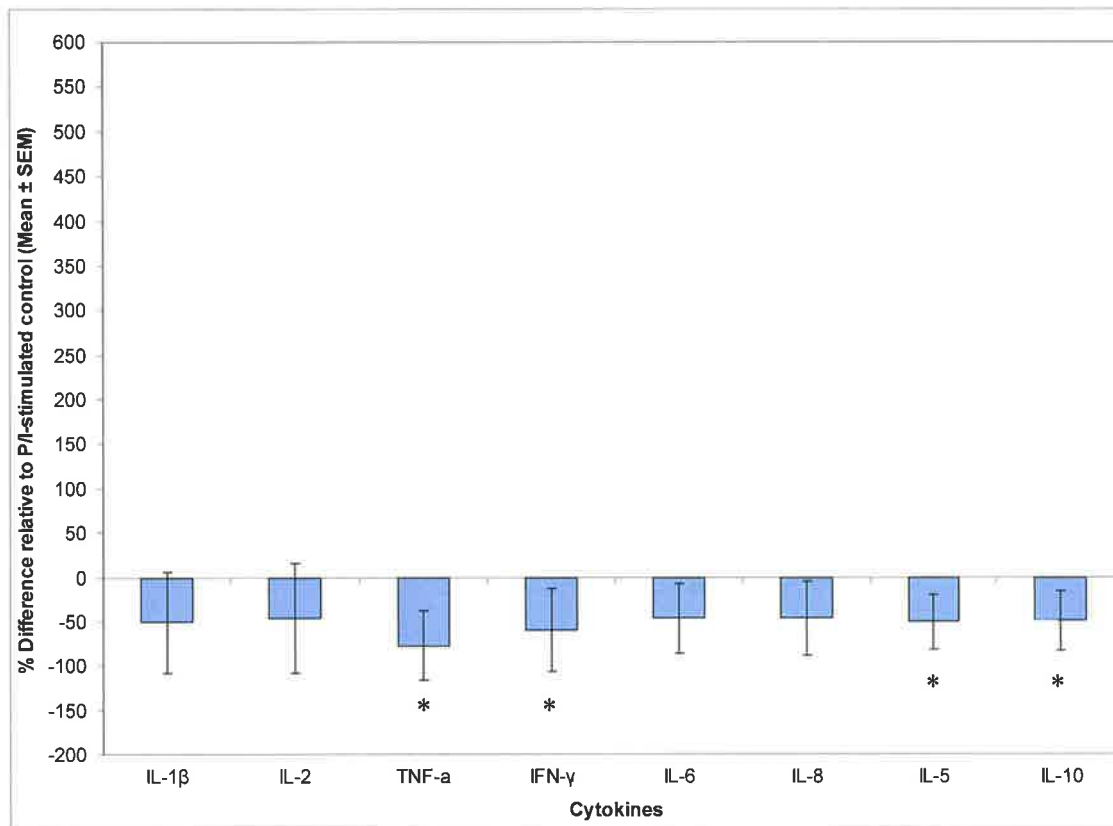


Figure 6.1 Percentage difference in cytokine expression following Atorvastatin addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Whole blood was incubated as previously described (Chapter 2) for 18 hours in the presence of atorvastatin [50 μ M] and PMA/Ionomycin for proliferation stimulation.

Whole blood supernatants were then pipetted off for multiplex analysis.

Each data point represents the mean percentage change in cytokine expression $\pm$ SEM.

Due to the variability between donors, data points from each experiment (minimum n=4) were normalized against the positive control allowing us to pool experiments (minimum n=4).

Statistical analysis was performed with the one-way ANOVA test. *p<0.05.

The Potential Immunomodulatory Effects of the Statins in Uveitis

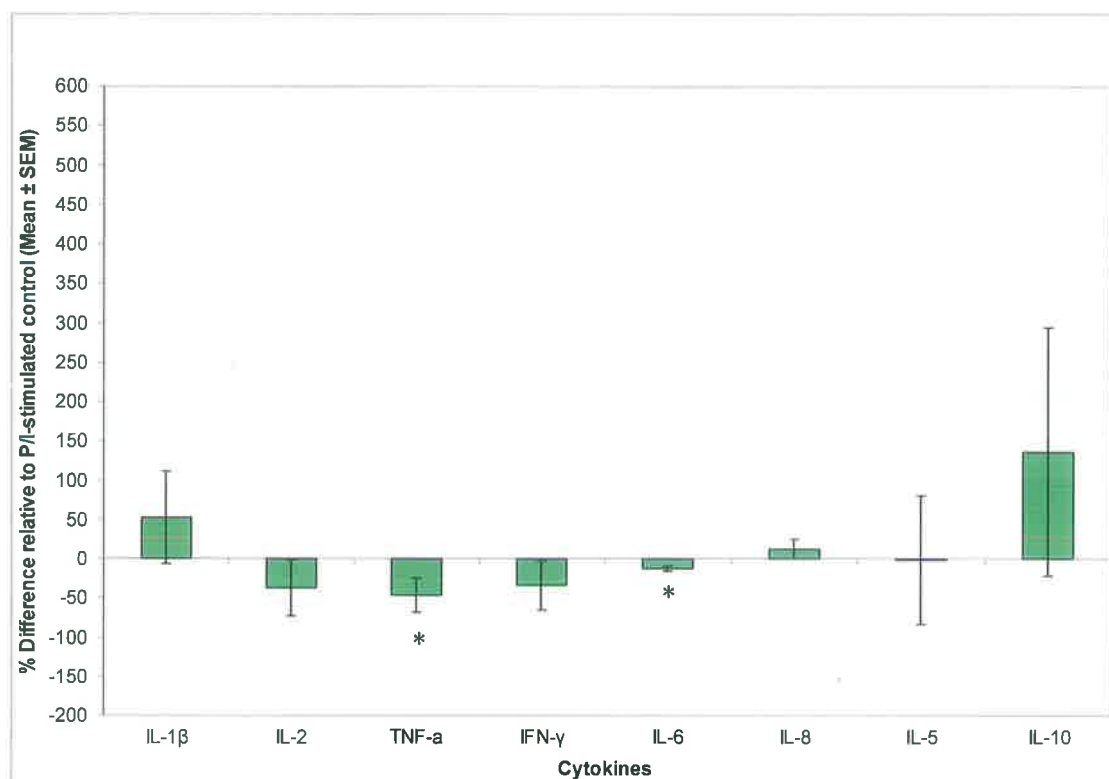


Figure 6.2 Percentage difference in cytokine expression following Lovastatin addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Whole blood was incubated as previously described (Chapter 2) for 18 hours in the presence of lovastatin [100 μ M] and PMA/Ionomycin for proliferation stimulation.

Whole blood supernatants were then pipetted off for multiplex analysis.

Each data point represents the mean percentage change in cytokine expression $\pm$ SEM.

Due to the variability between donors, data points from each experiment (minimum n=4) were normalized against the positive control allowing us to pool experiments (minimum n=4).

Statistical analysis was performed with the one-way ANOVA test. *p<0.01.

The Potential Immunomodulatory Effects of the Statins in Uveitis

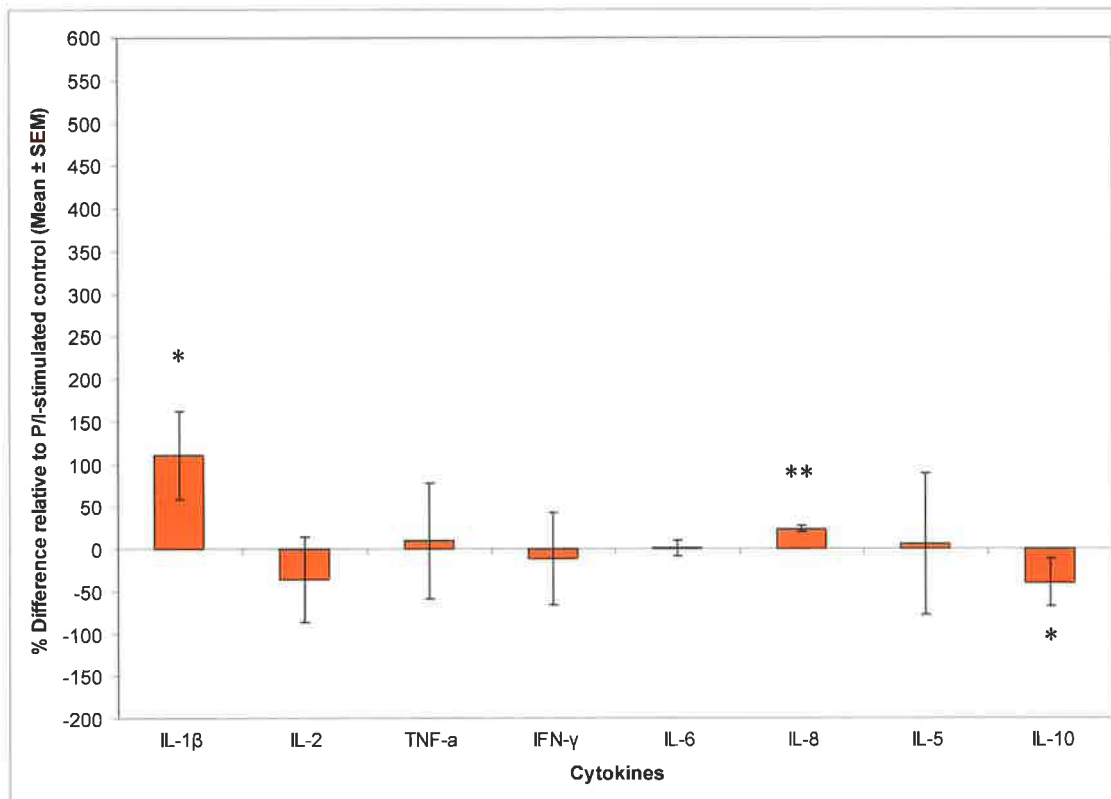


Figure 6.3 Percentage difference in cytokine expression following Simvastatin addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Whole blood was incubated as previously described (Chapter 2) for 18 hours in the presence of simvastatin [100 μ M] and PMA/Ionomycin for proliferation stimulation.

Whole blood supernatants were then pipetted off for multiplex analysis.

Each data point represents the mean percentage change in cytokine expression $\pm$ SEM.

Due to the variability between donors, data points from each experiment (minimum n=4) were normalized against the positive control allowing us to pool experiments (minimum n=4).

Statistical analysis was performed with the one-way ANOVA test. *p<0.05, **p<0.01.

The Potential Immunomodulatory Effects of the Statins in Uveitis

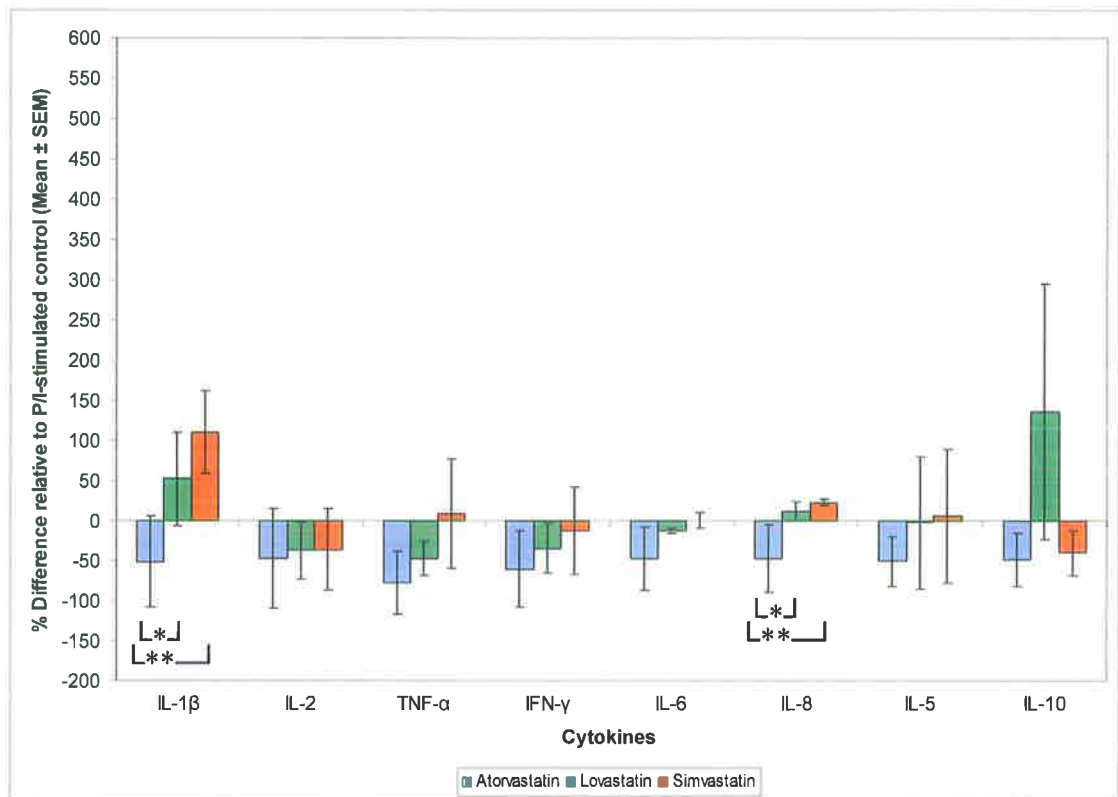


Figure 6.4 Comparison of percentage difference in cytokine expression between Atorvastatin, Lovastatin and Simvastatin following their addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Each data point represents the mean percentage change in cytokine expression $\pm$ SEM as previously shown in Figures 6.1, 6.2 and 6.3 respectively.

Statistical analysis was performed with the one-way ANOVA test. * $p < 0.05$ and ** $p < 0.01$.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Rapamycin was associated with a significant increase in IL-1 β of $29.3 \pm 20.8\%$ ($p < 0.05$; Figure 6.5). Rapamycin did increase the pro-inflammatory cytokines IL-2 by $6.4 \pm 66.3\%$ and IFN- γ by $26.0 \pm 70.4\%$ but did decrease TNF- α by $12.7 \pm 48.1\%$ and IL-6 by $9.6 \pm 8.9\%$. IL-8 expression was not significantly changed from control (Figure 6.5) in whole blood supernatants. The Th2 cytokine, IL-4, was increased (Figure 6.16) but IL-5 was decreased by $39.93 \pm 68.6\%$ below control (Figure 6.5). IL-10 was expressed at $5.9 \pm 55.6\%$ below baseline (Figure 6.5).

Mycophenolate was associated with an expression of IL-1 β at a level of $1.6 \pm 49.4\%$ (Figure 6.6). Mycophenolate decreased the pro-inflammatory cytokines IL-2 by $45.6 \pm 54.5\%$, TNF- α by $56.8 \pm 44.7\%$ ($p < 0.05$), IFN- γ by $10.4 \pm 58.6\%$ and IL-6 by $7.2 \pm 5.1\%$ ($p < 0.05$) but increased IL-8 by $11.6 \pm 13.4\%$ (Figure 6.6) in whole blood supernatants. The Th2 cytokines, IL-4 (Figure 6.16) and IL-5 were increased with the latter at $87.5 \pm 39.8\%$ reaching significance ($p < 0.01$, Figure 6.6). IL-10 was significantly increased by $99.1 \pm 71.7\%$ ($p < 0.05$; Figure 6.6).

The non-T cell specific, costimulatory cytokine for T cell activation, IL-1 β , was significantly increased by $43.6 \pm 9.4\%$ with CsA ($p < 0.01$, Figure 6.7). Pro-inflammatory cytokine expressions following CsA were, for IL-2, $15.7 \pm 15.5\%$, TNF- α , $3.4 \pm 50.2\%$, IFN- γ , $21.9 \pm 60.2\%$, IL-6, $10.6 \pm 12.0\%$ and IL-8 by $3.0 \pm 14.2\%$ above control (Figure 6.7). The Th2 cytokine, IL-4 was increased (Figure 6.16) but IL-5 showed no significant change in expression from control (Figure 6.7). IL-10, however, was significantly decreased by $73.18 \pm 13.03\%$ ($p < 0.01$, Figure 6.7).

The Potential Immunomodulatory Effects of the Statins in Uveitis

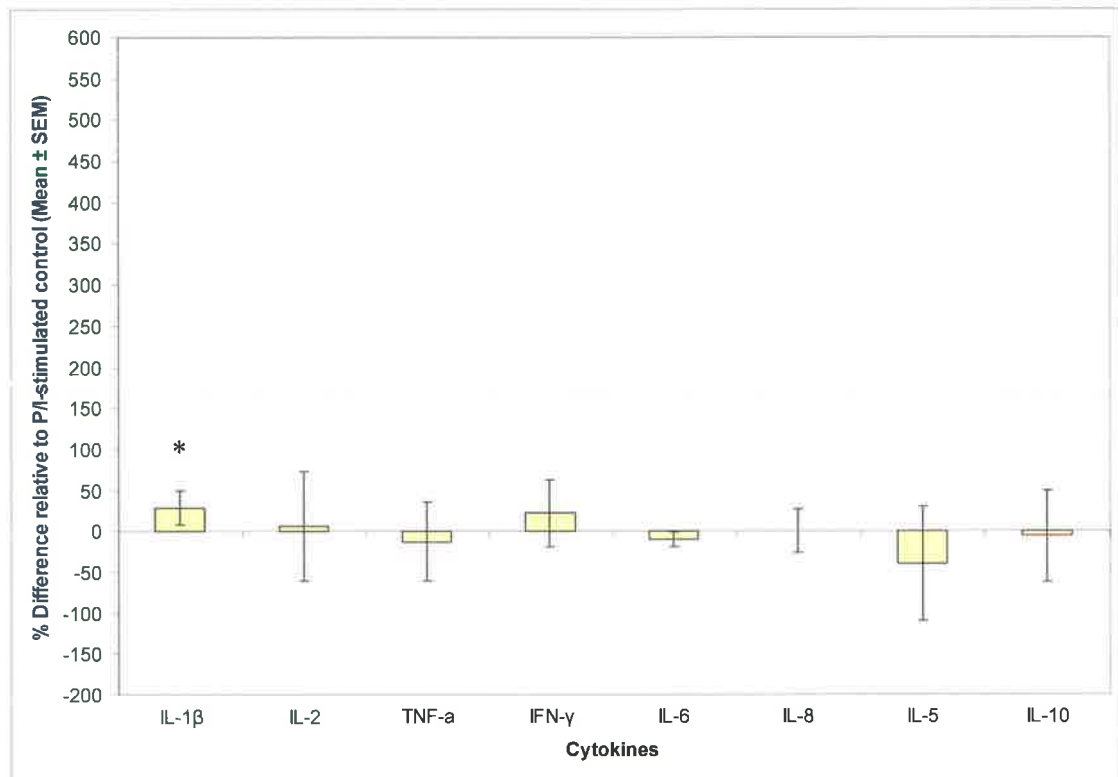


Figure 6.5 Percentage difference in cytokine expression following Rapamycin addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Whole blood was incubated as previously described (Chapter 2) for 18 hours in the presence of rapamycin [100 μ M] and PMA/Ionomycin for proliferation stimulation.

Whole blood supernatants were then pipetted off for multiplex analysis.

Each data point represents the mean percentage change in cytokine expression $\pm$ SEM.

Due to the variability between donors, data points from each experiment (minimum n=4) were normalized against the positive control allowing us to pool experiments (minimum n=4).

Statistical analysis was performed with the one-way ANOVA test. *p<0.05.

The Potential Immunomodulatory Effects of the Statins in Uveitis

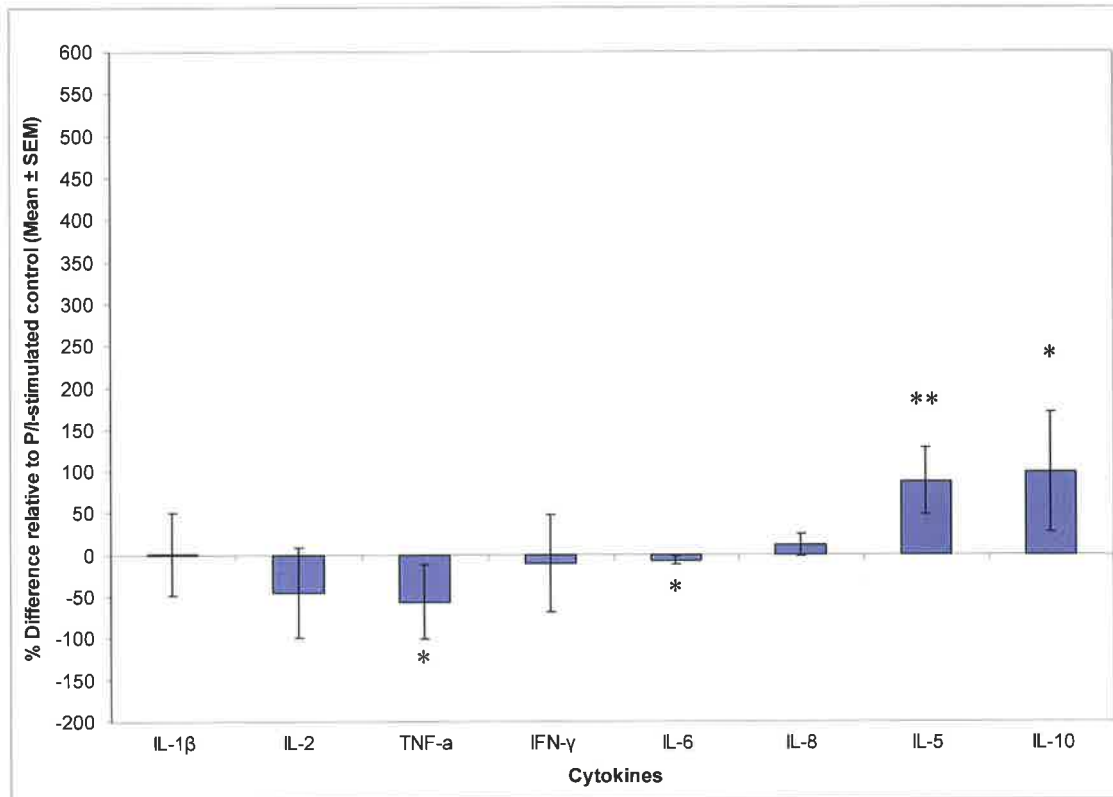


Figure 6.6 Percentage difference in cytokine expression following Mycophenolate addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Whole blood was incubated as previously described (Chapter 2) for 18 hours in the presence of mycophenolate [100 μ M] and PMA/Ionomycin for proliferation stimulation.

Whole blood supernatants were then pipetted off for multiplex analysis.

Each data point represents the average percentage change in cytokine expression $\pm$ SEM.

Due to the variability between donors, data points from each experiment (minimum n=4) were normalized against the positive control allowing us to pool experiments (minimum n=4).

Statistical analysis was performed with the one-way ANOVA test. *p<0.05 and **p<0.01.

The Potential Immunomodulatory Effects of the Statins in Uveitis

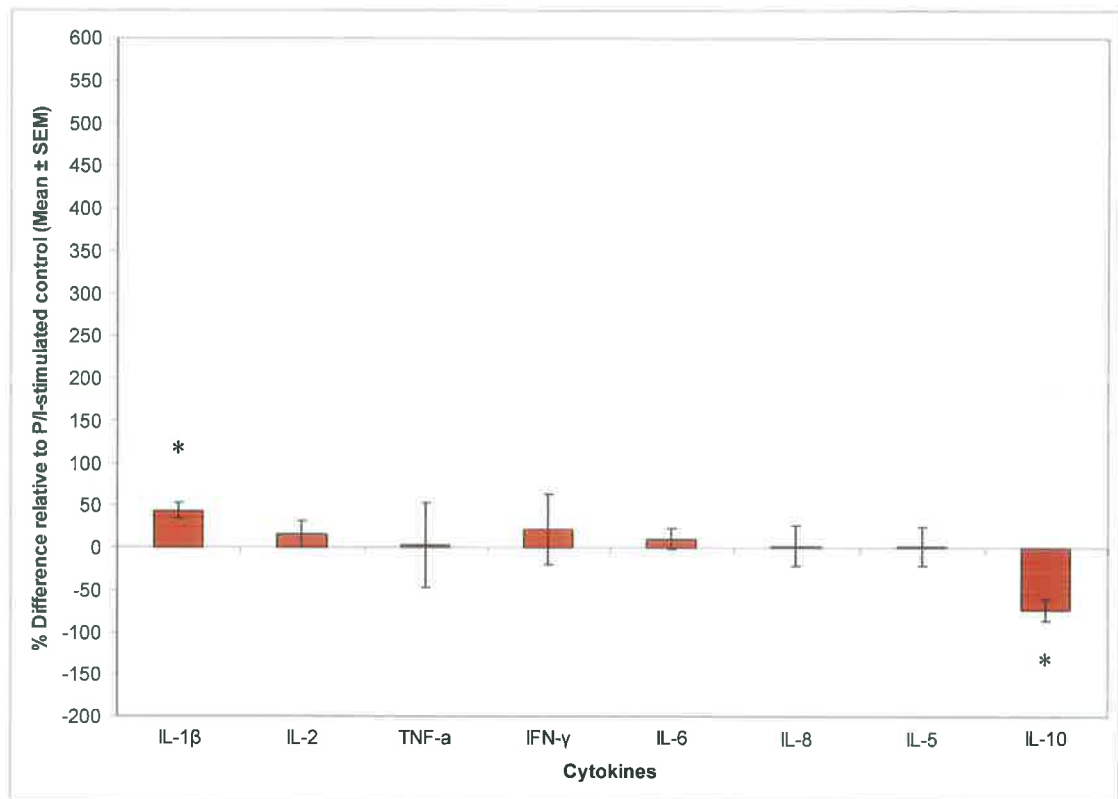


Figure 6.7 Percentage change in cytokine expression following CsA addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Whole blood was incubated as previously described (Chapter 2) for 18 hours in the presence of CsA [100 μ M] and PMA/Ionomycin for proliferation stimulation.

Whole blood supernatants were then pipetted off for multiplex analysis.

Each data point represents the average percentage change in cytokine expression $\pm$ SEM.

Due to the variability between donors, data points from each experiment (minimum n=4) were normalized against the positive control allowing us to pool experiments (minimum n=4).

Statistical analysis was performed with the one-way ANOVA test. *p<0.01.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Dexamethasone decreased IL-1 β by $31.4 \pm 33.0\%$ and significantly decreased the pro-inflammatory cytokines IL-2 by $87.7 \pm 24.3\%$ ($p < 0.01$), TNF- α by $82.7 \pm 6.0\%$ ($p < 0.01$), IFN- γ by $91.2 \pm 9.8\%$ ($p < 0.01$) and IL-6 by $16.1 \pm 11.4\%$ ($p < 0.05$) but increased IL-8 by $1.7 \pm 23.6\%$ (Figure 6.8) in whole blood supernatants. The Th2 cytokine, IL-4 was relatively unchanged from baseline (Figure 6.16) but IL-5 was decreased by $65.0 \pm 54.8\%$ (Figure 6.8). IL-10, however, was significantly increased by $129.78 \pm 76.4\%$ ($p < 0.01$, Figure 6.8).

Atorvastatin and rapamycin increased IL-1 β by $35.0 \pm 28.0\%$ (Figure 6.9) and the pro-inflammatory cytokines IL-2 by $181.8 \pm 330.2\%$, TNF- α by $11.7 \pm 34.3\%$, IFN- γ by $15.0 \pm 74.3\%$ and IL-8 significantly by $6.7 \pm 1.6\%$ ($p < 0.01$, Figure 6.9). IL-6, however, was significantly decreased by $7.7 \pm 6.2\%$ ($p < 0.05$) in whole blood supernatants. The Th2 cytokine, IL-4 was increased (Figure 6.16) along with IL-5 which was increased by $126.2 \pm 155.6\%$ (Figure 6.9). Expression of the Tr cytokine IL-10 was not significantly changed from control (Figure 6.9).

Atorvastatin and mycophenolate increased IL-1 β by $33.2 \pm 39.9\%$ and decreased the pro-inflammatory cytokines IL-2 by $31.4 \pm 46.5\%$, TNF- α by $59.9 \pm 46.3\%$ ($p < 0.05$), IFN- γ by $25.6 \pm 48.3\%$, IL-6 by $41.3 \pm 36.9\%$ and IL-8 by $34.9 \pm 37.15\%$ (Figure 6.10) in whole blood supernatants. The Th2 cytokine, IL-4 remained unchanged from baseline in 3 out of the 4 samples (Figure 6.16) with IL-5 relatively unchanged from baseline (Figure 6.10). IL-10 was decreased by $40.4 \pm 41.5\%$ (Figure 6.10).

The Potential Immunomodulatory Effects of the Statins in Uveitis

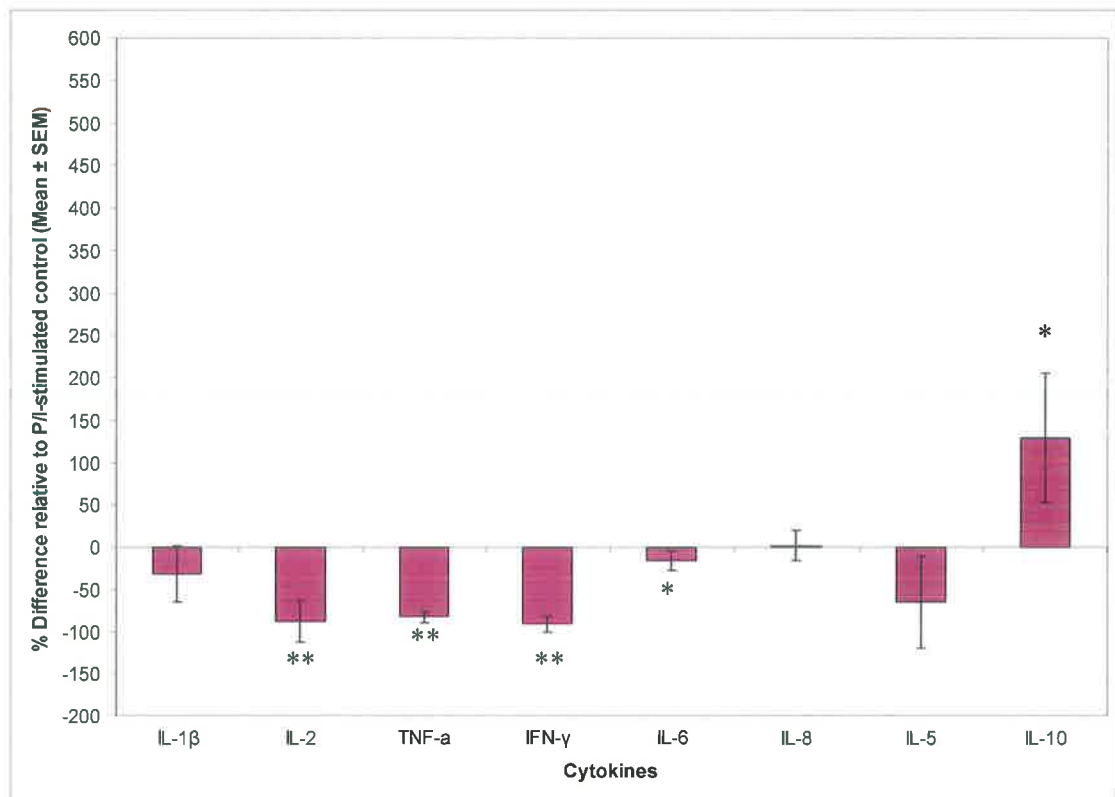


Figure 6.8 Percentage change in cytokine expression following Dexamethasone addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Whole blood was incubated as previously described (Chapter 2) for 18 hours in the presence of dexamethasone [400 μ g/ml] and PMA/Ionomycin for proliferation stimulation. Whole blood supernatants were then pipetted off for multiplex analysis.

Each data point represents the average percentage change in cytokine expression $\pm$ SEM. Due to the variability between donors, data points from each experiment (minimum n=4) were normalized against the positive control allowing us to pool experiments (minimum n=4).

Statistical analysis was performed with the one-way ANOVA test. *p<0.05 and **p<0.01.

The Potential Immunomodulatory Effects of the Statins in Uveitis

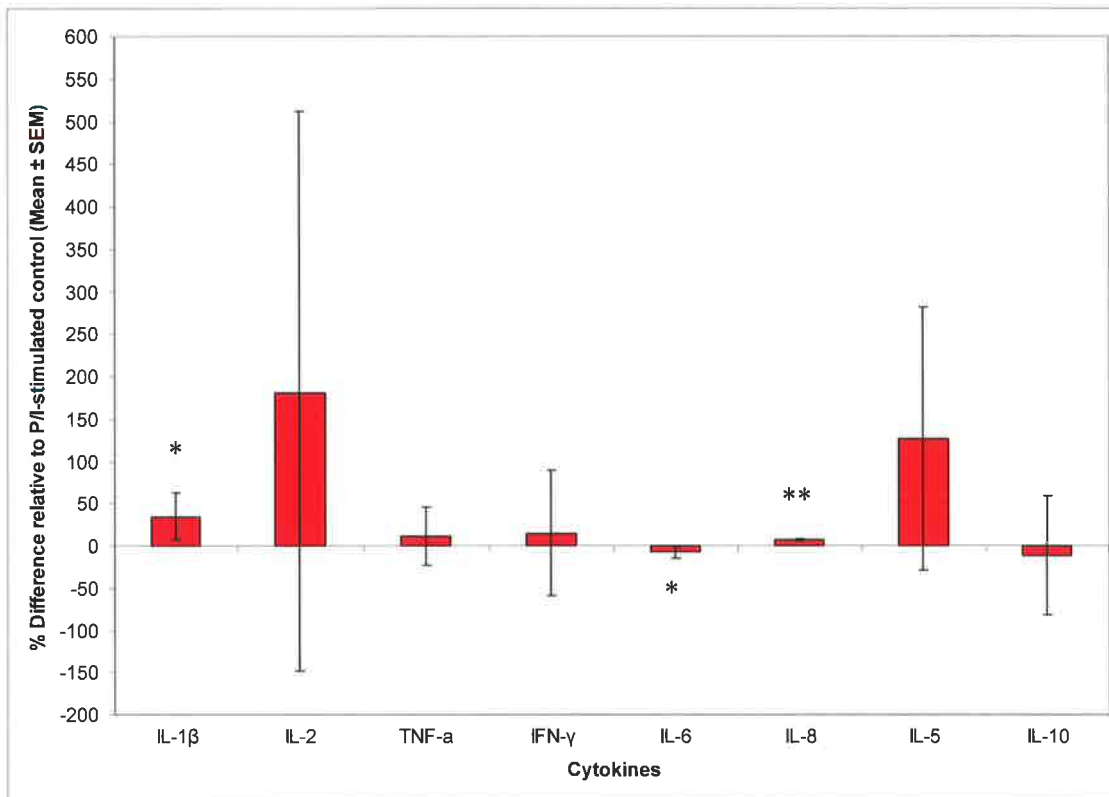


Figure 6.9 Percentage change in cytokine expression following Atorvastatin and Rapamycin addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Whole blood was incubated as previously described (Chapter 2) for 18 hours in the presence of Atorvastatin [50 μ M] and Rapamycin [100 μ M] and PMA/Ionomycin for proliferation stimulation.

Whole blood supernatants were then pipetted off for multiplex analysis.

Each data point represents the average percentage change in cytokine expression $\pm$ SEM.

Due to the variability between donors, data points from each experiment (minimum n=4) were normalized against the positive control allowing us to pool experiments (minimum n=4).

Statistical analysis was performed with the one-way ANOVA test. *p<0.05 and **p<0.01.

The Potential Immunomodulatory Effects of the Statins in Uveitis

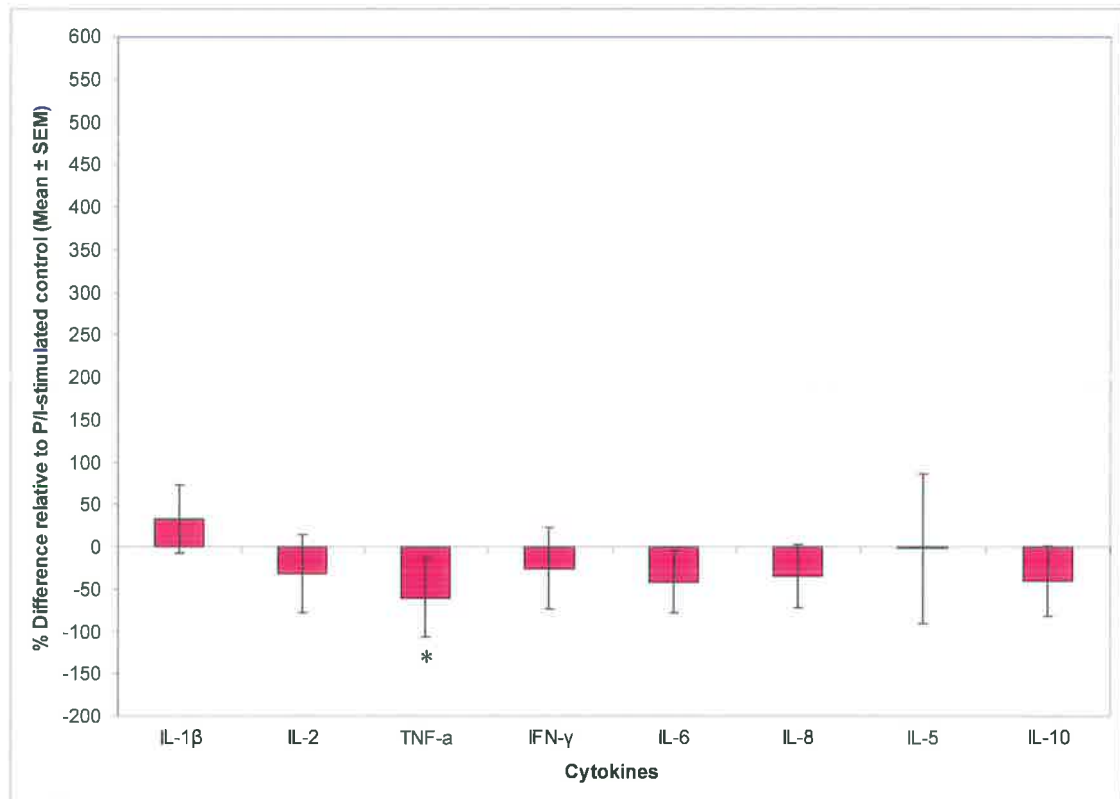


Figure 6.10 Percentage change in cytokine expression following Atorvastatin and Mycophenolate addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Whole blood was incubated as previously described (Chapter 2) for 18 hours in the presence of Atorvastatin [50 μ M] and Mycophenolate [100 μ M] and PMA/Ionomycin for proliferation stimulation.

Whole blood supernatants were then pipetted off for multiplex analysis.

Each data point represents the average percentage change in cytokine expression $\pm$ SEM. Due to the variability between donors, data points from each experiment (minimum n=4) were normalized against the positive control allowing us to pool experiments (minimum n=4).

Statistical analysis was performed with the one-way ANOVA test. *p<0.05.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Atorvastatin and CsA significantly increased IL-1 β by $49.1 \pm 22.2\%$ ($p < 0.01$; Figure 6.11) and decreased the pro-inflammatory cytokines IL-2 by $7.0 \pm 25.2\%$, TNF- α by $38.5 \pm 43.0\%$, IFN- γ by $35.9 \pm 44.1\%$, IL-6 by $20.5 \pm 24.5\%$ except for IL-8 which was increased by $15.5 \pm 8.0\%$ (Figure 6.11) in whole blood supernatants. The Th2 cytokine, IL-4 was increased (Figure 6.16) while IL-5 was decreased by 33.9 ± 36.2 (Figure 6.11). IL-10, however, was decreased significantly by $66.4 \pm 21.1\%$ ($p < 0.01$, Figure 6.11).

Atorvastatin and dexamethasone decreased IL-1 β by $55.9 \pm 33.9\%$ ($p < 0.05$; Figure 6.12) and significantly decreased the pro-inflammatory cytokines IL-2 by $99.7 \pm 0.4\%$ ($p < 0.01$), TNF- α by $88.9 \pm 9.3\%$ ($p < 0.01$), IFN- γ by $97.6 \pm 2.7\%$ ($p < 0.01$), IL-6 by $58.6 \pm 26.5\%$ ($p < 0.01$) and IL-8 by $31.4 \pm 42.8\%$ (Figure 6.12) in whole blood supernatants. The Th2 cytokine, IL-4 was increased (Figure 6.16) while IL-5 was increased by $110.9 \pm 100.1\%$ (Figure 6.12). IL-10 was increased by $163.1 \pm 143.5\%$ (Figure 6.12).

The Potential Immunomodulatory Effects of the Statins in Uveitis

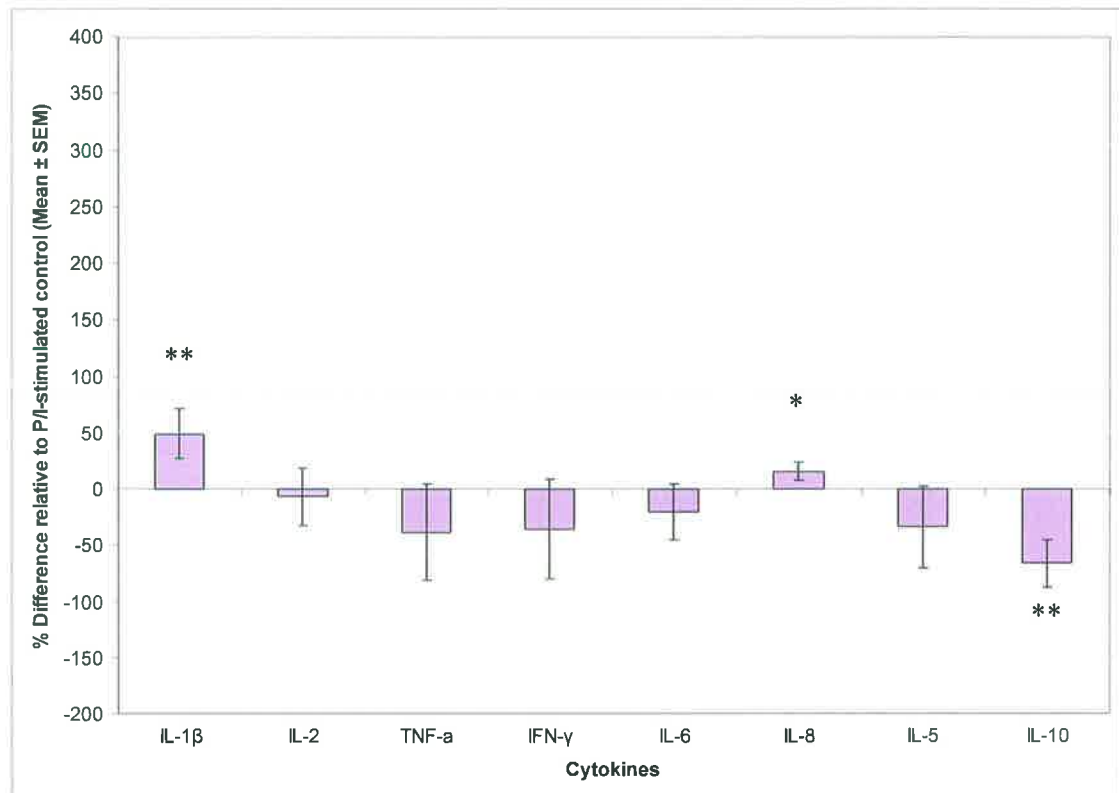


Figure 6.11 Percentage change in cytokine expression following Atorvastatin and CsA addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Whole blood was incubated as previously described (Chapter 2) for 18 hours in the presence of Atorvastatin [50 μ M] and CsA [100 μ M] and PMA/Ionomycin for proliferation stimulation.

Whole blood supernatants were then pipetted off for multiplex analysis.

Each data point represents the average percentage change in cytokine expression $\pm$ SEM.

Due to the variability between donors, data points from each experiment (minimum n=4) were normalized against the positive control allowing us to pool experiments (minimum n=4).

Statistical analysis was performed with the one-way ANOVA test. *p<0.01.

The Potential Immunomodulatory Effects of the Statins in Uveitis

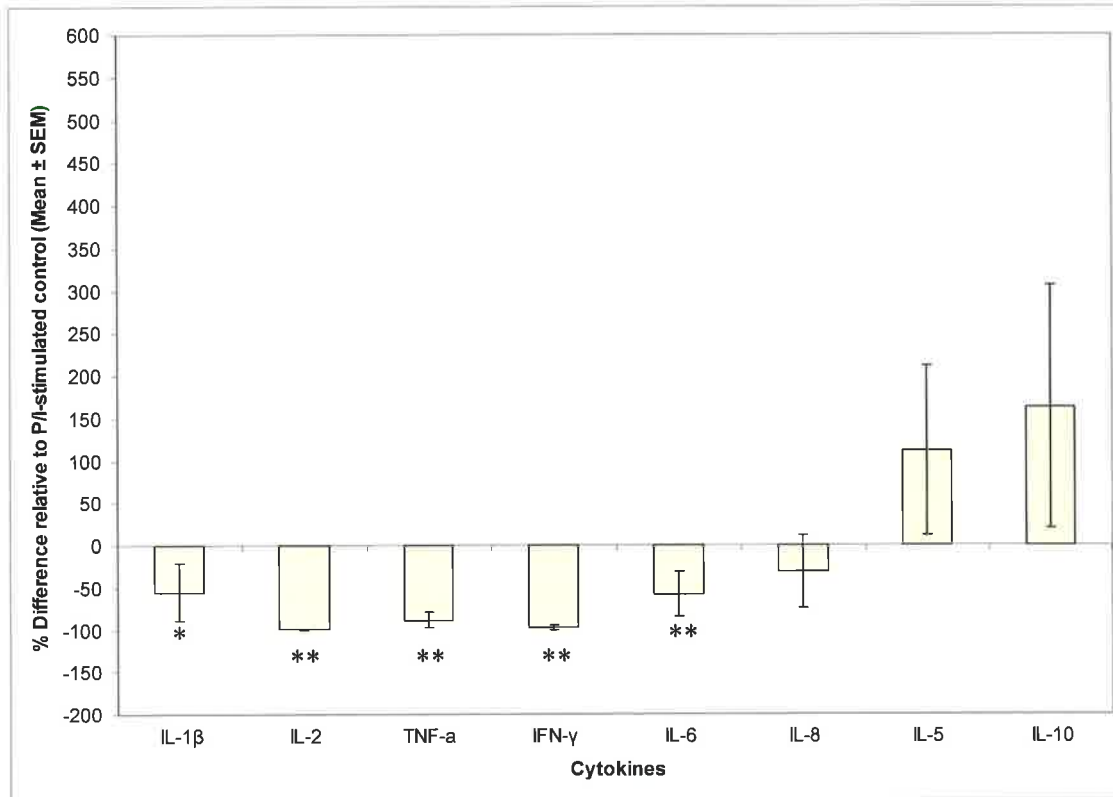


Figure 6.12 Percentage change in cytokine expression following Atorvastatin and Dexamethasone addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Whole blood was incubated as previously described (Chapter 2) for 18 hours in the presence of atorvastatin [50 μ M] and dexamethasone [400 μ g/ml] and PMA/Ionomycin for proliferation stimulation.

Whole blood supernatants were then pipetted off for multiplex analysis.

Each data point represents the average percentage change in cytokine expression $\pm$ SEM.

Due to the variability between donors, data points from each experiment (minimum n=4) were normalized against the positive control allowing us to pool experiments (minimum n=4).

Statistical analysis was performed with the one-way ANOVA test. *p<0.05 and **p<0.01.

The Potential Immunomodulatory Effects of the Statins in Uveitis

When comparing atorvastatin, dexamethasone and atorvastatin and dexamethasone together no significant differences were found in the reductions of IL-1 β , IL-2, TNF- α and IFN- γ though there was a trend towards the combination of atorvastatin and dexamethasone together producing the greatest and most consistent reduction of these cytokines. The combination of atorvastatin and dexamethasone, however, induced a significantly greater reduction in IL-6 as compared to dexamethasone alone ($p < 0.05$, Figure 6.13). There was also a trend for this combination to produce a greater reduction in IL-8 as compared to dexamethasone alone (Figure 6.13). This combination also increased IL-4 relative to dexamethasone alone (Figure 6.16) and significantly increased IL-5 as compared to dexamethasone alone ($p < 0.05$, Figure 6.13) and this increase also appeared to be greater than with atorvastatin alone but this was not statistically significant. Both dexamethasone alone and the combination of atorvastatin and dexamethasone significantly increased the level of IL-10 as compared to atorvastatin alone ($p < 0.01$ and $p < 0.05$ Figure 6.13). This combination also appeared to induce a greater amount of IL-10 than dexamethasone on its own (Figure 6.13).

Atorvastatin, dexamethasone and CsA together, decreased IL-1 β by $57.8 \pm 72.1\%$ (Figure 6.14) and decreased the pro-inflammatory cytokines IL-2 by $93.4 \pm 9.4\%$ ($p < 0.01$), TNF- α by $77.1 \pm 15.6\%$ ($p < 0.01$), IFN- γ by $95.8 \pm 2.0\%$ ($p < 0.01$) and IL-6 by $29.3 \pm 22.6\%$ but IL-8 was increased by $11.3 \pm 7.9\%$ ($p < 0.05$; Figure 6.14) in whole blood supernatants. The Th2 cytokine, IL-4 was increased (Figure 6.16) while IL-5 was increased by $55.2 \pm 100.0\%$ (Figure 6.14). IL-10 was significantly increased by $142.71 \pm 59.7\%$ ($p < 0.05$, Figure 6.14).

The Potential Immunomodulatory Effects of the Statins in Uveitis

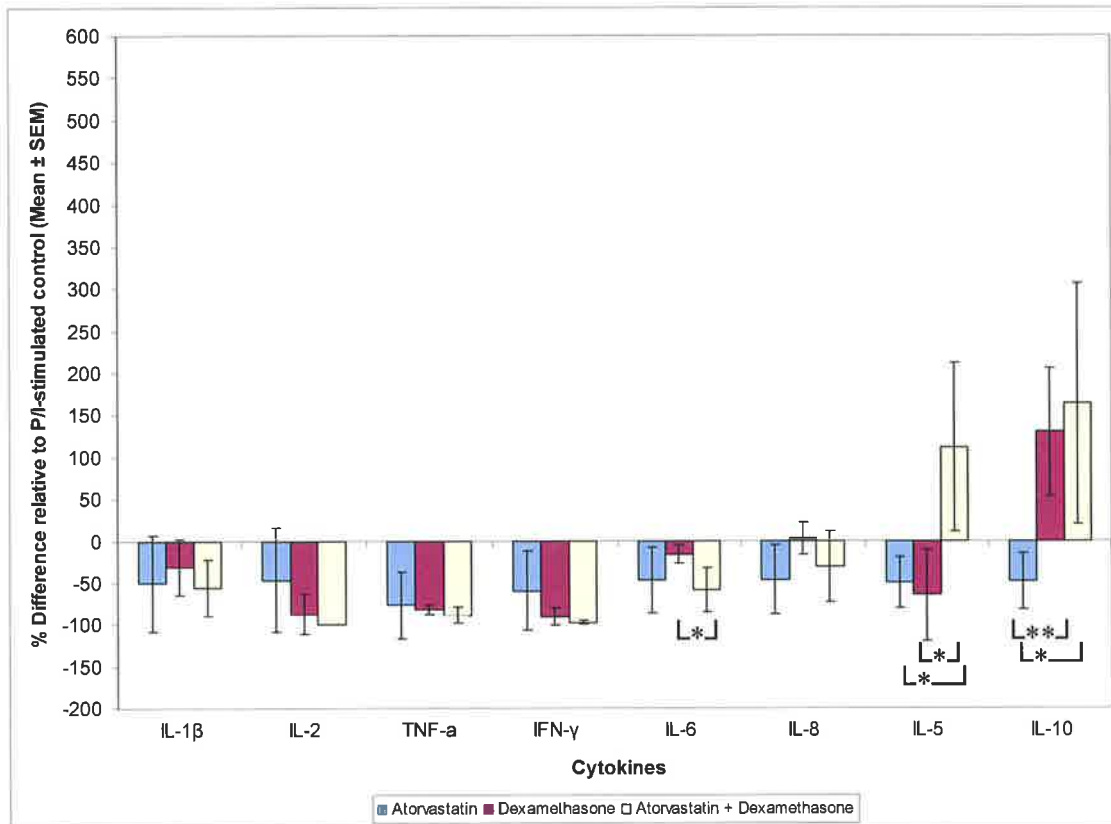


Figure 6.13 Comparison of percentage change in cytokine expression between Atorvastatin, Dexamethasone, Atorvastatin and Dexamethasone following their addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Each data point represents the mean percentage change in cytokine expression $\pm$ SEM as previously shown in Figures 6.1, 6.7 and 6.9 respectively.

Statistical analysis was performed with the one-way ANOVA test. * $p < 0.05$ and ** $p < 0.01$.

The Potential Immunomodulatory Effects of the Statins in Uveitis

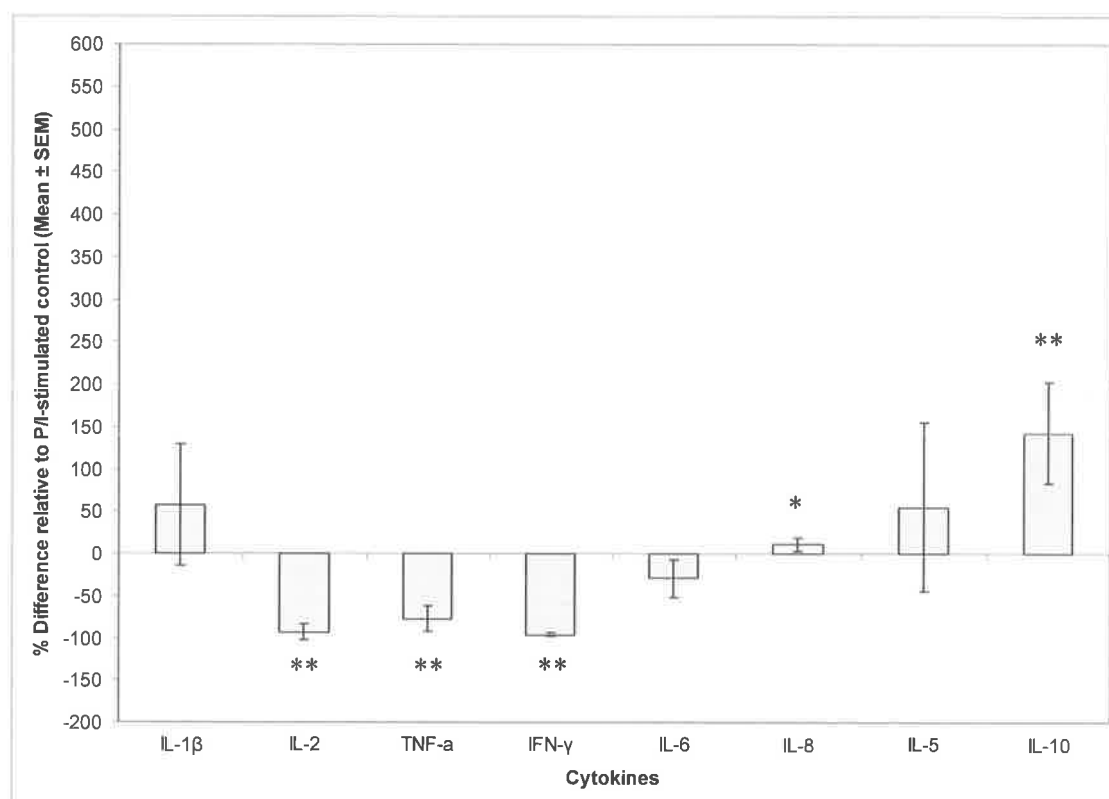


Figure 6.14 Percentage change in cytokine expression following Atorvastatin and Dexamaethasone and CsA addition to PMA/Ionomycin (P/I)-stimulated whole blood.

Whole blood was incubated as previously described (Chapter 2) for 18 hours in the presence of atorvastatin [50 μ M] and dexamethasone [400 μ g/ml] and CsA [100 μ M] and PMA/Ionomycin for proliferation stimulation.

Whole blood supernatants were then pipetted off for multiplex analysis.

Each data point represents the average percentage change in cytokine expression $\pm$ SEM.

Due to the variability between donors, data points from each experiment (minimum n=4) were normalized against the positive control allowing us to pool experiments (minimum n=4).

Statistical analysis was performed with the one-way ANOVA test. *p<0.05 and **p<0.01.

The Potential Immunomodulatory Effects of the Statins in Uveitis

In comparing the pairing of atorvastatin and dexamethasone with the combination of atorvastatin, dexamethasone and CsA no significant differences were found in the reductions of IL-2, IL-6, TNF- α and IFN- γ . Atorvastatin and dexamethasone alone, however, was associated with a significantly greater reduction in IL-1 β ($p < 0.05$; Figure 6.15). Atorvastatin and dexamethasone appeared to comparatively decrease IL-8 but this relative reduction was not significant (Figure 6.15). There were no significant differences in Th2 cytokine expression but there was a trend towards atorvastatin and dexamethasone alone inducing a greater expression of Th2 cytokines except for IL-4 (Figures 6.15 and 6.16).

The Potential Immunomodulatory Effects of the Statins in Uveitis

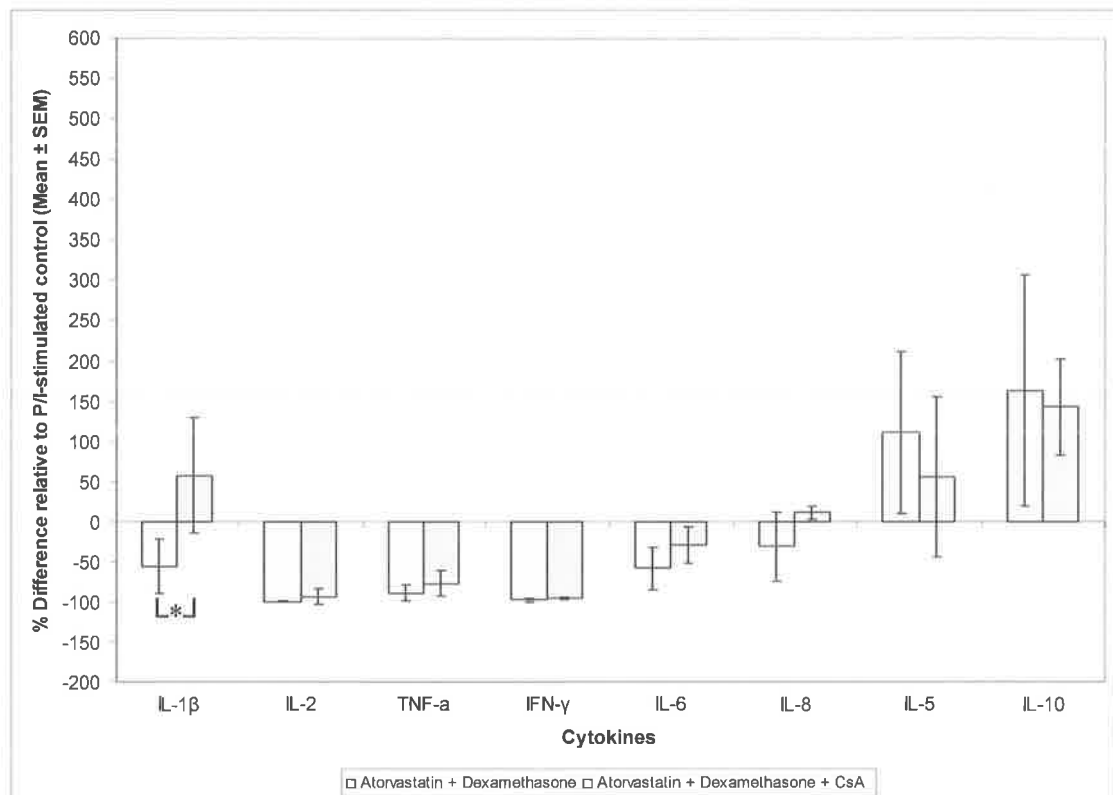


Figure 6.15 Comparison of percentage change in cytokine expression between Atorvastatin and Dexamethasone, Atorvastatin, Dexamethasone and CSA following their addition to PMA/Ionomycin stimulated whole blood.

Each data point represents the mean percentage change in cytokine expression $\pm$ SEM as previously shown in Figures 6.9 and 6.11 respectively.

Statistical analysis was performed with the one-way ANOVA test. *p<0.01.

The Potential Immunomodulatory Effects of the Statins in Uveitis

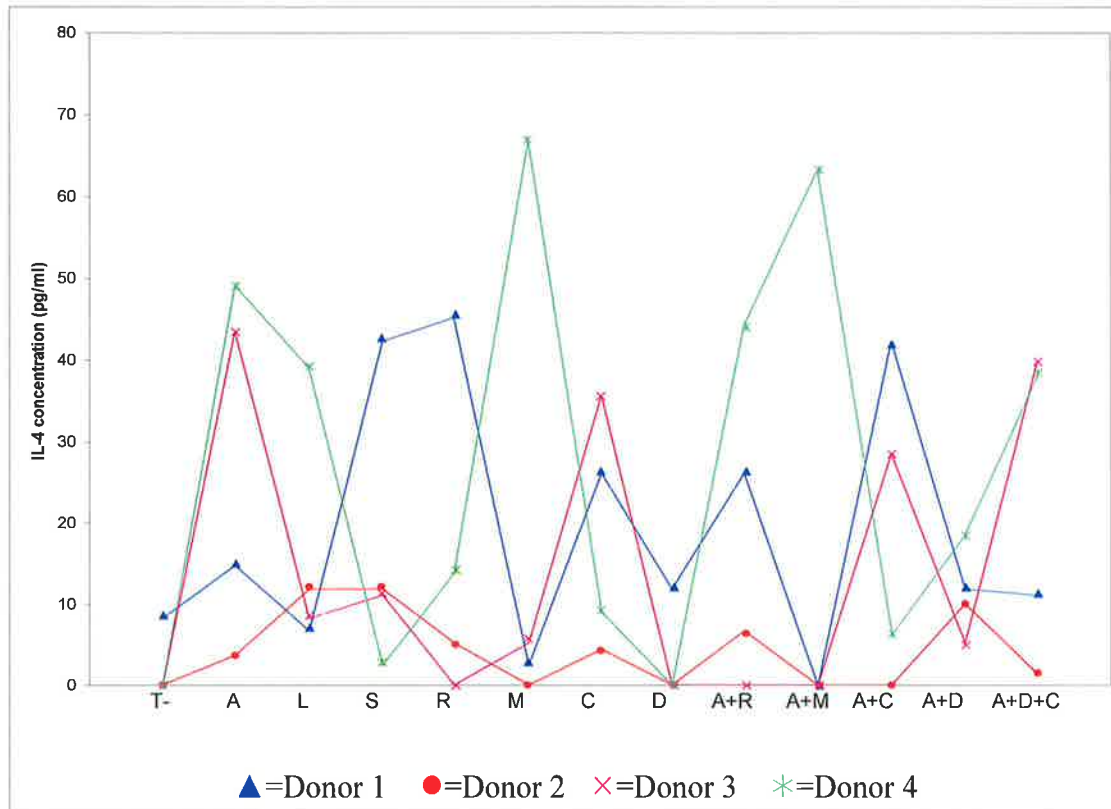


Figure 6.16 Comparison of IL-4 concentrations in whole blood stimulated with PMA/Ionomycin and treated with various drug combinations as detected by multiplex analysis. T=P/I stimulated T cells, A=Atorvastatin, L=Lovastatin, S=Simvastatin, R=Rapamycin, M=Mycophenolate, C=CsA, D=Dexamethasone.

Whole blood was incubated as previously described (Chapter 2) for 18 hours in the presence of various drug combinations and PMA/Ionomycin for proliferation stimulation.

Whole blood supernatants were then pipetted off for multiplex analysis.

The Potential Immunomodulatory Effects of the Statins in Uveitis

	P/I	D	C	M	R	A	L	S	A+R	A+M	A+C	A+D	A+D+C
IL-1β	1	N/S	<0.01	N/S	<0.05	N/S	N/S	<0.01	<0.05	N/S	<0.01	<0.05	N/S
IL-2	1	<0.01	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	<0.01	<0.01
TNF-α	1	<0.01	N/S	<0.05	N/S	<0.05	<0.01	N/S	N/S	<0.05	N/S	<0.01	<0.01
IFN-γ	1	<0.01	N/S	N/S	N/S	<0.05	N/S	N/S	N/S	N/S	N/S	<0.01	<0.01
IL-6	1	<0.05	N/S	<0.05	N/S	N/S	<0.01	N/S	<0.05	N/S	N/S	<0.01	N/S
IL-8	1	N/S	N/S	N/S	N/S	N/S	N/S	<0.01	<0.01	N/S	<0.05	N/S	<0.05
IL-4	1	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
IL-5	1	N/S	N/S	<0.01	N/S	<0.05	N/S	N/S	N/S	N/S	N/S	N/S	N/S
IL-10	1	<0.05	<0.01	<0.05	N/S	<0.05	N/S	<0.05	N/S	N/S	<0.01	N/S	<0.01

Table 6.1 Summary of P value significances in the evaluation of various drug combinations on cytokine expression as compared to control, within whole blood stimulated with PMA/Ionomycin. P/I=PMA/Ionomycin control with p value=1. D=Dexamethasone, C=CsA, M=Mycophenolate, R=Rapamycin, A=Atorvastatin, L=Lovastatin and S=Simvastatin. Significant increases in cytokine expression are highlighted in blue while significant decreases in cytokine expression are highlighted in red.

One-way ANOVA test p values are designated as N/S=not significant; <0.05 and <0.01=significantly and highly significantly decreased as compared to control (1); <0.05 and <0.01=significantly and highly significantly increased as compared to control (1)

The Potential Immunomodulatory Effects of the Statins in Uveitis

CsA added at time 0 decreased IL-1 β by $39.4 \pm 23.6\%$ ($p < 0.05$; Figure 6.17) and the expression of the pro-inflammatory cytokines IL-2 by $87.3 \pm 7.7\%$ ($p < 0.01$), TNF- α by $17.6 \pm 30.8\%$, TNF- β by $8.6 \pm 26.8\%$, IFN- γ by $55.1 \pm 17.5\%$ ($p < 0.01$), IL-6 by $16.5 \pm 28.2\%$ and IL-8 by $17.2 \pm 39.6\%$ (Figure 6.17). The Th2 cytokines IL-4 and IL-5 were reduced by $56.1 \pm 16.1\%$ ($p < 0.01$) and $86.9 \pm 7.7\%$ ($p < 0.01$) respectively while the Tr cytokine, IL-10, was reduced by $40.2 \pm 21.1\%$ ($p < 0.05$; Figure 6.17).

Whole blood pretreated by CsA for 2 hours prior to stimulation decreased IL-1b by $49.0 \pm 29.0\%$ ($p < 0.05$; Figure 6.17) and the expression of pro-inflammatory cytokines IL-2 by $83.3 \pm 8.5\%$ ($p < 0.01$), TNF- α by $21.8 \pm 36.2\%$, TNF- β by $0.9 \pm 13.9\%$, IFN- γ by $75.1 \pm 4.5\%$ ($p < 0.01$), IL-6 by $8.8 \pm 9.5\%$ and IL-8 by $21.3 \pm 35.4\%$ (Figure 6.17). The Th2 cytokines IL-4 and IL-5 were significantly reduced by $71.2 \pm 8.4\%$ ($p < 0.01$) and $82.7 \pm 8.0\%$ ($p < 0.01$) while IL-10 was reduced by $42.1 \pm 35.4\%$ (Figure 6.17).

When looking at whole blood pretreated by CsA for 2 hours prior to stimulation as compared to CsA added at time 0, there were no significant differences between the two time points in the percentages of expression of the cytokines assayed. There was, however, a trend towards IFN- γ being slightly more reduced.

The Potential Immunomodulatory Effects of the Statins in Uveitis

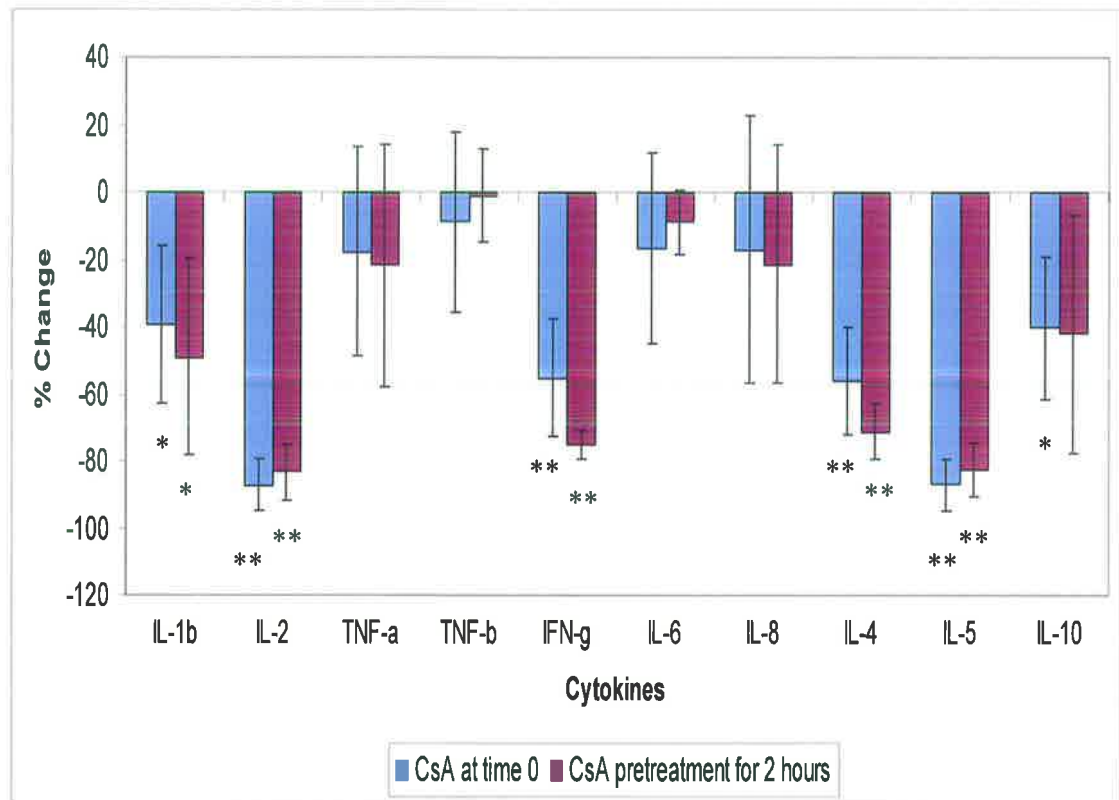


Figure 6.17 Comparison of percentage change of cytokine expression between whole blood treated with CsA at time of stimulation with PMA/Ionomycin (time 0) and whole blood pretreated with CsA for 2 hours prior to stimulation with PMA/Ionomycin.

Whole blood was incubated for 18 hours with either the presence of CsA (100 μ m) at time 0 or CsA (100 μ m) added 2 hours previously, and PMA/Ionomycin for multiplex analysis.

Whole blood supernatants were then pipetted off for FBI.

Each data point represents the average percentage change in cytokine expression $\pm$ SEM. Data points from each experiment (minimum n=3) were normalized against the positive control allowing us to pool experiments (minimum n=3).

Statistical analysis was performed with the one-way ANOVA test. *p<0.01 and *p<0.05

The Potential Immunomodulatory Effects of the Statins in Uveitis

6.4 Discussion

All cytokines in the first FBI experiment were well recovered except for TNF- β . This may have been due to a kit anomaly or a breach in kit protocol. The latter is unlikely as identical procedure was followed for the second FBI experiment with good TNF- β recovery on this occasion.

Atorvastatin reduced the expression of all the pro-inflammatory cytokines with significant reductions in TNF- α and IFN- γ . This pro-inflammatory attenuation is consistent with work done in hypercholesterolemic patients in which decreases in TNF- α , IL-1 and IL-6 were seen with the administration of oral atorvastatin (Ascer *et al* 2004). A decrease in IL-2 production from allogeneic T cells exposed to human ECs pretreated by atorvastatin has also been demonstrated (Kwak *et al* 2000). An atorvastatin decrease in *in vitro* IFN- γ production by human PBMCs has been demonstrated previously through a reduction in the IFN- γ -induced marker of inflammation, neopterin. In the same study a reduction in IFN- γ -induced tryptophan degradation and therefore kynurenine production which is linked with T cell activation, was demonstrated (Neurauter *et al* 2003). The neutrophil chemoattractant, IL-8, has also been shown to be decreased by atorvastatin in a rabbit model of atherosclerosis (Hernandez-Presa *et al* 2002). A similar IL-8 reduction from PBMCs was seen in coronary artery disease (CAD) patients treated with atorvastatin (Waehre *et al* 2003).

Atorvastatin also appeared to have a Th2 cytokine specific effect in that IL-5 and IL-10 were significantly reduced while only IL-4 expression was raised, albeit not

The Potential Immunomodulatory Effects of the Statins in Uveitis

reaching statistical significance due to the large variability in the increases above baseline. Atorvastatin appears, therefore, to have induced a specific Th1 to Th2 'switch' of cytokine production. This is partially consistent with a previous murine model of EAE in which decreases in TNF- α along with the Th1 cytokines, IFN- γ , IL-2 and IL-12 were demonstrated along with an increase in Th2 cytokines. In contrast to our study both Th2 cytokines, IL-4 and IL-5, were elevated. IL-10 and TGF- β were also elevated which was in contrast with our results where we found a decrease in IL-10 and did not investigate TGF- β . In this study atorvastatin was thought to prevent IFN- γ inducible MHC class II transactivator (CIITA) activation thereby inhibiting MHC II expression on APC thereby inhibiting Ag presentation to the TCR which may decrease the proliferation of pro-inflammatory cells producing Th1 cytokines (Youssef *et al* 2002).

Lovastatin did not significantly decrease any of the pro-inflammatory cytokines assayed except for TNF- α and IL-6. IL-1 β was shown to be significantly increased by lovastatin as compared with atorvastatin. With regards to the Th2 cytokines, IL-4 trended towards an increase but IL-5 showed little change. There was a trend for IL-10 to be increased in comparison with atorvastatin and simvastatin. Consistent with our results of significant reductions in TNF- α and IL-6, a recent rat model of EAE demonstrated that lovastatin treatment also decreased levels of IFN- γ as well as IL-6. IL-10 was also investigated and found to be elevated. It was concluded here that lovastatin induces a bias towards Th2 cytokines *ex vivo* (Stanislaus *et al* 2002). In a more recent mouse model of EAE, TNF- α as well as IFN- γ were found to be reduced by lovastatin. In addition an induction of IL-4, IL-5 and IL-10 was also demonstrated giving further evidence for a

The Potential Immunomodulatory Effects of the Statins in Uveitis

Th2 polarization with lovastatin (Nath *et al* 2004). DCs are powerful stimulators of cellular and humoral immune responses and are uniquely well equipped to activate naïve T cells (Banchereau and Steinman 1998). One recent study on the *in vitro* effects of lovastatin on DCs, showed via intracellular staining and ELISA techniques, that LPS stimulated DCs surprisingly exhibited an upregulation of the pro-inflammatory cytokines, TNF α , IL-6 and IL-12 by DCs with IL-10 not able to be detected. This was despite lovastatin being also found to be a potent inhibitor of DC maturation. These effects were reversible with mevalonate (Sun and Fernandes 2003). Even more recently, however, parenteral lovastatin was shown to attenuate the retinal pathology associated with a mouse model of EAU without a Th2 cytokine induction. IFN- γ was shown to be significantly reduced but IL-2 production was not changed and TNF- α was increased. In addition IL-4 and IL-5 levels were below the assay detection limit and IL-10 was significantly decreased (Gegg *et al* 2005).

Simvastatin failed to significantly decrease any of the proinflammatory cytokines. It was also found to increase the expression of TNF- α and IL-8 with the latter reaching significance. In a study conducted on patients with hypercholesterolemia who were given simvastatin in combination with or without aspirin, simvastatin alone was found to decrease TNF- α , in contrast with our results. Its IL-6 lowering effect, however, was reserved only for those patients with marked ($>6.5\text{mmol l}^{-1}$) hypercholesterolemia (Musial *et al* 2001). A similar study measuring the effects of simvastatin on hypercholesterolemic patients found small inconsistent effects on plasma levels of IL-6 as measured by ELISA (Wiklund *et al* 2002) which was consistent with our findings. The

The Potential Immunomodulatory Effects of the Statins in Uveitis

chemokine, IL-8, has been previously found to be decreased in the PBMCs of CAD patients by simvastatin. This was in contrast with our results but the investigators in this study did also observe that IL-8 reduction was less effective with simvastatin than with atorvastatin (Waehre *et al* 2003). In a murine model of collagen-induced arthritis, simvastatin was shown to effectively and specifically suppress pathologic Th1 and pro-inflammatory responses. IFN- γ was significantly suppressed in mice by simvastatin which which contrasted with our results. A reduction in serum IL-6 and TNF- α levels was also observed. Neither IL-4 nor IL-5 production was detected however and IL-10 production was shown to be suppressed. In the same study, the *in vitro* effects of simvastatin on anti-CD3/anti-CD28 stimulated PBMCs from rheumatoid arthritis patients were also investigated with IFN- γ being reduced but IL-4 and IL-5 undetectable and no significant change in IL-10 levels detected (Leung *et al* 2003). Apart from the reduction of IFN- γ and no appreciable effect on IL-10, once again our results contrasted with simvastatin inducing variable increases in IL-4 and a slight, inconsistent increase in IL-5.

Rapamycin showed a highly variable reduction in the pro-inflammatory cytokines, with TNF- α and IL-6 only being reduced. IFN- γ and IL-2 were slightly raised while IL-8 showed little change in expression. The non-T cell specific costimulatory cytokine for T cell activation, IL-1 β , however, was also significantly increased. The primary mode of immunosuppressive action of rapamycin, first recognized in T cell culture models, is an antiproliferative action reflecting the ability of the drug to disrupt signaling by T cell growth-promoting lymphokines such as IL-2 or IL-4 (Dumont *et al* 1990). Rapamycin acts on mTOR and does not inhibit the calcium/calcineurin-dependent transcriptional

The Potential Immunomodulatory Effects of the Statins in Uveitis

activation of lymphokine genes in T cells induced by TCR/CD3 triggering or PMA/ionomycin treatment (Dumont *et al* 1990, Tocci *et al* 1989). Mitogen- and antigen-stimulated T lymphocytes treated with rapamycin have been documented to have no effect on the production of IFN- γ , IL-2, IL-4, IL-5 as well as IL-3 and GM-CSF (Sehgal 2003). Certainly the levels of IFN- γ , and IL-2 as well as TNF- α that we found were inconsistent and overall not significantly changed from baseline. Further literature concerning IL-2 has been inconsistent with reports documenting T cell lymphokine gene transcription, 3-6 hours after activation, to be unaffected by rapamycin (Dumont *et al* 1990, Tocci *et al* 1989); however in another report, rapamycin diminished IL-2 and GM-CSF mRNA (Hanke *et al* 1992).

We documented a variable rise in IL-4 but found no significant reduction in IL-5 by rapamycin. The literature here is also controversial as there is a report which documents a dose-dependent suppression of IL-5 mRNA after a 16 hour exposure of PHA-stimulated T lymphocytes to rapamycin. However, at 4 and 8 hours, PHA-induced IL-5 mRNA was relatively resistant to rapamycin (Valentine and Sewell 1997). Flow cytometric detection of IL-5 induction after 18 hour PMA/ionomycin stimulation has also been reported but in this study the levels were elevated (Tkaczuk *et al* 1998). Thus the discrepancy between previously published results may be stimulation/sampling time-related. Tkaczuk *et al* (1998) also documented no significant change in expression of IL-10 as we did.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Mycophenolate treatment showed no significant reduction of the proinflammatory cytokines except for IFN- γ and IL-6. IL-2 was strongly inhibited but not to any level of significance once again probably due to the variable reductions between individuals. The significant reduction in IFN- γ showed the least consistency while the significant reduction in IL-6 was the most consistent but also the weakest. Mycophenolate has been shown to interfere with IFN- γ production in T cell activation models. In mycophenolate-treated BALB/c mice injected with staphylococcal enterotoxin B, IFN- γ was shown to be decreased (Izeradjene *et al* 2001). This study thereby suggested that in two independent *in vivo* models of T cell activation by bacterial superantigens and by anti-T cell antibodies, respectively, mycophenolate may decrease the two major synergistic proinflammatory cytokines, TNF- α and IFN- γ (Alegre *et al* 1990, Miethke *et al* 1992,). A downregulation in of TNF- α production accompanied by an upregulation of IL-10 *in vivo* by mycophenolate has also been reported following administration of LPS in mice which is in keeping with our findings of a significant reduction in TNF- α in conjunction with a significant increase in IL-10 (Durez *et al* 1999). In a Lewis rat EAE model, mycophenolate was shown to reduce levels of mRNA expression for Th1 (IL-2, IL-12R β 2, IFN- γ) and Th2 (IL-4, IL-10) cytokines as well as TNF- α and TGF- β (Tran *et al* 2001). One MLR study however did document a shift of Th1 to Th2 cytokines with allogeneic T cells stimulated by mycophenolate treated DCs expressing higher levels of Th2 cytokines such as IL-4 and IL-10 but lower levels of Th1 cytokines such as IL-2 and IFN- γ (Huang *et al* 2004). In our study in addition to the increase in IL-10 there were large increases in IL-4 and IL-5, with the latter reaching significance, indicating a Th1/Th2 shift. The combination of 1 α , 25-dihydroxyvitamin D3 and mycophenolate has

The Potential Immunomodulatory Effects of the Statins in Uveitis

been shown to induce CD4⁺CD25⁺ Tr cells in parallel with tolerance induction to fully mismatched mouse islet allografts. Perigraft DCs from tolerant mice were also found to induce only partial activation of alloreactive CD4⁺ cells, characterized by the absence of IFN- γ but conserved IL-2 and IL-4 secretion, as evidenced by intracellular cytokine staining (Gregori *et al* 2001).

CsA showed little overall effect on TNF- α and IL-8. No significant increases in IFN- γ , IL-2 and IL-6 were noted but a significant increase in IL-1 β was observed. IL-4 was variably elevated with IL-5 relatively unchanged but IL-10 significantly reduced. This was in contrast to the literature which has shown that CsA is associated with a reduction in flow cytometer measured cell surface staining for IL-2 in liver transplant patients and normal controls (van den Berg *et al* 1998). This IL-2 reduction was again demonstrated intracellularly and through cell surface staining, along with a reduction in IFN- γ , by the same group (van den Berg *et al* 1998). Apart from a reduction in IFN- γ , a reduction of IL-5 by CsA as measured by ELISA has also been documented in the PBMCs of nickel allergic patients cultured in the presence of allergen (Moed *et al* 2004). IL-5 mRNA expression induced by IL-2R signaling has also been found to be inhibited by CsA (Valentine and Sewell 1997). Furthermore, a reduction in IFN- γ , IL-2, TNF- α and IL-4 mRNA expression mediated by CsA has also been reported in normal human whole blood T lymphocytes pretreated for 2 hours with the CsA prior to CD28-mediated costimulation. Interestingly this picture was seen in only 3 of the 11 individuals in the sample population. In the remaining 8 an opposing picture was seen with elevated IFN- γ , IL-2, TNF- α and IL-4 mRNA. Notably in 7 of these IL-10 mRNA expression was

The Potential Immunomodulatory Effects of the Statins in Uveitis

increased (Härtel *et al* 2003). An earlier separate study has also found that CsA increases IFN- γ production by T cells when co-stimulated through CD28 (Rafiq *et al* 1998). These findings are more consistent with our PMA/ionomycin generated results. Härtel's group also demonstrated, however, that the resistance of IFN- γ production to the effect of CsA was not able to be extrapolated to PMA/ionomycin stimulation conditions. CsA blocked the effect of ionomycin as could be expected from the calcineurin-blocking activity of CsA (Härtel *et al* 2003). The relative ineffectiveness of CsA in reducing IFN- γ in our results might possibly be related to the relatively large significant decrease in IL-10 seen. A regulatory effect of IL-10 in IFN- γ production has previously been suggested in MLR experiments (Tadmori *et al* 1994) and in experiments *in vivo* (Grunig *et al* 1997).

Dexamethasone significantly decreased the expression of all the pro-inflammatory cytokines except IL-8. IFN- γ , TNF- α and IL-2 were all strongly reduced with the expression almost completely inhibited. The inhibition IL-6 expression was small in comparison but nevertheless fairly consistent. There was little overall effect on the expression of IL-8. A small but inconsistent rise in IL-4 was observed but there was a large decrease in IL-5 expression. This decrease, however, was offset by a significant and substantial increase in IL-10, the magnitude of which was not seen with any other drug combination. Dexamethasone, in previous PHA stimulated normal human whole blood cell cultures, has been shown, however, to decrease the expression of both Th2 cytokines, IL-4 and IL-10 and the Th1 cytokine IFN- γ . The immunoassays used in this study did show, however, that the inhibition of IL-4 and IL-10 was less strong than that of IFN- γ (Franchimont *et al* 1998). Recently it has been shown that dexamethasone enhanced IL-

The Potential Immunomodulatory Effects of the Statins in Uveitis

10 and reduced IL-4, IL-5 and IL-13 production in human CD4⁺ and CD8⁺ T cells (Richards *et al* 2000). Dexamethasone has also been shown to inhibit IL-5 but at a level less than the inhibition of IFN- γ , via flow cytometry, in the PBMCs of nickel allergic patients cultured in the presence of allergen (Moed *et al* 2004). GCs have also been found to induce upregulation of soluble receptors for IL-1 and TNF- α which attenuate inflammation because these antagonize the relevant cytokine (Munck and Náray Fejes Tóth 1994, Wilckens 1995). This was supportive of our work which showed a reduction in both IL-1 β and TNF- α with the addition of dexamethasone. IL-6 has been demonstrated to be very resistant to the suppressive effects of endogenous GCs as compared to IL-1 and TNF- α (DeRijk *et al* 1997, Wilckens 1995). Certainly in our results the level of IL-6 suppression was not as great as that of IL-1, IL-2, TNF- α and IFN- γ . Also in keeping with our results, experimental work has been conducted that shows a Th1/Th2 switch in cytokine production. In one study of human PBMCs stimulated *in vitro* with tetanus toxoid, dexamethasone was found to downregulate IFN- γ , subsequently permitting the increased production of IL-4 and IL-10 (Agarwal and Marshall 2001). Animal work with purified rat CD4⁺ T cells activated *in vitro* with ConA or by MLR, dexamethasone has also been shown to diminish the synthesis of IFN- γ and increase the production of IL-4 (Ramirez 1998).

The combination of atorvastatin and rapamycin produced a small but significant reduction in IL-6. There was an equally small but more significant increase in IL-8. There were small but non-significant increases in IFN- γ , TNF- α and IL-2. IL-1 β , however, was significantly increased. The addition of rapamycin to atorvastatin therefore

The Potential Immunomodulatory Effects of the Statins in Uveitis

appeared to detract from the pro-inflammatory cytokine reducing ability of atorvastatin on its own. There was an increase in IL-4 expression but again the combination of atorvastatin and rapamycin appeared to be less effective in increasing IL-4 compared to atorvastatin on its own. IL-5 expression was markedly increased although variable. This was in contrast to the reduction in this cytokine seen with atorvastatin and rapamycin individually and therefore suggests that the combination of atorvastatin and rapamycin may have an upregulatory effect on the production of IL-5. There was a small and inconsistent decrease in IL-10, much less than that observed with atorvastatin on its own but at a level consistent with the reduction seen with rapamycin on its own.

Atorvastatin and mycophenolate produced a moderate but quite variable reduction in all the pro-inflammatory cytokines except for TNF- α which showed a significant decrease. Apart from IL-6 the reduction of the pro-inflammatory was not as marked as with atorvastatin on its own. The increase in IL-4 produced by the combination of atorvastatin and mycophenolate was not as pronounced as that seen with atorvastatin alone. The marked elevation of IL-5 and IL-10 production seen by mycophenolate was reduced when atorvastatin was used in conjunction with mycophenolate. IL-5 levels showed overall no change and IL-10 was quite markedly reduced by comparison.

Atorvastatin and CsA used in combination led to a non-significant reduction in all the pro-inflammatory cytokines except IL-8 which showed a small but consistent and significant increase. This reduction was also not as great as that seen with atorvastatin alone. In addition, IL-1 β exhibited a highly significant increase in expression. There was

The Potential Immunomodulatory Effects of the Statins in Uveitis

an increase in IL-4 expression with atorvastatin and CsA but once again this did not reach levels seen with atorvastatin on its own. Moderate and marked reductions of IL-5 and IL-10 were seen respectively. Levels of IL-5 paralleled those seen with atorvastatin on its own while levels of IL-10 were more highly significant than those seen with atorvastatin or CsA on their own.

The pairing of atorvastatin and dexamethasone led to a significant reduction in all the pro-inflammatory cytokines except IL-8. The highly significant reductions in IL-2, TNF- α and IFN- γ were similar to those seen with dexamethasone and over and above those seen with atorvastatin. Reductions in IL-6 and IL-8 paralleled those seen with atorvastatin alone and were greater than those seen with dexamethasone alone, with IL-6 reaching a high level of significance. It has been suggested in the literature that GCs, with their associated IL-10 upregulation, in combination with IL-4 could achieve a Th-2 directed deviation beneficial in the treatment of autoimmune disease (Miossec *et al* 1996, Röcken *et al* 1996, van Roon *et al* 2002). Certainly the combination of atorvastatin and dexamethasone appeared to give rise to an increase in IL-4 although less than that seen with atorvastatin alone was much greater than the small rise seen with dexamethasone alone. There were also marked increases in IL-5 and IL-10. The upregulation in IL-5 production was surprising as both atorvastatin and dexamethasone on their own led to a decrease in IL-5. The increase in IL-10 was not as great as that seen with dexamethasone on its own. The mainly macrophage-derived pro-inflammatory cytokine, IL-1 β , showed an increase in its expression above baseline with all the drug combinations tested except for atorvastatin, dexamethasone and dexamethasone in combination with atorvastatin

The Potential Immunomodulatory Effects of the Statins in Uveitis

where a moderate decrease in its expression was noted. This reduction was slightly greater for the atorvastatin/dexamethasone combination and reached a level of significance which suggests that atorvastatin may potentiate the effect of dexamethasone on this cytokine.

The combination of atorvastatin, dexamethasone and CsA reduced all the pro-inflammatory cytokines except for IL-8 which exhibited a significant increase. Significant reductions, however, were only observed for IL-2, TNF- α and IFN- γ . The addition of CsA appeared to reduce the effectiveness of the atorvastatin/dexamethasone pairing, especially with regards to IL-6 and IL-8. There was an increase in IL-4 expression but this was not as great as that seen with atorvastatin alone. Interestingly IL-4 expression was greater than that seen with the atorvastatin/CsA and atorvastatin/dexamethasone combinations. IL-5 showed overall a moderate increase but with large variability. IL-10 was significantly raised but the mean value was less than that of the atorvastatin/dexamethasone combination with the CsA possibly detracting from the overall upregulatory effect.

Given the inconsistency of our CsA results when compared to the literature we therefore decided to compare the percentage change in cytokine expression between whole blood treated with CsA at time of stimulation with PMA/ionomycin and whole blood pretreated with CsA for 2 hours before stimulation with PMA/ionomycin. There was a reduction in the expression of all the cytokines assayed. The expression of IL-2 was reduced the most as is consistent with the literature (Ho *et al* 1996). Pretreatment

The Potential Immunomodulatory Effects of the Statins in Uveitis

with CsA led to slightly greater reductions in the pro-inflammatory cytokines IFN- γ , TNF- α and IL-8 when compared to CsA added at time of stimulation. The difference was greatest for IFN- γ . No significant differences, however, were observed between any of the cytokines in the pretreated as compared to the group with treatment at time 0. Also in keeping with the literature, reductions of the Th2 cytokines, IL-4 and IL-5 were present (Härtel *et al* 2003, Moed *et al* 2004). Their reduction did not appear to be any less than that of the Th1 cytokines thereby not favouring a Th2 shift. The documented regulation of the transcription of the cytokine genes for IFN- γ , IL-2, IL-3, IL-4, IL-5 but not IL-10 by NFAT, which is in turn activated by calcineurin is consistent with our results as CsA inhibits calcineurin (Rao 1994). The exception is IL-10 which in our study was also reduced with the reduction greater for pretreatment with CsA. A decrease in IL-10 mRNA expression has also been seen in some subjects in a study on CsA-treated normal human whole blood following anti-CD3/CD28 T cell costimulation (Härtel *et al* 2003). This same study found that the decreased IL-10 mRNA expression correlated with a CsA resistance for IL-2 mRNA expression suggesting that IL-10 may have a regulatory effect on the expression of other cytokines under the influence of CsA which was suggested previously (Rafiq *et al* 1998, Grunig *et al* 1997, Müller-Steinhardt *et al* 2001). We found no inverse correlation between the effects on IL-10 and the effects on IL-2. This may be accounted for by the fact that IL-10 is produced not only by T cells but also by other cells such as activated macrophages. The mainly macrophage-derived pro-inflammatory cytokine, IL-1 β , showed on this occasion a decrease in its expression below baseline however.

The Potential Immunomodulatory Effects of the Statins in Uveitis

The discrepancies in IL-2, IL4 and IL-10 detection between intracellular cytokine staining and microsphere assays could, on a theoretical basis, be accounted for by some time differences between intracellular production and extracellular secretion, especially during the very early phase, when transcription begins, and in the late phase, when synthesis is finished (Schuerwegh *et al* 2003). Optimum time points for detection have previously been reported as 6 hours of stimulation for lymphocytes (Jung *et al* 1993) and 8 hours for monocytes (Schuerwegh *et al* 2001). These were chosen on the basis of previously reported kinetic studies that observed a decrease in cytokine production after 12 hours of incubation with BFA (Mascher *et al* 1999). These time points, however, do vary from laboratory to laboratory. One must also be aware that the percentage of cytokine-producing T lymphocytes does not strictly reflect quantitative amounts of produced cytokine. For example, an increased level of IL-4 detected in plasma supernatants could be the result of an increased capacity of a few lymphocytes to secrete a huge amount of IL-4.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Chapter 7

The Potential Immunomodulatory Effects of the Statins in Uveitis

General Discussion

The Potential Immunomodulatory Effects of the Statins in Uveitis

7. General Discussion

The first hypothesis for this project was that given the emergence of flow cytometric bead-based technology as a means of sampling multiple cytokines within a small fluid sample, we hoped to more fully understand the cytokine profiles displayed within the AH of uveitis patients through the usage, and thereby comparison, of the techniques of ELISA and multiplex bead array assay. Secondly, given the recently discovered anti-inflammatory effects of statins, we decided to investigate their potential use in the treatment of uveitis by comparing their effects with other more well-known immunomodulators through T cell viability and proliferation (CFSE) studies as well as cytokine profiling using intracellular staining techniques and multiplex bead array assays on normal human whole blood samples.

In the initial experimental work, comparing ELISA and CBA, we demonstrated that the usage of this multiplex bead array assay kit to detect six cytokines simultaneously in a small AH volume was, for some cytokines, with greater efficacy than that of ELISA. We were then able to examine the cytokine patterns displayed in the AH of AU and PAN patients as a whole and then subdivide the analyses into AU and PAN patients with or without concomitant steroid usage and with or without associated systemic disease. Individual correlations of cytokine pairings were also able to be generated thereby enabling further previously unavailable in-depth analysis.

The Potential Immunomodulatory Effects of the Statins in Uveitis

First of all we found that levels of the pro-inflammatory cytokines IL-2 and TNF- α in AH of uveitis patients and controls were below the level of detection. This finding was both in keeping and at odds with the literature (Ongkosuwito *et al* 1998, Lacomba *et al* 2000, Lacomba *et al* 2001, Feys *et al* 1994). Generally we felt that these cytokines would be raised under these inflammatory conditions but their absence of detection may have been attributable to differences in sampling time or ongoing cytokine consumption as in the case of IL-2 or may be related to differing assay techniques and uveitis populations as well as the absence or presence of immunomodulators at time of sampling with regards to TNF- α . Certainly, however, the Th1 cytokine, IFN- γ , was found consistently at higher levels in both the uveitis groups than in the control group. This was true whether they were associated with a systemic disorder or not and regardless of steroid use or lack thereof. The increased levels of IFN- γ correlated with decreased levels of IL-4 and IL-5 thus demonstrating IFN- γ -mediated down-regulation of Th2 cytokines and thereby a Th1 shift. Polar differences were greatest in those with sarcoidosis and those with greater uveal tract involvement. In keeping with the literature, there were no statistical differences in IL-10 levels detected in any of the uveitis groups compared with the controls regardless of whether a systemic disease was present. Also concordant were the increased levels of IL-10 production in the panuveitis group taking steroids which reached statistical significance over the control group.

Interestingly in those uveitis entities in which infectious aetiologies are now being suspected, there appeared to be a slight difference in cytokine profiling with more Th2 cytokine expression. Concurrent infections have been reported in patients with AS and

The Potential Immunomodulatory Effects of the Statins in Uveitis

HLA-B27 and in our cohort of such patients, IL-5 expression was relatively increased as compared to the other autoimmune disorders. Interestingly in this group, TNF- α was also expressed at a greater level than the uveitis group as a whole. In FHC, where rubella has recently been identified in the AC as a possible culprit, the ratios of IFN- γ to IL-10 were equal as compared to the idiopathic uveitis group. Further work looking for specific serum and AH antibody titres and cytokine profiles in conjunction with linkage analyses would be useful in delineating the several disease-specific immunopathogenic mechanisms present thereby possibly leading to more tailored immunotherapies.

In the second part of the study the immunomodulatory properties of three statins (atorvastatin, lovastatin and simvastatin) were investigated by comparing them with established immunosuppressants (rapamycin, mycophenolate, CsA and dexamethasone) with regards to lymphocyte viability testing through the use of PI, and proliferation assays through the use of CFSE labeling. All three statins were demonstrated to reversibly inhibit lymphocyte proliferation without significantly affecting viability. Reversibility was achieved with the addition of mevalonate which restored proliferation rates to levels comparable with normal controls. This proved that the immunomodulatory effects of the statins were mediated by the inhibition of HMG-CoA reductase. Repeat titration experiments allowed the optimum statin concentrations for inhibition of lymphocyte proliferation with acceptable rates of viability to be determined. Similar optimum inhibitory concentrations were also obtained for rapamycin, mycophenolate, CsA and dexamethasone. PI enabled ease of viability detection through flow cytometry while CFSE proved an excellent means of tracking cell mitosis through the sequential

The Potential Immunomodulatory Effects of the Statins in Uveitis

loss of fluorescence intensity, as measured by flow cytometry, with each cell cycle progression. This avoided the need for the more expensive and labour-intensive [³H]-thymidine.

These optimum inhibitory concentrations were then used in the third set of experiments in which we compared the effects of the three statins and those of CsA, rapamycin and mycophenolate as well as dexamethasone, on intracellular cytokine staining conducted on whole blood. Combinations of atorvastatin in conjunction with CsA, rapamycin, mycophenolate, dexamethasone and CsA plus dexamethasone were also evaluated for synergistic effects on Th1 and Th2 cytokine production. Due to the nature of intracellular cytokine staining only 4 cytokines were able to be evaluated. There were significant reductions in the Th1 cytokine, IFN- γ , below baseline control expression for each of the statins with simvastatin statistically effecting a slightly more pronounced reduction. This was in contrast to the intracellular cytokine staining work done by Hakamada-Taguchi *et al* (2003) where simvastatin was demonstrated to be the least effective in promoting Th2 polarization through the inhibition of IFN- γ and the upregulation of IL-4 production. All the established immunosuppressants also showed a slightly greater inhibition of IFN- γ production as compared to the statin group as a whole. Dexamethasone appeared to induce the greatest reduction in IFN- γ . Combinations of atorvastatin and the other immunosuppressants did not result in any additive effect in terms of suppression with IFN- γ expression generally at levels either equivalent to or above that would be achieved by each of the individual drugs on their own. Levels of IL-2, IL-4 and IL-10 were not significantly altered above or below control levels and may

The Potential Immunomodulatory Effects of the Statins in Uveitis

have been related to the asynchronicity of cytokine production in these cells or poor binding of these particular antibodies in fixed and permeabilised cells (Openshaw *et al* 1995, Pala *et al* 2000).

In the final part of the study we employed FBI as another multiplex bead array system in an attempt to gauge the effects of the statins in comparison with the other known immunosuppressants on cell surface expression of a greater number of pro-inflammatory cytokines including the Th1- as well as the Th2-type cytokines. Atorvastatin was the only statin which decreased IL-1 β . All the statins, however, did reduce the pro-inflammatory cytokines IL-2 and TNF- α as well as IFN- γ (apart from simvastatin which only reduced IL-2 and IFN- γ) thereby favoring a Th2 phenotypic shift (Figure 7.1). Atorvastatin appeared to be the only statin which also decreased both IL-6 and IL-8. Lovastatin, however, did produce a small but significant decrease in IL-6. Atorvastatin was also the only statin to decrease the Th2 cytokine IL-5 and the Tr cytokine IL-10. The only statin to increase IL-10 production was lovastatin. All the statins, however, did upregulate the production of IL-4 with atorvastatin being the most efficacious and simvastatin the least. On balance simvastatin appeared to be the least effective at decreasing the proinflammatory and Th1 cytokines and increasing the Th2 cytokines. These results parallel more closely the intracellular cytokine staining work with IFN- γ and IL-4 in mice T cells by Hakamada-Taguchi *et al* (2003). Any steroid sparing *in vivo* studies with the statins might therefore benefit from having simvastatin as one of the latter options.

The Potential Immunomodulatory Effects of the Statins in Uveitis

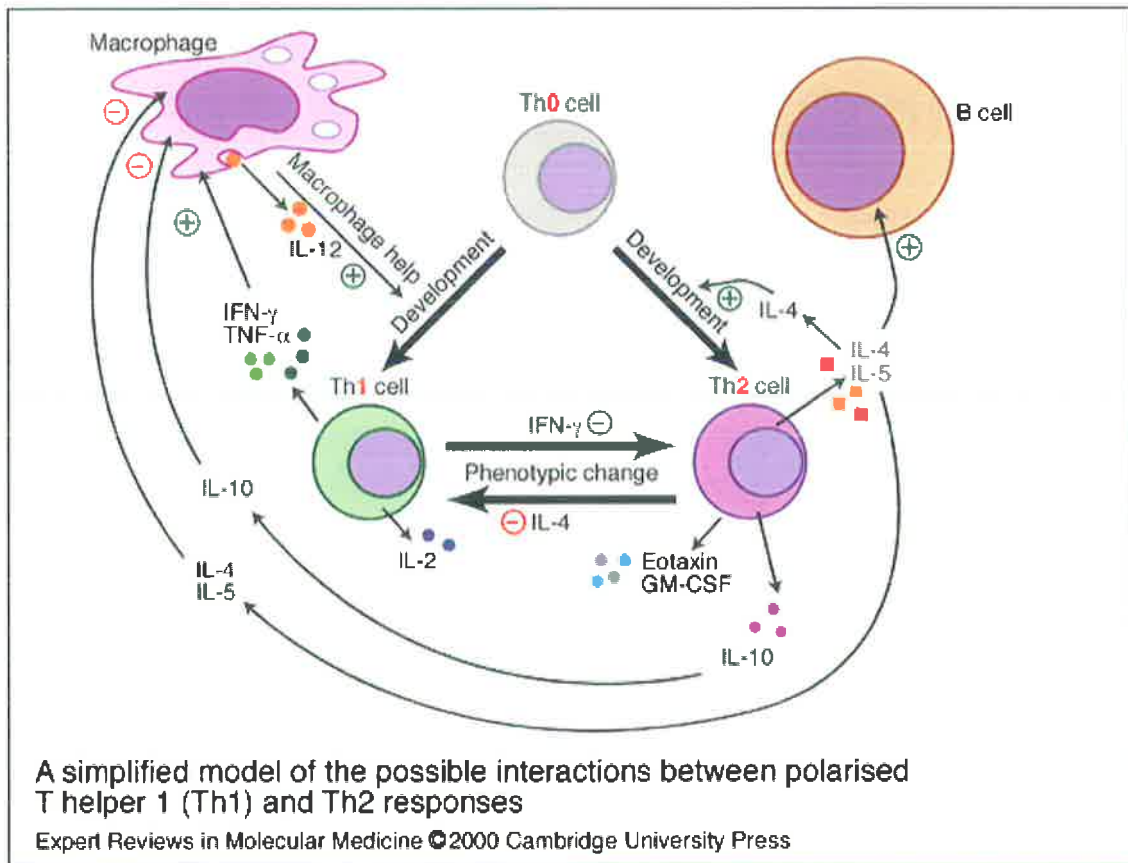


Figure 7.1 A simplified model of the possible interactions between polarised Th1 and Th2 responses. The model shows the cytokines produced by each cell type and how they positively (indicated by a plus sign) and negatively (indicated by a minus sign) regulate each other. It has been proposed that Th1 cells mitigate the autoimmune and alloimmune responses, while Th2 cells promote tolerance. The statins may induce a shift towards a Th2 phenotype through the inhibition of Th1 cytokines such as IFN- γ . Taken and adapted from Harber *et al* (2000)

The Potential Immunomodulatory Effects of the Statins in Uveitis

The cell surface cytokine characterization following rapamycin administration was more in keeping with the literature with respect to the general variability and paucity of cytokine expression. Mycophenolate extracellular cytokine profiling with FBI was also consistent with the literature with regards to a Th1 to Th2 shift in cytokine expression. Initial FBI analysis of CsA effects on cell surface cytokine expression showed inconsistent and overall negligible change in Th1 and Th2 cytokine expression inconsistent with the majority of the literature. A significantly reduced IL-10 was evident which may have had some bearing on the unexpected poor Th1 inhibitory effect. Repeat FBI was performed with a view to assess potential for increased cytokine effect with CsA pretreatment. Interestingly both whole blood supernatants pretreated for 2 hours with CsA and with CsA added at time 0 exhibited significant decreases in IL-1 β , IL-2 and IFN- γ and the Th2 cytokines IL-4, IL-5 and IL-10. No significant differences were noted between the two time points except for a greater reduction in IL-10 with pretreatment. Our results did mirror to some extent those of Härtel *et al* (2003) who also found quite marked differences in CsA effect on cytokine mRNA expression between individual subjects. Dexamethasone exhibited Th1 inhibitory properties consistent with the literature with the reductions in IL-2, IFN- γ and TNF- α being the most powerful of all the immunomodulators tested. It was also the only other immunosuppressant which reduced IL-1 β . Its upregulatory effect on IL-10 was also consistent with previous reports as were the minimal upregulatory effect on IL-4 and the marked inhibitory effect on IL-5.

Combinations of atorvastatin and another immunomodulatory drug resulted in, for the most part, somewhat disappointing non-additive effects with regards to pro-

The Potential Immunomodulatory Effects of the Statins in Uveitis

inflammatory and Th1 cytokine suppression. Generally the addition of another immunomodulator to atorvastatin detracted from the statin's own positive immunomodulatory effects with the exception of the combination of atorvastatin and dexamethasone. This coupling led to an impressive synergistic reduction of the pro-inflammatory and Th1 cytokines, as well as IL-1 β , with concomitant increase of all of the Th2 cytokines. There is little in the literature to suggest any contraindication to this particular drug pairing in terms of side effects and its use *in vivo* would be of interest. Lovastatin, with its upregulatory effect on IL-10, at least in our hands, as compared to the other statins would be of interest to test in conjunction with dexamethasone. Work with other cytokines such as IL-12, IL-13, IL-18, IL-23 and TGF- β would also be of interest.

Topical, periocular, intraocular and systemic corticosteroids have remained the gold standard of primary immunosuppressive therapy in uveitis because of their immediate efficacy. However, because of their increased risk of ocular and systemic side effects, independent of the administration route, with long term treatment (>3 months), steroid-sparing drugs should be added to the treatment regimen and should ideally even replace the use of corticosteroids or allow the use of corticosteroids below the Cushing-threshold (<10mg prednisone equivalent per day). Unfortunately the current available steroid-sparers themselves are associated with significant adverse effects and as such, form a major limitation to successful long-term treatment (Becker *et al* 2005). Cost is also an issue with mycophenolate and CsA for example costing approximately 14 and 23 euros each per day for maximal standard treatment. Monoclonal antibody treatments such as etanercept cost approximately 64 euros per day while infliximab costs at 144 euros

The Potential Immunomodulatory Effects of the Statins in Uveitis

per day for a period of 6 weeks with three infusions. At 9100 euros per cycle (5 days), intravenous immunoglobulin is the most costly (Becker *et al* 2005). Monoclonal antibody treatments themselves have side-effects with recurrences of tuberculosis associated with anti-TNF- α therapy; hypercholesterolemia associated with anti-IL-6 receptor antibodies and IL-1 receptor antagonists associated with injection site effects and a small decrease in neutrophil and platelet counts (Keane *et al* 2001, Nishimoto 2003, Fleishmann 2002).

The ideal steroid-sparer, therefore, would be of sufficient immunosuppressive strength while being both relatively cost-effective and free of side effects. Of great importance also are the ease and mode of drug delivery with ideal treatment being well-tolerated orally. Statins, in addition to being comparatively inexpensive, are given orally and are generally well tolerated (Baker *et al* 2003). Even in one study where pravastatin was used to manage familial hypercholesterolemia in children, no detectable effects upon growth, muscle or liver enzymes or endocrine function were documented (Wiegman *et al* 2004). However some reported side effects of statins include exercise intolerance, myalgia and myoglobinuria which are thought to be due to statin-induced inhibition of co-enzyme Q10 (CoQ10; Rundek *et al* 2004) which may be managed by dietary intervention or CoQ10 supplements (Pettit *et al* 2003).

Serum cytokine profiles in the uveitides have been observed to broadly parallel AH cytokine profiles in terms of a general increase in Th1 cytokine production with concomitant decrease in Th2 cytokine expression documented in some cases. Certainly our CBA data on a wide range of uveitides supports this phenomenon except in those

The Potential Immunomodulatory Effects of the Statins in Uveitis

with a suspected infectious aetiology. The cumulative viability, proliferation, intracellular and FBI work with the statins in comparison with the more accepted immunomodulators has shown that the statins, at least in normal human *in vitro* situations, can effect lymphocyte proliferation and decrease Th1 cytokine production without a significant loss in viability. Tested *in vitro* statin concentrations were chosen according to serum levels achieved with maximum allowable *in vivo* dosages. Further work with the statins on peripheral blood and AH samples of uveitis patients would appear to be warranted.

The concept of the statins being co-administered with corticosteroid therapy is also of interest and the combination of atorvastatin and dexamethasone in particular led to a synergistic effect on the inhibition of Th1 cytokines with an accompanying upregulation of Th2 cytokines. *In vivo* trials of this combination and atorvastatin on its own as a steroid-sparing agent would be of great interest. Further work with lovastatin on its own and in combination with other immunomodulators, in light of its possibly drug-specific potential upregulatory effect on IL-10 as compared to the other statins, would also be of interest. Statins therefore should be considered as a potential addition to the armamentarium of therapies to combat uveitis, and if steroid-sparing, would be an attractive option. This data constitutes the first known report of the combined use of proliferation studies with CFSE, intracellular cytokine staining and the multiplexed bead array assay to investigate the immunomodulatory properties of the statins and provides evidence for the potential of statins in the treatment of uveitis as a steroid-sparing monotherapy or as an element of combination immunotherapy.

The Potential Immunomodulatory Effects of the Statins in Uveitis

Chapter 8

The Potential Immunomodulatory Effects of the Statins in Uveitis

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The Potential Immunomodulatory Effects of the Statins in Uveitis

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