

**The role of complement and
complement regulatory proteins in
the progression of atherosclerosis**

PhD Thesis

Ruth D Lewis

UMI Number: U567228

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U567228

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Declaration and Statements

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed W.A.R. (candidate) Date 6.10.11

STATEMENT 1

This thesis is being submitted in partial fulfillment of the requirements for the degree of PhD

Signed W.A.R. (candidate) Date 6.10.11

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated.

Other sources are acknowledged by explicit references.

Signed W.A.R. (candidate) Date 6.10.11

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed W.A.R. (candidate) Date 6.10.11

STATEMENT 4: PREVIOUSLY APPROVED BAR ON ACCESS

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans **after expiry of a bar on access previously approved by the Graduate Development Committee.**

Signed (candidate) Date

Acknowledgments

I would like to thank both my supervisors, Professor B Paul Morgan and Dr Timothy Hughes, for their help and guidance throughout this thesis. The support and encouragement they have provided me with has been invaluable.

I thank my collaborator, Dr Christopher Jackson, for all his technical help and expertise during the project. I would also like to thank Dr Mark Perry for assisting with the DEXA measurements, Dr Irina Guschina for helping with the NEFA analysis, Dr James Neal for his help with the immunohistochemistry and the staff at Clinical Biochemistry Laboratories, Cardiff University Hospital for lipid analysis. To all my colleagues working on the 3rd floor of the Henry Welcome building and the Complement Biology Group that have provided help during this thesis, I am forever grateful. I would particularly like to thank Dr Claire Harris, Dr Valeria Ramaglia, Dr Marietta Ruseva, Dr Svetlana Hakobyan, Dr Meike Heurich, Dr Danielle Paixao-Cavalcante, Dr James Hindley, Dr Rhodri Turner and Dr Natalie Hepburn for all the help and advice you gave during my thesis.

To my father and mother, thank you for all your support within and outside the realms of this thesis. Thank you also to my father and mother in law for the support you have provided me with.

Finally I dedicate this thesis to my beloved husband, Philip, and my two little angels, Amelie and Zoe, for you are my everything.

Contents

Abstract	14
Publication list	15
List of abbreviations	16
Company address list	20
Chapter 1: Introduction	21
1. Atherosclerosis	21
1.1 An overview	21
1.2 A historic perspective	22
1.3 Risk factors for atherogenesis	22
1.4 Atherosclerotic lesion initiation	23
<i>1.4.1 Theory for atherosclerotic lesion initiation</i>	23
<i>1.4.2 Healthy arterial wall</i>	23
<i>1.4.3 Endothelial dysfunction</i>	24
<i>1.4.4 Intimal thickening</i>	25
1.5 Development of the fatty streak	27
<i>1.5.1 The role of innate immunity during fatty streak formation</i>	27
<i>1.5.2 Adaptive response of the immune system during fatty streak formation</i>	29
1.6 Progression towards a complex plaque	31
<i>1.6.1 Vascular smooth muscle cells (SMCs)</i>	31

1.6.2	<i>Macrophages</i>	31
1.6.3	<i>T-cells</i>	32
1.6.4	<i>Other inflammatory cells involved</i>	34
1.6.5	<i>Cellular necrosis and breakdown</i>	36
1.7	Plaque rupture and thrombosis	37
1.8	Animal models of atherosclerosis	39
1.9	Assessment of atherosclerosis in mice	43
1.9.1	<i>Quantitative assessment of atherosclerosis in mice</i>	43
1.9.2	<i>Qualitative assessment of atherosclerosis in mice</i>	44
1.10	Therapeutic perspectives	47
1.10.1	<i>Surgery</i>	47
1.10.2	<i>Statins</i>	47
1.10.3	<i>Anti-inflammatory drugs</i>	47
1.10.4	<i>Inflammatory biomarkers</i>	48
1.10.5	<i>Summary</i>	49
2.	The Complement system	50
2.1	Overview	50
2.2	Activation	51
2.2.1	<i>Classical pathway</i>	51
2.2.2	<i>Alternative pathway</i>	51
2.2.3	<i>MBL pathway</i>	52
2.2.4	<i>Other mechanisms of complement activation</i>	52
2.2.5	<i>Complement activation products</i>	52
2.3	Central component of the activation pathway	52
2.4	Terminal pathway	53

2.5 Function	54
2.6 Regulation	55
2.7 Complement receptors	57
2.8 Pathological role	58
3. The role of Complement in atherosclerosis	60
3.1 Initiation of complement activation during atherosclerosis	60
3.2 Effects of activation pathways of complement during atherosclerosis	62
3.3 Role of the terminal pathway	64
3.3.1 Localisation of MAC in the atherosclerotic plaque	64
3.3.2 MAC and endothelial cells	65
3.3.3 MAC and SMCs	66
3.3.4 MAC and macrophages	67
3.4 Complement regulation and atherosclerosis	67
3.4.1 Fluid phase regulators	67
3.4.2 Membrane bound regulators	69
3.5 Complement in animal models of atherosclerosis	71
3.6 Autoimmune disease and atherosclerosis	73
3.6.1 SLE and atherosclerosis	73
3.6.2 RA and atherosclerosis	74
4. Lipid Metabolism	75
4.1 Introduction	75
4.2 Lipoprotein structure and function	75
4.2.1 Lipoprotein structure	75
4.2.2 Function of lipids and lipoproteins	75

4.3 Lipid metabolism: an overview	76
4.3.1 Exogenous pathway	76
4.3.2 Endogenous pathway	78
4.4 The role of C3adesArg in lipid metabolism	80
4.5 Lipid metabolism alteration in atherosclerosis	84
4.6 Lipid metabolic disorders associated with the complement system	84
4.6.1 Obesity	85
4.6.2 Acquired partial lipodystrophy (PLD)	86
5. Summary and hypothesis	87
Chapter 2: Materials & Methods	91
1. Buffers and reagents	91
2. Mice	91
3. Induction of atherosclerosis	93
3.1 Experimental procedure	93
3.2 Termination procedure	93
4. Assessment of atherosclerosis	95
4.1 Antibodies	96
4.2 Immunohistochemistry	96
4.3 Fluorescence immunohistochemistry	96
4.3.1 Total C3 and C9/MAC immunostaining within the plaque using a fluorescent detection antibody	96
4.3.2 Plaque macrophage and smooth muscle-actin fluorescent staining	99

4.4 Histology	99
4.4.1 <i>Plaque lipid content</i>	99
4.4.2 <i>Elastin and collagen</i>	100
4.4.3 <i>Morphometric analysis</i>	100
5. Assessment of adiposity, plasma lipids and metabolites	101
5.1 Serum triglyceride, cholesterol and serum turbidity measurements	101
5.2 NEFA concentrations	102
5.3 Glucose levels	104
5.4 Measurement of C3adesArg by ELISA	104
5.5 In vivo quantification of fat content in mice using dual X-ray absorptiometry (DEXA)	106
6. Functional analysis of Complement activity	106
6.1 Production of mouse anti-rabbit erythrocyte antibody	106
6.2 Preparation of sensitised rabbit erythrocytes	107
6.3 Titre of mouse serum	108
6.4 CH50 assay	109
7. Protein analysis	110
7.1 Protein concentration	110
7.2 SDS-PAGE analysis	110
7.3 Coomassie staining	111
8. Statistical analysis	112
Chapter 3: Generation of genetically modified animals	113

1. Introduction	113
2. Specific methods	117
2.1 Screening of apoE, CD55 and CD59a deficiency	117
2.1.1 DNA isolation	117
2.1.2 PCR	118
2.1.3 Agarose gel electrophoresis	118
2.2 Screening of C6 deficiency	122
3. Results	123
3.1 Generation of apoE ^{-/-} /Cd59a ^{-/-} mice and their litter-matched apoE ^{-/-} /Cd59a ^{+/+} controls	123
3.2 Generation of apoE ^{-/-} /CD55 ^{-/-} mice and the apoE ^{-/-} controls	123
3.3 Generation of apoE ^{-/-} /C6 ^{-/-} mice and apoE ^{-/-} /C6 ^{+/+} controls	126
4. Discussion	131
Chapter 4: Role of the terminal pathway during atherosclerosis	134
1. Introduction	134
2. Unregulated MAC during atherosclerosis	137
2.1 A brief introduction	137
2.2 Specific methods	138
2.2.1 Histochemical staining of CD3+T-cells in atherosclerotic lesions	138
2.2.2 Macrophage immunostaining in atherosclerosis	138
2.2.3 SMC immunostaining in atherosclerosis	139

2.2.4 Immunostaining for complement regulators Crry, CD55 and CD59a	140
2.2.5 Nuclear staining using Mayer's haematoxylin	141
2.3 Results	141
2.3.1 Pathological observations	141
2.3.2 Plaque area is increased in apoE ^{-/-} /Cd59a ^{-/-} mice	142
2.3.3 Plaque lipid content	146
2.3.4 CD59a deficiency causes increased deposition of MAC in plaques	147
2.3.5 Infiltrating inflammatory cells in the atherosclerotic plaque	147
2.3.6 The absence of Cd59a is not compensated by up-regulation of other complement regulators	151
2.3.7 In the absence of Cd59a the MAC influences SMC proliferation and survival in atherosclerosis	151
2.3.8 Co-localisation of MAC on SMCs in an atherosclerotic artery of an apoE ^{-/-} mouse	153
2.3.9 Co-localisation of MAC with macrophages within the plaque	154
3. The role of complement C6 in atherosclerosis	158
3.1 A brief introduction	158
3.2 Results	159
3.2.1 Deficiency of C6 inhibits atherosclerotic plaque progression in apoE ^{-/-} mice	159
3.2.2 Terminal pathway activation is abolished in atherosclerotic plaques from apoE ^{-/-} /C6 ^{-/-} mice	163
3.2.3 Altered lipid metabolism in apoE ^{-/-} /C6 ^{-/-} mice	163

4. Complement therapy for the treatment of atherosclerosis	166
4.1 A brief introduction	166
4.2. Specific methods	167
4.2.1 BB5.1 Purification	167
4.2.1.1 <i>Antibody preparation</i>	167
4.2.1.2 <i>Purification of BB5.1 using Protein G affinity chromatography</i>	168
4.2.2 Mouse Ig purification	169
4.2.2.1 <i>Preparation of mouse Ig using Protein G affinity chromatography</i>	169
4.2.2.2 <i>In vitro characterisation of BB5.1 and mouse Ig</i>	169
4.2.2.3 <i>In vivo characterisation of BB5.1 and mouse Ig</i>	169
4.2.3 Experimental protocol	170
4.3 Results	171
4.3.1 Purification of anti-mouse C5 mAb and mouse Ig	171
4.3.2 BB5.1 inhibits complement haemolysis in mouse serum in vitro	172
4.3.3 Pilot experiment to determine function of purified BB5.1 on complement haemolytic activity in vivo	173
4.3.4 Treatment with C5 mAb inhibits complement activation in high fat fed apoE ^{-/-} mice	175
4.3.5 Treatment with anti-mouse C5 mAb has no effect on body weight, cardiac hypertrophy or lipid levels in apoE ^{-/-} mice	177
4.3.6 Complement inhibition at the level of C5 does not protect apoE ^{-/-} mice against atherosclerosis	178
4.3.7 Administration of BB5.1 anti-C5 mAb reduces terminal MAC deposition without affecting C3 deposition in atherosclerotic plaques	178

5. Discussion	183
---------------	-----

Chapter 5: The effect of CD55 deficiency on atherosclerosis in apoE

deficient mice 190

1. Introduction	190
-----------------	-----

2. Specific methods	193
---------------------	-----

2.1 Mean plaque area measurement	193
----------------------------------	-----

2.2 Activated C3 immunostaining	193
---------------------------------	-----

3. Results	194
------------	-----

3.1 Pathological observations	194
-------------------------------	-----

3.2 CD55 deficiency slows the progression of atherosclerosis	196
--	-----

3.3 Infiltrating SMCs and macrophage	196
--------------------------------------	-----

3.4 Complement C3 activation and MAC deposition correlate with severity of disease	199
---	-----

3.5 CD55 deficiency causes altered triglyceride profile and total serum cholesterol levels in a cholesterol rich environment	201
---	-----

3.6 CD55 deficiency is associated with increased C3 turnover and plasma C3adesArg levels	202
---	-----

3.7 CD55 regulates C3adesArg causing enhanced NEFA clearance	203
--	-----

3.8 CD55 deficiency is associated with increased body fat	206
---	-----

4. Discussion	209
---------------	-----

Chapter 6: Discussion and Conclusions 214

1. Summary of main findings	214
1.1 Studies on the Terminal Pathway and Atherosclerosis	214
1.2 CD59a is strongly anti-atherogenic	215
1.3 MAC deposition within the plaque is strongly pro-atherogenic	215
1.4 Therapeutic blockade of the terminal pathway	215
1.5 Regulation of C3 activation	216
2. Issues raised by thesis findings, and future directions	218
2.1 C6 and lipid metabolism	218
2.2 The use of apoE mouse model in studying the role of complement in atherosclerosis	220
2.3 Activation of C3 during lipid metabolism is regulated by CD55	221
2.4 Genetic factors affecting the complement system and knock on effects on obesity and cardiovascular disease	225
2.5 Targeting the terminal pathway as a therapy for atherosclerosis	228
2.6 Therapeutic targeting of C3	229
3. Conclusion	230
References	232
Appendices	293
Papers:	Molecular Immunology (primary research) American Journal of Pathology (primary research)
Abstracts and posters:	Cardiff University Postgraduate Research Day 2006 11 th European Complement Meeting 2007 i3-IRG Annual Summer Meeting 2009 XXIII International Complement Workshop 2010

Abstract

It is now accepted that atherosclerosis is an inflammatory disease involving both the innate and adaptive immune responses. Components of the complement system, a central component of the innate arm, have been found in the milieu of human coronary plaques and its exact role during the course of the disease is currently being investigated. Whilst evidence from human studies suggests that complement activation is pro-atherogenic, studies using animal models of the disease, including the low density receptor deficient ($ldlr^{-/-}$) and apolipoprotein E deficient ($apoE^{-/-}$) mouse models, contradict one another. The hypothesis underpinning this thesis is that the complement system contributes to disease pathology in atherosclerotic plaques of $apoE^{-/-}$ mice. The work focussed on the membrane attack complex (MAC) of the terminal pathway and the central component of the complement system, C3. I have shown that in the absence of the MAC regulator CD59a, $apoE^{-/-}$ mice had accelerated atherosclerosis compared to controls, accompanied by increased MAC activation within the plaques. In accordance, C6 deficiency was protective against atherosclerosis in $apoE^{-/-}$ mice, a result of absence of MAC in these mice. However, MAC inhibition using an anti-C5 antibody in $apoE^{-/-}$ mice did not inhibit progression of atherosclerosis. Surprisingly, in the absence of CD55, $apoE^{-/-}$ mice had smaller atherosclerotic lesions together with an anti-atherogenic lipoprotein profile and increased C3 activation product, C3adesArg, in their plasma. The data reveal a novel role for CD55 during lipid metabolism and, together with published data on the metabolic role of C3adesArg, highlight the need for further investigations into the role of complement during lipid metabolism.

Publication list

Lewis RD, Perry MJ, Guschina IA, Jackson CL, Morgan BP, Hughes TR

CD55 Deficiency Protects against Atherosclerosis in ApoE-Deficient Mice via C3a Modulation of Lipid Metabolism.
Am J Pathol. 2011 Aug 2

Lewis RD, Jackson CL, Morgan BP and Hughes TR

The membrane attack complex of complement drives the progression of atherosclerosis in apolipoprotein E knockout mice.
Mol Immunol. 2010 Feb;47(5):1098-105

Lewis RD, Jackson CL, Morgan BP and Hughes TR

A role for CD59a in the progression of atherosclerosis: The first direct evidence from an in vivo study.
Mol Immunol. 2007; 44(16):3939-3939

Coles B, Lewis R, Anning PB, Morton J, Baalasubramanian S, Morgan BP, O'Donnell VB

CD59 or C3 are not required for angiotensin II-dependent hypertension or hypertrophy in mice.
Immunology. 2007 Aug;121(4):518-25

Sivasankar B, Donev RM, Longhi MP, Hughes TR, Davies RD, Cole DS, Morgan BP, Marchbank KJ

CD59a deficient mice display reduced B cell activity and antibody production in response to T-dependent antigens.
Mol Immunol. 2007 Apr;44(11):2978-87

Danetz JS, Davies RD, Clemo HF, Baumgarten CM

Rabbit ventricular myocyte volume changes as a direct result of crystalloid cardioplegia in congestive heart failure induced by aortic regurgitation.
J Thorac Cardiovasc Surg. 2000 Apr;119(4 Pt 1):826-33

Danetz JS, Clemo HF, Davies RD, Embrey RP, Damiano RJ Jr, Baumgarten CM

Age-related effects of St Thomas' Hospital cardioplegic solution on isolated cardiomyocyte cell volume.
J Thorac Cardiovasc Surg. 1999 Sep;118(3):467-76

List of abbreviations

ApoE	Apolipoprotein E
ASP	Acylation stimulating protein
bp	Base Pairs
BSA	Bovine Serum Albumin
C3Nef	C3 nephritic factor
CCR#	chemokine receptor number #
CCL#	Chemokine ligand number #
CXCR#	C-X-C chemokine receptor type number #
CFD	Complement fixation diluent
CH50	Concentration of serum to cause 50% lysis of target cells
CRig	Complement receptor of the immunoglobulin superfamily
CRP	C-reactive protein
Crry	Complement receptor 1 related protein
DAB	Diaminobenzidine
DAF	Decayaccelerating factor
DEXA	Dual-energy X-ray absorpitometry
DNA	Deoxyribonucleic acid
dNTPs	Deoxy-nucleotide-tri-phosphates
E	Erythrocytes
EDTA	Ethylene diamine tetra-acetic acid
EVG	Miller's Elastin Van Gieson
fB	Factor B

FCHL	Familial combined hyperlipidemia
fD	Factor D
fH	Factor H
fI	Factor I
g	Grams
GPI	Glycosyl-phosphatidylinositol
HCl	Hydrochloric acid
HDL	High density lipoprotein
HRP	Horseradish peroxidase
HSP60	Heat shock protein 60
H ₂ O ₂	Hydrogen peroxide
ICAM-1	Intracellular adhesion molecule-1
IDL	Intermediate density lipoprotein
IEL	Inner elastic lamina
IFN- γ	Interferon- γ
IP#	interferon γ -induced protein number #
Ig	Immunoglobulin
IL-#	Interleukin number #
i.p	Intraperitoneal
LDL	Low density lipoprotein
Ldlr	Low density lipoprotein receptor
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAC	Membrane Attack Complex

MAPK	Mitogen activated protein kinase
MBL	Mannan-binding lectin
MCP	Membrane cofactor protein
MCP-1	Macrophage chemoattractant protein-1
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MIP-#	Macrophage inflammatory protein number #
MMPs	Metalloproteinases
mm LDL	Minimally modified LDL
NaCl	Sodium Chloride
NEO	Neomycin
NEFA	Non-esterified fatty acids
NK	Natural Killer
NF-κB	Nuclear Factor kappa B
NO	Nitric Oxide
NOS	Nitric oxide synthase
OEL	Outer elastic lamina
PAI-1	Plasminogen activator inhibitor 1
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide 3-kinase
PLA₂	Phospholipase A₂
PLD	Partial lipodystrophy
PRRs	Pattern recognition receptors

RA	Rheumatoid arthritis
rbE	Rabbit erythrocytes
rbE-A	Sensitised rabbit erythrocytes
rpm	Revolutions per minute
ROS	Reactive oxygen species
s.c	Subcutaneous
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
SMC	Smooth muscle cell
SR-A	Scavenger receptor A
TAE	Tris acetate EDTA
TBS	Tris buffered saline
TCR	T-cell receptor
TE	Tris-EDTA
TGF β	Transforming growth factor β
TLR#	Toll like receptor number #
Tris	tris (hydroxymethyl) aminomethane
VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very Low Density Lipoprotein
vWF	Von Willebrand factor
v/v	Volume to volume
w/v	Weight to volume
-/-	Deficient
+/+	Sufficient

Company address list

Abcam plc, Cambridge, UK

BD Pharmingen, San Diego, California, USA

Bioline Ltd, UK

Bio-Rad laboratories, Richmond, California, USA

BMG Labtech, Aylesbury, UK

CompTech, Texas, USA

Dako UK Ltd, Cambridgeshire, UK

Fisher Scientific, Loughborough, UK

GE Lunar Corporation, Madison, WI

Gene Tex Inc. California, USA

Hy-Cult Biotechnology, The Netherlands

Invitrogen Life Technologies, Carlsbad, California, USA

Jackson laboratories Inc, West Grove, Pennsylvania, USA

Lunar Corp., Madison, Wisconsin, USA

Media Cybernetics, Carlsbad, California, USA

Menarini Diagnostics, Berkshire, UK

Millipore, Watford, UK

National Diagnostics Ltd, Yorkshire, UK

New England Biolabs Inc, Beverly, Massachusetts, USA

Oxoid Limited, Basingstoke, Hampshire, UK

Santa Cruz Biotechnology Inc., California, USA

Serotec, Oxford, UK

Sigma Aldrich, Poole, Dorset, UK

Special Diet Services, Witham, UK

Vector laboratories, Peterborough, UK

Chapter 1: Introduction

1. Atherosclerosis

1.1 An overview

Atherosclerosis is the leading cause of death in the western world. Researchers in the field have been studying the pathology of atherosclerotic lesions for more than two centuries, charting the course of the disease and the factors affecting its progression from early lesions through to advanced, complex plaque formation which often results in myocardial infarction or stroke and death (Glass and Witztum, 2001). With the proven involvement of numerous inflammatory cells and an array of pro-inflammatory signalling molecules, atherosclerosis is now accepted as an inflammatory disease (Galkina and Ley, 2009; Hansson et al., 2002; Packard et al., 2009; Ross, 1999). Discovery of biomarkers in the disease have helped to identify the individuals at risk, whilst also helping in discovering therapeutic drugs in the clinic (Koenig and Khuseyinova, 2007; Ridker, 2007; White et al., 2010). Nevertheless, despite all efforts, the perfect drug to cure the disease is yet to be discovered. However, risk factors of atherosclerosis are being determined through epidemiological studies, highlighting both genetic and environmental risk factors involved in initiating the disease (Lusis et al., 2004a; Lusis et al., 2004b).

1.2 A historic perspective

The term “atheroma” was first proposed by Albrecht von Haller in 1755 to describe the degenerative process observed in the intima of arteries in the elderly. Later, in 1815, London surgeon Joseph Hodgson published his work on “Disease of Arteries and Veins”, and proposed that the underlying cause of atheromatous arteries was inflammation. Soon after, this view was discarded and most pathologists of the 19th century believed that atherosclerosis was a degenerative process, involving proliferation of connective tissue and calcification within the intima. This process was described by the term arteriosclerosis proposed by French pathologist Jean Lobstein in 1833.

However, German pathologist Rudolf Virchow continued to consider atheroma as a chronic inflammatory disease of the intima, that he called "chronic endarteritis deformans" (Virchow, 1856). In his opinion, the accumulation of lipids was a late manifestation of atheroma (Ratnoff et al., 1969; Sim et al., 1979). Finally in 1904, Felix Marchand introduced the term “atherosclerosis”, which since has been widely adopted and replaced arteriosclerosis, he suggested that atherosclerosis was responsible for almost all obstructive processes in the arteries (Marchand, 1904).

1.3 Risk factors for atherogenesis

Fatty streaks form in early childhood, never cause symptoms and present no direct threat to life. These early lesions either disappear with time or progress and develop into complex atherosclerotic plaques (Stary et al., 1992). Epidemiological studies have shown that the latter process is under the influence of various genetic and environmental risk factors, which can trigger further changes within the early fatty

streak lesion resulting in the formation of an advanced atherosclerotic plaque. Risk factors for atherosclerosis with a significant genetic component include hypertension, disorders of lipid metabolism and diabetes mellitus (Lusis et al., 2004b). In addition smoking, diet and exercise are examples of environmental risk factors that contribute to the development of the disease (Lusis et al., 2004a).

1.4 Atherosclerotic lesion initiation

1.4.1 Theory for atherosclerotic lesion initiation

The “response to injury” hypothesis, proposed by Russel Ross, is the most widely accepted of various theories for the initiation of atherosclerosis to date (Ross et al., 1977). This hypothesis was the first to describe endothelial denudation as an initiating event in atherosclerosis. In the 1990’s this hypothesis was updated and endothelial dysfunction is now widely regarded to be the initial step of atherosclerosis (Ross, 1993).

1.4.2 Healthy arterial wall

The vascular wall is comprised of three layers; intima, media and adventitia. The intima is the innermost layer of the artery wall, which is separated from the vascular lumen and blood components by only one layer of endothelial cells. The endothelial layer resides at the critical interface between the blood flow and the artery wall and functions by maintaining haemostatic functions. These include haemocompatibility, anti-inflammatory actions and regulation of vascular tone through the production of NO, prostacyclin and endothelin-1 (Libby et al., 2006). The intima of an artery is comprised of a proteoglycan-rich layer and a deeper musculoelastic layer and a continuous inner elastic lamina (IEL) separates the intima from the media. The latter consists of elastic

tissue and smooth muscle cells (SMCs) which are tightly arranged in a uniform manner. The outer elastic lamina (OEL) creates a boundary between the media and adventitia. Unlike the media, the adventitia consists mainly of fibroblasts, not SMCs.

Even though a healthy artery wall possesses numerous protective mechanisms, it can asymptotically thicken in response to small physiological changes, including disturbance of laminar shear stress. This process of intimal thickening can begin early in life (Stary et al., 1992).

1.4.3 Endothelial dysfunction

Atherosclerotic lesions are prone to develop at specific sites in the vascular tree. These sites tend to lie at bifurcations of the arteries where blood flow is turbulent and the effects of undisturbed laminar flow are lost.

Endothelial dysfunction triggers an inflammatory response causing infiltration of leukocytes, in particular monocytes and lymphocytes, into the subendothelial space, initiating the development of atherosclerosis. Recruitment of leukocytes into the vessel wall relies upon endothelial-leukocyte interactions which are mediated by the production of chemokines and adhesion molecules within the vessel wall. These factors are critical in atherogenesis since genetic deletion of endothelial adhesion molecules, leukocyte integrins or chemokines and their receptors result in attenuation of atherosclerosis in animal models (Boring et al., 1998; Cybulsky et al., 2001; Dansky et al., 2001; Dong et al., 2000; Dong et al., 1998; Gu et al., 1998; Shih et al., 1999; Veillard et al., 2005).

Leukocyte attachment to the endothelium of rabbit vessel wall occurs shortly after the introduction of an atherogenic diet (Poole and Florey, 1958). Initially, leukocytes bind to endothelial P- and E- selectins which results in the tethering and rolling of the cells along the vessel wall (Dong et al., 1998). Subsequently, the attachment to the endothelium of the rolling leukocyte becomes much more firm. A process which depends on the activation of leukocytes by chemoattractants, released from the tissue followed by binding of leukocyte integrins. The leukocyte integrins very late antigen-4 and lymphocyte function-associated antigen-1 bind to their endothelial counterparts, vascular adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) respectively. In the context of atherosclerosis, both VCAM-1 and ICAM-1, have been shown to be expressed on endothelial cells located in atherosclerotic prone regions of the vasculature (Cybulsky and Gimbrone, 1991; Nakashima et al., 1998). In addition, VCAM-1 and ICAM-1 are strongly expressed in the endothelial layer overlying an atherosclerotic plaque and weakly in the endothelium overlying the non-inflamed arterial wall (Davies et al., 1993a; Nakashima et al., 1998). Finally, in vitro studies have shown that oxidized low density lipoprotein (oxidised LDL) in the sub-endothelial space and haemodynamic stress can trigger the expression of adhesion molecules (Berliner et al., 1990; Libby et al., 2006; Vora et al., 1997; Walpola et al., 1995).

1.4.4 Intimal thickening

Once firmly attached to the vascular wall, leukocytes receive chemoattractant signals allowing penetration into the intima. For example, macrophage chemoattractant protein-1 (MCP-1) produced by the vascular wall directs the migration and diapedesis of adherent monocytes by binding to its receptor, chemokine receptor 2 (CCR2)

(Boring et al., 1998; Gu et al., 1998). Other chemokines are involved in this process namely, interleukin (IL)-8, eotaxin and fractalkine (Boisvert et al., 2000; Haley et al., 2000; Lesnik et al., 2003). In addition, matrix metalloproteinase 9 (MMP-9), which is secreted by monocytes, degrades the collagen within the extracellular matrix, allowing movement into the intima (Amorino and Hoover, 1998).

Once leukocytes are firmly attached to the endothelium, leukocytes transmigrate through the endothelial lining into the intima a process which involves numerous chemotactic molecules. These include chemokines, anaphylatoxins C3a and C5a and modified lipoproteins. Chemokines exert their biological effects by interacting with their receptors that are selectively found on the surfaces of their target cells. A wide repertoire of chemokines (for example, monocyte chemoattractant protein-1 (MCP-1), chemokine ligand 5 (CCL5), macrophage inflammatory protein (MIP)-1 α , MIP-1 β , growth regulated oncogene (GRO)- α , IL-8, interferon γ -induced protein (IP)-10, fractalkine, eotaxin) and their respective receptors (chemokine receptor (CCR)2, CCR3, C-X-C chemokine receptor type (CXCR)2, CX3CR1) have been shown to be involved in atherosclerosis (Hristov and Weber, 2009; Sheikine and Hansson, 2004). In addition, circulating platelets that interact with activated endothelial cells can release chemokines resulting in increased leukocyte recruitment onto the inflamed or atherosclerotic endothelium (von Hundelshausen et al., 2001). Furthermore, studies have shown a link between chemokine expression and increased atherosclerotic stimuli such as minimally modified low density lipoprotein (Miller et al., 2005), complement activation products (Torzewski et al., 1996) and alterations in shear stress (Shyy et al., 1994).

1.5 Development of the fatty streak

Fatty streaks are early lesions of atherosclerosis, which consist mainly of fat-filled macrophages known as foam cells. Fatty streaks are clinically silent; however, they have the potential to progress into more advanced lesions of atherosclerosis.

1.5.1 The role of innate immunity during fatty streak formation

Cellular and soluble components of innate immunity provide a fast but blunt response and serve as the first line of defence against infectious agents. Cells of the innate system respond to pathogens via pattern recognition receptors (PRRs). These receptors are able to recognise a restricted pattern of ligands called pathogen-associated molecular patterns (PAMPs). PRR are mainly expressed on macrophages and dendritic cells. Other components of innate immunity include the complement system, acute phase proteins, and cytokines.

Activated endothelial cells produce pro-inflammatory molecules such as macrophage colony-stimulating factor (M-CSF). This particular cytokine plays a critical role within the intima during fatty streak development, where it promotes proliferation and differentiation of monocytes into macrophages. The critical role of this pro-inflammatory molecule during atherosclerosis has been supported by a study showing that mice lacking M-CSF, on an atherosclerotic prone background (either apolipoprotein E (apoE) or low density lipoprotein receptor (ldlr) deficiency) show attenuation of plaque development with reduced macrophage accumulation when compared to their litter-matched controls (Rajavashisth et al., 1998; Smith et al., 1995).

M-CSF also has an influence on the expression of PRRs involved in innate immunity. Members of the PRR family include scavenger receptors, toll like receptors (TLRs),

pentraxins and natural antibodies. M-CSF induced expression of scavenger receptors, scavenger receptor A (SR-A) and CD36 on macrophages has been shown to be involved in the uptake of modified lipoproteins and hence, the transformation of macrophages to foam cells within the intima (see figure 1.1) (Febbraio et al., 2000; Goldstein et al., 1979; Suzuki et al., 1997). In addition, the uptake process by SR-A can also lead indirectly to inflammation through the presentation on MHC class II molecules for further recognition by cells of the adaptive immune system (Nicoletti et al., 1999). Furthermore, scavenger receptors can also recognise other antigens apart from modified lipoproteins, for example apoptotic cells and pathogenic organisms (Peiser et al., 2002).

TLRs, have also gained attention in recent studies of atherosclerosis. In a healthy arterial wall TLR-2 and TLR-4 are expressed on endothelial cells, and in an inflamed vessel macrophages also express TLRs (Janeway and Medzhitov, 2002). The increased expression of both TLR-2 and TLR-4 can result from either LPS or IFN- γ induced activation of human endothelial cells (Faure et al., 2001). Activation of TLRs can be triggered by various ligands within the fatty streak, including heat shock protein 60 (HSP60) (Ohashi et al., 2000), minimally modified LDL (Miller et al., 2003) and bacterial antigens (Hajishengallis et al., 2002; Prebeck et al., 2001). Subsequent ligation of TLRs elicits various effects that stimulate the progression of atherosclerosis through NF- κ B and the mitogen activated protein kinase (MAPK) pathway (Faure et al., 2000; Guha and Mackman, 2001). TLR activation also directly stimulates leukocyte recruitment, phagocytosis, production of reactive oxygen species (ROS), expression of MMPs (Janeway and Medzhitov, 2002) and cytokine production in human monocytes (Guha and Mackman, 2001). The detrimental role of PRRs in atherosclerosis has been

highlighted by animal studies showing that deletion of either scavenger receptors or TLR4 attenuates the progression of atherosclerosis (Björkbacka et al., 2004; Febbraio et al., 2000; Michelsen et al., 2004; Suzuki et al., 1997).

1.5.2 Adaptive response of the immune system during fatty streak formation

The adaptive arm of the immune system also plays an important role during the development of the fatty streak within the atherosclerotic vascular wall. In a normal healthy artery, T lymphocytes reside within the adventitia (Galkina and Ley, 2007). However, shortly after up-regulation of adhesion molecules (including VCAM-1), and in the presence of cytokines (such as M-CSF), T-cells migrate towards the intima where they can undergo antigen-dependent activation. Such antigens include fragments of oxidised LDL digested by macrophages (Stemme et al., 1995), HSP60 (Benagiano et al., 2005), β_2 -glycoprotein I (George et al., 2000) and the fragments of bacterial antigens (de Boer et al., 2000; Mosorin et al., 2000). T-cell-macrophage cross talk requires the presence of CD40 receptor on the surface of macrophage and CD40 ligand on the surface of T-lymphocytes (Phipps, 2000). The fatty streak contains only CD4⁺ T cells expressing $\alpha\beta$ T-cell receptor (TCR), which is indicative of an activation response to a limited set of local antigens (Hansson and Libby, 2006).

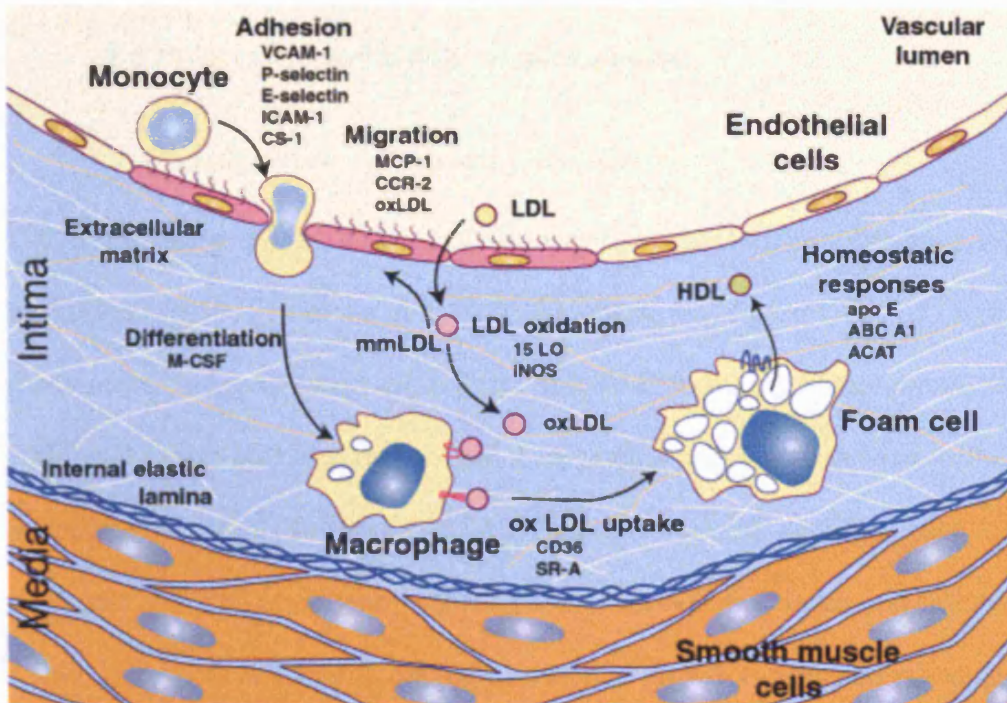


Figure 1.1 Development of a fatty streak. Initial injury to the endothelium wall leads to the expression of adhesion molecules, allowing circulating monocytes to attach and migrate into the intima, where they differentiate into macrophages. Within the intima, LDL may undergo oxidative modification, resulting in the production of either minimally modified low density lipoprotein (mmLDL) or oxidised LDL (ox LDL), causing the binding and uptake into macrophages via their scavenger receptors, which leads to the formation of fatty-filled foam cells. Figure taken from *Atherosclerosis: The Road Ahead* (Glass and Witztum, 2001).

1.6 Progression towards a complex plaque

1.6.1 Vascular smooth muscle cells (SMCs)

One of the characteristic features of plaque progression is the proliferation and migration of SMCs from the media into the intima (see figure 1.2). Although other cell types contribute to the production of the extracellular matrix of the intima, the vascular SMC is the main cell type responsible. In a healthy artery wall the extracellular matrix is composed mostly of types I and III collagen; in contrast, atherosclerotic matrix contains a mixture of proteoglycans together with type I collagen and fibronectin (Ross, 1999). In response to stimuli, including secreted growth factors and cytokines from surrounding inflammatory cells, SMCs also produce proinflammatory signalling molecules such as platelet derived growth factor, transforming growth factor β (TGF β), IFN- γ , MCP-1 and IL-1, exacerbating the inflammatory response within the vessel wall (Doran et al., 2008). During plaque progression, SMC proliferation also occurs within the media causing increased medial thickness and contributing to vascular remodelling. Although the adventitia lack SMCs, it does contain fibroblasts and other progenitor cells, which during plaque progression can differentiate to migratory myofibroblasts having a similar phenotype to SMCs (Siow and Churchman, 2007) and thus providing another source of cellular material for the inflamed intima.

1.6.2 Macrophages

Depending on their function, macrophages can be classified into two types: M1 and M2 (Gordon and Taylor, 2005). Both M1 and M2 macrophage sub-types reside within intima, where they exert pro- and anti-atherosclerotic effects respectively. The classically activated macrophage M1 exacerbates atherosclerosis by producing pro-

inflammatory cytokines such as TNF α , IL-16, IL12, and ROS. Conversely, the M2 macrophage produces anti-inflammatory cytokines, including IL-10 and TGF- β , which exert anti-atherogenic effects by promoting resolution of inflammation within the plaque (Shimada, 2009; Tabas, 2010).

1.6.3 T-cells

T cells constitute approximately 5-20% of the cells in an advanced atherosclerotic plaque (Hansson et al., 1989b; Jonasson et al., 1986). Interestingly, the percentage of T cells increases in the culprit lesions of patients with unstable angina and acute myocardial infarction (Hosono et al., 2003). Furthermore, there is evidence to suggest that the activation of T-lymphocytes may play an important role in plaque destabilisation since the T cells of unstable plaque showed signs of specific, antigen-driven activation (De Palma et al., 2006). In addition, the T cell response in unstable angina patients is directed against plaque antigens (Caligiuri et al., 2000). In agreement with these human findings, evidence from animal studies also found a correlation between T cell infiltration and the progression of atherosclerosis. Thus, apoE^{-/-}/CD4^{+/-} mice showed attenuation of the disease in comparison to the apoE^{-/-} controls (Zhou et al., 2005), while transfer of CD4⁺ T cells into immuno-deficient apoE^{-/-} mice accelerated atherosclerosis (Zhou et al., 2000).

As with macrophages, CD4⁺ T cells are grouped into sub-types. The sub-group of CD4⁺ T cells is defined according to the cytokines they secrete. In the context of the early atherosclerotic lesion, T cells largely have properties of Th1 sub-type and secrete the cytokines IL-2, IFN- γ , TNF- α , and TNF- β . These cytokines are pro-inflammatory and pro-atherogenic, causing activation of macrophages and vascular cells and

contributing to worsening of the disease. The Th1 sub-type dominates in early lesions of atherosclerosis, while in contrast, Th2 cytokines (IL-4, IL-13 and IL-10) are scarce and have anti-atherogenic properties (see figure 1.2) (Frostegård et al., 1999; Uyemura et al., 1996). Animal studies have shown a shift from Th1 to Th2 dominance within lesions as they progresses towards an advanced phenotype. Thus, atherosclerotic lesions in the apoE knockout mouse model contain Th2 subtype only in extreme hypercholesterolemic conditions, as reflected by increased Th2 cytokines within the plaque and in the spleen (Zhou et al., 1998).

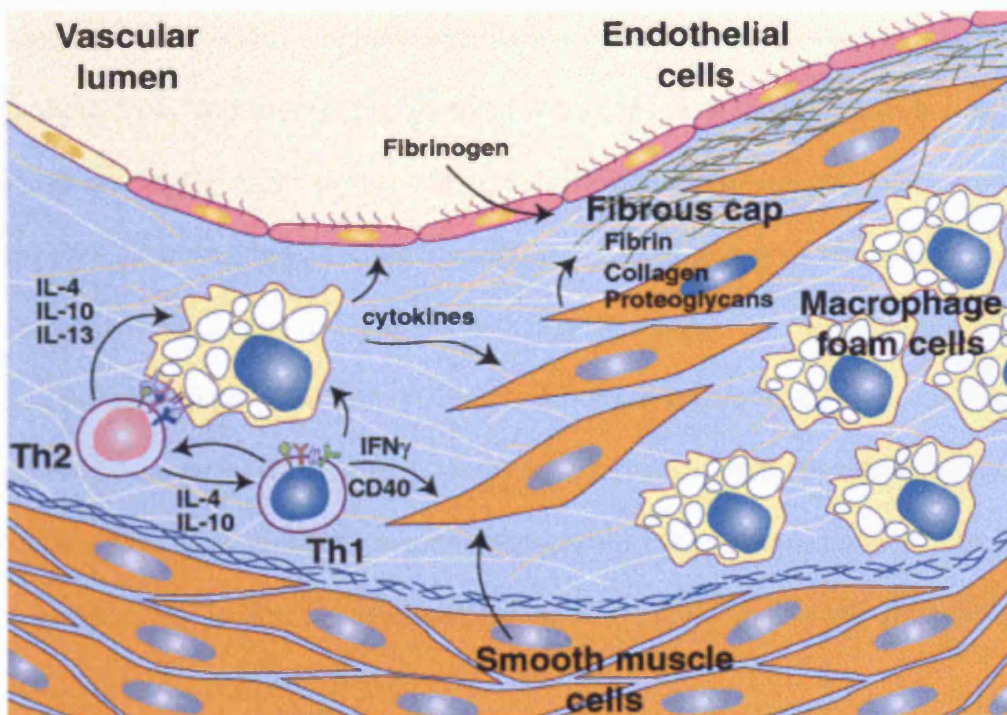


Figure 1.2 Progression towards a complex plaque. As the lesion progresses a chronic inflammatory process evolves, caused by further influx of inflammatory cells, including Th1 and Th2 cells. SMCs also migrate and proliferate from the media into the intima where they secrete extracellular matrix proteins that form a fibrous plaque. Figure taken from *Atherosclerosis: The Road Ahead* (Glass and Witztum, 2001).

1.6.4 Other inflammatory cells involved

Within the setting of advanced atherosclerosis, the lesion becomes more complex, with increased recruitment and stimulation of inflammatory cells including, B cells, dendritic cells, Natural killer (NK) cells, mast cells and neutrophils. These cells encourage cell-mediated cytotoxicity and release numerous cytokines, enzymes or antibodies, all of which are likely to contribute towards the progression of atherogenesis.

B cells produce natural antibodies that circulate in a healthy individual even in the absence of exogenous antigenic stimulation. Natural antibodies are mostly of the IgM isotype. They contain highly conserved sequences that recognise a wide range of exogenous antigens, as well as altered self structures. Natural antibodies are thus capable of providing protection against invading pathogens and in addition have been implicated in removal of senescent and apoptotic cells, thus providing protection from autoimmunity (Baumgarth et al., 2005). Within spleens of apoE^{-/-} mice, natural antibodies against oxidised LDL were identified and cloned. Subsequently researchers showed that these monoclonal antibodies (mAbs) were able to immunostain atherosclerotic lesions in rabbit and human and recognise epitopes of oxidised LDL in human plasma (Palinski et al., 1996).

NK cells are large lymphocytes derived from bone marrow. They function by releasing small cytoplasmic granules of proteins called perforin and granzyme which cause direct cell-mediated cytotoxicity by activating apoptosis. In addition, they can also produce pro-inflammatory cytokines such as IFN- γ . In the context of atherosclerosis, NK cells have been detected in early lesions in young adults (Millonig et al., 2002) and increased

circulating NK cells have been shown to correlate with severity of the disease (Clerc and Rouz, 1997).

Dendritic cells are highly effective antigen presenting cells, expressing both MHC class II molecules and co-stimulatory molecules on their surface for the activation of naïve T cells. Within a healthy artery wall, a small number of dendritic cells are present particularly within atherosclerotic prone areas of the vascular tree, and their numbers increase during the progress of atherosclerosis (Bobryshev and Lord, 1995). The role of dendritic cells within the atherosclerotic plaque is not well understood. However, dendritic cells have been detected with T cells in human aortic intima (Bobryshev and Lord, 1995), an observation which suggests that dendritic cells may play a role in antigen processing and presentation in vivo in atherosclerotic tissue. In addition, a histological analysis of human carotid artery specimens detected dendritic cells co-localised with T cells in the shoulder region of vulnerable plaques which were prone to rupture (Yilmaz et al., 2004), suggesting that dendritic cells may contribute to plaque destabilisation by activating T cells.

Mast cells are cells of the innate immune system which play a major role in allergy and host defence response (Galkina and Ley, 2009). Activation of mast cells generates a wide range of pro-inflammatory molecules, including, vasoactive substances such as histamine, cytokines (IFN- γ , TNF-alpha, IL-6), proteases and growth factors (Galkina and Ley, 2009). Although small in number, mast cells have been detected in the adventitia and within atherosclerotic lesions, in particular at the shoulder regions where they may contribute to plaque destabilisation (Lindstedt et al., 2007).

Neutrophils are phagocytic cells of the innate immune system and secrete a wide range of biologically active molecules such as ROS, myeloperoxidase, proteases and chemokines (Galkina and Ley, 2009). Neutrophils are rare in stable human atherosclerotic lesion, but abundant in unstable plaques which are prone to rupture (Naruko et al., 2002).

1.6.5 Cellular necrosis and breakdown

In order to resolve increased inflammation within the lesion, a series of processes must take place, including inhibition of inflammatory cell recruitment (Tabas, 2010), promotion of inflammatory cell egress (Llodrá et al., 2004) and clearance of apoptotic cells by phagocytes (efferocytosis) (Lawrence and Gilroy, 2007; Serhan et al., 2007). These effects are mediated by anti-inflammatory cytokines (including IL-10 and TGF- β) (Mosser and Zhang, 2008), signalling molecules (such as lipoxins, and resolvins) (Serhan et al., 2008) and transcription factors (such as PPAR) (Huang et al., 1999). These inflammatory resolution responses are critical in preventing the progression of an early lesion to an advanced complex plaque where these processes are impaired.

Decreased resolution of inflammation within atherosclerosis indicates defective efferocytosis, impaired egress of inflammatory cells and a persistent inflammatory state within the plaque. Animal studies have shown that in apoE^{-/-} mice, in a cholesterol rich environment, inflammatory cell egression is halted in atherosclerotic plaques.

Conversely, normal cholesterol levels were found to promote migration of inflammatory cells out through adventitial lymph vessels towards local lymph nodes (Trojan et al., 2006). This inability of an atherosclerotic plaque to clear apoptotic inflammatory cells leads to the enlargement of the necrotic core within the plaque.

Animal studies looking at the effects of macrophage apoptosis on lesion size have suggested that the necrotic core of an advanced plaque develops from the combined effect of defective efferocytosis and cellular apoptosis (Arai et al., 2005; Gautier et al., 2009; Liu et al., 2005; Tabas, 2010; Thorp et al., 2009). Further studies looking at efferocytosis impairment using mouse models of atherosclerosis have identified a few genes involved in this process (Ait-Oufella et al., 2007; Bhatia et al., 2007; Boisvert et al., 2006). These include C1q, a component of the early activation pathway of the complement system, which binds to apoptotic cells and plays an important role in their disposal, thus bridging the gap between apoptosis and efferocytosis (Korb and Ahearn, 1997; Nauta et al., 2002b; Navratil et al., 2001; Taylor et al., 2000). Human studies have shown that efferocytosis is impaired in advanced human atherosclerotic plaques (Schrijvers et al., 2005).

1.7 Plaque rupture and thrombosis

The worst clinical scenario produced from an advanced plaque is the sudden thrombotic occlusion of an artery (Libby and Aikawa, 2002), an event which is likely to be caused from rupture of the fibrous cap that overlies the lipid core of the plaque (Libby and Aikawa, 2002). Hence, the ability of a plaque to maintain stability is critical to avoid rupturing of the fibrous cap. Results from plaque rupture studies suggest that the culprit plaques in these instances may not be the largest, but rather those which are vulnerable to rupture. Hence, vascular events leading to damage to the heart, brain and lower extremities can occur without warning and may result in tragic consequences.

Pathological studies on plaques in human aorta and coronary arteries that have undergone unstable angina have been used to identify various characteristics of

vulnerable plaques (Annex et al., 1995; Davies et al., 1993b; Falk, 1989; Moreno et al., 1994). These characteristics of vulnerable plaques include; increased plaque macrophage content, decreased SMC content, a thin fibrous cap, large lipid core which occupies at least 50% of the overall plaque volume and increased tissue factor content.

Accumulation of these factors greatly increases the risk of plaque rupture, and the plaque is then termed “vulnerable”. Further studies on advanced plaques have identified various immunological factors that can cause or exacerbate these characteristics of a vulnerable plaque. For example, in a stable atherosclerotic plaque vascular SMCs secrete interstitial collagens, providing stability for the extracellular matrix; however, pro-inflammatory mediators within advanced plaques can favour destabilisation of the fibrous cap. IFN- γ , secreted by T_h1 cells, effects extracellular matrix de-stabilisation, through inhibiting the production of collagen by SMCs (Amento et al.) and by inhibiting the proliferation of vascular SMCs within the intima (Hansson et al., 1989a). In addition activated macrophages produce proteases, including members of the MMP family, which contribute to the breakdown of collagen within the matrix, further contributing to plaque de-stabilisation (Galis et al., 1994; Henney et al., 1991) (see figure 1.3).

Plaque rupture exposes the lipid core of the lesion to coagulation factors which then can cause the formation of a thrombus (as shown in figure 1.3). Tissue factor, a potent pro-coagulant expressed by a subpopulation of macrophages, aids platelet aggregation and thrombosis (Libby and Aikawa, 2002). In addition to T-cells, endothelial cells and SMCs, platelets also express CD40 ligand (Henn et al., 1998). In addition to its role in stimulating an immune response, CD40- CD40 ligand interaction between platelets can

cause platelet activation and hence, enhance thrombosis causing an increase in the rate of clot formation (Inwald et al., 2003; Prasad et al., 2003).

1.8 Animal models of atherosclerosis

Atherogenesis is a very complex and multi-factorial disease, and thus by its very nature it is difficult to define the molecular mechanisms behind the progression of the disease. Another problem is the inability to follow the changing characteristics of lesions within an individual patient, despite progress in imaging techniques (Hansson and Libby, 2006). Our current knowledge and understanding of the underlying mechanisms of atherosclerosis has grown rapidly in recent years owing to the generation and use of genetically modified mouse models of atherosclerosis. These have allowed researchers in the field to evaluate existing therapeutic drugs as well as discover new therapeutic targets (Jackson, 2007; Kinderlerer et al., 2006; Mason et al., 2002; Nakamura et al., 2009).

There are obvious drawbacks in using mice to study atherosclerosis. These include body size, lifespan, weight and importantly in terms of creating an animal model to study human atherosclerosis, the mouse has very different lipoprotein profiles. In humans, 75% of the cholesterol is carried around the body by LDL; in contrast, cholesterol in the mouse is carried by high density lipoprotein (HDL), which is protective against atherosclerosis in humans. Indeed, non-genetically modified mice on a normal chow diet, resist the pathological consequences of atherosclerosis.

Before the availability of gene targeting, the mouse model used to study atherosclerosis involved feeding inbred strains of mice with a very high fat diet. Some strains of mice,

such as C3H/HeJ, were resistant to atherosclerosis, while others, including the inbred strain C57BL/6, developed pathology, including several layers of fatty foam cells within the intima (Breslow, 1996; Liao et al., 1993; Paigen et al., 1985).

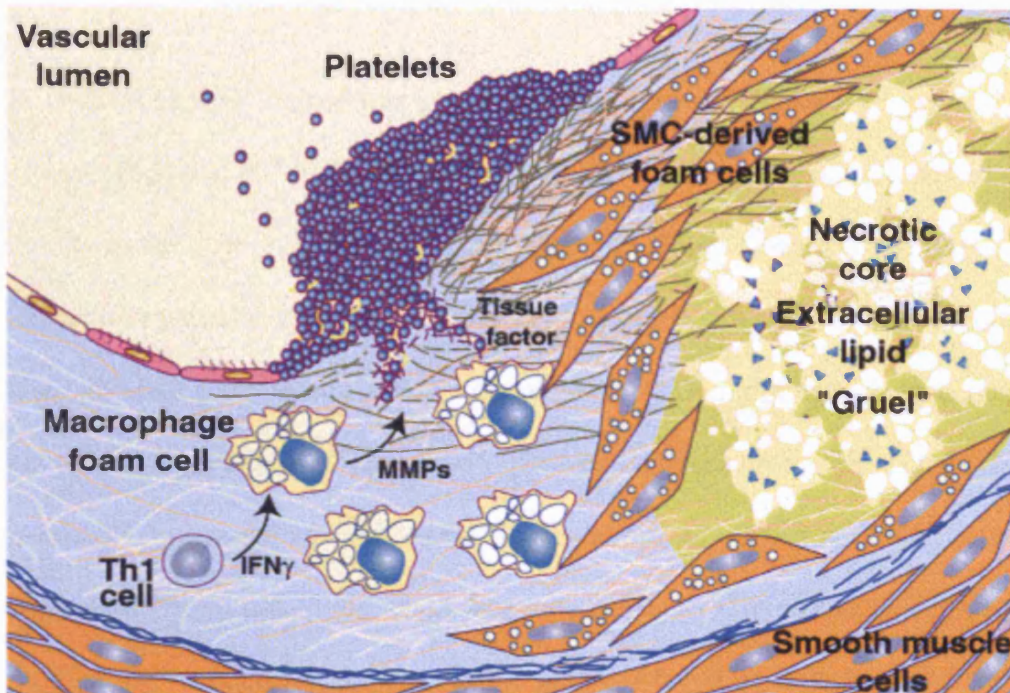


Figure 1.3 Plaque rupture and thrombosis. The final stage of atherosclerosis is initiated by necrosis of macrophage and fat-filled SMCs resulting in the production of a necrotic core. The fibrous plaque weakens as a result of MMP secretion (including MMP-1,-2, -3, -9 and -12) from residing macrophages and eventually the plaque will rupture, exposing blood components to tissue factor, initiating coagulation, recruitment of platelets, and the formation of a thrombus. Figure taken from *Atherosclerosis: The Road Ahead* (Glass and Witztum, 2001).

There were several disadvantages with this mouse model. Firstly, lesions were limited to the fatty streak stage of the disease and did not proceed to the more complex phenotype of advanced human plaques. Secondly, lesion formation was restricted to the

aortic valve leaflets whereas in humans plaques are formed at all branch points of major vessels. Thirdly, the diet used to induce atherosclerosis in these mice was unphysiological with regard to its extremely high cholesterol content (10–20 times more than in westernised diet) and the presence of cholic acid.

In 1992, the apoE^{-/-} mouse was generated simultaneously by two independent research groups (Plump et al., 1992; Zhang et al., 1992). ApoE is a glycoprotein produced mainly in the liver and brain of humans and mice. It is a surface constituent of all lipoprotein particles apart from LDL and functions as a ligand for receptors that clear chylomicrons and very low density lipoprotein (VLDL) remnants. Hence, apoE^{-/-} mice have delayed clearance of lipoprotein particles and even on a normal chow diet have increased cholesterol and triglyceride levels compared to their normal litter-matched controls (Jawień et al., 2004; Nakashima et al., 1994). This mouse model also shows progression of atherosclerosis beyond the fatty streak stage with lesions developing at vascular sites typically affected in human atherosclerosis (Nakashima et al., 1994).

In the apoE^{-/-} mouse the initial stages of atherosclerosis develop after 5-6 weeks on a normal chow diet, with attachment of monocytes to the endothelial wall followed by infiltration into the subendothelial space. At 10 weeks, fatty streaks appear and fibrous plaques develop at around 20 weeks of age. The progression of atherosclerosis in this model is accelerated when fed a high fat diet. The popularity of this model is due not only to its rapid plaque formation but also to the morphological similarity of these plaques to human lesions.

The characteristics of atherosclerosis shared by the apoE^{-/-} mouse model and humans are listed below:

- Distribution and size of lesion increases with age
- Lesions progress from fatty streak to advanced plaques
- Advanced intact plaques containing a necrotic core surrounded by a fibrous cap of SMCs, together with collagen and elastin fibres.
- Events demonstrating evidence of plaque rupture including;
 1. Formation of an acellular necrotic core
 2. Erosion of the necrotic core through to the lumen with exposure to the lumen
 3. Appearance of intraplaque haemorrhage
 4. Disruption of the fibrous cap
- Layering within the plaque implying multiple events (buried fibrous caps)

The second most widely used model of atherosclerosis is the ldlr^{-/-} mouse. This model was generated in 1993 by Ishibashi et al. (Ishibashi et al., 1993). Whilst both apoE^{-/-} and ldlr^{-/-} mouse models produce similar atherosclerotic lesions that progresses from the initial fatty streak to an advanced complex lesion, plasma lipid profiles differ between the two models during a high cholesterol diet. In the ldlr^{-/-} mice, triglyceride and cholesterol levels are increased during a high cholesterol diet, whilst in the apoE^{-/-} model, although cholesterol levels are increased, triglyceride levels do not change (Joven et al., 2007).

Some of the advantages of using atherosclerotic mouse models as a research tool are listed below.

- Reproducible pattern of plaque development
- Ability to use normal or modified diet
- Ability to control for environmental and genetic factors
- Rapid breeding
- Rapid onset of disease and progression
- Economical

Using mouse models of atherosclerosis as a research tool is pivotal in further understanding the complex pathophysiology and signalling mechanisms underlying this disease and will lead us closer towards a clinical breakthrough in the prevention and treatment of atherosclerosis.

1.9 Assessment of atherosclerosis in mice

1.9.1 Quantitative assessment of atherosclerosis in mice

Assessment of atherosclerosis has generally been made by quantifying the area of plaques, most often those present in the aorta or aortic root. Interestingly, studies mapping areas of atherosclerosis have shown that lesions at the aortic valve leaflet develop faster than in the aorta (Veillard et al., 2004), thus the location for assessing atherosclerosis is critical in determining the outcome of the analysis. Cross section analysis and *en face* methods are used to measure the extent of atherosclerosis. The *en face* method usually involves splitting the aorta open, exposing the luminal

endothelium and pinning it onto a standard black wax dissection pan before staining for lipid deposition with dyes such as Sudan IV. Cross sectional lesion area in the aortic root and brachiocephalic artery are also commonly quantified as a measure of atherosclerosis. Histology and immunohistochemistry for various markers can then be performed on cross sections for a multi-quantitative assessment of these arteries. Imaging software is then used in detailed and accurate quantification of atherosclerotic lesions for both *en face* and cross section methods.

1.9.2 Qualitative assessment of atherosclerosis in mice

While an assessment of plaque size yields useful information, this kind of analysis gives no indication as to the stability of the plaques present. Plaque stability is a critical parameter since it governs the likelihood of a rupture occurring, which could lead to occlusive thrombosis and death. Over the last few years, a growing body of data has indicated that the brachiocephalic artery may be a useful site to examine plaque stability in mice. The brachiocephalic artery, a known site of lesion development in humans (Krinsky et al., 2001; Nakajima et al., 2008), is a very short vessel arising from the arch of the aorta and branching into the right common carotid artery and the right subclavian artery (See figure 1.4). Typical length of a brachiocephalic artery from a 13 week old apoE^{-/-} mouse is approximately 660µm which doubles at 23 weeks of age (McAteer et al., 2004). High turbulence likely makes this vessel a site of predilection for plaque formation. The characteristics of an unstable human plaque include the presence of a large necrotic core surrounded by a thin fibrous cap (Rosenfeld et al., 2002). The brachiocephalic artery in the atherosclerosis-prone mouse has been shown to consistently provide advanced plaques (Seo et al., 1997), which have unstable

phenotypes similar to that seen in humans, including formation of a necrotic core, erosion of the necrotic mass through to the lumen, thrombotic occlusion (Williams et al., 2002). It has also been shown to develop spontaneous plaque rupture (where rupture has not been induced mechanically) (Johnson and Jackson, 2001) which some argue is an unqualified generic advantage of the model since it lacks end-stage atherosclerosis (Lutgens et al., 2003). However mouse model of spontaneous plaque rupture allows researchers in the field to determine the events which lead up to and trigger spontaneous plaque rupture.

Several different groups have published evidence of plaque rupture in mouse models of atherosclerosis (Rosenfeld et al., 2000; Williams et al., 2002). However, this is currently in dispute as some argue against the histopathologic criteria used for plaque rupture identification and disagree with interpretation of buried fibrous caps as indirect evidence for previous plaque ruptures (Falk et al., 2007; Schwartz et al., 2007).

In humans plaque rupture is defined as “an area of fibrous cap disruption whereby the overlying thrombus is in continuity with the lipid core” (Virmani et al., 2000) and in mice a rupture is defined as “disruption of the fibrous cap accompanied by the intrusion of blood products into the plaque itself” (Williams et al., 2002). The definition allows a true plaque rupture to be distinguished from artefacts caused during histological processing.

In apoE^{-/-} mice, evidence of plaque rupture has been reported with disturbed continuity of the fibrous cap, intraplaque haemorrhage and buried fibrous caps as evidenced by the presence of erythrocytes (Williams et al., 2002).

Neovasularisation, a key event which arises from the adventitia owing to the abundance of vasa vasorum at this location, in advanced plaques may cause intraplaque hemorrhage which may result in plaque destabilisation and rupture. Of note, intraplaque haemorrhage is not necessarily associated with plaque rupture and is defined as the deposition of blood products within an atherosclerotic lesion (Lutgens et al., 2003).

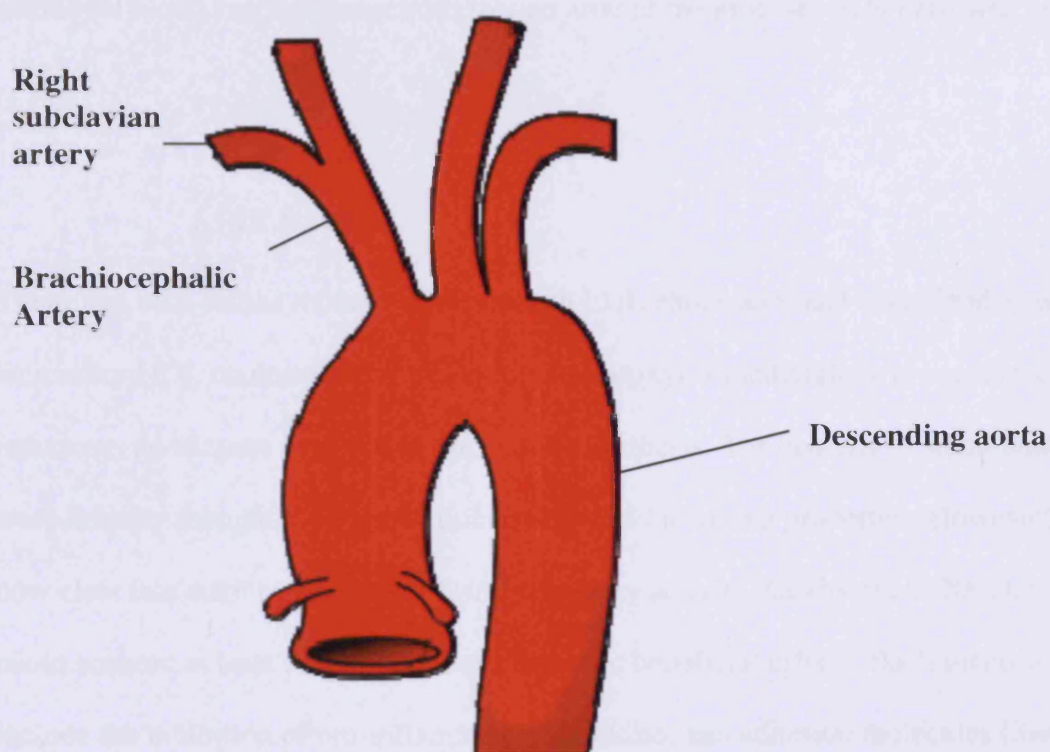


Figure 1.4 Localisation of the brachiocephalic artery. A schematic diagram showing the location of the brachiocephalic artery arising from the arch of the aorta and dividing into the right common carotid artery and right subclavian artery. Adapted from the original version: www.daviddarling.info/images/subclavian_artery.png

1.10 Therapeutic perspectives

1.10.1 Surgery

Typically; surgery is only performed in severe cases of atherosclerosis, and includes minimally invasive angioplasty procedures, for example insertion of stents to physically expand narrowed arteries, and major invasive surgery, such as bypass surgery, to create additional blood supply connections that go around the most severely narrowed areas.

1.10.2 Statins

Treatment with statins reduces levels of both LDL cholesterol and triglycerides, while increasing HDL cholesterol, by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase, an enzyme involved in cholesterol synthesis. The benefits of statin treatment were initially thought to be solely due to their lipid lowering properties. However, it is now clear that statins also have anti-inflammatory activity (Crisby et al., 2001), which could account at least partially for their dramatic beneficial effects. Such functions include the inhibition of pro-inflammatory cytokines and adhesion molecules (Steffens and Mach, 2006) and up-regulation of complement regulators (Kinderlerer et al., 2006; Mason et al., 2002), which will be discussed in more detail later. This anti-inflammatory effect is also supported by a recent clinical trial where atorvastatin was found to be beneficial in patients with rheumatoid arthritis (RA) (McCarey et al., 2004).

1.10.3 Anti-inflammatory drugs

The current systemic anti-inflammatory approach, which includes glucocorticoids and non-steroidal anti-inflammatory drugs, exerts many unwanted side effects rendering

them poor candidates for a long term therapeutic approach which is needed for the treatment of atherosclerosis (Libby et al., 2009). However, more specific anti-inflammatory agents are being considered, with lipoprotein associated phospholipase A₂ (PLA₂) inhibition currently in clinical trials (Boekholdt et al., 2008). PLA₂ releases fatty acids from phospholipids resulting in the production of arachidonic acid and lysophospholipids (Dennis, 1994). In the context of atherosclerosis, PLA₂ is bound to LDL whilst being transported around the body and thus enters the inflamed atherosclerotic lesion as a consequence of LDL transmigration into the vessel wall. Subsequently, LDL is oxidised and PLA₂ acts on the oxidised LDL releasing oxidised fatty acids and lysophosphatidylcholine that promotes inflammatory responses, including increasing monocyte adhesion, migration of vascular SMCs and up-regulating expression of growth factors, adhesion molecules, cytokines and chemokines by macrophages, T-cells and endothelial cells (Garza et al., 2007; Khakpour and Frishman, 2009).

Vaccination strategies are also being considered to induce protective immunity. In particular, animal studies have shown that vaccination with oxidised LDL, bacteria containing modified phospholipids or HSP60 are beneficial in preventing atherosclerosis (Binder et al., 2003; Maron et al., 2002; Palinski et al., 1995).

1.10.4 Inflammatory biomarkers

Work on specific anti-inflammatory drugs has not yet produced an ideal candidate for the clinic; however, inflammatory biomarkers have been identified and used to predict risk, monitor treatment and aid therapy. Such biomarkers include myeloperoxidase, Lp-PLA₂, pentraxin-3, various cytokines, proteases, C-reactive protein (CRP) and

complement proteins C3 and C4 (Engström et al., 2007; Libby et al., 2009). The most studied of these is CRP, which has been tested in many large-scale cohort studies, and reported to predict incidence of myocardial infarction, stroke and cardiovascular death (Ridker, 2007); however, this remains controversial (Mosca, 2002; Nordestgaard, 2009).

1.10.5 Summary

There remains an urgent need both for new inflammatory therapeutics targeted against atherosclerosis and also for more basic research aimed at unravelling and understanding the complex web of inflammatory interactions that give rise to atherosclerosis. Such an enhanced understanding of this multi-factorial disease can only help in the discovery of new therapeutics to aid in combating the increasing healthcare burden of atherosclerosis.

2. The Complement system

2.1 Overview

The complement system is part of the innate immune response. Originally described as a “complement” to adaptive immunity, today we understand it as a central effector mechanism of the innate immune system, defending the host against infections, bridging innate and adaptive immunity and disposing of immune complexes and apoptotic cells (Walport, 2001a; Walport, 2001b).

To date there are more than 30 soluble and membrane bound proteins which mediate activation and regulation of the proteolytic complement cascade to finely balance the elimination of invading pathogens and the protection of the host by limiting complement deposition on healthy tissue. However, if this delicate balance is disrupted, the complement system may cause injury and contribute to the pathogenesis of various diseases (Ffrench-Constant, 1994; Mead et al., 2004; Széplaki et al., 2009; Williams et al., 2004).

Activation of the complement system is rapid and efficient. Soluble complement components are present in the blood, body fluids and tissues to readily trigger a defence reaction against external (i.e. pathogens) or internal (i.e. autoimmunity) danger signals.

2.2 Activation

The complement system consists of three activation pathways: classical, mannose binding lectin (MBL) and the alternative pathway (see figure 3) (Morley and Walport, 2000).

2.2.1 Classical pathway

The classical pathway is activated by the recognition of an antigen-antibody complex by C1q, however other molecules can also trigger its activation including CRP (Siegel et al., 1974), nucleic acids (Jiang et al., 1992) or apoptotic cells (Nauta et al., 2002b). Upon binding, C1r cleaves C1s which in turn cleaves C2 and C4 into a small (C2b, C4a) and a large fragment (C2a, C4b) (as shown in figure 1.5). C2a and C4b together form the classical pathway C3 convertase, C4b2a.

2.2.2 Alternative pathway

The alternative pathway starts with spontaneous low-rate hydrolysis of C3 generating C3(H₂O) which binds to factor B (fB), permitting cleavage by factor D (fD) to form the fluid-phase C3 alternative pathway convertase C3(H₂O)Bb. This enzyme cleaves C3 and deposits C3b on surfaces where, in the absence of complement inhibitors such as factor H (fH), it binds and catalyses cleavage of fB to form surface bound alternative pathway C3 convertase C3bBb. The alternative pathway C3 convertase is stabilised by properdin (as shown in figure 1.5) (Morley and Walport, 2000) which promotes further cleavage of C3 molecules, thus creating a positive feedback. This C3b-Bb-fD loop is known as the “amplification loop” and is necessary for efficient activation of complement.

2.2.3 MBL pathway

Activation of the MBL pathway involves the binding of mannose-binding lectin or ficolins, which are typical pattern recognition receptors, to carbohydrate groups present on the surface of bacteria. Following this recognition process, MBL-associated serine proteases (MASPs) become activated and cleave C4 and C2 exactly as described for the classical complement pathway (as shown in figure 1.5) (Fujita et al., 2004).

2.2.4 Other mechanisms of complement activation

Recently, additional routes of complement activation have been proposed, including, direct cleavage of C3 and C5 by non-complement proteins such as lysosomal enzymes released from neutrophils, kallikrein (part of the kinin and fibrinolysis systems), or thrombin, the so-called “extrinsic protease” pathway (Markiewski and Lambris, 2007).

2.2.5 Complement activation products

Complement activation results in the production of biologically active molecules such as anaphylatoxins, opsonins and MAC that exert multiple functions which may lead to extensive cell triggering and to activation of cellular responses ranging from proliferation to cell death (Walport, 2001a).

2.3 Central component of the activation pathway

Irrespective of the pathway involved, complement activation leads to the cleavage of C3 resulting in two functionally different molecules: C3a and C3b. C3a is an anaphylatoxin, and in the context of atherosclerosis, it promotes the adhesion and infiltration of inflammatory cells into the vessel wall. However, C3a is rapidly

inactivated in plasma through the removal of the carboxy-terminal arginine by serum carboxypeptidase N (seen in figure 1.5). The cleavage of C3a results in the formation of C3adesArg, which is identical to acylation stimulating protein (ASP), a potent anabolic activator of triglyceride synthesis and glucose uptake (Maslowska et al., 2005). The larger cleavage product, C3b, is essential for terminal pathway activation and acts as an opsonin, which during atherosclerosis can promote the clearance of lipids and other debris.

2.4 Terminal pathway

At this stage, lack of regulation of the activation pathway may cause the cascade to proceed to the terminal pathway. This involves the cleavage of C5 into C5a and C5b by either the classical or alternative pathway C5 convertases, C4b2a3b and C3bBbC3b, respectively. Newly formed C5b, still attached to membrane bound C3b within the convertase, presents an acceptor site for the next sequential complement component, C6. Unlike the activation pathway where each step relies on enzymatic cleavage, the terminal pathway is dependent on conformational changes induced by binding. Thus, one molecule of C5b, C6, C7 bind to form a C5b67 complex which is released from the convertase enzyme and attaches to the adjacent cell membrane. C8 then binds forming the C5b678 complex which is then followed by binding and polymerisation of as many as 10-16 molecules of C9 to form a pore which is inserted within the lipid bilayer of the plasma membrane, the membrane attack complex (MAC) (as shown in figure 1.5). Insertion of MAC on cell membranes of invading pathogens causes cellular damage and death (Nauta et al., 2004). An overview of the complement system is shown in figure 1.5.

2.5 Function

The complement system has varied and wide-ranging functions for example it can act as a stimulus to inflammation through the production of small anaphylatoxic fragments C3a and/or C5a which bind to their receptors, expressed on target cells (Guo and Ward, 2005; Köhl, 2001). For example, anaphylatoxins can activate mast cells to release histamine, TNF- α , cytokines and chemokines, mediating vasodilation and leukocyte migration from the bloodstream to the site of inflammation (Lee et al., 2002). In addition C5a is a powerful chemoattractant for cells of both the innate and adaptive immune system, including macrophages (Aksamit et al., 1981), neutrophils (Ehrengruber et al., 1994), activated B cells (Ottonello et al., 1999) and T cells (Nataf et al., 1999), basophils (Lett-Brown and Leonard, 1977) and mast cells (Hartmann et al., 1997).

Recent findings provide evidence that complement also regulates adaptive immune responses (Carroll, 2004; Hawlisch and Köhl, 2006). In particular C3 breakdown products have been shown to regulate B cell immunity (Fearon and Locksley, 1996). Further studies showed that complement receptor 1 (CR1) and complement receptor 2 (CR2) are both involved in mediating the elimination of self-reactive B cells and amplification of the humoral responses to both thymus dependent and independent antigens (Carroll, 2004; Klos et al., 2009).

Another function of complement is to dispose of immune complexes, necrotic and apoptotic cells generated during an inflammatory reaction (Manderson et al., 2004; Walport, 2001a; Walport, 2001b). The clearance of immune complexes is facilitated by maintaining their solubility through the binding of C1, and fragments of C4 and C3,

opsonising the complex. This prevents a growth in the size of the immune complex stopping precipitation, and allows their recognition and removal by phagocytes. This complement aided process limits inflammation and the propagation of injury on neighbouring tissues. The removal of necrotic and apoptotic cells is critical for the termination of inflammation and prevention of autoimmunity. Dying cells undergo several changes to signal their removal to phagocytic cells. These changes involve modifications of the plasma membrane resulting in the exposure of self-antigens, normally sequestered within the viable cell, and the internalisation of proteins normally expressed on the cell surface. The presentation of “eat me” signals and the down-regulation of “don’t eat me” signals trigger the binding of a number of complement opsonins such as C4b and C3b to mediate the removal of dying cells. This is a key process in the maintenance of tissue homeostasis (Cole and Morgan, 2003; Flierman and Daha, 2007; Nauta et al., 2003).

2.6 Regulation

Sophisticated regulatory mechanisms allow the complement system to rapidly attack invading pathogens whilst protecting host cells from its detrimental effects. Regulation of the complement system exists at each level through the action of several complement regulators and inhibitors. Both membrane bound and fluid phase regulators control the complement cascade, ensuring cell and tissue integrity (Morley and Walport, 2000). See table 2.1 for specific functions of complement regulatory proteins.

Complement System: an overview

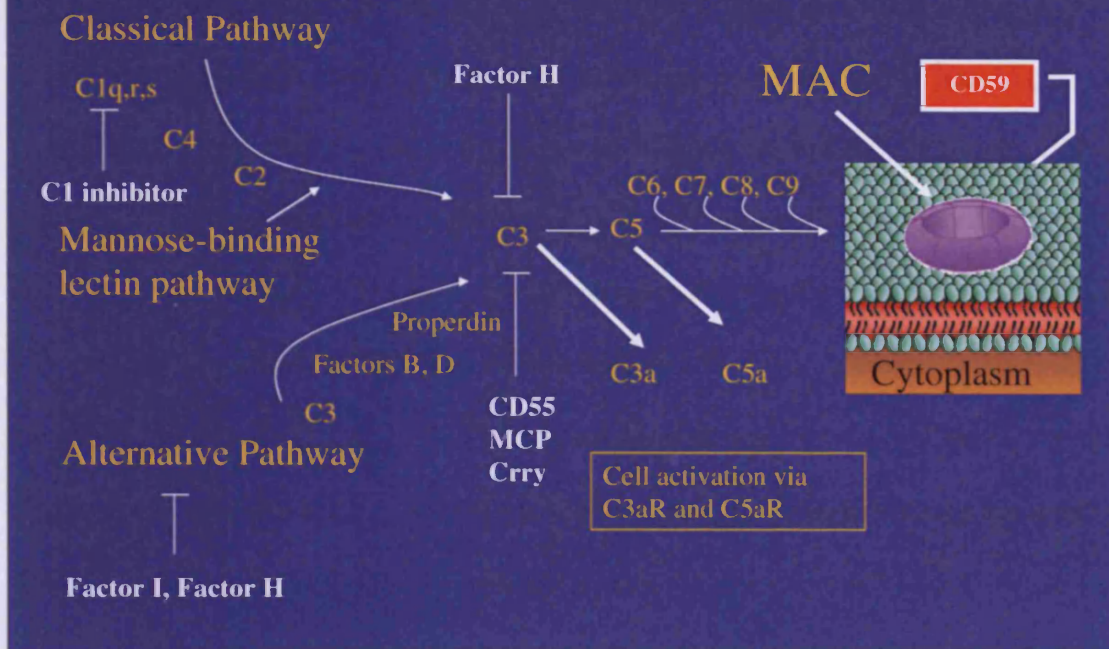


Figure 1.5 Complement activation cascade. The classical and mannose-binding lectin pathways are initiated by PAMPs (pathogen-associated molecular patterns) such as foreign antigens and mannose, respectively. The C1 complex of classical pathway consists of C1q, C1r and C1s. The alternative pathway is activated by spontaneous hydrolysis of C3 and is dependent on the presence of factor B (fB), D and properdin. The central component of the cascade, C3, is controlled by numerous regulators including factor I, factor H, CD55, MCP (membrane cofactor protein) and Crry (complement-receptor 1-related gene/protein γ). Activation of C3 can also lead to the activation of the terminal pathway *via* C5. Subsequent binding of C6, C7, C8 and C9 leads to the formation of the pore like structure, membrane attack complex (MAC). CD59 is the sole inhibitor of the MAC complex which prevents the binding of C9 to C8.

There are multiple fluid phase complement regulators which function at different level of the cascade depending on their specific target. Plasma based alternative pathway regulators are fH, fH like-1 (FHL1) and the activator protein properdin, whilst carboxypeptidase N, is involved in all three activation pathways (Zipfel and Skerka, 2009). The soluble classical and lectin pathway inhibitors include C1 inhibitor (C1INH) and C4 binding protein (C4BP). Soluble regulators of the terminal pathway include vitronectin and clusterin. Membrane bound complement regulators also have a crucial role in sustaining tissue homeostasis and protecting cells from self harm. These include the glycosyl-phosphatidylinositol (GPI) anchored membrane proteins, CD55 (otherwise known as decay-accelerating factor (DAF)) and CD59, and transmembrane proteins, complement receptor 1 (CR1) and membrane cofactor protein (MCP) (also known as CD46) (Harris et al., 1999; Wiesmann et al., 2006; Zipfel and Skerka, 2009). More recently, complement receptor of the immunoglobulin superfamily (CRig) has been shown to inhibit alternative pathway activation in a mouse model of intestinal ischemia/reperfusion induced injury (Chen et al., 2009). The complement regulators each act at specific points of the cascade. Thus, whilst CD55 is involved at the early stages of complement activation where it accelerates the decay of both C3 and C5 convertases (Fujita et al., 1987; Nicholson-Weller et al., 1982), CD59 acts on the terminal portion of the complement cascade where it binds to the forming MAC, blocking recruitment of multiple C9 molecules necessary for the formation of the MAC pore (Meri et al., 1990).

2.7 Complement receptors

Most of the potent biological effects of complement activation products require receptors; these include CR1, CR2, CR3 and CR4, through which C3 fragments induce

and modulate inflammation, control inflammatory and cellular responses and induce effector functions, such as tagging of cell surfaces and activation of phagocytosis (Zipfel and Skerka, 2009). Numerous receptors exist to elicit the responses of effectors such as C3a and C5a, C3b opsonised surfaces and tagged microorganisms. The intrinsic actions of anaphylatoxins C3a and C5a are mediated by receptors belonging to the seven transmembrane domain receptor family, C3aR, C5aR and the recently described C5a-receptor like 2 (C5L2) (Klos et al., 2009). The precise role of the latter remains to be determined, however it is thought to be a receptor for C5a, C5adesArg (Monk et al., 2007) and C3adesArg (Kalant et al., 2003). Currently the latter is a controversial topic.

2.8 Pathological role

Despite tight regulation of the complement system it can still cause self-harm and has been implicated in the pathogenesis of several inflammatory and immunological diseases, including RA (Linton and Morgan, 1999), multiple sclerosis (Morgan et al., 1984; Storch et al., 1998), glomerulonephritis (Welch, 2002) and atherosclerosis (Seifert and Kazatchkine, 1988). Although it may not be the initiating factor in these conditions, it is thought to promote and perpetuate inflammation resulting in acceleration of disease pathology. In such pathological conditions, whilst cell death is common, lysis may not be the dominant feature of complement activation *in vivo*. Instead complement activation may trigger cellular signalling events that contribute to worsening disease pathology.

Complement regulators	Complement mediated function	Reference
Soluble regulators		
C1NH	Binds and inactivates C1r and C1s and MASP2	(Davis et al., 2008)
C4BP	Accelerates the decay of the classical pathway C3 convertase and is a cofactor for factor I	(Blom et al., 2004)
Factor H	Accelerates the decay of the alternative pathway C3 convertase and is a cofactor for factor I	(Józsi and Zipfel, 2008)
FHL1	Accelerates the decay of the alternative pathway C3 convertase and is a cofactor for factor I	(Zipfel and Skerka, 1999)
Properdin	Stabilises the alternative pathway C3 convertases	(Hourcade, 2006)
Factor I	Cleaves and inactivates C3b and C4b in the presence of cofactor	(Seya et al., 1995)
Clusterin	Inhibits MAC assembly	(Tschopp and French, 1994)
Vitronectin	Inhibits MAC assembly	(Milis et al., 1993)
Membrane-bound regulator		
MCP	Cofactor for factor I	(Seifert and Hansson, 1989a)
CD55	Decays the acceleration of C3 convertase	(Davitz, 1987; Kim and Song, 2006)
CD59	Inhibits MAC assembly	(Kim and Song, 2006; Meri et al., 1990)
CR1	Decays the acceleration of the alternative and classical pathway C3 and C5 and is a cofactor for factor I	(Saito et al., 1992)

Table 1.1 Complement regulatory proteins and their function

3. The role of Complement in atherosclerosis

3.1 Initiation of complement activation during atherosclerosis

Normal arterial intima lacks complement activation products, however, several activators of the complement system are known to reside within the vessel wall during the progression of atherosclerosis. These include enzymatically modified LDL (E-LDL), immune complexes containing IgG or IgM, apoptotic cells and pentraxins (including CRP). In addition, other complement activators found within atherosclerotic lesions include bacterial pathogens *Chlamydia pneumoniae* (Campbell and Kuo, 2003; Megran et al., 1985), cholesterol crystals (Seifert and Kazatchkine, 1987) and cell debris (Pinckard et al., 1975).

Immune complexes are the most well defined activators of the classical pathway of complement but also they have been found to accelerate activation of the alternative pathway by stabilising the alternative pathway C3 convertase (Ji et al., 2002). Since there are antigens present within the atherosclerotic wall, against which a humoral response is generated, it is then reasonable to presume that immune complexes also reside within the lesion. In fact, both IgG and IgM have been detected in human atherosclerotic lesions (Hollander et al., 1979; Vlaicu et al., 1985b). In addition, autoimmune complexes against oxidised LDL are present within the atherosclerotic lesion and are able to activate the classical pathway of the complement cascade (Freire de Carvalho et al., 2007; Saad et al., 2006).

During the progression of atherosclerosis, lipoproteins that become trapped within the subendothelial space undergo modifications and trigger local complement activation.

Within human atherosclerotic plaques modified lipoproteins have been shown to activate complement via the alternative pathway and these lipids extracted from atherosclerotic lesions were bound to MAC (Seifert et al., 1990). In addition, LDL treatment with enzymes such as trypsin, cholesterol esterase, and neuraminidase transforms LDL to particles with properties akin to those of lipid extracted from atherosclerotic lesions (E-LDL), able to activate the alternative pathway of complement through to activation of the terminal pathway resulting in MAC formation (Bhakdi et al., 1995). Co-localisation of MAC with E-LDL has been found even at early stages of the disease (Torzewski et al., 1998b).

Another activator of the complement system, CRP, has also been shown to be present in human atherosclerotic lesions (Reynolds and Vance, 1987). CRP can bind to phosphocholine expressed on the surface of dead or dying cells in order to activate the classical complement activation pathway, via C1q (Gershov et al., 2000; Thompson et al., 1999). In early atherosclerotic lesions, re-modelling of LDL exposes phosphocholine head groups for CRP binding, which in turn activates the classical pathway (Bhakdi et al., 1999). In both instances, the effect of CRP mediated classical activation in the plaque results in the clearance and the removal of target structures by phagocytosis.

More recently the monomeric form of CRP (mCRP) has been implicated in modulating complement activation during atherosclerosis. mCRP is formed from native, pentameric CRP in certain conditions such as low pH, which may be found at sites of inflammation (Diehl et al., 2000). Activation of the classical pathway occurs by immobilisation of mCRP to modified lipoproteins, in contrast, fluid phase mCRP

prevents activation of the terminal pathway by binding to C1q and thus inhibiting its binding to other complement activators (Ji et al., 2006).

3.2 Effects of activation pathways of complement during atherosclerosis

All three activation pathways of the complement system have now been implicated in the pathogenesis of atherosclerosis (Niculescu and Rus, 2004). Evidence of complement proteins, regulators and receptors deposited in human atherosclerotic lesions is abundant. C3 was the first complement component to be detected in human atherosclerosis (Hollander et al., 1979). Since then, studies have shown deposition of numerous classical and alternative components and effector molecules within the upper intimal layer. These include C3a, C5a (Oksjoki et al., 2007c), C1q, C4 and C3c (Vlaicu et al., 1985b). Whilst the regulators of the classical and alternative pathways, C4b-binding protein (C4bBP) and fH respectively, are only present in the upper intimal layer, alternative pathway modulators including properdin have also been found in the deeper intimal layer (Torzewski et al., 1998b). Whilst CRP and immunoglobulins are thought to initiate the classical pathway at the upper intimal layer, E-LDL has been suggested to activate the alternative pathway at deeper sites within the lesion (Széplaki et al., 2009).

Anaphylatoxins C3a and C5a, key products of the activation pathways, have been shown to have important roles within the progressing atherosclerotic plaque. Both cause downstream signalling events by binding to their respective receptors, C3aR and C5aR. These receptors are expressed on many of the cellular lineages found within the plaque including macrophages, T cells, mast cells, endothelium and SMCs (Oksjoki et al., 2007c). Both C5a and C3a have potent chemotactic properties where they are

known to attract immune cells including monocytes, mast cells and T cells to the site of injury (Guo and Ward, 2005; Zwirner et al., 1998a; Zwirner et al., 1998b).

Other roles have been suggested for C5a and C3a during atherosclerosis apart from chemotaxis. These include the role of C5a in plaque stabilisation by up-regulating plasminogen activator inhibitor-1 (PAI-1) expression in human macrophages (Kastl et al., 2006). In vitro studies have also demonstrated a role for C3a in plaque growth, where C3a was shown to induce cellular proliferation in both macrophages and vascular SMCs (Verdeguer et al., 2007). However, in contrast to these results animal studies on C3 deficiency in an atherosclerotic prone mouse model showed a protective role for C3 in atherosclerosis (Buono et al., 2002; Persson et al., 2004).

Opsonins are products of the activation pathways of the complement system involved in facilitating the clearance of foreign particles and pathogens by phagocytic cells. This mechanism, also used to help maintain healthy tissues by clearing unwanted matter such as cell debris, involves coating the target surface with opsonins (breakdown products of both C4 and C3 such as C4b, C3b and iC3b) and subsequent binding and activation of complement receptors present on phagocytes, triggering phagocytosis (see table 3.1 for a list of complement receptors and ligands). In the context of atherosclerosis, CR1, CR3 and CRig are all expressed on macrophages (Saito et al., 1992; Seifert and Hansson, 1989a; Zipfel and Skerka, 2009). CRig has recently been implicated in causing plaque inflammation during atherosclerosis by stimulating MMP-9 and IL-8 secretion from macrophages through the binding of C3b (Lee et al., 2006).

3.3 Role of the terminal pathway

3.3.1 Localisation of MAC in the atherosclerotic plaque

In atherosclerosis, MAC deposition has been shown to correlate with disease severity (Niculescu et al., 1987a) and has been detected in atherosclerotic lesions of all stages of the disease (Oksjoki et al., 2007a; Torzewski et al., 1997; Vlaicu et al., 1985c). Similar to the early activation products of complement, MAC is absent from normal arterial wall. However, as first shown in hypercholesterolemic rabbits, MAC deposition within the intima is an early event during atherosclerosis, since in the subendothelium MAC deposition occurs temporally with cholesterol accumulation and this occurs before monocyte infiltration and foam cell formation (Seifert et al., 1989).

In the upper intima layer, several components of the early activation pathways but not the terminal pathway have been shown to localise. The absence of the terminal pathway at this locality is thought to be due to the presence of regulators of the activation pathways (Oksjoki et al., 2003; Oksjoki et al., 2007b; Széplaki et al., 2009), acting at the C3 stage, consequently preventing activation proceeding to the terminal pathway. Instead, complement activation is thought to only proceed to the terminal stage at deeper sites within the mucoelastic layer of the intima, (Torzewski et al., 1998b) where MAC was found to co-localise with the positive regulator of the alternative pathway properdin (Oksjoki et al., 2007a). A strong correlation was found between localisation of properdin and MAC deposition. In contrast, only weak staining for IgM or C4 was seen in areas that showed MAC deposition, suggesting that the terminal pathway is activated through the alternative pathway. Within the deep area of the intima, MAC has also been found to co-localise with SMCs (Torzewski et al., 1997), CRP (Torzewski et al., 1998a), apoptotic cells (Niculescu et al., 2004) and modified lipoproteins

(Torzewski et al., 1998b). SMCs may be the first target of MAC attack, since these cells are suggested to lack the MAC inhibitor, CD59 (Seifert et al., 1992); however, CRP, apoptotic cells and modified lipoproteins are all potential complement activators within the plaque (discussed in section 3.1 “Initiation of complement activation during atherosclerosis”).

Lysis of nucleated cells by MAC is a rare event *in vivo* since several protective mechanisms exist including the widespread expression of the MAC inhibitor CD59 (Meri et al., 1990) and exo- and endo-cytosis of the MAC complex (Morgan et al., 1987). However, most frequent, is the effect of non-lytic deposition of MAC, which may trigger several signalling cascades (Cole and Morgan, 2003; Fosbrink et al., 2006). In the context of atherosclerosis, cell signalling studies on the effects of sub-lytic MAC deposition have implied several roles for the terminal pathway in atherosclerotic plaque formation (see figure 1.6).

3.3.2 MAC and endothelial cells

Studies have demonstrated that sublytic amounts of MAC on endothelial cells induce the expression of the P- and E-selectins, intracellular adhesion molecules (such as ICAM-1 and VCAM-1), together with MCP-1 secretion, all of which are involved in recruitment of monocytes to the atherosclerotic vessel wall (Kilgore et al., 1997; Kilgore et al., 1995; Tedesco et al., 1997; Tran et al., 2002). The cytokines IL-1 β and IL-8 are also up-regulated in response to MAC on endothelial cells, contributing further to the inflammatory process (Kilgore et al., 1997; Saadi et al., 2000).

MAC is also thought to play a role in plaque neovascularisation, where a study using cultured aortic endothelial cells showed that pre-incubation with sublytic doses of MAC caused cell proliferation and migration (Fosbrink et al., 2006). Furthermore, with the use of specific inhibitors, they showed that this effect occurred through activation of the Akt pathway, and was dependent on the inactivation of the transcription factor forkhead box (subclass o1) (Fosbrink et al., 2006). Further evidence of the involvement of MAC in endothelial proliferation was suggested by studies which showed that the MAC caused the release of basic fibroblast growth factor and platelet derived growth factor (Benzaquen et al., 1994).

In addition, in vitro studies have shown that sublytic amounts of MAC on cultured human endothelial cells induce the release of both von Willebrand factor (vWF) (Hattori et al., 1989) and tissue factor (Tedesco et al., 1997), implicating a role for MAC in promoting platelet aggregation and thrombosis.

3.3.3 MAC and SMCs

The assembly of the MAC on vascular SMCs causes the release of MCP-1 (Torzewski et al., 1996) and IL-6 (Viedt et al., 2000). MAC was also found to promote SMC proliferation via Phosphoinositide 3-kinase (PI3K)/Akt pathway (Niculescu et al., 1999), and inhibit apoptosis of vascular SMC secreting insulin like growth factor 1 (Zwaka et al., 2003). In contrast, at high concentrations, MAC is capable of stimulating caspase activation and apoptosis (Nauta et al., 2002a), in addition, MAC co-localises with apoptotic cells in atherosclerotic lesions (Niculescu et al., 2004), thus implying that MAC may be involved in apoptosis of vascular cells.

3.3.4 MAC and macrophages

Macrophages, the ubiquitous cell type within a lesion, have also been found to co-localise with MAC within the plaque (Rus et al., 1988). Studies on the effect of MAC on phagocytes, such as macrophages, have shown that sublytic doses of MAC can cause profound activation, with the production and secretion of inflammatory mediators including, prostaglandins, leukotrienes and ROS (Hänsch et al., 1984), all of which are known to be involved in atherosclerosis (Cipollone, 2005; Jala and Haribabu, 2004; Kaneto et al., 2010).

3.4 Complement regulation and atherosclerosis

Both soluble and membrane bound regulators exist within atherosclerotic plaques (Niculescu et al., 1990; Seifert and Hansson, 1989a; Seifert et al., 1992; Yasojima et al., 2001). These regulators are critical to prevent complement from attacking host cells and protect host tissue from damage caused by uncontrolled complement activation.

Regulators of the complement system that are involved in atherosclerosis are listed in table 1.2.

3.4.1 Fluid phase regulators

Fluid phase inhibitors of both the activation and terminal stages of the complement system have been detected within atherosclerotic lesions. The fluid phase inhibitor of the classical pathway C4bBP is found in the upper intimal layer (Oksjoki et al., 2007b), while, deeper within the intimal layers, regulators of the alternative and terminal pathway can be found, including properdin, clusterin and S-protein (Mackness et al., 1997; Niculescu et al., 1987b; Oksjoki et al., 2007b).

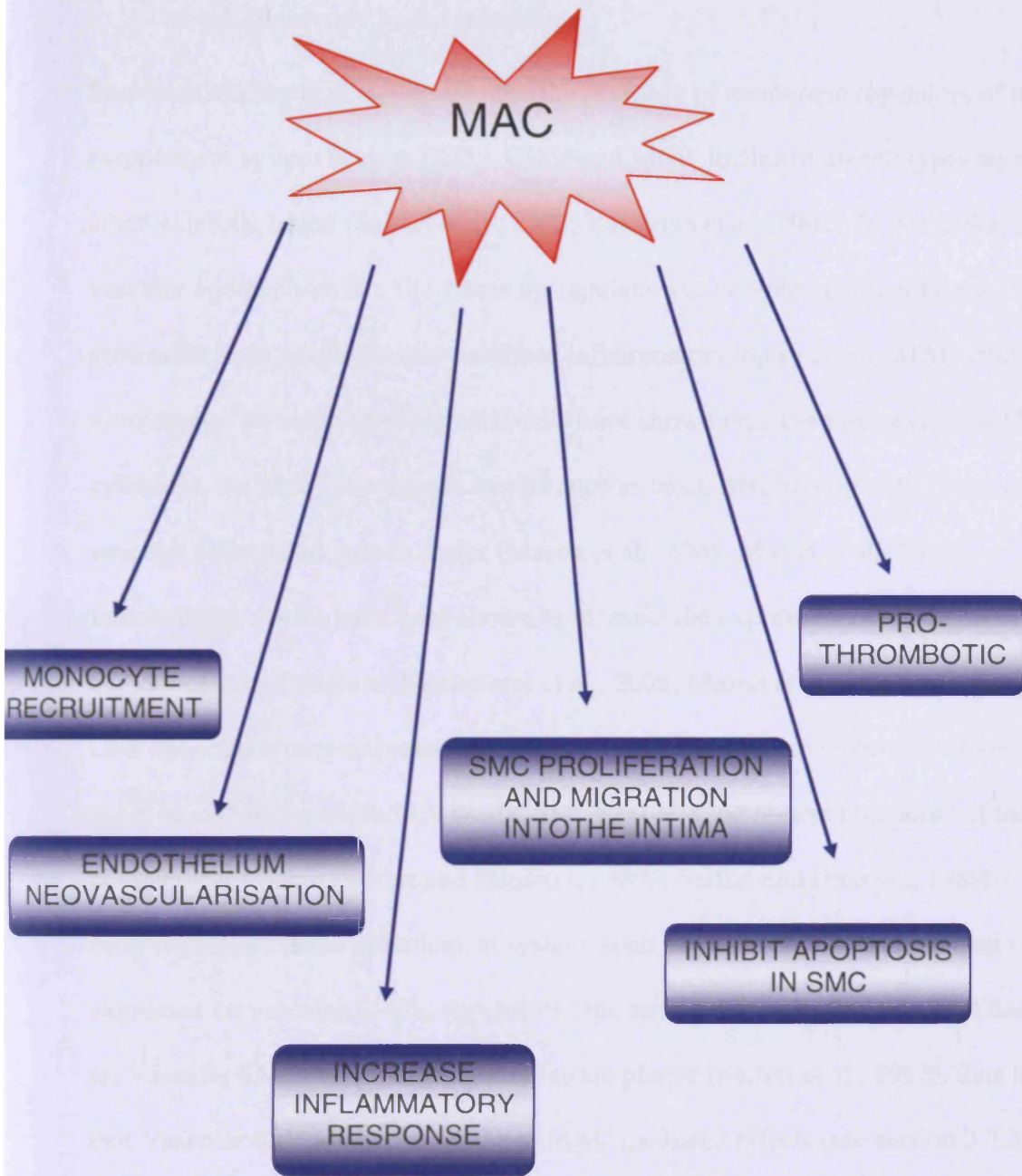


Figure 1.6 Summary of the diverse roles of MAC during the progression of atherosclerosis. A diagram illustrating the range of cellular effects of MAC during the progression of atherosclerosis.

3.4.2 Membrane bound regulators

Immunohistochemical studies showed the presence of membrane regulators of the complement system such as CD59, CD55 and MCP, in almost all cell types within the atherosclerotic lesion (Seifert et al., 1992; Yasojima et al., 2001). In particular, the vascular endothelium is a site where up-regulation of complement regulators offers protection from anaphylatoxin-mediated inflammatory injury and/or MAC attack. In vitro studies on cultured endothelial cells have shown increased expression of CD55 by cytokines, the MAC and growth factors such as basic fibroblast growth factor and vascular endothelial growth factor (Mason et al., 2001; Mason et al., 1999).

Interestingly, statins have been shown to increase the expression of CD55 and CD59 on the vascular endothelium (Kinderlerer et al., 2006; Mason et al., 2002). Furthermore, CRP can induce over-expression of CD55, CD59 and MCP in cultured endothelial cells (Li et al., 2004). In addition, vascular SMCs express membrane regulators of the complement system (Seifert and Hansson, 1989a; Seifert and Hansson, 1989b). While early regulators of the complement system, such as CD55 and MCP are constitutively expressed on vascular SMCs, regulator of the terminal MAC, CD59 was not found on all Vascular SMCs within the atherosclerotic plaque (Seifert et al., 1992), thus implying that Vascular SMCs are more prone to MAC mediated effects (see section 3.3.3).

Complement regulators	Atherosclerotic cell type	Reference
CD55	Endothelial cells	(Seifert and Hansson, 1989a)
	Macrophages	(Oksjoki et al., 2007a)
	Vascular SMCs	(Seifert and Hansson, 1989b)
CD59	Endothelial cells	(Meri et al., 1991; Seifert et al., 1992)
	Macrophages	(Ross, 1999; Seifert et al., 1992)
	Vascular SMCs	(Oksjoki et al., 2007a)
	T cells	(Seifert et al., 1992)
CR1	Macrophages	(Saito et al., 1992)
CRig	Macrophages	(Lee et al., 2006)
MCP	All nucleated cells	(Liszewski et al., 1991)

Table 1.2 Membrane bound complement regulators are present within cells that are involved in atherosclerosis.

3.5 Complement in animal models of atherosclerosis

Human studies have provided strong evidence of the involvement of complement during the progression of atherosclerosis; however, data from animal studies contradict one another (Bhatia et al., 2007; Buono et al., 2002; Geertinger and Soerensen, 1977; Lewis et al., 2010; Matthijsen et al., 2009; Pang et al., 1979; Patel et al., 2001; Persson et al., 2005; Persson et al., 2004; Schepers et al., 2006; Schmiedt et al., 1998; Seifert et al., 1989; Tanhehco et al., 2000; Thorbjornsdottir et al., 2005; Verdeguer et al., 2007). From early studies in hypercholesterolemic rabbits to the more recent apoE^{-/-} and ldlr^{-/-} mouse models of atherosclerosis, scientists fail to agree on whether complement overall plays an anti-atherogenic role, a pro-atherogenic role, or indeed if it plays any role at all in atherosclerosis.

In 1977, Geertinger and Sorensen were the first to demonstrate a pro-atherosclerotic role for complement, when they found that hypercholesterolemic rabbits on a C6 deficient background displayed reduced atherosclerosis compared to C6 sufficient animals (Geertinger and Soerensen, 1977). This was confirmed much later with data from another study using C6 deficient rabbits and with better controlled conditions (Schmiedt et al., 1998).

C5 deficiency had no effect on atherosclerosis in the aortic root of apoE^{-/-} mice after 18 weeks on a high fat diet in comparison to the control group. Lipoprotein profiles also remained the same between the two groups (Patel et al., 2001). In contrast, New Zealand white rabbits in an ex vivo model of ischemia and reperfusion showed a cardioprotective effect of C5a. In this study isolated hearts, mounted on a Langendorff apparatus, were subjected to 30 minutes of ischemia and one hour reperfusion prior to

20 minutes of treatment with either normal human plasma alone or in addition to anti-C5a monoclonal antibody (mAb), or recombinant C5a. They showed that sub-lytic doses of complement in human plasma increased protection against infarcts, which was abolished with anti-C5a antibody (Tanhehco et al., 2000).

C3 deposition was found in atherosclerotic lesions of hypercholesterolemic rabbits as early as 1979 (Pang et al., 1979). More recently however, deletion of C3 in both apoE^{-/-}/ldlr^{-/-} and in ldlr^{-/-} mice was shown to cause a worsening of the disease, implying that C3, the central orchestrator of all complement activation pathways, has an anti-atherogenic role (Buono et al., 2002; Persson et al., 2004). In addition, C3 deficiency caused altered lipoprotein profiles in the apoE^{-/-}/ldlr^{-/-} deficient mouse model. To delineate which activation pathway of the complement system was involved, the effect of fB deficiency in apoE^{-/-}/ldlr^{-/-} mice was examined (Persson et al., 2004). Whether at the aortic root or in *en face* preparations of the aorta, the extent of atherosclerosis was similar between test and control, and there was no difference in lipoprotein profiles. Taken together these data suggest that the atheroprotective C3 is either independent of complement or produced via the classical or MBL pathways, and causes alterations in lipid metabolism resulting in a reduction in plasma lipid levels, consequently reducing the amount of lipid build up within the vascular wall. In contrast, another group demonstrated a pro-atherosclerotic role for C3 during vein graft thickening in hypercholesterolemic apolipoprotein E3-leiden transgenic mice (Schepers et al., 2006).

The first component of the classical pathway, C1q, has also been shown to have a protective role in atherosclerosis in the aortic root of ldlr^{-/-} mice (Bhatia et al., 2007). In this study there was no significant difference in lipoprotein profiles between C1qa^{-/-}

ldlr^{-/-} and the *ldlr*^{-/-} controls. The authors concluded that the protective effect of C1q during atherosclerosis might be through accelerating the clearance of apoptotic cells.

3.6 Autoimmune disease and atherosclerosis

Atherosclerosis is now acknowledged as a common cause of mortality in patients suffering from autoimmune diseases such as systemic lupus erythematosus (SLE) and RA (Abou-Raya and Abou-Raya, 2006). In both SLE and RA, the classical risk factors of atherosclerosis such as smoking and hypertension, have been found to further increase the incidence of atherosclerosis (Dessein et al., 2007; Urowitz et al., 2007). In both SLE and RA patients increased systemic inflammatory modulators are present and are thought to be the mechanism linking these diseases to atherosclerosis (Abou-Raya and Abou-Raya, 2006).

3.6.1 SLE and atherosclerosis

SLE is an autoimmune rheumatic disease that has a late mortality phase owing mainly to accelerated atherosclerosis. Patients with deficiencies of components of the classical pathway of complement, such as C1, C2 and C4 develop an SLE-like illness (Manderson et al., 2004). This has been suggested to result, at least partly, from failure of complement dependent B-cell tolerance, and ineffective clearance of apoptotic cells and immune complexes (Carroll, 2004; Pickering et al., 2000). A study on the effect of C2 deficiency in the Swedish population found a significant increase in atherosclerosis; indeed, the correlation between atherosclerosis and C2 deficiency was greater than between atherosclerosis and the presence of SLE in the patients (Jönsson et al., 2005).

Of other complement deficiencies, genetic defects of MBL have also been associated with arterial events in SLE (Rugonfalvi-Kiss et al., 2002; Øhlenschlaeger et al., 2004).

3.6.2 RA and atherosclerosis

RA is another example of an autoimmune disease which is associated with higher risk of cardiovascular complications, including atherosclerosis, resulting in early mortality and excess morbidity (Tanasescu et al., 2009). In 1989, researchers looking into the role of complement in RA and its association with vascular complications found that RA patients with vasculitis, also called malignant RA, have increased consumption of complement compared to RA patients without vascular complications. In this study, malignant RA patients had decreased levels of complement components C3 and C4 in their serum, and an increase in the activation product of C3, C3d, compared to RA patients (Tomooka, 1989). Another study analysed the rate of increase of carotid intima medial thickness in patients with RA according to disease duration, showing a higher rate in more prolonged disease (Del Rincón et al., 2007).

4. Lipid Metabolism

4.1 Introduction

To fully understand and appreciate the role of complement during the pathogenesis of atherosclerosis, we must also look at its role in lipid metabolism since a defect in lipid metabolism is the most significant risk factor for coronary artery disease and stroke (Lusis et al., 2004b).

4.2 Lipoprotein structure and function

4.2.1 Lipoprotein structure

Lipoproteins are globular complexes and vary in size, density, lipid composition and apolipoprotein composition (Feher and Richmond, 2001) (see figure 1.7). The outer membrane is made of polar lipids such as phospholipids and unesterified (free) cholesterol and the inner core contain hydrophobic lipids (such as triglycerides and esterified cholesterol). They also contain apolipoproteins, which span from the inner core to the outer membrane, and a variety of enzymes, such as PLA₂ and cholesterol acyltransferase.

4.2.2 Function of lipids and lipoproteins

The main function of lipoproteins is to transport lipids such as cholesterol and triglycerides around the body. Both of these lipids have a variety of important roles in biological functions. Cholesterol is an integral component of cell membranes and a precursor for many biologically active molecules such as vitamin D, steroid hormones

and bile acid (Hegele, 2009). It has also been implicated in activating neurological signalling cascades involved in memory and learning (Kotti et al., 2006). Triglycerides provide a source of energy for the body (Hegele, 2009). Thus, due to the critical functions of these lipids, an efficient transport system within the body is crucial to maintain a balanced and well regulated system (Feher and Richmond, 2001).

The lipid transport system is thus made of several different lipoproteins, namely, chylomicrons, VLDL, intermediate low density lipoprotein (IDL), LDL and HDL, all of which have specific functions to carry out during lipid metabolism.

4.3 Lipid metabolism: an overview

4.3.1 Exogenous pathway

Lipid metabolism consists of two major pathways; exogenous and endogenous (see figure 1.8). Both are equally important but very different in their function.

Exogenous transport begins at the epithelial lining of the small intestine where lipids are absorbed from the diet. Eventually entering enterocytes, reconstituted triglycerides are packaged with cholesterol esters and apoB48 into chylomicrons. These chylomicrons are then secreted from the intestinal epithelial cells and enter the circulation. Subsequently, HDL particles present in the blood transfer apoC and apoE to the chylomicrons rendering the chylomicrons mature.

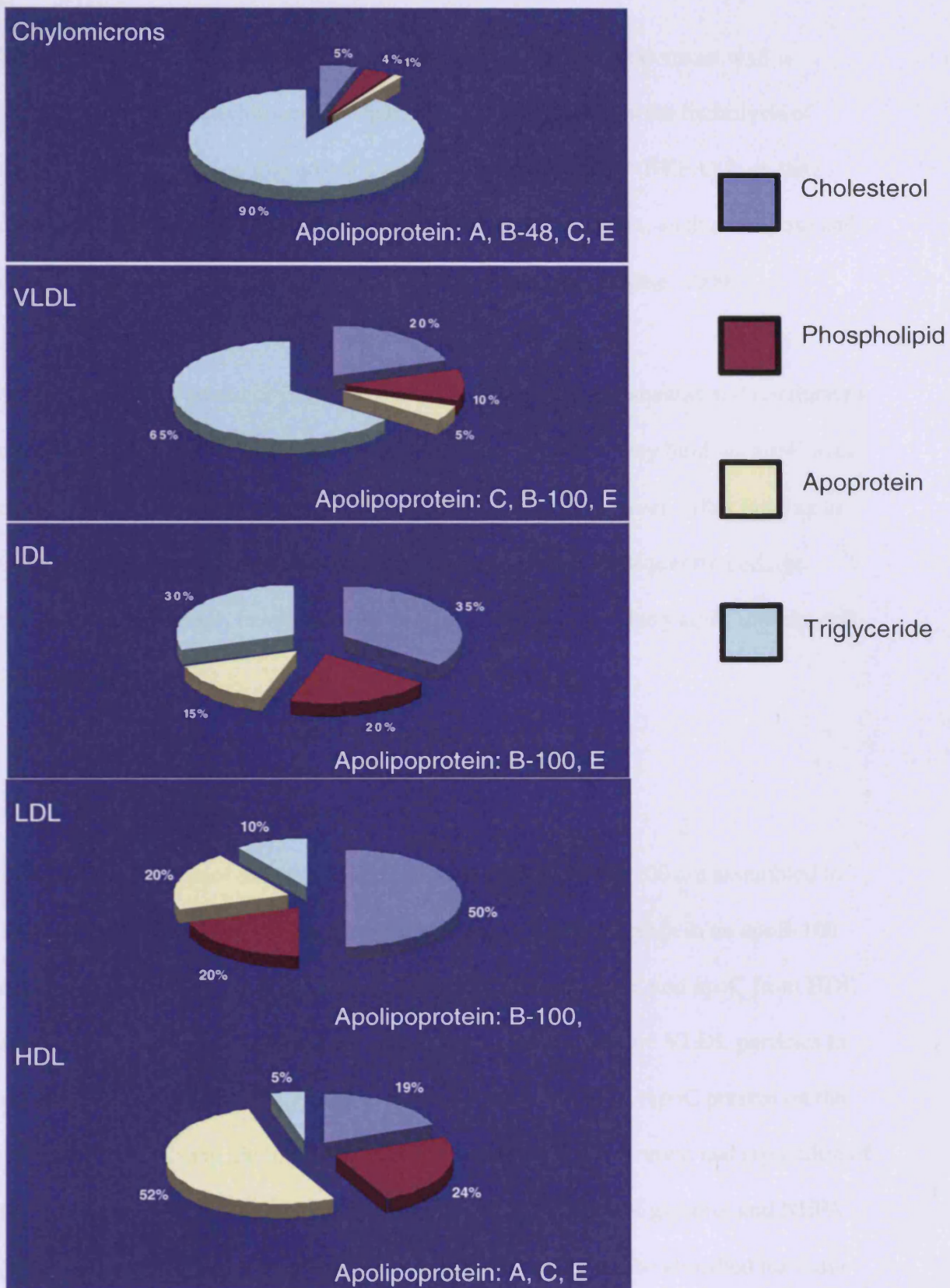


Figure 1.7 Lipoprotein composition. The approximate percentage composition of cholesterol, triglyceride, phospholipid and protein in chylomicrons, VLDL, IDL, LDL and HDL are shown.

Adapted from (Feher and Richmond, 2001).

Lipoprotein lipase (LPL) present on endothelial cells of the blood vessel wall is activated by mature chylomicrons. Upon activation, LPL causes the hydrolysis of triacylglycerol, releasing glycerol and non esterified fatty acids (NEFA) from the chylomicrons. These are then taken up into the peripheral tissues, such as adipose and muscle, where they can either be stored or used for energy (Hegele, 2009).

At this point, hydrolysed chylomicrons become chylomicron remnants and continue to circulate around the body. Circulating chylomicron remnants may bind via apoE with chylomicron remnant receptors which are found mostly in the liver. After binding to their receptors, chylomicron remnants are internalised and subsequently undergo lysosomal degradation, resulting in the release of glycerol and fatty acids into the cell (Hegele, 2009).

4.3.2 Endogenous pathway

In the liver, cholesterol and triacylglycerol together with apoB-100 are assembled to form VLDL particles, which are then released into the blood stream in an apoB-100 dependent manner. Once in the circulation, VLDL absorbs apoE and apoC from HDL and VLDL is then rendered “mature” (Havel et al., 1973). Mature VLDL particles in the circulation engage with LPL expressed on endothelial cells. ApoC present on the surface of VLDL (and chylomicrons) is responsible for the activation and regulation of LPL, causing hydrolysis of the VLDL particle and the release of glycerol and NEFA (Alipour et al., 2008). As mentioned above, these products can be absorbed for tissue utilization or storage and the hydrolysed VLDL particles become IDLs (or VLDL remnants). Circulating IDLs are absorbed by the liver via an interaction between apoE and the remnant receptor *ldlr*, or they can be further hydrolysed by hepatic lipase. In

this latter case, glycerol and fatty acids are released, leaving behind IDL remnants, or LDL particles, containing high amounts of cholesterol. Circulating LDL can be absorbed by the liver and peripheral cells by binding Ldlr. Once within the cell LDL undergoes lysosomal degradation; the apoB of the complex is hydrolysed to its constituent amino acids and the cholesteryl esters are hydrolysed to free cholesterol.

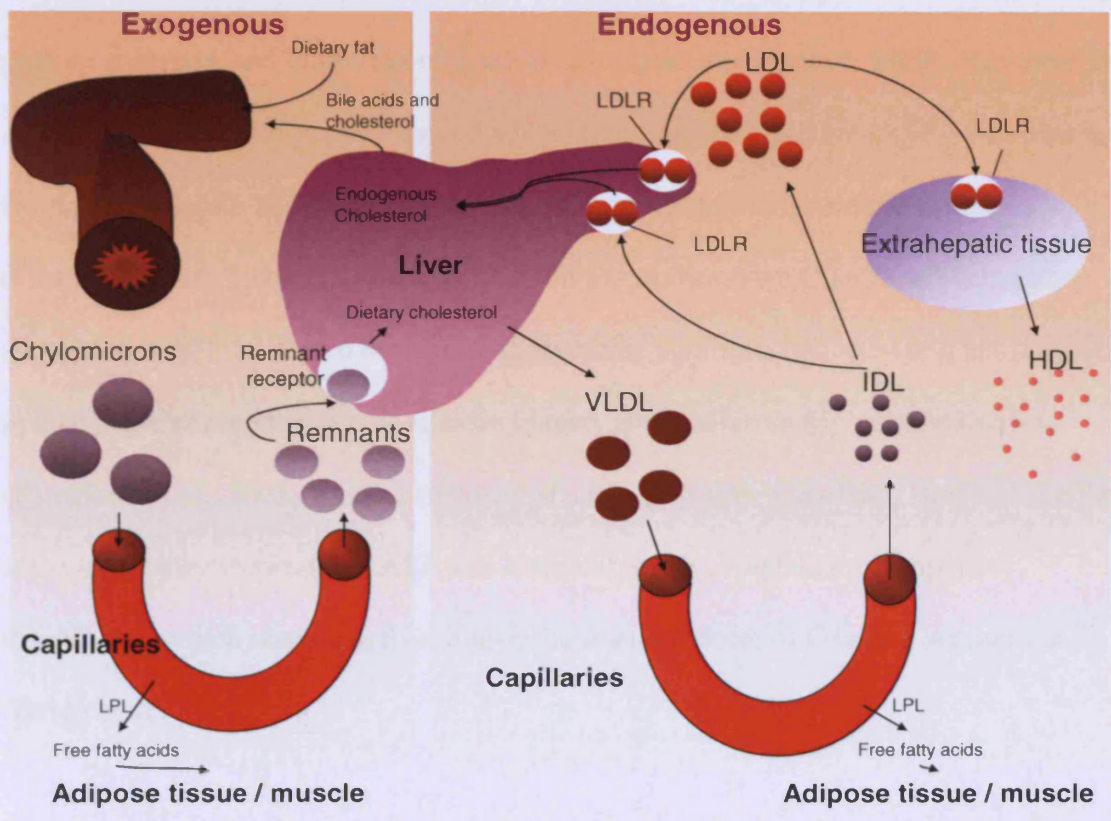


Figure 1.8 Overview of lipoprotein metabolism. A schematic diagram of the exogenous and endogenous lipoprotein transport system. Adapted from (Feher and Richmond, 2001).

4.4 The role of C3adesArg in lipid metabolism

Adipose tissue is the main site of the body for storing energy in the form of triglyceride (Lafontan, 2008). It also serves as an endocrine organ by producing hormones such as leptin, resistin and adiponectin (Hamdy, 2005). Until recently, the hormone insulin was believed to be the sole regulator of triglyceride synthesis in adipocytes. The action of insulin via the insulin receptor causes increased LPL activity (Sadur and Eckel, 1982), glucose transport and inhibition of lipolysis (Strålfors and Honnor, 1989). However, in 1989, a 76 amino acid peptide named acylation stimulating protein (ASP), was purified from human serum and shown to play an important role in triglyceride synthesis (Cianflone et al., 1999; Cianflone et al., 1989a). Studies from Cianflone's laboratory showed that ASP increased the rate of triglyceride synthesis by increasing net fatty acid uptake, accelerating the rate of glucose uptake, and inhibiting triglyceride lipolysis (Cianflone et al., 2003; Van Harmelen et al., 1999). A few years later, studies from the same laboratory showed that ASP was identical to the complement component C3adesArg, which is the inactive (non-inflammatory) form of C3a (see section 2.2.2) (Baldo et al., 1993).

C3 is expressed in adipose tissue along with other components of the complement system, including fB and fD, the latter is also termed adipsin in the context of lipid metabolism (Choy et al., 1992). Whilst, C3adesArg has lost the anaphylatoxic and chemotactic activities of its precursor C3a, it has gained new roles in triglyceride synthesis. Firstly, C3adesArg is responsible for stimulating triglyceride synthesis by activating the enzyme involved in the final step of triglyceride synthesis, diacylglycerol acyltransferase (DGAT) (Yasruel et al., 1991). Secondly, it increases glucose uptake into the cell (Germinario et al., 1993; Maslowska et al., 1997b) by causing translocation

of the glucose transporters (GLUT) 1, GLUT 3 and GLUT 4 from the cytoplasm to the plasma membrane (Germinario et al., 1993). Glucose is essential for triglyceride synthesis, since it is required for the production of glycerol-3-phosphate to which the free fatty acid is linked. Moreover, a series of in vitro experiments showed that exposure of cultured human skin fibroblasts, rat L6 muscle cell line and cultured human adipocytes to C3adesArg caused an increase in glucose uptake in an insulin independent manner (Germinario et al., 1993; Maslowska et al., 1997b; Tao et al., 1997). This research also provides evidence that the effect of C3adesArg on triglyceride synthesis occurs in both adipose tissue and muscle (Tao et al., 1997).

However, the exact mechanism by which C3adesArg stimulates its activities during triglyceride synthesis is unclear. Whilst some research groups are of the opinion that C3adesArg mediates its effects by binding to C5L2 receptor (Kalant et al., 2003); others suggest that C5L2 is merely a decoy receptor (Johswich et al., 2006).

However, signalling studies on the interactions between C3adesArg and C5L2 have shown that C3adesArg increased triglyceride synthesis by activating PI3K and phospholipase C, with downstream activation of PKC, Akt, MAPK/ERK(1/2), and PLA₂ (Maslowska et al., 2006). Using cultured human adipocytes, the same group have also shown that whilst insulin is able to stimulate the production of C3 and C3adesArg in the medium up to two fold, chylomicrons are able to increase their production by more than 100 fold (Maslowska et al., 1997a).

In humans, an increase in plasma C3adesArg is detected in several conditions such as obesity, insulin resistance, cardiovascular disease and dyslipidaemia (Cianflone et al.,

2008). In contrast, weight loss, increased exercise and hypothyroidism are all associated with decreased levels of plasma C3adesArg (Cianflone et al., 2008). Human studies have also demonstrated a direct correlation between fasting plasma C3adesArg levels and postprandial triglyceride and NEFA clearance (Cianflone et al., 2004).

In vivo, the effects of C3adesArg on lipid metabolism have also been highlighted in animal models of obesity. C3adesArg levels were increased in the obese rat model when compared with its lean control, suggesting that adipose tissue served as a generator from which increased amounts of C3adesArg could be produced (Boggs et al., 1998). In accordance with this, plasma C3adesArg levels were also significantly increased in the obese leptin deficient mouse model, when compared to C57BL/6 controls (Paglialunga et al., 2008).

Mouse models of obesity, including ob/ob mice and db/db mice (obese diabetic) have changes in their plasma lipid profiles (Nishina et al., 1994a; Saleh et al., 2001). Db/db mice have increased baseline levels of both triglyceride and glucose while total cholesterol and insulin levels are similar to their C57BL/6 controls, whilst ob/ob mice have increased baseline levels of total cholesterol, glucose and insulin levels compared to their C57BL/6 controls. To explore the cause of the altered plasma lipid profiles in these mice, the effects of C3adesArg on postprandial lipid levels were measured and compared to their C57BL/6 controls (Saleh et al., 2001). Both models of obese mice had delayed postprandial triglyceride and NEFA clearance after an oral fat feed. However, both postprandial plasma triglyceride and NEFA clearance were increased following an intraperitoneal injection (i.p) with C3adesArg (Saleh et al., 2001). In fact, the rate of clearance of both triglyceride and NEFA were comparable to the rate of

clearance in the C57BL/6 controls. Of interest, both db/db and ob/ob mice have increased resistance to the development of atherosclerosis when fed a high fat diet compared to the C57BL/6 controls (Nishina et al., 1994b).

The importance of C3adesArg in lipid metabolism is further highlighted in C3 deficient mice. These mice, also deficient in C3adesArg, have delayed postprandial plasma triglyceride clearance and increased fasting triglyceride levels (Murray et al., 1999a; Pagliialunga et al., 2008). The mice also have delayed NEFA clearance (Murray et al., 1999a) and increased energy intake with modest changes in insulin/glucose metabolism, implying increased insulin sensitivity. The mice are lean and specific adipose tissue depots are reduced by up to 26% in comparison to control mice on both low and high fat diets (Murray et al., 1999b).

Animal studies from Cianflone's research laboratory have shown further evidence implicating a role for C5L2 receptor in lipid metabolism (Pagliialunga et al., 2007). They demonstrated that the C5L2^{-/-} mice on a high fat diet had delayed postprandial triglyceride, insulin and leptin clearance, with increased fasting levels of triglyceride, insulin and leptin when compared with litter-matched controls. Of note, the same study also revealed that the C5L2^{-/-} mice on a low fat diet displayed no difference in postprandial triglyceride clearance and fasting lipid levels when compared with litter-matched controls (Pagliialunga et al., 2007).

As for the regulation of C3 and C3adesArg in adipose tissue, complement regulatory proteins such as Complement receptor 1 related protein (Crry) and fH are expressed in pre-adipocytes and were found to be down regulated post-adipocyte differentiation

(Choy and Spiegelman, 1996). Thus the pathway of regulation of C3adesArg production during lipid metabolism is still unknown.

4.5 Lipid metabolism alteration in atherosclerosis

The major lipid alterations associated with the progression of atherosclerosis include increases in concentrations of total and LDL cholesterol, and serum triglycerides, and a decrease in HDL cholesterol (Austin et al., 1998; Feher, 2003). Raised triglyceride levels have pro-atherogenic effects, including increased postprandial lipaemia and a shift in lipoprotein size and density (Alipour et al., 2008). An alteration in the size of LDL particles, from large less dense (anti-atherogenic) to small, dense particles (pro-atherogenic), allows movement of lipoprotein particles through the endothelium and into the vessel wall (Karpe et al., 1994). Furthermore, LDL, IDL, and chylomicron remnants that have high triglyceride content can more readily undergo oxidative modification, thus increasing foam cell production within the artery wall. Finally, increased susceptibility to oxidative modification in small/dense LDL particles attenuates the rate of clearance by its receptor, ldlr, consequently allowing prolonged residence in the plasma and exposure to the artery wall.

4.6 Lipid metabolic disorders associated with the complement system

Complement is known to play a key role in initiation and maintenance of inflammation and there is an increasing body of evidence to suggest that activation can have a profound effect on lipid metabolism. Components of the classical and alternative pathway are expressed in adipose tissue (Choy et al., 1992; Zhang et al., 2007), and all

three activation pathways of the complement system have been implicated in numerous disorders of lipid metabolism.

4.6.1 Obesity

Obesity, the most prevalent disease found in the western world, is closely associated with atherosclerosis. It is a chronic inflammatory disease that involves both innate and adaptive arms of the immune system (Rocha and Libby, 2009). Indeed, complement activation is thought to play a role, where activation of both the classical and alternative pathways has been implicated in the disease.

Whilst expression of the classical pathway C1q was up-regulated in adipose tissue in animal models of obesity (Zhang et al., 2007), mRNA levels of fD, of the alternative pathway, was drastically reduced in mouse models of obesity including, ob/ob, db/db and monosodium glutamate injected mice (Flier et al., 1987; Rosen et al., 1989).

Human studies have found increased expression of C1s and C1r detected in the adipose tissue of insulin-resistant humans when compared to insulin-sensitive control group (Zhang et al., 2007). In addition, serum levels of C3, fB, factor I (fI), fH and alternative pathway haemolytic activity (AP50) were increased in obese patients when compared to normal body weight controls (Pomeroy et al., 1997). Another study looking at the effect of obesity in young children found a positive correlation between plasma levels of C3 and its breakdown product C3adesArg and obesity (Cianflone et al., 2005).

4.6.2 *Acquired partial lipodystrophy (PLD)*

Another complement associated disease of lipid metabolism is PLD (also known as Barraquer-Simons syndrome). PLD is a rare form of lipodystrophy associated with fat loss around the face, upper trunk, and upper extremities. Simultaneously, fat hypertrophy occurs in the lower extremities. PLD is associated with activation of the alternative pathway and C3 deficiency (McLean and Hoefnagel, 1980), driven by an IgG autoantibody called C3 nephritic factor (C3Nef). C3Nef can bind and stabilise C3bBb, consequently accelerating both tickover and the alternative pathway amplification loop. In vitro studies showed that lysis of adipocytes can occur in the presence of both complement and C3Nef (Choy et al., 1992). Adipocytes synthesise C3, fB and fD allowing local production of the C3bBb without activating the lytic terminal pathway. However, C3NeF prevents the normally labile C3bBb from inactivation resulting in adipocyte lysis (Choy et al., 1992; Mathieson et al., 1993). The odd distribution pattern of fat loss is unexplained. However, fD, the limiting component of the alternative complement pathway (cleaves C3-bound fB to its active enzymatic form) (Choy et al., 1992), is expressed to a higher extent in adipose tissue in the upper half of the body than in the lower half. Regional differences in fD expression might thus explain why the pattern of fat loss is restricted to the upper half of the body (Mathieson and Peters, 1997). More recently, another form of PLD has been found with activation of the classical pathway and low levels of C4 (Savage et al., 2009).

5. Summary and hypothesis

Atherosclerosis, the leading cause of death in the western world, is a disease driven by chronic inflammation within the artery wall. Both the innate and adaptive immune systems are involved throughout disease progression; from initial stages of the disease where fatty streaks are formed within the artery wall, to the advanced stages where increased inflammation along with a large necrotic core and a thin fibrous cap causes the plaque to become “vulnerable” consequently leading to plaque rupture, thrombosis and even death.

Research into the field has greatly been aided through the use of animal models of atherosclerosis. In particular the apoE and ldlr deficient mouse models which, when fed appropriate diets, develop lesions with many of the characteristics of human atherosclerotic plaques. There are differences between the two mouse models of atherosclerosis. In particular plasma lipoprotein levels differ between the two models. On a normal chow, apoE^{-/-} mice have hypercholesterolaemia (~600mg/dl) in comparison with a slight increase in cholesterol levels in the ldlr^{-/-} mice (~250mg/dl). In addition, distribution of cholesterol differs between the two mouse models, with cholesterol accumulating in VLDL and IDL particles of apoE^{-/-} mice while in ldlr^{-/-} mice cholesterol is elevated in LDL particles. Metabolic response to dietary cholesterol is also different between the two mouse models of atherosclerosis (Joven et al., 2007).

In addition to genetic backgrounds, differences in strain, site of lesion and diet can also influence disease outcome. For instance, BALB/c mice are more resistant to atherosclerosis than C57BL/6 mice (Paigen et al., 1985). In addition, cholesterol

absorption can differ between various strains. For example, 129/Sv mice can more readily absorb cholesterol from their diet compared to C57/BL6 mice, thus resulting in hypercholesterolaemia which in turn influences the progression of atherosclerosis (Jolley et al., 1999). Differences in diet content can also influence progression of the disease. Joven *et al.* showed that changes in plasma cholesterol and triglyceride levels were dependent on the diet and strain (either apoE^{-/-} or ldlr^{-/-}) used (Joven et al., 2007). In addition, site of analysis at which atherosclerotic lesions are studied will also have an impact on the data. Lesion progression at the aortic sinus is evident in young apoE^{-/-} mice, whilst at 8 to 9 months of age, disease progression is throughout the arterial tree (Reddick et al., 1994).

Initially, this study set out to determine the role of complement during plaque rupture. Thus, a mouse model of atherosclerotic plaque rupture was chosen. Dr. Christopher Jacskon's laboratory at Bristol University has established this model using apoE^{-/-} mice. They have shown that the brachiocephalic arteries of these mice, which are on a mixed background (71% C57BL/6 and 29% 129/Sv), develop plaque rupture after 8 weeks of fat feeding (Johnson and Jackson, 2001). Of note, since the completion of this study it has been published that apoE^{-/-} mice on the C57BL/6 background can also be used as a model for plaque rupture (Reimers et al., 2011).

The involvement of complement in atherogenesis is evident from human studies that show deposition of complement proteins and activation products within the atherosclerotic plaque. However, data generated from animal studies have produced conflicting results. Whilst C3 deficient animals on an atherosclerotic prone background

have accelerated atherosclerotic plaque formation compared to their litter-matched controls, C6 deficient rabbits are protected against the disease.

Atherosclerosis is highly associated with defects in lipid metabolism. Both environmental and genetic risk factors can cause lipid disorders including familial combined hyperlipidemia (FCHL), obesity and type II diabetes. Such disorders usually present with increased LDL-cholesterol and triglyceride levels and decreased HDL-cholesterol levels allowing LDL cholesterol to build up in the artery wall. Complement has been associated with lipid metabolism. In particular C3adesArg is a key adipokine, increased levels of which are associated with elevated fasting and postprandial plasma triglyceride levels along with lipid disorders including obesity.

Pharmacological agents available for the treatment and prevention of atherosclerosis, such as statins, have many side effects. Thus the demand for a better drug is critical particularly with an increase in morbidity caused by vascular complications of obesity in the Western world.

The hypothesis for this study therefore is:

Both early activation and terminal pathways of the Complement system contribute to disease pathology in atherosclerotic plaques of apoE^{-/-} mice

The specific aims are twofold:

Part 1 aims to investigate the role of the MAC and its regulation in the progression of atherosclerosis. Firstly, by looking at the effect of dysregulated MAC formation in atherosclerosis, by generating apoE^{-/-}/CD59a^{-/-} mice and secondly, by studying the effect of suppression of MAC formation, by generating apoE^{-/-}/C6^{-/-} mice and by looking at the therapeutic effect of anti-C5 mAb.

Part 2 investigates the role of CD55 in regulating C3 activation in atherosclerosis and its involvement in lipid metabolism through the generation of apoE^{-/-}/CD55^{-/-} mice.

Chapter 2: Materials & Methods

1. Buffers and reagents

All standard laboratory chemicals were purchased from Sigma or Fisher Scientific.

2. Mice

Animals used for the thesis are listed below.

- B6.129-Cd59a^{tm1Bpm} (Cd59a^{-/-}) mice were generated as previously described (Holt et al., 2001) and back-crossed onto the C57BL/6 background for eight generations.
- C6-deficient mice, originally identified as a natural mutation in a wild mouse strain and bred onto the C3H/He background were back-crossed five generations onto C57BL/6 (Morgan et al., 2006).
- CD55 knockout (CD55^{-/-}) mice, were provided by Prof. Wenchao Song (University of Philadelphia) and back-crossed onto C57BL/6 for nine generations (Sun et al., 1999).
- ApoE knockout mice (apoE^{-/-}) mice were originally generated by J. Breslow (The Rockefeller University, New York) (Plump et al., 1992). The strain background of the apoE^{-/-} mice was 71% C57BL/6 and 29% 129/Sv.

Buffer name	Composition
PBS	8.2 mM Na ₂ HPO ₄ , 1.5mM KH ₂ PO ₄ , 137mM NaCl, pH 7.4
Oil red O (stock solution)	0.5% oil red O/isopropanol
Haematoxylin	50g aluminium potassium alum, 5g haematoxylin, 0.4g sodium iodate, 20ml glacial acetic acid make up to 1L dH ₂ O
Anti-coagulant solution	0.5M Ethylene diamine tetra-acetic acid (EDTA) pH8
CFD (Oxoid)	2.8mM Barbituric acid, 145.5mM NaCl, 0.8mM MgCl ₂ , CaCl ₂ , 0.9mM Sodium Barbital, pH 7.2
Diaminobenzidine (DAB) (Vector laboratories)	To 5.0 ml of dH ₂ O, add 2 drops of Buffer Stock Solution. Next add 4 drops of DAB Stock Solution. Finally, add 2 drops of the H ₂ O ₂ Solution. Between each step vortex the solution.
ELISA coating buffer	0.2M Na ₂ CO ₃ , pH9.6
SDS stacking buffer	0.5M Tris, 0.4% (w/v) SDS, pH 6.8
SDS resolving gel buffer	1.5M Tris, 0.4% (w/v) SDS, pH8.8
Non-reduced loading buffer	0.1M Tris, 10% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, pH6.8
Reduced loading buffer	5% (v/v) β mercaptoethanol in non-reduced loading buffer
Coomassie staining	0.2% (w/v) Coomassie blue R250 in 40% (v/v) methanol, 10% (v/v) acetic acid in dH ₂ O
De-staining solution	20% (v/v) methanol, 8% (v/v) acetic acid in dH ₂ O
Gel drying buffer	4% (v/v) glycerol, 20% (v/v) methanol in dH ₂ O

Table 2.1 Composition of buffers and reagents

ApoE^{-/-} mice were crossed with either CD59a^{-/-}, C6^{-/-} or CD55^{-/-} mice to generate apoE^{-/-}/CD59a^{-/-}, apoE^{-/-}/C6^{-/-} or apoE^{-/-}/CD55^{-/-} double knockout mice (see chapter 3 for the generation of these double knockouts).

Animals were housed in a specific pathogen-free environment. All studies and protocols were approved by the institutional Ethics Review Committee and by the United Kingdom Home Office and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3. Induction of atherosclerosis

3.1 Experimental procedure

Male mice were fed a high-fat diet containing 21% (wt/wt) pork lard and supplemented with 0.15% (wt/wt) cholesterol (Special Diet Services) for 8 or 12 weeks starting at 8 weeks of age.

3.2 Termination procedure

Animals were anaesthetised by i.p injection of sodium pentobarbitone and weighed before exsanguination by cardiac puncture followed by arterial perfusion via the left cardiac ventricle with phosphate buffered saline (PBS) at a constant pressure of 100 mmHg, with outflow through the incised jugular veins. Brachiocephalic arteries were removed with a piece of the aortic arch and the stump of the right subclavian artery still attached to aid orientation during histological processing. These were immediately

embedded in OCT compound (Raymond A Lamb Limited, Eastbourne, UK) and snap-frozen in liquid nitrogen (figure 2.1). The heart, spleen, kidney, liver and tibia were removed from each animal. The weight of each organ was recorded and the length of the tibia measured. These were then used to determine organ hypertrophy either by calculating organ: body weight or organ: tibia length ratio. In the initial experiments organ: body weight ratios were used to assess organ hypertrophy, however since body weight varied between animals, the more constant tibia length was used (Yin et al., 1982).

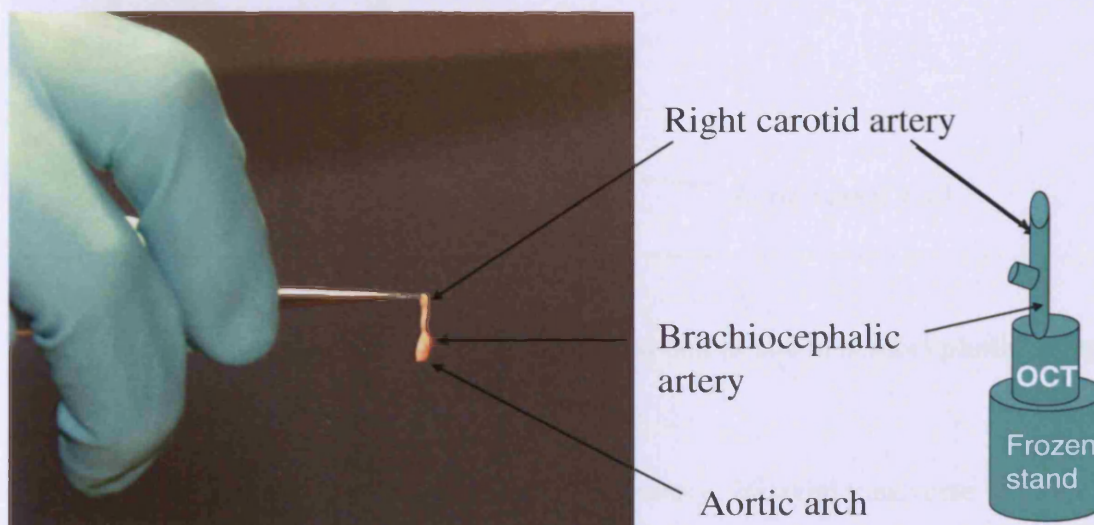


Figure 2.1 Photograph of a brachiocephalic artery isolated from the mouse and a schematic illustration of the mounting procedure. The freshly isolated brachiocephalic artery is rapidly mounted in OCT by suspending the artery over a frozen stand before carefully applying OCT.

4. Assessment of atherosclerosis

OCT embedded brachiocephalic artery tissue was mounted on a cryostat set at -22°C with the aorta facing towards the cryostat cutting blade. Sections through the aorta were then cut ($20\mu\text{m}$ thickness) until the proximal end of the brachiocephalic artery was reached. This was identified by visual closure of the brachiocephalic trunk, along with a cutting of the aorta as shown in figure 2.2.

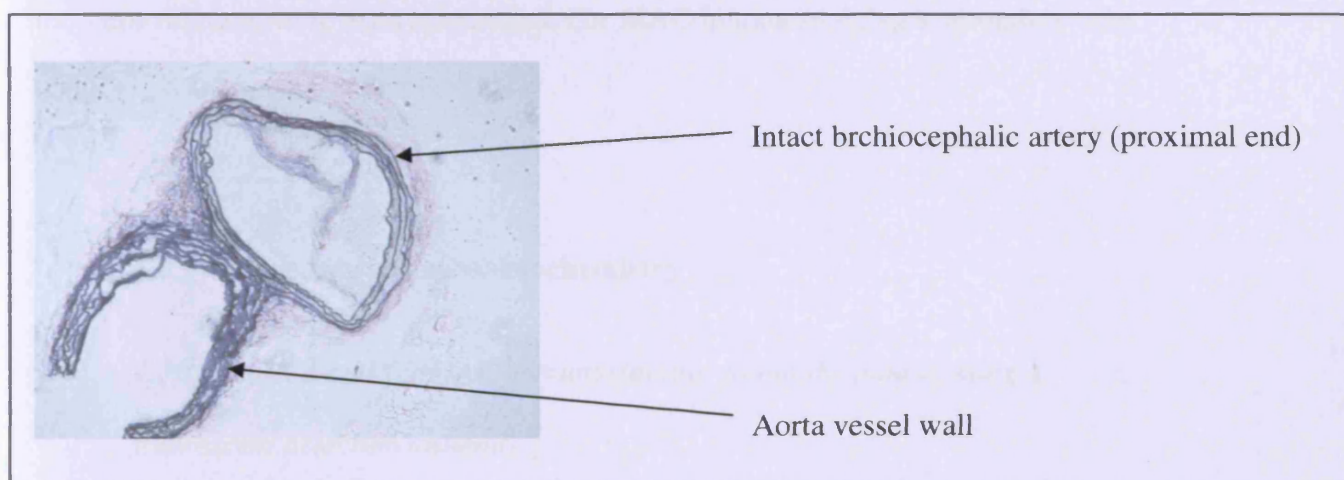


Figure 2.2 Cross section of the proximal end of the brachiocephalic artery.

Once at the proximal end of the brachiocephalic artery, 26 serial transverse sections, $7\mu\text{m}$ thickness, were cut and each placed on a separate glass slide clearly labelled with animal identification and section numeration from A to Z. Each glass slide contained a section from both control ($\text{apoE}^{-/-}$) and test group ($\text{apoE}^{-/-}/\text{complement protein}^{-/-}$) which were cut at the same point along the brachiocephalic artery. Sections were allowed to air dry before being stored at -80°C . Sections were subsequently stained for the protein of interest.

4.1 Antibodies

The primary and secondary antibodies used for immunohistochemistry are detailed in tables 2.2 and 2.3 respectively.

4.2 Immunohistochemistry

Negative controls, where the primary antibody was replaced with an isotype control at the same dilution, were always included. For MAC immunostaining a secondary only control was included.

4.3 Fluorescence immunohistochemistry

4.3.1 Total C3 and C9/MAC immunostaining within the plaque using a fluorescent detection antibody

Immunostaining was performed for complement components C3 (rat anti-mouse C3 mAb 11H9 (20 μ g/mL); Hy-Cult Biotechnology), and C9 (rabbit anti-rat C9 (2 μ g/mL); prepared in-house using standard immunisation procedures). For these, sections were blocked using 2% bovine serum albumin in PBS and bound antibodies were detected with either Alexa fluor 594-labelled donkey anti-rat IgG (20 μ g/mL) or Alexa fluor 594-labeled goat anti-rabbit IgG (20 μ g/mL) (both from Molecular Probes Inc). All sections were counter-stained using DAPI. For C3 staining, a negative control, where the primary antibody was replaced with rat IgG (of the same isotype) at the same concentration, was always included. Whilst a secondary only control was included for MAC staining.

Name	Antigen recognised	Species	Isotype	Supplier
MOMA2	mouse macrophage	Rat	IgG2b	Serotec
clone 1A4	mouse actin, α -Smooth muscle	Mouse	IgG2a	Sigma Aldrich
F4/80	mouse macrophage	Rat	IgG2a	Serotec
48-2B	mouse CD3 T-cells	Armenian Hamster	IgG1	Santa Cruz Biotechnology, Inc
mCD59a.7	mouse CD59a	Rat	IgG1	Dr. C. L. Harris (Cardiff University)
2C6	mouse CD55	Rat	IgG1 κ	Prepared In house using standard techniques
5D5	mouse Crry	Rat	IgG1	Dr. M. Holers (Denver, USA)
11H9	mouse native C3	Rat	IgG2a	Prepared In house using standard techniques
2/11	mouse C3b/iC3b/C3c	Rat	IgG1	Hy-Cult Biotechnology
MAC (affinity purified)	Rat/mouse MAC	Rabbit	Polyclonal	Prepared In house using standard techniques

Table 2.2 List of primary antibodies used for immunohistochemistry

Name	Antigen recognised	Species	Isotype	Supplier
Biotinylated anti-rat IgG	Rat IgG	Goat	Polyclonal	Vector laboratories
Biotinylated anti- mouse IgG reagent (M.O.M kit)	Mouse IgG	-	-	Vector laboratories
Biotinylated anti- Armenian Hamster IgG	Armenian hamster IgG	Goat	Polyclonal	Abcam plc,
Alexa Fluor 594-labelled anti-rat IgG	Rat IgG	Donkey	Polyclonal	Invitrogen Life Technologies
Alexa Fluor 488- labelled anti-rat IgG	Rat IgG	Goat	Polyclonal	Invitrogen Life Technologies
Alexa fluor 594-labeled anti-rabbit IgG	Rabbit IgG	Goat	Polyclonal	Invitrogen Life Technologies

Table 2.3 List of secondary antibodies used for immunohistochemistry

4.3.2 Plaque macrophage and smooth muscle-actin fluorescent staining

Macrophages and smooth muscle cells were identified using anti-murine macrophage antibody (F4/80, Serotec; diluted 1:100) and anti- α -smooth muscle actin (clone α -1-A4; Sigma-Aldrich; diluted 1:100). In brief, sections were fixed in ice-cold acetone and blocked using an avidin/biotin blocking kit (Vector Laboratories) followed by either 10% goat serum or the M.O.M mouse Ig blocking reagent (Vector Laboratories). Sections were then incubated with the appropriate biotinylated secondary antibodies: biotinylated goat anti-rat IgG (Vector Laboratories; 3.5 μ g/ml in 10% mouse serum) or biotinylated anti-mouse Ig reagent (diluted as instructed by vector M.O.M kit). Immunopositive cells were detected using Fluorescein Avidin D diluted 1:200 in 2%BSA in PBS (Vector Laboratories) and cell nuclei were stained using DAPI.

4.4 Histology

4.4.1 Plaque lipid content

Oil red O-stained sections were used to determine plaque lipid content. Sections were allowed to air dry at room temperature for 30 minutes prior to staining, then immersed in distilled H₂O (dH₂O) three times for 5 minutes each before being washed with 60% (v/v) isopropanol/dH₂O for 5 minutes at room temperature. Sections were then stained with oil red O working solution (stock oil red O diluted 2:3(v/v) in dH₂O) for 15 minutes at room temperature before being washed again with 60% (v/v) isopropanol/dH₂O. Sections were then rinsed well under running tap water for 5 minutes before being stained with haematoxylin for 3 minutes. Finally, the sections were 'blued' in tap water before being rinsed in dH₂O and mounted in aquamount. The total stained area in the plaque was expressed as a percentage of the total plaque area to give the fractional plaque lipid content.

4.4.2 Elastin and collagen

Firstly, sections were allowed to air dry at room temperature for 30 minutes before being dehydrated in a 5 step procedure for 5 minutes at each step as follows:

- 1) dH₂O
- 2) 70%ethanol
- 3) 90%ethanol
- 4)100%ethanol
- 5)100%ethanol

Sections were then immersed in Miller's Elastin Van Gieson (EVG) stain for 40 minutes at room temperature before being rinsed in absolute ethanol twice, (30 seconds each). This was followed by rinsing in dH₂O for a further 30 seconds before being placed into van Gieson solution for 2 minutes. The sections were then rinsed twice in absolute ethanol (1 minute each). Sections were then dehydrated (as procedure above) before being placed into histoclear solution twice (5 minutes each). Slides were then mounted with histomount.

4.4.3 Morphometric analysis

Morphometric analyses were carried out on elastin-stained sections. One section was quantified per mouse at the same position along the brachiocephalic artery, following the established method of Johnson *et al.* (Johnson et al., 2005) as described in section 4.0. Morphometry was performed using Image ProPlusTM software version 4.0 (Media Cybernetics). The lengths of the internal and external elastic lamina were traced and recorded. These were used to derive the media area by assuming them to be the circumferences of perfect circles. The plaque area was measured directly, by tracing either EVG/oil red O or fluorescently stained sections, this value was subtracted from

the area enclosed by the internal elastic lamina to derive the true lumen area see figure 2.3.

Percentage staining for specific antigens within the plaque was assessed using Image ProPlus™ software version 4.0. Plaque area for each section was measured as above and the area of staining was analysed by setting detection threshold and staining above threshold scored as positive. The percentage of the plaque area stained was then calculated by using the following equation:

$$\% \text{ stain in the plaque} = \text{Sum of area stained} / \text{Total plaque area} \times 100$$

Intensity of staining throughout plaque was not measured.

5. Assessment of adiposity, plasma lipids and metabolites

5.1 Serum triglyceride, cholesterol and serum turbidity measurements

Mouse blood (~1 mL) was collected into tubes without anticoagulant, allowed to clot at room temperature and then incubated on ice for one hour. Serum was separated by centrifugation and analysed for triglyceride, cholesterol and serum turbidity on an automated analyser (Clinical Biochemistry Laboratories, Cardiff University Hospital).

In brief, cholesterol esters were enzymatically hydrolysed by cholesterol esterase to cholesterol and free fatty acids. Free cholesterol, including that originally present, was then oxidised by cholesterol oxidase to cholest-4-ene-3-one and hydrogen peroxide. The hydrogen peroxide combines with hydroxybenzoic acid and 4-aminoantipyrine to form a chromophore (quinoneimine) which was quantitated by absorbance at 500nm.

Triglycerides were enzymatically hydrolysed by lipase to free fatty acids and glycerol. The glycerol was phosphorylated by ATP with glycerol kinase to produce glycerol-3-phosphate and ADP. Glycerol-3-phosphate was oxidised to dihydroxyacetone phosphate by glycerol phosphate oxidase, producing H_2O_2 . In a colour reaction catalysed by peroxidase, the H_2O_2 reacts with 4-aminoantipyrine and 4-chlorophenol to produce a red-coloured dye.

The absorbance of this dye is proportional to the concentration of triglyceride present in the sample. The triglyceride assay was also read at 500nm.

Both VLDL and chylomicrons effectively scatter light, causing turbidity. Serum turbidity, a measurement of circulating chylomicrons and VLDL particles, was determined as the difference between absorbance at 660 and 700 nm.

5.2 NEFA concentrations

Measurement of NEFA concentrations in the plasma was carried out by Dr. Irina Gushina, Cardiff University. In brief, lipids were extracted from plasma samples by a modified Folch method (Garbus et al., 1963). Non-polar lipids were separated using 1-dimensional thin layer chromatography. Free fatty acids were visualised with 0.05% (w/v) 8-anilino-4-naphthosulphonic acid in methanol and their compositions and contents were determined by gas chromatography.

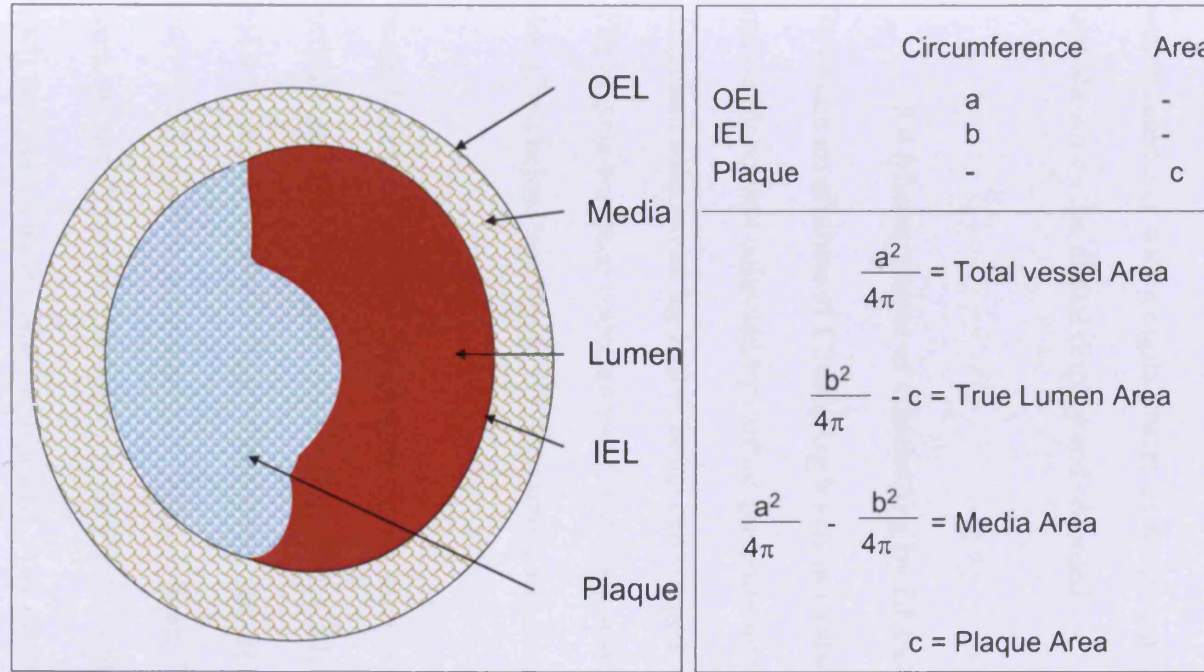


Figure 2.3 Morphometric analysis of atherosclerotic arteries. Using the above equations morphometric analyses of arteries can be made. OEL = Outer elastic lamina; IEL = Inner elastic lamina

5.3 Glucose levels

A blood glucose meter (Glucomen, A. Menarini Diagnostics) was used for measuring blood glucose level. Approximately 5µl of blood was placed on a disposable test strip which interfaces with a digital meter. After several seconds, the level of blood glucose was shown on the digital display and recorded.

5.4 Measurement of C3adesArg by ELISA

To obtain an estimate of C3adesArg levels in mouse plasma, approximately 1ml of mouse blood was collected by cardiac puncture into tubes containing 40µl of anti-coagulant solution on ice before being centrifuged at 4°C at 10,000 rpm for 10 minutes. The plasma was then collected and C3adesArg was measured by sandwich ELISA, described below (see figure 2.3 for a schematic diagram).

Nunc-Immuno™ ELISA plates were first coated with 50µl of purified monoclonal rat anti-mouse C3a (0.5mg/ml; BD pharmingen) in ELISA coating buffer (0.2M Na₂CO₃, pH 9.6) overnight at 4°C, then blocked with 100 µl per well of blocking buffer (2% (w/v) BSA in PBS), incubated at 37 °C for 1 hour. The plasma samples to be tested were diluted either at a dilution of 1:10 or 1:50 in blocking buffer and 50µl per well of each was placed on the plate in triplicate. As a control plasma from C3^{-/-} mice was used at the same dilution to show specificity of the assay. On each plate a linear standard curve was generated using 50µl per well of purified mouse C3a diluted in blocking buffer and carried out in duplicate (100ng/ml – 0.78ng/ml, and blocking buffer only as a negative control; BD pharmingen). This step was carried out at 37 °C for 1 hour. Subsequently, 50µl per well of biotinylated rat anti-mouse C3a antibody, diluted in

blocking buffer (2ug/ml; BD pharmingen) and incubated at 37 °C for 1 hour, was used to detect bound C3adesArg.

Following this step, the plate was incubated with 50µl per well of streptavidin-peroxidase diluted in blocking buffer (1:5000; Jackson ImmunoResearch Laboratories, Inc) and incubated for 37 °C for 30 minutes. Between incubations, plates were washed three times with 150µl per well of 0.1% (v/v) Tween in PBS. The ELISA was developed with 100µl per well of ELISA developing solution (TMB Substrate Reagent Set, BD Pharmingen) and stopped with 100µl per well of 10% (v/v) H₂SO₄ when background began to increase. Absorbance was read at 450 and 590nm using FLUOstar Optima (BMG LABTECH). Read out values at 450nm were then subtracted from 590nm using Excel.

Values were firstly subtracted from background value (negative control) and these values were then transferred into GraphPad Prism and a standard curve was generated by plotting known C3adesArg values on the x axis against absorbance values on the y axis. Unknown x values were determined for all unpaired y values by using lowess curve fitting model. The interpolated values were multiplied by the dilution factor in order to calculate C3adesArg concentrations. A typical standard curve is shown in figure 2.4.

Representative mouse C3adesArg standard curve

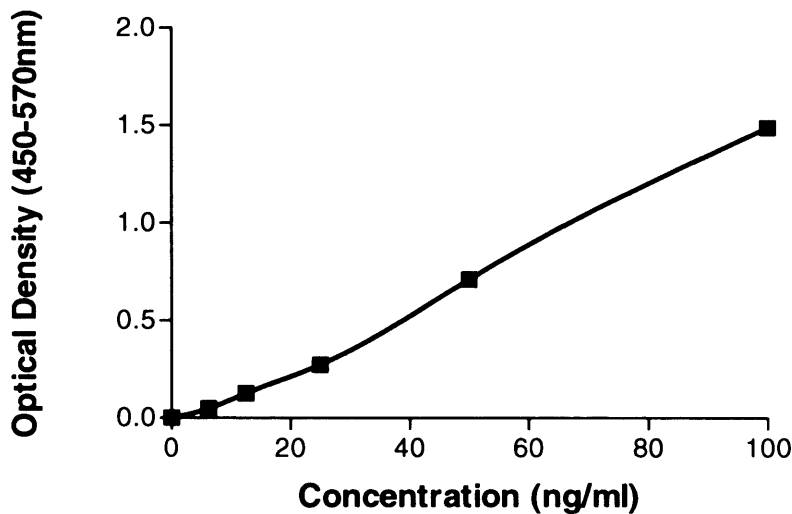


Figure 2.4 Representative graph of C3adesArg standard curve

5.5 In vivo quantification of fat content in mice using dual X-ray absorptiometry (DEXA)

Total percentage body fat was measured by DEXA using a PIXImus scanner with Lunar small animal software (version 1.45) (GE Lunar Corporation).

6. Functional analysis of Complement activity

6.1 Production of mouse anti-rabbit erythrocyte antibody

BALB/c mice were injected s.c with 100 μ l of rbE (packed cells) in Alsever's solution (an isotonic, balanced salt solution which comprises of NaCl (4.2g/L), citric Acid \cdot 3Na \cdot 2H $_2$ O (8.0g/L), citric Acid \cdot H $_2$ O (0.55g/L), D-Glucose (20.5g/L)) at two different sites. Four weeks post-immunisation, the mice were administered a booster immunisation with 200 μ l of rbE (packed cells) given by i.p injection. The succeeding

third and fourth immunisations were also given i.p one to two weeks apart from each other. After the final boost, the mice were terminated by a schedule 1 method and blood was collected for plasma. Each batch of polyclonal mouse anti-rabbit erythrocyte antibody was tested in a standard haemolytic assay, to monitor polyclonal batch variability. Of note, the laboratory now uses a monoclonal anti-rabbit erythrocyte antibody, recently produced in house, for haemolysis assays.

6.2 Preparation of sensitised rabbit erythrocytes

Sensitised rbE for use in various tests of mouse complement activity was prepared as follows: whole blood obtained by cardiac puncture from a rabbit under terminal anaesthesia was centrifuged at 1000rpm for 5 minutes. RbE from the cell pellet were re-suspended in 15ml of PBS and washed twice by centrifugation at 1000rpm for 5 minutes. A 2% suspension of rbE was made by taking 200 μ l of the packed, washed rbE pellet and re-suspending in 10ml of complement fixation diluent (CFD) was prepared from complement fixation diluent tablets (Oxoid). Mouse anti-rabbit erythrocyte antiserum (40 μ l) was diluted in 10ml CFD and mixed with the 2% suspension of rbE. This mixture was incubated at 37°C for 30 minutes on a rotator. Following incubation, the cells were washed three times in CFD (20ml each time) by centrifugation at 2000rpm for 5min to remove any unbound antibody. An illustration of the C3adesArg ELISA method is shown in figure 2.5.

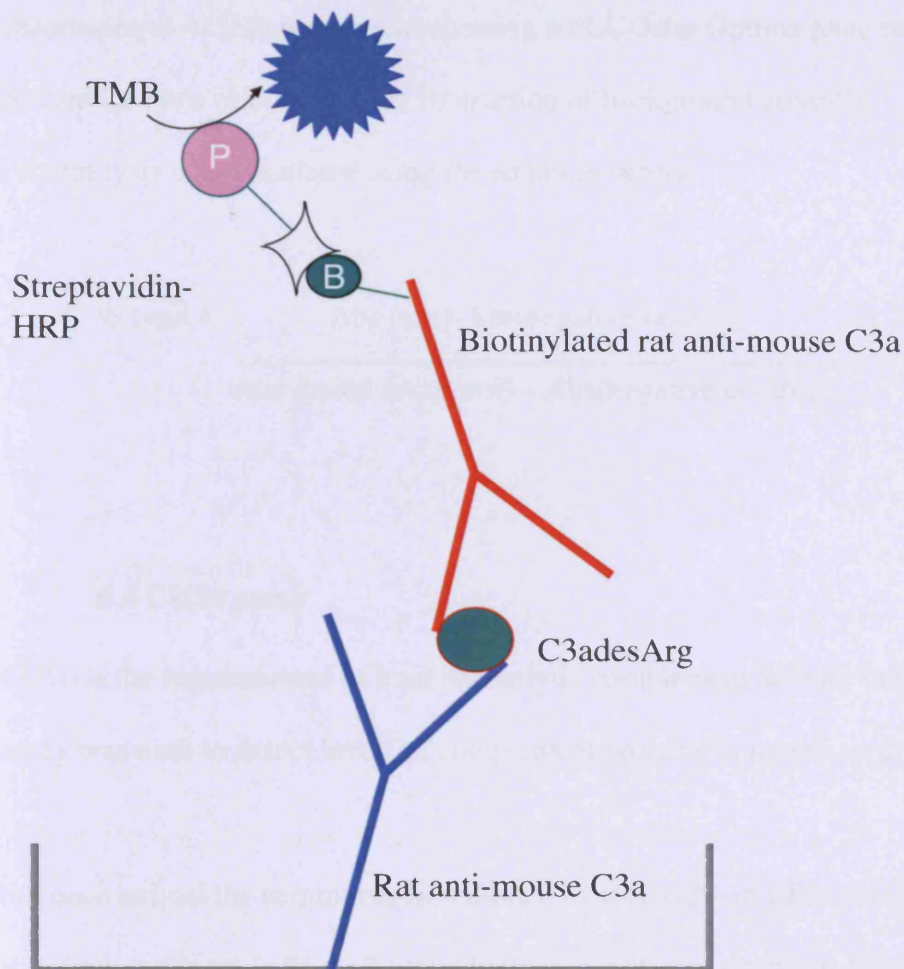


Figure 2.5 Schematic diagram of C3adesArg sandwich ELISA.

6.3 Titre of mouse serum

For lysis assays 96 well round bottomed plates were used. Sera under test were diluted 1:10 in CFD and aliquoted 50 μ l per well. Sensitized rbE-A (50 μ l) were added to each well, agitated briefly and the mixture incubated for 30 minutes at 37°C. The plate was then centrifuged at 1400rpm for 5 minutes and 80 μ l of supernatant transferred into wells of a 96 well flat bottomed plate. Controls of background lysis (CFD buffer) and 100% lysis (cells lysed in equivalent volume of H₂O) were also carried out. The

absorbance at 415nm was measured using a FLUOstar Optima plate reader and the percentage lysis calculated after subtraction of background controls.

Percent lysis was calculated using the equation below:

$$\% \text{ lysis} = \frac{\text{Abs (test)} - \text{Abs(negative control)}}{\text{Abs (positive control)} - \text{Abs(negative control)}} \times 100$$

6.4 CH50 assay

CH50 is the measurement of total haemolytic complement activity in the serum. This assay was used to detect levels of complement proteins in mouse serum.

For each animal the serum was first diluted to 10% (v/v) in CFD, this diluted serum was further diluted in 96 well round bottomed plates by doubling dilution (v/v). These samples of serum (50µl) were each incubated with 50µl of a 2% suspension of antibody coated rbE-A in round bottomed 96 well plates for 30 minutes at 37°C. 0% lysis was established by incubation of 50µl rbE-A with 50µl CFD and 100% lysis was achieved by incubation of 50µl of rbE-A and 50µl H₂O. Following the incubation period, the cells were pelleted by centrifugation at 1400 rpm for 5 minutes. 80 µl of supernatant from each well was transferred to a flat-bottomed 96 well plate and the absorbance at 415nm was determined using FLUOstar Optima plate reader. To calculate CH50 values for the various sera under test, plots of log x against log(y/(1-y)) were made where x was the volume of serum added (µl) and y the % haemolysis. The intercept on the x axis is log K, where K is the volume of serum giving 50% haemolysis.

The number of CH50 units (U/ml) of each mouse serum was determined from the following equation:

$$\text{CH50} = 10 \times (1000/K) \text{ (U/ml)}$$

The first multiplier corrects for the 1:10 original dilution.

7. Protein analysis

7.1 Protein concentration

The concentration of purified proteins was obtained by measuring their absorbance at 280nm in a spectrophotometer (Jenway). The concentration was calculated using the following equation: $C = A / \epsilon$ Where C is concentration (mg/ml), A is absorbance and ϵ is the extinction coefficient when the pathlength is 1cm. Extinction coefficient for both mouse Ig and anti-mouse C5 mAb is 1.4.

7.2 SDS-PAGE analysis

Electrophoretic separation of proteins was carried out using Biorad gel apparatus. All gels poured were with a 5% acrylamide stacking gel and a 10% acrylamide resolving gel. Recipes for the gels are found in table 2.4. 10 μ l of pre-stained protein marker, broad range (New England Biolabs, Inc) was run along side protein of interest.

	Percentage gel	
	5% (Stacking)	10% (Resolving)
Stacking gel buffer (ml)	1.2	
Resolving gel buffer (ml)		3.75
40% Acrylamide (ml)	0.63	3.75
10% ammonium Persulphate (μ l)	50	150
dH ₂ O (ml)	3.07	7.25
TEMED (μ l)	5	15

Table 2.4 Recipe for SDS-PAGE gels

The protein of interest was mixed (1:1) with either non-reducing or reducing loading buffer. All samples were boiled for 5 minutes and loaded into the wells of a prepared SDS-PAGE gel within a running tank containing running buffer. The gels were subjected to electrophoresis at 100V for approximately 90 minutes.

7.3 Coomassie staining

Coomassie blue gel staining solution was used to detect μ g quantities of protein in SDS-PAGE gels. Following electrophoresis, the gels were soaked in Coomassie stain for 1 hour with agitation. Unbound stain was removed by soaking gels in destain solution with agitation until background staining was minimal and the bands were clearly visible. Gels were then soaked in gel drying buffer for approximately 30

minutes before being placed between pre-soaked acetate gel drying films (Promega) and stretched within a gel drying frame until ready.

8. Statistical analysis

All calculations were carried out using Microsoft Excel and/or GraphPad Prism software version 5. Significance was tested by two-tailed unpaired Student's t-test, with significance assumed at $p < 0.05$.

Chapter 3: Generation of genetically modified animals

1. Introduction

The development of atherosclerosis in humans is a complex process and involves various genetic and environmental factors. Thus, the very nature of the disease impedes progress in its field of research. Animal models of atherosclerosis are therefore an obvious choice as a tool for researching into the field of atherosclerosis. Mouse models of atherosclerosis, including apoE^{-/-}, ldlr^{-/-} and apoE/ldlr double knockouts, have helped in elucidating atherosclerotic lesion development (Ishibashi et al., 1993; Ishibashi et al., 1994; Piedrahita et al., 1992), and are used to assess drug treatment of the disease (Zadelaar et al., 2007). These atherosclerosis prone mouse models have also proven useful in studying the effects of complement in atherosclerosis (Bhatia et al., 2007; Buono et al., 2002; Patel et al., 2001; Persson et al., 2004).

In our study we chose to use the apoE deficient mouse model to determine the role of complement during the progression of atherosclerosis. In 1992, Piedrahita and colleagues inactivated the endogenous apoE gene by using gene targeting in mouse embryonic stem cells through homologous recombination (Piedrahita et al., 1992) (see figure 3.1). These mice appeared to be healthy at three months old but upon further examination phenotypic changes in their lipid and lipoprotein profiles were revealed (Zadelaar et al., 2007).

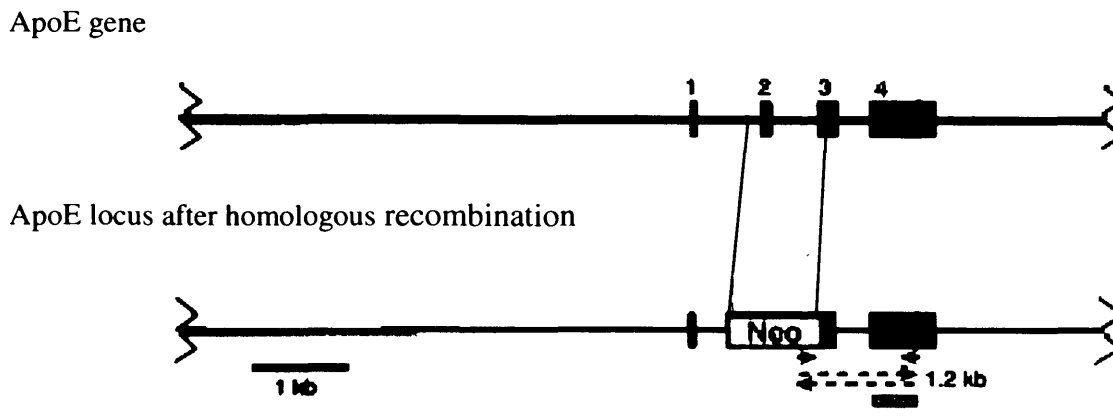


Figure 3.1 Targeting of the apoE gene. Construction of a gene-targeting vector, in which exon 2 and the majority of exon 3, the major coding portion of the protein were replaced by the neomycin resistance gene (Neo). Figure adapted from (Plump et al., 1992).

Complement regulator deficient mice have also been generated using the gene targeting approach. These include $CD59^{-/-}$ (Holt et al., 2001; Qian et al., 2000) and $CD55^{-/-}$ mice (Sun et al., 1999). Two genes exist in the mouse which encode for CD59 protein; Cd59a and Cd59b. In our laboratory, Cd59 deficient mice were generated by targeting the deletion of the widely expressed Cd59a gene (Holt et al., 2001) as opposed to Cd59b which is expressed mostly in the testis (Baalasubramanian et al., 2004). Standard gene-targeting methods in mouse embryonic stem cells were used, and the targeting vector designed removed the majority of the mature protein sequence by eliminating exon 3 (see figure 3.2) (Holt et al., 2001). Male $Cd59a^{-/-}$ mice were 2-3g lighter in weight when compared with their $Cd59a^{+/+}$ litter-matched controls however this difference was not observed in the females (Holt et al., 2001). Furthermore, $Cd59a^{-/-}$ mice of both sexes exhibited increased haemoglobin concentration in plasma and urine, indicating that these mice have spontaneous intravascular haemolysis and haemoglobinuria (Holt et al., 2001).

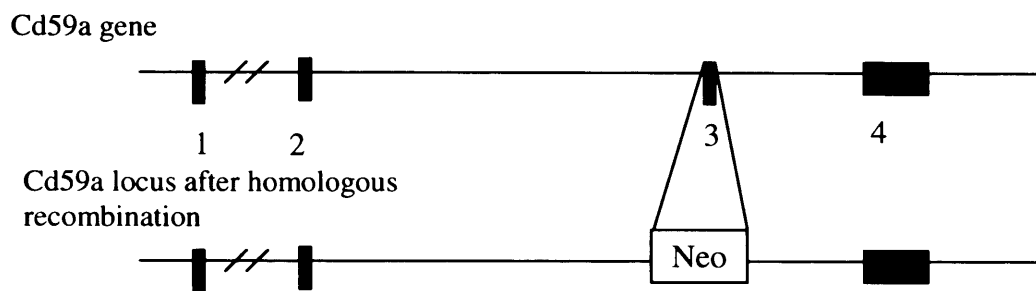


Figure 3.2 Targeted deletion of the Cd59a gene (adapted from Holt *et al.* 2000)

Construction of a gene-targeting vector, in which exon 3, the major coding portion of the protein, was replaced by the neomycin resistant gene (Neo).

Similar to CD59, mice also contain two genes for CD55 encoding transmembrane and GPI anchored forms of CD55. Due to the wider distribution of the latter, Sun and colleagues targeted the deletion of this gene by using homologous recombination in embryonic stem cells (See figure 3.3) (Sun *et al.*, 1999). The mice used for the work detailed in this thesis were obtained from this laboratory and hence deficient in the GPI anchored form of CD55. I refer to these mice here after as CD55^{-/-}. This knockout mouse model shows no sign of haemolytic anaemia or overt phenotypic abnormalities (Sun *et al.*, 1999).

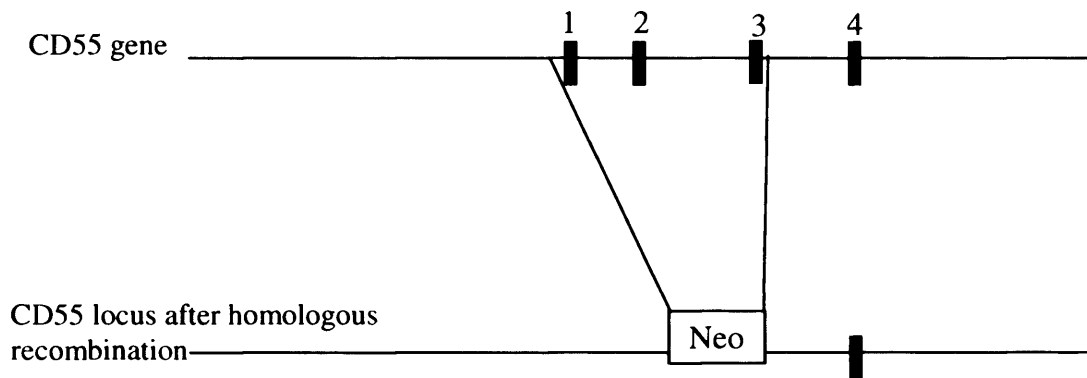


Figure 3.3 Targeted deletion of CD55 GPI gene (adapted from Sun et.al 1999).

Construction of a gene-targeting vector, in which the first 3 exons, were deleted and replaced with the NEO gene.

Complement protein deficient mice also exist including the C3H/He C6 deficient mouse strain which was established more than two decades ago (Orren et al., 1988). In 2004, Bhole and colleagues identified 7 point mutations within the C6 gene of these mice, four of which lead to amino acid substitutions causing disruption of the tertiary structure of the protein resulting in C6 deficiency (Bhole and Stahl, 2004). In our laboratory we have back-crossed these mice for six generations onto the C57BL/6 strain.

The aims of the work described in this chapter were to generate the following mouse strains from the knockouts described above, and establish breeding colonies for each:

- ApoE^{-/-}/CD55^{-/-} and apoE^{-/-}/CD55^{+/+} controls
- ApoE^{-/-}/C6^{-/-} and apoE^{-/-}/C6^{+/+} controls
- ApoE^{-/-}/Cd59a^{-/+}

Of note, apoE^{-/-}/Cd59a^{-/+} and apoE^{-/-}/Cd59a^{+/+} mice generated from the apoE^{-/-}/Cd59a^{-/+} breeding colony were pooled for controls.

The generation of these breeding colonies was core to the project and allowed further characterisation and assessment of atherosclerosis to be carried out in these mice.

2. Specific methods

2.1 Screening of apoE, CD55 and CD59a deficiency

2.1.1 DNA isolation

Tail tips were digested overnight at 65°C in 500µl of tail buffer (50mM Tris-hydrochloric acid (HCl) pH8, 100mM EDTA, 100mM NaCl, 1% (w/v) SDS) containing 15µl proteinase K. 250µl of 6M NaCl was mixed into the sample and centrifuged at 13,000 rpm for 10 minutes. Supernatant was removed, into a fresh tube containing 500µl isopropanol, mixed and centrifuged. The pellet was washed in ethanol and resuspended in 100µl TE buffer (10mM Tris pH 7.5, 1mM EDTA).

2.1.2 PCR

Isolated DNA was amplified by standard PCR conditions using various primers (see table 3.1). The following reagents were added into a PCR tube:

- 10 x NH₄ reaction buffer (Bioline Ltd; 160mM (NH₄)₂SO₄, 670mM Tris-HCl, 0.1% (v/v) Tween-20)
- 200μM deoxy-nucleotide-tri-phosphates (dNTPS) (Bioline Ltd)
- 1μl 50mM MgCl₂ (Bioline Ltd)
- 10pmol of each primer
- dH₂O added to bring the reaction volume to 22.5μl
- 2μl Isolated mouse DNA

The reaction was heated at 95°C for 5 minutes to denature the DNA and then 0.5μl TAQ polymerase (Bioline Ltd, 5U/ μl) was added.

The following PCR steps employed were dependent on the gene of interest. Table 3.2 and 3.3 show the PCR cycles optimised for CD55 wild type primers and CD55 NEO gene primers respectively. Table 3.4 and 3.5 show the PCR cycles optimised for ApoE and CD59 primers respectively.

2.1.3 Agarose gel electrophoresis

DNA fragments and PCR products were visualised by agarose gel electrophoresis. 2% (w/v) agarose (Invitrogen Life Technologies) in 1 x TAE buffer was melted in a microwave and allowed to cool before the addition of ethidium bromide (100ng/ml final concentration). Agarose gel was poured into a gel casting tray (Thermo Electron

Corporation), an appropriate size comb was inserted and the gel was allowed to set. The comb was removed and the gel immersed in 1 x TAE in an electrophoresis tank (Thermo Electron Corporation). DNA samples containing 10% (v/v) agarose gel loading dye were loaded along with DNA standard size markers. Gels were run at 60V for approximately 1 hour. The DNA within the gel was visualised using a transilluminator (Chemi-Doc (Bio-Rad laboratories), the images were captured on a digital camera using the Bio-Rad Gel Documentation system.

Primer name	Primer Sequence 5' – 3'
ApoE 901	CGC CGC TCC CGA TTC GCA GCG CAT CGC
ApoE 1753	CTC TGT GGG CCG TGC TGT TGG TCA CAT TGC TGA CA
ApoE 1754	CTC GAG CTG ATC TGT CAC CTC CGG CTC TCC C
Cd59 P1	GGT GAC CAA CTG GTG TTA ACA AAG GG
Cd59a P2	GAA CCT GCG TGC AAT CCA TCT TG
Cd59a P3	GCT ACC ACT GTT TCC AAC CGG TG
CD55 2A	TCA ATT AAC TGC GGC TCA AA
CD55 2B	GGA CAG CAG CAA CAG AGA CA
CD55 4A	CAG AAA GCG AAG GAG CAA AG
CD55 4B	TTC ATC CTC TGA GCC ACT GA

Table 3.1 Primer sequences used to amplify murine apoE, Cd59a and CD55.

Step #	Protocol for CD55 wild type primers
1.	Incubate at 95 ⁰ C for 1 minute
2.	Incubate at 58 ⁰ C for 1 minute
3.	Incubate at 72 ⁰ C for 30 seconds
4.	Cycle to step 2 for 4 more times
5.	Incubate at 95 ⁰ C for 1 minute
6.	Incubate at 60 ⁰ C for 30 seconds
7.	Incubate at 72 ⁰ C for 30 seconds
8.	Cycle to step 6 for 35 more times
9.	Incubate at 4 ⁰ C for 1 hour

Table 3.2 Protocol for CD55 wild type primers

Step #	Protocol for CD55 NEO gene primers
1.	Incubate at 95 ⁰ C for 1 minute
2.	Incubate at 64 ⁰ C for 30 seconds
3.	Incubate at 72 ⁰ C for 2 minutes
4.	Cycle to step 2 for 39 more times
5.	Incubate at 72 ⁰ C for 10 minutes
6.	Incubate at 4 ⁰ C for 1 hour

Table 3.3 PCR Protocol for CD55 NEO gene primers

Step #	Protocol for apoE gene primers
1.	Incubate at 95 ⁰ C for 1 minute
2.	Incubate at 64 ⁰ C for 1 minute
3.	Incubate at 72 ⁰ C for 3 minutes
4.	Cycle to step 2 for 34 more times
5.	Incubate at 72 ⁰ C for 10 minutes
6.	Incubate at 4 ⁰ C for 1 hour

Table 3.4 Protocol for apoE gene primers

Step #	Protocol for Cd59a gene primers
1.	Incubate at 95 ⁰ C for 1 minute
2.	Incubate at 60 ⁰ C for 1 minute
3.	Incubate at 72 ⁰ C for 1 minutes
4.	Cycle to step 2 for 394more times
5.	Incubate at 72 ⁰ C for 10 minutes
6.	Incubate at 4 ⁰ C for 1 hour

Table 3.5 Protocol for Cd59a gene primers

2.2 Screening of C6 deficiency

Mouse serum was collected to test for C6 deficiency. Both dot blot and haemolytic assay were then employed to confirm C6^{-/-}, C6^{+/-} and C6^{+/+} animals. In order to carry out the haemolytic assay, mouse anti-rabbit erythrocyte antibody was generated. See chapter 2 for detailed methodology.

Results from the haemolysis assay was able to distinguish between C6^{+/+} and C6^{+/-} mice, but not between C6^{-/+} and C6^{+/+} mice. Thus, to confirm C6 deficiency sera was also tested by dot blot. A grid was drawn on nitrocellulose membrane and 5 µl of a dilution of mouse serum was carefully placed in the centre of each square. The nitrocellulose membrane was then placed in a dot blot 96 system, which dries the membrane using a vacuum pump. Subsequently, the membrane was blocked with blocking buffer (5% non-fat dried milk in PBS) for 30 minutes before being incubated for an hour with the primary antibody, goat anti-human C6 (CompTech) (1:1000 diluted in 2.5% blocking buffer). The membrane was washed three times in 0.05% (v/v) Tween 20 in PBS then incubated with the secondary antibody, rabbit anti-goat IgG (Dako UK Ltd) (diluted 1:2000 in 2.5% (w/v) blocking buffer) for 30 minutes. Washing steps were then repeated before the detection of positive staining for C6 using chloronaphthol (1x tablet in 10ml methanol, 40ml 1xTBS and 5µl H₂O₂). The reaction was left to develop for 30 minutes and stopped by washing with distilled H₂O.

3. Results

3.1 Generation of apoE^{-/-}/Cd59a^{-/-} mice and their litter-matched apoE^{-/-}/Cd59a^{+/+} controls

ApoE^{-/-} and Cd59a^{-/-} mice (four pairs) were mated to generate F1 double heterozygote mice, which were subsequently crossed to produce F2 mice (n = 153) (figure 3.4). Screening of F2 mice was carried out successfully to identify gene deletions (figure 3.5 and figure 3.6), and allowed the generation of an apoE^{-/-}/Cd59a^{+/+} breeding colony. F2 generation mice revealed no significant deviation from anticipated Mendelian ratios for either apoE or Cd59a alleles [apoE^{-/-} : +/- : +/+ = 1.3 : 2.6 : 1.0; Cd59a^{-/-} : +/- : +/+ = 1.0 : 3.1 : 1.4], indicating that the combination of two mutations (apoE and Cd59a) was not fatal. F3 generated mice were used for experimental procedures with subsequent screening for Cd59a gene post-termination. This breeding strategy provided test and litter-matched control animals for a blinded experiment.

3.2 Generation of apoE^{-/-}/CD55^{-/-} mice and the apoE^{-/-} controls

In order to reduce animal numbers and to cut down the amount of genotyping needed for the study, it was decided to slightly alter the breeding strategy. Hence, colonies of test and control mice were generated from the F2 screening. Initially, apoE^{-/-} and CD55^{-/-} mice (three pairs) were mated to generate F1 mice, which were then crossed to produce F2 mice (n = 101) (figure 3.7).

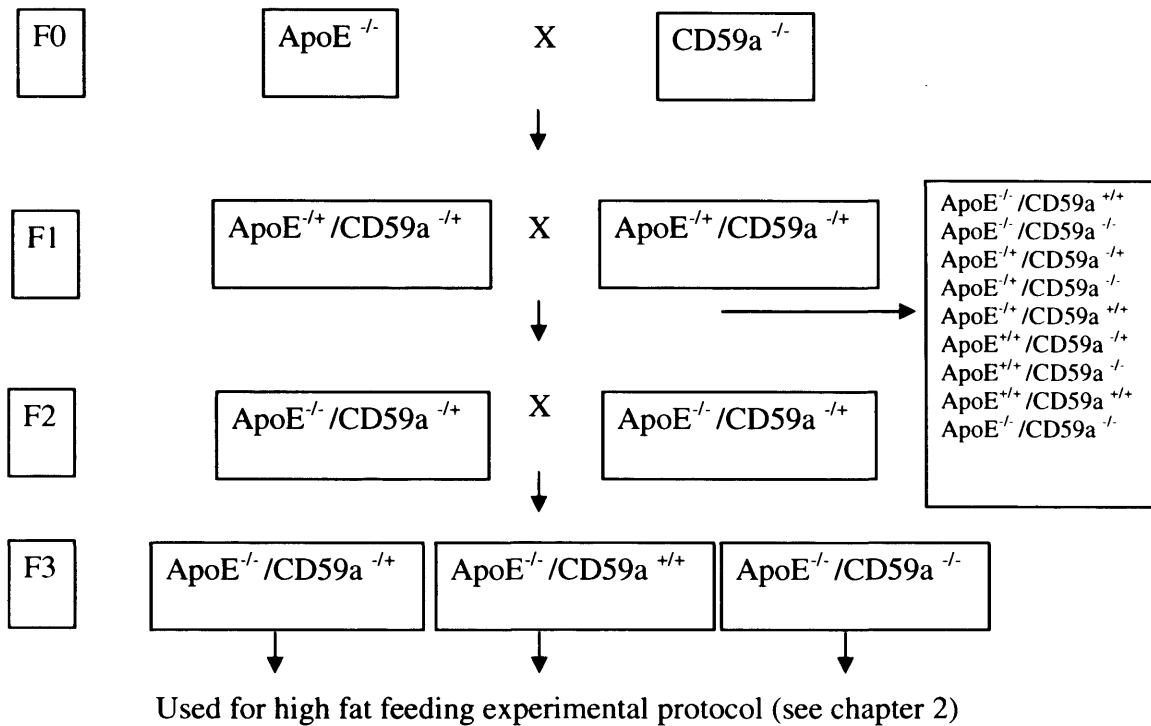


Figure 3.4 Mice breeding strategy. F0 apoE^{-/-} mice were crossed with Cd59a^{-/-} mice to generate F1 progeny that were heterozygous for both apoE and Cd59a gene. F1 mice were then crossed to produce the F2 generation which were subsequently screened for both genes and breeding colonies of apoE^{-/-}/Cd59a^{+/-} mice were set up. These produced apoE^{-/-}/Cd59a^{+/-}, apoE^{-/-}/Cd59a^{+/+}, and apoE^{-/-}/Cd59a^{-/-} mice which were subsequently used as experimental mice for the study.

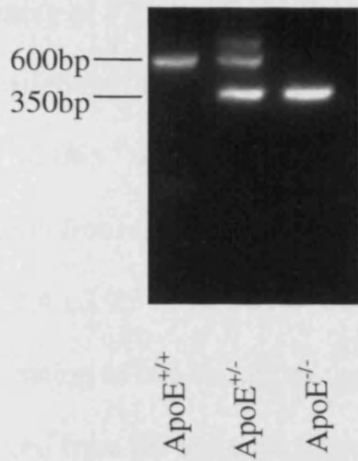


Figure 3.5 PCR genotyping of genomic DNA derived from the progeny of ApoE^{+/-}.

The 600-base pairs (bp) fragment amplified from the wild type allele (with primers ApoE 901 and ApoE 1754) and the 350-bp fragment amplified from the targeted allele (with primers ApoE 1754 and ApoE 1753) are seen in the appropriate lanes.

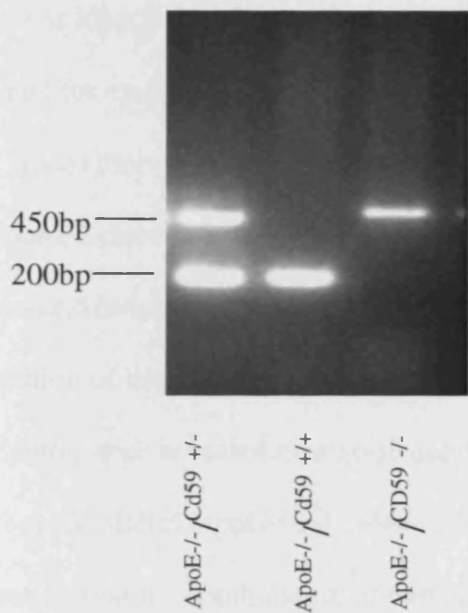


Figure 3.6 PCR genotyping of genomic DNA derived from the progeny of CD59^{+/-}

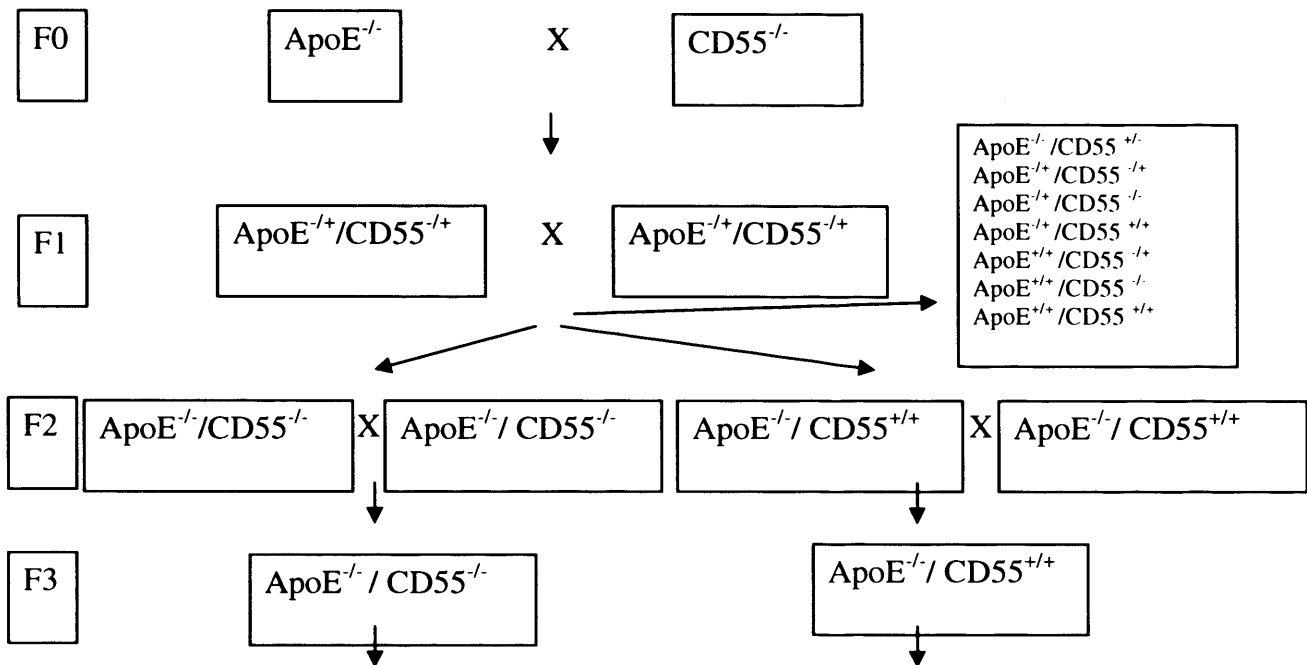
mice. The 200-bp fragment amplified from the wild type allele (with primers P1 and P3) and the 450-bp fragment amplified from the targeted allele (with primers P2 and P3) are seen in the appropriate lanes.

Screening of F2 mice was subsequently carried out and was successful in identifying gene deletions (see figure 3.8), allowing the generation of breeding colonies for both apoE^{-/-}/CD55^{-/-} and apoE^{-/-}/CD55^{+/+} mice. F2 generation mice revealed no significant deviation from anticipated Mendelian ratios at apoE or CD55 alleles [apoE ^{-/-} : ^{+/-} : ^{+/+} = 1.4 : 2.9 : 1.0; CD55 ^{-/-} : ^{+/-} : ^{+/+} = 1 : 1.3 : 1.8], indicating that the combination of two mutations (apoE and CD55) had no effect on fertility. Animals produced from the test and control breeding colonies were used in the experimental procedure (see chapter 2).

3.3 Generation of apoE^{-/-}/C6^{-/-} mice and apoE^{-/-}/C6^{+/+} controls

The breeding strategy employed for the generation of apoE^{-/-}/C6^{-/-} and apoE^{-/-}/C6^{+/+} mice was identical to that used for apoE^{-/-}/CD55^{-/-} and apoE^{-/-}/CD55^{+/+} above and was initiated for exactly the same reasons as stated in section 3.2. Consequently, apoE^{-/-} and C6^{-/-} mice (four pairs) were mated to generate F1 mice, which were then crossed to produce F2 mice (n = 65) (figure 3.9). Screening of F2 mice was subsequently carried out successfully identifying gene deletions (figure 3.10 and figure 3.11), allowing the generation of breeding colonies for both apoE^{-/-}/C6^{-/-} and apoE^{-/-}/C6^{+/+} mice. F2 generation mice revealed no significant deviation from anticipated Mendelian ratios at apoE or C6 alleles [apoE ^{-/-} : ^{+/-} : ^{+/+} = 1.5:3.3:1.0; C6 ^{-/-} : ^{+/-} : ^{+/+} = 0.9:2.4:1.0], indicating that the combination of two mutations (apoE and C6) was not fatal.

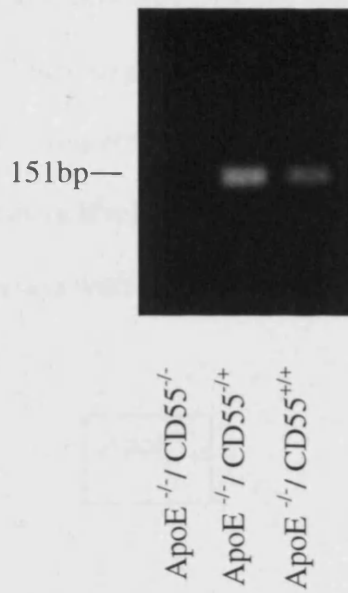
Since C6 deficiency can arise from 7 point mutations, screening for C6 deficiency by a molecular method is virtually impossible. Thus, screening for C6 deficiency was carried out by two methods.



Used for high fat feeding experimental protocol (see chapter 2)

Figure 3.7 Mice breeding strategy for $apoE^{-/-}/CD55^{-/-}$. F0 $apoE^{-/-}$ mice were crossed with $CD55^{-/-}$ mice to generate F1 progeny that were heterozygous for both $apoE$ and $CD55$. F1 progeny consisted of heterozygote $apoE^{-/+}/CD55^{-/+}$ mice which were backcrossed to produce F2 generation. The F2 generation were subsequently screened for both genes and breeding colonies of $apoE^{-/-}/CD55^{-/-}$ mice and $apoE^{-/-}/CD55^{+/+}$ mice were set up to provide F3 progeny which were subsequently used as experimental mice for the study.

A



B

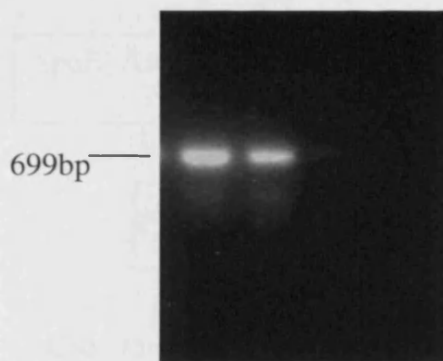


Figure 3.8 PCR genotyping of genomic DNA derived from the progeny of CD55^{-/+} mice. For this, separate PCR reactions were carried out. **(A)** The 151-bp fragment amplified from the wild type allele (with primers CD55 2A and CD55 2B) and **(B)** the 699-bp fragment amplified from the targeted allele (with primers CD55 4A and CD55 4B).

Firstly, measurement of complement activity in mouse serum was assessed by haemolysis assay, followed by a dot blot. Whilst homozygote for C6 along with C6 deficiency and heterozygotes can be detected by haemolysis assay (see figure 3.10), the dot blot method was employed to definitively determine C6 deficiency (see figure 3.11). Since complement levels in the serum differ between male and female, two separate haemolysis assays were carried out.

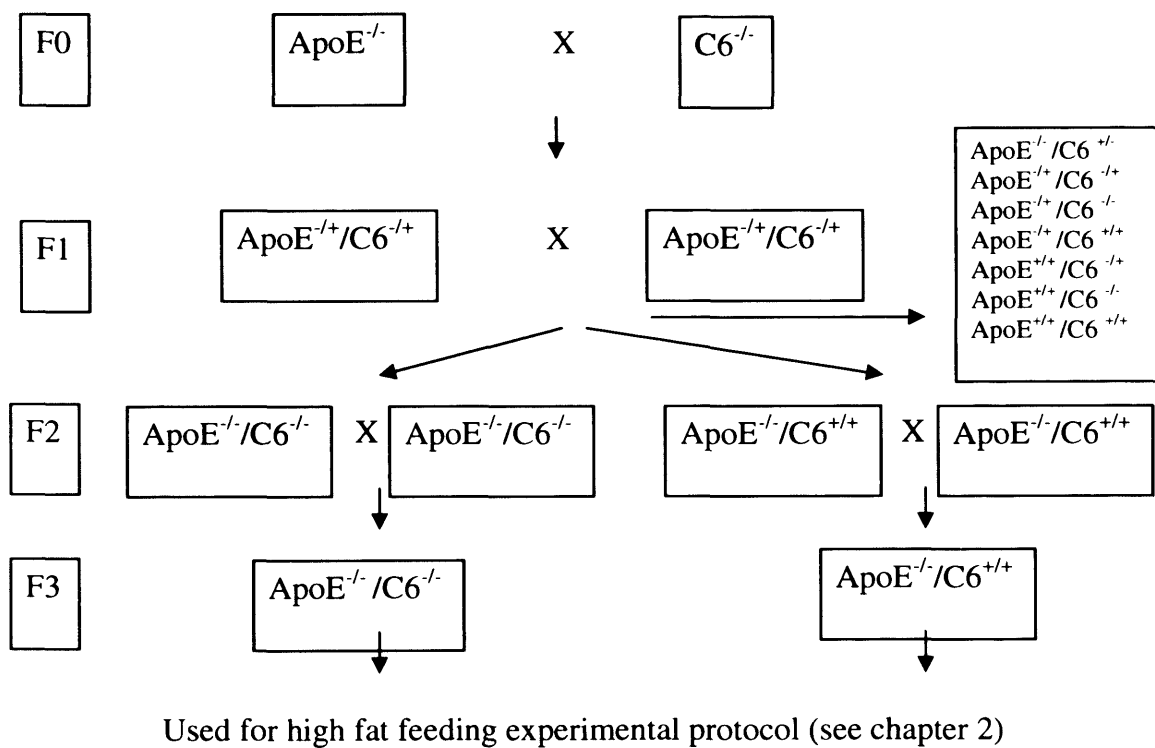
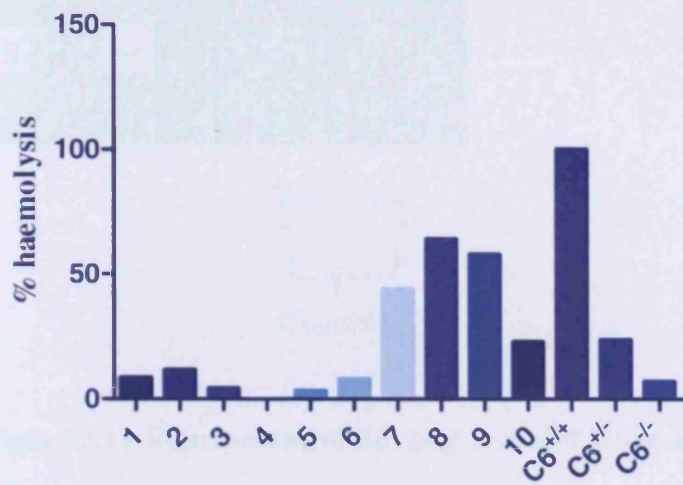


Figure 3.9 Mice breeding strategy for apoE^{-/-}/C6^{-/-} mice. F0 apoE mice were crossed with C6^{-/-} mice to generate F1 progeny that were heterozygous for both apoE and C6. F1 progeny were crossed to produce F2 generation. The F2 generation were subsequently screened for both apoE and C6 genes and breeding colonies of apoE^{-/-}/C6^{-/-} mice and apoE^{-/-}/C6^{+/+} mice were set up. The subsequent generation, F3, were used in the experimental protocol for the study.

A



B

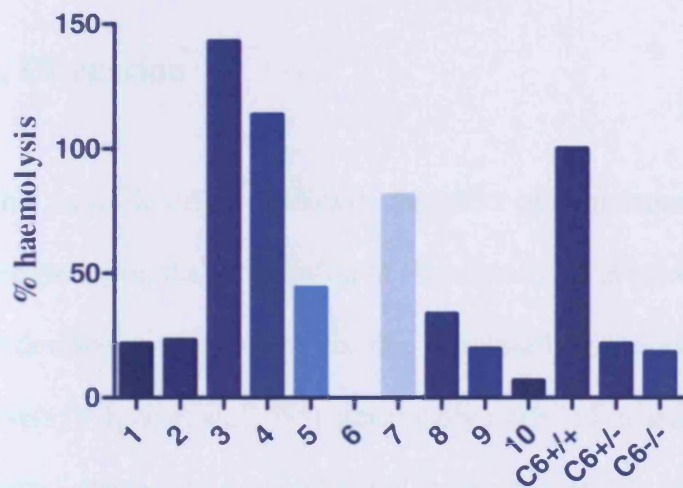


Figure 3.10 Percentage haemolytic activity in serum samples of (A) male apoE^{-/-}/C6^{-/-}, apoE^{-/-}/C6^{+/-} and (B) female apoE^{-/-}/C6^{+/-} mice. X-axis denotes sample numbers and control animals.

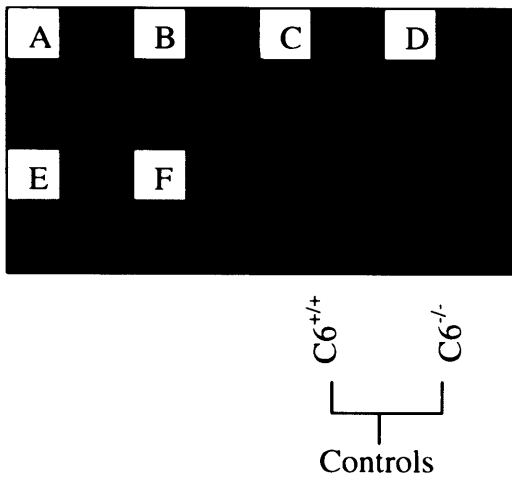


Figure 3.11 Representative dot blot result of C6 screening to generate apoE^{-/-}/C6^{-/-} mice and their apoE^{-/-} controls. Sample letters are noted on each corner (A-F) and serum control samples from C6^{+/+} and C6^{-/-} are always included.

4. Discussion

This thesis describes studies on the effect of complement and complement regulator deficiency on the progression of atherosclerosis in mice. Since mice are highly resistant to developing atherosclerosis, due to naturally low plasma cholesterol and triglyceride levels (Paigen et al., 1985), genetically modified mouse models of atherosclerosis has been a crucial and successful tool in the progress of the research to date. In particular, the apoE^{-/-} and ldlr^{-/-} mice are popular amongst researchers in the cardiovascular field due to their ability to rapidly produce atherosclerotic lesions within the vascular wall. In this study the apoE^{-/-} mouse model was chosen since it has been shown to develop complex plaques with the formation of an acellular necrotic core, erosion of the necrotic mass through to the lumen and intraplaque haemorrhage (Rosenfeld et al., 2000). These features mirror those seen in human atherosclerosis. In addition, when fed a diet containing 21% pork lard and 0.15% cholesterol spontaneous plaque rupture was

observed in the lesions present in the brachiocephalic artery (Johnson and Jackson, 2001). Thus, the use of this animal model allows the study of complement and its regulators during plaque progression and in plaque stability.

Naturally deficient C6 mice have 7 different point mutations (Bhole and Stahl, 2004) rendering PCR strategy unfeasible and thus genotyping was not employed for screening. Instead, haemolysis assay was used to detect any C6 activity and secondly C6 deficiency was confirmed by utilising the dot blot method. The apoE^{-/-} mice used were on a mixed background, 71% C57BL/6 and 29% 129/Sv, which are grey, white or black in appearance. All other mice used were on the C57BL/6 background.

The breeding strategy changed over the course of the project. In the first instance, during the generation of apoE^{-/-}/Cd59a^{-/-}, all F2 generation were put on a high fat diet and were used as experimental animals. Consequently both apoE^{-/-}/Cd59a^{+/+} and apoE^{-/-}/Cd59a^{+/-} animals were generated and used in the control group. This strategy had two drawbacks, firstly there were always more animals in the control group compared with the numbers of apoE^{-/-}/Cd59a^{-/-} mice and secondly it involved more time in genotyping and was less cost effective due to housing costs of the F2 generated animals. Following careful consideration the breeding strategy was revised so that two parallel colonies of F2 animals, test (apoE^{-/-}/X^{-/-}) and control (apoE^{-/-}/X^{+/+}) were generated. This breeding strategy reduced both the amount of time spent on genotyping and the number of animals used which is in line with a commitment to the principles of the 3R's ('Replacement, Refinement, and Reduction') in animal research in reducing the overall numbers of mice used.

All male mice from the colonies (test and control) were subjected to a period of high fat feeding as detailed in chapter 2. Group sizes were initially set at 10, though large experimental variation forced an increase in group size to around 15. It is possible that experimental variation may have been increased due to the use of mice on a mixed background and thus obtaining apoE^{-/-} on the C57BL/6 background may be of beneficial use for future experiments. However, the data produced in this thesis is in line with all published studies so far exploring the role of complement in murine atherosclerosis models. Thus the effect of strain background on disease outcome in this particular case is minimal.

Chapter 4: Role of the terminal pathway during atherosclerosis

1. Introduction

Over the last two decades it has become clear that atherosclerosis is associated with chronic inflammation (Fan and Watanabe, 2003; Nilsson and Hansson, 2008; Ross, 1999; Seifert and Kazatchkine, 1988). While the causes of the initial insult to the artery wall remain unclear, evidence has accumulated implicating both early and terminal complement activation during disease progression (Meuwissen et al., 2006; Niculescu et al., 2004; Vlaicu et al., 1985b; Yasojima et al., 2001).

Terminal pathway activation results in the production of C5a and C5b. Whilst, the latter associates with C6, C7, C8 and multiple C9 molecules (C5b-9) to form the MAC, C5a is a potent anaphylatoxin which regulates vasodilation, increases the permeability of small blood vessels and stimulates contraction of SMCs. It also acts as a chemoattractant for monocytes, B- and T-lymphocytes (Nataf et al., 1999; Schulman et al., 1988). In human atherosclerotic lesions, C5a was located around cholesterol clefts and necrotic cell debris where it was suggested to play an active role in plaque destabilisation by up-regulating the expression of MMP-1 and MMP-9 in macrophages (Speidl et al., 2010). C5a has also been shown to exert inflammatory responses through up-regulation of cytokines and related receptors (e.g. IL-6, IL-18 receptor) and adhesion molecules (e.g. P-selectin, E-selectin, ICAM-1 and VCAM-1) on endothelial and SMCs (Albrecht et al., 2004; Foreman et al., 1994; Monsinjon et al., 2003; Shagdarsuren et al., 2010). In addition, C5a also stimulates the release of TNF- α , IL-1

and oncostatin M from cultured macrophages (Kastl et al., 2008; Okusawa et al., 1987; Okusawa et al., 1988), and promotes activation of the coagulation cascade by stimulating the secretion of vWF and tissue factor by endothelial cells (Ikeda et al., 1997; Platt et al., 1991).

MAC is found deposited on atherosclerotic vessel walls in both early and advanced stages of the disease (Oksjoki et al., 2007b; Vlaicu et al., 1985a). Similar to the early activation products of complement, MAC is absent from normal arterial wall. However, as first shown in hypercholesterolemic rabbits, MAC deposition within the intima is an early event during atherosclerosis, since in the subendothelium MAC deposition occurs temporally with cholesterol accumulation and this occurs before monocyte infiltration and foam cell formation (Seifert et al., 1989). Complement activation is thought to only proceed to the terminal stage at deeper sites within the mucoelastic layer of the intima (Torzewski et al., 1998b), where MAC deposition was found co-localised with properdin (a positive regulator for the alternative pathway) in human coronary arteries (Oksjoki et al., 2007a).

Within the deep area of the intima, MAC has also been demonstrated to co-localise with SMCs (Torzewski et al., 1997), CRP (Torzewski et al., 1998a), apoptotic cells (Niculescu et al., 2004) and modified lipoproteins (Torzewski et al., 1998b). From these studies it can be seen clearly that MAC formation takes place in areas of the artery wall rich in moieties which activate complement. The effects of MAC deposition on cultured nucleated cells are many and varied depending on the cell type under examination; however, lysis of nucleated cells by MAC is a rare event in vivo, since several protective mechanisms exist including the widespread expression of CD59 (Meri et al.,

1990) and the ability of nucleated cells to exo- or endo-cytose the MAC complex (Morgan et al., 1987). In spite of this there are many important “non-lethal” events which can occur due to the deposition of the MAC on a nucleated cell.

MAC can trigger signalling cascades that lead to smooth muscle and endothelial cell proliferation (Fosbrink et al., 2006; Halperin et al., 1993; Niculescu et al., 1999). In the context of atherosclerosis, these processes can result in SMC infiltration into and proliferation within the intima and endothelium neovascularisation. Sublytic MAC formation on endothelial cells has also been shown to induce the release of growth factors (Benzaquen et al., 1994), increase expression of adhesion molecules (Kilgore et al., 1995), tissue factor (Saadi et al., 1995) and cyclooxygenase-2 (COX-2) (Bustos et al., 1997) and stimulate production of inflammatory cytokines (Kilgore et al., 1997). MAC deposition on SMCs can induce protection from apoptosis (Niculescu et al., 1999) and in macrophages MAC has been shown to promote foam cell formation in the presence of Cu-oxidized LDL (Wu et al., 2009).

Despite ample evidence suggesting a role for terminal pathway activation during progression of atherosclerosis, studies using animal models have so far produced conflicting results (Geertinger and Soerensen, 1977; Patel et al., 2001; Schmiedt et al., 1998). C5 deficiency had little or no effect in the apoE-deficient mouse model, leading the authors to suggest that MAC had no role in this model of atherosclerosis (Patel et al., 2001). However, contrasting data were obtained from studies in C6^{-/-} rabbits which, when fed a high-fat diet, developed less atherosclerosis than their C6^{+/+} controls (Geertinger and Soerensen, 1977; Schmiedt et al., 1998). MAC formation is blocked in C5^{-/-} mice and C6^{-/-} rabbits, suggesting that there may be species differences in the roles

of MAC in atherosclerosis; alternatively, it is possible that C5a, the other product of C5 cleavage, has a hitherto unrecognised protective role in atherosclerosis.

The work described in this chapter was initiated to clearly define the role of the terminal pathway during atherosclerosis. Section 2 and 3 of this chapter describe the role of MAC during the development of atherosclerosis by characterising the effect of CD59a and C6 deficiency on the progression of the disease in the apoE^{-/-} mouse model. Section 4 of this chapter details an investigation into the efficacy of anti-C5 treatment in apoE^{-/-} mice as a potential complement-mediated therapy for atherosclerosis.

2. Unregulated MAC during atherosclerosis

2.1 A brief introduction

Animal studies carried out so far investigating the role of the terminal pathway in atherosclerosis using the apoE^{-/-} mouse model have focused on individual complement components rather than complement regulators.

It was hypothesised that:

- 1. defective complement regulation in the vessel wall would enhance local complement activation.**
- 2. the resultant increase in inflammation would exacerbate atherosclerosis.**

To test these hypotheses, Cd59a^{-/-} mice were back crossed onto the apoE^{-/-} mouse background and the resulting double knock-outs (and their litter-matched controls) fed

a high fat diet at 8 weeks of age for 8 weeks. Atherosclerosis was assessed in the brachiocephalic artery.

2.2 Specific methods

2.2.1 Histochemical staining of CD3+T-cells in atherosclerotic lesions

T-cell staining was carried out on sections fixed in 4% formaldehyde and blocked with 10% goat serum in 2% (w/v) BSA in PBS. The presence of T-cells was revealed using hamster anti-mouse CD3 antibody (20 μ g/ml), followed by biotinylated goat anti-hamster IgG (18.5 μ g/ml), each diluted in PBS containing 2% (w/v) BSA and subsequent detection using a streptavidin-alkaline phosphatase conjugated antibody (BD Bioscience) followed by New Fuchsin solution (New Fuchsin Kit; Dako UK Ltd). Between each step, sections were washed in PBS three times (5 minutes each). Sections were finally washed in dH₂O for 2 minutes and counterstained with Mayer's haematoxylin. Subsequently, sections were mounted using aquamount (BDH Laboratory Supplies).

2.2.2 Macrophage immunostaining in atherosclerosis

Sections to be stained for macrophages were fixed in ice-cold acetone for 10 minutes. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide solution for 20 minutes at room temperature. Sections were then blocked with avidin/biotin blocking kit (Vector laboratories) incubated at room temperature for 10 minutes in each solution followed by a second blocking step using a mixture of 10% (v/v) goat and 2.5% (v/v) mouse serum diluted in PBS incubated at room temperature for 1 hour. Macrophages were detected using anti-murine macrophage antibody (0.1

$\mu\text{g/mL}$) (MOMA-2, Serotec) for 1 hour at room temperature. Sections were then incubated with biotinylated goat anti-rat Ig (Vector Laboratories) (diluted 1:250 in 1% (w/v) bovine serum albumin (BSA) in PBS) for 45 minutes at room temperature, followed by horseradish peroxidase-labelled Extravidin (Sigma-Aldrich) (diluted 1:500 in 1% (w/v) BSA in PBS) for 30 minutes at room temperature. Immunopositive cells were detected using diaminobenzidine (DAB) (Vector laboratories). All sections were counterstained with Mayer's haematoxylin.

2.2.3 SMC immunostaining in atherosclerosis

Sections to be stained were fixed in ice-cold acetone for 10 minutes at room temperature. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide solution at room temperature for 20 minutes. Sections were subsequently blocked with avidin/biotin blocking kit (Vector laboratories) and M.O.M mouse Ig blocking reagent (Vector laboratories).

Sections were incubated with 50 μl of mouse actin α -smooth muscle antibody diluted 1:400 in PBS containing 2% (w/v) BSA) at room temperature for 1 hour. Sections were then incubated with 50 μl of biotinylated goat anti-rat (Vector Laboratories) (diluted 1:250 in PBS containing 2% (w/v) BSA) and detected using Vectastain Elite ABC kit (Vector Laboratories). All sections were washed between each step in PBS for 5 minutes three times.

Finally sections were rinsed in dH_2O for 2 minutes before detection with DAB substrate (Vector laboratories). Brown colour was allowed to develop and the reaction was

stopped by rinsing in tap water. All sections were counterstained with Mayer's haematoxylin.

2.2.4 Immunostaining for complement regulators Crry, CD55 and CD59a

Sections to be stained for complement regulators were fixed in ice-cold acetone for 10 minutes. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide solution for 20 minutes at room temperature. Sections were then blocked with avidin/biotin blocking kit (Vector laboratories) (incubated at room temperature for 10 minutes in each solution) followed by a second blocking step using a mixture of 10% (v/v) goat and 2.5% (v/v) mouse serum in PBS, incubated at room temperature for 1 hour. Complement regulators Crry, CD55 and CD59a were subsequently detected using: rat anti-mouse Crry mAb 5D5 (2.6 µg/mL; a generous gift from Dr. M. Holers, Denver, USA) ; (Li et al., 1993); rat anti-mouse CD55 mAb 2C6 (13 µg/mL; prepared in house using standard immunisation procedures) (Spiller et al., 1999); and rat anti-mouse CD59a mAb mCD59a.7 (0.1 µg/mL; a kind gift from Dr. C. L. Harris, Cardiff University) respectively. Primary antibodies were incubated for 1 hour at room temperature. Sections were then incubated with biotinylated goat anti-rat (Vector Laboratories) (diluted 1:250 in 1% (w/v) bovine serum albumin (BSA) in PBS) for 45 minutes at room temperature, followed by horseradish peroxidase-labelled Extravidin (Sigma-Aldrich) (diluted 1:500 in 1% (w/v) BSA in PBS) for 30 minutes at room temperature. Immunopositive cells were detected using DAB (Vector laboratories). All sections were counterstained with Mayer's haematoxylin.

2.2.5 Nuclear staining using Mayer's haematoxylin

Sections were counterstained with Mayer's haematoxylin for 3 minutes and blued in running tap water. Sections were then dehydrated using a five step procedure, 5 minutes at each step:

- 1) dH₂O,
- 2) 70%ethanol
- 3) 90%ethanol
- 4) 100%ethanol
- 5) 100%ethanol.

Slides were placed into histoclear solution (National Diagnostics Ltd) twice for 5 minutes before mounted with histomount (National Diagnostics Ltd).

2.3 Results

2.3.1 Pathological observations

To examine the role of CD59 during atherosclerosis I generated mice deficient in both apoE and CD59a and subjected them (together with their controls) to a period of high fat feeding. These two groups of mice, (apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-}) were then characterised and compared, analysing various parameters including body weight, heart: body weight ratio (as a measurement of cardiac hypertrophy) and lipid profiles (cholesterol, triglyceride and serum turbidity) (see table 4.1).

2.3.2 Plaque area is increased in apoE^{-/-}/Cd59a^{-/-} mice

Next the role of the MAC and its regulator, CD59, was examined in relation to the development of atherosclerosis. Morphometric analyses of atherosclerotic plaques were carried out comparing fat-fed apoE^{-/-}/Cd59a^{-/-} and their litter-matched apoE^{-/-} controls. For this, one section of the brachiocephalic artery was taken at the same position for each mouse from the proximal end of the vessel and stained with EVG revealing that the average plaque size in apoE^{-/-}/Cd59a^{-/-} mice was double that seen in gender, age, strain and litter-matched apoE^{-/-} controls on an identical diet. Figures 4.1A and B show representative sections from apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice respectively. The pooled data are shown in figure 4.1C ($59.9 \pm 13.1 \times 10^3 \mu\text{m}^2$ versus $28.2 \pm 7.9 \times 10^3 \mu\text{m}^2$; $P < 0.05$). Table 4.2 summarises the morphometric analyses, including plaque, lumen, media and vessel area. Mean lumen area, media area and total vessel area were not significantly different between the groups.

Group	Body weight (g)	Heart: body ($\times 10^{-3}$)	Cholesterol (mmol/L)	Triglyceride (mmol/L)	Serum turbidity
ApoE ^{-/-} /Cd59a ^{-/-}	41 ± 2.3 (n=12)	4.8 ± 0.2 (n=12)	29.0 ± 1.7 (n=21)	2.7 ± 0.3 (n=22)	25.0 ± 3.7 (n=14)
ApoE ^{-/-}	44 ± 2.1 (n=16)	4.3 ± 0.2 (n=16)	34.0 ± 1.9 (n=25)	3.2 ± 0.3 (n=28)	36.8 ± 5.4 (n=16)
P value	0.240	0.140	0.080	0.230	0.090

Table 4.1 Body weight, heart: body weight ratio, lipaemia index, total cholesterol and triglyceride levels in 16 week old apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice. All animals were fed a high fat diet from 8 weeks old for 8 weeks. Data are shown as mean ± SEM.

Group	Vessel Area (x10³ μm²)	Plaque Area (x10³ μm²)	Media Area (x10³ μm²)	Lumen Area (x10³ μm²)
ApoE ^{-/-} /Cd59a ^{-/-} (n=14)	345.0 ± 25.0	*59.9 ± 13.1	97.0 ± 7.6	188.0 ± 10.8
ApoE ^{-/-} (n=15)	325.0 ± 20.0	28.2 ± 7.9	90.0 ± 8.1	207.0 ± 9.8
P value	0.520	0.045	0.530	0.200

Table 4.2 Brachiocephalic artery morphometric data in 16 week old apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice.

All animals were fed a high-fat diet from 8 weeks of age for 8 weeks. *P<0.05 versus apoE^{-/-} control.

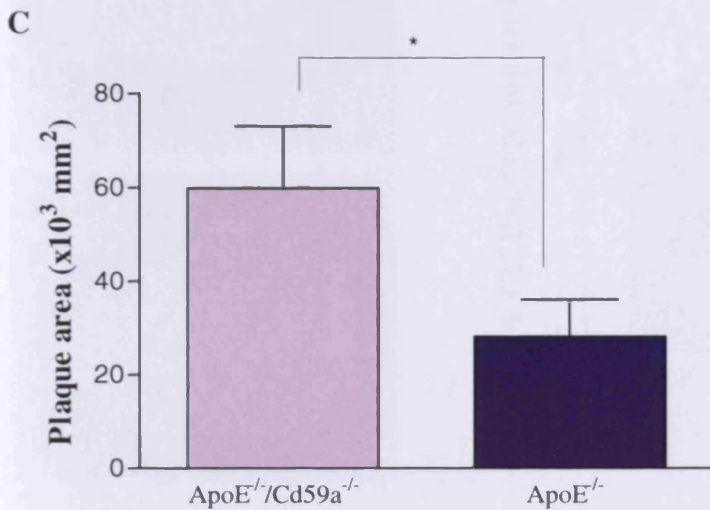
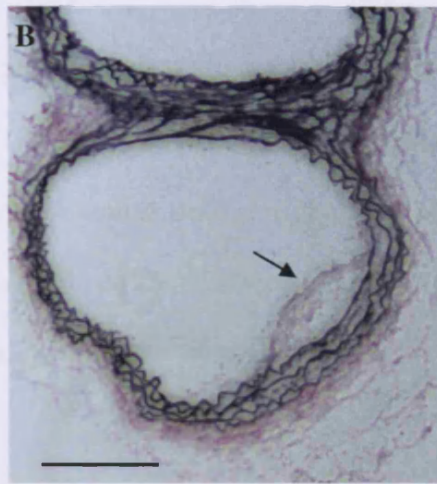
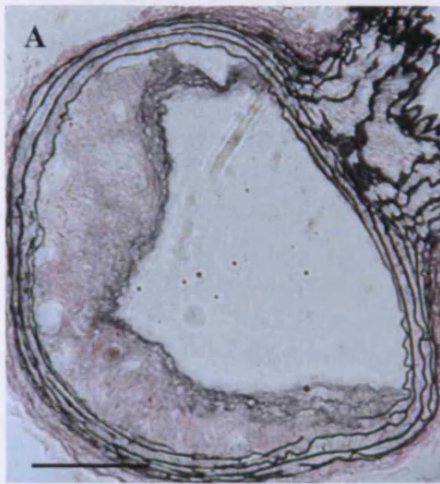


Figure 4.1 Mean plaque area measurement in brachiocephalic arteries of apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice after 8 weeks of high-fat feeding. Sections of the brachiocephalic artery were stained with EVG. The bars show group means ± SEM. Scale bars: 200µm.

- (A) Single large plaque occupying half of the vessel from an apoE^{-/-}/Cd59a^{-/-} mouse.
- (B) A small plaque (arrowed) from an apoE^{-/-} mouse.
- (C) Mean plaque area in apoE^{-/-}/Cd59a^{-/-} (n=14) and apoE^{-/-} (n=15) mice.

2.3.3 Plaque lipid content

As another index of plaque development, plaque lipid content and the effect of MAC on these parameters were measured in apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice. Brachiocephalic artery sections were stained with oil red O to visualise neutral triglyceride and lipid content. No difference was observed in plaque lipid content between apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice as demonstrated in figure 4.2.

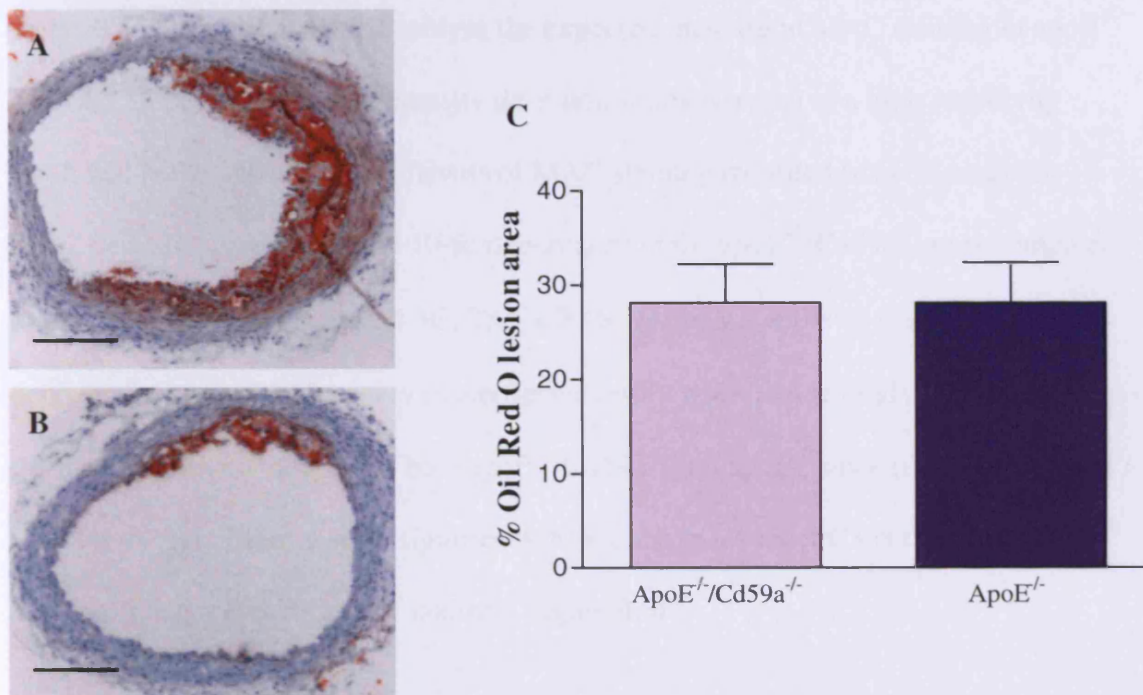


Figure 4.2 Measurement of plaque lipid content. Comparison of percentage of plaque area staining for lipid with oil red O between apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} animals after 8 weeks of high-fat diet. Sections of the brachiocephalic artery were stained with oil red O (red) and counterstained with haematoxylin (blue). Bars show group means \pm SEM. Scale bars: 200 μ m.

(A) Representative section from an apoE^{-/-}/Cd59a^{-/-} mouse. (B) Representative section from an apoE^{-/-} mouse. (C) Mean percentage staining with oil red O in the lesion area in apoE^{-/-}/Cd59a^{-/-} (n=9) and apoE^{-/-} (n=10) mice.

2.3.4 CD59a deficiency causes increased deposition of MAC in plaques

The extent of terminal complement pathway activation was examined by staining for C9 deposition as a surrogate marker of MAC. Complement activation was also assessed by staining for C3 fragment deposition. MAC staining was absent from unaffected vessel walls (i.e. those vessels with no plaque), but clearly present in early and late stage plaques obtained from both apoE^{-/-}/Cd59a^{-/-} (figure 4.3A) and apoE^{-/-} mice (figure 4.3B). To better visualise and analyse the expected increase in MAC staining in apoE^{-/-}/Cd59a^{-/-} mice, fluorescence intensity detection limits were set at a high sensitivity level, hence the apparently low levels of MAC staining recorded in apoE controls. MAC deposits were more than 10-fold increased in the apoE^{-/-}/Cd59a^{-/-} mice compared to the apoE^{-/-} controls (figure 4.3C; $28.9 \pm 9.4\%$ versus $2.7 \pm 0.8\%$; $P \leq 0.05$). C3 deposition was detected weakly in unaffected artery walls and strongly in plaques from the brachiocephalic arteries of both apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice (figures 4.3D and E respectively). There was no significant difference in levels of C3 between the apoE^{-/-}/Cd59a^{-/-} mice and their apoE^{-/-} controls (figure 4.3F).

2.3.5 Infiltrating inflammatory cells in the atherosclerotic plaque

To assess whether increased MAC deposition in apoE^{-/-}/Cd59a^{-/-} mice would cause an increase in the numbers of inflammatory cells, plaques were stained for macrophages and T cells. There were no significant differences in the proportion of plaque area staining for macrophages or T cells (as revealed by staining with MOMA-2 and CD3 respectively) between the two groups (figures 4.4 and 4.5 respectively).

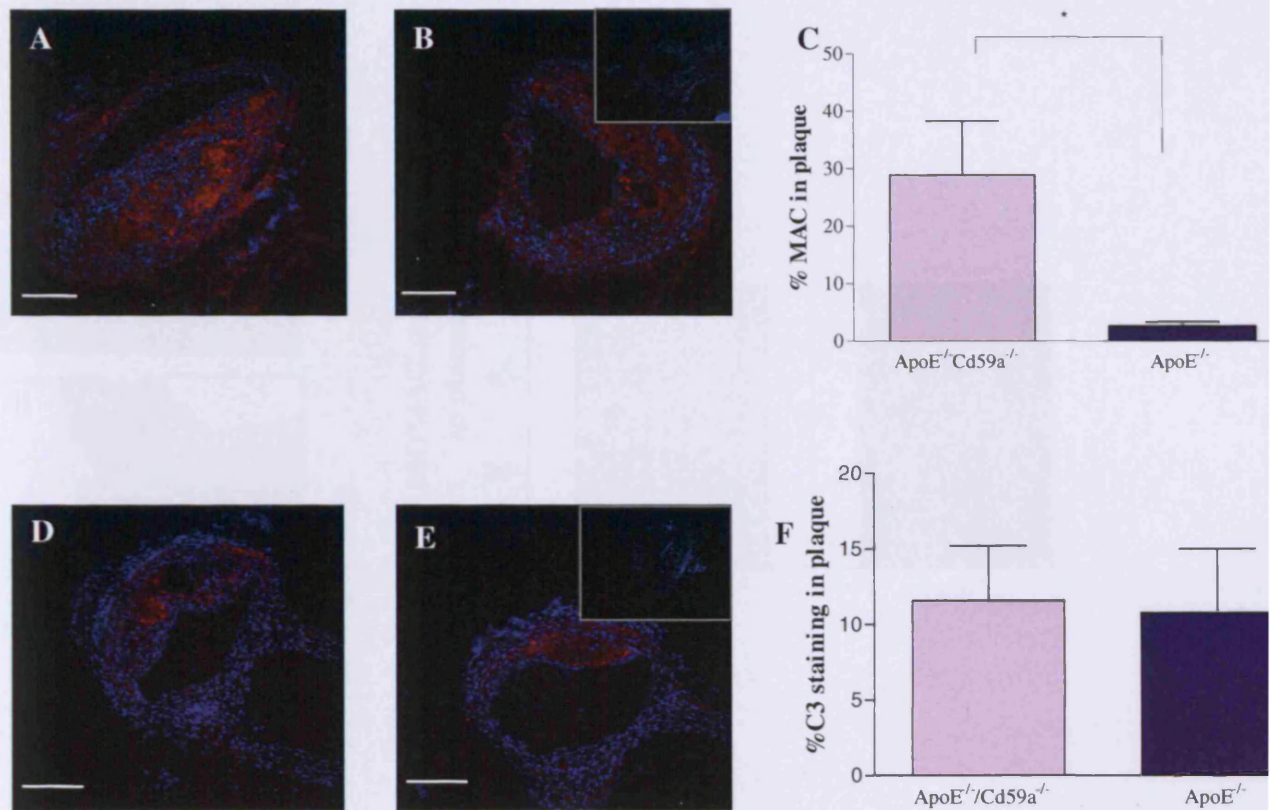


Figure 4.3 MAC deposition and lesional C3 levels in atherosclerotic brachiocephalic arteries. Comparison of percentage MAC staining (Panels A, B, C) or C3 staining (Panels D, E, F) in apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} animals at 16 weeks after 8 weeks of high-fat diet. Sections of the brachiocephalic artery were stained with rabbit anti-rat C9 for MAC or rat anti-mouse native C3 (red) and nuclei were stained with DAPI (blue). The bars show group means \pm SEM. Scale bars: 200 μ m.

- (A) Representative section from an apoE^{-/-}/Cd59a^{-/-} mouse.
- (B) Representative section from an apoE^{-/-} mouse. Inset shows negative control.
- (C) Mean percentage of lesional MAC staining in apoE^{-/-}/Cd59a^{-/-} (n=11) and apoE^{-/-} (n=9) mice.
- (D) Representative section from apoE^{-/-}/Cd59a^{-/-} mouse.
- (E) Representative section from apoE^{-/-} mouse. Inset shows negative control.
- (F) Mean percentage of lesional C3 staining in apoE^{-/-}/Cd59a^{-/-} (n=11) and apoE^{-/-} (n=11) mice.

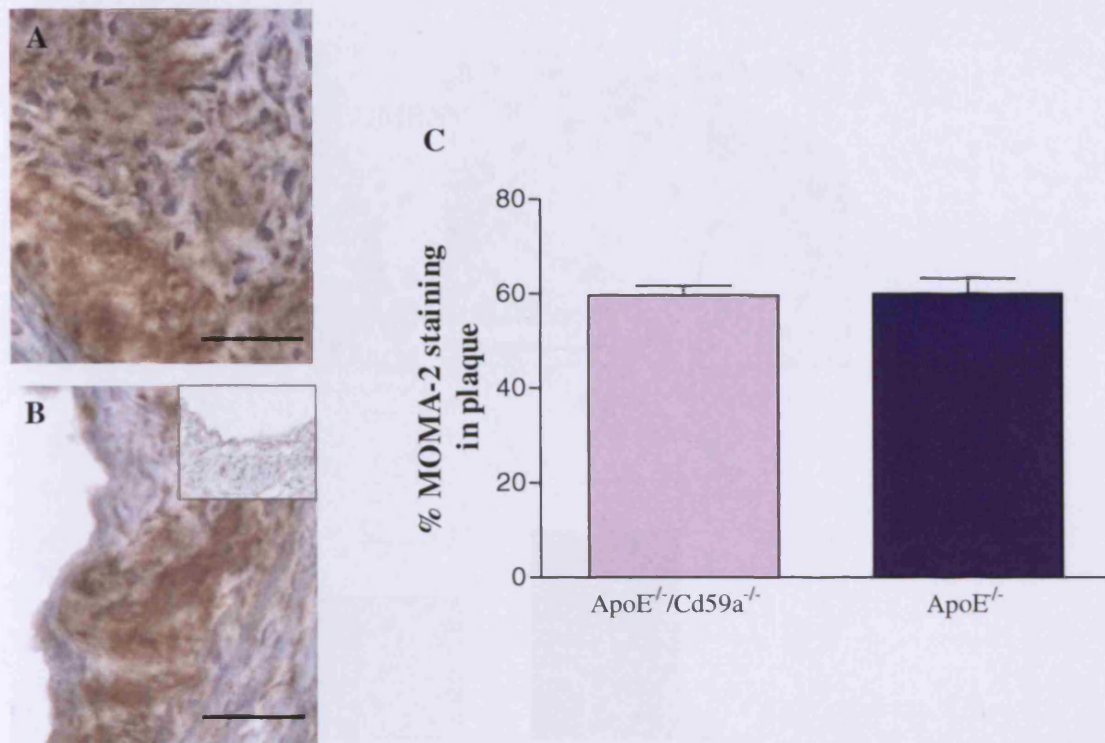


Figure 4.4 Macrophage staining in plaques. Comparison of percentage of plaque area staining with a macrophage marker in apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} animals after 8 weeks of high-fat diet. Sections were stained with MOMA-2 (brown) for macrophages and counterstained with haematoxylin (blue). The bars show group means \pm SEM. Scale bars: 50 μ m. (A) Representative sections from an apoE^{-/-}/Cd59a^{-/-} and (B) apoE^{-/-} mouse stained for MOMA-2, inset shows an isotype control stained section. (C) Mean percentage of plaque stained with MOMA-2 in apoE^{-/-}/Cd59a^{-/-} (n=7) and apoE^{-/-} (n=4) mice.

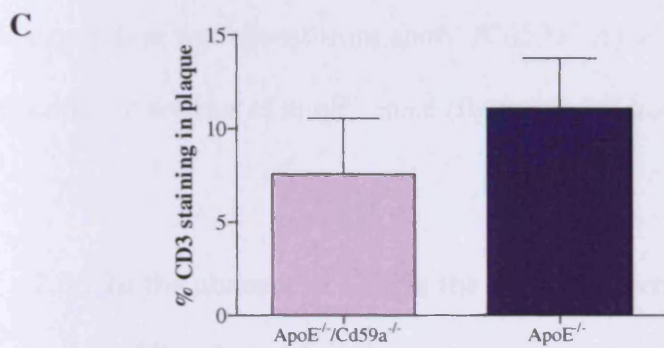
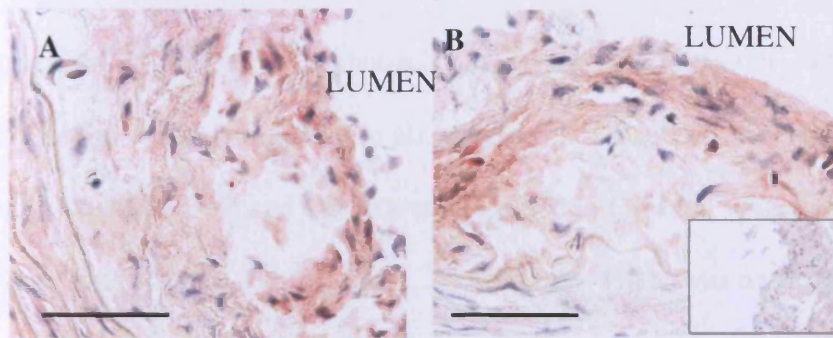


Figure 4.5 Measurement of T-cell staining in plaques. Comparison of percentage CD3-positive staining within the lesions of apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice after 8 weeks of high-fat diet. Sections of the brachiocephalic artery were stained with anti-CD3 antibody (red) and counterstained with haematoxylin (blue). Bars show group means \pm SEM. Scale bars for **A** and **B**: 50 μ m. Scale bars for **D** and **E**: 200 μ m

(A) Representative section from an apoE^{-/-}/Cd59a^{-/-} mouse.

(B) Representative section from an apoE^{-/-} mouse. Inset shows an isotype control stained section.

(C) Mean percentage CD3-stained lesion area in apoE^{-/-}/Cd59a^{-/-} (n=5) and apoE^{-/-} (n=7) mice.

2.3.6 The absence of Cd59a is not compensated by up-regulation of other complement regulators

Expression of the membrane-bound complement regulators Crry, CD55 and Cd59a were examined and compared in atherosclerotic vessels of both apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} controls. Crry and CD55 were expressed in normal vessel walls and plaques in both groups of mice (figures 4.6 A, B, D and E). There was no obvious difference in immunostaining by casual examination of either Crry or CD55 between the groups. Cd59a expression was absent from apoE^{-/-}/Cd59a^{-/-} mice and present within the brachiocephalic arteries of apoE^{-/-} mice (figures 4.6 C and F respectively).

2.3.7 In the absence of Cd59a the MAC influences SMC proliferation and survival in atherosclerosis

The MAC has been shown to co-localise with SMCs in human atherosclerotic plaques, where it plays a major role in SMC proliferation (Niculescu et al., 1999; Torzewski et al., 1997). To examine the role of the MAC and its regulator CD59 on plaque SMC content sections from the brachiocephalic arteries of apoE^{-/-} mice lacking the inhibitor of MAC formation, Cd59a were stained for α -actin (a marker of SMCs). Firstly SMC content in the plaques from apoE^{-/-}/Cd59a^{-/-} versus apoE^{-/-} controls was compared. In this case, the absence of Cd59a did not appear to alter plaque SMC content (figure 4.7).

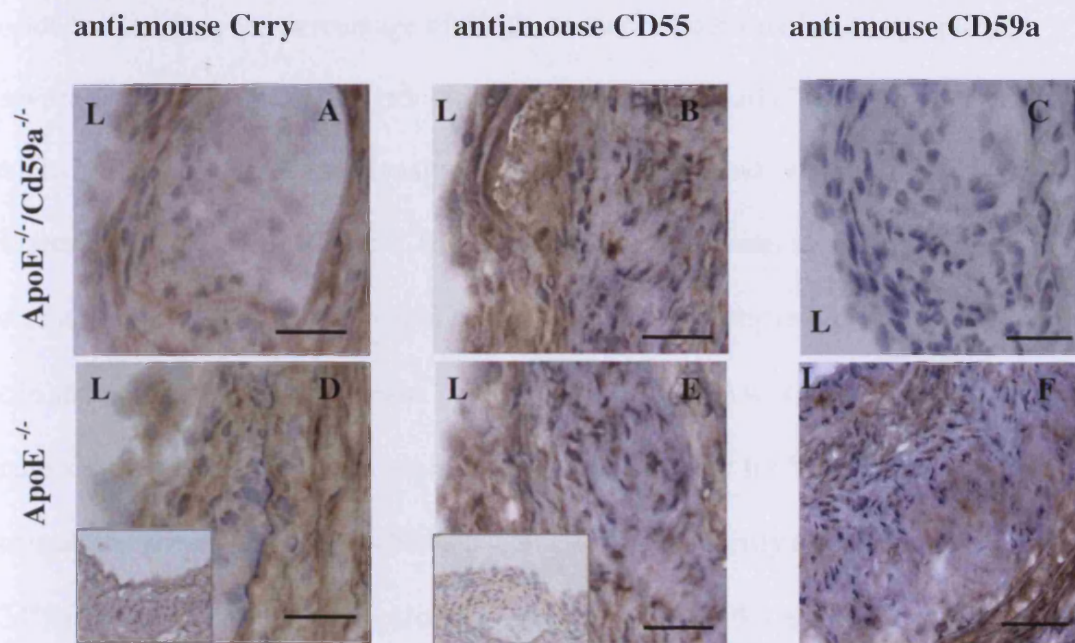


Figure 4.6 Immunolocalisation of complement regulators. *ApoE^{-/-}/Cd59a^{-/-}* and *apoE^{-/-}* mice were fed a high-fat diet for 8 weeks from 8 weeks of age. Sections of the brachiocephalic artery were stained for complement regulators using specific monoclonal antibodies (brown) and nuclei were stained with haematoxylin (blue). Scale bars: 50µm. Lumen is indicated by L.

(A), (B) and (C) Staining of Crry (rat anti-mouse Crry mAb (5D5)), CD55 (rat anti-mouse CD55 mAb (2C6)) and CD59a (rat anti-mouse CD59a mAb (mCD59a.7)) respectively in *apoE^{-/-}/Cd59a^{-/-}* mice. (D), (E) and (F) Staining of Crry, CD55 and CD59a respectively in *apoE^{-/-}* mice. Inset shows a negative stained section of an atherosclerotic brachiocephalic artery from an *apoE^{-/-}* mouse for Crry (D) and CD55 (E) staining respectively.

However, it was noted that plaque size varied greatly within both groups, thus it was decided to compare the percentage of SMC content in both small and large plaques between the two groups. After grouping the plaques into “early” (fatty streaks and fibrous plaques with cross sectional areas $<80 \times 10^3 \mu\text{m}^2$) and “advanced” (complex plaques with cross sectional areas $\geq 80 \times 10^3 \mu\text{m}^2$), a significant increase in the SMC content of “early” plaques from apoE^{-/-}/Cd59a^{-/-} mice as compared to apoE^{-/-} mice (% α -actin staining $62.0\% \pm 7.1\%$ versus $21.4 \pm 6.1\%$; $P < 0.01$) was found. Figures 4.8 A and B show representative pictures of early plaques stained for SMC α -actin. By contrast, in “advanced” plaques SMC content was significantly reduced in apoE^{-/-}/Cd59a^{-/-} mice versus apoE^{-/-} controls (% α -actin staining $15.3 \pm 4.8\%$ versus $36.6 \pm 6.7\%$; $P < 0.05$). Figures 4.8 C and D show representative pictures of advanced plaques stained for SMC α -actin. Combined data are presented in figure 4.8 F. The proportion of plaque staining for α -actin in the apoE^{-/-}/Cd59a^{-/-} mice fell more than three-fold between “early” and “advanced” plaques ($62.0\% \pm 7.1\%$ versus $15.3 \pm 4.8\%$; $P < 0.01$).

2.3.8 Co-localisation of MAC on SMCs in an atherosclerotic artery of an apoE^{-/-} mouse

To further interrogate the relationship between MAC and SMC plaque content in the mouse, brachiocephalic artery sections of high fat fed apoE^{-/-} mice were double stained with rat anti-rabbit C9 and α -smooth muscle actin. The data clearly show that MAC co-localises with SMC in the plaque, which is depicted in yellow (figure 4.9).

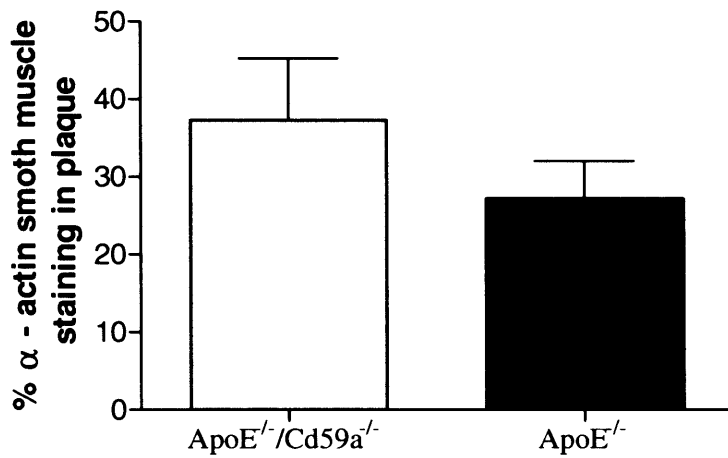


Figure 4.7 Percentage of SMC content in apoE^{-/-}/Cd59a^{-/-} versus apoE^{-/-} mice. Mice were fed a high fat diet at 8 weeks of age for 8 weeks. Brachiocephalic artery sections were stained with α -actin smooth muscle antibody. Graph shows percentage of plaque stained for smooth muscle α -actin in apoE^{-/-}/Cd59a^{-/-} (n=10) and apoE^{-/-} (n=13) mice.

2.3.9 Co-localisation of MAC with macrophages within the plaque

Although no difference was observed in percentage plaque macrophage content between apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice, we investigated whether there was any relation between MAC formation and resident macrophages within the plaque. We observed co-localisation of MAC with macrophages (figure 4.10 (depicted in yellow)) at deep intimal sites within the lesion.

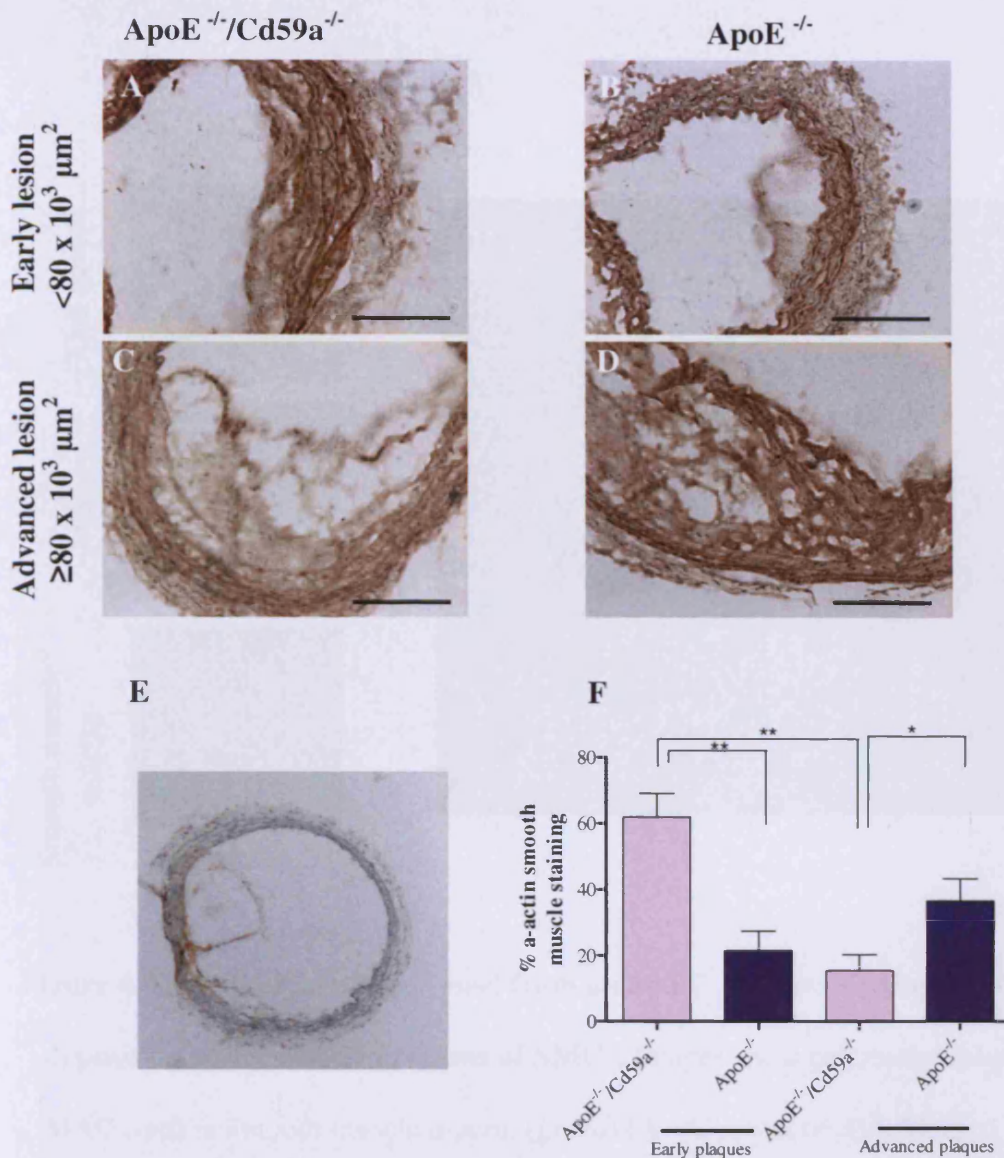


Figure 4.8 SMC content in early and advanced lesions. Comparison of percentage smooth muscle α -actin staining in early plaques ($< 80 \times 10^3 \mu\text{m}^2$) or advanced plaques (complex plaques with areas $\geq 80 \times 10^3 \mu\text{m}^2$) of apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} animals at 16 weeks, after 8 weeks of high-fat diet. Brachiocephalic artery sections were stained for SMCs with anti-smooth muscle α -actin and counterstained with haematoxylin (blue). The bars show group means \pm SEM. Scale bars = 200 μm . (A) Representative section of an early lesion from an apoE^{-/-}/Cd59a^{-/-} mouse. (B) Representative section of an early lesion from an apoE^{-/-} control. (C) Representative section of an advanced lesion from an apoE^{-/-}/Cd59a^{-/-} mouse. (D) Representative section of an advanced lesion from an apoE^{-/-} control. (E) A negative stained section of an atherosclerotic brachiocephalic artery from an apoE^{-/-} mouse. (F) Percentage of plaque stained for smooth muscle α -actin in early and advanced plaques of apoE^{-/-}/Cd59a^{-/-} (n=3 and 8 respectively) and apoE^{-/-} (n=5 and 5 respectively) mice.

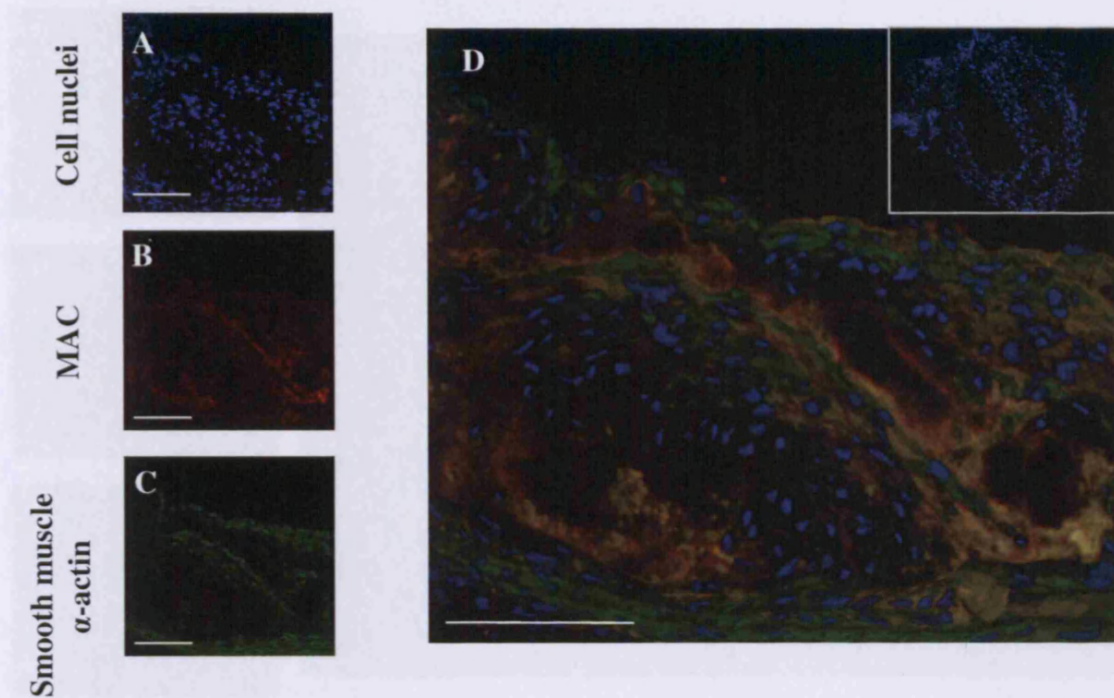


Figure 4.9 An atherosclerotic vessel from an $\text{apoE}^{-/-}$ mouse showing MAC deposition co-localised in regions of SMCs. Images show cell nuclei (blue), MAC (red) or smooth muscle α -actin (green) (A - C respectively). Merged images correspond to the overlay of cell nuclei, MAC and smooth muscle α -actin (Figure D). Co-localisation of MAC with smooth muscle α -actin staining appears in yellow. Inset shows negative control. Scale bar: $100\mu\text{m}$.

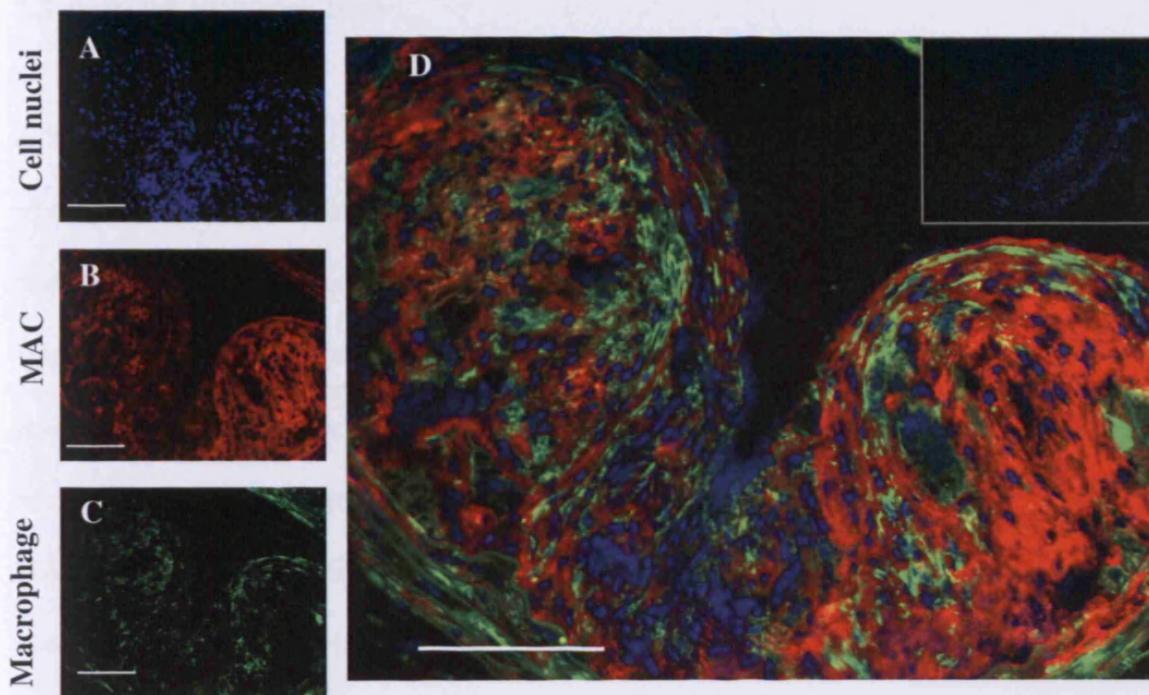


Figure 4.10 An atherosclerotic vessel from an apoE^{-/-} mouse showing MAC deposition co-localised in regions of macrophages. Images show cell nuclei (blue), MAC (red) or macrophage marker F4/80 (green) (A - C respectively). (D) Merged images correspond to the overlay of cell nuclei, MAC and macrophages, inset shows negative control. Co-localisation of MAC with macrophage is shown in yellow. Scale bar: 100µm.

3. The role of complement C6 in atherosclerosis

3.1 A brief introduction

In the first part of chapter 4, it is shown that MAC formation plays an important role in atherosclerotic plaque growth since in the absence of the MAC regulator, CD59a, the rate of progression of atherosclerosis was greatly accelerated in the apoE^{-/-} mouse model. To further explore this relationship, atherosclerotic burden was studied in the absence of functional terminal lytic pathway activation, using C6^{-/-} mice on the apoE^{-/-} background. C6^{-/-} mice are particularly relevant for investigating the pathogenic role of MAC, since they have previously been used to implicate MAC as a causative agent in a wide range of diseases, including reperfusion injury, glomerular damage, and xenograft hyperacute rejection (Falk et al., 1983; Fondevila et al., 2008; McCurry et al., 1995).

I hypothesised that: **deficiency of C6 would result in diminished terminal pathway activation in apoE^{-/-} mice causing protection against atherosclerosis.**

Two independent studies have already shown that deficiency of C6 was protective in a rabbit model of atherosclerosis (Geertinger and Soerensen, 1977; Schmiedt et al., 1998). However, studies using mouse models of atherosclerosis to determine the effect of terminal pathway activation have been controversial. Whilst C5 deficiency had no effect on atherosclerotic burden in apoE^{-/-} mice (Patel et al., 2001), inhibition of C5 using a neutralising anti-mouse C5 antibody (BB5.1) slowed the progression of atherosclerosis, assessed in aortic roots and by *en face* staining, of apoE^{-/-}/Cd59a/b^{-/-} mice (Wu et al., 2009).

3.2 Results

3.2.1 Deficiency of C6 inhibits atherosclerotic plaque progression in apoE^{-/-} mice

To delineate the role of MAC during atherosclerosis apoE^{-/-} mice were crossed with C6^{-/-} mice generating apoE^{-/-}/C6^{-/-} and controls (apoE^{-/-}/C6^{+/+}). Having no C6, these mice were incapable of MAC formation from birth. The mice were fed a high-fat diet for 12 weeks to induce severe disease, in the expectation that this would more clearly reveal differences between the test and control groups. Analyses of the brachiocephalic arteries revealed that the mean plaque cross-sectional area in apoE^{-/-}/C6^{-/-} mice was significantly decreased compared to the apoE^{-/-}/C6^{+/+} controls. Figures 4.11 A and B show representative sections from apoE^{-/-}/C6^{-/-} and apoE^{-/-} mice respectively, while figure 4.11 C shows the pooled data (66.0 ± 23.2 versus $179.5 \pm 14.1 \times 10^3 \mu\text{m}^2$; $P < 0.001$). Table 4.3 summarises the morphometric analyses, including plaque, lumen, media and vessel area. The mean lumen and media areas were not different between the groups. However, the total vessel area was significantly decreased in the apoE^{-/-}/C6^{-/-} mice compared to apoE^{-/-} controls (Table 4.3; 349.9 ± 39.6 versus $469.8 \pm 28.2 \times 10^3 \mu\text{m}^2$; $P < 0.05$). There were no significant differences in body weight, heart: tibia, kidney: tibia or spleen: tibia ratio between apoE^{-/-}/C6^{-/-} and apoE^{-/-} mice (Table 4.4).

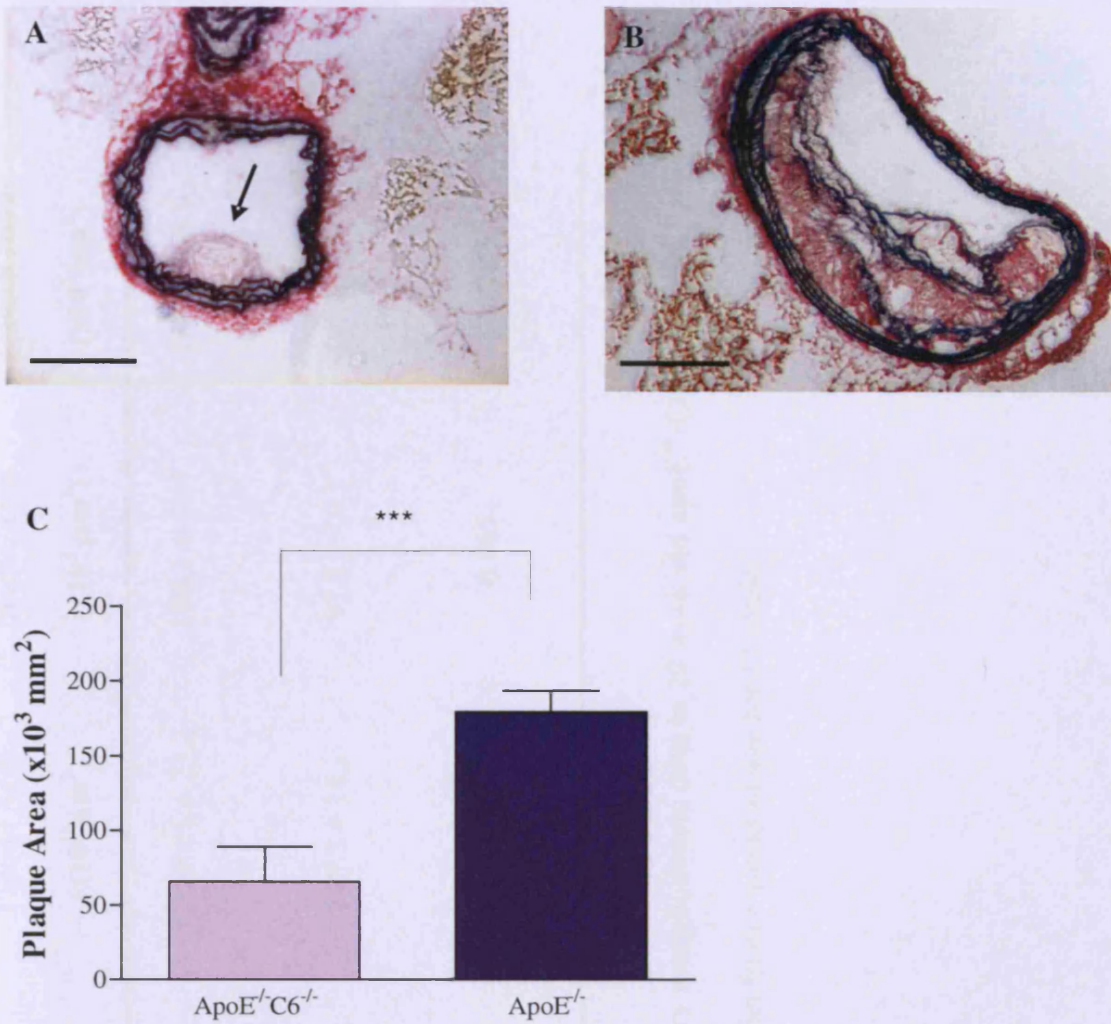


Figure 4.11 Mean plaque cross sectional area in brachiocephalic artery of *apoE^{-/-}/C6^{-/-}* and *apoE^{-/-}* control mice after 12 weeks of high-fat feeding. Sections of the brachiocephalic artery were stained with EVG. The bars show group means \pm SEM. Scale bars: 200 μm .

(A) A small plaque (arrowed) from an *apoE^{-/-}/C6^{-/-}* mouse.

(B) Single large plaque occupying half of the vessel from an *apoE^{-/-}* mouse.

(C) Mean plaque area in *apoE^{-/-}/C6^{-/-}* (n=8) and *apoE^{-/-}* (n=10) mice.

	Vessel Area (x10³ μm²)	Plaque Area (x10³ μm²)	Media Area (x10³ μm²)	Lumen Area (x10³ μm²)
ApoE ^{-/-} /C6 ^{-/-} (n=8)	*349.9 ± 39.6	***66.01 ± 23.2	100.2 ± 11.7	183.7 ± 15.9
ApoE ^{-/-} (n=10)	469.8 ± 28.2	179.5 ± 14.1	97.5 ± 10.1	192.8 ± 17.2
P value	0.022	0.001	0.865	0.708

Table 4.3 Brachiocephalic artery morphometric data in 20 week old apoE^{-/-}/C6^{-/-} and apoE^{-/-} mice.

All animals were fed a high-fat diet from 8 weeks of age for 12 weeks.

Group	Body weight (g)	Heart: tibia (x10⁻³)	kidney: tibia (x10⁻³)	Spleen: tibia (x10⁻³)
ApoE ^{-/-} /C6 ^{-/-}	39.7 ± 1.5 (n=25)	9.6 ± 0.6 (n=5)	13.7 ± 0.6 (n=5)	8.1 ± 1.0 (n=5)
ApoE ^{-/-}	42.85 ± 1.2 n=25	10.7 ± 0.5 (n=15)	12.2 ± 0.5 (n=10)	9.3 ± 0.5 (n=10)
P value	0.103	0.244	0.128	0.347

Table 4.4 Body weight and heart, kidney and spleen: tibia data in 20 week old apoE^{-/-}/C6^{-/-} and apoE^{-/-} mice. In order to assess organ hypertrophy, tibia length was measured and used to normalise heart, spleen and kidney weights. All animals were fed a high-fat diet from 8 weeks of age for 12 weeks.

3.2.2 Terminal pathway activation is abolished in atherosclerotic plaques from apoE^{-/-}/C6^{-/-} mice

It was hypothesised that attenuation of plaque progression observed in apoE^{-/-}/C6^{-/-} mice was due to absence of terminal complement activation and MAC formation within the plaque. To address this hypothesis, plaques were stained for the presence of MAC. MAC deposition was abundant in plaques of control apoE^{-/-} mice, both on the endothelium (inset, thick white arrow) and in the necrotic core (inset, large non-cellular area) (figure 4.12 B). In contrast, no staining for MAC above background levels was found in plaques from apoE^{-/-}/C6^{-/-} mice (figure 4.12 A). Figure 4.12 C shows the pooled data for MAC deposition (apoE^{-/-}/C6^{-/-}: 12.8 ± 3.5% versus apoE^{-/-}: 43.0 ± 6.5%; P ≤ 0.01).

3.2.3 Altered lipid metabolism in apoE^{-/-}/C6^{-/-} mice

Lipid profile measurements were carried out to assess whether lack of MAC formation in the plaque was solely responsible for attenuation of disease progression in apoE^{-/-}/C6^{-/-} mice. Since alteration of lipid profile can influence disease progression, serum cholesterol, triglyceride and serum turbidity were measured in apoE^{-/-}/C6^{-/-} mice. Glucose levels were also assessed for this reason. Surprisingly, a significant decrease in cholesterol, triglyceride and serum turbidity was observed in the serum of apoE^{-/-}/C6^{-/-} mice when compared to apoE^{-/-} controls (Figure 4.13 A; 33.0 ± 3.6mmol/L versus 44.0 ± 2.3mmol/L; P<0.05; B; 2.7 ± 0.7mmol/L versus 4.4 ± 0.4mmol/L; P<0.05; C; 31.4 ± 5.5mmol/L versus 61.77 ± 5.9mmol/L; P<0.01 respectively). C6 deficiency had no effect on glucose levels (figure 4.13 D).

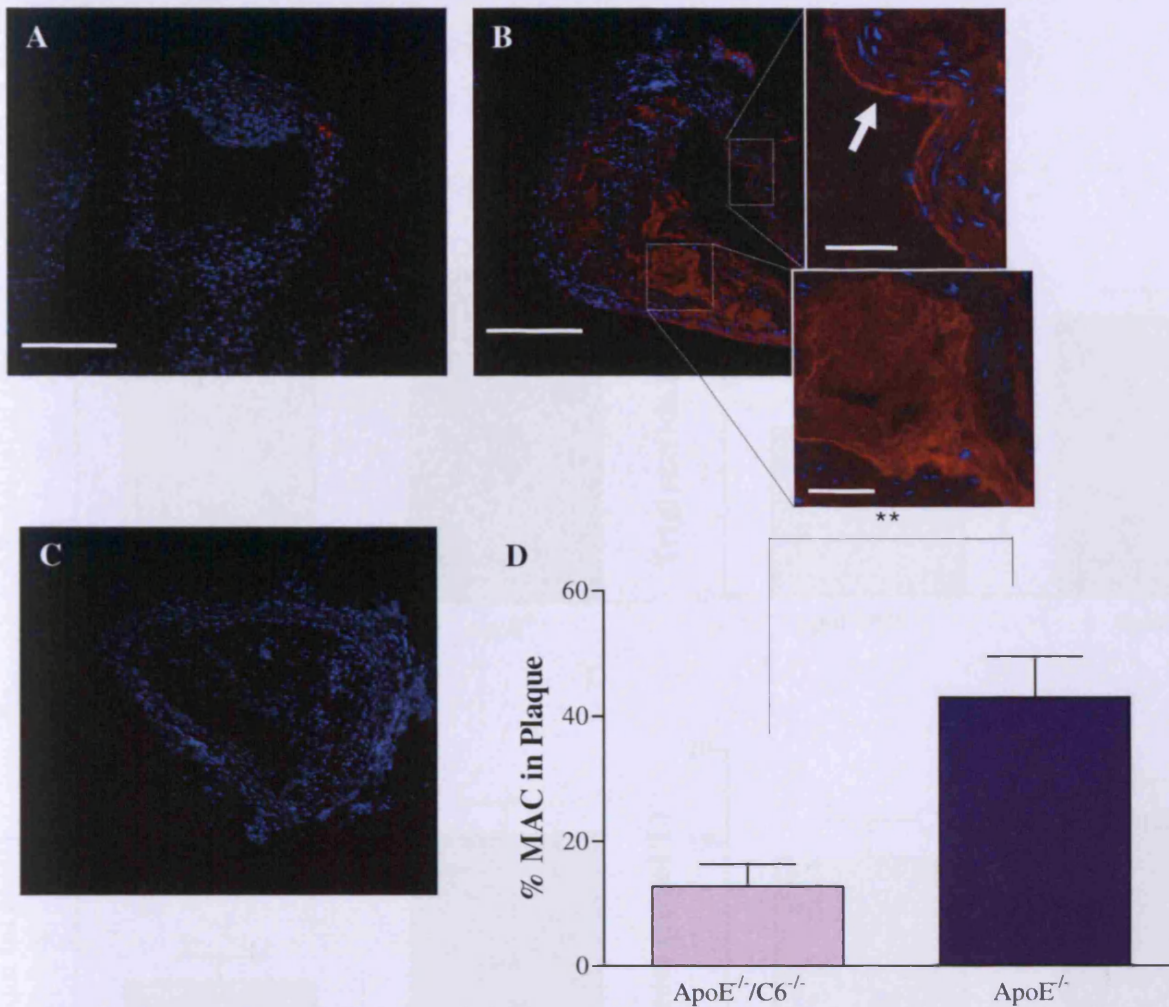


Figure 4.12 Terminal complement activation in apoE^{-/-}/C6^{-/-} and apoE^{-/-} mice

Brachiocephalic artery sections were stained with rabbit anti-rat C9 (red) for MAC deposition and nuclei were stained blue with DAPI.

(A) Minimal MAC staining in a large plaque from an apoE^{-/-}/C6^{-/-} mouse.

(B) MAC staining is abundant in an advanced plaque of an apoE^{-/-} mouse. Insets show higher magnification of areas where MAC deposition is localised within the plaque:

lining the endothelial wall (arrowed) and within the necrotic core of the plaque. (Inset scale bar: 50µm).

(C) Brachiocephalic artery section stained with secondary antibody only.

(D) Comparison of percentage MAC staining in apoE^{-/-}/C6^{-/-} (n=8) and apoE^{-/-} (n=9) mice.

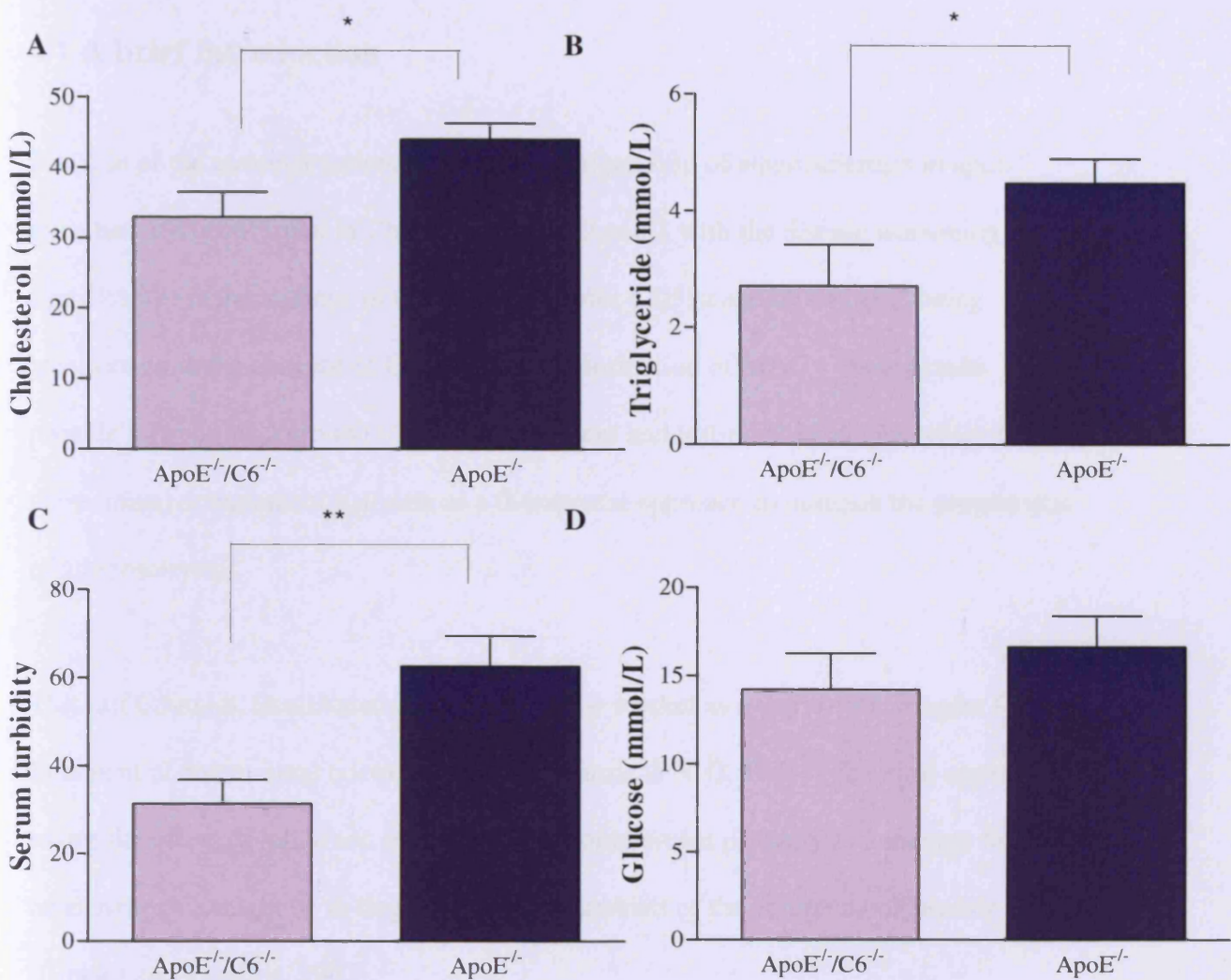


Figure 4.13 Serum lipid and glucose levels of apoE^{-/-}/C6^{-/-} and apoE^{-/-} mice after 12 weeks of high-fat feeding. (A) Serum cholesterol (n=8 and 16 respectively) (B) triglyceride (n=9 and 16 respectively) (C) serum turbidity (n=9 and 16 respectively) and (D) glucose levels (n=6 and 5 respectively) were measured at 20 weeks of age after 12 weeks of high fat diet in apoE^{-/-}/C6^{-/-} mice and apoE^{-/-} controls.

4. Complement therapy for the treatment of atherosclerosis

4.1 A brief introduction

The role of the terminal pathway during the progression of atherosclerosis in apoE^{-/-} mice has been confirmed in Chapter 4 section 2 and 3 with the disease worsening significantly in the absence of the MAC regulator CD59a and by contrast, being ameliorated in the absence of C6, vital for the formation of MAC . These results provide a strong background for the development and testing of strategies which target the terminal complement pathway as a therapeutic approach to mitigate the progression of atherosclerosis.

The anti C5 mAb, Eculizumab, is already on the market as a therapeutic reagent for the treatment of paroxysmal nocturnal hemoglobinuria (PNH). Thus, a practical approach to test the effect of inhibition of the terminal complement pathway as a therapy for atherosclerosis might be to target the fifth component of the complement cascade (Ricklin and Lambris, 2007).

It was hypothesised that: **inhibiting activation of the terminal pathway by administration of a C5 mAb in vivo would reduce disease severity in an atherosclerotic-prone mouse model.**

This study was carried out using the anti-mouse C5 mAb, BB5.1. This antibody, similar to Eculizumab, prevents cleavage of complement factor C5, thereby preventing C5b generation and MAC formation. In other mouse models, administration of BB5.1 has been shown to attenuate disease progression in experimental autoimmune uveoretinitis,

myocardial ischemia and reperfusion injury and antibody-induced glomerulonephritis (Busche and Stahl, 2010; Copland et al., 2010; De Vries et al., 2003; Huugen et al., 2007).

4.2. Specific methods

4.2.1 BB5.1 Purification

4.2.1.1 Antibody preparation

Anti-mouse C5 neutralising antibody, BB5.1, producing hybridoma cells (a kind gift from Professor B. Stockinger) were maintained in CELLline 1000 Integra flasks (Integra Bioscience). The flask consists of a lower cell compartment and an upper compartment for nutrient medium separated by a 10kDa semi-permeable membrane.

Cell compartment medium contained 15% v/v heat inactivated low bovine IgG foetal calf serum (Invitrogen Life Technologies) in RPMI-1640 (Invitrogen Life Technologies), 50U/ml penicillin/streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and 1% non-essential amino acids (Invitrogen Life Technologies).

Nutrient medium contained RPMI-1640 (Invitrogen Life Technologies), 50U/ml penicillin/streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and 1% non-essential amino acids (Invitrogen Life Technologies).

Initially, approximately 2×10^7 cells in 20 ml of tissue culture media were inoculated from static culture into the cell compartment of the CELLline 1000 integra flask.

Nutrient medium (1L) was placed in the nutrient medium compartment and the flask placed into a 5% CO₂, 37 ° C humidified tissue culture incubators.

During cell harvesting the nutrient medium was discarded and the contents of the cell compartment were then removed with a pipette. Cell numbers were determined by diluting and counting samples using a standard haemocytometer. Cells were diluted 3-4 fold determined by cell numbers in fresh cell compartment medium to 20 ml and returned to the cell compartment. Nutrient medium was removed and replaced by pouring during cell compartment harvest.

The residual harvested cell and antibody containing supernatant was centrifuged to remove cells and debris (6000g for 30 minutes at 4°C) and subsequent filtration, through a 0.45µm Stericup filter unit (Millipore).

4.2.1.2 Purification of BB5.1 using Protein G affinity chromatography

Culture supernatant prepared as above was applied to a HiTrap Protein G column on the Akta Prime chromatography system. All steps were performed at 4°C in order to minimise the loss of functional activity of BB5.1. The HiTrap column was connected to the AKTA prime, equilibrated with binding buffer (50mM NaPhosphate, 500mM NaCl, pH6) then the supernatant was loaded onto the column. The run-through supernatant was retained and the column washed with binding buffer. Bound antibody was eluted from the column using elution buffer (0.1 M glycine/HCl pH 2.5) and 1ml fractions were collected into neutralising buffer (300µl 1M Tris/HCl pH9). Eluted antibody was pooled and dialysed in PBS overnight at 4°C. The dialysed antibody was

then concentrated using an Amicon concentration system and stored at -20°C until use.

Purity of antibody was assessed by SDS PAGE.

4.2.2 Mouse Ig purification

4.2.2.1 Preparation of mouse Ig using Protein G affinity chromatography

Mouse serum (25ml; Gene Tex Inc.) was centrifuged at 10,000 RPM for 20 minutes at 4°C, filtered through a 0.45µm Stericup filter unit, and diluted 1:1 in PBS, to give a final volume of 50ml prior to application to a protein G column for antibody purification as above.

4.2.2.2 In vitro characterisation of BB5.1 and mouse Ig

The functional activity of BB5.1 and mouse Ig were tested in vitro. 50µl of rbE-A were incubated with 50µl mouse serum pre-treated with doubling dilutions of either BB5.1 or mouse Ig control starting at a concentration of 5µg/ml. After incubating for 30 minutes at 37°C cells were pelleted by centrifugation at 2400rpm for 5 minutes and the percentage haemolysis was calculated (for method see chapter 2). Measurement of haemoglobin release was expressed relative to 100% control comprising erythrocytes lysed using H₂O.

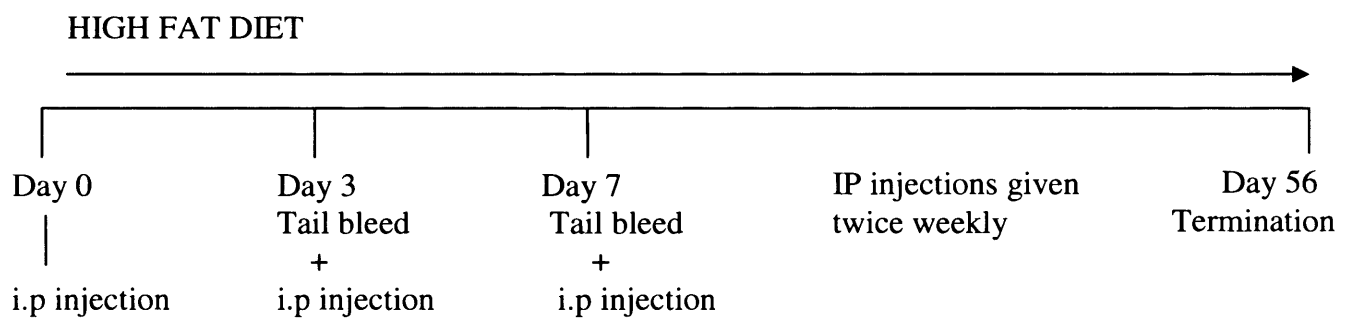
4.2.2.3 In vivo characterisation of BB5.1 and mouse Ig

A pilot experiment was set up in order to assess the dose of antibody required to inhibit complement lysis in apoE^{-/-} mice. 8 week old male apoE^{-/-} mice were given a single i.p injection of either 1.5mg/ml or 2mg/ml of BB5.1 or mouse Ig. 3 days post injection,

mice were sacrificed and blood harvested by cardiac puncture. Blood was allowed to clot, and the serum was obtained by centrifugation at 10,000rpm for 10 minutes at 4°C. Serum from the animals was then tested in a haemolysis assay using rbE-A.

4.2.3 Experimental protocol

Male apoE^{-/-} mice were placed on a high fat diet at 8 weeks of age for 8 weeks concurrent with antibody treatment before termination (see Chapter 2 for termination protocol). During the treatment period mice were given either 500µl of BB5.1 (2mg/ml) or 500µl of mouse Ig (2mg/ml), twice weekly by i.p injection. Blood samples were taken on days 0 (prior to administration of BB5.1), 3 and 7 (post BB5.1 treatment). This protocol is shown below in diagrammatic form. Subsequently, both percentage haemolysis and CH50 values were calculated in order to ensure complement inhibition was achieved in these animals (see chapter 2 for detailed method).



4.3 Results

4.3.1 Purification of anti-mouse C5 mAb and mouse Ig

In order to assess the efficacy of a strategy to block the terminal complement pathway through the use of therapeutic monoclonal antibodies, the anti C5 mAb BB5.1 was produced in large quantities and purified to homogeneity on Protein G column; mouse IgG was similarly purified (figure 4.14). One clear peak was visible in the elution fractions at 280nm, and the relevant fractions were collected. After 3 runs of the supernatant over the Protein G column, 88mg of BB5.1 was purified in a total volume of 14 ml, and 110mg of mouse Ig in a total volume of 22ml.

To assess the purity of the eluted BB5.1 and mouse Ig samples, SDS-PAGE was performed. Figure 4.14 A shows that one band was detected in the non-reduced BB5.1 sample and two bands were visible in the reduced BB5.1 sample of a Coomassie staining of an SDS-PAGE gel. Similarly, figure 4.14 B shows that one band is detected in the non-reduced mouse Ig sample and two bands were seen in the reduced mouse Ig sample. These results show that both BB5.1 and mouse Ig samples are pure with the native protein forming a band at 150 kDa. The reduced gel of both samples show a heavy chain band at 50kDa and a light chain at 25kDa.

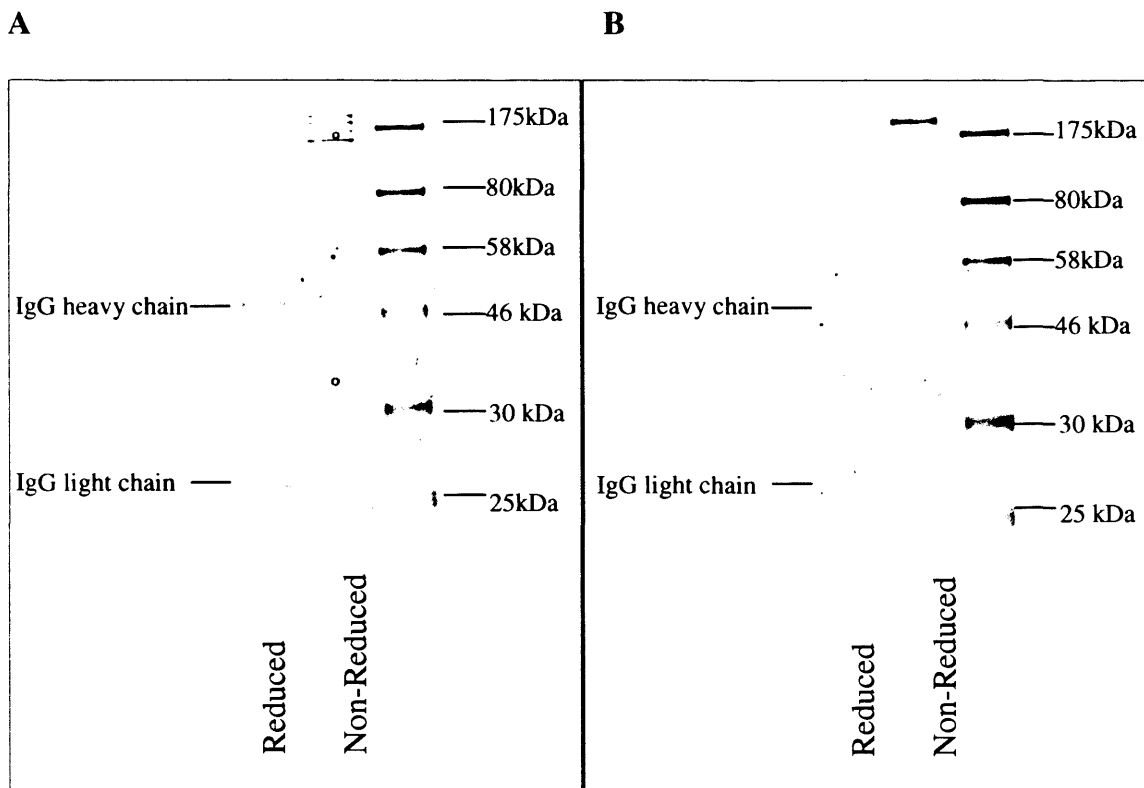


Figure 4.14 Purification of BB5.1 and mouse Ig. BB5.1 and mouse Ig was purified by using protein G chromatography (A) Coomassie stained 10% SDS-PAGE gel showing a band of 150kDa MW for non-reduced BB5.1 and two bands of 50kDa and 25kDa for reduced BB5.1. (B) Coomassie stained 10% SDS-PAGE gel showing a major band of 150kDa MW for non-reduced mouse Ig and two bands of 50kDa and 25kDa for reduced mouse Ig.

4.3.2 BB5.1 inhibits complement haemolysis in mouse serum in vitro

Before using BB5.1 to neutralise C5 in vivo, haemolysis assay was carried out to determine its functional activity. The haemolytic assay was carried out using mouse serum, which had been titrated to give 100% lysis of rb-EA at a dilution of 1:20. As shown in figure 4.15 addition of mouse Ig to mouse serum had no inhibitory effect on

the percentage haemolysis. However, the addition of 3.33 μ g/ml of BB5.1 to mouse serum resulted in a 72.5% inhibition of complement mediated haemolysis.

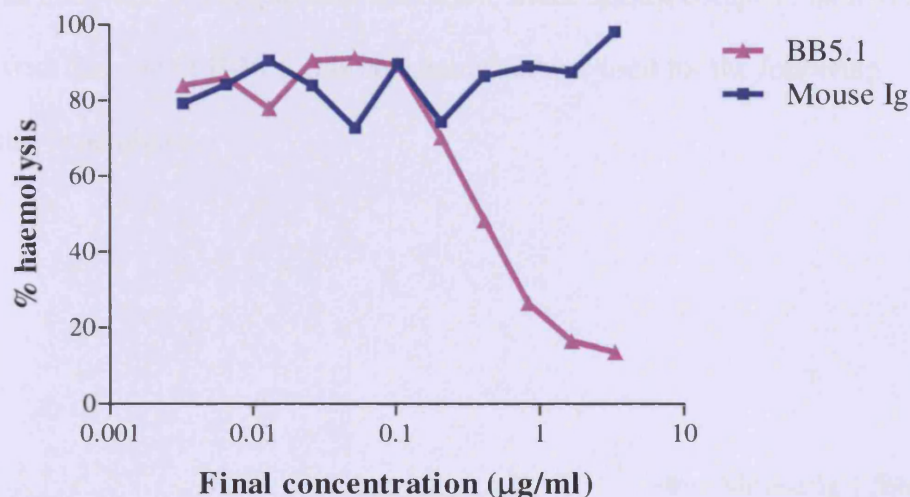


Figure 4.15 BB5.1 inhibits complement activation in vitro. RbE-A were incubated with mouse serum pre-treated with doubling dilutions of either the monoclonal anti mouse-C5 antibody BB5.1 or mouse Ig control. Measurement of haemoglobin release was expressed relative to 100% control erythrocytes lysed with H₂O.

4.3.3 Pilot experiment to determine function of purified BB5.1 on complement haemolytic activity in vivo

A pilot experiment was carried out in order to determine the optimal concentration of the antibody required to inhibit complement activation in vivo. ApoE^{-/-} mice received 0.75 mg (500 μ l of 1.5 mg/ml) or 1mg (500 μ l of 2 mg/ml) of either mouse Ig or BB5.1 by i.p injection. Three days post injection the animals were sacrificed and blood harvested by cardiac puncture. Blood was allowed to clot and the serum was collected after centrifugation. Doubling dilutions of the serum were added to rbE-A and

percentage haemolysis was calculated. The data from this experiment is shown in figure 4.16. Administration of mouse Ig in vivo, at either 1.5mg/ml or 2mg/ml, did not affect complement activity. In contrast, i.p injection of BB5.1 at either 1.5mg/ml or 2mg/ml resulted in inhibition of complement activation. Since almost complete inhibition was reached with 2mg/ml of BB5.1, this concentration was used for the following therapeutic experiment.

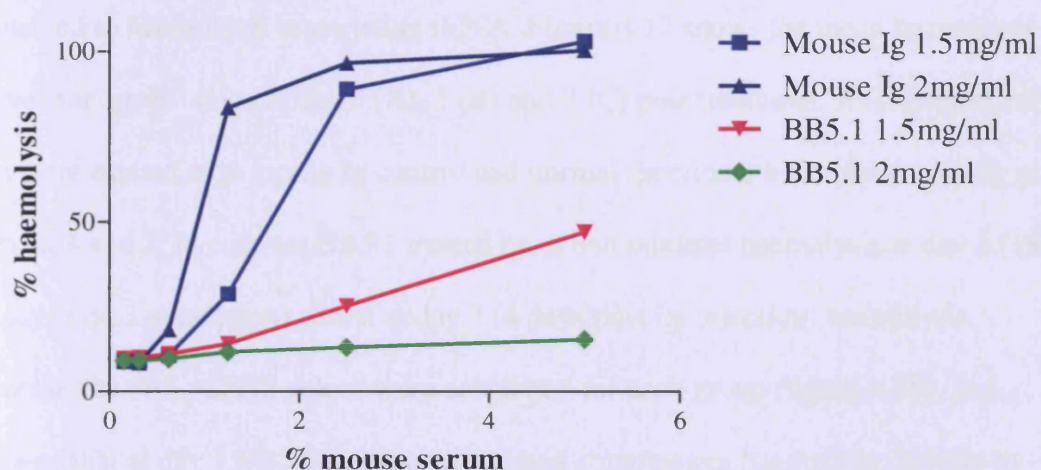


Figure 4.16 Neutralisation of C5 in mouse serum with BB5.1. To test the ability of BB5.1 to neutralise C5 in vivo different concentrations of BB5.1 or control mouse Ig were given to apoE^{-/-} mice. Treatment with BB5.1 inhibited the complement activity of mouse serum as reflected by the dramatic decrease of the percentage haemolysis.

4.3.4 Treatment with C5 mAb inhibits complement activation in high fat fed apoE^{-/-} mice

At 8 weeks of age apoE^{-/-} mice were placed on a high fat diet concurrent with administration of either BB5.1 (1mg/mouse) or control Ig at the same concentration. Mice were treated twice weekly with i.p injections of antibody. During the first week of treatment 50µl blood samples were collected from the tails of each mouse. Blood was allowed to clot and the serum was obtained by centrifugation. Following 8 weeks of high fat diet and antibody treatment, animals were terminated and tissue was processed for disease analysis. The mouse serum collected was taken at day 0, 3 and 7 and subjected to haemolysis assay using rbE-A. Figure 4.17 shows the mean haemolysis curves for apoE^{-/-} mice at day 0 (A), 3 (B) and 7 (C) post treatment. As expected, mice that were treated with mouse Ig control had normal functional haemolytic activity at days 0, 3 and 7. In contrast BB5.1 treated mice had minimal haemolysis at day 3 (18%) (3 days post i.p injection) whilst at day 7 (4 days post i.p injection) haemolysis increased to 60%. CH50 values were calculated for each group (figure 4.18), and showed that at day 3 BB5.1 treatment inhibited complement haemolytic activity by 98% when compared with the Ig control treatment (figure 4.18; 19.7 ± 11.1 U/ml versus 670.5 ± 82.8 U/ml; $P < 0.0001$). At day 7, inhibition of haemolytic activity was less in the BB5.1 treated mice compared to that seen on day 3. These data confirm that BB5.1 administration inhibits complement haemolytic activity in apoE^{-/-} mice. However treatment must be given every 3-4 days, to prevent restoration of complement activity.

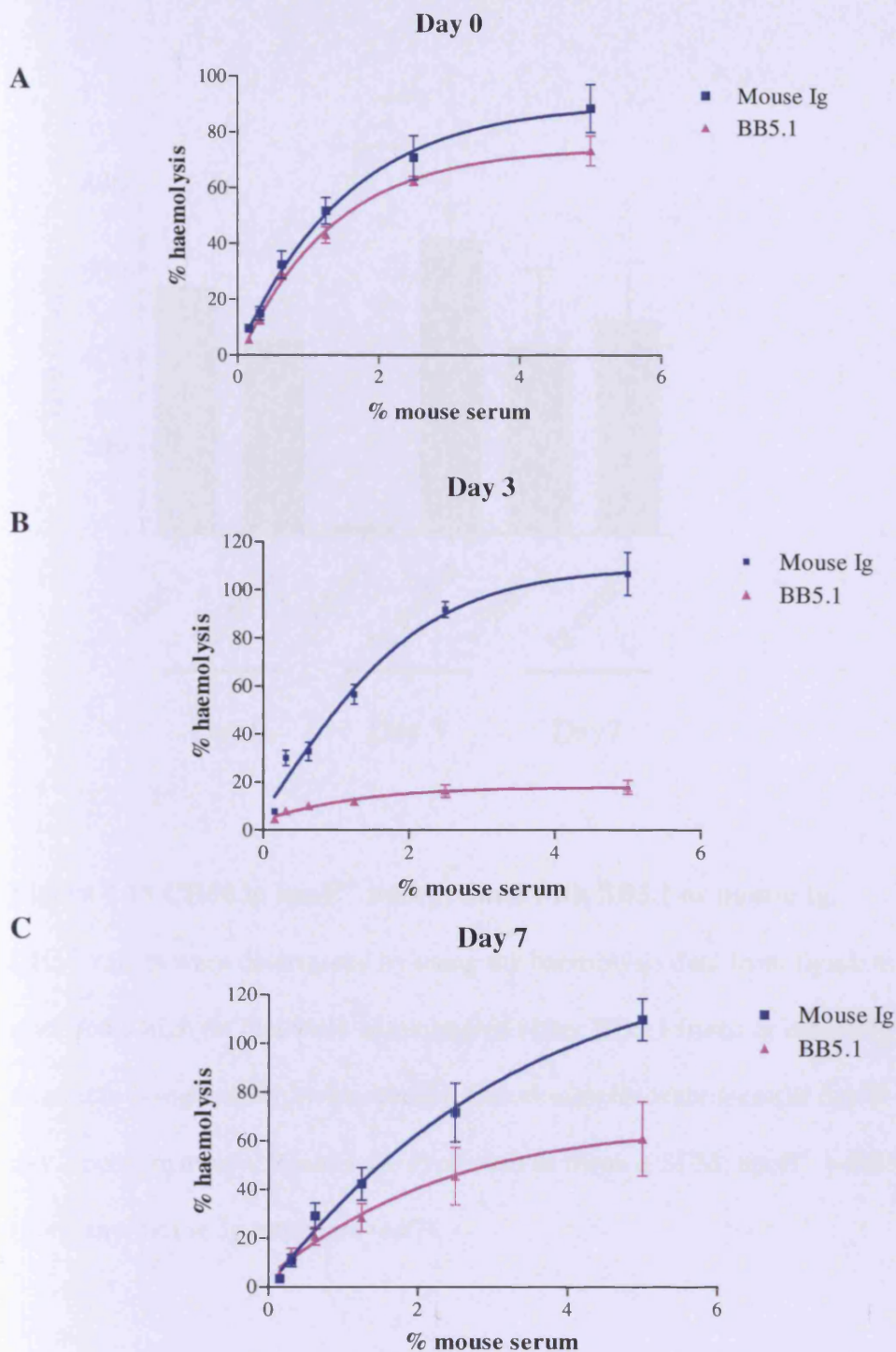


Figure 4.17 Effect of BB5.1 on haemolysis in apoE^{-/-} mice. 8 week old apoE^{-/-} mice were fed a high fat diet for 8 weeks in parallel with either BB5.1 or mouse Ig treatment. 100% haemolysis of rE-A was achieved by substituting mouse serum with H₂O. A-C show haemolysis curves for BB5.1 (n=6) and mouse Ig (n=7) treated animals at day 0 (A), 3 (B) and 7 (C). Results are expressed as mean haemolysis ± SEM.

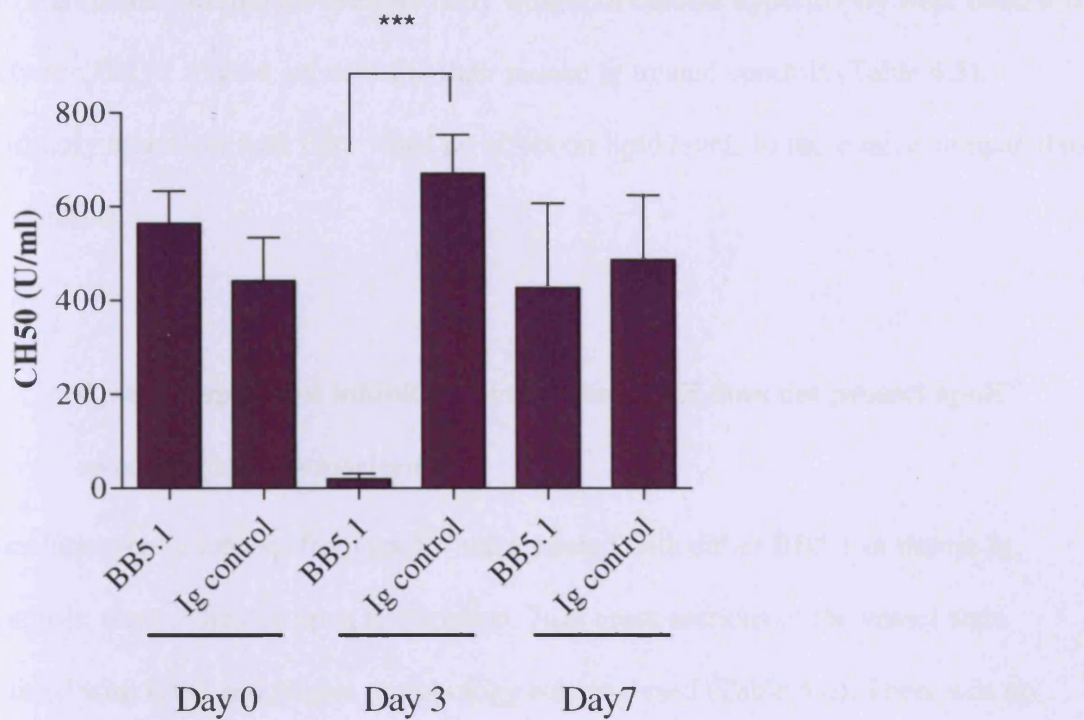


Figure 4.18 CH50 in apoE^{-/-} mice treated with BB5.1 or mouse Ig.

CH50 values were determined by using the haemolysis data from figure 4.17. ApoE^{-/-} mice fed a high fat diet were administered either BB5.1 (n=6) or mouse Ig (n=7) treatment (1mg/mouse) twice weekly. Blood samples were taken at day 0, day 3 and day 7 post-treatment. Results are expressed as mean ± SEM; apoE^{-/-} + BB5.1 treatment (n=6) and mouse Ig treatment (n=7).

4.3.5 Treatment with anti-mouse C5 mAb has no effect on body weight, cardiac hypertrophy or lipid levels in apoE^{-/-} mice

After 8 weeks of concurrent treatment with antibody and high fat diet mice were terminated and body and heart weights recorded. Cardiac hypertrophy was assessed by calculating the heart: body weight ratio for each mouse. Blood was also collected at termination by cardiac puncture, allowed to clot and serum obtained by centrifugation.

No significant differences in either body weight or cardiac hypertrophy were observed between BB5.1 treated animals and their mouse Ig treated controls (Table 4.5).

Similarly treatment with BB5.1 had no effect on lipid levels in these mice compared to Ig controls.

4.3.6 Complement inhibition at the level of C5 does not protect apoE^{-/-} mice against atherosclerosis

Brachiocephalic arteries from apoE^{-/-} mice treated with either BB5.1 or mouse Ig controls, were collected upon termination. 7µm cross sections of the vessel were stained with EVG and plaque morphology was analysed (Table 4.6). There was no significant difference in plaque area between the two groups. Figures 4.19A and B show representative pictures from each group. Figure 4.19 C shows pooled data: $34.7 \pm 18.3 \times 10^3 \mu\text{m}^2$ versus $29.7 \pm 9.6 \times 10^3 \mu\text{m}^2$.

4.3.7 Administration of BB5.1 anti-C5 mAb reduces terminal MAC deposition without affecting C3 deposition in atherosclerotic plaques

In order to assess the effect of BB5.1 treatment on complement levels within the plaque immunofluorescent staining for C3 and MAC deposition were carried out. C5 depletion by BB5.1 treatment did not alter C3 levels within atherosclerotic plaques of apoE^{-/-} mice when compared to controls (figures 4.20 A-C). However depletion of C5 by administration of BB5.1 antibody caused a ten-fold reduction in MAC deposition in the plaques of apoE^{-/-} mice when compared to mouse Ig treated controls. Figures 4.20D and E show representative plaques from each group, while figure 4.20 F shows the pooled data ($3.1 \pm 2.8\%$ versus $26.0 \pm 7.3\%$; $P \leq 0.05$).

Group	Body weight (g)	Heart: body weight (x10⁻³)	Cholesterol (mmol/L)	Triglyceride (mmol/L)
ApoE ^{-/-} + BB5.1 (n=10)	43.1 ± 4.2	4.3 ± 0.8	41.4 ± 10.2	4.957 ± 1.2
ApoE ^{-/-} + mouse Ig (n=10)	42.2 ± 4.2	3.9 ± 0.5	37.7 ± 15.3	4.9 ± 1.8
P value	0.640	0.200	0.590	0.940

Table 4.5 Body weight, cardiac hypertrophy and lipid measurements in 16 week old apoE^{-/-} mice after complement inhibitory treatment (BB5.1) or control (mouse Ig). All animals were fed a high-fat diet from 8 weeks of age for 8 weeks and treatment was given.

Group	Vessel Area (x10³ μm²)	Plaque Area (x10³ μm²)	Lumen Area (x10³ μm²)	Media Area (x10³ μm²)
ApoE ^{-/-} + BB5.1 (n=10)	355.6 ± 51.8	34.7 ± 18.3	216.4 ± 28.2	104.5 ± 17.1
ApoE ^{-/-} + mouse Ig (n=10)	341.4 ± 37.7	33.0 ± 10.0	195.7 ± 24.6	112.7 ± 15.7
P value	0.830	0.940	0.580	0.730

Table 4.6 Brachiocephalic artery morphometric data in 16 week old apoE^{-/-} mice after complement inhibitory treatment (BB5.1) or control (mouse Ig). All animals were fed a high-fat diet from 8 weeks of age for 8 weeks and treatment was given.

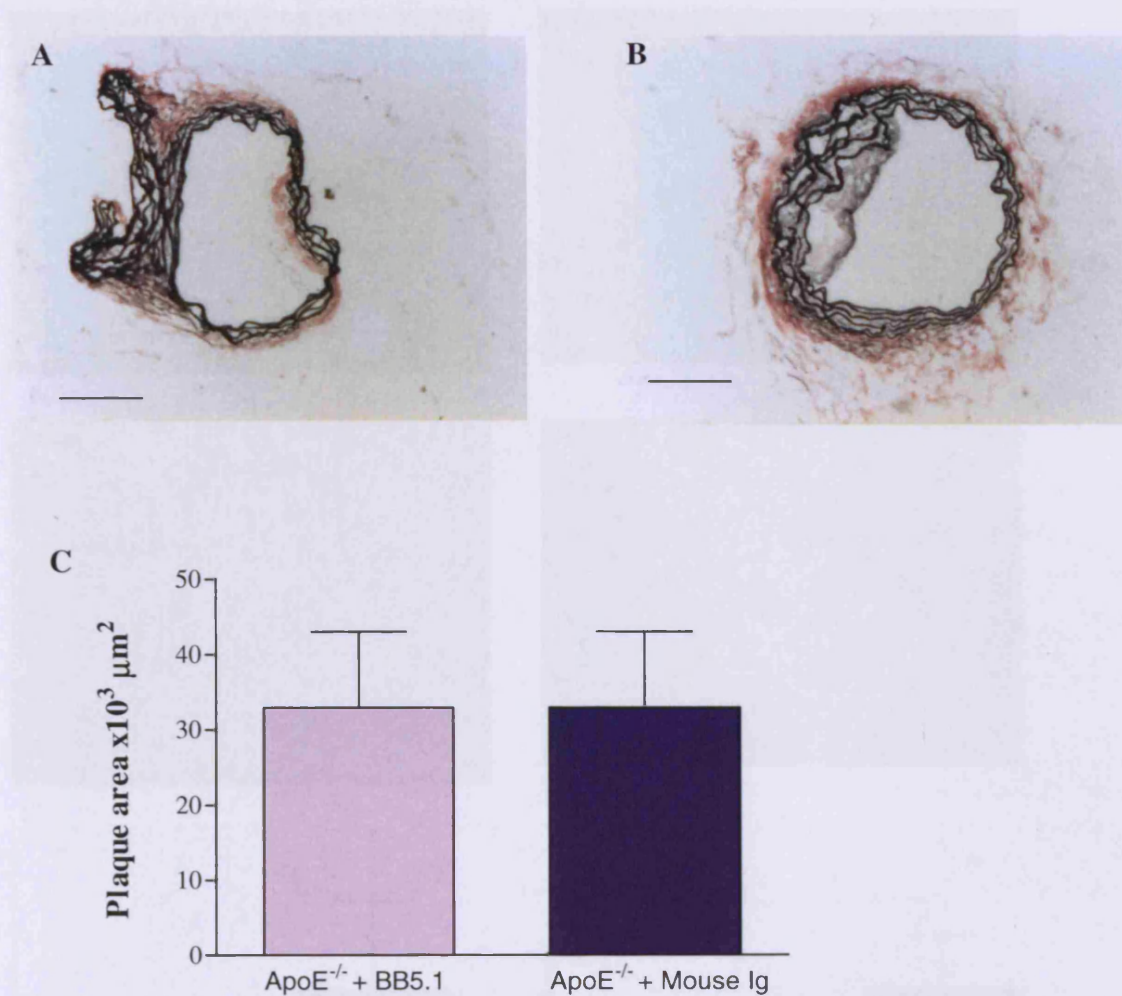


Figure 4.19 Atherosclerotic plaque area following administration of BB5.1 or mouse Ig controls in apoE^{-/-} mice. Sections of the brachiocephalic artery were stained with EVG. The bars show group means \pm SEM. Scale bars: 200 μ m.

(A) Atherosclerotic plaque from an apoE^{-/-} mouse following BB5.1 administration.

(B) A plaque from an apoE^{-/-} control mouse.

(C) Mean plaque area in apoE^{-/-} mice treated with BB5.1 (n=9) or mouse Ig control (n=9).

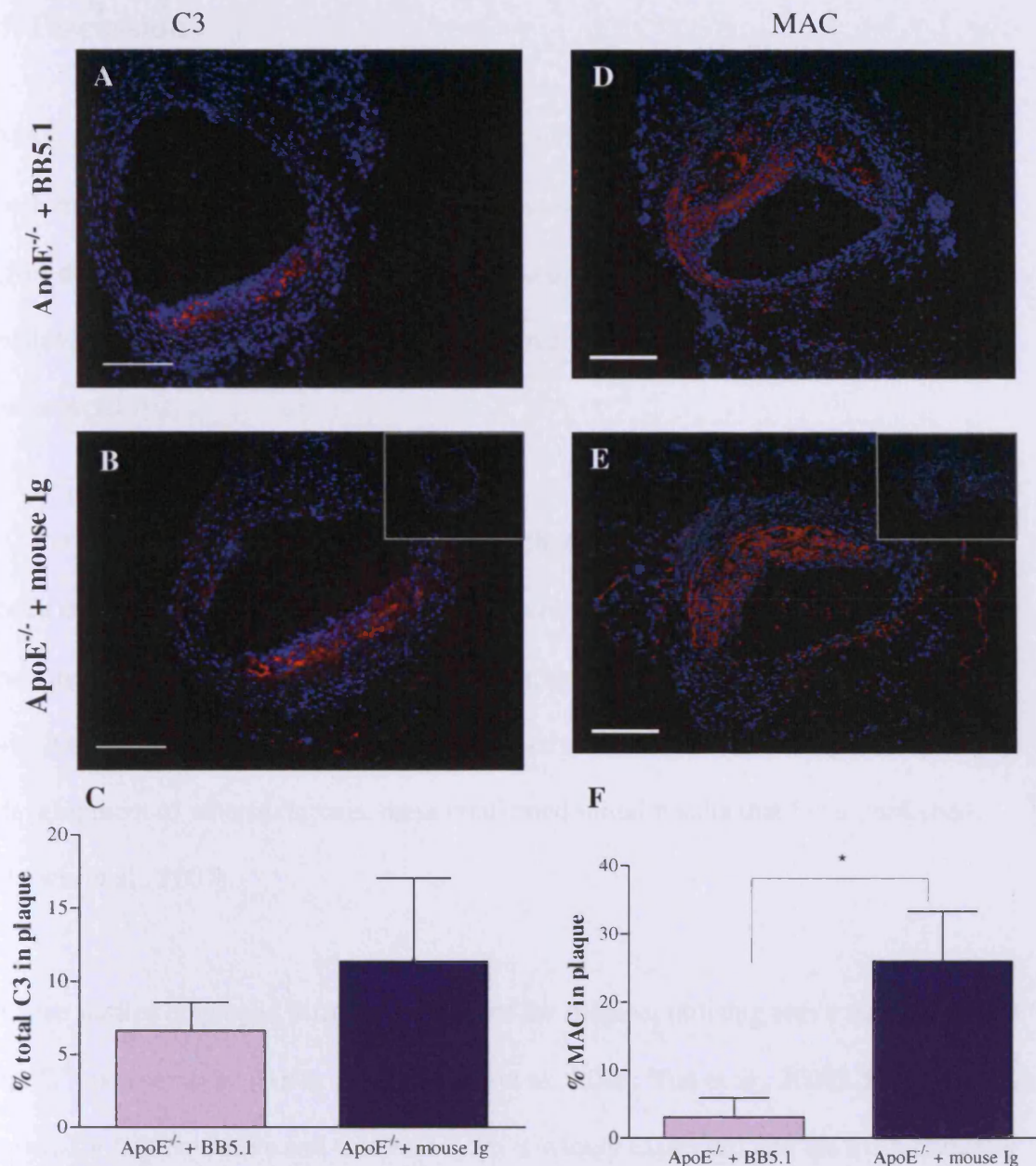


Figure 4.20 Lesional C3 levels and MAC deposition in atherosclerotic brachiocephalic arteries. Comparison of percentage plaque area stained for total C3 or MAC in *apoE*^{-/-} animals at 16 weeks of age after 8 weeks of high-fat diet in parallel with antibody treatment. Sections of the brachiocephalic artery were stained with rat anti-mouse C3 or rabbit anti-rat C9 for MAC (red) and nuclei were stained with DAPI (blue). The bars show group means \pm SEM. Scale bars: 200 μ m. (A) and (D) Representative section from an *apoE*^{-/-} mouse + BB5.1. (B) and (E) Representative section from an *apoE*^{-/-} mouse + mouse Ig, inset shows negative control. (C) and (F) Mean percentage area of lesional C3 or MAC staining respectively in *apoE*^{-/-} mice treated with BB5.1 (n=5) or mouse Ig (n=6).

5. Discussion

MAC deposition is a characteristic feature of human atherosclerotic lesions and the pattern of MAC deposition in murine plaques closely resembles that seen in humans (Rus et al., 1988). It is therefore reasonable to propose that complement terminal pathway activation contributes to plaque formation and progression in human atherosclerosis.

At the time when the work presented in this chapter was begun no animal studies had been carried out on CD59 deficiency and atherosclerosis. However as the work was nearing completion three independent studies were published (An et al., 2009; Wu et al., 2009; Yun et al., 2008) observing a protective role for CD59 during the development of atherosclerosis, these confirmed initial results that I had published (Lewis et al., 2007).

These studies employed different models of the disease, utilising either the *ldlr*^{-/-} or the *apoE*^{-/-} mouse model (An et al., 2009; Wu et al., 2009; Yun et al., 2008). Mice have two genes for CD59: *Cd59a* and *Cd59b*. *Cd59a* is widely expressed and the main regulator of MAC assembly in the mouse (Baalasubramanian et al., 2004) whilst *Cd59b* is highly expressed only in testis (Donev et al., 2008). Yun et al. tested *ldlr*^{-/-} mice deficient in *Cd59a* and showed increased plaque formation (Yun et al., 2008). Wu *et al.* examined *apoE*^{-/-} mice deficient in *Cd59a* and *CD59b* similarly demonstrating increased atherosclerotic burden in these mice, whilst protection was restored in transgenic endothelial and hematopoietic cell-selective over expression of CD59 in *apoE*^{-/-} mice (Wu et al., 2009).

During the first part of this chapter, the effect of MAC dysregulation was examined in apoE^{-/-} mice. ApoE^{-/-}/Cd59a^{-/-} mice were generated by crossing Cd59a^{-/-} mice with apoE^{-/-} mice. After 8 weeks of high-fat feeding the brachiocephalic arteries of these mice were found to contain plaques more than twice as large as those present in the apoE^{-/-} controls. To strengthen this observation and further probe the role of the complement terminal pathway in atherosclerosis, the effect of C6 deficiency was examined. ApoE^{-/-}/C6^{-/-} mice were generated and fed a high fat diet for 12 weeks. The number of weeks on high fat diet was increased in order to observe maximum effect of MAC inhibition on plaque development. This experiment showed clearly, for the first time, that deficiency of C6 is strongly protective against progression of atherosclerosis in apoE^{-/-} mice. This result is consistent with published studies in fat fed C6^{-/-} rabbits and strongly implicates the MAC in both of these models of atherosclerosis (Geertinger and Soerensen, 1977; Schmiedt et al., 1998). Together these data illustrate the contrasting effects of C6 and CD59a deficiencies on plaque development in apoE^{-/-} mice and clearly support the hypothesis that the MAC is an important contributor to atherosclerotic plaque development in the apoE^{-/-} mouse model.

It is well known that, as in humans (Hamasaki et al., 2000), atherosclerotic arteries of apoE^{-/-} mice can undergo remodelling, which maintains the lumen area of the vessel and consequently increases the total vessel area (Bentzon et al., 2003; Jackson, 2007). Complement has been implicated in this process, whereby sublytic doses of MAC on endothelial cells caused proliferation (Fosbrink et al., 2006). In agreement with these studies, we found the lumen area of apoE^{-/-}/Cd59a^{-/-} mice remain unchanged, despite a significant increase in plaque area, suggesting that remodelling had taken place in these

animals. Accordingly, total vessel area had an upward trend however this did not reach significance.

In fat fed apoE^{-/-}/C6^{-/-} mice, where plaque size was markedly reduced, staining for MAC deposition within the plaques was also significantly reduced; the converse was true in apoE^{-/-}/Cd59a^{-/-} mice with much larger plaques compared to controls and increased MAC deposition per unit area. Thus, the extent of disease in these mice correlated with the extent of MAC deposition. In contrast to the markedly elevated MAC deposition over controls seen in apoE^{-/-}/Cd59a^{-/-} mice, C3 fragments were abundant in plaques from both apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice. This is an observation which has been previously noted (An et al., 2009).

These results imply that the acceleration of disease progression observed in the apoE^{-/-}/Cd59a^{-/-} mice was due to increased activation of the terminal complement cascade. MAC deposition was observed in non-cellular regions of the plaque, where cell nuclei were absent. In this case MAC in the plaque is most likely to be associated with extracellular debris and lipid, which accumulate in the core of advanced plaques. Activation of complement at this location is probably due to the presence of oxidised and enzymatically modified LDL, known to activate complement through the alternative pathway (Bhakdi, 1998). Of note, staining for the other broadly expressed murine membrane complement regulators CD55 and Crry was not different between the groups, indicating that deficiency of CD59a was not compensated for by increased expression of these regulators. Plaque lipid content and macrophage and T cell infiltration were also similar in test and control groups.

Strong MAC deposition was also observed in regions of SMC accumulation in the plaque and much less frequently on macrophage cells. Co-localisation of MAC with macrophages has previously been reported in human atherosclerotic plaques (Rus et al., 1989). SMC proliferation is a characteristic feature of plaque formation. In early plaques from apoE^{-/-}/Cd59a^{-/-} mice, SMC accumulation was markedly increased compared to apoE^{-/-} controls, suggesting accelerated proliferation or infiltration arising from unregulated MAC formation. In addition an association between MAC deposition and regions of SMC within atherosclerotic plaques was observed. The MAC has previously been shown to cause aortic SMC proliferation in vitro (Niculescu et al., 1999) strengthening the hypothesis that the increases in SMC seen in the small early plaques from apoE^{-/-}/Cd59a^{-/-} mice was a MAC triggered event. In contrast, larger and more advanced plaques in apoE^{-/-}/Cd59a^{-/-} mice contained fewer SMCs than similar sized plaques from apoE^{-/-} controls, likely due to MAC-induced cytolysis. Reduced SMC number in these plaques will render them unstable and vulnerable to rupture. These observations are compatible with those of Wu *et al*, who noted that the plaques present in their apoE^{-/-}/Cd59a^{-/-}/Cd59b^{-/-} mice had a more vulnerable phenotype than those of the apoE^{-/-} controls (Wu et al., 2009).

Inhibition of MAC formation in fat-fed apoE^{-/-} mice by administration of a blocking anti-C5 mAb gave contradictory results in that, despite a highly significant reduction in MAC deposition in plaques, plaque size was not significantly affected, suggesting that MAC inhibition in this context had little effect on disease progression. Notably, a previous study had showed no effect of C5 deficiency on the levels of atherosclerosis in fat-fed apoE^{-/-} mice (Patel et al., 2001). Taken together with the results from apoE^{-/-}C6^{-/-}

^{-/-} mice, these data suggest that there are important differences between blockade of MAC at the stages of C5 and C6 which have significant effects on disease. This difference is not absolute because inhibition of C5 can be protective in some contexts as was apparently the case when anti-C5 was administered to fat-fed apoE^{-/-}/Cd59a^{-/-}/Cd59b^{-/-} mice, significantly reducing plaque development (Wu et al., 2009). However, there are clear gaps in available data here since the effects of anti-C5 on disease severity induced by fat feeding in the apoE^{-/-}/Cd59a^{-/-} mice in this study was not tested. Similarly, the study of Wu et al. did not examine the effect of BB5.1 treatment in apoE^{-/-} mice undergoing high fat diet.

It is possible that the effect of CD59 deficiency on plaque progression in apoE^{-/-} mice is due to a complement independent mediated effect. For instance, CD59 has been shown to inhibit T-cell activity in vivo and in vitro (Longhi et al., 2005). Thus, it is possible that the increase in lesion progression observed in apoE^{-/-}/CD59a^{-/-} is due to enhancement of T-cell activity in these mice. In order to delineate this hypothesis, one approach would be to generate T-cell specific CD59 depleted mice on the apoE^{-/-} background. The exact mechanism of the protective effect of CD59 during lesion progression could then be further examined.

However, the question remains as to why C6 deficiency is apparently more protective than either deficiency of C5 or C5 inhibition. Lack of either protein should block MAC formation, and indeed, MAC deposition in plaques was absent in apoE^{-/-}/C6^{-/-} and markedly reduced in anti-C5 treated apoE^{-/-} mice. It is possible that antibody-mediated MAC inhibition is incomplete in the tissues and the residual MAC deposition is sufficient to drive disease. However, this is unlikely given the very low levels of MAC

deposition in plaques of anti-C5-treated mice. Another explanation is that C6 plays a role in lipid metabolism, since apoE^{-/-}/C6^{-/-} mice had lowered lipid levels in comparison to apoE^{-/-} controls, consequently inducing protection against atherosclerosis. The only other difference is that C5 inhibition or deficiency will block not only MAC formation but also C5a generation, unaffected by C6 deficiency. A speculative interpretation of these findings would be that C5a plays a hitherto unsuspected anti-atherogenic role during the progression of the disease. Such “protective” roles have already been ascribed to C5a in other contexts (Addis-Lieser et al., 2005; Strey et al., 2003; Tanhehco et al., 2000). In atherosclerosis, important recent observations support this speculation: firstly, the C5a receptor is expressed in atherosclerotic plaques in man (Oksjoki et al., 2007c), secondly, C5a has been shown to up-regulate expression of MMP-1 and MMP-9 in plaque macrophages and PAI-1 in macrophages, smooth muscle cells and in human mast cells associating C5a in defence against plaque destabilisation and rupture (Kastl et al., 2006; Shagdarsuren et al., 2010; Speidl et al., 2010; Wojta et al., 2002).

Recently, it has been shown that in apoE^{-/-} mice, blocking C5a receptor with a specific antagonist or blocking mAb treatment for one week after carotid denudation injury caused a strong protective effect against atherosclerosis (Shagdarsuren et al., 2010). In contrast, long term (3 weeks) treatment promoted plaque stability through PAI-1, thus no difference was seen in neointimal plaque formation between the treated group and controls (Shagdarsuren et al., 2010). Since C5a receptor is known to bind to C5a with high affinity, it is plausible that the effect seen with long term blocking of C5a receptor was mirrored in this study, as the anti-C5 mAb treatment in this study was given for 8

weeks. This would further explain why deficiency of C5 in apoE^{-/-} mice had no effect on plaque size.

It can be further suggested that, in mice lacking CD59a, the amplification of damage caused by the MAC outweighs any protective effect of C5a and in this case inhibition of MAC formation by blockade of C5 is protective.

Another issue in comparing C6 deficiency with C5 inhibition is the fact that the deficient mice are never exposed to MAC formation, while for anti-C5, the mice are treated only for the 8 week duration of the experiment. In the latter case, MAC-induced damage to vessels may already have occurred in these atherosclerosis-prone mice prior to the start of treatment with anti-C5 and this might abrogate to some extent the protective effect of MAC inhibition.

Since MAC deposition is a characteristic feature of atherosclerotic lesions in both mice and humans (An et al., 2009; Lewis et al., 2010; Rus et al., 1988; Wu et al., 2009) it is reasonable to propose that MAC contributes to plaque formation and progression in human atherosclerosis in a similar manner to that which I and others have now demonstrated occurs in mice. Inhibition of MAC formation therefore remains a potential strategy for therapy of atherosclerosis and a realistic one given that terminal pathway inhibitors are already in use in the clinic (Davis, 2008) with more under development (Song et al., 2003). However, these results may suggest that inhibition of the MAC in this particular context should be targeted at C6 or later in MAC assembly rather than at the level of C5.

Chapter 5: The effect of CD55 deficiency on atherosclerosis in apoE deficient mice

1. Introduction

Although hypercholesterolemia has had significant attention in the pathogenesis of coronary artery disease, hypertriglyceridemia is also a key factor in the development of atherosclerosis (Austin et al., 1998; Ginsberg, 2001). Firstly, high circulating levels of triglycerides predict increased risk for atherosclerosis (Hokanson and Austin, 1996). Secondly, triglyceride-rich lipoproteins have been shown to penetrate the sub-endothelial layer of artery walls (Rapp et al., 1994) and initiate or aggravate atherosclerosis in a variety of ways, including promotion of endothelial dysfunction (Zhao et al., 2001), stimulation of MCP-1 expression (Shin et al., 2004), enhanced monocyte adherence (Kawakami et al., 2002), increased foam cell formation (Kawakami et al., 2005), oxidative modification (McEneny et al., 2002) and promotion of inflammatory response (de Man et al., 2000). Thirdly, hypertriglyceridemia is known to be associated with low levels of HDL and high levels of abnormally small dense LDL (Ginsberg, 2002).

The complement system has been shown to be important in lipid metabolism homeostasis through the action of its central component C3. When C3 is activated it is cleaved into two fragments C3a and C3b. The latter is involved in opsonisation and removal of bacterial cells and other unwanted particles. C3a, the smaller fragment is a

crucial player in the inflammatory response. The potent pro-inflammatory properties of C3a require that it is rapidly inactivated in the serum by carboxypeptidase N to form C3adesArg. C3adesArg, also known as acylation stimulating protein (ASP), is a stable product of C3 activation and a potent adipokine that stimulates uptake of triglycerides, glucose and NEFA, enhances triglyceride synthesis and storage and inhibits triglyceride lipolysis in adipose tissue, (Cianflone et al., 1989a; Germinario et al., 1993; Maslowska et al., 1997b).

Since postprandial hypertriglyceridemia is a risk factor for atherosclerosis (Cohn, 1998) and increased generation of C3adesArg through activation of C3 is an important factor aiding postprandial triglyceride clearance, complement activation may thus play a role in maintaining healthy arterial walls.

There is evidence to suggest that CD55, a 70kDa membrane protein that inhibits C3 activation by accelerating the decay of the C3 cleaving enzymes of the alternative (C3bBb) and classical/lectin (C4b2a) pathways, is present in the endothelium. Statins and thrombin have been shown to induce endothelial CD55 (Lidington et al., 2000; Mason et al., 2002). In particular, statins have been shown to act as inducers of membrane complement regulatory proteins on endothelial cells, thus increasing the resistance of these cells to anaphylatoxin-mediated inflammatory injury and/or C5b-9-induced activation (Mason et al., 2002). In addition, exposure of thrombin to cultured endothelial cells induced CD55 expression six hours post-stimulation, with maximal expression reached at 24 hours. Thrombin induced up regulation of CD55 and thus caused reduced C3 deposition and complement mediated lysis of endothelial cells (Lidington et al., 2000). Both sets of data suggest that endothelial cells, lining the artery

wall, up-regulate CD55 to offer extra protection of the vascular wall from complement injury.

However mouse studies examining the role of C3 in atherosclerosis have reported that deficiency of C3, but not fB, exacerbated plaque formation and caused hyperlipidemia on *ldlr*^{-/-} and *apoE*^{-/-}/*ldlr*^{-/-} backgrounds, (Buono et al., 2002; Persson et al., 2004).

CD55 knockout (*CD55*^{-/-}) mice are healthy, fertile, and do not develop spontaneous intravascular haemolysis, likely due to the presence of CD59a on erythrocytes which prevents MAC assembly and lysis (Sun et al., 1999). In humans, there is a single CD55 gene located on chromosome 1 (Lublin and Atkinson, 1989), whilst in the mouse two CD55 genes exist, these are named *daf1* and *daf2* which are located sequentially on chromosome 1 (Fukuoka et al., 1996; Song et al., 1996; Spicer et al., 1995). The mouse *daf1* gene encodes a GPI-anchored CD55 protein and is broadly expressed (Harris et al., 1999; Lin et al., 2001; Song et al., 1996; Spicer et al., 1995; Sun et al., 1999). In contrast *daf2* gene encodes a transmembrane CD55 variant (Spicer et al., 1995) which is predominately expressed in the testis (Lin et al., 2001; Spicer et al., 1995; Sun et al., 1999) and splenic dendritic cells (Lin et al., 2001).

In order to test the effect of CD55 deficiency on progression of atherosclerosis, *CD55*^{-/-} mice were back-crossed onto the *apoE*^{-/-} background and fed an atherogenic diet.

Based on the findings with the *apoE*^{-/-}/*CD59a*^{-/-} mice, I hypothesised that CD55 deficiency would exacerbate disease pathology in the *apoE*^{-/-} mouse model.

2. Specific methods

2.1 Mean plaque area measurement

Five sections were taken per mouse at the same relative position along the brachiocephalic artery, and mean plaque area assessed via image analysis using an established method (Johnson et al., 2005). See chapter 2 for detailed methodology.

2.2 Activated C3 immunostaining

Immunostaining for complement C3 activation utilised rat anti-mouse C3b/iC3b/C3c mAb clone 2/11 (5µg/mL; Hy-Cult Biotechnology). Firstly, sections were fixed in acetone at 4°C for 10 minutes prior to 1 hour incubation at room temperature in 10% goat serum diluted in 2% BSA/PBS. Sections were then incubated with the primary antibody diluted in 2% BSA/PBS for 1 hour at room temperature. The sections were then washed before incubation with Alexa fluor 488-labeled goat anti-rat IgG (20µg/mL) (Invitrogen Life Technologies). Nuclei were stained using DAPI. Negative controls included replacement of primary antibody with isotype control IgG.

3. Results

3.1 Pathological observations

To examine the role of CD55 during atherosclerosis I generated mice deficient in both apoE and CD55 and subjected them (together with their controls) to 12 weeks of high fat feeding. In order to characterise and compare the pathology of apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice, various parameters were studied. These included measurements of body, heart, spleen and kidney weight. I expressed these as both ratios of organ: body weight and also organ: tibia length, which is more consistent than body weight (Yin et al., 1982).

There were no significant differences in body weight between apoE^{-/-}/CD55^{-/-} and apoE^{-/-} control mice (see table 5.1). In addition, CD55 deficiency did not affect heart or kidney size in these mice (see table 5.1). In contrast, spleen weight was significantly reduced in apoE^{-/-}/CD55^{-/-} mice when compared to apoE^{-/-} controls when normalised against both body weight (table 5.1; $4.4 \pm 0.2 \times 10^{-3}$ versus $7.22 \pm 1.0 \times 10^{-3}$; P=0.003) or tibia length (table 5.1; $8.9 \pm 0.4 \times 10^{-3}$ versus $14.7 \pm 1.6 \times 10^{-3}$; P=0.0004).

	ApoE^{-/-}/CD55^{-/-}	ApoE^{-/-}	P value
Body weight (g)	38.5 ± 1.6 n = 19	40.6 ± 1.4 n = 20	0.312
Heart: body weight (x10 ⁻³)	5.3 ± 0.2 n=19	5.2 ± 0.2 n=18	0.562
Heart weight: tibia length (x10 ⁻³)	10.7 ± 0.4 n=19	10.7 ± 0.5 n=10	0.967
Kidney:body weight (x10 ⁻³)	6.5 ± 0.3 n=14	7.1 ± 0.4 n=10	0.229
Kidney weight:tibia length (x10 ⁻³)	13.1 ± 0.6 n=14	14.9 ± 1.2 n=10	0.153
Spleen: body weight (x10 ⁻³)	4.4 ± 0.16 n=14	7.2 ± 1.0 n=10	0.003
Spleen weight:tibia length (x10 ⁻³)	8.9 ± 0.4 n=14	14.7 ± 1.6 n=10	0.001

Table 5.1 Pathological observations in 20 week old apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice. All animals were fed a high fat diet from 8 weeks old for 12 weeks. Data are shown as mean ± SEM.

3.2 CD55 deficiency slows the progression of atherosclerosis

In order to assess atherosclerosis in the brachiocephalic arteries of apoE^{-/-}/CD55^{-/-} mice versus apoE^{-/-} controls mean plaque area was measured and analysed at multiple points along the vessel. Surprisingly, analysis revealed a three fold decrease in mean plaque cross sectional area from apoE^{-/-}/CD55^{-/-} versus controls ($54.7 \pm 11.2 \times 10^3 \mu\text{m}^2$ versus $155.2 \pm 16.8 \times 10^3 \mu\text{m}^2$; $P < 0.001$; figures 5.1A-C).

3.3 Infiltrating SMCs and macrophage

To further explore differences in plaque character, SMC content and macrophage infiltration into plaques were assessed. I found significantly less plaque SMC content in apoE^{-/-}/CD55^{-/-} mice versus apoE^{-/-} control. Representative pictures of SMC staining in both apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice are shown in figures 5.2A and B respectively. Pooled data is shown in figure 5.2C ($9.3 \pm 2.0\%$ versus $18.0 \pm 2.8\%$; $P < 0.05$). However, there was no significant change in percentage macrophage plaque content between the two groups. Figure 5.2D and E show representative staining of macrophage in apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice respectively. Pooled data is shown in figure 5.2F ($19.4 \pm 6.5\%$ versus $18.8 \pm 4.2\%$). Of note, percentage plaque macrophage content was lower in the apoE^{-/-} controls after 12 weeks of high fat feeding (figure 5.2 F; $18.8 \pm 4.2\%$) compared with apoE^{-/-} mice after 8 weeks of high fat feeding (figure 4.4; $60.1 \pm 3.2\%$). This is consistent with the notion that advanced plaques have lower percentage macrophage content compared with smaller sized plaques.

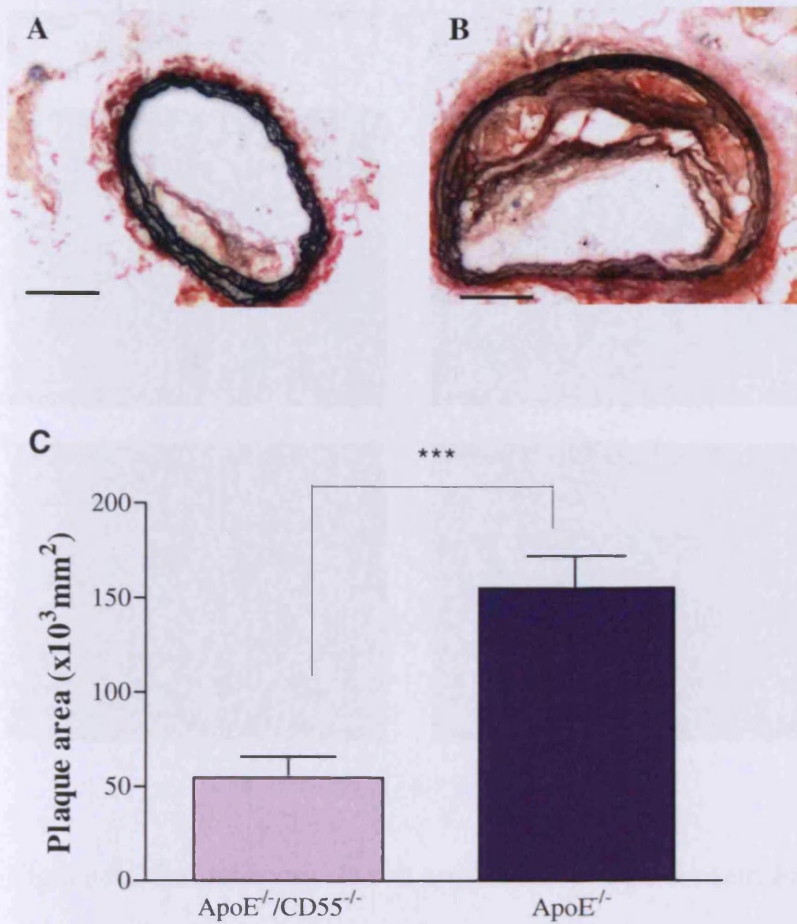


Figure 5.1 Plaque area measurement in brachiocephalic arteries of apoE^{-/-}/CD55^{-/-} and apoE^{-/-} control mice after 12 weeks of high-fat feeding. Sections of the brachiocephalic artery were stained with Miller's elastin/van Gieson. The bars in C show group means + SEM. Scale bars in A and B represent 200µm.

(A) A small plaque (arrowed) from an apoE^{-/-}/CD55^{-/-} mouse.

(B) Single large plaque occupying half of the vessel from an apoE^{-/-} mouse.

(C) Mean plaque area in apoE^{-/-}/CD55^{-/-} (n=13) and apoE^{-/-} (n=12) mice.

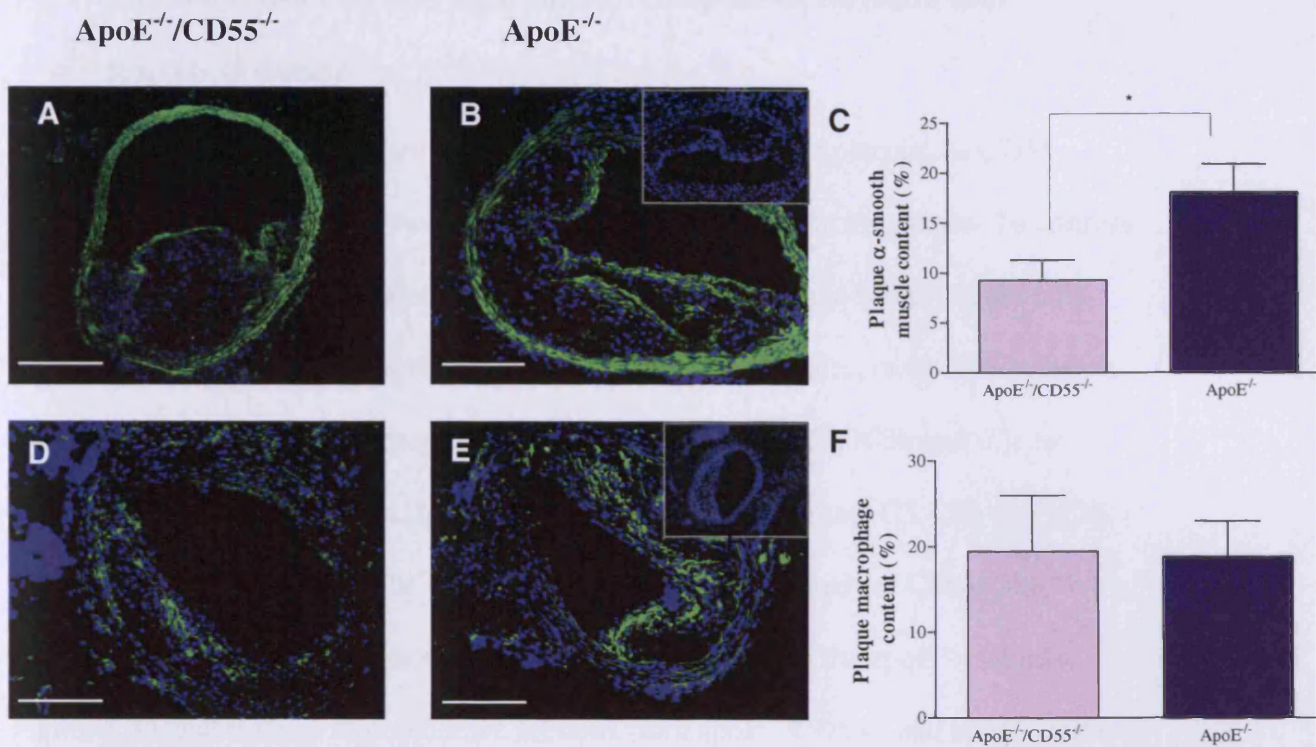


Figure 5.2 Smooth muscle cell and macrophage content in atherosclerotic lesions

in $\text{apoE}^{-/-}/\text{CD55}^{-/-}$ and $\text{apoE}^{-/-}$ mice. Comparison of percentage smooth muscle α -actin and macrophage staining in plaques of $\text{apoE}^{-/-}/\text{CD55}^{-/-}$ and $\text{apoE}^{-/-}$ animals at 20 weeks, after 12 weeks of high-fat diet. Brachiocephalic artery sections were stained for smooth muscle cells with anti-smooth muscle α -actin (green) (A-C) or macrophage (green) (D-F) and counterstained with DAPI (blue). Scale bars: 200 μm . The bars show group means \pm SEM. (A-B) Representative section of smooth muscle α -actin stained section from $\text{apoE}^{-/-}/\text{CD55}^{-/-}$ mice and $\text{apoE}^{-/-}$ control mice respectively, inset showing negative control. (C) Mean percentage of plaque stained for smooth muscle α -actin in $\text{apoE}^{-/-}/\text{CD55}^{-/-}$ (n=9) and $\text{apoE}^{-/-}$ (n=7) mice. (D-E) Representative section of macrophage stained vessel from $\text{apoE}^{-/-}/\text{CD55}^{-/-}$ mice and $\text{apoE}^{-/-}$ control respectively, inset showing negative control. (F) Mean percentage of plaque stained for macrophage of $\text{apoE}^{-/-}/\text{CD55}^{-/-}$ (n=8) and $\text{apoE}^{-/-}$ (n=6) mice.

3.4 Complement C3 activation and MAC deposition correlate with severity of disease

Next I examined the extent of complement activation within the plaques, as CD55 deficiency may cause altered local complement activation within the plaque. To address this question, plaques were stained for C3 activation products and MAC (figure 5.3). C3 product deposition was assessed using two different antibodies, mAb 2/11, which is fragment-specific (does not recognise intact C3) and detects C3b, iC3b and C3c in tissues (figures 5.3A-C), and 11h9, which recognise total C3; intact C3, C3b and iC3b fragments (figures 5.3D-F). The percentage of plaque area stained for C3b/iC3b/C3c fragments in apoE^{-/-}/CD55^{-/-} mice was significantly less than in the apoE^{-/-} controls. Figure 5.3A and B show representative sections from apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice respectively. The pooled data are shown in figure 5.3C (28.0% ± 8.2%, versus 57.3% ± 7.7%; P<0.05). In contrast, total C3 was present at similar levels in plaques of apoE^{-/-}/CD55^{-/-} animals and their controls (20.7% ± 4.8%, versus 19.9% ± 3.6%; P=0.89).

MAC deposition was also markedly reduced in plaques from apoE^{-/-}/CD55^{-/-} compared to controls. Figure 5.3G and H show representative images of apoE^{-/-}/CD55^{-/-} mice and apoE^{-/-} controls. Figure 5.3I shows pooled data (17.9 ± 3.5%, (n=7) versus 30.5 ± 4.1; P<0.05).

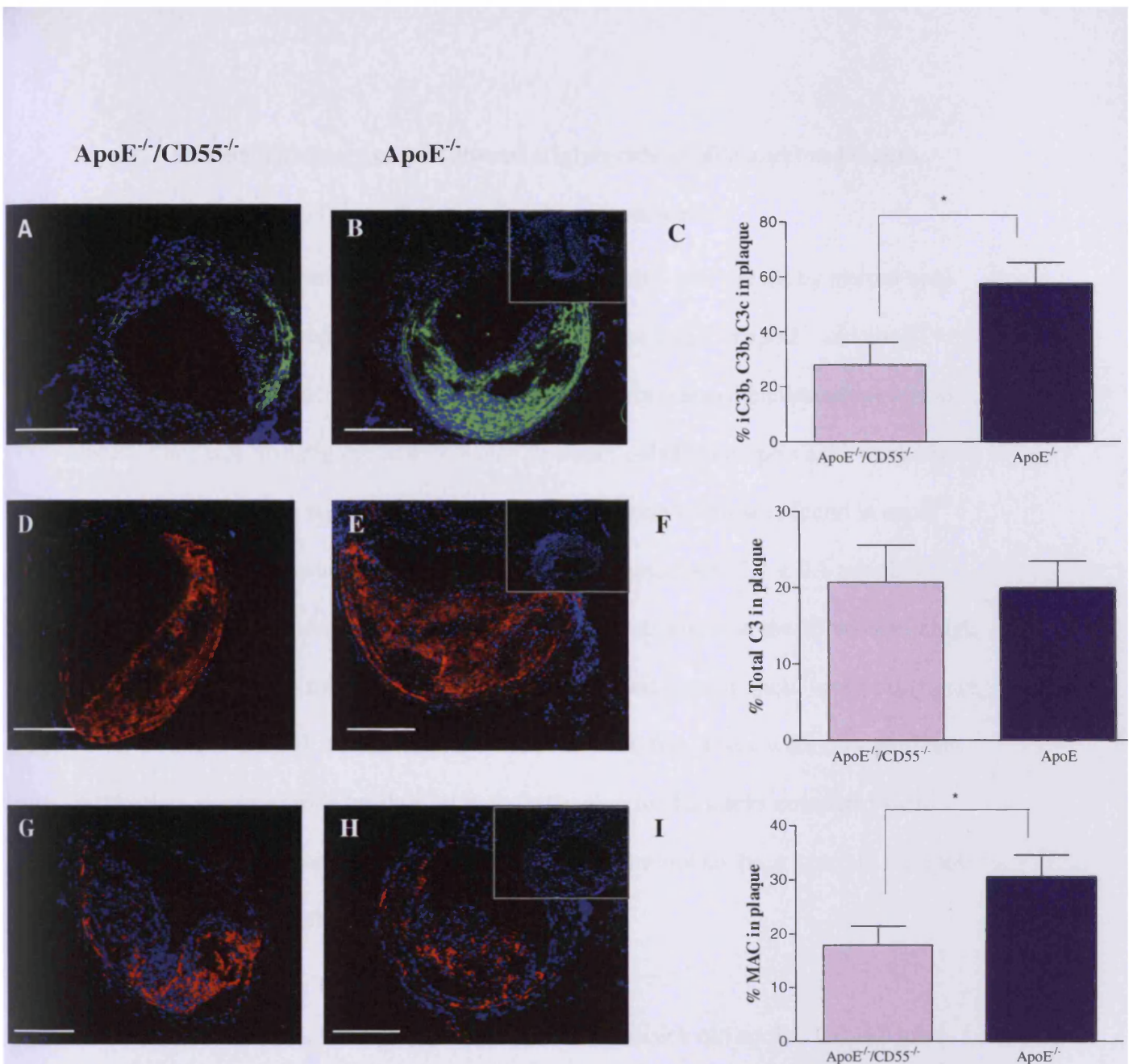


Figure 5.3 Complement activation in atherosclerotic brachiocephalic arteries. Comparison of C3 activation products (A-C), total C3 (D-F) or MAC staining (G-I) in apoE^{-/-}/CD55^{-/-} and apoE^{-/-} animals at 20 weeks of age after 12 weeks of high-fat diet. Sections of the brachiocephalic artery were stained with rat anti-mouse C3b/iC3b/C3c mAb (green), rat anti-mouse C3 (red) or MAC (red) and nuclei were stained with DAPI (blue). The bars show group means \pm SEM. Scale bars: 200 μ m. (A, D, G). Representative section from an apoE^{-/-}/CD55^{-/-} mouse. (B, E, H) Representative section from an apoE^{-/-} mouse, inset showing negative control. (C, F, I) Mean percentage of lesional staining in apoE^{-/-}/CD55^{-/-} (n=7 in each group) and apoE^{-/-} (n=6, 10 and 7 respectively) mice.

3.5 CD55 deficiency causes altered triglyceride profile and total serum cholesterol levels in a cholesterol rich environment

To test whether the observed effect on plaque progression was caused by altered lipid handling, I measured serum triglyceride and cholesterol levels in apoE^{-/-} and apoE^{-/-}/CD55^{-/-} mice. The effect of CD55 deficiency on triglyceride and cholesterol levels in apoE^{-/-} mice was investigated at both 8 and 20 weeks old (Pre and post a 12 week high fat diet). At 8 weeks a significant reduction in triglyceride levels was found in apoE^{-/-}/CD55^{-/-} mice when compared to the apoE^{-/-} group (figure 5.4A; 2.1 ± 0.1 mmol/L versus 5.1 ± 0.6 mmol/L; $P < 0.01$). Similarly, triglyceride levels in the 20 week old high fat fed apoE^{-/-}/CD55^{-/-} mice were halved when compared to their apoE^{-/-} controls (figure 5.4A; 1.7 ± 0.3 mmol/L versus 3.6 ± 0.5 mmol/L; $P < 0.01$). There were no significant differences in triglyceride levels after high fat feeding for 12 weeks compared with before within either group, an observation which has previously been noted in the apoE^{-/-} mice (Joven et al., 2007).

Cholesterol levels were not significantly different in 8 week old apoE^{-/-}/CD55^{-/-} mice when compared to apoE^{-/-} controls (figure 5.4B; 16.1 ± 1.4 mmol/L versus 18.2 ± 0.8). After 12 weeks of high fat feeding, cholesterol levels significantly increased in both groups. However levels in apoE^{-/-}/CD55^{-/-} mice at this point were significantly below those present in their apoE^{-/-} controls (figure 5.4B; 29.0 ± 1.8 mmol/L versus 38.3 ± 2.5 mmol/L; $P < 0.01$). Thus, in apoE^{-/-}/CD55^{-/-} mice at 8 weeks serum cholesterol levels were 16.1 ± 1.4 mmol/L while at 20 weeks serum cholesterol levels reached 29.0 ± 1.8 mmol/L ($P < 0.01$). At 8 weeks, apoE^{-/-} mice serum cholesterol levels were 29.0 ± 1.8 mmol/L, compared with 38.3 ± 2.5 mmol/L at 20 weeks.

3.6 CD55 deficiency is associated with increased C3 turnover and plasma

C3adesArg levels

C3adesArg, while losing any inflammatory activity, has significant roles in lipid metabolism (Cianflone et al., 2008). To investigate the possibility that CD55 deficiency affects C3adesArg levels during lipid metabolism, I compared serum C3adesArg levels in apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice at both 8 and 20 weeks of age (pre- and post-high fat diet respectively). A sandwich ELISA revealed a high fat diet dependent increase in C3adesArg levels in apoE^{-/-}/CD55^{-/-} mice when compared to the apoE^{-/-} control mice. Thus, after 12 weeks of high fat feeding serum C3adesArg levels were significantly increased in 20 week old apoE^{-/-}/CD55^{-/-} mice when compared to their apoE^{-/-} controls (figure 5.5A; 1.6 ± 0.1 $\mu\text{g/ml}$ versus 1.0 ± 0.2 $\mu\text{g/ml}$; $P < 0.01$). However, C3adesArg levels in 8 week old apoE^{-/-}/CD55^{-/-} mice, which were fed normal chow, showed no difference when compared to the apoE^{-/-} controls (figure 5.5A). Thus, CD55 deficiency caused an increase in serum C3adesArg levels after 12 weeks of high fat diet, which correlated with decreased cholesterol and decreased levels of disease.

To explore the relationship between fat intake and C3 turnover, levels of C3adesArg were also measured in fat-fed apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice after an overnight fast; C3adesArg levels were reduced 5-fold compared to non-fasted mice and there was no difference between fasted apoE^{-/-}/CD55^{-/-} and apoE^{-/-} groups (figure 5.5B).

3.7 CD55 regulates C3adesArg causing enhanced NEFA clearance

C3adesArg has been shown to increase NEFA clearance (Van Harmelen et al., 1999) and stimulate glucose uptake (Germinario et al., 1993; Maslowska et al., 1997b; Tao et al., 1997) into adipose tissue. Furthermore, C3^{-/-} mice have significant delays in triglyceride and NEFA clearance (Murray et al., 2000; Murray et al., 1999b). Thus, it was hypothesised that the increase in C3adesArg levels caused by CD55 deficiency would lower circulating NEFA and glucose levels in the apoE^{-/-} mice.

Circulating NEFA levels were measured from 20 week old apoE^{-/-}/CD55^{-/-} mice and apoE^{-/-} control mice (post a 12 week high fat diet). In keeping with the hypothesis, plasma NEFA levels were significantly lower in apoE^{-/-}CD55^{-/-} mice compared with apoE^{-/-} controls (0.34 ± 0.04 mg/ml versus 0.52 ± 0.04 mg/ml; $P < 0.001$; figure 5.6A).

Fasting and non-fasting blood glucose levels were measured in 20 week old apoE^{-/-}/CD55^{-/-} mice and apoE^{-/-} control mice after 12 weeks of high fat feeding. However, no significant difference was found in fasting or non-fasting glucose levels between the groups (figure 5.6B). Conversely, glucose measurements almost doubled in both groups when comparing fasting (4.3 ± 0.5 mmol/L versus 10.2 ± 0.8 mmol/L; $P < 0.0001$) and non-fasting blood samples (4.9 ± 0.3 mmol/L versus 9.3 ± 0.7 mmol/L; $P < 0.0001$).

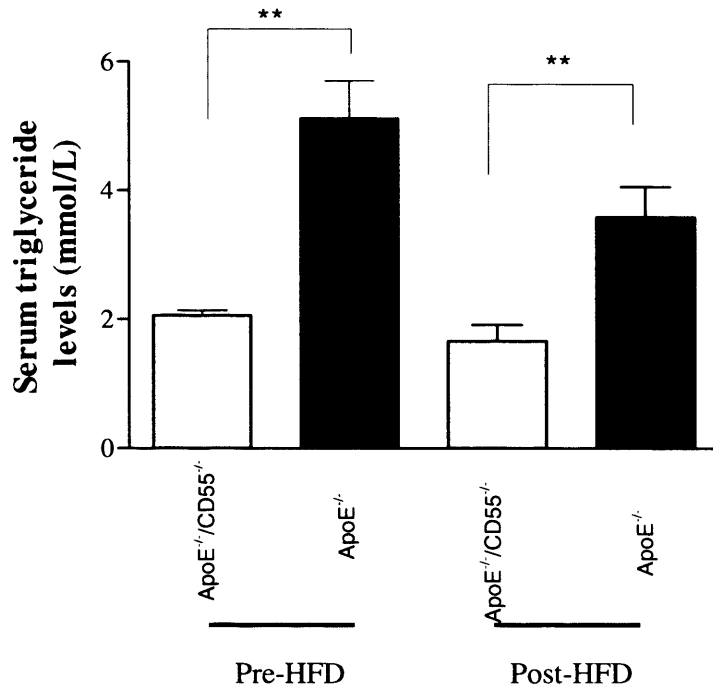
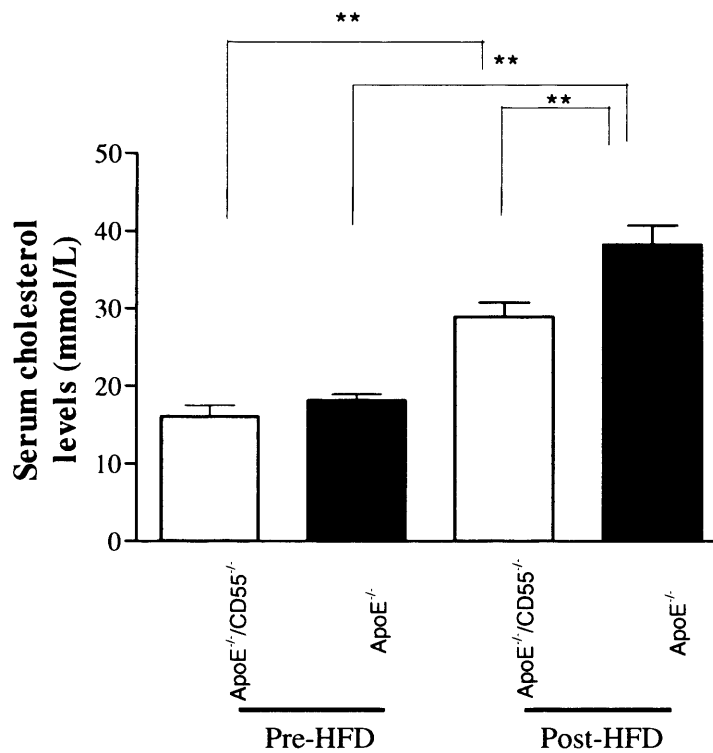
A**B**

Figure 5.4 Triglyceride and cholesterol levels in apoE^{-/-}/CD55^{-/-} mice pre-and post- high fat diet. (A) Serum Triglyceride and (B) cholesterol levels were measured at 8 weeks of age, pre-high fat diet (Pre-HFD) and at 20 weeks of age after 12 weeks of high fat diet (Post-HFD) in apoE^{-/-}/CD55^{-/-} mice (n=4 and 12 respectively) and apoE^{-/-} controls (n=6 and 15 respectively).

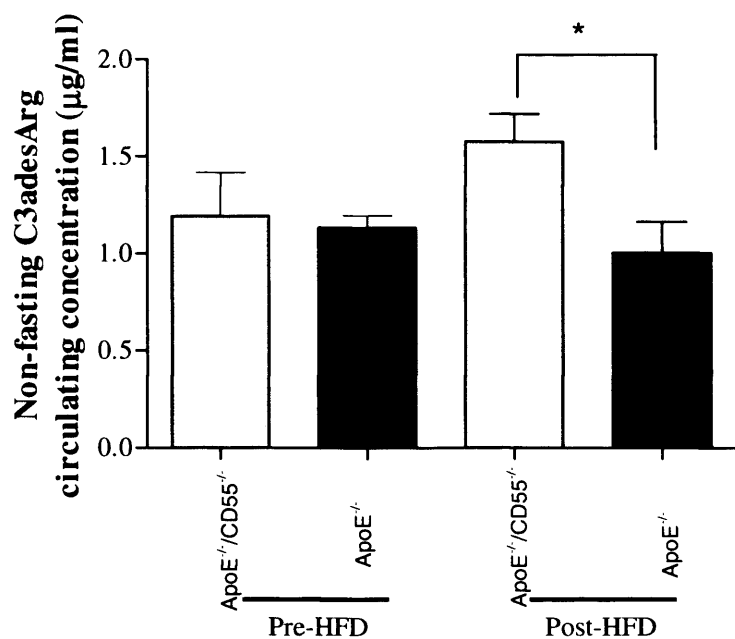
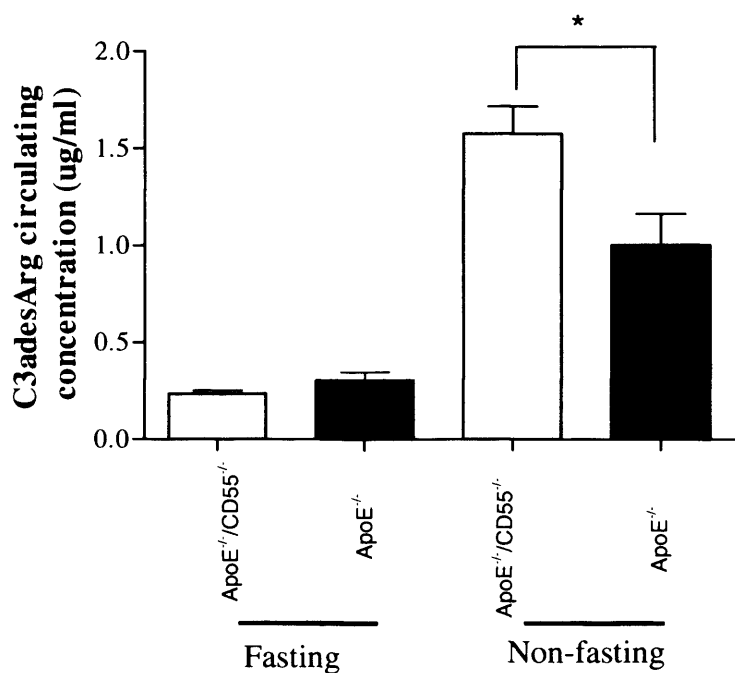
A**B**

Figure 5.5 Assessment of circulating C3adesArg levels in apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice. (A) Non-fasting C3adesArg levels were compared between apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice at 8 weeks old, pre-high fat diet (Pre-HFD) (n=8 and 8 respectively), and at 20 weeks after 12 weeks on high fat diet (Post-HFD) (n=11 and 12 respectively). (B) Comparison of C3adesArg levels in 20 week old (post-HFD) apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice in both fasting (n=11 in both groups) and non-fasting state (n=11 and 12 respectively).

3.8 CD55 deficiency is associated with increased body fat

It has previously been shown that 16 and 26 week old $C3^{-/-}$ mice on the $ldlr^{-/-}/apoE^{-/-}$ background have approximately two thirds lower body fat compared to control mice (Persson et al., 2004). Therefore, to further explore the hypothesis that CD55 is involved in regulating lipid metabolism through control of C3 activation, percentage body fat in high fat fed $apoE^{-/-}/CD55^{-/-}$ deficient mice was compared with $apoE^{-/-}$ controls. After 12 weeks of high fat feeding, $apoE^{-/-}/CD55^{-/-}$ mice had significantly more body fat by comparison with the $apoE^{-/-}$ controls (figure 5.7; $28.6 \pm 4.2\%$ versus $19.5 \pm 1.8 \%$ respectively; $p < 0.05$).

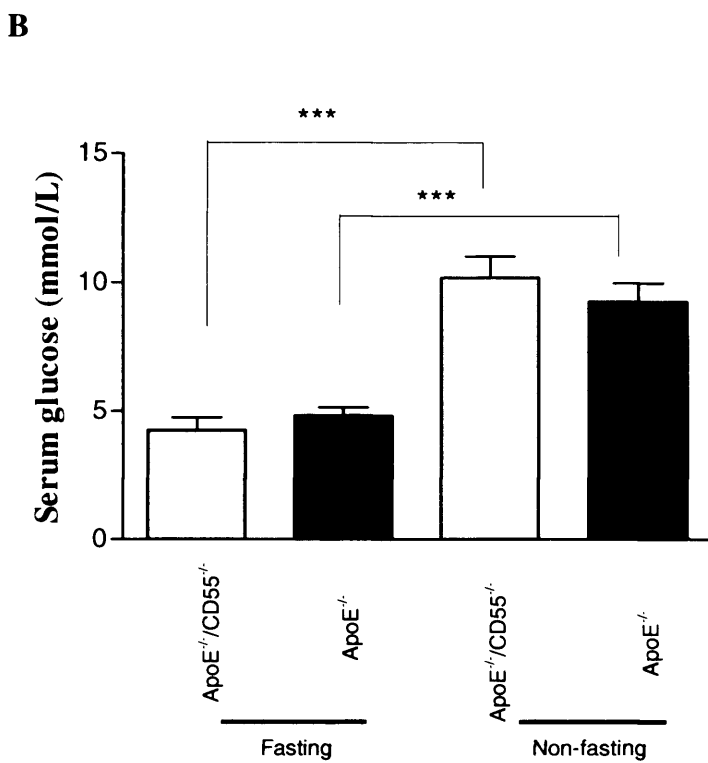
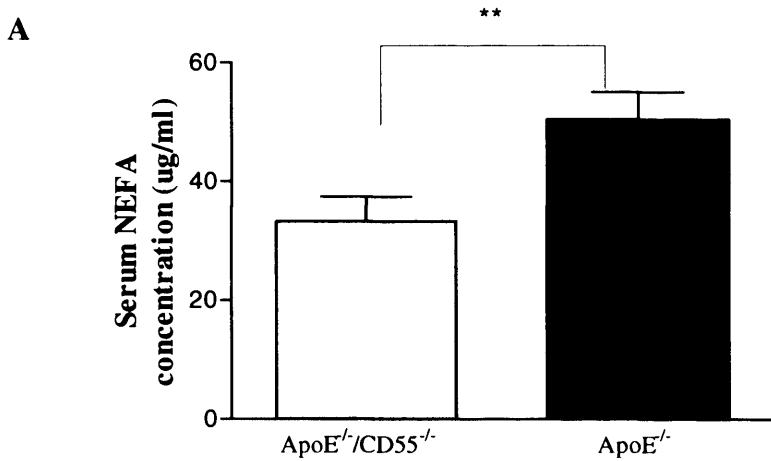


Figure 5.6 Circulating NEFA and glucose levels. NEFA and glucose measurements were compared between apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice at 20 weeks after 12 weeks on high fat diet. **(A)** Non-fasting NEFA levels (n=12 and 14 respectively). **(B)** Comparison of glucose levels in both fasting (n=12 and 11 respectively) and non-fasting state (n=6 and 10 respectively).

A

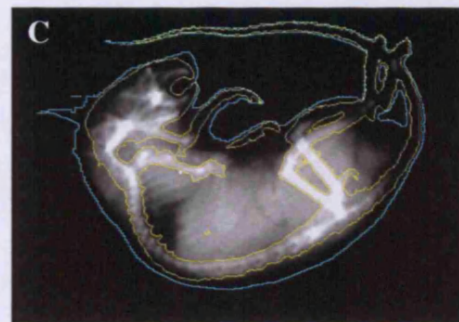
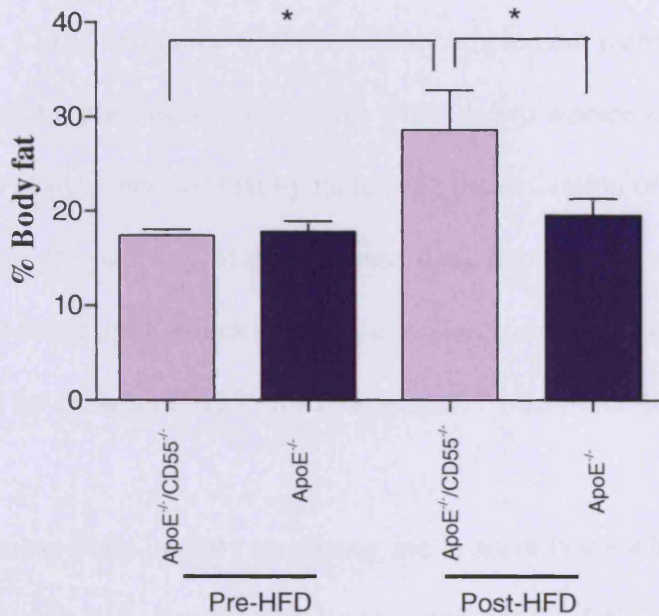


Figure 5.7 Percentage body fat in apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice. DEXA

measurements of percentage body fat compared between apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice at 8 weeks old, pre-high fat diet (Pre-HFD) (n=6 and 8 respectively), or at 20 weeks after 12 weeks on high fat diet (Post-HFD) (n=6 and 9 respectively).

(A) Mean percentage body fat in apoE^{-/-}/CD55^{-/-} and apoE^{-/-} mice. The bars show group means \pm SEM.

(B) A representative DEXA image of an apoE^{-/-}/CD55^{-/-} mouse.

(C) A representative DEXA image of an apoE^{-/-} mouse.

4. Discussion

In the previous chapter, it was demonstrated that by removing the complement regulator, CD59, activation of the terminal complement pathway was increased in atherosclerotic plaques of apoE^{-/-} mice which led to worsening of the disease. On this basis, it was hypothesised that by increasing the activation of the complement system earlier on in the pathway, at the C3 stage, disease progression would similarly accelerate in the apoE knock out mouse model. However in apoE^{-/-}/CD55^{-/-} mice, generated by crossing CD55^{-/-} mice with apoE^{-/-} mice, less disease was found.

These results, while initially surprising, are in accordance with two other independent studies. Buono et al. were first to demonstrate that C3 deficient mice on an ldlr^{-/-} background had a greater burden of atherosclerotic plaques, present in both the aortic arch and descending aorta (Buono et al., 2002). Another group using the apoE^{-/-}/ldlr^{-/-} mouse model similarly demonstrated that C3 was protective in atherosclerosis and by using fB deficient mice they also demonstrated that this was independent of the alternative pathway (Persson et al., 2004).

In the early stages of atherosclerosis, the dominant cell occupying the plaque is the macrophage, which differentiates into foam cells. However, no difference in macrophage cell content was found between apoE^{-/-}/CD55^{-/-} and apoE^{-/-} controls, despite a significant difference in plaque size between the two groups. This may have resulted from increased local production of C3b in CD55 deficient mice causing increased C3b opsonisation and hence clearance of apoptotic foam cells within the plaque. Foam cells in human atherosclerotic plaques are known to express C3b receptors such as CRig and CR1 together with the iC3b receptor, CR3 (Lee et al., 2006;

Saito et al., 1992). Further characterisation of the vessels revealed that the small plaques from apoE^{-/-}/CD55^{-/-} mice had less smooth muscle cell infiltration implicating that these plaques were early in composition.

However, when complement activation was assessed in the plaques of apoE^{-/-}/CD55^{-/-} a decrease in deposited MAC and C3 activation fragments were found when compared to apoE^{-/-} controls. This apparent reduction in complement activation is likely due to the fact that plaques formed in the absence of CD55 are early lesions and thus probably have less necrotic debris together with less free oxidized and modified lipid all known to activate complement (Bhakdi et al., 1999; Mevorach et al., 1998; Torzewski et al., 1998b). In contrast those plaques found in CD55-sufficient mice are larger, more advanced and complex and thus likely contain more complement activating material. These data implied that enhanced clearance of apoptotic foam cells through increased complement activation within the plaques was not the primary cause of the smaller plaques present in the apoE^{-/-}/CD55^{-/-} mice of this study.

The apoE^{-/-}/CD55^{-/-} mice in this study had smaller spleens compared to apoE^{-/-} controls, thus indicative of reduced disease in this model. During atherosclerotic disease progression, there is increased activation of adaptive immunity including B-cell dependent antibody production which occurs in the spleen. Hence an enlarged spleen within apoE^{-/-} mouse model may correlate with disease state.

Alongside the decreases in disease levels, apoE^{-/-}/CD55^{-/-} mice had significantly altered levels of circulating lipids. Both hypercholesterolemia and hypertriglyceridemia are recognised as independent risk factors for atherogenesis in man and models (Lusis et

al., 2004b). It was therefore hypothesised that the observed anti-atherogenic effect of CD55 deficiency was due to the combined effects of lower plasma lipid levels; however, the exact mechanism linking CD55 deficiency and altered lipoprotein profile remained to be defined. The rate of triglyceride clearance from plasma correlates directly with plasma levels of C3adesArg (Cianflone et al., 1989b). Thus, sustained changes in plasma levels of C3adesArg would be predicted to affect lipid profiles. In this study chronically elevated levels of C3adesArg were found in fat-fed apoE^{-/-}/CD55^{-/-} mice (50% increase compared to CD55-sufficient controls) and were accompanied by markedly decreased plasma triglyceride and cholesterol levels. These findings support studies *ex vivo* and *in vivo* that has implicated chylomicrons and/or VLDL as the primary physiological trigger for C3 activation resulting in C3adesArg production in adipose tissue (Maslowska et al., 1997a; Scantlebury et al., 1998). *Ex vivo*, exposure of adipose tissue to purified chylomicrons caused markedly increased synthesis of C3 and generation of C3adesArg, while *in vivo*, chylomicronaemia acutely increased plasma C3 and C3adesArg levels. The amount of C3adesArg generated will depend on the local activity and regulation of C3 convertase, in turn dependent on the presence and abundance of complement regulators. CD55 is expressed on adipose cells (Festy et al., 2005; Gronthos et al., 2001), and will therefore contribute to local regulation of the convertase; in its absence, the convertase will persist and generate more C3adesArg.

While triglycerides were lowered regardless of diet significantly lower levels of cholesterol in apoE^{-/-}/CD55^{-/-} mice only became apparent after 12 weeks of high fat diet. Together with these observations it was found that NEFA levels were also significantly lowered.

The data indicate that the observed lowering of lipid plasma in the apoE^{-/-}/CD55^{-/-} mice was due to increased cholesterol uptake and triglyceride synthesis occurring locally by adipocytes, and as a result an increase in percentage body fat was observed. In agreement with this finding, apoE^{-/-}/ldlr^{-/-}/C3^{-/-} mice had reduced body fat (Persson et al., 2004) which correlated with increased triglyceride levels; in this study the mice were fed normal chow and therefore there was no alteration in their cholesterol levels.

These results are surprising in that absence of an important complement regulator would be predicted to exacerbate injury in a disease characterised by complement activation. Two recently published studies tested effects of CD55 deficiency in mouse atherosclerosis models (An et al., 2009; Leung et al., 2009). In the first of these, female CD55^{-/-} mice on the ldlr^{-/-} background showed exacerbated disease and increased plaque size when compared to the ldlr^{-/-} controls. These contradictory results may be due in part to the different atherosclerosis-prone backgrounds; others have shown that results obtained from apoE^{-/-} and ldlr^{-/-} mice are often not comparable, perhaps because of the more generalised metabolic derangement accompanying ldlr deficiency (Joven et al., 2007). In particular, apoE^{-/-} mice have much higher serum triglyceride levels when compared to ldlr^{-/-} mice on the same diet (Joven et al., 2007). The use of female mice likely also contributes; it is well documented that complement levels are much greater in male mice than female in many inbred mouse strains (Beurskens et al., 1999; Holt et al., 2001). For this reason, when studying roles of complement in disease models, it is advantageous to use male mice only. In the second published study, apoE^{-/-}/CD55^{-/-} mice were generated and both male and female groups placed on a high fat diet for 8 or 16 weeks; plaque area was measured at end-point in the aortic arch. There was no significant difference in plaque size when apoE^{-/-}/CD55^{-/-} and apoE^{-/-} groups were

compared; however, when separated based on sex, there was a clear trend, un-remarked upon by the authors, towards smaller plaques in male apoE^{-/-}/CD55^{-/-} mice at 8 and 16 weeks. Group sizes after dividing by sex were small, and the differences observed were not significant. These published findings support the observation in this chapter, likely made significant in this study by the use of larger group sizes.

This chapter has demonstrated that CD55 deficiency in fat fed male apoE^{-/-} mice markedly attenuated the progression of atherosclerosis. Plaques were infrequent and small in comparison with their apoE^{-/-} controls; complement activation in the plaques was decreased with lower deposition of C3 fragments and MAC, likely reflecting the early plaque stage. ApoE^{-/-}/CD55^{-/-} mice had a markedly altered lipid profile, dominated by low circulating triglyceride, NEFA and cholesterol levels. These changes in lipid levels likely reflect increased triglyceride uptake into adipose tissue and fat synthesis in the apoE^{-/-}/CD55^{-/-} mice. All of these changes were likely to be caused by diminished capacity, in the absence of CD55, to regulate the C3 convertase, resulting in dysregulation of the ASP pathway and increased production of C3adesArg locally in adipose tissue in response to circulating chylomicrons or VLDL particles. The chain of events demonstrated here explains both atheroprotection and increased adiposity in the mice and also provides an explanation for the enigmatic observation that C3 deficient mice – lacking the capacity to generate C3adesArg – show accelerated disease in models of atherosclerosis (Buono et al., 2002; Persson et al., 2004). The data show that C3adesArg itself, or agents mimicking its lipid-modulating effects, might be of benefit in the treatment of atherosclerosis and related diseases.

Chapter 6: Discussion and Conclusions

The studies presented in this thesis describe my work to test the hypothesis outlined in the introduction that: **“The Complement system contributes to disease pathology in atherosclerotic plaques of apoE^{-/-} mice”**.

This was investigated by observing the effects of both the terminal and activation pathways of the complement system during plaque progression using the apoE^{-/-} mouse model of atherosclerosis.

1. Summary of main findings

1.1 Studies on the Terminal Pathway and Atherosclerosis

My work on the involvement of the terminal pathway of complement set out to unequivocally define the roles of the MAC and its regulator, CD59a in atherosclerotic plaque formation in the apoE^{-/-} mouse model. The data presented here demonstrating the contrasting effects of C6 and CD59a deficiencies on plaque development in apoE^{-/-} mice clearly support the hypothesis that:

“The MAC is an important contributor to atherosclerotic plaque development in the apoE^{-/-} mouse model”.

1.2 CD59a is strongly anti-atherogenic

ApoE^{-/-}/Cd59a^{-/-} mice developed much larger plaques on fat feeding compared to closely matched apoE^{-/-} controls; MAC deposition in the plaques was also markedly increased in apoE^{-/-}/Cd59a^{-/-} mice. Of note, the data from these experiments show that the SMC content of advanced plaques was significantly less in apoE^{-/-}/Cd59a^{-/-} mice than that seen in apoE^{-/-} controls. This finding suggests a possible mechanism whereby MAC formation could contribute not only to plaque development but also to plaque instability and hence the incidence of vessel ruptures and consequent acute infarcts or strokes.

1.3 MAC deposition within the plaque is strongly pro-atherogenic

For the first time, I have presented clear evidence that deficiency of C6 and lack of a functioning terminal pathway is strongly protective against the progression of atherosclerosis in apoE^{-/-} mice. This result is consistent with published studies both in fat fed C6-deficient rabbits and with other mouse models of atherosclerosis where Cd59a has been deleted (An et al., 2009; Geertinger and Soerensen, 1977; Schmiedt et al., 1998; Wu et al., 2009; Yun et al., 2008).

1.4 Therapeutic blockade of the terminal pathway

The therapeutic effect of an anti-C5 mAb was tested in the model. Anti-C5 treatment eliminated MAC deposition in the plaques of fat fed apoE^{-/-} mice, but did not significantly alter plaque size, suggesting that C5 and the MAC had little effect on the development of atherosclerosis. These data are not consistent with the above findings

and contrast with a recent report showing that the same anti-C5 reagent, BB5.1, did inhibit plaque formation in apoE^{-/-} mice deficient in Cd59a and Cd59b (Wu et al., 2009). However, it was notable that in this paper no data were presented from apoE^{-/-} mice rendering a direct comparison difficult. While these data conflict with my findings in CD59a and C6 deficiency, they are in line with the findings of Patel et al who failed to find any significant effect of C5 deficiency on the progression of atherosclerosis in the apoE^{-/-} mouse model (Patel et al., 2001). Taken together, these findings imply an anti-atherogenic role of C5 or its activation fragments. In our therapeutic model and in the apoE^{-/-}/C5^{-/-} mice of Patel et al it can be hypothesised that the absence of such a beneficial mechanism is balanced by blockade of the terminal pathway leading to no MAC production; hence, there is no net effect on disease levels.

1.5 Regulation of C3 activation

In the second part of my thesis, I describe investigations into the role of CD55 during the progression of atherosclerosis in apoE^{-/-} mice. Given that CD55 is up-regulated on both vascular SMC and macrophages in advanced plaques, and is functionally competent as a complement regulator *ex vivo* (Niculescu et al., 1990; Seifert and Hansson, 1989b), my initial hypothesis was that:

“CD55 deficiency will exacerbate disease pathology in apoE^{-/-} mice.”

However, strikingly, fat fed male apoE^{-/-}/CD55^{-/-} mice were strongly *protected* from developing atherosclerosis compared to apoE^{-/-} controls. Plaques were infrequent, small and morphologically simple by comparison with those present in apoE^{-/-} controls; complement activation in the plaques was decreased with lower deposition of C3

fragments and MAC, likely reflecting the early plaque stage and lack of complement activating material.

A second surprising finding from this experiment was that apoE^{-/-}/CD55^{-/-} mice had a markedly altered lipid profile, dominated by low circulating levels of triglycerides, NEFA and cholesterol. Alongside these changes in lipid levels there were significant rises in the systemic levels of C3adesArg, the metabolically active fragment of C3a, and also increased total body adiposity. Given the literature on this moiety (Baldo et al., 1993; Cianflone et al., 1989b; Cianflone et al., 2003; MacLaren et al., 2008) it was hypothesised that the observed beneficial changes in the absence of CD55 are due to increased lipid uptake into adipose tissue and/or increased triglyceride synthesis within adipocytes leaving less lipid available systemically to be taken up into the artery walls. It was further hypothesised that these changes were due to increased C3 activation and the increased systemic levels of C3adesArg arising through the lack of CD55 in these animals. Currently reagents are not available in my laboratory to test this hypothesis. However, the chain of events demonstrated here, may explain the atheroprotection and the increased adiposity observed in these mice. In addition it may also provide an explanation for the observation that C3^{-/-} mice – lacking the capacity to generate C3adesArg – show accelerated disease in models of atherosclerosis (Buono et al., 2002; Persson et al., 2004). Together, the data imply that, whilst agents targeted at inhibiting C3adesArg itself might be of benefit in the prevention of obesity, treatment with C3adesArg or agents mimicking its effects may help control atherosclerosis.

There are two other possible explanations for the observed protective effect; firstly the absence of CD55 may have caused accelerated activation of C3 within the atherosclerotic plaque, leading to increased production of C3b and iC3b. This effect causes better opsonisation of apoptotic cells, debris and immune complexes and phagocytosis via complement receptors CR1, CR3 and CR4 (Ricklin et al., 2010; Trouw et al., 2008) resulting in attenuation of plaque growth. Secondly, CD55 itself may exert a pro-atherogenic response directly. Both possibilities are reasonable and deserve further investigation, however, of note, there was no evidence of increased C3 activation within the plaques, in fact the reverse was true: significantly less C3 activation fragment deposition was seen in plaques from apoE^{-/-}/CD55^{-/-} mice in comparison with those from apoE^{-/-} controls (see figure 5.3C). Nevertheless there is a possibility that the activation fragments of C3 may have been consumed in the plaque; however, given that this is a model where exaggerated plasma cholesterol level drives disease pathology, it is likely that changes in lipid profiles would be the most obvious cause for protection against atherosclerosis.

2. Issues raised by thesis findings, and future directions

A number of interesting questions have been raised by the data generated in this thesis.

2.1 C6 and lipid metabolism

In addition to data implicating C6, and hence the terminal pathway, in the progression of atherosclerosis, it was also intriguingly found that the apoE^{-/-}/C6^{-/-} animals had altered serum lipid levels. In particular, serum triglyceride, and cholesterol levels and serum turbidity were significantly reduced in apoE^{-/-}/C6^{-/-} mice when compared to controls. Of note, serum cholesterol levels were not different in C6^{-/-} rabbits when

compared to C6 sufficient controls, however, serum triglyceride levels were not tested in these animals. These findings implicate a role for C6 in lipid metabolism in the apoE^{-/-} mouse model. In humans, complement component C6 expression was shown to be down regulated in omental adipose tissue of obese subjects when compared with lean controls (Hurtado Del Pozo et al., 2010). Unfortunately, measurements of percentage body fat in the apoE^{-/-}/C6^{-/-} mice and controls were not carried out in this instance due to reasons that were out of my control. Given that C6 has never previously been shown to have any effect on lipid metabolism this clearly warrants further investigation in both mice and humans into the mechanisms behind this phenomenon.

To determine the extent to which other complement components are involved in lipid metabolism in the apoE^{-/-} mouse model, further studies are warranted. Given that the effects might be diet dependent, such studies should include thorough investigations into the effect of different complement components on lipid levels in mice fed on diets with normal and high levels of fat. It is envisaged that this work would utilise various complement deficient mice whose lipid levels would be analysed after being fed either *ad libitum* or by giving a fat bolus by gavage. DEXA analysis would further reveal percentage body fat and fat distribution in these animals. To further delineate the mechanism involved in complement mediated lipid metabolism, microarray analysis could be utilised to compare the various knockouts with wild type background controlled animals. This would reveal differences in metabolic pathways existing in complement deficient strains.

2.2 The use of apoE mouse model in studying the role of complement in atherosclerosis

Since 1992, the apoE and ldlr mouse models have proved very useful in aiding experimental atherosclerosis research (Ishibashi et al., 1993; Piedrahita et al., 1992). Of note, the plasma lipid profiles vary widely between the two models, revealing differences in their metabolic activities (Joven et al., 2007). These differences between two key animal models of atherosclerosis may be one of the reasons behind the contradictory results from experiments investigating the role of complement in atherosclerosis.

In vivo, animal studies, using both the ldlr^{-/-} and apoE^{-/-} mouse models which have studied the role of Cd59a deficiency on the progression of atherosclerotic plaques are all in agreement that unregulated MAC caused worsening of the disease (An et al., 2009; Lewis et al., 2010; Wu et al., 2009; Yun et al., 2008) . These studies are also in agreement in that Cd59a deficiency did not alter lipid levels in either mouse model.

In contrast, studies looking at the effect of altered C3 activity, either by the removal of CD55, C3 or C3aR, on plaque size in atherosclerotic-prone mouse models contradict one another. In the ldlr^{-/-} mouse model, CD55 and C3 deficiency caused increased plaque size (Buono et al., 2002; Leung et al., 2009), whilst in the absence of apoE, I have shown that CD55 deficiency resulted in smaller plaque size. This is also in accordance with C3 deficiency, which caused an increase in lesion size (Persson et al., 2004). Of note the latter study was on a double apoE and ldlr deficient background. However, what is clear from these studies is that the removal of apoE along with either CD55 or C3 alters triglyceride levels and when a high fat diet is administered,

subsequent changes in cholesterol levels are observed. In contrast, despite differences in disease outcome, *ldlr* deficiency had no effect on lipid levels in the absence of CD55 or C3. It must be emphasised that another study using *apoE^{-/-}/CD55^{-/-}* versus *apoE^{-/-}* controls observed no difference in plaque size when fed a high fat diet; however, there was a clear trend towards a protective effect of CD55 deficiency which may have become significant had they used larger groups (An et al., 2009). Unfortunately, this group did not measure lipid levels in the mice. Another complication is that *fB* (required for alternative pathway activation) deficiency had no effect on lipid levels in *apoE^{-/-}/ldlr^{-/-}* mice (Persson et al., 2004).

In summary, the contrasting reports on the role of complement in atherosclerosis in different mouse models maybe due to the fact that one mouse model highlights the role of complement in lipid metabolism whereas the other does not. It may be that during lipid metabolism the beneficial effect of complement activation in reducing plasma lipid levels, and therefore protecting against atherosclerosis is dependent either on the presence of *ldlr*, hence in *ldlr^{-/-}* mice the role of complement in lipid metabolism is blocked, or has more of a role in the absence of *apoE*. Either way it is clearly important for both models to be used to study the role of complement during progression of atherosclerosis and lipid metabolism.

2.3 Activation of C3 during lipid metabolism is regulated by CD55

In this study, *C3adesArg* levels were significantly elevated in non-fasted *apoE^{-/-}/CD55^{-/-}* mice after 12 weeks on a high fat diet. It was also noted that plasma *C3adesArg* levels were increased more than 10-fold in the non-fasted state when compared to fasting levels in both *apoE^{-/-}* and *apoE^{-/-}/CD55^{-/-}* mice. These data imply that C3 activation

takes place post-prandially and that after a prolonged period of high fat feeding this increase in C3 activation is sustained. These findings are in accordance with those of Cianflone's group (Cianflone et al., 2003; Murray et al., 1999a; Saleh et al., 1998) who have demonstrated post prandial rises in C3adesArg levels which concomitantly affect lipid uptake and metabolism.

The exact mechanism of post-prandial C3 activation is not clearly defined. In vitro, both chylomicrons and insulin have been shown to stimulate the production of C3 and the generation of C3adesArg in cultures of differentiated human adipocytes and murine adipocyte cell line (3T3-L1) (Gao et al., 2011; Maslowska et al., 1997a). In addition, isolated human post-prandial chylomicrons have been shown to directly cause production of C3adesArg in normal human serum by accelerating C3 tick-over, resulting in activation of the alternative amplification loop (Fujita et al., 2007). Taken together these data suggest that in the post-prandial state local adipocyte production of C3 is increased and secreted locally into the plasma where it is activated by chylomicrons, increasing systemic levels of C3adesArg.

Little is known about the regulation of post-prandial C3 activation; one group has postulated that fH may play a role (Fujita et al., 2007), while other studies using differentiated 3T3-L1 cells have shown that sex hormones, such as testosterone and progesterone, may indirectly regulate the effect of C3adesArg on lipid metabolism by down regulating mRNA levels of C5L2, the proposed receptor for C3adesArg (Gao et al., 2010; Kalant et al., 2003; Kalant et al., 2005; Wen et al., 2008).

Based on published data to date regarding C3 activation and its role in lipid metabolism, together with the knowledge that CD55 is expressed on mature human adipocytes (Festy et al., 2005), I have proposed a mechanism for the anti-atherogenic effects observed in this study, summarised in figure 7.1.

To further examine the concept that CD55 regulates chylomicron induced production of C3adesArg in adipocytes the murine 3T3-L1 cell line which is often used in adipocyte studies, could be utilised as a model. The levels of CD55 could be determined and modified by transfection with expression plasmids or knock-down technology, followed by differentiation into adipocytes and stimulation with chylomicrons. A number of mechanistic issues could then be explored, including the effect of adipocyte CD55 on regulating C3 activation during chylomicron load, and induced adipocyte production of C3 in response to chylomicron uptake. Signalling events delineating the mechanism involved C3adesArg regulation by CD55 both intracellularly and at the cell surface could then be explored.

To support the hypothesis that increased C3 activation regulates lipid metabolism, which in turn protects apoE^{-/-}/CD55^{-/-} mice from atherosclerosis, a further study would be required.

Definitive experiments would involve giving apoE^{-/-}/CD55^{-/-} mice blocking antibodies against C3adesArg whilst on a high fat diet. In parallel, an isotype control antibody would be given to apoE^{-/-}/CD55^{-/-} mice at equal dose and for the same duration.

Adipose tissue

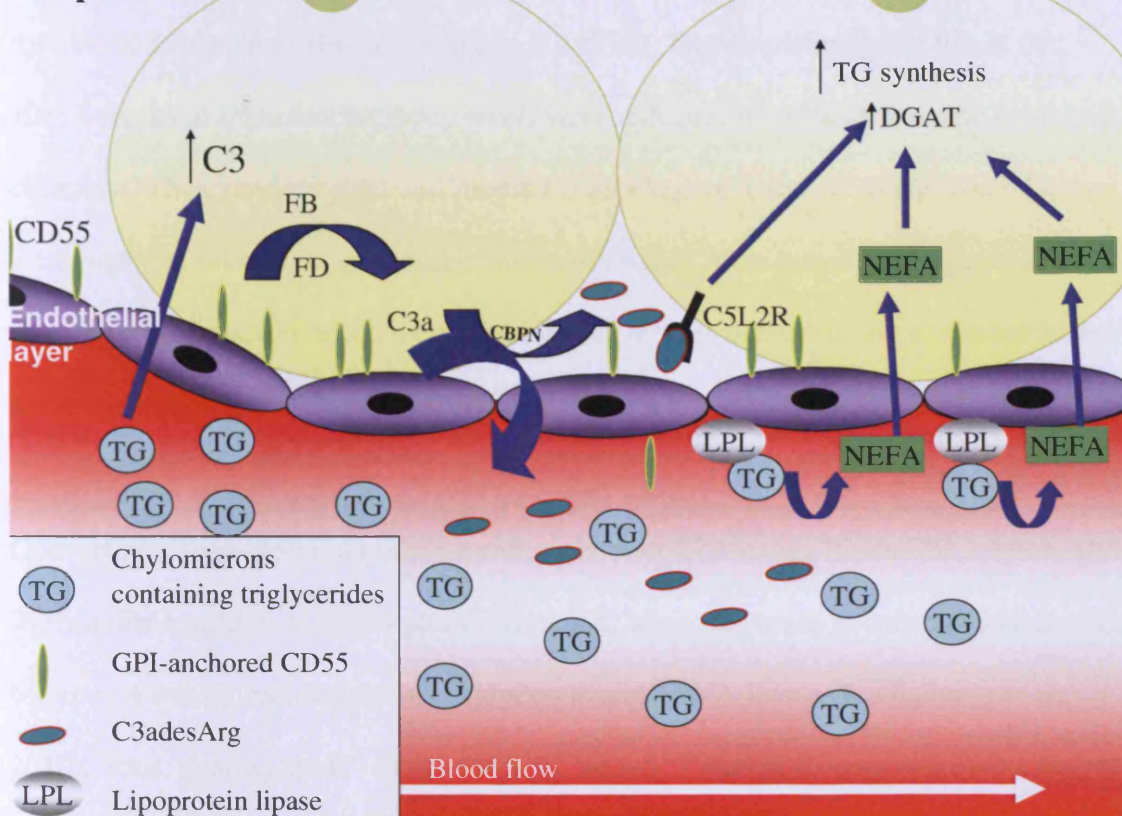


Figure 7.1 CD55 regulates local adipose C3 activation during lipid metabolism

1. High fat diet causes an increase in chylomicron production
2. Increased circulating chylomicrons stimulate the production of C3 and generation of C3adesArg
3. In apoE^{-/-}/CD55^{-/-} mice, local alternative pathway activation is dysregulated causing increased production of C3adesArg in the plasma
5. C3adesArg binds to C5L2 receptor on adipocytes which activates diacylglycerol acyltransferase (DGAT) thus stimulating triglyceride synthesis
6. C3adesArg indirectly activates LPL by protecting LPL from NEFA inhibition

Currently, researchers are using polyclonal rabbit antibody against human C3adesArg for in vivo murine experiments (Saleh et al., 1998). The disadvantage of this is that after 4 weeks of treatment antibody levels were reduced, suggesting immune mediated clearance. Thus, producing an anti-mouse C3adesArg mAb in C3^{-/-} mice would be the ideal intervention tool for generating antibodies which were non-immunogenic and thus providing reagents which could be used for long term treatment in mice without raising an immune response.

Converse to these experiments, recombinant human C3adesArg binds C5L2, stimulates triglyceride synthesis in adipocytes ex vivo and, when delivered at constant rate in vivo for up to 4 weeks, reduces plasma triglyceride and NEFA levels (Paglialunga et al., 2010). Thus, treating apoE^{-/-} mice with a recombinant murine C3adesArg over a longer period and examining the effects on atherosclerosis, body fat and blood lipid levels would further strengthen the hypothesis and delineate whether C3adesArg treatment can inhibit progression and/or reverse established disease in this model.

2.4 Genetic factors affecting the complement system and knock on effects on obesity and cardiovascular disease

Human studies in genetic deficiencies of complement proteins have associated certain complement polymorphisms with increased risk for cardiovascular disease (Berg and Heiberg, 1976; Dissing et al., 1972; Elston et al., 1976; Jylhävä et al., 2009; Kardys et al., 2006; Kristensen and Petersen, 1978). These common polymorphisms result in individuals having complement systems of varying activity. Given some of the findings presented in this thesis, this could offer an explanation as to why some individuals within the population are more at risk for obesity and/or cardiovascular disease.

Complement deficiencies might also predispose. A study in Sweden examining health records of C2 deficient patients found an association with increased rate of coronary heart disease which was independent of rheumatic manifestations (Jönsson et al., 2005; Nityanand et al., 1999). C4 deficiency and deficiency of the C4B variant have been implicated with a higher risk for myocardial infarction or stroke in Hungarian and Icelandic populations respectively (Arason et al., 2003; Kramer et al., 1994). However a population based study in Sweden, found no correlation between C4 null alleles and myocardial infarction patients compared to healthy controls (Lefvert et al., 1995). Subtotal MBL deficiency is common within the population and is associated with increased risk of infection but also increased incidence of cardiovascular disease and atherosclerotic plaque formation (Best et al., 2004; Hegele et al., 2000; Madsen et al., 1998).

Several studies have identified a polymorphism in fH, fH_{Y402H}, as a predicative indicator for age-related macular degeneration (AMD) (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005). Further studies on this polymorphism showed a positive correlation of the H variant with myocardial infarction (Jylhävä et al., 2009; Kardys et al., 2006). However, this is under debate as a study in the USA found an inverse correlation between the H variant of fH_{Y402H} and myocardial infarction, and no association was found in a meta-analysis of 48,000 participants (Pai et al., 2007; Sofat et al., 2010). Interestingly, within the meta-analysis study the authors did find a correlation between triglyceride levels and the fH polymorphic variants. Homozygote individuals who had the common Y402 allele had increased triglyceride levels compared to homozygote H402 allele.

Polymorphic variation is also found within the C3 gene. The substitution of a single DNA base pair in the C3 protein defines two allelic variants; C3S and C3F, named for their migration on an electrophoretic gel (slow and fast respectively), the result of an amino acid substitution C3_{R102G}. Whilst there is no evidence that this polymorphic variation causes altered plasma C3 levels, there are studies which have shown C3F variant may more readily activate (Welch et al., 1990). Interestingly, C3F variant has been associated with atherosclerosis, hypertension and hypercholesterolemia (Berg and Heiberg, 1976; Dissing et al., 1972; Elston et al., 1976; Kristensen and Petersen, 1978). However, the effect of this C3 polymorphism on C3adesArg production remains to be investigated.

Dr Claire Harris's laboratory at Cardiff University, has examined common polymorphic variants of complement proteins. The functional activities of C3, fH and fB polymorphic variants have been studied and compared (Heurich et al., 2011; Montes et al., 2009; Schmidt et al., 2008; Tortajada et al., 2009). Polymorphic risk variants in AMD (C3_{102G}, fB_{32R}, fH_{62V}) showed increased alternative pathway activation and thus if a complotype (combination of complement polymorphisms) of an individual can be determined, then their susceptibility to alternative pathway driven diseases can also be defined (Heurich et al., 2011). In the context of atherosclerosis, alternative pathway activation is almost certainly involved (Malik et al., 2010; Oksjoki et al., 2007b).

Although studies have looked at polymorphic variation of certain individual complement proteins and risk of coronary heart disease, no one has yet examined the effects of the complotype that confers most risk for atherosclerosis. Obesity and cardiovascular disease are growing problems in the Western population. Given that the alternative pathway of complement activation is involved in lipid metabolism and

components of the classical pathway are found in adipose tissue of obese subjects (Paglialunga et al., 2008; Xia and Cianflone, 2003; Zhang et al., 2007), it is reasonable to suggest that by determining the polymorphic risk variants in the population through screening individuals for their genotype, the likelihood of an individual becoming obese or developing cardiovascular complications could be predicted, such information would prove useful to general practitioners and cardiovascular specialists in advising patients and informing clinical decisions.

2.5 Targeting the terminal pathway as a therapy for atherosclerosis

Complement activation has been shown in vulnerable plaques which are prone to rupture and patients that present with unstable angina pectoris have been found to have increased plasma C5b-9 in comparison to patients with stable angina pectoris (Hoffmeister et al., 2002). In addition, evidence from animal studies have shown that deficiency of CD59 causes occlusive coronary disease, increased rate of mortality and vulnerable plaque phenotype (Wu et al., 2009). In agreement I have shown that that accelerated MAC formation in advanced lesions coincided with decreased SMC content, a characteristic of vulnerable plaques. The vulnerable plaque is more prone to rupture leading to cardiovascular complications including myocardial infarction and death. Developing therapeutic drugs targeted for the treatment of chronic cardiovascular disease is challenging. However, one strategy might be to inhibit the terminal pathway of complement to prevent identified vulnerable plaques from rupturing.

Eculizumab, a humanised anti-C5 mAb, is the first complement specific antibody on the market (Ricklin and Lambris, 2007) and provides the only approved therapy for

paroxysmal nocturnal hemoglobinuria (PNH), an acquired disorder of haematopoiesis (Rother et al., 2007). In the context of cardiovascular disease, Pexelizumab, a short-acting, single chain of Eculizumab, has been clinically tested for use in coronary artery bypass graft surgery and myocardial infarction (Whiss, 2002). At first the drug showed promising therapeutic effects, with a decrease in the rate of major cardiac events after myocardial infarction or coronary bypass surgery (Granger et al., 2003; Verrier et al., 2004). However, the APEX AMI trial, which included 5745 patients with acute ST-elevation myocardial infarction, showed no improvement in mortality or recurrent myocardial infarction with Pexelizumab (Armstrong et al., 2007). Similarly, I have shown that, targeting inhibition of the terminal pathway with an anti-C5 mAb failed to attenuate progression of the disease in apoE^{-/-} mice. Thus, targeting activation of the terminal complement pathway at the C5 level may not provide the ideal treatment for the prevention of the detrimental effects of end-stage atherosclerotic plaque.

In contrast, the terminal pathway at the level of C6 may provide a more inviting target since, as the data in this thesis demonstrate, apoE^{-/-}/C6^{-/-} deficient mice show a clear reduction in levels of atherosclerosis. Generation of an anti-C6 mAb in C6^{-/-} mice and the ability to therapeutically test this reagent in apoE^{-/-} animals might be a next logical step to be undertaken as a follow up from this project.

2.6 Therapeutic targeting of C3

I have shown that dysregulation at the C3 level slowed the progression of atherosclerosis in high fat fed apoE^{-/-} mice. This was paralleled by decreases in plasma lipid levels together with increased body fat. The role of C3 in obesity has also been highlighted in other studies. In rodents, obese Zucker rat model have significantly higher levels of plasma C3 compared to lean controls (Boggs et al., 1998). Furthermore,

C3a receptor^{-/-} mice have been shown to be protected from obesity when fed a high fat diet (Mamane et al., 2009). Human studies have similarly demonstrated a relationship between increased C3 and C3adesArg levels with body mass index and obesity (Cianflone et al., 2003; Pomeroy et al., 1997; Yang et al., 2006).

Therapeutically this poses a difficult problem in that design and use of agents to block the action of C3adesArg and thus prevent or reduce obesity may result in increased blood cholesterol and triglyceride levels in patients with a concomitant increased risk of atherosclerosis. In order to avoid this obvious problem, one strategy might be to combine blocking C3adesArg therapy with a stringent low fat diet regime

3. Conclusion

Atherosclerosis is a chronic inflammatory disease where the adaptive and innate arms of the immune system are activated in response to stress or damage of the endothelium. Complement activation has long been seen as critical during the progression of atherosclerosis and it is now emerging that its role in lipid metabolism may be equally important.

In this thesis, I have clearly demonstrated the importance of the terminal complement pathway in the progression of atherosclerosis and the vital role played by Cd59a in attenuating this effect. A second major finding was that enhanced C3 activation in the absence of CD55 was beneficial in attenuating disease progression. The mechanisms behind the latter effects remain to be definitively determined but the existing evidence

from this study strongly point towards C3 fragment, C3adesArg, and its role in modulating lipid homeostasis.

Further work is clearly warranted to understand the mechanistic implications of the discoveries made during this thesis and its relevance to human atherosclerosis which is a growing problem throughout the developed and developing world. Such investigations should allow a definitive analysis of the potential for using complement based therapeutics in a clinical setting to treat cardiovascular disease.

References

- Abou-Raya A. and Abou-Raya S., 2006. Inflammation: a pivotal link between autoimmune diseases and atherosclerosis. *Autoimmun Rev* 5, 331-7.
- Addis-Lieser E., Köhl J. and Chiaramonte M., 2005. Opposing regulatory roles of complement factor 5 in the development of bleomycin-induced pulmonary fibrosis. *J Immunol* 175, 1894-902.
- Ait-Oufella H., Kinugawa K., Zoll J., Simon T., Boddaert J., Heeneman S., Blanc-Brude O., Barateau V., Potteaux S., Merval R., Esposito B., Teissier E., Daemen M., Lesèche G., Boulanger C., Tedgui A. and Mallat Z., 2007. Lactadherin deficiency leads to apoptotic cell accumulation and accelerated atherosclerosis in mice. *Circulation* 115, 2168-77.
- Aksamit R., Falk W. and Leonard E., 1981. Chemotaxis by mouse macrophage cell lines. *J Immunol* 126, 2194-9.
- Albrecht E. A., Chinnaiyan A. M., Varambally S., Kumar-Sinha C., Barrette T. R., Sarma J. V. and Ward P. A., 2004. C5a-induced gene expression in human umbilical vein endothelial cells. *Am J Pathol* 164, 849-59.
- Alipour A., Elte J., van Zaanen H., Rietveld A. and Castro Cabezas M., 2008. Novel aspects of postprandial lipemia in relation to atherosclerosis. *Atheroscler Suppl* 9, 39-44.
- Amento E., Ehsani N., Palmer H. and Libby P., Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. *Arterioscler Thromb* 11, 1223-30.

- Amorino G. and Hoover R., 1998. Interactions of monocytic cells with human endothelial cells stimulate monocytic metalloproteinase production. *Am J Pathol* 152, 199-207.
- An G., Miwa T., Song W., Lawson J., Rader D., Zhang Y. and Song W., 2009. CD59 but not DAF deficiency accelerates atherosclerosis in female ApoE knockout mice. *Mol Immunol* 46, 1702-9.
- Annex B., Denning S., Channon K., Sketch M. J., Stack R., Morrissey J. and Peters K., 1995. Differential expression of tissue factor protein in directional atherectomy specimens from patients with stable and unstable coronary syndromes. *Circulation* 91, 619-22.
- Arai S., Shelton J., Chen M., Bradley M., Castrillo A., Bookout A., Mak P., Edwards P., Mangelsdorf D., Tontonoz P. and Miyazaki T., 2005. A role for the apoptosis inhibitory factor AIM/Spalpha/Ap16 in atherosclerosis development. *Cell Metab* 1, 201-13.
- Arason G. J., Bødvarsson S., Sigurdarson S. T., Sigurdsson G., Thorgeirsson G., Gudmundsson S., Kramer J. and Füst G., 2003. An age-associated decrease in the frequency of C4B*Q0 indicates that null alleles of complement may affect health or survival. *Ann N Y Acad Sci* 1010, 496-9.
- Armstrong P. W., Granger C. B., Adams P. X., Hamm C., Holmes D., O'Neill W. W., Todaro T. G., Vahanian A., Van de Werf F. and Investigators A. A., 2007. Pexelizumab for acute ST-elevation myocardial infarction in patients undergoing primary percutaneous coronary intervention: a randomized controlled trial. *JAMA* 297, 43-51.
- Austin M., Hokanson J. and Edwards K., 1998. Hypertriglyceridemia as a cardiovascular risk factor. *Am J Cardiol* 81, 7B-12B.

- Baalasubramanian S., Harris C., Donev R., Mizuno M., Omidvar N., Song W. and Morgan B., 2004. CD59a is the primary regulator of membrane attack complex assembly in the mouse. *J Immunol* 173, 3684-92.
- Baldo A., Sniderman A., St-Luce S., Avramoglu R., Maslowska M., Hoang B., Monge J., Bell A., Mulay S. and Cianflone K., 1993. The adipsin-acylation stimulating protein system and regulation of intracellular triglyceride synthesis. *J Clin Invest* 92, 1543-7.
- Baumgarth N., Tung J. and Herzenberg L., 2005. Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. *Springer Semin Immunopathol* 26, 347-62.
- Benaglio M., D'Elis M., Amedei A., Azzurri A., van der Zee R., Ciervo A., Rombolà G., Romagnani S., Cassone A. and Del Prete G., 2005. Human 60-kDa heat shock protein is a target autoantigen of T cells derived from atherosclerotic plaques. *J Immunol* 174, 6509-17.
- Bentzon J., Pasterkamp G. and Falk E., 2003. Expansive remodeling is a response of the plaque-related vessel wall in aortic roots of apoE-deficient mice: an experiment of nature. *Arterioscler Thromb Vasc Biol* 23, 257-62.
- Benzaquen L., Nicholson-Weller A. and Halperin J., 1994. Terminal complement proteins C5b-9 release basic fibroblast growth factor and platelet-derived growth factor from endothelial cells. *J Exp Med* 179, 985-92.
- Berg K. and Heiberg A., 1976. Linkage studies on familial hyperlipoproteinemia with xanthomatosis: normal lipoprotein markers and the C3 polymorphism. *Cytogenet Cell Genet* 16, 266-70.

- Berliner J., Territo M., Sevanian A., Ramin S., Kim J., Bamshad B., Esterson M. and Fogelman A., 1990. Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J Clin Invest* 85, 1260-6.
- Best L. G., Davidson M., North K. E., MacCluer J. W., Zhang Y., Lee E. T., Howard B. V., DeCroo S. and Ferrell R. E., 2004. Prospective analysis of mannose-binding lectin genotypes and coronary artery disease in American Indians: the Strong Heart Study. *Circulation* 109, 471-5.
- Beurskens F., Kuenen J., Hofhuis F., Fluit A., Robins D. and Van Dijk H., 1999. Sex-limited protein: in vitro and in vivo functions. *Clin Exp Immunol* 116, 395-400.
- Bhakdi S., 1998. Complement and atherogenesis: the unknown connection. *Ann Med* 30, 503-7.
- Bhakdi S., Dorweiler B., Kirchmann R., Torzewski J., Weise E., Tranum-Jensen J., Walev I. and Wieland E., 1995. On the pathogenesis of atherosclerosis: enzymatic transformation of human low density lipoprotein to an atherogenic moiety. *J Exp Med* 182, 1959-71.
- Bhakdi S., Torzewski M., Klouche M. and Hemmes M., 1999. Complement and atherogenesis: binding of CRP to degraded, nonoxidized LDL enhances complement activation. *Arterioscler Thromb Vasc Biol* 19, 2348-54.
- Bhatia V., Yun S., Leung V., Grimsditch D., Benson G., Botto M., Boyle J. and Haskard D., 2007. Complement C1q reduces early atherosclerosis in low-density lipoprotein receptor-deficient mice. *Am J Pathol* 170, 416-26.
- Bhole D. and Stahl G., 2004. Molecular basis for complement component 6 (C6) deficiency in rats and mice. *Immunobiology* 209, 559-68.

- Binder C., Hörkkö S., Dewan A., Chang M., Kieu E., Goodyear C., Shaw P., Palinski W., Witztum J. and Silverman G., 2003. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between *Streptococcus pneumoniae* and oxidized LDL. *Nat Med* 9, 736-43.
- Björkbacka H., Kunjathoor V., Moore K., Koehn S., Ordija C., Lee M., Means T., Halmen K., Luster A., Golenbock D. and Freeman M., 2004. Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways. *Nat Med* 10, 416-21.
- Bobryshev Y. and Lord R., 1995. Ultrastructural recognition of cells with dendritic cell morphology in human aortic intima. Contacting interactions of Vascular Dendritic Cells in athero-resistant and athero-prone areas of the normal aorta. *Arch Histol Cytol* 58, 307-22.
- Boekholdt S., de Winter R. and Kastelein J., 2008. Inhibition of lipoprotein-associated phospholipase activity by darapladib: shifting gears in cardiovascular drug development: are antiinflammatory drugs the next frontier? *Circulation* 118, 1120-2.
- Boggs R., McCumbee W., Cobbs S., Todd D., Kahle E., Stewart N., Bailey M. and Reichenbecher V., 1998. Increased expression of complement component C3 in the plasma of obese Zucker fa and LA/N fa(f) rats compared with their lean counterparts. *Obes Res* 6, 361-7.
- Boisvert W., Curtiss L. and Terkeltaub R., 2000. Interleukin-8 and its receptor CXCR2 in atherosclerosis. *Immunol Res* 21, 129-37.
- Boisvert W., Rose D., Boullier A., Quehenberger O., Sydlaske A., Johnson K., Curtiss L. and Terkeltaub R., 2006. Leukocyte transglutaminase 2 expression limits atherosclerotic lesion size. *Arterioscler Thromb Vasc Biol* 26, 563-9.

- Boring L., Gosling J., Cleary M. and Charo I., 1998. Decreased lesion formation in CCR2^{-/-} mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* 394, 894-7.
- Breslow J., 1996. Mouse models of atherosclerosis. *Science* 272, 685-8.
- Buono C., Come C., Witztum J., Maguire G., Connelly P., Carroll M. and Lichtman A., 2002. Influence of C3 deficiency on atherosclerosis. *Circulation* 105, 3025-31.
- Busche M. and Stahl G., 2010. Role of the complement components C5 and C3a in a mouse model of myocardial ischemia and reperfusion injury. *Ger Med Sci* 8.
- Bustos M., Coffman T. M., Saadi S. and Platt J. L., 1997. Modulation of eicosanoid metabolism in endothelial cells in a xenograft model. Role of cyclooxygenase-2. *J Clin Invest* 100, 1150-8.
- Caligiuri G., Paulsson G., Nicoletti A., Maseri A. and Hansson G., 2000. Evidence for antigen-driven T-cell response in unstable angina. *Circulation* 102, 1114-9.
- Campbell L. and Kuo C., 2003. Chlamydia pneumoniae and atherosclerosis. *Semin Respir Infect* 18, 48-54.
- Carroll M., 2004. The complement system in regulation of adaptive immunity. *Nat Immunol* 5, 981-6.
- Chen J., Crispín J. C., Dalle Lucca J. and Tsokos G. C., 2009. A Novel Inhibitor of the Alternative Pathway of Complement Attenuates Intestinal Ischemia/Reperfusion-Induced Injury. *J Surg Res*.
- Choy L., Rosen B. and Spiegelman B., 1992. Adipsin and an endogenous pathway of complement from adipose cells. *J Biol Chem* 267, 12736-41.
- Choy L. and Spiegelman B., 1996. Regulation of alternative pathway activation and C3a production by adipose cells. *Obes Res* 4, 521-32.

- Cianflone K., Lu H., Smith J., Yu W. and Wang H., 2005. Adiponectin, acylation stimulating protein and complement C3 are altered in obesity in very young children. *Clin Endocrinol (Oxf)* 62, 567-72.
- Cianflone K., Maslowska M. and Sniderman A., 1999. Acylation stimulating protein (ASP), an adipocyte autocrine: new directions. *Semin Cell Dev Biol* 10, 31-41.
- Cianflone K., Paglialunga S. and Roy C., 2008. Intestinally derived lipids: metabolic regulation and consequences--an overview. *Atheroscler Suppl* 9, 63-8.
- Cianflone K., Sniderman A., Walsh M., Vu H., Gagnon J. and Rodriguez M., 1989a. Purification and characterization of acylation stimulating protein. *J Biol Chem* 264, 426-30.
- Cianflone K., Vu H., Walsh M., Baldo A. and Sniderman A., 1989b. Metabolic response of Acylation Stimulating Protein to an oral fat load. *J Lipid Res* 30, 1727-33.
- Cianflone K., Xia Z. and Chen L., 2003. Critical review of acylation-stimulating protein physiology in humans and rodents. *Biochim Biophys Acta* 1609, 127-43.
- Cianflone K., Zakarian R., Couillard C., Delplanque B., Despres J. and Sniderman A., 2004. Fasting acylation-stimulating protein is predictive of postprandial triglyceride clearance. *J Lipid Res* 45, 124-31.
- Cipollone F., 2005. COX-2 and prostaglandins in atherosclerosis. *Lupus* 14, 756-9.
- Clerc G. and Rouz P., 1997. Lymphocyte subsets in severe atherosclerosis before revascularization. *Ann Intern Med* 126, 1004-5.
- Cohn J., 1998. Postprandial lipemia: emerging evidence for atherogenicity of remnant lipoproteins. *Can J Cardiol* 14 Suppl B, 18B-27B.

- Cole D. and Morgan B., 2003. Beyond lysis: how complement influences cell fate. *Clin Sci (Lond)* 104, 455-66.
- Copland D., Hussain K., Baalasubramanian S., Hughes T., Morgan B., Xu H., Dick A. and Nicholson L., 2010. Systemic and local anti-C5 therapy reduces the disease severity in experimental autoimmune uveoretinitis. *Clin Exp Immunol* 159, 303-14.
- Crisby M., Nordin-Fredriksson G., Shah P., Yano J., Zhu J. and Nilsson J., 2001. Pravastatin treatment increases collagen content and decreases lipid content, inflammation, metalloproteinases, and cell death in human carotid plaques: implications for plaque stabilization. *Circulation* 103, 926-33.
- Cybulsky M. and Gimbrone M. J., 1991. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* 251, 788-91.
- Cybulsky M., Iiyama K., Li H., Zhu S., Chen M., Iiyama M., Davis V., Gutierrez-Ramos J., Connelly P. and Milstone D., 2001. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin Invest* 107, 1255-62.
- Dansky H., Barlow C., Lominska C., Sikes J., Kao C., Weinsaft J., Cybulsky M. and Smith J., 2001. Adhesion of monocytes to arterial endothelium and initiation of atherosclerosis are critically dependent on vascular cell adhesion molecule-1 gene dosage. *Arterioscler Thromb Vasc Biol* 21, 1662-7.
- Davies M., Gordon J., Gearing A., Pigott R., Woolf N., Katz D. and Kyriakopoulos A., 1993a. The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. *J Pathol* 171, 223-9.
- Davies M., Richardson P., Woolf N., Katz D. and Mann J., 1993b. Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage, and smooth muscle cell content. *Br Heart J* 69, 377-81.

- Davis J., 2008. Eculizumab. *Am J Health Syst Pharm* 65, 1609-15.
- de Boer O., van der Wal A., Houtkamp M., Ossewaarde J., Teeling P. and Becker A., 2000. Unstable atherosclerotic plaques contain T-cells that respond to *Chlamydia pneumoniae*. *Cardiovasc Res* 48, 402-8.
- de Man F., Nieuwland R., van der Laarse A., Romijn F., Smelt A., Gevers Leuven J. and Sturk A., 2000. Activated platelets in patients with severe hypertriglyceridemia: effects of triglyceride-lowering therapy. *Atherosclerosis* 152, 407-14.
- De Palma R., Del Galdo F., Abbate G., Chiariello M., Calabró R., Forte L., Cimmino G., Papa M., Russo M., Ambrosio G., Giombolini C., Tritto I., Notaristefano S., Berrino L., Rossi F. and Golino P., 2006. Patients with acute coronary syndrome show oligoclonal T-cell recruitment within unstable plaque: evidence for a local, intracoronary immunologic mechanism. *Circulation* 113, 640-6.
- De Vries B., Matthijsen R., Wolfs T., Van Bijnen A., Heeringa P. and Buurman W., 2003. Inhibition of complement factor C5 protects against renal ischemia-reperfusion injury: inhibition of late apoptosis and inflammation. *Transplantation* 75, 375-82.
- Del Rincón I., O'Leary D., Freeman G. and Escalante A., 2007. Acceleration of atherosclerosis during the course of rheumatoid arthritis. *Atherosclerosis* 195, 354-60.
- Dennis E., 1994. Diversity of group types, regulation, and function of phospholipase A2. *J Biol Chem* 269, 13057-60.

- Dessein P., Norton G., Woodiwiss A., Joffe B. and Wolfe F., 2007. Influence of nonclassical cardiovascular risk factors on the accuracy of predicting subclinical atherosclerosis in rheumatoid arthritis. *J Rheumatol* 34, 943-51.
- Diehl E., Haines G. r., Radosevich J. and Potempa L., 2000. Immunohistochemical localization of modified C-reactive protein antigen in normal vascular tissue. *Am J Med Sci* 319, 79-83.
- Dissing J., Lund J. and Sorensen H., 1972. C3 polymorphism in a group of old arteriosclerotic patients. *Hum Hered* 22, 466-72.
- Donev R., Sivasankar B., Mizuno M. and Morgan B., 2008. The mouse complement regulator CD59b is significantly expressed only in testis and plays roles in sperm acrosome activation and motility. *Mol Immunol* 45, 534-42.
- Dong Z., Brown A. and Wagner D., 2000. Prominent role of P-selectin in the development of advanced atherosclerosis in ApoE-deficient mice. *Circulation* 101, 2290-5.
- Dong Z., Chapman S., Brown A., Frenette P., Hynes R. and Wagner D., 1998. The combined role of P- and E-selectins in atherosclerosis. *J Clin Invest* 102, 145-52.
- Doran A., Meller N. and McNamara C., 2008. Role of smooth muscle cells in the initiation and early progression of atherosclerosis. *Arterioscler Thromb Vasc Biol* 28, 812-9.
- Edwards A. O., Ritter R., Abel K. J., Manning A., Panhuysen C. and Farrer L. A., 2005. Complement factor H polymorphism and age-related macular degeneration. *Science* 308, 421-4.

- Ehrengruber M., Geiser T. and Deranleau D., 1994. Activation of human neutrophils by C3a and C5A. Comparison of the effects on shape changes, chemotaxis, secretion, and respiratory burst. *FEBS Lett* 346, 181-4.
- Elston R. C., Namboodiri K. K., Go R. C., Siervogel R. M. and Glueck C. J., 1976. Probable linkage between essential familial hypercholesterolemia and third complement component (C3). *Cytogenet Cell Genet* 16, 294-7.
- Engström G., Hedblad B., Janzon L. and Lindgärde F., 2007. Complement C3 and C4 in plasma and incidence of myocardial infarction and stroke: a population-based cohort study. *Eur J Cardiovasc Prev Rehabil* 14, 392-7.
- Falk E., 1989. Morphologic features of unstable atherothrombotic plaques underlying acute coronary syndromes. *Am J Cardiol* 63, 114E-120E.
- Falk E., Schwartz S. M., Galis Z. S. and Rosenfeld M. E., 2007. Putative murine models of plaque rupture. *Arterioscler Thromb Vasc Biol* 27, 969-72.
- Falk R., Dalmaso A., Kim Y., Tsai C., Scheinman J., Gewurz H. and Michael A., 1983. Neoantigen of the polymerized ninth component of complement. Characterization of a monoclonal antibody and immunohistochemical localization in renal disease. *J Clin Invest* 72, 560-73.
- Fan J. and Watanabe T., 2003. Inflammatory reactions in the pathogenesis of atherosclerosis. *J Atheroscler Thromb* 10, 63-71.
- Faure E., Equils O., Sieling P., Thomas L., Zhang F., Kirschning C., Polentarutti N., Muzio M. and Arditi M., 2000. Bacterial lipopolysaccharide activates NF-kappaB through toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells. *J Biol Chem* 275, 11058-63.

- Faure E., Thomas L., Xu H., Medvedev A., Equils O. and Arditi M., 2001. Bacterial lipopolysaccharide and IFN-gamma induce Toll-like receptor 2 and Toll-like receptor 4 expression in human endothelial cells: role of NF-kappa B activation. *J Immunol* 166, 2018-24.
- Fearon D. and Locksley R., 1996. The instructive role of innate immunity in the acquired immune response. *Science* 272, 50-3.
- Febbraio M., Podrez E., Smith J., Hajjar D., Hazen S., Hoff H., Sharma K. and Silverstein R., 2000. Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. *J Clin Invest* 105, 1049-56.
- Feher M., 2003. Lipid lowering to delay the progression of coronary artery disease. *Heart* 89, 451-8.
- Feher M. and Richmond W., 2001. *Lipids & Lipid disorders*, 3rd Edition. Harcourt Health Communications.
- Festy F., Hoareau L., Bes-Houtmann S., Péquin A., Gonthier M., Munstun A., Hoarau J., Césari M. and Roche R., 2005. Surface protein expression between human adipose tissue-derived stromal cells and mature adipocytes. *Histochem Cell Biol* 124, 113-21.
- Ffrench-Constant C., 1994. Pathogenesis of multiple sclerosis. *Lancet* 343, 271-5.
- Flier J., Cook K., Usher P. and Spiegelman B., 1987. Severely impaired adiponin expression in genetic and acquired obesity. *Science* 237, 405-8.
- Flierman R. and Daha M., 2007. The clearance of apoptotic cells by complement. *Immunobiology* 212, 363-70.

- Fondevila C., Shen X., Tsuchihashi S., Uchida Y., Freitas M., Ke B., Busuttill R. and Kupiec-Weglinski J., 2008. The membrane attack complex (C5b-9) in liver cold ischemia and reperfusion injury. *Liver Transpl* 14, 1133-41.
- Foreman K., Vaporciyan A., Bonish B., Jones M., Johnson K., Glovsky M., Eddy S. and Ward P., 1994. C5a-induced expression of P-selectin in endothelial cells. *J Clin Invest* 94, 1147-55.
- Fosbrink M., Niculescu F., Rus V., Shin M. and Rus H., 2006. C5b-9-induced endothelial cell proliferation and migration are dependent on Akt inactivation of forkhead transcription factor FOXO1. *J Biol Chem* 281, 19009-18.
- Freire de Carvalho J., Sherer Y. and Shoenfeld Y., 2007. The fine-tuning of anti-oxidized low-density lipoprotein antibodies in cardiovascular disease and thrombosis. *Thromb Haemost* 98, 1157-9.
- Frostegård J., Ulfgrén A., Nyberg P., Hedin U., Swedenborg J., Andersson U. and Hansson G., 1999. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* 145, 33-43.
- Fujita T., Endo Y. and Nonaka M., 2004. Primitive complement system--recognition and activation. *Mol Immunol* 41, 103-11.
- Fujita T., Fujioka T., Murakami T., Satomura A., Fuke Y. and Matsumoto K., 2007. Chylomicron accelerates C3 tick-over by regulating the role of factor H, leading to overproduction of acylation stimulating protein. *J Clin Lab Anal* 21, 14-23.
- Fujita T., Inoue T., Ogawa K., Iida K. and Tamura N., 1987. The mechanism of action of decay-accelerating factor (DAF). DAF inhibits the assembly of C3 convertases by dissociating C2a and Bb. *J Exp Med* 166, 1221-8.

- Fukuoka Y., Yasui A., Okada N. and Okada H., 1996. Molecular cloning of murine decay accelerating factor by immunoscreening. *Int Immunol* 8, 379-85.
- Galis Z., Sukhova G., Lark M. and Libby P., 1994. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest* 94, 2493-503.
- Galkina E. and Ley K., 2007. Leukocyte influx in atherosclerosis. *Curr Drug Targets* 8, 1239-48.
- Galkina E. and Ley K., 2009. Immune and inflammatory mechanisms of atherosclerosis (*). *Annu Rev Immunol* 27, 165-97.
- Gao Y., Gauvreau D. and Cianflone K., 2010. Hormone and pharmaceutical regulation of ASP production in 3T3-L1 adipocytes. *J Cell Biochem* 109, 896-905.
- Gao Y., Gauvreau D., Cui W., Lapointe M., Paglialunga S. and Cianflone K., 2011. Evaluation of chylomicron effect on ASP production in 3T3-L1 adipocytes. *Acta Biochim Biophys Sin (Shanghai)* 43, 154-9.
- Garbus J., Deluca H., Loomans M. and Strong F., 1963. The rapid incorporation of phosphate into mitochondrial lipids. *J Biol Chem* 238, 59-63.
- Garza C., Montori V., McConnell J., Somers V., Kullo I. and Lopez-Jimenez F., 2007. Association between lipoprotein-associated phospholipase A2 and cardiovascular disease: a systematic review. *Mayo Clin Proc* 82, 159-65.
- Gautier E., Huby T., Witztum J., Ouzilleau B., Miller E., Saint-Charles F., Aucouturier P., Chapman M. and Lesnik P., 2009. Macrophage apoptosis exerts divergent effects on atherogenesis as a function of lesion stage. *Circulation* 119, 1795-804.

- Geertinger P. and Soerensen H., 1977. On the reduced atherogenic effect of cholesterol feeding in rabbits with congenital complement (C6) deficiency. *Artery* 1, 177-184.
- George J., Harats D., Gilburd B., Afek A., Shaish A., Kopolovic J. and Shoenfeld Y., 2000. Adoptive transfer of beta(2)-glycoprotein I-reactive lymphocytes enhances early atherosclerosis in LDL receptor-deficient mice. *Circulation* 102, 1822-7.
- Germinario R., Sniderman A., Manuel S., Lefebvre S., Baldo A. and Cianflone K., 1993. Coordinate regulation of triacylglycerol synthesis and glucose transport by acylation-stimulating protein. *Metabolism* 42, 574-80.
- Gershov D., Kim S., Brot N. and Elkon K., 2000. C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. *J Exp Med* 192, 1353-64.
- Ginsberg H., 2001. Hypertriglyceridemia: new insights and new approaches to pharmacologic therapy. *Am J Cardiol* 87, 1174-80; A4.
- Ginsberg H., 2002. New perspectives on atherogenesis: role of abnormal triglyceride-rich lipoprotein metabolism. *Circulation* 106, 2137-42.
- Glass C. and Witztum J., 2001. Atherosclerosis. the road ahead. *Cell* 104, 503-16.
- Goldstein J., Ho Y., Basu S. and Brown M., 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U S A* 76, 333-7.
- Gordon S. and Taylor P., 2005. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 5, 953-64.

- Granger C. B., Mahaffey K. W., Weaver W. D., Theroux P., Hochman J. S., Filloon T. G., Rollins S., Todaro T. G., Nicolau J. C., Ruzyllo W., Armstrong P. W. and Investigators C., 2003. Pexelizumab, an anti-C5 complement antibody, as adjunctive therapy to primary percutaneous coronary intervention in acute myocardial infarction: the COMplement inhibition in Myocardial infarction treated with Angioplasty (COMMA) trial. *Circulation* 108, 1184-90.
- Gronthos S., Franklin D., Leddy H., Robey P., Storms R. and Gimble J., 2001. Surface protein characterization of human adipose tissue-derived stromal cells. *J Cell Physiol* 189, 54-63.
- Gu L., Okada Y., Clinton S., Gerard C., Sukhova G., Libby P. and Rollins B., 1998. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* 2, 275-81.
- Guha M. and Mackman N., 2001. LPS induction of gene expression in human monocytes. *Cell Signal* 13, 85-94.
- Guo R. and Ward P., 2005. Role of C5a in inflammatory responses. *Annu Rev Immunol* 23, 821-52.
- Hageman G. S., Anderson D. H., Johnson L. V., Hancox L. S., Taiber A. J., Hardisty L. I., Hageman J. L., Stockman H. A., Borchardt J. D., Gehrs K. M., Smith R. J., Silvestri G., Russell S. R., Klaver C. C., Barbazetto I., Chang S., Yannuzzi L. A., Barile G. R., Merriam J. C., Smith R. T., Olsh A. K., Bergeron J., Zernant J., Merriam J. E., Gold B., Dean M. and Allikmets R., 2005. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A* 102, 7227-32.

Haines J. L., Hauser M. A., Schmidt S., Scott W. K., Olson L. M., Gallins P., Spencer K. L., Kwan S. Y., Noureddine M., Gilbert J. R., Schnetz-Boutaud N., Agarwal A., Postel E. A. and Pericak-Vance M. A., 2005. Complement factor H variant increases the risk of age-related macular degeneration. *Science* 308, 419-21.

Hajishengallis G., Sharma A., Russell M. and Genco R., 2002. Interactions of oral pathogens with toll-like receptors: possible role in atherosclerosis. *Ann Periodontol* 7, 72-8.

Haley K., Lilly C., Yang J., Feng Y., Kennedy S., Turi T., Thompson J., Sukhova G., Libby P. and Lee R., 2000. Overexpression of eotaxin and the CCR3 receptor in human atherosclerosis: using genomic technology to identify a potential novel pathway of vascular inflammation. *Circulation* 102, 2185-9.

Halperin J. A., Taratuska A. and Nicholson-Weller A., 1993. Terminal complement complex C5b-9 stimulates mitogenesis in 3T3 cells. *J Clin Invest* 91, 1974-8.

Hamasaki S., Higano S. T., Suwaidi J. A., Nishimura R. A., Miyauchi K., Holmes D. R. and Lerman A., 2000. Cholesterol-lowering treatment is associated with improvement in coronary vascular remodeling and endothelial function in patients with normal or mildly diseased coronary arteries. *Arterioscler Thromb Vasc Biol* 20, 737-43.

Hamdy O., 2005. The role of adipose tissue as an endocrine gland. *Curr Diab Rep* 5, 317-9.

Hansson G., Hellstrand M., Rymo L., Rubbia L. and Gabbiani G., 1989a. Interferon gamma inhibits both proliferation and expression of differentiation-specific alpha-smooth muscle actin in arterial smooth muscle cells. *J Exp Med* 170, 1595-608.

- Hansson G., Holm J. and Jonasson L., 1989b. Detection of activated T lymphocytes in the human atherosclerotic plaque. *Am J Pathol* 135, 169-75.
- Hansson G. and Libby P., 2006. The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol* 6, 508-19.
- Hansson G., Libby P., Schönbeck U. and Yan Z., 2002. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res* 91, 281-91.
- Harris C., Rushmere N. and Morgan B., 1999. Molecular and functional analysis of mouse decay accelerating factor (CD55). *Biochem J* 341 (Pt 3), 821-9.
- Hartmann K., Henz B., Krüger-Krasagakes S., Köhl J., Burger R., Guhl S., Haase I., Lippert U. and Zuberbier T., 1997. C3a and C5a stimulate chemotaxis of human mast cells. *Blood* 89, 2863-70.
- Hattori R., Hamilton K., McEver R. and Sims P., 1989. Complement proteins C5b-9 induce secretion of high molecular weight multimers of endothelial von Willebrand factor and translocation of granule membrane protein GMP-140 to the cell surface. *J Biol Chem* 264, 9053-60.
- Havel R., Kane J. and Kashyap M., 1973. Interchange of apolipoproteins between chylomicrons and high density lipoproteins during alimentary lipemia in man. *J Clin Invest* 52, 32-8.
- Hawlich H. and Köhl J., 2006. Complement and Toll-like receptors: key regulators of adaptive immune responses. *Mol Immunol* 43, 13-21.
- Hegele R., 2009. Plasma lipoproteins: genetic influences and clinical implications. *Nat Rev Genet* 10, 109-21.
- Hegele R. A., Ban M. R., Anderson C. M. and Spence J. D., 2000. Infection-susceptibility alleles of mannose-binding lectin are associated with increased carotid plaque area. *J Investig Med* 48, 198-202.

- Henn V., Slupsky J., Gräfe M., Anagnostopoulos I., Förster R., Müller-Berghaus G. and Kroczeck R., 1998. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 391, 591-4.
- Henney A., Wakeley P., Davies M., Foster K., Hembry R., Murphy G. and Humphries S., 1991. Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization. *Proc Natl Acad Sci U S A* 88, 8154-8.
- Heurich M., Martinez-Barricarte, Nigel F., Dawn R., Rodriguez de Cordoba S., Morgan B. P. and Harris C., 2011. Common polymorphisms in C3, factor B and factor H collaborate to determine systemic complement activity and disease risk. Manuscript in review.
- Hoffmeister H. M., Ehlers R., Büttcher E., Kazmaier S., Szabo S., Beyer M. E., Steinmetz A. and Seipel L., 2002. Comparison of C-reactive protein and terminal complement complex in patients with unstable angina pectoris versus stable angina pectoris. *Am J Cardiol* 89, 909-12.
- Hokanson J. and Austin M., 1996. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. *J Cardiovasc Risk* 3, 213-9.
- Hollander W., Colombo M., Kirkpatrick B. and Paddock J., 1979. Soluble proteins in the human atherosclerotic plaque. With spectral reference to immunoglobulins, C3-complement component, alpha 1-antitrypsin and alpha 2-macroglobulin. *Atherosclerosis* 34, 391-405.
- Holt D., Botto M., Bygrave A., Hanna S., Walport M. and Morgan B., 2001. Targeted deletion of the CD59 gene causes spontaneous intravascular hemolysis and hemoglobinuria. *Blood* 98, 442-9.

- Hosono M., de Boer O., van der Wal A., van der Loos C., Teeling P., Piek J., Ueda M. and Becker A., 2003. Increased expression of T cell activation markers (CD25, CD26, CD40L and CD69) in atherectomy specimens of patients with unstable angina and acute myocardial infarction. *Atherosclerosis* 168, 73-80.
- Hristov M. and Weber C., 2009. Progenitor cell trafficking in the vascular wall. *J Thromb Haemost* 7 Suppl 1, 31-4.
- Huang J., Welch J., Ricote M., Binder C., Willson T., Kelly C., Witztum J., Funk C., Conrad D. and Glass C., 1999. Interleukin-4-dependent production of PPAR-gamma ligands in macrophages by 12/15-lipoxygenase. *Nature* 400, 378-82.
- Hurtado Del Pozo C., Calvo R., Vesperinas-García G., Gómez-Ambrosi J., Frühbeck G., Rubio M. and Obregon M., 2010. Expression Profile in Omental and Subcutaneous Adipose Tissue from Lean and Obese Subjects. Repression of Lipolytic and Lipogenic Genes. *Obes Surg.*
- Huugen D., van Esch A., Xiao H., Peutz-Kootstra C., Buurman W., Tervaert J., Jennette J. and Heeringa P., 2007. Inhibition of complement factor C5 protects against anti-myeloperoxidase antibody-mediated glomerulonephritis in mice. *Kidney Int* 71, 646-54.
- Hänsch G., Seitz M., Martinotti G., Betz M., Rauterberg E. and Gemsa D., 1984. Macrophages release arachidonic acid, prostaglandin E2, and thromboxane in response to late complement components. *J Immunol* 133, 2145-50.
- Ikeda K., Nagasawa K., Horiuchi T., Tsuru T., Nishizaka H. and Niho Y., 1997. C5a induces tissue factor activity on endothelial cells. *Thromb Haemost* 77, 394-8.
- Inwald D. P., McDowall A., Peters M. J., Callard R. E. and Klein N. J., 2003. CD40 is constitutively expressed on platelets and provides a novel mechanism for platelet activation. *Circ Res* 92, 1041-8.

- Ishibashi S., Brown M., Goldstein J., Gerard R., Hammer R. and Herz J., 1993.
Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest* 92, 883-93.
- Ishibashi S., Herz J., Maeda N., Goldstein J. and Brown M., 1994. The two-receptor model of lipoprotein clearance: tests of the hypothesis in "knockout" mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proc Natl Acad Sci U S A* 91, 4431-5.
- Jackson C., 2007. Is there life after plaque rupture? *Biochem Soc Trans* 35, 887-9.
- Jala V. and Haribabu B., 2004. Leukotrienes and atherosclerosis: new roles for old mediators. *Trends Immunol* 25, 315-22.
- Janeway C. J. and Medzhitov R., 2002. Innate immune recognition. *Annu Rev Immunol* 20, 197-216.
- Jawień J., Nastalek P. and Korbut R., 2004. Mouse models of experimental atherosclerosis. *J Physiol Pharmacol* 55, 503-17.
- Ji H., Ohmura K., Mahmood U., Lee D., Hofhuis F., Boackle S., Takahashi K., Holers V., Walport M., Gerard C., Ezekowitz A., Carroll M., Brenner M., Weissleder R., Verbeek J., Duchatelle V., Degott C., Benoist C. and Mathis D., 2002. Arthritis critically dependent on innate immune system players. *Immunity* 16, 157-68.
- Ji S., Wu Y., Potempa L., Liang Y. and Zhao J., 2006. Effect of modified C-reactive protein on complement activation: a possible complement regulatory role of modified or monomeric C-reactive protein in atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 26, 935-41.

- Jiang H., Cooper B., Robey F. and Gewurz H., 1992. DNA binds and activates complement via residues 14-26 of the human C1q A chain. *J Biol Chem* 267, 25597-601.
- Johnson J., George S., Newby A. and Jackson C., 2005. Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. *Proc Natl Acad Sci U S A* 102, 15575-80.
- Johnson J. and Jackson C., 2001. Atherosclerotic plaque rupture in the apolipoprotein E knockout mouse. *Atherosclerosis* 154, 399-406.
- Johswich K., Martin M., Thalmann J., Rheinheimer C., Monk P. and Klos A., 2006. Ligand specificity of the anaphylatoxin C5L2 receptor and its regulation on myeloid and epithelial cell lines. *J Biol Chem* 281, 39088-95.
- Jolley C. D., Dietschy J. M. and Turley S. D., 1999. Genetic differences in cholesterol absorption in 129/Sv and C57BL/6 mice: effect on cholesterol responsiveness. *Am J Physiol* 276, G1117-24.
- Jonasson L., Holm J., Skalli O., Bondjers G. and Hansson G., 1986. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis* 6, 131-8.
- Joven J., Rull A., Ferré N., Escolà-Gil J., Marsillach J., Coll B., Alonso-Villaverde C., Aragonés G., Claria J. and Camps J., 2007. The results in rodent models of atherosclerosis are not interchangeable: the influence of diet and strain. *Atherosclerosis* 195, e85-92.
- Jylhävä J., Eklund C., Pessi T., Raitakari O. T., Juonala M., Kähönen M., Viikari J. S., Lehtimäki T. and Hurme M., 2009. Genetics of C-reactive protein and complement factor H have an epistatic effect on carotid artery compliance: the Cardiovascular Risk in Young Finns Study. *Clin Exp Immunol* 155, 53-8.

Jönsson G., Truedsson L., Sturfelt G., Oxelius V., Braconier J. and Sjöholm A., 2005.

Hereditary C2 deficiency in Sweden: frequent occurrence of invasive infection, atherosclerosis, and rheumatic disease. *Medicine (Baltimore)* 84, 23-34.

Kalant D., Cain S., Maslowska M., Sniderman A., Cianflone K. and Monk P., 2003.

The chemoattractant receptor-like protein C5L2 binds the C3a des-Arg77/acylation-stimulating protein. *J Biol Chem* 278, 11123-9.

Kalant D., MacLaren R., Cui W., Samanta R., Monk P., Laporte S. and Cianflone K.,

2005. C5L2 is a functional receptor for acylation-stimulating protein. *J Biol Chem* 280, 23936-44.

Kaneto H., Katakami N., Matsuhisa M. and Matsuoka T., 2010. Role of reactive

oxygen species in the progression of type 2 diabetes and atherosclerosis. *Mediators Inflamm* 2010, 453892.

Kardys I., Klaver C. C., Despriet D. D., Bergen A. A., Uitterlinden A. G., Hofman A.,

Oostra B. A., Van Duijn C. M., de Jong P. T. and Witteman J. C., 2006. A common polymorphism in the complement factor H gene is associated with increased risk of myocardial infarction: the Rotterdam Study. *J Am Coll Cardiol* 47, 1568-75.

Karpe F., Steiner G., Uffelman K., Olivecrona T. and Hamsten A., 1994. Postprandial

lipoproteins and progression of coronary atherosclerosis. *Atherosclerosis* 106, 83-97.

Kastl S., Speidl W., Kaun C., Katsaros K., Rega G., Afonyushkin T., Bochkov V.,

Valent P., Assadian A., Hagmueller G., Hoeth M., de Martin R., Ma Y., Maurer G., Huber K. and Wojta J., 2008. In human macrophages the

complement component C5a induces the expression of oncostatin M via AP-1 activation. *Arterioscler Thromb Vasc Biol* 28, 498-503.

Kastl S., Speidl W., Kaun C., Rega G., Assadian A., Weiss T., Valent P., Hagmueller G., Maurer G., Huber K. and Wojta J., 2006. The complement component C5a induces the expression of plasminogen activator inhibitor-1 in human macrophages via NF-kappaB activation. *J Thromb Haemost* 4, 1790-7.

Kawakami A., Tanaka A., Nakajima K., Shimokado K. and Yoshida M., 2002. Atorvastatin attenuates remnant lipoprotein-induced monocyte adhesion to vascular endothelium under flow conditions. *Circ Res* 91, 263-71.

Kawakami A., Tani M., Chiba T., Yui K., Shinozaki S., Nakajima K., Tanaka A., Shimokado K. and Yoshida M., 2005. Pitavastatin inhibits remnant lipoprotein-induced macrophage foam cell formation through ApoB48 receptor-dependent mechanism. *Arterioscler Thromb Vasc Biol* 25, 424-9.

Khakpour H. and Frishman W., 2009. Lipoprotein-associated phospholipase A2: an independent predictor of cardiovascular risk and a novel target for immunomodulation therapy. *Cardiol Rev* 17, 222-9.

Kilgore K., Schmid E., Shanley T., Flory C., Maheswari V., Tramontini N., Cohen H., Ward P., Friedl H. and Warren J., 1997. Sublytic concentrations of the membrane attack complex of complement induce endothelial interleukin-8 and monocyte chemoattractant protein-1 through nuclear factor-kappa B activation. *Am J Pathol* 150, 2019-31.

Kilgore K., Shen J., Miller B., Ward P. and Warren J., 1995. Enhancement by the complement membrane attack complex of tumor necrosis factor-alpha-induced endothelial cell expression of E-selectin and ICAM-1. *J Immunol* 155, 1434-41.

- Kinderlerer A., Steinberg R., Johns M., Harten S., Lidington E., Haskard D., Maxwell P. and Mason J., 2006. Statin-induced expression of CD59 on vascular endothelium in hypoxia: a potential mechanism for the anti-inflammatory actions of statins in rheumatoid arthritis. *Arthritis Res Ther* 8, R130.
- Klein R. J., Zeiss C., Chew E. Y., Tsai J. Y., Sackler R. S., Haynes C., Henning A. K., SanGiovanni J. P., Mane S. M., Mayne S. T., Bracken M. B., Ferris F. L., Ott J., Barnstable C. and Hoh J., 2005. Complement factor H polymorphism in age-related macular degeneration. *Science* 308, 385-9.
- Klos A., Tenner A., Johswich K., Ager R., Reis E. and Köhl J., 2009. The role of the anaphylatoxins in health and disease. *Mol Immunol* 46, 2753-66.
- Koenig W. and Khuseyinova N., 2007. Biomarkers of atherosclerotic plaque instability and rupture. *Arterioscler Thromb Vasc Biol* 27, 15-26.
- Korb L. and Ahearn J., 1997. C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. *J Immunol* 158, 4525-8.
- Kotti T., Ramirez D., Pfeiffer B., Huber K. and Russell D., 2006. Brain cholesterol turnover required for geranylgeraniol production and learning in mice. *Proc Natl Acad Sci U S A* 103, 3869-74.
- Kramer J., Rajczy K., Hegyi L., Fülöp T., Mohácsi A., Mezei Z., Keltai M., Blaskó G., Ferenczy E. and Anh-Tuan N., 1994. C4B*Q0 allotype as risk factor for myocardial infarction. *BMJ* 309, 313-4.
- Krinsky G. A., Freedberg R., Lee V. S., Rockman C. and Tunick P. A., 2001. Innominate artery atheroma: a lesion seen with gadolinium-enhanced MR angiography and often missed by transesophageal echocardiography. *Clin Imaging* 25, 251-7.

- Kristensen B. O. and Petersen G. B., 1978. Association between coronary heart disease and the C3F-gene in essential hypertension. *Circulation* 58, 622-5.
- Köhl J., 2001. Anaphylatoxins and infectious and non-infectious inflammatory diseases. *Mol Immunol* 38, 175-87.
- Lafontan M., 2008. Advances in adipose tissue metabolism. *Int J Obes (Lond)* 32 Suppl 7, S39-51.
- Lawrence T. and Gilroy D., 2007. Chronic inflammation: a failure of resolution? *Int J Exp Pathol* 88, 85-94.
- Lee D., Friend D., Gurish M., Benoist C., Mathis D. and Brenner M., 2002. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science* 297, 1689-92.
- Lee M., Kim W., Kang Y., Jung Y., Kang Y., Suk K., Park J., Choi E., Choi B., Kwon B. and Lee W., 2006. Z39Ig is expressed on macrophages and may mediate inflammatory reactions in arthritis and atherosclerosis. *J Leukoc Biol* 80, 922-8.
- Lefvert A. K., Hamsten A. and Holm G., 1995. Association between circulating immune complexes, complement C4 null alleles, and myocardial infarction before age 45 years. *Arterioscler Thromb Vasc Biol* 15, 665-8.
- Lesnik P., Haskell C. and Charo I., 2003. Decreased atherosclerosis in CX3CR1^{-/-} mice reveals a role for fractalkine in atherogenesis. *J Clin Invest* 111, 333-40.
- Lett-Brown M. and Leonard E., 1977. Histamine-induced inhibition of normal human basophil chemotaxis to C5a. *J Immunol* 118, 815-8.
- Leung V., Yun S., Botto M., Mason J., Malik T., Song W., Paixao-Cavalcante D., Pickering M., Boyle J. and Haskard D., 2009. Decay-accelerating factor

suppresses complement C3 activation and retards atherosclerosis in low-density lipoprotein receptor-deficient mice. *Am J Pathol* 175, 1757-67.

Lewis R., Jackson C., Morgan B. and Hughes T., 2007. A role for CD59a in the progression of atherosclerosis: The first direct evidence from an in vivo study. *Molecular Immunology* 44, 3939-3939.

Lewis R. D., Jackson C. L., Morgan B. P. and Hughes T. R., 2010. The membrane attack complex of complement drives the progression of atherosclerosis in apolipoprotein E knockout mice. *Mol Immunol* 47, 1098-105.

Li B., Sallee C., Dehoff M., Foley S., Molina H. and Holers V., 1993. Mouse Crry/p65. Characterization of monoclonal antibodies and the tissue distribution of a functional homologue of human MCP and DAF. *J Immunol* 151, 4295-305.

Li S., Szmitko P., Weisel R., Wang C., Fedak P., Li R., Mickle D. and Verma S., 2004. C-reactive protein upregulates complement-inhibitory factors in endothelial cells. *Circulation* 109, 833-6.

Liao F., Andalibi A., deBeer F., Fogelman A. and Lusis A., 1993. Genetic control of inflammatory gene induction and NF-kappa B-like transcription factor activation in response to an atherogenic diet in mice. *J Clin Invest* 91, 2572-9.

Libby P. and Aikawa M., 2002. Stabilization of atherosclerotic plaques: new mechanisms and clinical targets. *Nat Med* 8, 1257-62.

Libby P., Aikawa M. and Jain M., 2006. Vascular endothelium and atherosclerosis. *Handb Exp Pharmacol*, 285-306.

Libby P., Ridker P. and Hansson G., 2009. Inflammation in atherosclerosis: from pathophysiology to practice. *J Am Coll Cardiol* 54, 2129-38.

- Lidington E., Haskard D. and Mason J., 2000. Induction of decay-accelerating factor by thrombin through a protease-activated receptor 1 and protein kinase C-dependent pathway protects vascular endothelial cells from complement-mediated injury. *Blood* 96, 2784-92.
- Lin F., Fukuoka Y., Spicer A., Ohta R., Okada N., Harris C., Emancipator S. and Medof M., 2001. Tissue distribution of products of the mouse decay-accelerating factor (DAF) genes. Exploitation of a Daf1 knock-out mouse and site-specific monoclonal antibodies. *Immunology* 104, 215-25.
- Lindstedt K., Mäyränpää M. and Kovanen P., 2007. Mast cells in vulnerable atherosclerotic plaques--a view to a kill. *J Cell Mol Med* 11, 739-58.
- Linton S. and Morgan B., 1999. Complement activation and inhibition in experimental models of arthritis. *Mol Immunol* 36, 905-14.
- Liszewski M., Post T. and Atkinson J., 1991. Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. *Annu Rev Immunol* 9, 431-55.
- Liu J., Thewke D., Su Y., Linton M., Fazio S. and Sinensky M., 2005. Reduced macrophage apoptosis is associated with accelerated atherosclerosis in low-density lipoprotein receptor-null mice. *Arterioscler Thromb Vasc Biol* 25, 174-9.
- Llodrá J., Angeli V., Liu J., Trogan E., Fisher E. and Randolph G., 2004. Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proc Natl Acad Sci U S A* 101, 11779-84.

- Longhi M., Sivasankar B., Omidvar N., Morgan B. and Gallimore A., 2005. Cutting edge: murine CD59a modulates antiviral CD4+ T cell activity in a complement-independent manner. *J Immunol* 175, 7098-102.
- Lublin D. and Atkinson J., 1989. Decay-accelerating factor: biochemistry, molecular biology, and function. *Annu Rev Immunol* 7, 35-58.
- Lusis A., Fogelman A. and Fonarow G., 2004a. Genetic basis of atherosclerosis: part I: new genes and pathways. *Circulation* 110, 1868-73.
- Lusis A., Mar R. and Pajukanta P., 2004b. Genetics of atherosclerosis. *Annu Rev Genomics Hum Genet* 5, 189-218.
- Lutgens E., van Suylen R. J., Faber B. C., Gijbels M. J., Eurlings P. M., Bijmens A. P., Cleutjens K. B., Heeneman S. and Daemen M. J., 2003. Atherosclerotic plaque rupture: local or systemic process? *Arterioscler Thromb Vasc Biol* 23, 2123-30.
- Mackness B., Hunt R., Durrington P. and Mackness M., 1997. Increased immunolocalization of paraoxonase, clusterin, and apolipoprotein A-I in the human artery wall with the progression of atherosclerosis. *Arterioscler Thromb Vasc Biol* 17, 1233-8.
- MacLaren R., Cui W. and Cianflone K., 2008. Adipokines and the immune system: an adipocentric view. *Adv Exp Med Biol* 632, 1-21.
- Madsen H., Videm V., Svejgaard A., Svennevig J. and Garred P., 1998. Association of mannose-binding-lectin deficiency with severe atherosclerosis. *Lancet* 352, 959-60.
- Malik T. H., Cortini A., Carassiti D., Boyle J. J., Haskard D. O. and Botto M., 2010. The alternative pathway is critical for pathogenic complement activation in

endotoxin- and diet-induced atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation* 122, 1948-56.

Mamane Y., Chung Chan C., Lavallee G., Morin N., Xu L. J., Huang J., Gordon R., Thomas W., Lamb J., Schadt E. E., Kennedy B. P. and Mancini J. A., 2009. The C3a anaphylatoxin receptor is a key mediator of insulin resistance and functions by modulating adipose tissue macrophage infiltration and activation. *Diabetes* 58, 2006-17.

Manderson A., Botto M. and Walport M., 2004. The role of complement in the development of systemic lupus erythematosus. *Annu Rev Immunol* 22, 431-56.

Marchand F., 1904. Ueber Atherosclerosis, Vol. 21 Kongresse.

Markiewski M. and Lambris J., 2007. The role of complement in inflammatory diseases from behind the scenes into the spotlight. *Am J Pathol* 171, 715-27.

Maron R., Sukhova G., Faria A., Hoffmann E., Mach F., Libby P. and Weiner H., 2002. Mucosal administration of heat shock protein-65 decreases atherosclerosis and inflammation in aortic arch of low-density lipoprotein receptor-deficient mice. *Circulation* 106, 1708-15.

Maslowska M., Legakis H., Assadi F. and Cianflone K., 2006. Targeting the signaling pathway of acylation stimulating protein. *J Lipid Res* 47, 643-52.

Maslowska M., Scantlebury T., Germinario R. and Cianflone K., 1997a. Acute in vitro production of acylation stimulating protein in differentiated human adipocytes. *J Lipid Res* 38, 1-11.

Maslowska M., Sniderman A., Germinario R. and Cianflone K., 1997b. ASP stimulates glucose transport in cultured human adipocytes. *Int J Obes Relat Metab Disord* 21, 261-6.

- Maslowska M., Wang H. and Cianflone K., 2005. Novel roles for acylation stimulating protein/C3adesArg: a review of recent in vitro and in vivo evidence. *Vitam Horm* 70, 309-32.
- Mason J., Ahmed Z., Mankoff R., Lidington E., Ahmad S., Bhatia V., Kinderlerer A., Randi A. and Haskard D., 2002. Statin-induced expression of decay-accelerating factor protects vascular endothelium against complement-mediated injury. *Circ Res* 91, 696-703.
- Mason J., Lidington E., Yarwood H., Lublin D. and Haskard D., 2001. Induction of endothelial cell decay-accelerating factor by vascular endothelial growth factor: a mechanism for cytoprotection against complement-mediated injury during inflammatory angiogenesis. *Arthritis Rheum* 44, 138-50.
- Mason J., Yarwood H., Sugars K., Morgan B., Davies K. and Haskard D., 1999. Induction of decay-accelerating factor by cytokines or the membrane-attack complex protects vascular endothelial cells against complement deposition. *Blood* 94, 1673-82.
- Mathieson P. and Peters D., 1997. Lipodystrophy in MCGN type II: the clue to links between the adipocyte and the complement system. *Nephrol Dial Transplant* 12, 1804-6.
- Mathieson P., Würzner R., Oliveria D., Lachmann P. and Peters D., 1993. Complement-mediated adipocyte lysis by nephritic factor sera. *J Exp Med* 177, 1827-31.
- Matthijsen R., de Winther M., Kuipers D., van der Made I., Weber C., Herias M., Gijbels M. and Buurman W., 2009. Macrophage-specific expression of mannose-binding lectin controls atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation* 119, 2188-95.

- McAteer M., Schneider J., Clarke K., Neubauer S., Channon K. and Choudhury R., 2004. Quantification and 3D reconstruction of atherosclerotic plaque components in apolipoprotein E knockout mice using ex vivo high-resolution MRI. *Arterioscler Thromb Vasc Biol* 24, 2384-90.
- McCarey D., McInnes I., Madhok R., Hampson R., Scherbakov O., Ford I., Capell H. and Sattar N., 2004. Trial of Atorvastatin in Rheumatoid Arthritis (TARA): double-blind, randomised placebo-controlled trial. *Lancet* 363, 2015-21.
- McCurry K., Kooyman D., Diamond L., Byrne G., Martin M., Logan J. and Platt J., 1995. Human complement regulatory proteins in transgenic animals regulate complement activation in xenoperfused organs. *Transplant Proc* 27, 317-8.
- McEneny J., McMaster C., Trimble E. and Young I., 2002. Rapid isolation of VLDL subfractions: assessment of composition and susceptibility to copper-mediated oxidation. *J Lipid Res* 43, 824-31.
- McLean R. and Hoefnagel D., 1980. Partial lipodystrophy and familial C3 deficiency. *Hum Hered* 30, 149-54.
- Mead R., Neal J., Griffiths M., Linington C., Botto M., Lassmann H. and Morgan B., 2004. Deficiency of the complement regulator CD59a enhances disease severity, demyelination and axonal injury in murine acute experimental allergic encephalomyelitis. *Lab Invest* 84, 21-8.
- Megran D., Stiver H. and Bowie W., 1985. Complement activation and stimulation of chemotaxis by *Chlamydia trachomatis*. *Infect Immun* 49, 670-3.
- Meri S., Morgan B., Davies A., Daniels R., Olavesen M., Waldmann H. and Lachmann P., 1990. Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. *Immunology* 71, 1-9.

- Meri S., Waldmann H. and Lachmann P., 1991. Distribution of protectin (CD59), a complement membrane attack inhibitor, in normal human tissues. *Lab Invest* 65, 532-7.
- Meuwissen M., van der Wal A., Niessen H., Koch K., de Winter R., van der Loos C., Rittersma S., Chamuleau S., Tijssen J., Becker A. and Piek J., 2006. Colocalisation of intraplaque C reactive protein, complement, oxidised low density lipoprotein, and macrophages in stable and unstable angina and acute myocardial infarction. *J Clin Pathol* 59, 196-201.
- Mevorach D., Mascarenhas J. O., Gershov D. and Elkon K. B., 1998. Complement-dependent clearance of apoptotic cells by human macrophages. *J Exp Med* 188, 2313-20.
- Michelsen K., Wong M., Shah P., Zhang W., Yano J., Doherty T., Akira S., Rajavashisth T. and Arditì M., 2004. Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. *Proc Natl Acad Sci U S A* 101, 10679-84.
- Miller Y., Viriyakosol S., Binder C., Feramisco J., Kirkland T. and Witztum J., 2003. Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells. *J Biol Chem* 278, 1561-8.
- Miller Y., Viriyakosol S., Worrall D., Boullier A., Butler S. and Witztum J., 2005. Toll-like receptor 4-dependent and -independent cytokine secretion induced by minimally oxidized low-density lipoprotein in macrophages. *Arterioscler Thromb Vasc Biol* 25, 1213-9.

- Millonig G., Malcom G. and Wick G., 2002. Early inflammatory-immunological lesions in juvenile atherosclerosis from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY)-study. *Atherosclerosis* 160, 441-8.
- Monk P., Scola A., Madala P. and Fairlie D., 2007. Function, structure and therapeutic potential of complement C5a receptors. *Br J Pharmacol* 152, 429-48.
- Monsinjon T., Gasque P., Chan P., Ischenko A., Brady J. J. and Fontaine M. C., 2003. Regulation by complement C3a and C5a anaphylatoxins of cytokine production in human umbilical vein endothelial cells. *FASEB J* 17, 1003-14.
- Montes T., Tortajada A., Morgan B. P., Rodríguez de Córdoba S. and Harris C. L., 2009. Functional basis of protection against age-related macular degeneration conferred by a common polymorphism in complement factor B. *Proc Natl Acad Sci U S A* 106, 4366-71.
- Moreno P., Falk E., Palacios I., Newell J., Fuster V. and Fallon J., 1994. Macrophage infiltration in acute coronary syndromes. Implications for plaque rupture. *Circulation* 90, 775-8.
- Morgan B., Chamberlain-Banoub J., Neal J., Song W., Mizuno M. and Harris C., 2006. The membrane attack pathway of complement drives pathology in passively induced experimental autoimmune myasthenia gravis in mice. *Clin Exp Immunol* 146, 294-302.
- Morgan B., Dankert J. and Esser A., 1987. Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis. *J Immunol* 138, 246-53.

- Morgan B. P., Campbell A. K. and Compston D. A., 1984. Terminal component of complement (C9) in cerebrospinal fluid of patients with multiple sclerosis. *Lancet* 2, 251-4.
- Morley B. and Walport M., 2000. *The Complement Facts Book*. Academic Press, London.
- Mosca L., 2002. C-reactive protein--to screen or not to screen? *N Engl J Med* 347, 1615-7.
- Mosorin M., Surcel H., Laurila A., Lehtinen M., Karttunen R., Juvonen J., Paavonen J., Morrison R., Saikku P. and Juvonen T., 2000. Detection of Chlamydia pneumoniae-reactive T lymphocytes in human atherosclerotic plaques of carotid artery. *Arterioscler Thromb Vasc Biol* 20, 1061-7.
- Mosser D. and Zhang X., 2008. Interleukin-10: new perspectives on an old cytokine. *Immunol Rev* 226, 205-18.
- Murray I., Havel P., Sniderman A. and Cianflone K., 2000. Reduced body weight, adipose tissue, and leptin levels despite increased energy intake in female mice lacking acylation-stimulating protein. *Endocrinology* 141, 1041-9.
- Murray I., Sniderman A. and Cianflone K., 1999a. Mice lacking acylation stimulating protein (ASP) have delayed postprandial triglyceride clearance. *J Lipid Res* 40, 1671-6.
- Murray I., Sniderman A., Havel P. and Cianflone K., 1999b. Acylation stimulating protein (ASP) deficiency alters postprandial and adipose tissue metabolism in male mice. *J Biol Chem* 274, 36219-25.
- Nakajima M., Yasaka M. and Minematsu K., 2008. Mobile thrombus from a ruptured plaque in the brachiocephalic artery. *J Stroke Cerebrovasc Dis* 17, 423-5.

- Nakamura K., Sasaki T., Cheng X., Iguchi A., Sato K. and Kuzuya M., 2009. Statin prevents plaque disruption in apoE-knockout mouse model through pleiotropic effect on acute inflammation. *Atherosclerosis* 206, 355-61.
- Nakashima Y., Plump A., Raines E., Breslow J. and Ross R., 1994. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler Thromb* 14, 133-40.
- Nakashima Y., Raines E., Plump A., Breslow J. and Ross R., 1998. Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. *Arterioscler Thromb Vasc Biol* 18, 842-51.
- Naruko T., Ueda M., Haze K., van der Wal A., van der Loos C., Itoh A., Komatsu R., Ikura Y., Ogami M., Shimada Y., Ehara S., Yoshiyama M., Takeuchi K., Yoshikawa J. and Becker A., 2002. Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation* 106, 2894-900.
- Nataf S., Davoust N., Ames R. and Barnum S., 1999. Human T cells express the C5a receptor and are chemoattracted to C5a. *J Immunol* 162, 4018-23.
- Nauta A., Daha M., Tijmsma O., van de Water B., Tedesco F. and Roos A., 2002a. The membrane attack complex of complement induces caspase activation and apoptosis. *Eur J Immunol* 32, 783-92.
- Nauta A., Daha M., van Kooten C. and Roos A., 2003. Recognition and clearance of apoptotic cells: a role for complement and pentraxins. *Trends Immunol* 24, 148-54.
- Nauta A., Roos A. and Daha M., 2004. A regulatory role for complement in innate immunity and autoimmunity. *Int Arch Allergy Immunol* 134, 310-23.
- Nauta A., Trouw L., Daha M., Tijmsma O., Nieuwland R., Schwaeble W., Gingras A., Mantovani A., Hack E. and Roos A., 2002b. Direct binding of C1q to

apoptotic cells and cell blebs induces complement activation. *Eur J Immunol* 32, 1726-36.

Navratil J., Watkins S., Wisnieski J. and Ahearn J., 2001. The globular heads of C1q specifically recognize surface blebs of apoptotic vascular endothelial cells. *J Immunol* 166, 3231-9.

Nicholson-Weller A., Burge J., Fearon D., Weller P. and Austen K., 1982. Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. *J Immunol* 129, 184-9.

Nicoletti A., Caligiuri G., Törnberg I., Kodama T., Stemme S. and Hansson G., 1999. The macrophage scavenger receptor type A directs modified proteins to antigen presentation. *Eur J Immunol* 29, 512-21.

Niculescu F., Badea T. and Rus H., 1999. Sublytic C5b-9 induces proliferation of human aortic smooth muscle cells: role of mitogen activated protein kinase and phosphatidylinositol 3-kinase. *Atherosclerosis* 142, 47-56.

Niculescu F., Hugo F., Rus H., Vlaicu R. and Bhakdi S., 1987a. Quantitative evaluation of the terminal C5b-9 complement complex by ELISA in human atherosclerotic arteries. *Clin Exp Immunol* 69, 477-83.

Niculescu F., Niculescu T. and Rus H., 2004. C5b-9 terminal complement complex assembly on apoptotic cells in human arterial wall with atherosclerosis. *Exp Mol Pathol* 76, 17-23.

Niculescu F. and Rus H., 2004. The role of complement activation in atherosclerosis. *Immunol Res* 30, 73-80.

Niculescu F., Rus H. and Vlaicu R., 1987b. Immunohistochemical localization of C5b-9, S-protein, C3d and apolipoprotein B in human arterial tissues with atherosclerosis. *Atherosclerosis* 65, 1-11.

- Niculescu F., Rus H. and Vlaicu R., 1990. Decay-accelerating factor regulates complement-mediated damage in the human atherosclerotic wall. *Immunol Lett* 26, 17-23.
- Nilsson J. and Hansson G., 2008. Autoimmunity in atherosclerosis: a protective response losing control? *J Intern Med* 263, 464-78.
- Nishina P., Lowe S., Wang J. and Paigen B., 1994a. Characterization of plasma lipids in genetically obese mice: the mutants obese, diabetes, fat, tubby, and lethal yellow. *Metabolism* 43, 549-53.
- Nishina P., Naggert J., Verstuyft J. and Paigen B., 1994b. Atherosclerosis in genetically obese mice: the mutants obese, diabetes, fat, tubby, and lethal yellow. *Metabolism* 43, 554-8.
- Nityanand S., Truedsson L., Mustafa A., Bergmark C. and Lefvert A. K., 1999. Circulating immune complexes and complement C4 null alleles in patients in patients operated on for premature atherosclerotic peripheral vascular disease. *J Clin Immunol* 19, 406-13.
- Nordestgaard B., 2009. Does elevated C-reactive protein cause human atherothrombosis? Novel insights from genetics, intervention trials, and elsewhere. *Curr Opin Lipidol* 20, 393-401.
- Ohashi K., Burkart V., Flohé S. and Kolb H., 2000. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol* 164, 558-61.
- Oksjoki R., Jarva H., Kovanen P., Laine P., Meri S. and Pentikäinen M., 2003. Association between complement factor H and proteoglycans in early human coronary atherosclerotic lesions: implications for local regulation of complement activation. *Arterioscler Thromb Vasc Biol* 23, 630-6.

- Oksjoki R., Kovanen P., Meri S. and Pentikainen M., 2007a. Function and regulation of the complement system in cardiovascular diseases. *Front Biosci* 12, 4696-708.
- Oksjoki R., Kovanen P., Mäyränpää M., Laine P., Blom A., Meri S. and Pentikäinen M., 2007b. Complement regulation in human atherosclerotic coronary lesions. Immunohistochemical evidence that C4b-binding protein negatively regulates the classical complement pathway, and that C5b-9 is formed via the alternative complement pathway. *Atherosclerosis* 192, 40-8.
- Oksjoki R., Laine P., Helske S., Vehmaan-Kreula P., Mäyränpää M., Gasque P., Kovanen P. and Pentikäinen M., 2007c. Receptors for the anaphylatoxins C3a and C5a are expressed in human atherosclerotic coronary plaques. *Atherosclerosis* 195, 90-9.
- Okusawa S., Dinarello C. A., Yancey K. B., Endres S., Lawley T. J., Frank M. M., Burke J. F. and Gelfand J. A., 1987. C5a induction of human interleukin 1. Synergistic effect with endotoxin or interferon-gamma. *J Immunol* 139, 2635-40.
- Okusawa S., Yancey K., van der Meer J., Endres S., Lonnemann G., Hefter K., Frank M., Burke J., Dinarello C. and Gelfand J., 1988. C5a stimulates secretion of tumor necrosis factor from human mononuclear cells in vitro. Comparison with secretion of interleukin 1 beta and interleukin 1 alpha. *J Exp Med* 168, 443-8.
- Orren A., Hayakawa J., Johnson J., Nash H. and Hobart M., 1988. Allotypes of mouse complement component C6 in inbred strains and some wild populations. *Immunogenetics* 28, 153-7.

- Ottonello L., Corcione A., Tortolina G., Airoidi I., Albesiano E., Favre A., D'Agostino R., Malavasi F., Pistoia V. and Dallegri F., 1999. rC5a directs the in vitro migration of human memory and naive tonsillar B lymphocytes: implications for B cell trafficking in secondary lymphoid tissues. *J Immunol* 162, 6510-7.
- Packard R. R., Lichtman A. H. and Libby P., 2009. Innate and adaptive immunity in atherosclerosis. *Semin Immunopathol* 31, 5-22.
- Paglialunga S., Fisette A., Munkonda M., Gao Y., Richard D. and Cianflone K., 2010. The effects of acylation stimulating protein supplementation VS antibody neutralization on energy expenditure in wildtype mice. *BMC Physiol* 10, 4.
- Paglialunga S., Fisette A., Yan Y., Deshaies Y., Brouillette J., Pekna M. and Cianflone K., 2008. Acylation-stimulating protein deficiency and altered adipose tissue in alternative complement pathway knockout mice. *Am J Physiol Endocrinol Metab* 294, E521-9.
- Paglialunga S., Schrauwen P., Roy C., Moonen-Kornips E., Lu H., Hesselink M., Deshaies Y., Richard D. and Cianflone K., 2007. Reduced adipose tissue triglyceride synthesis and increased muscle fatty acid oxidation in C5L2 knockout mice. *J Endocrinol* 194, 293-304.
- Pai J. K., Manson J. E., Rexrode K. M., Albert C. M., Hunter D. J. and Rimm E. B., 2007. Complement factor H (Y402H) polymorphism and risk of coronary heart disease in US men and women. *Eur Heart J* 28, 1297-303.
- Paigen B., Morrow A., Brandon C., Mitchell D. and Holmes P., 1985. Variation in susceptibility to atherosclerosis among inbred strains of mice. *Atherosclerosis* 57, 65-73.

- Palinski W., Hörkkö S., Miller E., Steinbrecher U., Powell H., Curtiss L. and Witztum J., 1996. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. *J Clin Invest* 98, 800-14.
- Palinski W., Miller E. and Witztum J., 1995. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc Natl Acad Sci U S A* 92, 821-5.
- Pang A., Katz A. and Minta J., 1979. C3 deposition in cholesterol-induced atherosclerosis in rabbits: a possible etiologic role for complement in atherogenesis. *J Immunol* 123, 1117-22.
- Patel S., Thelander E., Hernandez M., Montenegro J., Hassing H., Burton C., Mundt S., Hermanowski-Vosatka A., Wright S., Chao Y. and Detmers P., 2001. ApoE(-/-) mice develop atherosclerosis in the absence of complement component C5. *Biochem Biophys Res Commun* 286, 164-70.
- Peiser L., Mukhopadhyay S. and Gordon S., 2002. Scavenger receptors in innate immunity. *Curr Opin Immunol* 14, 123-8.
- Persson L., Borén J., Nicoletti A., Hansson G. and Pekna M., 2005. Immunoglobulin treatment reduces atherosclerosis in apolipoprotein E-/- low-density lipoprotein receptor-/- mice via the complement system. *Clin Exp Immunol* 142, 441-5.
- Persson L., Borén J., Robertson A., Wallenius V., Hansson G. and Pekna M., 2004. Lack of complement factor C3, but not factor B, increases hyperlipidemia and atherosclerosis in apolipoprotein E-/- low-density lipoprotein receptor-/- mice. *Arterioscler Thromb Vasc Biol* 24, 1062-7.

- Phipps R., 2000. Atherosclerosis: the emerging role of inflammation and the CD40-CD40 ligand system. *Proc Natl Acad Sci U S A* 97, 6930-2.
- Pickering M., Botto M., Taylor P., Lachmann P. and Walport M., 2000. Systemic lupus erythematosus, complement deficiency, and apoptosis. *Adv Immunol* 76, 227-324.
- Piedrahita J., Zhang S., Hagan J., Oliver P. and Maeda N., 1992. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proc Natl Acad Sci U S A* 89, 4471-5.
- Pinckard R., Olson M., Giclas P., Terry R., Boyer J. and O'Rourke R., 1975. Consumption of classical complement components by heart subcellular membranes in vitro and in patients after acute myocardial infarction. *J Clin Invest* 56, 740-50.
- Platt J. L., Dalmaso A. P., Lindman B. J., Ihrcke N. S. and Bach F. H., 1991. The role of C5a and antibody in the release of heparan sulfate from endothelial cells. *Eur J Immunol* 21, 2887-90.
- Plump A., Smith J., Hayek T., Aalto-Setälä K., Walsh A., Verstuyft J., Rubin E. and Breslow J., 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 71, 343-53.
- Pomeroy C., Mitchell J., Eckert E., Raymond N., Crosby R. and Dalmaso A., 1997. Effect of body weight and caloric restriction on serum complement proteins, including Factor D/adipsin: studies in anorexia nervosa and obesity. *Clin Exp Immunol* 108, 507-15.

- Poole J. and Florey H., 1958. Changes in the endothelium of the aorta and the behaviour of macrophages in experimental atheroma of rabbits. *J Pathol Bacteriol* 75, 245-51.
- Prasad K. S., Andre P., Yan Y. and Phillips D. R., 2003. The platelet CD40L/GP IIb-IIIa axis in atherothrombotic disease. *Curr Opin Hematol* 10, 356-61.
- Prebeck S., Kirschning C., Dürr S., da Costa C., Donath B., Brand K., Redecke V., Wagner H. and Miethke T., 2001. Predominant role of toll-like receptor 2 versus 4 in *Chlamydia pneumoniae*-induced activation of dendritic cells. *J Immunol* 167, 3316-23.
- Qian Y., Qin X., Miwa T., Sun X., Halperin J. and Song W., 2000. Identification and functional characterization of a new gene encoding the mouse terminal complement inhibitor CD59. *J Immunol* 165, 2528-34.
- Rajavashisth T., Qiao J., Tripathi S., Tripathi J., Mishra N., Hua M., Wang X., Loussararian A., Clinton S., Libby P. and Lusis A., 1998. Heterozygous osteopetrotic (op) mutation reduces atherosclerosis in LDL receptor- deficient mice. *J Clin Invest* 101, 2702-10.
- Rapp J., Lespine A., Hamilton R., Colyvas N., Chaumeton A., Tweedie-Hardman J., Kotite L., Kunitake S., Havel R. and Kane J., 1994. Triglyceride-rich lipoproteins isolated by selected-affinity anti-apolipoprotein B immunosorption from human atherosclerotic plaque. *Arterioscler Thromb* 14, 1767-74.
- Ratnoff O., Pensky J., Ogston D. and Naff G., 1969. The inhibition of plasmin, plasma kallikrein, plasma permeability factor, and the C'1r subcomponent of the first component of complement by serum C'1 esterase inhibitor. *J Exp Med* 129, 315-31.

- Reddick R. L., Zhang S. H. and Maeda N., 1994. Atherosclerosis in mice lacking apo E. Evaluation of lesional development and progression. *Arterioscler Thromb* 14, 141-7.
- Reimers G. J., Jackson C. L., Rickards J., Chan P. Y., Cohn J. S., Rye K. A., Barter P. J. and Rodgers K. J., 2011. Inhibition of rupture of established atherosclerotic plaques by treatment with apolipoprotein A-I. *Cardiovasc Res* 91, 37-44.
- Reynolds G. D. and Vance R. P., 1987. C-reactive protein immunohistochemical localization in normal and atherosclerotic human aortas. *Arch Pathol Lab Med* 111, 265-9.
- Ricklin D., Hajishengallis G., Yang K. and Lambris J. D., 2010. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* 11, 785-97.
- Ricklin D. and Lambris J., 2007. Complement-targeted therapeutics. *Nat Biotechnol* 25, 1265-75.
- Ridker P., 2007. C-reactive protein and the prediction of cardiovascular events among those at intermediate risk: moving an inflammatory hypothesis toward consensus. *J Am Coll Cardiol* 49, 2129-38.
- Rocha V. and Libby P., 2009. Obesity, inflammation, and atherosclerosis. *Nat Rev Cardiol* 6, 399-409.
- Rosen B., Cook K., Yaglom J., Groves D., Volanakis J., Damm D., White T. and Spiegelman B., 1989. Adipsin and complement factor D activity: an immune-related defect in obesity. *Science* 244, 1483-7.
- Rosenfeld M., Carson K., Johnson J., Williams H., Jackson C. and Schwartz S., 2002. Animal models of spontaneous plaque rupture: the holy grail of experimental atherosclerosis research. *Curr Atheroscler Rep* 4, 238-42.

- Rosenfeld M., Polinsky P., Virmani R., Kauser K., Rubanyi G. and Schwartz S., 2000. Advanced atherosclerotic lesions in the innominate artery of the ApoE knockout mouse. *Arterioscler Thromb Vasc Biol* 20, 2587-92.
- Ross R., 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362, 801-9.
- Ross R., 1999. Atherosclerosis--an inflammatory disease. *N Engl J Med* 340, 115-26.
- Ross R., Glomset J. and Harker L., 1977. Response to injury and atherogenesis. *Am J Pathol* 86, 675-84.
- Rother R. P., Rollins S. A., Mojcik C. F., Brodsky R. A. and Bell L., 2007. Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. *Nat Biotechnol* 25, 1256-64.
- Rugonfalvi-Kiss S., Endr sz V., Madsen H., Buri n K., Duba J., Proh szka Z., Kar di I., Romics L., G ncz l E., F st G. and Garred P., 2002. Association of *Chlamydia pneumoniae* with coronary artery disease and its progression is dependent on the modifying effect of mannose-binding lectin. *Circulation* 106, 1071-6.
- Rus H., Niculescu F., Poru iu D., Ghiurca V. and Vlaicu R., 1989. Cells carrying C5b-9 complement complexes in human atherosclerotic wall. *Immunol Lett* 20, 305-10.
- Rus H., Niculescu F. and Vlaicu R., 1988. Co-localization of terminal C5b-9 complement complexes and macrophages in human atherosclerotic arterial walls. *Immunol Lett* 19, 27-32.
- Saad A., Virella G., Chassereau C., Boackle R. and Lopes-Virella M., 2006. OxLDL immune complexes activate complement and induce cytokine production by MonoMac 6 cells and human macrophages. *J Lipid Res* 47, 1975-83.

- Saadi S., Holzkecht R., Patte C. and Platt J., 2000. Endothelial cell activation by pore-forming structures: pivotal role for interleukin-1alpha. *Circulation* 101, 1867-73.
- Saadi S., Holzkecht R. A., Patte C. P., Stern D. M. and Platt J. L., 1995. Complement-mediated regulation of tissue factor activity in endothelium. *J Exp Med* 182, 1807-14.
- Sadur C. and Eckel R., 1982. Insulin stimulation of adipose tissue lipoprotein lipase. Use of the euglycemic clamp technique. *J Clin Invest* 69, 1119-25.
- Saito E., Fujioka T., Kanno H., Hata E., Ueno T., Matsumoto T., Takahashi Y., Tochiwara T. and Yasugi T., 1992. Complement receptors in atherosclerotic lesions. *Artery* 19, 47-62.
- Saleh J., Blevins J., Havel P., Barrett J., Gietzen D. and Cianflone K., 2001. Acylation stimulating protein (ASP) acute effects on postprandial lipemia and food intake in rodents. *Int J Obes Relat Metab Disord* 25, 705-13.
- Saleh J., Summers L., Cianflone K., Fielding B., Sniderman A. and Frayn K., 1998. Coordinated release of acylation stimulating protein (ASP) and triacylglycerol clearance by human adipose tissue in vivo in the postprandial period. *J Lipid Res* 39, 884-91.
- Savage D., Semple R., Clatworthy M., Lyons P., Morgan B., Cochran E., Gorden P., Raymond-Barker P., Murgatroyd P., Adams C., Scobie I., Mufti G., Alexander G., Thiru S., Murano I., Cinti S., Chaudhry A., Smith K. and O'Rahilly S., 2009. Complement abnormalities in acquired lipodystrophy revisited. *J Clin Endocrinol Metab* 94, 10-6.

- Scantlebury T., Maslowska M. and Cianflone K., 1998. Chylomicron-specific enhancement of acylation stimulating protein and precursor protein C3 production in differentiated human adipocytes. *J Biol Chem* 273, 20903-9.
- Schepers A., de Vries M., van Leuven C., Grimbergen J., Holers V., Daha M., van Bockel J. and Quax P., 2006. Inhibition of complement component C3 reduces vein graft atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *Circulation* 114, 2831-8.
- Schmidt C. Q., Herbert A. P., Hocking H. G., Uhrin D. and Barlow P. N., 2008. Translational mini-review series on complement factor H: structural and functional correlations for factor H. *Clin Exp Immunol* 151, 14-24.
- Schmiedt W., Kinscherf R., Deigner H., Kamencic H., Nauen O., Kilo J., Oelert H., Metz J. and Bhakdi S., 1998. Complement C6 deficiency protects against diet-induced atherosclerosis in rabbits. *Arterioscler Thromb Vasc Biol* 18, 1790-5.
- Schrijvers D., De Meyer G., Kockx M., Herman A. and Martinet W., 2005. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. *Arterioscler Thromb Vasc Biol* 25, 1256-61.
- Schulman E., Post T., Henson P. and Giclas P., 1988. Differential effects of the complement peptides, C5a and C5a des Arg on human basophil and lung mast cell histamine release. *J Clin Invest* 81, 918-23.
- Schwartz S. M., Galis Z. S., Rosenfeld M. E. and Falk E., 2007. Plaque rupture in humans and mice. *Arterioscler Thromb Vasc Biol* 27, 705-13.
- Seifert P. and Hansson G., 1989a. Complement receptors and regulatory proteins in human atherosclerotic lesions. *Arteriosclerosis* 9, 802-11.

- Seifert P. and Hansson G., 1989b. Decay-accelerating factor is expressed on vascular smooth muscle cells in human atherosclerotic lesions. *J Clin Invest* 84, 597-604.
- Seifert P., Hugo F., Hansson G. and Bhakdi S., 1989. Prelesional complement activation in experimental atherosclerosis. Terminal C5b-9 complement deposition coincides with cholesterol accumulation in the aortic intima of hypercholesterolemic rabbits. *Lab Invest* 60, 747-54.
- Seifert P., Hugo F., Tranum-Jensen J., Zähringer U., Muhly M. and Bhakdi S., 1990. Isolation and characterization of a complement-activating lipid extracted from human atherosclerotic lesions. *J Exp Med* 172, 547-57.
- Seifert P. and Kazatchkine M., 1987. Generation of complement anaphylatoxins and C5b-9 by crystalline cholesterol oxidation derivatives depends on hydroxyl group number and position. *Mol Immunol* 24, 1303-8.
- Seifert P. and Kazatchkine M., 1988. The complement system in atherosclerosis. *Atherosclerosis* 73, 91-104.
- Seifert P., Roth I., Schmiedt W., Oelert H., Okada N., Okada H. and Bhakdi S., 1992. CD59 (homologous restriction factor 20), a plasma membrane protein that protects against complement C5b-9 attack, in human atherosclerotic lesions. *Atherosclerosis* 96, 135-45.
- Seo H., Lombardi D., Polinsky P., Powell-Braxton L., Bunting S., Schwartz S. and Rosenfeld M., 1997. Peripheral vascular stenosis in apolipoprotein E-deficient mice. Potential roles of lipid deposition, medial atrophy, and adventitial inflammation. *Arterioscler Thromb Vasc Biol* 17, 3593-601.

- Serhan C., Brain S., Buckley C., Gilroy D., Haslett C., O'Neill L., Perretti M., Rossi A. and Wallace J., 2007. Resolution of inflammation: state of the art, definitions and terms. *FASEB J* 21, 325-32.
- Serhan C., Chiang N. and Van Dyke T., 2008. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol* 8, 349-61.
- Shagdarsuren E., Bidzhekov K., Mause S. F., Simsekylmaz S., Polakowski T., Hawlisch H., Gessner J. E., Zerneck A. and Weber C., 2010. C5a receptor targeting in neointima formation after arterial injury in atherosclerosis-prone mice. *Circulation* 122, 1026-36.
- Sheikine Y. and Hansson G., 2004. Chemokines and atherosclerosis. *Ann Med* 36, 98-118.
- Shih P., Brennan M., Vora D., Territo M., Strahl D., Elices M., Lusis A. and Berliner J., 1999. Blocking very late antigen-4 integrin decreases leukocyte entry and fatty streak formation in mice fed an atherogenic diet. *Circ Res* 84, 345-51.
- Shimada K., 2009. Immune system and atherosclerotic disease: heterogeneity of leukocyte subsets participating in the pathogenesis of atherosclerosis. *Circ J* 73, 994-1001.
- Shin H., Kim Y., Kim K., Lee J. and Hong K., 2004. Remnant lipoprotein particles induce apoptosis in endothelial cells by NAD(P)H oxidase-mediated production of superoxide and cytokines via lectin-like oxidized low-density lipoprotein receptor-1 activation: prevention by cilostazol. *Circulation* 109, 1022-8.
- Shyy Y., Hsieh H., Usami S. and Chien S., 1994. Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 gene expression in vascular endothelium. *Proc Natl Acad Sci U S A* 91, 4678-82.

- Siegel J., Rent R. and Gewurz H., 1974. Interactions of C-reactive protein with the complement system. I. Protamine-induced consumption of complement in acute phase sera. *J Exp Med* 140, 631-47.
- Sim R., Reboul A., Arlaud G., Villiers C. and Colomb M., 1979. Interaction of 125I-labelled complement subcomponents C-1r and C-1s with protease inhibitors in plasma. *FEBS Lett* 97, 111-5.
- Siow R. C. and Churchman A. T., 2007. Adventitial growth factor signalling and vascular remodelling: potential of perivascular gene transfer from the outside-in. *Cardiovasc Res* 75, 659-68.
- Smith J., Trogan E., Ginsberg M., Grigaux C., Tian J. and Miyata M., 1995. Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. *Proc Natl Acad Sci U S A* 92, 8264-8.
- Sofat R., Casas J. P., Kumari M., Talmud P. J., Ireland H., Kivimaki M., Marmot M., Hughes A. D., Thom S., Ebrahim S., Whittaker J. C., Smeeth L., Lawlor D. A., Humphries S. E. and Hingorani A. D., 2010. Genetic variation in complement factor H and risk of coronary heart disease: eight new studies and a meta-analysis of around 48,000 individuals. *Atherosclerosis* 213, 184-90.
- Song H., He C., Knaak C., Guthridge J., Holers V. and Tomlinson S., 2003. Complement receptor 2-mediated targeting of complement inhibitors to sites of complement activation. *J Clin Invest* 111, 1875-85.
- Song W., Deng C., Raszmann K., Moore R., Newbold R., McLachlan J. and Negishi M., 1996. Mouse decay-accelerating factor: selective and tissue-specific induction by estrogen of the gene encoding the glycosylphosphatidylinositol-anchored form. *J Immunol* 157, 4166-72.

- Speidl W., Kastl S., Hutter R., Katsaros K., Kaun C., Bauriedel G., Maurer G., Huber K., Badimon J. and Wojta J., 2010. The complement component C5a is present in human coronary lesions in vivo and induces the expression of MMP-1 and MMP-9 in human macrophages in vitro. *FASEB J.*
- Spicer A., Seldin M. and Gendler S., 1995. Molecular cloning and chromosomal localization of the mouse decay-accelerating factor genes. Duplicated genes encode glycosylphosphatidylinositol-anchored and transmembrane forms. *J Immunol* 155, 3079-91.
- Spiller O., Harris C. and Morgan B., 1999. Efficient generation of monoclonal antibodies against surface-expressed proteins by hyperexpression in rodent cells. *J Immunol Methods* 224, 51-60.
- Stary H., Blankenhorn D., Chandler A., Glagov S., Insull W. J., Richardson M., Rosenfeld M., Schaffer S., Schwartz C. and Wagner W., 1992. A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler Thromb* 12, 120-34.
- Steffens S. and Mach F., 2006. Drug insight: Immunomodulatory effects of statins--potential benefits for renal patients? *Nat Clin Pract Nephrol* 2, 378-87.
- Stemme S., Faber B., Holm J., Wiklund O., Witztum J. and Hansson G., 1995. T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. *Proc Natl Acad Sci U S A* 92, 3893-7.
- Storch M., Piddlesden S., Haltia M., Iivanainen M., Morgan P. and Lassmann H., 1998. Multiple sclerosis: in situ evidence for antibody- and complement-mediated demyelination. *Ann Neurol* 43, 465-71.

- Strey C., Markiewski M., Mastellos D., Tudoran R., Spruce L., Greenbaum L. and Lambris J., 2003. The proinflammatory mediators C3a and C5a are essential for liver regeneration. *J Exp Med* 198, 913-23.
- Strålfors P. and Honnor R., 1989. Insulin-induced dephosphorylation of hormone-sensitive lipase. Correlation with lipolysis and cAMP-dependent protein kinase activity. *Eur J Biochem* 182, 379-85.
- Sun X., Funk C., Deng C., Sahu A., Lambris J. and Song W., 1999. Role of decay-accelerating factor in regulating complement activation on the erythrocyte surface as revealed by gene targeting. *Proc Natl Acad Sci U S A* 96, 628-33.
- Suzuki H., Kurihara Y., Takeya M., Kamada N., Kataoka M., Jishage K., Ueda O., Sakaguchi H., Higashi T., Suzuki T., Takashima Y., Kawabe Y., Cynshi O., Wada Y., Honda M., Kurihara H., Aburatani H., Doi T., Matsumoto A., Azuma S., Noda T., Toyoda Y., Itakura H., Yazaki Y. and Kodama T., 1997. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature* 386, 292-6.
- Széplaki G., Varga L., Füst G. and Prohászka Z., 2009. Role of complement in the pathomechanism of atherosclerotic vascular diseases. *Mol Immunol* 46, 2784-93.
- Tabas I., 2010. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol* 10, 36-46.
- Tanasescu C., Jurcut C., Jurcut R. and Ginghina C., 2009. Vascular disease in rheumatoid arthritis: from subclinical lesions to cardiovascular risk. *Eur J Intern Med* 20, 348-54.

- Tanhehco E., Lee H. and Lucchesi B., 2000. Sublytic complement attack reduces infarct size in rabbit isolated hearts: evidence for C5a-mediated cardioprotection. *Immunopharmacology* 49, 391-9.
- Tao Y., Cianflone K., Sniderman A., Colby-Germinario S. and Germinario R., 1997. Acylation-stimulating protein (ASP) regulates glucose transport in the rat L6 muscle cell line. *Biochim Biophys Acta* 1344, 221-9.
- Taylor P., Carugati A., Fadok V., Cook H., Andrews M., Carroll M., Savill J., Henson P., Botto M. and Walport M., 2000. A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. *J Exp Med* 192, 359-66.
- Tedesco F., Pausa M., Nardon E., Introna M., Mantovani A. and Dobrina A., 1997. The cytolytically inactive terminal complement complex activates endothelial cells to express adhesion molecules and tissue factor procoagulant activity. *J Exp Med* 185, 1619-27.
- Thompson D., Pepys M. and Wood S., 1999. The physiological structure of human C-reactive protein and its complex with phosphocholine. *Structure* 7, 169-77.
- Thorbjornsdottir P., Kolka R., Gunnarsson E., Bambir S., Thorgeirsson G., Kotwal G. and Arason G., 2005. Vaccinia virus complement control protein diminishes formation of atherosclerotic lesions: complement is centrally involved in atherosclerotic disease. *Ann N Y Acad Sci* 1056, 1-15.
- Thorp E., Li Y., Bao L., Yao P., Kuriakose G., Rong J., Fisher E. and Tabas I., 2009. Brief report: increased apoptosis in advanced atherosclerotic lesions of Apoe^{-/-} mice lacking macrophage Bcl-2. *Arterioscler Thromb Vasc Biol* 29, 169-72.
- Tomooka K., 1989. [Serum complement levels in patients with rheumatoid arthritis and vasculitis]. *Fukuoka Igaku Zasshi* 80, 456-66.

- Tortajada A., Montes T., Martínez-Barricarte R., Morgan B. P., Harris C. L. and de Córdoba S. R., 2009. The disease-protective complement factor H allotypic variant Ile62 shows increased binding affinity for C3b and enhanced cofactor activity. *Hum Mol Genet* 18, 3452-61.
- Torzewski J., Oldroyd R., Lachmann P., Fitzsimmons C., Proudfoot D. and Bowyer D., 1996. Complement-induced release of monocyte chemotactic protein-1 from human smooth muscle cells. A possible initiating event in atherosclerotic lesion formation. *Arterioscler Thromb Vasc Biol* 16, 673-7.
- Torzewski J., Torzewski M., Bowyer D., Fröhlich M., Koenig W., Waltenberger J., Fitzsimmons C. and Hombach V., 1998a. C-reactive protein frequently colocalizes with the terminal complement complex in the intima of early atherosclerotic lesions of human coronary arteries. *Arterioscler Thromb Vasc Biol* 18, 1386-92.
- Torzewski M., Klouche M., Hock J., Messner M., Dorweiler B., Torzewski J., Gabbert H. and Bhakdi S., 1998b. Immunohistochemical demonstration of enzymatically modified human LDL and its colocalization with the terminal complement complex in the early atherosclerotic lesion. *Arterioscler Thromb Vasc Biol* 18, 369-78.
- Torzewski M., Torzewski J., Bowyer D., Waltenberger J., Fitzsimmons C., Hombach V. and Gabbert H., 1997. Immunohistochemical colocalization of the terminal complex of human complement and smooth muscle cell alpha-actin in early atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 17, 2448-52.
- Tran G., Hodgkinson S., Carter N., Killingsworth M., Spicer S. and Hall B., 2002. Attenuation of experimental allergic encephalomyelitis in complement component 6-deficient rats is associated with reduced complement C9

deposition, P-selectin expression, and cellular infiltrate in spinal cords. *J Immunol* 168, 4293-300.

Trogan E., Feig J., Dogan S., Rothblat G., Angeli V., Tacke F., Randolph G. and Fisher E., 2006. Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc Natl Acad Sci U S A* 103, 3781-6.

Trouw L. A., Blom A. M. and Gasque P., 2008. Role of complement and complement regulators in the removal of apoptotic cells. *Mol Immunol* 45, 1199-207.

Urowitz M., Ibañez D. and Gladman D., 2007. Atherosclerotic vascular events in a single large lupus cohort: prevalence and risk factors. *J Rheumatol* 34, 70-5.

Uyemura K., Demer L., Castle S., Jullien D., Berliner J., Gately M., Warriar R., Pham N., Fogelman A. and Modlin R., 1996. Cross-regulatory roles of interleukin (IL)-12 and IL-10 in atherosclerosis. *J Clin Invest* 97, 2130-8.

Van Harmelen V., Reynisdottir S., Cianflone K., Degerman E., Hoffstedt J., Nilsson K., Sniderman A. and Arner P., 1999. Mechanisms involved in the regulation of free fatty acid release from isolated human fat cells by acylation-stimulating protein and insulin. *J Biol Chem* 274, 18243-51.

Veillard N., Steffens S., Burger F., Pelli G. and Mach F., 2004. Differential expression patterns of proinflammatory and antiinflammatory mediators during atherogenesis in mice. *Arterioscler Thromb Vasc Biol* 24, 2339-44.

Veillard N., Steffens S., Pelli G., Lu B., Kwak B., Gerard C., Charo I. and Mach F., 2005. Differential influence of chemokine receptors CCR2 and CXCR3 in development of atherosclerosis in vivo. *Circulation* 112, 870-8.

Verdeguer F., Castro C., Kubicek M., Pla D., Vila-Caballer M., Vinué A., Civeira F., Pocoví M., Calvete J. and Andrés V., 2007. Complement regulation in murine

and human hypercholesterolemia and role in the control of macrophage and smooth muscle cell proliferation. *Cardiovasc Res* 76, 340-50.

Verrier E. D., Shernan S. K., Taylor K. M., Van de Werf F., Newman M. F., Chen J. C., Carrier M., Haverich A., Malloy K. J., Adams P. X., Todaro T. G., Mojcik C. F., Rollins S. A., Levy J. H. and Investigators P.-C., 2004. Terminal complement blockade with pexelizumab during coronary artery bypass graft surgery requiring cardiopulmonary bypass: a randomized trial. *JAMA* 291, 2319-27.

Viedt C., Hänsch G., Brandes R., Kübler W. and Kreuzer J., 2000. The terminal complement complex C5b-9 stimulates interleukin-6 production in human smooth muscle cells through activation of transcription factors NF-kappa B and AP-1. *FASEB J* 14, 2370-2.

Virchow R., 1856. Phlogose und Thrombose im Gefassystem. In: *Gesammelte Abhandlungen zu r wissenschaftlichen Medizin*. F. Meidinger Sohn, Frankfurt am Main.

Virmani R., Kolodgie F. D., Burke A. P., Farb A. and Schwartz S. M., 2000. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 20, 1262-75.

Vlaicu R., Niculescu F., Rus H. and Cristea A., 1985a. Immunohistochemical localization of the terminal C5b-9 complement complex in human aortic fibrous plaque. *Atherosclerosis* 57, 163-77.

Vlaicu R., Rus H., Niculescu F. and Cristea A., 1985b. Immunoglobulins and complement components in human aortic atherosclerotic intima. *Atherosclerosis* 55, 35-50.

- Vlaicu R., Rus H., Niculescu F. and Cristea A., 1985c. Quantitative determinations of immunoglobulins and complement components in human aortic atherosclerotic wall. *Med Interne* 23, 29-35.
- von Hundelshausen P., Weber K., Huo Y., Proudfoot A., Nelson P., Ley K. and Weber C., 2001. RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. *Circulation* 103, 1772-7.
- Vora D., Fang Z., Liva S., Tyner T., Parhami F., Watson A., Drake T., Territo M. and Berliner J., 1997. Induction of P-selectin by oxidized lipoproteins. Separate effects on synthesis and surface expression. *Circ Res* 80, 810-8.
- Walpolo P., Gotlieb A., Cybulsky M. and Langille B., 1995. Expression of ICAM-1 and VCAM-1 and monocyte adherence in arteries exposed to altered shear stress. *Arterioscler Thromb Vasc Biol* 15, 2-10.
- Walport M., 2001a. Complement. First of two parts. *N Engl J Med* 344, 1058-66.
- Walport M., 2001b. Complement. Second of two parts. *N Engl J Med* 344, 1140-4.
- Welch T., 2002. Complement in glomerulonephritis. *Nat Genet* 31, 333-4.
- Welch T. R., Beischel L. and Kleesattel A., 1990. Functional consequences of the genetic polymorphism of the third component of complement. *J Pediatr* 116, S92-7.
- Wen Y., Wang H., MacLaren R., Lu H., Hu X. F. and Cianflone K., 2008. Sex steroid hormones induce acylation stimulating protein resistance in 3T3-L1 adipocytes. *J Cell Biochem* 105, 404-13.
- Whiss P. A., 2002. Pexelizumab Alexion. *Curr Opin Investig Drugs* 3, 870-7.
- White H., Held C., Stewart R., Watson D., Harrington R., Budaj A., Steg P. G., Cannon C. P., Krug-Gourley S., Wittes J., Trivedi T., Tarka E. and Wallentin L., 2010. Study design and rationale for the clinical outcomes of the

STABILITY Trial (STabilization of Atherosclerotic plaque By Initiation of darapLadIb TherapY) comparing darapladib versus placebo in patients with coronary heart disease. *Am Heart J* 160, 655-61.

Wiesmann C., Katschke K., Yin J., Helmy K., Steffek M., Fairbrother W., McCallum S., Embuscado L., DeForge L., Hass P. and van Lookeren Campagne M., 2006. Structure of C3b in complex with CRIg gives insights into regulation of complement activation. *Nature* 444, 217-20.

Williams A., Mizuno M., Richards P., Holt D. and Morgan B., 2004. Deletion of the gene encoding CD59a in mice increases disease severity in a murine model of rheumatoid arthritis. *Arthritis Rheum* 50, 3035-44.

Williams H., Johnson J., Carson K. and Jackson C., 2002. Characteristics of intact and ruptured atherosclerotic plaques in brachiocephalic arteries of apolipoprotein E knockout mice. *Arterioscler Thromb Vasc Biol* 22, 788-92.

Wojta J., Kaun C., Zorn G., Ghannadan M., Hauswirth A., Sperr W., Fritsch G., Printz D., Binder B., Schatzl G., Zwirner J., Maurer G., Huber K. and Valent P., 2002. C5a stimulates production of plasminogen activator inhibitor-1 in human mast cells and basophils. *Blood* 100, 517-23.

Wu G., Hu W., Shahsafaei A., Song W., Dobarro M., Sukhova G., Bronson R., Shi G., Rother R., Halperin J. and Qin X., 2009. Complement regulator CD59 protects against atherosclerosis by restricting the formation of complement membrane attack complex. *Circ Res* 104, 550-8.

Xia Z. and Cianflone K., 2003. Acylation-stimulating protein precursor proteins in adipose tissue in human obesity. *Metabolism* 52, 1360-6.

- Yang Y., Lu H. L., Zhang J., Yu H. Y., Wang H. W., Zhang M. X. and Cianflone K., 2006. Relationships among acylation stimulating protein, adiponectin and complement C3 in lean vs obese type 2 diabetes. *Int J Obes (Lond)* 30, 439-46.
- Yasojima K., Schwab C., McGeer E. and McGeer P., 2001. Complement components, but not complement inhibitors, are upregulated in atherosclerotic plaques. *Arterioscler Thromb Vasc Biol* 21, 1214-9.
- Yasruel Z., Cianflone K., Sniderman A., Rosenbloom M., Walsh M. and Rodriguez M., 1991. Effect of acylation stimulating protein on the triacylglycerol synthetic pathway of human adipose tissue. *Lipids* 26, 495-9.
- Yilmaz A., Lochno M., Traeg F., Cicha I., Reiss C., Stumpf C., Raaz D., Anger T., Amann K., Probst T., Ludwig J., Daniel W. and Garlichs C., 2004. Emergence of dendritic cells in rupture-prone regions of vulnerable carotid plaques. *Atherosclerosis* 176, 101-10.
- Yin F. C., Spurgeon H. A., Rakusan K., Weisfeldt M. L. and Lakatta E. G., 1982. Use of tibial length to quantify cardiac hypertrophy: application in the aging rat. *Am J Physiol* 243, H941-7.
- Yun S., Leung V., Botto M., Boyle J. and Haskard D., 2008. Brief report: accelerated atherosclerosis in low-density lipoprotein receptor-deficient mice lacking the membrane-bound complement regulator CD59. *Arterioscler Thromb Vasc Biol* 28, 1714-6.
- Zadelaar S., Klemann R., Verschuren L., de Vries-Van der Weij J., van der Hoorn J., Princen H. and Kooistra T., 2007. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler Thromb Vasc Biol* 27, 1706-21.

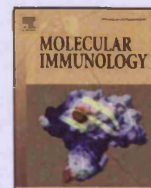
- Zhang J., Wright W., Bernlohr D., Cushman S. and Chen X., 2007. Alterations of the classic pathway of complement in adipose tissue of obesity and insulin resistance. *Am J Physiol Endocrinol Metab* 292, E1433-40.
- Zhang S., Reddick R., Piedrahita J. and Maeda N., 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 258, 468-71.
- Zhao S., Liu L., Gao M., Zhou Q., Li Y. and Xia B., 2001. Impairment of endothelial function after a high-fat meal in patients with coronary artery disease. *Coron Artery Dis* 12, 561-5.
- Zhou X., Nicoletti A., Elhage R. and Hansson G., 2000. Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. *Circulation* 102, 2919-22.
- Zhou X., Paulsson G., Stemme S. and Hansson G., 1998. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J Clin Invest* 101, 1717-25.
- Zhou X., Robertson A., Rudling M., Parini P. and Hansson G., 2005. Lesion development and response to immunization reveal a complex role for CD4 in atherosclerosis. *Circ Res* 96, 427-34.
- Zipfel P. and Skerka C., 2009. Complement regulators and inhibitory proteins. *Nat Rev Immunol* 9, 729-40.
- Zwaka T., Torzewski J., Hoeflich A., Déjosez M., Kaiser S., Hombach V. and Jehle P., 2003. The terminal complement complex inhibits apoptosis in vascular smooth muscle cells by activating an autocrine IGF-1 loop. *FASEB J* 17, 1346-8.

Zwirner J., Götze O., Sieber A., Kapp A., Begemann G., Zuberbier T. and Werfel T., 1998a. The human mast cell line HMC-1 binds and responds to C3a but not C3a(desArg). *Scand J Immunol* 47, 19-24.

Zwirner J., Werfel T., Wilken H., Theile E. and Götze O., 1998b. Anaphylatoxin C3a but not C3a(desArg) is a chemotaxin for the mouse macrophage cell line J774. *Eur J Immunol* 28, 1570-7.

Øhlenschlaeger T., Garred P., Madsen H. and Jacobsen S., 2004. Mannose-binding lectin variant alleles and the risk of arterial thrombosis in systemic lupus erythematosus. *N Engl J Med* 351, 260-7.

Appendices



The membrane attack complex of complement drives the progression of atherosclerosis in apolipoprotein E knockout mice

Ruth D. Lewis^a, Christopher L. Jackson^b, B. Paul Morgan^a, Timothy R. Hughes^{a,*}

^a Complement Biology Group, Department of Medical Biochemistry and Immunology, School of Medicine, Cardiff University, CF14 4XN, UK

^b Bristol Heart Institute, University of Bristol, Level 7, Bristol Royal Infirmary, Bristol BS2 8HW, UK

ARTICLE INFO

Article history:

Received 10 September 2009

Received in revised form 26 October 2009

Accepted 29 October 2009

Available online 2 December 2009

Keywords:

Atherosclerosis

Complement

Membrane attack complex

Apolipoprotein E knockout mouse

ABSTRACT

Aims: To examine the roles of the membrane attack complex of complement and its sole membrane regulator, CD59, in atherosclerosis.

Methods: C6 (C6^{-/-}) deficient and CD59a (Cd59a^{-/-}) knockout mice were separately crossed onto the apolipoprotein E knockout (apoE^{-/-}) background. The double knockout mice were fed high-fat diet in order to study the effects of absence of C6 or CD59a on the progression of atherosclerosis.

Results: C6 deficiency significantly reduced plaque area and disease severity. CD59a had the opposite effect in that deficiency was associated with a significant increase in plaque area, correlating with increased membrane attack complex (MAC) deposition in the plaque and increased smooth muscle cell proliferation in early plaques.

Conclusions: Our results demonstrate that the MAC contributes to the development of atherosclerosis, C6 deficiency being protective and CD59a deficiency exacerbating disease.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Over the last two decades it has become increasingly clear that atherosclerosis is associated with chronic inflammation (Fan and Watanabe, 2003; Nilsson and Hansson, 2008; Ross, 1999; Seifert and Kazatchkine, 1988). While the causes of the initial insult to the artery wall remain unclear, evidence has accumulated implicating the complement system in disease progression (Meuwissen et al., 2006; Niculescu et al., 2004; Vlaicu et al., 1985; Yasojima et al., 2001). Despite these findings, studies on the role of the complement system in atherosclerosis using animal models have so far produced conflicting results (Bhatia et al., 2007; Patel et al., 2001; Persson et al., 2004; Schmiedt et al., 1998). The deletion of C3 in apolipoprotein-E-deficient (apoE^{-/-}) and C1q in low density lipoprotein receptor (Ldl-r)-deficient mice caused a worsening of the disease in both cases, implying that C1q which initiates the classical pathway of complement, and C3, the central orchestrator of all complement activation pathways, have anti-atherogenic roles (Bhatia et al., 2007; Persson et al., 2004). In contrast a recent paper by Leung et al. looked at the effect of DAF deficiency in the Ldl-r mouse model of atherosclerosis. They found a worsening of disease in the absence of DAF (Leung et al., 2009). C5 deficiency had

little or no effect in the apoE-deficient model, leading the authors to suggest that the membrane attack complex (MAC) had no role in this model of atherosclerosis (Patel et al., 2001). Contrasting data were obtained from studies in C6-deficient rabbits which, when fed a high-fat diet, developed less atherosclerosis than their C6 sufficient controls (Schmiedt et al., 1998). MAC formation is blocked in both C5-deficient mice and C6-deficient rabbits, suggesting that there may be species differences in the roles of MAC in atherosclerosis; alternatively, it is possible that C5a, the other product of C5 cleavage, has a hitherto unrecognised protective role in atherosclerosis.

Regulation of the terminal pathway has also recently been examined in mice through the generation of animals deficient in CD59a, the major regulator of MAC formation in the mouse. CD59 functions by blocking the interaction of the C5b-8 complex with C9, thereby preventing the formation of the lytic membrane attack complex (MAC). These laboratories employed different models of the disease, utilising either the Ldl-r or the apoE knockout mouse respectively (Wu et al., 2009; Yun et al., 2008; An et al., 2009). Mice have two genes for CD59: Cd59a and Cd59b. Cd59a is widely expressed and the main regulator of membrane attack complex assembly in the mouse (Baalasubramanian et al., 2004), while Cd59b is highly expressed only in testis (Donev et al., 2008). Yun et al. (2008) tested Ldl-r^{-/-} mice deficient in Cd59a and showed increased plaque formation, while Wu et al. (2009) examined apoE^{-/-} mice deficient in Cd59a and Cd59b and similarly demonstrated a worsening of disease. The latter paper also showed that treatment of the triple-deficient mice while on high-fat diets with

* Corresponding author at: Department of Medical Biochemistry and Immunology, School of Medicine, 3rd Floor Henry Welcome Building, Heath Park, Cardiff University, Cardiff CF14 4XN, UK. Tel.: +44 2920687304.

E-mail address: HughesTR@cf.ac.uk (T.R. Hughes).

the anti-C5 monoclonal antibody BB5.1 caused a significant lessening of disease severity. This result contradicts the finding that deficiency of C5 in the apoE^{-/-} mouse did not inhibit progression of atherosclerosis (Patel et al., 2001).

This study was initiated to clearly define the role of MAC during the development of atherosclerosis by characterising the effect of C6 deficiency on the progression of atherosclerosis in the apoE^{-/-} mouse model. We have undertaken a direct comparison of the effects of C6 and CD59a deficiency in the same colony of apoE^{-/-} mice. C6-deficient mice are particularly relevant for investigating the pathogenic roles of MAC, since they have previously been used to implicate MAC as a causative agent in a wide range of diseases, including reperfusion injury, glomerular damage, and xenograft hyperacute rejection (Falk et al., 1983; Fondevila et al., 2008; McCurry et al., 1995). ApoE deficient mice lacking C6 were markedly protected from atherosclerosis compared to C6 sufficient controls. In contrast, CD59a deficiency significantly exacerbated atherosclerosis, in agreement with recent published studies and supportive of a key role of MAC in disease progression.

2. Methods

Additional methods are provided in Supplementary material.

2.1. Animals

Male apoE^{-/-}/C6^{-/-}; apoE^{-/-}/Cd59a^{-/-} mice together with litter-matched controls were fed high-fat diet containing 21% (wt/wt) pork lard and supplemented with 0.15% (wt/wt) cholesterol (Special Diet Services, Witham, UK) for 8 or 12 weeks starting at 8 weeks of age. Animals were housed in a specific pathogen-free environment. All studies and protocols were approved by the institutional Ethics Review Committee and by the United Kingdom Home Office and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Termination

Animals were anaesthetised and processed as previously described (Rosenfeld et al., 2002). Brachiocephalic arteries were removed with a piece of the aortic arch and the stump of the right subclavian artery still attached to aid orientation during histological processing. These were immediately embedded in optimum cutting temperature (OCT) compound (Raymond A Lamb Limited, Eastbourne, UK) and snap-frozen in liquid nitrogen.

2.3. Histology and immunohistochemistry

Serial transverse sections, of 7 µm thickness, were cut along the brachiocephalic artery, starting from the proximal end. Sections were stained with Miller's elastin/van Gieson or oil red O (both from Sigma-Aldrich, Poole, UK). Sections were immunostained for the following complement regulators: DAF (13 µg/mL) (rat anti-mouse DAF, 2C6, prepared in-house using standard immunisation procedures) (Spiller et al., 1999); Crry (2.6 µg/mL) (rat anti-mouse Crry mAb 5D5, a generous gift from Dr. M. Holers, Denver, USA) (Li et al., 1993); and Cd59a (10 µg/mL) (rat anti-mouse CD59a mAb, mCD59a.7, a kind gift from Dr. C.L. Harris, Cardiff University). Macrophages, smooth muscle cells and T cells were identified using anti-murine macrophage antibody (0.1 µg/mL) (MOMA-2, Serotec, Oxford, UK), anti-α-smooth muscle actin (diluted 1:400) (clone α-1-A4; Sigma-Aldrich) and hamster anti-CD3 (20 µg/mL) (48-2B; Santa Cruz) respectively.

Immunostaining was also performed for complement components C3 (20 µg/mL) (rat anti-mouse C3 mAb 11H9; Hy-Cult

Biotechnology, The Netherlands), and C9/MAC (2 µg/mL) (rabbit anti-rat C9 prepared in-house using standard immunisation procedures).

In each case positive staining was expressed as a percentage fractional area of the lesion as analysed by a computerised image-analysis program (Image ProPlus™ software version 6.3, Media Cybernetics, Carlsbad, CA, USA).

2.4. Histomorphometry

Morphometric analyses were carried out on elastin-stained sections. One section was quantified per mouse at the same position along the brachiocephalic artery, following the established method of Johnson et al. (2005). Morphometry was performed using Image ProPlus™ software as above. The lengths of the internal and external elasticae were recorded. These were used to derive the media area by assuming them to be the circumferences of perfect circles. The plaque area was measured directly and was subtracted from the area enclosed by the internal elastic lamina to derive the true lumen area.

2.5. Serum triglyceride and cholesterol concentrations

Mouse blood (~1 mL) was collected into tubes without anticoagulant, allowed to clot at room temperature and then incubated on ice for 1 h. Serum was separated by centrifugation and analysed for triglyceride, cholesterol and lipaemia index on an automated analyser (Clinical Biochemistry Laboratories, Cardiff University Hospital).

2.6. Plaque lipid content

Oil red O-stained sections were used to determine plaque lipid content. The total stained area in the plaque was expressed as a percentage of the total plaque area to give the fractional lipid content.

2.7. Statistical analysis

Data are expressed as mean ± SEM and significance tested by two-tailed unpaired Student's *t*-test (GraphPad Prism software version 3.0), with significance assumed at *P* < 0.05.

3. Results

3.1. Deficiency of C6 inhibits atherosclerotic plaque progression

To delineate the role of the MAC during atherosclerosis we crossed apoE^{-/-} mice with C6^{-/-} mice generating apoE^{-/-}/C6^{-/-} and litter-matched controls (apoE^{-/-}/C6^{+/+}). Having no C6, these mice were incapable of MAC formation from birth. The mice were fed a high-fat diet for 12 weeks to induce severe disease, in the expectation that this would amplify differences between the test and control groups. Analyses of the brachiocephalic arteries revealed that the mean plaque cross-sectional area in apoE^{-/-}/C6^{-/-} mice was significantly decreased compared to the apoE^{-/-}/C6^{+/+} controls. Fig. 1A and C shows representative sections from apoE^{-/-}/C6^{-/-} mice while Fig. 1B and D shows representative sections from apoE^{-/-} control mice. Additionally, (C) and (D) shows the area defined as "plaque", superimposed onto the image. While Fig. 1E shows the pooled data ($66.01 \pm 23.2 \times 10^3 \mu\text{m}^2$ versus $179.5 \pm 14.1 \times 10^3 \mu\text{m}^2$; *P* < 0.001). Table 1 summarises the morphometric analyses, including plaque, lumen, media and vessel area. The mean lumen and media areas were not different between the groups. However the total vessel area was significantly decreased in the apoE^{-/-}/C6^{-/-} mice compared to apoE^{-/-} controls ($349.9 \pm 39.6 \times 10^3 \mu\text{m}^2$ versus $469.8 \pm 28.2 \times 10^3 \mu\text{m}^2$;

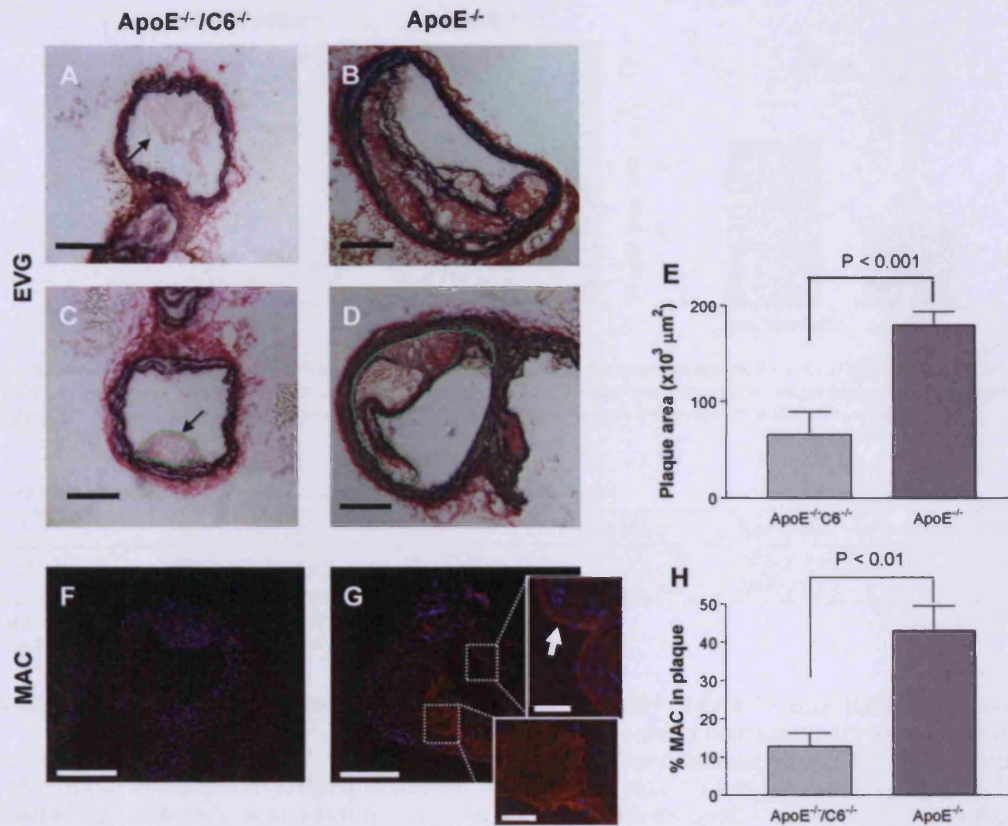


Fig. 1. Analysis of atherosclerosis in $\text{apoE}^{-/-}/\text{C6}^{-/-}$ mice. Plaque area measurement in brachiocephalic arteries of $\text{apoE}^{-/-}/\text{C6}^{-/-}$ and $\text{apoE}^{-/-}$ mice fed a high-fat diet from 8 weeks of age for 12 weeks. Sections of the brachiocephalic artery were stained with Miller's elastin/van Gieson. The bars show group means \pm SEM. Scale bars: 200 μm unless otherwise stated. (A and C) Small plaques (arrowed) from the brachiocephalic arteries of $\text{apoE}^{-/-}/\text{C6}^{-/-}$ mice. (C) Area defined as plaque using Image ProPlus™ software. (B and D) Large plaques from the brachiocephalic arteries of $\text{apoE}^{-/-}$ mice. (D) Area defined as plaque using Image ProPlus™ software. (E) Mean plaque area in $\text{apoE}^{-/-}/\text{C6}^{-/-}$ and $\text{apoE}^{-/-}$ mice. Brachiocephalic artery sections were stained with rabbit anti-rat C9 (red) for MAC deposition and nuclei were stained blue with DAPI. (F) Minimal C9 staining in a large plaque from an $\text{apoE}^{-/-}/\text{C6}^{-/-}$ mouse. (G) MAC staining is abundant in an advanced plaque of an $\text{apoE}^{-/-}$ mouse. Insets show higher magnification of areas where C9 deposition is localised within the plaque: lining the endothelial wall (arrowed) and within the necrotic core of the plaque (inset scale bar: 50 μm). (H) Comparison percentage membrane attack complex staining in $\text{apoE}^{-/-}/\text{C6}^{-/-}$ and $\text{apoE}^{-/-}$ mice.

$P < 0.05$). MAC deposition was abundant in plaques of control mice, both on the endothelium (inset, thick white arrow) and in the necrotic core (inset, large non-cellular area) (Fig. 1G). In contrast, no staining for MAC above background levels was found in plaques from $\text{apoE}^{-/-}/\text{C6}^{-/-}$ mice (Fig. 1F). Fig. 1H shows the pooled data for MAC deposition ($\text{apoE}^{-/-}/\text{C6}^{-/-}$: $12.8 \pm 3.5\%$ versus $\text{apoE}^{-/-}$: $33.0 \pm 6.5\%$; $P \leq 0.01$). Note that the absence of MAC staining in the $\text{apoE}^{-/-}$ mice provides verification for the use of the anti-C9 antiserum as a MAC marker because these mice have normal plasma levels of C9 (data not shown).

2. Plaque cross-sectional area is increased in $\text{apoE}/\text{Cd59a}$ double knockout mice

To examine the role of regulation of MAC formation during atherosclerosis we generated mice deficient in both

apoE and Cd59a and subjected them (together with their controls) to a period of high-fat feeding. Analysis of the brachiocephalic arteries revealed that average plaque size in $\text{apoE}^{-/-}/\text{Cd59a}^{-/-}$ mice was double that seen in gender, age, strain and litter-matched $\text{apoE}^{-/-}$ controls on an identical diet. Fig. 2A and B shows representative sections from $\text{apoE}^{-/-}/\text{Cd59a}^{-/-}$ and $\text{apoE}^{-/-}$ mice respectively. The pooled data are shown in Fig. 2C ($59.9 \pm 13.1 \times 10^3 \mu\text{m}^2$ versus $28.2 \pm 7.9 \times 10^3 \mu\text{m}^2$; $P < 0.05$). Table 2 summarises the morphometric analyses, including plaque, lumen, media and vessel area. The mean lumen area, the media area and the total vessel area were not different between the groups. There were no significant differences in body weight, heart:body weight ratios, serum triglyceride or cholesterol levels, the lipaemia index, or plaque lipid content between $\text{apoE}^{-/-}/\text{Cd59a}^{-/-}$ and $\text{apoE}^{-/-}$ mice (supplemental data, Table 1 and Fig. 1).

Table 1
Brachiocephalic artery morphometric data in 20-week-old $\text{apoE}^{-/-}/\text{C6}^{-/-}$ and $\text{apoE}^{-/-}$ mice.

Group	Vessel area ($\times 10^3 \mu\text{m}^2$)	Plaque area ($\times 10^3 \mu\text{m}^2$)	Media area ($\times 10^3 \mu\text{m}^2$)	Lumen area ($\times 10^3 \mu\text{m}^2$)
$\text{apoE}^{-/-}/\text{C6}^{-/-}$ (n=8)	349.9 ± 39.6	66.01 ± 23.2	100.2 ± 11.7	183.7 ± 15.9
$\text{apoE}^{-/-}$ (n=10)	469.8 ± 28.2	179.5 ± 14.1	97.5 ± 10.1	192.8 ± 17.2

All animals were fed a high-fat diet from 8 weeks of age for 12 weeks.

* $P < 0.05$ versus $\text{apoE}^{-/-}$ control.

** $P < 0.001$ versus $\text{apoE}^{-/-}$ control.

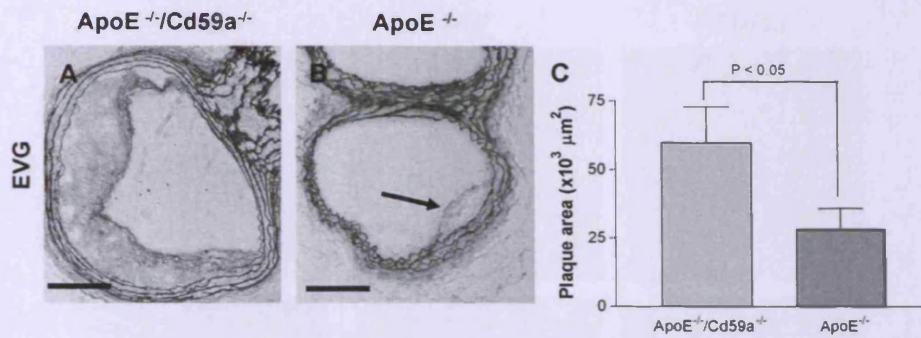


Fig. 2. Plaque area measurement in brachiocephalic arteries of apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} control mice after 8 weeks of high-fat feeding. Sections of the brachiocephalic artery were stained with Miller's elastin/van Gieson. The bars show group means \pm SEM. Scale bars: 200 μ m. (A) Single large plaque occupying half of the vessel from an apoE^{-/-}/Cd59a^{-/-} mouse. (B) A small plaque (arrowed) from an apoE^{-/-} mouse. (C) Mean plaque area in apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice.

Table 2
Brachiocephalic artery morphometric data in 16-week-old apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice.

Group	Vessel area ($\times 10^3 \mu\text{m}^2$)	Plaque area ($\times 10^3 \mu\text{m}^2$)	Media area ($\times 10^3 \mu\text{m}^2$)	Lumen area ($\times 10^3 \mu\text{m}^2$)
apoE ^{-/-} /Cd59a ^{-/-} (n = 14)	345.0 \pm 25.0	*59.9 \pm 13.1	97.0 \pm 7.6	188.0 \pm 10.8
apoE ^{-/-} (n = 15)	325.0 \pm 20.0	28.2 \pm 7.9	90.0 \pm 8.1	207.0 \pm 9.8

All animals were fed a high-fat diet from 8 weeks of age for 8 weeks.

* $P < 0.05$ versus apoE^{-/-} control.

3.3. CD59a deficiency causes increased deposition of MAC in plaques

The extent of terminal complement pathway activation was examined by staining for C9 deposition as a surrogate marker of MAC (verified as described above). Complement activation was also assessed by staining for C3 fragment deposition. MAC staining was absent from unaffected vessel walls (i.e. those with no plaque), but clearly present in early and late stage plaques obtained from both apoE^{-/-}/Cd59a^{-/-} (Fig. 3A) and apoE^{-/-} mice (Fig. 3B). To better visualise and analyse the expected increase in MAC staining

in apoE^{-/-}/Cd59a^{-/-} mice, fluorescence intensity detection limits were set at a high sensitivity level, hence the apparently low levels of MAC staining recorded in apoE controls. MAC deposits were more than 10-fold increased in the apoE^{-/-}/Cd59a^{-/-} mice compared to the apoE^{-/-} controls (Fig. 3C; $28.9 \pm 9.4\%$ versus $2.7 \pm 0.8\%$; $P \leq 0.05$). C3 deposition was detected weakly in unaffected artery walls and strongly in plaques from the brachiocephalic arteries of both apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice (Fig. 3D and E, respectively). There was no significant difference in levels of C3 between the apoE^{-/-}/Cd59a^{-/-} mice and their apoE^{-/-} controls (Fig. 3F). To assess whether increased MAC deposition in apoE^{-/-}/Cd59a^{-/-}

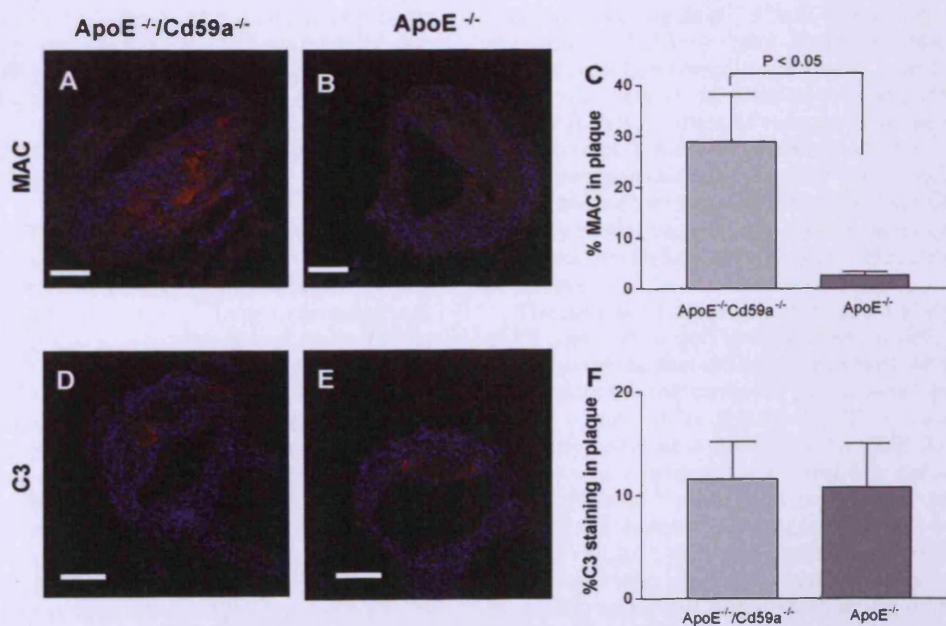


Fig. 3. MAC deposition and lesional C3 levels in atherosclerotic brachiocephalic arteries. Comparison of percentage MAC staining (panels A–C) or C3 staining (panels D–F) in apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} animals at 16 weeks after 8 weeks of high-fat diet. Sections of the brachiocephalic artery were stained with rabbit anti-rat C9 for MAC or rat anti-mouse C3 (red) and nuclei were stained with DAPI (blue). The bars show group means \pm SEM. Scale bars: 200 μ m. (A) Representative section from an apoE^{-/-}/Cd59a^{-/-} mouse. (B) Representative section from an apoE^{-/-} mouse. (C) Mean percentage of lesional MAC staining in apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice. (D) Representative section from apoE^{-/-}/Cd59a^{-/-} mouse. (E) Representative section from apoE^{-/-} mouse. (F) Mean percentage of lesional C3 staining in apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice.

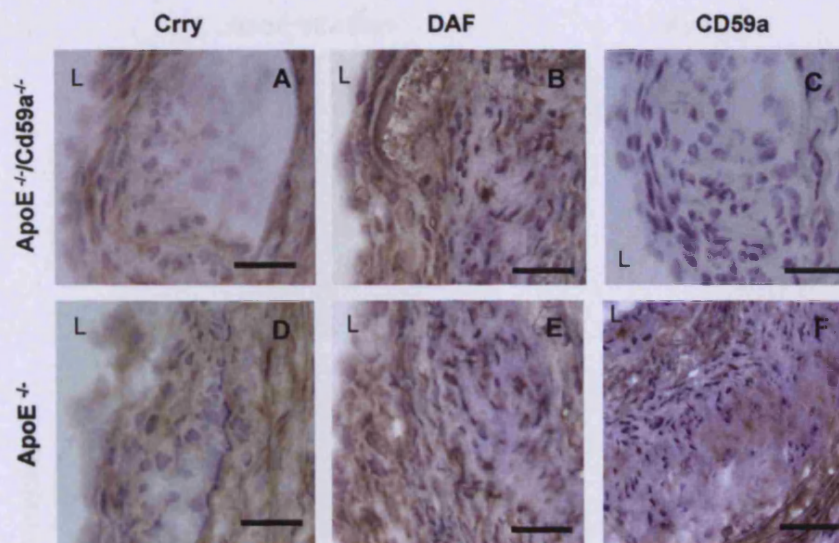


Fig. 4. Immunolocalisation of complement regulators. apoE/Cd59a and apoE deficient mice were fed a high-fat diet for 8 weeks from 8 weeks of age. Sections of the brachiocephalic artery were stained for complement regulators using specific monoclonal antibodies (brown) and nuclei were stained with haematoxylin (blue). Scale bars: 50 μ m. Lumen is indicated by L. (A–C) Staining of Crry (rat anti-mouse Crry mAb (5D5)), decay accelerating-factor (DAF) (2C6) and Cd59a respectively in apoE^{-/-}/Cd59a^{-/-} mice. (D–F) Staining of Crry, DAF and Cd59a respectively in apoE^{-/-} mice.

mice would cause an increase in the numbers of inflammatory cells within the plaque we stained for macrophages and T cells. There were no significant differences in the proportions of plaque area staining for macrophages or T cells (as revealed by staining with MOMA-2 and CD3, respectively) between the two groups (Supplemental Data Figs. 2 and 3).

3.4. Absence of CD59a is not compensated by up-regulation of other complement regulators

The membrane-bound complement regulators Crry and decay accelerating factor (DAF) were expressed in normal vessel walls and plaques in both groups of mice (Fig. 4A, B, D and E). There was no gross difference in the pattern or abundance of expression of either Crry or DAF between the groups. Cd59a expression was absent from apoE^{-/-}/Cd59a^{-/-} mice and present within the brachiocephalic arteries of apoE^{-/-} mice (Fig. 4C and F, respectively).

3.5. In the absence of CD59a the MAC influences smooth muscle cell proliferation and survival in atherosclerosis

Because of the known effect that the MAC has on smooth muscle cell proliferation (Benzaquen et al., 1994) we decided to quantify smooth muscle cell content by staining for α -actin in plaques from the brachiocephalic arteries of apoE^{-/-} mice lacking the MAC regulator CD59a. Grouping the plaques into “early” (fatty streaks and fibrous plaques with cross-sectional areas $<80 \times 10^3 \mu\text{m}^2$) and “advanced” (complex plaques with cross-sectional areas $>80 \times 10^3 \mu\text{m}^2$) we found a significant increase in the smooth muscle cell content of “early” plaques from apoE^{-/-}/Cd59a^{-/-} mice as compared to apoE^{-/-} mice (% α -actin staining $62.0 \pm 7.1\%$ versus $21.4 \pm 6.1\%$; $P < 0.01$). Fig. 5A and B shows representative pictures of early plaques stained for smooth muscle cell α -actin. By contrast, in “advanced” plaques smooth muscle cell content was significantly reduced in the plaques from apoE^{-/-}/Cd59a^{-/-} mice versus apoE^{-/-} controls (% α -actin staining $15.3 \pm 4.8\%$ versus $36.6 \pm 6.7\%$; $P < 0.05$). Fig. 5C and D shows representative pictures of advanced plaques stained for smooth muscle cell α -actin. Combined data are presented in Fig. 5E. The proportion of plaque staining for α -actin in the apoE^{-/-}/Cd59a^{-/-} mice fell more than three-fold between “early” and “advanced” plaques ($62.0 \pm 7.1\%$ versus $15.3 \pm 4.8\%$;

$P < 0.01$). Fig. 5F–I shows C9 deposition in smooth muscle cell regions of an atherosclerotic plaque from an apoE deficient mouse.

4. Discussion

In this paper we set out to unequivocally define the roles of the MAC in atherosclerotic plaque formation in the apoE^{-/-} mouse model. Importantly, we clearly show, for the first time, that deficiency of C6 is strongly protective against progression of atherosclerosis in apoE^{-/-} mice. This result is consistent with published studies in fat fed C6-deficient rabbits and strongly implicates the MAC in both of these models of atherosclerosis (Schmiedt et al., 1998). Secondly, we tested the effects of deficiency of the major murine regulator of MAC formation, CD59a, and showed that apoE^{-/-}/Cd59a^{-/-} mice developed much larger plaques on fat feeding when compared to closely matched apoE^{-/-} controls; MAC deposition in the plaques was also markedly increased in apoE^{-/-}/Cd59a^{-/-} mice. Of note we have also shown in these mice that the smooth muscle cell content of advanced plaques is significantly less than that seen in apoE^{-/-} controls. This finding suggests a possible mechanism whereby MAC formation could contribute not only to plaque development but also to plaque instability and hence the incidence of vessel rupture and consequent acute infarcts or strokes.

The data that we have presented here on the contrasting effects of C6 and CD59a deficiency on plaque development in apoE^{-/-} mice clearly support the hypothesis that the MAC is an important contributor to atherosclerotic plaque development in the apoE^{-/-} mouse model. In fat fed apoE^{-/-}/C6^{-/-} mice, plaque size was markedly reduced and staining for MAC deposition within the plaques was also significantly reduced; the converse was true in apoE^{-/-}/Cd59a^{-/-} mice with much larger plaques compared to controls and increased MAC deposition. The mean total vessel area in apoE^{-/-}/C6^{-/-} mice was significantly smaller than that seen in apoE^{-/-} controls, while the lumen area remained the same between the two groups. This is due to arterial remodelling which occurs during atherosclerosis and maintains the lumen area in the face of increased obstruction caused by plaque growth. The extent of disease in these mice correlated with the extent of MAC deposition. In contrast to the markedly elevated MAC deposition, C3 staining was seen strongly in plaques from both apoE^{-/-}/Cd59a^{-/-} and

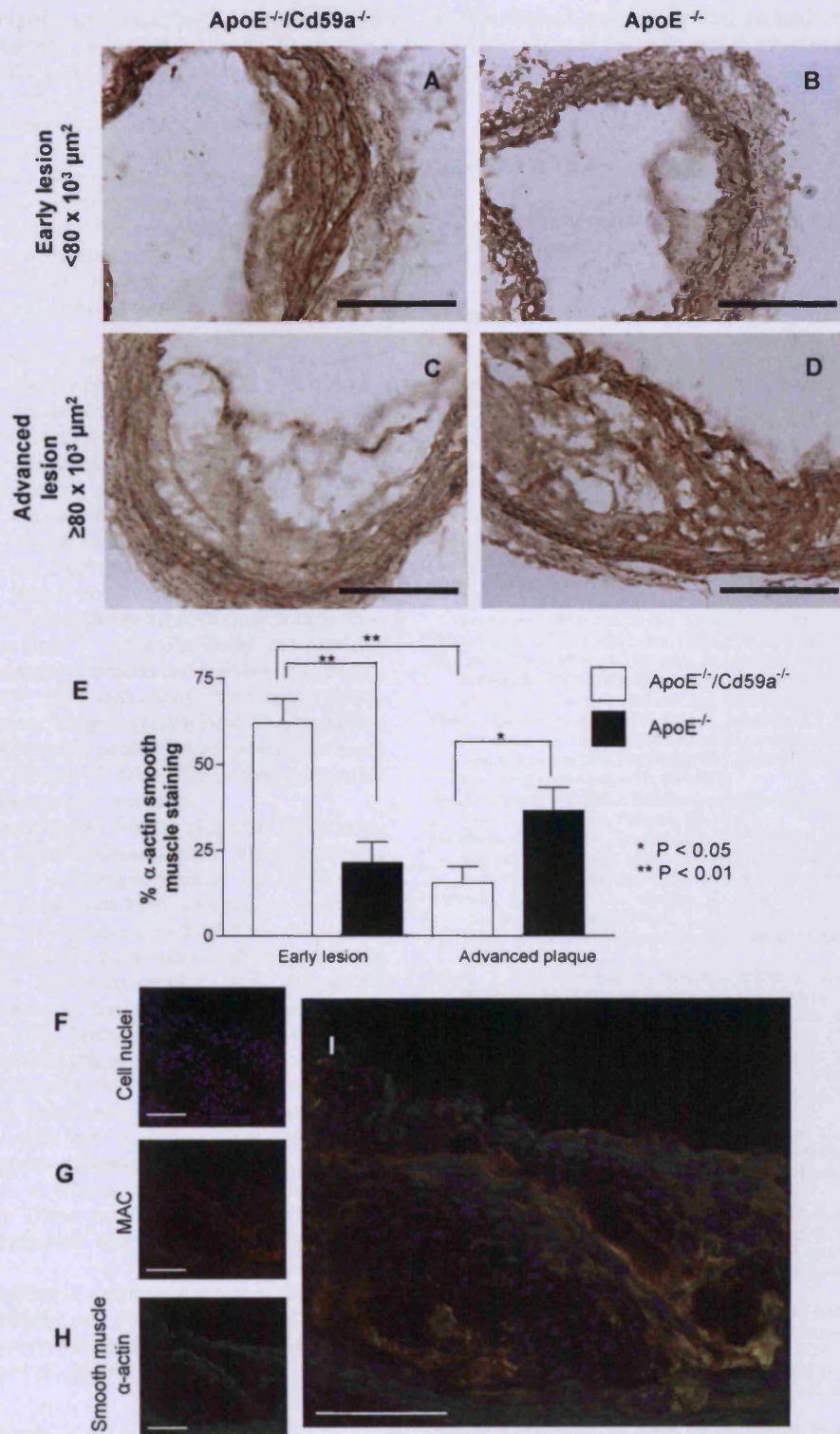


Fig. 5. Smooth muscle cell content in early and advanced lesions. Comparison of percentage smooth muscle α -actin staining in early plaques ($<80 \times 10^3 \mu\text{m}^2$) or advanced plaques (complex plaques with areas $\geq 80 \times 10^3 \mu\text{m}^2$) of apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} animals at 16 weeks, after 8 weeks of high-fat diet. Brachiocephalic artery sections were stained for smooth muscle cells with anti-smooth muscle α -actin and counterstained with haematoxylin (blue). The bars show group means \pm SEM. Scale bars for (A–D): 200 μm . (A) Representative section of an early lesion from an apoE^{-/-}/Cd59a^{-/-} mouse. (B) Representative section of an early lesion from an apoE^{-/-} control. (C) Representative section of an advanced lesion from an apoE^{-/-}/Cd59a^{-/-} mouse. (D) Representative section of an advanced lesion from an apoE^{-/-} control. (E) Percentage of plaque stained for smooth muscle α -actin in early and advanced plaques of apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice. (F–I) An atherosclerotic vessel from an apoE deficient mouse showing C9 deposition co-localised in regions of smooth muscle cells. Images show cell nuclei (blue), C9 (red) or smooth muscle α -actin (green) (F–H respectively). (I) Merged images correspond to the overlay of cell nuclei, C9 and smooth muscle α -actin (Fig. 1). Co-localisation of C9 with smooth muscle α -actin is shown in yellow. Scale bar = 100 μm .

apoE^{-/-} controls, an observation which has been previously noted (An et al., 2009). Most of the MAC deposited in the plaques is associated with extracellular debris and lipid, which accumulate in the core of advanced plaques. Activation of complement at this location is probably due to the presence of oxidised and enzymatically modified LDL, known to activate complement through the alternative pathway (Bhakdi, 1998). Of note, the expression of the other broadly expressed murine membrane complement regulators DAF and Crry was not different between the two groups, indicating that deficiency of CD59a was not compensated by increased expression of other regulators. Plaque lipid content and macrophage and T cell infiltration were also similar in test and control groups.

Smooth muscle cell proliferation is a characteristic feature of plaque formation. In small, simple plaques from apoE^{-/-}/Cd59a^{-/-} mice, smooth muscle cell accumulation was markedly increased compared to apoE^{-/-} controls, suggesting accelerated plaque development. In addition we observed an association between C9 deposition and regions of smooth muscle cell within atherosclerotic plaques. The MAC has previously been shown to cause aortic smooth muscle cell proliferation in vitro (Niculescu et al., 1999), which strengthens our impression that this is a MAC triggered event. In contrast, larger and more advanced plaques in apoE^{-/-}/Cd59a^{-/-} mice contained fewer smooth muscle cells than similar sized plaques from apoE^{-/-} controls, likely due to MAC-induced cytotoxicity. Reduced smooth muscle cell number in these late plaques in apoE^{-/-}/Cd59a^{-/-} mice will render the plaques unstable and vulnerable to rupture. These observations are compatible with those of Wu et al. (2009) who noted that the plaques present in their apoE^{-/-}/Cd59a^{-/-}/Cd59b^{-/-} mice had a more vulnerable phenotype than those of the apoE^{-/-} controls.

MAC deposition is a characteristic feature of human atherosclerotic lesions; the pattern of MAC deposition in murine plaques closely resembles that seen in humans (Rus et al., 1988). It is therefore reasonable to propose that MAC contributes to plaque formation and progression in human atherosclerosis in a similar manner to that which we and others have now demonstrated occurs in mice. Our data are in agreement with Wu et al., who also implicated the terminal pathway in the progression of atherosclerosis. More recently, Leung et al. (2009) have shown that DAF deficiency exacerbates disease in the ldl-r mouse model of atherosclerosis again implying that unregulated complement activation accelerates the progression of atherosclerosis. However, these findings are at odds with a previous study which had showed no effect of terminal pathway disruption (at the level of C5) on the progression of atherosclerosis in fat fed apoE^{-/-} mice (Patel et al., 2001). These contrasting findings highlight the need for further investigation into the role of C5 and C6 in atherosclerosis.

Inhibition of MAC formation is a potential strategy for therapy of atherosclerosis and a realistic one given that terminal pathway inhibitors are already in use in the clinic (Davis, 2008), with more under development (Song et al., 2003).

Conflict of interest statement

None declared.

Funding: This work was supported by the British Heart Foundation [Studentship number: FS/05/087/19466] and the Wellcome Trust [Programme Grant number: 068590 to BPM].

Acknowledgements

We thank Marieta Ruseva, Rhodri Turner, Dr Simone Meuter, Dr James Neal and Dr Claudia Calder for help and advice with

immunohistochemistry, Dr Paul Brennan and Dr Claire Harris for critical reading of the manuscript. We also thank all the staff at the Biomedical Services Unit at Cardiff University for technical support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2009.10.035.

References

- An, G., Miwa, T., Song, W., Lawson, J., Rader, D., Zhang, Y., Song, W., 2009. CD59 but not DAF deficiency accelerates atherosclerosis in female ApoE knockout mice. *Mol. Immunol.* 46, 1702–1709.
- Baalasubramanian, S., Harris, C., Donev, R., Mizuno, M., Omidvar, N., Song, W., Morgan, B., 2004. CD59a is the primary regulator of membrane attack complex assembly in the mouse. *J. Immunol.* 173, 3684–3692.
- Benzaquen, L., Nicholson-Weller, A., Halperin, J., 1994. Terminal complement proteins C5b-9 release basic fibroblast growth factor and platelet-derived growth factor from endothelial cells. *J. Exp. Med.* 179, 985–992.
- Bhakdi, S., 1998. Complement and atherogenesis: the unknown connection. *Ann. Med.* 30, 503–507.
- Bhatia, V., Yun, S., Leung, V., Grimsditch, D., Benson, G., Botto, M., Boyle, J., Haskard, D., 2007. Complement C1q reduces early atherosclerosis in low-density lipoprotein receptor-deficient mice. *Am. J. Pathol.* 170, 416–426.
- Davis, J., 2008. Eculizumab. *Am. J. Health Syst. Pharm.* 65, 1609–1615.
- Donev, R., Sivasankar, B., Mizuno, M., Morgan, B., 2008. The mouse complement regulator CD59b is significantly expressed only in testis and plays roles in sperm acrosome activation and motility. *Mol. Immunol.* 45, 534–542.
- Falk, R., Dalmaso, A., Kim, Y., Tsai, C., Scheinman, J., Gewurz, H., Michael, A., 1983. Neoantigen of the polymerized ninth component of complement. Characterization of a monoclonal antibody and immunohistochemical localization in renal disease. *J. Clin. Invest.* 72, 560–573.
- Fan, J., Watanabe, T., 2003. Inflammatory reactions in the pathogenesis of atherosclerosis. *J. Atheroscler. Thromb.* 10, 63–71.
- Fondevila, C., Shen, X., Tsuchihashi, S., Uchida, Y., Freitas, M., Ke, B., Busuttill, R., Kupiec-Weglinski, J., 2008. The membrane attack complex (C5b-9) in liver cold ischemia and reperfusion injury. *Liver Transpl.* 14, 1133–1141.
- Johnson, J., George, S., Newby, A., Jackson, C., 2005. Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. *Proc. Natl. Acad. Sci. USA* 102, 15575–15580.
- Leung, V., Yun, S., Botto, M., Mason, J., Malik, T., Song, W., Paixao-Cavalcante, D., Pickering, M., Boyle, J., Haskard, D., 2009. Decay-accelerating factor suppresses complement C3 activation and retards atherosclerosis in low-density lipoprotein receptor-deficient mice. *Am. J. Pathol.* 175, 1757–1767.
- Li, B., Sallee, C., Dehoff, M., Foley, S., Molina, H., Holers, V., 1993. Mouse Crry/p65. Characterization of monoclonal antibodies and the tissue distribution of a monoclonal homologue of human MCP and DAF. *J. Immunol.* 151, 4295–4305.
- McCurry, K., Kooyman, D., Diamond, L., Byrne, G., Martin, M., Logan, J., Platt, J., 1995. Human complement regulatory proteins in transgenic animals regulate complement activation in xenoperfused organs. *Transpl. Proc.* 27, 317–318.
- Meuwissen, M., van der Wal, A., Niessen, H., Koch, K., de Winter, R., van der Loos, C., Rittersma, S., Chamuleau, S., Tijssen, J., Becker, A., Piek, J., 2006. Colocalisation of intraplaque C reactive protein, complement, oxidised low density lipoprotein, and macrophages in stable and unstable angina and acute myocardial infarction. *J. Clin. Pathol.* 59, 196–201.
- Niculescu, F., Badea, T., Rus, H., 1999. Sublytic C5b-9 induces proliferation of human aortic smooth muscle cells: role of mitogen activated protein kinase and phosphatidylinositol 3-kinase. *Atherosclerosis* 142, 47–56.
- Niculescu, F., Niculescu, T., Rus, H., 2004. C5b-9 terminal complement complex assembly on apoptotic cells in human arterial wall with atherosclerosis. *Exp. Mol. Pathol.* 76, 17–23.
- Nilsson, J., Hansson, G., 2008. Autoimmunity in atherosclerosis: a protective response losing control? *J. Intern. Med.* 263, 464–478.
- Patel, S., Thelander, E., Hernandez, M., Montenegro, J., Hassing, H., Burton, C., Mundt, S., Hermanowski-Vosatka, A., Wright, S., Chao, Y., Detmers, P., 2001. ApoE^{-/-} mice develop atherosclerosis in the absence of complement component C5. *Biochem. Biophys. Res. Commun.* 286, 164–170.
- Persson, L., Borén, J., Robertson, A., Wallenius, V., Hansson, G., Pekna, M., 2004. Lack of complement factor C3, but not factor B, increases hyperlipidemia and atherosclerosis in apolipoprotein E^{-/-} low-density lipoprotein receptor^{-/-} mice. *Arterioscler. Thromb. Vasc. Biol.* 24, 1062–1067.
- Rosenfeld, M., Carson, K., Johnson, J., Williams, H., Jackson, C., Schwartz, S., 2002. Animal models of spontaneous plaque rupture: the holy grail of experimental atherosclerosis research. *Curr. Atheroscler. Rep.* 4, 238–242.
- Ross, R., 1999. Atherosclerosis—an inflammatory disease. *N. Engl. J. Med.* 340, 115–126.

- Rus, H., Niculescu, F., Vlaicu, R., 1988. Co-localization of terminal C5b-9 complement complexes and macrophages in human atherosclerotic arterial walls. *Immunol. Lett.* 19, 27–32.
- Schmiedt, W., Kinscherf, R., Deigner, H., Kamencic, H., Nauen, O., Kilo, J., Oelert, H., Metz, J., Bhakdi, S., 1998. Complement C6 deficiency protects against diet-induced atherosclerosis in rabbits. *Arterioscler. Thromb. Vasc. Biol.* 18, 1790–1795.
- Seifert, P., Kazatchkine, M., 1988. The complement system in atherosclerosis. *Atherosclerosis* 73, 91–104.
- Song, H., He, C., Knaak, C., Guthridge, J., Holers, V., Tomlinson, S., 2003. Complement receptor 2-mediated targeting of complement inhibitors to sites of complement activation. *J. Clin. Invest.* 111, 1875–1885.
- Spiller, O., Harris, C., Morgan, B., 1999. Efficient generation of monoclonal antibodies against surface-expressed proteins by hyperexpression in rodent cells. *J. Immunol. Methods* 224, 51–60.
- Vlaicu, R., Niculescu, F., Rus, H., Cristea, A., 1985. Immunohistochemical localization of the terminal C5b-9 complement complex in human aortic fibrous plaque. *Atherosclerosis* 57, 163–177.
- Wu, G., Hu, W., Shahsafaei, A., Song, W., Dobarro, M., Sukhova, G., Bronson, R., Shi, G., Rother, R., Halperin, J., Qin, X., 2009. Complement regulator CD59 protects against atherosclerosis by restricting the formation of complement membrane attack complex. *Circ. Res.* 104, 550–558.
- Yasojima, K., Schwab, C., McGeer, E., McGeer, P., 2001. Complement components, but not complement inhibitors, are upregulated in atherosclerotic plaques. *Arterioscler. Thromb. Vasc. Biol.* 21, 1214–1219.
- Yun, S., Leung, V., Botto, M., Boyle, J., Haskard, D., 2008. Brief report: accelerated atherosclerosis in low-density lipoprotein receptor-deficient mice lacking the membrane-bound complement regulator CD59. *Arterioscler. Thromb. Vasc. Biol.* 28, 1714–1716.

Supplemental Methods

Reagents

All standard laboratory chemicals were purchased from Sigma (Poole, Dorset, UK) or Fisher Scientific (Loughborough, UK).

Animals

B6.129-Cd59atm1Bpm (Cd59a^{-/-}) mice were generated as previously described, (Holt et al., 2001; Morgan et al., 2006) and back-crossed onto the C57BL/6 background for eight generations. C6-deficient mice, originally identified as a natural mutation in a wild mouse strain and bred onto the C3H/He background, were back-crossed five generations onto C57Bl/6. (Morgan et al., 2006) ApoE^{-/-} mice were originally provided by J. Breslow (The Rockefeller University, New York). The strain background of the apoE^{-/-} mice was 71% C57BL/6 and 29% 129. These apoE^{-/-} mice were crossed with either Cd59a^{-/-} or C6^{-/-} mice to generate apoE^{-/-}/Cd59a^{-/-} or apoE^{-/-}/C6^{-/-} double knockout mice. In both cases, apoE^{-/-} single knockout gender, strain, age and litter-matched mice provided the appropriate controls.

Genomic DNA was extracted from tail tips for genotyping by polymerase chain reaction (apoE, Cd59a), while C6 deficiency was identified either by assessing serum haemolytic activity in a classical pathway assay or detecting C6 protein in immunoblots using a cross-reactive anti-C6 antiserum. (Morgan, 2000)

Termination

Animals were anaesthetised by intraperitoneal injection of sodium pentobarbitone and weighed before exsanguination and arterial perfusion via the abdominal aorta with phosphate-buffered saline (PBS) at a constant pressure of 100 mmHg, with outflow through the incised jugular veins. The heart was removed from each animal to calculate the heart:body weight ratio. The brachiocephalic artery was chosen as the site for evaluation of plaque progression because it has been demonstrated to be a site of predilection for plaque development in the apoE^{-/-} mice, particularly prone to development of late plaques and plaque rupture.

Histology and Immunohistochemistry

Sections to be stained for macrophages and α -smooth muscle actin were fixed in ice-cold acetone and endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide solution. Sections were blocked with either an avidin/biotin blocking kit (Vector laboratories, Peterborough, UK) followed by a mixture of 10% goat serum and 2.5% mouse serum, or with the Mouse-On-Mouse kit (Vector Laboratories). Sections were then incubated with the appropriate biotinylated secondary antibodies: biotinylated goat and rat (Vector Laboratories) (diluted 1:250 in 1% (wt/vol) bovine serum albumin (BSA) in PBS) followed by horseradish peroxidase-labeled Extravidin (Sigma-Aldrich) (diluted 1:500 in 1% (wt/vol) BSA in PBS), Vectastain Elite ABC (Vector Laboratories). Immunopositive cells were detected using diaminobenzidine (DAB) (Vector laboratories). α -smooth muscle actin was also detected using Fluorescein Avidin D (Vector

Laboratories)(1:100). T-cell staining was carried out on sections fixed in 4% formaldehyde and blocked with 10% goat serum. The presence of T-cells was revealed with hamster anti-mouse CD3 antibody followed by biotinylated goat anti-hamster IgG and subsequent detection using avidin-biotin (DakoCytomation) followed by New Fuchsin solution (New Fuchsin Kit; DakoCytomation). All sections were counterstained with Mayer's haematoxylin. Negative controls, where the primary antibody was replaced with either mouse or rat IgG at the same dilution, were always included.

Sections were also immunostained for complement components C3 (rat anti-mouse C3 mAb 11H9; Hy-Cult Biotechnology, The Netherlands) and C9/MAC (rabbit anti-rat C9 prepared in-house using standard immunisation procedures). In this case, sections were blocked using 2% bovine serum albumin in PBS and bound antibodies were detected with either Alexa fluor 594-labelled donkey anti-rat IgG (20 µg/mL) or Alexa fluor 594-labeled goat anti-rabbit IgG (20 µg/mL) (both from Molecular Probes Inc, Eugene, OR, USA). All sections were counter-stained with DAPI. A negative control, where the primary antibody was replaced with either rabbit or rat IgG (of the same isotype) at the same concentration, was always included.

In each case positive staining was expressed as a percentage fractional area of the lesion as analysed by a computerised image-analysis program (Image ProPlus™ software version 4.0, Media Cybernetics, Carlsbad, California, USA).

Supplemental references

- Holt D., Botto M., Bygrave A., Hanna S., Walport M. and Morgan B. (2001)
Targeted deletion of the CD59 gene causes spontaneous intravascular hemolysis and hemoglobinuria. *Blood* **98**, 442-9.
- Morgan B. (2000) Measurement of complement hemolytic activity, generation of complement-depleted sera, and production of hemolytic intermediates. *Methods Mol Biol* **150**, 61-71.
- Morgan B., Chamberlain-Banoub J., Neal J., Song W., Mizuno M. and Harris C. (2006) The membrane attack pathway of complement drives pathology in passively induced experimental autoimmune myasthenia gravis in mice. *Clin Exp Immunol* **146**, 294-302.

Figure I. Measurement of lipid content in plaques. Comparison of percentage of plaque area staining for lipid with oil red O between apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} animals after 8 weeks of high-fat diet. Sections of the brachiocephalic artery were stained with Oil Red O (red) and counterstained with haematoxylin (blue). Bars show group means ± SEM. Scale bars: 200µm.

(A) Representative section from an apoE^{-/-}/Cd59a^{-/-} mouse.

(B) Representative section from an apoE^{-/-} mouse.

(C) Mean percentage staining with oil red O in the lesion area in apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice.

Figure II. Macrophage staining in plaques. Comparison of percentage of plaque area staining with a macrophage marker in apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} animals after 8 weeks of high-fat diet. Brachiocephalic artery sections were stained with MOMA-2 (brown) for macrophages and counterstained with haematoxylin (blue). The bars show group means ± SEM. Scale bars: 50µm. Representative sections from an apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mouse stained for MOMA-2 (**A** and **B** respectively). Mean percentage of plaque stained with MOMA-2 (**C**) in apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice.

Figure III. Measurement of T-cell staining in plaques. Comparison of percentage CD3-positive staining within the lesions of apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice after 8 weeks of high-fat diet. Sections of the brachiocephalic artery were stained with anti-CD3 antibody (red) and counterstained with haematoxylin (blue). Bars show group means ± SEM. Scale bars: 50µm.

(A) Representative section from an apoE^{-/-}/Cd59a^{-/-} mouse.

(B) Representative section from an apoE^{-/-} mouse.

(C) Mean percentage CD3-stained lesion area in apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice.

Table I. Body weight, heart:body weight ratio, lipaemia index, total cholesterol and triglyceride levels in 16 week old apoE^{-/-}/Cd59a^{-/-} and apoE^{-/-} mice. All animals were fed a high fat diet from 8 weeks old for 8 weeks. Data are shown as mean ± SEM.

Group	Body Weight (g)	Heart:Body Weight Ratio (x10 ⁻³)	Cholesterol (mmol/L)	Triglyceride (mmol/L)	Lipaemia Index
apoE ^{-/-} /CD59a ^{-/-}	41 ± 2.3 (n=12)	4.7 ± 0.2 (n=13)	29.0 ± 1.7 (n=21)	2.7 ± 0.3 (n=22)	25.0 ± 3.7 (n=14)
apoE ^{-/-}	44 ± 2.1 (n=16)	4.4 ± 0.2 (n=18)	34.0 ± 1.9 (n=25) P=0.08	3.2 ± 0.3 (n=28) P=0.22	36.8 ± 5.4 (n=16) P = 0.09

Short Communication

CD55 Deficiency Protects against Atherosclerosis in ApoE-Deficient Mice via C3a Modulation of Lipid Metabolism

Ruth D. Lewis,* Mark J. Perry,[†]
Irina A. Guschina,[‡] Christopher L. Jackson,[§]
B. Paul Morgan,* and Timothy R. Hughes*

From the Complement Biology Group,* Department of Infection, Immunity and Biochemistry, School of Medicine, and the School of Biosciences,[‡] Cardiff University, Cardiff; the Department of Anatomy,[†] University of Bristol Veterinary School, Bristol; and the Bristol Heart Institute,[§] University of Bristol, Bristol, United Kingdom

Atherosclerosis, the leading cause of death in the Western world, is driven by chronic inflammation within the artery wall. Elements of the complement cascade are implicated in the pathogenesis, because complement proteins and their activation products are found in the atherosclerotic plaque. We examined the role of CD55, a membrane inhibitor of the complement component 3 (C3) convertase, which converts C3 into C3a and C3b, in atherosclerosis. CD55-deficient (*CD55*^{-/-}) mice were crossed onto the atherosclerosis-prone apolipoprotein E (apoE)-deficient (*apoE*^{-/-}) background. High fat-fed male *apoE*^{-/-}/*CD55*^{-/-} mice were strongly protected from developing atherosclerosis compared with *apoE*^{-/-} controls. Lipid profiling showed significantly lower levels of triglycerides, nonesterified fatty acids, and cholesterol in *apoE*^{-/-}/*CD55*^{-/-} mice than that in controls after high-fat feeding, whereas body fat in *apoE*^{-/-}/*CD55*^{-/-} mice content was increased. Plasma levels of C3 fell, whereas concentrations of C3adesArg (alias acylation stimulating protein; ASP), produced by serum carboxypeptidase N-mediated desargination of C3a, increased in nonfasted high fat-fed *apoE*^{-/-}/*CD55*^{-/-} mice, indicating complement activation. Thus, complement dysregulation in the absence of CD55 provoked increased C3adesArg production that, in turn, caused altered lipid handling, resulting in atheroprotection and increased adiposity. Interventions that target complement activation in adipose tissue should be ex-

plored as lipid-decreasing strategies. (Am J Pathol 2011, 179:1601–1607; DOI: 10.1016/j.ajpath.2011.06.015)

Atherosclerosis, long considered a passive process of accumulation of lipid in blood vessel walls accompanied by smooth muscle proliferation and culminating in loss of endothelial integrity, is now recognized as an active process with immune cells and mediators accumulating in forming plaques from the earliest stages, and inflammation central to disease progression.^{1,2} Both innate immunity and adaptive immunity play roles, with mediators of both arms of the immune system present in the plaque.³ Among the innate immune components, complement (C) and its activation products are abundant and suggested to play critical roles in atherogenesis, both directly through local cell damage and indirectly by attracting and activating immune cells.^{4–9} C comprises three activation pathways, alternative, classical, and lectin, and activation of each has been shown in atherosclerosis.^{10–12} Effector molecules generated during C activation include anaphylactic and chemotactic fragments (C3a, C5a), opsonic fragments (C4b, C3b), and the cytotoxic membrane attack complex (MAC). C3a and C5a may promote infiltration of inflammatory cells into the plaque^{13,14}; this activity is regulated by carboxypeptidase N, which clips the carboxy-terminal arginine. Although C3adesArg is inactive as an inflammatory mediator, a growing body of literature reports that it has potent adipogenic activity, promoting lipid uptake, triglyceride synthesis, and storage in adipocytes.^{15,16} C3-deficient mice, which cannot generate C3adesArg, have delayed postprandial triglyceride clearance, together with higher

Supported by the British Heart Foundation studentship no. FS/05/087/19466 and by the Wellcome Trust programme grant 068590 (B.P.M.).

Accepted for publication June 3, 2011.

B.P.M. and T.R.H. contributed equally to this work.

Address reprint requests to Timothy R. Hughes, Ph.D., Complement Biology Group, Department of Infection, Immunity and Biochemistry, School of Medicine, Cardiff University, Cardiff CF144XN, United Kingdom. E-mail: hughestr@cardiff.ac.uk.

levels of nonesterified fatty acids (NEFAs), and significantly reduced adiposity than do wild types.^{17,18}

Animal models have contributed to establishing the relevance of C to atherosclerosis. Almost 40 years ago, studies in fat-fed C6-deficient rabbits showed that absence of C6, an essential component of the MAC, markedly inhibited plaque formation,¹⁹ findings replicated and extended more recently.²⁰ Atherosclerosis-prone mouse strains back-crossed onto C-deficient strains have been used to further explore roles of C. Fat-fed apolipoprotein E (*apoE*)-deficient (*apoE*^{-/-}) mice lacking C6 showed significantly attenuated disease, replicating findings in rabbits, whereas absence of CD59a, the principle murine regulator of MAC assembly, exacerbated disease.²¹⁻²³ In contrast, deficiency of C5, removing the capacity to form C5a and MAC, had no effect on atherosclerosis progression in *apoE*^{-/-} mice,²⁴ whereas deficiency of C3, but not factor B, exacerbated plaque formation and caused hyperlipidemia on *apoE*^{-/-}/*ldlr*^{-/-} or *ldlr*^{-/-} backgrounds.^{25,26}

CD55 (decay accelerating factor) is a 70-kDa membrane-bound C regulator that accelerates decay of the C3 convertase. To test the effect of CD55 deficiency on progression of atherosclerosis, *CD55*^{-/-} mice were back-crossed onto the *apoE*^{-/-} background and fed an atherogenic diet. Informed by our findings with CD59a deficiency, we anticipated that CD55 deficiency would exacerbate disease. Instead, deficiency of CD55 was highly protective for atherosclerosis; plaques were smaller and remained structurally simple. We here show that altered lipid handling resulting from C dysregulation is responsible for reduced atherogenesis in *CD55*^{-/-} mice. The demonstration that C activation products markedly affect lipid handling and plaque formation will influence future strategies for treatment of atherosclerosis.

Materials and Methods

Reagents and Animals

All chemicals were purchased from Sigma-Aldrich (Poole, UK) or Fisher Scientific (Loughborough, UK). Fatty acid and lipid standards were from Nu-Chek-Pre Inc. (Elysian, MN) and Sigma-Aldrich, respectively. Silica gel G plates were from Merck KGaA (Darmstadt, Germany).

CD55 knockout (*CD55*^{-/-}) mice were provided by Prof Wenchao Song (University of Philadelphia, Philadelphia, PA) and back-crossed onto C57BL/6 for nine generations. *ApoE*^{-/-} mice were originally provided by J. Breslow (Rockefeller University, New York, NY). The strain background of these original mice was 71% C57BL/6 and 29% 129. The *apoE*^{-/-} mice were crossed with *CD55*^{-/-} mice to generate *apoE*^{-/-}/*CD55*^{-/-} double knockouts along with *apoE*^{-/-} single knockouts; these sex-, strain-, and age-matched littermates provided the appropriate controls. Mice were genotyped by polymerase chain reaction with the use of genomic DNA extracted from tail tips.

Male mice aged 8 weeks were fed a high-fat diet, containing 21% (wt/wt) pork lard and supplemented with 0.15% (wt/wt) cholesterol (Special Diet Services, Witham,

UK), for 12 weeks. Animals were housed in a specific pathogen-free environment. Some mice were deprived of food for 16 hours overnight to obtain baseline levels of various parameters. All studies and protocols were approved by the institutional ethics review committee and the United Kingdom Home Office and conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Histology and Immunohistochemistry

Mice were anesthetized by intraperitoneal injection of sodium pentobarbitone and weighed before exsanguination by arterial perfusion via the abdominal aorta with PBS at a constant pressure of 100 mmHg, with outflow through the incised jugular veins. Brachiocephalic arteries were removed with a piece of the aortic arch and the stump of the right subclavian artery still attached to aid orientation during processing, immediately embedded in optimum cutting temperature compound (RA Lamb Ltd, Eastbourne, UK), and snap-frozen in liquid N₂.

Serial transverse 7- μ m sections were cut along the brachiocephalic artery, starting from the proximal end. Sections were stained with Miller's Elastic/Van Gieson (Sigma-Aldrich). Macrophages and smooth muscle cells were identified with anti-murine macrophage mAb (diluted 1:100; F4/80; Serotec, Oxford, UK) and anti- α -smooth muscle actin mAb (diluted 1:100; clone α -1-A4; Sigma-Aldrich), respectively. Sections were fixed in ice-cold acetone and blocked with either avidin/biotin blocking kit (Vector Laboratories, Peterborough, UK) followed by 10% goat serum or Mouse-on-Mouse kit (Vector Laboratories). Blocked sections were incubated with appropriate biotinylated secondary antibodies: goat anti-rat Ig (Vector Laboratories; 3.5 μ g/mL in 10% mouse serum) or anti-mouse Ig diluted as directed (Mouse-on-Mouse kit). Staining was developed with Fluorescein-Avidin D (diluted 1:200 in 2% bovine serum albumin in PBS; Vector Laboratories), and cell nuclei were counterstained with DAPI (Sigma-Aldrich).

Immunostaining for C activation used either rat anti-mouse C3b/iC3b mAb clone 2/11 (5 μ g/mL; Hycult Biotech) or affinity-purified rabbit anti-rat/mouse C9 generated in house, proven reactive with MAC in mouse tissues (2 μ g/mL).²³ For C3 and C9 staining, sections were fixed in acetone at 4°C, blocked in 2% bovine serum albumin in PBS, and, after staining with primary antibody, developed with either Alexa Fluor 488-labeled goat anti-rat (20 μ g/mL; Invitrogen, Carlsbad, CA) or Alexa Fluor 594-labeled goat anti-rabbit IgG (20 μ g/mL; Molecular Probes, Eugene, OR) respectively. Nuclei were counterstained with DAPI.

Negative controls included replacement of primary antibody with IgG isotype control. Staining was expressed as the percentage of lesion area staining positive, assessed by computerized image analysis (Image ProPlus 4.0; Media Cybernetics, Carlsbad, CA).

Histomorphometry

Five sections were taken per mouse at the same relative positions along the brachiocephalic artery and were assessed for the presence of plaque with the use of an established method.²⁵ Plaque area was calculated with image analysis as above.

Measuring Serum Triglycerides, Cholesterol, and NEFAs

Mice were sacrificed between 9 AM and 11 AM, blood (1 mL) was collected into tubes with or without EDTA, and serum or plasma was separated by centrifugation. Triglyceride and cholesterol levels were measured at the Clinical Biochemistry Laboratories, University Hospital Cardiff, on an Aeroset automated analyzer (Abbott Diagnostics, Berkshire, UK). For NEFAs, lipids were extracted and separated by one-dimensional thin-layer chromatography on 10 × 10-cm silica gel G plates, double developed with toluene/hexane/formic acid (140:60:1, v/v/v) for the entire plate, followed by hexane/diethyl ether/formic acid (60:40:1, v/v/v) to half height. Plates were sprayed with 0.05% (wt/v) 8-anilino-4-naphthosulphonic acid in methanol and viewed under UV light to show lipids. Free fatty acids were scraped from the plate and were identified and quantified by gas chromatography.

Measuring C3adesArg

C3adesArg was measured in a sandwich ELISA with the use of a pair of anti-mouse C3a mAbs, one unlabeled as capture (0.2 µg/mL), the other biotinylated as detection (0.5 µg/mL), and recombinant mouse C3a (100 to 0.78 ng/mL) as standards (all from BD Pharmingen, San Diego, CA). Appropriately diluted plasma samples were included. The assay was developed with streptavidin-peroxidase (1:5000; Jackson ImmunoResearch, West Grove, PA).

Measuring Mouse C3

Serum C3 levels were measured by ELISA essentially as previously described,²⁷ except that rat anti-mouse C3 (2 µg/mL; clone 11H9; Hycult Biotech) was used as the capture antibody. A standard curve of known concentrations, starting from 0.5 µg/mL, was produced with the use of purified mouse C3 (a kind gift from Dr Claire Harris; Cardiff University).

Body Fat Measurement

Percentage of body fat was measured by dual-energy X-ray absorptiometry scanning of whole animals with the use of a PIXImus scanner (Lunar Corp, Madison, WI) with small animal software.

Statistical Analysis

Data are expressed as mean ± SEM, and significance was tested by two-tailed unpaired Student's *t*-test (GraphPad Prism software, version 3.0; GraphPad Software Inc., San Diego, CA), with significance assumed at *P* < 0.05.

Results

Deficiency of CD55 Protects from Atherosclerosis in apoE^{-/-} Mice

Matched apoE^{-/-} and apoE^{-/-}/CD55^{-/-} mice were sacrificed at 20 weeks of age after 12 weeks on a high-fat diet, and the extent of atherosclerosis was assessed in the brachiocephalic arteries, a known site of predilection for plaque development.²⁸ Plaque cross-sectional area, assessed at multiple sites along the vessel, was reduced threefold in apoE^{-/-}/CD55^{-/-} mice compared with apoE^{-/-} controls (54.7 ± 11.2 × 10³ µm² versus 155.2 ± 16.8 × 10³ µm²; *P* < 0.001; Figure 1, A–C). Plaque stage and complexity were further explored by measuring smooth muscle cell content and macrophage infiltration; smooth muscle cells as a proportion of total cell number in apoE^{-/-}/CD55^{-/-} plaques were significantly lower than in apoE^{-/-} plaques (9.3% ± 2.0% versus 18.0% ± 2.8%; *P* < 0.05; Figure 1, D–F). Plaque macrophage content was similar in the groups (19.4% ± 6.5% versus 18.8% ± 4.2%; Figure 1, G–I).

To address whether CD55 deficiency influenced local C activation, plaques were stained for C3 fragments and MAC. C3 fragment deposition was assessed with mAb 3/26, a neoepitope-specific mAb that specifically detects C3b, iC3b, and C3c in tissues, whereas MAC was detected with affinity-purified anti-rat/mouse C9. Percentages of plaque area stained for C3b/iC3b/C3c and MAC were twofold reduced in plaques from apoE^{-/-}/CD55^{-/-} mice compared with apoE^{-/-} controls (C3b/iC3b/C3c: 28.0% ± 8.1% versus 57.3% ± 7.7%; *P* < 0.05; Figure 1, J–L; MAC: 17.9% ± 3.5% versus 30.5% ± 4.0%; *P* < 0.05; Figure 1, M–O).

CD55 Deficiency Is Associated with Reduced Serum Triglyceride and Cholesterol Levels

To test whether the absence of CD55 affected lipid handling, lipid levels were measured in apoE^{-/-}/CD55^{-/-} mice and in apoE^{-/-} controls at 8 weeks old on normal diet and at 20 weeks old after 12 weeks on a high-fat diet. At 8 weeks, triglyceride levels were markedly reduced in the apoE^{-/-}/CD55^{-/-} mice than in the apoE^{-/-} controls (2.1 ± 0.1 mmol/L versus 5.1 ± 0.6 mmol/L; *P* < 0.01; Figure 2A); cholesterol levels were not significantly different between these groups (16.1 ± 1.4 mmol/L versus 18.2 ± 0.8 mmol/L; Figure 2B). After 12 weeks of fat feeding, triglyceride levels were little changed, as expected in the apoE^{-/-} model,²⁹ and remained significantly lower in the apoE^{-/-}/CD55^{-/-} mice compared with the apoE^{-/-} controls (1.7 ± 0.2 mmol/L versus 3.6 ± 0.5 mmol/L; *P* < 0.01; Figure 2A). Cholesterol levels were increased after 12 weeks of fat feeding in both groups but were significantly lower in the apoE^{-/-}/CD55^{-/-} mice than in the apoE^{-/-} controls (29.0 ± 1.8 mmol/L versus 38.3 ± 2.5 mmol/L; *P* < 0.01; Figure 2B). Plasma levels of NEFAs were measured in mice before and after being fed the high-fat diet. Significant increases in NEFA concentrations were seen in both groups after 12 weeks of a

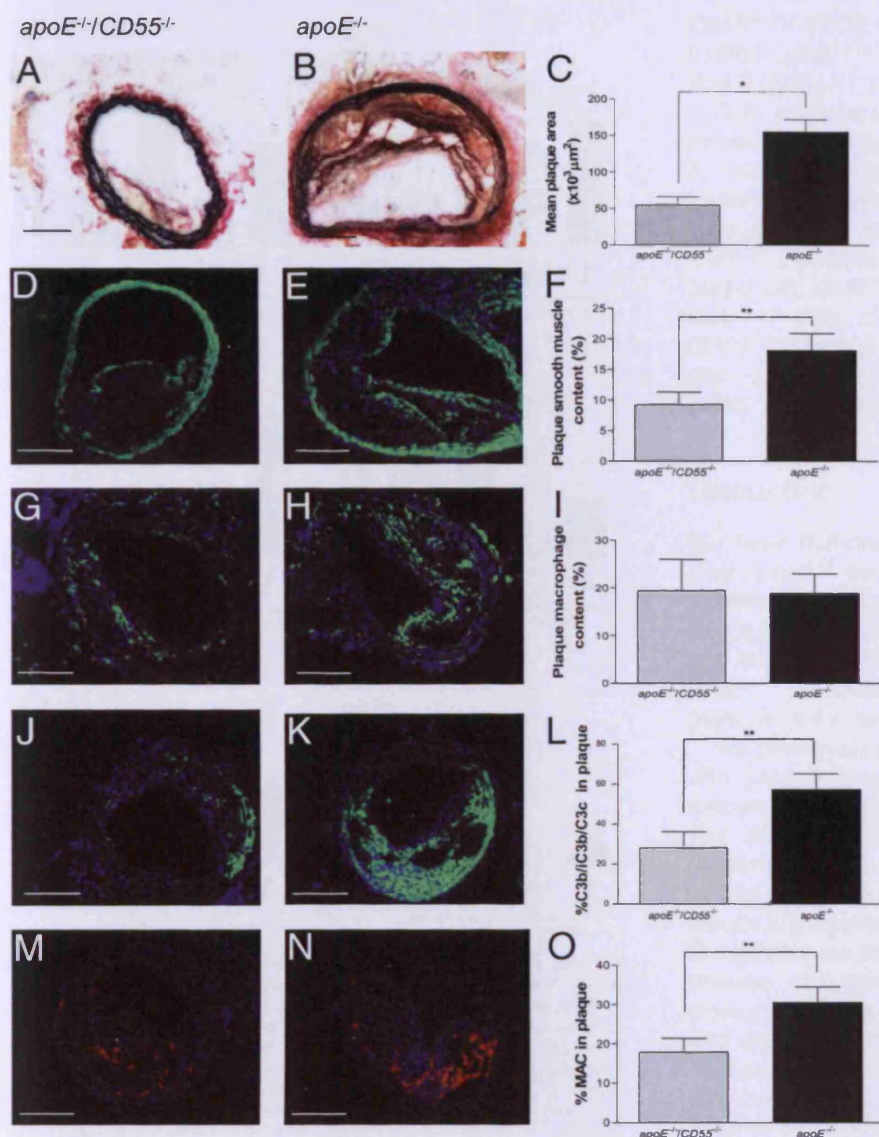


Figure 1. Assessment of atherosclerosis in brachiocephalic arteries of *apoE^{-/-}/CD55^{-/-}* and *apoE^{-/-}* control mice after 12 weeks of high-fat feeding. Histologic appearance of representative sections of brachiocephalic arteries from a single experiment that compared *apoE^{-/-}/CD55^{-/-}* mice (A, D, G, J, and M) with *apoE^{-/-}* control mice (B, E, H, K, and N); both groups had been fed a high-fat diet for 12 weeks. Sections were stained with Miller's/Elastic Van Gieson (A and B, *n* = 13 and 12, respectively); immunostained for α -smooth muscle actin (smooth muscle cells; D and E, *n* = 7 and 7, respectively); F/480 (macrophages; G and H, *n* = 8 and 6, respectively); C3 fragments (J and K; *n* = 7 and 6, respectively), or MAC deposition (M and N; *n* = 7 and 7, respectively). Scale bars: 200 μ m (A, B, D, E, G, H, J, K, M, and N). C, F, I, L, and O: Composite data from each group compiled from digital analyses of individual sections as described in *Materials and Methods*. C: Average plaque size for the two groups as determined from five individual sections taken from each mouse. F, I, L, and O: Mean percentage of plaque area staining for smooth muscle cells, macrophages, C3b/iC3b/C3c, and MAC, respectively. Mean values \pm SEM are represented. **P* < 0.0001, ***P* < 0.05.

high-fat diet (*apoE^{-/-}/CD55^{-/-}*: 0.153 ± 0.016 mg/mL versus 0.333 ± 0.041 mg/mL before and after the high-fat diet, respectively; *P* < 0.001; *apoE^{-/-}*: 0.121 ± 0.006 mg/mL versus 0.505 ± 0.046 mg/mL before and after the high-fat diet, respectively; *P* < 0.001; Figure 2C). Although no significant differences were observed in NEFA levels between *apoE^{-/-}/CD55^{-/-}* and *apoE^{-/-}* mice before fat feeding, levels were significantly lower in *apoE^{-/-}/CD55^{-/-}* mice than in *apoE^{-/-}* controls after 12 weeks on a high-fat diet (0.333 ± 0.041 mg/mL versus 0.505 ± 0.046 mg/mL; *apoE^{-/-}/CD55^{-/-}* and *apoE^{-/-}*, respectively; *P* < 0.01; Figure 2C).

CD55 Deficiency Is Associated with Increased C3 Turnover and Plasma C3adesArg Levels

C3adesArg, also known as ASP, is a stable product of C3 activation and a potent adipokine that stimulates uptake of triglycerides and NEFAs, enhances triglyceride synthesis and storage, and inhibits triglyceride lipolysis in

adipose tissue.³⁰⁻³² Circulating nonfasting C3 and its activation product C3adesArg (Figure 2, D and E, respectively) were measured before and after the high-fat feeding in *apoE^{-/-}/CD55^{-/-}* and *apoE^{-/-}* mice. C3 levels were significantly reduced in both *apoE^{-/-}/CD55^{-/-}* and *apoE^{-/-}* mice after 12 weeks of high-fat feeding compared with before the high-fat feeding values (*apoE^{-/-}/CD55^{-/-}*: 0.42 ± 0.03 mg/mL versus 0.3 ± 0.04 mg/mL; *P* < 0.01, respectively; *apoE^{-/-}*: 0.48 ± 0.05 mg/mL versus 0.22 ± 0.05 mg/mL; *P* < 0.05, respectively). C3 levels were not significantly different between *apoE^{-/-}/CD55^{-/-}* and *apoE^{-/-}* mice at either time point (Figure 2D). Plasma C3adesArg levels were markedly lower after being deprived of food overnight compared with not being deprived of food regardless of age, diet, and genotype of the mice (compare Figure 2, E with F). Fasting C3a levels were similar in all groups of mice (Figure 2F); in contrast, nonfasting C3adesArg levels were significantly higher in *apoE^{-/-}/CD55^{-/-}* mice than in *apoE^{-/-}* mice but only on the high-fat diet (Figure 2E; 1.58 ± 0.14

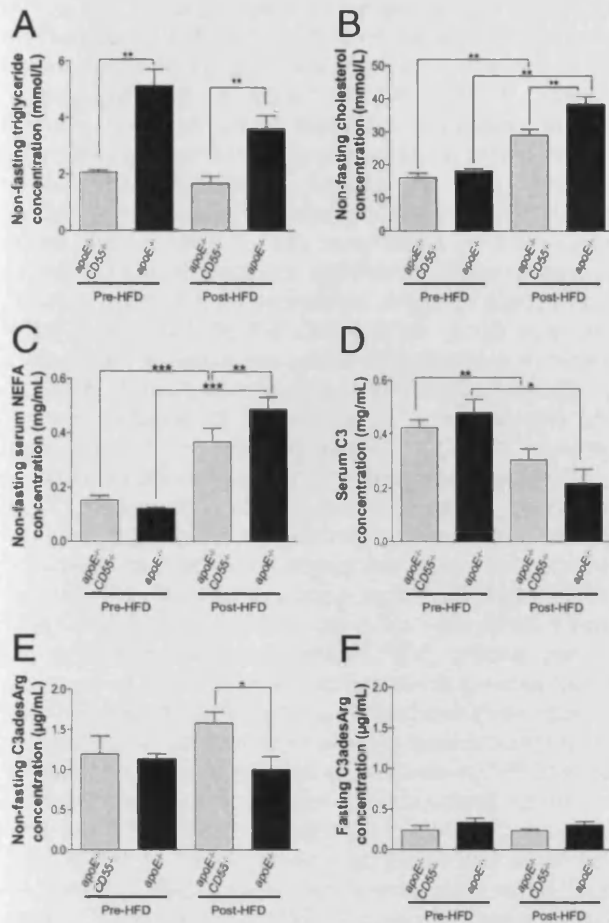


Figure 2. Assessment of lipid profile, C3 and C3adesArg levels in *apoE*^{-/-}/*CD55*^{-/-} and *apoE*^{-/-} mice. Serum levels of triglycerides, cholesterol, NEFAs, C3, and plasma C3adesArg were assayed in *apoE*^{-/-}/*CD55*^{-/-} and *apoE*^{-/-} mice at 8 weeks of age (before high-fat diet; Pre-HFD) and at 20 weeks after 12 weeks of high-fat diet (Post-HFD). **A:** Serum triglyceride levels (Pre-HFD, *n* = 4 and 6, respectively; Post-HFD, *n* = 12 and 15, respectively). **B:** Serum cholesterol levels (Pre-HFD, *n* = 4 and 6, respectively; Post-HFD, *n* = 12 and 15, respectively). **C:** Serum NEFA levels (Pre-HFD, *n* = 6 and 6, respectively; Post-HFD, *n* = 12 and 14, respectively). **D:** Total serum C3 levels (Pre-HFD, *n* = 6 and 6, respectively; Post-HFD, *n* = 6 and 4, respectively). **E:** Plasma nonfasting C3adesArg levels (Pre-HFD, *n* = 6 and 8, respectively; Post-HFD, *n* = 11 and 12, respectively). **F:** Plasma fasting C3adesArg levels (Pre-HFD, *n* = 8 and 7, respectively; Post-HFD, *n* = 11 and 11, respectively). Mean values ± SEM are represented. **P* < 0.05, ***P* < 0.01, and ****P* < 0.0001.

µg/mL versus 1.00 ± 0.16 µg/mL; *P* < 0.01). The data show that high-fat feeding in *apoE*^{-/-}/*CD55*^{-/-} mice is associated with increased C3 turnover and higher circulating levels of C3adesArg compared with *apoE*^{-/-} controls.

ApoE^{-/-}/*CD55*^{-/-} Mice Have Increased Adipose Tissue Content

To test whether the observed changes in circulating lipid levels and C3 activation in high fat-fed *apoE*^{-/-}/*CD55*^{-/-} mice affected fat storage, body weight and composition were compared between the groups. The high-fat diet caused significant increases in body weight for both groups, without any significant differences between *apoE*^{-/-}/*CD55*^{-/-} mice and *apoE*^{-/-} controls (*apoE*^{-/-}/*CD55*^{-/-}:

CD55^{-/-}: 27.32 ± 0.6 g versus 38.16 ± 2.12 g; *P* < 0.0001; *apoE*^{-/-}: 30.64 ± 0.39 g versus 41.38 ± 1.17 g; *P* < 0.0001).

Both adipose mass and percentage of body fat were measured with dual-energy X-ray absorptiometry (Figure 3, A and B, respectively). After 12 weeks of high-fat feeding CD55 deficiency was associated with increased adipose tissue mass and percentage of body fat, significant in the latter case, in *apoE*^{-/-}/*CD55*^{-/-} mice compared with *apoE*^{-/-} controls (Figure 3B; before high-fat diet: 17.43% ± 0.64%; 17.8% ± 1.11% *apoE*^{-/-}/*CD55*^{-/-} versus *apoE*^{-/-}, respectively; after high-fat diet: 28.63% ± 4.18%; 19.54% ± 1.77% *apoE*^{-/-}/*CD55*^{-/-} versus *apoE*^{-/-}, respectively; *P* < 0.05).

Discussion

We here demonstrate that CD55 deficiency in fat-fed male *apoE*^{-/-} mice markedly attenuated the progression of atherosclerosis. Plaques were infrequent, small, and structurally simple, having fewer smooth muscle cells and less neo-intimal thickening in comparison with their *apoE*^{-/-} controls, which displayed large, advanced plaques at the same time point.

We previously showed that MAC deposition correlates with plaque stage, probably reflecting the increased amount of C-activating cell debris in advanced lesions.²³ The data show that, despite global absence of the C regulator CD55, C activation is reduced in the early, simple plaques formed in *apoE*^{-/-}/*CD55*^{-/-} mice. These results are surprising in that the absence of an important C regulator would be predicted to exacerbate injury in a disease characterized by C activation; indeed, deficiency of CD59a markedly exacerbated MAC formation and disease in atherosclerosis-prone mice.²¹⁻²³ Two recent studies tested effects of CD55 deficiency in atherosclerosis models. Leung et al²⁷ showed exacerbated disease and increased plaque size in female *CD55*^{-/-}/*ldlr*^{-/-} mice compared with the *ldlr*^{-/-} controls. This contradictory result is probably because of the different atherosclerosis-prone background; deficiency of *ldlr* causes a more severe metabolic derangement and deranged lipid profiles compared with *apoE* deficiency.²⁹

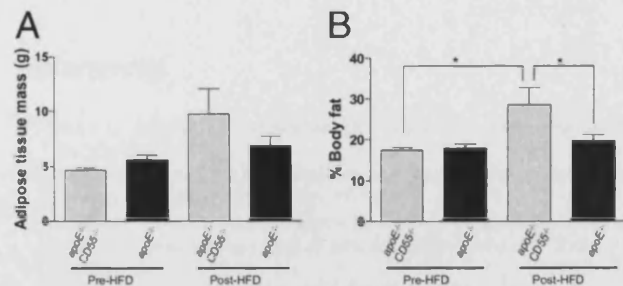


Figure 3. Measurement of adipose tissue mass and percentage of body fat in *apoE*^{-/-}/*CD55*^{-/-} and *apoE*^{-/-} mice. Adipose tissue mass (**A**) and percentage of body fat (**B**) were measured by dual-energy X-ray absorptiometry in *apoE*^{-/-}/*CD55*^{-/-} and *apoE*^{-/-} mice at 8 weeks of age (before high-fat diet; Pre-HFD; *n* = 6 and 8, respectively) and at 20 weeks of age after 12 weeks of high-fat diet (Post-HFD, *n* = 6 and 9, respectively). Mean values ± SEM are represented. **P* < 0.05.

An et al³³ placed male and female *apoE*^{-/-}/*CD55*^{-/-} mice on a high-fat diet for 8 or 16 weeks. Plaque area, measured at end point in the aortic arch, was not significantly different in *apoE*^{-/-}/*CD55*^{-/-} and *apoE*^{-/-} groups; however, when separated according to sex, there was a clear trend, not remarked on by the investigators, toward smaller plaques in male *apoE*^{-/-}/*CD55*^{-/-} mice after 16 weeks of the high-fat diet. Group sizes after dividing by sex were small, and the differences observed were not significant. These published findings support our observation, probably made significant in our study by the use of larger group sizes and male mice. Indeed, many inbred mouse strains, including C57BL/6, have a constitutively more active hemolytic C system because of differences in activation and lytic pathways.³⁴⁻³⁶ Remarkably, *apoE*^{-/-}/*CD55*^{-/-} mice had a markedly altered lipid profile, dominated by low circulating triglyceride, NEFA, and cholesterol levels; increased triglyceride uptake into adipose tissue and fat synthesis resulted in increased adiposity. Two hypotheses were considered to explain the above findings: first, that CD55 played a direct role in lipid metabolism independent of its role in regulating C, and, second, that the absence of CD55 caused increased C3 turnover that in turn influenced lipid handling. Others have shown that C3 deficiency on atherosclerosis-prone backgrounds exacerbated hyperlipidemia and atherogenesis.^{25,26} This observation favored a role for C3, provoking us to ask whether C3 turnover was altered in *CD55*^{-/-} mice and contributed to the observed lipid profile and atheroprotection. C3 levels were significantly reduced in both *apoE*^{-/-} and *apoE*^{-/-}/*CD55*^{-/-} mice when fed a high-fat diet, suggesting that a high-fat diet provoked C3 consumption. Remarkably, although C3adesArg levels were low in all mice after being deprived of food overnight, levels in mice not deprived of food were markedly higher and were significantly increased in high fat-fed *apoE*^{-/-}/*CD55*^{-/-} mice compared with *apoE*^{-/-} controls. These data imply that the observed changes in lipid profile were caused by diminished capacity, in the absence of CD55, to regulate the C3 convertase, resulting in dysregulation of the ASP pathway and increased production of C3adesArg/ASP in response to circulating chylomicrons or very low density lipoprotein particles.³⁷⁻³⁹ Although hypercholesterolemia has been the focus of most attention in atherosclerosis, elevated triglyceride and NEFA levels are recognized as independent risk factors for atherogenesis in humans and models.^{40,41} Indeed, the antiatherogenic effects of lipoic acid in the *apoE*^{-/-} model were shown to be due to its triglyceride-lowering properties.⁴² It is therefore probable that the observed antiatherogenic effect of CD55 deficiency is due to the combined effects of lower plasma levels of triglycerides, cholesterol, and NEFAs.

These findings support *in vitro* and *in vivo* studies that implicate chylomicrons and/or very low density lipoprotein as the primary physiological trigger for C3adesArg/ASP production from adipose tissue.^{43,44} *In vitro*, exposure of adipose tissue to purified chylomicrons switched on the ASP pathway, markedly increasing synthesis of precursor C3 and generation of C3adesArg, whereas, *in*

in vivo, chylomicronemia acutely increased plasma C3 and C3adesArg/ASP levels.^{43,44} The amount of C3adesArg/ASP generated will depend on the local activity and regulation of C3 convertase, in turn depending on the presence and abundance of C regulators. CD55 is expressed on adipose cells^{45,46} and will therefore contribute to local regulation of the convertase; in its absence, the convertase will persist and generate more C3adesArg/ASP.

Because C3adesArg/ASP is such a critical factor in maintaining lipid homeostasis, a persistent increase in local and circulating levels could be predicted to affect lipid profiles in precisely the manner observed in the *apoE*^{-/-}/*CD55*^{-/-} mice of the current study by provoking increased uptake of triglycerides and NEFAs into adipose tissue, resulting in reduced plasma levels. We chose to use male mice in this study because of proven differences in C activity and have suggested that failure to detect this effect of CD55 in previous studies was due to inclusion of female mice. A further potential confounder is the effect of sex hormones on the ASP pathway. Progesterone down-regulates the expression of C5L2, the receptor for C3adesArg, in 3T3 adipocytes, potentially rendering female mice less responsive to C3adesArg and blunting the atheroprotective effect of CD55 deficiency seen in male *apoE*^{-/-}/*CD55*^{-/-} mice.⁴⁷

The chain of events shown here explains both atheroprotection and increased adiposity in the mice and also provides an explanation for the enigmatic observation that C3-deficient mice, lacking the capacity to generate C3adesArg, show accelerated disease in models of atherosclerosis.^{25,26} The data show that C3adesArg itself, or agents mimicking its lipid-modulating effects, might be of benefit in the treatment of atherosclerosis and related diseases.

Acknowledgments

We thank Prof. Wenchao Song for providing the *CD55*^{-/-} mice. We also thank all of the staff at the Biomedical Services Unit at Cardiff University for technical support. We thank Dr. Marieta Ruseva, Dr. Meike Heurich, and Dr. Claire Harris for providing mouse C3. Finally, we thank the staff of the Clinical Biochemistry Laboratories, particularly Nigel Roberts and Stuart Pople (University Hospital Cardiff), for their support with our lipid analyses.

References

1. Glass C, Witztum J: Atherosclerosis. The road ahead. *Cell* 2001, 104:503-516
2. Jawien J: New insights into immunological aspects of atherosclerosis. *Pol Arch Med Wewn* 2008, 118:127-131
3. Hansson G, Libby P, Schönbeck U, Yan Z: Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res* 2002, 91: 281-291
4. Kostner K: Activation of the complement system: a crucial link between inflammation and atherosclerosis? *Eur J Clin Invest* 2004, 34:800-802
5. Haskard D, Boyle J, Mason J: The role of complement in atherosclerosis. *Curr Opin Lipidol* 2008, 19:478-482
6. Meuwissen M, van der Wal A, Niessen H, Koch K, de Winter R, van der Loos C, Rittersma S, Chamuleau S, Tijssen J, Becker A, Piek J:

- Colocalisation of intraplaque C reactive protein, complement, oxidised low density lipoprotein, and macrophages in stable and unstable angina and acute myocardial infarction. *J Clin Pathol* 2006, 59:196–201
7. Niculescu F, Niculescu T, Rus H: C5b-9 terminal complement complex assembly on apoptotic cells in human arterial wall with atherosclerosis. *Exp Mol Pathol* 2004, 76:17–23
 8. Vlaicu R, Niculescu F, Rus H, Cristea A: Immunohistochemical localization of the terminal C5b-9 complement complex in human aortic fibrous plaque. *Atherosclerosis* 1985, 57:163–177
 9. Yasojima K, Schwab C, McGeer E, McGeer P: Complement components, but not complement inhibitors, are upregulated in atherosclerotic plaques. *Arterioscler Thromb Vasc Biol* 2001, 21:1214–1219
 10. Matthijsen R, de Winther M, Kuipers D, van der Made I, Weber C, Herias M, Gijbels M, Buurman W: Macrophage-specific expression of mannose-binding lectin controls atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation* 2009, 119:2188–2195
 11. Madsen H, Videm V, Svejgaard A, Svennevig J, Garred P: Association of mannose-binding-lectin deficiency with severe atherosclerosis. *Lancet* 1998, 352:959–960
 12. Torzewski J, Bowyer D, Waltenberger J, Fitzsimmons C: Processes in atherogenesis: complement activation. *Atherosclerosis* 1997, 132:131–138
 13. Zwirner J, Werfel T, Wilken H, Theile E, Götz O: Anaphylatoxin C3a but not C3a(desArg) is a chemotaxin for the mouse macrophage cell line J774. *Eur J Immunol* 1998, 28:1570–1577
 14. Oksjoki R, Laine P, Helske S, Vehmaan-Kreula P, Mäyränpää M, Gasque P, Kovanen P, Penttinen M: Receptors for the anaphylatoxins C3a and C5a are expressed in human atherosclerotic coronary plaques. *Atherosclerosis* 2007, 195:90–99
 15. MacLaren R, Cui W, Cianflone K: Adipokines and the immune system: an adipocentric view. *Adv Exp Med Biol* 2008, 632:1–21
 16. Sniderman AD, Cianflone K, Summers L, Fielding B, Frayn K: The acylation-stimulating protein pathway and regulation of postprandial metabolism. *Proc Nutr Soc* 1997, 56:703–712
 17. Murray I, Sniderman A, Cianflone K: Mice lacking acylation stimulating protein (ASP) have delayed postprandial triglyceride clearance. *J Lipid Res* 1999, 40:1671–1676
 18. Murray I, Havel P, Sniderman A, Cianflone K: Reduced body weight, adipose tissue, and leptin levels despite increased energy intake in female mice lacking acylation-stimulating protein. *Endocrinology* 2000, 141:1041–1049
 19. Geertinger P, Soerensen H: On the reduced atherogenic effect of cholesterol feeding in rabbits with congenital complement (C6) deficiency. *Artery* 1977, 1:177–184
 20. Schmiedt W, Kinscherf R, Deigner H, Kamencic H, Nauen O, Kilo J, Oelert H, Metz J, Bhakdi S: Complement C6 deficiency protects against diet-induced atherosclerosis in rabbits. *Arterioscler Thromb Vasc Biol* 1998, 18:1790–1795
 21. Yun S, Leung V, Botto M, Boyle J, Haskard D: Brief report: accelerated atherosclerosis in low-density lipoprotein receptor-deficient mice lacking the membrane-bound complement regulator CD59. *Arterioscler Thromb Vasc Biol* 2008, 28:1714–1716
 22. Wu G, Hu W, Shahsafaie A, Song W, Dobarro M, Sukhova G, Bronson R, Shi G, Rother R, Halperin J, Qin X: Complement regulator CD59 protects against atherosclerosis by restricting the formation of complement membrane attack complex. *Circ Res* 2009, 104:550–558
 23. Lewis RD, Jackson CL, Morgan BP, Hughes TR: The membrane attack complex of complement drives the progression of atherosclerosis in apolipoprotein E knockout mice. *Mol Immunol* 2010, 47:1098–1105
 24. Patel S, Thelander E, Hernandez M, Montenegro J, Hassing H, Burton C, Mundt S, Hermanowski-Vosatka A, Wright S, Chao Y, Detmers P: ApoE(-/-) mice develop atherosclerosis in the absence of complement component C5. *Biochem Biophys Res Commun* 2001, 286:164–170
 25. Persson L, Borén J, Robertson A, Wallenius V, Hansson G, Pekna M: Lack of complement factor C3, but not factor B, increases hyperlipidemia and atherosclerosis in apolipoprotein E-/- low-density lipoprotein receptor-/- mice. *Arterioscler Thromb Vasc Biol* 2004, 24:1062–1067
 26. Buono C, Come C, Witztum J, Maguire G, Connelly P, Carroll M, Lichtman A: Influence of C3 deficiency on atherosclerosis. *Circulation* 2002, 105:3025–3031
 27. Leung V, Yun S, Botto M, Mason J, Malik T, Song W, Paixao-Cavalcante D, Pickering M, Boyle J, Haskard D: Decay-accelerating factor suppresses complement C3 activation and retards atherosclerosis in low-density lipoprotein receptor-deficient mice. *Am J Pathol* 2009, 175:1757–1767
 28. Williams H, Johnson J, Carson K, Jackson C: Characteristics of intact and ruptured atherosclerotic plaques in brachiocephalic arteries of apolipoprotein E knockout mice. *Arterioscler Thromb Vasc Biol* 2002, 22:788–792
 29. Joven J, Rull A, Ferré N, Escolà-Gil J, Marsillach J, Coll B, Alonso-Villaverde C, Aragones G, Claria J, Camps J: The results in rodent models of atherosclerosis are not interchangeable: the influence of diet and strain. *Atherosclerosis* 2007, 195:e85–e92
 30. Cianflone K, Sniderman A, Walsh M, Vu H, Gagnon J, Rodriguez M: Purification and characterization of acylation stimulating protein. *J Biol Chem* 1989, 264:426–430
 31. Germinario R, Sniderman A, Manuel S, Lefebvre S, Baldo A, Cianflone K: Coordinate regulation of triacylglycerol synthesis and glucose transport by acylation-stimulating protein. *Metabolism* 1993, 42:574–580
 32. Maslowska M, Sniderman A, Germinario R, Cianflone K: ASP stimulates glucose transport in cultured human adipocytes. *Int J Obes Relat Metab Disord* 1997, 21:261–266
 33. An G, Miwa T, Song W, Lawson J, Rader D, Zhang Y, Song W: CD59 but not DAF deficiency accelerates atherosclerosis in female ApoE knockout mice. *Mol Immunol* 2009, 46:1702–1709
 34. Holt D, Botto M, Bygrave A, Hanna S, Walport M, Morgan B: Targeted deletion of the CD59 gene causes spontaneous intravascular hemolysis and hemoglobinuria. *Blood* 2001, 98:442–449
 35. Beurskens FJ, Kuenen JD, Hofhuis F, Fluit AC, Robins DM, Van Dijk H: Sex-limited protein: in vitro and in vivo functions. *Clin Exp Immunol* 1999, 116:395–400
 36. Tanaka S, Suzuki T, Nishioka K: Assay of classical and alternative pathway activities of murine complement using antibody-sensitized rabbit erythrocytes. *J Immunol Methods* 1986, 86:161–170
 37. Gao Y, Gauvreau D, Cianflone K: Hormone and pharmaceutical regulation of ASP production in 3T3-L1 adipocytes. *J Cell Biochem* 2010, 109:896–905
 38. Gao Y, Gauvreau D, Cui W, Lapointe M, Pagliarunga S, Cianflone K: Evaluation of chylomicron effect on ASP production in 3T3-L1 adipocytes. *Acta Biochim Biophys Sin (Shanghai)* 2011, 43:154–159
 39. Murray I, Sniderman A, Cianflone K: Enhanced triglyceride clearance with intraperitoneal human acylation stimulating protein in C57BL/6 mice. *Am J Physiol* 1999, 277:E474–E480
 40. Lusis A, Mar R, Pajukanta P: Genetics of atherosclerosis. *Annu Rev Genomics Hum Genet* 2004, 5:189–218
 41. Singh U, Zhong S, Xiong M, Li T, Sniderman A, Teng B: Increased plasma non-esterified fatty acids and platelet-activating factor acetylhydrolase are associated with susceptibility to atherosclerosis in mice. *Clin Sci (Lond)* 2004, 106:421–432
 42. Zhang W, Bird K, McMillen T, LeBoeuf R, Hagen T, Frei B: Dietary alpha-lipoic acid supplementation inhibits atherosclerotic lesion development in apolipoprotein E-deficient and apolipoprotein E/low-density lipoprotein receptor-deficient mice. *Circulation* 2008, 117:421–428
 43. Maslowska M, Scantlebury T, Germinario R, Cianflone K: Acute in vitro production of acylation stimulating protein in differentiated human adipocytes. *J Lipid Res* 1997, 38:1–11
 44. Scantlebury T, Maslowska M, Cianflone K: Chylomicron-specific enhancement of acylation stimulating protein and precursor protein C3 production in differentiated human adipocytes. *J Biol Chem* 1998, 273:20903–20909
 45. Gronthos S, Franklin D, Leddy H, Robey P, Storms R, Gimble J: Surface protein characterization of human adipose tissue-derived stromal cells. *J Cell Physiol* 2001, 189:54–63
 46. Festy F, Hoareau L, Bes-Houtmann S, Péquin A, Gonthier M, Munstun A, Hoarau J, Césari M, Roche R: Surface protein expression between human adipose tissue-derived stromal cells and mature adipocytes. *Histochem Cell Biol* 2005, 124:113–121
 47. Wen Y, Wang H, MacLaren R, Lu H, Hu XF, Cianflone K: Sex steroid hormones induce acylation stimulating protein resistance in 3T3-L1 adipocytes. *J Cell Biochem* 2008, 105:404–413

Aim

To study the role of complement and complement regulatory proteins on the progression of atherosclerosis and atherosclerotic plaque stability in the apolipoprotein E-knockout mouse model

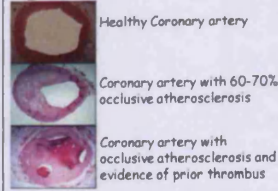
Introduction

Atherosclerosis:

- Is the leading cause of disease and death in the USA and most Western countries
- Is an inflammatory disease involving both the innate and adaptive immune system
- Consists of 3 phases (see figure. 1, 2 and 3):

1. Early atherosclerotic lesion
2. Lesion progression
3. Plaque rupture and thrombosis

Figure 1. (Glass Ck. et al. 2001)



Complement:

- Plays a central role in innate immunity and in the modulation of inflammatory response
- Consists of three activation pathways that converge into the terminal pathway leading to the assembly of a membrane attack complex (see figure 4.)
- Its activation can lead to cell lysis or more commonly in nucleated cells altered cell function
- It is regulated by complement regulatory proteins such as CD59 and decay accelerating factor (DAF)
- has been implicated in the pathogenesis of numerous diseases including atherosclerosis

Atherosclerosis and Complement: The evidence

Human studies

- Complement proteins have been shown to co-localise with known activators of Complement within the atherosclerotic wall

Animal studies

- Whilst studies using C6^{-/-} rabbits found that complement plays an integral part to the progression of atherosclerosis, animal studies involving atherosclerotic prone mouse models, low density lipoprotein (LDLR) and apolipoprotein (apoE)

Figure 2. Initiating events in the development of a fatty streak lesion (Glass Ck. et al. 2001)

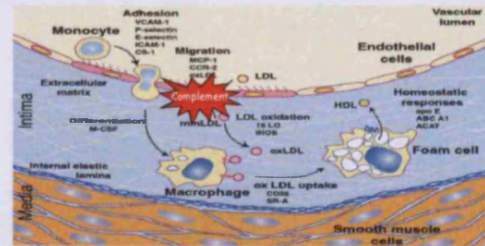


Figure 3. Lesion progression, plaque rupture and thrombosis (Glass Ck. et al. 2001)

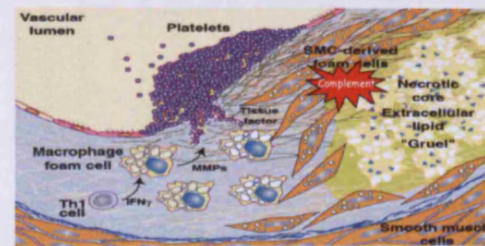
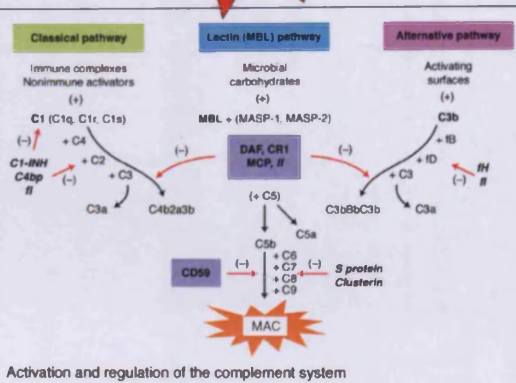
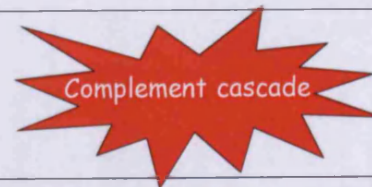


Figure 4.



Methods

Animal models:

- ApoE^{-/-}/DAF^{-/-}
- ApoE^{-/-}/CD59a^{-/-}
- ApoE^{-/-}/CD59a^{-/-}/DAF^{-/-}
- ApoE^{-/-}/C6^{-/-}

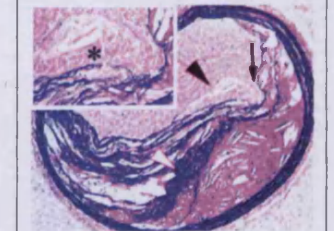
Experimental animals are fed a high fat diet for 8 weeks from the age of 8 weeks

Experimental parameters:

- Blood pressure
- Cardiac hypertrophy
- Triglyceride and cholesterol levels
- Haemolytic activity
- Complement deposition in brachiocephalic artery and aorta
- Frequency of plaque rupture in brachiocephalic artery (see figure 5)

Figure 5. (Johnson J. et al. 2005)

Fat feeding rapidly provokes plaque rupture in the brachiocephalic artery of apoE^{-/-} mice

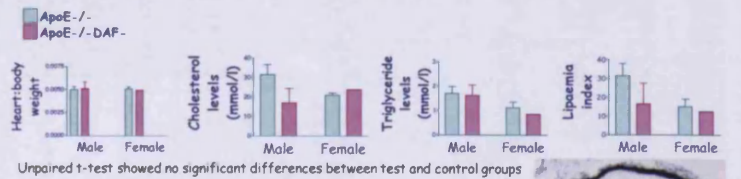


Thin elastin-rich cap (indicated by black arrowhead) over an advanced lesion in brachiocephalic artery of male apoE^{-/-} knock-out mouse that had been fat fed for 8 weeks, then euthanized and perfusion fixed. There is acute plaque rupture (arrow) with hemorrhage into lesion (asterisk). White arrowhead points to buried fibrous cap. Inset shows higher-power view.

Results

Figure 6.

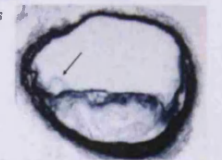
• Triglyceride and cholesterol levels, lipaemia index and heart:body weight ratio of apoE^{-/-} and apoE^{-/-}/DAF^{-/-} mice that had been fat fed for 8 weeks



Unpaired t-test showed no significant differences between test and control groups

Figure 7.

• Thin elastin-rich cap (indicated by black arrow) over an advanced lesion in brachiocephalic artery of male apoE^{-/-} mouse that had been fat fed for 8 weeks



Summary

ApoE^{-/-}/DAF^{-/-} and apoE^{-/-}/CD59a^{-/-} mouse models have been established and placed on a high fat diet. Data collection and analyses from these ongoing studies are currently underway. ApoE^{-/-}/CD59a^{-/-}/DAF^{-/-} and apoE^{-/-}/C6^{-/-} mouse models are being generated.

References

- Glass CK and Witztum JL. (2001). Atherosclerosis: The Road Ahead. Cell 104, 503-516.
- Johnson J, Carson K, Williams H, Karanam S, Newby A, Angelini G, George S, Jackson C. (2005). Plaque rupture after short periods of fat feeding in the apolipoprotein E-knockout mouse: model characterization and effects of

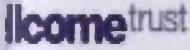


A role for CD59a in the progression of Atherosclerosis: the first direct evidence from an in vivo study.



Ruth D Lewis^a, Claudia Calder^a, Christopher L Jackson^b,
B Paul Morgan^a and Timothy R. Hughes^a

^aDepartment of Medical Biochemistry and Immunology; School of Medicine, Cardiff University, UK
^bBristol Heart Research Institute, Bristol University, Bristol, UK



Introduction

Atherosclerosis is the leading cause of death in the Western world. It is a chronic inflammatory disease involving both the innate and adaptive immune system.

Pathogenesis of the disease

The initial atherosclerotic lesion: formation of the fatty streak is caused by the entry of pro-inflammatory mediators and a variety of immune cells to the vessel wall in response to injury.



In the early stages of progression, monocyte derived macrophages become trapped in the vessel wall and take up lipid and differentiate into foam cells. Smooth muscle cells migrate into the plaque which acquires a 'fatty cap' and becomes stabilised.

Plaque rupture and thrombosis: the core of the plaque gradually becomes necrotic, and the vessel structure weakens becoming unstable. When this happens the vessel can rupture leading to the formation of a thrombus, which can lead to attack and death.

Complement System:

The complement system is a part of the innate immune system that results in death or damage to self or pathogens by using drastically altered cell function.

It is regulated by regulatory proteins such as CD59 and decay accelerating factor (DAF).

Defects in the complement system are associated with the pathogenesis of numerous diseases including atherosclerosis.

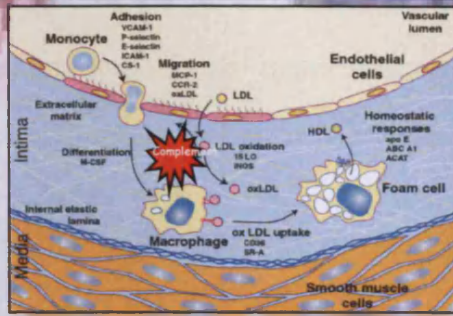


Figure 1. Initiating events in the development of a fatty streak lesion (Glass CK. *et al.* 2001)

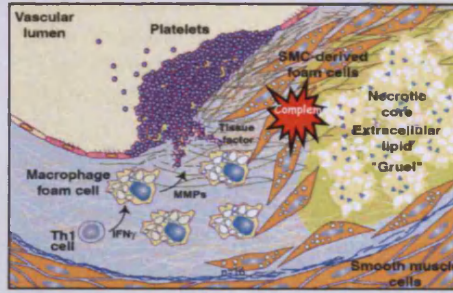


Figure 2. Lesion progression, plaque rupture and Thrombosis (Glass CK. *et al.* 2001)

Atherosclerosis and complement: the evidence

Human studies

- C5b-9 (MAC) found in atherosclerotic plaques.
- OxLDL and E-LDL have been shown to activate complement

Animal studies

- Rabbits deficient in C6 get less atherosclerosis
- C3 deficient mice get more atherosclerosis (*apoE/ldlr*)
- C5 deficient mice show no change (*apoE*)
- C1q deficient mice get more atherosclerosis (*ldlr*)

Our Aim: to examine the role of Complement and its Regulators in the ApoE mouse model of Atherosclerosis.

Knockout Animals generated or in progress:

- Early Activation Pathways *ApoE^{-/-}/DAF^{-/-}*
- Terminal Pathway Regulation *ApoE^{-/-}/CD59a^{-/-}*
- Whole Terminal Pathway *ApoE^{-/-}/C6^{-/-}*
- Absence of regulation in both activation and Terminal Pathways *ApoE^{-/-}/CD59a^{-/-}/DAF^{-/-}*

Experimental Design:

16 week old *ApoE^{-/-}/CD59a^{-/-}* mice and their controls were fed on a high fat diet for 8 weeks (25% lard + cholesterol).

Plasma was collected for assessment of lipid levels and complement activity.

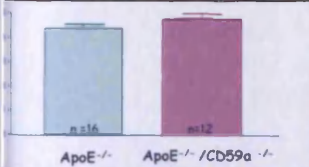
Brachiocephalic arteries were collected and frozen into OCT for sectioning and analyses.

Experimental Parameters:

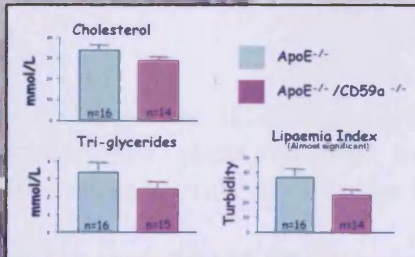
- Cardiac hypertrophy
- Triglyceride and cholesterol levels
- Plaque size (and incidence of rupture)
- Artery Size and Lumen Area
- Haemolytic activity
- Complement deposition in brachiocephalic artery and aorta
- Macrophage, smc, T-cell, collagen and elastin content of harvested plaques

Results

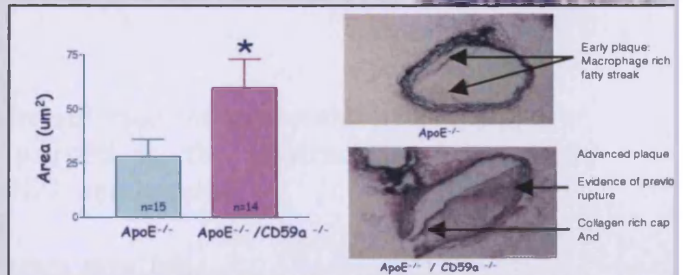
Cardiac hypertrophy is unaffected by CD59 deficiency in mice after 8 weeks of High Fat Diet.



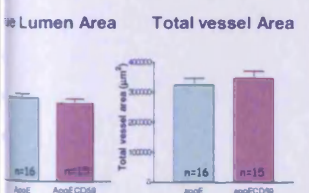
Blood Lipid Indices all show downward trends after 8 weeks on a High Fat Diet.



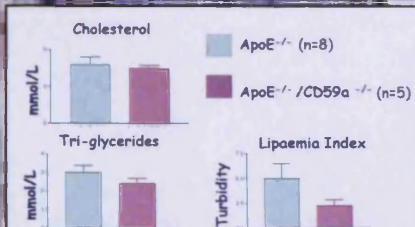
Plaque Sizes in the Brachiocephalic arteries of *ApoE^{-/-}/CD59a^{-/-}* are more than doubled by comparison with *ApoE^{-/-}* mice.



Artery Remodelling after 8 weeks of high fat diet maintains Lumen Area



Blood Lipid Indices after 22 weeks on Normal Chow Diet



Conclusions/Summary

- Mice deficient in ApoE and CD59 mouse have been generated.
- After 8 weeks of high fat diet the *ApoE/CD59* cross has shown an increased rate of plaque growth by comparison with the control *ApoE* mice.
- Data implies that CD59 has a protective role in the early development of the atherosclerotic plaque.
- Future Work
 - Frequency of plaque rupture in brachiocephalic artery
 - Blood Pressure
 - Indices of Apoptosis
 - T-cell infiltration

References

- Glass CK and Witztum JL, (2001). Atherosclerosis: The Road Ahead. *Cell* 104: 503-516.



Infection, Immunity & Inflammation (I³-IRG)

AN INTERDISCIPLINARY RESEARCH GROUP (IRG)

1. Abstract:

<p>TITLE:</p> <p>Authors:</p> <p>Affiliations:</p>	<p>The membrane attack complex of complement drives the progression of atherosclerosis in apolipoprotein E knockout mice.</p> <p>Ruth D Lewis, Christopher L Jackson, Paul Morgan and Timothy R. Hughes</p>
<p>Abstract:</p> <p>Abstract must fit in this box with Arial pt 10 font (approx. 250 words)</p>	<p>Aims To examine the roles of the membrane attack complex of complement and its sole membrane regulator, CD59, in atherosclerosis and to test whether terminal pathway inhibition has therapeutic potential.</p> <p>Methods C6 (C6^{-/-}) deficient and CD59a (Cd59a^{-/-}) knockout mice were separately crossed onto the apolipoprotein E knockout (apoE^{-/-}) background. The double knockout mice were fed high-fat diet in order to study the effects of absence of C6 or CD59a on the progression of atherosclerosis. The therapeutic potential of terminal pathway inhibition was investigated through the administration of a neutralising antibody against C5 to apoE^{-/-} mice fed a high-fat diet.</p> <p>Results C6 deficiency significantly reduced plaque area and disease severity. CD59a had the opposite effect in that deficiency was associated with a significant increase in plaque area, correlating with increased membrane attack complex (MAC) deposition in the plaque and increased smooth muscle cell proliferation in early plaques. Terminal pathway inhibition through the administration of an anti-C5 antibody dramatically reduced MAC deposition in the plaques of apoE^{-/-} mice but did not affect disease progression.</p> <p>Conclusions Our results demonstrate that the MAC contributes to the development of atherosclerosis, C6 deficiency being protective and CD59a deficiency exacerbating disease. Therapeutic intervention by blockade of MAC formation at the level of C5 was ineffective.</p>

or:

I am not submitting an abstract to accompany my registration:

Although a limited number of abstracts will be selected for presentation at the meeting, all submitted abstracts will be printed in the abstract booklet, highlighting the nature of your work to the I³-IRG membership.

Poster sessions are not held as part of the summer meeting.

CD55 deficiency inhibits disease in a mouse model of atherosclerosis by modulating lipid handling.

Ruth D Lewis^a, Mark Perry^b, Christopher L Jackson^b, B Paul Morgan^a and Timothy R. Hughes^a

^aDepartment of Infection, Immunity & Biochemistry; School of Medicine, Cardiff University, UK
^bBristol Heart Research Institute, Bristol University, Bristol UK

Introduction

Atherosclerosis

Atherosclerosis is the leading cause of death in the Western world. It is an inflammatory disease involving both the innate and adaptive immune systems.

It consists of three phases: Early, lesion progression, and plaque rupture and thrombosis.

Complement System

Over-activation results in death or damage to self-cells leading to drastically altered cell function.

It is implicated in the pathogenesis of numerous diseases including atherosclerosis.

It is controlled by regulatory proteins such as CD59 and CD55.

Atherosclerosis and complement: the evidence

Several studies:

• C3 (MAC) found in atherosclerotic plaques.

• C5L and E-LDL have been shown to activate complement.

Studies

• Mice deficient in C6 are atherosclerosis-resistant

• Mice deficient in C3 are atherosclerosis-prone (*apoE/Idlr*)

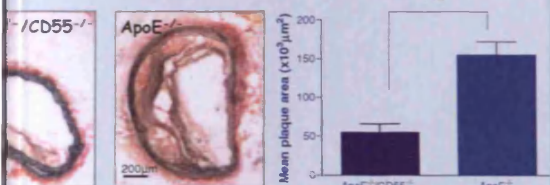
• Mice deficient mice show no change (*apoE*)

• Mice deficient mice are atherosclerosis-prone (*Idlr*)

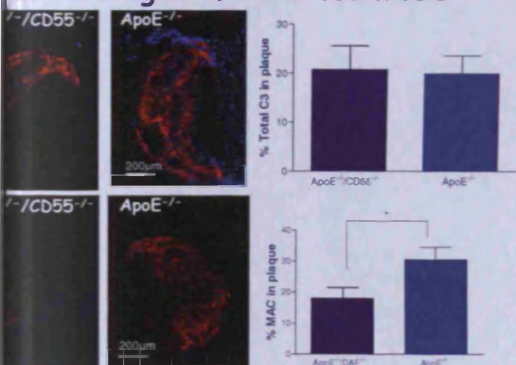
• Mice deficient mice are atherosclerosis-prone (*apoE/Idlr*)

Results

Deficiency of CD55 limits progression of atherosclerosis in *apoE*^{-/-} mice



Complement activation proceeds to advanced stages of atherosclerosis



Altered cellular composition in advanced atherosclerotic plaques

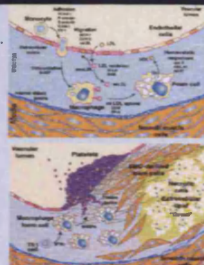
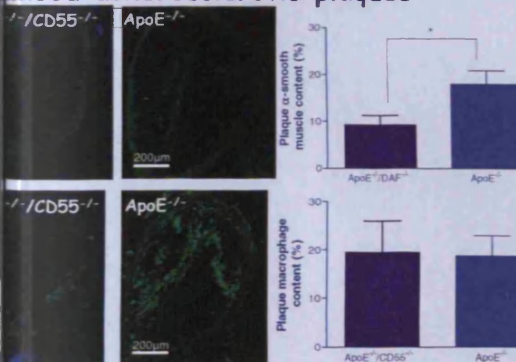


Figure 1. Initiating events in the development of a fatty streak.
 (Glass CK, et al 2001)



Figure 2. Lesion Progression plaque Rupture and thrombosis.
 (Glass CK, et al 2001)

Lipid metabolism

• Dietary lipids are packed into chylomicrons, secreted and primarily redistributed between adipose tissue for storage, immediate use in muscle and uptake into the liver.

• Alteration in lipid metabolism is an important risk factor for atherosclerosis.

• C3a is inactivated in plasma by carboxypeptidase N, which efficiently removes the carboxy-terminal arginine generating C3adesArg (also known as acylating stimulating protein (ASP))

• C3adesArg is a potent anabolic activator of triglyceride synthesis and glucose uptake.

Aim: to test the effect of CD55 deficiency on progression of atherosclerosis

Methods

Experimental Design:

• 8 week old *apoE*^{-/-}/*CD55*^{-/-} mice and their controls were placed on a high fat diet (HFD) for 12 weeks (0.15% cholesterol)

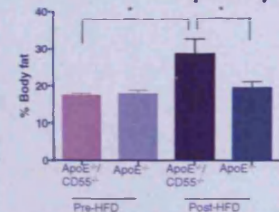
Assessment of lipid metabolism:

- Triglyceride and cholesterol levels
- Circulating C3adesArg concentration
- Adiposity

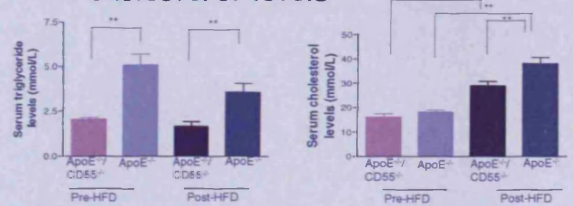
Assessment of atherosclerosis in the brachiocephalic artery:

- Plaque cross-sectional area
- Complement deposition in brachiocephalic artery
- Macrophage and smooth muscle content of harvested plaques

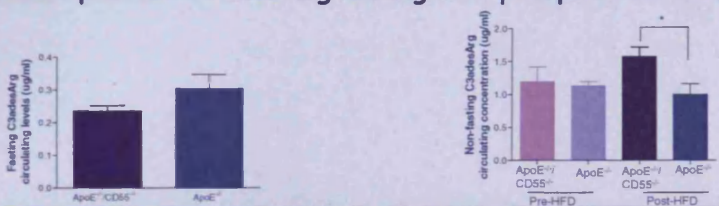
Fat fed *apoE*^{-/-}/*CD55*^{-/-} mice are obese with increased adiposity



Fat fed *apoE*^{-/-}/*CD55*^{-/-} mice have reduced serum triglyceride and cholesterol levels



Deficiency of CD55 causes increased turnover of C3 and increased plasma C3adesArg during the postprandial state



Summary

• CD55 deficiency in male *apoE*^{-/-} mice markedly attenuated the progression of atherosclerosis while on high fat diet for 12 weeks.

• C3 deposition in the plaques from *apoE*^{-/-}/*CD55*^{-/-} mice was similar to control but MAC deposition was significantly reduced.

• Plaques from *apoE*^{-/-}/*CD55*^{-/-} mice contained less infiltrating cells, indicative of early lesions.

• *apoE*^{-/-}/*CD55*^{-/-} mice have increased adiposity with altered lipoprotein profile which is dominated by low triglyceride levels.

Conclusions

• Chylomicrons stimulate C3 synthesis, and local production of C3adesArg which in turn causes increased triglyceride clearance. We propose that absence of CD55 increases alternative pathway cycling in adipose tissue, increasing C3adesArg production and further increasing triglyceride clearance. These changes explain increased adiposity and decreased plasma triglyceride levels.

• The marked decrease in triglyceride levels is highly protective for atherosclerosis.

• Others have proposed that complement mediated therapy might provide a novel strategy for the treatment of atherosclerosis. Here we show that C3adesArg itself or agents mimicking its lipid-modulating effects may be of benefit.

References

• Glass CK and Witztum JL. (2001). Atherosclerosis.