



<https://theses.gla.ac.uk/>

Theses Digitisation:

<https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/>

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses

<https://theses.gla.ac.uk/>
research-enlighten@glasgow.ac.uk

An investigation of the innate inflammatory properties of immune stimulating complexes (ISCOMS) and their importance for the generation of adaptive immunity

Rosemary Elizabeth Smith
BSc. MSc.

This thesis is being submitted for the degree of PhD

University of Glasgow

Department of Immunology, Western Infirmary, Glasgow

March 2000

ProQuest Number: 10390784

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.



ProQuest 10390784

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

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

GLASGOW
UNIVERSITY
LIBRARY

11973 (copy 2)

Acknowledgements

The time I spent working on my PhD has been one of the most enjoyable and happy times of my life. I feel extremely fortunate to have spent these few years in the inspiring and amiable environment that is the Department of Immunology, Western Infirmary, Glasgow. I now appreciate why so many former students strive to return to work in this wonderful city. By no means do I wish to detract from the blood, sweat and tears shed in the process of doing this study, but I would not have wished to have shared it with any other group of people.

In particular I would like to express my admiration and thanks to **Allan Mowat**, for cajoling, advising and grilling me throughout the course of this work, and teaching me the finer points of dancing at Scottish weddings! For my lab partners: **Anne Donachie**, who picked me at interview because I giggled, helped me with experiments and through our time working together has become a great and trusted friend; **Tinni Sharma**, a wonderful friend who has shared many times and even managed to drag me up one or two hills and **Andrew Leishman** who taught me almost everything I know about Rangers Football Club (and football in general) in between kicking my head in constantly! All of whom were true partners in crime, coffee and the odd gin and tonic! What laughs we had! I would also like to acknowledge the other members of staff in the department, especially **Paul Garside** for helpful advice and taking the mick on a regular basis, **Christina Ross** for FACS advice and the odd giggle or two!, **Jane Hare** for electron microscopy and **Rod Ferrier** for cutting all of my frozen sections, **Sandra Scatter** for attempting to teach me the finer points of RT-PCR and then consoling me when it didn't work! In addition I would like to mention the admirable efforts of **Maureen Deehan** and **Karen Smith** who in addition to other things, got myself and Anne to the gym three times a week! Also not forgetting **Annette**, **Steven Reid**, **Stephen Gauld**, **Elad**, **Kirsty**, **Clare**, **Dave Piedrafita**, **Dave Hunter**, **Damo**, **Will**, **Wanda**, **Jackie**, **Helen Arthur**, **Helen Goodridge**, **Kersten**, **Wei**, **Gui-jie**, **Bernard**, **Matt**, **Holger** and **Christina**, **Joan**, **Ana**, **Jeremy**, **David**, **Jimbo**, **Maggie**, all the clinical immunologists, **Helen Bailey** and **Anne McIlveen**, who all made my time in Glasgow so jolly!

It just leaves me to express my thanks to **Mum and Dad** for starting me down this path in the first place and giving me such love and support, to **Ed Carlyle** who proof read my manuscript (brave lad!) and most importantly to **Richard Humble**, who has been there for me throughout, listened to my continuous moaning during the write up and kept me sane (well nearly!) - sweetheart, we can go and have some fun now!!

List of Contents

	Page
Title Page	i
Acknowledgements	ii
List of Contents	iii
List of Figures and Tables	xi
List of Publications	xvi
List of Abbreviations	xvii
Summary	xx
<hr/>	
Chapter 1	1
Introduction	
<hr/>	
1.0 Overview of the Immune Response	2
1.1 Innate Immunity	2
i) Triggers of the Innate Immune System	2
ii) The Innate Inflammatory Cascade	3
iii) Soluble Mediators of the Inflammatory Cascade	4
1.2 The role of the Innate Cascade on the Development of Adaptive Immunity	8
1.3 Protective Immunity	9
2.0 The Common Mucosal Immune System	10
2.1 Immune Mechanisms of the GALT	11
2.2 Effector Immune Responses in the Mucosa	12
i) Humoral Immune Responses in the Mucosa	13
ii) Cellular Immune Responses in the Mucosa	14
2.3 Immune Regulation in the GALT	15
2.4 Compartmentalisation of the GALT	16

3.0 Mucosal Vaccination Strategies	17
3.1 Oral Vaccination Vectors	17
i) Live Attenuated Oral Vaccines	17
ii) Attenuated Organisms as Oral Vectors	18
3.2 Oral Adjuvants	19
i) Soluble Toxins as Adjuvants	19
ii) Particulate Oral Adjuvants	20
4.0 Immune Stimulating Complexes (ISCOMS) as Oral Vaccine Vectors	21
4.1 Structure of ISCOMS	21
4.2 ISCOMS as Parenteral Adjuvants	21
4.3 Induction of Protective Immunity by ISCOMS	23
4.4 ISCOMS as Mucosal Adjuvants	24
4.5 Mechanisms for ISCOMS Adjuvanticity	26
5.0 Specific Aims of Study	27

Chapter 2	29
------------------	-----------

Materials and Methods	
------------------------------	--

2.1 Animals	29
2.2 Anaesthesia	29
2.3 Antigens	29
3.4 Adjuvants	30
2.5 Production of OVA ISCOMS	30
i) Palmitification of OVA	30
ii) Preparation of OVA ISCOMS	31
iii) Quantification of OVA ISCOMS Protein Content	32
iv) Quantification of Endotoxin Content in OVA ISCOMS	33
2.6 Administration of OVA ISCOMS in vivo	33
2.7 Isolation of Peritoneal Exudate Cells	33

2.8	Histological Analysis of PEC by Cytospin	34
2.9	Isolation of Lymphoid Cells	34
2.10	Flow Cytometry	34
2.11	Stimulation of Cytokine Production in vitro	35
2.12	Removal of Adherent Cells in vitro	35
2.13	Measurement of Reactive Oxygen Intermediate Production	36
2.14	Measurement of Nitric Oxide Production	36
2.15	Measurement of Systemic Delayed Type Hypersensitivity (DTH) Responses	36
2.16	Measurement of OVA-Specific Antibodies in Serum	37
	i) Quantitation of Total IgG Antibodies	37
	ii) Quantitation of OVA-Specific Serum IgG Antibody Isotypes	38
2.17	Measurement of Intestinal Secretory IgA Antibodies	38
2.18	Measurement of OVA-Specific Proliferative Responses	39
2.19	Measurement of Cytokines by ELISA	40
2.20	Measurement of OVA-Specific Cytotoxic T Cell Responses	40
2.21	Statistics	41

Chapter 3 **45**

Parenteral and Oral Administration of ISCOMS induces an Inflammatory Cascade in vivo

Introduction	45
Experimental Protocol	45
Results	46
3.1 Recruitment of Local Inflammatory Cells after Intraperitoneal Administration of ISCOMS	46
3.2 Histological Examination of PEC after i.p. ISCOMS	46
3.3 Phenotypic Analysis of PEC Recruited by Parenteral Administration of ISCOMS	47

3.4 Parenteral Administration of ISCOMS Induces Production of Local Inflammatory Cytokines and Mediators	48
i) IL-1 α	49
ii) IL-6	49
iii) Immunoreactive IL-12	49
iv) γ IFN	50
v) TNF α	50
3.5 Stimulation of Nitric Oxide and Reactive Oxygen Intermediate Production after Parenteral Administration of ISCOMS	50
3.6 Secretion of Inflammatory Mediators by ISCOMS Induced PEC is Dependent on the Presence of Adherent Cells	51
3.7 Oral Administration of ISCOMS Induces Cellular Recruitment in the Gut Associated Lymphoid Tissues	52
3.8 Phenotypic Analysis of GALT Cells After Oral Administration of ISCOMS	53
i) Mesenteric Lymph Node	53
ii) Peyer's Patch	54
3.9 Effects of Oral Administration of ISCOMS on the Production of Inflammatory Mediators by GALT Cells	55
i) Mesenteric Lymph Node	55
ii) Peyer's Patch	56
Summary and Conclusions	57

Chapter 4	72
------------------	-----------

The Importance of ISCOMS Induced Inflammatory Mediators for the Generation of Antigen-Specific Immunity	
--	--

Introduction	72
Experimental Protocol	72
Results	73

4.1 Immune Responses to ISCOMS in Interleukin 12 Deficient Mice	73
4.1.1 Antigen-Specific DTH Responses	74
4.1.2 OVA-Specific Proliferative Responses	74
4.1.3 Antigen-Specific Cytokine Responses	74
4.1.4 OVA-Specific CTL Activity	75
4.1.5 Serum IgG Isotypes	75
4.1.6 Secretory Intestinal IgA Antibody Production	75
4.1.7 Summary	76
4.2 Immune Responses to ISCOMS in Interleukin 18 Deficient Mice	76
4.2.1 Antigen-Specific DTH Responses	77
4.2.2 Antigen-Specific Proliferative Responses	77
4.2.3 Antigen-Specific Cytokine Responses	77
4.2.4 OVA-Specific CTL Activity	78
4.2.5 Serum IgG Antibody Production	78
4.2.6 Summary	78
4.3 Immune Responses to ISCOMS in γInterferon-Receptor Deficient Mice	79
4.3.1 OVA-Specific DTH Responses	79
4.3.2 OVA-Specific Proliferative Responses	79
4.3.3 OVA-Specific Cytokine Responses	80
4.3.4 OVA-Specific CTL Activity	80
4.3.5 Antigen-Specific Serum IgG Production	81
4.3.6 OVA-Specific Intestinal IgA Production	81
4.3.7 Summary	81
4.4 Immune Responses to ISCOMS in Interleukin 4 Deficient Mice	81
4.4.1 Antigen-Specific DTH Responses	82
4.4.2 Antigen-Specific Proliferative Responses	82
4.4.3 Antigen-Specific Cytokine Responses	82
4.4.4 Antigen-Specific CTL Activity	83
4.4.5 Antigen-Specific Serum IgG Antibody Production	83

4.4.6 Secretory Intestinal IgA Antibody Production	83
4.4.7 Summary	83
4.5 Immune Responses to ISCOMS in Interleukin 6 Deficient Mice	84
4.5.1 Antigen-Specific DTH Responses	84
4.5.2 Antigen-Specific Proliferative Responses	84
4.5.3 Antigen-Specific Cytokine Responses	85
4.5.4 Antigen-Specific CTL Activity	85
4.5.5 Antigen-Specific Serum IgG Antibody Production	85
4.5.6 Summary	85
4.6 Immune Responses to ISCOMS in Inducible Nitric Oxide Synthase Deficient Mice	86
4.6.1 Antigen-Specific DTH Responses	86
4.6.2 Antigen-Specific Proliferative Responses	86
4.6.3 Antigen-Specific Cytokine Responses	87
4.6.4 Antigen-Specific Serum IgG Antibody Production	87
4.6.5 Summary	87
Summary and Conclusions	88
<hr/>	
Chapter 5	121
Role of Inflammatory Mediators Induced by ISCOMS in Regulating Innate Immune Responses	
<hr/>	
Introduction	121
Experimental Protocol	121
Results	122
5.1 Innate Responses to ISCOMS in Interleukin 12 Deficient Mice	122
5.1.1 ISCOMS Induced Inflammatory Infiltrate	122
5.1.2 Phenotypic Analysis of PEC	122
5.1.3 Inflammatory Cytokine Production	124

5.1.4 Secretion of Nitric Oxide	125
5.1.5 Summary	126
5.2 Innate Responses to ISCOMS in γInterferon-Receptor Deficient Mice	126
5.2.1 ISCOMS Induced Inflammatory Infiltrate	126
5.2.2 Phenotypic Analysis of PEC	127
5.2.3 Inflammatory Cytokine Production	128
5.2.4 Nitric Oxide Release	129
5.2.5 Summary	129
5.3 Innate Responses to ISCOMS in Interleukin 18 Deficient Mice	130
5.3.1 ISCOMS Induced Inflammatory Infiltrate	130
5.3.2 Phenotypic Analysis of PEC	130
5.3.3 Inflammatory Cytokine Production	131
5.3.4 Summary	132
5.4 Innate Responses to ISCOMS in Interleukin 6 Deficient Mice	132
5.4.1 Recruitment of PEC	133
5.4.2 Inflammatory Cytokine Production	133
5.4.3 Secretion of Nitric Oxide	134
5.4.4 Summary	134
5.5 Innate Responses to ISCOMS in Interleukin 4 Deficient Mice	134
5.5.1 Recruitment of PEC	135
5.5.2 Inflammatory Cytokine Production	135
5.5.3 Secretion of Nitric Oxide	136
5.5.4 Summary	136
Summary and Conclusions	136

Chapter 6 **159**

Discussion

ISCOMS Induce an Inflammatory Cascade **160**

The Importance of ISCOMS Induced Inflammatory Mediator Production **167**

Chapter 7 **182**

References

List of Figures and Tables

	Page
Chapter 1	
Figure 1.1	Ultrastructural appearance of OVA ISCOMS 28
Chapter 2	
Table I	Phenotypic marker antibodies used for flow cytometry 42
Table II	Capture antibodies used for sandwich ELISA 43
Table III	Recombinant cytokine used in sandwich ELISA 43
Table IV	Detection antibodies used in sandwich ELISA 44
Chapter 3	
Figure 3.1	ISCOMS Recruit Inflammatory Cells Following Local Injection 59
Figure 3.2	Histological Examination of PEC after ISCOMS Injection 60
Figure 3.3	Induction of Mast Cell Recruitment and Degranulation by ISCOMS 61
Figure 3.4	Phenotypic Analysis of ISCOMS Recruited PEC 62
Figure 3.5	ISCOMS Induce Local Secretion of Inflammatory Cytokines by PEC 63
Figure 3.6	ISCOMS Induced Nitric Oxide Production from PEC 64
Figure 3.7	ISCOMS Induced Production of Reactive Oxygen Intermediates 65
Figure 3.8	Adherent Cells are Required for the Production of ISCOMS Induced Inflammatory Mediators 66
Figure 3.9	Recruitment of Inflammatory Cells by a Single Feed of ISCOMS 67
Figure 3.10	Phenotypic Examination of MLN Cells Induced by Oral Administration of ISCOMS 68
Figure 3.11	Phenotypic Examination of PP Cells Induced by Oral Administration of ISCOMS 69

Figure 3.12	Secretion of Inflammatory Cytokines by MLN After Oral Administration of ISCOMS	70
Figure 3.13	Secretion of Inflammatory Cytokines by PP After Oral Administration of ISCOMS	71
 Chapter 4		
Figure 4.1	Antigen-Specific DTH in OVA ISCOMS Immunised IL-12 KO mice	89
Figure 4.2	Antigen-Specific <i>in vitro</i> Proliferation in OVA-ISCOMS Immunised IL-12KO Mice	90
Figure 4.3	Antigen-Specific T Cell Cytokine Production in OVA ISCOMS Immunised IL-12KO Mice	91
Figure 4.4	Antigen-Specific MHC Class I -Restricted CTL Activity in OVA ISCOMS Immunised IL-12KO Mice	92
Figure 4.5	Antigen-Specific Serum IgG Isotype in OVA-ISCOMS Immunised IL-12KO Mice	93
Figure 4.6	Antigen-Specific Intestinal IgA Production in OVA ISCOMS Immunised IL-12KO Mice	94
Figure 4.7	Antigen-Specific DTH in OVA ISCOMS Immunised IL-18KO mice	95
Figure 4.8	Antigen-Specific <i>in vitro</i> Proliferation in OVA-ISCOMS Immunised IL-18KO Mice	96
Figure 4.9	Antigen-Specific T Cell Cytokine Production in OVA ISCOMS Immunised IL-18KO Mice.	97
Figure 4.10	Antigen-Specific MHC Class I -Restricted CTL Activity in OVA ISCOMS Immunised IL-18KO Mice	98
Figure 4.11	Antigen-Specific Serum IgG Isotype in OVA-ISCOMS Immunised IL-18KO Mice	99
Figure 4.12	Antigen-Specific DTH in OVA ISCOMS Immunised γ IFN γ KO mice	100
Figure 4.13	Antigen-Specific <i>in vitro</i> Proliferation in OVA-ISCOMS Immunised γ IFN γ KO Mice	101

Figure 4.14	Antigen-Specific T Cell Cytokine Production in OVA ISCOMS Immunised γ IFN γ KO Mice	102
Figure 4.15	Antigen-Specific MHC Class I-Restricted CTL Activity in OVA ISCOMS immunised γ IFN γ KO Mice	103
Figure 4.16	Antigen-Specific Serum IgG Isotype in OVA-ISCOMS Immunised γ IFN γ KO Mice	104
Figure 4.17	Antigen-Specific Intestinal IgA Production in OVA ISCOMS Immunised γ IFN γ KO Mice	105
Figure 4.18	Antigen-Specific DTH in OVA ISCOMS Immunised IL-6 KO mice	106
Figure 4.19	Antigen-Specific <i>in vitro</i> Proliferation in OVA-ISCOMS Immunised IL-6 KO Mice	107
Figure 4.20	Antigen-Specific T Cell Cytokine Production in OVA ISCOMS Immunised IL-6 KO Mice	108
Figure 4.21	Antigen-Specific MHC Class I-Restricted CTL Activity in OVA ISCOMS Immunised IL-6 KO Mice	109
Figure 4.22	Antigen-Specific Serum IgG Isotype in OVA-ISCOMS Immunised IL-6 KO Mice	110
Figure 4.23	Antigen-Specific DTH in OVA ISCOMS Immunised IL-4 KO mice	111
Figure 4.24	Antigen-Specific <i>in vitro</i> Proliferation in OVA-ISCOMS Immunised IL-4 KO Mice	112
Figure 4.25	Antigen-Specific T Cell Cytokine Production in OVA ISCOMS Immunised IL-4 KO Mice	113
Figure 4.26	Antigen-Specific MHC Class I-Restricted CTL Activity in OVA ISCOMS Immunised IL-4 KO Mice	114
Figure 4.27	Antigen-Specific Serum IgG Isotype in OVA-ISCOMS Immunised IL-4 KO Mice	115
Figure 4.28	Antigen-Specific Intestinal IgA Production in OVA ISCOMS Immunised IL-4 KO Mice	116
Figure 4.29	Antigen-Specific DTH in OVA ISCOMS Immunised iNOS KO mice	117

Figure 4.30	Antigen-Specific <i>in vitro</i> Proliferation in OVA-ISCOMS Immunised iNOSKO Mice	118
Figure 4.31	Antigen-Specific T Cell Cytokine Production in OVA ISCOMS Immunised iNOSKO Mice	119
Figure 4.32	Antigen-Specific Serum IgG Isotype in OVA-ISCOMS Immunised iNOSKO Mice	120

Chapter 5

Figure 5.1	ISCOMS Induced Recruitment of PEC in IL-12KO Mice	139
Figure 5.2	Phenotypic Analysis of ISCOMS Recruited PEC from IL-12KO Mice.	140
Figure 5.3	Phenotypic Analysis of ISCOMS Recruited PEC from IL-12KO Mice	141
Figure 5.4	ISCOMS Induced Inflammatory Cytokine Production by PEC from IL-12KO Mice	142
Figure 5.5	ISCOMS Induced Nitric Oxide Production from IL-12KO PEC	143
Figure 5.6	ISCOMS Induced Recruitment of PEC in γ IFN RKO Mice	144
Figure 5.7	Phenotypic Analysis of PEC from γ IFN RKO mice after Injection of ISCOMS	145
Figure 5.8	Phenotypic Analysis of ISCOMS Recruited PEC from γ IFN RKO Mice	146
Figure 5.9	ISCOMS Induced Inflammatory Cytokine Production by PEC from γ IFN RKO Mice	147
Figure 5.10	ISCOMS Induced Nitric Oxide Production by PEC from γ IFN RKO Mice	148
Figure 5.11	ISCOMS Induced Recruitment of PEC from IL-18KO Mice	149
Figure 5.12	Phenotypic Analysis of ISCOMS Recruited PEC from IL-18KO Mice	150
Figure 5.13	Phenotypic Analysis of ISCOMS Recruited PEC from IL-18KO Mice	151
Figure 5.14	ISCOMS Induced Inflammatory Cytokine Production by PEC from IL-18KOMice	152
Figure 5.15	ISCOMS Induced Recruitment of PEC in IL-4KO Mice	153

Figure 5.16	ISCOMS Induced Inflammatory Cytokine Production by PEC from IL-4KO Mice	154
Figure 5.17	ISCOMS Induced Nitric Oxide Production by PEC from IL-4KO Mice	155
Figure 5.18	ISCOMS Induced Recruitment of PEC from IL-6KO Mice	156
Figure 5.19	ISCOMS Induced Inflammatory Cytokine Production by PEC from IL-6KO Mice	157
Figure 5.20	ISCOMS Induced Nitric Oxide Production by PEC from IL-6KO Mice	158

List of Publications

Smith R.E., Donachie A.M., Grdic D., Lycke N. & Mowat A. McI. (1999) Immune stimulating complexes induce an IL-12 dependent cascade of innate immune responses. *Journal of Immunology*. **162**: 5536-5546.

Grdic D., Smith R.E., Donachie A.M., Kjerrulf M., Hörnquist E., Mowat A.McI & Lycke N. (1999) The mucosal adjuvant effects of cholera toxin and ISCOMS differ in their requirement for IL-12, indicating different pathways of action. *European Journal of Immunology*. **29**: 1774-1784.

Mowat A.McI., Smith R.E., Donachie A.M., Furrle E., Grdic D. & Lycke N. (1999) Oral vaccination with immune stimulating complexes. *Immunology Letters*. **65**: 133-140.

Smith R.E., Donachie A.M. & Mowat A.McI. (1998) Preservation of Mucosal and Systemic Adjuvant Properties of ISCOMS in the Absence of Functional Interleukin 4 or γ Interferon. *Immunology*. **93**: 556-562.

Smith R.E., Maloy, K.J., Donachie A.M. & Mowat A.McI. (1998) Immune stimulating complexes as mucosal vaccines. *Immunology and Cell Biology*. **76**: 263-269.

Mowat A.McI. Maloy K.J., Smith R.E. & Donachie A.M. (1997) ISCOMS as mucosal vaccine vectors. In *Vaccine Design; The Role of Cytokine Networks*. Ed. Gregoriadis G. Plenum Press, London.

List of Abbreviations

Abbreviation	Meaning
APC	antigen presenting cell
CFA	complete Freund's adjuvant
CGD	chronic granulomatous disease
Cl ₂ MDP	dichloromethane diphosphate
Con A	concanavalin A
CT	cholera toxin
CTL	cytotoxic T lymphocyte
DEA	diethanolamine
DMSO	dimethyl sulphoxide
DTH	delayed type hypersensitivity
ELISA	enzyme linked immunosorbant assay
eNOS	endothelial nitric oxide synthase
FAE	follicular associated epithelium
FCS	foetal calf serum
FL3L	Flt3 ligand
GALT	gut associated lymphoid tissue
GC	germinal centre
HAO	heat aggregated ovalbumin
HBSS	Hank's balanced salt solution
HIV	human immunodeficiency virus
i.p.	intraperitoneal
ICE	interleukin-1 cleaving enzyme
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin

IL	interleukin
iNOS	inducible nitric oxide synthase
ISCOMS	immune stimulating complexes
KLH	keyhole limpet haemocyanin
KO	knockout
LP	lamina propria
LPS	lipopolysaccharide
LT	heat labile enterotoxin of <i>E. coli</i>
LTB4	leukotriene B4
MADCAM-1	mucosal addressin adhesion molecule-1
MALT	mucosa associated lymphoid tissue
MCP-1	monocyte chemoattractant protein-1
Mega-10	N-methylglucamide
MHC	major histocompatibility complex
MLN	mesenteric lymph nodes
NK	natural killer
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NPS	N-(palmitoyloxy) succinamide
OVA	ovalbumin
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PBST	phosphate buffered saline plus Tween 20
PEC	peritoneal exudate cells
PEG	polyethylene glycol
PGE2	prostaglandin E2
pIgR	poly immunoglobulin receptor
PLG	poly (lactide-co-glycosides)

PLN	popliteal lymph nodes
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl
PP	Peyer's patch
PRR	pattern recognition receptors
ROI	reactive oxygen intermediates
s.c.	subcutaneous
sIgA	secretory IgA
SOD	superoxide dismutase
TD	thoracic duct
TEM	transmission electron microscope
TGF	transforming growth factor
Th	T helper
TNF	tumour necrosis factor

Summary

Immune stimulating complexes (ISCOMS) are small cage like particles which form spontaneously upon agitation of phospholipid, cholesterol and the saponin adjuvant Quil A. It is possible to incorporate protein antigen into the ISCOMS structure and ISCOMS have been used to stimulate antigen-specific immune responses by both parenteral and mucosal routes. However, the mechanisms that underlie these adjuvant effects have not been fully elucidated. This project was undertaken in order to expand the knowledge of the adjuvant properties of ISCOMS and to identify the influence that these had on the ability of ISCOMS to drive an antigen-specific immune response.

My initial experiments concentrated on performing a detailed study of the innate effects of ISCOMS *in vivo*, when given initially as an intraperitoneal injection and later orally. The results presented demonstrate that ISCOMS have an ability to stimulate an innate inflammatory cascade, comprised of the recruitment and activation of an inflammatory infiltrate, secretion of inflammatory cytokines and mediators. Secretion of these factors was dependent on the presence of adherent cells indicating the possible role of macrophages in their production. A similar pattern of cellular recruitment and activation was measured in gut associated lymphoid tissues after oral administration of ISCOMS, but this was much less dramatic than had been observed earlier.

I then undertook an examination of the influence that these inflammatory mediators had on the generation of antigen-specific immunity as determined by antigen-specific DTH, proliferation, cytokine production, CTL activity serum IgG isotype production and intestinal IgA production, using gene-targeted knockout mice including IL-12KO, IL-6KO, IL-18KO, IL-4KO, iNOSKO and γ IFN γ KO. My experiments revealed that most of the components of the inflammatory response were not essential for the development of antigen-specific immunity to ISCOMS, but it did reveal that IL-12 played a particularly important role in the immunogenicity of ISCOMS, as immune responses were severely defective in these mice after either parenteral or oral immunisation with OVA ISCOMS.

I then went on to determine if the generation of normal antigen-specific immune response, in the absence of inflammatory cytokines, was due to the maintenance of the inflammatory cascade induced by ISCOMS. I therefore studied the cellular recruitment and inflammatory mediator release from ISCOMS injected gene targeted knockout mice. Despite most knockout animals retaining an intact innate cascade, the IL-12KO mice with decreased antigen-specific immune responses to ISCOMS also had an ablated innate response. However, unusually the γ IFNRRKO mice which had normal antigen-specific responses to ISCOMS also lacked an innate inflammatory cascade. This apparent dichotomy indicated that the role of the ISCOMS induced innate immune response for the generation of antigen-specific immunity is as complex as it is important and must be better understood if efficacious and potent vaccines are to be developed.

Chapter 1

Introduction

Infection with an invasive pathogen stimulates the development of a protective immune response and immunological memory. The purpose of vaccination is to mimic these effects of natural infection, without the morbidity and possible mortality associated with actual infection. Much time and energy has been invested in the development of vaccination strategies and, although there have been successes, such as measles, polio and smallpox, there is an ongoing need for effective vaccines that are readily available, easy to administer and capable of immunising against all the pathogens that endanger quality of life.

The majority of all antigenic encounters occur at mucosal surfaces such as the gut, nasal cavity and respiratory tract, eye and urogenital tract. These tissues have an extensive lymphoid system, which is distinct from the peripheral lymphoid system and so parenteral immunisations fail to generate protective immune responses in the mucosa, especially the gut. Therefore, it is important to generate vaccine strategies that are effective at mucosal sites. Subunit vaccines containing purified antigen are being investigated as immunisation strategies, as they retain the immunogenicity produced during a natural infection, but do not pose the threat of virulence. However, subunit vaccines are not efficacious when administered alone, especially by the oral route and therefore effective immunisation requires the co-administration of an adjuvant.

It has become clear recently, that the innate immune response has a strong, guiding influence over the initiation of adaptive immunity and determines the function of adjuvants. The purpose of the work described in this thesis was to explore the role of the innate immune response in the efficacy of the parenteral and oral vaccine vector ISCOMS.

1.0 Overview of the Immune Response

The mammalian immune response is a highly complex and delicately balanced process that comprises both innate and adaptive components. These two parts, although distinct from each other, are continually interacting.

1.1 Innate Immunity

Innate or natural immunity is considered phylogenetically to be the oldest form of host defence and forms of it exist in all species. It provides a rapid response to microorganisms which is not dependent on genetically variable, specific recognition elements. Instead this network exploits a mixture of highly conserved, invariant soluble factors and cell surface receptors, many of which can recognise non-protein structures such as carbohydrates or lipids, to monitor the normal environment and detect the presence of potentially harmful invading microorganisms.

i) Triggers of the Innate Immune System

The surface receptors on the host form a non-specific surveillance system for pathogen associated molecular patterns on invading organisms, a phenomenon that has been termed pattern recognition [Medzhitov and Janeway Jr, 1997b, Fearon and Locksley, 1996]. Three main families of pattern recognition receptors (PRRs) have been identified and classified according to their physical and functional properties. Firstly, there are PRRs that are plasma proteins and can opsonise microorganisms and therefore aid destruction by the complement membrane attack complex (C5-9). These include the collectins such as mannan binding lectin, conglutinin and lung surfactant proteins A and D [Sastry and Ezekowitz, 1993], or pentraxins such as C-reactive protein and serum amyloid protein [Gewurz et al., 1995]. The second family of PRRs comprises endocytic receptors which are found on the surface of cells such as macrophages and dendritic cells and bind directly to molecules present on invasive micro-organisms and activate entry into the endocytic pathway for

antigen processing and presentation, such as the macrophage mannose receptor [Stahl, 1992], macrophage scavenger receptor [Krieger and Herz, 1994]. The third family of PRRs is the Toll-like-receptors which are involved in both pattern recognition and signal transduction [Kopp and Medzhitov, 1999]. These were first identified in *Drosophila* and recently five Toll-like-receptor proteins have been discovered in humans [Rock et al., 1998]. Each contain a leucine rich extracellular domain and a cytoplasmic tail involved in signal transduction pathways, which activate NF- κ B [Kopp and Medzhitov, 1999]. Although many of the ligands for the Toll-like-receptors have yet to be identified, all known ones are involved in expression of co-stimulatory molecules and cytokine or soluble mediator secretion, via the induction of NF- κ B [Stancovski and Baltimore, 1997]. Most important of these is a receptor for LPS on monocytes and macrophages, which binds the lipopolysaccharide binding protein together with CD14 [Yang et al., 1998].

ii) The Innate Inflammatory Cascade

The ligation of PRRs on host cell surfaces or in serum, initiates a cascade of inflammatory events that comprises the release of chemotactic molecules, cellular recruitment and mediator release. These not only provide a rapid response to invasion, but also have a far reaching influence on subsequent antigen-specific immunity. The initial component is the release of chemokines, such as IL-8 or monocyte chemoattractant protein 1 (MCP-1), from activated phagocytes or inflamed endothelium, fibroblasts or platelets [Baggiolini, 1998]. Chemoattractants recruit neutrophils and monocytes, with neutrophils arriving first and being followed quickly by macrophages and dendritic cells, both of which are important for clearance of microbes and for processing and presentation of antigen to the lymphocytes which are the last of the recruited cells to arrive [Campbell et al., 1998]. The recruited neutrophils and macrophages become activated, increase their microbicidal activity, release soluble factors and macrophages show increased expression of co-stimulatory molecules, adhesion molecules and MHC class II. Mast cells are also involved in this early phase of the response. Classically these cells are described as the effector cells of IgE mediated immune responses, especially in the context of helminth infestations, but they are also capable of

secreting a number of preformed pro-inflammatory compounds, such as histamine, leukotriene B4 (LTB4), macrophage inflammatory protein-1 α (MIP-1 α) and MIP-1 β as well as TNF α [Mekori and Metcalfe, 2000] which can enhance the recruitment of other inflammatory phagocytes and lymphocytes, by upregulating the expression of surface adhesion molecules and thus providing a suitable site for the extravasation of leukocytes [Galli et al., 1999].

iii) Soluble Mediators of the Inflammatory Cascade

Pro-inflammatory mediators released during the acute phase response also induce a cascade of events that trigger metabolic processes in the host in an attempt to control infection, minimise damage and initiate repair to inflamed tissues. TNF α is released immediately as a preformed molecule from mast cells [Gordon and Galli, 1990], or synthesised by macrophages after stimulation by LPS or ingestion of bacteria or viruses, and after the binding of C5a complement component to the p55 and p75 TNF α receptors which are present on a wide variety of target cells and tissues [Adolf et al., 1994]. TNF α induces the synthesis and release of IL-1, which together with TNF α , synergise to induce IL-6 secretion [Baumann and Gauldie, 1994]. In association with IL-1, TNF α is also a potent pyrogen and appetite suppressant, inducing these effects by binding to receptors present on the cells of the hypothalamus [Tracey et al., 1988, Plata-Salaman et al., 1988]. TNF α can also stimulate γ IFN production by NK cells in synergy with IL-12 [Tripp et al., 1993]. Together with γ IFN, TNF α acts in an autocrine fashion to amplify the production of inducible nitric oxide (iNOS) and thus nitric oxide (NO) release from macrophages, increasing the microbicidal activity of these cells and increasing the blood flow to the inflamed site [Oswald and James, 1996].

IL-1 α and IL-1 β are produced by activated macrophages and many other cell types in an inactive form that is activated by IL-1 converting enzyme (ICE) which cleaves off a regulatory region [Black et al., 1988]. Active IL-1 binds to the type I and type II IL-1 receptors which are present on a wide variety of cells including T cells, B cells, endothelium, hepatocytes and the hypothalamus [Dinarello, 1998]. Many of the inflammatory effects of IL-1 are induced in combination with TNF α , including the stimulation of IL-6, fever and the

secretion of amyloid precursor proteins, as well as the release of chemotactic signals such as IL-8 and MCP-1 by mononuclear phagocytes and endothelium [Baumann and Gauldie, 1994]. IL-1 in synergy with IL-6, acts as a growth factor for CD4 T cells, inducing secretion of IL-2 production by T cells [Holsti and Raulet, 1989, Houssiau et al., 1988] and expression of the IL-2 receptor α chain (CD25) [Houssiau et al., 1989].

IL-6 is secreted from as part of the acute phase response by macrophages, fibroblasts and mast cells after activation by TNF α and IL-1 [Baumann and Gauldie, 1994]. It induces hepatocytes to secrete plasma proteins such as fibrinogen as part of the acute phase response and has many effects which overlap with those of TNF α and IL-1, including increasing body temperature, the expression of adhesion molecules and chemotactic factors on the surface of inflamed endothelium which aids the entry of leukocytes. IL-6 also plays a role in the antigen-specific immune response, as it promotes the growth and maturation of B cells and stimulates plasma cell differentiation [Kishimoto, 1989]. Recent studies have also implicated the direct involvement of IL-6 in the secretion of IgA [Beagley et al., 1989, Kunimoto et al., 1989], and IL-6 together with IL-10 has also been shown to maintain the secretion of IgA in the gut of IL-4KO mice [Okahashi et al., 1996]. IL-6KO mice have been reported to have defects in sIgA production and the recruitment of neutrophils, as well as being susceptible to intracellular organisms such as *Candida albicans* [Romani et al., 1996] or *Listeria monocytogenes* [Kopf et al., 1994]. However, other studies have disputed the involvement of IL-6 in generating antigen-specific mucosal immune responses [Bromander et al., 1996].

IL-12 is a heterodimeric cytokine secreted from macrophages, dendritic cells and B cells [Trinchieri, 1995]. The bioactive p70 heterodimer is comprised of disulphide linked [Gubler et al., 1991], 35kD and 40kD subunits [Kobayashi et al., 1989, Stern et al., 1990] whose genes are found on chromosomes 3 and 5 respectively [Sieburth et al., 1992]. IL-12 production is induced directly by bacterial and parasitic products including endotoxins, superantigens and heat shock proteins [Trinchieri, 1995], but also after cognate interactions between the CD40 and CD40 ligand (CD40L) [Kennedy et al., 1996, Stuber et al., 1996], present on APC and activated T cells respectively. The best known effect of IL-12 is to induce γ IFN production by NK cells and T cells and it is the principal factor controlling the

differentiation of Th1 CD4⁺ T cells [Wenner et al., 1996, Hsich et al., 1993]. However, IL-12 also upregulates the expression of IL-8 and thus enhances the recruitment of neutrophils to an inflamed site and the establishment of an inflammatory cascade [Nuame et al., 1993]. In addition, IL-12 increases the growth and cytotoxic activity of NK cells [Kobayashi et al., 1989, Robertson et al., 1992] and acts as a growth factor for activated NK cells [Nuame et al., 1993]. It has similar effects on CD8⁺ T cells [Gately et al., 1994, Chouaib et al., 1994], probably in conjunction with γ IFN. IL-12 alone can induce the upregulation of IL-2 and the expression of the IL-2 receptor α chain [Nuame et al., 1993], thus increasing responsiveness to IL-2 and the ability to clonally expand effector T cells.

The importance of IL-12 in infection has been shown in several models. Addition of rIL-12 enhances resistance to *Leishmania major* [Heinzel et al., 1993], *Listeria monocytogenes* [Hsieh et al., 1993, Tripp et al., 1994] and *Toxoplasma gondii* [Gazzinelli et al., 1993]. In the absence of IL-12, mice had defective protective immunity against *Mycobacterium tuberculosis* [Cooper et al., 1997]. IL-12 also protects against the pathology associated with normal granuloma response induced by *Schistosoma mansoni* eggs in liver and spleen [Wynn et al., 1994]. Because of its ability to enhance cytolytic activity, IL-12 has also been shown to be involved in the generation of anti-tumour CD8⁺ T cell activity in murine cancer models [Brunda et al., 1993].

IL-18 is a newly characterised cytokine which has several properties similar to IL-12 including the induction of γ IFN production. However IL-18 resembles IL-1 structurally and requires enzymatic cleavage by ICE to release the biologically active cytokine [Gu et al., 1997, Ghayur et al., 1997]. First identified as a product of Kupffer cells in the livers of *Propionibacterium acnes* infected mice [Okamura et al., 1995], IL-18 is now known to be produced by a number of different cell types, including keratinocytes [Stoll et al., 1997], osteoblasts [Udagawa et al., 1997], and macrophages [Okamura et al., 1995]. Like IL-12, IL-18 increases the cytolytic activity and induces secretion of γ IFN from NK cells and T cells [Okamura et al., 1998a] and enhances the development of Th1 cells [Kohno et al., 1997], and IL-12 and IL-18 may act together to generate a number of these effects. There is some recent

evidence that IL-12 may increase the expression of the IL-18R β 2 gene which would also increase the pressure to drive a Th1 mediated immune response [Xu et al., 1998].

The gaseous free radical nitric oxide (NO) is produced by the enzymatic action of nitric oxide synthase (NOS) on the amino acid arginine [Palmer et al., 1988]. There are three isoforms of the NOS enzyme which have two separate functions. Neuronal cell NOS (nNOS) and endothelial cell NOS (eNOS) are involved in the Ca²⁺ dependent, constitutive production of small amounts of NO which acts as a signal transduction molecule in the nervous system and as a vasodilatory molecule [Meffert et al., 1994, Moncada and Higgs, 1991]. Inducible NOS (iNOS) on the other hand, is found in macrophages after stimulation by inflammatory signals such as the phagocytosis of bacteria or recognition of conserved molecules on pathogens such as LPS [Griscavage et al., 1996]. The other major inducer of iNOS transcription is γ IFN [Kamijo et al., 1994], while TNF α , and IL-1 β can have similar effects [Green and Nacy, 1993]. The high levels of NO produced by activated macrophages are toxic to intracellular pathogens or tumour cells. These effects are due to the ability of NO to interfere with several intracellular metabolic processes, by targeting iron binding proteins including mitochondrial NADPH-ubiquinone, the succinate-ubiquinone oxidoreductases of the electron transport chain and the Kreb's cycle enzyme aconitase [Oswald and James, 1996]. NO also targets iron containing molecules of pathogens, forming nitrosyl-Fe molecules which disrupt the metabolic and proliferative activity of intracellular pathogens and inhibit the superoxide dismutase enzyme (SOD), which is capable of protecting the pathogen against the lethal effects of free radicles such as H₂O₂ and NO [Oswald and James, 1996]. NO has also been implicated in a number of disease conditions, the most important of which is septic shock, where it is responsible for dilation of the local and then later the systemic vasculature [Green and Nacy, 1993]. Thus the regulation of such a dangerous molecule is tightly controlled and NO production is inhibited by TGF β [Ding et al., 1990] and IL-10 [Cuncha et al., 1992] which decrease the stability of iNOS mRNA. Reactive oxygen intermediates (ROI) are a group of oxygen species including superoxide (O₂⁻), hydroxyl radicals (^oOH), and singlet oxygen species (^oO) which are formed following activation of the NADPH oxidase enzyme present in the lysosomal membrane of phagocytes

[Babior et al., 1988]. These molecules are all toxic and play an important part in the killing of intracellular pathogens, which is most clearly demonstrated in individuals with chronic granulomatous disease (CGD), who have a genetic defect in the ability to generate oxygen radicals. They are deficient in, or have an abnormal form of, one of the protein components that makes up the respiratory burst, for example NADPH oxidase [Clark, 1999]. These patients suffer recurrent infections of bacteria, parasites and fungal infections and develop widespread generation of granulomatous tissue which is formed in an attempt to control these infections [Clark, 1999].

1.2 The Role of the Innate Cascade on the Development of Adaptive Immunity

The mediators and cells described above not only play a crucial and often conclusive role in the early stages of infection, but also initiate and shape the ensuing antigen-specific immune response [Fearon and Locksley, 1996a]. Thus, the ligation of PRRs on resting dendritic cells increases the expression of costimulatory molecules, MHC class II and the antimicrobial activity of these cells [Medzhitov and Janeway Jr, 1997a, Medzhitov and Janeway Jr, 1997b, Janeway Jr, 1989], which enhances the ability to process and present antigen [Banchereau and Steinman, 1998]. In addition, these receptors and the pro-inflammatory cytokines IL-1, TNF α and IL-12, induces the migration of DC out of tissues and into the T cell dependent area of the secondary lymphoid organs where they can interact with naive T and B cells.

The nature of these ligand of PRR can also influence the ability of dendritic cells to polarise the eventual antigen-specific effector immune response. Recently it has been demonstrated that dendritic cells are capable of inducing both a Th1 and Th2 response from naive T cells, but this mechanism appears to be a consequence of the level of IL-12 production induced [Kalinski et al., 1999]. For example, a number of pathogens secrete IL-12 inhibiting factors such as prostanoids from some helminths [Meyer et al., 1996], or IL-10 induced by *Schistosoma mansoni* eggs [Velupillai and Harn, 1994], disruption of IL-12

production is also observed in HIV [Yoo et al., 1996] and measles virus infection [Karp et al., 1996].

Depending on the stimulus dendritic cells can also be immunogenic or tolerogenic. When resting myeloid derived dendritic cells were pre-cultured with necrotic fibroblasts *in vitro*, the dendritic cells, increased surface expression of costimulatory molecules and were capable of inducing proliferation of naive T cell clones [Gallucci et al., 1999]. In contrast, when resting dendritic cells were pre-cultured with apoptotic fibroblasts, no elevation of costimulatory molecules expression or naive T cell proliferation was measured [Gallucci et al., 1999]. This phenomenon was also detected *in vivo*, as OVA+apoptotic fibroblast immunised mice produced significantly lower OVA-specific DTH responses than OVA+necrotic fibroblast immunised mice [Gallucci et al., 1999].

Thus it appears that the nature of the antigen has a profound influence on the outcome of the immune response induced, or in fact whether immunity is stimulated at all.

1.3 Protective Immunity

There are three arms of the antigen-specific protective immune response, firstly CD4⁺ T lymphocytes which recognise antigen presented by MHC class II molecules and secrete cytokines, whose profile determines the nature of the effector immune response that is generated. γ IFN and IL-2 secretion are associated with Th1 CD4⁺ lymphocytes and the establishment of cell mediated immunity, whereas IL-4, IL-5, IL-10 and IL-13 are secreted by Th2 cells and promote humoral immune responses [Mosmann and Coffman, 1989, Mosmann, 1996]. Secondly, antigen-specific antibodies recognise structural patterns within the infecting agent, with CD4⁺ T cells assisting in the differentiation and antibody production by B cells [Parker, 1993]. Thirdly, antigen-specific CD8⁺ CTL, recognise and kill infected cells bearing processed antigenic peptides within MHC class I [Griffiths, 1995], by release of pore forming agents such as perforin and granzyme serine esterases that disrupt metabolic processes and induce apoptosis of the infected cell [Kagi et al., 1994]. CTL also induce apoptosis following ligation of the Fas ligand (FasL) expressed on CTL with Fas on the target

cell [Kagi et al., 1994]. The exact nature of the protective immune response depends on the infecting agent and thus if any vaccine immunotherapy is to be successful in generating a protective immune response it is vital that we understand and can mimic different immune mechanisms induced by various infecting organisms or disease processes. Viral infections generally involve the ability of MHC class I restricted CTL to kill infected cells, but also induce the production of neutralising antibodies to limit the dissemination of the viral pathogen. Intracellular pathogens other than viruses are either bacteria, protozoal parasites or fungi. Most of these organisms rely on active uptake into host cells following ligation of PRR and induction of phagocytosis and thus these pathogens generally target macrophages [Aderem and Underhill, 1999]. Because these organisms reside within the cell, they are concealed from immune surveillance, and most have evolved evasion strategies which not only prevents killing by normal antimicrobial processes of the cell but also prevents their discovery by the immune system [Brodsky et al., 1999]. However, after recognition by CD4⁺ T cells, the infected cells are activated by γ IFN, with enhancement of microbicidal activity of the macrophage and clearance of the pathogen. In addition, there may be secretion of TNF α and IL-1 as well as activation of CD8⁺ CTL [Orme et al., 1993]. Extracellular pathogens are continuously exposed to potential attack by the adaptive immune response and Th2 dependent humoral immune responses are the major mechanism for the elimination of such organisms. Small extracellular pathogens such as bacteria can be opsonised and cleared by macrophage phagocytosis, while larger multicellular organisms such as helminths or nematodes which have been opsonised by antigen-specific antibody can be cleared by antibody dependent complement or cell mediated cytotoxicity. Extracellular organisms may also secrete toxins that are harmful, and thus neutralising antibodies are also a powerful mechanism for the capture and clearance of these soluble molecules, by immune complex formation and clearance in the liver [Schfferli et al., 1986].

2.0 The Common Mucosal System

The tissues of the mucosae encounter the majority of antigens that enters the host and infections of mucosal surfaces such as the digestive tract, nasal cavity, respiratory tract, eye and urogenital tract and breast are the most common cause of morbidity and mortality in humans [Bloom, 1989]. These infections are caused by viruses such as rhinoviruses, influenza virus, poliovirus, and human immunodeficiency virus (HIV), bacteria such as *E. coli*, *S. typhi*, *V. cholerae*, *Bordetella pertussis* and *Mycobacterium tuberculosis*; protozoal parasites such as *Giardia lamblia* and *Cryptosporidium parvum*, as well as helminthic parasites such as *Trichuris trichura*, *Ascaris lumbricoides*, and *Haematobium solis*. The mucosal surfaces contain considerable amounts of mucosa-associated lymphoid tissue (MALT) that are anatomically and functionally distinct from the peripheral immune system [Mowat and Vincy, 1997]. Of these, the gut associated lymphoid tissues (GALT) are the most intricate and specialised, containing organised lymphoid structures such as the Peyer's patches (PP) and mesenteric lymph nodes (MLN), as well as large numbers of scattered lymphocytes both in the epithelium (intraepithelial lymphocytes (IEL)) and in the lamina propria (LP).

2.1 Immune Mechanisms of the GALT

The induction of intestinal immune responses is believed to occur first in the PP, which are submucosal nodules of organised lymphoid tissue in the small intestine, containing defined T and B cell areas including germinal centres (GC) [McGhee et al., 1992]. Above the T and B cell areas is the dome area of the PP which contains T and B cells, macrophages and dendritic cells, and bulges into the lumen of the small intestine, being covered by a single layer of follicular associated epithelium (FAE). This contains specialised epithelial cells (M cells), that have microfolds on their luminal surface and which are able to take up particulate materials such as bacteria and viruses from the intestinal lumen [Neutra et al., 1996]. M cells do not process antigen, but appear to transfer it to professional APC [Wolf and Bye, 1984],

which reside in an epithelial pocket created by the M cell, or in the dome area immediately below the FAE [Keren, 1992, Mowat and Viney, 1997]. The majority of these cells are dendritic cells, but they differ from those found in other tissues [Kelsall and Strober, 1996]. Most dendritic cells found beneath the M cells are immature and have been shown to capture antigenic peptides *in vivo* [Kelsall and Strober, 1996, Ruedl et al., 1996]. These cells are then thought to migrate to the MLN where they are able to present antigen to naive T and B cells [MacPherson et al., 1995]. Although the initial encounter with antigen is thought to occur in the PP, cells migrate away to other mucosal sites before becoming effector cells. For example B cells primed in the PP show preferential class switching to IgA production [McGhee et al., 1992], but before full differentiation, these leave the PP via the efferent lymphatics and recirculate to mucosal effector sites such as the LP, by which time they have differentiated into high affinity IgA secreting plasma cells [Brandtzaeg et al., 1999]. The production of IgA secreting cells occurs because of the influence of the local cytokine environment in the PP, with TGF β being a crucial switch factor for IgA production [Brandtzaeg et al., 1999].

T cells are also primed in the PP and like B cells, migrate from the PP to the mesenteric lymph node (MLN) via the afferent lymphatics before differentiating fully [McGhee et al., 1992]. The MLN has a normal secondary lymphoid architecture and appears to form an interface between the mucosal and peripheral immune systems [Mowat and Viney, 1997]. After a few days, these T and B cells leave the MLN and gain access to the thoracic duct (TD), from where they enter the blood stream and exit preferentially in the mucosal surfaces because of expression of the $\alpha 4 \beta 7$ integrin which associates with the mucosal addressin adhesion molecule-1 (MADCAM-1), found predominantly only on mucosal vasculature [Mowat and Viney, 1997]. In addition to access via M cells, some soluble antigen may also cross the villus epithelium directly and be transported to the MLN in the lymphatics or carried on an APC such as dendritic cells, which can lead to the priming of T cells in the MLN itself [Mowat and Viney, 1997].

2.2 Effector Immune Responses in the Mucosa

The effector phase of the mucosal immune response occurs in the wall of the gut itself. Here are found B cells, T cells, macrophages, dendritic cells and mast cells, which are scattered within the LP or as dispersed T cells within the epithelium (IEL). These cells are capable of making a wide range of immune responses, which are able to combat the variety of pathogens encountered at this site.

i) Humoral Immune Responses in the Mucosa

The principal humoral immune defence in the gut is mediated by secretory IgA (sIgA) antibodies [Brandzaeg, 1989]. Over 90% of all antibody in the gut is sIgA, which is produced by plasma cells in the LP as a dimer linked by a joining (J) chain. This dimer binds to the poly Ig receptor (pIgR) on the basal surface of the enterocytes in the crypt, triggering endocytosis and then transport through the epithelial cell in a transport vesicle [Mostov, 1994]. This vesicle ultimately fuses with the luminal surface of the epithelial cell, where the pIgR is partially cleaved and releases sIgA that contains a small fragment of the pIgR still attached (secretory component)[Mostov, 1994]. sIgA is found only in mucosal secretions and because of its multivalent binding sites, is ideally suited to interfering with binding and entry of pathogens into mucosal tissues [Czinn et al., 1993, Mazanec et al., 1995]. sIgA bound to luminal antigen can either be excreted or taken back up through the epithelium, where it can enter the liver via the portal bloodstream and be cleared through phagocytosis by hepatic macrophages (Kupffer cells) or excreted in the bile. As IgA does not bind complement, sIgA mediated clearance of pathogens does not cause inflammation in the delicate tissues of the gut mucosa [Russell-Jones et al., 1981, Lamm et al., 1995].

Despite the predominance of sIgA in mucosal secretions, the absence of sIgA is a relatively common immunodeficiency and usually does not incur major complications or infection [Mbawuike et al., 1999]. In this situation the function of sIgA may be replaced by IgM, which also contains a J chain, and thus can bind to the pIgR and undergo transcytosis across the epithelium. IgG is found in secretions only under conditions of inflammation,

while IgE production may contribute to protective responses against helminth infection, by binding to FcεR on eosinophils and mast cells elevated at times of expulsion and releasing toxic granule contents into the lumen of the gut [Galli, 1997].

ii) Cellular Immune Responses in the Mucosa

Effector T cells are localised throughout the organised and diffuse compartments of the GALT. The CD4⁺ T cells that are found within these tissues all show an activated memory phenotype [Mowat and Viney, 1997] and they normally secrete cytokines such as γIFN, IL-4, IL-5 and TGFβ [Mowat and Viney, 1997] which are thought to directly influence the isotype switching of antibodies from B cells. IL-5 is a required cytokine if an augmented sIgA response is to be generated [Harriman et al., 1988], and TGFβ also directs the class switching to IgA [Brandtzaeg et al., 1999].

The T cells in the IEL are very different to those found in the periphery. Up to 80% of these cells are CD8⁺, and conventional CD4αβ⁺ T cells are rare [Guy-Grand and Vassalli, 1993]. There are many different subsets of IEL and of the CD8⁺ IEL, only a small subset are classical CD8αβ⁺ αβ TCR⁺ CTL, with another population of IEL being CD8αα⁺ [Guy-Grand and Vassalli, 1993]. Of these cells some are αβ TCR⁺ but some are also γδ TCR⁺ [Lefrancois, 1991]. These γδ TCR⁺ cells are thought to have developed extrathymically [Rocha et al., 1994] and some γδ TCR⁺ IEL exhibit NK cell like lytic activity but this is not generated to MHC class I restricted antigens [McGhee et al., 1992]. The role, if any, that these cells have in protective mucosal immune responses is yet to be fully defined as they normally do not exhibit classical CTL activity and do not proliferate well to mitogens or antigen [Mowat and Viney, 1997], even though a large expansion of CD8αα⁺ γδ TCR⁺ cells is found in coeliac patients [Halstensen et al., 1989, Savilahi et al., 1990]. There is some recent evidence that these γδ TCR⁺ IEL may play some regulatory role in maintaining gut homeostasis and tolerance to intestinal antigens [Mowat and Viney, 1997], in addition to a possible role in providing help for mucosal IgA secretion [Fujihashi et al., 1996]. Many of the IEL have been identified as containing cytoplasmic granules and perforin [Guy-Grand et al., 1991] and some CD8αβ⁺ IEL have been shown to have antigen-specific cytotoxic activities

against *Toxoplasma gondii* [Charles et al., 1994b]. Nearly all subsets of IEL express the $\alpha E\beta 7$ integrin, which associates with E-cadherin on enterocytes and so is thought to be responsible for the accumulation and maintenance of IEL in the villus epithelium [Cepek et al., 1994].

2.3 Immune Regulation in the GALT

Although the immune apparatus of the gut must generate strong protective immune responses against pathogens, the majority of antigen encountered by the GALT is harmless, such as dietary proteins and commensal bacteria. Active immune responses against these materials would be dangerous as it can lead to chronic inflammatory diseases such as Crohn's disease, ulcerative colitis and coeliac disease [Mowat, 1987]. For these reasons, the default response to harmless antigen is the induction of systemic immunological unresponsiveness, known as oral tolerance [Mowat, 1987]. This long lasting phenomenon can affect all aspects of the systemic immune response and in addition to being of physiological importance, it is a potential obstacle to the development of orally active vaccines containing purified protein antigens [Mowat, 1987].

Several mechanisms have been implicated in mediating oral tolerance, but these may be dependent on the feeding regime used to induce tolerance. Thus it is proposed that single or continuous feeds of high doses of antigen may lead to clonal anergy or perhaps deletion of antigen-specific T cells, whereas multiple feeds of low doses of antigen may induce the generation of regulatory T cells such as the CD4⁺ Th3 subset or Tr1 subsets of T cells, which may suppress other T cell responses by secretion of TGF β , IL-4 or IL-10 [Faria and Weiner, 1999]. In all cases, CD4⁺ T cells appear to be the most important targets and mediators of oral tolerance [Mowat and Viney, 1997]. The induction of tolerance is preceded by limited activation of these cells in tissues of the GALT such as the MLN, and it appears that orally administered soluble antigens may be presented by APC lacking the appropriate costimulatory molecules such as B7.1 and B7.2, which leads to partial activation of the CD4⁺ T cells, followed by unresponsiveness (anergy) upon subsequent restimulation with antigen in

an immunogenic form [Mueller et al., 1989]. Of the APC that might be involved, recent attention has focused on the resting dendritic cells present in the gut. Administration of the dendritic cell growth factor flt3 ligand (FL3L) increases the numbers of dendritic cells in the GALT and other lymphoid organs and dramatically enhances the development of oral tolerance [Viney et al., 1998]. These FL3L induced dendritic cells express low levels of B7 and CD40 costimulatory molecules, which may indicate dendritic cells in the gut are normally in a resting state and present antigen in a tolerogenic fashion [Viney et al., 1998]. In addition, recent evidence suggests that the gut is rich in immunomodulatory cytokines, such as IL-10, TGF β and PGE $_2$, which may inhibit the activation of APC and thus suppress T cell induction [Khoury et al., 1992]. Overcoming or modulating these mechanisms is an important consideration for designing oral vaccines.

2.4 Compartmentalisation of the GALT

One of the most important features of the mucosal immune system is its compartmentalisation and isolation from the peripheral immune system. T and B cells primed in the GALT recirculate preferentially to mucosal surfaces, whereas T and B cells stimulated in other peripheral lymphoid tissues are unable to enter mucosal tissues [Phillips-Quagliata and Lamme, 1988]. This phenomenon is due to the fact that lymphocytes primed in the MALT lose L-selectin, and increase their expression of $\alpha 4\beta 7$ which interacts with the mucosal addressin adhesion molecule-1 (MAdCAM-1), found mainly on endothelium of mucosal vasculature [Bargatze et al., 1995]. This provides the molecular basis of why a mucosally delivered vaccine is necessary to generate protective immune responses in these tissues. Despite this separation from the peripheral immune system, there is dissemination of lymphocytes between the different mucosal tissues, which all express MAdCAM-1. This is termed the common mucosal immune system and its existence is important for the development of mucosal vaccines as it implies that lymphocytes can be primed at an easily accessible mucosal site, such as the gut or nasal cavity, and then disseminate to other distant mucosal tissues as effector cells [Mestecky et al., 1994].

3.0 Mucosal Vaccination Strategies

It is important that a successful vaccine is able to prime all the arms of the immune response that would normally be stimulated by natural infection, as well as inducing effective memory. The majority of current vaccines are administered parenterally, but these are generally ineffective against mucosal pathogens, due to the inability of primed lymphocytes to enter the mucosal compartment [Phillips-Quagliata and Lamme, 1988]. Therefore there is a pressing need for the development of vaccines which are active at mucosal sites and it is widely accepted that a single, orally administered recombinant vaccine inducing long lasting mucosal and systemic immunity is a principal goal of vaccine research.

3.1 Oral Vaccination Vectors

i) Live Attenuated Oral Vaccines

The simplest means of generating an appropriate, long lasting immune response to a pathogen is by pre-exposure to the living organism. However, as the principle of vaccination is to protect the host without inducing disease, it is vital that the infecting micro-organism is made harmless before administration. Therefore, the first vaccines consisted of whole organisms which were killed, one of the most successful being the Salk polio vaccine [Smith, 1992]. However, although killed vaccines share most antigens with the live pathogen, they often do not induce the same range of innate and specific immune responses due to their inability to invade. Therefore several attenuated vectors have been investigated to induce protective responses. Of these, attenuated *Salmonella* strains, with mutations in growth genes such as *surA* have been very successful at generating protective immune responses [Sydenham et al., 2000], as have *Shigella* strains with mutated genes *aroA* and *virG* genes [Kotloff et al., 1996, Noriega et al., 1996].

ii) Attenuated Organisms as Oral Vectors

Attenuated bacteria and viruses have also been used as delivery vectors for the generation of immune responses against incorporated DNA of other pathogens. For example, *Salmonella* [VanCott et al., 1996, Steger and Pauza, 1997], *Shigella* [Fennelly et al., 1999], *Listeria* [Ikonomidis et al., 1997] and BCG bacteria [Fennelly et al., 1995] have been studied as potential mucosal vaccine vectors, after incorporation of genes from a number of different pathogens including influenza [Ikonomidis et al., 1997], measles [Fennelly et al., 1995], simian immunodeficiency virus (SIV) [Steger and Pauza, 1997], and Tetanus Toxoid [VanCott et al., 1996]. Viruses have also been used as vaccine vectors, for example attenuated vaccinia virus [Moss et al., 1984] and Venezuelan equine encephalitis virus [Caley et al., 1997] have been studied for their ability to deliver in the most part other viral genes. This strategy is particularly appropriate in the defence against viral pathogens as it is capable of generating CTL responses. However, there are several problems that have hindered the development of such recombinant bacterial and viral vectors. The bacterial carrier itself can induce an immune response in the GALT, which if a booster regime is required means that the second immunisation may be blocked by an anti-vector response [McGhee et al., 1992]. Also the expression of the foreign gene may be unstable and therefore lost and there is a possibility of a reversal of the attenuating processes of the carrier organism [McGhee et al., 1992]. These types of vaccines are also not particularly stable at fluctuating temperatures and so limit the potential market to that of more temperate climates. Finally, the use of the inactivated carrier limits the immune response generated to that normally induced to the carrier and so may not stimulate a response relevant for clearance of the pathogen [McGhee et al., 1992]. Thus research has been undertaken to search for non living mucosal vaccine carriers that generate all of the required protective immune responses, both mucosally and systemically, but which also avoid the problems associated with using attenuated organisms.

3.2 Oral Adjuvants

i) Soluble Toxins as Adjuvants

A number of non-viable mucosal adjuvants have been described, of which cholera toxin (CT) produced from *Vibrio cholerae* and the homologous heat labile enterotoxin from *E. coli* (LT) are the most widely studied. These toxins are comprised of five attachment units or B subunits surrounding the enzymatically active A subunit, which is divided into A1 and A2 [Spangler, 1992]. The B subunits bind to the GM1 ganglioside receptor found on the surface of many cells, including intestinal enterocytes and following binding, the A subunit can translocate into the cell [Holmgren, 1981]. The CTA1 and LTA1 subunits have ADP-ribosyl transferase activity, which activates GTP binding proteins and Gs proteins, thus increasing adenylate cyclase activity and cAMP levels [Holmgren, 1981]. In addition to being responsible for the massive fluid and electrolyte loss seen in the relevant infections, the ADP-ribosylase activity is crucial for the adjuvant activity of CT, although it may not be required for that of LT [Lycke, 1997].

CT was first recognised as a potential adjuvant when it was found that the holotoxin stimulated active mucosal and systemic immune responses, both to itself and to co-administered unrelated protein antigen when given orally [Lycke and Holmgren, 1986]. Since this discovery, the use of CT as an adjuvant has become more refined and it has been shown that CT is capable of increasing both humoral and cell mediated immunity mediated by both CD4 and CD8 T cells. For example, feeding of low doses of CT together with keyhole limpet haemocyanin (KLH), could induce both KLH-specific antibody and cell mediated immune responses. Feeding OVA+CT generates antigen-specific DTH, proliferation, Th1 and Th2 cytokine secretion as well as high levels of mucosal sIgA and IgG [Grdic et al., 1999].

There are several ways in which in which CT or LT may act as adjuvants, including increasing the uptake of luminal antigen via epithelial cells, increasing expression of B7.2 on APC [Cong et al., 1997] and stimulation of high levels of inflammatory cytokines such as IL-1 and IL-6 [Grdic et al., 1999]. However, the mucosal adjuvant effects of CT are dependent on the presence of IL-4 [Vajdy et al., 1995]. These results have been interpreted as showing

that the local adjuvant effects of CT require presentation of antigen in a manner which involves intimate cell-cell contacts in the follicular areas of Peyer's Patches (PP), which are defective in IL-4KO mice [Vajdy et al., 1995]. Despite the potent adjuvant effects of CT and LT, their practical use as vaccine vectors has been limited by their high toxicity in man. In addition, they also carry the same potential risk of inducing immune responses to themselves as with live vectors. Recent work has attempted to develop a variety of non toxic forms of CT and LT and these may prove useful in the future [Agren et al., 1997].

ii) Particulate Oral Adjuvants

A large number of orally active adjuvant systems are particulate in form. This may be because these are targeted preferentially to M cells, or because they protect protein antigen in the hostile environment of the gut. One such example are microspheres constructed from biodegradable polymers such as poly(lactide-co-glycosides) (PLG). These can be made to many different sizes, from $\geq 1\mu\text{m}$ up to 3 mm in diameter and can be formulated to degrade at different rates, releasing antigen in a constant flow over a few days to up to 2 years [Morris et al., 1994], which could eliminate the need for booster immunisations. Oral administration of microparticles induces a wide range of antigen-specific systemic and mucosal immune responses, including sIgA responses and systemic CTL and serum IgG responses [Maloy et al., 1994]. However, this approach has proved difficult as large amounts of antigen are required and the microencapsulation process may alter the antigenicity and stability of the incorporated antigen [Gupta and Siber, 1995].

Simple liposomes are another particulate vaccine carrier. Like PLG, their size can be varied and it is possible to incorporate protein antigen. Although preliminary studies have shown that sIgA responses can be stimulated by oral immunisation with liposomes containing antigen [Michalek et al., 1989], liposomes are fairly unstable in the intestine and thus it would be desirable to have other stable, non-living mucosal adjuvant systems that induce a wide range of immune responses by the oral route. This prompted the investigation of lipophilic immune stimulating complexes (ISCOMS) containing Quil A.

4.0 Immune Stimulating Complexes (ISCOMS) as Oral Vaccine Vectors

4.1 Structure of ISCOMS

ISCOMS were first described by Morein and colleagues in 1984. An ISCOM is a hydrophobically bonded micelle that forms spontaneously on mixing cholesterol, a lipid such as phosphatidyl choline and the mixture of saponins which comprise Quil A [Lovgren and Morein, 1988]. Each ISCOM particle consists of identical 12nm subunits which combine to form rigid icosahedral matrices of about 40nm diameter [Morein, 1988] (Figure 1.1). During formation of the ISCOMS, it is also possible to incorporate other hydrophobic molecules such as glycoproteins from cell membranes. The first ISCOMS to be described used crude mixtures of glycoproteins found in the membranes of viruses and bacteria. These included membrane glycoproteins of viruses such as parainfluenza-3 virus [Morein et al., 1984], influenza A virus [Sundquist et al., 1988] measles virus [Varsanyi et al., 1987], feline leukaemia virus [Osterhaus et al., 1985] and some bacterial antigens for example, *Neisseria gonorrhoeae* [Kersten et al., 1988a]. However, it was difficult to incorporate purified hydrophilic protein antigens, thus limiting the potential usefulness of the approach. This can be overcome by chemical modification of proteins by acidification to reveal hydrophobic groups [Morein et al., 1990], or by addition of hydrophobic regions by chemical linkage of palmitic acid tails [Mowat and Reid, 1992]. Using the latter approach, a variety of proteins have been incorporated into ISCOMS including ovalbumin (OVA) [Reid, 1992], cytochrome c [Reid, 1992] gp120 from HIV [Browning et al., 1992] and somatostatin-avidin complexes [Estrada et al., 1995].

4.2 ISCOMS as Parenteral Adjuvants

Initial experiments showed that parenteral immunisation with ISCOMS containing viral and bacterial membrane glycoproteins induced antigen-specific immune responses, but these were assessed mainly by the levels of serum antibody [Morein et al., 1984, Ertürk et al.,

1989, Lovgren et al., 1988, Kersten et al., 1988b, Nagy et al., 1990] The antibody levels stimulated by ISCOMS were at least equivalent to those found after immunisation with conventional adjuvants such as Complete Freund's Adjuvant (CFA) or alum, and parenteral immunisation with ISCOMS induced all subclasses of IgG [Lovgren, 1988]. Later studies investigated the effect of ISCOMS on other immunological parameters and found that cell mediated immune responses were also induced. These included delayed type hypersensitivity (DTH) reactions *in vivo* [Mowat et al., 1991a, Grdic et al., 1999], antigen-specific proliferative responses [Mowat et al., 1991a, Fossum et al., 1990, Maloy et al., 1995, Grdic et al., 1999] and cytokine production *in vitro* [Maloy et al., 1995, Sjolander et al., 1997] These responses are characteristically CD4⁺ T cell dependent and the profile of IgG isotypes secreted after immunisation with ISCOMS, indicated that both Th1 and Th2 subsets of CD4⁺ T cells were primed by parenteral immunisation [Maloy et al., 1995, Sjolander et al., 1997]. This finding was confirmed by the pattern of antigen-specific cytokine production which encompassed a Th1 and Th2 profile, including IL-2 [Fossum et al., 1990, Villacres-Eriksson et al., 1992, Maloy et al., 1995, Villacres-Eriksson, 1995], IL-4 [Maloy et al., 1995, Villacres-Eriksson, 1995, Sjolander et al., 1997], IL-5 [Maloy et al., 1995], IL-10 [Sjolander et al., 1997, Villacres-Eriksson, 1995] and γ IFN [Villacres-Eriksson et al., 1992, Maloy et al., 1995, Sjolander et al., 1997, Villacres-Eriksson, 1995]. A surprising and important additional property of ISCOMS was the finding that antigen entrapped within the ISCOMS structure was able to enter the endogenous antigen processing pathway and hence prime class I MHC-restricted CTL. CTL activity has been shown with a number of antigens [Mowat et al., 1991a, Heeg et al., 1991, Jones et al., 1988, Trudel et al., 1992, Mowat et al., 1993], and the OVA-specific CTL have been identified as CD8⁺ lymphocytes that recognise the immunodominant epitope OVA 257-264 expressed from endogenously synthesised protein in H-2K^b cells [Heeg et al., 1991] The basis for this capacity of ISCOMS to prime for CTL activity is unknown, but could reflect the detergent effect of Quil A allowing direct entry of protein into the cytoplasm through the cell membrane or after ingestion into an endosomal compartment [Campbell and Peerbayc, 1992]. Previous studies have shown that the induction of CTL by ISCOMS requires functionally active macrophages, as CTL generation can be inhibited by

the lysosomal toxin dichloromethylene diphosphate (Cl₂MDP), or with silica, both of which paralyse the function of macrophages [Maloy, 1996]. This is consistent with recent findings that the phagocytic pathway in macrophages is highly efficient at driving class I processing in other systems [Debrick et al., 1991, Kovacsovics-Bankowski and Rock, 1995, Harding and Song, 1994].

4.3 Induction of Protective Immunity by ISCOMS

The essential property of any vaccine is that it can generate a highly specific protective response to subsequent challenge infections. Although the primary response to ISCOMS peaks 2-3 weeks after immunisation, memory T cell responses can be recalled for up to 8 months after a single subcutaneous immunisation with OVA ISCOMS [Maloy, 1996] indicating their potential to generate long-lasting memory.

The ability of parenteral administration of antigen loaded ISCOMS to confer resistance to a challenge with a pathogen has been tested in a number of animal models of infection including influenza A [Lovgren et al., 1990], canine distemper virus [Visser et al., 1992, de Vries et al., 1988], pseudorabies virus [Tulman and Garmendia, 1994], rabies virus [Fekadu et al., 1992], simian immunodeficiency virus (SIV) [de Vries et al., 1994] and respiratory syncytial virus [Trudel et al., 1992], *Salmonella* [Charles et al., 1994a], *Yersinia enterocolitica* [Noll and Autenreith, 1996], *Trypanosoma cruzi* [Araujo and Morein, 1991], *Leishmania major* [Papadopoulou et al., 1998] and *Toxoplasma gondii* [Overnes et al., 1991]. Most encouraging is an ISCOMS based parenteral vaccine against equine influenza virus which is currently being used in Sweden. Trials of a human flu vaccine using ISCOMS technology have also been initiated. These findings have recently been published and indicate that a single intramuscular injection of an ISCOM based influenza vaccine was tolerated well and induced an increased level of influenza-specific CTL activity in 50-60% of the volunteers, as compared to those who received the standard influenza vaccine [Ennis et al., 1999]. Such positive outcomes of early human trials indicates that ISCOMS which retain immunogenicity can be formulated for human use.

4.4 ISCOMS as Mucosal Adjuvants

Early studies showed that intranasal administration of ISCOMS containing influenza A virus envelope glycoproteins stimulated serum antibody titres equivalent to those found after subcutaneous administration of ISCOMS [Lovgren, 1988]. Subsequent work using the same vector demonstrated local cellular and humoral immune responses in the lung after intranasal immunisation [Jones et al., 1988], as well as immunity to challenge with live influenza A virus [Lovgren et al., 1988]. By using a fusion peptide of paramyxovirus linked to a measles virus derived CTL epitope from measles nucleoprotein, ISCOMS were also shown to prime nucleoprotein-specific CTL after an intranasal immunisation [Hsu et al., 1996]. Although initial studies suggested that ISCOMS containing influenza proteins were ineffective when given by the oral route [Lovgren, 1988], ISCOMS are exceptionally stable structures, and can resist degradation by exposure to low pH and bile salts [Kersten, 1990]. Furthermore, it has been known for many years that the saponins themselves are effective adjuvants when given orally, inducing serum antibodies, antigen-specific proliferation and CTL activity [Chavali and Campbell, 1987b, Chavali and Campbell, 1987a]. These indications that ISCOMS could be an effective oral vaccine were confirmed by initial findings from this laboratory that a single feed of ISCOMS containing OVA could induce primary OVA-specific IgG, DTH and class I MHC-restricted CTL responses in mice [Mowat et al., 1991a]. In addition, feeding OVA ISCOMS did not induce the systemic tolerance to subsequent challenge which is the normal consequence of feeding equivalent amounts of soluble OVA. Thus ISCOMS appear to bypass the regulatory mechanisms which normally inhibit responses to orally administered proteins. Although the dose of OVA ISCOMS needed to induce primary systemic responses orally was ten fold greater than that given parenterally [Mowat et al., 1991a], as little as 50µg OVA could induce detectable DTH responses [Mowat et al., 1991a]. However, it was more difficult to prime serum antibody responses in this way and six feeds of 50µg - 100µg OVA ISCOMS were found to be optimal for inducing serum IgG antibody responses and CTL activity in the spleen. This protocol also

stimulates local immune responses, as shown by the fact that CTL could be demonstrated in draining mesenteric lymph node (MLN) and by the presence of high levels of secretory IgA antibodies in gut lavage fluid [Mowat et al., 1993]. Interestingly, optimal IgA production was dependent more on the frequency of oral immunisation, rather than the amount of antigen, as IgA levels increased progressively after 1, 3 or 6 feeds, but 10µg OVA/dose was as effective as 100µg [Mowat et al., 1993]. No IgA antibodies could be found in serum after oral immunisation alone, but these could be stimulated by combined oral and parenteral immunisation with OVA ISCOMS (Personal Communication A.M. Donachie). Later studies showed that repeated oral immunisation with OVA ISCOMS resulted in both Th1 and Th2 dependent immune responses, with OVA-specific proliferative responses and the production of IL-2, γIFN and IL-5 in the MLN and spleen [Maloy et al., 1995]. Oral OVA ISCOMS also primed for both IgG1 and IgG2a serum antibody production after challenge with OVA parenterally [Maloy et al., 1995]. Thus, orally administered ISCOMS are strong inducers of both local and systemic immunity, prime both MHC class I and MHC class II restricted responses, induce cellular and humoral responses and do not bias towards either the Th1 or Th2 cells. Subsequent work has shown that the oral immunogenicity of ISCOMS is not confined to the model antigen OVA. Local and systemic immune responses which protected against challenge were seen after oral administration of ISCOMS containing subunit antigens derived from influenza virus [Ghazi et al., 1995, Scheepers and Becht, 1994], and surface sporozoite antigens from *Eimeria falciformis* [Kazanji et al., 1994].

These studies also have important practical implications for the use of ISCOMS as vaccine vectors. Firstly, the toxicity often found due to the Quil A component of ISCOMS given by other routes is reduced when given orally [Phillips et al., 1979]. Secondly, oral immunisation with OVA ISCOMS does not induce susceptibility to immunopathology in the intestine upon subsequent oral exposure to antigen [Mowat et al., 1993], as is found in other circumstances when oral tolerance is broken. Together these results suggest that ISCOMS will be safe and effective when given as an oral or nasal vaccine.

There have been few studies of the immunogenicity of ISCOMS given by other mucosal routes. However it has been shown that local antigen-specific antibody responses

can be generated when ISCOMS containing antigens from sheep erythrocytes are administered into the vagina or the upper reproductive tracts of female mice. [Thapar et al., 1991].

Thus, especially after the encouraging start to human trials of an intramuscular ISCOMS based influenza vaccine, the development of oral human ISCOMS based vaccines is a second priority.

4.5 Mechanisms for ISCOMS Adjuvanticity

Little is known currently of how ISCOMS act. However, previous work in our laboratory had suggested that Quil A and the particulate nature of ISCOMS played an important role [Maloy, 1996]. Quil A itself is capable of inducing similar antigen-specific immune responses by both parenteral and oral routes, with the same range of CD4⁺ and CD8⁺ T cell responses, as well as local and systemic antibody production [Campbell and Peerbaye, 1992]. However, much larger amounts of both Quil A and antigen are required to generate immune responses equivalent to those induced by ISCOMS themselves [Maloy, 1996]. Thus it seems that delivery of the Quil A adjuvant in the particulate form of the ISCOMS is central to their adjuvant effects. Other work has suggested that ISCOMS may influence the innate immune response, with reports of increased recruitment of neutrophils [Watson et al., 1989] and the production of IL-1 [Villacres-Eriksson et al., 1993] IL-6 [Behboudi et al., 1997] and IL-12 [Villacres-Eriksson et al., 1997] after parenteral administration. In addition recent evidence has indicated that ISCOMS induce an upregulation of the expression of B7.2 [Sambhara et al., 1998]. However, these properties have not been studied in detail, nor is it known if they are essential for the adjuvant effects of ISCOMS on specific immune responses. Finally no studies have been undertaken in the mucosa. These were the aims of the current work.

5.0 Specific Aims of Study

In the first part of this thesis, I assessed the innate responses induced by a single administration of ISCOMS. To do this, I studied the effects of injecting ISCOMS into the peritoneum, as this provided an easily accessible and discrete compartment from which inflammatory cells could be removed. I monitored the number and phenotypic populations of cells that were recruited and determined their functional activity by measuring the production of inflammatory cytokines and mediators. Having obtained data from this site, I then performed equivalent studies on the innate immune responses induced in the secondary lymphoid tissues of the small intestine following a single oral dose of ISCOMS. The results of these studies are described in Chapter 3.

As these experiments showed that ISCOMS stimulated a large range of innate immune responses, I then went on to determine how some of these factors influenced the induction of antigen-specific immune responses to ISCOMS using gene-targeted knock out mice. The results of this work are described in Chapter 4.

As these studies indicated that only certain components of the innate immune response were required for the immunogenicity of ISCOMS, finally, I examined how defects in these factors correlated with differences in the early inflammatory responses induced by ISCOMS. These results are presented in Chapter 5.

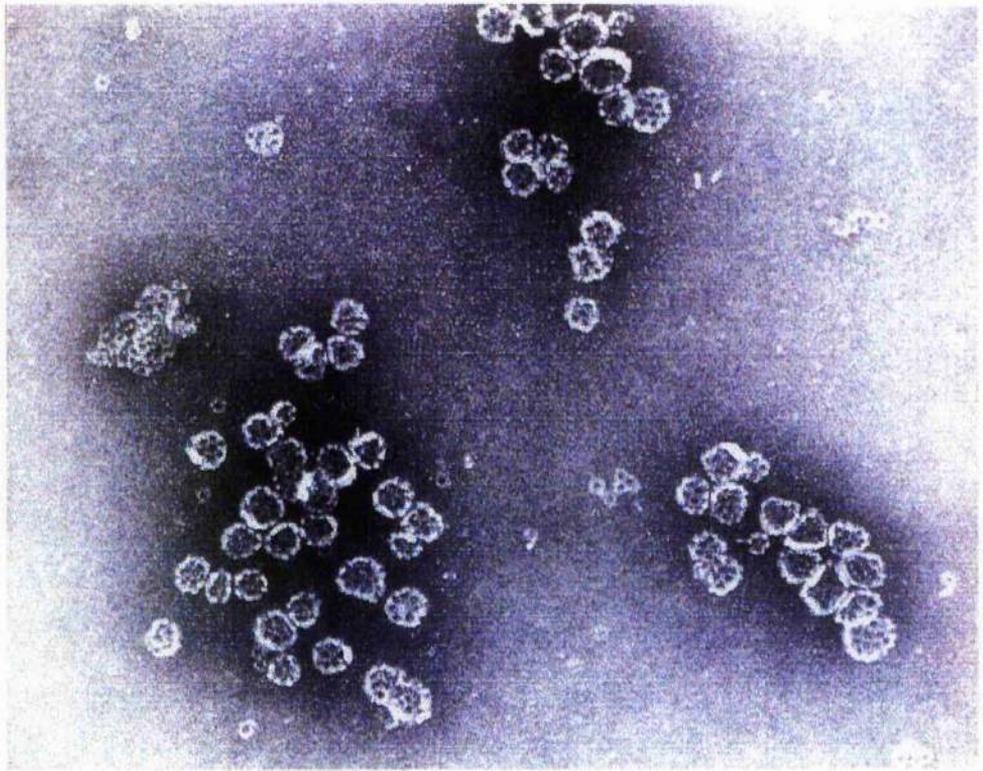


Figure 1.1 Ultrastructural appearance of OVA ISCOMS.

A transmission electron micrograph (magnification $\times 73\,000$) of ISCOMS after negative staining, with 3% phosphotungstic acid, showing the characteristic cage-like structure.

Chapter 2

Materials and Methods

2.1 Animals

Female C57Bl/6 (B6) mice were purchased from Harlan Olac (Bicester, Oxon, UK.) while female BALB/c mice were bred at the University of Glasgow. 129/Sv γ IFN-type II-receptor knockout (γ IFNRKO) [Huang et al., 1993] mice and (B6x129) F2 IL-4 knockout (IL-4KO) [Kopf et al., 1993] mice were originally obtained from Dr H. Bluethmann (Hoffmann-La Roche, Basal, CH). p40 IL-12 deficient (IL-12KO) BALB/c and C57BL/6 [Magram et al., 1996] mice were obtained from Dr. J. Magram, Roche Pharmaceuticals, Piscataway, NJ, USA. Inducible nitric oxide synthase deficient (iNOSKO) MF1 mice [Wei et al., 1995] and IL-18 deficient (MF1x129) mice were a gift from Prof. F.Y. Liew, Department of Immunology, University of Glasgow. All these mice were bred and maintained at the University of Glasgow under SPF conditions and first used at 6-8 weeks of age.

Female IL-6 deficient (IL-6KO) SV mice [Kopf et al., 1994] were obtained from Prof. J. Alexander, Department of Immunology, University of Strathclyde, Glasgow and were bred and maintained at the University of Strathclyde.

2.2 Anaesthesia

For some procedures, animals were anaesthetised using 5% halothane BP (Rhône Merieux, Harlow, Essex, U.K.)

2.3 Antigens

Ovalbumin (OVA, Grade V) was obtained from Sigma (Poole, Dorset, U.K.) as a lyophilised powder and stored at 4°C until required.

Heat-aggregated OVA (HAO) was prepared by heating a 2% (w/v) solution of OVA in sterile saline (Baxter Healthcare Ltd, Thetford, Norfolk, U.K.) at 70°C for 60 minutes in a waterbath. The precipitated OVA was then centrifuged for 10 minutes at 450g at 4°C and the supernatant removed. The pellet was then resuspended and washed with cold saline and re-centrifuged. The pelleted HAO was resuspended in sterile saline at a concentration of 20mg/ml and stored in aliquots at -20°C until required. For use as an *in vivo* challenge, HAO was resuspended at a concentration of 2mg/ml in sterile saline and sonicated for 20 minutes until a colloidal suspension was obtained.

2.4 Adjuvants

Quil A (Spikoside) produced by Iscotec AB, Lulea, Sweden, was kindly provided by Dr. K. Lövgren (Department of Veterinary Medical Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden.) and whole cholera toxin (CT) derived from *Vibrio cholerae* was purchased from Sigma, resuspended in sterile saline to a concentration of 1mg/ml and stored at 4°C until required.

2.5 Production of OVA ISCOMS

OVA ISCOMS were prepared using a modified protocol of that described previously [Mowat and Reid, 1992]. In order to incorporate the non-hydrophobic OVA protein into the lipophilic ISCOMS matrix, OVA was chemically modified by attachment of hydrophobic palmitic acid groups via a succinimide esterification reaction. Palmitified OVA was then mixed with cholesterol and phosphatidyl choline in the presence of Quil A and the ISCOMS formed spontaneously.

i) Palmitification of OVA

20mg OVA was dissolved in 2.5ml of 0.05M carbonate buffer (pH 9.2) and passed through a Sephadex PD10 column (Pharmacia, Uppsala, Sweden.) to remove low molecular

weight peptides. Whole OVA was eluted from the column with 0.05M carbonate buffer and 200µl fractions collected in a Falcon flexible 96 well assay plate (Becton Dickinson Labware, Oxnard, CA, USA.). Protein content was assessed by the Bradford assay and the protein containing fractions pooled. The concentration of protein was adjusted to 1mg/ml by addition of 0.05M carbonate buffer containing 5% sodium deoxycholate (BDH Ltd, Poole, Dorset, U.K.) and 10% dimethyl sulfoxide (DMSO) (Sigma).

Palmitification of the resulting purified OVA was achieved by dropwise addition of 314µl of a 10mg/ml N-(palmitoyloxy) succinimide (NPS) (Sigma) solution dissolved in DMSO giving a 20:1 molar ratio of palmitic acid to OVA. Both NPS and OVA were prewarmed to 37°C and the reaction performed with constant shaking to prevent precipitation of NPS. The mixture was then incubated overnight at 37°C in a shaking incubator with a stroke rate of 100/minute. The palmitification reaction was stopped by addition of 1ml Tris (hydroxymethyl methylamine (TrisHCl, pH 8.5), followed by a further incubation for 30 minutes at 37°C. Excess TrisHCl prevents palmitification by providing free amino groups.

Free palmitic acid was separated from palmitified OVA by ultracentrifugation. A 40% (w/v) solution of sucrose (BDH) was prepared in phosphate buffered saline (PBS) and 8ml pipetted into six Ultraclear tubes (Beckman Ltd, High Wycombe, Bucks, U.K.). 1g sucrose was added to the protein/NPS mixture so as to give a 5% w/v solution, and 4ml of this was overlaid on to the 40% sucrose layer in each tube. 1ml of a 2% (w/v) solution of decanoyl N-methylglucamide (Mega-10) (Sigma) in PBS was added carefully to the top of each tube, which were then centrifuged at 40,000 rpm for 4 hours at 4°C using a SW41.14 rotor (Sorvall (UK) Ltd, Stevenage, Herts, U.K.) in an ultracentrifuge (Sorvall). Following ultracentrifugation, the top layer of PBS containing the free palmitic acid was discarded and the palmitified OVA harvested from the 5% sucrose layer.

ii) Preparation of OVA ISCOMS

A mixture of cholesterol and phosphatidyl choline (both Sigma) was prepared by adding 50mg of each to 1ml chloroform (Aldrich Chemical Co., Poole, Dorset, U.K.) and adding this to 5 ml of distilled H₂O containing 20% (w/v) Mega-10. The chloroform was

evaporated under a stream of nitrogen until the mixture was completely clear, leaving a solution containing 10mg/ml of each lipid. The lipid mixture was dispensed in 1ml aliquots and stored at -20°C until required.

To prepare OVA ISCOMS, 1ml of the lipid mixture (giving a final concentration of 0.5mg/ml) and 50mg Quil A (giving a final concentration of 0.1% (w/v)) was added to the palmitified OVA solution. The solution was sonicated for 20 minutes, transferred into dialysis tubing (Visking, Medicell International Ltd, London, U.K.) and dialysed for 4-5 days at 20°C against 4 litres of 50mM TrisHCl or PBS which was changed every 12 hours. The formation of ISCOMS was observed by a cloudiness of the dialysate.

The ISCOMS were separated from free reaction products by ultracentrifugation over a discontinuous sucrose gradient prepared in Ultraclear tubes. The sucrose gradient was produced by pipetting 4mls of 40% sucrose (w/v) solution in PBS into Ultraclear tubes (Beckman) and overlaying 4mls of 10% sucrose (w/v) solution in PBS. 5mls of the ISCOMS mixture was carefully layered on to the top of each tube and these were then centrifuged at 40,000 rpm for 16 hours at 4°C using the SW41.14 rotor (Sorvall) and a Sorvall ultracentrifuge. The ISCOMS were visualised as a white band at the interface between the 10% and 40% sucrose layers and were harvested and dialysed for 24 hours against 4 litres of PBS to remove excess sucrose.

ISCOMS were then sterilised by filtration through a 0.2µm filter (Acrodisc, Gelman Sciences, Ann Arbor, MI, U.S.A.), aliquoted and stored at -20°C until required. The formation of intact ISCOMS was confirmed by negatively staining with 3% phosphotungstic acid (pH 6.6) and analysis on a Philips CM10 transmission electron microscope (TEM) at a final magnification of 73000x (Mrs Jane Hare, Department of Pathology, Western Infirmary, Glasgow).

iii) Quantification of OVA ISCOMS Protein Content

The concentration of OVA in samples of purified protein and in OVA ISCOMS was measured using Bradford's Assay. Briefly, 20µl of test sample were added to 180µl of a 20% (v/v) solution of Bradford's reagent (Coomassie Blue Dye in 55% phosphoric acid with 15%

methanol) (Bio-Rad, Hemel Hempstead, Bucks. U.K.) in dH₂O. After an incubation for 20 minutes at room temperature, the O.D. 570nm of the samples was read using a MR5000 automatic plate reader (Dynatech Laboratories Ltd., Billingshurst, West Sussex, U.K.) and the concentration of OVA calculated by comparison with a standard curve generated from dilutions of OVA in PBS.

iv) Quantification of Endotoxin Content in OVA ISCOMS

The content of lipopolysaccharide (LPS) in OVA ISCOMS was quantified using the Limulus lysate assay (Sigma) and was found to be very low (<0.5µg/ml), equivalent to 0.5ng/dose of ISCOMS.

2.6 Administration of OVA ISCOMS *in vivo*

ISCOMS were administered parenterally either as a subcutaneous (s.c.) injection in a volume of 50µl sterile saline into the hind footpad under light halothane anaesthesia or as an (i.p.) injection in a total volume of 200µl sterile saline.

Oral immunisation of ISCOMS was performed by feeding 200µl volumes using a rigid steel gavage needle placed in the oesophagus. Control mice were injected with saline and killed after 3.5 hours.

2.7 Isolation of Peritoneal Exudate Cells

Peritoneal exudate cells (PEC) were removed by lavage. Mice were injected i.p. with 6mls of RPMI 1640 (Gibco BRL, Paisley, U.K.), the abdomen gently massaged and fluid withdrawn using a 19 gauge needle. The cells were washed by centrifuging in RPMI 1640 at 450g for 5 minutes, resuspended in fresh RPMI 1640 and viable cell counts performed in a Neubauer haemocytometer (Weber Scientific, Middlesex, U.K.) using a phase contrast microscope at x400 magnification (Nikon Labophot, UK). The cells were then centrifuged again at 450g and the pellet resuspended at a concentration of 1×10^7 PEC/ml in the appropriate medium and stored on ice until required.

2.8 Histological Analysis of PEC by Cytospin

Aliquots of 5×10^5 cells were spun onto glass microscope slides at 200 rpm for 5 minutes using a cyto-centrifuge (Shandon, Runcorn, UK) and fixed in a 50:50 methanol in acetone solution for 2 minutes. The slides were allowed to air dry and washed in PBS before being immersed in Geimsa stain (Sigma) for 30 minutes. The slides were rinsed in distilled water, allowed to air dry and then mounted in Histamount (Sigma) under glass.

The cells were then assessed histologically at x400 magnification using a Nikon Labophot microscope. To perform qualitative analysis of cell numbers, 10 fields/cytospin were assessed and the average calculated.

2.9 Isolation of Lymphoid Cells

Popliteal lymph nodes (PLN), spleens (SP), Peyer's patches (PP) and mesenteric lymph nodes (MLN) were removed after sacrifice and cleaned of any surrounding tissue. The lymphoid organs were then gently crushed using a sterile 2ml syringe plunger (Plastipak, Becton Dickinson, Mountain View, CA, USA) in a Petri dish (Costar, Northumbria Biologicals, Cramlington, U.K.) containing RPMI 1640 medium. The cells were then pipetted several times through a sterile glass pipette to obtain a single cell suspension which was then passed through sterile nylon mesh (Nitex, Cadich and Sons Ltd. London, U.K.) to remove debris. The cells were washed by centrifuging at 450g for 5 minutes, resuspended in fresh RPMI 1640, counted, resuspended at an appropriate concentration and stored on ice until use.

2.10 Flow Cytometry

Lymphoid cells and PEC were analysed phenotypically by flow cytometry using the surface markers listed in Table I. Aliquots of 10^6 cells were washed twice in FACS Buffer (PBS containing 2% FCS (Gibco BRL) and 0.02% sodium azide) and all staining reactions

performed for 45 minutes on ice in a total volume of 50µl FACS Buffer containing antibodies at 1:25 dilutions. Between staining steps, cells were washed twice in cold FACS Buffer and finally resuspended in 500µl of FACSflow (Becton Dickinson). For analysis, a Becton Dickinson FACScan® flow cytometer (Becton Dickinson) was used which consisted of single argon laser of 488nm wavelength. Data from 10,000 cells were acquired and analysed using Lysis II software.

2.11 Stimulation of Cytokine Production *in vitro*

For the assessment of cytokine production, 4×10^6 cells were cultured *in vitro* in 24 well plates (Costar) in 1ml of RPMI 1640 containing 10% heat inactivated FCS, 100U/ml penicillin, 100µg/ml streptomycin, 50µg/ml Fungizone (all Gibco BRL) and 0.05µM 2-mercaptoethanol (Sigma) (culture medium) in the presence or absence of 10µg/ml LPS (*S. enteritidis*, Sigma) plus 20ng/ml recombinant murine γ IFN (PharMingen) or with 10µg/ml concanavalin A (Con A). For the measurement of antigen-specific cytokine secretion, similar aliquots of 4×10^6 cells were cultured with or without 10µg/ml Con A or 1mg/ml OVA. After culture at 37°C in 5% CO₂ in air, the supernatants were removed and centrifuged for 5 minutes at 13000g before being frozen at -20°C until required.

2.12 Removal of Adherent Cells *in vitro*

Adherent cells were removed from PEC populations by adding 4×10^6 PEC in 1ml culture medium to 24 well plates (Costar) and incubating at 37°C in a humidified incubator containing 5% CO₂ in air for 3 hours. Non-adherent cells were then gently aspirated using a sterile glass pipette and transferred into fresh wells.

2.13 Measurement of Reactive Oxygen Intermediate Production

Reactive oxygen intermediates (ROI) released during the respiratory burst were measured by a chemiluminescence assay [Easmon et al., 1980]. Briefly, cells were washed in Hank's Balanced Salts Solution (HBSS) (Gibco BRL) without phenol red, and warmed to 37°C in a water bath. 100µl of 0.1mM 5-amino-2,3-dihydro-1,4-phthalazinedione solution (Luminol) (Sigma) was added to 2×10^6 cells in 400µl of the warmed HBSS and these were then stimulated by addition of 1µg/ml phorbol 12-myristate 13-acetate (PMA) (Sigma). The suspensions were mixed at 37°C and the chemiluminescence measured using a 1250 Luminometer (LKB Wallac, Turku, Finland) every 10 seconds for a total of 10 minutes. All measurements were expressed as mV and the background chemiluminescence was assessed by incubating cells in the absence of PMA.

2.14 Measurement of Nitric Oxide Production

The concentration of nitric oxide (NO) present in culture supernatants was determined by detection of nitrite, a stable metabolite of NO, using the Griess reaction. Briefly, the Griess reagent was made by mixing equal volumes of 5% orthophosphoric acid containing 1% sulphanilamide (Sigma) and 0.1% naphthylethylenediamine (Sigma) and kept at 4°C until required. 50µl culture supernatant or standard dilutions of sodium nitrite in RPMI 1640 containing 10% FCS were added to 96 well Immulon 4 microtitre plates (Dynatech) in triplicate and 50µl of the Griess reagent was then added. The colour generated by the reaction was measured spectrophotometrically at 570nm using a MR5000 microtitre plate reader (Dynatech).

2.15 Measurement of Systemic Delayed Type Hypersensitivity (DTH) Responses

Systemic OVA-specific DTH responses were assessed 7 days after immunisation with OVA ISCOMS by measuring the increment in footpad thickness 24 hours after a

subcutaneous challenge with 100µg HAO, using callipers (Kroplin Ltd, Kingston-upon-Thames, Surrey, U.K.) . Background responses were determined by challenging naive mice with HAO and were subtracted to obtain antigen-specific DTH levels.

2.16 Measurement of OVA-Specific Antibodies in Serum

Blood was obtained from the retro-orbital plexus using heparinised capillary tubes (Hawksley & Sons Ltd, Lancing, Sussex, U.K.) under light halothane anaesthesia and the serum was separated by centrifugation at 500g for 10 minutes. Samples were stored at -20°C until required and the levels of serum antibody were then analysed by ELISA, [Garside et al., 1995].

i) Quantification of Total IgG Antibodies

Immulon 4 ELISA plates (Dynatech) were coated overnight at 4°C with 50µl of a 10µg/ml solution of OVA in 50mM carbonate buffer. The plates were then washed three times with PBS containing 0.05% polyoxyethylenesorbitan monolaurate (Tween 20) (Sigma) (PBST). 100µl of samples diluted 1:400 in PBST were added to the plate in triplicate and incubated for 2.5 hours at room temperature in a humid environment. The plates were then washed three times with PBST and 100µl of a 1:1000 dilution of alkaline phosphatase conjugated goat-anti-mouse IgG (Sigma) was added and the plates left for a further 3 hours at room temperature in a humid environment. Plates were then washed three times with PBST and 100µl of a 1mg/ml solution of phosphatase substrate (p-nitrophenyl phosphate) (Sigma) dissolved in dH₂O containing 10% diethanolamine (DEA) (Aldrich) was added to each well. The O.D. 450nm of the samples was read using an MR5000 automatic microplate reader (Dynatech) after an incubation for 10-30 minutes at room temperature. The results from individual mice were expressed either as an O.D. 450nm, or as the dilution that gave an equivalent O.D. 450nm of a 5% concentration of an hyperimmune standard of ammonium sulphate-purified anti-OVA IgG which had been obtained from mice immunised twice with 100µg OVA in CFA.

ii) Quantitation of OVA-Specific Serum IgG Antibody Isotypes

Immulon 4 plates were coated overnight at 4°C with 50µl of a 10µg/ml OVA solution, then washed three times with PBST. The plates were blocked with 3% (w/v) bovine serum albumin (BSA) (Sigma) in PBST for 1 hour at room temperature and then washed again three times with PBST. A 1:400 dilution of hyperimmune OVA-specific serum as standard or serum samples were diluted either 1:400 in PBST for IgG1 or 1:20 in PBST for IgG2a and added to the plates in a volume of 50µl and then serially diluted 1:1 in PBST. The plates were incubated for 2.5 hours at room temperature and then washed three times with PBST. IgG1 was detected by adding 50µl of a 1:16,000 dilution of biotinylated rat-anti-mouse IgG1 antibody (Serotec) in PBST and IgG2a by the addition of 50µl of a 1:500 dilution of biotinylated rat-anti-mouse IgG2a (Serotec) in PBST. The plates were then incubated at room temperature for 1 hour and then washed three times with PBST. 100µl of a 1:1000 dilution of extravidin peroxidase (Sigma) in PBST was added to each well and incubated for 1 hour at room temperature. The plates were washed several times with PBST and 100µl of the chromophore 3, 3', 5, 5' - tetramethylbenzidine (TMB) and H₂O₂ (Dynamech) added to each well. After an incubation of 10 minutes at room temperature the plates were read at 630nm on a MR5000 microtitre plate reader (Dynamech). The level of IgG isotypes present in test wells was calculated the dilution that gave an equivalent O.D. 450nm \geq 5% of the hyperimmune standard.

2.17 Measurement of Intestinal Secretory IgA Antibodies

Intestinal secretions were collected using a lavage technique modified from that published previously [Elson et al., 1984]. Mice were given four feeds of 200µl 16% polyethylene glycol (PEG) (Sigma) solution in PBS within an hour. The small intestine and caecum were then removed and rinsed through with 6ml of ice cold PBS containing 0.1mg/ml soyabean trypsin inhibitor and 50mM ethylenediametetraacetic acid (EDTA) (both Sigma). The fluid was clarified by centrifugation at 1500g for 10 minutes and 3ml of the

supernatant was removed. Then 30 μ l of 100mM phenylmethylsulfonyl (PMSF) (Sigma) in 95% ethanol (Aldrich) was added to each aliquot, which were then centrifuged in Eppendorf tubes at 27000g for 15 minutes at 4°C. 10 μ l of PMSF solution, 10 μ l of a 1% (w/v) solution of sodium azide (Sigma) in PBS and 50 μ l of FCS were then added to 1ml of the resulting supernatants which were then stored at -20°C until required.

OVA-specific intestinal IgA responses were also assessed by ELISA. Immulon 4 ELISA plates (Dynatech) were coated overnight at 4°C with 50 μ l of a 10 μ g/ml solution of OVA in 50mM carbonate buffer. The plates were then washed thoroughly with PBST and 100 μ l of undiluted sample added in triplicate, incubated for 2.5 hours at room temperature in a humid environment and then washed three times with PBST. 100 μ l of a 1:1000 dilution of alkaline phosphatase conjugated goat-anti-mouse IgA (Sigma) was added and the plates left for a further 3 hours at room temperature in a humid environment. The plates were then washed three times with PBST and 100 μ l of a 1mg/ml solution of phosphatase substrate (Sigma) dissolved in dH₂O containing 10% DEA (Aldrich) was added to each well. The O.D. 450nm of the samples was read using an MR5000 automatic microplate reader (Dynatech) after an incubation of 10-30 minutes at room temperature. The results from individual mice were expressed as an O.D. 450nm.

2.18 Measurement of OVA-Specific Proliferative Responses

Single cell suspensions from spleen or popliteal lymph node (PLN) were cultured in flat bottomed 96 well microtitre plates (Costar) in quadruplicate. Cells were cultured at a concentration of 2.5×10^5 /well in culture medium with or without 1mg/ml OVA or 10 μ g/ml Con A at 37°C in 5% CO₂ and humidified air. Eighteen hours prior to harvesting, the cells were pulsed with 1 μ Ci ³H-thymidine (West of Scotland Radionucleotide Dispensary, Western Infirmary, Glasgow) and harvested onto glass fibre filter mats using a Betaplate 96 well harvester (LKB Wallac, Turku, Finland). DNA bound ³H-thymidine was then counted using a Betaplate scintillation counter (LKB Wallac).

2.19 Measurement of Cytokines by ELISA

Culture supernatants were assayed for cytokine levels by sandwich ELISAs, already established in the laboratory [Garside et al., 1995]. Immulon 4 plates (Dynatech) were coated with an appropriate concentration of rat-anti-cytokine antibody (See Table II) diluted in 0.1M carbonate buffer and incubated overnight at 4°C. The plates were then washed three times with PBST and blocked for 1 hour with 200µl of 10% FCS in PBS. The plates were then washed twice with PBST and 50µl of samples, or serial dilutions of recombinant murine cytokines as standards, added in triplicate to the plate (See Table III). The plates were then incubated at 37°C for three hours before being washed three times with PBST. 50µl of the appropriate biotinylated-detection antibody was added to the plate and incubated for 1 hour at 37°C (See Table IV). The plates were then washed three times with PBST before 100µl of a 1:1000 dilution of extravidin peroxidase (Sigma) in PBS + 10% FCS was added to each well and incubated for 1 hour at 37°C. The plates were washed several times with PBST and 100µl of the chromophore TMB and H₂O₂ (Dynatech) added to each well. After an incubation of 10 minutes at room temperature, the plates were read at 630nm on a MR5000 microtitre plate reader (Dynatech). The level of cytokine present in test wells was calculated from the standard curve.

2.20 Measurement of OVA-Specific Cytotoxic T Cell Responses

OVA-specific CTL activity was measured in spleens of mice 7 days after i.p. immunisation or 7 days after the final feed of OVA ISCOMS as described previously [Mowat et al., 1991b]. 3×10^7 spleen cells from immunised animals were restimulated *in vitro* with 1.5×10^6 OVA transfected EG7.OVA cells which had been treated for 60 minutes at 37°C with 50µg of mitomycin c per 1×10^7 cells in a volume of 1 ml of culture medium containing 5% NCTC-135 medium (Gibco) (CTL medium). The cells were co-cultured in 25cm² culture flasks (Costar) for 5 days at 37°C in 5% CO₂ and humidified air. After culture, the effector cells were washed three times and assayed for CTL activity using EG7.OVA cells that had

been labelled with ^{51}Cr by incubating 2.5×10^6 EG7.OVA cells in 1ml of RPMI 1640 containing 5% newborn calf serum (NCS)(Gibco) and 2MBq $\text{Na}_2^{51}\text{CrO}_4$ (West of Scotland Radionucleotide Dispensary) for 60 minutes at 37°C in 5% CO_2 and humidified air. The target cells were then washed five times to remove excess $\text{Na}_2^{51}\text{CrO}_4$ by centrifugation at 450g for 5 minutes and added to V-bottomed 96 well microtitre plates (Costar) in RPMI 1640+5% NCS at a concentration of 2×10^4 cells/well. Effector cells were added to target cells in quadruplicate at various concentrations so as to give effector:target (E:T) ratios of 100:1, 50:1 and 25:1 in a total volume of 200 μl . The cultures were then incubated at 37°C in 5% CO_2 for 4 hours before 100 μl of supernatant was removed from the top of each well and ^{51}Cr release counted on a Compugamma counter (LKB Wallac). The percentage of OVA-specific CTL lysis of target EG7 cells was calculated by using the following formula:

$$\% \text{ Cytotoxicity} = \frac{(\text{Experimental Release} - \text{Spontaneous Release})}{(\text{Total Release} - \text{Spontaneous Release})} \times 100\%$$

In all experiments, 10% Triton X (BDH Laboratory Supplies, Poole, UK) was used to obtain the maximum release and medium or naive spleen cells restimulated with EG7.OVA cells was used to obtain the spontaneous release. ^{51}Cr -labelled EL4 cells were used as non-OVA expressing negative control target cells.

2.21 Statistics

Unless otherwise stated, all results are expressed as means \pm 1 standard deviation (1SD) and were compared using Student's t-test.

Table I. Phenotypic marker antibodies used for flow cytometry

MARKER	CLONE	ISOTYPE	HOST	CONJUGATE	DILUTION	COMPANY
CD4	RM4-5	IgG2ak	Rat	PE	1:25	Pharmingen
CD8	53-6.7	IgG2ak	Rat	FITC	1:25	Pharmingen
B220	RA3-6.B2	IgG2ak	Rat	Biotin	1:50	Pharmingen
Whole Neutrophils	5120- 26.1/11	IgG2a	Rat	Biotin	1:10	Serotec
F4/80	C1;A3-1	IgG2b	Rat	Biotin	1:10	Serotec
CD11c	HL3	IgG1	Hamster	PE	1:25	Pharmingen
I-A ^{bd}	25-9-17	IgG2ak	Mouse	FITC	1:25	Pharmingen
CD25	7D4	IgMκ	Rat	FITC	1:25	Pharmingen
CD16/ CD32	2.4G2	IgG2bκ	Rat	-	1:25	Pharmingen
Streptavidin	-	-	-	PE	1:10	Vector

Table II. Capture antibodies used for sandwich ELISA

CYTOKINE	CLONE	ISOTYPE	HOST	CONCENTRATION (ng/ml)	COMPANY
IL-1 α	ALF161.1	IgG	Hamster	10 μ g/ml	Genzyme
IL-5	TRFK5	IgG1	Rat	4 μ g/ml	Pharmingen
IL-6	MP5- 20F3	IgG1	Rat	1 μ g/ml	Pharmingen
IL-12	C17.15	IgG2a	Rat	5 μ g/ml	Genzyme
γ IFN	XMG1.2	IgG1	Rat	2 μ g/ml	Pharmingen

Table III. Recombinant cytokine used in sandwich ELISA

CYTOKINE	TOP STANDARD	BOTTOM STANDARD	COMPANY
IL-1 α	20.000 ng/ml	31.250 pg/ml	Genzyme
IL-5	200.000 U/ml	0.313 U/ml	Genzyme
IL-6	10.000 ng/ml	15.625pg/ml	Genzyme
IL-12	10.000 ng/ml	15.625 pg/ml	Genzyme
γ IFN	40.000 ng/ml	62.500 ng/ml	Genzyme

Table IV. Detection antibodies used in sandwich ELISA

CYTOKINE	CLONE	ISOTYPE	CONJUGATE	HOST	CONCENTRATION ($\mu\text{g/ml}$)	COMPANY
IL-1 α	Polyclonal	-	Biotin	Rabbit	5 $\mu\text{g/ml}$	Genzyme
IL-5	TRFK4	IgG2a	Biotin	Rat	4 $\mu\text{g/ml}$	Pharmingen
IL-6	MP5- 32C11	IgG2a	Biotin	Rat	1 $\mu\text{g/ml}$	Pharmingen
IL-12	Polyclonal	-	Biotin	Rabbit	5 $\mu\text{g/ml}$	-
γ IFN	XMG1.2	IgG1	Biotin	Rat	2 $\mu\text{g/ml}$	Pharmingen

Chapter 3

Parenteral and Oral Administration of ISCOMS induces an Inflammatory Cascade *in vivo*.

Introduction

Although ISCOMS are well known to induce a wide range of antigen-specific immune responses *in vivo* by both parenteral and mucosal routes, the basis for their adjuvant properties has not been studied in any detail. As it is now clear that an ability to stimulate the innate immune response exerts a powerful influence over the type of acquired immunity generated to an antigen [Medzhitov and Janeway Jr, 1997a, Fearon and Locksley, 1996], it seemed appropriate to explore the effects of ISCOMS on the early non-specific immune response. Therefore, in the experiments described in this chapter, I investigated if ISCOMS were capable of inducing an innate response after parenteral or oral administration.

Experimental Protocol

In the first experiments, mice were injected i.p. with 5 μ g OVA ISCOMS in saline. The peritoneum was chosen as it provided a discrete, relatively localised compartment for sampling, that was also easily accessible. In addition, previous studies in our laboratory had shown that ISCOMS were immunogenic by this route [Mowat et al., 1991a, Maloy et al., 1995]. At various times after injection, PEC were removed by lavage, counted and examined by cytopins and flow cytometry. The release of ROI was determined by PMA induced chemiluminescence of Luminol and the production of nitric oxide was measured by the Greiss reaction after *in vitro* culture in the presence or absence of γ IFN+LPS. Inflammatory cytokines were assessed by ELISA analysis of supernatants in response to γ IFN+LPS. After studying the responses to i.p. ISCOMS, I went on to perform similar experiments using Peyer's patches and mesenteric lymph nodes taken from mice fed 200 μ g OVA ISCOMS. In all experiments, control mice received saline alone.

Results

3.1 Recruitment of Local Inflammatory Cells after Intraperitoneal Administration of ISCOMS.

Intraperitoneal administration of ISCOMS induced recruitment of an inflammatory infiltrate as determined by the total number of PEC obtained in peritoneal lavage. This was preceded by an early reduction in the number of PEC, with a 50% decrease in the total number of PEC being observed at 3.5 hours after administration of ISCOMS as compared with saline injected controls. The numbers of PEC remained depressed until 48 hours after ISCOMS injection, by which time there was a marked and significant increase in the number of PEC to levels three times that of control mice. The number of PEC was still significantly elevated at 72 hours (Figure 3.1).

3.2 Histological Examination of PEC after i.p. ISCOMS

I next explored the nature of the inflammatory infiltrate by histological analysis of cytopins prepared of PEC isolates. PEC from control mice consisted mostly of small resting lymphocytes and macrophages, together with a small number of mast cells which were identified by the presence of multiple dense granules (Figure 3.2a). The decrease in total PEC early after injection of ISCOMS was accompanied by an increase in the absolute number of polymorphonuclear leukocytes at 3.5 hours (Figure 3.2b) and 7 hours (Figure 3.2c). Polymorphonuclear cells were still present at 24 hours, although in much lower numbers, (Figure 3.2d), and by 48 and 72 hours, polymorphonuclear cell numbers had returned to control levels (Figures 3.2e and f). Mast cell numbers also increased soon after injection of ISCOMS and showed evidence of degranulation. This was seen as early as 3.5 hours (Figure 3.2b), when a substantial number of these cells had degranulated. There was a second rise in mast cell numbers at 48 hours, although no degranulation was seen at this time (Figure 3.3).

Mast cell numbers then returned to control levels at 72 Hours (Figure 3.3). There was no change in the numbers of macrophages from PEC isolated at early timepoints after injecting ISCOMS, but at 48 hours, there was an influx of large mononuclear cells, many with a foamy appearance consistent with activated macrophages (Figure 3.2e). In parallel, there was a steady increase in the numbers of lymphoid cells from control levels to a peak at 48 hours, when many were lymphoblastoid in appearance (Figure 3.2e). At 72 hours after ISCOMS injection, lymphocyte morphology was returning to that observed in control cytopins (Figure 3.2f).

3.3 Phenotypic Analysis of PEC Recruited by Parenteral Administration of ISCOMS.

To analyse the inflammatory infiltrate in more detail, I used flow cytometry to assess the expression of cell surface markers specific for neutrophils, macrophages, dendritic cells and T and B lymphocytes. In addition, I determined the activation status of these cells by assessing the levels of MHC class II on macrophages and dendritic cells and the IL-2R α chain (CD25) on CD4 $^{+}$ and CD8 $^{+}$ T cells. Unfortunately, no statistical analysis of these results were feasible as I had to pool the PEC cells from injected mice.

Consistent with the histological appearances I had observed previously, the decrease in PEC numbers early after injection of ISCOMS was accompanied by a large increase in the absolute numbers and proportions of neutrophils which peaked at 3.5 hours and declined rapidly thereafter. The number of F4/80 expressing macrophages began to increase by 7 hours, reached a peak at 48 hours and then declined (Figure 3.4a), again confirming the microscopic analysis of cytopins.

The numbers of dendritic cells, as determined by expression of the CD11c surface marker, were low in control PEC but, after a further drop at early timepoints, these increased to a peak at 72 hours. These CD11c $^{+}$ cells were observed to be virtually all MHC class II $^{+}$, consistent with them being mature dendritic cells (Figure 3.4d).

The numbers of CD4 $^{+}$ and CD8 $^{+}$ T cells declined rapidly over the first 7 hours after ISCOMS injection (Figure 3.4b). Thereafter, the numbers of CD4 $^{+}$ T cells returned toward

control levels, whereas CD8⁺ T cells remained low until 72 hours, before rising to a level that was twice that of control mice (Figure 3.4b). The numbers of B220⁺ B cells followed a similar pattern, with a rapid early decrease followed by recovery to levels which were eventually double that of control values at 48 hours after administration of ISCOMS (Figure 3.4c).

I next determined the activation status of the inflammatory infiltrate by examining the expression of CD25 and MHC class II antigens and their co-expression on T cells and macrophages respectively. MHC class II⁺ F4/80⁺ macrophages were not found in control PEC, but their numbers increased dramatically from 7 hours until 48 hours, when 25% of macrophages were MHC class II⁺. By 72 hours, few MHC class II⁺ macrophages remained (Figure 3.4c). There were also virtually no CD4⁺ T cells coexpressing CD25 in control PEC. Although the rise in the absolute numbers of CD4⁺ T cells was not dramatic after injection of ISCOMS, these CD4⁺ T cells were clearly becoming activated, as evidenced by their expression of CD25, with 26% of CD4⁺ T cells being CD25⁺ by 48 hours (Figure 3.4e). As the numbers were much smaller, I was unable to assess the level of CD25⁺ CD8⁺ T cells.

Thus i.p. injection of ISCOMS induced a rapid, local recruitment of inflammatory cells, consisting initially of neutrophils and mast cells, followed by macrophages and DC and later by both T and B lymphocytes. Once recruited, many of these cells become activated as indicated by their increased expression of MHC class II and CD25.

3.4 Parenteral Administration of ISCOMS Induces Production of Local Inflammatory Cytokines and Mediators

I next assessed the functional consequences of the cellular recruitment observed after an i.p. injection of ISCOMS by measuring IL-1, TNF α , IL-6 and IL-12 as products of activated macrophages and dendritic cells and γ IFN as a lymphocyte product.

i) IL-1 α

PEC from control mice showed very low levels of spontaneous production of IL-1 α , but spontaneous IL-1 α production was significantly enhanced 48 hours after ISCOMS injection, when PEC produced more than 20 times that of controls, before decreasing to undetectable levels (Figure 3.5a). An almost identical pattern of increased IL-1 α production by ISCOMS induced PEC, following stimulation *in vitro* with γ IFN+LPS was found (Figure 3.5a).

ii) IL-6

PEC from control animals produced moderate levels of IL-6 both spontaneously and after stimulation with γ IFN+LPS *in vitro* (Figure 3.5b). Immediately after i.p. injection of ISCOMS both the spontaneous and γ IFN+LPS induced production of IL-6 fell to undetectable levels, before increasing again after 24 hours, to levels more than double those of controls at 48 and 72 hours (Figure 3.5b).

iii) Immunoreactive IL-12

Control PEC cultures did not produce IL-12 spontaneously and only small amounts of γ IFN+LPS induced IL-12 (Figure 3.5c). Spontaneous IL-12 production rose gradually from 24 hours after administration of ISCOMS, plateauing after 48 hours at levels significantly above those of control PEC. The γ IFN+LPS stimulated production of IL-12 declined to approximately 20% that of controls within 3.5 hours, but rose sharply to a peak at 72 hours, when levels were twice that of controls (Figure 3.5c).

iv) γ IFN

There was no spontaneous production of γ IFN from control PEC, but low levels were detected after stimulation with Con A. Significant levels of spontaneous γ IFN production were detected 24 hours after injection of ISCOMS but not thereafter. Con A stimulated PEC showed a small, but significant increase in γ IFN production 3.5 hours after injection with ISCOMS. This fell back to control levels at 7 hours, but increased again to peak at 24 hours, when γ IFN production was twice that of control PEC (Figure 3.5d).

v) TNF α

No TNF α production was found in the supernatants from any PEC preparation either spontaneously or after stimulation with γ IFN+LPS (data not shown).

Thus, the local infiltration by macrophages, dendritic cells and T cells with activated phenotypes following injection of ISCOMS is accompanied by the production of inflammatory cytokines, indicating that recruited cells are not only phenotypically but also functionally activated by exposure to ISCOMS.

3.5 Stimulation of Nitric Oxide and Reactive Oxygen Intermediate Production After Parenteral Administration of ISCOMS

In view of the marked recruitment of activated phagocytic cells such as macrophages and neutrophils, I next examined the production of two other characteristic mediators of such cells, nitric oxide and reactive oxygen intermediates. The concentration of NO in PEC culture supernatants was determined from the concentration of its stable breakdown product nitrite, as assessed by the Griess Reaction. There was very little spontaneous NO production by

control PEC, but this was increased by stimulation with γ IFN+LPS (Figure 3.6). Spontaneous production of NO was detectable by 24 hours after injection of ISCOMS and substantial amounts were still present at 48 hours (Figure 3.6). Immediately after injection with ISCOMS, γ IFN+LPS stimulated production of NO fell, but recovered from 7 hours onwards, reaching a peak at 48 hours which was significantly above control levels (Figure 3.6).

PMA stimulated production of ROI was measured by chemiluminescence of Luminol. Control PEC produced little or no ROI, but within 6 hours after injection of ISCOMS, ROI production was greatly increased (Figure 3.7). PEC taken 24 hours after ISCOMS produced even more ROI, with levels more than twenty times that of control cells. Thereafter, there was a decline in ROI production and by 72 hours after injection of ISCOMS, ROI production had returned to control levels (Figure 3.7).

Thus the recruitment of neutrophils and macrophages by ISCOMS is accompanied by increased production of ROI and NO respectively, underlining the possibility that these cells are activated following administration of ISCOMS.

3.6 Secretion of Inflammatory Mediators by ISCOMS Induced PEC is Dependent on the Presence of Adherent Cells

I next investigated the role of adherent cells in the production of inflammatory cytokines and nitric oxide, by depleting these cells from populations of PEC removed 48 hours after administration of ISCOMS. As before, ISCOMS stimulated PEC produced small amounts of spontaneous IL-12, NO and IL-1 α at this time, which were all increased by stimulation with γ IFN+LPS (Figures 3.8a, b and c). There was no spontaneous production of IL-6 in this experiment, but marked amounts occurred after stimulation in vitro with γ IFN+LPS (Figures 3.8d). In all cases, both the spontaneous and stimulated production of these mediators was reduced virtually to background levels when adherent cells were depleted (Figures 3.8a, b, c, d). Removal of adherent cells also abolished the spontaneous production of γ IFN and significantly reduced the Con A stimulated production of γ IFN by the same cells (Figure 3.8e). These results are consistent with the critical role of adherent cells,

perhaps macrophages, in the production of inflammatory mediators after injection of ISCOMS.

3.7 Oral Administration of ISCOMS Induces Cellular Recruitment in the Gut Associated Lymphoid Tissues.

As one of the important features of ISCOMS is that they are active via mucosal routes, I went on to investigate whether oral administration of ISCOMS induced inflammatory responses in intestinal lymphoid tissues similar to those found in the peritoneum. Therefore I performed phenotypic and functional studies on PP and MLN cells at various times after a single feed of 200 μ g OVA ISCOMS. I was again unable to perform statistical analysis on these data, because I pooled the cells from each group of animals.

Oral administration of ISCOMS induced cellular recruitment in both PP and MLN as evidenced by total cell numbers in these tissues (Figures 3.9a and b). This was less marked in PP than in the MLN and was less pronounced compared with that found following an i.p. injection of ISCOMS (Figure 3.1). Nevertheless, a small increase in cell yield was found in both MLN and PP early after feeding ISCOMS, at 3.5 and 7 hours respectively, and after a subsequent fall, the numbers of MLN cells began to rise steadily, so that by 48 hours they were 2.5 times that of controls (Figure 3.9a). The total number of PP cells also increased from 48 hours onwards, but when this peaked at 72 hours, it was only 50% above that of controls (Figure 3.9b).

3.8 Phenotypic Analysis of GALT Cells After Oral Administration of ISCOMS.

In order to assess the nature of the inflammatory infiltrate induced by ISCOMS, I analysed cell-specific surface markers and the expression of cellular activation molecules in PP and MLN cells.

i) Mesenteric Lymph Nodes

In contrast to the peritoneal cavity, there was no change in the numbers of neutrophils found in MLN at any time after oral administration of ISCOMS (Figure 3.10a). However, the numbers of F4/80⁺ macrophages increased steadily to a maximum 48 hours after feeding ISCOMS, at which time the numbers of F4/80⁺ macrophages were six times that of controls (Figure 3.10a). The numbers of F4/80 cells then began to decline towards control levels by 72 hours. An early increase in the numbers of MLN derived CD11c⁺ dendritic cells occurred 3.5 hours after feeding ISCOMS, but this was transient and the numbers quickly decreased to 50% of controls by 24 hours after feeding ISCOMS. However, the numbers of dendritic cells then increased again, reaching a maximum by 48 hours after ISCOMS, at which time numbers were 2.5 times those in control mice, before returning to background levels by 72 hours (Figure 3.9d). The majority of these CD11c⁺ cells were also MHC class II⁺, indicating that these cells were fully differentiated dendritic cells (Figure 3.10b)

The numbers of CD4⁺ T cells in MLN rose transiently immediately after feeding ISCOMS, fell thereafter and then increased steadily to a maximum at 48 hours, by which time numbers had increased to three times that of control animals (Figure 3.10c). An identical pattern was observed for CD8⁺ T cells (Figure 3.10c). The numbers of both CD4⁺ and CD8⁺ T cells returned towards background levels by 72 hours. A similar trend was noted for the numbers of B lymphocytes, with a small spike in the numbers of B220⁺ cells occurring 3.5 hours after feeding ISCOMS, followed by a more sustained recruitment after 24 hours, with peak numbers of B220⁺ B cells being noted at 48 hours, before decreasing towards control levels by 72 hours (Figure 3.10d).

I used the coexpression of MHC class II on macrophages and CD25 on CD4 lymphocytes to determine the degree of activation of these cells after oral administration of ISCOMS. After a small increase in the numbers of MHC class II⁺ F4/80 macrophages at 7 hours, however these numbers fell at 24 hours after feeding ISCOMS, when they were 50% that of control mice. This was despite the increase in total macrophages numbers at this time. However, the numbers of activated macrophages then quickly rose to a peak at 48 hours, by which time they were 3.5 times greater than controls (Figure 3.10e). There was a more gradual, but sustained, increase in the numbers of activated CD4 lymphocytes, as measured by coexpression of the CD25. These reached a maximum level at 72 hours after feeding ISCOMS, by which time the numbers of CD4⁺ CD25⁺ cells were twice that of controls (Figure 3.10c). It was not possible to measure the numbers of CD8⁺ CD25⁺ lymphocytes derived from the MLN, as the numbers were so low as to make accurate quantitation impossible.

ii) Peyer's Patch

As in MLN, there was no increase in the numbers of neutrophils recovered from the PP at any time after feeding ISCOMS (Figure 3.11a). The numbers of F4/80⁺ macrophages, increased markedly at 3.5 and 7 hours, to levels nearly four times that of controls, but had returned to control levels by 24 hours (Figure 3.11a). The numbers of CD11c⁺ dendritic cells in PP changed very little throughout the experiment, and although there was some evidence of a transient increase at 3.5 hours, only 50% of these cells expressed MHC II in contrast to the dendritic cells in other tissues I examined (Figure 3.11b).

A small and transient increase in the numbers of CD4⁺ T cells was noted at 3.5 hours but this then declined before rising gradually to a peak at 72 hours when there was a 50% increase in the numbers of CD4⁺ lymphocytes compared with controls (Figure 3.11c). There were no differences in CD8⁺ T cell numbers at any time after feeding ISCOMS (Figure 3.11c). There was a small increase in the numbers of B220⁺ B cells early after feeding

ISCOMS, but by 24 hours B cells numbers had returned to control levels before rising again to reach a plateau from 48 hours onwards, when their numbers were increased by 45% above control levels (Figure 3.11d).

The numbers of activated MHC class II⁺ macrophages paralleled those of total F4/80⁺ cells, with an early increase up to 7 hours, followed by a return to control levels by 24 hours and thereafter (Figure 3.11e). There was no alteration in the numbers of CD25⁺ CD4⁺ lymphocytes at any time after feeding ISCOMS (Figure 3.10e).

Therefore, although oral administration of ISCOMS induces an inflammatory response in intestinal lymphoid tissues, this is less intense and more restricted in scope than that found after intraperitoneal injection. In addition, an inability to perform statistical analysis means that caution is required when interpreting the results.

3.9 Effects of Oral Administration of ISCOMS on the Production of Inflammatory Mediators by GALT Cells

After observing that there was some recruitment of inflammatory cells into the GALT, I went on to determine if these cells were functionally activated as I had found with PEC. Therefore, I isolated MLN and PP cells at different times after a single feed of ISCOMS and cultured them *in vitro* in the presence or absence of γ IFN+LPS to examine for production of inflammatory mediators.

i) Mesenteric Lymph Node

Control MLN produced a relatively large level of IL-6 spontaneously and this was only slightly elevated after stimulation *in vitro* with γ IFN+LPS (Figure 3.12a). Spontaneous production of IL-6 was completely ablated after a single feed of ISCOMS and did not recover to control levels at any point thereafter (Figure 3.12a). The γ IFN+LPS induced production of IL-6 from MLN was also severely reduced within 3.5 hours of feeding ISCOMS and never

recovered to that of controls, despite a small rise after 24 hours (Figure 3.12a). Control MLN produced some IL-12 spontaneously and this was more than doubled in the presence of γ IFN+LPS (Figure 3.12b). Spontaneous IL-12 production was decreased within 3.5 hours of feeding ISCOMS to 40% of control levels and did not rise thereafter. γ IFN+LPS induced IL-12 production was reduced by 50% immediately after a feed of ISCOMS and continued lower than normal throughout the time course (Figure 3.12b). Spontaneous production of γ IFN by control MLN cells was very low, but after restimulation with Con A, γ IFN secretion was more than 10 times greater (Figure 3.12c). Spontaneous production of γ IFN did not vary from control levels at any time after feeding ISCOMS (Figure 3.12c). However within 7 hours, the Con A induced γ IFN levels were reduced by 90%, before steadily returning to control levels by 72 hours (Figure 3.12c). No IL-1 α or NO production from MLN cells was found at any time after feeding ISCOMS (Data not shown).

Thus despite the fact that ISCOMS induced recruitment of inflammatory cells into the MLN, these cells fail to produce inflammatory cytokines in this environment.

ii) Peyer's Patch

Control PP produced measurable levels of IL-12 which were not significantly elevated after stimulation *in vitro* with γ IFN+LPS (Figure 3.13a). There was a decrease in the production of spontaneous IL-12 seven hours after feeding ISCOMS, but this was not maintained and levels returned to that of controls within 24 hours (Figure 3.13a). After feeding ISCOMS, a slow decrease in γ IFN+LPS induced IL-12 production culminated in a 60% reduction by 24 hours (Figure 3.12a). However, IL-12 levels then began to rise, and by 48 hours, a very small but significant increase above controls was noted, before levels fell to that of controls once more (Figure 3.13a). Spontaneous production of γ IFN was not detectable in control PP, but Con A induced γ IFN could be measured (Figure 3.13b). There was a small increase in the level of spontaneous γ IFN production early after feeding ISCOMS, but this returned to control levels by 24 and 48 hours, before evidence of a further increase by 72 hours (Figure 3.13b). In contrast, the Con A induced production of γ IFN was significantly reduced below control levels immediately after feeding ISCOMS and remained

so for the duration of the experiment (Figure 3.12b). There was no spontaneous or stimulated production of NO, IL-1 α or IL-6 detectable in the PP cells at any time after feeding ISCOMS (Data not shown). These data reflected the small changes in PP cell populations as determined by flow cytometry. However, a small increase in activated F4/80 macrophages was noted at 7 hours and this may be responsible for the small increase in IL-12 secretion at later times. This cytokine production observed in the PP of ISCOMS fed mice was similar to that of the MLN, in that the majority of factors were either undetectable or unchanged after the administration of ISCOMS.

Summary and Conclusions

The results presented in this chapter indicate that ISCOMS induce intense local activation of the innate immune system with the recruitment of a wide variety of inflammatory cells including neutrophils, mast cells, dendritic cells, macrophages and lymphocytes. This inflammatory cascade is most evident when ISCOMS are administered parenterally, where many of the recruited cells were activated, as evidenced by expression of surface activation markers and the production of cytokines and other mediators. Oral administration of ISCOMS also induced an inflammatory cascade in the GALT, with recruitment of activated macrophages, dendritic cells, activated CD4⁺, CD8⁺ T cells and B cells. However there was no neutrophilia and although these recruited cells showed signs of phenotypic activation, very little or no increase in the production of inflammatory cytokines and mediators was measured after oral administration of ISCOMS.

In the next chapter, I will describe experiments conducted to determine which if any of the inflammatory mediators induced by ISCOMS were important in the induction of antigen-specific immunity.

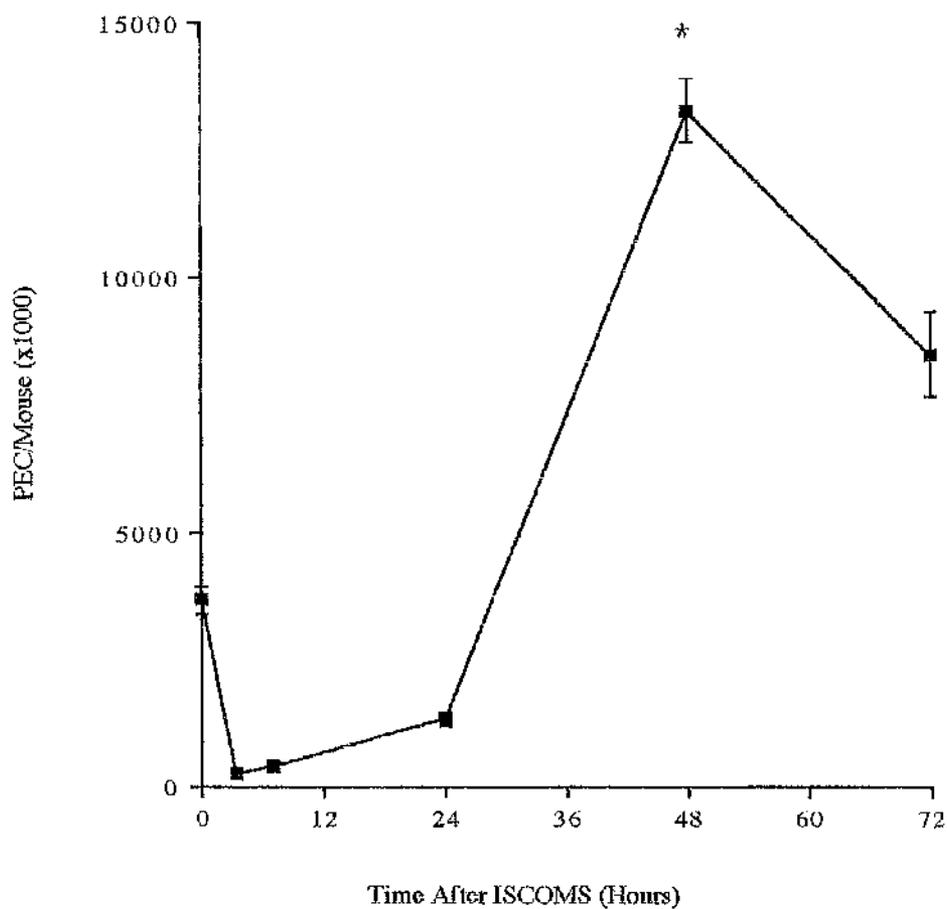


Figure 3.1 ISCOMS Recruit Inflammatory Cells Following Local Injection.

The graph shows PEC recruitment following an i.p. injection of 5µg OVA ISCOMS. PEC were removed by lavage and cell counts of PEC from the individual mice performed. The results shown are mean PEC numbers for three mice \pm 1 SD ($p < 0.005$ * versus saline injected mice).

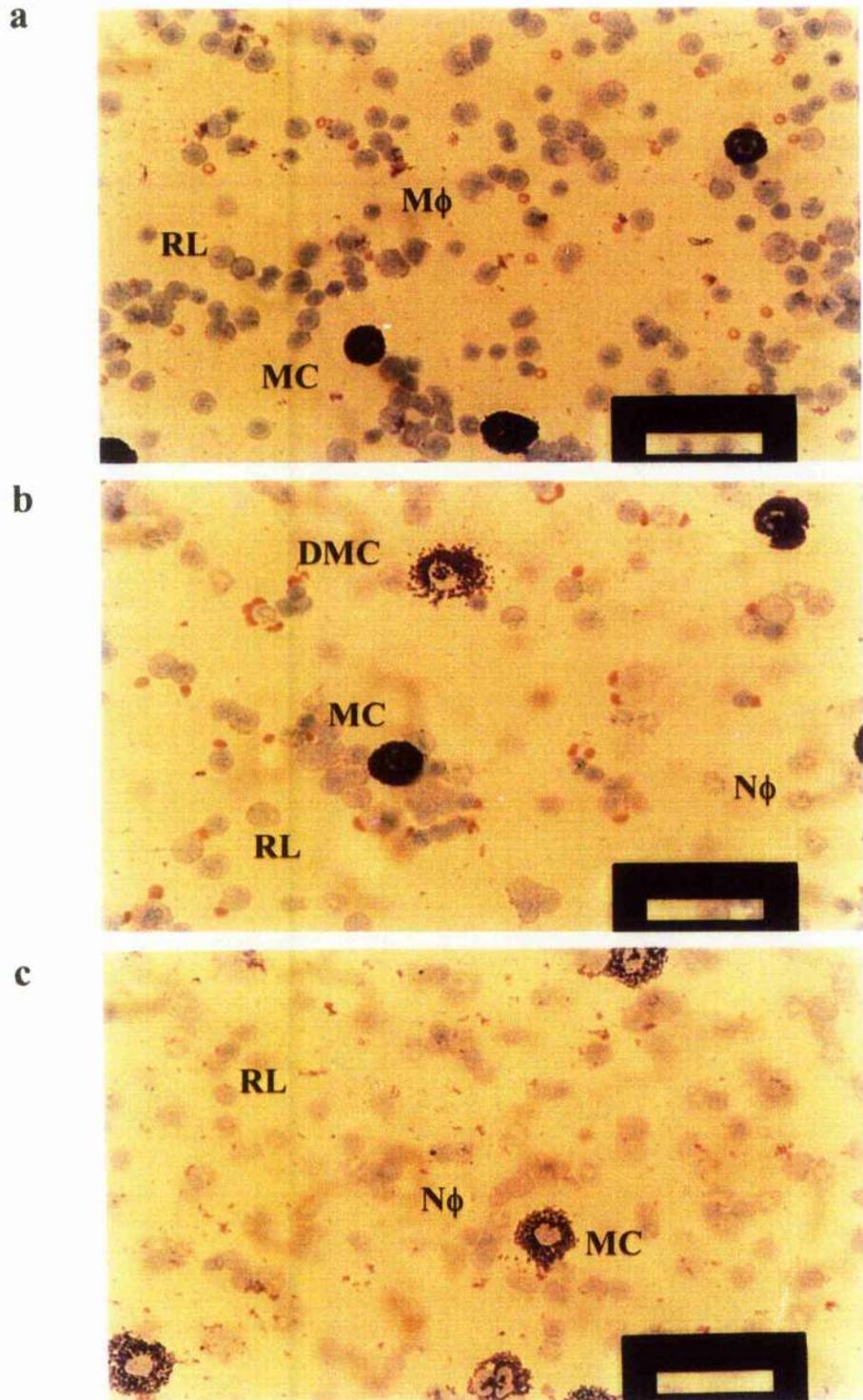


Figure 3.2 Histological Examination of PEC after ISCOMS Injection

Cytopsin appearances of pooled PEC removed from (a) saline injected mice or (b) 3.5 hours, (c) 7 hours after i.p. injection of 5 μ g OVA ISCOMS. Giemsa stained cytopsin were examined by microscopy at x400 magnification. (Key: RL - resting lymphocyte, MC - mast cell, M ϕ - macrophage, N ϕ - neutrophil, DMC - degranulating mast cell.)

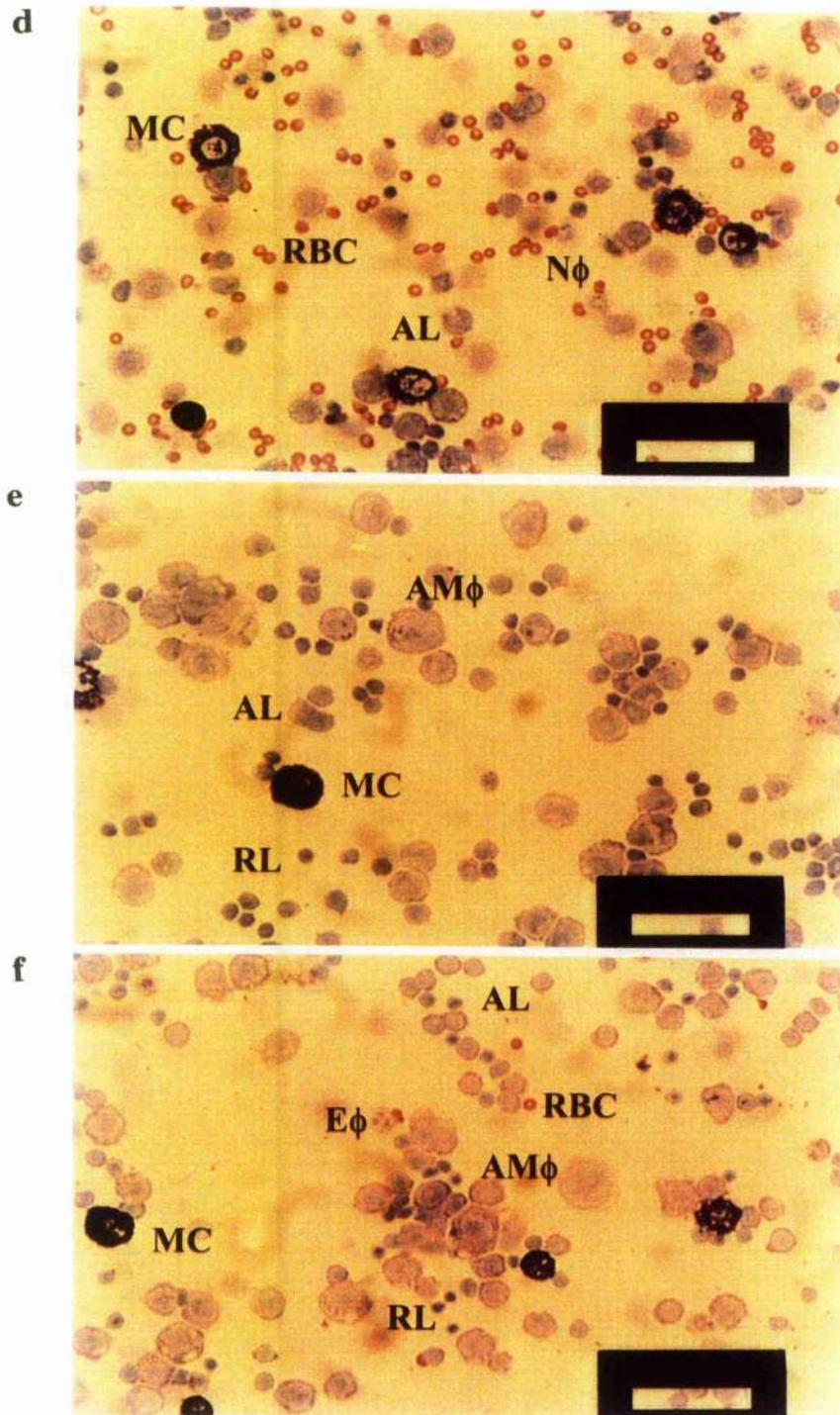


Figure 3.2 Histological Examination of PEC after ISCOMS Injection

Cytospin appearances of pooled PEC removed from (d) 24 hours, (e) 48 hours and (f) 72 hours after i.p. injection of 5 μ g OVA ISCOMS. Giemsa stained cytopsin were examined by microscopy at x400 magnification. (Key: RBC - red blood cell, AL - activated lymphocyte, MC - mast cell, N ϕ - neutrophil, AM ϕ - activated macrophage, RL - resting lymphocyte and E ϕ - eosinophil).

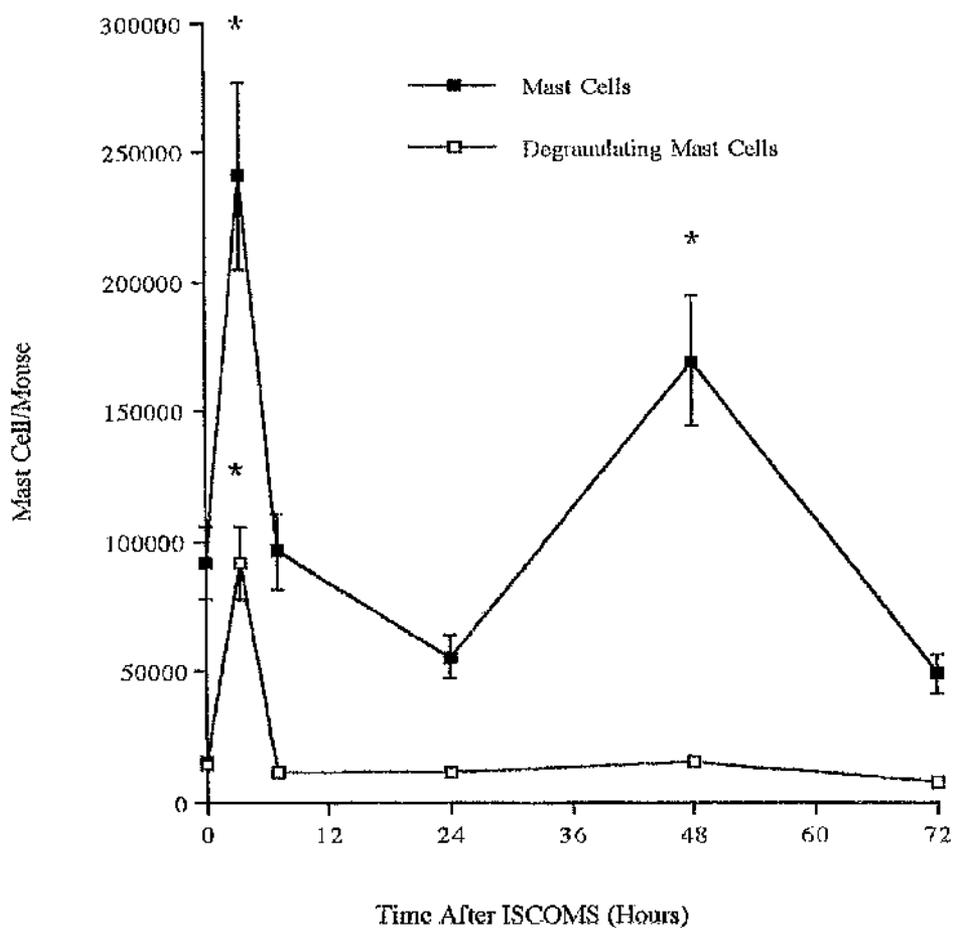


Figure 3.3 Induction of Mast Cell Recruitment and Degranulation by ISCOMS

Mast cells were counted on cytopins of PEC removed at various times after i.p. injection of 5 μ g OVA ISCOMS. The results shown are the mean numbers of mast cells and degranulating mast cells in ten microscope fields at x400 magnification \pm 1 SD (* p<0.01 versus saline injected mice).

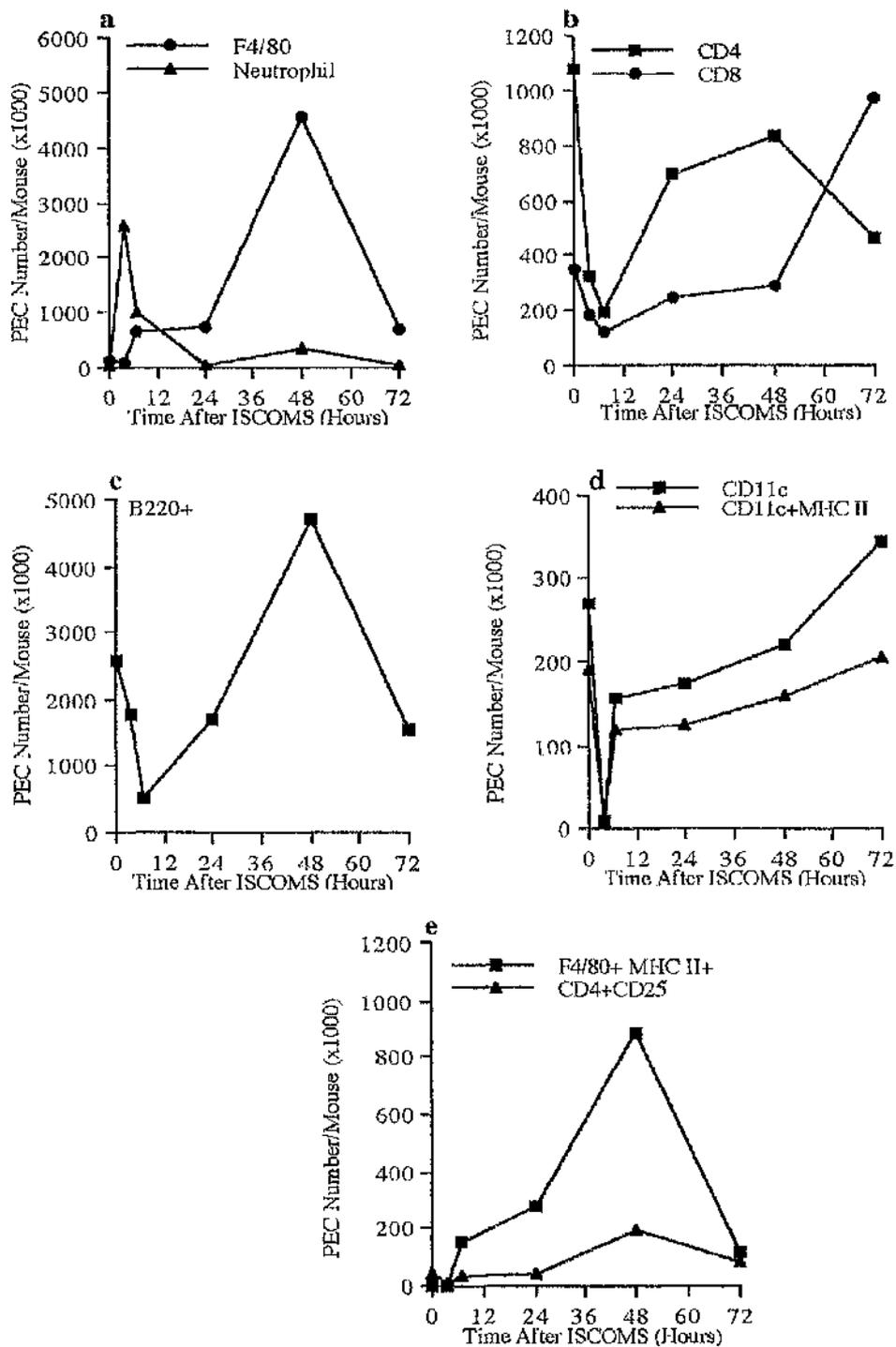


Figure 3.4 Phenotypic Analysis of ISCOMS Recruited PEC.

PEC were removed by lavage after an i.p. injection of 5 μ g OVA ISCOMS and analysed by flow cytometry. The results shown are the numbers of (a) neutrophils and F4/80⁺ macrophages, (b) CD4 and CD8 T cells, (c) B220⁺ B cells, (d) CD11c⁺ dendritic cells, (e) MHC class II⁺ F4/80 macrophages and CD25⁺ CD4 T cells. The results are expressed as the number of cells per mouse which were calculated from the percentage of cells positive for each marker in the PEC pooled from 5 animals per group. Control mice received saline alone.

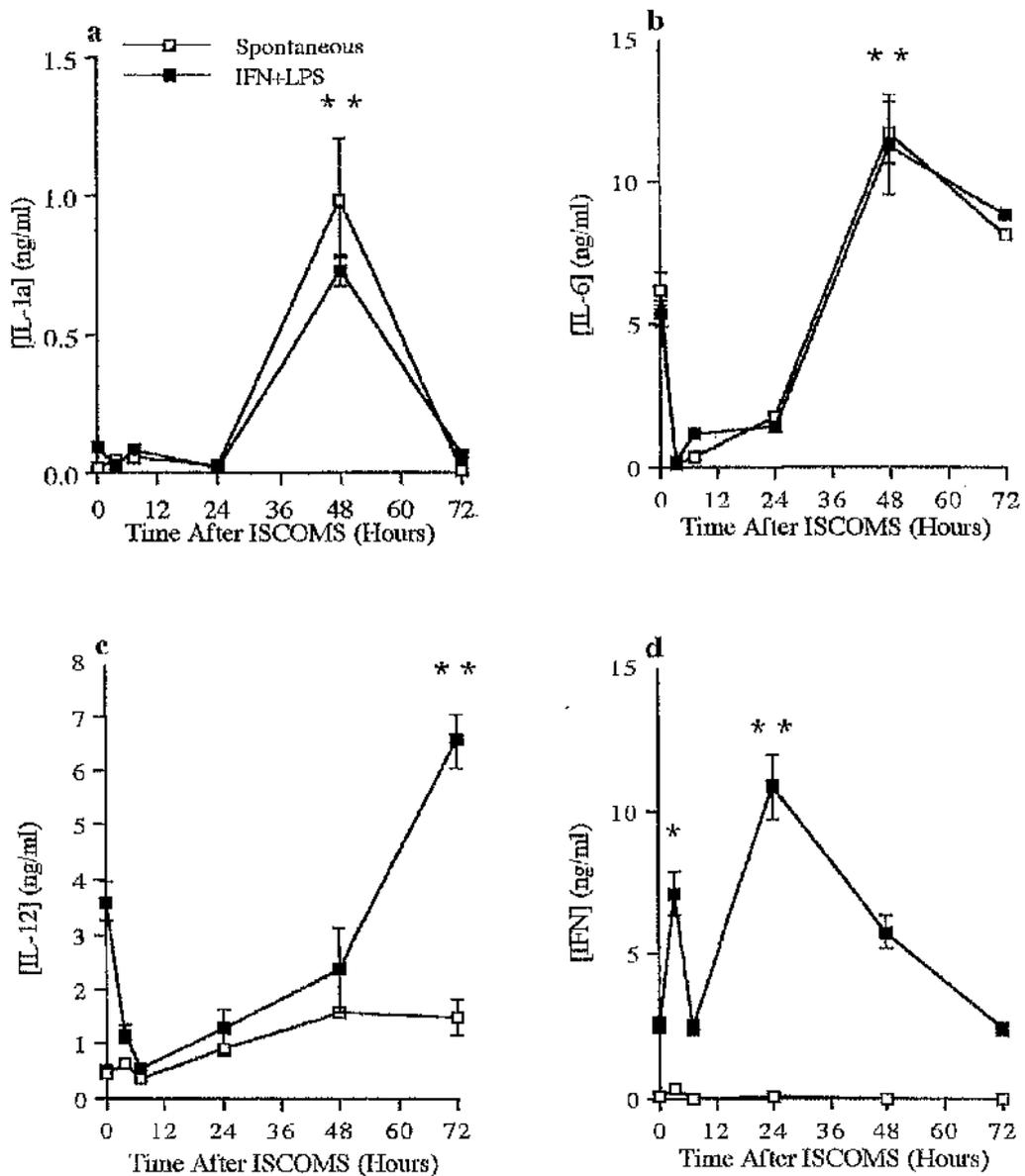


Figure 3.5 ISCOMS Induce Local Secretion of Inflammatory Cytokines by PEC.

Production of (a) IL-1 α , (b) IL-6, (c) immunoreactive IL-12 and (d) γ IFN by PEC pooled from 5 mice given an i.p. injection of 5 μ g ISCOMS 3.5-72 hours before. Control mice received saline alone. The results shown are the mean concentration of cytokines measured in triplicate after 48 hours stimulation in the presence or absence of γ IFN+LPS (a-c) or the presence or absence of Con A (d) \pm 1 SD (* $p < 0.05$ versus saline injected mice; ** $p < 0.005$ versus saline injected mice).

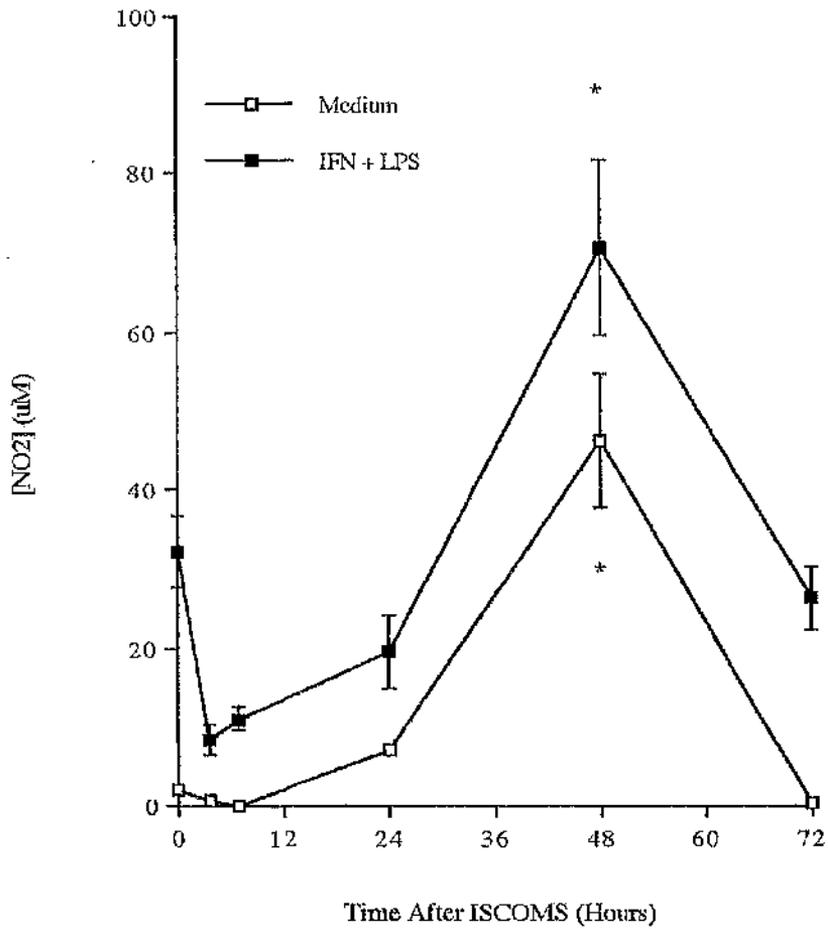


Figure 3.6 ISCOMS Induced Nitric Oxide Production from PEC

Production of nitric oxide from PEC pooled from 5 mice after injection of 5µg ISCOMS or saline. The results shown are the mean concentration of nitrite measured in triplicate after culture for 48 hours in the presence or absence of γIFN+LPS ± 1 SD (* p<0.01 versus saline injected mice).

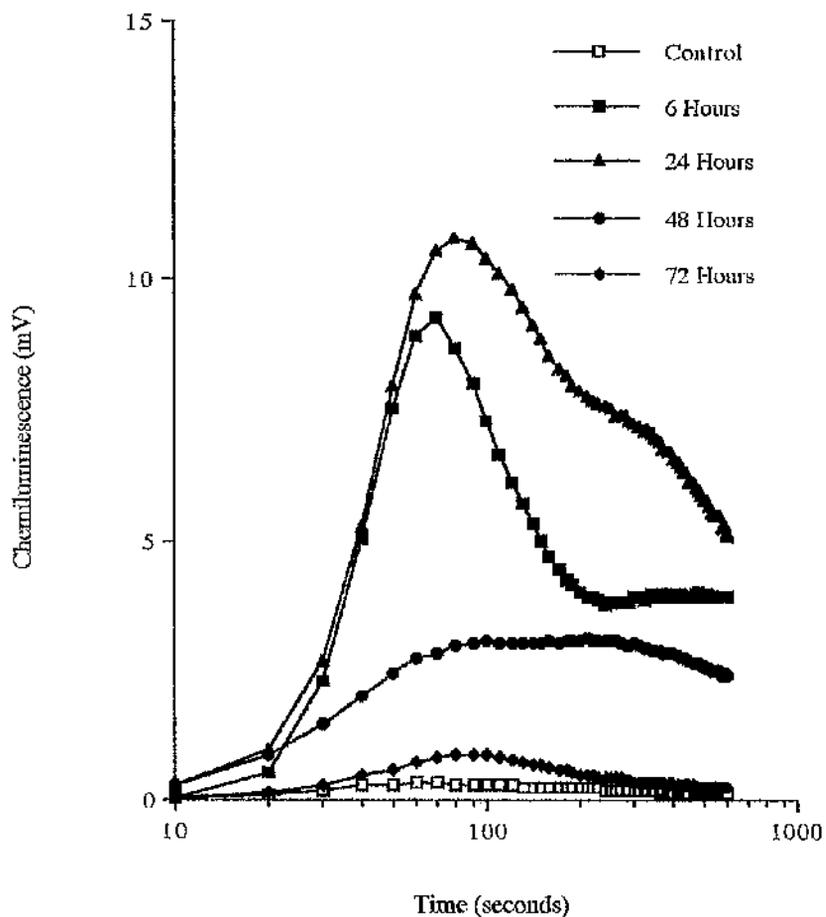


Figure 3.7 ISCOMS Induced Production of Reactive Oxygen Intermediates

Production of reactive oxygen intermediates from PEC pooled from 5 animals after injection with 5 μ g ISCOMS or saline. The results show the release of ROI from 1×10^6 PEC as measured by the PMA stimulated chemiluminescence of Luminol and are expressed in mV.

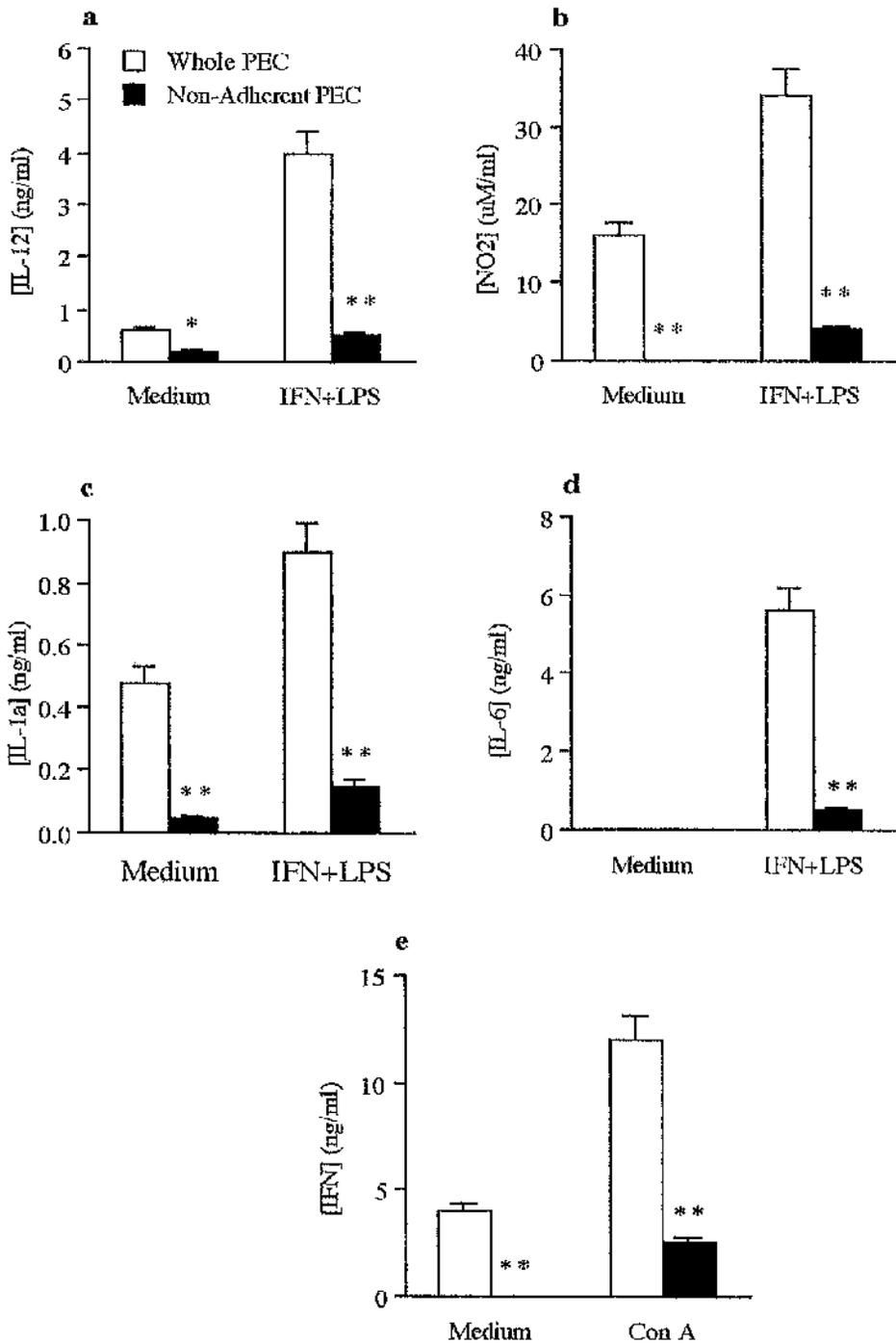


Figure 3.8 Adherent Cells are Required for the Production of ISCOMS Induced Inflammatory Mediators

Production of (a) IL-12, (b) NO, (c) IL-1 α , (d) IL-6 and (e) γ IFN by PEC pooled from 5 mice given an i.p. injection of 5 μ g ISCOMS 48 hours earlier. The results shown are the mean concentration of cytokines from supernatants of either whole PEC cultures or of PEC depleted of adherent cells after 48 hours stimulation in the presence or absence of γ IFN+LPS (a-d) or the presence or absence of Con A (e) \pm 1 SD (* p<0.05 versus whole PEC cultures; ** p<0.005 versus whole PEC cultures).

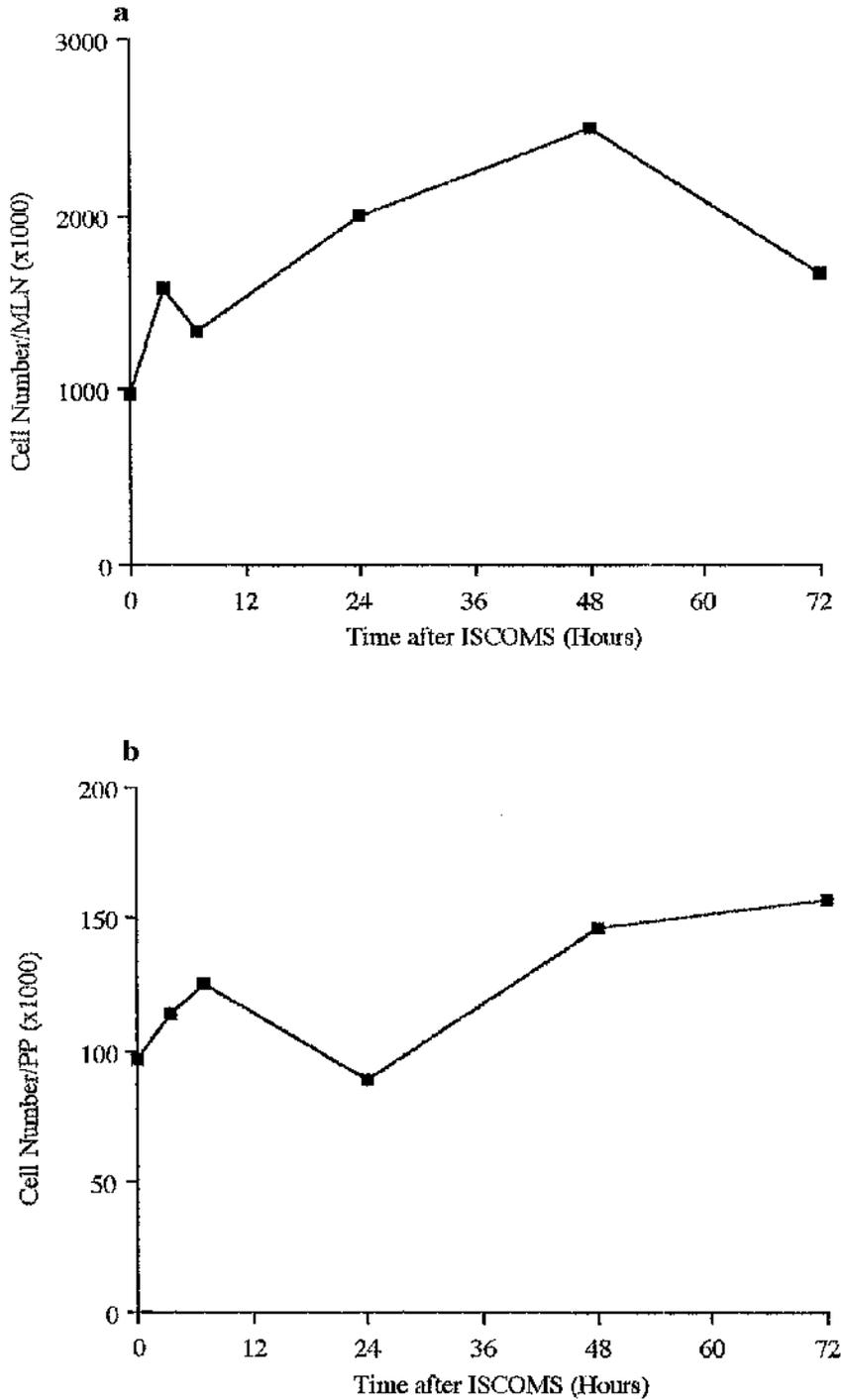


Figure 3.9 Recruitment of Inflammatory Cells by a Single Feed of ISCOMS

The graph shows recruitment of (a) MLN and (b) PP cells after a single feed of 200 μ g OVA ISCOMS. MLN and PP tissues were removed from five mice at various times after oral ISCOMS and cell counts performed. The results shown are the mean cell numbers per MLN or PP, calculated from pooled samples. Control mice received saline alone.

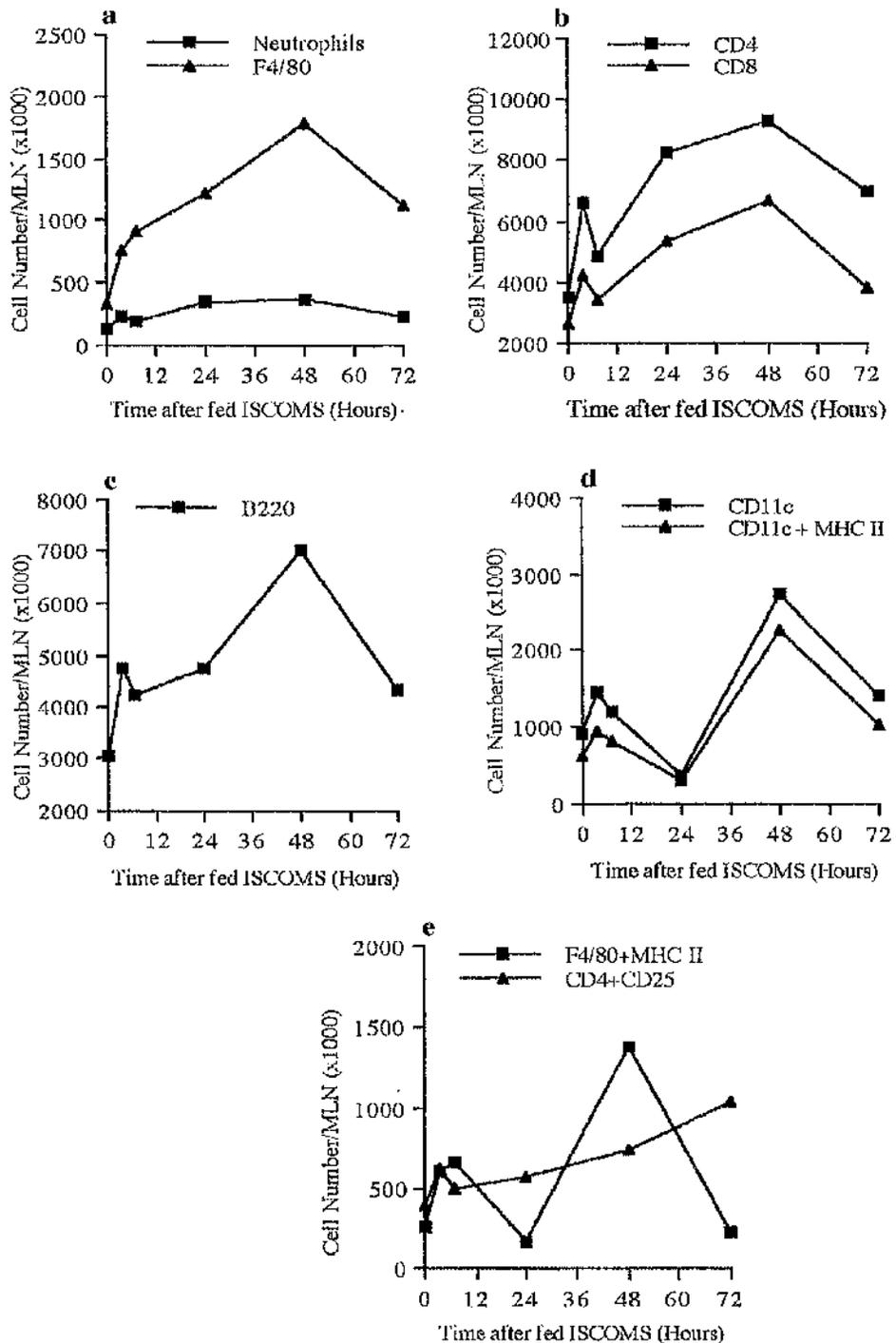


Figure 3.10 Phenotypic Examination of MLN Cells Induced by Oral Administration of ISCOMS

MLN were removed at various times after a single feed of 200µg OVA ISCOMS, pooled and analysed by flow cytometry. The results shown are the numbers of (a) neutrophils and F4/80⁺ macrophages, (b) CD4 and CD8 T cells, (c) B220⁺ B cells, (d) CD11c⁺ dendritic cells and MHC class II⁺ CD11c⁺ dendritic cells, (e) MHC class II⁺ F4/80 macrophages and CD25⁺ CD4 T cells. The results are expressed as the number of cells per MLN, calculated from the percentage of cells positive for each marker. Control mice received saline alone.

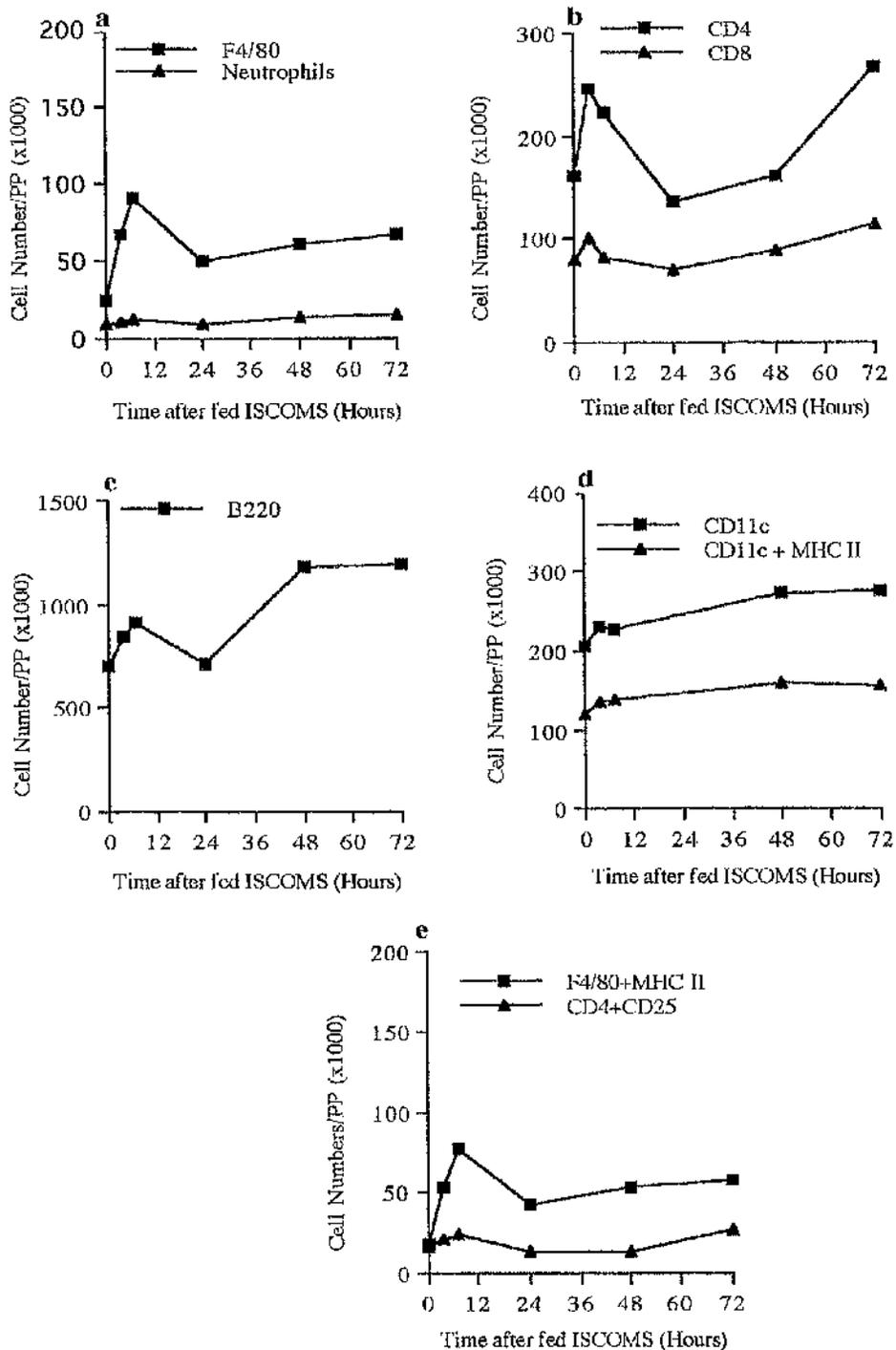


Figure 3.11 Phenotypic Examination of PP Cells Induced by Oral Administration of ISCOMS

PP were removed at various times after a single feed of 200 μ g OVA ISCOMS, pooled and analysed by flow cytometry. The results shown are the numbers of (a) neutrophils and F4/80⁺ macrophages, (b) CD4 and CD8 T cells, (c) B220⁺ B cells, (d) CD11c⁺ dendritic cells and MHC class II⁺ CD11c⁺ dendritic cells, (e) MHC class II⁺ F4/80 macrophages and CD25⁺ CD4 T cells. The results are expressed as the number of cells per MLN, calculated from the percentage of cells positive for each marker. Control mice received saline alone.

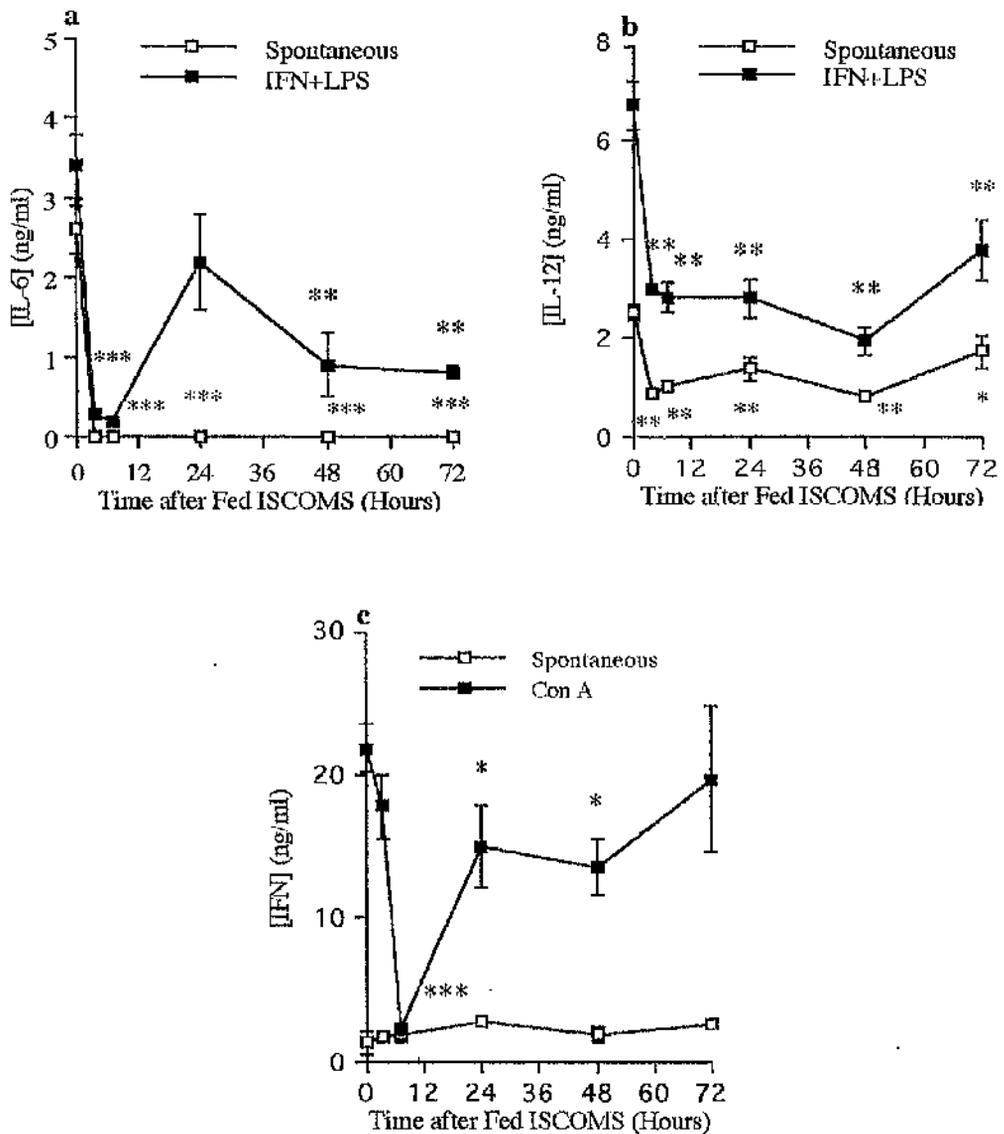


Figure 3.12 Secretion of Inflammatory Cytokines by MLN After Oral Administration of ISCOMS

Production of (a) IL-6, (b) IL-12 and (c) γ IFN by MLN pooled from 5 mice given an i.p. injection of 5 μ g ISCOMS 3.5-72 hours before. Control mice received saline alone. The results shown are the mean concentration of cytokines measured in triplicate after 48 hours stimulation in the presence or absence of γ IFN+LPS (a, b) or the presence or absence of Con A (c) \pm 1 SD (* $p < 0.05$ versus saline injected mice; ** $p < 0.005$ versus saline injected mice; *** $p < 0.001$ versus saline injected mice).

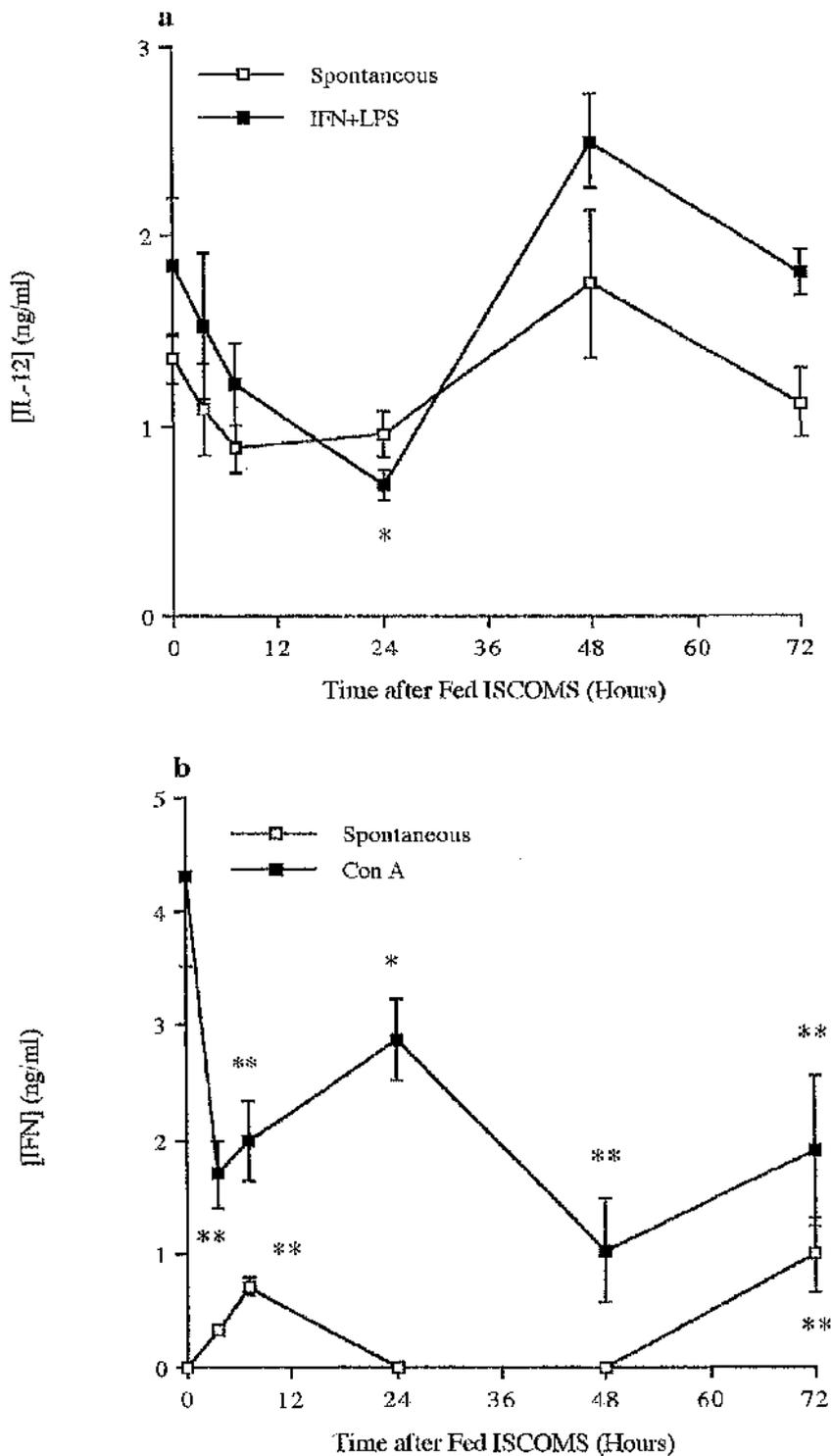


Figure 3.13 Secretion of Inflammatory Cytokines by PP After Oral Administration of ISCOMS

Production of (a) IL-12 and (b) γ IFN by PP pooled from 5 mice after a single feed of 200 μ g ISCOMS 3.5-72 hours before. Control mice received saline alone. The results shown are the mean concentration of cytokines measured in triplicate after 48 hours stimulation in the presence or absence of γ IFN+LPS (a), or the presence or absence of Con A (b) \pm 1 SD (* $p < 0.05$ versus saline injected mice; ** $p < 0.01$ versus saline injected mice).

Chapter 4

The Importance of ISCOMS Induced Inflammatory Mediators for the Generation of Antigen-Specific Immunity

Introduction

It is currently believed that adjuvants act principally by stimulating different components of the innate immune system, with consequent upregulation of costimulatory molecules and production of accessory cytokines [Khortus and Jenkins, 1996]. In the previous chapter, I showed that ISCOMS are no exception to this theory, being capable of inducing a rapid and diverse range of inflammatory effects after parenteral or oral administration. In particular, there was a notable increase in the production of a number of proinflammatory mediators including γ IFN, IL-6, IL-12 and NO after an i.p. injection or feeding of ISCOMS. I felt that it was essential to determine if any of these mediators played a role in the generation of antigen-specific immune responses to ISCOMS associated antigen.

We had demonstrated previously that oral or parenteral immunisation with OVA-ISCOMS induced a wide range of antigen-specific responses, as measured by a number of *in vitro* and *in vivo* parameters, including delayed type hypersensitivity (DTH), MHC class I restricted cytotoxic T cells, as well as antigen-specific proliferation, cytokine and antibody production [Maloy et al., 1995, Mowat et al., 1993, Mowat et al., 1991b]. Therefore, in the experiments described in this chapter, I monitored these antigen-specific responses in gene-targeted knockout animals deficient in IL-6, γ IFN, IL-12, iNOS, IL-18 and IL-4.

Experimental Protocol

Female KO mice and appropriate wild type mice all of 6-8 weeks of age, were immunised subcutaneously (s.c.) in the rear footpad with 5 μ g of OVA ISCOMS in a volume of 50 μ l saline, intraperitoneally (i.p.) with 5 μ g of OVA ISCOMS in a volume of 200 μ l saline, or orally with six consecutive feeds of 100 μ g OVA ISCOMS in 200 μ l saline on days

1, 2, 3, 8, 9, and 10. Control mice were immunised with saline alone. OVA-specific DTH was measured in mice 24 hours after subcutaneous challenge of 100µg heat aggregated OVA (HAO) given in the footpad, seven days after s.c. immunisation or the final feed of OVA ISCOMS. Antigen-specific proliferation and cytokine production in response to *in vitro* restimulation with OVA was measured in popliteal lymph nodes removed from animals 14 days after s.c. immunisation or spleens taken at the same time after the final feed of OVA ISCOMS. OVA-specific CTL activity was measured in spleens of mice 7 days after i.p. immunisation or 14 days after oral administration of ISCOMS. Antigen-specific antibody production was assessed in serum taken 7 days after challenge with HAO. Antigen-specific secretory IgA production was measured in intestinal secretions collected 7 days after the final feed of ISCOMS.

Results

4.1 Immune Responses to ISCOMS in Interleukin 12 Deficient Mice

I have described earlier that production of IL-12 by ISCOMS recruited PEC was greatly enhanced immediately after injection with ISCOMS. IL-12 triggers the production of γ IFN from NK cells [Trinchieri, 1995] and is responsible for driving the development of γ IFN producing Th1 CD4⁺ T cells, as well as the differentiation and activation of CTL [Abdi and Herrmann, 1997]. IL-12 also regulates the expression of the IL-2R α and β subunits [Robertson et al., 1992, Nuame et al., 1993] on the surface of lymphocytes, meaning that it could potentially control T cell proliferation to an immune signal. IL-12 has also been implicated in the induction of cell surface IL-8 [Nuame et al., 1993] and so could also be involved in initiating the chemotactic compartments of an inflammatory cascade. For these reasons I was interested to investigate how IL-12 might regulate ISCOMS induced antigen-specific immune responses. To do this, IL-12KO mice on the BALB/c background were used for the assessment of antigen-specific DTH, antibody, proliferation and cytokine production

and IL-12KO mice on the C57BL/6 background were used to measure antigen-specific CTL activity.

4.1.1 Antigen-Specific DTH Responses

As anticipated, wild type BALB/c mice showed a good level of OVA-specific DTH following s.c. immunisation with OVA ISCOMS (Figure 4.1a) and there was an even better response in orally immunised mice (Figure 4.1b). IL-12KO mice had dramatically decreased DTH responses after s.c. immunisation (Figure 4.1a) and these were also reduced by 50% in orally immunised IL-12KO mice (Figure 4.1b).

4.1.2. OVA-Specific Proliferative Responses

Lymph node cells from wild type BALB/c mice immunised with OVA ISCOMS s.c or orally generated good proliferative responses on restimulation with OVA *in vitro*. These were reduced by 40-60% in IL-12KO mice (Figure 4.2a and b).

4.1.3. Antigen-Specific Cytokine Production

OVA ISCOMS induced antigen specific production of γ IFN from wild type BALB/c PLN after s.c. immunisation (Figure 4.3a), and from the spleen after oral immunisation (Figure 4.3b). Wild type mice also produced OVA-specific IL-5 after s.c. (Figure 4.3c) and oral immunisation (Figure 4.3d). As expected, IL-12KO mice produced virtually no OVA-specific γ IFN in response to s.c. or oral immunisation (Figure 4.3a and b), but interestingly, these animals also had a 40% reduction in antigen-specific IL-5 production after s.c. immunisation (Figure 4.3c). However, orally immunised IL-12KO animals showed significantly increased levels of IL-5, which were twice that of wild types (Figure 4.3d).

4.1.4 OVA-Specific CTL Activity

Wild type C57Bl/6 mice generated good OVA-specific CTL responses after i.p. immunisation (Figure 4.4a). In IL-12KO animals, OVA-specific CTL activity was significantly reduced compared to that in wild type mice, with responses in the order of 50-60% that of normal animals (Figure 4.6.4a). There was no lysis of non OVA-expressing EL4 cells by any cell population. Orally immunised wild type animals produced strong OVA-specific CTL responses (Figure 4.4b), with IL-12KO mice showing a slight reduction in the CTL activity at high effector : target cell ratios compared with wild type mice, but when less effector cells were used, this difference was not so obvious (Figure 4.4b).

4.1.5 Serum IgG Isotypes

Wild type BALB/c animals produced large amounts of OVA-specific IgG2a and IgG1 antibodies after s.c. immunisation with ISCOMS (Figure 4.5a and b). IgG2a levels were significantly decreased in IL-12KO mice but the mice had normal production of IgG1 antibodies, (Figure 4.5a and b). Orally immunised wild type animals produced OVA-specific IgG1 antibodies in serum and these responses were significantly increased in IL-12KO animals (Figure 4.5c). Neither wild type nor IL-12KO mice produced any IgG2a antibodies after oral immunisation in this experiment.

4.1.6 Secretory Intestinal IgA Antibody Production

Wild type BALB/c and IL-12KO BALB/c mice produced equivalent amounts of secretory IgA antibodies in gut washes after oral immunisation (Figure 4.6).

4.1.7 Summary

IL-12KO mice have a severely impaired antigen-specific immune response to OVA ISCOMS compared with wild type controls. The defect in OVA-specific immunity is more dramatic after s.c. immunisation, where IL-12KO mice generally produced less than half the level of immunity of their wild type counterparts for all the parameters measured *in vivo* and *in vitro*, including both Th1 and Th2 type responses. A similar defect was seen in orally immunised IL-12KO mice, but in this case immune responses were generally only reduced by approximately 30-40%. In addition some responses, such as OVA-specific IgA, IL-5 and IgG1 production, were either equivalent to, or increased compared with wild type controls after oral immunisation. Thus IL-12 plays an important role in the generation of ISCOMS induced OVA-specific immunity, but this is partly dependent on the route of immunisation used, apparently being important when ISCOMS are given subcutaneously than orally. The residual responses present in IL-12KO mice show that IL-12 is clearly not the sole factor required for the generation of antigen-specific immunity and so I went on to investigate other factors induced during an ISCOMS induced inflammatory cascade, which might replace or synergise with IL-12.

4.2 Immune Responses to ISCOMS in IL-18 Deficient Mice

IL-18 is a recently described cytokine which is part of the IL-1 family of proteins. It is secreted early in inflammation by a wide variety of cells including APC, keratinocytes and epithelial cells [Kohno and Kurimoto, 1998]. It is known to induce the activation of γ IFN from NK cells and T cells, thus having similar properties to IL-12 [Okamura et al., 1998a, Okamura et al., 1995]. I had not been able to measure the presence of IL-18 after ISCOMS injection, because of the absence of a suitable ELISA, but the fact that IL-12KO mice had persistent responses to ISCOMS suggested that it would be useful to investigate whether IL-18 played a role. Mice deficient in IL-18 (IL-18KO) had been recently generated in this department by Dr. X-Q. Wei and I therefore performed a preliminary examination of antigen-

specific immune responses to ISCOMS in wild type MF1, heterozygous (IL-18^{+/-}) and IL-18KO animals.

4.2.1 Antigen-Specific DTH Responses

A single s.c. immunisation of OVA ISCOMS in the footpad induced a large DTH response in wild type MF1 animals, but homozygous IL-18KO animals showed almost complete ablation of the OVA-specific DTH response and IL-18^{+/-} heterozygous mice also had significantly reduced DTH responses compared with wild types (Figure 4.7a). Six feeds of OVA ISCOMS also induced a strong DTH response in wild type animals, but in contrast to s.c. immunised animals, orally immunised homozygous IL-18KO and IL-18^{+/-} heterozygous mice had normal DTH responses (Figure 4.7b).

4.2.2 Antigen-Specific Proliferative Responses

Wild type mice immunised with s.c. OVA ISCOMS produced a good level of antigen-specific proliferation on restimulation with OVA *in vitro* and identical responses were obtained in homozygous and heterozygous IL-18KO mice (Figure 4.8). Unfortunately, I obtained no results from the proliferation assays set up from spleen cells taken from orally immunised mice.

4.2.3. Antigen-Specific Cytokine Production

Wild type animals immunised s.c. with OVA ISCOMS produced large amounts of γ IFN when restimulated with OVA *in vitro*, as did heterozygous and homozygous IL-18KO mice (Figure 4.9a). Subcutaneously immunised wild type mice also produced a large amount of OVA-specific IL-5 and again there was no difference in the heterozygous or homozygous IL-18KO mice (Figure 4.9b). Wild type animals immunised orally with ISCOMS produced a large amount of γ IFN, but neither heterozygous nor homozygous IL-18KO mice produced

significant levels of γ IFN (Figure 4.9c). Wild type mice also produced OVA-specific IL-5 and this was elevated to nearly three times that of wild type levels in both heterozygous and homozygous IL-18KO mice (Figure 4.9d).

4.2.4. Antigen-Specific CTL Activity

Wild type animals were primed for good antigen specific CTL activity after six feeds of OVA ISCOMS (Figure 4.10). There was no difference in the degree of cytotoxicity between either homozygous or heterozygous IL-18KO animals and wild types (Figure 4.10). Unfortunately there was not enough mice at this time to perform parallel experiments in parenterally immunised IL-18KO mice.

4.2.5. Serum IgG Antibody Production

Wild type animals were primed for IgG1 and IgG2a antibody production after s.c. immunisation with OVA ISCOMS and equivalent secondary responses occurred in heterozygous and homozygous IL-18KO mice (Figures 4.11a and b). Wild type mice given six feeds of OVA ISCOMS were also primed for IgG1 and IgG2a antibody production, as were heterozygous and homozygous IL-18KO mice (Figures 4.11c and d).

4.2.6 Summary

Thus IL-18KO mice appear to have some defect in immune responses to ISCOMS. However, no consistent pattern was observed for either route of administration, or for individual components of the immune response, even those which IL-18 is thought to be important, such as γ IFN production. Unfortunately, no further mice were available for these experiments to be repeated during the course of my project, but in view of the defects of IL-12KO mice and possibly IL-18KO mice, I went on to investigate the role of mediators downstream of these cytokines, beginning with γ IFN, using γ IFNRKO mice.

4.3. Immune Responses to ISCOMS in γ Interferon-Receptor-Deficient Mice

γ IFN is essential for many aspects of effector immune responses, being central to the maintenance of the Th1 polarised response [Maggi et al., 1992] and assisting the generation of class I MHC-restricted CTL [Scott and Kaufman, 1991]. As a result, it is often difficult to induce such responses or protective immunity to intracellular pathogens, in mice lacking γ IFN or its receptor [Buchmeier and Schreiber, 1985, Scott, 1991, Schijns et al., 1994]. ISCOMS are potent inducers of Th1 dependent responses *in vivo*, as well as CTL activity [Maloy et al., 1995, Mowat et al., 1991b, Mowat et al., 1993] and I showed in Chapter 3 that γ IFN production was one of the components of early inflammatory response to ISCOMS. Therefore it seemed important to determine if γ IFN was required for the induction of local and systemic immune responses by ISCOMS. To do this I used γ IFNRKO mice which lack functional γ IFN.

4.3.1 OVA-Specific DTH Responses

Subcutaneously immunised wild type mice produced low but significant antigen-specific DTH responses upon challenge with HAO and unexpectedly, s.c immunised γ IFNRKO mice had a significantly increased DTH response compared with wild type mice which was four times greater than wild type mice (Figure 4.12a). Wild type animals fed OVA ISCOMS produced good DTH responses and there was no significant difference in the DTH responses in orally immunised γ IFNRKO mice as compared to wild type mice (Figures 4.12b).

4.3.2 OVA-Specific Proliferative Responses

Wild type mice immunised with OVA ISCOMS generated strong proliferative responses on restimulation with OVA *in vitro* after both subcutaneous or oral immunisation

(Figure 4.13a and b). Proliferative responses were significantly enhanced in s.c. immunised γ IFN γ RKO mice (Figure 4.13a), but these were significantly decreased in orally immunised γ IFN γ RKO animals (Figure 4.13b).

4.3.3 OVA-Specific Cytokine Production

Subcutaneous immunisation with OVA ISCOMS induced a low but significant level of antigen specific γ IFN from wild type mice and this was significantly enhanced in γ IFN γ RKO mice (Figure 4.14a). Subcutaneous immunisation with OVA ISCOMS also primed antigen-specific IL-5 production in wild type mice and this was nearly twice as high in γ IFN γ RKO mice (Figure 4.14b). In general, the antigen-specific cytokine production was much less in orally immunised mice than following s.c. immunisation with ISCOMS. Oral immunisation with OVA ISCOMS induced a low level of antigen-specific γ IFN production from wild type mice and this was increased in γ IFN γ RKO mice to three times that of wild type animals (Figure 4.14c). Six consecutive feeds of OVA ISCOMS also primed low levels of antigen-specific IL-5 production in wild type mice and γ IFN γ RKO mice produced IL-5 responses that were approximately 6 times greater than wild type animals (Figure 4.14d).

4.3.4. OVA-Specific CTL Responses

Wild type mice generated good OVA-specific CTL responses after i.p. immunisation, as did γ IFN γ RKO mice, which showed normal levels of OVA-specific CTL activity (Figures 4.15a). Wild type animals also produced good OVA-specific CTL responses after feeding OVA ISCOMS and these were somewhat elevated in γ IFN γ RKO mice fed ISCOMS (Figure 4.15b).

4.3.5 Antigen-Specific Serum IgG Production

OVA ISCOMS primed for good secondary humoral immune responses in wild type mice when administered orally or as a s.c. immunisation. As expected, γ IFN^{RKO} mice showed increased production of IgG1 and reduced IgG2a antibodies when ISCOMS were given subcutaneously (Figure 4.16a and b).

4.3.6 OVA-Specific Intestinal IgA Production

Wild type mice had significant induction of IgA antibodies in gut washes following six consecutive feeds of OVA ISCOMS and these were greatly increased in the absence of functional γ IFN (Figure 4.17).

4.3.7 Summary

Thus, immune responses to either oral or parenteral immunisation with OVA ISCOMS are essentially normal, or even increased, in the absence of functional γ IFN. Despite the high level of inflammatory γ IFN released immediately after exposure to ISCOMS, as well as some evidence for the role of the γ IFN inducing cytokines IL-12 and IL-18, it appeared that γ IFN did not directly promote the generation of the antigenic immunity induced by OVA ISCOMS. Therefore I carried out further investigations into the involvement of other cytokines that are known to direct the immune response.

4.4 Immune Responses to ISCOMS in Interleukin 6 Deficient Mice

In Chapter 3, I showed that ISCOMS induced a large non-specific increase in secretion of IL-6 after a parenteral injection. This is consistent with the role of this cytokine in the early acute inflammatory response [Baumann and Gauldie, 1994, Kopf et al., 1994] and also with reports that IL-6 is involved with the generation of protective antigen-specific

immune responses *in vivo* against *Candida albicans* [Romani et al., 1996], *Listeria monocytogenes* [Kopf et al., 1994] and *Mycobacterium avium* [Appelberg et al., 1994]. In addition, it has been suggested that IL-6 acts as an early inducer of Th2 responses [Rincon et al., 1997] and there has been disputed evidence that mucosal IgA responses in IL-6KO mice may be defective [Ramsay et al., 1994] [Ramsay et al., 1994, Bromander et al., 1996]. Thus I investigated if IL-6 played any part in the development of acquired immune responses to OVA ISCOMS using IL-6 deficient (IL-6KO) mice. In these studies, I first performed preliminary experiments using the subcutaneous route and, in view of the results obtained and the difficulty in obtaining access to the mice, I carried out very few oral immunisation studies.

4.4.1 Antigen-Specific DTH Responses

Wild type 129/Sv animals produced good antigen-specific DTH responses seven days after a s.c. immunisation of OVA ISCOMS and there was no significant difference in the DTH response in IL-6KO mice (Figure 4.18).

4.4.2 Antigen-Specific Proliferative Responses

Wild type mice immunised with OVA ISCOMS subcutaneously generated good proliferative responses on restimulation with OVA *in vitro* and identical results were obtained in IL-6KO mice (Figure 4.19).

4.4.3 Antigen-Specific Cytokine Production

Subcutaneous immunisation with OVA ISCOMS primed very low antigen-specific γ IFN (Figure 4.20a) and IL-5 (Figure 4.20b) production in wild type mice. Normal γ IFN production was measured after s.c. immunisation in IL-6KO mice (Figure 4.20a), but OVA-specific IL-5 in IL-6KO mice was twice that of the wild type animals (Figure 4.20b).

4.4.4 Antigen-Specific CTL Activity

Wild type SVJ mice generated good OVA-specific CTL responses after i.p. immunisation with OVA ISCOMS and identical responses were found with IL-6KO mice (Figure 4.21). There was no killing of the non-antigen expressing EL4 cells.

4.4.5 Antigen-Specific Serum IgG Antibody Production

Wild type mice produced OVA-specific IgG1 and IgG2a when immunised subcutaneously with OVA ISCOMS (Figure 4.22a and b). There was no difference in the levels of either IgG isotype obtained in IL-6KO mice (Figure 4.22a and b).

4.4.6 Summary

Thus the absence of IL-6 does not impair the generation of antigen-specific immune responses after immunisation with OVA ISCOMS. In light of this information I went on to investigate the influence of another of the Th2 cytokines, IL-4.

4.5. Antigen-Specific Responses to ISCOMS in Interleukin 4 Deficient Mice

Previous results had shown that ISCOMS primed a number of Th2 responses, including antigen-specific cytokines as well as serum IgG1 antibodies [Maloy et al., 1995] and recently, the oral adjuvant properties of cholera toxin (CT), another potent mucosal vaccine vector, were found to be entirely dependent on the presence of IL-4 *in vivo* [Vajdy et al., 1995]. Therefore it was important to investigate the role of this cytokine in ISCOMS induced immunogenicity .

4.5.1. Antigen-Specific DTH Responses

As expected, wild type mice had significant DTH responses when challenged 7 days after subcutaneous immunisation with OVA ISCOMS. These were significantly greater in IL-4KO mice (Figure 4.23a). Wild type animals fed OVA ISCOMS also produced strong DTH responses when challenged with HAO seven days after the final feed, but there was no significant difference in the DTH results obtained in orally immunised IL-4KO mice (Figures 4.23b).

4.5.2. Antigen-Specific Proliferative Responses

Wild type mice immunised with OVA ISCOMS subcutaneously generated good proliferative responses from PLN cells after restimulation with OVA *in vitro* and these were significantly enhanced in IL-4KO mice to 5 times of those in wild type animals (Figure 4.24a). A similar pattern was observed in the spleens of orally immunised wild type mice and IL-4KO mice (Figure 4.24b).

4.5.3. Antigen-Specific Cytokine Production

Subcutaneous immunisation with OVA ISCOMS primed good antigen-specific production of γ IFN from wild type splenocytes and, as expected, IL-4KO mice showed a dramatic increase in the level of antigen-specific γ IFN production (Figure 4.25a). OVA-specific IL-5 production was also induced in wild type mice, and as expected, this was significantly reduced in IL-4KO mice (Figure 4.25b). Six feeds of OVA ISCOMS primed only low levels of antigen-specific γ IFN production in wild type mice (Figure 4.25c) and no OVA-specific IL-5 production (Figure 4.25d). As before, orally immunised IL-4KO mice had significantly increased production of γ IFN (Figure 4.25c), but unexpectedly, also showed some residual production of IL-5, although this was very low in comparison to that obtained in s.c. immunised animals (Figure 4.25d).

4.5.4. Antigen-Specific CTL Activity

Wild type mice generated good OVA-specific CTL responses after i.p. immunisation with OVA ISCOMS (Figure 4.26a) and also significant CTL activity after oral immunisation (Figure 4.26b). IL-4KO mice were primed for identical levels of OVA-specific CTL activity after i.p. immunisation (Figure 4.26a), and also had CTL activity after oral immunisation, although the levels that were approximately 30% less than those in wild type animals (Figure 4.26b). There was no killing of the non-OVA expressing EL4 cell line.

4.5.5 Antigen-Specific Serum IgG Antibody Production

Wild type animals produced antigen-specific IgG1 and IgG2a antibodies after both subcutaneous and (Figures 4.27a, b). As expected, IL-4KO mice had decreased levels of IgG1 and elevated IgG2a antibody in serum after either subcutaneous or oral immunisation with OVA ISCOMS (Figures 4.27a, b).

4.5.6. Increased Production of Intestinal Secretory IgA in IL-4KO Mice

Fourteen days after the last of six feeds of OVA ISCOMS, wild type mice had good OVA-specific secretory IgA production as measured in gut washes. This was significantly increased in orally immunised IL-4KO mice (Figure 4.28).

4.5.7 Summary

Thus, apart from the expected defects in Th2 activity, IL-4KO mice generate normal immune responses to either oral or parenteral immunisation with ISCOMS. I therefore went on to examine the last of the inflammatory mediators induced by injection of ISCOMS, namely NO.

4.6 Immune Responses to ISCOMS in Inducible Nitric Oxide Synthase Deficient Mice

In the previous chapter, I showed that NO levels increased dramatically after an injection of ISCOMS into the peritoneum. During inflammation, NO is produced mainly via the action of inducible nitric oxide synthase (iNOS) and it plays an important part in regulating several aspects of inflammation, including blood supply [Moncada et al., 1991], macrophage microbicidal activity [Nathan and Hibbs, 1991] and IL-12 production [Huang et al., 1998]. There has recently been evidence that iNOS deficient mice are unable to control *L. major* infection [Wei et al., 1995]. Therefore I investigated the role of inflammatory NO in the development of antigen-specific immune responses to OVA ISCOMS using iNOS deficient mice (iNOSKO). The experiments shown, only examined the responses to subcutaneous immunisation, as these mice were being bred onto an inbred background whilst my project was being concluded and before I could perform studies of oral immunisation.

4.6.1 Antigen-Specific DTH Responses

Wild type animals given a s.c. immunisation of OVA ISCOMS produced a good OVA-specific DTH response when challenged with HAO one week later and there was no significant difference in the DTH response obtained in iNOSKO mice (Figure 4.29).

4.6.2 Antigen-Specific Proliferative Responses

Wild type MF1 mice immunised with OVA ISCOMS subcutaneously generated good proliferative responses upon restimulation with OVA *in vitro* (Figure 4.30). Although, there was a slightly reduced proliferative response in iNOSKO mice, this was not statistically significant (Figure 4.30).

4.6.3 Antigen-Specific Cytokine Production

Subcutaneous immunisation with OVA ISCOMS induced antigen-specific production of γ IFN from wild type mice and this was significantly reduced in iNOSKO mice (Figure 4.31a). Subcutaneous immunisation also primed antigen-specific IL-5 production in wild type mice, although this was low compared with that found in other experiments. iNOSKO mice showed reduced levels of OVA-specific IL-5 production which were four times lower than those in wild type animals (Figure 4.31b).

4.6.4 Antigen-Specific Serum IgG Antibody Production

Wild type and iNOSKO mice produced low, but similar levels of both IgG1 and IgG2a antibodies after challenge with HAO (Figure 4.32a and b).

4.6.5 Summary

The absence of functional iNOS and thus a reduction in the production of inflammatory NO did not affect many of the antigen-specific immune responses generated by subcutaneous immunisation with OVA ISCOMS. The only alteration in OVA-specific immunity in iNOS mice was the reduction in cytokine production which affected IL-5 and γ IFN equally. Therefore, there is a possible defect in these mice but, given that these were preliminary experiments, it is a priority to repeat studies with the iNOSKO mice with measurement of a full range of immune parameters after both parenteral and oral immunisation.

Summary and Conclusions

The results presented in this chapter show that even though ISCOMS induce a wide range of non-specific inflammatory mediators *in vivo*, their mucosal and systemic adjuvant

effects are expressed normally in mice lacking the function of most of these factors, including IL-4, IL-6 and γ IFN. Systemic DTH, class I MHC-restricted CTL responses and humoral immunity *in vivo*, as well as antigen-specific proliferation and cytokine production *in vitro*, together with intestinal secretory antibody responses where measured, were induced normally after either oral or parenteral immunisation of mice deficient in these factors.

However, the adaptive immune response to ISCOMS was at least partly dependent on the presence of IL-12, as mice deficient in IL-12 appeared to have significantly decreased immune responses when immunised with OVA ISCOMS parenterally. The majority of responses were also significantly reduced in orally immunised IL-12KO mice, but the reduction under these circumstances was not as dramatic as with s.c. immunised animals and some responses such as intestinal IgA secretion were entirely normal. Preliminary studies in IL-18KO mice suggested that some immune responses may also have been dependent on the complimentary cytokine IL-18, although the pattern was less clear and the experiments need repeated and extended. The study in iNOSKO mice also suggests that some aspects of the OVA-specific response may be altered in these animals, but these experiments were also preliminary and need to be repeated and extended.

Together, these findings emphasise that the scope of action of ISCOMS may not be limited by the absence of many individual regulatory cytokines, perhaps because they have an ability to exploit redundant cytokines. To try and explore in more detail how these inflammatory mediators help to regulate the induction adaptive immune responses to ISCOMS, in the next chapter I will describe the innate immune responses which were induced in the different knockout mice and will indicate how these correlate with antigen-specific immunity in the same animals.

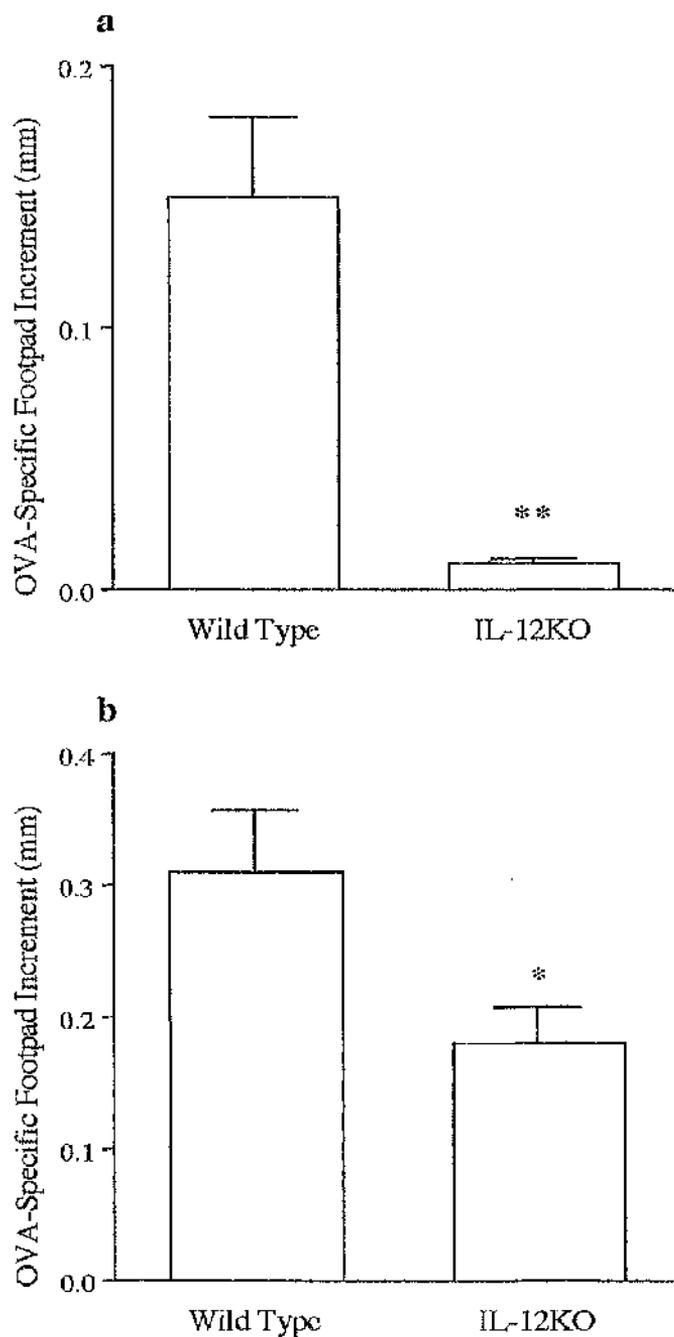


Figure 4.1 Antigen-Specific DTH in OVA ISCOMS Immunised IL-12 KO mice.

The graph shows OVA specific DTH responses measured 24 hours after subcutaneous challenge into the footpad with 100 μ g HAO. Mice were immunised with either (a) 5 μ g OVA ISCOMS in 50 μ l saline subcutaneously into the rear footpad or (b) six feeds of 100 μ g OVA ISCOMS in 200 μ l saline. The results shown are the mean specific increments in footpad thickness of 5 animals per group \pm 1 SD, measured 7 days after the last immunisation (** p <0.005, * p <0.05 verses wild type BALB/c mice). The background footpad increment obtained in unimmunised animals has been subtracted.

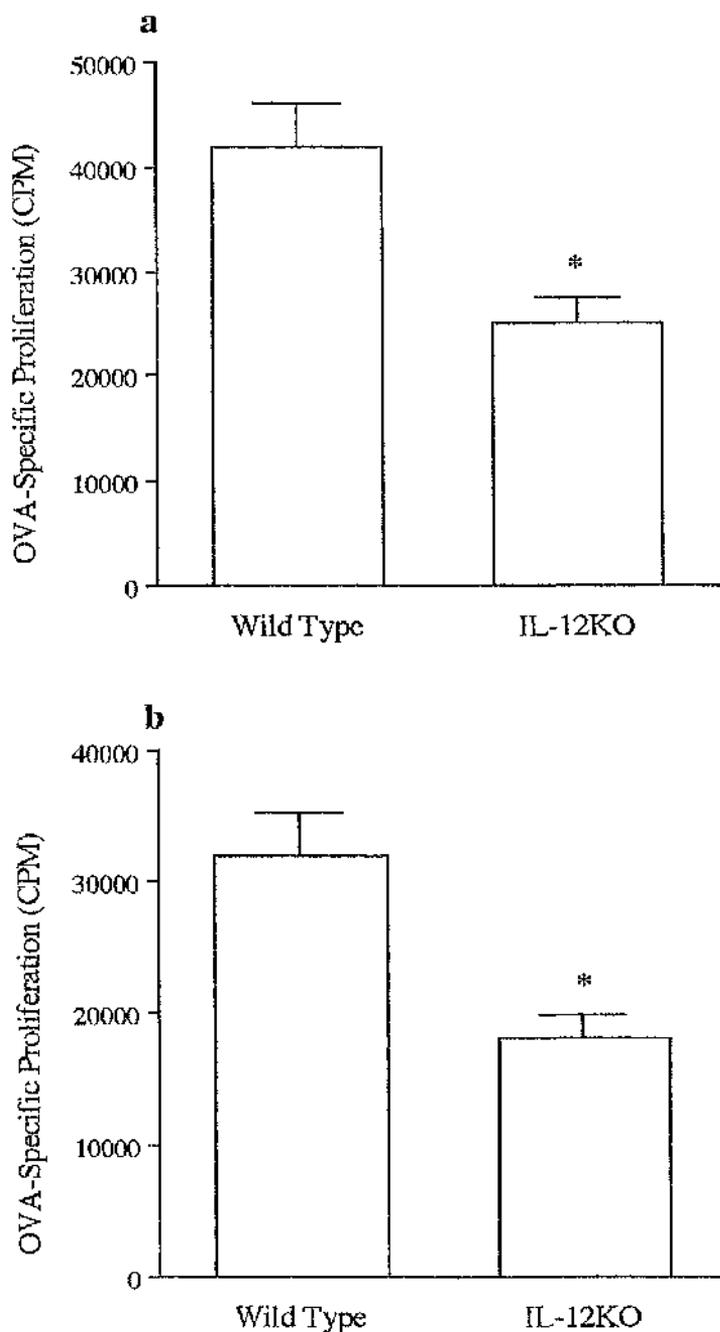


Figure 4.2 Antigen-Specific *in vitro* Proliferation in OVA-ISCOMS Immunised IL-12KO Mice.

OVA specific proliferative responses were measured in PLN removed (a) 14 days after a single subcutaneous immunisation in the rear footpad with 5 μ g OVA ISCOMS in 50 μ l saline or (b) 14 days after the last of 6 feeds of 100 μ g OVA ISCOMS in 200 μ l saline. The graph shows the mean OVA-specific proliferation following a 96 hour culture with 1mg/ml OVA as determined by incorporation of [3 H]thymidine into DNA after 16 hours incubation. Results are shown as mean counts of quadruplicate cultures of cells pooled from 5 animals \pm 1 SD (* p <0.05 verses wild type BALB/c mice). Background proliferation from control animals has been subtracted.

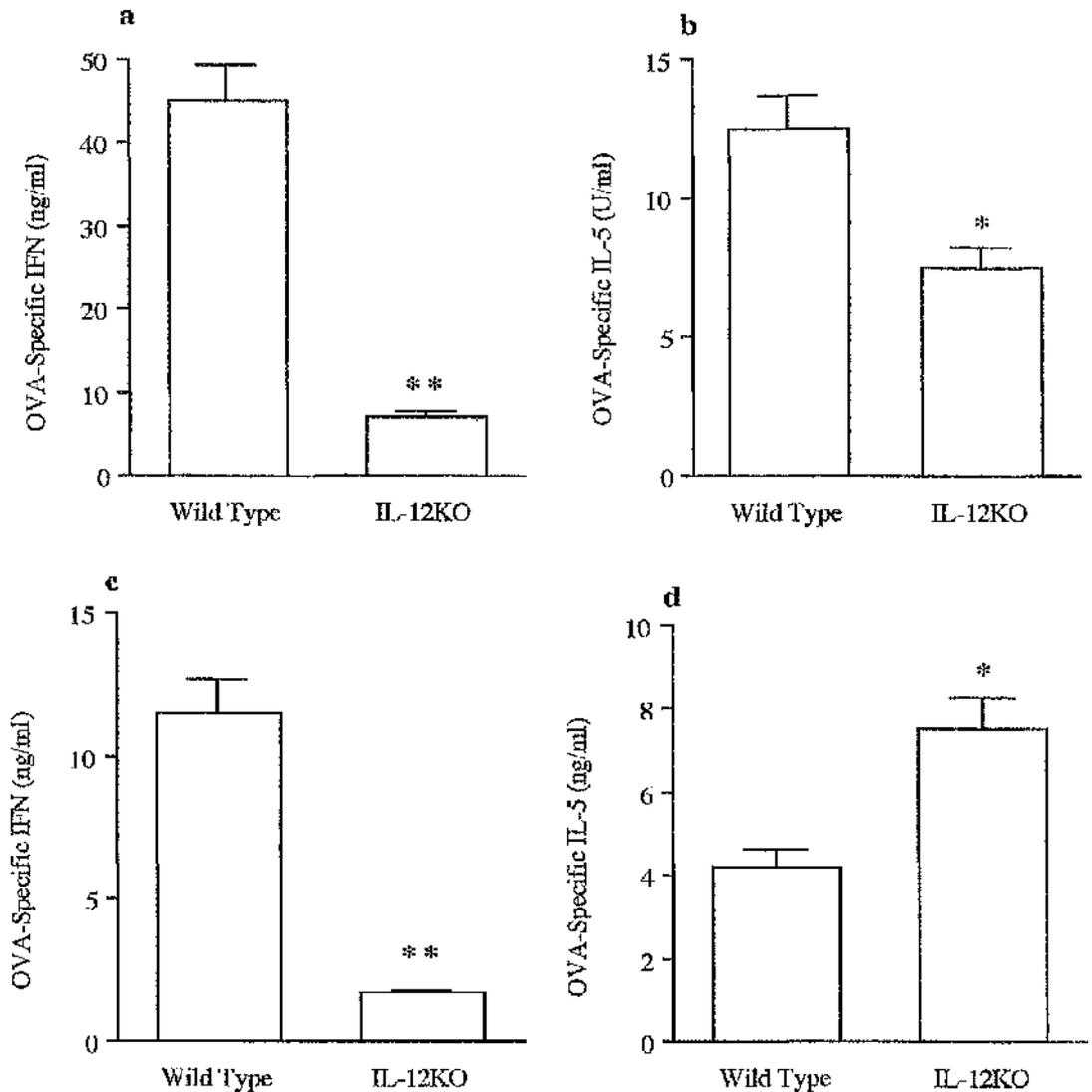


Figure 4.3 Antigen-Specific T Cell Cytokine Production in OVA ISCOMS Immunised IL-12KO Mice.

OVA-specific γ IFN (a) and IL-5 (b) production after s.c. immunisation with 5 μ g OVA ISCOMS. OVA-specific γ IFN (c) and IL-5 (d) production following six feeds of 100 μ g OVA ISCOMS. The results are shown as means \pm 1 SD of triplicate cultures using cells pooled from 5 mice per group, 14 days after the last immunisation. Background cytokine production from control animals has been subtracted. (* p <0.05, ** p <0.001 versus wild type BALB/c mice).

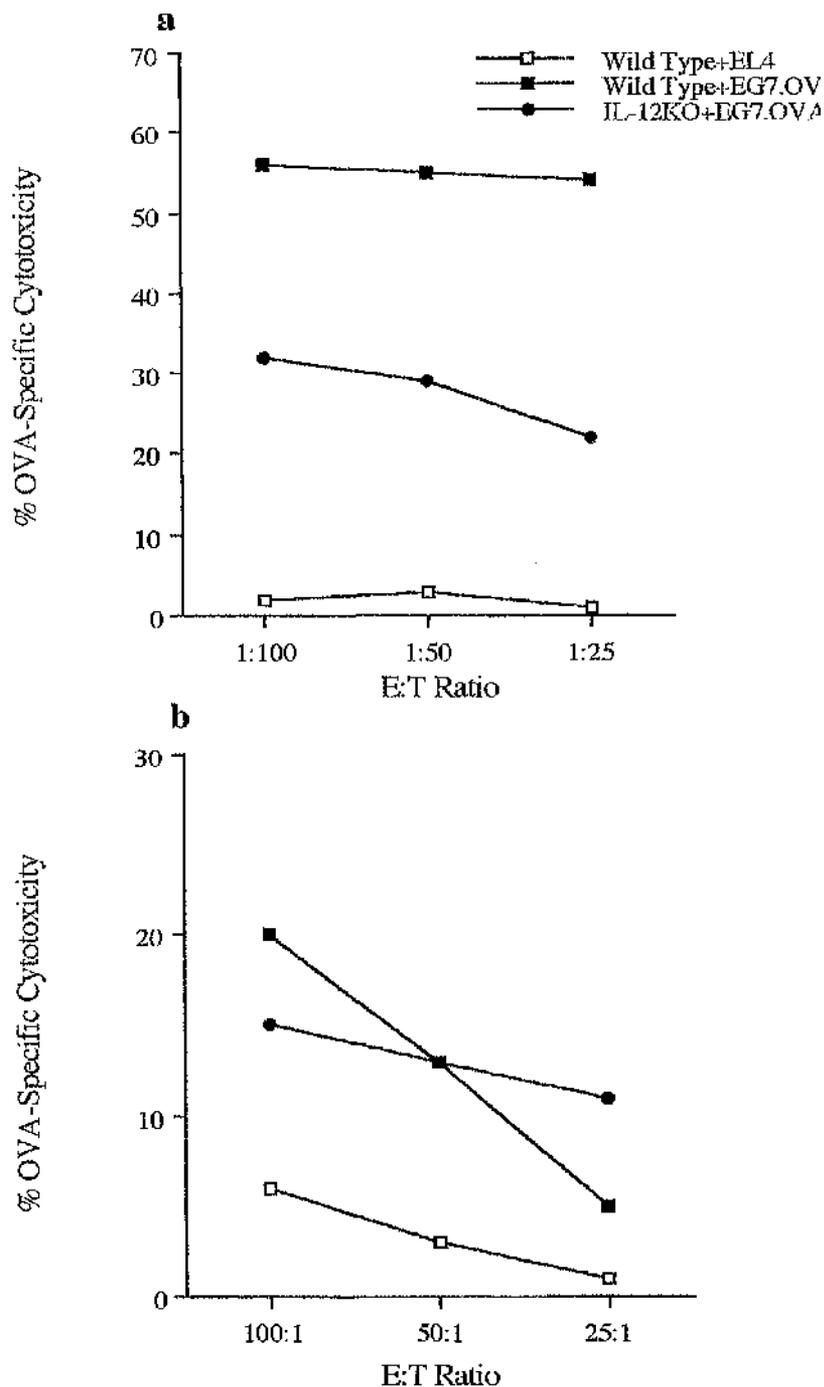


Figure 4.4 Antigen-Specific MHC Class I -Restricted CTL Activity in OVA ISCOMS immunised IL-12KO Mice.

OVA-specific CTL activity in spleens of IL-12KO or C57Bl/6 wild type mice, seven days after the last immunisation with either (a) a single i.p. immunisation of 5µg OVA ISCOMS in 200µl saline or (b) 14 days after the final of 6 feeds of 100µg OVA ISCOMS in 200µl saline. Spleens from 5 animals were pooled and 3×10^7 cells restimulated with 1.5×10^6 mitomycin c treated OVA expressing EG7.OVA cells. The results show the % cytotoxicity calculated from quadruplicate samples. Antigen-specificity was determined by the degree of lysis of non-OVA expressing EL4 cells.

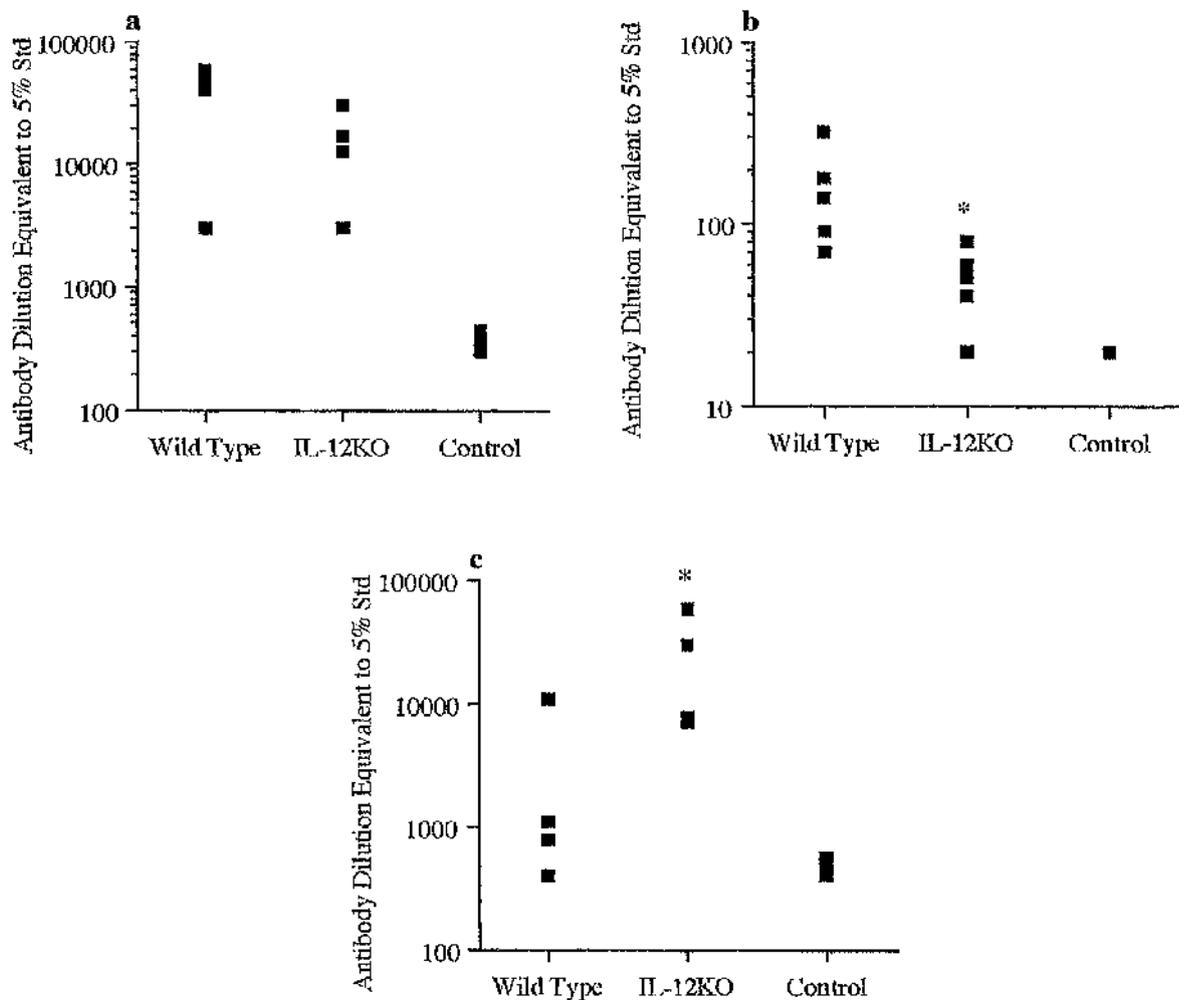


Figure 4.5 Antigen-Specific Serum IgG Isotype in OVA-ISCOMS Immunised IL-12KO Mice.

OVA-specific IgG1 (a and c) and IgG2a (b) antibody levels in serum, 7 days after s.c. challenge of HAO in mice immunised s.c. (a and b) or orally (c) with OVA ISCOMS. The results show the reciprocal dilutions of serum from individual mice which produced the equivalent O.D. 630nm to a 5% dilution of a standard anti-OVA serum (* $p < 0.05$ verses wild type mice). The control levels are from mice that received HAO only.

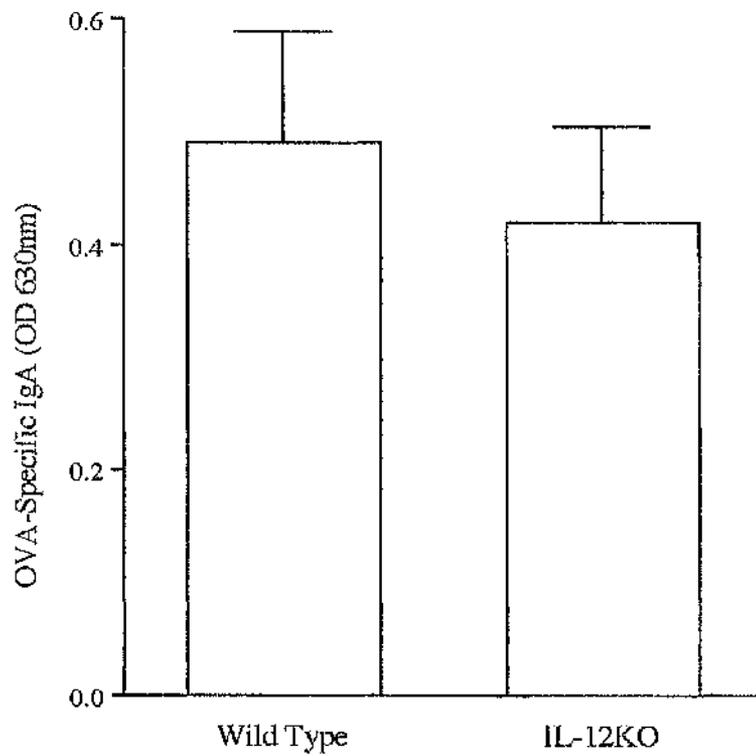


Figure 4.6 Antigen-Specific Intestinal IgA Production in OVA ISCOMS Immunised IL-12KO Mice.

IgA secretion from the small intestine was assessed in gut washes by ELISA seven days after the last six feeds of 100 μ g OVA ISCOMS. The results shown are the mean OD 405nm of triplicate samples from 5 mice \pm 1SD.

Background IgA production from control animals has been subtracted.

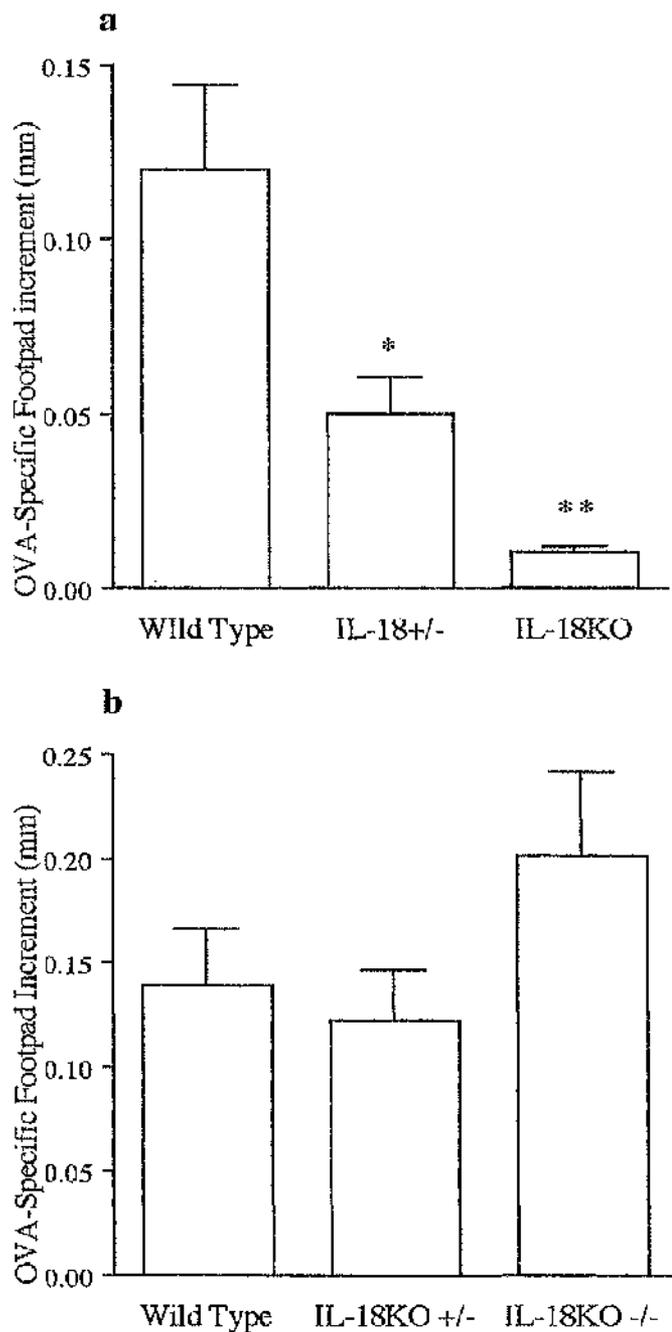


Figure 4.7 Antigen-Specific DTH in OVA ISCOMS Immunised IL-18KO mice.

The graph shows OVA specific DTH responses measured 24 hours after subcutaneous challenge into the footpad with 100µg HAO. Heterozygous (IL-18KO^{+/-}), homozygous (IL-18KO^{-/-}) or wild type mice were immunised with either (a) 5µg OVA ISCOMS in 50µl saline subcutaneously into the rear footpad or (b) six feeds of 100µg OVA ISCOMS in 200µl saline. The results shown are the mean specific increments in footpad thickness of 5 animals per group ± 1 SD, measured 7 days after the last immunisation (**p<0.001, * p<0.01 verses wild type mice). The background footpad increment obtained in unimmunised animals has been subtracted.

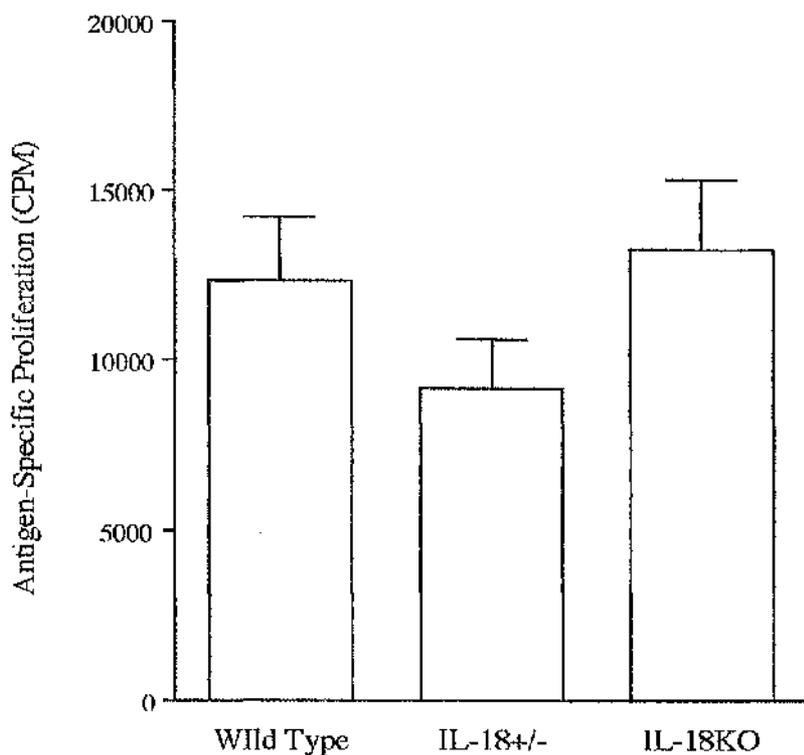


Figure 4.8 Antigen-Specific *in vitro* Proliferation in OVA-ISCOMS Immunised IL-18KO Mice.

OVA specific proliferative responses were measured in PLN removed from heterozygous (IL-18KO^{+/-}), homozygous (IL-18KO^{-/-}) or wild type mice 14 days after s.c. immunisation with 5µg OVA ISCOMS in 50µl saline. The graph shows the mean OVA-specific proliferation following a 96 hour culture with 1mg/ml OVA as determined by incorporation of [³H]thymidine into DNA after 16 hours incubation. Results are shown as mean counts of quadruplicate cultures of cells pooled from 5 animals ± 1 SD (*p<0.05 verses wild type mice). Background proliferation from control animals has been subtracted.

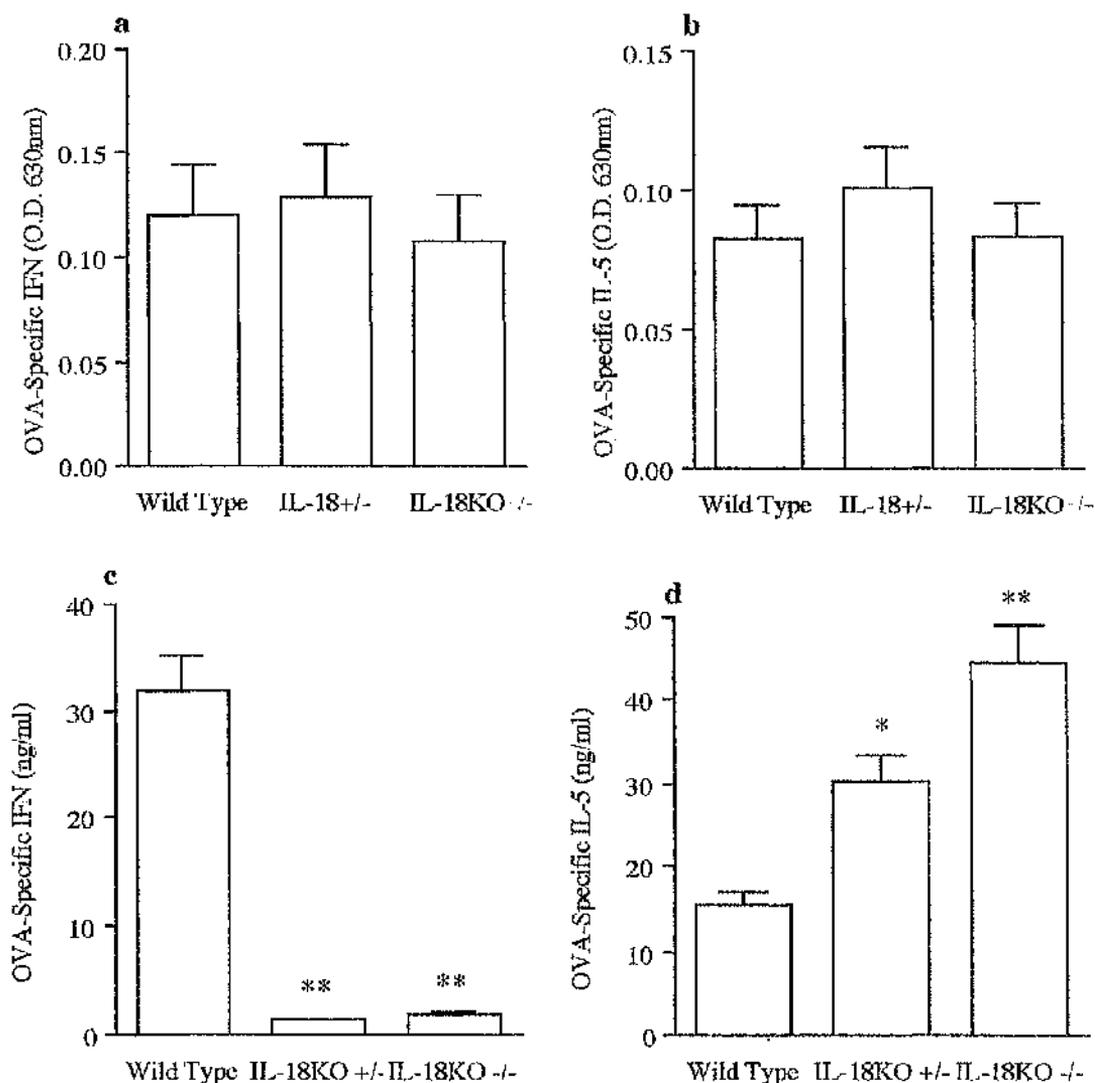


Figure 4.9 Antigen-Specific T Cell Cytokine Production in OVA ISCOMS Immunised IL-18KO Mice.

OVA-specific γ IFN (a) and IL-5 (b) production after s.c. immunisation with 5 μ g OVA ISCOMS. OVA-specific γ IFN (c) and IL-5 (d) production following six feeds of 100 μ g OVA ISCOMS. The results are shown as means \pm 1 SD of triplicate cultures using cells pooled from 5 mice per group, 14 days after the last immunisation. Background cytokine production from control animals has been subtracted. (* p <0.05, ** p <0.001 vs wild type mice).

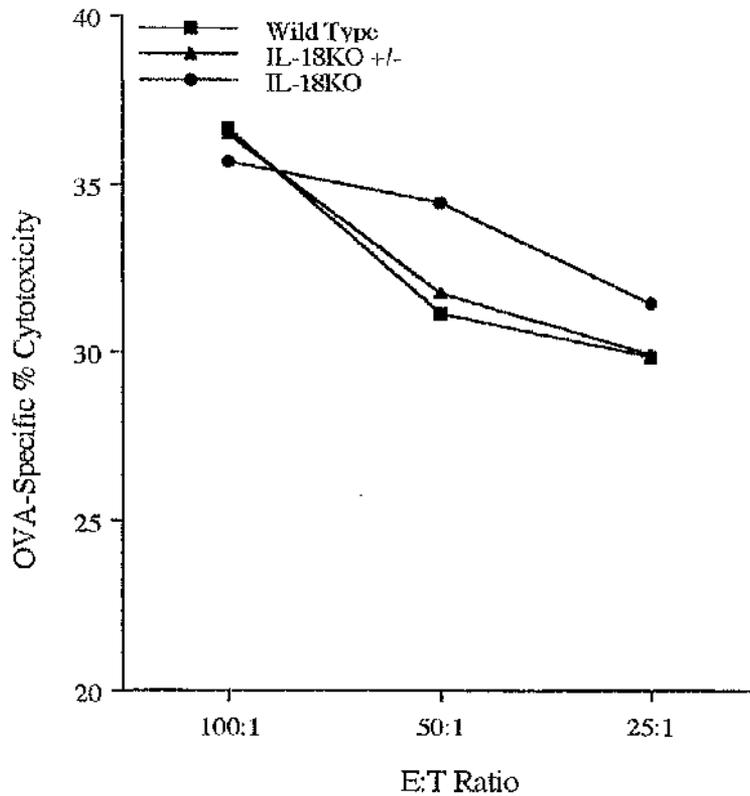


Figure 4.10 Antigen-Specific MHC Class I -Restricted CTL Activity in OVA ISCOMS immunised IL-18KO Mice.

OVA-specific CTL activity in spleens 14 days after the final of 6 feeds of 100µg OVA ISCOMS in 200µl saline. Spleens from 5 animals were pooled and 3×10^7 cells restimulated with 1.5×10^6 mitomycin c treated OVA expressing EG7.OVA cells. The results show the % cytotoxicity calculated from quadruplicate samples.

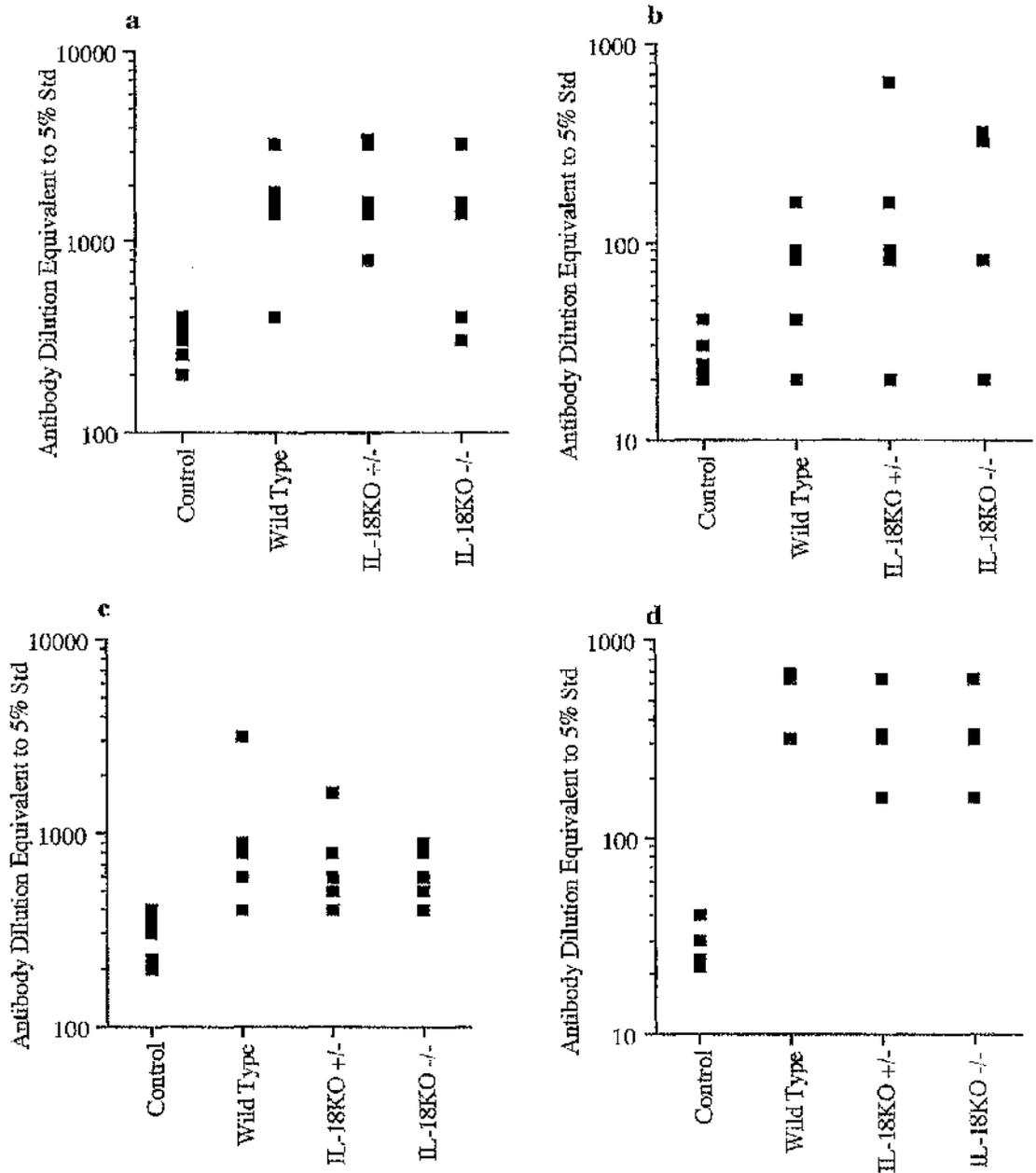


Figure 4.11 Antigen-Specific Serum IgG Isotype in OVA-ISCOMS Immunised IL-18KO Mice.

OVA-specific IgG1 (a and c) and IgG2a (b and d) antibody levels in serum, 7 days after s.c. challenge of HAO in mice immunised s.c. (a and b) or orally (c and d) with OVA ISCOMS. The results show the reciprocal dilutions of serum from individual mice which produced the equivalent O.D. 630nm to a 5% dilution of a standard anti-OVA serum. The control levels are from mice that received HAO only.

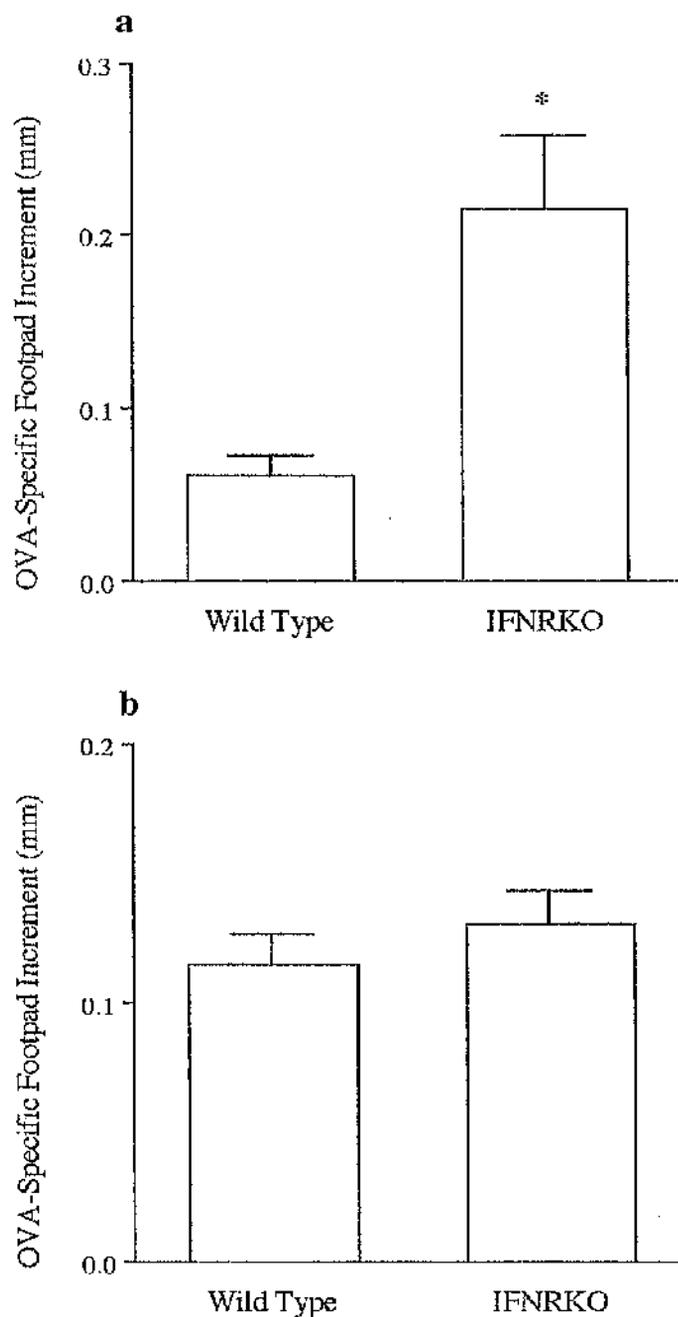


Figure 4.12 Antigen-Specific DTH in OVA ISCOMS Immunised γ IFNRKO mice.

The graph shows OVA specific DTH responses measured 24 hours after subcutaneous challenge into the footpad with 100 μ g HAO. Mice were immunised with either (a) 5 μ g OVA ISCOMS in 50 μ l saline subcutaneously into the rear footpad or (b) six feeds of 100 μ g OVA ISCOMS in 200 μ l saline. The results shown are the mean specific increments in footpad thickness of 5 animals per group \pm 1 SD, measured 7 days after the last immunisation (* p <0.01 versus wild type C57Bl/6 mice). The background footpad increment obtained in unimmunised animals has been subtracted.

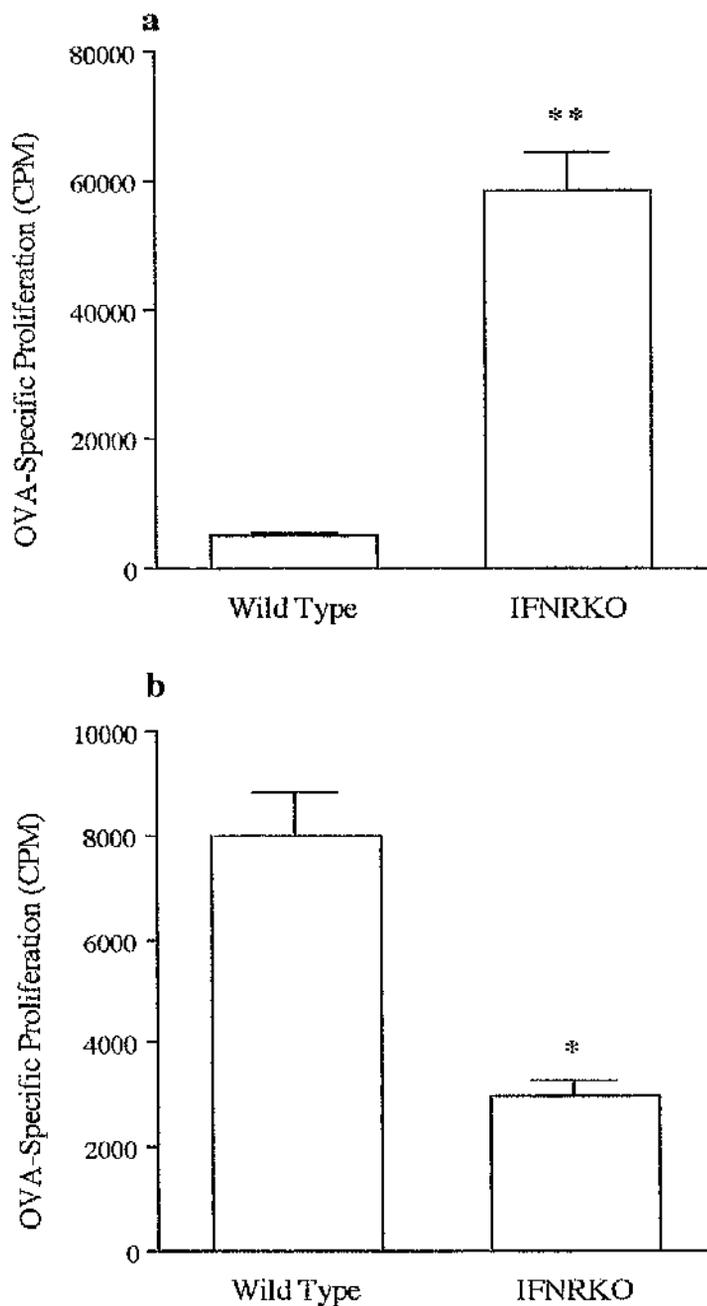


Figure 4.13 Antigen-Specific *in vitro* Proliferation in OVA-ISCOMS Immunised γ IFNRKO Mice.

OVA specific proliferative responses were measured in PLN removed (a) 14 days after a single subcutaneous immunisation in the rear footpad with 5 μ g OVA ISCOMS in 50 μ l saline or (b) 14 days after the last of 6 feeds of 100 μ g OVA ISCOMS in 200 μ l saline. The graph shows the mean OVA-specific proliferation following a 96 hour culture with 1mg/ml OVA as determined by incorporation of [3 H]thymidine into DNA after 16 hours incubation. Results are shown as mean counts of quadruplicate cultures of cells pooled from 5 animals \pm 1 SD (* p <0.05, ** p <0.001 verses wild type C57Bl/6 mice). Background proliferation from control animals has been subtracted.

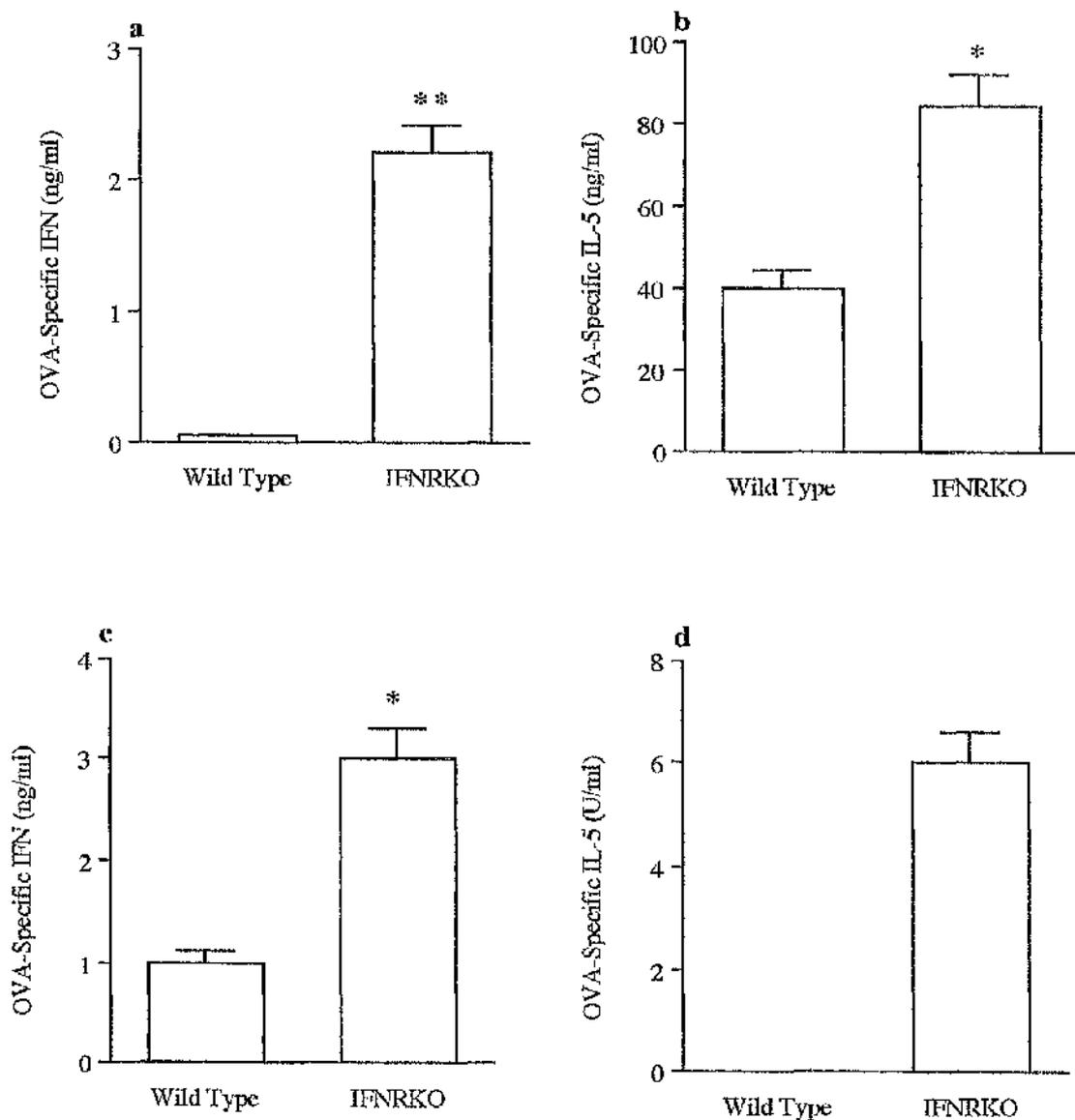


Figure 4.14 Antigen-Specific T Cell Cytokine Production in OVA ISCOMS Immunised γ IFN̳KO Mice.

OVA-specific γ IFN̳ (a) and IL-5 (b) production from PLN after s.c. immunisation with 5 μ g OVA ISCOMS, and OVA-specific γ IFN̳ (c) and IL-5 (d) production from spleen following six feeds of 100 μ g OVA ISCOMS. The results are shown as means \pm 1 SD of triplicate cultures using cells pooled from 5 mice per group, 14 days after the last immunisation. Background cytokine production from control animals has been subtracted. (*p<0.05, **p<0.005 verses wild type C57Bl/6 mice).

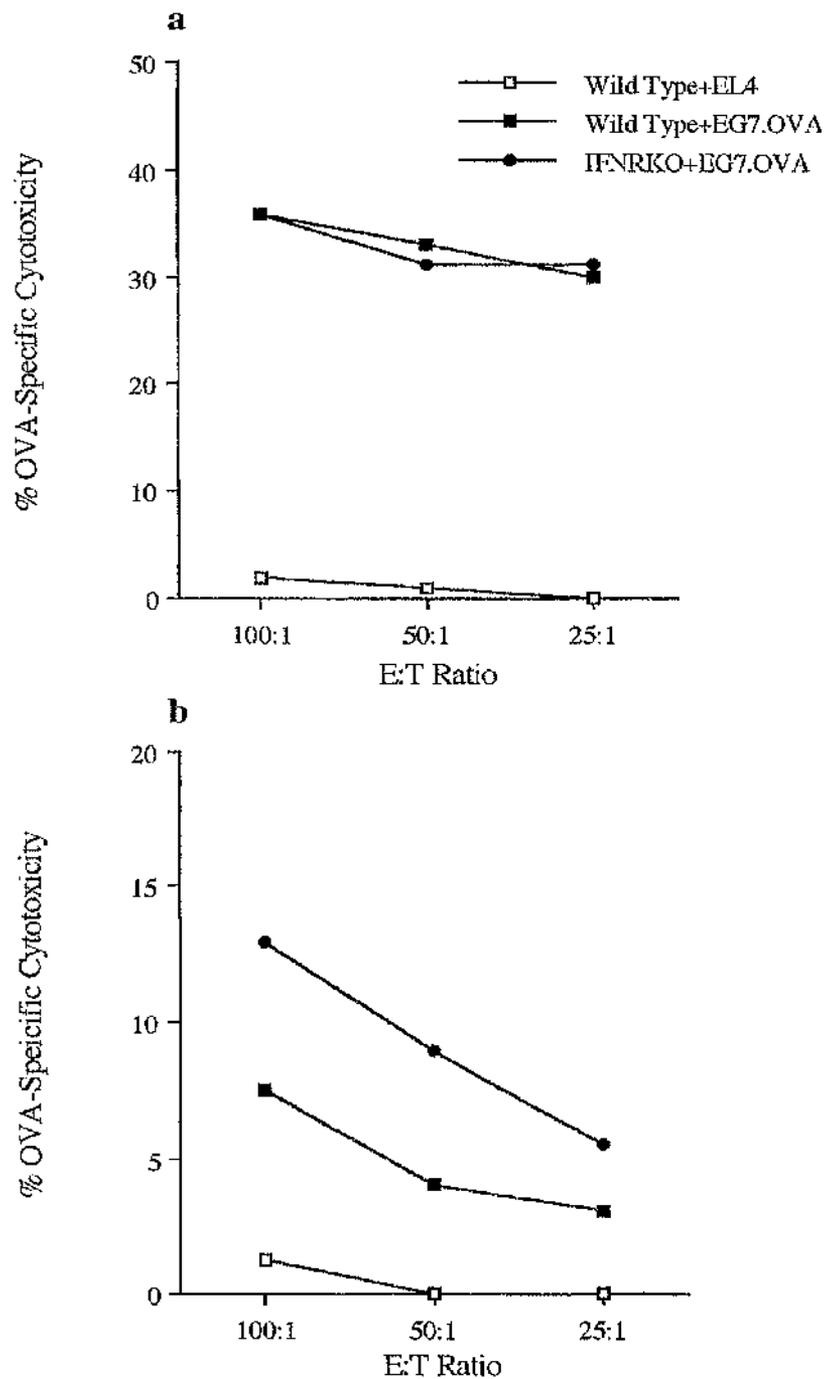


Figure 4.15 Antigen-Specific MHC Class I-Restricted CTL Activity in OVA ISCOMS immunised γ IFNRIKO Mice.

OVA-specific CTL activity in spleens seven days after the last immunisation with either (a) a single i.p. immunisation of 5 μ g OVA ISCOMS in 200 μ l saline or (b) 14 days after the final of 6 feeds of 100 μ g OVA ISCOMS in 200 μ l saline. Spleens from 5 animals were pooled and 3 x 10⁷ cells restimulated with 1.5 x 10⁶ mitomycin c treated OVA expressing EG7.OVA cells. The results show the % cytotoxicity calculated from quadruplicate samples. Antigen-specificity was determined by the degree of lysis of non-OVA expressing EL4 cells.

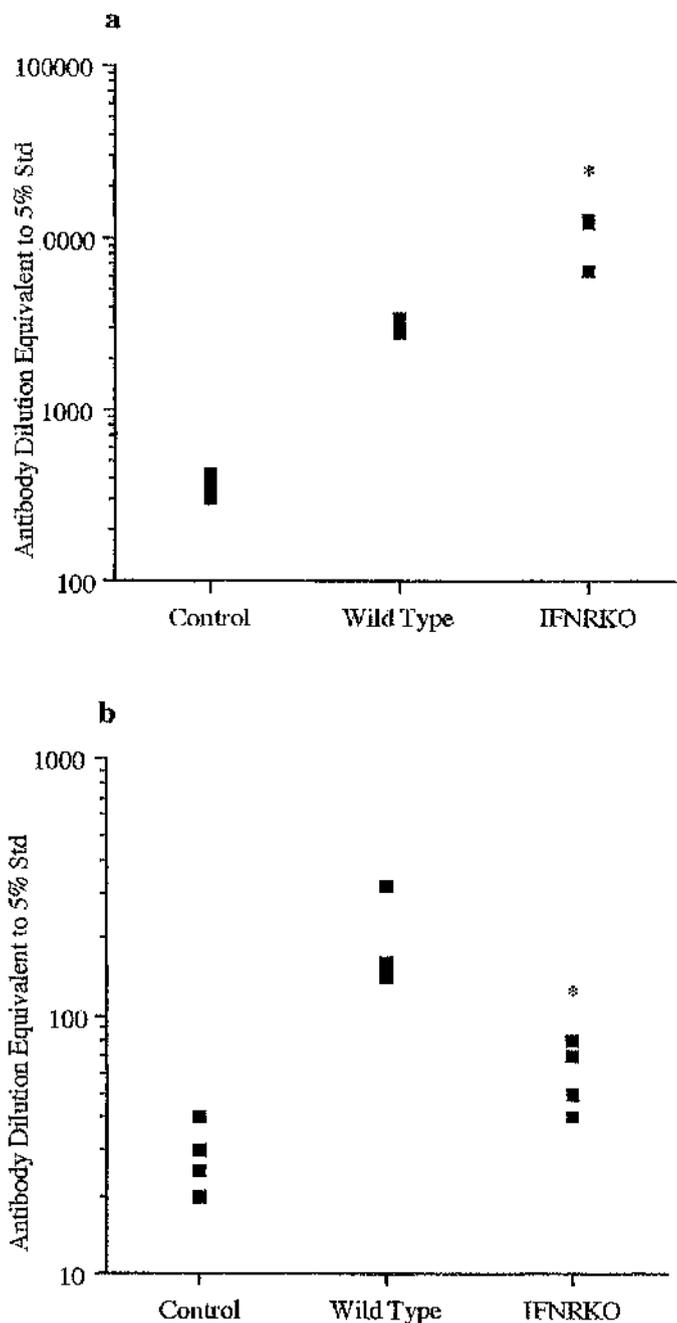


Figure 4.16 Antigen-Specific Serum IgG Isotype in OVA-ISCOMS Immunised γ IFNRKO Mice.

OVA-specific IgG1 (a) and IgG2a (b) antibody levels in serum, 7 days after s.c. challenge of HAO in mice immunised s.c with OVA ISCOMS. The results show the reciprocal dilutions of serum from individual mice which produced the equivalent O.D. 630nm to a 5% dilution of a standard anti-OVA serum ($p < 0.005$ versus wild type mice). The control levels are from mice that received HAO only.

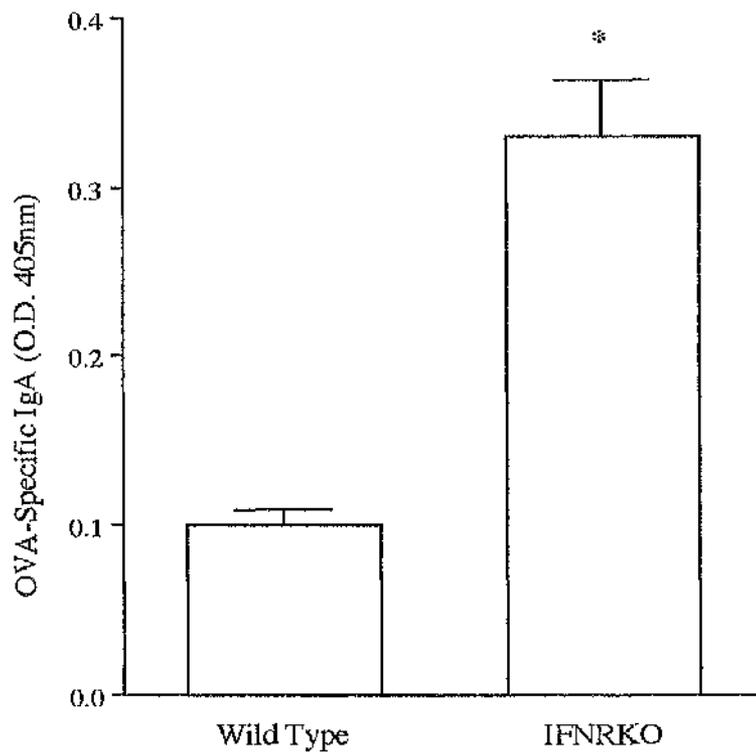


Figure 4.17 Antigen-Specific Intestinal IgA Production in OVA ISCOMS Immunised γ IFN̳RKO Mice.

IgA secretion from the small intestine was assessed in gut washes by ELISA seven days after the last six feeds of 100 μ g OVA ISCOMS. The results shown are the mean OD 405nm of triplicate samples from 5 mice \pm 1SD (* p <0.01 versus wild type C57Bl/6 mice). Background IgA production from control animals has been subtracted.

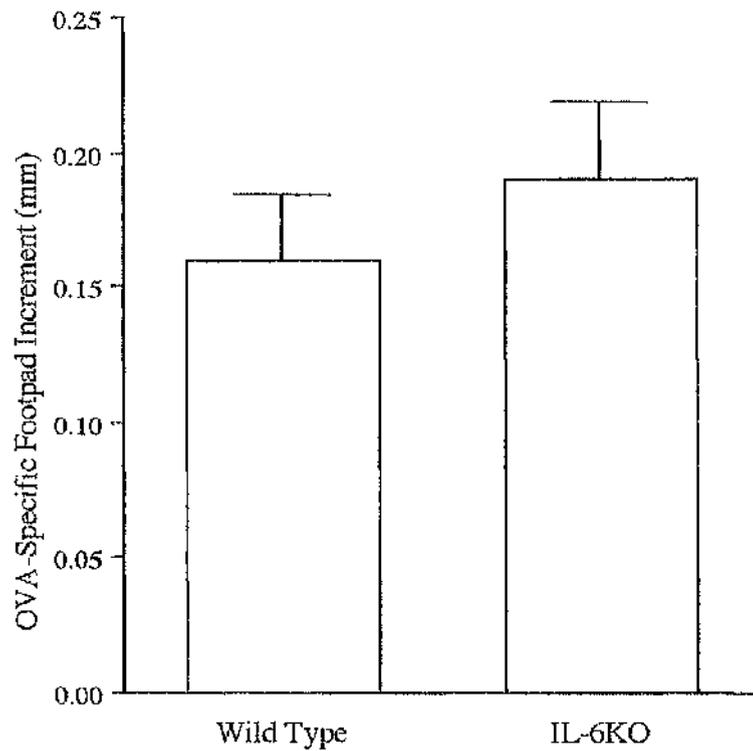


Figure 4.18 Antigen-Specific DTH in OVA ISCOMS Immunised IL-6KO mice.

The graph shows OVA specific DTH responses measured 24 hours after subcutaneous challenge into the footpad with 100 μ g HAO. Mice were immunised with 5 μ g OVA ISCOMS in 50 μ l saline subcutaneously into the rear footpad. The results shown are the mean specific increments in footpad thickness of 5 animals per group \pm 1 SD, measured 7 days after the last immunisation. The background footpad increment obtained in unimmunised animals has been subtracted.

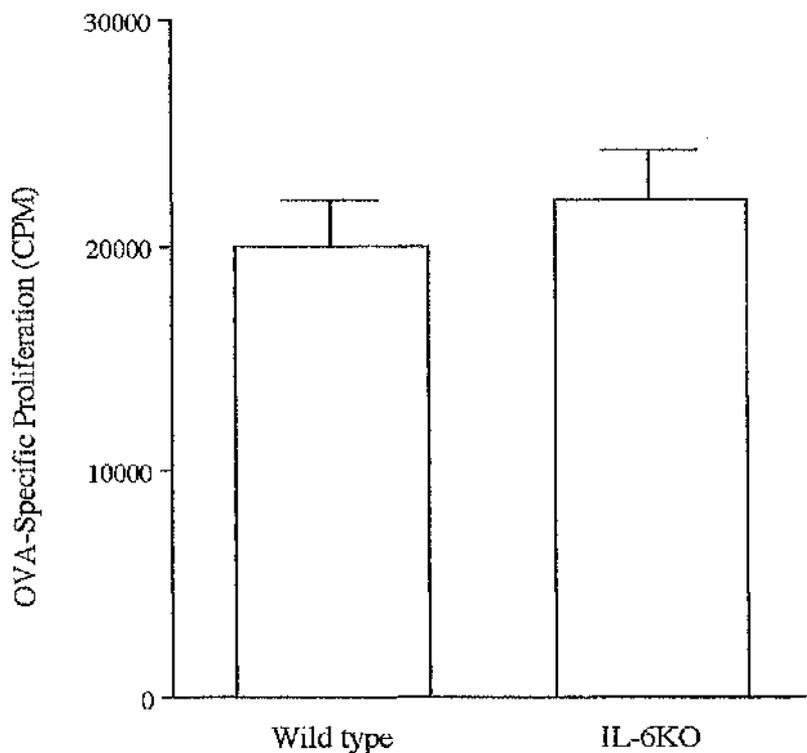


Figure 4.19 Antigen-Specific *in vitro* Proliferation in OVA-ISCOMS Immunised IL-6KO Mice.

OVA specific proliferative responses were measured in PLN removed 14 days after a single subcutaneous immunisation in the rear footpad with 5 μ g OVA ISCOMS in 50 μ l saline. The graph shows the mean OVA-specific proliferation following a 96 hour culture with 1mg/ml OVA as determined by incorporation of [3 H]thymidine into DNA after 16 hours incubation. Results are shown as mean counts of quadruplicate cultures of cells pooled from 5 animals \pm 1 SD. Background proliferation from control animals has been subtracted.

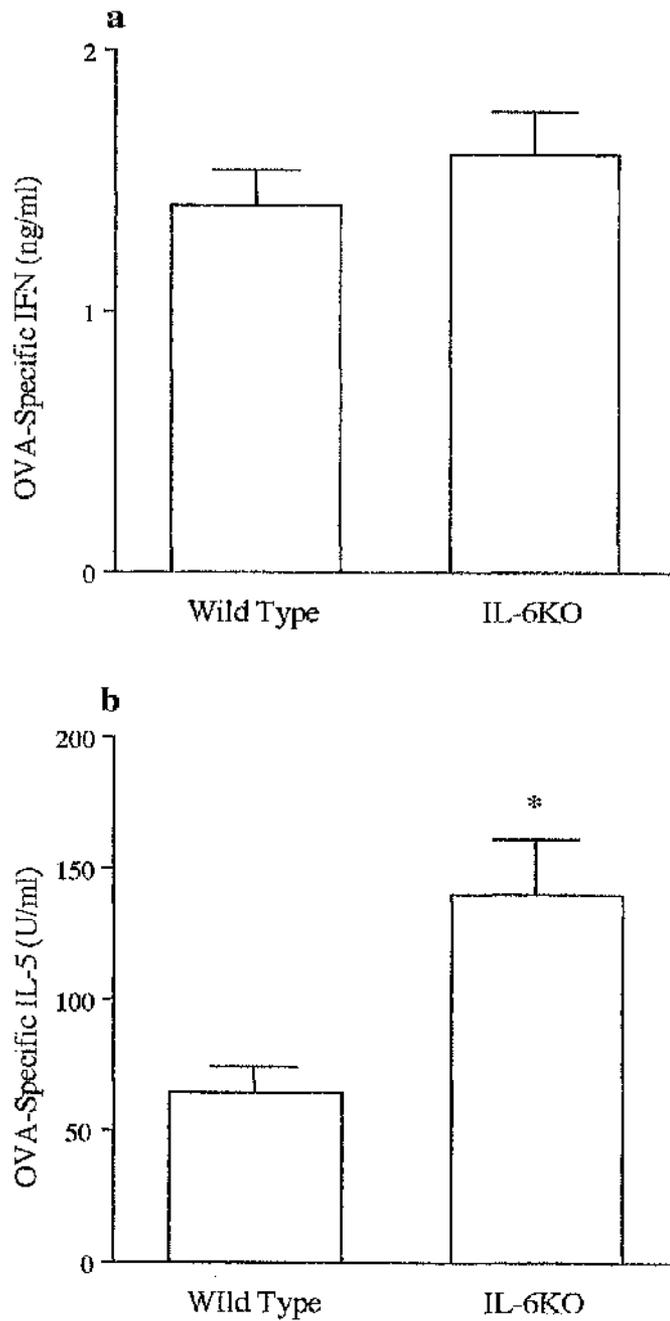


Figure 4.20 Antigen-Specific T Cell Cytokine Production in OVA ISCOMS Immunised IL-6KO Mice.

OVA-specific γ IFN (a) and IL-5 (b) production after s.c. immunisation with 5 μ g OVA ISCOMS. The results are shown as means \pm 1 SD of triplicate cultures using cells pooled from 5 mice per group, 14 days after the last immunisation. Background cytokine production from control animals has been subtracted. (*p<0.05 verses wild type 129/Sv mice).

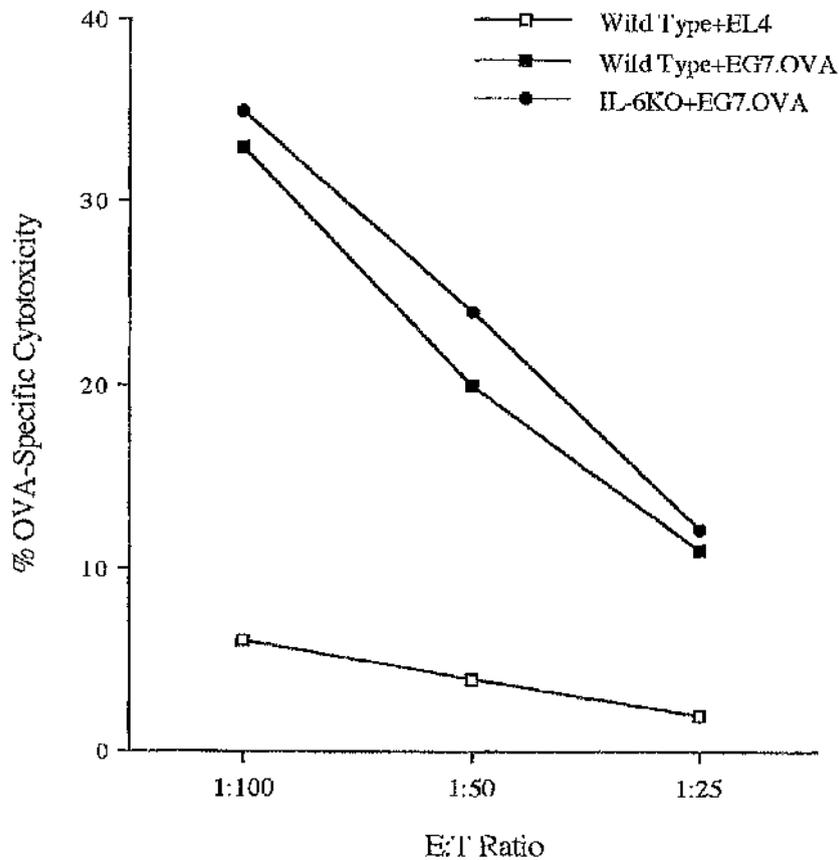


Figure 4.21 Antigen-Specific MHC Class I-Restricted CTL Activity in OVA ISCOMS immunised IL-6KO Mice.

OVA-specific CTL activity in spleens of IL-12KO or C57Bl/6 wild type mice, seven days after the last immunisation with a single i.p. immunisation of 5µg OVA ISCOMS in 200µl saline. Spleens from 5 animals were pooled and 3×10^7 cells restimulated with 1.5×10^6 mitomycin c treated OVA expressing EG7.OVA cells. The results show the % cytotoxicity calculated from quadruplicate samples. Antigen-specificity was determined by the degree of lysis of non-OVA expressing EL4 cells.

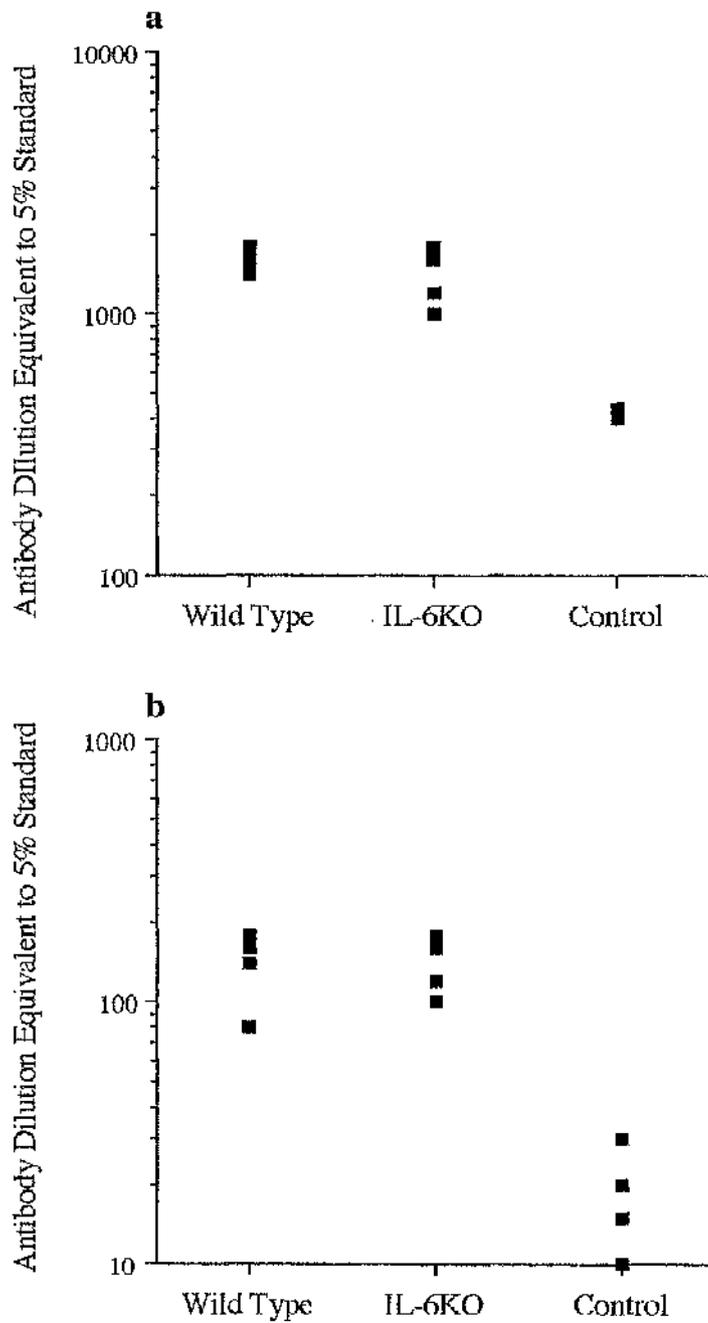


Figure 4.22 Antigen-Specific Serum IgG Isotype in OVA-ISCOMS Immunised IL-6KO Mice.

OVA-specific IgG1 (a) and IgG2a (b) antibody levels in serum, 7 days after s.c. challenge of HAO in mice immunised s.c. with OVA ISCOMS. The results show the reciprocal dilutions of serum from individual mice which produced the equivalent O.D. 630nm to a 5% dilution of a standard anti-OVA serum. The control levels are from mice that received HAO only.

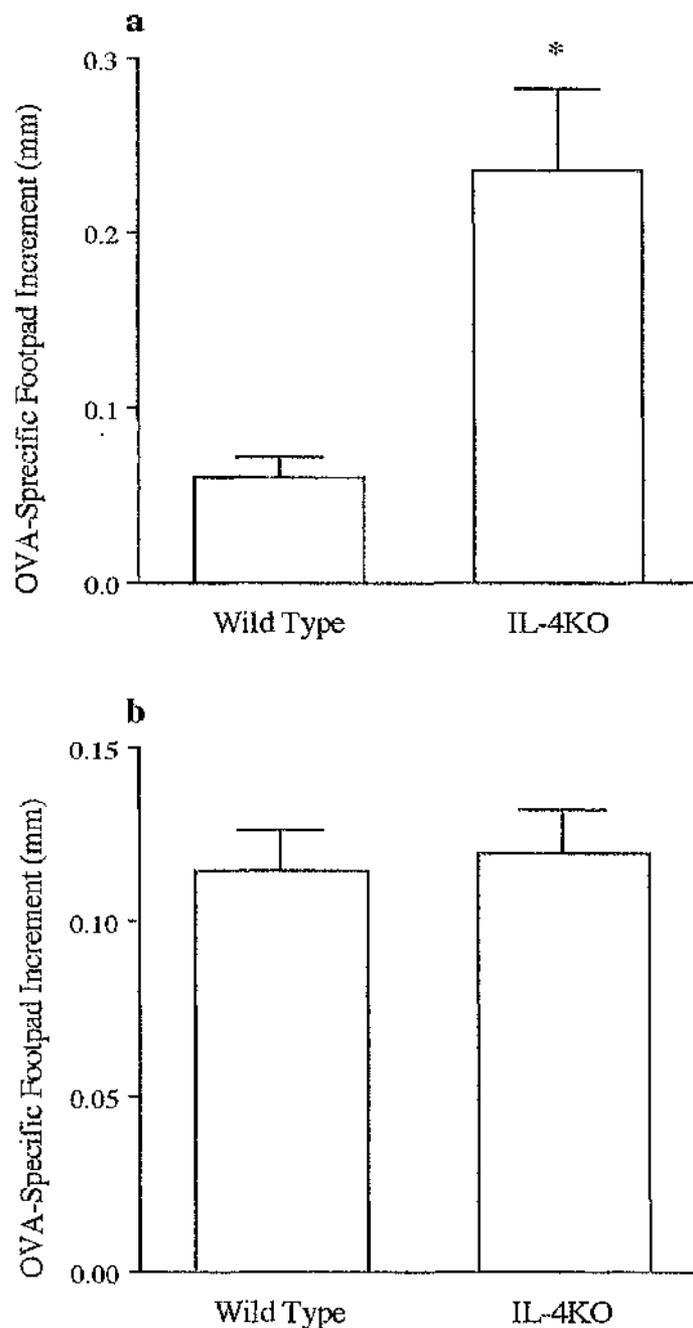


Figure 4.23 Antigen-Specific DTH in OVA ISCOMS Immunised IL-4KO mice.

The graph shows OVA specific DTH responses measured 24 hours after subcutaneous challenge into the footpad with 100 μ g HAO. Mice were immunised with either (a) 5 μ g OVA ISCOMS in 50 μ l saline subcutaneously into the rear footpad or (b) six feeds of 100 μ g OVA ISCOMS in 200 μ l saline. The results shown are the mean specific increments in footpad thickness of 5 animals per group \pm 1 SD, measured 7 days after the last immunisation (* p <0.05 verses wild type C57Bl/6 mice). The background footpad increment obtained in unimmunised animals has been subtracted.

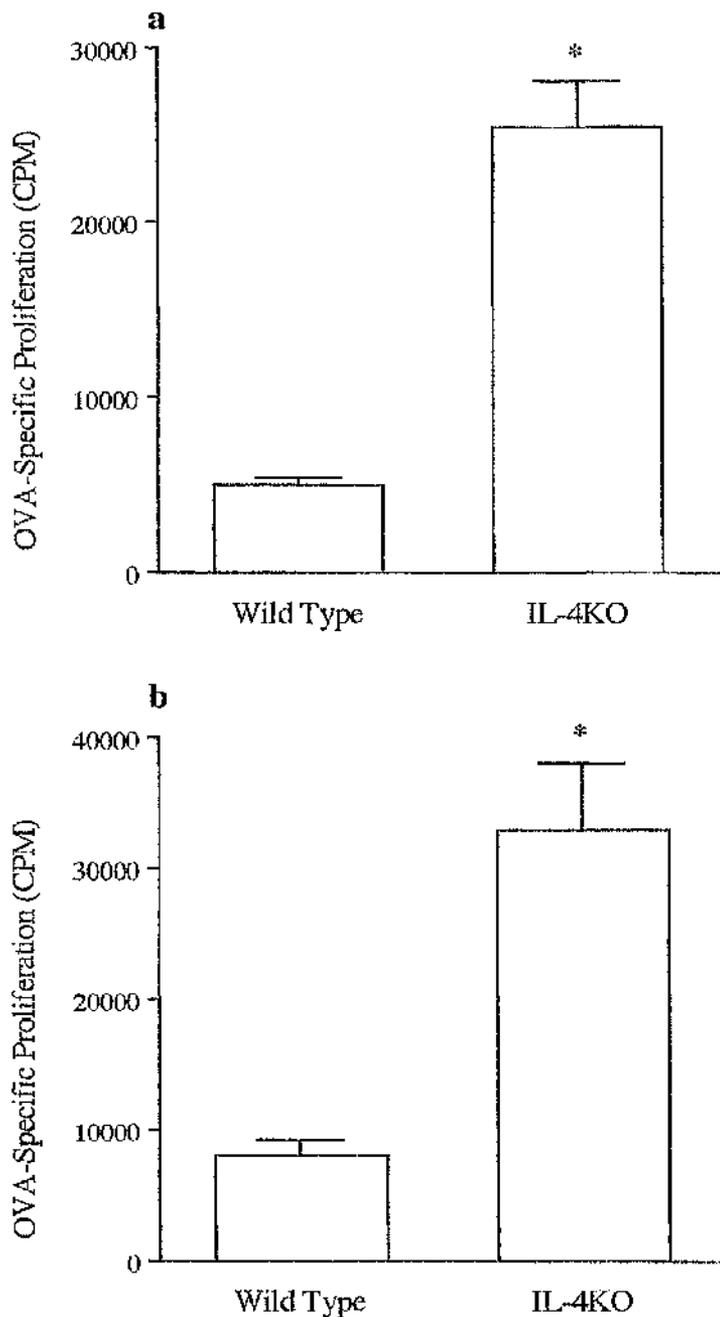


Figure 4.24 Antigen-Specific *in vitro* Proliferation in OVA-ISCOMS Immunised IL-4KO Mice.

OVA specific proliferative responses were measured in PLN removed (a) 14 days after a single subcutaneous immunisation in the rear footpad with 5 μ g OVA ISCOMS in 50 μ l saline or (b) 14 days after the last of 6 feeds of 100 μ g OVA ISCOMS in 200 μ l saline. The graph shows the mean OVA-specific proliferation following a 96 hour culture with 1mg/ml OVA as determined by incorporation of [3 H]thymidine into DNA after 16 hours incubation. Results are shown as mean counts of quadruplicate cultures of cells pooled from 5 animals \pm 1 SD (* p <0.005 verses wild type C57Bl/6 mice). Background proliferation from control animals has been subtracted.

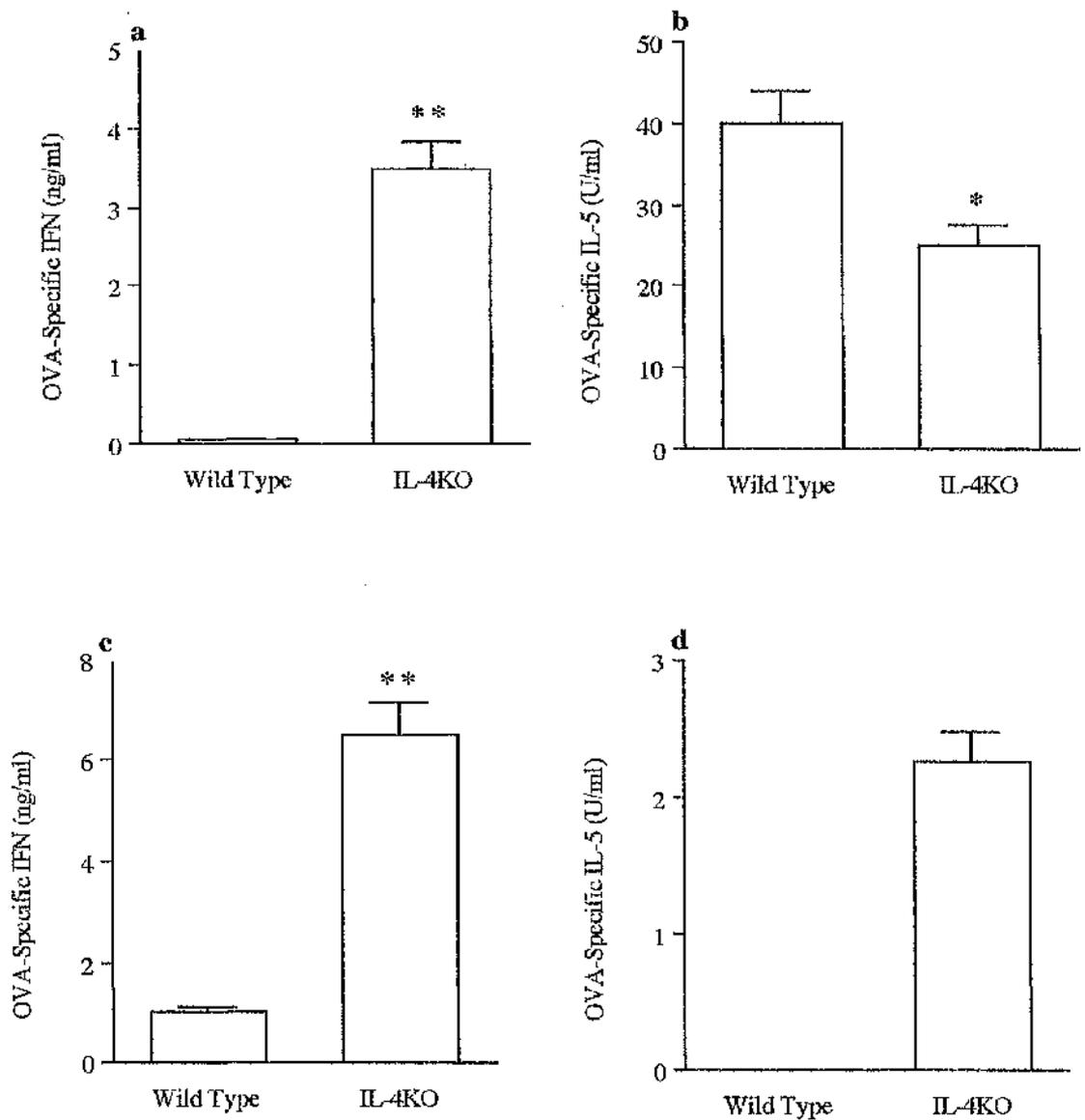


Figure 4.25 Antigen-Specific T Cell Cytokine Production in OVA ISCOMS Immunised IL-4KO Mice.

OVA-specific γ IFN (a) and IL-5 (b) production after s.c. immunisation with 5 μ g OVA ISCOMS. OVA-specific γ IFN (c) and IL-5 (d) production following six feeds of 100 μ g OVA ISCOMS. The results are shown as means \pm 1 SD of triplicate cultures using cells pooled from 5 mice per group, 14 days after the last immunisation. Background cytokine production from control animals has been subtracted. (* p <0.05, ** p <0.005 verses wild type C57Bl/6 mice).

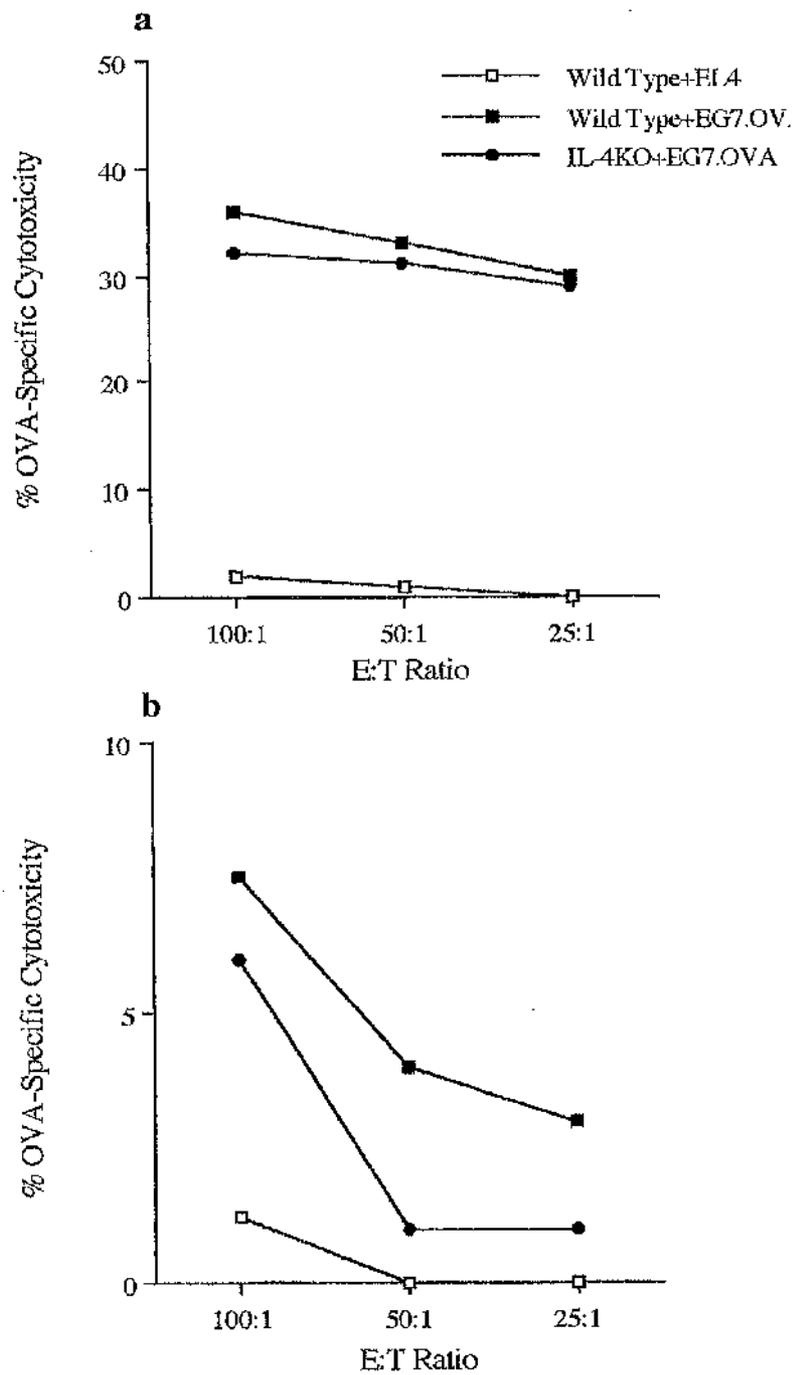


Figure 4.26 Antigen-Specific MHC Class I-Restricted CTL Activity in OVA ISCOMS immunised IL-4KO Mice.

OVA-specific CTL activity in spleens of IL-14KO or C57Bl/6 wild type mice, seven days after the last immunisation with either (a) a single i.p. immunisation of 5 μ g OVA ISCOMS in 200 μ l saline or (b) 14 days after the final of 6 feeds of 100 μ g OVA ISCOMS in 200 μ l saline. Spleens from 5 animals were pooled and 3 x 10⁷ cells restimulated with 1.5 x 10⁶ mitomycin c treated OVA expressing EG7.OVA cells. The results show the % cytotoxicity calculated from quadruplicate samples. Antigen-specificity was determined by the degree of lysis of non-OVA expressing EL4 cells.

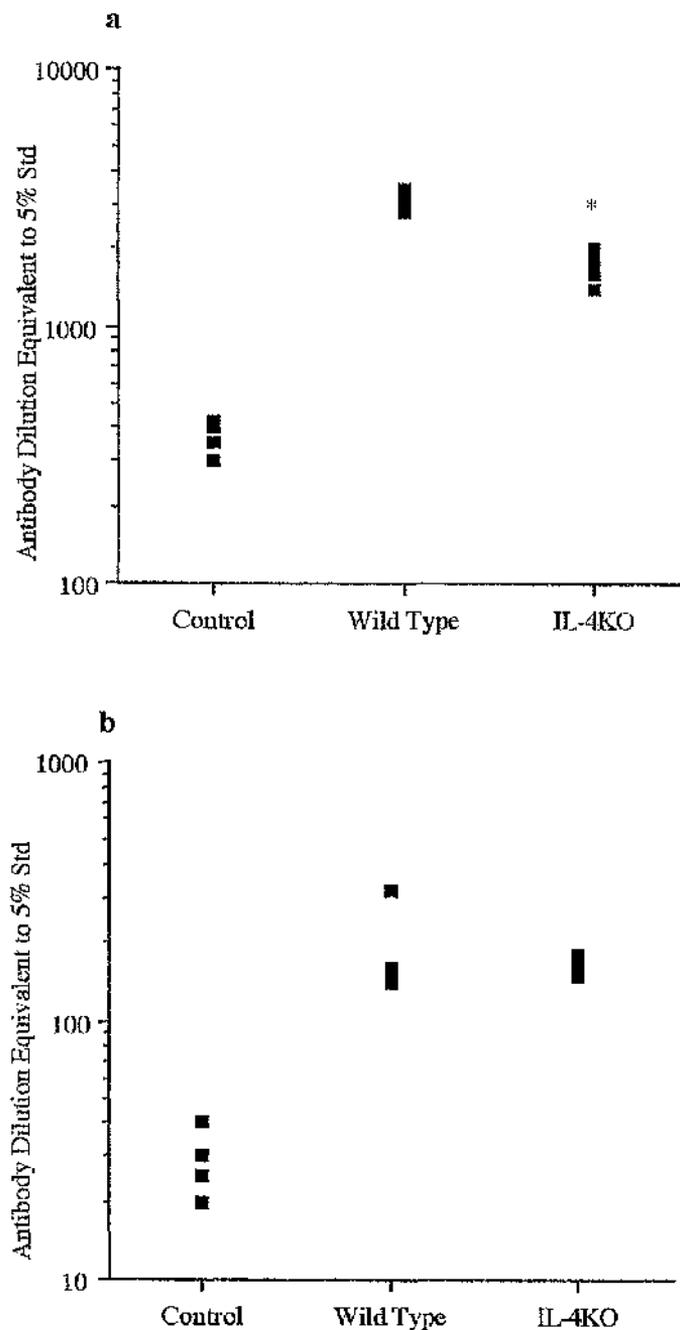


Figure 4.27 Antigen-Specific Serum IgG Isotype in OVA-ISCOMS Immunised IL-4KO Mice.

OVA-specific IgG1 (a) and IgG2a (b) antibody levels in serum, 7 days after s.c. challenge of HAO in mice immunised s.c. with OVA ISCOMS. The results show the reciprocal dilutions of serum from individual mice which produced the equivalent O.D. 630nm to a 5% dilution of a standard anti-OVA serum (* $p < 0.05$ versus wild type mice). The control levels are from mice that received HAO only.

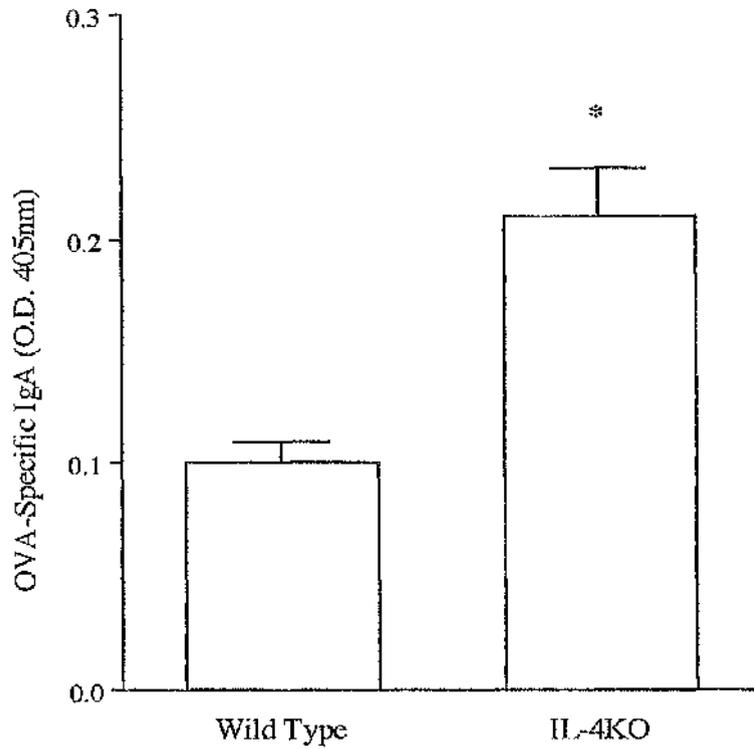


Figure 4.28 Antigen-Specific Intestinal IgA Production in OVA ISCOMS Immunised IL-4KO Mice.

IgA secretion from the small intestine was assessed in gut washes by ELISA seven days after the last six feeds of 100 μ g OVA ISCOMS. The results shown are the mean OD 405nm of triplicate samples from 5 mice \pm 1SD (* p <0.01 versus C57Bl/6 wild type mice). Background IgA production from control animals has been subtracted.

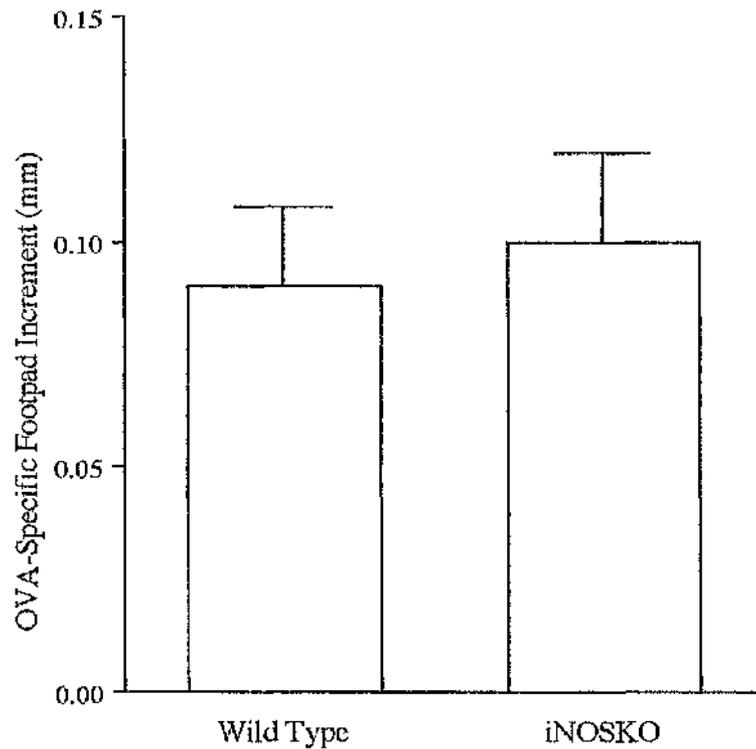


Figure 4.29 Antigen-Specific DTH in OVA ISCOMS Immunised iNOSKO mice.

The graph shows OVA specific DTH responses measured 24 hours after subcutaneous challenge into the footpad with 100 μ g HAO. Mice were immunised with 5 μ g OVA ISCOMS in 50 μ l saline subcutaneously into the rear footpad. The results shown are the mean specific increments in footpad thickness of 5 animals per group \pm 1 SD, measured 7 days after the last immunisation. The background footpad increment obtained in unimmunised animals has been subtracted.

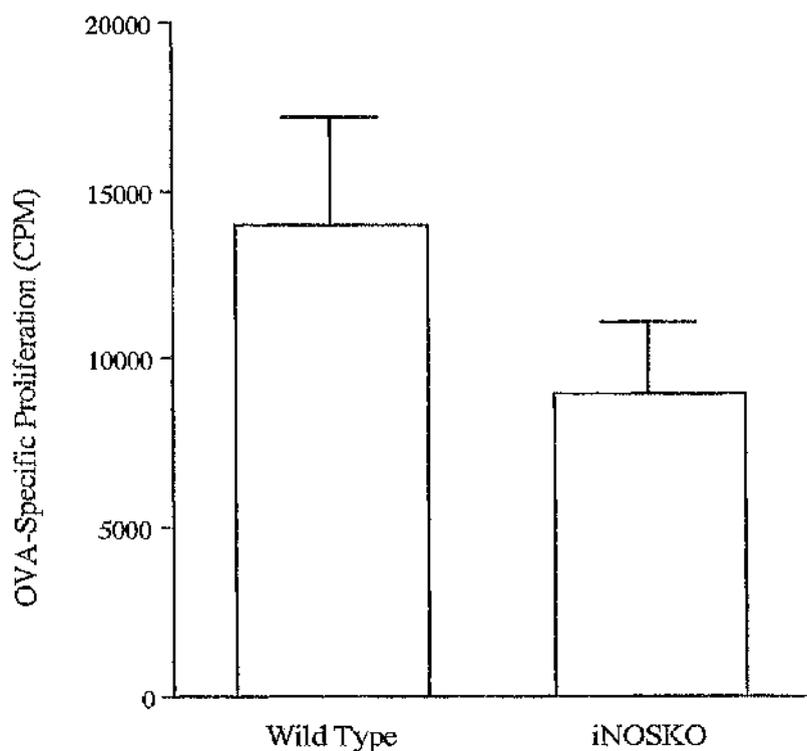


Figure 4.30 Antigen-Specific *in vitro* Proliferation in OVA-ISCOMS Immunised iNOSKO Mice.

OVA specific proliferative responses were measured in PLN removed 14 days after a single subcutaneous immunisation in the rear footpad with 5µg OVA ISCOMS in 50µl saline. The graph shows the mean OVA-specific proliferation following a 96 hour culture with 1mg/ml OVA as determined by incorporation of [³H]thymidine into DNA after 16 hours incubation. Results are shown as mean counts of quadruplicate cultures of cells pooled from 5 animals ± 1 SD. Background proliferation from control animals has been subtracted.

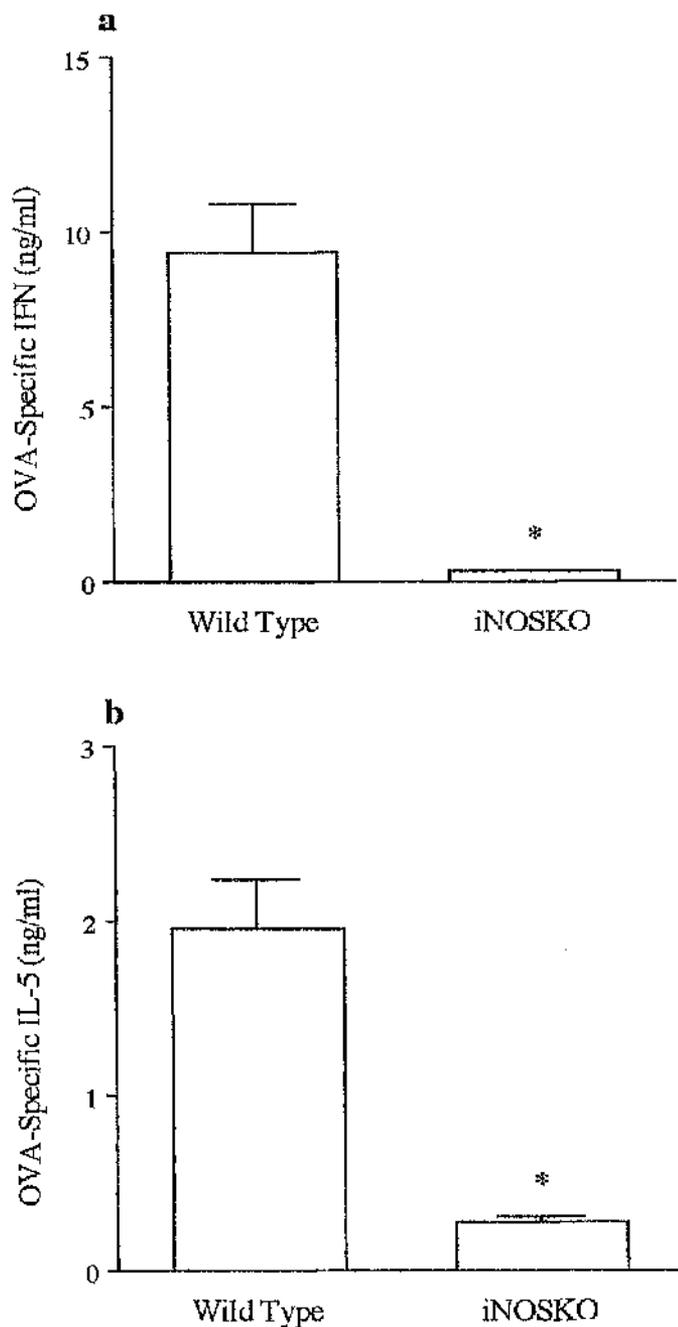


Figure 4.31 Antigen-Specific T Cell Cytokine Production in OVA ISCOMS Immunised iNOSKO Mice.

OVA-specific γ IFN (a) and IL-5 (b) production after s.c. immunisation with 5 μ g OVA ISCOMS. The results are shown as means \pm 1 SD of triplicate cultures using cells pooled from 5 mice per group, 14 days after the last immunisation. Background cytokine production from control animals has been subtracted. (* p <0.005 verses wild type MF1 mice).

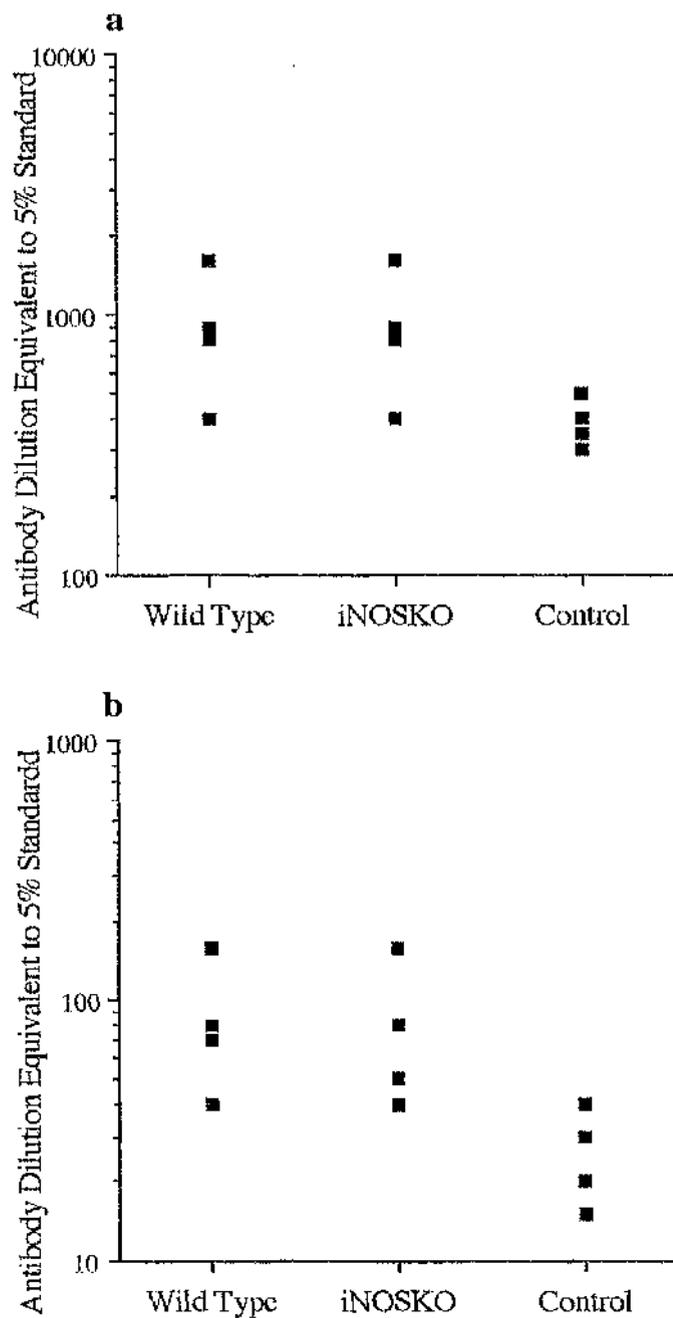


Figure 4.32 Antigen-Specific Serum IgG Isotype in OVA-ISCOMS Immunised iNOSKO Mice.

OVA-specific IgG1 (a) and IgG2a (b) antibody levels in serum, 7 days after s.c. challenge of HAO in mice immunised s.c. with OVA ISCOMS. The results show the reciprocal dilutions of serum from individual mice which produced the equivalent O.D. 630nm to a 5% dilution of a standard anti-OVA serum. The control levels are from mice that received HAO only.

Chapter 5

Role of Inflammatory Mediators Induced by ISCOMS in Regulating Innate Immune Responses.

Introduction

As I have already described in Chapter 3, parenteral or oral administration of ISCOMS induces a wide range of innate immune responses, including the recruitment and activation of inflammatory cells and the production of several inflammatory mediators. In Chapter 4 I determined, by utilising gene-targeted knockout mice, which of these mediators were important for the generation of an antigen-specific immune response to OVA ISCOMS. I found that although most of these factors were not essential for antigen-specific immunity to OVA ISCOMS, mice lacking functional IL-12 had abnormal immune responses when immunised with OVA ISCOMS. It is now generally accepted that the development of an acquired response is influenced by the initiation of innate cascades, and so in this chapter I have attempted to investigate if these defects in adaptive immunity were related to abnormalities in the activation of the different components of the early inflammatory response. To do this, I explored the inflammatory responses induced in cytokine deficient mice, using the model of PEC recruitment I described in Chapter 3.

Experimental Protocol

Gene targeted IL-12KO, IL-18KO, γ IFN α 1KO, IL-6KO and IL-4KO mice, together with wild type controls, were injected i.p. with a single dose of 5 μ g OVA ISCOMS in 200 μ l saline. At various times after injection of ISCOMS, PEC were removed from the animals by peritoneal lavage, and examined phenotypically by FACS. The production of cytokines and nitric oxide was measured after 48 hour culture *in vitro* in the presence or absence of various stimuli. In all experiments control mice were injected with saline.

Results

5.1 Innate Responses to ISCOMS in Interleukin 12 Deficient Mice

In Chapters 3 and 4, I have shown that IL-12 was not only induced as part of the inflammatory cascade, but was also vital for the generation of antigen-specific immunity to OVA-ISCOMS. As IL-12 is an early inflammatory factor which has been linked to the initiation of cellular recruitment through its regulation of the chemokine IL-8 [Nuame et al., 1993], I was interested to examine if the absence of this cytokine affected the innate inflammatory cascade, and if so, whether this could account for the importance of IL-12 in the generation of antigen-specific immunity.

5.1.1. ISCOMS Induced Inflammatory Infiltrate.

Wild type B6 mice produced the usual pattern of PEC recruitment following i.p. injection of ISCOMS. Initially, PEC numbers were reduced, with the minimum occurring at 7 hours after administration of ISCOMS. However PEC numbers then began to increase to a peak at 48 hours by which time they were 1.5 times greater than in uninjected mice. PEC numbers were beginning to return to background levels by 72 hours (Figure 5.4.1). In contrast, IL-12KO mice showed an early spike in the numbers of PEC after administration of ISCOMS, which then rapidly declined below that of the control saline injected animals and remained so until 72 hours, when PEC numbers returned to normal (Figure 5.1).

5.1.2. Phenotypic Analysis of PEC

As I found in earlier experiments, the number of neutrophils in PEC increased dramatically in wild type B6 mice soon after injection of ISCOMS, with peak levels being seen at 3.5 hours. Neutrophil numbers then returned to control levels (Figure 5.2a). IL-12KO mice also showed an immediate increase in the numbers of neutrophils after injection of

ISCOMS, but the increase was only 20% of that in wild type mice and the peak numbers were recorded later, at 7 hours. At all other times, no neutrophils could be found in IL-12KO mice (Figure 5.2a).

The number of F4/80⁺ macrophages also increased after an injection of ISCOMS into wild type mice, with maximal levels being seen at 48 hours, returning towards control levels by 72 hours (Figure 5.2b). In contrast, IL-12KO mice had higher baseline numbers of F4/80⁺ macrophages before injection and these cells were almost completely lost within 3.5 hours of administration of ISCOMS. Thereafter, the levels of F4/80⁺ cells remained very much lower than in control mice for the duration of the experiment (Figure 5.2b).

Wild type animals showed a steady increase in the numbers of activated F4/80⁺ macrophages from a baseline of zero, as indicated by the expression of the MHC class II molecule. Peak levels of activated macrophages were observed at 48 hours, when 25% of all macrophages were MHC class II⁺ (Figure 5.2c). In addition to the failure to recruit macrophages, IL-12KO mice also did not show this pattern of activation. Class II MHC⁺ macrophages were present in uninjected mice, but then there was a marked drop in their numbers with no recovery thereafter (Figure 5.2c).

The numbers of dendritic cells in wild type B6 mice as determined by expression of CD11c, were low in control PEC, but after a further drop at early timepoints after injection of ISCOMS, these made a gradual return to baseline levels by 72 hours (Figure 5.2d). IL-12KO mice had a much higher level of baseline CD11c⁺ dendritic cells compared with wild type mice and these increased numbers were present throughout the experiment. However, the numbers of CD11c⁺ dendritic cells from IL-12KO mice fell dramatically within 3.5 hours after injection of ISCOMS and although their numbers began to increase at 48 hours, they were still below baseline numbers and even fell again slightly at 72 hours (Figure 5.2d).

Wild type mice showed an immediate reduction in the number of CD4⁺ lymphocytes in PEC after injection of ISCOMS, before returning towards control levels by 48 hours. IL-12KO mice had slightly higher numbers of CD4⁺ T cells before injection of ISCOMS, but these fell rapidly after injection of ISCOMS and there was no subsequent recovery in their numbers at later times (Figure 5.3a).

Although there was no dramatic increase in CD4⁺ T cells in wild type B6 mice after injection of ISCOMS, these cells were becoming activated as shown by their expression of CD25 and by 48 hours, 26% of CD4⁺ T cells were CD25⁺ (Figure 5.3b). In contrast, IL-12KO mice did not show any increase in the expression of this activation marker and in fact, the levels of CD25⁺ CD4 cells were lower than those in the uninjected controls throughout the experiment (Figure 5.3b).

A small decrease in the number of CD8⁺ lymphocytes was also noted immediately after administration of ISCOMS to wild type animals, but these began to recover rapidly and by 72 hours, CD8 cell numbers were more than twice those of uninjected controls (Figure 5.3c). IL-12KO mice showed the same immediate loss of CD8⁺ cell numbers, but these did not recover above baseline levels during the experiment (Figure 5.3c). It was not possible to determine the activation state of these cells, as numbers were so low.

The numbers of B220⁺ B cells in wild type B6 mice showed a rapid early decrease, followed by recovery to levels which were eventually double that of control values at 48 hours after administration of ISCOMS (Figure 5.3d). Although IL-12 KO mice had baseline numbers of B cells that were greater than those in wild type mice, these fell constantly after injection of ISCOMS and remained suppressed below that of the control animals throughout the experiment (Figure 5.3d).

5.1.3. Inflammatory Cytokine Production

In parallel with the numbers of activated macrophages, PEC from saline injected wild type mice produced little or no γ IFN+LPS stimulated IL-1 α and this remained the case until 48 hours after administration of ISCOMS, when IL-1 α secretion was nearly eight times greater than baseline levels, before falling back to zero (Figure 5.4a). Consistent with the presence of class II MHC⁺ macrophages, IL-12KO mice had a slightly higher basal level of IL-1 α production, but this was quickly reduced to below control levels after injection of ISCOMS and remained so until 72 hours (Figure 5.4a).

There was some basal γ IFN+LPS stimulated IL-6 production by PEC removed from saline injected wild type mice. However, this was reduced almost to zero within 3.5 hours of injection of ISCOMS, remaining very low until 48 hours, when IL-6 production was nearly double that of control mice and then returned towards normal levels by 72 hours (Figure 5.4b). PEC from IL-12KO mice produced nearly double the background IL-6 levels compared with wild type PEC, but this too was reduced to extremely low levels within 3.5 hours of injection of ISCOMS. Unlike wild type mice, this low level of IL-6 production then persisted throughout the experiment (Figure 5.4b).

PEC from wild type B6 mice injected with ISCOMS produced two peaks of γ IFN production when restimulated with Con A *in vitro*, both of which were approximately 6 times greater than that in uninjected mice. The first peak was at 3.5 hours and the second at 48 hours, but γ IFN production had returned to control levels by 72 hours (Figure 5.4c). PEC taken from IL-12KO mice and cultured *in vitro* with Con A showed a similar level of basal γ IFN production, but immediately after administration of ISCOMS, this was reduced by half, before returning to control levels by 24 hours. γ IFN production then remained at this level until 72 hours (Figure 5.4c).

As anticipated, no IL-12 was measurable in IL-12KO mice, although wild type B 6 mice showed the characteristic early decrease followed by an increase to a peak in IL-12 production by 72 hours (data not shown).

5.1.4. Secretion of Nitric Oxide

PEC from saline injected wild type mice secreted high levels of NO after stimulation *in vitro* with γ IFN+LPS. This was reduced by 80% within 3.5 hours of injection of ISCOMS. The depressed level of NO production was maintained until 48 hours, when levels rose to 1.5 times of those in control mice, before returning to normal by 72 hours (Figure 5.5). PEC from IL-12KO mice produced a similar level of baseline NO production as wild types. This also decreased soon after administration of ISCOMS, but this suppressed level persisted for most of the experiment, only returning to normal levels by 72 hours (Figure 5.5).

5.1.5. Summary

Thus, the absence of IL-12 severely inhibits the generation of the inflammatory cascade induced by injection of ISCOMS. Apart from some neutrophilia and a higher than normal level of macrophages present before injection of ISCOMS, IL-12KO mice were unable to recruit inflammatory cells into the peritoneum and the production of inflammatory mediators was also abolished.

5.2 Innate Responses to ISCOMS in γ Interferon-Receptor Deficient Mice

γ IFN is another of the inflammatory cytokines that I showed in Chapter 3 to be upregulated after injection of ISCOMS. However, I showed in Chapter 4 that unlike IL-12, γ IFN appeared not to be required for the generation of antigen-specific immunity to OVA-ISCOMS. Therefore I decided to explore the innate cascade induced by ISCOMS in γ IFNRKO mice to determine if this might shed light on the different patterns of antigen-specific immune responses to ISCOMS found in these two strains.

5.2.1 ISCOMS Induced Inflammatory Infiltrate.

Wild type B6 mice showed the characteristic profile of cellular recruitment after i.p. injection of ISCOMS, with an early decrease in PEC numbers, followed by an increase at later times, peaking at 48 hours when numbers had increased by approximately two fold (Figure 5.6). Control γ IFNRKO mice had similar numbers of PEC and showed the same early loss of PEC numbers after injection of ISCOMS. However, PEC remained low in these animals and did not return to control levels throughout the timecourse of the experiment (Figure 5.6).

5.2.2. Phenotypic Analysis PEC

The detailed phenotypic analysis of the inflammatory infiltrates induced by ISCOMS in γ IFN RKO mice was performed at the same time as that in IL-12KO mice, using the same group of wild type B6 mice as controls. These data are shown again for comparison in the relevant figures. γ IFN RKO mice showed the early neutrophilia soon after injection of ISCOMS, but the numbers were much reduced compared with those in controls and they peaked later than in wild type mice (Figure 5.7a).

Saline injected γ IFN RKO mice had a larger baseline number of F4/80 $^{+}$ macrophages than wild type mice, but this decreased rapidly to very low levels within 3.5 hours of an injection of ISCOMS and remained low for the duration of the experiment (Figure 5.7b). In parallel with the failure to recruit macrophages to the same extent as in wild type mice, F4/80 $^{+}$ macrophages in ISCOMS injected γ IFN RKO mice did not express MHC class II at any time during the experiment (Figure 5.7c).

γ IFN RKO mice showed a similar reduction of dendritic cell numbers seen in the first 7 hours after injection of ISCOMS as that seen in wild type mice. However some CD11c $^{+}$ cells remained and the numbers were actually greater than baseline by 24 hours, before decreasing again at later times, when numbers were steadily increasing in wild type animals (Figure 5.7d).

Despite the numbers of CD4 $^{+}$ PEC in saline injected γ IFN RKO mice being much lower than in wild types, γ IFN RKO CD4 numbers began a steady increase immediately after administration of ISCOMS, peaking after 48 hours, when there was over twice the number of CD4 $^{+}$ cells than in control animals and then began to decline back to control levels by 72 hours (Figure 5.8a). Although the numbers of CD4 $^{+}$ T cells were much lower, this recruitment was different to that seen in wild types, as no increase in CD4 $^{+}$ T cell numbers was observed.

Whereas wild type mice injected with ISCOMS showed a dramatic increase in the level of activated CD4 $^{+}$ T cells expressing the IL-2R α chain CD25, the level of CD25 expression on CD4 $^{+}$ T cells removed from γ IFN RKO mice remained at baseline levels

throughout the experiment (Figure 5.8b). CD8⁺ lymphocytes in γ IFN γ IRKO mice increased steadily from very low levels in control mice to a maximum by 48 hours when their numbers were nearly 6 times greater than baseline. However these numbers were still much reduced compared with those in injected wild type mice (Figure 5.8c).

The numbers of B cells in control γ IFN γ IRKO mice were approximately 50% lower than in wild type mice and fell further immediately after injection of ISCOMS, remaining below the baseline level for a further 24 hours and then returning to normal levels by 48 hours. However, unlike the subsequent recruitment seen in wild type mice, there was no increase in B cell numbers above baseline levels in γ IFN γ IRKO mice at any time and the numbers were always lower than those in wild type animals (Figure 5.8d).

5.2.3. Inflammatory Cytokine Production

As described earlier in Chapter 3, PEC from uninjected wild type mice produced a low but significant level of IL-1 α in response to stimulation with γ IFN+LPS, and this was increased to more than 5 times greater than uninjected levels at 48 hours after injection of ISCOMS, before decreasing quickly to baseline levels (Figure 5.9a). γ IFN γ IRKO mice did not produce any IL-1 α at any time before or after administration of ISCOMS (Figure 5.9a).

PEC from saline injected wild type mice produced a large amount of IL-6 after stimulation with γ IFN+LPS, which was reduced by 75% within 3.5 hours of injection with ISCOMS (Figure 5.9b). IL-6 production then returned to normal and, in this experiment, continued at control levels for the remainder of the study. PEC from saline injected γ IFN γ IRKO mice produced significantly higher levels of IL-6 compared with PEC from wild type mice, but IL-6 levels then fell rapidly to 50% of baseline within 3.5 hours and continued at low levels thereafter (Figure 5.9b).

PEC from saline injected wild type mice produced IL-12 after stimulation *in vitro* with γ IFN+LPS and this declined soon after injection of ISCOMS, so that by 7 hours, it was half that of unimmunised mice. However, the levels of IL-12 production began to rise at later times and by 72 hours, ISCOMS stimulated PEC produced more than twice the amount of IL-

12 compared with uninjected animals (Figure 5.9c). PEC from saline injected γ IFN RKO mice secreted very small amounts of IL-12 and although this increased slightly by 3.5 hours after injection of ISCOMS, the levels of IL-12 from γ IFN RKO mice did not change thereafter and remained significantly lower than those of wild type mice until the duration of the experiment (Figure 5.9c).

As I found in Chapter 3, PEC from saline injected wild type mice produced two peaks of Con A stimulated γ IFN production, both of which were approximately 6 times greater than those of uninjected control mice. The first peak was at 3.5 hours, and the second peak occurred at 48 hours (Figure 5.9d). γ IFN RKO mice on the other hand, did not produce any γ IFN in response to Con A at any time either before or after injection of ISCOMS (Figure 5.9d).

5.2.4 Nitric Oxide Release

As I observed in Chapter 3, PEC from saline injected wild type animals produced substantial amounts of NO in response to γ IFN+LPS stimulation *in vitro* before injection of ISCOMS and this was reduced to zero immediately after administration of ISCOMS. NO production returned to control levels by 24 hours and by 48 hours was increased significantly by approximately 20% above baseline levels, before declining to below control levels by 72 hours (Figure 5.10). There was no measurable production of NO from γ IFN RKO PEC either before or after administration of ISCOMS (Figure 5.10).

5.2.5. Summary

Thus γ IFN RKO mice showed a major defect in the inflammatory response induced by ISCOMS. There was a dramatic yet selective failure to recruit cells into the peritoneum after injection of ISCOMS, as seen by reduced numbers of neutrophils, macrophages and activation of CD4⁺ T cells and macrophages. However, normal levels of B cells, CD4⁺ T cells and dendritic cells were observed in PEC from γ IFN RKO mice. However, a major

defect in inflammatory mediator production from ISCOMS injected γ IFN^{RKO} PEC was noted as cells were not activated nor secreting inflammatory mediators.

5.3 Innate Responses to ISCOMS in Interleukin 18 Deficient Mice

As I showed in Chapter 4, antigen-specific immune responses were not completely defective after immunisation of IL-12KO mice with OVA ISCOMS and some antigen-specific responses were also defective in mice lacking the functionally related cytokine IL-18. Therefore I thought that it was important to determine if IL-18KO mice shared the defective innate immune response seen in IL-12KO mice. Only a small number of IL-18KO mice were available for these studies and no wild type or heterozygous littermate animals at all were available. However, in view of the potential importance of the cytokine and because I had found relatively consistent inflammatory responses to ISCOMS in the wild type animals used in other experiments, I decided to proceed with a preliminary experiment.

5.3.1. ISCOMS Induced Inflammatory Infiltrates

Immediately after injection of ISCOMS into IL-18KO mice, the number of PEC fell by half and remained below baseline levels until 72 hours, by which time the numbers returned to baseline levels (Figure 5.11). However, the numbers did not rise above the background, in contrast to what I had observed earlier in wild type mice.

5.3.2. Phenotypic Analysis of PEC

The numbers of neutrophils decreased to zero immediately after injection of ISCOMS into IL-18KO mice and these reduced levels of neutrophils continued until 72 hours, when neutrophil numbers had returned to normal (Figure 5.12a). The numbers of F4/80⁺ macrophages also dropped to zero within 3.5 hours after injection of ISCOMS and remained so for the duration of the experiment (Figure 5.12b). This contrasts with the increase in

neutrophils I had observed previously using wild type mice injected with ISCOMS. In addition, there was also no evidence of the usual macrophage activation in IL-18KO mice (Figure 5.12c).

The numbers of CD11c⁺ dendritic cells were also reduced to background numbers in IL-18KO mice immediately after injection with ISCOMS and this suppressed level was maintained until 72 hours (Figure 5.12d), in contrast to the increased recruitment of CD11c⁺ dendritic cells I observed previously in wild type animals.

The numbers of CD4⁺ lymphocytes in IL-18KO PEC also decreased soon after administration of ISCOMS and failed to recover at any time thereafter and ISCOMS also failed to activate these T cells in IL-18KO mice as identified by expression of CD25 (Figure 5.13a). The numbers of CD8⁺ T lymphocytes followed the same pattern as CD4⁺ T lymphocytes, with an immediate decrease which was sustained until the end of the experiment (Figure 5.13b). Such a pattern contrasts with the profile of CD4 and CD8 recruitment I had found in wild type mice injected with ISCOMS.

The numbers of B cells from IL-18KO mice dropped by 50% immediately after injection of ISCOMS, but as previously observed in wild type mice, this then returned to normal before increasing to five times the baseline numbers at 72 hours (Figure 5.13d).

5.3.3. Inflammatory Cytokine Production

PEC from saline injected IL-18KO mice did not produce any IL-1 α after *in vitro* stimulation with γ IFN+LPS and this was the case until 72 hours after injection of ISCOMS, when IL-1 α production was significantly increased above background levels (Figure 5.14a).

Saline injected IL-18KO PEC secreted significant amounts of IL-6 after *in vitro* stimulation with γ IFN+LPS, but in contrast to what I had found previously with wild type mice, there was no significant change in the production of IL-6 from PEC removed from ISCOMS injected IL-18KO mice at any time during the experiment (Figure 5.14b).

Saline injected IL-18KO PEC produced very little IL-12 when stimulated *in vitro* with γ IFN+LPS, but within 6 hours of administration of ISCOMS, IL-12 production was

significantly increased. By 24 hours, levels were more than twenty times greater than in uninjected mice and continued to a peak at 72 hours, when IL-12 production was 25 times greater than background (Figure 5.14c). Although the early increase contrasts with the normal decrease in IL-12 after injection of ISCOMS in wild type animals, maximal production of IL-12 from IL-18KO PEC coincided with that normally seen with wild type animals.

PEC from saline injected IL-18KO mice produced a small amount of γ IFN after *in vitro* stimulation with Con A, and within 6 hours of administration of ISCOMS, γ IFN production more than doubled, reaching a peak at 24 hours when γ IFN levels were nearly five times greater than control animals and remained high until 72 hours (Figure 5.14d). This pattern of sustained increase in γ IFN production is in contrast to the biphasic pattern normally seen in wild type animals.

5.3.4 Summary

Thus, in the absence of IL-18, no recruitment of PEC occurred in response to ISCOMS, but there appeared to be normal or even increased production of γ IFN and IL-12 as well as an abnormal pattern of IL-1 α secretion, with the only defective cytokine measured being IL-6 as the levels were unchanged after injection with ISCOMS.

5.4. Innate Responses to ISCOMS in Interleukin 4 Deficient Mice

As I had observed normal antigen-specific immune responses in IL-4KO mice immunised with OVA-ISCOMS, I wished to see if innate immune responses were also normal in these mice and if correlating these responses in the different mice would provide information on how the innate response regulates adaptive immunity. Again, because I found that these mice retained normal antigen-specific responses to ISCOMS, I decided to use representative timepoints only.

5.4.1. Recruitment of PEC

PEC numbers from wild type B6 mice were normal 24 hours after injection of ISCOMS in this study. Thereafter PEC numbers increased by approximately two fold at 72 hours and remained at an elevated level at 168 hours (Figure 5.15). An identical pattern of PEC recruitment was seen in IL-4KO mice at all times after injection of ISCOMS (Figure 5.15).

As there were no gross differences in the pattern of ISCOMS recruited PEC in IL-4KO mice and their antigen-specific responses were entirely normal, phenotypic studies were not performed on PEC in this study and only selected functional studies were undertaken.

5.4.2. Inflammatory Cytokine Production

No detectable levels of IL-1 α were measured in ISCOMS injected wild type or IL-4KO mice, but this was not unexpected, given that this timecourse did not include the times when I found IL-1 α to be maximal (data not shown).

As with other wild type mice, PEC from saline injected animals produced detectable IL-6 production in response to γ IFN+LPS and this fell markedly by 24 hours after injection of ISCOMS, remaining depressed throughout the remainder of the experiment. (Figure 5.16a). Again this may be because the usual peak of IL-6 production was missed. PEC from IL-4KO mice show a decrease in γ IFN+LPS stimulated IL-6 production at 24 hours, but in these mice, there was an increase in IL-6 production at 72 hours, when levels were significantly greater than in wild type animals. IL-6 levels then decreased below background levels by 168 hours (Figure 5.16a).

As before, wild type B6 mice produced low levels of IL-12 which initially decreased at early timepoints, but then increased markedly to a peak at 72 hours after injection of ISCOMS, when levels of IL-12 secretion were 20 times greater than the saline injected controls. IL-12 production then returned to background levels by 168 hours (Figure 5.16b). IL-4KO mice showed a similar pattern of γ IFN+LPS stimulated IL-12 secretion, which also

peaked by 72 hours, when it was significantly greater than that in wild type mice, before returning to control levels by 168 hours (Figure 5.16b).

PEC from saline injected wild type B6 mice produced a significant amount of Con A stimulated γ IFN and after an initial decrease in γ IFN secretion 24 hours after injection of ISCOMS, this increased steadily to a peak at 72 hours at a level more than twice that of controls (Figure 5.16c). IL-4KO mice showed an identical pattern of γ IFN production, with an early decrease compared with background levels, followed by a peak at 72 hours which returned to control levels by 168 hours. There were no significant differences between Con A induced γ IFN production from IL-4KO and wild type mice at any time (Figure 5.16c).

5.4.3. Secretion of Nitric Oxide

NO production in wild type mice fell soon after injection of ISCOMS to zero, before levels returned to those of saline injected control mice by 72 hours. (Figure 5.20). An identical pattern was found in ISCOMS injected IL-4KO mice, although a significant decrease was noted at 168 hours compared with wild types (Figure 5.20).

5.4.4 Summary

Together these results indicate that the absence of IL-4 has no effect on the ability of ISCOMS to induce an inflammatory cascade.

5.5 Innate Responses to ISCOMS in Interleukin 6 Deficient Mice

I had shown in Chapters 3 and 4 that large amounts of IL-6 were induced by an injection of ISCOMS, but IL-6 was not important for the induction of antigen-specific immune responses to ISCOMS. As I found discrepancies between the innate and adaptive immune responses induced by ISCOMS in γ IFN γ NRKO mice, I was interested to see if a similar dichotomy occurred in IL-6KO mice. In these experiments I only assessed two representative

timepoints, 24 hours and 72 hours, as well as an extended timepoint at 168 hours in order to assess if there might be a more delayed response in these animals.

5.5.1 Recruitment of PEC

The recruitment of PEC in wild type 129/Sv mice after injection of ISCOMS induced a steady increase in cell numbers which reached a peak at 72 hours when PEC numbers had nearly doubled before returning towards control levels by 168 hours. The recruitment of PEC in IL-6KO mice followed an almost identical pattern to that found in wild type mice (Figure 5.18). Because of this and because I found that the IL-6KO mice had normal antigen-specific responses, I did not conduct further experiments to assess individual populations of PEC phenotypically.

5.5.2. Inflammatory Cytokine Production

There was no measurable production of γ IFN+LPS stimulated IL-1 α in either 129/Sv or IL-6KO mice, but this is not unexpected given the lack of a 48 hour timepoint when IL-1 α has been shown previously to be maximal (Data not shown). No γ IFN+LPS stimulated IL-6 production was measured in IL-6KO mice, although SV/129 wild types produced a normal profile of IL-6 with decreased levels detected at 24 hours which then returned to control levels by 72 and 168 hours (Data not shown).

Wild type 129/Sv mice produced increased levels of γ IFN+LPS stimulated IL-12 at 24 hours after injection of ISCOMS, which were approximately 4 times greater than in saline injected control 129/Sv mice. However as in my previous experiments, the maximal production of IL-12 occurred at 72 hours, by which time IL-12 production was 5 times that of control levels (Figure 5.19a). IL-12 production was also increased in ISCOMS injected IL-6KO mice, but this peaked at the earlier time of 24 hours and remained high at 72 hours. The peak of IL-12 production in IL-6KO mice was twice that in wild type mice. At later times,

IL-12 production from both 129/Sv and IL-6KO mice returned to control levels (Figure 5.19a).

In these experiments, uninjected wild type 129/Sv mice had high baseline levels of Con A stimulated γ IFN production, but this decreased rapidly after injection of ISCOMS and remained depressed until 168 hours (Figure 5.19b). Saline injected IL-6KO mice produced very low amounts of γ IFN and this was not increased after injection of ISCOMS. (Figure 5.19b).

5.5.3 Secretion of Nitric Oxide

PEC from wild type 129/Sv mice stimulated with γ IFN+LPS produced a large amount of NO before injection with ISCOMS and this decreased rapidly by 90% within 24 hours of administration of ISCOMS. NO production then gradually returned to background levels by 168 hours (Figure 5.20). IL-6KO mice had significantly lower levels of NO production than wild type controls and these remained very low until 168 hours, when there was a dramatic increase in NO production which was now significantly greater than wild types (Figure 5.20).

5.5.4. Summary

IL-6KO mice did not show any defect in cellular recruitment after injection of ISCOMS and were capable of sustaining inflammatory responses after an injection of ISCOMS, but there were some differences in the ability to produce inflammatory mediators in particular γ IFN.

Summary and Conclusions

The experiments in this chapter were designed to investigate how the absence of individual inflammatory mediators influenced the induction of innate immune responses by

ISCOMS and to explore how these correlated with the generation of antigen-specific immunity in these mice.

My previous results had shown that antigen-specific immune responses to ISCOMS were dependent on the presence of IL-12 and here I found that IL-12KO mice had severely crippled innate responses to ISCOMS, lacking recruitment or activation of an inflammatory infiltrate and with little or no inflammatory mediator production. Thus, this cytokine plays a crucial role in the initiation of the inflammatory cascade by ISCOMS.

γ IFN^RKO mice proved to be the most interesting of the knockout animals. Although I had previously found that these mice had intact antigen-specific immune responses to OVA ISCOMS, the innate immune response was unexpectedly and severely deficient in γ IFN^RKO mice. The most dramatic abnormality in these mice was the complete absence of cellular recruitment or activation especially of macrophages or CD4⁺ lymphocytes, but numbers of B cells and dendritic cells were unaltered. However, these mice had severely defective inflammatory mediator production following injection with ISCOMS.

In Chapter 4 I found that certain antigen-specific responses to ISCOMS were defective in IL-18KO mice and in this chapter, I found that IL-18KO mice also appeared to have defective cellular recruitment into the peritoneum, with B cells being the only cell type which seemed to be recruited in the same way as in wild type mice. However, IL-18KO mice actually retained the ability to produce high levels of IL-12, γ IFN and IL-1 α , occasionally at levels somewhat higher than I had found previously with wild type animals. However, IL-1 responses were delayed and there was no induction of IL-6. The interpretation of these experiments was limited by the unavailability of appropriate controls.

IL-6 is a member of the inflammatory cascade and was increased after administration of ISCOMS in wild type animals. However IL-6 was shown not to be involved in the generation of OVA-specific immunity to ISCOMS and in this chapter I found that this cytokine is not important for the innate immune response, as IL-6KO mice had normal cellular recruitment and there was no gross defect in the inflammatory mediator production. A similar pattern was seen in IL-4KO mice which had normal antigen-specific immune responses to OVA ISCOMS and an intact inflammatory response. One problem with these

experiments was that they were conducted before I had determined the optimal time courses for the induction of inflammatory cytokine production and therefore some of the responses I observed were variable or suboptimal. Nevertheless, as there was no gross defect in the inflammatory cascade and because specific immune responses were normal for both IL-4KO and IL-6KO, I decided that it was inappropriate to perform further work in these animals.

In conclusion, individual components of the immune system showed different patterns of involvement in antigen-specific and innate immunity. Whereas IL-4 and IL-6 appear not to be required for either aspect of the immune response, IL-12 is important for both. In contrast, the absence of γ IFN had dramatic effects on the induction of innate inflammatory cascade, but was not required for antigen-specific immunity, whereas IL-18 appeared to have variable effects on both aspects of the immune response. Therefore, these experiments show that the links between the innate and adaptive immune system are regulated by a highly complex and delicately balanced system.

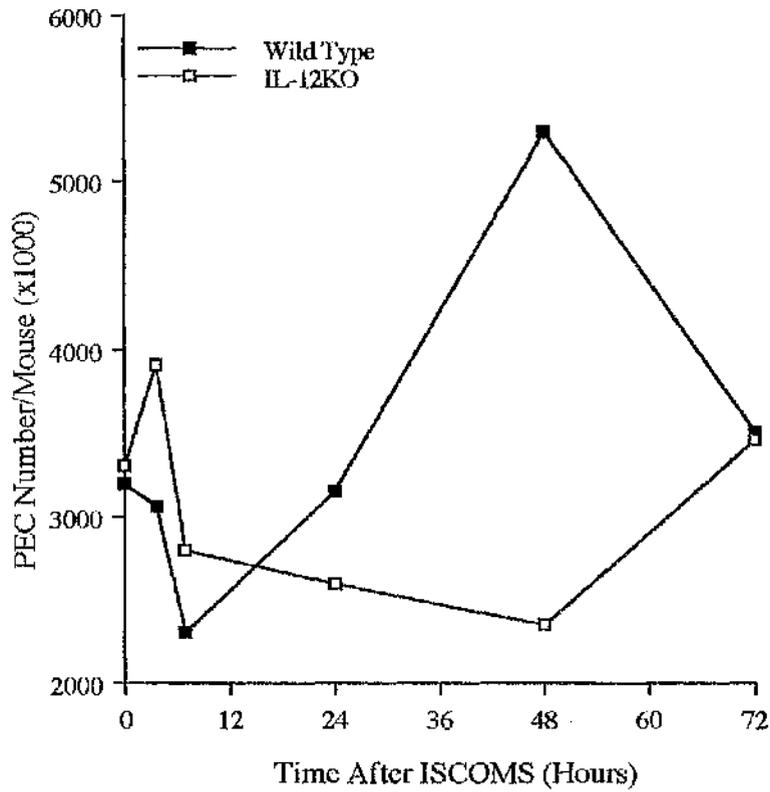


Figure 5.1 ISCOMS Induced Recruitment of PEC in IL-12KO Mice.

The graph shows PEC recruitment following an i.p. injection of 5 μ g OVA ISCOMS. PEC were removed from five mice by lavage, pooled and cell counts performed. The results shown are mean PEC numbers for wild type mice (closed squares) and IL-12KO mice (open squares). Saline injected mice from each strain were used as controls.

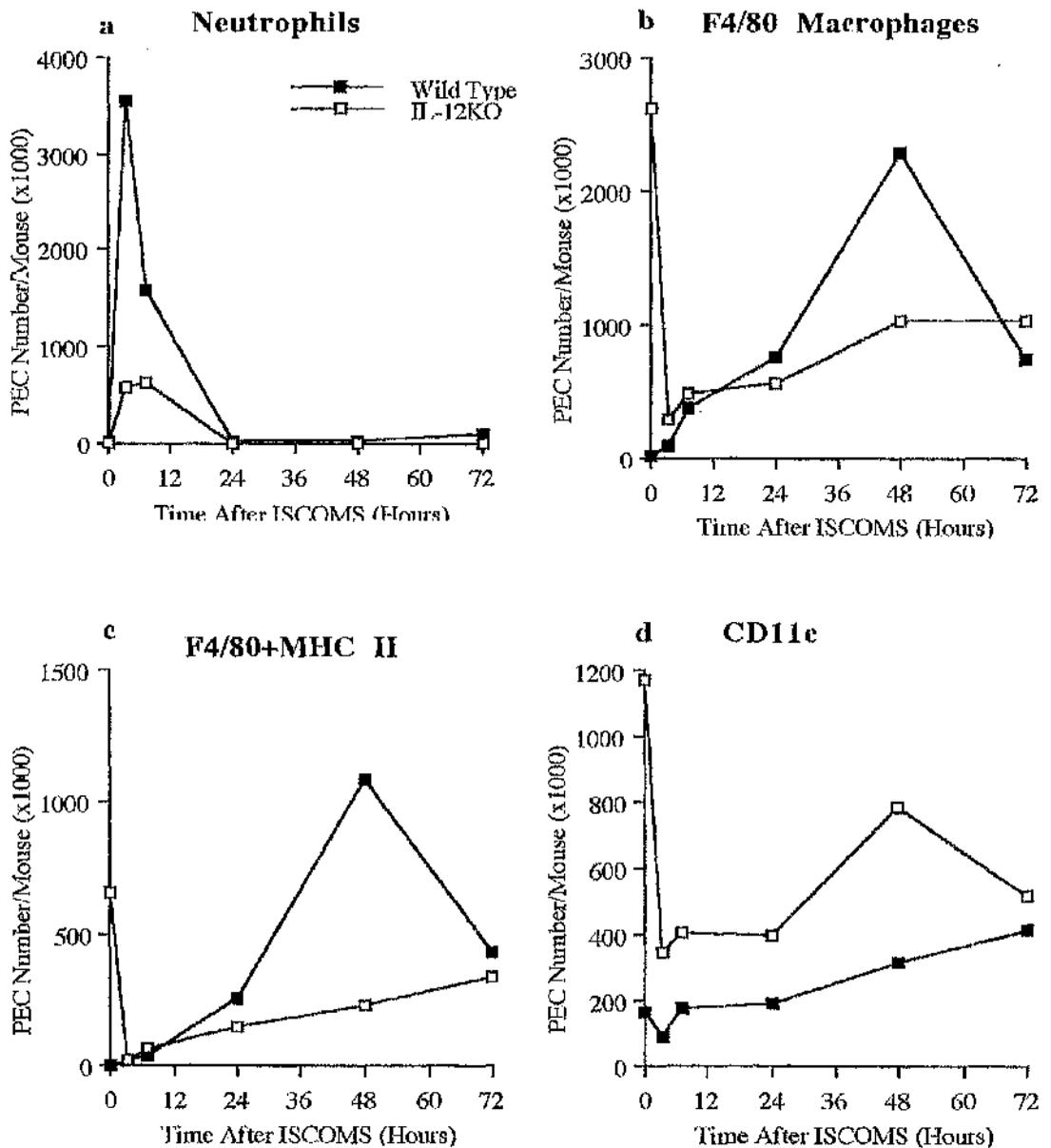


Figure 5.2 Phenotypic Analysis of ISCOMS Recruited PEC from IL-12KO Mice.

PEC were removed by lavage after an i.p. injection of 5 μ g OVA ISCOMS and analysed by flow cytometry. The results shown are the numbers of (a) neutrophils, (b) F4/80⁺ macrophages, (c) MHC class II⁺ F4/80 macrophages and (d) CD11c⁺ dendritic cells. The results are expressed as the number of cells per peritoneum which were calculated from the percentage of cells positive for each marker in the PEC pooled from 5 animals per group for wild type mice (closed squares) and IL-12KO mice (open squares). Saline injected animals of each strain were controls.

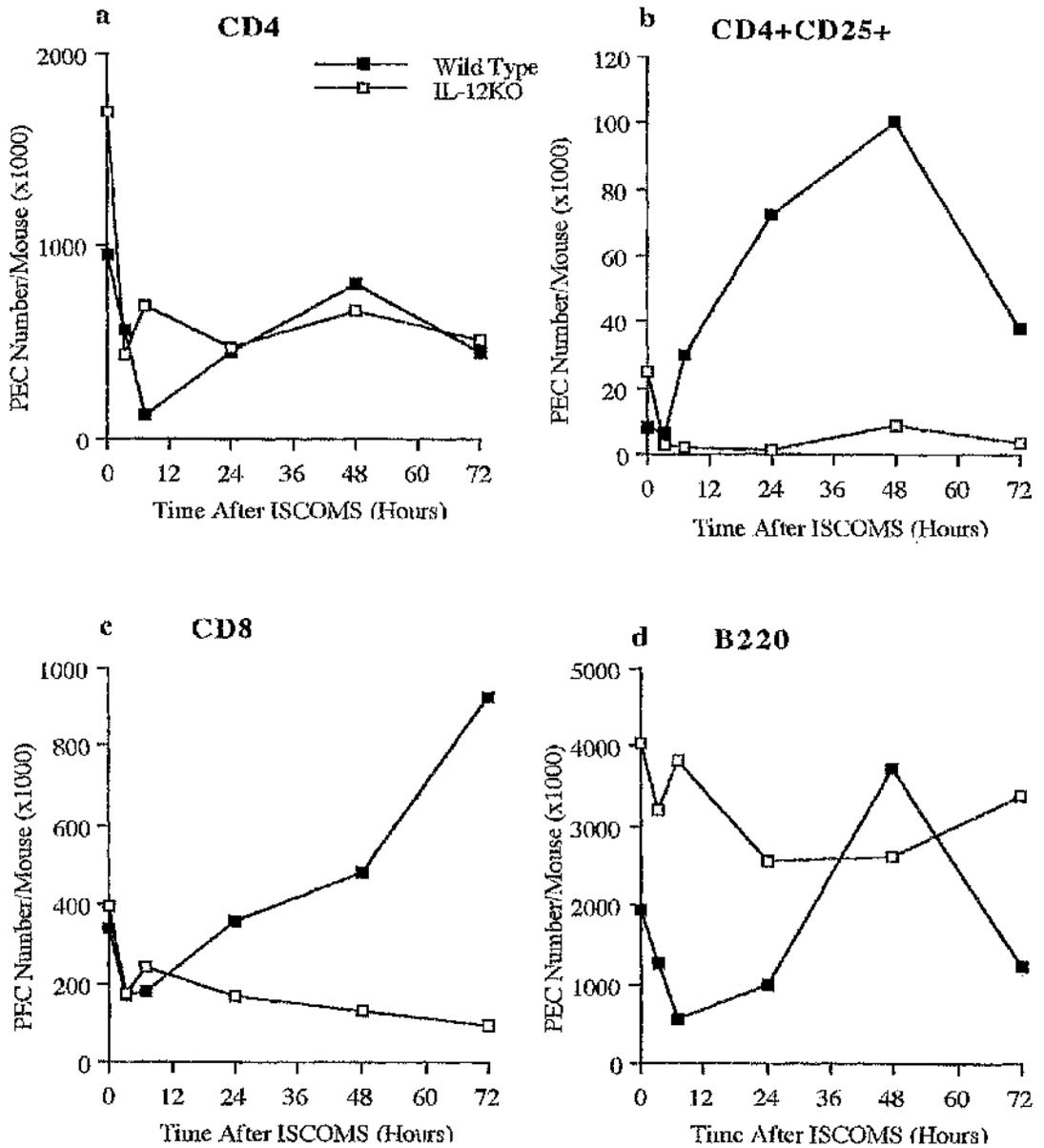


Figure 5.3 Phenotypic Analysis of ISCOMS Recruited PEC from IL-12KO Mice.

PEC were removed by lavage after an i.p. injection of 5 μ g OVA ISCOMS and analysed by flow cytometry. The results shown are the numbers of (a) CD4⁺ lymphocytes, (b) CD4⁺ lymphocytes, (c) CD8⁺ lymphocytes and (d) B220⁺ B cells. The results are expressed as the number of cells per peritoneum which were calculated from the percentage of cells positive for each marker in the PEC pooled from 5 animals per group for wild type mice (closed squares) and IL-12KO mice (open squares). Control mice were saline injected animals of each strain.

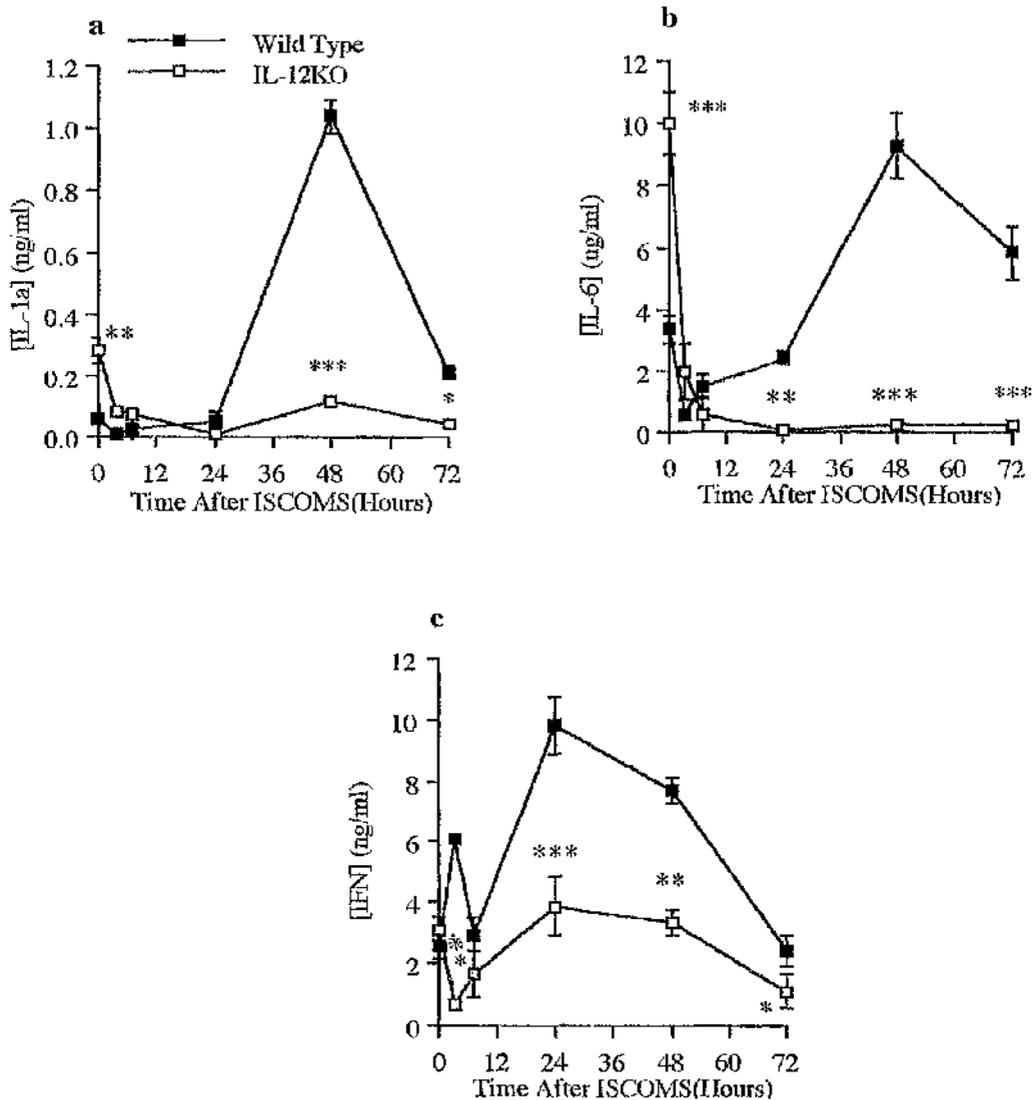


Figure 5.4 ISCOMS Induced Inflammatory Cytokine Production by PEC from IL-12KO Mice

Production of (a) IL-1 α , (b) IL-6, (c) γ IFN by PEC pooled from 5 mice given an i.p. injection of 5 μ g ISCOMS 3.5-72 hours before. The results shown are the mean concentration of cytokines from wild type mice (closed squares) or IL-12KO mice (open squares), measured in triplicate after 48 hours stimulation in the presence or absence of γ IFN+LPS (a and b) or the presence or absence of Con A (c) \pm 1 SD (* p <0.05 versus wild type mice; ** p <0.005; *** p <0.001 versus wild type mice). Control mice were uninjected animals of each strain.

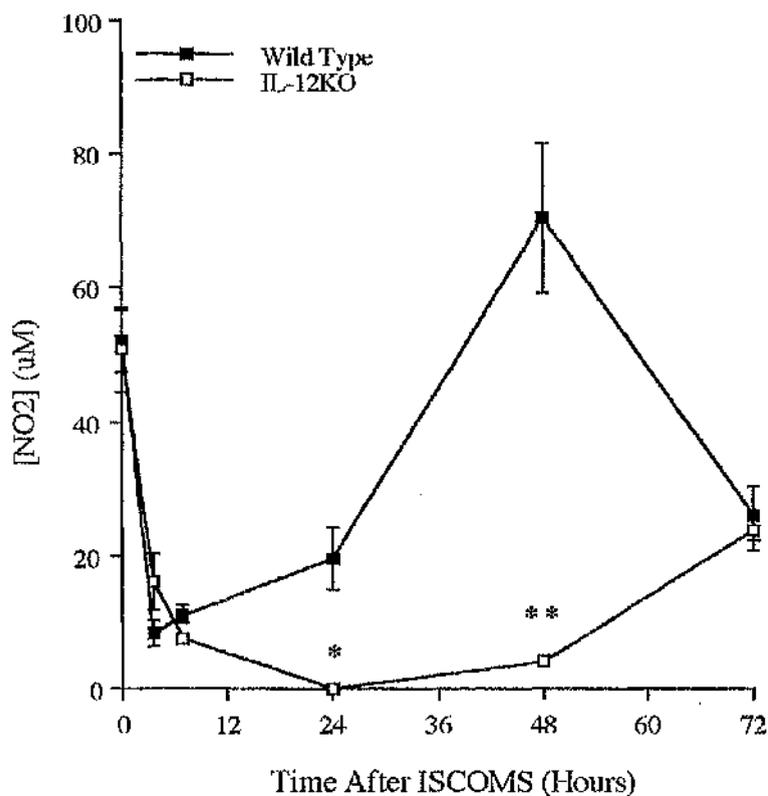


Figure 5.5 ISCOMS Induced Nitric Oxide Production from IL-12KO PEC

Production of nitric oxide from PEC pooled from 5 mice following injection of 5μg ISCOMS. The results shown are the mean concentration of nitrite measured in triplicate from wild type mice (closed squares) or IL-12KO mice (open squares), after culture for 48 hours in the presence or absence of γIFN+LPS ± 1 SD (* p<0.05 versus wild type mice; ** p<0.005 versus wild type mice). Control mice were uninjected animals of each strain.

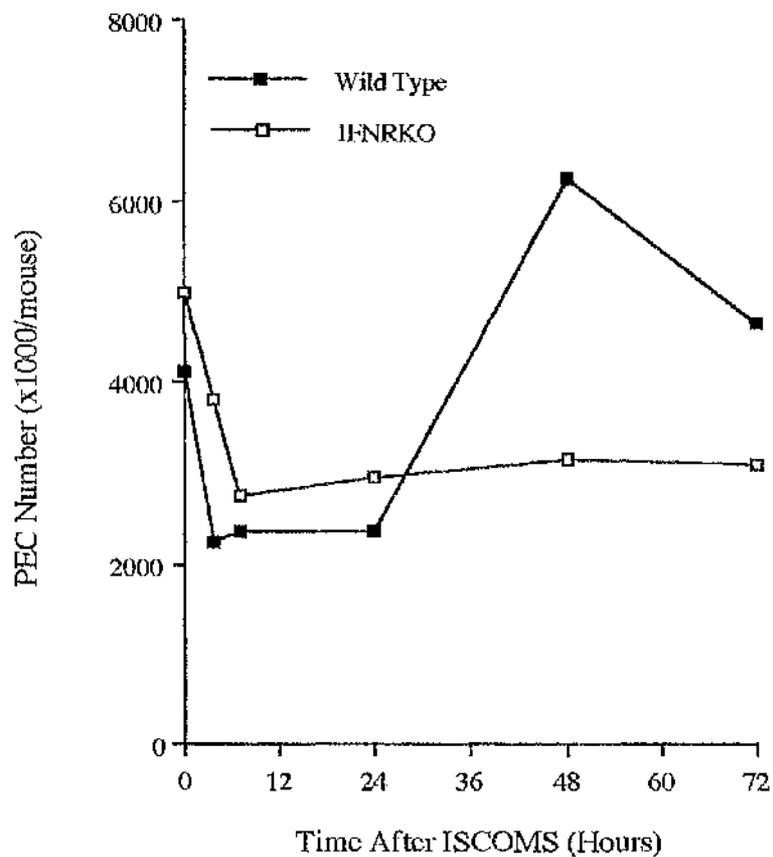


Figure 5.6 ISCOMS Induced Recruitment of PEC in γ IfNRRKO Mice.

The graph shows PEC recruitment following an i.p. injection of 5 μ g OVA ISCOMS. PEC were removed from five mice by lavage, pooled and cell counts performed. The results shown are mean PEC numbers for wild type mice (closed squares) and IfNRRKO mice (open squares). Saline injected mice from each strain were used as controls.

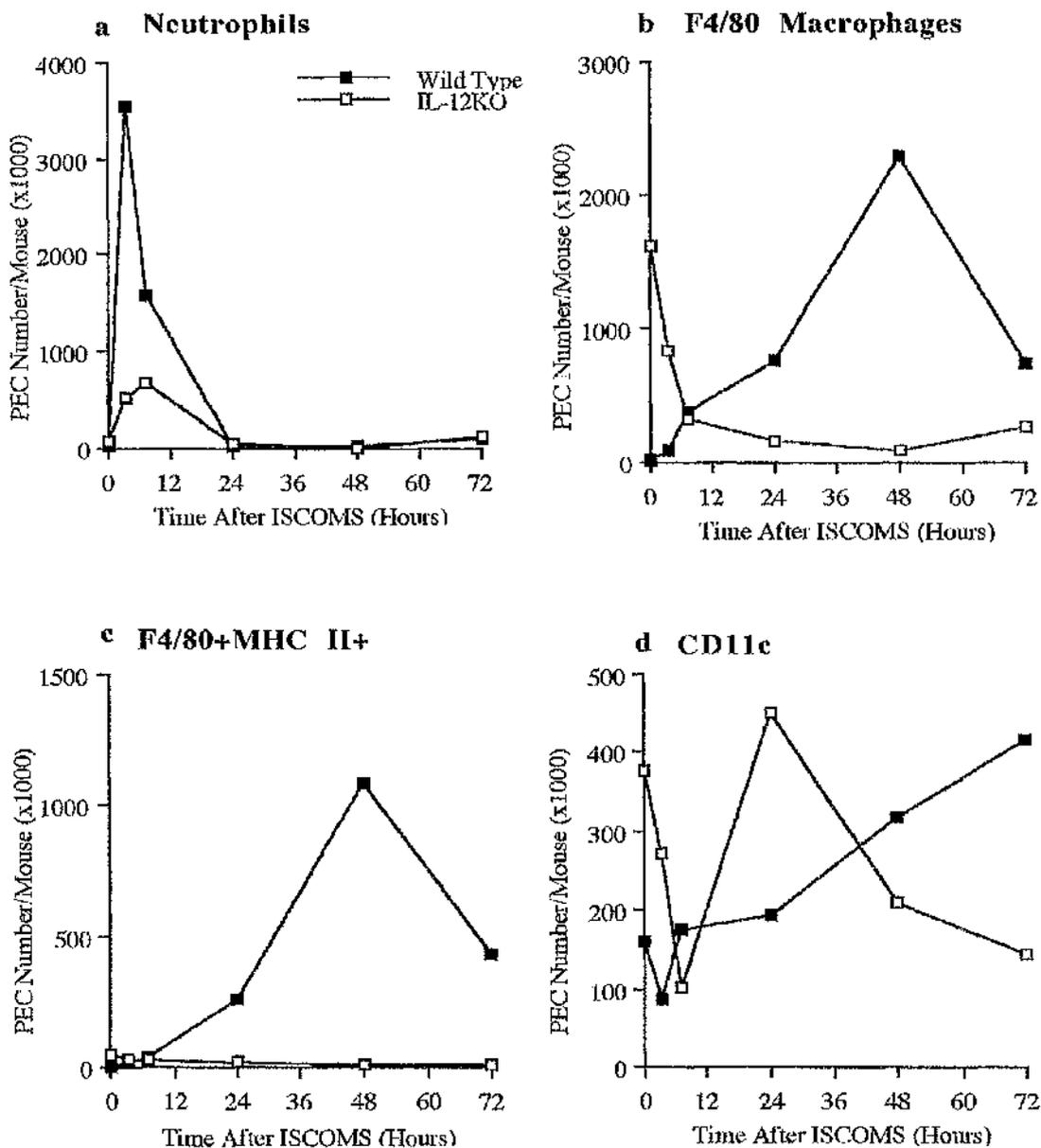


Figure 5.7 Phenotypic Analysis of PEC from γ IFN RKO mice after Injection of ISCOMS.

PEC were removed by lavage after an i.p. injection of 5 μ g OVA ISCOMS and analysed by flow cytometry. The results shown are the numbers of (a) neutrophils, (b) F4/80⁺ macrophages, (c) MHC class II⁺ F4/80 macrophages and (d) CD11c⁺ dendritic cells. The results are expressed as the number of cells per peritoneum which were calculated from the percentage of cells positive for each marker in the PEC pooled from 5 animals per group for wild type mice (closed squares) and γ IFN RKO mice (open squares). Control mice were uninjected.

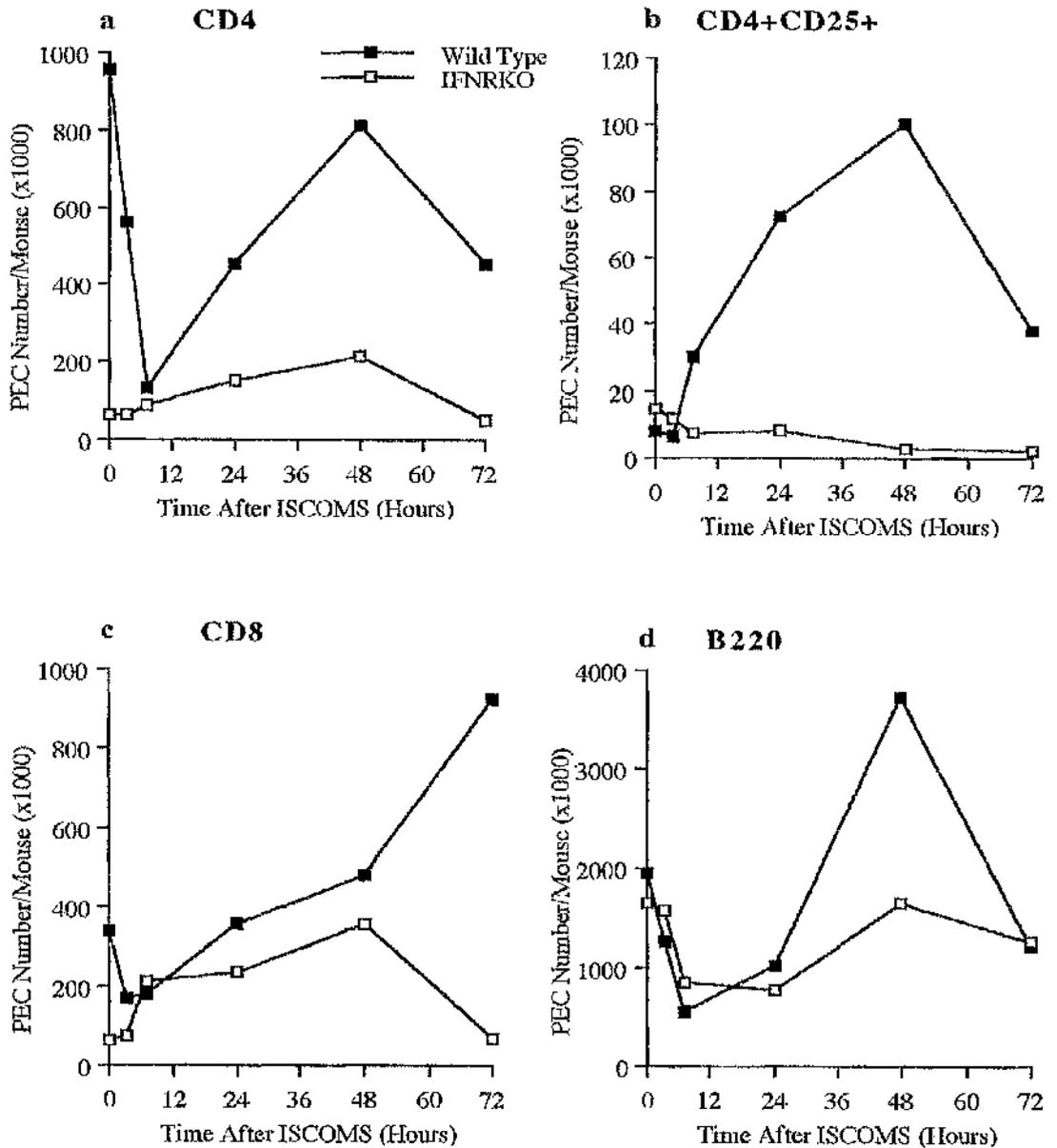


Figure 5.8 Phenotypic Analysis of ISCOMS Recruited PEC from γ IFN RKO Mice.

PEC were removed by lavage after an i.p. injection of 5 μ g OVA ISCOMS and analysed by flow cytometry. The results shown are the numbers of (a) CD4⁺ lymphocytes, (b) CD25⁺ CD4 lymphocytes, (c) CD8⁺ lymphocytes and (d) B220⁺ B cells. The results are expressed as the number of cells per peritoneum which were calculated from the percentage of cells positive for each marker in the PEC pooled from 5 animals per group for wild type mice (closed squares) and γ IFN RKO mice (open squares). Control mice were injected with saline.

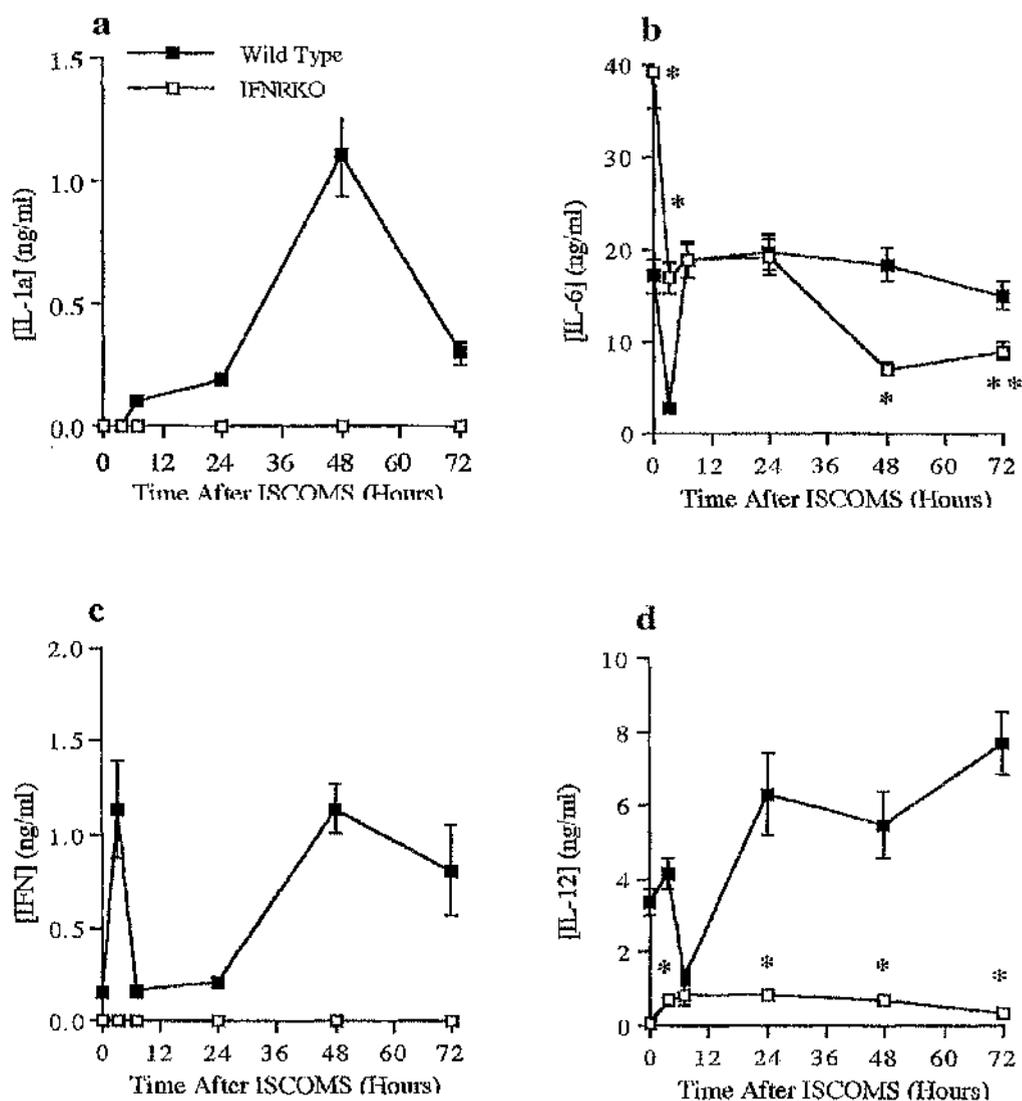


Figure 5.2 ISCOMS Induced Inflammatory Cytokine Production by PEC from γ IFN RKO Mice.

Production of (a) IL-1 α , (b) IL-6, (c) immunoreactive IL-12 and (d) γ IFN by PEC pooled from 5 mice given an i.p. injection of 5 μ g ISCOMS 3.5-72 hours before. The results shown are the mean concentration of cytokines from wild type mice (closed squares) or γ IFN RKO mice (open squares), measured in triplicate after 48 hours stimulation in the presence or absence of γ IFN+LPS (a-c) or the presence or absence of Con A (d) \pm 1 SD (*p<0.001 versus wild type mice; **p<0.05 versus wild type mice). Control mice were saline injected animals of each strain.

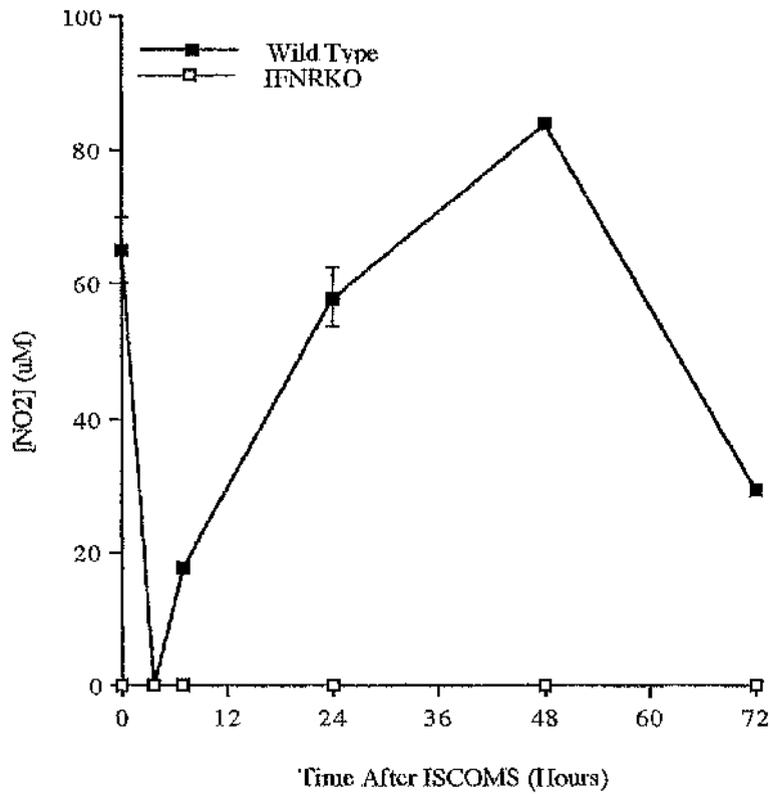


Figure 5.10 ISCOMS Induced Nitric Oxide Production by PEC from γ IFNRRKO Mice .

Production of nitric oxide from PEC pooled from 5 mice following injection of 5 μ g ISCOMS. The results shown are the mean concentration of nitrite measured in triplicate from wild type mice (closed squares) or γ IFNRRKO mice (open squares), after culture for 48 hours in the presence or absence of γ IFN+LPS \pm 1 SD. Control mice were saline injected animals of each strain.

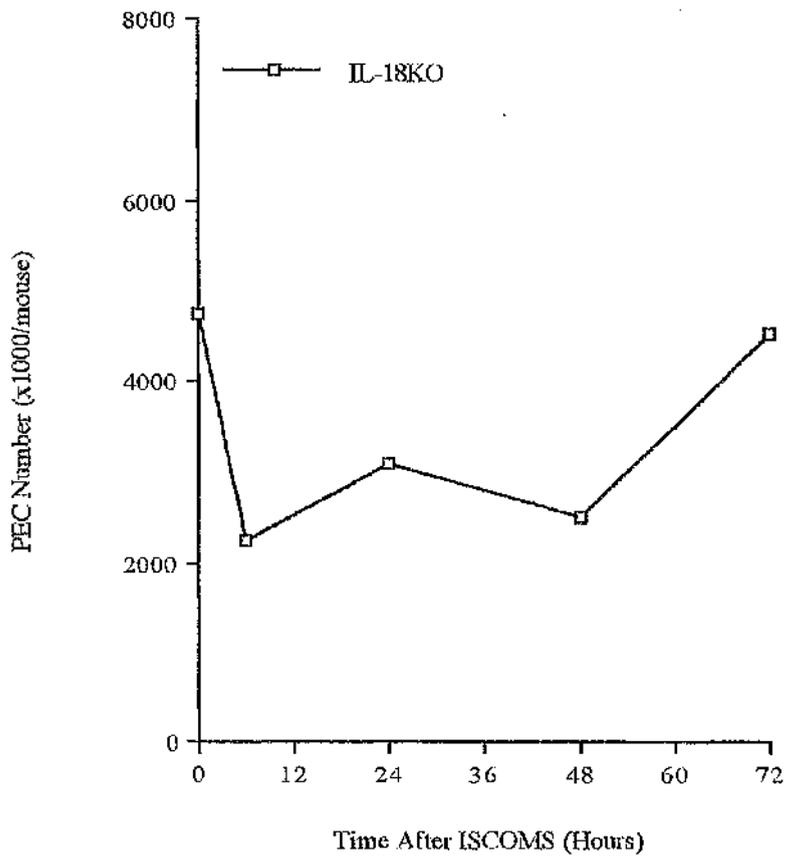


Figure 5.11 ISCOMS Induced Recruitment of PEC from IL-18KO Mice.

The graph shows PEC recruitment following an i.p. injection of 5µg OVA ISCOMS. PEC were removed from five mice by lavage, pooled and cell counts performed. The results shown are mean PEC numbers for IL-18KO mice (open squares). Saline injected mice were used as controls.

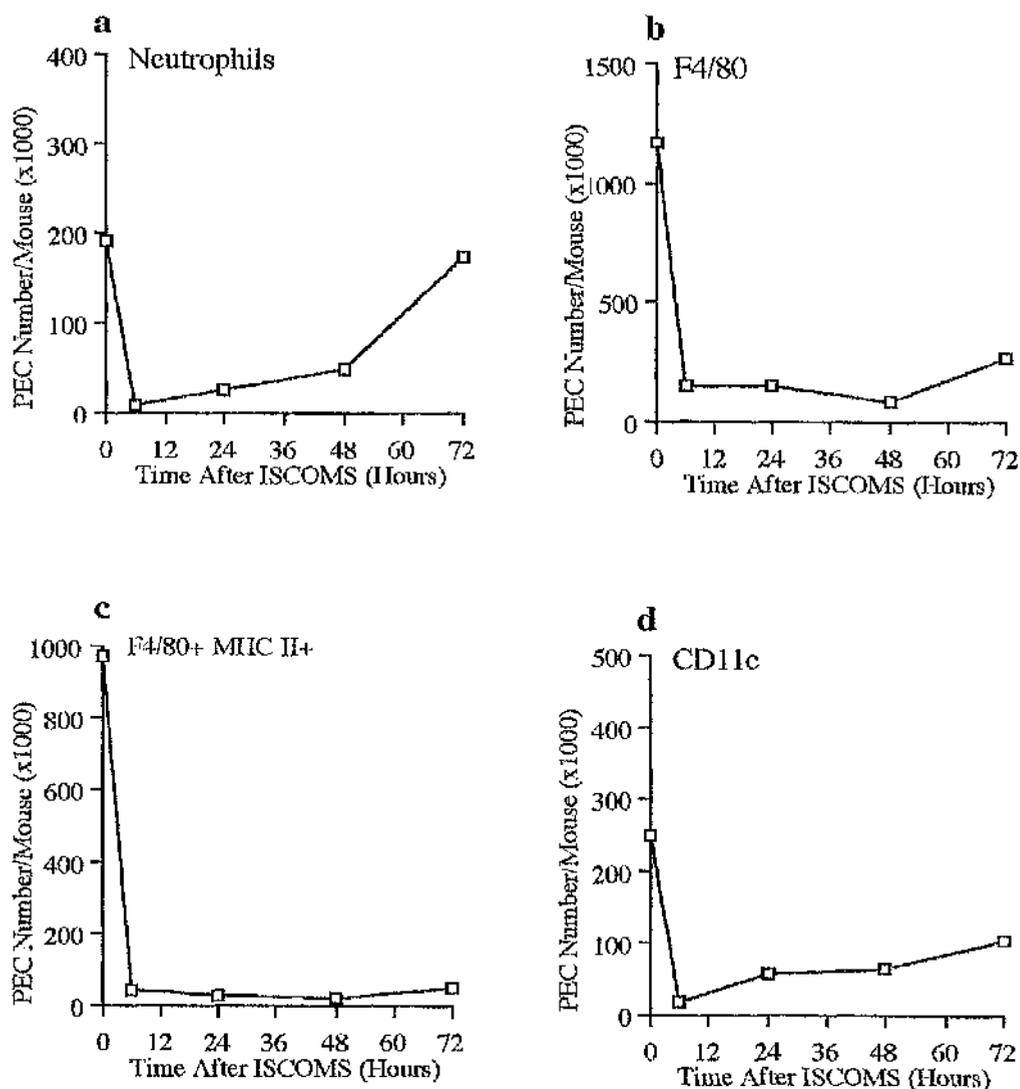


Figure 5.12 Phenotypic Analysis of ISCOMS Recruited PEC from IL-18KO Mice.

PEC were removed by lavage after an i.p. injection of 5 μ g OVA ISCOMS and analysed by flow cytometry. The results shown are the numbers of (a) neutrophils, (b) F4/80⁺ macrophages, (c) MHC class II⁺ F4/80 macrophages and (d) CD11c⁺ dendritic cells. The results are expressed as the number of cells per peritoneum which were calculated from the percentage of cells positive for each marker in the PEC pooled from 5 animals per group for IL-18KO mice (open squares). Control mice were saline injected animals.

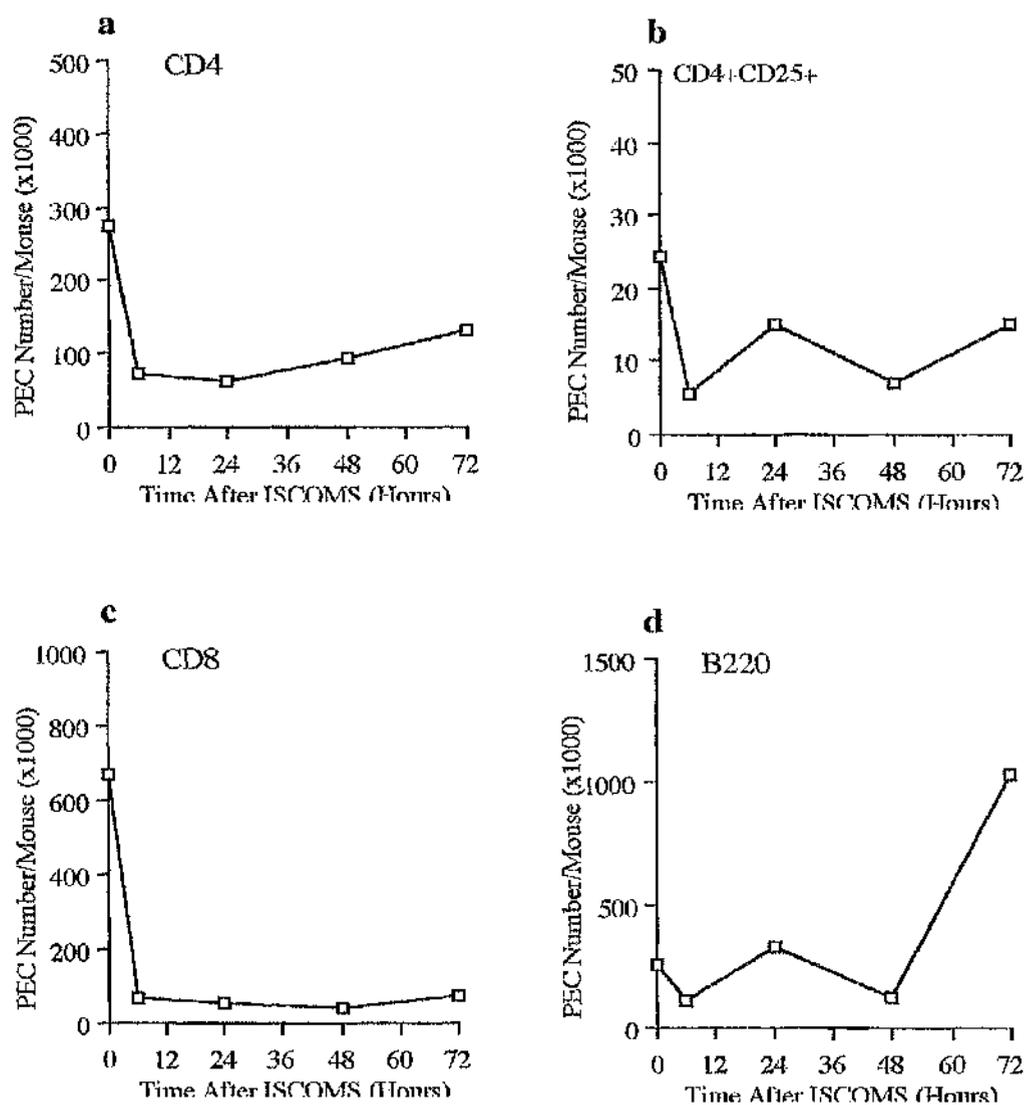


Figure 5.13 Phenotypic Analysis of ISCOMS Recruited PEC from IL-18KO Mice.

PEC were removed by lavage after an i.p. injection of 5 μ g OVA ISCOMS and analysed by flow cytometry. The results shown are the numbers of (a) CD4⁺ lymphocytes, (b) CD25⁺ CD4 lymphocytes, (c) CD8⁺ lymphocytes and (d) B220⁺ B cells. The results are expressed as the number of cells per peritoneum which were calculated from the percentage of cells positive for each marker in the PEC pooled from 5 animals per group for IL-18KO mice (open squares). Control mice were saline injected animals.

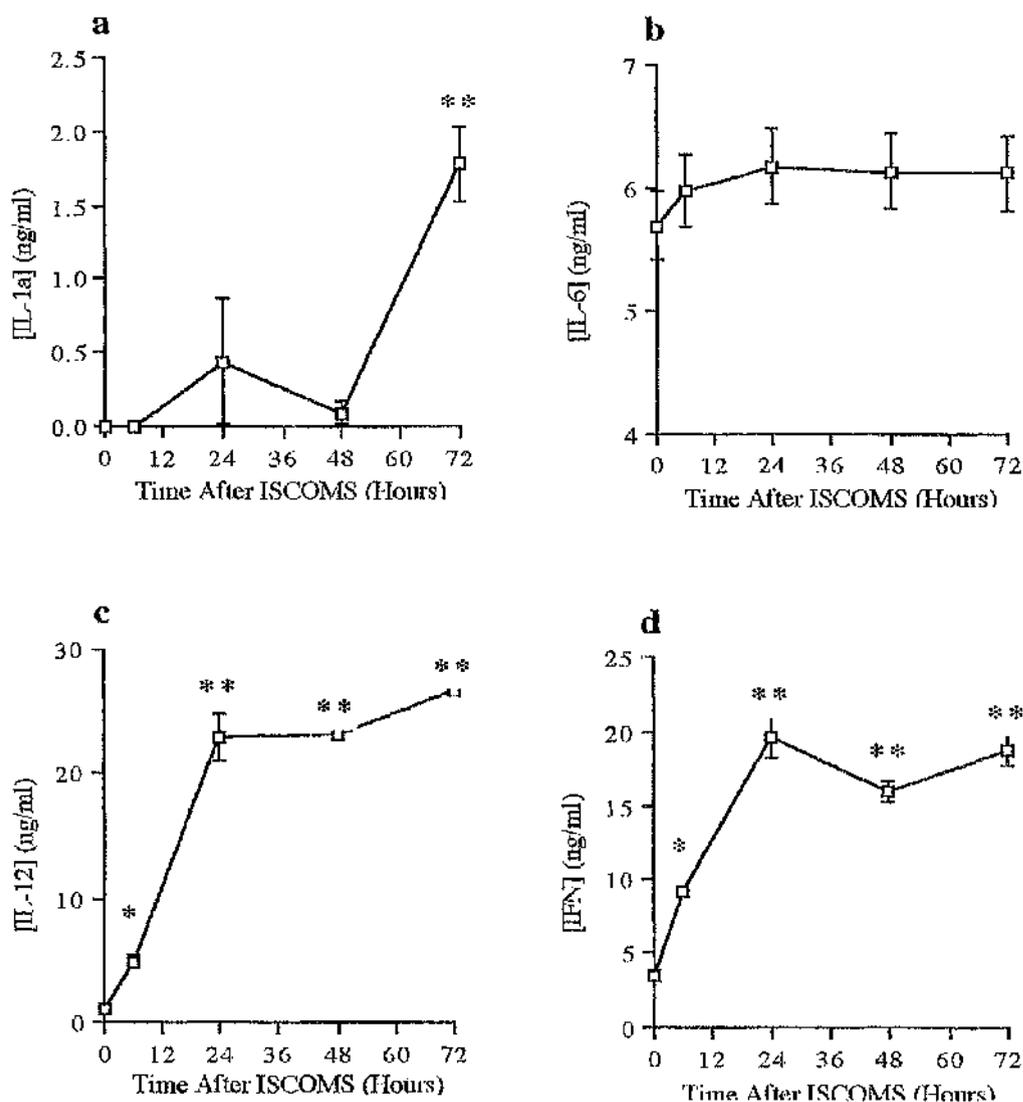


Figure 5.14 ISCOMS Induced Inflammatory Cytokine Production by PEC from IL-18KO mice.

Production of (a) IL-1 α , (b) IL-6, (c) immunoreactive IL-12 and (d) γ IFN by PEC pooled from 5 mice given an i.p. injection of 5 μ g ISCOMS 3.5-72 hours before. The results shown are the mean concentration of cytokines from IL-18KO mice, measured in triplicate after 48 hours stimulation in the presence or absence of γ IFN+LPS (a-c) or the presence or absence of Con A (d) \pm 1 SD (* $p < 0.05$ versus wild type mice; ** $p < 0.001$ versus wild type mice). Control mice were saline injected animals of each strain.

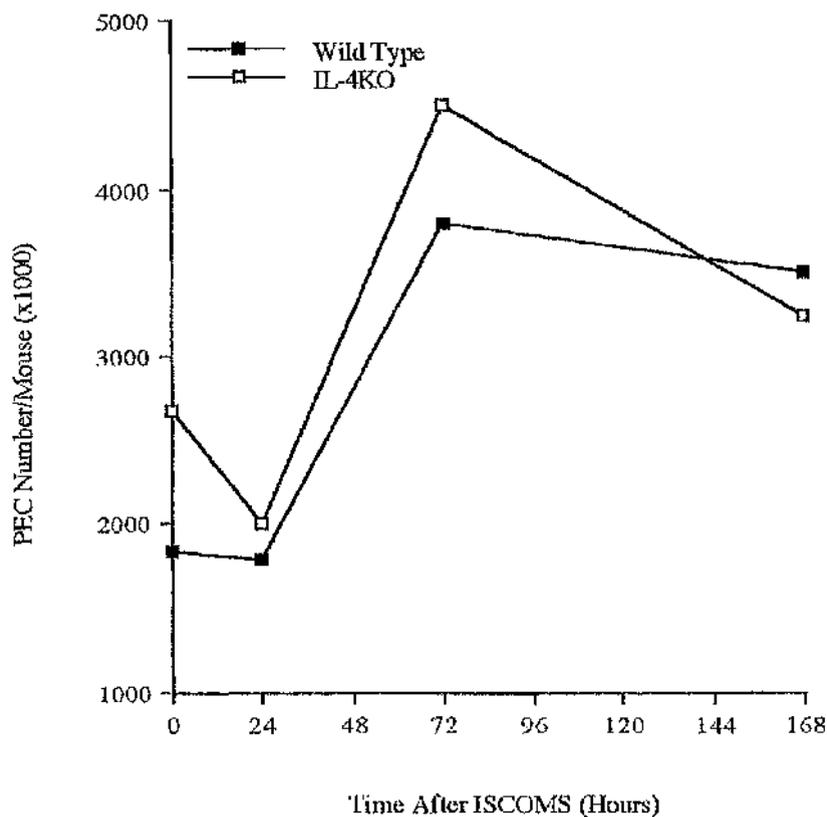


Figure 5.15 ISCOMS Induced Recruitment of PEC in IL-4KO Mice.

The graph shows PEC recruitment following an i.p. injection of 5µg OVA ISCOMS. PEC were removed from five mice by lavage, pooled and cell counts performed. The results shown are mean PEC numbers for wild type mice (closed squares) and IL-4KO mice (open squares). Saline injected mice from each strain were used as controls.

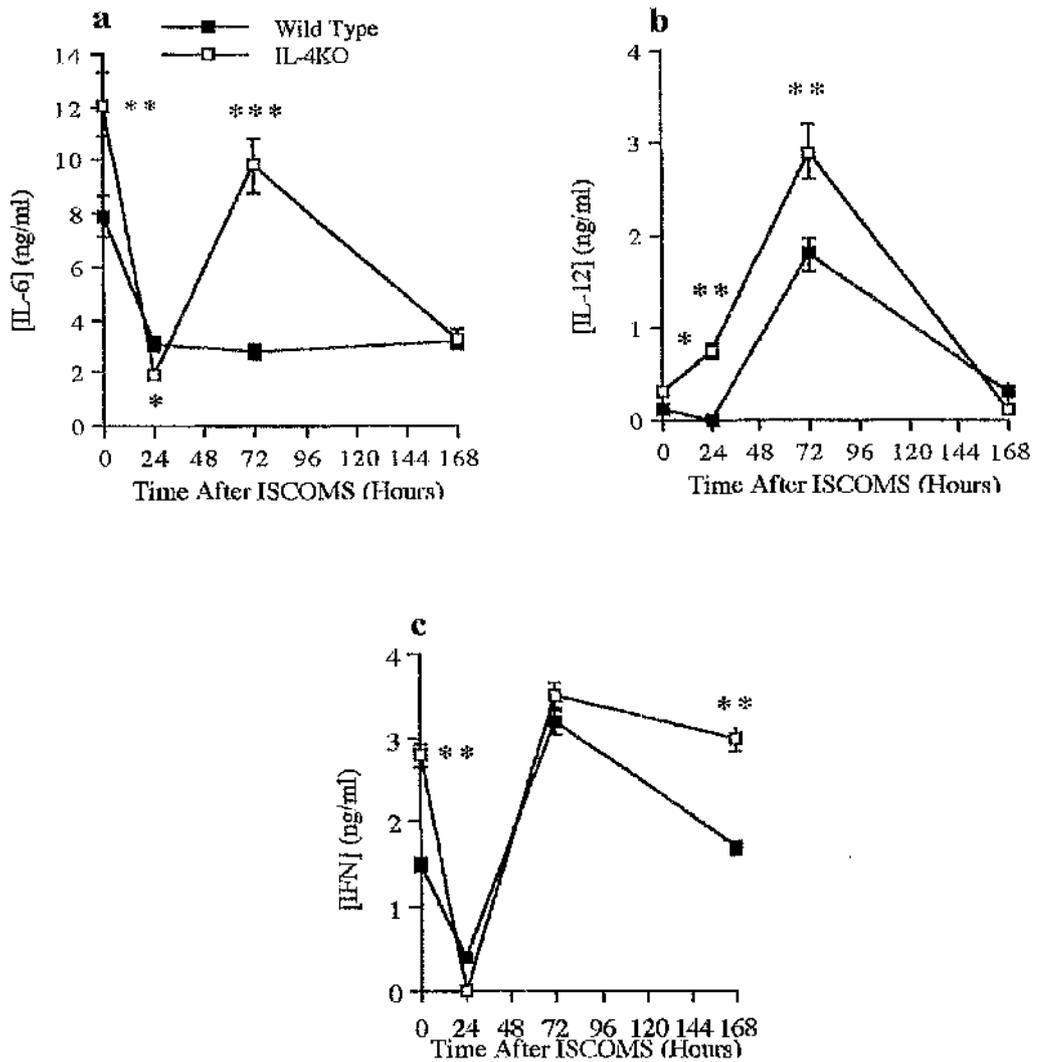


Figure 5.16 ISCOMS Induced Inflammatory Cytokine Production by PEC from IL-4KO Mice.

Production of (a) IL-6, (b) immunoreactive IL-12 and (c) γ IFN by PEC pooled from 5 mice given an i.p. injection of 5 μ g ISCOMS. The results shown are the mean concentration of cytokines from wild type mice (closed squares) or IL-4KO mice (open squares), measured in triplicate after 48 hours stimulation in the presence or absence of γ IFN+LPS (a-b) or the presence or absence of Con A (c) \pm 1 SD (* p<0.05 versus wild type mice; ** p<0.005 versus wild type mice; *** p<0.001 versus wild type mice). Control mice were saline injected animals of each strain.

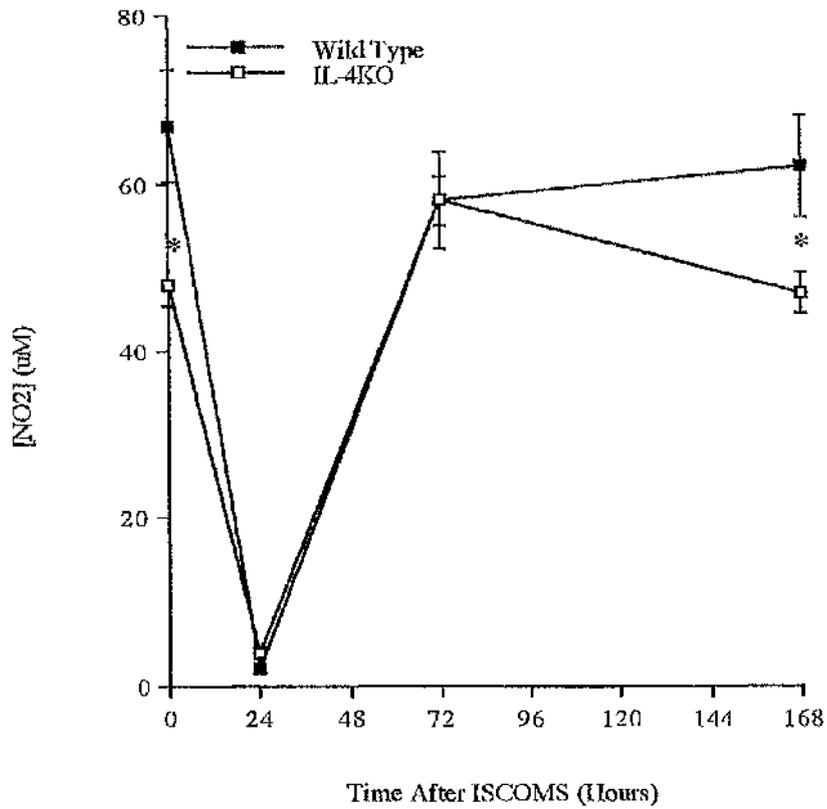


Figure 5.17 ISCOMS Induced Nitric Oxide Production by PEC from IL-4KO Mice

Production of nitric oxide from PEC pooled from 5 mice following injection of 5µg ISCOMS. The results shown are the mean concentration of nitrite measured in triplicate from wild type mice (closed squares) or IL-4KO mice (open squares), after culture for 48 hours in the presence or absence of γ IFN+LPS \pm 1 SD (* p<0.01 versus wild type mice). Control mice were saline injected animals of each strain.

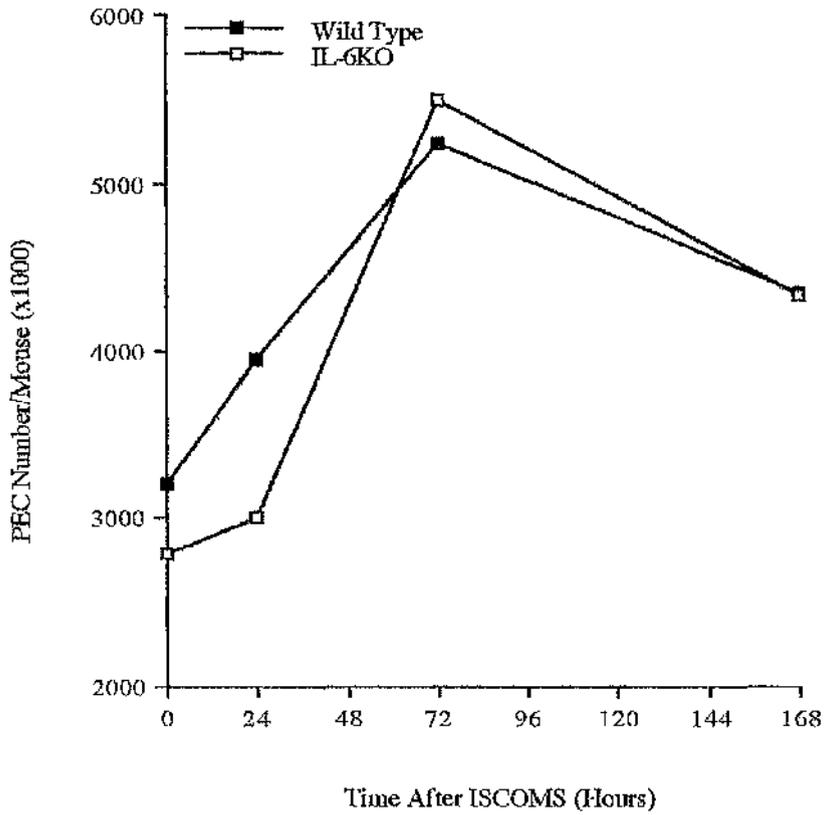


Figure 5.18 ISCOMS Induced Recruitment of PEC from IL-6KO Mice.

The graph shows PEC recruitment following an i.p. injection of 5 μ g OVA ISCOMS. PEC were removed from five mice by lavage, pooled and cell counts performed. The results shown are mean PEC numbers for wild type mice (closed squares) and IL-6KO mice (open squares). Saline injected mice from each strain were used as controls.

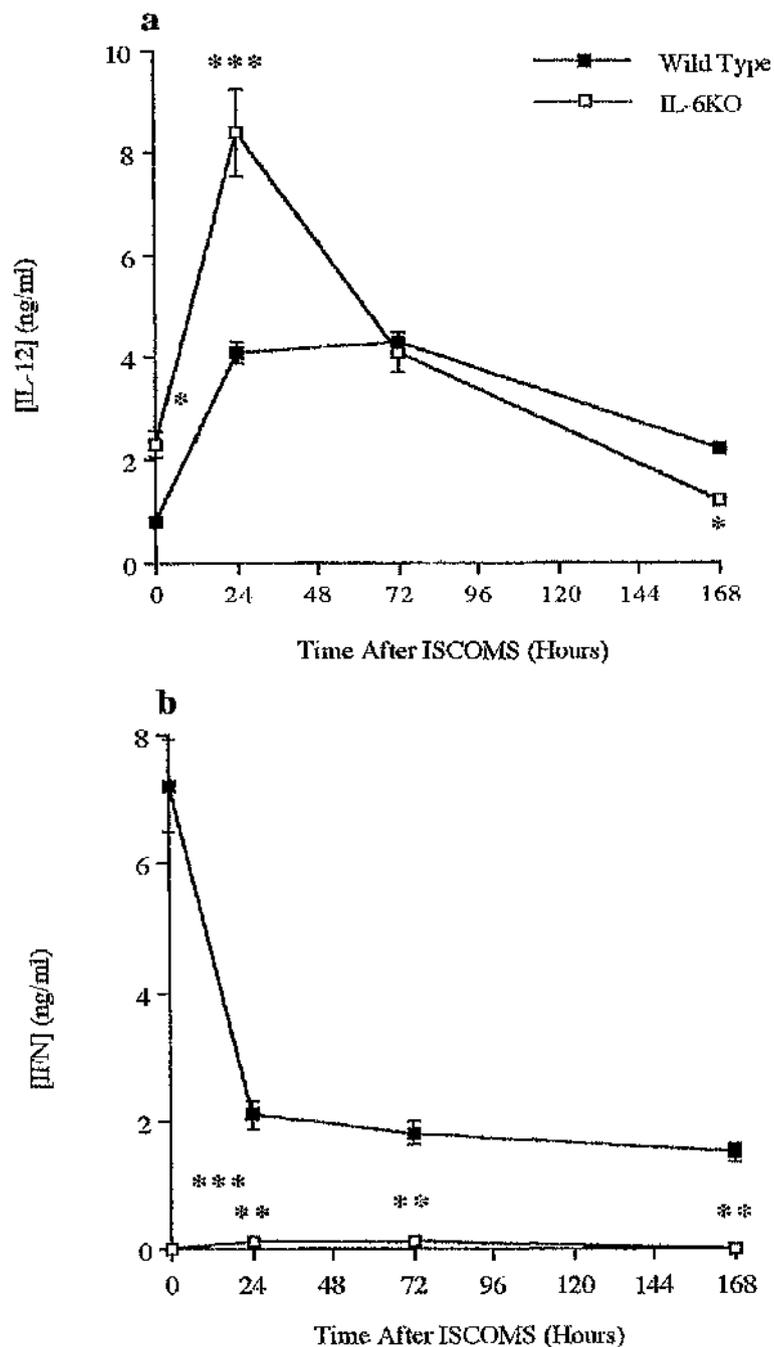


Figure 5.19 ISCOMS Induced Inflammatory Cytokine Production by PEC from IL-6KO Mice.

Production of (a) immunoreactive IL-12 and (b) γ IFN by PEC pooled from 5 mice given an i.p. injection of $5\mu\text{g}$ ISCOMS 3.5-72 hours before. The results shown are the mean concentration of cytokines from wild type mice (closed squares) or IL-6KO mice (open squares), measured in triplicate after 48 hours stimulation in the presence or absence of γ IFN+LPS (a) or the presence or absence of Con A (b) \pm 1 SD (* $p < 0.05$ versus wild type mice; * $p < 0.01$ versus wild type mice; *** $p < 0.001$ versus wild type mice). Control mice were saline injected animals of each strain.

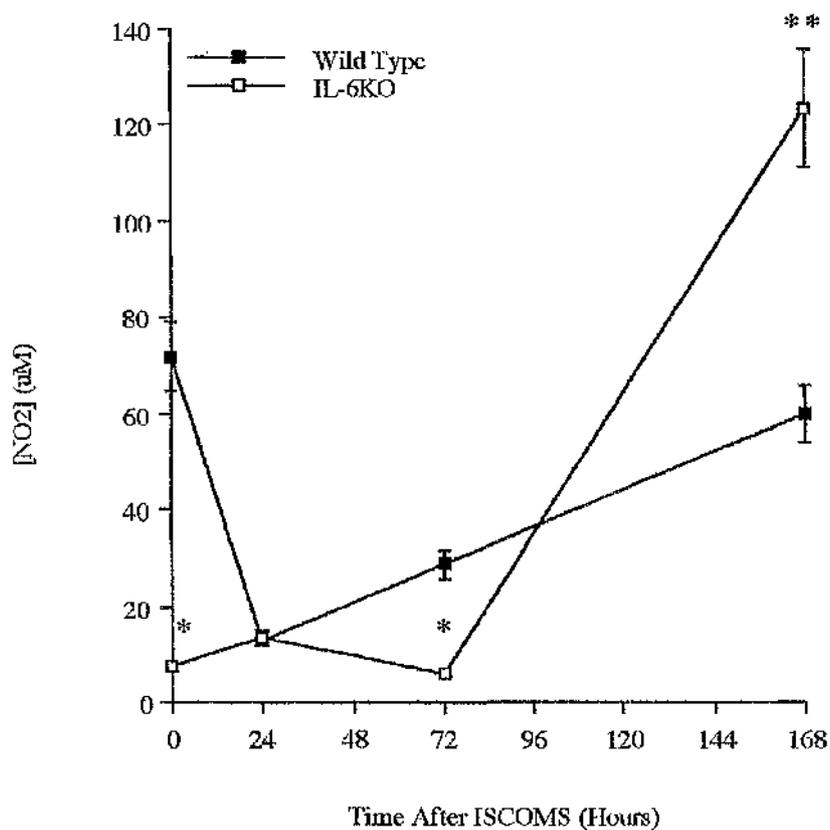


Figure 5.20 ISCOMS Induced Nitric Oxide Production by PEC from IL-6KO Mice

Production of nitric oxide from PEC pooled from 5 mice following injection of 5 μ g ISCOMS. The results shown are the mean concentration of nitrite measured in triplicate from wild type mice (closed squares) or IL-6KO mice (open squares), after culture for 48 hours in the presence or absence of γ IFN+LPS \pm 1 SD (* $p < 0.05$ versus wild type mice; ** $p < 0.005$ versus wild type mice). Control mice were saline injected animals of each strain.

Chapter 6

Discussion

Although ISCOMS have been used to stimulate antigen-specific immune responses by parenteral and mucosal routes, the mechanisms that underlie these adjuvant effects have not been elucidated. The results presented in this thesis have demonstrated that the adjuvant properties of ISCOMS are associated with an ability to stimulate an innate inflammatory cascade, and that only certain components of this response are essential for the antigen-specific effects of ISCOMS. My work therefore extends previous investigations into the non-specific properties of ISCOMS [Watson et al., 1989, Villacres-Eriksson et al., 1993, Behboudi et al., 1996, Behboudi et al., 1997, Villacres-Eriksson et al., 1997] and showed that IL-12 played a particularly important role in the immunogenicity of ISCOMS. A clearer understanding of how adjuvants like ISCOMS exert their effects may lead to more efficiently designed and effective vaccines that are active parenterally and orally.

My initial experiments examined which, if any, inflammatory effects were induced after an injection of ISCOMS. I decided to focus primarily on following the response after a parenteral injection of ISCOMS into the peritoneum, which provided an easily accessible and discrete site to collect and assess the functional activity of any cellular infiltrate. Once this protocol had been established, I then extended the work to include an assessment of the inflammatory responses occurring at mucosal sites after oral administration of ISCOMS. These experiments showed that ISCOMS induced a cascade of inflammatory events characterised by recruitment and activation of cells to the injection site and release of several inflammatory mediators. To assess the role of these factors in the generation of antigen-specific immune responses to immunisation of ISCOMS, I immunised gene-targeted knockout mice and monitored their specific immune responses as determined by parameters already established in the laboratory. These studies identified IL-12 as the only mediator of all of the factors induced by ISCOMS which was necessary for specific immune responses to ISCOMS. Responses were either completely normal or only partially defective in the absence of the other mediators.

Once this had been established, I then went on to determine how the same inflammatory cytokines influenced the inflammatory cascade induced by ISCOMS. In this case the innate cascade was lacking in both IL-12KO and γ IFN α 1bKO mice, despite the fact that antigen-specific immune responses were lacking only in the IL-12KO animals. This apparent dichotomy indicated that the role of the ISCOMS induced innate immune response in the generation of antigen-specific immunity is as complex as it is important and must be better understood if efficacious and potent vaccines are to be developed.

ISCOMS Induce an Inflammatory Cascade

Over the past few years, the view that the innate immune response was merely a retention of an immune mechanism utilised by primitive evolutionary ancestors, has been redefined. As a result, it has been realised that these non-specific inflammatory processes both initiate and co-ordinate the antigen-specific immune response by recruiting and activating immune cells, as well as providing appropriate cytokines and costimulatory molecules necessary for promoting adaptive immunity. The innate immune response therefore plays a number of crucial roles in the induction and shaping of protective immunity [Medzhitov and Janeway Jr, 1997a, Fearon and Locksley, 1996].

In Chapter 3, I showed that intraperitoneal injection of ISCOMS induced intense local activation of the innate immune response, which consisted of recruitment of a wide variety of inflammatory cells including neutrophils, mast cells, dendritic cells, macrophages and lymphocytes. Many of these cells were activated as evidenced by expression of surface activation markers and the production of cytokines and other mediators.

The first effect of injecting ISCOMS was a pronounced decrease in the numbers of PEC which was retained up to 48 hours after administration of ISCOMS. The saponin derivative Quil A has long been known to have haemolytic properties which are due to its ability to disrupt membranes by binding to cholesterol and inducing pore formation [Kensil, 1996, Ronnberg et al., 1997]. As ISCOMS have some of the haemolytic nature of Quil A [Gupta et al., 1993], the sudden decrease in total PEC cell numbers that occurred immediately

after administration of ISCOMS could be due to cell death because of the toxic effects of Quil A. However I found no evidence of cell death in PEC at any time after injection of ISCOMS as determined by histological examination. As this may have been because I did not examine PEC removed from mice at an early enough time after administration of ISCOMS, it would be important to assess PEC isolated at earlier timepoints. However, at present I did not find any evidence that cell death is responsible for the loss in numbers. An alternative mechanism which may explain this observed phenomenon was that ISCOMS induced some cells to leave the peritoneum and migrate to draining lymph nodes. Experiments to confirm this possibility should be part of any future work and could be performed by labelled PEC prior to injection of ISCOMS and then monitoring where these cells travel to after injection.

In the next 48 hours after injection of ISCOMS there was a large increase in the numbers of cells recovered from the peritoneum. This local inflammatory infiltrate consisted of an initial emigration of neutrophils, followed by macrophages and dendritic cells, and eventually lymphoid cells. Neutrophils are not normally present in the peritoneum, but at 3.5 and 7 hours after administration of ISCOMS, I observed a large increase in the numbers of polymorphonuclear cells. Histological and phenotypic studies identified that these were mostly neutrophils, confirming previous observations that neutrophils are recruited to the site of injection with ISCOMS [Watson et al., 1989, Watson et al., 1992]. In other models of inflammation, neutrophils are recruited by chemokines such as IL-8, neutrophil chemotactic factor (NCF), the C5a fragment of complement, prostaglandin D₂ (PGD₂) and leukotriene B₄ (LTB₄) [Baggiolini, 1995]. None of these factors were examined after injection of ISCOMS and this would be an important aspect of future studies.

The neutrophilia was accompanied by the recruitment of mast cells 6 hours after administration of ISCOMS, when there was morphological evidence of mast cell degranulation. A second, larger recruitment of mast cells was observed at 48 hours. It would be important to establish the functional implications of these findings by determining the production of mast cell products such as histamine, or mouse mast cell protease-1 (MCP-1). Together with their ability to produce arachidonic acid metabolites and preformed cytokines such as TNF α , mast cells could play an important role in initiating the early phase of the

inflammatory response. Mast cell products have been implicated as being important for the induction of several cell mediated immune responses *in vivo* such as DTH and contact sensitivity [Church and Levi-Schaffer, 1997], and also act as sources of chemotactic agents for other leukocytes. For example TNF α and LTB $_4$ are important for the recruitment of macrophages and monocytes [Wershil et al., 1991]. It is not clear how ISCOMS could recruit and activate mast cells, although mast cells do accumulate early in other forms of acute inflammation, and are known to be recruited by chemotactic signals such as C3 complement fragments [Prodeus et al., 1997]. Although the most usual way that mast cells are activated is by crosslinking of IgE bound to Fc ϵ R by multivalent antigen [Galli et al., 1999], this could not occur so quickly after administration of ISCOMS. Therefore, an alternative mechanism must be initiating the activation of these cells and one possibility is that a secretory factor produced by neutrophils or another cell type may induce degranulation. This is consistent with other findings that TNF α [Brzezinska-Blaszczyk and Pietrzak, 1997] and stem cell factor (SCF) [Wershil et al., 1992] were able to induce mast cell degranulation and may implicate neutrophils as the cells from which mast cell stimulators are released. However, the role of mast cells in the induction of specific immunity by ISCOMS remains to be clarified, although this could be achieved using mast cell deficient W/W^V mice.

Macrophages were next population to appear in the recruited PEC, reaching maximal numbers 48 hours after injection of ISCOMS. Macrophages were identified histologically on cytopins by their characteristic kidney-shaped nuclear morphology, and phenotypically by the expression of the pan metalophilic tissue macrophage marker F4/80. The macrophages may have been recruited to the injection site by neutrophil derived chemotactic factors such as macrophage chemotactic factor-1 (MCP-1) and TNF α . The ISCOMS recruited macrophages were shown to be activated both by their large and foamy morphological appearance and by their increased expression of MHC class II. These observations were consistent with other laboratories' findings of increased MHC class II expression after ISCOMS treatment [Watson et al., 1992, Bergstrom et al., 1990]. Despite this and in contrast to earlier findings of elevated CD86 on APC removed from ISCOMS vaccinated mice [Sambhara et al., 1998], in preliminary studies I found little or no alteration in CD40, CD80

or CD86 expression on ISCOMS recruited macrophages. This suggests that these cells may play an inflammatory role, rather than being directly involved in presenting antigen to T cells. This is consistent with evidence from this and other laboratories using silica or the liposomal toxin dichloromethylene diphosphate (Cl₂MDP) to block macrophage function, that macrophages are not important for the priming of most specific immune responses by ISCOMS [Maloy, 1996, Claassen et al., 1995]. Nevertheless, ISCOMS appear to be taken up efficiently by macrophages, presumably reflecting their particulate nature [Claassen et al., 1995, Claassen et al., 1995] and it has been shown that eliminating macrophages markedly inhibits the induction of OVA-specific CD8⁺ CTL responses *in vivo* [Maloy, 1996] as well as a reduction in viral specific CTL activity in other systems [Wijburg et al., 1997]. Thus the role of macrophages in the uptake and preservation of ISCOMS associated antigen needs to be examined directly.

Increased numbers of dendritic cells, determined as cells expressing CD11c were also observed in the recruited PEC population. Dendritic cells were present in low numbers in control PEC populations and disappeared soon after injection of ISCOMS. The loss of dendritic cells at early timepoints may be a result of cell death, or that dendritic cells are migrating away from the peritoneum after ISCOMS injection, which would be consistent with their proposed immune surveillance role. The number of dendritic cells then increased to a maximum at 72 hours, and these cells were mature as determined by their co-expression of MHC class II [Cella et al., 1997]. It is now considered that dendritic cells are the principle APC that can prime naive T cells, due to their efficient ability to take up and process antigen, as well as their expression of high levels of MHC class II and of costimulatory molecules such as CD40, CD80 and CD86 in conditions of inflammation [Gallucci et al., 1999]. I did perform preliminary experiments to explore the expression of CD80 and CD86 costimulatory molecules on ISCOMS recruited dendritic cells, but I did not detect any expression either before or after administration of ISCOMS at any timepoint. This could reflect that I looked at the wrong time and that the relevant dendritic cells were either recruited at later timepoints or had already left the injection area, and those I examined were recently recruited, immature cells. Recent studies conducted in the laboratory since I left support this latter possibility, as

they show that there is a very early increase in dendritic cell numbers at times before I looked, 1-2 hours after injection of ISCOMS and that PEC contain antigen-loaded APC at this time (Allan Mowat personal communication). In addition, other work using the cytokine flt3 ligand (FLt3L) to recruit dendritic cells, has shown that OVA-specific responses are enhanced in FLt3L treated mice immunised with ISCOMS (Allan Mowat personal communication). More work is clearly warranted to investigate fully the role of dendritic cells in the establishment of immune responses to ISCOMS.

At the same time that activated macrophages and dendritic cells were observed in PEC populations, lymphocyte numbers were also increasing. A small number of CD4⁺ T cells were identified in PEC from control mice, but within a few hours of administration of ISCOMS, this population decreased before steadily returning towards background levels by 48-72 hours. At this time a large percentage of CD4⁺ T cells were activated, as determined by their co-expression of the IL-2 receptor α chain (CD25). A similar pattern of CD8⁺ T cell recruitment was also observed, although the low numbers of these cells present, meant that it was not possible to assess their expression of CD25. As ISCOMS are strong inducers of CD8-restricted CTL activity [Mowat et al., 1991a, Heeg et al., 1991, Smith et al., 1998], it would have been useful to have examined this in more detail. The fact that lymphocytes were the last cell types to be recruited into the PEC suggested that these cells might include antigen-specific T cells destined to respond to the OVA incorporated in the ISCOMS. To examine this, I undertook preliminary experiments in which OVA-specific TcR transgenic CD4⁺ and CD8⁺ lymphocytes were transferred into syngeneic recipients, before i.p. injection with ISCOMS. These studies indicated that OVA-specific CD4⁺ and CD8⁺ T cells were part of the lymphocyte recruitment and that these cells showed signs of activation such as an increase in size (data not shown). More detailed timecourse studies of the appearance and functions of these transgenic T cells would provide information on the relationships between ISCOMS induced inflammation and the priming of specific T cells.

Although ISCOMS can prime antigen-specific antibody responses [Lovgren et al., 1988, Morein, 1991, Sjolander et al., 1996], little is known about how ISCOMS promote these responses. I found that ISCOMS also recruited B cells into the peritoneum, although

whether these cells were activated or producing OVA-specific antibody was not determined. It would also have been interesting to examine if the recruited B cells played any part in the induction of primary immune responses to ISCOMS, but μ MT KO mice lacking B cells were not available to examine this.

Oral administration of ISCOMS also induced an inflammatory infiltrate in the lymphoid tissues of the gut. However alterations of the cell populations in these tissues was less marked than in the peritoneum, perhaps indicating tighter regulation of potentially dangerous inflammation in this vulnerable organ, as well as the fact that the gut is not a closed compartment like the peritoneum. A single feed of ISCOMS did not induce the decrease in cell numbers in either PP or MLN that I had observed in PEC. Instead, cell numbers increased steadily in both tissues to peak at 48-72 hours. In addition there was no recruitment of neutrophils in either MLN or PP at any time after feeding ISCOMS, perhaps reflecting the fact that neutrophils are rare in these tissues. It may be important to control rigorously the recruitment of these potent inflammatory cells to such delicate tissues.

Despite the absence of neutrophilia in the gut, recruitment and activation of macrophages was still noted in both MLN and PP. The numbers of F4/80⁺ macrophages in MLN resembled the pattern of recruitment observed in PEC, with numbers increasing gradually to a peak almost 4 times that of controls at 48 hours. These cells were almost all activated as indicated by their co-expression of MHC class II. The profile of F4/80⁺ macrophage recruitment in PP was completely different, as it was characterised by an immediate increase at 7 hours and levels above background for the duration of the experiment. Again nearly all these macrophages expressed MHC class II. Thus neutrophils are not essential for the recruitment of macrophages into tissues in response to ISCOMS. MHC class II expressing mature dendritic cell numbers were increased in MLN, with the peak seeming at 48 hours after feeding ISCOMS. However, there was no increase in dendritic cell numbers in the PP and it would be interesting to determine whether this reflected emigration to the MLN by the time I examined the cells. Afferent lymph draining from the gut mucosa and PP contains many dendritic cells [Lui and MacPherson, 1995] which are

known to constantly migrate to the MLN, especially after exposure to inflammatory stimuli [MacPherson et al., 1995]. This could have accounted for the increase in dendritic cells numbers I found in the MLN after feeding ISCOMS.

MLN tissue showed an almost identical pattern of T cell recruitment as that found in PEC. CD8⁺ lymphocytes increased to a peak at 48 hours and CD4⁺ lymphocytes also increased gradually until a maximum at 72 hours, and by this time 15% were CD25⁺. In PP there was no real change in CD8 numbers after feeding ISCOMS, but a small increase in CD4⁺ T cells was observed at 3.5 hours and again at 72 hours, although these cells did not express CD25. B cell numbers were also increased in MLN after feeding ISCOMS, peaking by 48 hours. This pattern although smaller was also present in PP.

Together these results suggest that there is more recruitment of cells after feeds of ISCOMS in the MLN and they are consistent with the idea that the increased numbers of dendritic cells, lymphocytes and macrophages in MLN are derived from cells emigrating from the PP and the intestinal mucosa. However, this needs to be confirmed in more detailed timecourse studies using OVA-specific TcR transgenic T cells to define where and when ISCOMS first initiates inflammation and then activation of specific T cells. I did undertake preliminary studies to investigate the localisation of orally administered ISCOMS and these suggested that the ISCOMS could be detected rapidly in cells both in the PP and the intestinal LP. This partly which confirms other studies showing localisation of ISCOMS to the PP after feeding [Claassen et al., 1995], but I was unable to establish the identity of the cells involved. It would be of interest to determine the nature of these cells and their subsequent migration pathways *in vivo*.

Together, these results show that ISCOMS recruit many components of the innate and adaptive immune responses. This recruitment is not restricted to the periphery and although cellular changes are less pronounced in the GALT, the same pattern of recruitment is present at least in the MLN. I then went on to examine the functional significance of this inflammatory infiltrate, by exploring the pattern of inflammatory mediator secretion after administration of ISCOMS.

The Importance of ISCOMS Induced Inflammatory Mediator Production

The cytokines and other inflammatory mediators, produced during the early inflammatory response are critical for initiating and shaping the primary antigen-specific immune response [Fearon and Locksley, 1996b, Medzhitov and Janeway Jr, 1997a]. I therefore examined the pattern of inflammatory cytokine recruitment after administration of ISCOMS and also, how the absence of some of these affected antigen-specific and innate immune responses, using gene-targeted knockout mice.

Although previous studies had demonstrated that *in vitro* administration of ISCOMS induced IL-1 secretion from spleen cells [Villacres-Eriksson et al., 1993], other reports had suggested that no IL-1 was detected *in vivo* after administration of ISCOMS [Behboudi et al., 1996]. PEC from control mice did not secrete IL-1 α , but 48 hours after injection of ISCOMS, large quantities of IL-1 α were present in PEC supernatants. Interestingly, the amount of IL-1 produced by ISCOMS induced PEC was the same irrespective whether the PEC were stimulated with γ IFN+LPS or not, suggesting ISCOMS may have primed maximal amounts of IL-1 production. This IL-1 was dependent on the presence of adherent cells, consistent with the source being activated macrophages whose recruitment was also maximal at this time. I was unable to determine the role of IL-1 in ISCOMS mediated antigen-specific immunity, because no viable IL-1 deficient mice or depleting antibodies were available. However, this cytokine is an important component of the acute phase, regulating the recruitment and activation of a number of inflammatory cells, as well as increasing the expression of adhesion molecules on vascular endothelium and assisting in the activation of T cells. Thus a role for IL-1 in the induction of antigen-specific immune responses to ISCOMS cannot be excluded without direct investigation.

IL-6 production was also enhanced in ISCOMS stimulated PEC and again this correlated with maximal numbers of activated macrophages and was dependent on the presence of adherent cells. This observation confirms earlier findings that ISCOMS induced elevated serum IL-6 levels [Behboudi et al., 1997]. IL-6 is an important mediator for effector immune responses, having a role in the promotion of T cell proliferation in synergy with IL-1

[Houssiau and Van Snick, 1992] and also differentiation to Th2 CD4⁺ T cells [Rincon et al., 1997], as well as promoting B cell differentiation and antibody responses [Takatsuki et al., 1988]. Therefore I investigated the ability of ISCOMS to induce antigen-specific immune responses in IL-6KO mice. Previous studies in these mice showed them to have defective protective immune responses to intracellular infections such as vaccinia virus [Kopf et al., 1994], *L. major* [Kopf et al., 1994] and *C. albicans* [Romani et al., 1996], as well as defects in mucosal IgA secretion [Ramsay et al., 1994], IgG production and CTL activity [Kopf et al., 1994]. However, I found that IL-6KO mice developed entirely normal antigen-specific immune responses to parenteral immunisation with OVA ISCOMS, as determined by DTH, proliferation, CTL activity, cytokine and antibody profiles. The retention of antigen-specific immune responses in IL-6KO mice is consistent with other studies in these animals which showed normal responses to CT + KLH given orally or nasally and normal IgA responses to an infection of *Helicobacter felis* [Bromander et al., 1996]. I was unable to examine the status of secretory immunity in IL-6KO mice immunised orally with OVA ISCOMS due to the fact that these mice were no longer available to me. However, I can conclude that IL-6 is not required for the majority of responses induced by ISCOMS.

The innate immune cascade induced by ISCOMS was also normal in IL-6KO mice as far as I examined. IL-6KO mice had normal recruitment of total PEC and production of inflammatory mediators. However, in light of the previous reports of the defects in neutrophil recruitment that these mice exhibit [Kopf et al., 1994], it would have been interesting to undertake a phenotypic study of the PEC recruited after injection of ISCOMS, so as to clarify the importance of this cytokine for the recruitment and activation of individual inflammatory cells after injection of ISCOMS.

An i.p. injection of ISCOMS led increased production of γ IFN by Con A stimulated PEC. This showed a biphasic pattern, with an early but transient increase first occurring at 3.5 hours. After a subsequent fall, γ IFN production rose again to levels four times greater than baseline by 24 hours. As γ IFN activates transcription of the genes that encode the enzymes of the NADPH oxidase system [Cassatella et al., 1990], the early spike in γ IFN production may have been responsible for the production of H₂O₂ by neutrophils recruited to the peritoneum

after injection with ISCOMS. I did not determine the source of the ISCOMS induced γ IFN at either timepoint. Although the production of γ IFN required the presence of adherent cells, it is unlikely that these cells themselves are producing γ IFN. CD4⁺ T cells, CD8⁺ T cells and NK cells can all produce γ IFN [Heinzel et al., 1994]. However, NK cell derived γ IFN requires accessory cell derived cytokines such as IL-12 and TNF α [Tripp et al., 1993], which are both produced by among other cell types, adherent macrophages. CD4⁺ and CD8⁺ T cells were rare at the time of I found maximal levels of γ IFN. Thus it may be that NK cells were involved, an idea that is consistent with evidence from other systems in which NK cells producing γ IFN early in the immune response [Billiau, 1995]. One other possibility is the small subset of NK1.1⁺ CD4⁺ T cells which recognise lipid antigens derived from microbes in association with non-classical restriction elements such as CD1, and produce large amounts of IL-4 together with other cytokines such as γ IFN [Vicari and Zlotnik, 1996]. CD1 is capable of presenting non-peptidic antigens such as lipids and glycolipids [Porcelli et al., 1998], and as ISCOMS contain many lipid elements, it is feasible that these could be capable of binding to CD1. It would be interesting to define the cells producing γ IFN early after administration of ISCOMS, for example by intracellular cytokine staining.

γ IFN has a number of properties which could influence immune responses and inflammation induced by ISCOMS, including activation of phagocytic activity and increasing MHC class II expression on APC, the development of Th1 responses, the establishment of CTL activity [Roth et al., 1991] and switching to an IgG2a antibody isotype [Finkelman et al., 1988]. I therefore examined the requirement for γ IFN in immunity to parenterally and orally administered ISCOMS using γ IFNRKO mice. Surprisingly, I found that γ IFNRKO mice had normal or even increased DTH responses after immunisation with OVA ISCOMS. This is in contrast to other observations in the laboratory which had shown that γ IFNRKO mice had impaired responses to OVA and CFA [Mowat et al., 1999]. As I measured a classical DTH response which peaked at 24-48 hours after challenge, the reasons for this retention of DTH to OVA ISCOMS in the absence of functional γ IFN are unclear. However, it could reflect the ability of ISCOMS or Quil A to induce mediators which may compensate for the usual requirement of γ IFN. Thus it would be of interest to examine whether the DTH

response in γ IFN RKO mice is identical in all respects to that in normal animals and if it is affected by neutralisation of other cytokines. Overall, there was no evidence for a defect in antigen-specific immunity to OVA ISCOMS in γ IFN RKO mice. Although I found a decrease in OVA-specific proliferative responses after oral immunisation of these mice and others have reported defective mucosal immune responses to orally administered CT [Kjerrulf et al., 1997] and *Salmonella* in γ IFN RKO mice [Hess et al., 1996], other responses to oral OVA ISCOMS, were normal or increased, including sIgA production. In addition, all responses to OVA ISCOMS were normal after parenteral immunisation. Antigen specific proliferative responses were actually increased after subcutaneous immunisation with OVA-ISCOMS, a finding that may be due to the known cytostatic properties of this cytokine [Jeong, 1995, Hansson et al., 1989]. The cytokine profiles of both s.c. and orally immunised γ IFN RKO mice showed a greatly increased level of antigen-specific IL-5 production, consistent with the known ability of γ IFN to suppress Th2 dependent responses [Wange et al., 1994]. Similar findings were seen with the IgG1 and IgG2a antibody isotypes, as γ IFN RKO mice had suppressed IgG2a and elevated IgG1 antibodies. I also found that γ IFN RKO mice produced levels of γ IFN which were above that of the wild type mice, confirming earlier findings have been reported by others [Dalton et al., 1993]. This indicates that the production of γ IFN *in vivo* does not require γ IFN signalling and suggests that γ IFN may normally down-regulate its own production. As γ IFN has been identified as a potential cofactor for the generation of CD8 restricted CTL activity [Roth et al., 1991], it was somewhat surprising that CTL responses were normal in OVA ISCOMS immunised γ IFN RKO mice, or even increased. These results indicate that other factors must be involved in the generation of OVA-specific CTL by immunisation with ISCOMS.

Despite the mostly normal specific responses in γ IFN RKO mice, the development of a full innate inflammatory cascade was dependent on the presence of functional γ IFN, as γ IFN RKO mice failed to generate normal cellular recruitment after injection of ISCOMS. These mice showed reduced recruitment of neutrophils, lowered recruitment of B220⁺ lymphocytes and CD4⁺ lymphocytes and no recruitment of macrophages. However, relatively normal CD8 recruitment was noted and an increase in dendritic cell numbers was

also observed. In addition to the defective inflammatory responses seen in γ IFN^{RKO} mice, the inflammatory cells that were recruited showed virtually no evidence of activation as assessed by expression of cell surface markers, or the secretion of inflammatory cytokines and mediators. As antigen-specific immune responses to ISCOMS were normal after both parenteral and oral immunisation of IFN^{RKO} mice, the remaining innate responses in these animals may provide enough of an innate trigger to induce adaptive immunity. Alternatively other factors whose activity I did not measure in γ IFN^{RKO} mice given ISCOMS, may have been important, such as mast cells. It is possible that a relatively normal range of innate responses did occur in these mice, but that these were delayed beyond the 72 hour timecourse that I examined. Thus a longer time course measuring more innate factors is warranted in these animals to attempt to understand the dichotomy between the innate and adaptive responses to ISCOMS in the absence of functional γ IFN.

The cytokine for which I obtained the most conclusive evidence as being involved in the immunogenicity of ISCOMS was IL-12. PEC from ISCOMS injected mice showed elevated IL-12 production at 72 hours, extending recent findings of elevated serum IL-12 levels after injection of ISCOMS [Villacres-Eriksson et al., 1997]. The increased levels of IL-12 in PEC and PP coincided with the appearance of cells such as macrophages, dendritic cells and B cells, all of which can secrete IL-12 [Trinchieri, 1995]. The production of IL-12 *in vitro* required the presence of adherent cells and recent studies have indicated that ISCOMS can directly stimulate production of IL-12 mRNA from PEC adherent cells [Grdic et al., 1999], suggesting an involvement of macrophages in this process. It would be interesting to determine the source of this IL-12 more directly, for example by observing coexpression of internal IL-12 in phenotypically labelled cells either by flow cytometry or immunohistochemistry *in vivo* [Reis e Sousa et al., 1997]. Although ISCOMS may indeed induce IL-12 production directly from macrophages, recent studies suggest that a substantial proportion of IL-12 *in vivo* is derived from dendritic cells, often requiring activation by CD40-CD40 ligand (CD40L) interactions with antigen-specific T cells [Stuber et al., 1996, Kennedy et al., 1996]. This could be explored by a combination of the approaches described above, together with the use of CD40LKO mice.

When I examined the requirement of IL-12 in antigen-specific immune responses to ISCOMS, I found that IL-12KO mice had substantial defects in these responses. This defect was more pronounced in parenterally immunised mice, but was also present after oral immunisation. In contrast to the published evidence that IL-12KO mice have only selective defects in Th1 and CD8 T cell activity [Trinchieri, 1995], I found that virtually all immunological parameters were decreased in IL-12KO mice immunised with OVA ISCOMS. In addition to the expected defects in DTH, γ IFN production and CTL activity, I also found that there was a 40% reduction in OVA-specific proliferation in IL-12KO mice. This may reflect the role of IL-12 in inducing the expression of the IL-2R α and β subunits [Robertson et al., 1992, Nuame et al., 1992], an idea supported by the my observation that IL-12KO mice failed to increase expression of CD25 on peritoneal CD4⁺ T cells following administration of ISCOMS. More surprisingly, s.c. immunised IL-12KO mice also had defective IL-5 production, supporting further the possibility that IL-12 plays an important role in the priming of all T cells, rather than just Th1 cells. Nevertheless, it should be noted that IL-12KO mice had a selective defect in IgG2a responses, whereas the Th2 dependent IgG1 response was normal or even enhanced. This supports other studies which have revealed that IL-12 regulates the production of IgG isotypes after immunisation [McKnight et al., 1994, German et al., 1995, Buchanan et al., 1995] IL-12KO mice also had a 50% reduction in CTL activity compared with wild types after i.p. immunisation, extending previous studies on the role of IL-12 in the generation of antigen-specific CTL [Abdi and Herrmann, 1997] and the increased susceptibility of these animals to intracellular pathogens such as *Leishmania major* [Mattner et al., 1996], *Mycobacterium tuberculosis* [Cooper et al., 1997] and *Listeria monocytogenes* [Gateley et al., 1998]. Thus IL-12 plays an important and wide ranging role in the induction of systemic immune responses by parenterally administered ISCOMS. However, its influence on the mucosal immune responses seemed less, with entirely normal secretion of intestinal IgA antibodies in IL-12KO mice. This may indicate that the GALT may use alternative mechanisms for generating inflammation, to compensate for the lack of IL-12.

In addition to the defective antigen-specific systemic immune response to ISCOMS in IL-12KO mice, these animals also had a severely impaired innate response to ISCOMS, with low levels of recruitment of neutrophils, macrophages, CD4⁺ or CD8⁺ T cells and B cells, being the only changes observed. The macrophages and CD4⁺ T cells which were recruited also failed to show evidence of activation, such as production of inflammatory mediators and expression of MHC class II or CD25 respectively. Interestingly, the baseline numbers of dendritic cells were increased in these mice, although no additional recruitment of these cells was observed, indicating that IL-12 may in some way regulate proliferation of dendritic cells. The marked lack of an inflammatory cascade in IL-12KO mice could reflect an absence of chemotactic factors such as IL-8 whose production may be dependent on IL-12 [Nuame et al., 1993]. The lack of MHC class II on macrophages presumably reflects the expected absence of γ IFN, while the absence of CD25 on T cells supports the suppressed proliferative responses in ISCOMS immunised IL-12KO mice. Despite the severely altered innate immune responses to ISCOMS, it is important to note that antigen-specific responses were not completely lost in IL-12KO mice, indicating that there are other factors which govern the generation of adaptive immunity in the absence of IL-12. These may be factors which I did not measure, such as mast cells and other inflammatory mediators and chemokines, which can replace in part, the function of IL-12. One candidate for this is IL-18, which has many properties that are similar to IL-12, including the generation of NK activity and γ IFN secretion [Kohno and Kurimoto, 1998], and is also secreted from activated macrophages [Okamura et al., 1998b].

As a suitable ELISA did not become available until the end of my project and I was unable to establish PCR methods for assessing IL-18 mRNA levels, I could not determine whether ISCOMS stimulated the production of IL-18 *in vitro*. However, during the course of my work, IL-18KO mice were developed in the department and I was able to perform a preliminary experiment in which specific immune responses were examined in the absence of IL-18. However, conflicting results were obtained. Although, homozygous IL-18^{-/-} mice had dramatically reduced antigen-specific DTH responses after subcutaneous immunisation and a partial decrease was also noted in heterozygous IL-18^{+/-} mice, other responses were normal.

S.c immunised IL-18KO mice had normal OVA-specific IL-5 and more surprisingly, normal γ IFN responses, contrasting with earlier findings that IL-18 is closely implicated in the induction of γ IFN production [Okamura et al., 1998a, Kohno and Kurimoto, 1998]. Similarly all of the IL-18KO mice also had normal IgG1 and IgG2a antibody responses. When these mice were immunised orally, conflicting data were also obtained. In contrast to s.c. immunised mice, homozygous and heterozygous IL-18KO mice had normal DTH and antigen-specific proliferative responses, but had severely defective OVA-specific γ IFN secretion and enhanced IL-5 production. This pattern supports the role of this cytokine in the establishment of Th1 cell activity but contrasts with my earlier observations in s.c. immunised animals. Orally immunised IL-18KO mice also developed normal OVA-specific cytotoxicity, as well as IgG1 and IgG2a antibody responses. These results need confirmation in a larger series of studies in IL-18KO mice.

I also undertook a preliminary experiment to explore the ISCOMS induced inflammatory cascade in IL-18KO animals. Although no wild type animals of the appropriate strain were available at the time I carried out this experiment and the results must be interpreted with caution, but i.p. injection of IL-18KO mice with ISCOMS did not induce the usual pattern of cellular recruitment that I found in previous experiments in wild type mice. There was no increase in total PEC numbers and there was a failure to recruit neutrophils, CD4⁺ T cells and macrophages. There was also no evidence of increased MHC class II expression on macrophages, or of CD25 expression on CD4⁺ T cells. However there were elevated numbers of CD8⁺ T cells, B cells and mature dendritic cells. These features were somewhat similar to those I found in IL-12KO and γ IFNRKO mice. Nevertheless, PEC from ISCOMS injected IL-18KO mice had some evidence of functional activity, as they produced increased amounts of IL-1 α , IL-12 and γ IFN, although IL-6 did not increase above control levels. Again it would be important to repeat these studies using more IL-18KO and wild type mice in parallel. However, my preliminary results suggest that the role of IL-18 in the innate and adaptive responses to ISCOMS warrants further study, both by more extensive confirmation of my findings, as well as by determining if IL-18 is induced by ISCOMS as

part of an early innate cascade. It would also be useful to determine if IL-12 and IL-18 play overlapping roles using IL-12 x IL-18 double knockout mice.

IL-4 has potent immunomodulatory properties, including the activation or inhibition of APC activity, as well as promoting the differentiation of Th2 cells [Noble et al., 1993, Nabors and Farrell, 1994]. As my results confirmed, previous work in the laboratory had shown that ISCOMS were capable of inducing antigen-specific Th2 responses [Maloy et al., 1995, Maloy, 1996] and others had shown that mucosal adjuvant properties of CT were dependent on IL-4 [Vajdy et al., 1995]. Therefore I attempted to assess whether IL-4 was important for the induction of immune responses by ISCOMS, first by measuring the secretion of IL-4 immediately after injection of ISCOMS. However, I could not detect any IL-4 protein in supernatants from PEC taken from ISCOMS injected mice by ELISA. Therefore I tested the ability of ISCOMS to induce specific immune responses by oral and parenteral routes in IL-4KO mice.

IL-4KO mice immunised s.c. with OVA ISCOMS produced antigen-specific DTH responses above that of wild type mice, and orally immunised IL-4KO mice retained normal DTH responses. OVA-specific proliferation was increased both in s.c and orally immunised IL-4KO mice, and this normal or enhanced response may be consistent with the ability of IL-4 dependent Th2 cells to suppress Th1 dependent immune responses normally [Maggi et al., 1992]. As expected, both s.c and orally immunised ISCOMS immunised IL-4KO mice had elevated production of OVA-specific γ IFN, and s.c. immunised IL-4KO mice produced significantly less IL-5 than wild types. Although orally immunised IL-4KO mice also produced low levels of IL-5, surprisingly these were higher than those in wild type animals. This may be consistent with early reports that IL-5 production could be partially preserved in IL-4KO mice [Kuhn et al., 1991, Kopf et al., 1993]. I found that antigen-specific CTL responses were also retained in IL-4KO mice after both oral and parenteral immunisation at levels similar to or greater than those in the wild type mice, despite evidence that IL-4 may be a growth factor for CTL *in vitro* [Liang et al., 1992, Horvat et al., 1991]. However, this is the first study to examine antigen-specific CTL induction in IL-4KO animals *in vivo* and the preserved activity may reflect the ability of other cytokines such as IL-2 [Horvat et al., 1991,

Ley et al., 1991], γ IFN [Roth et al., 1991] and IL-15 [Ye et al., 1996] to compensate as CTL growth factors *in vivo*. Th2 cells and, in particular IL-4, have also been associated with regulating the production of sIgA in the intestine [Murray et al., 1987, Harriman et al., 1988], but I found that the total sIgA antibody production was actually enhanced in IL-4KO mice compared with controls. These results contrast with the defective IgA responses found in IL-4KO mice immunised orally with antigen and cholera toxin (CT) [Vajdy et al., 1995], but similar findings of normal mucosal responses in the absence of IL-4 have been made using *Salmonella* as a mucosal adjuvant [Okahashi et al., 1996]. The local immune response to CT itself is also intact in IL-4KO mice [Vajdy et al., 1995]. The innate immune responses I measured in IL-4KO mice were also normal after injection of ISCOMS indicating that IL-4 is not essential for either specific or innate immune responses induced by ISCOMS. However, this may be due to the activity of compensatory cytokines such as IL-6, IL-10 or TGF β [Okahashi et al., 1996]. As described previously, I investigated the role of IL-6 in the generation of antigen-specific immune responses to ISCOMS and found it not to be required. Therefore it would be important to establish the role of IL-10 and TGF β , in compensating for the absence of IL-4. IL-13 would be a further important molecule to examine shares many of the biological functions of IL-4 [Chomarat and Banchereau, 1998]. Its role could be determined using IL-4 x IL-13 double knockout mice or in IL-4R α KO mice [Mohrs et al., 1999].

An i.p. injection of ISCOMS produced large increases in the respiratory burst, as measured by H₂O₂ and also increased NO production. The production of oxygen radicals and H₂O₂ are crucial innate mechanisms for the control of intracellular microbes [Clark, 1999] and are properties of activated macrophages and neutrophils. After injection of ISCOMS, the peak production of ROI coincided with the maximal neutrophilia and occurred at a time when other potential ROI producing cells were virtually absent from the PEC population. In parallel, there was no neutrophil recruitment or ROI production in tissues of the GALT after feeding ISCOMS. These results suggest that neutrophils are the source of ROI after injection of ISCOMS and confirm that these cells are activated.

Although it is unlikely that these ROI could contribute to the induction of specific immunity to ISCOMS, nitric oxide was also increased after injection of ISCOMS. This corresponded with the maximum recruitment of activated macrophages and the production of NO was totally dependent on the presence of adherent cells, supporting other evidence that activated macrophages are the major source of iNOS derived NO [Nathan and Hibbs, 1991]. The peak production of NO also coincided with the maximum secretion of γ IFN which is a known promoter of iNOS activity [Xie et al., 1992] and its rapid loss may reflect the fact that NO can regulate its own production [Huang et al., 1998]. There was no NO production was observed in the gut after feeding ISCOMS, perhaps because the repeated exposure to low doses of LPS, which occurs in the gut may cause a state of unresponsiveness of iNOS induction which has been demonstrated in macrophages pre-incubated with small concentrations of LPS *in vitro* [Severn et al., 1993]. Mice deficient in iNOS have been shown previously to be susceptible to a range of intracellular and extracellular infections such as *L. major*, herpes simplex virus [McClellan et al., 1998], as well as having decreased susceptibility to endotoxic shock [Wei et al., 1995]. In addition to this critical role as a microbicidal factor, other reports have suggested that NO may also influence the outcome of antigen-specific immune responses. First, NO can decrease the expression of MHC class II [Sicher et al., 1994] as well as the proliferation of T lymphocytes [Wei et al., 1995]. In addition, it can down regulate the production of γ IFN *in vitro* and promote a more Th2 like T cell phenotype [Huang et al., 1998]. Thus I attempted to examine specific immune responses to ISCOMS in a small cohort of iNOSKO mice that had been generated in the department. In these preliminary studies, OVA-specific DTH responses were normal after s.c. immunisation, as was antigen-specific proliferation as well as IgG1 and IgG2a antibody production. However, iNOSKO mice had markedly reduced levels of both OVA-specific γ IFN and IL-5 production. Thus it appeared that the loss of functional NO may have had some minor effects on the induction of antigen-specific immunity by ISCOMS, but no clear pattern was observed and it would be important to extend these studies when additional mice become available.

When I examined the inflammatory cytokine secretion in GALT after oral administration of ISCOMS a markedly different pattern was observed that compared with that

seen in PEC cells removed after an i.p. injection of ISCOMS. I was unable to detect IL-1 α production in either MLN or PP tissues at any time, IL-6 production was reduced in MLN and absent in PP cells, IL-12 production was reduced in MLN after administration of oral ISCOMS but a small but not significant increase in IL-12 was seen in PP, γ IFN production was also reduced in MLN and also PP. Apart from the elevated levels of IL-12 in γ IFN+LPS induced PP cells, I did not observe any increase in inflammatory mediator secretion. This is surprising given that there are populations of activated macrophages recruited into both tissues after feeding ISCOMS, although at a lower level that was observed in PEC. The lack of cytokine production may reflect a more tightly regulated inflammatory response in the GALT, or that there are different populations of macrophages secreting different cytokines in different tissues. Therefore, it would be important to assess if feeding ISCOMS induce inflammatory mediator secretion from other GALT tissues such as LP or epithelium, and this could be performed quickly by RT-PCR.

The dichotomy between the specific and innate immune responses induced by ISCOMS in IL-12KO mice and γ IFNRKO mice may provide some clues to how inflammatory mediators regulate the establishment of antigen-specific immunity. Although the defective antigen-specific immunity to OVA ISCOMS in IL-12KO mice was accompanied by a decrease in innate immunity, γ IFNRKO mice had normal or even enhanced antigen-specific immune response to OVA ISCOMS, but almost completely absent innate immunity. The only difference in the innate immune responses found in the two strains was that γ IFNRKO mice retained some recruitment of B cells and an early increase in CD11c⁺ dendritic cells, suggesting that one of these cell types could be involved in the induction of immunity to ISCOMS. Although both can produce IL-12, this appears to be a function mostly of dendritic cells *in vivo* [Trinchieri, 1995]. In addition, dendritic cells are generally considered to be the predominant APC able to prime T cells [Gallucci et al., 1999]. Nevertheless, B cells are also capable of acting as APC, under some conditions [Yang and Brunham, 1998, Evans et al., 2000] and it has been shown previously that splenic B cells can present ISCOMS associated antigen to T cells *in vitro*, almost to the same extent as dendritic

cells [Villacres-Eriksson, 1995]. As noted earlier, the adjuvant effects of another mucosal adjuvant, CT and its derivatives, appear to be dependent on B cells [Vajdy et al., 1995]. In most cases, no role for B cells in priming T cells has been identified but this needs to be tested directly in the case of ISCOMS associated antigen using B cell KO mice. There are no specific models of dendritic cell deficiency *in vivo* available, but an alternative approach is to expand the dendritic cell numbers using the selective growth factor Flt3L. Preliminary experiments suggest that Flt3L does enhance immune responses and the activation of antigen-specific T cells after immunisation with ISCOMS (Allan Mowat personal communication). Therefore dendritic cells may be important in the establishment of ISCOMS immunity.

One cell that my studies seems to exclude in being central to the induction of immune responses to ISCOMS is the macrophage, as both IL-12KO and γ IFNRKO mice had equally deficient activated macrophages in PEC after injection of ISCOMS, although γ IFNRKO mice had normal antigen-specific immunity despite this. In other studies, ISCOMS have been seen to accumulate within marginal metallophilic macrophages (MMM) in the spleen after i.v. injection [Claassen et al., 1995]. Depletion of these macrophages *in vivo* ablated T cell proliferative responses in the spleen, but did not significantly alter humoral immunity to ISCOMS [Claassen et al., 1995]. Macrophages are known to preferentially take up particulate antigens and have excellent antigen-presenting properties. Therefore the particulate nature of ISCOMS may target them to macrophages and also in the process of phagocytosis, activate them. In the presence of ISCOMS, macrophages have been shown to increase expression of MHC class II *in vitro* [Watson et al., 1992, Bergstrom et al., 1990], and I observed this phenomenon *in vivo*. However, in this laboratory, ablation of macrophages either by silica or toxin loaded liposomes did not significantly alter the generation of cell mediated responses [Maloy, 1996] which parallels my findings in the γ IFNRKO mice.

In order to address the mechanism by which ISCOMS exert their adjuvant effect several questions need to be addressed. Firstly, how do ISCOMS gain access to APC? One possibility is that ISCOMS directly associate with APC by binding to and stimulating receptors on the cell surface. As ISCOMS are very rich in carbohydrates because of the fact

that Quil A contains a mixture of triterpenoids [Higuchi et al., 1988], it is feasible that these may bind to receptors such as the mannose receptor found on macrophages and dendritic cells and thus be internalised [Aderem and Underhill, 1999]. Alternatively, because of their lipophilic nature, it is also possible that ISCOMS may simply diffuse through the plasma membrane and into cells.

The second important question is how do ISCOMS establish the early inflammatory response? Although, at the times I examined PEC histologically, there was no evidence of cell debris or dead cells, it is possible that the haemolytic nature of ISCOMS causes cell lysis, with release of inflammatory cytoplasmic contents into the local micro-environment, as well as ISCOMS particles themselves. This could recruit inflammatory cells, as well as dendritic cells, which have been shown recently to be able to phagocytose necrotic cells including macrophages followed by the priming of specific immune responses [Gallucci et al., 1999], may then be attracted to this site and phagocytose cell debris and ISCOMS. This possibility has recently been emphasised by observations of increased numbers of dendritic cells within 1 hour of administration of ISCOMS (Allan Mowat personal communication), which may reflect the recruitment of these cells very early to clear cell debris.

Thus I propose that ISCOMS are internalised by macrophages *in vivo* and that these cells are lysed due to the haemolytic nature of Quil A. The subsequent release of inflammatory cytoplasmic material, together with intact ISCOMS induces the release of chemoattractants and chemokines, and consequently the recruitment and degranulation of local mast cells followed by the release of other inflammatory molecules such as histamine. This process recruits further inflammatory cells which include dendritic cells and macrophages, which process and present the ISCOMS associated antigen and are induced to secrete large quantities of IL-12. The resulting release of γ IFN then positively feeds back to increase the activation of macrophages and elevate still further the expression of surface MHC class II. IL-12 is also a direct stimulus to T cells and can induce the activity of CD8⁺ T cells. As a result, I predict that IL-12 derived possibly from dendritic cells may be the central factor in the ability of ISCOMS to prime such a wide range of specific immune responses in mice.

In conclusion, my results confirm and extend previous reports that ISCOMS generate wide ranging inflammatory cascades comprising recruitment of cells and release of inflammatory mediators. In addition, the mucosal and systemic immunogenicity of ISCOMS encompasses a wide range of immune effector responses, including both Th1 and Th2 dependent responses. However, the antigen-specific responses to ISCOMS are not limited by the absence of the majority of these innate factors thought to be critical for initiating these responses, γ IFN, IL-4, IL-6 and iNOS respectively, but these results do indicate that there is a role for ISCOMS induced IL-12 and to a lesser extent IL-18 in the development of antigen-specific immune responses following ISCOMS immunisation. These findings distinguish ISCOMS from other mucosal adjuvant vectors such as *Salmonella* or CT and underline the complexity of the factors which may determine the induction of immune responses via mucosal surfaces. My results have identified key components of the innate immune response, namely IL-12, whose presence may be crucial for the establishment of protective immunity by ISCOMS. The identification of such components has led to a better understanding of the mechanism by which ISCOMS exert their adjuvanticity, and hopefully may allow for their better exploitation in practical vaccine studies in the future.

Chapter 7

References

- Abdi, K. and Herrmann, S. H. (1997) CTL generation in the presence of IL-4 is inhibited by free p40: evidence for early and late IL-12 function. *Journal Of Immunology*, **159**: 3148.
- Aderem, A. and Underhill, D. M. (1999) Mechanisms of phagocytosis in macrophages. *Annual Review of Immunology*, **17**: 593.
- Adolf, R.A., Grell, M. and Scheurich, P. (1994) Tumour necrosis factor, In *Epidermal Growth Factors and Cytokines*(Eds, Luger, T. A. and Schwarz, T.) Marcel Dekker Inc., New York, pp. 63.
- Agren, L.C., Ekman, L., Lowenadler, B. and Lycke, N.Y. (1997) A genetically engineered nontoxic vaccine adjuvant that combines B cell targeting with immunomodulation by Cholera toxin A1 subunit. *Journal of Immunology*, **158**: 3936.
- Appelberg, R., Castro, A.G., Pedrosa, J. and Minoprio, P. (1994) Role of interleukin-6 in the induction of protective T cells during mycobacterial infections in mice. *Immunology*, **82**: 361.
- Araujo, F.G. and Morein, B. (1991) Immunization with *Trypanosoma cruzi* epimastigote antigens incorporated into iscoms protects against lethal challenge in mice. *Infection and Immunity*, **59**: 2909.
- Babior, B. M., Kuper, R. and Curnutte, J. T. (1988) Kinetics of activation of the respiratory burst oxidase in a fully soluble system from human neutrophils. *Journal of Biological Chemistry*, **263**: 1713.

- Baggiolini, M. (1995) Activation and recruitment of neutrophil leukocytes. *Clinical and Experimental Immunology*, **101**: 5.
- Baggiolini, M. (1998) Chemokine and leukocyte traffic. *Nature*, **329**: 565.
- Banchereau, J. and Steinman, R.M. (1998) Dendritic cells and the control of immunity. *Nature*, **392**: 245.
- Bargatze, R.F., Jutila, M.A. and Butcher, E.C. (1995) Distinct roles of L-selectin and integrins $\alpha 4\beta 7$ and LFA-1 in lymphocyte homing to Peyer's patch-HEV *in situ*: the multistep model confirmed and refined. *Immunity*, **3**: 99.
- Baumann, H. and Gauldie, J. (1994) The acute phase response. *Immunology Today*, **15**: 74.
- Beagley, K., Eldridge, J., Lee, F., Kiyono, H., Everson, M., Koopman, W., Hirano, T., Kishimoto, T. and McGhee, J. (1989) Interleukins and IgA synthesis: human and murine interleukin 6 induce high rate IgA secretion in IgA-committed B cells. *Journal of Experimental Medicine*, **169**: 2133.
- Behboudi, S., Morein, B. and Villacres-Eriksson, M. (1996) *In vitro* activation of antigen-presenting cells (APC) by defined composition of *Quillaja saponaria molina* triterpenoids. *Clinical and Experimental Immunology*, **105**: 26.
- Behboudi, S., Morcin, B. and Villacres-Eriksson, M. (1997) *In vivo* and *in vitro* induction of IL-6 by *Quillaja saponaria molina* triterpenoid formulations. *Cytokine*, **9**: 682.
- Bergstrom, M., Fossum, C., Akerblom, L. and Morein, B. (1990) Iscoms enhance the expression of class-II MHC antigens. *Scandinavian Journal Of Immunology*, **32**: 384.

- Billiau, A. (1995) Interferon- γ : Biology and role in pathogenesis, *Advances in Immunology*, **62**: 61.
- Black, R.A., Kronheim, S.R., Cantrell, M., Deeley, M.C., March, C.J., Prickett, K.S., Wignall, J., Conion, P.J., Cosman, D. and Hopp, T.P. (1988) Generation of biologically active interleukin-1 beta by proteolytic cleavage of the inactive precursor. *Journal of Biological Chemistry*, **263**: 9437.
- Bloom, B.R. (1989) Vaccines for the Third World. *Nature*, **342**: 115.
- Brandtzaeg, P., Baekkevold, E.S., Farstad, I.N., Jahnsen, F.L., Johansen, F-E., Nilsen, E.M. and Yamanaka, T. (1999) Regional specialisation in the mucosal immune system: what happens in the microcompartments. *Immunology Today*, **20**: 141.
- Brandzaeg, P. (1989) Overview of the mucosal immune system. *Current Topics in Microbiology and Immunology*, **146**: 13.
- Brodsky, F.M., Lem, L., Solachc, A. and Bennett, E.M. (1999) Human pathogen subversion of antigen presentation. *Immunological Reviews*, **168**: 199.
- Bromander, A.K., Ekman, L., Kopf, M., Nedrud, J.G. and Lycke, N.Y. (1996) IL-6-deficient mice exhibit normal mucosal IgA responses to local immunisations and *Helicobacter felis* infection. *Journal of Immunology*, **156**: 4290.
- Browning, M., Reid, G., Osborne, R. and Jarrett, O. (1992) Incorporation of soluble-antigens into ISCOMS: HIV gp120 ISCOMS induce virus neutralizing antibodies. *Vaccine*, **10**: 585.

Brunda, M.J., Luistro, L., Warriar, R.R., Wright, R.B., Hubbard, B.R., Murphy, M., Wolf, S. F. and Gately, M.K. (1993) Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *Journal of Experimental Medicine*, **178**: 1223.

Brzezinska-Blaszczyk, E. and Pietrzak, A. (1997) Tumor necrosis factor alpha (TNF- α) activates human adenoïdal and cutaneous mast cells to histamine secretion. *Immunology Letters*, **59**: 139.

Buchanan, J.M., Vogel, L.A., Van Cleave, V.H. and Metzger, D.W. (1995) Interleukin 12 alters the isotype restricted antibody response of mice to hen eggwhite lysozyme. *International Immunology*, **7**: 1519.

Buchmeier, N.A. and Schreiber, R.D. (1985) Requirement for endogenous interferon- γ production for resolution of *Lysteria monocytogenes* infection. *Proceedings of the National Academy of Sciences, USA*, **82**: 7404.

Caley, I.J., Betts, M.R., Irlbeck, D.M., Davis, N.L., Swanstrom, R., Frelinger, J.A. and Johnston, R.E. (1997) Humoral, mucosal and cellular immunity in response to a human immunodeficiency virus type 1 immunogen expressed by a Venezuelan equine encephalitis virus vaccine vector. *Journal of Virology*, **71**: 3031.

Campbell, J.B. and Peerbaye, Y.A. (1992) Saponin. *Research In Immunology*, **143**: 526.

Campbell, J.J., Hedrick, J., Zlotnik, A., Siana, M.A., Thompson, D.A. and Butcher, E.C. (1998) Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science*, **279**: 381.

Cassatella, M.A., Bazzoni, F., Flynn, R.M., Dusi, S., Trinchieri, G. and Rossi, F. (1990) Molecular basis of interferon- γ and lipopolysaccharide enhancement of phagocyte respiratory

burst capability. Studies on the gene expression of several NADPH oxidase components. *Journal of Biological Chemistry*, **265**: 2002.

Cella, M., Sallusto, F. and Lanzavecchia, A. (1997) Origin, maturation and antigen presenting function of dendritic cells. *Current Opinion in Immunology*, **9**: 10.

Cepek, K.L., Shaw, S.K., Parker, C.M., Russell, G. ., Morrow, J.S., Rimm, D.L. and Brenner, M.B. (1994) Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and an integrin $\alpha E\beta 7$. *Nature*, **372**: 190.

Charles, S.D., Hussain, I., Choi, C.U., Nagaraja, K.V. and Sivanandan, V. (1994a) Adjuvanted subunit vaccines for the control of *Salmonella enteritidis* infection in turkeys. *American Journal Of Veterinary Research*, **55**: 636.

Charles, T., Buzoni-Gatel, D., Lepage, A., Bernard, F. and Bout, D. (1994b) *Toxoplasma gondii* oral infection induces specific cytotoxic CD8 α/β^+ Thy1 $^+$ intraepithelial lymphocytes, lytic for parasite-infected enterocytes. *Journal of Immunology*, **153**: 4596.

Chavali, S R. and Campbell, J. (1987a) Adjuvant effects of orally administered saponins on humoral and cell-mediated immune responses in mice. *Immunobiology*, **174**: 347.

Chavali, S.R. and Campbell, J. (1987b) Immunomodulatory effects of orally administered saponins and non-specific resistance against rabies infection. *International Archives of Allergy and Applied Immunology*, **84**: 129.

Chomarat, P. and Banchereau, J. (1998) Interleukin 4 and interleukin 13: their similarities and discrepancies. *International Review of Immunology*, **17**: 1.

Chouaib, S., Chehimi, J., Bani, L., Genetet, N., Tursz, T., Gay, F., Trinchieri, G. and Mami-Chouaib, F. (1994) Interleukin 12 induces the differentiation of major histocompatibility complex class I-primed cytotoxic T lymphocyte precursors into allospecific cytotoxic effectors. *Proceedings of the National Academy of Science, USA*, **91**: 12659.

Church, M.K. and Levi-Schaffer, F. (1997) The human mast cell. *Journal Of Allergy and Clinical Immunology*, **99**: 155.

Claasen, I., Osterhaus, A., Boersma, W., Schellekens, M. and Claasen, E. (1995) Fluorescent labelling of virus, bacteria and ISCOMS: *in vivo* systemic and mucosal localisation patterns. *Advances in Experimental Medicine and Biology*, **317B**: 1485.

Claassen, I., Osterhaus, A. and Claassen, E. (1995) Antigen-detection *in-vivo* after immunization with different presentation forms of rabies virus-antigen: involvement of marginal metallophilic macrophages in the uptake of immune-stimulating complexes. *European Journal Of Immunology*, **25**: 1446.

Clark, R.A. (1999) Activation of the neutrophil respiratory burst oxidase. *Journal of Infectious Diseases*, **179**: S309.

Cong, Y.C., Weaver, C. and Elson, C. (1997) The mucosal adjuvanticity of Cholera toxin involves enhancement of co-stimulatory activity by selective up-regulation of B7.2 expression. *Journal of Immunology*, **159**: 5301.

Cooper, A.M., Magram, J., Ferrante, J. and Orme, I.M. (1997) Interleukin-12 (IL-12) is crucial to the development of protective immunity in mice infected with *Mycobacterium tuberculosis*. *Journal of Experimental Medicine*, **186**: 39.

Cuncha, F.Q., Moncada, S. and Liew, F.Y. (1992) IL-10 inhibits the induction of nitric oxide synthase by IFN γ murine macrophages. *Biochemical and Biophysical Research Communications*, **182**: 1155.

Czinn, S.J., Cai, A. and Nedrud, J.G. (1993) Protection of germ free mice from infection by *Helicobacter felis* after oral or passive IgA immunisation, *Vaccine*, **11**.

Dalton, D.K., Pitts-Meck, S., Kershav, S., Figari, I.S., Bradley, A. and Stewart, T.A. (1993) Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science*, **259**: 1739.

de Vries, P., Heeney, J.L., Boes, J., Dings, M.E.M., Hulskotte, E.G.J., Dubbes, R., Koomstra, W., Tenhaaft, P., Akerblom, L., Eriksson, S., Morein, B., Norley, S. and Osterhaus, A. (1994) Protection of *Rhesus macaques* from SIV infection by immunization with different experimental SIV vaccines. *Vaccine*, **12**: 1443.

de Vries, P., Uytdehaag, F.G.C.M. and Osterhaus, A.D.M.E. (1988) Canine distemper virus (CDV) immune stimulating complexes (ISCOMS), but not measles virus ISCOMS, protect dogs against CDV infection. *Journal of General Virology*, **69**: 2071.

Debrick, J.E., Campbell, P.A. and Staertz, U.D. (1991) Macrophages as accessory cells for class I MHC-restricted immune responses, *Journal of Immunology*, **147**: 2846.

Dinarello, C.A. (1998) Interleukin-1. In *The Cytokine Handbook* Academic Press Ltd., , pp. 35.

Ding, A., Nathan, C.F., Graycar, J., Derynck, R., Stuehr, D.J. and Srinivasan, S. (1990) Macrophage deactivating factor and TGF- β 1, - β 2, - β 3 inhibit induction of macrophage nitrogen oxide synthesis by IFN γ . *Journal of Immunology*, **145**: 940.

Easmon, C.S.F., Cole, P.J., Williams, A.J. and Hastings, M. (1980) The measurement of opsonic and phagocytic function by Luminol-dependent chemiluminescence. *Immunology*, **41**: 67.

Elson, C.O., Falding, W. and Lefkowitz, J. (1984) A lavage technique allowing repeated measurement of IgA antibody in mouse intestinal secretions. *Journal of Immunological Methods*, **43**: 574.

Ennis, F.A., Cruz, J., Jameson, J., Klein, M., Burt, D. and Thippahawong, J. (1999) Augmentation of human influenza A virus specific cytotoxic T lymphocyte memory by influenza vaccine and adjuvanted carriers (ISCOMS). *Virology*, **259**: 256.

Ertürk, M., Jennings, R., Hockley, D. and Potter, C.W. (1989) Antibody responses and protection in mice immunised with herpes simplex virus type I antigen immune-stimulating complex preparations. *Journal of General Virology*, **70**: 2149.

Estrada, A., Laarveld, B., Li, B. and Redmond, M.J. (1995) Induction of systemic and mucosal immune-responses following immunization with somatostatin avidin complexes incorporated into ISCOMS. *Immunological Investigations*, **24**: 819.

Evans, D.E., Munks, M.W., Purkerson, J.M. and Parker, D.C. (2000) Resting B lymphocytes as APC for naive T lymphocytes: dependence on CD40 ligand/CD40. *Journal of Immunology*, **164**: 688.

Faria, A.M.C. and Weiner, H.L. (1999) Oral tolerance: mechanisms and therapeutic applications. *Advances in Immunology*, **73**: 153.

Fearon, D.T. and Locksley, R.M. (1996) The instructive role of innate immunity in the acquired immune response. *Science*, **272**: 50.

Fekadu, M., Shaddock, J.H., Ekstrom, J., Osterhaus, A., Sandcrin, D.W., Sundquist, B. and Morein, B. (1992) An immune stimulating complex (ISCOM) subunit rabies vaccine protects dogs and mice against street rabies challenge. *Vaccine*, **10**: 192.

Fennelly, G.J., Flynn, J.L., ter Meulen, V., Liebert, U.G. and Bloom, B.R. (1995) Recombinant Bacille Calmette-Guérin priming against measles. *Journal of Infectious Diseases*, **172**: 698.

Fennelly, G.J., Khan, S.A., Abadi, M.A., Wild, T.F. and Bloom, B.R. (1999) Mucosal DNA vaccine immunisation against measles with a highly attenuated *Shigella flexneri* vector. *Journal of Immunology*, **162**: 1603.

Finkelman, F.D., Katona, I.M., Mosmann, T.R. and Coffman, R.L. (1988) IFN- γ regulates the isotypes of Ig secreted during *in vivo* humoral immune responses. *Journal of Immunology*, **140**: 1022.

Fossum, C., Bergstrom, M., Lovgren, K., Watson, D.L. and Morein, B. (1990) Effect of iscoms and their adjuvant moiety (matrix) on the initial proliferation and il-2 responses - comparison of spleen-cells from mice inoculated with iscoms and or matrix. *Cellular Immunology*, **129**: 414.

Fujihashi, K., McGhee, J.R., Kweon, M-N., Cooper, M.D., Tonegawa, S., Takahashi, I., Hiroi, T., Mestecky, J. and Kiyono, H. (1996) γ/δ T cell deficient mice have impaired mucosal immunoglobulin A responses. *Journal of Experimental Medicine*, **183**: 1929.

Galli, S.J. (1997) The mast cell: a versatile effector cell for a challenging world. *International Archives Of Allergy and Immunology*, **113**: 14.

Galli, S.J., Maurer, M. and Lantz, C.S. (1999) Mast cells as sentinels of innate immunity, *Current Opinion in Innate Immunity*, **11**: 53.

Gallucci, S., Lolkema, M. and Matzinger, P. (1999) Natural adjuvants: endogenous activators of dendritic cells. *Nature Medicine*, **5**: 1249.

Garside, P., Steel, M., Worthey, E., Satoskar, A., Alexander, J., Liew, F. and Mowat, A. (1995) Th2 cells are subjected to high dose oral tolerance and are not essential for its induction. *Journal of Immunology*, **154**: 5649.

Gateley, M.K., Renzetti, L.M., Magram, J., Stern, A.S., Adorini, L., Gubler, U. and Presky, D.H. (1998) The interleukin 12/interleukin 12 receptor system: role in normal and pathologic immune responses. *Annual Review of Immunology*, **16**: 495.

Gately, M.K., Warrior, R.R., Honasoge, S., Carvajal, D.M., Faherty, D.A., Connaughton, S.E., Anderson, T.D., Sarmiento, U., Hubbard, B.R. and Murphy, M. (1994) Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN- γ *in vivo*. *International Immunology*, **6**: 157.

Gazzinelli, R.T., Hieny, S., Wynn, T.A., Wolf, S. and Sher, A. (1993) Interleukin 12 is required for the T-lymphocyte independent induction of interferon- γ by an intracellular parasite and induces resistance in T deficient hosts. *Proceedings of the National Academy of Science, USA*, **90**: 6115.

German, T., Bongartz, M., Dlugonska, H., Hess, H., Schmidt, E., Kolbe, L., Kölsch, E., Podlaski, F. J., Gately, M.K. and Rüde, E. (1995) Interleukin 12 profoundly up-regulates the

synthesis of antigen specific complement fixing IgG2a, IgG2b and IgG3 antibody subclasses *in vivo*. *European Journal of Immunology*, **25**: 823.

Gewurz, H., Zhang, X-H. and Franklin Lint, T. (1995) Structure and function of the pentraxins. *Current Opinion in Immunology*, **7**: 54.

Ghayur, T., Banerjee, S., Hugunin, M., Butler, D., Herzog, L., Carter, A., Quin, L., Sekut, L., Talanian, R., Paskind, M., Wong, W., Kamen, R., Tracey, D. and Allen, H. (1997) Caspase-1 processes IFN- γ -inducing factor and regulates LPS-induced IFN- γ production. *Nature*, **386**: 619.

Ghazi, H.O., Potter, C.W., Smith, T.L. and Jennings, R. (1995) Comparative antibody-responses and protection in mice immunized by oral or parenteral routes with influenza-virus subunit antigens in aqueous form or incorporated into ISCOMS. *Journal Of Medical Microbiology*, **42**: 53.

Gordon, J.R. and Galli, S.J. (1990) Mast cells as a source of both preformed and immunologically inducible TNF- α /cachetin. *Nature*, **346**: 274.

Grdic, D., Smith, R., Donachie, A., Kjerrulf, M., Hornquist, E., Mowat, A. and Lycke, N. (1999) The mucosal adjuvant effects of Cholera toxin and immune-stimulating complexes differ in their requirement for IL-12, indicating different pathways of action. *European Journal of Immunology*, **29**: 1774.

Green, S.J. and Nacy, C.A. (1993) Antimicrobial and immunopathologic effects of cytokine-induced nitric oxide synthesis. *Current Opinion in Infectious Diseases*, **6**: 384.

Griffiths, G.M. (1995) The cell biology of CTL killing. *Current Opinion in Immunology*, **7**: 343.

Griscavage, J.M., Wilk, S. and Ignarro, L.J. (1996) Inhibitors of the proteasome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of transcription factor NF- κ B. *Proceedings of the National Academy of Science, USA*, **93**: 3308.

Gu, Y., Kuida, K., Tsutsui, H., Ku, G., Hsiao, K., Fleming, M., Hayashi, N., Higashino, K., Okamura, H., Nakanishi, K., Kurimoto, M., Tanimoto, T., Flavell, R., Sato, V., Harding, M., Livingston, D. and Su, M. (1997) Activation of interferon-gamma inducing factor mediated by interleukin 1beta converting enzyme. *Science*, **275**: 206.

Gubler, U., Chua, A.O., Schoenhaut, D.S., Dwyer, C.M., McComas, W., Motyka, R., Nabavi, N., Wolitzky, A.G., Quinn, P.M., Familletti, P.C. and Gately, M.K. (1991) Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proceedings of the National Academy of Science, USA*, **88**: 4143.

Gupta, R.K., Relyveld, E.H., Lindblad, E.B., Bizzini, B., Benefraim, S. and Gupta, C.K. (1993) Adjuvants: a balance between toxicity and adjuvanticity. *Vaccine*, **11**: 293.

Gupta, R.K. and Siber, G.R. (1995) Adjuvants for human vaccines: current status, problems and future prospects. *Vaccine*, **13**: 1263.

Guy-Grand, D., Malassis-Seris, M., Briottet, C. and Vassalli, P. (1991) Cytotoxic differentiation of mouse gut thymus dependent and independent intraepithelial T lymphocytes is induced locally. Correlation between functional assays, presence of perforin and granzyme transcripts, and cytoplasmic granules. *Journal of Experimental Medicine*, **173**: 1549.

Guy-Grand, D. and Vassalli, P. (1993) Gut intraepithelial T lymphocytes. *Current Opinion in Immunology*, **5**: 247.

- Halstensen, T., Scott, H. and Brandtzaeg, P. (1989) Intraepithelial T cells of the TcR γ/δ^+ CD8⁻ and V δ 1/J δ 1⁺ phenotypes are increased in coeliac disease. *Scandinavian Journal of Immunology*, **30**: 665.
- Hansson, G.K., Hellstrand, M., Rymo, I., Rubbia, I. and Gabbiani, G. (1989) Interferon gamma inhibits both proliferation and expression of differentiation-specific alpha smooth muscle actin in arterial smooth muscle cells. *Journal of Experimental Medicine*, **170**: 1595.
- Harding, C.V. and Song, R. (1994) Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class-I MHC molecules. *Journal Of Immunology*, **153**: 4925.
- Harriman, G.R., Kunimoto, D.Y., Elliot, J.F., Paetkau, V. and Strober, W. (1988) The role of IL-5 in IgA B cell differentiation. *Journal of Immunology*, **140**: 3033.
- Heeg, K., Kuon, W. and Wagner, H. (1991) Vaccination of class-I major histocompatibility complex (MHC) - restricted murine CD8⁺ cytotoxic T lymphocytes towards soluble-antigens - immunostimulating-ovalbumin complexes enter the class-I MHC-restricted antigen pathway and allow sensitization against the immunodominant peptide. *European Journal Of Immunology*, **21**: 1523.
- Heinzel, F.P., Rerko, R.M., Ling, P., Hakimi, J. and Schoenhaut, D.S. (1994) Interleukin 12 is produced *in vivo* during endotoxemia and stimulates synthesis of gamma interferon. *Infection and Immunity*, **62**: 1155.
- Heinzel, F.P., Schoenhaut, D.S., Rerko, R.M., Rosser, L.E. and Gately, M.K. (1993) Recombinant interleukin 12 cures mice infected with *Leishmania major*. *Journal of Experimental Medicine*, **177**: 1505.

Hess, J., Ladell, C., Miko, D. and Kaufmann, S.H.E. (1996) *Salmonella typhimurium aroA*-infection in gene targeted immunodeficient mice: major role of CD4⁺ TCR alpha-beta cells and IFN- γ in bacterial clearance. *The Journal of Immunology*, **156**: 3321.

Higuchi, R., Tokimitsu, Y. and Komori, T. (1988) An acylated triterpenoid saponin from *Quillaja saponaria*. *Phytochemistry*, **27**: 1165.

Holmgren, J. (1981) Action of cholera toxin and the prevention and treatment of cholera. *Nature*, **292**: 413.

Holsti, M.A. and Raulat, D.H. (1989) IL-6 and IL-1 synergize to stimulate IL-2 production and proliferation of peripheral T cells. *Journal of Immunology*, **141**: 151.

Horvat, B., Loukides, J.A., Ancandian, I., Brewer, E. and Flood, P.M. (1991) Production of interleukin 2 and interleukin 4 by immune CD4⁺CD8⁺ cells and their role in the generation of antigen-specific cytotoxic T cells. *Journal of Immunology*, **21**: 1863.

Houssiau, F. and Van Snick, J. (1992) IL-6 and the T cell response. *Research Immunology*, **143**: 740.

Houssiau, F.A., Coulie, P.G., Olive, D. and Van Snick, J. (1988) Synergistic activation of human T cells by interleukin 1 and interleukin 6. *European Journal of Immunology*, **18**: 653.

Houssiau, F.A., Coulie, P.G. and Van Snick, J. (1989) Distinct roles of IL-1 and IL-6 in human T cell activation. *Journal of Immunology*, **143**: 2520.

Hsieh, C., Macatonia, S.E., Tripp, C.S., Wolf, S.F., O'Garra, A. and Murphy, K.M. (1993) *Listeria* induced Th1 development in $\alpha\beta$ -TCR transgenic CD4⁺ T cells occurs through macrophage production of IL-12. *Science*, **260**: 547.

Hsieh, C.-S., Macatonia, S. F., Tripp, C. S., O'Garra, A. and Murphy, K. M. (1993) Development of Th1 CD4⁺ T cells through IL-12 produced by *Listeria* induced macrophages. *Science*, **260**: 547.

Hsu, S.C., Schadeck, E.B., Delmas, A., Shaw, M. and Steward, M.W. (1996) Linkage of a fusion peptide to a CTL epitope from the nucleoprotein of measles-virus enables incorporation into ISCOMS and induction of CTL responses following intranasal immunization. *Vaccine*, **14**: 1159.

Huang, F-P., Niedbala, W., Wei, X-Q., Xu, D., Feng, G-J., Robinson, J.H., Lam, C. and Liew, F.Y. (1998) Nitric oxide regulates Th1 cell development through the inhibition of IL-12 synthesis by macrophages. *European Journal of Immunology*, **28**: 4062.

Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H.R.K., Vilcek, J., Zinkernagel, R.M. and Aguet, M. (1993) Immune response in mice lacking the interferon- γ receptor. *Science*, **259**: 1742.

Ikonomidis, G., Portnoy, D.A., Gerhard, W. and Paterson, Y. (1997) Influenza specific immunity induced by recombinant *Listeria monocytogenes* vaccines. *Vaccine*, **15**: 433.

Janeway Jr, C.A. (1989) Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor Symposium on Quantitative Biology*, **54**: 1.

Jeong, G. (1995) Suppressed proliferation of mouse hepatoma cells by conditioned media from interferon treated hepatocytes. *Biochemistry and Molecular Biology International*, **36**: 136.

Jones, P.D., Hla, R.T., Morein, B., Lovgren, K. and Ada, G.L. (1988) Cellular immune-responses in the murine lung to local immunization with influenza-A virus glycoproteins in micelles and immunostimulatory complexes (iscoms). *Scandinavian Journal Of Immunology*, **27**: 645.

Kagi, B., ledermann, K. and Burki, R. (1994) Molecular mechanisms of lymphocyte mediated cytotoxicity and their role in immunological protection and pathogenesis *in vivo*. *Annual Review of Immunology*, **12**: 207.

Kalinski, P., Hilkens, C.M.U., Wierenga, E.A. and Kapsenberg, M.L. (1999) T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunology Today*, **20**: 561.

Kamijo, R., Harada, H., Matsuyama, T., Bosland, M., Gerecitano, J., Shapiro, D., Le, J., Im, K.S., Kimura, T., Green, S., Mak, T.W., Taniguchi, T. and Vilcek, J. (1994) Requirements for transcription factor IRF-1 in NO synthase induction in macrophages. *Science*, **263**: 1612.

Karp, C.L., Wysocka, M., Wahl, L.M., Ahearn, J.M., Cuomo, P.J., Sherry, B., Trinchieri, G. and Griffin, D.E. (1996) Mechanism of suppression of cell mediated immunity by measles virus. *Science*, **273**: 228.

Kazanji, M., Laurent, F. and Pery, P. (1994) Immune-responses and protective effect in mice vaccinated orally with surface sporozoite protein of *Eimeria falciformis* in ISCOMS. *Vaccine*, **12**: 798.

Kelsall, B. and Strober, W. (1996) Distinct populations of dendritic cells are present in the subepithelial dome and T cell regions of the murine Peyer's patch. *Journal of Experimental Medicine*, **183**: 237.

Kennedy, M.K., Picha, K.S., Fanslow, W.C., Grabstein, K.H., Alderson, M.R., Clifford, K.N., Chin, W.A. and Mohler, K.M. (1996) CD40/CD40 ligand interactions are required for T cell-dependent production of interleukin 12 by mouse macrophages. *European Journal of Immunology*, **26**: 370.

Kensil, C.R. (1996) Saponins as vaccine adjuvants. *Critical Reviews In Therapeutic Drug Carrier Systems*, **13**: 1.

Keren, D.F. (1992) Antigen processing in the mucosal immune system. *Seminars in Immunology*, **4**: 217.

Kersten, G.F.A. (1990) *Aspects of ISCOMS. Analytical, pharmaceutical and adjuvant properties*. PhD Thesis, University of Utrecht.

Kersten, G.F.A., Teerlink, T., Derks, H., Verkleij, A.J., Vanwezel, T.L., Crommelin, D.J.A. and Beuvery, E.C. (1988a) Incorporation of the major outer-membrane protein of *Neisseria gonorrhoeae* in saponin-lipid complexes (iscoms): chemical analysis, some structural features, and comparison of their immunogenicity with 3 other antigen delivery systems. *Infection and Immunity*, **56**: 432.

Kersten, G.F.A., Vandeput, A.M., Teerlink, T., Beuvery, E.C. and Crommelin, D.J.A. (1988b) Immunogenicity of liposomes and iscoms containing the major outer membrane protein of *Neisseria gonorrhoeae*: influence of protein content and liposomal bilayer composition. *Infection and Immunity*, **56**: 1661.

Khortus, A. and Jenkins, M.K. (1996) Studying immunological tolerance by physically monitoring antigen-specific T cells *in vivo*. *Annals of the New York Academy of Science*, **778**: 72.

Khoury, S.J., Hancock, W.W. and Wiener, H.L. (1992) Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with downregulation of inflammatory cytokines and differential up-regulation of transforming growth factor β , interleukin 4 and prostaglandin E expression in the brain. *Journal of Experimental Medicine*, **176**: 1355.

Kishimoto, T. (1989) The Biology of interleukin 6. *Blood*, **74**: 1.

Kjerrulf, M., Grdic, D., Ekman, L., Schon, K., Vajdy, M. and Lycke, N. (1997) Interferon- γ deficient mice exhibit impaired gut mucosal immune responses but intact oral tolerance. *immunology*, **92**: 60.

Kobayashi, M., Fitz, L., Ryan, M., Hewick, R.M., Clark, S.C., Chan, S., Loudon, R., Sherman, F., Perussia, B. and Trinchieri, G. (1989) Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple effects on human lymphocytes. *Journal of Experimental Medicine*, **170**: 827.

Kohno, K., Kataoka, J., Ohtsuki, T., Suemoto, Y., Usui, M., Ikeda, M. and Kurimoto, M. (1997) IFN- γ -inducing factor (IGIF) is a costimulatory factor on the activation of Th1 but not Th2 cells and exerts its effect independently of IL-12. *The Journal of Immunology*, **158**: 1541.

Kohno, K. and Kurimoto, M. (1998) Interleukin 18, a cytokine which resembles IL-1 structurally and IL-12 functionally but exerts its effect independently of both. *Clinical Immunology and Immunopathology*, **86**: 11.

Kopf, M., Baumann, H., Freer, G., Freudenberg, M., Lamers, M., Kishimoto, T., Zinkernagel, R., Bluethmann, H. and Köhler, G. (1994) Impaired immune and acute phase responses in interleukin-6-deficient mice. *Nature*, **368**: 339.

- Kopf, M., Gros, G.L., Bachmann, M., Lamers, M.C., Bluethmann, H. and Kohler, G. (1993) Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature*, **362**: 245.
- Kopp, E.B. and Medzhitov, R. (1999) The Toll-receptor family and control of innate immunity. *Current Opinion in Immunology*, **11**: 13.
- Kotloff, K.L., Noriega, F., Losonsky, G.A., Sztein, M.B., Wasserman, S.S., Nataro, J.P. and Levine, M.M. (1996) Safety, immunogenicity and transmissability in humans of CVD 1203, a live oral *Shigella flexneri* 2a vaccine candidate attenuated by deletions in *aroA* and *virG*. *Infection and Immunity*, **64**: 4542.
- Kovacsovics-Bankowski, M. and Rock, K. (1995) A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science*, **267**: 243.
- Krieger, M. and Herz, J. (1994) Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor related protein (LRP). *Annual Review of Biochemistry*, **63**: 601.
- Kuhn, R., Rajewsky, K. and Muller, W. (1991) Generation and analysis of interleukin 4 deficient mice. *Science*, **254**: 707.
- Kunimoto, D., Nordan, R. and Strober, W. (1989) IL-6 is a potent cofactor of IL-1 in IgM synthesis and of IL-5 in IgA synthesis. *Journal of Immunology*, **143**: 2230.
- Lamm, M.E., Mazaneca, M.B., Nedrud, J.G. and Kaetzl, C.S. (1995) New functions for mucosal IgA. *Advances in Experimental Medical Biology*, **371A**: 647.

- Lefrancois, L. (1991) Intraepithelial lymphocytes of intestinal mucosa: curiouser and curiouser. *Seminars in Immunology*, **3**: 99.
- Ley, V., Longlade Demoyen, P., Kourilsky, P. and Larsson Sciard, E.L. (1991) Interleukin 2-dependent activation of tumour-specific cytotoxic T lymphocytes *in vivo*. *European Journal of Immunology*, **21**: 851.
- Liang, S.M., Lee, N., Finbloom, D.S. and Liang, C.M. (1992) Regulation by glutathione of interleukin 4 activity on cytotoxic T cells. *Immunology*, **75**: 435.
- Lovgren, K. (1988) The serum antibody response distributed in subclasses and isotypes after intranasal and subcutaneous immunisation with influenza virus immunostimulating complexes. *Scandinavian Journal of Immunology*, **27**: 241.
- Lovgren, K., Kaberg, H. and Morein, B. (1988) Influenza virus ISCOMS: antibody response in animals. *Vaccine*, **82**: 435.
- Lovgren, K., Kaberg, H. and Morein, B. (1990) An experimental influenza subunit vaccine (iscom): induction of protective immunity to challenge infection in mice after intranasal or subcutaneous administration. *Journal of Experimental Immunology*, **82**: 435.
- Lovgren, K. and Morein, B. (1988) The requirement of lipids for the formation of immunostimulating complexes (iscoms). *Biotechnology and Applied Biochemistry*, **10**: 161.
- Liu, L. and MacPherson, G.G. (1995) Rat intestinal dendritic cells: immunostimulatory potency and phenotypic characterisation. *Immunology*, **85**: 88.
- Lycke, N. (1997) The mechanism of cholera toxin adjuvanticity. *Research Immunology*, **148**: 504.

Lycke, N. and Holmgren, J. (1986) Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. *Immunology*, **59**: 301.

MacPherson, G.G., Jenkins, C.D., Stein, M.J. and Edwards, C. (1995) Endotoxin-mediated dendritic cell release from the intestine. Characterisation of released dendritic cells and TNF dependence. *Journal of Immunology*, **154**: 1317.

Maggi, E.P., Parronchi, R., Manetti, C., Simonelli, C., Piccinni, M.P., Rugui, F.S., De-Carli, M., Ricci, M. and Romagnani, S. (1992) Reciprocal regulatory effects of IFN- γ and IL-4 on the *in vitro* development of human Th1 and Th2 clones. *Journal of Immunology*, **148**: 2142.

Magram, J., Connaughton, S.E., Warriar, R.R., Caravajal, D.M., Wu, C-Y., Ferrante, J., Stewart, C., Sarmiento, U., Faherty, D.A. and Gately, M.K. (1996) IL-12 deficient mice are defective in IFN- γ production and type I cytokine responses. *Immunity*, **4**: 471.

Maloy, K.J. (1996) *The basis of the oral and parenteral adjuvant properties of immune stimulating complexes (ISCOMS)*. PhD Thesis, University of Glasgow.

Maloy, K.J., Donachie, A.M. and Mowat, A.M. (1995) Induction of Th1 and Th2 CD4⁺ T-cell responses by oral or parenteral immunization with ISCOMS. *European Journal Of Immunology*, **25**: 2835.

Maloy, K.J., Donachie, A.M., Ohagan, D.T. and Mowat, A.M. (1994) Induction of mucosal and systemic immune-responses by immunization with ovalbumin entrapped in poly(lactide-co-glycolide) microparticles. *Immunology*, **81**: 661.

Mattner, F., Magram, J., Ferrante, J., Launois, P., Di Padova, K., Behin, R., Gately, M.K., Louis, J.A. and Alber, G. (1996) Genetically resistant mice lacking interleukin 12 are

susceptible to infection with *Leishmania major* and mount a polarized Th2 cell response. *European Journal of Immunology*, **26**: 1553.

Mazanec, M.B., Kaetzel, C.S., Lamm, M.E., Fletcher, D., Peterra, J. and Nedrud, J.G. (1995) Intracellular neutralisation of Sendai and influenza viruses by IgA monoclonal antibodies. *Advances in Experimental Medicine*, **371A**: 651.

Mbawuikc, I.N., Pacheco, S., Acuna, C.L., Switzer, K.C., Zhang, Y. and Harriman, G.R. (1999) Mucosal immunity to Influenza without IgA: an IgA knockout mouse model. *Journal of Immunology*, **162**: 2530.

McClellan, A., Wei, X-Q., Huang, F-P., Al-Alem, U.A.H., Chan, W.L. and Liew, F.Y. (1998) Mice lacking inducible nitric oxide synthase are more susceptible to herpes simplex virus infection despite enhanced Th1 cell responses. *Journal of General Virology*, **79**: 825.

McGhee, J.R., Mestecky, J., Dertzbaugh, M.T., Eldridge, J.H., Hirasawa, M. and Kiyono, H. (1992) The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine*, **10**: 75.

McKnight, A.J., Zimmer, G.J., Fogelman, I., Wolf, S.F. and Abbas, A.K. (1994) Effects of IL-12 on helper T cell-dependent immune responses *in vivo*. *Journal of Immunology*, **152**: 2172.

Medzhitov, R. and Janeway Jr, C.A. (1997a) Innate immunity: impact on the adaptive immune response. *Current Opinion in Immunology*, **9**: 4.

Medzhitov, R. and Janeway Jr, C.A. (1997b) Innate immunity: the virtues of a nonclonal system of recognition. *Cell*, **91**: 295.

Meffert, M.K., Premack, B.A. and Schulman, H. (1994) Nitric oxide stimulates Ca^{2+} independent synaptic vesicle release. *Neuron*, **12**: 1235.

Mekori, Y.A. and Metcalfe, D.D. (2000) Mast cells in innate immunity. *Immunological Reviews*, **173**: 131.

Mestecky, J., Abraham, R. and Ogra, P.L. (1994) Common mucosal immune system and strategies for the development of vaccines effective at the mucosal surfaces. In *Handbook of Mucosal Immunology* (Eds, Ogra, P.L., Mestecky, J., Lamm, M.E., Strober, W. and McGhee, J.R.) Academic Press Inc., San Diego, pp. 357.

Meyer, D.J., Muimo, R., Thomas, M., Coates, D. and Isaac, R.E. (1996) Purification and characterisation of prostaglandin-H E-isomerase, a sigma-class glutathione S-transferase from *Ascaridia galli*. *Biochemistry Journal*, **313**: 223.

Michalek, S.M., Childers, N.K., Katz, J., Denys, F.R., Berry, A.K. and Eldridge, J.H. (1989) Liposomes as oral adjuvants. *Current Topics in Microbiology and Immunology*, **146**: 51.

Mohrs, M., Ledermann, B., Kohler, G., Dorfmueller, A., Gessner, A. and Brombacher, F. (1999) Differences between IL-4 and IL-4-receptor-alpha-deficient mice in chronic leishmaniasis reveal a protective role for IL-13 receptor signalling. *Journal of Immunology*, **162**: 7302.

Moncada, S. and Higgs, E.A. (1991) Endogenous nitric oxide: physiology, pathology and clinical relevance. *European Journal of Clinical Investigation*, **21**: 361.

Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991) Nitric oxide: physiology and pharmacology. *Pharmacology Review*, **43**: 109.

Morein, B. (1988) The iscom antigen-presenting system. *Nature*, **332**: 287.

Morein, B. (1991) Induction of antibody and cell mediated immune response by iscoms. *Aids Research and Human Retroviruses*, **7**: 152.

Morein, B., Ekstrom, J. and Lovgren, K. (1990) Increased immunogenicity of a nonamphipathic protein (BSA) after inclusion into iscoms. *Journal Of Immunological Methods*, **128**: 177.

Morein, B., Sundquist, B., Hoglund, S., Dalsgaard, K. and Osterhaus, A. (1984) Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. *Nature*, **308**: 457.

Morris, W., Steinhoff, M.C. and Russell, P.K. (1994) Potential of polymer microencapsulation technology for vaccine innovation. *Vaccine*, **12**: 5.

Mosmann, T.R. and Coffman, R.L. (1989) Different patterns of cytokine secretion lead to different functional properties. *Annual Review of Immunology*, **7**: 145.

Moss, B., Smith, G.L., Gerin, J.L. and Purcell, R.H. (1984) Live recombinant vaccinia virus protects chimpanzees against hepatitis B. *Nature*, **311**: 67.

Mostov, K.E. (1994) Transepithelial transport of immunoglobulins. *Annual Review of Immunology*, **12**: 63.

Mowat, A.M. (1987) The regulation of immune responses to dietary protein antigens. *Immunology Today*, **8**: 93.

Mowat, A.M., Donachie, A.M., Reid, G. and Jarrett, O. (1991a) Immune-stimulating complexes containing Quil-A and protein antigen prime class-I MHC-restricted T lymphocytes *in vivo* and are immunogenic by the oral route. *Immunology*, **72**: 317.

Mowat, A.M., Donachie, A.M., Reid, G. and Jarrett, O. (1991b) Induction of class-I MHC-restricted T-cells and stimulation of mucosal immunity by incorporation of soluble-protein in ISCOMS. *Faseb Journal*, **5**: A1460.

Mowat, A.M. and Reid, G. (1992) Preparation of immune stimulating complexes (ISCOMS) as adjuvants. In *Current Protocols in Immunology*, Vol. 1 (Eds, Coligan, E., Kruisbeak, M., Margulies, D.H., Shervach, E.M. and Strober, W.) Wiley Interscience, pp. 2.11.1.

Mowat, A.M., Maloy, K.J. and Donachie, A.M. (1993) Immune-stimulating complexes as adjuvants for inducing local and systemic immunity after oral immunization with protein antigens. *Immunology*, **80**: 527.

Mowat, A.M. and Viney, J.L. (1997) The anatomical basis of intestinal immunity. *Immunological Reviews*, **156**: 145.

Mowat, A.M., Steel, M., Leishman, A.J. and Garside, P. (1999) Normal induction of oral tolerance in the absence of a functional IL-12-dependent IFN γ signalling pathway. *Journal of Immunology*, **163**: 4728.

Mueller, D.L., Jenkins, M.K. and Schwartz, R.H. (1989) Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annual Review of Immunology*, **7**: 445.

Murray, P.D., McKenzie, D.T., Surain, S.L. and Kagnoff, M.F. (1987) Interleukin 5 and interleukin 4 produced by Peyer's patch T cells selectively enhance immunoglobulin A expression. *Journal of Immunology*, **139**: 2669.

Nabors, G.S. and Farrell, J.P. (1994) Depletion of IL-4 in Blab/c mice with established *Leishmania major* infections increases the efficacy of antimony therapy and promotes Th1 like responses. *Infection and Immunity*, **62**: 5498.

Nagy, B., Hoglund, S. and Morein, B. (1990) Iscom (immunostimulating complex) vaccines containing monovalent or polyvalent pili of enterotoxigenic *Escherichia coli*: immune-response of rabbit and swine. *Journal Of Veterinary Medicine*, **37**: 728.

Nathan, C.F. and Hibbs, J.B. (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. *Current Opinion in Immunology*, **3**: 65.

Neutra, M.R., Frey, A. and Kraehenbueh, J-P. (1996) Epithelial M cells: gateways for mucosal infection and immunisation. *Cell*, **86**: 345.

Noble, A., Staynor, D.Z. and Kemeny, D.M. (1993) Generation of rat Th2 like cells *in vitro* is interleukin 4 dependent and inhibited by interferon-gamma. *Immunology*, **79**: 562.

Noll, A. and Autenreith, I.B. (1996) Immunity against *Yersinia enterocolitica* by vaccination with *Yersinia* HSP60 immunostimulating complexes or *Yersinia* HSP60 plus IL-12. *Infection and Immunity*, **64**: 2955.

Noriega, F.R., Wang, J.Y., Losonsky, G., Maneval, D.R., Hone, D.M. and Levine, M.M. (1996) Construction and characterisation of attenuated *aroA*, *virG* *Shigella flexneri* 2a strain CVD 1203, a prototype live oral vaccine. *Infection and Immunity*, **62**: 5168.

Nuame, B., Gately, M. and Espevik, T. (1992) A comparative study of IL-12 (cytotoxic lymphocyte maturation factor), IL-2 and IL-7 induced effects on immunomagnetically purified CD56⁺ NK cells. *Journal of Immunology*, **148**: 2429.

Nuame, B., Johnsen, A-C., Espevik, T. and Sundan, A. (1993) Gene expression and secretion of cytokines and cytokine receptors from highly purified CD56⁺ natural killer cells stimulated with interleukin 2, interleukin 7 and interleukin 12. *European Journal of Immunology*, **23**: 1831.

Okahashi, N., Yamamoto, M., Vancott, J.L., Chatfield, S.N., Roberts, M., Bluethmann, H., Hiroi, T., Kiyono, H. and McGhee, J.R. (1996) Oral immunization of interleukin-4 (IL-4) knockout mice with a recombinant *Salmonella* strain or cholera-toxin reveals that CD4⁺ Th2 cells producing IL-6 and IL-10 are associated with mucosal immunoglobulin-A responses. *Infection and Immunity*, **64**: 1516.

Okamura, H., Kashiwamura, S-I., Tsutsui, H., Yoshimoto, T. and Nakanishi, K. (1998a) Regulation of interferon- γ production by IL-12 and IL-18. *Current Opinion in Immunology*, **10**: 259.

Okamura, H., Tsutsui, H., Kashiwamura, S-I., Yoshimoto, T. and Nakanishi, K. (1998b) Interleukin-18: a novel cytokine that augments both innate and acquired immunity. *Advances in Immunology*, **70**: 281.

Okamura, H., Tsutsui, H., Komatsu, T., Yatsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Okura, T., Nukada, Y. and Hattori, K. (1995) Cloning of a new cytokine that induces IFN- γ production by T cells. *Nature*, **378**: 88.

Orme, I., Flynn, J.L. and Bloom, B.R. (1993) The role of CD8⁺ T cells in immunity to tuberculosis. *Trends in Microbiology*, **1**: 77.

Osterhaus, A., Weijer, K., Uytdehaag, F., Jarrett, O., Sundquist, B. and Morein, B. (1985) Induction of protective immune response in cats by vaccination with feline leukemia-virus iscom. *Journal Of Immunology*, **135**: 591.

Oswald, I.P. and James, S.L. (1996) Nitrogen oxide in host defence against parasites. *Methods: A Companion to Methods in Enzymology*, **10**: 8.

Overnes, G., Nesse, L.L., Waldeland, H., Lovgren, K. and Gudding, R. (1991) Immune-response after immunization with an experimental *Toxoplasma gondii* iscom vaccine. *Vaccine*, **9**: 25.

Palmer, R., Ashton, D.S. and Moncada, S. (1988) Vascular endothelial cells synthesise nitric oxide from L-arginine. *Nature*, **333**: 664.

Papadopoulou, G., Karagouni, E. and Dotsika, E. (1998) ISCOMS vaccine against experimental leishmaniasis. *Vaccine*, **16**: 885.

Parker, D.C. (1993) T cell-dependent B-cell activation. *Annual Review of Immunology*, **11**: 331.

Phillips, J.C., Butterworth, K.R., Gaunt, I.F., Evans, J.G. and Grasso, P. (1979) Long-term toxicity study of *Quilaja* extract in medicine. *Food Cosmetic Toxicology*, **17**: 23.

Phillips-Quagliata, J.M. and Lamme, M.E. (1988) Migration of lymphocytes in the mucosal immune system. In: *Migration and homing of lymphoid cells*(Ed, Husband, A. J.) CRC Press, Boca Raton, pp. 53.

Plata-Salaman, C.R., Oomuta, Y. and Kai, Y. (1988) Tumour necrosis factor and interleukin 1 β : suppression of food intake by direct action in the central nervous system. *Brain Research*, **448**: 106.

Porcelli, S A., Segelke, B.W., Sugita, M., Wilson, I.A. and Brenner, M.B. (1998) The CD1 family of lipid antigen-presenting molecules. *Immunology Today*, **19**.

Prodeus, A.P., Zhou, X., Maurer, M., Galli, S.J. and Carroll, M.C. (1997) Impaired mast cell-dependent natural immunity in complement C3-deficient mice. *Nature*, **390**: 172.

Ramsay, A.J., Husband, A.J., Ramshaw, I.A., Bao, S., Matthaei, K.I., Koehler, G. and Kopf, M. (1994) The role of interleukin-6 in mucosal IgA antibody responses *in vivo*. *Science*, **264**: 561.

Reid, G. (1992) Soluble-proteins incorporate into ISCOMS after covalent attachment of fatty-acid. *Vaccine*, **10**: 597.

Reis e Sousa, C., Hieny, S., Scharon-Kersten, T., Jankovic, D., Charest, H., Germain, R.N. and Sher, A. (1997) *In vivo* microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. *Journal of Experimental Medicine*, **186**: 1819.

Rincon, M., Anguita, J., Nakamura, T., Fikrig, E. and Flavell, R.A. (1997) Interleukin (IL)-6 directs the differentiation of IL-4 producing CD4⁺ T cells. *Journal of Experimental Medicine*, **185**: 461.

Robertson, M.J., Soiffer, R.J., Wolf, S.F., Manley, T.J., Donahue, C., Young, D., S.H. and Ritz, J. (1992) Response of human natural killer cells to NK cell stimulatory factor (NKSF):

cytolytic activity and proliferation of NK cells are differentially regulated by NKSF. *Journal of Experimental Medicine*, **175**: 779.

Rocha, B., Vassili, P. and Guy-Grand, D. (1994) Thymic and extrathymic origins of gut intraepithelial lymphocyte populations in mice. *Journal of Experimental Medicine*, **180**: 681.

Rock, F.L., Hardiman, G., Timans, J.C., Kastelein, R.A. and Bazan, J.F. (1998) A family of human receptors structurally related to *Drosophila* Toll. *Proceedings of the National Academy of Science, USA*, **94**: 14614.

Romani, L., Menacacci, A., Cenci, E., Spaccapelo, R., Toniatti, C., Puccetti, P., Bistoni, F. and Poli, V. (1996) Impaired neutrophil response and CD4⁺ T helper cell 1 development in interleukin-6-deficient mice infected with *Candida albicans*. *Journal of Experimental Medicine*, **183**: 1345.

Ronnberg, B., Fekadu, M., Behboudi, S., Kenne, L. and Morein, B. (1997) Effects of carbohydrate modification of *Quillaja saponaria* Molina QH- B fraction on adjuvant activity, cholesterol-binding capacity and toxicity. *Vaccine*, **15**: 1820.

Roth, A.D., Hornicek, F.J. and Gerstner, C.G. (1991) Effects of interferon-gamma and tumour necrosis factor alpha on the development of cytotoxic T lymphocytes in autologous mixed lymphocyte tumour cultures with human melanoma. *Clinical and Experimental Immunology*, **86**.

Rudl, C., Rieser, C., Bock, G., Wick, G. and Wolf, H. (1996) Phenotypic and functional characterization of CD11c⁺ dendritic cell population in mouse Peyer's patches. *European Journal of Immunology*, **26**: 1801.

Russell-Jones, G.J., Ey, P.L. and Reynolds, B.L. (1981) Inhibition of cutaneous anaphylaxis and Arthus reactions in the mouse by antigen-specific IgA. *International Archives of Allergy and Applied Immunology*, **66**: 316.

Sambhara, S., Kurichh, A., Miranda, R., Tamane, A., Arpino, R., James, O., McGuinness, U., Kandil, A., Underdown, B., Klein, M. and Burt, D. (1998) Enhanced immune responses and resistance against infection in aged mice conferred by Flu-ISCOMS vaccine correlate with up-regulation of costimulatory molecule CD86, *Vaccine*, **16**: 1698.

Sastry, K. and Ezekowitz, R.A. (1993) Collectins: pattern recognition molecules involved in first line host defence. *Current Opinion in Immunology*, **5**: 59.

Savilahti, E., Arato, A. and Verkasalo, M. (1990) Intestinal γ/δ receptor-bearing T lymphocytes in coeliac disease and inflammatory bowel disease in children. Constant increase in coeliac disease. *Paediatric Research*, **28**: 579.

Scheepers, K. and Becht, H. (1994) Protection of mice against an influenza-virus infection by oral vaccination with viral nucleoprotein incorporated into immunostimulating complexes, *Medical Microbiology and Immunology*, **183**: 265.

Schfferli, J.A., Ng, Y.C. and Peters, D.K. (1986) The role of complement and its receptor in the elimination of immune complexes. *New England Journal of Medicine*, **315**: 488.

Schijns, V.E.C., Haagmans, B.L., Rijke, E.O., Huang, S., Aguet, M. and Horzinek, M.C. (1994) IFN γ -receptor-deficient mice generate antiviral Th1-characteristic cytokine profiles but also altered antibody responses. *The Journal of Immunology*, **153**: 5649.

Scott, P. (1991) IFN γ modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *Journal of Immunology*, **147**: 3149.

- Scott, P. and Kaufman, S.E. (1991) The role of T-cell subsets and cytokines in the regulation of infection. *Immunology Today*, **12**: 346.
- Severn, A., Xu, D., Doyle, J., Leal, L.M.C., O'Donnell, C.A., Brett, S., Moss, D.W. and Liew, F.Y. (1993) Pre-exposure of murine macrophages to lipopolysaccharide inhibits the induction of nitric oxide synthase and reduces leishmanicidal activity. *European Journal of Immunology*, **23**: 1711.
- Sicher, S.C., Vazquez, M. and Lu, C.Y. (1994) Inhibition of macrophage Ia expression by nitric oxide. *Journal of Immunology*, **153**: 1293.
- Sieburth, D., Jabs, E.W., Warrington, J.A., Li, X., Lasota, J., Laforgia, S., Kelleher, K., Huebner, K., Wasmuth, J.J. and Wolf, S.F. (1992) Assignment of genes encoding a unique cytokine (IL-12) composed of two unrelated subunits to chromosomes 3 and 5. *Genomics*, **14**: 59.
- Sjolander, A., Bengtsson, K.L., Johansson, M. and Morein, B. (1996) Kinetics, localization and isotype profile of antibody-responses to immune stimulating complexes (iscoms) containing human influenza virus envelope glycoproteins. *Scandinavian Journal Of Immunology*, **43**: 164.
- Sjolander, A., Vant Land, B. and Bengtsson, K.L. (1997) Iscoms containing purified *Quillaja* saponins upregulate both Th1-like and Th2-like immune responses. *Cellular Immunology*, **177**: 69.
- Smith, J.S. (1992) Suspend judgment: remembering the role of Thomas Francis Jr. in the design of the 1954 Sslk vaccine trial. *Control of Clinical Trials*, **13**: 181.

Smith, R.E., Maloy, K.J., Donachie, A.M. and Mowat, A.M. (1998) Immune stimulating complexes as mucosal adjuvants. *Immunology and Cell Biology*, **76**: 263.

Spangler, B.D. (1992) Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiology Review*, **56**: 622.

Stahl, P.D. (1992) The mannose receptor and other macrophage lectins. *Current Opinion in Immunology*, **4**: 49.

Stancovski, I. and Baltimore, D. (1997) NF- κ B Activation: The I κ B Kinase Revealed? *Cell*, **91**: 299.

Steger, K.K. and Pauza, C.D. (1997) Immunisation of *Mucaca mulatta* with *aroA* attenuated *Salmonella typhimurium* expressing SIVp27 antigen. *Journal of Medical Primatology*, **26**: 44.

Stern, A.S., Podlaski, F.J., Hulmes, J.D., Pan, Y.C., Quinn, P.M., Wolitzky, A.G., Familletti, P.C., Stremlo, D.L., Truitt, T., Chizzonite, R., Wolf, S.F. and Gately, M.K. (1990) Purification to homogeneity and partial characterisation of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proceedings of the National Academy of Science, USA.*, **8**: 6808.

Stoll, S., Muller, G., Kurimoto, M., Saloga, J., Tanimoto, T., Yamauchi, H., Okamura, H., Knop, J. and Enk, A.H. (1997) Production of IL-18 (IFN- γ inducing factor) messenger RNA and functional protein by murine keratinocytes. *Journal of Immunology*, **159**: 298.

Stuber, E., Strober, W. and Neurath, M. (1996) Blocking the CD40L-CD40 interaction *in vivo* specifically prevents the priming of T helper 1 cells through the inhibition of interleukin 12. *Journal of Experimental medicine*, **183**: 693.

- Sundquist, B., Lovgren, K. and Morein, B. (1988) Influenza-virus iscoms: antibody-response in animals. *Vaccine*, **6**: 49.
- Sydenham, M., Douce, G., Bowe, F., Ahmed, S., Chatfield, S. and Dougan, G. (2000) *Salmonella enterica* serovar typhimurium *surA* mutants are attenuated and effective live oral vaccines. *Infection and Immunity*, **83**: 1109.
- Takatsuki, F., Okano, A., Suzuki, C., Chieda, R., Takahara, Y., Hirano, T., Kishimoto, T., Hamuro, J. and Akiyama, Y. (1988) Human recombinant IL-6/B cell stimulatory factor 2 augments murine antigen-specific immune responses *in vitro* and *in vivo*. *The Journal of Immunology*, **141**: 3072.
- Thapar, M.A., Parr, E.L., Bozzola, J.J. and Parr, M.B. (1991) Secretory immune-responses in the mouse vagina after parenteral or intravaginal immunization with an immunostimulating complex (iscom). *Vaccine*, **9**: 129.
- Tracey, K.J., Vlassara, H. and Cerami, A. (1988) Cachectin/tumour necrosis factor induces cachexia, anemia and inflammation. *Journal of Experimental Medicine*, **167**: 1211.
- Trinchieri, G. (1995) Interleukin-12: a proinflammatory cytokine with immuno-regulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annual Reviews of Immunology*, **13**: 251.
- Tripp, C.S., Gately, M.K., Hakimi, J., Ling, P. and Unanue, E.R. (1994) Neutralization of IL-12 decreases resistance to *Listeria* in SCID and CB-17 mice. *Journal of Immunology*, **152**: 1883.
- Tripp, C.S., Wolf, S.F. and Unanue, E.R. (1993) Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe

combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiological antagonist. *Proceedings of the National Academy of Science, USA*, **90**: 3725.

Trudel, M., Nadon, F., Seguin, C., Brault, S., Lusignan, Y. and Lemieux, S. (1992) Initiation of cytotoxic T-cell response and protection of Balb/c mice by vaccination with an experimental ISCOMS respiratory syncytial virus subunit vaccine. *Vaccine*, **10**: 107.

Tulman, E.R. and Garmendía, A.E. (1994) Delivery of pseudorabies virus envelope antigens enclosed in immunostimulating complexes (ISCOMS) - elicitation of neutralizing antibody and lymphoproliferative responses in swine and protection in mice. *Vaccine*, **12**: 1349.

Udagawa, N., Horwood, N.J., Elliot, J., Mackay, A., Owens, J., Okamura, H., Kurimoto, M., Chambers, T.J., Martin, T.J. and Gillespie, M.T. (1997) Interleukin 18 (interferon- γ -inducing factor) is produced by osteoblasts and acts via granulocyte/macrophage colony stimulating factor and not via interferon- γ to inhibit osteoclast formation. *Journal of Experimental Medicine*, **185**: 1005.

Vajdy, M., Koscovilbois, M.H., Kopf, M., Kohler, G. and Lycke, N. (1995) Impaired mucosal immune responses in interleukin 4 targeted mice. *Journal Of Experimental Medicine*, **181**: 41.

VanCott, J.L., Staats, H.F., Pascual, D.W., Roberts, M., Chatfields, S.N., Yamamoto, M., Coste, M., Carter, P.B., Kiyono, H. and McGhee, J.R. (1996) Regulation of mucosal and systemic antibody responses by T helper subsets, macrophages and derived cytokines following oral immunisation with live recombinant *Salmonella*. *The Journal of Immunology*, **156**: 1504.

Varsanyi, T.M., Morein, B., Löve, A. and Norrby, E. (1987) Protection against lethal measles-virus infection in mice by immun-stimulating complexes containing the haemagglutinin or fusion protein. *Journal of Virology*, **61**: 3896.

Velupillai, P. and Harn, D.A. (1994) Oligosaccharide-specific induction of interleukin 10 production by B220⁺ cells from schistosome infected mice: a mechanism for regulation of CD4⁺ T cell subsets. *Proceedings of the National Academy of Science, USA*, **91**: 18.

Vicari, A.P. and Zlotnik, A. (1996) Mouse NK1.1⁺ T cells: a new family of T cells. *Immunology Today*, **17**: 71.

Villacres-Eriksson, M. (1995) Antigen presentation by naive macrophages, dendritic cells and B cells to primed T lymphocytes and their cytokine production following exposure to immunostimulating complexes. *Clinical and Experimental Immunology*, **102**: 46.

Villacres-Eriksson, M., Behboudi, S., Morgan, A.J., Trinchieri, G. and Morein, B. (1997) Immunomodulation by *Quillaja saponaria* adjuvant formulations: *In vivo* stimulation of interleukin 12 and its effects on the antibody response. *Cytokine*, **9**: 73.

Villacres-Eriksson, M., Bergstrom-Mollaoglu, M., Kaberg, H., Lovgren, K. and Morein, B. (1993) The induction of cell-associated and secreted IL-1 by iscoms, matrix or micelles in murine splenic cells. *Clinical and Experimental Immunology*, **93**: 120.

Villacres-Eriksson, M., Bergstrommollaoglu, M., Kaberg, H. and Morein, B. (1992) Involvement of interleukin-2 and interferon-gamma in the immune response induced by influenza-virus iscoms. *Scandinavian Journal Of Immunology*, **36**: 421.

- Viney, J.L., Mowat, A.M., O'Malley, J.M., Williamson, E. and Fanger, N.A. (1998) Expanding dendritic cells *in vivo* enhances the induction of oral tolerance. *Journal of Immunology*, **160**: 5815.
- Visser, I.K.G., Vedder, E.J., van der Bildt, M.W.G., Orvell, C., Barrett, T. and Osterhaus, A.D.M.E. (1992) Canine distemper virus ISCOMS induce protection in harbour seals (*Phoca vitulina*) against phocid distemper but still allow subsequent infection with phocid distemper virus-1. *Vaccine*, **10**: 435.
- Wange, Z-E., Reiner, S., Zheng, S., Dalton, D.K. and Locksley, R.L. (1994) CD4⁺ effector cells default to the Th2 pathway in IFN γ deficient mice. *FASEB JOURNAL*, **8**: A250.
- Watson, D.L., Lovgren, K., Watson, N.A., Fossum, C., Morein, B. and Hoglund, S. (1989) Inflammatory response and antigen localization following immunization with influenza-virus iscoms. *Inflammation*, **13**: 641.
- Watson, D.L., Watson, N.A., Fossum, C., Lovgren, K. and Morein, B. (1992) Interactions between immune-stimulating complexes (iscoms) and peritoneal mononuclear leukocytes. *Microbiology and Immunology*, **36**: 199.
- Wei, X-Q., Charles, I.G., Smith, A., Ure, J., Feng, G-J., Huang, F., Xu, D., Muller, W., Moncada, S. and Liew, F.Y. (1995) Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature*, **375**: 408.
- Wenner, C.A., Güler, M.L., Macatonia, S.E., O'Garra, A. and Murphy, K.M. (1996) Roles of IFN γ and IFN α in IL-12 induced T helper cell-1 development. *Journal of Immunology*, **156**: 1442.

Wershil, B.K., Tsai, M., Geissler, E.N., Zsebo, K.M. and Galli, S.J. (1992) The rat c-kit ligand, stem cell factor, induces c-kit receptor dependent mouse mast cell activation *in vivo*. Evidence that signalling through the c-kit receptor can induce expression of cellular function. *Journal of Experimental Medicine*, **175**: 245.

Wershil, B.K., Wang, Z-S., Gordon, J.R. and Galli, S.J. (1991) Recruitment of neutrophils during IgE-dependent cutaneous late phase responses in the mouse is mast cell dependent: Partial inhibition of the reaction with antiserum against tumour necrosis factor alpha. *Journal of Clinical Investigation*, **87**: 446.

Wijburg, O.L., Heemskerk, M.H., Boog, C.J. and Van Rooijen, N. (1997) Role of spleen macrophages in innate and acquired immune responses against mouse hepatitis virus strain A59. *Immunology*, **92**: 252.

Wolf, J.L. and Bye, W.A. (1984) The membranous epithelial (M) cell and the mucosal immune system. *Annual Review of Medicine*, **35**: 95.

Wynn, T.A., Eltoun, I., Oswald, I.P., Cheever, A.W. and Sher, A. (1994) Endogenous interleukin 12 (IL-12) regulates granuloma formation induced by eggs of *Schistosoma mansoni* and exogenous IL-12 both inhibits and prophylactically immunises against egg pathology. *Journal of Experimental Medicine*, **179**: 1551.

Xie, Q-W., Cho, H., Calaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D., Ding, A., Troso, T. and Nathan, C. (1992) Cloning and characterisation of inducible nitric oxide synthase. *Proceedings of the National Academy of Science, USA*, **256**: 225.

Xu, D., Chan, W.L., Leung, B.P., Hunter, D., Carter, W., McInnes, I.B., Robinson, J.H. and Liew, F.Y. (1998) Selective expression and functions of interleukin 18 receptor on T helper (Th) type 1 but not Th2 cells. *Journal of Experimental Medicine*, **188**: 1485.

Yang, R.B., Mark, M.R., Gray, A., Huang, A., Xie, M.H., Zhang, M., Goddard, A., Wood, W.I., Gurney, A.L. and Godowski, P.J. (1998) Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature*, **395**: 284.

Yang, X. and Brunham, R.C. (1998) Gene knockout B cell deficient mice demonstrate that B cells play an important role in the initiation of T cell responses to *Chlamydia trachomatis* (Mouse pneumonitis) lung infection. *Journal of Immunology*, **161**: 1439.

Ye, W., Zheng, I.M., Young, J.D.E. and Liu, C.C. (1996) The involvement of interleukin 15 (IL)-15 in regulating the differentiation of granulated metrial gland cells in mouse pregnant uterus. *Journal of Experimental Medicine*, **184**: 2405.

Yoo, J., Chen, H., Kraus, T., Hirsch, D., Polyak, S., George, I. and Sperber, K. (1996) Altered cytokine production and accessory cell function after HIV-1 infection. *Journal of Immunology*, **157**: 1313.