ROLE OF Th1 AND Th2 CYTOKINES IN THE PATHOGENESIS OF SYSTEMIC AUTOIMMUNE DISEASES

Ehsanollah Esfandiari (MD)

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

This thesis dedicated to:

my parents

and

my wife, Mastaneh for her encouragement, patience, enthusiasm and love

and

my daughters, Sahar and Sarah

Abstract

Systemic lupus erythematosus (SLE), is a prototypic systemic autoimmune disease characterised by multi-system involvement, female preference, activation of T-cells, B-cell hyper-activity, autoantibody production and immune complex deposition. The origin of the defects leading to pathogenicity in systemic lupus erythematosus, is still controversial. The therapeutic strategies today for treatment of lupus disease are mainly based on a general suppression of the immune system with uncertainty about their long-term effects. The underlying mechanism for the development of the disease is yet to be clarified. Cytokines play a critical role in regulating the quantitative and qualitative responses of T cells, B cells, macrophages, and other cell types. Many cytokine disorders have been reported in both SLE patients and the animal models but findings are often difficult to reconcile especially differences between data from the *in vitro* and *in vivo* studies. In the murine model, it was suggested that the balance of Th1/Th2 cytokines related to the pathogenesis of SLE. Recent evidence clearly demonstrates that Th1 cytokines are involved in the immuno-pathogenesis of SLE. Several factors are required for optimal induction of Th1 activity, chief among them are IL-12 and IL-18. IL-12 promoted IFN-γ dependent renal injury in MRL/*lpr* mice, which develop spontaneous lupus-like autoimmune disease. In order to understand the mechanism of immune regulation in SLE, I carried out detailed analysis of the nature and pathological relevance of Th1 and Th2 cytokines, IL-12 and IL-18 in particular, in the pathogenesis of SLE.

I found that serum from patients with SLE contained significantly higher concentrations of IL-18 than normal individuals. To investigate the potential role of IL-18 in SLE, I studied the effect of recombinant-IL-18 on MRL/*lpr* mice, which develop spontaneous lupus-like autoimmune disease. MRL/*lpr* mice produced significantly more IL-18 as disease progressed compared with the wild-type MRL/++ mice. MRL/*lpr* mice injected daily with IL-18 or IL-18 + IL-12 resulted in accelerated proteinuria, glomerulonephritis, and vasculitis. In contrast, the treatment

had no effect on the control MRL/++ mice. IL-18 and IL-18 + IL-12-treated MRL/*lpr* mice produced more inflammatory cytokines (IFN-γ, TNF-α and IL-6) compared with untreated MRL/*lpr* mice. IL-18-treated MRL/*lpr* mice also exhibited the butterfly facial rashes characteristic of clinical SLE. In contrast, MRL/*lpr* mice treated with a combination of IL-18 and IL-12, while showing more severe vasculitis than those treated with IL-18 alone, did not present any facial rash. Histological analysis of the facial lesion revealed extensive epidermal thickening with intense inflammatory cell infiltrate and immunoglobulin deposition accompanied by extensive apoptosis in the IL-18-treated mice compared with control or IL-12 + IL-18 treated mice. IL-18 may thus be a novel target for therapeutic intervention of spontaneous autoimmune diseases.

Elevated levels of IL-12 (p40/p70) have been reported in MRL/*lpr* serum and have been linked to increased nitric oxide production and disease activity. Therefore, studies were also performed to determine whether IL-12 and nitric oxide (NO) play a significant role (similar to MRL/*lpr* mice) in induction of the disease in NZB/W mice a lupus-like model with different genetic backgrounds from MRL/*lpr* mice and with intact Fas. The results demonstrate that serum of NZB/W F1 mice contains higher level of total IL-12 (p40/p70) than control mice and IL-12 is increased in correlation with disease of this lupus-like strain. In humans, the serum level of total IL-12 is significantly higher in SLE patients than control individuals. Whole blood culture from SLE patients also showed higher IL-12 production, when cultured with LPS and IFN-γ, compared with control individuals. Results presented in this thesis demonstrate that IL-18 and IL-12 play important roles in the induction of SLE through the activation of Th1 cells.

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List of Contents **Page**

Abstract 2

Acknowledgements 4

List of contents 5

List of figures 10

List of tables 13

Abbreviations 14

Chapter 1 General Introduction 17

1.1 Introduction to Autoimmunity 18

1.1.1 Tolerance 18

1.1.2 Peripheral Tolerance 19

1.1.3 B-cell Tolerance 22

1.1.4 Cytokines in autoimmunity 22

1.1.5 Classification of autoimmune diseases 24

1.2 Systemic lupus erythematosus (SLE) 24

1.2.1 History 25

1.2.2 Epidemiology and aetiology 25

1.2.3 Clinical features of SLE 27

1.2.4 Serological abnormalities in SLE 31

i) Anti-DNA autoantibodies 31

ii) Circulating Immune Complexes 33

1.2.5 SLE diagnostic Criteria 33

1.2.6 Treatment 36

1.3 Murine models of SLE 36

1.3.1 Lupus Models 37

1.3.2 Glomerulonephritis in lupus mice 38

1.3.3 Other histological changes in lupus-like models 39

1.3.4 serological changes in lupus-like models 40

1.4 Immunopathogenesis of SLE 41

1.4.1 B cells in SLE 41

1.4.2 T-cells in SLE 42

1.4.3 Cytokines in SLE 43

1.5 Interleukin-18 52

1.5.1 Biology of IL-18 52

1.5.2 IL-18 receptor 54

1.5.3 IL-18 expression and function in inflammatory diseases 55

1.6 Interleukin -12 57

1.6.1 Biology of IL-12 57

1.6.2 IL-12 receptor 60

1.6.3 Clinical function of IL-12 60

1.7 Aims of the thesis 62

Chapter 2 Materials and Methods 63

2.1 Patients and samples 64

2.2 Ethical considerations 64

2.3 Reagents /buffers 64

2.4 Plasma and tissue samples 65

2.4.1 Human plasma and serum 65

2.4.2 Mouse sera 65

2.5 Tissue sections 65

2.51 Frozen section 65

2.5.2 Paraffin-embedded tissue 66

2.6 Cell culture 66

2.6.1 Peripheral blood mononuclear cell preparation 66

2.6.2 Whole blood cell culture 67

2.6.3 Proliferation and cytokine production by human cells 67

2.6.4 Murine spleen cell and peritoneal cell preparation 68

2.6.5 Murine spleen cell and peritoneal cell activation 68

2.7 Immunohistochemistry 69

2.7.1 Peroxidase staining 70

2.7.2 Immunofluorescence staining 71
2.7.3 Tunnel staining 71
2.8 The measurement of nitric oxide (NO) 73
2.8.1 Griess reaction 73
2.8.2 Nitrite / Nitrate level in the serum and fluid bodies 73
2.8.2a Chemiluminescence assay for nitrite / nitrate 73
2.8.2b NO measurement by Modified Griess reaction 74
2.9 Enzyme linked immunosorbent assay (ELISA) 75
2.9.1 General ELISA protocol 75
2.9.2 Cytokines ELISA 76
2.9.3 ds-DNA antibody measurement by ELISA 76
2.10 FACS analysis Human cell subset analysis 77
2.11 RT-PCR for mRNA expression in tissues 78
2.11.1 RNA extraction 78
2.11.2 mRNA reverse transcription 79
2.11.3 cDNA Polymerase Chain Reaction (PCR) 79
2.11.4 Analysis of PCR products 80
2.12 Recombinant murine IL-18 purification 80
2.12.1 Rapid screening for recombinant IL-18 protein expression 80
2.12.2 Large scale purification of rmIL-18 81
2.12.3 Measurement of protein concentration 82
2.12.4 SDS-PAGE gel electrophoresis 82
2.12.5 Coomassie Blue staining of SDS-PAGE gels 83
2.13 Evaluation proteinuria in murine lupus-like models 83
2.14 Statistical Analysis 83
Chapter 3 Role of IL-12 and nitric oxide in the pathogenesis of SLE 85
Introduction 86
Results
3.1 Detection of IL-12 (p40/70) and IL-12 (p70) 87
3.2 Total IL-12 serum level in the different ages of NZB/W mice 90
3.3 Cytokine production by spleen cells from mice 94
3.4 IL-12 (p70) and IL-12 (p40/p70) in the serum of SLE patients 96
3.5 correlation between IL-12 level and clinical data in SLE patients 99
3.6 IL-12 production by PBMC and whole blood 101

cells from SLE patients
3.7 Assay for nitrite in biological fluids 105
3.8 Nitrite / Nitrate in the serum of NZB/W mice 107
3.9 NO production by peritoneal and spleen cells from lupus-like mice 109
3.10 NO production by PBMC and whole blood cells from SLE patients 111
3.11 NO production by PBMC and whole blood cells 113
Discussion 115

Chapter 4 Association of IL-18 with SLE 119
Introduction 120
Results
4.1 Detection of IL-18 in mouse and human serum 121
4.2 IL-18 in the serum of SLE patients 123
4.3 IL-18 level in the serum of MRL/*lpr* mice compared to MRL/++ 126
4.4 IL-18 production by MRL/*lpr* peritoneal and spleen cells 128
4.5 *In vitro* effects of recombinant IL-18 on MRl/*lpr* mice 130
Discussion 133

Chapter 5 Recombinant IL-18 accelerates disease in a lupus-like model 135
Introduction 136
Results
5.1 Bioactivity of recombinant IL-18 in vitro 137
5.2 Recombinant IL-18 accelerates disease in MRL/*lpr* mice 140
5.3 Histological analysis of the kidney 146
5.4 Vasculitis in the treated group 149
5.5 IL-18 treated mice developed facial rash 151
5.6 Immune-complex deposition and development of glomerulonephritis and vasculitis 153
5.7 Serum cytokines level in different treated group of mice 155
5.8 Level of IgG anti-DNA autoantibody isotypes in MRL/*lp* mice 157
5.9 In vitro immunologic consequences of in vivo IL-18 and IL-12 administration 159
5.10 *In vivo* and *in vitro* experiment in MRL/++ mice 163
Discussion 165

Chapter 6 Recombinant IL-18 induces a facial rash on MRL/*lpr* mice 171

Introduction 172

Results

6.1 recombinant IL-18 induces a facial rash in MRL/*lpr* mice 174

6.2 Skin histopathology 176

6.3 *In vitro* immunologic consequences of in vivo treatment 180

6.4 Autoantibodies and cytokine levels in the serum 182

6.5 Cytokines profiles and autoantibody levels after two weeks treatment 184

6.6 Cytokines profile and autoantibody levels after four weeks treatment 188

6.7 Assessment of IgG deposition in the skin of different group of mice 192

6.8 Apoptosis in the skin lesion of IL-18 treated mice 194

Discussion 197

Chapter 7 General Discussion 202

Introduction 203

7.1 Role of IL-12 and NO in SLE. 204

7.2 Association of IL-18 with SLE 206

References 212

Appendix I Preparation of buffers 260

Appendix II Publications arising 263

List of Figures

Chapter 3

Figure 3.1	Standard curve for murine IL-12 ELISA.	88
Figure 3.2	Standard curve for human IL-12 ELISA.	89
Figure 3.3	Elevated serum IL-12 levels in MRL/*lpr* mice.	91
Figure 3.4	Enhanced IL-12 levels in the serum of NZB/W mice.	92
Figure 3.5	Total IL-12 was elevated in NZB/W lupus strain.	93
Figure 3.6	Spleen cells from NZB/W mice produced higher concentrations of IL-12 in response to IFN-γ and LPS.	95
Figure 3.7	IL-12 (p40/p70) concentrations in sera obtained from SLE patients.	97
Figure 3.8	IL-12 (p70) concentration in serum from SLE patients.	98
Figure 3.9	Correlation between IL-12 serum levels and SLEDAI.	100
Figure 3.10	IL-12 (p40/p70) production by PBMC and peripheral whole blood cells from SLE patients.	103
Figure 3.11	IL-12 levels in culture supernatants from peripheral whole blood cells of SLE patients.	104
Figure 3.12	Standard curves for NO measurement.	106
Figure 3.13	Level of nitric oxide (nitrite/nitrate) in the serum of different ages of NZB/W and BALB/c mice.	108
Figure 3.14	NO production by peritoneal and spleen cells from NZB/W mice did not correlate with the age or disease.	110
Figure 3.15	Nitric oxide (nitrite / nitrate) levels in the serum of SLE patients by chemiluminescence assay.	112
Figure 3.16	IL-12 did not induce NO production by peripheral blood from SLE patients.	114

Chapter 4

Figure 4.1 Standard curve for IL-18 ELISA. 122

Figure 4.2 Serum level of IL-18 in SLE patients and healthy controls. 124

Figure 4.3 Serum IFN-γ and IL-5 levels in SLE patients and controls. 125

Figure 4.4 Serum levels of IL-18 in MRL/*lpr* and MRL/++ mice. 127

Figure 4.5 IL-18 production by cultured spleen and peritoneal cells from MRL/++ and MRL/*lpr* mice. 129

Figure 4.6 *In vitro* production of Th1 and Th2 cytokine production by spleen cells from 6 week old MRL/*lpr* mice. 131

Figure 4.7 *In vitro* production of Th1 and Th2 cytokine production by spleen cells from 13-14 week old MRL/*lpr* mice. 132

Chapter 5

Figure 5.1 The purity of recombinant IL-18. 140

Figure 5.2 Bioactivity of recombinant murine IL-18. 140

Figure 5.3 The protocol used to assess the role of rmIL-18 in the development of lupus disease in MRL/*lpr* mice. 143

Figure 5.4 The onset and progression of nephritis in SLE 144

Figure 5.5 Photomicrograph of kidney sections from different treated groups of MRL/*lpr* mice. 147

Figure 5.6 A quantitative estimate of kidney histopathology 148

Figure 5.7 Vasculitis in different groups of treated MRL/*lpr* mice. 150

Figure 5.8 Recombinant IL-18 treatment induced a facial rash in MRL/*lpr* mice. 152

Figure 5.9 Immune complex deposition in the kidney of MRL/*lpr* mice 154

Figure 5.10 Anti-DNA antibody isotype in the serum of MRL/*lpr* 156

Figure 5.11 Serum cytokine and NO_2/NO_3 levels in MRL/*lpr* mice 158

Figure 5.12 Th1 & Th2 related cytokine production by spleen cells from MRL/*lpr* treated mice. 161

Figure 5.13 NO_2 and IL-18 production by peritoneal cells from treated 162

MRL/*lpr* mice.

Figure 5.14 Cytokine levels in the serum and culture supernatants of MRL/++ mice. 163

Chapter 6

Figure 6.1 Malar rash in systemic lupus erythematosus. 172

Figure 6.2 Protocol used to assess the role of rmIL-18 in the development of skin lesion in MRL/*lpr* mice. 175

Figure 6.3 and 6.4 Recombinant IL-18 induces a facial rash in MRL/*lpr* mice 177

Figure 6.5 Th1 and Th2 cytokines production by spleen cells from MRL/*lpr* mice after facial rash appearance 181

Figure 6.6 Effect of rmIL-18 or rmIL-18 plus rmIL-12 treatment on the serum cytokine and anti-DNA antibody levels. 183

Figure 6.7 Levels of serum cytokine IFN-γ and IL-5 and anti-DNA antibody of IgG2a and IgG1 isotypes in MRL/*lpr* mice after two weeks cytokine treatment with IL-18, IL-12 plus IL-18 or PBS control. 186

.**Figure 6.8** *In vitro* cytokine production in the supernatants of culture spleen cells from MRL/*lpr* mice after 2 week cytokine treatment. 187

Figure 6.9 Cytokine levels in the serum and culture supernatants of MRL/*lpr* mice after 4 week cytokine 190

Figure 6.10 Anti-DNA antibody isotype distribution in the serum and culture supernatant of MRL/*lpr* mice after 4 weeks treatment. 191

Figure 6.11 IgG deposition in MRL/*lpr* treated groups 193

Figure 6.12 TUNEL staining of apoptosis in the facial skin lesion of IL-18 treated MRL/*lpr* mice. 195

List of Tables

Table 1.1	The 1982 revised criteria for classification of SLE	35
Table 2.1	Antibodies for immunohistochemistry	84

Abbreviations

2-ME	2-Mercaptoethanol
^{3}H-TdR	Tritiated thymidine
Ab	Antibody
ANA	Anti neucleotic antibody
AP	Alkalin phosphatase
APC	Antigen presenting cell
BBS	Borate - buffered saline
BSA	Bovine serum albumin
bp	Base pairs
CD	Cluster determinant
cDNA	Complementary deoxyribonucleic acid
CDR	Complementary-determining region
CM	Culture medium
ConA	Concanavaline A
CFU	Colony forming unit
cpm	Counts per minute
CRP	C-reactive protein
CTLL	Cytotoxic T lymphocyte line
DMARD	Disease modifying anti-rheumatic drug
ddH2O	Double distilled water
dH2O	Distilled water
DMSO	Dimethyl sulphoxide
DN	Double negative
DNA	Deoxyribose nucleic acid
dNTPs	2'-Deoxyribonucleoside 5'-triphosphate
ds	Double strand
EAE	Experimental allergic encephalomyelitis
EBV	Epstein Barr Virus
EDTA	Ethylenediaminotetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ENA	Epithelial neutrophil activating peptide
ESR	Erythrocyte sedimentation rate
FAD	Flavin adenine dinucleotide
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage - colony stimulating factor
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase

ICAM	intercellular adhesion molecule
IDDM	Insulin dependent diabetes mellitus
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin-
IU	International unit
kD	Kilo-Dalton
LFA	Lymphocyte function antigen
lpr	Lymphoproliferation
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metallo proteinase
mRNA	messenger ribonucleic acid
NADPH	ß-Nicotiamide adenine dinucleotide phosphate
NBT	p-nitroblue tetrazolium chloride
NGS	normal goat serum
NK	natural killer
NO	nitric oxide
$NO2^-$	Ntirite
$NO3^-$	Nitrate
NOD	Non obese diabetic
NOS	Nitric oxide synthase
NRS	Normal rabbit serum
NSAID	Non steroidal anti-inflammatory drug
OA	Osteoarthritis
OD	Optical density
PBL	Peripheral blood lymphocyte
PBMN	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBT	Peripheral blood T cell enriched
PE	Phycoerythrin
PFA	Paraformaldehyde
PG	Prostaglandin
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
ppb	Parts per billion
RA	Rheumatoid arthritis

RF	Rheumatoid factor
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SCID	Severe combined immuno-deficiency
SD	Standard deviation
SEM	Standard error of the mean
SE	Staphylococcal enterotoxin
SF	Synovial fluid
SLE	Systemic lupus erythematosus
ss	Single strand
Taq	Thermus aquaticus
TBE	Tris-borate / EDTA electrophoresis buffer
TBS	Tris-buffered saline
TCR	T-cell receptor
TE	Tris-EDTA
TGF	Transforming growth factor
Th	Helper T-lymphocyte
TNF	Tumour necrosis factor
Tris	Tris (hydroxymethyl)methylamine
TU	Titration unit
U	Units
v/v	Volume per volume
vs	Versus
M	Molar
mM	millimolar
mg	milligram
ml	millilitre
μg	microgram
μl	microlitre
μm	micrometre
μM	micromolar
ng	nanogram
nM	nanomolar
pg	picogram

Chapter 1

Introduction

1.1 Introduction to Autoimmune Diseases

Autoimmune diseases are characterised by the failure of the immune system to distinguish between self and non-self. The normal response of an adaptive immune system against foreign antigens is the clearance of antigens from the body. Immunological tolerance, or unresponsiveness, to self antigens is induced either by the encounter of immature lymphocytes with self antigens in the generative lymphoid organs, i.e. the bone marrow and thymus (central tolerance), or by exposure of mature lymphocytes to self antigens under particular conditions in peripheral tissues (peripheral tolerance) (Male et al., 1987; Miller et al., 1993). The breakdown of tolerance to self antigens that are often not encountered in the thymus, can result in autoimmune diseases in human. This breakdown in tolerance leads to undesirable clinical consequences, often including a series of pathological effects and organ destruction. Autoimmune diseases may also be characterised by the presence of either organ-specific autoantibodies, e.g. autoantibody to thyroid tissue in Graves disease, or by the presence of autoantibodies to different organs without organ specificity, in systemic lupus erythematosus (SLE).

1.1.1 Tolerance

The mechanism of self-tolerance is crucial in establishing a normal immune system, and central to which is clonal deletion of self-reactive lymphocytes. The original hypothesis of Burnet and Fenner (1949) dealing with unresponsiveness to self antigen, stated that all anti-self lymphocytes were eliminated before maturity. Tolerance to self antigens in healthy individuals can largely be explained by clonal deletion of self-reactive T cells in the thymus (McFarland 1996). However, *in vitro* studies on antigen reactivity as well as immune manipulations (Sakaguchi et al., 1985; Mason et al., 1992) that result in the development of autoimmune diseases have demonstrated that autoreactive T cells are still present in normal hosts. The observation that autoreactive T cells can be found in normal individuals indicates

that negative selection in the thymus is not absolute and suggests that other mechanisms operate in the periphery to actively maintain tolerance to self, or at least to inhibit the autoaggressive potential of T cells. Peripheral tolerance to self proteins is induced because these antigens are presented to T lymphocyes under conditions that do not allow effective immune responses to develop, or because the responses of these specific T cells are tightly regulated. Mechanisms of tolerance induction and maintenance in the periphery have evolved to avoid reactivity to self antigen (Ohashi et al., 1991; Oldstone et al., 1991).

1.1.2 Peripheral Tolerance

Peripheral tolerance is the mechanism which maintains unresponsiveness to antigens that are present only in peripheral tissues and not in the generative lymphoid organs. Peripheral mechanisms may also inactivate or kill lymphocytes that are specific for ubiquitous self antigens but escape central tolerance. The consequence of antigen recognition, i.e. activation or tolerance, depends mainly on two factors: how the antigen is presented to lymphocytes (its concentration, tissue location and persistence, and the nature of the cells that present the antigen), and how the response of specific lymphocytes to that antigen are regulated. The principal mechanisms of peripheral tolerance are activation-induced cells death (AICD), anergy and T suppressor cell activity regulatory T cells (Shevach, 2000).

Activation-induced cell death Activation-induced cell death (AICD) is a process of apoptosis induced by repeated activation of T lymphocytes by their cognate antigen but without certain co-stimulation, necessary for full development of activation. In $CD4^+$ T cells the principal mechanism of AICD is the co-expression of Fas (CD95) and Fas ligand (FasL, CD90), followed by engagement of Fas and delivery of a death-inducing signals (Nagata et al., 1994). The importance of this mechanism is illustrated by the fatal lupus-like systemic

autoimmune disease that develops in mice homozygous for mutations in either Fas or FasL, and by the similar disease seen in humans with mutations in Fas (Lenardo, 1996). From experiments on MRL-Mp-*lpr-lpr* (MRL/*lpr*) mice, which is extensively used as a lupus-like model, it is concluded that Fas does not play a significant role in central tolerance, but is crucial for deletion of mature T cells (van Parijs et al., 1998). The major pathway of AICD-dependent self-tolerance in mature $CD4^+$ T cells appears to involve Fas-FasL interactions. The main physiological role of Fas-mediated AICD is to eliminate T cells that are repeatedly stimulated by high concentrations of persistent antigens, e.g. self-antigens (Singer & Abbas, 1994; Singer et al., 1994; van Parijis et al., 1998). It has also been suggested that tumour necrosis factor (TNF), which is homologous to FasL, may participate in AICD in a mature $CD4^+$ T cells (Sytwu et al., 1996).

Anergy The second mechanism of peripheral T cell tolerance is anergy in which T cells become unresponsive to antigen. This was first described in mouse T cell clones, and shown to be due to a block in antigen receptor-generated signals as a results of antigen recognition in the absence of co-stimulation and IL-2 (Schwartz, 1990, 1997). Functional responses to antigen (signal 1) require additional signals, provided by co-stimulators and/or growth factors, and signal 1 alone leads to functional anergy. If T cells from TCR transgenic mice are exposed to their cognate peptide antigen in the absence of co-stimulation or growth factors, they undergo passive cell death (van Parijs et al., 1996, 1998). Exposure to tolerogenic peptide (high dose without adjuvant) leads to an initial expansion of peptide-specific T cell clones, but these T cells then become unresponsive to subsequent re-stimulation with antigen *in vivo* or *in vitro* (Perez et al., 1997).

Co-stimulators of the B7 family play critical roles in regulating the choices between T cell survival, proliferation and differentiation on one hand, and anergy or apoptosis on the other.

If antigen is presented by antigen-presenting cells (APC_S) in the presence of B7 antagonists, the antigen is effectively ignored by specific T cells. This results in passive cell death (non-Fas mediated AICD). If, however, T cells use the CTLA-4 receptor to interact with B7 molecules at the time of antigen recognition, the result is T cell anergy, whereas CD28-B7 interactions trigger functional T cell activation. What determines the choice between CTLA-4 or CD28 recognition of B7 is not established (van Parijs et al., 1997; Perez et al., 1997; Waterhouse et al., 1995).

The elucidation of T cell death and pathways and mechanisms of anergy has provided important clues about how peripheral tolerance to self-antigen is maintained. These concepts have been strongly reinforced by the identification of genetic mutations that lead to autoimmunity. Thus, the available evidence indicates that mutations in Fas/FasL and IL-2/IL-2Rα and IL-2Rß interfere with AICD, and this may lead to autoimmunity. There is a possibility that AICD is responsible for tolerance to abundant and widely disseminated protein antigens, which may be presented by competent APC_S and trigger the Fas pathway. In contrast, tissue antigens may be presented by resting APC_S in the absence of inflammatory cytokines, leading to CTLA-4-mediated anergy (van Parijs et al., 1998).

Regulatory T cells Convincing evidence now exists demonstrating that self tolerance, at least to tissue-specific antigens, is not a passive process but instead is an active dynamic state in which potentially pathogenic autoreactive T cells are prevented from causing disease by other T cells called regulatory T cells (Nishizuka & Sakakura, 1969; Fowell et al., 1993; Powrie et al., 1995). The evidence that $CD4^+$ T cells are functionally heterogeneous, as a result of their different cytokine profiles (Mosmann et al., 1986; 1989), has offered an explanation for the ability of certain T cells to produce immunopathology (i.e. autoimmunity) and others to regulate these autoreactive T cells (Liblau et al., 1995; O'Garra et al.,1993; Powrie et al.,

1996). *In vivo* and *in vitro* studies suggest that the $CD4^+$ $CD25^+$ population represents a unique lineage of suppressor T cells and play a critical role in regulating autoimmune disease in a large number of animal models (Taguchi & Takashi, 1996; reviewed by: Shevach 2000).

1.1.3 B-cell tolerance

The mechanisms of B-cell tolerance are not understood as well as those of T-cell tolerance. Tolerance in B cells may reflect an absence of B-cell reactivity (deletion or anergy), failure of T-helper cell function, T-cell mediated suppression or control of specific antibody production by B cells. B-cell development and tolerance also take place at the central and peripheral level. At the central level, a "pre-immune" repertoire of B-cell clones is generated by the diversity of variable joining regions of immunoglobulin due to gene recombinations that occur in the developing bone marrow or liver B-cell populations. This gives rise to a vast heterogenous B-cell population that produces low-affinity antibodies (Male et al., 1987). At the peripheral level, contact of these pre-immune B cells with specific antigen in the presence of helper T cells results in clonal expansion of B cells that produce high affinity antibody through somatic mutation. Since most pre-immune B-cell clones express "antibodies" with low affinity, it is unlikely that self-reactive antigens are present in very high levels (Gutierrez-Ramos et al., 1990; Moller et al., 1987; Martinez et al., 1988). Clonal deletion may occur if pre-immune low-affinity B cells encounter large amounts of antigens early in their development in the bone marrow or liver (high dose tolerance). Clonal anergy or functional inactivation may be induced in mature high-affinity peripheral B cells by contact with low amounts of antigen (low dose tolerance).

1.1.4 Cytokines in autoimmunity

CD4+ T helper (Th) cells include at least two subsets called Th1 and Th2 in mice and humans based on the profile of cytokines they secrete on activation. Th1 cells produce IL-2,

IFN-γ, lymphotoxin and are involved in cell-mediated immune responses directed at mainly intracellular infections. Th2 cells produce IL-4, IL-5, IL-10 and IL-13 and are involved in helping B cells to produce IgM, IgA, IgG1 and mainly IgE which is a strictly Th2-dependent antibody isotype (Mosmann et al., 1987; Powrie & Coffman 1993; Fowell et al., 1991; Romagnani et al., 1994).

The cytokines that are present in the vicinity of the CD4+ T cell at the time it comes into contact with a peptide antigen are crucial. IL-12 is a dominant cytokine directing Th1 development and is produced by activated macrophages and dendritic cells (Hesieh et al., 1993; Trinchieri et al., 1995; Macatonia et al., 1995). IFN-γ, produced by Natural Killer (NK) cells under the influence of IL-12 and TNF-α, and IL-18 are also required for IL-12-driven Th1 development. By contrast, IL-4 is the cytokine that plays a key role in the differentiation of the precursor CD4+ T cell towards a Th2 phenotype, accompanied by the production of IL-4, IL-5, IL-13 and IL-10 (Hsieh et al., 1992; Abbas et al., 1994). The two subsets are reciprocally regulated by the cytokines they produce. IFN-γ inhibits the proliferation of Th2 cells and antagonises some of the effects of IL-4 on B cell such as the increase in MHC class II and CD23 expression and isotype switching to IgE and IgG1 (D'Andrea et al., 1995). IL-10, IL-13 and IL-4 inhibit Th1 development by acting on macrophages.

The innate immune response and the accompanying Th1-reponse are thought to have evolved for the eradication of microbial pathogens (Sher et al., 1992); however, it has been proposed that if inflammatory Th1 responses are inappropriately directed against self-antigens this may lead to tissue destruction and pathology (Liblau et al., 1995; Powrie et al., 1993; O'Garra et al., 1997). Most destructive organ-specific autoimmune diseases are probably initiated by Th1 cells (O'Garra et al., 1997). The differentiation of T-helper lymphocytes into Th1 or Th2

cells is crucial in terms of susceptibility or resistance to infectious diseases such as *Leishmania major* infection in mice (Liew, 1989) and probably also play an important role in terms of susceptibility or resistance to the development of numerous autoimmune diseases

1.1.5 Classification of autoimmune diseases

Autoimmune disease can be classified according to the mechanism by which immune-mediated tissue damage is induced. (1) Humoral-mediated autoimmune diseases. Examples of these are myasthenia gravis and immune thrombocytopenia purpura (ITP). (2) Cell-mediated autoimmune diseases. Examples of these are, insulin-dependent diabetes mellitus, Hashimoto's thyroiditis, and rheumatoid arthritis. The division between the two is not clear cut, and evidence for the involvement of T-cells in pathology of antibody-mediated disease and vice versa has been documented.

Diseases associated with autoimmune phenomena tend to distribute in a spectrum of the number of organs afflicted. Thus, there are organ-specific diseases and multi-system diseases. At one pole, there is Hashimoto's disease, in which antibodies react against thyroid gland. At the other end of the spectrum, there is systemic lupus erythematosus (SLE) which is a systemic disease with multi-organ involvement (Roitt et al., 1996).

1.2 Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a non-organ specific autoimmune disease with unknown aetiology. It affects the joints, skin, serous membranes (pleura, pericardium, and the peritoneum), the kidneys, gastrointestinal tract, cardiovascular system, the brain, the bones and the clotting system. The clinical manifestations vary depending largely on which organ system, or systems, is affected and ranges from the mildest form of skin rash to life-threatening internal organ involvement.

1.2.1 History

Lupus, a term attributed to the 13th century physician Rogeriou, is Latin for "wolf". Rogeriou used it to describe the erosive facial lesions that were reminiscent of a "wolf's bite" (Blotzer, 1983). The term Lupus Erythematosus was first applied by the Frenchman Cazenava in 1851 (Potter, 1993). In 1845 von Hebra, a Viennese physician, used "butterfly" to describe the distribution of the malar rash of the disease. Kaposi recognised the visceral involvement of the disease in 1872 which then came to be known as *"acute disseminated lupus erythematosus"* (Benedek, 1997). In the 1920s and 1930s SLE was identified as a distinct clinical entity, largely because of the work of pathologists who had described the morbid anatomic changes that were characteristic of SLE. An example of this was the atypical nonbacterial endocarditis described by Emmanuel Libman and Benjamin Sacks in 1924 (Blotzer, 1983, Smith, 1988).

1.2.2 Epidemiology and aetiology

SLE is recognised worldwide. Its prevalence has ranged from 12/100,000 in Britain to 39/100,000 in Sweden (Hochberg, 1990). SLE is more prevalent in women, particularly in their reproductive years. In most studies, 90% of patients are women. For the 14-64 year age group, the ratio of age-specific and sex-specific incidence rates show 6-10 fold female excess (Lathia et al., 1981). This effect of age and sex probably indicates a role for hormonal factors in its pathogenesis. This proposal is supported by the abnormalities of sex hormone metabolism, causing elevated 16-α-hydroxyestrone and prolactin levels in SLE (Talal et al., 1987; Lavalle et al., 1987). The most likely role of these sex hormones in predisposing to SLE relates to their immuno-stimulatory effect on humoral immune function. Ostrogen binds directly to receptors on T-cytotoxic cells, inhibiting their activity and resulting in increased antibody production (Ansar Ahmed et al., 1985).

Genetic contribution The predisposition to SLE has been studied in twins. Dizygotic pairs have a concordance rate that is similar to that for other family members but is still six to eight times greater than that for unrelated individuals. On the other hand, the concordance rate in monozygotic twins is between 30 and 50 percent. These findings provide strong evidence of genetic factors influencing disease, they also suggest that environmental factors are of additional importance (Block et al., 1976; Arnett et al., 1976).

Of the genetic loci implicated in SLE, one of the most important for the manipulation of the autoreactive immune response is class II region of the major histocompatibility complex (MHC). Multiple MHC and non-MHC genes appear to predispose to the pathogenesis of SLE. Several studies have found that HLA-DR7 was associated with more severe disease (Welch et al., 1988). There is an increased incidence of HLA-DR2 and/or HLA-DR3, as well as inherited deficiencies of complement due to alleles for complement C4 loci. The frequency of certain autoantibodies is related to HLA-B8 and DR3, for anti-La (SSB), and HLA-DR4 for anti-RNP(Sm) (Hartung et al.,1989). The class III region of MHC contains the C4A and C2 genes of the complement system. Heterozygous or homozygous deletions of the C4A are common among SLE patients (Kemp et al., 1987), and C2 deficiencies are associated with the development of SLE (Roberts et al., 1978).

Environment Contribution The role of environmental factors in SLE has attracted much attention, but there is no clear etiologic agent yet identified. Some drugs (diphenylhydantoin, isoniazid, hydralazin, and procainamide) have been shown to cause symptoms similar to SLE (SLE-like syndrome). Exacerbation of SLE skin rash, occur after viral or bacterial infections, and changes in disease activity occur after administration of exogenous hormones. It seems that an abnormal humoral antibody response to an infection may lead to formation of antibodies that cross-react with a wide variety of human tissues in individuals who are

genetically predisposed to produce these autoantibodies (Strand and August, 1974; Panem et al., 1976).

1.2.3 Clinical features of SLE

SLE is characterised clinically by a mullet-system involvement affecting a variety of tissues and organs. 80% of patients with SLE will present with involvement of the skin or joints. A common presenting complaint is a photosensitive rash often with alopecia. Alternatively, patients may present with arthralgia or frank arthritis. However, patients may present with fever accompanied by single organ involvement, such as inflammatory serositis, glomerulonephritis, neuropsychiatric disturbance or haematological disorder (i.e. autoimmune haemolytic anaemia or thrombocytopenia). Patients can present rarely with severe, generalised acute lupus crisis with multi-organ involvement.

The wide clinical manifestations of SLE can make the diagnosis difficult. The patient's condition is often misdiagnosed as rheumatoid arthritis, fever of unknown origin, fibromyalgia, or even a psychsomatic disorder (Hoffman, 1978; Wilke, 1995; Perry & Miller, 1992).

Systemic Effects

Constitutional 90% of patients with SLE experience fatigue. Arthralgia and myalgia often accompany complaints of malaise. A less common but more problematic constitutional feature of SLE is persistent fever and weight loss. The fever of lupus is of a low grade and rarely exceeds 39°C (Stahl et al., 1979).

Musculoskeletal Approximately 90% of patients with SLE have musculoskeletal symptoms. One of the most common presenting symptoms of lupus is arthritis. The typical clinical

manifestation is arthralgia. The joints most commonly involved are the proximal interphalangeal, metacarpophalangeal, wrist, and knees. In contrast to rheumatoid arthritis, however, lupus is rarely accompanied by frank articular erosions. When arthritis occurs in SLE it usually is the consequence of periarticular inflammation with involvement or tendons. This can lead to Jaccoud's arthropathy, which is notable for reducible deformities. Myalgias are another common feature of SLE. Less common is frank inflammatory myositis which occurs occasionally during the course of SLE (Feldman et al., 1992; Isenberg, 1982).

Muco-cutaneous Approximately 80% of patients with SLE have dermatological manifestations during the course of their illness. The acute cutaneous eruption is manifest as a photosensitive rash, which often has a butterfly appearance by virtue of involving the bridge of the nose and malar areas of the face. A characteristic feature of this rash is sparing of the nasolabial folds. Photosensitivity is less common in black patients but occurs in 40-50% of all patients with SLE. The malar rash is acute in onset and usually heals without scarring. The "lupus-band test", which measures immunoglobulin and complement deposition at the dermal-epidermal junction in non-lesional skin is positive in more than 60% percent of patients. The rash of subacute cutaneous lupus is observed in anti-Ro antibody positive patients. This eruption is intermediately photosensitive and can either have an annular, polycyclic appearance or a more papulosquamous, pityriasiform, or psoriasiform appearance. 25% of patients with SLE have discoid skin lesions. These lesions are often on the face with a predilection for the inner pinna of the ear but are not photosensitive. These lesions are characterised clinically by follicular plugging, skin atrophy, scaling, telangectasia and skin erythema. Mucosal ulcers and raynauds phenomenons are frequent complication of lupus, occurring in 30% of patients.

Renal system Although the majority of patients with SLE may have glomerulopathy clinically relevant kidney disease occurs in about 50% of patients. This is usually the consequence of the deposition of immune complexes containing anti-DNA in the kidney (Appel et al., 1978; Estes et al., 1971; Kelley et al., 1997). Renal biopsy evidence of immune complex deposition is found in the kidney of all patients with SLE (Mahajan et al., 1977). Urine protein is a useful measure of renal lupus activity. Incremental changes of 500 mg of protein excretion is significant to renal pathology. Serum antibodies to DNA are a marker for the development of renal disease. Hypocomplementemia is often a harbinger of active renal disease. Mesangial lupus nephropathy is generally associated with an excellent prognosis. Proliferative lupus nephropathy, especially diffuse proliferative, often has a nephritic picture with hypertension, urinary red cell casts and can be accompanied by significant deterioration in renal function. Nephrotic syndrome in the absence of hypertension, active urinary sediment, or significant hypocomplementemia suggests membranous lupus nephropathy (Golbus & McCune, 1994; Gladman et al., 1989; Kelley et al., 1997).

Serositis Inflammatory serositis of the pleura, pericardium and peritoneum occurs in 50% of patients with SLE. This may produce pleuritis, pericarditis and medical peritonitis. These may occur in the absence of any significant effusion and represent a non-effusive serositis. Alternatively, patients can develop large pleural effusions, pericardial effusions or ascites. These effusions are typically inflammatory and exudative (Wang et al., 2000).

Hematological Anaemia of chronic inflammation is a common feature of SLE. Coombs positive hemolytic anemia with an acute declining hematocrit and reticulocytosis is a characteristic but not especially common occurrence in SLE. Autoimmune thrombocytopenia purpura can be a presenting feature of SLE or occur at any time in the course of the illness. Thrombocytopenia as a consequence of the antiphophsolipid antibody

syndrome has also been described in SLE. Leukopenia with lymphopenia is also a characteristic feature of SLE. Interestingly, when this occurs in the absence of cytotoxic drug therapy of the illness, it is not a significant risk for infection (Isenberg et al.,1982).

Central Nervous System (CNS) Neuropsychiatric complications can occur in up to 66% of SLE patients, and include acute and chronic, as well as focal and diffuse manifestations (McCune & Golbus, 1988). CNS manifestations include seizures, psychiatric illness, and disorder of cranial nerves. Cerebral vascular accidents are the consequence of either inflammatory or non-inflammatory, thrombotic vasculopathy in the central nervous system. Seizures complicate the course in 10-20% of patients with lupus (Sergent et al., 1975; Kohen et al., 1993). Diffuse cerebral dysfunction is manifest as an organic effective disorders, personality disorder, psychosis, or coma. Vascular or migraine headaches occur in 10% of lupus patients. Recurrent involvement of the central nervous system may result in an organic brain syndrome and dementia (Feinglass et al., 1976; Kelley et al., 1997).

Gastrointestinal Medical peritonitis with or without ascites is a manifestation of lupus serositis involving the peritoneum. Less common manifestations of lupus involving the gastrointestinal tract include mesenteric ischemia from mesenteric vasculitis and pancreatitis. The latter can be a manifestation of disease activity, or less commonly, a consequence of disease treatment as with steroids. Non-specific inflammatory liver disease has been described in lupus (Hallegua et al., 2000).

Ocular . Patients with lupus may develop anterior uveitis or iridocyclitis. Frank retinal vasculitis has been described, as well as central retinal artery occlusion, central retinal vein occlusion and ischemic optic neuropathy. Xerostomia with keratoconjunctivitis sicca is seen in 10% of patients (Wong et al., 1981).

1.2.4 Serological abnormalities in SLE

There are many serological abnormalities found in SLE, the most notable and widely recognised are serum autoantobodies, circulating immune complexes and hypocomplementaemia.

i) Anti-DNA autoantibodies in SLE

The common denominator among SLE patients is immunoglobulin G (IgG) autoantibody production, and the hallmark of this disease is elevated serum levels of antibodies to nuclear constituents (i.e., anti-nuclear antibodies).

Anti-nuclear antibodies (ANA) were first identified in the serum of patients with SLE over 40 years ago in four different laboratories (Isenberg et al., 1997). Among the myriad of autoantibodies produced in SLE, principal targets include certain protein-nucleic acid complexes, chromatin, the U1 and Sm small nuclear ribonucleoprotein (snRNP) particles, and the Ro/SSA and La/SSB RNP complexes, phospholipids, cytoplasmic and cell surface components, and even IgG rheumatoid factor (Tan, 1989; Kotzin and O'Dell, 1995).

Anti-dsDNA antibodies IgG autoantibodies to double-stranded DNA appear to play a prominent role in the immune complex glomerulonephritis of SLE (Kotzin and O'Dell, 1995). The likely involvement of these antibodies in the pathogenesis of human SLE, and in animal models of SLE, is indicated by

(1) the close links between disease activity and serum levels of anti-dsDNA antibodies (Spronk, et al. 1995);

(2) the elution (removal and collection) of these antibodies from the kidneys of patients with SLE and lupus-prone mice;

(3) direct evidence of these antibodies being associated with pathogenicity in isolated rat perfusion systems (in which kidneys are dissected from the rat and their function is maintained artificially for a few hours) and mice with severe combined immunodeficiency (SCID); and

(4) the fact that although antibodies to single-stranded DNA (ssDNA) are frequently found in healthy relatives of patients with SLE, those that bind to dsDNA are virtually never detected (Andrzejewski et al. 1981).

Clinical anti-dsDNA antibody studies Many studies have concluded that levels of anti-dsDNA antibodies, quantified by enzyme-linked immunosorbent assay (ELISAs) or radioimmunoassay (RIA), generally reflect clinical disease activity (Spronk et al. 1995). This observation appears to be particularly true of renal disease, and most of the evidence that anti-dsDNA antibodies are pathogenic has been derived from studies of the kidney.

High levels of high affinity anti-dsDNA antibodies, and low values of functional complement CH50 were found predominantly in lupus patients (Swaak et al., 1979; Lioyd and Schur, 1981). In contrast, antibodies to ssDNA are not specific for patients with SLE, being present, for example, in many individuals with infectious diseases. A prospective study of 72 patients showed that active lupus nephritis was usually associated with high titres of anti-dsDNA antibodies (ter Borg et al., 1990). More recently, a close relationship was seen between renal disease activity (assessed by biopsy) and the ds-DNA antibody isotype (Okamura et al., 1993). Disease activity correlated with IgG against dsDNA but not with IgG against ssDNA or with IgM reactive against either dsDNA or ssDNA. Bootsma and colleagues (1995), using the concept of a rise in levels of anti-dsDNA antibodies as a means of predicting a clinical relapse, showed that treating such patients with high levels of prednisolone (30 mg/day)

reduced the relapse rate, compared with a control group who were treated with either lower doses of prednisolone or no steroids.

ii) Circulatory Immune Complexes

Immune complexes (IC) are commonly observed in both SLE patients and lupus mouse models. IC_S have been shown to consist of nucleic acid and serum antibodies of corresponding specificity. Three types of IC_S identified are ds-DNA-anti-dsDNA, ssDNA-anti-ssDNA and ssDNA-anti-dsDNA (Brentjens and Andres, 1982). It is believed that deposition of immune complexes in tissues, such as skin, kidneys, blood vessels and brain, may initiate some inflammation. DNA antigens and associated antibodies have been demonstrated in the immune complexes deposited in kidneys showing proliferative and membranous lupus nephritis (Angelo et al., 1976; Andres et al., 1975). Antibody avidity and class, antigen/antibody ratio and size of DNA have been shown to be important in determining the deposition of these immune complexes (Morrow et al., 1982, 1983).

1.2.5 SLE diagnostic Criteria

For a disease with such protean manifestations and variable course as SLE, the need for classification criteria, which would allow comparison of patients, is quite clear. To assist in the diagnosis of SLE the American Rheumatism Association has define a list of 11 criteria and if a patient has 4 more of these over a period of time, they may have Lupus (Tan et al., 1982). The 1982 revised ACR criteria for SLE are listed in Table 1.1.

Criteria for disease activity in SLE Over sixty systems to assess clinical disease activity in SLE have been devised, but there have been only a few which have been validated (Klipple et al., 1998). Three of these, the SLE Disease Activity Index (SLEDAI), the British Isles Lupus

Assessment Group (BILAG) and the SLE Activity Measure (SLAM) distinguished among patients, and correlate highly with each other (Gladman et al., 1994).

Table 1.1 The 1982 revised criteria for classification of systemic lupus erythematosus.

Criterion	Definition
1. Malar Rash	Fixed erythema, flat or raised. Over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous raised patches with adherent keratonic scaling and follicular plugging.
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight.
4. Oral ulcers	Oral or nasopharyngial ulceration, usually painless, observed by a physician.
5. Arthritis	Nonerosive arthritis involving two or more peripheral joints, characterised by tenderness, swelling or effusion.
6. Serositis	(a) Pleuritis, convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion. (b) Pericarditis, documented by ECG or rub or evidence of pericardial effusion.
7. Renal disorders	(a) Persistent proteinuria greater than 0.5 grams per day or greater than 3+ if quantitation not performed. (b) Cellular casts, may be red cell, hemoglobin, granular, tubular or mixed.
8. Neurological disorder	(a) Seizures, in the absence of offending drugs or known metabolic disorders: e.g Uremia, ketoacidosis, or electrolyte imbalance. (b) Psycosis, in the absence of offending drugs or known metabolic disorders: e.g Uremia, ketoacidosis, or electrolyte imbalance.
9. Hematologic disorder	(a) Hemolytic anemia, with reticulocytosis (b) Leukopenia, less than 4000 mm^3 total on two or more occasions. (c) Lymphopenia, less than 1500 mm^3 on 2 or more occasions (d) Thrombocytopenia, less than 100,000/mm^3 in the absence of offending drugs.
10. Immunologic disorder	(a) positive LE cell preparation. (b) Anti-DNA antibody to native DNA in abnormal titre (c) Anti-Sm:presence of antibody to Sm nuclear antigen (d) False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by Treponema pallidum immobilization or florescent Treponemal antibody absorption test.
11. Antinuclear antibody	an abnormal titre of antinuclear antibody by immunofluoresence or an equivalent assay and in the absence of drugs known to be associated with "drug-induced lupus" syndrome.

For the purpose of identifying patients in clinical studies, a person shall be said to have systemic lupus erythematosus if any of 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.

1.2.6 Treatment

Although SLE is a chronic rheumatic syndrome, its clinical course is typically one of relapse and remission. Since the cause of the disease is still unknown, clinical treatment for SLE have been mainly based upon achieving symptomatic relief. Management includes interventions directed at acute flares of the disease, that occasionally may be life-threatening, maintenance therapies and close monitoring of chronic progressive disease, and minimal or no treatment during periods of remission. There are four main groups of drugs including the non-steroid anti-inflammatory drugs (NSAID), anti-malaria and cytotoxic drugs (Klipple, 1998) and corticosteroids (Kimberly, 1992). The therapeutic strategies are usually designed for a general suppression of the immune system

The prognosis for patients with SLE has greatly improved over the last few decades with at least 80-90% of all patients surviving ten years. Thereafter life expectancy approximates to that of age matched healthy controls. This improvement reflects the general advancements in health care (i.e. dialysis, antibiotics, antihypertensives, newer immunosuppressives with more favourable efficacy to toxicity ratio) and also the specialised care available for patients with SLE (Strand, 2000).

1.3 Murine models of Systemic Lupus Erythematosus

The pathogenesis of autoimmune diseases cannot easily be studied and manipulated without appropriate animal models. Inbred mice that develop a lupus-like disease similar to human systemic lupus erythematosus (SLE) have been used extensively to elucidate the etiopathogenesis of SLE. The main clinical and immunological abnormalities, which are related to the human disease, also appear in these mouse models.

Most studies on SLE have been on three different genetic background strains of mice, F1 hybrids of New Zealand Black (NZB) and New Zealand White (NZW) mice (NZBxNZW)F1, MRL, and BXSB mice.

1.3.1 Lupus models

(NZBxNZW) F1 hybrids were the first described experimental model, which spontaneously develop lupus-like autoimmune disease (Howie and Heyler, 1965, 1968). The NZB ($H2^d$) strain which inherits haemolytic anaemia by mating with the phenotypically normal NZW ($H2^z$) changed to the hybrids (NZBxNZW)F1 that underwent changes remarkably similar to human lupus nephritis (Howie and Helyer, 1965). The NZB/W ($H2^{d/z}$) strain has been used as one of the best models of human SLE studies.

Two other mice strains, MRL and BXSB, were developed in 1976 by Murphy and Roths at the Jackson Laboratory (Murphy and Roths, 1979). The MRL strain originated by a series of crosses involving inbred strains AKR/J, C57BL/6J, C3H/Di and LG/J. In the 12th. generation of inbreeding, some of the offspring developed massive general lymphodenopathy. These were termed MRL/n and the other offspring, which did not develop lymphodenopathy, were termed MRL/l. Breeding tests and reciprocal backcross between MRL/l and MRL/n mice indicated that the massive lymphoproliferation was controlled by a single autosomal recessive gene, termed *lpr* (lymphoproliferation). The original substrain MRL/n was redesigned MRL/Mp-+/+ at F28. By 1980, the *lpr* gene had been transferred by 10 cycles of cross-intercross mating to MRL/Mp-+-+ with an estimated residual difference of less than 0.1% (Theofilopoulos and Dixon, 1985). Mice homozygous for *lpr* develop autoimmune syndromes characterised by the formation of autoantibodies to nuclear antigens and the marked accumulation of an abnormal double negative (DN) $CD4^-/CD8^-$ T cell population in the lymph node and spleen (Cohen and Eisenberg. 1991). The *lpr* mutation

interferes with Fas transcription and prevents expression of Fas on the cell surface, (Watanabe-Fukunaga et al.,1992).

The BXSB strain ($H2^b$), a recombinant inbred strain derived from a cross between C57BL/6 (B6) females and SB/Le males, spontaneously develops an autoimmune syndrome with features of SLE that affects male animals much earlier than females (Murphy & Roths 1978; Andrews et al., 1978). Yaa gene (Y chromosome-linked autoimmune acceleration) present in the Y chromosome of the BXSB mouse is responsible for the accelerated autoimmune abnormalities and immunopathological lesions (Murphy & Roths 1978; Hang et al., 1981; Izui et al., 1984). A unique cellular abnormality associated with the Yaa gene is monocytosis (Wofsy et al., 1984; Izui et al., 1995).

Esterogens accelerate disease in (NZB/W F1)mice, slightly accelerate disease in MRL/*lpr* mice and they have no effect on BXSB mice (Stoll & Gavalchin 2000).

1.3.2 Glomerulonephritis in the lupus mice

The (NZB X NZW)F1, MRL-*lpr/lpr*, and BXSB mice all develop a progressive severe glomerulonephritis and are primarily models of diffuse proliferative lupus nephritis. As in human SLE, all these mice develop high levels of IgG autoantibodies to nuclear antigens, including dsDNA. These autoantibodies mediate nephritis, probably as a result of in situ immune complex formation in the glomerulus. Lupus-prone strains produce antibodies to another self-antigen, the endogenous xenotropic viral glycoprotein, gp70, and these autoantibodies have also been implicated in the pathogenesis of murine lupus nephritis. Extra-renal disease manifestations variably occur in these models and include lymphoproliferation with both splenomegaly and lymphadenopathy, haemolytic anaemia, autoimmune thrombocytopenia, vasculitis, thrombosis, and arthritis (in MRL/*lpr*). All of

these lupus-prone strains also exhibit premature thymic atrophy, the significance of which, however, is unknown (Vyse and Kotzin 1998; Theofilopoulos & Dixon 1985; Izui et al., 1995).

The major cause of death in NZB/W, MRL/*lpr* and BXSB mice is glomerulonephritis. This is an exudative and proliferative acute form in the BXSB mice, a subacute proliferative form in the MRL/*lpr* mice and a chronic obliterative form in the NZB/W mice. Glomerular lesions in the MRL/*lpr* mice consist of the accumulation of monocytes and the proliferation of both endothelial and mesangial cells, with occasional crescent formation and basement membrane thickening. The obliterative lesion in NZB/W female mice is mesangial with occasionally intravascular proteinaceous deposits, moderate proliferation of all glomerular cells elements and crescent formation (Theofilopoulos & Dixon 1985; Andrews 1978).

1.3.3 Other histological changes in the lupus-like models

A severe cortical thymic atrophy is a feature of SLE pathology in all strains of lupus mice (Andrew et al., 1978; Theofilopoulos and Dixon, 1981). In female NZB/W mice the thymic atrophy is appeared by the fourth month of age, but by 6-7 months of age they lose 60-70% of their cortexes. In BXBS and MRL/*lpr* mice, the thymic atrophy and cystic necrosis appears by 2 months of age and a complete loss of cortex is occurred by 4.5 and 3.5 months of age, respectively (Theofilopoulos and Dixon, 1985).

Marked splenic and lymph node hyperplasia exists in all murine lupus strains, and lymphoid infiltrates may also occur in the lungs, kidneys, liver, saliva glands, and bone marrow (Theofilopoulos and Dixon, 1985). Lymph node size can be up to 2-3 times normal in older NZB/W mice; 10 to 20 times more than normal in older BXSB males; and up to 100 times normal in 4-5 months old MRL/*lpr* mice (Andrew et al., 1978; Murphy,1981; Theofilopoulos

and Dixon, 1981). There is an approximate 7-fold enlargement in the spleen of older MRL/*lpr* mice. A similar 8-fold splenomegaly is observed in older male BXSB mice (Theofilopoulos and Dixon, 1985).

1.3.4 Serological changes in lupus-like models

Elevated serum Immunoglobulin (Ig) concentrations, anti-nuclear antibodies (ANA), anti-ds and anti-ssDNA antibodies, anti-retroviral envelop gp70 antibodies, immune complexes and reduced complement levels are common in all SLE strains.

Antibodies against nuclear constituents are characteristic of SLE in humans and mice (Tan, 1982). Among lupus-like models ANA titres are highest in MRL/*lpr*, then NZB/W females, and then BXBS males (Andrews et al., 1978). In all strains a peripheral or rim pattern of nuclear fluorescence is always at the highest positive serum dilution, whereas the homogeneous pattern is sometimes seen at lower dilutions. Anti-DNA antibodies are classified into four groups (reviewed by Tan, 1982):

(1) Antibodies reactive with double-strand DNA recognise mainly the deoxyribose phosphate backbone and are reactive with both double and single-strand DNA. Therefore, antibodies against dsDNA are usually cross-reactive with ssDNA.

(2) Antibodies reactive only with ssDNA. It appears that this clone of antibody is directed against purine or pyrimidine bases and does not react with dsDNA because the bases are buried within the double helix. These antibodies appear in several different diseases including, SLE, drug-induced lupus, chronic active hepatitis, infectious mononucleosis, and rheumatoid arthritis.

(3) Antibodies which recognise the sugar-phosphate backbone and therefore recognise both dsDNA and ssDNA. Some of these antibodies also react with polynucleotides and phospholipids, including cardiolipin.

(4) Antibodies against "left-handed" or Z-DNA.

Because of differences in epitope and disease association, anti-DNA assays must clearly distinguish between ssDNA and dsDNA substrates. Two methods are available: (1) digestion with S1 nuclease, which removes overhanging ssDNA, and (2) chromatography on a hydroxyapatite column, which separate large single-stranded segments from dsDNA. However, native DNA may spontaneously denature, especially when bound to plastic ELISA plates. Two other additional assays offer greater assurance for anti-dsDNA testing. (1) The Farr radioimmunoassay, which resembles immunopercipitation assays, involves the binding of autoantibodies to radiolabelled dsDNA in solution. (2) *Crithidia Luciliae* immunofluorescence test that provides an inherently reliable dsDNA analysis. Farr and *Crithidia Luciliae* immunofluorescence tests thus provide effective, complementary mechanisms to distinguish anti-ssDNA from anti-dsDNA activities (Kelley et al., 1997).

1.4 Immunopathogenesis of SLE

Numerous immunological disorders featuring functional abnormalities of lymphoid cells are related to SLE (Tsokos, 1992). These include hyperactivity of B cells and T cells, and the failure of immunoregulatory mechanisms to down-regulate these responses. The consequence of this unregulated lymphocyte activation are production of pathogenic autoantibodies, formation of immune complexes, and T cell dysfunction.

1.4.1 B cells in SLE

B-cell hyperactivity is one of the immunological markers of lupus disease. B cells from the blood of SLE patients spontaneously secrete large amounts of immunoglobulins including antibodies to self-antigens (Jasin and Ziff, 1975; Budman et al., 1977). Upon B-cell mitogen stimulation, these cells also secrete higher levels of immunoglobulin than cells from normal subjects (Delfraissy et al., 1986; Flescher et al., 1990). B cells are abnormal in

both human and murine SLE (Reininger et al., 1996; Clark et al., 1996). In people with SLE, there is a marked increase in the number of plasma cells in the peripheral blood that are secreting immunoglobulin, as well as B cells at all stages of activation. Pathogenic IgG autoantibody production in SLE is selective for only certain self-antigens and the autoreactive B cells are stimulated by self-antigens. In SLE and lupus mice, a subset of anti-DNA antibody-producing B cells are clonally expanded and their immunoglobulin genes are modified by somatic mutation (reviewed by Radic et al., 1994). This process indicates a normal T cell-dependent response to foreign antigen, involving common mechanisms of somatic mutation, affinity maturation, and IgM to IgG class switching (Kotzin, 1996).

1.4.2 T-cells in SLE

Studies on peripheral and thymic T-cells have revealed that cellular and functional abnormalities of T-cells exist in SLE patients as well as in lupus animal models. The main evidence for defects in the T-cell compartment is the abnormal activities of these cells. T-cell lymphopenia is characteristic of patients with lupus and its severity correlates with disease activity (Steinberg et al., 1991). The association of SLE with particular class II major histocompatibility complex (MHC) alleles and the affinity maturation of IgG autoantibody production in this disease also strongly suggest that CD4 T cells are important in the pathogenesis of SLE (Kotzin, 1996).

T-cells from lupus patients and several mouse models display severely impaired abilities to proliferate and to produce cytokines in response to T-cell mitogens (Kroemer and Wick, 1989; Kroemer and Martinez-A, 1991). Decreased autologous mixed lymphocyte reaction (AMLR), and reduced activities of cytotoxic T-cells and NK cells have been common findings both in human lupus and lupus-like models (Kuntz et al., 1979; Theofilopoulos, 1992). Furthermore, in all of the major murine models, treatment with anti-CD4 antibodies

can ameliorate IgG autoantibody production and disease (Steinberg et al., 1980; Peng et al., 1996; Singh and Hahn, 1998). Additional studies indicate that blocking T cell activation or T cell-B cell interactions will also prevent autoantibody production and disease (Finck et al., 1994; Mohan et al., 1995). T-cells that lack both CD4 and CD8 molecules in SLE patients (Steinberg et al., 1991) and in lupus mice models (Datta, 1989) which are not MHC restricted, may provide clues to the regulation of the pathogenic autoantibody secreting B-cells. In addition, $CD4^+DR^+$ and $CD8^+DR^+$ T-cells isolated from SLE patients were also reported to support polyclonal IgG production and autoantibody synthesis (Linker-Israeli et al., 1990). Therefore, the B-cell hyper-responsiveness may be largely attributed to lack of, or abnormal, T-cell regulation.

1.4.3 Cytokines in SLE

In normal individuals the expression and production of cytokines are under tight regulatory control. The presence or absence and the concentration of a particular cytokine can have profound effects on the regulation of the immune system. In recent years, the role of cytokine regulation in autoimmune diseases has been widely investigated. *In vitro* and *in vivo* evidence to date indicates the involvement of cytokine dysregulation in the development of autoimmunity. Abnormal cytokine production and defective responsiveness of lymphocytes to various cytokines have been demonstrated in many of the autoimmune disorders including SLE.

The structure, expression and functional status of cytokines in SLE have been investigated regarding the possible utility of their agonists and antagonists as therapeutic agents. In fact, mouse strains predisposed to lupus (MRL/*lpr,* NZBxW, BXSB) are the primary source of information in the role of cytokines in autoimmunity.

Th1 lymphocytes secrete IFN-γ and are considered to mediate cell mediated immunity (CMI) and it has been hypothesised that induce organ specific autoimmune diseases such as IDDM. Th2 cytokines are considered to mediate humoral immunity and antibody mediated autoimmune diseases such as lupus. Such a paradigm seems to be too simplistic in lupus because both Th1 and Th2 cytokines have been found to exert profound effects on spontaneous mouse models of this disease.

Th1 cytokines

IL-2 The earliest cytokine defect identified in all lupus mouse strains was a reduced production of, and response to, IL-2 (Altman et al., 1981; Dauphinee et al., 1981). This defect appears at 4-6 weeks of age in MRL/*lpr* and BXSB mice, and somewhat later in the (NZBxNZW)F_1 mice. Thereafter, this defect becomes more pronounced with disease advancement. The cause of this defect is unknown, but several possibilities have been considered. These include impaired T cell receptor (TCR) signal transduction, IL-2R structural defects, abnormalities in IL-2 or IL-2R gene transcription factors, and exhaustion of IL-2 production subsequent to excessive and repetitive activation *in vivo* (Tanaka et al., 1993; Liang et al., 1998).

When peripheral mononuclear cells (PBMCs) from patients with SLE are stimulated with LPS and PHA in culture, basal and induced levels of supernatant IL-2 are significantly correlated with the clinical SLAM index (Segal et al., 1997). High IL-2 levels were detected in the sera of 50% of patients with active disease. However, there are also increased levels of the soluble receptor for IL-2 (CD25) found in the sera of patients with active disease which may antagonise IL-2 deficiency (Huang et al., 1988).

The relation of the IL-2 defect to the disease process remains unclear. Several reports have shown correction of the *in vitro lpr* T cell defective proliferation and apoptosis by exogenous IL-2 (Clements et al., 1994; Radvanyi et al., 1998). In a recent study (Huggins et al., 1999) MRL/*lpr* mice infected orally by gavage with an attenuated strain of *Salmonella typhimurium* transfected with the IL-2 gene (administered at 6 weeks of age and repeated every three weeks to 15 weeks of age) were shown to have reduced double negative T cells, autoantibody levels, glomerulonephritis (GN) and vasculitis. In contrast, intramuscular injections of an IL-2 encoding cDNA expression vector in MRL/*lpr* mice were reported to increase autoantibody production and disease (Raz et al., 1995). Another study (Owen et al., 1989) reported no effect on disease progression and severity in (NZBxNZW)F_1 mice treated with low or high doses of human recombinant IL-2, while others found suppression of nephritis in (NZBxNZW)F_1 mice treated with anti-IL-2R mAb (Bocchieri et al., 1984).

IFN-γ Among the many cytokine abnormalities found in lupus mice, the most consistent has been high expression of IFN-γ (1995; Davidson et al., 1991; Shiraie et al., 1995). The production of IFN-γ by PBMCs from patients with SLE is significantly correlated with the Systemic Lupus Activity Measure (SLAM) (Viallard et al., 1999; Sturfelt et al., 1997). *In vitro* production of IgG by PBMCs from patients with SLE was decreased by exogenous IFN-γ (Braude et al., 1988). Funauchi and colleagues suggested that IFN-γ might be one of the factors that promote polyclonal B cell activation in SLE (1991).

The importance of this cytokine in murine lupus pathogenesis was initially suggested by the demonstration that (NZBxNZW)F_1 mice treated with IFN-γ showed accelerated disease. Conversely, treatment with anti-IFNγ antibody (Jacob et al., 1987) or soluble IFNγ-R (Ozmen et al., 1995) early in life significantly delayed disease progression. A study on a long-lived

substrain of MRL/*lpr* mice also showed reduced IFN-γ levels compared with the parental strain. The reduced IFN-γ is concomitant with a shift of Ig isotype from the complement-fixing IgG2a and the cryogenic-nephritogenic IgG3 to the less pathogenic IgG1 isotype (Takahashi et al., 1996). Moreover, MRL/*lpr* mice crossed with an IFN-γ gene deleted mouse (Peng et al., 1997), MRL/*lpr* mice rendered congenic for deletions in either the IFN-γ (Balomenos et al., 1998) or the IFNγ-R (Hass et al., 1997; Balomenos et al., 1997), and (NZBxNZW)F_1 mice congenic for the IFNγ-R deletion (Haas et al., 1998) all showed significant reduction in humoral and histological characteristics of the disease.

In one of these studies (Balomenos et al., 1998), the following observations were made: Hypergammaglobulinaemia was maintained in IFN-/- mice with a switch from IgG2a to IgG1 predominance. Along with this, there was a highly significant decrease in levels of the dominant IgG2a anti-dsDNA autoantibodies but there was no increase IgG1 subclasses. This finding suggested that therapeutic interventions to reduce IFN-γ levels in lupus may selectively affect certain pathogenic autoimmune responses without significantly compromising the person's capacity to respond to exogenous antigens.

IL-12 IL-12 production by PBMCs is lower in SLE patients than in healthy controls and this seems to be due to decreased IL-12 production from monocytes (Liu et al., 1998). IL-12 production is also lower in-patients with active disease when compared with those with inactive disease (Liu et al., 1998). Treatment with recombinant anti-IL-10 antibody reversed the apparent deficiency in IL-12 production by PBMCs from patients with SLE but had no effect on PBMCs from healthy controls. This finding suggests that the low IL-12 production seen in patients with SLE may be attributable to the excessive IL-10 production that is known to occur in the disease.

The role of IL-12 in murine lupus has also been investigated. An intrinsic defect in the production of IL-12 *in vitro* by endotoxin activated macrophages from MRL-+/+ and (NZBxNZW)F_1 mice has been reported (Alleva et al., 1998). Other studies, however, found that peritoneal macrophages of MRL/*lpr* mice hyperproduce IL-12 after stimulation with IFN-γ and/or LPS, and exhibit high concentrations of IL-12 in the serum (Huang et al., 1996) as well as kidney (Fan et al., 1997). Moreover, daily injections of recombinant IL-12 led to increased serum levels of IFN-γ and nitric oxide (NO) metabolites, and accelerated GN in this model (Huang et al., 1996). These findings, together with previous reports that NO synthase inhibitors can ameliorate autoimmune disease in MRL/*lpr* mice (Weinberg et al., 1994), suggest that the high production and response to IL-12 by these mice may be important in disease pathogenesis. Treatment of (NZBxNZW)F_1 mice with anti-IL-12, however, was ineffective in preventing the onset or severity of glomerulonephritis (Nakajima et al., 1997).

Th2 cytokines

IL-4 Reduced levels of IL-4 have been found in MRL/*lpr* and (NZBxNZW)F_1 mice, resulting in an increased IFN-γ to IL-4 ratio (Shirai et al., 1995). Interestingly, in contrast with the expected paradigm of systemic autoimmunity being causally related to Th2 response, glomerulonephritis development was completely abrogated in (NZWxC57BL/6.Yaa) F_1 mice rendered transgenic for the IL-4 gene under the control of the IgH enhancer (Santiago et al., 1997). This protection was retention of IgG anti-DNA autoantibodies, but there was a significant reduction in the nephritogenic IgG3 and IgG2a isotype. Other studies, in contrast, found that transfer of IL-4 stimulated splenocytes from 5 month old (NZBxNZW)F_1 mice into syngeneic recipients increased the production of IgG anti-dsDNA antibodies, and administration of anti-IL-4 before disease onset inhibited this production (Nakajima et al., 1997). Moreover, anti-IL-4 treatment alone prevented

glomerulonephritis, while anti-IL-12 alone was ineffectual. It is noteworthy that combined treatment with both antibodies abrogated the beneficial effect of anti-IL-4 (Nakajima et al., 1997). Finally, IL-4 gene deletion (Peng et al., 1998) as well as recombinant mouse IL-4R or anti-IL-4 mAb treatments (Schorlemmer et al., 1995) led to significantly reduced lymphadenopathy and end organ disease in MRL/*lpr* mice.

IL-10 Serum IL-10 levels are higher in patients with SLE when compared with controls (Lacki et al., 1997). This increase is mainly attributable to an increase in IL-10 production by monocytes, a subset of B cells and possibly CD4+CD45 RO+ memory T cells. Serum titres of IL-10 are positively correlated with anti-ds DNA antibody titres and the SLEDAI score and negatively correlated with complement C3 levels (Lacki et al., 1997; Houssiau et al., 1995; Park et al., 1998). IL-10 increases IgG production by PBMCs from patients with SLE (Llorente et al., 1994). In healthy controls, IL-10 stimulates B cell proliferation and IgG synthesis and this is increased when the cells are activated through CD-40 before stimulation (de-Waal-Malefyt et al., 1992).

Reduced autoantibody levels and kidney disease was observed in (NZBxNZW)F_1 mice treated with an IL-10 inhibiting immuno-modulator (AS101) (Kalechman et al., 1997), and conversely increased Ig and autoantibody production was observed in IL-10 treated peripheral blood lymphocytes SLE patients. The inference has been also made that IL-10 promotes systemic autoimmunity by increasing Fas/FasL mediated apoptosis (Georgescu et al., 1997).

IL-6 patients with lupus nephritis were shown to have increased plasma concentrations of IL-6 and sIL-6R as compared with normal controls (Tezar et al., 1998). The ratio of IL-6/sIL-6R is increased in lupus nephritis, suggesting a raised effective level of IL-6 in SLE

patients with lupus nephritis. In addition, both IL-6 protein and mRNA have been found in 52% of kidney biopsy specimens taken from 19 patients with lupus nephritis (Herrera-Esparza et al., 1998). IL-6 is detectable in the urine of patients with lupus nephritis and may constitute a useful diagnostic marker (Horii et al., 1993).

TNF-α Several studies have also shown that human lupus is characterised by high serum levels of TNF-α and soluble TNF-R that parallel disease activity (Aderka et al., 1993; Studnicka-Benke et al., 1996).

Relatively low levels of TNF-α production have been described in some patients with SLE (Jacob et al., 1990). Stimulated PBMCs or enriched monocyte populations taken from patients with SLE or healthy controls who are DQw1 and DR2 positive, produce lower levels of TNF-α than DR3 or DR4 positive subjects. There is a strong association between the MHC class II DR2, DQw-1 and/or DR3 alleles and SLE. However, this association may be because of linkage disequilibrium with TNF-α alleles as the TNF-α gene lies within the class III region of the major histocompatibility complex. It therefore seems possible that a strong TNF-α response is protective against lupus nephritis.

Further supportive evidence for a protective role of TNF-α in SLE was given by measurement of increased plasma levels of TNF-α and its soluble receptor TNF-sR2 in patients with active lupus nephritis compared with controls (Tezar et al., 1998). The ratio of TNF-α to its soluble receptor was decreased in patients compared with healthy controls suggesting a lower effective level of TNF-α despite the higher titre.

Despite this apparent protective role of TNF-α, 52% of renal biopsy samples from patients with lupus nephritis had TNF-α protein deposited along the glomeruli and tubules as demonstrated by immunofluorescence (Herrera-Esparza et al., 1998). In situ hybridisation and RT-PCR amplification showed local expression of these cytokines in the biopsy specimens indicating that they are synthesised in the kidneys of patients with nephritis. This finding implicates TNF-α in the pathology of lupus nephritis but in the absence of measurements of the level of TNFα-sR it is not possible to determine whether TNF-α exerts a proinflammatory effect.

TGF-ß Transformin Growth Factor-beta (TGF-ß) is produced by T cells and NK cells and has a powerful inhibitory effect on the *in vitro* production of IL-6, IL-1 and TNF-α by macrophages (Kitamura et al., 1996). TGF-ß also suppresses B lymphocyte secretion of IgG (Horwitz et al., 1997; Ohtsuka et al., 1999). Constitutive and stimulated levels of TGF-ß are lower in patients with SLE and this is probably a consequence of the high levels of IL-10 known to suppress TGF-ß production by NK cells. Addition of TGF-ß and IL-2 to PBMCs from SLE patients reverses the upregulated IgG production (Lacki et al., 1997; Ohtsuka et al., 1999). It therefore seems that the high IgG production seen in patients with SLE is attributable, in part, to low levels of TGF-ß and inadequate suppression of IgG production.

Male BXSB and MRL/*lpr* mice show increased levels of TGF-ß (Prud'homme et al., 1995). This was shown in MRL/*lpr* mice to adversely affect host defence against both Gram negative and positive bacterial infections (Lowrance et al., 1994). Such findings provide an explanation for the increased risk of such infections in SLE patients. Nevertheless, direct injections of a TGF-ß cDNA expression vector into the skeletal muscle was reported to reduce autoantibody levels in MRL/*lpr* mice (Raz et al., 1995), while infection with a non-

pathogenic strain of *Salmonella typhimurium* carrying the TGF-β gene was without effect (Huggins et al., 1999).

From the data discussed the imbalance in the levels of cytokines and their receptors found in SLE is clearly crucial to the development of the pathology of the disease. These molecules would be expected to exert inhibitory or promoting effects on the initiation and perpetuation of systemic lupus erythematosus (SLE). The balance of Th1/Th2 cytokines is related to the pathogenesis of SLE (Takhashi et al., 1996; Yoshii et al., 1995). Despite the popular notion that a predominant Th2 phenotype is essential for SLE, recent evidence suggests that CD4+ T cells involved in SLE might also induce a Th1 subset (Takahashi et al., 1996; Reininger et al., 1996). Th1-derived cytokines are regulated mainly by IL-12 (Heinzel et al., 1993). Previous studies in professor Liew's group have shown that IL-12 and nitric oxide (NO) play a role in disease pathogenesis in lupus-prone MRL/*lpr* mice (Huang et al., 1996). Studies were thus performed to determine if IL-12 involved in the pathogenesis of human SLE and lupus-like models with different genetic background from MRL/*lpr* mice.

IL-18 is a novel cytokine with potent IFN-γ inducing activities and plays an important role in the Th1-mediated immune response in synergy with IL-12. There is, however, no convincing data to show a role of IL-18 in SLE pathogenesis. Therefore, I studied in details the role of Th1 related cytokines in SLE and in particular to determine if IL-18 plays a significant role in the pathogenesis of SLE.

1.5 Interleukin 18 (IL-18)

Interleukin-18 (IL-18), previously named Interferon-gamma-inducing factor (IGIF), was purified and cloned in 1995 from the liver of mice inoculated with *Propionibacterium acnes* and challenged with lipopolysaccharide to induce toxic shock (Okamura et al., 1995; Okamura et al., 1998; Dinarello et al., 1997). IL-18 is mainly produced and released by APC activated macrophages, Kupffer cells, dendritic cells, Langerhans cells and B cells. This may imply that IL-18 acts at the early steps of the immune response and accounts for polarization of the immune response. Investigations have revealed that IL-18 mRNA is expressed in a wide range of cells including Kupffer cells, macrophages, T cells, B cells, osteoblasts, keratinocytes, dendritic cells, astrocytes and microglia (Udagawa, et al., 1997; Stoll et al., 1997; Okamura et al., 1998).

1.5.1 Biology of IL-18

Pro-IL-18 is cleaved by IL-1 β-converting enzyme (ICE; Caspase 1) to yield an active 18 kDa glycoprotein (Ghayur et al., 1997), alternate processing by caspase 3 or 4, or proteinase 3, is also postulated (Fantuzzi et al., 1999).

IL-18 shares biological properties with IL-12 such as stimulation of IFN-γ production, enhancement of natural killer (NK) cell cytotoxicity, and stimulation of Th1 cell differentiation. Despite their functional similarity, IL-18 is not structurally related to IL-12. Bazan and colleagues (1996) predicted that murine IGIF would show similarities to IL-1 in its three-dimensional structure of β-pleated sheets forming a barrel configuration. Therefore, the name of IL-1γ was proposed but, as IGIF does not bind to the type I IL-1-receptor (IL-1RI) or signal through it, the name of IL-18 was adopted.

Like IL-1, IL-18 is synthesised as an inactive precursor lacking a typical signal peptide and pro-IL-18 requires cleavage by IL-1ß converting enzyme (ICE, caspase 1) to release an active 18 kDa glycoprotein (Ghayur et al., 1997). ICE-deficient ($ICE^{-/-}$) mice lacking both mature IL-18 and IL-1ß are protected against lethal endotoxemia (Fantuzzi et al., 1997). Pro-IL-18 is produced in a wide range of cells including Kupffer cells and macrophages. As it has been shown that LPS activates ICE (Schuamann et al., 1998). LPS-dependent ICE activation might contribute to LPS induction of IL-18 secretion. It shares some of the biological activities with IL-12, but without significant structural homology, and serves as a costimulatory factors in the activation of Th1 cells (Takeda et al., 1998). IL-18 has other functions in addition to those it shares with IL-12. Treatment of athymic nude mice sensitised to *P. acnes* with anti-IL-18 antibody can prevent LPS-induced liver injury, indicating that IL-18 is involved in the pathogenesis of endotoxin-induced liver injury (Okamura et al., 1995; Tsutsui et al., 1997). IL-18 up-regulates Fas ligand (FasL) expression on NK cells (Tsutsui et al., 1996). These results suggest that hepatic NK cells participate in IL-18-induced liver injury as effector cells through the Fas-FasL system. The role of IL-18 in LPS-induced liver injury and endotoxic shock in mice primed with *P. acnes* was examined using the IL-18-deficient mice (Sakao et al., 1999). IL-18-deficient mice primed with *P. acnes* showed resistance to LPS-induced liver injury, as expected from the results of the experiment with anti-IL-18.

It is noteworthy that IL-12 and IL-18 promptly and synergistically induce naive T cells to develop into IFN-γ producing cells without engaging their antigen receptors (Yoshimoto et al., 1998). Compared with Th1 cells after stimulation with anti-CD3 plus IL-2, in which IL-18R mRNA was induced transiently, IL-12-stimulated T cells strongly and continuously expressed IL-18R mRNA. T cells stimulated with IL-12 plus IL-18 without anti-CD3 produce strikingly IFN-γ. The physiological relevance of these IFN-γ producing T cells is

uncertain. As they promptly and strikingly produce IFN-γ in response to IL-12 plus IL-18 without developing into memory cells, they might play an important role in the innate immune response. IFN-γ was thought to be produced solely by activated T cells and NK cells. Although either IL-12 and IL-18 alone induce low levels of IFN-γ mRNA transcripts, the combined stimulation of murine bone-marrow-derived macrophages with both cytokines leads to the efficient production of IFN-γ protein, suggesting a novel pathway of autocrine macrophage activation (Munder et al., 1998).

Although IL-18 is involved in Th1 cell development, a recent study revealed a strong induction of the Th2 cytokine, IL-13 by IL-18 in NK and T cells in synergy with IL-2 (Hoshino et al., 1999). IL-12 did not enhance the IL-13 production induced by IL-2 alone; moreover, in the absence of IFN-γ (i.e. IFNγ-/- mice), IL-2 plus IL-18-induced IL-13 expression in purified NK and T cells were greater than that seen in purified cells from normal controls. These results suggest that IFN-γ levels may endogenously regulate IL-13 expression induced by IL-2 plus IL-18 *in vivo*. When IFN-γ is suppressed, IL-18 can be a cofactor in the development of the humoral immune response by inducing IL-13. Depending upon the cell type, IL-18 might act as strong co-inducer of Th1 or Th2 cytokines (Xu et al., 2000).

1.5.2 IL-18 receptor

IL-18 recognises a heterodimer receptor (IL-18R) comprising unique a ligand-binding subunit (IL-1Rrp) and non-binding β (ACPL) signalling chains (Akira, 2000). This receptor is widely expressed on cells implicated in both innate and specific immune responses, and signals through a pathway that involves my88 (myeloid differentiation 88),

IRAK (IL-1 receptor-associated kinase), TRAF6 (Tumor necrosis factor receptor-associated factor 6) and NF-$_k$B (nuclear factor kappa B).

The IL-1 receptor system is composed of two subunits: the IL-1RI and the IL-1 receptor accessory protein (IL-1RAcP). Although IL-1RAcP does not bind IL-1 directly, it is involved in the formation of a high affinity receptor complex as well as in IL-1 signalling. As IL-1RAcP mRNA is constitutively expressed in a variety of cell types, the IL-1 responsiveness of a given cell is mainly determined by the expression of IL-1RI (Hoshino et al., 1999).

IL-1Rrp is essential for IL-18 binding as well as IL-18-mediated functions administration of IL-18BP to mice abrogated circulating IFN-γ following treatment with LPS. Thus, IL-18BP functions as an inhibitor of the early Th1 cytokine response (Dinarello's et al., 1997, 2000). IL-18-binding protein (IL-18BP), which are distinct from IL-18R, may be present in high concentrations in the extracellular milieu where they can bind to IL-18 with high affinity and neutralize its effector function (Kim et al., 2000).

1.5.3 IL-18 expression and function in inflammatory diseases

IL-18 acts in synergy with IL-12 to promote development of T helper 1 (Th1) responses. IL-18-deficient mice exhibit impaired Th1 responses to intracellular bacteria, including *Propionibacterium acnes*, *Mycobacterium bovis* and *Staphylococcus aureus*, as well as parasites such as *Leishmania major* (Wei et al., 1999; Takeda et al., 1998). Such responses are further impaired in IL-12/IL-18 double-knockout mice. Similarly, neutralization of IL-18 impairs host defence against several infectious species, including *Cryptococcus*, *Salmonella* and *Yersinia* (Kawakami et al., 1997, 2000; Bohn et al., 1998). The situation in autoimmune models has been less-well characterised. IL-18 mRNA is up-

regulated in the pancreas of non-obese diabetic (NOD) mice and the murine gene encoding IL-18 maps to the Idd2 susceptibility locus, suggesting a potential role in Th1-mediated autoimmunity. However, IL-18 supplementation in NOD mice retards the clinical onset of hyperglycaemia and modifies the transition from Th2 to Th1 cytokine mRNA expression in pancreatic islets (Rothe et al., 1999). Moreover, IL-18 has also been shown to enhance Th2 cytokine production, eosinophilia and allergic sensitisation in a ragweed-antigen-induced allergy model (Wild et al., 2000)

IL-18 has recently been detected in the synovial compartment of patients with rheumatoid arthritis (RA) (Gracie et al., 1999). Whereas IL-18 mRNA was found in both RA and osteoarthritis (OA) synovial membranes, IL-18 protein was reproducibly detected by histology and ELISA only in RA-derived tissues. IL-18 also induced nitric oxide (NO) release by RA synovial membranes *in vitro*. DBA/1 mice injected with type II collagen (CII) in incomplete Freund's adjuvant (FA) normally develop only low-grade arthritis. Co-administration of recombinant IL-18 induced development of severe, inflammatory, erosive arthritis (Leung et al., 2000), and IL-18 deficient DBA mice show less arthritis (Wei et al., 2001)

Elevated IL-18 expression has also been reported in inflammatory bowel disease, particularly Crohn's disease (Pizarro et al., 1999). IL-18 mRNA and protein have been detected in mucosal biopsies by RT-PCR, immunochemistry, western blotting and bioactivity assays (Monteleone et al., 1999).

1. 6 Interleukin-12 (IL-12)

Interleukin-12 (IL-12) is a pivotal cytokine in driving the immune system towards a T helper (Th)1 type response and preventing a Th2 type immune profile. Therefore, IL-12 is indispensable in the defence against certain, mainly intracellular pathogens, but overproduction of this cytokine is crucially involved in the etiology of several inflammatory and autoimmune diseases.

1.6.1 Biology of IL-12

IL-12 was originally identified in the culture supernatants of Epstein-Barr virus (EBV)-transformed human B cell lines due to its ability to activate NK cells to produce IFN-γ, and was initially known as NK cell stimulatory factor (NKSF; Kobayashi et al., 1989; Stern et al., 1996). However, it was subsequently found to be produced mainly by phagocytic cells (monocytes, macrophages and neutrophils) in response to both Gram negative and positive bacteria, bacterial products such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA), viruses and intracellular parasites (D'Andrea et al., 1992; Cleveland et al., 1996; Kanangat et al., 1996). Bacterial DNA has also been shown to be a potent inducer of IL-12 (Halpern et al., 1996). Dendritic cells, polymorphonuclear cells and mast cells also produce IL-12 (Smith et al., 1994; Cassatella et al., 1995; Cella et al., 1996; Heufler et al., 1996; Kang et al., 1996; Koch et al., 1996).

Bioactive IL-12 is a heterodimeric 70 kDa glycoprotein consisting of a 40 kDa subunit and a 35 kDa subunit linked by disulphide bonds that are essential for the biological activity of IL-12. p35 is ubiquitously and constitutively expressed in a variety of cell types, whereas p40 expression is more restricted and highly inducible. The two subunits of IL-12 are not related to any other known proteins. p40 shows some homology with the extracellular domain of the

receptor for IL-6 (Gearing and Cosman, 1991) , and p35 appears to be a homologue of IL-6 (Merberg et al., 1992).

The gene encoding the p40 subunit of IL-12 maps to human chromosome 5q31-q33 in the same region that also harbors other cytokine genes. The gene encoding the p35 subunit of IL-12 maps to human chromosome 3p12-q13.2. The expression of the two genes is regulated independently of each other (Sieburth et al., 1992). The production of p40 exceeds the production of p70 by from 10 fold to more than 500 fold depending on the experimental system (Wysocka et al., 1995; Snijders et al., 1996; Haskó et al., 1998). Five to forty per cent of this is secreted as a homodimer called p(40)2. The p(40)2 homodimer has been shown to exert antagonistic activity on the IL-12 receptor in both *in vitro* (Gilessen et al., 1995; Ling et al., 1995) and *in vivo* (Heinzel et al., 1997; Mattner et al., 1997; Rothe et al., 1997) systems. On the other hand, the p(40)2 homodimer stimulates the differentiation of CD8+ T cells with type 1 cytokine profile demonstrating agonistic properties (Gateley et al., 1998). The p35 subunit lacks any biological activity.

The *in vivo* induction of IL-12 can be either T cell-independent or -dependent. Infection with bacteria or intracellular parasites results in rapid IL-12 production by direct stimulation of phagocytes; indeed, T cell-deficient SCID mice produce bioactive IL-12 upon infection (Gazzinelli et al., 1994; Tripp et al., 1994). However, T cell-dependent mechanisms have also been demonstrated IL-12 production occurred in response to presentation of T cell-dependent antigens such as OVA via triggering of CD40 molecules on antigen presenting cells and was dependent on TCR ligation (DeKruyff et al., 1997; Maruo et al., 1997). CD40-CD40L interaction plays a critical role in bioactive IL-12 production by regulating p40 but not p35 mRNA accumulation (Kato et al., 1996).

IL-12 is a key mediator of innate immunity and is also involved in the establishment of adaptive immune responses (reviewed by Trinchieri, 1995). It directs the differentiation of helper T cells towards a type 1 phenotype, which is characterised by the production of IFN-γ and down-regulation of IL-4. IL-12 also stimulates IFN-γ production by NK cells, and thus establishes a positive feedback loop resulting in enhanced activation of macrophages, including stimulation of NO production. This is especially important for the effective removal of intracellular pathogens such as *Leishmania major* (reviewed by Ma et al., 1996). Animals treated with neutralising doses of monoclonal antibodies against IL-12 p40, or lacking either the IL-12 p40 or p35 gene are highly susceptible to such intracellular pathogens (Biron and Gazzinelli, 1995; Trinchieri and Scott, 1995).

IL-12 is involved probably also in the selection of immunoglobulin isotypes. At picomolar concentrations IL-12 markedly inhibits the synthesis of IgE by peripheral blood mononuclear cells stimulated with IL-4 also in the presence of antibodies directed against IFN-gamma .

Both subunits must be co-expressed in the same cell to generate bioactive heterodimer and since p35 is constitutively-expressed in a variety of cell types, it was originally assumed that p70 generation was mainly controlled at the level of p40 transcription. However, there is now much evidence of regulation of p35 transcripts (Hayes et al., 1995; Snijders et al., 1996; Aste-Amezaga et al., 1998; Kincy-Cain and Bost, 1997), and it is therefore more likely that p35 is the limiting subunit. Indeed, the formation of antagonistic $p40_2$ may predominate even under optimal conditions (Hayes et al., 1995; Snijders et al., 1996) and it has therefore been suggested that a temporal balance between $p40_2$ and bioactive p70 determines the IL-12 response (Schultze et al., 1999).

Several cytokines have activatory or suppressive effects on the stimulation of IL-12 production by phagocytic cells. While IFN-γ and GM-CSF enhance IL-12 (Cassatella et al., 1995; Kubin et al., 1994; D'Andrea et al., 1993), IL-10, IL-4, IL-13 and TGF- β inhibit IL-12 production by suppressing both p40 and p35 accumulation (Kubin et al., 1994; D'Andrea et al., 1995).

1.6.2 IL-12 receptor

IL-12 exerts its effects by binding to specific cell surface receptors on its target cells. The high affinity IL-12 receptor is formed by the co-expression of two sub-units, the IL-12Rß1 (Chua et al., 1994) and IL-12Rß2 (Presky et al., 1996). While both the IL-12Rß1 and IL-12Rß2 are responsible for providing the binding energy, the IL-12Rß2 is essential for signal transduction (Gately et al., 1998). The expression of IL-12Rß2 appears to be confined to Th1 cells (Rogge et al., 1997; Szabo et al., 1997), which may provide a selective therapeutic target for altering the Th1/Th2 balance in immuno-pathological conditions. Similar to the production of IL-12, the expression of both IL-12Rß1 and IL-12Rß2 is regulated by cytokines. While IL-2, IL-7, IL-15 and IFN-γ enhance IL-12 receptor expression, IL-4, IL-10 and TGF-ß down-regulate IL-12 receptors and IL-12 responsiveness (Gollob et al., 1997; Rogge et al., 1997; Szabo et al., 1997; Wu et al., 1997; Himmelreich et al., 1998).

1.6.3 Clinical function of IL-12

IL-12 has been shown to be directly and prominently involved in the induction of the pathophysiology of several autoimmune diseases including multiple sclerosis (Leonard et al., 1995), inflammatory bowel disease (Neurath et al., 1995), insulin dependent diabetes mellitus (Trembleau et al., 1995), glomerulonephritis (Kitching et al., 1999), systemic lupus erythematosus (Huang et al., 1996), and rheumatoid arthritis (Germann et al., 1995). The

overproduction of IL-12 is also an important pathogenetic factor in inflammatory states such as septic shock (Wysocka et al., 1995) and the generalized Shwartzman reaction (Ozmen et al., 1994). Furthermore, a potential role for IL-12 was suggested in the promotion and maintenance of inflammation in atherosclerotic or psoriatic lesions (Uyemura et al., 1996; Yawalkar et al., 1998).

In contrast to the immunopathological role of over-expression of IL-12 in Th1 driven responses, IL-12 deficiency can contribute to an overactive Th2 type immune phenotype. This was best shown by the fact that IL-12 treatment reversed the airway hyper-responsiveness and decreased IL-4 and IL-5 expression in a murine model of asthma, a disease associated with a hyperreactive Th2 immune response (Gavett et al., 1995). IL-12 deficiency has been associated with tumour growth, while this cytokine has been successfully administered in-patients with cancer (Lotze et al., 1996). Finally, treatment with IL-12 has been proposed for controlling viral infections such as chronic hepatitis or AIDS (Gately, 1997).

1.7 Aims of the thesis

The aim of the study was to investigate the mechanisms of immune regulation, particularly the roles of cytokine disorders, in the development of lupus disease. The study included both *in vitro* (Chapter 3 and Chapter 4) and *in vivo* (Chapter 5 and 6) approaches based on two murine models of SLE and human SLE samples.

The immunopathological processes in SLE resemble an ongoing immune response. Components of humoral and cell mediated arms of the immune system are represented in the immunopathological process in SLE although their relative contributions remain controversial. Regardless of whether these process are primary, or secondary to an unidentified insult, evidence from animal and human studies indicates that Th1 and Th2 regulatory cytokines play prominent role in pathogenicity of SLE. Th1 regulatory cytokines are of particular interest to the author and the main aims of study were:

1) To investigate IL-12 and NO production in (NZB/W)F1 lupus-like mice and their levels in human SLE Blood samples and to determine their relation with disease development.

2) *In vitro* study to investigate whether there was any association between IL-18 and SLE in human and lupus-like models.

3) *In vivo* studies to examine the role of IL-18 in the development of systemic autoimmune disease in MRL/*lpr* mice.

Chapter 2

Material and Methods

2.1 Patients and samples

Samples were collected from SLE patients attending the Centre for Rheumatic Diseases, Glasgow Royal Infirmary, the Rheumatology Department, Gartnavel General Hospital, the Connective Tissue Diseases Clinic, Glasgow Royal Infirmary and the Department of Rheumatology, Nottingham Hospital. Kidney and skin pathology sections for immunohistochemical studies were obtained from Dr. George Lindop (the Pathology Department, Glasgow Western Infirmary).

SLE patients satisfied the American College of Rheumatology diagnostic criteria (Arnett et al., 1988; Tan et al., 1982). Clinical data were obtained from the case record and included age, gender, family history, medical past history, disease duration, laboratory tests, drug therapy and concurrent disease.

2.2 Ethical consideration

Blood samples were collected only when clinically indicated, and informed consent was obtained from patients prior to research commencing. All animal experiments were performed under project licences provided following UK Home Office review and were used only to address questions not amenable to *in vitro* study of human diseases. Experimentation was performed under Project Licence 60/12045, procedure 1 and Project Licence 60/1311, procedure 5.

2.3 Reagents / buffers

The source of reagents either purchased or donated is given in the text and tables. Details of preparation of buffers and reagents, where appropriate, are contained in Appendix I.

2.4 Plasma and serum samples

2.4.1 Human plasma and serum

SLE plasma and sera were obtained from blood samples taken from diagnosed SLE patients. Normal human plasma and sera were collected from healthy donors through provided by the Blood Transfusion Unit, Western Infirmary, Glasgow.

2.4.2 Mouse sera

Blood samples were taken from all experimental mouse models including lupus strains, MRL/lpr-lpr, NZB/NZW mice and MRL/++ with BALB/c mice as controls. Mice were bled by aorta artery puncture, or tail bleeding before removing the spleen.

All serum and plasma samples were kept at -70°C before assaying. Plasma samples were supplied in the presence of anticoagulant either Potassium EDTA (BS 4851 sample container) or heparin (10 I.U./ml).

2.5 Tissue sections

Freshly isolated mouse kidneys, livers, joints, skin, lymph nodes and spleen were sliced and processed as follow for histological and immunocytochemical examinations:

2.5.1 Frozen section

Blocks of tissue were snap-frozen in liquid nitrogen and kept at -70 °C. Before each assay, 5 μm sections were cut from the frozen tissue, mounted on washed plain glass slides, and kept at -70°C.

2.5.2 Paraffin-embedded tissue

Blocks of tissues were fixed in neutral buffered formalin. After being embedded in paraffin wax, 4 μm sections were cut and mounted on washed plain glass slides.

2.6 Cell culture

All culture media and supplements were supplied from Gibco BRL (Paisley, Scotland, UK). RPMI was supplemented with 100 I.U./ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (complete RPMI) and heat inactivated foetal bovine serum (FBS 10%). Dulbecco's modified Eagle medium was supplemented with penicillin, streptomycin, L-glutamine and FBS as above. Iscove's modified Dulbecco's medium was supplemented with L-glutamin, penicillin and streptomycin and was used for whole blood culture. FBS was mycoplasma free and heat inactivated at 56°C for 30 minutes in a water bath and then stored in 50 ml aliquots at -20°C. Cell viability was determined by Trypan-blue exclusion assay, using, 0.1% Trypan-blue (Sigma) and 0.1% acetic acid (BDH Lab. supplies, Leicestershire, UK). Cells were counted directly using a Neubauer haemocytometer (Weber Scientific International Ltd, UK) on a Nikon Labphot microscope. Cell cultures were performed at 37°C in a humidified incubator with 5% CO_2.

2.6.1 Peripheral blood mononuclear cell preparation (PBMC)

Mononuclear cell populations were obtained by density gradient centrifugation. Venous blood was collected into heparinised sterilised universals (10 I.U./ml preservative free heparin, Leo laboratories Ltd, Bucks, UK) and was diluted 1:2 in complete Dulbecco's MEM. The diluted blood (5 ml) was layered over an equal volume of Lymphoprep (Nycomed Pharma, Oslo, Norway) in a 13 ml conical tube and spun at 500 x g for 30 minutes at 22°C. The mononuclear layer was collected and washed three times in PBS before adjustment to the required cell

concentration. Cellular percentage in PBMC was assessed by FACS analysis (section 2.10).

2.6.2 Whole blood cell culture

Blood was withdrawn, heparinised (10 I.U./ml) and immediately diluted 1:5 in complete Iscove's medium. After the addition of phytohaemagglutinin (PHA, 1 μg/ml), LPS (3 μg/ml), recombinant human-IFNγ (10 ng/ml), rh-IL-12 (10 ng/ml), or rh-IL-18 (20 ng/ml) to the diluted blood, duplicate cultures (500 μl each) were set up in 24-well culture plates and incubated for 24, 48 and 72 hours. Supernatants were collected and stored at –20°C until assayed for cytokine and nitric oxide concentration.

2.6.3 Proliferation and cytokine production by human cells

Proliferation assays were performed in triplicate in complete RPMI and 10% FBS. PBMC ($1x10^6$ in 100 μl) were incubated in U-bottom 96 well culture plates (Nunclon microwell, Nunc, Denmark) for 24, 48 and 72 hours. Stimulatory reagents or medium alone were added in a further 100 μl at twice the desired final concentration, 30 minutes after seeding the cells to culture plates. ^{3}H-thymidine (Amersham Life Science, UK), 1 μCi in 25 μl complete RPMI containing 10% FBS was added to each well during the final 8 hours of culture before harvesting onto a glass fibre filter (Packard, CT, USA) using a Micromate 196 Harvester (Packard). Proliferation was expressed as a stimulation index derived as follows: SI= (mean counts per minute test culture) / (mean counts per minute medium alone).

Parallel cultures were performed in 24 well plates for analysis of the PBMC and whole blood cytokine production following stimulation. Culture supernatants were frozen at -70°C for cytokine concentration by ELISA. Reagents used for stimulation included phytohaemagglutinin (PHA, Murex, Diagnostics Ltd, UK), human recombinant interleukin-gamma (IFNγ, a gift from Dr. Adolf, Vienna),

lipopolysaccaride (LPS, *Salmonella enteritidis*, Sigma, UK), and recombinant interleukin-12 (Immunex, USA), recombinant interleukin-18 (rh-IL-18, PeproTech, UK).

2.6.4 Murine spleen cell and peritoneal cell preparation

Mouse spleens were aseptically removed and immersed in 20 ml serum-free RPMI, then weighed, cut into fragments in 10-cm Petri dish (Sterlin, Middlesex, UK) and then forced through a sterile tea strainer, using a rubber plunge of a 10 ml syringe. The cell suspension was washed three times in complete RPMI medium, passed through Nytex membrane to remove cell debris and clumps. After one more wash, the cell pellet was re-suspended in 10 ml RPMI-1640 containing 10% FBS. Cell viability was determined by the Trypan-blue exclusion assay (Section 2.6).

To prepare peritoneal cells, mice were injected into the peritoneum with 5 ml RPMI medium supplemented with penicillin and streptomycin, and cells removed by peritoneum washing. The cell suspension was washed two times in complete RPMI medium, passed through a Nytex membrane to remove debris and clumps and washed once more before re-suspending in 10 ml RPMI-1640 containing 10% FBS. Cell viability and counting was determined as previously mentioned (Section 2.6). The normal peritoneal cell population is composed of about 50% macrophages. To purify the macrophages further, the cell suspension was placed into a 75 cm^2 flask (Costar) and incubated for 1.5 hours at 37°C. Non-adherent cells were discarded and the adherent cells were detached with cold PBS. After washing once with complete RPMI the pellet was re-suspended in 10 ml RPMI-1640 containing 10% FBS and used in the required concentration after counting and determining their viability.

2.6.5 Murine spleen cell and peritoneal cell activation

Proliferation assays for spleen cells were performed in triplicate in U-bottom 96 well culture plates (Nunclon) at $1x10^6$ cells/ml in 100 µl complete RPMI with

10% FBS. Stimuli were added in 100 μl giving a final culture volume of 200 μl. Stimuli included 0.1 to 10 μg/ml concanavalin A (Sigma) as positive control. 1 μCi of ^{3}H-thymidine was added during the final 6 hours of culture and plates were harvested as described in section 2.6.3. Cytokine production by spleen cells was measured by incubating $4x10^{6}$ cells in 1 ml of complete RPMI, and 10% FBS for various times in 48-well culture plates, in the presence or absence of stimuli. Supernatants were frozen at -20°C before being assayed for cytokine production. Stimuli included lipoplysaccharide (LPS, 100 ng/ml) and recombinant IFNγ (50 U/ml) for peritoneal macrophages or concanavalin A (ConA, 2.5 μg/ml) for T-cell activation.

To measure cytokine production by spleen cells in some of the experiments, anti-CD3 pre-coated plates were used. Mouse anti-CD3 (150 μl, 4 μg/ml, Pharmingen), in phosphate buffer saline (PBS, pH 7.3) was applied to 24-well plates (Nunc) and incubated at 37°C for 2 hours. These were washed twice with cold PBS and then dried under sterile conditions. Spleen cells ($2x10^{6}$ cell/ml in 1 ml complete RPMI with 10% FBS) were added to each well and after 30 minutes were stimulated with rm-IL-18, or rm-IL-12. Supernatant were collected after 24, 48, 72 hours and frozen at -20°C before being assayed for cytokine concentration.

2.7 Immunohistochemistry

Tissue from SLE patients undergoing renal biopsy and renal transplant rejected was collected from Pathology Department of Glasgow Western Infirmary. Kidneys from lupus-like mice and the normal controls were dissected immediately after sacrificing, placed in mounting medium (Cryo-M-Bed, Bright Instrument Company Ltd, Cambs) and snap frozen in liquid nitrogen. Samples were then stored at –70°C until required.

Glass microscope slides were soaked overnight in 2% (v/v) Decon 90 (Decon Labs Ltd, UK), then rinsed in tap water for 3 hours before air drying. Slides were dipped in 2% (v/v) silane (3-aminopropyltriethoxysilane, Sigma) in acetone for 4 minutes, rinsed in running tap water for 6 minutes and then air dried. 4-6 μm frozen sections of tissues were cut onto the silane coated slides at -20°C using either a Bright 5030 Microtome or a Leica CM1800 cryostat and immediately fixed by immersion in acetone (BDH Lab Supplies, Poole, UK) at 4°C for 15 minutes. Sections were then air dried for 10 minutes and stored at -20°C in an air / moisture tight container until required.

The primary, secondary and negative control antibodies used are detailed in tables 2.1 (page 84).

2.7.1 Peroxidase staining

Both formalin-fixed and frozen sections were used for immunohistochemical examination. Details of the panel of antibodies used are given in Table 2.1. Formalin-fixed sections were passed through graded concentration of alcohol to PBS, whilst frozen sections were re-hydrated in PBS for 10 minutes. Endogenous peroxidase activity was blocked by incubation with hydrogen peroxidase 0.5% (v/v) in 50% (v/v) methanol for 30 minutes [formalin-fixed sections were then either microwaved in citrate buffer (pH 6.0, 10 mM) for 9 minutes (with a 650 W microwave), or incubated with 0.1% (w/v) trypsin solution pH 7.8 for 30 minutes] to enhance antigen retrieval. Both frozen and formalin-fixed sections were then incubated for 30 minutes with 5% (v/v) rabbit or goat serum (depending on secondary antibody) to reduce non-specific background binding. The sections were then incubated sequentially with primary antibody, secondary antibody, and (for biotynilated secondary antibodies) streptavidin-biotin horseradish peroxidase complexes (StreptABC, DAKO, UK) were then performed. All incubations were made at room temperature for 1 hour, except for monoclonal antibodies (overnight at

4°C) and StreptABC (30 minutes at room temperature). Sections were then washed three times in PBS between each incubation stage. All dilutions were made in PBS, and primary and secondary antibodies (with the exception of those specific for the immunoglobulin classes) were pre-incubated by appropriate dilutions of 5% (v/v) goat serum. Binding was visualised by the addition of 3,3'-diaminobenzidine tetrahydrochloride 0.05% (w/v) and hydrogen peroxidase 0.01% (v/v) in PBS and sections were counter-stained with Mayer's haematoxylin.

2.7.2 immunofluorescence staining

Frozen sections or dewaxed paraffin sections were rinsed in PBS for 10 minutes, and treated with pontamine sky blue 0.5% (w/v) in PBS (pH 7.4) for 20 minutes which stains the elastic tissue red and diminishes background. This was washed off by flooding with PBS. To reduce non-specific reaction, sections were blocked with 2% (w/v) BSA in PBS. Excess BSA was drained off, and the sections were incubated with detecting antibodies at a 1:50 dilution in 2% BSA/PBS, for 30 minutes for frozen sections, or 1 hour for paraffin sections, in a full humidity environment at room temperature. To detect immune complexes, a FITC-conjugated goat anti-mouse IgG (Vector Lab.) was used to detect autoantibody binding. To assess autofluorescene and non-specific staining, sections which were incubated with diluent alone or with normal goat serum were included as negative controls. Sections were washed in PBS after each antibody incubation.

2.7.3 Tunel staining

DNA fragmentation of skin biopsy specimens in paraffin section were examined by TUNEL staining according to manufacture's instruction (TdT-FragEL^{TM}, Oncogene). In this assay terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals and catalyses the addition of biotin-labelled and unlabeled deoxynucleotides. Biotinylated nucleotides are detected using a streptavidin-horseradish peroxidase

(HRP) conjugate. Diaminobenzidine reacts with the labelled sample to generate an insoluble coloured substrate at the site of DNA fragmentation. Counter-staining with methyl green aids in the morphological evaluation and characterisation of normal and apoptotic cells. Briefly, after deparaffinization and rehydration by xylene and ethanol, the slides were rinsed with 1 x TBS. The specimen was permeabilised with proteinase K and endogenous peroxidase was inactivated with 3% H_2O_2 in methanol at room temperature for 5 minutes. Slides were rinsed with 1 x TBS and covered with 100 µl of 1 x TdT Equilibration Buffer at room temperature for 10 to 30 minutes. After blotting the 1xTdT Equilibrqtion Buffer from specimen, 60 µl of TdT labelling reaction mixture was immediately applied onto each specimen and incubated in a humidified chamber at 37°C for 1.5 hours. Slides were rinsed with 1 x TBS and labelling reaction stopped with 5 minutes incubation at room temperature with stop solution. After rinsing the slides the specimen was blocked with blocking buffer for 10 minutes. Then 100 µl of conjugate applied for 30 minutes at room temperature. After rinsing with 1 x TBS the entire specimen was covered with 100 µl of DAB solution for 10-15 minutes and then the slides were rinsed with dH_2O. Immediately, the entire specimen was covered with methyl counterstain solution and incubated at room temperature for 3 minutes. Then slides were dipped 2-4 times into 100% ethanol and after blotting on an absorbing towel, the slides were dipped 2-4 times into xylene and then mounted. Tonsil stained with TdT-FragEL™ DNA Fragmentation Detection Kit was used as a positive control, and the negative control was from IL-18 treated group skin lesion which was kept in 1x reaction buffer during the labelling step and other steps performed as described.

2.8 The measurement of nitric oxide (NO)

The level of nitric oxide in the serum and culture supernatants was estimated by the Griess reaction, which measures the level of nitrite (NO_2^-) which is the oxidative products of NO (Archer, 1993).

2.8.1 Griess reaction

Griess reaction is an assay to measure the level of nitrite (Green et al., 1982; Ding et al., 1998). The Griess solution consists of: 0.1% naphtylene diamine dihydrochloride in distilled water (Solution A) and 1% sulphanilamide (Sigma) in 5% (v/v) phosphoric acid (Sigma) (solution B). Both the stock solutions A and B were stored in the dark at 4°C for up to 2 months. The Griess solution was prepared by mixing an equal volume of solution A and B immediately before use. A sodium nitrite stock solution (Sigma) was used as a standard to determine nitrite concentration, which is equivalent to NO production. The assay was performed in triplicate. An equal volume of the Griess solution was added to the test samples or standard dilution of sodium nitrite in identical medium in a 96-well flat bottomed plate . The plate was incubated for 10 minutes at room temperature in the dark, and thereafter, the colorimetric reaction was measured at 570 nm (reference filter at 630 nm) in an MRX microplate reader (Dynex Technology, Chantilly, USA).

2.8.2 Nitrite / Nitrate level in the serum and fluid bodies

2.8.2a Chemiluminescent assay

NO has a very short half-life in the serum or plasma and is rapidly converted to nitrate (NO_3^-). To measure serum NO levels, a conversion of nitrate to nitrite by nitrate reductase was first carried out. However, the presence of high protein concentrations in serum and body fluids leads to the interference with colorimetric assessment. Therefore, a chemiluminescent assay for nitrite was also used (Aoki, 1990; Palmer et al., 1987). To convert serum nitrate to nitrite 25 µl of the serum

sample was incubated with an equal volume of reaction buffer containing 5 mg/ml NADPH (Sigma), 41.5 mg/ml FAD (Sigma), 0.5 M KH_2PO_4 and 35 mg/ml of nitrate reductase (Sigma) which are prepared immediately before using. The conversion was carried out at 37°C for 1 hour in a 96-well bottomed flat plate. Nitrate and nitrite standards were also run in the same time. A Disbi Chemiluminescence NO analyser (Model 2107) was used to measure NO concentration in the serum samples. A reflux reaction was created by continuously boiling 75 ml glacial acetic acid (BDH) with 25 ml 6% (w/v) sodium iodide (BDH) in a 250 ml Pyrex reaction flask, through which was passed a low flow of nitrogen gas. Fifty or 75 μl of converted test samples, or standard nitrite solution prepared as above, was injected directly into the reaction flask using a Hamilton syringe (Sigma). NO_2^- in the sample is immediately reduced to NO•, which is carried in gaseous phase through a condenser and a cold trap, created with a glass U-tube surrounded by "dry ice" to remove acetic acid vapour, and on to a chemiluminescence NO analyser (Disbi Environmental Corporation, Japan). NO reacts with ozone causing photoemission which may be detected and converted to a digital readout. The photomultiplier signal is proportional to the nitrite concentration in the original sample, allowing the generation of a standard curve and estimation of nitrite concentration in test samples. Standard curves for sodium nitrite and sodium nitrate were included in the reductase reaction to provide a control for the efficiency of the reduction. This was calculated for the conversion of 100 μM sodium nitrate to sodium nitrite as shown below and was routinely >75%.

$$\% \text{ reduction nitrate} = (\text{ppb nitrite} / \text{ppb nitrate}) \times 100$$

All standard and test samples were assayed in triplicate and calculated for the mean and standard deviation.

2.8.2b NO measurement in the serum by modified Griess reaction

To measure total NO in the biological fluids in an easier and faster way a modified Griess reaction was developed (Moshage et al., 1995). All serum samples

were diluted in PBS (optimum dilution 1:4) and assayed in 96-well microplate. $NaNO_2$ and $NaNO_3$ standards were used (diluted 1:4 starting with 200 μM). The conversion of nitrate to nitrite was carried out by the addition of NADPH (Sigma, N-7505), FAD (Sigma; F-6625) and nitrate reductase (Sigma, N-7265) to the serum samples and standards to yield final concentrations of 50 μM, 5 μM and 200 μM respectively. After one hour incubation at 37°C, Lactase dehydrogenase (final concentration of 10 mg/ml) from rabbit muscle (Boehringer Mannhiem) and sodium pyruvate (10 mM) were added and further incubated for 5 minutes at 37 °C to stop the conversion and to remove NADPH. Zinc sulphate (final concentration of 15 g/ml) was added to deproteinize the serum samples. After 20 minutes centrifugation at 600 x g the supernatant from each (80 μl) was transferred into an Immulon 2 plate (Dynatech) and equal volumes of Griess reagents were added. After 10 minutes of colour development at room temperature, the absorbance was measured at 570 nm.

2.9 Enzyme linked immunosorbent assay (ELISA)

2.9.1 General ELISA protocol

96-well ELISA plates (Immunol 4, Dynatech Laboratories, Chantilly, USA) were coated overnight at 4°C with capture antibody or recombinant protein at 2-4 μg/ml in 0.1 M bicarbonate coating buffer (pH 8.3). Plates were then washed twice with PBS/0.05% (v/v) Tween 20 (PBS/Tween) and blocked by adding 200 μl/well 10% FBS/PBS for 2 hours at 37°C. The plates were then washed twice as before and standard and samples were added at graded dilution or concentrations. After incubation at 37°C for 2 hours, the plates were washed four times with PBS/Tween and then the detecting antibody was added at the appropriate dilution, either recommended by the manufacturer or determined experimentally. After incubation for 2 hours at room temperature, the plates were washed six times with PBS/Tween. For HRP-conjugated detecting antibodies, 50 μl/well TMB microwell peroxidase substrate (Kirkegaard & Perry Laboratories, MA, USA) was added and the optical density measured at 630 nm. For biotinylated detecting antibodies, 50 μl/well

peroxidase-conjugated extravidin (1:2000 Sigma, USA) was added for 30 minutes at room temperature and the plate was then washed six times with PBS/Tween prior to adding TMB substrate. Where indicated, secondary detecting antibodies were used.

2.9.2 Cytokine ELISA

Murine TNFα, IFNγ, IL-4, IL-6, IL-5 and IL-10 were assayed using paired antibodies (PharMingen, San Diego, CA) according to the manufacturer's instructions. Lower limits of detection were as follows: IL-4, IL-6, and TNF α were all at 10 pg/ml; IL-10 was at 50 pg/ml; and IFNγ was at 30 pg/ml. Briefly, capturing antibody (2-4 μg/ml) was diluted in carbonate-bicarbonate buffer (pH 8.2) and coated on a 96-well Dynatech micro-ELISA plate (Immunol 4) overnight at 4 °C. After coating, the plates were washed three times with PBS containing 0.05% (v/v) Tween-20 (PBS-Tween) and blotted dry. Remaining binding sites on the plates were blocked by adding 200 μl/well of 1% BSA/PBS and 10% FBS- Tween and incubated at 37°C for 2 hour. Following two washes with PBS-Tween, a wide range of concentration standard and samples was added, and incubated for 3 hours. The plates were then rinsed three times followed by a 1 hour incubation at room temperature with detecting antibody (3-4 μg/ml). Bound total IgG was detected with HRP-conjugated goat anti-mouse IgG (Genzyme). IgG1 and IgG2a were detected with biotin-conjugated anti-mouse IgG1 and IgG2a (PharMingen) respectively, and developed as described above in section 2.9.1. Plates were read at 630 nm.

2.9.3 Antibodies against ds-DNA measurement by ELISA

This was carried out using a modification of previously described method (Luzuy et al., 1986). Briefly, Immunol 2 Flat-bottom plates (Dynatech, Alexandria, USA) were first coated with poly-L-lysine (sigma, 20 μg/ml in PBS), 1.5 hours at 37°C. After washing with PBS/0.05% Tween 20, plates were coated with 10 μg/ml of Calf thymus DNA (Sigma) in PBS and incubated over night at 4 °C. Plates were then washed twice with PBS/0.05% (v/v) Tween 20 (PBS/Tween) and blocked by

adding 200 µl/well 1%BSA/PBS for 1.5 hours at room temperature to block remaining binding sights on the plates. The plates were then washed twice as before and 100 µl of serum samples (started at 1/100 dilution) or culture supernatant were serially diluted and added to the plates and incubated for 1.5 hours at room temperature. After washing, total Immunoglobulin (Ig) bound was measured by adding HRP-conjugated goat anti-mouse Ig (Dako). IgG, IgG1 and IgG2a isotypes were measured using HRP-conjugated goat anti-mouse IgG (Sigma) or anti-IgG1 and anti-IgG2a (PharMingen). Secondary antibodies were used at a dilution of 1/3000 in 0.5% BSA in PBS . After 1 hour incubation at room temperature, the wells were washed and substrate solution added. Results are expressed in U/ml in reference to a standard curve obtained with human ds-DNA and a reference standard of pooled sera from 20 weeks old MRL-*lpr* mice. Although this method detected predominantly anti-dsDNA antibodies, it may also detect low levels of ss-DNA antibodies. ds-DNA for antigen coating was prepared by S1 nuclease digestion and phenol extraction (Sambrook et al., 1989).

2.10 FACS analysis Human cell subset analysis

Double immunofluorescence staining of peripheral blood lymphocytes, was performed by FACS analysis. Samples blood (100 µl) placed in a 12 x 75 mm polyprolyne tube (Falcon 2052, Becton Dickinson, UK). Primary antibodies employed were as follows: CD3 (FITC and PE), CD16 (PE), CD20 (FITC), CD4 (FITC), CD8 (PE) (all Becton Dickinson). Primary antibody (10 µl) were added to the cells for 30 minutes at 4°C. Negative control primary antibodies (IgG1 FITC, IgG1 PE; DAKO) of appropriate isotype and conjugates were added to parallel tubes and were similarly processed. FACS^R Brand Lysing Solution (2 ml) was added (1:10 dilution of commercial stock contained 5% diethylene glycol, 1.5% (v/v) formaldehyde; Becton Dickinson, UK), the tubes were vortexed for 5 seconds and then the cells were incubated for 10 minutes at room temperature in the dark to facilitate erythrocyte lysis. Leukocytes were recovered by spinning at 300 x g for 5

minutes, then washed with 5 ml FACS Flow (Becton Dickinson, UK) at 200 x g for 5 minutes. Cells were re-suspended in 200 µl FACSFlow and analysed on a FACScan (Becton Dickinson). Gates were set for lymphocytes and monocytes using forward and side light scatter parameters. The percentage of FITC or PE positive cells, or of double labelled cells, were generated for lymphocytes within this region using Lysis II software (Becton Dickinson, UK).

2.11 RT-PCR for mRNA expression in spleen cells and PBMC

Mouse spleen cells were prepared as described previously (2.6.4). Spleen cells and kidney cells from each of the treated groups of mice were pooled and immediately re-suspended in 800 µl RNAzol™ (Biogenesis, Bournemouth, UK) before being stored at -70°C. PMBC and whole blood cells from SLE patients and from normal control were immediately, after receiving, re-sespunded in 800 µl RNAzol prior to storage at -70°C.

2.11.1 RNA extraction

Total mRNA was extracted using RNAzol™ (Biogenesis) as described (Chomczynski & Sacchi, 1987). The cell pellet was re-suspended in RNAzol. To each tube 1/10 volume of chloroform (Sigma) was added and the sample vortexed vigorously for 15 seconds before being incubated on ice for 5 minutes. This was followed by centrifugation at 12,000 x g for 15 minutes at 4°C. The colourless upper aqueous phase was added to an equal volume of isopropanol (Sigma) and incubated at 4°C for 15 minutes. After centrifugation at 12,000 x g for 20 minutes at 4°C, RNA precipitates formed a pellet at the bottom of the tube. The supernatant was removed and the pellet was re-suspended in 800 µl ice-cold 75% ethanol, centrifuged at 12,000 x g for 8 minutes, dried under vacuum for 10 minutes and then re-suspended in 20 µl of ice-cold TE buffer (10 mM Tris.Hcl, 1 mM EDTA, pH 7). RNA concentration was determined by its optical density at 260 nm and 280 nm (Sambrook et al., 1989) and calculated as follow:

RNA (μg/ml) = [(62 x OD_{260}) - (36 x OD_{280})] x dilution factor

2.11.2 mRNA reverse transcription

cDNA was synthesised by reverse transcription. RNA (2 μg) in 8.5 μl was heated to 90°C for 5 minutes and quickly chilled on ice to break up secondary structures and added to the following mixture: 0.5 μl 40 I.U./μl RNasinR RNase inhibitor (Promega), 2 μl containing 0.5 μg random primers (Promega), 4 μl 5 x RT buffer (375 mM KCl, 15 mM $MgCl_2$, 250 mM Tris-HCl, pH 8.3), 2 μl 10 mM dNTP (Promega), 2 μl 10 mM DTT (Promega) and 1 μl containing 200 units Moloney murine leukaemia virus reverse transcriptase (Gibco BRL). Samples were then mixed and centrifuged for 1 minute at 7500 x g before incubation at room temperature for 5-10 minutes. The samples were incubated at 37 °C for 1 hour, and the reaction stopped by heating at 75 °C for 10 minutes then cooled quickly on ice.

2.11.3 cDNA Polymerase Chain Reaction (PCR)

cDNA (2 μl) was amplified in a 0.5 ml microtube in a reaction mix containing 0.2-0.3 μM of upstream and downstream specific primers, 10 μl 10 x reaction buffer (500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatine, 100 mM Tris-HCl pH 8.3), 4 μl containing 40 μM dNTP (Promega), and made up to 99.5 μl with water. This mixture was heated to 95°C for 5 minutes, cooled on ice before addition of 0.5 μl Taq DNA polymerase (Promega) and overlaid with 50 μl mineral oil (Sigma) to prevent evaporation during thermal cycling. PCR was performed in a DNA thermal cycler (Techne PHC-3 Dri-Block Cycler) which was programmed for 30-35 cycles : (1) denaturing for 1 minute at 94°C; (2) primer annealing for 2 minutes at 56 °C; (3) primer extension for 3 minutes at 72°C. At the end of the programme, the primer extension was continued for a further 8 minutes at 72 °C.

2. 11.4 Analysis of PCR products

After the PCR, the mineral oil was removed and 20 µl of the reaction product was visualised by electrophoresis on 1% (w/v) agarose gel in 0.5 x tri-borate EDTA (TBE, appendix I) containing 0.5 µg/ml ethidium bromide (Sigma) at 80 mA for 45 minutes. 1 µg of DNA 1 kb ladder (Gibco *BRL*, UK) was run in parallel as molecular weight standard. Negative control included amplification of a sham reverse transcription without reverse transcriptase to assess any contribution of contaminating genomic DNA in RNA samples; and amplification of a reaction mix with no added cDNA to assess contamination by DNA from other sources.

2.12 Recombinant IL-18 purification

2.12 .1 Rapid screening for recombinant IL-18 protein expression

To produce recombinant protein the *E. coli* strain M15 was transformed with the pQE30 expression vector carrying an insert encoding IL-18 (Collaboration with Dr. Wei, Department of Immunology, Glasgow University). Single bacterial colonies were obtained by plating transformed bacteria on LB-agar (Gibco BRL) containing 100 µg/ml ampicillin. Twelve single colonies were picked and cultured in 2 ml LB medium containing 100 µg/ml ampicillin with shaking at 37°C overnight. Each culture was then duplicated by adding 500 µl into 2 x 1.5 ml of fresh, pre-warmed LB (containing ampicillin). The cultures were shaken for another 30 minutes until the OD was between 0.7 and 0.9 when compared to fresh medium alone. 20 µl of 200 mM IPTG (final concentration of 1-2 mM) were added to one set of cultures, the other half serving as non-induced controls. Incubation was continued at 37°C for 3-5 hours after which time cultures were transferred to fresh 2 ml Eppendorf tubes. After centrifugation at 3,000 x g the supernatant was aspirated and the bacterial pellet re-suspended in 200 µl of buffer **B**. The re-suspended bacterial suspension was then frozen at –70°C for 20 minutes, thawed at room temperature whilst inverting several times to allow complete bacterial lysis. The samples were then centrifuged at 15,000 x g for 10 minutes and the supernatant transferred to a

fresh tube. Forty µl of 50% slurry nickel-agarose (Ni-NTA, Qiagen) were added and the samples mixed at room temperature for 30 minutes. The nickel-agarose was then spin-washed 3 times with buffer **C** at 13,000x g before adding 20 µl of buffer **C** containing 100 mM EDTA. The samples were mixed carefully and spun at 13,000 x g to recover the supernatant. Twenty µl supernatant from IPTG-induced and the non-induced samples were mixed with an equal volume of 2 x SDS-loading buffer and heated to 95°C for 10 minutes before analysis via SDS-PAGE. Induced and non-induced samples of the same bacterial clones were loaded next to each other on the gel for comparison.

2.12.2 Large scale purification of IL-18

LB-broth medium (100 ml containing 100 µg/ml ampicillin) was inoculated with a single colony of *E. coli*-M15 transformed with the pQE30 vector carrying an insert encoding IL-18. The culture was incubated with shaking at 37 °C overnight and added to 900 ml of fresh LB (Gibco BRL), containing antibiotic, pre-warmed to 37°C. The culture was further incubated with shaking for approximately 2 hours until OD_{600} reaches 0.7-0.9. IPTG (10 ml of 200 mM final concentration of 1-2 mM) was added and incubation with shaking was continued at 37°C for 5 hours. The bacterial suspension was then pelleted by centrifugation in a JA10 rotor (Beckman, USA) at 4000 x g, 4°C for 20 minutes. The supernatant was removed and the pellet re-suspended in 20 ml of buffer **B** (or stored at -70°C). The suspension was incubated with stirring at room temperature for two hours (overnight) followed by centrifugation at 14000 x g in a JA17 rotor (Beckman, USA). The supernatant was recovered and 1 ml (for 10 mg protein about 1 ml Ni-NTA is enough) of 50% slurry nickel agarose (Ni-NTA, Qiagen) was added. Recombinant protein carrying a 6 x His tag was allowed to bind to Ni-NTA for one hour by gently shaking the samples at room temperature. The Ni-NTA was then spin-washed 3 times with buffer **B** before loading onto a filter column (Qiagen). The Ni-NTA was allowed to settle by gravity and was washed by applying buffer **B** until the OD_{280} of the flow-through was below

0.001. The Ni-NTA was then washed with buffer **C** until $OD_{280} < 0.001$. It was then washed with 80% buffer C and 1 x PBS then 50% buffer C plus 1xPBS and 10% glycerol and finally 20% buffer C in 1 x PBS and 10% glycerol. One column volume (approximately 10 ml) of buffer **C** containing 20 mM imidazole and 10% glycerol was added to elute non-specific protein bound to Ni-NTA. Recombinant IL-18 bound to Ni-NTA was eluted by adding 2-3 ml buffer **C** containing 400 mM imidazole and dialysed in 2000 ml PBS containing 50 mM 2-ME overnight.

2.12.3 Measurement of protein concentration

The concentration of protein samples was measured using the Coomassie blue Method. Reagents were purchased from Pierce, Illinois, following the protocol provided by the manufacturer.

2.12.4 Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE ranging from 10-15% acrylamide was performed according to the molecular weight of the protein sampled. The Acrylamide solution was made as a mixture of acrylamide and bis-acrylamide in a ratio of 29:1. Resolving (lower) gels ranging from 10% to 15% were made in resolving gel buffer containing 1% (w/v) ammonium persulphate (BDH) and 0.1% (v/v) TEMED (Sigma). The mixed gels were then poured between glass plates in a SDS-PAGE gel chamber (BDH) and allowed to polymerise. Stacking (upper) gels were prepared in a similar way using stacking gel buffer containing ammonium persulfate and TEMED. Electrophoresis was performed in SDS-PAGE buffer. Protein samples were mixed in equal volumes with 2 x concentrated SDS-sample buffer and heated to 100 °C for 10 minutes prior to loading on gels. Protein molecular weight markers (Rainbow marker, range 14-200 kD, Amersham) were loaded on the gel to compare molecular weight of specific proteins within the samples. Electrophoresis was carried out at 5 mA/cm-gel length

and allowed to continue until that the bromophenol blue dye of the SDS sample buffer reached the bottom end of the gel.

2.12.5 Coomassie Blue staining of SDS-PAGE gels

SDS-PAGE gels were stained in 0.5% (w/v) Coomassie brilliant blue R250 (Sigma) containing 40% methanol and 10% glacial acetic acid for 1 hour at room temperature. The gels were then destained in 50% methanol and 10% glacial acetic acid for 4-5 hours, changing the destaining solution several times during this period until the gel-background was clear and protein bands were clearly visible. The stained gels were transferred to filter paper (Whatman 3MM) and dried at 80 °C under vacuum.

2.13 Evaluation proteinuria in murine lupus-like models

Proteinuria was assessed using a commercially available kit (Multistix, Bayer, Cambridge, UK) and graded according to the manufacturers instructions. A scoring system of 0 to + 4 was used as follows: 0/trace, ~<30 mg/dl; 1 +, ~30 mg/ml; + 2, ~100 mg/ml; + 3, ~300 mg/ml; and + 4, ~>500 mg/ml. A score of 2 + or greater was considered indicative of severe proteinuria, and mice exhibiting severe proteinuria on three or more successive occasions or at the final evaluation before sacrificing were considered positive for renal disease.

2.14 Statistical Analysis

Data were collected and statistical analysis performed using Minitab software for Macintosh. Cytokine and NO measurements are displayed as means and standard error of triplicate samples unless otherwise indicated. Parametric and non-parametric statistical tests were then used as appropriate including general linear models procedures, paired and non-paired T tests, Mann-Whitney U tests as well as Fisher test, Pearson correlation coefficients and Kaplan-Meier survival curve. Significance was accepted at $p<0.05$.

Table 2.1 Antibodies for immunohistochemistry

Antibody	Specificity	Host (Dilution)	Source
Polyclonal	Mouse Ig	Rabbit (1:200)	DAKO
Polyclonal	Rabbit IgG	Goat (1:500)	DAKO
Polyclonal	Human-IL-18	Mouse 10 μg/ml	R&D
Polyclonal (FITC)	Mouse IgG	Rabbit (1:100)	DAKO

Chapter 3

Role of IL-12 and Nitric Oxide in the pathogenesis of Systemic Lupus Erythematosus

Introduction

There are now data suggesting that NO plays an important role in autoimmune pathology. Inducible NOS expression and increased NO production have been implicated in experimental allergic encephalomyelitis and rheumatoid arthritis (reviewed by Zhao et al., 1999; Kolb & Kolb-Bachofen 1992; McInnes et al., 1996). It has been shown that MRL/*lpr* mice excrete significantly higher concentrations of urinary nitrate/nitrite than age-matched normal C3H mice (Weinberger et al., 1994). Furthermore, MRL/*lpr* mice showed markedly reduced proteinuria and minimal glomerular proliferation when treated orally with $_{L}$-N^{G} monomethyl argininie ($_{L}$- NMMA), an inhibitor of nitric oxide synthase (NOS) (Weinberger et al., 1994). Oral administration of the nitric oxide synthase inhibitor (NMMA) before the onset of clinical disease significantly decreases renal and joint pathology in MRL-*lpr* mice. After the onset of the disease oral NMMA and restricted dietary arginine reduce joint pathology scores in MRL-*lpr* mice and reduced renal pathology scores in NZB/W mice (Weinberger et al., 1994).

Serum from MRL/*lpr* mice contains more nitrite/nitrate than serum from age-matched control MRL/MP-++ (MRL/+), BALB/c or CBA/6J mice. Spleen and peritoneal cells from MRL/*lpr* mice, when cultured with IFN-γ and LPS, also produce significantly more NO than those from control mice (Huang et al., 1996). Furthermore, peritoneal cells from MRL/*lpr* mice produce markedly higher concentrations of IL-12 than control mice and cells from MRL/*lpr* produce high concentrations of NO when cultured with IL-12 and LPS (Huang et al., 1996). These findings were supported by the observation that daily injection of recombinant IL-12 increased serum levels of IFN-γ and NO metabolites, and accelerated glomerulonephritis in young MRL/*lpr* mice but not in MRL/+ (Huang et al., 1996). In SLE patients iNOS expression is increased in renal biopsy specimens from patients

with immune complex deposition (Oates et al., 1997) and elevated NO serum levels have also been reported (Levrtowsky et al., 1995; Gilkeson et al., 1995).

Studies were thus performed to determine whether the same mechanism was involved in the pathogenesis of other lupus-like models with different genetic backgrounds and intact Fas. New Zealand black/white (NZB/W) F1 hybrid mice display a variety of autoimmune SLE-like phenotypes, characterised by hyper-responsive B cells, autoantibody production, cytokine dysregulation and glomerulonephritis (Jongstra-Bilen et al., 1997); they carry no Fas mutation and have a different genetic background from MRL/*lpr* mice. This strain of mice seemed to be a suitable model to extend the search for the role of IL-12 and NO in the pathogenesis of SLE. Finally, because the aim of animal experiments was to elucidate the mechanism of human disease, the experiments described in the present chapter set out to establish whether SLE patients indeed produced more NO and IL-12 than normal individuals.

3.1 Detection of IL-12 (p40/p70) and IL-12 (p70) in mouse and human

Interleukin 12 p40/p70 (total IL-12) concentrations in the serum and peripheral blood culture supernatants were measured with a standard sandwich ELISA. A monoclonal anti-IL12 (p40/p70) (PharMingen) as capture antibody and biotin anti-mouse IL-12 (p40) (PharMingen) as detecting antibody were used and all plate standards were prepared with recombinant murine IL-12 (Genetic Institute). To detect murine IL-12 (p70) a monoclonal anti-IL12 (p35/p70) antibody (PharMingen) was used as capture antibody and the rest of reagents were the same as used for the total IL-12 assay. Figures 3.1a and 3.1b show the murine total IL-12 and IL-12 (p70) ELISA standard curve.

Concentrations of IL-12 (p70) in the serum and culture supernatant from SLE patients and normal control individuals were measured by commercially available

ELISA kit (R&D). Total IL-12 (p40/p70) was also measured by ELISA. Figure 3.2a and Figure 3.2b show human IL-12 (p70) and total human-IL12 standard curves.

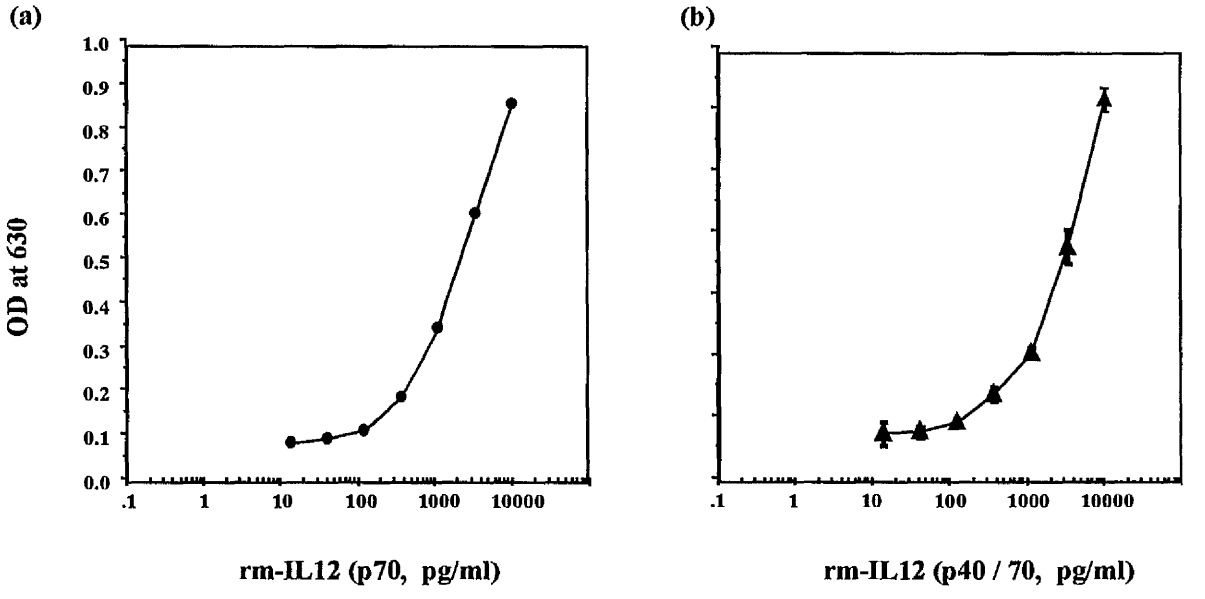


Figure 3.1 Standard curve for murine IL-12 ELISA. (a) Sensitivity for IL-12 (p70) ELISA was 25 pg/ml. (b) Sensitivity for IL-12 (40/p70) assay was 40 pg/ml.

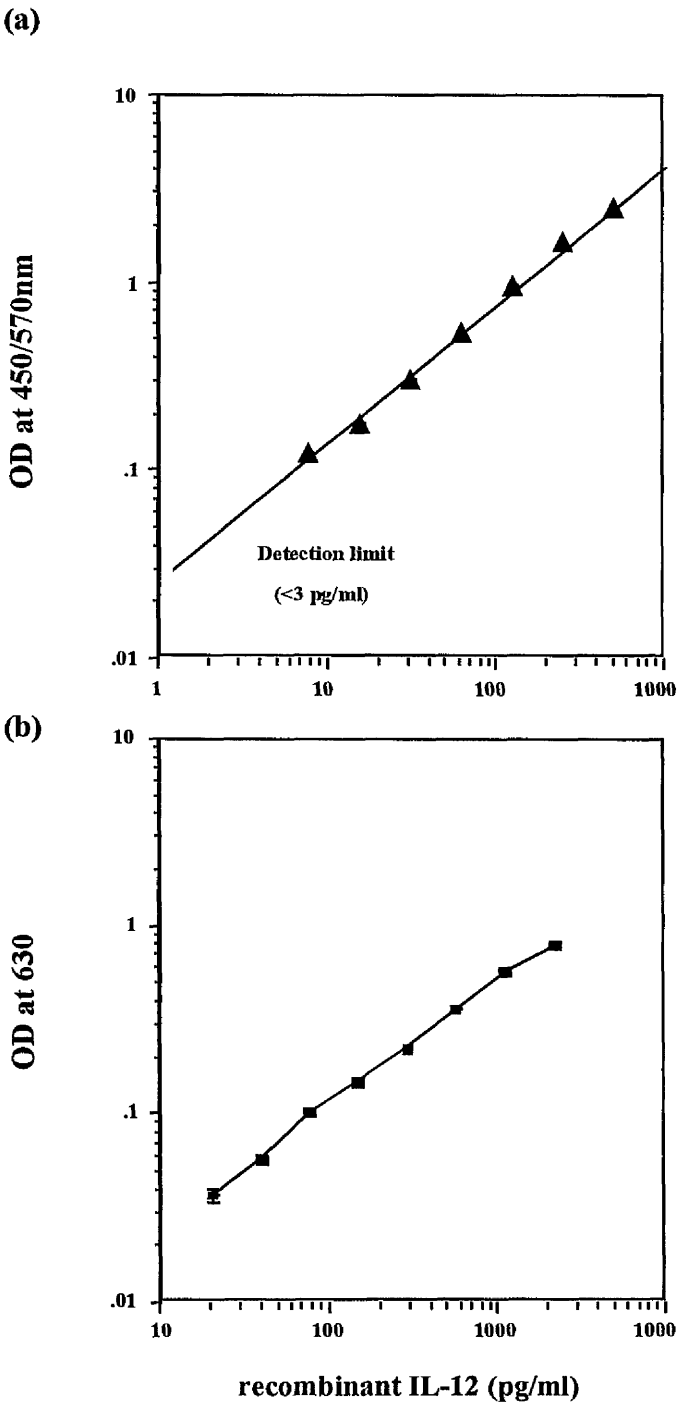


Figure 3.2 Standard curve for human IL-12 ELISA. (a) Concentrations of bioactive IL-12 (p70) in the serum and culture supernatant from SLE patients and normal control individuals were measured by an ELISA kit purchased from R&D. Sensitivity for the ELISA was 3 pg/ml. (b) Total IL-12 (p40/p70) was measured by ELISA kit (Donovet, Genzyme). Sensitivity for the ELISA was 20 pg/ml.

3.2 Total IL-12 serum levels in different ages of NZB/W mice

Sera from 30 NZB/W mice and 20 BALB/c mice (as a control group) of various ages were analysed for the levels of IL-12 (p40/p70). MRL/*lpr* sera were used as a positive control (Figure 3.3). Figure 3.4a shows that the concentration of total IL-12 in the serum of NZB/W mice increased markedly at the age of 7-9 months, with the onset of proteinuria and glomerulonephritis. However, in the sex- and age-matched BALB/c mice, no changes in IL-12 levels were detected. The mean concentration of IL-12 (p40/p70) measured from 7-9 months old NZB/W mice was 3100 ± 500 pg/mg, significantly higher ($p<0.02$) than 1-3 month old mice 1250 ± 220 pg/ml (mean ± SEM, Mann-Whitney, Figure 3.5a). There was a significant correlation between proteinuria and the level of IL-12 in 7-9 month old mice ($r=0.689$, $p<0.05$) compared with younger mice (Figure 3.5b). The serum level of IL-12 between the two age groups from BALB/c mice did not differ ($p<0.376$, Figure 3.4a). IL-12 levels in NZB/W mice at 10-12 month old (with severe glomerulonephritis) were lower; it is possible that severe kidney disease leads to excretion of IL-12 through altered glomerular permeability. These data taken together indicate that the increase in serum levels of IL-12 (p40/p70) in NZB/W mice correlated with age and disease.

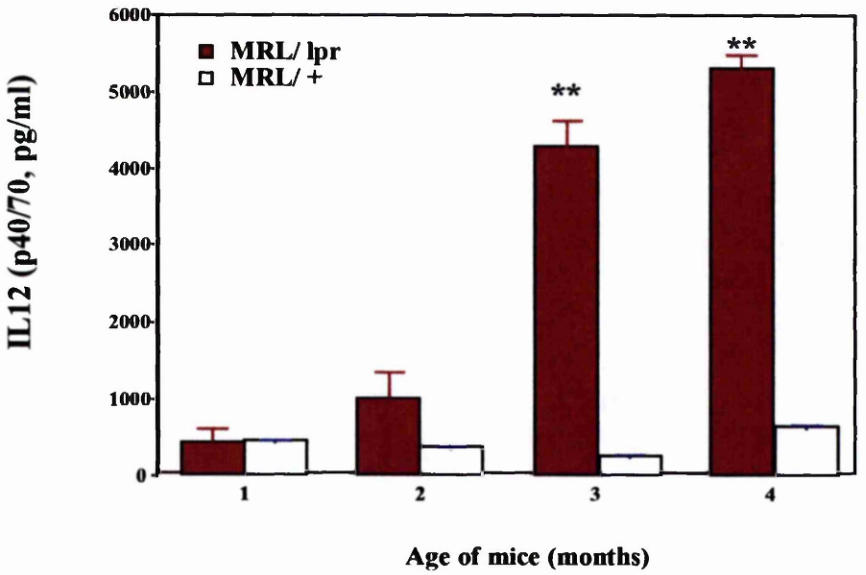


Figure 3.3 Elevated serum IL-12 levels in MRL/*lpr* mice. IL-12 (p40/70) levels were measured by ELISA in pooled sera from different ages of MRL/*lpr* lupus strain and MRL/++ controls (15 mice in each group of ages). Serum IL-12 levels were markedly higher in *lpr* mice, especially in older mice with clinical disease compared with controls (**$p<0.001$). Serum IL-12 levels increased with age in MRL/*lpr* mice.

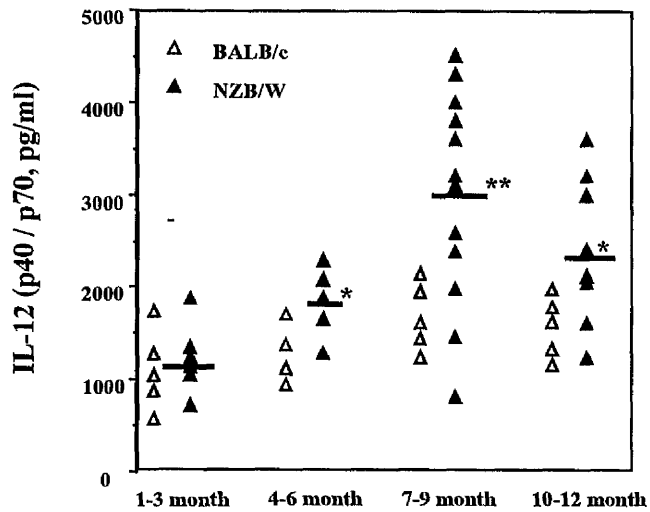


Figure 3.4 Enhanced IL-12 levels in the serum of NZB/W mice. (a) Serum IL-12 (p40/p70) levels in (NZB/W) F1 lupus strain (n=30) and BALB/c strain control group (n=20) of mice at different ages. Total IL-12 concentrations in serum were determined by ELISA. Serum IL-12 levels were markedly higher in the NZB/W mice, especially in the 7-9 month compared with control groups (**P<0.001, *P<0.05). (b) IL-12 (p40/p70) serum levels in NZB/W mice were higher than BALB/c control group (p<0.003, Mann-Whitney). IL-12 (p70) serum levels were under sensitivity level of ELISA.

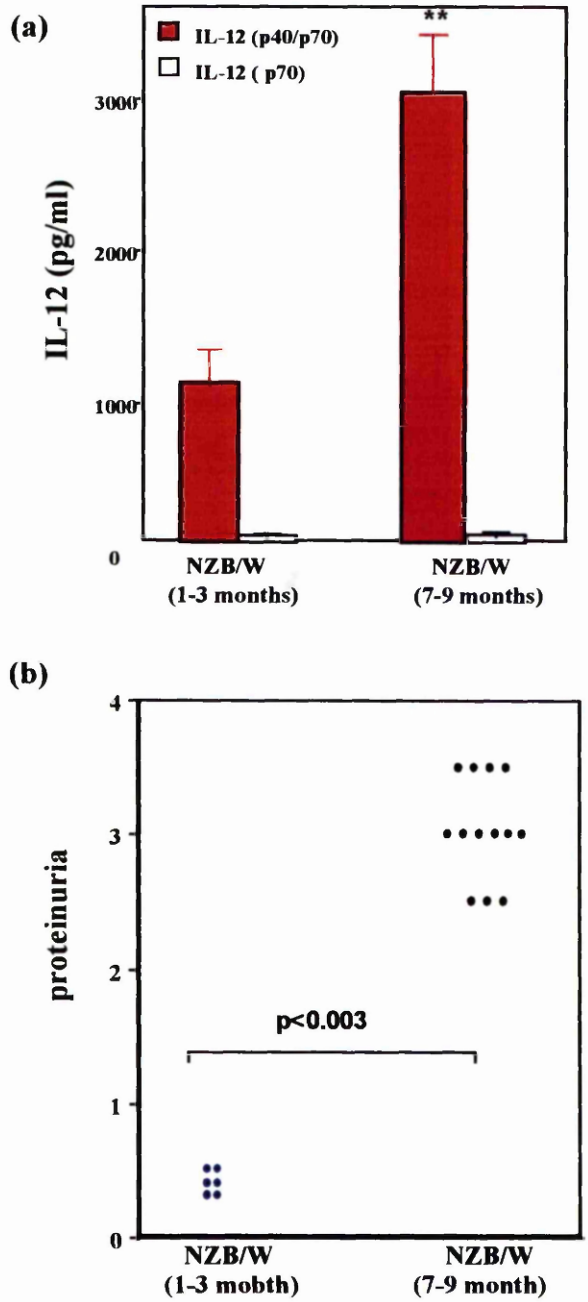


Figure 3.5 Total IL-12 was elevated in NZB/W lupus strain. (a) Total IL-12 serum levels in NZB/W mice were increased with age. The highest level of IL-12 was at 7-9 month old (about 3-fold more than 1-3 month old). (b) IL-12 (p40/p70) increased was correlated with proteinuria (as a surrogate for glomerolunephritis, $p<0.003$).

3.3 Cytokine production by spleen cells from NZB/W lupus-like mice

Spleen cells from NZB/W mice 2.5 or 7 months old, and age- and sex-matched BALB/c mice were cultured with ConA (5 μg/ml) or LPS (100 ng/ml) and IFNγ (50 U/ml). Culture supernatants were collected after 48 hours incubation, and IL-12 levels were measured. Spleen cells from 7-month-old NZB/W mice produced approximately 2-3 times more total IL-12 than 2.5 month old NZB/W mice (Figure 3.6b). There was no significant difference between BALB/c mice aged 7 months or 3 months. There were also no significant differences between 2.5 month old NZB/W mice and 2 month old BALB/c mice (Figure 3.6a). IL-12 (p70) was not detectable in the supernatants.

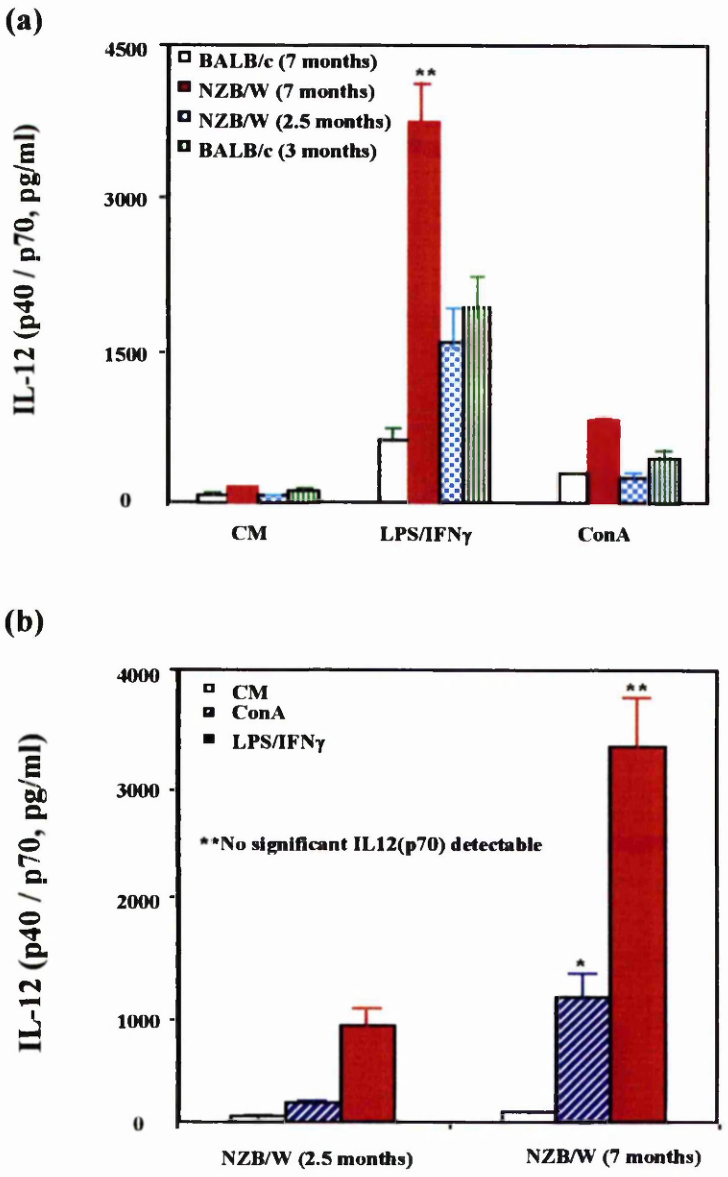


Figure 3.6 Spleen cells from NZB/W mice produced higher concentrations of IL-12 in response to IFN-γ and LPS. Pooled spleen cells from NZB/W mice (n=5, 2.5 and 7 month old), and BALB/c mice (n=5, 3 and 7 month old) were stimulated in 96-well culture plates with or without fixed doses of ConA (5 μg/ml) or LPS (100 ng/ml) and IFN-γ (50 U/ml) or only with culture medium (CM). Culture supernatants were collected after 48 hours and IL-12 levels measured by ELISA. (a) Spleen cells from NZB/W mice produced more total IL-12 than spleen cells from control group (*$p<0.05$, **$p<0.001$). (b) IL-12 production by NZB/W spleen cells was age-dependent (*$p<0.05$).

3.4 IL-12 (p70) and IL-12 (p40/p70) in the serum of SLE patients

Finally, because the aim of animal experiments is to elucidate mechanisms of human disease, I set out to establish whether SLE patients indeed produced more IL-12 than normal individuals.

Serum samples were collected from 50 patients who attended the Connective Tissue Diseases Clinic at Glasgow Royal Infirmary, Rheumatology Clinic in Nottingham and Rheumatology Clinic in Glasgow Gartnaval Hospital. All fulfilled at least four of the ARA criteria for the classification of SLE (Tan et al., 1982) [Table 1.1]. In particular data concerning disease activity, selected organ involvement, presence of infection, and therapy were collected. The SLE Disease Activity Index (SLEDAI) [Bombardier et al., 1992] or SLAM (Liang et al., 1989) was calculated for all patients. Serum samples from 30 healthy blood donors of comparable age and gender, were also studied.

The levels of IL-12 (p40/p70) in the sera of SLE patients were significantly higher than in control groups $p<0.006$ (Figure 3.7). The level of total-IL-12 in the samples from different clinics was analysed separately. SLE patients in all three clinics had significantly higher levels of total IL-12 compared with normal controls. Because the level of IL-12 (p70) generally fell below the limit of sensitivity of the ELISA, it was not possible to correlate expression of this with SLE activity (Figure 3.8).

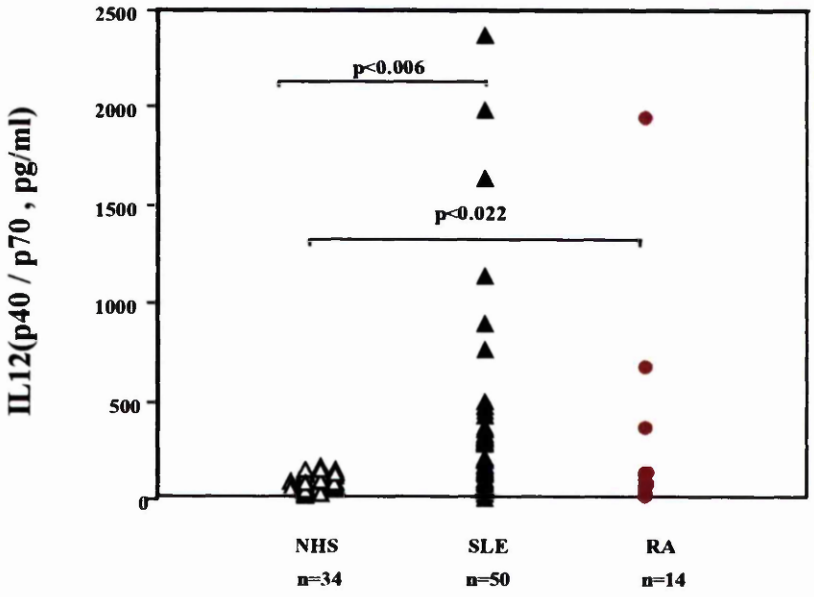


Figure 3.7 IL-12 (p40/p70) concentrations in sera obtained from SLE patients. Total IL-12 serum levels were assayed using a commercial ELISA kit. IL-12 levels in serum from SLE patients (n=50) and normal human serum (NHS, n=34) were compared. Significant increments of total IL-12 levels were observed in SLE patients ($p<0.006$, Mann-Whitney). Although patients with rheumatoid arthritis had less IL-12 than SLE patients, IL-12 levels were higher in the serum of RA patients than normal controls ($p<0.022$, Mann-Whitney U-test)

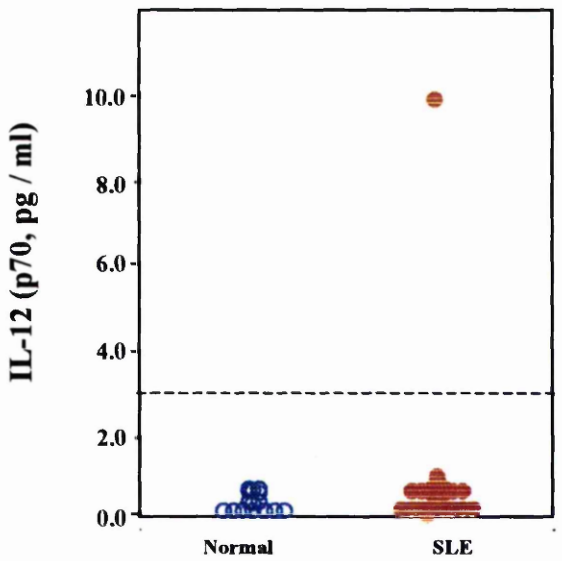


Figure 3.8 IL-12 (p70) concentration in serum from SLE patients.

There was no difference between IL-12 (p70) in the serum of SLE patients (n=27) and healthy controls (n=19). IL-12 (p70) levels in most of the patients were less than sensitivity of the kit assay (<3pg/ml).

3.5 Correlation between IL-12 levels in the SLE patients and clinical profile

To investigate the correlation between total IL-12 levels and disease, clinical details of SLE patients were collected at the time of sample taking. Disease activity (SLAM or SLEDAI), organ involvement, anti-DNA antibodies, C-reactive protein, ESR (erythrocyte sedimentation rate), and drugs were analysed (Table 3.1). There was no significant correlation between IL-12 levels in the serum and the SLE Disease Activity Index ($r=0.265$, Figure 3.9). Autoantibodies, ESR and CRP did not show any correlation with total IL-12 serum levels.

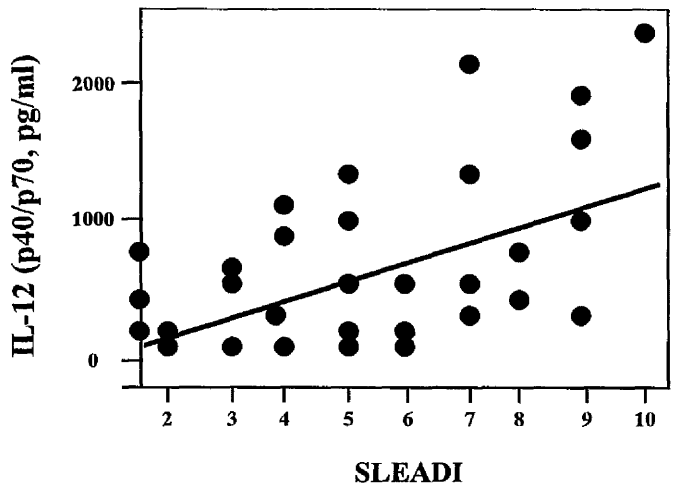


Figure 3.9 Correlation between IL-12 serum levels and SLEDAI. Clinical data from 30 SLE patients was compared with their IL-12 (p40/p70) serum levels. There was no correlation between total IL-12 serum levels and SLEDAI (n=30, r=0.265, Pearson test).

3.6 IL-12 production by PBMC and whole blood cells from SLE patients

Twelve follow-up patients were included in this study. All were diagnosed as SLE by the revised American Rheumatism Association Criteria (Tan et al., 1982) in Glasgow Royal Infirmary Connective Tissue Diseases Clinic. Patients did not receive any steroid or cytotoxic drugs within 1 month of sampling. Seven healthy controls matched for age and sex were also studied. PBMC were prepared from patients with different disease activity (SLE Disease Activity Index). Cultures were stimulated by LPS (3 μg/ml), *Staphylococcal enterotoxin* B (SEB, 2 μg/ml) and IFN-γ (100 U/m) or PHA (1 μg/ml). Culture supernatants were collected after 48 hours and stored at -20°C until assay for IL-12.

Results in Figure 3.10a showed that IL-12 production by PBMC in SLE patients was significantly lower than normal controls ($p<0.05$). These data were apparently contradictory with serum data. Therefore, further *in vitro* experiments were performed on whole blood cells. Blood was withdrawn, heparinised (10 I.U/ml) after differential WBC count, immediately diluted 1:5 in complete Iscove's medium. After addition, LPS, SEB and IFN-γ, duplicate cultures (1 ml each) in 24-well culture plates were established, and culture supernatants were collected after 48 hours incubation and stored at -20°C until assay for IL-12. Unstimulated, diluted blood was withheld as "time 0" negative control, and after centrifugation supernatant was stored at -20°C prior to IL-12 estimation by ELISA. Total IL-12 levels in the SLE supernatant were significantly higher than controls ($p<0.05$, Figure 3.11b). Figure 3.11b also shows that LPS (3 μg/ml) and IFN-γ (100 u/ml) were the best stimulators.

The experiments on NZB/W F1 mice, a lupus-like model with intact Fas, and results from SLE patients were consistent with those previously observed in MRL/*lpr* mice (Huang et al., 1996) in which higher levels of IL-12 were correlated with disease. The following experiments were carried out to investigate whether high capacity IL-

12 production in NZB/W F1 and SLE patients led to higher output of NO (Huang et al., 1996).

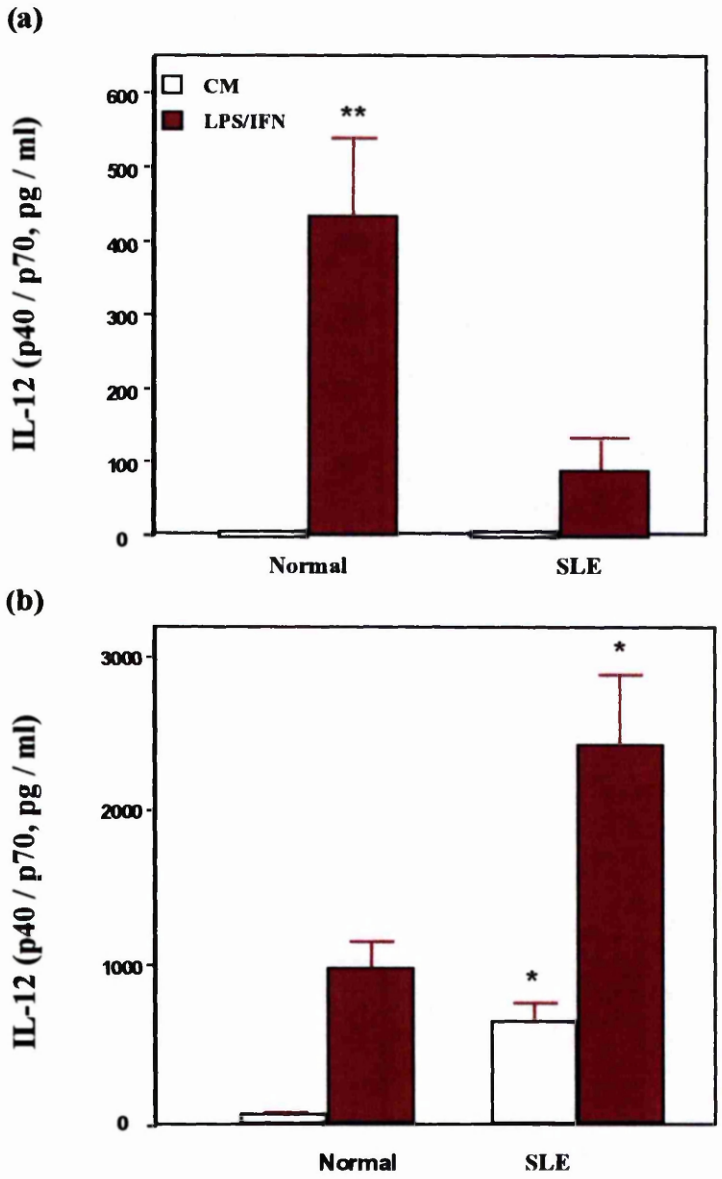


Figure 3.10 IL-12 (p40/p70) production by PBMC and peripheral whole blood cells from SLE patients. (a) Peripheral blood mononuclear cells (PBMC) from normal control (n=7), when cultured with LPS (3 μg/ml) and IFN-γ (100 U/ml), produced more IL-12 than lupus patient (n=15) peripheral blood cells ($p<0.02$, Mann-Whitney). (b) Peripheral whole blood cells from SLE patients and normal controls after differential WBC count were diluted (1:5) in Iscove's medium and cultured with LPS (3 μg/ml) and IFN-γ (100 U/ml). Supernatants from lupus patients contained significantly more IL-12 (p40/p70) than normal controls ($p<0.05$, Mann-Whitney).

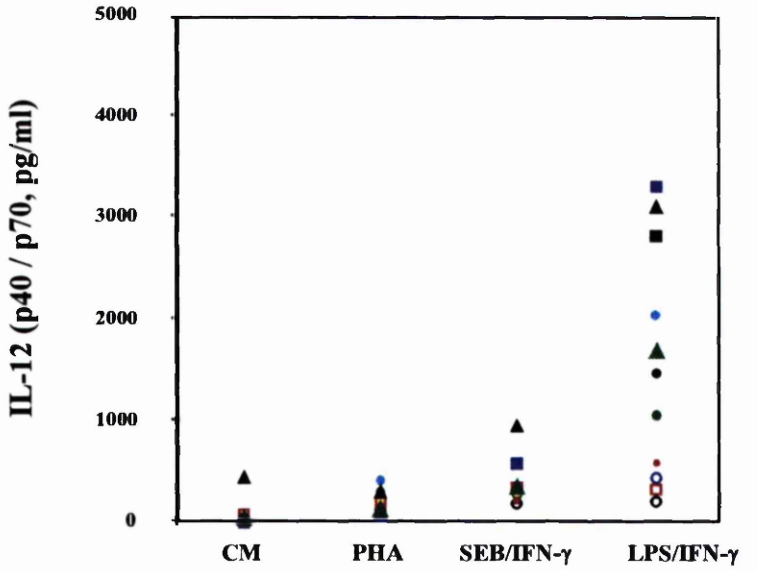


Figure 3.11 IL-12 levels in culture supernatants from peripheral whole blood cells of SLE patients. Heparinised whole blood cells from 12 SLE patients were cultured in Iscove's medium (1:5 dilution) and stimulated with LPS, SEB, IFN-γ, PHA or only culture medium (CM). Culture supernatants were collected after 48 hours. LPS (3 μg/ml) and IFN-γ (100 U/ml) were the best stimulants for IL-12 production.

3.7 Assay for nitrite in biological fluids

In culture supernatants and serum samples, nitric oxide production was estimated by the concentration of its oxidative products nitrite and nitrate ions. NO concentration in peritoneal and spleen cell culture supernatants was measured by the Griess reaction, with typical sensitivity of 2 μM (Figure 3.12a).

Total serum or plasma nitrite levels were estimated by two methods.

(a) The Griess reaction develops at acid pH raising the possibility of protein precipitation in test samples. Total serum nitrite levels were therefore measured by chemiluminescence, after reduction of nitrate to nitrite using nitrate reductase. $NaNO_2$ and $NaNO_3$ standard curves are shown in Figure 3.12b, demonstrating sensitivity of 2 μM.

(b) Modified Griess reaction was developed to measure total NO in biological fluids (Chapter 2.8). To all serum samples after reduction of nitrate to nitrite and deproteinization, equal amounts of Griess reagents were added. The standard curve of modified Griess reaction is compared with chemiluminescence assay in Figure 3.12.

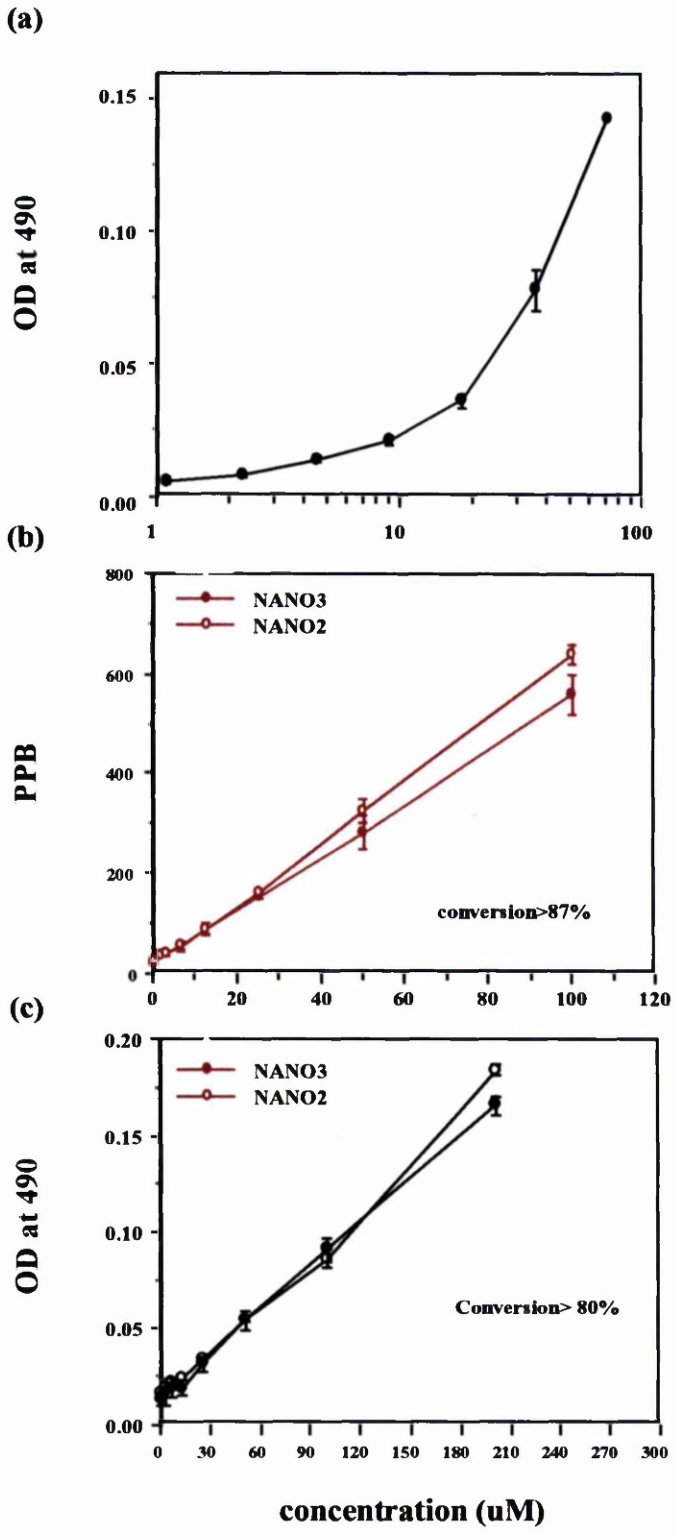


Figure 3.12 (a) The standard curves for NO2 measurement by Griess reaction with 2 μM sensitivity. (b) Standard curve for Nitrite / Nitrate measurement in the serum by chemiluminescence assay. (c) Standard curve to measure Nitrite / Nitrate in the serum by modified Griess reaction.

3.8 Nitrite / Nitrate in the serum from different ages of NZB/W mice

Serum from NZB/W (n=30) mice and BALB/c mice (n=20, as control group) of various age was analysed for NO metabolites by converting nitrate to nitrite and determining the total nitrite by Griess reaction. Figure 3.13a shows that serum from BALB/c mice contained significantly higher concentrations of nitrate and nitrite than those from age- and sex-matched NZB/W mice ($p<0.02$; Mann-Whitney). Serum NO levels in NZB/W mice did not correlate with age or glomerulonephritis (Figure 3.13). These data showed that the level of NO in the serum of NZB/W mice, was not higher than normal controls and there was no significant change in NO serum level with age or disease development.

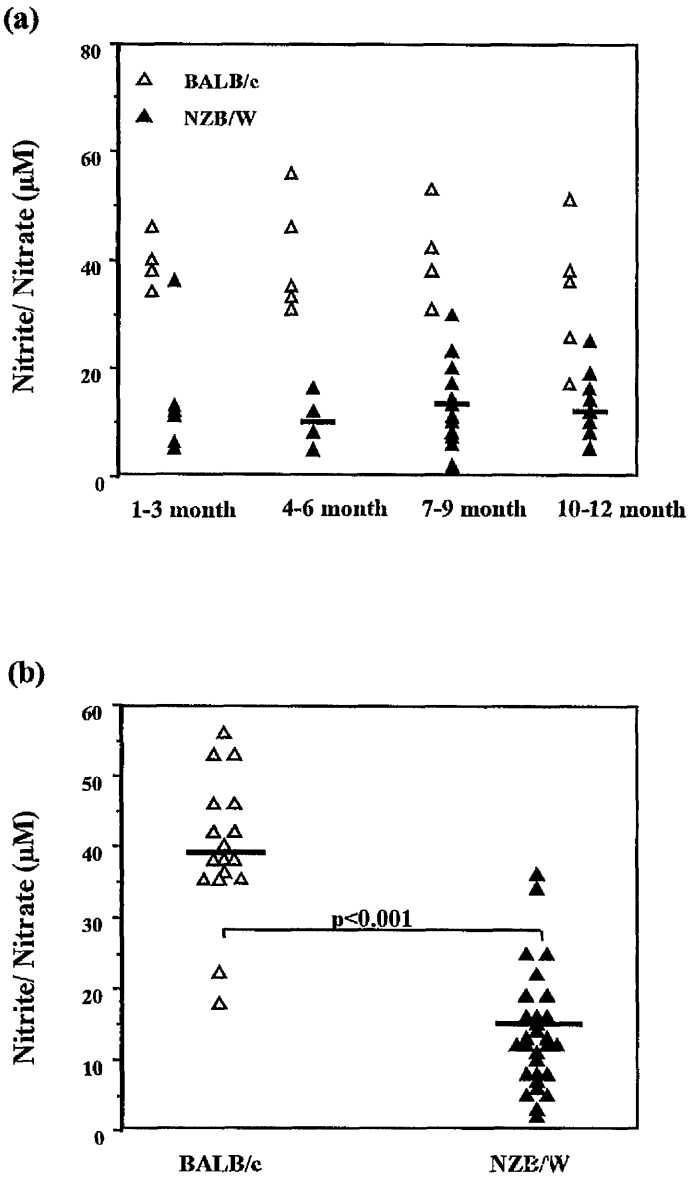


Figure 3.13 Level of nitric oxide (nitrite/nitrate) in the serum of different ages of NZB/W and BALB/c mice. (a) NO was generally higher in the serum of different ages of BALB/c mice compared with NZB/W mice. There was no significant correlation between NO serum levels with the age or disease in NZB/W mice. (b) NO level in the serum of BALB/c mice (normal control) is significantly higher than NZB/W mice ($p<0.001$, Mann-Whitney).

3.9 Peritoneal and spleen cell culture in lupus-like mice

Peritoneal and spleen cells pooled from five NZB/W or BALB/c mice, all 7 months old, were cultured with LPS (100 ng/ml), IFN-γ (50 U/ml) or IL-12 (10 ng/ml) for up to 72 hours. Concentration of NO in the culture supernatants was measured by the Griess method. Cells from NZB/W showed no significant differences in NO production from BALB/c mice when cultured with LPS and IFN-γ (Figure 3.14a). There were also no significant differences when peritoneal cells were cultured with LPS and IL-12 (Figure 3.14a). NZB/W mice at age 7 months or 3 months did not differ in NO production (Figure 3.14b). Spleen and peritoneal cells from MRL/*lpr* mice were used as a positive control in these experiments.

Taken together these results were contradictory to these obtained from MRL/*lpr* mice. There was no disease related NO enhancement in NZB/W mice and it was demonstrated that IL-12 did not induce more NO production spleen and peritoneal cells from NZB/W mice with established disease compared with young and disease free NZB/W mice.

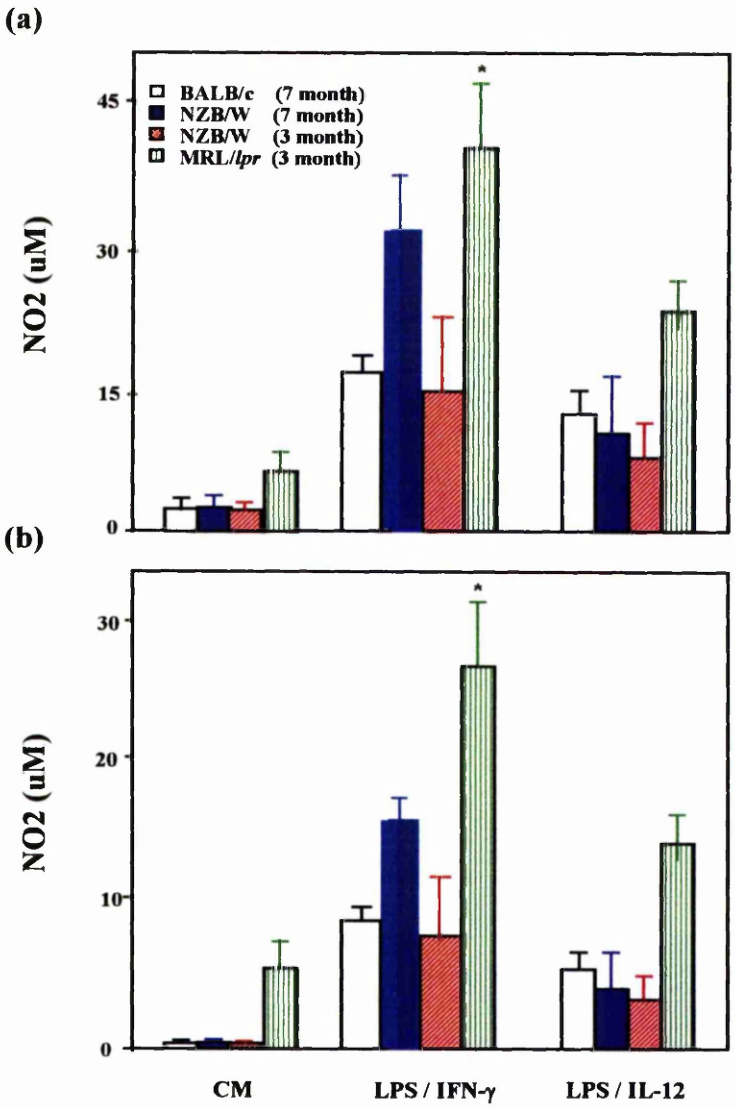


Figure 3.14 NO production by peritoneal and spleen cells from NZB/W mice did not correlate with the age or disease. Peritoneal (a) and spleen (b) cells from different ages of NZB/W mice were stimulated in 96-well culture plates with or without r-IL-12 (10 ng/ml) and LPS (100 ng/ml). Culture supernatants were collected after 72 hours and nitrite level measured by the Griess method. There were no differences in NO production between 7 month old and 3 month old NZB/W mice. MRL/*lpr* mice were used as positive control group.

3.10 Nitric oxide level in the serum of SLE patients

Serum samples were collected from SLE patients with various disease activities. The samples were stored at -70°C prior assay for nitrite / nitrate concentration using two methods (a) chemiluminescence and (b) modified Griess reaction. Figures 3.15a and 3.15b show that the mean concentration of nitrite / nitrate, representing total NO level, did not differ from age- and gender-matched controls by either method.

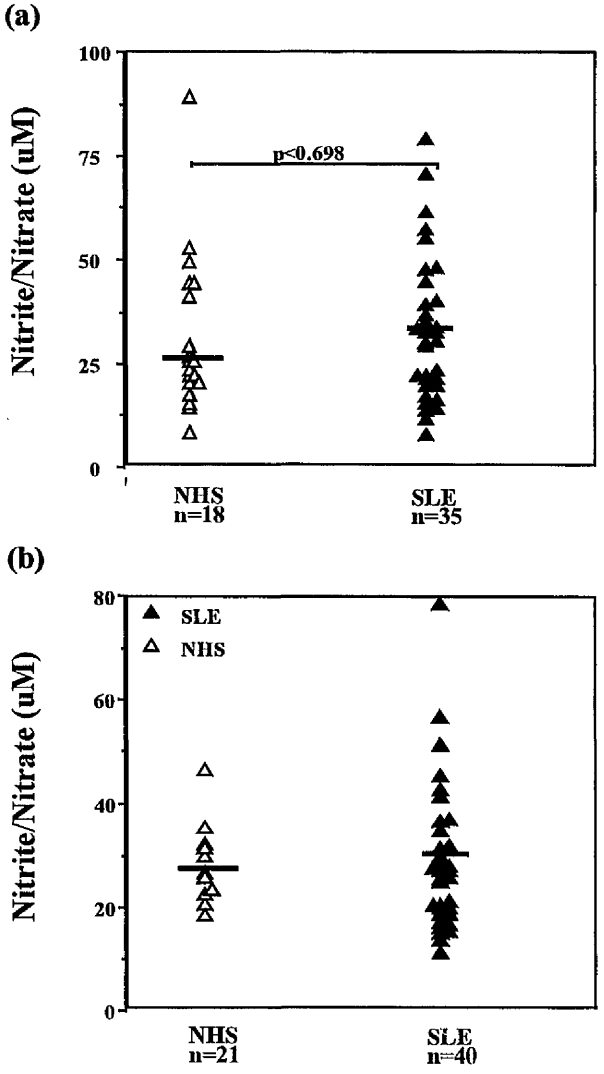


Figure 3.15 (a) Nitric oxide (nitrite / nitrate) levels in the serum of SLE patients by chemiluminescence assay. NO level in the serum from 35 SLE patients did not differ from normal controls (Mann-Whitney U-test). (b) NO level was measured by modified Griess reaction and there was no significant difference between two groups.

3.11 NO production by PBMC and whole blood cells from SLE patients

Twelve SLE patients were included in this study. Seven health controls matched for age and sex were also studied. PBMC were prepared from patients with different disease activity (SLE Disease Activity Index, SLEDAI) and stimulated by LPS (3 μg/ml), SEB (2 μg/ml) and IFN-γ (100 U/m) or IL-12 (10 ng/ml) for NO production. Culture supernatants were collected after 72 hours and stored in -20 °C until assay for NO. NO production by PBMC in the SLE patients and normal controls were about or less than the sensitivity level of the assay (2 μM). IL-12 did not induce NO production in PBMC from SLE patients or normal controls.

Whole blood culture was also used to investigate whether the changes shown in the level of inducible IL-12 could be observed for NO production (section 3.6). However, as Figure 3.16 shows there were no significant changes between SLE patients and control individuals in NO production.

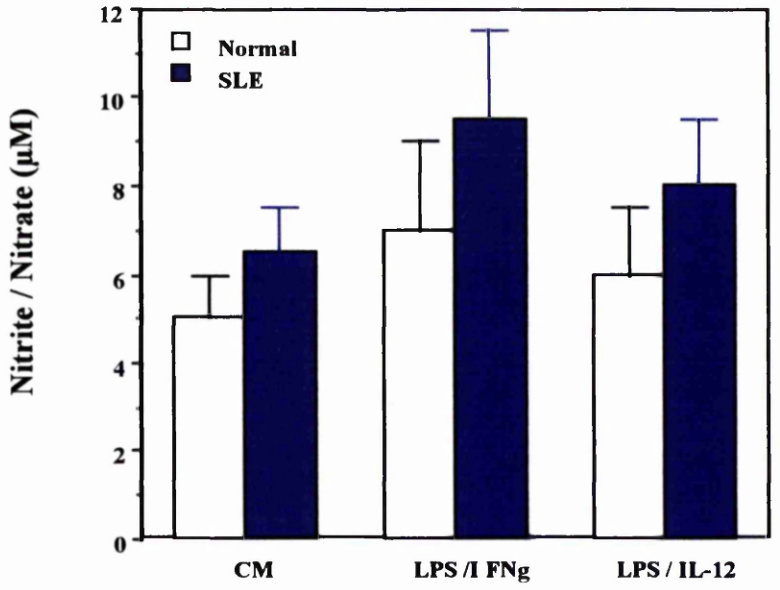


Figure 3.16 IL-12 did not induce NO production by peripheral blood from SLE patients. Heparinised whole blood cells from 15 SLE patients and 7 healthy controls were cultured in 24-well culture plates and stimulated with LPS (3 µg/ml) and IL-12 (10 ng/ml), LPS (3 µg/ml) and IFN-γ (100 U/ml) or only culture medium (CM). Culture supernatants were collected after 72 hours. There were no significant changes in NO production in the supernatants from SLE patients and controls (Student's t-test).

Discussion

Elevated levels of IL-12 (p40/p70) have been reported in MRL/*lpr* serum and have been linked to increased nitric oxide production (Huang et al., 1996). Amelioration of disease by administering inhibitors of the NO pathway (Weinberger et al., 1994), and exacerbation by recombinant IL-12 injections indicate a role for both of these molecules in the pathogenesis of SLE (Huang et al., 1996). The aim of this study was to investigate whether IL-12 and NO have the same effect in a lupus-like model with intact Fas (NZB/W) F1 mice and in human SLE. The results demonstrate that in NZB/W F1 mice total IL-12 (40/p70) serum levels are higher than in control mice. IL-12 is increased in correlation with disease of this lupus-like strain. Increase in IL-12 coincides well with the time of renal disease onset at around 7-month age (Theofilopoulos et al., 1986). The data are supported by the results from MRL/*lpr* mice (Huang et al., 1996).

The human data show that the serum level of total IL-12 is significantly higher in SLE patients than control individuals. However, PBMC from SLE patients produced less IL-12 (p70/p40) than control PBMC in contrast with the serum data. However, whole blood culture from SLE patients showed higher IL-12 production, when cultured with LPS and IFN-γ, compared with control individuals, consistent with the serum data. The reason for different results in PBMC and whole blood cell culture might be because of the role of granulocytes (especially neutrophils), which are deleted in PBMC, or possibly because of other factors such as different proteins or cytokines which are found in the plasma of patients and washed away during PBMC purification. The important role of cell contacts in immunological reaction is another possible explanation for different results obtained from PBMC and whole blood cell cultures. Macrophages from young disease free NZB/W mice show an enhanced capacity for IL-10 production (Alluvia et al., 1997) and higher level of IL-10 in younger NZB/W (Kalechman et al., 1997). Several papers reported higher level of

IL-10 in the serum of lupus patients and it's relation with disease activity (Houssiau et al., 1995; Lacki et al., 1997). IL-10 is closely associated with Th2 type immune responses and being produced by various non-T cells, only Th0 and Th2 cells secrete IL-10 in mice, whereas Th1 cells can also secrete IL-10 in humans (Del Prete et al., 1993; Street et al., 1991). In the peripheral blood of lupus patients, monocytes and B cells appear to be the main source of IL-10 (Llorente et al., 1994). On the other hand IL-12 is a strong IL-10 inducer (Meyaard et al., 1996; Jeannin et al., 1996) and it seems that an IL-10 / IL-12 immunoregulatory circuit controls susceptibility to autoimmune disease (Segal et al., 1997).

IL-12 is a disulphide-linked heterodimeric glycoprotein, which is composed of a heavy chain 40 kDa and a light chain of 35 kDa. The two chains, encoded by separate genes, become covalently linked to form the active p70 (p35/p40) heterodimer (Wolf et al., 1991). The p40 chain is overproduced relatively to the p35 chain. The p40 (p40/p40) chain then forms homodimers that bind to the IL-12 receptor and compete with the bioactive p70 heterodimer for receptor occupancy, thus serving as a competitive receptor inhibitor (Gilessen et al., 1995). In MRL/*lpr* mice both total IL-12 and IL-12 p70 are increased disease related, but in NZB/W F1 mice and human SLE we detected just total IL-12 enhancement and IL-12 p70 was undetectable. IL-12 p70 in concentration 100-500 times less than p40 homodimer can show bioactivity. Therefore, undetectable p70 (under sensitivity level of ELISA assay) may still be bioactive. Another possibility is that in the patients and lupus like models there are high level of p40 homodimer which act as antagonists to bioactive IL-12 (p70) and inhibit Th1 cytokines to reduce the inflammatory response in SLE. However , to elucidate the precise role of IL-12 in SLE, further investigation should be focused on the bioactive IL-12 p70 itself and the potential pathogenic role it may play.

We should note that there were wide deviations in the levels of IL-12 among the patients. However, there was no correlation between IL-12 serum level and activity of the disease and despite a correlation between level of IL-12 and proteinuria and kidney involvement in lupus-like models (NZB/W F1 and MRL/*lpr* mice), in human SLE patients no significant correlation between IL-12 level and kidney involvement or C3, C4 level was found. However, about six patients with high levels of IL-12 had the highest scores for disease activity.

It is demonstrated that corticosteroids inhibit IL-12 production in human monocytes (Vieira et al., 1998), therefore, the effect of corticosteroids in the management of lupus patients might be in part because of their inhibitory roles on IL-12 production.

The serum level of Nitric oxide in MRL/*lpr* mice appeared to be in correlated with age and disease (Huang et al., 1996). However, the level of NO in the serum of SLE patients and NZB/W mice were measured by two different methods and they showed no significant differences from normal controls. *In vitro* studies did not show any significant changes for NO production when stimulated with LPS and IFN-γ or IL-12 in both human SLE patients and NZB/W mice. Therefore, the data could not show the same phenomenon observed in MRL/*lpr* mice.

To investigate the possible role of *lpr* in IL-12 and NO production, we measured the level of IL-12 and NO in different *lpr* transgenic mice from different backgrounds, this showed no significant role for Fas deficiency in NO or IL-12 production(data was not shown). Taken together our studies shows similar serum NO level in SLE patients and healthy controls which is supported by other reported studies (Gonzalez-Crespo et al., 1998; Wigan et al., 1997). However, NO must play a role in the disease development as shown by the fact that NOS II inhibitors reduced renal pathology (Oates et al., 1997). There are a few reports that the serum levels of NO were significantly higher than the normal individuals and that the increase was

disease related (Levrtowsky et al., 1995; Gilkeson et al., 1996). This is in contrary to the results we obtained. The difficulties in techniques to measure NO in the body fluids specially in the serum and plasma with high level of proteins, and the effect of nutrients on the level of NO in the serum might be possible reasons for disparity. Another possible explanation why no increase in serum NO level was detected particularly among 7-9 months old NZB/W mice could be that at the time of sacrificing the mice NO level had returned to normal. However, that is unlikely since this would imply that NO was only important during disease onset and not during the chronic stage of the disease. This would be in contrast to findings that the modulation of NO production after disease onset could significantly reduce renal pathology (Oates et al., 1997). Urinary nitrite and nitrate excretion which is noticed with disease progression (Gilkeson et al., 1996) might be an explanation for unchanging serum level of NO during kidney involvement. We postulate that local tissue specific NO production might be important, especially for the development of renal pathology. Cells most likely to contribute to local NO production in the kidney are vascular endothelial cells and mesangial cells (Weinberger et al., 1998; Furusu et al., 1998) which were not examined in this experiment.

Chapter 4

Association of IL-18 with SLE

Introduction

A number of studies have described Th1 or Th2 cytokine abnormalities, such as IFN-γ,IL-10, IL-4, IL-12, TNF-α, in systemic lupus erythematosus (Huang et al., 1988; Linker-Israeli et al., 1991; Al-Janadi et al., 1993; Llorente et al., 1994; Houssiau et al., 1995; Klinman et al., 1995; Huang et al., 1996; Peng et al., 1997). In the lupus mice model, it was suggested that the balance of Th1/Th2 cytokines related to the pathogenesis of SLE (Takashi et al., 1996; Yoshii et al., 1995). Th1-derived cytokine synthesis is regulated mainly by IL-12 (Heinzel et al., 1993) and IL-12 is a pivotal cytokine in determining the nature and efficacy of immune responses. It plays a critical role in the promotion of IFN-γ synthesis, generation of Th1 cells and suppression of Th2 lymphocytes development. IL-12 is a major trigger for IFN-γ-dependent renal injury in MRL/*lpr* mice (Huang et al., 1996; Schwarting et al., 1999). In chapter 3 it was shown that levels of IL-12 were increased in the serum of SLE patients and in the serum of the NZB/W lupus-like mouse model compared with normal controls. Therefore, it is also possible that an abnormality of other T-cell regulatory cytokines, which modulate Th1 lymphocyte development, contributed to SLE pathogenesis.

Like IL-12, IL-18 is a member of the Th1-inducing family of cytokines. IL-18 is an IL-1-like cytokine, which was first identified by its ability to induce high levels of IFN-γ secretion by both NK and T cells (Okamura et al., 1995). IL-18 mediates other important functions, including enhancement of NK cell activity and stimulation of proliferation of activated T-cells (Okamura et al., 1995). IL-18 affects the development of cellular immunity (Th1 response) following antigen presentation (Robinson et al., 1997). It also potentiates IL-12 driven Th1 development in BALB/c mice and, synergistically with IL-12, promotes IFN-γ production from Th1 cells. Unlike IL-12, IL-18 alone does not drive Th1 development. Studies of IL-18 deficient mice support the notion that IL-18 plays an important supportive role in Th1 development (Takeda et al., 1998; Wei et al., 1998). IL-18 expression also has

been reported in several human diseases, including rheumatoid arthritis (Gracie et al., 1999; Leung et al., 2000) and inflammatory bowel disease (Pizarro et al., 1999).

The aim of this chapter was to investigate whether there was any association between IL-18 and SLE. To achieve this we determined:

a) IL-18 serum levels in a cross-section study of lupus patients and in the MRL/*lpr* lupus-like mouse model.
b) relations between IL-18 levels and clinical profile.
c) IL-18 production by spleen and peritoneal cells from lupus-like model.
d) the *in vitro* effects of IL-18 on different stages of disease development in MRL/*lpr* mice.

4.1 Detection of IL-18 in mouse and human serum

Human IL-18 in serum was measured by sandwich enzyme immunoassay (ELISA) using paired antibodies (R&D, Oxon, UK). The assay was performed according to the manufacture's instruction. A typical standard curve shows the lower detection limit at around 40 pg/ml (Figure 4.1a).

Concentrations of IL-18 in the serum and culture supernatant from MRL/*lpr* mice and MRL/++ mice were similarly measured by ELISA. Murine IL-18 was assayed with paired antibodies (R&D, Oxon, UK). The assay was performed according to manufacturer's instruction. A typical standard curve shows the lower detection limit for murine IL-18 at 30 pg/ml (Figure 4.1b).

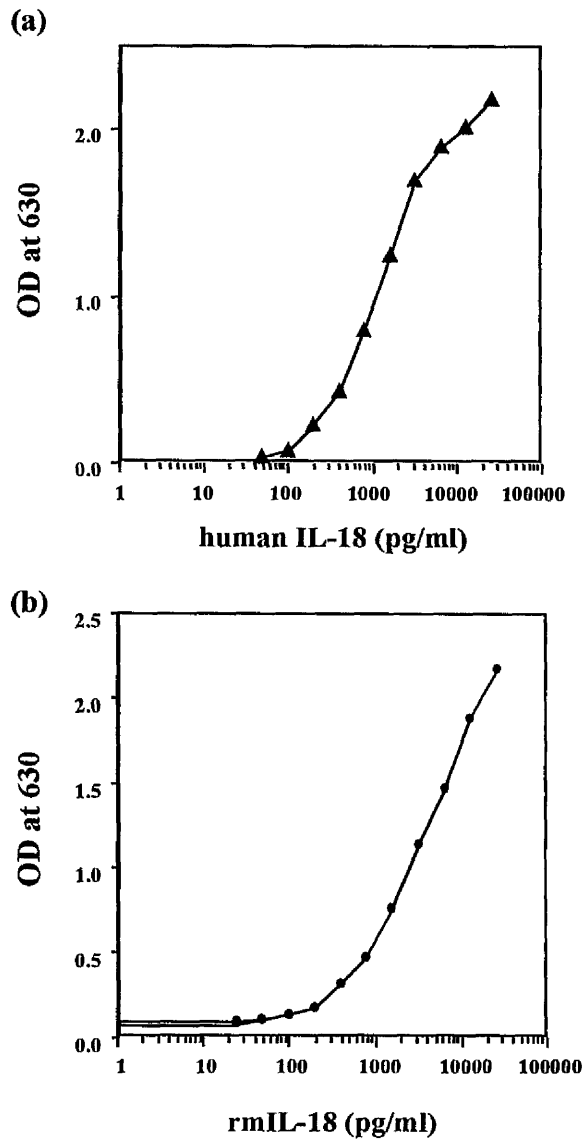


Figure 4.1 Standard curve for IL-18 ELISA. (a) Sensitivity for human IL-18 ELISA was 40 (pg/ml). (b) Sensitivity for murine IL-18 was 30 (pg/m).

4.2 IL-18 in the serum of SLE patients

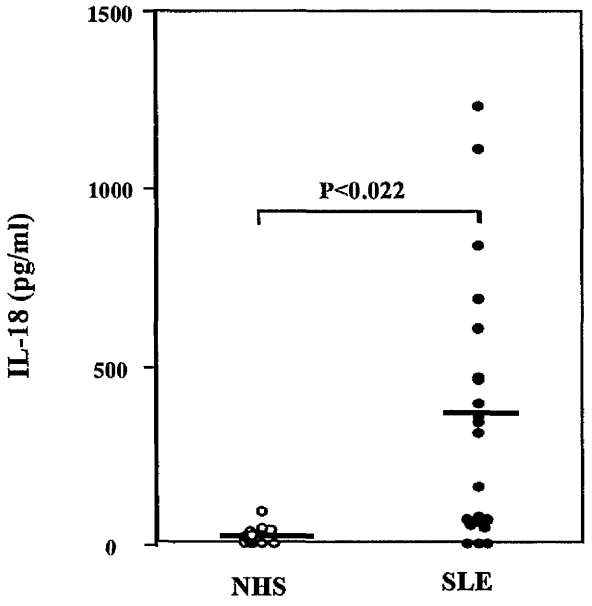
Serum samples were collected from 30 patients who attended the Connective Tissue Diseases Clinic at Glasgow Royal Infirmary, all fulfilled at least four of the ARA criteria for the classification of SLE. Special attention was paid to disease activity, selected organ involvement, presence of infection, and therapy. The SLE Disease Activity Index (SLEDAI) was applied to all patients. Serum samples from 20 healthy blood donors of comparable age and gender, were also studied.

As shown in Figure 4.2a, IL-18 serum levels in lupus patients were significantly higher than normal controls (mean ± SEM, patients 325 ± 65 pg/ml, and controls 43.2 ± 7.4, $p<0.02$).

The relationship between IL-18 serum levels and clinical profile in SLE patients was examined. There was no significant relation to SLEDAI (Figure 4.2b) and there was no correlation with nephropathy, musculoskeletal dysfunction nor anti-dsDNA antibodies, CRP or ESR levels.

IL-18 acts in synergy with IL-12 to promote development of Th1 responses. The levels of both IL-12 (shown in Chapter3) and IL-18 in the serum of SLE patients were higher than normal controls. Therefore, IFN-γ, IL-4 and IL-5 levels were measured in the serum of SLE patients to investigate whether high levels of IL-12 and IL-18 had any effects on the serum levels of other Th1 or Th2 cytokines. There were no significant differences in serum IFN-γ levels and serum IL-5 levels between SLE patients and normal controls (Figure 4.3). IL-4 was undetectable in the serum of the both groups.

(a)



(b)

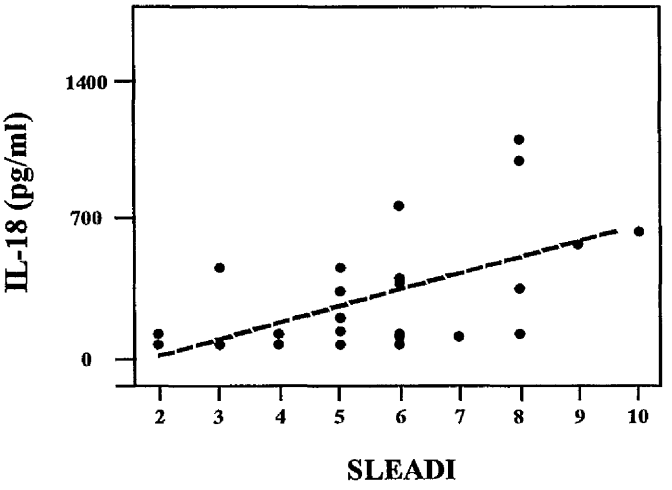


Figure 4.2 (a) Serum level of IL-18 in SLE patients (n=30) and healthy controls (n=20, $p<0.022$). The line shows the mean value in each population. (b) There was no significant correlation between serum IL-18 level and SLE disease activity (SLEDAI score) (n= 30, r = 0.215).

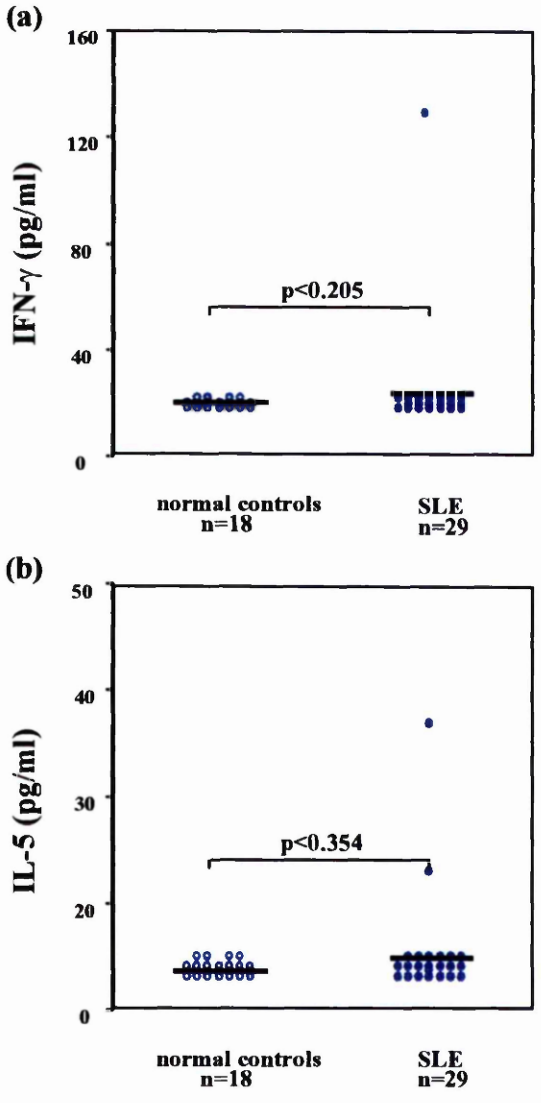


Figure 4.3 Serum IFN-γ (a) and IL-5 (b) levels in SLE patients and controls. Serum samples from SLE patients and healthy controls were collected and the levels of IL-5 and IFN-γ were measured by ELISA (Mann-Whitney).

4.3 IL-18 level in MRL/*lpr* mice with clinical disease

Murine SLE is a good model of human SLE as most of the immunological abnormalities apparently fundamental to the human disease also appear to be operative in the mouse (Theofilopoulos and Dixon, 1985). Among the SLE mice, the MRL/*lpr* strain has been extensively used as a lupus-like model. MRL/*lpr* mice are early-life lupus model and the major cause of death, like all lupus-like models and human SLE, is glomerulonephritis. These mice after 3 month of age start showing an obvious proteinuria, which is the onset of glomerulonephritis.

To determine the potential pathogenic role of IL-18 in SLE, we investigated IL-18 production in MRL/*lpr* mice. The serum of female MRL/*lpr* mice at 4-6 month age, with obvious proteinuria and kidney involvement, contained significantly more IL-18 than those of age- and sex-matched wild-type MRL/++ mice (Figure 4.4). Mean ± SEM, 462.5 ± 106 vs. 183.4 ± 49 respectively, $p<0.022$.

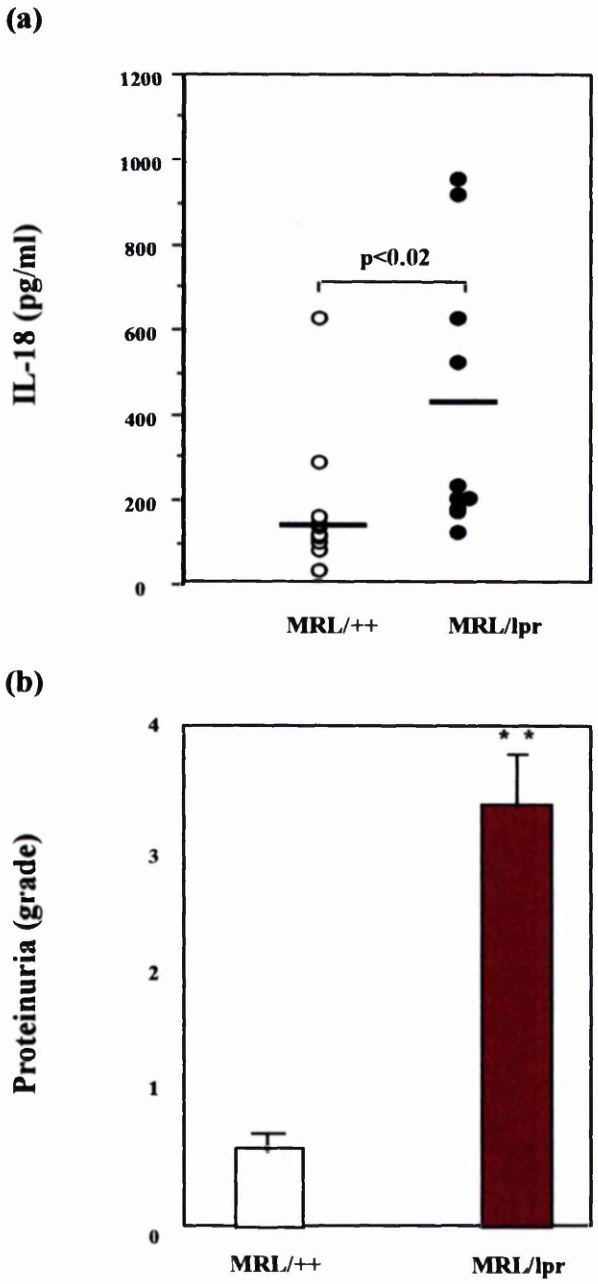


Figure 4.4 Serum levels of IL-18 in MRL/lpr and MRL/++ mice. The level of IL-18 in the serum of MRL/*lpr* mice (n=10) at age of 4-6 months (with obvious kidney involvement) was significantly higher than MRL/++ control mice (n=11, $p<0.02$, Mann-Whitney). (b) The proteinuria was measured, semi-quntitatively, by dipstick. The MRL/*lpr* mice had significant proteinuria (mean grade 3.3 ± 0.5), but MRL/++ control mice did not have any proteinuria (normal or trace).

4.4 IL-18 production by spleen and peritoneal cells from MRL/*lpr* mice

IL-18 production by spleen and peritoneal cells from MRL/*lpr* (n=5) or MRL/++ (n=5) mice at 14-15 weeks was measured. Cells were cultured with or without LPS (10 ng/ml) and IFN-γ (50 U/ml) in 96-well plates for up to 48 hours. The concentrations of IL-18 in the culture supernatants were determined.

As Figure 4.5a shows, the peritoneal macrophages and spleen cells from MRL/*lpr* mice spontaneously produced more IL-18 than MRL/++ control mice ($p<0.001$, Student's t-test). Exogenous LPS and IFN-γ down-regulated the IL-18 production by spleen or peritoneal cells.

Spleen cells spontaneously produced more IL-18 than peritoneal cells. This suggested several possibilities including cells other than macrophages may be the main source of IL-18 production and that contact between macrophages and other cells in spleen is required to secrete more IL-18.

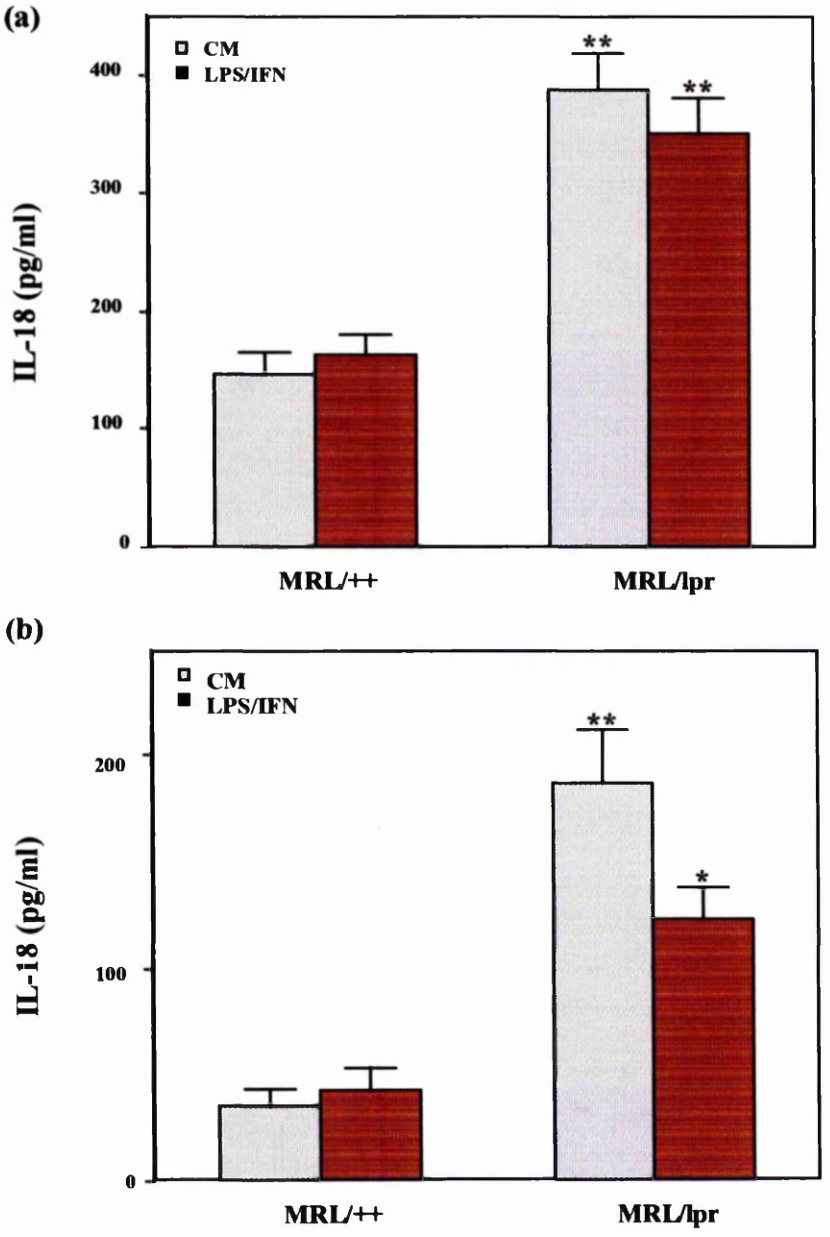


Figure 4.5 IL-18 production by cultured spleen (a) and peritoneal (b) cells from MRL/++ and MRL/*lpr* mice. Spleen or peritoneal cells from MRL/*lpr* mice and MRL/++ control mice were stimulated in 96-well culture plates with or without LPS (100 ng/ml) and recombinant IFN-γ (50 U/ml). Culture supernatants were collected after 48 hours and IL-18 levels were measured by ELISA. Unstimulated cells from MRL/*lpr* mice produced spontaneously more IL-18 than MRL/++ control mice (**$p<0.001$, *$p<0.05$, Student's t-test)

4.5 *In vitro* effects of IL-18 on different ages of MRL/*lpr* mice

It has been shown that serum IL-18 levels in MRL/*lpr* mice were higher than MRL/++ control mice, and that spleen cells from MRL/*lpr* mice spontaneously produced more IL-18 than controls. To further investigate the role of IL-18 in the pathogenesis of SLE, *in vitro* studies was taken on the MRL/*lpr* spleen cells at different stages of disease development. Spleen cells from MRL/*lpr* mice (n=5) were pooled and cultured in 24-well plates coated with anti-CD3 antibody (4 μg/ml) in the presence of different doses of recombinant IL-18.

Firstly, the effect of recombinant murine IL-18 together with immobilised anti-CD3 antibody was analysed on the spleen cells from 6-week old MRL/*lpr* mice. Culture supernatant were collected up to 48 hours and analysed for IL-4, IL-5, IL-10, IL-13 and IFN-γ by ELISA. Cells cultured with anti-CD3 alone produced significant levels of these cytokines. As expected the levels of IFN-γ was significantly increased by IL-18 in a dose-dependent manner ($p<0.001$). IL-18 and anti-CD3 were able also to induce spleen cells from MRL/*lpr* 6 week old (without any evidence of glomerulonephritis) to produce IL-5, IL-10, IL-13 in a dose-dependent manner (Figure 4.6).

We then investigated whether the effect of IL-18 in the present system could be affected by the age and disease development. Spleen cells from 13-14 week MRL/*lpr* mice, with obvious proteinuria and the onset of the disease, were cultured with immobilised anti-CD3 antibody in the presence of different doses of IL-18. The results in Figure 4.7 show that IL-18 in combination with anti-CD3 antibody induced significant IFN-γ production ($p<0.001$, Student's t-test), but suppressed IL-4, IL-10 and IL-13 production in the mice with the onset of glomerulonephritis.

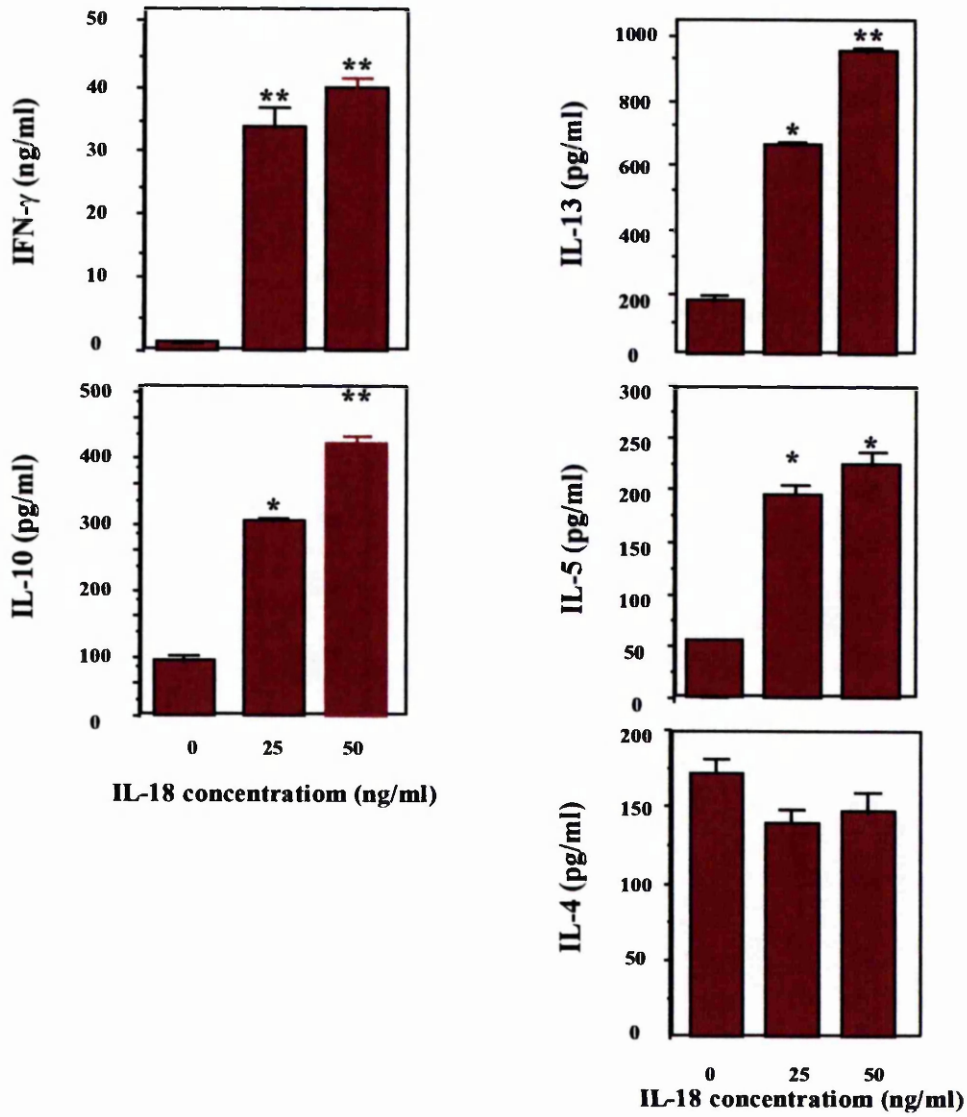


Figure 4.6 *In vitro* production of Th1 (IFN-γ) and Th2 (IL-5, IL-10, and IL-13) cytokine production by spleen cells from 6-week-old MRL/*lpr* mice. Pooled spleen cells from MRL/*lpr*, 6 weeks old, cultured with anti-CD3 and different doses of recombinant murine IL-18 and culture supernatants were collected at 48 hours. There was marked enhancement of IFNγ, IL-5, IL-13 and IL-10 production by IL-18 stimulation in a dose-dependent manner (*$p<0.05$, **$p<0.001$, Student's t-test).

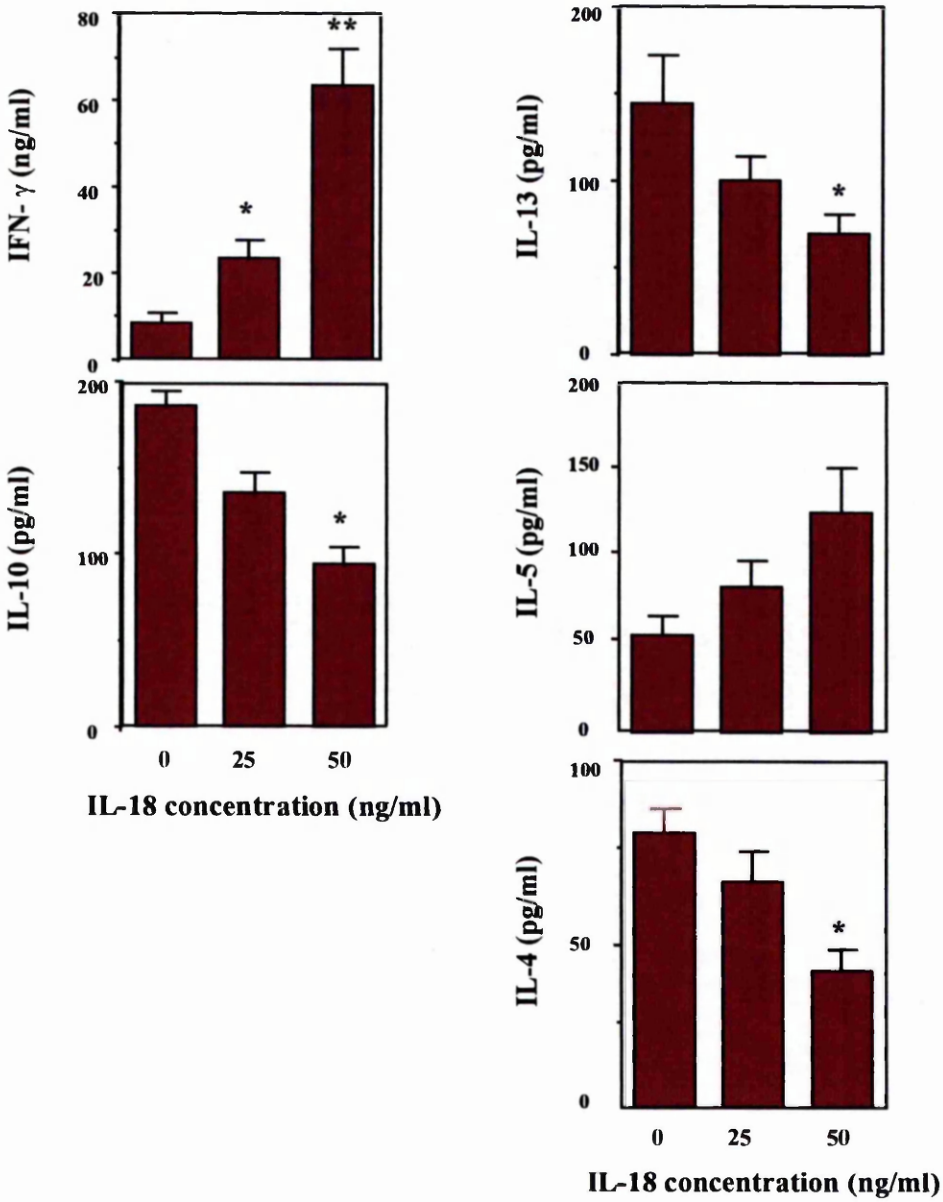


Figure 4.7 *In vitro* production of Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-10, IL-13) cytokine production by spleen cells from 13-14 week old MRL/*lpr* mice. Pooled spleen cells from 14-15 week old MRL/*lpr* mice cultured with anti-CD3 and IL-18. IL-18 induced a dose-dependent enhanced IFN-γ production (**$p<0.001$) and suppressed IL-4, IL-10 and IL-13 production (*$p<0.05$).

Discussion

IL-18 is an important cytokine in promoting Th1-mediated immune response in collaboration with IL-12. Imbalance towards Th1 predominance is associated with an acceleration of lupus-like autoimmune syndrome in MRL/*lpr* mice (Takashi et al., 1996). However, a functional role of IL-18 in clinical or murine SLE is unknown. To determine the potential pathogenic role of IL-18 in SLE, we investigated IL-18 production in SLE patients and in MRL/*lpr* mice, which develop spontaneous lupus-like autoimmune disease.

Data presented in this chapter exhibited significantly elevated IL-18 serum levels compared with controls. This result therefore suggests that the production of IL-18 may be associated with the pathogenesis of SLE.

Next we examined the relation between IL-18 serum levels and clinical manifestation. There was no significant relation between IL-18 levels and disease activity indices (SLEDAI). IL-18 did not have a significant correlation with nephropathy, C4 and C3 levels, and anti-dsDNA antibody levels. Clinical manifestations of SLE are extremely diverse and variable. Therefore, the number of patients was not enough to analyse relation between each clinical index with IL-18 serum levels. The change of each cytokine varied among the SLE patients, possibly because of the diversity in the disease and effect of drug treatments (Huang et al., 1988; Linker-Israeli et al., 1991). Thus, the serum levels of cytokines in SLE are not ever simple. Another possibility is that the IL-18, which was detected in the serum of SLE patients, was mainly pro-IL-18. Therefore, they need to be cleaved by IL-1ß-converting enzyme (ICE, caspase 1) to yield an active IL-18 (Gu et al., 1999). Therefore, environmental or unknown effects may activate this process and trigger an active disease.

Animal models have contributed to understanding the immunological aspects of immunology and autoimmune diseases. Lupus-like models are a good reflection of

human SLE because the main immunological abnormalities, which are related to the human disease also, appear in these mouse models. Therefore, to determine the potential pathogenic role of IL-18 in SLE, we investigated IL-18 production in MRL/*lpr* mice, which develop spontaneous lupus-like autoimmune disease.

Serum of MRL/*lpr* mice at the age of 4-6 months (with obvious glomerolunephritis) contained significantly higher concentration of IL-18 compared with those of control MRL/++ mice. In addition, spleen and peritoneal cells from MRL/*lpr* mice produced spontaneously higher concentration of IL-18 than cells from MRL/++ mice. These results are therefore consistent with that of clinical SLE and re-enforced a major role for IL-18 in spontaneous autoimmune disease. Then, we investigated the *in vitro* effects of IL-18 on Th1 type and Th2 type cytokine regulation from MRL/*lpr* mouse spleen cells at different stages of the disease development. *In vitro* studies showed that IL-18 and anti-CD3 were able to induce spleen cells to produce IFN-γ (Th1) and IL-5, IL-13, IL-10 (Th2 cytokines) in a dose-dependent-manner in young animals with no SLE. However, after the disease was establishmed in older MRL/*lpr* mice, IL-18 induced only IFN-γ production and down-regulated Th2 cytokine production by spleen cells. Several recent reports show that IL-18-mediated effects on T cells, in the absence of IL-12 may extend beyond Th1 differentiation to include type 2 cytokine production (Yoshimoto et al., 2000; Wild et al., 2000; Hoshino et al., 2000).

We speculate here those different effects of IL-18 on spleen cells from different age and disease of MRL/*lpr* mice is because of endogenous level of IL-12. As it was shown previously in Chapter 3, IL-12 is increased with age in MRL/*lpr* mice. Therefore at the early stages of disease, when there was low level of IL-12, IL-18 induced both Th1 and Th2 cytokines. The level of IL-12 was increased when the mice getting older, therefore synergistic effect of IL-18 and IL-12 induced higher amounts of IFN-γ that inhibits Th2 cytokines production. These data taken together strongly suggested that production of IL-18 might be associated with the pathogenesis of SLE.

Chapter 5

IL-18 accelerates autoimmune disease in MRL/*lpr* mice

Introduction

The foregoing data in Chapter 4 clearly indicated an association between IL-18 and SLE, and suggested a potential role for this cytokine in the pathogenesis of this disease. This potential role was next investigated *in vivo* using appropriate animal models. Several experimental models, which closely resemble human SLE, have been characterised as a means to understanding the pathophysiology and identifying new strategies for treatment.

Murine SLE is a model of human SLE because most of the immunological abnormalities fundamental to the human disease also appear to be operative in the mouse (Theofilopoulos and Dixon, 1985). There are many advantages in using lupus mice to study the disease development.

Firstly, lupus mice start to develop clinical disease at well-defined ages. This offers opportunities to study any existing defects in the mice, not only during but also before development of the clinical disease.

Secondly, variations in clinical presentation, diagnosis and treatment, as well as the effects of drug therapy may complicate the pathogenesis of human SLE. These are avoided in the mouse model.

Thirdly, some lupus mouse models like MRL/lpr are inbred mutant strains. These mice are genetically identical however, there may be some minor individual variation due to environmental influences or the stochastic process of mouse generation.

In addition, although most of the serological and histopathological abnormalities are common to all SLE mouse models, the onset of the disease differs considerably between strains. These strain-dependent differences in the onset of disease allow

analysis of the relationship between disease kinetics and those immunological or physiopathological changes identified experimentally.

Thus, understanding the pathogenesis and aetiology of murine lupus should lead to a better comprehension of the human disease and of autoimmunity and immuno-regulation in general.

The aim of this chapter was to investigate the role of IL-18 in the evolution of the disease process of SLE in MRL/*lpr* mice. To generate sufficient IL-18, a recombinant murine IL-18 was produced and after analysing the bioactivity, it was used for *in vivo* studies. The aims of these *in vivo* studies were to examine the role of IL-18 in:

a) disease development including kidney pathology, vasculitis and skin lesion.

b) serum autoantibody profiles.

c) immune-complex deposition in the kidney.

d) Th1 & Th2 cytokine production.

5.1 Production and bioactivity of recombinant murine IL-18

Recombinant murine IL-18 (rmIL-18) was produced by *Escherichia coli* M15 (Qiagen) transfected with a pQE-30 expression vector (Qiagen, Dorking, UK) carrying an insert encoding IL-18. This was generated in collaboration with Dr. Xiao qing Wei (Dept. of Immunology, University of Glasgow). The primer set pairs (Genosys, Cambs, UK) used to clone IL-18 from the cDNA of J774 cells was:

Sense GACACCATGGGCCGACTTCACTGTACAACCGC

Antisense CCTAAGATCTATGTAAGTTAGTGAGAGTGA

The protein was extracted under native conditions following induction with isopropyl-D-thiogalactoside (Bioline, London, UK) and purified as a 6 x histidine tagged fusion protein using a nickel agarose purification system (Qiagen). It was purified according to the manufacturer's recommendation with some modification. Purity was assessed by SDS-PAGE electrophoresis and Coomassie blue protein staining which showed a single band at 19 kDa (Figure 5.1).

Recombinant IL-18 bioactivity was analysed by its ability to induce IFN-γ production. Murine spleen cells were cultured in micro-plates pre-coated with anti-CD3 antibody (2 μg/ml) and with rmIL-18 at different concentrations. The supernatant was collected after 48 hours and the level of IFN-γ was measured by ELISA (Figure 5.2). Recombinant IL-18 induced significant IFN-γ production in a dose-dependent manner. IL-18 used for *in vivo* studies were endotoxin free as showed by the limulus amoebocyte assay (Sigma). In some experiments, murine IL-18 purchased from Pepro Tech EC Ltd (London, UK) was used.

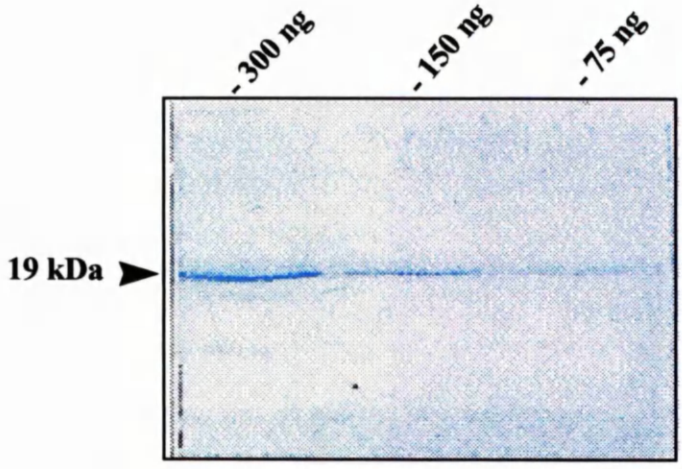


Figure 5.1 The purity of recombinant IL-18. The purified rIL-18 was assessed by SDS-PAGE electrophoresis and stained with Coomassie blue, which showed a single band at 19 kDa (arrow).

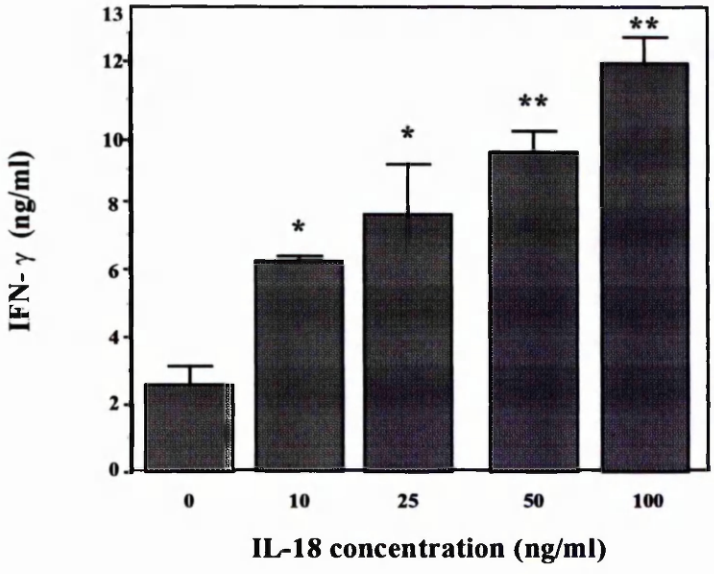


Figure 5.2 Bioactivity of recombinant murine IL-18. Murine spleen cells from 6-week-old BALB/c mice were cultured in 96-well plate pre-coated with anti-CD3 antibody. The cells were stimulated with increasing concentrations of rmIL-18 (10-100 ng/ml). Recombinant IL-18 induced IFN-γ production in a dose-dependent manner. Data are mean ± SEM (n=3, *$p<0.05$, **$p<0.001$ compared with control no IL-18).

5.2 Recombinant IL-18 accelerates autoimmune disease in MRL/*lpr* mice

To investigate the role of IL-18 in the induction and development of autoimmune disease, this cytokine was administered to lupus MRL/*lpr* mice and the onset of disease monitored. Numerous experimental models, which closely imitate human SLE, have been characterised as a means to understanding disease pathophysiology and identify new treatment strategies. The three animal models that have been studied in greatest detail are MRL/*lpr* mice, NZB/W F1 mice and BXSB mice. MRL/*lpr* mice were used in this study because: 1) MRL/*lpr* mice develop a spontaneous autoimmune disease and have been used extensively as a model for clinical SLE. 2) It has already been shown by our group that rm-IL-12 accelerated disease in MRL/*lpr* mice (Huang et al., 1996). 3) At the time I planned to start rmIL-18 treatment, MRL/*lpr* mice were the only available murine lupus strain that could be obtained commercially.

Preliminary experiments were performed to define the appropriate doses for *in vivo* studies. The initial doses of cytokines in one preliminary experiment were chosen according to our *in vitro* experiments and the experience of our group (Huang et al., 1996). Preliminary experiment was performed on four groups of mice (n=5). The IL-18 group was treated with 500 ng/mouse/day of rmIL-18 intraperitonealy (i.p), the IL-12 group was treated with 250 ng/mouse/day i.p of IL-12, while the third group was treated with a combination of IL-18 (500 ng/mouse/day) and IL-12 (250 ng/mouse/day). Finally, the control group was treated with the same volume of PBS alone. After 5 days, all the mice in the IL-12 + IL-18 group showed weight loss, diarrhoea and muscle wasting and one of the mice died. High concentration of IFN-γ was detected in the serum of these mice. The rest of these mice recovered in three days after stopping the treatment the surviving mice. Therefore, the dose of IL-12 was reduced to 100 ng/mouse/day and it was well tolerated.

The final protocol was as follows (Figure 5.3): 30 young (4 week old) MRL/*lpr* mice were divided randomly into three groups. One group was given daily intraperitoneal injections of recombinant IL-18 (500 ng/mouse/day) for 60 days. The second group of mice was injected with the same volume (100 µl/mouse/daily) of PBS diluent. A third group received a combination of rmIL-18 (500 ng/mouse) and rmIL-12 (100 ng/mouse/day), because IL-12 is a major trigger for IFNγ-dependent renal injury in MRL/*lpr* mice (Huang et al., 1996; Schwarting et al., 1999). Moreover, IL-18 synergies with IL-12 in IFN-γ production (Robinson et al., 1997).

The same protocol was used with wild-type MRL/++ as control group (5 mice in each group).

Glomerulonephritis is a severe complication of the renal involvement which is the major cause of pathology and death in SLE (Theofilopoulos, 1992; Corrien et al., 1985). The onset and progression of SLE was assessed by renal function as measured by grade of proteinuria. Proteinuria were monitored daily using commercial dipsticks and graded semi-quantitatively (0, trace, +1, +2, +3, +4). Mice were sacrificed when they were 12-13 week old and the histopathology of the kidney was examined.

A score of 2 + or greater was considered indicative of severe proteinuria, and mice exhibiting severe proteinuria on three or more successive occasions or at the final evaluation before sacrificing were considered positive for renal disease.

The effect of treatment on development of SLE is shown on Figure 5.4. Control MRL/*lpr* mice injected with PBS developed the expected spontaneous disease; proteinuria appeared at around 9 weeks and progressed steadily throughout the study period. MRL/*lpr* mice treated

with rmIL-18 developed accelerated proteinuria compared with the PBS group. Significant divergence occurred as early as 32 days after treatment compared with the PBS and the IL-18 + IL-12 groups (Figure 5.4a).

The mice treated with a combination of rm-IL-18 and rmIL-12 developed proteinuria, which was indistinguishable from that of the PBS group until day 43 after treatment. Thereafter, the level increased rapidly at a similar rate to the IL-18 treated group (Figure 5.4 a).

At the end of treatment, day 60, the rmIL-18 group and the IL-18 + IL-12 group had significantly greater proteinuria (renal disease) than the PBS control group ($p<0.008$, Log rank test, Kaplan-Meier survival analysis). There were no significant differences between IL-18 and IL-18 + IL-12 group. None of these cytokine treatments affected the renal function of wild-type MRL/++ mice (Figure 5.4b).

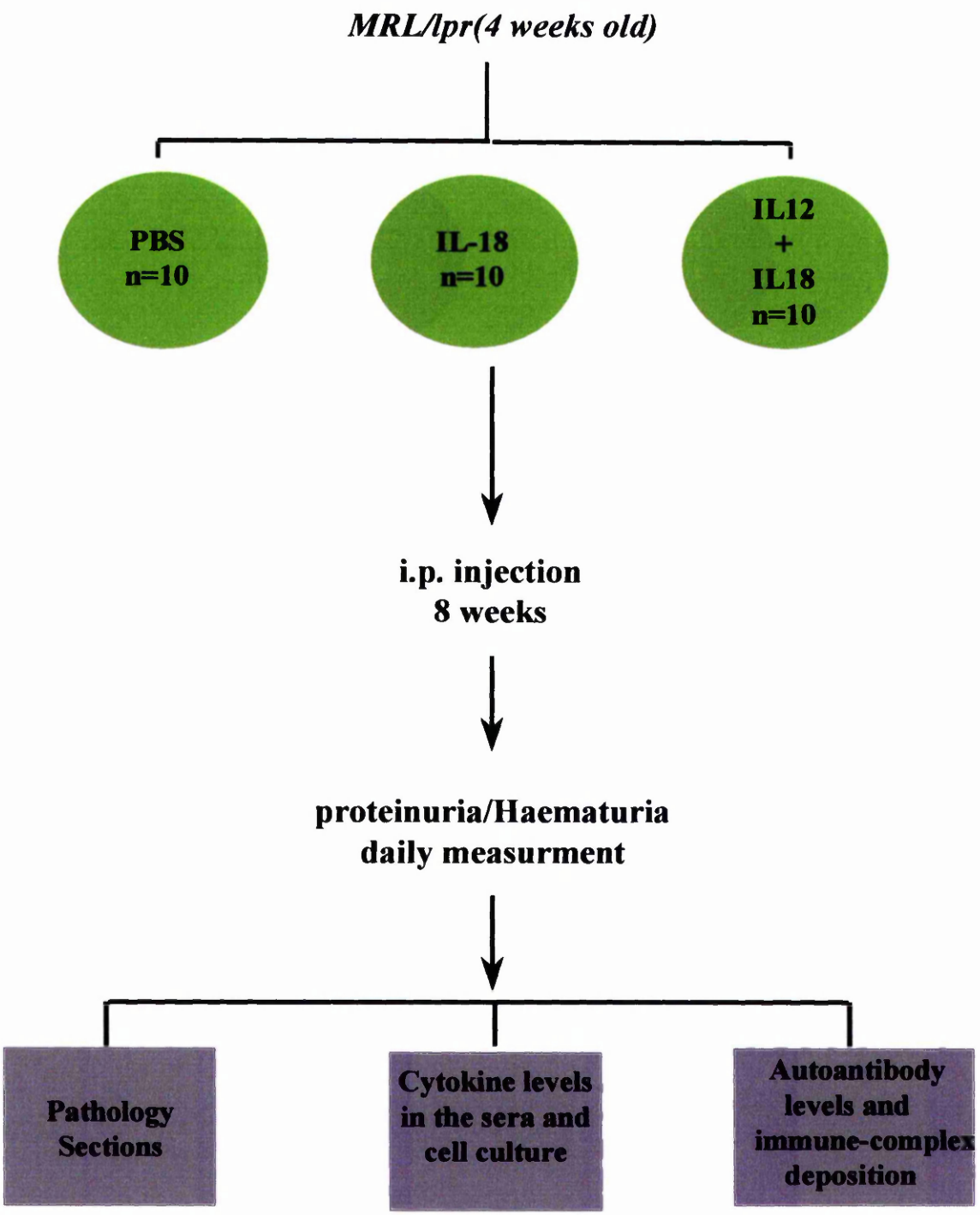


Figure 5.3 The protocol used to assess the role of rmIL-18 in the development of lupus disease in MRL/*lpr* mice.

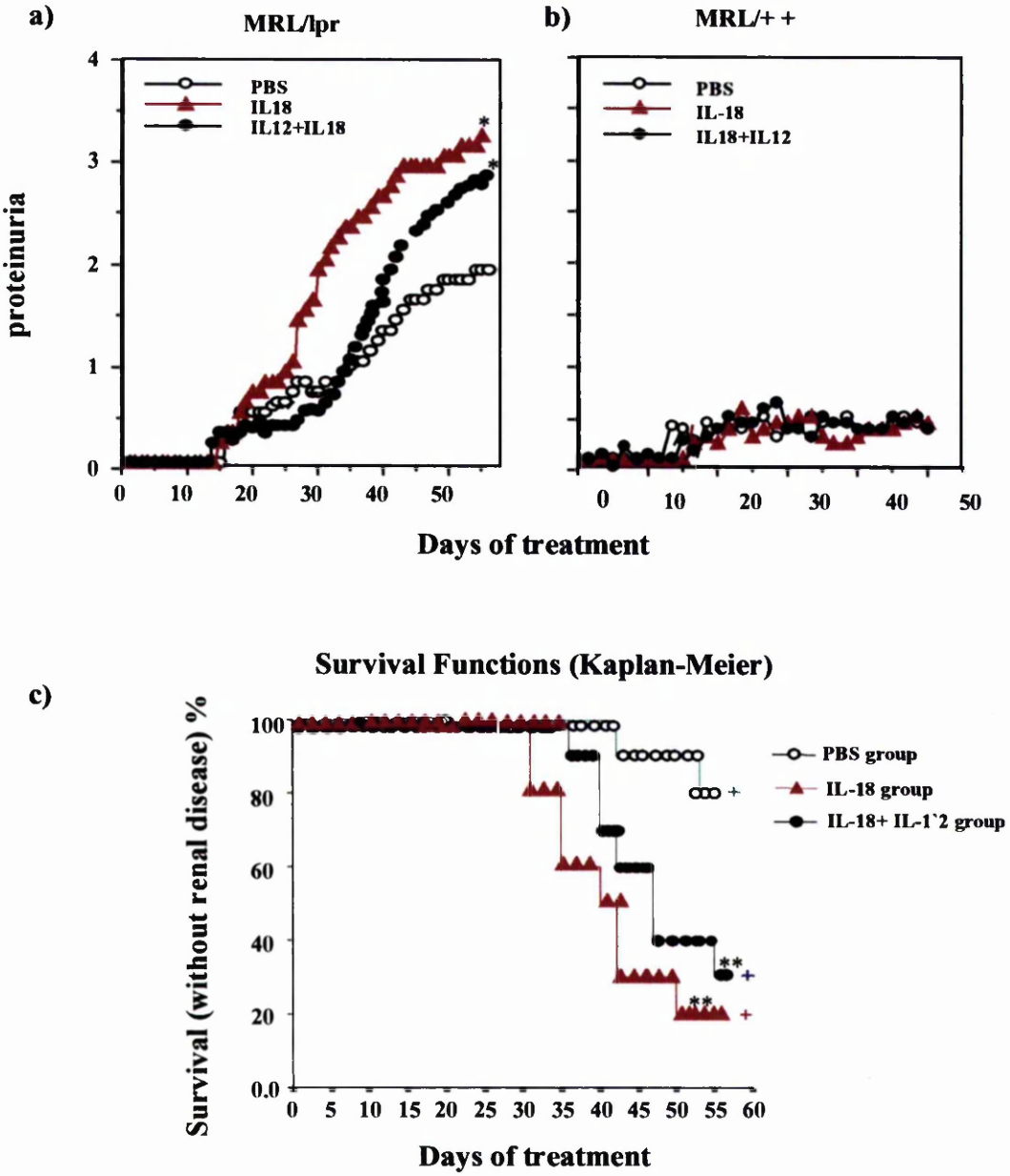


Figure 5.4 The onset and progression of SLE was assessed by renal function: grade of proteinuria. Proteinuria was measured daily in MRL/*lpr* mice and MRL/++ control mice treated with daily i.p. injections of PBS (controls) or rmIL-18 or combination of rmIL-18 and rmIL-12.

(a) At the end of experiment both IL-18-treated mice and IL-12 plus IL-18-treated mice had significantly higher proteinuria than the PBS controls (*$p<0.05$, ANOVA).

(⟶ Figure 5.4 legend continued):

(b) Recombinant IL-18 or a combination of rIL-12 and rIL-18 had no effect on renal function of MRL/++ mice.

A score of greater than 2 + was considered indicative of severe proteinuria, and development of severe proteinuria in treated mice followed for 56 days. Mice exhibiting severe proteinuria on three or more successive occasions or at the final evaluation before sacrificing were considered positive for renal disease. The differences in disease frequency are statistically significant (Kaplan-Meier, Log-Rank: **$p<0.008$).

5.3 Histological analysis of the kidney after administration of rmIL-18

After 60 days treatment (12-13 weeks of age) mice from different groups were sacrificed and the kidneys resected. Kidneys were bisected then fixed in neutral buffered formalin and embedded in paraffin wax. Haematoxylin and eosin (H & E) stained sections were coded and the severity of glomerulonephritis was graded on an arbitrary five-point scale by an experienced histopathologist (Dr. GBM Lindop, Department of Pathology, Glasgow University), on two different occasions. Kidney damage was assayed by the " kidney pathology index" (KPI). Sections from both halves of the kidney were also screened for vasculitis and rated positive or negative. All sections were assessed blind, without the knowledge of the experimental group to which the animals belonged.

All mice at different stages of the disease showed typical lupus nephritis including segmental and global mesangial hypercellularity increased mesangial matrix, some capillary inflammatory cells, apoptotic bodies and tuft-to-capsule adhesions. The most severely damaged tissue also contained fibrin deposits, focal and segmental necrosis and crescents. Typical examples are shown in Figure 5.5.

The group of MRL/*lpr* mice treated with rmIL-18 showed significantly more severe glomerulonephritis compared with the PBS control group (mean ± SEM, 3.9 ± 0.27 vs. 2.9 ± 0.23, $p<0.03$, Figure 5.6a). Figure 5.6a also shows that the rmIL-18 + rmIL-12 group had more severe kidney involvement than the PBS group (4 ± 0.33, $p<0.02$, Mann-Whitney U-test). There were no significant differences between the IL-18 and the IL-18 + IL-12 groups. The histopathology results were compatible with the proteinuria levels in the different groups of the mice. The cytokine treatment had no pathological effects on the kidney of wild-type MRL/++ mice (Figure 5.6b).

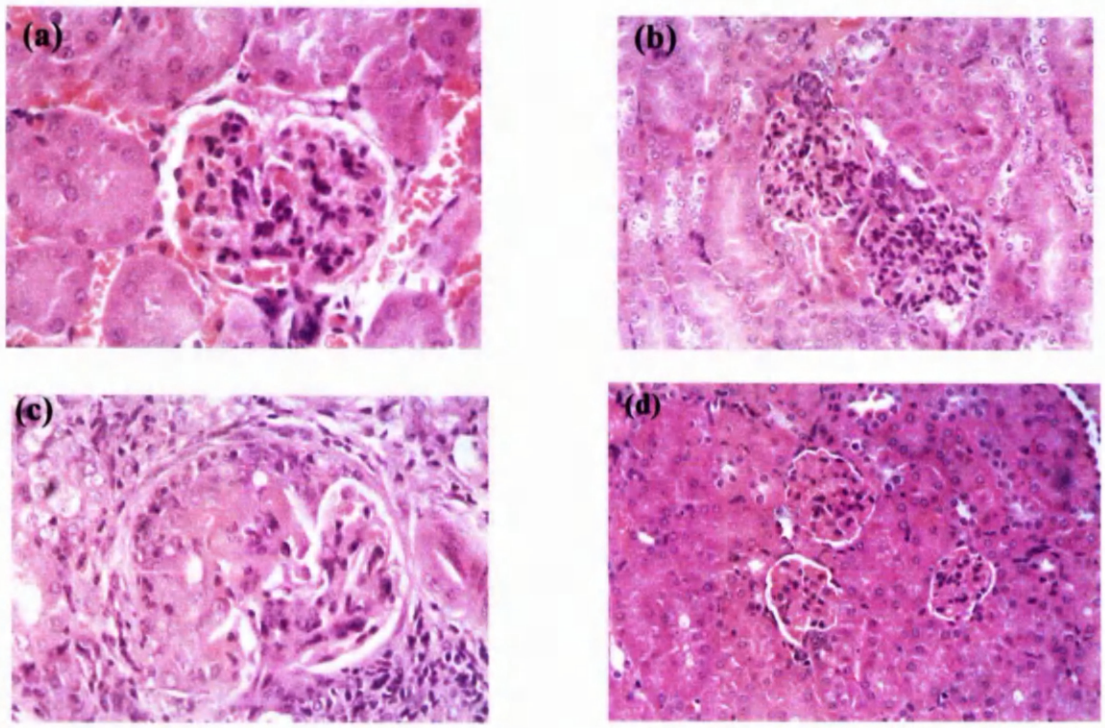


Figure 5.5 Photomicrograph of kidney sections from different treated groups of MRL/*lpr* mice. Haematoxylin and eosin stained section representing:

(a) kidney involvement in PBS treated control group. This shows an enlarged glomeruli with global mesangial hypercellularity and containing a few inflammatory cells (grade 3 glomerulonephritis).

(b) representing the rmIL-18 treated group showing global mesangial hypercellularity, more inflammatory cells, increased mesangial matrix and tuft-to-capsule adhesions (glomerolunephritis grade +4).

(c) representing the rmIL-12/rmIL-18 treated group showing a large glomerulus with addition focal necrosis, apoptotic bodies and a large crescent (grade 5 glomerulonephritis).

(d) representing MRL/++ control mice which were treated with IL-12/IL-18 and at the end of experiment showing a grade 1 of glomerulonephritis.

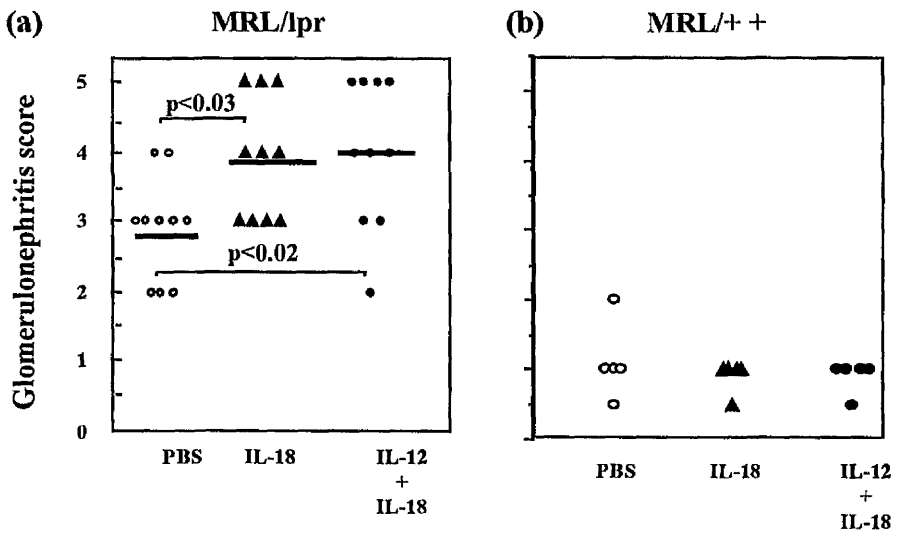


Figure 5.6 A quantitative estimate of kidney histopathology in different treated groups of (a) MRL/*lpr* mice and (b) MRL/++ mice. At the end of experiment (day 60), kidneys were removed, formalin-fixed and H & E sections were prepared. Histologic appearances in kidney were blindly scored (0-5) by an experienced pathologist on two different occasions. The mean of each group is presented by a line. (p value calculated by Mann-Whitney U-test). (b) There was no pathological effect on the kidney of wild-type MRL/++ mice.

5.4 Vasculitis after administration of rmIL-18

One of the clinical manifestations of human SLE and lupus mouse models is vasculitis. Vasculitis in MRL/*lpr* mice involving mostly medium-sized arteries of the kidney, genital organs and heart (Andrews et al., 1978). Sections from both halves of the kidney were assessed for vasculitis and rated positive or negative

Figure 5.7a shows that all the mice treated with a combination of rmIL-18 and rmIL-12 had vasculitis; 6 out of 10 of the mice treated with IL-18 had vasculitis. In the control PBS group just 4 of 10 mice had vasculitis (Figure 5.7a). The rmIL-18 + rmIL12 treated group had significantly more vasculitis than PBS ($p<0.001$, Fisher test). There was no significant difference between PBS and IL-18 treated groups.

Vasculitis predominantly affected the origins of the radial arteries in the deep cortex. There was fibrinoid necrosis in the arterial media and prominent periarteritis (Figure 5.7b). These changes occurred in a background of focal chronic pyelonephritis and lymphoproliferative disease that occurs spontaneously in this model. Figure 5.7c shows a positive immunofluorescent staining for IgG in similar kidney with severe vasculitis from a mouse treated with IL-12 plus IL-18 shown in the Figure 5.7b.

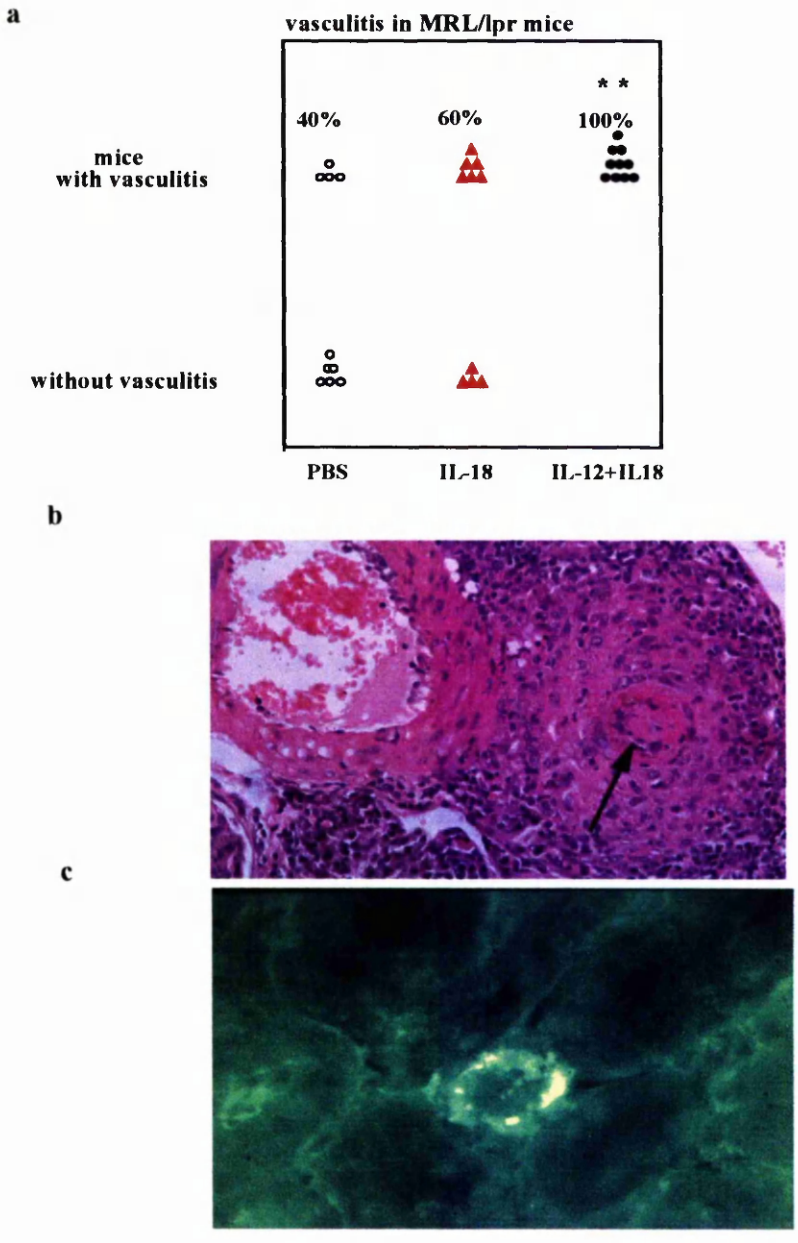


Figure 5.7 Vasculitis in different groups of treated MRL/*lpr* mice.

(a) All the mice treated with a combination of rmIL-18 and rmIL-12 had some vasculitis (**$p<0.001$,*$p<0.05$, non-parametric Fisher test). (b) This H & E stained section from a mouse treated with rmIL-12 plus rmIL-18 shows a large renal artery and a smaller radial artery (arrow) with vasculitis and florid periateritis (c) Positive immunofluorescent staining for IgG in similar kidney section in Figure 5.7b.

5.5 IL-18 treated MRL/*lpr* mice developed lupus-associated facial rash

About a third of patients with SLE have cutaneous involvement that commonly takes the form of a photosensitive erythematosus rash with malar distribution described as a butterfly rash. There are no reports of a similar facial skin lesion in MRL/*lpr* mice resembling the malar rash of human SLE.

The PBS control group did not show any skin lesion (Figure 5.8a). Six of 10 MRL/*lpr* mice injected with rmIL-18 developed skin rashes, most prominently on the malar region of the whisker pad (Figure 5.8b). This occurred after 34 days treatment and with variable severity. MRL/*lpr* mice treated with the combination of rmIL-18 and rmIL-12 showed no facial lesions despite developing more severe glomerulonephritis and vasculitis (Figure 5.8c). The facial skin lesions in the IL-18 treated mice peaked around 9 weeks old and did not disappear by the time the mice were sacrificed. None of the treated wild-type MRL/++ mice developed skin lesions (pathology analysis in chapter 6).

There are other skin manifestations which spontaneously develop in MRL/*lpr* mice and these have been evaluated as a dermatological model of human systemic lupus erythematosus (Furukawa et al., 1982; Horiguchi et al., 1984). The skin lesions are mainly on the ears, back and abdominal skin of the mice, and do not appear before 14 week age (Furukawa et al., 1984).

At the age of 12 weeks when the mice were sacrificed about 50% of the IL-18 treated MRL/*lpr* mice (5 of 10 mice) had lesions on the ears, back and abdominal skin, which was early for this age and some of them were very severe. However, they were the typical skin lesion of MRL/lpr mice (Figure 5.8d).

(a) (b) (c)

(d)

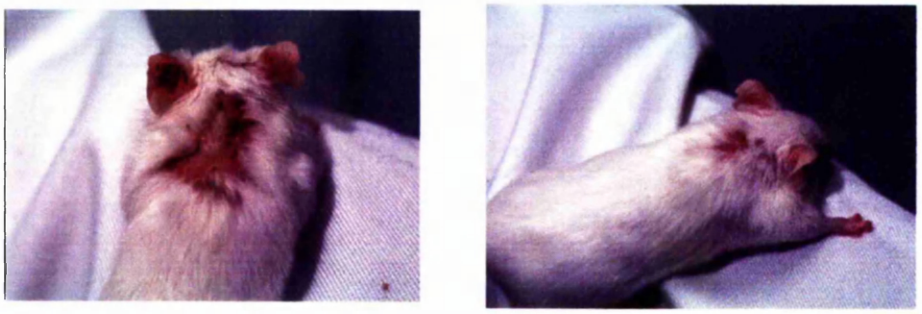


Figure 5.8 Recombinant IL-18 treatment induced a facial rash in MRL/*lpr* mice.

(a) In the PBS MRL/*lpr* control group there were no skin lesion.

(b) Six of 10 MRL/*lpr* mice after 5 weeks treatment with rmIL-18, developed spontaneous skin rashes, most prominently at the malar region of the whisker pad.

(c) MRL/*lpr* mice treated with rmIL-18 + rmIL-12 showed no sign of facial skin lesions despite developing more severe glomerulonephritis and vasculitis.

(d) At 12 weeks when the mice were sacrificed, 5 of 10 MRL/*lpr* mice in the IL-18 treated group developed skin lesions on the back, ears or abdomen.

5.6 Immune complex deposition and development of glomerulonephritis

Glomerulonephritis in SLE is believed to be due to immune complex (IC) deposition and complement activation. It is the major cause of pathology and death therefore reflecting severity of the disease. Immunofluoresence staining was performed on frozen sections of kidney from different groups of the mice to assess whether treatment with rmIL-18 and rmIL-12 had any effect on immune complex deposition.

To detect IgG deposits, the tissues were snap frozen in an isopentane bath cooled in liquid nitrogen. Frozen sections embedded in OCT (optimised cutting temperature) were cut at 5 μm thickness. Sections were then incubated with FITC conjugated goat anti-mouse IgG (Dako). The section was read blind and intensity of positive staining assessed according the number of positively stained glomeruli.

Figure 5.9 shows that stronger fluorescent staining was observed in the sections which had a higher degree of glomerulonephritis. The more severe glomerulonephritis in each group showed stronger staining for IgG deposition.

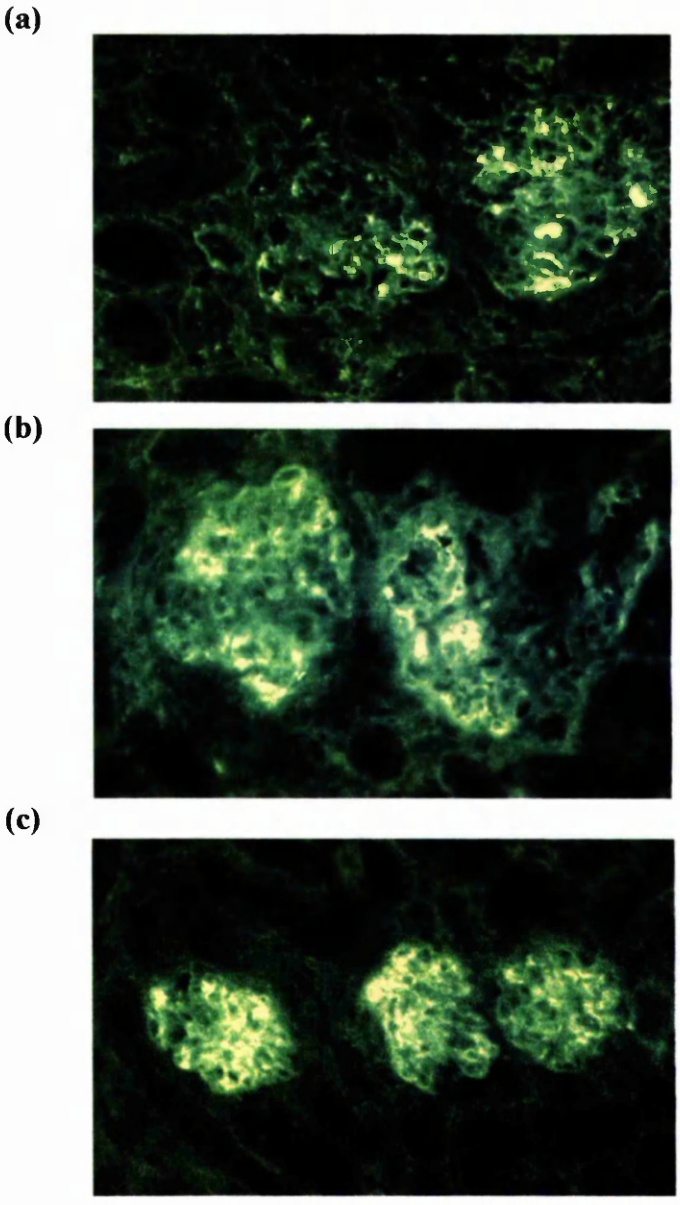


Figure 5.9 Immune complex deposition in the kidney of MRL/*lpr* mice in different treated groups. Immunofluoresence staining was performed on frozen sections of kidney in the different groups of mice.

(a) kidney with grade 2 of glomerolunephritis.

(b) kidney with grade 4 glomerulonephritis had stronger fluorescent staining than grade 2.

(c) The strongest staining was observed with more severe kidney involvement (grade 5).

5.7 Serum IgG anti-ds-DNA antibody isotypes in MRL/*lpr* mice

Measurement of anti-nuclear antibodies (ANA) has been a basic laboratory test for many years and the presence of these antibodies is a standard criterion for the diagnosis of SLE. Among the autoantibodies that are present in the serum of patients with SLE, those that bind to double-strand DNA (ds-DNA) remain of paramount interest. Many papers (Swaak et al., 1997; Spronk et al., 1995) have concluded that the levels of anti-dsDNA antibodies generally reflect clinical disease activity; though not in all patients (Gharavi et al., 1998). A pathological role for anti-ds-DNA antibodies appears to be particularly true for renal disease, and most of the evidence that anti-ds-DNA antibodies are pathogenic has been collected from studies of the kidney. Okumora and colleagues (1993) have demonstrated that disease activity is correlated with IgG against ds-DNA but not IgG against (single strand) ss-DNA or IgM against either ds-DNA or ss-DNA. As in human lupus, the lupus-like mouse models produce elevated levels of total immunoglobulins and autoantibodies (such as anti-dsDNA) and are thought to develop nephritis and arteritis as a result of deposition of immune complexes involving autoantibodies in the kidney or arteries.

Sera from different treatment groups of MRL/*lpr* mice were assessed for levels of auto-antibodies against ds-DNA (Figure 5.10). The results showed no significant differences between the groups of treated mice for the total anti-dsDNA antibody production. However, as Figures 5.10 b and 5.10c show, rmIL-18 and rmIL-12/rmIL-18 treated mice produced significantly higher level of IgG2a anti-ds DNA antibodies ($p<0.05$), which are typically produced during a Th1 response, compared with controls. Titres of anti-ds-DNA IgG1 were also elevated in IL-18 recipients compared with IL-12/IL-18 treated group (Figure 5.10c, $p<0.05$).

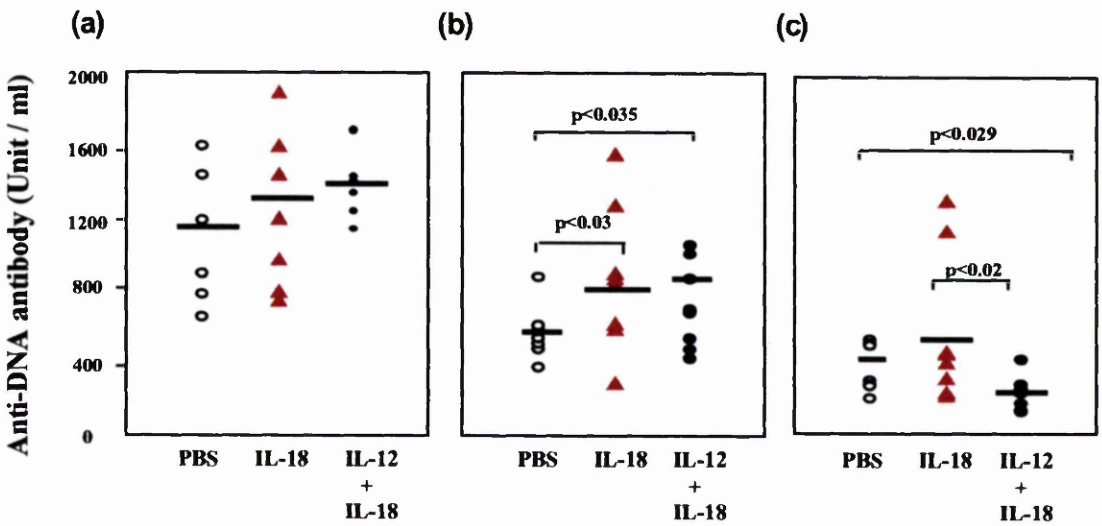


Figure 5.10 Anti-DNA antibody isotype in the serum of different treatment groups of MRL/*lpr* mice. Anti-DNA antibody isotypes, total IgG (a), IgG2a (b), IgG1 (c), were measured at the end of treatment (day 60) by ELISA. Data are expressed as arbitrary units/ml (compared with standard serum from 5 month old MRL/*lpr* mice, Mann-Whitney U-test).

5.8 Serum cytokine levels in different treated group of mice

To study the influence of IL-18 and IL-18 plus IL-12 on cytokine profiles in MRL/*lpr* mice, serum samples were analysed for the presence of IFNγ, IL-10, IL-4, IL-5, TNF-α and Nitrite / Nitrate (NO) at various time points associated with the disease. Serum cytokine levels were measured by ELISA and NO_2/NO_3 serum levels were assayed by a modified Griess method (Chapter 2).

The levels of serum cytokines and NO in control PBS treated MRL/*lpr* mice and in mice treated with rmIL-18 or a combination of rmIL-18 and rmIL-12 are shown on Figure 5.11. IFN-γ was significantly increased in the IL-12 + IL-18 group (mean ± SEM, 121.3 pg/m ± 17.8, $p<0.002$) and the rmIL-18 treated group (78.8 ± 11.3, $p<0.04$) compared with the PBS control group (45.8 ± 9.6). Although the sera of the IL-12 plus IL-18-treated mice contained more IFN-γ than the IL-18 group, the difference between them was not significant. Significant reductions of IL-10 were observed in the IL-18+IL12 group (59.6 ± 2.3, $p<0.001$) and in the IL-18 group (60.9 ± 3.2, $p<0.001$) compared with the PBS treated mice (217.2 ± 27.9). Serum IL-4 and IL-5 was undetectable in all groups of mice.

The level of TNF-α in the IL-18 plus IL-12-treated group was significantly higher than PBS group (263 ± 63.7 versus 103 ± 24.9, $p<0.041$) but the increment in TNF-α in the rmIL-18 treated group was not significant (192.5 ± 74.9) compared with the PBS treated group. A similar enhancement of NO levels was observed in the IL-12 + IL-18-treated group (93.4 ± 12.1 μM, $p<0.003$) compared with the PBS group (39.8 ± 3) but there was no significant increase in NO in the IL-18-treated mice (73.2 ± 16.8, $p<0.087$).

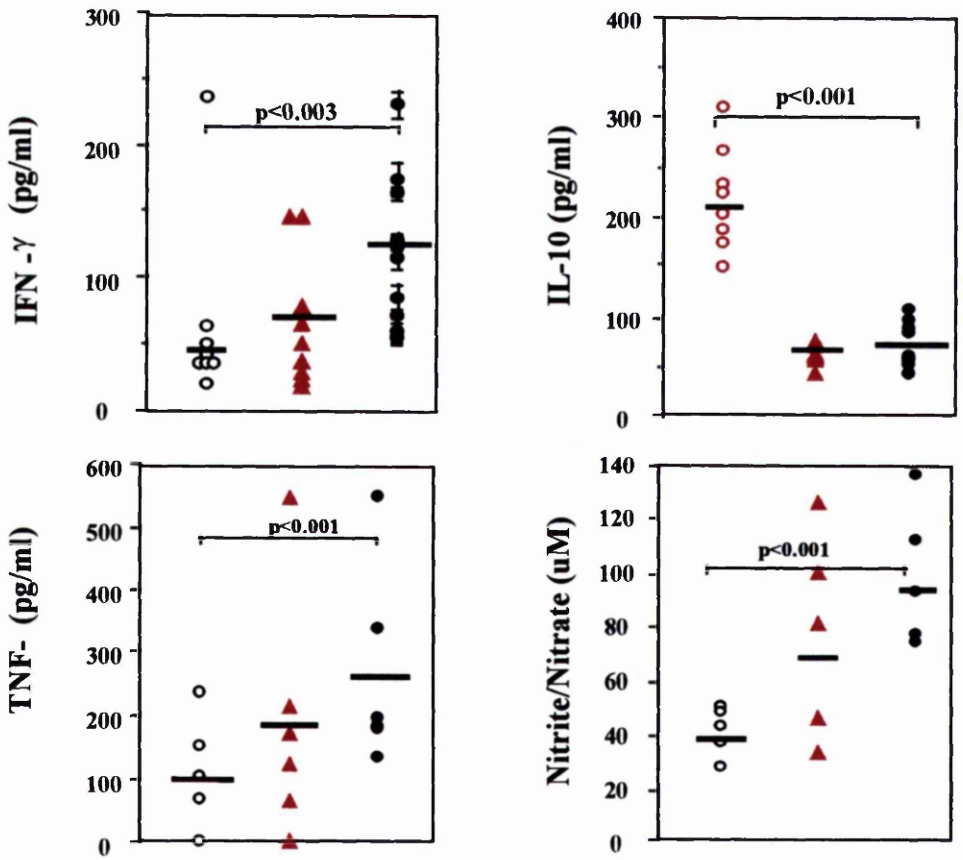


Figure 5.11 Serum cytokine and NO_2/NO_3 levels in MRL/*lpr* mice and the effect of IL-18 or rmIL-18 plus rmIL-12 treatment. Serum cytokine levels from different groups of mice were collected at the end of treatment (day 60). Serum cytokine levels were measured by ELISA and NO_2/NO_3 serum levels were analysed by a modified Griess method (p value was calculated by Mann-Whitney U-test).

5.9 Does *in vivo* rmIL-18 treatment result in a phenotypic shift of T-helper subsets.

We next determined whether the altered disease phenotype in rmIL-18 and rmIL-18 plus rmIL-12 treated mice was due to the a shift of Th1 or Th2 related cytokines. Spleen cells ($4x10^6$/ml) were harvested from mice (n=10) at the end of experiment (day 60) and stimulated with the mitogen concanavalin A (Con A). After 48 hours supernatants were collected and cytokine levels measured by ELISA.

IFN-γ Spleen cells from the IL-18-treated mice produced more IFN-γ (mean ± SEM, 2890 ± 98 pg/ml, $p<0.05$) than control mice (1627 ± 78 pg/ml) in response to Con A (Figure 5.12). Synergistic enhancement of IFN-γ production by spleen cells was evident in IL-18/IL-12 treated mice (5720 ± 390 pg/ml, $p<0.001$).

IL-10 Spleen cells from the IL-18-treated mice produced less IL-10 (59 ± 16pg/ml, $p<0.001$) than control mice in response to Con A, indicating suppression of Th2 responses (Figure 5.12). There were no significant differences between the rmIL-18 treated group and the rmIL-12/rmIL-18 treated mice (71 ± 18 pg/ml) for IL-10.

IL-5 As Figure 5.12 shows, the IL-18 group was not significantly differ from the PBS group in IL-5 production. IL-5 production was further and significantly inhibited by IL12/IL-18 treatment (mean ± SEM, 25 ± 2 pg/ml). IL-4 was undetectable in all treated groups.

TNF-α TNF-α production was significantly increased in IL-12/IL-18 treated mice (375 ± 30, $p<0.05$) but there were no significant differences between the levels in the IL-18 treated group (260 ± 19 pg/ml) and control mice (185 ± 25).

After sacrifice, peritoneal cells from each MRL/*lpr* cytokine treatment group were pooled and stimulated with LPS and IFN-γ to assess NO_2 and IL-18 production. As Figure 5.13a shows peritoneal cell from IL-18 (mean ± SEM, 62±10 μM, $p<0.05$) and IL12 plus IL-18 treated mice (79 ± 11μM, $p<0.05$) produced significantly more NO than PBS group (34 ± 9 μM). IL-18 and IL-12 / IL-18 group spontaneously (without stimulation) produced more IL-18 than PBS treated mouse (Figure 5.13b, $p<0.05$).

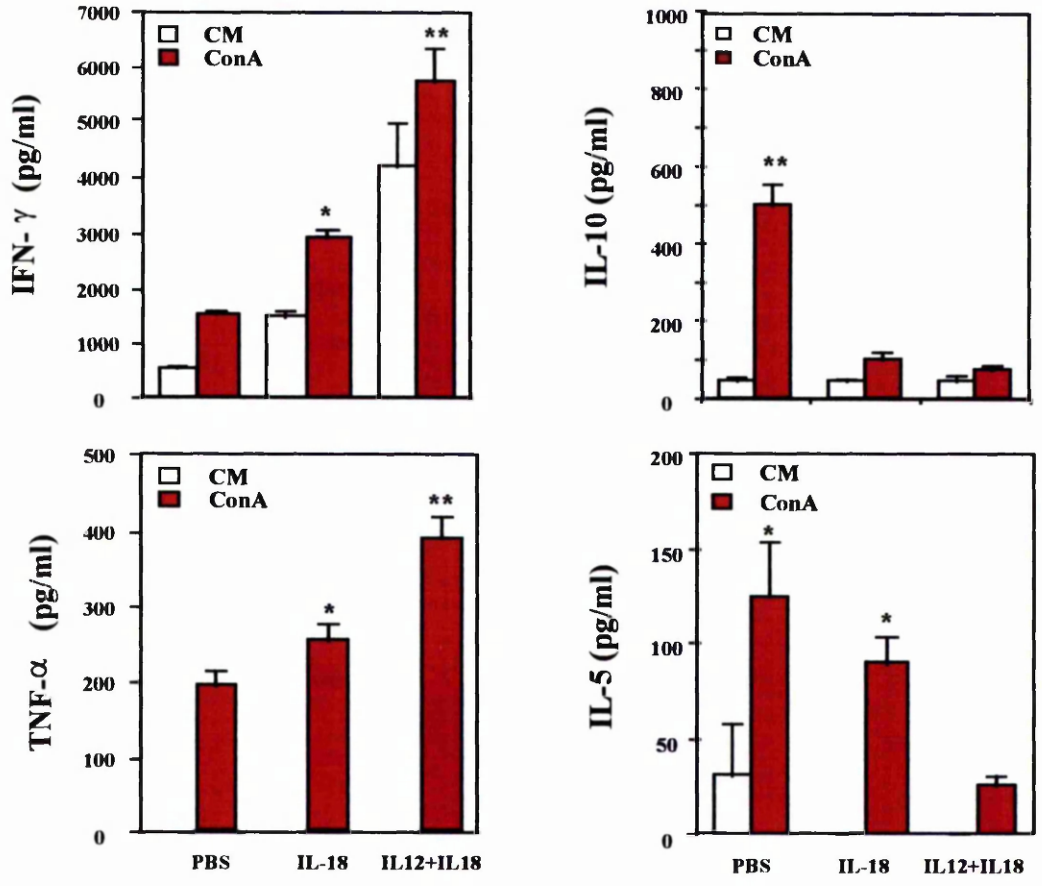


Figure 5.12 Th1 & Th2 related cytokine production by spleen cells from MRL/*lpr* treated mice. Pooled spleen cells were harvested from different treated groups (5 mice in each group) and stimulated with concanavalin A (Con A) or culture medium (CM) control. Cytokine levels in the culture supernatants at 48 hours were measured by ELISA. IL-4 was not detected in any of the supernatants.

Data are mean ± SEM of triplicate cultures (*$p<0.05$; **$p<0.001$, by Student's t-test).

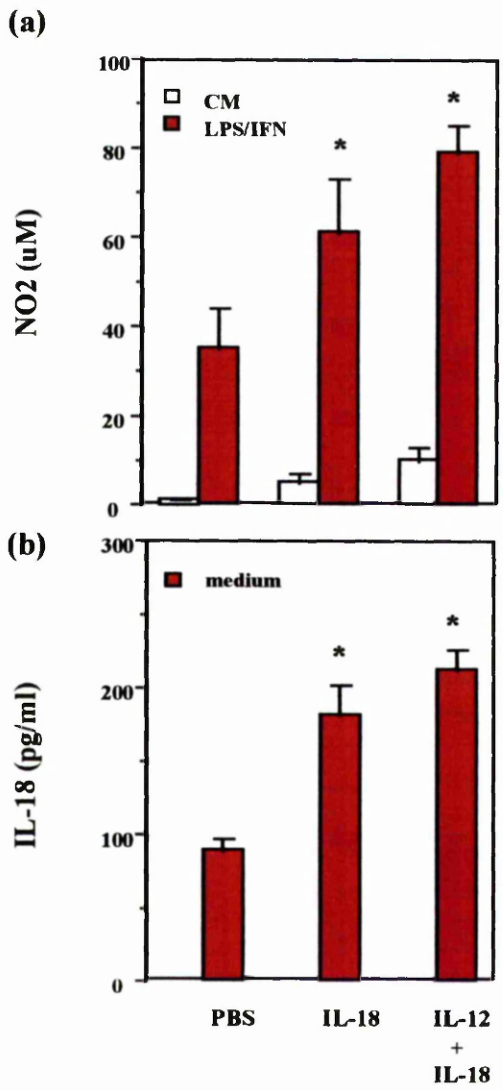


Figure 5.13 NO_2 and IL-18 production by peritoneal cells from MRL/lpr treated groups. Pooled peritoneal cells from different groups (5 mice in each group) were cultured with or without LPS and IFN-γ. Culture supernatants were collected after 72 hours. IL-18 was measured by ELISA and NO2 measured by Griess reaction.
(Data expressed by mean ± SEM (*$p<0.05$, Student's t-test).

5.10 Cytokine levels in the serum and spleen culture supernatants from MRL/++ treated control groups

MRL/*lpr* mice differ from the MRL/++ mice in the impairment of transcription of the gene encoding Fas antigen. Therefore, MRL/++ mice is a very ideal control group for MRL/*lpr* mice experiment.

The level of IFN-γ in the IL-12 + IL-18 treated group was higher than PBS control group (165 ± 21 versus 82 ± 28, $p<0.05$). There were no significant differences between the IL-18 group (110 ± 16) and the PBS group (Figure 5.14a). The level of IL-10 in the IL-12 + IL-18 group was significant decrease ($p<0.002$), (mean ± SEM, 32 ± 5 versus 108 ± 14) compared with PBS treated mice. IFN-γ was not significantly different between the PBS and the IL-18 group (Figure 5.14a). IL-4 and IL-5 levels were undetectable in the serum of the MRL/++ mice.

Pooled spleen cells from each group of treated MRL/++ mice (5 in each group) were cultured with or without Con A (5 μg/ml). Culture supernatants were collected after 48 hours to assess cytokine production by ELISA. Cells from the IL-12/IL-18 treated mice produced more IFN-γ than the PBS control mice (mean ± SEM: 890 ± 46 vs. 310 ± 15 ng/ml). There was no significant difference between the IL-18 group and the PBS group. IL-12 / IL-18 treatment significantly suppressed IL-10 production by MRL/++ spleen cells ($p<0.001$, Figure 5.14b).

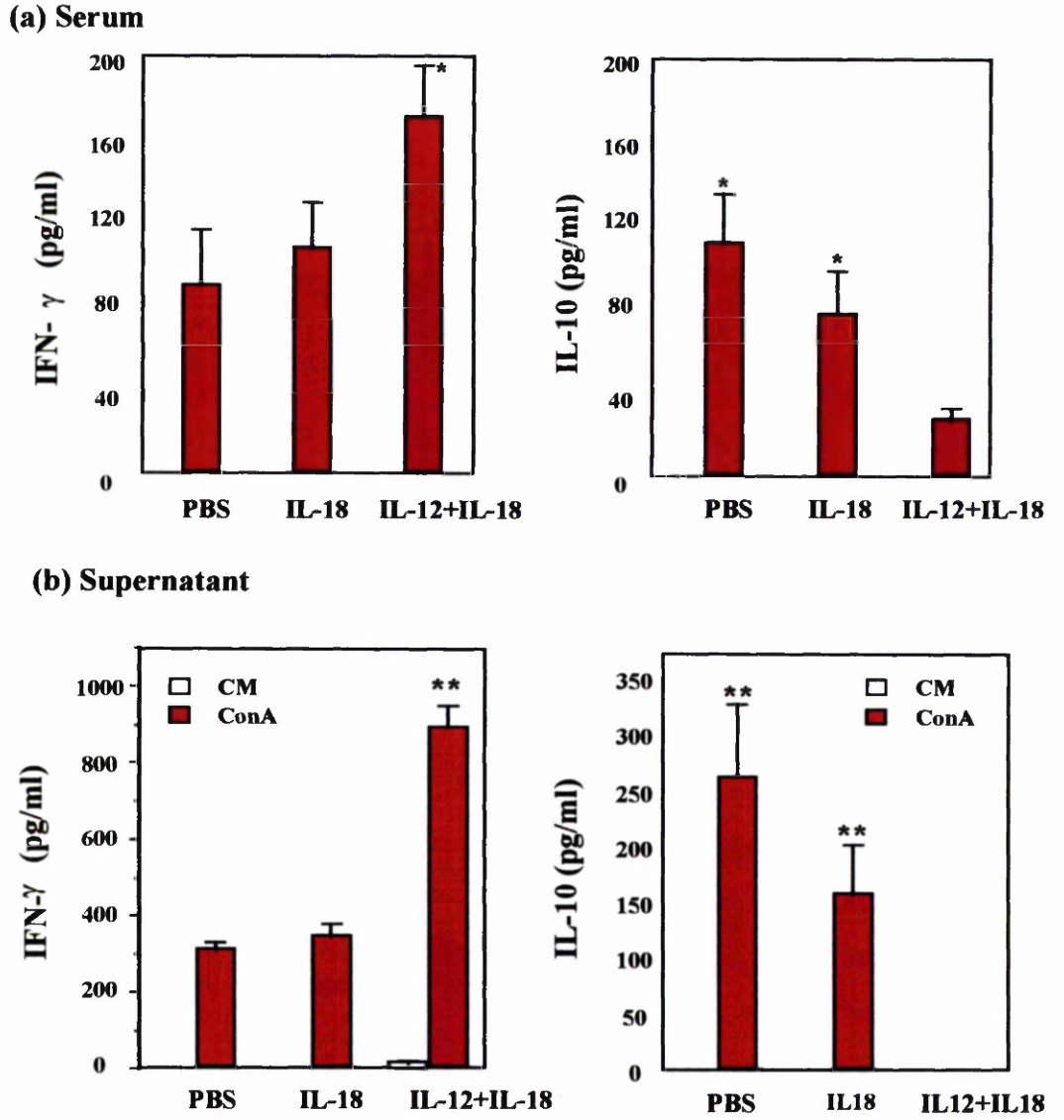


Figure 5.14 Cytokine levels in the serum and culture supernatants of MRL/++ mice. (a) Pooled sera (n=5) were collected at the end of treatment (day 60) and cytokines analysed by ELISA. Data are mean ± SEM (*$p<0.05$ and, Mann-Whitney). (b) Spleen cells were collected and pooled (n=5) from different treated groups and cultured with Con A for up to 48 hours. Cytokines were measured by ELISA. IL-4 and IL-5 were undetectable in culture supernatants. Data are expressed as mean ± SEM (**$p<0.001$, Student's t-test).

Discussion

The role of IL-18 in systemic autoimmunity was investigated here by studying the effects of a recombinant IL -18 on the development of autoimmune disease in the (MRL/*lpr*) murine model of SLE. We tried to stimulate constitutive IL-18 levels by daily intraperitoneal injection of rmIL-18, intraperitoneally, into young MRL/*lpr* mice. Our data suggest that rmIL-18 promotes the spontaneous development of lupus-like glomerulonephritis, vasculitis and skin lesions. Recombinant IL-18 also induced a facial rash, resembling malar rash in human SLE, at the early stage of the disease. This acceleration of the disease occurred in association with marked changes in the Th1 and Th2 cytokines and changes in the IgG subclasses rather than in the total levels of anti-DNA antibodies. This was accompanied by a similar modulation of the IgG subclass of T cell-dependent antibody responses, but not T cell-independent antibody responses, suggesting a causal link between IFN-γ, up-regulation of Th1 autoimmune responses, and acceleration of SLE disease.

Although polarised T helper 1 (Th1) responses have been implicated in the pathogenesis of organ-specific autoimmune diseases, little is known about the role of cytokines produced by Th-cell subsets in the development of auto-antibody mediated disease. Because Th2 cytokines are associated with B-cell help, the pathogenic effect of auto antibodies in SLE favours the theory that a humoral Th2 response is responsible for the disease rather than a cell-mediated Th1 response (Horwitz et al., 1998; Funauchi et al., 1998). However, despite the popular notion that a predominant Th2 phenotype is essential for such disease, recent evidence suggests that CD4+ T cells involved in SLE might also induce a Th1 subset (Takahashi et al., 1996; Reininger et al., 1996).

IL-18 has been identified as a critical regulatory factor in the evolution of Th1 immune responses, usually acting in synergy with IL-12 (Takeda et al., 1998). At the end of experiment, in the serum of IL-18 treated mice IFN-γ increment and IL-10 decreasing were noticed. IL-18 had a synergistic effect with IL-12 when injected i.p. in IFN-γ production. Despite higher level of IFN-γ by IL-12/ IL-18 group, they did not show significantly more severe glomerulonephritis than the groups treated only with IL-18. It shows that the mechanism by which SLE disease prompted is unlikely to be just due to Th1 response. Proteinuria in IL-18 treated group began earlier than the IL-12 / IL-18 group, and at the early stages of treatment IL-12/ IL-18 group shows less proteinuria even less than PBS control group. The isotype, of anti-DNA antibody response in IL-18 recipients was not restricted to IgG2a, suggesting effects beyond Th1-mediated B cell help. These data suggest *in vivo* effects for IL-18 in the development of glomerolunephritis in the lupus-like model that extend beyond T cell differentiation.

From these data it might be speculated that at the early stages of the disease a strong Th1 response could delay proteinuria in IL-12 / IL-18 treated mice. Possibly because a strong Th1 response caused more Th2 suppression and it seems that Th2 cytokines are necessary mainly at the early stages of the disease to help auto-reactive B cells to produce autoantibody. However, the amount of IFN-γ induced by combination of rmIL-12 and rmIL-18 was not enough to suppress Th2 response completely. Therefore, they still produced anti-DNA antibodies but less than the two other groups. However, when autoantibodies reached to a threshold level, a stronger Th1 response, which is necessary for destruction of the target tissues, accelerated the disease.

The isotype, of anti-DNA antibody response in IL-18 recipients was not restricted to IgG2a, suggesting effects beyond Th1-mediated B cell help. The reason that why in IL-18 treated mice the proteinuria appeared earlier than IL12/IL-18 group might be explained by the hypothesis that IL-18 can induce Th2 cytokines in the absence of endogenous IL-12 at the early stages of the disease (Figure 3.1). Therefore, IgG anti-DNA antibodies were produced in larger amounts at the earlier stage of disease in IL-18 treated mice, and when the level of endogenous IL-12 rose in an synergistic effect, it was switched to more Th1 (IFN-γ) response which is necessary for organ destruction. This theory is consistent with the data showing both Th1 (IFN-γ) and Th2 (IL-4) cytokines are necessary in the pathogenesis of SLE (Peng et al., 1997).

Another possible mechanism for the involvement of IL-18 in SLE is due to IFN-γ production. IL-18 induces IFN-γ production that up-regulates MHC class II on antigen presenting cells, leading to effective presentation of T-cell epitopes and the activation of T-cells (Halloran et al., 1992). The results from IFN-γ -/- MRL/*lpr* and mercury-treated IFNγ -/- mice show that this cytokine is required for auto-antibody generation of either Th1 or Th2 associated isotypes, probably by enhancing autoantigen presentation (Balmenos et al., 1998; Haas et al., 1998).

Both Th1 and Th2 cytokines activate B cells to secrete antibodies, one possible answer to why Th1 pathway is essential for the development of SLE, is through differential effects on the CD27-CD27L costimulatory pathway during cognate T and B-cell interaction (Hintzen et al., 1994). This pathway plays a crucial role in the humoral immune response. CD27 is a disulphide-linked 120 kDa transmembrane glycoprotein expressed on the T and B cells that has homology to the tumour necrosis factor (TNF) family of molecules (Goodwin et al.,

1993). CD27-CD27L costimulaion leads to B-cell activation and differentiation of B cells into Ig-producing plasma cells (Jacqout et al., 1997; Kobata et al., 1995). An interesting recent study showed that the Th1 cytokine IFN-γ up-regulates, whereas IL-4 down-regulates the expression of CD27L on B cells, thereby differentially modulating the CD27-CD27L costimulation pathway (Hartwig et al., 1997). Overexpression of IL-4 transgene by B cells completely prevented the development of lethal lupus-like glomerulonephritis by reducing the Th1-predominant IgG2a and IgG3 Antibodies in a murine model of systemic lupus erythematosus (Santiago et al., 1997). The differential regulation of CD27L on B cells by Th1 (IFN-γ) and Th2 (IL-4) cytokines might represent the point at which the Th1 and Th2 cytokine responses differentially modulate the pathogenic humoral immune response.

It is significant that a relatively enhanced activation of Th1 vs. Th2 type cells, leads to increased production of IgG2a and IgG3, but to a diminished production of IgG1. It is associated with acceleration of lupus nephritis in MRL mice bearing the *lpr* or *Yaa* gene. This is highly relevant to the immunopathogenesis of lupus nephritis. Since murine IgG2a, but not IgG1, antibodies activate for better the complement system, the complement activating IgG2a autoantibodies can be more nephritogenic than IgG1 autoantibodies (Takahashi et al. 1996; Berney et al., 1992; Takahashi et al., 1991). Therefore, IL-18 alone or in synergistic effects with IL-12 enhance glomerolunephritis in lupus mice might be due to IgG2a anti-DNA Antibodies increment.

The promotional effect of rmIL-18 treatment is thus likely to be a result of a up-regulation of Th1 responses involved in autoantibody production in this murine SLE model. This notion is supported by a recent observation that the progression of lupus nephritis in MRL mice bearing the *Yaa* or *lpr* (Fas) mutation correlates with an increased production of IgG2a and

IgG3 versus IgG1 antibodies in parallel to an enhanced expression of IFN-γ versus IL-4 mRNA by CD4+ T cells (Takahashi et al., 1996). In addition, it has been shown that repeated injections of recombinant IFNγ can accelerate the development of SLE, and treatment with anti-IFN-γ monoclonal antibody (mAb) or soluble IFN-γ receptors can inhibit the progression of SLE in (NZB x NSW)F1 mice (Jacob et al., 1987; Ozmen et al., 1995). In addition, the complement activating IgG2a antibodies may be more nephritogenic than IgG1 antibodies.

It should not be assumed that autoimmune manifestations of the SLE disease are based solely on a Th1 type autoimmune response, since Th2 type cytokines such as IL-6 and IL-10 are apparently involved (Finck et al., 1994; Ishida et al., 1994), perhaps in relation to cytokines' ability to augment the overall production of pathogenic autoantibodies by promoting the terminal differentiation of activated B cell (Hakomota et al., 1986; Kishimoto, 1989; Rousset et al., 1992). In fact, many immune responses do not reflect an absolute Th1 or Th2 pattern, but seem to be functionally dominated either by Th1 (IFN-γ) or Th2 (IL-4) cytokines (Paul et al., 1994), and therefore, as suggested by a study of Klinman and Steinberg (Klinman et al., 1995), an altered ratio of IFN-γ / IL-4 producing T-cells could determine the nature, strength, and duration of systemic autoimmune responses. It should also be noted that the spontaneous production of IgG anti-DNA autoantibodies in lupus-prone (NZB x NSW)F1 mice was shown to reflect the intrinsic abnormality of B cells, as well as their hyperactivity to normal levels of immunostimulatory cytokines such as IL-5 (Herron et al., 1988; Reininger et al., 1992).

Among the non-immunologic mechanisms, enhanced monocyte activation and phagocytosis, oxidative stress and nitric oxide (NO) productions are likely candidates. With regard to NO, it

has been shown increased production in MRL/*lpr* mice (Huang et al., 1996; Weinberg et al., 1994), and prevention of glomerulonephritis upon oral administration of a nitric oxide synthase (NOS) inhibitor (Weinberg et al., 1994). Our studies show a significant NO increment in the serum of IL-12 / IL-18 treated mice, but not in IL-18 treated group. However, peritoneal cells from IL-18 treated mice produce significantly more NO than PBS control. Serum NO level in IL-12 / IL-18 treated group had a significant correlation with vasculitis ($r=0.438$) but not with glomerulonephritis ($r=0.194$). Our results are consistent with the study that shows reduction in vasculitis in iNOS -/- mice (Gileson et al., 1997). However, We postulate that local tissue specific NO production might be important, especially for the development of renal pathology. Cells most likely to contribute to local NO production in the kidney are vascular endothelial cells and mesangial cells (Lincoln et al., 1997).

In conclusion, in conjunction with previous investigations (Jacob et al., 1987; Ozmen et al., 1995; Takahashi et al., 1996; Huang et al., 1996 and Balomenos et al., 1998) this study strongly points to the significant contribution of heightened IFN-γ production in the pathogenesis of murine lupus. Other cytokines such as TNF-α (Jacob et al., 1988), IL-12 (Huang et al., 1996) and NO (Weinberger et al., 1997) have also been experimentally manipulated to affect serologic and histologic manifestation of murine lupus. In this regard, IL-18 as a cytokine that can regulate NO and pro-inflammatory cytokines might be important candidate in SLE pathogenesis.

Chapter 6

Recombinant IL-18 induces a facial rash on MRL/*lpr* mice resembling Malar rash in human SLE

Introduction

The most characteristic skin manifestation of SLE is a "butterfly" rash on the face (Figure 6.1), which is an erythematous or maculopapular eruption, which appears over the malar regions and the bridge of the nose giving a butterfly shape. It is usually aggravated by exposure to sunlight or to ultraviolet light. Pathology of the skin demonstrates immunoglobulin and complement deposits along the dermal-epidermal junction (Petri, 1996). It is most likely because of interactions of nuclear proteins, autoantibodies to nuclear proteins, and complement. The antigen-antibody interactions at the derma-epidermal junction may also involve autoantibodies to other skin elements such as the epidermal basement membrane and to the keratinocytes (Aiba et al., 1989).

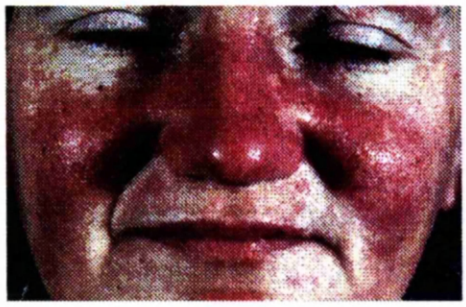


Figure 6.1 Malar rash in systemic lupus erythematosus.

The pathogenesis of the skin lesion is unknown. One possible contributing factor is exposure to sunlight and ultraviolet light which may enhance epidermal cell death and apoptosis, in particular in an SLE background where apoptotic regulatory defects are evident (Cascicol-Rosen and Rosen, 1997). One mechanism proposed for this disease process involves the formation of UV induced apoptotic blebs containing DNA released in the skin might act as autoantigens, and become a target autoantibody production with the development of a local, or systemic, inflammatory

response. This hypothesis is supported by the observation that ultraviolet B light increases the binding of autoantibody pro to Ro(SS-A) and La(SS-B) nucleo-protein antigens on keratinocytes in a dose-dependent manner (Emerit & Michelson, 1984; Furukawa et al., 1990).

MRL/Mp-*lpr* mice are known to have many clinical characteristics similar to human SLE. Their skin lesions develop spontaneously and this has been used as a dermatological model of human systemic lupus erythematosus (Furukawa et al., 1982; Furukawa et al., 1984; Horiguchi et al., 1984; Furukawa et al., 1984). The skin lesions are mainly on the ears, back and abdominal skin, and rarely appears before 14 week age (Furukawa et al., 1984). The lesions consist of hair loss, scab formation, and bleeding.

The immunofluorescent lupus band pattern produced on the skin of MRL/*lpr* mice is different from that produced on human SLE skin. Lupus band test is seen beneath the acanthotic epidermis of the lesional skin and they are a continuous granular reaction. The basal membrane zone (BMZ) shows a relatively weak reaction minimal deposition of complement components in the skin (Horiguchi et al., 1986).

Facial skin lesions in MRL/*lpr* mice resembling the human malar rash have not been reported. However, observations reported in this thesis (Chapter 5) demonstrate that sixty percent of MRL/*lpr* mice injected with IL-18 developed skin rashes, most prominently at the malar region, about 34 days after starting the treatment (9 week-old, Figure 5.13). MRL/*lpr* mice treated with a combination of IL-18 and IL-12 showed no sign of skin rash. This suggested that the balance of function of IL-18 and IL-12 might have an important role in the immunopathogenesis of the facial skin rash and perhaps other clinical aspects of SLE.

To further investigate the role of IL-18 in the lupus skin lesion, we repeated the protocol outlined in Chapter 5 (Figure 6.2). The mice were sacrificed just after the appearance of the lesions to assess:

i) Whether a commercial IL-18 causes facial skin lesion in MRL/*lpr* mice.

ii) the histology of the lesions immediately after appearance the facial lesions.

ii) Immune-complex depositions in the skin by immunofluorescence or Immunocytochemistry

iii) the role of apoptosis in the skin lesions.

iv) the effect of IL-18 treatment on *in vivo* cytokine profiles and anti-DNA antibodies.

6.1 Recombinant IL-18 induces a facial rash in MRL/*lpr* mice

Forty-five young (4-week-old) MRL/*lpr* mice were divided randomly into three groups. Recombinant murine IL-18 (IL-18) was purchased from PerproTech (UK). One group of the mice was injected daily with IL-18 (500 ng/mouse/day, i.p.). Control mice were injected with the same volume (100 ul/mouse/daily) of PBS. The third group received the combination of IL-18 (500 ng/mouse) and rmIL-12 (100 ng/mouse/day).

The IL-18 treated mice developed skin rashes, most prominently on the malar region of whisker pad (Figure 6.3 & 6.4). This occurred 40 days after treatment in 4 of 5 mice with different severity. The facial skin was maximum at 10-11 weeks of age and the mice were sacrificed at that time to assess the pathology changes in the skin lesions. Control MRL/*lpr* mice treated with PBS or IL-18+IL-12 showed no facial skin lesions (Figure 6.3 & 6.4).

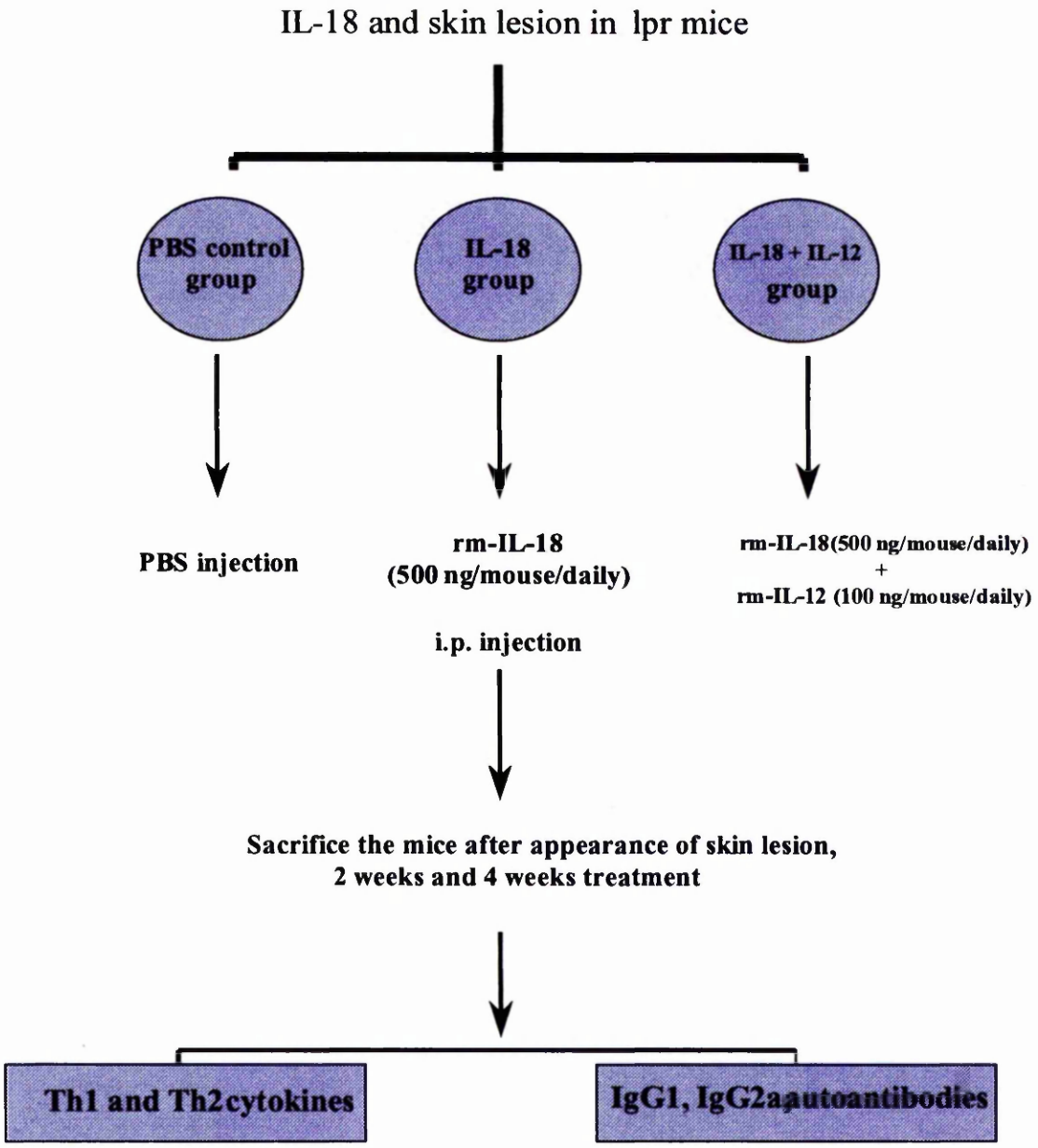


Figure 6.2 Protocol used to assess the role of rmIL-18 in the development of facial skin lesion in MRL/*lpr* mice resembling malar rash in human SLE.

6.2 Skin histopathology

After 42 days treatment, at 11 weeks, the mice were sacrificed. Skin tissue was fixed in neutral buffered formalin and embedded in paraffin wax. Sections were stained with haematoxylin and eosin and periodic acid schiff. The sections were analysed by two experienced pathologists.

The skin lesions showed thickened epidermis with acanthosis and hyperkeratosis. In occasional animals the skin was excoriated; however apart from those areas, the basal layers of the epidermis was intact. The dermis contained an intense inflammatory cell infiltrate consisting mainly of a lympho-histocytic population. Polymorphs were also present even in cases where the skin was not broken. In contrast, untreated mice had a minimal increase in chronic inflammatory cells mainly small lymphocyts (Figure 6.3 and 6.4). In the IL-18 plus IL-12 treated group there were some infiltering lymphocytes and these tended to form loose aggregates associated with the skin appendages and more hyperkeratosis than the PBS control group (Figure 6.3b and 6.4b). Vasculitis was only identified in the skin of one animal in the IL-12 / IL-18 group.

Figure 6.3 IL-18 induces a facial skin lesion in MRL/lpr mice.

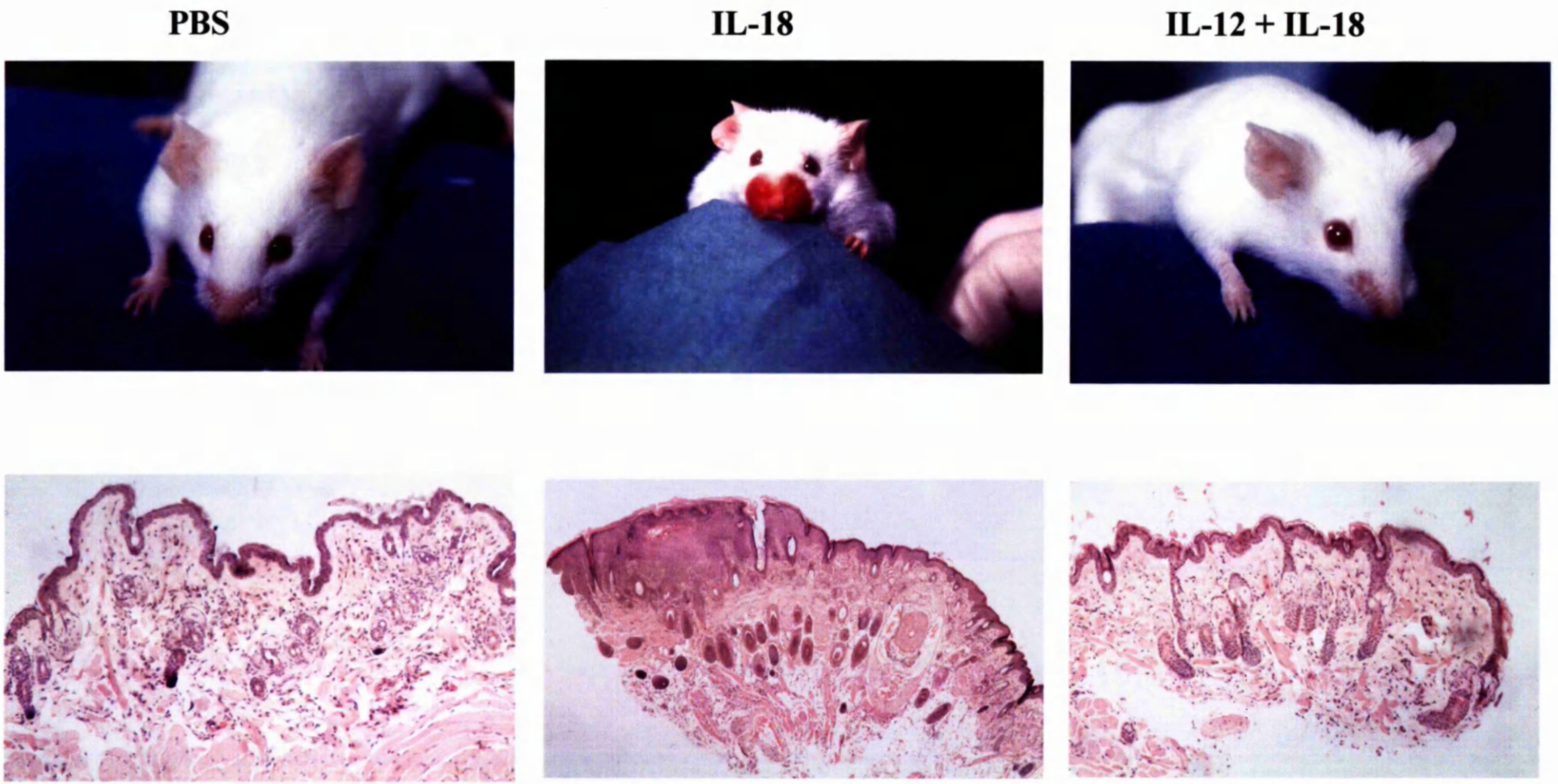
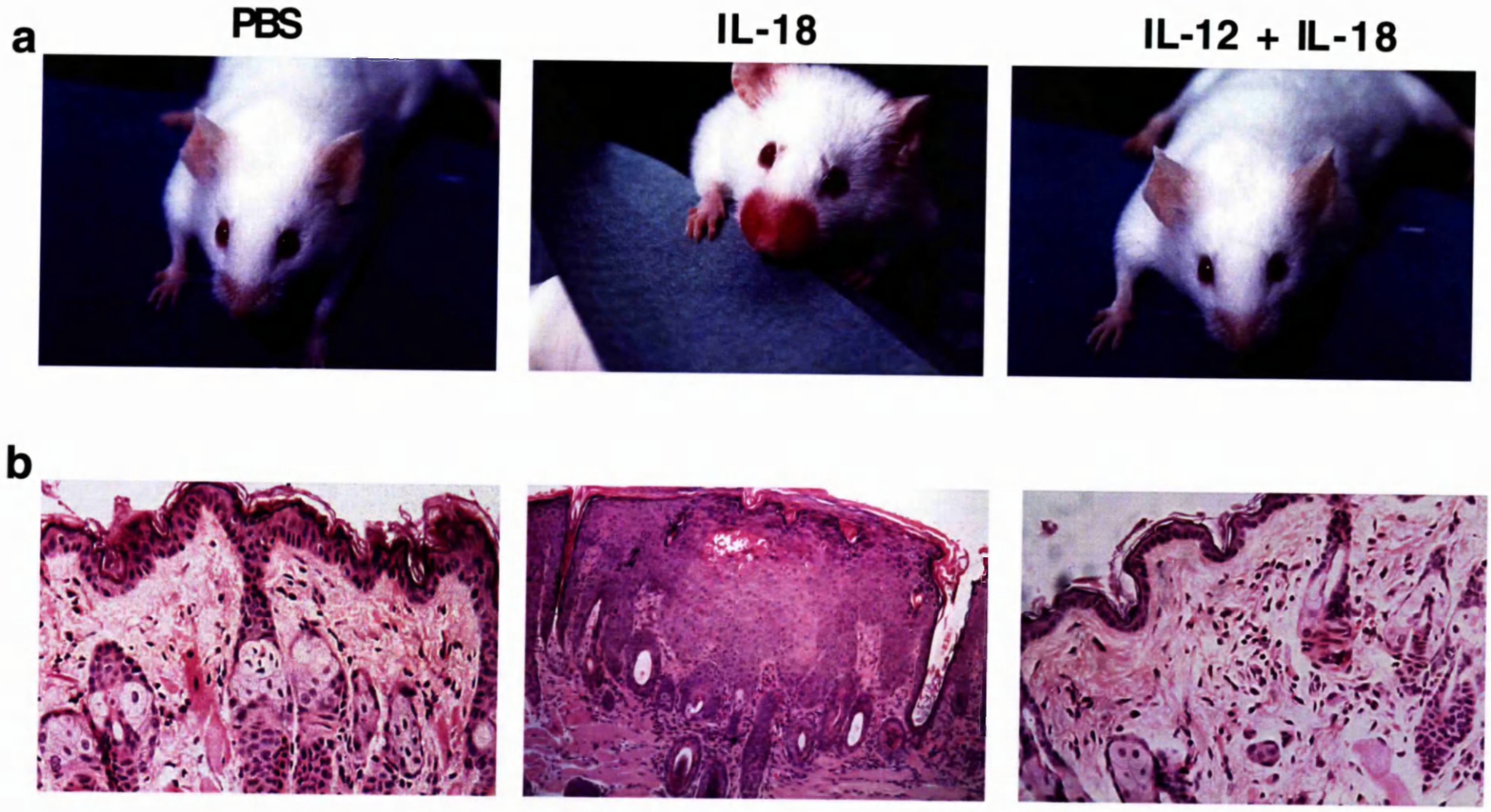


Figure 6.4 IL-18 induces a facial skin lesion in MRL/lpr mice.



Legend for Figure 6.3 and 6.4:

Recombinant IL-18 induces a facial rash in MRL/*lpr* mice.

(a) Upper panel shows the characteristic red "butterfly" symmetrical skin rash in mice treated with IL-18 (centre) and none of the untreated mice (left) or those treated with IL-12+IL-18 (right). Mice injected with IL-18 developed spontaneous skin rashes, most prominently at malar region of the whisker pad with total disappearance of hair in the affected area.

(b) The lower panel shows representative histological sections (Fig. 6.3: magnification x 100 ; Fig. 6.4: magnification x 200). The IL-18-treated mice showed thickened epidermis with acanthosis and hyperkeratosis. The dermis contained an intense lympho-histocytic inflammatory. Control mice showed no inflammatory changes (left) whereas in the IL-12+IL-18 group there was inflammatory infiltrate and a slight thickening of the epidermis.

6.3 *In vitro* cytokine production after *in vivo* IL-18 treatment

Spleen cells from each group were pooled and cultured as a single-cell suspension ($2x10^6$ viable cells/ml) in 24-well plates pre-coated with anti-CD3 antibody (2 μg/ml) in standard culture conditions. After 48 hours, supernatant was collected to assess the production of the cytokines IL-4, IL-5, IL-6, IL-10, IFN- γ and TGF-β by ELISA (Figure 6.5).

Spleen cells from IL-18-treated mice produced more IFN-γ than control mice (mean ± SEM, 890 ± 40 pg/ml vs. 470 ± 10 pg/ml, $p<0.05$) in response to anti-CD3 antibody. Synergistic enhancement of IFN-γ production by spleen cells was evident in IL-18/IL-12 treated mice (1450 ± 60 pg/ml, $p<0.001$).

Spleen cells from the IL-18-treated mice produced significantly less IL-10 (36 ± 6 pg/ml, $p<0.05$) than control mice in response to anti-CD3, indicating suppression of Th2 responses. The suppression of IL-10 production was more pronounced in spleen cells from IL-12/IL-18 treated mice.

IL-5 and IL-4 production was detectable only in the control PBS-treated group (respectively, 27 ± 3 and 12 ± 3 pg/ml) and this was abrogated by IL-18 and IL-18/IL-12 treatment in. In contrast, IL-6 production was significantly increased in the IL-18 (350 ± 5 pg/ml, $p<0.05$) and IL-12/IL-18-treated animals (730 ± 40, $p<0.001$) compared to controls (180 ± 15). TGF-ß was undetectable in any of the groups. These data suggest that IL-18 may shift the balance of T helper-cells toward Th1 activity.

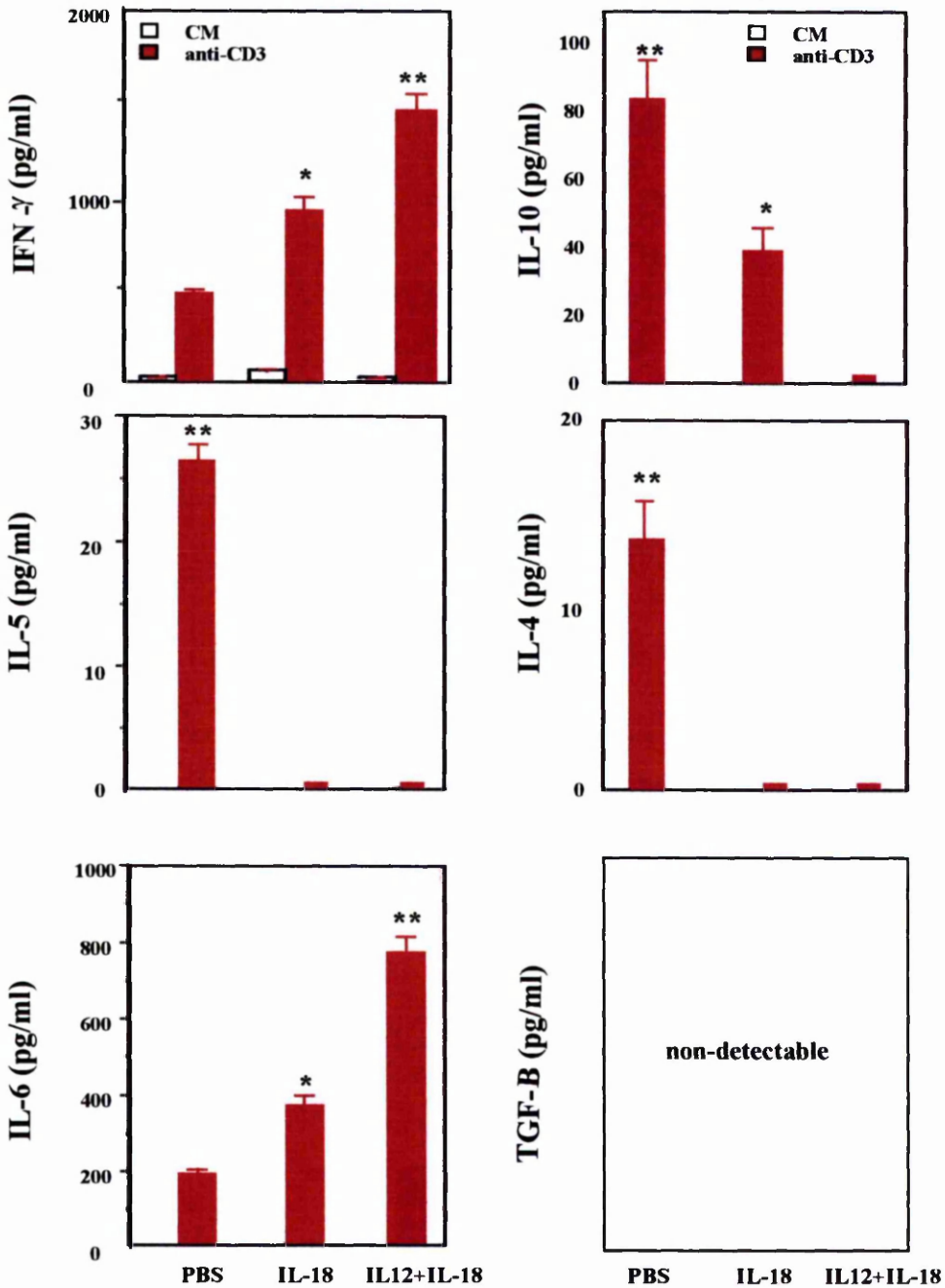


Figure 6.5 Th1 and Th2 cytokines production by spleen cells from MRL/*lpr* mice after facial rash appearance (10-11 weeks old).

Pooled spleen cells were harvested from the different treatment groups (5 mice in each group) and stimulated with anti-CD3 antibody or culture medium. Cytokine levels in the culture supernatants at 48 hours were measured by ELISA. Data are mean ± SEM of triplicate cultures (*$p<0.05$, **$p<0.001$ compared with control group, student's t-test).

6.4 Serum cytokine and auto-antibody levels after IL-18 treatment

To study the influence of IL-18 and IL-18 plus IL-12 treatment on cytokine profiles, serum samples were analysed for the presence of IFN-γ, IL-10, IL-4, IL-5, and autoantibodies to DNA (Figure 6.6). There were no detectable levels of IL-4 and IL-5 in any group.

IFN-γ was significantly increased in the IL-12/IL-18 mice (mean ± SEM, 139.3 ± 15.6 pg/m, $p<0.002$) and the IL-18 mice (67.2 ± 11.3, $p<0.05$) compared with the PBS control group (41.2 ± 9.6). Significant reduction of IL-10 was observed in the IL-18/IL-12 group (61.6 ± 4.3) and the IL-18 group (71 ± 3.2) compared with the PBS treated mice (187.2 ± 17.9).

Figures 6.6b demonstrates that IL-18 and IL-12/IL-18 treated mice produced significantly higher levels of IgG2a anti-dsDNA antibodies ($p<0.05$), compared with control group. IgG2a antibody is typical of Th1 type response and IgG1 antibody is typical of a Th2 response. The titres of anti-dsDNA IgG1 antibody were unaffected by IL-18 treatment but was significantly reduced by IL-12/IL-18 treatment ($p<0.05$).

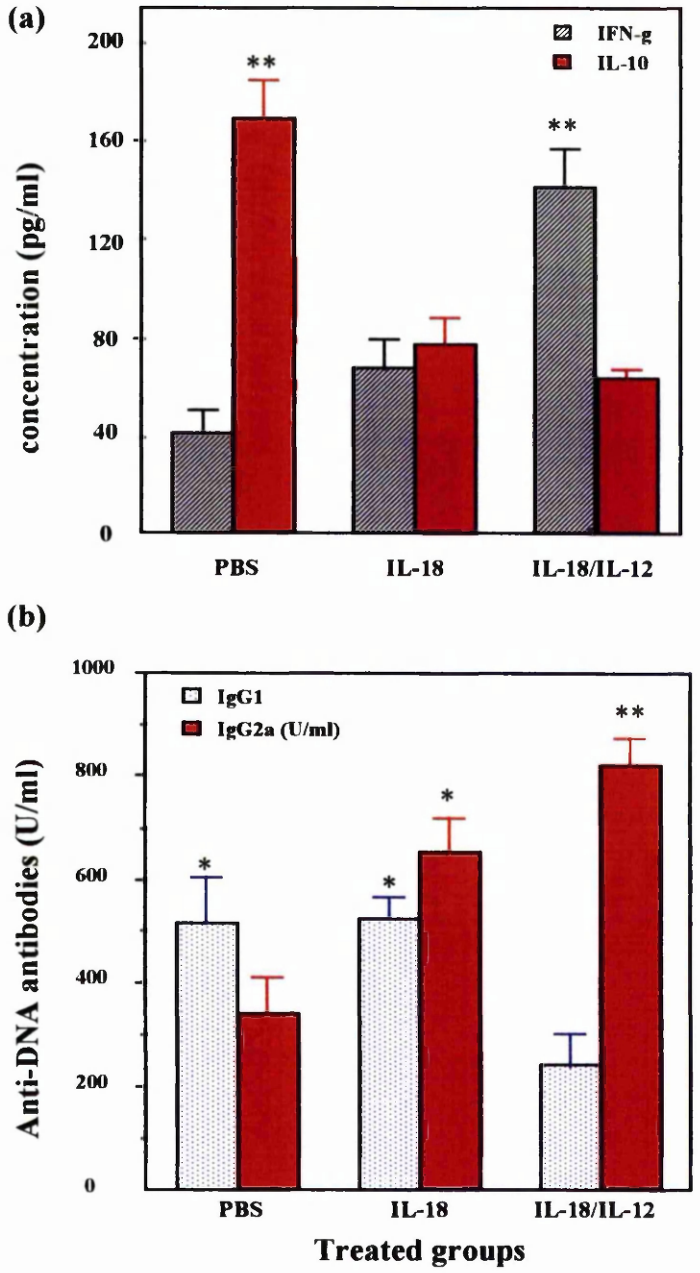


Figure 6.6 Effect of IL-18 or IL-18 plus IL-12 treatment on the serum cytokine and anti-DNA antibody levels. (a) Serum from different groups of mice collected at the end of treatment (6 week treatment) were measured by ELISA (b) anti-DNA antibody isotype distribution in the serum of different treated groups of mice were measure by ELISA. Data are mean ± SEM., *$p<0.05$, **$p<0.005$ compared to PBS control group, Mann-Whitney U-test.

6.5 Serum cytokine profiles and autoantibody level after two week treatment with rmIL-18

To investigate *in vivo* effect of IL-18 in the early stages of the pathogenesis of the facial skin lesion, 4 mice in each treatment group were treated for two weeks then sacrificed at the age of 6 weeks.

Serum samples were collected and levels of IL-4, IL-5, IFN-γ and autoantibodies were measured (Figure 6.7). IFN-γ was detected only in the serum of IL-12 plus IL-18 group (mean ± SD, 510 ±105 pg/ml) and IL-5 was detected only in the group of mice which were treated with IL-18 (145 ± 48 pg/ml). IL-4 was undetectable in the serum of any of the mice.

The serum level of autoantibody to ds-DNA is shown on Figure 6.7b. The level of IgG2a anti-dsDNA antibodies measured in the serum of the IL-18 group (50 U/ml) was significantly higher ($p<0.05$) than both PBS group and IL-12 plus IL-18 treated group. IgG1 anti-DNA antibodies was detected only in the serum of IL-18 treated mice.

Single cell suspensions of spleen cells from mice from each treatment group were cultured ($2x10^6$ viable cells/ml) in 24-well plates pre-coated with anti-CD3 antibody (2 μg/ml) in standard culture condition. After 48 hours the supernatant was taken to assess *in vitro* cytokine production by ELISA (Figure 6.8).

Spleen cells from IL-18-treated mice produced more IFN-γ (4020 ± 520 pg/ml, mean ± SD, $p<0.05$) than control mice (1370 ± 110 pg/ml) in response to anti-CD3. Synergistic enhancement of IFN-γ production by spleen cells was evident in IL-18/IL-12 treated mice (8450 ±1060 pg/ml, $p<0.001$).

Spleen cells from the rmIL-18-treated mice produced more IL-10 (136 ± 36 pg/ml, $p>0.05$) than control mice in response to anti-CD3 antibody (Figure 6.8). IL-10 production by spleen cells was suppressed in the IL-12/IL-18 treated mice compared with the treated PBS mice.

These data show that IL-18 in the younger mice after two weeks treatment increased Th2 cytokines and induce IgG1 and IgG2a anti-DNA antibodies in this model of lupus disease.

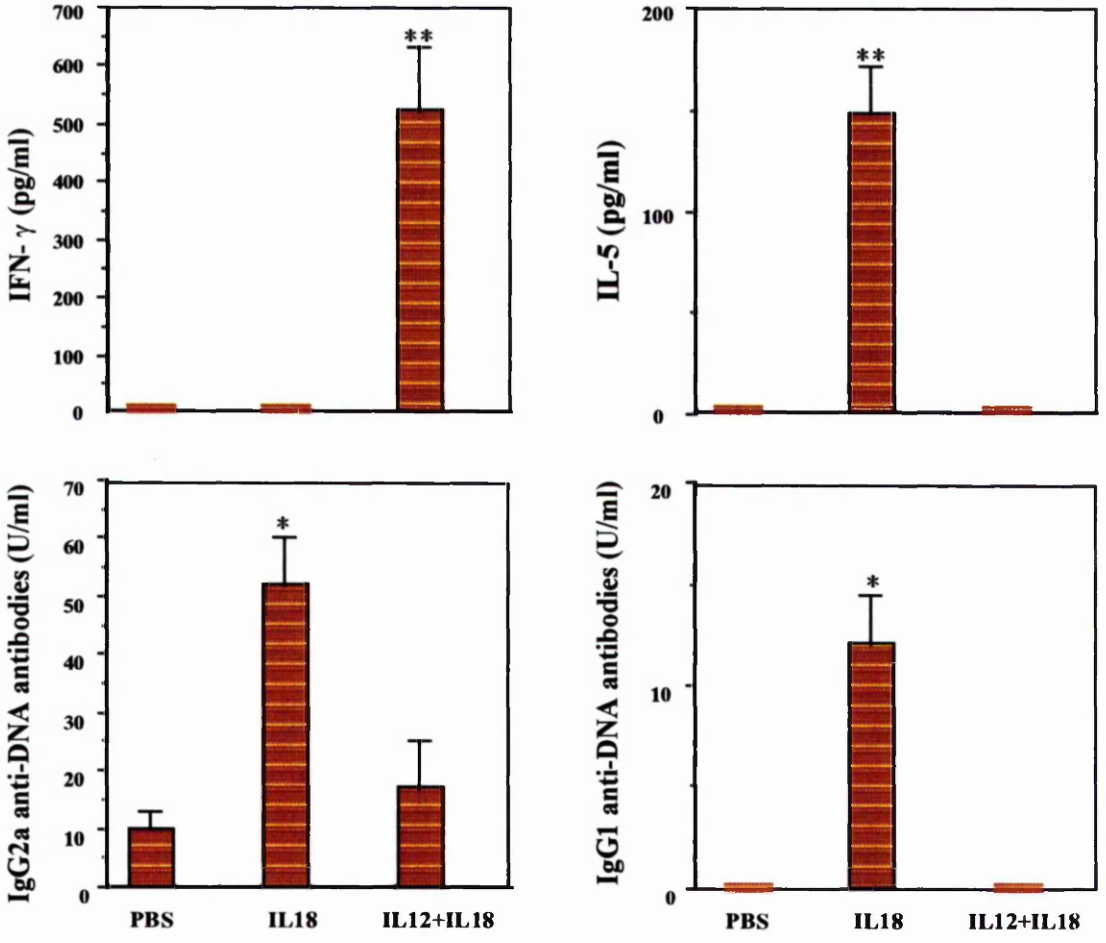


Figure 6.7 Serum IFN-γ, IL-5 and anti-DNA antibody (IgG2a and IgG1 isotypes) in MRL/*lpr* mice after two weeks cytokine treatment with IL-18, IL-12 plus IL-18 or PBS control.

IFN-γ was detected at significantly high levels in the IL-12+IL-18 group. In contrast, IL-5 was detected only in the group treated with IL-18. IL-4 was undetectable in any of the mice. The levels of IgG2a anti-dsDNA antibodies in the IL-18 group were significantly higher than both the PBS and IL-12+IL-18 groups (**$p<0.001$). IgG1 anti-DNA antibodies were detected only in the serum of IL-18 treated mice (*$p<0.05$).

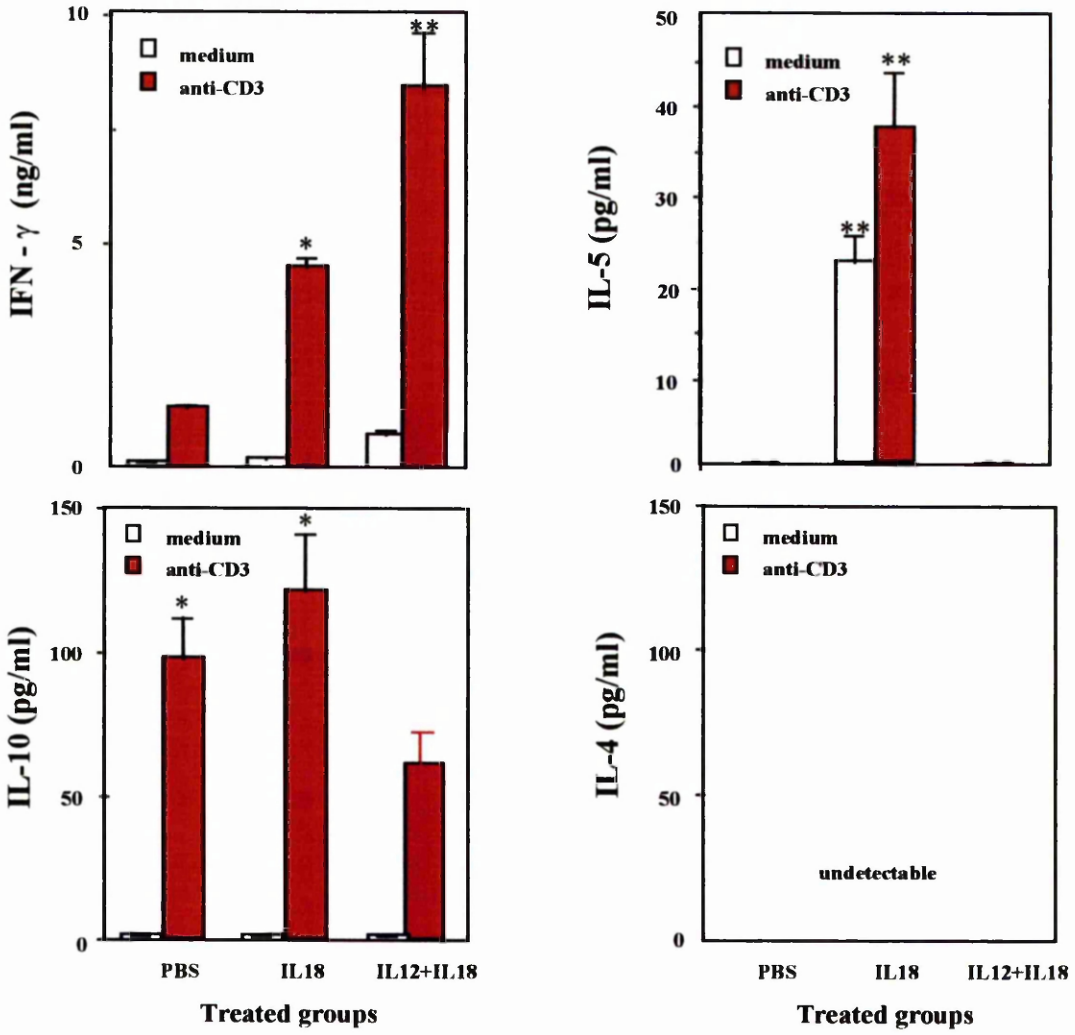


Figure 6.8 ***In vitro*** **cytokine production by spleen cells from MRL/*lpr* mice after 2-week cytokine treatment.** Cytokine levels were measured by ELISA. IL-4 was not detected in any of the groups. *$p<0.05$, **$p<0.001$.

6.6 Cytokine profiles and autoantibody level after four weeks treatment with IL-18

Four mice in each treatment group aged 4 weeks and were treated for 4 weeks were sacrificed at the age 8 weeks. Serum was collected and IFN-γ and IL-10 were measured (Figure 6.9a).

The serum levels of IFN-γ were significantly higher in the IL-18 treated group than the PBS group ($p<0.05$), IL-12 in synergy with IL-18 induced significantly more IFN-γ than the IL-18 group ($p<0.05$). However, IL-10 was significantly higher in the serum of IL-18 treated group than both the PBS control and the IL-12/IL-18 groups. IL-4 and IL-5 were undetectable in of any of the groups.

Spleen cells from each group were cultured with anti-CD3 antibody (2 μg/ml). After 48 hours supernatant was taken to assess *in vitro* cytokine production by ELISA (Figure 6.9b).

Spleen cells from IL-18 plus IL-12 treated mice produced more IFN-γ (mean ± SD 16020 ± 1420, $p<0.05$) than control mice (7370 ± 410 pg/ml). Spleen cells from the IL-18 treated group also produced more IFN-γ (10,050 ± 1200) than the PBS control group ($p<0.068$). Spleen cells from the IL-18 and the PBS treated mice produced more IL-10 (for both $p<0.05$) than the IL-12/IL-18 treated group.

IL-5 production was increased by IL-18 treatment, and IL-12 plus IL-18 treatment significantly suppressed IL-5 production compared with control or IL-18 treatment. IL-12 in synergy with IL-18 suppressed IL-4 production compared with control group.

The serum autoantibody are shown on Figure 6.10. Serum levels of IgG2a anti-DNA antibodies were significantly higher in both the IL-12+ IL-18 and IL-18 groups than

in the PBS-treated group. There was a significant increase in serum levels of IgG1 anti-DNA in the IL-18 group than in both the PBS and the IL-12/IL-18.

IgG2a anti-DNA antibodies produced by spleen cells from IL-18 and IL-12 plus IL-18 groups were significantly higher than the PBS group and spleen cells from the IL-18 group produced more IgG1 anti-DNA antibody than other groups (Figure 6.10b).

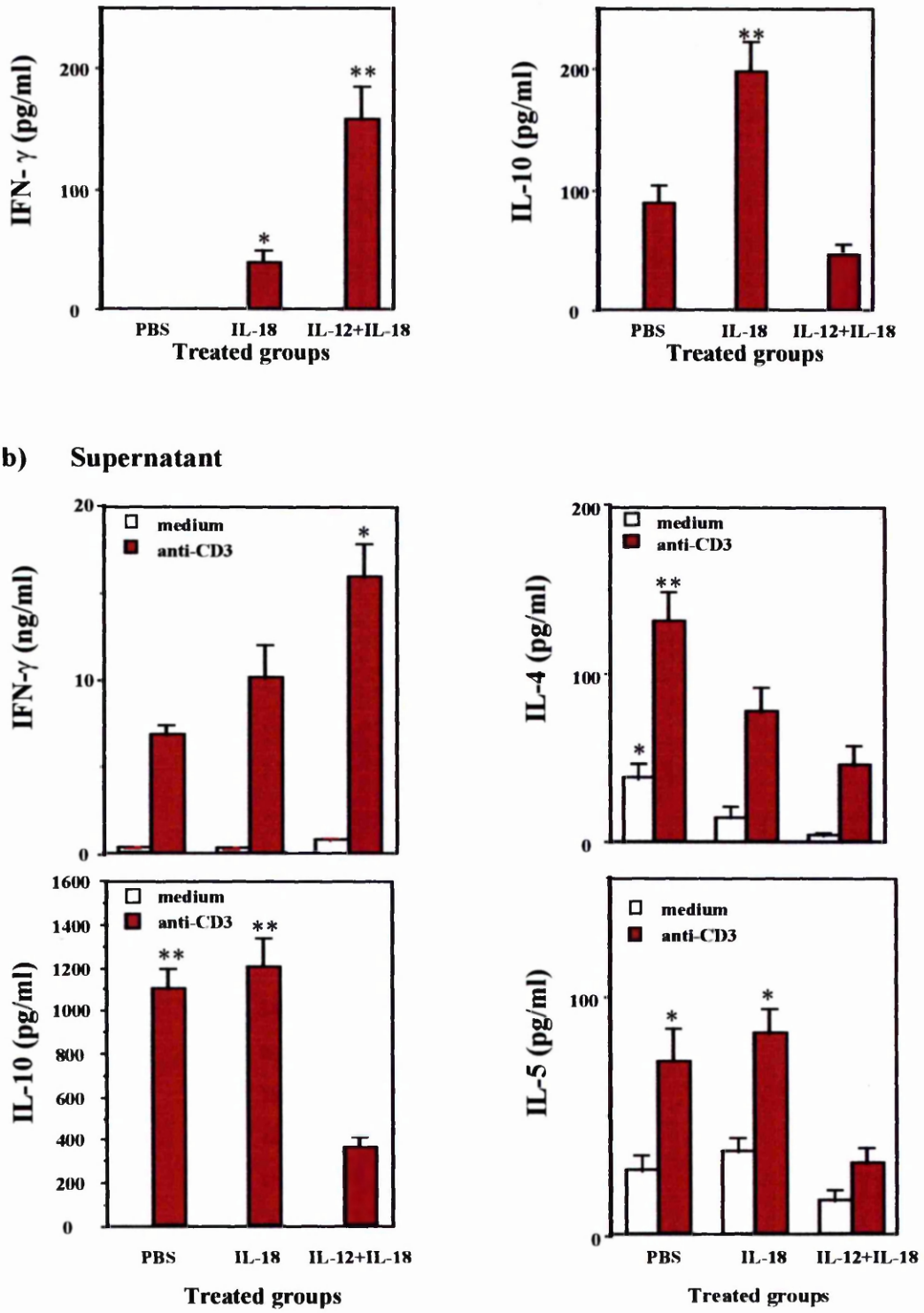


Figure 6.9 Cytokine levels in the serum (a) and culture supernatants (b) of MRL/*lpr* mice after 4-week cytokine treatment. Cytokine levels were measured by ELISA. Data are mean ± SEM of triplicate cultures (*$p<0.05$; **$p<0.001$, by Student's t-test).

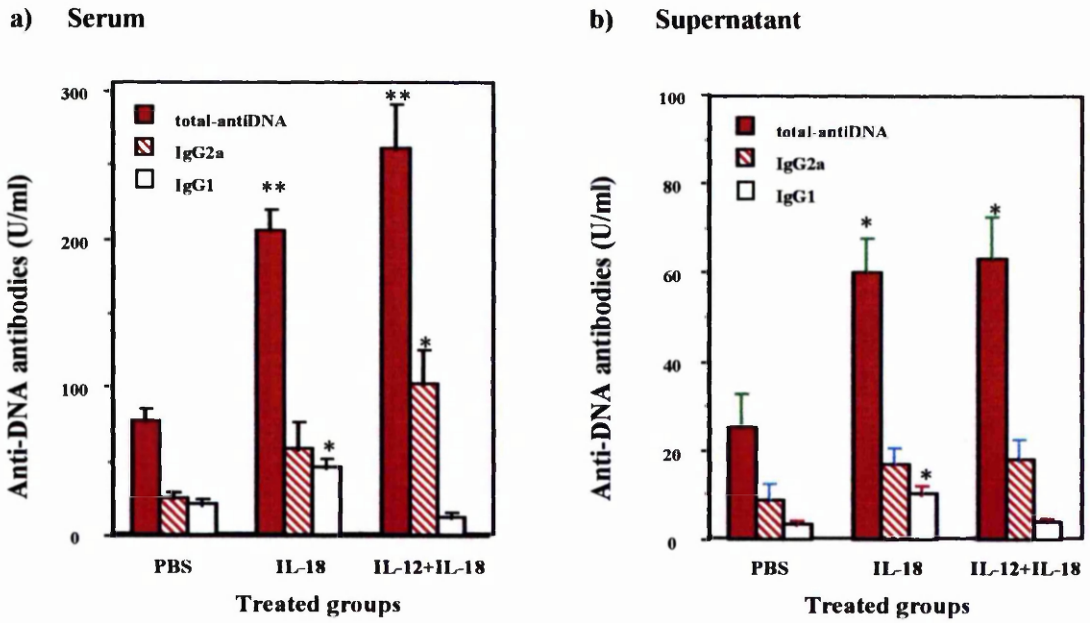


Figure 6.10 Anti-DNA antibody isotype distribution in the serum (a) and culture supernatant (b) of MRL/*lpr* mice after 4 weeks treatment. Anti-DNA antibody isotypes, total, IgG2a, IgG1, were measured after by ELISA. Data are expressed as arbitrary units/ml (compared with standard serum from 5 month old MRL/*lpr* mice (*$p<0.05$, **$p<0.001$ Student's t-test).

6.7 Assessment of IgG deposition in the skin of different group of mice

The presence of a malar rash is associated with SLE and is commonly accompanied by other inflammatory manifestations of the disease. Pathologically, the skin lesion shows non-specific inflammation, although by immunofluorescence the classic deposits of immunoglobulin and complement at the dermal-epidermal junction may be seen. The presence of theses immune deposits may also be seen in un-involved skin.

Immunofluorescence staining was performed on frozen sections of skin biopsies from the different treatment groups of mice to assess whether IL-18 affected immune complex deposition. The tissues were snap frozen in an isopentane bath cooled in liquid nitrogen. Frozen sections embedded in OCT were cut at 5 μm thickness. Sections were then incubated with FITC conjugated goat anti-mouse IgG. After thoroughly washing in PBS, stained sections were mounted. No fluorescent staining was detected in any of the treated groups.

Immune complex deposition in the skin was assessed also by immuno-histochemistry of paraffin section of skin. Immunoglobulin (Ig) deposition was increased in the whole skin from the IL-18 and IL-18/IL-12 treated mice (**$p<0.001$, *$p<0.05$) compared with the PBS control mice (Figure 6.11). The skin lesions from the IL-18 treated mice contained more Ig deposition in dermo-epidermal junction (DEJ) than the IL-12/IL-18 and the PBS treated mice ($p<0.001$). Samples were examined with a Leitz DRMB microscope linked to a Panasonic F15 CCD video camera. Images first transferred to an IBM-compatible computer by means of Neotech Image Grabber software (version 1.21; Neotech Ltd., Eastleigh, UK) and cell counts were made with computer image analysis software (Count Gem; ME Electronics, Ltd, Reading, UK).

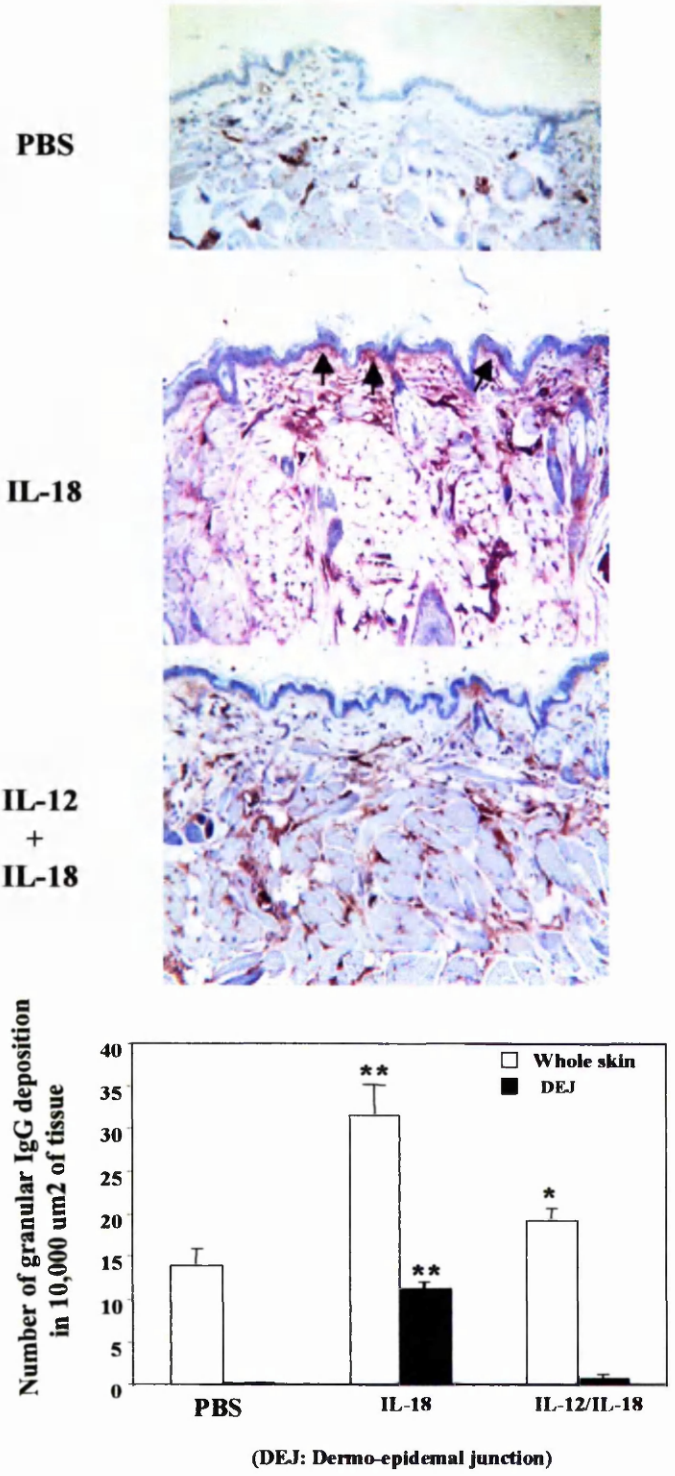


Figure 6.11 IgG deposition in the skin of different treated groups of mice after 4 weeks treatment. Arrows indicate Ig deposition in the Dermo-epidermal junction of mice treated with IL-18. This is largely absent in the other two groups. Ig deposition was quantified by Image analysis and presented as number of granular IgG deposition in 10 nm^2 of tissue. Vertical bars are SD of mean, n=4, **p<0.001, *p<0.05.

6.8 Apoptosis in the skin lesion in the IL-18 treated mice

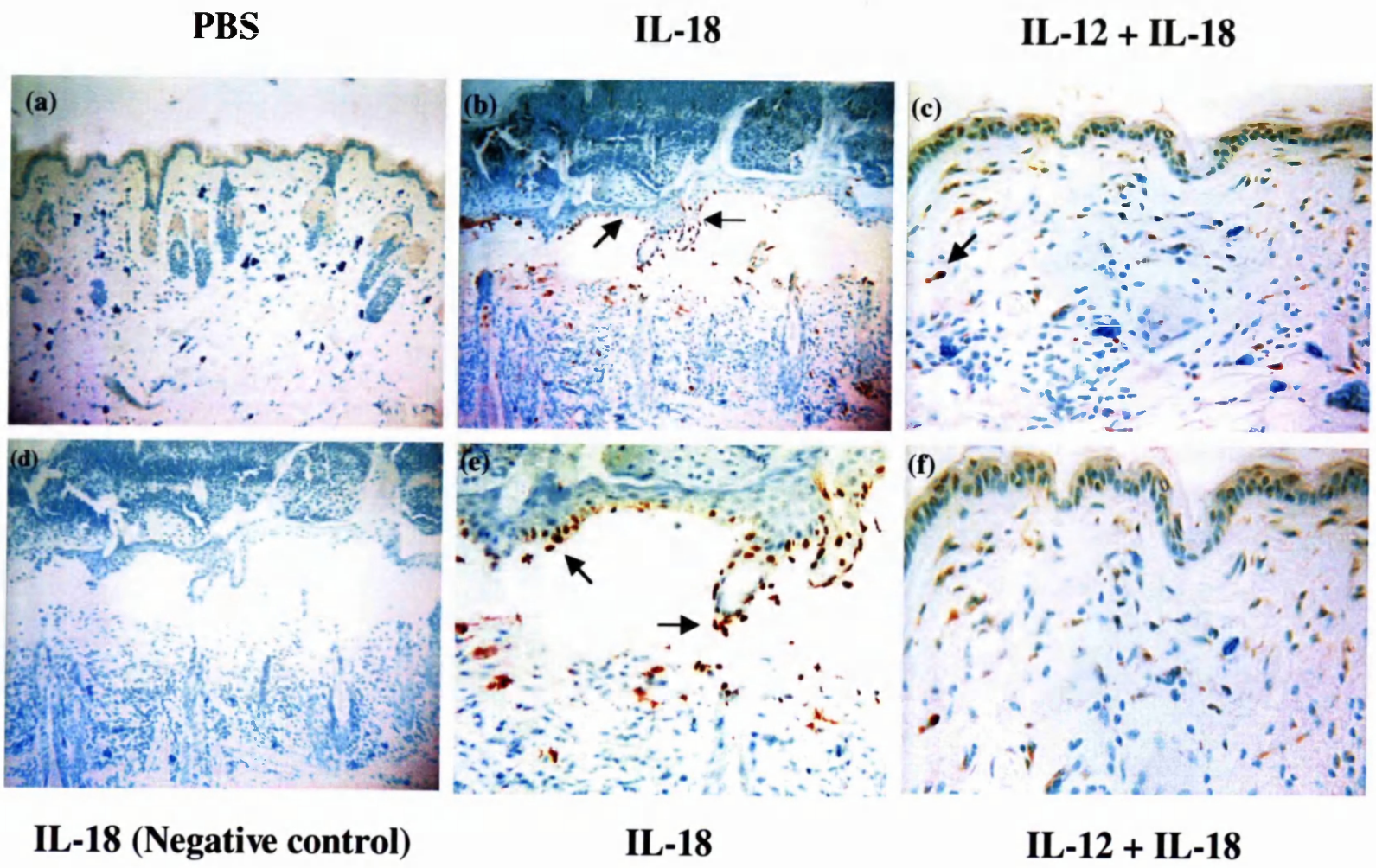
Apoptosis is of fundamental biomedical interest and is of particular importance in the pathogenesis of systemic autoimmune diseases. Apoptosis is an active process that leads to the ordered destruction of cells, avoiding the release of intracellular contents into the extracellular microenvironment, where they may have a powerful inflammatory effect. Apoptotic cells undergo a series of changes of the surface lipid membrane, cytoskeletal disruption, cell shrinkage and a characteristic pattern of DNA fragmentation. It has been long recognised that DNA and histones are major auto-antigens in SLE, but only more recently it was recognised that the DNA-histone complex, i.e., nucleosomes, are the preferred targets of autoantibodies (Mohan et al., 1993).

One possible explanation for antigen selection autoantibody mediated diseases is apoptosis. During apoptosis, the cell membrane forms cytoplasmic blebs, some of which are shed as apoptotic bodies. UV-induced apoptosis of keratinocytes leads to redistribution of several nuclear proteins and DNA to the apoptotic blebs where they can become auto-antigen (Cascicola-Rosen et al., 1994).

To test whether apoptotic cells can be a source of autoantigens and whether immune-complex deposition can be involved in the pathogenesis of the skin lesion observed in the IL-18 treatment group, DNA fragmentation in skin biopsy specimens was examined by TUNEL staining as previously explained in Chapter 2.

As Figure 6.12 shows skin lesions from IL-18 treated mice revealed apoptotic cell death in the epidermis, dermis and epidermo-dermis junction and marked leukocyte infiltration in the dermis (Figure 6.12e).

Figure 6.12 **(Legend next page)**



Legend for Figure 6.12 TUNEL staining of apoptosis in the facial skin lesion of IL-18 treated MRL/*lpr* mice. Skin lesion from IL-18 treated mice revealed apoptotic cell death in the epidermis, dermis and epidermal-dermis junction, and marked leukocyte infiltration in the dermis (b and e). The negative control (d) from an IL-18-treated with reaction buffer containing no protein. DNA fragmentation was not detectable in the PBS-treated mice (a). Skin from IL-12+IL-18 treated group showed significantly less apoptotic cells in the epidermis and dermis than IL-18 treated skin lesions.

Discussion

The aetiology and pathogenesis of autoimmune diseases cannot be readily analysed without appropriate animal models. Skin lesions are one of the commonest manifestations of human lupus erythematosus. However, animal models rarely show these skin lesions (Furukawa et al., 1984). The MRL/*lpr* mouse is model which spontaneous develop skin lesions in some aspects similar to those seen in human lupus erythematosus (Furukawa et al., 1984; Horiguchi et al., 1984; Provost et al., 1993; Furukawa, 1997). Macroscopically, these skin lesions show alopecia and scab formation on the upper dorsal region (Andrews et al., 1978). Immuno-pathological studies have revealed hyperkeratosis, acanthosis, hypergranulosis, liquifiction-like changes in basal keratinocytes, dermal T-cell infiltration, mononuclear cell infiltration into the dermis and epidermis, and vasodilatation by the age of 5 month (Furukawa et al., 1984). Ultrastructural changes show similarity to those of human lupus erythematosus skin lesions (Horiguchi et al., 1984, 1986). Immunohistological studies show immunoglobulin deposition at the dermal and dermal-epidermal junction (DEJ) to un-involved skin in MRL/*lpr* mice over 5 month old (Furukawa et al., 1984). The malar rash of human SLE has not been described on any lupus-like experimental models. Furthermore, there are no reports of any cytokine inducing skin rashes in the lupus-like models. For the first time we report that IL-18 induces a skin lesion on the face of MRL/*lpr* mice resembling human malar rashes. IL-18 also accelerates the development of the typical SLE skin lesions on the upper dorsal skin of MRL/*lpr* mice.

Two separate experiments investigated the role of IL-18 in the induction of the skin lesions in MRL/*lpr* mice. 60-80% of the *lpr* mice injected with IL-18 showed facial skin lesions at age of 9-11 weeks. The *lpr* mice treated with PBS or IL-18 + IL-12 did not show any skin lesions. The skin lesions in IL-18 treated mice showed loosened hairs and scab formation. Light microscopy showed hyperkeratosis, acanthosis, and infiltration of mononuclear cells into the epidermis and the dermis.

vasodilatation and some bleeding in the dermis. The MRL/++ control mice did not show any skin lesions when treated with IL-18.

IL-18 also accelerated the glomerulonephritis in the MRL/*lpr* mice. However the peak of skin lesions on the malar whisker pads occurred earlier than the appearance of the glomerulonephritis. The cytokine analysis after 11 weeks of age showed a strong Th1 response increased (IFN-γ and IgG2a-antiDNA autoantibodies) and Th2 cytokine suppression (reduced IL-10, IL-5) compared with control PBS treated mice. The cause of the skin lesions cannot be simply this pattern of cytokine profile changes, because the IL-18/IL-12 treated mice which showed a stronger Th1 responses and more Th2 suppression (IL-10, IL-5 and IgG1 anti-DNA antibodies) than IL-18 treated mice, did not show any skin lesions. However, IL-18/IL-12 treated mice showed an accelerated glomerulonephritis even more than treated IL-18 mice. Therefore, it can be concluded that the skin lesions in SLE have a different pathogenesis from the glomerolunephritis and vasculitis.

To investigate why the skin lesions appeared only in the IL-18 treated mice but not in the treated IL-18/IL-12 mice despite more severe glomerolunephritis, we investigated the effect of IL-18 on the cytokine pattern and different sub-classes of anti-DNA antibodies after two weeks and four weeks treatment.

After two weeks treatment, serum from MRL/*lpr* mice injected with IL-18 contained a significantly raised level of IL-5. This was undetectable in the serum of the PBS or IL-12 + IL-18 treated groups. In contrast, mice treated with IL-12 + IL-18 produced high levels of IFN-γ, which was undetectable in the PBS or IL-18-treated mice. IL-18 treated groups also produced markedly higher levels of anti-DNA antibodies compared with the other two groups. Thus, it shows that IL-18 administration augmented the Th2 responses, whereas the combination of IL-12 and IL-18 induced a predominant Th1 activity. However, after 8 weeks treatment, the cytokine profile

changed. At the end of treatment (aged 11-12 weeks, Chapter 5), IL-18 treated group were the same as the IL-18 plus IL-12 treated group developed a predominant Th1 type of response and produced more pro-inflammatory cytokines compared with the control mice.

These data indicates that IL-18 at the early stages of administration induced a strong Th2-type cytokine response in. This shifted to a more Th1 dominant response over time. We speculate here that the effects of IL-18 on different age of MRL/*lpr* mice vary because of endogenous level of IL-12. As shown previously in Chapter 3 and by Huang and colleagues (1996), IL-12 increases with age in MRL/*lpr* mice. Therefore at the early stages of the disease, when there was low levels of IL-12, IL-18 induced a Th2 dominant response. When the mice were older, because of higher level of endogenous IL-12 in a synergistic effect with IL-18, they induced higher amount of IFN-γ. The results from the IL-18 plus IL-12 treated mice support this hypothesis. The mice in this group at all stages of disease showed a Th1 dominant response. Several recent reports now show that IL-18-mediated effects on T cells may extend beyond Th1 differentiation to include induction of type 2 cytokine production (Yoshimoto et al., 2000; Wild et al., 2000; Hoshino et al., 2000; Xu et al., 2000).

Patients with cutaneous manifestations of lupus are more likely to have mRNA for IL-2, IL-4 and IL-5 in skin biopsy specimens than healthy controls where mRNA for IL-5, IL-4 and IL-2 is undetectable (Sauder et al., 1993; Nuremberg et al., 1995). IgG1 subclass antibodies are believed to be important in the pathogenesis of skin lesions in SLE (Furukuwa et al., 1983; 1984). From these findings, we conclude that one reason why the skin lesion occurs only in the IL-18 group at this stage of the disease is because of induction Th2 type responses at an early stage of disease development. Since IL-18 induces significantly higher amounts of IgG1 anti-DNA compared with the PBS and IL-12 + IL-18, the skin lesion is induced only in this IL-

18 treated group. However, for glomerulonephritis IgG2a antibodies are more pathogenic than IgG1, therefore, this is accelerated in both the IL-18 treated and the IL-18/IL-12 group.

In immunohistochemical studies, there was more Ig deposition in the skin sections from the IL-18 and IL-18/IL-12 groups than the PBS group. One possible reason that despite Ig deposition in the IL-18/IL-12 group, the skin lesion was induced only in the IL-18 treated mice, is that the Ig subclasses in the IL-18 group were mainly IgG1 sub-class but in the IL-18/IL-12 group more IgG2a. Therefore, because of the important role of IgG1 in skin lesion the lesions were induced only in IL-18 treated mice. It can also be inferred that the Ig deposition is a necessary factor in the skin lesion induction but it is not sufficient and other factors are also necessary to induce skin lesion in SLE.

Although early studies suggested that SLE was associated with defective apoptosis (Cohen & Eisenberg, 1991; Elkon, 1994), current evidence suggests the opposite (Mysler et al., 1994; Salmon & Gordon, 1999; Petri et al., 2000; Pickering et al., 2001). It has been shown that the presence of large numbers of apoptotic cells can evoke an immune response. Mevorach and colleagues demonstrated that the intravenous injection of apoptotic thymocytes resulted in the production of autoantibodies to nuclear antigens in the majority of normal mice (Mevorach et al., 1998). In addition, the consequences of a disturbance in the removal of apoptotic cells have been addressed in several studies. The important role that C1q plays in the removal of apoptotic cells was demonstrated in C1q knockout mice (Botto et al., 1998). C1q-/- mice had higher titres of autoantibodies and higher mortality compared with strain-matched controls. Similar findings have been reported in SAP-deficient mice. In mice with a targeted deletion of the SAP gene an autoimmune disease developed, which characterised by the presence of autoantibodies to DNA and chromatin and severe glomerulonephritis (Bickerstaf et al., 1995). Further indications that the removal of apoptotic cells and their antigenic structures is

relevant to the pathogenesis of SLE are delivered by a recent study performed with Dnase1-deficient mice (Napirei et al., 2000).

Keratinocyte apoptosis has been considered a potential mechanism for the induction of the skin lesions and autoantibody production in SLE (Casicola-Rosen and Rosen, 1997). DNA-containing apoptotic blebs can be processed by APC as foreign antigen and directed to class II MHC molecules and may then provide a substrate for autoantibody production with the development of a local, or systemic, inflammatory response. Skin lesions from IL-18-treated mice revealed apoptotic cell death in the epidermis, dermis and epidermo-demal junction. Skin from the IL-12 + IL-18 treated mice showed much less apoptotic cells than the IL-18 treated skin. Therefore, this is another possible reason why skin lesions appear only in the IL-18 treated mice. It is important to appreciate that the increase in apoptosis observed in *lpr* mice, who are *Fas* deficient, may be due to IL-18 inducing apoptosis through pathways rather than Fas/FasL or more defect in the removal of apoptotic cells. These data are supported by Yamanaka and colleagues (2000) who showed that skin-specific caspace-1 transgenic mice have cutaneous apoptosis, independent of Fas/FasL pathway, accompanied by a high serum level of IL-18.

Keratinocytes from SLE patients express more IL-18 than those from normal individuals and it is reported that keratinocytes constitutively produce pro-IL-18 (Stoll et al., 1997). Therefore, one may speculate that a possible mechanism in the pathogenesis of skin lesions in lupus patients is higher production of pro-IL-18 by keratinocytes. Sun, ultraviolet light or other environmental factors may change pro-IL-18 to bioactive IL-18 ,which then induces apoptosis and thus activate skin lesion in lupus. Taken together, it seems that IL-18 is an important cytokine in the pathogenesis of lupus skin lesion and may be a novel therapeutic target.

Chapter 7

General discussion

Introduction

Systemic lupus erythematosus (SLE), the prototypic systemic spontaneous autoimmune disease, has the potential to involve multiple organ systems directly with extremely diverse and variable clinical manifestations (Kotzin and O'Dell, 1996). Some patients demonstrate predominantly skin rash and joint pain others suffer from severe and progressive glomerulonephritis, the common denominator being elevated serum antibodies to nuclear constituents. Studies using animal models have contributed greatly to the elucidation of SLE pathogenesis. MRL/MP-lpr-lpr (MRL/*lpr*) mice develop a spontaneous autoimmune disease and have been used extensively as a model for clinical SLE. The disease is characterised by lymphadenopathy, autoantibody production, and inflammatory manifestations such as nephritis, vasculitis, and arthritis (Andrew et al., 1978; Cohen and Eisenberg, 1991). The cause of the disease is likely multifactorial, including a single gene mutation (*lpr*) of the *fas* apoptosis gene on mouse chromosome 19 (Watanabe-Fukunaga et al., 1992; Watson et al., 1992) and background genes from the MRL strain (Andrew et al., 1978; Watson et al., 1992).

An impressive range of clinical and experimental evidence supports a critical role of T cells in disease manifestation of SLE. MRL/*lpr* mice deficient in T cells did not produce autoantibody and glomerulonephritis (Steinberg et al., 1980; Singh et al., 1998). Disruption of T cell activation by blocking CD28-B7 (Finck et al., 1994; Nakajima et al., 1995) or CD40-CD40L (Datta and Kalled, 1997; Diakh et al., 1997) interactions prevented SLE in the mouse. Furthermore, CD4+ T cells appear to be of paramount important as CD4-deficiency (Chesnutt et al., 1998) and anti-MHC class II-TCR antibody (Adelman et al., 1983; Santoro et al., 1988) blocked autoantibody production and ameliorated disease progression. However, the relative role of Th1 and Th2 cell in the pathogenesis of SLE remains controversial. Although IL-4 has been implicated in disease pathogenesis (Shirai et al., 1995; Santiago et al., 1997; Nakajima et al., 1997), elevated levels of IFNγ have been consistently associated with SLE (Tsokos et al., 1986; Takahashi et al., 1996). IFNγ and IFNγR knockout mice have

significantly delayed onset and reduced severity in disease development (Balomenos et al., 1998; Haas et al., 1998), whereas administration of rmIFNγ accelerated the disease progression (Jacob et al., 1987). Nitric oxide (NO) (Weinberg et al., 1994; Huang et al., 1996) and IL-12 have also been shown to play a pathological role in murine SLE. Thus IL-12 induces the differentiation of Th1 cells which produce IFNγ that activates macrophages to produce high levels of NO which, at least in part, causes the pathology in SLE (Weinberg et al., 1994).

7.1 Role of IL-12 and NO in SLE

The aim of this study in chapter 3 was to investigate whether IL-12 and NO have the same effect in a lupus-like model with intact Fas (NZB/W) F1 mice and in human SLE. The results demonstrate that in NZB/W F1 mice total IL-12 (40/p70) serum levels are higher than in control mice. IL-12 is increased in correlation with disease of this lupus-like strain. Increase in IL-12 coincides well with the time of renal disease onset at around 7 months age (Theofilopoulos et al., 1986). The data are supported by the results from MRL/*lpr* mice (Huang et al., 1996).

The human data show that the serum level of total IL-12 is significantly higher in SLE patients than control individuals. However, PBMC from SLE patients produced less IL-12 (p70/p40) than control PBMC in contrast with the serum data. However, whole blood culture from SLE patients showed higher IL-12 production, when cultured with LPS and IFN-γ, compared with control individuals, consistent with the serum data . The reason for different results in PBMC and whole blood cell culture might be because of (1) the role of granolucytes (specially neutrophils), which are deleted in PMBC, or (2) possibly because of other factors such as different proteins or cytokines which are found in the plasma of patients and washed away during PBMC purification, and (3) the important role of cell contacts in immunological reaction is another possible explanation for different results obtained from PBMC and whole blood cell cultures. In MRL/*lpr* mice both total IL-12 and IL-12 p70 increased and disease related, but in NZB/W F1 mice and human SLE we detected just total

IL-12 enhancement and IL-12 p70 was undetectable. It might be because of very low level of p70 that can show its bioactivity even in concentration 100 times less than p40 homodimer. Another possibility is that in the patients and lupus like models there are high level of p40 homodimer which act as antagonists to bioactive IL-12 (p70) and inhibit Th1 cytokines to reduce the inflammatory response in SLE. However , to elucidate the precise role of IL-12 in SLE, further investigation should be focused on the bioactive IL-12 p70 itself and the potential pathogenic role it may play. We should note that there were wide deviations in the levels of IL-12 among patients. However, there was no correlation between IL-12 serum level and activity of the disease and despite a correlation between level of IL-12 and proteinuria and kidney involvement in lupus-like models (NZB/W F1 and MRL/*lpr* mice). In human SLE patients no significant correlation between IL-12 level and kidney involvement or C3, C4 level was found. However, about six patients with high levels of IL-12 had the highest scores for disease activity.

The serum level of Nitric oxide in MRL/lpr mice appeared to be correlated with age and disease (Huang et al., 1996). However, the level of NO in the serum of SLE patients and NZB/W mice were measured by two different methods and they showed no significant differences from normal controls. In vitro studies did not show any significant changes for NO production when stimulated with LPS and IFN-γ or IL-12 in both human SLE patients and NZB/W mice. Therefore, the data could not show the same phenomenon observed in MRL/*lpr* mice. We postulate that local tissue specific NO production might be important, especially for the development of renal pathology. Cells most likely to contribute to local NO production in the kidney are vascular endothelial cells and mesangial cells (Lincoln et al., 1997) which were not examined in this experiment.

Because of the diversity of the disease and effects of drug treatments, the interpretation of cytokine serum levels is not easy and it is difficult to relate directly the increase of IL-12 in this study to the pathogenesis of SLE. However, we should consider the indirect effect of

higher levels of IL-12 and to investigate its relation with other Th1 & Th2 cytokines abnormality in the pathogenesis of SLE.

7.2 Association of IL-18 and SLE

Like IL-12, IL-18 is a member of the Th1-inducing family of cytokines. Recent evidence clearly demonstrates that several factors are required for optimal induction of Th1 activity, chief among them are IL-12 and IL-18. IL-18 is a member of the IL-1 cytokine family (Kohno and Kurimoto et al., 1998). Pro-IL-18 is cleaved by IL-1 β-converting enzyme (ICE, caspase 1) to yield an active 18 kDa glycoprotein (Ghayur et al., 1997) that recognises a heterodimeric receptor, consisting of unique α (IL-1Rrp) and non-binding β (AcPL) signalling chains (Torigoe et al., 1997; Dinarell, 1999) that are widely expressed on cells implicated in both innate and adoptive immunity. IL-18 is expressed in various cell types, including macrophages, dentritic cells, keratinocytes, osteoblasts, pituitary gland cells, adrenal cortical cells, intestinal epithelial cells, skin cells and brain cells (Okamura et al., 1995; Stoll et al., 1997, 1997; Udagawa et al., 1997; Olee et al., 1999; Conti et al., 1999). IL-18 is capable of promoting proliferation and IFNγ production by Th1, CD8+ and NK cells in mice and in human (Okamura et al., 1995). It shares some of the biological activities with IL-12, but without significant structural homology, and serves as a costimulatory factor in the activation of Th1 cells (Kohno et al., 1997). It does not drive Th1 cell development but induces IL-12R expression (Xu et al., 1998) and thus synergies with IL-12 for IFNγ production (Robinson et al., 1997).

IL-18 expression has been reported in several human diseases, including rheumatoid arthritis (Stoll et al., 1998; Gracie et al., 1999) and inflammatory bowel disease (Pizarro et al., 1999; Monteleone et al., 1999). However, a functional role of IL-18 in clinical or murine SLE is

unknown. The aim of studies in chapters 4,5 and 6 was to investigate whether there was any association between IL-18 and the pathogenesis of SLE.

To determine the potential pathogenic role of IL-18 in SLE, we investigated IL-18 production in SLE patients and in MRL/*lpr* mice, which develop spontaneous lupus-like autoimmune disease.

The presented data showed significantly elevated IL-18 serum levels in SLE patients compared with healthy controls. However, there was no significant relation between IL-18 levels and disease activity indices (SLEDAI) which can be because of: (1) Clinical manifestations of SLE are extremely diverse and variable. Therefore, the number of patients was not enough to analyse relation between each clinical indices with IL-18 serum levels. (2) The change of each cytokine varied among the SLE patients, possibly because of the diversity in the disease and effect of drug treatments (Huang et al., 1988; Linker-Israeli et al., 1991). (3) Another possibility is that the IL-18, which was detected in the serum of SLE patients, was mainly pro-IL-18. Therefore, it needs to be cleaved by IL-1ß-converting enzyme (ICE, caspase 1) to yield an active IL-18 (Gu et al., 1999).

Animal models have contributed greatly to understanding of the immunological aspects of autoimmune diseases. Lupus-like models are a good reflection of human SLE because the main immunological abnormalities, which are related to the human disease also, appear in these mouse models. Therefore, to determine the potential pathogenic role of IL-18 in SLE, we investigated IL-18 production in MRL/*lpr* mice, which develop spontaneous lupus-like autoimmune disease.

In vitro study presented in this thesis shows that serum of MRL/*lpr* mice at the age of 4-6 months (with obvious glomerolunephritis) contained significantly higher concentration of IL-18 compared with those of control MRL/++ mice. In addition, spleen and peritoneal cells from MRL/*lpr* mice produced spontaneously higher concentration of IL-18 than cells from

MRL/++ mice. These results are therefore consistent with that of human SLE and re-enforced a major role for IL-18 in spontaneous autoimmune disease.

Potential role of IL-18 in the pathogenesis of SLE was next investigated *in vivo* using MRL/*lpr* lupus mice. A recombinant murine IL-18 was used for *in vivo* studies. My data suggest that rmIL-18 promotes spontaneous development of lupus-like glomerulonephritis, vasculitis and skin lesions. Recombinant IL-18 also induced a facial rash, resembling malar rash in human SLE, at the early stage of the disease. This acceleration of the disease occurred in association with marked changes in the Th1 and Th2 cytokines and in the IgG subclasses of anti-DNA antibodies.

After two weeks treatment, serum from MRL/*lpr* mice injected with IL-18 contained significantly raised level of IL-5. This was undetectable in the serum of the PBS or IL-12 + IL-18 treated groups. In contrast, mice treated with IL-12 + IL-18 produced high levels of IFN-γ, which was undetectable in the PBS or IL-18-treated mice. IL-18 treated groups also produced markedly higher levels of anti-DNA antibodies compared with the other two groups. Thus, it shows that IL-18 administration augmented the Th2 responses, whereas the combination of IL-12 and IL-18 induced a predominant Th1 activity. However, after 8 weeks treatment, the cytokine profile changed. Although IFN-γ was higher than the control PBS group it showed a shift more towards Th1 response, the level of IgG1 anti-DNA antibodies in the serum of the IL-18 treated group was significantly higher than both the PBS and IL-12/IL-18 groups. IL-18, therefore has a synergistic effect with IL-12 when injected i.p. in IFN-γ production.

These data indicates that IL-18 at the early stages of administration induced a strong Th2-type cytokine response in *lpr* mice. This shifted to a more Th1 dominant response over time. We speculate here that the effects of IL-18 on different age of MRL/*lpr* mice vary because of endogenous level of IL-12. As shown previously in Chapter 3 and by Huang and colleagues

(1996), IL-12 increases with age in MRL/*lpr* mice. Therefore at the early stages of the disease, when there was low levels of IL-12, IL-18 induced a Th2 dominant response. When the mice were older, because of higher level of endogenous IL-12 in a synergistic effect with IL-18, they induced higher amount of IFN-γ. The results from the IL-18 plus IL-12 treated mice support this hypothesis. Mice in this group at all stages of disease showed a Th1 dominant response. Several recent reports now show that IL-18-mediated effects on T cells may extend beyond Th1 differentiation to include induction of type 2 cytokine production (Yoshimoto et al., 2000; Wild et al., 2000; Hoshino et al., 2000).

It is significant that a relatively enhanced activation of Th1 vs. Th2 type cells leads to increased production of IgG2a and IgG3, but to a diminished production of IgG1. It is associated with lupus nephritis in MRL mice bearing the *lpr* or *Yaa* gene. This is highly relevant to the immunopathogenesis of lupus nephritis and vasculitis. Since murine IgG2a, but not IgG1, antibodies activate the complement system, the complement activating IgG2a autoantibodies can be more nephritogenic than IgG1 autoantibodies (Takahashi et al., 1996; Berney et al., 1992; Takahashi et al., 1991). Therefore, IL-18 alone or in synergism with IL-12 enhances glomerolunephritis in lupus mice by increasing IgG2a anti-DNA Antibodies. Another possible mechanism for the involvement of IL-18 in lupus nephritis and vasculitis is due to IFN-γ production. IL-18 induces IFN-γ production that up-regulates MHC class II on antigen presenting cells, leading to effective presentation of T-cell epitopes and the activation of T-cells (Halloran et al., 1992). The results from IFN-γ -/- MRL/*lpr* and mercury-treated IFNγ (-/-) mice show that this cytokine is required for auto-antibody generation of either Th1 or Th2 associated isotypes, probably by enhancing auto-antigen presentation (Balmenos et al., 1998; Haas et al., 1998).

In conclusion, in conjunction with previous investigations (Jacob et al., 1987; Ozmen et al., 1995; Takahashi et al., 1996; Huang et al., 1996 and Balomenos et al., 1998) this study strongly points to the significant contribution of heightened IFN-γ production in the pathogenesis of murine lupus nephritis and vasculitis. Other cytokines such as TNF-α (Jacob

et al. 1988), IL-12 (Huang et al., 1996) and NO (Weinberger et al., 1997) have also been experimentally manipulated to affect serologic and histologic manifestation of murine lupus. In this regard, IL-18 as a cytokine that can regulate NO and pro-inflammatory cytokines might be important candidate in SLE pathogenesis.

MRL/*lpr* mice injected with IL-18 showed facial skin lesions at age of 9 weeks. The MRL/*lpr* mice treated with PBS or IL-18 + IL-12 did not show any skin lesions. The IL-18/IL-12 treated mice who showed a stronger Th1 responses and more Th2 suppression (IL-10, IL-5 and IgG1 anti-DNA antibodies) than IL-18 treated mice, did not show any skin lesions. However, IL-18/IL-12 treated mice showed an accelerated glomerulonephritis and vasculitis even more than treated IL-18 mice. Therefore, it can be concluded that the skin lesions in SLE have a different pathogenesis from the glomerolunephritis and vasculitis.

Patients with cutaneous manifestations of lupus are more likely to have mRNA for IL-2, IL-4 and IL-5 in skin biopsy specimens than healthy controls where mRNA for IL-5, IL-4 and IL-2 is undetectable (Sauder et al., 1993; Nuremberg et al., 1995). IgG1 subclass antibodies are believed to be important in the pathogenesis of skin lesions in SLE (Furukuwa et al., 1983; 1984). From these findings, we conclude that one reason why the skin lesion occurs only in the IL-18 group at this stage of the disease is because of induction Th2 type responses at an early stage of disease development. Since IL-18 induces significantly higher amounts of IgG1 anti-DNA compared with the PBS and IL-12 + IL-18, the skin lesion is induced only in this IL-18 treated group. However, for glomerulonephritis IgG2a antibodies are more pathogenic than IgG1, therefore, this is accelerated in both the IL-18 treated and the IL-18/IL-12 group.

In immunohistochemical studies, there was more Ig deposition in the skin sections from the IL-18 and IL-18/IL-12 groups than the PBS group. One possible reason that despite Ig deposition in the IL-18/IL-12 group, the skin lesion was induced only in the IL-18 treated

mice, is that the Ig subclasses in the IL-18 group were mainly IgG1 sub-class but in the IL-18/IL-12 group more IgG2a. Therefore, because of the important role of IgG1 in skin lesion the lesions were induced only in IL-18 treated mice. It can also be inferred that the Ig deposition is a necessary factor in the skin lesion induction but it is not sufficient and other factors are also necessary to induce skin lesion in SLE.

Despite the studies which consider SLE as a disease of defective apoptosis (Cohen & Eisenberg, 1991; Elkon, 1994), evidence suggests the opposite (Mysler et al., 1994; Salmon & Gordon, 1999). Keratinocyte apoptosis has been considered a potential mechanism for the induction of the skin lesions and autoantibody production in SLE (Casicola-Rosen and Rosen, 1997). Skin lesions from IL-18-treated mice revealed apoptotic cell death in the epidermis, dermis and epidermo-demal junction. Skin from the IL-12 + IL-18 treated mice showed less apoptotic cells than the IL-18 treated skin. Therefore, this is another possible reason why skin lesions appear only in the IL-18 treated mice. It is important to appreciate that the increase in apoptosis observed in *lpr* mice, which are *Fas* deficient, may be due to IL-18 inducing apoptosis through pathways rather than Fas/FasL. These data are supported by Yamanaka and colleagues (2000) who showed that skin-specific caspace-1 transgenic mice have cutaneous apoptosis, independent of Fas/FasL pathway, accompanied by a high serum level of IL-18.

Keratinocytes from SLE patients express more IL-18 than those from normal individuals and it is reported that keratinocytes constitutively produce pro-IL-18 (Stoll et al., 1997). Therefore, one may speculate that a possible mechanism in the pathogenesis of skin lesions in lupus patients is higher production of pro-IL-18 by keratinocytes. Sun, ultraviolet light or other environmental factors may change pro-IL-18 to bioactive IL-18, which then induces apoptosis, and thus activate skin lesion in lupus. Taken together, it seems that IL-18 is also an important cytokine in the pathogenesis of lupus skin lesion and may be a novel therapeutic target.

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Appendix

Appendix I Preparation of buffers

Buffers

1. PBS (x 10 stock)

80g NaCl
11.6g NaH_2PO_4
2g KCl
2g KH_2PO_4
Make up to 1000 ml with dH_2O

2. PBS / Tween

0.5 ml Tween 20
1000 ml PBS (x1)

3. Coating buffer (ELISA)

0.1M $NaHCO_3$, pH 8.2

4. Vector Kit buffer

0.1M Tris-HCl, pH 8.2

5. TBS (ICC Wash buffer)

900 ml 0.9% NaCl
100 ml 50mM Tris-HCl, pH 3.6
50 mM Tris-HCl
- Add conc HCl to 25 ml 0.2M tris-HCl to pH 7.36
Make up to 100 ml with dH_2O

6. Paraformaldehyde

1g PFA
100 ml PBS
100 µl 2M NaOH
Heat to 60°C then gently mix until solution clears.

7. Fast red solution

A 100 mg naphthol-AS-MX phosphate
5 ml N.N.-dimethylformamide
100 ml TBS, pH8.2

B 0.1M levamisole in dH_2O

8. NSE stain phosphate buffers

A 9.08 g/l KH_2PO_4 (0.067M)

B 11.9 g/l Na_2HPO_4, $2H_2O$ (0.067M)

Add 98.5 ml of A to 1.5 ml of B.

9. Griess Reaction

A 0.1% α-naphthyl-amine in dH_2O

B 1% sulfanilamide in 5% phosphoric acid

Mix equal volumes for Griess reagent
Store away from light

10. Solution B

For 200 ml solution

10 M	Urea	(160 ml)
2 M	Tris. Hcl pH=8.0	(2 ml)
2M	NaH2PO4	(10 ml)
Adjust pH to 8.0 with NaOH (2N)		
Distilled water		(28 ml)

11 Buffer C (200 ml)

10 M	Urea	(160 ml)
2M	NaH2PO4	(10 ml)
2 M	Tris-Hcl Ph 8.0	(2 ml)
Adjust Ph to 6.3		
Add distilled water		(28 ml)

12. Elution Buffer

Buffer C plus 100 mM EDTA