CYTOKINE REGULATION IN SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

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ABBREVIATIONS

+ve	Positive control
-ve	Negative control
2-ME	2-Mercaptoethanol
^{3}H-TdR	Tritiated thymidine
<	Less than
>	Greater than
AEC	3-amino-9-ethyl carbazole
BCIP	5-bromo-4-chloro-3-indoyl phosphate
AP	Alkaline phosphatase
B-cell	Bone marrow derived lymphocyte
BBS	Borate-buffered saline
BSA	Bovine serum albumin
CD	Cluster determinant
CFA	Complete Freund's adjuvant
Con A	Concanavalin A
cpm	Counts per minute
CTLL	Cytotoxic T lymphocyte line
dATP	2'-Deoxyadenosine 5'-triphosphate
dCTP	2'-Deoxycytidine 5'-triphosphate
ddH2O	Double distilled water
dGTP	2'-Deoxyguanosine 5'-triphosphate
dH2O	Distilled water
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
DN	Double negative
DNA	Deoxyribose nucleic acid
dNTPs	2'-Deoxyribonucleoside 5'-triphosphates
ds	Double stranded
DTT	1, 4-dithiothreitol
dTTP	2'-Deoxythymidine 5'-triphosphate
EDTA	Ethylenediaminotetraacetic acid
ELISA	Enzyme linked immunosorbent assay
F(ab')2	Antigen-binding fragment of an Ig
FACS	Fluorescence-activated cell sorter
FCS	Foetal calf serum

FAD	Flavin adenine dinucleotide
FITC	Fluorescein isothiocyanate
HBSS	Hanks' balanced salt solution
HEPES	N-2-[hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
HRP	Horseradish peroxidase
i.u.	International units
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-2R	IL-2 receptor
KPI	Kidney Pathology Index
lpr	Lymphoproliferation
LPS	Lipopolysaccharide
M	Molar
M-MLV	Moloney murine leukemia virus
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NADPH	β-Nicotinamide adenine dinucleotide phosphate
NBT	p-nitroblue tetrazolium chloride
NGS	Normal goat serum
NK	Natural Killer
NO	Nitric oxide
$NO2^-$	Nitrite
$NO3^-$	Nitrate
NSS	Normal sheep serum
OD	Optical density
OPD	Orthophenylene diamine
p-NPP	p-nitrophenyl phosphate
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PMA	Phorbol myristic acetate
ppb	Parts per billion
PWM	Pokeweed mitogen
R	Receptor
RNA	Ribonucleic acid

RNasin	Ribonuclease inhibitor
rpm	Revolutions per minute
RT	Room temperature
SD	Standard deviation
SLE	Systemic lupus erythematosus
ss	Single stranded
T-cell	Thymus derived lymphocyte
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
TUI	Tumour Index
U	Units
v/v	Volume per volume
vs	Versus

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SUMMARY

The origin of the defects leading to pathogenicity in systemic lupus erythematosus (SLE), an autoimmune disorder characterised by multi-system involvement, female preference, B-cell hyperactivity, autoantibody production and immune complex deposition, 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 being beneficial or rather harmful to the patients. These treatments that may achieve a temporary symptomatic relief do not cure the disease and are often followed by problematic side effects including infections. The underlying mechanism for the development of the disease is yet to be clarified. There is, however, some evidence that defects in the ability of lymphocytes to produce or respond to cytokines may disturb the finely balanced immune system resulting in abnormal B-cell regulation. In contrast to the B-cell hyperactivity, deficient T-cell functional activity is another prominent feature of the disease. 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 order to understand the mechanism of immune regulation in SLE, experiments were designed, in the present study, to give a detailed analysis of the nature and pathological relevance of those immunological abnormalities, the T-helper cell functional defects in particular.

Much of the work focused on and described in this thesis was based mainly on two well established murine models of SLE including an early-life (MRL/lpr) model and a later-life (NZB/W) disease model. Both the NZB/W and the MRL/lpr strains are mutant mice which spontaneously develop a disease similar to human SLE. Female mice of different ages were used in the study and the normal control strains were sex and age-matched BALB/c and CBA mice. Results from studies using lymphocytes and

serum samples from SLE patients are also described and compared with the studies on the mouse models. The study included both *in vitro* and *in vivo* approaches.

Results from the *in vitro* studies indicate that T-cell, particularly T-helper, functions are severely impaired in the lupus mice. These include abnormal production of IL-2, IFN-γ and IL-4, inability of T-cells to express functional high affinity IL-2R and to proliferate in response to Con A stimulation, confirming and extending previous studies by other investigators. Since the stage of development at which these defects occur is not clearly understood, efforts have been devoted to analysis of lymphocytes from lupus mice at different ages, in relation to the disease activity. Attention has been particularly focused on young lupus mice including mice of one week old, before the onset of clinical disease. Evidence is given indicating an early onset of the T-helper functional defects and the central role of IL-2 deficiency in the defective T-cell activation. The hyporesponsiveness of lupus T-cells to Con A is due to the inability of T-helper cells to produce IL-2 and this can be bypassed by exogenous IL-2 *in vitro*. Addition of IL-2 during Con A activation restores fully the ability of lupus T-cells to proliferate, upregulates IL-2R expression and increases the frequency of IFN-γ secretors resulting in normalised levels of IFN-γ secretion. T-cell phenotype analysis indicates that IL-2 preferentially promotes the expansion of the functionally more mature single positive cell population while inhibiting the growth of the characteristic $CD3^+CD4^-CD8^-$ (double negative) cells in MRL/lpr mice.

Addition of IL-1 during Con A stimulation of T-cells did not restore the IL-2 production defect in the lupus mice. To assess the function of accessory cells in the lupus mice, replacement of the adherent cells from MRL/lpr mice by adherent cells from H-2 compatible normal mice also failed to bypass the T-cell activation defect. However, some serum factors which affect the growth of the IL-2 dependent CTLL cell line were found in sera and plasma from SLE patients with active disease and some lupus mice. At high concentrations, these factors inhibit CTLL cell proliferation in

response to IL-2 but, at low concentrations they synergise with IL-2 to stimulate CTLL cells to proliferate. Characterisation of the serum components shows that these factors bind to CTLL cells but not to Protein A, and are heat labile but not neutralised by IL-2. In addition, serum nitric oxide levels were increased in MRL/lpr mice, but decreased in NZB/W mice, compared to the BALB/c controls at all ages.

The study of cytokine mRNA expression by PCR shows that spleen cells freshly isolated from both lupus and normal mice do not spontaneously express mRNA for IL-2 or IL-4 but do express IFN-γ. IL-6 mRNA was only detected in the cells from BALB/c and MRL/lpr but not NZB/W mice. However, Con A-induced mRNA expression for all the above T-cell cytokines was detectable in these mice. There was no increased expression for any of these cytokines in the two strains of lupus mice compared to the normal controls, although the quantitative information provided by PCR may be limited and is to be confirmed. On the contrary, Con A-induced mRNA expression for IL-2 and IL-4 was clearly reduced in the MRL/lpr and to less degree in the NZB/W mice (25 weeks old).

To determine the *in vivo* effects of IL-2 and TGF-β on the development of lupus disease in MRL/lpr mice, a non-virulent live *S. typhimurium aro*$^-$ mutant has been used as a vector to deliver cytokines by oral administration of IL-2 or TGF-β1 gene transfected bacteria. Preliminary results from small scale pilot experiments show that TGF-β1 suppressed Con A-induced T-cell proliferation, IL-2 and IFN-γ production in normal and lupus mice. MRL/lpr mice had delayed onset of glomerulonephritis. However, TGF-β1-treated MRL/lpr mice later developed increased titres of anti-ss and anti-dsDNA and glomerulonephritis. In contrast, MRL/lpr mice treated with IL-2 showed reduced levels of serum anti-ssDNA, a significant decrease in the abnormal double negative cell population, suppressed IFN-γ production and reduced glomerulonephritis.

In general, the study indicates a crucial role for the Th1 functional defect and therapeutic potential of IL-2 in SLE. It sheds some doubt on the current clinical treatments by non-specific immunosuppressive drugs and favours approaches which may enhance the endogenous capacity of the immune system to carry out its proper functions in maintaining self-tolerance. The need for new insights into the underlying mechanism of the disease and more consideration of the immune deficient aspects of autoimmune phenomena as a whole for therapeutic strategies are discussed.

Chapter 1

INTRODUCTION

1.1 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE), formerly named 'disseminated lupus erythematosus', is a non-organ-specific autoimmune disease of unknown aetiology. It is one of a group of related systemic rheumatic diseases including rheumatoid arthritis and Sjogren's syndrome. The disease has been recognised for centuries and yet has been studied in depth only in relatively recent years largely owing to the establishment of good animal models.

1.1.1 Clinical repertoire and the natural history of SLE

SLE is characterised clinically by a multisystem involvement affecting a variety of tissues and organs. One of the typical clinical manifestations of the disease is erythematosus, the so called butterfly rash. In Latin, the term 'lupus' means wolf and it has been used since mediaeval times to described the erythemic ulcerations which can 'eat away' the face (Morrow and Isenberg, 1987). Many other organs and tissues, such as joints, blood vessels, muscles, nerves, kidneys, lung, heart, and spleen may also be affected during disease development causing a variety of disorders. Glomerulonephritis is a severe complication of the renal involvement which is believed to be caused by deposition of immune complexes and complement activation. In human SLE, although glomerulonephritis is not necessarily manifested in the clinical features of many patients it is one of the major contributions to death (Corriea *et al.*, 1984). The full spectrum of the disease has been described in details by Morrow and Isenberg (1987) illustrating a broad and complex clinical picture. As the disease may be presented in many ways, it makes SLE potentially difficult to diagnose. The criteria set by the American Rheumatism Association (ARA) for the classification and diagnosis of SLE (Tan, 1982; Tan et al., 1983) include the number of clinical manifestations which reflect the features of multisystem involvement and non-organ-specificity of the disease.

The disease has a high incidence in young women, particularly in females of child-bearing age or after puberty. In general, it has been estimated that approximately 9 out of 10 SLE sufferers are females suggesting a possible role of sex hormones in the disease development. In a SLE mouse model (MRL/*lpr*), disease-limiting effects of an aromatase inhibitor, 4-OHA (4-hydroxyandrostenedione) which blocks oestrogen formation, has been reported (Greenstein *et al.*, 1993). However, there are some families in which the disease occurs mainly in males (Lahita *et al.*, 1983). The heritability of the disease in humans is still not certain. Several family occurrence studies have described a higher incidence of SLE in close family relatives of patients (Brunjes *et al.*, 1961), even in twins (Bloch *et al.*, 1976), but results from other reports suggest little or no such relationship (Winchester, 1983). Ethnic variation has also been observed as another feature of the disease. According to the study by Serdula and Rhoads (1979), the prevalence rates per million population at risk are: 241 in ethnic Chinese, 204 in mixed Hawaiian, 199 in ethnic Filipino, 182 in ethnic Japanese and 58 in Caucasian. Among females, the prevalence rates per million may vary from 4000 in Jamaica to 232 in New Zealand (Morrow and Isenberg, 1987). Lupus may affect young people and it has been reported in children as young as 3 years old. The clinical manifestations of juvenile lupus resemble their adult counterpart, except that it is often more severe. Although women with lupus may have normal fertility, children born to mothers with lupus have been shown to have an increased risk of congenital heart disease (Esscher and Scott, 1979).

Since the cause of the disease is still unknown, clinical treatments for SLE have been mainly based upon achieving symptomatic relief rather than cure. There are four main groups of drugs including the non-steroid anti-inflammatory drugs (NSAID), the anti-malarial and cytotoxic drugs (Klippel, 1992) and corticosteroids (Kimberly, 1992). The therapeutic strategies are usually designed for a general suppression of the immune

system because of the prominent features of B-cell hyperactivity and the non-specific organ involvements.

1.1.2 Serological abnormalities

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

A diversity of antibodies to self antigens present in the blood of lupus patients and the animal models is one of the most prominent features in SLE. These autoantibodies may bind to an extensive array of molecules including nuclear antigens (DNA, RNA, RNP, Sm, histones and non-histone chromatin proteins), phospholipids, cytoplasmic and cell surface components, and even immunoglobulin G (rheumatoid factor). Among them, the anti-nuclear antibodies (ANA) have attracted most attention and therefore been most extensively studied. It is now nearly forty years since the coincidental discovery of anti-DNA antibodies in the serum of lupus patients by many independent research groups and laboratories (Friou, 1957; Cepellini *et al.*, 1957; Miescher and Straessle, 1957; Robbins *et al.*, 1957; Seiligmann, 1957). Clinically, anti-dsDNA are diagnostic of SLE since they can be detected in majority of lupus patients and rarely occur in other conditions (Buskila and Shoenfeld, 1992). In order to understand their pathological and pathogenic significance, much effort has been made to analyse structurally and functionally the nature of these antibodies.

SLE has been long regarded as a polyclonal autoimmune disease due to the variety of antibody specificities detected. However, recent studies using isoelectric focusing (IEF) technique have revealed that the anti-DNA antibodies in the sera of SLE patients and the mouse models (Stott *et al.*, 1986; 1988, 1990), as well as the serum anti-Sm antibodies in MRL/*lpr* mice (Williams *et al.*, 1986), are clonally restricted. In

agreement with this notion, the anti-DNA B-cells in the MRL/*lpr* mice were also found to be clonally restricted (Shlomchik *et al.*, 1990). More recently, the anti-DNA-secreting B-cell clones of SLE patients have been shown to be not only restricted in number, but also stable and long lived (Stott, 1992). The findings are striking in regard to the wide range of antibody specificities and clinical features of the systemic involvement of the disease. Since monoclonal anti-DNA antibodies derived from lupus mice and SLE patients exhibit extensive cross-reactivity, a possibly false impression of polyclonal activation is therefore suggested. A study by Raz et al. (1993) showed that anti-DNA, but not anti-RNA or anti-histone, antibodies secreted by hybridoma cell lines derived from spleen cells of NZB/W F1 female mice bound to membrane proteins of several different cell types. By opposing the hypothesis of an environmental mitogen-driven B-cell polyclonal activation, the evidence indicates an intrinsic immunological defect in SLE. It is postulated that such a clonally restricted autoimmune response might be due to somatic mutation of clones from anti-non-self to anti-self, or otherwise due to some limited number of potentially self-reactive clones which escape from a defective immune tolerant mechanism (Stott, 1992).

Circulating immune complexes (CICs) are commonly observed in both SLE patients and lupus mouse models. CICs have been shown to consist of nucleic acid and serum autoantibodies of corresponding specificity. Three types of CICs identified are dsDNA-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 inflammatory mechanisms. These include complement fixation and recruitment of inflammatory cells resulting in tissue damage. There have been many attempts to elucidate the role of immune complexes in the pathogenesis of renal damage. DNA antigens and antibodies have been demonstrated in immune complexes in kidneys with proliferative and membranous lupus nephritis (Agnello *et al.*, 1973, 1976; Andres *et al.*, 1970). The determinants responsible for the deposition, in particular the precise location of immune complexes,

are still not clear. However, antibody avidity and class, antigen/antibody ratios and size of DNA have been shown to be important in determining the deposition of immune complexes (Morrow and Isenberg, 1987).

Changes in serum complement levels, reduced early reacting components (C1-4) but increased final products (C5-9), are associated with the lupus disease activity. This has been suggested to be a consequence of complement activation. Many studies have demonstrated that complement is consumed by immune complexes and localised in the tissues, particularly the kidney. In addition, in contrast to patients with rheumatoid arthritis demonstrating high levels of serum acute phase proteins, C-reactive protein (CRP) is low in SLE unless there is a superimposed infection. Other acute phase proteins like amyloid P component are elevated in many other autoimmune diseases but not in SLE (Pepys et al. 1978). Although the serological abnormalities share many common features with other autoimmune diseases, these are well recognised outstanding characteristics of lupus.

1.1.3 Immunopathological features and aetiological considerations

i) Adoptive transfer of SLE

The origin of the defect leading to autoaggression in SLE is still an unsolved puzzle. However, in the mouse models of SLE, it has been clearly demonstrated that lupus is caused solely by a defect within the lymphoid system. The evidence is that the disease can be transferred by transplantation of the lymphoid cells (spleen, liver or bone marrow cells) between lupus and H-2 compatible lethally irradiated normal mice. On the basis of many experimental models, several studies have shown that, as well as its transferable nature, many features of disease expression by donors are also adoptable by the recipients, such as differences in the onset of clinical disease, the pace of disease development and the sex preferences (Theofilopoulos and Dixon, 1985). Conversely,

lethally irradiated lupus strain of mice (NZB) which received normal bone marrow from a H-2 compatible mice has been shown to possess all the characteristics of the normal donor (Jyonouchi *et al.*, 1981). Another very important finding was that spleen cells from old lupus mice with clear-cut disease do not produce disease in recipients any faster than transfer of bone marrow from premorbid mice (Eisenberg *et al.*, 1980). Thus, the defect seems to exist at stem cell level and is carried through the animals' life. In addition, a similar study by Akizuki et al. (1978) showed that the disease could even be transferred into H-2 incompatible nonautoimmune lethally irradiated recipients when the lupus mouse (NZB/W) bone marrow cells were pre-treated with anti-Thy 1.2 serum plus complement to eliminate differentiated T-cells. These studies indicate that murine lupus haematopoietic stem cells or lymphoid processor cells are inherently defective and possess all that is necessary for the expression of the disease. A variety of studies has been carried out since to determine the exact nature of the lymphoid defect.

ii) Cellular and functional abnormalities of lymphoid cells

Numerous immunological disorders featuring cellular and functional abnormalities of lymphoid cells are related in SLE (Tsokos, 1992). The two most prominent features are B-cell hyperactivity on one hand reflected by massive autoantibody production, and defective T-cell mediated immunity on the other.

B-cell hyperactivity is one of the immunological markers of the lupus disease. B-cells from the blood of SLE patients spontaneously secrete large amounts of immunoglobulin including antibodies to self antigens in cultures (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. SLE B-cell proliferate vigoriously in the presence of 50 kD BCGF or a recombinant 12 kD BCGF even in the absence of mitogen, although not in the presence of IL-2 and the 20 kD BCGF (Delfrassy et al., 1986; Flescher et al., 1990). The B-cell hyperactivity *in vitro*

resembles its *in vivo* state as indicated by the increased antibody and autoantibody levels in the sera from patients. However, in spite of the increased immune activity in the B-cell compartment, infection is known to be one of the potential causes for disease exacerbation. Although there has not been sufficient evidence to suggest a general increase in susceptibility of lupus patients to infections, it has been shown that lupus B-cells are indeed unable to respond to some antigens by mounting a normal immune response. By sensitising cells with specific antigen *in vitro*, Pelton et al. (1982) showed a decreased production of anti-influenza antibodies by B-cells from SLE patients. A B-cell defect *per se* in lupus is evident.

Owing to the increasing knowledge about the crucial role of T-cells in modulating the B-cell response, the importance of T-cell abnormalities in the development of lupus disease has become more and more emphasised. 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 the existence of defects in the T-cell compartment is the abnormal functional activities of the cells. 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). T-cell lymphopenia is characteristic of patients with lupus and its severity correlates with disease activity (Steinberg *et al.*, 1991). An impaired T-cell-mediated cellular immunity is a prominent immunological feature in both human SLE and the animal models. Decreased autologous mixed lymphocyte reaction (AMLR), and reduced activities of cytotoxic T-cells and NK cells have been common findings in both human lupus and the mouse models (Kuntz *et al.*, 1979; Theofilopoulos and Dixon, 1985; Theofilopoulos, 1992). On the other hand, however, recent studies have identified certain T-cell subsets, T-cells that lack both CD4 and CD8 molecules in SLE patient (Steinberg *et al.*, 1991) and in lupus mouse models (Datta, 1989) which are not MHC restricted, may provide excessive help to 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 is now largely attributed to lack of, or abnormal, T-cell regulation.

iii) Environmental and genetic factors

A number of environmental factors, such as drugs, exposure to UV light, and viral infections, are able to induce or are associated with the disease. Many viruses have been shown to produce various abnormalities found in lupus. Among them, type C RNA viral particles have been suggested as an aetiological agent responsible for SLE. These particles were not only found in large numbers in a lupus strain (NZB) of mice (Mellors and Huang, 1966; East *et al.*, 1967) but also in SLE patients (Strand and August, 1974; Panem *et al.*, 1976). However, the basis for the increased sensitivity and susceptibility of lupus patients and mice to these environmental influences is not clear. Since this virus was later found in some normal strains of mice too, its relation to lupus disease has become questionable.

Genetic analysis of disease susceptibility has been used to study the role of the major histocompatibility complex (MHC) class I and II antigens in SLE. In human SLE, evidence from a large body of studies has established an association of SLE with the class II HLA markers DR2 and DR3. Although the association has been found to be inconsistent in different ethnic groups and between various studies, C4 null alleles are now considered to be a strong genetic determinant which is relatively common in various ethnic groups (Pisetsky, 1991). In the MRL/*lpr* mouse model of SLE, lupus disease has been found to be linked with a defect in a gene controlling Fas antigen which is a cell surface protein involved in cell apoptosis in the thymus (Watanabe-Fukunaga *et al.*, 1992).

1.2 Lupus mice: the animal models of SLE

The availability of animal models of SLE has been an enormous boost in studies of the disease in the last decades. In particular, several lupus strains of mice have been well established and proved to be the best models of the human disease because of their similar clinical, serological and immunological characteristics. These mice spontaneously develop lupus-like symptoms characterised by vasculitis, arthritis, glomerulonephritis and hypergammaglobulinaemia. Previous studies using murine SLE models have provided a good understanding of the disease in many aspects and results from studies by different investigators show considerable agreement (reviewed by: Theofilopoulos and Dixon, 1985; Morrow and Isenberg, 1987; Steinberg *et al.*, 1991; Theofilopoulos, 1992).

1.2.1 The mouse strains

Among several murine strains, the F1 hybrids produced by the mating of New Zealand Black (NZB) with New Zealand White (NZW) mice, [(NZB/NZW) F1] (NZB/W), have been suggested to be one of the best models of human lupus. The NZB ($H\text{-}2^d$) is the first described animal model which develops lupus-like autoimmune disease (Bielschowsky *et al.*, 1959; Helyer and Howie, 1963). This mouse strain is primarily a model for autoimmune haemolytic anaemia. As it manifests many clinical features of lupus disease, such as glomerulonephritis, lymphomas, autoantibodies and immune complexes, the NZB is also considered to be a lupus-prone strain. The NZW ($H\text{-}2^z$) does not develop overt autoimmunity, but its hybrid with the NZB undergoes changes very closely resembling human SLE. Therefore, the NZB/W ($H\text{-}2^{d/z}$) strain has been an popular animal model used in many of the previous studies of SLE.

Two other best known lupus mouse models are MRL/MP-*lpr*/*lpr* (MRL/*lpr*) and BXSB/Mp (BXSB) developed by Murphy and Roths (1979) at the Jackson

Laboratory, USA. The MRL (H-2^k) strain originated as a by-product of a series of crosses involving several inbred strains AKR/J (H-2^k), C57BL/6J (H-2^b), C3H/Di (H-2^k), and LG/J (H-$2^{d/f}$). During the inbreeding, some of the offspring of the 25th generation developed massive generalised lymph node enlargement early in life, while others did not. With subsequent inbreeding, one subline that developed lymphadenopathy was termed MRL/*lpr*, and the other that did not express the lymphoid abnormality was named MRL/n. Although MRL/*lpr* and MRL/n share at least 89% of their genome, a single autosomal recessive gene named lymphoproliferation (*lpr* mapped to Fas) and possibly from the AKR ancestors, has been suggested to control the lymphoproliferation in MRL/*lpr* mice. An advantage of the MRL over the NZB/W is that, unlike the latter whose H-$2^{d/z}$ haplotype is difficult to match with other strains of mice in some studies, the MRL/*lpr* is an inbred strain with one homozygous H-2^k haplotype. It enables studies like the adoptive lymphoid cell transfer where such matching is crucial. The BXSB (H-2^b) is also a recombinant inbred strain derived from a cross between a C57BL/6J (H-2^b) female and a SB/Le (H-2^b) male. A trait or gene(s) on, or linked to, the Y chromosome of BXSB mice derived from SB/Le, was found to profoundly enhance autoimmunity in this strain of lupus mice (cited in: Theofilopoulos and Dixon, 1985).

The NZ, the MRL and the BXSB are three main types of SLE mouse models. Other less known lupus mice include the Moth-eaten (Me) strain developed from a mutation (recessive moth-eaten trait) in the C57BL/6J (Schultz and Zurier, 1978), the Palmerstron-North (PN) strain derived in New Zealand at the Palmerston North Hospital (Walker *et al.*, 1978), and Swan strain that gets its name from Swiss Anti-nuclear as it was originally developed from a Swiss line and selected for breeding according to ANA positivity (Morrow and Isenberg, 1987).

1.2.2 Natural history and immunopathological characteristics

Murine SLE parallels its human counterpart in a wide range of clinical, serological and immunopathological features and therefore provides a good basis for the studies of the disease. Clinically, murine lupus displays features of multi-organ involvement and exhibits symptoms resembling the active phase of human SLE at late stages. Many manifestations such as skin lesions, serum autoantibodies and immune complexes, and glomerulonephritis are found in common among these strains. As mentioned previously, one of the findings from the studies of murine lupus is that the disease is caused by genetically determined abnormalities of the haematopoietic stem or lymphoid cells. These abnormalities are commonly expressed as a generalised B-cell hyperactivity leading to hypergammaglobulinaemia, early IgM to IgG switching, production of autoantibodies against self antigens, and formation of immune complexes. In contrast to lymphoid system hyperplasia, another common feature of lupus mice is, however, early thymic atrophy. The B-cell hyperactivity and the early recessive thymic function have been the two most intriguing features consistently found in all lupus mice, but their possible link in terms of pathological significance is still controversial.

Various SLE mouse models also show strain-related characteristics such as sexual preference. Similar to human SLE, the disease that develops in some lupus strains, like NZB/W and BXSB, is sex-linked, while others, like NZB and MRL/*lpr*, are not. Female NZB/W and male BXSB mice display clinical disease in its most severe form including glomerulonephritis with heavy proteinuria, but the pathological changes in the opposite sex can be less severe and are developed much later. In spite of the general feature of female dominance in human SLE, the male preference in BXSB mice is interesting because, as mentioned previously, there are some families in which the disease occurs mainly in fathers and sons (Lahita *et al.*, 1983).

The difference in the onset, pace and degree of the disease development between strains is another feature of the mouse models. Two forms of murine SLE, the 'early-life' and the 'late life' models, have been described by many early investigators (Murphy and Roths, 1979; Theofilopoulos and Dixon, 1985; Warner, 1977; Howie and Helyer, 1968). The MRL/*lpr* is a typical early-life disease model. Some immunological abnormalities become observable as early as one or two months in the mice (Altman *et al.*, 1981). The life span of the animals, both male and female, is about 5-7 months according to Morrow and Isenberg (1987), or the mean survival time (50% mortality) at 5 months as observed by Theofilopoulos and Dixon (1985). The onset and pace of disease development in BXSB male mice is similar to that of MRL/*lpr*, but the female mice have a much longer life span with a 50% mortality of more than 20 months. In comparison, the NZB/W strain is a later-life model. The clinical manifestations and detectable serological abnormalities do not normally appear until about 4 months of age. The 50% mortality is approximately 8 months for the female mice (Theofilopoulos and Dixon, 1985). The differences in the disease onset and pace are still not clear, but it has been suggested that, apart from the genetic defect that determines B-cell hyperactivity, some kinds of accelerating factors could be controlling the development of the disease. These might be exogenous factors such as viruses and bacterial products that may activate B-cells (Rook and Stanford, 1992), or endogenous factors like female hormones and cytokine disorders (Kroemer and Martinez-A, 1991).

1.2.3 Treatment of murine SLE

Regarding the analogies between human SLE and the mouse models, lupus mice are valuable animal models for therapeutic experimentation to assess effects of various clinical interventions in the disease. Much effort has been made towards the control of lupus-like symptoms, serological and pathological changes, and some studies concerned the regulation of T and B-cell functions. Similar to treatments in human

SLE, most of the approaches to the therapeutic studies of lupus mice have been targeted at the disease-associated inflammatory processes and hyperactivity of the immune system. These include immunosuppressive and cytostatic drugs. Corticosteroids, such as methylprednisolone which is still currently the treatment of choice in SLE patients, have been tested on NZ mice with some effects in reducing disease symptoms and increasing life expectancy (Fan *et al.*, 1979; Fessel, 1980; Isenberg *et al.*, 1982). Cytotoxic agents, such as azathioprine (Gelfand and Steinberg, 1972) and cyclophosphamide (Chia *et al.*, 1981; McCune et al., 1988) have been shown to prevent or to arrest the disease progression in lupus mice. Cyclosporin A is a powerful immunosuppressant. It inhibits both spontaneous and LPS-induced anti-DNA antibody production by lymphocytes from lupus-prone mice *in vitro* (Klaus, 1981). However, none these immunosuppressive agents cure the disease. Therefore, the control of the clinical disease requires continuous use of the drugs, but long-term treatment with some of these drugs has been reported to increase malignant tumours in the animals. In NZB mice, it has been shown that treatment with azathioprine may cause a considerable increase in the appearance of thymic lymphomas (Marrow and Isenberg, 1987). In addition, predisposition to infections is a possible side effect of the immunosuppressive drugs which may in turn escalate the progression of the disease. Interestingly, in contrast to its effect on lupus mice, azathioprine causes leucopenia in human SLE. Cyclosporin A has also been reported to induce autoimmune disease in mice (Bucy *et al.*, 1993).

On the other hand, paradoxically, some drugs which have immunostimulatory rather than anti-inflammatory effects were also reported to be beneficial to the disease. For example, N-(2-carboxyphenyl)-4-Chloroanthranilic acid disodium was found to have significant effects on increasing 50% survival time of female NZB/W mice (Ohsugi *et al.*, 1978). This is in agreement with the observation in the *in vitro* study of lymphocytes from SLE patients that isoprinosine, an anti-viral drug with

immunostimulatory activity, enhances T-cell mitogenic responses and IL-2 production, but reduces immunoglobulin production (Nakamura *et al.*, 1983).

There are other forms of experimental treatments which have been more specific, being directed primarily against the T, B or accessory cell compartments. Injection of prostaglandin E1 considerably increases the survival of NZB/W (Winkelstein and Kelly, 1980) and MRL/*lpr* (Izui *et al.*, 1980) mice and protects the mice against nephritis, which have been attributed to its selective effect on B lymphocytes. However, the treatments did not affect the production of anti-DNA antibodies although deposits of immune complexes in the kidneys were reduced in the mice. Administration of specific antibodies to CD4 molecules has been shown to prevent and reverse spontaneous autoimmune disease in three genetically unrelated strains of lupus-prone mice including NZB/W (Wofsy and Seaman, 1985; 1987), BXSB (Wofsy, 1986) and MRL/*lpr* (Santoro *et al.*, 1988); and in other autoimmune diseases such as non-obese diabetic mice (Shizuru *et al.*, 1988), experimental allergic encephalomyelitis (EAE) (Waldor *et al.*, 1985), and collagen-induced arthritis (CIA) (Ranges *et al.*, 1985). However, like those non-specific immunosuppressive drugs, the effect is transient unless such treatment is continuously and chronically given. Upon a short course of anti-CD4 treatment, even combined with thymectomy, severe autoimmune disease still develops with autoantibody levels, proteinuria and mortality comparable with the controls, despite significant reduction of $CD4^+$ cells (Connolly *et al.*, 1992). Therefore, such a general depletion of T-helper cells also remains questionable both in theory and in practice.

Another therapeutic development that has shown disease-limiting effect is the anti-idiotype suppression. Administration of syngeneic anti-DNA monoclonal antibodies (Hahn and Ebling, 1983; 1984) or syngeneic anti-DNA IgG with a synthetic immunoadjuvant (Zouali *et al.*, 1985) is able to induce anti-idiotype antibodies which suppress the corresponding idiotype. Since anti-idiotypic therapy may have the

undesired effect of increasing idiotype expression (Hahn and Ebling, 1984), anti-idiotypic antibodies conjugated with a cytotoxic agent neocarzinostatin, have been reported to eliminate anti-DNA antibody-producing cells selectively (Saski *et al.*, 1986). Following the demonstration of beneficial effects of this specific treatment on lupus mice, similar therapy for treating SLE patients by using a human-mouse or human-human hybridoma approach is being developed. In addition, many immune regulators such as thymic hormones and cytokines have also been used to treat lupus mice. One of the most notable approaches is the *in vivo* delivery of IL-2 by live *vaccinia* recombinant viruses in MRL/*lpr* mice which showed prolonged survival, decreased autoantibody levels and absence of glomerulonephritis (Gutierrez-Ramos *et al.*, 1990, 1991), although direct injection of recombinanat IL-2 in NZB/W mice showed no effect on the disease in an earlier study (Owen et al., 1989)

1.3 Immune regulation and the role of cytokines

A property that is universal to all biological systems is the need for regulation. This also applies to the immune system which may otherwise malfunction and cause disease. The mechanism of immune regulation is important not only in the control of the magnitude of an immune response once it is initiated, but also to establish a stable, non self-responsive environment, during the development of the immune system, which should then be properly maintained throughout.

1.3.1 T-cell-mediated regulation

i) "Help" and "suppression"

Many studies have shown that the regulation of the immune response is a complex network involving distinct populations and sub-populations of regulatory cells. These cells interact among themselves and with populations of effector cells to up-regulate (help) or down-regulate (suppress) the immune response (Hodes, 1989).

"T-helper", or "T-inducer", refers to a special subset of T-cells important in modulating the normal immune response. It is well known that, through specific cellular recognition, these cells can provide 'help' for many types of cells by producing a variety of cytokines, or more precisely lymphokines, which are themselves non-specific immune regulators. In antigen specific, T-dependent responses, T-helper cells are known to control the maturation of B cells. After activation, the B cells become sensitive to T cell-derived cytokines which drive them to proliferate and differentiate. This T-B cell co-operation has been recognised as an essential feature involved in immune regulation. Playing a major role in the T-B cell communication, IL-2, as well as many other lymphokines are a group of non-specific helper T-cell derived cell regulators inducible by stimulation either with antigens or mitogens such as Concanavalin A. Numerous evidence has been presented indicating their cell regulatory activity (Balkwill and Burke, 1989), and their dysfunctions implicated in the pathophysiology of many diseases (Kromemer and Martinez-A, 1991). T-cell help has also been demonstrated in other immune responses including T-cell-mediated immunity, such as the generation of cytotoxic lymphocytes (Hodes, 1989).

However, T-cells have been found to have the capacity to suppress immune responses as well. T-cell mediated suppression is a phenomenon observed for many years, but progress in clarifying the cellular nature of the so called 'suppressor cells' has been slow and the results controversial (Mitchison, 1989). This is partially due to the lack of specific markers necessary for distinguishing such a unique T-cell subset. Many immunologists still argue about the existence of suppressor T-cells, but the phenomenon of immune suppression has been continuously demonstrated. These suppressive regulatory events in the immune system have now largely been ascribed to the inhibitory actions of products of helper or inducer cells, such as IL-4, IL-10 and TGF-β.

ii) Cytokines, T-helper phenotypes and their functional properties

Cytokines are peptidic or glycopeptidic mediators secreted by a array of cells that, similar to "classical" hormones, exert their effects via specific receptors expressed on the surface of target cells but within short distances. Although most cytokines are produced by several different cell types, a few of them are specific products of lymphocytes also called "lymphokines". Cytokines are well known to be integrated in a regulatory network of mutual interactions (Balkwill and Burke, 1989). Their effects are pleiotropic in the sense that they participate in induction of many different kinds of responses in cells of multiple types. They may induce or suppress the secretion of others so regulating each other.

In mice, on the basis of two essentially non-overlapping sets of characteristic lymphokines that they secrete, T-helper cells ($CD4^+$) have been phenotypically divided into two sub-populations. T-cells that secrete IL-2 and IFN-γ are designated as Th1, and those that secrete IL-4, IL-5, IL-6, IL-7, IL-8 and IL-10 are termed Th2 (Mosmann and Coffman, 1989). In addition, Th0 is a third T-helper phenotype described recently, referring to cells that have the ability to secrete lymphokines overlapping both Th1 and Th2 subsets (Street *et al.*, 1990), and most of the $CD4^+$ cell clones in human have been found to possess the Th0 phenotype in their secretion of lymphokines.

The significance of the phenotypic division is mainly about the functional property of the different cytokines that are associated with the regulation of various immune responses. It is suggested that Th2 cells which produce lymphokines like IL-4, IL-5 and IL-6 are associated with B-cell differentiation (Finkelman *et al.*, 1990; Harriman *et al.*, 1988), thus regulating humoral immunity. Lymphokines of the Th1 phenotype are important in the regulation of cellular immunity since they are found to be associated with the generation of delayed-type-hypersensitivity responses. Evidence has been shown that in some pathological states including both infectious and allergic

diseases, the spectrum of lymphokine production by the $CD4^+$ cells tends to shift towards a Th2 phenotype (Romagnani, 1990). However, such functional division is not absolute as IFN-γ has also been shown to enhance antibody responses in both mice and humans (Snapper *et al.*, 1988); and an effective response by cytotoxic $CD8^+$ cells may require regulation and augmentation by a combination of cytokines of both Th1 and Th2 phenotypes (Carreno *et al.*, 1992).

In fact, many studies have suggested that the functions of 'help' or 'suppression' of a given cell type or their derivatives (cytokines) are often relative rather than absolute depending on many factors which ensure a dynamically balanced immune system. For example, IFN-γ may enhance both murine IgG2a and human IgG1 responses but it inhibits many of the functions of IL-4 on murine B-cells including the capacity of IL-4 to cause Ig class switching (Paul, 1989). Conversely, although IL-4 synergies with the Th1-derived cytokine IL-2 in promoting the IL-2-dependent growth of CTL cells, it has also been shown to down-regulate the activity of IL-2 on the induction of lymphokine-activated killer cells mediated through monocytes (Brooks *et al.*, 1992). On the other hand, cytokines do exert particular and predictable effects on the immune system as they interact with defined targets. One way that determines the specificity of different cytokine's actions is the expression of specific receptors for each cytokine by target cells. It also depends how these receptors are linked to the distinct pathways of intracellular signalling that regulate cell behaviour (Swain, 1991).

1.3.2 Interleukin-2 and its central role in immune regulation

In the cytokine family, IL-2 produced by T-cells is believed to provide a universal signal for proliferation of antigen or mitogen-activated T-cells through its binding to specific cell receptors (Swain, 1991). IL-2 is the first of a series of lymphocytotropic hormones and the one best characterised both in terms of molecular biochemistry and functions. The production of this 15.5 KD effector glycoprotein is thought to be

confined to $CD4^+$ periphery T-cells of the inflammatory phenotype (Th1) and certain thymocyte subsets. IL-2 was first studied for its key role in supporting clonal expansion of T-cells therefore named previously as 'T-cell growth factor' (Morgan *et al.*, 1976). It stimulates T-cells to undergo cell cycle progression via its specific receptors (IL-2R). Subsequently, many other important functions of IL-2 have been disclosed including modulating activation, functional differentiation and division of B-cells, natural killer (NK) and lymphokine activated killer cells (LAK) (Smith, 1988, 1990). Under normal circumstances, IL-2 acts either as an autocrine growth factor (Meuer *et al.*, 1984) (e.g. Th1 and pro-T-cells) or in a paracrine fashion, where specific Th1 cells release IL-2-containing vesicles in apposition to the contact site with the interacting cells (Sitkovsky and Paul, 1988). IL-2 and its mRNA, and signals that induce the IL-2 gene transcription are short-lived (Mier and Gallo, 1982). IL-2 is subjected to proteolytic process and antagonised by soluble inhibitors (Kucharz et al., 1988). It is also rapidly cleared by the kidney and has a serum half-live of 3.7 ($\pm$ 0.8) mins after i.v. injection (Donohue and Rosenberg, 1983).

While resting T-cells lack the ability to produce IL-2 and do not express anti-Tac reactive components of the IL-2R, exposure to lectin or antigen in the presence of accessory cells induces T-cells to release IL-2 (Meuer *et al.*, 1984) and display immunoreactive IL-2R (Williams *et al.*, 1985). The interaction of IL-2 with the IL-2R promotes progression of T-cells through the S phase of the cell cycle (Gillis *et al.*, 1977; Leonard, 1992). Studies employing accessory cell depletion have shown several stimuli required for induction of IL-2 and IL-2R. Transcription of IL-2 mRNA, production of IL-2 protein, and expression of membrane-bound IL-2R are dependent upon T-cells receiving signals from antigen in conjunction with co-stimulator from accessory cells. IL-1, IL-6 and used to be proposed as the candidates responsible for co-stimulation (Hadden, 1988; Majumdar et al, 1990). More recently, an increasing number of accessory molecules mediating the process has been identified and many of the ligands for the accessory molecules are cell surface proteins expressed on lymphoid

cells (Hivroz-Burgaud and Cantrell, 1992. The interaction of CD28 molecules of T-cells and a B-cell molecule B7/BB1 has now been considered to be the major co-stimulatory pathway for IL-2 production (Norton *et al.*, 1992).

The central role of IL-2 in the regulation of the immune system has been well documented. It has been established since the early 80s that the presence of IL-2 is both necessary and sufficient for the expansion of most T-cells, natural killer cells and B-cells following activation. Interference of IL-2 activity, with antibodies to IL-2 or its receptors for examples, blocks the development of antibody-secreting B-cells, and the development of precursors into cytolytic effectors. Later it was shown that blocking IL-2 production in the thymus interrupted T-cell development (Tentori *et al.*, 1988); and blocking with anti-IL-2R *in vivo* prevented the development of virus-specific cytotoxic T-cells (Leist *et al.*, 1989). IL-2 may also induce other changes in cell behaviour. It stimulates the expression of IL-2R p55 chain (Smith, 1989), as well as up-regulating other cytokine receptors on lymphocytes such as that for tumour necrosis factor (TNF)-α (Owen-Schaub *et al.*, 1989).

1.3.3 Immune regulation, tolerance and ontogeny of the immune system

Apart from the function of generating immune responses to foreign antigens, another fundamental aspect of the immune system is tolerance to self tissues. In healthy individuals, self-reactive B cells and low titre detectable autoantibodies are not uncommon. The origin of the self-reactive B cells is not clear but, to maintain unresponsiveness to self, the immune system is believed to be capable of preventing autoimmunity through specific deletion of self-reactive T and B-cell clones or by suppression. The term 'tolerance' defines a state of specific immunological unresponsiveness to an antigen which arises after an initial encounter with the antigen (Male *et al.*, 1987, Miller *et al.*, 1993). "A state of indifference or non-reactivity towards a substance that would normally be expected to excite an immunological

response" described R.D. Owen (1945) almost 50 years ago. The concept of tolerance today includes 'acquired immunological tolerance' that is the unresponsiveness arising as a result of administration of exogenous antigen, and 'natural immunological tolerance' or 'self-tolerance' defining the immunological unresponsiveness to endogenous antigens.

The mechanism of self-tolerance is crucial in establishing a normal immune system, which included clonal deletion of self-reactive immunocytes. The hypothesis of Burnet and Fenner, dealing with unresponsiveness to self antigens, stated that all anti-self lymphocytes were eliminated before maturity (Burnet and Fenner, 1949). On the other hand, autoimmune states can also be regulated by the induction of a state of acquired immunological unresponsiveness. The mechanisms of tolerance induction are still hypothetical. One mechanism that has been proposed to explain B-cell tolerance is exhaustive terminal differentiation which might result in clonal deletion of the B-cell. It has been established that many more precursors for a response against a particular autoantigen are present in the pre-B-cell pool than in the mature B-cell pool (cited from Male et al., 1987). It was therefore interpreted that a process of purging occurs that leads to elimination of the autoreactive B-cells during ontogeny. A similar conclusion can be drawn when the clonal deletion mechanism is extended to the T-cell repertoire where the so called 'thymic negative selection' process is involved to prevent release of potentially autoreactive T-cell clones into the circulation. It is now belived that the mechanism of T-cell selection is related to the T-cell receptor rearrangements and the affinity of the receptors for self-MHC molecules (Lydyard and Grossi, 1993). T-cells which may be negatively selected include cells not making successful TCR gene rearrangements, and those which have successfully rearranged their genes but have produced receptors which either no affinity, or very high affinity for MHC molecules. This programed cell death occurs by apoptosis achieved by activating endogenous nucleases, causing DNA fragmentation.

Another mechanism for induction of tolerance has been attributed to T suppressor cell activity or, alternatively, immunosuppression. It was first shown by Gershon and Kondo (1972) that when animals were given a large dose of sheep red blood cells they became tolerant to this antigen. The importance of this finding is that such tolerance can only be induced in the presence of T-cell help. Since splenic T-cells could transfer this unresponsive state to other mice, the tolerance was suggested to be mediated by T suppressor cells. Similar studies have subsequently been demonstrated using different antigens. In some early reports, absence of a suppressor T cell subset was suggested to lead to B cell hyper-reactivity and to be linked with elevated levels of autoantibodies in SLE (Abdou *et al.*, 1976, Bresnihan and Jasin, 1977). Morimoto et al. (1987) found that lack of $CD4^{+}2H4^{+}$ cells, a suppressor-inducer T-helper subset, is characteristic in patients with SLE. Although the role of suppressor cells in autoimmune diseases still remains controversial, a $CD45RC^{low}$ Th2-like phenotype has recently been found responsible for suppression of diabetes in rat (Fowell, 1993). A reduced CD4 T-cell-mediated suppression of autoantibody response rat RBC has also been observed in NZB mice (Roitt, 1993)

The role of cytokines in the induction of tolerance is controversial. The ability of IL-2 to modulate tolerance induction is particularly interesting but paradoxical. It has been suggested that the presence of IL-2 interferes with the generation of a state of tolerance (Male *et al.*, 1987). The reasons are, firstly, the fact that neonatal spleen cells do not produce IL-2 following Con A stimulation; secondly, stimuli known to trigger IL-2 production (the lectin Con A, and graft-versus-host reactions) block, whereas reagents which suppress IL-2 production, such as cyclosporin A and anti-CD4 (L3T4), favour tolerance induction; and thirdly, *in vivo* administration of IL-2 blocks the development of tolerance to alloantigens and the generation of antigen specific T suppressor cells by haptenated cells. However, the thymus, which is known to be an important organ where the tolerisation of T-cells takes place, produces a considerable number of peptides influencing T-cell development. These are thymus-derived

hormones including thymosin, thymopoietin, thymulin and thymus humoral factor, some of which have been shown to promote the differentiation of IL-2 producing T-helper cells. The role of IL-2 in intrathymic T-cell selection is not clear, but normal thymus does not lack $CD4^+$ mature T-cells which are capable of producing IL-2. In addition, IL-2 is known to be essential for the maintenance of all T-cell lines. It is conceivable that IL-2 might be necessary for the generation of suppresser T-cells which is one of the important mechanisms for induction of tolerance. Most recent evidence has indicated that IL-2 induces apoptosis in mouse thymocytes (Migliorati *et al.*, 1993).

1.4 Cytokine regulation in autoimmunity

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

1.4.1 Cytokine disorders in SLE and other autoimmune diseases

As mentioned previously, one of the main T-cell functional abnormalities in SLE is the abnormal production of cytokines. A defective IL-2/IL-2R system, in particular, has been implicated in the autoimmune phenomena. In both human SLE (Linker-Israeli *et al.*, 1983; Tsokos and Balow, 1984; Murakawa *et al.*, 1985; Huang *et al.*, 1986, 1988; Hishikawa et al., 1990) and lupus-prone mice (Altman *et al.*, 1981; Blank et al., 1981; Wofsy *et al.*, 1981; Theofilopoulos *et al.*, 1985; Santoro *et al.*, 1987; Zubler and

Miescher, 1990), it has been shown that there exists a defect in the ability of T-helper lymphocytes to produce IL-2. By stimulating T-cells *in vitro* with different T-cell mitogens such as Con A or PHA, or allogeneic stimulator cells, these studies all showed a reduced level of IL-2 activity in the cell culture supernatants when compared to normal controls. The *in vitro* IL-2 production defect has also been found to be common in some other autoimmune diseases. A similar abnormality is present in rheumatoid arthritis (RA) (Combe., 1985), Sjogren's syndrome (Leventhal *et al.*, 1967) and active multiple sclerosis (Merrill *et al.*, 1984). In addition, it has been demonstrated that T-cells from MRL/*lpr* mice also lack ability to respond to IL-2. Altman (1981) showed that cells from lymph nodes and spleens of 6-month old MRL/*lpr* mice were unable to proliferate in response to Con A even in the presence of IL-2. This was later attributed to a defect in IL-2R expression (Wofsy et al., 1984). Since IL-2 is known to be required for up-regulation of functional IL-2R expression, it is still not clear whether the defect in IL-2R expression is secondary to the defective IL-2 activity in the disease. However, in MRL/lpr mice, it has been shown that the abnormally accumulated DN T-cells were unable to generate functional IL-2R (Rosenburg *et al.*, 1989; Tanaka *et al.*, 1993).

Early studies have suggested that autoimmunity might be regulated in part by IFN modulating macrophage Ia antigen expression resulting in enhanced production of cell antigens (Steeg *et al.*, 1982). In organ-specific autoimmune diseases, it was shown that IFN-γ increases expression of class II MHC antigens leading to inappropriate presentation of self antigens, and inhibits IL-4 activity by altering the balance of Ig isotype expression or by Fcγ receptor expression (Bottazzo *et al.*, 1986). It has been proposed that the immune activating lymphokine may have deterious effects in autoimmune diseases (Rosenberg et al., 1984). However, peripheral mononuclear cells from patients with rheumatoid arthritis exhibit deficient production of IFN-γ *in vitro* (Hasler *et al.*, 1983), and clinical studies indicate that IFN-γ has some efficacy in the treatment of this disease (Browning, 1989; Machold et al., 1992). In SLE, studies

on production of IFN-γ are not in agreement with each other too. Impaired ability of SLE mononuclear cells to produce IFN-γ in response to some viruses has been reported (Neighbour and Grayzel, 1981; Strannegard et al., 1982). Some studies also showed decreased mitogen-induced production of IFN-γ in both SLE patients (Tsokos et al., 1988) and lupus mice (Kofler *et al.*, 1984), but others described normal production of IFN-γ in the disease (Santoro et al., 1983; Mckenna et al., 1988).

The LPS- and/or IFN-γ-triggered *in vitro* secretion of TNF-α by macrophages from several autoimmune-prone strains of mice has been found to be significantly lower than that produced by MHC-matched controls (Jacob *et al.*, 1988, 1990; Malave *et al.*, 1989). Using restriction fragment length polymorphism (RFLP) technique to analyse TNF-α gene, Jacob revealed abnormalities that correlated with the defective TNF-α production in the NZW strain of mice, known to carry a gene responsible for induction of autoimmune disease. In later studies, although it was shown that many normal inbred strains of mice exhibit a similar RFLP too (Richter *et al.*, 1989), a polymorphic microsatellite in the TNF-α promoter has been shown to identify an allele unique to the NZW mouse strain (Jongeneel *et al.*, 1990).

Moreover, abnormal production of many other cytokines are found to be correlated with autoimmune diseases. Linker-Israeli (1983) showed that, apart from their inability of IL-2 production in response to PHA or allogeneic Daudi cells, peripheral blood mononuclear cells from SLE patients produced lower levels of IL-1 in response to phorbol myristic acetate (PMA). This was confirmed by Dinarello (1989) who showed that blood monocytes from SLE patients produce less IL-1 than controls but, in contrast, monocytes from patients with active RA produced elevated amounts of IL-1. Recently, Levine et al. (1993) have shown evidence that IL-1 dysregulation is an intrinsic defect in macrophages from MRL/*lpr* mice. Since IL-1 used to be suggested as a co-stimulator required for IL-2 production, the inability of macrophages to produce IL-1 was considered to be a possible cause for low *in vitro* IL-2 production in SLE.

However, by adding exogenous IL-1 *in vitro* Linker-Israeli et al. (1983) did not restore ability of T-cells from lupus patients to produce IL-2. Defective LPS-induced expression of IL-1α, IL-1β and IL-6 by peritoneal macrophages has also been demonstrated in autoimmune-prone mice (Donnelly *et al.*, 1990). Furthermore, T-cells from MRL mouse strain display defective IL-3 production in response to pokeweed mitogen (PWM) (MacNeil *et al.*, 1986) and Con A. Santoro (1987) showed evidence suggesting that, the defective Con A-induced IL-3 as well as IL-2 production was not only in the DN but also in the $CD4^+$ cell subsets in the *lpr*-bearing MRL/*lpr* and C57BL/6 mice.

1.4.2 Controversy and hypothetical mechanisms

In SLE and other autoimmune situations, B cells are hyperactive, proliferating and secreting large amounts of immunoglobulin, as if they were receiving excessive stimulatory signals. T-cell help has been shown to be essential for induction of B-cells to produce an anti-DNA responses in mouse chimaera models of SLE (Stott *et al.*, 1988) and in NZB.H-2^{bm12} mice (Naiki et al., 1992). However, the deficient production of IL-2 and other cytokines are difficult to reconcile with the elevated B-cell activity. Although the *in vitro* IL-2 hyposecretion has been consistently demonstrated in many studies, there have been different views on its nature. It has been suggested that the inability of lupus T-cells to secrete IL-2 might be an outcome of the development of the autoimmune lesions (Kroemer and Martinez-A, 1991). Paradoxically, autoimmune diseases like SLE (Huang *et al.*, 1988) and multiple sclerosis (Adachi *et al.*, 1989; Trotter *et al.*, 1988) associated with defective *in vitro* IL-2 production are reported to have elevated serum IL-2 levels. Huang and his colleagues found that sera from 50% of the SLE patients contained higher levels of IL-2 compared to normal controls. Most interestingly, he also showed that the ability of lupus T-cells to produce IL-2 could be restored when these cells were rested for 2 to 3 days in culture before stimulation (Huang *et al.*, 1986). The authors suggested that the *in vitro* IL-2

hyposecretion could be due to a transient exhaustion of T-cells that had been over activated *in vivo*. Alternatively, others suggested that the suppressed cytokine activity and cell proliferative responses could be a consequence of non-specific immune suppression resulted from the hyperactivated immune system. It may therefore reflect some kinds of operating negative feedback mechanism that attempts to restore the immune homeostasis. In MRL/*lpr* mice, since the disease is associated with the expansion of phenotypically immature and functionally unknown double-negative $CD3^+$ lymphocytes, lack of IL-2 activity in culture supernatants of Con A-stimulated spleen cells has been attributed to a 'cell dilution' effect by the proliferating non-IL-2 or other cytokine secretors (Kroemer and Wick, 1989). Moreover, release of soluble IL-2R which prevents function of IL-2 and is elevated in SLE (Cuadrado *et al.*, 1993) and other autoimmune diseases such as multiple sclerosis (Greengery *et al.*, 1988), is also thought to be a possible cause responsible for the reduced cytokine levels in cultures.

One of the important questions is whether abnormalities in cytokine production or responsiveness may predispose to the development of the autoimmune disease. Several studies have suggested that deficient IL-2 activity may be intrinsic to SLE lymphocytes because the defect was observed in SLE patients regardless of the disease activity or corticosteroid therapy (Linker-Israeli *et al.*, 1983; Tsokos and Balow, 1984; Murakawa *et al.*, 1985). However, the evidence for a possible intrinsic nature of IL-2 deficiency is still lacking. Nevertheless, the defective lymphokine production and responsiveness indicate the functional abnormalities of the lymphocytes. Although the original defect that causes these functional disorders is still to be clarified, it has led to speculation that an imbalance in the normal T-cell-mediated regulatory processes of the immune system might cause abnormal B-cell regulation thereby contributing to the development of autoimmune diseases.

1.4.3 Aim and scope 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* (Chapters 3 to 6) and *in vivo* (Chapters 7 to 8) approaches based on mainly two murine models of SLE. The thesis focuses on the roles of T-helper cells and the nature of various lymphocyte functional defects in association with different immunological and serological abnormalities, and the characteristic pathological changes in SLE.

As part of the *in vitro* study, the third chapter begins the investigation by looking at the abnormal IL-2 production in lupus mice at different ages and the natural history of murine lupus disease. This is to determine the onset of this T-helper cell functional abnormality and its relationship to the kinetics of disease. Subsequent experiments described in the fourth chapter were designed to analyse other functions of T-cells from lupus mice, and from SLE patients in selected experiments. These included Con A-induced T-cell proliferation, IL-2R expression, production of IFN-γ and IL-4, as well as mRNA expression of Th1 and Th2 phenotypes for IL-2, IFN-γ, IL-4 and IL-6. The role of T-cell activation co-stimulators including effects of IL-1 and adherent cell function was also studied. To understand the role of IL-2 deficiency in the defective T-cell functions, the fifth chapter demonstrates the effects of exogenous IL-2 on lupus T-cell activation. In addition, the *in vitro* effects of IL-2 on autoantibody secretion, and the responsiveness of different T-cell subsets from MRL/*lpr* mice to IL-2 were also discussed. Concerning serological abnormalities in SLE, the sixth chapter describes mainly a finding of serum factors which are increased in SLE patients with active disease, and in some lupus mice. The serum factors which affect the growth of IL-2 dependent CTLL cells were partially characterised in an attempt to determine their nature and the possible role in the immune disorders.

Based on the evidence from the *in vitro* observations, the following two chapters explore the possibility of treatment of lupus disease by cytokine intervention. A new approach using *Salmonella typhimurium aro*$^-$ mutant as a vector to deliver cytokines *in vivo* was carried out in the small scale pilot studies. The *in vivo* effects of TGF-β1 and IL-2 on lupus and normal mice are described in the seventh and the eighth chapters respectively.

Chapter 2

MATERIALS AND METHODS

2.1 Animals and materials

2.1.1 Animals

i) Mouse Models

MRL-Mp-*/lpr/lpr* (MRL/*lpr*) and [NZBxNZW]F1 (NZB/W) mice, were bred in the Glasgow University Animal House from pairs purchased from *Harlan Olac Ltd., UK* Female mice were used in the study. Sex and age matched normal BALB/c and CBA control mice were also purchased from *Harlan Olac Ltd., UK* BALB/c mice were used as controls throughout the study and, in addition, CBA mice were used in selected experiments. All mice were kept in the Animal House, Gartnavel Hospital, Glasgow.

The lupus mice were divided into groups according to age. NZB/W mice aged between 7 and 9 months or MRL/*lpr* mice aged between 4 to 6 months were old mice which exhibited obvious clinical symptoms with elevated serum autoantibodies. Young mice of different ages before the development of overt autoimmune lesions were used at 3, 2, 1-month and 1-week old as indicated.

ii) Rabbits

Two NZB female rabbits (005865, 004896), weighing 2.5 and 3 kg, were purchased from *Froxfield U.K.* and kept in the Animal House, Gartnavel Hospital, Glasgow.

2.1.2 Plasma and serum samples

i) Mouse sera

Blood samples were taken from all experimental mouse models including lupus strains, MRL/*lpr* and NZB/W, and normal control BALB/c and CBA mice. Ether anaesthesia was used and the mouse was bled by common iliac artery puncture prior to the removal of its spleen.

ii) Rabbit sera and antisera

Normal rabbit sera and rabbit anti-r-human IL-2 antisera were obtained by bleeding the ear vein before and after immunisation with antigen.

iii) Human plasma and sera

SLE plasma and sera were obtained from blood samples from diagnosed SLE patients with active disease, kindly provided by Dr J. Hunter in the Department of Rheumatology, Gartnavel Hospital. Normal human plasma and sera were obtained from healthy donors and provided by the Blood Transfusion Unit, Western Infirmary, Glasgow.

All serum and plasma samples were kept at -20°C before assaying. Plasma samples were supplied in the presence of anticoagulant either potassium EDTA (BS 4851 5ml sample container) or heparin (10 i.u./ml).

2.1.3 Tissue and sections

Freshly isolated mouse kidneys were sliced into halves longitudinally and processed as follow for histological and immunocytochemical examinations:

i) Frozen tissue

Blocks of tissue were fast-frozen in liquid nitrogen and kept at -70 °C. 24 to 48 hours before each assay, 5 μm sections were cut from the frozen tissue, mounted on washed plain glass slides, and kept at 4 °C.

ii) Paraffin-embedded fixative-fixed tissue

Blocks of tissue were fixed in neutral buffered formalin or in Bouin's fixative. After being embedded in paraffin wax, 4 μm sections were cut and mounted on washed plain glass slides.

2.1.4 Cell lines and maintenance

i) CTLL cell line

The mouse CTLL (cytotoxic T lymphocyte line, Gillis & Smith, 1977) cell line was kindly provided by Dr. Richard Grencis at the University of Manchester. The IL-2 dependent mouse CTLL cells were routinely maintained at 37°C/5%CO2 in a humidified incubator in RPMI-1640 complete culture medium containing 10%FCS, supplemented with r-human IL-2 at 10 ng/ml and 2-mercaptoethanol at 50 μM. The cells were passaged regularly and rigorously every 2 to 3 days. To split cells, 80-85% volume of the cell culture was replaced with fresh medium that contained IL-2.

ii) Mink Lung cell line (CCL-64)

The CCL-64 mink lung cell line was kindly provided by Professor F.Y. Liew and his research group at the Research Laboratory of Immunobiology, Wellcome Foundation, UK. The cells were adherent cells and routinely maintained at 37 °C/5%CO2 in a humidified incubator in RPMI-1640, or DMEM (Dulbecco's Modified Eagle's Medium) complete culture medium containing 0.45% glucose and 10% FCS in 25-cm² culture flasks. Culture medium was changed every 3 days. Two days before each assay, the cells were propagated in a new flask of appropriate size depending on cell number required. The cells were detached from culture flasks by trypsinisation (Holley *et al.*, 1983). After two washes with warm serum-free culture medium, the cells were incubated with 0.025% trypsin in calcium and magnesium free tris buffer saline containing 0.5 mM Na_2EDTA for 2 to 3 minutes at 37 °C. Cells were then suspended by gently patting the wall of the flask and the enzyme activity stopped by addition of culture medium which contained 10% FCS. An aliquot of the cells was re-seeded in fresh culture medium in a new flask or 96-well assay plates.

Both the CTLL and mink lung cell lines have been screened for mycoplasma and found to be free of mycoplasma contamination.

2.1.5 Reagents and Chemicals (see Appendix I)

2.2 Antibody production, quantitation, purification and conjugation

2.2.1 Production of antibodies to rIL-2 in rabbits

i) Antigen preparation

Recombinant human IL-2 (*Glaxo*)10 μg/ml dissolved in PBS, was mixed and emulsified with an equal volume of Complete Freund Adjuvant (CFA) for primary immunisation, or Incomplete Freund Adjuvant (IFA) for subsequent booster injections,

in a 10 ml sealed syringe on a Griffin Flask Shaker. After 1 to 2 hours vigorous shaking, the antigen emulsion was checked by its ability to form stable droplets on the surface of water.

ii) Immunisation of rabbits

Prior to the primary immunisation, 5 ml blood was taken from the ear vein of each of two normal rabbits (005865, 004896), 5 months of age, and the sera were kept at -20 °C for assay controls. Each rabbit was injected subcutaneously with the antigen at 8 sites, 0.2 ml/site (about 8 μg rIL-2 per animal). Subsequent booster injections of the antigen in IFA were given 4 weeks after the primary immunisation and at 2 week intervals thereafter. Blood samples were taken immediately before each injection and tested for titres of specific antibodies to r-human IL-2 by ELISA. In antisera with high titres of specific antibodies, the immunoglobulin fraction was purified by ammonium sulphate precipitation.

iii) Titration of antisera

A HRP-conjugated goat anti-rabbit IgG was used to monitor specific antibodies to r-human IL-2 raised in the rabbits. r-Human IL-2 (Glaxo) was diluted to 5 μg/ml in carbonate coating buffer and 100 μl/well plated on a 96-well *Dynatech* micro-ELISA plate (Immulon 2) before incubation at 4 °C overnight. Although IL-2 concentrations at up to 10 μg/ml have been tested for coating, it did not reach saturation (according to a preliminary assay of antigen titration). A 5 μg/ml coating concentration was chosen because it satisfied the assay purpose of antibody titration. After washing 4 times with tris-Tween buffer, the remaining binding sites on the plate were blocked by adding 200 μl of blocking buffer, 0.5% (w/v) bovine serum albumin (BSA) in PBS, to each well. This was incubated at room temperature for 30 minutes, and then washed 4 times in tris-Tween buffer. Serial dilutions of the rabbit antisera in PBS were then added to each well (100 μl/well, duplicates) and incubated at 4 °C for 2 hours. Negative

controls were provided by using normal sera taken from the same rabbits before immunisation. After incubation, the plates were washed 3 times. 100 µl of the HRP-conjugated goat anti-rabbit IgG diluted 1:1000 in 0.5% (w/v) BSA in PBS, was added to each well and incubated at room temperature for 30 minutes. Following 5 washes in tris-Tween buffer, 100 µl of substrate, orthophenylene diamine (OPD, *Sigma*) diluted to 0.4 mg/ml in McIlwaine's buffer containing 0.32 µl/ml hydrogen peroxide, was added and incubated for 30 minutes in the dark at room temperature. The reaction was stopped by adding 100 µl per well of 4N sulphuric acid, and the optical density was read on a 'Multiscan' spectrophotometer, at 492 nm.

iv) Immune responses of the rabbits to IL-2

Figure 2.1 shows the specific immune responses of the two rabbits. Four weeks after the primary immunisation, both rabbits 005865 and 004896 showed an immune response to the antigen given. The antiserum titres increased linearly after subsequent boost injections and reached a peak level at week 8, 2 weeks after the second boost injection. Rabbit 004896 was killed at 9 weeks due to a serious skin infection around the sites where antigen was injected.

2.2.2 Quantitation of autoantibody production in the mouse models

i) Quantitation of anti-ssDNA and anti-dsDNA antibodies by ELISA

Calf thymus DNA (*Sigma*), single (ss-) or double (ds-) stranded, was used as antigen. To obtain ssDNA, the calf thymus DNA at 1 mg/ml in borate buffered saline (BBS, pH 8.5) was boiled for 10 minutes and put on ice. dsDNA for antigen coating was prepared by S1 nuclease digestion and phenol extraction (Sambrook *et al.*, 1989) before using for coating plates. The dsDNA used in this study was kindly prepared and given by Dr. R. Quinn in the department.

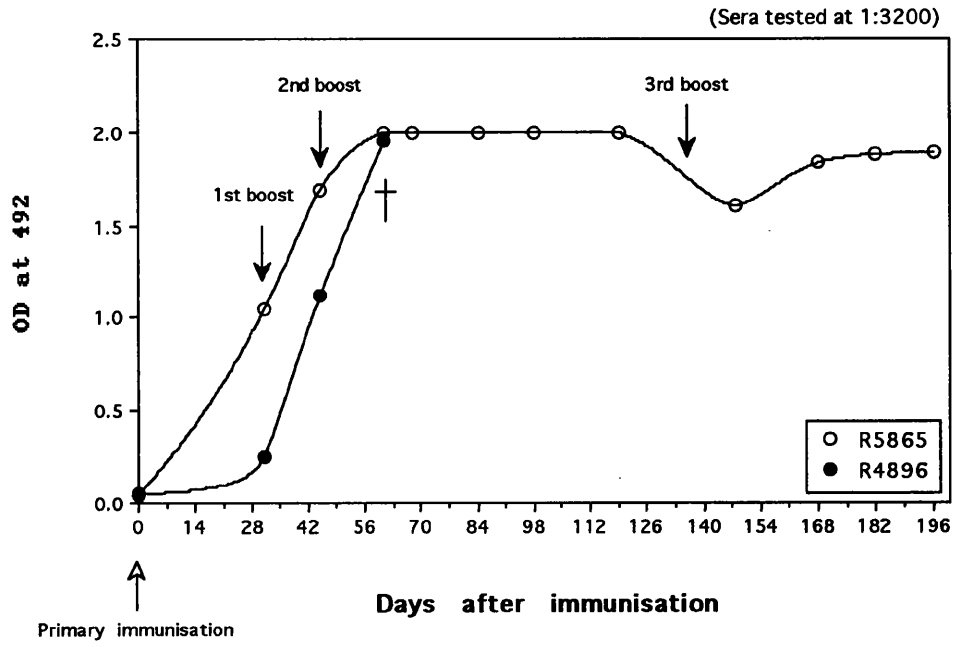


Figure 2.1 Immune responses of rabbits to r-human IL-2. Serum samples taken from 2 rabbits (R5865, R4896) at different time points after immunisation with IL-2 were assayed by ELISA. One rabbit (R4896) was killed at 9 weeks after the primary immunisation because of severe skin infection. The titration curves show specific antibody titres in serum samples diluted at 1:3200.

A 96-well Dynatech micro-ELISA plate (Immulon 2) was used in the assay. Before antigen coating, the plates were treated with poly-L-lysine (*Sigma*) 50 μg/ml in BBS, 100 μl/well incubated at 37 °C for 1 hour then at 4 °C overnight. After 3 washes in BBS containing 0.02% Tween-20, ssDNA or dsDNA diluted 10 μg/ml in BBS was coated, 50 μl/well, onto the plates. The plates were incubated 3 hours at 37 °C or overnight at 4 °C and unbound antigen was washed off 4 times with BBS-Tween. Remaining binding sites on the plates were blocked by adding 200 μl of blocking buffer, 0.5% (w/v) bovine serum albumin (BSA) in BBS, to each well. This was incubated at room temperature for one hour, and the plates were either stored with BSA/BBS in the wells at -20 °C or used for immediate assaying.

After 3 washes with BBS-Tween, serial dilutions of standard and test samples in diluent, 2% BSA/ BBS/0.05% Tween-20 (for anti-dsDNA 10% normal goat serum was added), were then added to the plates (50 μl/well, duplicates) and incubated at 4 °C overnight. After incubation, the plates were washed 3 times with BBS-Tween and blotted dry. An alkaline phosphatase conjugated goat anti-mouse immunoglobulin antibody (γ-chain specific, *Sigma*) was used as a detector antibody, 1/1000 diluted in the diluent and incubated at room temperature for 30 minutes. Following 5 washes with BBS-Tween, the substrate, p-nitrophenyl phosphate (p-NPP, *Sigma*) 1 mg/ml in the p-NPP substrate buffer (see Appendix I), 50 μl per well, was added and incubated for 30 to 45 minutes in the dark at room temperature. The reaction was stopped by adding 100 μl per well of 1 M sodium hydroxide, and the optical density was read on a 'Multiscan' spectrophotometer, at 492 nm.

For titration purposes, pooled sera from MRL/*lpr* mice (5 months) was used as a standard for anti-ss and -dsDNA antibody quantitation. The sera had been shown in preliminary assays to have high titres of anti-ss and -dsDNA antibodies. For each assay, a series of two-fold dilutions of the standard serum from 1/50 to 1/102,400 was included in parallel with test samples. One titration unit (T.U.) is arbitrary defined as

the amount of antibody present in a fixed dilution of the standard serum, which is 1/10,000 for anti-ssDNA and 1/1,000 for anti-dsDNA antibodies in the study. From the standard curve of OD against dilutions, the equivalent dilution (1/x) of standard serum giving the same OD as the test sample was read off. Let the dilution of the test sample be 1/D. Therefore:

$$\text{No. of T.U. in sample} = \frac{10^4}{X} \times D \qquad \text{(anti-ssDNA)} \quad \text{---------- (1)}$$

$$\text{No. of T.U. in sample} = \frac{10^3}{X} \times D \qquad \text{(anti-dsDNA)} \quad \text{---------- (2)}$$

ii) Determination of the frequency of autoantibody secreting cells by ELISPOT assay

The assay was based on the method developed by Czerkinsky et al.(1988) to determine the frequency of antibody secreting cells. This technique was modified in the present study to detect autoantibody secreting cells.

Calf thymus ssDNA or dsDNA was prepared as previously described for the ELISA above, and diluted in 20x SSC (see Appendix I) at 10 μg/ml. The antigen was coated, 100 μl/well, onto a sterile Millipore Millititer 96-well plate (5THA 096NS) with HA 0.45 μm nitrocellulose membrane fixed to bases of wells. After 1 hour standing at room temperature, the plate was emptied, blotted dry and baked at 80°C for 2 hours. After coating, the plates were washed 3 times with PBS containing 0.05% Tween-20. Remaining binding sites on the plates were blocked by adding 200 μl/well of 0.5%BSA/PBS and incubated at 37 °C for 30 minutes. Following 3 washes with PBS, the plates were pre-filled with culture medium (50 μl/well) and kept in an incubator at 37°C/5% CO_2 until the cell suspension was ready. Splenic lymphocytes were washed and diluted in culture medium at the required concentrations of viable cells. 10^4 to 10^6 viable cells per well in 100 μl culture medium were incubated overnight (16 hrs) at

37°C/5% CO2. The assay was carried out under sterile conditions until this step. The plates were then rinsed 5 times followed by immersing in PBS-Tween for 5 minutes.

A HRP-conjugated rabbit anti-mouse IgG (at 1/200, *the Laboratory*) and an alkaline phosphatase conjugated rabbit anti-mouse IgM (at 1/400, *the Laboratory*) were used as detecting antibodies. After an one hour incubation at 37°C, the plates were washed 4 times with PBS-Tween (without azide), immersed in 0.05 M tris buffered saline (TBS, pH 8.0) for 5 minutes and dried using a *Millipore* vacuum water pump system.

5-bromo-4-chloro-3-indoyl phosphate (BCIP, *Sigma*) and p-nitroblue tetrazolium chloride (NBT, *Sigma*) were used as substrate for alkaline phosphatase; and 3-amino-9-ethyl carbazole (AEC, *Sigma*) was used as substrate for horseradish peroxidase. To prepare these substrates, BCIP (3mg) and NBT (6mg) dissolved separately in 0.2ml dimethylformamide (DFM) were added to 20 ml of 0.1 M NaHCO3 containing 1 mM MgCl2 (pH 9.8). AEC (5mg) was dissolved in 0.4 ml DMF and added to 19 ml 0.05M NaOAc (pH 5.0). The relevant substrates were filtered and added to the wells (100 µl/well) followed by a 20 to 30 minute development at room temperature. To stop the reaction, the plates were rinsed with PBS and blotted dry on paper towel. The number of spots was counted using a *Carl Zeiss* stereo zoom microscope. The spots were clearer and easier to count when the membrane of the plate was completely dry (i.e. when left overnight at room temperature).

2.2.3 Antibody purification

i) Ammonium sulphate precipitation of immunoglobulins

The whole procedure was carried out at 4 °C. To 1 ml cold serum, an equal volume of saturated ammonium sulphate (*Fisons*) was added dropwise with slow stirring for 1 hour. After centrifuging at 10,000 g for 10 minutes, the pellet was

resuspended in 10 ml 50% saturated ammonium sulphate and centrifuged as before. The pellet was then dissolved in 1 ml distilled water and dialysed with 3 changes in PBS. The sample OD was read at 280 nm and the immunoglobulin concentration calculated:

$$\text{Concentration of IgG (mg/ml)} = \frac{OD_{280nm} \times \text{dilution}}{1.3}$$

ii) Protein A column separation of human serum IgG

To characterise the unknown serum factor(s) found in SLE patients (Chapter 6), which affected the IL-2 dependent CTLL cells growth, a protein A column was used to separate IgG from other serum proteins.

Protein A coupled to Sepharose 4B fast flow (*Sigma*) in suspension form was loaded in an 1ml syringe with a glass bead at the bottom end of the column. The 1ml protein A column has a capacity for IgG binding of 35 mg. After a continuous wash with 10 times the column volume of PBS (0.02% sodium azide), 0.4 ml of serum sample was loaded and eluted with PBS. Protein concentration in the eluent was determined by its optical density measured at 280 nm. Unbound proteins (first peak) were collected in a series of tubes (0.2 ml/tube). Following a thorough wash with PBS until $E^{280} = 0$, the IgG fraction (second peak) was eluted from the column with 0.1 M glycine/HCl (pH 2.8), and 0.2 ml fractions collected in a series of tubes containing 20 µl of 3 M tris (pH 8.5). Sample fractions in the first or the second peak giving high OD were pooled, dialysed in PBS (no azide) with 3 changes at 4 °C, and the final protein concentration in the samples measured. After each use, the column was washed with 0.1 M glycine/HCl, 2 M urea, and then PBS extensively. The Protein A column was stored in PBS containing 0.02% sodium azide at 4 °C for reuse.

iii) Antiserum absorption by liver powder

All antisera and the fluorescein conjugates used in the immuno-staining for immune complexes in mouse kidney tissue sections were pre-absorbed with sheep or donkey liver powder to remove non-specific binding antibodies and proteins. To 2 grams of sheep or donkey liver powder (*Sigma*) in an universal, 20 ml PBS were added and mixed gently to dissolve. The suspension was then spun at 1000 g for 5 minutes at 4 °C and the pellet resuspended in fresh PBS. The washing was repeated, about 3-5 times, until the supernatant became colourless. The washed liver powder was then resuspended in 20 ml cold PBS and 2 ml suspensions aliquoted in small flat-bottom bijou bottles. Finally, these were spun again at 1000 g for 5 minutes and the pellets stored at -20 °C for serum absorption. To an aliquot of washed liver powder in a small bijou bottle, 1 ml antiserum from the same species was added and mixed gently to suspend. The bottle was well sealed and rotated on a *Matburn* mixer for 1 hour at room temperature. The suspension was spun at 1000 g for 5 minutes and the supernatant transferred to a fresh aliquot of washed liver powder to repeat the absorption procedure once. The absorbed serum was stored in aliquots at -20 °C.

2.2.4 Antibody conjugation

i) Fluorescein-antibody conjugation for B-cell marker in FACS analysis

Equal amounts of the IgG fractions of rabbit anti-mouse IgG (κ-chain, 5563) and anti-mouse IgM (RC406/8/9) were combined to give a protein concentration of 11.58 mg/ml. At a ratio of 1:20, FITC (fluorochrome isothiocyanate) powder was added to the immunoglobulin preparation and mixed at 4 °C overnight. The mixture was then applied onto a G-25M Sephadex column (*Pharmacia, UK*) preabsorbed with 2 ml 0.5% BSA in PBS, and eluted with PBS. Two colour bands were visible in 5 minutes. The first one (conjugate) was collected in a series of 10 tubes, 0.5 ml/tube; and the second band (unbound fluorochrome) discarded. Samples from tubes 4-6 showing strong yellow green colour were therefore combined. To determine its fluorescein/protein

molar ratio, the conjugate was diluted 1:50 in PBS and the OD measured at 280 and 495 nm. The molar ratio of the conjugate was 0.97.

ii) Antibody biotinylation for IL-2 ELISA

The IgG fraction, isolated from the rabbit anti-rIL-2 (005865, the *Laboratory*) by ammonium sulphate precipitation, was diluted at 5 mg/ml in PBS. At a biotin/protein molar ratio of 200:1, 5 mg IgG was mixed with 2.13 mg of biotin (long arm, *Sigma*) pre-dissolved in 100 µl of dimethylformamide. The reaction took 2 hours at room temperature followed by extensive dialysis in PBS. The conjugate was stored at 4 °C.

2.3 Cell culture and stimulation

2.3.1 Mouse spleen cell preparation

Under ether anaesthesia, mouse spleens were aseptically removed and immersed in 5 ml serum-free Hanks' Balanced Salt Solution (HBSS, *Gibco*), then transferred into 10 ml fresh HBSS containing 1% FCS in a plastic universal. The spleen tissue was forced through a sterile tea strainer into a petri dish in the buffer by the rubber plunge of a 10ml-syringe. Using the syringe without needle, the cell suspension was drawn up and down 3 times. Finally, this was expelled through a 21-gauge needle into a conical centrifuge tube and allowed to settle for 2-3 minutes. The resulting cell suspension was transferred into a 25ml-universal bottle and centrifuged at 1300 g for 5 minutes. After 3 washes with the Hanks' buffer, the spleen cell pellet was resuspended in 10 ml RPMI-1640 containing 10% FCS. Cell viability was determined by staining dead cells with 0.05% trypan blue.

2.3.2 Cell culture

RPMI-1640 (*Gibco, UK*) was used for all cell cultures. This medium was supplemented with heat inactivated (56 ^{0}C, 30 minutes) foetal calf serum (5%), penicillin (50 U/ml), streptomycin (50 μg/ml) and 2-mercaptoethanol (50 μM). Mouse spleen cells were cultured in 25-cm^2 culture flasks (*Nunc, U.K.Ltd.*) at 10^6 viable cells/ml in 10 ml culture medium with and without addition of Con A (2.5 μg/ml, *SIGMA*, U.K.) at 37 ^{0}C/5%CO2. Samples were collected at intervals and tested for IL-2R cell surface expression, cell proliferation and cytokine activity in the medium.

2.3.3 T-cell activation

Polyclonal activators, or mitogens, are widely used as agents to activate T and B lymphocytes. When it was established that lymphocytes could be separated into T and B-cells, it was discovered that Concanavalin A (Con A) and phytohaemagglutinin (PHA) stimulated T-cells (Stobo *et al.*, 1972); whereas lipopolysaccharide (LPS) stimulated B-cells (Andersson *et al.*, 1972). These mitogens induce effector functions and the cellular requirements for mitogen-induced responses have been shown to be similar to those of antigen specific induction (Habu and Raff, 1977; Hadden, 1988).

In the present study, Con A has been used to assess T-cell functions in lupus disease. To obtain optimum conditions for *in vitro* mitogen stimulation of mouse lymphocytes, the optimal concentration of Con A was determined. The effect of Con A-induced T-cell activation was evaluated by the T-cell proliferative responses and IL-2 production (see Chapter 3, Fig.3.1). The Con A concentration of 2.5 μg/ml in medium containing 5% FCS was chosen for T-cell activation in subsequent experiments in the study. In selected experiments, PHA (5 μg/ml) was also used to stimulate T-cells in cultures.

Recombinant human IL-2 (kindly donated by Dr. J.G. Shields), recombinant human IL-1, recombinant mouse IL-4 and recombinant mouse IFN-γ were used to test effects of exogenous cytokines on the *in vitro* mitogenic activation of lupus and normal T-cells. Cells were cultured in 25-cm^2 flasks with or without Con A plus various concentrations of IL-2 or the other cytokines. Proliferative responses from day 0 up to day 7 were determined by ^{3}H-thymidine incorporation and lymphocyte surface IL-2 receptor expression by 'FACS' analysis.

2.3.4 Lymphocyte proliferation assay

A tritiated thymidine incorporation assay was used to measure the rate of cell DNA synthesis. Stimulated and unstimulated mouse spleen cells were pulsed in 24-well plates (0.5 ml/well, *Falcon, USA*) with tritiated thymidine (0.5 μCi/well, *Amersham International, UK*) for 3 hours and harvested on to glass fibre filters. Incorporated radioactivity was determined in 'Ecosint A' scintillation fluid using a liquid scintillation counter and calculated as means (cpm) of duplicates.

2.4 Cytokine assays

2.4.1 Cytokine quantitation

i) Quantitation of IL-2 bio-activity (CTLL bio-assay)

The CTLL bio-assay was used to measure IL-2 activity in culture supernatants, and to study serological abnormalities in SLE (Chapter 6). The concentration of IL-2 in samples was determined by its ability to induce CTLL cell proliferation. After 3 washes with HBSS containing 1% FCS, the indicator cells, $4X10^3$ viable cells per well, were incubated with serial dilutions of test or control samples in complete culture medium containing 10% FCS in 96-well plates (*Nunc, UK Ltd*) for 24 hrs at 37°C/5%CO2 and pulsed with ^{3}H-thymidine (0.5 μCi/well, *Amersham International,*

UK) for the last 4 hours. The cells were then harvested on to glass fibre filter paper and the incorporated radioactivity was measured using a 'Beta Plate' scintillation counter (*Pharmacia UK*). The IL-2 concentration was determined by comparison with a standard curve using r-human IL-2 at 50% maximum or, if lower, on the linear part of the curve of thymidine uptake (cpm) in every assay, and expressed as equivalent activity. The assay sensitivity has a detection limit below 10 pg/ml (±2SD). Figure 2.2a shows a typical rIL-2 standard curve. The IL-2 dependent cell line has been tested and showed no response to r-mouse IL-4 or IL-4 synergistic effect.

ii) IL-2 quantitation by ELISA

A 'sandwich' ELISA method was designed to quantify soluble immunoreactive IL-2 present in biological samples. This included a mouse monoclonal antibody (1E2, in ascites kindly provided by Dr. C.D. Benjamin, *Biogen*) to r-human IL-2 as capturing antibody for coating, and a rabbit polyclonal antibody (005865, IgG fraction, the *Laboratory*) to r-human IL-2 used as a detecting antibody. Since the r-human IL-2 exhibited high cross-reactivity in promoting the growth of mouse CTLL cells (IL-2 bio-assay), the human IL-2 ELISA was also intended to measure IL-2 in the mouse system. Two assay systems were developed:

IL-2 ELISA I

In this system, the 1E2 monoclonal antibody was used as a capture antibody for coating and the polyclonal rabbit anti-human IL-2 antibody as detector antibody. To determine the sensitivity of the assay, the system was first of all optimised. Briefly, 1E2 antibody, 3 to 30 μg/ml, was diluted in carbonate-bicarbonate buffer (pH 9.6, Appendix I) and coated on a 96-well Dynatech micro-ELISA plate (Immulon 2) overnight at 4 ^{0}C. After coating, the plates were washed 3 times with PBS containing 0.05% Tween-20 (PBS-T) and blotted dry. Remaining binding sites on the plates were blocked by adding 200 μl/well of 2%BSA/PBS-T and incubated at room temperature for 1 hour. Following 3 washes with PBS-T, a wide range of concentrations of r-

human IL-2 in RPMI-1640 containing 5%FCS was added, 100 µl/well, and incubated at room temperature for 3 hours. The plates were then rinsed 3 times followed by a 1 hour incubation at room temperature with detecting antibody, the rabbit anti-human IL-2 antibody at various concentrations (from 5 to 100 µg/ml tested) in antibody diluent (2%BSA/PBS-T). The plates were washed again 5 times and incubated at room temperature for 1 hr with a goat anti-rabbit IgG alkaline phosphatase conjugated antibody (*Sigma*), at 2 µg/ml (1:1000) in the same diluent. The enzyme substrate was p-nitrophenyl phosphate (p-NPP) tablets (*Sigma*), 1 mg/ml dissolved in 1 M tris buffer containing 3 mM MgCl2. The enzyme substrate reaction was carried out at room temperature for 30 to 60 minutes and stopped by addition of 1 M sodium hydroxide (100 µl/well). Optical density was read on a 'Dynatech MR 700' ELISA reader at 410 nm. Negative controls were culture medium alone during antigen incubation, and replacement of the second antibody with normal rabbit serum.

IL-2 ELISA II

The procedure of System II was basically the same as that of System I except the coating antibody was replaced by the polyclonal rabbit anti-human IL-2 antibody. In addition, the biotinylated same rabbit antibody (see 2.2.4ii) was used as the detector which was followed by an HRP-conjugated avidin amplification. Orthophenylene diamine (OPD, *Sigma*) diluted to 0.4 mg/ml in McIlwaine's buffer containing 0.32 µl/ml hydrogen peroxide, was used as the enzyme substrate, developed in the dark at room temperature. The reaction was stopped by adding 100 µl per well of 4N sulphuric acid, and the optical density was read at 492 nm.

However, although both the IL-2 ELISA Systems I and II were able to detect r-human IL-2 at nanogram levels, it showed no detectable cross-reactivity to murine IL-2 in the culture supernatants of Con A activated mouse spleen cells. Figure 2.2c is a representative IL-2 standard curve derived from the ELISA System II using r-human IL-2.

iii) IFN-γ ELISA

A sensitive ELISA method has been developed to quantify murine IFN-γ. The assay employed a rat monoclonal antibody (R46AT) to mouse IFN-γ and a rabbit anti-mouse IFN-γ polyclonal antibody. r-Mouse IFN-γ was used as the standard to quantify IFN-γ concentrations in test samples.

R46AT rat monoclonal antibody at 10 μg/ml diluted in PBS was coated, 50 μl/well, onto a 96-well Dynatech micro-ELISA plate (Immulon 2) overnight at 4 ^{0}C. After coating, the plates were washed 3 times with PBS containing 0.05% Tween-20 (PBS-T) and remaining binding sites on the plates were blocked by adding 150 μl/well of PBS containing 10% FCS and incubated at room temperature for 30 minutes. Serial dilutions of IFN-γ standards (0 to 10 ng/ml) and test samples were prepared in RPMI-1640 containing 10% FCS. The samples, 50 μl/ml, were incubated at room temperature for 2 hours after 3 washes of the plates with PBS-T. The plates were then rinsed 6 times followed by a 1 hour incubation at room temperature with detecting antibody, the rabbit anti-mouse IFN-γ antibody, 100 μl/well at 5 μg/ml in PBS-T containing 5% FCS. The plates were washed again 6 times and incubated with an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (*Sigma*) at 2 μg/ml (1:1000) in the same diluent at room temperature for 1 hour. The enzyme substrate was p-nitrophenyl phosphate (p-NPP) tablets (*Sigma*), 1 mg/ml dissolved in 1 M tris buffer containing 3 mM MgCl2. The enzyme substrate reaction was carried out at room temperature for 30 to 60 minutes and stopped by addition of 1 M sodium hydroxide (100 μl/well). Optical density was read on a *Dynatech MR 700* ELISA reader at 410 nm. Negative controls were culture medium alone during antigen incubation, and replacement of the second antibody with normal rabbit serum. The assay has a detection limit below 0.1 ng/ml (± 2SD). Figure 2.2d shows a typical r-IFN-γ standard curve.

iv) IL-4 ELISA

A sensitive ELISA method has been developed to quantify murine IL-4. The assay employed a rat monoclonal antibody (Clone BVD4-1D11, IgG2b *Phamingen*) to mouse IL-4 and a biotinylated rat anti-mouse IL-4 monoclonal antibody (Clone BVD6-24G2, IgG1, *Phamingen*). Recombinant mouse IL-4 was used as standard for quantification of the cytokine in test samples.

Purified antibody BVD4-1D11 at 2 μg/ml diluted in 0.1 M NaHCO3 was coated, 50 μl/well, onto a 96-well *Dynatech* micro-ELISA plate (Immulon 4) overnight at 4 ^{0}C. After coating, the plates were washed 3 times with PBS containing 0.05% Tween-20 (PBS-T) and remaining binding sites on the plates were blocked by adding 150 μl/well of PBS containing 10% FCS and incubated at room temperature for 30 minutes. Serial dilutions of rIL-4 standards (0 to 10 ng/ml) and test samples were prepared in RPMI-1640 containing 10% FCS. The samples, 50 μl/ml, were incubated at room temperature for 2 hours after 3 washes of the plates with PBS-T. The plates were then rinsed 6 times followed by a 1 hour incubation at 37 ^{0}C with detecting antibody, the biotinylated rat anti-mouse IL-4 monoclonal antibody (BVD6-24G2), 100 μl/well at 1 μg/ml in PBS-T containing 10% FCS. The plates were washed again 6 times and a extravidin-peroxidase, at 2 μg/ml in the same diluent, was incubated at 37 ^{0}C for 1 hour. After further extensive washes (X 8), TMB Microwell Peroxidase Substrate *(Dynatech)* solution, 100 μl/well was finally added. The reaction was developed at RT for 30 minutes and the optical density read on a *Dynatech MR 700* ELISA reader at 492 nm. Negative controls were culture medium alone during antigen incubation. The assay has a detection limit below 0.05 ng/ml (± 2SD). Figure 2.2e shows a typical r-IL-4 standard curve.

v) TNF-α ELISA

XT22.11 monoclonal antibody at 10 μg/ml diluted in carbonate-bicarbonate buffer (pH8.5) was coated, 100 μl/well, onto a 96-well *Dynatech* micro-ELISA plate

(Immulon 2) overnight at 4 ^{0}C. After coating, the plates were washed 3 times with PBS containing 0.05% Tween-20 (PBS-T) and remaining binding sites on the plates were blocked by adding 200 µl/well of PBS containing 20% FCS and incubated at room temperature for 30 minutes. Serial dilutions of r-mouse TNF-α standards (0 to 100 U/ml) and test samples were prepared in RPMI-1640 containing 10% FCS. The samples, 100 µl/ml, were incubated at room temperature for 2 hours after 3 washes of the plates with PBS-T. The plates were then rinsed 8 times followed by a 1 hour incubation at room temperature with detecting antibody, the rabbit anti-mouse TNF-α antibody, 100 µl/well at 10 µg/ml in PBS-T containing 5% FCS. The rest of the procedure was the same as that of IFN-γ ELISA including the goat anti-rabbit IgG alkaline phosphatase conjugated antibody (*Sigma*) and the enzyme substrate development (see 2.4.1iii). The assay has a detection limit below 4 U/ml (±2SD). Figure 2.2f shows a typical r-TNF-α standard curve.

vi) TGF-β bio-assay (MvILu)

Two days before assaying, the CCL-64 mink lung cells (*Wellcome, UK*) were propagated in new flasks and the cells were detached from the culture flasks by trypsinisation (see 2.1.4). After washing, the cells were seeded in 96-well flat bottom culture plates at 10^5 cells per well. After an overnight incubation at 37^{0}C/5% CO2, non-adherent cells were removed by washing with warm plain culture medium once. r-human TGF-β2 was used as the standard in every assay. Samples were incubated with the cells in 100 µl complete culture medium in triplicate wells overnight in a cell culture incubator. Cells were washed again twice and pulsed with tritiated thymidine (0.5 µCi/well, *Amersham International, UK*) overnight. Finally, the cells were washed once with plain medium, dissolved in 1 N sodium hydroxide at 100 µl/well for 30 minutes and harvested on to glass fibre filter paper. Incorporated radioactivity was measured using a 'Beta-Plate' scintillation counter (*Pharmacia, UK*). Figure 2.2b shows a typical r-TGF-β2 standard curve.

Recombinant cytokines as well as the specific antibodies used in the IFN-γ and TNF-α ELISA assays, and the Mink lung cell line used in the TGF-β bioassay were kindly provided by Professor F.Y. Liew. Recombinant human TGF-β2 was kindly provided by Dr Paul Garside.

2.4.2 Determining frequency of cytokine-secreting cells by ELISPOT assays

The ELISPOT technique (Hutching *et al.*, 1989) was also introduced in the study to quantify the frequency of IFN-γ and IL-2-secreting cells *in vitro*. This is a 'sandwich' assay and the assay protocol was a combination based principally on the ELISPOT assay for detection of autoantibody secreting cells and ELISAs for quantitation of IFN-γ and IL-2, which have been described previously (see 2.2.2ii, 2.4.1ii & iii).

Briefly, the R46AT rat anti-murine IFN-γ at 10 μg/ml, or the 005865 rabbit anti-human IL-2 antibody (IgG fraction, at up to 100 μg/ml), diluted in PBS were used as capture antibodies for coating, 100 μl/well, onto a sterile Millipore Millititer, HA 0.45 μm nitrocellulose membrane based, 96-well plate (5THA 096NS). After incubation overnight at 4 ^{0}C, the plates were washed 3 times with PBS and remaining binding sites on the plates blocked by adding 150 μl/well of RPMI-1640 containing 10% FCS and incubated at 37 ^{0}C for 30 minutes. Unstimulated and Con A-stimulated mouse spleen cells were washed and cultured, 10^{4} to 10^{6} viable cells per well in 200 μl culture medium, at 37 ^{0}C/5% CO_2 for about 16 hours. The plates were then rinsed 5 times and immersed in PBS-Tween for 5 minutes. The procedure was followed by a 1 hour incubation at room temperature with detecting antibodies, the rabbit anti-mouse IFN-γ antibody at 5 μg/ml or the biotinylated rabbit anti-IL-2 antibody at 10 μg/ml, 100 μl/well in PBS-T containing 10% FCS. After washing the plates 5 times, enzyme conjugates: a goat anti-rabbit IgG alkaline phosphatase conjugated antibody (*Sigma*), at

2 μg/ml; or avidin-alkaline phosphatase conjugate (Vector Laboratory) at 1/5000, were added. The conjugates were diluted in PBS-T containing 10% FCS and incubated for 1 hour at room temperature. Finally, the plates were washed 5 times with PBS-Tween, immersed in 0.05M tris buffered saline (TBS, pH 8.0) for 5 minutes and dried using a *Millipore* vacuum water pump system..

5-bromo-4-chloro-3-indoyl phosphate (BCIP, *Sigma*) and p-nitroblue tetrazolium chloride (NBT, *Sigma*) were used as substrate (see 2.2.2ii) to generate an insoluble colour product, blue spots on the nitrocellulose membrane. After the membrane was dry, the number of spots was counted using a *Carl Zeiss* stereo zoom microscope.

2.4.3 Cytokine assay evaluation

The CTLL IL-2 bio-assay showed high sensitivity and consistency in detecting both the r-human and nature murine IL-2 in Con A-stimulated spleen cell culture supernatants. The long term maintenance of the CTLL cell line by the pure recombinant IL-2 might explain why these cells do not response to IL-4. This assay has, therefore, been used to detect and to quantify biologically active IL-2 throughout the study. The assay has a upper detection detection limit which is an equivalence of about 1000 pg per ml of the r-human IL-2 used in the study. It was noted that IL-2 at concentrations above the upper limit had strong over-dose inhibitory effects on the growth of these IL-2 dependent CTLL cells (Fig. 2.3), although IL-2 used in the cultures of mouse spleen cell at much higher doses still showed stimulatory effects (see Chapter 5). Therefore, all samples tested for IL-2 activity were assayed at least in serial doubling dilutions from undiluted up to 1:256. In addition, the possibility of IL-2 inhibitors (see Chapter 6) present in the test samples might have interfered, to some extent, with the results.

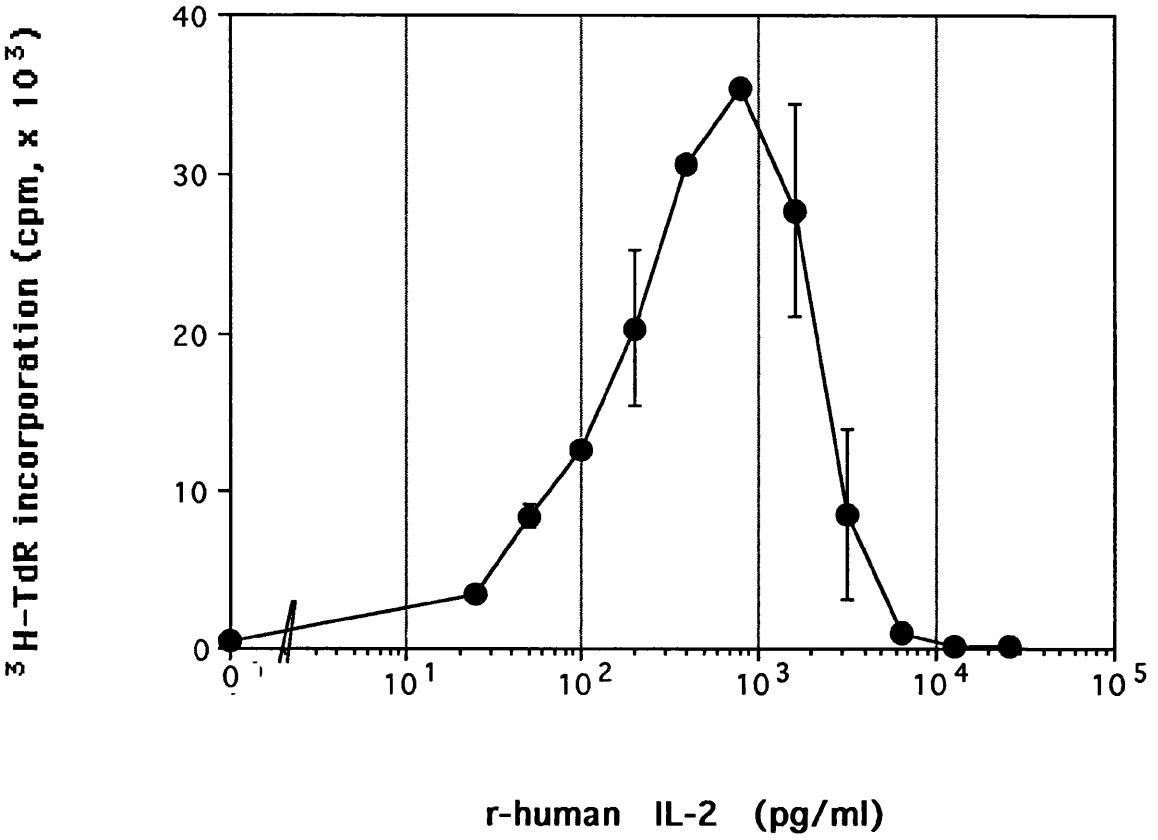


Figure 2.3 High dose inhibitory effects of IL-2 on the CTLL cell line. CTLL cells, at 4 x 10^3 cells per well in a 96-well plate, were incubated with different concentrations of r-human IL-2 in culture medium containing 10%FCS for 24 hours and pulsed with ^{3}H-TdR, 0.5 μCi per well, for the last 4 hours. Cells in the cultures containing IL-2 over 1000 pg per ml had reduced thymidine incorporation. Data shown are means (±SD) of duplicate cultures.

Using antibodies specific for r-human IL-2 in an highly optimised IL-2 ELISA assay, it was able to detect r-human IL-2 at a concentration below 0.1 ng/ml (Fig.2.2c). However, it failed to detect mouse IL-2 in the spleen cell culture medium by ELISA (both the system I and II), and IL-2 secreted by viable Con A-stimulated mouse spleen cells in ELISPOT assays. Although human IL-2 showed strong biological cross-reactivity in supporting growth of the mouse CTLL cells, the antibodies raised in mouse and rabbits specific for r-human IL-2 did not appear to cross-react immunologically (or react very weakly) with mouse IL-2. In addition, the antibodies were also found to block only weakly function of r-human IL-2 in the CTLL assay.

The ELISA assays for IFN-γ and IL-4 showed high sensitivity in detecting both recombinant and mouse spleen cell-derived cytokines with reproducible results. The TNF-α ELISA is also a reproducible assay but detected no TNF-α in all culture supernatants of Con A-stimulated mouse spleen cells from normal and lupus mice, suggesting negative effect of Con A in the induction of TNF-α production. The IFN-γ ELISPOT assay detects readily IFN-γ secretion by Con A stimulated mouse spleen cells. However, the attempt of using same antibodies as that used in the IL-4 ELISA assay has failed to detect IL-4 secretion by the IL-4 ELISPOT technique presumably due to the low amount of the cytokine secreted in cultures. The TGF-β bio-assay also showed high sensitivity in the cytokine detection. TGF-β neutralising antibody has not been used in the MvILu bio-assay to determine the specificity of the assay in the present study, but this has been demonstrated elsewhere (*Wellcome Lab, UK*).

2.5 FACS analysis of cell surface markers and receptor expression

2.5.1 Analysis of IL-2R (CD25) expression on T and B-cells

Surface IL-2R positive cells were detected by using a monoclonal rat anti-mouse CD25 antibody (IgG2a, Clone AMT13, *Boehringer Mannheim, Germany*) specific for mouse IL-2R α-chain conjugated with R-phycoerythrin (PE). Pan-T cell marker was a hamster anti-mouse CD3 FITC-conjugated monoclonal antibody (IgG, Clone 145-2C11, *Boehringer Mannheim, Germany*). Pan-B cell marker was a mixture of rabbit anti-mouse IgG Kappa-chain (MR11 and MR13, the Laboratory) and rabbit anti-mouse IgM (RC406/8/9, the Laboratory), which were conjugated with FITC (see 2.2.4i).

To label the cells, approximately one million splenocytes, stimulated and unstimulated, were incubated with the conjugated anti-CD25 antibody (1 μg in 100 μl) as well as the T or B-cell marker at 4 °C for 30 minutes. Contaminated red cells were lysed with 1ml of 0.144M ammonium chloride in 17 mM Tris buffer (pH 7.2) for 5 minutes at room temperature. The cells were then washed twice with cold PBS and resuspended in 1ml of PBS containing 0.5%BSA. IL-2R cell surface expression was analysed using a *Becton & Dickinson* fluorescence-activated cell sorter (LYSYS II program). 10^4 viable cells were analysed from each sample. The number of CD25 positive cells is expressed as '% cell frequency'. The fluorescence intensity indicates density of IL-2R expressed on the cell surface. To eliminate non-specific binding, cells were preincubated, prior to antibody staining, with 0.5%BSA/PBS containing 0.02% sodium azide and 1% normal rat serum. An isotype control, rat myeloma IgG2a conjugated with R-phycoerythrin and FITC (*Caltag, USA*), was used to identify false positivity due to Fc binding. Propidium iodide, which stains dead cells, was used at 1 μg/ml to assess cell viability, and to eliminate dead cells during acquisition which might otherwise interfere with subsequent analysis.

2.5.2 T-cell phenotyping

To study T-cell phenotype changes in the disease development of lupus mice, the expression of functional T-cell markers CD4, CD8 and CD3 were analysed by using antibodies to these markers. The antibodies used in the analysis were: PE-conjugated rat anti-mouse L3T4 (CD4) (4 μl/test, *Becton & Dickinson*), FITC-conjugated or biotinylated rat anti-mouse Lyt 2 (CD8) (4 μl/test, *Becton & Dickinson*), and the pan-T cell marker hamster anti-mouse CD3 FITC-conjugated (Clone 145-2C11, 0.5 μg/test, *Boehringer Mannheim, Germany*). Mouse spleen cells were dual-labelled as follow:

Unstimulated and stimulated cells were washed 2 times with ice-cold PBS containing 0.1% sodium azide. For each sample, cells were prepared in 3 separate tubes, about one million cells per tube. Cells in each of the 3 tubes were incubated for 30 minutes at 4 °C, with 2 of the above antibody conjugates at different combinations: anti-CD4 (PE) + anti-CD8 (FITC), anti-CD4 (PE) + anti-CD3 (FITC), anti-CD8 (biotin) + anti CD3 (FITC). Cells labelled with antibodies of the third combination were further incubated with PE-conjugated avidin (*Vector Laboratory*). The cells were then washed twice with cold PBS and resuspended in 1ml of PBS containing 0.5%BSA for FACS analysis.

2.6 PCR-assisted cytokine Message Amplification Phenotyping (MAPPing)

The rapid, highly sensitive technique developed by Brenner et al (1989) to simultaneously analyse the array of cytokine messenger RNAs made by small numbers of cells has been modified to study cytokine gene expression in lupus mice. The technique incorporates a micro-procedure for isolating total cellular RNA, reverse transcription of mRNA to produce cDNA (complementary DNA), and enzymatic

amplification of cytokine-specific DNA fragments using the polymerase chain reaction (PCR).

One day before the operation, double distilled water (ddH2O), TE buffer, microtubes (1.5ml and 0.5ml) and pipette tips were autoclaved.

2.6.1 Cellular RNA extraction

i) Cell preparation

Mouse spleen cells were prepared as described previously (2.3.1). Spleen cells from 3 to 5 mice of the same strain and age were pooled. The splenic lymphocytes used for cellular RNA extraction included cells that were freshly isolated from mouse spleens and cells cultured without added stimulus (unstimulated), and those that were subsequently stimulated *in vitro*. For T-cell activation, spleen cells were cultured in $25cm^2$ culture flasks, at a density of 1×10^7 viable lymphocytes per millilitre, in RPMI-1640 complete culture medium containing 5% FCS with or without Con A (2.5 μg/ml, *Sigma*). Cell samples were collected into 1.5ml microtubes, 2×10^7 cells/sample, at 0 (freshly isolated), 3, 6, 9 and 16 hours of incubation and pelleted by centrifugation for 5 minutes at 1300 g, 4 ^{0}C. After contaminated red cells were lysed by exposure to distilled water for 30 seconds, the cell pellets were washed with ice-cold PBS twice and stored at -70 ^{0}C until all samples were ready for RNA extraction.

ii) RNA extraction

Total cellular RNA preparation was performed using a RNAzol^{TM} B method (Chomczynski and Sacchi, 1987). To the cell pellet of each sample, 0.8 ml of RNAzol^{TM} B (*Biogenesis*) was added to lyse the cells, and the cellular RNA was solubilised by passing the lysate a few times through a blue-tip pipette. To each tube, 80 μl of chloroform (*Sigma*) was added and the mixture was shaked vigorously for 15 seconds with the tube tightly covered. The samples were allowed to stand on ice for 5

minutes followed by centrifugation at 12000 g for 15 minutes at 4 ^{0}C. The colourless upper aqueous phase, which contained purified soluble RNA , was transferred into fresh tubes. The RNA was then precipitated by addition of an equal volume (about 0.4 ~ 0.5 ml) of anhydrous isopropanol (*Sigma*) and the samples stored for at least 15 minutes at 4 ^{0}C. After centrifugation at 12000 g for 20 minutes at 4 ^{0}C, RNA precipitates formed a white-yellow pellet at the bottom of the tube and the supernatant was removed. To wash the pellet, 1.5 ml cold 75% ethanol was added. After a brief vortexing, the samples were repelleted at 7500 g for 8 minutes and the supernatant removed. The RNA samples in the residue ethanol was finally dried under vacuum for 10 minutes (not completely dry) at room temperature, resuspended with 50 µl of ice-cold TE buffer (10 mM Tris, 1mM EDTA, pH 7) and heated at 90 ^{0}C for 5 minutes. RNA concentration was determined by its optical density at 260 nm and 280 nm and estimated as follow:

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

RNA samples were kept at -20 ^{0}C up to 1 month, or at -70 ^{0}C for longer periods.

2.6.2 mRNA reverse transcription

cDNA was synthesised by mRNA reverse transcription. Three micrograms of total cellular RNA in each sample were reverse transcribed using either oligo-dT_{15} or random hexanucleotides as primers in a 20-µl reaction. The reaction mixture contained 20 units of RNasin^R RNase inhibitor (*Promega*), 0.8 µg of oligo-dT_{15} (*Promega*) or 0.09 units of random hexanucleotides (*Gibco BRL*), 0.25 mM each dNTP (dATP, dCTP, dGTP, dTTP), 4 µl of 5x RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 10 mM DTT and 200 units Moloney murine leukemia virus (M-MLV) reverse transcriptase (*Gibco BRL*). The RNA sample was heated at 90 ^{0}C for 5 minutes and quickly chilled on ice to break up secondary structures and aggregates

before addition to the reaction mixture (Innis and Gelfand, 1990). Samples were then mixed by spinning for 1 minute at 7500 g and standing at room temperature for 5 to 10 minutes. The reaction was carried out at 37 ^{0}C for 1 hour, and stopped by heating at 75 ^{0}C for 10 minutes then chilling quickly on ice.

2.6.3 cDNA PCR-amplification

i) Primers

Murine IL-2, IFN-γ and β-actin specific primer pairs were synthesised by *Genosys* (USA) according to primer sequences designed by Ehlers et al. (1992) for IL-2, IFN-γ, IL-4 and IL-6; and by Barral-Netto et al. (1992) for β-actin (Table 2.1).

Table 2.1 Oligonucleotide sequences of cytokine primers for PCR

IL-2 (320bp)	
sense	5'-ATG-TAC-AGC-ATG-CAG-CTC-GCA-TCC-TGT-GTC-A-3'
antisense	5'-AGT-CAA-ATC-CAG-AAC-ATG-CCG-CAG-AGG-TCC-A-3'
IFN-γ (388bp)	
sense	5'-GAA-AGC-CTA-GAA-AGT-CTG-AAT-AAC-T-3'
antisense	5'-ATC-AGC-AGC-GAC-TCC-TTT-TCC-GCT-T-3'
IL-4 (351bp)	
sense	5'-ACA-AAA-ATC-ACT-TGA-GAG-AGA-TCA-T-3'
antisense	5'-AGT-AAT-CCA-TTT-GCA-TGA-TGC-TCT-T-3'
IL-6 (600bp)	
sense	5'-CTG-GTG-ACA-ACC-ACG-GCC-TTC-CCT-A-3'
antisense	5'-ATG-CTT-AGG-CAT-AAC-GCA-CTA-GGT-T-3'
β-actin (420bp)	
sense	5'-GAC-TTC-GAG-CAG-GAG-ATG-GCC-AC-3'
antisense	5'-CTA-CAC-CTA-GTC-GTT-CGT-CCT-C-3'

ii) PCR-amplification

The conditions of the PCR assay were optimised in different preliminary experiments, including concentrations of magnesium, dNTPs and primers, as well as temperature for cDNA denaturation, primer annealing and extension. The results showed that these conditions were important in obtaining sensitive amplification with

high specificity. In particular, it was found that the concentration of magnesium used in the PCR reaction was crucial, which was optimal at about 1.5 mM within a narrow range between 1 to 2 mM under the following conditions.

All procedure was performed on ice unless where indicated otherwise. An aliquot of 2 microlitres of the heat-chill-treated cDNA (equivalent to 0.3 μg of total cellular RNA) was amplified in a 0.5ml microtube in the presence of 0.2 μM final concentration of upstream and downstream specific primers (Table 2.1), 0.1 mM each dNTP, 1.25 units Thermus aquaticus thermostable (Taq) DNA polymerase (*Promega*), and PCR buffer containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, and 0.1% Triton X-100) in a final volume of 50 μl. Samples were then mixed by spinning for 1 minute at 7500 g, and overlaid with a drop of light mineral oil (*Sigma*) to prevent evaporation during thermal cycling. PCR was performed in a DNA thermal cycler (*Techne PHC-3, UK*) for 30-35 cycles: (1) denaturing for 1 minute at 94 ^{0}C; (2) primer annealing for 2 minutes at 56 ^{0}C; and (3) primer extending for 3 minutes at 72 ^{0}C. At the end of the programme, the primer extension was allowed a further 8 minutes at 72 ^{0}C.

iii) Analysis of amplification products

After amplification, the mineral oil was removed. The PCR reaction product was visualised by electrophoresis of 10 μl of the reaction mix (equivalent to 30 ng of total cellular RNA origin) at 80 mA for 45 minutes in 1% agarose (Electrophoresis grade, *Bethesda Research Laboratory, USA*) in 0.5 x tris-borate-EDTA (TBE, see Appendix I) buffer containing 0.5 μg/ml ethidium bromide. One microgram of 1kb DNA ladder (*Gibco BRL, UK*) was run in parallel as m.w. markers giving bands at 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517, 506, 396, 344, 298, 220, 201, 154, 134 and 75bp, for size estimates of the products. Negative controls included amplification of a sham reverse transcription without Taq DNA polymerase to assess any contribution of contaminating genomic DNA in the

RNA samples; and amplification of a reaction mix with no added cDNA to assess contamination by plasmid or other sources of DNA.

2.7 Kidney histopathology and immunohistochemistry

2.7.1 Preparation of tissue sections

i) Cryostat sections

Cryostat sections were prepared from the frozen kidney tissue for immunofluorescence staining of immune complex deposition. 24-48 hours before screening, 5 μm cryostat tissue sections were cut and stored at 4 ^{0}C (see 2.1.3). After 30 minutes at room temperature, the sections were washed in PBS in a Coplin jar on a shaker with 2 changes of PBS over 20 minutes. The section were then fixed in cold ether ethanol (50: 50) for 10 minutes and followed by 20 minutes in methylated spirits.

ii) Paraffin sections

5 μm thick Bouin's-fixed, paraffin-embedded kidney tissue sections were dewaxed in xylene for 8 minutes, rehydrated through graded alcohols and into water. For histopathological examination, the sections were stained by the periodic-acid Schiff technique (Lendrum *et al.*, 1962) and examined by light microscopy. To detect kidney immune complex deposition, the dewaxed paraffin sections were processed for immunofluorescence staining.

2.7.2 Immunofluorescence staining

Alcohol-fixed cryostat 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 nonspecific reaction, the sections were

blocked with 2% (w/v) BSA in PBS. Excess BSA was drained off, and the sections were incubated with the detecting antibodies at 1:10 diluted in 2% BSA/PBS, 30 minutes for cryostat sections or 1 hour for paraffin sections, in a full humidity environment at room temperature. To detect immune complexes, a FITC-conjugated sheep anti-mouse IgG (*Sapu*) was used to detect autoantibody binding; and a goat $(Fab)_2$ anti-mouse C3 antibody (*Cappel, USA*) was used to observe complement activation, which was followed by a FITC-conjugated donkey anti-goat/sheep IgG antibody (*Sapu*) at 1:20 in the same diluent. To assess autofluorescence 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. All antisera used for the staining had been pre-absorbed with normal sheep or donkey liver powder to minimise non-specific binding (see 2.2.3iii). Finally, the sections were mounted in 'Citifluor' mounting fluid and examined using a fluorescence microscope.

2.8 Measurement of serum nitric oxide (NO) levels

2.8.1 The nitrate reductase assay (Bartholomew, 1984)

i) Nitrate (NO_3^-) ---> Nitrite (NO_2^-) conversion

Nitric oxide has a very short half-life and is rapidly converted to nitrate (NO_3^-) in plasma. To measure serum NO levels, a conversion of NO_3^- to NO_2^- by nitrate reductase must first be carried out. To convert, 30 µl of the serum sample was incubated with an equal volume of reaction buffer containing 5 mg/ml NADPH, 41.5 mg/ml FAD, 0.5 M KH_2PO_4 and 35 mg/ml of the nitrate reductase (*Sigma*) added immediately before used. The conversion was carried out at 37 ^{0}C for 2 hours in a 96-well ELISA plate. NO standards were also run in parallel with the test samples.

ii) NO quantitation

A Disibi Chemiluminescence NO Analyser (Model 2107) was used to measure NO concentration in the serum samples. A boiling solution containing 75 ml of concentrated acetic acid, 25 ml 6% sodium iodide (kept dark until used) in distilled water (dH2O) was added to a round bottomed flask. Once the analyser had stabilised (about 1/2 hour), nitrite NO_2^- standards were injected starting with the highest dose. The standard and test samples were propelled through the system by a flow of nitrogen, after being boiled off from a mixture of acetic acid/sodium iodide. Oxygen, extracted from air was converted to O3 in the reaction chamber at 42 ^{0}C. Breakdown of the O3 to O2 excited NO_2^-. On its return to ground state, light was evolved. A reading of each sample at its peak value was taken and allowed to return to baseline before adding the next sample. All measurements were recorded in conjunction with a background reading which was subtracted from the sample reading.

For NO quantitation, standards were prepared from known amounts of stock NO_3^- and NO_2^- and run in parallel with test samples in every assay. The standards were made up freshly in dH2O every 6 weeks, including 300, 100, 30, and 10 μM for both NO_3^- and NO_2^-. All standard and test samples were assayed in triplicate and calculated as mean and standard deviation. For the negative control, the serum sample was replaced by distilled water in the same buffer. Figure 2.4 shows typical standard curves for both nitrate and nitrite. The assay had a detection limit below 5 μM. Sample NO level was estimated from the NO_3^- and NO_2^- standard curves.

2.8.2 The Greiss method (Green *et al.*, 1982; Drapier and Hibbs, 1988)

i) Preparation of the Greiss solution

The Greiss solution was prepared fresh immediately before use. The solution was obtained by mixing equal volume of 0.1% alpha-naphtyl-amine in distilled water (Solution A) and 1% sulfanilamide in 5% phosphoric acid (Solution B). Both of the

stock Solutions A and B were kept in dark (light sensitive) at 4 ^{0}C up to a maximum of 2 months.

ii) NO quantitation by the Greiss reaction

To 50 μl per well of test samples in 96-well ELISA plates, an equal volume of the Greiss solution was added. After 10 minutes of development at room temperature, the plates were read by an ELISA reader at 550 nm. Sample NO levels were estimated by the nitrite standard curve which was established by doubling dilutions of a sodium nitrite stock solution (10 μg/ml) in every assay. For NO quantification by the Greiss reaction, nitrite secretion in culture supernatants were measured directly. However, for measurement of serum samples, the conversion procedure described in Section 2.8.1i was also carried out before assaying.

2.9 The construction of BRD509/TGF-β1 and BRD509/IL-2

The *S. typhimurium* mutant used in the present study, BRD509, was an *aro A*$^-$ *aro D*$^-$ double mutant strain derived from *S. typhimurium* SL1344 (*Salmonella Genetic Stock Centre, Calgary, Canada*). Mouse cytokine cDNA (TGF-β1 and IL-2) was purchased from *DNAX, UK.* The expression plasmid pKK-TGF-β1 and pKK-IL-2 were constructed by Dr D-M Xu in the Department, using commercially available expression vehicle pKK-233-2 (*Pharmacia, Uppsala, Sweden*).

2.9.1 The construction procedure (brief)

The procedure for the construction has been described previously in detail by Strugnell (1992). Briefly, the leading sequence of the cytokine cDNA was deleted and the cDNA reconstructed by PCR to make it suitable for expression in *Salmonella.* Table 2.2 shows the primer sequences used in the PCR reconstruction for murine TGF-β1 and IL-2. The DNA fragments derived were then purified by the standard method

and inserted into the expression vector pKK-233-2. The recombinant plasmids were screened by restriction enzyme mapping and the positive plasmid were transformed into *E. coli* strain TG1. The expression of TGF-β1 and IL-2 was then determined by western blot. A brief outline of the molecular construction and transformation is illustrated in Figure 2.5 (provided by D-M Xu).

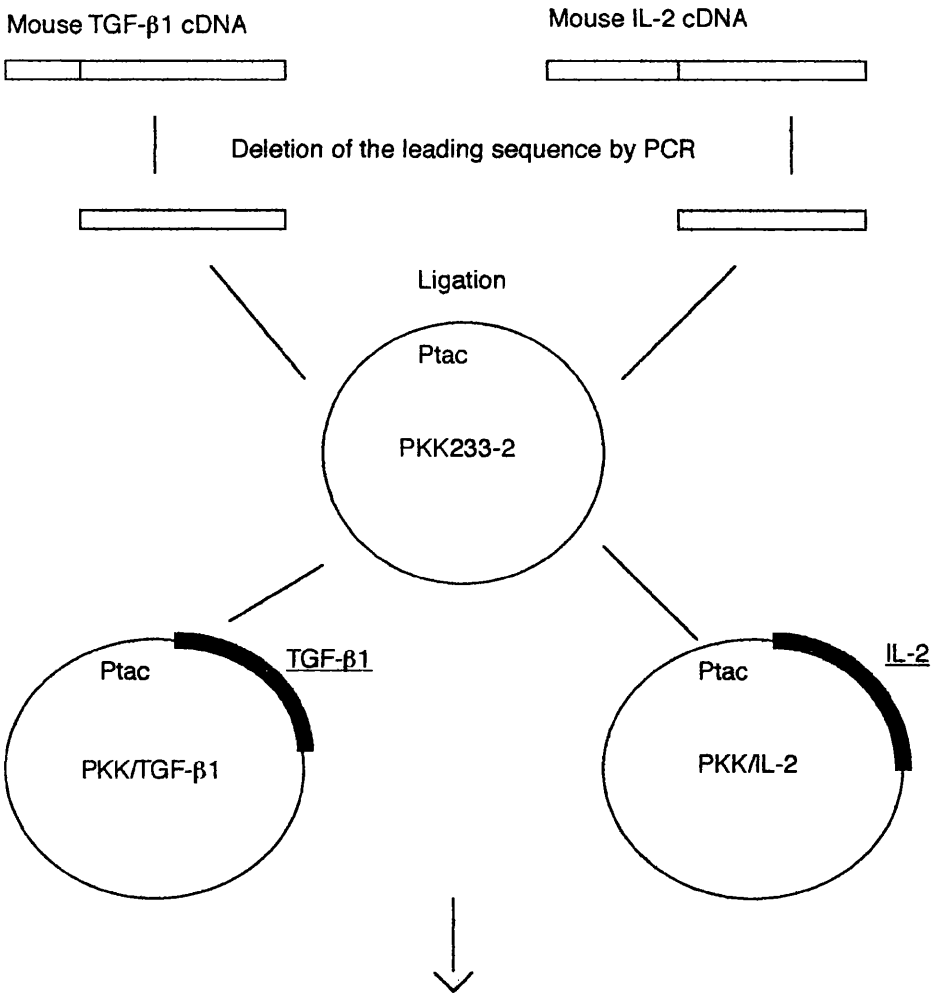
Table 2.2

Primer sequences for cytokine cDNA reconstruction

TGF-β1	
sence	5'-CTGCAGAGCTCACGGCACCCCATGGCCCTGGATACCAAC-3'
anti-sence	5'-GGCGGGGGCGGGGCCAAGCTTGCCGGGAGGGGCGGG-3'
IL-2	
sence	5'-ACACTTGTGCTCCTTGGATCCATGGCACCCACTTCAAGC-3'
anti-sence	5'-CATATTTATCATCTGCAGACTAGTAGTTAC-3'

2.9.2 Propagation of the *Salmonella* bacteria for treatment of mice

One day before the treatment, the cytokine gene transfected and the wild-type bacteria were grown statistically overnight in 500 ml L-broth bulk cultures from a starting OD of 0.05 to a final OD of 0.2 (about 10^8 bacteria per ml). The bacteria were then harvested and concentrated by centrifugation at room temperature, for 7 minutes at 9000g (Beckman centrifuge, JA10 rotor), and the pellet resuspended in small volume of PBS, at 5×10^{10} per ml. For oral treatment of mice, 10^{10} live *Salmonella* per dose in 0.2 ml of the bacterial suspension was given to each mouse by a gavage tube. In addition, aliquots of the bacterial samples were also assessed for cytoplasmic expression of the cytokines by bio-assays.



Transformed into E.Coli and identified by Western blot and bioassays

Preparation of the plasmid from the postive clone and transformation of the plasmid into *S. typhimurium* BRD 5O1O

Transduction of the plasmid from BRD5010 into *S. typhimurium Aro A⁻,Aro D⁻* BRD 509 by phage22 and identification of the ampicillin resistant colonies were identified by Western blot and bioassays

Figure 2.5 Diagram illustrating a brief procedure for the construction of BRD509/cytokines

(provided by D-M Xu, the Department)

2.9.3 Extraction of cytoplasmic cytokines expressed by *S. typhimurium*

To confirm the expression of TGF-β1 and IL-2 in the gene transfected *S. typhimurium*, the cytokines were isolated from cytoplasm of the bacteria following an extraction procedure. To extract, 10^8 bacteria in 1 ml culture were centrifuged at 12000g for 2 mins and 200 µl of 7M urea added. After a 4 h incubation at RT, the samples were centrifuged at 12000g for 5 mins and the supernatants collected. 1.02 ml of saturated ammonium sulphate was then added to the supernatant, and mixed at 4 °C overnight. Precipitated proteins were spun down at 4 °C, 12000g for 5 mins, and resuspended in 100 µl of RPMI 1640. The suspension was left at 4 °C for 1.5 hrs before being tested by the MvILu TGF-β bioassay for TGF-β activity, or by the CTLL IL-2 bioassay for IL-2 activity.

2.10 Statistical analysis

The Mann Whitney Test was the only statistical method used in the study. Results which were subjected to the test are indicated (P value) and described in each of the figure legends as appropriate.

Chapter 3

Early, progressive IL-2 defect and the natural history of disease kinetics of murine lupus

3.1 Introduction

The early finding of the *in vitro* mitogen-induced IL-2 hypoproduction in SLE has led to many contradictory interpretations over its *in vivo* significance for the development of lupus disease (Kroemer and Martinez-A, 1991). It is not clear whether or how this T-cell defect might be contributing to the origins of abnormalities leading to B-cell hyperactivation and autoaggression. The nature of the T-cell functional defect therefore remains to be clarified.

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). There are many advantages of using lupus mice over the human system to study the disease development. Firstly, unlike studies in human system, the mouse system may have less problems in the interpretation of results due to clinical diagnosis or treatment, such as drug effects. Secondly, some lupus mouse models like MRL/*lpr* and BXSB are inbred mutant strains. These mice are, in theory, genetically identical in spite of some individual variations due to environmental influences or the stochastic process of mouse generation. Another important aspect, thirdly, of using the mouse models is the natural history of murine lupus. Like other animal disease models, lupus mice start to develop clinical disease only at certain ages. It offers opportunities to study any existing defects in the mice not only during but also before the development of the clinical disease. 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. However, these strain-dependent differences in disease onset allow analysis of the relationship or possible link between disease kinetics and those experimentally found immunological or physiopathological changes.

To study the nature of the previously reported *in vitro* IL-2 hypoactivity in SLE, the first two questions addressed in the present study were: "whether the cytokine system is indeed defective in lupus mouse models;" and if so, "at what stage of the disease development does it occur ?"

3.2 Con A-induced IL-2 production in normal and lupus mice

3.2.1 Determination of the optimum concentration of Con A for T-cell activation

The ability of T-cells to produce IL-2 was assessed by *in vitro* T-cell mitogen stimulation. Con A was used throughout the study, and the optimum concentration for stimulation of T-cell proliferation and IL-2 production was determined. Figure 3.1 shows the dose-dependent response of BALB/c splenic T-cells to Con A measured by secretion of IL-2 and incorporation of ^{3}H-thymidine. The results indicate that the optimum dose of Con A depended on the concentration of foetal calf serum (FCS) added in the culture medium. To induce IL-2 secretion, the optimum concentration of Con A is 2.5 and 5 μg/ml in cultures containing 5 and 10% FCS respectively (3.1a, arrows). Although for a peak proliferative response Con A was optimum at 0.625 - 1.25 and 2.5 - 5 μg/ml in cultures containing 5 and 10% FCS respectively, the T-cells responded well to a relatively broader range of Con A concentration (3.1b). At 2.5 μg/ml it induced satisfactory T-cell proliferation in either culture conditions containing 5 or 10% FCS. Considering both IL-2 production and cell proliferation, therefore, Con A was used at 2.5 μg/ml, in cultures containing 5% FCS, for the *in vitro* mouse T-cell activation in all subsequent experiments.

3.2.2 Kinetics of Con A-induced IL-2 production

In adult mice, similar kinetics of Con A-induced IL-2 production were observed between the two lupus strains and BALB/c control mice according to repeated time course studies. In the culture supernatants of Con A-stimulated mouse spleen cells

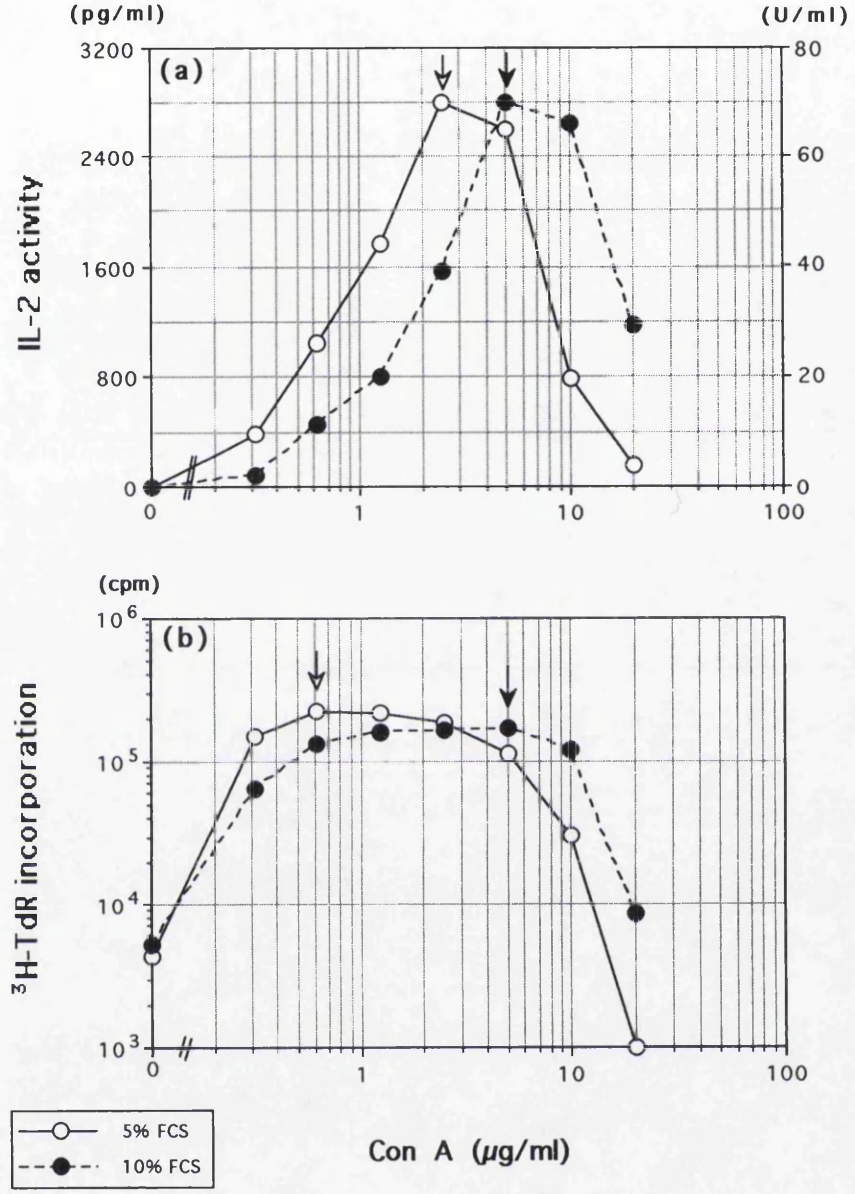


Figure 3.1 Con A dose-response curves for T-cell activation. Spleen cells from BALB/c mice were cultured at 10^6 cells/ml in RPMI complete culture medium containing 10 or 5% of FCS in the presence of different concentrations of Con A as indicated. (a) IL-2 activities presented in the culture supernatants were measured at 24 hrs by the CTLL assay; (b) cell proliferative responses were determined at 48 hrs by tritiated thymidine incorporation. Data are shown as the means of triplicates. The peaks of the responses were indicated by arrows.

from these 3 strains, IL-2 activity was detectable after 2h, and it increased rapidly and normally reached a peak at 24h. However, individual variations exist particularly in the lupus strains. An earlier fall or sustained levels of the IL-2 activity were seen in some cases (Fig.3.2). However, peak IL-2 production by spleen cells from CBA mice appeared to be relatively late, at between 48 and 72h (Fig.3.3). IL-2 activity was undetectable in the culture supernatants of spleen cells without Con A stimulation. In addition, there were age-related differences in the kinetics of IL-2 production between young mice. The peak IL-2 activity was delayed by 1 to 2 days in mice under 2-months old, compared with mice of 3 months or older (Fig.3.4).

Therefore, to determine IL-2 production by spleen cells from different strains and ages, the results presented were IL-2 activity detected at its peak time point, for each age and strain of mouse.

3.2.3 IL-2 activity in old NZB/W and MRL/*lpr* mice with clinical disease

MRL/*lpr* is a typical early-life lupus disease mouse model with a 50% mortality rate (female and male) at 5 months of age (Theofilopoulos, 1992). In contrast, NZB/W mice develop disease later, and the 50% mortality rate is at 8 and 12 months for the female and male mice respectively. According to their age at peak disease activities, the mice chosen for this experiment were female MRL/*lpr* of 4-6 months and female NZB/W of 7-9 months. All of the mice used in the experiment showed various degrees of clinical manifestations including skin lesion, enlarged spleens, and tumours (MRL/*lpr* strain, due to lymphoproliferation).

IL-2 activity, determined by the CTLL bioassay, was significantly reduced in culture supernatants of Con A-stimulated spleen cells from old lupus mice (Fig.3.5). In spite of considerable individual variation and strain differences, the IL-2 hypoactivity in lupus mice was consistently found to be highly significant in every experiment, in

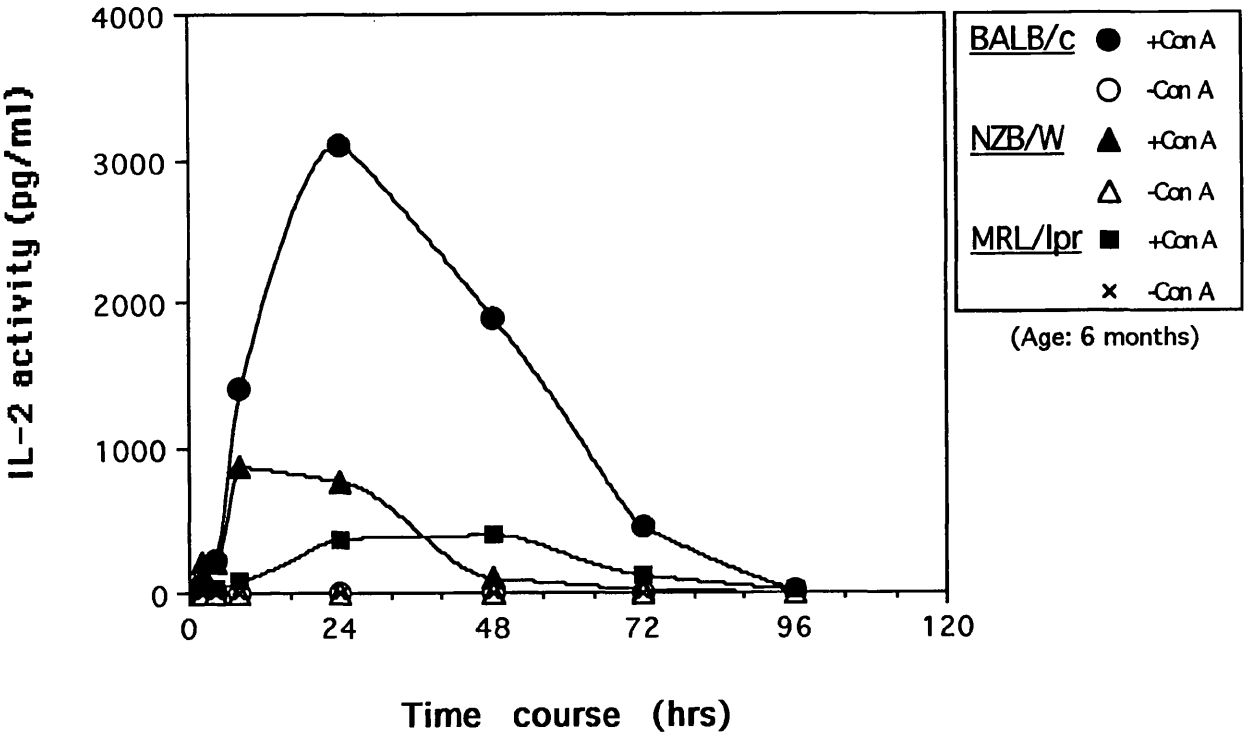


Figure 3.2 Kinetics of Con A-induced IL-2 secretion by spleen cells from BALB/c, NZB/W and MRL/lpr mice. Spleen cells from 2 mice of each strain were pooled and cultured at 10^6 cells/ml in RPMI complete culture medium containing 5% of FCS in the presence or absence of Con A (2.5 μg/ml). IL-2 activities in the culture supernatants were measured at 0, 2, 4, 8, 24, 48, 72 and 96 hrs by the CTLL assay.

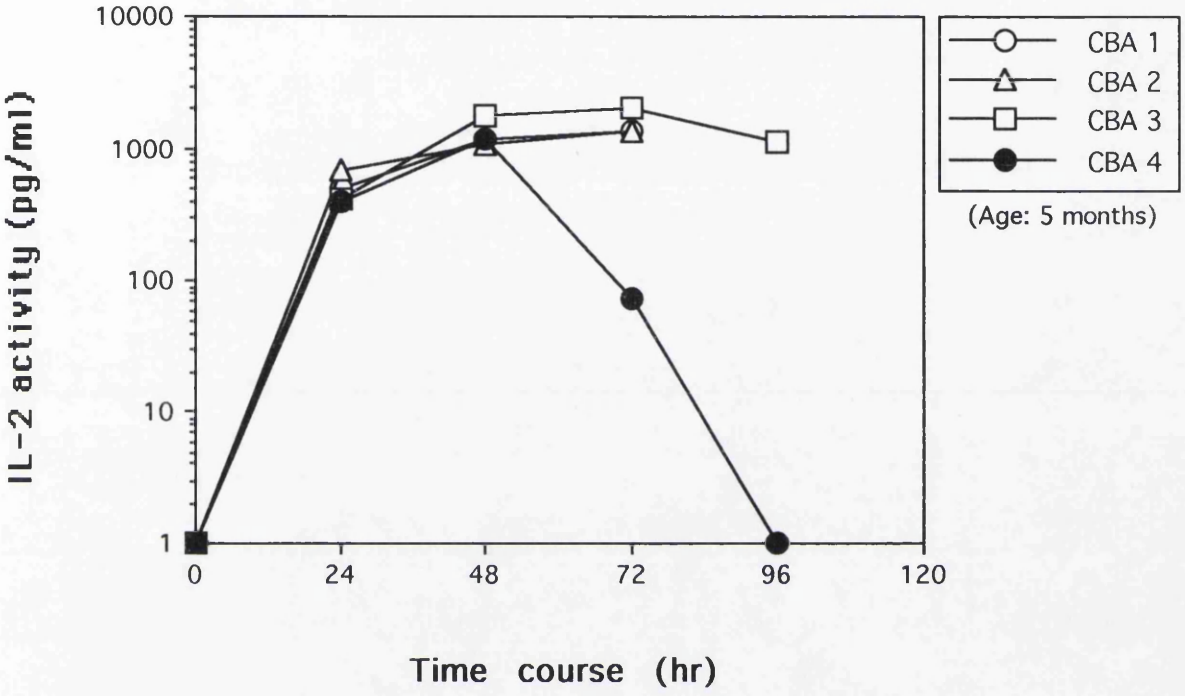


Figure 3.3 Kinetics of Con A-induced IL-2 secretion by spleen cells from 4 individual CBA mice (CBA 1-4).

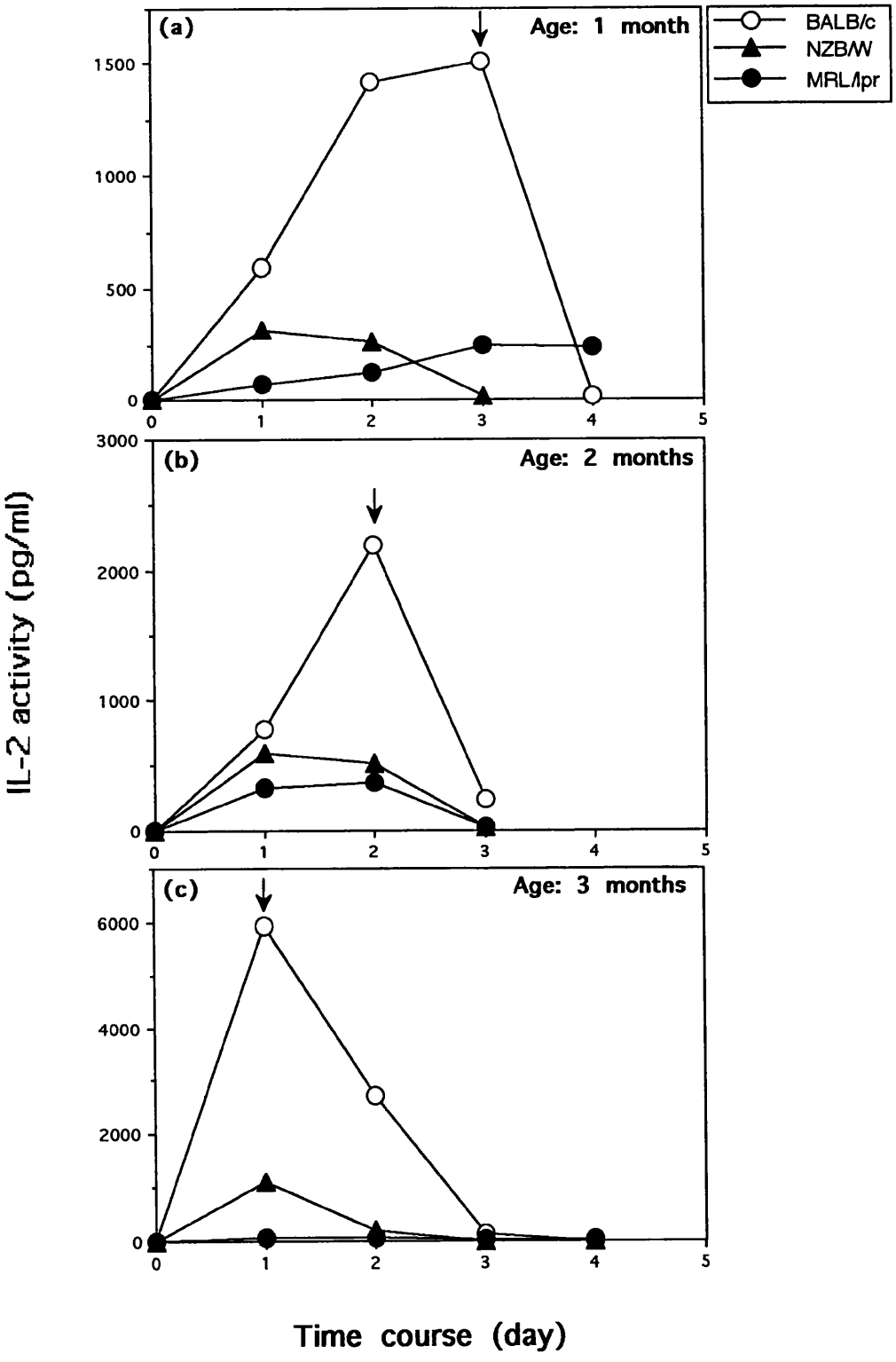


Figure 3.4 Kinetics of Con A-induced IL-2 secretion in young BALB/c, NZB/W and MRL/lpr mice. Spleen cells from 2 mice of each strain and age group were pooled and cultured at 10^6 cells/ml in RPMI complete culture medium containing 5% of FCS in the presence of Con A (2.5 μg/ml). Culture supernatants were collected at daily intervals and IL-2 activities measured by the CTLL assay.

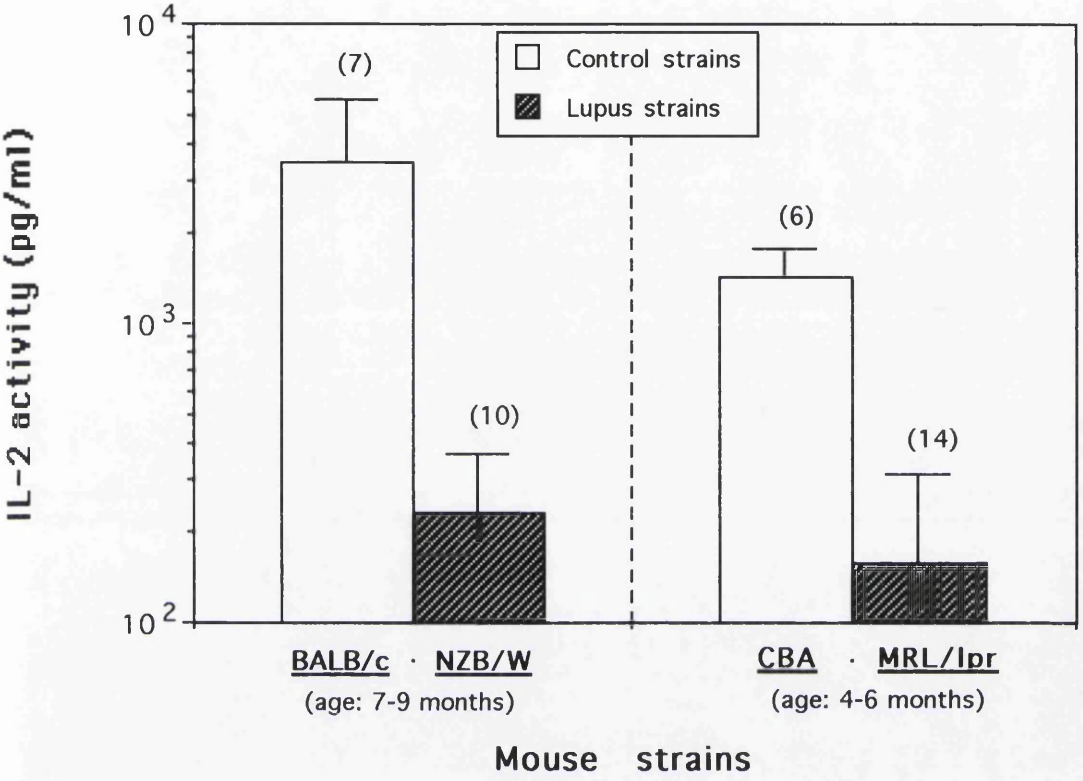


Figure 3.5 Defective Con A-induced IL-2 production in old lupus mice with active disease. Spleen cells from individual mice of each strain and age group were cultured at 10^6 cells/ml in RPMI completed culture medium containing 5% of FCS in the presence of Con A (2.5 μg/ml). IL-2 activities were measured at daily intervals and data shown are means (±SD) of the peak values obtained for each mouse. Number of mice in each group is indicated on the top of each column.

comparison to the sex-age-matched BALB/c and CBA control mice ($P<0.01$). Since the data shown were the peak IL-2 activity present in the culture supernatants, the defective IL-2 production by lupus T-cells was not due to kinetic differences from control mice or between strains. There was no spontaneous IL-2 secretion by the spleen cells cultured without Con A detected in both the lupus and the normal control mice.

3.2.4 IL-2 deficiency: its early onset in lupus mice

To determine whether the IL-2 defect found in old lupus mice was a consequence of the autoimmune lesions, the next step of the study was to assess T-cell function in mice of different ages from as young as 1-week old, preceding the onset of disease activity. However, IL-2 hypoactivity was evident in all age groups studied except the 1-week old mice (Fig.3.6). Relatively higher IL-2 activity was noted in cultures from younger NZB/W mice than in old NZB/W mice and both young and old MRL/*lpr* strain mice. In general, the older the mice the greater the difference observed between the control and the lupus mice.

IL-2 activity was not measurable in culture supernatants of Con A stimulated spleen cells from 6-day old BALB/c mice though low levels of IL-2 (<30 pg/ml) could be detected in the two lupus strain mice at the same age (Fig.3.6). This was due to a lack of T-cells in mouse spleens ($CD3^+ < 4\%$) at this very early age (see Figs.3.8, 3.9).

3.3 The age-related lupus disease activities: serological changes, cellular abnormalities and clinical manifestations

As mentioned previously, the onset and age relation of the disease in many lupus models have been well studied with considerable agreement between different

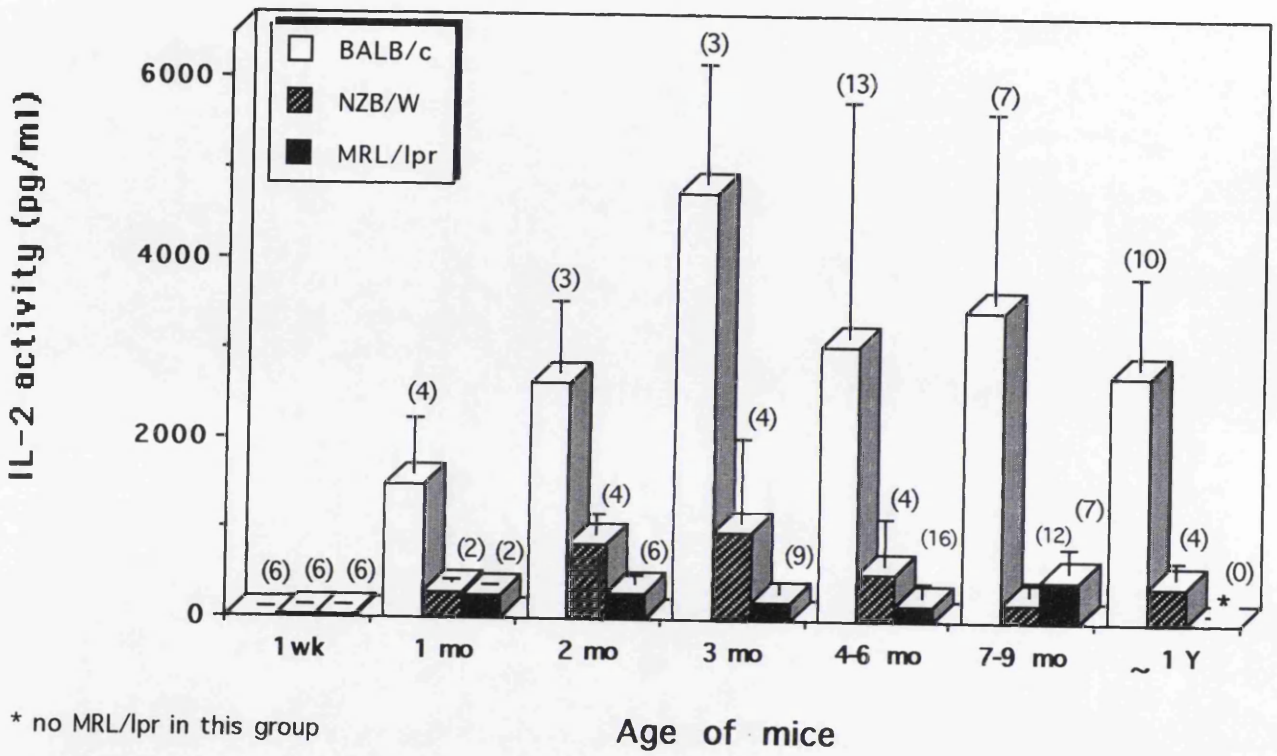


Figure 3.6 **Early, progressive IL-2 hypoactivity in lupus mice.** The results show Con A-induced IL-2 production by spleen cells from normal and lupus mice at different ages. IL-2 activities were measured by the CTLL bio-assay, and data shown are means (±SD) of the peak values obtained in a daily time course. Number of mice in each group is indicated on the top of each column. The difference between control and lupus mice of either strain is highly significant ($P<0.01$), except the 1-week group, as judged by the 'Mann Whitney Test'. For statistical analysis, data from the young mouse groups (1-3 months) were combined.

laboratories (Theofilopoulos and Dixon, 1985; Theofilopoulos, 1992). In the present study for the purpose of confirmation, some aspects of the possible pathological activities of the disease in the lupus mice used were studied.

3.3.1 Autoantibody production

Screening 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. Although there has been some controversy regarding the correlation between the autoantibodies and lupus disease activity, the dsDNA binding test has remained the single the most important laboratory test for SLE as approximately 75%, or higher depending on the assay methods used, of patients with the disease are seropositive (Morrow *et al.*, 1982; Buskila and Shoenfeld, 1992). As in human lupus, serum autoantibodies to an extensive array of molecules including the nucleic acids can be detected in different strains of lupus mice. These antibodies vary in their life span, time course and specificities (Stott *et al.*, 1990). As one of the indicators of disease development, autoantibody production may reflect the characteristic B-cell hyperactivation, autoaggression and onset of the disease.

Sera taken from lupus and normal mice at different ages were assessed for the production of antibodies against ss- and ds-DNA. The results showed low detectable levels of anti-ss and ds-DNA in normal BALB/c mice, but an age-dependent significant increase of autoantibody production was found in both MRL/*lpr* and NZB/W mice (Fig.3.7). High levels of anti-ss (>1000 TU average) and anti-ds (>200 TU average) DNA antibodies were detectable in MRL/*lpr* mice of 3 months or older. In the NZB/W strain, the increase of serum autoantibody titre appeared to occur much later (after 6 months), and the levels of the anti-ssDNA were relatively lower compared with MRL/*lpr* mice. Variations in the serum autoantibody levels were observed especially in the 4 to 6 month old MRL/*lpr* mice, ranging between 1000 to 100,000 TU and 50 to

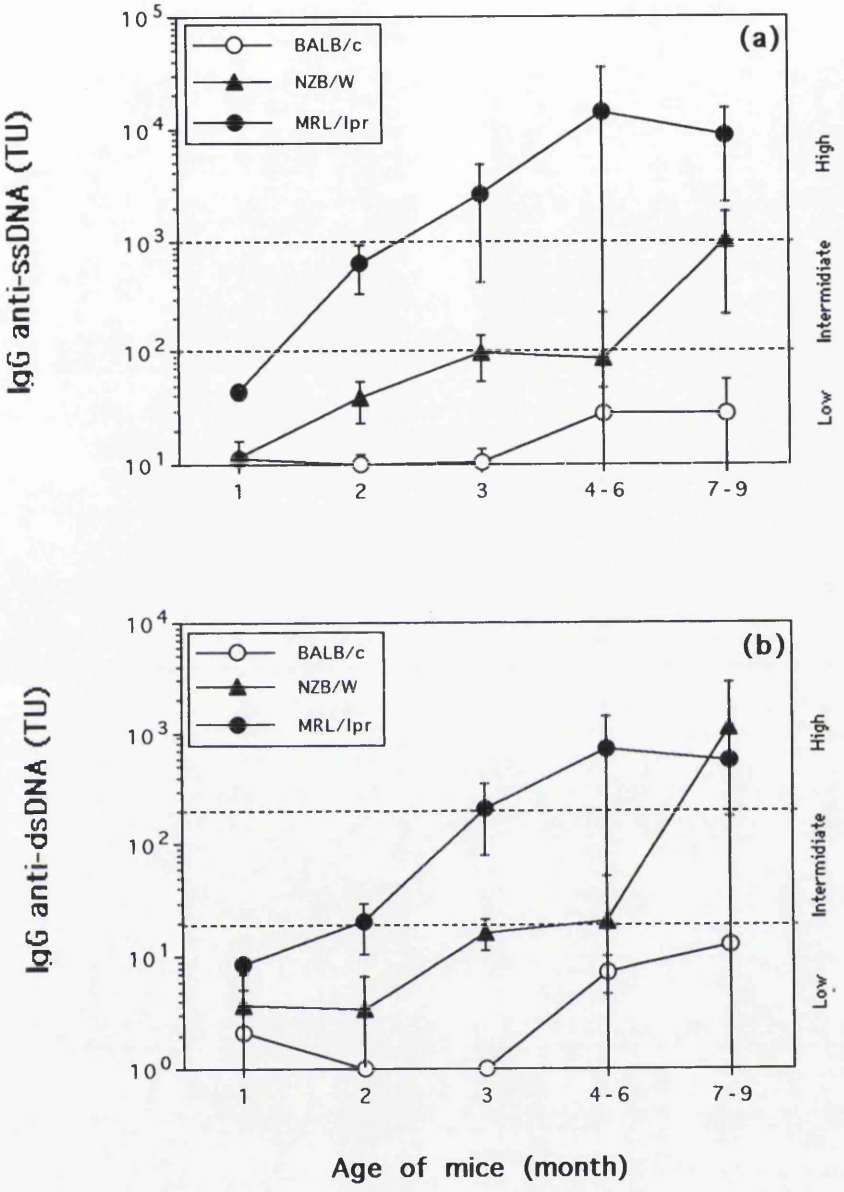


Figure 3.7 Serum levels of anti-ssDNA (a) and anti-dsDNA (b) antibodies in BALB/c, NZB/W and MRL/lpr mice at different ages. A total of 59 serum samples from BALB/c (19), NZB/W (19) and MRL/lpr (21) mice of different ages were titrated for anti-ss and anti-dsDNA by ELISA. Data are shown as the mean values (±SD) obtained from individual sera in different age groups. The relative levels (low, intermediate or high) of the autoantibodies were assigned arbitrarily as indicated in the graphs. Number of mice in each group is as follow:

Age groups	1	2	3	4-6	7-9 (mo)
BALB/c	2	2	2	10	3
NZB/W	3	4	2	4	6
MRL/lpr	3	3	3	10	5

2000 TU for anti-ss and ds-DNA respectively. However, in the young lupus mice, normal or only slightly increased levels of serum anti-DNA antibodies (anti-ssDNA < 100 TU; anti-dsDNA < 20 TU average) were observed at least until 4-month old in NZB/W, and until 2 month old in MRL/*lpr* mice. Intermediate levels of anti-ssDNA (100 - 1000 TU average) were detected in the 2-month old MRL/*lpr* mice. The observations have also been confirmed by results from ELISPOT assays specific for antibodies to ss and ds DNA.

3.3.2 Lymphoproliferation

Lymphoproliferation is another feature of murine lupus, particularly in MRL/*lpr* mice. Old lupus mice show enlarged spleens and lymph nodes. Figure 3.8 shows age-dependent changes in total numbers of spleen cells and $CD3^+$ cells in NZB/W and MRL/*lpr* mice. The lymphoproliferation begins at 3 months in MRL/*lpr* mice with a massive increase in the total and $CD3^+$ cell number (3-7.5 fold), peaking at 4 to 6 months. In NZB/W mice, the lymphoproliferation was only moderate (about 2 fold at the peak) and it appeared much later at 7 to 9 months. However, there was no evidence for such lymphoproliferation in young lupus mice until 4 and 2 months of age in NZB/W and MRL/*lpr* strains respectively. The average total number of spleen cells and $CD3^+$ cells were within the normal range of the BALB/c controls.

Figure 3.9 shows that there was a gradual increase in the percentage of splenic $CD3^+$ cells in young mice between 1 week and 2 months in all three strains. It peaked at 2 months (open arrow), and then declined before a relatively constant level was established after 3 months in BALB/c and NZB/W mice. However, the percentage of $CD3^+$ cells expanded rapidly after 3 months and reached a second peak at 4-6 months (solid arrow) in MRL/*lpr* mice.

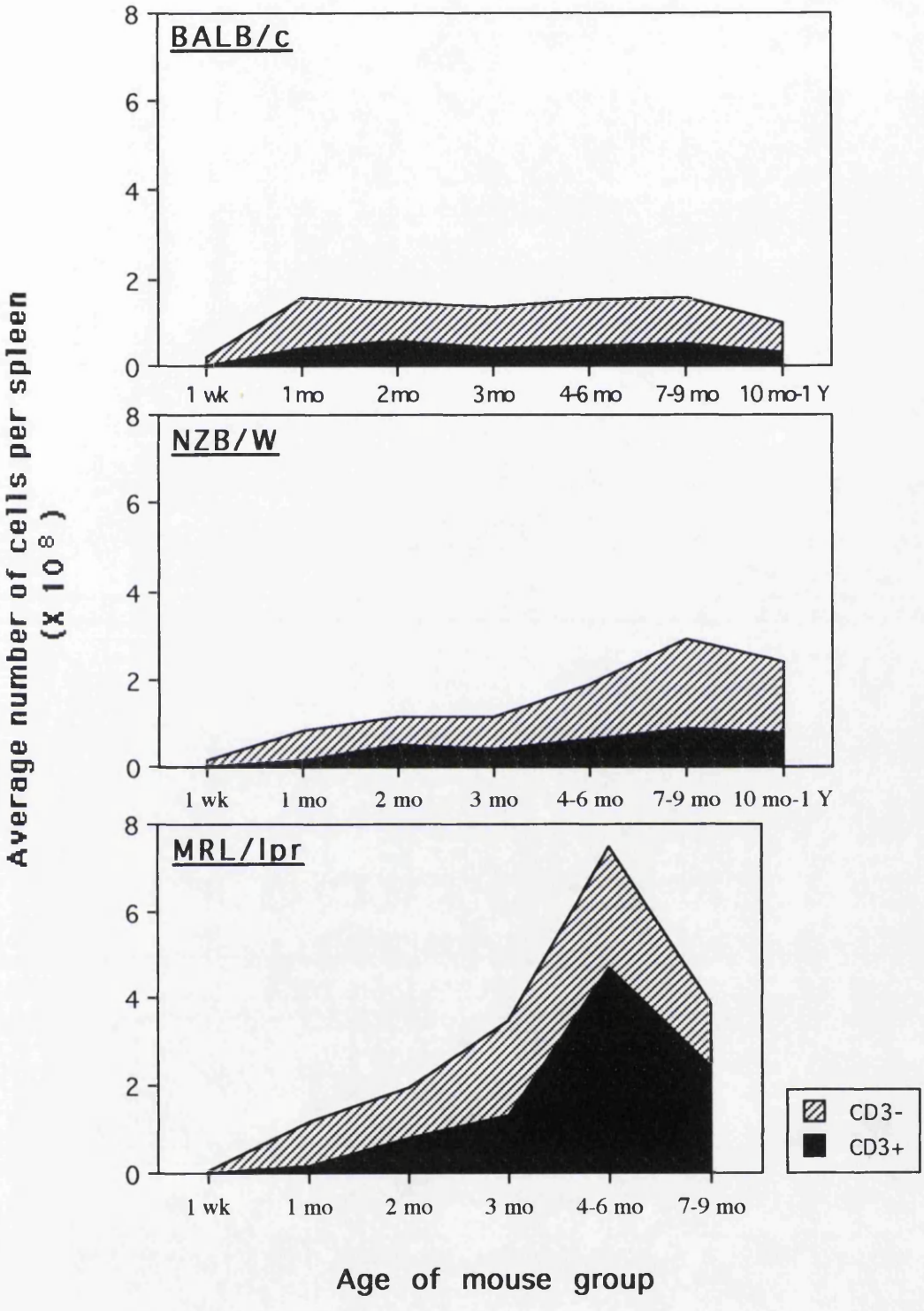


Figure 3.8 Flow cytometry analysis of the total number of splenic cells and the number of CD3⁺ cells in BALB/c, NZB/W and MRL/lpr mice of different ages. Freshly isolated spleen mononuclear cells were stained with a hamster anti-mouse CD3 antibody conjugated with FITC and 10^4 viable cells were analysed in each sample using a Becton & Dickinson cell sorter. Data are shown as means of individual mice in each of the age groups.

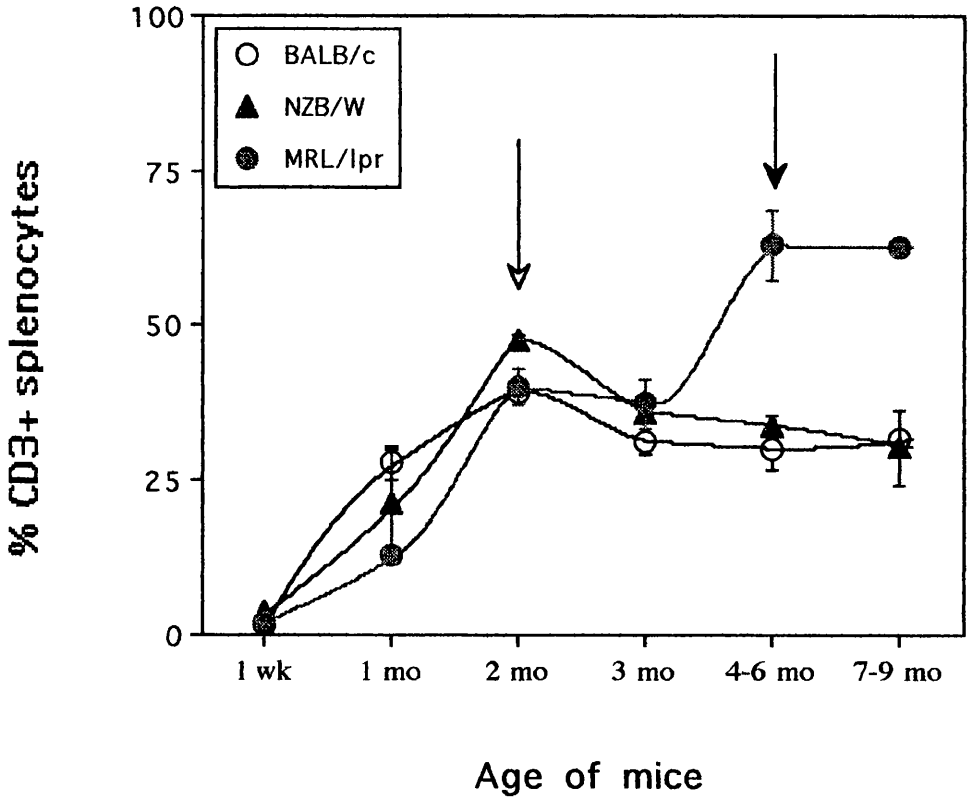


Figure 3.9 Age-dependent changes in the percentage of splenic CD3+ cells in BALB/c, NZB/W and MRL/lpr mice. The open arrow indicates a peak of percentage CD3+ cells in all three mouse strains at 2 months of age, while the closed arrow points at a second peak that appears only in the MRL/lpr strain of 4 months of age or older. Data are shown as the mean values (±SD) obtained from individual mice. Number of mice in each group is as follow:

Age groups	1wk	1mo	2mo	3mo	4-6mo	7-9mo
BALB/c	6	4	2	3	8	2
NZB/W	6	2	2	1	2	5
MRL/lpr	6	2	3	6	10	2

3.3.3 T-cell phenotypical abnormalities in MRL/*lpr* mice

A spontaneously proliferating abnormal $CD3^+$ subset, the so called 'double negative' (DN) cells, which lacks the functional T-cell markers CD4 and CD8, is a prominent feature in MRL/*lpr* mice. By using monoclonal antibodies specific to mouse T-cell markers, the percentage of the 4 T-cell subsets, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD4^+CD8^+$ and $CD3^+CD4^-CD8^-$, in spleens of MRL/*lpr* mice of different ages, was studied by FACS analysis. Figure 3.10 shows that this phenotypic abnormality peaks at 5 to 6 months. However, it does not become significant in the spleens of young MRL/*lpr* mice until 3 months of age. The DN cells made up less than 5% of total $CD3^+$ cells in 6-month old BALB/c and NZB/W mice.

3.3.4 Immune complex deposition and development of glomerulonephritis

Glomerulonephritis in SLE is believed to be due to immune complex deposition and complement activation. It is the major cause of pathology and death, therefore reflecting severity of the disease. Figures 3.11 and 3.12 show typical results from histological and immunocytochemical studies of the renal changes in 3 and 5 months MRL/*lpr* mice. Pathological changes were prominent in the kidneys of 5-month old MRL/*lpr* mice and included severe focal glomerulonephritis, pyelonephritis, vasculitis, urinary protein casts, mesangial proliferation and adhesion (Fig.3.11**c**). Deposition of immunoglobulin and complement (C3) on the glomerular basement membrane was readily detectable (Fig.3.12a, **b**, **c**). In the 3-month old MRL/*lpr*, although pyelonephritis was also observed , these other pathological changes were much less severe or not detectable.

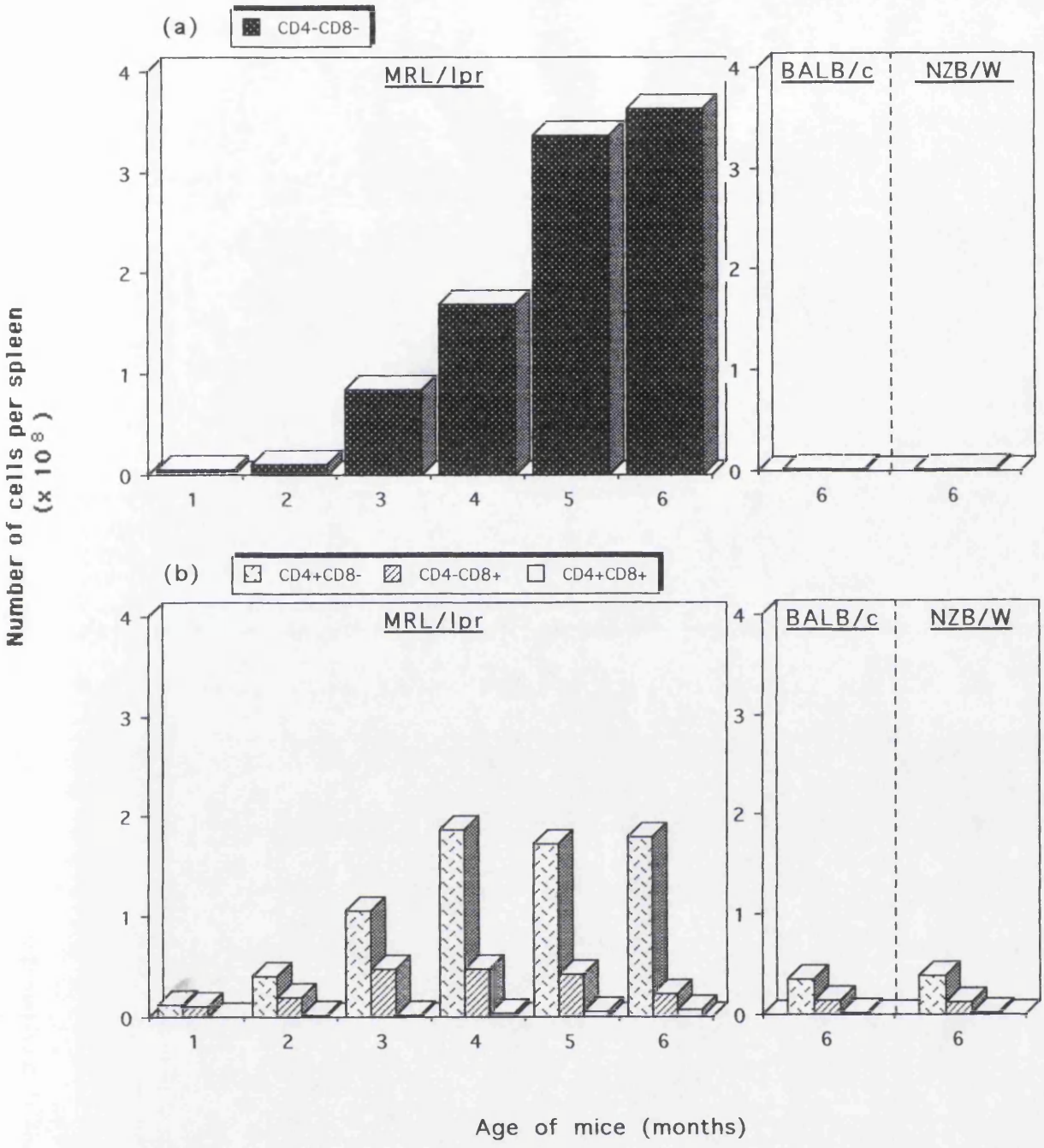


Figure 3.10 Age-dependent changes in the number of the double negative (a) and other (b) T-cell populations in spleens of MRL/lpr mice. Freshly isolated spleen mononuclear cells were counted for total cell number. The cells were then dual-labelled with a rat anti-mouse CD4 or a rat anti-mouse CD8 monoclonal antibodies and a pan-T cell marker. All antibodies were pre-conjugated with either R-PE or FITC. 10^4 viable cells were analysed in each sample using a Becton & Dickinson fluorescence-activated cell sorter. All cells gated for analysis were $CD3^+$. Data are shown as average total number of cells per spleen. At least three mice were included in each of the age groups.

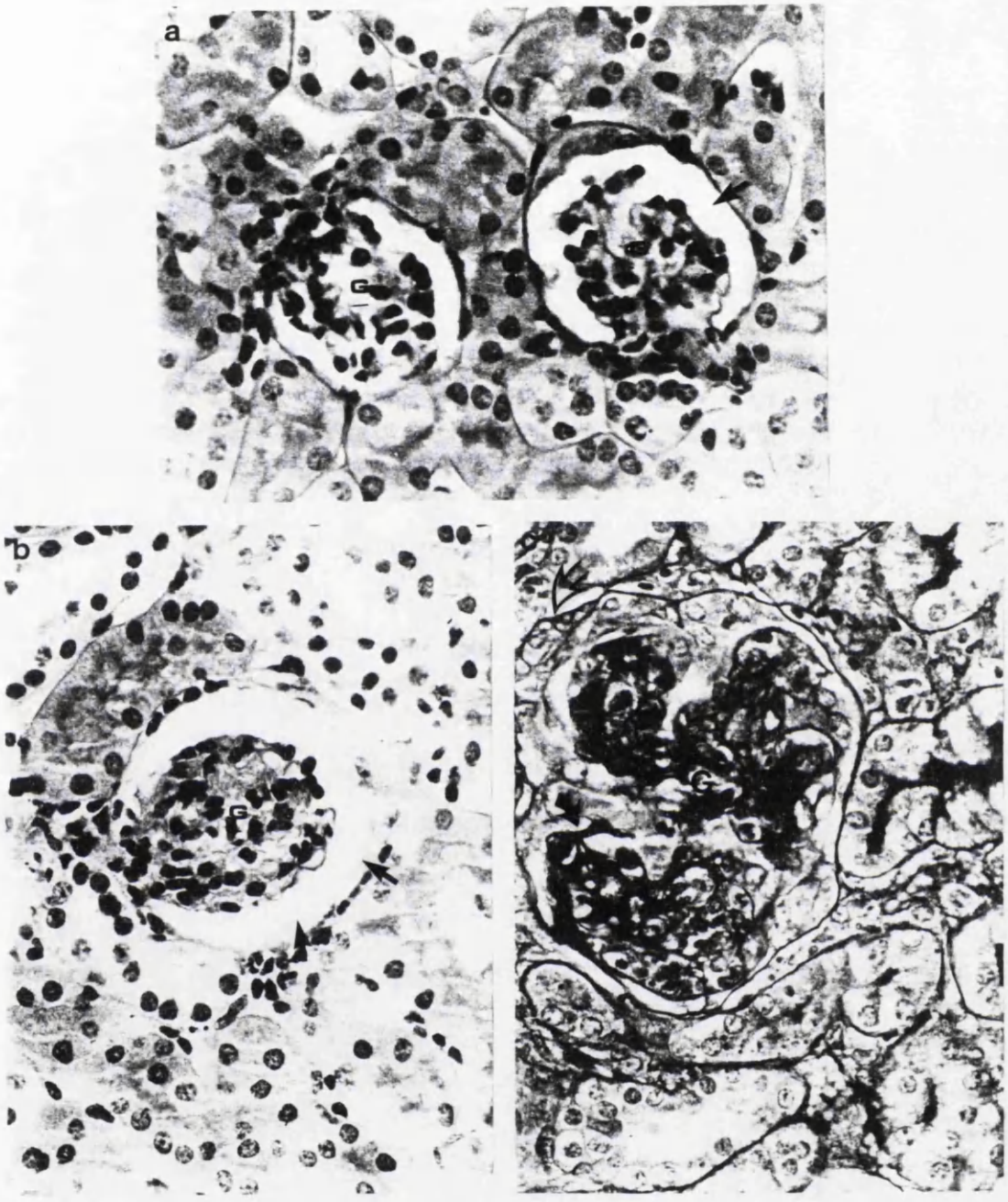


Figure 3.11 Renal histopathological changes in 3 and 5 months old MRL/*lpr* mice. Representative photomicrographs of kidney sections from: **a**). a BALB/c mouse showing normal renal structure, and **b**). a 3-month old MRL/*lpr* mouse showing relatively normal structure of glomeruli [G] and Bowman's space [▲]; compared with **c**). a 5-month old MRL/*lpr* mouse showing an enlarged and severely damaged glomerulus with cellular hyperplasia, focal glomerulonephritis [▲] and thicken Bowman's capsule ['crescent' like change, ▼] with little Bowman's space. PAS stain, x480.

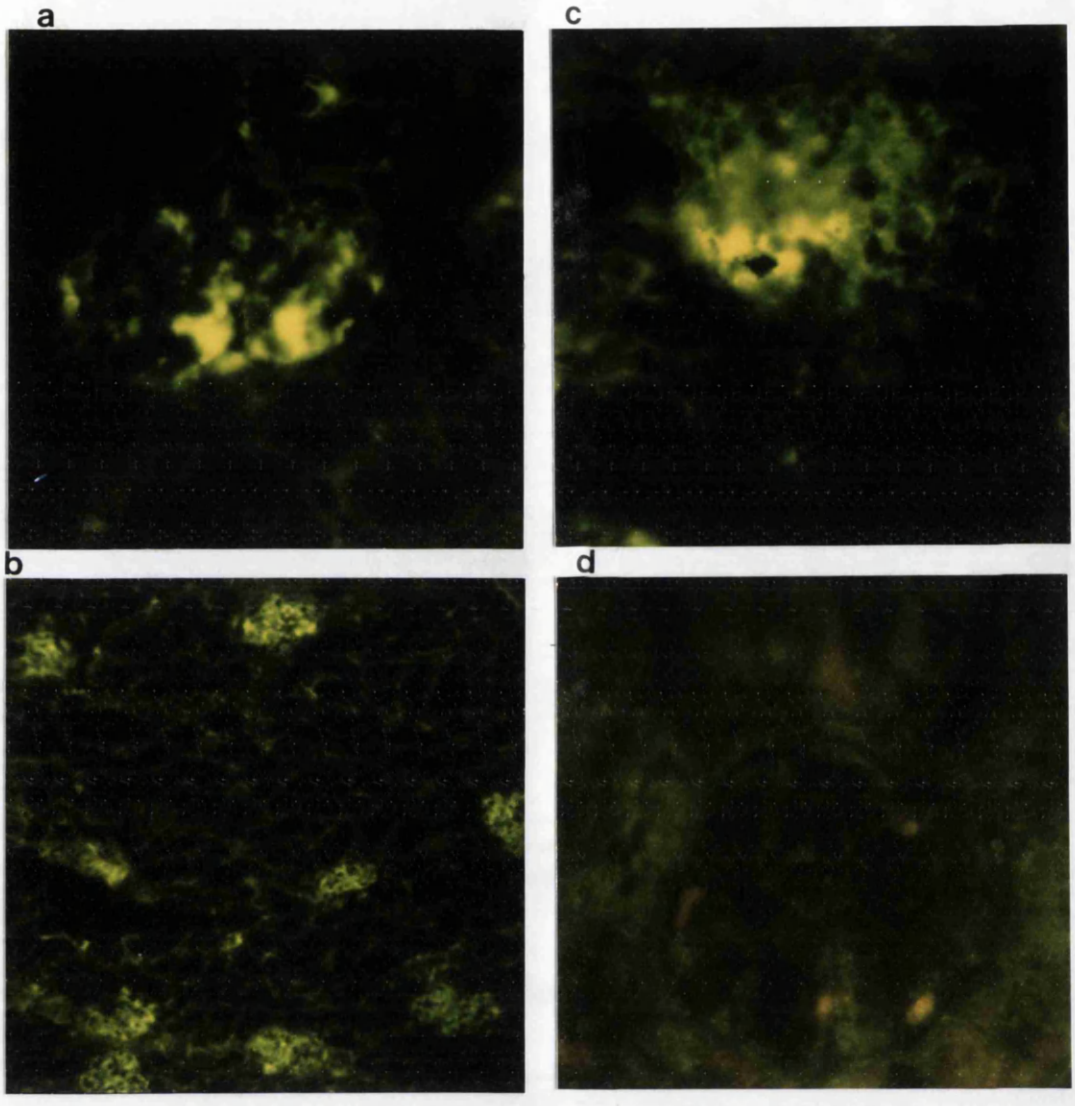


Figure 3.12 Immunofluorescent staining of renal immune complex deposition in a 5-month old MRL/*lpr* mouse. Representative photomicrographs of kidney sections showing specific staining of mouse C3 (**a, b**) and IgG (**c**) (frozen sections). (**b**) a low power graph (x 100) showing strong positive staining for mouse IgG in many glomeruli. (**a**) and (**c**) high power graphs (x 400) showing strong fluorescent staining is mainly located at the glomerular lacis region (mesangial cells). Negative control (**d**) is a kidney section from the same mouse incubated with normal goat serum (x 400).

3.4 Summarising remarks

The study so far has demonstrated that there exists an early, progressive IL-2 deficiency in NZB/W and MRL/*lpr* mice. The results show that the T-cell defect exists not only in old lupus mice with overt clinical disease but also in young mice as early as 4 weeks or younger. In aged MRL/*lpr* mice, IL-2 hyposecretion may be partially due to dilution of $CD4^+$ cells by the proliferating DN cells. However, spleen cells from NZB/W and especially from young MRL/*lpr* mice are also unable to produce IL-2 although expression of CD4 is normal in these mice. Preceding disease activity, this defect exists in young mice at a stage before the rise of serum autoantibodies, lymphoid hyperplasia and kidney pathology. The abnormal IL-2 secretion is not due to kinetic difference of T-cell activation between strains. Although young mice of one to two month old display late responses to the stimulation, reduced IL-2 activity were detected at every time point in the lupus strains compared to age-matched normal mice. IL-2 is known to be crucial in modulating the maturation, functional differentiation, activation and clonal expansion of T-cells (Toribio *et al.*, 1989). In conjunction with other cytokines, IL-2 also plays an important role in the regulation of B-cells and other cell types such as NK effector cells. The observation that the T cell defect precedes B-cell hyperactivity emphasises its significance in relation to autoimmunity. It is conceivable that such an early impaired T-helper function may disturb the normal development of the immune system resulting in immune regulatory disorders and failure.

Chapter 4

The defective T-cell activation in lupus disease

4.1 Introduction

In contrast to B-cell hyperactivity, an impaired T-cell mediated immunity is another feature in SLE. The defective Con A-induced IL-2 production suggests a functional abnormality of the Th1 phenotype. Although the early onset of the IL-2 defect emphasises its role in the abnormal T-cell functions, defects in other cell compartments or phenotypes have also been reported in the disease (Tsokos, 1992). It is therefore necessary to have a detailed analysis of other functional activities of lupus T-cells, including their ability to proliferate, to express cytokine receptors and to produce other characteristic lymphokines of T-cells. In addition, the role of co-stimulators in the defective lupus T-cell activation is not well understood. Complete T-cell activation are known to require co-stimulatory signals provided by accessory cells. In the absence of the co-stimulative signal, T-cells make only a partial response, and more importantly, to enter an unresponsive state, called 'clonal anergy' in which the T-cells are incapable of producing its own growth hormone, IL-2 (Schwartz, 1990). Production of IL-1, an important co-stimulator for T-cell activation derived mainly from adherent cell populations, has been shown to be deficient in lupus disease (Linker-Israeli, 1983; Steinberg *et al.*, 1991). In order to investigate the nature of the defect in lupus T-cell activation, the effect of exogenous IL-1 and the function of lupus adherent cells was also assessed. Finally, to identify whether any defect at the level of cytokine gene transcription could be involved in the defective cytokine production found in lupus mice, mRNA expression for the characteristic T-cell-derived lymphokines, including IL-2, IFN-γ, IL-4 and IL-6, was analysed and described in this chapter.

4.2 Con A-induced lupus T-cell proliferative responses

The optimum concentration of Con A (2.5 μg/ml) for T cell activation in the mouse has been determined in the preliminary experiments (Chapter 3). In agreement with previous findings, the Con A-induced proliferative response of T-cells was

deficient in old NZB/W and MRL/*lpr* mice with clinical disease. Although freshly isolated unstimulated lupus spleen cells usually show higher thymidine uptake than the controls, they responded poorly (in general below 50% of the thymidine uptake by cells from control mice) to subsequent Con A stimulation. Figure 4.1 shows the defective proliferative responses of splenic T-cells from old MRL/*lpr* (8 month) and NZB/W (10 months) mice, in comparison to BALB/c (10 months) control mice. The time course study also confirmed that the reduced T-cell proliferative response was not due to kinetic difference between strains. The Con A-induced T-cell proliferation in mice of 2 months or older usually peaked between 2 to 3 days after stimulation, and exceptionally some delay was observed in the lupus strains.

The defective T-cell proliferation was also observed in young lupus mice. MRL/*lpr* mice begin to display such a proliferative defect as young as 4 weeks old and it is progressive with age. Although this early proliferative defect could sometimes be observed in young NZB/W mice too, it is not consistently established in most of the cases until 6 months (see Chapter 5). A reduced cell proliferative response is often associated with low IL-2 activity in the culture which is usually below 1 ng per ml (peak activity).

T-cells from SLE patients also showed abnormal proliferative responses to Con A. Fig.4.2 shows the Con A-induced proliferation of T-cells from peripheral blood of 3 SLE patients with active disease, in a whole blood cell culture. The cells were stimulated with a wide range of serial concentration of Con A, but no response was observed in the samples from two patients while the other showed a relatively enhanced response in comparison to the normal control.

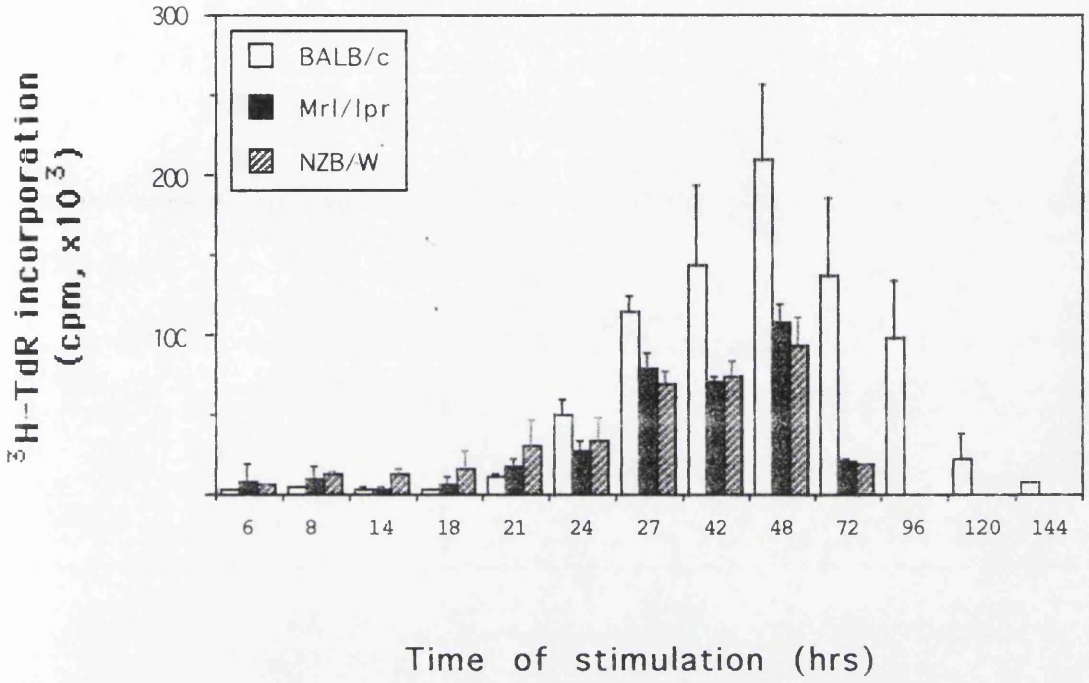


Figure 4.1 Con A-induced proliferative responses of splenic T-cells from normal and lupus mice. Spleen cells from 2 mice (8 to 10 month old) of each strain were pooled and cultured in the presence of Con A (2.5 μg/ml). Cell proliferative responses were measured at different times by ^{3}H-thymidine incorporation. Data are presented as means of duplicates (±2SD)

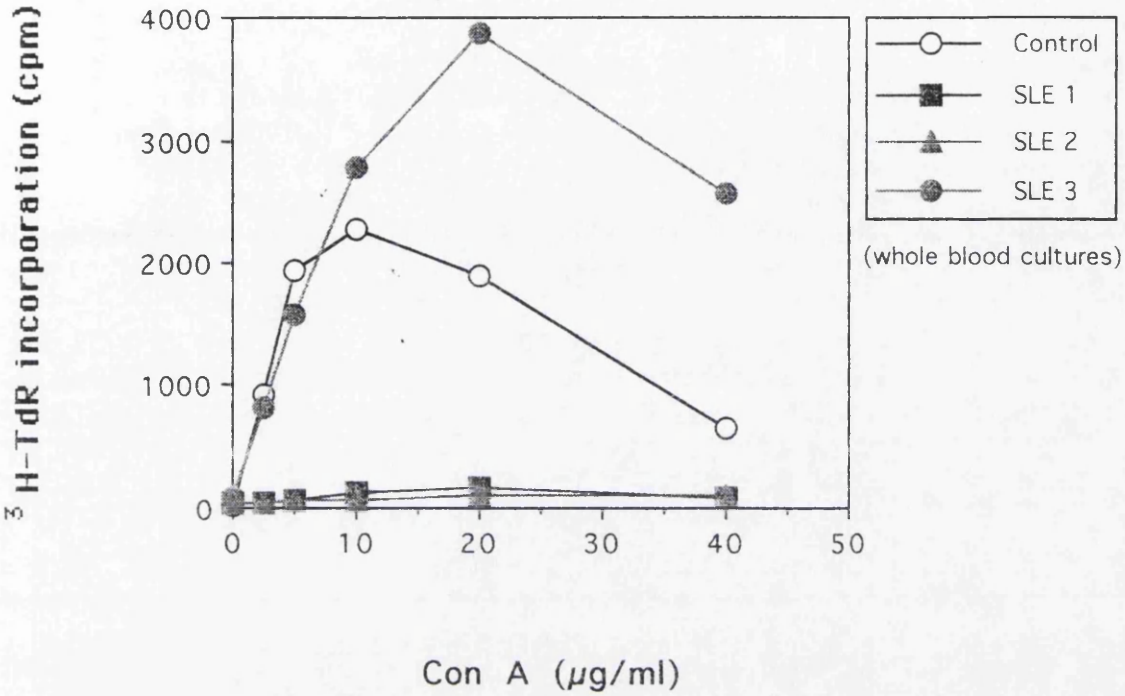


Figure 4.2 Con A-induced proliferative responses of T-cells from peripheral blood of normal and SLE patients. Blood samples from 3 SLE patients with active disease were diluted 1:1 with RPMI containing 5% FCS and cultured in 96-well culture plates (0.2 ml/well) in the presence of different concentrations of Con A. After 48 hrs of stimulation, the cultures were pulsed with ^{3}H-thymidine (0.5 μCi/well) for further 6 hrs and the incorporated radio-activity measured using a Beta Plate scintillation counter. Data are presented as means of triplicates. Control: blood sample from a normal donor.

4.3 IL-2 receptor expression

Optimum Con A-induced T-cell ($CD3^+$) IL-2R (α chain, CD25) expression in mice was found between two to three days of stimulation. This was indicated by the frequency of $CD3^+$ cells expressing IL-2R and the density of cell surface IL-2R expression (mean fluorescence intensity). Figure 4.3 shows representative results from a 6-day time course study on Con A-induced T-cell IL-2R expression and $CD3^+$ cell proliferation in BALB/c mice. There was no significant difference in the kinetics of Con A-induced T-cell IL-2R expression between a 4-month and an 8-month old mouse tested. It was noted, in addition, that the peak IL-2R expression (a & b) occurred immediately before T-cell division (c & d). After 2 to 3 days of Con A stimulation in culture, a rapid increase in the percentage (d) and number (c) of $CD3^+$ cells and, reduced T-lymphoblasts (c), was accompanied by a sharp decline in the T-cell IL-2R expression (a, b).

Surface expression of IL-2 receptor (CD25) on freshly isolated unstimulated splenic $CD3^+$ T-cells was under 6% in all mice studied, except the one week old BALB/c mice, with no significant difference between the lupus and the control strains. T-cells from the one week old BALB/c mice showed relatively higher CD25 expression (14.1±3.47 %) than lupus mice of the same age, i.e. 3.4 ±0.99% in NZB/W and 2.9 ±1.1% in MRL/*lpr*. In addition, IL-2R expressing splenic T-cells cultured without Con A was under 10% of total $CD3^+$ cells and with low density.

However, Con A-induced T-cell surface IL-2R expression was significantly decreased in old lupus mice compared with the age-sex-matched control BALB/c and CBA mice (Fig.4.4a). Figure 4.4b shows typical FACS profiles of the Con A-induced T-cell IL-2R expression in lupus (MRL/*lpr*) and normal control (BALB/c) mice. The defective Con A-induced IL-2R expression was also evident in young lupus mice (Fig.4.5), both in

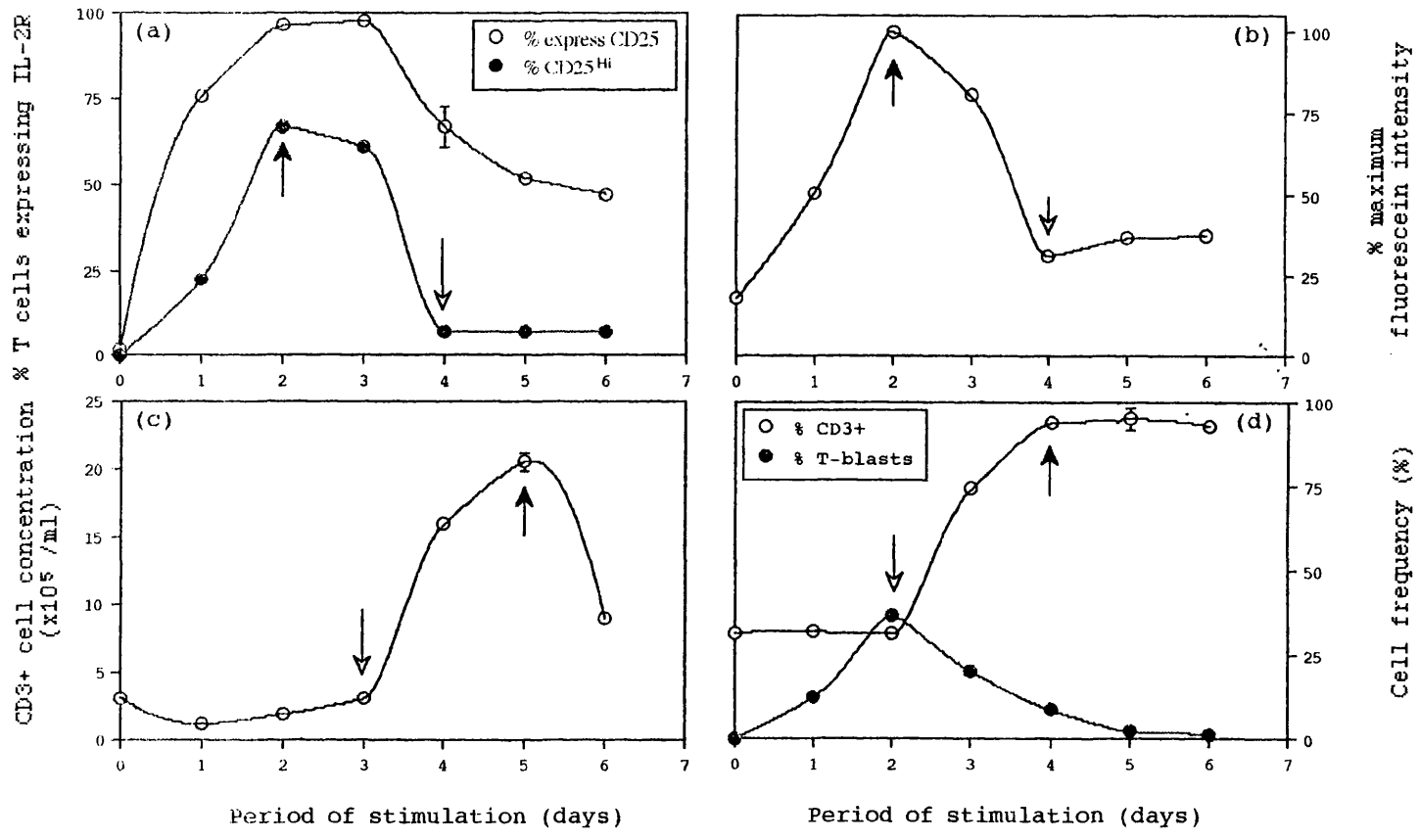
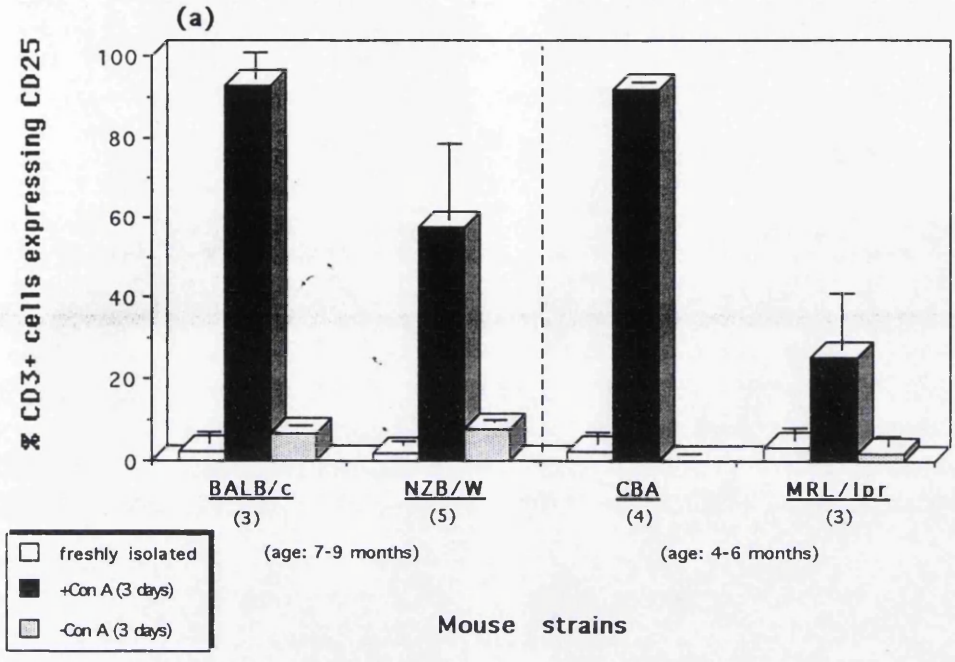


Figure 4.3 FACS analysis of kinetics of Con A-induced splenic T-cell activation in BALB/c mice: relationship between T-cell IL-2R expression, proliferation and division. Spleen cells from a 4 and a 7-month old BALB/c mice were cultured in 25-cm^2 culture flasks in the presence of Con A (2.5 μg/ml), and cell samples were taken at daily intervals during a 6-day time course. Results from the two mice of different ages were similar and data shown were from the 7-month old BALB/c mouse: (a) frequencies of total CD3$^+$ cells expressing CD25 (open circles) and CD3$^+$ cells expressing CD25 with high density (fluorescein intensity >100, closed circles); (b) average fluorescein intensity (expressed as percentage of the peak expression of CD25); (c) CD3+ cell concentrations in cultures; (d) CD3$^+$ cells expressed as percentage of total cells in cultures (open circles) and T-blasts (CD3$^+$ large cells, closed circles) as percentage of total CD3$^+$ cells. Arrows indicate the peaks or time points at which sharp or transitional changes occur. Please see Materials and Methods for details of the antibody markers, staining procedure and FACS analysis.



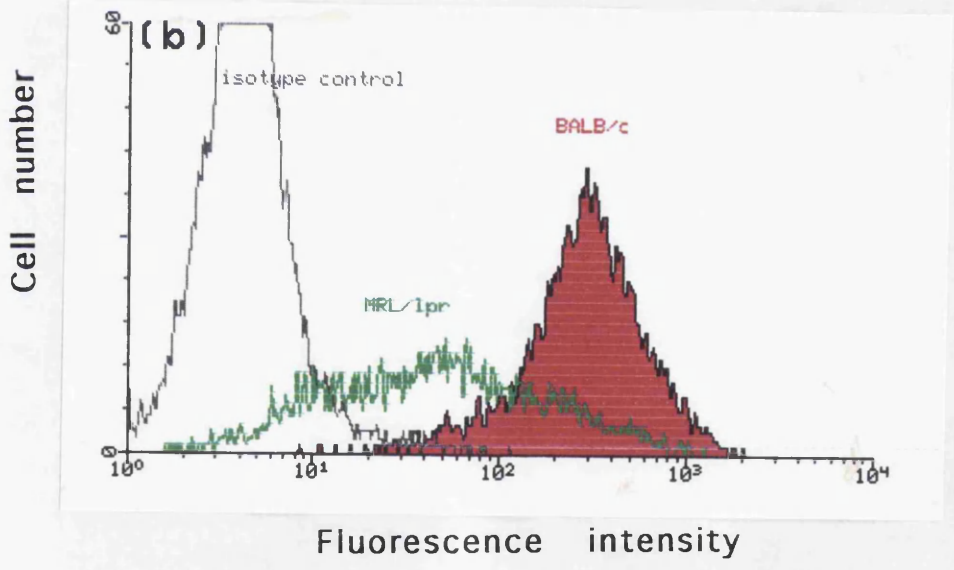


Figure 4.4 Defective surface IL-2R (CD25) expression by splenic T-cells from old lupus mice with active disease. Spleen cells freshly isolated as well as cells cultured with and without Con A (2.5 μg/ml) for 3 days were analysed by FACS using specific antibodies to mouse CD25 and CD3. (a) Percentage of total $CD3^+$ cells expressing CD25 (cell frequency) in lupus and age-sex-matched BALB/c or CBA control mice. The ages and number of mice in each group are indicated in brackets. (b) Typical FACS profile of CD25 expression by Con A-stimulated splenic $CD3^+$ cells from 5-month old MRL/lpr and BALB/c mice. Isotype control: rat myeloma IgG2a R-PE conjugated. Cells gated for the analysis were >95% viable after 'PI' exclusion.

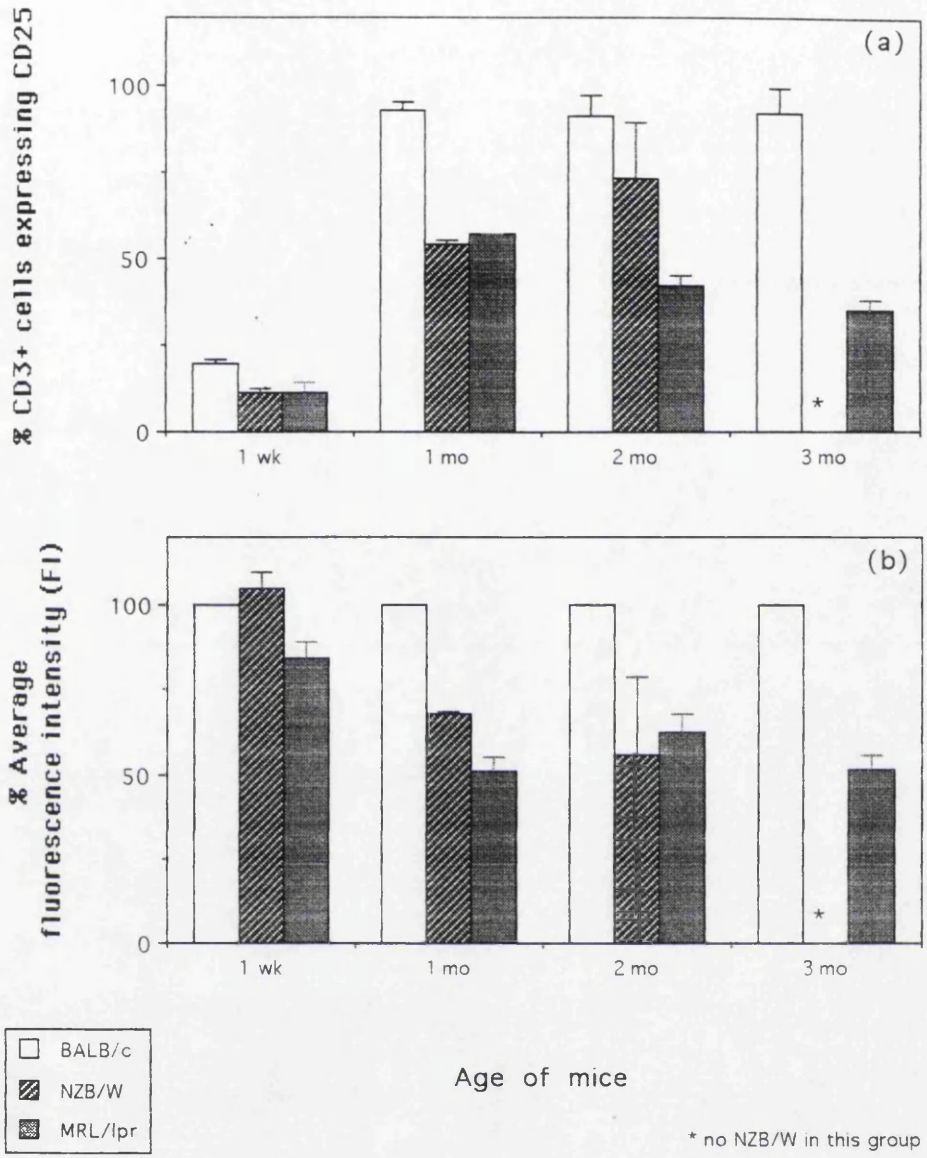


Figure 4.5 Con A-induced IL-2R expression by splenic T-cells from young mice. Spleen cells from individual lupus and normal control mice of different ages were stimulated with Con A (2.5 μg/ml) in cultures for 60 hrs before being analysed by FACS. Data are expressed as means (±SD). These include percentage of total $CD3^+$ cells expressing CD25 (a), and percentage average fluorescein intensity of the control BALB/c mice (b). CD25 expression on cultured cells without Con A was under 10% (not shown).

terms of frequency of T-cells expressing IL-2R, and average density (relative fluorescence intensity).

4.4 T-cell activation co-stimulators

4.4.1 Effects of exogenous IL-1 on IL-2 production

Due to the availability at the time of study, the IL-1 used in the experiment was also a r-human protein (*Wellcome Laboratory, UK*). The effects of exogenous IL-1 on T-cell activation in lupus mice were examined by adding r-human IL-1 during initial Con A stimulation. Spleen cells were cultured at 10^6 cells/ml with or without Con A (2.5 μg/ml) and serial concentrations of IL-1, at 0, 50, 100 and 200 ng/ml. Culture supernatants were collected at daily intervals, and IL-2 production measured by the CTLL assay. No significant effect of exogenous IL-1 was observed on IL-2 production in either lupus or normal mice (Fig.4.6). The mice used in this experiment were 3 month old. Results shown were peak IL-2 activity present in the culture supernatants (all at 24h).

4.4.2 Adherent cell function in MRL/*lpr* mice

Adherent and non-adherent spleen cells from MRL/*lpr* and H-2 haplotype compatible normal CBA mice (H-2k) were separated by plastic adhesion in $25cm^2$ culture flasks, at 37^0C, $5\%CO_2$ in complete culture medium containing 5% FCS for 2 hours. Non-adherent cells were collected by gentle pipetting and rinsing once with warm medium. The cells were then spun down, resuspended in the original volume of fresh medium and counted. Non-adherent cells obtained by this method made up about 66 to 79% of the total spleen cells in the original cultures. Adherent cells were washed twice more with warm medium. In different combinations, the non-adherent cells from MRL/*lpr* and CBA mice were recombined or exchanged with the adherent cells in

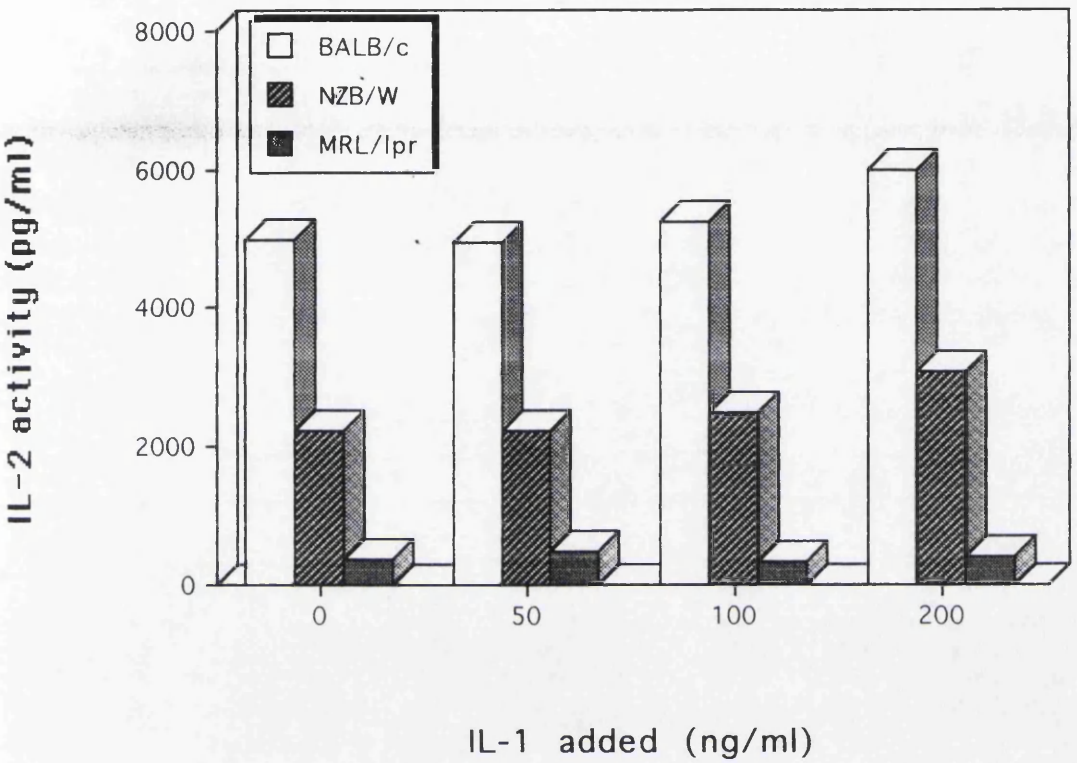


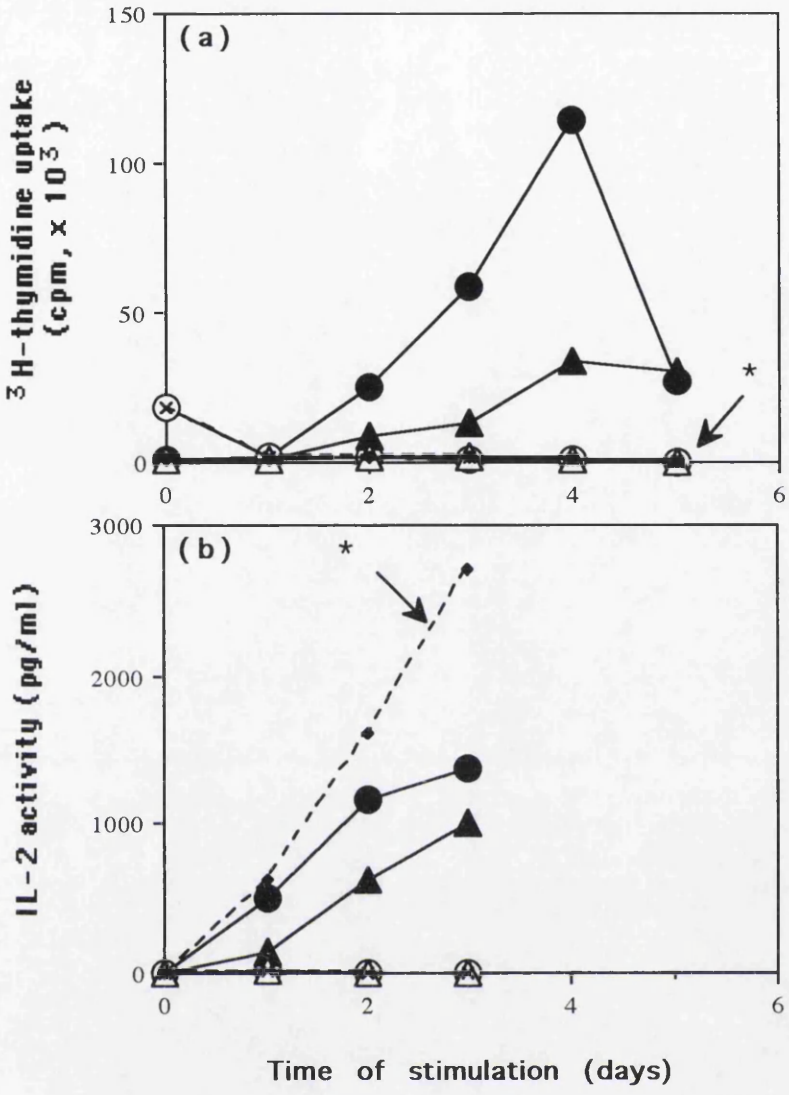
Figure 4.6 Effects of exogenous rIL-1 on Con A-induced IL-2 secretion. Spleen cells from 2 mice (3-month old) of each strain were pooled and cultured in the presence of Con A (2.5 μg/ml) with various doses of r-human IL-1. Culture supernatants were collected at daily intervals and assayed by the CTLL bio-assay. Results shown are peak IL-2 activity (24 hrs) obtained in a 3-day time course.

appropriate flasks, and cultured in the presence of Con A. At daily intervals, culture supernatant samples were collected for measurement of IL-2 production and the cell proliferation assessed by thymidine incorporation. T-cell IL-2R expression was tested at 60h. Mice used in these experiment were 4 to 5 months old.

Results from the first experiment showed that, in normal CBA mice, non-adherent spleen cells alone responded relatively poorly to Con A. The ability of T-cells to proliferate and to produce IL-2 was much reduced without the presence of adherent cells (Fig.4.7). Lupus T-cells from MRL/*lpr* mice, however, responded equally poorly to Con A either with or without adherent cells from the same strain, compared to that of normal CBA mice. Replacement of lupus adherent cells with the adherent cells from CBA mice did not restore the ability of lupus T-cells to proliferate or to produce IL-2. Surprisingly, it was noted that the proliferative response of non-adherent cells from CBA mice was completely suppressed when these cells were cultured with adherent cells from MRL/*lpr* mice (Fig.4.7a, *). This was found to be in contrast to a significantly enhanced IL-2 activity detected in the culture supernatants (Fig.4.7b, *).

However, results from 4 subsequent repeat experiments were not consistent (Fig.4.8). They failed to reproduce the conflicting suppressive and stimulatory effects of lupus adherent cells on normal T-cell activation. In spite of similar procedures and culture conditions, the T-cell proliferative response in the control groups (Fig.4.8a, groups I and II) also varied considerably. This was not due to the cell separation procedure as shown by the control cell cultures with and without separation procedure in the same experiment .

From results of the 5 separate experiments, the only phenomenon that appeared to be clear and consistent was that adherent cells from normal CBA mice were not capable of restoring the ability of lupus T-cells either to proliferate, to produce IL-2 or to express IL-2 receptors (Fig.4.8c, Group VI).



Key to symbols		CBA (H-2K)		MRL/lpr (H-2K)	
		adherent	non-adherent	adherent	non-adherent
●	I	+	+		
○	II			+	+
▲	III		+		
△	IV				+
* ◆	V		+	+	
✕	VI	+			+

Figure 4.7 Analysis of adherent cell functional activity in Con A-induced T-cell proliferation (a) and IL-2 secretion (b) in normal and lupus mice (Experiment 1). Before Con A stimulation, mouse spleen adherent and non-adherent cells were separated by adhesion to plastic. The two types of cells from the H-2 compatible MRL/lpr and CBA mice were then re-grouped by different combinations as indicated by the key to symbols and Con A at 2.5 μg/ml was added. Cell proliferative responses and IL-2 activity in the culture supernatants were assayed at daily intervals and the data shown are peak responses observed. Arrows indicate a suppressed proliferative response (a) of CBA splenic T (non-adherent) cells by the adherent cells from a 4 -month old MRL/lpr mouse in contrast to the enhanced IL-2 activity detected in the culture supernatants (b).

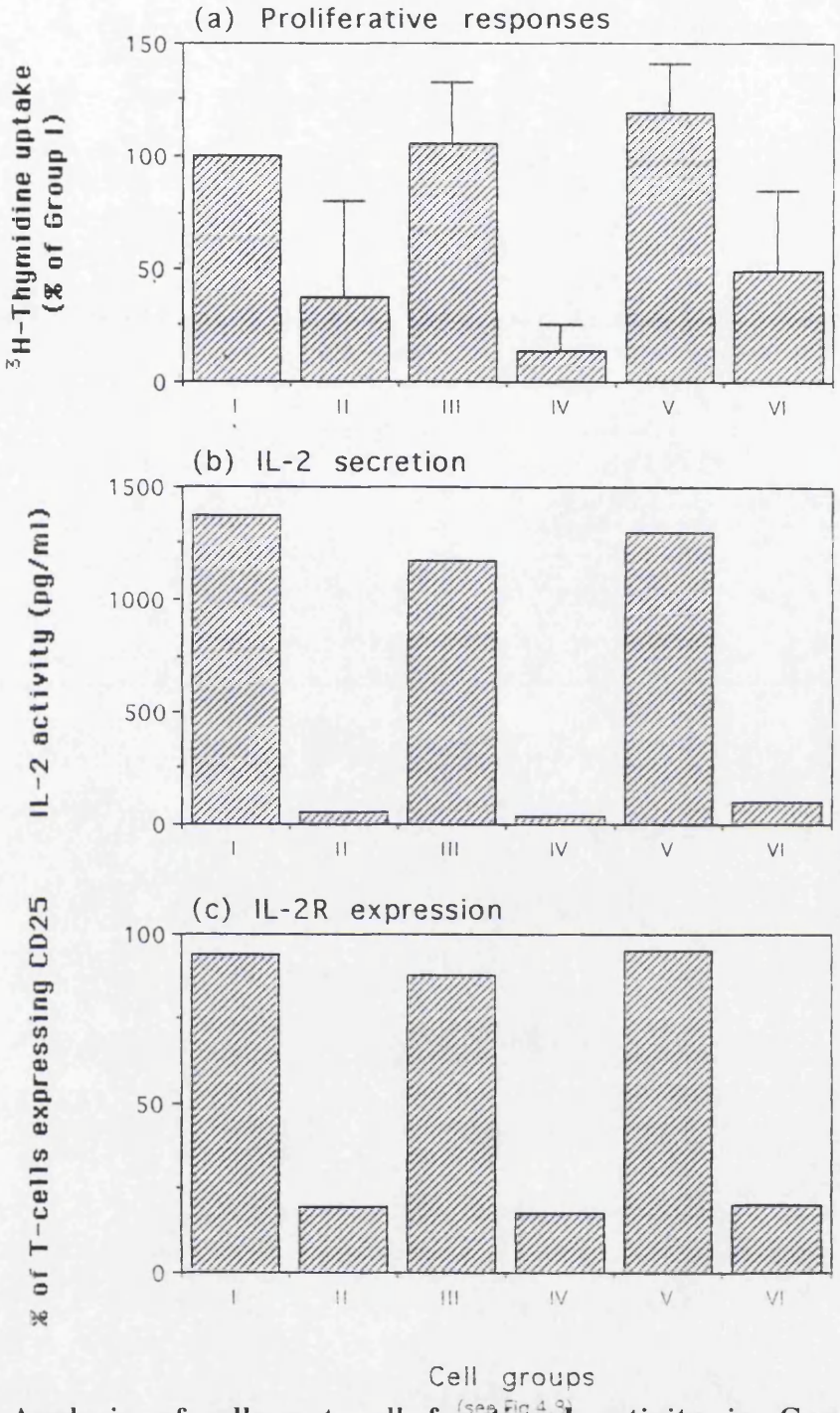


Figure 4.8 Analysis of adherent cell functional activity in Con A-induced T-cell proliferation (a), IL-2 secretion (b) and IL-2R expression (c) in normal and lupus mice (Experiments 2 to 5). The procedure for cell separation, re-combination group and stimulation, and the subsequent functional assessments was same as that described for the Experiment 1 in Figure 4.8. The data of cell proliferation (a) shown are mean value obtained from results of the 4 repeat experiments (±2SD). The data of IL-2 secretion (b) were from the Experiment 2 only. Additionally, in Experiment 4 at day 3, the cells were also assessed for IL-2R (CD25) expression by FACS (c). All mice used were 4 to 5-month old.

4.5 Other T-cell derived lymphokines

4.5.1 Production of IFN-γ

The IL-2 defect indicates a defective Th1 cell function in lupus mice. Since IFN-γ is also produced by the Th1 subset, the ability of splenic T-cells from lupus mice to produce this lymphokine was therefore assessed. Immuno-reactive IFN-γ in cell culture supernatants was quantitated by a sensitive ELISA method (Chapter 2). In a time course study, Con A-induced IFN-γ secretion peaked at 3 days in BALB/c mice. In NZB/W mice, the IFN-γ activity in culture supernatants declined after 2 days of stimulation. However, in MRL/*lpr* mice, it was relatively level throughout 1 to 5 days with a gradual increase (Fig.4.9). As shown in Figure 4.9, Con A-induced IFN-γ production was relatively reduced in 4-month old lupus mice, especially the MRL/*lpr* strain, compared to BALB/c mice of the same age. No significant spontaneous secretion of IFN-γ by unstimulated cultured spleen cells was detected.

A modified ELISPOT assay has also been developed for detection of IFN-γ secreted by viable culture cells in the present study. It enables determination of the frequency of the cytokine secretors, and is a semi-quantitative assessment of IFN-γ secretion on an individual cellular basis. Insoluble membrane-bound immune complexes formed by the secreted IFN-γ and the specific antibodies were visualised by cleavage of the substrate (BCIP-NBT) by the conjugated alkaline phosphatase. This resulted in dark blue colour spots on the membrane which varied in size with a condensed centre and diffuse edge. The spots were readily counted at low magnification (2 X 12.5) under a stereo zoom microscope. According to results from the ELISPOT assay, IFN-γ hyposecretion in old lupus mice was not due to less IFN-γ secreted per cell, as shown by the size of spots (Fig.3.10c, d), but due to a reduced frequency of IFN-γ secretors (Fig.3.10a, b). The frequency of Con A-induced IFN-γ secreting cells was 1:1585 (± 215) in a group of 4-

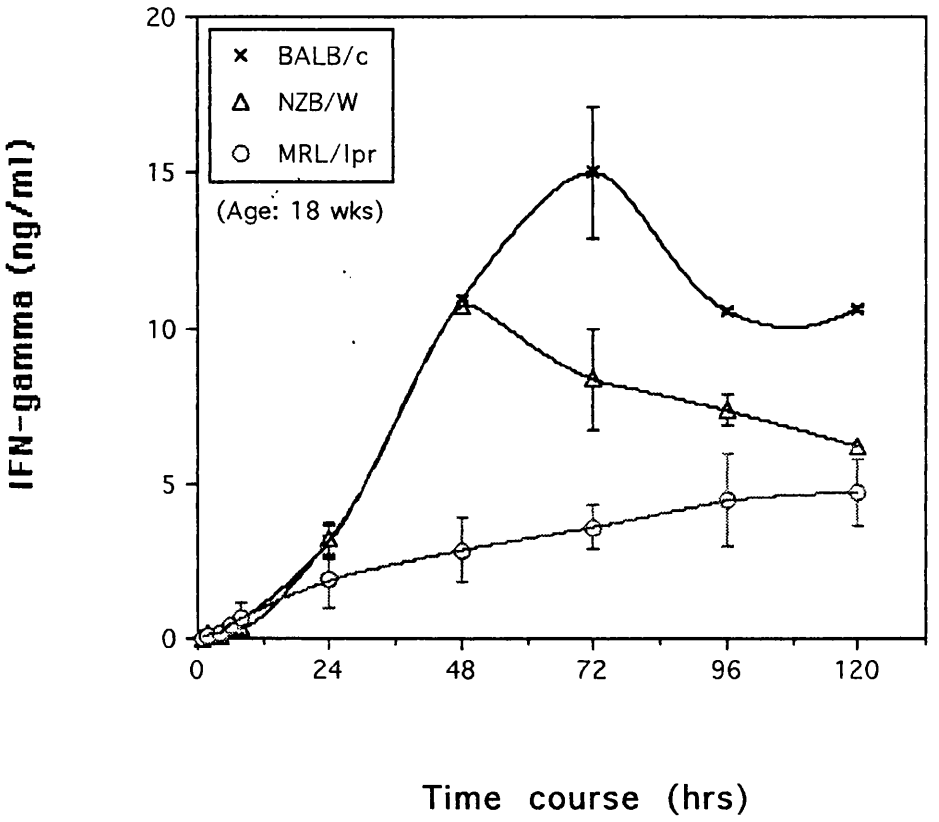


Figure 4.9 Kinetics of Con A-induced IFN-γ production by spleen cells from BALB/c, NZB/W and MRL/lpr mice. IFN-γ activities in the supernatants of Con A-stimulated and unstimulated mouse spleen cell cultures at 0, 2, 4, 6, 8, 24, 48, 72, 96 and 120 hrs were measured by mouse IFN-γ specific ELISA. Data are expressed as means of triplicates.

BALB/c MRL/lpr

x 9

x 32

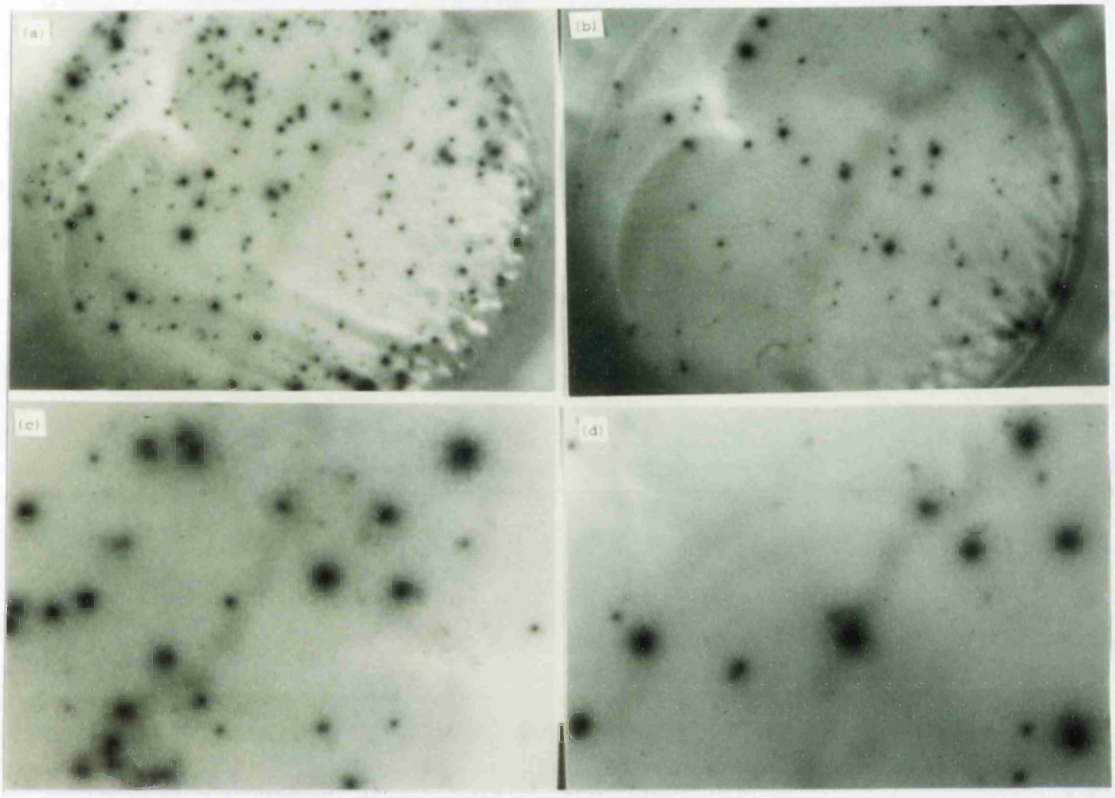


Figure 4.10 ELISPOT analysis of Con A-induced IFN-γ secretion by spleen cells from normal and lupus mice (age: 20 wks). Spleen cells from BALB/c (a, c) and MRL/lpr (b, d) mice were stimulated with Con A (2.5 μg/ml), and washed before a further incubation for 16 hrs at a cell concentration of 1 x 10^5 cells per well in 96-well nitrocellulose membrane based plates pre-coated with a rat anti-mouse IFN-γ antibody. Results presented are representative microphotographs at low (x 9) and high (x 32) magnifications showing decreased frequency of IFN-γ secretors from a 20-week MRL/lpr mouse (**b**) compared with that of a sex-age-matched BALB/c mouse (**a**), and no significant difference in the size of the spots between the two strains (c, d).

month old MRL/*lpr* mice, which was 2.65 times lower than that of BALB/c control mice (1:594 ± 115).

Therefore, subsequent experiments were carried out to look at the defect of IFN-γ production in lupus mice of different ages. However, the reduction of IFN-γ production in lupus mice was found to be inconsistent and not well established until 6-9 months old in both NZB/W and MRL/*lpr* mice (Fig. 4.11). Con A-stimulated spleen cells from the 1-week old lupus and BALB/c mice did not secrete detectable level of IFN-γ. In the one month age group, MRL/*lpr* mice but not NZB/W mice showed reduced IFN-γ production, compared to BALB/c controls. In addition, it was observed that spleen cells from three 1-year old NZB/W mice which had no clinical symptoms produced levels of IFN-γ comparable to that of BALB/c mice of the same age (Fig. 4.11, arrow). Also, in selective experiments, considerable strain difference in the production of IFN-γ was observed between the control BALB/c and CBA strains. Con A stimulated spleen cells from 5-month old CBA mice produced significantly higher levels of IFN-γ (228 ± 170.9 ng/ml, n = 5), compared to BALB/c mice of the same age (43.2 ± 17 ng/ml, n = 8).

4.5.2 Production of IL-4

Con A-induced production of IL-4 by mouse spleen cells in cultures was measured by an ELISA method developed to study Th2 cell functional activity in lupus mice. The detection limit of the assay was below 10 pg/ml (± 2SD). Figure 4.12 shows the kinetics of IL-4 production by Con A-induced spleen cells from 4-month old BALB/c and lupus mice. Both NZB/W and MRL/*lpr* mice showed reduced IL-4 production, although increased IL-4 secretion was observed at the earlier time points in NZB/W mice. In 106106MRL/*lpr* mice, the IL-4 production was almost completely suppressed. The results indicate that IL-4 production is also deficient in lupus mice at this age.

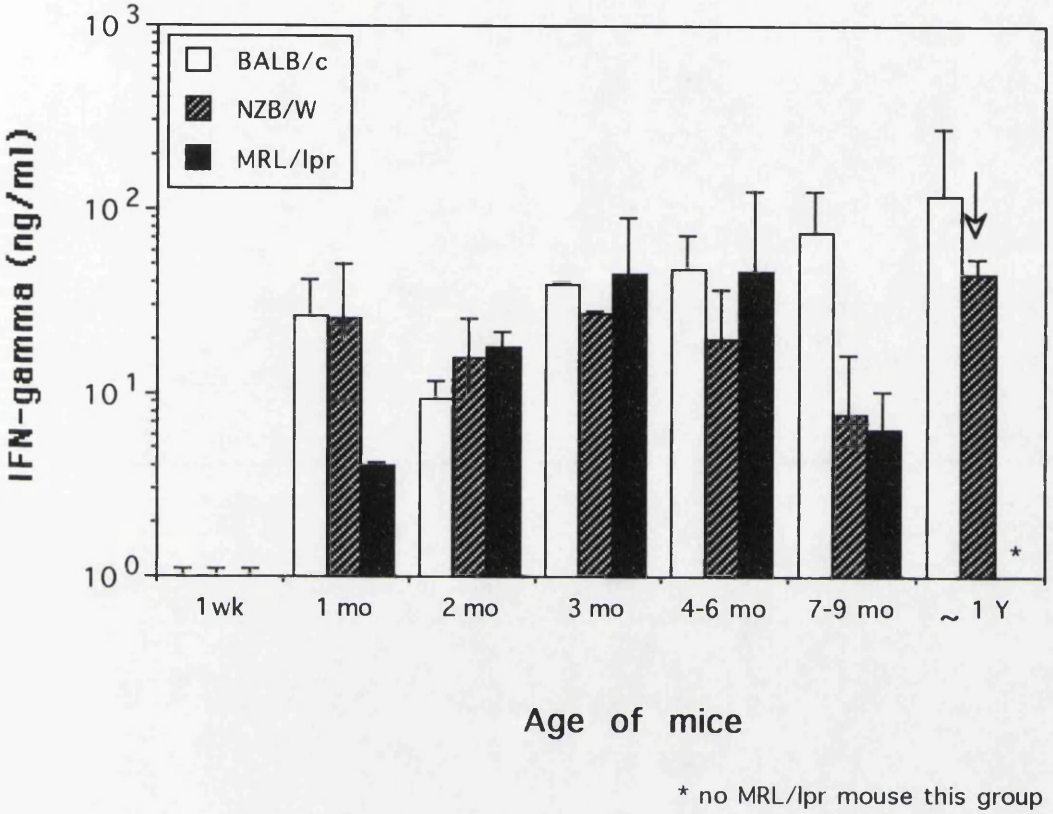


Figure 4.11 Con A-induced IFN-γ production by spleen cells from BALB/c, NZB/W and MRL/lpr mice at different ages. IFN-γ activities in the supernatants of Con A-stimulated mouse spleen cell cultures were measured at daily intervals and data shown are means of the peak values obtained for individual mice in each group, except the one-week old groups in which spleen cells from 6 mice of each strain were pooled. In the one-year old mouse group, NZB/W mice showed no sign of lupus disease activity. Number of mice in each group is as follow:

Age groups	1wk	1mo	2mo	3mo	4-6mo	7-9mo	~1yr
BALB/c	6	4	2	5	10	3	3
NZB/W	6	2	3	2	4	6	2
MRL/lpr	6	2	3	6	10	2	0

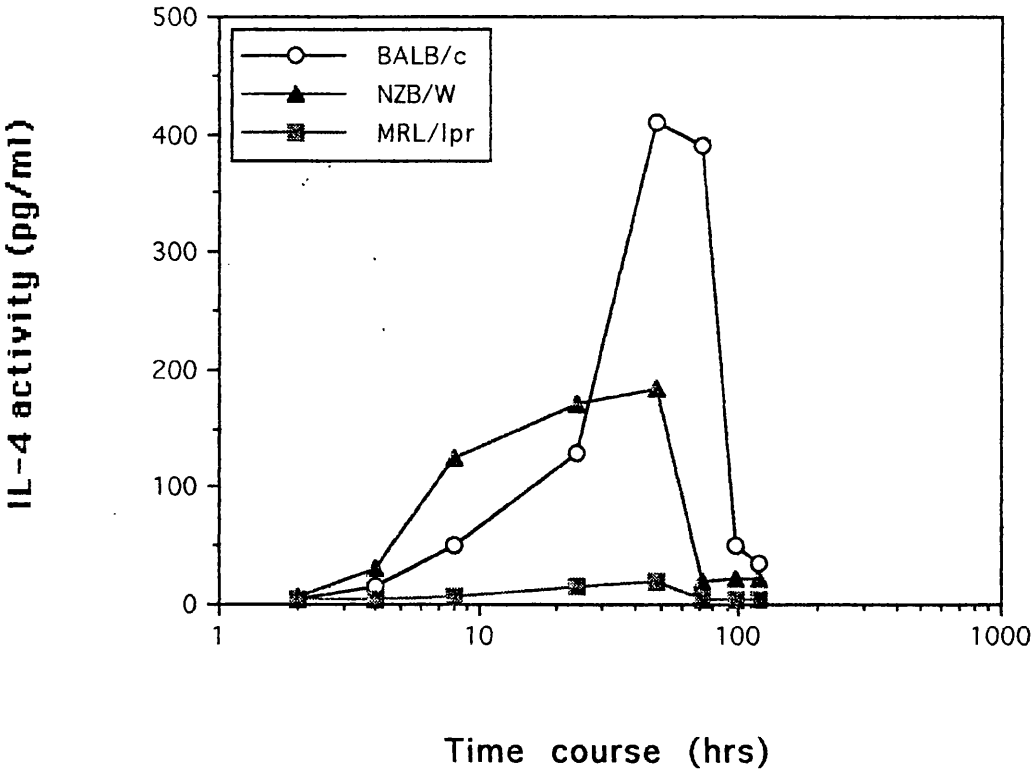


Figure 4.12 Kinetics of Con A-induced IL-4 production by spleen cells from BALB/c, NZB/W and MRL/lpr mice. IL-4 activities in the supernatants of Con A-stimulated mouse spleen cell cultures at 2, 4, 8, 24, 48, 72, 96 and 120 hrs were measured by mouse IL-4 specific ELISA. Data are expressed as means of triplicates.

The IL-4 ELISPOT assay, which used the same antibodies as in ELISA, failed to detect IL-4 secretion by individual cells in cultures.

4.6 T-cell derived cytokine gene expression

The PCR-assisted cytokine message amplification phenotyping (MAPPing) technique involved a reversed transcription of total cellular mRNA followed by PCR amplification of the cDNA using specific primers for each cytokine. The conditions of the PCR assay were optimised in preliminary experiments (see Chapter 2, Section 2.6).

Expression of cytokine genes in freshly isolated and Con A-stimulated spleen cells from lupus mice was studied to determine which lymphokines might be transcribed by these cells *in vivo*, and *in vitro* after stimulation. Time course study showed some kinetic delay of the Con A-induced cytokine gene transcription in the lupus mice, particularly IL-2 and IL-4 in MRL/*lpr* mice, and IL-6 in the NZB/W mice. However, the relative peak expression for all these cytokines appeared between 6 to 9 hours after stimulation in all three strains of mice (Fig.4.13). The results showed no significantly detectable mRNA expression in the unstimulated cells for either IL-2 (Fig.4.13a) or IL-4 (Fig.4.13c) in both lupus and normal control mice. However, compared to control BALB/c, Con A-induced mRNA expression for IL-2 was considerably, and for IL-4 moderately, reduced in the MRL/*lpr* strain, but only marginally reduced in NZB/W mice. Low levels of IL-6 mRNA were detected in freshly isolated spleen cells from BALB/c and MRL/*lpr*, but not NZB/W mice (Fig.4.13d). After Con A stimulation, the NZB/W mice also seem to have a relatively low level of IL-6 mRNA expression. In contrast, a considerable amount of IFN-γ mRNA was detected in the splenic lymphocytes from mice of the three strains not only of the Con A-stimulated cells but also the unstimulated cells (Fig.4.13b). The mRNA expression for IFN-γ was very much comparable between the control BALB/c and lupus mice, except for a marginal decrease in the MRL/*lpr* strain (Fig. 4.13b).

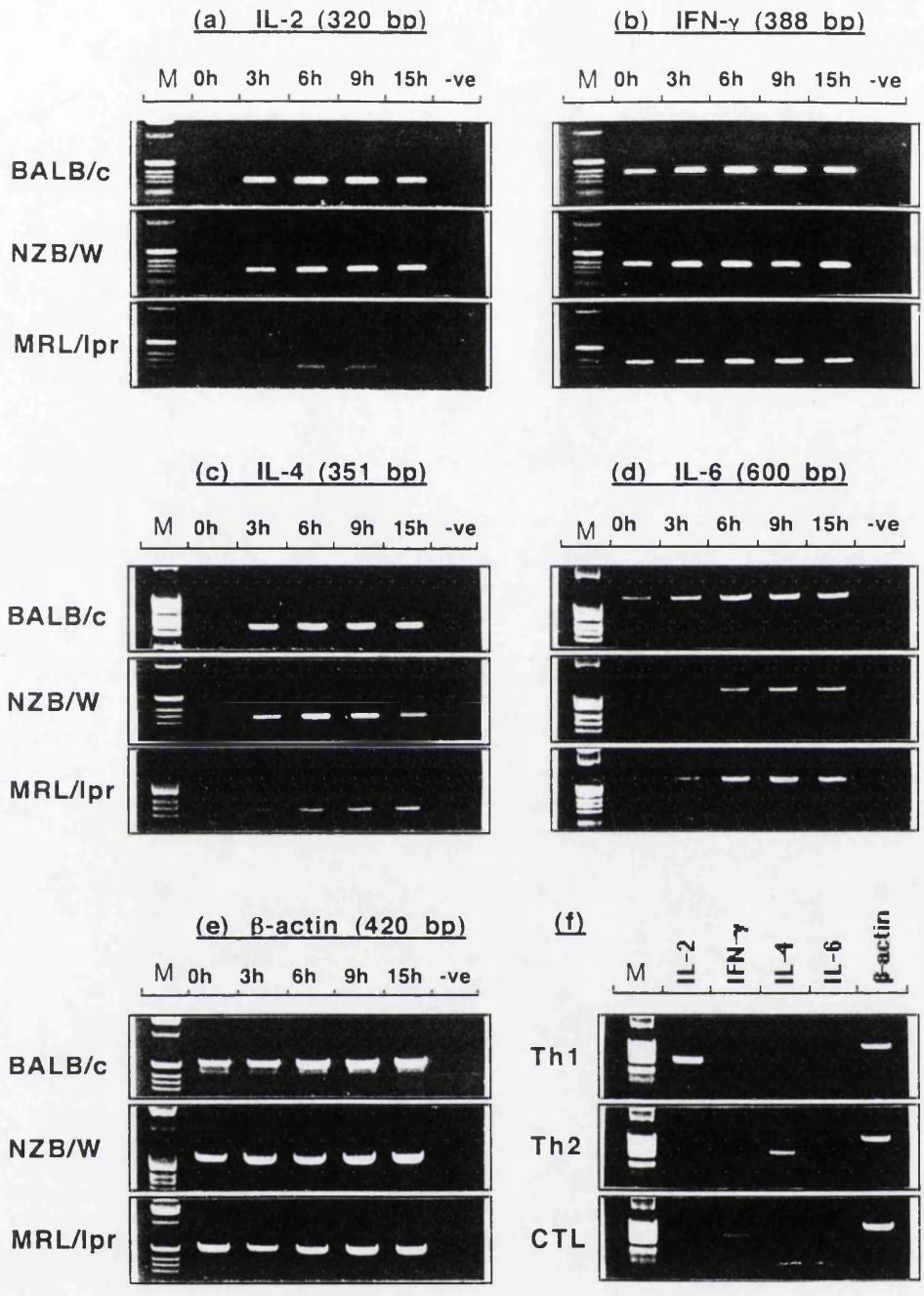


Figure 4.13 PCR-assisted analysis of the kinetics of Con A-induced cytokine mRNA expression by spleen cells from BALB/c, NZB/W and MRL/lpr mice (age: 25 wks). Total RNA was extracted from mouse spleen cells unstimulated (0 hr) and Con A-stimulated for 3, 6, 9 and 15 hrs. After reverse transcription, specific messages for IL-2 (a), IFN-γ (b), IL-4 (c), IL-6 (d) and β-actin control (e) were amplified by PCR subjected to 35 cycles. RNA samples extracted from mouse Th1 (WEP 999) and Th2 (WEP 988) cell clones (malaria specific), and CTLL cells were run in parallel to establish the cytokine expression patterns of the cells (f) and to confirm the specificity of the primers. The negative controls shown were samples amplified without cDNA templates. M: 1Kb DNA markers.

The oligonucleotide sequence of each of the cytokine primers was described in the Materials and Methods chapter. Each pair of these cytokine primers had been tested on RNA samples extracted from Con A stimulated murine Th1 (WEP999, *Wellcome, UK*), Th2 (WEP988,*Wellcome, UK*) and CTLL cell clones to confirm the specificity of primers and the PCR amplification (Fig.4.13f). It shows that only one of the 4 different cytokine message could be detected in each of the three T-cell clones. The Th1 cells expressed IL-2, Th2 cells expressed IL-4 and CTL cells expressed IFN-γ alone. No IL-6 mRNA was detected in any of the T-cell clones, though it could be readily detected in the Con A-stimulated mouse spleen cells (Fig.4.13d). The mRNA expression for β-actin was tested to assess the integrity of the RNA preparations from each of the original samples, and also gave positive control signals in each case. To some extent, it reflects semi-quantitatively the experimental error as well as the accuracy of the test. There was no false positivity detected in the negative controls in which sham reverse transcription reactions were run without enzyme, or without DNA.

In the study, it was taken into account that the conventional PCR technique does not produce sufficient information for quantitation. As a complementary measure, the final PCR-amplified cytokine cDNA was therefore examined by serial dilutions. Figure 4.14 shows the 6-hour (optimum) Con A-induced cytokine mRNA expression in such a semi-quantitative way. The results suggest that there is no significant difference (less than 2 folds) in the amount of PCR-amplified IL-2, IFN-γ, IL-4 and IL-6 gene transcripts between lupus and normal mice except for an 8 fold reduction of IL-2 gene expression in MRL/*lpr* mice.

The overall results show that Con-induced mRNA expression for IL-2, IL-4, IL-6 and IFN-γ were all detectable in spleen cells from both normal and lupus mice. There was no increased expression of these cytokines at transciptional levels in either the stimulated or unstimulated spleen cells in the two lupus strains of mice of 6 months old. Although the

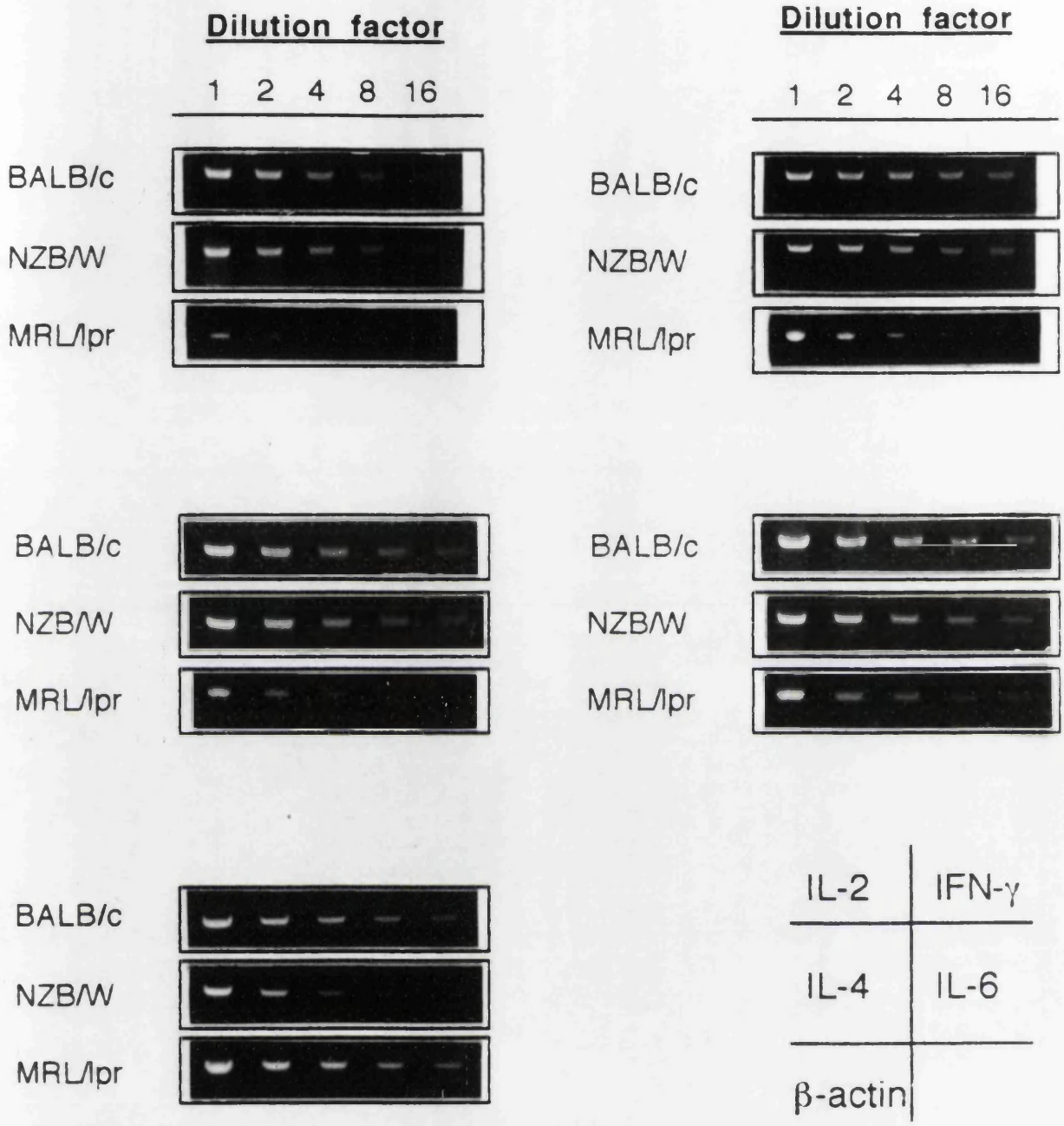


Figure 4.14 Semi-quantitative analysis of the PCR-amplified cytokine mRNA expression by spleen cells from BALB/c, NZB/W and MRL/lpr mice. Cytokine mRNA expression for IL-2, IFN-γ, IL-4 and IL-6 in the mouse spleen cells after 6 hrs of Con A stimulation was partially quantified by serial dilution of the PCR-amplified products, in comparison to the β-actin controls of each of the corresponding samples.

quantitative information derived from the PCR assay might be limited, the reduction of Con A-induced IL-2 mRNA expression in the MRL/*lpr* mice is clear.

4.7 Discussion

T-cell mitogens such as Con A and PHA are known to induce normal T-cells to enter 'G1' phase from a resting 'Go' phase of the cell cycle, resulting in enlargement of the cells and synthesis of protein and RNA. However, DNA synthesis occurs only when a second signal, IL-2, is present (Hadden, 1988; Majumdar *et al.*, 1990). The failure of T-cells from lupus mice to respond to Con A might be controlled by this second signalling process. The defective IL-2 production is apparently accountable for the inability of lupus T-cells to proliferate which prevents a complete cell cycling upon mitogen stimulation. In addition, it was observed that the proliferative defect appeared earlier in the MRL/*lpr*, later in the NZB/W and progressive with age in both strains. This correlates with the degree of IL-2 hypoactivity found in the two mouse strains at different ages.

Hadden (1988) suggested that the second signalling process might be initiated by IL-1 produced by adherent accessory cells resulting in production of IL-2 by primed T lymphocytes. Thus, the inability of lupus T-helper cells to respond to an activation signal by secreting IL-2 could be due to either a defect in the accessory cell function or a defect in the T-helper cell itself. However, addition of IL-1 did not correct the defect in IL-2 production. It is possible that human IL-1 might not be physiologically active for mouse, but previous studies using human IL-1 also failed to restore the defect in T-cell activation in human SLE (Linker-Israeli, 1983). Furthermore, since replacement of lupus adherent cells with normal adherent cells did not correct the defect in Con A-induced IL-2 production either, it suggests that the defective lupus T-cell activation could not be simply attributed to a lack of co-stimulative signal from adherent cells. More recent studies showed that responsiveness to IL-1 is restricted to memory $CD4^+$

cells which are mainly IL-4 but not IL-2 secretors (Luqman *et al.*, 1992), but a different APC-derived signal has been proposed for triggering IL-2 synthesis and secretion by T-helper cells of Th1 phenotype. It is now suggested that the co-stimulative signal responsible for IL-2 secretion is mainly generated by interaction of CD28 molecule on T-cells with the B-cell restricted B7/BB1 ligand (Norton *et al.*, 1992; Costello *et al.*, 1993). The present study did not rule out the possibility of a defect within this co-stimulation, because no attempt was made to separate and to replace B-cells in the cultures.

Stimulation of T-lymphocytes with antigen or mitogen results in the expression of surface IL-2 receptor molecules that, upon interaction with IL-2, promote clonal expansion of T-cells (Smith, 1980). Con A-induced T-cell IL-2 receptor expression are defective in NZB/W and MRL/*lpr* mice. This is in agreement with Zubler's (1986) finding in human SLE patients. Since IL-2 is known to be capable of upregulating expression of its own receptors (both α and β-chains) resulting in enhancement of the frequency of IL-2 responsive cells (Reem *et al.*, 1985), it is conceivable that the inability of lupus T-cells to express IL-2R may also be due to IL-2 deficiency. However, It has been shown that the abnormally expanded DN population in MRL/*lpr* mice does not express IL-2R β-chain (p75) therefore is unable to generate functional high-affinity IL-2R (α and β-chains) (Rosenberg, 1989). Although this has been controversial as study by Gutierrez-Ramos et al (1989) demonstrated a 75-kDa IL-2 binding molecule identified on the surface of *lpr* T-cells, a most recent report (Tanaka *et al.*, 1993) favours Rosenberg's finding. It was interesting to note that a rapid decline in the IL-2R expression of activated normal T-cells occurred almost immediately after cell division. This might be due to the release of IL-2R after activation, or a transient inability of these post-activated or dividing cells to express IL-2R. However, it is not clear, whether this phenomenon might be reflecting any kind of *in vivo* post-activated state of a dominated T-cell population in lupus mice.

IFN-γ is another characteristic Th1-derived lymphokine, which has also been involved in autoimmune phenomenon. Results from early studies on production of IFN-γ in SLE have been contradictory and inconclusive (Santoro *et al.*, 1983; Kofler *et al.*, 1984, Umland *et al.*, 1989). In the present study, Con A-induced IFN-γ production by lupus T-cells was decreased in old lupus mice possibly due to reduced frequency of the secretors, in spite of individual variation and strain differences. However, unlike the deficient Con A-induced IL-2 production, the IFN-γ production defect is not consistently established in young lupus mice. Since IFN-γ were not only secreted by Th1 cells ($CD4^+$) but also by $CD8^+$ and other cells, the production of such a cytokine could be more complex and may not only reflect Th1 function. In addition, kinetic studies are often necessary to provide a basis for a valid comparison between mouse models of different strains and ages. The difference in IFN-γ production between lupus and normal mice, which peaked at 3 days, would not have been observed if the samples had been taken up to 24 hours only like in an earlier study (Umland, 1989).

IL-4 is produced by T-helper cells of the Th2 phenotype. Production of IL-4 may reflect functional activity of Th2 cells. Although IL-4 is known as a B-cell growth factor it also has pleiotropic effects on other cell types such as T-cells, mast cells and macrophages (O'Garra *et al.*, 1988). Evidence has been recently shown that this Th2-derived lymphokine may down-regulate IL-2 activity via its action on monocytes (Brook *et al.*, 1992), and it can also suppress production of IFN-γ by human mononuclear cells (Peleman et al., 1992). Since liver of MRL/lpr mice contain interleukin-4-producing lymphocytes and accessory cells that support the proliferation of Th2 helper T lymphocyte clones (Magilavy et al, 1992), an increased Th2 functional activity could explain the defective Th1 phenotype. However, the present study shows that Con A-induced IL-4 production was also deficient in lupus mice.

The present study on cytokine mRNA expression demonstrates that gene transcription for IL-2, IFN-γ, IL-4 and IL-6 can all be induced in lupus mice. However, compared to normal mice, Con A-induced expression of some of these cytokines was reduced to different extent in the lupus mice. The results were largely in agreement with Umland's study (1989) on MRL/*lpr* and BXSB mice using the RNase protection assay to assess gene expression for IL-2, IL4 and IFN-γ. The reduced mRNA expression for IL-2 and IL-4 in MRL/*lpr* mice might suggest that the deficient production of the cytokines is due to some defect at gene transcriptional level. However, in NZB/W mice, IL-2 and IL-4 genes are expressed comparably to control BALB/c mice although production of these cytokines is also defective, suggesting that a different mechanism might be involved. It is worth pointing out that, in the 5-month old MRL/*lpr* mice, although Con A-stimulated spleen cells showed reduced levels of IL-2 and IL-4 mRNA it might have been a cell dilution effect if the spleen enlargement (3-7.5 times) due to lymphoproliferation was taken into account. These together with the quantitative limitation of the assay are in general inadequate to provide any conclusive answer to whether the defect responsible for the reduced cytokine production was in the gene or post-transcriptional levels. The significance of the reduced expression of IL-6 mRNA in NZB/W mice is also not clear. Nevertheless, it is important to know that there is no enhancement of the cytokine mRNA expression, particularly in freshly isolated spleen cells, in lupus mice. This suggests that T-helper cells are not in a generally activated state *in vivo*. Further study of cytokine gene expression in young mice will be more informative.

Collectively, all the results described here have further confirmed the functional abnormalities of lupus T-cells and their defective responses to activation. The origin of the defect causing these T-cell abnormalities is not yet revealed, but it is clear that the early, progressive IL-2 deficiency would have prevented the normal process for T-cell activation.

Chapter 5

Effects of exogenous IL-2 and other cytokines on T-cell activation in lupus mice and a SLE patient *in vitro*

5.1 Introduction

The results presented in the previous two chapters have demonstrated a deficient IL-2 activity accompanied by various abnormal T-cell functions in SLE. The causes responsible for these T-cell abnormalities remain to be elucidated. The defective IL-2 activity found in young lupus mice which have not yet developed symptoms of autoimmune disease suggests the possible intrinsic nature of the Th1 functional defect. However, it is well established that IL-2 stimulates activated T-cells to proliferate by binding to specific cell-surface receptors. Since Con A did not induce lupus T-cells to express normal levels of IL-2R, it is necessary to identify whether the defective T-cell activation is simply due to a lack of IL-2 or also because of these other T-cell defects such as the inability of cells to express IL-2 receptors. The next important question to be answered is, therefore, "can lupus T-cells respond normally to IL-2 ?" In other words, if the lupus T-cells were not able to produce sufficient endogenous IL-2 upon activation, could it be by-passed by addition of exogenous IL-2 allowing a normal Con A response ?

5.2 Effects of exogenous cytokines on proliferative responses of T-cells from lupus mice and a SLE patient

5.2.1 Dose-dependent effects of IL-2 on old lupus mice

The defective proliferative response of splenic T-cells from lupus mice to Con A stimulation was completely restored by addition of recombinant IL-2 to the cultures (Fig. 5.1). The IL-2 effect was dose-dependent (Fig. 5.1d, f) and 5 to 10 ng/ml was sufficient to restore the response to that of the BALB/c or CBA control cells (Fig. 5.1c, e). The effect was synergistic since the same dose of IL-2 alone did not stimulate the cells to proliferate although at higher doses it had a marginal effect (Fig. 5.1a, b). IL-2 alone has been tested at up to 30 ng/ml in all strains with similar results. Exogenous

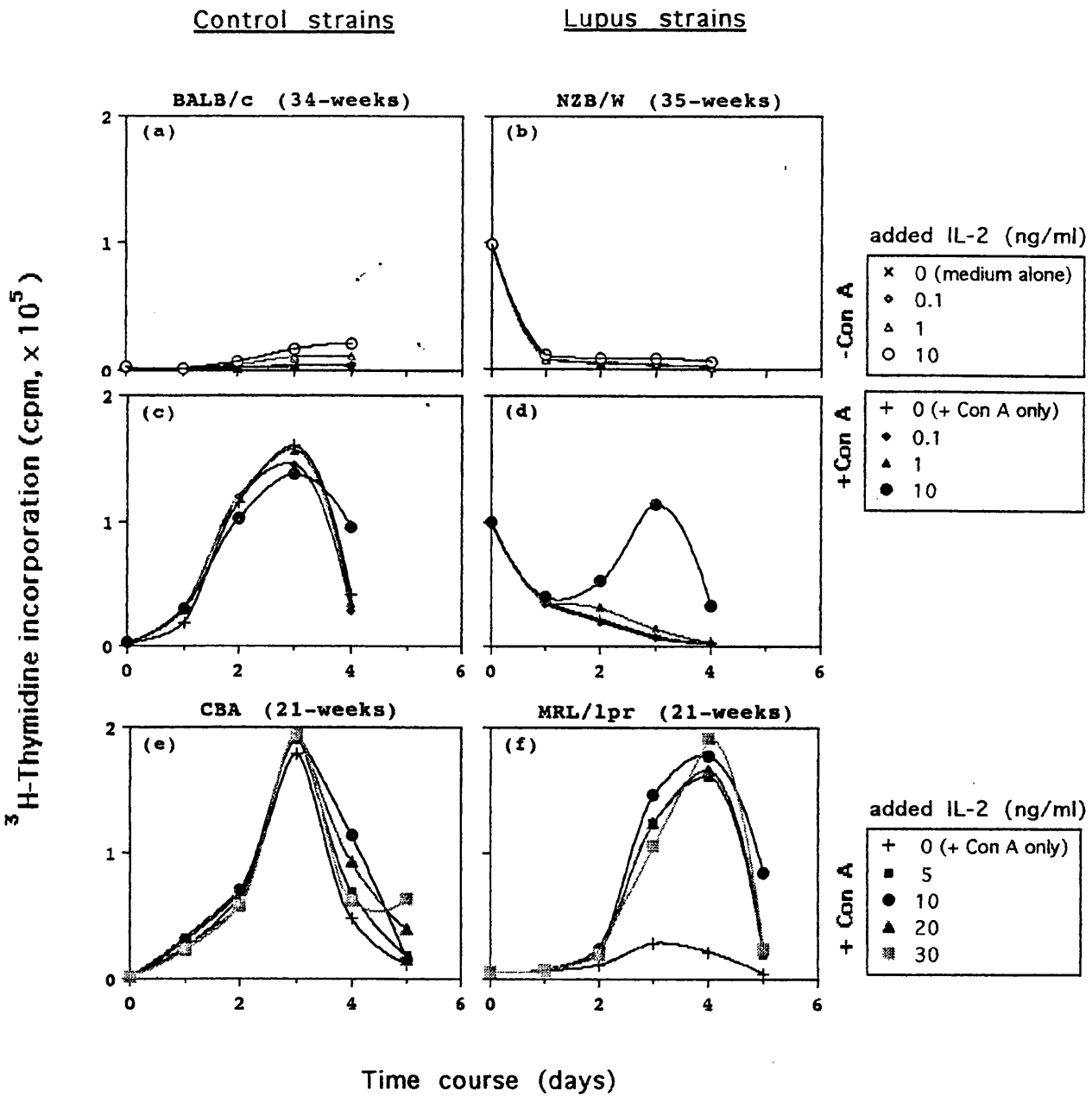


Figure 5.1

Dose-dependent exogenous IL-2 effects on Con A-induced lupus T-cell proliferative responses. Spleen cells from lupus and sex-age-matched normal BALB/c or CBA mice were cultured (10^6cells per ml) with or without Con A (2.5 μg/ml) and various amounts of r-human IL-2 as indicated. Cell proliferation was measured at daily intervals by the proliferation assay. The lupus mice, NZB/W at 35-weeks and MRL/lpr at 21-weeks, were at their peak stages of disease development (50% mortality: NZB/W ~8 months; MRL/lpr ~4 months). Cells were cultured with medium alone, or with increasing doses of rIL-2 at 0.1, 1, or 10 ng/ml in the absence of Con A (a, b); or with Con A alone (c-f), Con A plus rIL-2 at 0.1, 1 or 10 ng/ml (c, d) or Con A plus IL-2 at 5, 10, 20 or 30 ng/ml (e, f).

IL-2 did not significantly affect the mitogenic responses of T-cells from normal BALB/c and CBA mice other than a slight prolongation of cell proliferation (Fig. 5.1c, e).

5.2.2 Effects of IL-2, IL-4 and IFN-γ on young lupus mice

The effects of exogenous IL-2 on T-cell activation was also determined in young lupus mice of different ages. The defective Con A-induced T-cell proliferation was evident in MRL/*lpr* mice as young as 4 weeks old and it is consistent and progressive with age (Fig.5.2c, f, i. closed triangles). This is in comparison with the responses of T-cells from sex-age-matched normal BALB/c mice (Fig.5.2a, d, g). Although the early proliferative defect could sometimes be observed in young NZB/W mice too (not shown), it was not consistently established in most of the cases until 6-8 months (Fig.5.2b, e, h; Fig.5.1d).

Recombinant IL-2 was used at 10 ng/ml in these subsequent experiments. Figure 5.2 also shows typical patterns of the exogenous IL-2 effects (closed circles) on the proliferative responses of splenic T-cells from young lupus and control mice between 1 to 12 weeks of age. In the lupus mice of 4-weeks or older, addition of r-IL-2 during Con A stimulation by-passed the IL-2 hypoproduction defect where it existed, and induced the T-cells to proliferate in full. Again, these mouse spleen cells from both lupus and normal strains did not spontaneously proliferate in the absence of Con A (Fig.5.2, open triangles), and only marginally responded to IL-2 alone (open circles). In the one week old mouse group, BALB/c spleen cells did not respond to Con A stimulation even in the presence of exogenous IL-2 (Fig. 5.2j). Since less than 4% of cells in the spleens from the one-week old mice of the three strains were $CD3^+$, the inability to respond to Con A stimulation is probably due to a lack of T-cells at this very early stage. However, although these early spleen cells from lupus mice also remained

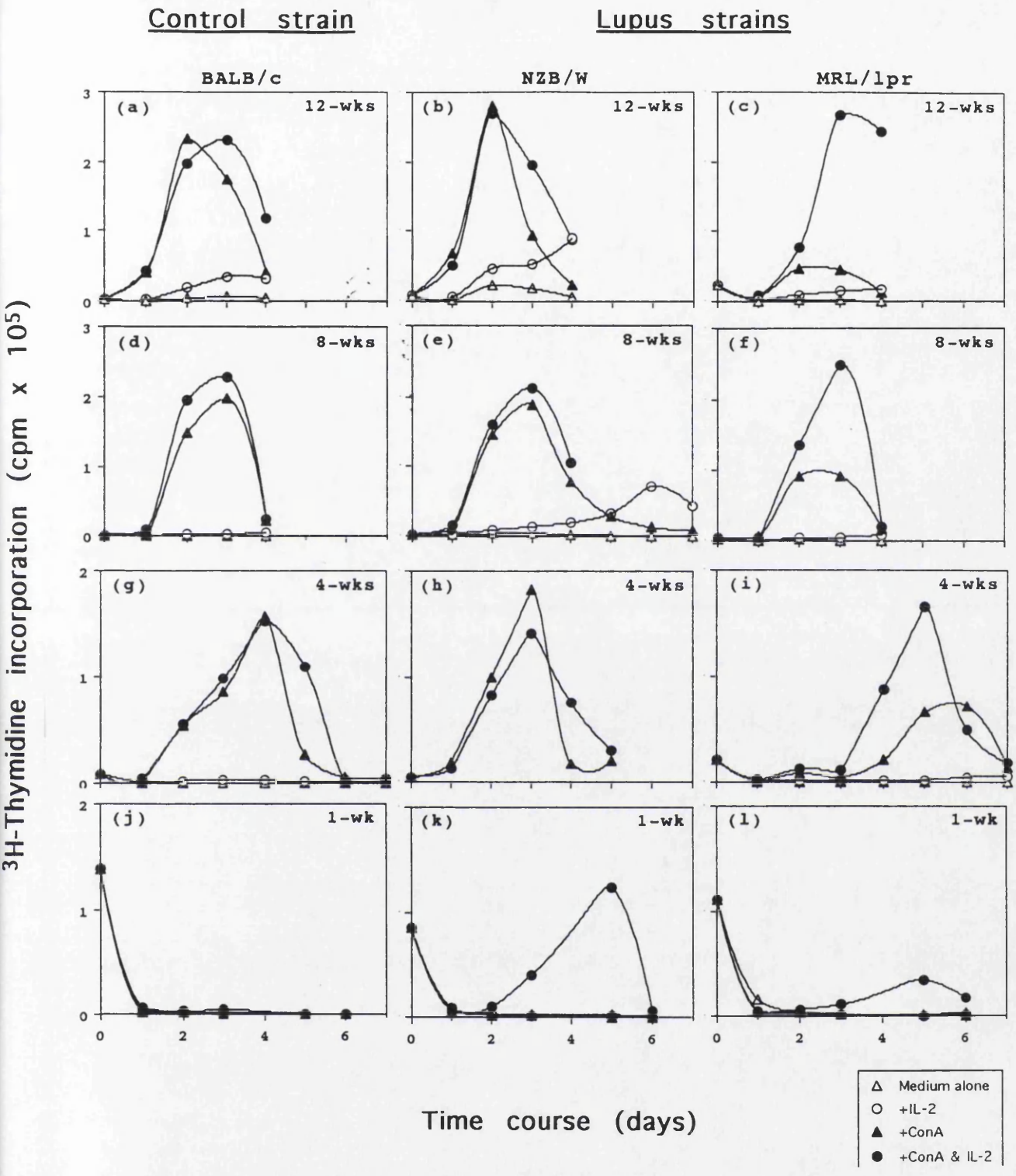


Figure 5.2 Exogenous IL-2 effects on Con A-induced proliferative responses of spleen cells from young lupus and normal mice between 1 and 12 weeks old. Recombinant human IL-2 was used at 10 ng/ml. Data are presented with representative results from individual mice of different ages and strains, except the 1 and 4-week mouse groups (g-l) in which spleen cells from more than 2 mice of the same age and strain were pooled. The age of the mice is indicated at the top right hand corner of each individual graph.

unresponsive to Con A alone, their T cells showed earlier responsiveness to exogenous IL-2 during Con A stimulation (Fig. 5.2k, l).

Figure 5.3a shows effects of exogenous IL-2 on the proliferative response of T-cells from a 3 month old MRL/*lpr* mouse, by using supernatants from cultures of Con A stimulated (24h) BALB/c spleen cells as a source of IL-2. The natural murine IL-2 derived from BALB/c spleen cells (supernatant used neat) also effectively restored the lupus T-cell proliferative response to Con A (close triangles). In addition, it was noted that the culture supernatants of Con A-stimulated (24h) spleen cells from the 3-month old MRL/*lpr* mice had certain suppressive effects on the proliferative response of spleen cells from a BALB/c mouse (close circles). Exchange of spleen cell culture supernatants between a 3 months old NZB/W and a BALB/c mouse 24 hours after Con A stimulation did not affect the subsequent thymidine uptake by the cells, and the young NZB/W mouse showed a normal proliferative response to Con A (Fig. 5.3b).

To determine effects of other cytokines on the T-cell activation, recombinant murine IL-4 and IFN-γ were also tested on spleen cells from MRL/*lpr* and BALB/c mice. At 10 ng per ml, while IL-2 significantly enhanced the proliferative response of lupus cells to Con A, the effect of IL-4 was only marginal and IFN-γ had no effect (Fig.5.4). The immunoreactivities of the recombinant mouse IL-4 and IFN-g used in the experiments have been confirmed by the specific ELISA techniques, but no efforts were made to determine their bio-physiological activities.

5.2.3 Effects of IL-2 and IL-1 on spleen cells from a SLE patient

A human spleen from a SLE patient (female, 47 years old) with active disease was kindly provided by Dr M Fields, Department of Medicine, Royal Infirmary, Glasgow. The spleen was surgically removed due to its hyper-hemocidal activity resulting in thrombocytopenia in the patient.

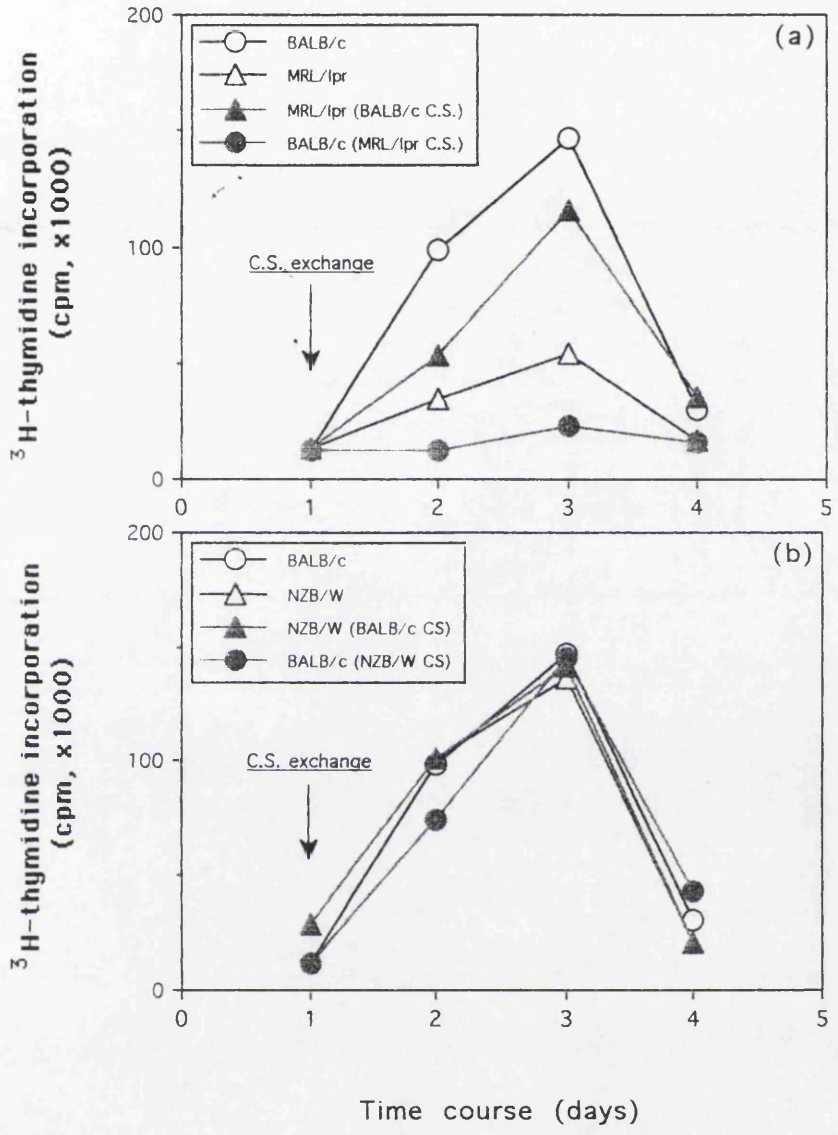


Figure 5.3 Effects of supernatants from Con A-stimulated spleen cell cultures as the source of IL-2 on subsequent T-cell proliferation in normal and lupus mice. Spleen cells (10^6 cells per ml) were stimulated with Con A for 24 hrs followed by exchange of culture supernatants between normal (BALB/c) and lupus, MRL/lpr (a) or NZB/W (b), mice (closed symbols). The proliferative responses were measured by ^{3}H-thymidine incorporation and compared with the control cell cultures in which supernatants were unchanged (open symbols).

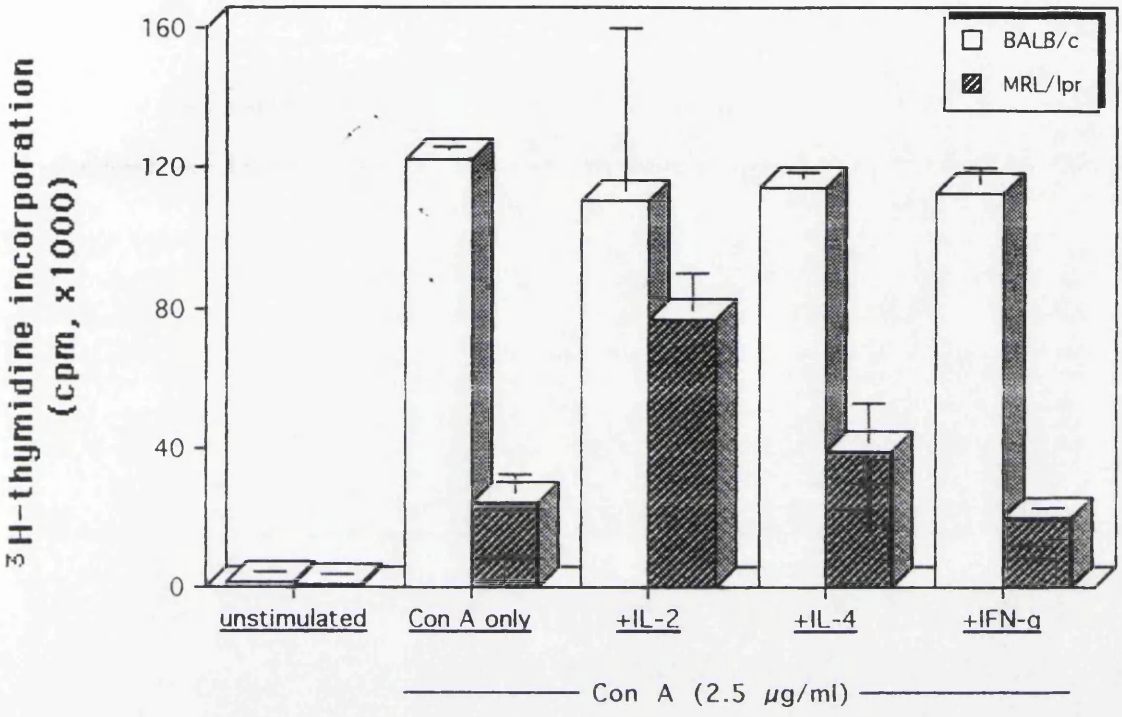


Figure 5.4 Different effects of r-human IL-2, r-mouse IL-4 and r-mouse IFN-γ on Con A-induced mouse T-cell proliferation. Exogenous cytokines were used at 10 ng per ml. Proliferation of the spleen cells pooled from 2 mice (4 month old) in each strain, stimulated or unstimulated, were all measured at 3 days by ^{3}H-thymidine uptake. Data shown are means (±SD) of duplicate cultures.

Cells were prepared from the spleen as previously described for dealing tissue from mouse spleens, and stimulated with Con A (2.5 μg/ml) or PHA (5 μg/ml) in 96-well cell culture plates. The cell cultures were supplemented with recombinant human IL-2 or IL-1 at various concentrations. After 66 hours of incubation at 37^0C, 5%CO2, the cultures were pulsed with ^{3}H-thymidine for 6 hours and the incorporated radioactivity measured. No significant effect of exogenous IL-1 (0-100 ng/ml tested) was observed on the proliferative responses of the cells to Con A or PHA (Fig. 5.5a). In addition, the cells did not respond to IL-1 alone either. However, similar to its effects on mouse spleen cells, IL-2 enhanced proliferative responses of lupus spleen cells from the SLE patient to both of Con A and PHA. It was noted with interest that unlike the mouse, the human lupus splenocytes responded strongly to IL-2 alone at high doses. At doses above 10 ng/ml, there was a much higher rate of increase of the IL-2 stimulating effects in the cultures with IL-2 alone compared to cultures with IL-2 and either Con A or PHA. At the highest dose (100 ng/ml) of IL-2 tested, there was even a 2-fold higher thymidine uptake by the cells cultured with IL-2 alone compared to cells cultured with the same dose of IL-2 in the presence of mitogen (Fig. 5.5b).

5.3 Effects of IL-2 on mouse T-cell IL-2R expression

IL-2 dependent recovery of the T-cell response is also illustrated by the surface IL-2 receptor (CD25) expression on T-cells ($CD3^+$). Figure 5.6 shows that exogenous IL-2 restores the responsiveness of splenic T-cells from lupus mice to Con A by increasing frequency and density of IL-2 receptor expression. Recombinant IL-2 significantly upregulates IL-2R expression on T-cells from lupus mice of both strains despite some kinetic delay. It was noted that the frequency reached normal levels at day 3 (Fig. 5.6a, b), but the average density of surface IL-2R was still below normal (Fig. 5.6c, d). In addition, the effect of exogenous IL-2 on old lupus mice was relatively poor compared to young mice, particularly in the MRL/*lpr* strain. Figure 5.7 shows

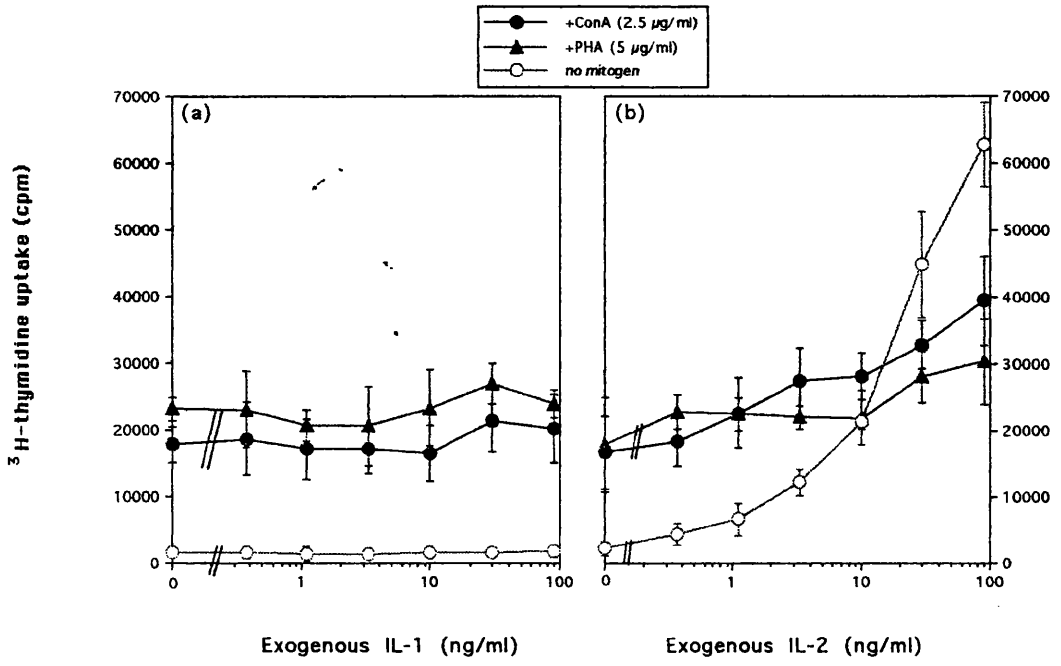


Figure 5.5 Effects of Con A, PHA and exogenous IL-1 and IL-2 on proliferative responses of splenocytes from a SLE patient with active disease activity. Spleen cells were cultured for 66 hrs with or without mitogen and different concentrations of r-human IL-1 or r-human IL-2 as indicated. The proliferative responses of the cells were then measured by pulsing for a further 6 hrs with ^{3}H-thymidine at 0.5 μCi per well.

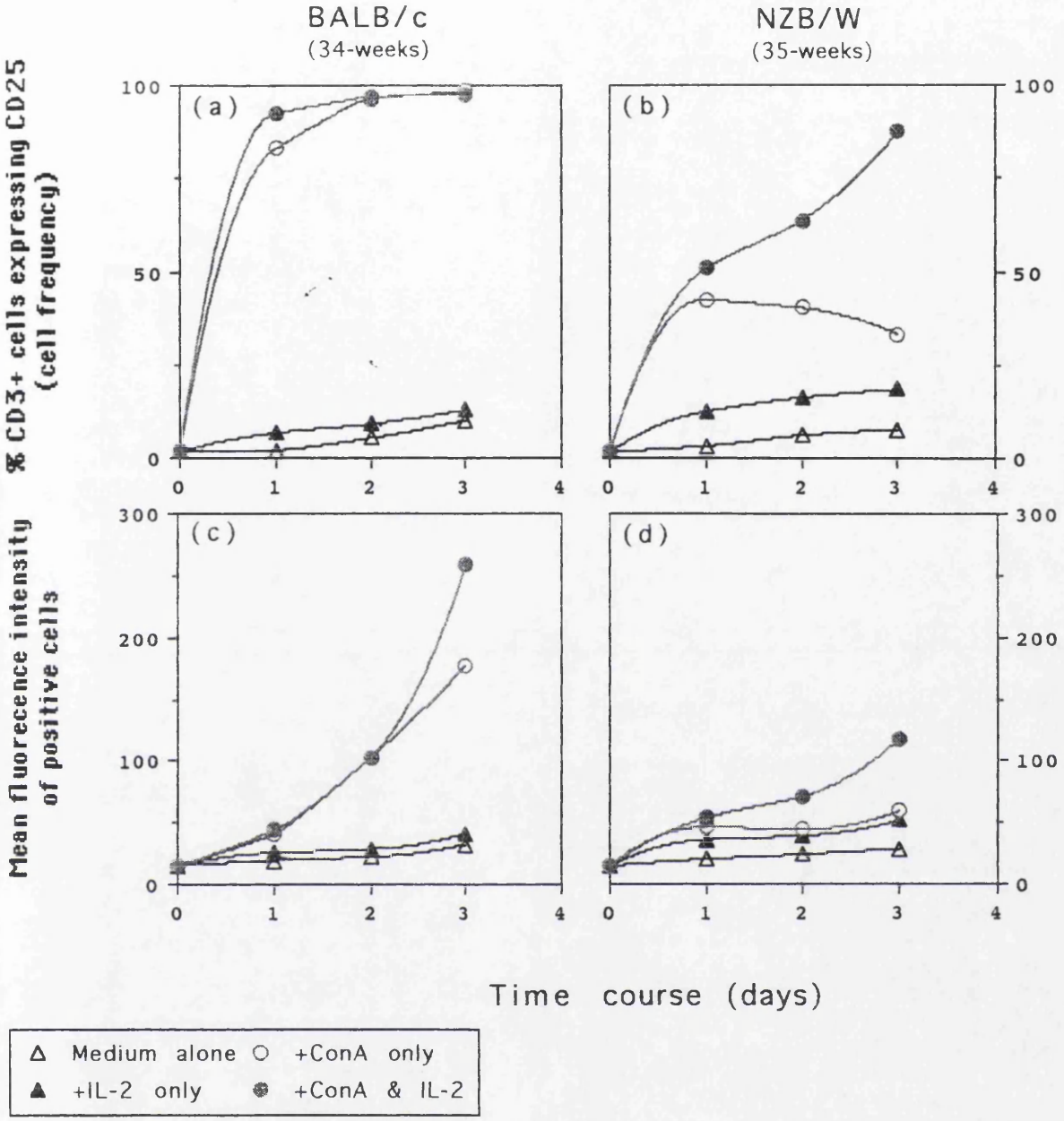


Figure 5.6 Effects of exogenous IL-2 on restoring ability of T-cells ($CD3^+$) from an old NZB/W mouse to express IL-2R (CD25) in response to Con A-stimulation. The results shown are from a three-day time course and rIL-2 was used at 10 ng per ml and Con A at 2.5 μg per ml. Data are presented as: (a, b) percentage of CD3+ cells expressing IL-2R (frequency of CD25 positive cells); and (c, d) mean fluorescence intensity of positive cells (cell surface IL-2R density).

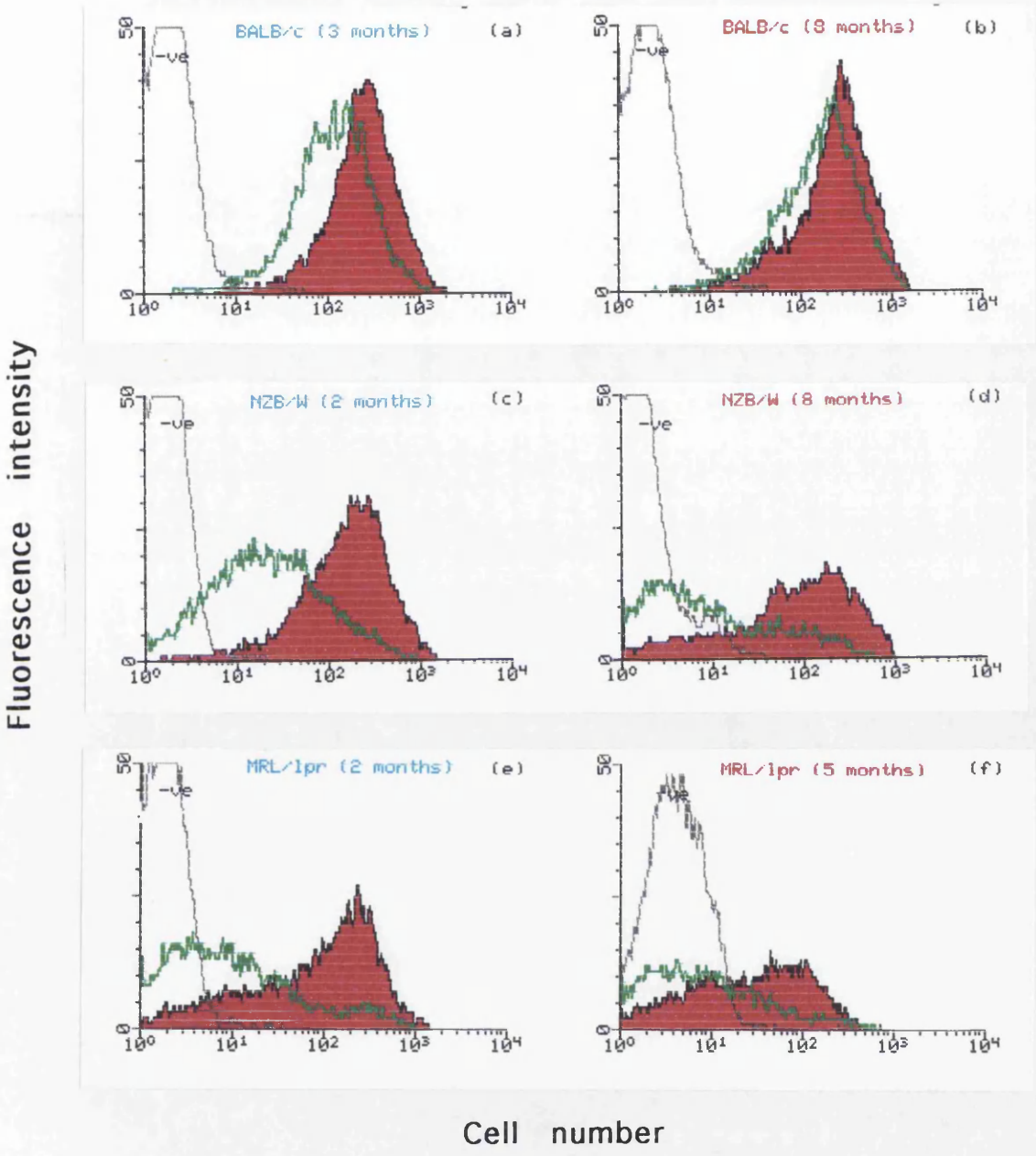


Figure 5.7 Typical flow cytometry profile showing effects of IL-2 on Con A-induced T-cell IL-2R expression on young and old mice. Spleen cells from BALB/c (a, b), NZB/W (c, d) and MRL/lpr (e, f) mice were stimulated with Con A (2.5 μg/ml) in the presence (red filled profile) or absence (green open profile) of IL-2 (10 ng/ml) for 3 days, before being labelled with a R-PE conjugated rat monoclonal anibody specific to mouse CD25. Pan-T cell marker is a FITC-conjugated hamster anti-mouse CD3. All cells gated for the analysis are $CD3^+$ and viable. -ve: isotype negative control.

typical FACS profile of the effects of exogenous IL-2 on Con A-induced T-cell IL-2R expression in young (2 to 3 months) and old (5 to 8 months) NZB/W, MRL/*lpr* and BALB/c mice.

The effect of exogenous IL-2 on IL-2R expression by splenic B-cells in the cultures was marginal and no significant difference was observed between normal and lupus mice.

5.4 Effects of IL-2 on Con A-induced IFN-γ secretion in MRL/*lpr* mice

As previously described in Chapter 4, Con A-induced IFN-γ production was reduced in older lupus mice because of decreased frequency of IFN-γ secretors. This may also be attributed to the IL-2 deficiency as it can be restored by addition of exogenous IL-2 *in vitro* (Fig 5.8). Supplementing IL-2 during Con A stimulation increased IFN-γ secretion by upregulating the frequency of IFN-γ secretors. The effect was synergistic because IL-2 alone did not stimulate IFN-γ secretion in both BALB/c and MRL/*lpr* mice.

5.5 Effects of IL-2 on autoantibody production by mouse spleen cells in cultures

Figure 5.9 shows that spleen cells from old lupus mice spontaneously secrete autoantibodies into cell cultures, but the autoantibody secretion is not significantly enhanced by addition of IL-2, at least in the short term (6 day) cultures. Although the result was just from one experiment and is to be confirmed, it shows exogenous IL-2 did not have an accountable effect on either young (3 month old) MRL/*lpr* mice which secreted low levels of antibodies, or old (8 month old) NZB/W mice which secreted high levels of antibodies to ss or dsDNA.

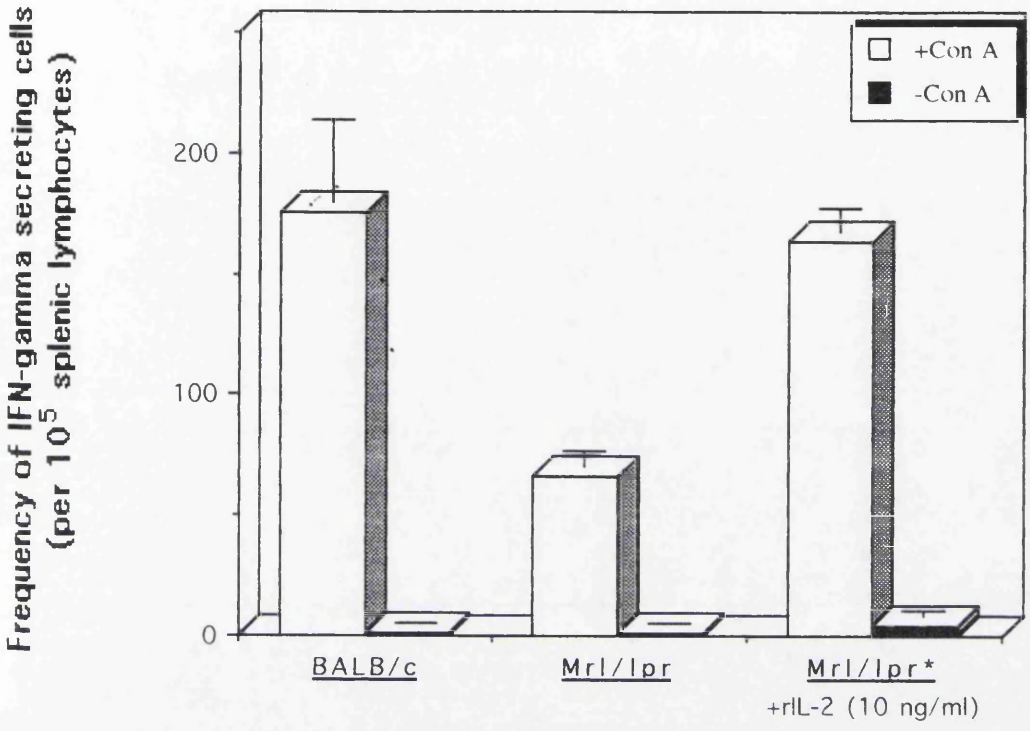


Figure 5.8 Effects of IL-2 on Con A-induced IFN-γ secretion by spleen cells from a MRL/lpr mouse (ELISPOT assay). The figure shows a reduced frequency of IFN-γ secreting spleen cells from a 20-week old MRL/lpr (b) in response to Con A alone compared to the age-sex-matched BALB/c control (a). However, addition of rIL-2 (10 ng/ml) during the stimulation upregulated the production of IFN-γ in the MRL/lpr mouse as shown by the increased number of IFN-γ secreting cells (c).

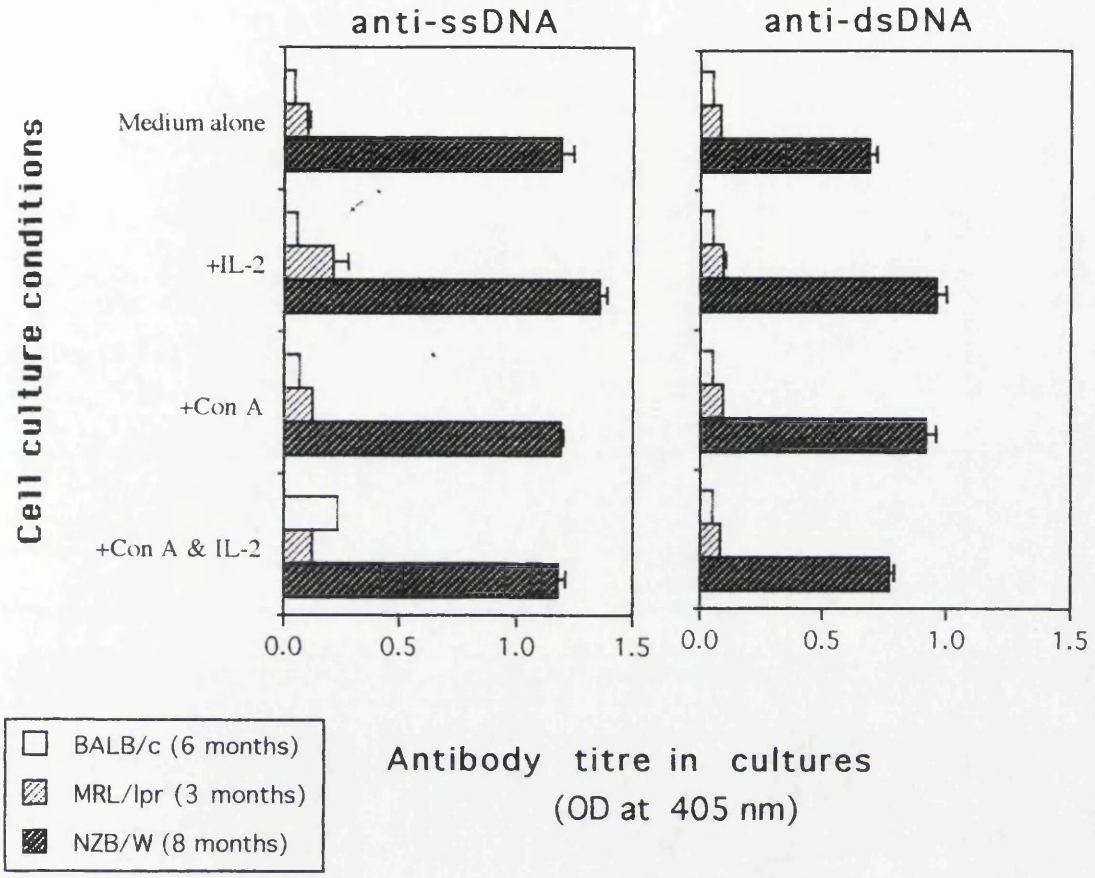


Figure 5.9 ***In vitro*** **spontaneous production of anti-ssDNA and anti-dsDNA antibodies by spleen cells from lupus mice and the effects of Con A (2.5 μg/ml) and rIL-2 (10 ng/ml) on the autoantibody titres.** Spleen cells from BALB/c (6 months), MRL/lpr (3 months) and NZB/W (8 months) mice were cultured with or without Con A (2.5 μg/ml) and/or IL-2 (10 ng/ml) as indicated. Antibodies to ss and dsDNA present in the culture supernatants undiluted were measured at day 5.

5.6 T-cell subsets and the IL-2 responders *in vitro* in MRL/*lpr* mice

To assess the *in vitro* effects of exogenous IL-2 on the abnormal DN T-cells in MRL/*lpr* mice, and the phenotypes of the IL-2 responders, stimulated and unstimulated (freshly isolated) spleen cells from MRL/*lpr* mice were analysed by using specific antibodies to T-cell surface markers. Figure 5.10 shows a selective expansion of $CD8^+$ lupus T-cells with a sharp decline in the percentage of abnormal $CD3^+CD4^-CD8^-$ cells, from 64.94 to 14.32%, after 3 days of Con A stimulation in the presence of exogenous IL-2. There was a 5-fold increase in total number of CD3+ cells compared to cells in the original cultures at day 0, while the absolute number of DN cells remained unchanged. The results indicate that these DN cells did not proliferate in respond to IL-2 *in vitro*.

5.7 Discussion

One of the arguments discounting the primary importance of the IL-2 defect was the failed attempt of earlier investigators to restore lupus T-cell activation by exogenous IL-2. Using IL-2 preparations derived from cultures of Con A-stimulated spleen cells of C3H/St mice and rats, previous investigators (Altman *et al.*, 1981) were not able to correct the defective response of lymph node and spleen cells to Con A in old MRL/*lpr* mice. The authors attributed this to an inability of lupus T-cells to respond to IL-2. However, a later study by Warrington et al. (1989) using limiting dilution analysis techniques showed that addition of exogenous IL-2 during the initial step of mitogen stimulation might increase precursor frequencies of IL-2 responders of peripheral blood lymphocytes from SLE patients. In agreement with Warrington's observation, the present study has demonstrated clearly that exogenous IL-2 by-passes the *in vitro*

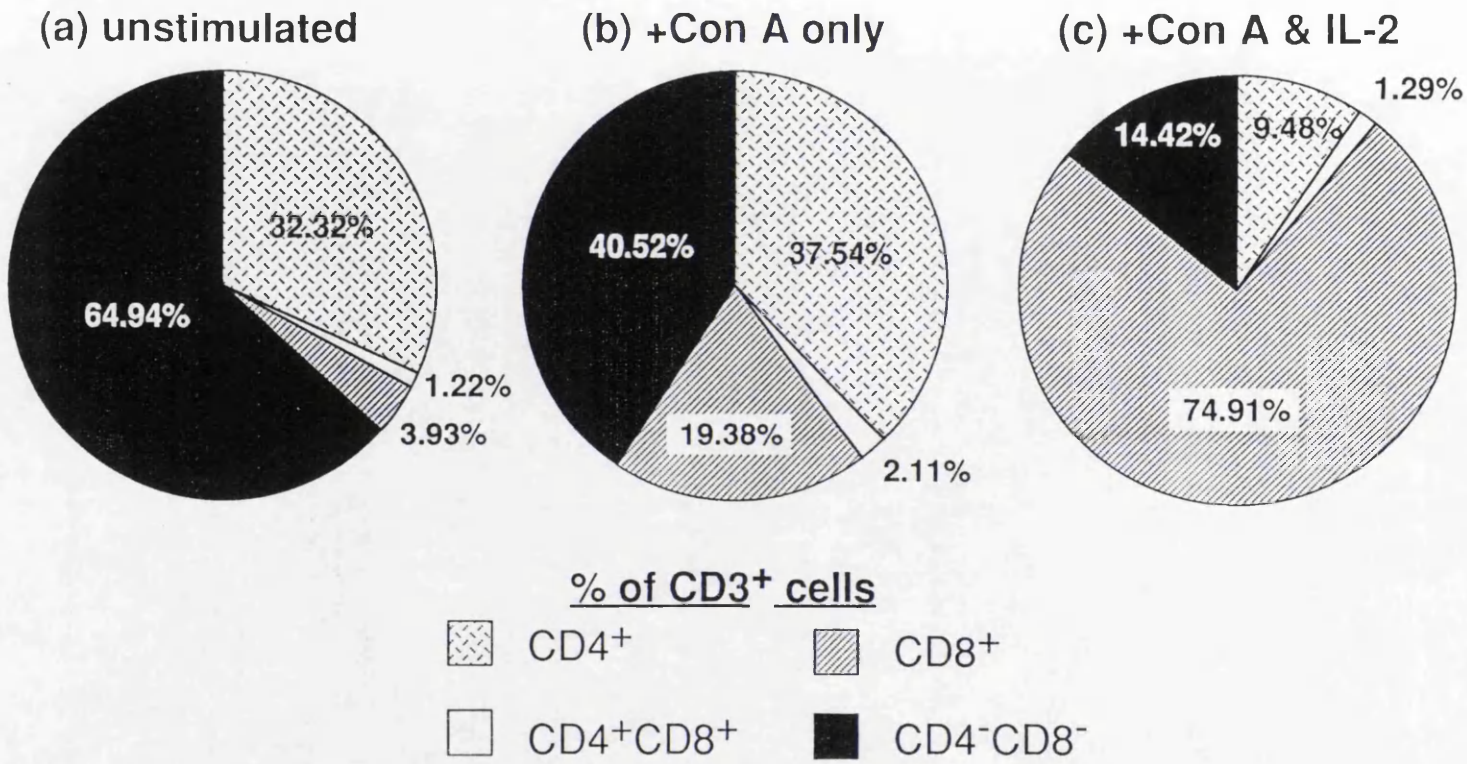


Figure 5.10 FACS analysis of the effects of IL-2 *in vitro* on the proliferation of CD4+, CD8+, CD4+CD8+ and CD4-CD8- T-cell subsets from an old MRL/lpr mouse (5 months). The analysis included spleen cells freshly isolated (a), stimulated by Con A without (b) or with IL-2 at 10 ng per ml (c) for 3 days. Mouse CD4 and CD8 specific monoclonal antibodies pre-conjugated with either FITC or R-PE were used and the Pan-T cell marker was a hamster anti-mouse CD3. Proportion of the T-cell subsets are expressed as percentage of total $CD3^+$ cells.

activation defects in NZB/W and MRL/*lpr* mice, and enhances the proliferative response in the SLE patient.

Under the *in vitro* culture conditions, exogenous IL-2 is capable of restoring the ability of lupus T-cells to respond to Con A or PHA, to proliferate, to express IL-2R and to secrete IFN-γ. In contrast, normal T-cells from BALB/c and CBA control mice are activated by the mitogen and undergo complete cell cycling in the absence of exogenous IL-2 as they are able to secrete sufficient endogenous IL-2. The experimental evidence indicates that IL-2 deficiency is the major and prime cause of an unsuccessful T-cell activation induced by the T-cell mitogens. Therefore, many of the T-cell functional abnormalities in lupus disease may be secondary to the IL-2 deficiency. The results also indicate that T-cells in lupus mice and the SLE patient are highly responsive to exogenous IL-2 when the first signal is generated by Con A. These cells do not appear to be in an 'exhausted' unresponsive state as suggested previously by other investigators (Huang *et al.*, 1986; 1988). Majority of the cells from lupus mice are not in an activated state either as they do not respond to IL-2 alone. Spleen cells from one week old mice did not respond to either Con A or IL-2 alone. This could be explained by the lack of T-cells, especially T-helper cells at this early stage. However, it is not clear why T-cells from both lupus strains had an increased response to Con A plus IL-2 when compared to cells from BALB/c mice of same age. Presumably, these cells, which should have been eliminated during normal thymic selection, might have escaped the immune control process and were released to peripheral lymphoid organs.

Exogenous IL-2 is able to upregulate IFN-γ secretors of old lupus mice to produce normal levels of IFN-γ also suggesting the secondary nature of the abnormal IFN-γ production, but not a lack of precursors. However, addition of exogenous IL-2 in short-term cultures does not enhance the autoantibody secretion. Response to exogenous IL-2 during T-cell activation resulted in selective expansion of the mature

$CD8^+$ cell population but not the immature DN cells from MRL/*lpr* mice in cultures. It suggests that a normal T-cell activation which requires IL-2 may be important in limiting proliferation of the self-reactive DN cells which provide factors supporting production of autoantibodies (Datta, 1989) and are related to disease progression.

Before recombinant IL-2 was available, the only source of IL-2 was the conditioned medium derived from stimulated lymphocyte cultures (Morgan *et al.*, 1976). It contains not only IL-2 but also many other complex biological components including suppressive factors for IL-2. The measurable IL-2 in the culture supernatants is also a residual activity remaining after consumption by the stimulated cells. One of the possible explanations for the previous failure in restoring lupus T-cell activation might be that the level of exogenous IL-2 in the supernatant was inadequate to elicit a full response. The present study has shown that a minimum concentration of IL-2 of 5 to 10 ng/ml is required to restore T-cell responsiveness, which is two to three times more activity than is normally detected in cultures of Con A stimulated normal spleen cells. An IL-2 preparation from stimulated BALB/c spleen cells tested in the present study was used without dilution and this also showed effects on lupus T-cells. In addition, the IL-2 effect peaked at 3 days and it had only a marginal effect at 2 days (Fig.5.3a). However, in Altman's study, the IL-2 preparation was only tested at 1:10 dilution in the 'MLR' assay, or at a maximum of 1:2 dilution in the proliferation assay but measured at 48 hours. In addition, the present kinetic study has shown that Con A-induced IL-2 secretion by mouse spleen cells normally peaks at 24 hours and decline rapidly thereafter (Chapter 3, Fig.3.2a). The crude IL-2 used in Altman's study, which was derived from rat spleen cell cultures, was taken 48 hours after Con A stimulation. It might not necessarily contain peak IL-2 activity. Therefore, it is not surprising that there was no significant effect observed.

The significance of the finding that spleen cells from the SLE patient responded more strongly to IL-2 alone at high doses than to IL-2 plus Con A or PHA is not clear. It is likely that the majority of the potential IL-2 responders might have been B-cells which were hyperactive and bearing IL-2 receptors. When T-cells were activated in the presence of T-cell mitogen and exogenous IL-2, the significantly lower thymidine uptake, compared to cells cultured with IL-2 alone at the same dose, suggests the presence of suppressive factor(s) as a consequence of T-cell activation. In mice, in spite of the incomplete T-cell activation, culture supernatants derived from Con A-stimulated MRL/*lpr* spleen cells also showed a suppressive effect on the proliferative response of cells from BALB/c mice. In addition, the effect of IL-2 on recovery of IL-2R expression was poorer in old lupus mice which is possibly due to other defect(s) developed along with the disease activity. These support the hypothesis that some mechanism of feedback suppression might be operative during the development of lupus disease, reflecting attempts of the immune system to restore homeostasis (Kroemer and Martinez-A, 1991). As a consequence, it may also nonspecifically suppress IL-2 activity even further.

Chapter 6

Serological abnormalities in SLE

6.1 Introduction

There has already been a multitude of serological abnormalities found in both human SLE and the mouse models, with common features in many aspects as well as differences between species or strains. Among them, production of a variety of autoantibodies, and the presence of circulating immune complexes (ICs) with reduced levels of complement components are the main but complex manifestations which have been studied in detail for many years (Morrow and Isenberg, 1987; Theofilopoulos and Dixon, 1985; Buskila and Shoenfeld, 1992). Recently, nitric oxide and cytokines that induce nitric oxide synthesis have been suggested to play a role in autoimmune process. Elevated levels of reactive nitrogen intermediates (RNI) have been reported in sera of patients with rheumatic disease (Miesel and Zubler, 1993).

In this chapter, an unknown serum factor or factors, discovered in human sera and plasma and increased in SLE patients with active disease, is described. The factor(s) have adverse effects on the proliferation of IL-2 dependent CTLL cells, and does not appear to be either immunoglobulin (IgG) or an IC. Similar inhibitor(s), but with lower titre, were also found in some sera from MRL/*lpr* mice. In addition, serum levels of nitric oxide (NO) in BALB/c, CBA, NZB/W and MRL/*lpr* mice were analysed during the study.

6.2 SLE serum factors affecting the growth of IL-2 dependent CTLL cells

The initial aim of this study was to measure IL-2 activity in serum and plasma samples by the CTLL IL-2 bio-assay. In the CTLL assay, IL-2 activity is determined by its ability to induce CTLL cell proliferation (Chapter 2). The attempt to measure IL-2 in either sera or plasma by the bio-assay, however, repeatedly failed due to the presence of an inhibitor in the serum or plasma samples. No positive IL-2 activity was

detected in any of the untreated serum or plasma samples by CTLL cell growth. On the contrary, the cells incubated with serum or plasma samples showed even lower thymidine uptake compared to cells cultured in medium alone (negative controls). The inhibitory effect was concentration dependent and the unknown inhibitor was found at higher concentrations in SLE sera. To understand the role of this serum factor in the defective IL-2 activity in lupus disease, the purpose of the follow-up study was to investigate the nature of the serum factor that inhibits the growth of IL-2 dependent cells.

6.2.1 Concentration dependent effects on CTLL cell proliferation

To determine activities of the CTLL cell growth inhibitor in SLE, serial dilutions of the serum and plasma samples from SLE patients with active disease as well as normal donors were cultured with the indicator cells in the presence of a fixed amount of human r-IL-2. IL-2 was used at 100 pg/ml in the assay because the growth of CTLL cells at this dose is sensitive to changes of IL-2 activity according to IL-2 standard curves established in preliminary experiments. It normally gives about 40 to 80% of maximum thymidine incorporation, which is within the most linear part of the IL-2 standard curves (Chapter 2, Fig.2.2a). Surprisingly, the results revealed not only a significantly higher inhibitory titre but also a potential stimulatory activity in the sera and plasma from SLE patients at different dilutions compared with normal human sera and positive control samples without added serum.

Figure 6.1 shows the concentration-dependent but opposite effects of SLE sera on CTLL cell proliferative responses to IL-2: (i) at low dilutions -- inhibition; (ii) at high dilutions -- stimulation. The serum factor(s) inhibit the IL-2-induced CTLL cell growth at high concentrations. Although the inhibitor(s) could be found in the sera from both normal and SLE patients, it was significantly higher in the latter (up to 8 fold). The effect of inhibition disappeared at 1:32 to 1:64 for samples from normal

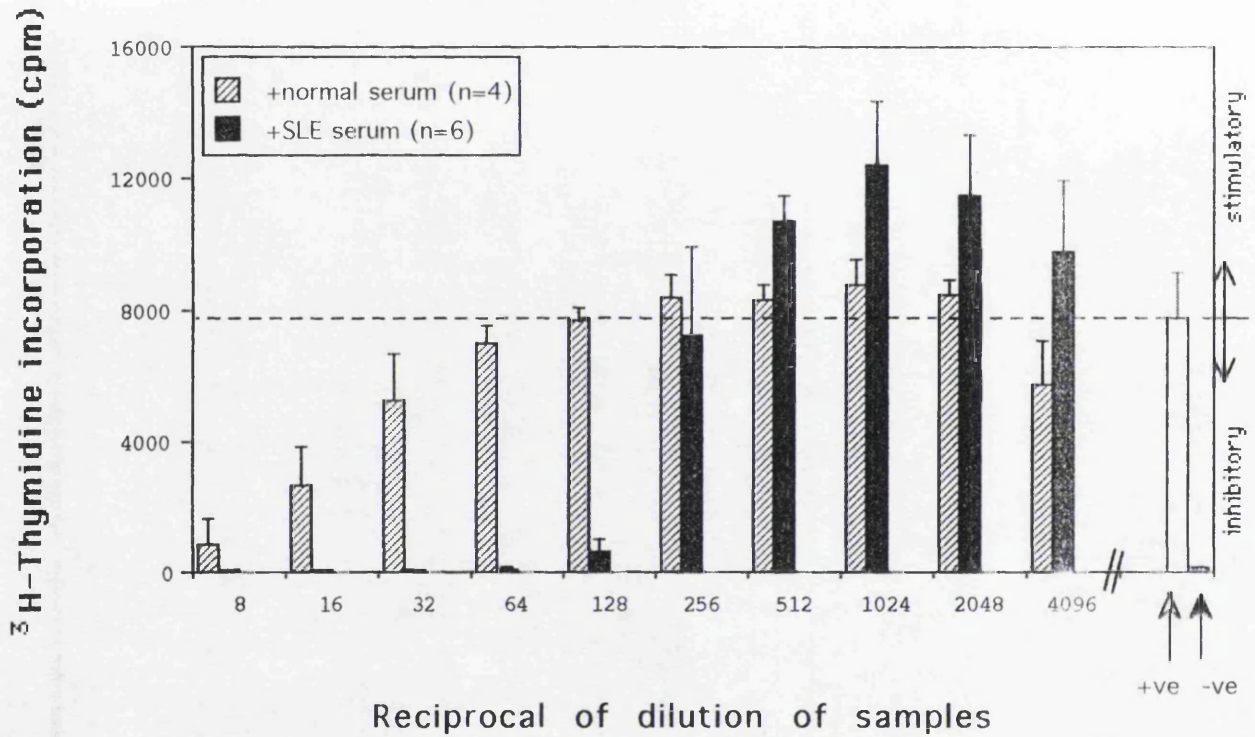


Figure 6.1 **Serum factor(s) affecting IL-2 dependent CTLL cell proliferation: a serological abnormality in SLE (human).** Serial dilutions of serum samples from healthy donors and SLE patients with active disease were incubated with CTLL cells at 37 ^{0}C/5% CO2. All assay cultures contained r-human IL-2 at 100pg/ml except negative controls (-ve, closed arrow). Proliferative responses of the cells were measured at 24 hrs including 3 hrs of pulsing with ^{3}H-thymidine (0.5 μCi/well). The horizontal line is set to define the stimulatory (upper) and the inhibitory (lower) effects of added sera on CTLL cells according to the positive controls (+ve, open arrow) which were cultures without test serum. Data shown are mean value (±SD) obtained from individual serum samples of normal donors (n=4) and SLE patients (n=6).

individuals, but at 1:256 or even higher dilutions for samples from SLE patients. Although the activity at high titre could occasionally be found in normal sera, group studies confirmed that the difference between the SLE patients and normal controls was highly significant (at 1:64, $P < 0.001$, Fig.6.2). In addition, what is a more interesting finding is some stimulatory activity of SLE sera at low concentrations on the CTLL cell growth. As shown in both Figures 6.1 and 6.2, serum samples from SLE patients, at dilutions between 1:512 to 1:4096, enhanced thymidine uptake by the IL-2-induced CTLL cells. The stimulatory effect was synergistic as the cells incubated with the samples but without IL-2 showed only growth suppression. In the samples from normal controls, this cell growth enhancer was not detectable, or only marginally so. The difference between the SLE sera and normal controls was also highly significant (at 1:1024, $P < 0.001$, Fig.6.2).

The CTLL cell growth modulators were also found in plasma samples and results were similar to that of sera. There was no correlation between the titres of the inhibitor and stimulator in each of the individual samples. Freezing and thawing were found to degrade their activity, particularly the stimulatory effect. In addition, a similar inhibitor with increased titres, but no stimulator, was also observed in the sera from some MRL/*lpr* mice. However, the titres were low and only 2 out of 4 MRL/*lpr* mice tested (6 months old) showed about 2-fold increased activity in comparison with BALB/c controls (Fig.6.3).

6.2.2 Functional characterisation of the SLE serum factors

The serum factors were partially characterised to study their nature and the mechanism of interaction by which they influence the IL-2 dependent growth of the CTLL cells. Serum samples from SLE patients which showed high levels of both the inhibitory and stimulatory activities, and from normal donors as controls, were selected for the characterisation. The results show that: (i) the inhibitory effect is not neutralised

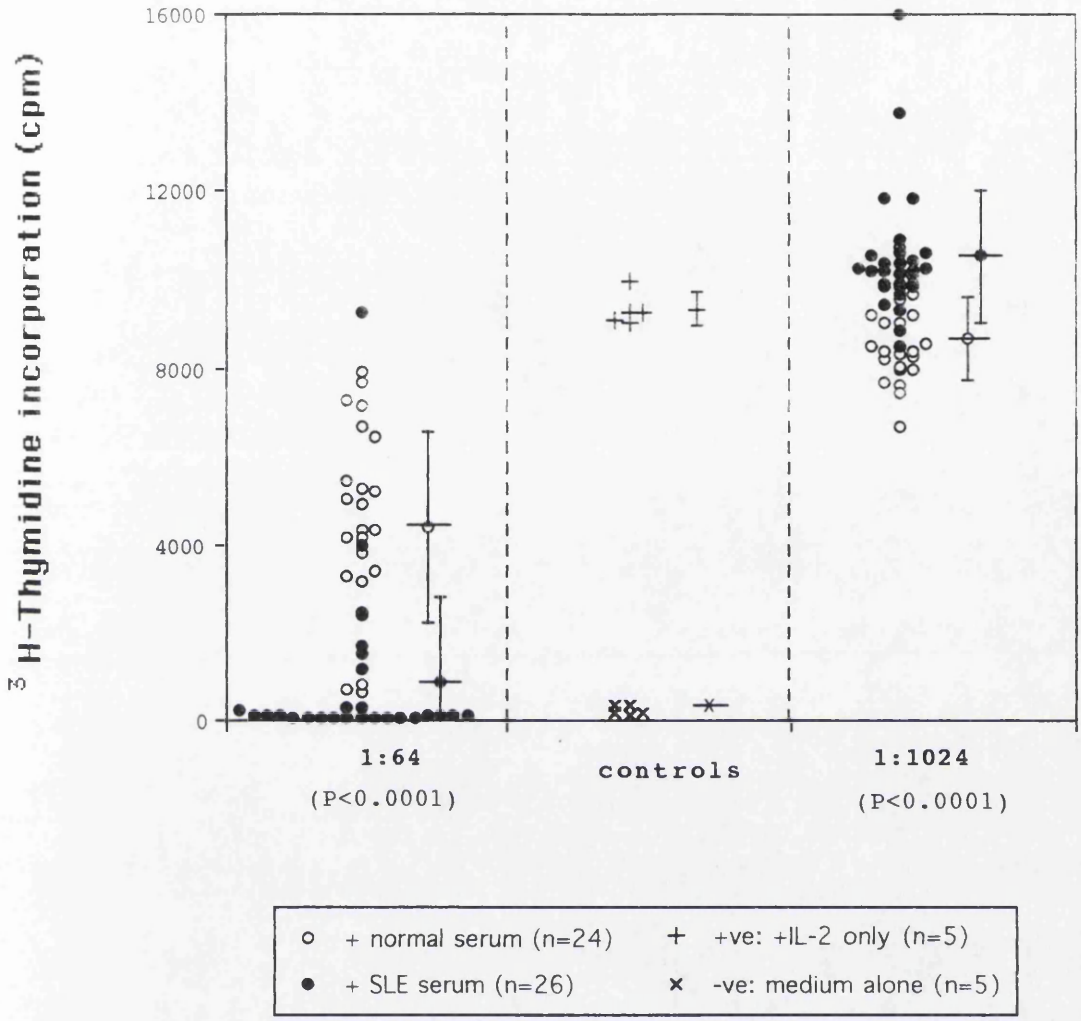


Figure 6.2 Statistical analysis of the concentration dependent inhibitory and stimulatory activities in sera from 26 SLE patients. This is a repeat experiment similar to that shown in Figure 6.2, but the samples, 24 normal or 26 SLE human sera, were tested at two dilutions of 1:64 and 1:1024. Each of the open or closed circles represents the mean cpm of duplicate cultures from one serum sample tested. The mean (±2SD) of all individual samples in each test group was also indicated in the graph. The difference between the SLE and control groups was highly significant (P<0.0001) as judged by the 'Mann Whitney Test'.

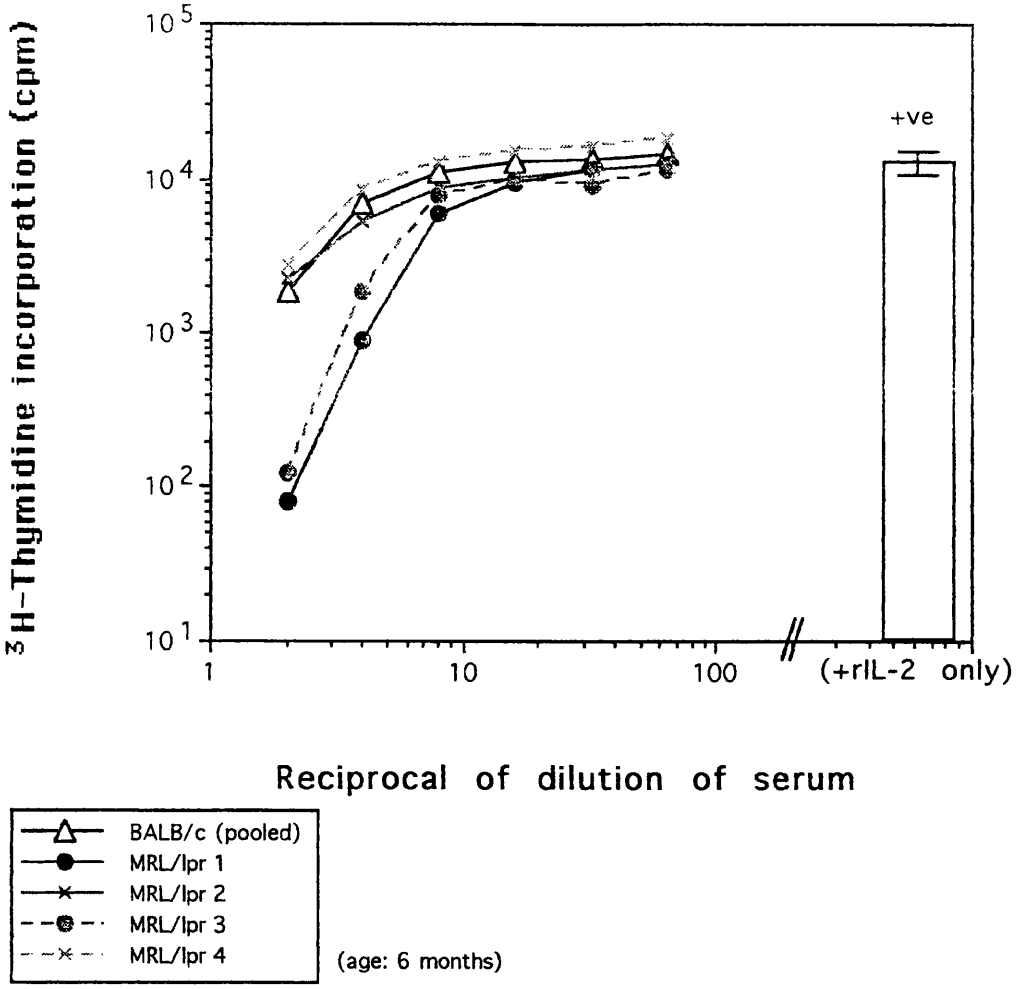


Figure 6.3 The CTLL cell growth inhibitor(s) in mouse sera. Serial dilutions of serum samples from four 6-month old MRL/*lpr* mice were tested in the assay as described in Figure 6.1 for human samples. Normal control was pooled sera from BALB/c mice of same age. Positive controls (+ve) were CTLL cells cultured with IL-2 alone at 100 pg per ml without test serum. Data shown are means of duplicated cultures.

by IL-2; (ii) the inhibitor binds to CTLL cells, (iii) is heat labile and (iv) does not bind to Protein A.

In the first experiment, CTLL cells were cultured with a fixed concentration (1:32) of plasma samples, and different doses of r-human IL-2. By increasing the dose of the IL-2 added, at up to 20 ng/ml, it did not neutralise the inhibitory effect of SLE plasma on the CTLL cell proliferation (Fig.6.4a). The normal control plasma did not have a significant inhibitory effect at a 1:32 dilution compared to the no serum controls, but it enhanced the cell proliferation when higher doses of IL-2 were added. In the second experiment, a serum from a SLE patient was tested at 1:50, 1:100 and 1:1000 (Fig.6.4b). IL-2 which was used at 0.8, 4, 20, 100 and 500 ng/ml had no effect on the inhibitory activity of human SLE serum on the growth of CTLL cells at any of the serum dilutions.

However, pre-absorption of the serum samples with viable CTLL cells removes the inhibitory effect. The degree of absorption was cell concentration-dependent (Fig. 6.5). The number of cells required to absorb the serum factor varied from sample to sample, which reflects different levels of activities in the sera. Incubation of 4×10^7 CTLL cells in 1 ml of neat SLE serum for 30 minutes at 37 °C eliminated the inhibitory activity fully in most samples tested. As indicated in Figure 6.5, the inhibitory effect could also be eliminated by pre-heating the samples prior to cell culture. In all SLE serum samples tested, the inhibitor was completely inactivated after 40 minutes incubation at 60 °C. All pre-absorbed and pre-heated samples were tested at the low dilutions only to determine effects of cell absorption and heating on the serum inhibitory activity. The results shown in Figure 6.5 were combined data from two similar experiments in which samples were used at 1:50, but sample dilutions at 1:25 and 1:32 were also tested with consistent agreement. No attempt has been made to assess the effects of these pretreatments on the stimulatory activity in the samples, which is present at high dilutions of the untreated SLE sera (Figs.6.1 and 6.2).

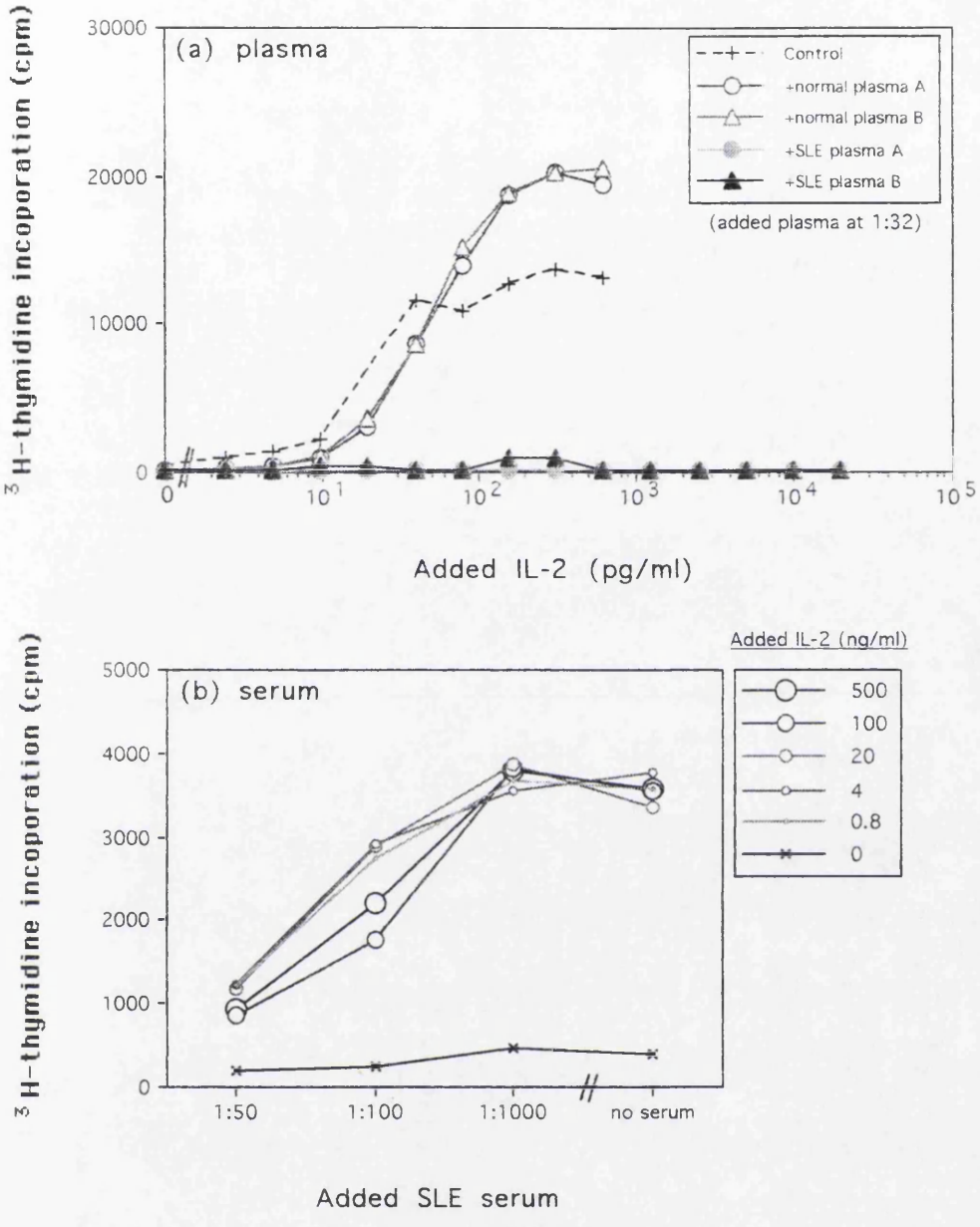


Figure 6.4 Effects of IL-2 on CTLL cell growth inhibitor(s) in plasma and sera from SLE patients and normal donors: (a) two plasma samples from both SLE patients and normal donors were tested at 1:32; and (b) a SLE serum sample was tested at 1:50, 1:100, 1:1000 and no serum. These cultures were supplemented with different doses of r-human IL-2 as indicated. Other assay procedure were as described in Figure 6.1.

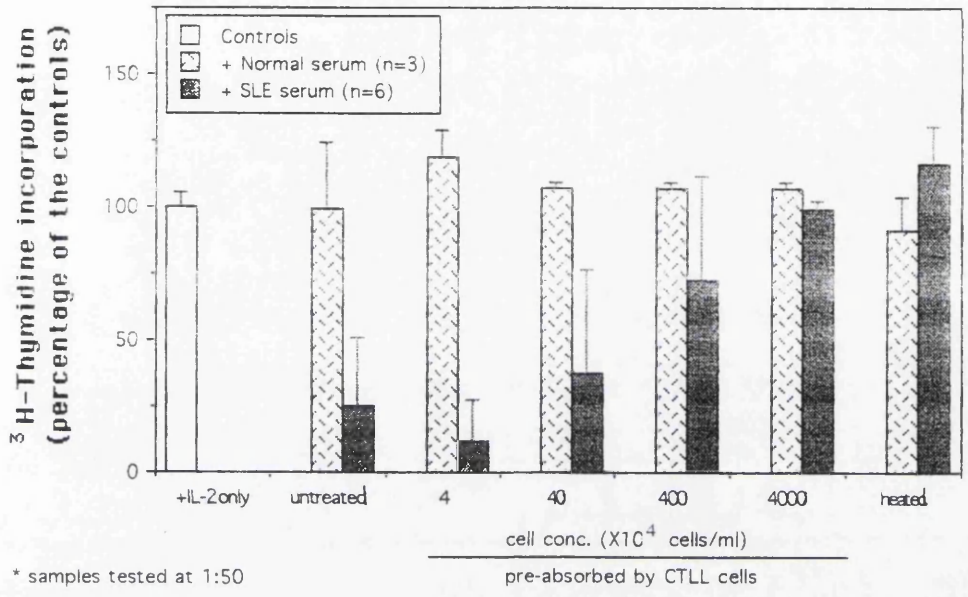


Figure 6.5 Characterisation of the unknown serum factor(s) by pre-absorption and heat-inactivation. Serum samples from 6 SLE patients and 3 normal donors were: (i) pre-absorbed by incubation with viable CTLL cells at different concentrations of cells for 30 mins at 37 ^{0}C/5% CO2; or (ii) pre-heated at 60 ^{0}C for 30 mins. The treated and untreated samples were then tested at 1:50 dilution for their inhibitory activities on the CTLL cells. Data shown are means (±SD) of the individual samples expressed as percentage of the positive controls (cells cultured with IL-2 alone at 100 pg/ml).

To identify the unknown serum factor(s), Protein A-Sepharose which binds specifically to human IgG, was used to separate serum IgG molecules from other components. Two human sera (0.4 ml each), one SLE and one normal control, were fractionated by the Protein A column (IgG binding capacity: 35 mg). The SLE serum was from a patient with active disease and has been tested in the previous experiment showing both the inhibitory and stimulatory activities. Details of the fractionation procedure have been described in Section 2.2.3ii, Chapter 2.

Figure 6.6 shows the serum protein levels in the sample fractions separated by the Protein A column. For each serum sample, the first peak represents the amount of unbound proteins and the second peak records the amount of IgG eluted from the Protein A column by glycine-HCl (pH 2.8). For each peak, adjacent fractions giving an OD of 0.2 or higher at 280 nm diluted at 1:20 in PBS, were combined. The total protein recovered in the first and second peaks from the SLE serum was 24.5 mg, about 59.2% of the original amount of protein (41.4 mg in 0.4 ml serum). The rest of the sample fractions containing low or no proteins were therefore discarded.

After dialysis in PBS buffer at 4 ^{0}C, extensive precipitation appeared in the sample of the second peak from the SLE serum. The precipitated proteins were then removed by centrifugation. The soluble form of IgG remaining in the supernatant was about 52.8% of the total IgG. The actual amounts of soluble proteins recovered in each sample for the first and second peaks respectively were, 18.65 and 2.65 mg for the SLE serum and, 18.19 and 3.70 mg for the control serum. All samples were adjusted to a final volume of 0.7 ml (dilution factor: 1.75). In addition, samples of unseparated sera (0.5 ml each) were also dialysed in PBS at 4 ^{0}C for 3 days, then sterile-filtered, the volume adjusted to 0.7 ml (dilution factor: 1.4). These samples were stored at -20 ^{0}C before assaying.

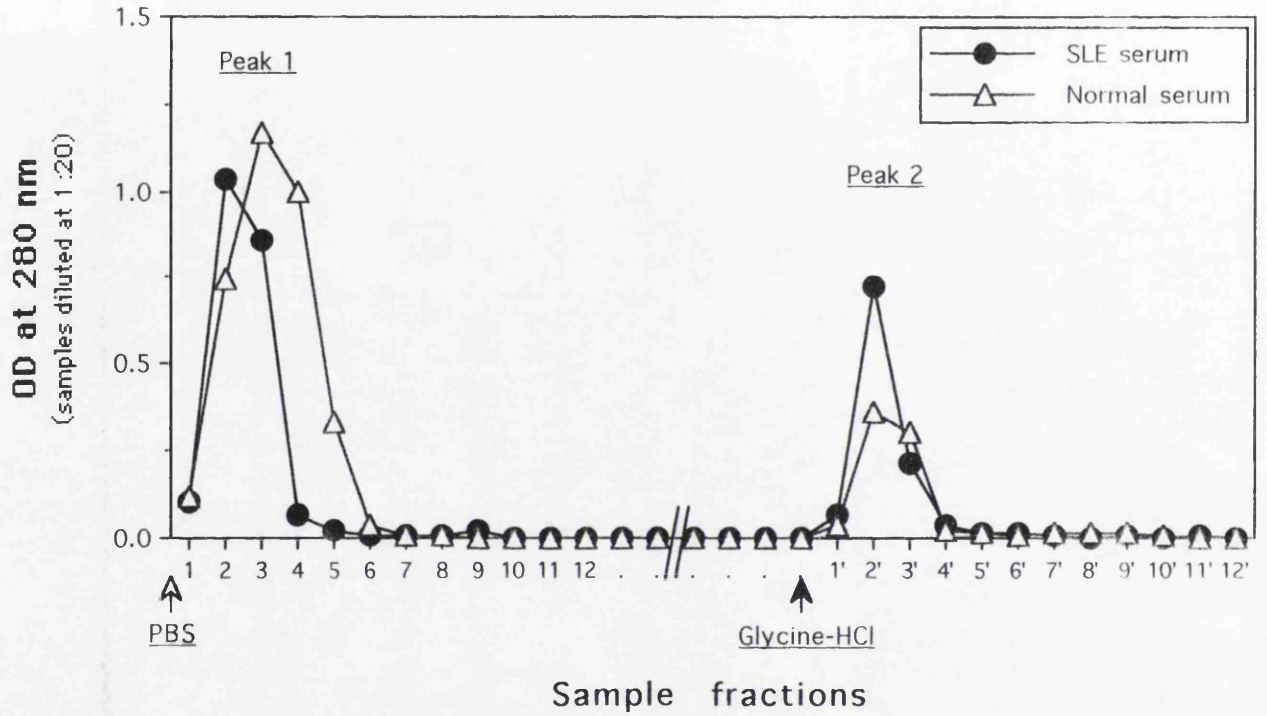


Figure 6.6 **Serum fractionation by Protein A column.** One serum sample from a SLE patient which contained high levels of both inhibitory and stimulatory activities on CTLL cell growth and another serum sample from a normal donor were subjected to Protein A fractionation to isolate IgG antibodies from other serum proteins (see Methods and Materials). Peak 1 contains the unbound proteins, and Peak 2 represents IgG antibodies eluted from the column with glycine-HCl (pH 2.8).

Figure 6.7a shows the activities of the SLE serum factor(s) in samples before and after Protein A separation. In this assay, the untreated serum did not show any stimulatory activity presumably due to freezing and thawing of the sample, but it still had a high level of inhibitory activity. The inhibitory activity was also detected in the dialysed serum with only a slight reduction after the extensive dialysis in PBS buffer. In the Protein A fractionated samples, the immunoglobulin fraction (peak 2) did not have any significant effect on the growth of CTLL cells. However, the activity existed in the first peak, although it was much lower when compared to the untreated or dialysed serum. In the normal control serum, inhibitory activity was evident in the untreated sample, but it was not detected in the Protein A fractionated samples from both peaks. On the contrary, low stimulatory activity was observed in the second peak (Fig.6.7b).

To determine the possible role of anti-T-cell antibodies in the inhibition of the T-cell growth, normal rat serum was used as a source of complement. CTLL cells were cultured with samples from the second peaks (IgG) supplemented with 10% rat serum. However, there was no inhibitory but a strong stimulatory effect for both SLE and control samples. Since the same effect was also observed in cells cultured with rat serum alone (Fig.6.7a,b), the stimulation was considered to be due entirely to component(s) from the rat serum itself. In Figure 6.7, all data presented referred to adjusted dilutions taking into account the differences between original serum volume and the final adjusted volume after the various pre-treatments described.

6.3 Serum nitric oxide levels in NZB/W, MRL/*lpr*, BALB/c and CBA mice

To study serological abnormalities in SLE, plasma levels of nitric oxide in lupus and normal mice were measured. Eighty-one sera from mice of two lupus strains, MRL/*lpr* (24) and NZB/W (24), and two normal strains, BALB/c (27) and CBA (6), at

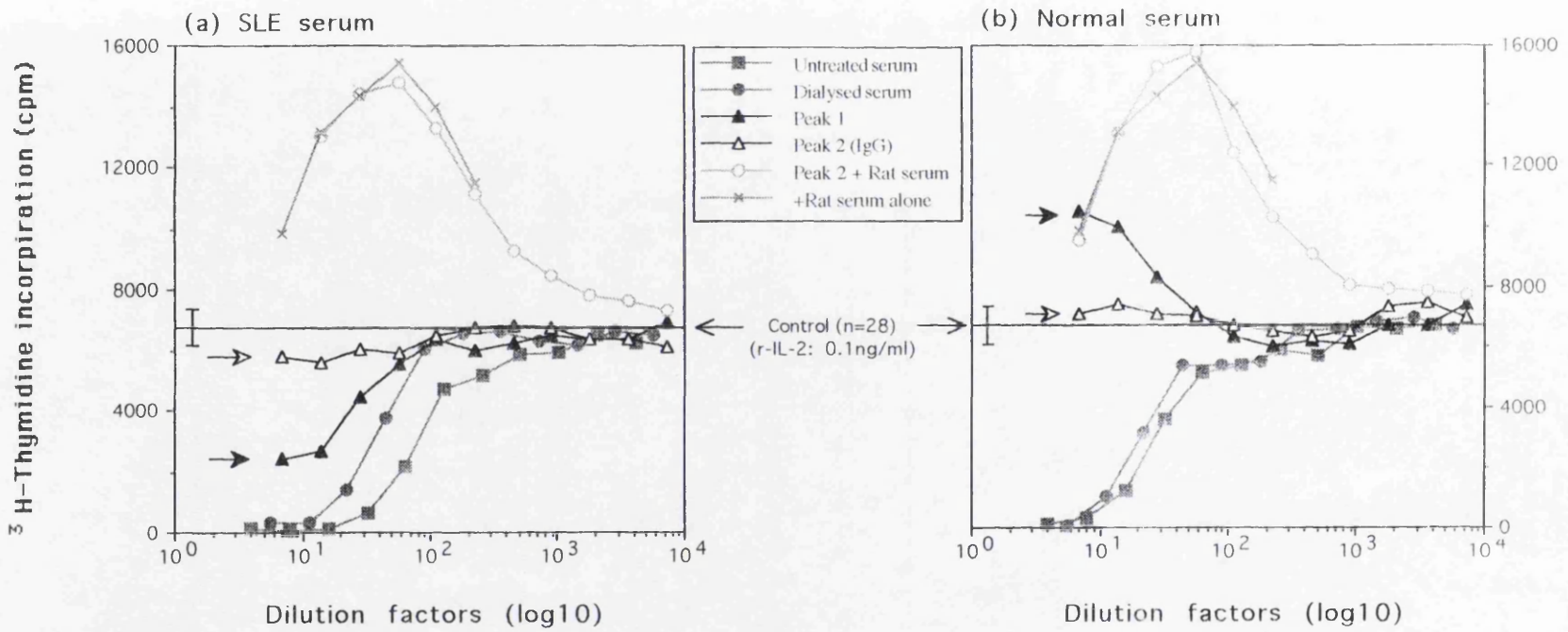


Figure 6.7 Assessment of the serum factor activity after Protein A fractionation and dialysis. The Protein A-fractionated serum samples (see Figure 6.6) were assayed for their activities on the growth of CTLL cells, in comparison with the untreated and PBS-dialysed samples. Normal rat serum (10%) was added to the IgG fractions (Peak 2 + Rat serum) to reconstitute complement components. The dilutions of all samples were adjusted to their original volume of the untreated sera. The horizontal line is the mean incorporation of ^{3}H-thymidine by CTLL cells cultured in medium containing IL-2 (10 ng/ml) alone. Data shown are means of triplicate cultures.

different ages were tested. The sera had been collected periodically and stored at -20 ^{0}C before assaying.

The serum NO level was significantly increased in MRL/*lpr* mice compared to both BALB/c and CBA control mice, in every age group (Fig.6.8). In the control BALB/c and CBA mice, the serum NO levels seem to correlate with the age of the mice, peaking at between 4 to 6 months. However, the increased serum NO levels in MRL/*lpr* mice did not seem to have a clear correlation with age although the average activity was slightly higher in the 1-2, and 4-6 months age groups. In contrast, NZB/W mice showed overall reduced serum NO levels. The reduction was consistent in most of the age groups except the 1-2 month old and the 10-12 month old age groups. In addition, CBA mice of the 2-4 month old group also had low serum NO activity.

6.4 Discussion

A major finding from these experiments is a serological abnormality in SLE, *viz.* a soluble serum component, or components, which affect IL-2 activity on CTLL cell proliferation. It is already known that rodent serum contains soluble factors that inhibit IL-2 activity (Hardt *et al.*, 1981; Male *et al.*, 1985). Of interest is the observations that sera and plasma from SLE patients with active disease contain significantly higher levels of soluble factors that exhibit not only inhibitory but also stimulatory effects on the same target cells depending on sample dilutions.

Djeu and her colleagues (1986) have also described previously a 60,000 - 70,000 molecular weight IL-2 inhibitor in human sera, which is also heat-labile and inhibits IL-2-induced CTLL growth. In contrast to the present finding, they found that such IL-2 inhibitor was reduced in patients with SLE and other autoimmune diseases. This seems difficult to be interpreted. However, Djeu's conclusion drawn from the comparison of

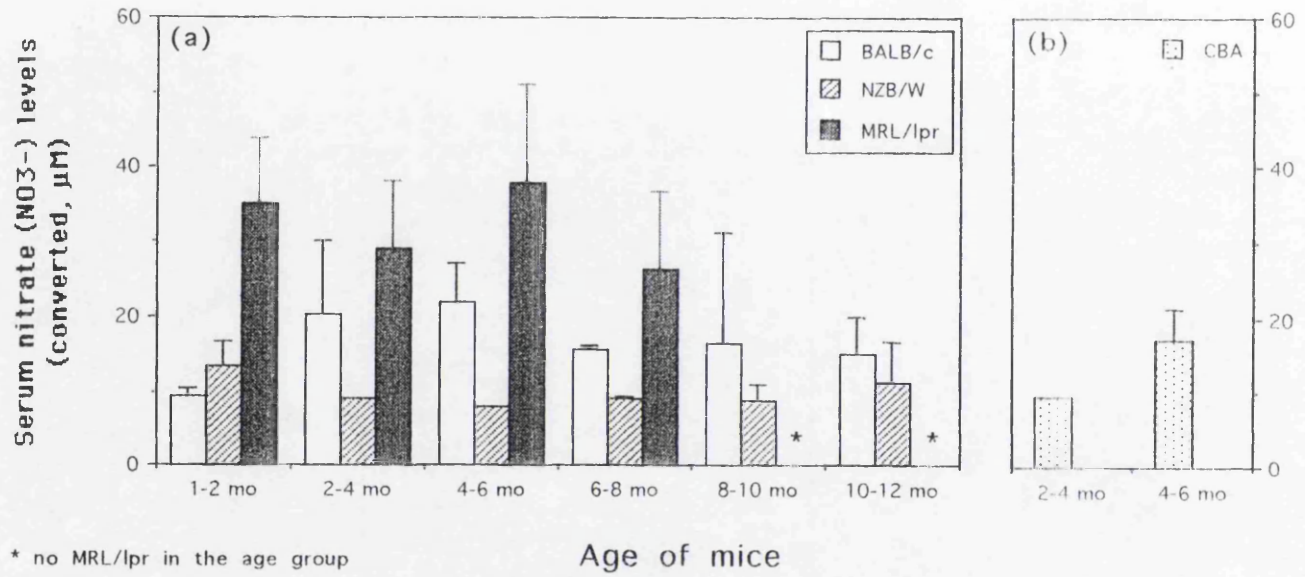


Figure 6.8 **Serum NO levels in normal and lupus mice.** The results show nitric oxide levels, tested after conversion from NO_3^- to NO_2^-, in sera from 24 MRL/*lpr*, 24 NZB/W, 27 BALB/c (a) and 6 CBA (b) female mice of different ages. Data are shown as means (±SD) of individual sera in each of the age groups. At least 3 mice were tested in each group. (*Not tested).

the inhibitory activities between normal and SLE sera were based on a single dilution of serum samples at 1:10, and expressed only as percentage of inhibition. The present study were based on assays of a wide range of sample dilutions. The results showed that, in cultures with either normal or SLE serum at such a high concentration (1:10), thymidine uptake by CTLL cells was extremely low (Fig.6.1). More importantly, the present study used pure recombinant IL-2 at 100 pg/ml (about 2 U/ml) which is a concentration most sensitive to changes of the IL-2 activity in the culture, because it is within the most linear part of response curve (see Fig.2.2a). The IL-2 used in Djeu's study was either culture supernatants from the EL4 cell line or Con A/PMA-stimulated human mononuclear cells, which was used at 100 U/ml. First of all, changes of the cell responses to such high dose of IL-2 might be very different. In addition, those supernatants Djeu used as a source of IL-2 might contain other factors such as mitogens complicating the results. It is worth pointing out that IL-2 at wide range up to 500 ng (about 1000 U) per ml was also tested in the present study and showed consistent results (Fig.6.4).

An immediate consideration of the nature of these factors is the possible influence of drugs. The human SLE serum or plasma samples were taken from patients, most of whom had been receiving or had received treatment before the samples were taken. The routinely used immunosuppressive drugs like cyclosporin A or Corticosteroids might have complicated the events. However, the fact that extensive dialysis of the serum samples failed to remove the inhibitory activity does not support such a simple explanation. In addition, although some plasma samples were collected in the presence of EDTA which could have some effect on the CTLL cells, serum samples, especially mouse sera free of such contaminant, also displayed these activities.

Secondly, it is necessary to consider whether these activities were due to soluble serum IL-2 receptors. It has been widely reported that sera from patients with SLE (Wolf and Brelsford, 1988; Tokano *et al.*, 1989) and rheumatoid arthritis (Campen *et*

al., 1988; Wood *et al.*, 1988; Keystone *et al.*, 1988) have increased levels of circulating soluble IL-2 receptors that correlate with disease activity. These soluble receptors may bind to IL-2 preventing its functions. However, the cell absorption experiments showed that these serum factors bound directly to CTLL cells. In particular, since exogenous IL-2 (up to 500 ng/ml) did not neutralise activity of the serum factors (at 1:32 and 1:50), the possibility of the inhibitory effects on CTLL cells being due to soluble IL-2 receptors present in the test sera or plasma may be excluded.

The over-dose toxic effect of IL-2 on CTLL cell proliferation has been described previously in Chapter 2. Therefore, the inhibitory effect of the serum samples on CTLL cells might be explained by a high or increased serum level of IL-2, which became stimulatory when being highly diluted. However, this does not appear to be a good explanation either, because addition of serum alone at any dilution did not stimulate the growth of CTLL cells. Since the stimulatory activity of the SLE serum samples at high dilutions can only be observed when IL-2 is present, the effect is clearly synergistic. Despite this, it is still possible that the two concentration dependent opposite effects are due to one factor which acts as an IL-2 enhancer at optimal doses but becomes inhibitory when the concentration exceeds a certain level. Therefore, in *in vivo* situations, the factor may serve as a T-cell stimulator during the early development of the disease and may subsequently become inhibitory as its serum concentration rises. However, in some SLE and normal sera which contained significant levels of inhibitor, while serially diluting the samples diminished the inhibitory activity, there was no observable stimulatory effect. The fact that the inhibitory activity did not correlate with the stimulatory activity in each of the individual samples suggests that these effects were more likely to be due to different factors.

Another possibility is anti-T-cell antibodies or immune complexes. It has been reported that in the sera from as many as 80% of SLE patients and many family members, lymphocytotoxic antibodies (LCA) were detectable (De Horatius and

Messner, 1975; Tanaka *et al.*, 1989; reviewed by Winfield and Mimura, 1992). The cell absorption experiments showed binding of the serum factors to CTLL cells, but the Protein A isolated SLE IgG fraction contained no inhibitory activity. Since the factor did not bind to Protein A and the inhibitory activity presented in the first peak, it is also unlikely to be ICs. Supplementing the IgG fraction with rat serum as a source of complement did not show any inhibitory effect either, except for a stimulatory effect which was due entirely to the rat serum itself. However, as most of the T-cell antibodies found in SLE are of the IgM class (Morrow and Isenberg, 1987), it does not therefore exclude the possibility that such T-cell antibodies might be IgM which does not bind to Protein A. It is not clear why the Protein A fractionated normal serum had lost its inhibitory activity, and rather became stimulatory in the first peak in which IgG was removed. The mechanism for these different effects appears to be rather complex.

The pathological significance of these serum factors is still to be investigated. The fact that these serum factors were found not only in SLE patients but also in the lupus mouse model MRL/*lpr* mice might suggest some relevance to lupus disease. However, the activity was low and not detectable in some of the mice. It will be necessary to characterise the mouse serum factor before any conclusion can be drawn. The significance of the fact that serum NO levels are increased in MRL/*lpr* but decreased in NZB/W mice is also not clear. Since the changes are not related to age of mice and disease activities, the pathological importance is unknown. NZB serum factor (NZB-SF) is a 60-kDa glycoprotein initially isolated from sera of young NZB mice treated with *Corynebacterium parvum.* It was demonstrated that the factor enhanced maturation and proliferation of sIg^- pre-B cells in bone marrow, but was distinguishable from many known cytokines. Since a similar factor could also be found at very low levels in the sera from mice of several normal strains treated in the same way, its physiological and pathological importance has been suggested (Jyonouchi *et al.*, 1991). In another study by Tomura et al. (1989), a factor derived from the cell culture supernatants of B cells from SLE and RA patients has been shown

to enhance CTLL cell proliferation, and has therefore been named "B cell derived-growth enhancing factor-2 (BGEF-2)". It is not clear whether the serum factor found in the present study which also stimulated CTLL cell growth could be the same factor found by Tomura in the B-cell culture supernatants. However, after excluding many possibilities mentioned above, these biologically potent, heat-unstable, T-cell binding serum factors are likely to be protein effectors or cytokines (possibly still unknown), which act as antagonists or agonists regulating IL-2 action on T-cells. Further characterisation of these factors is therefore recommended.

Chapter 7

Delivery of murine TGF-β1 by *Salmonella typhimurium* *aro*$^-$ mutant in MRL/*lpr* and BALB/c mice

7.1 Introduction

7.1.1 TGF-β: its multiple, diverse immune regulatory functions

Transforming growth factor-β (TGF-β) is a group of structurally related polypeptides belonging to the 'TGF superfamily', which are believed to participate in the regulation of cell proliferation and differentiation during development and tissue repair (Sporn and Roberts, 1989). TGF-β was initially found to be able to induce phenotypic transformation of mammalian cells in culture (Roberts *et al.*, 1983). It has since been shown to have diverse effects on a variety of cells. Its activity, either stimulatory or inhibitory, depends on the cell type, the state of differentiation and the culture conditions. The expression of this 25-kD homodimeric peptide can be detected in many types of cells and tissues including activated T and B lymphocytes (Kehrl *et al.*, 1986a, b) with relatively high levels found in platelets; and it is particularly prominent in embryonic tissues. Three TGF-β isoforms, TGF-β1, TGF-β2 and TGF-β3, have now been identified in mammals (Derynck *et al.*, 1988).

Many studies have demonstrated that TGF-β is an important immunoregulator critically involved in the regulation of the immune and inflammatory systems. Functioning generally as an immunosuppressive agent, TGF-β has been shown to suppress B-cell proliferation, and immuglobulin (IgG and IgM) formation and secretion (Kehrl *et al.*, 1986, 1991; Straub and Zubler, 1989; Shalaby and Ammann, 1988; Quere and Thorbecke, 1990). It also inhibits T-cell function and cytokine production (Kehrl *et al.*, 1986b; Ristow, 1986; Espevik *et al.*, 1987; Chantry *et al.*, 1989). However, the multipotential, diverse features of TGF-β activities are also evident for its roles in immune regulation. Results from *in vitro* studies suggest that the inhibition of T-cell function is not absolute but selective. For example, it has been shown that TGF-β suppresses the development of cells with a Th2-like phenotype but, on the other hand, promotes the development of IL-2 and IFN-γ secreting cells (Swain, 1991a, b).

The author suggested, therefore, that TGF-β may act on resting CD4 cells, and direct them to commit or to differentiate to a particular subset or pattern of lymphokine expression. In another study, Karpus and Swanborg (1991) identified a 'recovery associated' $CD4^+$ T suppressor cell that inhibits experimental allergic encephalomyelitis (EAE) *in vivo* and can abrogate IFN-γ but not IL-2 production in target effector cells *in vitro*. The mechanism of action of this $CD4^+$ suppressor was also found to involve TGF-β. The importance of TGF-β1 as an immunoregulatory cytokine has been further confirmed by a study of TGF-β1 gene disruption in murine embryonic stem cells (Shull *et al.*, 1992). Mouse chimaeras carrying the disrupted TGF-β1 gene were shown to develop a fast, severe and multifocal inflammatory disease leading to organ failure and death.

The role of TGF-β in autoimmunity is however controversial. By using an animal model of acute mesangial proliferative glomerulonephritis, Border et al. (1990) showed that the induction of glomerulonephritis in the rat by antithymocyte serum is associated with increased production and activity of TGF-β1. Administration of anti-TGF-β1 at the time of induction attenuated the renal pathological changes. In contrast, both studies by Kuruvilla et al. (1991) and by Thorbecke et al. (1992) showed protective effects of TGF-β1 on collagen type II-induced arthritis (CIA) in DBA/1 mice which is another well established experimental autoimmune animal model. A recent study of gene therapy in MRL/lpr mice has suggested that direct intramuscular injection of expression virus vectors encoding TGF-β1 results in decreased proteinuria and deceased levels of serum anti-chromatin antibodies (Raz et al., 1993). The mechanisms for the development of these diseases may be different, but all these studies suggest the possible involvement of TGF-β1 in the pathology of the diseases.

7.1.2 *S. typhimurium aro*⁻ mutant as a vector for antigen and therapeutic protein delivery

Aromatic amino acid dependent *S. typhimurium* mutants are non-virulent live vaccines against salmonellosis. They were first developed by Hoiseth and Stocker (1981) by introducing defined mutations into genes of virulent *Salmonella* strains. These genes are known to be involved in aromatic biosynthesis essential for the organism to establish a clinically significant infection. As a result of the gene insertion, the mutants become auxotrophic for two compounds, *p*-aminobenzoic acid (*p*AB) and 2,3-dihydroxybenzoate (DHB), which are not available in vertebrate tissues. For this reason, their ability to grow in such tissue is limited.

For many years now, *S. typhimurium* mutants have also been widely used as carriers for delivery of many heterologous protein antigens. It has been proven clinically and experimentally that they are a good transport model system for antigens like β-galactosidase (Brown *et al.*, 1987), heat labile enterotoxin of Escherichia coli (Maskell *et al.*, 1987), M protein of streptococcus pyogenes (Poirier *et al.*, 1988), the circumsporozoite antigen of plasmodium (Sadoff *et al.*, 1988; Aggarwal *et al.*, 1990), influenza A virus nucleoprotein (Tite *et al.*, 1990), tetanus toxin C fragment (Fairweather *et al.*, 1990), gp63 of Leishmania major (Yang *et al.*, 1990), and the P69 antigen of Bordetella pertussis (Strugnell *et al.*, 1992).

More recently, for the first time, the possibility of using this system for transfer of therapeutic recombinant proteins *in vivo* has been explored with success (Carrier *et al.*, 1992). It was shown that recombinant human IL-1β delivered by the *Salmonella* vector in BALB/c mice was adequately expressed *in vivo*, functionally active and well recognised by the immune system. The transfected bacterial vaccine may be administered by both injection and oral routes of inoculation. Although it is at present unclear how cytokine is delivered to the appropriate tissues, it was suggested that the

cytoplasmically expressed cytokine might be liberated as a result of cell lysis. The release of the effector proteins by the vector is a relatively gradual process. Since most cytokines have very short half-lives and low physiological doses, this approach might have advantages over the direct injection of cytokines for reducing side effects and dose control.

7.1.3 Purposes of the study

In view of the immune suppressive effects of TGF-β, as well as its selective regulatory activities on the T-helper cell functions, the main intention of the study described in this chapter was to investigate the role of TGF-β1 in the development of lupus disease. This was a pilot study of cytokine intervention *in vivo* in a lupus mouse model by the new approach of using *Salmonella* mutant as the cytokine carrier. Groups of small number of MRL/*lpr* mice were employed. Groups of BALB/c mice were also included as controls in the treatment scheme.

7.2 Materials, mouse groups and treatment scheme

7.2.1 TGF-β1 gene expression in the transfected *S. typhimurium*

The expression plasmid pKK-TGF-β1 gene transfected *S.typhimurium* BRD509 was constructed and kindly provided by Dr D-M Xu in the department (see Materials and Methods). Before being used for treating mice, the TGF-β1 gene transfected *Salmonella* was assessed to determine the cytoplasmic expression of the cytokine. Figure 7.1 shows the TGF-β activity detected in the cytoplasmic extracts of the TGF-β1 gene transfected *Salmonella* by the TGF-β bio-assay. The average TGF-β activity generated from one million recombinant bacteria was about 100 - 180 pg.

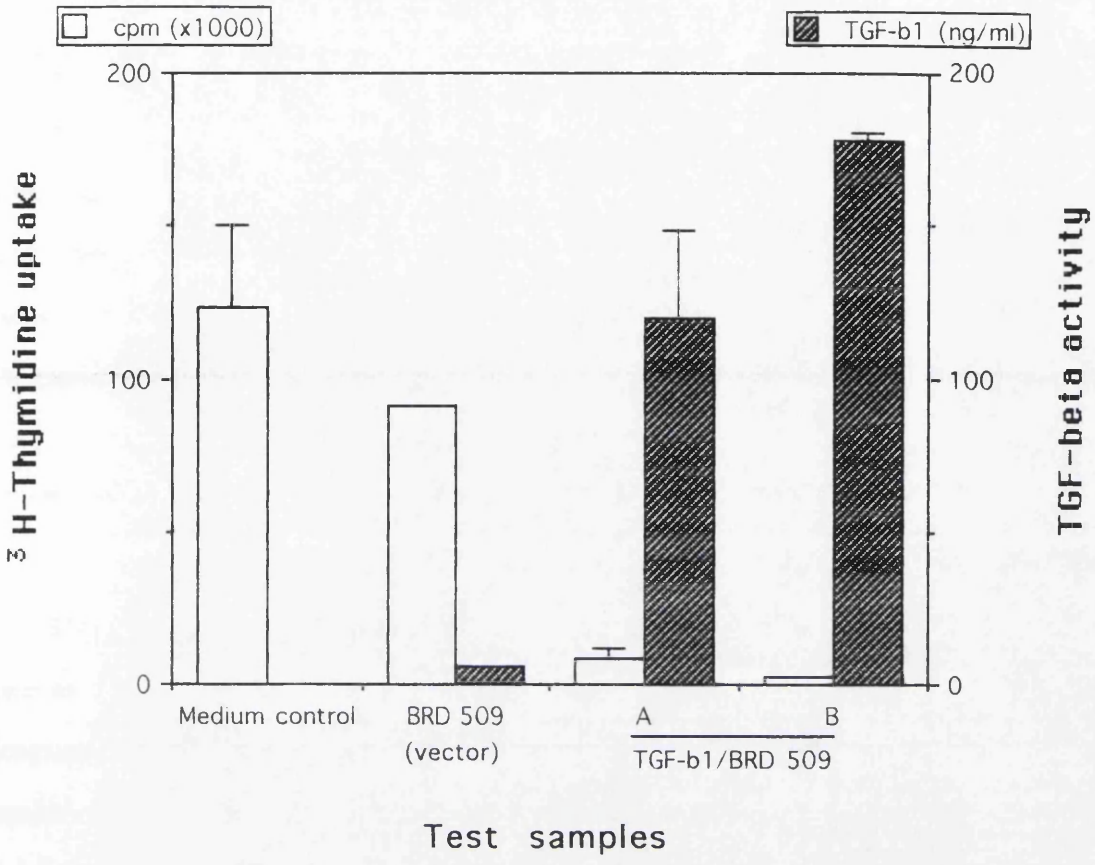


Figure 7.1 Cytoplasmic expression of TGF-β in transfected *S.typhimurium*. TGF-β activity in the cytoplasmic extract from the TGF-β1 gene transfected *S.typhimurium* (TGF-β1/BRD509, duplicate samples A and B) and the wild type control bacteria (BRD509) was measured by the TGF-β bio-assay (MvILu, Section 2.4.1). Data show the suppression of ^{3}H-thymidine incorporation by mink lung cells (open bars) by TGF-β in the test samples compared to the cells cultured in medium alone, as well as the equivalent TGF-β activity (hatched bars) estimated from the standard curve using r-TGF-β1.

The level of *in vivo* TGF-β1 expression by the bacterial carriers in mice depends on both growth and killing of the bacteria in the host and not simply the initial dose given. No attempt was made to determine the optimum dose of the vaccine in this pilot study. With reference to the dose used in a previous study (Carrier *et al.*, 1992), however, a single dose was used at about 10^{10} bacteria per mouse. The concentration of bacteria was determined by optical density using a spectrophotometer at 600 nm.

7.2.2 Animals and treatment scheme

The experiment employed MRL/*lpr* mice as the lupus mouse model, and BALB/c mice as the control strain. The mice were bred in the Animal House, Gartnavel Hospital, Glasgow. All mice used were female and the treatment started at the age of 9 weeks. This was a small scale pilot experiment. Fifteen mice of each strain were divided equally into three treatment groups (Groups I, II and III). Group III was treated with TGF-β1 gene transfected *Salmonella.* The two control groups were mice treated with the bacterial vector alone (Group II), or untreated (Group I) (Fig.7.2).

The mice were treated by oral administration of the live bacteria. The first treatment was given to the mice at 9 weeks old. After the first boost at 12 weeks, the mice in each treatment group were sub-divided into 2 groups: one sacrificed at 15 weeks of age after the two doses, and the other sacrificed at 20 weeks of age, 4 weeks after the second boost. A summary of the treatment scheme is shown in Figure 7.2.

7.3 Effects of TGF-β1 treatment on normal and lupus mice

During the treatment period, one MRL/*lpr* mouse in group II (vector alone treated) died one day after the primary immunisation, and one MRL/*lpr* mouse in Group III (IL-2/vector treated) died overnight after the first boost. The accidental mouse death was

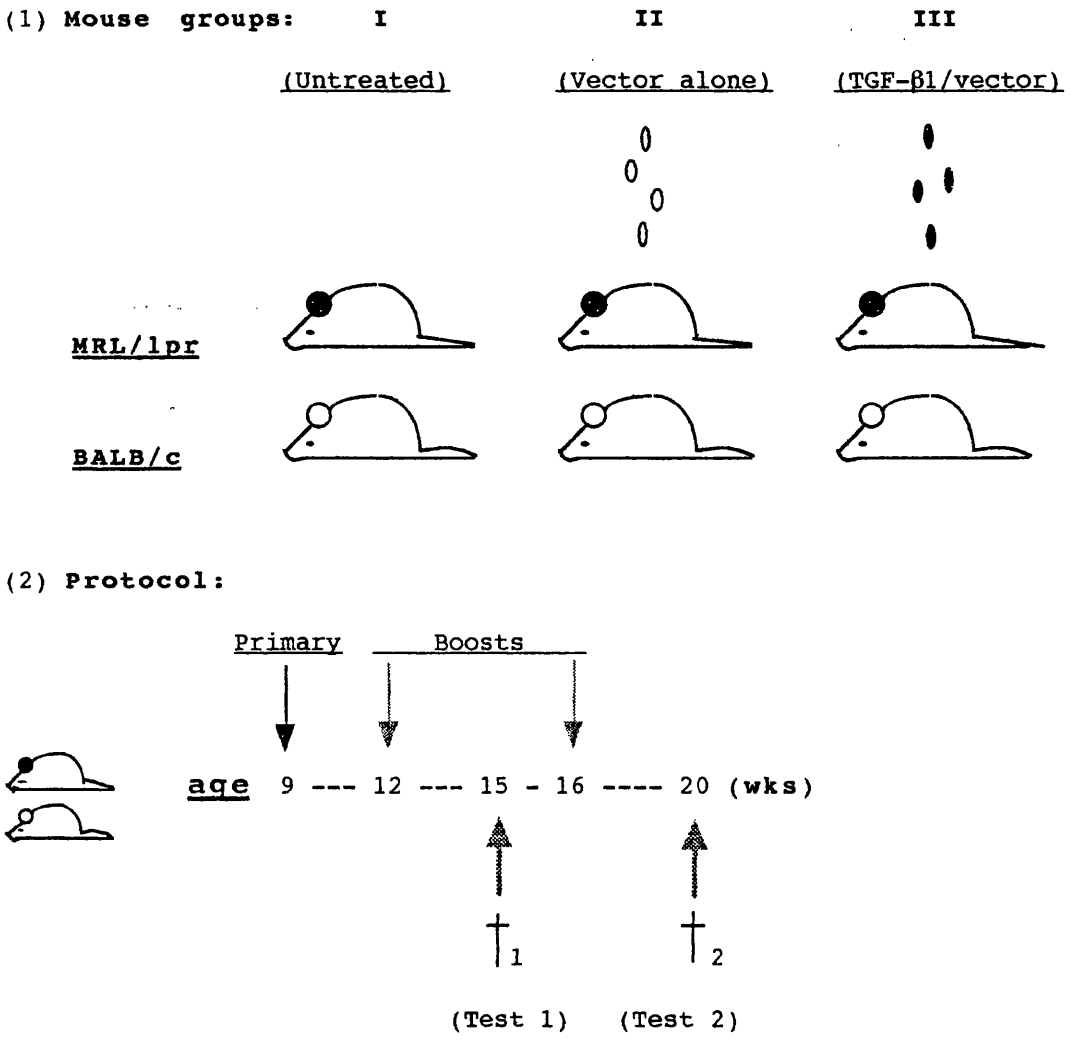


Figure 7.2 Schematic diagram representing the plan for treatment of BALB/c and MRL/lpr mice with TGF-β1 gene transfected *S.typhimurium*.

caused by the feeding procedure possibly due to respiratory infection. In addition, in the untreated MRL/*lpr* mouse group (Group I), one mouse died at 19 weeks old but because it was found dead over a weekend, no examination was given. There was no accidental death in the BALB/c mouse groups.

7.3.1 Changes in the cytokine profile

i) Serum TGF-β activity

The serum samples were taken from the mice at the time when the they were sacrificed, and kept at -20 ^{0}C before being tested by the TGF-β bio-assay. These included mice of 15 weeks old (Test 1) 3 weeks after the second dose, and mice of 20 weeks old (Test 2) 4 weeks after the third dose.

There was no significant change of serum TGF-β activity between the treated and untreated mice of either MRL/*lpr* or BALB/c strain at the age of 15 weeks (Fig.7.3). In the 20 week old BALB/c groups, although the average level of TGF-β was higher in sera from mice of the TGF-β/vector treated group, the differences were not statistically significant, due to sample variation and the small number of cases (three in each group). However, the untreated old MRL/*lpr* mice showed spontaneously increased levels of serum TGF-β at 20 weeks of age. Compared with the untreated controls, MRL/*lpr* mice treated with either TGF-β/vector or vector alone had reduced serum TGF-β activities rather then being increased (Fig.7.3b).

ii) Production of IL-2

Con A-induced IL-2 production by spleen cells was significantly suppressed in mice after treatment with TGF-β (Fig.7.4). The suppression was effective on mice tested at the age of 15 weeks (Fig.7.4a, b) and became less obvious at 20 weeks

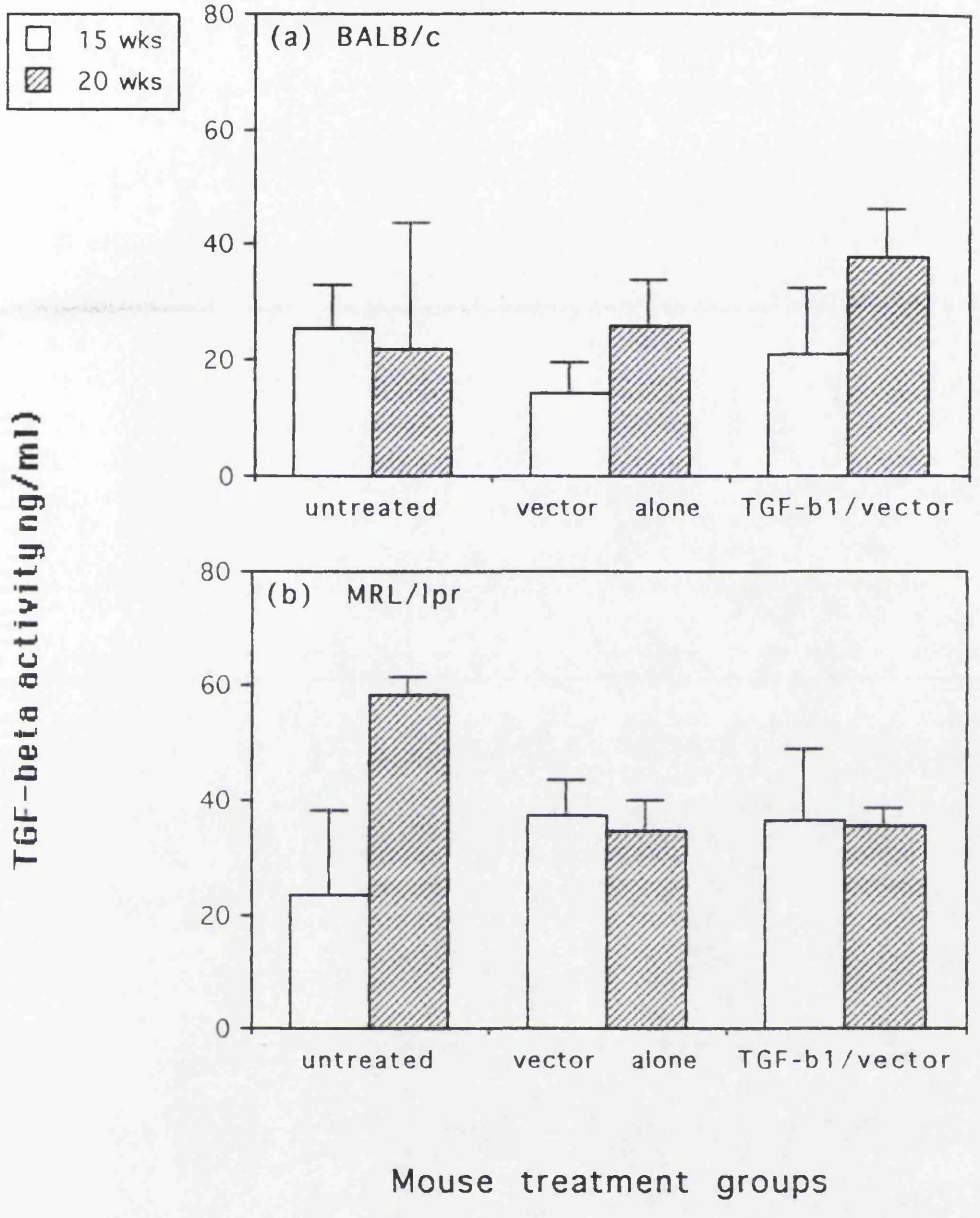


Figure 7.3 Serum TGF-β levels in BALB/c (a) and MRL/*lpr* (b) mice of TGF-β1-treated and control groups. Serum samples were taken from the mice at ages of 15 and 20 weeks, after 2 and 3 doses of the treatment respectively, and tested by the MvILu assay. Data are expressed as mean value (±SD) obtained from sera of 2 or 3 individual mice in each group.

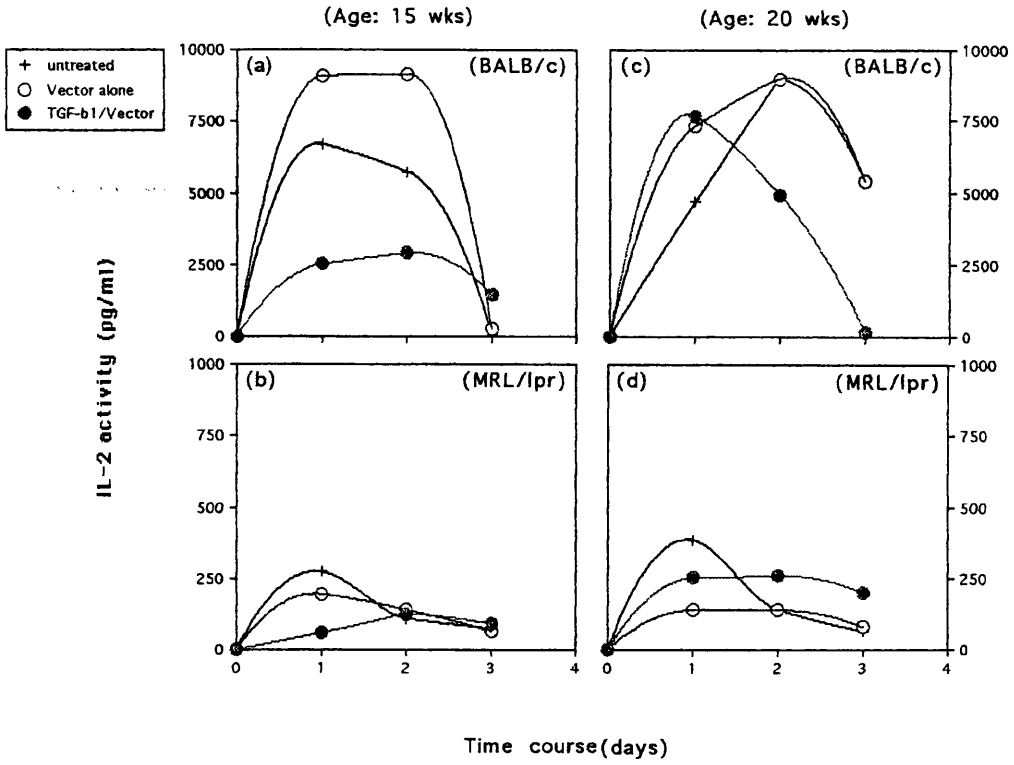


Figure 7.4 ***In vivo*** **effects of TGF-β1 treatment on Con A-induced IL-2 production in BALB/c and MRL/*lpr* mice.** Data show IL-2 activities in cultures of Con A-stimulated spleen cells from mice which had been treated with TGF-β1 transfected *S.typhimurium* for 2 or 3 doses (closed circles) at age of 15 and 20-weeks respectively. Control groups included mice treated with the bacterial vector alone (open circles) or untreated (+). The cells from 2 or 3 mice in each group were pooled and, the culture supernatants were collected at daily intervals and tested by the CTLL bio-assay. (Note scale differences in the graphs).

(Fig.7.4c, d). In the untreated control groups, IL-2 activity in cultures of Con-A stimulated spleen cells from MRL/*lpr* mice was twenty times lower compared with the BALB/c mice. However, the already deficient IL-2 secretion appeared to have been depressed even further after the treatment (Fig.7.4b). In addition, the vector showed some stimulatory effect on IL-2 production by spleen cells from BALB/c mice, but was rather slightly suppressive for the MRL/*lpr* mice (Fig.7.4d). Since IL-2 activity was so low in all MRL/*lpr* groups, it is not clear whether the effect was due to TGF-β treatment, or to the vector, or that there was no effect at all, only individual variations. There was no detectable spontaneous secretion of IL-2 by the cells from any of the mouse groups studied.

iii) Production of IFN-γ

TGF-β treatment had different effects on Con A-induced IFN-γ secretion depending on the stages of the treatment and mouse strains (Fig.7.5). Reduced IFN-γ levels were detected in the culture supernatants of Con A-stimulated spleen cells from the TGF-β treated mice at 15 weeks of age (Test 1), compared to the untreated groups and the vector treated groups which even showed increased levels (Fig.7.5a, b). The suppression was more effective in BALB/c, and just marginal in MRL/*lpr* mice possibly because of the already suppressed IFN-γ secretion as shown in the untreated MRL/*lpr* controls. Results from the ELISPOT assay also indicated a decreased frequency of IFN-γ secretors in the TGF-β treated BALB/c mice (15-week old groups only) confirming the data from ELISA assays. In contrast, the vector-treated BALB/c mice had increased frequency of IFN-γ secretors. However, in MRL/*lpr* mice, the frequency of IFN-γ secretors was low in the untreated group and there was no observable change after the treatments both with vector alone or with TGF-β/vector (Fig.7.6).

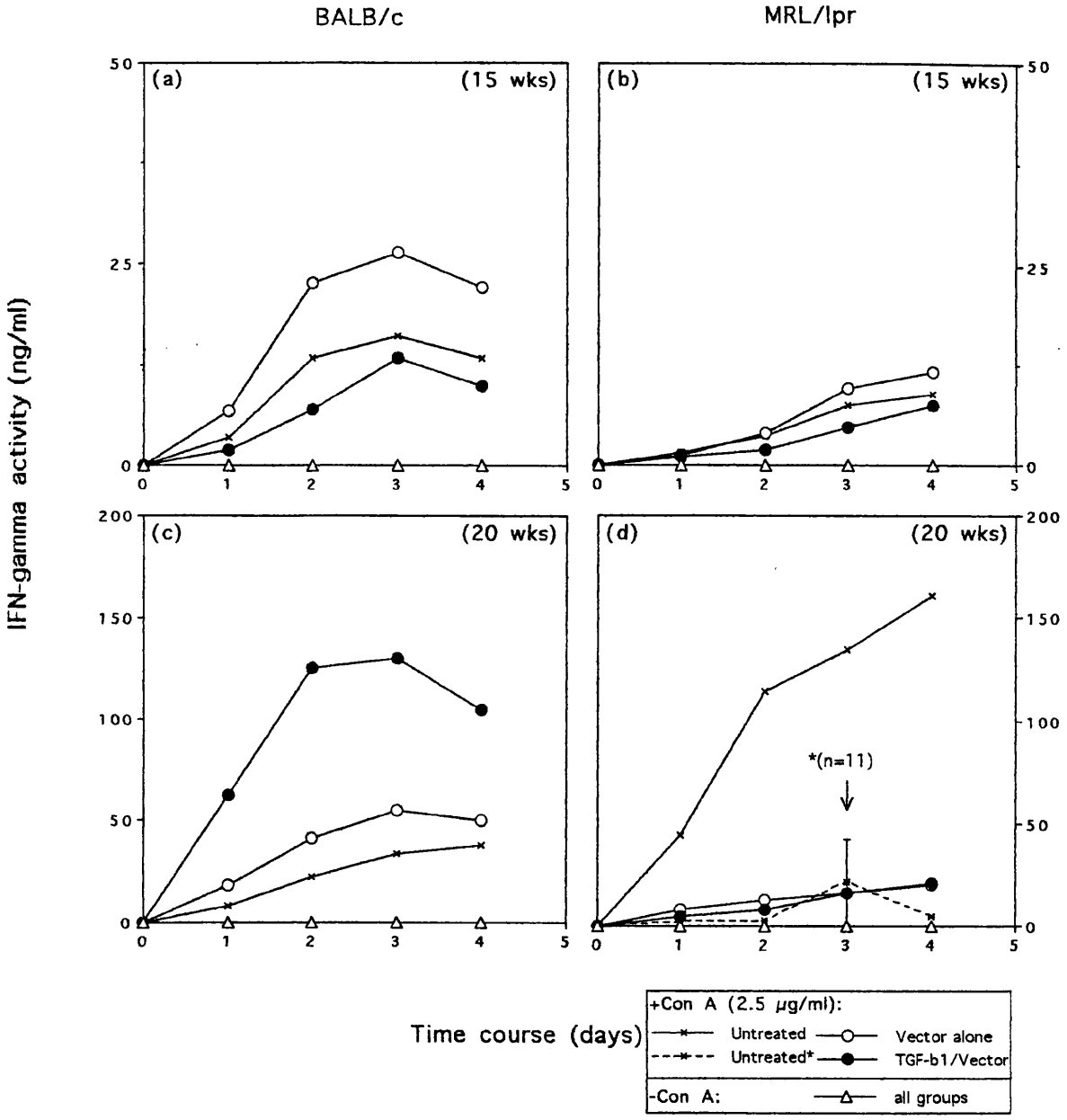


Figure 7.5 ***In vivo*** **effects of TGF-β1 treatment on Con A-induced IFN-γ production in BALB/c and MRL/*lpr* mice.** Data show IFN-γ activities detected in cultures of unstimulated (open triangles) and stimulated (closed circles) spleen cells from mice which had been treated with TGF-β1 transfected *S.typhimurium* for 2 or 3 doses at age of 15 and 20-weeks respectively. Control groups included mice treated with the bacterial vector alone (open circles) or untreated (x). Age of mice is indicated at the upper right hand corners. The cells from 2 or 3 mice in each group were pooled and, the culture supernatants were collected at daily intervals and tested by IFN-γ specific ELISA. In graph (d), the additional dotted line (untreated*) represents data established in separate experiments, of which data shown at day 3 are mean value (±SD) from 11 individual MRL/*lpr* mice between 18 to 21 weeks old. (Note scale differences in the graphs).

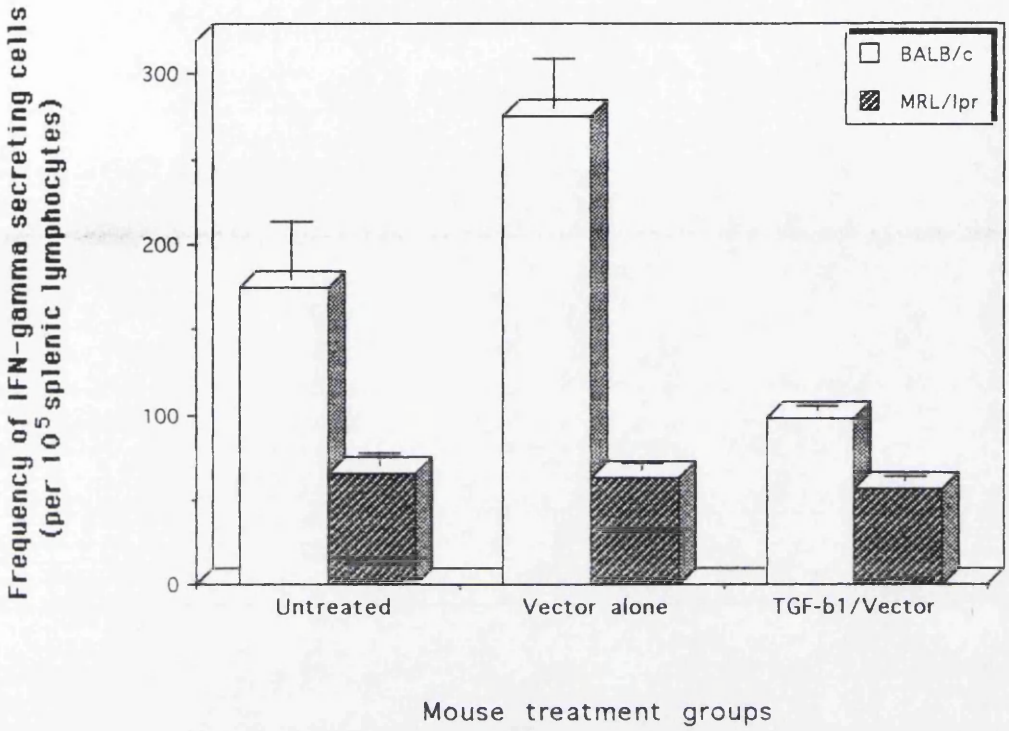


Figure 7.6 **ELISPOT assay showing effects of TGF-β1 treatment on Con A-induced IFN-γ production (cell frequency) by spleen cells from BALB/c and MRL/*lpr* mice at 15 weeks of age.**

However, a largely increased IFN-γ secretion was detected in the culture supernatants of Con A-stimulated spleen cells from the TGF-β treated BALB/c mice at 20 weeks (Test 2, Fig.7.5c). There was no such increase found in MRL/*lpr* mice, but the level of IFN-γ detected in the samples from the untreated MRL/*lpr* group (2 mice) was much higher than that normally detected in other untreated MRL/*lpr* mice at this age (Fig.7.5d). In Figure 7.5d, the curve shown as a dotted line is a time course of IFN-γ secretion established in a separate experiment using untreated MRL/*lpr* mice of the same age (20 wks). In addition, the data at day 3 (arrow) is the mean (±SD) of IFN-γ activities detected by same method in a total of eleven individual MRL/*lpr* mice between 18 to 21 weeks old.

There was no detectable IFN-γ activity in the unstimulated cell cultures in any of the groups.

7.3.2 Effects of TGF-β1 on Con A-induced T-cell proliferative responses

The effects of TGF-β treatment on T-cell proliferative responses are shown in Figure 7.7. In the 15-week (Test 1) mouse groups, the treatment did not show an obvious effect on the Con A-induced cell proliferative response, except for a kinetic delay in the TGF-β treated BALB/c group (Fig.7.7a) and a modest stimulation in both TGF-β/vector and vector alone treated MRL/*lpr* groups (Fig.7.7b). At the later stage (Test 2, 20 weeks), however, the treatment showed adverse effects on the two different strains of mice. While T-cells from the TGF-β treated BALB/c mice displayed a significantly increased responsiveness to Con A compared to both untreated and vector alone treated mouse groups (Fig.7.7c), the response of the T-cells from MRL/*lpr* mice was completely suppressed by the TGF-β treatment (Fig.7.7d). In addition, it was noted that the vector alone treatment also had such effects, but to a less degree, on the mice of two different strains (Fig.7.7c, d). No significant difference in the thymidine

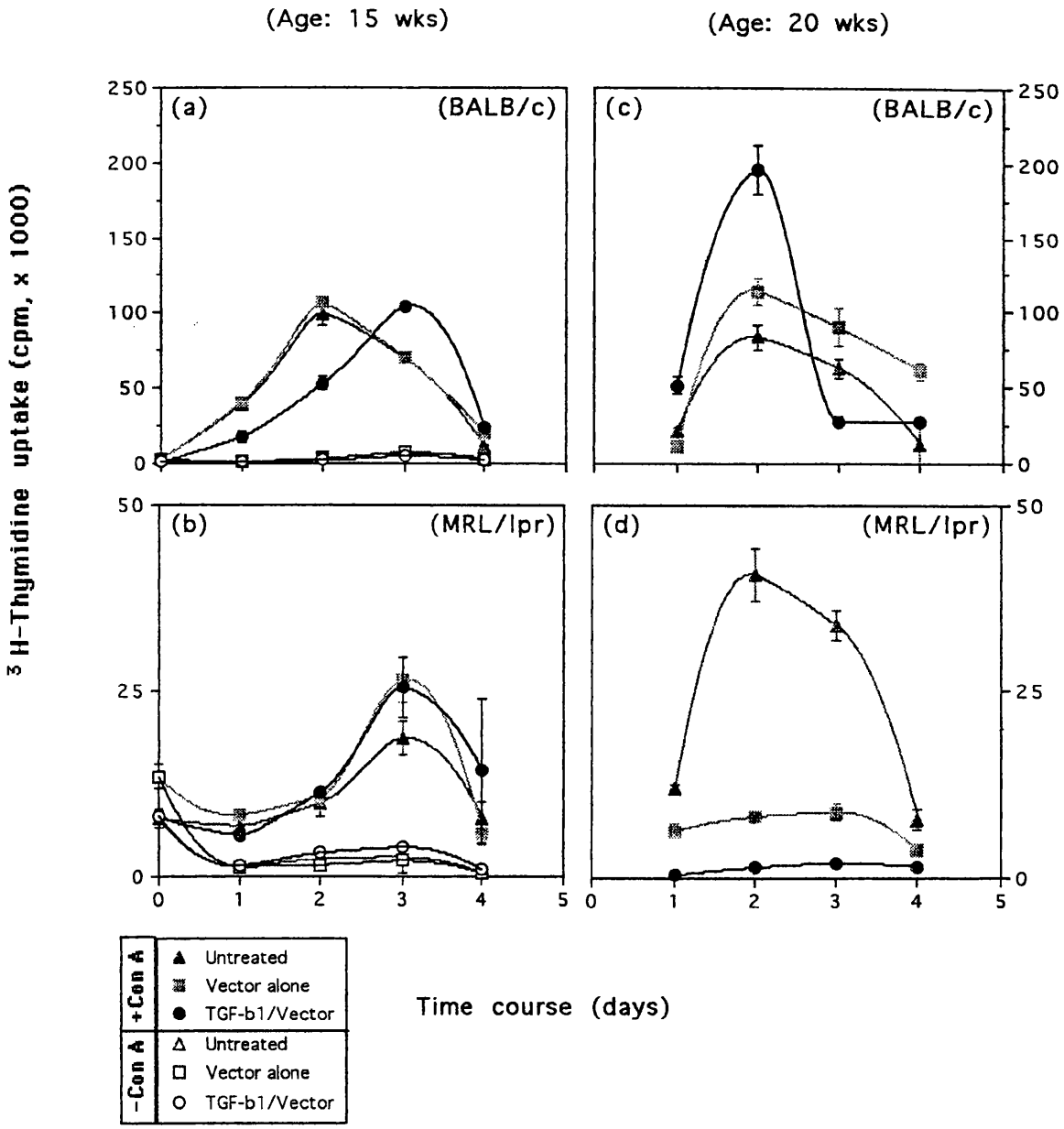


Figure 7.7 ***In vivo*** **effects of TGF-β1 treatment on T-cell proliferative responses in BALB/c and MRL/*lpr* mice.** Two or three mice in each group treated with TGF-β1 transfected *S.typhimurium* (circles), the bacterial vector alone (squares) or untreated controls (triangles) at age of 15 (a, b) and 20-weeks (c, d) were included. Mouse spleen cells were cultured (10^6 cells per well) in the presence or absence of Con A (2.5 μg/ml) in a 4-day time course, and the cell proliferation was measured by ^{3}H-thymidine incorporation at daily intervals. Data expressed are means (±SD) of triplicates. Note scale differences.

uptake by unstimulated cells was observed between groups of each strain (Fig.7.7a, b; Test 1 only).

7.3.3 Effects of TGF-β1 on lymphoproliferation and splenic cell phenotypes in MRL/*lpr* mice

Macroscopically, the size of spleens and the total number of spleen cells obtained from the 15-week old mice was similar between groups in each strain. However, spleens from the 20-week old MRL/*lpr* mice in both TGF-β/vector and vector alone treated groups were smaller compared with the untreated control group. In the 20-week mouse groups, the average total numbers of mononuclear cells ($x10^8$ per spleen; cells from 2 spleens in each group were pooled) obtained were: 8.73, 4.55 and 4.81 for MRL/*lpr*; 1.59, 1.33 and 1.0 for BALB/c in the untreated, the vector-treated and the TGF-β/vector-treated groups respectively.

FACS analysis of splenocytes from the treated and untreated BALB/c and MRL/*lpr* mice showed that the treatments did not significantly alter the total percentage of $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ subsets in the spleens. In MRL/*lpr* mice, the percentage of splenic $CD4^+$ cells was slightly higher in mice of the TGF-β/vector treated group (26.2%) compared to the untreated group (19.75%), but a similar increase was also found in the mice treated with vector alone (26.5%). Figure 7.8 shows the absolute number (average per spleen) of $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and other (cells do not express either CD4 or CD8) spleen cells. Regrettably, due to shortage of a pan-T cell marker at the time of assaying, the results do not include data on the percentage or total number of T-cell and the DN T-cell populations.

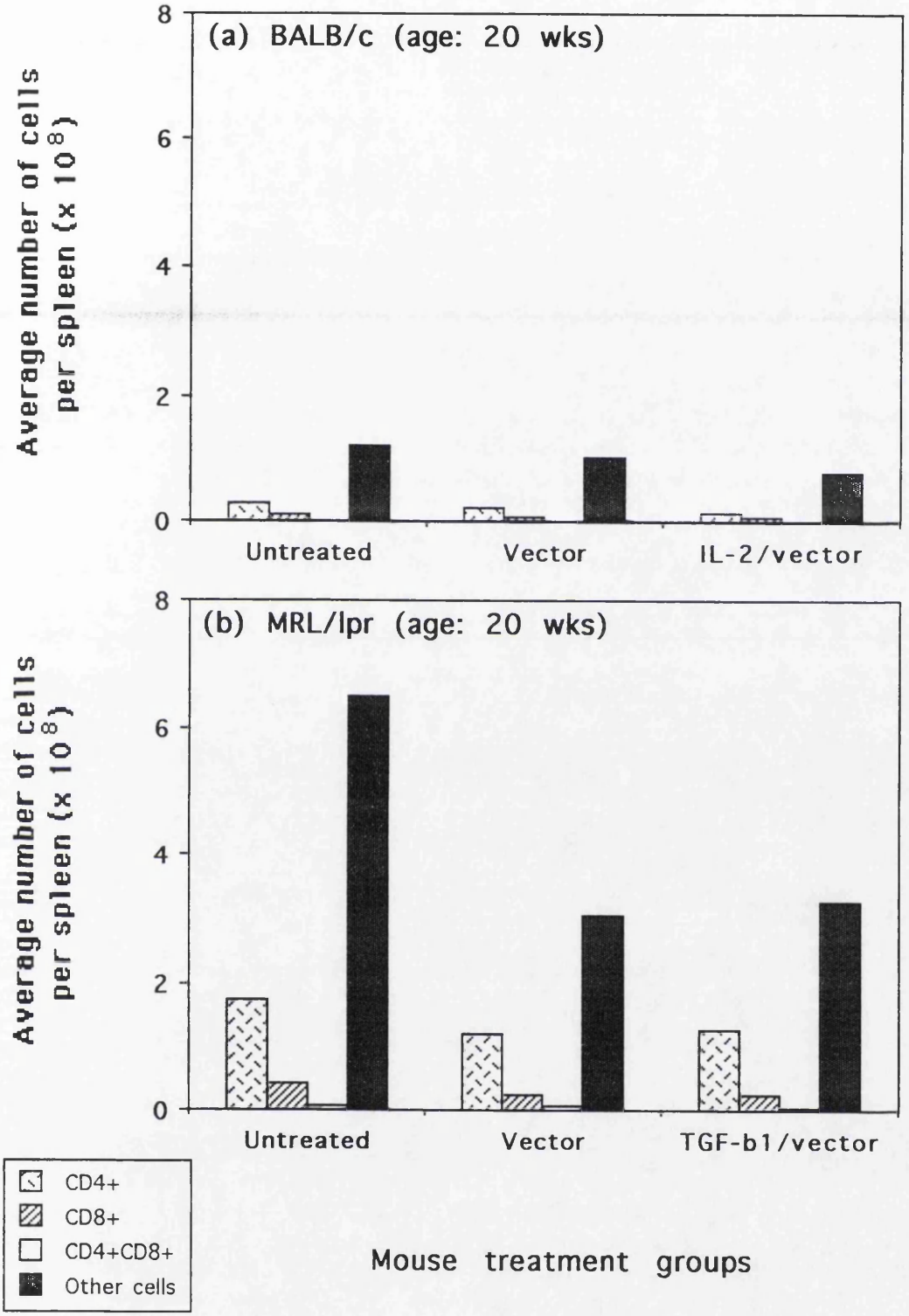


Figure 7.8 Changes of splenic mononuclear cell number and phenotypes in BALB/c and MRL/*lpr* mice treated with TGF-β1 or the vector alone, or the untreated controls. Total number of spleen mononuclear cells was counted microscopically and cell phenotypes were analysed by FACS using antibodies specific to mouse CD4 and CD8. Cells from 2 or 3 mice in each of the groups were pooled and the data presented are average cell number per spleen.

7.3.4 Effects of TGF-β1 on autoantibody production and renal pathology in MRL/*lpr* mice

To monitor effects of TGF-β treatment on MRL/*lpr* mice, serum autoantibody levels, kidney immune complex deposition and renal histological changes in the mouse kidneys were assessed as indications for the disease progression. The results are summarised in Table 7.1.

As shown in Table 7.1, the serum levels of autoantibodies (IgG) to ss- and ds-DNA are variable among individual mice. However, in general, old (20 wks) MRL/*lpr* mice showed higher titres of anti-dsDNA serum antibodies compared with the 15-week old groups. Since there were only 2 mice in each of the experimental groups, the results have no statistical significance, but 2 sera from the TGF-β treated MRL/*lpr* mice seem to have higher anti-ssDNA antibodies at 20 weeks compared with the controls. In the control BALB/c mice, serum titres of both anti-ss and -dsDNA were lower than 60 and 10 TU respectively in all groups.

For renal histological assessment, mouse kidney sections, prepared from the paraffin embedded tissues after Bouin's/formalin fixation and periodic acid-Schiff (PAS) staining procedure, were examined by routine light microscopy. The sections (4-5 μm) from the mice of different groups were randomly labelled, and examined by an experienced renal pathologist, Dr G.B.M. Lindop, in the University Department of Pathology. The assessment was a blind but subjective test. Severity of the kidney damage was assessed by taking into account the extent of enlargement and structural changes of glomeruli, interstitial and glomerular cellular proliferation, membrane adhesion, vasculitis, protein casts etc. By combining different degrees of these abnormalities, 0 to 5 pluses were given to score each section for statistical comparison. It must be pointed out that pyelonephritis with mononuclear cell infiltration in the

Age	Mouse groups	Auto-Ab Titre (IgG, TU) anti-ssDNA	anti-dsDNA	Kidney pathology *KPI*	C3
15-wks	**I**	11000	140	**++++**	++++
(Test 1)	(Untreated)	9000	50	-	++++
	II	2700	28	**++++**	++++
	(Vector)	8100	73	**++++**	++++
	III	2000	5	-	-
	(TGF-β1)	9800	45	-	-
20-wks	**I**	4800	1000	**++**	ND
(Test 2)	(Untreated)	4800	400	**+++++**	ND
	II	3300	190	**++++**	ND
	(Vector)	5300	170	**+++++**	ND
	III	6500	1800	**++**	ND
	(TGF-β1)	8000	150	**+++++**	ND

Table 7.1 Effects of rTGF-β1 treatment on autoantibody production and renal pathological changes in MRL/*lpr* mice. Mouse serum antibodies to ss and dsDNA were measured by ELISA and expressed as titration units (TU). Kidney sections were prepared from individual mice for light and immunofluorescence microscopic examinations (see Methods). Degrees of renal pathological changes were scored, zero to 5 pluses on a blind basis, according to the severity of kidney damage (KPI, Kidney Pathological Index, see Materials and Methods); intensity of immunofluorescent staining for kidney deposition of complement component 3 (C3). ND: not done.

medulla was common in all MRL/*lpr* groups examined and this was discounted for the assessment. Some of the kidney sections were also studied by immunostaining using antibodies specific to mouse C3 (see Materials and Methods).

Table 7.1 also summarises results from the histopathological and immunopathological studies. Among the 12 MRL/*lpr* mice of different groups, some variable degrees of kidney damage, indicated by the 'kidney pathology indices (KPI)', were evident in 5 out of the 6 groups of mice. However, glomerulonephritis and the other renal changes mentioned above were absent in the two mice in the TGF-β treated group at 15-weeks. This was in agreement with results from the complement C3 immunostaining of the kidney sections, although there was no positive staining for IgG on these formalin-fixed, paraffin embedded tissue sections. It is not certain whether the apparent delay of renal pathological change was due to the effect of the treatment or individual variations since one out of two untreated MRL/*lpr* mice also showed absence of glomerulonephritis at this age. In addition, there was no correlation between the serum autoantibody levels (IgG) and the degrees of kidney damage.

7.4 Discussion

First of all, it must be pointed out that this was a pilot study within a small scale of experimentation. The preliminary results described in this chapter are to be further confirmed.

Cytoplasmic expression of TGF-β in the recombinant *Salmonella* bacteria was an important precondition for the study. It ensured that the transfected bacteria used for treatment did carry the gene of interest and expressed murine TGF-β in its cytoplasm. However, its ability to deliver TGF-β *in vivo* depends on whether these antigenically modified bacteria were able to penetrate the immunological barriers of the host, to multiply and to release TGF-β. A recent similar study using the same model system to

deliver IL-1β in BALB/c mice has shown evidence demonstrating the presence of viable *S. typhimurium* expressing IL-1β *in vivo* in the lymphoid organs after oral immunisation (Carrier *et al.*, 1992).

The present study included BALB/c mice which were used not only as a control strain for the lupus disease model, but also as a test model to demonstrate or to confirm the *in vivo* effects of TGF-β on the normal immune system, which are still not fully understood. As a positive control, it was also important to be able to verify that the bacteria expressed and released biologically active TGF-β *in vivo*. The TGF-β treatment has shown clear immunosuppressive effects on the BALB/c mice at the early stage of treatment. It reduced the ability of spleen cells to produce IL-2 in response to Con A stimulation. Suppression was also indicated by decreased IFN-γ production and the delayed proliferative response to Con A at 15 weeks (Test 1). However, at the later stage (20 wks, Test 2), the inhibitory effects were not only diminished, but also followed by a greatly enhanced responsiveness of the cells to Con A to secrete IFN-γ and to proliferate. The 'rebound' might suggest the presence of some feedback mechanism as a result of the early inhibitory effects of TGF-β. This observation is in agreement with the *in vitro* finding that, in long term cell cultures, TGF-β increases activities of IFN-γ secreting cells (Swain, 1991a, b), but induces no such increase in the IL-2 activity.

In MRL/*lpr* mice, the effects of TGF-β treatment were weak and variable, possibly due to two factors. One is the already suppressed T-cell responsiveness in the untreated mice. The fact that serum TGF-β activity was increased in the untreated old MRL/*lpr* mice (20 wks) might suggest an on-going mechanism of spontaneous suppression *in vivo*. The effects of further treatment with TGF-β exogenously could therefore be masked. Unlike the effects of TGF-β on BALB/c mice, the lack of the post-treatment 'rebound' in MRL/*lpr* mice indicates the complex mechanism of the

spontaneous suppression in lupus disease. It might suggest an inability of self-regulation in the mice. Another factor involved could be the influence of the bacterial vector stimulation. In MRL/*lpr* mice treated with TGF-β/vector or vector alone, the equally reduced, rather than increased, serum TGF-β activities suggest an adverse, but nonspecific effect of the bacterial vector. In addition, the reduced size of the spleens, decreased number of total spleen cells and increased percentage of $CD4^+$ cells in both of the treated groups also support the notion of this 'vector effect'. Presumably, these were due some antigenic or mitogenic substances released by the lysis of the bacterial vector, such as LPS or superantigens.

The levels of serum autoantibodies to ss and dsDNA, which were variable among all MRL/*lpr* groups, did not correlate with the kidney pathological changes. This suggests the importance of other factors which may be involved in the formation and deposition of ICs resulting in kidney damage, conditions necessary for complement activation for example, as supported by the agreement of results from the kidney histological assessments and C3 staining. Both of the observations showed a possible delay of the renal pathological changes in the MRL/*lpr* mice after treatment with TGF-β. This might be explained by the early immune suppressive function of the cytokine. However, due to the limited number of cases and the fact that one untreated mouse was also free of such changes, the results are to be confirmed.

Through this pilot study, much useful information has been gained, although the scale of the experiment was too small to draw any definite conclusion. The new approach for cytokine intervention may be an applicable tool for future *in vivo* studies of cytokine regulation in autoimmune and other diseases. For undertaking further investigations, larger numbers and different strains of mice would have to be used and factors such as the optimum dose, the kinetics and the vector effect would need to be carefully considered.

Chapter 8

Delivery of murine IL-2 by *Salmonella typhimurium aro*$^-$ mutant in MRL/*lpr* mice

8.1 Introduction

The *in vitro* observations about the possible intrinsic nature of the IL-2 defect in lupus mice have provided a basis for undertaking further an *in vivo* investigation. In the previous chapters, it has been demonstrated that there exists an early, progressive defect in responsiveness to T-cell activation in SLE. It was then shown that exogenous IL-2 was able to by-pass the defect *in vitro*. Thus, what impact IL-2 intervention might have on the development of the disease *in vivo* becomes the next most important question to answer.

As reviewed by Kroemer et al. (1989, 1991) and Tsokos (1992), the role of IL-2 in autoimmune diseases is still controversial. In SLE, the focus of arguments has been the interpretation of the *in vitro* findings over their clinical relevance. Seemingly, the B-cell hyperactivation and the T-cell functional hypoactivity are mutually exclusive. IL-2 treatment of lupus disease, in which the immune system is thought to be already in an overactivated state, would therefore appear to be theoretically unfavourable. For this reason, presumably, as well as the early failed attempts in the *in vitro* study using exogenous IL-2 (Altman *et al.*, 1981), there have been few studies of such an *in vivo* cytokine intervention being carried out. IL-2 interventions by injection of cytokine which has a very short half-life may have a major drawback for being difficult to maintain its lasting effects *in vivo* (Owen et al., 1989), or otherwise running into problems of high-dose side effects. The possibility of using vector system to deliver IL-2 might be able to solve this problem. A previous approach using recombinant vaccinia virus expressing human IL-2 to treat MRL/*lpr* mice has shown positive effects on the recovery of lupus mice from autoimmunity (Gutierrez-Ramos *et al.*, 1990).

The present study has explored a new vector system for delivering cytokines by *S.typhimurium aro⁻ mutant* in lupus mice. As part of the pilot study using the same

model system as that for delivering TGF-β1 (Chapter 7), a reconstructed live *S. typhimurium* expressing mouse IL-2 was used to study the *in vivo* effect of IL-2 in MRL/*lpr* mice.

8.2 Materials, mouse groups and treatment scheme

8.2.1 IL-2 gene transfected *S.typhimurium*

The expression plasmid pKK-IL-2 gene transfected *S.typhimurium* BRD509 was also constructed and kindly provided by Dr D-M Xu in the department (see Chapter 2). Before being used for treating mice, the transfected *Salmonella* were assessed to determine expression of IL-2 in the cytoplasm. After the extraction procedure which is described in Chapter 2 (Section 2.9.2), IL-2 activity in the cytoplasmic extract was measured by the CTLL IL-2 bioassay.

High levels of IL-2 activity (equivalent to 440 ng/ml of r-human IL-2 in the extract, or about 44 ng/ml of the original bacterial culture) were detected in the cytoplasmic extract of IL-2 gene transfected *Salmonella*, but not in that of untransfected control bacteria (Figure 8.1). The average IL-2 activity generated from a million of the recombinant bacteria was about 440 pg. The expression of IL-2 was confirmed using different samples taken from repeated treatments.

8.2.2 Animals and treatment scheme

Thirty MRL-Mp-*lpr*/*lpr* (MRL/*lpr*) female mice of three week old were purchased from Harlan Olac Ltd, UK. The treatment was carried out in the Animal House, Gartnavel Hospital, Glasgow. The mice were divided into three treatment groups (Groups I, II and III). Group I (8 mice) received no treatment; Group II (11 mice) was

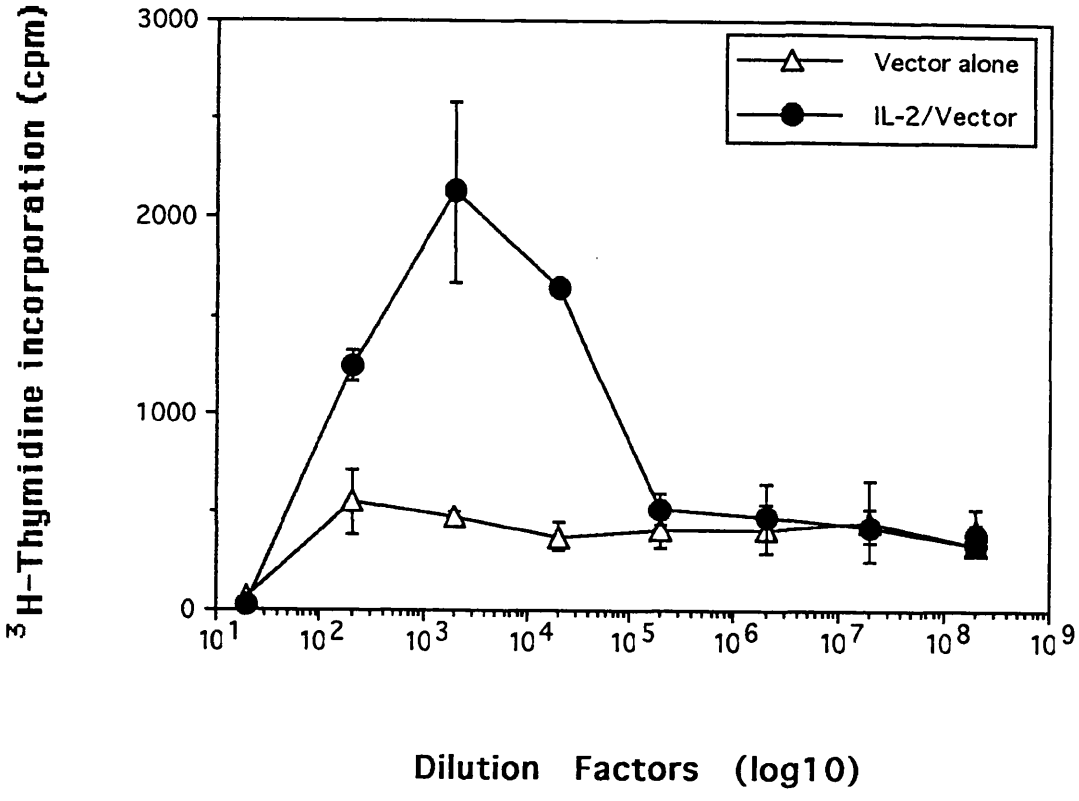


Figure 8.1 Cytoplasmic expression of IL-2 in the gene transfected *S.typhimurium*. IL-2 activity in the cytoplasmic extract from the IL-2 gene transfected *S.typhimurium* (IL-2/Vector) is shown by its ability to promote the proliferation of the IL-2 dependent CTLL cells. The wild type bacteria were used as control (Vector alone).

treated with the bacterial vector alone, and Group III (11 mice) with IL-2 gene transfected *Salmonella.*

Figure 8.2 shows a diagram of the treatment scheme in summary. The early onset of IL-2 deficiency is one of the features of the immunological abnormalities in lupus mice. The fact that an IL-2 defect exists in young mice, at an age when thymic influence is important for establishing a normal immune system was the reason for starting the IL-2 treatment at an early age. The first treatment was given to the mice at 3 weeks old, and subsequent boosts at 3-week intervals. The mice in each group were then divided into 2 sub-groups, one sacrificed after 4 doses of the treatment at 14 weeks of age, and the other sacrificed after 5 doses at 19 weeks of age. For the same reasons mentioned in Chapter 7, there was no attempt to determine the optimum dose of the treatment. Each single dose of 10^{10} bacteria in 0.2 ml PBS was given per mouse orally using a gavage tube. The 5th dose was doubled because of the increased body weight of the mice.

Since accidental mouse death could be caused by the feeding procedure mainly due to respiratory infection and 2 mice (one in Group II and another in Group III) which developed severe lupus disease at ages between 17 to 18 weeks, the numbers of mice in each test group were not even. In addition, it was noted that mice of the untreated group used in this experiment developed lupus disease earlier compared to that in the study of TGF-β treatment. This was the reason for the earlier sacrifice of the mice at 14 and 19 week old, rather than at 15 and 20 week as the TGF-β experiment (Chapter 7).

8.3 Effects of IL-2 treatment on MRL/*lpr* mice

8.3.1 Cellular aspects: effects of IL-2 on lymphoproliferation *in vivo*

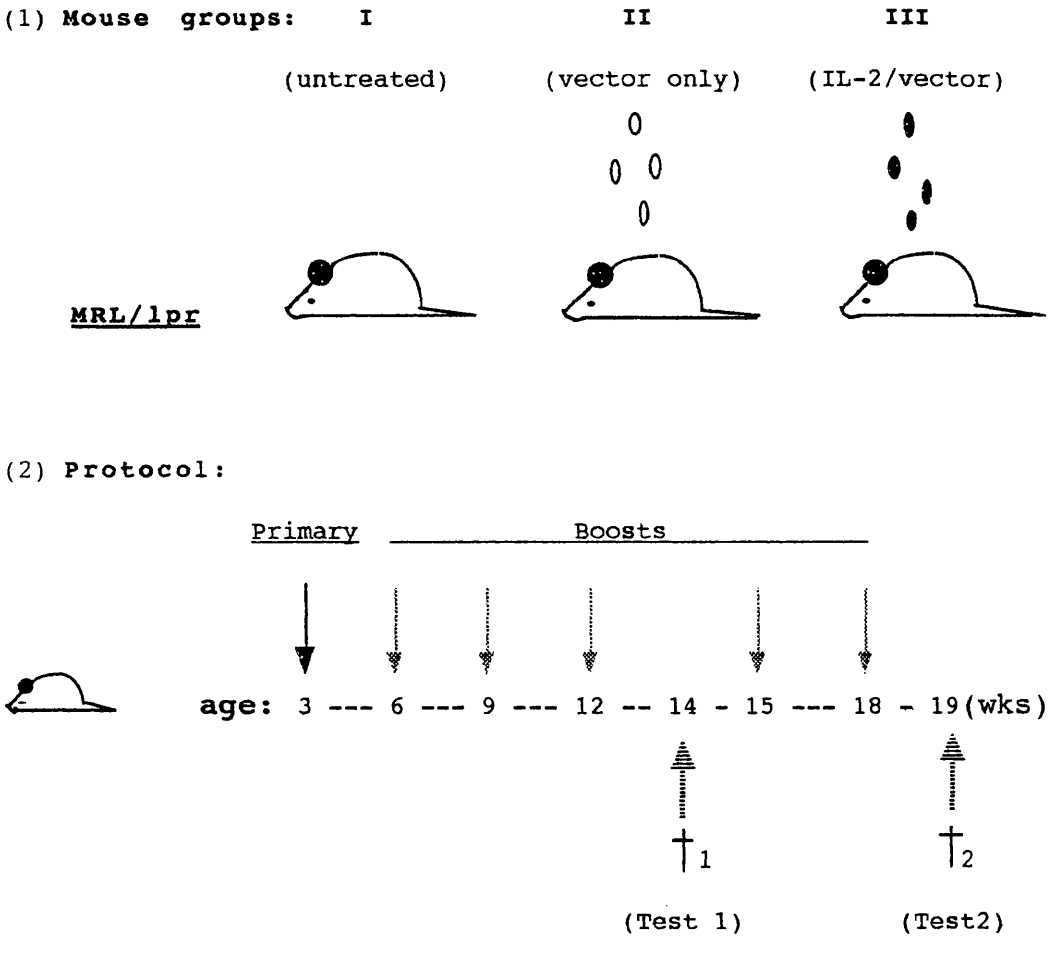


Figure 8.2 Schematic diagram representing the plan for treatment of MRL/lpr mice with IL-2 gene transfected *S.typhimurium*.

Lymphadenopathy develops in the MRL/*lpr* mice. As described previously, cellular hyperplasia in peripheral lymphoid organs with accumulation of the immature DN T-cells is a feature of the MRL/*lpr* disease resulting in enlarged spleen and lymph nodes, and development of sub-dermal lymphoid tumours (lymphocytoma). To assess the effect of IL-2 treatment on the abnormal lymphoproliferation in the MRL/*lpr* mice, degrees of the development of these lymphoid abnormalities were compared between the mouse groups

i) Effects on the development of lymphoid tumours in the mice

The assessment was kindly done by Mr Derek Milroy and Mr Kevin Jones, 2 experienced workers in the animal house, combining measurements of number and size of the sub-dermal lymphoid tumours. The results are expressed as 'Tumour Index (TI)'. Table 8.1 shows the rating criteria and scale.

Table 8.1 Scoring criteria for the assessment of sub-dermal lymphoid tumour development in MRL/*lpr* mice

Score	number/size of tumours
-	no tumour
+	1 to 2 small (< 0.5 cm^3) tumours
++	3 small or 2 including 1 large (>0.5 cm) tumours
+++	4 small or 2 to 3 large tumours
++++	5 to 6 tumours
+++++	more than 6 tumours

Sub-dermal lymphoid tumours were observable at 14-weeks of age in all of the experimental groups. However, differences in the number and size of the lymphoid tumours between the IL-2 treated group and the 2 control groups were observed. In spite of considerable variations in all three mouse groups, the IL-2 treated mice showed

an overall reduction in the number and size of lymphoid tumours compared to the control group treated with vector only (Fig.8.3). The difference between the tumour indexes for the 2 mouse groups is statistically significant (1.4 vs 2.2, $P < 0.05$). Although the difference between IL-2-treated and untreated mouse groups was not statistically significant ($P>0.05$), the mean 'TI' was also lower in the IL-2-treated group (1.4 vs 1.875).

ii) T-cell number and phenotype changes in the mouse spleens

The size of the spleens of the 14-week old mice were similar in all 3 groups measuring about 2.5 x 0.7 x 0.3 cm. In the 19-week old mouse groups, all mice showed enlarged spleen, but the spleens of the IL-2 treated mice were relatively smaller compared to the ones in the 2 control groups. The average total numbers of mononuclear cells obtained from the spleens of the 19-week old mice were 5.85, 6.25 and 5.53 (x 10^8) per spleen in the untreated, vector alone treated and IL-2/vector treated mice respectively.

To monitor effects of IL-2 treatment on lymphoproliferation and the peripheral accumulation of DN cells, spleen cells from the IL-2-treated and control mice were analysed by T-cell phenotyping. The mice at 14-weeks had shown the beginning of spontaneous T-cell proliferation and appearance of the double negative cells, but with no significant difference between groups (Fig.8.4a). The average total numbers of splenic T-cells ($CD3^+$) were 2.39, 2.869 and 2.023 (x 10^8) per spleen in the untreated, vector alone treated and IL-2/vector treated mice respectively. However, when the T-cell abnormalities became more apparent in 19-week old mice, a much lower percentage and number of the DN cells, as well as a reduction in total number of $CD3^+$ cells, was observed in the IL-2 treated group (Fig.8.4b). Due to reduction of the DN subset, the percentage of single positive ($CD4^+CD8^-$, $CD4^-CD8^+$) T-cell populations was relatively increased, though the absolute cell number of these 2 subsets remained unchanged.

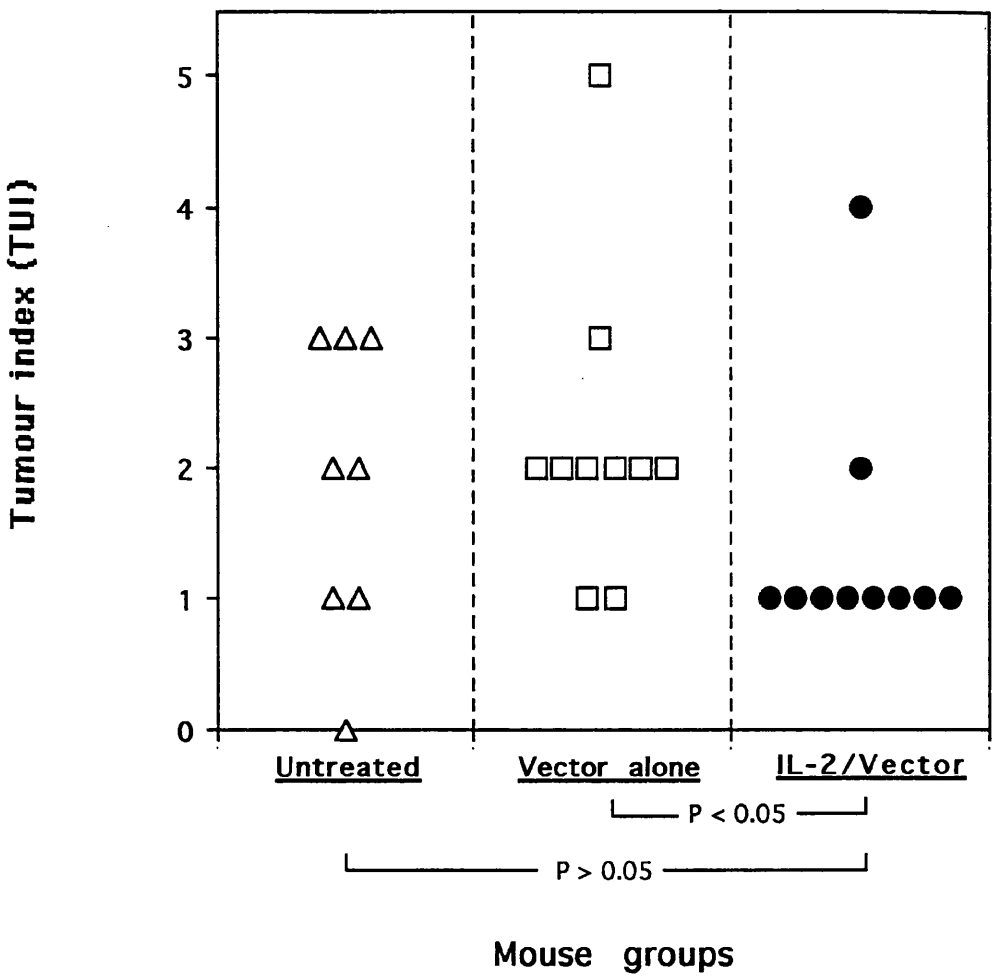


Figure 8.3 Effects of IL-2 treatment on lymphoid tumour development in MRL/*lpr* mice. Degree of lymphadenopathy developed in the MRL/*lpr* mice treated with IL-2/vector (n=10) or vector alone (n=10) for 4 doses, and the untreated control mice (n=8), at an age of 14 weeks was kindly assessed by Mr. D. Milroy and Mr. K. Jones in the Departmental Animal House. The severity of tumour development in individual mice is indicated by 'Tumour Index (TUI)' according to the scoring criteria described in Table 8.1 .

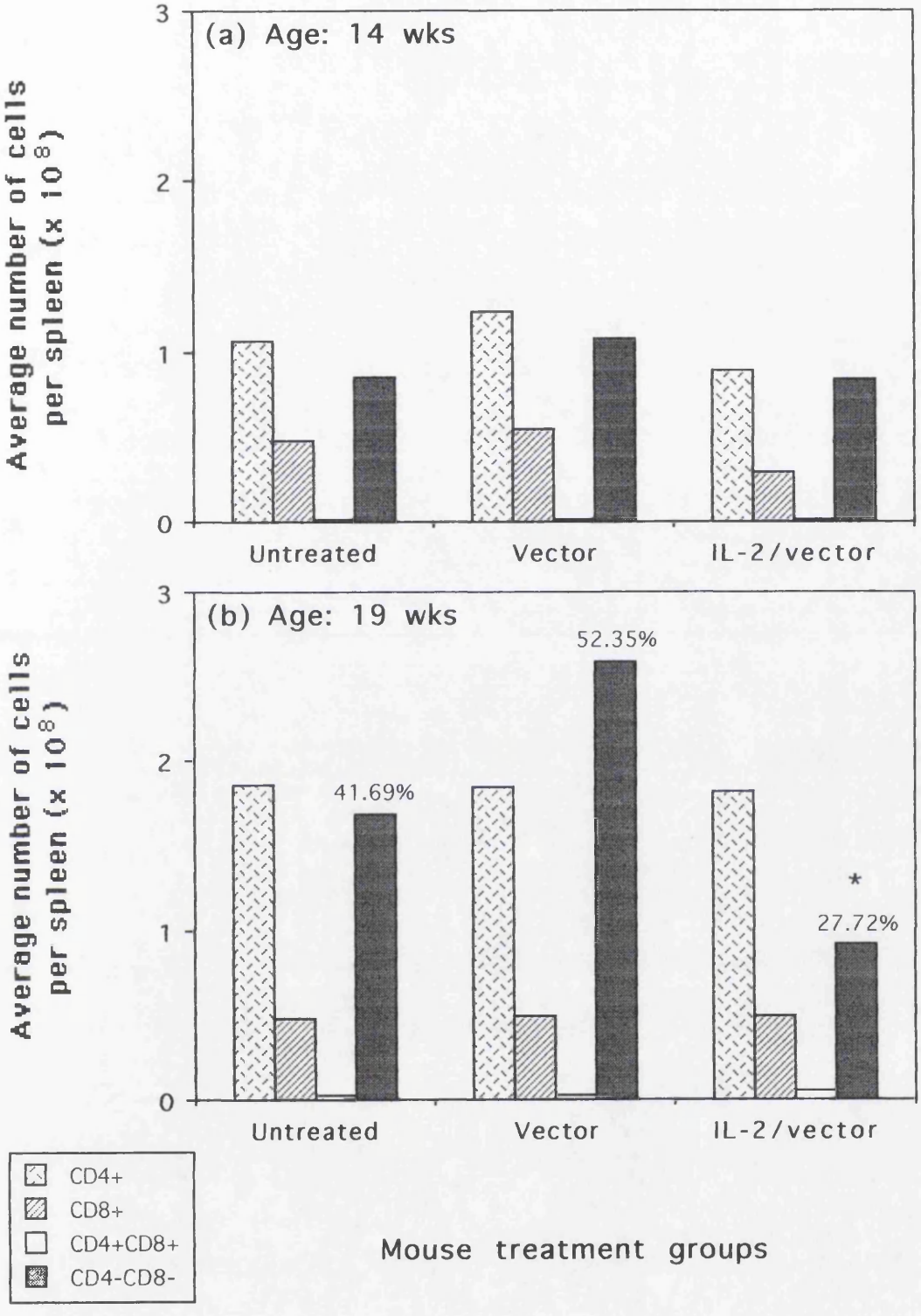


Figure 8.4 Effects of IL-2 treatment on total number of splenic T-cells ($CD3^+$) and phenotype changes in MRL/*lpr* mice by flow cytometry. T-cell phenotypes were analysed using antibodies specific to mouse CD3, CD4 and CD8. Cells from 2 or 3 mice in each of the groups were pooled and the data presented are average number of cells ($CD3^+$) per spleen. The percentage indicated in the 19-wk mouse groups refers to the double negative cells in the entire $CD3^+$ populations. Age of the mice is indicated at the upper left hand corner of the graphs. (The double positive cell population in all groups was below 1.5%).

The average total numbers of splenic T-cells ($CD3^+$) in the 19-week old mice were 4.06, 4.94 and 3.27 (x 10^8) per spleen in the untreated, vector alone treated and IL-2/vector treated mice respectively.

8.3.2 Effects of IL-2 on T-cell functional activities

i) Production of IL-2 and IFN-γ

After IL-2 treatment, the ability of the mouse T-cells to produce IL-2 and IFN-γ in response to Con A was assessed. At the age of 14 and 19-weeks, spleen cells from 3 to 5 mice of each group were prepared, pooled and stimulated with Con A as previously described. Culture supernatants were taken at daily intervals for the cytokine assays. The samples from all of the 6 mouse groups showed low levels of IL-2 activities (< 300 pg/ml). There was no kinetic difference in Con A-induced IL-2 secretion between groups of the same age. IL-2 activity in culture supernatants of spleen cells from the IL-2 treated mice of 14-week old was similar to that of the untreated control group, although it was slightly higher if compared with the vector alone treated group (Fig.8.5a). The IL-2 treatment did not increase the ability of lupus T-cells to produce IL-2. On the contrary, IL-2 activity was further depressed in the 19-week old mice, in both IL-2/vector and vector alone treated groups (Fig.8.5b).

IFN-γ production was assayed at 3 days after Con A stimulation, its peak activity in cultures according to the time course established previously . The levels of IFN-γ in culture supernatants of spleen cells from 14-week old mice of the 3 groups were comparable. However, a clear suppression of IFN-γ secretion was observed in the IL-2 treated mice at age of 19-weeks compared to the 2 control groups (Fig.8.6).

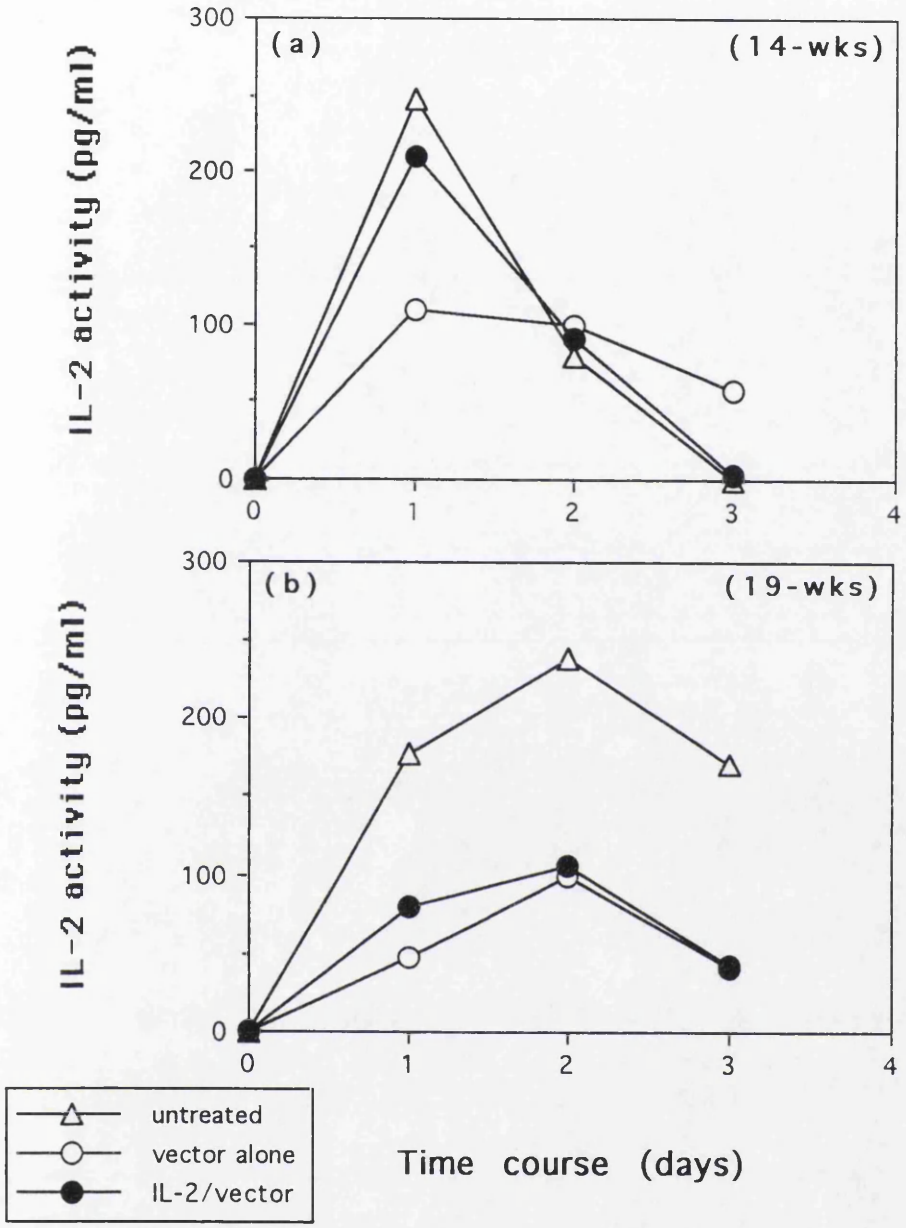


Figure 8.5 ***In vivo*** **effects of IL-2 treatment on Con A-induced IL-2 production** ***in vitro*** **in MRL/*lpr* mice.** Data show IL-2 activities in cultures of Con A-stimulated spleen cells from mice which had been treated with IL-2 transfected *S.typhimurium* for 4 or 6 doses (closed circles) at 14 and 19-weeks of age respectively. Control groups included mice treated with the bacterial vector alone (open circles) or untreated (open triangles). The cells from 3 to 5 mice in each group were pooled and, the culture supernatants were collected at daily intervals and tested by the CTLL bio-assay.

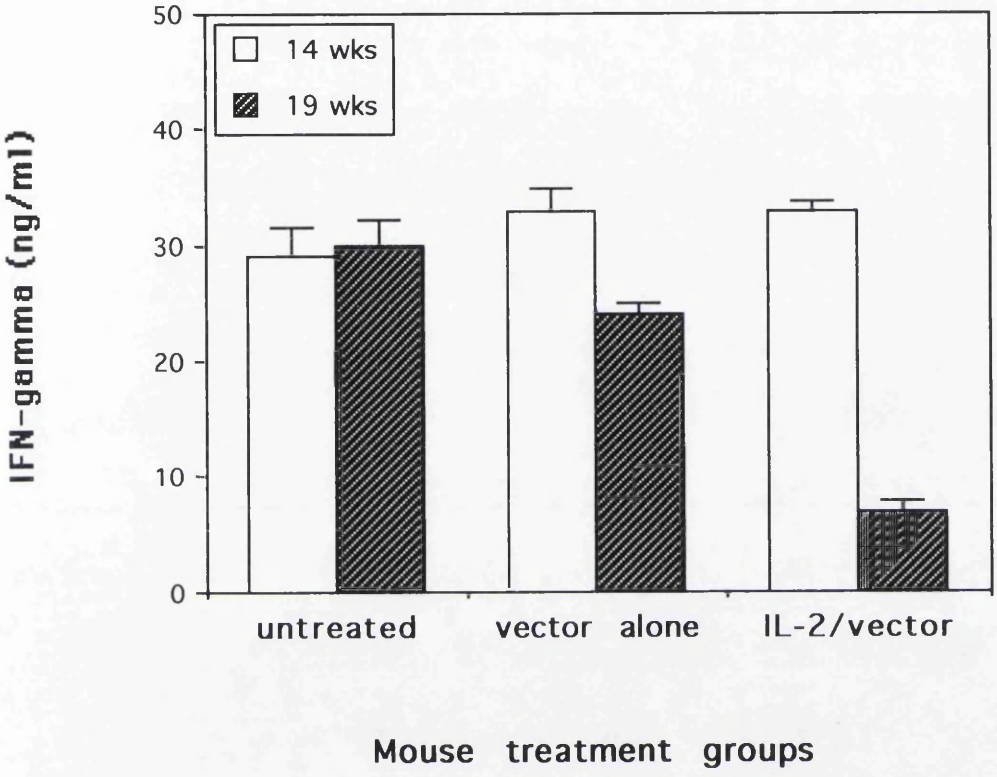


Figure 8.6 ***In vivo*** **effects of IL-2 treatment on Con A-induced IFN-γ production in MRL/*lpr* mice.** Data show IFN-γ activities detected in cultures of Con A-stimulated spleen cells from mice which had been treated with IL-2 transfected *S.typhimurium* or the bacterial vector alone for 4 or 6 doses (at age of 14 and 19-weeks respectively), and from mice of the untreated groups. The cells from 3 to 5 mice in each group were pooled and, the culture supernatants were assayed at day 3 by IFN-γ specific ELISA. Data expressed are means (±SD) of triplicate samples.

ii) Cytokine mRNA expression

In the 14-week old mouse groups, low levels of IL-2 mRNA were detectable in freshly isolated spleen cells from the IL-2/vector and the vector alone treated mice, but not from the untreated mice (Fig.8.7(1)a), which might suggest an *in vivo* activated state of these cells. Spontaneous gene expression for IFN-γ was detected in all freshly isolated spleen cells. In contrast to the protein assays (Fig.8.5; 8.6), analysis of cytokine gene expression showed increased Con A-induced mRNA expression for both IL-2 and IFN-γ in the IL-2/vector treated mice compared with the untreated group (Fig.8.7(1)c). The results do not include the vector control group because of accidental loss of the RNA sample during the extraction procedure. Therefore it is not clear whether the increase of mRNA expression was due to IL-2 treatment or simply a vector effect. In addition, since expression of β-actin mRNA in these samples was not included in the assay, the results are not intended for much quantitative comparison.

In the 19-week old mouse groups, IFN-γ mRNA was detected in freshly isolated spleen cells from the IL-2/vector treated, vector treated and untreated mice with slightly increased levels in the two treated groups (Fig.8.7(2)a), but it disappeared after 6 hours of culture without Con A (Fig.8.7(2)b). Spontaneous mRNA expression for IL-2, IL-4 and IL-6 were not detected in cells freshly isolated or cultured without Con A, except some weak expression of IL-6 gene (just identifiable on the gels) in both of the treated groups (Fig.8.7(2)a,b; II & III). After 6 hrs of Con A stimulation, the spleen cells from the 3 groups of mice had readily detectable mRNA for IL-2, IFN-γ, IL-4 and IL-6 (Fig.8.7(2)c). However, a slightly reduced expression of mRNA for these 4 cytokines by the Con A-stimulated spleen cells was observable in both IL-2/vector treated and vector alone treated mice in comparison with the untreated controls.

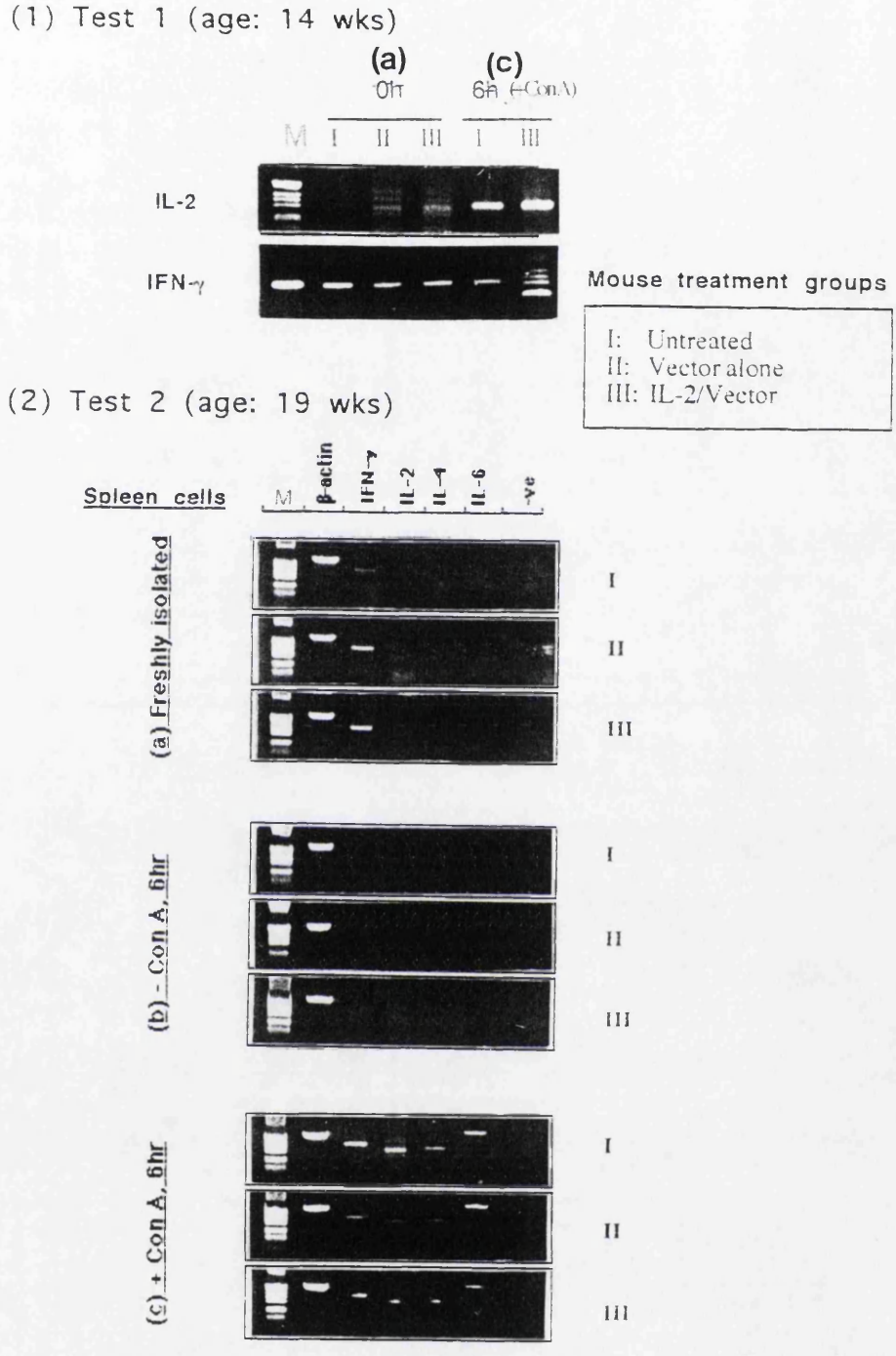


Figure 8.7 PCR analysis of the effects of IL-2 treatment on cytokine mRNA expression in MRL/*lpr* mice. Total RNA extracted from spleen cells from 14 (1) and 19 (2) weeks old mice freshly isolated (a), cultured without Con A (b, 19-wks only) and with Con A for 6 hours (c) were reversed transcribed into cDNA. Specific cDNA coding for IFN-γ, IL-2, IL-4, IL-6 and β-actin was amplified by PCR technique for 30 cycles using pairs of primers listed in Materials and Methods.

iii) Con A-induced T-cell proliferative response

Figure 8.8 shows a time course of Con A-induced proliferative responses of spleen cells from the treated and untreated mice at ages of 14 and 19 weeks. Splenic T-cells from both the IL-2/vector treated and vector alone treated mice showed reduced proliferative responses to Con A, compared to the untreated mice. The suppression was observable in the 14-week old group (Fig.8.8a) and became obvious in the 19-week old group (Fig.8.8b). No significant difference was observed between groups in the thymidine uptake by unstimulated cells (day 0).

8.3.3 Effects of IL-2 on autoantibody production and kidney pathology

To monitor effects of IL-2 treatment on lupus disease progression, serological and renal pathological changes in the IL-2 treated and control MRL/*lpr* mice were studied. Serum levels of autoantibodies (IgG) were measured by specific antibodies to ss and dsDNA by ELISA methods. For renal immunohistopathological and histophological studies, the procedure including tissue section preparation and scoring criteria was the same as that described in Chapter 7. The histopathological assessment was kindly done by Dr GBM Lindop, in the University Department of Pathology. Since this was entirely a subjective judgement, a repeat examination of all of the sections was carried out one week after the first assessment, and results from the 2 assessments compared for reproducibility. Among 23 kidney sections examined, 14 (58.3%) of them were scored exactly the same, and the rest had only a one plus difference, in 2 separate assessments done on different dates.

There were considerable individual variations of the serum levels of autoantibodies to ss and dsDNA in the mice (Fig.8.9). The results showed that the mean serum level of anti-ssDNA was higher in both of the treated groups compared to

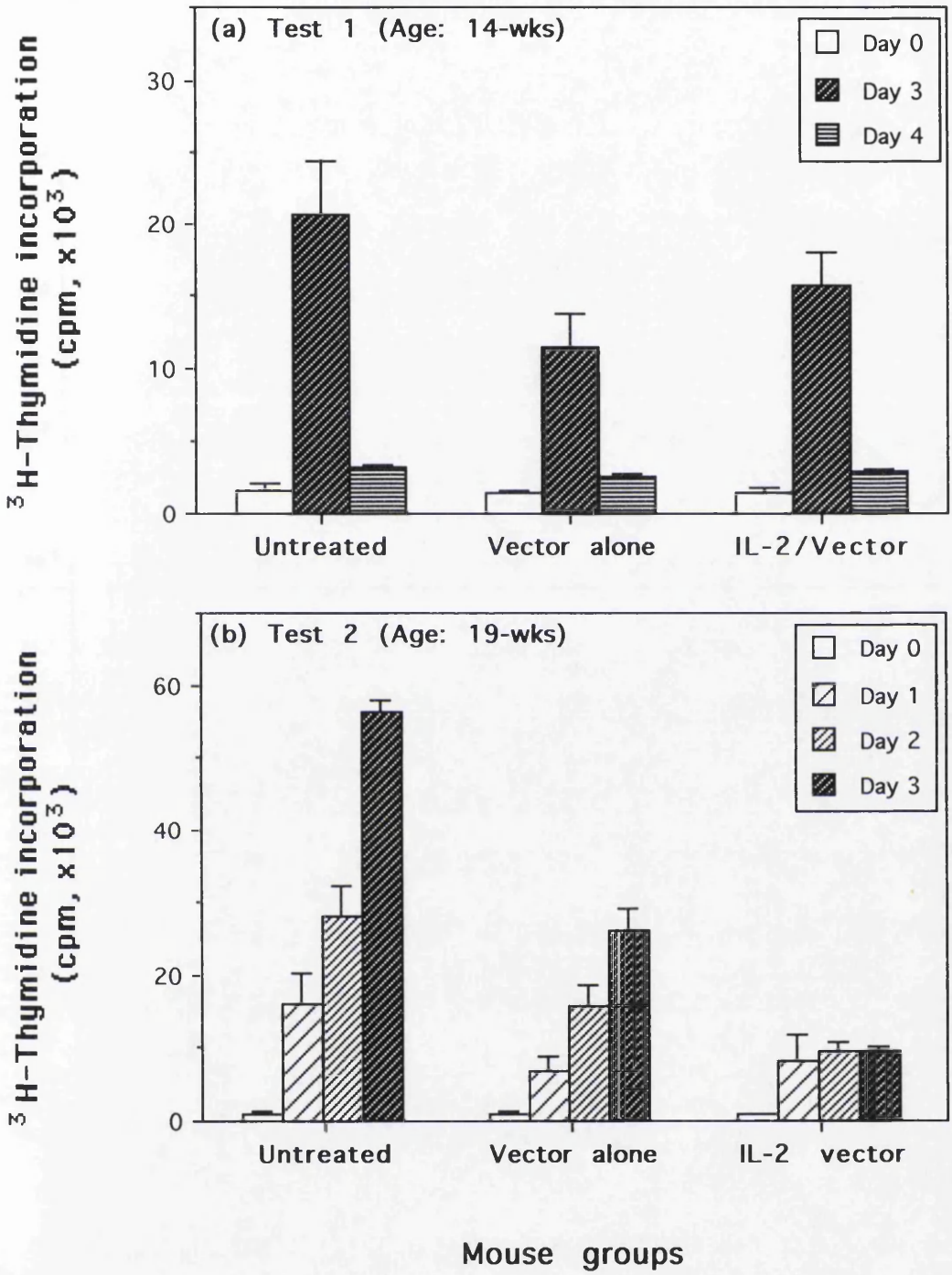


Figure 8.8 Effects of IL-2 treatment on T-cell proliferative responses in MRL/*lpr* mice. Mouse spleen cells from 3 to 5 mice in each group, treated with IL-2 transfected *S.typhimurium* or with the bacterial vector alone for 4 or 6 doses (at age of 14 and 19-weeks respectively) and the untreated controls, were pooled and cultured (10^6 cells per well) in the presence of Con A (2.5 μg/ml). Cell proliferation was measured by ^{3}H-thymidine incorporation at daily intervals. Data expressed are means (±SD) of triplicate cultures (note scale differences).

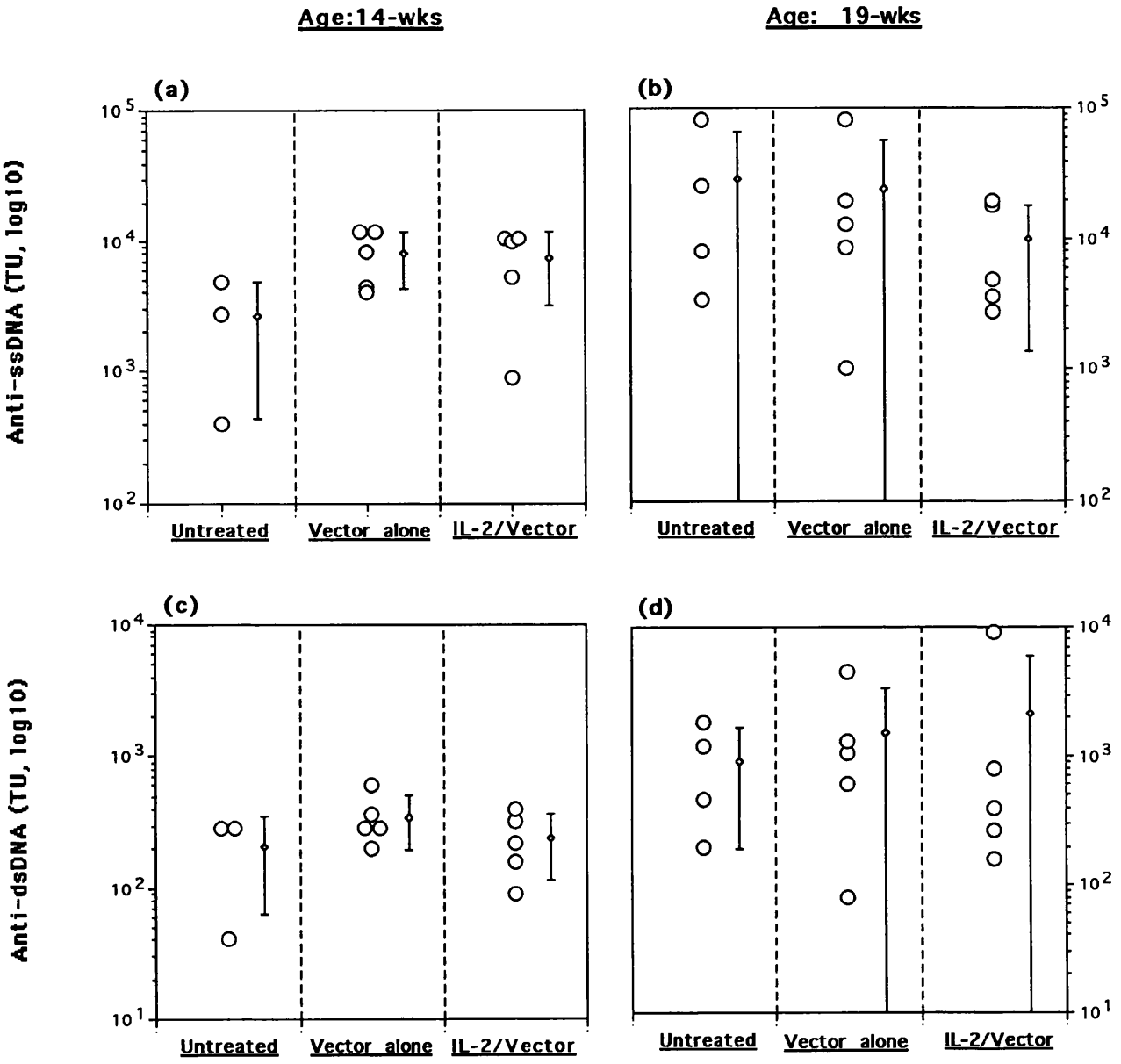


Figure 8.9 Serum anti-ssDNA and anti-dsDNA antibody levels in MRL/*lpr* mice after *in vivo* IL-2 treatment. Serum samples were taken from MRL/*lpr* mice in the IL-2/vector or vector alone treated groups, and the untreated control group at 14 (a, c) and 19-wks (b, d) of age. Serum levels of antibodies to ssDNA (a, b) and dsDNA (c, d) were measured by ELISA. The data are expressed as titration units (TU) obtained from individual mice as well as the mean values (±SD) from 3 to 5 mice of the same group. All differences between the IL-2 treated and the two corresponding control groups are not statistically significant ($p > 0.05$).

the untreated group at 14-weeks, although the anti-dsDNA was similar between the groups. In the 19-week mouse groups, the mean serum level of anti-ssDNA was lower, but anti-dsDNA was higher, in the IL-2 treated mice compared to the vector treated and untreated controls. However, due to the individual variations, the differences are not statistically significant ($p > 0.05$). In addition, it was also noted that, in the IL-2-treated mice of 19-week old group, there were 1 or 2 out of 5 mice showed significantly higher serum levels of anti-ssDNA (Fig.8.9b) and anti-dsDNA (Fig.8.9d) than the rest of mice in the same group.

Degrees of kidney damage indicated by the 'kidney pathology index (KPI)' were also variable among individual mice of each group except the untreated mice at 19-weeks (Table 8.2). In the 14-week old mice, KPI varied between 0 to 4 in the untreated group, 0 to 5 in the vector treated group and 0 to 3 in the IL-2/vector treated group, with no statistically significant difference between groups. In the 19-week old mouse groups, however, KPI was significantly lower in the IL-2 treated mice compared to the untreated controls ($P = 0.05$), but not if compared to the vector alone treated mice ($P > 0.05$). Mice in the vector treated group showed considerable variation between individuals in their kidney pathological changes (KPI: 0 to 5 pluses). Some of these mice showed severe glomerulonephritis while others in the same group were free of the pathological changes. Figure 8.10 shows representative pictures of the mouse kidney histology in the 19-week old mice, with contrast between the reduced renal pathology in the IL-2 treated group (a, b) and the severe glomerulonephritis in the untreated control group (c, d). The pictures shown in Figure 8.10(c) and (d) in the vector treated group are selected ones from a mouse which had most severe renal damage (+++++) in the group.

Results from the immunohistopathological studies are shown in Table 8.2 with no clearly cut difference between groups, except a relative low C3 staining observed in the IL-2 treated mice at 19-weeks compared to the vector treated and untreated mice. In

Age	Mouse groups	Auto-Ab Titre (IgG, TU)		Kidney pathology		
		anti-ss	anti-ds	***KPI***	IgG	C3
14-wks (Test 1)	I (Untreated)	400	40	**++++**	++++	+++++
		2700	290	-	+++	++++
		4800	290	-	++	++
		(NT)*	(NT)*	(NT)*	(NT)*	(NT)*
	II (Vector)	4000	290	**+++++**	++++	+++++
		4400	600	**++**	++	++++
		8200	290	**+**	+++	+++++
		12000	200	**+**	++	+++
		12000	370	-	++++	+++++
		(NT)*	(NT)*	(NT)*	(NT)*	(NT)*
	III (IL-2)	900	90	**+++**	+++++	+++++
		10500	330	**++**	++	++
		10500	160	**+**	+++	+++
		10000	400	-	++	++++
		5200	220	-	+	+++++
		(NT)*	(NT)*	(NT)*	(NT)*	(NT)*
19-wks (Test 2)	I (Untreated)	3400	200	**+++++**	+	++++
		8000	1200	**+++++**	+++++	+++++
		81000	460	**+++++**	++	+++++
		26000	1800	**+++**	++	++++
	II (Vector)	1000	80	**+++++**	+++++	+++++
		8500	4600	**++**	++	+++++
		20000	1300	-	+++	+++++
		81000	1050	-	+	+++
		13000	600	(NT)**	(NT)**	(NT)**
	III (IL-2)	18000	9000	**++**	++	+++
		4800	270	**+**	++++	+++
		3600	400	**+**	+	++++
		2700	160	(NT)*	(NT)*	(NT)*
		20000	810	(NT)**	(NT)**	(NT)**

(NT)*: not tested, died due to feeding prdcedure, no lupus symptom
(NT)**: not tested, killed due to severe lupus disease

Table 8.2 Comparison of autoantibody production, renal immune complex deposition and pathological changes in the IL-2 treated MRL/*lpr* mice. Mouse serum antibodies to ss and dsDNA were measured by ELISA and expressed as titration units (TU). Kidney sections were prepared from individual mice for light and immunofluorescence microscopic examinations (see Methods). Degrees of renal pathological changes were scored, zero to 5 pluses on a blind basis, according to the severity of kidney damage (KPI, Kidney Pathological Index); intensity of immunofluorescent staining for kidney deposition of complement component 3 (C3) and immunoglobulin G (IgG). The data shown are intended to illustrate the relationship between KPI (shown as bold) and other parameters of changes in individual mice corresponded.

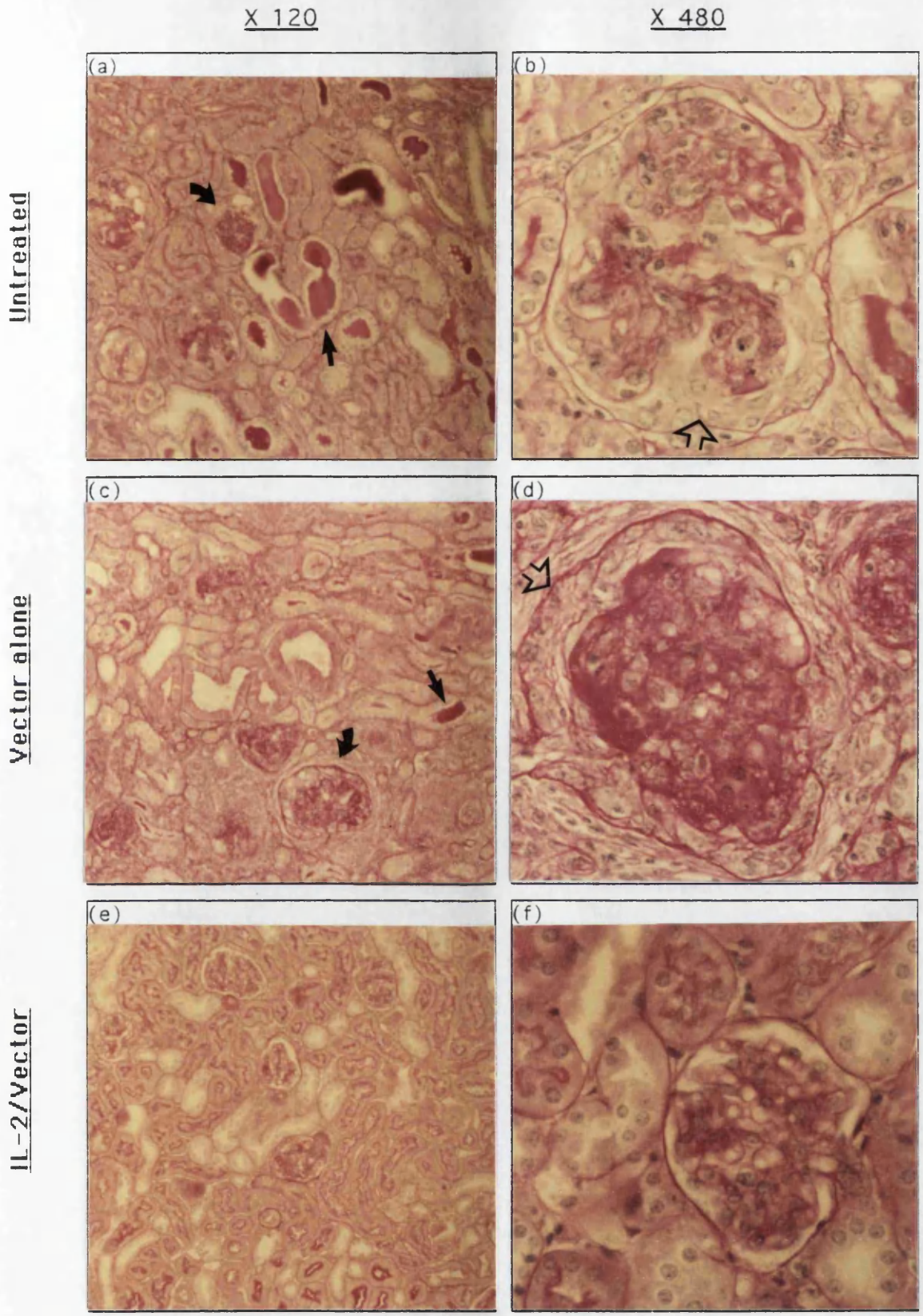


Figure 8.10 Renal histopathology. Representative photomicrographs of kidney sections from MRL/*lpr* mice (19 week old) showing severe kidney damage (+++++) in the two control groups (a-d) in comparison with the relatively normal kidney structure (+) in the IL-2 treated mice (e-f). (a, c) pictures at low power (x120) showing severe glomerulonephritis (↘) and protein casts in the tubules (↑); (b, d) pictures at high power (x 480) showing details of the damaged glomeruli including their enlargement and structural change, cellular proliferation and Bowman's capsule thickening, the 'crescent' like changes (⌐).

addition, as shown in Table 8.2, there is no any correlation between serum autoantibody levels, including both anti-ssDNA and anti-dsDNA, and the pathological changes in the kidneys of the individual mice.

8.4 Discussion

Delivery of exogenous IL-2 *in vivo* did not restore the ability of lupus T-cells to produce IL-2. On the contrary, mice given exogenous IL-2 showed even further suppressed IL-2 activity compared with untreated MRL/*lpr* mice. Since the suppression was also observed in the mice treated with vector alone, it was probably due to a feedback suppressive mechanism of the host to *Salmonella* challenge. As a secondary effect of IL-2 hypoactivity, this may also explain the proliferative hyporesponsiveness of the T-cells after the treatment, as well as the decreased mRNA expression for the other T-cell derived cytokines. Although relatively increased IL-2 mRNA expression was observed in the IL-2 treated mice at 14-weeks compared to untreated controls, it is not clear if this was also due to the "vector effect". However, levels of IL-2 activity in the culture supernatants of the Con A-stimulated cells were still low, suggesting that the lack of endogenous IL-2 is not merely due to a defect in gene activation. There might exist some other defects of the T-cells at post-transcriptional levels, such as protein synthesis or secretion.

The significance of IL-2 treatment on IFN-γ production is not clear. In contrast to its *in vitro* effects, IL-2 treatment effectively depressed the ability of T-cells to produce IFN-γ. The level of this lymphokine in culture supernatants of Con A-stimulated spleen cells from IL-2 treated mice was about 3 to 4 fold lower compared to both vector treated and untreated groups. It was not simply due to low IL-2 activity in the cell culture because IFN-γ was not reduced in the vector treated group in spite of similar level of IL-2 in the culture (Fig.8.5b). In addition, the suppression did not occur at 14 weeks, but at 19 weeks when peak disease activity developed in the control groups

suggesting the importance of the immune suppression mechanism in relation to outcome of the disease.

Similar to the observations in the study of TGF-β treatment, individual variation has been a problem in judging effects of the cytokine delivery on the mice, particularly in the pilot studies of such small scale. The variations may be due to differences in the susceptibility of the mice, such as differences in the state of immunisation to *Salmonella* Carrier et al. (1992) or stomach pH of the mice (Schorr *et al.*, 1991). However, in spite of these, the IL-2 treatment did appear to have some effect on reducing glomerulonephritis and other renal changes in MRL/*lpr* mice. It is encouraging to note that the IL-2 treatment brought about a significant reduction of the abnormal DN cells which characterise MRL/*lpr* disease. It reduced the size of spleens and total number of splenic T-cells indicating the effect on blocking lymphadenopathy. This might also explain why these mice had developed lymphoid tumours to a less degree compared to the control groups. The results have not only confirmed the *in vitro* observation that the proliferation of DN cells is not IL-2 dependent, but also suggested a possible role of IL-2 in suppressing or eliminating these pathogenic and autoreactive cells. Although there was no correlation between serum anti-ssDNA or anti-dsDNA of IgG class and kidney pathology, complement activation might be another important factor determining the deposition of immune complexes. It addition, it is still to be determined whether there may be any effect on the IgM class.

In summary, the pilot study has shown encouraging effects of *in vivo* IL-2 treatment of lupus disease, confirming the previous study by Gutierrez-Ramos et al. (1990; 1991). However, the results were limited for a comprehensive interpretation of their clinical significance. For undertaking further investigations, larger numbers and different strains of mice would have to be used and other factors such as the optimal dose and the kinetics would need to be considered.

Chapter 9

GENERAL DISCUSSION

Immune regulation is a complex network governing the functions of the immune system in a self-modulating manner. Numerous factors are involved in the network, interacting with, balancing or limiting each other. Abnormal expression of a particular factor during the development of disease can be the cause or outcome of another factor, or a reflection of the self-balancing processes in response to physiological feedback or pathological changes. Therefore, one must avoid a narrow interpretation of experimental data from *in vitro* or *in vivo* findings without considering all aspects of the system that may be involved.

9.1 The functional defects of lupus T-cells *in vitro*

The present study has demonstrated a variety of immunological defects in SLE. Many of them, particularly the functional abnormalities of T-helper cells, indicate a regulatory disorder confirming previous findings by other investigators. Lupus T-cells are hyporesponsive to mitogenic stimulation including an inability of T-cells to proliferate and failure to express high affinity functional IL-2 receptors. These together with the abnormal production of IL-2, IL-4 and IFN-γ *in vitro* appear to be a generalised activation defect. Lymphokine disorders reflect a malfunctioning state of T-helper cells. In spite of controversial views about its origin, the inability of T-cells to produce IL-2 *in vitro* has been consistently demonstrated in both lupus patients and the mouse models (Kroemer and Martinez-A, 1991). In the present study, the defect in the production of IL-2 was found in lupus mice of both NZB/W and MRL/*lpr* strains and the reduction was highly significant in all age groups, except the one-week old mice that lack CD4 cells (Chapter 3). Since the presence of IL-2 as a second signal is required for a complete activation of T-cells by Con A, a deficient IL-2 activity could be directly responsible for the activation failure.

The inability of T-cells to proliferate parallels with the kinetics of the disease, occurring much earlier in the MRL/*lpr* mouse which is a typical early-disease model than in the NZB/W mouse (late-life model). However, in both of the lupus mouse models, the inability of lupus T-cells to proliferate precedes the onset of clinical disease. This suggests the unlikelihood that such a defect results from autoimmune lesions, at least in young lupus mice. On the other hand, spleen cells from some young NZB/W mice had normal proliferative responses to Con A alone while IL-2 activities in the cultures were reduced. This might be explained by a threshold of minimum IL-2 activity that is required for a full proliferative response. An impaired T-cell proliferation is usually observed when IL-2 activity in the culture is below a certain level. Indeed, following a further suppressed IL-2 activity in the older mice, proliferative hyporesponsiveness of T-cells becomes established in both NZB/W and MRL/*lpr* mice. Taken together, it is probably sufficient to conclude that the inability of lupus T-cells to proliferate in response to Con A is a secondary event due to IL-2 deficiency. In confirming this, a demonstration that exogenous IL-2, but not IL-1, IL-4 or IFN-γ, is able to bypass such a defect *in vitro* has provided convincing evidence (Chapter 5). Addition of IL-2 during Con A activation not only restores fully the ability of lupus T-cells to proliferate, but also upregulates the expression of IL-2R and increases the frequency of IFN-γ secretors resulting in normal levels of IFN-γ secretion. This suggests that IL-2 deficiency may also play a primary role in the abnormal production of IFN-γ and the defective IL-2R expression.

However, the defect in IFN-γ production is not consistently established in both NZB/W and MRL/*lpr* mice until peak disease activity is developed at old age. It is not clear whether, in the young lupus mice, the seemingly normal production of IFN-γ in contrast to the reduced IL-2 activity might reflect an unbalanced functional activity within the Th1 phenotype, or this could be because of the complication of abnormal production of this cytokine by other cell types. An interesting finding described in Chapter 4 shows that the WEP999 Th1 cell clone expresses IL-2 but not IFN-γ, the

WEP988 Th2 cell clone expresses IL-4 but not IL-6, and the CTLL cell line expresses IFN-γ but not IL-2, IL-4 or IL-6 (Fig.4.13f). It is therefore also possible that there might be some T-cell subsets *in vivo* which secrete IL-2 or IFN-γ but not both. To clarify this, development of a 2-colour ELISPOT assay would be very useful.

The effects of exogenous IL-2 on upregulation of IL-2R expression *in vitro* were weaker in old mice than young mice, especially in the MRL/*lpr* strain. Addition of rIL-2 *in vitro* only partially restored the ability of the T-cells to express surface IL-2R with increased positive cell frequency but low density and delayed kinetics. This indicates that there may also be T-cells which are intrinsically defective in their ability to express IL-2 receptors. One of the explanations is that the IL-2 responders might be of T-cell subsets other than the abnormally expanded DN cells because the latter do not express IL-2R β-chains and are hence incapable of generating the high affinity αβ IL-2 receptors (Rosenberg, 1989; Tanaka *et al.*, 1993). To examine this further, it may be necessary to study the effect of IL-2 on purified DN cells. However, the results from cell phenotypic analysis have indicated that IL-2 does not promote but rather suppresses the expansion of the abnormal cell population both in the *in vitro* (Chapter 5) and *in vivo* (Chapter 8) conditions.

9.2 The nature of IL-2 deficiency in SLE: a primary or secondary defect ?

A focus of arguments on the defect in IL-2 production concerns its nature. One of the questions is how the *in vitro* data can be used to explain what may actually happen *in vivo*, especially when elevated serum levels of IL-2 were found in patients with SLE (Huang *et al.*, 1988). Huang's other finding (Huang *et al.*, 1986) which shows restoration of IL-2 production by resting cells in culture before stimulation has been regarded as evidence in favour of the view that the defective IL-2 production may be

secondary to autoimmune lesions. These T-cells, which might otherwise be too "exhausted" to respond to stimulation due to their post-activated state *in vivo*, are thought to be able to restore their responsiveness during the resting period. This has been proposed to be the most easily acceptable interpretation that explains both the hyperactive B-cell state and the mutually contradictory *in vivo* and *in vitro* data. Indeed, results from the present study show that the defect in IL-2 production is progressive with age and it is most prominent at the peak of clinical disease expression, suggesting some association with the autoimmune activity.

9.2.1 Are T-cells from lupus mice in an activated, post-activated or exhausted state ?

The 'exhaustion theory' is obviously based on the assumption that T-cells are polyclonally activated in SLE. This is because T-cell mitogens, such as Con A, stimulate T-cells non-specifically. Thus, if the defective mitogen-induced IL-2 production is due to T-cells being exhausted, it would have to be a generalised state of T-cell exhaustion.

However, what the 'theory' does not explain is, firstly, the normal responsiveness of the majority of T-cells from lupus mice of different ages to IL-2 upon Con A stimulation. These T-cells have the ability to respond to the signals generated by Con A providing a sufficient level of IL-2 is present. Secondly, the fact that there was no significantly increased responsiveness of the T-cells to IL-2 without the presence of Con A indicates that they were not in an ongoing activated stage either. Thus, even if some T-cells were in an activated or post-activated exhausted state, they would have to be a minority of the T-cell population. It is possible that T-cell activation *in vivo* in lupus disease is also clonally restricted, similar to the oligoclonal nature of autoantibody secreting B-cells (Stott, 1992). In addition, freshly isolated spleen cells from lupus mice do not spontaneously express IL-2, IFN-γ, IL-4 and IL-6 which are

characteristic T-cell derived lymphokines usually considered as cell activation markers. It would be difficult to interpret that the IL-2 secretors are in a post-activated state because of a non-specific T-cell activation while the majority of other T-cells are apparently resting.

The pathogenic DN T-cells which are a characteristic feature of MRL/*lpr* mice are in a state of active proliferation. However, these cells lack the ability to express IL-2R and can proliferate in the absence of IL-2. Therefore, the expansion of this cell population is unlikely to have resulted from IL-2 hyperactivity *in vivo*. On the other hand, it is obvious that the defective IL-2 activity in young lupus mice, including young MRL/*lpr* mice with normal T-cell phenotypes, is not due to the expansion of DN cells. Lymphoid cells from *lpr* gene-bearing mice are known to produce spontaneously factors other than IL-2, which induce B-cell differentiation and immunoglobulin synthesis (Prud'homme *et al.*, 1983; Dobashi *et al.*, 1987; Ashiba *et al.*, 1987a,b). More recent studies have also shown that double negative T-cells isolated from MRL/*lpr* , NZB/W and NZB/SWR mice (Datta, 1989), as well as from patients with active SLE (Shivakumar *et al.*, 1989) can secrete soluble factors providing help to B-cells necessary for the production of pathogenic autoantibodies. However, the DN cell-derived factors responsible for the autoantibody production, and the factors that control the proliferation of the DN cells have still not been well characterised. Interestingly, exogenous IL-2 inhibits the growth of the DN cells while allowing those phenotypically more mature, single positive cell subsets to expand. My results support Prud'homme's view that defective IL-2 production might be a necessary prerequisite for the increased production of B-cell differentiation factor(s), reflecting an imbalance in cytokine regulation.

9.2.2 The role of co-stimulatory factors in lupus T-cell activation

The present study has also investigated the role of costimulators and accessory cells in the defective T-cell activation in lupus mice. Monocytes from both SLE patients and the lupus mouse models have reduced activation with abnormally low IL-1 secretion (Linker-Israel *et al.*, 1983; Dinarello *et al.*, 1989; Doelly *et al.*, 1990; Steinberg *et al.*, 1991). The IL-1 defect has been detected in MRL mice at birth (Levine *et al.*, 1993). Such IL-1 deficiency may underlie IL-2 secretion abnormalities, because of the important role of the APC-derived cytokine as a costimulatory factor in antigen-dependent T-cell activation. However, addition of r-human IL-1 did not bypass the T-cell activation defect in either NZB/W or MRL/*lpr* mice, which confirms the previous study in SLE patients by Linker-Israel et al. (1983). Replacement of adherent cells from H-2 compatible normal mice also failed to restore the ability of T-cells from MRL/*lpr* mice to proliferate, to secrete IL-2 and to express IL-2R in response to Con A, indicating that the defect responsible for the abnormal T-cell activation exists in the non-adherent cells (Chapter 4). Further study is needed to answer the question whether there might be a defect in providing co-stimulatory signals necessary for T-cell activation through the CD28/B7 (Norton *et al.*, 1992) or CTLA-4/GL1 (Hatchcock *et al.*, 1993) interactions. However, although it is possible that the defect could be in the B-cell compartment which provides B7 and GL1 molecules, DN T-cells isolated from MRL/*lpr* mice do not lack CD28 expression (Giese *et al.*, 1993; Clements *et al.*, 1993).

Another point which has been taken into consideration is whether the effects of exogenous IL-2 on T-cell activation observed in lupus mice might have been due to a re-activation of anergised cells. It is possible that autoreactive cells in the autoimmune mice are in an anergised or refractory state (Giese *et al.*, 1993; Clements *et al.*, 1993), rendered by the immune system to avoid immune responses to self. Clonal anergy which results from the absence of co-stimulatory signals may be reversed by IL-2-driven cell division (Jenkins, 1992). However, the present study has shown that the lupus spleen cells, especially the DN cells from MRL/*lpr* mice, did not proliferate in

response to exogenous IL-2, and IL-2-treated MRL/*lpr* mice had significantly reduced DN cells both in terms of percentage and total cell number. Moreover, addition of IL-2 to the cultures of spleen cells from the lupus mice did not increase the spontaneous secretion of autoantibodies to ss and dsDNA.

9.2.3 An unbalanced T-helper function or mechanisms of feedback suppression?

T-helper cells exhibit reciprocal negative regulation between Th1 and Th2 phenotypes by secreting distinct soluble cytokine mediators. This is probably one of the important mechanisms that the normal immune system relies on to maintain self-balance. However, in situations where cytokines or other functional activities of either of the phenotypes are abnormally produced, the balance may be broken leading to pathology (Romagnani, 1990). Alternatively, where an intact immune system has the capacity to regulate itself efficiently, a new balance may be reached instead to avoid pathology. In SLE, the suppressed IL-2 activity suggests a deficient function of Th1 phenotype. This is accompanied by a concomitant hyperactivity of B-cells that indicates an increased B-cell help. Th2 cells produce IL-4, IL-5 and IL-6 which are known as B-cell growth or differentiation factors, and IL-10 which has been given the name of 'cytokine synthesis inhibitory factor' (Moore *et al.*, 1990). Since both IL-4 (Brooks *et al.*, 1992) and IL-10 (Malefyt *et al.*, 1991) may down-regulate IL-2 activity via their action on monocytes, an increased Th2 activity could well explain the immunological features in the disease. Results from the present study did not suggest an increased IL-4 activity in lupus mice but this does not rule out the role of IL-10 or other cytokines such as the DN cell-derived factors that enhance autoantibody production (Datta *et al.*, 1989), and the SLE serum factor that affects the IL-2 dependent CTLL cells. The balance between Th1 and Th2 functional activities is relative. An increased Th2 activity may also be due to a deficiency of the Th1 phenotype. In other words, enhanced B-cell help could be due not only to an increased

production of a given cytokine but also to a lack of negative control of its functional activity on target cells.

It is noteworthy that exogenous IL-2 was able to restore the ability of splenic T-cells from lupus mice to respond to mitogen in cultures at physiological doses, but increasing concentrations of IL-2 failed to neutralise the inhibitory activity of the serum factor, or factors, even at high doses. However, as the serum factor binds to T-cells, the inhibition is more likely to be through a direct effect on the cells rather than binding to IL-2. Thus, routine washing of cells before culturing could remove serum that contains such inhibitor. This might explain why pre-culture of the spleen cells increased responsiveness of T-helper cells to mitogen stimulation (Huang *et al.*, 1986), because the lasting effects of the inhibitor might be further limited during the pre-culture period. The factor which inhibits IL-2 activity on the proliferation of CTLL cells is elevated in patients with active SLE and some old lupus mice. It is possible that the serum factor might also bind to T-helper cells inhibiting their function. Therefore, the fact that IL-2 activity is suppressed progressively with disease activity may partially be explained by the factor. Thus, although the defect in IL-2 production that exists in young lupus mice prior to clinical disease could be intrinsic, some secondary suppressive mechanism that contributes to the abnormality at later stages is not excluded. In addition, the serum factor can also be detected at low levels in normal individuals suggesting its possible physiological role. It is likely that the rise in the factor(s) acting as an antagonist of IL-2 could be due to a mechanism of feedback suppression in response to some pathological changes such as autoimmune lesions. Its presence may reflect an active process, or a passive 'frustrated' attempt, at down-regulating the self-destructive process as suggested previously (Kroemer and Martinez-A, 1991). Consequently, it further suppresses IL-2 activity non-specifically.

9.2.4 The early onset and origin of IL-2 deficiency in lupus mice

The most strong evidence that counters the 'exhaustion hypothesis' is the early inability of T-helper cells from lupus mice to produce IL-2. This T-cell defect is detectable in mice of both lupus strains as early as 4 weeks of age or younger, which is at a stage long before the rise in serum autoantibodies, clinical symptoms and lymphoid hyperplasia. The CD4/CD8 T-cell ratio and total numbers of spleen cells in these young mice were similar to the normal control mice. Therefore IL-2 deficiency in lupus disease cannot simply be attributed to a "cell dilution effect" by the DN cells either. However, the results do not rule out the possibility that the absolute number of Th1 cells or IL-2 producers might be abnormal in these mice, and the "cell dilution effect" may have some role at later stages of the disease.

Taken together, many aspects of the study discussed so far have exclusively pointed out that the T-cell functional abnormalities are possibly due to a defect in the T-helper cell itself. To address the question concerning at which level the defect may exist, the study has been extended to an analysis of cytokine expression at the transcriptional level. Different degrees of reduction in cytokine gene expression for IL-2, IL-4, IL-6 and IFN-γ were observed, especially in the MRL/*lpr* mice. Although the quantitative information provided by the PCR technique is limited, the reduction of Con A-induced IL-2 and IL-4 mRNA expression in the 25-week old MRL/*lpr* mice was unambiguous. However, the question still remains as to whether such a reduction might be due to some immunosuppressive mechanism that regulates the transcriptional factors at the cell activation stage, or to a defect in the gene. In these 25 weeks old MRL/*lpr* mice, the lack of gene expression for IL-2 and other lymphokines could have been a cell dilution effect due to proliferation of the DN cells which were significantly increased in the old mice. In addition, it is not clear whether the abnormal cytokine

production was due to the lack of cytokine gene expression only, or if there is also a defect in the process of protein synthesis or secretion. To explore further, other studies including cytoplasmic expression of the cytokines, cellular signalling transduction and gene analysis, especially in young MRL/*lpr* mice before the lymphoproliferation occurs, will be needed.

In conclusion, the defective IL-2 production does not appear to be simply due to a 'passive T-cell exhaustion' or lack of co-stimulatory signals from adherent cells. The early onset of the IL-2 defect rather suggests the possibility of an intrinsic and phenotypically restricted T-helper functional defect.

9.3 The possible pathological significance of the T-helper cell functional defect in the development of lupus disease

To discuss the mechanism of the development of autoimmunity, one of the fundamental issues to be considered is why and how those self-reactive clones are allowed to be selected, activated and expanded in the first place. The presence of these cells, which should be otherwise eliminated or inactivated in an intact immune system, is the evidence that indicates the failure of self-tolerant mechanisms during the establishment and development of the immune system.

9.3.1 A role in the early establishment of lymphocyte repertoire ?

The early onset of IL-2 deficiency in lupus mice gives rise to the possibility that the T-helper functional abnormality might prevent the normal development of the lymphoid system. This refers particularly to immune regulatory events during the early establishment of the lymphocyte repertoire.

Differentiation and maturation of T-cell precursors occur in the thymus, and T-cells recognising self-components are deleted before maturity while T-cells responding to foreign antigens are exported to the peripheral T-cell pool (Marrack and Kappler, 1990). To establish a normal T-cell repertoire, T-cells are believed to undergo a vigorous process of selection in the thymus where the majority of cell clones are deleted due to their self-reactivity. The early T-cell development and process of T-cell maturation therefore depend critically on the functions of the thymus. However, a rapidly declining thymic function, known as the early thymic atrophy found in all lupus mice, is a prominent feature of murine lupus disease. In MRL/*lpr* mice, it is accompanied by the accumulation of the DN cells. It has been suggested that the DN cells are immature T-cells which are arrested at a stage of development similar to foetal thymocytes (Katagiri *et al.*, 1987). Since the *lpr* gene is associated with the Fas antigen which mediates apoptosis, the escape of these autoreactive cells to the peripheral circulation may be a result of a defect in negative selection (Matsumoto *et al.*, 1991; Watanabe-Fukunaga *et al.*, 1992; Watson *et al.*, 1992).

The role of IL-2 in the process of immunocyte selection is not fully understood, but recent evidence indicates that IL-2 is involved in the induction of thymocyte apoptosis (Migliorati *et al.*, 1993). IL-2 has been shown to induce an active process of cell death on immature mouse thymocytes *in vitro*. Therefore, a deficient IL-2 activity may interfere with the normal thymic functions impairing the mechanism of self-tolerance. In addition, a targetted disruption of the IL-2 gene in mice has been shown to lead to severe inflammatory lesions associated with the presence of immature B-cells in peripheral lymphoid organs (Sadlack *et al.*, 1993). Thus, it is conceivable that the potentially autoreactive T or B-cells that escape from normal selection process and are released into the peripheral circulation may seed a pre-determined immune repertoire with an autoimmune tendency.

9.3.2 Failure in maintaining other mechanisms of self-tolerance ?

Apparently, a failed mechanism of thymic selection does not itself explain the autoimmune responses in the periphery. The existence of potentially autoreactive cells which remain unresponsive in normal individuals indicates that some other mechanisms are also operational in dealing with those immunocytes which may be at the edge of self tolerance (Mitchison, 1993). Thus, conditions that allow the activation and expansion of autoreactive clones initiating autoimmune responses can be envisaged as a result of impaired mechanisms of post-thymic tolerance.

Suppression of immune responses as a phenomenon in immunology has been long observed and established in adoptive transfer experiments. It is believed to play an important role in the mechanisms of tolerance in the peripheral immune system. Although many cloned suppressor cells have been found to have no specific gene rearrangement or deletion in their TcR (Hedrick et al., 1985), as an alternative explanation, the phenomenon of suppression is now believed to be explained by the interaction of different soluble mediators or cytokines (Bloom *et al.*, 1992), such as IL-4, IL-10, IFN-γ and TGF-β, that deliver negative signals. Since IL-2 deficiency is primarily responsible for many abnormal T-cell functional activities in lupus mice, links between the deficient T-helper function, the lack of T-suppressor activity (Bresnihan and Jasin, 1977), and the commonly observed impaired cell-mediated immunity cannot be excluded. The effects of exogenous IL-2 on expansion of $CD8^+$ cells (Chapter 5), a phenotype traditionally associated with suppressor activities, suggests its role in maintaining immune suppression. It provides some explanation for the effect of IL-2 treatment that down-regulates the pathogenic immature DN cells in MRL/*lpr* mice. However, the mechanism of such an IL-2-mediated suppression, particularly the role of

IFN-γ that suppresses the Th2 phenotype, is not clear. It seems contradictory that IL-2 upregulated IFN-γ production *in vitro* while spleen cells from the IL-2-treated MRL/*lpr* mice had reduced IFN-γ production. Perhaps, the stimulatory effect of exogenous IL-2 on IFN-γ secretion may induce some mechanism of negative feedback *in vivo* or, in this case, the "exhaustion" mechanism may apply.

There is evidence that apoptosis is not limited to immature thymocytes. Normal mature splenic T-lymphocytes have been observed to undergo such programmed cell death too (Lenardo, 1991; Perandones *et al.*, 1993; Kabelitz *et al.*, 1993), although the regulatory mechanism might differ from that of the intrathymic process (Perandones *et al.*, 1993). It appears therefore that the activation-induced cell death can also occur in T-cells that have escaped intrathymic selection. Since mice bearing the *lpr* mutation are defective in Fas which mediates apoptosis, it has been recently suggested that the age-related accumulation of DN in the mice might be because cells die less rapidly than do their normal counterparts (Cohen, 1993). Therefore, Cohen used the term 'lymphoaccumulation' instead to describe what has been called 'lymphoproliferation' in these mice.

The role of IL-2 in peripheral apoptosis is controversial. It has been suggested that IL-2 programmes mature mouse T-cells for apoptosis and provides an essential second signal for anti-CD3-stimulated apoptosis of murine T-cell clones (Ucker *et al.*, 1992; Lenardo, 1991). However, Kabelitz and Wesselborg (1992) showed that such a T-cell death may occur following anti-CD3 signalling in the absence of exogenous IL-2. In addition, T-cell clonal anergy which does not involve clonal deletion can render cells unresponsive to self antigens or superantigens, but the mechanism of self-tolerance by the functional inactivation of autoreactive T-cells can also be overridden by the presence of IL-2 (Jenkins, 1992). On one hand, IL-2 may favour the tolerance process by mediating apoptosis, but on the other it might also prevent the induction of tolerance by different mechanisms, such as reversal of anergised lymphocytes. Thus, it

is probably inappropriate to conclude unilaterally that IL-2 would either favour or block the induction of tolerance without the consideration of particular circumstances. Like many other cytokines, IL-2 is an immune modulator with pleiotropic functions, and its inhibitory or stimulatory activity is often related to certain conditions as well as cell types. IL-2 induces apoptosis of immature thymocytes but withdrawal of IL-2 results, for example, in apoptotic changes of CTLL cells (Fadok et al., 1992). In addition, the serum IL-2 inhibitor which is increased in SLE patients has completely opposite effects on CTLL cells at different concentrations.

It is possible that, as a non-specific immune regulator, IL-2 could prevent or even reverse anergy by providing costimulation which is otherwise missing during the anergy induction. In the meantime, however, IL-2 may induce an active suppressive function by promoting $CD8^+$ T suppressor cells or other tolerant mechanisms which are otherwise lacking in a deficient immune system. Circumstances under which autoreactive clones are allowed to be activated will then depend on the nature of the original defect that determines the disease and balance of the regulatory mechanisms that influences it. It is also likely that the escape of these cells from the process of thymic negative selection is due to a deficient immune recognition, or the presence of some apoptosis-inhibitors (Perandones *et al.*, 1993). The active process of IL-2-mediated apoptosis in the thymus is known to require protein synthesis (Perandones *et al.*, 1993). An induction of the refractory DN cells (Giese *et al.*, 1993; Clements *et al.*, 1993) could be necessary for eliminating more effectively the hidden clones which are otherwise difficult for the immune control mechanisms to recognise. Although the functional inactivation of self-reactive cells may be an important complementary mechanism of tolerance in normal individuals, the nature of this type of tolerance is relatively passive. These anergised cells will remain potential risks, particularly in autoimmune situations where mechanisms of suppression are often absent. In other words, self-tolerance will not be properly maintained unless an intact immune regulatory network is established. Nevertheless, the regulatory mechanisms for

tolerance induction might differ from one another, but what is most important is the outcome of the regulation that may eventually limit, or escalate, autoaggression.

9.4 Approaches to treatment of SLE with new insights into the underlying mechanisms of autoimmunity

The ultimate aim of studying and understanding mechanism of a disease is to develop effective therapeutic approaches that may eventually cure the disease. In return, the outcome effects of a treatment of the disease can also help to gain a better understanding of the mechanism of the disease development. On the basis of the *in vitro* findings, the *in vivo* pilot studies of treatment of lupus mice by delivering TGF-β1 and IL-2 using *Salmonella* vectors have provided some preliminary but useful information. The issue is now how we can use this information to explain the disease and what we may learn from it.

As described in Chapter 7, TGF-β1 treatment *in vivo* inhibited functional activities of T-cells in both normal and lupus mice. In MRL/*lpr* mice, it seems to result in a delay in the disease kinetics. This might be attributed to the immunosuppressive function of TGF-β1 that has been suggested to have a cyclosporin A-like activity. Interestingly, a 'rebound' effect was observed later in which BALB/c mice had an enhanced immune responsiveness, and the MRL/*lpr* mice developed glomerulonephritis similar to controls and even increased levels of serum autoantibodies. It is possible that the repeated doses might have triggered the production of antibodies against the bacterial vector thus decreasing the effectiveness of the treatment. However, such a 'rebound' was not observed in the IL-2 treated mice who received two or three more doses in each of the corresponding groups. An alternative explanation may be that, in the BALB/c mice, the enhanced T-cell responsiveness including production of IFN-γ and cell proliferation at the later stage of treatment could reflect a negative feedback mechanism in response to the suppressive action of exogenous TGF-β1. Therefore,

the lack of such responses in the MRL/*lpr* mice might also indicate an incapacity for self-regulation.

The *in vivo* effects of IL-2 treatment of lupus mice were much in agreement with the previous study using recombinant IL-2/*vaccinia* viruses (Gutierrez-Ramos et al. 1990; 1991). This includes the phenotypic, serological and histological normalisation of autoimmune MRL/*lpr* mice. It is interesting that amelioration of autoimmune disease, as judged by the renal histopathology, was observed in MRL/*lpr* mice of both groups treated with either IL-2 or TGF-β1, however, the mice treated with IL-2 showed suppressed disease activity at a later stage than the TGF-β1-treated mice. Unlike the latter which had only a transient delay in disease onset, the reduced kidney damage in the mice after IL-2 therapy appeared to be more sustainable even at the peak of disease kinetics expressed by the control mice. Most notably, in agreement with the *in vitro* results, the IL-2 treatment of mice starting at 3 weeks of age resulted in a reversal of T-cell phenotypic abnormalities. It can be predicted that the mechanism of this IL-2-mediated suppression of the autoreactive DN cells could also be important during thymic selection in the early life of mice when the normal process of clonal deletion is taking place.

It must be pointed out again that the preliminary results are still to be confirmed in a future study. The main limitation of these results is probably the size of the experimental groups and the individual variations, which affect the statistical analysis of the data. The variations may be partially due to differences in the susceptibility of the mice. Individuals who are already immune to *Salmonella* would be expected to eliminate the organisms more quickly than the others. Using a similar model system, Carrier et al. (1992) have shown differences of IL-1β-mediated radioprotection effect between three routes of administration. They found that the effects of oral or i.p. treatment tended to be more variable than by the i.v. route of administration. It was

suggested that the i.v. route may lead to more effective colonisation of the *Salmonella* construct in the liver and spleen, generating a higher concentration of the cytokine. The present study used the oral route because it has the advantage of being simple, but it will be necessary to compare these other routes in future experiments. It is also possible that one of the factors which might account for the variations between individual mice could be the low pH in the stomach possibly preventing the penetration of the live bacteria in the gut. Therefore, neutralisation of the stomach pH in the mice before feeding them with the bacteria may be essential (Schorr *et al.*, 1991).

The "vector effect" observed in both the TGF-β1 and IL-2 treatments is however interesting. It is surprising to note that some of the vector alone treated MRL/*lpr* mice showed little or no sign of renal pathology while others suffered from severe glomerulonephritis. The reason for the 'vector effect' is not clear. Presumably, this is due to an effect of non-specific stimulation of the immune system by the vector, as might be induced by superantigen and bacteria-derived LPS. Production of endogenous cytokines by an activated endothelial reticular system could then be responsible. In fact, IL-2 itself is a non-specific immune regulator. The clue from this notion is that a general immune stimulation might trigger active immune suppressive functions which would require production of IL-2 and possibly other cytokines. IL-2 treatment of these IL-2 deficient mice might be able to restore or enhance such a process. Therefore, a good vector system which can deliver controllable physiological doses of the cytokine over a certain period is needed. However, before this approach of using *Salmonella* vector to deliver cytokines can be applied to human disease, a detailed analysis of this vector stimulation with particular attention to possible consequences of bacterial lysis which may release large amount of antigenic or mitogenic substances, is absolutely necessary.

Nevertheless, the hint from the finding that IL-2 therapy might have disease limiting rather than escalating effects is important because it calls our attention to the

need for fresh insights into the underlying mechanisms of autoimmune phenomena. Essential features of an intact immune system require both the ability to generate immune responses to foreign antigens and to maintain tolerance to self. The "Ying-Yang theory" (relatively 'Ying' refers to inhibition and 'Yang' refers to activation) on which traditional Chinese medicine has been long based emphasises the importance of an internal balance of the body system. In particular, the theory stresses the necessity of discriminating the nature of an imbalance between 'Ying' and 'Yang', where a disease could be due to either a hyperactivity or deficiency of 'Ying' or 'Yang', or the two. This is considered to be crucial for therapeutic strategies in deciding whether to suppress or to compensate one or the other, or both, so that a re-balance may be reached at an appropriate level. Autoaggression, which could be due to a deficient immune function that controls self-tolerance usually gives an impression of an overt immunity. Clinically, attention is often focused on developing strategies to suppress, though non-specifically and passively, the autoimmune responses using immunosuppressive agents rather than treatments that help patients to restore their own immune regulatory function. While immunosuppressive therapy temporarily suppresses the autoimmune responses in the recipients, it might further undermine their self-regulatory capability. The importance of this concept lies in the necessity that therapeutic efforts should be made to restore the active mechanisms of self-tolerance rather than a passive and non-specific immunosuppression, or to combine the two approaches. A strengthened immune system may also be beneficial if slow infections are the causes of some autoimmune diseases as proposed by Rook and Stanford (1992). The hypothesis is that, in other words, some autoimmune disorders could be just another form of expression of immunodeficiency.

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APPENDICES

I. REAGENTS AND CHEMICALS

i) Antibodies and antibody conjugates

Donkey anti-goat/sheep IgG
FITC-conjugated (*Sapu*)

Goat (Fab')2 anti-mouse C3
FITC-conjugated (*Cappel*)

Goat anti-mouse immunoglobulin
alkaline phosphatase conjugated (γ-chain specific, *Sigma*)

Goat anti-rabbit IgG
alkaline phosphatase conjugated (*Sigma*)

Hamster monoclonal antibody (Clone 145-2C11, IgG2)
anti-mouse CD3, FITC-conjugated (*Boehringer Mannheim, Germany*)

Mouse monoclonal antibody (Clone IE2, ascites)
anti-human IL-2 (*Biogen*)

Rabbit anti-human IL-2 (R005865, R004896)
antisera, IgG fractions or biotinylated (*The Laboratory*)

Rabbit anti-mouse IFN-γ (*Wellcome Lab, UK*)

Rabbit anti-mouse IgG (κ-chain specific, MR11 and MR13)
IgG fractions, HRP-conjugated or FITC-conjugated (*The Laboratory*)

Rabbit anti-mouse IgM (RC406/8/9)
IgG fraction, AP-conjugated, or FITC-conjugated (*The Laboratory*)

Rat monoclonal antibody
anti-mouse CD4, PE or FITC-conjugated (*Becton & Dickinson*)

Rat monoclonal antibody
anti-mouse CD8, FITC or PE-conjugated (*Becton & Dickinson*)

Rat monoclonal antibody (Clone AMT13, IgG2a)
anti-mouse CD25 (IL-2R α-chain specific), PE-conjugated
(*Boehringer Mannheim, Germany*)

Rat monoclonal antibody (Clone R46AT)
anti-mouse IFN-γ (*Wellcome Lab, UK*)

Rat monoclonal antibody (Clone BVD6-24GA, IgG1)
anti-mouse IL-4 (*Pharmingen*)

Rat monoclonal antibody (Clone XT22.11)
anti-mouse TNF-α (*Wellcome Lab, UK*)

Sheep anti-mouse IgG
FITC-conjugated (*Sapu*)

ii) Buffers and solutions

Borate-buffered saline:
0.1 M Boric acid, 0.025 M di-sodium tetraborate (Borax), 0.075 M sodium chloride in distilled water, pH 8.3-8.5.

Carbonate coating buffer:
0.1 M sodium carbonate in distilled water, pH 8.2.

Carbonate-bicarbonate coating buffer:
14.15 mM sodium carbonate 34.88 mM sodium hydrogen carbonate, 3.08 mM sodium azide in distilled water, pH 9.6.

Blocking buffers:
2%, 1% and 0.5% (w/v) BSA, or 20%, 10% and 5% FCS, in PBS or BBS as indicated in different assay protocols.

Greiss solution:
obtained by mixing equal volume of 0.1% alpha-napthyl-amine in distilled water (Solution A) and 1% sulphanilamide in 5% phosphoric acid (Solution B). Kept dark.

McIlwaine's buffer:
17.9 mM citric acid, 64.2 mM disodium hydrogen orthophosphate dihydrate, pH 6.0.

Nitrate-nitrite conversion reaction buffer:
5 mg/ml NADPH, 41.5 mg/ml FAD, 0.5 M potassium dihydrogen orthophosphate and 35 mg/ml nitrate reductase (*Sigma*)

Phosphate-buffered saline:
0.15 M sodium chloride, 1.98 mM potassium dihydrogen orthophosphate, 7.96 mM disodium hydrogen orthophosphate, pH 7.3.

Tris-buffered saline:
0.05 M tris, 0.15 M sodium chloride in distilled water, adjusted to pH 8.0 with HCl.

Tris-Tween washing buffer:
0.2 M tris-HCl, 0.19 M sodium chloride, 0.05% (v/v) Tween 20, adjusted to pH 7.4 with HCl.

Urea buffer:
10% (w/v) SDS, 8 M urea, 10 mM Tris in ditilled water, adjusted to pH 7.0 with HCl and sterilised by autoclaving.

20x SSC (Sodium chloride, sodium citrate)
3 M sodium chloride, 0.3 M trisodium citrate in distilled water, adjusted to pH 6.5 with HCl.

Acetic buffer:
0.05 M sodium acetate in ditilled water, adjusted to pH 5.0 with acetic acid.

iii) Cytokines

Recombinant human IL-1β (*Wellcome Lab., UK*)

Recombinant human IL-2 (*Glaxo*)

Recombinant murine IL-4 (*Immunex, USA*)

Recombinant murine IFN-γ (*Bender Wien, Austria*)

Recombinant murine TNF-α (*Bender Wien, Austria*)

Recombinant murine TGF-β2 (from Dr Joe Carlino, *Palo Alto, USA*)

iv) Enzymes

S1 nuclease (*Boehringer Mannheim, Germany*)

Trypsin-EDTA (For resuspending adherent cells)
0.025% EDTA (*Sigma*) in calcium and magnesium-free tris buffered saline containg 0.5 mM Na_2EDTA.

v) Enzyme substrates

For ELISA assays:
Orthophenylene diamine (OPD) HRP substrate (*Sigma*)
2.21 mM OPD dissolved in McIlwaine's buffer, and 0.032% (v/v) hydrogen peroxide (added immediately before use, kept in dark).

TMB Microwell Peroxidase Substrate solution (*Dynatech*)

p-NPP alkaline phosphatase substrate tablets (*Sigma*)
1 mg/ml dissolved in the p-NPP substrate buffer.

For ELISPOT assays:
BCIP/NBT alkaline phosphatase substrate
3 mg of 5-bromo-4-chloro-3-indoyl phosphate (BCIP, *Sigma*), and 6 mg of p-nitroblue tetrazolium chloride (NBT, *Sigma*) (dissolved separately in 0.2 ml dimethylformamide), in 20 ml 0.1 M NaHCO3 containing 1 mM $MgCl_2$ (pH 9.8).

AEC HRP substrate
5 mg of 3-amino-9-ethyl carbazole (AEC, *Sigma*) (dissolved in 0.4 ml dimethylformamide), in 19 ml 0.05 M NaOAc (pH 5.0).

vi) Media

RPMI 1640 medium (*Gibco*)
For cell culture, the complete culture medium was RPMI 1640 containing 2 mM L-Glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin, and foetal calf serum at 5%, 10% or 20%. (stored at 4 ^{0}C)

HBSS (Hanks' balanced salt solution, with pyruvate, *Gibco*)

DMEM (Dulbecco's Modified Eagle's Medium)
Containing 1 mg per ml glucose, 100 units/ml penicillin, 100 ug/ml streptomycin, and 10% foetal calf serum, stored at 4 ^{0}C (*Gibco*).

Medium for freezing cells
contained 15% (v/v) DMSO (Koch-Light Ltd.) in heat-inactivated foetal calf serum.

vii) Molecular biological reagents

Agarose (Electrophoresis grade, *Bethesda Research Laboratory, USA*)
dissolved in 0.5 x TBE buffer containing 0.5 μg/ml ethidium bromide.

Anhydrous isopropanol (*Sigma*)

dATP, dCTP, dGTP and dTTP (*Gibco BRL*)

1kb DNA ladder (*Gibco BRL, UK*)
m.w. markers giving bands at 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517, 506, 396, 344, 298, 220, 201, 154, 134 and 75bp

DTT (*Gibco BRL*)

Moloney murine leukemia virus (M-MLV) reverse transcriptase (*Gibco BRL*)

Oligo-dT_{15} (*Promega*)

10x PCR buffer (*Promega*):
15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl, and 1% Triton X-100.

Primer pairs for mouse IL-2, IFN-γ, IL-4, IL-6 and β-actin:
synthesised by *Genosis* (*USA*). For the oligonucleotide sequences see Tables 2.1, 2.2 (Materials and Methods).

Random hexanucleotides (*Gibco BRL*)

5x Reverse transcrition buffer (*Gibco BRL*):
250 mM Tris HCL (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$.

RNasin^R RNase inhibitor (*Promega*)

RNAzol^{TM} B (*Biogenesis*)

0.5x Tris-borate-EDTA electrophoresis (TBE) buffer:
0.045 M Tris-borate, 0.001 M EDTA in distilled water, pH 8.0.

TE (Tris-EDTA) buffer:
10 mM Tris, 1mM EDTA in double distilled water, pH 7.0.

Thermus aquaticus thermostable (Taq) DNA polymerase (*Promega*)

viii) Other serum products

Foetal calf serum (*Gibco*)
inactivated for cell culture by heating at 56 ^{0}C for 45 minutes.

Bovine serum albumin
Fraction V, 96-99% albumin (*Sigma*)

Normal goat serum (*Sapu*)

Normal sheep serum (*Sapu*)

vii) Other reagents

Avidin AP-conjugated (*Vector Laborarory*)

Avidin HRP-conjugated (*Vector Laborarory*)

Avidin PE-conjugated (*Vector Laborarory*)

Biotin (long arm, *Sigma*)

Concanavalin A, Type IVs(*Sigma*)

Calf thymus DNA (*Sigma*)

Complete Freund's Adjuvant (*DIFCO Laboratories*)

Fluorochrome isothiocyanate (*Sigma*)

Incomplete Freund's Adjuvant (*DIFCO Laboratories*)

2-Mercaptoethanol, cell culture grade (*Sigma*)

Penicillin/Streptomycin (*Gibco*)

Phytohaemagglutinin (*Wellcom Lab. UK*)

Poly-L-lysine (*Sigma*)

Protein A column Sepharose 4B fast flow (*Sigma*)

Propidium iodide (*Sigma*)

Tritiated thymidine (*Amersham International, UK*)

G-25M Sephadex column (*Pharmacia*)

Sheep liver powder (*Sigma*)

Donkey liver powder (*Sigma*)