A Transgenic Mouse Model of Systemic Lupus Erythematosus

by

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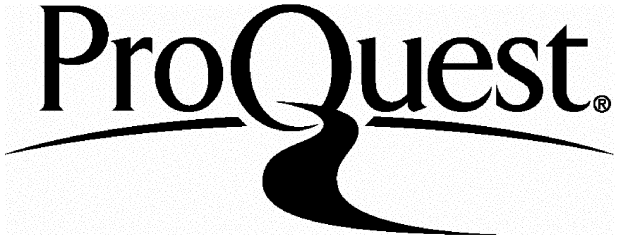

Abstract

Systemic lupus erythematosus (SLE) is a relatively common autoimmune syndrome characterised by widespread organ damage secondary to autoantibody production against a broad range of self antigens. The disease primarily affects women and varies markedly between patients both in the pattern of organ involvement and the severity of damage to involved organs. SLE carries a significant mortality primarily as a result of antinuclear antibody mediated glomerulonephritis. The factors triggering and the mechanisms underlying autoantibody production are the subject of intense research. We have developed an IFN-γ transgenic mouse model of SLE and in this thesis I provide evidence that the skin immune system is at least capable of playing the central role in disease pathogenesis. In addition, dissection of the mechanisms involved in autoantibody production in these mice suggests that antinuclear antibodies arise via an antigen driven T cell-dependent process. Apoptotic keratinocytes may act as the source of self nuclear antigen and the mechanism of autoantibody production is relevant to the naturally occurring human disease. We investigated the efficacy of anti-apoptotic agents in IFN-γ transgenic mice and show that these agents may profoundly alter the pattern of autoantibody production in this lupus model. Our findings may aid in the development of new therapeutic options for this potentially devastating disease.

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Chapter 1

Introduction

1.1 Systemic lupus erythematosus

1.1.1 The clinical syndrome

Patients with SLE are predisposed to a staggering range of pathologies, the most prominent of which are listed in Table 1.1. These disease manifestations can occur in isolation or in practically any combination. In addition, the antinuclear autoantibodies characteristic of lupus can occur in a variety of other disorders, albeit at a lower frequency. It is not surprising, therefore, that the diagnosis is often missed and in other cases patients are incorrectly labelled as suffering from SLE (Lahita, 1999). Such confusion has implications not only for patients but also for epidemiological and pathological studies of the disease. The American College of Rheumatology (ACR) has designated eleven criteria for the diagnosis of SLE (Table 1.2) (Tan et al, 1982). The presence of four or more of these criteria makes the diagnosis of SLE with 96% specificity and sensitivity (Lahita, 1999). Patients who fail to meet four of these criteria are said to have a "lupus-like syndrome".

Although the disease is tremendously variable in its manifestations, some occur at sufficient frequency (Table 1.1) to allow one to describe a typical case scenario. The patient, usually female, presents with oligoarticular arthritis or arthralgia. On clinical examination an erythematous rash (Figure 1.1) and peripheral lymphadenopathy may be prominent. Systemic symptoms are common: primarily fatigue, weight loss and fever. Laboratory investigation will show a positive fluorescent antinuclear antibody test (FANA) and leukopenia/lymphopenia (Hochberg et al, 1985). Clinically significant renal involvement has been reported in 30% to 75% of patients with SLE (the higher published figures may be due to "referral bias" in specialist centres) and the severity of the kidney lesions remains the key determinant of overall disease prognosis (Huong et al, 1999).

Table 1.1. Frequency of clinical manifestations of SLE

Manifestation	Frequency (%)
Arthritis	80-95
Cutaneous	80-90
Malar rash	20-60
Alopecia	37-45
Photosensitivity	10-90
Dermal vasculitis	20-30
Discoid lesions	15
Rheumatoid nodules	12
Oral ulcers	7-23
Fever	80
Serositis	65
Neuropsychiatric	50-60
Renal	30-75
Nephrotic syndrome	15-30
Hypertension	50
Pulmonary	5-10
Cardiac	5-15
Antiphospholipid syndrome	10
Haematological	90
Anaemia	60-80
Leukopenia	60
Neutropenia	50
Lymphocytopenia	85
Lympadenopathy	50

Data from Estes and Christian, 1971; Hochberg et al 1985;. Sontheimer and Provost 1996, Hasan et al, 1997; Harvey et al, 1954; Dubois, 1976; Eisner et al, 1996; Asherson and Cervera, 1994.

Table 1.2. The revised ACR criteria for SLE diagnosis

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over malar eminence, spares nasolabial folds
2. Discoid rash	Erythematous raised patches with scaling and follicular plugging; atrophic scarring in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physicians
5. Arthritis	Nonerosive, involving two or more peripheral joints, with tenderness, swelling or effusion
6. Serositis	Pleuritis or Pericarditis
7. Renal disorders	Persistent proteinuria > 0.5 g/day or Cellular casts
8. Neurological disorder	Unexplained Seizures or Psychosis
9. Haematological disorder	Haemolytic anaemia or Leukopenia or Lymphopenia or Thrombocytopenia
10. Immunological disorder	Anti-DNA or Anti-Sm or Positive finding of anti-phospholipid antibody
11. ANA	An abnormal titre of ANA excluding "drug induced" lupus syndrome

A person has SLE if any four or more of the 11 criteria are present, serially or simultaneously, during any period of observation (Tan et al, 1982).

1.1.2 Antinuclear antibodies (ANA) and organ damage

Production of high titre IgG autoantibodies against components of the cell nucleus (antinuclear antibodies: ANA) is the pathological hallmark of SLE. Approximately 50 antinuclear antibody specificity's have been described in the disease. Different combinations of these autoantibodies occur in different patients. However, in an individual patient, autoantibodies against individual components of a given nuclear ribonucleoprotein complex tend to occur together (e.g. antihistone and anti-dsDNA antibodies). The occurrence of antinuclear antibodies in such "linked sets" has lead to the proposal that intact ribonucleoprotein particles act as the initial autoantigens in SLE (see section 4.4). The major anti-nuclear autoantibodies of SLE and their putative antigenic targets are listed in Table 1.3. Autoantibodies against cytoplasmic and cell surface molecules are also common and may play a role in organ damage (vide infra).

Table 1.3. Major antinuclear autoantigen-autoantibody groups in SLE

Antigen	Autoantibodies
Nucleosome	α-DNA, α-histone
U1 snRNP	α-Sm, α-nRNP, α-U1 RNA
DNA-PK	α-Ku, α-DNA-PK_{cs}
Y5 RNP	α-Ro(SSA), α-La(SSB), α-Y5 RNA

In broad terms organ damage in SLE is mediated by two mechanisms. Immune complexes containing antinuclear antibodies deposit or form in the organs and in the walls of the blood vessels supplying them. The organs primarily affected by this process (e.g. the kidney and the choroid plexus) have anatomical and physiological characteristics predisposing them to immune complex deposition. As the severity of immune complex nephritis is the key determinant of prognosis in SLE most research has focused on immune complex mediated kidney damage. There is little doubt that deposition of anti-dsDNA antibodies in the glomerulus plays the key role in inducing kidney damage in SLE (Berden, 1997). There is evidence to suggest that anti-dsDNA may be deposited in the glomerulus by two mechanisms: direct binding to the glomerular basement membrane (GBM) (Foster and Kelley, 1999) or binding to the GBM mediated by nucleosomes (Kramers et al, 1994). Which mechanism is operative in a given patient may depend on the precise properties of the anti-dsDNA antibodies present.

Immune complexes containing IgG anti-dsDNA antibodies activate both the classical and alternative complement pathways. The generation of chemotactic factors results in an influx of phagocytic cells into the tissue. Phagocytosis of immune deposits results in the release of enzymes and other factors which contribute to the damaging effects of inflammation. In addition, complement components of the membrane-attack complex have been demonstrated in the glomeruli of patients with lupus and likely contribute to cellular damage (Biesecker et al, 1982, Biesecker et al, 1981). Interestingly, studies in experimental animals have shown that the binding of complement components to the subepithelial deposits in glomeruli initiates the proteinuria associated with these lesions (Salant et al, 1980).

The clinical consequences of renal immune complex deposition are highly variable, ranging from mild proteinuria to a rapidly progressive glomerulonephritis (GN) fatal in weeks in the absence of therapy (Adler et al, 1996). The reasons for this variability are not determined. There is some

evidence that defective clearance of, or pathophysiological response to immune complexes may play a role in determining disease severity in some patients. For example, a significant decrease in the frequency of a particular Fc gamma receptor allele (FcγRIIa-H131) has been demonstrated in African-American patients with lupus nephritis (Duits et al, 1995). The FcγRIIa-H131 allele encodes the only FcγR which binds IgG2 efficiently and may therefore play the key role in clearing immune complexes containing this Ig subclass (IgG2 is a potent activator of the complement cascade) (Salmon et al, 1996).

Autoantibodies against cell surface components particularly in the haematological system but possibly also in the CNS, bind to cells and either promote their destruction or alter their function. For example, autoimmune haemolytic anaemia (AIHA) affects approximately one-third of SLE patients at some point during the disease (Simantov et al, 1999). Anti-erythrocyte autoantibodies of the "warm type" are responsible for red cell destruction. Autoantibodies coat the erythrocyte resulting in autoagglutination in the splenic pulp. This haemoconcentration increases the likelihood of a destructive encounter between the antibody-sensitised erythrocyte and phagocytic cells (Frank, 1977).

The main focus of research in SLE is into understanding the origin of the wide range of autoantibodies present in the disease and delineation of the pathogenic mechanisms giving rise to them.

1.1.3 Geography and gender in SLE

The disease occurs in all parts of the world. However, prevalence varies with race and geographical location. The true prevalence rates in the U.K. probably lies between 20 to 40 per 100,000 (Gladman and Hochberg, 1999). In the U.S.A., the figure is in the region of 100 per 100,000 (Gladman and Hochberg, 1999). The rate of disease is much higher in females than males: the ratio of affected females to males is approximately nine to one (Kotzin, 1996; Hochberg, 1990). SLE is more common in blacks and mortality is highest in this group

(Reveille et al, 1990). Longitudinal studies of patients with SLE indicate that survival has improved considerably over the past four decades in the developed world. In the 1950s, Merrell and Shulman reported a 51% cumulative survival rate at four years after diagnosis (Merrell and Shulman, 1955). At the present time, 20 year survival figures approach 70% (Abu-Shakra et al, 1993). In the developing world, much poorer survival figures are reported (Seleznick and Fries, 1991; Nossent, 1993). However, even in developed countries with the advent of transplantation and renal replacement therapy, the presence of renal involvement still predicts poor outcome. A 20 year survival rate of approximately 50% has been reported in the Mayo clinic cohort of lupus nephritis patients (Donadio et al, 1995).

The strong female predominance of SLE strongly implies that sex hormones play a central role in disease pathogenesis (Wilder, 1995). Several studies have suggested an imbalance between androgen and oestrogen levels in the plasma of lupus patients, possibly secondary to an increase in aromatase activity. Aromatase activity in various tissues correlates directly with oestrogen levels and inversely with disease activity (Folomeev et al, 1992). Furthermore, males with SLE have a higher prevalence of hypoandrogenism than controls (Sequeira et al, 1993). Hence an imbalance between androgens and oestrogen's seems to play a role in the pathogenesis of SLE. Androgens protect against and oestrogen's promote the development of the disease. Although sex hormones are known to have powerful immunomodulatory functions, the mechanisms involved in their effects on autoimmunity have not been determined (Homo-Delarche et al, 1991).

1.2 A brief history of lupus

The skin lesions of SLE were recognised as a distinct entity centuries before the associated lesions of the internal organs were demonstrated. Several authors

have suggested that lupus may have been included in the skin diseases Hippocrates (460-375 BC?) termed herpes esthiomenos (Ravogli, 1926). The first written references containing the term lupus (Latin for wolf) appear in mediaeval times. The term was loosely applied to a range of skin diseases which like the wolf "had the ability to devour flesh" probably including what we understand today as lupus erythematosus (Smith and Cyr, 1988). An account of the miraculous healing of Eraclius, Bishop of Liege, at the shrine of St Martin in Tours is the first written description of lupus. Herbernus of Tours, c. 916 AD recorded the event in his *Miracles of St Martin* and a more detailed account is contained in the Foundation Charter of the College of Cannons of St. Martin of Liege, c 963 AD.

"He (Eraclius) was seriously afflicted and almost brought to the point of death by the disease called lupus.....The location of the disease.....was not to be seen, nonetheless, a sort of thin red line remained as a mark of the scar" (Du Cange, 1845)

In 1851 Cazenave was the first to use the term lupus erythematosus (Talbot et al, 1974) to distinguish the lesion from the tuberculous lupus vulgaris.
Almost a thousand years after the first recorded descriptions of the rash, the systemic manifestations of the disease were described by Kaposi (Kaposi, 1872). Kaposi proposed that two types of lupus erythematosus existed; the discoid and the disseminated forms. He listed several characteristics of the disseminated form; subcutaneous nodules, arthritis, lymphadenopathy, fever, weight loss, anaemia and CNS involvement. After the publication of his work in 1872 the disease became known as acute disseminated lupus erythematosus (Kaposi, 1872). Kaposi did not make the connection between lupus and renal disease, the key determinant of prognosis in SLE. In 1904, Sir William Osler described two women developing renal failure within 10 months of the

appearance of facial erythema, although he did not give a specific diagnosis at the time (Osler, 1904). Indeed confusion reigned well into the 20th century with many considering SLE a variant of tuberculosis (Reifenstein, 1939)

SLE was defined as a distinct clinical entity in the 1920s and 30s largely due to the description of the morbid anatomical changes characteristic of the disease by pathologists such as Emmanuel Libmann, Benjamin Sacks and George Baeher. In 1935, Baeher described the classical "wire loop" lesions of the glomeruli in an autopsy series of 23 cases. He is often also credited with the first description of the solar sensitivity characteristic of the disease. This is erroneous, photosensitivity was originally described by Hutchinson in the nineteenth century (Hutchinson, 1879). So by the mid 1930s, SLE was established as the distinct clinical entity known today; an acute erythematous disease affecting primarily young women with a poor prognosis secondary to widespread lesions of the internal organs. In 1936, Friedberg, Gross and Wallache established, again in a post mortem series, that the systemic manifestations of the disease could occur in the absence of a rash. These detailed pathological examinations of the 1920s and 1930s finally excluded tuberculosis as a factor in disease pathogenesis (Lahita, 1999a).

The serological abnormalities characteristic of SLE have been the subject of intense research since the first reports of the false positive syphilis test in patients with the disease in the early 1940s. In 1948 Hargraves, Richmond and Morton first described the "LE cell" in the marrow of patients with SLE. The demonstration of this abnormality in the peripheral blood remains a useful diagnostic adjunct to the present day. In the original "LE cell" test, whole blood is incubated and stained smears of leucocytes are examined for LE cells, which consist of neutrophil polymorphs containing ingested homogenous nuclear material (Catto, 1985). It's description stimulated the search for antibodies reacting with self components-autoantibodies. In 1957, Friou modified the Coombes test to search for such reactivities and demonstrated the positive

fluorescent antinuclear antibody test (FANA), positive in 95% to 98% of lupus patients (Friou, 1957). The presence of autoantibodies to DNA in patients serum was demonstrated directly at around the same time (Deicher, 1959). The positive LE cell test reflects the presence of these antinuclear antibodies in patients serum as they react with the nuclei of leucocytes dying during the incubation period and allow their opsonisation by neutrophils.

1.3 The origin of antinuclear antibodies in SLE

1.3.1 SLE and the skin immune system (SIS)

Exposure of patients skin to UVB radiation exacerbates both the cutaneous and the systemic manifestations of SLE (Casciola-Rosen and Rosen, 1997). The ability to modify the course of the disease by UV irradiation of the skin implies that the skin immune system (SIS) may play a crucial role in lupus pathogenesis. Interestingly, one retrospective analysis suggested that polymorphous light eruption (PLE) preceded the onset of SLE by several years in some patients (Nyberg et al, 1997). The skin and the joints are the most frequently affected sites in lupus patients. Different series have indicated that skin disease occurs in 80 to 90% of patients with SLE at some point during the course of their illness (Lahita, 1999b).

The skin lesions of lupus are grouped together under the term cutaneous lupus erythematosus (CLE), inflammatory disorders of the skin which may occur in isolation or in association with the systemic manifestations of lupus. CLE is subdivided into three distinctive forms identifiable on the basis of their clinical appearance and duration of disease: chronic cutaneous lupus eryrthematosus (CCLE), subacute cutaneous lupus erythematosus (SCLE) and acute cutaneous lupus erythematosus (ACLE) (Gilliam and Sontheimer, 1981). The three subtypes of CLE share a pathognomonic histological appearance with a

mononuclear dermal inflammatory infiltrate composed predominantly of CD4+ T cells and macrophages (Andrews 1986) and characteristic degenerative changes in the basal layer of the epidermis (David-Bajar and Davis, 1997) (Figure 1.2). Indeed the basal layer of the epidermis appears to be the principle site of injury in all three subtypes of CLE, with loss of normal organisation and orientation of basal cells, oedema with vacuole formation between and sometimes within basal cells, partial obliteration of the dermal-epidermal junction and thickening of the basement membrane.

The mechanism underlying epidermal damage in CLE is controversial. Deposition of a thick band of Ig and complement components at the dermal-epidermal junction is a classical immunohistochemical finding in the majority of CLE cases (David-Bajar and Davis, 1997) and the demonstration of these deposits in non-lesional skin (the lupus band test) is a useful diagnostic test in atypical cases (Sontheimer 1996, David-Bajar and Davis, 1997). It has been argued that this phenomenon may be directly responsible for the damage to the epidermal basal layer. However, this seems unlikely as the deposits present in non-lesional skin from patients with SLE are not associated with subsequent development of LE skin lesions.

In terms of histology the subtypes differ only in the severity of the changes listed above (CCLE>SCLE>ACLE). Clinically, CCLE and SCLE are commonly seen in the absence of systemic manifestations of lupus. In contrast, ACLE in the absence of SLE is rare and the classic light sensitive "butterfly rash" characteristic of ACLE (Figure 1.1) is reported to occur in 35 to 60% of SLE patients (Cervera et al, 1993, Watson, 1989) at some point during the course of their disease.

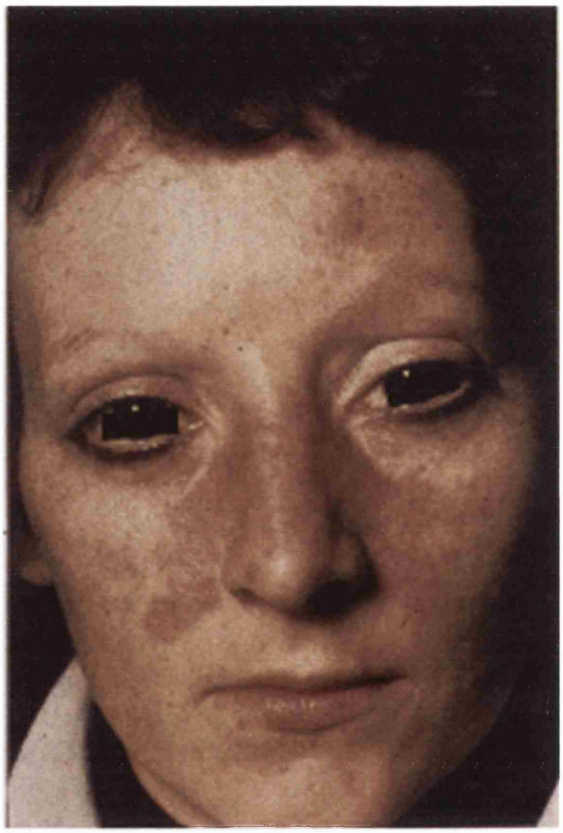


Figure 1.1 The classical "butterfly" rash of SLE.

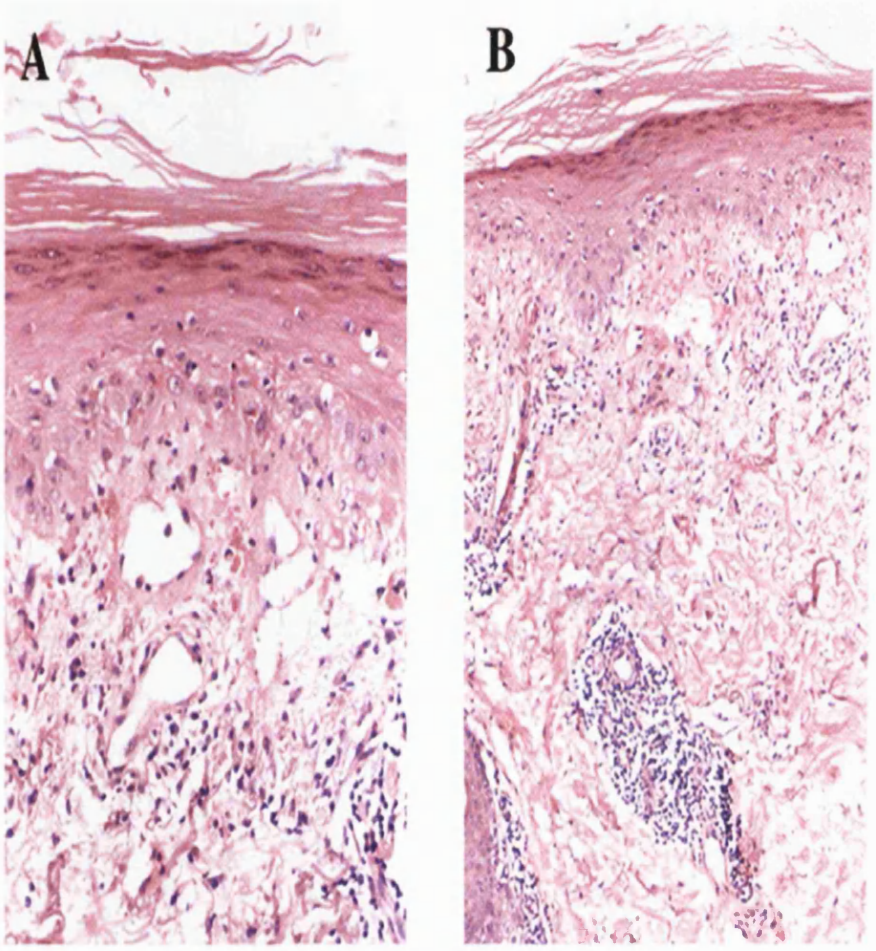


Figure 1.2 Skin histology in ACLE. Note the characteristic destruction of the basal layer of the epidermis in (A) and the mononuclear dermal inflammatory infiltrate in (B).

1.3.1.1 Photosensitivity in SLE

The mechanisms of photosensitivity in SLE are not understood. Clearly there are two, probably related, phenomena to explain; changes in the skin rash and alterations in systemic autoantibody titres.

1.3.1.1.1 Cutaneous photosensitivity

The actual incidence of cutaneous photosensitivity in established SLE is controversial. Figures ranging from 11% to 94% were reported in older studies, the wide range probably arising from different diagnostic criteria and failure to control for racial variation in incidence (Hasan et al, 1997). A controlled study of white patients with SLE placed the figure at approximately 70% (Hasan et al, 1997).

It seems likely that multiple mechanisms contribute to cutaneous photosensitivity in lupus patients: UV induced cytokine and nitric oxide release and direct effects of specific subsets of ANA have received most attention. Almost all UVB radiation is absorbed in the first 0.3 mm of skin tissue on surface illumination. A photoinduced skin reaction begins with absorption of photons by cellular chromophores, DNA, RNA, proteins, porphyrins and lipids (Walchner et al, 1997). Pyrmidines and purines have absorption spectra between 250 and 300 nm and DNA is, therefore, primarily affected by these wavelengths (Becker and Wang, 1989). After activation by UV rays chromophores become unstable and return to their original energy level by transferring energy to secondary molecules. This results in the formation of reactive oxygen intermediates (ROI) (Peak et al, 1987). ROI have potent effects on cellular transcription leading to upregulation of numerous mediators including pro-inflammatory cytokines (Baeuerle and Henkel, 1994).

After the formation of ROI several steps follow, upregulation of cell surface proteins, keratinocyte apoptosis, migration of Langerhans cells (LC) and formation of inflammatory infiltrates. Cytokines released by keratinocytes are

key mediators of skin reactions following UV irradiation (Sauder et al, 1993; Furukawa et al; 1989; Furukawa et al, 1994). TNF-α is particularly important. TNF-α and -β promoters contain UV-responsive elements, for example recognition sequences binding the nuclear factor (NF)-κB, which is indirectly activated by ROI (Baeuerle and Henkel, 1994; Messer et al, 1990; Bazzoni et al, 1994). UV irradiation also triggers IL-1 and IL-6 release leading to local inflammation and increased keratinocyte intracellular adhesion molecule-1 (ICAM-1) expression which could facilitate damaging interactions with immunocytes (Kupper et al, 1987; Urbanski et al, 1990; Krutmann et al, 1990; Norris, 1993). In addition, UV irradiation can increase endothelial E-selectin thereby promoting dermal infiltration of memory and activated T cells (Norris et al, 1991).

Recent evidence has implicated excessive UV induced nitric oxide (NO) production in lupus associated photosensitivity. UVB acts as a potent stimulus to human keratinocyte and endothelial cell NO release and *in vivo* studies using inhibitors of NO synthesis strongly imply that this mediator plays a central role in UVB induced erythema (Deliconstantinos et al, 1997). Furthermore, it has been shown that keratinocyte and endothelial cell expression of inducible nitric oxide synthase (iNOS) is markedly elevated in skin biopsies from SLE patients compared to controls. This is associated with elevated serum levels of NO in these patients (Belmont et al, 1997). Clearly exaggerated release of this mediator could contribute to UV induced erythema in SLE patients and elevated serum levels might alter systemic immune function. Interestingly, in skin biopsies taken four to 20 days after UV irradiation, epidermal iNOS expression (assessed at the level of both protein and mRNA) rose rapidly in normal subjects before subsiding after 48 hours. In LE patients the opposite pattern was observed, iNOS-specific signal was undetectable in keratinocytes for two days after irradiation but became positive on day 3 and persisted for up to 25 days in the evolving skin lesions (Kuhn et al, 1998).

In terms of UV induced exacerbation of lupus erythema, it has been proposed that anti-Ro/SSA autoantibodies play a key role. (Norris, 1993). Following UVB irradiation keratinocytes become apoptotic with Ro/SSA antigen expression in discrete surface blebs which appear to be associated with sites of oxidative modification (Casciola-Rosen et al, 1994). Autoantibody binding to this translocated antigen can be demonstrated in vitro and could result in antibody-dependent cell-mediated cytotoxicity (ADCC) *in vivo* with worsening of inflammation (Norris and Lee, 1985). Serum positivity for anti-Ro/SSA has been associated with photoinduced lesions and may confer increased risk compared to other antibodies (Mond et al, 1989; Thompson et al, 1993). However, it should be noted that there is no difference in the frequency of anti-Ro/SSA antibodies in patients with positive and negative phototest reactions (Wolska et al, 1989; Nived et al, 1993; Thompson et al, 1993).

1.3.1.1.2 UV irradiation and systemic disease manifestations

The response of the systemic disease to UV irradiation of the skin is complex. Clinical evidence suggests that UVB irradiation of the skin can aggravate the systemic manifestations of SLE (Casciola-Rosen and Rosen, 1997). However, a double blind placebo controlled study has shown that UVA exposure may be therapeutic in SLE. Diminuation of the systemic manifestations of the disease was demonstrated in association with a significant fall in anti-dsDNA titres following low-dose UVA irradiation (McGrath et al, 1996). These results confirmed previous anecdotal reports (McGrath, 1994).

UV light has multiple effects on cutaneous immune function which may contribute to its systemic effects. UV induced keratinocyte cytokine release and the ability of UVB to activate a skin derived immunosuppressor agent, cis-urocanic acid, provide an obvious link with systemic immunity (Noonan and DeFabo, 1992). In addition, UVB may affect LC function in several ways. For example irradiation decreases the ability of LC to stimulate T cells, particularly

CD4+Th1, thus resulting in unopposed Th2 stimulation of B cells (Stingl et al, 1981; Simon et al, 1992).

These factors may well be important, however, it is tempting to speculate that the ability of UV light to alter the systemic disease reflects an interaction with processes more fundamental to lupus pathogenesis. All UV wavelengths induce apoptosis (Godar and Lucas, 1995) and in this context it is of interest that UVA at high dose levels also exacerbates lupus (Lee et al, 1986, Golan et al, 1992). Clearly, if apoptotic keratinocytes were the source of autoantigen this would form an obvious point at which UV irradiation could aggravate the condition. UV light induces keratinocyte apoptosis by at least two mechanisms. There is no doubt that DNA damage induced by UV light can lead to p53 mediated keratinocyte apoptosis ("sunburn cells") and mice devoid of p53 develop almost no sunburn cells following UV exposure (Ziegler at al, 1994). Interestingly, a recent histological analysis of CLE demonstrated high numbers of apoptotic cells in the diseased basal layer (Pablos et al, 1999). Furthermore, both apoptotic and non-apoptotic keratinocytes in diseased areas expressed increased levels of p53 (Pablos et al, 1999). There is also evidence that UV light can directly activate Fas in keratinocytes in the absence of FasL. Apoptosis secondary to UV induced Fas activation is caspase dependent and inhibited by the peptide caspase inhihitor Z-VAD (Aragane et al, 1998).

At low doses, the effects of UVB and UVA on events in the cell nucleus may be antagonistic. UVB wavelengths are readily absorbed by DNA (Setlow, 1974) and the resulting generation of cyclobutane thymidine dimers enhances DNA immunogenicity (Davis et al, 1976). UV damaged DNA is immunogenic resulting in anti-DNA antibodies and immune complex GN when injected into New Zealand albino rabbits (Natali and Tan, 1972). In contrast UVA photons are poorly absorbed by DNA and promote photoreactivation, a process directed at the repair of DNA cyclobutane pyrmidine dimers (McGrath, 1994). Such a mechanism might explain the proposed therapeutic efficacy of UVA in SLE.

1.3.2 SLE as an autoimmune response to apoptotic keratinocytes

1.3.2.1 Apoptotic cells as autoantigen source in SLE

The demonstration of increased circulating levels of oligonucleosomes in SLE patients suggested that apoptotic cells may be the source of nuclear autoantigen in the disease (Rumore and Steinman 1990, Amoura et al, 1997). Several groups have shown that the major autoantigenic nucleoprotein particles recognised by autoantibodies in lupus patients undergo specific modifications during the apoptotic process. Components of these particles including DNA-dependent protein kinase (DNA-PK), poly(ADP-ribose) polymerase (PARP) and the 70kDa protein component of the U1 small nuclear riboprotein are proteolytically cleaved early during the apoptotic process (Casciola-Rosen et al, 1994; Casciola-Rosen et al, 1995; Casciola-Rosen et al, 1994b; Casiano et al, 1996). Furthermore, inhibition studies directly implicate the caspases in this phenomenon (Casciola-Rosen et al, 1995). In addition, it has been shown that sera from SLE patients, but not patients with other autoimmune disorders, can immunoprecipitate several phosphoproteins present only in cells undergoing stress induced apoptosis (Utz et al, 1997). Indirect evidence implicates the stress activated serine/threonine kinases (SAP kinases) in this phenomenon (Utz et al, 1997). Although by no means definitive evidence, these observations imply that apoptosis plays some undefined role in lupus pathogenesis, perhaps as a source of self antigen (vide infra).

Apoptotic cells as autoimmunogen could also offer an antigen source to drive the production of anti-phospholipid antibodies, a group of autoantibodies responsible for disordered blood clotting, a common problem in SLE (Hughes et al, 1986). Early during apoptosis, anionic phospholipids, particularly phosphatidylserine (PS), redistribute from the inner to the outer surface of the plasma membrane bilayer generating a pro-coagulant external cell surface (Casiola-Rosen et al, 1996). The available evidence implies that the humoral

immune response to anionic phospholipids in SLE patients actually recognises complexes of the phospholipid and one of several PS-binding proteins (Matsuura et al, 1994, Koike et al 1995, Igarashi et al, 1996). The demonstration that these phospholipid-binding proteins coat the apoptotic cell surface is at least consistent with the presence of immunogenic phospholipid-protein complexes on the surface of these cells in vivo (Casiola-Rosen et al, 1996). In support of this contention IgG purified from patients with antiphospholipid antibody syndrome bind to the apoptotic cell surface and completely block its procoagulant activity (Casiola-Rosen et al, 1996).

Hence the major autoantigens of SLE are concentrated on the surface of apoptotic cells in an altered state. Furthermore, there is some evidence to suggest that apoptotic rates may be high in patients with SLE, at least in lymphocytes. Cultured and freshly isolated lymphocytes from patients with the disease show higher levels of apoptosis compared to lymphocytes isolated from controls (Emlen, 1994) In addition, apoptotic rates of lymphocytes correlated with disease activity (Emlen, 1994). It has been proposed that some autoreactive T cells escape tolerance induction because they are directed against cryptic determinants on self antigens which are not efficiently generated during antigen processing in the thymus (Sercarz et al, 1993). Taken together these observations have led to the proposal that alterations of self proteins and macromolecular complexes during the apoptotic process may expose previously cryptic epitopes which can stimulate autoreactive T cells which have escaped intrathymic deletion (Casciola-Rosen et al, 1995).

Although appealing, the evidence for such a mechanism is purely circumstantial. To date, only a small number of the autoantigens of SLE have been definitively shown to undergo modifications during apoptosis (Casciola-Rosen and Rosen, 1997). In addition, increased rates of lymphocyte apoptosis are also found in other autoimmune disorders not characterised by antinuclear antibody production (Lorenz et al, 1997). Clearly increased numbers of apoptotic cells of itself is not

sufficient stimulus for antinuclear antibody production. In support of this, Mevorach et al have shown that although exposure of the immune system to excess numbers of apoptotic cells under non-physiological conditions can lead to ANA production, the full gamut of pathogenic antinuclear autoantibodies is never produced. Intravenous injection of syngeneic apoptotic thymocytes in non-autoimmune mice resulted in IgG antinuclear, anticardiolipin and anti-ssDNA autoantibody production. This was associated with immune complex deposition in the kidney (Mevorach et al, 1998). These findings are remarkable as autoantibodies are rarely produced by immunisation with self antigens in the absence of special adjuvants (Schwartz and Stollar, 1985) and the intravenous route is normally considered to be tolerogenic (Schwartz, 1993), However, nephritogenic IgG anti-dsDNA antibodies were not produced. It would seem probable, therefore, that elaboration of the full spectrum of pathogenic IgG ANA would require some further "non-physiological" alterations in the nucleoprotein complexes to generate neo-epitopes.

Apoptotic cells arising in an inflamed organ could act as a source of altered self antigen in SLE particularly if they occur in an organ rich in antigen presenting cells (APC) (Mevorach et al, 1998). Furthermore, in an inflammatory milieu, damage to nucleoproteins by oxygen species released by activated phagocytes may enhance immunogenicity (vide infra). Long standing clinical observations and experimental work suggest one such source of apoptotic nuclei in SLE; the skin.

1.3.2.2 Apoptosis in CLE

Apoptotic keratinocytes are present in abundance in all cell layers of ACLE, SCLE and CCLE. Interestingly, bcl-2 is constitutively expressed in the basal layer of normal epidermis and levels of this "survival factor" are markedly reduced in the basal layer of epidermis affected by CLE (Chung et al, 1998). Evidence that apoptotic keratinocytes could act as a source of self antigen in SLE

is provided by the observation that the putative autoantigens of the disease are contained in two distinct groups of surface blebs of keratinocytes rendered apoptotic by UVB irradiation. The two populations of blebs are numerous in apoptotic keratinocytes and differ in size. The smaller blebs contain membranous vesicles of uniform size probably originating from the endoplasmic reticulum and contain ribosomes and the autoantigen Ro. The larger blebs form from apoptotic bodies and contain nucleosomal DNA, Ro and La and the small ribonucleoproteins surrounded by nuclear membrane (Casciola-Rosen et al, 1994). Interestingly, Ro, La and snRNPs become concentrated around the rim of apoptotic bodies (i.e. nuclear membrane). In addition, Ro is in close proximity to ER membrane in the small blebs. This observation may be of great significance as after apoptosis cells sustain progressive lipid peroxidation, reflecting the generation of lipid-diffusible reactive oxygen species (Hockenbery et al, 1993) and the ER and nuclear membrane are two of the major sites of free radical generation (Hockenbery et al, 1993; Boveris and Chance, 1973; Cross and Jones, 1991).

Hence, during keratinocyte apoptosis the major nuclear autoantigens of SLE are in close proximity to sites of free radical generation. Free radical induced modifications of proteins have been described that may reveal cryptic determinants, including fragmentation (Davis, 1987; Wolff and Dean, 1986), amino acid modifications (Davis, 1987) and novel sensitivity to protease attack (Levine et al, 1981). Under normal conditions, presumably this potential problem is controlled by the mechanisms responsible for disposing of apoptotic remnants. However, it is possible that in conditions of excess apoptosis or altered immune function these control mechanisms are overcome.

Critically, apoptotic keratinocytes in SLE occur in an inflammatory milieu; dermal inflammation and inflammatory destruction of the epidermal basal layer are key features of all three forms of CLE. The inflammatory process may further alter the autoantigens of lupus, for example via reaction with oxygen

species released by phagocytes, rendering them immunogenic (Casciola-Rosen et al, 1994). DNA damaged by reactive oxygen species in vitro becomes immunogenic triggering an anti-dsDNA antibody response when injected into rabbits (Cooke et al, 1997). The process may be self-amplifying as recent evidence suggest that opsonisation of apoptotic cells and uptake by macrophages may illicit an inflammatory response (Manfredi et al, 1998a, Manfredi et al, 1998b). It has been shown that binding of anti-phospholipid antibodies to apoptotic cells facilitates their phagocytosis with subsequent release of large amounts of TNF-α (Manfredi et al, 1998).

Failure to clear apoptotic keratinocytes has been suggested as the central abnormality in SLE complicating complement deficiencies (particularly C1q deficiency) (Korb and Ahern, 1997). All patients with C1q deficiency develop SLE irrespective of gender and a photosensitive rash is a prominent feature of this syndrome (Bowness et al, 1994, Slingsby et al, 1996). Human keratinocytes rendered apoptotic by UVB or infection with Sindbis virus specifically and directly bind C1q in the absence of antibody. The binding of C1q to apoptotic keratinocytes could have profound effects on the immunological response to these cells. Firstly, it is possible that bound C1q may protect against autoimmunisation by promoting antibody independent phagocytosis and clearance of apoptotic keratinocytes. Secondly, ligation of C1q receptors on T and B cells may alter their function. For example, it has been shown that ligation of C1q receptors on T cells dramatically reduces their response to mitogens (Chen et al, 1994). Alternatively, C1q binding may activate the classical complement pathway with covalent attachment of C3 and C4 on the apoptotic cell surface. There is evidence that C3 is indeed deposited on apoptotic keratinocytes following UVB exposure (Rauterberg et al, 1993). It has recently been reported that C3b suppresses IL-12 production by primary human monocytes via ligation of membrane cofactor protein (CD46) (Karp et al, 1996). Hence, the presence of C3 proteolytic fragments on the surface of apoptotic keratinocytes could critically

alter the balance between Th1 and Th2 responses in the skin immune system. Intriguingly, Hammerberg et al, have presented evidence that ligation of CD11b by iC3b molecules, formed from C3 activation in UV-exposed skin, suppresses the ability of cutaneous APCs to initiate a primary immune response but promotes their ability to induce tolerance (Hammerberg et al, 1998). A photosensitive rash is a prominent feature of SLE complicating C1q deficiency (Slingsby et al, 1996) and loss of complement promoted tolerogenic mechanisms in the skin could play a role in the origin of ANA in this syndrome.

1.3.2.3 Evidence of antigen presentation in CLE

The skin is an immune organ capable of triggering an array of local and systemic immune phenomena in response to physical, chemical and biological agents (Williams and Kupper, 1996). The consequences of skin immune system (SIS) activation depend on a co-ordinated cytokine-mediated interaction between multiple cell types. Langerhans cells (LC) are the professional APC of the epidermis (Streilein and Bergstresser, 1984). LC possess specialised mechanisms permitting efficient capture and uptake of exogenous antigens and presentation of processed peptides to T cells in the context of MHC class II. In response to inflammatory stimuli, LC migrate from the epidermis to draining regional lymph nodes via the dermal lymphatics (Kripke et al, 1990). During this migration LC upregulate expression of class II MHC and the costimulatory molecules B7-1(CD80) and B7-2(CD86) increasing their effectiveness at stimulating naive antigen-specific T cells (Larsen et al, 1994; Kawamura and Furue, 1995).

Immunohistochemical studies have identified a number of interesting immune-related abnormalities in CLE. Although purely descriptive these findings can be tentatively linked with autoantibody generation in lupus. For example, there is evidence of LC migration in the skin lesions of lupus. In a study of the immunopathology of light induced skin lesions in DLE and SCLE, decreased

numbers of LC in the epidermis was demonstrated (Velthuis et al, 1990) . In the dermis increased numbers of LC occur in association with CD4+ T cells (Mori, et al 1994). Interestingly, identical changes in LC distribution have been described in the chronic inflammatory skin lesions of the MRL-*lpr* mouse (Kanauchi et al, 1991).

Keratinocyte surface expression of ICAM-1 is detectable in a diffuse pattern throughout the epidermis in SCLE (Bennion et al, 1995), whereas it is undetectable in the epidermis of normal skin. In addition, HLA-DR expression is markedly upregulated in basal keratinocytes particularly in lesions of SCLE (Kind et al, 1993). Keratinocytes have the ability both to recruit and expand T cell infiltrates. IL-7 is produced constituitively at low levels by keratinocytes (Heufler et al, 1993) and is a potent stimulus to the growth of immature B and T cells as well as mature T cells (Namen et al, 1988; Morrissey et al, 1989; Murray et al, 1989). Keratinocytes can also produce IL-15, at least in response to UV irradiation (Mohamadzadeh et al, 1995). IL-15 is a potent chemotactic and chemokinetic factor for T cells (Wilkinson and Liew, 1995).

Whether keratinocytes can act as APCs for CD4+ T cells is controversial. Studies using mouse or human Th1 clones have shown that presentation of antigenic peptides by class II MHC molecules of keratinocytes results in T cell anergy (Gaspari and Katz, 1988; Bal et al, 1990). In addition, injection of haptenated class II positive keratinocytes into syngeneic mice induced T cell hyporesponsiveness to subsequent hapten immunisation (Gaspari and Katz, 1991). Induction of anergy may result from failure of keratinocytes to express costimulatory molecules. Transgenic mice expressing B7-1 on keratinocytes show amplified contact hypersensitivity responses (Williams et al, 1994; Nasir et al, 1994) implying that keratinocytes can promote T cell activation when they provide an appropriate second signal. It has also been shown that keratinocytes activated in vitro with IFN-γ and a phorbol ester express a CD28 counterligand distinct from B7-1 and B7-2 recognised by the BB1 monoclonal antibody

(Nickoloff et al, 1993; Augustin et al, 1993; Fleming et al, 1993; Boussiotis et al, 1993) and that phorbal ester-treated keratinocytes can provide a second signal capable of supporting T cell growth (Simon et al, 1991).

Hence, keratinocytes have intimate access to the autoantigens of SLE, are known to express MHC class II and T cell adhesion molecules in CLE, can recruit T cells to the skin and may be capable of providing a second signal. Furthermore, recent evidence suggests that these cells can also direct a T cell response towards either a Th1 or Th2 profile and hence promote the development of either a humoral or cell mediated immune response (Mosmann and Coffman, 1989; Romagnani, 1994). Keratinocytes can produce both IL-10 (Enk and Katz, 1992) and IL-12 (Muller et al, 1994). Since IL-12 promotes (Hsieh et al, 1993) and IL-10 inhibits Th1 development (Howard and O'Garra, 1992), keratinocytes may be capable of conditioning the cutaneous milieu to favour Th1 or Th2 development by selective IL-10 or IL-12 production. It is possible, therefore that the keratinocyte not only provides the autoantigens of lupus but could also play the key and controlling role in initiating the T cell dependent autoimmune response.

To date there is no definitive evidence that apoptotic keratinocytes are the source of self nuclear antigen in SLE. However, it is clear from the data outlined above that many circumstantial observations support this hypothesis and that such a source could explain several key features of the disease.

1.4 Murine models of SLE

Much of our current knowledge concerning the origins of antinuclear antibodies in lupus is derived from studies of murine models of the disease. These models have been particularly useful in studying the "downstream mechanisms" involved in generating these antibodies. It is fair to say that in general, the

genetic defects triggering autoantibody production in these mice are probably not relevant to idiopathic SLE in man. A summary of the murine lupus models reported to date is contained in Table 1.4 and the best characterised models are briefly discussed in the following sections.

Table 1.4. Murine models of lupus (Theofilopoulos and Kono, 1999).

- Spontaneous
  - NZ strains and derivatives
    - NZB
    - NZW
    - (NZBxNZW) F1
    - (NZBxSWR) F1, NZB^{bm12}, (NZBxNZW) RI lines (NZM/Aeg lines), (NZBxSM) RI lines, (NZBxC58) RI
  - FAS and FASL defective mice and derivatives
    - MRL-*lpr/lpr*
    - MRL-+/+
    - MRL-*Fasl*^{pr} .ll (long-lived substrain)
    - MRL-*Fas*^{lpr}.*Yaa* (MRL-*lpr* with the Yaa accelerator of male BXSB)
    - MRL-*FasL*^{gld}
    - CH3-lpr, SJL-lpr, C57BL/6-lpr, AKR-lpr
    - CBA-*lpr*^{cg}/*lpr*^{cg}
    - C3H-HeJ-*gld/gld*
    - MRL-*gld* (and other *gld* homozygous strains)
  - Y chromosome accelerator mice and derivatives
    - BXSB
    - BXSB.ll (long-lived substrain)
    - (NZBxBXSB) F1, (NZWxBXSB) F1, (NZBxSB/Le) F1
  - (SWRxSJL)F1
  - Motheaten strains
    - *me/me*
    - *me*^{v}/*me*^{v}
  - Palmerston north strain
  - "Flaky skin" (fsn/fsn) mice
- Induced models
  - Chronic graft versus host disease
  - Dominant idiotype
  - Anti-DNA peptide
  - Drug-induced, Pristane, Xenobiotic-induced (mercury chloride)
  - Transgenic and gene knockout models
    - Transgenics (*Bcl*-2, *IL*-4, *IFN*-γ, *Fli*-1, *Pim*1)
    - Knockouts (*TGF*β1, Lyn, *IL*-2*R*β, *IL*-2*R*α, *IL*-2, *Zfp*-36, *CD*22, *C1q*)

1.4.1 Spontaneous murine-lupus models

Strains of mice which spontaneously develop key components of the lupus complex have been available for study for almost four decades. The first spontaneous murine model of lupus described was the (NZBxNZW)F1 mouse [abbreviated to (BxW)F1] (Heyler and Howie, 1963, Howie and Helyer, 1968). In the mid 1970s two further strains of lupus mice were reported: the MRL (H-2k) and BXSB (H-2b) strains (Murphy, 1981, Murphy and Roths, 1979, Andrews *et al*, 1978). The MRL mice were further subdivided into two substrains: MRL+/+ with mild lupus like disease, and MRL-*lpr/lpr* (*lpr*: lymphoproliferation mutation), a strain in which females develop a severe lupus like disease early in life. MRL-*lpr/lpr* mice (abreviated to MRL-*lpr* mice) develop massive lymphoid organ enlargement due to the presence of a greatly expanded $CD4^{-}8^{-}B220^{+}$ αβ T cell population.

Early in life all spontaneous lupus strains develop severe glomerulonephritis associated with glomerular Ig antinuclear antibody and C3 deposition (Theofilopoulos, 1999). Indeed, these strains are primarily models of ANA mediated glomerulonephritis. Delineation of the factors triggering autoantibody production in lupus strains is an area of intense research. There is strong evidence implicating abnormalities of apoptosis in autoreactive T and B cell deletion. The molecular basis of lupus pathogenesis in (BxW)F1 and male BXSB has not been defined. Autoantibody production may result from defective B and T cell apoptosis and at least in the case of (BxW)F1 there is evidence that defective apoptosis is a multigenic trait (Kozono et al, 1996).

MRL-*lpr* mice carry a mutation in the Fas gene associated with insertion of an early retroviral transposon (Etn) in the second intron leading to aberrant RNA splicing, a frame shift mutation and premature termination of the long terminal repeat of the Etn (Watanabe-Fukunaga et al, 1992, Nagata and Suda, 1995, Adachi et al, 1993, Kobayashi et al, 1993, Chu et al, 1993). That this mutation is responsible for the lupus phenotype in these animals has been established by

correction of the phenotype in MRL-*lpr* transgenic for Fas (Wu et al, 1994). The mechanism by which mutated Fas leads to autoantibody production is not determined but may relate to abnormalities in activation-induced cell death (AICD) of T cells following antigenic challenge (Ju et al, 1995, Brunner et al, 1995, Dhein et al, 1995 Wong et al, 1997).

Reduced T and B cell apoptosis is also the central abnormality in several of the gene knockout and transgenic models of SLE developed in recent years. For example, transgenic mice which overexpress the apoptosis regulatory proteins bcl-2 (under the control of an Ig enhancer) and Fli-1 show prolonged survival of T and B cells with antinuclear antibody production and glomerulonephritis (Strasser, A et al, 1991; Zhang, L et al, 1995). However, it is important to note that reduced rates of T and B cell apoptosis probably do not play a role in human SLE. Mutations of FAS do not occur in the naturally occurring human disease. A syndrome secondary to deficient apoptosis has been described in man, the Canale-Smith Syndrome. These patients cannot eliminate T cell clones arising during an immune response and although they do develop autoimmune phenomena they do not develop SLE (Mysler et al, 1994). In fact, freshly isolated B and T cells from patients with the disease show increased levels of FAS expression compared to controls and mitogenic stimulation of both these cells types results in normal Fas antigen upregulation (Mysler et al, 1994, Ohsako et al, 1994). Furthermore, cultured and freshly isolated lymphocytes from patients with SLE show higher levels of apoptosis compared to lymphocytes isolated from controls (Emlen, 1994) In addition, apoptotic rates of lymphocytes correlated with disease activity (Emlen, 1994).

Two murine models developing a lupus-like syndrome apparently secondary to alterations in B cell receptor function have been described: *lyn* knockout mice and mice carrying a mutation in the Shp1 protein tyrosine phosphatase (*motheaten* (*me/me*) and viable *motheaten* (me^v/me^v)). Shp1 associates with CD22 (Lankester et al, 1995) and FcγRIIB1 (D'Ambrosio et al, 1995) and

results in dephosphorylation of the Igα component of the B cell receptor complex (D'Ambrosio et al, 1995). Shp1 may, therefore, play a role in downregulating antigen-induced B cell activation (D'Ambrosio et al, 1995; Cyster and Goodnow, 1995). There is some evidence from transgenic studies (Chen et al, 1995) and studies in man (Suzuki et al, 1997) that defective B cell receptor editing plays a role in the emergence of antinuclear antibodies in SLE. One study showed that several SLE patients were unable to edit a particular Vk gene that is frequently associated with a cationic anti-DNA antibody whereas normal B cells appear capable of editing out this light chain (Suzuki et al, 1997). However, it should be noted that the autoantibodies produced in *lyn* knockout mice and Shp1 mutants are predominantly IgM in both cases and systemic disease is mild (Hibbs et al 1995, Nishizumi et al, 1995, Yu et al, 1996).

Lupus, like other autoimmune diseases is a complex genetic trait with contributions from multiple genes both MHC and non-MHC related. Each gene confers a small increased risk to disease susceptibility and different loci may determine the nature and levels of autoantibody production and the severity of systemic disease produced in response to the presence of these autoantibodies (Vyse and Kotzin, 1996). For example, transfer of the *lpr* mutation into normal strains of mice results in autoantibody production and lymphoid enlargement but not tissue damage (Izui et al, 1984, Kelley and Roths, 1985).

In recent years, the development of techniques to map the position of disease susceptibility loci in genome-wide screens has greatly advanced genetic susceptibility research (Dietrich et al, 1996). These techniques are now being applied to murine lupus. Although multiple loci have now been positioned in murine lupus the intervals containing the aetiological alleles are large, hence reducing the chance of identifying a candidate gene. To date, none of the susceptibility genes at these loci have been identified. However, ongoing studies are reducing these intervals mainly using congenic mice and identification of genes may be imminent.

1.5 Transgenic models of skin disease

Transgenic technology provides a means by which the long term *in vivo* effects of excess levels of biologically active molecules can be studied. Promoters exist which can target transgene expression to the epidermis and this approach has proved useful in the study of both pathological and physiological processes (Fuchs and Byrne, 1994; Zhou et al, 1995). The effects of epidermal overexpression of several individual cytokines have been studied in transgenic mice primarily using the K14 promoter (Table 1.5). The surprising conclusion from these studies is that overexpression of individual proinflammatory cytokines such as IL-6 (Turksen et al, 1992), TGF-α (Vassar and Fuchs, 1991), KGF (Guo et al, 1993) and VEGF (Detmar et al, 1994) in general results in little cutaneous inflammation and minimal changes in the epidermis. Dermal macrophage infiltrates associated with slightly decreased epidermal LC numbers were seen in some animals expressing an IL-1α transgene under the control of the K14 promoter (Groves et al, 1995). Mice expressing K10/BMP-6 developed either a limited psorriform phenotype or decreased keratinocyte proliferation depending on the level of transgene expression (Blessing et al, 1996).

The pathogenesis of inflammatory skin disorders is a long standing interest of our laboratory. In order to define the role of IFN-γ in the pathogenesis of inflammatory skin lesions, transgenic mice expressing the mouse cytokine under the control of the human involucrin promoter were developed (Carroll et al, 1997). Involucrin is a component of the keratinocyte cell envelope and it's expression is essentially limited to the suprabasal layers of stratified squamous epithelia, although recent data has also confirmed expression in urothelium (Prowse et al, in press). The involucrin promoter has been used to target integrin and whn (winged-helix nude) expression to the suprabasal layers of the epidermis resulting in a psoriatic and hyperplastic phenotype respectively (Carroll et al, 1995, Prowse et al, in press).

Table 1.5 Pathology of transgenic models of cytokine induced skin disease

Transgene	AC/HP	T cell infiltrate	Neutrophil infiltrate	Reference
K14/IL-1a	+/S	+/S	+/S	Groves et al, 1995
K14/IL-6	+	-	-	Turksen et al, 1992
K14/TGFα	+	+/S	+/S	Vasser and Fuchs, 1991
K14/KGF	+	-	-	Guo et al, 1993
K10/BMP-6	+	+	+	Blessing et al, 1996
K14/VEGF	-	n.d.	-	Detmar et al, 1998

AC/HP: presence of epidermal acanthosis/hyperproliferation, +/S: positive in some severely affected animals, n.d: not done.

1.6 IFN-γ and SLE

At the time of starting this study, IFN-γ had not been targeted to the epidermis of transgenic mice, however there is a considerable amount of evidence that both the SIS (reviewed above) and IFN-γ play a central role in lupus pathogenesis.

1.6.1 IFN-γ in murine and human lupus

Of all the abnormalities of the cytokine network described in murine and human lupus the most consistent and convincing evidence implicates IFN-γ in the pathogenesis of the disease. Administration of IFN-γ accelerates systemic disease while administration of anti-IFN-γ antibody or soluble IFN-γR early in life significantly delayed disease progression in (BxW)F1 mice (Jacob et al, 1987; Ozmen et al, 1995). In addition, a long-lived substrain of MRL-*lpr* mice showed reduced IFN-γ levels compared to the parental strain concomitant with a shift of Ig isotypes from the complement-fixing IgG2a and the nephritogenic IgG3 to the less pathogenic IgG1 isotype (Takahashi et al, 1996). MRL-*lpr* mice congenic for either the IFN-γ or INF-γR gene deletions and (BxW)F1 congenic

for the IFN-γR deletion showed significant reduction in autoantibody levels and systemic disease (Balomenos et al, 1998, Haas et al, 1997, Haas et al, 1998) Surprisingly, renal disease was also prevented in IFN-$\gamma^{+/-}$ mice despite no overall change in anti-dsDNA levels and persistent immune complex deposition in the kidney. This beneficial effect was associated with a switch in the dominant antibody subclass from IgG2a to IgG1 with no overall change in systemic antibody levels and no compensatory increase in Th2 related immunoglobulin classes (Balomenos et al, 1998)

These findings imply that therapeutic interventions to reduce IFN-γ levels in SLE patients may be effective in controlling the disease without complete reduction in IFN-γ levels and probably without a significant reduction in the ability of the individual to fight infection. Interestingly, a similar separation of autoantibody production and histological kidney disease has been reported in (BxW)F1 mice deficient in certain Fcγ receptors (lacking FcγRI and FcγRIII but retaining FcγRIIB) (Clynes et al, 1998). As in the case of IFN-$\gamma^{+/-}$ MRL-*lpr* mice, $Fc\gamma R^{-/-}$ (BxW)F1 mice demonstrated reduced histological renal disease despite continued autoantibody production and immune complex deposition in the kidney.

The beneficial effects of reduced IFN-γ levels in murine lupus may be mediated by one of several mechanisms. Reduced expression of MHC class I and class II molecules both on APCs and target organs (Balomenos et al, 1998) and reduced T cell proliferation (Novelli et al, 1996) may play a role. In addition, it has been shown that administration of IL-12 to MRL-*lpr* mice results in exacerbation of the systemic disease in association with increased serum levels of IFN-γ and NO metabolism (Huang et al, 1996). Increased NO levels have been demonstrated in renal tissue in MRL-*lpr* mice (Weinberg et al, 1994). IFN-γ is known to induce NO formation and it is possible that the renal protective effects of reduced IFN-γ relate to reduced tissue levels of NO.

There is interesting anecdotal evidence suggesting that increased levels of interferon might play a role in the pathogenesis of SLE in man. The emergence

of SLE, with *de-novo* anti-dsDNA antibody production, has been described in some patients receiving systemic treatment with interferon-α or -γ (Ronnblom et al, 1990, Graninger et al, 1991) suggesting that excess amounts of the cytokine can trigger an autoimmune response relevant to SLE in susceptible individuals.

1.6.2 IFN-γ transgenic mice

Prior to my arrival in the laboratory, three independent founder lines expressing IFN-γ under the control of the involucrin promoter had been established. In situ hybridisation analysis confirmed appropriate expression of IFN-γ mRNA in the suprabasal layers of the epidermis. Grossly elevated expression at the protein level in transgenic skin was confirmed using a number of techniques. Sonicated extracts of $2cm^2$ areas of transgenic skin contained markedly elevated levels of IFN-γ in all three founder lines. No IFN-γ was detected in the skin of non-transgenic littermate controls. Flow cytometry of keratinocytes isolated directly from transgenic skin, permeabilised and stained for IFN-γ confirmed expression of the transgene in these animals. Again no IFN-γ was detected in keratinocytes isolated from non-transgenic littermate controls. Importantly for subsequent work, systemic levels of the cytokine, as determined by ELISA of serum and estimation of cytokine levels in renal tissue extracts, were not elevated in transgenics.

1.6.2.1 Gross skin phenotype

All transgenic mice appeared normal at birth. However, by 8 d many animals exhibited marked growth retardation and retarded hair growth. By two weeks of age all transgenics exhibited hypopigmentation of the hair. DOPA staining of skin sections from transgenics showed a marked reduction in melanocyte numbers in the hair follicles of transgenic animals. By three weeks of age the most severely affected transgenics (5 to 20% depending on the founder line) showed extreme erythema, growth retardation, hair loss over large areas of the

trunk and pustular lesions of the skin, especially around the limb joints (Figure 1.3). Mildly affected animals exhibited slight erythema. In general, with age the skin lesions became more severe. Upon dissection, all transgenics had enlarged superficial lymph nodes and splenic size was usually greatly enlarged (on average twice the weight of spleens from control animals).

1.6.2.2 Skin histology in IFN-γ transgenics

Histological examination of skin from mildly affected transgenic mice showed evidence of a dermal mononuclear inflammatory infiltrate with dilatation of dermal capillaries and spongiosis affecting all layers of the epidermis. In more severely affected animals there was marked mononuclear infiltration of the dermis with evidence of epidermal hyperplasia and hyperkeratosis. Disruption of the epidermal basal layer was evident in some of the more severely affected transgenics with separation of the epidermis from the underlying dermis with infiltration of lymphocytes and macrophage into the resulting space (Figure 1.4).

1.6.2.3 Upregulation of ICAM-1 and MHC class II in IFN-γ transgenics

It has been shown that keratinocytes treated in vitro with IFN-γ express MHC class II and ICAM-1 (Dustin et al, 1988; Griffiths et al, 1989). In normal mouse skin MHC class II expression is confined to LC. All epidermal layers in IFN-γ transgenics were positive for MHC class II on immunostaining. In addition, the basal layer of transgenic epidermis was positive for ICAM-1 expression. No ICAM-1 could be demonstrated on keratinocytes in non-transgenic littermate controls.

Figure 1.3 Gross phenotype of IFN-γ transgenic mice. Two week old IFN-γ transgenic littermates (top and bottom) and nontransgenic littermate control. Note the variation in disease severity from severe skin inflammation with alopecia and growth retardation to minor inflammation with loss of hair colour (present in all transgenics).

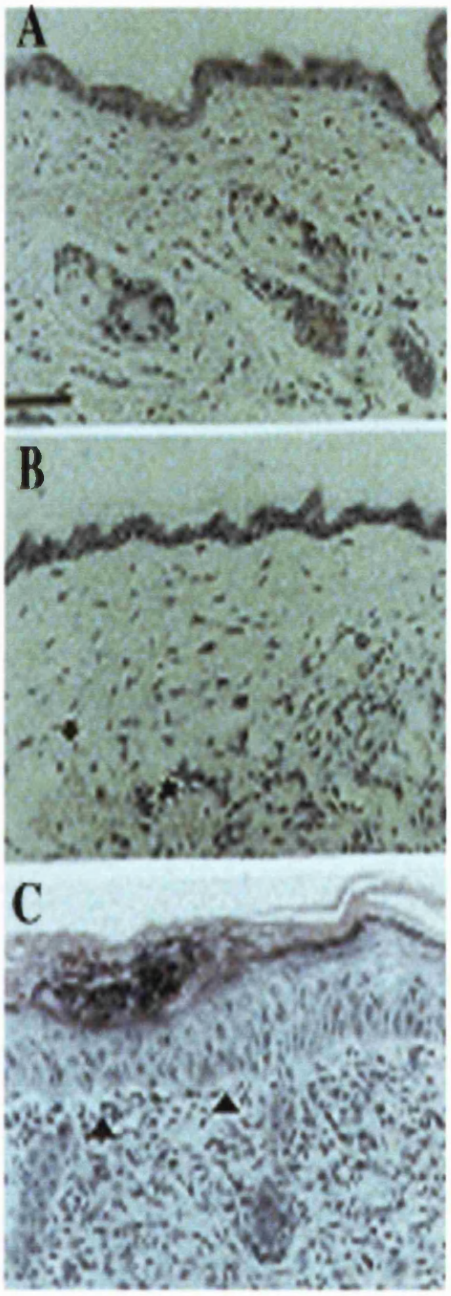


Figure 1.4 Histology of IFN-γ transgenic mouse skin. Haematoxylin and eosin stain of back skin from non-transgenic littermate control (A), mildly affected transgenic mouse (B) and severely affected transgenic mouse (C). Note the dermal inflammatory infiltrate in (B) and (C) accompanied by enlarged capillaries. A split at the dermo-epidermal junction was a characteristic finding in severely affected transgenics (C, arrow). Scale bar in (A): 60 μm (A = B = C)

1.6.2.4 The dermal inflammatory infiltrate in IFN-γ transgenics

On immunohistochemical analysis the majority of the immunocytes in the dermis of IFN-γ transgenics were of the macrophage/monocyte lineage with a lesser but significant amount of T cells. There was little evidence of epidermal inflammation; however, in the most severely affected animals occasional CD4 positive cells were detected. Interestingly, staining with an antibody to Mac1 (anti-CD11b) showed a complete absence of LC in IFN-γ transgenic epidermis. Loss of LC from the epidermis was confirmed on FACS analysis of disaggregated epidermal cells stained with NLDC-145 (a marker of nonlymphoid dendritic cells). Furthermore, the number of NLDC-145 positive cells in the superficial lymph nodes draining the skin was elevated in transgenics compared to controls (approximately 40% of cells in lymph nodes of transgenics were NLDC-145 positive compared to 5% in controls). These observations are at least consistent with an IFN-γ induced migration of LC from the epidermis to the draining lymph nodes; an observation which was central to latter research.

It is clear from the above description that skin disease in IFN-γ transgenic mice shares many features with skin disease in human lupus. In view of the known association of IFN-γ with organ specific autoimmune disease in other tissues we examined IFN-γ transgenics for autoantibody production. To our surprise these animals produced non-organ specific autoantibodies characteristic of SLE (Carroll et al, 1997).

Chapter 2

Materials and Methods

2.1 Tissue culture

All reagents used were of tissue culture grade and kept sterile. Cells were grown in a humidified incubator at 37°C with 5% CO_2 on tissue culture grade plastic.

2.1.1 Tissue culture media and general solutions

"ICRF" indicates that the reagent was prepared by the Central Cell Services Unit at the Imperial Cancer Research Fund London.

2.1.1.1 Phosphate Buffered Saline (PBS, ICRF)

8g NaCl, 2.5g KCl, 1.43g NaH_2SO_4 and 0.25g KH_2PO_4 were dissolved in 1l distilled water (d/w), the pH was adjusted to 7.2 and the solution autoclaved. For immunofluorescence staining PBS was supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBSABC).

2.1.1.2 Trypsin Solution (5X stock, ICRF)

O.25% pig trypsin (Difco) was dissolved in sterile d/w and then adjusted to 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris, pH 7.7, 0.1% glucose, 0.0015% phenol red, 0.006% penicillin and 0.01% streptomycin.

2.1.1.3 EDTA solution (Versene, ICRF)

8g NaCl, 0.2g KCl, 1.15g Na_2HPO_4, 0.2g KH_2PO_4, 0.2g ethyldiaminotetracetic acid EDTA and 1.5 ml 1% phenol red solution were dissolved in d/w, the pH adjusted to 7.2 and the volume made up to 1l with d/w.

2.1.1.4 Keratinocyte culture medium (ICRF)

Keratinocyte growth medium (FAD medium) comprises a 3 plus 1 mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F12 medium supplemented with 1.8 x 10^{-4} M adenine. It was prepared from FAD powder (Imperial Laboratories), supplemented with 36.5 mM $NaHCO_3$, 100 IU/l penicillin (Gibco BRL) and 100 μg/l streptomycin (Gibco BRL) and was adjusted to acidic pH with CO_2. Prior to use, the medium was supplemented with 10% (v/v) batch tested foetal calf serum (FCS; Imperial laboratories) and 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10^{-10} M cholera toxin and 10 ng/ml epidermal growth factor (HICE) (Rheinwald and Green, 1975; Watt, 1994). Insulin was prepared as a 5 mg/ml stock solution in 5 mM HCl and stored at -20°C. Hydrocortisone, cholera enterotoxin and epidermal growth factor were stored at -20°C as a 1000x cocktail, prepared by adding 100 μl of 10^{-5} M cholera enterotoxin (ICN) in d/w, 1 ml of 5 mg/ml hydrocortisone (Calbiochem) in 95% ethanol, and 1 ml of 100 ng/ml recombinant human epidermal growth (Austral Biologicals) in 0.1 M acetic acid (BDH) to 7.9 ml FAD medium containing 10% FCS.

2.1.1.5 J2-3T3 growth medium (ICRF)

Dulbecco's Modified Eagle Medium (DMEM; ICN). Prior to use, the medium was supplemented with 10% (v/v) donor calf serum (DCS; Imperial Laboratories).

2.1.2 Standard Keratinocyte Culture

Keratinocytes were grown on a feeder layer of 3T3 cells, clone J2 (Rheinwald 1989). 3T3 cells were cultured in DMEM/DCS until confluent, treated for two hours with 4 μg/ml mitomycin C, harvested with versene and split 1:3 (i.e. to

give a one third confluent cell density) into 25 cm^2 or 75 cm^2 tissue culture flasks (Falcon) containing FAD/HICE/FCS. Keratinocytes were seeded at densities of $2x10^4$ - $1x10^5$ cells per flask, and cultured in a humidified 37°C incubator in a 5% CO_2 atmosphere. The medium was changed every 2-3 days. Keratinocytes were harvested by removing the 3T3 cells with versene, adding trypsin/versene (1 volume of 5x trypsin to 4 volumes of versene), incubating for 10-15 minutes at 37°C, and adding 9 volumes of FAD/HICE/FCS. The cell suspension was then centrifuged at 1000xg for 5 minutes and the keratinocytes resuspended in FAD/HICE/FCS.

2.1.3 Culture of mouse keratinocytes

Mouse epidermal keratinocytes were cultured according to the method described by Romero (Romero et al, 1999). Adult mice (2-10 months) were killed, shaved and sequentially washed in providone solution (0.75% stock solution, diluted 1:10 in d/w) then in d/w followed by 70% ethanol and finally in d/w. The skin was removed, placed in PBS containing 200 U/ml penicillin, 200 µg/ml streptomycin and 100 U/ml nystatin and all of the subcutaneous tissue was scrapped off. The skin was then incubated in 0.25% trypsin for 2 hours at 32°C. The epidermis was separated from the dermis and stirred in FAD medium containing 10% FCS, 200 U/ml penicillin and 200 µg/ml streptomycin for 20 minutes at room temperature. Cells were filtered through a 70 µm Teflon mesh, centrifuged and resuspended in complete FAD medium. Cells were seeded onto fibronectin/collagen-coated dishes (100 ml FAD, 1 mg fibronectin, 10 ml of 1 mg/ml BSA solution, 1 ml Vitrogen collagen solution, 1 ml 2M HEPES, 1 ml 116 mM $CaCl_2$; Morris, 1994) in the presence of a feeder layer of J2-3T3 cells at a density of $2\text{-}4x10^6$ cells per 6 cm diameter dish. Cells were

then incubated at 32°C. Cells were subsequently handled as described for human keratinocytes.

2.2 IFN-γ transgenic mice

2.2.1 Preparation of transgenic mice

Transgenic mice expressing IFN-γ in the epidermis were developed by Dr Joseph Carroll (Carroll et al 1997). The cDNA for murine IFN-γ (kindly provided by Nora Sarvetnick) was excised from the vector pSVEmuγ as an *Eco*RI/*Bam*HI fragment (Gray and Goeddel, 1983; Sarvetnick et al, 1988), blunt ended and ligated to *Not*I linkers. The cDNA was then cloned into the *Not*I site of the involucrin expression cassette consisting of 2.5 kb of the human involucrin upstream region, the involucrin intron, an SV40 intron and an SV40 polyadenylation sequence. The 3.7 kb upstream sequence contains all the necessary information for high level expression in the suprabasal layers of transgenic mouse epidermis (Carroll et al, 1993). The transgene was excised from the expression vector by cutting with *Sal*I, purified (Carroll et al, 1993) and resuspended in sterile PBS at a concentration of 5 mg/ml for oocyte injection.

The transgene was injected into fertilised oocytes from (CBA x C57/BL10) F1 mice. Three independent founder lines were generated. The transgene copy number was determined by Southern analysis of genomic DNA isolated from tail snips and digested with *Hind*III/*Eco*RV. Founder line 1205D contains two copies of the transgene; 1205C contains 6 copies and 1212F contains 32 copies. Mice in all three founder lines had the same phenotype (Carroll et al 1997),

although in general animals from the 1212F founder line had the most severe disease.

2.2.2 Screening for the presence of the transgene

The presence of the IFN-γ transgene was detected using PCR on genomic DNA from ear or tail snips. The PCR was carried out by my collaborator Dr. Eddie Wang in the Lymphocyte Laboratory at the ICRF as previously described (Carroll et al, 1995). Genomic DNA was isolated by standard techniques (Sambrook et al, 1989). PCR was carried out with primers specific for SV40 intronic sequences (5'-TACGGAAGTGTTACTTCTGCTCTAAAAGCTG-3') and for the 5' end of the IFN-γ gene (5'-CAAGATGCAGTGTGTAGCGTTCATTGTCTC-3') under the following PCR conditions: 1 cycle of 95°C for 5 minutes, 54°C for 30 s, 72°C for 30 s; 30 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 30 s; 1 cycle of 95°C for 30 s, 54°C for 30 s, 72°C for 10 minutes. DNA from mice heterozygous for the transgene produced a 200 bp band, visualised by ethidium bromide in 1% agarose gels, which was absent in non transgenic littermate controls.

2.2.3 Serum and renal IFN-γ ELISA.

Serum from transgenic mice was tested for the presence of IFN-γ using a murine IFN-γ Cytoscreen immunoassay kit (Bio-Source Intl.). Recombinant mouse IFN-γ (Genzyme) in the range 10-500 pg per ml was used to standardise for known amounts of protein. Extracts of renal tissue were prepared by sonication on ice of slices of renal cortex in 1 ml phosphate PBS containing 0.2 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM pepstatin. IFN-γ levels in two renal extracts from each animal tested were measured using the IFN-γ Cytoscreen immunoassay kit and expressed per mg of total protein present.

Experiments were performed in triplicate and chromogenic results were read on a spectrophotometer at OD_{450}.

2.3 αβ and γδ T cells deficient IFN-γ transgenic mice

2.3.1 Generation of IFN-γ transgenic mice deficient in αβ or γδ T cells

Mombaerts et al. generated mice congenitally deficient in αβ T cells from embryonic stem cells (ES) carrying a genomic deletion of 15 kb encompassing the TCRβ locus from Dβ1.3 to downstream of Cβ2. Mice homozygous for the mutation ($TCR\beta^{-/-}$) are completely deficient in αβ T cells (Mombaerts et al 1992). $TCR\delta^{-/-}$ mice are also available. These mice were generated by disruption of the TCR δ constant gene segment (Cδ) using a gene targeting approach in ES cells. Homozygotes are completely deficient in γδ T cells (Itohara et al 1993). Importantly, the development of γδ T cells is not affected by the absence of αβ T cells and visa versa (Mombaerts et al 1992; Itohara et al 1993). $TCR\beta^{-/-}$ and $TCR\delta^{-/-}$ mice, referred to as $\beta^{-/-}$ and $\delta^{-/-}$ mice respectively, were obtained from The Jackson Laboratories, Bar Harbor, Maine, USA. The $\beta^{-/-}$ and $\delta^{-/-}$ mice used in this study were on a C57BL/6 background. All mice were maintained and bred at the ICRF animal facility.

Male mice heterozygous for the IFN-γ transgene (founder line 1205D) were crossed with $\beta^{-/-}$ or $\delta^{-/-}$ deficient females. From these litters males heterozygous for both the IFN-γ transgene and the β or δ chain deletion were crossed with $\beta^{-/-}$ and $\delta^{-/-}$ T cell deficient females respectively. From the resulting litters, female animals heterozygous for the interferon transgene and homozygous for either

TCR deletion were obtained and used throughout this study. Age and sex matched littermates from this generation positive for the transgene and heterozygous for the relevant TCR deletion acted as controls, since the available evidence suggests that T cell function is normal in animals heterozygous for either the β or δ chain deletion (Mombaerts et al 1992, Itohara et al 1993). As the males used to breed the test litters were heterozygous for the IFN-γ transgene, approximately 25% of the females in each litter were not transgenic and were heterozygous for the relevant TCR deletion and were used to assess baseline autoantibody levels and renal histology. These animals are subsequently referred to as "littermates negative for the transgene" and the term "control" is limited to animals positive for the transgene and heterozygous for the relevant TCR deletion.

2.3.2 Genotyping of T cell deficient mice

TCRβ genotyping was performed by flow cytometry of peripheral blood lymphocytes stained with anti-mouse αβ TCR-FITC conjugated monoclonal antibody (Becton Dickinson) on a FACS Profile flow cytometer with CellQuest software (Becton Dickinson). TCRδ genotyping was carried out by PCR of genomic DNA using the following primers: TCRδ-F - 5'-AGATAATGAAAAACTACCAGAACC-3'; TCRδ -R - 5'-AATATGAAGTGACCAATTCTTACC-3' under the following PCR conditions: 1 cycle of 94°C for 5 minutes, 50°C for 30 s, 72°C for 30 s; 30 cycles of 94°C for 20 s, 50°C for 30 s, 72°C for 30 s; 1 cycle of 94°C for 20 s, 50°C for 30 s, 72°C for 10 minutes. DNA from heterozygous TCRδ$^{+/-}$ mice produced a 600 bp band, visualised by ethidium bromide in 1% agarose gels, which was absent in TCRδ$^{-/-}$ mice.

2.4 Immunohistochemical Methods

2.4.1 Reagents

2.4.1.1 Gelvatol

2.4g of gelvatol (Monsanto) was mixed with 6g of glycerol (Sigma) and vortexed. 6ml of d/w was added and the mixture left to stand for 90 minutes at room temperature. 12.5 ml of 200 mM Tris pH 8.5 was then added and the solution vortexed, heated to 50^{o}C and vortexed again. Heating and vortexing were repeated 3 times and the solution then placed on an end over end mixer overnight at room temperature. The solution was then centrifuged at 400xg for 10 minutes at room temperature and stored in aliquots at 4^{o}C.

2.4.2 Immunofluorescence detection of kidney deposits

Kidney tissue from transgenic mice and negative control littermates was snap frozen in an isopentane bath cooled in liquid nitrogen. Frozen sections embedded in OCT (Tissue Tek$^{(TM)}$, Miles Inc.) were cut at 5-8 μm thickness. Sections were air dried and blocked for 30 minutes with goat serum. Sections were then incubated for 45 minutes with Texas red-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories) diluted at 1:100. Each antibody incubation was carried out at room temperature and was followed by thorough washing in PBS. Stained sections were mounted in Gelvatol (Monsanto) and examined using a Zeiss Axiophot microscope.

2.4.3 Indirect immunofluorescence on normal mouse skin and oesophagus

Serum samples from transgenic mice and negative control littermates were screened for the presence of autoantibodies by indirect immunofluorescence on

normal mouse tail skin or oesophagus. Unfixed, 8 μm thick frozen sections of normal mouse skin/oesophagus were allowed to air-dry at room temperature for 30 min before staining. Sections were blocked for 30 minutes in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ (PBSABC) and 10% FCS (Imperial Laboratories) followed by incubation with mouse serum at a dilution of 1:10 in PBSABC for 45 minutes. Sections were then incubated for 45 minutes with Texas red-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories) at a dilution of 1:100 in PBSABC.

2.4.4 Immunofluorescence staining of cultured keratinocytes

Mouse and human keratinocytes grown on coverslips were fixed and permeabilised for 10 minutes using a 50:50 methanol/acetone solution at -20^{0}C or in 3.7% formaldehyde for 10 minutes at room temperature, followed by 0.1% Triton X-100 in PBS for 5 minutes at room temperature. The same staining patterns were observed with each fixation technique. The coverslips were incubated with transgenic mouse serum diluted 1:10 in PBSABC for 45 minutes. After washing in PBS, coverslips were incubated with Texas red-conjugated goat anti-mouse IgG for 45 minutes. In some experiments, coverslips were incubated with rabbit anti-desmoglein antibody 919 (recognising the cytoplasmic repeat region of desmoglein-1 and -2) (Wheeler et al 1991) (kindly provided by Dr Anthony Magee, National Institute for Medical Research, U.K), followed by Texas red conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories). All incubations and PBS washes were carried out at room temperature. Stained cells were mounted in Gelvatol and examined using a Zeiss Axiophot microscope.

2.4.5 Immunofluorescence staining of tissue sections

Tissue was frozen in an isopentane (BDH) bath in liquid nitrogen, embedded in OCT compound (BDH) and cryosectioned into 6 μm sections. Frozen sections of oesophageal mucosa were incubated with 10% foetal calf serum in PBSABC for 30 minutes. Sections were then incubated for 45 minutes with primary antibody followed by extensive washing in PBS. Sections were subsequently incubated with FITC conjugated secondary antibody (Jackson Immunoresearch Laboratories), again washed extensively in PBS and finally mounted in Gelvatol. Tissue sections were examined using a Zeiss Axiophot microscope.

2.4.6 Staining paraffin sections

Tissues were fixed overnight in formol saline and embedded in paraffin. Sections were cut at 6-8 μm. For staining, sections were dewaxed and taken down to 100% alcohol. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide (BDH) in methanol followed by rehydration in sequential alcohols to water. Slides were blocked by incubation with 10% FCS in PBS for 30 minutes followed by incubation with primary antibody for 45 minutes. After washing, sections were incubated with biotinylated anti-mouse or biotinylated anti-rabbit antibodies for 35 minutes. Slides were again washed in PBS followed by incubation for 30 minutes with Streptavidin-peroxidase (DAKO). Staining was visualised by incubation with DAB solution i.e. 5 mg of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 10 ml of PBS containing 20 μl of 30% hydrogen peroxide.

For detection of nuclear Ki67 expression, rehydrated sections were placed in 0.01 M sodium citrate solution and boiled in a 700 W microwave oven for 20 minutes (i.e. 2 x 10 min). The staining procedure was then carried out as outlined above using rabbit polyclonal IgG anti-Ki67 (Novacastra)

2.5 Electron microscopy

Tissue from the renal cortex of transgenic and non-transgenic controls was fixed in cold 2.5% gluteraldehyde in 0.1 M phosphate buffer, pH 7.4, post fixed in 1% osmium tetroxide in the same buffer and embedded in araldite. Sections were cut on a Reichert Ultracut, stained with uranyl acetate and lead citrate and viewed in a Zeiss EM 10 CR.

2.6 Protein biochemistry

2.6.1 Extraction of Triton-soluble proteins

Confluent cultured mouse and human keratinocytes were lysed on ice in extraction buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.1% Triton, 2 mM PMSF and 0.01% leupeptin. The stock PMSF solution was a 100 mM solution in acetone and stored at 4°C. The leupeptin stock solution was a 100 μg/ml solution in dH_2O and stored at -20°C. Cells were washed twice in ice cold PBS followed by extraction for 10 minutes at 4°C on a rocking platform. The cells were scraped from the tissue culture plastic and cellular debris was pelleted by centrifugation at 14,000 rpm for 10 minutes at 4°C. The supernatant was assayed for protein content using the Bradford assay, aliquoted and stored at -70°C. Before use the lysates were thawed on ice and centrifuged at 14,000 rpm for 10 min at 4°C in order to remove insoluble material.

2.6.2 Bradford assay for measuring protein concentration

The assay was carried out using the Biorad *DC* Protein Assay kit (Biorad) according to the manufacturers instructions. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. Copper-treated proteins effect a reduction of the Folin reagent producing several species with a characteristic blue colour with a maximum absorbance at 750 nm and minimum absorbance at 405 nm. A standard curve was prepared by making triplicate dilutions of a standard protein solution (BSA, 2 mg/ml, Pierce). Lysis buffer was used as a control "blank" and to dilute samples. Samples were applied neat, 1:2 dilution and 1:5 dilution. 5 µl protein standard or sample was added into triplicate wells in a 96-well plate (Immunolon Dynatech). The assay was carried out according to the manufacturer's instructions: 20 µl reagent S was added to 1 ml reagent A (an alkaline copper tartrate solution) to make working reagent A. 25 µl working reagent A was added to each well. 200 µl reagent B (a dilute Folin reagent) was then added to each well. The plate was gently agitated and incubated for 15 minutes at room temperature. Any bubbles present in the wells were dispersed. The OD690 was measured for the contents of each well on a Titretek Multiskan MCC/340 MKII spectrophotometer. The protein concentration of sample was determined against the standard curve.

2.6.3 Polyacrylamide Gel Electrophoresis

2.6.3.1 Laemmli sample buffer

125 mM Tris, pH 6.8, 2% (w/v) SDS, 20% (w/v) glycerol and 0.02% (w/v) bromophenol blue.

2.6.3.2 SDS-PAGE

Vertical gel electrophoresis apparatus systems (Model SE400, Hoefer Scientific Instruments) were used. 1.5 mm thick gels were prepared between glass plates using the method of Laemmli (Laemmli, 1970). The solutions used to prepare the gels are detailed in Table 2.1 and the composition of the gels is presented in Table 2.2. After pouring resolving gel solution, 1 ml of 0.1% SDS solution (Table 2.3) was applied immediately to ensure a level interface as well as to eliminate an air-acrylamide interface. Gels were allowed to polymerise at room temperature for a minimum of one hour. O.1% SDS solution was discarded, and the stacking gel solution was poured. a 1.5 mm thick comb was inserted to create wells and then the gel was left to polymerise. After the gel had set, the comb was removed and the wells were flushed with SDS-PAGE running buffer which comprised 50 mM Trizma base, 384 mM glycine and 0.1% SDS. Samples were then applied to the wells. 10 µl of pre-stained rainbow molecular weight markers (Amersham) were added to 10 µl of Laemmli sample buffer, boiled for 5 minutes and loaded into one of the wells. Samples were electrophoresed overnight at 40-50V.

Table 2.1 Solutions for preparation of SDS-PAGE gels

Solution	Composition
A	30% acrylamide/0.8% bisacrylamide (Millipore), 4°C
B	3M Tris-HCl, pH 8.8 (BDH)
C	10% SDS
D	2M Tris-HCl, pH 6.8 (BDH)
AP	10% ammonium persulphate (Bio-Rad) in dH2O, freshly prepared
Temed	N,N,N',N'-tetramethylethylenediamine (Bio-Rad)

Table 2.2 Resolving gels (30ml)

	Stock solutions					
	A	B	C	dH_2O	TEMED	AP
5%	5 ml	3.75 ml	0.3 ml	20.95 ml	30 µl	300 µl
7.5%	7.5 ml	3.75 ml	0.3 ml	18.45 ml	30 µl	300 µl
10%	10 ml	3.75 ml	0.3 ml	15.95 ml	30 µl	300 µl

Table 2.3 Stacking gel (10ml)

A	C	D	dH_2O	TEMED	AP
1.5 ml	100 µl	0.625 ml	7.775 ml	10 µl	100 µl

2.6.3.3 Western blotting

Human and mouse keratinocyte protein lysates in extraction buffer were centrifuged at 14,000 rpm for 5 minutes and the pellets discarded. The supernatants were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis on 5% or 10% slab gels (Table 2.2), followed by transblotting to nitrocellulose paper. Transblotting was carried out in 48 mM Tris, 387 mM glycine, 3.5 mM sodium dodecyl sulphate containing 20% methanol for 2 hours at 35V followed by two hours at 70V. The blots were blocked overnight in 5% skim milk powder in PBS containing 0.01% Tween (PBS/T), then incubated for 1 hour with serum from transgenic or control mice diluted at 1:200 in PBS/T containing 0.1% bovine albumin (Sigma). Nitrocellulose strips were incubated for 1 hour with horse radish peroxidase (HRP)-conjugated rabbit anti mouse Ig (DAKO) diluted 1:5000 in PBS/T containing 0.1% BSA. Between antibody incubations strips were washed three times with PBS/T. Additional strips were incubated with rabbit anti-desmoglein antibody 145 (raised against the repeat region of desmoglein-1 and recognising all three

desmogleins, kindly provided by Dr Anthony Magee) followed by HRP-conjugated donkey anti-rabbit Ig (Amersham). All incubation and washing steps were performed at room temperature. The peroxidase activity was visualised by chemiluminescence (ECL, Amersham).

2.6.4 Radioactive-labelling of cultured cells

Human and mouse keratinocytes were incubated in the presence of 50 μCi/ml [^{35}S]-cysteine and methionine (specific activity > 100 Ci/mmol; Translabel, ICN) for 20 hours under standard culture conditions. The labelling medium was then discarded and the flasks were rinsed three times with cold PBS. The cells were then lysed in situ with the Triton X-100 extraction buffer at 4°C.

2.6.5 Trichloroacetic acid (TCA) precipitation

TCA precipitation was used to estimate [^{35}S]-cysteine and -methionine incorporation into proteins. 2-5 ml of the protein lysate was incubated with 1 ml 10% (w/v) TCA solution for one hour at 4°C. Triplicate solutions were prepared. The solutions were applied onto glass microfibre filters (Whatman GF/C) which had been pre-wetted with 10% TCA solution and fitted on a Millipore manifold apparatus. The solutions were drawn by vacuum suction through the filters which trapped precipitates. The filters were washed twice with 5 ml 10% TCA solution at 4°C, followed by 5 ml absolute alcohol and air dried. The filters were immersed in 5 ml scintillation fluid (Ecolume, ICN) and then counted in a β-counter (Beckman L81 801). Stock TCA solution was 100% (w/v) and was prepared by dissolving 500g TCA (Sigma) in 227 ml of dH_20.

2.6.6 Protein immunoprecipitation

Protein G-Agarose (Sigma) was supplied as pre-swollen in a solution of 70% ethanol. The beads were hydrated in excess PBS, mixing end over end for 30 minutes at room temperature. To use, the supernatant was discarded and protein G-Agarose beads were washed three times with PBS and a 1:1 (v/v) slurry of beads and PBS was prepared and this was used in immunoprecipitation.

2.6.6.1 Immunoprecipitation protocol

For the immunoprecipitation of radioactive labelled protein lysates, up to 10^7 cpm of TCA-precipitable material were used per immunoprecipitation. Lysates were pre-cleared with Protein G-Agarose beads for 2 hours at 4°C. The beads were discarded and the lysates were incubated with 1 µl of serum from test animals (IFN-γ transgenics and non-transgenic controls) in 50 µl of the protein G/PBS suspension and incubated at room temperature for 5 hours. The beads were washed six times in PBS, resuspended in an equal volume of 2x Laemmli sample buffer, boiled for 5 minutes and centrifuged for 10 minutes. The supernatant was applied to polyacrylamide SDS gels under reducing conditions.

2.6.6.2 Autoradiography

After electrophoresis, the gel was removed from the glass plates and immersed in a fixing solution which comprised isopropanol:water:acetic acid (25:65:10) for 30 minutes. Gels were further incubated for 30 minutes in a fluorographic agent (Amplify; Amersham) before drying onto Whatman 3MM filter paper under vacuum at 80°C. The dried gel was exposed to Kodak XAR-5 film (Eastman Kodak Co.) for an appropriate period of time.

2.7 Serological techniques

2.7.1*Crithidia luciliae* staining.

Serum samples from transgenic and negative control littermates were screened for the presence of anti-dsDNA antibodies using a commercially available *Crithidia luciliae* dsDNA kit (The Binding Site Ltd.). *C. luciliae* contains an organelle (the kinetoplast), a giant mitochondrion containing dsDNA but apparently free of histones and other mammalian nuclear antigens (Aarden et al, 1975). Slides coated with *C. luciliae* were incubated for thirty minutes at room temperature with serum samples diluted 1:10 in PBS. The slides were washed in PBS and then incubated for 30 minutes with FITC-conjugated goat anti mouse IgG. After further washing the slides were mounted in Gelvatol and viewed either using a Zeiss Axiophot Microscope (Carl Zeiss Ltd.) or a confocal laser scanning microscope (Model MRC-1000, Bio Rad). A series of 1 μm optical sections through the specimens was obtained with a confocal microscope and a composite image (Z series) was constructed.

2.7.2 Assay for antibodies against extractable nuclear antigens (ENA)

Serum samples were screened for the presence of antibodies against extractable nuclear antigens (Sm, U1 RNP, SS-A (Ro), SS-B (La), Jo1 and Scl-70) using a commercially available ENA screening counter current immunoelectrophoresis (CIE) kit (The Binding Site Ltd.). This procedure was performed by Miss Karen Walker, University of Birmingham. Transgenic serum found to be positive on this screen was further characterised using an ENA typing CIE kit specific for the same antigens (The Binding Site Ltd.). For ENA screening, 120 μl of ENA extract (buffered sheep spleen extract preserved in 100 mM PMSF and 10 mM mercaptoethanol) and 20 μl of test serum were applied to the

surface of an agarose gel by means of an application mask (15 to 20 µl of test serum and appropriate positive controls for ENA typing). For both procedures electrophoresis was carried out at 50V for 1 hour 15 minutes on a Beckman Paragon power pack. Gels were dried and then stained in Acid Blue 29 in 5% v/v acetic acid for 2 seconds and destained in 5% v/v acetic acid for 5 minutes. Gels were dried completely by placing in a 45°C incudryer for 15 minutes and then examined for a visible immunoprecipitate.

2.7.3 Antihistone, anti-dsDNA and antinucleosome ELISA

2.7.3.1 Chromogenic substrate solution

ELISAs were developed using the following chromogenic substrate solution: 10 mg of O-phenylene diamine (Sigma) in 29.5 ml of 0.15 M citrate-phosphate buffer pH 5.0 containing 6% hydrogen peroxide (BDH). 200 µl of chromogenic substrate solution was added to each well of the ELISA plate following the final wash. The chromogenic reaction was stopped by adding 50 µl of 3 M sulphuric acid and the absorbance read at 492 nm in a Titertek Multiskan MCC/340 spectrophotometer.

2.7.3.2 ELISA protocol

The levels of total Ig and IgG antihistone and anti-dsDNA antibodies in serum were measured using a modification of previously described methods (Kotzin et al 1987). Calf thymus histones (Sigma) were diluted in PBS to a concentration of 2.5 µg/ml and 0.2 ml of this antigen solution was added to each well of an Immulon II microtitre plate (Dynatech Laboratories, Inc.). After overnight incubation at 4°C, wells were coated with 0.4 ml gelatin (1 mg/ml in PBS) for at least 24 hours at 4°C. After washing, 0.2 ml of serum

samples diluted 1/100 to 1/1000 in 0.1% Tween, 1mg/ml gelatin and 0.5% bovine serum albumin in PBS were added and incubated for 1.5 hours at room temperature. After washing, total Ig bound was measured by adding HRP-conjugated rabbit anti-mouse Ig (DAKO) or IgG was measured using HRP-conjugated goat anti-mouse IgG (Sigma). Both secondary antibodies were used at a dilution of 1/4000 in 0.1% Tween in PBS. After 1.5 hours incubation at room temperature the wells were washed and substrate solution added. The optical density (OD) was then read with an automated spectrophotometer at 492 nm.

To measure anti-dsDNA antibody levels, wells were coated with dsDNA (Sigma). To attach dsDNA, microtitre wells were first coated with poly-L-lysine (Sigma) at 5.0 μg/ml in H_2O for 1.5 hours at 37°C. After washing, dsDNA was added at 5.0 μg/ml in PBS and incubated overnight at 4°C. After washing, serum samples diluted 1/100 to 1/1000 were added as described above.

Levels of IgG antinucleosome antibodies were measured by ELISA as previously described (Amoura et al 1994). These assays were carried out by Dr. Sophie Koutouzov, Hopital Necker, Paris, France. Briefly, Purified mononucleosomes prepared as previously described (Koutouzov et al 1996) were dissolved in PBS at 5 μg/ml and 100 μl added to Luxlon microtitre plates (CML). Plates were incubated overnight at 4°C. Wells were washed with PBS-0.1% Tween, pH 7.4 (PBST) and postcoated for 2 hours with 0.1 ml of PBS-10% FCS, pH 7.4. After washing, sera (1/100) diluted in PBS/T were added and reacted for 2 hours. Bound antibodies were detected with peroxidase-conjugated goat anti-mouse Fc antisera (Sigma). Binding was measured by adding ABTS substrate (Southern Biotechnology), and the OD was read at 405 nm by an automated spectrophotometer (Dynatech Laboratories, Inc.).

A serum sample known to be positive for anti-ssDNA and negative for anti-dsDNA showed no significant binding in the dsDNA ELISA (OD 0.00+/-0.05). All sera were also tested on uncoated ELISA plate plastic. Non-specific binding to plastic was low (OD on uncoated wells ranged from 0 to 5% of values on antigen coated wells). The antihistone and anti-dsDNA ELISA tests were run in triplicate and anti-nucleosome ELISA in duplicate. In addition, serum samples were checked for non-specific binding to control wells lacking antigen. We have previously shown that this anti-dsDNA ELISA system shows no cross reactivity with ssDNA.

2.8 Characterisation of lymph node and splenic composition in IFN-γ transgenic mice

2.8.1 Immunohistochemistry of lymph nodes and spleen

For immunohistochemistry, lymph nodes and spleens were snap frozen and sections cut onto glass slides and stored at -70°C. Before antibody staining, sections were fixed in acetone at -20°C for 5 minutes, and endogenous peroxidase activity blocked by incubation for 15 minutes at room temperature in 0.6% hydrogen peroxide. For double immunolabelling with anti-B220 and anti-CD3, sections were blocked with rabbit serum (Sigma) for 15 minutes at room temperature at 1:25 dilution before addition of primary antibodies (see below; used as recommended by Pharmingen). Germinal centres were stained with peanut agglutinin (PNA)-biotin (1 μg/ml). Secondary reagents were combinations of the following: rabbit anti-rat IgG-alkaline phosphatase (AP) (Sigma) diluted 1:50; streptavidin-horse radish peroxidase (HRP) (DAKO) diluted 1:400; or streptavidin-AP (DAKO) diluted 1:100. AP was visualised in

blue using the Vector Blue AP Substrate kit III (Vector Laboratories); HRP in red using 3-amino 9-ethyl carbazole (AEC) as described (Boenisch 1989). Photographs of sections were taken with an Olympus LB 1x KDC System attached to a Leica microscope. Images were transferred onto Adobe Photoshop 5.0.2 using Kodak DCS Acquire (version 5.5.9) software.

2.8.2 Flow cytometry of lymph node and splenic cells

Single cell suspensions were obtained by teasing spleens or lymph nodes and filtering the resultant cell mix through a sterile, glass wool-plugged pasteur pipette to remove stromal debris. Splenocytes or lymphocytes were incubated for 30 minutes on ice in DMEM supplemented with 5% fetal calf serum (E4-5), with combinations of the following pre-conjugated monoclonal antibodies: anti-CD3-FITC, anti-B220-PE, anti-IgM-biotin, anti-IgD-FITC, anti-CD11c-biotin, anti-TCRβ-biotin, anti-γδTCR-FITC (all from Pharmingen) or anti-monocyte (F4/80)-FITC (Caltag Laboratories). Stained cells were washed twice with E4-5 before incubation with second step reagents as above. Biotin conjugated antibodies were visualised using streptavidin-Tricolor (Caltag Laboratories). Twenty thousand events were collected per sample and analysed on a FACSCalibur using Cell Quest software (Becton Dickinson).

2.9 Demonstration of apoptosis in IFN-γ transgenic mice

2.9.1 Fluorescent TUNEL staining of skin sections

TUNEL staining of paraffin sections of skin was carried out using a commercially available kit (Apoptosis Detection System, Fluorescein, Promega). Formalin fixed skin sections were deparaffinised and rehydrated

sequentially in graded ethanol. Sections were then washed in 0.85% NaCl followed by PBS. Tissue sections were fixed in 4% methanol-free formaldehyde in PBS for 15 minutes at room temperature followed by extensive washing in PBS. Sections were subsequently incubated with Proteinase K (20 mg/ml in 100 mM Tris-HCl containing 50 mM EDTA, pH 8.0) for 8 minutes at room temperature. Sections were again formalin fixed and after washing in PBS were incubated for 10 minutes at room temperature with "equilibration buffer" (200 mM potassium cacodylate, 25 mM Tris-HCl, 0.2 mM DTT, 0.25 mg/ml BSA and 2.5 mM cobalt chloride, pH 6.6). The reaction mixture (equilibration buffer containing 5 mM fluorescein-12-dUTP, 10 mM dATP, 100 mM EDTA and 0.5 units/ml terminal transferase) was added and incubated at 37^0C for one hour. The reaction was stopped by immersing the slides in 2X SSC for 15 minutes at room temperature. Following extensive washing in PBS, sections were counterstained with propidium iodide solution (1 μg/ml in PBS). After washing in deionized water stained sections were mounted in Gelvatol (Monsanto) and examined using a Zeiss Axiophot microscope.

2.10 Statistics

Optical densities obtained with serum on ELISA from different groups of animals were compared using the Mann-Whitney *U* test. The significance of differences in the incidence of renal disease at dissection was assessed using Fisher's exact test. The relationship between severity of renal disease and autoantibody levels was defined by calculating the Pearson product moment correlation coefficient.

Chapter 3

Murine Lupus in IFN-γ Transgenic Mice

3.1 Introduction

The demonstration of antinuclear antibodies in IFN-γ transgenic mice and the similarities between IFN-γ induced inflammatory skin disease and CLE raised the possibility that these animals developed a lupus-like syndrome. Such a model might allow dissection of the putative role of the SIS in the pathogenesis of SLE. I, therefore, investigated these animals for the presence of lupus-related systemic disease.

3.2 Mice studied

The nature of the autoimmune response and the possible presence of related systemic disease in IFN-γ transgenic mice was investigated in a study of thirty four transgenic animals and 12 non-transgenic littermate controls. Three founder lines with varying copy number of the transgene were available for study, 1212F: 32 copies, 1205C: 6 copies, 1205D: 2 copies. All three founder lines were represented in this study. Tables 3.1 and 3.2 list the age and sex of each animal and summarise several of the parameters measured. The majority of animals analysed in our initial description of the IFN-γ associated skin phenotype were under 5 months of age (Carroll et al, 1997). However, the mean age of the transgenics and negative control littermates in the present study was greater: 8.1 months and 9.6 months, respectively.

We previously reported that IFN-γ was readily detectable in the skin of transgenic but not control mice, the 1212F line having the highest concentration (33 pg/cm^2) (Carroll et al, 1997). None of the transgenics in the early study had detectable levels of circulating IFN-γ (Carroll et al, 1997). I used the same ELISA method to test the serum of 15 of the present group of transgenics and five negative littermate controls (Tables 3.1 and 3.2). All five

controls and 11 of the transgenic animals had no detectable serum IFN-γ, but 4 male transgenics had small amounts (15-40 pg/ml; limit of detection in the assay is 5 pg/ml).

There is evidence that the involucrin promoter is active in urothelium (Prowse et al, in press). Increased levels of IFN-γ in the parenchyma of the kidney might have contributed to the pathogenesis of glomerulonephritis in IFN-γ transgenics. We, therefore, measured IFN-γ levels in kidney tissue extracts of two transgenic females from this founder line (no.s 33 and 34, Table 3.2) and two littermate controls (C11 and C12, Table 3.1). The level of IFN-γ detected was 4 pg/mg total protein in the transgenics and 6 pg/mg in the controls, implying that increased levels of the cytokine do not play a significant role in the pathogenesis of GN.

3.3 IFN-γ transgenic mice produce non-organ specific antinuclear autoantibodies.

Previous studies in transgenic models have shown that local overproduction of IFN-γ can result in tissue-specific autoimmunity (Sarvetnick et al, 1990. Gu et al, 1995). However, indirect immunofluoresence testing on skin and oesophagus using serum from IFN-γ transgenic mice produced staining of multiple cell types: keratinocytes, stromal fibroblasts, muscle cells and endothelial cells could all be identified. Positive staining of all cell types was observed, (Table 3.2, Figure 3.1A) with serum from 14 of 17 transgenic mice over the age of 3 months. Serum from 6 of 7 littermate controls was negative.

Autoantibodies directed against membrane antigens are a feature of several autoimmune skin diseases, the antigens frequently being proteins involved in cell-cell or cell-extracellular matrix adhesion (Iwatsuki et al, 1994, Liu et al, 1993). The cellular distribution of the antigens recognised by antibodies in the

serum of the IFN-γ transgenics was examined by staining cultured mouse and human keratinocytes. In the 10 serum samples examined (Table 3.2) there was intense staining of the nucleus, with no evidence of membrane staining (Figure 3.2A,C). Autoantibodies stained nuclei of both mouse and human cells. For comparison keratinocytes were stained with an antibody to desmogleins, the autoantigens of pemphigus vulgaris and pemphigus foliaceus, which mediate intercellular adhesion at desmosomes; as illustrated in Figure 3.2B the staining pattern was quite distinct from that observed with autoantibodies from the IFN-γ transgenics.

Table 3.1 Gender, age, autoantibodies and serum IFN-γ levels in negative control littermates of IFN-γ transgenic mice.

Animal	Sex	Age	Serum IFN-γ pg/ml	IIF	CL	Kidney DIF
C1	M	12		-	-	-
C2	F	10		-	-	-
C3	F	3		-	-	
C4	F	10		-	-	-
C5	M	13	UN	-	-	-
C6	M	13	UN	-	-	-
C7	M	13		+	-	
C8	F	12	UN		-	
C9	F	11	UN		-	
C10	M	4	UN		-	
C11	F	7			-	
C12	F	7			-	

Age is quoted in months. M: male, F: female, IIF: indirect immunofluorescence on normal mouse skin/oesophagus, DIF = direct immunofluorescence, CL: serum tested on *C. luciliae*, UN = undetectable.

Table 3.2. Gender, age, autoantibodies and serum IFN-γ levels in IFN-γ transgenic mice.

Animal	Sex	Age	Serum IFN-γ pg/ml	IIF	Staining pattern on CK	Western blot	CL	ENA	Kidney DIF
1	M	9			N	-	+	-	+
2	M	5		+		-	+	-	
3	M	12	UN	+			+		
4	M	4	15						
5	M	4	UN						
6	M	5	40	+	N	-	+	-	-
7	M	12					+		++
8	M	9	UN	+		-	+		+++
9	M	9		+	N	-	+	-	-
10	F	12	UN				+		
11	F	7	UN	+	N	-	+		++
12	F	6			N	-	+	-	+
13	F	10					+		++
14	F	10	UN	+			+		+++
15	F	11	UN				+		
16	F	4	UN		N	-	+	Sm	+++
17	-	4		+	N	-	+	-	-
18	M	12	15				-		
19	M	5	30	+	N	-	+	-	+++
20	M	5		+	N	-	-	-	-
21	M	8		+					
22	M	8		+	N	-			+++
23	M	8		+					
24	M	9		+					
25	M	5		-					
26	M	4		-					
27	M	13					-		
28	F	3		-					
29	M	13	UN						
30	F	12	UN						
31	F	12	UN						
32	F	12							
33	F	7					+		
34	F	7					+		

Table 3.2. Age is quoted in months. M: male, F: female, IIF: indirect immunofluorescence on normal mouse skin or oesophagus, CK: cultured keratinocytes, CL: serum tested on *C. luciliae*, ENA: antibodies against extractable nuclear antigens, DIF: direct immunofluoresence, UN: undetectable, N: nuclear staining on cultured mouse and/or human keratinocytes. On direct immunofluorescence of kidneys, glomerular staining intensity ranged from none (-), mild/focal (+), moderate (++) to intense (+++). The presence of subendothelial-mesangial deposits was confirmed by electron microscopy in mice 13 and 16. There was histological evidence of severe proliferative glomerulonephritis in animals 12, 14 and 16 (see Table 3.3).

3.3.1 Antinuclear antibodies in IFN-γ transgenics are specific for dsDNA and histones

In order to identify proteins recognised by the autoantibodies from IFN-γ transgenic mice, extracts of cultured mouse and human keratinocytes were resolved on SDS-PAGE and subjected to immunoblotting (Table 3.2). 10 of the 12 serum samples recognised a single band with an apparent molecular mass of about 100kD. However, serum from two non-transgenic BALB/c control mice also recognised the band, indicating that it was not specific to the transgenics. As predicted from the immunofluorescence staining shown in Figure 3.2B, the 100 kD band was not recognised by a pan-desmoglein antibody (data not shown). Furthermore, the mobility is distinct from that of the bullous pemphigoid antigens (180kD) (Labib et al, 1986). In addition, serum from transgenic animals did not immunoprecipitate specific proteins (data not shown).

We next tested the serum samples from 9 female transgenics for antibodies to extractable nuclear antigens (ENA) using counter-current immunoelectrophoresis, performed by Miss Karen Walker , Department of Immunology, Birmingham University. Increased levels of autoantibodies against the ENA screened (Sm, U1 RNP, SS-A (Ro), SS-B (La), Jo1 and Scl-70) were detected in one animal only (no. 16 in Table 3.2). On further characterisation this mouse was found to be positive for anti-Sm autoantibodies.

In order to examine whether antibodies to dsDNA were present, we screened serum for reactivity to the kinetoplast of the flagellate organism *Crithidia luciliae*. Indirect immunofluorescence testing of serum on *C. luciliae* has been used as a specific test for the presence of anti-dsDNA autoantibodies (Sontheimer and Gilliam, 1978). Serum samples from 21 transgenic mice and 12 negative littermate controls were tested. Eighteen samples from transgenic animals produced definite staining of the kinetoplast (Figure 3.3, Table 3.2). All littermate controls were negative on this test (Table 3.1).

We used ELISA assays to quantitate the levels of anti-dsDNA autoantibodies and to determine whether antihistone antibodies were also present. Sera from male (mice no.s 1-9 and 19, mean age: 7.4 months, Table 3.2) and female transgenic mice (mice no.s 10-16, mean age: 8.6 months, Table 3.2) and negative control littermates (mice C1-C6 for anti-dsDNA and mice C1-C4, C9 and C10 for antihistone, Table 3.1), were measured and are shown in Figure 3.4A,B and Table 3.3. Compared to littermate controls (mean OD: 0.038) both male (mean OD: 0.081, $p<0.01$) and female (mean OD: 0.232, $p<0.003$) transgenics showed evidence of anti-dsDNA antibody production. Levels were significantly higher in females than males ($p<0.007$). Four 6 month old female MRL-*lpr* mice known to produce high levels of antinuclear antibodies (kindly provided by Dr C.T. Ravirajan, Bloomsbury Rheumatology Unit, University College, London) were included as positive controls. All four MRL-*lpr* mice

produced higher levels of anti-dsDNA antibody than the transgenic females (mean OD: 0.848 v 0.232, respectively, (Figure 3.4).

Female transgenics tested produced antihistone antibodies at levels comparable to the MRL-*lpr* positive controls (mean OD 0.313 and 0.478 respectively, Figure 3.4B). Anti-histone antibody levels in serum from male transgenics did not differ significantly from negative littermate controls (mean OD: 0.011 and 0. 013 respectively, Figure 3.5). Antinuclear autoantibodies were detected in female animals from all three transgenic founder lines. Interestingly, mice which produced the highest levels of anti-dsDNA tended to produce low levels of antihistone antibodies and visa versa (Table 3.3).

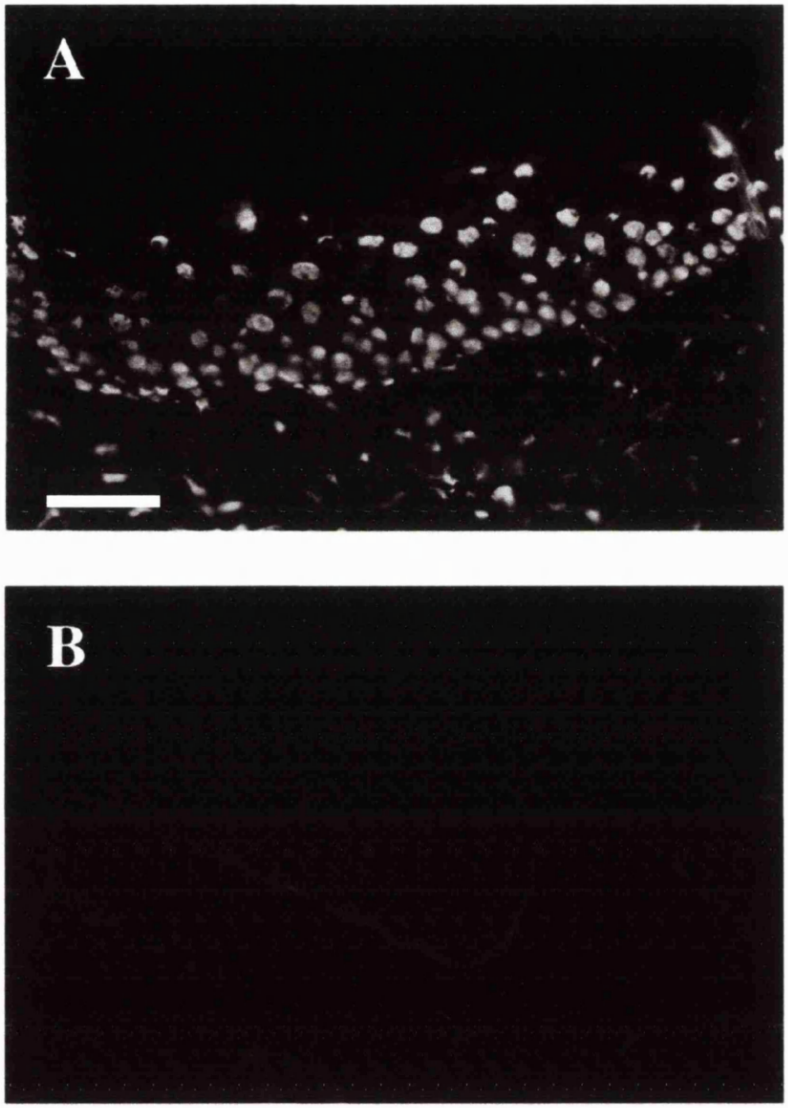


Figure 3.1 Indirect immunofluorescence on normal mouse tail skin using serum (1:10 in PBS) from female IFN-γ transgenic mouse (A) and non-transgenic female littermate control (B). Scale bar (A = B): 60μm. Note the nuclear staining in (A) is not confined to keratinocytes but is also present in cells in the dermis.

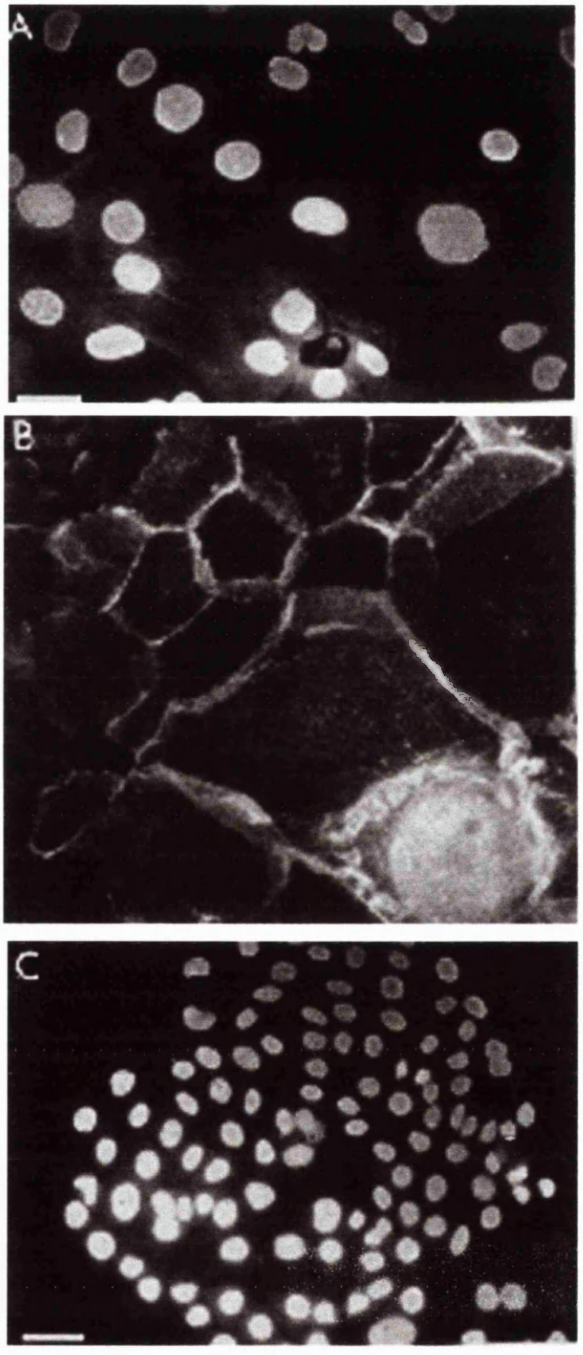


Figure 3.2 Indirect immunofluorescence staining of cultured keratinocytes (A and B) mouse and (C) human keratinocytes. (A and C) Stained with serum from a transgenic mouse (1:10 in PBS). (B) Stained with antidesmoglein antibody. Scale bar (A and B): 30μm; (C): 60μm.

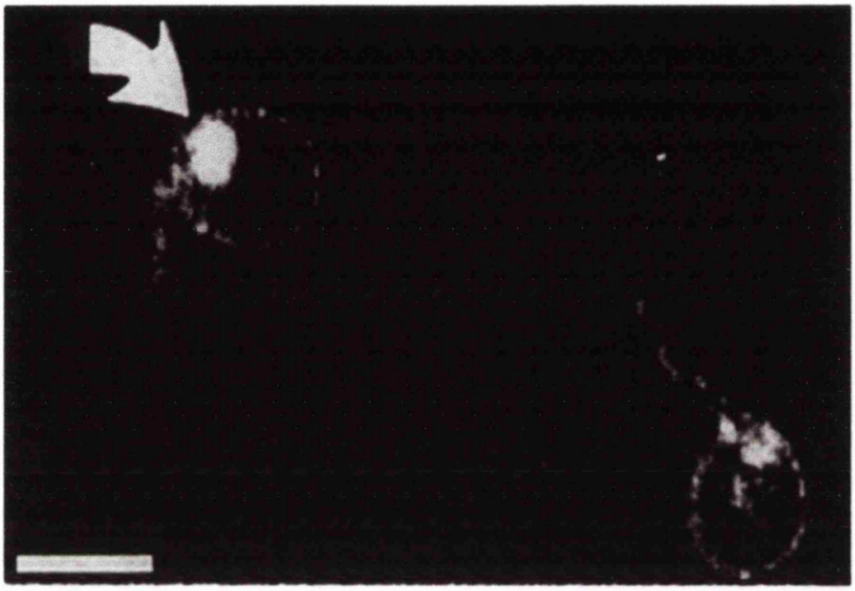


Figure 3.3 *C. luciliae* assay. Staining with serum (1:10 in PBS) from IFN-γ transgenic mice produces intense staining of the kinetoplast of the organism (arrow), consistent with the presence of anti-dsDNA antibodies. A series of 1 μm optical sections through the specimens was obtained with a confocal microscope and a composite image (Z series) was constructed. Scale bar: 25 μm

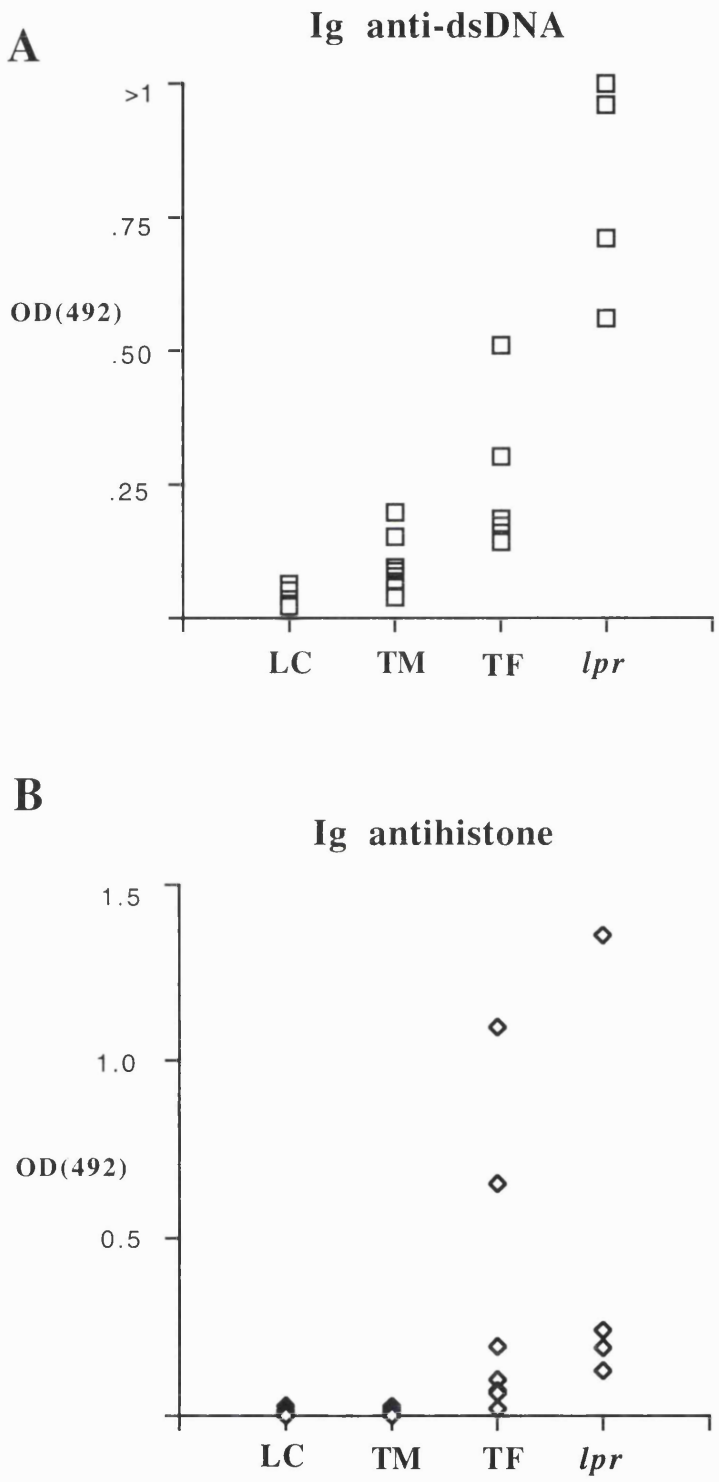


Figure 3.4 (A) Anti-dsDNA and (B) antihistone antibody levels in transgenic mouse serum. Sera from 17 transgenic mice and 6 negative littermate controls were tested individually against dsDNA at 1:250 and histones at 1:1000 dilution. Each OD value represents the mean of three measurements. LC, littermate controls; TM, transgenic males; TF, transgenic females. Four female MRL-*lpr* mice aged 6 months and known to produce high levels of ANA are included as positive controls.

3.4 Evidence of systemic disease in IFN-γ transgenic mice

3.4.1 IFN-γ transgenics develop immune complex glomerulonephritis

Anti-dsDNA antibodies are known to deposit in the kidneys of 60-70% of SLE patients and to cause glomerulonephritis (GN) (Appel et al, 1978 , Itoh et al, 1993). We, therefore, examined the kidneys of the transgenic mice for evidence of autoantibody deposition and organ damage (Fig. 3.5). The degree of renal damage was assessed by Dr Victoria Cattell (consultant histopathologist) at St. Mary's Hospital, London. Immunohistochemistry of kidneys showed dense deposits of IgG within the glomeruli in all female IFN-γ transgenic mice examined (n = 5, Table 3.2). As in human lupus-nephritis, both mesangial (Figure 3.5A) and capillary (Figure 3.5 B) patterns of Ig deposition were found. Five of eight male mice tested also had evidence of Ig deposits in the glomeruli (Table 3.2).

Histological examination of kidney tissue (16 males and 11 females) demonstrated clear evidence of GN in female mice only. The severity of the lesion varied from mild mesangial nephritis (not shown) to severe diffuse proliferative GN (Figure 3.5C). The former was observed in mice from two founder lines 1205C and 1212F and overall occurred in approximately 25% of female animals examined (no. 12, 14, 16 Tables 3.2 and 3.3). Subendothelial-mesangial deposits were confirmed by electron microscopy (Figure 3.5E) and the immunopathology closely corresponded with the findings in spontaneous murine lupus-like syndromes (Andrews et al, 1978). Interestingly, severe proliferative GN occurred in female transgenics with high levels of anti-dsDNA (Table 3.3). Control littermates had normal kidneys (n=10) (Figure 3.5D).

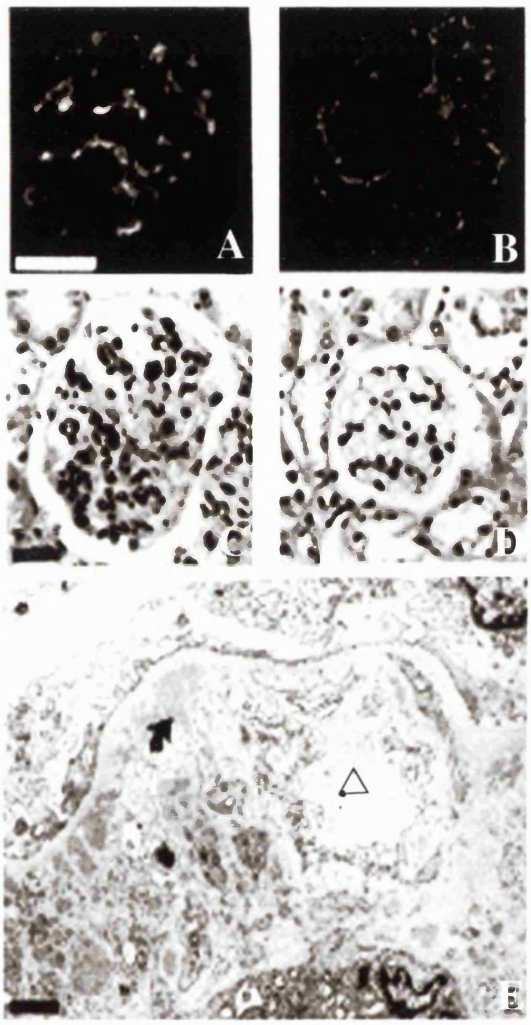


Figure 3.5. Glomerular pathology in female transgenic mice. Direct immunofluorescence staining of renal tissue demonstrated two patterns of immune complex deposition in these animals, (A) mesangial and (B) capillary. Haematoxylin and eosin stained glomerulus from a 10 month old female transgenic mouse (C) and a glomerulus from an age and sex matched negative littermate control (D). Note the hypercellularity in (C). (E) Electron micrograph of kidney tissue from a 4 month old female transgenic shows electron dense subendothelial-mesangial deposits (arrow) and a narrowed capillary lumen (triangle). Scale bar (A-D): 60 μm; (E): 1.5 μm.

Table 3.3. Kidney pathology and relative levels of antinuclear antibodies in female IFN-γ transgenic mice.

Animal no.	Anti-dsDNA	Antihistone	Kidney pathology
10	0.143	0.019	Mild mesangial GN
11	0.186	0.194	Mild mesangial GN
12	0.148	0.070	Severe diffuse proliferative GN
13	0.160	0.653	Mild mesangial GN
14	0.303	0.101	Severe diffuse proliferative GN
15	0.173	1.094	Mild mesangial GN
16	0.510	0.063	Severe diffuse proliferative GN

Antibody levels are expressed as optical density (OD) measured at 492 nm. Anti-dsDNA and antihistone ELISAs were carried out at dilutions of 1/250 and 1/1000 respectively. GN: glomerulonephritis.

3.5 Discussion

3.5.1 The lupus-like syndrome of IFN-γ transgenics closely resembles SLE in humans

Targeting an IFN-γ transgene to the suprabasal epidermal layers via the involucrin promoter results in a clear lupus-like syndrome with production of antihistone and anti-dsDNA autoantibodies and immune complex nephritis. Lupus in IFN-γ transgenic mice mirrors many features of the naturally occurring human disease. The highest levels of both antihistone and anti-dsDNA autoantibodies were detected in female animals and histological evidence of kidney pathology was only observed in female transgenics (Figure 3.5). The severity of the lupus phenotype varied markedly between IFN-γ transgenic female mice from low autoantibody levels associated with minor increases in glomerular cellularity to high levels of anti-dsDNA antibodies with

severe proliferative GN. The severity of the disease also varies markedly in man. Indeed affected monozygotic twins have been described with the same serology but marked differences in systemic disease manifestations indicating variable penetrance even in the presence of all susceptibility determining loci (Reichlin et al, 1992).

As in humans two patterns of immune complex deposition were observed in the kidney. In some animals deposition occurred purely in the measangium whereas in others Ig was detected both in the mesangium and subendothelial region deposits (i.e. capillary loop involvement) (Figure 5A,B). Clinical experience indicates that there may be two forms of mesangial nephropathy, one confined to the mesangium itself with a subsequent benign course (stable WHO class II) and a second with both mesangial and subendothelial immune leading to progressive proliferative glomerular lesions with poor prognosis (Who Classes III to IV). Interestingly, the two transgenic mice with the most severe proliferative renal disease demonstrated Ig deposition in the glomerular capillary loops (no. 14 and 16, Table 3.2).

In addition, the inflammatory skin disease of these animals shares several features with cutaneous lupus in man; keratinocyte MHC and ICAM-1 expression, a dermal mononuclear infiltrate and the migration of dendritic cells from the epidermis. Furthermore, the separation of epidermis from dermis with infiltration of haemopoietic cells is reminiscent of the hydropic degeneration of basal cells that is characteristic of SLE (Lever and Lever 1983). The ability of IFN-γ to reproduce the histological features of CLE may relate to its ability to induce iNOS expression in keratinocytes (Arany et al, 1996). As discussed in section 1.3 excess iNOS activity may play a role in UVB induced erythema in SLE patients. In addition, the skin disease occurs in association with marked superficial lymphadenopathy and splenomegaly.

3.5.2 The absence of autoantibodies against ENA in IFN-γ transgenic mice

With the exception of one animal, increased levels of anti-dsDNA antibodies were not accompanied by production of autoantibodies against the ENA tested. Interestingly, the single mouse with anti-ENA antibodies was found to be positive for anti-Sm autoantibodies, a serological finding considered pathognomonic SLE (Tan, 1989). However, the consistent generation of high levels of anti-histone and anti-dsDNA antibodies in our transgenic mice in the absence of significant autoreactivity to ENA supports the concept that there are as yet unidentified differences in the pathogenic mechanisms underlying the generation of the two types of autoantibody (Ma et al, 1996). For example, Ma et al, showed that MRL*lpr* mice congenitally deficient in CD40 ligand fail to produce IgG anti-dsDNA and do not develop glomerulonephritis. However, they continue to produce IgG anti-snRNP antibodies. This may indicate that production of anti-snRNP antibodies is less dependent on cognate T-B cell interaction. Some IgG anti-Sm antibodies do indeed have a germline Ig-variable region sequences and their production may be less dependent on T cell help (Sanz et al, 1989).

3.5.3 The consequences of IFN-γ overexpression are tissue specific

With the benefit of hindsight the development of antinuclear antibodies in these mice is not surprising. There is good evidence that both IFN-γ and the SIS are involved in the pathogenesis of SLE (see sections 1.4 and 1.7). However, previous observations in transgenic mice in which IFN-γ is overexpressed in specific tissues have suggested that local overproduction of IFN-γ is primarily involved in the pathogenesis of organ specific autoimmunity. Transgenic overexpression of IFN-γ in pancreatic islets, neuromuscular junction and the CNS results in autoimmune diseases resembling insulin dependent diabetes mellitus, myasthenia gravis and multiple sclerosis respectively (Sarvetnick et al, 1990; Gu et al, 1995; Horwitz et al, 1997). However, even these studies

strongly implied that the consequences of IFN-γ overexpression may vary with tissue type. Transgenic expression of IFN-γ in beta-cells results in a cell mediated immune destruction of pancreatic islets (Sarvetnick et al, 1990), whereas overexpression in the neuromuscular junction elicits a humoral response with no evidence of cell mediated damage (Gu et al, 1995).

It has been argued that the response of antigen-presenting cells to cytokines may differ in different tissue types, leading to different patterns of T cell activation (Sarvetnick, 1996). Certainly, there is evidence that the skin immune system has distinctive properties, both in terms of its APC and keratinocyte functions (Bos and Kapsenberg, 1993). Perhaps most prominent among these distinctive properties is the ability of the keratinocyte to act as a potent immunomodulator. Keratinocytes produce a wide array of cytokines which can profoundly alter immunocyte function. For example, recent evidence suggests that keratinocytes can direct a T cell response towards either a Th1 or Th2 profile and hence promote the development of either a humoral or cell mediated immune response via production of IL-10 and IL-12 (Mosmann and Coffman, 1989; Romagnani, 1994, Enk and Katz, 1992, Muller et al, 1994, Hsieh et al, 1993, Howard and O'Garra, 1992). In addition, keratinocytes can profoundly alter APC function. GM-CSF produced by keratinocytes is a potent survival factor for LC (Witmer-Pack, 1987) and also promotes LC maturation during migration to local nodes, in particular the upregulation of B7-1 and B7-2 expression (Larsen et al, 1994).

Interestingly, it has also been shown that the phenotype of T cells in cutaneous inflammatory infiltrates is distinctive compared to other sites. For example, the majority of T cells in cutaneous infiltrates express cutaneous lymphocyte antigen, a marker rarely detected in other sites of inflammation (Picker et al, 1990). These differences may reflect the fact that several ligands including E-selectin, NCAM and CD36 are expressed to a greater extent on endothelial cells in inflamed skin compared to other anatomical regions (Picker et al, 1991;

Mizutani et al, 1994; Swerlick et al, 1992). In addition, the SIS contains a population of γδ T cells restricted to the epidermis (dendritc epidermal T cells: DETC) in adult mice expressing an invariant T cell receptor composed of Vγ5 and Vδ1 chains with no junctional diversity (Asarnow et al, 1988). Vγ5Vδ1 transfected Jurkat cells produced IL-2 when stimulated with mouse keratinocytes suggesting that the γδ T cell receptor of these T cells recognises a keratinocyte self antigen (Havran et al, 1991). Several lines of evidence suggest that DETC function to suppress autoreactivity. Recovery from cutaneous graft versus host (GVH) reactions in mouse skin is associated with increased numbers of DETC and protection against subsequent development of GVH (Shiohara et al, 1990). Furthermore, compared to controls, a cutaneous GVH reaction is initiated more easily in C δ null mice which lack γδ DETC (Shiohara et al, 1996). Hence, the SIS has many distinctive properties which could influence it's response to overexpression of IFN-γ.

Skin lesions are one of the classical clinical manifestations of SLE. Characterisation of IFN-γ transgenic mice provides evidence consistent with a central role both for this cytokine and the skin immune system in the pathogenesis of the systemic complications of the disease. I next set about elucidating the mechanisms involved in the generation of antinuclear autoantibodies by the skin immune system and attempted to identify possible sources of autoantigen.

Chapter 4

Pathogenic Mechanisms in IFN-γ-related Murine Lupus.

4.1 Introduction

There is convincing evidence that production of pathogenic IgG ANA by autoreactive B cells in human lupus requires autoantigen-specific cognate and contact dependent CD4+ αβ T cell help. Autoreactive CD4+ αβ T cells responsive to histones or nucleosomes (Desai-Mehta et al, 1995, Rajagopalan et al, 1990), U1 snRNP antigens and the ribosomal P antigen (Crow et al, 1994) have been isolated from humans with lupus. T cells isolated from SLE patients with the ability to help pathogenic anti-dsDNA production proliferate when mixed with B cells in a class II MHC-restricted and CD40-CD40 ligand-dependent fashion (Desai-Mehta et al, 1995; Desai-Mehta et al, 1996; Koshy et al, 1996). Detailed sequence analysis of antinuclear autoantibodies provides further convincing evidence that they arise via T cell dependent mechanisms. Analysis of V-regions in human anti-dsDNA antibodies shows a high frequency of replacement versus silent mutations in a non-random distribution, indicative of affinity maturation of the autoimmune response and strongly suggestive of antigen driven T cell dependent somatic hypermutation (Radic and Weigert 1994). For example, disease-associated IgG anti-DNA antibodies often have cationic amino acid substitutions in complementarity-determining regions (CDR). These substitutions enhance binding to DNA and in some instances result in conversion of reactivity from single-stranded to double-stranded DNA (Diamond et al, 1992; Radic and Weigert 1994).

The limited range of autoantibodies detected in IFN-γ mice suggested to me that they too arise by a specific antigen driven process. One way of testing this hypothesis is to assess the effects of T cell subset deficiencies on the lupus phenotype in IFN-γ transgenics. I, therefore, generated female IFN-γ transgenic mice congenitally deficient in either αβ or γδ T cells and measured the effects of these deficiencies on each aspect of the lupus phenotype.

4.2 The role of αβ and γδ T cells in IFN-γ related murine lupus

4.2.1 Animals studied

All mice used were females from the 1205D founder line and 5 months of age at the time of study. Female IFN-γ transgenic mice heterozygous for either the TCRβ or TCRδ chain deletion were used as controls since T cell function is essentially normal in these animals (Mombaert et al, 1992, Itohara et al, 1993). In addition, animals negative for IFN-γ, referred to as littermates negative for the transgene, were used to assess baseline autoantibody levels and renal histology.

4.2.2 γδ T cells do not play an essential role in IFN-γ induced lupus

Three of eight $\delta^{-/-}$ IFN-γ and three of eleven $\delta^{+/-}$ IFN-γ transgenic females examined developed the full IFN-γ associated lupus syndrome with IgG anti-dsDNA autoantibody production and proliferative glomerulonephritis on histology (Table 4.1). Renal disease, when present, tended to be more severe in $\delta^{-/-}$ IFN-γ transgenics (Table 4.1); however, the relatively low incidence of light microscopic renal lesions in these animals prevented statistical testing of this observation.

Serum from the mice was screened for autoantibodies to dsDNA, histones and nucleosomes. There was no difference in IgG anti-dsDNA titres between $\delta^{+/-}$ and $\delta^{-/-}$ IFN-γ transgenics (mean OD: $\delta^{-/-}$: 0.21; $\delta^{+/-}$: 0.16; p = NS) (Figure 4.1). Levels of both IgG antinucleosome and IgG antihistone antibodies were low in these litters. There appeared to a tendency towards higher levels of anti-nucleosomal antibody production in $\delta^{+/-}$ compared to $\delta^{-/-}$ transgenics, however, this did not reach statistical significance (mean OD: $\delta^{+/-}$: 0.10; $\delta^{-/-}$: 0.05; p = NS) (data not shown). Transgenic animals from the litters produced levels of IgG antihistone autoantibodies comparable to littermates negative for the

transgene (mean OD: $\delta^{-/-}$: 0.02; $\delta^{+/-}$: 0.01; littermates negative for the transgene: 0.02; p = NS) (Table 4.1). The occurrence of anti-dsDNA antibody production in the absence of anti-histone antibodies is interesting in view of the antigen spread hypothesis. Separation of linked sets of autoantibodies has been described in other models (Fatenejad et al, 1993) and presumably relates to unidentified genetic factors in these litters.

Table 4.1 Renal pathology in γδ T cell deficient IFN-γ transgenic mice.

Animal	Glomerular Pathology			
	GL/HC	Fibrin	Crescents	PMN
γδ Het				
1	-	-	-	-
2	-	-	-	-
3	-/+	-	-	-
4	++	-	-	-
5	-/+	-	-	-
6	-	-	-	-
7	+	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
11	+	-	-	-
γδ KO				
1 2	-/+	-	-	-
13	-/+	-	-	-
14	+++	-	-	+
15	-/+	-	-	-
16	++	-	-	-
17	-	-	-	-
18	-	-	-	-
19	++	-	-	-

γδ Het and γδ KO: IFN-γ transgenic animals heterozygous and homozygous for the TCRδ chain deletion, respectively. GL/HC, glomerular hypercellularity (-/+: equivocal; +: mild; ++: moderate; +++: severe); PMN: infiltrate of polymorphonuclear leukocytes.

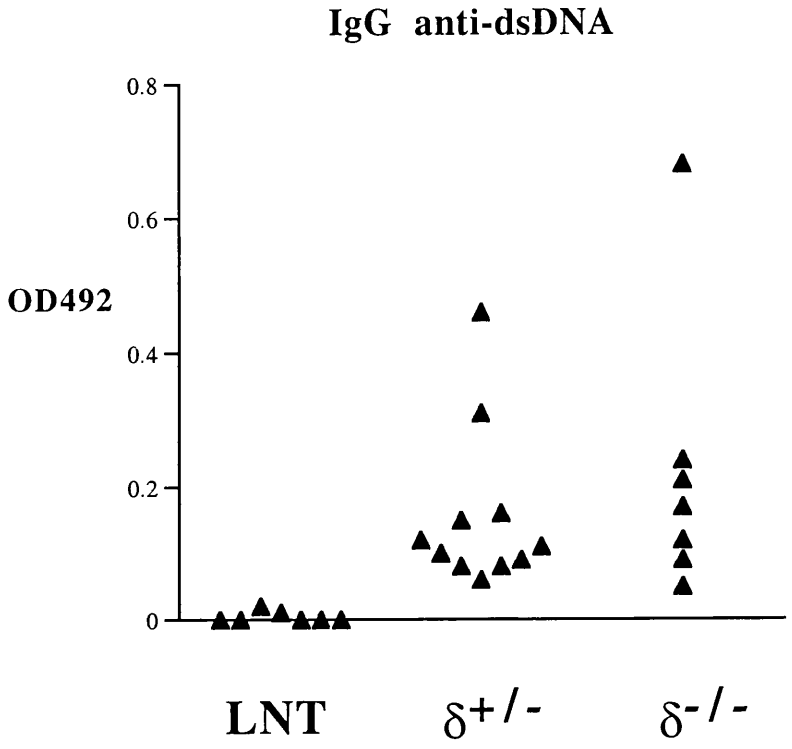


Figure 4.1 Anti-dsDNA IgG autoantibody levels in γδ T cell deficient IFN-γ transgenic mouse serum. Sera from 11 $\delta^{+/-}$, 8 $\delta^{-/-}$ IFN-γ transgenic mice and 7 littermates negative for the transgene (LNT) were tested individually against dsDNA at 1/100 dilution. The OD value for each sample represents the mean of three measurements.

4.2.3 IFN-γ induced lupus is αβ T cell dependent

We used ELISA assays to quantitate the levels of IgG anti-dsDNA, IgG antihistone and IgG antinucleosome autoantibodies in IFN-γ transgenic $\beta^{+/-}$ and $\beta^{-/-}$ mice. The six $\beta^{+/-}$ transgenics examined produced high levels of IgG antinuclear antibodies (mean ODs: 0.29, 0.20 and 0.63 for IgG anti-dsDNA, antihistone and antinucleosome autoantibodies, respectively) (Figure 4.2 and 4.3). Compared to the $\beta^{+/-}$ animals levels of all three antinuclear antibodies tested were significantly reduced in $\beta^{-/-}$ IFN-γ transgenics (mean ODs: 0.01; $p < 0.003$, 0.04; $p < 0.004$ and 0.02; $p < 0.003$ for IgG anti-dsDNA, IgG antihistone and IgG antinucleosome autoantibodies, respectively). Indeed serum from the 11 $\beta^{-/-}$ transgenic mice tested showed antinuclear antibody levels no higher than age and sex matched littermates negative for the transgene (Figure 4.2 and 4.3). In addition, levels of total Ig anti-dsDNA antibodies (i.e. all Ig classes) in $\beta^{-/-}$ IFN-γ transgenic mice were comparable to those in matched littermates negative for the transgene (mean OD: 0.03 and 0.02 respectively at 1/100 dilution, p = NS) (mean OD in $\beta^{+/-}$ IFN-γ transgenics: 0.21). Interestingly, serum from two littermates negative for the transgene showed low but significant levels of IgG antinucleosome autoantibodies (mean ODs: 0.28 and 0.30 at 1/100 dilution) suggesting a background susceptibility to autoantibody production, as has previously been reported in C57/BL mice (Seery et al, 1997, Botto et al, 1998). Mice negative for the IFN-γ transgene never produce anti-dsDNA or antihistone antibodies and never developed kidney disease (n = 30, in all studies to date).

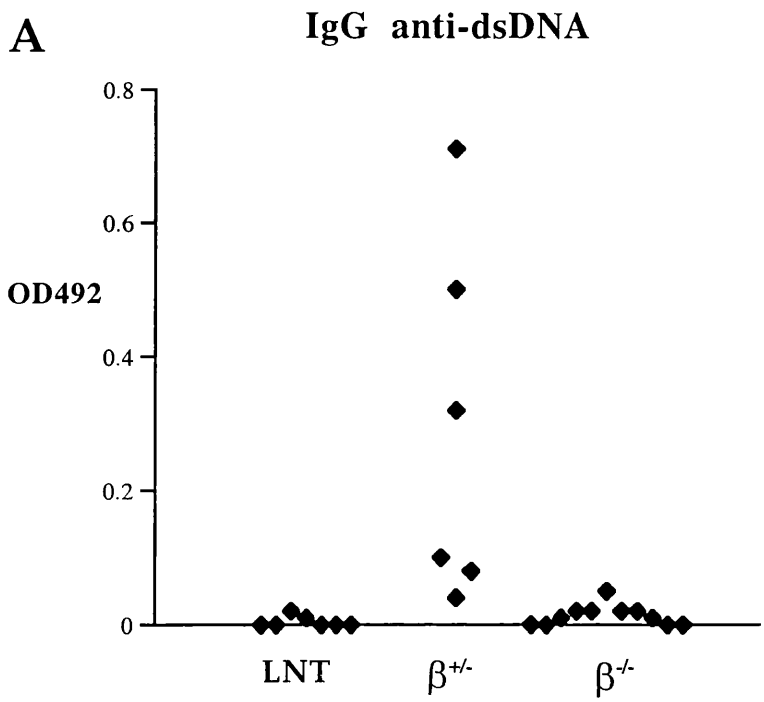
Three of the six $\beta^{+/-}$ female transgenics examined had proliferative glomerulonephritis. In contrast, renal histology was normal in the 15 $\beta^{-/-}$ IFN-γ transgenic animals examined (Table 4.2, p = 0.002). On direct immunofluorescence both mesangial and capillary patterns of glomerular IgG deposition were observed in $\beta^{+/-}$ IFN-γ transgenics (Table 4.2). Glomerular

deposition of IgG could not be demonstrated in any of 7 $\beta^{-/-}$ IFN-γ transgenic females tested (Table 4.2, $p = 0.008$). The two $\beta^{+/-}$ IFN-γ transgenic mice with nucleosome restricted autoantibodies showed evidence of IgG deposition in the kidney but no definite evidence of glomerulonephritis on light microscopy (no.s 4 and 6, Table 4.2)

Table 4.2. Renal pathology and autoantibody levels in αβ T cell deficient IFN-γ transgenic mice.

Animal	Glomerular Pathology				
	IF	GL/HC	Fibrin	Crescents	PMN
αβ Het					
1		++	-	-	+
2		+++	+	10%	+
3		-	-	-	-
4	+/M	-	-	-	-
5	+/C	+++	-	-	+
6	+/M	-/+	-	-	-
αβ KO					
7	-	-/+	-	-	-
8	-	-	-	-	-
9	-	-	-	-	-
10		-	-	-	-
11		-	-	-	-
12	-	-	-	-	-
13	-	-	-	-	-
14	-	-/+	-	-	-
15		-	-	-	-
16	-	-	-	-	-
17		-	-	-	-
18		-	-	-	-
19		-	-	-	-
20		-	-	-	-
21		-	-	-	-

αβ Het and αβ KO: IFN-γ transgenic animals heterozygous and homozygous for the TCRβ chain deletion, respectively. IF, direct immunofluorescence; M and C, mesangial and capillary patterns, respectively, of IgG deposition in glomerulus, GL/HC, glomerular hypercellularity (-/+: equivocal; +: mild; ++: moderate; +++: severe); PMN: infiltrate of polymorphonuclear leukocytes.



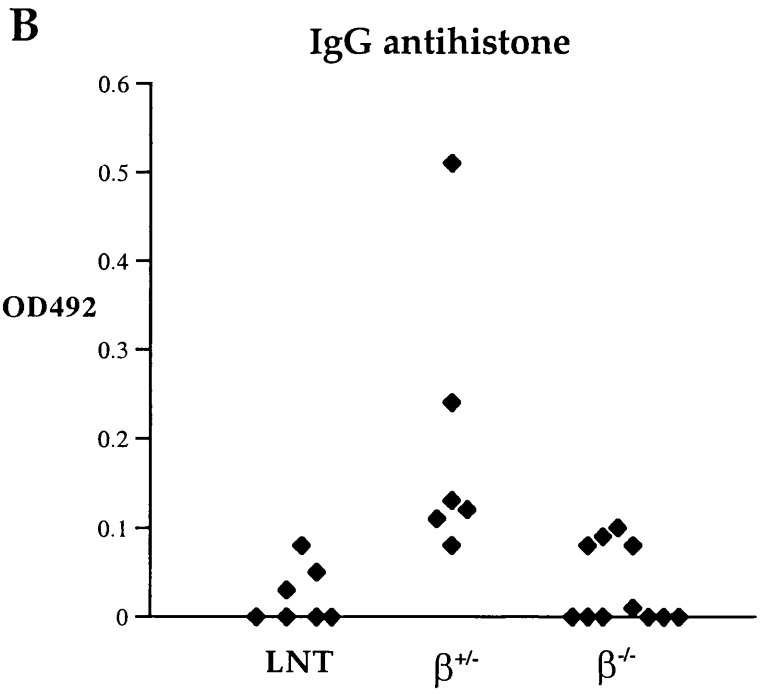


Figure 4.2 A) Anti-dsDNA and B) antihistone IgG autoantibody levels in αβ T cell deficient IFN-γ transgenic mouse serum. Sera from 6 $\beta^{+/-}$, 11 $\beta^{-/-}$ IFN-γ transgenic mice and 7 littermates negative for the transgene (LNT) were tested individually against dsDNA and histones at 1/100 dilution. Each OD value represents the mean of three measurements.

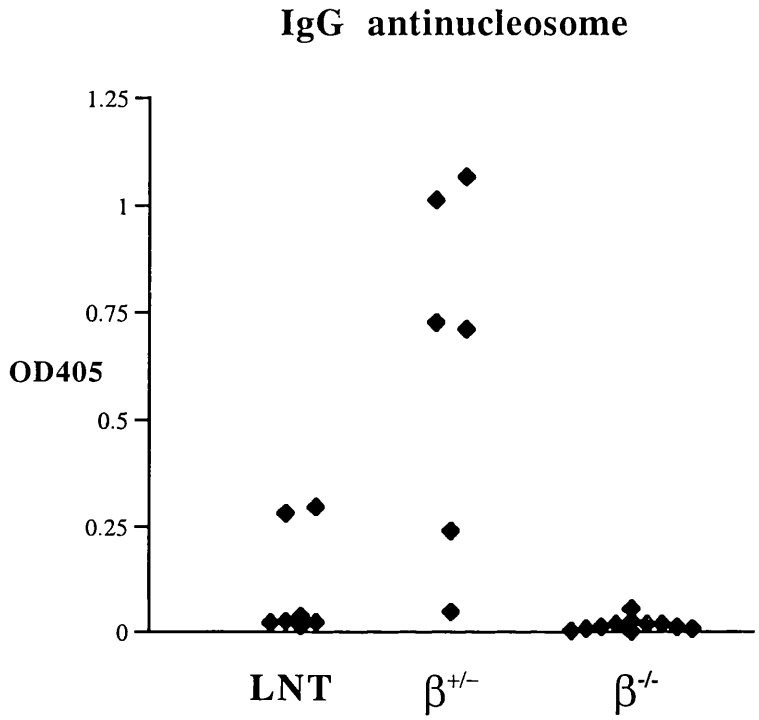


Figure 4.3 Antinucleosome IgG autoantibody levels in αβ T cell deficient IFN-γ transgenic mouse serum. Sera from 6 $\beta^{+/-}$, 11 $\beta^{-/-}$ IFN-γ transgenic mice and 7 littermates negative for the transgene (LNT) were tested individually against dsDNA and histones at 1/100 dilution. Each OD value represents the mean of two measurements.

4.2.3 IFN-γ associated inflammatory skin disease, lymphadenopathy and splenomegaly do not depend on the presence of either αβ or γδ T cells

All IFN-γ transgenic $\delta^{-/-}$ and $\beta^{-/-}$ mice developed the skin phenotype previously described in association with IFN-γ overexpression in the epidermis (Carroll et al, 1997). Early in the neonatal period, T cell deficient transgenic animals exhibited marked hypopigmentation of the hair. A proportion of these mice developed hair loss, cutaneous erythema and flaking, all of which were particularly marked around the limb joints. Histological examination of skin from both $\delta^{-/-}$ and $\beta^{-/-}$ IFN-γ transgenics demonstrated a range in the severity of lesions from focal spongiosis in the epidermis with dermal inflammation to epidermal hyperplasia associated with hyperproliferation and a predominantly mononuclear, dermal inflammatory infiltrate as previously described (Carroll et al, 1997).

On internal examination, gross peripheral lymphadenopathy and splenomegaly was obvious in the majority of IFN-γ transgenics, irrespective of the animals T cell status. Detailed histological analysis of the lymph nodes and spleen from one of the $\beta^{-/-}$ IFN-γ transgenics, (carried out by Dr. Eddie Wang, The Lymphocyte laboratory, I.C.R.F.) showed a complete absence of T cell zones and germinal centres (Figure 4.3). We have previously shown that superficial lymph nodes in IFN-γ transgenics contain markedly elevated numbers of dendritic cells, consistent with migration of these APCs from the skin to draining nodes (Carroll et al, 1997). This phenomenon persisted in $\beta^{-/-}$ IFN-γ transgenics. On FACS analysis of grossly enlarged lymph nodes from $\beta^{-/-}$ mice, cells positive for F4/80 and CD11c positive comprised 4.9% of all non-erythrocyte cells present compared to 1.5% in lymph nodes from littermates negative for the transgene (data not shown).

There is evidence that congenital absence of T cells may result in impaired B cell development associated with loss of B cell CD23 expression (Gonzalez et

al, 1995). We found no evidence of such a phenomenon in $\beta^{-/-}$ IFN-γ transgenics. B cells from lymph nodes and spleen of $\beta^{-/-}$, $\beta^{+/-}$ IFN-γ transgenics and littermates negative for the transgene showed comparable levels of CD23 expression (Figure 4.5).

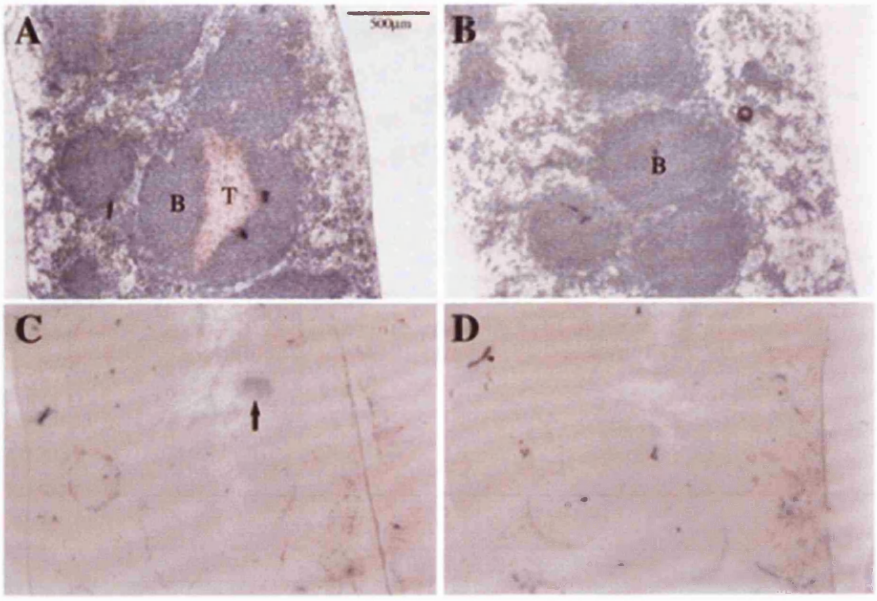


Figure 4.4 Absence of T cell regions and germinal centres in IFN-γ transgenic β-/- mice. Spleens from IFN-γ transgenic, β+/- (A,C) or β-/- (B,D) mice were stained for B (antiB220) and T cells (antiCD3) (A,B) or germinal centres (PNA) (C,D). (A) Distinct T cell regions (red, marked T) were seen within B cell regions (blue, marked B) in β+/- spleens, but were absent inβ-/- spleens (B). (C) Germinal centres were present inβ+/- spleens (arrow), but (D) absent fromβ-/- spleens. Scale bar (A = B = C =D: 500μm)

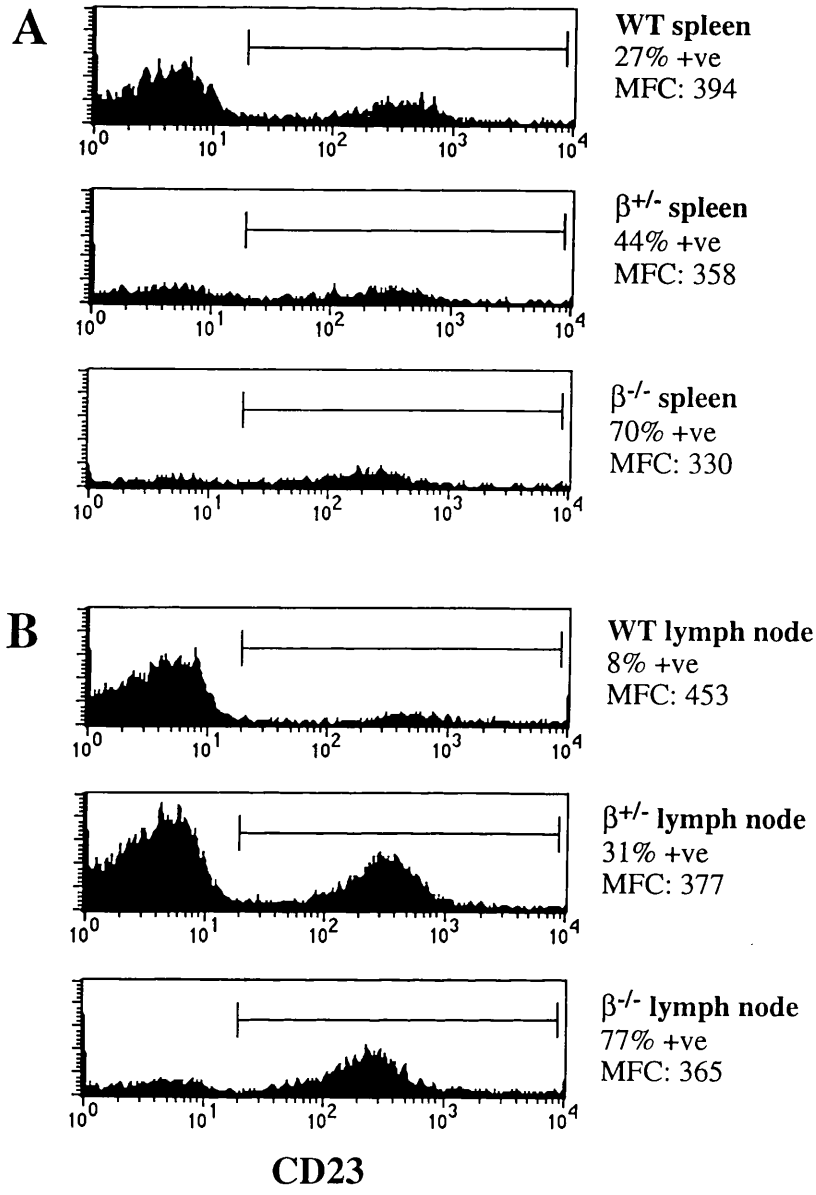


Figure 4.5 Splenocyte CD23 expression in IFN-γ transgenic mice. CD23 cell surface expression on splenocytes (A) and lymph node cells (B) of IFN-γ transgenic, $\beta^{+/-}$ or $\beta^{-/-}$ mice and littermates negative for the transgene (WT) was investigated by FACS analysis using standard forward versus side scatter lymphocyte gates. There was a significantly greater proportion of both (A) splenocytes, and (B) lymph node cells, expressing CD23 in $\beta^{-/-}$ compared to $\beta^{+/-}$ mice. There were no differences in cell surface expression as estimated by the mean fluorescence channel (MFC) of $CD23^{+}$ cells.

4.3 The severity of renal disease correlates with IgG anti-dsDNA autoantibody levels in IFN-γ transgenic mice

In the process of studying the role of T cells in the pathogenesis of IFN-γ related lupus we generated three groups of animals producing significant titres of anti-dsDNA antibodies ($\delta^{+/-}$, $\delta^{-/-}$ and $\beta^{+/-}$ IFN-γ transgenics, n = 25). This afforded an opportunity to study the relationship between autoantibody levels and the severity of renal disease. Seven of the animals in these three groups developed moderate to severe glomerular hypercellularity. All seven had relatively high levels of anti-dsDNA antibodies (no. 4,14,16,19 in Table 4.1 and no. 1,2,5 in Table 4.2). There was a positive correlation between the degree of glomerular hypercellularity and anti-dsDNA antibody levels with a Pearson product moment correlation coefficient of 0.77 ($p < 0.01$). There was no correlation between the degree of glomerular hypercellularity and the level of IgG antinucleosome levels (Pearson product moment correlation coefficient of 0.40, p = NS).

4.4 The source of nuclear autoantigens in IFN-γ transgenics

The demonstration of an absolute requirement for αβ T cells in the development of lupus disease in IFN-γ transgenic mice strongly implies that the production of autoantibodies is antigen driven. I next tried to identify the source of autoantigen. Apoptotic keratinocytes have been suggested as the source of self nuclear antigens in patients with SLE (Casciola-Rosen and Rosen, 1997). In addition, IFN-γ is known to facilitate keratinocyte apoptosis in vitro (see section 4.4). The mechanism is controversial but may include facilitation of FAS dependent pathways (Matsue et al, 1995; vide infra). This suggested that apoptotic keratinocytes were a possible clinically relevant source of self nuclear antigen in IFN-γ transgenics.

4.4.1 The skin of IFN-γ transgenic mice contains foci of apoptotic cells

I examined H&E stained skin sections from IFN-γ transgenic mice for the presence of apoptotic nuclei. Abnormal clusters of morphologically apoptotic cells were seen in the hair follicles and interfollicular epidermis in transgenics (Figure 4.6A and B). Apoptotic nuclei were observed in the skin of mice expressing the IFN-γ transgene, regardless of the T cell status of the animals.

The observation of increased numbers of apoptotic cells in the skin of IFN-γ transgenics was confirmed on TUNEL-staining of skin sections. TUNEL-positive cells were present in the hair follicles and interfollicular epidermis in IFN-γ transgenic mice. The distribution of these positive cells was patchy (the number of positive cells in IFN-γ transgenic interfollicular epidermis varied from 0 to 3 compared to 0 in all high powered fields examined from non-transgenic littermates). In addition, regions of the dermis in these animals contained large quantities of TUNEL-positive material (Figure 4.6C and D) which often had the appearance of having been phagocytosed (Figure 4.6D). No TUNEL-positive cells or material were seen in the interfollicular epidermis or dermis of littermates negative for the transgene (Figure 4.6F). Occasional hair follicles from transgene negative animals contained TUNEL-positive cells but this is not unexpected as apoptosis may play a central role in hair follicle regression (catagen) (data not shown) (Lindner et al, 1997).

Immunofluorescence staining of skin sections showed definite expression of Fas in the basal and immediate suprabasal layers of epidermis from transgenic mice and non-transgenic littermate controls (Figure 4.7A). Furthermore, on immunohistochemical staining occasional cells in the dermis (presumably immunocytes) stained positive for FasL (Figure 4.7B). Interaction between Fas and FasL in the IFN-γ induced inflammatory infiltrate is responsible for the apoptosis in the epidermis of transgenic mice. Interestingly, most of the apoptotic cells in the interfollicular epidermis were present in the basal layer (Figure 4.6C).

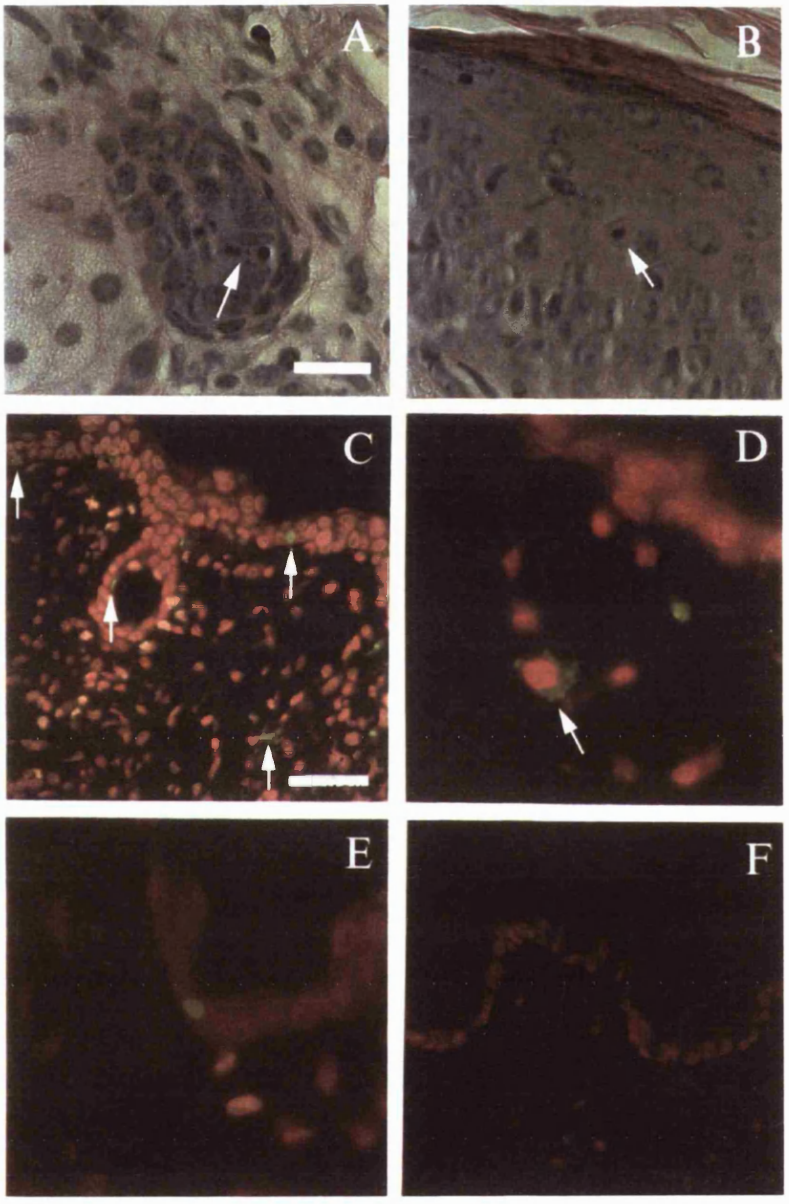


Figure 4.6 Apoptotic nuclei and TUNEL-positive cells in the skin of IFN-γ transgenic mice (A,B). H&E stained sections of A) hair follicle B) interfollicular epidermis in a transgenic mouse. Arrows indicate apoptotic nuclei. C - F: TUNEL-labelling of IFN-γ transgenic (C-E) and littermate nontransgenic (F) skin. Note the TUNEL-positive material in the hair follicle, interfollicular epidermis and dermis of IFN-γ transgenic skin (C, arrows). Much of the TUNEL-positive material in the dermis appeared to have been phagocytosed (D, arrow). TUNEL-positive interfollicular basal cell is shown in (E). No TUNEL-positive cells or material are present in skin from littermates negative for the transgene (F).
(Scale bar; A = B = D =E: 180 μm, C = F: 50 μm).

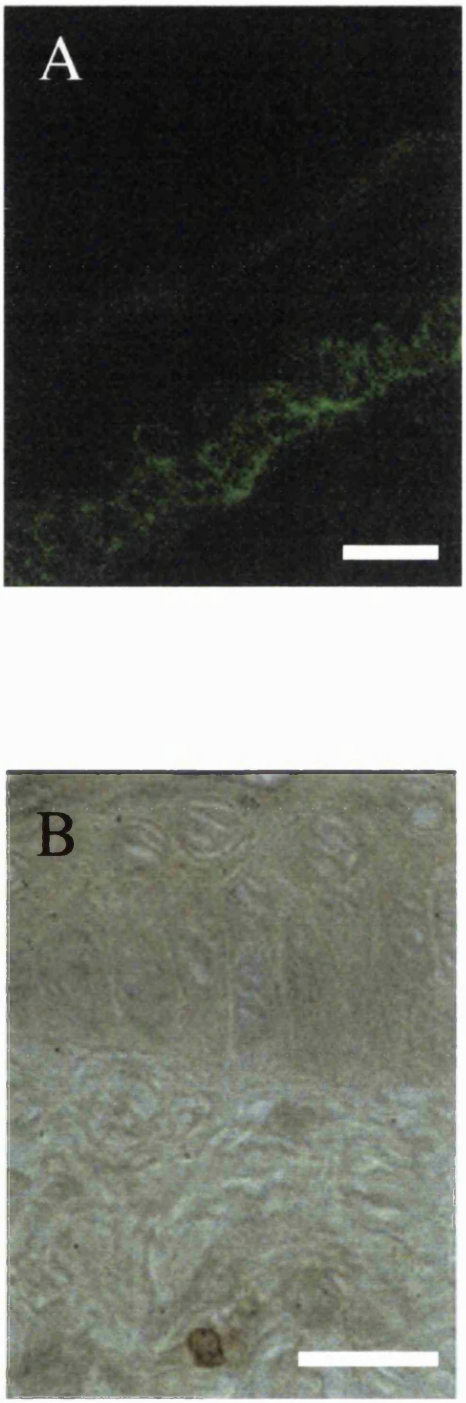


Figure 4.7 Fas/FasL expression in the skin of IFN-γ transgenic mice. (A) Staining with anti-Fas antibody demonstrates expression in the basal layer of the epidermis. (B) Staining with anti-FasL antibody reveals occassional scattered cells in the dermis which express the molecule. Scale bar (A = 60 μm, B = 40 μm).

4.5 Discussion

4.5.1 T-B cell interactions in the pathogenesis of lupus in IFN-γ transgenic mice

Production of pathogenic IgG antinuclear autoantibodies and end organ disease in the IFN-γ transgenic lupus model are clearly critically dependent on the presence of αβ T cells. This observation is consistent with antigen driven, αβ T cell activation of specific autoantibody producing B cells and emphasises the relevance of the model to the human disease.

An alternative explanation is that B cell development is abnormal in αβ T cell deficient animals since it has been shown that in thymectomised mice complete absence of T cells results in impaired B cell development and defective T-B-cell interactions (Gonzalez et al, 1995, Chan and Shlomchik, 1998). There is evidence that this phenomenon is secondary to a marked reduction in B cell CD23 expression (Gonzalez et al, 1995). However, this mechanism is unlikely to be playing a role in $\beta^{-/-}$ IFN-γ transgenic mice for a number of reasons. $\beta^{-/-}$ IFN-γ transgenics are not completely T cell deficient and the available evidence suggests that γδ T cells can substitute for αβ T cells in driving B cell development (Wen and Hayday, 1997). In support of this contention B cell CD23 expression in $\beta^{-/-}$ IFN-γ transgenics was comparable to that in littermates negative for the transgene (Figure 4.4).

4.5.2 The comparative effects of congenital T cell deficiency in murine lupus models

Our findings are in keeping with the results of several lines of investigation demonstrating an essential role for $CD4^+$ αβ T cells in the pathogenesis of lupus in murine models of the disease (Jevnikar et al, 1994, Steinberg et al, 1980, Yamamoto et al, 1990). However, our results contrast with the data of Peng *et al*, who showed that MRL-*lpr* mice deficient in αβ T cells develop

murine lupus with antinuclear autoantibodies and immune complex renal disease albeit of reduced severity (Peng et al, 1996). They postulated that γδ T cells could substitute for αβ T cells in autoantibody generation and mediation of end organ damage. We found no evidence of such a phenomenon, as $\beta^{-/-}$ IFN-γ transgenic mice developed none of the extra-cutaneous features of murine lupus.

The contrasting findings may reflect differences in the two murine lupus models. For example, B cell function is known to be intrinsically abnormal in MRL-*lpr* mice with polyclonal activation possibly rendering antibody secretion less dependent on T cell help (Perkins et al, 1990, Sobel et al, 1991). It has been argued that pathogenic antinuclear antibodies arise by a two stage process in the MRL-*lpr* model. In the first stage T cell independent polyclonal B cell activation initially results in production of low affinity IgM autoantibodies. In the second stage an αβ T cell dependent process results in a class switch to high affinity IgG autoantibody production (Desai-Mehta et al, 1995, Wen et al, 1994). We could find no evidence of a T cell independent stage in IFN-γ transgenic mice as we could not demonstrate autoantibodies of any class in αβ T cell deficient transgenics up to an age of 6 months, at which time αβ TCR deficient MRL-*lpr* mice have already developed them (Peng et al, 1996).

It has also been shown that mice congenitally deficient in αβ T cells due to a deletion mutation in the TCRα subunit spontaneously develop a lupus like syndrome (Wen et al, 1994). However, it should be noted that the presence of α^{-}/β^{+} T cells in the periphery in these animals may act as an alternative source of T cell help (Eichelberger et al, 1995). This T cell subset is not present in $\beta^{-/-}$ mice and the two lines of mice are, therefore, not directly comparable.

There is evidence that germinal centres can form in $\beta^{-/-}$ mice in response to infectious agents (Pao et al, 1996). We could not demonstrate germinal centres in lymph nodes or spleen from $\beta^{-/-}$ transgenics, although we did not carry out

immunisations in our mice, and T cell zones were completely absent. We have previously shown that lymph node enlargement in IFN-γ transgenics is associated with a marked increase in dendritic cell content (Carroll et al, 1997). This phenomenon persisted in $\beta^{-/-}$ transgenics which is at least consistent with IFN-γ induced migration of dendritic cells from the skin to the draining lymph nodes independent of T cell function.

4.5.3 Antigen presentation to T cells in the SIS of IFN-γ transgenic mice

As in ACLE, keratinocytes in IFN-γ transgenics express both MHC class II and ICAM-1 (Carroll et al, 1997). As outlined in section 1.3, keratinocytes may act as antigen presenting cells and could, in theory, present nuclear autoantigens to autoreactive T cells in these animals. However, as T cell infiltration of the epidermis is not a major feature of the skin disease, we think this is unlikely (Carroll et al, 1997). Immunohistochemical data and FACS analysis of disaggregated lymph nodes suggest that migration of Langerhans cells from the epidermis to draining lymph nodes is a major feature of the inflammatory skin disease in IFN-γ transgenics (Carroll et al, 1997). It is reasonable to suggest that, in IFN-γ transgenic mice, autoantigens from the skin are taken up by Langerhans cells and presented to antigen specific autoreactive αβ T cells in the draining lymph nodes with consequent stimulation of antinuclear antibody producing B cells. The demonstration of apoptotic cells in the epidermis and TUNEL-positive material in the dermis of IFN-γ transgenics suggests a possible, and again relevant, source of autoantigen. Apoptotic cells in epithelia are known to be rapidly phagocytosed by macrophages and we have previously shown that the majority of infiltrating cells in the dermis of IFN-γ transgenics are of the macrophage lineage (Carroll et al, 1997; Metcalfe and Streuli, 1997). Therefore, the demonstration of large quantities of TUNEL-positive material in the dermis may reflect phagocytosis of apoptotic material from the overlying epidermis.

4.5.4 Mechanism of excess apoptosis in IFN-γ transgenic mice

The cell growth inhibitory effects of the interferons are well known and form the basis of their use to treat malignant disease (Grander et al, 1997). It is now clear that in addition to direct cytotoxic effects interferons can also induce or facilitate apoptosis in a variety of cell types, including keratinocytes, although the mechanisms mediating this effect are ill-defined. Keratinocytes constituitively express Fas, a member of the TNF receptor family, *in vivo* and *in vitro* (Sayama et al, 1994). However, FasL is not expressed in the skin and apoptosis does not occur in normal non-inflamed epidermis (Gandarillas et al, 1999). Therefore, it has been argued that keratinocyte apotosis will only occur in vivo via the Fas pathway when T cells infiltrate the epidermis or dermis and provide a source of FasL. Furthermore, in vitro studies using anti-Fas monoclonal antibody have suggested that engagement of Fas is not sufficient to induce apoptosis unless the keratinocytes have been pre-treated with IFN-γ (Sayama et al, 1994; Matsue et al, 1995). Precisely how IFN-γ is facilitating Fas mediated apoptosis in this situation is unclear but may relate to the cytokines ability to cause marked upregulation of keratinocyte Fas expression, (Sung et al, 1997). Hence, IFN-γ overexpression in the epidermis could in theory stimulate keratinocyte apoptosis by upregulating their Fas expression in addition to providing a source of FasL by stimulating dermal inflammation. Also, it could condition the keratinocytes to favour a "productive" interaction of Fas and FasL. There is strong circumstantial evidence that a similar mechanism underlies the successful treatment of basal cell carcinoma with intralesional injection of IFN-α (Buechner et al, 1997). It is also of interest that dermal immunocytes positive for FasL occurring in association with apoptotic cells have been demonstrated in lesional skin from patients with SLE, occurring most commonly in the hair follicles (Nakajima et al, 1997).

It has been shown that overexpression of both interferon induced protein kinase and (PKR) and RNaseL can induce apoptosis in mammalian cells (Diaz-Guerra

et al, 1997; Lee et al, 1997). Furthermore, the effects of the two systems may be additive as coexpression of 2-5A-synthetase enhances the apoptotic effect of RNase L (Diaz-Guerra et al, 1997). The mechanisms involved in this process have not been determined. Induction of apoptosis by PKR occurs upstream of bcl-2 and is associated with cleavage of the death substrate poly(ADP-ribose) polymerase (PARP) (Lee et al, 1997). To what extent these mechanisms interact with the Fas pathway is not known. However, there is some evidence that PKR may phosphorylate the nuclear factor for IL-6 expression with subsequent activation of the Fas gene (Takizawa et al, 1996).

4.5.5 Apoptotic keratinocytes as a source of nuclear antigen in IFN-γ transgenic mice

Apoptosis in INF-γ transgenic mice occurs in association with tissue inflammation. As outlined in section 1.3, apoptotic cells arising in such an environment, particularly one rich in APCs, are an attractive possible source of autoantigen as oxidative damage may enhance the immunogenicity of their nuclear components. Nucleosomes are released from apoptotic cells and it has been shown that "nucleosome restricted" antibodies are the first to emerge during the course of murine lupus followed by diversification of the autoimmune response to its individual components (Burlingame et al, 1993; Amoura et al, 1994). This has lead to the suggestion that the nucleosome may be the initial driving immunogen in the lupus autoimmune response and that anti-histone and anti-dsDNA antibodies may subsequently arise by the process of "epitope spreading" (Amoura et al, 1994; Burlingame et al, 1993). Indeed any model of lupus pathogenesis must explain one central paradox in the disease: how does a T cell dependent autoantibody response develop against DNA when the available evidence suggests that T cells are incapable of binding DNA and APC cannot process or present the macromolecule (Craft

and Fatenejad, 1997). Epitope spreading to individual components of multicomponent complexes can resolve this apparent paradox.

B cells can internalise intact multicomponent particles with great efficiency following reaction of their surface Ig with exposed epitopes of the particle (Mamula et al, 1993). Furthermore, following internalisation, these cells can process and present diverse antigenic components of the particle (i.e. unrelated to the specificity of the surface Ig) to relevant T cells. These T cells can then provide help to B cells to differentiate into plasma cells and produce antibodies directed against different components of the particle (Roth et al, 1996). In this way an anti-dsDNA producing B cell could internalise a nucleosome via binding to nucleosomal dsDNA and then present processed histone epitopes to histone-specific T cells in the context of MHC class II. Cognate T-B cell help, involving cytokine release and costimulatory molecule engagement, then results in the proliferation and differentiation of the B cell with anti-DNA antibody production (Craft and Fatenejad, 1997).

It has been shown that autoreactive B cells with Ig directed against DNA or against surface exposed histones can internalise and process nucleosomes in endosomal-lysosomal pathways, with presentation of histone peptides by class II molecules to autoreactive T cells derived from SNF_1 mice (Mohan et al, 1995; Kaliyaperumal et al, 1996) as well as from patients with SLE (Desai-Mehta et al, 1995). In the setting of cytokine release and appropriate costimulatory molecules these T cells can provide help to anti-dsDNA producing B cells with resultant linkage of the two antibodies (Craft and Fatenejad, 1997).

The demonstration of nucleosome restricted antibodies in IFN-γ transgenics and the occurrence of antinucleosome, antihistone and anti-dsDNA together in most animals with intact T cell function is at least in keeping with such a process in these animals.

4.5.6 ANA and organ damage in IFN-γ transgenic mice

The presence of anti-dsDNA antibodies seems to be a prerequisite for the development of severe renal disease in IFN-γ transgenic mice. The importance of these autoantibodies in the pathogenesis of renal disease in IFN-γ transgenics is supported by the correlation between the severity of glomerular hypercellularity and IgG anti-dsDNA antibody levels in the three groups of animals with intact αβ T cell function ($\beta^{+/-}$, $\delta^{+/-}$, $\delta^{-/-}$). It seems likely that loss of these autoantibodies is a key factor in the absence of renal disease in $\beta^{-/-}$ IFN-γ transgenics. Nevertheless, there is strong evidence that antinucleosome antibodies are capable of inducing lupus nephritis (Burlingame et al, 1993, Jovelin et al, 1998) and nucleosome-specific T helper cells have been shown to play a key role in triggering nephritis in murine lupus (Kaliyaperumal et al, 1996). The mechanism by which antinucleosome antibodies cause glomerulonephritis is controversial, but may involve deposition of nucleosome/antinucleosome antibody complexes in the glomerular basement membrane relatively early in the disease (Kramers et al, 1994). Therefore, loss of this reactivity in $\beta^{-/-}$ transgenics may be important in the abolition of renal disease. However, it is of interest to note that the two $\beta^{+/-}$ IFN-γ transgenics with IgG antinucleosome restricted autoantibodies did not show definite histological evidence of kidney disease, although it is possible that lesions might have developed with age (no. 4 and 6, Table 4.2). The development of proliferative glomerulonephritis in the absence of IgG antihistone antibodies (e.g. mice no. 4,7,11,16,19, Table 4.1) demonstrates that antihistone autoantibodies do not play an essential role in the pathogenesis of lupus nephritis.

The occurrence of IFN-γ-associated inflammatory skin disease, lymphadenopathy and splenomegaly in the absence of systemic complications of the disease indicates that these processes are separable in our lupus model. Although uncommon, ACLE can occur in the absence of systemic autoimmune

disease in man (David-Bajar and Davis, 1997) indicating a further parallel between the transgenic model and the human disease.

4.5.7 The role of γδ T cells in the pathogenesis of IFN-γ-related lupus

It has been reported that deficiency of γδ T cells in MRL-*lpr* mice results in a rise in anti-dsDNA titres and worsening of renal disease (Peng et al, 1996). There also appeared to be a trend towards more severe renal disease in $\delta^{-/-}$ IFN-γ transgenics (Table 4.1). In contrast to $\delta^{-/-}$ MRL-*lpr* mice, this phenomenon was observed in the absence of any significant rise in anti-dsDNA levels, thus raising questions about the mechanisms involved. There is evidence that CD4+ T cells play a role in triggering glomerular hyperproliferation and crescent formation in animal models of glomerulonephritis (Tipping et al, 1998). In addition, several studies suggest that γδ T cells antagonise αβ T cell function resulting in reduced tissue injury in both autoimmune and pathogen induced inflammation (Mukasa et al, 1995, Fu et al, 1994, Roark et al, 1996, D'Souza et al, 1997). It is conceivable, therefore, that the possible worsening of renal disease in $\delta^{-/-}$ IFN-γ transgenic mice results from loss of a direct inhibitory effect of γδ T cells on glomerular damage.

In conclusion, the data presented here are consistent with a specific αβ T cell mediated, antigen driven process giving rise to pathogenic IgG antinucleosome, anti-dsDNA and antihistone autoantibodies in IFN-γ transgenic mice. This further emphasises the relevance of the model to human SLE. Circumstantial evidence implies that apoptotic keratinocytes could be the source of autoantigen in IFN-γ transgenics and humans with the disease. Pharmacological agents are now available which inhibit the apoptotic process. IFN-γ transgenic mice could prove invaluable for studying the effects of anti-apoptotic therapies aimed at SLE.

Chapter 5

Pharmacological Inhibition of Apoptosis in IFN-γ Transgenic Mice

"All who partake of this remedy recover in a short time except those whom it does not help, who all die. Therefore, it is obvious that it fails only in incurable cases."

Galen, 2nd century AD

5.1 Introduction

As detailed in the preceding sections apoptosis may play a key role in the pathogenesis of SLE. Apoptotic keratinocytes may be the source of autoantigen in the disease. In addition, aberrant programmed cell death may play a role in organ damage including skin disease, renal damage and haematological deficiencies of SLE (vide infra). We have provided circumstantial evidence that excessive keratinocyte apoptosis may drive antinuclear antibody production in IFN-γ transgenic mice. Furthermore, as in humans with SLE, systemic organ damage in IFN-γ transgenics seems to be directly related to anti-dsDNA levels.

Apotosis is a highly ordered process triggered by a variety of stimuli (Ashkenazi and Dixit, 1998; Thornberry and Lazebnik, 1998). Immunohistochemical analysis of skin from IFN-γ transgenics and the available in vitro data on keratinocyte apoptosis suggested to me that Fas-FasL interaction was the most likely cause of excess apoptosis in these transgenics (see sections 4.3 and 4.4). The effector pathway mediating apoptosis following ligation of Fas is a proteolytic cascade mediated by a group of cysteine proteases, the caspases. The caspases mediate the effector phase of apoptosis by hydrolysing key structural and housekeeping proteins generally at one site and resulting in loss or gain of function (Thornberry and Lazebnik, 1998). In addition, a different group of caspases are involved in some of the upstream apoptosis signalling pathways where they become activated and in turn activate

the effector caspases. The caspases are amongst the most specific of proteases described with a near absolute requirement for aspartic acid in the S1 subsite (Howard et al, 1991; Sleath et al, 1990). There is an equally stringent requirement for specific sequences of the four amino acids to the left of the cleavage site, the precise optimal recognition sequence differing for different caspases. Primary sequence recognition is a necessary requirement for catalysis (Howard et al, 1991).

A wide variety of cysteine protease inhibitors are now available and this raises the possibility of pharmacological intervention to reduce aberrant excessive apotosis. Several classes of electrophiles that form both reversible and irreversible adducts with the active site cysteine residue have been developed. Reversible inhibitors include aldehydes, ketones and nitriles whereas irreversible inhibitors are generally α-substituted ketones (diazomethylketones, halomethylketones and acyloxymethylketones) (Rich, 1986; Shaw, 1990). The most potent inhibitors contain a peptide which is the optimal recognition sequence of the target caspase (Nicholson et al, 1995) (Figure 5.2).

In view of the putative central role of apoptosis in human SLE and IFN-γ transgenics, I decided to study the effects of the tetrapeptide caspase inhibitor Z-VAD-fmk (carbobenzoxy - valyl - alanyl - aspartyl - (beta-o-methyl) - fluoromethylketone) on the lupus phenotype of IFN-γ transgenic mice. zVAD-fmk is a pan-caspase inhibitor resulting in irreversible inhibition of enzymatic function and is effective in blocking apoptosis in a variety of cell types (Dodel et al, 1998; Pronk et al, 1996; Slee et al, 1996).

5.2 Experimental design

Eighteen female IFN-γ transgenic mice were divided into two groups to receive either Z-VAD-fmk (10 mg/kg, n = 9) or diluent control (n = 9). Two transgenic

animals, which did not produce either anti-dsDNA and antihistone autoantibodies were included in each group (no. 3,9,12,13, Table 5.1). Z-VAD-fmk was diluted in normal saline containing 2% DMSO at a concentration of 1mg/ml and administered subcutaneously on a daily basis for three weeks. Each animal, therefore, received a daily subcutaneous injection of 100μl per 10g body weight (generally 200 to 300 μl). The age, weight, daily dose and founder line of each animal in the study is listed in Table 5.1. I had planed to include a further control group of animals treated with the negative control inhibitor Z-FA-fmk. However, this was not possible due to the insolubility of this agent.

To measure the effects of treatment on anti-dsDNA and antihistone levels, blood samples were taken four days before starting treatment, two, nine and sixteen days after commencing treatment and at the time of sacrifice (3 weeks). All serum samples were stored at -20°C and individual serum samples from the same animal were analysed on the same ELISA plate.

The effects of Z-VAD-fmk on IFN-γ induced skin disease was assessed by histological analysis of site matched samples from animals in the treatment and control groups and on clinical examination. In addition, TUNEL-staining was carried out on five non-sequential skin sections from three animals from the treatment (no. 4, 5 and 8, Table 5.1) and control (no. 10, 14 and 15, Table 5.1) groups. Changes in the reticuloendothelial system were assessed by weighing the spleen on dissection. The severity of renal disease in the two groups was assessed by histology at the time of sacrifice.

Clinical assessment of skin disease severity was carried out using a scoring system (1: hypopigmentation only; 2: hypopigmentation with small areas of macroscopically obvious inflammation; 3: hypopigmentation with extensive areas of macroscopic inflammation and hair loss). In total 14 animals were dissected at the end of the study period. Two animals in each group (no.s 2, 3, 16 and 18 Table 5.1) were left alive for future study.

Table 5.1 Characteristics of IFN-γ transgenics used in Z-VAD-fmk study.

ANIMAL	FOUNDER LINE	AGE (months)	DAILY DOSE (μg)
Z-VAD-fmk			
1	1212F	7	200
2	1212F	6	200
3	1205C	11	300
4	1205C	7	250
5	1205C	11	350
6	1212F	8	200
7	1205C	10	250
8	1205C	7	200
9	1205C	6	250
		MEAN: 8.1	
CONTROLS			
10	1212F	8	200
11	1205C	9	250
12	1205C	7	250
13	1205C	6	300
14	1212F	7	200
15	1205C	7	250
16	1205C	6	350
17	1212F	6	200
18	1205C	9	350
		MEAN: 8.4	

5.3 Z-VAD-fmk inhibits cutaneous apoptosis in IFN-γ transgenic mice

Treatment with Z-VAD-fmk did not affect the severity of skin disease in IFN-γ transgenics as assessed on clinical examination. The index of skin disease

severity did not vary significantly in the treatment group over the period of study. The mean score in these mice was 1.7 before and after treatment, p = NS (Table 5.2). In addition, on H&E examination, the dermal mononuclear infiltrate characteristic of IFN-γ related skin disease (Figure 5.1) persisted in these animals.

However, analysis of TUNEL-stained skin sections from animals in the treatment group showed a marked reduction in the level of TUNEL-positive material present, particularly in the dermis (Figure 5.1). As previously reported the bulk of the TUNEL-positive material was present in the dermis in the control group having been apparently phagocytosed by dermal cells (Figure 5.1). Interestingly, the skin of one animal treated with Z-VAD-fmk and sacrificed four days after discontinuation of the drug showed evidence of markedly increased apoptosis with abundant apoptotic material in the dermis suggesting a "rebound" in cell death rate following resynthesis of functional caspases (Figure 5.1).

5.4 Renal histology in IFN-γ transgenics treated with Z-VAD-fmk

It has been proposed that the proliferative changes in the glomerular mesangium in lupus nephritis may be secondary to reduced levels of apoptosis in this site. If this is the case then one might expect an apoptosis inhibitor to aggravate the renal manifestations of lupus. However, examination of renal tissue at the end of the study period showed no evidence of this phenomenon. In fact of 7 animals examined in the control group 3 had evidence of severe proliferative GN versus one of 7 animals in the treatment group. Overall the mean mesangial cellularity scores did not differ between control and Z-VAD-fmk treated transgenics (2.3 and 1.7 respectively p = NS, Table 5.2).

Table 5.2. Skin and renal disease in IFN-γ transgenics treated with Z-VAD-fmk and diluent-treated controls.

Spleen	D/A	S/D Grade Pre	S/D Grade Post	D/I	GL/HC	Spleen Weight (g)
Z-VAD-fmk						
1	dsDNA	2	2	Y	++	0.1
2	BO	2	2	nd	nd	nd
3	AN	1	1	nd	nd	nd
4	BO	2	2	Y	++	0.4
5	dsDNA	2	1	Y	+	0.1
6	dsDNA	3	3	Y	+++	0.2
7	Histone	1	1	Y	+	0.1
8	Histone	1	2	Y	++	0.2
9	AN	1	1	Y	+	0.1
						M: 0.17
CONTROLS						
10	dsDNA	3	3	Y	+++	0.2
11	Histone	2	2	Y	+	0.2
12	AN	1	1	Y	++	0.1
13	AN	2	2	Y	++	0.3
14	dsDNA	3	3	Y	+++	0.3
15	dsDNA	1	2	Y	++	0.1
16	BO	1	1	nd	nd	nd
17	dsDNA	2	2	Y	+++	0.3
18	dsDNA	2	2	nd	nd	nd
						M: 0.21

D/A: dominant autoantibody at start of treatment, BO: high levels of anti-dsDNA and antihistone antibodies, AN: autoantibody negative, S/D grade: macroscopic skin disease severity pre- and post-treatment (1: hypopigmentation only; 2: hypopigmentation with small areas of macroscopically obvious inflammation; 3: hypopigmentation with extensive areas of macroscopic inflammation and hair loss), D/I: dermal inflammation (Y: present), GL/HC: glomerular hypercellularity (-/+: equivocal; +: mild; ++: moderate; +++: severe), nd: not done, M: mean.

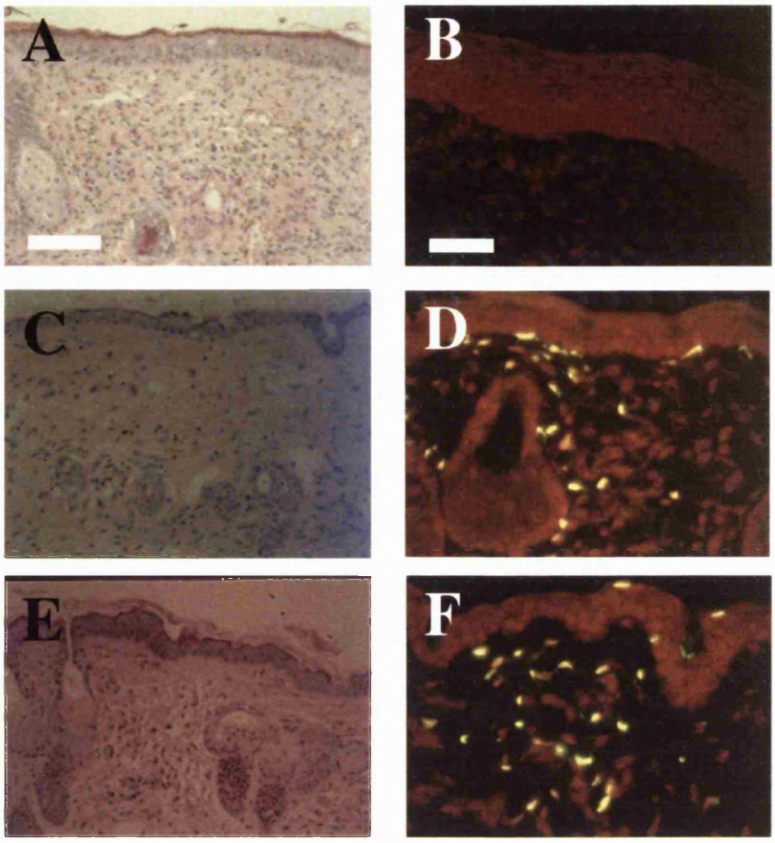


Figure 5.1 H&E stained skin sections (A, C, E) and TUNEL-stained sections (B, D, F) of skin from Z-VAD-fmk treated IFN-γ transgenic mice (A, B, E, F) and diluent treated control (C, D). Note the complete absence of TUNEL-positive material in the dermis of Z-VAD-fmk treated animal sacrificed on the final day of a three week course of treatment (B) despite persistence of a dense dermal inflammatory infiltrate (A). A diluent treated control shows the presence of TUNEL-positive material in association with the dermal infiltrate (B, D). Skin from a Z-VAD-fmk treated transgenic obtained four days after stopping treatment shows greatly increased amounts of TUNEL-positive material in the dermis (F). Scale bar (A): 50 μm (A = C = D), (B): 180 μm (B = D = F).

5.5 Untreated transgenics show a steady rise in autoantibody levels with time

With one exception (no. 15, Table 5.1), all control animals showed a gradual rise in autoantibody levels as assessed by ELISA. Overall the average rise in optical density over the four week period of observation was 41%.

5.6 Z-VAD-fmk has dramatic effects on autoantibody production in IFN-γ transgenics

On dissection, the characteristic superficial lymphadenopathy and splenomegaly characteristic of IFN-γ transgenics persisted to the same extent in Z-VAD-fmk treated animals and diluent-treated controls (mean splenic weight: 0.17 g and 0.21 g respectively and 0.1 g in 5 non-transgenic littermate controls).

The steady rise in autoantibody levels observed in control animals was not observed in the treatment group. The two animals included in this group which did not produce autoantibodies at the beginning of Z-VAD-fmk treatment (no.s 3 and 9 Table 5.1) remained negative for autoantibodies over the duration of study. Of the other seven animals treated with Z-VAD-fmk, three showed striking and rapid swings in autoantibody production after starting the drug (animals no. 2, 5 and 8 Table 5.1 and Figure 5.2). We have previously reported what appears to be a reciprocal relationship between the level of anti-dsDNA and antihistone autoantibodies in IFN-γ transgenics (Seery et al, 1997 and section 3.3). Transgenic animals producing high levels of anti-dsDNA antibodies tend to produce low levels of antihistone antibodies and visa versa. Three animals (no. 2, 5 and 8, Table 5.1 and Figure 5.2) showed a rapid reduction in the level of the "dominant" autoantibody after starting treatment.

This was accompanied by a striking rise in the level of the "secondary" antibody (Figure 5.2). This phenomenon was observed irrespective of the specificity of the initial dominant autoantibody (anti-dsDNA in no. 2 and 5, antihistone in no. 8, Table 5.2).

The remaining four animals in the treatment group did not demonstrate a consistent effect on autoantibody levels. Over the duration of treatment, animals 1 and 6 (Table 5.1) showed an 6% and 16% fall in optical density while animals 4 and 7 (Table 5.1) showed a 6% and 20% rise in optical densities on ELISA.

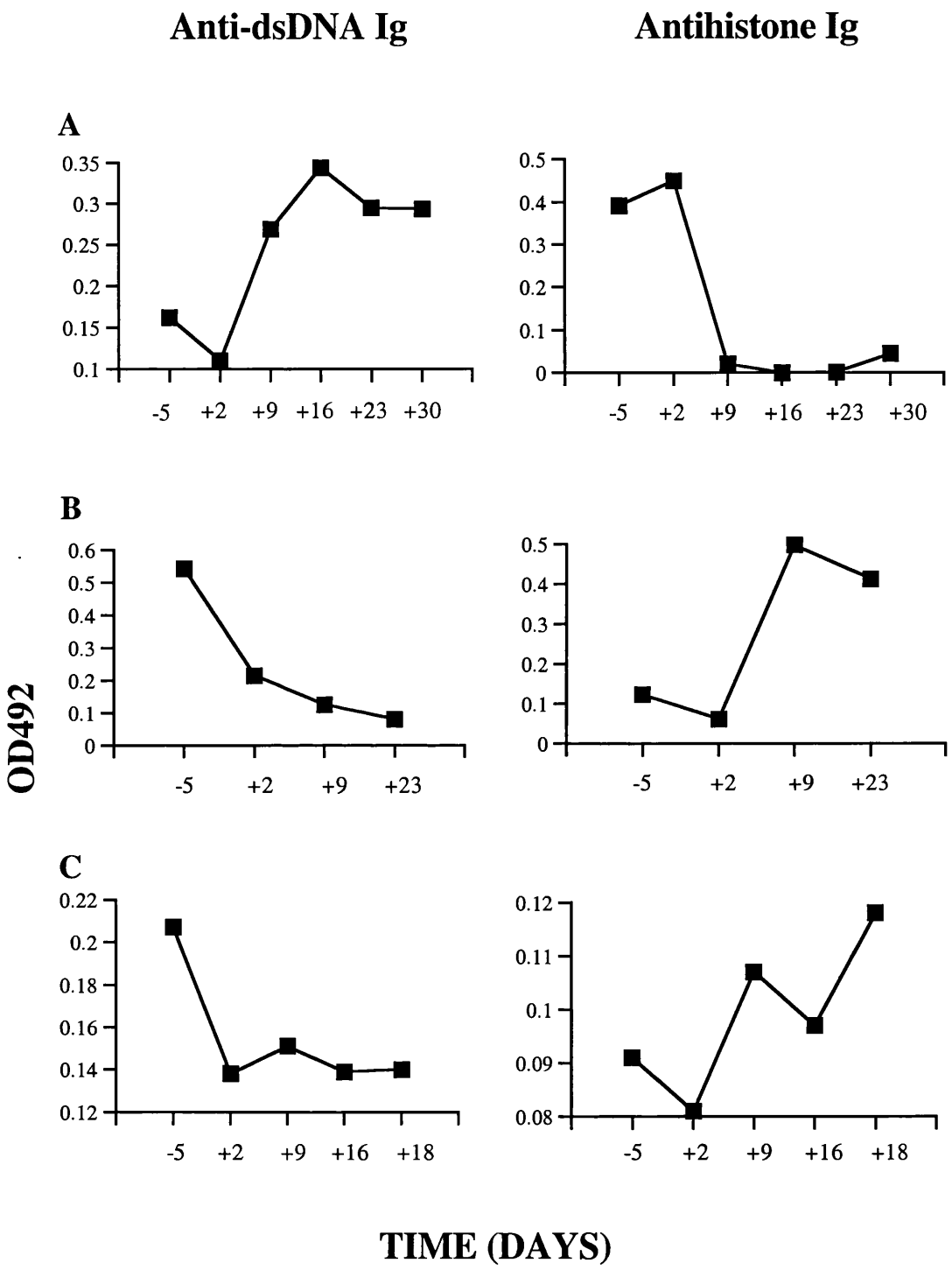


Figure 5.2 Levels of antinuclear antibodies as assessed on ELISA in Z-VAD-fmk treated IFN-γ transgenic mice. Note the reciprical rise and fall in antihistone and anti-dsDNA levels. Treatment was stopped on day 23 in animals (A) and (B) and on day 18 in animal (C).

5.7 Discussion

5.7.1 Fas-FasL mediated apoptosis in IFN-γ transgenics

The effects of Z-VAD-fmk on apoptosis are critically dependent on the nature of the stimulus triggering the apoptotic process. There is evidence that direct induction of apoptosis by IFN-γ in epithelial cells is caspase independent and cell death is mediated by an alternative cell execution pathway involving the *PML* gene product (Quignon et al, 1998). Interestingly, one report indicated that caspase inhibitors can stimulate this alternative pathway in susceptible cells and augment IFN-γ induced cell death in vitro (Quignon et al, 1998). Therefore, the finding that Z-VAD-fmk markedly reduces IFN-γ induced apoptosis supports the concept that Fas-FasL interactions are triggering apoptosis in the skin of these animals. As outlined in section 4.5, there is evidence to suggest that a similar mechanism may be involved in triggering the excessive epidermal apoptosis observed in SLE. In addition, there is evidence that keratinocyte apoptosis following UV irradiation is mediated by the direct activation of Fas and is sensitive to caspase inhibitors (Aragane et al, 1998), further emphasising the relevance of this model to pathological processes in lupus patients.

5.7.2 A therapeutic role for apoptosis inhibitors in SLE?

Several studies have shown that clinical flares in SLE can be predicted on the basis of rising anti-dsDNA titres up to ten weeks before the flare manifests (ter Borg et al, 1990). Flares are a source of mortality in SLE patients and presumably their cumulative effects contribute considerably to end stage organ damage. It has been shown that aggressive intervention with high dose steroids, at the time when anti-dsDNA titres begin to rise, is effective in preventing minor but not major relapses (Bootsma et al, 1995). These results imply that a major opportunity exists to prevent significant morbidity and mortality by

careful follow up of patients. However, it cannot be taken advantage of because of the ineffectiveness of current therapies. We currently lack a readily useable therapeutic agent which can affect the humoral autoimmune response rapidly at acceptable doses. Corticosteroids are unpredictable in their effects on humoral immunity (Markham et al, 1978; Settipane et al, 1978). In addition, T cell specific immunosuppressants used so successfully in transplantation (e.g. cyclosporin) have negligible effects on autoantibody levels (Fox and McCune, 1994). The ability of Z-VAD-fmk to rapidly alter autoantibody levels in IFN-γ transgenics is, therefore, potentially of great interest. The fact that anti-dsDNA reduction might be accompanied by a rise in antihistone antibody levels is not necessarily important. This autoantibody type is not pathogenic and indeed there is some evidence that they may protect against renal damage by preventing nucleosome deposition in the glomerulus (Tax et al, 1995). In this regard it is of interest that the transgenic animal with the highest level of anti-dsDNA in this study developed mild renal disease only (no. 4, Table 5.2). This animal also produced high levels of antihistone autoantibodies and it is possible that these antibodies exerted a protective effect.

5.7.3 The relationship between anti-dsDNA and antihistone antibody production

Although both antihistone and anti-dsDNA autoantibodies are present at high levels in IFN-γ transgenic mice consistent with their occurrence as a linked set, we had previously reported an inverse relationship between their levels in individual animals (Seery et al, 1997). The demonstration that inhibition of apoptosis can reduce the level of one autoantibody in a linked set while increasing the level of the other raises questions as to the relationship between them. Even though the amount of putative autoantigen (cutaneous apoptosis) is reduced on treatment it seems unlikely that the effects of Z-VAD-fmk on autoantibody levels result from altered supply of antigen. Firstly, the effect is

seen in animals initially producing high levels of either anti-dsDNA or anti-histone antibodies, excluding some form of differential effect on the source of antigen for the two antibodies. In addition, the effect of Z-VAD-fmk on autoantibody levels is rapid, occurring within 10 days of commencing treatment. Removal of the source of antigen would be expected to reduce antibody levels over a period of weeks to months, not days. It seems more likely that the effects of Z-VAD-fmk are mediated by inhibition of apoptosis in a regulatory element of the immune system although precisely what this is will require further study.

If the levels of antihistone antibodies have an inhibitory regulatory effect on the levels of anti-dsDNA antibodies this could provide an explanation for an apparent clinical paradox. The central role of anti-dsDNA antibodies in the pathogenesis of systemic organ damage in SLE has been established in several studies (Berden, 1997) and it would seem logical that reduction of the levels of this antibody would improve disease outcome, yet it does not. Trials of regular plasmaphoresis have failed to show any clinical benefit in patients with SLE (Doria et al, 1994). This lack of effectiveness has been associated with a rapid rise in anti-dsDNA antibody between treatment sessions (van Vollenhoven, 1999). Plasmapheresis is unselective, reducing levels of all antibodies and removal of a negative regulator could explain the marked rise in anti-dsDNA levels after treatment.

5.7.4 Inhibition of apoptosis and lupus nephritis

If apoptosis inhibitors were to find a role in the treatment of human SLE then the effects of these agents on the renal histology will be critical. In theory these agents may have the ability to aggravate lupus nephritis. In lupus nephritis proliferative glomerular lesions carry the worst prognosis with a high risk of progression to end stage renal failure (Baldwin et al, 1970). Some studies have indicated that apoptosis may play a beneficial role in clearing infiltrating

leukocytes and excess glomerular cells which have accumulated due to earlier proliferation (Baker et al, 1994; Shimizu et al, 1995). Indeed there is evidence that increased expression of bcl-2 by mesangial cells may play a role in generating glomerular hypercellularity in human nephritis (Takemura et al, 1995). However, these conclusions have been challenged. Evidence has been presented that excessive apoptosis may be central to the loss of renal function in a variety of glomerulonephritidies. Sugiyama et al., demonstrated a significant correlation between the number of glomerular TUNEL-positive cells per glomerular cross section in cases of IgA nephropathy and histological scores of both proliferation and sclerosis and confirmed a similar relationship between apoptosis and glomerular sclerosis in lupus nephritis (Sugiyama et al, 1996). This work and studies on adult polycystic kidney disease (Woo, 1995) suggests that excess apoptosis in the glomeruli may be central to the progression to end stage renal disease (Savill et al, 1996). In my short term study treatment with a caspase inhibitor did not lead to a significant worsening of histologically assessed renal disease. In fact fewer animals in the treatment group had evidence of significant hyperproliferative glomerular disease at the end of the study period than in the control group. Whether or not this is a real beneficial effect of the drug will need to be analysed in a larger long term study.

In summary, Z-VAD-fmk inhibited apoptosis in the skin of IFN-γ transgenic mice, supporting the hypothesis that Fas-FasL interactions give rise to excessive programmed cell death in these animals. In addition, the effects of these agents on autoantibody levels in some transgenic mice suggests a possible therapeutic capability for caspase inhibitors which is worthy of further study.

Chapter 6

General Discussion

I have shown that IFN-γ overexpression in the suprabasal layer of the epidermis results in a lupus-like syndrome in transgenic mice. This is consistent with the hypothesis that the SIS plays the central role in the pathogenesis of naturally occurring SLE. Furthermore, I have provided evidence that the mechanisms involved in autoantibody production in IFN-γ mice are relevant to the human disease: ANA arise by a T cell dependent, antigen driven process with apoptotic cells in the epidermis possibly acting as a source of self nuclear antigen.

6.1 Skin disease as the primary abnormality in SLE

The demonstration that IFN-γ specifically overexpressed in the skin can trigger a lupus-like syndrome in transgenic mice raises the question as to whether the skin disease of SLE is the primary abnormality in the condition. This is clearly possible from the evidence presented in the thesis and is further supported by the observation that UV irradiation of the skin affects the entire disease complex, not only in man but also in all murine models studied (Casciola-Rosen and Rosen, 1997; Furukawa, 1997). One obvious objection to the hypothesis is that clinically overt skin disease is not seen in all patients with SLE (Venables, 1993a). Nevertheless, although (BxW)F1 and BXSB lupus-mice do not develop clinically overt skin lesions, UV irradiation of the skin can alter ANA titres, accelerate systemic disease and increase mortality in both models (Natali et al, 1978; Ansel et al, 1985; McGrath et al, 1987). It would, therefore, seem possible that the SIS can still function in the production of ANA even in the absence of clinically detectable skin disease.

6.2 Could excessive IFN-γ production be the cause of SLE in patients?

The role of IFN-γ in the naturally occurring human disease is a matter of controversy. There are data to suggest that low levels of this cytokine or at least a reduced ratio of IFN-γ to IL-10 (Hagiwara et al, 1996) may play a role in the pathogenesis of SLE, since it has been reported that peripheral mononuclear cells from patients with SLE exhibit deficient production of IFN-γ on stimulation with PHA or IL-2 (Tsokos et al, 1988). However, the latter findings have been challenged (McKenna et al, 1988). If reduced IFN-γ levels played a role in the pathogenesis of SLE it would fit with the long held belief that an alteration in T cell activity towards a Th2 profile is likely to be involved in the pathogenesis of humoral autoimmunity (Reeves et al, 1999). However, this concept does not rest easily with the observation that administration of exogenous interferon to patients can trigger de novo and severe SLE with classical lupus nephritis and skin disease (Graninger et al, 1991). In addition, as outlined in section 1.7 there is evidence that manipulations which reduce the level of this cytokine in murine lupus models result in significant disease amelioration. Taken together these findings are at least consistent with a role for elevated levels of IFN-γ in the pathogenesis of the disease. Our studies suggest that the key issue may not be the systemic levels of the cytokine but rather local levels in the skin affected by LE. Although keratinocytes have not been shown to produce IFN-γ (Williams and Kupper, 1996) the immunocytes in the dermal infiltrate certainly would be expected to have this capability. Demonstrations of reduced IFN-γ production by total populations of peripheral mononuclear cells in SLE patients are not necessarily relevant to this issue as, for example, dermal inflammatory infiltrates are highly polarised towards particular T cell subsets (see section 3.5). Clearly, this an issue worthy of further study.

6.3 What triggers SLE in humans?

The available epidemiological evidence strongly implies that SLE results from the action of an, as yet, unidentified environmental agent or agents on a genetically susceptible individual (Arnett et al, 1972; Kaplan, 1984). Numerous putative causative agents have been implicated in the breakdown of self tolerance, including retroviruses (Griffiths et al, 1999), pathogens mimicking or altering self epitopes (Grayzel et al, 1991; Kaplan and Tan, 1968; Venables, 1993b) and environmental chemicals which modify nuclear complexes (Pollard et al, 1997). Clearly, a transgenic model of the disease as described in this thesis can tell us little or nothing about the actual trigger of the disease. Yet medicine is full of instances where improved understanding of the pathophysiology of a condition has lead to highly effective therapies in the absence of any knowledge of its underlying cause. An obvious example is the successful treatment of peptic ulcer disease with acid suppressive therapy years before the isolation of *H. pylori*. I think that it is in the development of new therapies for SLE that the model described in this thesis could be most useful.

6.4 New therapies in SLE

There is tremendous interest in experimental therapies for SLE. This is primarily as a result of the lack of effectiveness of currently available treatments. The efficacy of new therapies in this disease is not easily assessed due to the heterogeneity of the condition and large multicentre studies are required. Recently, there has been considerable interest in the use of immunomodulatory techniques aimed directly at the immunological mechanisms involved in the pathogenesis of autoimmune disease (Kalden et al, 1998). The feasibility of such approaches in the treatment of lupus has been demonstrated in preliminary trials

of the DNA-toleragen LJP394. LJP394 is a triethylene glycol-derived platform with four 20-mer oligonucleotides, consisting of alternating deoxycytidine and deoxyadenosine nucleotides covalently attached. This molecule has high affinity for anti-DNA antibodies and decreases anti-oligonucleotide antibodies in oligonucleotide immunised mice (Coutts et al, 1996). In dose-ranging studies, patients with SLE given 50 mg of LJP394 intravenously once weekly for 16 weeks showed a 50% reduction in anti-DNA titres and this reduction persisted in a post-treatment two month follow-up period (Weisman et al, 1997). The initial decrease in autoantibody levels may reflect binding of the molecule to the autoantibodies followed by clearance of the immune complexes formed. The persistent effect on autoantibody levels after withdrawal of treatment is obviously of great interest and may result from B cell anergy induced by binding of soluble antigen to the B cell in the absence of a costimulatory signal from T cells.

Techniques such as antigen-based tolerance induction and TCR peptide vaccination raise the possibility of definitive treatment for SLE, re-establishing self tolerance with minimal side effects (Kalden et al, 1998). However, testing and refinement of these new therapies in relevant animal models may be critical. For example, vaccination with attenuated AChR-specific T cell lines in experimental autoimmune myasthenia gravis induces AChR-specific suppresser cells in the spleen but paradoxically enhances anti-AChR antibody responses (Kahn et al, 1990). Furthermore, testing will require the use of animal models which accurately mirror the pathogenesis of the disease in question. The emergence of the lupus phenotype in the most widely used murine models of lupus to date depends primarily on unchecked proliferation of autoreactive T and B cells secondary to defects in apoptosis. These mechanisms are not operative in human SLE. On the contrary, the available evidence strongly implies that autoreactive B cell proliferation develops as a result of classical antigen driven, T cell dependent mechanisms. In this context, the demonstration that anti-nuclear autoantibodies in IFN-γ mice arise by such a process is a major strength of the

model and these animals could act as a powerful tool to study these new therapeutic options.

Clearly, our study of the effects of apoptosis inhibitors on the lupus phenotype in IFN-γ transgenics is preliminary. However, there is now tremendous interest world-wide in the therapeutic potential of these agents in diverse conditions. For example, apoptosis is believed to play a significant role in neuronal loss during meningitis and the efficacy of Z-VAD-fmk in an animal model of this condition has been demonstrated (Braun et al, 1999). Before these agents can be tested in patients the issue of toxicity, particularly in terms of the risk of neoplasia, will have to be addressed. However, the experimental data available from experimental animals suggests that toxicity of cysteine protease inhibitors may be surprisingly low (Roose and Noorden, 1995).

The demonstration that Z-VAD-fmk is capable of altering the autoantibody profile of IFN-γ transgenics over a timescale of days to weeks will need to be confirmed in further studies. If confirmed, this is potentially of great interest as systemic disease flares in SLE can be predicted several weeks in advance by rising ANA titres (Ter Borg et al, 1990). In addition, as this caspase inhibitor was capable of turning off apoptosis in the skin of transgenics, it will be interesting to study the medium to long term effects of this agent on autoantibody levels and kidney disease in these animals. Our findings illustrate the potential of this model to aid in the development of effective therapies for this devastating disease.

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Appendix I. List of suppliers

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Austral Biologicals, San Ramon, California, USA.

BDH Laboratory Supplies Inc., Hemel Hempstead, Hertfordshire, UK.

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Appendix II. List of abbreviations

AChR	acetylcholine receptor
ACLE	acute cutaneous lupus erythematosus
ACR	American College of Rheumatology
AD	anno domini
ADCC	antibody-dependent cell-mediated cytotoxicity
AIHA	autoimmune heamolytic anaemia
ANA	antinuclear antibodies
AP	alkaline phosphatase
APC	antigen presenting cell
AICD	activation induced cell death
BC	before Christ
BSA	bovine serum albumin
CCLE	chronic cutaneous lupus erythematosus
cDNA	complementary deoxyribonucleic acid
CDR	complementarity-determining regions
CLE	cutaneous lupus erythematosus
CNS	central nervous system
DAB	3,3'-diaminobenzidine tetrahydrochloride
DCS	donor calf serum
DETC	dentritic epidermal T cells
DLE	discoid lupus erythematosus
DMEM	Dulbecco's modification of Eagle's medium
DNA	deoxyribo nucleic acid
DNA-PK	deoxyribonucleic acid-dependent protein kinase
DOPA	dihydroxyphenylalanine
DIF	direct immunofluorescence

DTT	dithiolthreitol
dATP	deoxyadenine triphosphate
dUTP	deoxyuridine triphosphate
ECL	enhanced chemiluminescence
EDTA	ethyldiaminotetracetic acid
ELISA	enzyme-linked immunosorbent assay
ENA	extractable nuclear antigens
ER	endoplasmic reticulum
ES	embryonic stem
etn	early retroviral transposon
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
fmk	fluoromethylketone
GBM	glomerular basement membrane
GN	glomerulonephritis
GM-CSF	granulocyte/macrophage-colony stimulating factor
GVH	graft versus host
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HLA	human leukocyte antigen
HRP	horse radish peroxidase
ICAM	intracellular adhesion molecule
ICRF	Imperial Cancer Research Fund
Ig	immunoglobulin
IFN-γ	interferon-gamma
IFN-γR	interferon-gamma receptor
IIF	indirect immunofluorescence
IL	interleukin
iNOS	inducible nitric oxide synthase

K14, 10	keratin 14, 10
LC	Langerhans cells
LE	lupus erythematosus
LNT	littermates negative for the transgene
MFC	mean fluorescence channel
MHC	major histocompatibility complex
mRNA	messanger ribonucleic acid
NCAM	neural cell adhesion molecule
NF-κB	nuclear factor-kappaB
NO	nitric oxide
NS	not significant
NZB	New Zealand black
NZW	New Zealand white
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PARP	poly(ADP-ribose) polymerase
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PBS/T	phosphate buffered saline/Tween
PHA	phytohaemagglutinin
PLE	polymorphous light eruption
PMN	polymorphonuclear leukocytes
PMSF	phenylmethylsulfonylfluoride
PNA	peanut agglutinin
PS	phosphatidylserine
RNA	ribonucleic acid
ROI	reactive oxygen intermediate
rpm	revolutions per minute
SAP kinase	serine/threonine kinase

SCLE	subacute cutaneous lupus erythematosus
SDS	sodium dodecyl sulphate
SIS	skin immune system
SLE	systemic lupus erythematosus
snRNP	small ribonucleoprotein
SSC	salt sodium citrate buffer
ssDNA	single stranded deoxy ribonucleic acid
TNFα,β	tumour necrosis factor alpha, beta
TCA	trichloroacetic acid precipitation
TCR	T cell receptor
TUNEL	terminal deoxynucleotidyl transferase-mediated nick end labeling
UVA, B	ultraviolet A, B
V	Volts
W	Watts
WHO	world health organisation
Z-FA	Z-Phenylalanine-Alanine
Z-VAD	Z-Valine-Alanine-Aspartate
α-DNA	anti-deoxyribo nucleic acid antibody
α-DNA-PKcs	anti-deoxyribo nucleic acid-dependent protein kinase antibody
α-La(SSB)	anti-La(Sjögren's syndrome B) antibody
α-nRNP	anti-nuclear ribonucleoprotein particle antibody
α-Ro(SSA)	anti-Ro(Sjögren's syndrome A) antibody
α-U1 RNA	anti-U1 ribonucleic acid antibody
α-Y5 RNA	anti-Y5 ribonucleic acid antibody