The effect of *Mycobacterium bovis*-BCG infection on longevity and autoimmune disease in MRL/*lpr* mice

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Abstract

Systemic lupus erythematosus (SLE) is a debilitating autoimmune disease affecting 1/1000 Canadians, primarily women of childbearing age. *Mycobacterium bovis*-BCG, the tuberculosis vaccine, is a potent immunomodulator and has been used to treat other autoimmune diseases. To study the impact of *M. bovis*-BCG on disease progression in the MRL/*lpr* mouse model of lupus, groups of MRL/*lpr* and background control MRL/+ mice were infected with BCG at 16-18 weeks of age, and their longevity, kidney function and serologic characteristics examined. MRL/*lpr* mice infected with BCG showed a slight improvement in longevity, and were protected from kidney failure. This was accompanied by a delay in the class-switching from IgM to pathogenic IgG anti-double-stranded DNA autoantibodies, and a delay in epitope spreading among autoantibodies to spliceosomal proteins. BCG infection of lupus mice thus provided transient protection from autoimmune disease, possibly through effects on cytokine production.

Résumé

Le lupus érythémateux disséminé (LED) est une maladie auto-immune débilitante qui affecte un canadien sur mille, particulièrement les femmes en âge de procréer. *Mycobacterium bovis*-BCG, le vaccin contre la tuberculose, est un puissant immunomodulateur utilisé pour traiter certaines maladies auto-immunes. Afin d'étudier l'impact de *M. bovis*-BCG sur le modèle murin de lupus MRL/*lpr*, des groupes de souris MRL/*lpr* et contrôles MRL/+ âgées de 16-18 semaines ont été infectées avec le BCG. Leur longévité, leur fonction rénale ainsi que leurs caractéristiques sérologiques ont été étudiées. Une légère augmentation de la longévité ainsi qu'une protection contre l'insuffisance rénale ont été observées chez les souris MRL/*lpr* infectées avec BCG. De plus, l'apparition des auto-anticorps IgG anti-ADN ainsi que la diffusion des épitopes reconnus sur les protéines du spliceosome ont été retardées. L'infection des souris lupiques avec le BCG les protège de façon transitoire contre leur maladie auto-immune, possiblement en modulant la production de cytokines.

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List of Abbreviations

Ag – antigen

Ab – antibody

ALPS – autoimmune lymphproliferative syndrome

APC – antigen presenting cell

BCG – Bacille Calmette Guérin

BUN – blood urea nitrogen

CD – cluster(s) of differentiation

CFU – colony forming unit

CNS – central nervous system

DC – dendritic cell

DC-SIGN – DC-specific intercellular adhesion molecule-3 grabbing non-integrin

DN – double negative

DNA – deoxyribonucleic acid

dsDNA – double-stranded DNA

EAE – experimental autoimmune encephalomyelitis

EEA1 – early endosomal antigen 1

ELISA – enzyme linked immunosorbent assay

HLA – human leukocyte antigen

IDDM – insulin-dependent diabetes mellitus

IFN-γ – interferon gamma

Ig – immunoglobulin

IL – interleukin

lpr – lymphoproliferation

ManLAM – mannose-capped lipoarabinomannan

MHC – major histocompatability complex

mRNA – messenger RNA

MS – multiple sclerosis

NK – natural killer

NO – nitrous oxide

NOD – non-obese diabetic

NRAMP – natural resistance-associated macrophage protein

PI3 – phosphatidylinositol 3

PPD – purified protein derivative

RA – rheumatoid arthritis

RF – rheumatoid factor

RNA – ribonucleic acid

RNP – ribonucleoprotein

SLE – systemic lupus erythematosus

Sm – Smith antigen

TGFβ – transforming growth factor beta

Th1 – T helper type 1

Th2 – T helper type 2

TLR – Toll-like receptor

TNFα – tumour necrosis factor alpha

Treg – regulatory T cell

UV – ultraviolet

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Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease primarily affecting women of childbearing age (9:1 female:male ratio between ages 15-50). There is also a higher prevalence within some ethnic groups, such as African-Americans, Hispanics, North-American aborigines and Orientals (Winchester, 1992). Major organ systems affected can include the central nervous system, heart and kidneys, as well as the skin and joints. Although the disease can now be managed through drug regimens that often include steroids and broad-spectrum immunosuppressors, these drugs have numerous side-effects, and research into less harmful alternative treatments is ongoing. Nevertheless, even with modern-day treatment, SLE is still the cause of significant morbidity and mortality within the North American population, and world-wide (Schoenfeld, 1992), and thus a better understanding of the pathophysiology of this disease would aid in efforts to combat it.

Studies using murine SLE models are useful in furthering knowledge regarding the causes and progression of this disease. The MRL/*lpr* mouse model is a well-established model of human SLE. Disease onset occurs at 3-4 months of age, and is characterized by: lymphadenopathy and splenomegaly due to the accumulation of abnormal double-negative T cells; development of autoantibodies to various self antigens, including double-stranded DNA and spliceosomal proteins; proliferative glomerulonephritis; occasional arthritis reminiscent of human rheumatoid arthritis, complete with rheumatoid factor production; and skin

lesions (Theofilopoulos, 1992). Although the lymphoproliferation (*lpr)* mutation, which consists of a point mutation in the apoptosis-mediator Fas, accelerates disease progression, mice of the MRL background without this mutation still develop lupus-like disease symptoms at 12 months of age, and the *lpr* mutation on non-autoimmune background strains does not cause nephritis (Vidal et al, 1998); therefore, as yet unidentified genes in the MRL background are also important for the development of autoimmune disease in this strain.

SLE is characterized by the production of large amounts of autoantibodies to nuclear antigens. These include antibodies to DNA and other nucleic acids, histones, and spliceosomal components. How tolerance to these ubiquitous antigens is broken is as yet unknown: Presentation of self antigens modified during apoptosis, defective clearance of apoptotic cells, molecular mimicry and failure in peripheral tolerance mechanisms are all thought to contribute to either disease onset or progression. Generation of high titres of autoantibodies leads to the formation of large immune complexes, which overwhelm the clearance capacity of the affected individual and subsequently deposit on the walls of small blood vessels, particularly in the kidney. There, they can activate the complement pathway and cause an inflammatory response, which is maintained due to the persistence of the antigen in the body and continued immune complex formation (Crow and Christian, 1992).

The combined impact of the humoral and inflammatory responses in SLE has led to debate as to which arm of the immune response is

responsible for disease. Inflammatory, or Th1, cytokines have been shown to be important in end-organ tissue destruction, but conversely, Th2 cytokines are required for B cell activation and autoantibody production. Studies in mouse models using various cytokine knock-out strains have shown that both Th1 and Th2 cytokines are important for disease to occur, for removal of cytokines of either type can improve the course of disease, but does not eliminate it (Theofilopoulos and Lawson, 1999).

The kidney is the organ most often studied with respect to SLE, as lupus nephritis is the most severe disease manifestation in terms of mortality. Anti-native DNA autoantibodies, particularly those of the IgG2a isotype, are implicated in the development of nephritis in mouse models (Theofilopoulos, 1992). It has recently been shown that IFN-γ stimulation of B cells leads to production of IgG2a antibodies through activation of the transcription factor T-bet (Peng et al, 2002). Conversely, IL-4 stimulation of B cells leads to class switching to IgG1 antibodies through the activation of STAT6 (Singh et al, 2003). Therefore, pathogenic IgG2a anti-dsDNA antibodies could arise in response to IFN-γ stimulation, but as disease is not completely abrogated in the absence of IFN-γ (Peng et al, 1997), other factors and antibodies of other specificities cannot be disregarded.

Many factors have been associated with the development of SLE, including genetics, hormonal and environmental influences, and infection history. Decreased infection with endogenous mycobacteria and other organisms has been proposed to be partially responsible for the increase in autoimmune and allergic diseases observed in the industrialized world,

the "hygiene hypothesis". Infection with saprophytic mycobacteria, helminthes and lactobacilli may provide required stimulation for regulatory T cells and other immune regulatory cells important in the prevention of the immune system dysregulation characteristic of autoimmune and allergic diseases (reviewed in Rook et al, 2004).

Mycobacterium bovis-BCG is the only vaccine currently available for tuberculosis, despite controversy surrounding its efficacy. It has also been investigated with respect to various autoimmune diseases. Treatment with BCG ameliorated disease in both the mouse models of diabetes and multiple sclerosis, through different mechanisms. In non-obese diabetic (NOD) mice, mycobacterial treatment protected mice from developing diabetes through the activation of regulatory T cells (Martins and Aguas, 1999); in mice with experimental autoimmune encephalomyelitis (EAE), BCG treatment prevented accumulation of T cells in the central nervous system by diverting T cell traffic to BCG granulomas (Sewell et al, 2003). Infections with other bacteria have also shown protective effects on mouse models of SLE: infection of lupus-prone NZW/NZB F1 mice with *Toxoplasma gondii* at an early disease time point protected the mice from kidney disease and significantly improved survival (Chen et al, 2004).

In contrast to these findings, Ratkay et al (1993) found that injection of MRL/*lpr* mice with Complete Freund's adjuvant (CFA) enhanced the development of arthritis in this strain. Treatment with CFA, which contains mycobacterial antigens emulsified in oil, caused the number of mice

developing arthritis to increase from 12% of untreated to 72% of treated mice, and also caused earlier disease onset. To examine whether BCG infection would also accelerate the development of arthritis in MRL/*lpr* mice, groups of young (4-6 weeks) and old (16-18 weeks) mice were infected with BCG and monitored for the development of arthritis. Surprisingly, the mice did not show an increased incidence of arthritis, but the mice infected at the later time point were found to be somewhat protected from the lupus-like disease, showing a lower titre of anti-dsDNA autoantibodies and decreased kidney damage. Older MRL/*lpr* mice were also more efficient at clearing the BCG infection than their MRL/+ counterparts (Newkirk MM and Radzioch D, unpublished observations). The current study was undertaken to confirm and expand on these preliminary results. We hypothesized that infection with BCG at a critical time during disease onset would cause a shift in the cytokine profile, which would lead to the amelioration of lupus-like disease symptoms and increased longevity. We observed a slight increase in longevity in infected MRL/*lpr* mice, accompanied by protection from kidney disease. We also observed a delay in the development of pathogenic IgG anti-dsDNA antibodies, and in epitope spreading from anti-RNP to anti-Sm antibodies. The BCG infection also induced the rheumatoid factor response in the MRL/+ mice. These studies therefore shed light on the complex interactions that occur between host and pathogen.

LITERATURE REVIEW

1. Autoimmunity and Autoimmune Disease

In the early 20th century, as our understanding of the immune system was developing, Ehrlich introduced the concept of autoimmunity, or *horror autotoxicus* as he termed it, recognizing that the powerful effector mechanisms of the human immune system could be devastating if turned against the host (Ehrlich and Morgenroth, 1906). This observation naturally led immunologists to search for the mechanisms which prevent this from occurring, and to the discovery of autoimmune diseases, in which these mechanisms are dysfunctional. However, not all autoimmune individuals will develop autoimmune disease. Several conditions such as infection, close kinship with an autoimmune diseased individual or aging can lead to the development of high titres of antibodies to self antigens, and yet no disease will manifest itself in most of these individuals. Clearly, additional factors are required to cause the progression from autoimmunity to autoimmune disease, which is defined mainly by the chronic, adaptive nature of the autoimmune response (Schoenfeld, 1992).

Genetics, infection history, hormonal and immune system defects, and exposure to environmental stimuli may all play a role in the induction of autoimmune disease. The major histocompatability complex (MHC) genes, particularly the MHC II human leukocyte antigen (HLA) alleles, have been implicated in several autoimmune diseases, including insulin-dependent diabetes mellitus (IDDM), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), through twin and family studies

(Schoenfeld, 1992). Infection with certain viruses, including hepatitis B, cytomegalovirus and Epstein-Barr virus (James et al, 2001), has been proposed to contribute to the development of autoimmune disease, possibly through molecular mimicry mechanisms. Infection with certain environmental bacteria, in contrast, has been associated with decreased autoimmune disease incidence (Rook et al, 2004). Since most autoimmune diseases, such as RA, SLE and multiple sclerosis (MS) show distinct sex-based risk association, researchers have begun to investigate hormonal influences in the etiology of these diseases (Janeway, 4th ed., 1999).

Autoimmune diseases have been classified based on their immunopathogenic mechanism. Type II diseases are mediated by antibodies to cell surface or matrix antigens, and include rheumatic fever and Goodpasture's syndrome. Type III contains the immune-complex mediated diseases and includes systemic lupus erythematosus (SLE). The type IV diseases are T-cell mediated and include IDDM and RA (Janeway, 4th ed., 1999). However, autoimmune diseases often share diagnostic criteria, as in the case of SLE and mixed connective tissue disease, and conversely, patients diagnosed as having the same disease can present with very different symptoms and pathologies involving both the B cell and T cell arms of the adaptive immune response. Thus, it is difficult to classify and diagnose these illnesses (Schoenfeld, 1992).

2. **Systemic Lupus Erythematosus**

a) Pathology and Prevalence

Systemic lupus erythematosus (SLE), or lupus, is a multifactorial type III autoimmune disorder with a prevalence in Canada of approximately 1/1000 individuals. It occurs at a much higher frequency in women of reproductive age (9:1 ratio to men of the same age group), with the juvenile and geriatric forms showing a much more even sex distribution. There is also a higher risk for the development of SLE associated with some ethnic backgrounds, namely African-American, Hispanic, North American aboriginal and Oriental (Hochberg, 1992). This suggests a genetic contribution to the development of SLE, which is confirmed by the higher concordance rate in monozygotic twins (25%) vs. dizigotic twins (5%) (Pisetsky, 1997). Several genes have been linked to the occurrence of SLE, but no one mutation or polymorphism has been found to control susceptibility (Winchester, 1992). SLE is thus a complex polygenic disease, with several susceptibility-conferring mutations being required in any given individual to cause disease.

Genetic susceptibility, multifactorial or not, is still not enough to cause disease. Infection history and environmental stimuli also play a role in the induction of the immune dysregulation characteristic of this disorder. The specific trigger(s) for SLE is as yet unknown. Bacterial or viral infections, dietary factors, exposure to UV light or to certain drugs, and hormonal imbalances have all been proposed to contribute to the

development of active disease. No compelling data supporting a direct cause and effect mechanism has yet been offered (Mok and Lau, 2003).

What is clear is that exposure of a genetically susceptible individual to one of these agents, or an as yet undetermined substance, causes the individual's B cells to break tolerance to self antigens. Autoreactive T and B cells are generated in normal individuals, but are generally either deleted during development or rendered anergic in the periphery by immune regulatory mechanisms. In autoimmune individuals, these cells become active and are therefore capable of mounting an acquired, chronic immune response to self antigens such as single- and double-stranded DNA, spliceosomal components, histones, ribosomal proteins and many others. Mutations in genes involved in the generation and maintenance of tolerance, such as the *fas* gene, have been observed in mouse models of SLE, but not in human patients (Crow and Christian, 1992).

The process whereby normally sequestered antigens become available to the immune system is also unclear. Defective clearance of apoptotic bodies, which often contain modified, immunogenic forms of cellular antigens, has been proposed as one mechanism. In support of this theory, mutations in genes responsible for clearance of apoptotic bodies, such as C1q, have been identified in a subset of lupus patients and also in mouse models of SLE (Crow and Christian, 1992; Winchester, 1992).

Once tolerance to one antigen has been broken, the immune system of the patient begins to recognize many different but related antigens in a process known as epitope or determinant spreading (Crow

and Christian, 1992). This usually occurs with proteins (or nucleic acids, in the case of DNA and histones) that are physically associated within the cell and are therefore taken up together by B cells and other professional antigen presenting cells. In this way, B cells specific for one antigen can receive help from T cells specific for a related antigen, by presenting peptides derived from both on their surfaces, and thus antibodies can be produced against both antigens. This process has been documented in mice, rabbits, and in patients, specifically with respect to the subunits of the spliceosome (James et al, 1995).

Autoantibody production can begin years before the onset of clinical disease. A recent report by Arbuckle et al (2003) showed that certain autoantibodies can develop nine to ten years before diagnosis occurs, and that patients usually acquire autoantibodies of three different specificities before they are diagnosed with lupus. This lends support to the hypothesis that high titres of autoantibodies are required before the onset of clinical symptoms, which include: arthritis, malar or butterfly facial rash, CNS involvement, cardiac damage and kidney disease (Crow and Christian, 1992). The mechanism behind autoantibody-mediated tissue damage is generally thought to be as follows: high titres of autoantibodies are generated against ubiquitous self antigens, which leads to the formation of large numbers of small, antigen-antibody immune complexes. These complexes are not cleared from the circulation, due perhaps in part to mutations in proteins responsible for clearance, but also because the sheer number of complexes produced may overwhelm clearance capacity.

These immune complexes are therefore deposited in small blood vessels throughout the body, but especially in the skin, kidneys and joints. There, they cause the activation of complement, which in turn causes the recruitment of inflammatory cells and the production of cytokines that mediate tissue damage (Crow and Christian, 1992).

Lupus nephritis is historically the most life-threatening aspect of lupus. Before the introduction of modern therapies in the 1970's, the 5 year survival rate for lupus patients diagnosed with proliferative glomerulonephritis was 25% - now, that figure ranges from 45% to 70% (Pollack and Kant, 1992).

b) Autoantibodies in SLE

Autoantibodies are thought to play a primary role in lupus pathogenesis. At least 28 different specificities have been documented in sera from lupus patients, including antibodies to nucleosomes, histones, single- and double-stranded DNA, spliceosomal components (the Smith antigen (Sm) and ribonucleoprotein (RNP)), phospholipids, cellular matrix proteins and others (Buskila and Schoenfeld, 1992). Of all of these, the antibodies to double-stranded (ds)DNA are thought to be the most pathogenic. Clinically, high titres of these autoantibodies are reliably diagnostic for SLE (Buskila and Shoenfeld, 1992). Especially high titres are observed in nephritis patients, and these have been shown to correlate with kidney disease activity. Precisely because of such high titres, IgG anti-dsDNA antibodies have a high potential for passive deposition in the kidney, but also, certain IgG anti-dsDNA antibodies have

been shown to bind specifically to proteins expressed in the kidney, such as alpha-actinin and alpha-enolase (Mason et al, 2004; Migliorini et al, 2002).

Autoantibodies of other specificities are assumed to be pathogenic as well, although little data exists to support this assumption. Clinically, high autoantibody titres are associated with the onset of active disease in SLE patients. Generally, higher levels of autoantibodies of all specificities could lead to increased immune complex formation and deposition, and thus end organ damage. Specifically, besides the association between anti-dsDNA antibodies and nephritis described above, associations between anti-ribosomal P antibodies and neurological disease and between anti-Ro antibodies and cardiac and skin manifestations have been described (Mok and Lau, 2003). Arbuckle (2003) found that, although autoantibodies of certain specificities (ANA, anti-Ro, anti-La, antiphospholipid) could be detected in the sera up to 9 years before clinical diagnosis of SLE, appearance of antibodies to dsDNA, Sm and RNP occurred only months before diagnosis, at the initial onset of symptoms, thus strengthening the association between production of these autoantibodies and disease pathogenesis. The same group also noted that accrual of new autoantibody specificities seemed to halt at the time of diagnosis, although whether this was due to the commencement of treatment or was actually a part of the natural progression of the disease is unclear. More indirect evidence for the role of autoantibodies in disease pathogenesis comes from the recent success of B cell depletion therapies

using anti-CD20 antibodies in treating severe cases of SLE (Kazkaz and Isenberg, 2004).

The significance of epitope spreading with respect to clinical progression of SLE is also poorly understood. Although it has been shown to occur, primarily among the Sm and RNP antigens, in both animal models and human SLE patients, and many groups are involved in mapping B and T cell epitopes within these proteins (for review see Monneaux and Muller, 2002), only three studies have shown induction of clinical disease by immunization with protein sequences derived from epitope mapping studies (James et al, 1995; James et al, 1998; Arbuckle et al, 1998) using three different model systems (rabbits, mice and primates). These results have not been duplicated. Thus, the exact role of epitope spreading in disease pathogenesis, beyond contribution to the overall autoantibody burden, remains controversial.

c) Treatment of SLE

SLE is a many-faceted disease and treatment regimens must reflect the individual symptoms manifested by each patient, the severity of those symptoms, and the different stages of the disease cycle (i.e. flare vs. maintenance treatment). Lupus nephritis is currently treated with broad spectrum immunosuppressive drugs such as cyclophosphamide and corticosteroids such as prednisone, but not all patients respond well to these treatments and alternatives must be considered. Current alternatives include nucleoside analogues which act against resting and dividing lymphocytes, anti-CD20 antibodies which deplete B cells, and

intravenous immunoglobulin, of which the exact mechanism of action remains controversial. However, all of these treatments have an immunosuppressive effect, leading to complications such as infections. A treatment strategy that more specifically targets B and T cells that cause disease should be developed (Mok, Wong and Lai, 2003).

3. The MRL/*lpr* Murine Model of SLE

a) Deficiencies in Fas and FasL

Fas (CD95) is a ubiquitously expressed 48kDa type I membrane protein of the TNF receptor family. When Fas is bound by its ligand, FasL (CD95L), cells expressing Fas are induced to undergo apoptosis through the activation of its intracellular death domain and subsequent downstream events (Suda et al, 1994). FasL is expressed mainly by lymphoid cells such as activated T cells (Suda et al, 1994) and NK cells (Oshimi et al, 1996), by cells in immune privileged sites such as the eye (Griffith et al, 1995), and by some tumour cells (Strand et al, 1996). It is a type II membrane protein of 40 kDa which can be cleaved to a soluble, 28 kDa form (Kayagaki et al, 1995).

Fas/FasL-mediated apoptosis is important for several processes in the body, including lymphocyte homeostasis, tumour surveillance, and cytotoxic T-lymphocyte and NK cell-mediated killing of infected cells (reviewed in Lee and Ferguson, 2003). Many of these roles were discovered through the study of the spontaneous mutations which occurred in the autoimmune-prone MRL mouse strain. These mice exhibit a phenotype which strongly resembles the human disease systemic lupus erythematosus (SLE, reviewed in section 2), which consists of lymphadenopathy, including the accumulation of an abnormal subset of CD3+,CD4-,CD8-, B220+ alpha-beta T cells (double-negative (DN) T cells), splenomegaly, development of autoantibodies, and nephritis (Andrews et al, 1978). The lymphoproliferation (*lpr*) mutant allele has a

retroviral insertion in the Fas gene, which causes premature transcription termination (Watanabe-Fukunaga et al, 1992). The *lpr-cg* allele carries a point mutation in Fas (Watanabe-Fukunaga et al, 1992), and the generalized lymphoproliferative disease (*gld*) mutation is also caused by a point mutation, but in FasL (Takahashi et al, 1994). All three mouse strains develop the same phenotype when the alleles are homozygous on the original MRL background. The *lpr* and *gld* mutations have been transferred to other mouse strains, which develop autoantibodies and accumulate abnormally high DN T cell populations but do not develop kidney disease (Kelley et al, 1985), highlighting the contribution of the MRL background. Also, MRL mice with normal Fas and FasL function develop autoantibodies and kidney disease, but at a later age than mice with the mutations (12 months vs. 3-4 months of age), and do not exhibit lymphoproliferation (Theofilopoulos, 1992). Thus, the combination of the MRL background with defective Fas function is vital for full disease manifestation.

How defects in Fas-mediated apoptosis contribute to autoimmunity is not clearly understood. Initially, it was thought that Fas/FasL signalling was important during negative selection of autoreactive T cells in the thymus, but this is not the case (Singer et al, 1994). Fas/FasL interactions are important in the maintenance of peripheral tolerance in T cells, and in homeostasis of the lymphocyte population (Ju et al, 1995). T cell help is vital for the development of anti-DNA autoantibodies, which are a major pathologic subset in SLE, and so lack of tolerance could contribute in this

fashion (Sobel et al, 1993). Similarly, a role for Fas-mediated apoptosis has been proposed for the maintenance of peripheral B cell tolerance. B cells chronically desensitized by restimulation with self antigen may be deleted through Fas/FasL interactions with T cells (Rathmell et al, 1995).

Fas and FasL mutations have been documented in humans, but these people do not develop lupus. Instead, they develop a condition called autoimmune lymphoproliferative syndrome (ALPS). ALPS patients show early onset (usually before 5 years of age) of splenomegaly, lymphoproliferation with accumulation of DN T cells, and development of autoantibodies to a broad spectrum of proteins and lipids. They are classified based on the homozygosity or heterozygosity of their mutation and whether the mutation is in Fas, FasL or in one of the downstream effectors of the Fas pathway, such as caspase 10 (reviewed in Rieux-Laucat et al, 2003).

b) Other Genetic Abnormalities

As previously mentioned, the MRL genetic background contributes substantially to the development of lupus-like disease in MRL/*lpr* mice. MRL mice were derived from breeding of other inbred strains (current genome composition: 75% LG/J, 13% AKR/J, 12% C3H/Di, 0.3% C57BL/6J; Theofilopoulos, 1992). Putative susceptibility loci have been identified in genetic analyses of this strain (Vidal et al, 1998), but no functions have as yet been assigned to these loci. Interestingly, it has recently been shown that MRL/*lpr* mice share an IL-2 allele coding for a hypoactive variant of IL-2 with NOD and SJL/J mice, which are also

autoimmune-prone strains (Choi et al, 2002). Defective IL-2-mediated MRL T cell activation may thus play a role in the observed resistance to activation-induced cell death of MRL/+ T cells (independent of Fas function). MRL T cells also express lower levels of the high-affinity IL-2 receptor component CD25 (Choi et al, 2002), and some groups have observed proliferation defects in these cells when stimulated with IL-2 *in vitro* (Altman et al, 1981; Cameron and Waterfield, 1986).

c) Cytokine Profile

The role of different cytokine subsets and even of individual cytokines is highly controversial in the MRL/*lpr* mouse model. As it is widely accepted that autoantibody deposition in the kidney is responsible for the observed nephritis in these mice, it is logical to expect that T-helper type 2 (Th2) cytokines such as IL-4, which promote antibody responses by stimulating B cells, would be involved in disease pathogenesis, and there is evidence for such a role (Peng et al, 1997; de Albuquerque et al, 2004). Conversely, cytokines traditionally associated with inflammatory Th1 responses, such as IFN-γ, TNF-α, and IL-12 have been implicated in the progression of renal disease in MRL/*lpr* mice (Peng et al, 1997; Kim et al, 2002; Xu et al, 2001). Thus, it remains unclear whether lupus nephritis in MRL/*lpr* mice is a predominantly Th1 or Th2-driven response. One possible explanation for this observed discrepancy is that different cytokines may be required for the induction than are required for the maintenance of disease. Th2 cytokines may be more important early in the disease, for the generation of autoantibodies which start the process of

kidney damage, but Th1 cytokines may be important later in disease, to mediate the recruitment of inflammatory cells and tissue destruction observed in the kidneys of these mice.

Table 1. Cytokines and their involvement in lupus nephritis of MRL/*lpr* mice.

Cytokine	Observations
IL-10	• Knock-out mice have aggravated disease; administration of rIL-10 to KO mice reduced IgG2a anti-dsDNA autoantibodies [3]
IL-12	• Knock-out mice have reduced disease phenotype; rIL-12 injections increase IFN-γ and NO [6]
IL-18	• Upregulated in kidneys of MRL/*lpr* mice; Injections of IL-18 cause aggravated disease [2]
IL-4	• Knock-out mice have reduced lymphadenopathy and end-organ disease; reduced IgG1 and IgE [4]
IL-6	• Neutralising mAb to IL-6R beneficial [1]
IL-1	• Increased in all lupus mice; rIL-1R treatment decreased disease symptoms; IL-1R antagonist had no effect [1]
TNF-α	• Upregulated in diseased kidneys; inhibition improves pulmonary but not renal disease [5]
IFN-γ	• Knock-out mice have reduced lymphadenopathy and end-organ disease; reduced IgG2a and IgG2b and autoantibodies [4]
TGF-β	• Increased endogenous levels; increases host susceptibility to bacterial infection – anti-TGFβ mAb therapy ameliorated survival in lethally infected mice [1]

1 Theofilopoulos and Lawson, 1999
2 Esfandiari et al, 2001
3 Yin et al, 2002
4 Peng et al, 1997
5 Kim et al, 2002
6 Kikawada et al, 2003

d) Autoantibody Profile

MRL/*lpr* mice produce a wide range of autoantibodies against nuclear antigens. These include: anti-ssDNA and anti-dsDNA, anti-Sm and anti-RNP, anti-histone, and IgM and IgG rheumatoid factors, among others (Theofilopoulos, 1992). As in human SLE, anti-dsDNA antibodies are the most often associated with disease severity, particularly kidney dysfunction, in these mice. Anti-dsDNA antibodies of the IgG2a isotype are among the most pathogenic (Peng et al, 1997). IgM and IgG rheumatoid factors may assist in the formation of the immune complexes that aid in inflammation; specifically, IgG3 anti-IgG2a RF have been found in MRL/*lpr* mice and can cause cryoglobulin formation, associated with both kidney disease and skin lesion formation in this strain (Theofilopoulos, 1992). Anti-Sm and anti-RNP antibodies form to components of the U1 subunit of the spliceosome, a complex of proteins responsible for splicing introns from pre-mRNA to form mature mRNA. These autoantibodies are most frequently of the IgG2a isotype (Fatenejad et al,1994), which is the most highly represented subclass in the MRL/*lpr* diseased kidney; thus, these antibodies may also play a role in kidney disease in these mice by contributing to immune complex formation (Theofilopoulos, 1992).

e) Other Mouse Models of SLE

There are two other strains of mice that spontaneously develop lupus-like symptoms and have been used to research the pathogenesis of lupus. In the BXSB strain of mice, it is the males who develop the accelerated form of the disease, reaching 50% mortality by 5 months of age, whereas the females develop a delayed form of the disease and live a normal lifespan. The other strain is the F1 cross of New Zealand Black (NZB) and New Zealand White (NZW) mice. Interestingly, the NZW parental strain shows no disease phenotype, whereas the NZB parental strain develops haemolytic anaemia. The F1 males show a mild, late-onset disease, similar to that observed in the NZB parental strain, and the F1 females have the acute, early-onset disease phenotype (50% mortality at 7 months). All of the strains develop lupus nephritis, but are variable in other elements of disease such as specificity of autoantibodies and immune system abnormalities (Lahita et al, 1992).

4. ***Mycobacterium bovis*-BCG and the Host Immune Response**

a) Autoimmune Disease and Allergy

BCG is a potent immunomodulator and it's utility as a therapy for various diseases has been and continues to be explored. BCG infection induces a strong type 1 T cell helper response, stimulating the production of inflammatory cytokines such as IL-12, IFN-γ and TNF-α, among others. This property has been exploited for therapeutic use in models of allergic disease, which is generally characterised by a Th2 type response, as Th1 cytokines down-regulate Th2 responses (and vice versa). CD4+ Th2 cells provide help to allergen-specific B cells which produce IgE and IgG1 antibodies, which in turn cross link receptors on eosinophils and mast cells and promote their degranulation, causing allergic inflammation. Several studies have examined whether the presence of a strong Th1 host environment could prevent the development of Th2 allergic responses in mice. Airway administration of live or heat-killed BCG has been shown to protect against the induction of airway eosinophilia in mice (Major et al, 2002). Similarly, mice immunized with a recombinant *Mycobacterium vaccae* or BCG vector expressing an allergenic house dust mite peptide mounted a lower allergic response than non-immunized mice when challenged by the same peptide (Janssen et al, 2001). These studies showed that prior or simultaneous treatment with BCG had an ameliorative effect on allergy development in mice. In humans, some studies have shown an inverse correlation between BCG vaccination in childhood and

later development of allergy (Shirakawa et al, 1997). However, other studies have found no such correlation (Strannegard et al, 1998). The discrepancies may perhaps be explained by genetic variation between populations, differences in mean age at vaccination, in the vaccine strain and/or the mode of vaccination.

BCG therapy has also been investigated with respect to several autoimmune diseases. In mice, BCG infection has been shown to have a protective effect on the induction of experimental autoimmune encephalomyelitis (EAE), the mouse model of multiple sclerosis (MS) (Sewell et al, 2003). BCG has also proved effective in preventing the development of diabetes mellitus (IDDM) in non-obese diabetic (NOD) mice (Qin et al, 1998). Exposure to *Mycobacterium avium* was shown to protect against diabetes in NOD mice (Martins and Aguas, 1999). BCG has also been shown to induce protection from adjuvant arthritis induced in Lewis rats (Esaguy et al, 1996). Several mechanisms have been proposed to explain these seemingly counterintuitive results, as EAE and IDDM are both predominantly Th1-mediated diseases. Sewell et al. showed that MOG-specific T cells trafficked preferentially to BCG infection foci instead of to the central nervous system, which prevented the onset of disease through immune damage to the CNS. The activation of T-regulatory cell subsets, which inhibit autoreactive T cells in the periphery, has also been suggested as a mechanism for the protective effect of mycobacteria in IDDM and other diseases (Martins and Aguas, 1999; Gazda et al, 1996). Potential side effects of BCG therapy have also been

noted. In one study, mice protected from the development of diabetes by BCG later developed a pathologic antibody response resembling systemic lupus erythematosus (Baxter et al, 1994).

BCG vaccination and/or exposure to environmental mycobacteria have also been linked to protection from autoimmune disease in humans. Andersen et al. (1981) reported an inverse relationship between incidence of multiple sclerosis and exposure to environmental mycobacteria, as measured by spontaneous positive tuberculin skin test. BCG has been used as a therapy for MS in clinical trials, and was shown to lead to a 51% reduction in lesion size in the brain (Ristori et al, 1999). BCG vaccination in children has also been associated with a reduced incidence of early onset diabetes in the Netherlands (Classen and Classen, 1999).

b) Bladder Cancer Therapy

BCG has been the primary treatment for superficial bladder cancers since 1976, when Morales, Eidinger and Bruce first reported success using BCG as an immunomodulatory therapy. BCG is applied intravesicularly and has been associated with reduced tumour progression and less frequent post-therapy relapse rates. Studies in patients and in animal models have clarified some of the immune mechanisms responsible for this effect. Studies using animal models have shown that mycobacteria introduced into the bladder bound to the urothelial lining through a fibronectin attachment protein-fibronectin interaction (Ratliff et al, 1987; Zhao et al, 2000). This binding was required for successful

therapy and seemed to have a direct effect on tumour cell proliferation and viability. BCG has been shown to activate urothelial and resident antigen presenting cells, which then produced cytokines and chemokines which attracted granulocytes and other immunocompetent mononuclear cells. Once recruited, these cells released many pro-inflammatory cytokines. T cells were also recruited to the bladder, and contributed to the production of cytokines, serving a required accessory function. In this way, a largely Th1 milieu is established, as well as granuloma-like cellular foci. Cytokines, especially the IFN family, have direct tumouricidal effects, and NK cells are also very important, in an effector cell capacity and as cytokine producers. Induction of NOS has also been detected in bladder cancer cells of some patients and in a rat bladder cancer model (reviewed in Bohle and Brandau, 2003).

5. **Tuberculosis**

a) Prevalence and Pathology

According to the World Health Organization, tuberculosis causes approximately 2 million deaths worldwide each year. Up to one third of the world population, or 2 billion people, are currently latently infected with the causative agent, *Mycobacterium tuberculosis*. Ten percent of those will develop active tuberculosis during their lifetime, as their immune systems lose the ability to control the growth and spread of the bacteria due to immunosuppression caused by HIV co-infection, malnutrition, old age and/or immunosuppressive chemotherapy. The emergence of multi-drug resistant strains of *M. tuberculosis* has also made control and treatment problematic (WHO TB fact sheet, 2002).

Despite the high prevalence of infection, development of active disease relies heavily on several mitigating factors, and the majority of people exposed to the bacteria are able to successfully control the infection. The prevailing immune status of the host is perhaps the most important factor, but host genetics also play a role. In humans and mice, the natural resistance-associated macrophage protein 1 (Nramp1; see section 6) has been associated with the ability to control infection by *M. tuberculosis* and other intracellular pathogens (Greenwood et al, 2000; Vidal et al, 1996).

M. tuberculosis is a highly successful intracellular pathogen because of its ability to induce a persistent, latent infection in the host. Infection generally occurs through the inhalation of bacteria-containing

aerosols generated by the coughing or sneezing of patients with active pulmonary tuberculosis. A very low inoculum is required for infection to occur. Mycobacteria are then phagocytosed by alveolar macrophages, which, instead of killing the bacteria, become infected, as this pathogen has evolved numerous mechanisms which allow it to survive within the phagosomes of these cells (to be discussed later). Pro-inflammatory cytokines and chemokines are released by the infected cells, which recruit mononuclear cells from the surrounding blood vessels. Infected macrophages and recruited mononuclear cells form the core of the granuloma, or tubercule, the characteristic lesion of tuberculosis. The core is surrounded by foamy (Langhans) giant cells, which are aggregates of highly activated macrophages, with a peripheral concentration of T and B lymphocytes. The granuloma provides a physical barrier which contains the bacteria and prevents dissemination and active infection. However, the bacteria remain viable, and if the host's immune system becomes suppressed the granuloma can undergo caseation, break open and release the bacteria, which can then spread and cause active disease (reviewed in Russell, 2001 and Zahrt, 2003).

B cell-mediated humoral immunity does not seem to play a vital role in the control of *M. tuberculosis* in murine infection studies, although B cells are recruited to granulomas and may play a role in regulation of chemokine and/or adhesion protein expression (Johnson et al, 1997; Bosio et al, 2000). In contrast, T cell-mediated cellular immune responses are necessary for the control of infection. CD4+, CD8+, γδ and CD1-

restricted T cells have all been shown to play a role in regulating the immune response to tuberculosis (reviewed in Kaufmann, 2002). Interferon-gamma (IFN-γ) secreting Th1 polarized CD4+ cells are the most important of these, as CD4+ cells are twice as abundant as CD8+ cells in both mice and humans infected with *M. tuberculosis* (Gonzalez-Juarrero et al, 2001; Randhawa, 1990), and mice lacking IFN-γ show a much more rapid progression of disease than mice lacking IL-4, a typical Th2 cytokine (North et al, 1998). Dendritic cells (DC) are the primary antigen presenting cells in the T cell response to tuberculosis, as resident tissue DC are responsible for activating and recruiting T cells to the site of infection in the lung (Jiao et al, 2002).

The innate arm of the immune system is also important, especially in the initial response to tuberculosis infection. Mycobacterial products can bind to and stimulate surface Toll-like receptors TLR2 and possibly TLR4 on macrophages and dendritic cells, which leads to a cascade of events which ultimately induces TNF-α production and mycobacterial killing through NO production (reviewed in Senger et al, 2002). Production of TNF-α and NO by activated macrophages was thought to play an important role in granuloma maintenance, but may actually play a more important role in negative regulation of the Th1 immune response (Zganiacz et al, 2004).

Apoptosis of infected macrophages may also be important in the generation of acquired immunity to mycobacteria. It has been well established in the literature that *in vitro* infection of macrophages with

mycobacteria or exposure to mycobacterial products causes the cells to undergo apoptosis. This deprives the bacteria of their growth milieu and prevents the potentially damaging inflammation caused by necrotic death of infected cells, as well as containing the bacteria within apoptotic bodies instead of allowing them to disseminate. Apoptosis may also allow mycobacteria contained within apoptotic bodies to be phagocytosed by bystander cells, which can become activated and present mycobacterial antigens to T lymphocytes through MHC II on macrophages (Fratazzi et al, 1997) or MHC I and CD1 on dendritic cells (Schaible et al, 2003). The precise mechanism of induction of apoptosis by mycobacteria remains unclear. Lopez et al (2003) have reported that binding of the 19kDa *M. tuberculosis* protein (p19) to TLR2 mediated apoptosis in THP-1 cells, but other groups have shown no such effect of p19 using the same cell line (Gehring et al, 2003).

As mentioned above, mycobacteria have evolved several mechanisms which allow them to survive within macrophages. These include inhibition of phagosome-lysosome fusion and inhibition of bacterially-induced apoptosis. Upon binding to one of the mycobacterial receptors expressed on the surface of macrophages (e.g. mannose receptor, CD11b, CD11c, complement receptors), mycobacteria are engulfed (Tailleux et al, 2003). Bacteria engulfed by macrophages are normally processed through the early endocytic pathway and are killed by fusion of the bacteria-containing vesicle with the lysosome, a compartment that contains degradative enzymes and vacuolar ATPases which acidify

the compartment though influx of H^+ ions. This process also allows antigenic peptide generation and loading of major histocompatability complex II (MHCII), which is then trafficked to the cell surface for antigen presentation to CD4+ T lymphocytes. Mycobacteria have evolved a mechanism which arrests the maturation of the phagosome before fusion with the lysosome, allowing them to replicate within the phagosome of the host cell. Specifically, it has been shown that the mycobacterial surface component ManLAM (mannose-capped lipoarabinomannan) can inhibit the phosphorylation of phosphatidylinositol 3 (PI3) by the PI3 kinase hVPS34 in J774 cells (Vergne et al, 2003). PI3 phosphate interacts with early endosomal autoantigen 1 (EEA1), which plays a vital role in vesicle tethering and fusion (Christoforidis et al, 1999); therefore, inhibition of this pathway could explain the failure of phagolysosome formation in cells infected with mycobacteria. DC can also be infected with mycobacteria through binding of the DC-specific C-type lectin DC-SIGN (*DC*-specific *i*ntercellular adhesion molecule-3 *g*rabbing *n*onintegrin), which leads to suppression of DC function, possibly by interfering with Toll-like receptor signalling. DC can then become an important reservoir for *M. tuberculosis* (Geijtenbeek et al, 2003; Tailleux et al, 2003).

Mycobacteria have also evolved mechanisms to prevent apoptosis of their resident macrophages. The virulent *M. tuberculosis* strain H37Rv has been shown to induce less apoptosis than its avirulent counterpart H37Ra in macrophages (Keane et al, 1997; Danelishvili et al, 2003). The mycobacterial component(s) responsible for this effect remain undefined,

but it is clearly associated with infection with live bacteria, as exposure of macrophages to purified protein derivative (PPD) alone induces the same amount of apoptosis as observed in cells infected with attenuated bacteria (Gil et al, 2003).

b) Vaccine Development

The only vaccine currently approved for use against tuberculosis in humans is the attenuated *Mycobacterium bovis*-Bacille Calmette Guérin strain, developed in the 1920's by Calmette and Guérin at the Institut Pasteur. This vaccine is effective in preventing childhood tuberculosis, and therefore is still administered in countries where *M. tuberculosis* is endemic, but it fails to protect against the adult form of the disease (Wang et al, 2002). Also, there appears to be considerable inter-strain variability in the protection induced by different geographical strains of the vaccine (Behr et al, 2001). Therefore, attempts to develop improved vaccines are underway. Recombinant BCG strains that overexpress immunodominant antigens, such as Ag 85B (Dhar et al, 2002), or different Th1 cytokine genes have been developed (O'Donnell et al, 1994; Murray et al, 1996), but generally fail to improve on the capacity of BCG to induce Th1 responses in mice. Conjugation of modified mycobacterial components such as arabinomannan oligosaccharides to tetanus toxoid or immunodominant antigens has also been examined, but again did not show improved protection over the existing vaccine (Hamasur et al, 2003).

6. Summary

Autoimmune disease and allergy are growing causes of mortality and morbidity in the Western world, concomitant with the decrease in public health problems from infectious disease. Exposure to environmental organisms may be key in preventing autoimmune diseases by stimulating the developing immune system. In support of this hypothesis, early exposure to mycobacteria and other environmental organisms has been shown to correlate negatively with the incidence of autoimmunity (Rook et al, 2004). In murine models of autoimmune disease such as MS and IDDM, infection with *M. bovis*-BCG has been shown to cause an improvement in autoimmune disease pathology (Sewell et al, 2003; Martins and Aguas, 1999). The impact of BCG infection on SLE had not yet been examined. As current treatment protocols for SLE involve broad-spectrum immunosuppressive drugs with high levels of detrimental side effects, such as increased susceptibility to opportunistic infection, it was of interest to us to examine whether BCG could improve the pathogenesis of SLE in a murine model. BCG is already approved for use in humans as a vaccine for tuberculosis, and is also used for treatment of bladder cancer in humans with few observed side effects, thus making it an interesting candidate for SLE therapy.

Materials and Methods

Mice

Four to six week old MRL/*lpr* and MRL/+ mice were obtained from the Jackson Labs (Bar Harbor, Maine) and aged to 16 weeks in our facility. Ten mice of each genotype were then injected with 1.0×10^5 colony forming units (c.f.u.) *Mycobacterium bovis*-Bacille Calmette Guérin (BCG) (Montréal strain). Blood samples were taken from the lateral saphenous vein every two weeks (100µl) and serum was collected and stored at -20°C for future use. Mice were monitored twice weekly for skin lesion formation, arthritic changes (swelling and redness in hind limb joints) and signs of distress such as: weight loss >15% of total body mass, lethargy, ruffled fur, cyanosis, laboured breathing, etc., and euthanised accordingly. At 50% mortality within an experimental group, the remaining mice were sacrificed for blood and tissue collection. Mice were anaesthetized by CO_2 inhalation, and blood was collected by cardiac puncture (1 mL). Sera were collected and stored at -20°C until use. Urine samples were collected for kidney function analysis overnight prior to sacrifice and stored at -20°C until use. All experimental procedures using animals were approved by the Facility Animal Care Committee of McGill University.

Mycobacterium bovis-*BCG*

M. bovis-BCG Montréal strain was generously provided by Dr. Danuta Radzioch. 1.2×10^9 c.f.u. in 1mL phosphate-buffered saline (PBS)-0.05% Tween 80 were inoculated into 50 mL Middlebrook 7H9 broth (Fisher,

Nepean, ON) supplemented with 10% OADC enrichment (Becton Dickinson, Sparks, MD) and grown at 37°C with rotation until the optical density at 600 nm (OD_{600}) reached 0.5 (~5 days). Bacteria were then resuspended in PBS-0.05% Tween 80 and 1 mL aliquots were frozen at -70°C until use. One aliquot was serially diluted and plated on Dubos Oleic agar (Fisher) supplemented with 10% OADC enrichment to determine c.f.u. For injection, bacteria were resuspended in sterile PBS at 1.0×10^5 c.f.u./200 µl.

Enzyme-Linked Immunosorbent Assay

All antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), unless otherwise specified in the text. High-binding, flat-bottomed 96 well ELISA plates (Greiner Bio-one, Germany) were coated overnight at 4°C with 100 µl/well antigen in carbonate-bicarbonate buffer (pH 9.6). Plates were washed 3 times with PBS-0.1% Tween 20 (PBS/T) and incubated for 2 hrs. at 37°C with 100 µl/well of individual mouse sera at the specified dilution in PBS-1% BSA (Calbiochem, La Jolla, California). Samples were plated in duplicate with PBS-1% BSA as a negative control. One serum sample was chosen as an internal standard and run on all plates. Plates were again washed 3x with PBS/T and incubated for 1 hr. at 37°C with 100 µl/well horseradish peroxidase (HRP)-conjugated anti-mouse antibody at the specified dilution. Plates were subjected to a final wash and incubated with 100 µl/well of the HRP substrate orthophenylenediamine (OPD, Sigma, St. Louis, MO) (2mM in

0.02M citric acid, 0.05M Na_2HPO_4, 0.012% H_2O_2, pH 5.0) for 15 minutes at room temperature. The reaction was stopped by the addition of 50 µl of 4M H_2SO_4 to each well. Plates were read at 492 nm in an ELISA plate reader (SLT labinstruments, Austria). Values that were above the mean plus two standard deviations of the MRL/+ uninfected group were considered positive.

Rheumatoid Factor Assay (IgM)

Plates were coated with 2µg/mL mouse IgG, whole molecule and incubated with individual mouse sera at 1/100 dilution in PBS-1% BSA. F(ab')2 fragments of IgM diluted 1/5000 in PBS/T were used for detection. Values that were above the mean plus three standard deviations of the MRL/+ uninfected group were considered positive.

RF Assay (IgG)

Plates were coated with 2µg/mL human IgG Fc fragment generated by papain digestion of human monoclonal antibody EV2.7 and purified by affinity chromatography and ion exchange chromatography (Newkirk, 1990). Plates were then incubated with individual mouse sera at 1/100 dilution in PBS-1% BSA. A biotin-conjugated F(ab)'2 goat anti-mouse IgG Fab diluted 1/5000 in PBS/T was used for detection. Plates were washed again and incubated for 30 minutes at room temperature with 100 µl/well HRP-avidin diluted 1/10,000 in PBS/T before the addition of substrate.

Anti-double stranded DNA Antibody Assay

Plates were coated with 50 µl/well 2µg/mL solution of calf thymus nDNA (Sigma) in PBS-0.5% casein (Sigma). Plates were blocked 3 times for 5 minutes with PBS-0.5% casein and incubated for 3 hrs. at 37°C with 50 µl/well individual mouse sera at 1/50 dilution in PBS-0.5% casein. Plates were washed and incubated for 1 hr. at 37°C with 75 µl/well horseradish peroxidase (HRP)-conjugated donkey F(ab')2 fragments of anti-mouse IgM or HRP-conjugated goat F(ab')2 fragments of anti-mouse IgG diluted 1/5000 in PBS/T before addition of substrate. Values that were above the mean plus three standard deviations of the MRL/+ uninfected group were considered positive.

Anti-Sm/anti-RNP Antibody Assay

Plates (Greiner Bio-one) were coated with 2µg/mL solution of calf thymus Sm antigen or RNP antigen (Immunovision, Springdale, AR). Plates were washed and incubated with 100 µl/well individual mouse sera at 1/500 dilution in PBS/T. Plates were washed and incubated with donkey F(ab')2 fragments of anti-mouse IgM or goat F(ab')2 fragments of anti-mouse IgG diluted 1/15,000 in PBS/T before the addition of substrate.

Total IgG/IgG Subclass Assay

Plates were coated with 0.5µg/mL solution of goat anti-mouse IgG+IgM. Plates were washed and blocked for 1 hr. at room temperature with 10% fetal calf serum (Sigma) in PBS/T. Plates were again washed and incubated with individual mouse sera at 1/25,000 dilution in PBS/T. Plates were washed and incubated for 1 hr. at room temperature with goat

F(ab')2 fragments of anti-mouse IgG, goat anti-mouse IgG1 (Southern Biotech, Birmingham, AL) or goat anti-mouse IgG2a (Southern Biothech) diluted 1/10,000 in PBS-0.4% BSA before addition of substrate.

Blood Urea Nitrogen Assay

The blood urea nitrogen assay was purchased as a kit from Sigma and performed according to the manufacturer's instructions.

Proteinuria Assay

Ten microlitre aliquots of urine samples collected prior to sacrifice were plated in duplicate in a Corning 96 well plate (Fisher) with PBS as a negative control. 300 µl of Coomassie Protein Plus Assay reagent (Pierce, Rockford, IL) was added to each well and allowed to develop for 30 seconds. The plate was then read in an ELISA plate reader at 550 nm. Bovine serum albumin (BSA) diluted in PBS (1.5 mg/mL) was serially diluted and plated (10 µl) to serve as a standard curve. Protein concentrations (mg/mL) in the urine samples were extrapolated from the curve and corrected for total volume to yield total protein levels.

Statistical Analysis

Statistical analysis was performed using GraphPad Instat© software. All values reported were obtained by using the Mann-Whitney U test for nonparametric data.

Results

Longevity

In the current study, MRL/*lpr* mice infected with BCG survived for 2 weeks longer than their uninfected counterparts (22 vs. 20 weeks of age), or approximately 10% of their lifespan (Figure 1). However, since the mice were euthanised when 50% mortality within an experimental group was reached, these data do not represent a true measure of longevity. MRL/+ mice were sacrificed at the same time as their MRL/*lpr* counterparts to serve as controls, as these mice do not develop disease until much later in their life (12 months vs. 4 months).

Physical Characteristics

There was no difference in body mass observed between infected and uninfected mice at sacrifice, in either the MRL/*lpr* or MRL/+ groups (Table 2). Interestingly, MRL/*lpr* mice infected with BCG had a lower incidence of necrotising skin lesions than uninfected mice (70% vs. 50%, Table 2); however, this result is not likely significant due to the fact that some mice in the uninfected group began to develop skin lesions before the injection time point. MRL/+ mice did not develop any evidence of skin lesions during the course of this experiment. No evidence of arthritis was observed.

Total Immunoglobulin G (IgG) and IgG Subclass Production

The impact of BCG infection on total antibody titres was assessed by measuring total IgG, IgG1 and IgG2a titres at the time of sacrifice by ELISA (Figure 2). BCG infection induced production of significantly higher levels of IgG, IgG1 and IgG2a antibodies in MRL/*lpr* mice as compared to uninfected MRL/*lpr* mice (p=0.0159, 0.0079 and 0.0317, respectively), and IgG1 and IgG2a antibodies in MRL/+ mice (p=0.0148 and 0.0047).

Autoantibody Production

To ascertain whether the prolonged lifespan of the MRL/*lpr* mice was due to a decrease in pathogenic autoantibody production, several types of autoantibodies associated with lupus-like disease were measured by ELISA.

IgM and IgG Anti-dsDNA Antibodies

Pre-infection (pre-bleed) IgM anti-dsDNA antibody levels were similar between mice of the same genotype in the infected and uninfected groups (Figure 3A). IgM anti-dsDNA antibodies were significantly elevated in MRL/*lpr* mice vs. MRL/+ mice in both the infected and uninfected groups (p=0.0079 and 0.0004, respectively). Two weeks after BCG infection, levels of IgM anti-dsDNA antibodies were similar to those observed prior to infection in the MRL/*lpr* mice. The same was true for mice of the MRL/+ genotype. At four weeks post-infection, IgM anti-dsDNA antibodies were significantly elevated (p=0.05) in the infected MRL/*lpr* group as compared to the uninfected group. Four of six mice in this group

had individual levels of IgM anti-dsDNA antibodies above the cut-off value (mean plus 3 standard deviations above the mean of MRL/+ uninfected pre-bleed sera), compared to one of five mice in the uninfected group. Infected MRL/+ mice also showed a significant increase (p=0.0056) in IgM anti-dsDNA antibodies at this time point as compared to uninfected mice. At six weeks post-infection, MRL/*lpr* and MRL/+ serum levels of IgM anti-dsDNA antibodies returned to levels comparable with pre-infection levels, where most individual mice had levels below the cut-off value (two of six and zero of eight, respectively). Overall, there was a delay in the decrease of IgM anti-dsDNA antibodies over time in BCG infected mice.

IgG anti-dsDNA antibodies were significantly increased (p=0.0022) in the MRL/*lpr* uninfected group when compared with the infected group at two weeks post-infection (Figure 3B). Two of six mice in this group also had individual levels of IgG anti-dsDNA antibodies above the cut-off value, whereas none of the six mice in the infected group had levels above the cut-off. However, IgG anti-dsDNA antibodies were significantly elevated in this group pre-infection (p=0.0016, 1/9 vs. 0/8). At four weeks post-infection, levels of IgG anti-dsDNA antibodies remained above the cut-off value in the uninfected MRL/*lpr* group, whereas MRL/*lpr* infected mice had values below the cut-off (4/6 vs. 2/6). However, this difference was not statistically significant (p=0.4848). At six weeks post-infection, IgG anti-dsDNA antibodies in MRL/*lpr* infected mice remained below the cut-off value (two of six mice), and were comparable to those observed at four weeks post-infection. Therefore, BCG infection of MRL/*lpr* mice caused a

delay in the appearance of high titres of IgG anti-dsDNA antibodies, from 18 weeks to 20 weeks of age.

IgM and IgG Rheumatoid Factors

IgM rheumatoid factor (RF) levels were comparable in pre-infection sera of mice of each genotype in the infected and uninfected groups (Figure 4A). IgM RF levels were significantly elevated in the MRL/*lpr* mice as compared to the MRL/+ mice in both the infected and uninfected groups (p=0.0007 and 0.0001, respectively). At two weeks post-infection, IgM rheumatoid factor levels were not significantly different in the uninfected mice of both genotypes as compared to the infected mice. All six of the uninfected MRL/*lpr* mice had levels over the cut-off (mean + 3 standard deviations of MRL/+ uninfected mice) whereas five of the six infected mice had elevated IgM RF levels at this time point. At four weeks post-infection, IgM RF levels were significantly elevated in infected MRL/+ mice as compared to uninfected MRL/+ mice (p=0.0002), whereas there was no significant difference between infected and uninfected mice of the MRL/*lpr* genotype (4/6 vs. 4/6 above cut-off, respectively). At six weeks post-infection, IgM RF levels in infected MRL/*lpr* mice were increased compared to those at four weeks post-infection (6/6 vs 4/6 mice above cut-off). MRL/+ mice had significantly decreased levels compared to those at four weeks (p=0.0006), and were comparable to the levels observed in uninfected MRL/+ mice at four weeks. BCG infection thus appears to have induced an IgM RF response in MRL/+ mice. A corresponding response

was not observed in infected MRL/*lpr* mice, possibly due to the already elevated levels of IgM RF produced by these mice.

IgG RF levels were also comparable between infected and uninfected mice of the same genotype before infection with BCG (Figure 4B). Interestingly, IgG RF levels in the MRL/*lpr* mice were already above the cut-off value at this early time point (9/9 uninfected and 7/7 infected mice). At two weeks post-infection, infected MRL/+ mice had significantly elevated levels of IgG RF as compared to uninfected MRL/+ mice (p=0.0043), whereas there was no difference in the levels of IgG RF between infected and uninfected MRL/*lpr* mice. At four weeks post-infection, there were no significant differences between infected and uninfected mice of the same genotype; however, IgG RF levels were elevated in MRL/+ mice as compared to MRL/*lpr* mice. At six weeks post-infection, IgG RF levels in infected MRL/+ mice were significantly decreased as compared to both infected and uninfected MRL/+ mice at four weeks post-infection (p=0.0011 and p=0.0207, respectively). IgG RF levels in infected MRL/*lpr* mice were not significantly different from those observed in MRL/*lpr* mice at four weeks post-infection. BCG infection seems to have induced an IgG RF response to occur earlier in MRL/+ mice (18 vs. 20 weeks of age), whereas it had no effect on the IgG RF response in MRL/*lpr* mice.

IgG Anti-Sm and Anti-RNP Antibodies

- Anti-RNP

IgG anti-ribonucleoprotein antigen (anti-RNP) antibodies were similar between mice of the same genotype before BCG infection in the infected and uninfected groups (Figure 5A). MRL/*lpr* mice had significantly elevated levels of IgG anti-RNP antibodies at this time point as compared to MRL/+ mice (p=0.0076 and p=0.0003 for the uninfected and infected groups, respectively). At two weeks post-infection, BCG-infected MRL/*lpr* mice showed a decrease in IgG anti-RNP antibodies, although this difference was not statistically significant (p=0.1797). Uninfected MRL/*lpr* mice had an average IgG anti-RNP antibody level above the cut-off value, whereas the mean for the infected MRL/*lpr* group was below the cut-off. In contrast, BCG-infected MRL/+ mice showed an increase in IgG anti-RNP antibodies, although again this difference did not reach statistical significance (p=0.0931). At four weeks post-infection, IgG anti-RNP antibodies were significantly elevated in uninfected MRL/+ mice as compared to infected MRL/+ mice (p=0.0206). IgG anti-RNP levels in MRL/*lpr* mice were not different (p=0.6282). At six weeks post-infection, infected MRL/*lpr* and MRL/+ mice had IgG anti-RNP levels comparable to those observed at four weeks in those groups. Overall, BCG infection caused a transient decrease in IgG anti-RNP antibodies in MRL/+ mice, and a trend towards decrease in MRL/*lpr* mice.

- Anti-Sm

IgG anti-Smith antigen (anti-Sm) antibodies were similar between mice of the same genotype before BCG injection in the infected and uninfected groups (Figure 5B). At two weeks post-infection, levels of IgG anti-Sm antibodies remained comparable to those observed pre-infection in all mice. At four weeks post-infection, IgG anti-Sm antibodies were significantly elevated in the uninfected MRL/*lpr* mice as compared to their infected counterparts (p=0.0023). A similar effect was observed in uninfected MRL/+ mice as compared to infected MRL/+ mice (p=0.0055). At six weeks post-infection, infected MRL/*lpr* and MRL/+ mice had IgG anti-Sm levels comparable to those observed in uninfected MRL/*lpr* and MRL/+ mice at four weeks, respectively. Thus, BCG infection seems to have had a transient protective effect on the development of IgG anti-Sm antibodies in both MRL/*lpr* and MRL/+ mice.

Epitope spreading from the RNP response to a Sm response is known to occur in the MRL/*lpr* model (Fatenejad et al, 1994). With respect to the mice used in this study, it would appear that epitope spreading took place in the MRL/*lpr* uninfected mice, as the RNP response was elevated prior to the development of the Sm response. In MRL/*lpr* mice infected with BCG, epitope spreading seems to have been decreased. Though the infected mice mounted a significant anti-RNP response pre-infection, that response was decreased at two weeks post-infection and remained decreased throughout the rest of the study. Anti-Sm antibodies also remained below the cut-off in these mice until 6 weeks post-infection.

Thus, BCG infection seems to have induced a delay in the spread of the anti-RNP response to the anti-Sm response in MRL/*lpr* mice.

Kidney Function Analysis: Blood Urea Nitrogen and Proteinuria

Blood urea nitrogen (BUN) levels in sera collected from uninfected MRL/*lpr* mice at 20 weeks were significantly elevated as compared to levels in sera from infected MRL/*lpr* mice, both at 20 and at 22 weeks of age (4 and 6 weeks post-infection) (p=0.0317, Figure 6A). BUN levels were also significantly elevated in uninfected MRL/*lpr* mice as compared to uninfected MRL/+ mice at 20 weeks (p=0.0010), whereas infected MRL/*lpr* mice did not have BUN levels that were significantly different from control infected MRL/+ mice at 20 or at 22 weeks (p=0.8857 and p=0.2141). Also, uninfected MRL/*lpr* mice (n=5) all had BUN values over or approaching the cut-off value of 20 mg/dL (mean plus 2 standard deviations of the uninfected MRL/+ mice), whereas all other mice tested had values below or slightly above 20 mg/dL. Therefore, BCG infection seems to have had a significant protective effect on the development of elevated BUN levels, and by extension kidney dysfunction, in MRL/*lpr* mice.

Median total protein levels in the urine of MRL/*lpr* and MRL/+ mice at the end of their life spans (22 weeks for infected mice and 20 weeks for uninfected mice) were not significantly different (p=0.4857, Figure 6B). Total protein in urine samples taken from infected MRL/*lpr* mice at 20 weeks of age was also not different from the uninfected MRL/*lpr* mice

(p=0.6857). MRL/*lpr* mice in both the infected and uninfected groups had total protein levels that were significantly elevated as compared to their respective control MRL/+ mice (p=0.0095 and p=0.0286, respectively), but there was no significant difference between infected and uninfected MRL/+ mice. Overall, MRL/*lpr* mice had elevated urinary protein as compared to mice of the MRL/+ genotype, indicating more advanced kidney damage in the MRL/*lpr* mice. However, the protective effect of BCG infection on kidney dysfunction in MRL/*lpr* mice was not observed using this assay. Possible explanations for this discrepancy include: assay sensitivity (BUN is a more sensitive measure of kidney damage), small sample size and population heterogeneity among the MRL/*lpr* mice.

Discussion

This study was undertaken in order to confirm preliminary results indicating that BCG infection had an ameliorative effect on symptoms of autoimmune disease in aged MRL/*lpr* mice, and to ascertain what, if any, effect this improvement would have on the survival of these mice. The survival curve generated by Theofilopoulos (1992) in his initial studies characterizing the MRL/*lpr* and MRL/+ strains indicates 50% mortality by 20 weeks, or five months of age, for the MRL/*lpr* strain. This corresponds well with the survival curve obtained for the uninfected MRL/*lpr* mice in this experiment. BCG-infected MRL/*lpr* mice reached 50% mortality at 22 weeks of age, showing a slight improvement in life expectancy. MRL/+ mice, whether infected or uninfected, had no mortality during the course of this experiment.

No significant differences were observed between infected and uninfected MRL/*lpr* mice in total body mass, and the values obtained in this experiment again correlate well with the average body mass observed at five months by Theofilopoulos (48 g for MRL/*lpr* and 43 g for MRL/+).

MRL/*lpr* mice develop a spontaneous arthritis resembling human rheumatoid arthritis, including the induction of a rheumatoid factor response, and have been used as a model of this disease. Initially, the rate of occurrence of arthritis in MRL/*lpr* mice was observed to be from 20-25% of old, sick mice (Theofilopoulos, 1992) to 75% of mice by 5-6 months of age (Hang et al, 1982). More recently, the incidence of

spontaneous arthritis has been reported to be approximately 30% (Kamogawa et al, 2002) to 12% (Ratkay et al, 1993). Injection of *Staphylococcus* bacterial enterotoxin or Complete Freund's adjuvant, which contains heat-killed mycobacteria (Edwards et al, 1993; Ratkay et al, 1993), can reliably enhance the development of arthritis in this strain of mice, and thus cleaner animal colonies may account for the lower rates of arthritis observed by some groups. In this context, it is interesting that none of the BCG-infected mice, or any of the uninfected mice, developed arthritis, but given the low incidence this may only reflect small sample size. Also, we cannot rule out the possibility that arthritis would have occurred if the mice had survived longer.

The development of skin lesions in MRL/*lpr* mice is also well-documented. Small vessel vasculitis leading to skin lesion formation has been associated with the formation of cryoglobulins, particularly those containing IgG3 subclass antibodies, including rheumatoid factors, and injection of monoclonal or polyclonal IgG3 induced formation of lesions in non-autoimmune mice (Theofilopoulos, 1992). Skin lesion formation in MRL/+ mice has also been associated with exposure to ultraviolet light (Furukawa, 2003). Daily injection of recombinant IL-18 led to enhanced vasculitis and formation of a facial rash in MRL/*lpr* mice, whereas injection of IL-12 and IL-18, although enhancing vasculitis even further, did not lead to facial rash formation (Esfandiari et al, 2001). Although a difference in skin lesion formation was observed between infected and uninfected MRL/*lpr* mice in this study, it is not possible to attribute any significance to

this finding, due to the appearance of skin lesions before the infection time point in both groups of mice.

BCG infection induced a significant elevation in the overall production of antibodies of IgG, IgG1 and IgG2a isotypes in MRL/*lpr* mice, and IgG1 and IgG2a in MRL/+ mice. The overall increase in IgG in MRL/*lpr* mice is not surprising, as the mice must have mounted an immune response to the infection, but the increase in IgG1 antibodies, which are induced by IL-4 stimulation of B cells, is unexpected given the strong Th1 cytokine profile traditionally associated with BCG infection. MRL/+ mice did not mount a significantly higher IgG response to BCG infection, but the lack of significance may be attributable to assay sensitivity, as the IgG1 and IgG2a titres are both significantly elevated in infected mice of this genotype.

Anti-dsDNA autoantibodies of the IgG isotype have been shown to be directly involved in the development of lupus nephritis in both mouse models and human patients (Cortes-Hernandez et al, 2004). Significant IgG and C3 deposits can be observed by immunofluorescence in the glomeruli of MRL/*lpr* mice starting at 2 months of age. Renal eluates from MRL/*lpr* mice show a six fold increase of anti-dsDNA antibodies in the kidney over serum concentrations of anti-dsDNA antibodies (Theofilopoulos, 1992). Renal IgG from these mice is predominantly of the IgG2a or IgG2b isotype (Theofilopoulos, 1992), and production of anti-dsDNA antibodies of those isotypes correlates with observed kidney pathology (Peng et al, 1997). IgG2a antibodies are produced by B cells

stimulated by IFN-γ producing Th1 T cells to undergo isotype switching through the activation of the transcription factor T-bet (Peng et al, 2002); Th2 cells that produce IL-4 induce switching to IgG1 or IgE isotypes through the activation of the STAT6 protein (Singh et al, 2003). Thus, it may be that the delay in the IgG anti-dsDNA response observed in this study is due to an alteration in the cytokine profile of the mice caused by BCG infection.

IgM rheumatoid factors are induced not only in autoimmune disease situations, but in response to bacterial infections, where they aid in the opsonization and clearance of bacterial pathogens (Newkirk, 2002). The transient IgM RF response observed in the infected MRL/+ mice is most likely of this second type, despite its late appearance, as these mice have significant numbers of BCG in their spleens at three weeks post-infection (unpublished observations, Newkirk MM and Radzioch D). As the IgM RF response was induced in the MRL/*lpr* mice prior to infection, there would be no need for them to upregulate IgM RF in response to bacterial challenge. In fact, the early presence of high titres of IgM RF may contribute to the observed improved clearance of bacteria in the MRL/*lpr* mice as compared to MRL/+ mice (unpublished observation, Newkirk MM and Radzioch D).

The early elevation of IgG RF in infected MRL/+ mice may also be attributable to an anti-bacterial response. However, uninfected MRL/+ mice also showed a significant induction of IgG RF, albeit two weeks later than the infected mice of the same genotype. This difference was not

apparent in the MRL/*lpr* strain. The induction of cytokines by the BCG infection may have caused RF+ B cells to class switch to an IgG response earlier than is usual; no data could be found on exactly when these mice normally start to produce IgG RF, as most studies tend to focus on IgM RF. In terms of disease pathogenesis, IgG RF in MRL/*lpr* mice tends to be of the IgG1 or IgG3 isotype, and bind to IgG2a antibodies (Bond et al, 1988). Therefore, they have a high potential to contribute to cryoglobulin formation, which have been shown to contribute to skin manifestations and kidney dysfunction in these mice (Theofilopoulos, 1992). Perhaps the early elevated IgG RF response in the MRL/+ mice would have contributed to early disease manifestations in those mice, had they been followed for a longer period of time.

The pathogenic significance of anti-Sm and anti-RNP autoantibodies is unclear. Although high titres of anti-Sm antibodies are diagnostic of SLE in human patients, their involvement in pathologic manifestations of disease such as nephritis or facial rash is controversial. In the MRL/*lpr* mouse model, anti-Sm and anti-RNP antibodies are mainly of the IgG2a isotype, which suggests an association with renal disease (Theofilopoulos, 1992), as antibodies of the IgG2a subclass are most prominent among the IgG eluted from the kidney; however, no studies could be found that directly examine the existence of these antibodies in the kidney. The anti-Sm response in MRL/*lpr* mice is concurrent with the observed response in MRL/+ mice (4-5 months of age), which argues against a direct role in pathogenesis, since MRL/+ mice do not develop

disease until 12 months (Theofilopoulos, 1992). As previously mentioned with respect to epitope spreading, Fatenejad et al (1994) found that the anti-RNP response appears before the anti-Sm response; specifically, antibodies were found to bind to the A and D proteins of the U1-small nuclear RNP before binding the Sm-B'/B and Sm-D proteins of the same complex. This pattern is reflected in our study, where the uninfected MRL/*lpr* mice developed an anti-RNP response before the appearance of the anti-Sm response. The appearance of the anti-Sm response was delayed in the MRL/*lpr* mice infected with BCG; this delay could be because of multiple factors. One possibility is that, as with the anti-dsDNA response, alteration in the cytokine profile may play a role – anti-Sm B cells, which are relatively rare (Theofilopoulos, 1992), may not be receiving adequate Th1 T cell stimulation if T cell traffic is directed elsewhere to combat the BCG infection. The same situation could be affecting the MRL/+ mice.

Lupus nephritis is one of the most severe disease manifestations of SLE, and is modeled well by MRL/*lpr* mice; most of the high mortality observed in this strain is due to kidney failure. As mentioned above, lupus nephritis is correlated with the development of autoantibodies, especially IgG anti-dsDNA antibodies, in both human patients and mouse models of SLE. The decrease in IgG anti-dsDNA antibodies observed in this study is thus interesting in light of the observed impact of BCG infection on kidney function in MRL/*lpr* mice. Although the blood urea nitrogen assay, which measures accumulation of urea in the blood, indicated protection from

kidney disease, the level of protein in the urine did not. Besides the technical reasons for this discrepancy already discussed, this may reflect the transient nature of the protective effect, as observed in the autoantibody kinetics – autoantibody production was often delayed, but never stopped entirely. Thus, the improvement on kidney function may also be transient, and reflected only in one type of assay for kidney function. MRL/*lpr* mice develop high urinary protein as early as 3 months of age (Theofilopoulos and this study), but do not immediately succumb to renal failure; elevated BUN seems to be a more accurate predictor of incipient kidney failure.

Due to the importance of the humoral response in SLE, it is easy to assign high importance to Th2 cytokines in the pathogenesis of this disease. However, Th1 cytokines such as IL-12, IL-18, IFN-γ and TNF-α have all been shown to play a role in disease progression, especially in end-organ disease manifestations (Theofilopoulos and Lawson, 1999); for instance, knocking out both IFN-γ and IL-4 has beneficial effects in the MRL/*lpr* mouse model. Thus, lupus appears to be a complicated disease that does not conform to either a Th1 or Th2 paradigm. It may be that different cytokines are required at different times during disease initiation and progression, or that complex interactions involving multiple cytokines and chemokines are involved in disease pathogenesis. Recent work that provides support for this latter concept is the discovery that BCG infection in mice with experimental autoimmune encephalomyelitis (EAE, the mouse model of multiple sclerosis) exerts a protective effect on this

disease by diverting T cell traffic away from the central nervous system, the organ system most affected by this disease (Sewell et al, 2003). Thus, induction of some as yet undetermined chemokine(s) is most likely the protective factor in this situation. Other studies using BCG or mycobacteria in the context of other autoimmune diseases such as diabetes have suggested a role for regulatory T cells in the observed protection from disease (Qin et al, 1998; Martins and Agua, 1999); interestingly, some NOD mice protected from diabetes by BCG later developed a lupus-like syndrome, highlighting the complex interplay of factors involved in the development of autoimmune disease (Baxter et al, 1994). Recently, infection of NZW/NZB F1 lupus-prone mice with *Toxoplasma gondii* at disease onset was shown to have an ameliorative effect on nephritis and longevity through effects on both IL-10 and IFN-γ (Chen et al, 2004).

In this study, we failed to see a long-term protective effect on longevity and disease manifestations in the MRL/*lpr* mice. Perhaps the bacterial infection was cleared too rapidly to exert any lasting effect on the overall cytokine expression in the mice, or perhaps the critical time point at which the mice could be diverted from their disease course had passed before infection (some mice did fall ill and were euthanised before the infection time point). Although IFN-γ expression in the spleen and liver of infected and uninfected mice was compared by Northern blot analysis (data not shown), no differences were found; indeed, IFN-γ expression was undetectable in most of the samples. In light of the observed changes in autoantibody levels, it would probably be more informative to examine

tissue cytokine expression at earlier time points during expression, and not at sacrifice, as was performed.

A few points of interest remain to be addressed. The isotype of the IgG anti-dsDNA, RF anti-Sm and anti-RNP responses should be determined, to gain insight into which cytokines were most prevalent at the time of their induction. Although we attempted to develop an ELISA assay that would measure the IgG isotype response to BCG antigens, this proved to be difficult due to the high prevalence of low-affinity polyreactive antibodies in MRL/*lpr* sera. This assay would be instrumental in determining which specificities are responsible for the observed overall increase in IgG1 and IgG2a antibodies in sera of MRL/*lpr* mice. The cytokine profile in the tissues should also be further examined. Analysis of cytokines other than IFN-γ, such as IL-4, IL-10, IL-12, and others, should be performed, and samples from mice euthanised at earlier time points should be examined. Finally, studies investigating the long-term kinetics of BCG clearance in MRL/*lpr* and MRL/+ mice could provide valuable insights into host-pathogen interactions in an autoimmune context.

In summary, BCG infection of MRL/*lpr* mice caused a slight increase in longevity accompanied by protection from kidney failure. In terms of autoantibody production, there was a delay in the class-switch from IgM to IgG anti-dsDNA antibodies, and also in epitope spreading from anti-RNP to anti-Sm antibodies. BCG infection also induced both IgM and IgG RF in the MRL/+ mice. Studies such as these highlight the

important, often complex interactions between the host immune system and pathogenic organisms.

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Figure 1.

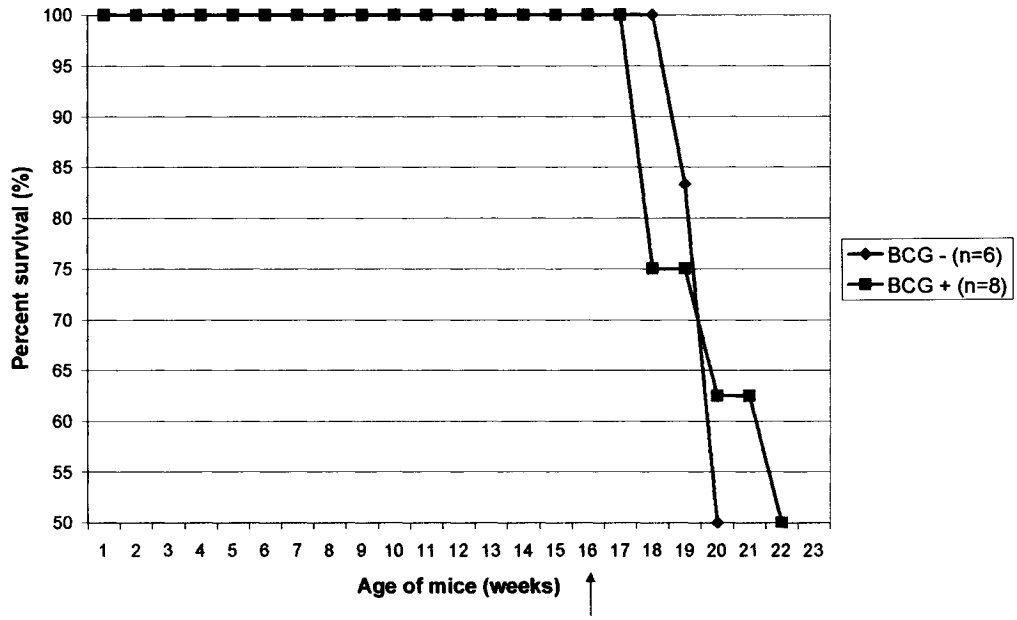


Figure 1. Percent survival of BCG-infected and uninfected MRL/*lpr* mice over the course of the experiment. Arrow indicates the time of infection.

Table 2. Physical characteristics of MRL/+ and MRL/*lpr* mice at 20 and 22 weeks of age (mean ± S.D.) Mice were infected with BCG at 16 weeks of age (n.d.= no data).

	MRL/+		MRL/lpr	
	BCG + (n=9)	**BCG - (n=9)**	**BCG + (n=8)**	**BCG - (n=10)**
Weight (g)	48.35 ± 4.80	45.01 ± 3.82	41.9 ± 5.18	41.29 ± 4.39
Skin Lesions	0/9 (0%)	0/9 (0%)	4/8 (50%)	7/10 (70%)
Arthritis	0/9 (0%)	n.d.	0/8 (0%)	n.d.

Figure 2A.

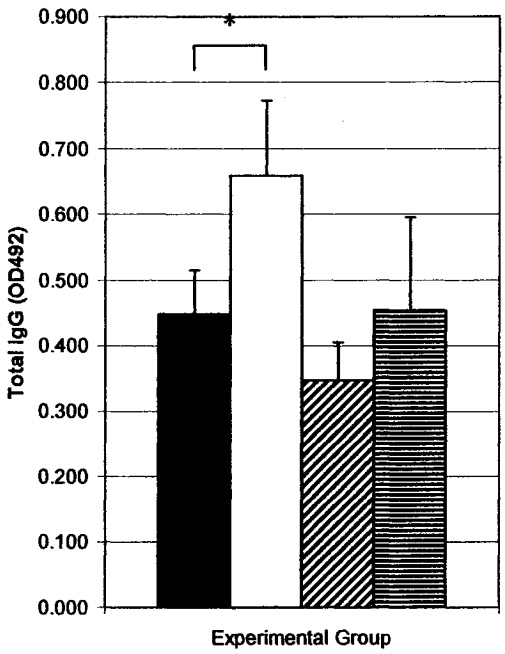
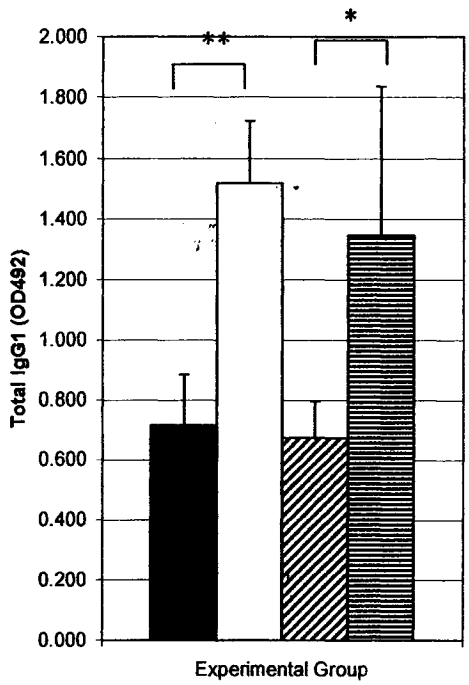


Figure 2B.



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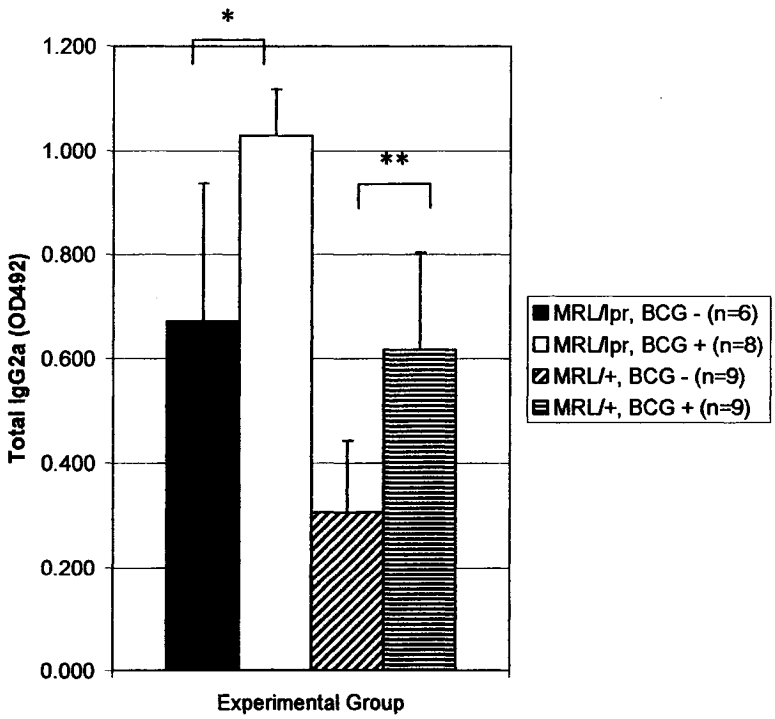

Figure 2. Total IgG (A), IgG1 (B) and IgG2a (C) in MRL/*lpr* and MRL/+ mouse sera at 22 (infected) and 20 (uninfected) weeks of age. Mice were infected with $1.0x10^5$ c.f.u. BCG at 16-18 weeks of age. Total antibody levels were measured by ELISA and are expressed as the mean OD 492 + S.D..

*: $p < 0.05$; **: $p < 0.01$ (Mann Whitney)

Figure 3A.

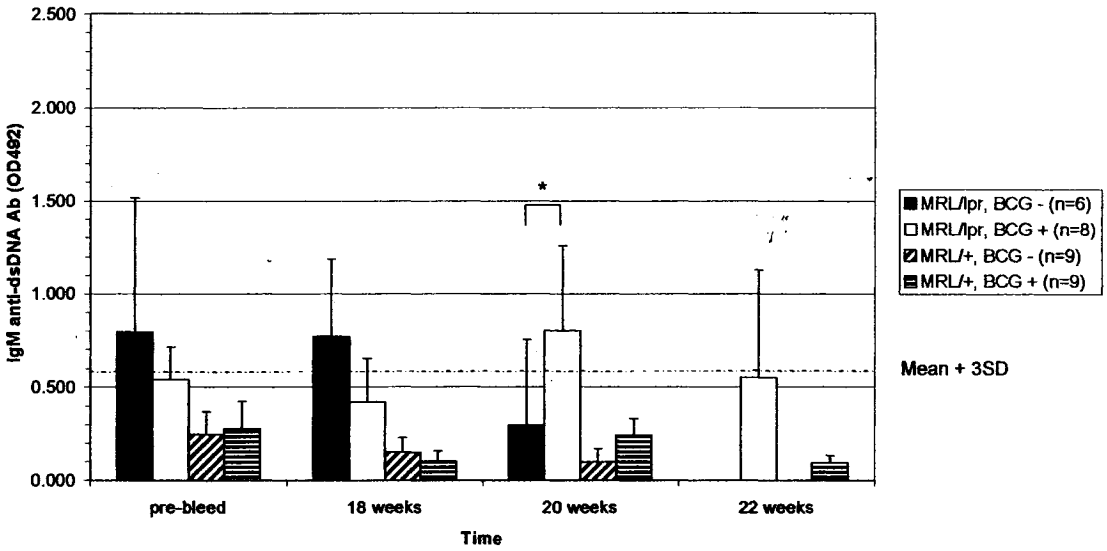

Figure 3B.

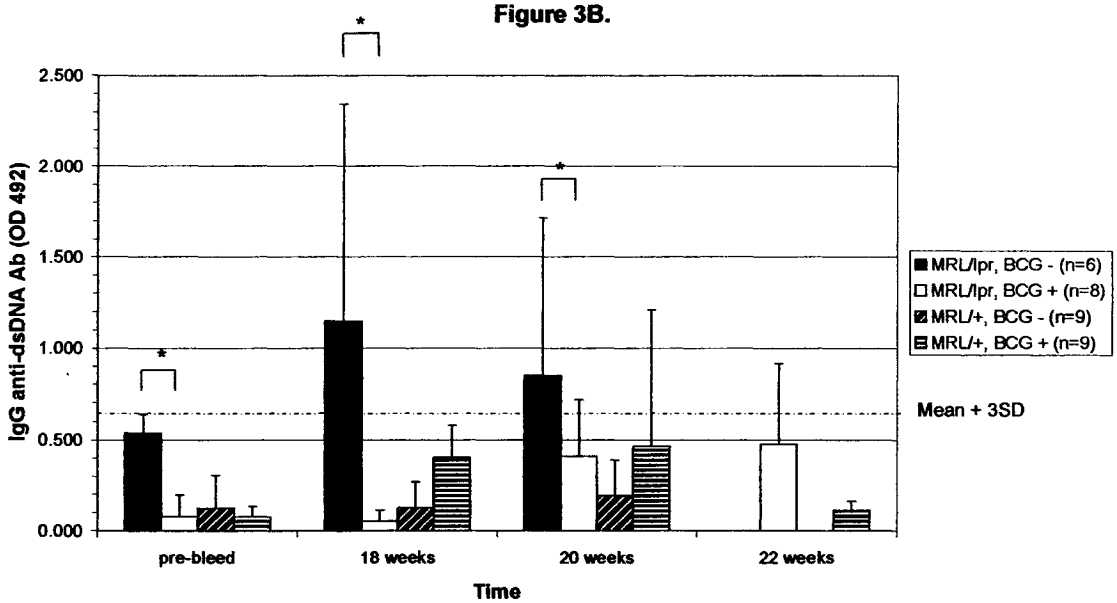

Figure 3. Changes over time in IgM (A) and IgG (B) anti-dsDNA antibody levels in serum from MRL/*lpr* and MRL/+ mice. Mice were left uninfected or infected with $1.0x10^5$ c.f.u. BCG at 16-18 weeks of age, and anti-dsDNA antibodies were measured by ELISA. Data are expressed as mean OD 492 + S.D. All values above the mean plus 3 standard deviations of the MRL/+ uninfected group were considered positive.

*: $p \leq 0.05$ (Mann-Whitney)

Figure 4A.

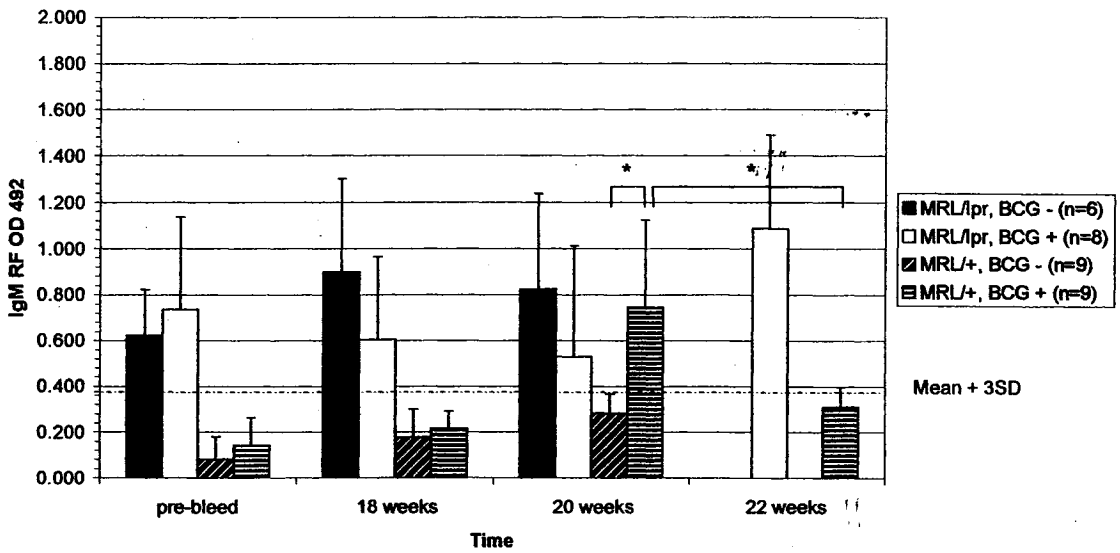

Figure 4B.

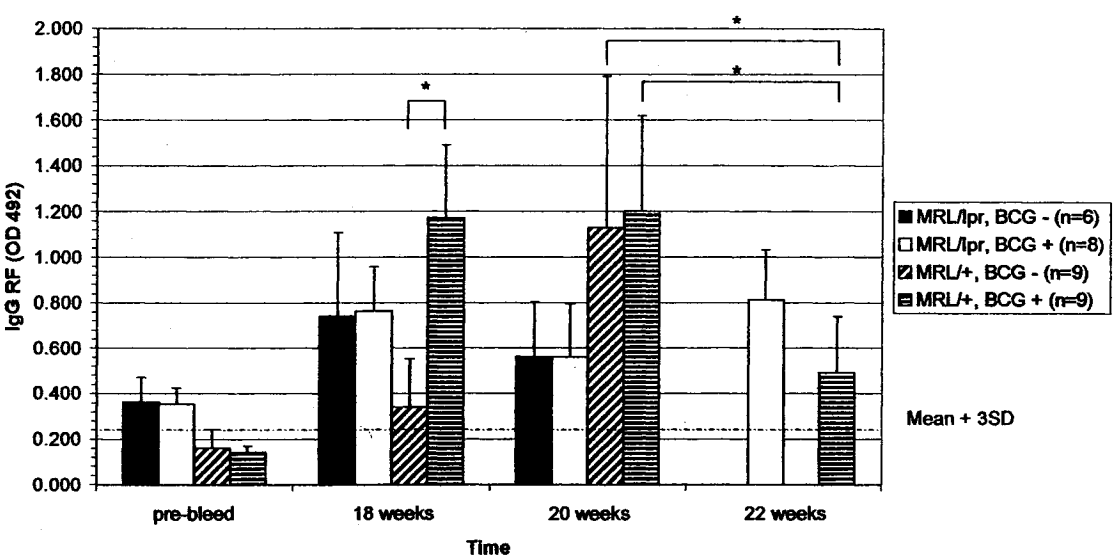

Figure 4. Changes over time in IgM (A) and IgG (B) rheumatoid factor levels in serum from MRL/*lpr* and MRL/+ mice. Mice were left uninfected or infected with $1.0x10^5$ c.f.u. BCG at 16-18 weeks of age, and rheumatoid factors were measured by ELISA. Data are expressed as mean OD 492 + S.D. All values above the mean plus 3 standard deviations of the MRL/+ uninfected group were considered positive.

*: $p < 0.05$ (Mann-Whitney)

Figure 5A.

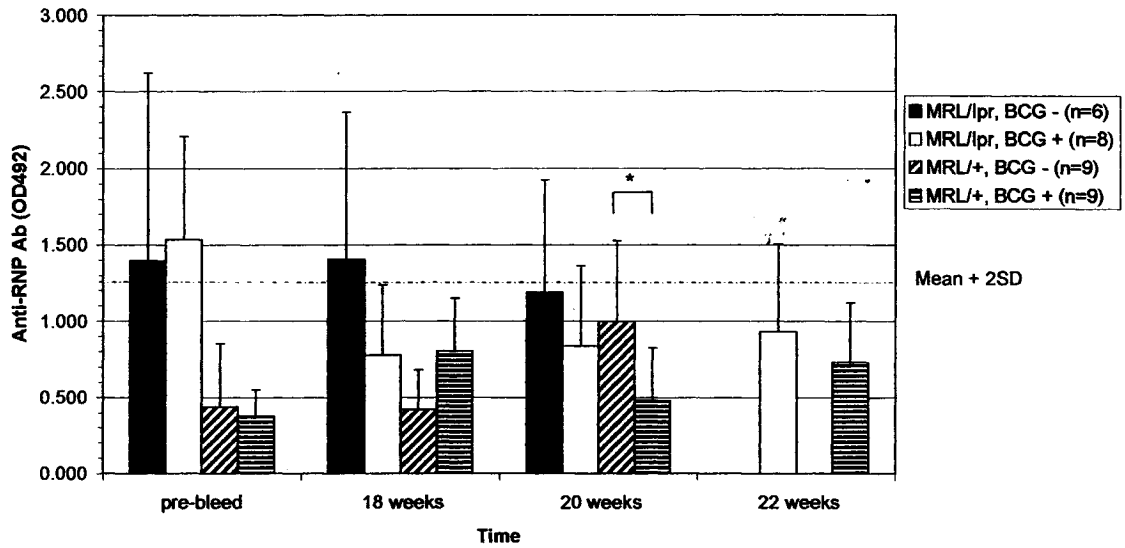

Figure 5B.

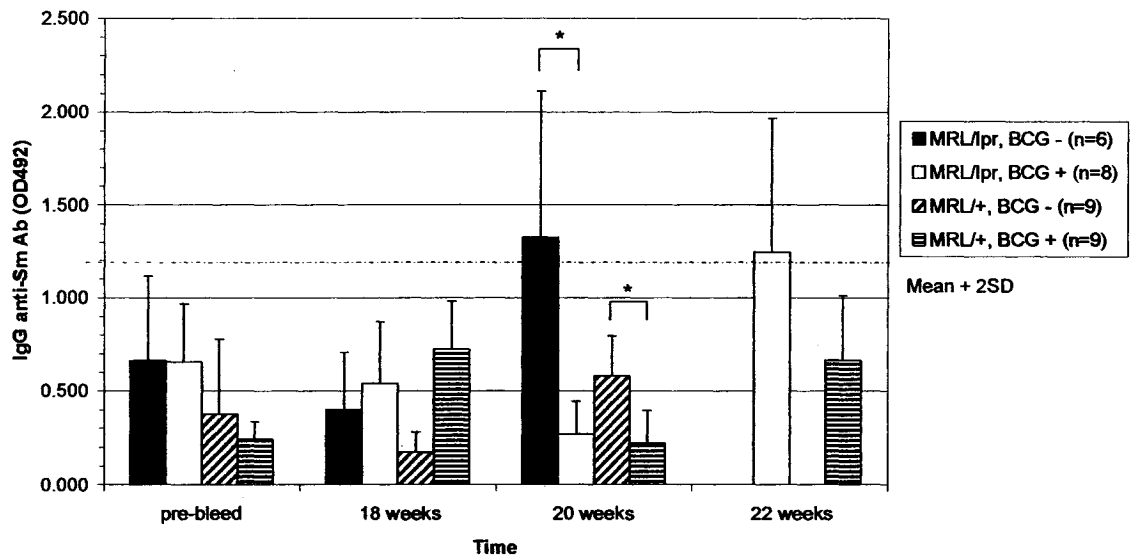

Figure 5. Changes over time in IgG anti-RNP (A) and anti-Sm (B) antibody levels in serum from MRL/*lpr* and MRL/+ mice. Mice were left uninfected or infected with $1.0x10^5$ c.f.u. BCG at 16-18 weeks of age, and anti-dsDNA antibodies were measured by ELISA. Data are expressed as mean OD 492 + S.D. All values above the mean plus 2 standard deviations of the MRL/+ uninfected group were considered positive.

*: $p < 0.05$ (Mann-Whitney)

Figure 6A.

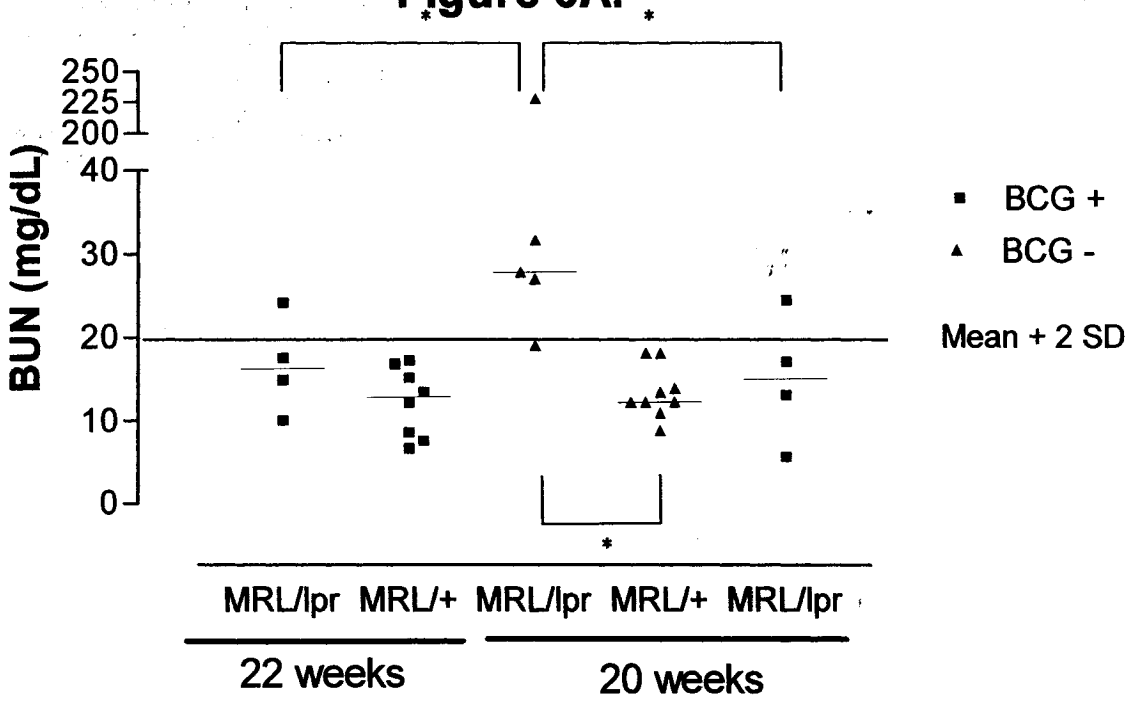

Figure 6B.

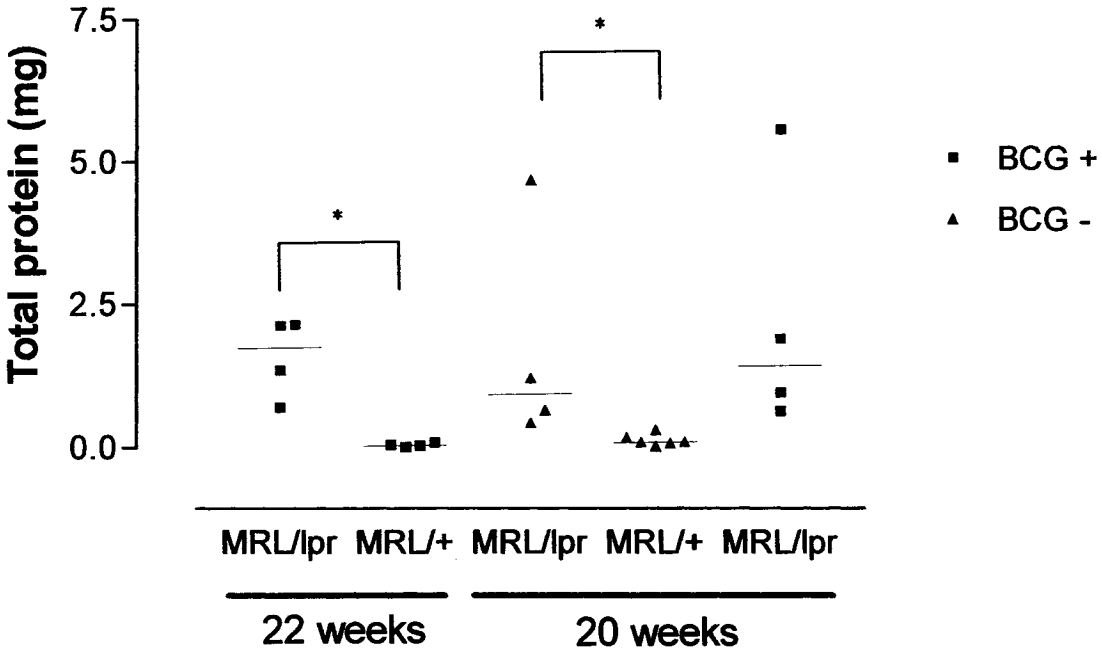

Figure 6. Kidney function analysis of MRL/*lpr* and MRL/+ mice at 20 and 22 weeks of age. Blood urea nitrogen (BUN) (A) and total urinary protein (B) levels were obtained from serum and urine samples, respectively, as described in Materials and Methods. Mice were left uninfected or infected with $1.0x10^5$ c.f.u. BCG at 16-18 weeks of age. Bars indicate the median value for each experimental group. In Figure 6A, all values above the mean plus 2 standard deviations (20mg/dL) of the MRL/+ uninfected group were considered positive.

*: $p < 0.05$ (Mann-Whitney)