Aus der Medizinischen Klinik und Poliklinik IV
der Ludwig-Maximilians-Universität München
Direktor: Prof. Dr. med. Martin Reincke

Immunoregulatory Proteases in Systemic Lupus Erythematosus and Lupus Nephritis

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

Vorgelegt von

Khader Valli Rupanagudi
aus
Proddatur, India
2013

Mit Genehmigung der Medizinischen Fakultät
der Universität München

1. Berichterstatter:	Prof. Dr. med. Hans-Joachim Anders
1. Mitberichterstatter:	Prof. Dr. Dr. Christian Sommerhoff
2. Mitberichterstatter:	Priv. Doz. Dr. Bianca Schaub
3. Mitberichterstatter:	Prof. Dr. jörg Christoph Prinz
Mitbetreuung durch den Promovierten Mitarbeiter:	Dr. med. Julia Lichtnekert
Dekan:	Prof. Dr. med. Dr. h.c. M. Reiser, FACR, FRCR

Tag der mündlichen Prüfung: 12.11.2013

Dedicated to

My wife Mrs. Sunitha Rupanagudi
My sons - Mahesh Rupanagudi &
Krishna Teja Rupanagudi

Khader Valli Rupanagudi M. Sc.
Medizinische Klinik und Poliklinik IV,
Klinikum der Universität Muenchen,
Nephrologisches Zentrum,
Arbeitsgruppe Klinische Biochemie,
Schillerstraße-42, Munich-80336, Germany.
Tel. 0049/89/2180-75857
khaderbio@gmail.com

DECLARATION

I hereby declare that the present work embodied in this thesis was carried out by me under the supervision of Prof. Hans Joachim Anders, Medizinische Klinik und Poliklinik IV- Innenstadt, Klinikum der Universität München. This work has not been submitted in part or full to any other university or institute for any degree or diploma.

Publications

Julia Lichtnekert, Khader Valli Rupanagudi, Onkar P. Kulkarni, Murthy Narayana Darisipudi, Ramanjaneyulu Allam and Hans-Joachim Anders. Activated Protein C Attenuates Systemic Lupus Erythematosus and Lupus Nephritis in MRL-Fas(lpr) Mice. The Journal of Immunology, 2011 Sep 15; 187(6):3413-21.

Khader Valli Rupanagudi, Onkar P. Kulkarni, Julia Lichtnekert, Murthy Narayana Darisipudi, Shrikant R. Mulay, Brigitte Schott, Sabine Gruner, Wolfgang Haap, Guido Hartmann and Hans-Joachim Anders. Cathepsin S inhibition abrogates immune complex glomerulonephritis because cathepsin S is essential for MHC class II-mediated CD4 T cell and B cell priming. (submitted)

Poster presentation

Julia Lichtnekert, Khader Valli Rupanagudi, Onkar Kulkarni and Hans J. Anders. Activated Protein C Attenuates Systemic Lupus Erythematosus and Lupus Nephritis in MRL-Fas(lpr) Mice - Presented at ASN Kidney Week 2011, November 8 - 13, 2011, Philadelphia, USA.

Khader Valli Rupanagudi, Onkar P. Kulkarni, Julia Lichtnekert, Murthy Narayana Darisipudi, Shrikant R. Mulay, Brigitte Schott, Sabine Gruner, Wolfgang Haap, Guido Hartmann and Hans-Joachim Anders. Cathepsin S inhibition abrogates immune complex glomerulonephritis because cathepsin S is essential for MHC class II-mediated CD4 T cell and B cell priming. (Submitted to ERA-EDTA 50^{TH} Congress, which will be held in May 2013, Istanbul, Turkey)

Part of the work was done by collaborators, as mentioned below:

Dr. Guido Hartmann and Dr. Brigitte Schott, Roche Pharmaceuticals, Basel, Switzerland.
They have provided in-situ hybridization images, in-vitro enzyme inhibition assay data, plasma levels of R05461111 data, as well as R05461111 dose dependent p10 accumulation data. These data are presented in the results part II (table 7, figure 21 and 23) of this thesis.

Khader Valli Rupanagudi.

Date: 5-3-2013

Acknowledgements

First and foremost, I am deeply grateful to my advisor Prof. Hans-Joachim Anders who has guided me in a constructive manner by providing crucial inputs and encouraging ideas, in making my thesis. I am thankful for his support, both financial and academic, contribution of time, in making my Ph.D. experience more productive and outstanding. The joy and enthusiasm he has for research is contagious and motivated me to pursue my research work with a similar attitude. His ability to select and approach compelling research problems, high scientific standards, and hard work set an example in my research work.

I would like to express my heartfelt thanks to Dr. Julia Lichtnekert, for providing me the platform to do research in this laboratory as a Ph.D. fellow. To work with her has been a real pleasure to me.

My special thanks to Dr. Ramanjaneyulu Allam for being my friend for so many years, without whose support I would not have achieved my dream. My sincere thanks to Dr. Onkar Kulkarni for his help and support.

I wish to thank all the staff members and colleagues of the department of Klinische Biochemie for their help, cooperation and for providing a friendly environment. I would like to thank Dr. Volker Vielhauer, Dr. Bruno Luckow and Dr. Peter Nelson for their constant encouragement and suggestions. My special thanks to Katharina for her help. I would like to acknowledge the skillful technical assistance provided by Dan Draganovici and Janina Mandelbaum.

I have been surrounded by wonderful colleagues, and I would like to thank them, who have been extremely supportive whilst pursuing my PhD studies. I would like to thank my past and current colleagues of my lab - Narayana Murthy Darisipudi, Shrikant Mulay, Santosh Kumar, Kirstin Andersen, Nuru Eltrich, Maciej Lech, Sufyan Sayyed, Adriana Migliorini, Simone Romoli, Mi Ryu, Heni Eka Susanti, Dana Thomasova, Ewa Radomska, Stephanie Käser, Anne Kuhn, Christina Scherbaum, Jan Henrik Hagemann, Georg Lorenz, Marc Weidenbusch, Martin Bernhard Müller, Ingo Hartter, Andrei Bideak, Hauke Bruns, Roman Günthner, John Hoppe for their valuable help, support and friendly attitude.

This thesis would not be possible without the love and support of my family. I would like to thank my wife Mrs Sunitha Rupanagudi for her love and encouragement. Many thanks to my mother, brother and sister for their love and support. My heartfelt thanks to all of them.

I am indebted to many people for making the time working on my Ph.D. an unforgettable experience. My time in Munich was made enjoyable in large part due to the many friends Sudhakar Reddy, Naresh, Vignesh, Vidhya Sankar, Harish, Satish and Praneeth. I am grateful to my friends who spent valuable time with me during my memorable trips in and around Germany. I would like take this opportunity to thank my friends Raghavan Vallur, Shahul Hameed, Vivek Sharma and Praseeda for their support in providing scientific papers to write my thesis.

I am grateful to everybody who has been part of my life and helped in some way or the other, may I failed to mention any of their names, I still thank each and every one of them. It is my duty to express my tearful acknowledgement to the animals, which have been sacrificed for the betterment of human being.

And last, but not the least, I am grateful to Prof. Vijayalakshmi Ravindranath for rekindling my dreams. I thank her from the bottom of my heart for her support and encouragement.

Khader Valli Rupanagudi, 2013

Table of contents

1. Introduction

1.1 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease that is characterized by the loss of tolerance to nuclear self-antigens, the production of pathogenic autoantibodies and damage to multiple organ systems. SLE was first described by the French dermatologist Beitt in 1828[1]. Lupus is characterized by flares and remissions and affects at least 5 million people worldwide. The disease can affect all ages but most commonly between 20-45 years of age. Statistics demonstrate that lupus is somewhat more frequent in African Americans and people of Chinese and Japanese descent. People with SLE can develop different combinations of symptoms and organ involvement. The clinical manifestations of SLE include autoimmune inflammation of the skin, joints, kidney, lungs, heart, blood vessels, nervous system, intestine, liver, etc. Common symptoms include fatigue, low-grade fever, headache, anemia, loss of appetite, muscle aches, hair loss (alopecia), arthritis, ulcers of the mouth and nose, facial rash ("butterfly rash"), unusual sensitivity to sunlight (photosensitivity), inflammation of the lining that surrounds the lungs (pleuritis) and the heart (pericarditis) and poor circulation to the fingers and toes with cold exposure (Raynaud's phenomenon). It is more common in women. The female to male ratio in adults is approximately 9:1. The impact of SLE on pregnancies can be severe. A meta-analysis calculated that 16% of pregnancies in women with SLE resulted in spontaneous abortion and 6.1% resulted in stillbirth or neonatal death[2].

One of the most challenging issues for people with SLE is receiving a timely and accurate diagnosis. Misdiagnosis is common, and there can be a 2-year delay between the onset of symptoms and a diagnosis of SLE. Since individuals with SLE can have a wide variety of symptoms and different combinations of organ involvement, no single test establishes the diagnosis of systemic lupus. The American Rheumatism Association established 11 classification criteria, which are closely related to the symptoms discussed above (Table 1). When a person has four or more of these criteria, the diagnosis of SLE is strongly suggested. Until March 2011, the only drugs that were approved by the Food and Drug Administration (FDA) for the treatment of lupus were corticosteroids (1955), hydroxychloroquine (1955) and aspirin (1948). The recent breakthrough in the treatment

Table 1. American college of Rheumatology criteria for the diagnosis of systemic lupus erythematosus

Criterion	Definition
Malar rash	A rash on the cheeks and nose, often in the shape of a butterfly
Discoid rash	A rash that appears as red, raised, disk-shaped patches
Photosensitivity	A reaction to sunlight that causes a rash to appear or get worse
Oral ulcers	Sores in the mouth
Arthritis	Joint pain and swelling of two or more joints
Serositis	Inflammation of the lining around the lungs (pleuritis) or inflammation of the lining around the heart that causes chest pain, which is worse with deep breathing (pericarditis)
Kidney disorder	Persistent protein or cellular casts in the urine
Neurological disorder	Seizures or psychosis
Blood disorder	Anemia (low red cell count), leukopenia (low white cell count), lymphopenia (low level of specific white cells), or thrombocytopenia (low platelet count)
Immunologic disorder	Positive test for anti–double stranded DNA, anti-Sm, or antiphospholipid antibodies
Abnormal antinuclear Antibodies	Positive antinuclear-antibody test

Four of the 11 criteria are needed for the formal diagnosis of SLE.
Adapted from Rahman *et al.*[3]

of lupus was the approval of belimumab by the FDA in 2011. Current therapeutic regimen for SLE mainly comprised of corticosteroids, antimalarials, nonsteroidal anti-inflammatory drugs (NSAIDS) and immune-suppressive drugs[4]. In milder, non-organ threatening disease, antimalarials, NSAIDS and lower dose steroids are usually effective. For an organ threatening disease that is not responding to steroids, immunosuppressants are initiated. Survival of patients with SLE has improved substantially in the past 5 decades as a result of earlier disease detection and advances in the treatment. Five-year survival in patients with SLE has improved from approximately 50% in the 1950s to >90% in the 2000s[5]. However, overall survival of patients with SLE has not improved much since the 1980s; the mortality still remains high compared with the general population. The most common causes of death in SLE include, SLE induced complications and immunosuppressant induced infections. Management of refractory disease, especially nephritis, cutaneous and neuropsychiatric manifestations, remains unsatisfactory.

Pathogenesis of SLE

The major immunological features of lupus are, loss of self-tolerance to autoantigens, the presence of autoreactive B and T cells, with polyclonal activation of B-cells, the conse-

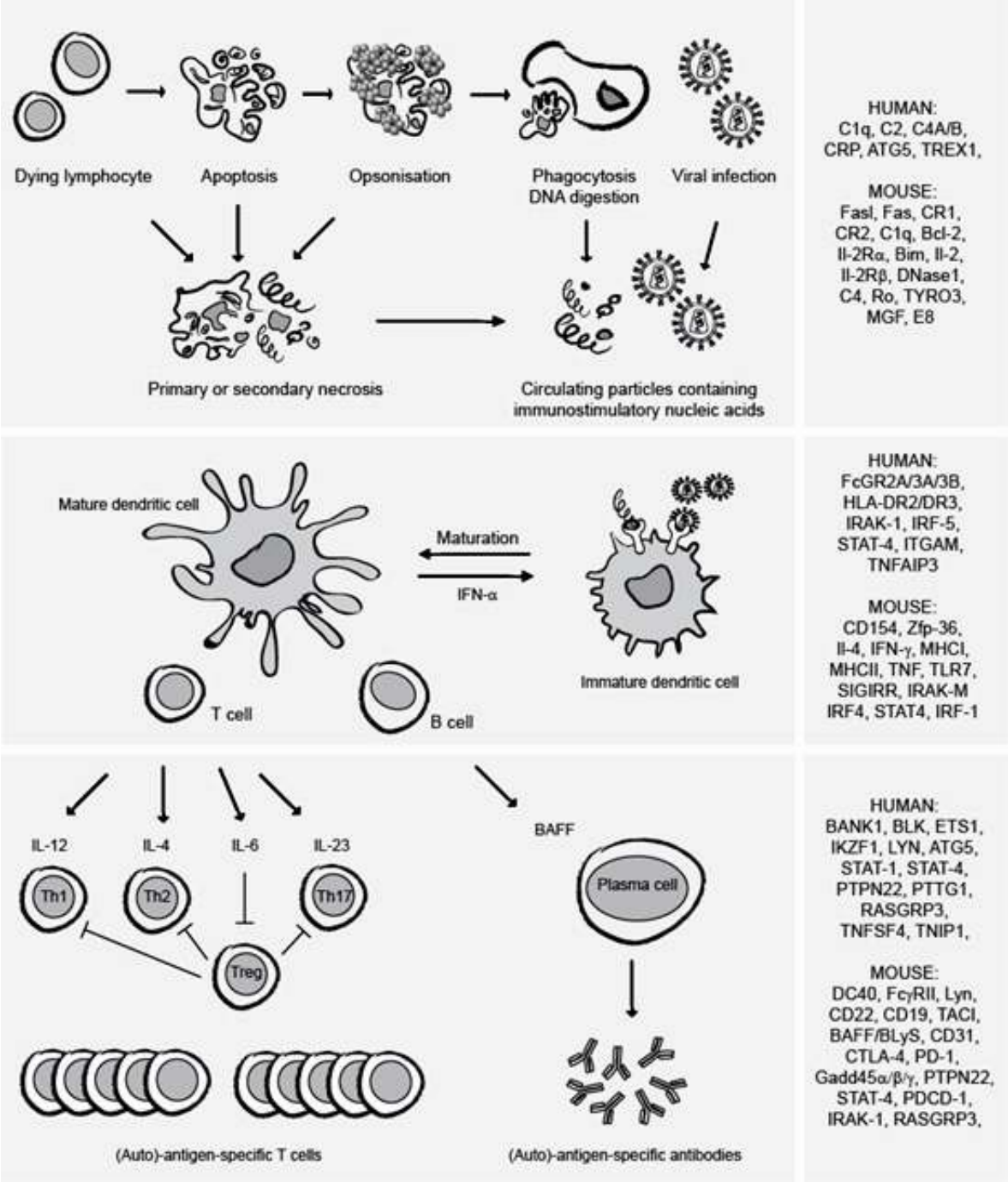


Figure 1. Pathogenesis of SLE. Increased production of autoantigens because of increased rates of apoptotic cell death coupled with defects in their removal contributes to lymphocyte hyperactivity and production of pathogenic autoantibodies. (The image was kindly provided by Dr. Maciej Lech)

quent production of autoantibodies by plasma cells (Figure 1). Anti-double stranded DNA antibodies (anti-dsDNA) are probably the most pathogenic type of antibody produced. The formation of immune complexes, the activation of the complement pathway and the defective clearance of immune complexes also play an important role in the pathogenesis of lupus. A more recent link between the innate and adaptive immune system in SLE

includes the neutrophil, which can be primed by interferon-α and autoantibodies to release neutrophil extracellular traps as an additional source of immunogenic DNA, histones, and neutrophil proteins. In SLE, three main immune pathways have been identified: aberrant clearance of nucleic acid containing debris and immune complexes, excessive innate immune activation involving Toll like receptors (TLRs) and type I interferons (IFNs) and abnormal T and B lymphocyte activation. Research has demonstrated evidence that a key enzyme's failure to dispose of dying cells may contribute to the development of SLE. The enzyme, DNase1 normally eliminates DNA by chopping them into tiny fragments for easier disposal and mice without DNase1 showed signs of SLE[6]. Thus, a genetic mutation in a gene that could disrupt the body's cellular waste disposal may be involved in the initiation of SLE. Autoantigens are released by both necrotic and apoptotic cells. Defects in the clearance of apoptotic cells have been described in this disorder and these defects could lead to aberrant uptake by macrophages, which then present the self-antigens to T and B cells, thus driving the autoimmune process. There are a few drugs that have been reported to trigger SLE. However, more than 90% of cases of "drug-induced lupus" occurs as a side effect of one of the following six drugs: hydralazine, quinidine, procainamide, phenytoin, isoniazid and d-penicillamine. These drugs are known to stimulate the immune system and cause SLE. Fortunately, drug-induced SLE is infrequent (accounting for less than 5% of all people with SLE) and usually resolves when the medications are discontinued[7].

Treatments of SLE aim to relieve symptoms and protect organs mainly by suppressing the underlying abnormal immune activation, systemic inflammation, and immunopathology of affected organs. To date, SLE treatment still depends on unspecific immunosuppressive or immunomodulatory agents. Despite this armamentarium, several problems still remain. First, all of the aforementioned drugs can have significant side effects and potent immunosuppression, as it is necessary to treat highly active or life-threatening SLE, which is often associated with serious infectious complications. Second, even intense immunosuppressive regimens are often unable to suppress highly active SLE. For example, when the Aspreva Lupus Management Study trial compared high-dose cyclophosphamide plus steroids with high-dose mycophenolate mofetil plus steroids in patients with diffuse proliferative lupus nephritis, >40% of patients in both groups did not reach partial remission with either of the treatments[8]. Therefore, finding novel drugs that can suppress SLE and lupus nephritis without major side effects are important.

Lupus nephritis

Lupus nephritis is a common manifestation of SLE and affects 50% of patients with significant impact on morbidity and mortality. Lupus nephritis is more prevalent and more aggressive in Afro-Americans and Hispanics, who exhibit a greater incidence of proliferative nephritis than Caucasians. Standard treatment includes corticosteroids such as prednisone or prednisolone, to reduce inflammation in the kidneys. The treatment of lupus nephritis has improved over the last few decades. Mycophenolate mofetil or cyclophosphamide are usually used as the first line treatment for nephritis, and in either case, corticosteroids are added. For maintenance therapy, either mycophenolate mofetil or azathioprine is preferred. Although effective in the majority of patients, cyclophosphamide was associated with serious adverse effects including infections, malignancy and infertility. Novel drugs that more specifically interfere with the disease pathomechanisms are needed to improve lupus nephritis management. By light and immunofluorescence microscopy, renal biopsies in patients with SLE display mesangial cell proliferation, inflammation, basement membrane abnormalities, and immune complex deposition, with immunoglobulins and complement components[9]. On electron microscopy, these deposits can be visualized in the mesangium and the subendothelial or subepithelial surface of the basement membrane. The International Society of Nephrology (ISN) classified the various forms of lupus nephritis according to the light, immunofluorescent and electron microscopic changes (Table 2).

Recent reports have identified some of the inflammatory mediators which play important role in chronic tissue inflammation associated with SLE. We assume that targeting inflammatory mediators without affecting systemic immunity can be an excellent therapeutic approach for SLE. In this study we evaluated the effect of two different proteases on murine lupus. In the first study we administered serine protease activated protein C (aPC) to the MRL-Fas(lpr) mice, a murine model of SLE. In the second study we inhibited cysteine protease Cathepsin S (Cat S) using a specific inhibitor R05461111 in MRL-Fas(lpr). Before describing the observed findings of the study, known details of SLE pathogenesis and current therapy, followed by a brief introduction on aPC and Cat S are reviewed in this introductory section.

Table 2. International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of lupus nephritis

Class I	***Minimal mesangial lupus nephritis***
	Normal glomeruli by light microscopy, mesangial immune deposits by immunofluorescence
Class II	***Mesangial proliferative lupus nephritis***
	Mesangial hypercellularity or matrix expansion, with mesangial immune deposits on immunofluorescence
Class III	***Focal lupus nephritis***
	Glomerulonephritis involving <50% of glomeruli, typically with subendothelial immune deposits
Class III (A)	Active lesions: focal proliferative lupus nephritis
Class III (A/C)	Active and chronic lesions: focal proliferative and sclerosing lupus nephritis
Class III (C)	Chronic inactive lesions with glomerular scars: focal sclerosing lupus nephritis
Class IV	***Diffuse lupus nephritis***
	Glomerulonephritis involving >50% of glomeruli, typically with subendothelial immune deposits. Can be segmental of global.
Class IV-S (A)	Active lesions: diffuse segmental proliferative lupus nephritis
Class IV-G (A)	Active lesions: diffuse global proliferative lupus nephritis
Class IV-S (A/C)	Active and chronic lesions: diffuse segmental proliferative and sclerosing lupus nephritis
Class IV-G (A/C)	Active and chronic lesions: diffuse global proliferative and sclerosing lupus nephritis
Class IV-S (C)	Chronic inactive lesions with scars: diffuse segmental sclerosing lupus nephritis
Class IV-G (C)	Chronic inactive lesions with scars: diffuse global sclerosing lupus nephritis
Class V	***Membranous lupus nephritis***
	Global of segmental sub-epithelial immune deposits
Class VI	***Advanced sclerosing lupus nephritis***
	>90% of glomeruli globally sclerosed without residual activity

Adapted and modified from Weening *et al.*[10]

1.1.1 Predisposing factors associated with SLE

A search of the precise immunopathogenesis of SLE has remained a challenge to many research groups all around the globe. Several mechanisms lead to a loss of self-tolerance and organ dysfunction in SLE. This has led to lots of conceptual theories which are published in recent times. Several studies in humans and several murine models of SLE have shown that a variety of predisposing factors play important roles in the pathogenesis of SLE. Predisposing factors that may increase risk of SLE include genetic, environmental, sex and hormonal factors.

Genetic factors

SLE is considered to be complex multi-genetic autoimmune disease. Genetic factors confer a predisposition to the development of SLE. Genes that contribute to the pathogenesis of systemic lupus are classified as follows: 1) Genes that cause a break in tolerance for the self-antigens. 2) Genes that lead to immune dysregulation. It is also suggested that multiple mutations (inherited or somatic) may be needed before a self-reactive clone B and T lymphocytes bypasses sequential tolerance check points resulting in the emergence of autoimmune disease[4]. CCL5 is important for the recruitment of lymphocytes, monocytes and eosinophils to the sites of inflammation. In a study of 146 Han Chinese patients with SLE, CCL5 polymorphism (–403 G/G) was found to be considerably more frequent in those with renal damage than in those without (79% versus 33%)[11]. Although in rare cases SLE may be associated with the deficiency of a single gene (e.g., the complement components C1q and C4), the disease more commonly results from the combined effect of variants in a large number of genes. Lack of C4 has been linked to decreased elimination of self-reactive B cells, whereas lack of C1q leads to deficient elimination of necrotic (waste) material. Each allele contributes only minimally, and the cumulative effect of several genes is necessary to substantially increase the risk of SLE.

Certain single nucleotide polymorphisms (SNPs) linked to SLE were reported for genes whose products may contribute to abnormal T-cell function in SLE (CD3-ζ[12] and PP2Ac[13]). HLA antigens and genes (particularly HLA class II) have been associated with SLE. Graham *et al.* have identified haplotypes carrying the HLA class II alleles DRB1*0301 and DRB1*1501 are clearly associated with SLE[14]. Recent genome-wide association study identified two novel genetic loci C8orf13-BLK and ITGAM-ITGAX that contribute to the risk of SLE[15]. A recent large scale replication study confirmed some of these associations and identified TNIP1, PRDM1, JAZF1, UHRF1BP1, and IL-10 as risk loci for SLE. Another large study of individuals and multi-case families with SLE suggested that an SNP within the programmed cell death 1 gene (PDCD1) is associated with the development of lupus in both European and Mexican populations[16]. Although these findings are promising, the loci identified so far can account for only about 15% of the heritability of SLE[17]. Polymorphisms in the low-affinity Fcγ receptors have been associated with susceptibility to a number of autoimmune diseases, including SLE. Low copy number of FCGR3B[18] gene has been associated with SLE.

Environmental factors

Sunlight is the most obvious environmental factor that may exacerbate SLE. Ultraviolet light (UV) induces apoptosis of keratinocytes and has been implicated in the skin manifestations of lupus. Drugs like procainamide, hydralazine and quinidine can lead to drug-induced lupus erythematosus. Epigenetic changes such as DNA hypomethylation have been attributed to medications known to cause SLE. Sulfonamide antibiotics can induce idiopathic SLE. Subacute cutaneous lupus erythematosus (SCLE) is associated with thiazides, calcium channel blockers and angiotensin-converting enzyme inhibitors. The possibility that viruses may trigger SLE has been considered during the past 40 years. Epstein Barr virus (EBV) has also been identified as a possible factor in the development of lupus. EBV may reside in and interact with B cells. To enter B cells, viral glycoprotein gp350 binds to cellular receptor CD21. Then, viral glycoprotein gp42 interacts with cellular HLA class II molecules. This triggers fusion of the viral envelope with the cell membrane, allowing EBV to enter the B cell. Gross *et al*.[19] found a high frequency of EBV infected B cells in lupus patients compared to controls and these infected cells are predominantly memory B cells. Amongst the other factors which are also associated with SLE are toxic exposures to silica and mercury[20].

Sex and Hormonal factors

SLE is a disease affecting women of childbearing age and there have been many anecdotal reports of exogenous estrogens exacerbating lupus or increasing the risk of developing this disorder. Oral contraceptive use in the Nurses' Health Study[21] was associated with a slightly increased risk of disease with a relative risk for users versus never users of 1·9. Sex hormones were shown to affect T cells and B cells. In particular estrogen has been suggested to predispose women to SLE. Estrogen has multiple effects on the immune system, which upregulates Bcl2, thus blocking tolerance induction of naive B cells[22]. Either an increase in estrogen or prolactin can break tolerance of high-affinity DNA-reactive B cells. Estrogen promotes the survival and activation of the T-independent marginal zone B cell subset[23]. In SLE T cells estrogen increases expression of the calcineurin[24], which promotes the proliferation and activation of T cells. The topical calcineurin inhibitors, tacrolimus and pimecrolimus, have been used to treat resistant cutaneous lupus. Estrogen can activate DCs[25]. Thus, estrogen may facilitate the maturation of pathogenic naive autoreactive B cells, whereas hampering a potentially protective autoreactive B-cell repertoire[26].

Hormones (estrogen, progesterone) contribute through unknown mechanisms to the increased prevalence of SLE among women. The X chromosome may contribute independently from hormones because in castrated female and male mice that have been genetically manipulated to express XX, XO (female), XY, or XXY (male) combinations, the presence of two X chromosomes increases the severity of SLE[27]. The X-chromosome has many genes involved in immune functions such as TLR7, FOXP3, Bruton's tyrosine kinase (BTK), CD40L, IRAK1[28] which contribute to the onset of SLE. High estrogen levels resulting from pregnancy may aggravate SLE. DHEA (dehydroepiandrosterone) is a hormone secreted by the adrenal gland. DHEA serves as a precursor to male and female sex hormones, treatment with DHEA significantly reduced the number of SLE flares[29].

1.1.2 Cell death: Implications in SLE and Lupus nephritis

Cell death is the most likely phenomenon to supply autoantigens. There are many different types of cell death mechanisms in living organisms, but the major types of cell death are apoptosis and necrosis. Apoptosis is an active, programmed cellular process, which appears under both physiological and pathological conditions in all tissues. One of the hallmarks of apoptosis is that the cell membrane stays intact. In healthy individuals, apoptotic cells are efficiently removed by macrophages before debris can be formed, without eliciting inflammation, whereas in SLE patients the clearance of apoptotic cells by macrophages is impaired. Clearance deficiency results in the initiation and maintenance of the systemic autoimmune reactions and ensuing chronic inflammation in SLE. There is growing evidence for a clearance deficiency of early apoptotic cells in mouse models of SLE[30] and in humans[31].

If apoptotic cells are not cleared on time, they lose their membrane integrity and enter the stage of secondary necrosis. Secondary necrotic cells can release DNA-containing nucleosomes together with dangerous inflammatory signals towards immune system cells[32]. Apoptotic bodies are a source of self-antigen, which in turn leads to release of pathogenic autoantibodies into the circulation. Autoantibodies then form immune complexes and get deposited in various organs that leads to tissue inflammation. Disordered regulation of both apoptosis and the clearance of apoptotic products have been implicated in the pathogenesis of SLE and lupus nephritis[33].

Under normal circumstances, activity against self-antigens is prevented by several mechanisms, including the Fas pathway of apoptosis, which was shown to be involved in the process of immune tolerance by deletion of unwanted autoreactive T cells and B cells[34]. Defective apoptosis leading to the prolonged survival of lymphocytes, particularly autoreactive lymphocytes was thought to be one disease mechanism for SLE. This hypothesis was supported by observations in murine lupus models. Defective Fas mediated apoptosis in MRL-Fas(lpr) mice results in massive lymphoproliferation and the development of a severe lupus-like disease with immune glomerulonephritis (GN). However, there are several problems with this model. First, the massive lymphoproliferation resulting from defective apoptosis seen in the MRL-Fas(lpr) mice is not characteristic of human SLE, which conversely is often associated with profound lymphopenia. Second, the expression of both Fas and FasL is normal in patients with SLE. Lastly, apoptosis of peripheral lymphocytes in patients with SLE has been shown to be increased compared with controls.

The reasons for the defective clearance of apoptotic cells in SLE are not clear. It could be the result of quantitative or qualitative defects of the early complement proteins, such as C2, C4, or C1q. Patients with homozygous deficiencies in these complement components develop a severe lupus-like disease early in life. The C1q receptors on the surface of phagocytes constitute an extremely important mechanism for the clearance of apoptotic cells. Patients or mice with homozygous C1q deficiency develop autoantibodies and a lupus-like syndrome apparently because of the inability to eliminate apoptotic cells.

So we know that apoptotic bodies are one of the major sources of autoantigens. If we look at the physiology of cell, every cell undergoes apoptosis, but at the same time in a healthy individual clearing mechanisms are appropriately placed to clear the debris. In addition to that, healthy immune system is tolerant to self. But in SLE the immune system is deregulated and increased generation of autoreactive B and T cells takes place along with the reduction of regulatory T cells. Circulating autoantigens or immune-complexes which are non-antigenic in healthy conditions are recognized as danger signals, which further lead to excessive activation of immune system in SLE[4,35]. The SLE patients have greater levels of circulating nucleosomes than in normal healthy individuals[36]. Increased rates of apoptosis and/or reduced clearance of neoantigens created by apoptosis might lead

to increased autoantibody production. One mechanism by which direct renal damage might occur in lupus nephritis is by increased rates of apoptosis among resident cells. In a murine model of lupus nephritis, caspase inhibitor therapy reduced glomerular injury[37].

Cell death by necrosis, on the other hand, occurs when external factors strike cells. Necrotic cell death is characterized by cytoplasmic and organelle swelling, followed by the loss of cell membrane integrity and release of the cellular contents into the surrounding extracellular space. This phenomenon is often triggered by an infectious agent, heat, ischemia, low ATP levels or a mechanical injury. No matter if cells die through apoptosis or necrosis, they must quickly be eliminated from tissues in order to prevent further damage. Early apoptotic cells are cleared by phagocytosis without eliciting either inflammation or immune response. Necrotic cells induce inflammation and favour the initiation of immune responses[38]. HMGB1 is passively released from necrotic cells and mediates inflammation and immune activation. In contrast, during apoptotic cell death, nuclear HMGB1 becomes tightly attached to hypo-acetylated chromatin and is not released into the extracellular milieu, thereby preventing an inflammatory response[39].

1.1.3 Role of dendritic cells in SLE

Dendritic cells (DCs) are a heterogeneous group of bone marrow-derived cells that function in immunosurveillance, antigen presentation and tolerance. The general categories of DCs are the conventional dendritic cells (cDC) and the plasmacytoid dendritic cells (pDC). The cDC include those found in the skin, in secondary lymph nodes and the spleen. One of the most studied cDCs is the skin DCs, known as Langerhans cells. Upon exposure to pathogens or insult, immature DC can recognize foreign material and phagocytize them. This phagocytosis is not as efficient as professional phagocytes such as macrophages; however, the main function of DCs carrying antigenic cargo is to migrate to secondary lymph nodes to activate T cells, particularly naïve T cells. pDCs have the prominent feature of producing type I interferons (IFNs) and specifically they are the major producers of IFN-α. Accumulating evidence has highlighted pDCs as the culprit for SLE pathogenesis, mainly through type I interferons production. In SLE patients, immune complexes consisting of autoantibodies bound to self-DNA and RNA can stimulate production of type I IFNs through TLR7 and TLR9 signaling of pDCs. Guiducci *et al*.[40]

have suggested that inhibitors of TLR7 and 9 signalling could be effective corticosteroid-sparing drugs. The primary anti-inflammatory mechanism of glucocorticoids is thought to be NF-kB inhibition. Chronic stimulation of pDCs through TLR7 and 9 by nucleic acid-containing immune complexes activates the NF-kB pathway essential for pDC survival. Glucocorticoids do not affect NF-kB activation in pDCs, preventing glucocorticoid induction of pDC death and the consequent reduction of systemic IFN-α levels. The pathogenic role of type I IFN in SLE is mediated in part by its ability to induce immune activation, including a positive feedback loop that induces plasma cell maturation and increases autoantibody formation[41]. pDCs infiltrating inflamed peripheral tissues represent the main source of type I IFNs in patients with SLE.

DCs in general are highly phagocytic and serve as sentinels to continuously detect danger signals from the environment. They uniquely express a plethora of innate pattern-recognition receptors (PRRs) including TLRs, C-type lectin receptors (CLRs), RIG-I-like receptors and NOD-like receptors (NLRs) which capture antigens through binding to the pathogen-associated molecular patterns (PAMPs) of microbes or damage-associated molecular patterns (DAMPs) of endogenous tissues. In humans, mDCs are classically characterized by the high expression of CD11c, CD1a and HLA-DR with the distinguishing morphology of protruding dendrites. pDCs, as suggested by its name, are plasma-cell like, negative for both CD11c and CD1a and express the comparatively lower level of HLA-DR.

DCs could influence SLE in several ways: presentation of self-antigen to autoreactive T cells; secretion of proinflammatory cytokines; and promotion of B cell autoantibody production, either directly or indirectly. DCs are widely considered to be critical for initiating T cell responses in infections[42]. Based on this notion, it could be assumed that they are the primary antigen-presenting cells (APCs) to induce T cell autoimmunity. However, depending on their activation state, DCs might also support peripheral T cell self-tolerance instead of T cell immunity. During the onset of autoimmunity, DCs are thought to be critical for priming of self-reactive T cells that have escaped tolerance induction. However, DCs can also induce T cell tolerance and DC-depleted mice showed increased frequencies of CD4+ thymocytes and infiltration of $CD4^+$ T cells into peripheral tissues. These mice developed spontaneous autoimmunity

characterized by reduced body weight, splenomegaly, autoantibody formation, neutrophilia, high numbers of Th1 and Th17 cells, and inflammatory bowel disease[43].

1.1.4 Role of T cells in SLE

T cells contribute to the initiation and perpetuation of autoimmunity in SLE. T cells play an important role, because the production of pathogenic autoantibodies in SLE is a T-cell-dependent process[44]. As major contributors to the disease, T cells in SLE display multiple abnormalities that reflect and partly explain some aspects of the complex disease process. The cytokine expression pattern is uniquely characterized by decreased expression of interleukin (IL)-2 and increased production of IL-17 and related cytokines. Moreover SLE T cells show inappropriate tissue homing and promote inflammation by secreting cytokines and activating DCs and B cells. T cells provide excessive help to B cells in SLE and mount inflammatory responses.

T cells are functionally and phenotypically heterogeneous, and can be divided into subgroups. On the basis of the expression of the CD4 and CD8 antigens, thymocytes could be subdivided into several subpopulations: ~5% express neither CD4 nor CD8 (double-negative T cells); ~80% express both CD4 and CD8 (double-positive T cells); ~10% express only CD4 ($CD4^+$ T cells); and ~5% express only CD8 ($CD8^+$ T cells). Double-negative T cells are significantly expanded in patients with SLE and induce anti-DNA antibody production by autoreactive B-cells. Recent studies showed that they also secrete other cytokines such as IL-1β and IL-17, and are found in the kidneys of patients with lupus nephritis. T cells can also be divided into regulatory T cells and effector T cells. Regulatory T cells suppress the activation or effector activities of other immune cells, especially self-reactive T cells; they are low and functionally abnormal in patients with SLE. In contrast, effector T cells proliferate in response to antigen stimulation, secrete cytokines and help the function of cytotoxic T cells or B cells for the production of antibodies. Effector T cells are divided into T helper (Th) 1, Th2 and Th17 cells, depending on the major cytokines that they produce (Figure 2). Th1 cells mainly produce IFN-γ, but also IL-2, tumor necrosis factor-α (TNF-α) and lymphotoxin (which favour the elimination of pathogens). Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 (which associate with allergy and humoral immunity, especially in B cell activation and

immunoglobulin class switch) and Th17 cells play a role in host defense against extracellular pathogens and produce the proinflammatory cytokine IL-17.

$CD4^+$ T cells and their cytokines play a crucial role in the induction and propagation of the inflammatory conditions. With the help of T cells, activated B cells migrate into lymphoid follicles of lymphoid organs and form germinal centers (GC). Recently, follicular helper T (Tfh) cells, a novel $CD4^+$ T subset, have been found to be present in GCs, which regulate the development of antigen-specific B-cells. Tfh cells provide help to GC B cells and play an essential role in mediating long-lived antibody responses.

Administration of antibodies that non-specifically deplete or inactivate helper $CD4^+$ T cells suppresses the production of antibodies to DNA and prevents or reverses glomerulonephritis in mice with SLE[45]. Inhibition of co-stimulation between APCs and T cells has therapeutic potential in SLE. CD86 and CD80 are expressed on activated antigen presenting cells (APCs) including B cells, DCs and macrophages. The interaction of CD86/80 with its ligands CD28 and CD152 (CTLA4) plays a critical role in the induction and regulation of immune responses. Binding of CD86/80 to CD28 on T cells results in the transaction of costimulatory signals required for activation or proliferation of T cells. In contrast, binding of CD86 to CTLA4 regulates T-cell activation and diminishes the immune response. Abatacept (CTLA4–Ig) prevents the CD28–CD80/86 interaction and shown to be effective in improving disease activity in lupus-prone mice[46,47], but failed in human clinical trials. Critical interactions between APCs that express CD40 and T cells expressing the CD40L are thought to provide important co-stimulatory signals required for activation and survival of B cells, enhanced APC function and T-cell activation. Monoclonal antibodies to CD40L have been used to treat patients with SLE, but enthusiasm for their use has been tempered by the tendency for patients treated with this antibody to experience thromboembolic complications[48].

Spontaneous apoptosis is increased in T cells from SLE patients. On the other hand, upon exposure to oxidizing agents, SLE T cells undergo necrosis rather than apoptosis. Increased T cell necrosis and apoptosis represents a source of nuclear material, which amplifies the inflammatory response in patients with SLE. T cells display various

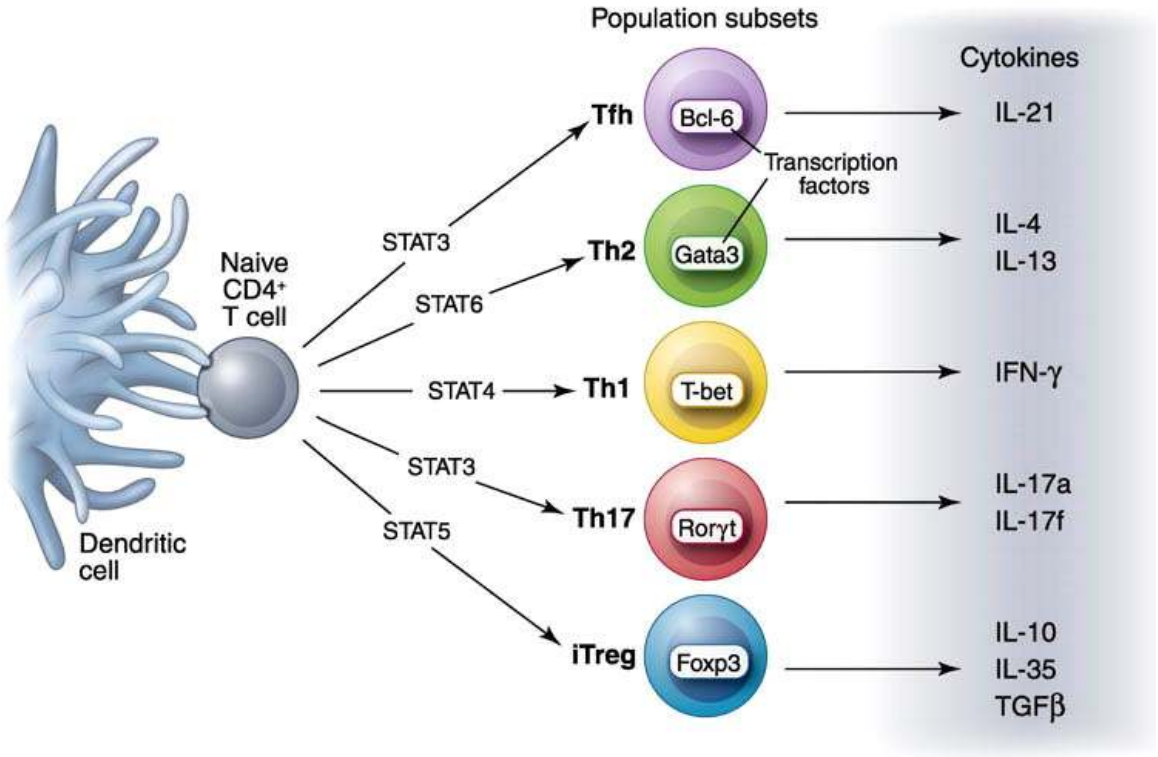


Figure 2. Helper T cell differentiation (adapted and modified from O'Shea *et al.*[49])

chemokine receptors that allow them to respond to inflammatory signals and migrate out of the circulation into the tissue. SLE T cells show altered migratory activity in states of inflammation. For example, the migration of T cells in response to CXCL12 is faster in SLE[50]. This may be due to increased expression of CXCR4, the receptor for CXCL12. In addition, kidneys from patients with lupus nephritis show high levels of CXCL12, further supporting the role of the CXCR4/CXCL12 axis in T cell homing to the kidney.

The $CD8^+$ T cells in SLE are impaired. Most of the disturbed T cell homeostasis in SLE seems to depend on aberrant mechanisms of peripheral control, and it is generally thought that central tolerance may not be affected or can only partly influence T cell autoimmunity in SLE[51,52]. SLE T cells display spontaneously increased activation associated with a reduced threshold of activation to self-antigens, yet they are hyporesponsive to further antigenic stimulation[53]. At the molecular level, T cell receptor (TCR) stimulation in SLE T cells associates with an increased signaling protein phosphorylation and a sustained increase in free intracellular Ca^{++} compared to control T

cells[54]. Additional molecular mechanisms that could contribute to impaired T-cell functions in SLE include histone acetylation and methylation, as shown by the finding that treatment with histone deacetylase inhibitors can suppress murine lupus[55].

1.1.5 Role of B cells in SLE

Growing evidence shows that B cells not only produce antibodies but also secrete interleukins (IL), act as antigen-presenting cells (APCs), contribute to T-cell activation, and modulate DCs. Several abnormalities in SLE B-cell populations have been identified, supporting the hypothesis that B cells play a central role in the pathogenesis of this disease. Thus, recent advances in SLE treatment have evolved to target B cells in the expectation that patients would have fewer side-effects than those which cause generalized immunosuppression. B cell hyperactivity has significant effects on SLE pathogenesis via autoantibody production, antigen presentation to T cells, production of cytokines including IL-10 and IL-6, and abnormal B cell signaling.

Recent findings in both human and mouse models suggest that TLR7 and TLR9 may play a central role in the progression of the disease by activating B cells to produce autoantibodies[56]. The role of the B cell in the pathogenesis of immune mediated glomerulonephritis has traditionally been viewed as limited to that of antibody producer. However, it is increasingly appreciated that B cells contribute to the pathogenesis of glomerulonephritis in many other ways. They can function as potent antigen-presenting cells (APCs), regulate T cells and DCs through the production of cytokines. Evidence for an antibody-independent role for B cells comes from animal models, lupus prone MRL-Fas(lpr) mouse with nonsecretory plasma cells (without antibody production) developed SLE[57], whereas B cell-deficient MRL-Fas(lpr) mice did not[58]. The role of the B lymphocyte as an APC is also likely to be essential in the development of autoimmunity[57,59].

To investigate the role of B cells in lpr-induced autoimmunity, Shlomchik *et al*.[58] crossed the "Jh knockout" mutation onto the autoimmune MRL-Fas(lpr) background. As expected, these animals lack B cells and show no signs of autoimmune kidney destruction

or vasculitis, in spite of carrying the lpr/lpr mutation. In contrast, lpr/lpr littermates that had B cells had severe nephritis and vasculitis, as well as autoantibodies. These results demonstrate a primary role for B cells and/or (auto) antibodies in initiating several types of autoimmune manifestations in MRL-Fas(lpr) mice. Another B-cell-related functions likely to be important in the pathogenesis of SLE is cytokine release, particularly IL-10, TNF-α, IL-6 and BLyS/BAFF (B lymphocyte stimulator/B-cell activating factor) are produced in high levels in SLE[60]. Ultimately, activated B cells can aggregate into ectopic lymph node-like structures containing plasmablasts, memory B cells, and plasma cells are observed in sites with chronic inflammation.

Several strategies that target B cells are currently available. The approaches include mainly inhibition of survival factors, interruption of co-stimulatory factors and depletion of B cells. Rituximab is a genetically engineered chimeric monoclonal antibody that targets B-lymphocyte surface marker CD20 (Figure 3). The mechanisms by which rituximab induces depletion probably include antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and apoptosis[61]. Because CD20 is only expressed from the pre-B stage to the mature B cells, rituximab spares haematopoietic stem cells, pro-B and plasma cells, allowing B-cell regeneration. Given the generally encouraging results in the open-labeled studies, it was most disappointing to learn that the EXPLORER study in the USA, comparing rituximab with placebo in non-renal lupus, did not reach its primary endpoints.

In contrast to rituximab, the experience with the use of epratuzumab in SLE is very limited. Epratuzumab is a humanized recombinant monoclonal antibody that targets CD22 (Figure 3), which is a co-receptor of the B-cell-antigen receptor (BCR) that regulates B-cell activation and interaction with T cells. It is expressed in pro-B, pre-B and mature B cells, but is absent from plasma cells. This drug has shown safety in short-term studies and potential for clinical efficacy, and warrants further exploration for the treatment of SLE[62].

B-lymphocyte stimulator (BLyS) is a cytokine that is involved in the survival of B cells, GC formation, and T cell-dependent and T cell-independent immunoglobulin-class switching. It binds to the surface of B cells and acts with the B-cell receptor in signal transduction. Studies in mice have shown a role of BLyS in the expression of lupus[63].

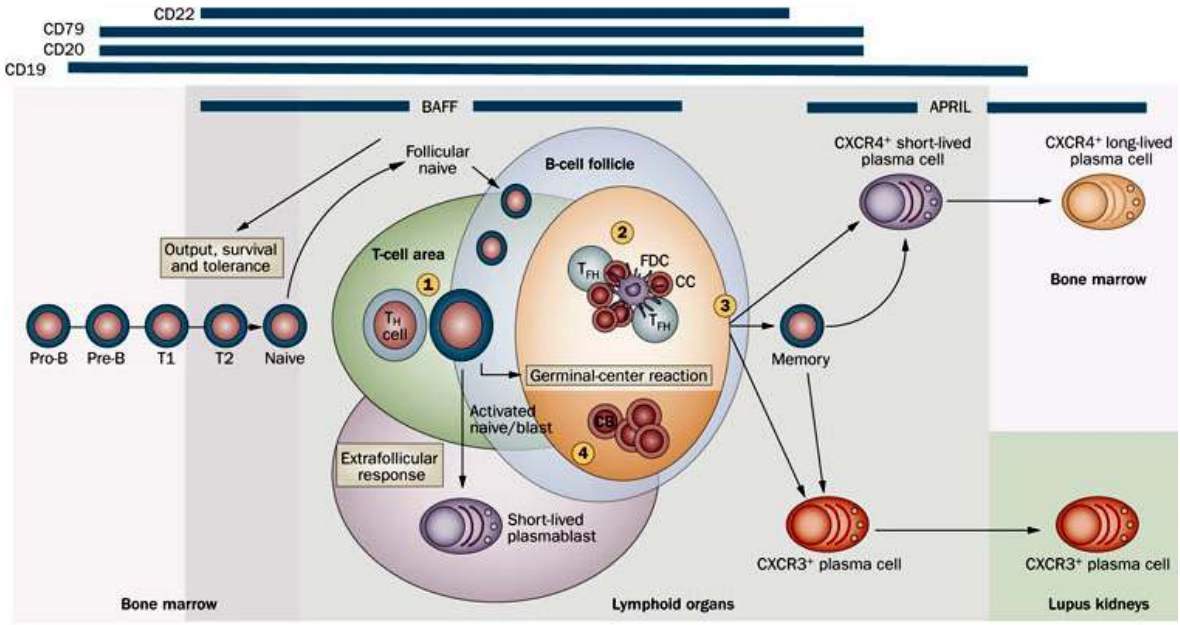


Figure 3. Strategies for B‑cell targeting in SLE (adapted from Sanz *et al.*[64]).

Blockade of BLyS with an anti-BLyS antibody resulted in a significant beneficial clinical effect within the first year of treatment in patients with mild or moderate disease. This antibody (belimumab) was approved by the Food and Drug Administration for use in the treatment of lupus.

1.1.6 Autoantibodies in SLE

Autoantibodies are antibodies that react with self-antigens. Self-antigens are internalized by APCs such as, DCs, macrophages and B cells. After internalization, APCs present self-antigens to $CD4^+$ T cells via major histocompatibility complex class II (MHC-II) molecules and activate the adaptive immune system in lupus. SLE is characterized by autoantibodies (Table 3), especially against nuclear antigens such as anti-dsDNA antibodies and anti-histone antibodies. Increased production of anti-dsDNA antibodies predicts periods of active clinical disease.

Anti-DNA antibodies constitute a subgroup of antinuclear antibodies (ANA) that bind single stranded DNA (ss-DNA), ds-DNA, or both. Antibodies targeted against ds-DNA were first described in 1957[65]. They might be IgM antibodies or any of the

subclasses of IgG antibodies. Anti-dsDNA antibodies are thought to play a crucial role in the pathogenesis of lupus nephritis[66]. In many patients with SLE, increased renal disease activity is associated with rising titers of anti-DNA antibodies. Although anti-dsDNA was once thought to cause glomerulonephritis by forming complexes with DNA that are passively trapped in the glomeruli, many investigators now believe that anti dsDNA antibodies are pathogenic to the kidney via direct (cross-reactivity) or indirect (via a nuclear antigen bridge) binding to glomerular structures. A series of studies by Chan *et al*.[67,68] have shed light on the potential pathogenicity of anti-DNA binding to glomerular cells in lupus nephritis. Subsets of anti-DNA antibodies from patients with SLE were shown to bind to human mesangial cells and endothelial cells, and the cellular binding of these autoantibodies correlated with disease activity.

In addition to cell binding, there is convincing evidence to show that anti-dsDNA antibodies penetrate into living cells. Administration of certain anti-dsDNA antibodies to non-autoimmune mice *in vivo* leads to cell penetration and intranuclear Ig deposits in the kidney and other organs[69]. Apart from glomerular cells, renal tubular cells, hepatocytes, neuronal cells, fibroblasts and mononuclear cells are all susceptible to penetration by anti-dsDNA antibodies. One intracellular effect of anti-DNA antibodies is to enhance cell growth and proliferation, or conversely induce apoptosis[70]. Madaio *et al*.[71] reported that nuclear localizing anti-DNA antibodies bind to DNAse I in living cells and inhibit the activity of this enzyme, making the cells more resistant to apoptosis. This observation might explain the finding of glomerular hypercellularity in mice injected with penetrating antibodies. Abetimus (LJP-394) is an intravenously administered tetrameric oligonucleotide conjugate that safely reduces anti-dsDNA antibodies. Anti-dsDNA antibodies are present in the majority of lupus patients and have been linked to the pathogenesis of LN. Abetimus is composed of four identical strands of anti-dsDNA and it is supposed to bind to the circulating anti-dsDNA antibodies.

Generation of nucleosomes *in vivo* requires apoptosis[72]. These nucleosomes and intracellular debris appear as blebs on apoptotic cell surfaces, and might incite T cell-driven stimulation of B cells. The injection of syngeneic apoptotic cells into normal mice has been shown to generate antinuclear antibodies and immune deposition in kidneys[73]. Kalaaji *et al*. have shown exposed glomerular basement membrane-associated

nucleosomes as target structures for nephritogenic autoantibodies *in vivo*[74,75]. It would be reasonable to assume that nucleosomes are one of the major autoantigens in lupus nephritis.

The presence of anti-Ro antibodies, anti-La antibodies, or both during pregnancy of lupus patients confers a 1 to 2% risk of fetal heart block. Ro antigens are exposed on the surface of fetal (but not maternal) cardiac myocytes as the heart undergoes remodeling by apoptosis, and maternal anti-Ro antibodies that cross the placenta interact with these antigens. Both anti-Ro and anti-nucleosome antibodies may play a role in cutaneous lupus. Anti-Ro antibodies are associated with an increased risk of the development of a photosensitive rash. Anti-Sm is an antibody specific against Sm, a ribonucleoprotein found in the cell nucleus. Anti-Sm antibodies are usually found only in SLE patients.

Antibodies against the N-methyl-d-aspartate receptor (NMDAR) may be important in central nervous system lupus. NMDA is an excitatory amino acid released by neurons. Kowal *et al*. have shown that in patients with lupus, the serum with antibodies against DNA and NMDA receptors caused cognitive impairment and hippocampal damage when given intravenously to mice. They also showed that anti-NMDA-receptor antibodies are present in the brain tissue of patients with cerebral lupus[76]. Antibodies against complement component C1q are associated with SLE and LN disease activity.

Table 3 – Pathogenic autoantibodies in SLE

Antigen specificity	**Prevalence %**	**Main clinical effects**
Anti-dsDNA	70-80	Kidney disease, skin disease
Nucleosomes	60-90	Kidney disease, skin disease
Ro	30-40	Skin disease, kidney disease, fetal heart problems
La	15-20	Fetal heart problems
Sm	10-30	Kidney disease
NMDA receptor	33-50	Brain disease
Phospholipids	20-30	Thrombosis, pregnancy loss
α-Actinin	20	Kidney disease
C1q	40-50	Kidney disease

* Modified from Rahman *et al.*[3]

1.1.7 Role of Complement in SLE

The role of complement in the pathogenesis of SLE is paradoxical. On the one hand, complement components appear to mediate autoantibody-initiated tissue damage. On the other hand, the complement system appears to have protective features as hereditary deficiencies of some complement components are associated with an increased risk for SLE. The kidney is an important source of complement synthesis. Activation of complements by immune complexes through classical pathway is supposed to be the main mechanism of tissue injury. Blocking the final common pathway C5-C9 of both the classical complement pathway and the alternative complement pathway could be an attractive approach for treating patients with SLE because both these pathways are activated in this disease. Moreover, those patients with hereditary deficiencies of complement proteins of the classical pathway are at increased risk for systemic lupus erythematosus. Wang *et al*. demonstrated decreased renal disease and increased survival in NZB/W F1 mice, a mouse model of SLE, after treatment with a monoclonal antibody specific for C5 component of complement. This antibody blocks the cleavage of C5 and thus prevents the generation of the potent proinflammatory factors C5a and C5b-9[77].

The involvement of the complement system in SLE occurs in three steps, known as the 'waste disposal hypothesis'. The first step is the failure to clear autoantigens i.e. defective waste disposal[78]. This is the stage at which complement deficiency might have a pathogenic role. The second step is the uptake of autoantigen by immature DCs in the presence of inflammatory cytokines, which causes these cells to mature into antigen-presenting cells, allowing the presentation of autoantigens to T cells. The third step is the pathogenesis of lupus nephritis provision of help by T cells to autoreactive B cells, which have taken up autoantigen by means of their immunoglobulin receptors. Such B cells mature into plasma cells that secrete autoantibodies. It is likely that in the majority of patients, SLE develops only in the presence of abnormalities in more than one of these steps.

The formation of an antibody–antigen complex (immune complex) is the principal way of activating the classical pathway of the complement system. C1q, an integral part of the first component of complement (C1), triggers the activation process when it docks onto

antibodies within these immune complexes. In this way, C1q acts to bridge the innate and adaptive immune systems. It has been widely accepted that the activation of complement by immune complexes is an important contributor to tissue injury in patients with SLE.

At the same time hereditary deficiencies in the complement components of the classical pathway increase the risk of lupus and lupus like disease. Deficiencies in C1, C2, C4 and CR1 predispose an individual to the development of SLE. For example, the complement component C1q eliminates necrotic cellular waste (apoptotic material) in healthy individuals. In patients with SLE, a possible deficiency of the C1q component can lead to disease expression. C1q-deficient mice developed renal injury with vascular thrombosis, proteinuria and renal failure. The increased IgG deposits and apoptotic cells in the glomeruli of C1q-deficient mice suggest that the exacerbation of disease may be due to a defect in the clearance of immune complexes from the kidneys[79]. Animal studies have produced conflicting data as to whether C3 confers protective or harmful effects[80,81]. A second example of genetic variance is a possible deficiency of the C4 complement, a component identified in the elimination of self-reactive B cells. At present, it is difficult to reconcile the potential positive and negative impacts of complement in SLE.

1.1.8 Role of cytokines and chemokines in SLE

Cytokines and chemokines may act as key players in the immunopathogenesis of SLE. Some cytokines and chemokines levels correlate with SLE disease activity. Certain cytokines such as the IL-6, IL-10, IL-17A, BLys, type I IFNs, TNF-α and chemokines such as CCL2, CCL5, CXCL10, CXCL12 are closely linked to the pathogenesis of SLE. The delineation of the role played by these cytokines and chemokines not only fosters our understanding of this disease but also provides a sound rationale for various therapeutic approaches.

Cytokines

Serum levels of interferon-α are also elevated in patients with active lupus[82], and microarray studies showed that 13 genes regulated by interferon were up-regulated in peripheral-blood mononuclear cells from patients with lupus, as compared with similar cells from healthy controls[83]. In studies of lupus-prone NZB/W F1 mice, nephritis

developed 15 to 20 weeks earlier in mice continuously exposed to IFN-α from a young age than in control mice not subject to this exposure[84].

Serum and urine levels of IL-6 were significantly elevated in active SLE patients and correlated with the SLE activity index. Monocytes secrete IL-6, which, together with transforming growth factor (TGF) β, promotes the differentiation of TH17 cells. IL-6 is very important in the stimulation of differentiation and proliferation of B-cells[85]. It has been shown that activated B cells express IL-6 receptors and IL-6 induces the differentiation to plasma cells[86]. Inhibition of IL-6 or IL-6 receptor (IL-6R) using specific antibodies resulted in clinical improvements in lupus-prone mice. The fully humanized monoclonal anti-IL6R antibody tocilizumab has shown promise in a phase I trial of SLE. SLE patients produce abnormally large amounts of IL-10 that correlate with disease activity[87]. IL-10 is produced by activated macrophages and $CD4^+$ T cells. IL-10 has a number of biologic effects, including stimulation of polyclonal populations of B lymphocytes. Blocking this cytokine could reduce the production of pathogenic autoantibodies. In a SLE mouse model, anti-IL-10 mAb treatment led to a significant reduction in autoantibody production. A small trial with a single injection of the anti-IL-10 mAb, B-N10, was conducted in lupus patients with active disease. Although there was an overall improvement in disease activity, all patients developed anti-murine antibodies[88].

The role of TNF-α in lupus is controversial. This cytokine may be protective in patients with lupus, since giving TNF-α to lupus-prone NZB/W F1 mice delayed the development of lupus[89]. The protective effect is specific to that mouse strain, and the mechanism is unknown. In some patients with rheumatoid arthritis who were treated with anti–TNF-α antibodies, anti–dsDNA antibodies developed[90], and lupus erythematosus developed in a few of these patients[91]. TGF-β could exert a bidirectional effect similar to the effects of the classical Th2 cytokines, with less inflammation on the one hand, but more fibrosis on the other. TGF-β may also play a role for regulatory T cell action. In lupus nephritis, Increased expression of TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) were found in glomeruli and the tubulointerstitium[92]. In MRL lpr/lpr mice, where TGF-β is likewise over expressed, additional TGF-β was beneficial with regard to autoantibody formation, kidney disease and survival[93], but its role in fibrosis has been suggested to be critical.

Elevated serum levels of IL-17A and increased numbers of T cells secreting IL-17 have been detected in SLE patients and in murine lupus models[94]. IL-17A is a potent neutrophil chemoattractant and it is the primary form of IL-17 secreted by Th17 cells. In lupus, Th17 cells traffic to inflamed tissues, such as the kidney and promote inflammation by enhancing cytokine production, which can in turn activate B cell antibody production, activate DCs, and stimulate resident cells in the target tissues.

IL-1α and IL-1β are primarily synthesized by activated monocytes and macrophages. IL-1 induces activation of T cells and promotes chemotaxis of polymorphonuclear cells, leukocytes, lymphocytes and monocytes and enhances the infiltration of these molecules into inflamed tissue. High levels of IL-1β were reported in the sera and cerebrospinal fluid of lupus patients. Anakinra, a recombinant human IL-1 receptor antagonist, is used in the treatment of rheumatoid arthritis. Two very small uncontrolled studies of three and four SLE patients suggested that anakinra might have beneficial effects on lupus-related manifestations. IL-18, belongs to the IL-1 cytokine family, has a variety of effects on DCs, T cells and natural killer (NK) cells. IL-18 was associated with the pathogenesis of systemic lupus erythematosus.

The B-lymphocyte stimulator (BLyS) is a member of the TNF-ligand superfamily. It promotes the proliferation and survival of B lymphocytes. Circulating levels of BLyS are elevated in several other conditions, including rheumatoid arthritis and Sjögren's syndrome, as well as in lupus. The overexpression of BLyS has been detected in both humans with lupus and lupus-prone mice[63].

Chemokines

The regulatory role of chemokines in leukocyte recruitment during inflammatory tissue injury has gained more interest over the last decade. In the kidney, all types of renal cells have been shown to produce inflammatory chemokines such as CCL2, CCL5, CXCL10 and CXCL12 upon various kinds of injury. Local production of these chemokines initiates recruitment of macrophages and T cell subsets, which leads to subsequent glomerulonephritis and interstitial nephritis. Autoimmune mice with targeted deletion of certain chemokines or chemokine receptors, moreover interventional studies with specific antagonists are protected from renal autoimmune tissue injury.

CCL2 (MCP-1), a potent chemoattractant for monocytes and T cells, and is secreted from many kinds of cells. A significant number of T cells and macrophages infiltrate the kidneys of patients with lupus nephritis. There is an increasing body of evidence that CCL2 plays a major role in the pathogenesis of lupus nephritis. Deletion of the CCL2 gene dramatically reduces lupus nephritis in MRL-Fas(lpr) mice[95]. Inhibition of CCL2 ameliorates lupus nephritis in MRL-Fas(lpr) mice[96]. These findings suggest that overexpression of CCL2 plays an important role in the pathogenesis of lupus nephritis in humans and in animal models. Serum CCL5 levels are significantly elevated in SLE patients when compared with normal controls. CCL5 is critical for glomerular macrophage recruitment, as CCL5 blockade reduces glomerular macrophage counts during immune complex glomerulonephritis[97]. CXCL10 is a chemokine that binds to the receptor CXCR3 and regulates immune responses through the activation and recruitment of leukocytes, such as, T cells, eosinophils, and monocytes. Elevated serum CXCL10 levels were observed in SLE patients. CXCL12 (SDF-1) is a chemokine that attracts T cells, B cells, pre-B cells and monocytes. Its receptor, CXCR4, is the most broadly expressed chemokine receptor, as it is present on the surface of T cells, B cells, monocytes, neutrophils and DCs. Monoclonal antibodies against CXCL12 hampered autoantibody production and development of nephritis in NZB/W mice. Blocking the CXCR4/CXCL12 axis represents another possible therapeutic approach for SLE treatment, and this has already shown some promise in mouse models of disease[98].

1.2 Current therapies of SLE

There is no cure for systemic lupus erythematosus. The goal of treatment is to relieve symptoms and protect the organs by decreasing inflammation and/or the level of autoimmune activity in the body. The precise treatment is decided on an individual basis. Mild flares are treated with nonsteroidal anti-inflammatory drugs and anti-malarials, while more serious flares are treated with corticosteroids and immunosuppressants.

Pain and inflammation are common problems in patients with SLE, and NSAIDs are usually the drugs of choice for patients with mild SLE with little or no organ involvement. NSAIDs are helpful in reducing inflammation, pain and fever. Examples include aspirin, ibuprofen, naproxen and sulindac. Since the individual response to

NSAIDs varies, it is common for a doctor to try different NSAIDs to find the most effective one with the fewest side effects. The most common side effects are stomach upset, abdominal pain, ulcers, and even ulcer bleeding.

Corticosteroids are hormones secreted by the cortex of the adrenal gland and are more potent than NSAIDs in reducing inflammation and restoring function when the disease is active. These drugs are used to treat many of the symptoms of lupus that result from inflammation. Administration of corticosteroids results in rapid depletion of circulating T-cells. Corticosteroids are particularly helpful when internal organs are affected. Prednisone is a corticosteroid that is often used to treat lupus. Unfortunately, corticosteroids have serious side effects when given in high doses over prolonged periods. Side effects of corticosteroids include weight gain, thinning of the bones, infection and diabetes. Although corticosteroids have potentially serious side effects, they are highly effective in reducing inflammation, pain, high blood pressure, fatigue, and suppressing the immune system. Corticosteroid creams are available to treat skin rashes caused by lupus.

Hydroxychloroquine (plaquenil), chloroquine and mepacrine are antimalarial drugs that are used to treat lupus. Hydroxychloroquine is the most commonly used antimalarial. These drugs are particularly useful for those patients with skin rashes, joint pain, fever, fatigue, pleurisy and especially those patients with discoid lupus. They may provide sufficient lupus treatment for a patient with moderately active lupus to avoid steroids. Consistently taking plaquenil can prevent flare-ups of lupus. Side effects include diarrhea, indigestion and may impair vision. Hydroxychloroquine is considered to be safe for use in pregnancy and breast feeding period. Antimalarials are not a sufficient treatment for more severe lupus symptoms such as kidney disease and nervous system. Antimalarial medications help to control lupus in several ways by modulating the immune system without predisposing you to infection. Antimalarial drugs are one of the mainstays of treatment for SLE as multiple studies have shown they can delay onset of SLE, reduce the symptoms, and prevent flares[99].

Immunosuppressives are medications that help suppress the immune system and are used for treating people with more severe manifestations of SLE, such as damage to internal organ(s). They are almost always taken along with corticosteroids and, in fact, are

Table 4. Medications for the management of SLE

Drug	Suggested dose
NSAIDs	
Ibuprofen	20-40 mg/kg per day
Naproxen	10-25 mg/kg per day
Aspirin	3.6 - 5.4 g/day
Corticosteroids	
Prednisone	Up to 2 mg/kg per day
Methyl prednisone (intravenous)	10-30mg per dose
Methyl prednisone (oral)	Up to 2 mg/kg per day
Antimalarials	
Hydroxychloroquine	5-7 mg/kg per day
Chloroquine	250 mg per day
Immunosuppressants	
Cyclophosphamide (oral)	0.5-2 mg/kg per day
Cyclophosphamide (intravenous)	500-1000 mg/m^2
Cyclosporine	2.5-4.5 mg/kg per day
Azathioprine	0.5-2.5 mg/kg per day
Rituximab	375 mg/m^2 per dose
Methotrexate	15-20 mg/kg per day
Belimumab	10 mg/kg
Others	
Mycophenolate mofetil	0.5-3 g/day

often used to help gradually reduce the dose of corticosteroids. Examples of immunosuppressive medications include methotrexate, leflunomide, azathioprine, cyclophosphamide, chlorambucil, cyclosporine, rituximab and belimumab. One of the most widely used immunosuppressive drugs for lupus, azathioprine works by blocking immune cell function. Side effects can include nausea, lowered blood cell counts, and liver inflammation. Methotrexate is used to control skin rash and joint pain caused by lupus and not recommended for severe lupus. Cyclosporine modifies the immune system without decreasing blood cell counts. Leflunomide is an anti-inflammatory medication that can help to reduce the pain and swelling of joints. Cyclophosphamide inhibits cell division and growth, and it is a strong immunosuppressive drug. Cyclophosphamide in lupus treatment is reserved for very serious kidney disease or other internal organ involvement. All immunosuppressive medications can seriously depress blood-cell counts and increase risks of infection, bleeding, liver damage, decreased fertility and an increased risk of cancer.

Despite the fact that the mortality and morbidity of patients with SLE has improved significantly during the last few decades, mortality rates remain approximately three times those of the age-matched and sex-matched population in most studies. The need for more

effective therapies with less toxic side effects has propelled interest in targeted biologic therapies based on an expanding understanding about SLE disease pathogenesis. B cells play a central role in the pathogenesis of lupus, making them a logical therapeutic target. The BAFF (B-cell-activating factor) is a critical survival factor for transitional and mature B cells. Belimumab (monoclonal antibody against BAFF) blocks the stimulation of the B cells and is indicated for the treatment of adult patients with active, autoantibody-positive SLE who are receiving standard therapy. It is important to note that the efficacy of belimumab has not been evaluated in patients with severe active lupus nephritis. Side effects include nausea, diarrhea and fever. Belimumab was approved by the FDA and the European medicines agency (EMA) for the treatment of SLE in 2011. Most recent research is indicating benefits of rituximab in treating lupus. Rituximab is a genetically engineered chimeric monoclonal antibody that targets B-lymphocyte surface marker CD20. Rituximab suppresses the B cells by decreasing their number in the circulation. Because rituximab does not eliminate plasma cells, it does not markedly reduce immunoglobulin levels. A large number of open-label studies documented the efficacy of rituximab in refractory lupus nephritis. Unfortunately, the placebo-controlled Phase II/III EXPLORER and LUNAR trials of rituximab in SLE failed to meet the primary and secondary endpoints.

In recent years, mycophenolate mofetil (CellCept) has been used as an effective medication for lupus, particularly when it is associated with kidney disease. Its lower side-effect profile has advantage over traditional immunosuppressive medications. In SLE patients with serious lupus cerebritis or lupus nephritis, plasmapheresis is sometimes used to remove antibodies and other immune substances from the blood to suppress immunity. Plasmapheresis is a process of removing blood and passing the blood through a filtering machine, then returning the blood to the body with its antibodies removed. End-stage kidney damage from SLE requires dialysis and/or a kidney transplant.

SLE treatment has moved from the use of conventional drugs such as hydroxychloroquine, NSAIDS, corticosteroids and non-specific immunosuppressants to targeting selective components of the immune system in the hope that they can be more effective and reduce undesired side-effects. Our increased understanding of SLE immunopathogenesis has led to the introduction of several new agents over the last 10 years, a move from therapeutic serendipity to sense. Biological agents targeting B cells and T cells are under development. Drugs under current research in SLE are directed against B

cell surface molecules (CD20, CD22 and CD19), co-stimulation (CTLA-4, CD40/CD40L, ICOS/B7-H2), complement inhibition, toleragens, TLR inhibitors, as well as cytokines and chemokines.

People with lupus are more likely to experience infection and infection-related complications. This is because their immune system is weakened by both the disease and the medications used to treat it. In particular corticosteroids and immunosuppressive drugs, affect the immune system. The most common infections in people with lupus include those of the respiratory tract, skin and urinary system. Immunosuppressive drugs used to treat lupus can also enhance the risk of cancer. Thus the main goal of research is to find drugs to treat lupus more specifically, without systemically suppressing the immune system. The development of targeted therapies that specifically address disease pathogenesis and progression has lagged, resulting in a limited therapeutic armamentarium of broad spectrum immuno-suppressive agents that have substantial toxicities and are not always adequate to control symptoms or prevent disease flares. Recent clinical trials based on rational hypotheses and robust preclinical effects on mouse SLE models have tested the efficacy of new biologic drugs in combination with standard-of-care therapies but have met with only limited success in recent years. Even though T cell and B cell targets seem to be promising, clinical trial results for these therapies do not look encouraging. This leaves scope for the development of new strategies to encounter SLE and lupus nephritis. Treatments, that are more specific in modifying particular subsets of immune cells (e.g. T/B cells), or the activity of cytokine they secrete have been gaining attention.

The development and approval of new treatments for SLE remain a challenge for rheumatologists and healthcare providers as the disease is multisystemic, remitting and relapsing, and usually requires corticosteroids as baseline therapy. Previous clinical trials with promising new drugs, such as rituximab and abatacept, have failed. However, these medications are responsible for the untargeted immune suppression and may increase the risk of infections and bone marrow suppression, hepatotoxicity and other adverse events. The currently available treatments for lupus have been shown to be ineffective in inducing/maintaining a low disease activity state or remission in some patients. Thus, there are unmet needs in SLE treatment for new drugs that can target specific pathways and reduce or eliminate disease activity without predisposing to serious adverse events.

1.3 Role of Proteases

Proteases are proteolytic enzymes that catalyze the breakdown of proteins by hydrolysis of peptide bonds. Proteases play a critical role in many physiological and pathophysiological processes by controlling the activation, synthesis and turnover of proteins. Proteases are involved in a large number of key physiological processes such as cell-cycle progression, cell proliferation, cell death, DNA replication, tissue remodeling, haemostasis, wound healing and immune responses. Different types of proteases have different biological functions. Proteases are mainly classified into five distinct classes: serine, cysteine, aspartic, threonine and metalloproteases; depending on the nature of the functional group at the active site.

The scope of the thesis is to study in detail roles of a serine protease (Activated protein C) and a cysteine protease (Cathepsin S) in SLE and lupus nephritis.

1.3.1 Activated protein C

Activated protein C (aPC), a vitamin K-dependent serine protease and is generated from the protein C (PC) zymogen by proteolytic activation by thrombin. Originally thought to be synthesized exclusively by the liver, recent reports have shown that PC is also produced by endothelial cells, smooth muscle cells, keratinocytes and some leukocytes. The molecule was discovered in 1976 by Johan Stenflo who purified a protein in pool C of a chromatography elute derived from bovine plasma[100]. The function of this protein was revealed a few years later when Charles Esmon and colleagues discovered that its activated form was a physiological anticoagulant. In contrast to the various procoagulant plasma factors, aPC has anticoagulant and cytoprotective activities. Normal activation of protein C by thrombin requires two membrane receptors, thrombomodulin and endothelial protein C receptor (EPCR). PC is activated on the endothelial surface when thrombin binds to thrombomodulin and cleaves the activation peptide (Figure 4). This conversion to aPC is augmented by the EPCR. Clinical studies reveal that deficiency of PC lead to microvascular thrombosis. PC circulates in plasma at 70 nM whereas aPC is present in much lower concentrations (40 pM or ~ 2.3 ng/ml).

aPC is a protein with multiple functions in the regulation of homeostasis. It acts as a natural anticoagulant by inactivating the coagulation factors Va and VIIIa. In addition to its anticoagulation functions, aPC also has cytoprotective effects such as anti-inflammatory, pro-fibrinolytic, anti-apoptotic, and endothelial barrier protection. aPC has been shown to be protective in severe human sepsis. In fact, a recombinant form of human aPC (Drotrecogin Alfa activated; commercially known as Xigris; Lilly Deutschland, Bad Homburg, Germany) was approved in 2001 by the U.S. Food and Drug Administration for the treatment of severe sepsis associated with a high risk of mortality[101]. On October 25th, 2011, Eli Lilly & Co. withdrew Xigris from the market after a major study (PROWESS-SHOCK trial) showed no efficacy for the treatment of sepsis. As a consequence, the FDA has recommended that aPC treatment should not be started in new patients.

There are four major moieties that make up the PC molecule; the Gla domain, two EGF-like regions, a small activation peptide, and the active serine protease domain. Upon binding with EPCR, thrombin-thrombomodulin complexes activate PC by removing 14 amino acids[100] from activation peptide (Figure 4). Cytoprotective actions generally require EPCR and protease activated receptor-1 (PAR-1). aPC has following cytoprotective activities.

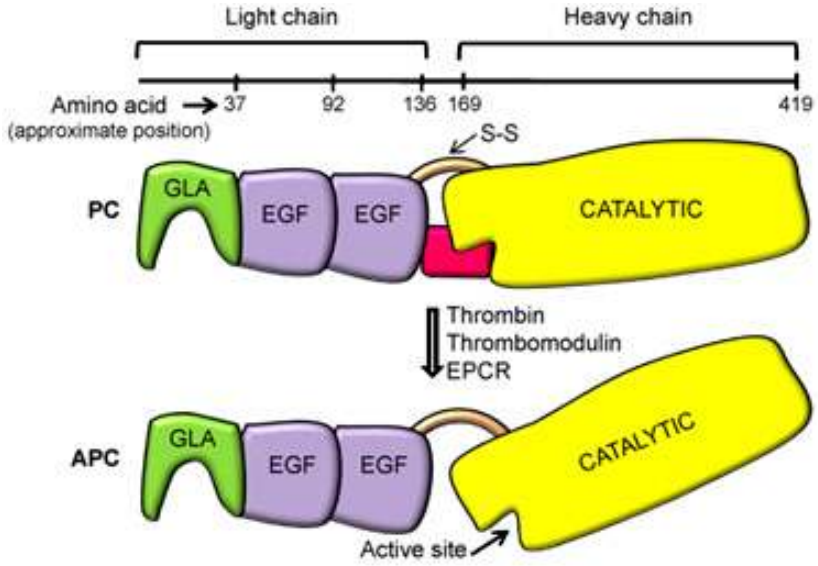


Figure 4. Structure of PC. GLA: γ-carboxy-glutamic acid-rich domain; EGF: epidermal growth factor-like moiety; S–S: disulphide bond. Crimson moiety represents the activation peptide. Bar shows approximate amino acid sequential numbers. (Adapted from Christopher J. Jackson and Meilang Xue[100])

Anticoagulant activity

In the presence of its cofactors such as protein S, phospholipids and glycosphingolipids aPC degrades the coagulation factor Va and VIIIa and inhibits thrombin generation. In addition, aPC promotes fibrinolysis by binding to plasminogen activator inhibitor which prevents inhibition of plasminogen conversion to plasmin.

Anti-apoptotic activity

Gene expression profiling pattern studies revealed modulation of gene expression by aPC for some major genes of inflammation and apoptosis, with the general effect of down-regulation of proinflammatory and pro-apoptotic pathways and up-regulation of anti-inflammatory and anti-apoptotic pathways. Notably, aPC decreases pro-apoptotic p53 and Bax expression and increases expression of anti-apoptotic genes such as Bcl-2.

Endothelial barrier stabilization

aPC is well-known for its ability to protect the endothelial barrier, which is mediated by EPCR-dependent PAR-1 activation. aPC activated PAR-1 can stimulate sphingosine kinase-1 (SphK-1) and enhance sphingosine-1-phosphate (S1P) production, which is involved in endothelial barrier protection. PAR-1 activation by thrombin destabilizes the endothelial barrier, while activation of PAR-1 by aPC enhances barrier function.

Anti-inflammatory activity

aPC inhibits nuclear translocation of nuclear factor (NF)-kB, which blocks expression of downstream TNF-α, IL-6, IL-8, IL-1β, MCP-1 and TNF-α, and cell adhesion molecules, intracellular adhesion molecule 1, vascular cell adhesion molecule 1 and E-selectin. In addition to downregulation of proinflammatory cytokines, aPC was shown to increase the expression of anti-inflammatory cytokine IL-10. Tissue factor (TF) is an important factor that links inflammation and coagulation, aPC decreases TF expression in an EPCR-dependent manner. Proinflammatory effects of the coagulation pathway are primarily mediated by thrombin, inhibition of thrombin formation indirectly by aPC through its inhibition of FVa and FVIIIa can reduce thrombin's proinflammatory activities.

Although aPC can be considered as a potentially useful clinical tool, its ugly side still remains (i.e. the bleeding risk associated with its anticoagulant activity). Site-directed mutagenesis has made it possible to engineer PC variants that are devoid of anticoagulant activity while preserving cell signaling properties. aPC variants with reduced anticoagulant

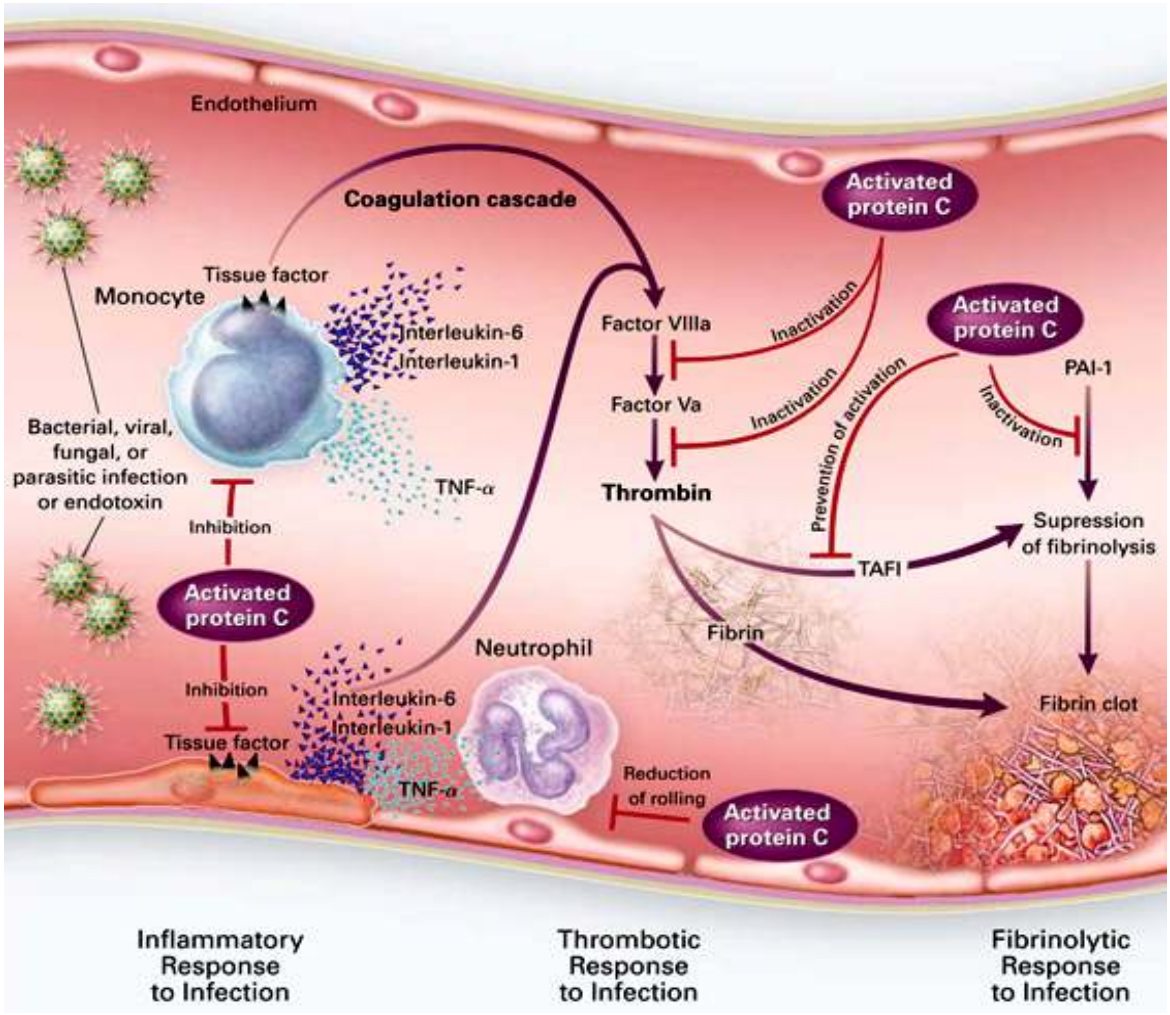


Figure 5. Proposed actions of activated protein C
Infectious agents and inflammatory cytokines such as TNF-α and interleukin-1 activate coagulation by stimulating the release of tissue factor from monocytes and the endothelium. The presentation of tissue factor leads to the formation of thrombin and a fibrin clot. Inflammatory cytokines and thrombin can both impair the release of plasminogen-activator inhibitor 1 (PAI-1) from platelets and the endothelium. PAI-1 is a potent inhibitor of tissue plasminogen activator, the endogenous pathway for lysing a fibrin clot. In addition, the thrombin suppresses the endogenous fibrinolytic system by activating thrombin-activatable fibrinolysis inhibitor (TAFI). The conversion of protein C, by thrombin bound to thrombomodulin, to the aPC is impaired by the inflammatory response. aPC can intervene at multiple points during the systemic response to infection. It exerts an antithrombotic effect by inactivating factors Va and VIIIa, limiting the generation of thrombin. As a result of decreased thrombin levels, the inflammatory, procoagulant, and anti-fibrinolytic response induced by thrombin is reduced. aPC exerts an anti-inflammatory effect by inhibiting the production of inflammatory cytokines (TNF-α, IL-1, and IL-6) by monocytes and limiting the rolling of monocytes and neutrophils on injured endothelium by binding selectins. aPC indirectly increases the fibrinolytic response by inhibiting PAI-1. (Adapted from Bernard *et al*.[101])

action but normal cytoprotective actions hold the promise of reducing bleeding risk because of attenuated anticoagulant activity while reducing mortality based on

cytoprotective effects. For example, safe aPC variants with reduced bleeding risk may permit therapies using higher aPC doses for shorter times. Higher doses of aPC may stabilize stressed cells at risk for excessive inflammation or apoptosis and prevent organ failure. aPC variants have been successfully tested in endotoxemia, ALS and ischemic stroke models[102,103].

Studies in animal models have demonstrated that aPC shows promise in a variety of pathologies. Although the precise way of action has not yet been elucidated, the immunosuppressive and cytoprotective effects of aPC have now emerged as a potential treatment for a number of autoimmune and inflammatory diseases that are associated with excessive immune responses such as acute respiratory distress syndrome, multiple sclerosis, rheumatoid arthritis, brain injury, lung injury, spinal cord injury, asthma, stroke, and chronic wounds[104]. aPC appears to have protective effects against ischemia/reperfusion-induced renal injury not by inhibiting coagulation, but by inhibiting the activation of leukocytes. The mechanism of action of aPC in wound healing is complex, and involves a unique combination of inhibition of inflammation, stimulation of angiogenesis and re-epithelialisation as well as anti-apoptotic properties. A recent study reported that aPC was also effective in protecting from diabetic nephropathy by inhibiting endothelial and podocyte apoptosis, where it modulates the mitochondrial apoptosis pathway via the PAR-1 and the EPCR[105]. aPC also had protective effects on chronic inflammatory bowel disease by reducing intestinal microvascular inflammation[106]. Given these beneficial effects of aPC on multiple hyperinflammatory and autoimmune disease states, we speculated that aPC may also suppress SLE and lupus nephritis. To address this question, we treated autoimmune and nephritic MRL-Fas(lpr) mice with recombinant human aPC and characterized the therapeutic effects on systemic autoimmunity, lupus nephritis, and other organ manifestations of SLE.

1.3.2 Cathepsin S

Cathepsin S (Cat S) is a lysosomal cysteine protease, plays an important role in antigen presentation and matrix degradation. Cat S has also been suggested to be involved in the generation of certain antigenic epitopes[107]. It is mainly found in lysosomal/endosomal compartments of APCs, such as B cells, macrophages and DCs and

can also be induced in many types of cells including adipocytes and smooth muscle cells[108]. It is also found in the 'non-professional' APC such as epithelial cells. Among the lysosomal proteases, Cat S possesses special characteristics, including its tissue-specific constitutive expression, its IFN-γ-dependent transcriptional activation and its activity over a broad pH range that extends to alkaline pH[109,110]. Unlike some other cathepsins, which are ubiquitously expressed, Cat S has a restricted tissue distribution and it is expressed mostly in the spleen, lymph, heart and lung. These findings suggest a role in immune and inflammatory processes. Its inhibition is expected to result in immune-suppression, making this enzyme an attractive target to potentially treat autoimmune and inflammatory diseases.

Cat S plays a major role in the degradation of the invariant peptide chain associated with the major histocompatibility complex and thus affects antigen presentation. Antigen presentation is the process whereby extracellular antigens, are taken up by professional and non-professional antigen presenting cells, and mostly degraded in the lysosomal compartment. The resulting peptides are displayed on the cell surface after the formation of MHC-II-peptide complexes. The molecular mechanism leading to the formation of class II peptide complexes and presentation of antigen on the cell surface begins with the synthesis of the class II α β heterodimers in the endoplasmic reticulum. These dimers are assembled together with the assistance of the Ii chaperone molecule to form a nanomeric complex αβ-Ii. This complex is then delivered to the endosomes where Ii is degraded in a stepwise fashion by the lysosomal proteases, allowing the antigen-binding site of MHC-II to be exposed. In B-cells and DCs, Cat S is the single enzyme that cleaves the Ii p10; a 10-kDa fragment of the lysosomal MHC-II bound invariant chain to form 24 amino-acid CLIP fragments (Figure 6). HLA-DM in humans and H-2M in mice catalyze the replacement of the MHC-II bound CLIP peptide with an antigenic peptide[111].

Active Cat S was detected in B cells, DCs and peritoneal macrophages. By contrast, cathepsin L (Cat L) was detected only in macrophages and cortical thymic epithelial cells, where a defect resulted in severely impaired $CD4^+$ T cell selection. In addition to Cat S, Cat L and cathepsin F (Cat F) have been implicated in Ii degradation and antigen presentation in alveolar macrophages. In macrophages, Cat S deficiency does not severely impair Ii degradation and antigen processing as Cat L and F can compensate for it[112].

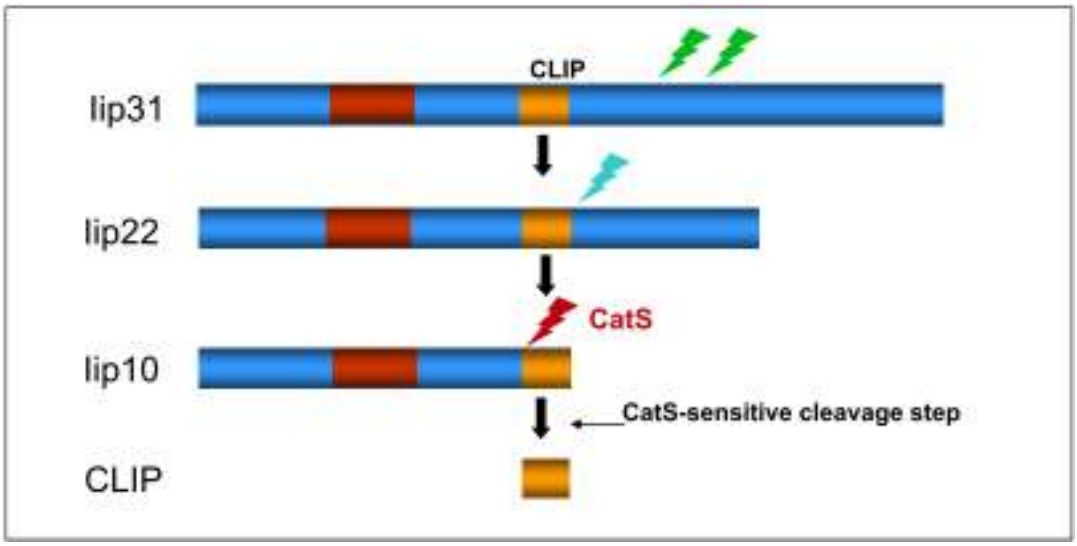


Figure 6. Role of cathepsin S in invariant chain processing. (Adapted from Virobay Inc. website)

Cat S has emerged as an attractive target for inhibiting immune responses, as its prime target is the disruption of antigen presentation. The continuous presentation of antigenic self-peptides is thought to perpetuate the autoimmune disease process. Inhibitors of Cat S block the presentation of autoantigens and may hold great promise for a novel immunosuppressive therapy. In APCs, deficiency of the Cat S enzyme, as a result of gene knock-down or inhibition by a small molecular compound, reduces degradation of the invariant chain, a crucial chaperon which also blocks peptide-binding by MHC-II molecules, thereby decreasing antigen presentation to $CD4^+$ T cells. Cat S deficient mice showed diminished activation of $CD4^+$ T cells, reduced production of antibodies, and are resistant to collagen-induced arthritis (CIA)[113]. Similar findings are observed in mice and rats dosed with Cat S inhibitors. Such inhibition reduced antigen dependent pulmonary inflammation in mice and decreased inflammation in the rat adjuvant-induced arthritis model. Cat S inhibitors have been found to block the rise in immunoglobulin E titers and eosinophil infiltration in the lungs, in a mouse model of pulmonary hypersensitivity. This suggests that Cat S may be involved in asthma. The possibility of using a Cat S inhibitor to regulate overactive autoimmune responses was confirmed, with the use of Cat S inhibitor CLIK-60 in a murine model for Sjögren's syndrome. Intraperitoneal administration of CLIK-60 showed elimination of the autoimmune manifestations by preventing the presentation of organ specific antigen α-fodrin by MHC-II[114]. *In vivo*, mice deficient in Cat S are healthy and normal in most respects but exhibit defects in immune function.

Furthermore, Cat S-deficient mice showed abnormal angiogenesis, defective microvessel development during wound healing.

It has been shown that extracellular Cat S is critical for the initiation and/or maintenance of neuropathic pain in peripheral nerve injured mice and rats. Intrathecal delivery of an irreversible Cat S inhibitor, morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone (LHVS) was anti-hyperalgesic and anti-allodynic in neuropathic rats and attenuated spinal microglia activation, leading to alleviation of chronic pain associated with spinal cord injury (SCI)[115]. Moreover, it was demonstrated that the pronociceptive role of extracellular Cat S might be mediated by the release of the transmembrane chemokine fractalkine in neurons. Cat S has a pivotal role in atherogenesis. Thus, a deficiency in Cat S has been shown to reduce atherosclerosis in mice, indicated by plaque size and stage of development[116]. In a novel potential therapeutic use of Cat S inhibitors, Novartis recently claimed their use for the treatment of chronic pain. The rationale is based on the discovery of Cat S mRNA upregulation in animal models of chronic pain and that inhibitors act as analgesics in these models.

A huge unmet medical need exists for a safe, oral medication for the treatment of SLE. In searching for novel therapeutic options, it has become clear that intervention at the stage of antigen presentation may be a viable option. Cat S has a non-redundant role in antigen presentation that selectively affects $CD4^+$ T cells and leaves $CD8^+$ T cells largely intact. This should provide therapeutic advantages over non-selective immunosuppressive compounds. Current therapies for lupus are often only partially effective and are accompanied by undesirable side effects. Thus targeting the antigen presentation pathway, responsible for triggering autoimmune diseases such as SLE, may provide a novel immunotherapy. Cat S inhibitors are more specific, safe and effective than the inhibitors with broad specificity.

Multiple genome-wide association studies performed on large cohorts of patients with lupus nephritis, membranous glomerulonephritis, or IgA nephropathy now confirmed that the pathogenesis of these common forms of immune complex glomerulonephritis (IC-GN) is tightly linked to the human leukocyte antigen genes that encode for MHC-II[15,117,118]. This implicates an essential role of MHC-II-mediated priming of adaptive

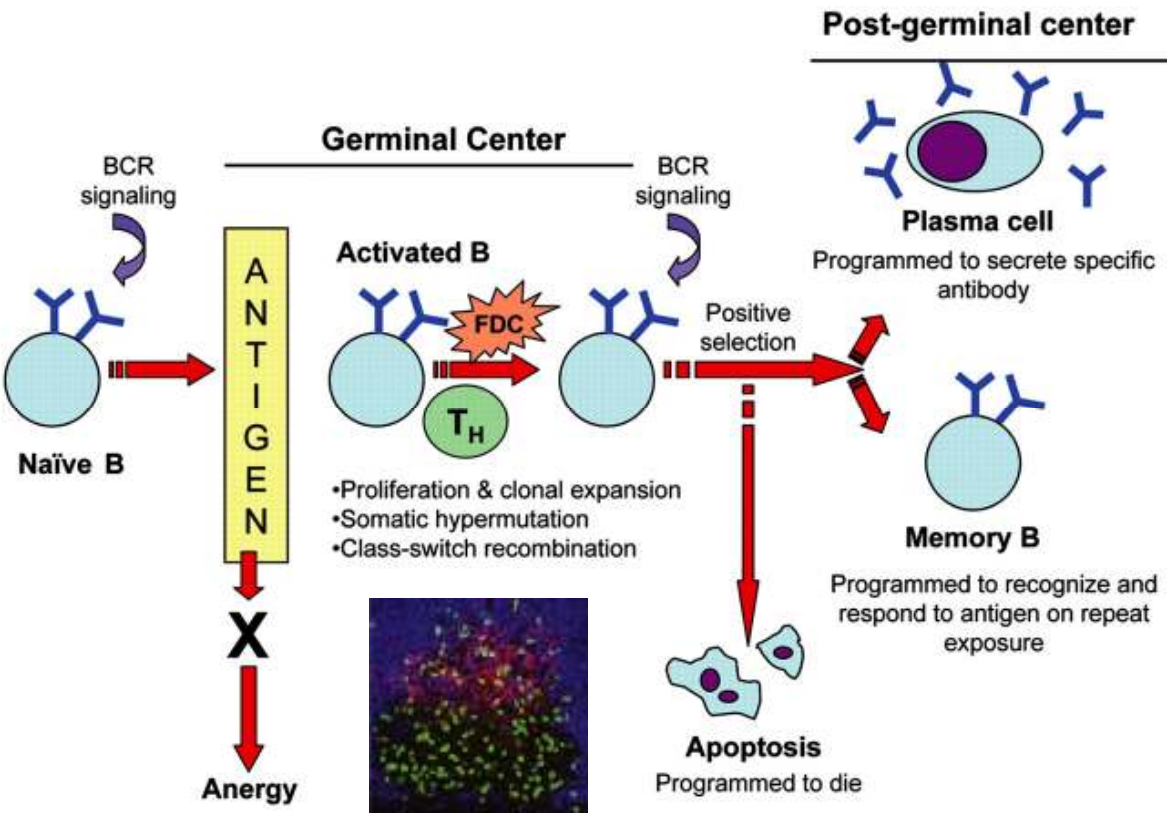


Figure 7. Schematic representation of normal B-cell ontogeny in the GC. Naïve B-cells encounter antigen and undergo proliferation, clonal expansion, somatic hypermutation and class-switch recombination in the GC microenvironment. The resulting B-cell clones with high affinity antibodies are positively selected to differentiate into plasma cells and secrete large quantities of specific antibodies or become memory B-cells that recognize and respond to repeat antigen exposure. The remaining B-cell clones are eliminated by apoptosis. (Adapted and modified from ASH Education Program Book 2007[119])

immunity in the development of IC-GN. The presentation of foreign or self-antigenic peptides within MHC-II in the context of TLR-dependent costimulation activates antigen-specific $CD4^+$T helper cells for their clonal expansion and polarization into the various $CD4^+$ T cell subsets. These cells then activate antigen-specific B cells undergo clonal expansion inside the GC of the lymphoid follicles (Figure 7). The GC environment is also needed for driving the somatic hypermutation of activated B cells, which involves affinity maturation and Ig class switch from immunoregulatory IgM-producing B cells into high affinity and complement-binding IgG antibody producing plasma cells[120]. Since formation and maintenance of GCs is T cell-dependent, altered T cell activation may be due to either Cat S-mediated disruption of antigen presentation or the unavailability of proper DC due to Cat S-mediated DC migration defects.

This process contributes to antigen-induced adaptive immunity as demonstrated by vaccinating mice with myelin oligodendrocyte glycoprotein 35e55 peptide[112], bovine type II collagen[112] or ovalbumin[121], even though Cat S was not required for thyroid stimulating hormone receptor-induced autoimmune thyroiditis[122]. As, in addition, Cat S degrades autoantigenic peptides favoring autoreactive $CD4^+$ T cells to escape thymic selection[123], there is a strong rationale for Cat S being a mediator of autoimmune IC-GN. To address this concept we first characterized Cat S expression in female MRL-(Fas)lpr mice with spontaneous lupus-like systemic autoimmunity and IC-GN. To study the functional contribution of Cat S and its suitability as a therapeutic target, we developed a specific, orally available, Cat S inhibitor that dose-dependently suppresses the proteolytic activity of murine and human Cat S *in vitro* and *in vivo*. By treating diseased female MRL-(Fas)lpr mice with this compound we not only demonstrate a non-redundant role of Cat S in IgG autoantibody production and glomerulonephritis but also identify Cat S as a novel therapeutic target for IC-GN.

1.4 MRL-Fas(lpr) mice - mouse model of SLE

MRL-Fas(lpr) mice develop an autoimmune disease that reflects pathologies of human SLE, including lymph node enlargement, increased IgG levels, antinuclear antibody production, proteinuria, and kidney failure. Female MRL-Fas(lpr) mice die at an average age of 17 weeks and male mice at 22 weeks. This accelerated phenotype was attributed to a recessive autosomal mutation termed lymphoproliferation (lpr). The lpr mutation, located on chromosome 19, alters transcription of the Fas receptor[124]. MRL-Fas(lpr) mice lack functional expression of the apoptosis-inducing receptor Fas, thereby accelerating the manifestation of the autoimmune disease, which also develops in wild-type MRL mice, albeit, with a slower kinetic. Studies on B-cells and T-cells from MRL-Fas(lpr), which both express Fas, confirmed a defect in apoptosis due to the lack of functional Fas receptor.

MRL-Fas(lpr) mice spontaneously develop lupus-like disease beginning at twelve weeks of age and often succumb to death from glomerulonephritis[125]. MRL-Fas(lpr) mice develop immune complex glomerulonephritis, associated with mononuclear cell infiltration, endothelial and mesangial cell proliferation, and crescent formation. MRL-

Fas(lpr) mice show systemic autoimmunity, massive lymphadenopathy associated with proliferation of aberrant $CD3^{+}B220^{+}CD4^{-}CD8^{-}$ T cells and immune complex-derived glomerulonephritis. Starting at approximately 3 months of age, levels of circulating immune complexes, such as those from spontaneously generated anti-dsDNA antibodies, rise dramatically in MRL-Fas(lpr) but not in wild-type MRL controls. The spleen weight of MRL- Fas(lpr) mice is sevenfold larger than that of controls. The lymph node weight of MRL-Fas(lpr) increases approximately 75-fold over controls and renal pathology occurs extensively in MRL-Fas(lpr) at approximately 4 months of age and die usually before 6 months of age.

Serologically, MRL-Fas(lpr) mice have fivefold increase in gamma-region proteins. Twofold increases in IgA, IgM, and IgG2b and six fold increases in IgG1 and IgG2a have been identified by radial immunodiffusion. Sera of MRL-Fas(lpr) mice are uniformly positive for antinuclear autoantibodies by 12 weeks of age. The presence of anti-single stranded DNA (anti-ssDNA), anti-dsDNA and anti-Smith (anti-Sm) autoantibodies is characteristic. Retroviral gp70 immune complexes and IgM and IgG rheumatoid factors are found in significant concentrations in MRL-Fas(lpr) mice[126].

The lungs of MRL-Fas(lpr) mice show extensive perivascular and peribronchial lymphocytic infiltration with only occasional atelectasis and exudates. Joints of MRL-Fas(lpr) mice show arthritic changes resembling those of rheumatoid arthritis[126]. Coronary artery disease and myocardial infarction can be a contributing cause of death. Near the end of their lifespan, MRL-Fas(lpr) mice develop erythematous skin lesions, necrosis of the ears, swollen feet, and, frequently, generalized edema. MRL-Fas(lpr) mice spontaneously develop lacrimal gland inflammatory lesions and are a model of human Sjogren's syndrome.

2. Research objectives

Current treatment for SLE including nonsteroidal anti-inflammatory drugs, corticosteroids, antimalarials and immunosuppressive drugs has significant side effects. Recent clinical trials for SLE using novel biological drugs have failed to achieve desired results. There is an unmet medical need for new drugs for SLE with a better safety profile. Considering all these findings we evaluated the role of two proteases namely aPC (serine protease) and Cat S (cysteine protease) as a novel therapeutic targets in the pathogenesis of SLE.

1. Our first objective was to study the effect of exogenous administration of aPC on systemic inflammation in MRL-Fas(lpr) mouse lupus model. Systemic lupus erythematosus (SLE) is a chronic autoimmune disease leading to inflammatory tissue damage in multiple organs. The role of aPC in inflammatory conditions is well known. aPC is a natural anticoagulant, in addition it has cytoprotective activities like anti-inflammatory, anti-apoptotic, profibrinolytic and protects endothelial barrier. Recombinant aPC was recently shown to suppress a number of hyperinflammatory and autoimmune states; hence, we had hypothesized that recombinant aPC may also suppress SLE and lupus nephritis.

2. Our second objective was to study the effect of cathepsin S inhibitor R05461111 on the pathogenesis of lupus in MRL-Fas(lpr) mouse model. SLE is characterized by loss of self-tolerance and abnormally activated B cells, T cells and antigen presenting cells. Cat S is a lysosomal cysteine protease and is responsible for the final proteolysis of MHC-II associated chaperone invariant chain (Ii) in antigen-presenting cells, thus plays an important role in antigen presentation. Cat S has emerged as an attractive target and inhibitors of Cat S block the presentation of autoantigens. Since abnormal antigen presentation leads to autoimmunity, we hypothesized that Cat S inhibitor would suppress SLE and lupus nephritis.

3. Materials and Methods

3.1 Materials

Equipments

Balances:

Analytic Balance, BP 110 S	Sartorius, Göttingen, Germany
Mettler PJ 3000	Mettler-Toledo, Greifensee, Switzerland

Cell Incubators:

Type B5060 EC-CO 2	Heraeus Sepatech, München, Germany

Centrifuges:

Heraeus Minifuge T	VWR International, Darmstadt, Germany
Heraeus Biofuge primo	Kendro Laboratory Products GmbH, Hanau, Germany
Heraeus Sepatech Biofuge A	Heraeus Sepatech, München, Germany
Eppendorf centrifuge 5417 R	Eppendorf AG, Hamburg, Germany

ELISA-Reader:

Tecan, GENios Plus	Tecan, Crailsheim, Germany

Fluorescence Microsocopes

Leica DC 300F	Leica Microsystems, Cambridge, UK
Olympus BX50	Olympus Microscopy, Hamburg, Germany

Spectrophotometer:

Beckman DU ® 530	Beckman Coulter, Fullerton, CA, USA
Nanodrop	Thermoscientific, Wilmington, DE, USA

Real time PCR:

LightCycler480	Roche diagnostics, Mannheim, Germany

Other Equipments:

Cryostat RM2155	Leica Microsystems, Bensheim, Germany
Cryostat CM 3000	Leica Microsystems, Bensheim, Germany
Homogenizer ULTRA-TURRAX T25	IKA GmbH, Staufen, Germany
Microtome HM 340E	Microm, Heidelberg, Germany
pH meter WTW	WTW GmbH, Weilheim, Germany
Thermomixer 5436	Eppendorf, Hamburg, Germany
Vortex Genie 2™	Bender&Hobein AG, Zurich, Switzerland
Water bath HI 1210	Leica Microsystems, Bensheim, Germany

Chemicals and materials

Chemicals for molecular biology techniques	
RNeasy Mini Kit	Qiagen GmbH, Hilden, Germany
qPCR primers	Metabion, Germany

Cell culture

RPMI-1640 medium	GIBCO/Invitrogen, Paisley, Scotland, UK
FSC	Biochrom KG, Berlin, Germany
Dulbecco's PBS (1×)	PAA Laboratories, Cölbe, Germany
Penicillin/Streptomycin (100×)	PAA Laboratories, Cölbe, Germany

Antibodies

anti-Mac-2	Cederlane, Ontario, Canada
anti-CD3	BD Pharmingen, Heidelberg, Germany
anti-CD4	BD Pharmingen, Heidelberg, Germany
anti-CD8	BD Pharmingen, Heidelberg, Germany
anti-CD25	BD Pharmingen, Heidelberg, Germany
anti-CD11c	BD Pharmingen, Heidelberg, Germany
anti-F4/80	AbD Serotec, Düsseldorf, Germany
anti-MHC-II	BD Pharmingen, Heidelberg, Germany
anti-B220	BD Pharmingen, Heidelberg, Germany

Miscellaneous

Needles	BD Drogheda, Ireland
Pipette's tip 1-1000µl	Eppendorf, Hamburg, Germany
Plastic histosettes	NeoLab, Heidelberg, Germany
Pre-separation filters	Miltenyi Biotec, Bergish Gladbach, Germany
SuperFrost® Plus Microscope slides	Menzel-Gläser, Braunschweig, Germany
Silver Impregnation Kit	Bio-Optica, Milano, Italy
Syringes	Becton Dickinson GmbH, Germany

Chemicals:

Acetone	Merck, Darmstadt, Germany
Bovine Serum Albumin	Roche, Mannheim, Germany
DEPC	Fluka, Buchs, Switzerland
DMSO	Merck, Darmstadt, Germany
EDTA	Calbiochem, SanDiego, USA
Ethanol	Merck, Darmstadt, Germany
Formalin	Merck, Darmstadt, Germany
HCl (5N)	Merck, Darmstadt, Germany
Isopropanol	Merck, Darmstadt, Germany
Potassium chloride	Merck, Darmstadt, Germany
Potassium dihydrogenphosphate	Merck, Darmstadt, Germany
Potassium hydroxide	Merck, Darmstadt, Germany
MACS-Buffer	Miltenyi Biotec, Bergisch Gladbach, Germany
Mercaptoethanol	Roth, Karlsruhe, Germany
Sodium acetate	Merck, Darmstadt, Germany
Sodium chloride	Merck, Darmstadt, Germany
Sodium citrate	Merck, Darmstadt, Germany
Penicillin	Sigma, Deisenhofen, Germany
SSC (Saline-sodium citrate Buffer)	Sigma, Deisenhofen, Germany
Tissue Freezing Medium	Leica, Nussloch, Germany
Trypan Blue	Sigma, Deisenhofen, Germany

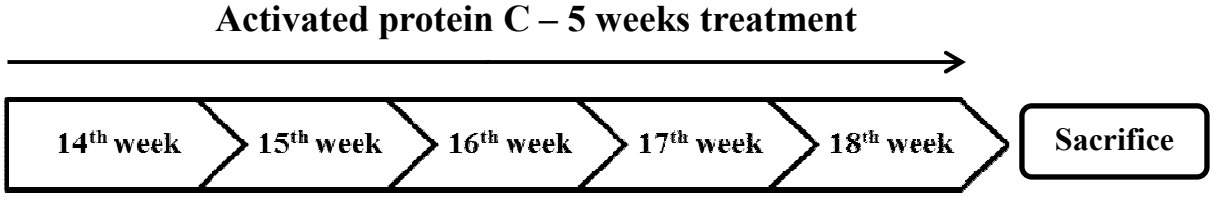
3.2 Methods

3.2.1 Animal experiments

Eight-week-old female MRL-Fas(lpr) mice were obtained from Harlan Winkelmann (Borchen, Germany) and kept under pathogen-free and normal housing conditions in a 12-h light and dark cycle. All experiments were performed according to German animal protection laws and had been approved by the local government authorities.

Experimental protocol - I

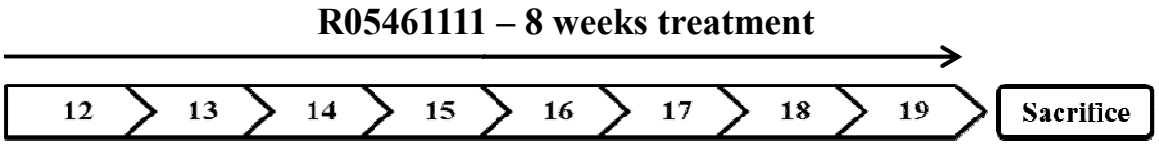
At the age of 14 wk, groups of 15 mice each started to receive either 5 mg/kg body weight mouse recombinant human aPC (Xigris™; Lilly Deutschland) or vehicle PBS (50 mM Tris, 150 mM NaCl, pH 7.6) only by i.p. injection on alternate days. Water and standard chow (Ssniff, Soest, Germany) were available *ad libitum*. All mice were sacrificed by cervical dislocation at the end of week 18 of age. Wild-type (wt) MRL mice were used as healthy controls. Blood and urine samples were collected from each mouse at the end of the study period.



Experimental protocol – II

At the age of 11 wk, proteinuria was assessed and only proteinuric mice were randomized to receive from week 12 either a medicated diet formulated by mixing the Cat S inhibitor (R05461111, 262.5mg/kg chow) or standard diet (vehicle). The mice consumed approximately 5 g food per day (1.31 mg drug per day) with no difference in food intake or body weight between the two groups. All mice were sacrificed by cervical dislocation at 20 weeks of age. Blood and urine samples were collected from each mouse during the 4th week of the study and at the end of the study period (8th week). For sheep IgG immunization study 8 week old male C57BL/6 mice were immunized on day 0

subcutaneously (s.c) in the both hind foot pads with 10µg of sheep IgG (Jackson immunoresearch laboratories, New market, Suffolk, UK) in an emulsion containing 50% (v/v) sterile PBS and 50% (v/v) Complete Freund's Adjuvant (sigma). On day 7, mice were re-challenged with 10µg of sheep IgG using retro-orbital injection. Ten days after immunization, the mice were sacrificed by cervical dislocation to collect blood and spleen tissue.



3.2.2 Quantification of activated protein C levels

The endogenous levels of aPC were estimated in plasma samples using an ELISA kit for aPC from Uscn Life Science (Wuhan, China). In this experiment pre-coated microplate plate has been used. The microplate used in this experiment has been pre-coated with an antibody specific to aPC. Standards or samples were added to the appropriate wells and incubated for 2h at 37ºC. Next, 100ul detection reagent A (Biotin-conjugated antibody specific for aPC) was added to the each well and incubated for 1h at 37ºC. After washing 3 times, 100ul detection reagent B (Avidin conjugated to Horseradish Peroxidase (HRP)) was added to the each well and incubated for 30 min at 37ºC. After washing 5 times, 90ul of the TMB substrate solution was added to each well and incubated for 15 min resulting in the development of a blue color. The color development was stopped using 50ul of stop solution. Then, using microplate reader absorbance was measured at 450nm. The concentration of aPC in the samples was then determined by comparing the O.D. of the samples to the standard curve.

3.2.3 Cathepsin S Inhibitor RO5461111

RO5461111 (CAS 1252637-46-9) is a competitive inhibitor of the active site of Cat S. The nitril function of RO5461111 allows covalent reversible inhibition of Cat S. It was provided by F. Hoffmann-La Roche, Ltd., Basel, Switzerland. The synthesis and drug development of RO5461111 has been described in WO 2010121918.

Recombinant cathepsins were produced in house or purchased from commercial vendors and enzymatic assay performed using the fluorogenic substrate (Z-Val-Val-Arg-AMC for human and mouse Cat S, human Cat L & Cat B, Z-Leu-Arg-AMC for human Cat K and Cat V). The generation of fluorescence over 10 min was measured using 10 μM of substrate and 1 nM of enzyme in the presence of increasing concentrations of RO5461111 to determine IC50 values. The cellular activity of RO5461111 was tested on B-cell lines of human (RAJI) or mouse (A20) origin. Increasing concentrations of RO5461111 was incubated overnight and then protein extracts prepared for detection of p10 upregulation by Cat S inhibition on western blots.

For the determination of *in vivo* enzyme inhibition activity of Cat S by RO5461111 we used normal BALB/c mice. After oral dosing of 0.1 mg/kg to 100 mg/kg of RO5461111 mice were sacrificed after 7 hours and spleens harvested. Protein extracts were separated by SDS-gel electrophoresis for the determination of p10 upregulation by Cat S inhibition as described below.

3.2.4 Morphological evaluation

Kidneys, spleens and lungs from all mice were fixed in 10% buffered formalin, processed and embedded in paraffin. Two-micrometer paraffin sections for periodic acid-Schiff (PAS) stains were prepared from spleen, lung and kidney tissue samples following routine protocols as described[127]. The severity of the renal lesions was graded using the indices for activity and chronicity as described for human lupus nephritis[128]. Cat S, IgG, C3c and Mac-2 immunostaining staining was performed using the following primary Abs: anti-cathepsin S antibody (Abcam, Cambridge, UK), anti-mouse IgG (Caltag Laboratories, Burlingame, CA), anti-mouse C3c (Nordic Immunological Laboratories, Tilburg, Netherlands) and anti-mouse Mac-2 (Cedarlane Laboratories, Toronto, Ontario, Canada). IgD immunostaining was performed on spleen tissue sections using rat anti-mouse IgG antibody (BD) to detect mantle B cells. Spleen tissue sections were also stained with the lectin peanut agglutinin (PNA) (Vector labs) as described[107], which binds to immature, proliferating B cells within the GC. The severity of the peribronchial inflammation was graded semi quantitatively from 0–4 as described[129].

3.2.5 In situ hybridization

In situ hybridization was performed using Quantigene ViewRNA ISH Tissue Assay Kit (Affymetrix / Panomics Solutions). The assay uses proprietary chemistry for the target specific probe sets and branched-DNA signal amplification for detection of a specific signal. Specific probe sets for mouse CTSS and EMR1 mRNAs were designed by Affymetrix based on the following sequences: NM_001267695 and NM_021281 for CTSS, NM_010130 for EMR1. Tissue sections of 4 μm prepared from Formalin Fixed Paraffin Embedded (FFPE) mouse kidney and spleen tissues, were mounted onto micro slides (X-tra^{TM} Adhesive, Leica) and processed according to manufacturer's instructions. Briefly, slides were baked for 1h at 60^{o}C (Thermobrite, Abbott Molecular) and fixed in 10% formaldehyde for 1h at RT. Deparaffinization was achieved using Histo-Clear reagent (National Diagnostics). Pre-hybridization conditions were found to be optimal with 10 minutes of boiling at 95^{o}C and 10 minutes incubation at 40^{o}C with Protease QF diluted 1/100x. Target probe set hybridization (Type-6 or Type-1 probe sets for blue or red staining respectively) was carried out for 2h at 40^{o}C. Hybridized probes were amplified using PreAmplifier Mix QT and Amplifier Mix QT oligonucleotides incubating slides at 40^{o}C for 25 and 15 minutes respectively. Slides were then exposed to Label Probe conjugated with alkaline phosphatase (Label Probe 6-AP or 1-AP for blue or red staining respectively) for 15 minutes at 40^{o}C and to Fast Blue or Fast Red Substrates at RT for 15 min or at 40^{o}C for 30 minutes respectively. When 2-Plex assays were carried-out, incubations with Label Probe 6-AP and Fast Blue Substrate were carried –out before incubations with Label Probe 1-AP and Fast Red Substrate. Slides were counterstained with Gill's Hematoxylin stain for 5-10 seconds at RT. Between each incubation, slides were washed 2-3 times in washing solution, PBS or water according to the manufacturer's instructions. Imaging was performed under brightfield with a Zeiss microscope equipped with an Axiocam MRC camera.

3.2.6 Plasma cytokines

The plasma levels of IL-6, IL-10, IL-12p40 and CCL2/MCP-1 were measured using commercial kits from BD (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Plasma levels of IL-2, IFN-γ and TNF-α were measured using

commercial kits from BioLegend (Fell, Germany). Absorbance was determined using a Tecan Microplate Reader (Biotek, Winooski, VT).

3.2.7 Serological analysis

For the detection of IgG and its isotypes, plates were precoated with anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG2c and IgG3 (Bethyl Laboratories, Montgomery, TX), and sera were applied at dilutions of 1:10,000 to 1:50,000. For the detection of IgM, plates were precoated with anti-mouse IgM (Bethyl Laboratories), and sera were applied at dilutions of 1:5000. The assay was developed with HRP-labeled goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG2c, IgG3 and IgM Abs. The plates were developed by adding TMB substrate to the wells and incubating for 15 min. The reaction was stopped by adding 2N H_2SO_4 and absorbance was measured at 450 nm. A standard curve was derived with purified mouse IgG, IgG1, IgG2a, IgG2b, IgG2c, IgG3 and IgM Abs.

Analysis of serum anti-dsDNA autoantibodies by ELISA

Serum dsDNA autoantibodies were determined by using commercial ELISA kits using the following antibodies: anti-mouse IgG, IgM, IgG1, IgG2a, IgG2b, IgG2c and IgG3 following the manufacturer's protocols. First, the NUNC maxisorp 96 well flat bottom ELISA plate was coated with 100 µl of poly-L-Lysine (Trevigen, Gaithersburg, MD, USA) and incubated for 1 hour at room temperature. After incubation, the plate was washed 5 times with PBS. After washing I added 100 µl of dsDNA (1 µg/ml) which was diluted in SSC buffer (1X) to the wells and incubated for overnight at 4ºC. For standards, the wells were coated with the capture antibody in coating buffer. After washing the plate 5 times with wash buffer, blocking solution was added to the wells and incubated for one hour. After incubation, diluted standards and samples were added in respective wells and incubated for 2 hours. After washing the plate the wells were incubated for 1 hour with secondary antibody. The plate was developed by adding TMB substrate to the wells and incubated for 15 min. The reaction was stopped by adding 2N H_2SO_4 and absorbance was measured at 450 nm.

Anti-Sm and anti Sm-RNP antibodies

NUNC maxisorp ELISA plates were coated with 1:250 diluted Smith (Sm) antigen (Immunovision, Springdale, AR, USA). An anti-Sm IgG (Y12) antibody was used as

standard. A horseradish peroxidase-conjugated goat anti-mouse IgG (2 mg/ml) was used as secondary antibody at a dilution of 1 µl in 50 ml in PBS (pH 7). After that TMB substrate was added to develop color and kept for 20 min in the dark. Later on the reaction was stopped by using 2N H_2SO_4. Absorbance was measured at 450 nm using a microplate reader (Tecan). The same procedure was followed for anti-SmRNP ELISA. Here the ELISA plates were captured with Sm-RNP complex (1000 units/ml) (Immunovision) at a dilution of 1:250 instead of Sm antigen.

Rheumatoid factor

NUNC maxisorp ELISA plates were coated with rabbit IgG at a concentration of 10µg/ml (Jackson Immunoresearch, West Grove, PA, USA) overnight at 4ºC. Serum samples were diluted 1:100 in PBS (pH 7), 10 weeks old C57BL/6 mouse serum was used as negative control. HRP conjugated goat anti-mouse IgG (2 mg/ml, Rockland Immunochemicals Research, Gilbertsville, PA, USA) was used as secondary antibody at a dilution of 1 µl in 50 ml of PBS (pH 7). TMB Substrate was used to develop the color and reaction was stopped by using 2N H_2SO_4. The absorbance was measured at 450 nm by using a microplate reader (Tecan).

3.2.8 Antinuclear antibody and Crithidia staining

Antinuclear antibody (ANA) is a general term used to describe autoantibodies against various cell nuclear proteins. ANA staining uses the indirect fluorescent antibody technique first described by Weller and Coons. Patient samples are incubated with antigen substrate to allow specific binding of autoantibodies to cell nuclei. If ANA's are present, a stable antigen-antibody complex is formed. After washing to remove non-specifically bound antibodies, the substrate is incubated with an anti-human antibody conjugated to fluorescein. When the results are positive, there is the formation of a stable three-part complex consisting of fluorescent antibody bound to a human antinuclear antibody, which binds to nuclear antigen. This complex can be visualized with the aid of a fluorescent microscope. In positive samples, the cell nuclei will show an apple-green fluorescence with a staining pattern characteristic of the particular nuclear antigen distribution within the cells. If the sample is negative for ANA, the nucleus will not show a clearly discernible pattern of nuclear fluorescence.

Serum samples were diluted to 1:50, 1:100 and 1:200 with PBS. Kallestad HEp-2 slide (ANA staining) or Kallestad *Crithidia luciliae* (*Crithidia* staining) slide was removed from the pouch and serum samples (20-25 µl) were added to the wells. The slide was incubated 30 minutes at room temperature in a moist covered chamber (a petri dish with moistened paper toweling will be adequate). The slide was removed from incubator tray and rinsed briefly with PBS using a squirt bottle. The slide was washed 10 minutes with PBS in a Coplin jar. The PBS wash solution was discarded after use. The slide was removed from PBS and dipped 3-5 times in distilled water. The slide was tapped on its side against bibulous paper or paper toweling to remove excess water. The slide was returned to the incubation chamber and covered the wells completely using fluorescent antibody reagent (Goat anti-mouse IgG-FITC, Invitrogen). The slide was incubated 30 minutes at room temperature in a moist chamber. During incubation, incubation chamber was covered with a paper towel to prevent exposure to light. The slide was removed from incubator tray and rinsed briefly with PBS. The slide was washed 10 minutes with PBS in a Coplin jar. The slide was removed from PBS and dipped 3-5 times in distilled water. The slide was tapped on its side against bibulous paper or paper toweling to remove excess water. 4-5 drops of mounting medium with DAPI was added along midline of each slide. The coverslip was placed on the slide carefully, avoiding air pockets, by gently lowering the coverslip from one end of the slide to the other. The slide was observed under fluorescent microscope.

3.2.9 Western blotting

Invariant chain p10 accumulation was detected in spleen tissue by western blotting. Spleen tissues from the vehicle and treated animals were homogenized in RIPA buffer with protease inhibitors. Lysates were electrophoresed and proteins were transferred to PVDF membrane. Following transfer, I blocked membrane with 5% nonfat dry milk for 30 min at room temperature. The membrane was incubated with CD74 primary antibody (BD Pharmingen, Heildelberg, Germany) at 4ºC overnight. After washing 3 times with TBST, the membrane was incubated with anti-rabbit HRP secondary antibody. After washing 5 times with TBST, the membrane was developed with ECL (GE Healthcare, Buckinghamshire, UK). The chemiluminescent signal was detected and recorded by exposure of the membrane to a light-sensitive X-ray film.

3.2.10 Renal function analysis

Urinary albumin to creatinine ratio, plasma creatinine and plasma BUN

Urinary albumin levels were determined using an albumin Elisa kit from Bethyl laboratories following manufacturer's instructions. Generally albumin levels in urine samples from MRL-Fas(lpr) mice were quite high, so urine samples were diluted to 2000 times with water before estimation. In short, capture antibody (Anti-mouse albumin, 1:100 dilution) was coated on polyethylene flat bottom 96 well plates (Nunc plates) using carbonate-bicarbonate (pH 9.6) coating buffer. After overnight incubation of the capture antibody at 4ºC, the plate was washed 3 times with wash buffer (50mM Tris, 0.14M NaCl, 0.05% Tween 20, pH 8) and blocked with blocking solution (50mM Tris, 0.14M NaCl, 1% BSA, pH 8) at room temperature for 1 hour. After blocking, the plate was washed 5 times with wash buffer and then diluted samples/standards were added to the respective wells and further incubated for 1 hour. After incubation, the plate was washed 5 times with wash buffer and diluted HRP-conjugated detection antibody (using the suggested dilution) was added and the plate was incubated in dark for 1 hour. After incubation, the plate was washed 5 times with wash buffer and TMB reagent (freshly prepared by mixing equal volumes of two substrate reagents) was added and incubated in the dark till color reaction was completed followed by addition of stop solution (2N H_2SO_4). The absorbance was read at 450 nm. The albumin content in each sample was determined using the equation of regression line generated by plotting absorbance of different standards against their known concentrations.

Urinary creatinine and plasma creatinine levels were measured using Jaffe´s enzymatic reaction using a Creatinine FS kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). Urine samples were diluted 10 times with distilled water whereas plasma samples were used undiluted. Different dilutions of standard were prepared using the stock provided with the kit. Working monoreagent was prepared by mixing 4 parts of reagent 1 (R1) and 1 part of reagent 2 (R2) provided with the kit. Then, 10 µl of each of the diluted samples and standards were added to a 96 well plate with flat bottom (Nunc maxisorb plate). The monoreagent (200 µl) was added to each well and the reaction mixture was incubated for one minute before measuring the absorbance at 492 nm immediately after and 1 (A1) and 2 (A2) min of addition using an ELISA plate reader. The

change in absorbance (ΔA) was calculated as ΔA = [(A2 – A1) sample or standard] – [(A2 – A1) blank]. The creatinine content of the samples was calculated as:

Creatinine (mg/dL) = ΔA sample /ΔA standard * Concentration of standard (mg/dL)

Plasma BUN levels were measured using an enzymatic reaction using a Urea FS kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). Different dilutions of standard were prepared using the stock provided with the kit. Working monoreagent was prepared by mixing 4 parts of reagent 1 (R1) and 1 part of reagent 2 (R2) provided with the kit. Then, 2 µl of each of the sample and standards were added to a 96 well plate with flat bottom (Nunc maxisorb plate). The monoreagent (200µl) was added to each well and the reaction mixture was incubated for one minute before measuring the absorbance at 360 nm immediately after and 1 (A1) and 2 (A2) min of addition using an ELISA plate reader. The change in absorbance (ΔA) was calculated as ΔA = [(A1 – A2) sample or standard] – [(A1 – A2) blank]. The BUN content of the samples was calculated as:

BUN (mg/dL) = ΔA sample /ΔA standard * Concentration of standard (mg/dL)*0.467

Urinary albumin to creatinine ratio was calculated after converting values for albumin and creatinine to similar units (mg/dL). The albumin content for each sample calculated (mg/dL) was divided by creatinine content (mg/dL) for the same sample.

Glomerular filtration rate (GFR)

Mice were anesthetized using Isoflurane, which for approximately 20 seconds. 5% FITC-inulin was dissolved in two ml of 0.9% NaCl - facilitated by heating the solution in boiling water. 5% FITC-inulin (3.74µl /g body weight) was injected retroorbitally under anesthesia within 10 seconds. Under general anaesthesia, blood was drawn from the retro orbital plexus at 5, 10, 15, 20, 35, 60 and 90 minutes post administration. Since pH significantly affects FITC fluorescence value, each plasma sample was buffered to pH 7.4, by mixing 10 µl of plasma with 40 µl of 500 mM HEPES (pH 7.4). The titrated samples were then loaded onto a 96-well plate, 50µl sample/well. Fluorescence was determined with 485 nm excitation, and read at 538 nm emission.

A two - compartment clearance model may be employed for the calculation of GFR. In the two-compartment model used, depicted in Figure 8, the initial, rapid decay phase represents redistribution of the tracer from the intravascular compartment to the extracellular fluid. Systemic elimination also occurs, but the distribution process is relatively dominant during this initial phase. During the later, slower decay in concentration of the tracer systemic clearance of the tracer from the plasma predominates. At any given time (t_x), the plasma concentration of the tracer (Y) equals to

$$Ae^{-\alpha tx} + Be^{-\beta tx} + Plateau$$

The parameters of above equation could be calculated using a non-linear regression curve-fitting program (GraphPad Prism, GraphPad Software, Inc., San Diego, CA). GFR was calculated using the equation:

$$GFR = I/(A/\alpha + B/\beta),$$

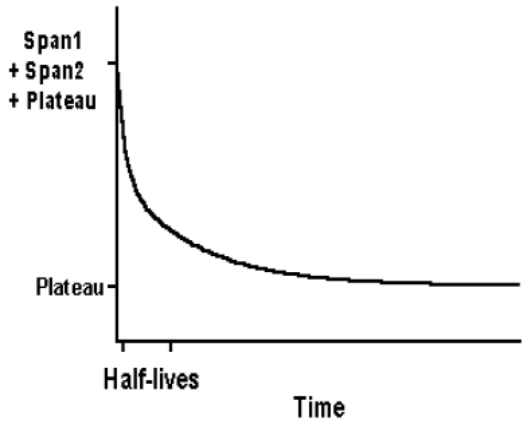


Figure 8: Representation two phase regression curve

where I is the amount of FITC-inulin delivered by the bolus injection; A (Span1) and B (Span2) are the y-intercept values of the two decay rates, and α and β are the decay constants for the distribution and elimination phases, respectively.

3.2.11 Flow cytometry

Spleen and kidney cells were obtained from mice of all groups at the end of the study for FACS analysis.

Isolation and preparation of murine spleen cells

Spleen was isolated under sterile conditions from the MRL-Fas(lpr) mice. Spleen was placed in a sterile petri plate containing 2-3 ml of ice-cold media (RPMI + 10% FCS + 1% PS) on ice. Then it was mashed using forceps in the petri plate itself and broken into fine pieces of tissues. Then the cell suspension was passed through 70 µm cell strainer (BD Biosciences). This single cell suspension was collected into a 15 ml Falcon tube and centrifuged at 1500 rpm at 4°C for 5 min. The supernatant was decanted and the pellet was resuspended in 3 ml of sterile 0.3M NH_4Cl (RBC lysis buffer) and incubated at room temperature 5 min. Then it was centrifuged at 1500 rpm at 4°C for 5 min. Supernatant was decanted and the pellet was resuspended in ice cold PBS and passed through 30 µm cell strainer (BD Biosciences). The flow through was centrifuged at 1500 rpm at 4°C for 5 min. Supernatant was decanted and the pellet was resuspended in ice-cold FACS buffer (PBS, 0.2% BSA, 0.1% sodium azide).

Isolation and preparation of murine kidney cells

Kidneys were isolated under sterile conditions from MRL-Fas(lpr) mice. The capsule was removed from the kidneys and placed in a petri dish containing 3ml of digestion buffer (collagenase + DNAse I). Inject digestion buffer into the kidneys several times and incubate at 37°C for 30 min. After incubation the kidneys were mashed with the help of a plunger and incubated again for 15 min. Single cell suspension was then prepared by repeatedly passing it through (5 times) 20G, 25G and 26G needles. The cell suspension was filtered through a 70 µm cell strainer and transferred into a 15ml falcon tube and the volume was adjusted to 10 ml with PBS and centrifuged at 1500 rpm for 5 min at 4°C. After centrifugation supernatant was discarded and the pellet was resuspended in PBS and filtered through a 30 µm cell strainer. The flow through was centrifuged again at 1500 rpm for 5 min at 4°C. After centrifugation supernatant was discarded and the pellet was resuspended in FACS buffer.

Staining of spleen and kidney cells for FACS

Cell suspensions were stained with the following Abs from BD Pharmingen (Heidelberg, Germany): anti-mouse CD4-allophycocyanin, CD8-PerCP, CD3-FITC, CD25-PerCP, CD45R-allophycocyanin, CD11c-FITC, B220-Alexa Fluor 647, IgD-FITC, IgM-PE, CD23-PE, CD21-FITC, CD40-FITC, and κ light Chain-PE (intracellular). The following Abs were procured from Serotec (Oxford, U.K.): F4/80-allophycocyanin, CD19-FITC, and MHC-II-FITC. Siglec-H is from eBioscience (San Diego, CA). Intracellular IFN-γ and IL-17A staining was performed as described elsewhere[130]. Briefly, splenocytes were isolated by standard protocols and incubated with PMA (5 ng/ml; Sigma-Aldrich) and ionomycin (1 mg/ml; Calbiochem) for 5 h. After 30 min, brefeldin A (10 mg/ml; Sigma-Aldrich) was added. After washing, cells were stained for CD3 and CD4. After permeabilization of the cells with Cytofix/Cytoperm (BD Biosciences), cells were stained with IFN-γ –PE (eBioscience) and IL-17A–Alexa Fluor 647 (BD Biosciences). To analyze the effect of aPC on short-lived and long-lived plasma cells, we injected BrdU (FITC-BrdU, BD Biosciences) in mice 5 days before sacrifice, which allowed us to differentiate short- from long-lived plasma cells.

3.2.12 RNA isolation, cDNA synthesis and Real-time PCR

RNA isolation

Mice were sacrificed on termination of the study, tissue samples from each mouse were preserved in RNA-later at -20°C until processed for RNA isolation. Total RNA was isolated from spleen and kidney tissue using PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA). In short, tissues (30 mg) preserved in RNA-later were homogenized using blade homogenizer for 30 seconds at 14500 rpm in lysis buffer (600 µl) containing β-mercaptoethanol (10 µl/ml). The homogenate was centrifuged at 14000 rpm for 3 min and 500 µl of supernatant was transferred to fresh DEPC-treated tube and to this tube equal amount (500 µl) of 70 % ethanol was added. The solution was mixed thoroughly by vortexing to disperse any visible precipitate that form after adding ethanol. 700 µl of whole mixture was transferred to the spin catridge (with the collection tube) and centrifuged at 12,000 x g for 15 seconds at room temperature. Flow-through was discarded and reinserted the spin cartridge into the same collection tube. Repeated the above step until the entire sample has been processed. Next, 350 µl of wash buffer I was added to the spin cartridge

containing the bound RNA and centrifuged at 12,000 x g for 15 seconds at room temperature. Flow-through and the collection tube were discarded. The spin cartridge was inserted into a new collection tube. 80 µl PureLink® DNase mixture (10 µl DNase + 70 µl reaction buffer) was added directly onto the surface of the spin cartridge membrane and incubated at room temperature for 15 minutes. After incubation, 350 µl wash buffer I was added to the spin cartridge and centrifuged at 12,000 x g for 15 seconds at room temperature. Flow-through and the collection tube were discarded and inserted the spin cartridge into a new collection tube. Next, 500 µl wash buffer II with ethanol was added to the spin cartridge and centrifuged at 12,000 x g for 15 seconds at room temperature. Flow-through was discarded and reinserted the spin cartridge into the same collection tube. Again 500 µl wash buffer II with ethanol was added to the spin cartridge and centrifuged at 12,000 x g for 15 seconds at room temperature. Flow-through was discarded and reinserted the spin cartridge into the same collection tube. The spin cartridge was centrifuged at 12,000 x g for 1–2 minutes to dry the membrane with bound RNA. Collection tube was discarded and inserted the spin cartridge into a recovery tube. 30-50µl RNase-free water was added to the center of the spin cartridge. Spin catridge was incubated at room temperature for 2 minutes. After the incubation, the spin cartridges were centrifuged for 2 minutes at 8000 x g at room temperature to elute the RNA from the membrane into the recovery tube. Isolated RNA was stored at -80°C until further used.

For quantification isolated RNA samples, 1µl of RNA sample was added on to the nanodrop and absorbance was measured at two wavelengths as 260 nm and 280 nm. The ratio of optical densities at 260 nm and 280 nm is an indicator of RNA purity (indicative of protein contamination in the RNA samples). Only samples with a ratio of 1.8 or more were considered to be of acceptable quality.

A further quality check was performed using a denaturing RNA gel. In short 2 % Agarose gel with Ethidium-bromide was cast, RNA samples were mixed with RNA loading buffer (4:1 ratio) (Sigma) and were loaded on the gel. Electrophoresis was carried out at constant volt (70-100 V) using MOBS running buffer for 1 hour and the gel was read on a gel documentation apparatus under a UV lamp. RNA samples showing a single bright band were considered to be of good quality. Loss of RNA integrity could be detected as smear formation in the agarose gel.

cDNA synthesis

cDNA was synthesized from 1 µg total RNA by using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen). The enzyme is used to synthesize first-strand cDNA and it can generate cDNA up to 12.3 kb. Random Hexamers are short oligodeoxyribonucleotides of random sequence. Random hexamer primers bind throughout the entire length of RNA, ensuring reverse transcription of all RNA sequences due to their random structure. Each reaction tube contained 4 µl of 5X First strand buffer, 0.5 µl of SuperScript II Reverse Transcriptase, 0.5 µl of RNase, 2µl of 0.1M DTT, 0.5 µl of dNTP, 0.5µl of random hexamers, 1µg of total RNA and made up the volume to 20 µl with sterile milli-Q water. After mixing the contents of the tube gently, tubes were incubated at 25°C for 10 min. The cDNA synthesis reaction took place at 42°C for 1 hr and then the reaction was inactivated by incubating at 70°C for 10 min. This cDNA was used as a template for amplification in real time PCR.

Real time PCR using SYBR Green I

SYBR Green I Dye detection system was used for quantitative PCR (qPCR) on LightCycler® 480 (Roche Diagnostics, Mannheim, Germany). The sequences of the gene-specific primers (Metabion, Martinsried, Germany) used are listed in Table 5. The conditions for real-time PCR were as follows: 95˚C for 3 min (1 cycle), 94˚C for 20 sec, 60˚C for 20 sec, and 72˚C for 40 sec (45 cycles), followed by melting curve program. Each reaction consisted of 10 µl of 2X SYBR Green I mix, 0.5 µl of 10 mM forward primer, 0.5 µl of 10 mM reverse primer, 0.2 µl (1 unit) of Taq DNA polymerase, 4 µl of cDNA and made up the volume to 20 µl with milli-Q water. 96 well PCR reaction plate was centrifuged briefly to spin down the contents and to eliminate air bubbles before starting the reaction. Real time PCR analysis was done using LightCycler®480 software. All reactions were performed in triplicate, and negative controls contained no template DNA. We used 18S rRNA as an endogenous control for normalization. To verify that the used primer pair produced only a single specific product, a dissociation protocol was added after thermocycling, to determine the dissociation of the PCR products from 60˚C to 95˚C. Data were analyzed using the comparative threshold cycle (ΔΔCt) method.

Table 5. Primer sequences used for Real Time - PCR

Gene	*Accession number*	*Primer Sequence*
CCL2/MCP-1	NM_011333	F : 5'- CCTGCTGTTCACAGTTGCC -3' R : 5'- ATTGGGATCATCTTGCTGGT -3'
IL-6	NM_031168	F : 5'- TGATGCACTTGCAGAAAACA -3' R : 5'- ACCAGAGGAAATTTTCAATAGGC -3'
IL-12p40	NM_008352	F : 5'- AGCAGTAGCAGTTCCCCTGA -3' R : 5'- AGTCCCTTTGGTCCAGTGTG -3'
18S rRNA	NR_003278	F : 5'- GCAATTATTCCCCATGAACG -3' R : 5'- AGGGCCTCACTAAACCATCC -3'
CXCL10	NM_021274	F : 5'- GGCTGGTCACCTTTCAGAAG -3' R : 5'- ATGGATGGACAGCAGAGAGC -3'
TNF	NM_013693.2	F : 5'- CCACCACGCTCTTCTGTCTAC -3' R : 5'- AGGGTCTGGGCCATAGAACT -3'
IL-12p35	NM_001159424.1	F : 5'- CTAGACAAGGGCATGCTGGT -3' R : 5'- GCTTCTCCCACAGGAGGTTT-3'
IL-10	NM_021274	F : 5'- TGTCAAATTCATTCATGGCCT -3' R : 5'- ATCGATTTCTCCCCTGTGAA -3'
IL-1β	NM_008361	F : 5'- TTCCTTGTGCAAGTGTCTGAAG -3' R : 5'- CACTGTCAAAAGGTGGCATTT -3'
IL-23p19	NM_031252.2	F : 5'- AATAATGTGCCCCGTATCCAGT -3' R : 5'- GCTCCCCTTTGAAGATGTCAG -3'
TGF-β1	NM_011577.1	F : 5'- CAACCCAGGTCCTTCCTAAA -3' R : 5'-GGAGAGCCCTGGATACCAAC -3'
CXCL12	NM_001012477.2	F : 5'- GCGCTCTGCATCAGTGAC -3' R : 5'- TTTCAGATGCTTGACGTTGG -3'
IFN-γ	NM_008337.3	F : 5'- ACAGCAAGGCGAAAAAGGAT -3' R : 5'- TGAGCTCATTGAATGCTTGG-3'
April	NM_001159505.1	F : 5'- GTTGCTCTTTGGTTGAGTTGGG -3' R : 5'- GTTGGATCAGTAGTGCGACAGC -3'
BAFF	NM_033622.1	F : 5'- CCACCGTGCCTCTGTTTTTGC -3' R : 5'- AGCCAACTGGTACAAGGACATCG -3'
Taci	NM_021349.1	F : 5'- GGTCCAGGATTGAGGCTAAGTA -3' R : 5'- GGAGAGTTTGCTTGTGACCCA -3'
Rorc	NM_011281.2	F : 5'- GGTGATAACCCCGTAGTGGA -3' R : 5'- ACAGAGACACCACCGGACAT -3'
Gata3	NM_008091.3	F : 5'- CTCGGCCATTCGTACATGGAA -3' R : 5'- GGATACCTCTGCACCGTAGC-3'
Foxp3	NM_001199347.1	F : 5'- CCCATCCCCAGGAGTCTTG -3' R : 5'- ACCATGACTAGGGGCACTGTA -3'
T-bet	NM_019507.2	F : 5'- CACTAAGCAAGGACGGCGAATGT -3' R : 5'- CTGGGTGGACATATAAGCGGTTC-3'
CCL5	NM_013653.3	F : 5'- CCACTTCTTCTCTGGGTTGG -3' R : 5'- GTGCCCACGTCAAGGAGTAT -3'
Cathepsin S	NM_001267695.1	F : 5'- AAGCGGTGTCTATGACGACCC -3' R : 5'- GAGTCCCATAGCCAACCACAAG -3'

Cathepsin L	NM_009984.3	F : 5'- GTGGACTGTTCTCACGCTCA -3'
		R : 5'- TATCCACGAACCCTGTGTCA-3'
Cathepsin B	NM_007798.3	F : 5'- GCAGGCTGGACGCAACTTCTAC -3'
		R : 5'- GGTCTCTAATCTGTCCAATGGTCG -3'
Cathepsin C	NM_009982.4	F : 5'- GAAGTTGGATACTGCCTACGACGAG -3'
		R : 5'- CACATCATGGACCCACCCAGTC -3'
Cathepsin D	NM_009983.2	F : 5'- CGCCTAAGACCACGGAGCCA -3'
		R : 5'- ACTTATGGTGGACCCAGCAGGC -3'
Cathepsin H	NM_007801.2	F : 5'- GCTGACCGTGAACGCCATAGA -3'
		R : 5'- GCTGTACGTCTTTTGATGCTGTTTCA -3'

3.2.13 Statistical analysis

Data are expressed as means ± SD. The statistical differences between two groups were performed using a Student *t* test, and comparison of three groups was performed using one-way ANOVA. A p value < 0.05 was considered statistically significant. All statistical calculations were performed using commercially available statistical software GraphPad Prism (version 5.0; GraphPad, San Diego, CA). GraphPad Prism combines scientific graphing, comprehensive curve fitting (nonlinear regression), understandable statistics, and data organization.

4. Results

4.1 Part I – Activated protein C in SLE

4.1.1 Recombinant aPC suppresses renal pathology in MRL-Fas(lpr) mice.

We addressed our hypothesis by observing MRL-Fas(lpr) mice until the first signs of lupus nephritis occurred (i.e., albuminuria of >3 µg/dL, which was evident in almost all animals at 14 wk of age). Note that serum levels of aPC in MRL-Fas (lpr) mice were significantly lower compared with MRL wt mice at 14 wk of age (Figure 9). Albuminuric MRL-Fas(lpr) mice were randomized to two treatment regimens with i.p. injections given on alternate days for 5 weeks. One group was treated with recombinant aPC at a dose of 5 mg/kg in PBS, whereas the other group received PBS (vehicle) injections only. At 18 wk of age, vehicle-treated MRL-Fas(lpr) mice developed diffuse proliferative glomerulonephritis, which was associated with diffuse mesangial matrix expansion, profound mesangial cell proliferation, and focal segmental glomerulosclerosis. Cellular crescents and global glomerulosclerosis were often seen (Figure 10A). Most animals revealed profound tubulointerstitial inflammation as characterized by periglomerular and diffuse interstitial leukocyte infiltrates, tubular atrophy, and intraluminal cast formation. On immunostaining, diffuse proliferative glomerulonephritis was associated with extensive glomerular IgG and complement factor C3c deposits presenting in mesangial and capillary staining patterns altogether, indicating diffuse proliferative lupus nephritis as the renal manifestation of SLE in MRL-Fas(lpr) mice (Figure 10A, 10B).

Treatment with recombinant aPC suppressed lupus nephritis as documented by a significant reduction of the lupus nephritis activity index that encompasses glomerular cell proliferation, matrix deposition, leukocyte infiltration, focal necrosis, crescent formation, and tubulointerstitial inflammation (Figure 10B). There was no significant difference in the chronicity index that encompasses glomerular sclerosis, fibrous crescents, tubular fibrosis and interstitial fibrosis (Figure 10B). Glomerular crescents and globally sclerotic glomeruli were no longer observed (Figure 10A). This improvement of renal structure was associated with a significant reduction in plasma BUN levels and a trend towards less proteinuria.

Serum creatinine levels in our model were low and not significantly reduced in aPC-treated mice (Figure 11). Together, recombinant aPC attenuates lupus-like renal pathology in MRL-Fas(lpr) mice.

4.1.2 Recombinant aPC reduces renal immune complex disease in MRL-Fas(lpr) mice

In SLE, renal damage and dysfunction result from immune complex glomerulonephritis, in which glomerular deposits of immune complexes activate the components of the classical complement pathway, which finally leads to the assembly of the C5b-9 multimer membrane attack complex[131]. The favorable effects of recombinant aPC treatment on markers of active lupus nephritis were associated with a significant reduction of glomerular IgG deposits as well as the deposition of complement factor C3c, a marker of intraglomerular complement activation (Figure 10A, 10B). Together, we conclude that aPC treatment reduces SLE-associated immune complex glomerulonephritis and glomerular complement activation.

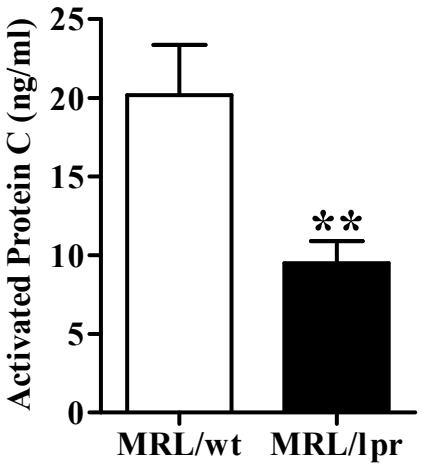


Figure 9. Plasma levels of aPC was quantified by ELISA in the wild type MRL and MRL-Fas(lpr) mice. **p<0.01. (Wild type MRL n=5, MRL-Fas(lpr) n=15).

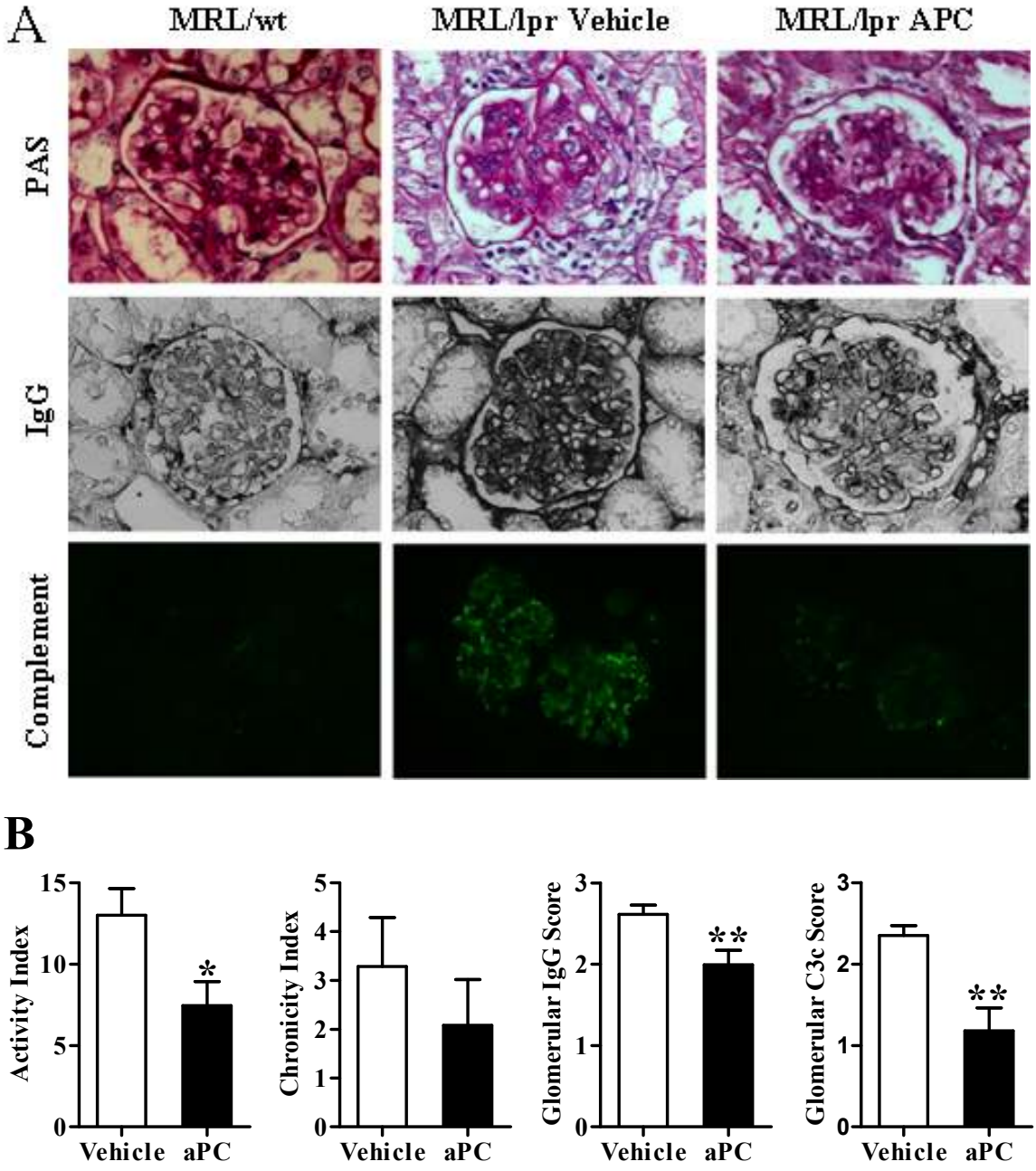


Figure 10. Recombinant aPC suppresses renal pathology in MRL-Fas(lpr) mice. (A) Renal sections of 18-wk-old MRL-Fas(lpr) mice were stained with PAS. Representative PAS staining kidney sections showed glomerular damage in vehicle-injected mice when compared with aPC-injected mice. MRL control mice showed no glomerular abnormalities. Renal sections of 18-wk-old MRL-Fas(lpr) mice were stained with Abs for IgG and complement C3c as indicated. Note that aPC-injected mice show less glomerular IgG and C3c deposits. Images are representative for 15 mice in each treatment group and 5 MRL wt mice. Original magnification x400 (PAS and IgG) or x200 (C3c). (B) Quantitative measures of lupus nephritis in MRL-Fas(lpr) mice. The lupus nephritis disease activity index (score ranging from 0–24) and the lupus nephritis chronicity index (score ranging from 0–12) were determined as markers of kidney damage in lupus nephritis. Glomerular IgG and complement C3c deposition was compared between vehicle- and aPC-treated groups (score ranging from 0–3). Data are expressed as means ± SEM. *$p<0.05$, **$p<0.01$ versus vehicle group (vehicle n=15; aPC n=15).

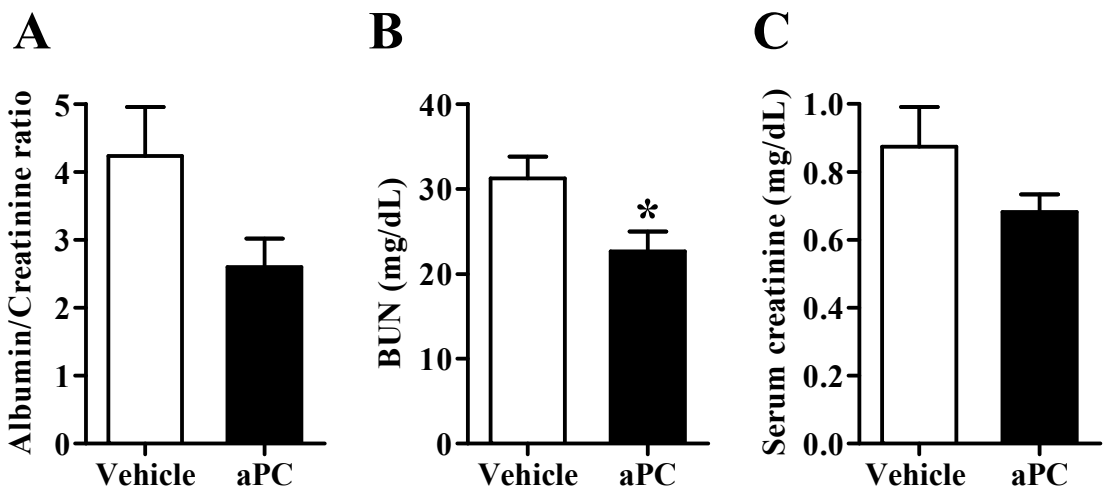


Figure 11. Proteinuria, blood urea nitrogen (BUN) and serum creatinine levels in MRL-Fas(lpr) mice. (A) Proteinuria was determined at 18 weeks of age. Urinary albumin/creatinine ratio is a functional marker of damage to the glomerular filtration barrier. The albumin/creatinine ratio was reduced substantially in aPC-injected mice when compared to vehicle-injected mice. (B) BUN levels at 18 weeks of age were significantly reduced in aPC-treated mice. (C) Serum creatinine levels were low in MRL-Fas(lpr) mice. There was no significant reduction in aPC treated mice. Data are expressed as means ± SEM. *$p<0.05$ versus vehicle group (Vehicle n=15, aPC n=15).

4.1.3 Recombinant aPC reduces intrarenal inflammation in MRL-Fas(lpr) mice

Local complement activation has the potential to activate (and eventually kill) renal cells; therefore, we next evaluated the intrarenal expression of pro-inflammatory mediators. Recombinant aPC treatment reduced mRNA expression levels of the proinflammatory cytokines IL-6 and IL-12p40 and of the chemokine CCL2/MCP-1 (Figure 12A), which correlated with a significant reduction of Mac-2 positive glomerular macrophages (Figure 12B). Together, we conclude that aPC treatment reduces intrarenal inflammation.

4.1.4 Recombinant aPC improves cutaneous lupus in MRL-Fas(lpr) mice

Is the therapeutic effect of recombinant aPC limited to renal manifestations of SLE? To address this question, we carefully monitored the skin of MRL-Fas(lpr) mice throughout the duration of the study. Skin disease is the most common organ manifestation in SLE affecting up to 85% SLE patients[3]. Skin disease includes three major subtypes: chronic lupus erythematosus, subacute lupus erythematous and acute lupus erythematosus.

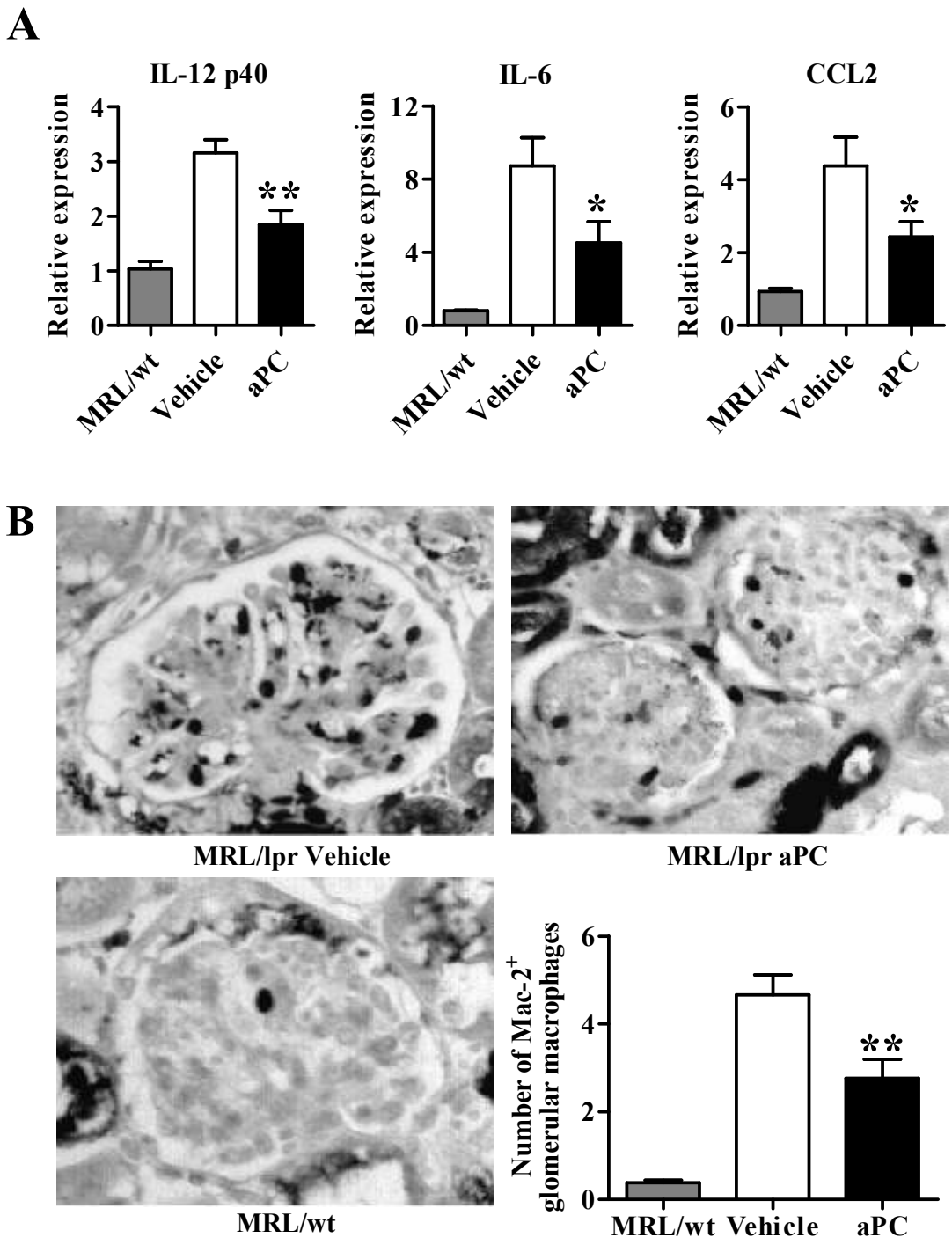


Figure 12. Kidney qPCR and glomerular macrophages. (A) Renal mRNA expression levels of IL-12p40, IL-6 and CCL2/MCP-1 in MRL-Fas(lpr) mice were determined by qPCR. Data are expressed as a mean ratio to the respective 18S rRNA mRNA expression level ± SEM. (B) Renal sections of 18-wk-old MRL-Fas(lpr) mice were stained with an anti–Mac-2 antibody. Representative images show Mac-2 positive glomerular macrophages and quantification of Mac-2 positive glomerular macrophages. Images are representative for 15 mice in each treatment group and 5 mice in MRL wt group. Data are expressed as means ± SEM. Original magnification x100. *p<0.05, **p<0.01 versus vehicle group (wt MRL n=5; Vehicle n=15; aPC n=15).

Treatment with recombinant aPC significantly delayed the onset of facial dermatitis as well as of neck skin ulcerations as compared with vehicle-treated MRL-Fas(lpr) mice (Figure 13A). During that period, all vehicle-treated MRL-Fas(lpr) mice developed a skin disease characterized by typical lesions in the facial area (Figure 13B). In contrast, among aPC-treated MRL-Fas(lpr) mice, 20% developed skin lesions. Thus, recombinant aPC protects MRL-Fas(lpr) mice not only from lupus nephritis but also from cutaneous lupus.

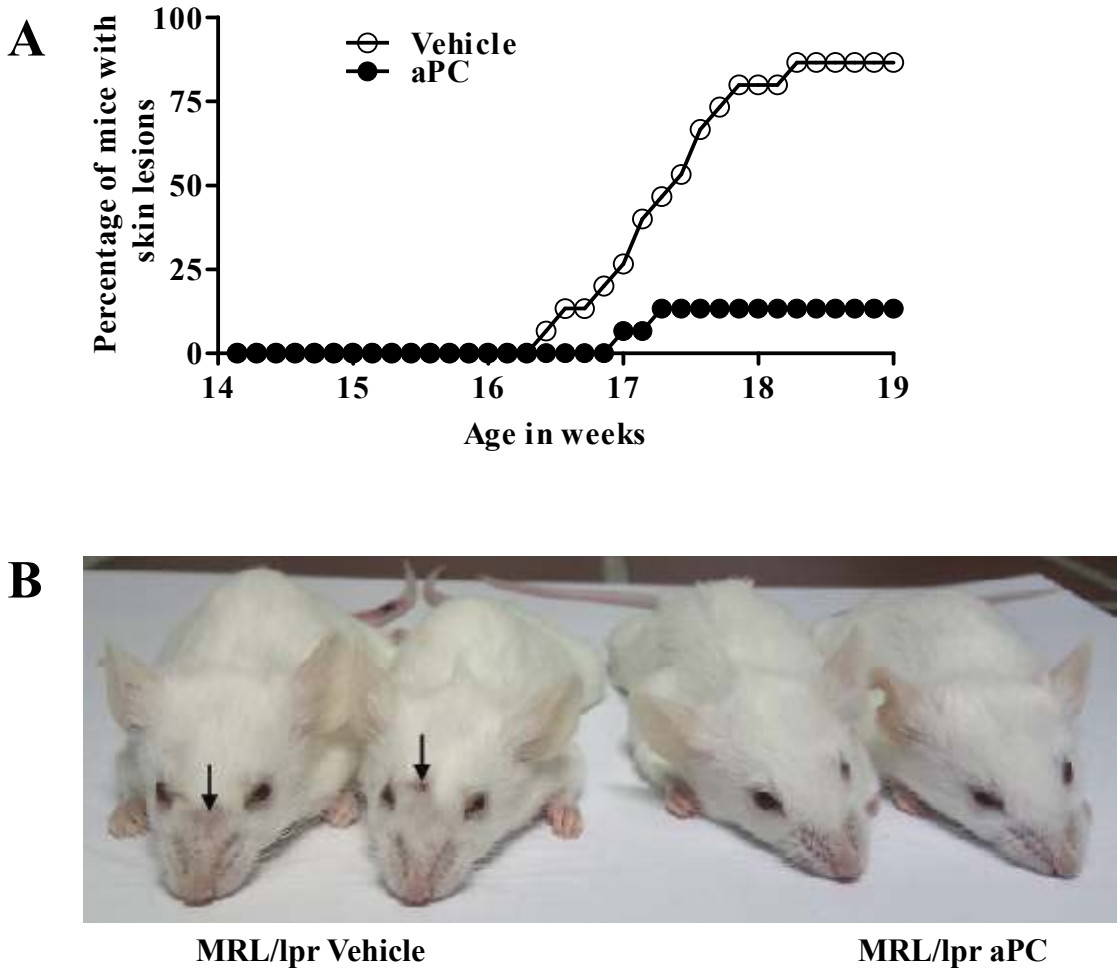


Figure 13. Recombinant aPC improves cutaneous lupus in MRL-Fas(lpr) mice. (A) The plot shows the percentage of mice developing skin lesions during the treatment period. Mice of both groups were regularly checked for cutaneous lupus manifestations, which typically occur in the facial or neck/back area and were less common in aPC-injected mice. (B) The image shows representative mice from both groups at 18 wk of age.

4.1.5 Recombinant aPC improves lung disease in MRL-Fas(lpr) mice

The autoimmune lung disease is another manifestation of SLE[132]. More than 50% of people with lupus have an autoimmune lung disease. Inflammation of the lining of the lung (pleurisy) is the most common problem. The lungs of MRL-Fas(lpr) mice show extensive perivascular and peribronchial lymphocytic infiltration with occasional atelectasis and exudates. We carefully analyzed the lungs of MRL-Fas(lpr) mice upon sacrifice. Vehicle-treated MRL-Fas(lpr) mice displayed focal areas of peribronchial and perivascular lymphocyte infiltrates. Such infiltrates were significantly decreased in 18-wk-old aPC-treated MRL-Fas(lpr) mice. This was evaluated by a semiquantitative lung injury

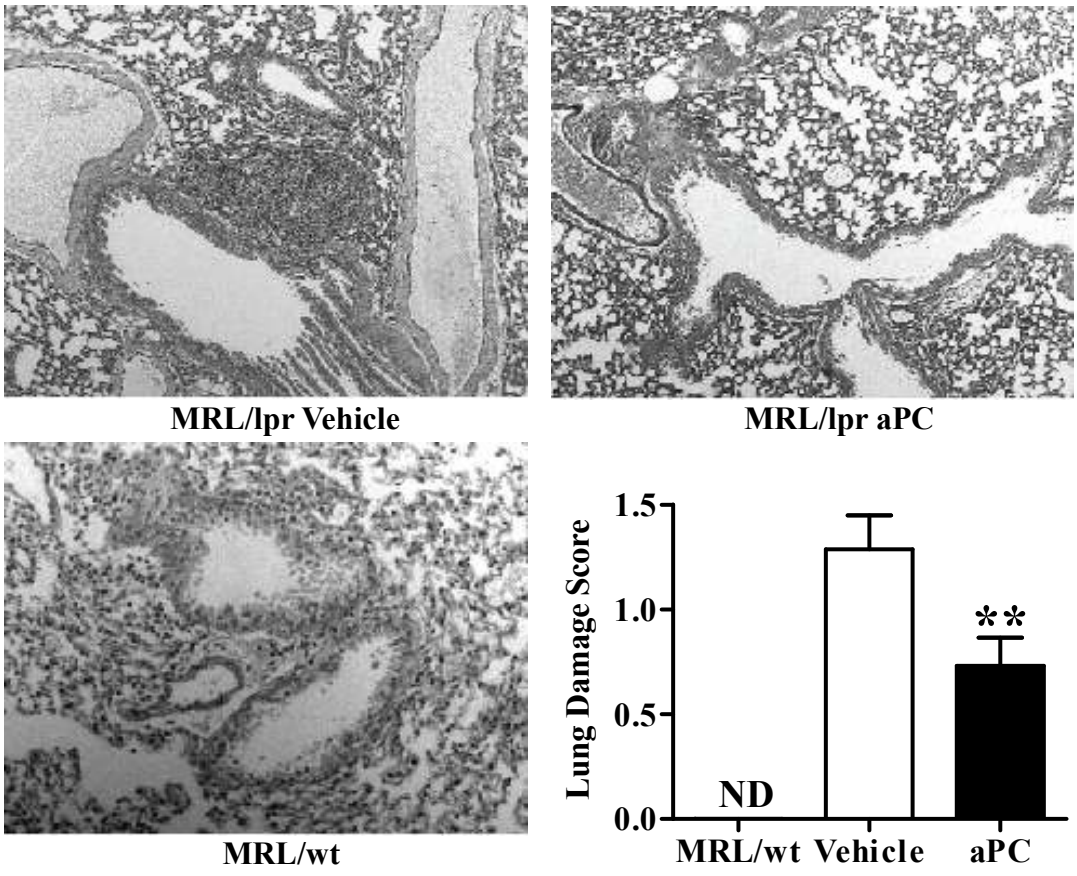


Figure 14. Recombinant aPC improves lung disease in MRL-Fas(lpr) mice. Lung sections of 18-wk-old MRL-Fas(lpr) mice of all groups were stained with PAS. Treatment with aPC reduced peribronchial inflammation in MRL-Fas(lpr) mice. Images are representative for 15 mice in each treatment group and 5 MRL/wt mice. Original magnification x100. Morphometry was used to quantify the peribronchiolar and perivascular inflammation by using a score ranging from 0–3 and scored semiquantitatively as described in Materials and Methods section. The data represent mean scores ± SEM. **p <0.01 versus vehicle group.

score ranging from 0–3 (Figure 14). Thus, recombinant aPC protects MRL-Fas(lpr) mice not only from lupus nephritis but also from autoimmune lung disease.

4.1.6 Recombinant aPC suppresses the abnormal humoral immunity in MRL-Fas(lpr) mice

As treatment with recombinant aPC reduced glomerular immune complex deposition, the question arises whether aPC modulates systemic autoimmunity and lupus autoantibody production in MRL-Fas(lpr) mice. At the age of 18 wk, MRL-Fas(lpr) mice displayed significant hypergammaglobulinemia, which was significantly reduced by aPC treatment (Figure 15A). This effect related especially to IgG of the IgG2a and IgG3 isotype, which are known to activate complement in murine lupus nephritis (Table 6). Plasma IgM levels were affected the same way (Figure 15B). Furthermore, aPC reduced the amount of circulating lupus autoantibodies as evidenced by a marked reduction in diffuse ANA staining intensity on Hep2 cells as well as quantitative plasma anti-dsDNA IgG and rheumatoid factor (anti-IgG) measured by ELISA (Figure 15C–E). Serum IgG as well as autoantibodies derive from Ig-producing plasma cells. Therefore, we performed flow cytometry for CD138 and kappa light chain-positive plasma cells in spleen of both treatment groups. aPC treatment significantly reduced the absolute numbers of total plasma cells (Figure 16A). To analyze the effect of aPC on short-lived and long lived plasma cells, we injected Bromodeoxyuridine (BrdU) in mice 5 days before sacrifice, which allowed us to differentiate short- from long-lived plasma cells. BrdU is a thymidine analog that is used in cell proliferation studies. BrdU is incorporated into newly synthesized DNA by cells entering and progressing through the DNA synthesis phase of the cell cycle. aPC treatment also significantly reduced the short- and long-lived spleen plasma cells (Figure 16B). Similarly, follicular B cell ($CD23^{high}$ $CD21^{low}$), marginal zone B cells ($CD23^{low}$ $CD21^{high}$), and mature B cells (IgM^{+} IgD^{+}) were reduced by aPC treatment (Figure 16C), whereas other major spleen T cell populations (i.e., $CD4^{+}$ T cells, $CD8^{+}$ T cells, and CD4/CD8 double-negative T cells) remained unaffected (Table 6). Interestingly, aPC treatment reduced the Th1 and Th17 polarization of T cells (Table 6). Together, treatment with recombinant aPC specifically reduces B cells and plasma cells as well as Th1 and Th17 polarization of T cells, which decreases overproduction of IgGs and lupus autoantibodies causing lupus nephritis in MRL-Fas(lpr) mice.

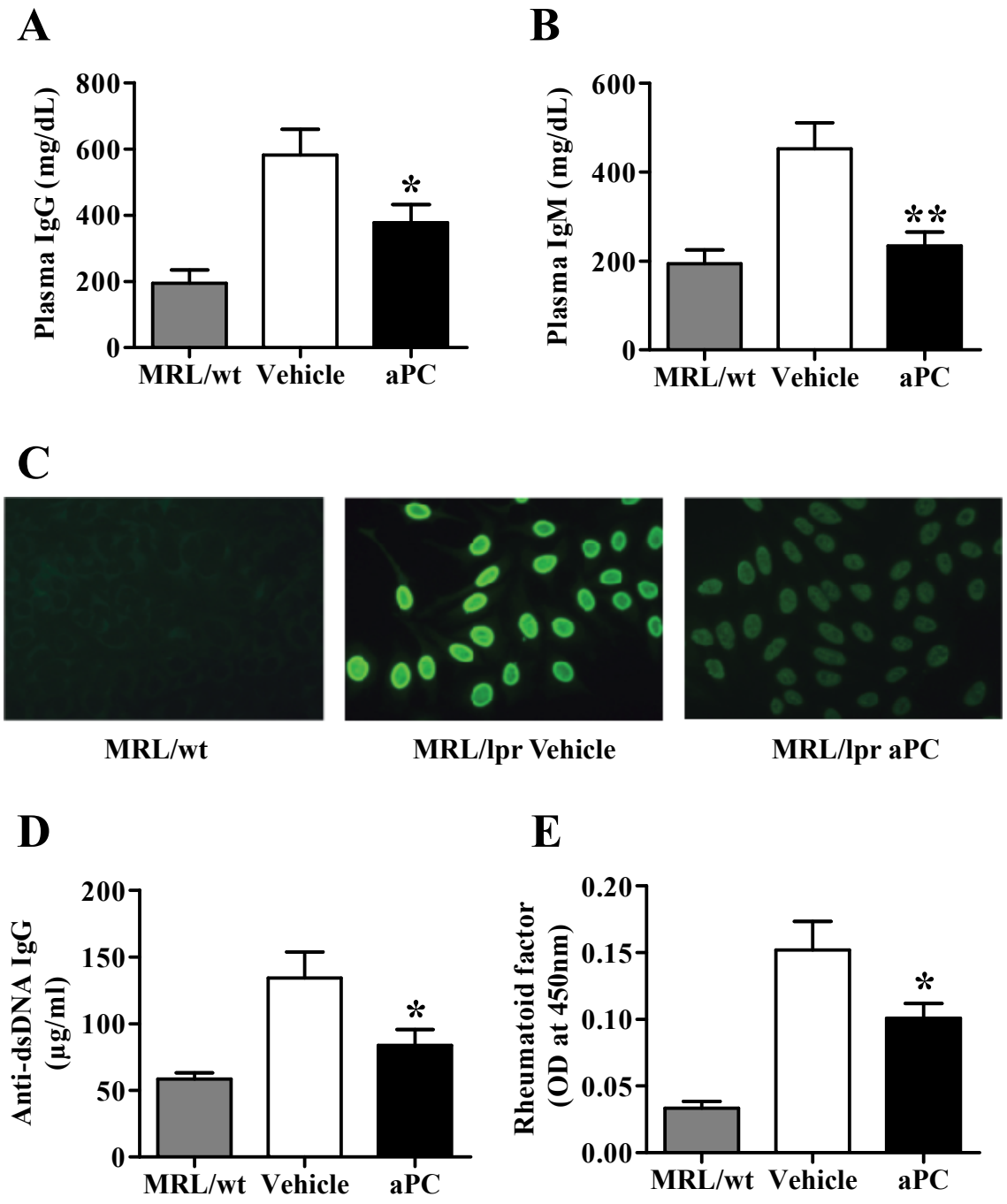


Figure 15. Recombinant aPC reduces autoantibodies in MRL-Fas(lpr) mice. Plasma levels of total IgG (A) and total IgM (B) were determined by ELISA. (C) ANA staining patterns on Hep-2 human epithelial cells for serum, derived from MRL wt, vehicle-treated and aPC treated MRL-Fas(lpr) mice at 1:200 dilution. Original magnification x1000. Plasma levels of total IgG against dsDNA (D) or rheumatoid factor (E) were determined by ELISA. Data are expressed as means ± SEM (n=15 each treatment group; n=5 MRL/wt). *$p<0.05$, **$p<0.01$ versus vehicle group.

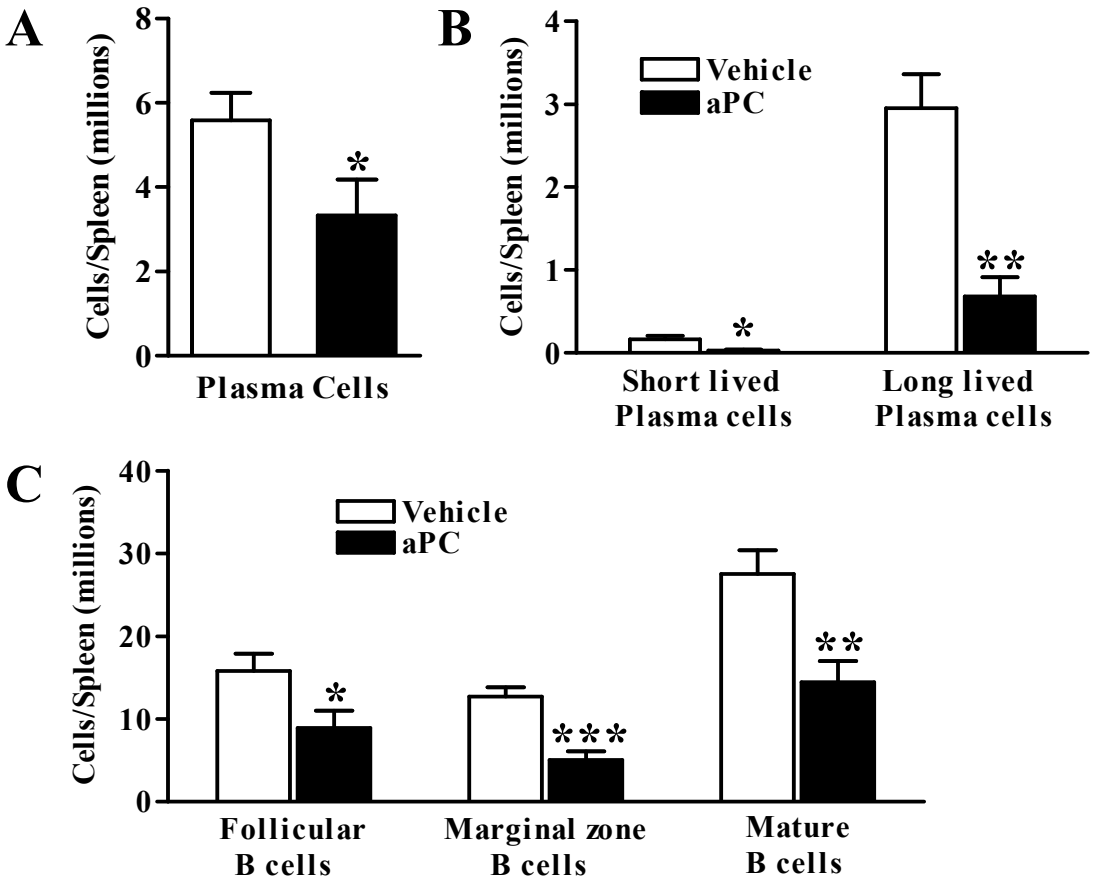


Figure 16. Recombinant aPC affects plasma cells and B cells in MRL-Fas(lpr) mice. (A) FACS analysis of the absolute number of total spleen plasma cells. (B) FACS analysis of the absolute number of short- and long-lived spleen plasma cells. (C) FACS analysis of the absolute number of the follicular B cells, marginal zone B cells and mature B cells from spleen. Data are expressed as means ± SEM. (n=10 each treatment group). *p<0.05, **p<0.01, ***p<0.001 versus vehicle group.

Table 6. Phenotype of SLE in MRL-Fas(lpr) mice

	Vehicle	*aPC*	*p value by t-test*
FACS (cells/spleen (millions))			
$CD3^+CD4^+$	53.39 ± 5.24	45.17 ± 7.09	0.3873
$CD3^+CD8^+$	14.75 ± 2.36	17.18 ± 3.69	0.5875
$CD3^+CD4^-CD8^-$	27.57 ± 5.02	24.14 ± 3.72	0.6073
$CD3^+CD4^+CD25^+$	8.44 ± 0.44	6.715 ± 0.768	0.0680
Th1 cells	14.40 ± 1.994	9.428 ± 0.785	0.0488
Th17 cells	0.122 ± 0.037	0.034 ± 0.007	0.0478
Serum IgG isotypes			
IgG1	591.1 ± 180.0	344.3 ± 94.98	0.2382
IgG2a	142.1 ± 3.866	130.1 ± 2.795	0.0194
IgG2b	20.95 ± 3.824	14.70 ± 1.564	0.1443
IgG2c	0.828 ± 0.067	0.806 ± 0.079	0.8328
IgG3	0.313 ± 0.010	0.279 ± 0.006	0.0124

4.1.7 Recombinant aPC treatment suppresses the activation of antigen presenting cells in MRL-Fas(lpr) mice

The activation of antigen presenting cells is a major stimulus for B cell proliferation and autoantibody production in SLE[43,133]. Therefore, we analyzed the activation of spleen DCs by flow cytometry in MRL-Fas(lpr) mice of both treatment groups. aPC treatment significantly reduced the total numbers of $CD11c^+$ cells including $CD4^+CD11c^+$ and $CD8^+CD11c^+$ cells (Figure 17A). aPC treatment also reduced dendritic cell activation as demonstrated by a significant reduction in CD11c-positive cells that were also positive for the activation markers CD40 or MHC-II (Figure 17B).

In addition, aPC decreased activation marker CD40 in $CD11c^+CD4^+$and $CD11c^+CD8^+$ cells (Figure 17C). Consequently, the spleens of aPC-treated MRL-Fas(lpr) mice expressed much lower mRNA levels of proinflammatory cytokines (IL-6 and IL-12p40) and chemokines (CXCL10 and CCL2/MCP-1) (Figure 18), using primers listed in Table 5. Spleen mRNA levels of TNF remained unaffected by aPC treatment (Figure 18). Thus, treatment with recombinant aPC reduced the activation of splenic DCs in MRL-Fas(lpr) mice.

4.1.8 Recombinant aPC treatment suppresses systemic inflammation in MRL-Fas(lpr) mice

Systemic lupus erythematosus is a systemic inflammatory autoimmune disease which can affect multiple organs of the human body. We measured systemic inflammation by estimating protein levels of proinflammatory cytokines in the plasma. aPC significantly reduced plasma protein levels of IL-6, IL-12p40 and CCL2/MCP-1. Plasma protein levels of TNF remained unaffected by aPC treatment (Figure 19). Thus, treatment with recombinant aPC suppressed systemic inflammation in MRL-Fas(lpr) mice.

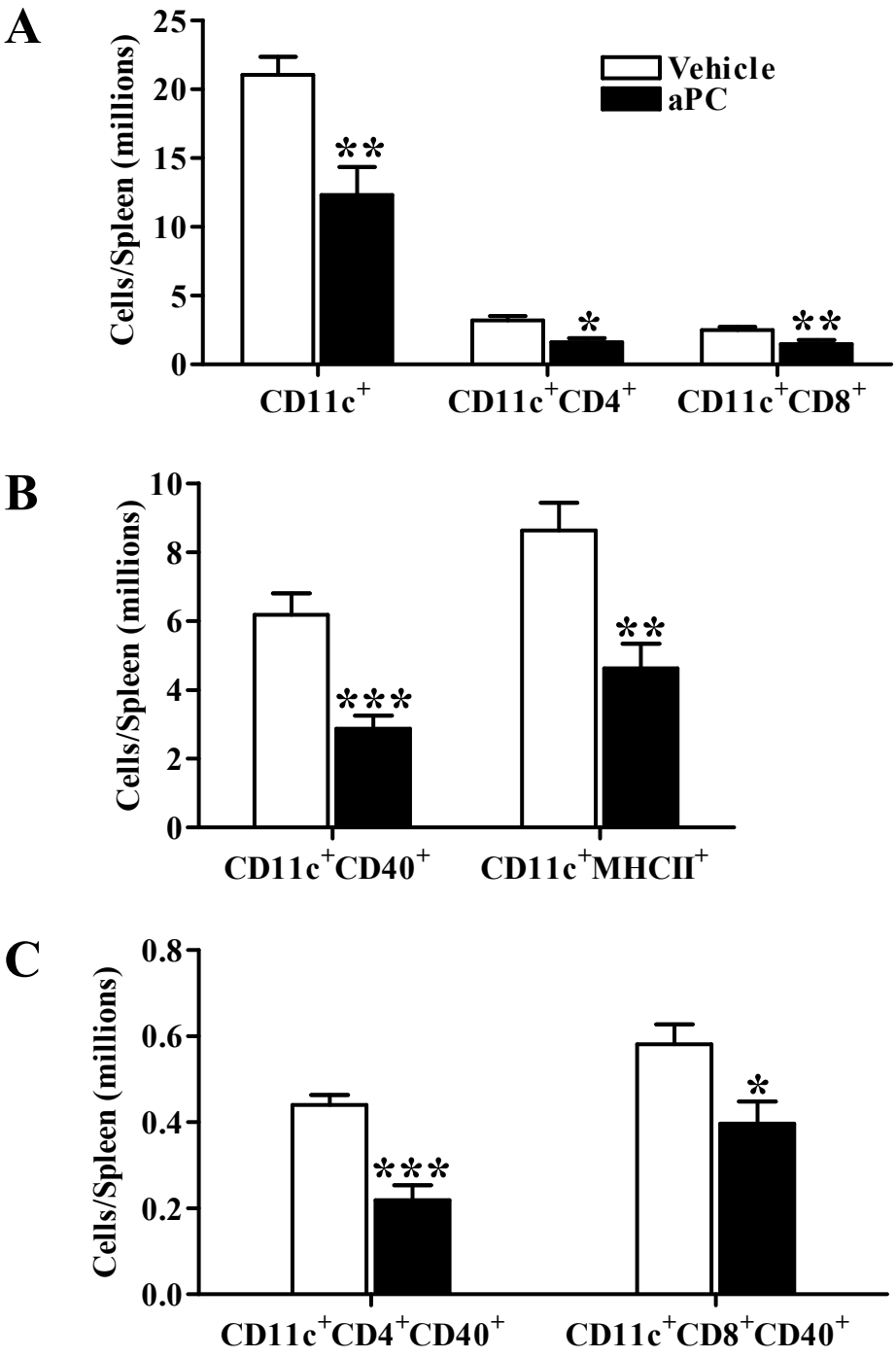


Figure 17. Recombinant aPC suppresses dendritic cell activation in MRL-Fas(lpr) mice. Spleen cell suspensions were prepared for flow cytometry by using specific antibodies that identify subsets of DCs. (A) FACS analysis of the absolute numbers of CD11c, $CD11c^+CD4^+$, and $CD11c^+$ $CD8^+$ cells. (B) FACS analysis of total numbers of CD11c-positive cells that were also positive for the activation markers CD40 or MHC-II. (C) FACS analysis of total numbers of $CD11c^+CD4^+$ or $CD11c^+CD8^+$ positive cells that were also positive for the activation marker CD40. Data are expressed as means ± SEM (n=10 each treatment group). *p<0.05, **p<0.01, ***p<0.001 versus vehicle group.

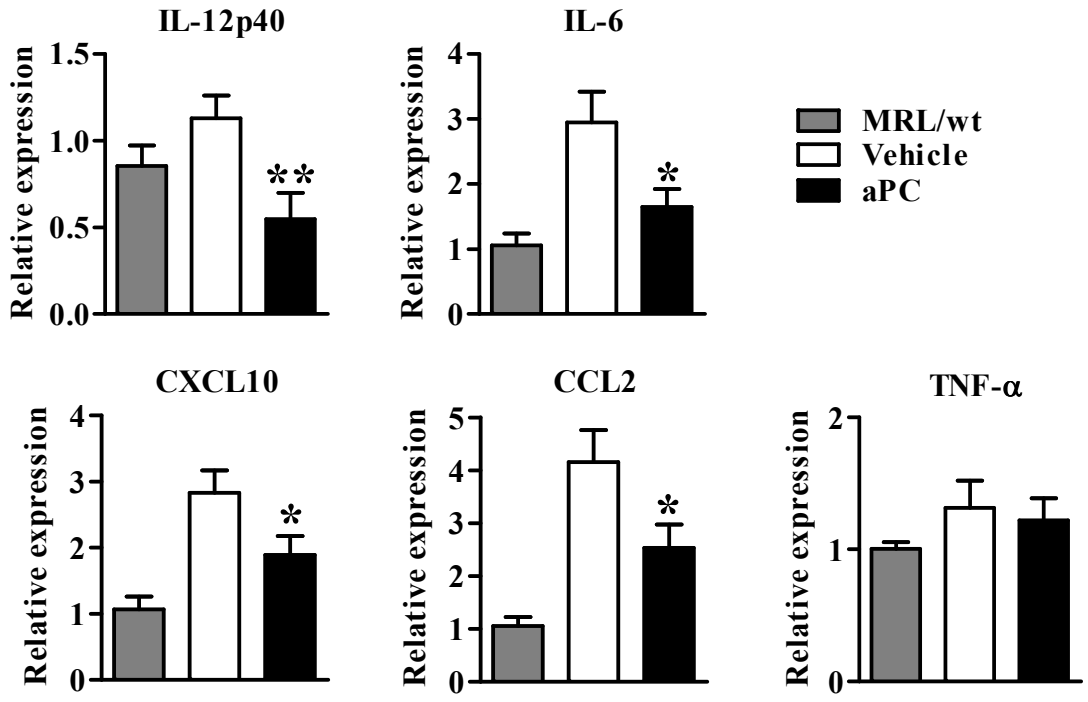


Figure 18. The spleen mRNA expression levels were determined for the IL-12p40, IL-6, CXCL10, CCL2 and TNF by qPCR. Data are expressed as mean of the ratio versus the respective 18S rRNA level ± SEM (MRL/wt n=5; vehicle n=15; aPC n=15). *p<0.05, **p<0.01 versus vehicle group.

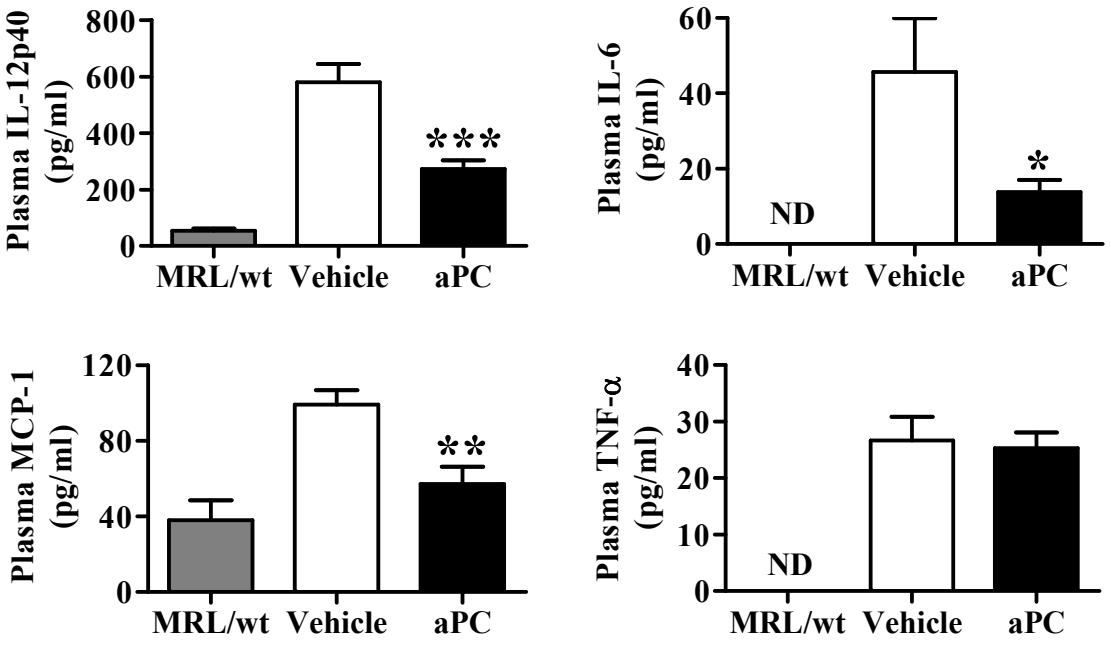


Figure 19. Plasma protein levels of IL-12p40, IL-6, MCP-1 and TNF-α were estimated by ELISA. Data are expressed as means ± SEM (MRL/wt n=5; vehicle n=15; aPC n=15). *p<0.05, **p<0.01, ***p<0.001 versus vehicle group. ND, not detectable.

4.2 Part – II Cathepsin S in SLE

4.2.1 Cathepsins expression in MRL-Fas(lpr) mice

First we quantified the mRNA expression levels of Cat-B, -C, -D, -H, -L, and -S in kidneys and spleens of 6, 10, and 14 week old female MRL-Fas(lpr) mice with SLE by real-time PCR. Age- and sex-matched MRL wild-type mice served as controls. In the kidney, among all the cathepsins only Cat S, -B, and -C were induced only at 14 weeks of age (Figure 20A). By contrast, all cathepsins were induced in spleen starting from 10 weeks of age (Figure 20B). In-situ hybridization localized Cat S mRNA expression to F4/80+ myeloid antigen-presenting cells but not to $CD20^{+}$ B cells or $CD247^{+}$ T cells in spleen, lung, and kidney of female MRL-Fas(lpr) mice (Figure 21A). Cat S immunostaining was consistent with that cell type-specific expression pattern in spleen and lung, while in kidney, epithelial cells of proximal tubuli stained positive, implying that Cat S protein gets reabsorbed from the glomerular filtrate (Figure 21B). In the kidney, Cat S positivity was also seen in the DCs of the tertiary lymphoid tissue formed in the perivascular area of larger arteries (Figure 22). Together, Cat S is expressed exclusively in myeloid antigen-presenting cells inside and outside the lymphoid tissue.

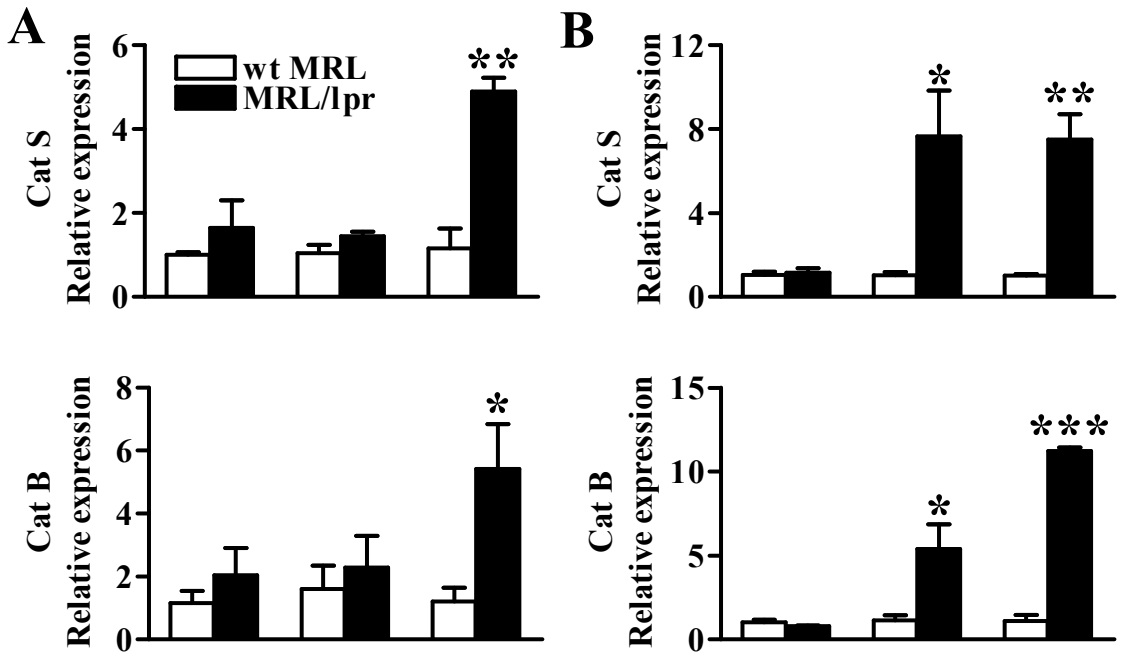


Figure continued...

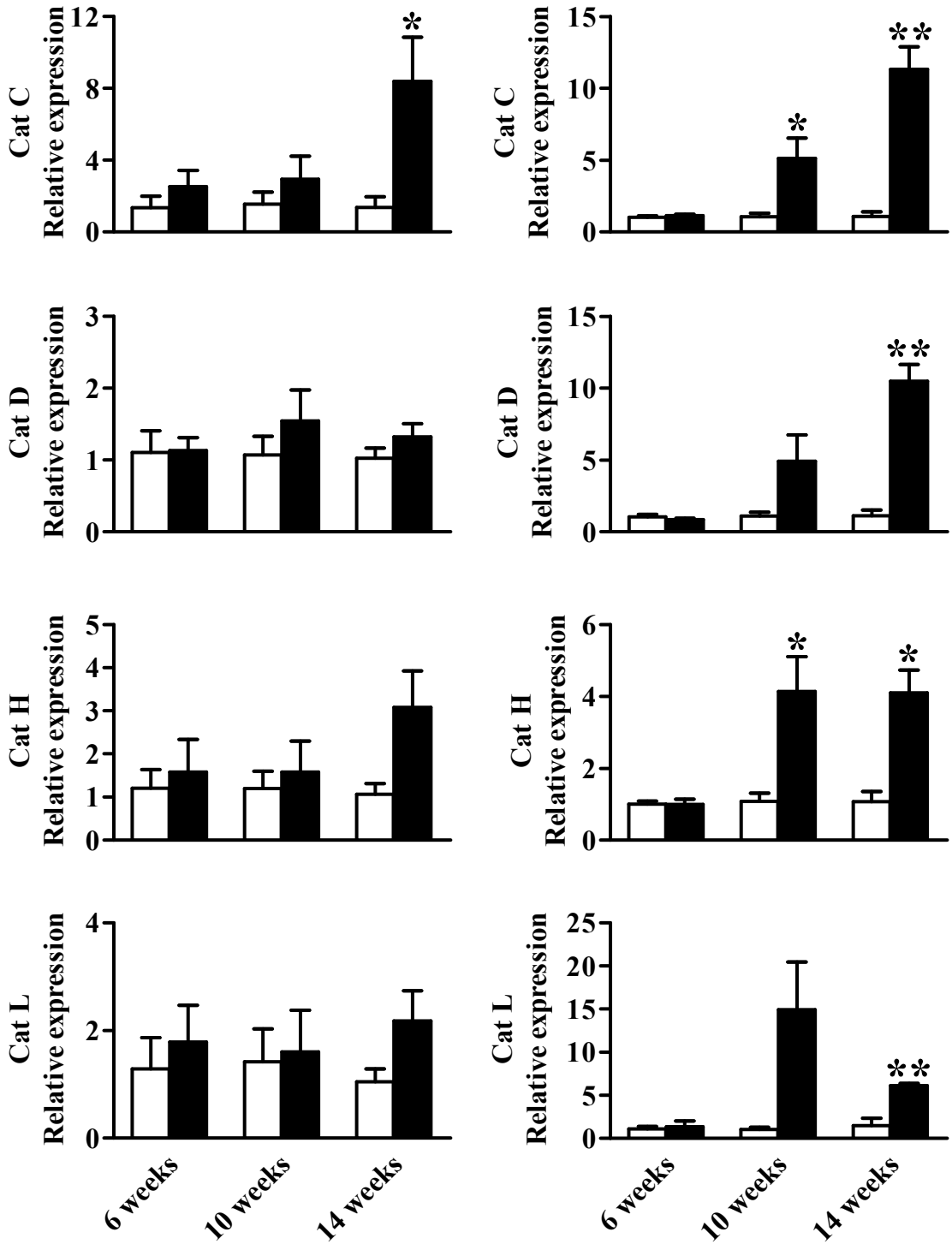


Figure 20. Cathepsin S, B, C, D, H and L mRNA expression levels in the kidney (A) and spleen (B) were quantified in wildtype MRL and in MRL-Fas(lpr) mice by real time PCR. Data are expressed as a mean ratio to the respective 18S rRNA mRNA expression level ± SEM. *p<0.05, **p<0.01. ***p<0.001 (wildtype MRL n=3, MRL-Fas(lpr) n=3).

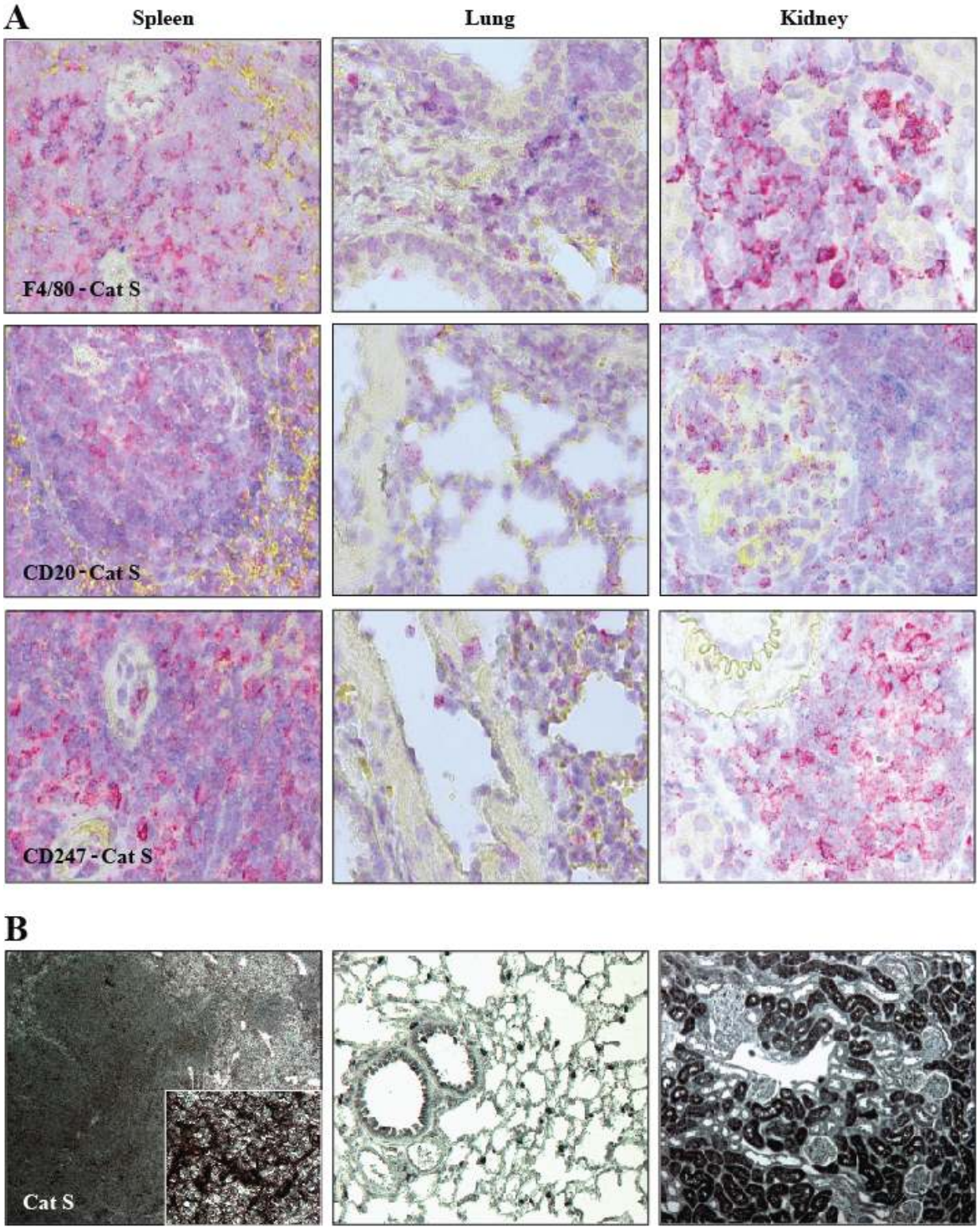


Figure 21. Cat S in-situ hybridization and immunohistochemistry. Spleen, lung, and kidney sections were prepared for in-situ hybridization (A) and Cat S immunostaining (B) as described in methods. Cat S mRNA expression is indicated by red colour. Co-staining by either F4/80 (myeloid DCs), CD20 (B cells), or CD247 (T cells) is shown in blue colour, respectively. Representative images are shown here for all organs at original magnifications of 100x, 200x or 400x (insert).

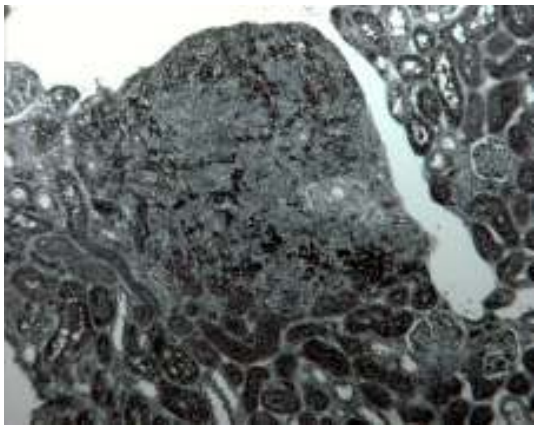


Figure 22. Cat S immunostaining. Kidney sections were stained for Cat S as described in methods. Representative image of intrarenal tertiary lymphoid tissue and is shown at an original magnification of 200x.

4.2.2 Pharmacodynamics and pharmacokinetics of RO5461111 in mice

To test the functional contribution of Cat S in systemic autoimmunity we used the Roche Cat S Inhibitor RO5461111 (Figure 23A). RO5461111 is a potent and selective Cat S inhibitor. In enzymatic assays it inhibits human Cat S with an IC50 of 0.4 nM. The mouse Cat S activity is comparable to the human activity with an IC50 of 0.5 nM. No submicro-molar inhibition of any other cathepsin (Cat B, Cat K, Cat L and Cat V) tested was detected (Table 7). In addition, RO5461111 has been tested in the diversity panel of CEREP consisting of nearly 100 receptor binding and enzymatic assays at 10 µM concentration. Here, RO5461111 showed ≤ 30% inhibition for all assays tested. The capability of RO5461111 to inhibit Cat S in cells was tested with the Iip10 accumulation assay in a human and mouse B-cell line and a potent induction of Iip10 was determined with EC50 of 17 nM and 8 nM respectively was determined (data not shown). The acute

Table 7. In vitro enzyme inhibition assay

Cathepsin	**IC50**
Human cathepsin S	0.4 nM
Mouse cathepsin S	0.5 nM
Human cathepsin K	>25 µM
Human cathepsin L	49 µM
Human cathepsin B	44 µM
Human cathepsin V	1.3 µM

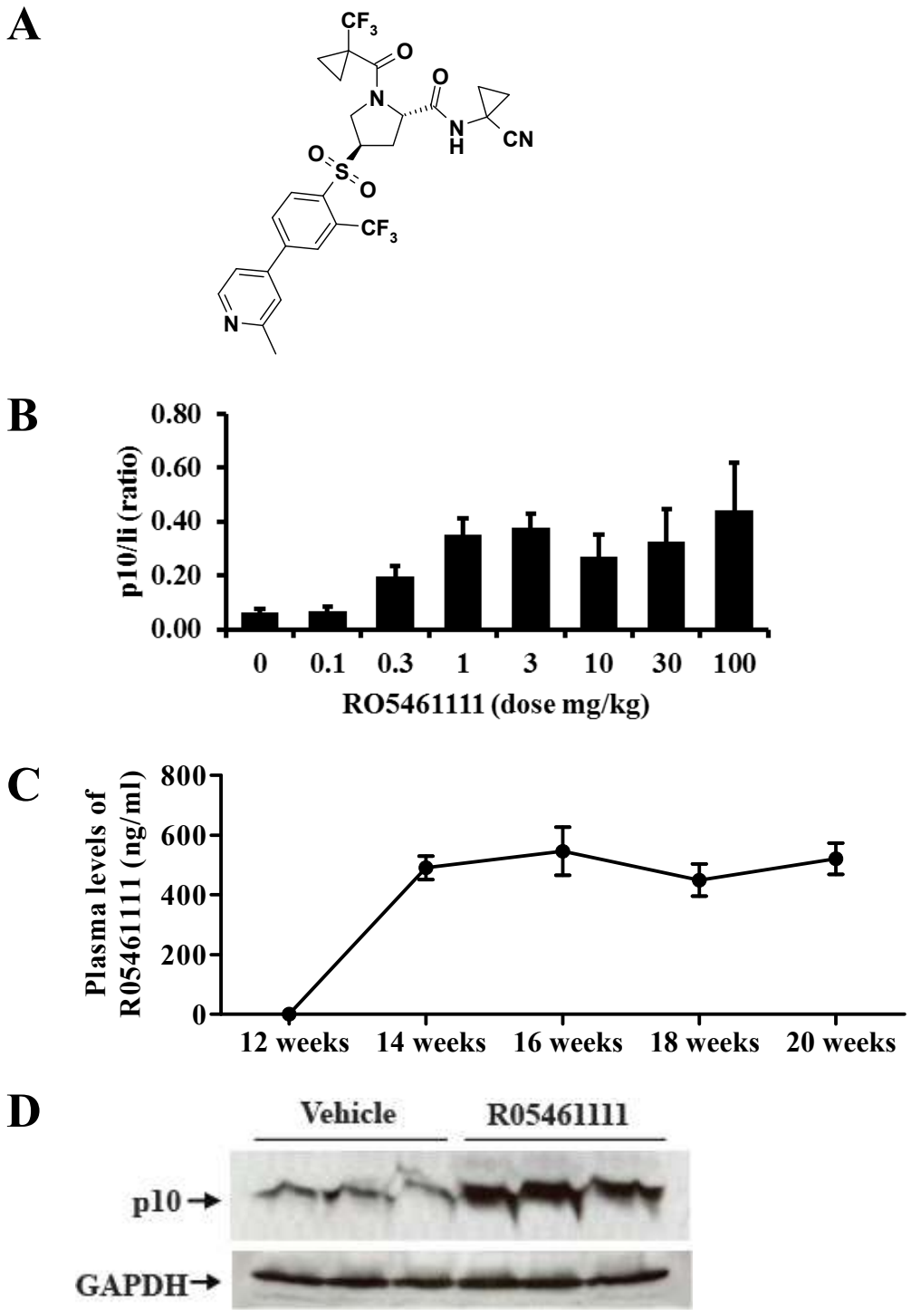


Figure 23. Pharmacokinetics of Cat S inhibitor R05461111 in MRL-Fas(lpr).(A) Chemical structure of R05461111. (B) For the determination of in-vivo Cat S activity inhibition by RO5461111, we used BALB/c mice. After oral dosing of 0.1 mg/kg to 100 mg/kg of RO5461111 mice were sacrificed after 7 hours and spleens were harvested. Protein extracts were separated by SDS-gel electrophoresis for the determination of p10 upregulation by Cat S inhibition. (C) Concomitant pharmacokinetic studies were performed for R05461111 in female MRL Fas(lpr) mice during the course of treatment from 12 to 20 wk of age. Plasma sampling was carried every two weeks and R05461111 plasma levels were determined by modification of a sandwich hybridization procedure. (R05461111 n=5). (D) Invariant chain p10 levels were estimated from spleen tissue using western blotting.

pharmacodynamics effect of RO5461111 was tested after oral gavage at doses from 0.1-100 mg/kg. Splenic induction of p10 was used as measured of enzyme inhibition and a strong p10 upregulation was measured with maximal induction at low doses as 1 mg/kg (Figure 23B). Oral administration of RO5461111 by food admix (262 mg/kg) to female MRL-(Fas)lpr mice results in a dose of 30 mg/kg and stable plasma levels of RO5461111 at 400-600 ng/ml over a period of 8 weeks and resulted in robust p10 accumulation in the spleen (Figures 23C and 23D). Furthermore, oral treatment of C57BL/6 mice with RO5461111 suppressed T cell priming upon vaccination with sheep IgG and also treatment with R05461111 significantly reduced anti-sheep IgG antibodies (Figure 24A and 24B). Together, RO5461111 is a specific small molecule Cat S antagonist with favorable pharmacodynamic and pharmacokinetic profiles to efficiently block Cat S over prolonged periods of time in mice, which has the potential to suppress antigen-induced B and T cell priming.

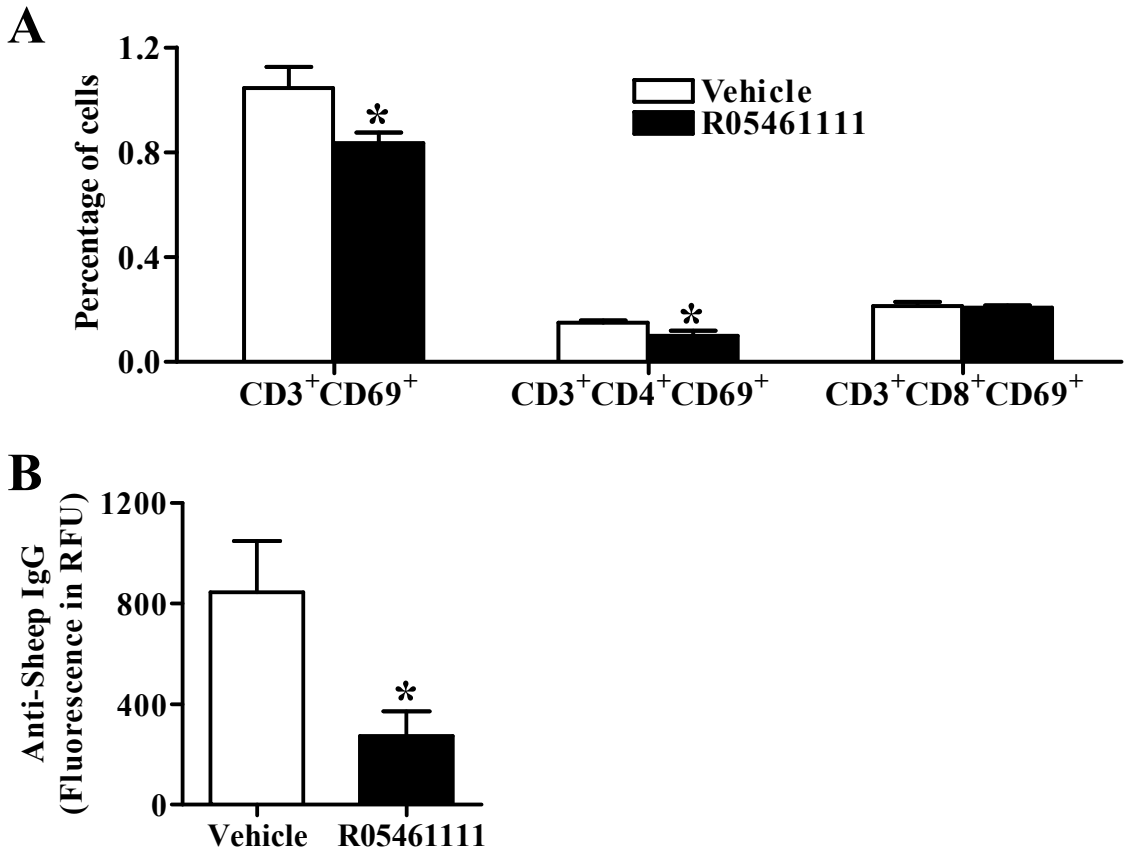


Figure 24. (A) Spleen FACS analysis of the percentage of total $CD3^+CD69^+$ T cells, $CD3^+CD4^+CD69^+$ T cells and $CD3^+CD8^+CD69^+$ T cells. (B) Anti-sheep IgG antibodies were estimated by ELISA. Data are expressed as means ± SEM. *$p<0.05$, versus vehicle group. (Vehicle n=6, R05461111 n=6).

4.2.3 Cat S blockade with RO5461111 reduces plasma levels of IL-10 and TNF in MRL-(Fas)lpr mice

To test the effects of therapeutic Cat S inhibition in autoimmune IC-GN, 12 week old albuminuric female MRL-Fas(lpr) mice were randomized to two groups that received either normal chow (vehicle) or a food admix with RO5461111. Food intake and weight gain were identical during the following 8 weeks until all mice were sacrificed (data not

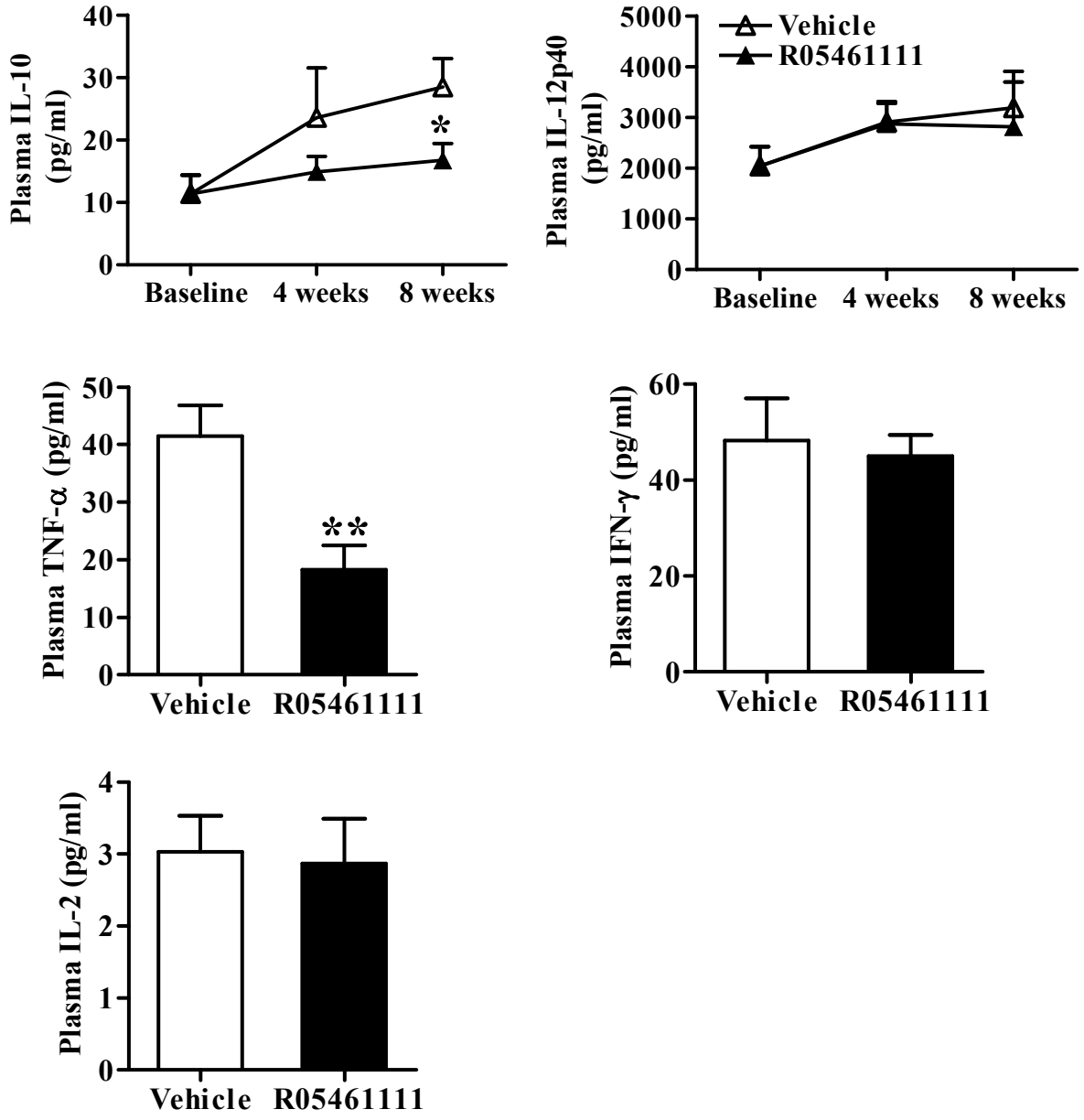


Figure 25. Plasma protein levels of IL-10, IL-12p40, TNF-α, IL-2 and IFN-γ were estimated by ELISA. Data are expressed as means ± SEM (n=15 each treatment group). *p<0.05, **p<0.01, versus vehicle group.

Table 8. Spleen and lymph node weight in MRL-Fas(lpr) mice

	Vehicle	**R05461111**	***P value* by t-test**
Spleen	1.777 ± 0.1152	1.505 ± 0.1111	0,1026
Lymph node	2.369 ± 0.2555	1.913 ± 0.1509	0,1364

shown). The same applied to spleen and lymph node weights (table 8). Plasma samples were obtained at baseline (12 weeks of age), at 4 and 8 weeks of treatment (16 and 20 weeks of age). We first measured IL-10 and IL-12p40 as major dendritic cell-derived cytokines that promote B and T cell activation and proliferation. IL-10 is a potent growth and differentiation factor for activated B cells. The Cat S blockade had significantly reduced IL-10 (not IL-12p40) levels but only after 8 weeks of treatment (Figure 25). Therefore, additional cytokines were measured at 8 weeks only. Cat S blockade significantly reduced TNF-α levels, while IL-2 and IFN-γ levels remained unaffected. IL-2 is a cytokine primarily produced by T cells and is necessary for T cell activation and proliferation. It has been reported that production of IL-2 is decreased in patients with systemic lupus erythematosus. High IFN-γ serum levels have been reported in SLE. IFN-γ receptor knockout lupus prone mouse strain has suggested that IFN-γ is essential for the development of nephritis in mice[134]. Thus, Cat S promotes the systemic expression of IL-10 and TNF-α in MRL-(Fas)lpr mice.

4.2.4 Cat S blockade with RO5461111 reduces the activation and expansion of spleen DCs in MRL-(Fas)lpr mice

Autoimmune IC-GN involves overactivation of adaptive immunity. Inappropriately stimulated monocytic antigen-presenting cells are a source for systemic cytokine release as well as immune dysregulation[43,133]. As immunostaining had localized Cat S positivity predominantly to antigen-presenting cells we performed splenocyte flow cytometry on MRL-(Fas)lpr mice. RO5461111 treatment reduced the percentages of $CD11c^{+}MHC\text{-}II^{+}$ and $CD11c^{+}CD40^{+}$ activated DCs in MRL-(Fas)lpr mice (Figure 26A). This was associated with lower spleen mRNA expression levels for IL-1β, IL-10, IL-23p19, and CXCL12, while the levels of IL-6, IL-12p35, IFN-γ, TGF-β, CCL2, and CCL5 were not reduced (Figure 26B and 26C).

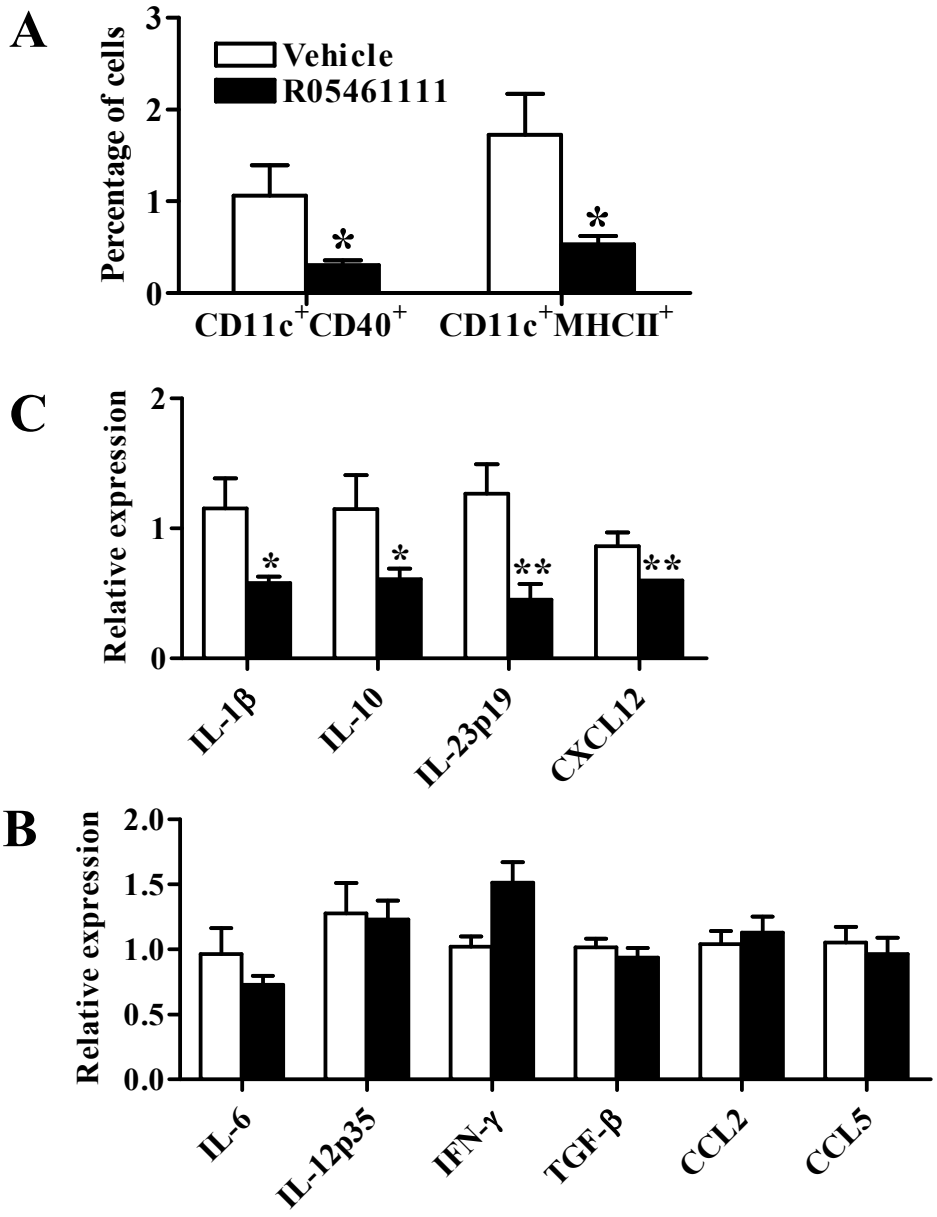


Figure 26. FACS analysis of splenic DCs. Spleen cell suspensions were prepared for flow cytometry by using specific antibodies that identify subsets of DCs. (A) FACS analysis of total numbers of CD11c positive cells that were also positive for the activation markers MHC-II or CD40. Data are expressed as mean percentages of all splenocytes ± SEM. (B) Splenic mRNA expression levels of IL-1β, IL-10, IL-23p19 and CXCL12 were determined by qPCR. (C) Splenic mRNA expression levels of IL-6, IL-12p35, IFN-γ, TGF-β, CCL2 and CCL5 were determined by qPCR. Data are expressed as mean of the ratio versus the respective 18S rRNA level ± SEM. (n=15 each treatment group). *p<0.05, **P<0.01 versus vehicle group.

4.2.5 Cat S blockade with RO5461111 reduces the activation and expansion of CD4 and autoreactive T cells in MRL-(Fas)lpr mice

Less spleen dendritic cell activation and cytokine expression may affect T cell priming. RO5461111 significantly reduced the amount of spleen CD3 cells as well as of activated ($CD69^+$) CD3 cells (Figure 27A). Treatment with inhibitor specifically significantly reduced $CD4^+$ cells and CD4/CD8 double negative 'autoreactive' T cells as well as activated ($CD69^+$) $CD4^+$ T cells and CD4/CD8 double negative T cells (Figure 27A). $CD4^+$ cell polarization is driven by distinct transcription factors, e.g. T-bet (Th1), Gata3 (Th2), Rorc (Th17) and Foxp3 (Treg). Cat S inhibition significantly specifically reduced spleen mRNA levels of Gata3 and Rorc (Figure 28). In contrast to RO5461111`s effect on $CD4^+$ cells, it did not affect the amount and the activation stage of $CD8^+$ T cells (Figure 27B). Together, Cat S promotes the activation and expansion of spleen DCs, $CD4^+$ T cells and autoreactive T cells in MRL-(Fas)lpr mice, a process that can be blocked with RO5461111.

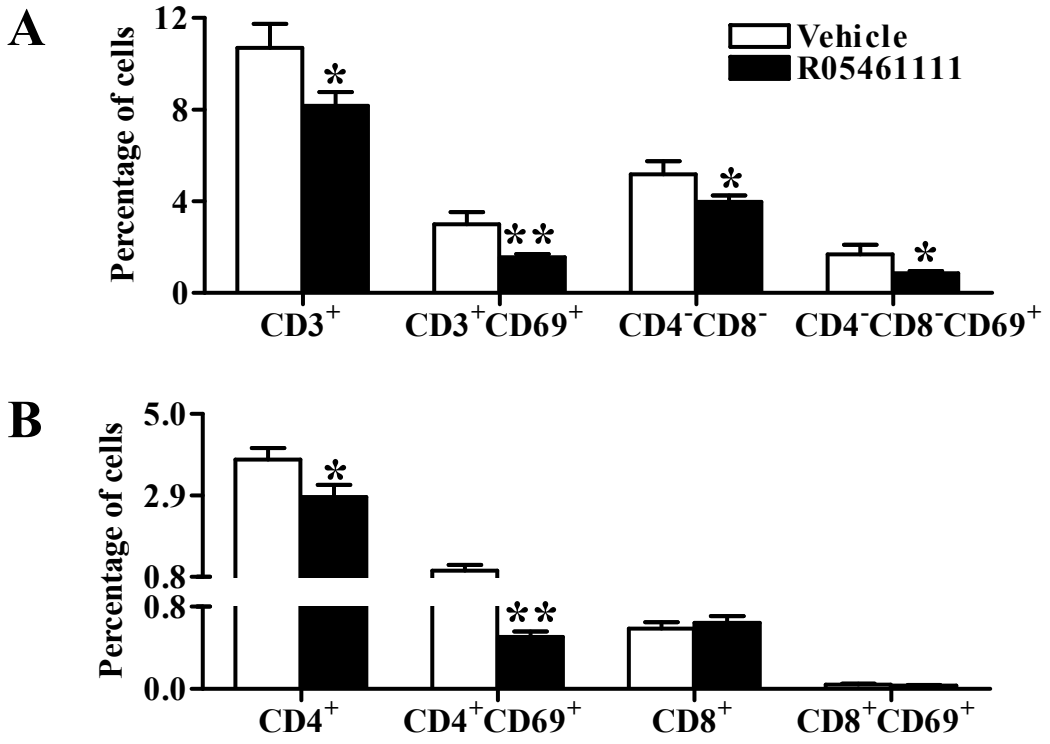


Figure 27. FACS analysis of splenic T cells. (A) FACS analysis of the percentage of total $CD3^+$, $CD3^+CD69^+$ T cells, double negative T cells and $CD69^+$ double negative T cells. (B) Percentage of total $CD4^+$, $CD4^+CD69^+$ T cells, $CD8^+$ and $CD8^+CD69^+$ T cells. Data are expressed as mean percentages of all splenocytes ± SEM. *p<0.05, **P<0.01 versus vehicle group.

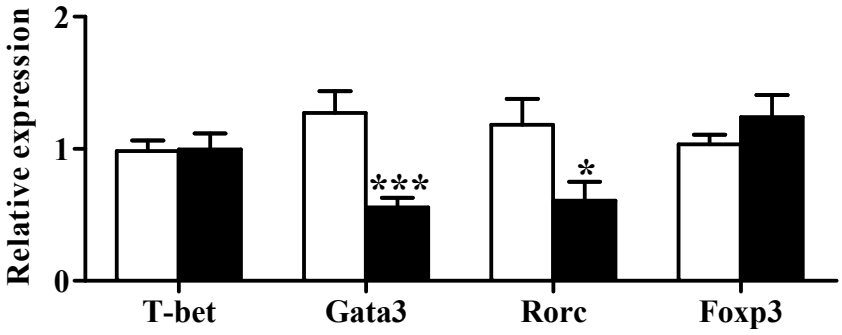


Figure 28. Splenic mRNA expression levels of T cell transcription factors like T-bet, Gata-3, Rorc and Foxp3 were determined by real time PCR. (n=15 each treatment group). *p<0.05, ***P<0.001 versus vehicle group.

4.2.6 Cat S blockade with RO5461111 reduces B cells and plasma cells in MRL-(Fas)lpr mice

DCs and activated T helper cells both regulate antigen-specific B cell and plasma cell responses. The inhibitor significantly reduced B220+, B220+CD69+ cells but not the percentages of mature ($B220^+IgM^+IgD^+$), marginal zone ($B220^+CD21^+CD23^-$), and follicular B cells ($B220^+CD21^-CD23^+$), while total plasma cells were significantly reduced (Figures 29A; 29D). Beyond the cytokines mentioned above, RO5461111 did not affect the expression of April, BAFF and Taci (Figure 29C). However, RO5461111 treatment significantly reduced the numbers of $CD138^+$ k Light $chain^+$ plasma cells (Figure 29B). Together, Cat S promotes the activation and expansion of splenic B cells and plasma cells in MRL-(Fas)lpr mice, a process that can be blocked with RO5461111.

4.2.7 Cat S blockade with RO5461111 disrupts germinal centers in MRL-(Fas)lpr mice

Systemic autoimmunity depends on the complex interaction of myeloid cells and lymphocytes to promote the clonal selection of autoreactive lymphocytes as well as Ig class switch and affinity maturation of autoantibodies[120,4,3]. These processes involve peanut agglutinin $(PNA)^+$ follicular B cells and takes place in the GC of the lymphoid tissue[107]. Vice versa, disruption of GC formation counteracts autoimmunity[120]. PNA immunostaining revealed that Cat S inhibition altered the distribution of follicular B cells

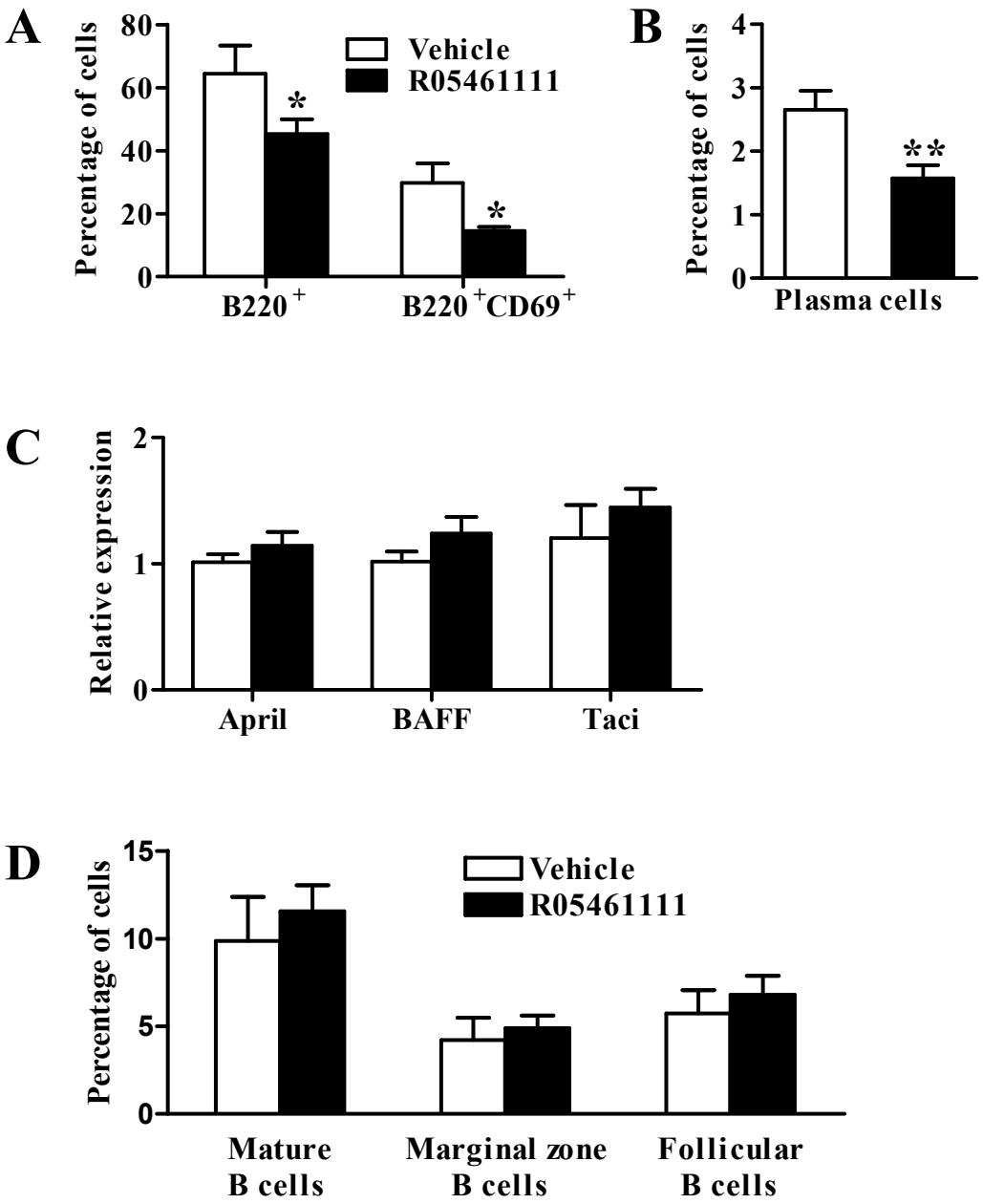


Figure 29. FACS analysis of splenic B cells and plasma cells. Spleen cell suspensions were prepared for flow cytometry by using specific antibodies that identify subsets of B cells and plasma cells. (A) FACS analysis of the percentage of total splenic $B220^+$ and $B220^+CD69^+$ cells. (B) FACS analysis of the percentage of total spleen plasma cells. (C) Splenic mRNA expression levels of April, BAFF and Taci were determined by qPCR. Data are expressed as a mean ratio to the respective 18S rRNA mRNA expression level ± SEM. (D) FACS analysis of the percentage of total mature B cells ($B220^+IgM^+IgD^+$), marginal zone B cells ($B220^+CD21^+CD23^-$) and follicular B cells ($B220^+CD21^-CD23^+$) from spleen tissue. Data are expressed as mean percentages of all splenocytes ± SEM (n=15 each treatment group). *p<0.05, **P<0.01 versus vehicle group.

away from lymphoid follicles to the perifollicular area in spleens of MRL-(Fas)lpr mice (Figure 30), implying that Cat S inhibition disrupted the spatial organization of GC. IgD immunostaining revealed naïve B cells, these cells are present in the light zone of GC. These findings were consistent with a RO5461111-related significant reduction of spleen CXCL12 mRNA expression that is required for GC formation (Figure 30). Cat S inhibition by R05461111 completely eliminated IgD^+ B cells from GC (Figure 31). Together, Cat S is required for GC formation in MRL-(Fas)lpr mice, which can be suppressed by RO5461111.

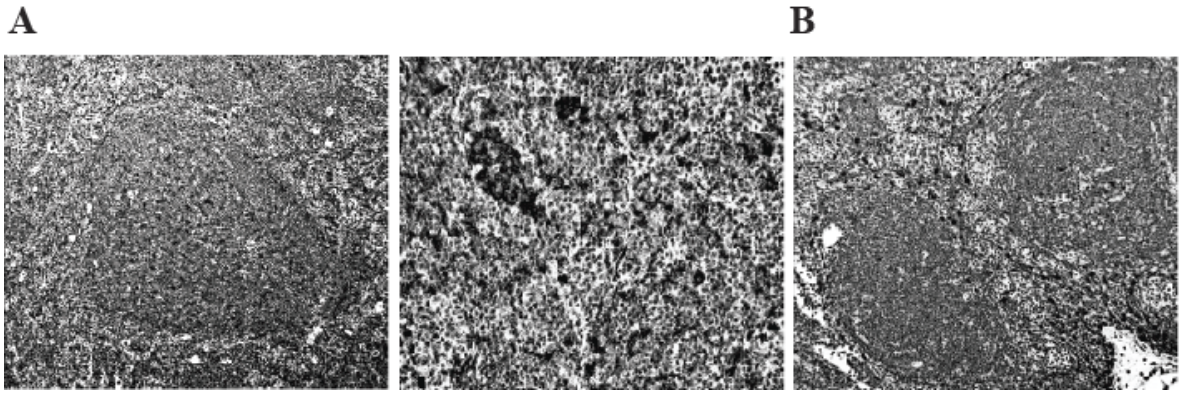


Figure 30. PNA immunostaining was performed in the spleens of both groups to identify follicular B cells in the GC of lymph follicles. (A) Vehicle (B) R05461111. Note that Cat S inhibition led to a redistribution of PNA^+ cells to the perifollicular area.

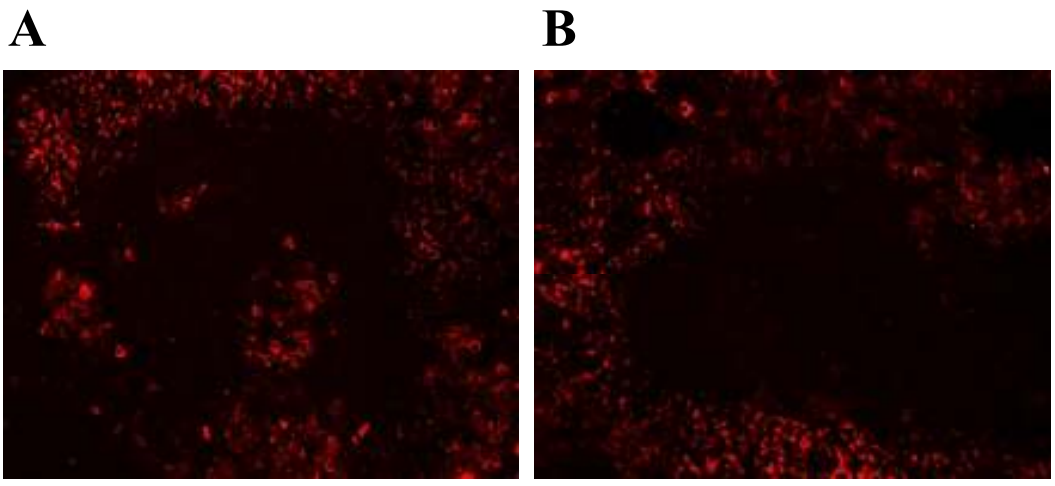


Figure 31. IgD immunostaining was performed in spleens of both groups to identify mantle B cells of the lymph follicles. (A) Vehicle (B) R05461111. Note that Cat S inhibition led to absence of IgD^+ B cells from the light zone of GC.

4.2.8 Cathepsin S blockade with RO5461111 reduces hypergammaglobulinemia in MRL-(Fas)lpr mice

B cell maturation and plasma cell expansion account for hypergammaglobulinemia and the production of high affinity IgG autoantibodies in autoimmunity. In vehicle-treated MRL-(Fas)lpr mice total IgG plasma levels increased from week 12 to 20 of age, which mostly related to IgG1 and IgG2a (Figure 32A). RO5461111 treatment significantly reduced total IgG, IgG1, IgG2a, IgG2b, and IgG2c levels already after 4 weeks of treatment but there was a progressive decline of especially IgG1 and IgG2a below baseline along treatment (Figure 32A). In contrast, IgG3 and IgM levels remained unaffected (Figures 32A and 32B). Thus, Cat S drives hypergammaglobulinemia in MRL-(Fas)lpr mice, a process that can be reversed by RO5461111.

4.2.9 Cat S blockade with RO5461111 reduces autoantibody production in MRL-(Fas)lpr mice

B cell maturation and plasma cell expansion account for the production of high affinity IgG autoantibodies in autoimmunity. 20 week old MRL-(Fas)lpr mice displayed strong positivity for antinuclear antibodies in a diffuse nuclear staining pattern consistent with the presence of anti-DNA antibodies (Figure 33A). This was validated by kinetoplast staining of the monocellular organism *Critidiae luciliae* that contains pure dsDNA (Figure 33B). Cat S blockade with RO5461111 drastically reduced ANA as well as *Critidiae luciliae* kinetoplast positivity (Figures 33A, 33B). Quantitative assessment of anti-dsDNA IgG and IgM during the treatment period confirmed that RO5461111 treatment reduced anti-dsDNA IgG but not IgM plasma levels (Figure 33C). This effect on Ig class switch related to all subclasses of IgG except for IgG2c (Figure 34A). It is of note that Cat S blockade lead to a progressive decline below baseline levels during the treatment period (Figure 34A) suggesting that Cat S is needed to maintain autoantibody production in existing clones of autoreactive plasma cells. RO5461111 treatment also significantly reduced the plasma levels of anti-nucleosome IgG, anti-smRNP IgG, and rheumatic factor in MRL-(Fas)lpr mice, while the respective IgM autoantibodies remains unaffected (Figure 34B, 35). Thus, Cat S drives the production of antinuclear IgG autoantibody production in MRL-(Fas)lpr mice, a process that can be reversed by RO5461111.

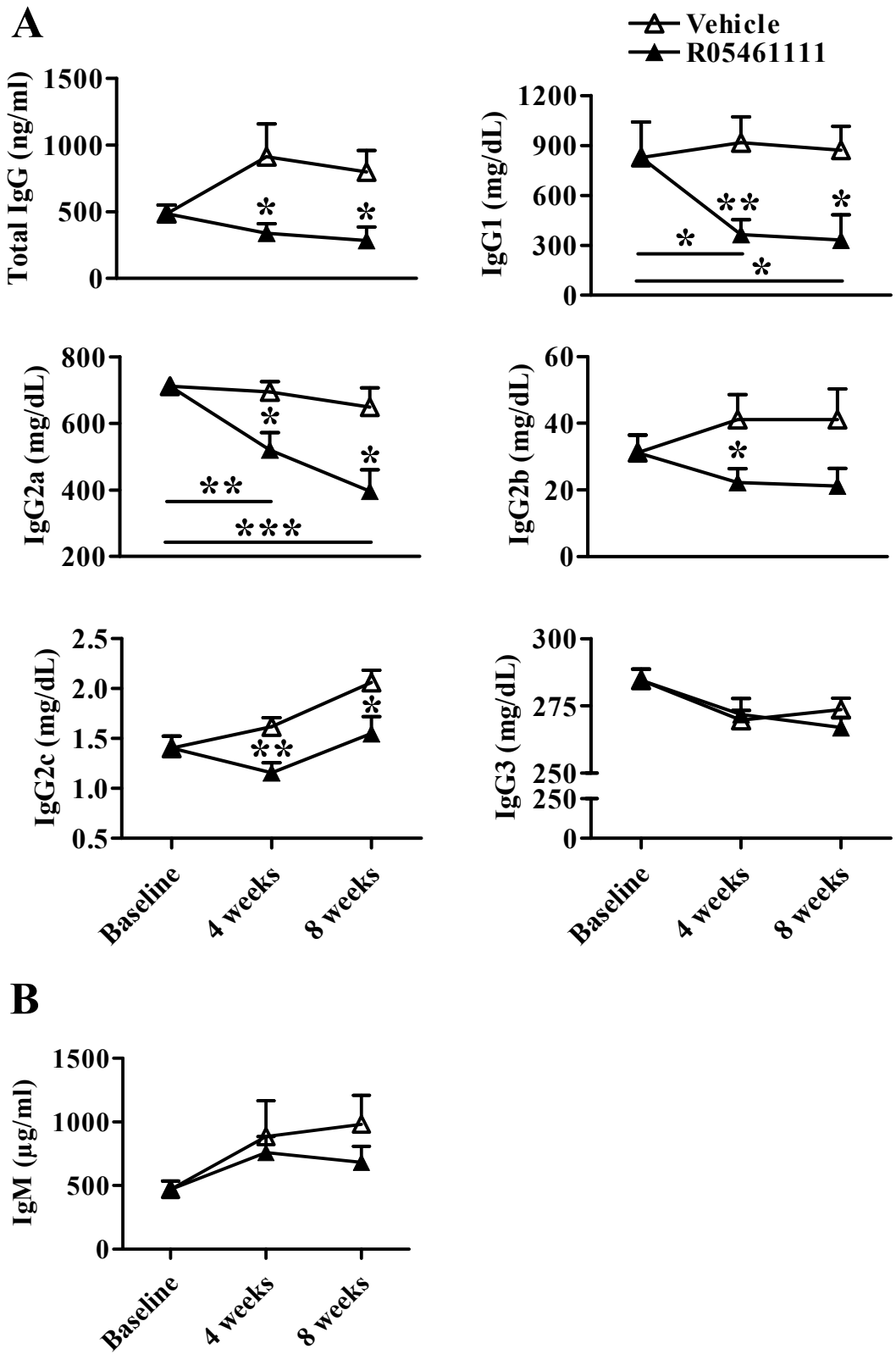


Figure 32. R05461111 reduces IgG antibodies in MRL-Fas(lpr) mice. (A) Plasma levels of total IgG and its isotypes IgG1, IgG2a, IgG2b, IgG2c and IgG3were determined by ELISA. (B) Plasma levels of IgM antibodies were determined by ELISA. Data are expressed as means ± SEM (n=15 each treatment group,). *p<0.05, **p<0.01, ***p<0.001 versus vehicle group.

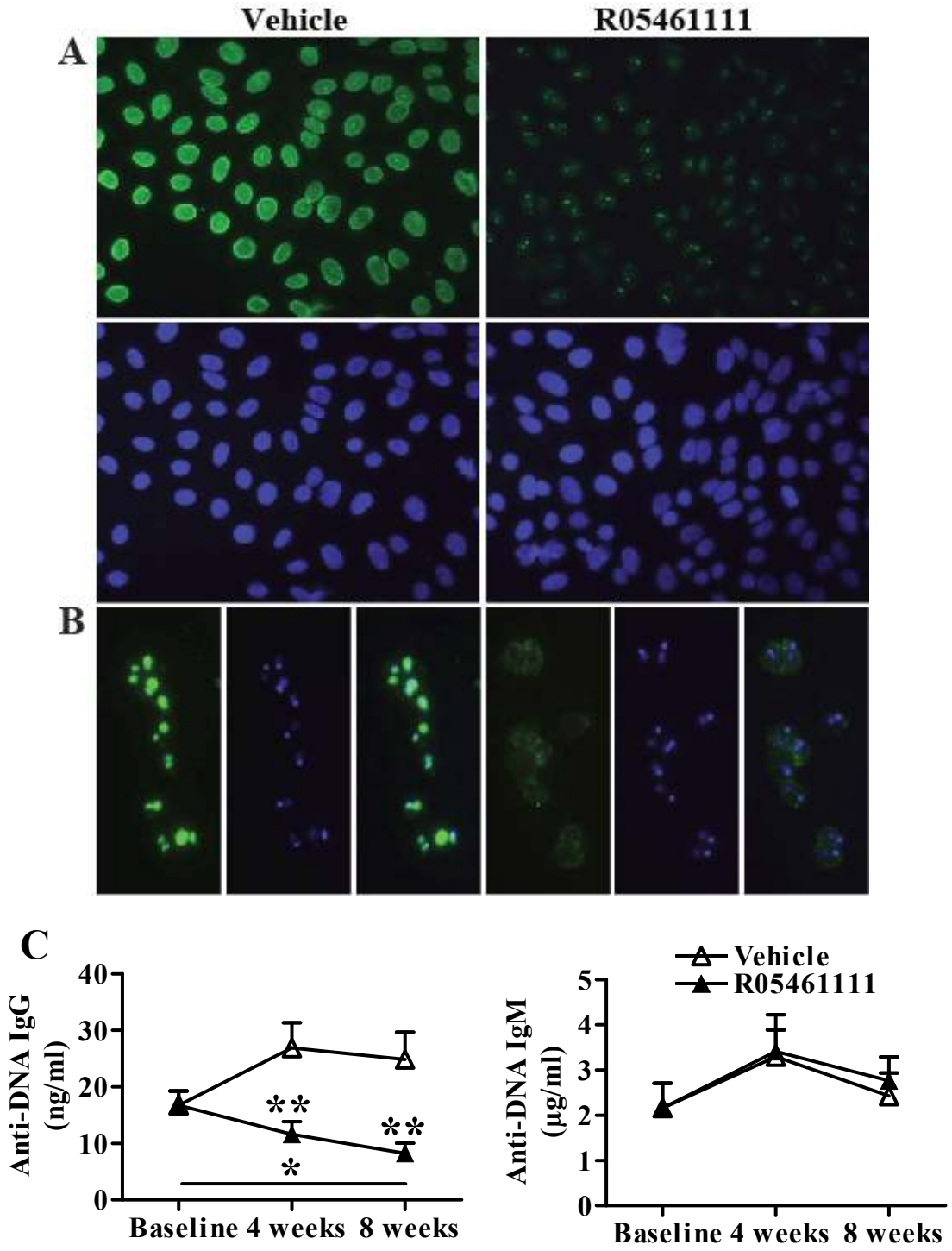


Figure 33. R05461111 reduces autoantibodies in MRL-Fas(lpr) mice. (A) ANA staining patterns on Hep2 human epithelial cells for plasma, derived from vehicle-treated and R05461111 treated MRL-Fas(lpr) mice at 1:200 dilution. (B) *Crithidia luciliae* slides were incubated with 1:50 diluted plasma of 20 weeks old mice from both treatment groups, and autoantibody binding to the flagellate's kinetoplast was detected using an FITC-labeled goat anti-mouse IgG. Images on the left show anti-dsDNA IgG in green, and staining the kinetoplast DNA itself with DAPI is in blue was shown in the middle. The merged pictures are shown on the right side demonstrating that FITC positivity matches with the kinetoplast at the flagella pole of *C. luciliae*. (C) Plasma levels of anti-dsDNA IgG and IgM antibodies were determined by ELISA. Data are expressed as means ± SEM (n=15 each treatment group). *p<0.05, **p<0.01 versus vehicle group.

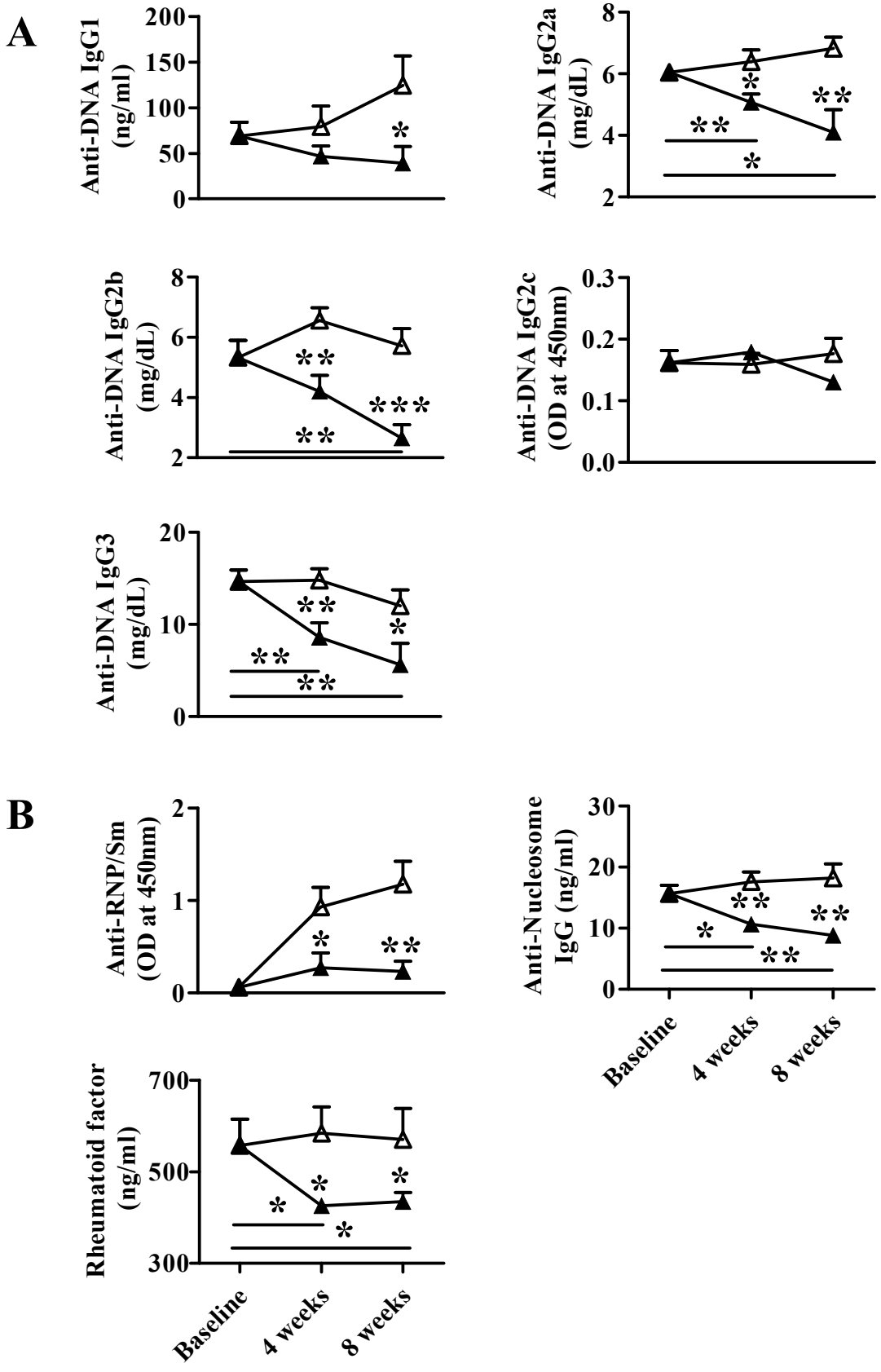


Figure 34. (A) Plasma levels of IgG isotypes such as IgG1, IgG2a, IgG2b, IgG2c and IgG3 against ds-DNA were determined by ELISA. (B) Plasma levels of anti-RNP/Sm, anti-nucleosome antibodies and rheumatoid factor were determined by ELISA. Data are expressed as means ± SEM (n=15 each treatment group). *p<0.05, **p<0.01, ***p<0.001 versus vehicle group.

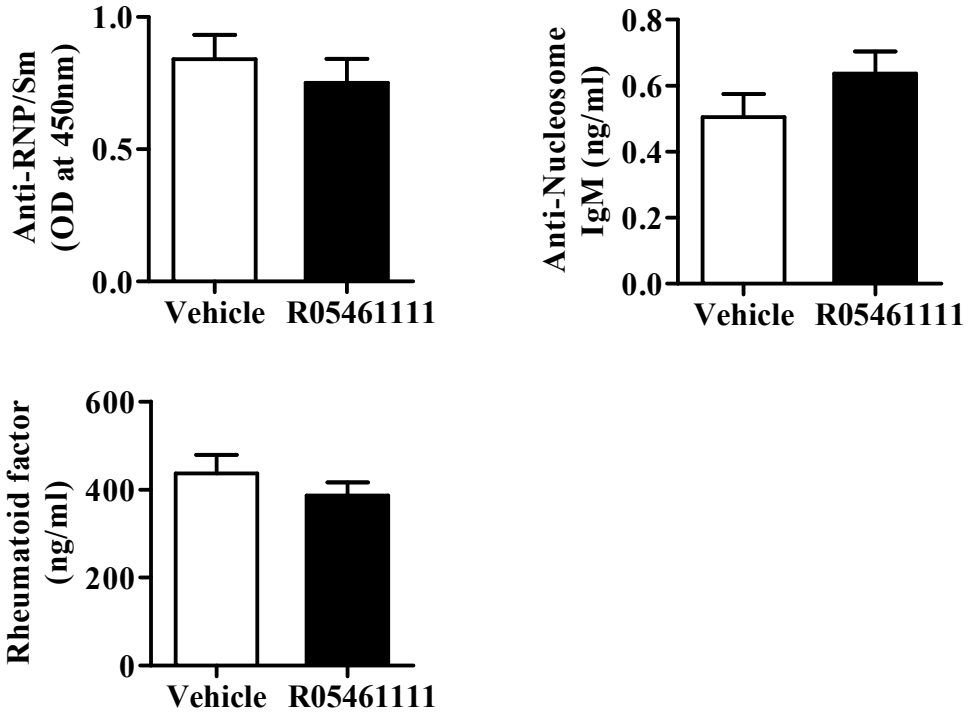


Figure 35. R05461111 does not affect IgM autoantibodies in MRL-Fas(lpr) mice. Plasma levels of IgM autoantibodies (smith antigen, anti-nucleosome, Rheumatoid factor) were determined by ELISA. Data are expressed as means ± SEM (n=15 each treatment group).

4.2.10 Cat S blockade with RO5461111 improves lupus nephritis in MRL-(Fas)lpr mice

Autoimmune IC-GN develops from intrarenal immune complex deposition and local complement activation[135]. Vehicle-treated 20 week old female MRL-Fas(lpr) mice displayed strong intraglomerular IgG and complement C3c deposits, which was associated with diffuse mesangial matrix expansion, profound mesangial cell proliferation, and focal segmental glomerulosclerosis (Figure 36A). Cellular crescents and global glomerulosclerosis were often seen. Most animals revealed profound tubulointerstitial inflammation as characterized by periglomerular and diffuse interstitial leukocyte infiltrates, tubular atrophy and intraluminal cast formation. Cat S blockade with RO5461111 significantly reduced glomerular IgG and C3c deposits (Figure 36B) and the glomerular as well as the tubulointerstitial immunopathology as quantified by the lupus nephritis activity index that encompasses glomerular cell proliferation, matrix deposition, leukocyte infiltration, focal necrosis, crescent formation, and tubulointerstitial inflamm-

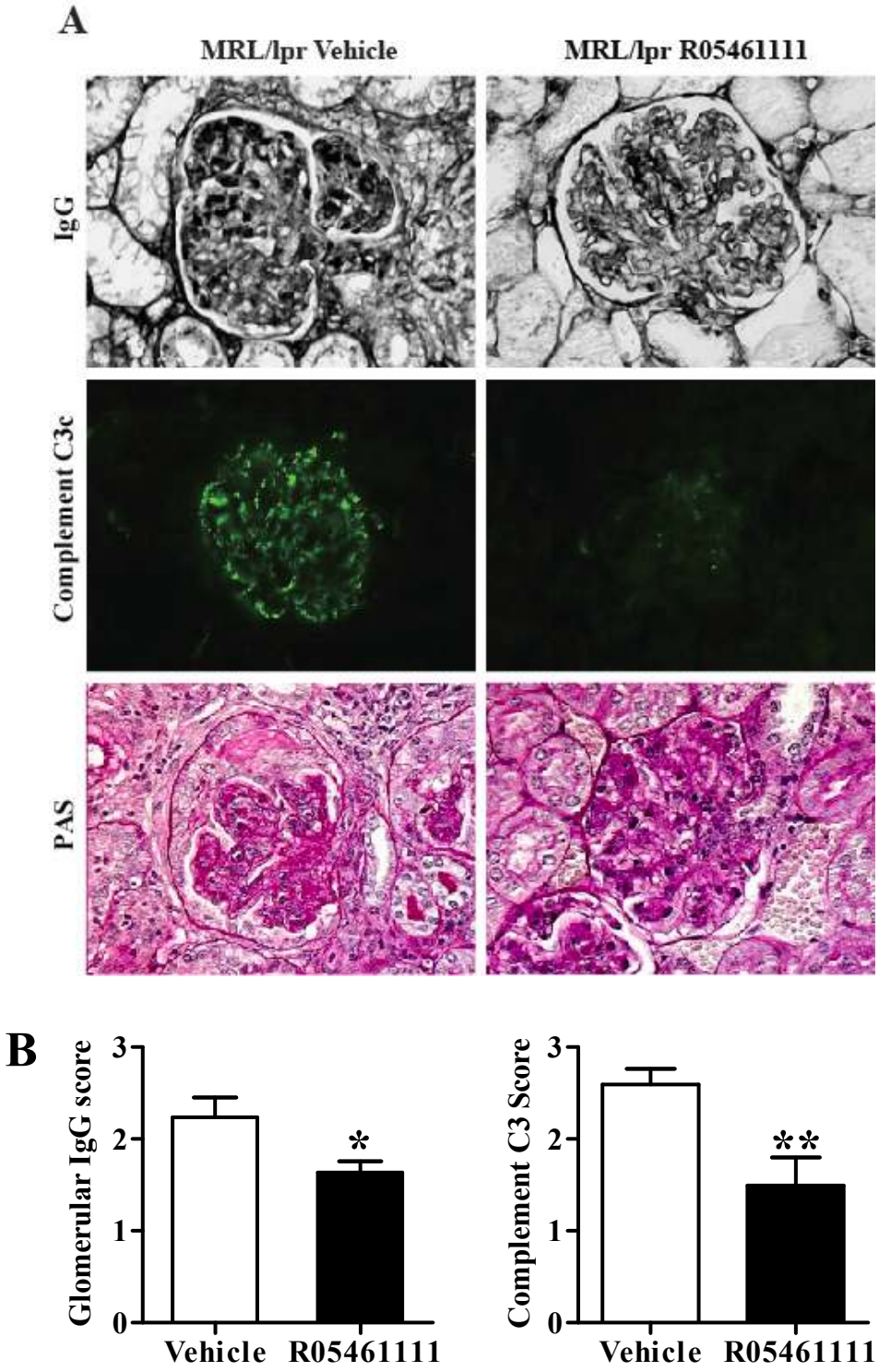


Figure 36. Cat S inhibitor R05461111 suppresses renal pathology in MRL-Fas(lpr) mice. (A) Renal sections of 20 week old MRL-Fas(lpr) mice were stained with antibodies for IgG and complement C3c as indicated. Note that inhibitor treated mice showed less glomerular IgG and C3c deposits. Renal sections of 20 week old MRL-Fas(lpr) mice were stained with periodic acid-Schiff (PAS). Representative PAS staining kidney sections showed glomerular damage in vehicle-treated mice when compared with inhibitor treated mice. Images are representative for 15 mice in each treatment group. Magnification, ×400 or ×200. (B) Glomerular IgG and complement C3c deposition was compared between vehicle- and inhibitor-treated groups (score ranging from 0 to 3). *$p<0.05$, **$p<0.01$, versus vehicle group. (Vehicle n=15, R05461111 n=15).

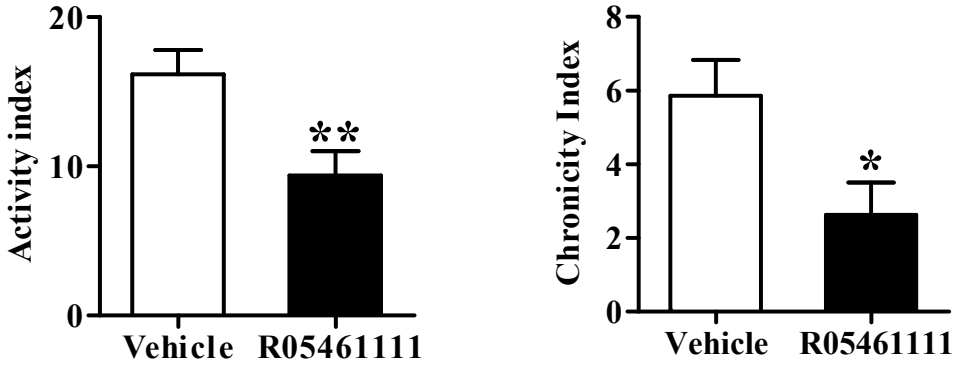


Figure 37. The lupus nephritis disease activity index (score ranging from 0 to 24), and the lupus nephritis chronicity index score ranging from 0 to 12) were determined as markers of kidney damage in lupus nephritis. Data are expressed as means ± SEM. *p<0.05, **p<0.01, versus vehicle group. (Vehicle n=15, R05461111 n=15).

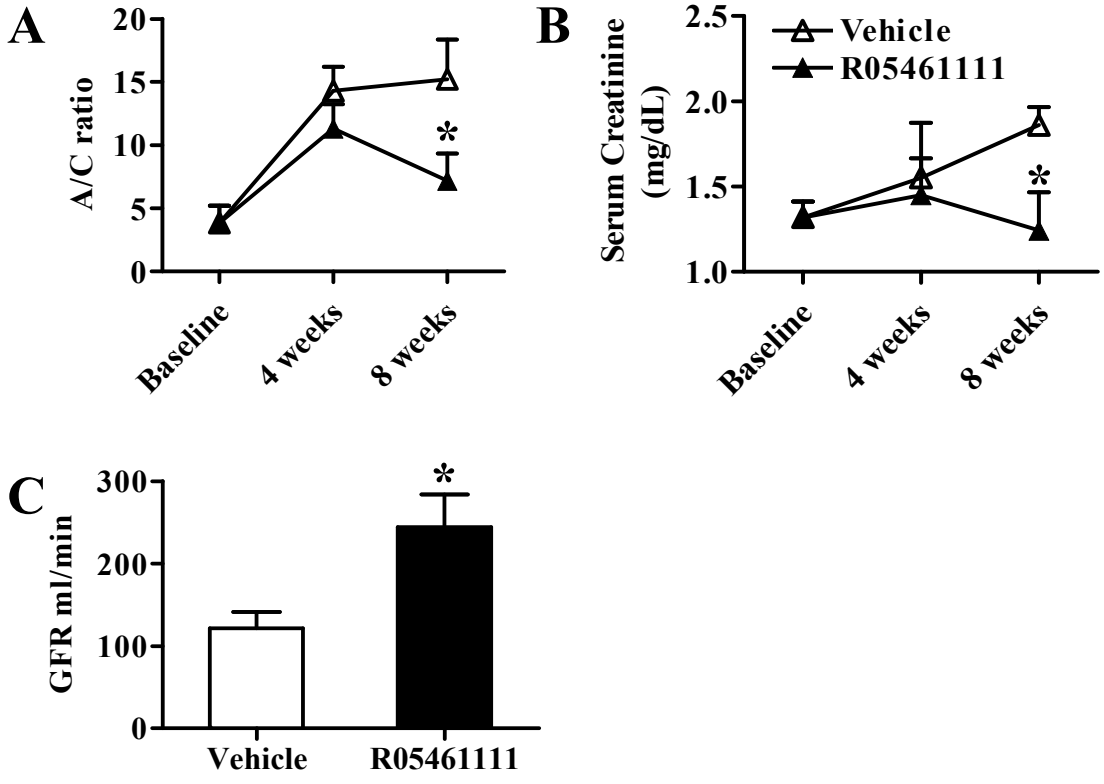


Figure 38. Renal functional parameters like Proteinuria (A), plasma creatinine (B) and glomerular filtration rate (C) were determined from 15 mice in the each treatment group at 20 weeks of age. Data are expressed as means ± SEM. *p<0.05 versus vehicle group. (Vehicle n=15, R05461111 n=15).

tion. Treatment with RO5461111 suppressed IC-GN as documented by a significant reduction of the nephritis chronicity index that encompasses glomerular sclerosis, fibrous crescents, tubular fibrosis and interstitial fibrosis (Figure 37). This improvement of renal structure was associated with a significant reduction of proteinuria and serum creatinine levels after 8 weeks of treatment (Figures 38A and 38B). Glomerular filtration rate, as assessed by FITC-inulin clearance kinetics at the end of the study, was severely impaired in vehicle-treated MRL-Fas(lpr) mice but significantly increased upon RO5461111 treatment (Figure 38C). These effects were associated with significantly reduced mRNA expression levels of IL-1β, IL-6, IL-10 and IL-12p35, while IFN-γ and TGF-β1 remained unaffected (Figure 39A). RO5461111 significantly reduced the number of glomerular Mac-2^+ macrophages (Figure 39B and 39C). FACS analysis of kidney has shown significantly reduced the number of activated DCs (Figure 39D). FACS analysis of F4/80^+ macrophages has shown a trend towards less macrophages in the kidneys of R05461111 treated mice (Figure 39E). Thus, Cat S contributes to the progression of IC-GN in MRL-Fas(lpr) mice, a process that can be prevented by RO5461111.

4.2.11 Cat S blockade with RO5461111 reduces lung inflammation in MRL-(Fas)lpr mice

The lung is another organ frequently affected by systemic autoimmunity in MRL-Fas(lpr) mice. Vehicle-treated MRL-Fas(lpr) mice developed prominent peribronchial immune cell infiltrates that compressed and destroyed lung tissue (Figure 40A; 40B). Such infiltrates were significantly decreased in 20-wk-old RO5461111-treated MRL-Fas(lpr) mice together with less mRNA expression levels of IL-6, IL-12p40 and CCL2 (Figure 40C), and lower numbers of Mac-2^+ macrophages (Figure 40D). Thus, Cat S also promotes pulmonary inflammation and damage in MRL-Fas(lpr) mice. Obviously, late onset of RO5461111 therapy has beneficial effects on immune deregulation, autoantibody production as well as IC-GN and lung disease in MRL-Fas(lpr) mice with systemic autoimmunity.

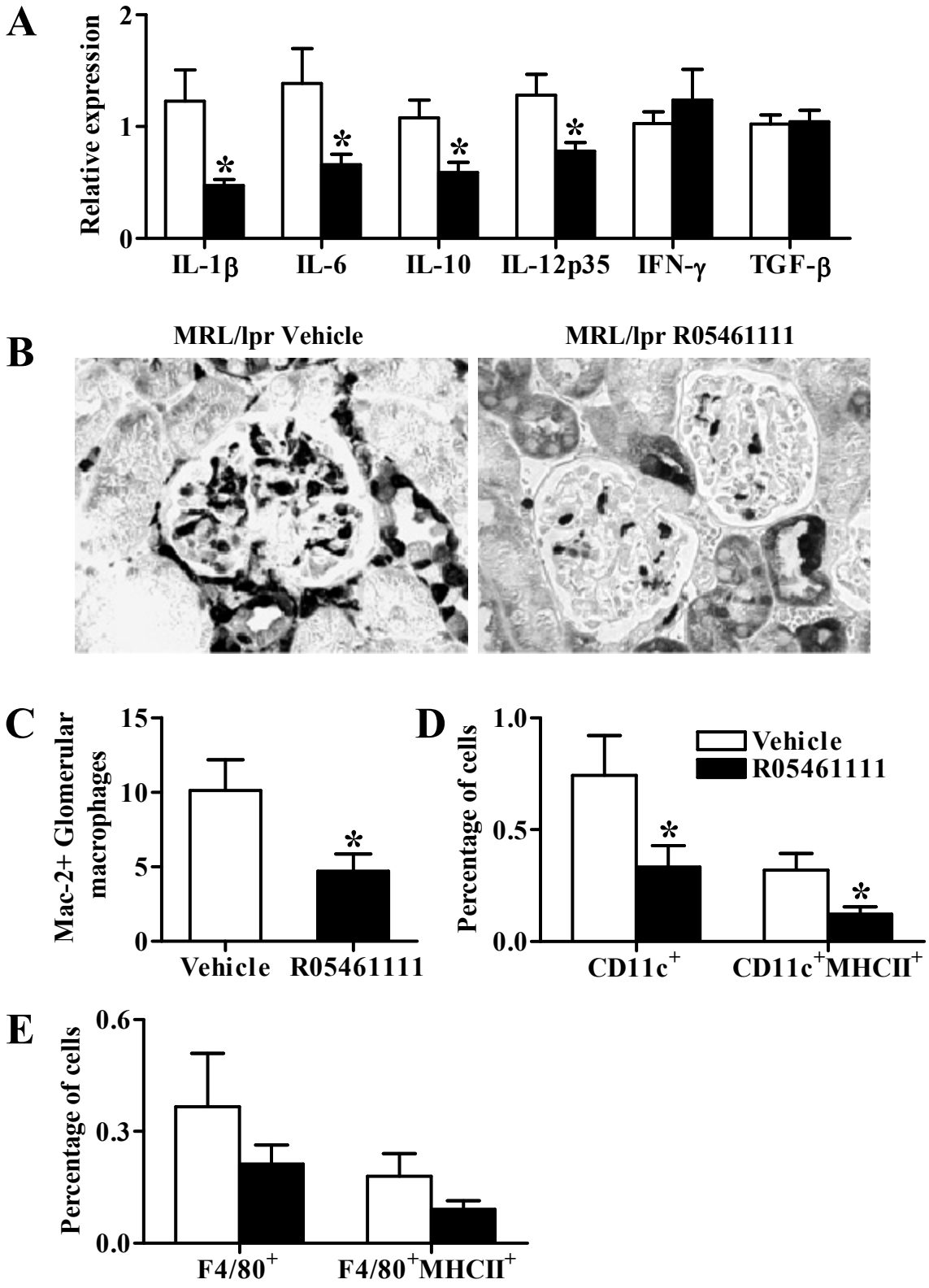


Figure 39. (A) Renal mRNA expression of IL-1β, IL-6, IL-10, IL-12p35, IFN-γ and TGF-β were determined by real time PCR. Data are expressed as a mean ratio to the respective 18S rRNA mRNA expression level ± SEM. (B) Renal sections of 20 weeks old MRL-Fas(lpr) mice were stained with an anti-Mac-2 antibody. (C) Quantification of Mac-2 positive glomerular macrophages. (D) FACS analysis of renal DCs and activated DCs. (E) FACS analysis of renal macrophages and activated macrophages. Data are expressed as means ± SEM. *$p<0.05$ versus vehicle group. Original magnification, ×100. (Vehicle n=15, RO5461111 n=15).

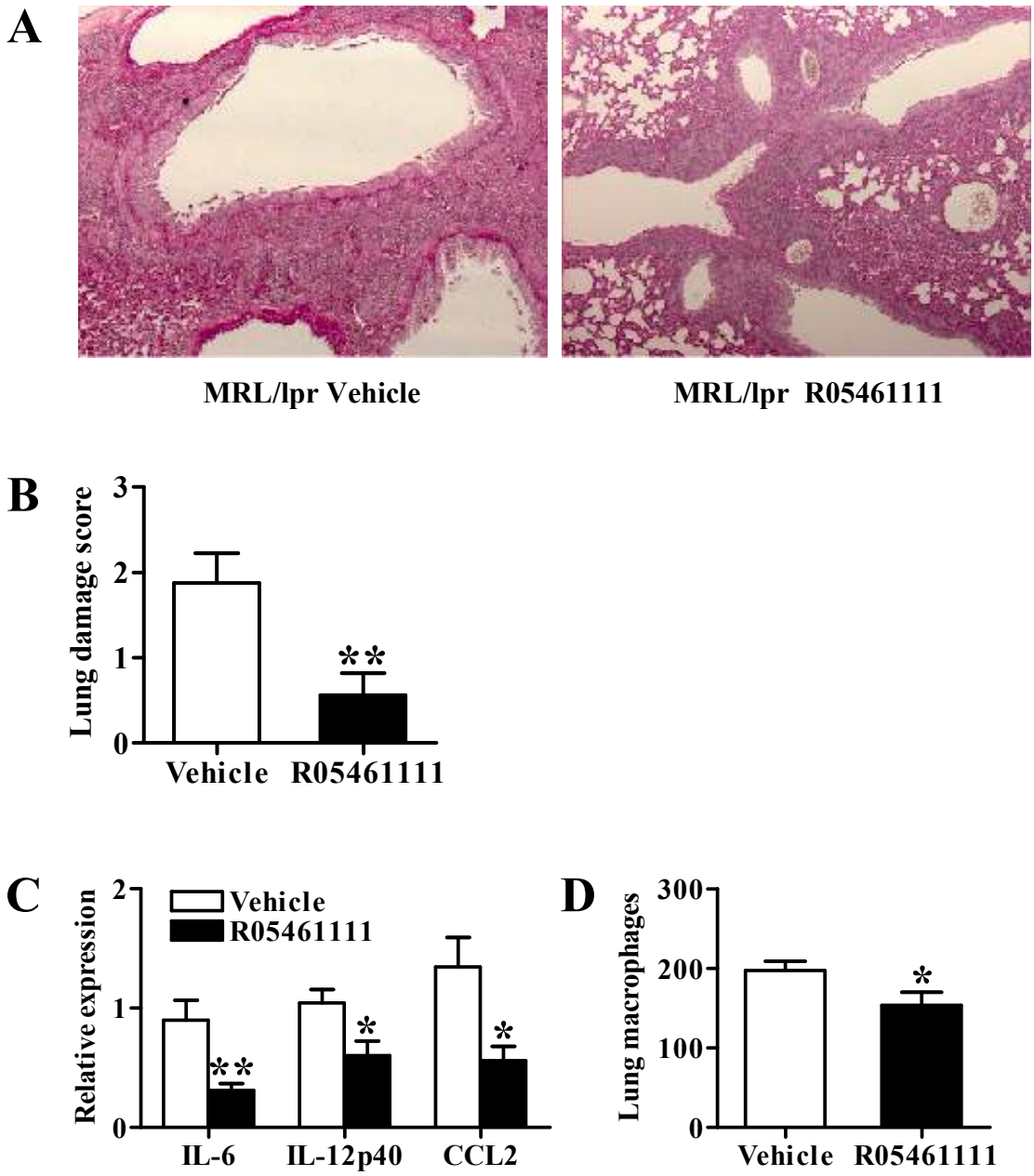


Figure 40. Cat S inhibitor R05461111 improves lung disease in MRL-Fas(lpr) mice. (A) Lung sections of 20 week old MRL-Fas(lpr) mice of both groups were stained with Periodic acid Schiff. Note the peribronchial and perivascular lymphocyte infiltrates were less in RO5461111 mice when compared with vehicle mice. Images are representative for 15 mice in each treatment group. Original magnification, ×100. (B) Morphometry was used to quantify the peribronchiolar and perivascular inflammation by using a score ranging from 0 to 3 and scored semiquantitatively as described in the methods section. (C) Lung mRNA expression levels of IL-6, IL-12p40 and CCL2 in MRL-Fas(lpr) mice of both groups were determined by qPCR. (D) Quantification of lung macrophages. The data represent mean scores ± SEM. *p<0.05, **p<0.01 versus vehicle group. (Vehicle n=15, RO5461111 n=15).

5. Discussion

5.1 Part I - Activated protein C in SLE

Recombinant aPC was recently shown to suppress a number of hyperinflammatory and autoimmune states; hence, we had hypothesized that recombinant aPC may also suppress SLE and lupus nephritis. Our experiments using autoimmune and nephritic MRL-Fas(lpr) mice now document that recombinant human aPC can suppress lupus nephritis as well as other organ manifestations of SLE such as cutaneous lupus and lung disease. Lupus nephritis is triggered by glomerular immune complex deposits that activate complement, increased intrarenal expression of proinflammatory cytokines and chemokines, and subsequent recruitment of macrophages and other immune cells that contribute to renal inflammation and damage. Therefore, active lupus nephritis in humans and MRL-Fas(lpr) mice is characterized by diffuse mesangioproliferative glomerulonephritis and often associated with fibrinoid tuft necrosis, focal adhesions and crescent formation.

In this study, we used Drotrecogin alfa activated (Xigris®; Lilly Deutschland GmbH), a human recombinant aPC that was shown to be effective in mice. Xigris® had a neuroprotective effect in murine neuronal and brain endothelial cell injury models[136]. Extracellular histones are cytotoxic toward endothelium in-vitro and are lethal in mice. aPC cleaves histones and reduces their cytotoxicity. Administration of aPC prevented septic-like reactions after injection of histones into mice[137]. Lethal toxin (LT) is a major virulence factor of *Bacillus anthracis* that plays a vital role in pathogenesis and the suppression of the host immune response. Sprague Dawley rats manifested pronounced lung edema and shock after LT treatments, resulting in high mortality. The activation of the coagulation cascade is involved in LT-mediated pathogenesis in rats. The anticoagulant aPC significantly reduced mortality in LT-treated rats[138]. Tissue plasminogen activator (tPA) is the only approved therapy for acute ischemic stroke. However, tPA has a brief therapeutic window. Its side effects include intracerebral bleeding and neurotoxicity. The tPA and 3K3A-APC (aPC variant with reduced anticoagulant activity) combination therapy reduced the infarct volume and improved behavior in models of ischemic stroke in rodents[139]. Thus, aPC and its variants have protective role.

In sepsis, it was initially thought that aPC has antithrombotic and profibrinolytic functions. Other agents with more potent anticoagulation functions were not effective in treating severe sepsis[140,141]. Recently, it has been postulated that cytoprotective effects of aPC are responsible for reduced mortality. Treatment with aPC variants with diminished anticoagulant activity could still have cytoprotective effects in a mouse model of sepsis[142]. This suggests that the effects of aPC in MRL-Fas(lpr) mice are due to cytoprotective signaling properties. aPC variants with reduced bleeding risk may permit therapies using higher aPC doses for shorter times. Higher doses of aPC may stabilize stressed cells at risk for excessive inflammation or apoptosis and prevent organ failure.

In addition, we could detect lower serum aPC levels in MLR-Fas(lpr) mice compared with MRL wt mice, indicating a suppression of the protein C pathway during SLE. In septic patients, reduced aPC levels have been associated with an increased risk of death[143,144]. The brain is one of the first organs affected clinically in sepsis. Microcirculatory alterations are suggested to be a critical component in the pathophysiology of sepsis. aPC treatment significantly improved pial microcirculation by reducing leukocyte adhesion and increasing functional capillary density in experimental endotoxemia in Lewis rats[145]. A pathogenic role for high-mobility group box 1 (HMGB1) protein has been postulated in severe sepsis. aPC not only inhibited HMGB1 release but also down-regulated the cell surface expression of all three HMGB1 receptors in endothelial cells. Thus, anti-inflammatory activities of aPC play important role in the suppression of severe sepsis. The protective effects of aPC were mediated through EPCR and PAR-1[146].

Aberrations of the monocyte/macrophage phenotype and function are increasingly recognized in SLE and animal models of the disease. Cao *et al*.[147]. showed that the anti-inflammatory activity of aPC on macrophages is dependent on integrin CD11b/CD18, but not on EPCR. They showed that CD11b/CD18-bound aPC, facilitated cleavage and activation of PAR-1, leading to enhanced production of sphingosine-1-phosphate and suppression of the proinflammatory response of activated macrophages[147]. aPC decreases the release of the MIP-1 a from the monocytic cell line THP-1 and from human monocytes of septic patients[148]. aPC also inhibits LPS-induced nuclear translocation of NF-kB, TNF-α, IL-1β, IL-6, and IL-8 production in the THP-1 cells[149,150]. Thus, treatment with aPC inhibits proinflammatory effects of monocytes/macrophages.

Treatment with recombinant aPC over a period of 5 weeks suppressed all of the aforementioned histopathological abnormalities of lupus nephritis. Renal function parameter serum BUN was significantly reduced by aPC treatment. Other renal function parameters like proteinuria and serum creatinine showed non-significant trends toward improvement of secretory and barrier functions, indicating that recombinant aPC is not as potent as pulse dosing with cyclophosphamide, which we previously reported to almost completely abrogate kidney disease in this model[151]. What are the mechanisms by which aPC treatment suppresses kidney disease in MRL-Fas(lpr) mice? aPC can elicit direct cytoprotective effects on glomerular endothelial cells and podocytes by inhibiting the intrinsic mitochondrial apoptosis pathways in mice with diabetic nephropathy[105]. aPC preserves endothelial cells via a PAR-1 and EPCR-dependent mechanism. Conversely, in podocytes, aPC inhibits apoptosis through proteolytic activation of PAR-3 independent of EPCR. PAR-3 is not signaling competent itself as it requires aPC-induced heterodimerization with PAR-2 (human podocytes) or PAR-1 (mouse podocytes)[152]. aPC also has anti-oxidant effects. Recently, Bock *et al*. have shown that aPC inhibits the expression of the redox-regulating protein $p66^{Shc}$, which is primarily associated with mitochondrial ROS generation and apoptosis. $p66^{Shc}$ deficient mice are protected against diabetic nephropathy. Treatment with aPC ameliorated diabetic nephropathy by inhibiting mitochondrial dysfunction in podocytes[153]. These studies identify mechanisms underlying the cytoprotective effects of aPC. These effects could contribute to the protective effects on glomerular damage in MRL-Fas(lpr) mice.

Recombinant aPC has been reported to prevent microvascular inflammation in autoimmune bowel disease[106]. Microvascular inflammation is a central element of infectious as well as autoimmune tissue damage and mediated by the activation of endothelial cells. A recent report first described that extracellular histones, which are released from dying cells, elicit a direct killing effect on endothelial cells followed by microvascular inflammation, a process that is inhibited by recombinant aPC, because the proteolytic activity of aPC degrades histones to immunologically inactive peptides[137]. The immunostimulatory effect and cytopathic effects of extracellular histones likely contribute also to lupus nephritis, at least in areas of focal necrosis, because histones are an essential element of neutrophil extracellular traps that were recently shown to mediate glomerular inflammation in anti-neutrophil cytoplasmic autoantibody-associated renal vasculitis[154].

Thus, aPC has protects endothelium by cleaving histones and thereby reducing their cytotoxicity.

aPC is a serine protease derived from its inactive zymogen protein C, and the fact that kidney, skin, and lung disease were all improved similarly suggests that recombinant aPC might suppress systemic disease mechanisms of SLE. In fact, our analysis revealed that aPC treatment affected many elements of autoimmunity. For example, lupus nephritis and most other tissue pathologies in SLE develop secondary to immune complex disease involving a polyclonal expansion of autoreactive B cells, plasma cells, and anti-nuclear autoantibody production[4]. aPC significantly reduced all of these elements of humoral autoimmunity as well as subsequent glomerular IgG deposits, indicating that protection from severe lupus nephritis is due to a systemic suppression of immune complex disease. The proliferation of autoreactive B cells in lupus depends on autoantigen-presentation and costimulatory signals from DCs[133]. Therefore, it is of note that recombinant aPC suppressed the activation of spleen DCs, which is an important determinant of SLE disease activity[133,40]. The activation of DCs also contributes to systemic inflammation; hence, we attribute the aPC-mediated suppression of plasma cytokine levels to its capacity to suppress DCs. Pretreatment with aPC in isolated macrophages activated *in vitro* with LPS showed less release of IL-6, whereas aPC-pretreated T cells activated with CD3/CD28 showed inhibition of NF-kB signaling[155]. Therefore, we conclude that, treatment with aPC suppressed the activation splenic DCs, leading to the reduction in B cells and consequently resulting in less plasma cells.

The signaling effects of aPC on B cells are still unknown. In mouse endotoxemia and sepsis models, mortality reduction requires the cell signaling function of aPC, mediated through PAR1 and EPCR. A recent study discovered that the expression of EPCR was limited to the $CD8^+$ subset of spleen conventional DCs and is sufficient to suppress immune responses during sepsis. In addition, aPC (5A-aPC) inhibited the inflammatory response of conventional DCs independent of EPCR and suppressed IFN-γ production by natural killer-like DCs[156]. Our own data are in line with these observations, as recombinant aPC reduced the activation of $CD4^+$ and $CD8^+$ spleen dendritic cell subsets in MRL-Fas(lpr) mice. As such, we assume that aPC did not have direct effects on T and B cells, but rather that these are secondary to aPC-mediated suppression of dendritic cell activation. Interestingly, recombinant aPC did not have an effect on spleen T cell counts,

and its suppressive effect was limited to Th1 and Th17 T cells, two effector T cell subpopulations that play a dominant role in the cellular component of autoimmunity in human SLE as well as in SLE of MRL-Fas(lpr) mice[130]. Also, in a mouse model of multiple sclerosis, the amelioration of disease after aPC treatment was accompanied by inhibition of Th1 and Th17 cytokines[155]. Recently Kulkarni *et al*. have shown that cyclophosphamide or Mycophenolate mofetil cause broad T cell ablation in MRL-Fas(lpr) mice, like in humans, which should be a causative factor for the infectious complications that are associated with such therapies[142,157]. In this regard, recombinant aPC may have a better toxicity profile as T cell ablation was not observed. The most frequent toxicity that occurred in the recombinant aPC sepsis trials was bleeding[101,158], notifying aPC's biological function as a natural anticoagulant. It is of note that bleeding or hemorrhages were not observed in aPC-treated MRL-Fas(lpr) mice, macroscopically or on histopathological assessment of the lungs, skin, and kidneys.

In summary, recombinant aPC improves lupus nephritis and lupus-related skin and lung disease mainly by suppressing the abnormal autoimmunity of SLE.

5.2 Part II - Cathepsin S in SLE

The central role of MHC-II-mediated adaptive immunity for the several forms of IC-GN and the non-redundant role of Cat S in peptide loading and MHC-II assembly prompted us to speculate that Cat S would be an essential mediator and potential therapeutic target for IC-GN. During the primary immune response low affinity antibodies of the IgM subclass are produced. Subsequently, driven by antigen stimulation through the B-cell antigen receptor and CD40, naïve B-cells enter the GC microenvironment, where they begin to proliferate and undergo clonal expansion. Germinal centers are specialized structures in which B lymphocytes undergo clonal expansion, class switch recombination, somatic hypermutation, and affinity maturation. Autoimmune IC disease develops from the presentation of autoantigens in the context of costimulation, followed by an expansion of autoreactive lymphocytes and the clonal selection of high affinity IgG autoantibody-producing plasma cells, a process that takes place in the GC of lymphoid follicles[120]. Somatic hypermutation and class switch recombination occur at the centroblast stage of B-cell maturation and together are responsible for generating high-affinity antibodies of

different subclasses that are capable of mediating specific immune responses. Our data confirm previously published reports that Cat S inhibition interferes with all of these processes upon vaccination with foreign or self-antigens[159,123,112,107], most likely by limiting Ii-dependent peptide loading onto MHC-II.

The novel aspects of our study are three-fold. First, here we first report a novel Cat S antagonist (R05461111) that is potentially suitable for therapy in humans. Second, we first document the contribution of Cat S in autoimmune IC-GN involving lupus-like spontaneous systemic autoimmunity and antinuclear antibody production. This process specifically involves priming of $CD4^+$ T cell subsets (but not $CD8^+$ T cells) and affinity maturation towards IgG autoantibody-producing plasma cells within the GC. Third, Cat S blockade initiated as late as upon 'clinically' detectable signs of IC-GN, such as proteinuria and/or elevated serum creatinine, was effective in suppressing hypergammaglobulinemia and autoantibody production below baseline and in preventing IC disease progression.

The possibility of using a Cat S inhibitor to regulate overactive autoimmune responses was confirmed, with the use of Cat S inhibitor CLIK-60 in a murine model for Sjoegren's syndrome. Intraperitoneal administration of CLIK-60 showed elimination of the autoimmune manifestations by preventing the presentation of organ specific antigen α-fodrin by MHC-II. The remission of lesions in salivary and lachrymal glands was not observed with a cathepsin B or L inhibitor[114]. A highly selective Cat S inhibitor, CSI-75, was tested in a murine model of multiple sclerosis and in a murine model of rheumatoid arthritis. When given orally, CSI-75 caused a significant reduction in disease score in both disease models. These studies have revealed that Cat S inhibitor, CSI-75 caused a selective suppression of the Th1 and Th17 cytokines[112]. Thus, indicating a promising role for Cat S inhibitors in the treatment of autoimmune diseases.

Cat S inhibitors or Cat S knockout mice have shown protection in some animal models of disease other than autoimmune diseases. Deschamps *et al*. have used genetic and pharmacological approaches to illustrate that Cat S is critically required for antigen-induced lung inflammation. Oral administration of Cat S inhibitor compound 7 has been found to block the rise in immunoglobulin E titers and eosinophil infiltration in the lungs, in a mouse model of ovalbumin-induced pulmonary inflammation. Cat S knockout mice

did not develop ovalbumin-induced pulmonary inflammation, consistent with a role for Cat S in the development of the allergic response. This suggests that Cat S is involved in asthma[121]. Zheng *et al*. have used selective Cat S inhibitor 05141 in IFN-γ-induced alveolar remodeling and emphysema. Cat S-dependent epithelial cell apoptosis is a critical event in the pathogenesis of IFN-γ-induced alveolar remodeling and emphysema. Selective Cat S inhibition with oral administration of 015141 or a null mutation of Cat S decreased IFN-γ-induced apoptosis, emphysema and inflammation. In conclusion, these studies demonstrated that Cat S-dependent epithelial cell apoptosis is a critical event in the pathogenesis of IFN-γ-induced alveolar remodeling and emphysema[160]. Thus, Cat S inhibition has therapeutic potential in the treatment of emphysema.

Human atherosclerotic lesions overexpress the lysosomal cysteine protease Cat S. Compared with $LDLR^{-/-}$ mice, double-knockout mice ($CatS^{-/-}LDLR^{-/-}$) developed significantly less atherosclerosis, as indicated by plaque size. These findings establish a pivotal role for Cat S in atherogenesis and Cat S deficient mice developed significantly less atherosclerosis[116]. Samokhin *et al*.[161] have investigated the effect of a specific Cat S inhibitor on atherosclerotic plaque progression in the brachiocephalic artery. Male and female Apoe-/- mice on a cholate-containing high-fat diet containing or lacking a specific Cat S inhibitor were evaluated for the remodeling of atherosclerotic lesions. The size of atherosclerotic plaques in inhibitor-treated mice was reduced by 36% in male and 68% in female mice. In conclusion, the inhibition of Cat S showed a strong atheroprotective activity. Thus, Cat S inhibition through specific inhibitors or genetic elimination of the gene has therapeutic potential.

Cat S inhibition with the novel, orally available, and highly selective Cat S inhibitor RO5461111 was effective in-vivo and had profound effects on the SLE-like systemic autoimmunity that spontaneously develops in MRL-Fas(lpr) mice. The most striking therapeutic effect was the suppression of IgG but not IgM (auto-) antibody production, which was consistent for all ANA specificities tested. This effect on Ig class switch implies that Cat S contributes to the affinity maturation of the respective antibody-producing cells, which was consistent with a significant suppression of spleen plasma cells upon Cat S inhibition. Affinity maturation takes place in the GC of lymphoid follicles and requires an orchestrated interaction of antigen-presenting cells and lymphocytes such as follicular DCs, follicular B cells, and antigen-specific $CD4^+$ T helper cells[120]. Cat S

inhibition disrupted the typical spatial organization of the GC in MRL-Fas(lpr) mice, as it was reported by previous vaccination studies using Cat S-deficient mice or Cat S antagonists[107]. Obviously, compromising appropriate MHC-II assembly is sufficient to impair GC formation, e.g. by suppressing CXCL12 expression, a factor needed for the spatial organization of follicular B cells to the dark zone of GC and their and the maturation towards plasma cells that produce high affinity IgG autoantibodies[162,163].

By contrast, Cat S is not involved in peptide loading to MHC-I, hence, Cat S inhibition did not affect $CD8^+$ T cells in MRL-Fas(lpr) mice. This implies that therapeutic Cat S inhibition should spare MHC-I and $CD8^+$ T cell-mediated immunity, which should reduce the risks of therapy-associated infections as compared to global immunosuppressants such as steroids, cyclophosphamide or mycophenolate mofetil. Of note, we did not observe any infectious complications in the mice studied with the Cat S inhibitor.

We designed our study also to test the therapeutic potential of Cat S inhibition; hence, treatment was initiated not before 'clinically' detectable signs of autoimmune tissue injury, such as proteinuria and/or elevated serum creatinine, became evident. It is of note that Cat S inhibition suppressed hypergammaglobulinemia and autoantibody production below the baseline levels, consistent with a reversal of the underlying systemic autoimmune process. However, at the tissue level Cat S inhibition was effective only in terms of preventing a further increase in serum creatinine, which implies a stabilization of renal function and not a reversal of tissue remodeling. Even when admitting that the reversal of tissue remodeling may take more time than we studied here, our data would favor to initiate Cat S blockade at an early stage of IC-GN disease to normalize aberrant immunity and to prevent disease progression.

In summary, Cat S is a non-redundant mediator of autoimmune IC-GN. Cat S inhibition with RO5461111 protects from progressive IC-GN.

There are some limitations to the conclusions drawn from the present two studies. 1. Both studies are performed in a murine model of SLE, therefore they lack supporting evidence from human experiments. 2. Only a fixed dose of aPC (5 mg/kg) or Cat S inhibitor R05461111 (30 mg/kg) was used throughout the study. But it might be possible that higher doses are even more effective. 3. We have treated mice for 5 weeks in aPC-SLE study and 8 weeks in Cat S inhibitor-SLE study. But it might be possible that different treatment durations are even more effective. 4. The mouse models of SLE are highly homogeneous in terms of disease development and progression, whereas in humans, it is highly variable from person to person. 5. The conclusions were drawn from studying only one animal model of SLE. To mimic the genetic and pathological heterogeneity in humans, it is crucial to use various mouse models in therapeutic studies. It has been shown that same treatment can have variable effects on different mouse models. Thus, multiple models should be used to ensure the applicability of drugs on the more complicated human genetic background. It might be possible that exogenous administration of aPC or blockade of cat S lead to different results in other disease models. 6. There is no mouse model that can mimic the complexity of human lupus. Thus, drugs validated in MRL-Fas(lpr) mouse model may only be effective for a subset of symptoms or patients. 7. Recombinant aPC used in the present study is a human specific protein. 8. Cat S inhibition-related immunosuppression may impair host defense, which was not addressed here. However, there is currently little data available on infection risk in the Cat S KO or in animals treated with Cat S inhibitors. 9. In the present studies we compared the treatment to placebo rather than the standard of care. 10. We did not evaluate survival benefit in both studies, which would have required an additional set of experiments with drug exposure until death. Such studies were not permitted by the ethical committee.

6. Summary and Conclusion

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease leading to inflammatory tissue damage in multiple organs (e.g., lupus nephritis). Current treatments including steroids, antimalarials, and immunosuppressive drugs have significant side effects. There is an unmet medical need for new drugs with less side effects. We evaluated the role of two proteases, namely activated protein C (aPC) and cathepsin S (Cat S), in the pathogenesis of SLE in MRL-Fas(lpr) mice.

aPC is a serine protease and acts as a natural anticoagulant. In addition to its anticoagulation functions, aPC also has cytoprotective activities such as anti-inflammatory, anti-apoptotic, pro-fibrinolytic and endothelial barrier protection. The immunosuppressive and cytoprotective effects of aPC have now emerged as a potential treatment for a number of autoimmune and inflammatory diseases that are associated with excessive immune responses such as acute respiratory distress syndrome, multiple sclerosis, rheumatoid arthritis, brain injury, lung injury, spinal cord injury, asthma, stroke, ischemia/reperfusion-induced renal injury, diabetic nephropathy and chronic wounds.

Based on immunosuppressive and cytoprotective effects of aPC, we hypothesized that recombinant aPC would suppress SLE and lupus nephritis. To test this concept, autoimmune female MRL-Fas(lpr) mice were injected with either vehicle (PBS) or recombinant human aPC for a period of 5 weeks, from 14-18 weeks of age. Treatment with Recombinant aPC improves lupus nephritis and lupus-related skin and lung disease mainly by suppressing the abnormal autoimmunity of SLE. aPC treatment significantly suppressed lupus nephritis as evidenced by a decrease in activity index, glomerular IgG and complement C3 deposits, macrophage counts, as well as intrarenal IL-12 expression. In addition, aPC attenuated cutaneous lupus and lung disease as compared with vehicle-treated MRL-Fas(lpr) mice. In addition, parameters of systemic autoimmunity, such as plasma cytokine levels of IL-12p40, IL-6, and CCL2/MCP-1, and numbers of B cells and plasma cells in the spleen were suppressed by aPC. The latter was associated with lower total plasma IgM and IgG levels as well as lower titers of anti-dsDNA IgG and rheumatoid factor. Our analysis supports previous findings that aPC acts as a suppressor of activated $CD4^+$ or $CD8^+$ DCs without causing T cell ablation. In conclusion, recombinant aPC

appears to be suitable to block the abnormal immune activation of SLE and thereby prevent lupus nephritis and other lupus manifestations.

Cat S is a lysosomal cysteine protease, which plays an important role in antigen presentation and extracellular matrix degradation. It is mainly expressed in antigen presenting cells (APCs) such as B cells, DCs and macrophages. Cat S has been implicated in the pathophysiology of a number of auto-immune and allergic conditions where $CD4^+$ T-cells are believed to play a role, namely rheumatoid arthritis, multiple sclerosis, psoriasis and bronchial asthma. In addition, Cat S in its secreted form is implicated in the degradation of the extracellular matrix, which may contribute towards initiation and propagation of a number of diseases, including atherosclerosis and chronic obstructive pulmonary disease.

Genetic studies consistently suggest the most common forms of immune complex glomerulonephritis (IC-GN) develop from polymorphisms in HLA genes, implying a role for MHC class II-mediated priming of (auto-) antibody production. Cat S degrades the invariant peptide chain during MHCII assembly with antigenic peptide in APCs, therefore, we hypothesized that Cat S inhibition would be therapeutic in IC-GN. We developed a highly specific small molecule, orally available, Cat S antagonist, RO5461111, with suitable pharmacodynamic and pharmacokinetic properties that efficiently suppressed antigen-specific T cell and B cell priming *in-vitro* and *in-vivo*. We treated MRL-Fas(lpr) mice with Cat S inhibitor R05461111, for a period of 8 weeks, from 12-20 weeks of age. RO5461111 significantly reduced the activation of spleen DCs and the subsequent expansion and activation of $CD4^+$ T cells and CD4/CD8 double negative 'autoreactive' T cells. Cat S inhibition impaired the spatial organization of germinal centers, suppressed follicular B cell maturation to plasma cells, and Ig class switch and subsequent production of high affinity IgG autoantibodies. This reversed hyergammaglobulinemia and significantly suppressed the plasma levels of numerous IgG (but not IgM) autoantibodies below baseline, including anti-dsDNA. This effect was associated with less glomerular IgG deposits, which protected kidneys from IC-GN. In conclusion, Cat S promotes IC-GN by driving MHCII-mediated T and B cell priming, germinal center formation, and B cell maturation towards plasma cells. These afferent immune pathways can be specifically reversed with the Cat S antagonist RO5461111, which prevents IC-GN progression even when given after disease onset. This novel therapeutic strategy could correct a common

pathomechanism of several IC-GNs. This very specific therapeutic intervention, e.g. sparing $CD8^+$ T cell-mediated adaptive immunity, holds the potential to avoid the unfavorable side effects of unselective immunosuppressive drugs currently used for the treatment of IC-GN.

Based on our investigation of the therapeutic potential of aPC and Cat S in a mouse model of SLE, we propose that aPC and Cat S could be novel therapeutic targets for SLE.

7. Zusammenfassung und Fazit

Der Systemische Lupus Erythematodes (SLE) ist eine chronische Autoimmunerkrankung, die mit entzündungsvermittelten Organschäden einhergeht (z.B. Lupusnephritis). Die derzeitigen Therapieregime beinhalten Steroide, Antimalarika und immunsuppressive Medikamente. Sie alle haben beachtliche Nebenwirkungen. Benötigt werden daher neue, nebenwirkungsärmere Substanzen. Wir haben die Rolle der Proteasen Protein C (aPC) und Cathepsin S (Cat S) bei der Pathogenese des SLE in MRL-Fas(lpr) Mäusen untersucht. aPC ist eine Serinprotesase und fungiert als endogenes Antikoagulanz. Außerdem hat aPC anti-entzündliche, anti-apoptotische und pro-fibrinolytische zytoprotektive Funktionen und schützt Endothelbarrieren. Die immunsuppressiven und zytoprotektiven Effekte des aPC liefern möglicherweise einen neuen Behandlungsansatz für eine Reihe von autoimmunen und entzündlichen Erkrankungen, die mit exzessiven Immunantworten einhergehen, wie ARDS, MS, RA, Gehirnschäden, Lungenverletzung, Rückenmarksverletzung, Asthma, Schlaganfall, Ischämie/Reperfusion induzierte Nierenschädigung, diabetische Nephropathie und chronische Wunden.

Basierend auf dem immunsuppressiven und zytoprotektiven Effekt von aPC vermuteten wir, dass rekombinantes aPC die Klinik des SLE und der Lupusnephritis verbessern würde. Um dies zu überprüfen, verabreichten wir MRL-Fas(lpr) Mäusen entweder PBS oder rekombinantes humanes aPC über einen Zeitraum von 5 Wochen (Woche 14-18). Die Behandlung mit aPC verbessert die Lupusnephritis und Lupus bedingte Haut- und Lungenläsionen. Dieser Effekt beruht hauptsächlich auf einer Unterdrückung der abnormen Autoimmunität beim SLE. Die aPC Behandlung reduzierte signifikant den Schweregrad der Lupusnephritis, was in einem reduzierten Aktivitätsindex, weniger glomerulären IgG und C3 Ablagerungen, reduzierter Makrophagenanzahl und intrarenaler IL-12 Expression Ausdruck fand. Außerdem senkte aPC kutane und pulmonale Manifestationen in MRL-Fas(lpr) Mäusen und es fanden sich eine Reduktion der Plasma IL-12p40, IL-6, MCP-1/CCL2 Spiegel und eine verminderte Anzahl an milzresidenten B- und Plasma Zellen in aPC behandelten Tieren - Parameter, die mit systemischer Autoimmunität korrelieren. In der Folge der zellulären Befunde fanden sich daher erniedrigte IgM, IgG, Rheumafaktor, und anti-dsDNA IgG Plasmaspiegel. Unsere Studie stützt vorherige experimentelle Befunde, dass aPC als ein Suppressor-aktivierter $CD4^+$ oder $CD8^+$ DCs fungiert, ohne dabei T-Zell Depletion zu bewirken. Daher scheint

rekombinantes aPC ein nützliches Therapeutikum, um abnorme Immunzellaktivierung beim SLE zu blockieren und dadurch Lupusnephritis und andere Organmanifestationen vorzubeugen.

Cat S ist eine lysosomale Cysteinprotease, die eine wesentliche Rolle bei der Antigenpräsentation und beim Abbau extrazellulärer Matrix spielt. Hauptsächlich wird sie in Antigen - präsentierenden Zellen (APCs), wie B Zellen, dendritischen Zellen und Makrophagen exprimiert. Cat S hat pathophysiologisch relevante Rollen unter zahlreichen autoimmunen und allergischen Zuständen, bei denen $CD4^+$ T Zellen beteiligt zu sein scheinen, z.B.: RA, MS, Psoriasis und Asthma bronchiale. Zudem ist die sezernierte Form von Cat S am Abbau extrazellulärer Matrixkomponenten beteiligt, die möglicherweise zur Initiation und Aufrechterhaltung von zahlreichen Erkrankungen, wie z.B. Atherosklerose oder COPD beitragen.

Genetische Analysen deuten darauf hin, dass sich die häufigsten Formen der Immunkomplex-Glomerulonephritiden (IC-GN) auf dem Boden von Polymorphismen in den HLA-Genen entwickeln. Dies suggeriert eine Rolle für MHCII vermitteltes Priming der (Auto-) Antikörper Produktion bei der Pathogenese dieser Erkrankungen. Cat S spaltet in APC die invariante Peptidkette des MHCII Moleküls während dessen Bindung an ein Antigen. Daher vermuteten wir, dass Cat S Inhibition in IC-GN therapeutisch nützlich sein würde. Wir entwickelten / benutzten einen hochspezifischen, oral verfügbaren Cat S Antagonisten, RO5461111, mit adäquaten pharmakokinetischen und -dynamischen Eigenschaften, der effektiv antigenspezifisches Priming von T und B Zellen in-vitro und in-vivo supprimierte. Über 8 Wochen (Woche 12-20) behandelten wir MRL-Fas(lpr) Mäuse mit dem Cat S Inhibitor RO5461111. Die Aktivität von DCs in der Milz und die Anzahl sowie die Aktivierung von $CD4^+$ T- und CD4/CD8 doppelnegativen ‘autoreaktiven’ T Zellen fand sich signifikant erniedrigt. Cat S Inhibition beeinträchtigte die räumliche Organisation der Keimzentren, unterdrückte die Reifung von B Zellen zu Plasmazellen und hemmte den Ig Klassenwechsel und die Affinitätsreifung. Die Folge waren ein Rückgang der Hypergammaglobulinämie und eine signifikante Reduktion der IgG - aber nicht IgM - Autoantikörper unter die Ausgangswerte, wie z.B. Anti-ds DNA. Assoziiert damit waren eine Reduktion der glomerulären IgG Ablagerungen, was die Niere vor IC-GN schützte. Zusammenfassend aggraviert Cat S durch seinen fördernden Beitrag zur MHCII abhängigen T und B Zell Aktivierung, Keimzentrumsbildung und

Plasmazellreifung den Verlauf der IC-GN. Diese immunologischen Signalwege können spezifisch mit dem Cat S Antagonist RO5461111 inhibiert werden, was den Verlauf der IC-GN bessert, auch wenn erst nach Erkrankungsbeginn therapiert wird. Dieser neue therapeutische Ansatz könnte einen, verschieden IC-GN gemeinsamen, pathomechanistischen Defekt adressieren und korrigieren. Diese sehr selektive therapeutische Intervention könnte damit, z.B. durch reduzierte CD8 vermittelte adaptive Immunität, im Vergleich zu bisherigen unselektiven Therapieregimen weniger unerwünschte Nebenwirkungen aufweisen.

Aus unseren Untersuchungen des therapeutischen Potentials von aPC und Cat S im Mausmodell des SLE folgern wir, dass aPC und Cat S neue therapeutische Angriffspunkte für die Behandlung von SLE darstellen könnten.

8. References

1. Hall RC, S.S. Medical and psychiatric features of systemic lupus erythematosus. *Psychiatr Med.* **Sep;1(3):**, 287-301. (1983).
2. Smyth, A.*, et al.* A Systematic Review and Meta-Analysis of Pregnancy Outcomes in Patients with Systemic Lupus Erythematosus and Lupus Nephritis. *Clinical Journal of the American Society of Nephrology* **5**, 2060-2068 (2010).
3. Rahman, A. & Isenberg, D.A. Systemic Lupus Erythematosus. *New England Journal of Medicine* **358**, 929-939 (2008).
4. Goodnow, C.C. Multistep Pathogenesis of Autoimmune Disease. *Cell* **130**, 25-35 (2007).
5. Lateef, A. & Petri, M. Unmet medical needs in systemic lupus erythematosus. *Arthritis Research & Therapy* **14**, S4 (2012).
6. Martínez Valle, F., Balada, E., Ordi-Ros, J. & Vilardell-Tarres, M. DNase 1 and systemic lupus erythematosus. *Autoimmunity Reviews* **7**, 359-363 (2008).
7. Vedove, C., Giglio, M., Schena, D. & Girolomoni, G. Drug-induced lupus erythematosus. *Arch Dermatol Res* **301**, 99-105 (2009).
8. Appel, G.B.*, et al.* Mycophenolate Mofetil versus Cyclophosphamide for Induction Treatment of Lupus Nephritis. *Journal of the American Society of Nephrology* **20**, 1103-1112 (2009).
9. Tumlin, J.A. Lupus nephritis - Histology, diagnosis and treatment. *Bulletin of the NYU hospital for joint diseases* **66**, 188-194 (2008).
10. Weening, J.J.*, et al.* The Classification of Glomerulonephritis in Systemic Lupus Erythematosus Revisited. *Journal of the American Society of Nephrology* **15**, 241-250 (2004).
11. Ye, D.*, et al.* Polymorphisms in the promoter region of RANTES in Han Chinese and their relationship with systemic lupus erythematosus. *Arch Dermatol Res* **297**, 108-113 (2005).
12. Gorman, C.L.*, et al.* Polymorphisms in the CD3Z Gene Influence TCRζ Expression in Systemic Lupus Erythematosus Patients and Healthy Controls. *The Journal of Immunology* **180**, 1060-1070 (2008).
13. Tan, W.*, et al.* Association of PPP2CA polymorphisms with systemic lupus erythematosus susceptibility in multiple ethnic groups. *Arthritis & Rheumatism* **63**, 2755-2763 (2011).
14. Graham, R.R.*, et al.* Specific combinations of HLA-DR2 and DR3 class II haplotypes contribute graded risk for disease susceptibility and autoantibodies in human SLE. *Eur J Hum Genet* **15**, 823-830 (2007).
15. Hom, G.*, et al.* Association of Systemic Lupus Erythematosus with C8orf13–BLK and ITGAM–ITGAX. *New England Journal of Medicine* **358**, 900-909 (2008).
16. Prokunina, L.*, et al.* A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. *Nat Genet* **32**, 666-669 (2002).
17. Manolio, T.A.*, et al.* Finding the missing heritability of complex diseases. *Nature* **461**, 747-753 (2009).
18. Niederer, H.A., Clatworthy, M.R., Willcocks, L.C. & Smith, K.G.C. FcγRIIB, FcγRIIIB, and systemic lupus erythematosus. *Annals of the New York Academy of Sciences* **1183**, 69-88 (2010).

19. Gross, A.J., Hochberg, D., Rand, W.M. & Thorley-Lawson, D.A. EBV and Systemic Lupus Erythematosus: A New Perspective. *The Journal of Immunology* **174**, 6599-6607 (2005).
20. Mok, C.C. & Lau, C.S. Pathogenesis of systemic lupus erythematosus. *Journal of Clinical Pathology* **56**, 481-490 (2003).
21. Sanchez-Guerrero, J.*, et al.* Past use of oral contraceptives and the risk of developing systemic lupus erythematosus. *Arthritis & Rheumatism* **40**, 804-808 (1997).
22. Bynoe, M.S., Grimaldi, C.M. & Diamond, B. Estrogen up-regulates Bcl-2 and blocks tolerance induction of naïve B cells. *Proceedings of the National Academy of Sciences* **97**, 2703-2708 (2000).
23. Grimaldi, C.M., Jeganathan, V. & Diamond, B. Hormonal Regulation of B Cell Development: 17β-Estradiol Impairs Negative Selection of High-Affinity DNA-Reactive B Cells at More Than One Developmental Checkpoint. *The Journal of Immunology* **176**, 2703-2710 (2006).
24. Rider, V., Jones, S.R., Evans, M. & Abdou, N.I. Molecular Mechanisms Involved in the Estrogen-Dependent Regulation of Calcineurin in Systemic Lupus Erythematosus T Cells. *Clinical Immunology* **95**, 124-134 (2000).
25. Hughes, G.C. & Clark, E.A. Regulation of dendritic cells by female sex steroids: Relevance to immunity and autoimmunity*. *Autoimmunity* **40**, 470-481 (2007).
26. Grimaldi, C.M. Sex and systemic lupus erythematosus: the role of the sex hormones estrogen and prolactin on the regulation of autoreactive B cells. *Current Opinion in Rheumatology* **18**, 456-461 (2006).
27. Smith-Bouvier, D.L.*, et al.* A role for sex chromosome complement in the female bias in autoimmune disease. *The Journal of Experimental Medicine* **205**, 1099-1108 (2008).
28. Jacob, C.O.*, et al.* Identification of IRAK1 as a risk gene with critical role in the pathogenesis of systemic lupus erythematosus. *Proceedings of the National Academy of Sciences* **106**, 6256-6261 (2009).
29. Chang, D.-M., Lan, J.-L., Lin, H.-Y. & Luo, S.-F. Dehydroepiandrosterone treatment of women with mild-to-moderate systemic lupus erythematosus: A multicenter randomized, double-blind, placebo-controlled trial. *Arthritis & Rheumatism* **46**, 2924-2927 (2002).
30. Licht, R., Dieker, J.W.C., Jacobs, C.W.M., Tax, W.J.M. & Berden, J.H.M. Decreased phagocytosis of apoptotic cells in diseased SLE mice. *Journal of Autoimmunity* **22**, 139-145 (2004).
31. Herrmann, M.*, et al.* Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. *Arthritis & Rheumatism* **41**, 1241-1250 (1998).
32. Rovere, P., Fazzini, F., Sabbadini, M. & Manfredi, A. *Apoptosis and systemic autoimmunity: the dendritic cell connection*, (2009).
33. Kamradt, T. & Mitchison, N.A. Tolerance and Autoimmunity. *New England Journal of Medicine* **344**, 655-664 (2001).
34. Nagata, S. & Golstein, P. The Fas death factor. *Science* **267**, 1449-1456 (1995).
35. Goodnow, C.C., Sprent, J., de St Groth, B.F. & Vinuesa, C.G. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature* **435**, 590-597 (2005).
36. Erbacher, A.I.M., Rönnefarth, V.M. & Decker, P. Circulating nucleosomes due to a defective clearance of dying cells: Consequences for systemic lupus erythematosus. *Autoimmunity* **40**, 311-314 (2007).

37. Seery, J.P., Cattell, V. & Watt, F.M. Cutting Edge: Amelioration of Kidney Disease in a Transgenic Mouse Model of Lupus Nephritis by Administration of the Caspase Inhibitor Carbobenzoxy-Valyl-Alanyl-Aspartyl-(β-o-methyl)-Fluoromethylketone. *The Journal of Immunology* **167**, 2452-2455 (2001).
38. Green, D.R., Ferguson, T., Zitvogel, L. & Kroemer, G. Immunogenic and tolerogenic cell death. *Nat Rev Immunol* **9**, 353-363 (2009).
39. Voll RE, U.V., Herrmann M, Kalden JR. High mobility group box 1 in the pathogenesis of inflammatory and autoimmune diseases. *The Israel Medical Association Journal* **10**, 26-28 (Jan 2008).
40. Guiducci, C.*, et al.* TLR recognition of self nucleic acids hampers glucocorticoid activity in lupus. *Nature* **465**, 937-941 (2010).
41. Jego, G.*, et al.* Plasmacytoid Dendritic Cells Induce Plasma Cell Differentiation through Type I Interferon and Interleukin 6. *Immunity* **19**, 225-234 (2003).
42. Mellman, I. & Steinman, R.M. Dendritic Cells: Specialized and Regulated Antigen Processing Machines. *Cell* **106**, 255-258 (2001).
43. Ohnmacht, C.*, et al.* Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. *The Journal of Experimental Medicine* **206**, 549-559 (2009).
44. Datta, S.K., Patel, H. & Berry, D. Induction of a cationic shift in IgG anti-DNA autoantibodies. Role of T helper cells with classical and novel phenotypes in three murine models of lupus nephritis. *The Journal of Experimental Medicine* **165**, 1252-1268 (1987).
45. Wofsy, D. & Seaman, W.E. Reversal of advanced murine lupus in NZB/NZW F1 mice by treatment with monoclonal antibody to L3T4. *The Journal of Immunology* **138**, 3247-3253 (1987).
46. Finck BK, L.P., Wofsy D. Treatment of murine lupus with CTLA4Ig. *Science* **Aug 26;265(5176)**, 1225-1227 (1994).
47. Cunnane, G.*, et al.* Prevention of renal damage in murine lupus nephritis by CTLA-4Ig and cyclophosphamide. *Arthritis & Rheumatism* **50**, 1539-1548 (2004).
48. Isenberg, D. & Rahman, A. Systemic lupus erythematosus[mdash]2005 annus mirabilis? *Nat Clin Pract Rheum* **2**, 145-152 (2006).
49. O'Shea, J.J. & Paul, W.E. Mechanisms Underlying Lineage Commitment and Plasticity of Helper CD4+ T Cells *Science* **327**, 1098-1102 (2010).
50. Wang, A.*, et al.* Dysregulated expression of CXCR4/CXCL12 in subsets of patients with systemic lupus erythematosus. *Arthritis & Rheumatism* **62**, 3436-3446 (2010).
51. Wither, J. & Vukusic, B. Autoimmunity Develops in Lupus-Prone NZB Mice Despite Normal T Cell Tolerance. *The Journal of Immunology* **161**, 4555-4562 (1998).
52. Fatenejad, S., Peng, S.L., Disorbo, O. & Craft, J. Central T Cell Tolerance in Lupus-Prone Mice: Influence of Autoimmune Background and the lpr Mutation. *The Journal of Immunology* **161**, 6427-6432 (1998).
53. Dawisha, S.M., Gmelig-Meyling, F. & Steinberg, A.D. Assessment of Clinical Parameters Associated with Increased Frequency of Mutant T Cells in Patients with Systemic Lupus Erythematosus. *Arthritis & Rheumatism* **37**, 270-277 (1994).
54. Vassilopoulos, D., Kovacs, B. & Tsokos, G.C. TCR/CD3 complex-mediated signal transduction pathway in T cells and T cell lines from patients with systemic lupus erythematosus. *The Journal of Immunology* **155**, 2269-2281 (1995).

55. Mishra, N., Reilly, C.M., Brown, D.R., Ruiz, P. & Gilkeson, G.S. Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse. *The Journal of Clinical Investigation* **111**, 539-552 (2003).
56. Barrat, F.J., Meeker, T., Chan, J.H., Guiducci, C. & Coffman, R.L. Treatment of lupus-prone mice with a dual inhibitor of TLR7 and TLR9 leads to reduction of autoantibody production and amelioration of disease symptoms. *European Journal of Immunology* **37**, 3582-3586 (2007).
57. Chan, O.T.M., Hannum, L.G., Haberman, A.M., Madaio, M.P. & Shlomchik, M.J. A Novel Mouse with B Cells but Lacking Serum Antibody Reveals an Antibody-independent Role for B Cells in Murine Lupus. *The Journal of Experimental Medicine* **189**, 1639-1648 (1999).
58. Shlomchik, M.J., Madaio, M.P., Ni, D., Trounstein, M. & Huszar, D. The role of B cells in lpr/lpr-induced autoimmunity. *The Journal of Experimental Medicine* **180**, 1295-1306 (1994).
59. O'Neill, S.K.*, et al.* Antigen-Specific B Cells Are Required as APCs and Autoantibody-Producing Cells for Induction of Severe Autoimmune Arthritis. *The Journal of Immunology* **174**, 3781-3788 (2005).
60. Martin, F. & Chan, A.C. B CELL IMMUNOBIOLOGY IN DISEASE: Evolving Concepts from the Clinic. *Annual Review of Immunology* **24**, 467-496 (2006).
61. Cartron, G., Watier, H., Golay, J. & Solal-Celigny, P. From the bench to the bedside: ways to improve rituximab efficacy. *Blood* **104**, 2635-2642 (2004).
62. Traczewski, P. & Rudnicka, L. Treatment of systemic lupus erythematosus with epratuzumab. *British Journal of Clinical Pharmacology* **71**, 175-182 (2011).
63. Cancro, M.P., D'Cruz, D.P. & Khamashta, M.A. The role of B lymphocyte stimulator (BLyS) in systemic lupus erythematosus. *The Journal of Clinical Investigation* **119**, 1066-1073 (2009).
64. Sanz, I. & Lee, F.E.-H. B cells as therapeutic targets in SLE. *Nat Rev Rheumatol* **6**, 326-337 (2010).
65. Holborow EJ, W.D., Johnson GD. A serum factor in lupus erythematosus with affinity for tissue nuclei. *British Medical Journal.* **2**, 732-734 (1957).
66. Hahn, B.H. Antibodies to DNA. *New England Journal of Medicine* **338**, 1359-1368 (1998).
67. Chan, T.M. & Cheng, I.K.P. Identification of Endothelial Cell Membrane Proteins that Bind Anti-DNA Antibodies from Patients with Systemic Lupus Erythematosus by Direct or Indirect Mechanisms. *Journal of Autoimmunity* **10**, 433-439 (1997).
68. Chan, T.-M., Leung, J.K.-H., Ho, S.K.-N. & Yung, S. Mesangial Cell-Binding Anti-DNA Antibodies in Patients with Systemic Lupus Erythematosus. *Journal of the American Society of Nephrology* **13**, 1219-1229 (2002).
69. Madaio, M.P. & Yanase, K. Cellular Penetration and Nuclear Localization of Anti-DNA Antibodies: Mechanisms, Consequences, Implications and Applications. *Journal of Autoimmunity* **11**, 535-538 (1998).
70. Hsieh, S.C.*, et al.* Monoclonal anti-double stranded DNA antibody is a leucocyte-binding protein to up-regulate interleukin-8 gene expression and elicit apoptosis of normal human polymorphonuclear neutrophils. *Rheumatology* **40**, 851-858 (2001).
71. Madaio, M.P., Fabbi, M., Tiso, M., Daga, A. & Puccetti, A. Spontaneously produced anti-DNA/DNase I autoantibodies modulate nuclear apoptosis in living cells. *European Journal of Immunology* **26**, 3035-3041 (1996).

72. Casciola-Rosen, L. & Rosen, A. Ultraviolet light-induced keratinocyte apoptosis: A potential mechanism for the induction of skin lesions and autoantibody production in LE. *Lupus* **6**, 175-180 (1997).
73. Mevorach, D., Zhou, J.L., Song, X. & Elkon, K.B. Systemic Exposure to Irradiated Apoptotic Cells Induces Autoantibody Production. *The Journal of Experimental Medicine* **188**, 387-392 (1998).
74. Kalaaji, M., Mortensen, E., Jørgensen, L., Olsen, R. & Rekvig, O.P. Nephritogenic Lupus Antibodies Recognize Glomerular Basement Membrane-Associated Chromatin Fragments Released from Apoptotic Intraglomerular Cells. *The American Journal of Pathology* **168**, 1779-1792 (2006).
75. Kalaaji, M.*, et al.* Glomerular apoptotic nucleosomes are central target structures for nephritogenic antibodies in human SLE nephritis. *Kidney Int* **71**, 664-672 (2007).
76. Kowal, C.*, et al.* Human lupus autoantibodies against NMDA receptors mediate cognitive impairment. *Proceedings of the National Academy of Sciences* **103**, 19854-19859 (2006).
77. Wang, Y.*, et al.* Amelioration of lupus-like autoimmune disease in NZB/WF1 mice after treatment with a blocking monoclonal antibody specific for complement component C5. *Proceedings of the National Academy of Sciences* **93**, 8563-8568 (1996).
78. Walport, M.J. Complement. *New England Journal of Medicine* **344**, 1140-1144 (2001).
79. Robson, M.G.*, et al.* Accelerated Nephrotoxic Nephritis Is Exacerbated in C1q-Deficient Mice. *The Journal of Immunology* **166**, 6820-6828 (2001).
80. Einav, S., Pozdnyakova, O.O., Ma, M. & Carroll, M.C. Complement C4 Is Protective for Lupus Disease Independent of C3. *The Journal of Immunology* **168**, 1036-1041 (2002).
81. Sekine, H.*, et al.* Complement Component C3 Is Not Required for Full Expression of Immune Complex Glomerulonephritis in MRL/lpr Mice. *The Journal of Immunology* **166**, 6444-6451 (2001).
82. Ronnblom, L. & Alm, G. Systemic lupus erythematosus and the type I interferon system. *Arthritis Res Ther* **5**, 68 - 75 (2003).
83. Baechler, E.C.*, et al.* Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proceedings of the National Academy of Sciences* **100**, 2610-2615 (2003).
84. Mathian, A., Weinberg, A., Gallegos, M., Banchereau, J. & Koutouzov, S. IFN-α Induces Early Lethal Lupus in Preautoimmune (New Zealand Black × New Zealand White)F1 but Not in BALB/c Mice. *The Journal of Immunology* **174**, 2499-2506 (2005).
85. Kishimoto, T. INTERLEUKIN-6: From Basic Science to Medicine—40 Years in Immunology. *Annual Review of Immunology* **23**, 1-21 (2005).
86. Kishimoto, T. The biology of interleukin-6. *Blood* **74**, 1-10 (1989).
87. Houssiau, F.*, et al.* Serum interleukin 10 titers in systemic lupus erythematosus reflect disease activity. *Lupus* **4**, 393-395 (1995).
88. Llorente, L.*, et al.* Clinical and biologic effects of anti–interleukin-10 monoclonal antibody administration in systemic lupus erythematosus. *Arthritis & Rheumatism* **43**, 1790-1800 (2000).
89. Jacob, C.O. & McDevitt, H.O. Tumour necrosis factor-[alpha] in murine autoimmune 'lupus' nephritis. *Nature* **331**, 356-358 (1988).

90. Charles, P.J., Smeenk, R.J.T., De Jong, J., Feldmann, M. & Maini, R.N. Assessment of antibodies to double-stranded DNA induced in rheumatoid arthritis patients following treatment with infliximab, a monoclonal antibody to tumor necrosis factor α: Findings in open-label and randomized placebo-controlled trials. *Arthritis & Rheumatism* **43**, 2383-2390 (2000).
91. Mohan, A.K., Edwards, E.T., Coté, T.R., Siegel, J.N. & Braun, M.M. Drug-induced systemic lupus erythematosus and TNF-α blockers. *The Lancet* **360**, 646 (2002).
92. Yamamoto, T.*, et al.* Expression of transforming growth factor-[beta] isoforms in human glomerular diseases. *Kidney Int* **49**, 461-469 (1996).
93. Raz, E.*, et al.* Modulation of disease activity in murine systemic lupus erythematosus by cytokine gene delivery. *Lupus* **4**, 286-292 (1995).
94. Ouyang, W., Kolls, J.K. & Zheng, Y. The Biological Functions of T Helper 17 Cell Effector Cytokines in Inflammation. *Immunity* **28**, 454-467 (2008).
95. Tesch, G.H., Maifert, S., Schwarting, A., Rollins, B.J. & Kelley, V.R. Monocyte Chemoattractant Protein 1–Dependent Leukocytic Infiltrates Are Responsible for Autoimmune Disease in Mrl-Faslpr Mice. *The Journal of Experimental Medicine* **190**, 1813-1824 (1999).
96. Kulkarni, O.*, et al.* Spiegelmer Inhibition of CCL2/MCP-1 Ameliorates Lupus Nephritis in MRL-(Fas)lpr Mice. *Journal of the American Society of Nephrology* **18**, 2350-2358 (2007).
97. Anders, H.-J.*, et al.* CC Chemokine Ligand 5/RANTES Chemokine Antagonists Aggravate Glomerulonephritis Despite Reduction of Glomerular Leukocyte Infiltration. *The Journal of Immunology* **170**, 5658-5666 (2003).
98. Chong, B.F. & Mohan, C. Targeting the CXCR4/CXCL12 axis in systemic lupus erythematosus. *Expert Opinion on Therapeutic Targets* **13**, 1147-1153 (2009).
99. Lee, S.-J., Silverman, E. & Bargman, J.M. The role of antimalarial agents in the treatment of SLE and lupus nephritis. *Nat Rev Nephrol* **7**, 718-729 (2011).
100. Jackson, C.J. & Xue, M. Activated protein C—An anticoagulant that does more than stop clots. *The International Journal of Biochemistry & Cell Biology* **40**, 2692-2697 (2008).
101. Bernard, G.R.*, et al.* Efficacy and Safety of Recombinant Human Activated Protein C for Severe Sepsis. *New England Journal of Medicine* **344**, 699-709 (2001).
102. Kerschen, E.J.*, et al.* Endotoxemia and sepsis mortality reduction by non-anticoagulant–activated protein C. *The Journal of Experimental Medicine* **204**, 2439-2448 (2007).
103. Guo, H.*, et al.* Neuroprotective activities of activated protein C mutant with reduced anticoagulant activity. *European Journal of Neuroscience* **29**, 1119-1130 (2009).
104. Jackson, C.*, et al.* New therapeutic applications for the anticoagulant, activated protein C. *Expert Opinion on Biological Therapy* **8**, 1109-1122 (2008).
105. Isermann, B.*, et al.* Activated protein C protects against diabetic nephropathy by inhibiting endothelial and podocyte apoptosis. *Nat Med* **13**, 1349-1358 (2007).
106. Scaldaferri, F.*, et al.* Crucial role of the protein C pathway in governing microvascular inflammation in inflammatory bowel disease. *The Journal of Clinical Investigation* **117**, 1951-1960 (2007).
107. Shi, G.-P.*, et al.* Cathepsin S Required for Normal MHC Class II Peptide Loading and Germinal Center Development. *Immunity* **10**, 197-206 (1999).

108. Gupta, S., Singh, R.K., Dastidar, S. & Ray, A. Cysteine cathepsin S as an immunomodulatory target: present and future trends. *Expert Opinion on Therapeutic Targets* **12**, 291-299 (2008).
109. Nakagawa, T.Y. & Rudensky, A.Y. The role of lysosomal proteinases in MHC class Il-mediated antigen processing and presentation. *Immunological Reviews* **172**, 121-129 (1999).
110. Villadangos, J.A.*, et al.* Proteases involved in MHC dass II antigen presentation. *Immunological Reviews* **172**, 109-120 (1999).
111. Riese, R.J.*, et al.* Cathepsin S activity regulates antigen presentation and immunity. *The Journal of Clinical Investigation* **101**, 2351-2363 (1998).
112. Baugh, M.*, et al.* Therapeutic dosing of an orally active, selective cathepsin S inhibitor suppresses disease in models of autoimmunity. *Journal of Autoimmunity* **36**, 201-209 (2011).
113. Nakagawa, T.Y.*, et al.* Impaired Invariant Chain Degradation and Antigen Presentation and Diminished Collagen-Induced Arthritis in Cathepsin S Null Mice. *Immunity* **10**, 207-217 (1999).
114. Saegusa, K.*, et al.* Cathepsin S inhibitor prevents autoantigen presentation and autoimmunity. *The Journal of Clinical Investigation* **110**, 361-369 (2002).
115. Anna K. Clark, F.M., Marta D' Auria, Meirion Davies, John Grist, Marzia Malcangio and Stephen B. McMahon. Cathepsin S Inhibition Attenuates Neuropathic Pain and Microglial Response Associated with Spinal Cord Injury. *The Open Pain Journal* **3**, 117-122 (2010).
116. Sukhova, G.K.*, et al.* Deficiency of cathepsin S reduces atherosclerosis in LDL receptor–deficient mice. *The Journal of Clinical Investigation* **111**, 897-906 (2003).
117. Feehally, J.*, et al.* HLA Has Strongest Association with IgA Nephropathy in Genome-Wide Analysis. *Journal of the American Society of Nephrology* **21**, 1791-1797 (2010).
118. Stanescu, H.C.*, et al.* Risk HLA-DQA1 and PLA2R1 Alleles in Idiopathic Membranous Nephropathy. *New England Journal of Medicine* **364**, 616-626 (2011).
119. Appendix: Color Figures. *ASH Education Program Book* **2007**, 509-520 (2007).
120. Victora, G.D. & Nussenzweig, M.C. Germinal Centers. *Annual Review of Immunology* **30**, 429-457 (2012).
121. Deschamps, K.*, et al.* Genetic and Pharmacological Evaluation of Cathepsin S in a Mouse Model of Asthma. *American Journal of Respiratory Cell and Molecular Biology* **45**, 81-87 (2011).
122. Kala, M.*, et al.* Cathepsin S is not crucial to TSHR processing and presentation in a murine model of Graves' disease. *Immunology* **116**, 532-540 (2005).
123. Stoeckle, C.*, et al.* Cathepsin S dominates autoantigen processing in human thymic dendritic cells. *Journal of Autoimmunity* **38**, 332-343 (2012).
124. Perry, D., Sang, A., Yin, Y., Zheng, Y.-Y. & Morel, L. Murine Models of Systemic Lupus Erythematosus. *Journal of Biomedicine and Biotechnology* **2011**(2011).
125. Tsokos, L.A.L.a.G.C. The IL-2 Defect in Systemic Lupus Erythematosus Disease Has an Expansive Effect on Host Immunity. *Journal of Biomedicine and Biotechnology* **2010**(2010).
126. Andrews, B.S.*, et al.* Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. *The Journal of Experimental Medicine* **148**, 1198-1215 (1978).

127. Patole, P.S.*, et al.* Coactivation of Toll-like receptor-3 and -7 in immune complex glomerulonephritis. *Journal of Autoimmunity* **29**, 52-59 (2007).
128. Austin, H.A., III, Muenz, L.R., Joyce, K.M., Antonovych, T.T. & Balow, J.E. Diffuse proliferative lupus nephritis: Identification of specific pathologic features affecting renal outcome. *Kidney Int* **25**, 689-695 (1984).
129. Allam, R.*, et al.* Viral 5′-triphosphate RNA and non-CpG DNA aggravate autoimmunity and lupus nephritis via distinct TLR-independent immune responses. *European Journal of Immunology* **38**, 3487-3498 (2008).
130. Steinmetz, O.M.*, et al.* CXCR3 Mediates Renal Th1 and Th17 Immune Response in Murine Lupus Nephritis. *The Journal of Immunology* **183**, 4693-4704 (2009).
131. Biesecker, G., Katz, S. & Koffler, D. Renal localization of the membrane attack complex in systemic lupus erythematosus nephritis. *The Journal of Experimental Medicine* **154**, 1779-1794 (1981).
132. Pego-Reigosa, J.M., Medeiros, D.A. & Isenberg, D.A. Respiratory manifestations of systemic lupus erythematosus: old and new concepts. *Best practice & research. Clinical rheumatology* **23**, 469-480 (2009).
133. Teichmann, L.L.*, et al.* Dendritic Cells in Lupus Are Not Required for Activation of T and B Cells but Promote Their Expansion, Resulting in Tissue Damage. *Immunity* **33**, 967-978 (2010).
134. Haas, C., Ryffel, B. & Le Hir, M. IFN-γ Receptor Deletion Prevents Autoantibody Production and Glomerulonephritis in Lupus-Prone (NZB × NZW)F1 Mice. *The Journal of Immunology* **160**, 3713-3718 (1998).
135. Kulkarni, O.P. & Anders, H.-J. Lupus nephritis. How latest insights into its pathogenesis promote novel therapies. *Current Opinion in Rheumatology* **24**, 457-465 (2012).
136. Wang, Y.*, et al.* Differential Neuroprotection and Risk for Bleeding From Activated Protein C With Varying Degrees of Anticoagulant Activity. *Stroke* **40**, 1864-1869 (2009).
137. Xu, J.*, et al.* Extracellular histones are major mediators of death in sepsis. *Nat Med* **15**, 1318-1321 (2009).
138. Kau, J.-H.*, et al.* Activated protein C ameliorates Bacillus anthracis lethal toxin-induced lethal pathogenesis in rats. *Journal of Biomedical Science* **19**, 98 (2012).
139. Wang, Y.*, et al.* An Activated Protein C Analog With Reduced Anticoagulant Activity Extends the Therapeutic Window of Tissue Plasminogen Activator for Ischemic Stroke in Rodents. *Stroke* **43**, 2444-2449 (2012).
140. Abraham E, R.K.O.S. & et al. Efficacy and safety of tifacogin (recombinant tissue factor pathway inhibitor) in severe sepsis: A randomized controlled trial. *JAMA* **290**, 238-247 (2003).
141. Warren Bl, E.A.S.P. & et al. High-dose antithrombin iii in severe sepsis: A randomized controlled trial. *JAMA* **286**, 1869-1878 (2001).
142. Griffin, J.H., FernÁNdez, J.A., Gale, A.J. & Mosnier, L.O. Activated protein C. *Journal of Thrombosis and Haemostasis* **5**, 73-80 (2007).
143. Yan, S.B., Helterbrand, J.D., Hartman, D.L., Wright, T.J. & Bernard, G.R. Low Levels of Protein C Are Associated With Poor Outcome in Severe Sepsis*. *CHEST Journal* **120**, 915-922 (2001).
144. Fisher, C.J.J. & Yan, S.B. Protein C levels as a prognostic indicator of outcome in sepsis and related diseases. *Critical Care Medicine* **28**, S49-S56 (2000).
145. Zhou, J.*, et al.* Activated protein C improves pial microcirculation in experimental endotoxemia in rats. *Microvascular Research* **83**, 276-280 (2012).

146. Bae, J.-S. & Rezaie, A.R. Activated protein C inhibits high mobility group box 1 signaling in endothelial cells. *Blood* **118**, 3952-3959 (2011).
147. Cao, C.*, et al.* The efficacy of activated protein C in murine endotoxemia is dependent on integrin CD11b. *The Journal of Clinical Investigation* **120**, 1971-1980 (2010).
148. Brueckmann, M.*, et al.* Activated protein C inhibits the release of macrophage inflammatory protein-1-alpha from THP-1 cells and from human monocytes. *Cytokine* **26**, 106-113 (2004).
149. White, B.*, et al.* Activated protein C inhibits lipopolysaccharide-induced nuclear translocation of nuclear factor κB (NF-κB) and tumour necrosis factor α (TNF-α) production in the THP-1 monocytic cell line. *British Journal of Haematology* **110**, 130-134 (2000).
150. Stephenson, D.A., Toltl, L.J., Beaudin, S. & Liaw, P.C. Modulation of Monocyte Function by Activated Protein C, a Natural Anticoagulant. *The Journal of Immunology* **177**, 2115-2122 (2006).
151. Kulkarni, O.P.*, et al.* 4SC-101, A Novel Small Molecule Dihydroorotate Dehydrogenase Inhibitor, Suppresses Systemic Lupus Erythematosus in MRL-(Fas)lpr Mice. *The American Journal of Pathology* **176**, 2840-2847 (2010).
152. Madhusudhan, T.*, et al.* Cytoprotective signaling by activated protein C requires protease-activated receptor-3 in podocytes. *Blood* **119**, 874-883 (2012).
153. Bock, F.*, et al.* Activated protein C ameliorates diabetic nephropathy by epigenetically inhibiting the redox enzyme p66Shc. *Proceedings of the National Academy of Sciences* **110**, 648-653 (2013).
154. Kessenbrock, K.*, et al.* Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med* **15**, 623-625 (2009).
155. Han, M.H.*, et al.* Proteomic analysis of active multiple sclerosis lesions reveals therapeutic targets. *Nature* **451**, 1076-1081 (2008).
156. Kerschen, E.*, et al.* Activated protein C targets CD8+ dendritic cells to reduce the mortality of endotoxemia in mice. *The Journal of Clinical Investigation* **120**, 3167-3178 (2010).
157. Kulkarni, O.*, et al.* Anti-Ccl2 Spiegelmer Permits 75% Dose Reduction of Cyclophosphamide to Control Diffuse Proliferative Lupus Nephritis and Pneumonitis in MRL-Fas(lpr) Mice. *Journal of Pharmacology and Experimental Therapeutics* **328**, 371-377 (2009).
158. Abraham, E.*, et al.* Drotrecogin Alfa (Activated) for Adults with Severe Sepsis and a Low Risk of Death. *New England Journal of Medicine* **353**, 1332-1341 (2005).
159. Germain, R.N. MHC-dependent antigen processing and peptide presentation: Providing ligands for T lymphocyte activation. *Cell* **76**, 287-299 (1994).
160. Zheng, T.*, et al.* Role of cathepsin S-dependent epithelial cell apoptosis in IFN-γ-induced alveolar remodeling and pulmonary emphysema. *The Journal of Immunology* **175**, 2026 (2005).
161. Samokhin, A.O., Lythgo, P.A., Gauthier, J.Y., Percival, M.D. & Brömme, D. Pharmacological Inhibition of Cathepsin S Decreases Atherosclerotic Lesions in Apoe-/- Mice. *Journal of Cardiovascular Pharmacology* **56**, 98-105 110.1097/FJC.1090b1013e3181e1023e1010 (2010).
162. Allen, C.D.C.*, et al.* Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol* **5**, 943-952 (2004).
163. Victora, G.D.*, et al.* Identification of human germinal center light and dark zone cells and their relationship to human B-cell lymphomas. *Blood* **120**, 2240-2248 (2012).

9. Abbreviations

ANA	Antinuclear antibodies
APC	Antigen presenting cell
aPC	Activated protein C
BCR	B cell receptor
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
Cat S	Cathepsin S
cDNA	Complementary DNA
CCL2	Chemokine C-C motif ligand 2
CCL5	Chemokine C-C motif ligand 5
CXCL10	Chemokine C-X-C motif ligand 10
CXCL12	Chemokine C-X-C motif ligand 12
CT	Cycle threshold
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
GFR	Glomerular filtration rate
IC	Immune complex
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
IL	Interleukin
IFN-γ	Interferon-γ
kDa	kilo Dalton
lpr	Lymphoproliferation
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
mRNA	messenger ribonucleic acid
O.D.	Optical density
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDCs	plasmacytoid dendritic cells
PE	Phycoerythrin
RNA	Ribonucleic acid
Rnase	ribonuclease
RPM	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	Reverse transcriptase
SLE	Systemic lupus erythematosus
Sm	Smith antigen
SNP	Single nucleotide polymorphism
SnRNP	Small nuclear ribonucleoprotein
TGF-β	transforming growth factor-β
TLR	Toll-like receptor

10. Appendix

Collagenase / DNAse solution:
1 mg/ml Collagenase, 0.1 mg/ml DNAse in 1X HBSS (without Ca, Mg)
For 10 ml:
10mg Collagenase (type I) (Sigma C0130)
1ml 1 mg/ml DNAse stock solution
9 ml 1X HBSS

To be preheated in 37ºC water bath before use.
Caution: Prepare freshly every time (Stable only for few days)

Collagenase solution:

1 mg/ml Collagenase in 1X HBSS (without Ca, Mg)
For 10 ml:
10 mg Collagenase (type I)
10ml HBSS (with Ca, Mg)

To be preheated in 37ºC water bath before use.
Caution: Prepare freshly every time (Stable only for few days)

FACS buffer:

500 ml DPBS
500 mg Sodium azide
1 g BSA

10X HBSS (Hank's Balanced Saline Solution) without Ca, Mg:

4 g KCl
0.6 g KH_2PO_4
80g NaCl
0.621g $Na_2HPO_4.2H_2O$

Dissolve in 1000 ml and autoclave.

1X PBS

8 g NaCl
0.2 g KCl
1.44 g Na_2HPO_4
0.24 g KH_2PO_4

Dissolve in 800 ml of distilled H_2O.
Adjust the pH to 7.4 with HCl. Add H_2O to 1 liter.

Curriculum Vitae

Rupanugudi Khader Valli

Date of birth	:	15th May 1979
Marital status	:	Married
Nationality	:	Indian
Languages	:	English, Hindi, Telugu

Education

2010-in progress	**Research Fellow**, Ludwig Maximillians University, AG Prof. Hans-Joachim Anders, Klinische Biochemie, Munich, Germany
1999-2001	**M.Sc. Biotechnology** with **71%** from Sri Venkateswara University, Tirupati, India.
1996-1999	**B.Sc. (Botany, Zoology, Chemistry)** with **74%** from Sri Venkateswara University, Tirupati, India

Research Experience

03/2007 – 03/ 2010	**Technical Officer** National Brain Research Centre, Haryana, India
02/2006 - 02/2007	**Project Assistant** National Brain Research Centre, Haryana, India
02/2002 - 08/2004	**Research Fellow** Sri Venkateswara University, Andhra Pradesh, India

Publications

1. Shrikant R Mulay, Onkar P. Kulkarni, **Khader Valli Rupanagudi**, Adriana Migliorini, Murthy Narayana Darisipudi, Akosua Vilaysane, Daniel Muruve, Yan Shi, Fay Munro, Helen Liapis and Hans-Joachim Anders. "Calcium oxalate crystals induce renal inflammation by NLRP3-mediated IL-1β secretion". The journal of clinical investigation. December 2012.

2. Ramanjaneyulu Allam, Christina Rebecca Scherbaum, Murthy Narayana Darisipudi, Shrikant R. Mulay, Holger Hägele, Julia Lichtnekert, Jan Henrik Hagemann, **Khader Valli Rupanagudi,** Mi Ryu, Claudia Schwarzenberger, Bernd Hohenstein, Christian Hugo, Bernd Uhl, Christoph A. Reichel, Fritz Krombach, Marc Monestier, Helen Liapis,Kristin Moreth, Liliana Schaefer and Hans-Joachim Anders. "Histones from Dying Renal Cells Aggravate Kidney Injury via TLR2 and TLR4". Journal of the american society of nephrology. June 2012.

3. Neha Sehgal, Alok Gupta, **Rupanagudi Khader Valli**, Shanker Datt Joshi, Jessica T Mills, Edith Hamel, Pankaj Khanna, Subhash Chand Jain, Suman S Thakur and Vijayalakshmi Ravindranath. "Withania somnifera reverses Alzheimer's disease pathology by enhancing low-density lipoprotein receptor-related protein". PNAS. January 2012.

4. Lalitha Durgadoss, Prakash Nidadavolu, **Rupanagudi Khader Valli**, Uzma Saeed, Mamata Mishra, Pankaj Seth and Vijayalakshmi Ravindranath. "Redox modification of Akt mediated by the

dopaminergic neurotoxin, MPTP, in mouse midbrain, leads to downregulation of pAkt". The FASEB Journal. December 2011.

5. Julia Lichtnekert, Onkar P. Kulkarni, Shrikant R. Mulay, **Khader Valli Rupanagudi**, Mi Ryu, Ramanjaneyulu Allam, Volker Vielhauer, Dan Muruve,, Maja T. Lindenmeyer, Clemens D. Cohen and Hans-Joachim Anders. "Anti-GBM glomerulonephritis involves IL-1 but is independent of NLRP3/ASC inflammasome-mediated activation of caspase-1". PLoS ONE. October 2011.

6. Julia Lichtnekert, **Khader Valli Rupanagudi**, Onkar P. Kulkarni, Murthy Narayana Darisipudi, Ramanjaneyulu Allam and Hans-Joachim Anders. "Activated protein C attenuates systemic lupus erythematosus and lupus nephritis in MRL-Fas(lpr) mice". Journal of Immunology. September 2011.

7. Neha Sehgal, Varsha Agarwal, **Rupanagudi Khader Valli**, Shanker Dutt Joshi, Leposava Antonovic, Henry W. Strobel and Vijayalakshmi Ravindranath. "Cytochrome P4504f, a potential therapeutic target for limiting neuroinflammation". Biochemical Pharmacology. July 2011.

8. Murthy Narayana Darisipudi, Ramanjaneyulu Allam, **Khader Valli Rupanagudi** and Hans-Joachim Anders. "Polyene Macrolide Antifungal drugs Trigger Interleukin-1β Secretion by Activating the NLRP3 Inflammasome". PLoS ONE. May 2011.

9. Ramanjaneyulu Allam, Murthy Narayana Darisipudi, **Khader Valli Rupanagudi**, Julia Lichtnekert, Jurg Tschopp and Hans-Joachim Anders. "Cutting Edge: Cyclic Polypeptide and Aminoglycoside Antibiotics Trigger IL-1β Secretion by Activating the NLRP3 Inflammasome". The Journal of Immunology. January 2011.

10. Uzma Saeed, Ajit Ray, **Rupanagudi Khader Valli**, Madan Ram Kumar and Vijayalakshmi Ravindranath. "DJ-1 loss by glutaredoxin but not glutathione depletion triggers Daxx translocation and cell death". Antioxidants & Redox Signaling. July 2010.

11. Smitha Karunakaran, Uzma Saeed, Mamata Mishra, **R. Khader Valli**, Shanker Datt Joshi, Durga Praveen Meka, Pankaj Seth and Vijayalakshmi Ravindranath. "Selective Activation of p38 Mitogen-Activated Protein Kinase in Dopaminergic Neurons of Substantia Nigra Leads to Nuclear Translocation of p53 in 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine -Treated Mice". The Journal of Neuroscience. November 2008.

12. Uzma Saeed, Lalitha Durgadoss, **R. Khader Valli**, Dinesh C. Joshi, Preeti G. Joshi and Vijayalakshmi Ravindranath. "Knockdown of Cytosolic Glutaredoxin 1 Leads to Loss of Mitochondrial Membrane Potential: Implication in Neurodegenerative Diseases". PLoS ONE. June 2008.

13. Varsha Agarwal, Reddy P. Kommaddi, **Khader Valli**, Daniel Ryder, Thomas M. Hyde, Joel E. Kleinman, Henry W. Strobel and Vijayalakshmi Ravindranath. "Drug Metabolism in Human Brain: High Levels of Cytochrome P4503A43 in Brain and Metabolism of Anti-Anxiety Drug Alprazolam to Its Active Metabolite". PLoS ONE. June 2008.

Poster Presentations:

1. Ajit Ray, Uzma Saeed, **R. Khader Valli**, Madan Ram Kumar and V. Ravindranath. Perturbation of protein thiol homeostasis through downregulation of glutaredoxin, a protein disulfide oxidoreductase results in proteolysis and loss of DJ1 - Presented at the WFN XVIII World Congress on Parkinson's Disease and Related Disorders, which was held in Miami Beach Florida, December 13-16, 2009.

2. Julia Lichtnekert, **Khader Valli Rupanagudi**, Onkar Kulkarni and Hans J. Anders. Activated Protein C Attenuates Systemic Lupus Erythematosus and Lupus Nephritis in MRL-Fas(lpr) Mice - Presented at ASN Kidney Week 2011, November 8 – 13, 2011, Philadelphia, USA.