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Functional role of Toll-like receptor-7 in experimental lupus nephritis

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Dedicated to,

my mother and father

"Only a life lived for others is a life worth while ."

Albert Einstein

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Research publications related to dissertation

1. **Pawar RD**, Ramanjaneyulu A, Kulkarni OP, Lech M, Segerer S, Anders HJ. Inhibition of Toll-Like Receptor-7 (TLR-7) or TLR-7 plus TLR-9 attenuates glomerulonephritis and lung Injury in experimental lupus. ***J Am Soc Nephrol.*** 2007 Jun;18(6):1721-31.
2. **Pawar RD,** Patole PS, Ellwart A, Lech M, Segerer S, Schlöndorff D, Anders HJ. Ligands to nucleic acid-specific toll-like receptors and the onset of lupus nephritis. ***J Am Soc Nephrol.*** 2006 (12) 3365-73.
3. **Pawar RD**, Patole PS, Zecher D, Segerer D, Kretzler M, Schlöndorff D, Segerer S, Anders HJ. Toll-like receptor-7 modulates immune complex glomerulonephritis. ***J Am Soc Nephrol.*** 2006 Jan; 17(1): 141-9.

Additional research publications from this lab

4. **Pawar RD**, Castrezana-Lopez L, Allam R, Kulkarni OP, Segerer S, Radomska E, Meyer TN, Schwesinger CM, Akis N, Gröne HJ, Anders HJ. Bacterial lipopeptide triggers massive albuminuria in murine lupus nephritis by activating Toll-like receptor 2 at the glomerular filtration barrier. ***Immunology*** 2008 Oct 24 [Epub ahead of print].
5. Allam R, **Pawar RD**, Kulkarni OP, Hornung V, Hartmann G, Segerer S, Akira S, Endres S, Anders HJ. Viral 5'-triphosphate RNA and non-CpG DNA aggravate autoimmunity and lupus nephritis via distinct TLR-independent immune responses. ***Eur J Immunol***. 2008 Dec;38(12):3487-98.
6. Kulkarni O, Eulberg D, Selve N, Zöllner S, Allam R, **Pawar RD**, Pfeiffer S, Segerer S, Klussmann S, Anders HJ. Anti-Ccl2 Spiegelmer permits 75% dose reduction of cyclophosphamide to control diffuse proliferative lupus nephritis and pneumonitis in MRL-Fas(lpr) mice. ***J Pharmacol Exp Ther***. 2009 Feb;328(2):371-7.
7. Kulkarni O, **Pawar RD**, Purschke W, Eulberg D, Selve N, Buchner K, Ninichuk V, Segerer S, Vielhauer V, Klussmann S, Anders HJ. Spiegelmer inhibition of CCL2/MCP-1 ameliorates lupus nephritis in MRL-(Fas)lpr mice. ***J Am Soc Nephrol.*** 2007 Aug;18(8):2350-8.
8. Patole PS, **Pawar RD**, Lech M, Zecher D, Schmidt H, Segerer S, Ellwart A, Henger A, Kretzler M, Schlöndorff D, Anders HJ.. Expression and regulation of Toll-like receptors in lupus-like immune complex glomerulonephritis of MRL-Fas(lpr) mice. ***Nephrol Dial Transplant*** 2006 Nov;21(11):3062-73.
9. Patole PS, **Pawar RD**, Lichtnekert J, Lech M, Kulkarni OP, Ramanjaneyulu R, Segerer S, Anders HJ. Coactivation of Toll-like receptor-3 and -7 in

immune complex glomerulonephritis. ***J Autoimmun***. 2007 Aug;29(1):52-9.

10. Patole PS, Zecher D, **Pawar RD**, Gröne HJ, Schlöndorff D, Anders HJ. G-rich DNA suppresses systemic lupus. ***J Am Soc Nephrol*** 2005 Nov;16(11):3273-80.
11. Emina Savarese, Christian Steinberg, **Rahul D. Pawar**, Wolfgang Reindl, Shizuo Akira, Hans-Joachim Anders, Anne Krug. TLR7-dependent anti-snRNP antibody production and glomerulonephritis in pristane-induced autoimmunity. ***Arthritis Rheum***. 2008 Apr;58(4):1107-15.

Presentations of parts of thesis work

A. Oral presentation at Deutsche Gesellschaft für Innere Medizin

Pawar RD, Segerer S, Anders HJ. Therapie der Lupusnephritis mit DNA. Blockade von Toll-like Rezeptor-7 mit inhibitorischen Oligonukleotiden. Abstract presented at Deutsche Gesellschaft für Innere Medizin (DGIM), Wiesbaden, 14-18 April 2007.

- *Award*

2nd prize as young investigator, for the research work presented at DGIM, Deutsche Gesellschaft für Innere Medizin (DGIM) (German congress of internal medicine), Wiesbaden, Germany, 14-18 April 2007.

B. *Poster presentations*

1. **Rahul D. Pawar**, Prashant S. Patole, Daniel Zecher, Stephan Segerer, Matthias Kretzler, Detlef Schlöndorff and Hans-Joachim Anders. Toll like receptors via interaction with nucleic acids of bacterial and viral origin play a significant role in aggravating autoimmunity and lupus like immune complex glomerulonephritis in MRL*/lpr/lpr* mice*.* Poster presented at TOLL-2006 Recent Advances in Pattern Recognition, Salvador, Brazil, 4-7, March 2006.

2. **Pawar RD**, Anders HJ. Poster: Toll-like receptor (TLR)-7 a novel target for systemic lupus. “Pattern-recognition receptors in human disease Meeting 2007”, Queens' College, University of Cambridge, UK, 8 - 10 August 2007.

Abbreviations

ANA	Antinuclear antibodies
ATF	Activating transcription factor
BCR	B cell receptor
BMDC	Bone marrow derived dendritic cells
CCL	Chemokine ligand
CCR	Chemokine receptor
CDC	Conventional dendritic cells
CNS	Central nervous system
CVS	Cardiovascular system
DC	Dendritic cells
dsRNA	Double stranded RNA
FLT-3	FMS-like tyrosine kinase 3
IC	Immune-complexes
IFN	Interferon
IKK	IkappaB kinase
IL	Interleukin
IRAK	Interleukin 1 receptor-associated kinase
IRF	Interferon regulatory factor
IRS	Immunoregulatory sequences
lpr	Lymphoproliferative
LPS	Lipopolysaccharide
MDA-5	Melanoma differentiation-associated gene -5
MCP	Monocyte chemoattractant protein
MDC	Myeloid dendritic cells
MyD88	Myeloid differentiation factor-88
ODN	Oligodeoxynucleotide
PAMP	Pathogen associated molecular pattern
PDC	Plasmacytoid dendritic cells
PRR	Pattern recognition receptor
RIG-I	Retinoic acid inducible gene- I
SLE	Systemic lupus erythematosus
Sm	Smith antigen
SnRNP	Small nuclear ribonucleoprotein
ssRNA	Single stranded RNA
NF_{KB}	Nuclear factor kappaB
NLR	Nod like receptor
NOD	Nuclear oligomerization domain
TCR	T cell receptor
TIR	Toll interleukin like receptor domain
TIRAP	TIR-associated protein
TLR	Toll like receptor
TNF-α	tumor necrosis factor-alpha
TRAF	TNF receptor associated factor
TRAM	toll-like receptor-associated molecule
TRIF	Toll-IL-1-receptor domain containing adaptor inducing IFN-beta
WT	Wild type

Contents

1. Introduction

1.1. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that affects multiple organ systems (musculoskeletal, dermatologic, hematologic, cardiac, pulmonary, renal and CNS). In SLE, the immune system is impaired, accompanied by loss of central and peripheral tolerance, which leads to formation of immune-complexes which are stimulatory and get deposited in different organs and leads to their damage (Isenberg *et al*, 2007; Manson *et al*, 2006; Childs *et al*, 2006; Marshak-Rothstein and Rifkin, 2006). SLE is one of the major autoimmune diseases which is often associated with high titers of IgG autoantibodies reactive with ubiquitously expressed intracellular autoantigens. Such autoantibodies and immune-complex containing autoantibodies accumulate in joints, kidneys, and other tissues, where they provoke inflammatory responses leading to tissue damage and destruction (Marshak-Rothstein, 2006). In many patients affected with SLE, autoantibodies are directed against relatively limited set of nucleoprotein particles (Plotz *et al*, 2003). Considerable circumstantial evidence links both onset and recurrence of systemic disease with various types of infections (Phillips *et al*, 1972; Denman *et al*, 2000). The theories claimed for the lupus are as follows-

1) At cellular level apoptosis is significantly higher and the clearance of apoptotic bodies is decreased. 2) The second theory is interferon signature. Gene microarray studies have demonstrated that 100 interferon responsive genes are activated in SLE.

1.2. Incidence and prevalence

SLE is more predominant in females than in males and the predicted female : male ratio is 9:1 for the incidence of the disease. A study from Birmingham, UK, found the prevalence in the general population to be 27.7 in 100,000 and nine times higher in Afro-Caribbean females. A study by NHS in USA has found the prevalence of 53.6 in 100,000. SLE is more common in Hispanic Americans, Asians, and African-Carribeans. On an average the age of onset for SLE is usually in the early 20s or 30s and the 10 year survival probability is 92%, which gets reduced to 88% in the patients with nephropathy (Manson *et al*, 2006; Isenberg *et al*, 2007). Mean age at death was found to be 44 yrs (varied from 18-81 yrs). Renal lupus was accounted for the largest number of deaths of SLE patients with less than 5 yrs of disease (Manson *et al*, 2006; Childs *et al*, 2006; Isenberg *et al*, 2007; Simard *et al*, 2007). Renal lupus shows immune-complex glomerulonephritis as a prominent feature (Anders *et al*, 2007a, Anders *et al* 2007b).

1.3. Pathological implications and symptoms

Table 1: Systems affected in SLE correlated with percentage of patients.

Affected organ system	% Of patients	Signs and symptoms
Constitutional	50-100	Fatigue, fever, weight loss
Skin	73	Butterfly rash, photosensitivity rash, mucus membrane lesion, Reynaud's phenomenon
Musculoskeletal	62-67	Arthritis, artharlgia
Renal	16-38	Hematuria, proteinuria, nephrotic syndrome, cellular casts
Hematologic	36	Anaemia, leucopoenia
Reticuloendothelial	7-23	Lymphadenopathy, splenomegaly
Neuropsychiatric	12-21	Psychosis, Seizures
Gastrointestinal	18	Abdominal pain, vomiting
Cardiac	15	Peri-, endo-, myo-carditis
Pulmonary	2-12	Pleurisy, pulmonary hypertension

(Gill *et al*, 2003)

1.4. Causative factors for SLE

a) Genetical

Genes that contribute to the pathogenesis of systemic lupus are classified as follows: 1) Genes that cause break in tolerance for the self-antigens. 2) Genes that lead to immune dysregulation (loss of control of regulatory lymphocytes over the autoreactive lymphocytes). 3) Genes that lead to immune complex deposition (RNA and DNA

associated immune complexes and others) (Simard *et al*, 2007; Mok *et al*, 2003) (Michelle Petri, 2006). 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 emergence of autoimmune disease (Goodnow, 2007).

b) Hormonal

SLE is predominantly a postpubertal female disease however till date the hormonal reasons behind the disease remain complex. In men with SLE, the androgens worsen the disease (Simard, 2007; Mock, 2003). The incidence of lupus in women is higher as compared to men, female: male ratio is 9:1 (Isenberg, 2007).

c) Immunological

The immune system is dysregulated and increased generation of autoreactive B and T cells takes place alongwith the reduction in regulatory T cells. The regulatory T cells loses its control on the expansion of T cells. Also peripherally circulating autoantigens or immune-complexes which are not antigenic in healthy conditions in normal individuals whereas in lupus such autoantigens are recognised as danger signals which further lead to excessive activation of immune system. (Goodnow, 2007; Marshak-Rothstein, 2006 ; Simard *et al*, 2007; Mok *et al*, 2003). In immune system many tolerance checkpoints exist to prevent self antigens from stimulating the relentless growth of self-reactive B and T lymphocytes (Goodnow, 2007). These checkpoints when bypassed leads to the emergence of autoimmune disease.

d) Environmental

Ultraviolet (UV), UV-A and UV-B, light has been classified as the classic environmental precipitant of SLE. Drugs like minocycline, and anti-TNF biologics, can lead to drug induced lupus erythematosus (DILE). Sulfonamide antibiotics can induce idiopathic SLE. Subacute cutaneous lupus erythematosus (SCLE), is associated with thiazides, calcium channel blockers and angiotensin-converting enzyme inhibitors. Epstein Barr viral infection has been strongly associated with SLE, in both children and adults in multicase SLE families. Amongst the other factors which are also associated with SLE are toxic exposures to silica and mercury (Simard *et al*, 2007; Mok *et al*, 2003).

1.5. Morbidity and mortality

Organ damage in SLE progresses over time (Gladman *et al*, 2000)). A cohort study (Rivest, 2000) found that within seven years of diagnosis, 61% of patients developed clinically detectable organ damage, with neuropsychiatric (20.5%), musculoskeletal (18.5%) and renal (15.5%) organ systems most commonly affected. Infections and diseases of CVS, renal, pulmonary, CNS are frequent causes of death in patients with SLE (Carneiro *et al*, 1999, Jonsson *et al*, 1989, Stahl-Hallengren *et al*, 2000, Cervera *et al*, 1999, Mok *et al*, 1999). Since the 1950s, five year survival rate for patients with SLE has increased from 50% to 91-97% (Carneiro *et al*, 1999; Jonsson *et al*, 1989; Stahl-Hallengren *et al*, 2000; Mok *et al*, 1999, Uramoto *et al* 1999, Urowitz *et al* 1997). Higher mortality rates for SLE are particularly high in children. In a retrospective study of Brazilian children, overall mortality during 16 years of follow up was 24% (Marini *et al*, 1999). Death occurred because of infection (58%) CNS disease (36%), renal disease

(7%). When disease onset was before 15 years, renal involvement and hypertension predicted mortality (Simard *et al*, 2007).

1.6. Targets used till date for the treatment of SLE

Targets used for treatment in clinic practice are the ones which can suppress the immune system, although these are not selective to eliminate the cause of end organ damage in SLE. Furthermore these drugs have limitations and/or adverse effects too, for e.g. immunomodulating drugs (cyclophosphamide, methotrexate), azathioprine, corticosteroids (prednisone), mycophenolate mofetil, antimalarials (chloroquine or hydroxychloroquine), non-steroidal anti-inflammatory drugs (Buhaescu *et al*, 2007; Davidson *et al*, 2006). The recent targets have been evolved to be more specific, targeting either the B cells (rituximab- anti CD-20 antibody), or TNF-α blockade (infliximab, etanercept), or by competitively antagonising autoantibodies (using abetimus sodium- LJP 394), or blocking the cytotoxic T-lymphocyte antigen- 4 (abatacept and belatacept - CTLA4-Ig) (Schneider *et al*, 2007; Waldman *et al*, 2006; Buhaescu *et al*, 2007). Recent experimental data have shown the trend to selectively block the receptor responsible for the progression of lupus or autoimmunity such as TLR9 binding to DNA-immune complex, or blocking Complement 5A with corresponding antagonist (Patole, 2005; Anders *et al*, 2005; Ponticelli, 2006; Schneider, 2007; Buhaescu *et al*, 2007).

1.7. Pathogen recognition and innate immune system

Pattern recognition receptors (PRRs) are the novel key players of immune responses in humans by recognizing pathogen associated molecular patterns (PAMPs). PRRs play a major role in host defence against viral, bacterial, and fungal pathogens by initiating both

innate and adaptive immune responses. PRR activation can differentially modulate immune responses in cases of infection and autoimmune disorders. These receptors are classified as Toll like receptors, nucleic acid specific-cytoplasmic receptors (RIG like helicases) and NOD like receptors (Lee and Kim, 2007, Anders *et al*, 2007a).

1.7.1. Toll like receptors

Toll-like receptor (TLR) superfamily of pattern recognition receptors is comprised of 13 members characterized in mice (TLR1-13) and 11 in humans (TLR1-11). All TLRs known in mammals are type 1 integral membrane glycoproteins containing an extracellular domain with leucine rich repeats (LRRs) responsible for ligand recognition and cytoplasmic toll/IL-1R homology (TIR) domain required for initiating signalling (Akira *et al*, 2004). TLRs are generally classified into cell membrane receptors and endosomal receptors. Majority of TLRs are expressed by cells of immune system. Till date number of the exogenous and endogenous ligands of TLRs have been found out (Marshak-Rothstein, 2006).

a) TLR1/2/6

TLR1, TLR2 and TLR6 are expressed on cell membrane of immune and non-immune cells and either of them need to be heterodimerized for the recognition of the ligands from pathogenic microorganisms. TLR1/2 dimer can recognize triacylated lipopeptide such as Pam3CSK4, TLR2/6 dimer can recognise diacylated lipopeptide such as MALP-2 (mycoplasma-derived macrophage-activating lipopeptide. TLR2, in concert with dectin-1, can recognize zymosan (Brown *et al*, 2006). Upon ligand recognition, TLR2 recruits

both the sorting adaptor TIRAP and the signalling adaptor MyD88, and initiates the MyD88-dependent pathway, leading to inflammatory cytokine production (Beutler *et al*, 2006).

b) TLR4

TLR4 is present on the cell membrane and is the only receptor which in association with CD14 and MD2 can initiate signals upon recognition of lipopolysaccharide (LPS) in humans and mice (Akira *et al*, 2006). TLR4 can activate two separate downstream pathways, MyD88 dependent and TRIF-dependent (Akira *et al*, 2006). The main role of MyD88-dependent pathway downstream of TLR4 is to induce the expression of inflammatory cytokines such as IL-6, IL-12, and TNF-alpha. The TRIF-dependent pathway induces the upregulation of costimulatory molecules and expression of type I IFNs (Kawai *et al*, 2005; Hobe *et al*, 2003a; Hoebe *et al*, 2003b). The MyD88-dependent pathway in TLR4 and TLR2 requires the recruitment of TIRAP and MyD88 via homophilic TIR-TIR interactions (Akira *et al*, 2006).

c) TLR3

TLR3 is the endosomal TLR which for the downstream signalling needs an adaptor molecule TRIF. TLR3 is a receptor for double stranded RNA (dsRNA), which is a viral PAMP, and its synthetic analogue poly (I:C). Recently it was reported that synthetic ssRNA such as polyinosinic acid can also stimulate the TLR3 (Marshall-Clarke *et al*, 2007). The signalling pathways of TLR3 are initiated by recruitment of TRIF, which then activates the TRIF-dependent pathway, leading to the subsequent production of

inflammatory cytokines and type 1 IFNs and to the upregulation of costimulatory molecules. Poly (I:C) can stimulate the myeloid DCs to express type I IFNs and inflammatory cytokines, whereas their expression is reduced but not abolished, in cells from TLR3-/- mice (Alexopoulou *et al*, 2001). This indicates that TLR3 is a receptor for dsRNA but additional sensors might also exist, which have been recently discovered and referred as CARD helicases. A recent finding describes an important *in vivo* role of TLR3, which is to promote cross-priming for initiation of a CD8+ T cell response against viruses that do not directly infect DCs (Schulz *et al*, 2005).

d) TLR 7/8/9

TLR7/8/9 are the endosomal TLRs and they have similar downstream signalling pathways via adaptor MyD88. TLR7 can recognize ssRNA derived from ssRNA viruses such as vesicular stomatitis virus (VSV), as well as synthetic ssRNA40 and poly-U RNA (Alter *et al*, 2007; Fink *et al*, 2006; Marshall-Clarke *et al*, 2006; Heil *et al*, 2004, Savarese *et al*, 2006). TLR7 can also recognize synthetic small molecules of the imidazoquinoline family such as resiquimod, imiquimod, gardiquimod. TLR8 is active in humans but not in mouse (Kawai *et al*, 2006). TLR9 recognizes synthetic CpG oligonucleotides of type A, B and C and unmethylated CpG motifs in bacterial and viral DNA. TLR7 and TLR9 are highly expressed in pDCs, a subset of DCs, but not in cDCs. They induce the rapid secretion of large quantity of IFN-alpha and production of inflammatory cytokines, upon stimulation with the viral nucleic acids (Kawai *et al*, 2004; Liu *et al*, 2005). Both responses absolutely depend on the TIR adaptor MyD88 and subsequently on IRAK4 (Figure 1). Two signalling pathways of TLR7/9 are thought to

induce inflammatory cytokine expression: the MyD88-TRAF6-TAK1-MAPK/IKK-AP-1/NF-kB pathway because TAK1-/- B cells show reduced activation of NF_{kB} and MAP kinases upon TLR9 stimulation (Sato *et al*, 2005; Takaoka *et al*, 2005). IRF7 constitutively expressed in pDCs can be incorporated into a multiprotein complex comprising at least MyD88, IRAK4, IRAK1, TRAF6 and TRAF3 is severely impaired in IRAK1-/- and TRAF3-/- cells and is abolished in IRF7-/- cells, but the production of other inflammatory cytokines is not defective in these cells (Oganesyan *et al*, 2006; Hacker *et al*, 2006; Kawai *et al*, 2004; Honda *et al*, 2004; Honda *et al*, 2005; Uematsu *et al*, 2005). Furthermore IRF7 activation is impaired in IRAK-/- pDCs upon TLR9 stimulation. TRAF3 associates with MyD88 and IRAK1, and synergises with them in activating IRF7 upon overexpression (Hacker *et al*, 2006; Uematsu *et al*, 2005). These data together suggest that TRAF3 and IRAK1 are responsible for IRF7 activation and subsequent IFN-alpha production downstream of TLR7-MyD88-IRAK4 and TLR9-MyD88-IRAK4 (Figure 1). Recently it has been reported that IKK_{α} is required for the production of IFNα downstream of TLR7 and TLR9 but not for other inflammatory cytokines. In addition it has been reported that IKKα is also associated with the phosphorylation of IRF7 (Hoshino *et al*, 2006). It has been reported recently that the TLR9-MyD88-IRF7- dependent IFNα production depends partly on the ability of pDCs to retain CpG-DNA in endosomes for a long time before it is translocated to the lysosomes for degradation. This suggests that the location and duration of ligand exposure to the TLRs influence the final activation response. The role of TLRs in mediating the pathogen recognition in case of viral infection is not much clear since even the mice lacking the MyD88 or TLR9 can still control replication of DNA and RNA

viruses and TLR independent type I IFN production in response to viral infection (Krug *et al*, 2004; Kato *et al*, 2005; Kato *et al*, 2006, Figure 1). Since the nucleic acid specific receptors are located in the endosomal compartments it prevents the exposure of the self nucleic acids to it and the generation of autoimmune reactions by self RNA and DNA molecules; also due to various modifications of the host RNA it is lesser potent in activating the DCs than unmodified RNAs (Kariko *et al*, 2005).

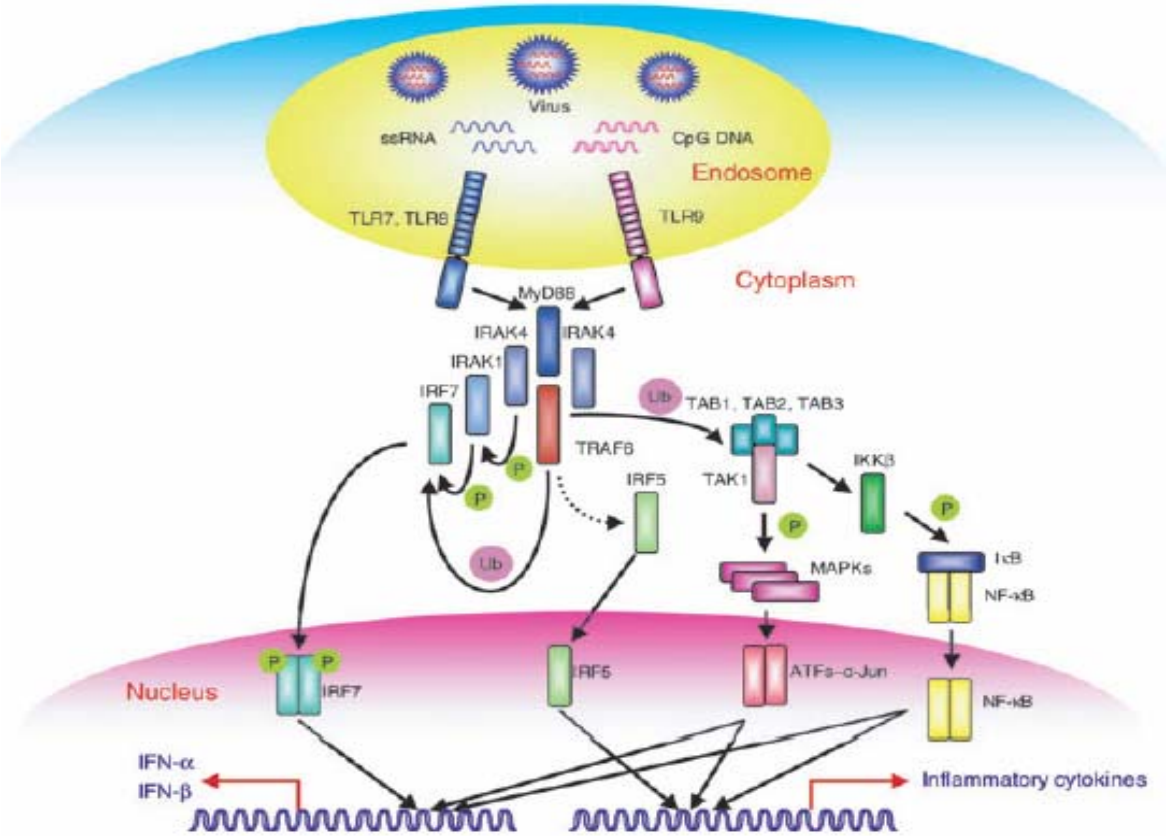


Figure 1: *Signaling pathways downstream of TLR7 and TLR9 in immune cells and their microbial ligands are shown in this figure (adopted from Akira et al, 2006).*

1.7.2. Cytoplasmic RNA sensors or CARD helicases

These are the PRRs which can detect the viral nucleic acids other than by endosomal TLRs. Retinoic acid inducible gene- I (RIG-I) and melanoma differentiation associated factor-5 (MDA-5) are RNA sensors, which has Dead/H box RNA helicase domain responsible for ligand recognition and two CARD domains essential for downstream signalling (Yoneyama *et al*, 2004; Andrejeva *et al*, 2004). RIG-I is essential for the production of interferons in response to RNA viruses including paramyxoviruses, influenza virus and Japanese encephalitis virus (Kato *et al*, 2006). MDA5 has been recently reported as a sensor of poly (I:C)- synthetic dsRNA and is critical for poly (I:C) induced type 1 IFN response, in murine embryonic fibroblasts, bone marrow derived DCs, bone marrow derived macrophages (Kato *et al*, 2006). MDA-5 is also critical for the antiviral response to the infection by picornaviruses, such as encephalomyocarditis virus (EMCV) (Kato *et al*, 2006). LGP2, a homolog of RIG-I/MDA5 lacking CARD domains, is thought to have a negative role in signalling from this helicases (Rothenfusser *et al*, 2005).

1.7.3. NOD-like recptors

Nod like receptors (NLRs) constitute a distinct family of 20 members of cytoplasmic PRRs in mammals (Inohara *et al*, Ting *et al*, 2006; Martinon *et al*, 2005). Subfamilies of NLRs have been grouped based on their effector functions such as NODs, NALPs, CIITA, IPAF, NAIPs. NODs and IPAF contains CARD effector domains, whereas NALPs contain pyrin (PYD) domains and NAIPs contain BIR domains (Martinon *et al*, 2005). The function of NLRs is to recognise the cytoplasmic microbial PAMPs and/or

endogenous ligands, initiating immunological responses.

1.8. Potential role of TLRs in SLE pathogenesis

Significantly high proportion of autoantibodies which can bind to DNA or RNA or RNA associated proteins are associated with systemic autoimmune diseases such as SLE, Sjögrens syndrome, scleroderma. The mechanisms of how such epitopes, which are considered as harmless under normal healthy conditions turns out to be danger signals in autoimmune diseases remains poorly understood. Out of the numerous possibilities, one might be that these intracellular autoantigens become visible to immune system when they accumulate on cell surface during apoptotic cell death (Cascieola Rosen *et al*, 1994) and subsequent uptake of immune-complexes by activated APCs leads to loss of tolerance. Another possibility is that apoptosis results in cleavage of such molecules by granzyme B, proteases, nucleases (Rosen *et al*, 1999), thereby creating neoepitopes which can be recognised as foreign material by cells of adaptive immune system. Neoepitopes could also be generated by other forms of post-translational factors (Utz *et al*, 2000; Plotz *et al*, 2000). Till date many studies have accumulated the data in support of the idea that autoantigens are autoadjuvants which have the capacity to activate the innate immune system directly and promote self directed immune responses. Many adjuvants have been confirmed to stimulate the innate immune system through PRRs, and it is becoming increasingly evident that many of the same PRRs are also involved in response to injury, the clearance of apoptotic cell debris, and the repair of damaged tissues.

Endogenous danger signals in autoimmunity

The major cellular coordinators of autoimmunity have recently been studied in great detail and these are the ligands acting through TLR7 and TLR9 (Figure 2). The drugs which inhibit the endosomal acidification and/or maturation are also able to inhibit the signalling cascades initiated by activation of TLR7 and 9. These two receptors are constitutively expressed by B cells, plasmacytoid dendritic cells, and macrophages, which are playing a major role in the pathogenesis in SLE and other systemic diseases through the production of autoantibodies, IFN-alpha, and other proinflammatory cytokines (Figure 2). It has been reported that DNA immune complexes are recognised by TLR9 and RNA immune complexes by TLR7, respectively (Marshak-Rothstein and Rifkin, 2007, Anders *et al*, 2005).

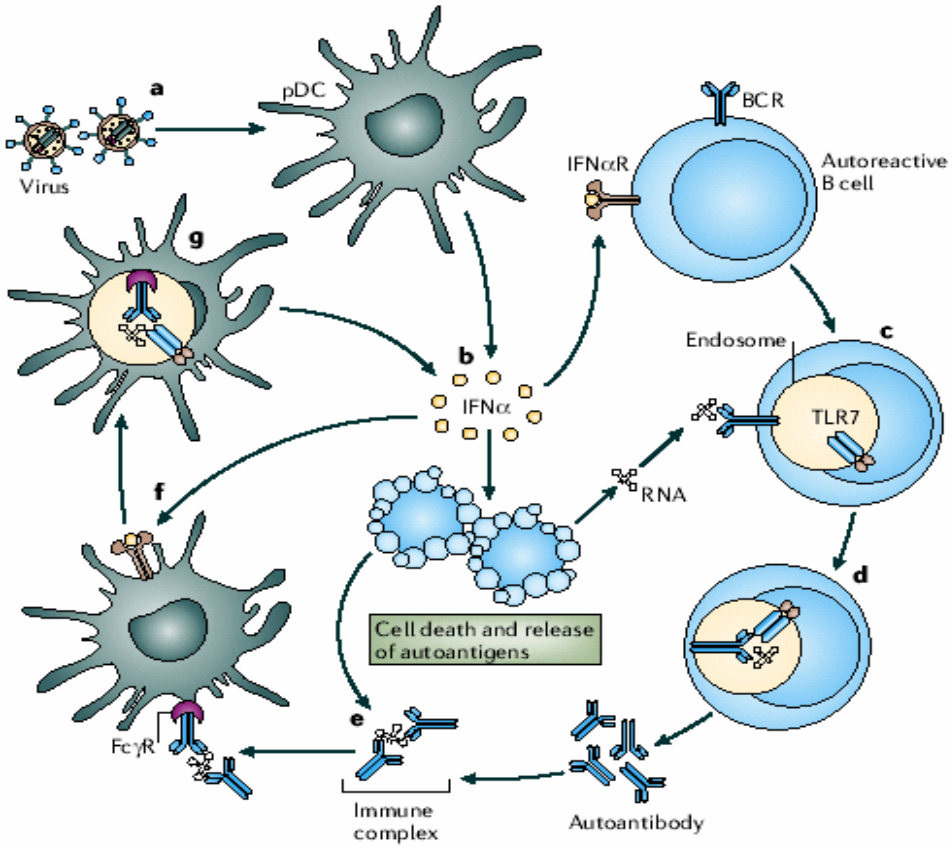


Figure 2: *Cycle of responses by immune cells to autoantigens released by cell death- a. virus infection, b. IFN-α upregulates TLR7 in B cells, and produces other actions, c. Autoreactive B cells with TLR7, d. B cell receptors deliver RNA autoantigen to TLR7, e. B cells produce autoantibody, f. immune complexes bind to Fc receptors. g. autoantigen internalization* (figure adopted from Marshak-Rothstein, *2006).*

Lerner *et al* had discovered that the RNP and Sm antigens recognized by lupus erythematosus antibodies are located on discrete particles containing single small nuclear RNA's complexed with proteins, the antigens Ro and La are also on ribonucleoproteins (Lerner et al, 1981). The small RNA's in ribonucleoproteins with Ro are discrete, like those associated with RNP and Sm; in contrast ribonucleoproteins with La contain a striking highly banded spectrum of small RNA's from uninfected cells as well as virus associated RNA from adenovirus-infected cells (Lerner *et al*, 1981). U1 snRNP (small nuclear ribonuclear protein) which is a potent autoantigen in systemic autoimmune disease such as SLE (van Venrooij *et al*, 1995, Figure 3) and is now found to be the TLR7 ligand from the laboratory of Anne Krug (Savarese, 2006). Stark *et al* for the first time described the three dimensional structure of human U1 SnRNP by single particle electron microscopy in 2001 (Stark *et al*, 2001).

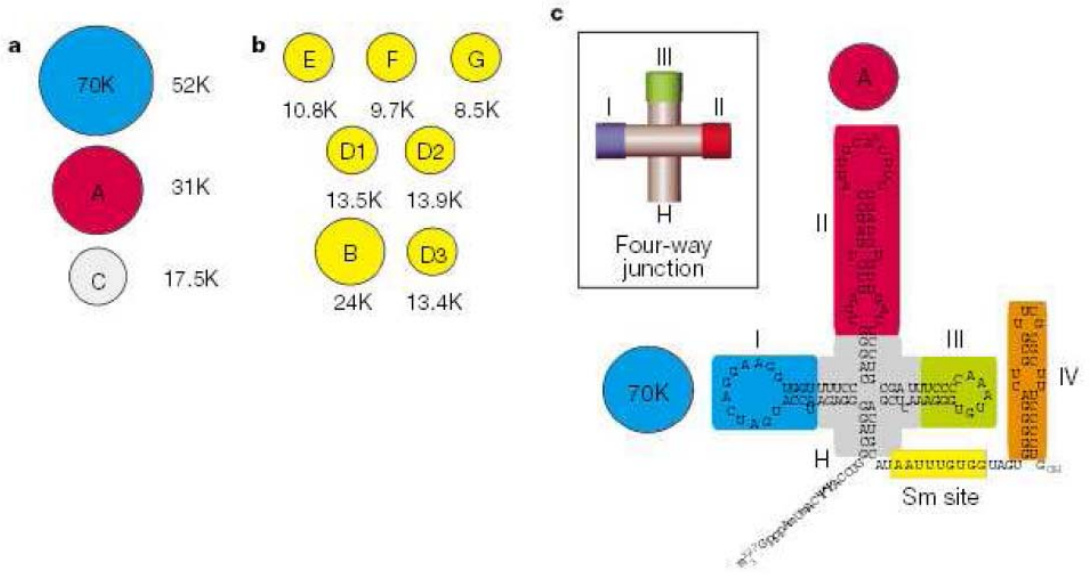


Figure 3. *The RNA and protein components of U1snRNP: a. The three proteins specific to U1snRNP, b. Seven Sm proteins common to all snRNPs. The Sm proteins bind to the Sm-site RNA to generate Sm core RNP. c. Numbers indicate the relative molecular masses, d. The secondary structure of RNA and the sites at which proteins U1-70K and U1-A bind to the RNA. The Sm site is yellow and the four way junction of four double helical strands is grey (Adopted from Stark et al, 2001).*

DNA containing immune-complexes are the endogenous ligands of TLR9. Synthetically prepared oligodeoxynucleotides using following sequences on phosphorothioate (stable) or phosphodiester backbone were reported to be TLR9 stimulatory or inhibitory *(Marshak-Rothstein, 2006, Patole et al, 2005, Anders et al, 2005).*

Table 2: Sequences found to be TLR9 stimulatory and inhibitory in vitro.

Type of ODN	***Name***	***Sequence***
Stimulatory	*ODN1585 (type A. mouse)*	*5'- ggGGTCAACGTTGAgggggg -3' (20 mer)*
	1826 (type B. mouse)	*5'-tccatgacgttcctgacgtt-3' (20 mer)*
	ODN2216 (type A. Human	*5'-ggGGGACGA:TCGTCgggggg-3' (20 mer)*
	2006 (type B. Human)	*5'-tcgtcgttttgtcgttttgtcgtt-3' (24 mer)*
Inhibitory	*2088 (mouse)*	*5'-tcctggcggggaagt-3'*
	ODN TTAGGG (Human)	*5'-tttagggttagggttagggttaggg-3'*

1.8.2. Cross talk of TLR7, TLR9 cross talk with B cell receptor

A functional link between TLR9 expression and B cell activation was first shown in studies involving B cell receptor transgenic murine cells. The AM14 transgenic cell line express receptor specificity commonly found in IgG autoantibody repertoire of FAS deficient MRL autoimmune prone mice (Schlomchik *et al*, 2003). These rheumatoid factor antibodies recognise $IgG_{2a}{}^{a/j}$ which in original host is autologus $IgG_{2a,}$ and B cells

expressing this receptor escape tolerance inducing mechanisms that eliminate more avid self-reactive cells. DNA associated IC induces a stimulatory and proliferative response in AM14 cells and it is dependent on expression of both AM14 BCR and TLR9 (Leadbetter *et al*, 2002; Busconi *et al*, 2007). IgG_{2a} monoclonal antibodies reactive with RNA or RNA associated protein are also stimulatory immune-complex (IC) for primary B cells and these responses are enhanced by type 1 IFN (Lau *et al*, 2005). Although the BCR/TCR paradigm was originally identified by IC activation of IgG_{2a} reactive B cells, additional *in vitro* studies indicate that TLR engagement is involved in activation of B cells that bind DNA or RNA or other autoantigens and therefore play an important role in early stages of autoantibodies production (Figure 4).

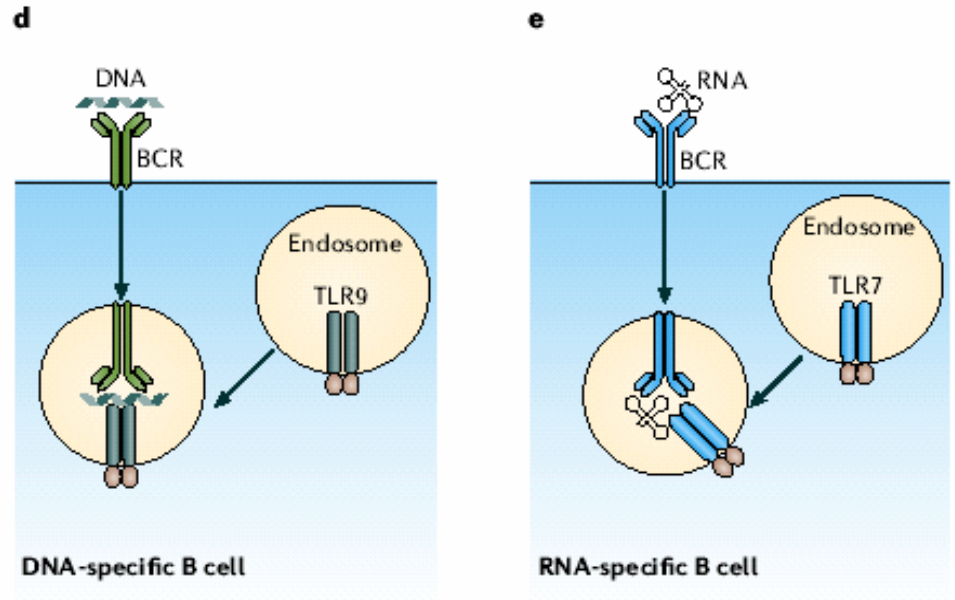


Figure 4: *RNA and DNA specific B cells which are able to internalize the RNA or DNA either endogenous or exogenous and later able to bind to endosomal TLR7 or 9 respectively (adopted from Marshak-Rothstein, 2006).*

1.8.3. Contribution of FcγR to TLR signals

DNA and RNA containing ICs can bind to low affinity FcγRs on various APCs which can internalize the IC and such DNA and RNA upon reaching endosomes can activate TLRs (Table 3, Figure 5). In recent studies carried out by using sera of SLE patients or other systemic autoimmune disorders, such sera can induce pDCs to produce high amounts of type 1 IFN, IFN-α (Figure 5; Vallin *et al*, 1999; Ronnblom *et al*, 2001). BL6 mice lacking the inhibitory FcγRIIB receptor were found to produce IgG autoantibodies and develop glomerulonephritis (Bolland *et al*, 2002, Figure 5). Similar results were reported by mixing IgG antibodies purified from patient sera with either apoptotic or necrotic cell debris, as by mixing same antibodies with CpG-DNA or Sm-RNP (Vollmer *et al*, 2005; Lovgren *et al*, 2004; Magnusson *et al*, 2004; Lovgren *et al* 2006; Barrat *et al*, 2005). Blocking antibodies to FcγRIIa could inhibit IFNα production. Further reports using TLR deficient murine PDCs as well as inhibitory ODNs and inhibitors of endosome acidification have linked these responses to TLR7 and TLR9 (Vollmer *et al*, 2005; Barrat *et al*, 2005; Bave *et al*, 2003; Savarese *et al*, 2006).

Table 3: Different types of cells expressing FcγRs and TLRs

Cells expressing FcγRs	TLR7/8	TLR9
pDCs (Human and murine)	+	+
CD8+ cDCs (Murine)		+
CD8- cDCs (murine)	+	+
Skin derived mast cells	+	+
Langerhans cells		+
Monocytes DC (human)	+	
Myeloid DCs (human)	+	
Activated neutrophils (human)	+	+
Platelets (murine, human)		+

(Marshak-Rothstein, Rifkin, 2006)

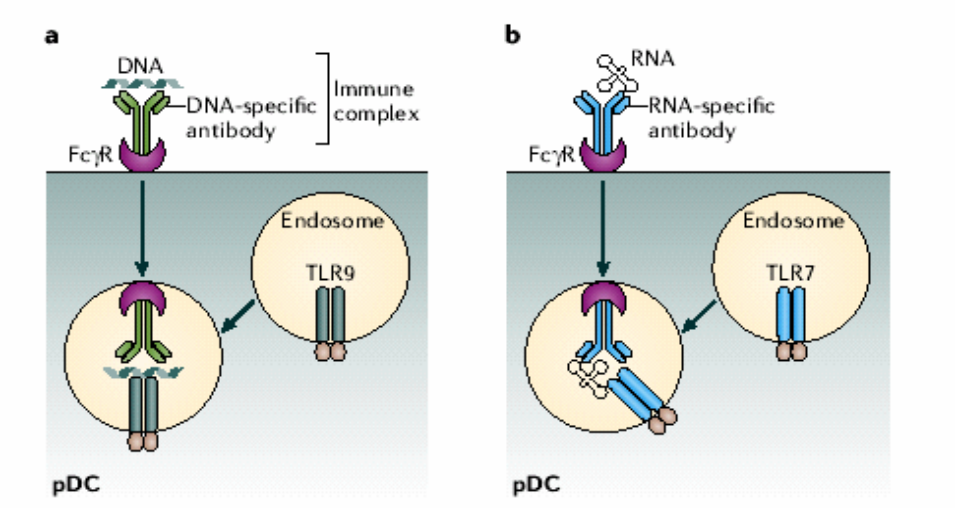


Figure 5: *FcγR mediated internalization of immune-complexes in plasmacytoid dendritic cells followed by binding to endosomal TLR7 or 9 (adopted from Marshak-Rothstein, 2006).*

1.8.4. Systemic lupus and role of endosomal TLRs in induction of IFNα

It is well known that IFNα is an inducer of clinical features of SLE (Preble *et al*, 1982). IFNα family has 13 subtypes each of them have diverse functions associated with immune responses to viral infections (Theofilopoulos, 2005). The causal relationship of IFNα and SLE become evident from the observation that repeated administration of recombinant IFNα to patients with various malignancies or chronic viral infections, could lead to production of antinuclear antibodies (ANA), and occasionally development of clinical symptoms associated with SLE or other autoimmune diseases (Ronnblom *et al*, 1990, Gota *et al*, 2003). Recent studies which examined gene expressions have identified IFN signature in most patients with active SLE. IFNα can contribute significantly to disease pathogenesis through various mechanisms, including direct and indirect effects on APCs, T cells, B cells (Banchereau *et al*, 2004). Another study showed that NZB mice lacking type 1 IFN receptor have reduced titers of DNA antibodies and less severe renal disease (Lian *et al*, 2004; Santiago-Raber *et al*, 2003).

A. Novel findings linking IFN signalling to genetic predisposition in systemic lupus

Several genetic factors contribute for development of SLE. Single nucleotide polymorphisms are associated with type 1 IFN signalling pathway. IFN-regulatory factor-5 (IRF-5) is constitutively expressed by B cells and PDCs and has crucial role in TLR induced transcription of proinflammatory cytokines (Takaoka *et al*, 2005). In human cells, IRF5 regulates expression of type 1 IFNs downstream of TLR7 but not TLR3 (Schoenemeyer *et al*, 2005).

B. IFNα and plasmacytoid dendritic cells

It is known that majority of the amounts of IFNα is produced by PDCs which were originally known as IFN producing cells. It was reported that the immune complexes (ICs) isolated from lupus patients sera could be potent stimulus for IFNα production, especially by PDCs, that have been previously exposed to type 1 IFNs or granulocyte/macrophage colony stimulating factor. Similar to other APCs, PDCs express receptors for Fc pattern of IgG (FcγRs) which can bind and internalize IgG containing immune complexes and function as activating receptors (Amigorena *et al*, 1998). Immune complexes from patients with SLE cannot stimulate PDCs that have been protected with blocking antibodies directed against FcγRIIa (or CD32). In summary certain immune complexes have IFNα inducing activity and is dependent on FcγRIIa receptors.

1.8.5. Pathogenic role of immune-complexes via TLR7 and TLR9

Many of the common autoantigens released by dead and dying cells becomes available to immune system as a result of excessive cell damage or injury or inappropriate clearance of apoptotic cell debris (Rifkin *et al*, 2005). Self reactive or autoreactive B cells can produce the autoantibodies which constitutes 5- 20 % of naïve B cell repertoire in most individuals (Wardemann *et al* 2003). Defects in early B cell tolerance might lead to an even greater percentage of self-reactive B cells in patients who are prone to development of autoimmune disease (Yurasov *et al* 2005). Several mechanisms that may or may not be linking to each other probably accounts for activation and differentiation of these cells which are normally quiescent.

It is reported that viral and bacterial infections are frequently associated with the onset of systemic autoimmune disease and subsequent clinical flares (Chen *et al*, 2005; Banchereau *et al*, 2006), these infections might play a pivotal role in loss of tolerance and production of autoantibodies. It has been reported (Rönnblom *et al*, 2001) that autoantigens upon presentation by APCs can stimulate potentially autoreactive T cells which in turn can potentiate the activation, clonal expansion and differentiation of autoreactive B cells. Under appropriate conditions these B cells undergo somatic hypermutation of their BCR and switch to expression of pathogenic immunoglobulin classes. The high affinity autoreactive B cells clones are selected by autoantigens that become increasingly available during the course of infection, owing partly to increased rate of cell death, caused by inflammatory cells and cytotoxic effector cells. B cell differentiation might also be driven by recognition of virus infected B cells by T cells (Hunziker *et al*, 2003). Excessive amounts of cell debris and autoantibodies then form immune complexes which can activate PDCs and also establish a feedback loop which further aggravates the disease process.

1.8.6. Triggers of autoantibodies generation

Certain autoantigens can possibly play a proactive role in loss of tolerance mediated through PRRs. BCR mediated endocytosis, is a route available not only to microorganisms but also to endogenous autoantigens that are recognized by autoreactive BCR. Therefore self-antigens when in excessive quantities can effectively engage both the BCR and TLR7 or 9 and might stimulate autoreactive B cells (Table 4). Similarly aberrant expression and regulation of such TLRs might render B cells hyperresponsive to

such endogenous ligands and therefore predispose the individual to development of systemic autoimmune disease (Marshak-Rothstein, 2006). Several studies have shown that the bacterial or viral DNA or synthetic DNA rich in hypomethylated CpG motifs can produce significant adjuvant activity (Krieg *et al*, 1995; Messina *et al*, 1991; Sun *et al*, 1997).

By contrast purified mammalian genomic DNA has remarkably poor adjuvant activity which probably is due to lack of hypomethylated CpG motifs and presence of sequences that inhibit effective activation of TLR9 by agonist motifs (Stacey *et al*, 2003; Gursel *et al*, 2003, Table 2). Hence the reasons why DNA/RNA containing immune complexes in lupus patients sera activate PDCs, can be possibly due to FcγR mediated uptake of ICs and delivery to intracellular compartment in PDCs and DNA/RNA binding B cells (Marshak-Rothstein, 2006, Rev, Yasuda *et al*, 2007) which might be more prevalent in patients with SLE.

Particular features of mammalian RNA subtypes are also likely to confer adjuvant activity. Agonists for TLR7 include ssRNA that is rich in uridine or combination of uridine and guanosine (Diebold *et al*, 2006; Heil *et al*, 2004). Many of the SnRNPs that are frequently targeted by autoantibodies consist of proteins that are bound to small UG rich RNAs. The adjuvant activity of RNA probably gets lost upon the post translational modification, which renders mammalian RNA harmless or inactive to TLR7. Based on reduced levels of modification such as oxidation might increase the adjuvant properties of mammalian RNA (Busconi *et al*, 2006) and UV light can also cause oxidative damage to RNA and DNA (Emerit *et al*, 1981) and can generate covalent RNA-protein complexes (Andrade *et al*, 2005), and can induce apoptotic or necrotic cell death (Carrichio *et al*,

2003), all such factors can promote release of potent TLR7 and 9 ligands.

To understand the role of exogenous bacterial or viral infections in an established lupus nephritis, synthetic mimic of bacterial DNA (Tlr9 ligand) and viral dsRNA (Tlr3 ligand) was injected in 16 weeks old lpr mice (Anders *et al*, 2004, Patole *et al*, 2005), the results showed that both the ligands based upon their binding affinity to the respective Tlrs could induce different immunological responses systemically as well as at the site of kidney. Injections with CpG DNA led to increase in levels of serum autoantibodies to DNA, and aggravated the renal injury in lpr mice (Anders *et al*, 2004). This proactive role of TLR9 in aggravation of lupus nephritis was confirmed later in the studies conducted in our lab, using a TLR9 inhibitory oligo G-rich DNA 2114. This ODN was injected in lupus mice for 13 weeks which led to reduction in the levels of serum dsDNA-specific IgG2a as well as the renal immune complex deposits (Patole *et al*, 2005). Viral dsRNA mimic poly(I:C), upon injection in lpr mice increased serum IL-12p70, IL-6, and IFN-alpha levels, although serum DNA autoantibody levels were unaltered (Patole *et al*, 2005). However the role of Tlr7 activation by an exogenous ligand which either mimics viral ssRNA or small molecule which binds Tlr7, was unexplored in experimental systemic lupus in the literature.

Table 4: ***Microbial and endogenous ligands of endosomal toll like receptors and their disease association (Marshak-Rothstein, 2006).***

TLR	Microbial ligand	Endogenous ligand		Disease association
		Autoantigen	**Natural source of autoantigen**	
TLR7 and TLR8	Single stranded RNA (viruses)	Single stranded RNA	ssRNA from dead or dying cells, SnRNPs	SLE, scleroderma, Sjögrens syndrome
TLR9	DNA	DNA	DNA from dead or dying cells	SLE
TLR3	Double stranded RNA (viruses)	Double stranded RNA	Necrotic cells	Arthritis

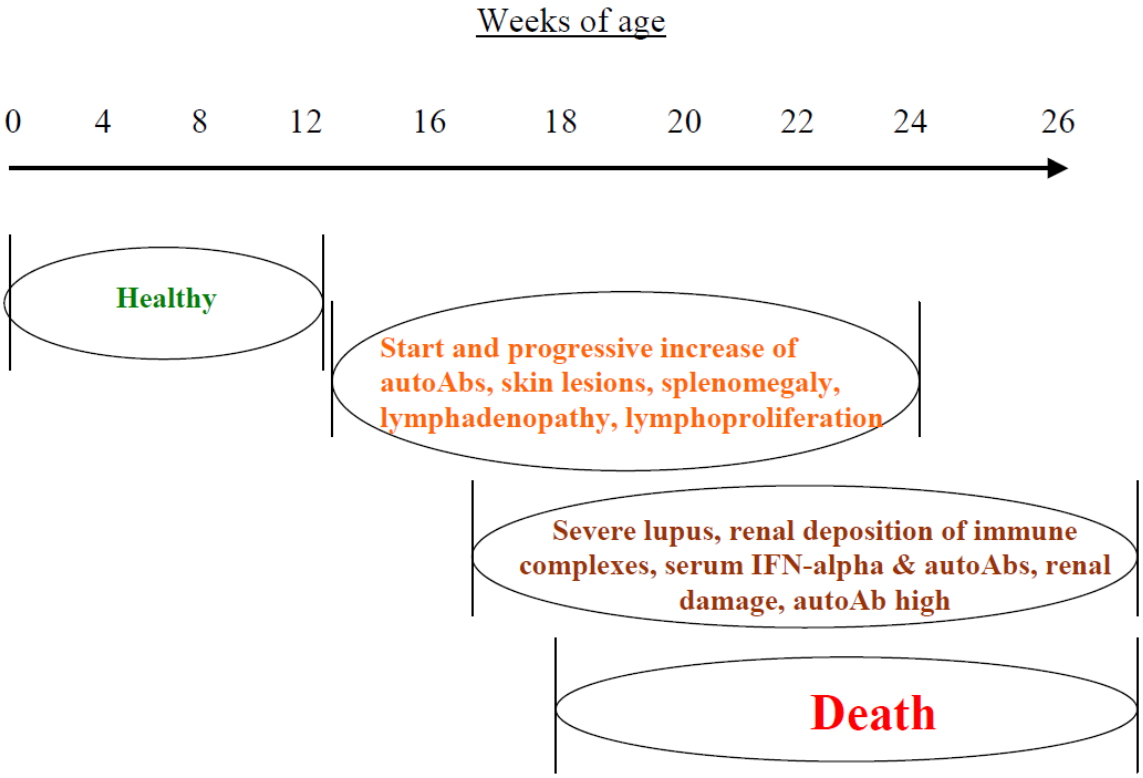
1.8. Experimental mouse model of SLE used in this thesis

The MRL/MpJ mice are large and docile mice with albino appearance and they are the parent and control strain for MRL/MpJ-*Fas*^{lpr}. Despite carrying the normal *Fas* gene, MRL/MpJ mice also exhibit autoimmune disorders, but symptoms are manifested much later in life compared to those the MRL/MpJ-*Fas*^{lpr} mice. Starting at about three months of age, levels of circulating immune complexes rise greatly in the MRL-*Fas*^{lpr} mouse but not in the wildtype control, MRL/MpJ. Also beginning at 3 months *Fas*^{lpr} mice exhibit very severe poliferative glomerulonephritis, whereas in the MRL/MpJ controls usually only mild glomerular lesions are detected. MRL/MpJ inbred female typically die at 73 weeks of age and males die at 93 weeks. This compares to a lifespan of 17 weeks in the female and 22 weeks for males in the mouse homozygous for *Fas*^{lpr}. See MRL/MpJ-*Fas*^{lpr} for additional information. As a strain developed as the control for MRL/MpJ-*Fas*^{lpr}, MRL/MpJ mice are useful in the study of their comparable defects and diseases.

The MRL/MpJ lymphoproliferation wildtype strain was generated by Jackson laboratory from a series of crosses with strains C57BL/6J (0.3%), C3H/HeDi (12.1%), AKR/J (12.6%) and LG/J (75%) and then followed by inbeeding. During development of this strain by Jackson Laboratory, at F12, the spontaneous mutation *Fas*^{lpr} was found. MRL/MpJ-*Fas*^{lpr} and the MRL/MpJ control are kept congenic with each other by backcrosses to the MRL/MpJ wildtype every 5-10 inbred generations. (Ref. http://jaxmice.jax.org/strain/000486.html)

For convenience, MRL/MpJ-*Fas*^{lpr} will be termed as MRLlpr/lpr and MRL/MpJ mice will be termed as MRL/WT in this thesis.

Diagrmatic representation of Life span of MRLlpr/lpr mice



2. Hypothesis

Here we gradually designed 3 hypotheses one after the other based on the results of the previous one for analyzing the role of Tlr7. These hypotheses were as follows-

1. The recognition of exogenously exposed synthetic mimic of viral ssRNA or small molecule via Tlr7 may represent an important mechanism of virus-induced autoimmunity, e.g. in lupus. Hence it was hypothesised to characterize the expression of Tlr7 in experimental lupus and to study the effects of Tlr7 ligation on lupus disease activity as a model for intercurrent viral infection in SLE.

2. In view of the reported *in vitro* studies it was hypothesized, whether transient exposure to Tlr7 ligand, imiquimod, would trigger lupus nephritis *in vivo* or not. Hence we planned to expose young lupus-prone MRL*lpr/lpr* mice or MRL wild-type mice to these compounds, so that we can also address the role of the *lpr* mutation for Tlr7 mediated effects if any on autoimmune tissue injury in MRL mice.

3. According to the recent published studies, Tlr7- or Tlr9-deficiency in lupus mice have opposing effects on experimental lupus it became necessary to dissect therapeutic blockade of these Tlrs by specific antagonists. Such antagonists were identified by Barrat, *et al.* who characterized the inhibitory effects of synthetic oligodeoxynucleotides with immunoregulatory sequences (IRS) on Tlr7 signaling *in vitro* (Barrat *et al*, 2005). Based on the present evidence of a proinflammatory role of Tlr7 for lupus, it was hypothesized that antagonism of Tlr7 would have beneficial effects on experimental lupus.

3. Materials and methods

3.1. Materials and methods Part 1

3.1.1. Animals and Experimental Protocol

Ten week old female MRL*lpr/lpr* mice were obtained from Harlan Winkelmann (Borchen, Germany) and kept in filter top cages under a 12 hour light and dark cycle. Water and standard chow (Sniff, Soest, Germany) were available *ad libitum*. All experimental procedures were approved by the local government authorities. At the age of 16 week, female MRL*lpr/lpr* mice were distributed into three groups that received intraperitoneal injections every alternate day as follows: I. 25 μg of imiquimod, a compound of the imidazoquinoline family and Tlr7 agonist (Sequoia Research Products Ltd, Oxford, UK) in 100 μl 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Steinheim, Germany), II. 100 μl 10% dimethyl sulfoxide (Merck, Darmstadt Germany), III. 100 μl normal saline. All mice were sacrificed by cervical dislocation at the end of week 18 of age. To assess the renal distribution of ssRNA 3'-rhodamine-labeled ssRNA40, known to ligate mTlr7 (Heil et al, 2004), was injected intravenously into MRLlpr/lpr mice at the age 16 weeks. Renal tissue was collected 2 hours later and subjected to further analysis as described below. For the identification of 3'-rhodamine-labeled renal cells costaining was performed using rat anti-F4/80 (Serotec, Oxford, UK, 1:50). In order to assess renal Tlr7 mRNA expression, kidneys were harvested from each group of treated mice at the end of 18 wks of age and also from 5 and 20 week old female MRL*lpr/lpr* mice.

3.1.2. Evaluation of autoantibodies and glomerulonephritis

Blood and urine samples were collected from each animal at the end of the study period

from each group of mice. Blood samples were collected under anesthesia by retroorbital puncture using capillaries and collected in 1.5 ml micro centrifuge tubes. Serum was collected after a settle time of 15 min at room temperature followed by centrifugation of blood at 10000 rpm for 5 min. Proteinuria and creatinine were determined using an automatic autoanalyzer (Integra 800, Roche Diagnostics, Germany).

A) Analysis of serum autoantibodies

Serum dsDNA autoantibodies were determined by using commercial ELISA kits (Bethyl Labs, Montgomery, TX, USA) using the following antibodies: anti-mouse IgG1 and IgG2a following the manufacturer's protocol with some modifications as follows:

1. First calculate the number of sample wells required. Coat the sample wells in NUNC maxisorp 96 well flat bottom ELISA plate with 50 µg/ml (dilute in PBS) of the aqueous solution of 100 µl of Poly-L-Lysine (Trevigen, Gaithersburg, MD, USA) for 1 hour at room temperature.
2. Wash once with washing buffer, tap dry on blotting paper and coat the sample wells only with 1 µg/ml of dsDNA, which can be diluted in SSC buffer (1X), pH 7 for making the required dilution.
3. For standard wells, capture the wells with the capture antibody in coating buffer as described in the protocol.
4. Incubate the plate overnight at 4°C.
5. Next day wash the plate with the wash buffer 3 times and then block it with blocking solution and follow the protocol as provided by the bethyl labs from this blocking step onwards.
6. Add the standard and samples in respective wells in required dilution and allow to

7. Wash the plate 3 times and then add secondary antibody at given dilution as per the protocol and incubate it for 1 hour at room temperature.
8. Wash the plate again as before and add TMB substrate (1:1 mixture of substrate A and B) in each well and incubate in dark for 5-20 minutes.
9. Stop the reaction with 1 M H_2SO_4 and measure the absorbance at 450 nm.

B) Morphological and histological processing

From all mice, the tissues isolated from kidneys or spleens and placed in plastic histocassettes and immediately fixed in 10% buffered formalin (formaldehyde in PBS) for overnight and next day processed, with automatic tissue-processor (Thermo-Shandon), which processes tissues in following manner- 70% Ethanol (for 5 hours), 96 % ethanol (2 hrs), 100 % ethanol 3.5 hrs, Xylene (2.5 hrs), paraffin (4 hrs). Then the histocassettes are taken out and the paraffin blocks are prepared with hot liquid paraffin using a machine (Microm- EC 350). The prepared blocks are allowed to cool down and later separated from the steel holders. These blocks are later used for making fine sections for different stainings (PAS or immunohisto). The fine sections (5µm) are prepared using the microtome (Microm- HM340E) and mounted gently on a glass frosted slide. Such slides are later allowed to dry for 2 hours in an oven at 50°C temperature. Deparaffinisation followed by dehydration was carried out by incubating the sections in xylene, 100% absolute ethanol, 95%, 80% and 50% ethanol followed by rinsing with PBS (2 changes, 3 minutes each). 5 µm sections for silver and periodic acid-Schiff stains were prepared following routine protocols for histopathology. From each mouse tissue

collected as mentioned above, 2 µM thick paraffin-embedded sections were cut and processed for immunohistochemical staining performed on paraffin-embedded sections.

C) Immunostaining

Immunostaining was either performed on paraffin-embedded or frozen sections as follows: using the following primary antibodies: anti-mouse Tlr7 (1:50, IMG581, Imgenex, San Diego, CA), anti-mouse ER-HR3 (1:50, monocytes/macrophages, DPC Biermann, Bad Nauheim, Germany), anti-mouse CD11c (1:50, clone HL3, BD), anti-mouse CD3 (1:100, clone 500A2, BD), anti-mouse smooth muscle actin (1:100, myofibroblasts, clone 1A4, Dako, Carpinteria, CA), anti-mouse CCL5 (1:50, clone VL1, Peprotech, Rocky Hill, NJ), anti-mouse Ccl2/MCP-1 (1:50, polyclonal, Santa Cruz Biotechnology), anti-mouse Mac-2 (1:50, monocytes/macrophages, BD Pharmingen, Hamburg, Germany), anti mouse B220 (1:400, early Pro-B to mature B cells, clone RA3-6B2, BD), anti-mouse Ki-67 (1:100, cell proliferation, Dianova, Hamburg, Germany); anti-mouse IgG1 (1:100, M32015, Caltag Laboratories, Burlingame, CA, USA), anti-mouse IgG2a (1:100, M32215, Caltag), anti-mouse C3 (1:20, GAM/C3c/FITC, Nordic Immunological Laboratories, Tilburg, Netherlands). Negative controls included incubation with a respective isotype antibody. For quantitative analysis glomerular cells were counted in 10 cortical glomeruli per section. Semiquantitative scoring of glomerular IgG and C3c deposits from 0-3 plus was performed on 15 cortical glomerular sections. Semiquantitative scoring of glomerular IgG deposits from 0 to 3 was performed on 15 cortical glomerular sections using a semiquantitative index as follows: 0 = no signal, 1 = low signal, 2 = moderate signal, and 3 = strong signal intensity. Peribronchial and

pulmonary inflammation was arbitrarily graded from 0 (no inflammation) to 3 (severe inflammation).

3.1.3. Isolation of primary cells

A. Isolation and preparation of murine bone marrow derived dendritic cells (BMDCs)

Tibia and femur bones from the fore and hind limbs of MRL-*lpr/lpr* mice were isolated after sacrificing 4-5 mice, using all sterilized materials and sterile conditions for the isolation in an aseptic hood with HEPA filters. Bones were freed from the adhering tissues and both the ends of each bone were punctured with the 24 gauge needle and then the RPMI media was flushed (using sterile surgical syringe with needle) through the bones to isolate bone marrow in a sterile Petri plate containing the 2-3 ml of ice-cold media (RPMI+ 10 %FCS + 1 % PS) on ice. Then it was homogenously resuspended using 1 ml pipette. This cell suspension was taken in a 15 ml Falcon tube using 1 ml pipette and centrifuged at 1600 rpm at 4°C for 6 min. and washed with 1x PBS (cell culture grade) for 2 times. Then it was passed through the 40 μm sterile filters to obtain single cell suspension and centrifuged as before. The supernatant was decanted and the pellet was resuspended in 4 ml of sterile 0.3M NH_4Cl and kept at room temperature for 5 min. Then it was centrifuged as above and supernatant was decanted and pellet was resuspended in ice cold (cell culture use) PBS and washed 2 times with PBS as before. The pellet was resuspended in RPMI media (2-4 ml) and the no. of cells were counted using Neubar's chamber under the microscope after suitable dilution. Adequate no. of cells (5 million) in 12 well plates (NUNC cell culture plates), each well containing 1.5 ml of complete RPMI media containing 100 ng/ml recombinant murine Flt3 ligand

(Immunotools, Friesoyth, Germany). The plates were incubated at 37°C for 8 days to obtain a lineage of mixture of plasmacytoid dendritic cells and conventional dendritic cells (pDC and cDC). After 4 days of incubation 1 ml of fresh media (with 100 ng/ml Flt3 ligand) was added in each well. After 8 days of the incubation, 2 ml of fresh media was replaced for the old media by careful removal only from the top of each well. Then the cells were stimulated with ligands or media. The plates were again incubated for 24 hrs at 37°C. After incubation the supernatants were carefully isolated in microcentrifuge tubes. The cells from each well were lysed using the required amount of RLT buffer containing β-mercaptoethanol (350 µl per well) and collected in DEPC treated sterile microcentrifuge tubes and stored immediately at –80°C, which were used for RNA isolation. The supernatants were separated from the residual amount of cells by centrifuging it at 6000 rpm for 10 min. And immediately stored them at –20°C. These supernatants were analyzed for the various cytokines, and chemokines by ELISA.

B. Isolation and preparation of murine spleen monocytes

Spleen was isolated from the MRL*lpr/lpr* or MRL/WT mice after sacrificing it, using all sterilized materials and sterile conditions for the isolation in an aseptic hood with HEPA filters. Spleen was collected 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 petriplate itself and broken into fine pieces of tissues. Then the cell suspension was passed through 70 µm plastic filter (BD biosciences) using the 1 ml pipette and collected in another sterile plate. This single cell suspension was later collected in a 15 ml Falcon tube with pipette and centrifuged at 1600 rpm at 4°C for 6 min. The supernatant was decanted and

the pellet was resuspended in 4 ml of sterile 0.3M NH_4Cl and kept at room temperature 5 min. Then it was centrifuged at 1600 rpm at 4°C for 6 min. Supernatant was decanted and pellet was resuspended in ice cold (cell culture use) PBS and centrifuged at 1600 rpm at 4°C for 6 min. The pellet was resuspended in RPMI media (2-4 ml) and the no. of cells were counted using Neubauer chamber under the microscope after suitable dilution. Adequate no. of cells (5 million) were added in 12 well plates (NUNC cell culture plates), each well containing 1.5 ml of RPMI media (RPMI Glutamax + 10 %FCS + 1 % PS). The plates were then incubated for 24 hrs in the incubator at 37°C. After 24/36 hrs of the incubation, fresh media was replaced for the old media by careful removal of the supernatant. Then the cells were stimulated with ligands or media. The plates were again incubated for 20-24 hrs at 37°C. After incubation the supernatants were carefully isolated in microcentrifuge tubes.

The cells from each well were lysed using the required amount of RLT buffer containing 10 µl/ml of β-mercaptoethanol (350 µl per well) and collected in DEPC treated sterile Eppendorff tubes and stored immediately at –80°C, which were later used for RNA isolation. The sups were separated from the residual amount of cells by centrifuging it at 6000 rpm for 10 min. and immediately stored them at –20°C. These supernatants were analyzed for the various cytokines, and chemokines by ELISAs.

3.1.4. Cell culture conditions and cytokine ELISAs

Flt-3 derived bone marrow dendritic cells and cell culture plate adherent spleen monocytes (from MRL*lpr/lpr* mice), processed and cultured as described above. Spleen monocytes were treated with medium control or imiquimod 3 µg/ml, RNA40 30 µg/ml

along with DOTAP (1µg/5µg) (Roche, Mannheim, Germany), and pI:C-RNA 30 µg/ml for 24 hours. Tlr9 ligand B-type CpG-DNA-1668 at a concentration of 1 µg/ml was used as a positive control in selected cases. After a period of 24 hours incubation culture supernatants were collected for cytokine measurements and cells were prepared for flow cytometric analysis. Dendritic cells and spleen monocytes were stimulated as above for 24 hours and cells were harvested for RNA isolation as described above. J774 mouse macrophages (American Type Culture Collection, Rockville, MD, USA) were grown in RPMI 1640 containing 1mM HEPES, 10% heat-inactivated bovine serum, 100 units/ml penicillin and 100µg/ml streptomycin (Biochrom KG, Berlin, Germany). A murine mesangial cell line was maintained in Dulbecco's modified Eagle's medium (DMEM, Biochrom KG, Berlin, Germany) supplemented with 2.5% fetal calf serum and 1% penicillin-streptomycin 100 U/ml and 100 µg/ml, respectively. Cells were incubated for 24 hours without serum supplements before stimulation. Cytokine levels were determined using commercial ELISA kits: Il-6, Il-12p70, Il-12p40, Mcp-1 (all OptEiA, BD Pharmingen), Tnf-alpha (Biolegend, San Diego, CA, USA), Ifn-α (PBL Biomedical Labs, USA) following the protocol provided by the manufacturers.

The protocol for ELISAs in brief was as follows

The NUNC ELISA plate wells were captured overnight at 4°C with the capture antibody in coating buffer. Next day the wells were washed 3 times with the washing buffer as given in protocol for 3 times and blocked with the blocking solution or assay diluent for 1 hour or as specified. Again the washings were repeated 3 times followed by addition of standards, samples and sample diluent as blank into the wells of tap dried plate and

incubated at RT for 2 hours and followed by washings for 5 times or as specified and then addition of HRP/AP conjugated secondary antibody diluted in assay diluent. The wells were washed again for 5-7 times or as specified and incubated with the 100 µl of substrate A and B (1:1 mixture) for 25-30 min in dark to develop colour. The reaction was stopped by addition of 100 µl 1 M H_2SO_4. The reading of the absorbance was taken at 450 nm and the reference wavelength was 620 nm using a spectrophotometer (TECAN-Genios Plus).

3.1.5. Flow cytometry

The mesangial cells and J774 macrophages were grown in cell culture and then used for the receptor expression analysis by flow cytometry. Cells were carefully removed from the culture plates by resuspending them in PBS using 1 ml pipette in a 15 ml Falcon tube and washed with PBS (cell culture grade) for 2 times by centrifuging it at 1300 rpm at 4 °C for 6 min. The cells were maintained on ice from here onwards and approximately 100, 000 cells were separated into 1.5 ml Eppendorff tubes each in a volume of 300 µl in PBS and 5 groups of tubes were used in duplicates for the staining with the Tlr receptor antibodies, isotype and unstained. All the groups of tubes were processed at a uniform time and with uniform processing conditions. The cells were resuspended in a volume of 200 µl of cold PBS (sterile) and stained with the primary antibodies for Tlrs and isotype antibodies for 40 min by incubating them on ice, followed by washing with PBS for 2 times. Later the cells were incubated in a volume of 200 µl, with the secondary antibodies conjugated with PE or FITC for 30 min. and washed 1 time followed by addition of streptavidin-APC, kept on ice for 20 min protected from light from this point onwards.

Then the cells were washed for 2 times with PBS, and finally resuspended in special transparent FACS tubes (5 ml round bottom poly propylene tubes, FALCON, Franklin Lakes, NJ, USA), kept on ice and always protected from light until analysis.

The following primary antibodies were used to detect Tlrs on mesangial cells and macrophages: anti-mouse Tlr3 (1:50, IMG516, Imgenex, San Diego, CA), Tlr7 (1:50), anti-mouse Tlr9 (1:50, IMG516, Imgenex, San Diego, CA). A biotinylated rabbit anti-mouse IgG antibody (1:100) and streptavidin-APC (1:100, BD Pharmingen) was used for detection and a rabbit IgG (BD pharmingen) was used as isotype control.

3.1.6. RNA isolation, cDNA synthesis and real time RT-PCR

A. Isolation of RNA from cultured cells

Cell lines as well as primary cell cultures in stimulation experiments were harvested for RNA isolation using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the protocol provided. Firstly, adherent cells to be harvested from 6 well culture plates were washed with sterile PBS to remove residual medium after collection and the wash-through was aspirated. 350 µl RLT buffer containing 10µg/ml beta-mercaptoethanol was added directly over adherent cells on the plate surface and pipetted vigorously so as to lyse cells. This produced a viscous fluid which was collected and placed in microcentrifuge tubes and frozen at –80 °C until RNA isolation. At the time of RNA isolation the samples collected were thawed, 350 µl of 70 % ethanol made in 1% Diethyl pyrocarbonate treated water (DEPC water) was added to it and mixed well. This mixture was then loaded onto RNeasy mini columns held in 2 ml collection tubes and centrifuged at 8000 g for 30 seconds. The flow-through was discarded and the columns were loaded

with 700µl of buffer RW1 and centrifuged at 8000 g for 30 seconds. The collection tubes were discarded together with the flow-through and the columns were transferred to fresh 2 ml collection tubes and 500 µl of Buffer RPE was pipetted onto the column, was centrifuged at 8000 g for 30 seconds and the flow-through was discarded. This step was repeated again and the column was rendered dry by centrifugation, placed in a 1.5 ml fresh collection tube, 40 µl of RNase free water was pipetted directly on the silica-gel membrane and was centrifuged to collect the RNA solution. No quality check was necessary in case of the kit isolation, as empirically the RNA obtained upon kit isolation was of good quality standards.

B. Isolation of RNA from tissues

The RNA isolation protocol was suitably modified from Chomczynski's method (Chomczynski 1987). 3 ml of solution D containing 10µl of beta-mercaptoethanol/ml was taken in a 15 ml Falcon tube, to which a small piece of tissue from which RNA had to be isolated, was placed. The tissue was homogenised using ULTRA-TURRAX T25 (IKA GmbH, Staufen, Germany) at speed level 2 and placed on ice. To this 300µl 2M sodium acetate solution was added and mixed gently, followed by addition of 3 ml Roti-Aqua-Phenol (Carl Roth GmbH, Karlsruhe, Germany) and gentle mixing. A 1.6 ml mixture of chloroform/isoamyl alcohol (49:1) was added to the contents of the Falcon and vortexed for 20 seconds untill a milky white suspension resulted. The Falcon tube was then placed on ice for 15 min and centrifuged at 4000 g at 4 °C. The upper phase (approximately 3 ml) was collected carefully in a fresh Falcon tube, to which 3 ml isopropanol was added, incubated for 30 minutes at –20° C and centrifuged for 15 minutes at 4000 g at 4 °C. The supernatant was then discarded carefully to avoid loss of pellet and the Falcon tube was

inverted on a tissue paper to drain of the remaining isopropanol. The pellet was then dissolved in 0.5 ml solution D and the solution was transferred to a fresh DEPC-treated tubes and 0.8 ml Isopropanol was added to it, mixed and placed at –20 for 30 minutes. This was followed by centrifugation for 15 minutes at 4000 g at 4 °C, the supernatant was discarded carefully to retain the pellet. The pellet was then washed with 80 % ethanol made in DEPC water, and vortexed again for 15 minutes at 4000 g at 4 °C. The supernatant was discarded and the tubes were inverted to drain of residual ethanol and the semi-dried pellet was dissolved in 100 µl DEPC water. A 10µl aliquot was used for the quality check and remaining RNA solution was stored at -80 °C until cDNA synthesis. The RNA was quantified and quality was determined by taking 2µl of the RNA solution diluted 50 times in DEPC water for calculating ratios 260/280 nm spectrophotometric OD measurement. The formula used was extinction x dilution to obtain number of µg/ml of RNA per sample and a ratio value approximately close to 1.9 was considered to be of acceptable quality. Further quality check (if necessary) was performed using a denaturing RNA gel, ran at 70-100 V for 1 hour and the gel was then read on a gel documentation apparatus.

C. cDNA synthesis and real-time RT-PCR

The RNA samples isolated according to the procedure detailed above were diluted in DEPC water to a concentration of 1µg/20µl. A master mix was prepared with reagents including 9 µl of 5x buffer (Invitrogen, Karlsruhe, Germany), 1µl of 25mM dNTP mixture (Amersham Pharmacia Biotech, Freiburg, Germany), 2 µl of 0.1 M DTT (Invitrogen, Karlsruhe, Germany), 1 µl of 40U/µl RNasin (Promega, Mannheim,

Germany), 0.5µl of 15µg/ml linear acrylamide (Ambion Ltd, Cambridgeshire, UK), 0.5µl of Hexanucleotide (Roche, Mannheim, Germany), 1µl of Superscript (Invitrogen, Karlsruhe, Germany) or ddH_2O in case of the controls. The master mix was made to a volume of 15µl and added to 2µg/30µl RNA samples were taken in separate DEPC treated microcentrifuge tubes, which were mixed and placed at 42 °C on a thermal shaker incubator for 1 hour. After 1 hour the cDNA samples were collected and stored at -20 °C until use for real-time RT-PCR analysis. The cDNA samples prepared as described above were diluted 1:10 a dilution for the real-time RT-PCR. The real-time RT-PCR was performed on a TaqMan® ABI Prism 7000 or 7700 (Applied Biosystems, Darmstadt, Germany). The quantitative PCR for mRNA is based on the employment of sequence-specific primers and likewise sequence-specific probes. The latter is tagged at both ends with a fluorescent molecule. The quencher absorbs TAMRA (at the 3'-End) the fluorescence of the other reporter tagged material such as FAM or VIC at the 5'-End. The TaqMan® universal PCR master mix (Applied Biosystems, Darmstadt, Germany) contained Taq polymerase possessing a 5'→3' polymerase activity and a 5'→3 ' exonuclease activity. During the elongation phase of the PCR, specifically bound probe was hydrolyzed by the exonuclease and the 5'-tag was set free. With every newly synthesized DNA strand fluorescent tag material was set free and the resulting fluorescence was measured at 488nm. The resulting fluorescence signal is directly proportional to the quantity of DNA synthesized. The CT value (= "Cycle Threshold") was computed for each sample. This is the cycle number, with which the reporter fluorescence signal breaks through a user-defined threshold. The TaqMan® universal PCR master mix containing, the forward primers and Reverse primers (final

concentration of 300 nM) and the probe (final concentration of 100 NM) was placed on ice. In the TaqMan® universal PCR master mix contained are PCR buffers, dNTPs and the AmpliTaqGold® previously mentioned (Taq polymerase without 3' → 5' exonuclease activity). 18µl of the mastermix was pipetted into each well of a 96-well plate and 2µl of template (DNA dilution) was added to each of these wells. The plate was sealed and centrifuged at 280 xg and analyzed using TaqMan® ABI PRISM 7000 or 7700. For the TaqMan® RT-PCR the following temperature settings were used: The first incubation was carried out for 2 minutes at 50°C followed by 95°C for 10 minutes so as to activate the polymerase. Templates were amplified during 40 cycles each comprising 15 seconds incubation at 95°C followed by 1 minute incubation at 60°C. The RT-PCR for the housekeepers (18S rRNA or GAPDH) was carried out under similar conditions. The CT values were recorded using the ABI PRISM Sequence Detection software (version 1.0) and the results were evaluated in relation the respective housekeepers. In all cases controls consisting of ddH_2O were negative for target and housekeeper genes.

Oligonucleotide primer (300 nM) and probes (100 nM) were from Applied Biosystems (Darmstadt, Germany):

Primers for Ccl2, Ccl5, and 18s rRNA were predeveloped TaqMan assay reagent from PE Biosystems.

Table 5. Probes used for real-time RT-PCR

	Accession-number	Sequence
Tlr3	AF355152,	Forward primer: 5'- CGAAAGT-TGGACTTGTCATCAAATC-3' Reverse primer: 5'-ACTTGCCAATTGTCTGGAAACAC–3' 6 FAM : 5'-CACTTAAAGAGTTCTCCC–3'
Tlr7	AY035889	Forward primer: 5'-TGCCACC-TAATTTACTAGAGCTCTATCTTTAT-3' Reverse primer: 5'-TAGGTCAAGAACTTGCAACTCA-TTG– 3' 6 FAM : 5' - CCAAGAAAATGATTTTAATAAC –3'
Tlr9	NM 031178	Forward primer: 5'-CAATCTGACCTCCCTTCGAGTACTT-3' Reverse primer: 5'-GCCACATTCTATAC-AGGGATTGG–3' 6 FAM : 5'- ATTGCCGTCGCTGCGACCATG –3'

3.1.7. Statistical analysis

Statistics were done using GraphPad Prism (4.03 version). Data were expressed as mean ± SEM. Data were analysed using unpaired two-tailed t-test for comparison between two groups. One-way ANOVA followed by post-hoc Bonferroni`s test was used for multiple comparisons. For nonparametric analysis of two groups Two-tailed Fisher's exact test and Mann Whitney U test were performed.

3.2. Materials and Methods- Part 2

3.2.1. Animals and experimental protocol

Five week old female MRL-*lpr/lpr* or MRL/WT mice were obtained from Harlan Winkelmann (Borchen, Germany) and maintained throughout in filter top cages under a 12 hour light and dark cycle. Autoclaved water and standard chow (Sniff, Soest, Germany) were available *ad libitum*. At 8 weeks of age MRL/WT and MRL-*lpr/lpr* mice were divided randomly into 2 groups of five mice that received intraperitoneal injections of the following compounds on every alternate days: Saline or 25 µg imiquimod (Sequoia Research Products Ltd, Oxford, UK) in 100 µl 0.25% sodium acetate (Merck, Germany). All mice were sacrificed by cervical dislocation at the end of week 10 of age. The experimental procedure had been approved by the local government authorities.

3.2.2. Evaluation of glomerulonephritis

Blood and urine samples were collected from each animal at the end of the study period. Urine albumin concentration was determined using ELISA kits by Bethyl labs (Montgomery, TX, USA). Urinary creatinine concentrations were determined using an automatic autoanalyzer (Integra 800, Roche Diagnostics, Germany). Serum dsDNA autoantibodies were determined by ELISA following the protocol mentioned before in part I (using ELISA kits by Bethyl labs). From all mice, kidneys were fixed in 10% buffered formalin, processed, and embedded in paraffin. Two µm sections for periodic acid-Schiff stains were prepared following routine protocols. The severity of the renal lesions was graded using the indices for activity and chronicity as described for human lupus nephritis (Austin *et al*, 1984) as described before in part 1.

3.2.3. Cell culture conditions and cytokine ELISAs

Bone marrow-derived dendritic cells were isolated from MRL/WT and MRL-*lpr/lpr* mice, processed, and cultured as described in part I. Cells were stimulated with pI:C RNA 30 μg/ml, imiquimod 3 μg/ml, CpG-ODN 1 μg/ml or medium control for 24 hours. Cytokine levels were determined in cell culture supernatants using ELISA kits for IL-6, IL-12p40 (OptEiA, BD Pharmingen), and IFN-α (PBL Biomedical Labs, USA). ELISAs were performed according to the protocol mentioned in part 1.

3.2.5. Isolation of primary B cells from the spleen of MRL-lpr/lpr and MRL/WT mice

B Cell Isolation Kit (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) was used for the isolation of B cells from spleens of female MRL-*lpr/lpr* or MRL/WT mice following the manufacturer's protocol with some additions and using ice cold conditions and solutions as follows:

Spleens were isolated from 2 female mice of either MRL-*lpr/lpr* or MRL/WT of 6-8 weeks of age after sacrificing them by cervical dislocation, using all sterilized materials and sterile conditions in an aseptic hood with HEPA filters. Spleen was collected in a sterile Petri plate containing the 2-3 ml of ice-cold media (RPMI+ 10 %FCS + 1 % PS) on ice. Then it was mashed using forceps in the petriplate itself and broken into fine pieces of tissues. Then the cell suspension was collected in a 15 ml Falcon tube using 1 ml pipette and centrifuged at 1600 rpm at 4°C for 6 min. The supernatant was decanted and pellet was resuspended in 4 ml of sterile 0.3M NH_4Cl solution in water and kept at room temperature for 5 min. Then it was Centrifuged at 1600 rpm at 4°C for 6 min. Supernatant was decanted and pellet was resuspended in ice cold (cell culture grade) PBS

and centrifuged at 1600 rpm at 4°C for 6 min. Now the cell suspension was passed through the 30 µm sterile pre-separation filter from Miltenyi biotec to obtain single cell suspension. Then it was centrifuged as above and the pellet was resuspended in 2 ml of buffer (PBS, pH 7.2 containing 0.5% BSA and 2 mM EDTA) (the buffer was prepared freshly and degassed by sonication, kept ice cold). A part of this cell suspension (100 µl) was used for counting the cell number after suitable dilution using Neubar's chamber. Then the cells were centrifuged at 300 g for 10 min. the supernatant was pipetted out and pellet was resuspended in 40 µl of buffer per 10^7 total cells. Then 10 µl of biotin antibody cocktail per 10^7 total cells was added and mixed with gentle pipetting and incubated for 10 min at 4-8 °C. Then 30 µl of buffer and 20 µl of anti biotin microbeads per 10^7 total cells was added, mixed and incubated for 15 min at 4-8°C. Later the cells were washed with buffer with 10-20 X labelling volume and centrifuging at 300 g for 10 min. the supernatant was pipetted off completely and the pellet was resuspended in 500 µl of buffer per 10^8 total cells. MACS LS column was placed in MiniMACS separator with column adapter and the column was rinsed with 500 µl of buffer. The cell suspension was applied in suitable amount of buffer onto the column (500-1000 µl). The cells were passed through the column and the effluent was collected as fraction with unlabelled cells, representing enriched B cell fraction. The column is washed with the appropriate amount of buffer (3 X 500 µl) and entire effluent is collected in same tube.

The cells count was done after suitable dilution in buffer and then the cells were plated in a 24 well or 96 well plate for stimulation or proliferation (1×10^5 or 3×10^5 cells per well) respectively. Purity was determined by FACS analysis using CD45/B220-PE or Rat IgG2a as an isotype (BD Biosciences, Hamburg, Germany) revealed 97 % B cells after

each isolation.

3.2.6. B-cell proliferation assay

Proliferation of B-cells was assessed using CellTiter 96 Proliferation Assay (Promega, Mannheim, Germany). In brief, 1 x 10^5 B-cells were incubated in 96-well plates in 100 µl RPMI medium containing 10 % FCS, 100 units/ml penicillin, and 100µg/ml streptomycin (Biochrom KG, Berlin Germany) with Tlr ligands as above for a period of 72 hours. To each well 20 µl CellTiter 96 Aqueous One Solution (Promega) was added and incubated at 37°C for 4 hours. The optical density was measured at 492 nm using a spectrophotometric plate reader.

3.3 Materials and Methods- Part 3

3.3.1. Phosphothioate IRS oligodeoxynucleotides (ODN) and other Tlr ligands

The following endotoxin-free ODN purchased from TIB Molbiol, Berlin, Germany were used for *in vitro* or *in vivo* studies: IRS661: 5'-TGCTTGCAAGCTTGCAAGCA-3'; IRS954: 5'- TGCTCCTGGA-GGGGTTGT-3'; CpG-DNA 1668: 5`-TCGATGACGTTCCTGATGCT-3`. Control ODNs were 5‘-TCCTGCAGGTTAAGT-3‘ and 5’-TCCTGGCGGAAAAGT-3‘. Poly (I:C)-RNA, LPS ultra-pure were purchased from Invivogen (San Diego, USA) and Imiquimod was purchased from Sequoia Research Products Ltd (Oxford, UK).

3.3.2. Studies with spleen monocytes

Adherent spleen monocytes were prepared from 10 week old female MRL-*lpr/lpr* mice and kept in culture as previously described in part 1 of materials and methods. Cells were stimulated with either medium or various concentrations of imiquimod or CpG-DNA 1668 or LPS or poly (I:C) with or without inhibitory oligos, IRS661 or IRS954 as indicated. Supernatants were harvested after 24 hours for ELISA.

3.3.3. Animal studies and experimental protocol

Ten weeks old female MRL-*lpr/lpr* mice (stock. 000485) were obtained from Jackson Laboratories (Bar Harbor, MA, USA) and were kept in filter top cages under a 12 hour light and dark cycle. Autoclaved water and standard chow (Sniff, Soest, Germany) were available *ad libitum*. To assess the distribution of injected IRS661, 100 µg 3'-rhodamine-labeled IRS661 or IRS954 was injected i.v. in a volume of 100 µl into 16 weeks old

MRL-*lpr/lpr* mice. Tissues were collected 2 hours after injection and subjected to further analysis. Different groups of mice were treated with either saline, IRS661 or IRS954 at a dose of 40 µg i.p. on alternate days from week 11 to 24 of age. Blood samples were collected after 3 hours of the last injection by retroorbital puncture. Mice were sacrificed by cervical dislocation under anesthesia. Blood and urine samples were collected from each animal at the end of the study period and the urine albumin concentration was determined using ELISA kits (Bethyl Labs), urine creatinine was determined as previously described (in part 2). All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities.

3.3.4. Autoantibody analysis

A. Anti-nuclear antibodies (ANA)

ANA were determined by incubating serum samples (1:200) with Hep-2 slides (Biosystems S.A. Costa Brava, Barcelona, Spain). The fluorescent patterns of ANA were classified as homogenous nuclear, speckled nuclear, cytoplasmic and mitotic pattern as described (Whichever staining pattern was observed predominant such as 80-90 % of the focused areas per well of Hep-2 slide was considered as that class of pattern). The secondary antibody used was fluorescein labelled Goat anti mouse IgG (Invitrogen, Karlsruhe, Germany) in 1:50 dilution in PBS. Serum of 6-10 week old C57BL/6 mice was used as negative control. Homogenous nuclear pattern- entire nucleus stained in a continuous manner, Speckled nuclear- staining of nucleus in a speckled manner, like many dots in and on the border of the nucleus, mitotic pattern- staining of the nucleus

when it is in division stage and can see both the nucleus together.

B. Anti-dsDNA ELISA assay

NUNC maxisorp ELISA plates were coated with poly-L-lysine (Trevigen, Gaithersburg, MD, USA) and followed by mouse embryonic stem cell dsDNA overnight. After incubation with mouse serum samples for 2 hours specific secondary antibody IgG, IgG1, IgG2a, IgG2b, IgG3 was used for detection of isotype by ELISA (Bethyl Labs, Montgomery, TX, USA) following the protocol as mentioned before in Part-1.

C. Crithidia luciliae assay

Immunofluorescence on specific slides of Crithidia Luciliae (BioRad Laboratories, Munich, Germany) was performed with 1:100 dilution of serum in PBS (pH 7) and scored for the intensity of kinetoplast DNA from 0 to 4. The secondary antibody used was fluorescein labeled Goat anti mouse IgG (Invitrogen, Karlsruhe, Germany) in 1:50 dilution in PBS (pH 7). For scoring, 0 – considered for no visually detectable fluorescence, 1 – considered for mild fluorescent signal from the kinetoplast, 2- considered for moderate signal, 3- considered for moderately higher signal, 4- considered for very high intense signal.

D. Anti-Sm and anti Sm-RNP

NUNC maxisorp ELISA plates were coated with Smith (Sm) antigen (stock- 5 mg/ml) (Immunovision, Springdale, AR, USA) at a dilution of 1:250. The Sm IgG (Y12) antibody (GeneTex, San Antonia, TX, USA) was used for standard curve and the

dilutions were made in PBS. A horseradish peroxidase-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 in PBS (pH 7). The same procedure was followed for anti-SmRNP as for anti-Sm except the ELISA plates were captured with Sm-RNP complex (1000 units/ml) (Immunovision) at a dilution of 1:250 instead of Sm antigen. For the ELISAs, equal volumes of Substrate A and B were mixed for the required number of wells and added for reaction completion (100 µl per well) and kept for 30 min in dark and followed by 100 µl of 1M H_2SO_4 for stopping the reaction. The reading of the absorbance was taken at 450 nm using spectrophotometer (TECAN).

E. Rheumatoid factor

NUNC maxisorp ELISA plates were coated with rabbit IgG at a concentration of 10 µg/ml (Jackson Immunoresearch, West Grove, PA, USA) overnight at 4° C. Serum samples were diluted 1:100 in PBS (pH 7), 10 week 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 (pH7). Substrate A and B were used for reaction completion for about 25-30 min to develop the colour in dark and followed by 1M H_2SO_4 for stopping the reaction. The absorbance was measured at 450 nm with reference wavelength as 620 nm at spectrophotometer as mentioned before.

3.3.5. Flow cytometry for spleen cells

Spleens were isolated from the treated MRL-lpr/lpr mice of each group (IRS661, IRS954,

Saline) after sacrificing it, and 3 subgroups were made by pooling 3, 3 & 4 spleens from each treatment group. Spleen was collected in a sterile Petri plate containing the 2-3 ml of ice-cold media (RPMI+ 10 %FCS + 1 % PS) on ice. Then it was mashed using forceps in the petriplate itself and broken into fine pieces of tissues. Then the cell suspension was passed through 70 µm plastic filter (BD biosciences) using the 1 ml pipette and collected in a 15 ml Falcon tube with pipette and centrifuged at 1600 rpm at 4°C for 6 min. The supernatant was decanted and the pellet was resuspended in 4 ml of sterile 0.3M NH_4Cl solution and kept at room temperature 5 min. Then it was Centrifuged at 1600 rpm at 4°C for 6 min. Supernatant was decanted and pellet was resuspended in ice cold (cell culture use) PBS and centrifuged at 1600 rpm at 4°C for 6 min. Pellet was resuspended in ice cold PBS and passed through 70 µm plastic filter and collected in new Falcon and again centrifuged as before. The pellet was resuspended in PBS (2-4 ml) and the no. of cells were counted using Neubar's chamber under the microscope after suitable dilution. The cells suspension was suitably diluted to obtain 1 million cells/ml, later cells were subdivided into different eppendorff tubes in a volume of 200 µl for different staining with the isotype, T cell marker, or unstained. Cells suspension in eppendorff tubes were incubated with labelled antibody for 30 min in dark, followed by washings (2 times) and then transfer in special FACS tubes in a volume of 400 µl of PBS and protected from light until flow cytometry analysis.

The following antibodies were used to identify T cell subsets: FITC-labelled hamster anti-mouse CD3 (clone 145-2C11, BD, Biosciences, Heidelberg, Germany), APC-labelled rat anti-mouse CD4 (clone RM4-5, BD), PerCP-labelled rat anti-mouse CD8 (clone 53-6.7, BD), PerCP-labelled rat IgG2a (clone R35-95, BD), and APC-labelled rat

IgG2a (clone R35-95, BD), PE-labelled rat IgG2a (clone R35-95, BD) were used as isotype controls, respectively.

4.0. Results

4.1. Results- Part 1

4.1.1 Toll like receptor 7 expression in experimental lupus nephritis

Based on the previous studies from our lab, it was found that the expression of Tlr7 is increased in the kidneys of 20 wks old female MRL-lpr/lpr mice compared to their young counterparts (Figure 6). In order to localize the source of renal Tlr7 expression a polyclonal antibody specific for murine Tlr7 and performed double staining for either ER-HR3 or CD11c in renal sections of 16 weeks old MRL-lpr/lpr mice. Approximately 30% of interstitial ER-HR3 macrophages and CD11c dendritic cells (ratio 90%: 10% respectively) stained positive for Tlr7 (Figure 7). Staining for Tlr7 appeared in a speckled pattern indicating that Tlr7 is localized in an intracellular compartment. Intrinsic renal cells were negative for Tlr7 and the macrophages found to be present in the glomerular compartment were rarely positive for Tlr7. Together these data suggest that in nephritic kidneys of MRL-lpr/lpr mice, Tlr7 is expressed mainly by infiltrating interstitial macrophages but not by intrinsic renal cells.

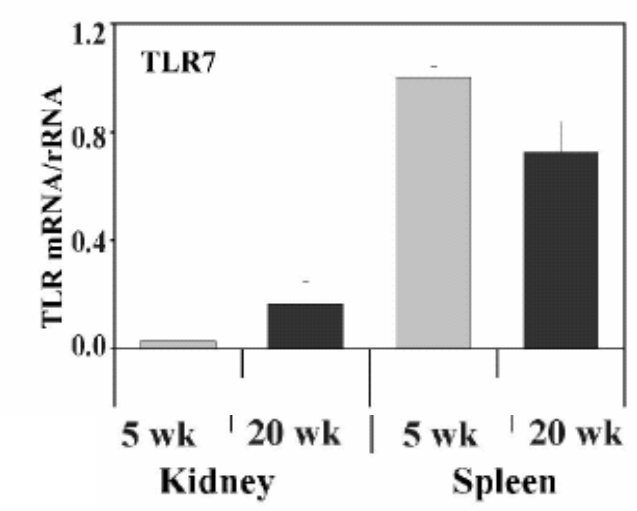


Figure 6. Tlr7 expression in MRL-lpr/lpr mice. *Expression of Tlr mRNA was assessed by real-time RT-PCR in duplicates using RNA isolated from spleens and kidneys from 7 MRL-lpr/lpr mice each at 5 weeks and 20 weeks of age as described in methods. Tlr mRNA expression is expressed as ratio to the respective 18S rRNA expression ± SEM.*

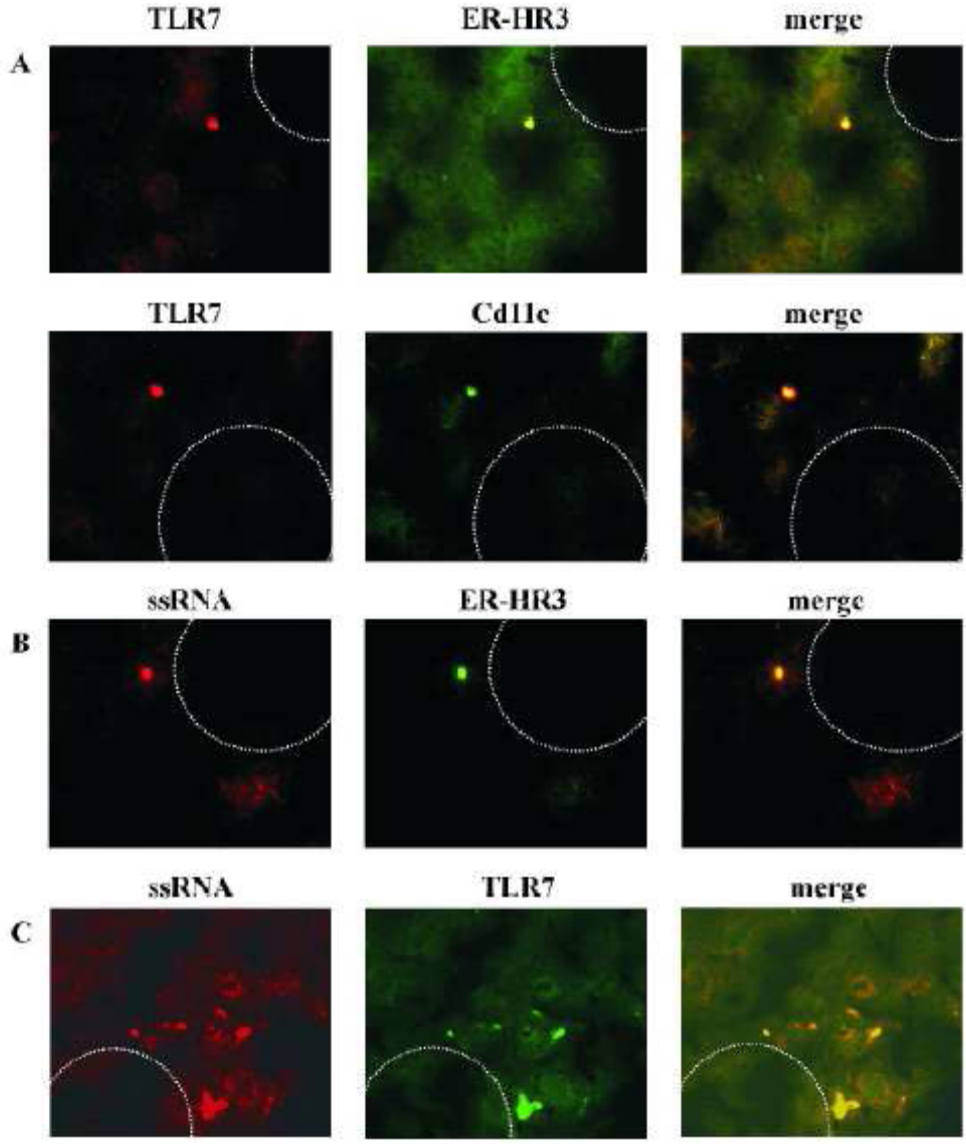


Figure 7. Tlr7 immunostaining and uptake of labelled ssRNA in kidneys of MRL-lpr/lpr mice*. A: mTlr7 specific antibody was used on renal sections of 18 week-old nephritic MRL-lpr/lpr mice. A PE-labelled secondary antibody was used for detection. Positive signals colocalized with ER-HR3 positive macrophages or CD11c positive dendritic cells, both detected by a FITC-labelled secondary antibody. B: Rhodamine-labelled ssRNA 40 was intravenously injected to 18 weeks old MRL-lpr/lpr mice and kidney tissue was harvested two hours later. Fluorescence imaging of frozen sections showed uptake of ssRNA 40 (red) into ER-HR3 positive macrophages (green). C:*

Costaining of rhodamine-labelled cells (red) for Tlr7 (green) demonstrates uptake of RNA 40 into Tlr7 positive cells. Original magnification of all images 530x.

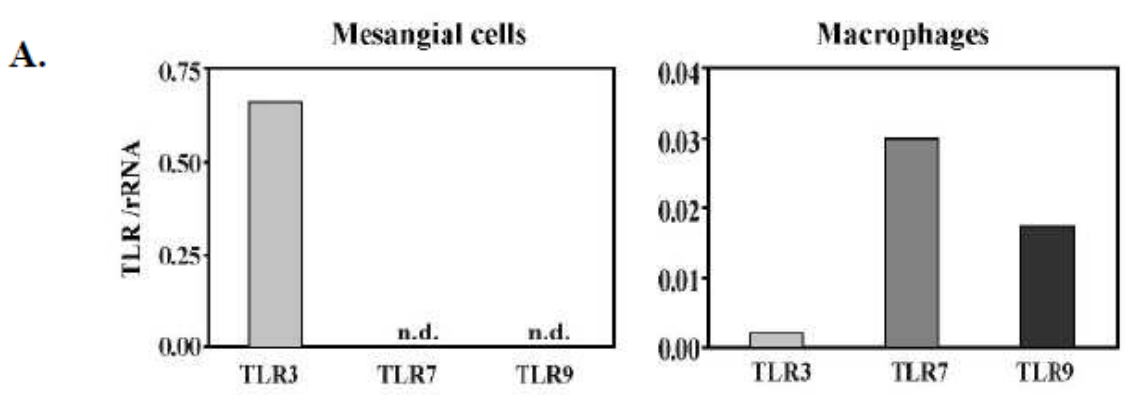
4.1.2. In vivo localization of labelled ssRNA after IV injection in MRL-lpr/lpr mice

In order to examine whether circulating ssRNA localizes to nephritic kidneys of MRL-lpr/lpr mice, rhodamine-labeled ssRNA40 was injected intravenously to 20 weeks old MRL-lpr/lpr mice. Consistent with Tlr7 immunostaining in the kidney, the labelled ssRNA was found in infiltrating cells in a granular intracellular staining pattern (Figure 7). Double labelling with an ER-HR3-specific antibody identifying these cells as renal macrophages (Figure 7). MRL-lpr/lpr mice injected with rhodamine only did not localize in the kidney. Double labelling for Tlr7 confirmed that injected ssRNA was taken up into Tlr7 positive cells (Figure 7). MRL-lpr/lpr mice injected which rhodamine only did not localize in the kidney. Taken together, in kidneys of MRL-lpr/lpr mice injected ssRNA colocalizes in an intracellular granular pattern with Tlr7 positive cells, i.e. infiltrating macrophages and DCs, but not with intrinsic renal cells.

4.1.3. In vitro stimulation of cultured macrophages and mesangial cells by Tlr7 ligand

In order to confirm the respective Tlr7 expression, murine cell lines for macrophages (J774) and mesangial cells were cultured. Under basal culture conditions mesangial cells expressed Tlr3 mRNA, while mRNAs for Tlr7, and –9 were not detected (Figure 8). By contrast, J774 macrophages expressed all three receptors (Figure 8). The subcellular localization of Tlr3, -7, and -9 was assessed by flow cytometry. In mesangial cells Tlr3 and in macrophages Tlr3, -7, and -9 were expressed intracellularly while surface

expression was absent (Figure 8). Next it was questioned whether mesangial cells and macrophages respond to microbial nucleic acids corresponding to their specific Tlr expression profile. Both cell types were stimulated with synthetic mimics of microbial nucleic acids: pI:C-RNA (Tlr3) or imiquimod or RNA40 (Tlr7), or CpG-DNA-1668 (Tlr9). In accordance with their respective Tlr expression profile, mesangial cells produced the CC-chemokine MCP-1 (CCL2) only after exposure to mimics of viral dsRNA, whereas J774 macrophages responded to all Tlr agonists tested (Figure 8). Together, these data indicate that macrophages, but not mesangial cells, express Tlr7 in an intracellular compartment and produce CCL2 upon exposure to ssRNA or imiquimod *in vitro*.



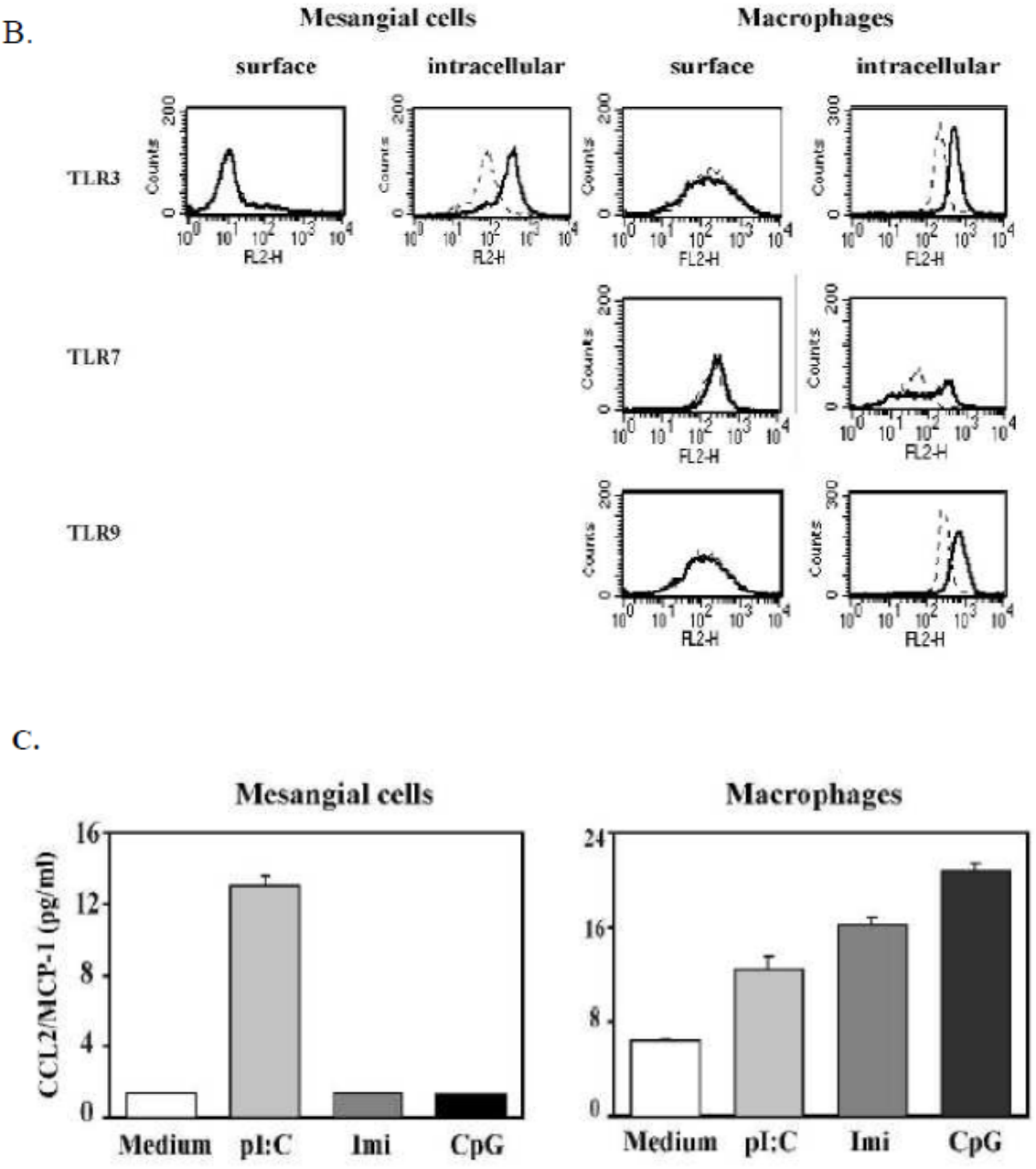


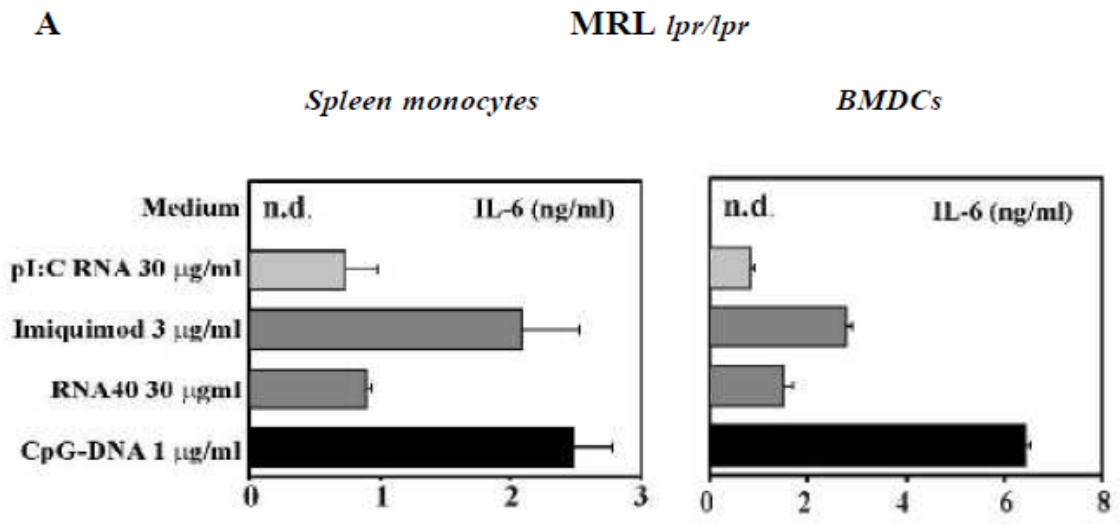
Figure 8. Nucleic acids activate cultured mesangial cells and J774 macrophages consistent with their respective Tlr expression profile. *Murine mesangial cells and J774 macrophages were cultured as described in methods. A: Expression of Tlr mRNA was assessed by real-time RT-PCR in duplicates using RNA isolated from cultured cell lines. Tlr mRNA expression is expressed as ratio to the respective 18S rRNA expression ± SEM (* $p < 0.05$). B: Flow cytometry for Tlrs before and after permeabilization for intracellular staining was performed as indicated. Expression of Tlr (solid line) is*

demonstrated by a fluorescence shift compared to the isotype control antibody (dotted line). C: Cultured cells were incubated with either pI:C RNA, imiquimod (IMI) or RNA 40 or CpG-DNA or standard medium without supplements for 24 hours as indicated. CCL2 production was measured in supernatants by ELISA. Results shown are representative of two comparable experiments each performed in duplicate.

4.1.4. Primary spleen monocytes and dendritic cells isolated from MRL-lpr/lpr mice responds to stimulation with Tlr7 agonists

As Tlr7 appears to be expressed by ER-HR3 positive macrophages and CD11c positive dendritic cells in nephritic kidneys of MRL-lpr/lpr mice, hence in order to characterize the factors produced by these cells in response to agonists of Tlr7 in comparison to other nucleic acid-like Tlr agonists, in-vitro experiments were planned. Spleen monocytes and bone-marrow dendritic cells were isolated from MRL-lpr/lpr mice as described in methods. ER-HR3 positive population comprised approximately 90% of the spleen monocytes, which were found to be negative for CD11c. Likewise, approximately 85% of the bone marrow derived dendritic cells were found to be positive for CD11c, which were found to be negative for ER-HR3 as determined by flow cytometry. Both cell types were incubated with either pI:C-RNA or RNA40 or imiquimod or CpG-DNA-1668. In spleen monocytes both, RNA40 and imiquimod, induced production of IL-6, IL12p70, IFN-α, and MCP-1 (CCL2) (Figures 9A-D). In bone marrow-derived dendritic cells the observed responses were similar except for increased IL12-p70 and IFN-α production upon exposure to pI:C RNA (Figure 9A-D) as compared to stimulation of spleen monocytes. These data suggest that Tlr7 agonists induce the production of IL-12p70, IL-6, CCL2, and IFN-α in both spleen monocytes and dendritic cells isolated from MRL-

lpr/lpr mice.


A
MRL lpr/lpr
Spleen monocytes
BMDCs
Medium
pI:C RNA 30 μg/ml
Imiquimod 3 μg/ml
RNA40 30 μgml
CpG-DNA 1 μg/ml
n.d.
IL-6 (ng/ml)
0
1
2
3
n.d.
IL-6 (ng/ml)
0
2
4
6
8

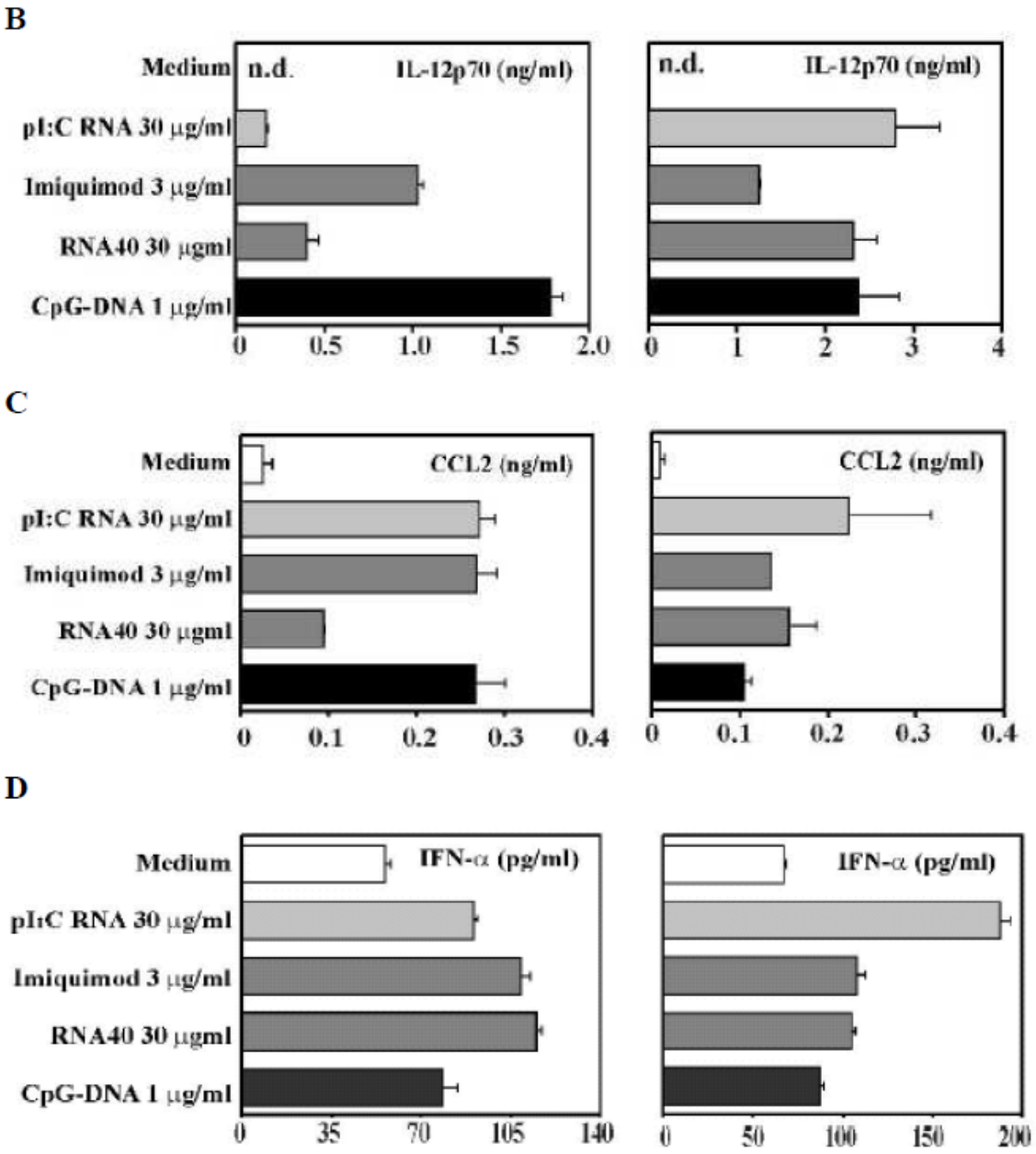


Figure 9. Tlr7 agonists activate ER-HR+ monocytes and CD11c+ dendritic cells isolated from MRL-lpr/lpr mice. *ER-HR3+/CD11c- spleen monocytes and ER-HR3-/CD11c+ dendritic cells (DCs) from spleens and bone marrows (BM) respectively of MRLlpr/lpr mice were cultured and incubated with either pI:C-RNA or RNA40 or imiquimod or CpG-DNA or standard medium for 24 hours as indicated. IL-6 (A), IL-12p70 (B), CCL2/MCP-1 (C), and IFN-α (D) were measured in supernatants by ELISA. Results shown are from one of three comparable experiments. For each experiment cells*

were pooled from 3 mice. Values represent means ± SEM, n.d. = non detectable.

4.1.5. Imiquimod increases serum IFN-alpha, IL-6, and IL-12p70 levels in MRLlpr/lpr mice.

Circulating IFN-alpha, IL-6, and IL-12p70 levels are markers of disease activity in lupus. Thus, having demonstrated the effect of Tlr7 ligation on IFN-alpha, IL-6, and IL-12p70 secretion in antigen-presenting cell subsets isolated from MRL*lpr/lpr* mice *in vitro*, serum levels of these factors were studied 6 hours after intraperitoneal injection of 25 μg of imiquimod or vehicle or saline to 16 weeks old MRL*lpr/lpr* mice. Injection of imiquimod significantly increased serum levels of IL-12p70, IL-6, and IFN-alpha in MRL*lpr/lpr* mice as compared to vehicle-injected controls (Figure 10).

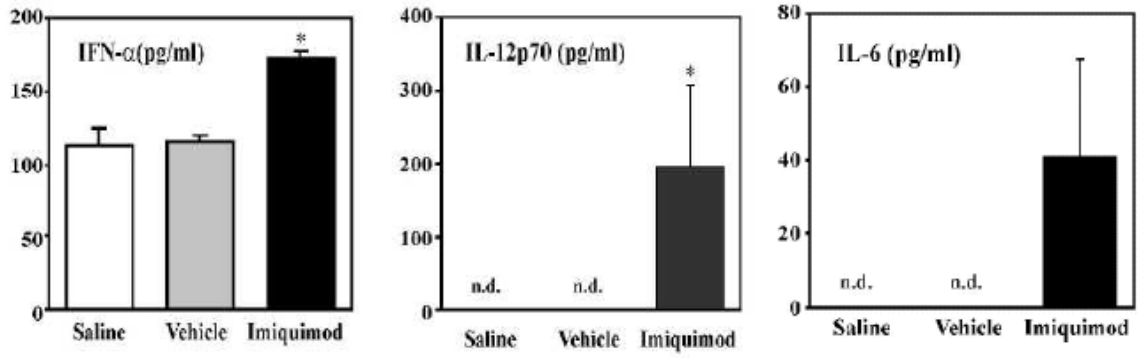


Figure 10. Serum IFN-α, IL-12p70, and IL-6 levels in MRLlpr/lpr mice. *Serum was obtained from 16 weeks old MRLlpr/lpr mice 6 hours after the first intraperitoneal injection of either saline or vehicle or 25 μg imiquimod as indicated (n=5-10). Serum levels were determined by ELISA. Data are mean ± SEM. * $p < 0.05$ vs saline.*

4.1.6. Imiquimod aggravates autoimmune tissue injury in MRL lpr/lpr mice.

From the above results one would predict that ligation of Tlr7 would be associated with more severe autoimmune tissue injury in MRL*lpr/lpr* mice. Therefore different groups of lupus mice were treated with intraperitoneal injections of either 25 μg imiquimod or vehicle or saline on alternate days from week 16 to 18 of age. Based on the results of *in vitro* studies with macrophages and dendritic cells, it was hypothesized that Tlr7 ligation would trigger local chemokine expression in nephritic kidneys of MRL *lpr/lpr* mice. Thus, immunostaining for CCL2 was performed. Taken together, imiquimod aggravated autoimmune tissue injury in MRL*lpr/lpr* mice associated with increased local expression of CCL2 in areas of inflammatory cell infiltrates and tissue damage in nephritic kidneys of MRL*lpr/lpr* mice.

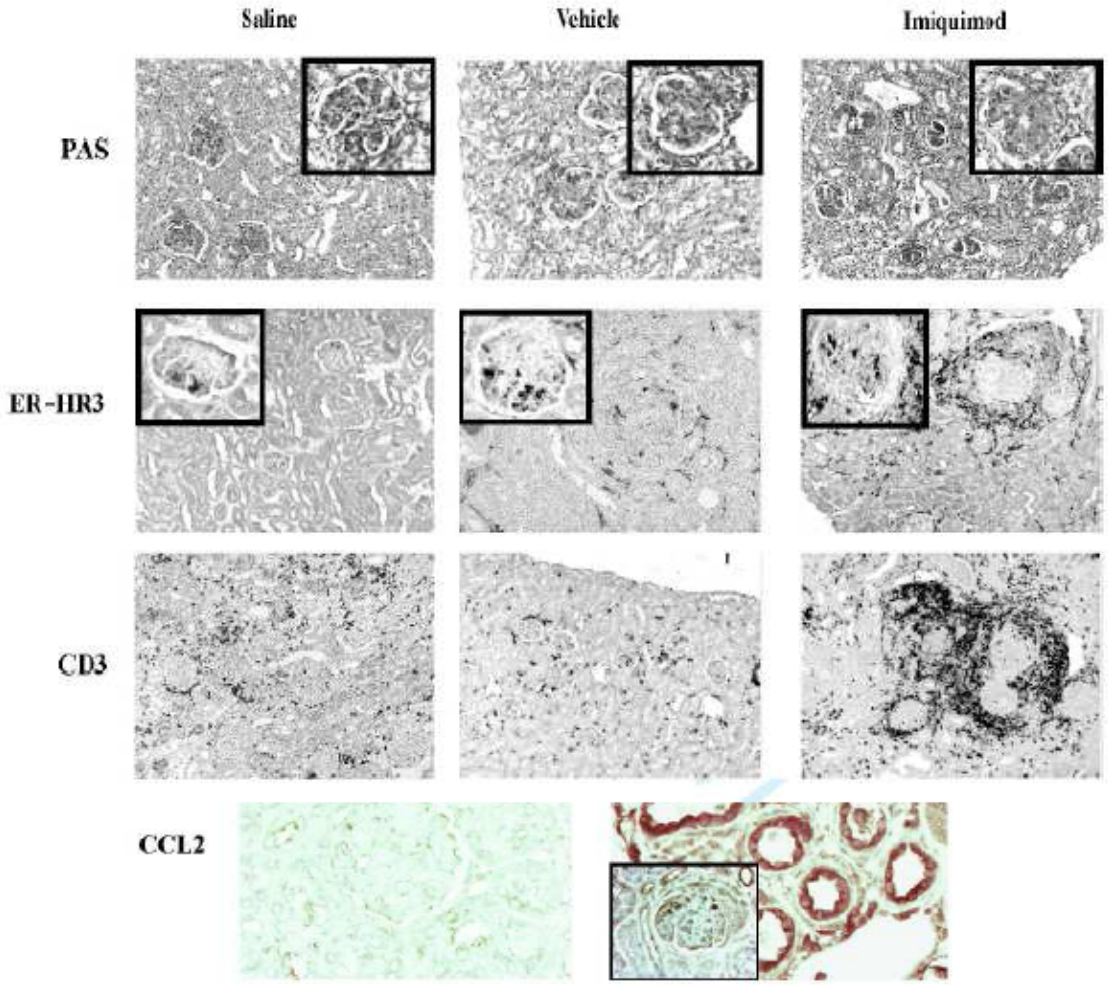


Figure 11. Renal histopathology. *A: Renal sections of 18 weeks old MRLlpr/lpr mice from all groups were stained with periodic acid Schiff, antibodies for ERHR-3 (macrophages), CD3 (lymphocytes), and SMA (smooth muscle antigen for myofibroblasts) as indicated. Insert in PAS stained sections of imiquimod-treated MRLlpr/lpr mice illustrates glomerular tuft necrosis and crescent formation not detected in mice of the other groups (Original magnification x400). Inserts in ERHR-3 and CD3-stained sections show respective glomeruli at a magnification of x530). Images are representative for 8-10 mice in each group. B: Renal sections of 18 weeks old vehicle or imiquimod-treated MRL$^{lpr/lpr}$ mice were stained for CCL2/MCP-1. Arrows indicate CCL2 positive glomerular and interstitial cells. Note CCL2 positivity also in tubular epithelial cells in imiquimod-treated mice (original magnification x400). Images are representative for 5 mice in each group.*

Table 7. Urinary finding in treated MRL*lpr/lpr* mice.

	Saline	Vehicle	Imiquimod
Proteinuria as Albumin/creatinine (mg/mg.)	5.4 ± 2.5	3.3 ± 0.9	17.8 ± 6.2[a]

Values are means ± SEM, [a] $p < 0.05$ imiquimod vs. Vehicle

4.1.7. Imiquimod increases renal immune complex deposition in MRL lpr/lpr mice

Based on the results of previous studies in our lab it was observed that the activation of Tlr9 as well as Tlr3 aggravated lupus nephritis in MRL*lpr/lpr* mice. Hence, the effects of Tlr7 ligation were investigated on serum dsDNA autoantibody levels and glomerular IgG deposits in MRL*lpr/lpr* mice. Imiquimod somewhat increased serum total IgG as well as IgG_1-, and IgG_{2a}-dsDNA autoantibodies as compared to vehicle-treated MRL*lpr/lpr* mice although this was not statistically significant (Figure 12A). However, imiquimod-treated MRL*lpr/lpr* mice showed increased glomerular capillary and mesangial deposits of total IgG, IgG_1, and IgG_{2a} (Figure 12B, Table 7). This was associated with increased glomerular capillary and mesangial deposits of complement factor C3c in imiquimod-treated MRL*lpr/lpr* mice (complement deposit score imiquimod: 2.4 ± 0.3 vs. vehicle: 1.3 ± 0.1, $p < 0.01$, Figure 12C). Together, imiquimod-induced aggravation of lupus nephritis in MRL*lpr/lpr* mice is associated with increased glomerular immune complex deposition.

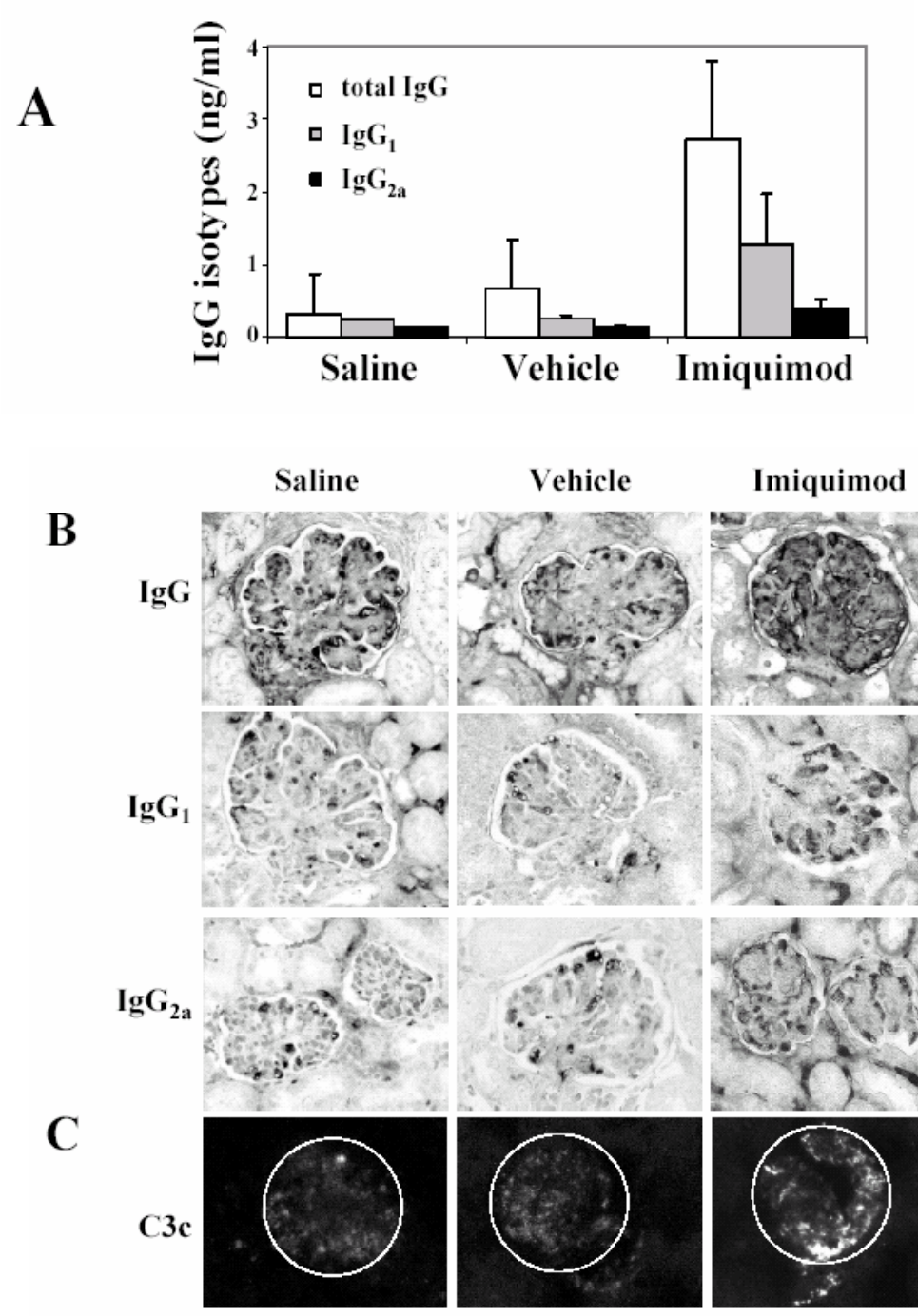


Figure 12. DNA autoantibodies and immune complex deposits in MRL*lpr/lpr* **mice.** *A: Serum dsDNA autoantibody levels were determined by ELISA (n=5-10). Data are mean ± SEM. p was < 0.05 vs vehicle for IgG and both IgG isotypes. B and C: Immunostaining for total IgG, IgG_1, IgG_{2a} and complement factor C3c were performed on renal sections of MRLlpr/lpr mice as described in Methods (original magnification 400x).*

4.2. Results Part 2

4.2.1 Tlr7 ligand induced effects on spleen and DNA autoantibodies in young MRL and MRLlpr/lpr mice

At 8 weeks of age no structural abnormalities were detected in kidney and spleens of MRL wild-type mice as observed using light microscopy. Spleens of age-matched MRL*lpr/lpr* mice displayed lymph follicle hyperplasia with enlarged B-cell zones and increased spleen weight (Figure 13A, Figure 14B). Renal morphology did not show any abnormalities in MRL*lpr/lpr* mice of this age. Given the pathogenic role of lymphoproliferation and autoantibody production for the pathogenesis of lupus nephritis it was first tested whether exposure to imiquimod induces splenomegaly and the production of DNA autoantibodies in young MRL or MRL*lpr/lpr* mice. Serum dsDNA autoantibodies were determined by ELISA in all groups of mice at 10 weeks of age. In saline-treated MRL wild-type mice, serum dsDNA antibodies were absent and imiquimod did not induce significant levels of dsDNA IgG autoantibodies. In contrast, 10 week old saline-treated MRL*lpr/lpr* mice had detectable levels of total IgG and IgG_1 dsDNA antibodies (Figure 13B). Exposure to imiquimod increased serum levels of total IgG dsDNA antibodies. However the ELISA kits for detection of IgG_{2a} and IgG_{2b} dsDNA antibodies were observed to be less sensitive compared to total IgG ELISA kit.

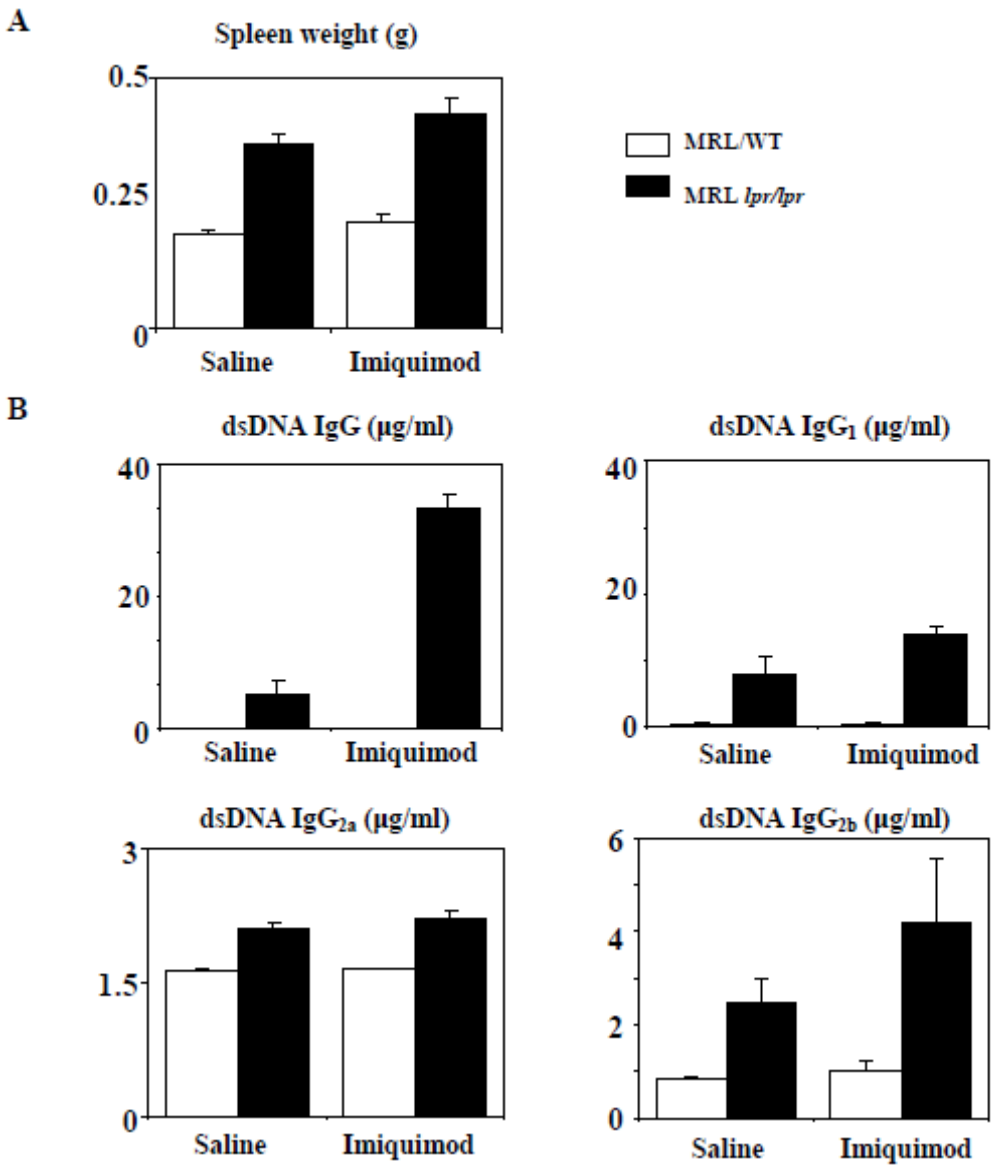
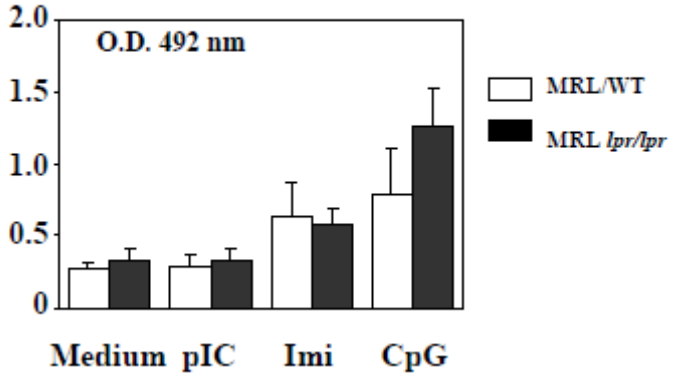


Figure 13. Lymphoproliferation and serum dsDNA autoantibodies in MRL and MRLlpr/lpr mice. *A: Spleen weights were assessed at time of sacrifice at 10 weeks of age in MRL and MRLlpr/lpr mice treated with saline or imiquimod, as indicated (n=5). B: Serum levels of dsDNA autoantibodies of the IgG, IgG_1, IgG_{2a} and IgG_{2b} isotypes were determined by ELISA. Imi = imiquimod. Data are mean ± SEM. * $p < 0.05$ vs. saline, # $p < 0.05$ vs. MRL wild-type mice. n.d. = not detectable.*

4.2.2 In vivo and in vitro B-cell responses by Tlr7 ligand in MRL and MRL-lpr/lpr mice

The potential of microbial nucleic acids to induce autoantibody production in MRL$^{lpr/lpr}$ mice may be linked to their potential to activate B-cells. The proliferation of medium-treated cultured primary B-cells isolated from MRL and MRL*lpr/lpr* mice was comparable (Figure 14A). Consistent with previously published data, the effect of imiquimod was less prominent in the absence of additional costimuli and did not significantly affect the proliferation of B-cells of either mouse strain. Imiquimod-induced IL-12p40 production was less potent, but in the presence of 5000 u/ml IFN-α imiquimod markedly induced IL-12p40 (Figure 14C). Thus, by virtue of its effect on spleen size and autoantibody production imiquimod per se was not found to be a potent B-cell mitogen in MRL*lpr/lpr* mice.

A



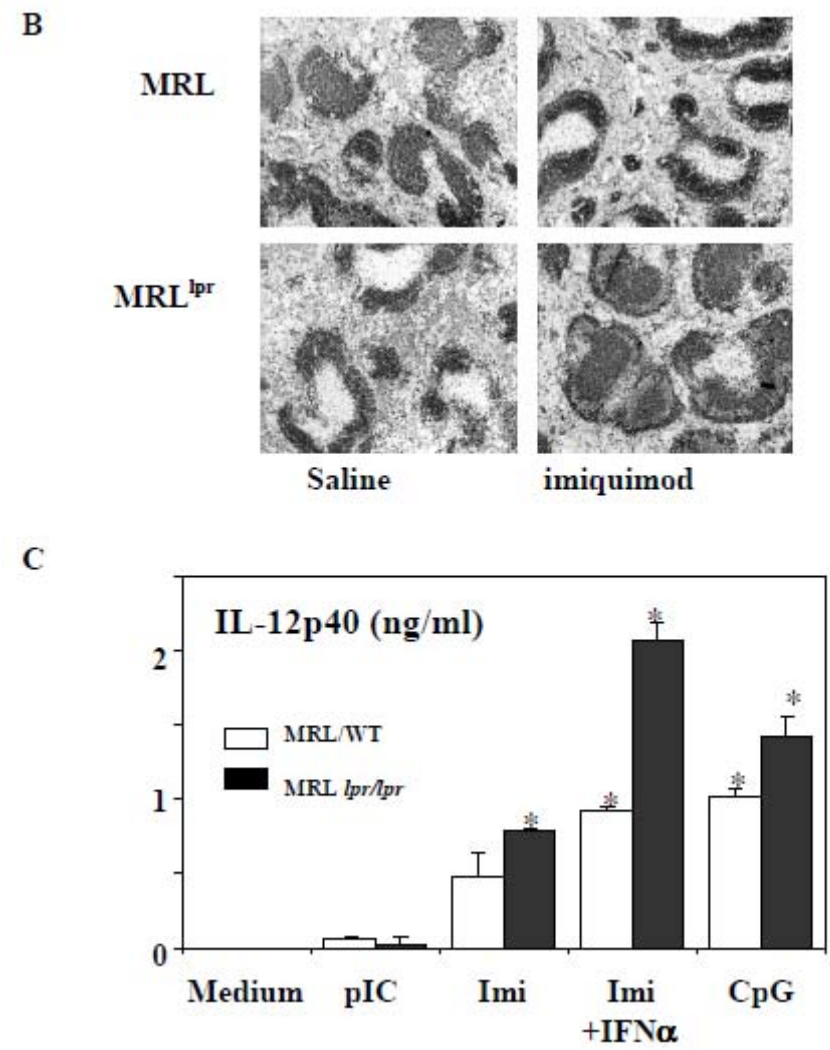


Figure 14. B-cells in MRL and MRL-*lpr/lpr* ***mice.*** *A: CD19+ B cells were prepared from spleens of MRL and MRLlpr/lpr mice and incubated with either pI:C RNA or imiquimod or CpG-DNA-1668 or standard medium as indicated. After 72 hours of incubation B-cell proliferation was assessed by CellTiter 96 proliferation assay. The optical density (O.D.) was read at 490 nm. B: Spleen sections were prepared from mice of all groups and stained for B220+ B-cells. Images are representative for 5 mice in each group (original magnification x50). C: CD19+ B cells were prepared from spleens of MRL and MRLlpr/lpr mice and incubated with either pI:C RNA or imiquimod or CpG-DNA or standard medium in the presence or absence of 5000 u/ml murine IFN-α as indicated. IL-12p40 were measured in supernatants by ELISA after 24 hours of incubation with the respective Tlr ligands. Results shown as mean ± SEM from three*

*comparable experiments. * $p<0.05$ vs medium, n.d. = non detectable.*

4.2.3. Serum levels of cytokines after challenge with Tlr7 ligand in young MRL and MRLlpr/lpr mice

Circulating IL-12p40, IL-6, and IFN-α levels are markers of disease activity in lupus. Thus, it was questioned to study imiquimod induced changes in the serum cytokine levels in 10 weeks old MRL and MRL*lpr/lpr* mice. Serum IL-12p40, IL-6, and IFN-α levels were determined at 3, 6, 12, and 24 hours after a single agonist injection in MRL and MRL*lpr/lpr* mice. Baseline levels of all cytokines were low in saline-treated MRL and MRL*lpr/lpr* mice (Figure 15). Il-12p40 was induced by imiquimod in both strains with a maximum at 6 hours after injection (Figure 15). Serum IFN-α levels were low in all groups of mice and did not respond significantly to imiquimod (Figure 15). These data suggest that IL-12p40 levels are induced upon exposure to imiquimod.

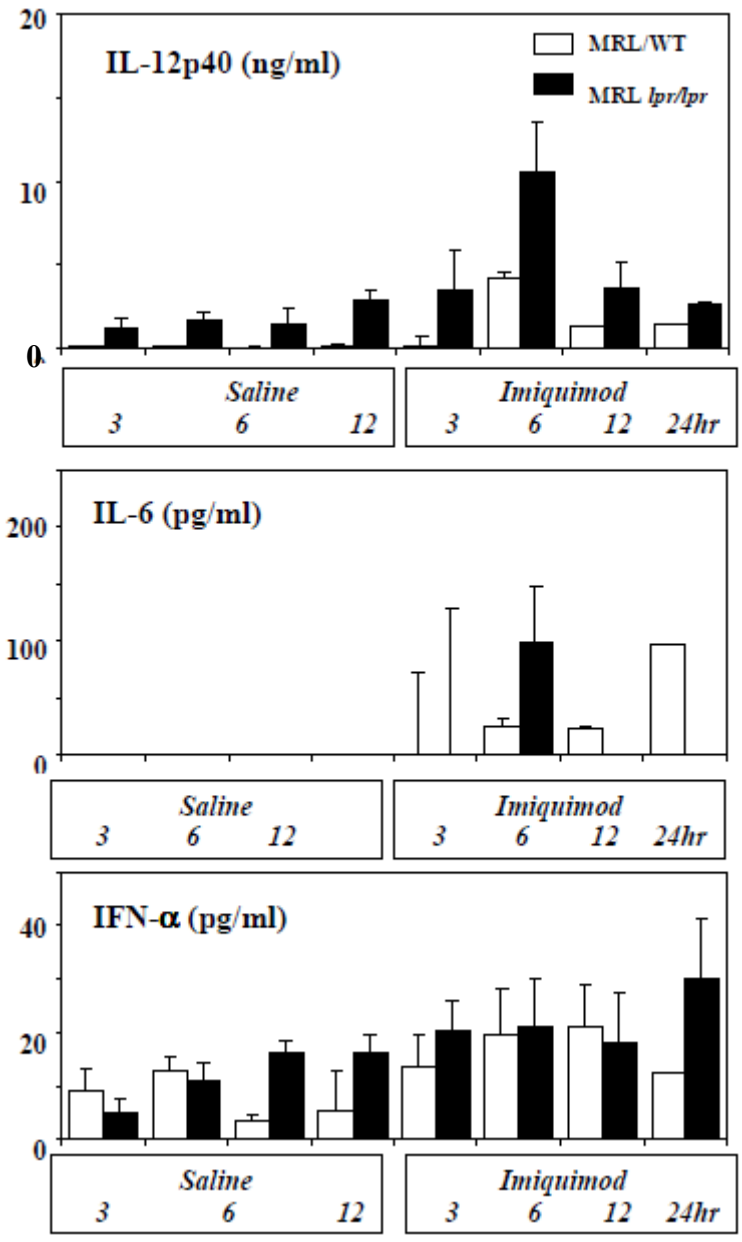


Figure 15. Serum IL-12p40, IL-6, and IFN-α levels in MRL and MRLlpr/lpr mice. *Serum was obtained from 10 weeks old MRL and MRLlpr/lpr mice at different time points after single injection with pI:C RNA, imiquimod, and CpG-DNA as indicated (n=5). Serum levels were determined by ELISA. Data are mean ± SEM. * $p < 0.05$ vs saline, # $p < 0.05$ vs. MRL wild-type mice, n.d. = not detectable.*

4.2.4. Tlr7 mediated activation of primary dendritic cells isolated from MRL and MRLlpr/lpr mice

Dendritic cells coordinate adaptive immune responses during antimicrobial immunity as well as autoimmunity. Hence it was examined whether Flt3-ligand-induced, CD11c positive ER-HR3 negative dendritic cells prepared from MRL or MRL*lpr/lpr* mice release proinflammatory cytokines when incubated with different doses of either medium or pI:C-RNA or imiquimod or CpG-DNA. All three Tlr agonists induced IL-12p40 and IL-6 in dendritic cells (Figure 16). Imiquimod and pI:C RNA were less potent in inducing IL-12p40 and IL-6 release in dendritic cells from MRL or MRL*lpr/lpr* mice and higher doses of imiquimod did not further increase cytokine release due to cytotoxicity. Imiquimod and CpG-DNA but not pI:C RNA induced IFN-α. No differences between dendritic cells from MRL and MRL*lpr/lpr* mice were detected except for high doses of CpG-DNA.

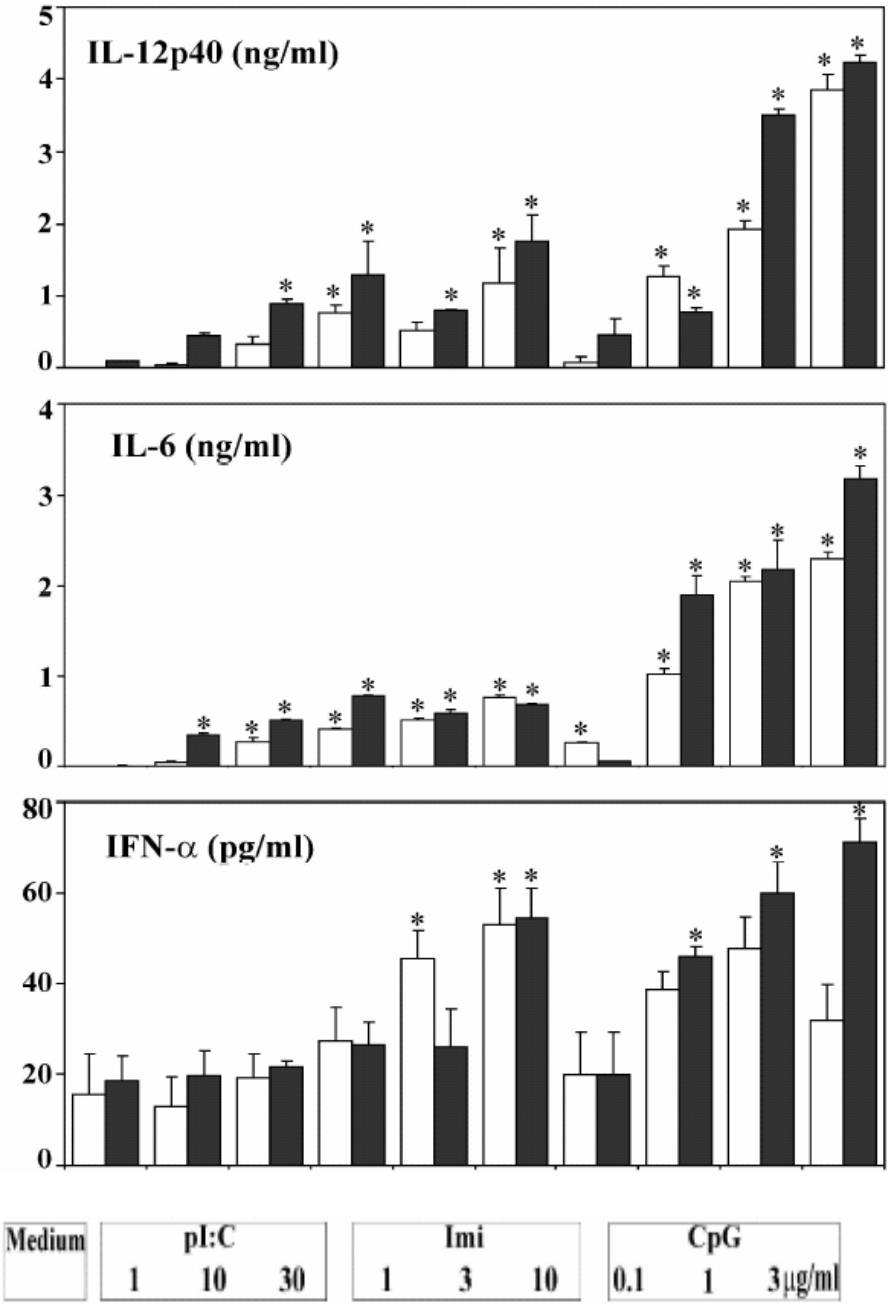


***Figure 16**. **Activation of dendritic cells isolated from MRL and MRLlpr/lpr mice.** ER-HR3-/CD11c+ dendritic cells were prepared from bone marrows of MRL and MRLlpr/lpr mice and incubated with standard medium or different doses of either pI:C-RNA or imiquimod or CpG-DNA for 24 hours as indicated. IL-12p40, IL-6, and IFN-α were measured in supernatants by ELISA. Results were shown as mean ± SEM from three comparable experiments, * $p<0.05$. n.d. = non detectable.*

Table 8. Histological and urinary findings in MRL/WT and MRLlpr/lpr mice.

	Saline	Imiquimod
Urinary albumin/creatinine ratio		
MRL	0.04 ± 0.01	0.07 ± 0.02
$MRL^{lpr/lpr}$	0.07 ± 0.01	0.12 ± 0.09

N = 5 per group, values are mean ± SEM.

4.2.5. Imiquimod modulates lupus nephritis in young MRL lpr/lpr and MRL mice

The induction of DNA autoantibody production could be associated with respective renal immune complex deposition and subsequent lupus nephritis. Hence, glomerular immune IgG_1 and IgG_{2a} deposition were quantified by immunohistochemistry. In MRL wild-type mice of all groups glomerular IgG_1 and IgG_{2a} deposits were not detected. Saline-treated MRL*lpr/lpr* mice had mild glomerular IgG_1 and IgG_{2a} deposits, predominantly in a capillary staining pattern (Figure 17). In MRL*lpr/lpr* mice injections of imiquimod did not significantly increase the amount of glomerular IgG_1 and IgG_{2a} deposits (Figure 17). Glomerular immune complexes cause glomerular damage through local complement activation. Glomerular C3 deposits in MRL*lpr/lpr* mice was hardly detectable in imiquimod-treated MRL*lpr/lpr* mice and absent in MRL wild-type mice (Table 8). Immunostaining for CD3 lymphocytes and Mac-2 macrophages in renal sections revealed no change in glomerular macrophages in different groups (Figure 17, Table 8). CD3 lymphocytes were not found at significant numbers in kidneys of MRL and MRL*lpr/lpr* mice. Imiquimod injected mice also had no change proteinuria indicated by urinary albumin/creatinine ratio (Table 8). These data indicate that exposure to imiquimod does not induce lupus nephritis in MRL wild-type mice.

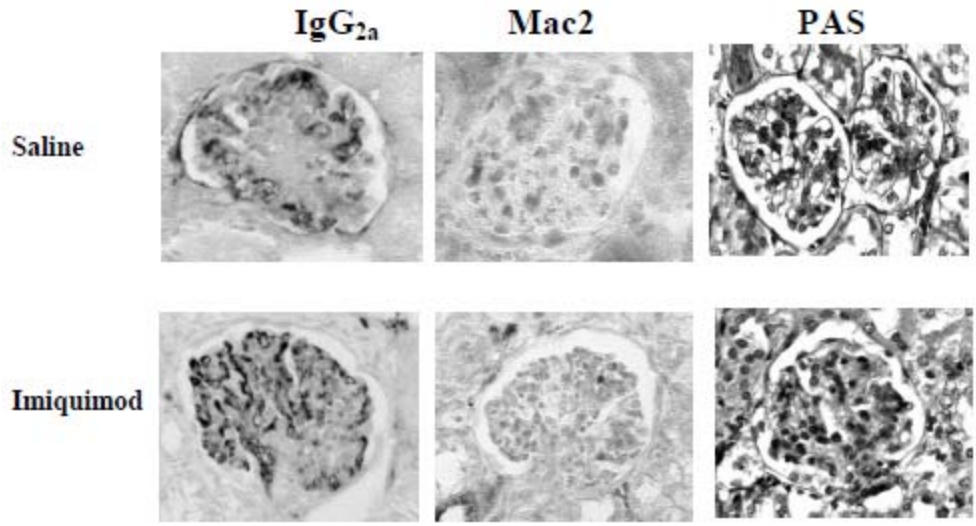
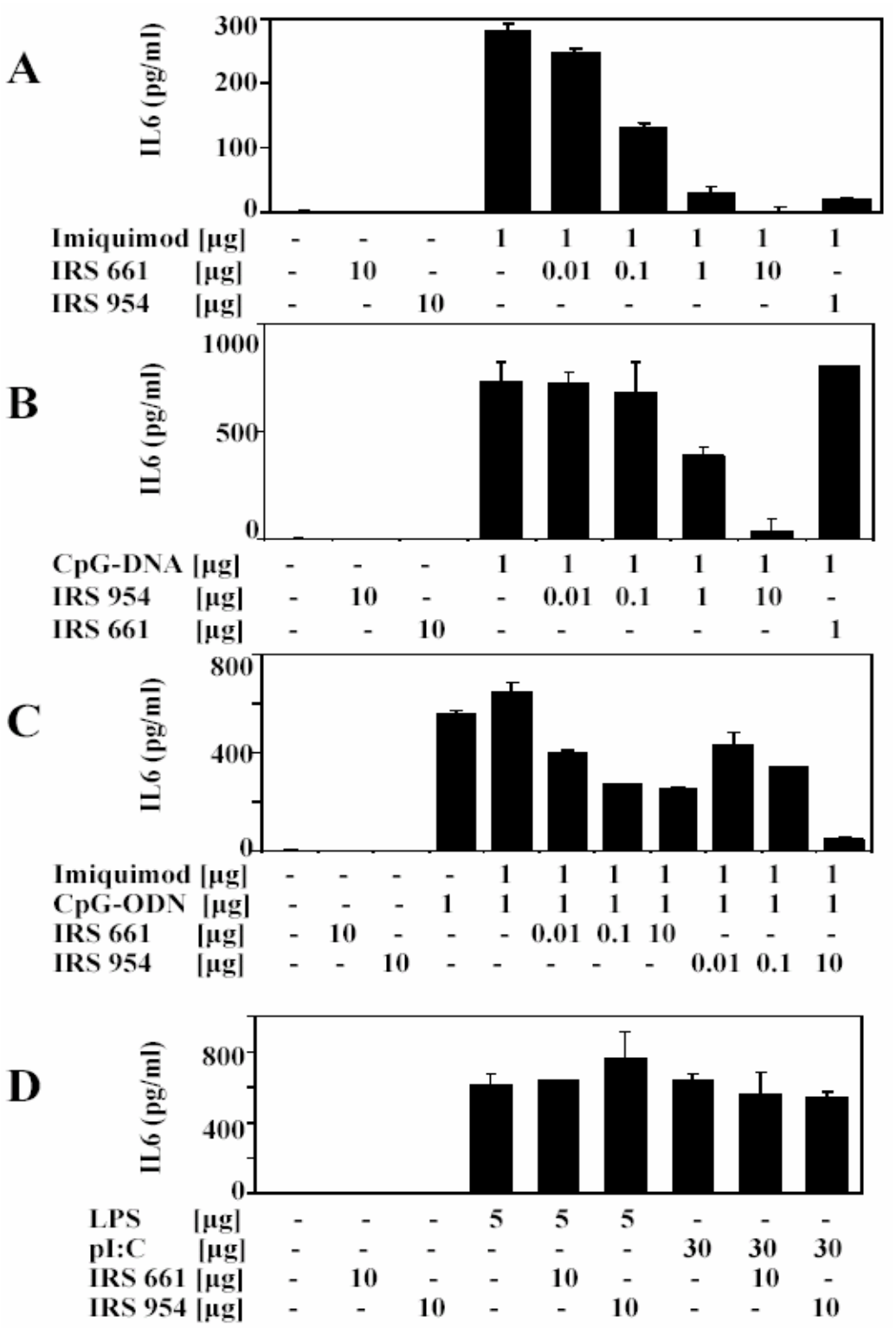


Figure 17. Renal histopathology in MRL*lpr/lpr* ***mice.*** *Renal sections of 10 weeks old MRLlpr/lpr mice from all groups were stained with antibodies for IgG_{2a} and Mac-2 (macrophages), PAS as indicated. Glomeruli are encircled. Images are representative for 5 mice in each group (original magnification x400).*

4.3. Results Part 3

4.3.1. Tlr7 blockade with IRS661 inhibits the imiquimod-induced release of IL-6 and TNF-α; and Tlr7/Tlr9 blockade with IRS954 inhibits imiquimod- & CpG-ODN-induced release of IL-6 & TNF-α in spleen monocytes from MRLlpr/lpr mice

Oligodeoxynucleotides with immunoregulatory sequences (IRS) specifically block Tlr7- (IRS661) or Tlr7/Tlr9-mediated interferon-α production (IRS954) *in vitro* (Barrat *et al*, 2005). To ascertain the rational to use these IRS in MRL*lpr/lpr* mice, spleen monocytes from MRL*lpr/lpr* mice were stimulated with respective Tlr7 or Tlr9 ligands, i.e. imiquimod or CpG-ODN-1668. IRS661 dose-dependently blocked imiquimod-induced production of IL-6 and TNF-α (Figure 18A, 19A). IRS954 but not IRS661 dose-dependently inhibited CpG-DNA-induced production of IL-6 and TNF-α (Figure 18B, 19B). IRS954, but not IRS661, showed significant dose dependent inhibition of IL-6 and TNF-α production when the cells were incubated with imiquimod plus CpG-DNA (Figure 18C, 19C). By contrast, neither IRS affected IL-6 or TNF-α production induced by LPS or pI:C RNA (Figure 18D, 19D). The same set of experiments were repeated for the two control oligos. Both the control oligos did not show any inhibition of the IL-6 and TNF-α release (Figure 18E-H, Figure 19E-H). These data confirms that IRS661 and IRS954 act as specific antagonists for either Tlr7- or Tlr7/Tlr9-induced activation of monocytes *in vitro.*


A
IL6 (pg/ml)
300
200
100
0
Imiquimod [µg] - - - 1 1 1 1 1 1
IRS 661 [µg] - 10 - - 0.01 0.1 1 10 -
IRS 954 [µg] - - 10 - - - - - 1
B
IL6 (pg/ml)
1000
500
0
CpG-DNA [µg] - - - 1 1 1 1 1 1
IRS 954 [µg] - 10 - - 0.01 0.1 1 10 -
IRS 661 [µg] - - 10 - - - - - 1
C
IL6 (pg/ml)
800
400
0
Imiquimod [µg] - - - - 1 1 1 1 1 1 1
CpG-ODN [µg] - - - 1 1 1 1 1 1 1 1
IRS 661 [µg] - 10 - - - 0.01 0.1 10 - - -
IRS 954 [µg] - - 10 - - - - - 0.01 0.1 10
D
IL6 (pg/ml)
800
400
0
LPS [µg] - - - 5 5 5 - - -
pI:C [µg] - - - - - - 30 30 30
IRS 661 [µg] - 10 - - 10 - - 10 -
IRS 954 [µg] - - 10 - - 10 - - 10

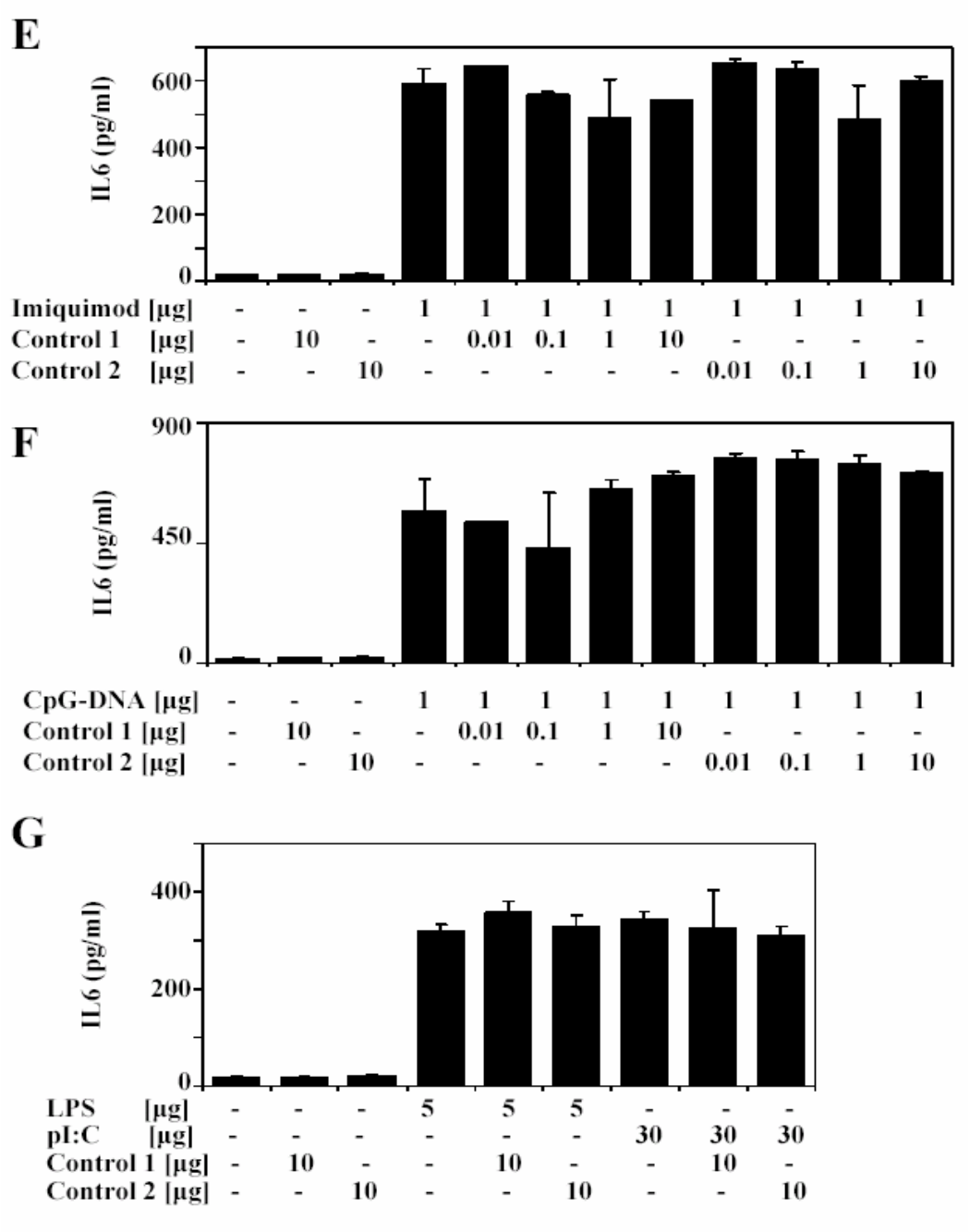
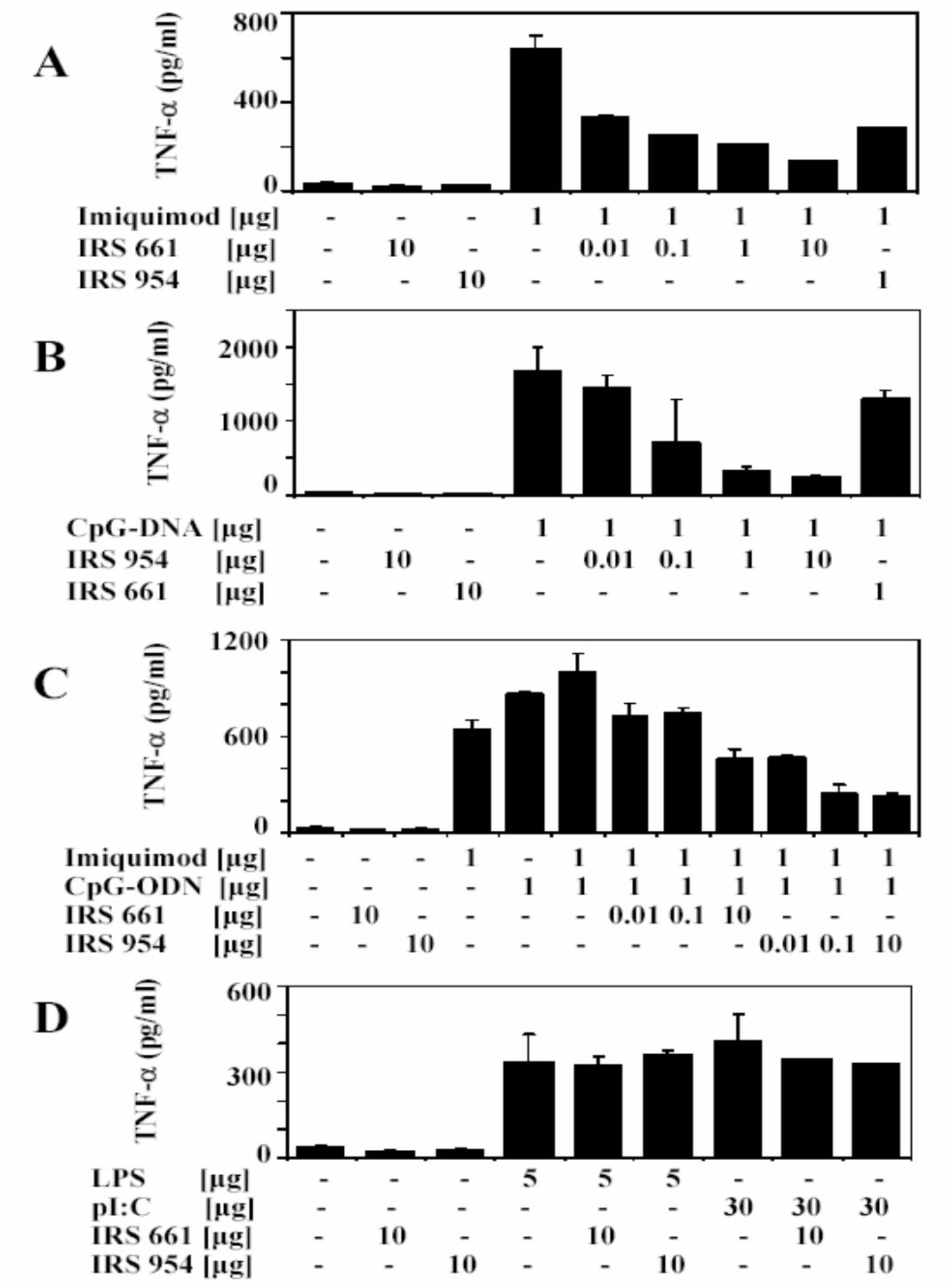


Figure 18. IRS661 and IRS954 specifically block specific TLR ligand induced IL-6 release in spleen monocytes from MRLlpr/lpr mice in vitro. *Spleen monocytes from MRLlpr/lpr mice were incubated with imiquimod and IRS661 at different concentrations as indicated or standard medium without supplements for 24 hours as indicated. A-C: Spleen monocytes were incubated with either imiquimod or CpG-DNA alone or imiquimod plus CpG-DNA. D: Cells were stimulated with either ultrapure LPS or pIC-*

*RNA. In A-D IL-6 production was measured in supernatants by ELISA. Results shown are mean ± SEM from two identical experiments. * Indicates $p < 0.05$ vs. imiquimod or CpG stimulation, by one-way ANOVA followed by post hoc Bonferroni's test.*


A
TNF-α (pg/ml)
800
400
0
Imiquimod [µg] - - - 1 1 1 1 1 1
IRS 661 [µg] - 10 - - 0.01 0.1 1 10 -
IRS 954 [µg] - - 10 - - - - - 1
B
TNF-α (pg/ml)
2000
1000
0
CpG-DNA [µg] - - - 1 1 1 1 1 1
IRS 954 [µg] - 10 - - 0.01 0.1 1 10 -
IRS 661 [µg] - - 10 - - - - - 1
C
TNF-α (pg/ml)
1200
600
0
Imiquimod [µg] - - - 1 - 1 1 1 1 1 1 1
CpG-ODN [µg] - - - - 1 1 1 1 1 1 1 1
IRS 661 [µg] - 10 - - - - 0.01 0.1 10 - - -
IRS 954 [µg] - - 10 - - - - - - 0.01 0.1 10
D
TNF-α (pg/ml)
600
300
0
LPS [µg] - - - 5 5 5 - - -
pI:C [µg] - - - - - - 30 30 30
IRS 661 [µg] - 10 - - 10 - - 10 -
IRS 954 [µg] - - 10 - - 10 - - 10

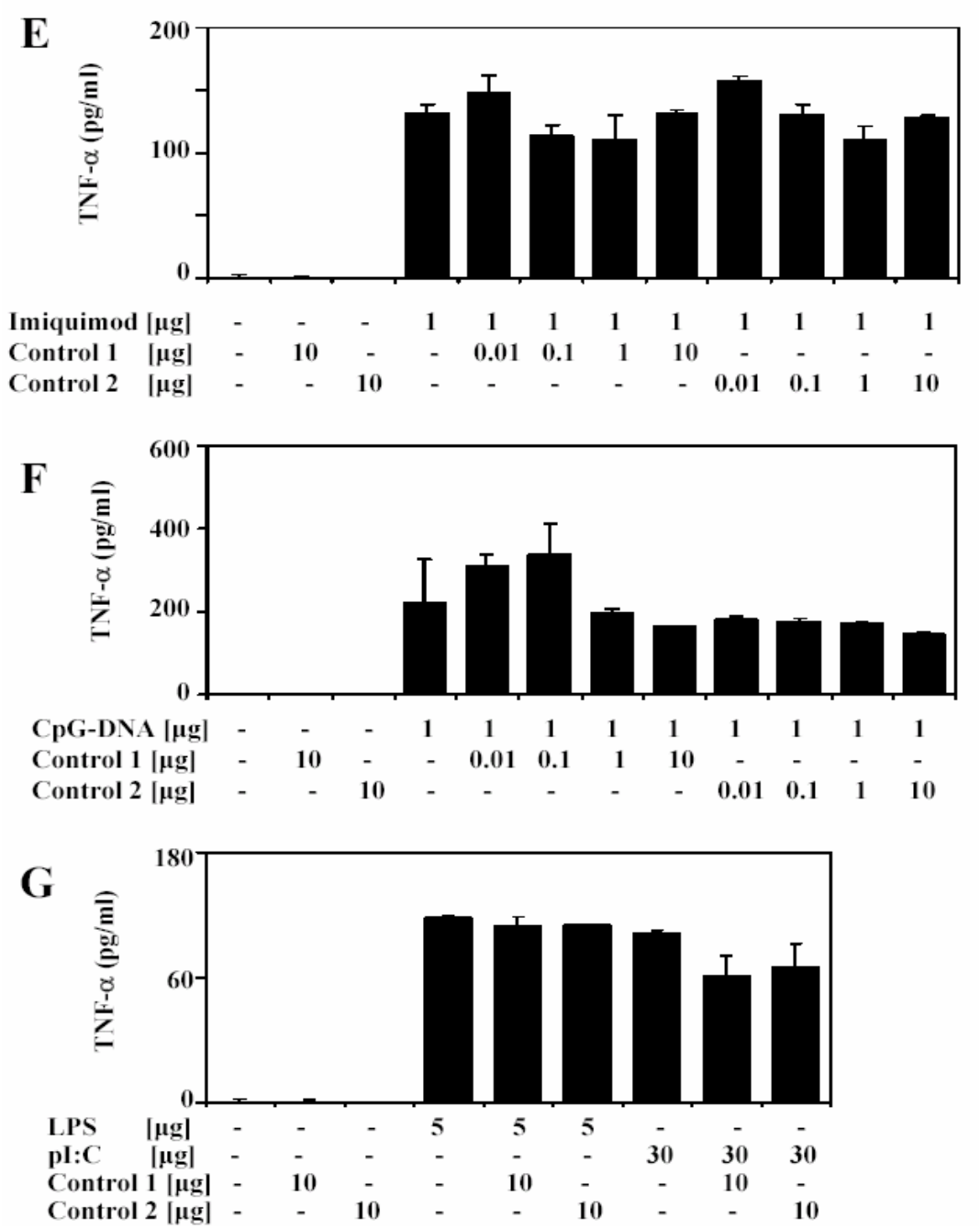


Figure 19. IRS661 and IRS954 specifically inhibits imiquimod- and CpG-DNA-induced TNF-α release in spleen monocytes from MRLlpr/lpr mice in vitro. *Spleen monocytes from MRL**lpr/lpr** mice were incubated with imiquimod and IRS661 at different concentrations as indicated or standard medium without supplements for 24 hours as indicated. A-C: Spleen monocytes were incubated with either imiquimod or CpG-DNA alone or imiquimod plus CpG-DNA. D: Cells were stimulated with either ultrapure LPS*

*or pIC-RNA. In A-D TNF-α production was measured in supernatants by ELISA. Results shown are mean ± SEM from two identical experiments. * indicates $p < 0.05$ vs. imiquimod or CpG stimulation, by one-way ANOVA followed by post hoc Bonferroni's test.*

4.3.2. Inhibitory oligos block imiquimod- or CpG-ODN-induced IL-12p40 serum levels in MRLlpr/lpr mice

In view of the *in vitro* efficacy of IRS661 and IRS954 to block imiquimod- or CpG-DNA-induced cytokine production in spleen monocytes, it was questioned whether these effects do also apply to MRL-*lpr/lpr* mice *in vivo*. Five groups of 14 week old MRL-*lpr/lpr* mice (n=5-8) were injected intraperitoneally with a single dose of either saline, 25 µg imiquimod or 40 µg CpG-DNA in the presence or absence of a single dose of IRS661 or IRS954 (40 µg) 30 min before the administration of the agonist. Serum IL-12p40 levels were determined after 3 hours of agonist administration by ELISA. Imiquimod and CpG-DNA significantly induced the serum levels of IL-12p40 as compared to saline-injected control mice (Figure 20). IRS661 and IRS954 both significantly reduced the imiquimod-induced induction of IL-12p40. IRS954 significantly reduced the CpG-DNA-induced induction of IL-12p40. Thus, 40 µg of IRS661 and IRS954 was observed to block Tlr7 or Tlr9 mediated effect *in vivo*.

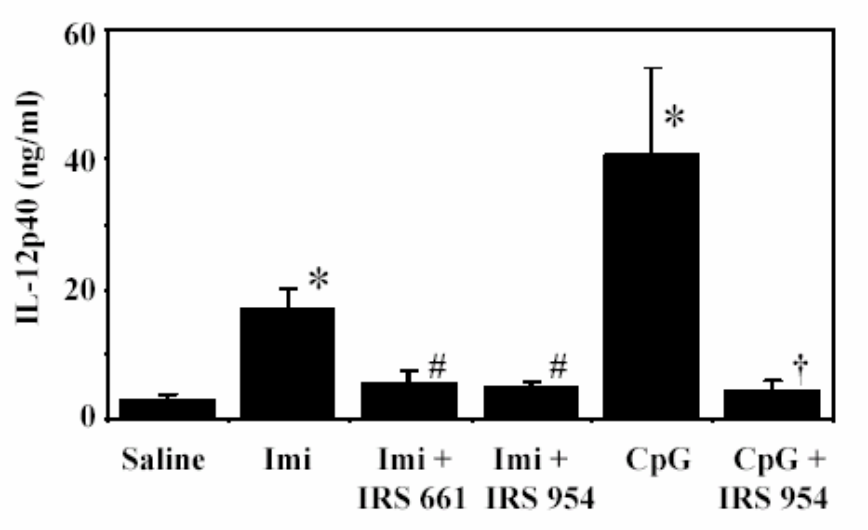


Figure 20. IRS661 and IRS954 block imiquimod- and CpG-DNA-induced IL-12p40 production in vivo. *Groups of 14 week old female MRL$^{lpr/lpr}$ mice (n=5) were injected with saline or a single dose of 25 µg imiquimod (imi) or 40 µg CpG-DNA (CpG) or 40 µg IRS as indicated. Serum IL-12p40 levels were determined after 3 hours by ELISA. Data represent means ± SEM. * $p < 0.05$ vs. saline, # $p < 0.05$ vs. imiquimod, † $p < 0.05$ vs. CpG-DNA.*

4.3.3. Serum cytokine levels of MRLlpr/lpr mice injected with Tlr7 and Tlr7/Tlr9 inhibitory oligos from week 11 to 24

It is known that Tlr7 and Tlr9 signaling activates the expression of proinflammatory cytokines and type I interferons (Akira *et al*, 2006). Hence, it was assessed whether injections with IRS661 or IRS954 from week 11 to 24 of age affected the serum levels of these mediators in MRL*lpr/lpr* mice by ELISA. We observed that, both IRS661 and IRS954 significantly reduced the serum levels of TNF as compared to saline-injected MRL*lpr/lpr* mice (Table 9). IL-6 levels were also decreased with IRS954, the trend did not reach statistical significance for IRS661 (Table 9). Contrarily, serum IL-12p40 levels were found to be only reduced with IRS661 injections but not with IRS954. Interferon-α levels were not affected by either IRS oligo (Table 9).

Table 9. Serum factors and lymphoproliferation in 24 week old MRL lpr/lpr mice.

		Saline	IRS661	IRS954
Serum IL-6	[pg/ml]	90.3 ± 31.3	58.1 ± 16.8	26.9 ± 3.1*
TNF	[pg/ml]	23.9 ± 4.0	4.4 ± 2.3*	5.8 ± 4.4*
IL-12p40	[ng/ml]	5.3 ± 1.1	3.4 ± 0.7*	4.6 ± 0.7
IFN-α	[pg/ml]	35.4 ± 8.3	30.6 ± 7.5	37.5 ± 7.9
IgG_1	[mg/ml]	38.4 ± 4.7	29.4 ± 4.8	34.4 ± 4.0
IgG_{2a}	[mg/ml]	12.0 ± 1.3	9.1 ± 1.7	12.9 ± 1.1
IgG_{2b}	[mg/ml]	9.2 ± 2.1	6.1 ± 1.1	5.8 ± 1.4
IgG_3	[mg/ml]	3.2 ± 0.2	3.3 ± 0.3	2.9 ± 0.2
Anti-dsDNA IgG_1	[µg/ml]	15.0 ± 6.3	14.7 ± 6.2	34.2 ± 12.7
IgG_{2a}	[µg/ml]	38.0 ± 7.1	18.9 ± 2.7*	56.8 ± 12.9#
IgG_{2b}	[µg/ml]	46.3 ± 9.5	18.2 ± 2.3*	34.8 ± 7.2#
IgG_3	[µg/ml]	27.7 ± 3.7	21.8 ± 2.1	24.1 ± 2.2
Anti-Sm IgG	[µg/ml]	8.8 ± 3.4	4.4 ± 1.1*	10.2 ± 2.8#
Rheumatoid factor	OD (450 nm)	0.88 ± 0.14	0.86 ± 0.13	0.81 ± 0.13
Lymph node weight	[mg/g BW]	16.7 ± 1.7	8.5 ± 1.6*	9.1 ± 0.6*
Spleen weight	[mg/g BW]	8.9 ± 0.8	6.7 ± 0.5*	6.1 ± 0.4*
CD4+ cells	% spleen	18.1 ± 3.4	17.6 ± 4.8	19.4 ± 4.3
CD8+ cells	% spleen	8.7 ± 1.7	9.7 ± 2.7	9.0 ± 2.0
CD4-/CD8-	% spleen	62.3 ± 1.4	51.7 ± 3.4*	57.5 ± 1.5*

N = 10 per group, values are means ± SEM, * $p < 0.05$ IRS661 or IRS954 vs. saline, # $p < 0.05$ IRS954 vs. IRS661 by unpaired *t* test

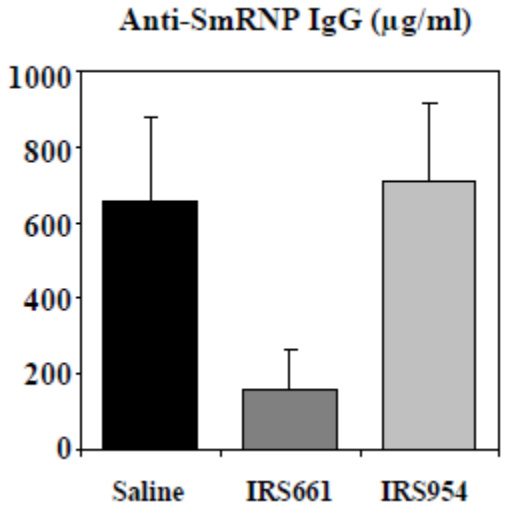


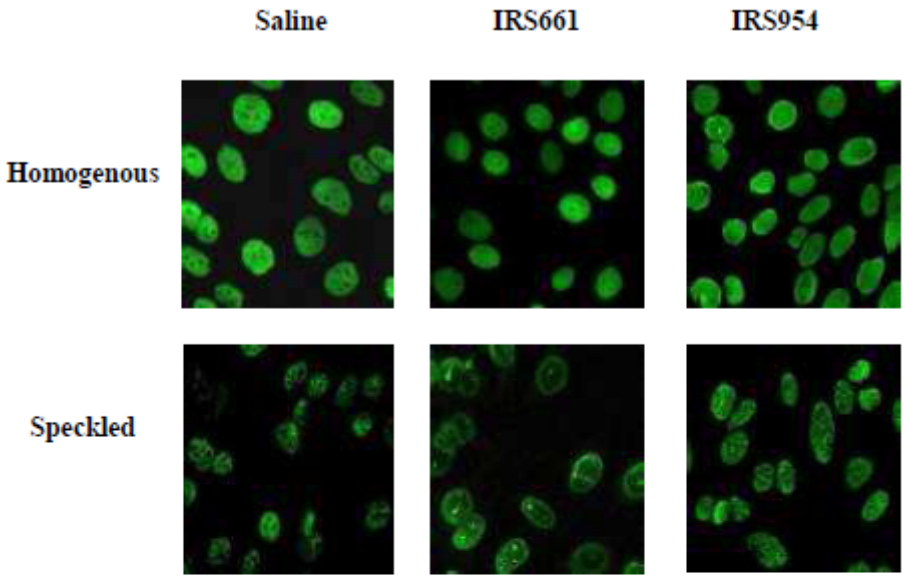
Figure 21. Serum Anti-SmRNP IgG levels in treated mice: *3 Groups of 24 weeks old female MRLlpr/lpr mice (n=10) were treated for 13 weeks (from week 11 to 24) with saline or IRS661 or IRS954. Serum anti-SmRNP IgG levels were determined by ELISA. Data represent mean ± SEM.*

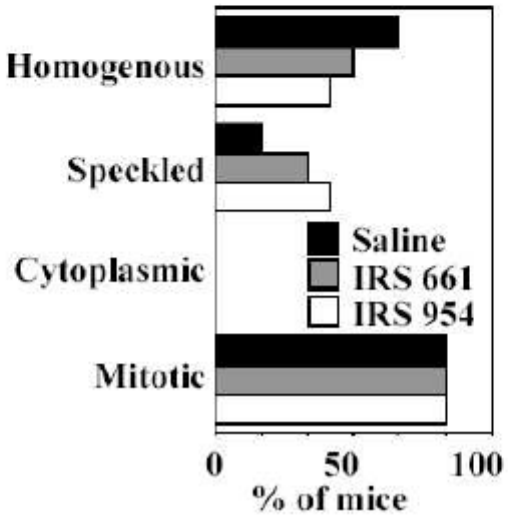
4.3.4. Tlr7 inhibitory oligo reduces serum autoantibody levels in MRLlpr/lpr mice

Tlr7 and Tlr9 were shown to have distinct effects on the production of antinuclear antibodies in MRL*lpr/lpr* mice (Christensen *et al*, 2006). Hence, it was assessed whether injections with IRS661 and IRS954 from week 11 to 24 of age affected serum IgG and autoantibody levels. Serum levels of IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 were not significantly affected by either IRS (Table 9). Antinuclear antibody staining on Hep2 cells revealed positive nuclear signals in each MRL*lpr/lpr* mouse of all groups (Figure 22A). In saline-treated MRL*lpr/lpr* mice a homogenous nuclear staining was most common. Positivity of condensed chromosomal areas of mitotic cells was indicative for autoantibodies directed against chromatin. A speckled nuclear staining pattern was less frequent and a cytoplasmic staining pattern was not observed. In IRS661-treated MRL*lpr/lpr* mice the

homogenous pattern was less frequent and speckled pattern was somewhat more frequent as compared to saline treated mice. Sera of IRS954 treated mice showed an equal distribution of homogenous and speckled nuclear staining patterns (Figure 22A). Antinuclear antibodies specific for dsDNA were studied by the specific binding to kinetoplast DNA of *Crithidia luciliae.* IRS661 but not IRS954 significantly reduced the indirect immunofluorescence intensity of mouse serum (at a dilution of 1:100) incubated with *Crithidia luciliae* substrates (Figure 22B). Assessing the binding of serum to polylysine-linked dsDNA by ELISA is another method to determine the specificity of antinuclear antibodies. IRS661, but not IRS954, significantly reduced serum levels of dsDNA-specific IgG_{2a} IgG_{2b}, and anti-Sm IgG antibodies as compared to saline-treated MRL*lpr/lpr* mice (Table 9). Anti-Sm IgG and anti-Sm RNP IgG were reduced upon IRS661 treatment to the same extent (Table 9, Figure 21). Anti-dsDNA IgG_1, and IgG_3 levels as well as rheumatoid factors were not affected by either IRS. These findings are suggestive of an inhibitory effect of IRS661 on the production of anti dsDNA IgG_{2a} and IgG_{2b} antibodies, which are known to induce glomerular pathology in MRL*lpr/lpr* mice by activating complement (Lucisano *et al*; Ehlers *et al*, 2006). Interestingly, glomerular deposits of IgG_{2a} and complement factor C3c were significantly reduced in both IRS661- and IRS954-injected MRL*lpr/lpr* mice (Figure 23, Table 10). Together these data indicate that injections with IRS661 but not with IRS954 reduce the production of selected autoantibodies. Glomerular complement activation was reduced with either IRS, independent of circulating IgG_{2a} isotype levels, suggesting that IRS can modulate immune complex deposition and complement activation independent of circulating autoantibody levels in MRL*lpr/lpr* mice.

A


Saline
IRS661
IRS954
Homogenous
Speckled


Homogenous
Speckled
Cytoplasmic
Mitotic
Saline
IRS 661
IRS 954
0
50
100
% of mice

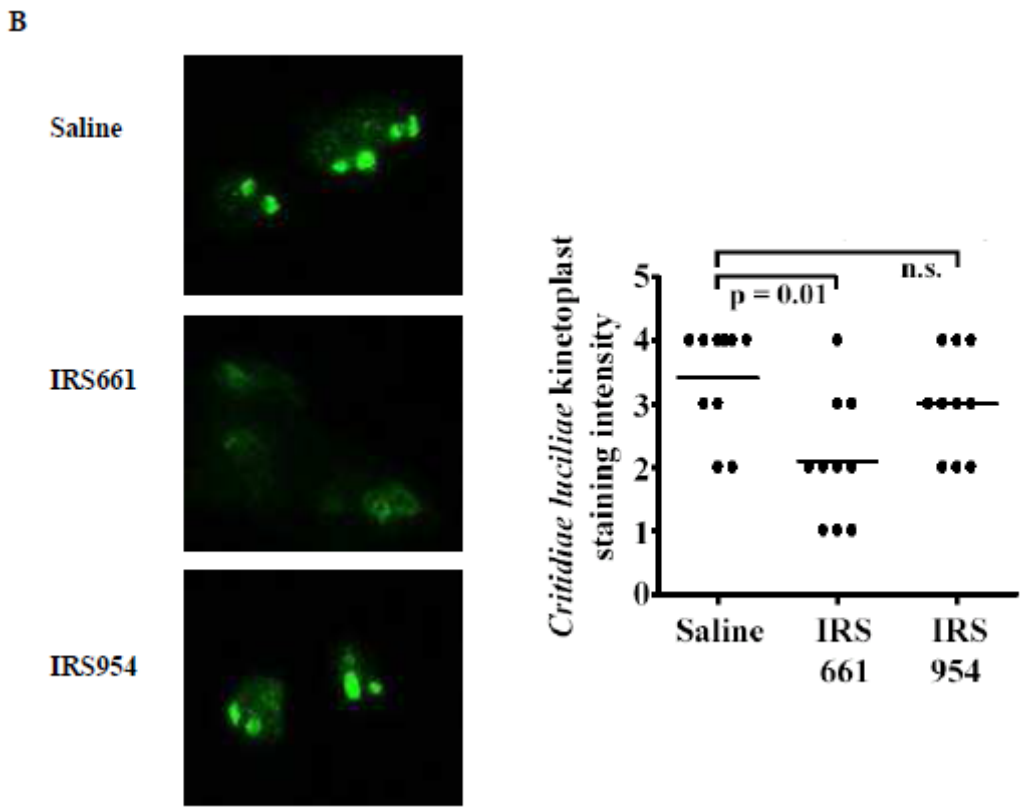


Figure 22. IRS661 and IRS954: Antinuclear antibodies and glomerular immune complex deposits in MRL*lpr/lpr* **mice.** *A: Serum antinuclear antibodies were detected by Hep2 slide staining as described in methods. Staining patterns were classified as homogenous, speckled or cytoplasmic. Positive staining of mitotic chromatin was assessed independently of the other staining patterns. Data shown on the right represent the staining pattern present in percent of mice of each group as indicated. B: Serum antibodies against Crithidia luciliae kinetoplast DNA were analysed as described in methods. Data represent staining intensity of sera from individual MRLlpr/lpr mice from each group. * p < 0.01 vs saline by two-tailed Mann Whitney U test.*

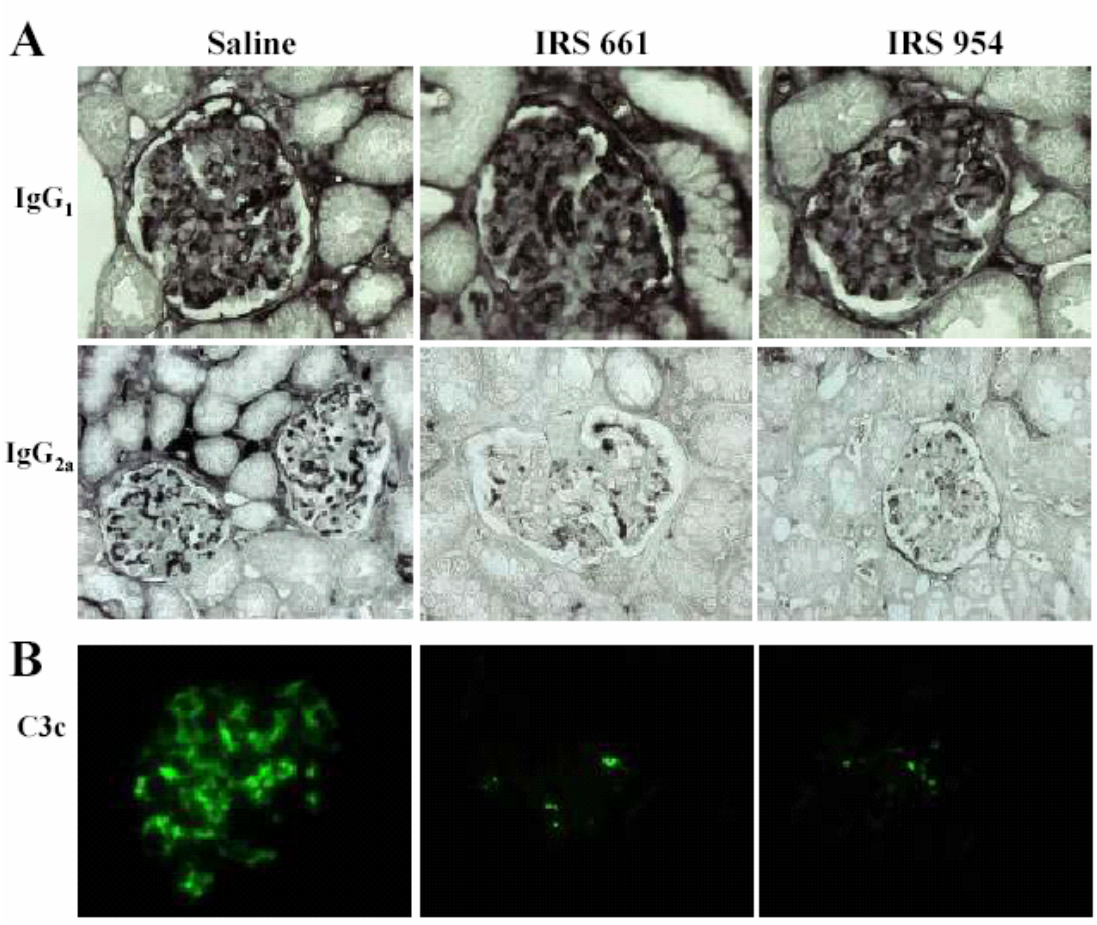


Figure 23. Glomerular immune complex and C3c deposits in MRLlpr/lpr mice. *Paraffin-embedded renal sections were stained for IgG_1 and IgG_{2a} as indicated (original magnification 400x). In saline-treated mice IgG_1 and IgG_{2a} localize to the mesangium and the glomerular capillary wall. Note that IRS661- and IRS954-treated MRLlpr/lpr mice show less IgG_{2a} deposits. Frozen renal sections were stained for complement factor C3c. Note the robust glomerular complement activation in saline-treated $MRL^{lpr/lpr}$ mice which was much lower detectable in IRS661- and IRS954-treated MRLlpr/lpr mice. Images are representative for 10 mice in each group (original magnification 400x).*

4.3.5. Tlr7 and Tlr9 inhibition reduce lymphoproliferation and protect MRL*lpr/lpr* mice against autoimmune tissue injury

Based on the reported phenotype of *Tlr7*-deficient MRL*lpr/lpr* mice (Christensen *et al*, 2006) and our own data with IRS661, a course of injections with IRS661 from week 11 to 24 of age might have beneficial effects on experimental lupus of MRL*lpr/lpr* mice. Compared to the phenotype of *Tlr9*-deficient MRL*lpr/lpr* mice (Christensen *et al*, 2006) the effects of IRS954 on experimental lupus were found to be quite opposite in this study. IRS661 and IRS954 significantly reduced the weight of spleens and mesenteric lymph nodes in MRL*lpr/lpr* mice as compared to saline-treated controls (Table 9). IRS661 and IRS954, also reduced the numbers of CD4/CD8 double negative CD3+ T cells in spleen while the numbers of spleen CD4+ cells and CD8+ cells remained unaffected (Table 9). These data indicate that both IRS reduce lymphoproliferation and the expansion of CD4/CD8 double negative 'autoreactive' T cells in MRL$^{lpr/lpr}$ mice. Both, IRS661 and IRS954, also significantly reduced the numbers of glomerular and interstitial Mac-2 macrophages and of interstitial CD3 lymphocytes (Table 10, Figure 25). Proteinuria is a clinically important marker of lupus nephritis but usually it shows a high interindividual variability in female MRL*lpr/lpr* mice. Thus, the trend to decreased proteinuria in IRS661- and IRS954-treated MRL*lpr/lpr* mice did not reach statistical significance. Furthermore, we evaluated extrarenal autoimmune tissue injury in lungs of MRL*lpr/lpr* mice. Lungs of IRS-treated MRL*lpr/lpr* mice showed less peribronchiolar and perivascular inflammatory cell infiltrates compared with saline-treated controls (Table 10, Figure 25). Together these data shows that IRS661 and IRS954 both reduce autoimmune tissue injury in lungs and kidneys of MRL*lpr/lpr* mice.

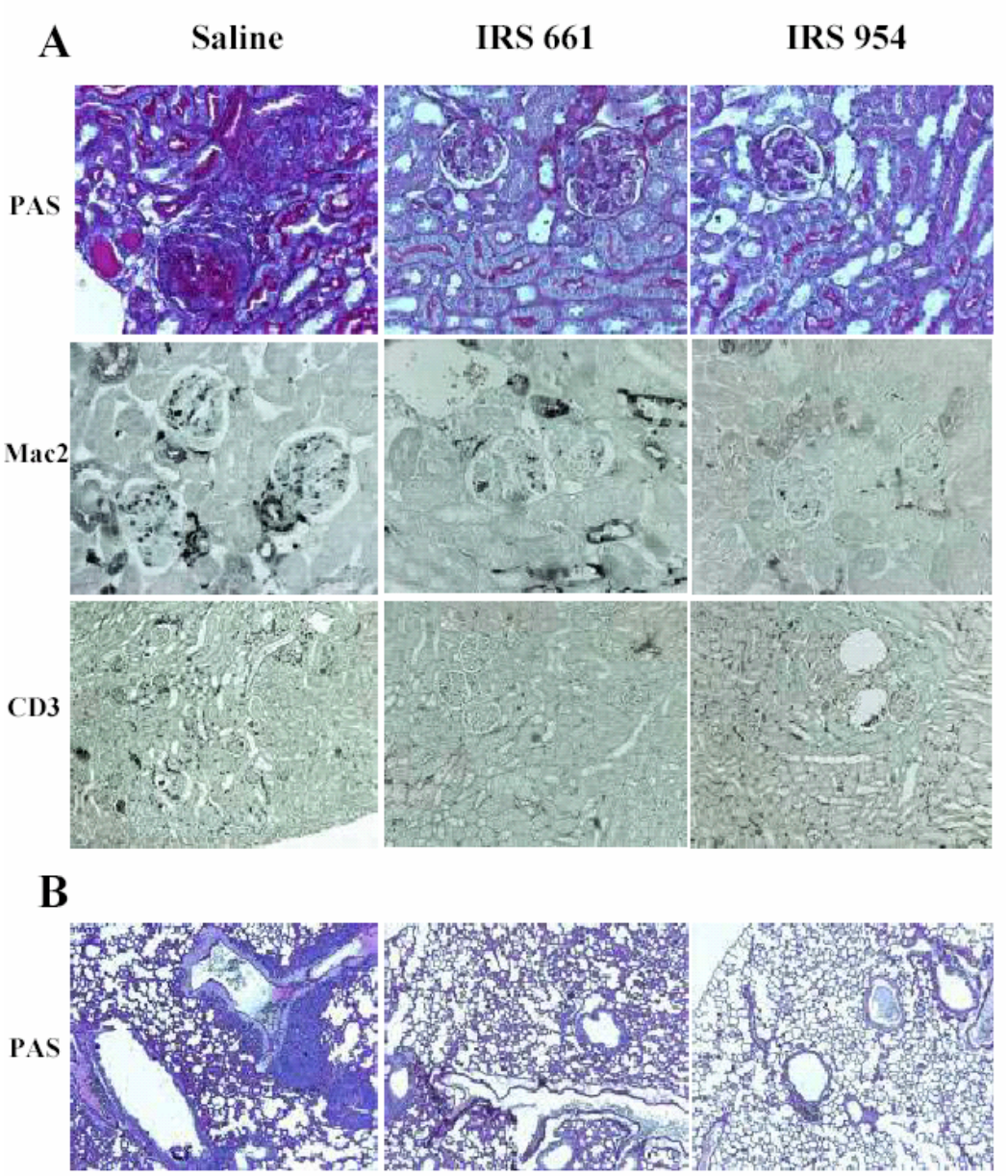


Figure 25. Lupus nephritis and lung injury in MRLlpr/lpr mice. *A. Renal sections were stained with PAS, Mac-2, and CD3 as indicated (original magnification 200x). Note that IRS661- and IRS954-treated MRLlpr/lpr mice show less periglomerular, and interstitial inflammatory cell infiltrates as compared to saline-treated MRLlpr/lpr mice. B: Lungs sections taken from 24 weeks old MRLlpr/lpr mice of all groups were stained with PAS. Note that IRS661- and IRS954-treated MRLlpr/lpr mice show less peribronchiolar and perivascular inflammatory cell infiltrates as compared to saline-injected MRLlpr/lpr mice. Images are representative for 10 mice in each group (original magnification 200x).*

Table 10. Urinary finding in 24 weeks old MLR *lpr/lpr* mice.

		Saline	IRS661	IRS954
U. albumin/ creat.	[mg/mg]	4.3 ± 1.6	1.7 ± 0.8	2.6 ± 0.7

N = 10 per group, values are mean ± SEM, Glom. = glomerular, Int. = interstitial,

* $p < 0.05$ IRS661 or 954 vs. saline, by unpaired *t* test

5. Discussion

5.1. Ligation of TLR7 did not lead to induction of lupus nephritis

In vitro studies suggest that the microbial nucleic acids can modulate autoimmunity through multiple mechanisms but their impact on the onset of lupus nephritis in MRL$^{lpr/lpr}$ mice at young age was not known (Lau *et al*, 2005; Leadbetter *et al*, 2002; Means *et al*, 2005, Pasare *et al*, 2003; Pasare *et al*, 2005, Rifkin *et al*, 2005, Krieg *et al*, 1995, Krieg *et al*, 2005). In this study young MRL*lpr/lpr* and MRL wild-type mice were used to study the effects of an intermittent exposure to Tlr7 ligand, imiquimod, on lymphoproliferation and autoantibody production in genetically predisposed and non-predisposed hosts. It was found that Imiquimod did not cause DNA autoantibody production or glomerulonephritis in MRL wild-type or MRL*lpr/lpr* mice. These data confirms that although Tlr7 is an important receptor for the pathogenesis of lupus but an exposure to the Tlr7 ligand at the given dose in young healthy age do not lead to induction of lupus in genetically predisposed and non-predisposed mice.

5.2. Ligation of TLR7 leads to significant aggravation of established lupus

Viral infections can aggravate disease activity in preexisting SLE, but the role of TLR7 ligation by viral ssRNA or it's synthetic mimic was not yet proven mechanistically in a mouse model of lupus nephritis. Hence in this project MRL*lpr/lpr* mice were used to study the effects of intermittent exposure to imiquimod, a synthetic Tlr7 ligand with immunostimulatory effects comparable to viral ssRNA (Heil et la, 2004; Hemmi *et al*, 2002). In this study we found evidence for an intracellular expression of Tlr7 on renal macrophages and dendritic cells in nephritic lesions of MRL*lpr/lpr* mice. Spleen

monocytes and bone marrow-derived dendritic cells produce proinflammatory cytokines, chemokines, and type I interferons upon ligation of Tlr7. The immunostimulatory effects of ssRNA relates to the cell type-specific expression pattern of Tlr7. These data may provide a new understanding of viral infection-induced exacerbation of lupus nephritis as well as other types of glomerulonephritis.

Antiviral host defense requires activation of innate immunity including the local production of type I interferons and chemokines. The finding that injected ssRNA localized to Tlr7 positive macrophages in kidneys of MRL*lpr/lpr* mice suggests a role for Tlr7 in activating renal macrophages in the kidney. Our studies with spleen monocytes isolated from MRL*lpr/lpr* mice favours Tlr7 activation and its effect on tissue macrophages contributing to the local production of proinflammatory mediators including Il-12, Il-6, Ccl2, Ifn-α which are known to contribute to the progression of lupus nephritis. As Tlr7 positive macrophages were mainly detected in the renal interstitium, we suggest that the aggravation of glomerular injury in imiquimod-treated MRL*lpr/lpr* mice could be secondary to increased glomerular immune complex deposition. Furthermore, a low number of CD11c positive dendritic cells were found to express Tlr7 in the kidney. The functional role of intrarenal CD11c positive dendritic cells for the progression of nephritis in MRL*lpr/lpr* mice was unclear. Our *in vitro* data with CD11c positive bone marrow-derived dendritic cells suggest that this cell type contributes to local production of proinflammatory mediators, i.e. Il-6, Il-12, Ccl2, and Ifn-α. For example, Il-6 produced by either renal macrophages or CD11c positive dendritic cells may suppress regulatory T cells that control autoreactive T cells and proliferation of T cells in total (Pasare *et al*, 2003). In the present study exposure to

imiquimod increased serum levels of the aforementioned mediators and aggravated autoimmune tissue injury in kidneys and lungs of the MRL*lpr/lpr* mice. This was associated with enhanced renal production of the chemokine Ccl2 and increased interstitial macrophage and T cell infiltrates. Based on similar effects of Tlr7, -3, and -9 ligands on cytokine and chemokine production by monocytes and dendritic cells, these data add to the concept of how nucleic-acid-specific Tlrs on antigen-presenting cells can modulate autoimmunity, e.g. lupus nephritis (Rifkin *et al*, 2005; Krieg *et al*, 2002).

Immunostaining for Tlr7 revealed that intrinsic renal cells do not express Tlr7. In addition, labelled ssRNA did not localize to intrinsic renal cells after injection. These observations were consistent with our findings in cultured mesangial cells, that did not express Tlr7 mRNA and did not respond to imiquimod. So far Tlr7 has not been reported to be expressed in any non-immune cell type (Nishimura *et al*, 2005). Thus, the expression of Tlr7 appears to be restricted to antigen-presenting cells in mice and humans (Hornung *et al*, 2002). These data indicate that imiquimod-induced aggravation of nephritis in MRL*lpr/lpr* mice is unrelated to direct activation of intrinsic renal cells.

Lupus is characterized by polyclonal autoantibody production and B cell proliferation, the latter expressing Tlr7 at intermediate levels (Hornung *et al*, 2002). Tlr7 ligation with repeated injections of imiquimod in MRL*lpr/lpr* mice, was associated with a trend towards increased serum dsDNA autoantibody levels, a significant increase in glomerular immune complex deposits and complement activation. The latter may explain, why imiquimod aggravated glomerular injury in MRL*lpr/lpr* mice despite having no effect on glomerular macrophage counts. The finding that imiquimod had only a moderate effect on autoantibody production is supported by a recent study, showing that B cells do not

respond to Tlr7 ligands unless their B cell sensitivity is enhanced due to IFN-α released by dendritic cells (Berkeredjian-Ding *et al*, 2005). Tlr7 ligands alone cannot induce B cell activation because interferon-producing dendritic cells control Tlr7 sensitivity of B cells. We assume that imiquimod had a moderate effect on B cell-dependent production of dsDNA autoantibodies in MRL*lpr/lpr* mice because B cells localize in close proximity to interferon-producing dendritic cells in lymphoid organs and imiquimod injection increased serum IFN-α levels in MRL*lpr/lpr* mice. It was found that, imiquimod and ssRNA induce IFN-α production in bone marrow-derived dendritic cells isolated from MRL*lpr/lpr* mice. Unlike CpG-Tlr9-mediated activation of B cells, Tlr7 ligation requires additional cofactors, e.g. IFN-α, in order to mount B cell activation and subsequent DNA autoantibody production. Thus, Tlr7 ligation has a moderate effect on autoantibody production in MRL*lpr/lpr* mice.

In this study it was found that the Tlr7 could activate B cells only in the presence of additional cofactors, which result in an intermediate increase of autoantibody production and glomerular immune complex deposition. In addition, Tlr7 induce dendritic cell maturation towards an antigen-presenting phenotype as well as production of proinflammatory cytokines, chemokines, and type I interferons by renal macrophages. These results also suggest that endogenous TLR7 ligand plays a role of adjuvant rather than a causative factor for aggravation of autoimmunity. However the role of TLR7 or endogenous TLR7 ligands such as smRNP, in breaking the tolerance checkpoints which leads to relentless growth of self reactive B and T lymphocytes (Goodnow, 2007) is not yet known. It is suggested that multiple genetic mutations might be needed before self-reactive clone bypasses sequential tolerance checkpoints resulting in emergence of

autoimmune disease (Goodnow, 2007). Hence it might be possible that TLR7 plays a role of amplification factor in lupus.

In summary, these results contribute to the understanding of the broad clinical spectrum and universal serum markers of infection-associated disease activity of lupus nephritis and possibly other types of immune complex glomerulonephritis.

5.3. Blockade of TLR7 or double blockade of TLR7 and TLR9 in lupus nephritis is protective

Nuclear lupus autoantigens trigger autoantibody production and the release of proinflammatory mediators by activating Tlr7 and Tlr9 in B cells and dendritic cells *in vitro* (Leadbetter *et al*, 2002, Lau *et al*, 2005; Means *et al*, 2005; Barrat *et al*, 2005; Lovgren *et al*, 2004; Savarese *et al*, 2006; Vollmer *et al*, 2005). Blocking Tlr9 signaling with injections of specific oligodeoxynucleotides could ameliorate lupus nephritis in MRL*lpr/lpr* mice (Patole *et al*, 2005; Dong *et al*, 2005), hence we hypothesized that antagonism of Tlr7 or Tlr7 plus Tlr9 may be effective *in vivo*.

Injections with TLR7 antagonist IRS661 were started from week 11 of age till 24 weeks. This could substantially reduced autoimmune tissue lung and kidney injury at week 24 of age which is also consistent with the phenotype of *Tlr7*-deficient MRL*lpr/lpr* mice (Christensen *et al*, 2006). These data support a role of Tlr7 for the mechanisms that foster the progression of autoimmune tissue injury in MRL*lpr/lpr* mice, e.g. the expansion of the autoreactive CD4/CD8 double negative autoreactive T cell population, immune complex-mediated local complement activation, and the local inflammatory response involving macrophage and T cell recruitment (Kotzin *et al*, 1996; Lipsky *et al*, 2001;

Singh *et al*, 2006). TLR7 antagonist, IRS661 significantly reduced the number of CD4/CD8 double negative T cells in spleen, a population which continuously expands in MRL*lpr/lpr*, because of the inability to delete autoreactive and activated T cells in mice via the interaction of Fas with the Fas-ligand in these mice (Cohen *et al*, 1991). Autoantibody production is another characteristic feature of MRL*lpr/lpr* mice and contributes to autoimmune tissue injury via immune complex formation and local complement activation. Interestingly, Tlr7 is particularly required to generate anti-Sm RNP IgG (Christensen *et al*, 2006) and serum levels of anti-Sm RNP IgG were also reduced with injection of IRS661. Furthermore, we found that IRS661 reduced the serum levels of anti-dsDNA IgG_{2a} and IgG_{2b}. We also observed that, the glomerular deposits of IgG_{2a} and complement factor C3c were both reduced in IRS661-treated mice, indicating a role for Tlr7 in the production of selected nephritogenic autoantibodies and immune complex-glomerulonephritis in MRL*lpr/lpr* mice. In this study we used identical assay systems as reported by Christensen *et al*, and we found that the data from both the labs consistently show that blockade or lack of Tlr7 does not substantially reduce homogenous nuclear and mitotic ANA staining of Hep2 cells (Christensen *et al*, 2006). In reports from same lab (Christensen *et al*, 2005 and 2006), 20-30 % of lpr mice showed speckled nuclear pattern indicating the presence of anti-SmRNP, autoantibodies which was confirmed by western blots for Sm binding activity, which supports our results that inhibition of Tlr7 lupus mice leads to decrease in levels of anti sm-RNP or anti-Sm antibodies. Our data is consistent and supported by results from other groups recently (Christensen *et al*, 2006) which showed that Tlr7 deficient lpr mice produced anti-DNA/anti-chromatin autoantibodies but not anti Sm-RNP antibodies. The Tlr7 deficient

lpr mice also have lower IgG2a and IgG titres than their Tlr-sufficient wild-type littermates as well as reduced numbers of activated T cells and PDCs (nevertheless, renal disease was decreased only moderately) (Christensen *et al*, 2006). Another study also showed similar results with lpr mice deficient in MyD88 failed to produce both DNA and Sm-reactive antibodies (Lau *et al*, 2005). Tlr7 also appears to play a role in activation of B cells that express transgene encoded 564 Igi B cells spontaneously produce antibodies that give cytoplasmic Hep-2 staining pattern and form ICs that deposit in kidney. Tlr7 deficient 564Igi B cells no longer spontaneously produce 564 Igi antibodies (Berland *et al*, 2006).

Together, the beneficial effects of Tlr7 blockade with IRS661 on autoimmune tissue injury of MRL*lpr/lpr* mice were associated with a reduction of CD4/CD8 double negative T cells, reduction in levels of pathogenic autoantibodies, and reduction in levels of proinflammatory chemokines in kidney, known to mediate immune cell recruitment into the tissue.

When we were performing these studies in our lab, some new results were published about Yaa mice, as reported by Subramanian *et al* (2006) and Pisitkun *et al* (2006), transgenic models with autoimmune accelerator on Y chromosome. The Yaa loci consisting of Tlr7 and 16 other genes from X chromosome which were duplicated on Y chromosome in these mice. Another study had reported Tlr7 transgene founder lines in transgenic BL6 mice, which were showed to have Tlr7-gene dosage dependent disease severity of lupus in such mice and Tlr7 gene dependent increase in RNA antibodies and increased nucleolar and speckled nucleolar patterns by such mice sera in Hep-2 slides (Deane *et al*, 2007). It also had severe effects on survival of these mice dependent on Tlr7

gene dosage compared to WT-BL6 mice. Altogether the results suggest that Tlr7 is responsible in progression and development of lupus.

Given the opposing effects of *Tlr7* and *Tlr9* deficiency in MRL*lpr/lpr* mice (Christensen *et al*, 2006; Wu *et al*, 2006) the effects of Tlr7/Tlr9 dual antagonist IRS954 were quite different. Injections with IRS954 from week 11 to 24 of age improved kidney and lung disease in MRL*lpr/lpr* mice and no additive effects were observed as compared to IRS661. The effects of IRS954 on lupus nephritis and lung injury are comparable to what has been observed with oligonucleotide antagonists specific for Tlr9 only in the same lupus model (Patole *et al*, 2005) or in NZB/NZW mice (Dong *et al*, 2005). These findings support the concept that recognition of endogenous DNA and RNA molecules via Tlr7 and Tlr9 contribute to the progression of autoimmune tissue injury. This concept was developed from studies showing that lupus autoantigens in immune complexes with IgG prepared from autoimmune mice or lupus patients can activate B cells and dendritic cells *in vitro* (Leadbetter *et al*, 2002; Lau *et al*, 2005; Means *et al*, 2005; Savarese, 2006; Vollmer *et al*, 2005). These *in vitro* experiments and *in vivo* studies with Tlr9 antagonists are inconsistent with the aggravated phenotype of *Tlr9*-deficient MRL*lpr/lpr* mice, and have questioned the specificity of the antagonists and the assay systems used for the analysis of serum autoantibodies (Christensen *et al*, 2006; Lartigue *et al*, 2006; Wu *et al*, 2006, Yu *et al*, 2006; Marshak Rothstein *et al*, 2006). Similar to our results, other groups have found out that Tlr9 deficient FcγRIIB deficient 56R mice have decreased IgG2a and IgG2b titers (Ehlers *et al*, 2006).

The data reported by Barrat, *et al*, and our own study have demonstrated the specificity of IRS954 for Tlr7 and Tlr9 (Barrat *et al*, 2005). Furthermore, by applying identical assay

systems as in the study reported by Christensen *et al*, we show that IRS954 has distinct effects on serum autoantibody levels as compared to IRS661 (Christensen *et al*, 2006). For example, in contrast to IRS661, IRS954 did not affect serum dsDNA autoantibodies, and may be Tlr9 antagonism provided by IRS954 'neutralized' the suppressive effect of on Tlr7-mediated anti-dsDNA IgG_{2a}, IgG_{2b}, anti-Sm and anti-Sm-RNP IgG production. Furthermore, IRS954 injections were associated with fewer mice with homogenous nuclear staining on Hep2 cells which was consistent with what has been observed in *Tlr9*-deficient $MRL^{lpr/lpr}$ mice (Christensen *et al*, 2006). The finding that IRS954 did not reduce dsDNA autoantibodies is potentially interesting because we previously found that another oligo ODN2114, blocking Tlr9 only, reduced these antibodies in $MRL^{lpr/lpr}$ mice (Patole *et al*, 2005). These reports suggests that the roles of Tlr7 and Tlr9 for the evolution of specific autoantibodies may even be more complex and requires a detailed analysis of immune cell subsets from Tlr7 and Tlr9 double-knock out cells which are not yet available. Our results were also supported by the results from another group about TLR7 and TLR9 dual inhibition and amelioration of disease symptoms in lupus mice (Barrat *et al*, 2007). Recent studies published about 5 separate patient cohorts have identified atleast two SNPs associated with IRF as high risk factor for SLE (Sigurdsson *et al*, 2005 and 2007; Graham *et al*, 2007), and since activation of Tlr7 and Tlr9 mediates the Type 1 IFN production via IRF5 and IRF7, role of these 2 receptors in pathogenesis of lupus has become the major contributing factor.

In summary these data suggest that delayed onset of oligonucleotide-based inhibition of Tlr7 reduces autoantibody production and prevents autoimmune tissue injury in experimental lupus. Combined blockade of Tlr7 and Tlr9 has no additive effects. This

data support the concept that endogenous ligands of Tlr7 contribute to the pathogenesis of autoantibody production and autoimmune tissue injury in SLE and propose Tlr7 blockade as a novel therapeutic target for lupus.

6. ZUSAMMENFASSUNG

Die vorliegende Studie trägt zum besseren Verständnis des breiten klinischen Spektrums und des Fehlens universeller Serum- Marker der infektionsassoziierten Krankheitsaktivität der Lupus- Nephritis und möglicherweise anderer Formen der Immunkomplexnephritis bei.

Exogener Kontakt mit TLR 7- Liganden hat die Entwicklung der Lupus- Nephritis bei jungen, gesunden MRL- Wildtyp und MRL*lpr/lpr* Mäusen nicht getriggert und hatte keinen signifikanten Effekt auf die Krankheitsaktivität bei diesen jungen Mäusen.

Bei alten Lupus- Mäusen führte eine ähnliche Exposition jedoch zu einem merklichen Anstieg der Serumspiegel proinflammatorischer Zytokine und von IFNα, sowie zu einer Infiltration der Nierenglomeruli 18 Wochen alter MRL*lpr/lpr* Mäuse mit Makrophagen und einer (nicht signifikanten) Erhöhung der Autoantikörperspiegel. Diese Daten unterstützen die Theorie, dass dem Toll- like Rezeptor 7 eine Rolle bei den Mechanismen, die das Fortschreiten der autoimmunen Gewebsschädigung in MRL*lpr/lpr* Mäusen fördern, zukommt.

Basierend auf den Ergebnissen der funktionellen Rolle von TLR 7 in Lupus- Mäusen und in Primärzellen, die aus Lupusmäusen isoliert wurden, sahen wir in der TLR 7- Blockade ein mögliches neues Ziel, um die schädlichen Effekte des Signalings über Immunkomplexe und endogene Liganden zu begrenzen.

Es zeigte sich, dass die Blockade von TLR 7 und TLR 7 + TLR 9 die Gewebsschäden in Nieren und Lunge signifikant reduzieren konnte. Eine TLR 7 Antagonisierung mit dem Oligodeoxyribonukleotid IRS661 senkte die Menge an Autoantikörpern (insbesondere anti- SM, anti- dsDNA, IgG_{2a}, IgG_{2b}), entzündlichen Zytokine und Chemokinen im

Serum, glomerulären Ablagerungen von IgG_{2a} und Komplementfaktor C3c deutlich. Die Hemmung von TLR 7 reduzierte ebenfalls die CC- Chemokin- gesteuerten Makrophagen- und T- Zell- Infiltrate in den Nieren. Dies zeigte sich durch erniedrigte Spiegel von Ccr2, Ccr5, Ccl2 und Ccl5 in den Nieren behandelter Tiere.

Diese Ergebnisse unterstützen das Konzept, dass endogene TLR 7- Liganden zur Pathogenese der Autoantikörperproduktion und der autoimmun vermittelten Gewebsschädigung des SLE beitragen. Die TLR 7- Blockade könnte ein neues therapeutisches Konzept beim Lupus erythematodes sein.

6. Conclusion

In summary, exogenous administration of Tlr7 ligand in young, healthy MRL-wild type and MRL*lpr/lpr* mice did not trigger the development of lupus nephritis, and did not have any significant effect on disease activity. Upon similar exposure in well established lupus produced marked increase in serum levels of proinflammatory cytokines and IFNα, infiltration of macrophages in glomeruli of kidneys of 18 weeks old MRL*lpr/lpr* mice and trend towards increased autoantibodies level. These data support a role of Tlr7 for the mechanisms that foster the progression of autoimmune tissue injury in MRL*lpr/lpr* mice. Hence these studies might suggest that TLR7 is an adjuvant amplification factor rather than a causative factor for autoimmunity.

Based on the results of functional role of Tlr7 in lupus mice and the primary cells isolated from the lupus mice suggested a new potential target of blocking Tlr7 to restrict the harmful effects of its downstream signalling via the immune-complexes and endogenous ligands. The blockade of Tlr7 and dual blockade of Tlr7 & Tlr9 resulted in efficient and

significant reduction of tissue injury in kidneys and lungs of lupus mice. Tlr7 antagonism with the oligodeoxyribonucleotide IRS661, significantly reduced the levels of autoantibodies (specifically anti-Sm, anti-dsDNA IgG_{2a}, IgG_{2b}), inflammatory cytokines, chemokines in serum, the glomerular deposits of IgG_{2a} and complement factor C3c. Inhibition of Tlr7 also reduced CC-chemokine-driven macrophage and T cell infiltrates in kidneys as replicated by decreased levels of Ccr2 and Ccr5 and Ccl2 and Ccl5 in treated kidneys. These results support the concept that endogenous ligands of Tlr7 such as smRNP and RNA associated immune-complexes contribute to the pathogenesis of autoantibody production and autoimmune tissue injury in SLE and propose TLR7 blockade as a novel therapeutic target for lupus. Although the role of TLR7 in escape of B and T cells from the tolerance checkpoints to become self-reactive and loss of anergy needs to be studied in future.

7. References

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8. Curriculum vitae

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Education details

1. **(Sep. 2004- June 2008) Ph.D. Fellow**: Nephrological centre, Medical Policlinic, faculty of medicine, Ludwig Maximilians University, Munich, Germany (Registered for Ph.D. in human biology). **Principal investigator**: PD Dr. H. J. Anders, M.D., Med. Policlinic, LMU, Munich. Research project: "Characterization of the functional role of toll like receptor-7 (TLR7) in experimental lupus nephritis."
2. **(Jul 2000- Dec 2001) Master of science** (Pharmacology): with GPA of 7.36 on 10 point scale: Thesis research work on "Antinociceptive effects of adenosine A2a and A3 receptor agonists in rat model of diabetic neuropathic pain" under the guidance of Associate Prof. Dr. S. S. Sharma, Dept. of Pharmacology and Toxicology, NIPER, India.
3. **(1996-2000) Bachelor** of Pharmaceutical sciences was completed at Bombay College of Pharmacy, University of Mumbai, Mumbai (Bombay), India.
4. **(1994-1996) Diploma** of pharmacy was completed at Bharati Vidyapeeth's college of Pharmacy, University of Mumbai, India.

5. **(1992-1994)** Higher secondary certificate, University of Mumbai, India.
6. **(1983-1992)** Primary and secondary school, University of Mumbai, India

Present employment

Research associate (Sep.2008 –Till date)

Division of Clinical Immunology and Rheumatology, School of Medicine, University of Alabama at Birmingham, Alabama, US.

Principal investigator: Prof. Dr. John D. Mountz, M.D. Ph.D.

Additional Research Experience in R & D

(Sep. 2002-Aug 2004) As "Research Associate" in Division of Pharmacology, New Drug Discovery Research, Ranbaxy Research Laboratories Ltd., India. During this, worked on the "Standardizing of the in vivo and in vitro models for the screening of NCE's in Asthma and COPD models using various animal species". Safety Pharmacology studies.

Research publications

1. **Pawar RD**, Ramanjaneyulu R, Kulkarni OP, Lech M, Segerer S, Anders HJ. Inhibition of Toll-Like Receptor-7 (TLR-7) or TLR-7 plus TLR-9 Attenuates Glomerulonephritis and Lung Injury in Experimental Lupus. *J Am Soc Nephrol.* 2007 Jun;18(6):1721-31.
2. **Pawar RD**, Patole PS, Ellwart A, Lech M, Segerer S, Schlöndorff D, Anders HJ. Ligands to nucleic acid-specific toll-like receptors and the onset of lupus nephritis*. J Am Soc Nephrol.* 2006 (12) 3365-73.
3. **Pawar RD**, Patole PS, Zecher D, Segerer D, Kretzler M, Schlöndorff D, Segerer S, Anders HJ. Toll-like receptor-7 modulates immune complex glomerulonephritis. ***J Am Soc Nephrol****.* 2006 Jan; 17(1): 141-9.
4. **Pawar RD**, Castrezana-Lopez L, Allam R, Kulkarni OP, Segerer S, Radomska E, Meyer TN, Schwesinger CM, Akis N, Gröne HJ, Anders HJ. Bacterial lipopeptide triggers massive albuminuria in murine lupus nephritis by activating Toll-like receptor 2 at the glomerular filtration barrier. ***Immunology*** 2008 Oct 24. [Epub ahead of print].
5. Allam R, **Pawar RD**, Kulkarni OP, Hornung V, Hartmann G, Segerer S, Akira S, Endres S, Anders HJ. Viral 5'-triphosphate RNA and non-CpG DNA aggravate autoimmunity and lupus nephritis via distinct TLR-independent immune responses.

Eur J Immunol. 2008 Dec;38(12):3487-98.

6. Kulkarni O, Eulberg D, Selve N, Zöllner S, Allam R, Pawar RD, Pfeiffer S, Segerer S, Klussmann S, Anders HJ. Anti-Ccl2 Spiegelmer permits 75% dose reduction of cyclophosphamide to control diffuse proliferative lupus nephritis and pneumonitis in MRL-Fas(lpr) mice. ***J Pharmacol Exp Ther***. 2009 Feb;328(2):371-7.
7. Kulkarni O, **Pawar RD,** Purschke W, Eulberg D, Selve N, Buchner K, Ninichuk V, Segerer S, Vielhauer V, Klussmann S, Anders HJ. Spiegelmer inhibition of CCL2/MCP-1 ameliorates lupus nephritis in MRL-(Fas)lpr mice. ***J Am Soc Nephrol.*** 2007 Aug;18(8):2350-8.
8. Emina Savarese, Christian Steinberg, **Rahul D. Pawar**, Wolfgang Reindl, Shizuo Akira, Hans-Joachim Anders, Anne Krug. TLR7-dependent anti-snRNP antibody production and glomerulonephritis in pristane-induced autoimmunity. ***Arthritis Rheum.*** 2008 Apr;58(4):1107-15.
9. Patole PS, **Pawar RD**, Lech M, Zecher D, Schmidt H, Segerer S, Ellwart A, Henger A, Kretzler M, Schlöndorff D, Anders HJ.. Expression and regulation of Toll-like receptors in lupus-like immune complex glomerulonephritis of MRL-Fas(lpr) mice. ***Nephrol Dial Transplant.*** 2006 Nov;21(11):3062-73.
10. Patole PS, **Pawar RD,** Lichtnekert J, Lech M, Kulkarni OP, Ramanjaneyulu R, Segerer S, Anders HJ. Coactivation of Toll-like receptor-3 and -7 in immune complex glomerulonephritis. ***J Autoimmun***. 2007 Aug;29(1):52-9.
11. Patole PS, Zecher D, **Pawar RD**, Gröne HJ, Schlöndorff D, Anders HJ. G-rich DNA suppresses systemic lupus. ***J Am Soc Nephrol*** 2005 Nov;16(11):3273-80.

Abstracts and conference proceedings

1. **Rahul D. Pawar** and Hans-Joachim Anders. Activation of TLR2 or TLR4 modulates the responses of intrinsic renal and immune cells in systemic lupus. Poster presented at “Pattern-recognition receptors in human disease”, Queens' College, University of Cambridge, UK, 8 - 10 August 2007
2. **Rahul D. Pawar**, Prashant S. Patole, Daniel Zecher, Stephan Segerer, Matthias Kretzler, Detlef Schlöndorff and Hans-Joachim Anders. Toll like receptors via interaction with nucleic acids of bacterial and viral origin play a significant role in aggravating autoimmunity and lupus like immune complex glomerulonephritis in MRLlpr/lpr mice. Poster presented at TOLL 2006 Recent Advances in Pattern Recognition - March 4-7 2006.

3. **Pawar RD**, Segerer S, Anders HJ. Therapie der Lupusnephritis mit DNA. Blockade von Toll-like Rezeptor-7 mit inhibitorischen Oligonukleotiden. Abstract presented at Deutsche Gesellschaft für Innere Medizin (DGIM), Wiesbaden, 14-18 April 2007.
4. Paul-Martini-Workshop 2006, Toll-like receptor-based drug development, Universität-Bonn, Bonn, attended on September 15-16 2006,
5. **RD Pawar**, CL Kaul and SS Sharma, "Protective effect of 5'-N-Ethyl carboxamido adenosine in diabetic neuropathic pain", at 53rd Indian Pharmaceutical Congress, held on Dec. 21-23, 2001 in New Delhi, India.

Reviews

1. Molecular mimicry in innate immunity? The viral RNA recognition receptor TLR7 accelerates murine lupus. Anders HJ, Krug A, Pawar RD. Eur J Immunol. 2008 Jul;38(7):1795-9.
2. Anders HJ, Zecher D, Pawar RD, Patole PS. Molecular mechanisms of autoimmunity triggered by microbial infection. Arthritis Res and Ther. 2005 2005 (7): 215-224
3. Pawar RD, Patole PS, Wörnle M, Anders HJ. Microbial nucleic acids pay a Toll in kidney disease. Am J Physiol Renal Physiol 2006 Sep;291(3):F509-16.

Academic Attainments

1. **2nd prize as young investigator**, for the research work presented at **DGIM**, Deutsche Gesellschaft für Innere Medizin (DGIM) (German congress of internal medicine), Wiesbaden, Germany, 14-18 April **2007**.
2. Qualified GRADUATE APTITUDE TEST FOR ENGINEERING (**GATE**-2000), India, with 80.03 %.

Fellowship awarded

1. Received GRAKO stipend (GRK-1202- Oligonucleotides in cell biology and therapy) since 2006- till date. http://gkoligo.web.med.uni-muenchen.de/
2. Received NIPER Fellowship after qualifying the National level entrance examinations conducted by NIPER for the M.S. (Pharm.) course.
3. Research assistant scholarship of LMU for international Ph.D. fellows was awarded for 3 months from March-2008 to May 2008.

Editorial assistance

Former member of the editorial assistance team for the Quarterly Research Bulletin (Current research in Pharmaceutical Sciences) CRIPS of NIPER, India.

Research guidence

Research guidance was provided whenever required to the medicine and new PhD students in the LMU-Nephrological centre associated laboratory of Dr. H.J.Anders during my Ph.D. tenure.

References

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2. Prof. Dr. Med. Detlef Schlöndorff,
   Ex-Medical Director, Medical policlinic, LMU, Pettenkoferstr. 8a, 80336 Munich, Germany, currently at Mount Sinai School of medicine, as visiting Professor,
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Declaration: The above stated particulars are true to the best of my knowledge.

(Mr. Rahul D. Pawar, 22nd September, 2009)