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# The function and regulation of single immunoglobulin IL-1-related receptor (SIGIRR) in kidney disease

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LIST OF ABBREVIATIONS:

AP-1 – activator protein 1

APC – antigen presenting cell

ATF – activating transcription factor

BMDC – bone marrow dendritic cell

BSA – bovine serum albumin

CAM – cell adhesion molecule

cDNA – complementary DNA

DC – dendritic cell

DEPC – diethylene pyrocarbonate

DMEM – Dulbecco's modified Eagle's medium

DNA – deoxyribonucleic acid

DNTPs – deoxynucleotide triphosphates

ECD – extracellular domain

ECM – extracellular matrix

EGF – epidermal growth factor

ELISA – enzyme-linked immuno sorbent assay

FAS (APT1) – apoptosis antigen 1

FCS – fetal calf serum

FITC – fluorescein isothiocyanate

Flt3L – FMS-like tyrosine kinase 3 ligand

GMCSF – granulocyte-macrophage colony-stimulating factor

HEK – human epithelial kidney cells

HMGBP1 – high mobility group box protein 1

IC – immune complex

ICD – intracellular domain

IFN – interferon

Ig – immunoglobulin

IL-1R – interleukin 1 receptor

IRAK – interleukin-1 receptor associated kinase

IRF – interferon regulatory factor

ITS – insulin/transferrine/selenium

JNK – C-jun N-terminal protein kinase

KC – keratinocyte-derived chemokine

LB – luria-bertani broth

LBP – LPS binding protein

LPS – lipopolysacharide

MCP-1 (CCL2) – monocyte chemoattractant protein-1

MD-2 – myeloid differentiation protein 2

MDA5 – melanoma differentiation associated protein 5

MHC – major histocompatibility complex

MIP-2 (CXCL2) – macrpphage inflammatory protein 2

MODS – multiple organ disfunction syndrome

MOF – multiple organ failure

MyD88 – myeloid differentiation protein 88

NADH – nicotinamidadenindinukleotid

NF-κB – nuclear factor κB

NLS – nuclear localization sequence

NOD – nucleotide-binding oligomerization domain

PAMPs – pathogen-associated molecular patterns

PBS – phosphate-buffered saline

PCR – polimerase chain reaction

PE – phycoerythrin

PGE-1 – prostaglandin 1

PI3K – phosphoinositide 3-kinase

PRR – pattern recognition receptors

PS – penicillin/streptomycin

RIG – retinoic acid-inducible gen 1

RNA – ribonucleic acid

RT – reverse transcriptase

SARM – sterile alpha and HEAT/armadillo motif protein

SIGIRR – single immunoglobulin IL-1-related receptor

SLE – systemic lupus erythematosus

SNP – single nucleotide polymorphism

snRNP – small nuclear ribonucleoproteins

SOCS-1 – suppressor of cytokine signaling 1

ss/ds – single-, double-stranded

TBS – tris buffered saline

TIR8 – toll interleukin-1 receptor 8

TIZ – TRAF6-inhibitory zinc finger protein

TLR – toll-like receptor

TMB – 3,3',5,5'-tetramethylbenzidine

TNF – tumor necrosis factor

TRAF – TNF receptor associated factor

TRAM – TRIF-related adaptor molecule

TRIF – TNF receptor-inhibitory factor

U - unit

ZCCHC11 – Zinc finger CCHC domain containing protein 11

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# 1. INTRODUCTION

## 1.1. INNATE AND ADAPTIVE IMMUNE SYSTEM

We are constantly exposed to infectious organisms but our immune system enables us to resist infections. The immune system consists of the innate or nonspecific immune system and the adaptive or specific immune system (Table 1). The innate system is evolutionary older and it is a dominant part of immunity in plants, fungi or insects. It is the first line of defense against invading organisms. The adaptive immune system developed relatively late in vertebrates. It requires certain time to response to pathogens, whereas the innate immune system is constitutively ready to deal with infections. The innate system is not antigen specific and reacts equally well with different organisms and unlike the adaptive immune system it does not develop an immunological memory.

*Table 1.* **Differences between activation of immune and adaptive immune responses**

| | Innate Immunity | Adaptive Immunity |
|---|---|---|
| Recognition receptors | | |
| type of receptors | Pattern recognition receptors (complement, mannose, immunoglobulins, TLR) | T cell receptors, B cell receptors |
| clonality of receptors | Non-clonal | Clonal |
| genetical structure | Single gene | Encoded in gene segments |
| receptor rearrangement | Not required | Required |
| recognition patterns | Conserved molecular patterns | Details of (secondary) structure |
| Self-foreign discrimination | Selected by evolution | Selected individually |
| Time to effector activation | Immediate activation | Delayed activation |
| Effector response | Opsonization, activation of complement and coagulation cascades, phagocytosis, proinflammatory cytokines and chemokines | Clonal expansion or anergy of antigen-specific B and T cells |

*taken from HJ Anders et.al. J Am Soc Nephrol 15: 854–867, 2004*

The innate immune system is composed of the cells and mechanisms that defend the host in a non-specific manner. One of the most important functions of this kind of immunity is recognition of pathogen-associated molecular patterns (PAMPs) and recruitment of immune cells to sites of

infection and inflammation, via the production of cytokines and chemokines. The nonspecific immunity affects also the activation of growth factors which control cell differentiation and proliferation as well as the complement system. Finally, it activates the adaptive immune system via antigen presentation.

The components of the innate immune system include:

1. Anatomical barriers to infections are mechanical factors (epithelial surfaces, skin, tract, tears and saliva), chemical factors (fatty acids in sweat, lysozyme and phospholipase in tears, saliva and nasal secretions, low pH of sweat and gastric secretions) and biological factors (competition of the normal flora of the skin and in the gastrointestinal tract with pathogenic bacteria).

2. Humoral barriers to infection are the complement prone, coagulation system, interferons and finally the interleukin 1 (IL-1) which induces fever and activates acute phase proteins and some antibacterial proteins like lactoferrin and transferrin (bind iron) or lysozyme (damage of bacterial cell wall).

3. Soluble mediators of the innate system such as pentraxins, collectins, ficolins, defensins and opsonins can bind to structures on pathogens, leading to agglutination, interference with receptor binding, opsonization, neutralization, direct membrane damage and recruitment of additional soluble and cellular elements through inflammation.

4. Cell-surface-associated pattern recognition receptors (PRR) such as the mannose receptor, scavenger receptors, complement receptors or Fc receptors which participate in the removal of foreign substances and waste materials but also activate cellular responses. Also Toll-like receptors (TLRs) belong to this group of molecules.

5. Intracellular factors such endosomal TLRs, the nucleotide-binding oligomerization domain (NOD) family of cytosolic proteins (Chamaillard 2003; Girardin 2003) or new cytosolic defence mechanisms against the viral components such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated protein 5 (MDA5; Yoneyama 2004, Kang 2004).

6. Cellular factors are the cells of non-specific immune system, which have the ability to phagocytose the invading organisms and nonspecifically kill virus-infected and tumor cells.

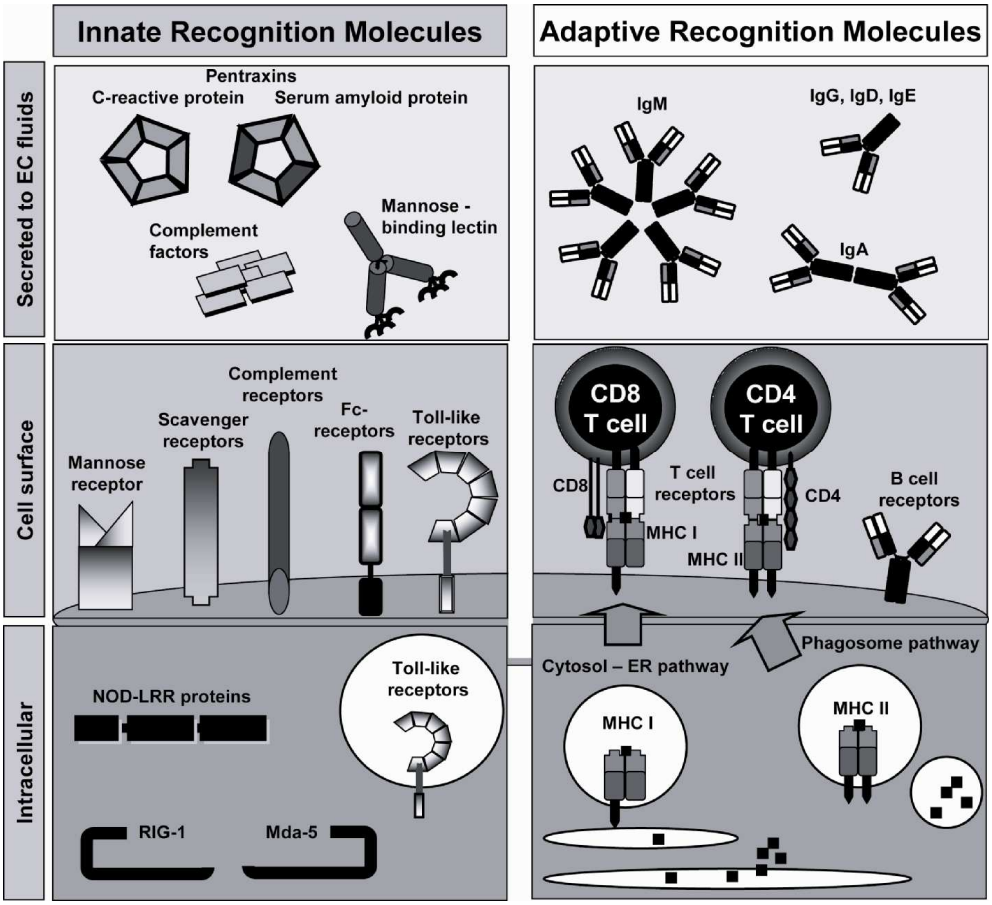


*Figure 1*. **Recognition molecules of innate and adaptive immune system**

## 1.2. TOLL-LIKE RECEPTOR/INTERLEUKIN-1 RECEPTOR (TLR/IL-1R) SUPERFAMILY SIGNALING

Toll-like receptors (TLRs) are a family of innate immune receptors whose critical role involves the recognition of invading pathogens. They are evolutionarily conserved; their homologs were found in mammals, plants and insects. Microbial infections rapidly induce activation of the innate immune system via receptors that either recognize pathogen-associated molecules or amplify proinflammatory cytokine signals, e.g. interleukin-1 (IL-1), tumour necrosis factor (TNF) or IL-18 (Janeway 2002). The TLR/IL-1R superfamily triggers robust inflammation in response to both classes of potential stimuli, microbial molecules as well as cytokines (Takeda 2003). In contrast to common intracellular signaling pathways, the members of the TLR and the IL-1R families differ in the structure of their extracellular domains (Figure 2). The discovery of the TLRs identified a group of innate immune receptors provided explanations to many phenomena of innate immunity. The TLRs reveal homologies to the *Drosophila melanogaster* Toll molecule, an important component of an antifungal defense mechanism of the fruit fly (Gay 1991, Medzhitov 1997). TLRs recognize molecules that are broadly expressed by pathogens: pathogen-associated molecular patterns (PAMPs). The TLRs appear to be one of the most conserved components of the immune system. Observations and studies made in the 90s strongly suggested that each of the TLRs in mammals might recognize conserved molecules produced by microbes (Table 2). The conserved character of these molecules is due to the fact, that they are necessary for the pathogen's survival and function. The leucin-rich repeat-containing extracellular domains of the TLRs bind defined PAMPs, e.g. diacyl- or triacyl-lipoproteins (heterodimers of TLR1/2 or TLR2/6), lipopolysaccharide (TLR4), flagellin (TLR5), double- or single-stranded viral RNA (TLR3 and -7/8) or CpG-DNA (TLR9) (Takeda 2003; Table 2). The multiple roles of TLRs in the initiation and regulation of innate and adaptive antimicrobial immune responses are increasingly recognized (Akira 2003, Schnare 2001). By contrast, the immunoglobulin domain-containing extracellular part of the IL-1 receptor family recruit a number of cytokine ligands including IL-1α, IL-1β and IL-18, which have important role in immunity. IL-1 subsequently activates the expression of MIP-2, KC and C-reactive protein whereas IL-18 plays a role in natural

killer cell activation and in Th1 cell polarization (Sims 2002, Nakanishi 2001). As for intracellular signaling, the members of this superfamily of type I transmembrane receptors share the intracellular Toll-IL-1R (TIR) domain at the C-terminus (Martin 2002). The TIR domain transmits ligand binding by the extracellular domain of TLR/IL-1Rs to intracellular signal transduction.

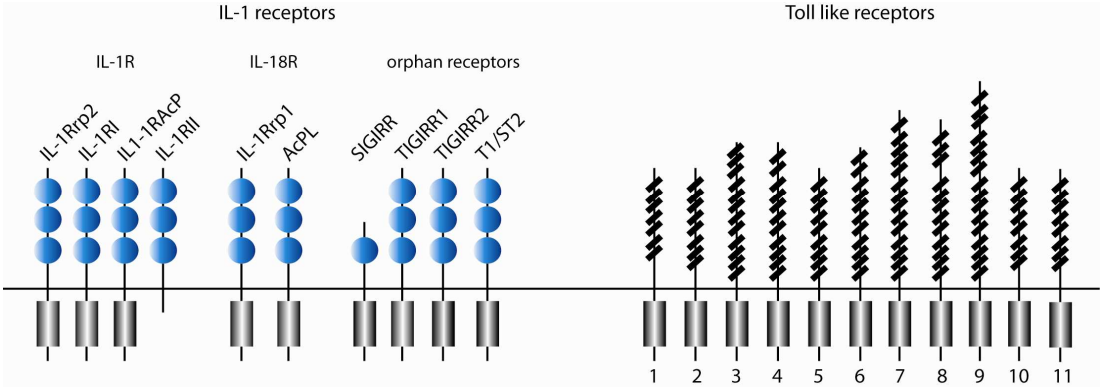


*Figure 2.* **The Toll / Interleukin-1 Receptor (TIR) Family:** Immunoglobulin domain; TIR domain; leucine rich repeats.

The intracellular TIR domain is present in all transmembrane members of the TLR/IL-1R family except for the decoy receptor IL-1RII (Mantovani 2001). The TIR domain consists of 135-160 residues that form a central five-stranded parallel beta-sheet that is surrounded by a total of five helices on both sides (Xu 2000). This domain is crucial for signal transduction of all the TLR/IL-1Rs that mediate outside-in signaling upon recognition of a specific ligand. Members of the TLR/IL-1R superfamily tend to homo- or heterodimerize via their TIR domain. The individual TIR domains are also required to form the signaling complex between the receptor and the adaptor molecules (Martin 2002). Deletion of single, essential amino acids within the TIR domain results in loss of signal transduction (Xu 2000, Radons 2002).

With the exception of TLR2, TLRs initiate signaling by homodimerization. TLR2 forms heterodimers with TLR1 or TLR6. Others form complexes with other co-receptors; TLR4 recognition of LPS requires MD-2, CD14 and LPS binding protein (LBP).

Activation of TLRs by pathogen associated molecular patterns induces the signaling cascades that lead to expression of genes required for effective pathogen-specific immune responses. The transcription factors such as NF-κB, interferon regulatory factor 3 (IRF3), IRF5 and IRF7, activated by TLRs enhance the expression of many immunoregulatory molecules, including type I interferons, chemokines and inflammatory cytokines. Five TIR domain– containing adaptor proteins have been identified, and so far four of those, MyD88, Mal, TRIF and TRAM are responsible for transcription factors activation, whereas fifth namely SARM is a specific negative regulator of TRIF signalling (Carty 2006). Further signaling molecules specific for the TLR/IL-1R superfamily include TNF receptor associated factors (TRAFs) and members of the interleukin-1 receptor associated kinase (IRAK) family (Beutler 2004, Akira 2004). While the members of the TLR and the IL-1R families share intracellular signaling pathways, they separate into two subfamilies by the structure of their extracellular domains. TLR-dependent activation leads to the translocation of NF-κB to the nucleus, which results in transcription of genes encoding for cytokines, chemokines, adhesion molecules, and antimicrobial peptides.

IL-1 pathway leads to activation of the transcription factors NF-κB, ATF and AP-1 (Dinarello 2006). IL-1 signaling involves adaptor protein MyD88 (Wesche 1997), IRAK4 (Li 2002; Suzuki 2002), IRAK (Cao 1996; Li 1999), TRAF6 (Cao 1996) and many others. However, unlike the IL-1 signalling pathway, TLRs can use MyD88 dependent or independent signalling. For TLR3 and TLR4 was shown that both can use TRIF (the TIR domain-containing adapter inducing interferon-β).TRIF deficient mice lack the TLR3 signalling and the TLR4 signalling is almost completely abolished (part of the NF-κB activation pathway goes via MyD88; Yamamoto 2003).

*Table 2.* **Toll-like Receptors and their ligands.**

| **Toll-like receptor** | **Exogenous ligands** | **Endogenous Ligand** |
|---|---|---|
| TLR1 & 2 | Tri-acyl lipopeptides (bacteria and mycobacteria) | |
| TLR2 | Peptidoglycan (Gram-positive bacteria)<br>Lipoteichoic acid (Gram-positive bacteria)<br>Lipoarabinomannan (mycobacteria)<br>Glycophospholipids (Trypanosomes)<br>Glycolipids (*Treponema*)<br>Porins (*Neisseria*)<br>Zymogen (fungi)<br>Phospholipomannan (*Candida albicans*)<br>tGPI-mutin (*Trypanosoma*)<br>hemagglutinin protein (*Measles virus*)<br>ND (HCMV,HSV1)<br>Lipopeptides | Heat shock proteins<br>High mobility group box protein 1 |
| TLR3 | Double-stranded RNA (virus) | mRNA |
| TLR4 | Lipopolysaccharides, lipid A (Gram-negative bacteria)<br>Taxol (plant)<br>Protein F (respiratory syncytial virus)<br>Hyphae (*Aspergillus*)<br>HSP60 (*Chlamydia*)<br>Envelope proteins (MMTV, RSV)<br>Mannan (*Candida albicans*) | Heat shock proteins<br>High mobility group box protein 1<br>Fibronectin extra domain A<br>Fibrinogen<br>Lung surfactant protein A<br>Low density lipoprotein<br>Heparan sulphate<br>Hyaluronan fragments |
| TLR5 | Flagellin (bacteria) | |
| TLR6 & 2 | Di-acyl lipopeptides (Mycoplasma)<br>LTA (Group B *Streptococcus*) | |
| TLR7 | U-rich single-stranded RNA (viral) | |
| TLR8 | Single-stranded RNA (viral) | |
| TLR9 | Unmethylated CpG DNA (bacteria & viruses) | Unmethylated CpG DNA |
| TLR11 | Uropathogenic *Escherichia coli*,<br>profilin (*Toxoplasma gondii*) | |

## 1.3. REGULATION OF TLR/IL-1R SUPERFAMILY SIGNALING

Inapproapriate cytokine release can cause major organ failure or death, e.g. in septic shock or systemic inflammatory response syndromes. It is therefore of great importance, that TLR/IL-1R-mediated immune activation is tightly regulated (Figure 3). Various superfamily members act as decoys for the receptors. IL-1RII and IL-18-binding protein are for example negative regulators of IL-1 and IL-18 signalling (Mantovani 2001, Colotta 1994, Novick 1999). Both released and intracellular form of IL-1ra (Arend 1993) and IL-1RII (Colotta 1993 and 1994; Mantovani 2001) are the negative regulators of IL-1 signaling. IL-1ra acts as an antagonist of IL-1R. IL-1RII interacts with IL-1 and forms the complex with IL-1RAcP, which is essential for IL-1R mediated signal transduction (Lang 1998, Malinowsky 1998).

Some of the inhibitors are working intracellularly, like IRAK-M (Kobayashi 2002), IRAK2 (Hardy 2004), MyD88s (Janssens 2002, Burns 2003), SOCS-1 (Kinjyo 2002) or Triad3A (Chuang 2004). IRAKM expression is induced upon TLR stimulation and inhibits TLR signaling. This molecule prevents IRAK and IRAK4 from dissociation from MyD88 and formation of IRAK-TRAF6 complex. Splicing variants of IRAK2; IRAK2 and IRAK2d have also inhibitory effect on Toll-IL-1R superfamily-mediated signaling (Hardy 2004), similar to splicing version of MyD88 (MyD88s), which prevents recruitment of IRAK4 (Janssens 2002, Burns 2003). Triad3A was reported to be a modulator of TLRs signaling by regulation of their ubiquitination and proteolytic degradation (Chuang 2004). Some soluble TLRs were identified to be negative regulators of TLR signalling (e.g. sTLR2, sTLR4). Activation of some TLRs may lead immediately to reduction of their surface expression or to reduction of expression of co-stimulatory molecules needed for the signalling. LPS stimulation results in reduced expression of LPS binding molecules TLR4 and MD-2 (Nomura 2000, Akashi 2000). SARM is a newly discovered TIR-containing adaptor protein, which puts a break on TRIF dependent pathway. The new group of zinc finger proteins such as A20, an A20-like protein Cezanne, TRAF6-inhibitory zinc finger protein TIZ, FLN29 and ZCCHC11 were shown to bind TRAF6 and negatively regulate TLR-TRAF6-induced NF-κB activation (Heyninck 1999, Evans 2001, Shin 2002, Mashima 2005, Minoda 2006). These proteins differs in the structure of zinc finger

repeats and in the cell expression profile, which suggest distinct modulation in different kind of cells. Some of the molecules like phosphoinositide 3-kinases (PI3Ks) represent an early phase of negative regulation of TLRs in innate immunity (Fukao 2002 and 2003; Guha 2002). Expression of IRAK-M and SOCS-1 (suppressor of cytokine signalling-1) is induced after the first activation of TLRs and these molecules act as negative regulators during the second stimulation by TLR agonists (Kobayashi 2002, Kinjyo 2002, Nakagawa 2002). By contrast, PI3K is constitutively expressed in innate immune cells and activated immediately after detection of pathogens (Fukao 2002, Herrera-Velit 1997). This dual-phase negative regulation of innate immune responses mechanism may exist in order to inhibit rapidly reacting cells, whereas the other mechanisms may be more common in non immune cells. In case all these inhibitory mechanisms fail the cell can still activate the programmed cell death in order to escape the inflammation.

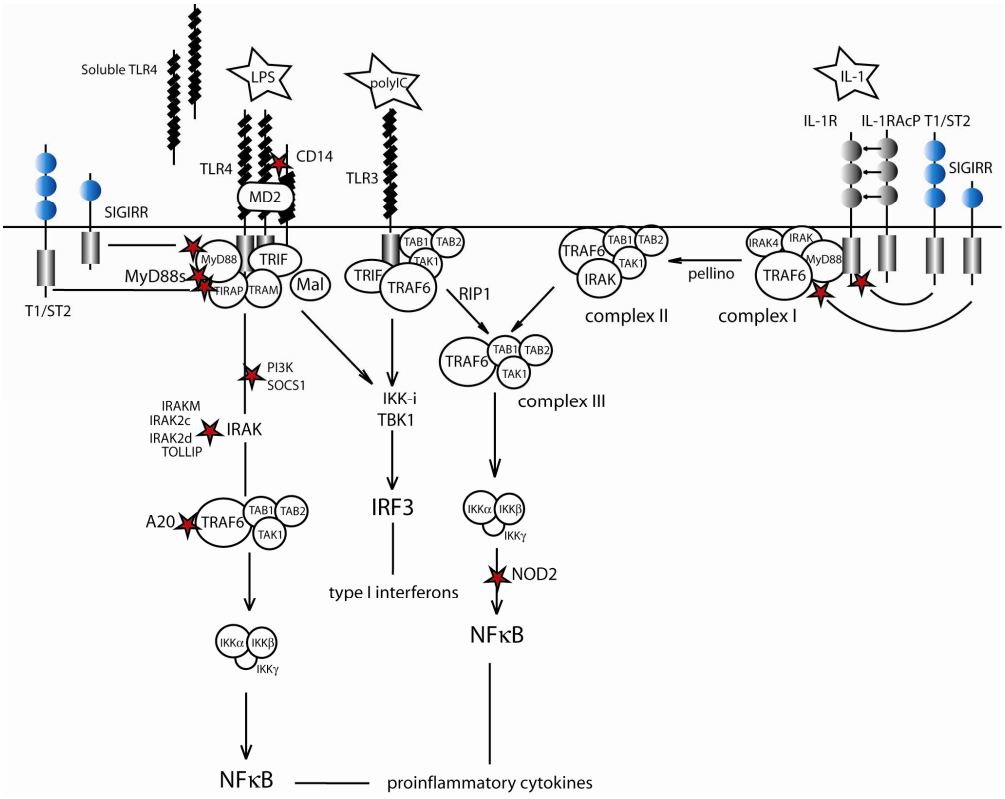


*Figure 3*. **Regulation of TLR signaling:** immunoglobulin domain; TIR domain; leucine rich repeat; negative regulation.

Also membrane-bound proteins containing the TIR domain, such as orphan receptor SIGIRR (single immunoglobulin IL-1 receptor-related molecule), T1/ST2 and toll-interacting protein, Tollip negatively regulate the TLR signalling (Brint 2004, Wald 2003, Zhang and Ghosh 2002). T1/ST2 and SIGIRR belong to Ig domain subgroup, which also includes IL-1R1 and IL-18R. They are negative regulators of Toll-IL-1R mediated signalling (Brint 2004, Garlanda 2004).

## 1.4. STRUCTURE AND FUNCTION OF SIGIRR

SIGIRR, also known as TIR8 (Toll interleukin-1 receptor 8) was identified by searching EST databases for TIR domain-containing sequences of yet unknown members of the TLR/IL-1R family (Thomassen 1999). Despite its genetic sequence homology to the other members of the IL-1R family located on chromosome 2, the *Tir8* gene is located on chromosome 11 at 11p15 (Thomassen 1999, Sims 1995). Four different transcripts (4.4 kb, 2.4 kb, 1.5 kb, and 0.9 kb) were found in humans (Thomassen 1999), whereas in mice only one transcript was found (Polentarutti 2003, Garlanda 2004). The protein length predicted from the open reading frame is 409 amino acids in mice and 410 amino acids in humans (Figure 4). Similar to ST2, hSIGIRR contains four and mSIGIRR contains five putative glycosilation sites and molecular weight of the glycosylated protein is between 50-80 kD (Thomassen 1999). In contrast to all other known members of the IL-1R family the extracellular domain of SIGIRR contains only one Ig domain of 118 amino acids (Figure 4). This extracellular part is too short to fold and therefore lacks a potential ligand binding site (Barclay 2003). Till now no soluble form of SIGIRR has been described. The transmembrane region links the single Ig-like domain to the 268 amino acids-long intracellular part which is 77 amino acids longer than that of the typical IL-1Rs (Thomassen 1999). Although, SIGIRR protein was found in the membrane fractions of COS cells transfected with SIGIRR but it is yet not clear, whether SIGIRR is expressed on the cell surface or in an intracellular, e.g. endosomal, compartment (Thomassen 1999).


*Figure 4.* **The amino acid sequence and structure of SIGIRR**

SIGIRR mRNA is expressed in most fetal and adult human as well as murine organs with the highest levels were detected in kidney, liver, and the gastrointestinal tract (Thomassen 1999, Polentarutti 2003, Wald 2003). In brain, heart, testis, muscle, endothelia or fibroblasts SIGIRR is virtually absent (Polentarutti 2003, Garlanda 2004, Wald 2003). SIGIRR expression in solid organs largely originates from epithelial cells. In the kidney for example SIGIRR is expressed at high levels in tubular and collecting duct epithelial cells of the renal cortex and medulla, but is undetectable in glomerular mesangial cells (Polentarutti 2004). By contrast, cells of the monocyte/macrophage lineage and neutrophils express SIGIRR mRNA at low levels, whereas T cells or B cells do not express SIGIRR at all (Polentarutti 2003).

Injection of LPS downregulates SIGIRR mRNA levels in various tissues (Polentarutti 2003, Garlanda 2004). The same occurs in cultured neutrophils or monocytes upon stimulation with LPS (Polentarutti 2003). This appears to be a LPS-specific mechanism because stimulation with IL-1β, IFN-γ, TNF, IL-4 or CpG-DNA had no effect on SIGIRR mRNA levels in these cells (Polentarutti 2003).

The observation, that the orphan receptors lack cytokine ligands or, in the case of SIGIRR, lack ligand binding sites, suggests a regulatory function on TLR/IL-1R signaling. The single extracellular Ig domain of SIGIRR may not have a function but modulatory effects could well mediated by the prominent intracellular domain. Indeed, SIGIRR was found to mediate negative regulatory effects on TLR/IL-1R signaling.

SIGIRR molecules form homodimers which interact with TLR/IL-1Rs (Wald 2003). The intracellular TIR domain is crucial for this interaction, and therefore SIGIRR dimers also interact with TIR

domain-containing adapter proteins involved in TLR/IL-1R signaling, i.e. MyD88 and Mal/TIRAP (Figure 3), (Wald 2003, Leung 2004). However, TIR complexes involving SIGIRR do not activate NF-κB and JNK but rather suppress TLR/IL-1R signaling. This relates to the fact that SIGIRR lacks two essential amino acids (Ser447 and Tyr536) in its TIR domain highly conserved in other members (Thomassen 1999, Wald 2003). SIGIRR interacts also with the IL-1R but, interestingly, and in contrast to interaction with the TLRs, this process involves both the intracellular and the extracellular domains (Figure 3). The single extracellular Ig domain of SIGIRR suppresses IL-1 signaling by interfering with the heterodimerization of IL-1R and IL-1RAcP (Qin 2005). SIGIRR-deficient cells show enhanced inflammatory responses to LPS, CpG-DNA, and IL-1 but not to TNF (Wald 2003). Similar effects were observed in immature dendritic cells but not in bone marrow macrophages, which lack SIGIRR expression (Garlanda 2004). Additionally, overexpression of SIGIRR specifically inhibits IL-1R and IL18R signaling in Jurkat and HepG2 cells (Wald 2003). Consistent with an inhibitory effect on TLR/IL-1R signaling SIGIRR-deficient mice are more susceptible to lethal septic shock after LPS challenge (Wald 2003). In fact, cytokine and chemokine production was markedly enhanced and prolonged in SIGIRR-deficient mice after challenge with LPS and IL-1 but not with TNF (Wald 2003). Similarly, SIGIRR-deficient mice are more susceptible to dextran sulphate sodium-induced chronic colitis (Garlanda 2004). In this model of chronic inflammatory bowel disease, SIGIRR deficient mice showed increased loss of body weight and of intestinal blood compared to wild type mice. This was associated with more severe damage of intestinal mucosa and inflammatory cell recruitment (Garlanda 2004). Thus, despite lacking cytokine or microbial ligand interaction, SIGIRR modulates TLR and IL-1 signaling on selected cell subsets. Regulation of these signalling pathways has important implications for inflammatory diseases such as sepsis or inflammatory bowel disease.

TLR/IL-1R superfamily members are involved in pathogenesis of autoimmune, inflammatory and infectious diseases (Cook 2004). TLRs and their signalling pathways might be partially responsible for development of diseases like: dilated cardiomyopathy, atherosclerosis, diabetes, experimental autoimmune encephalomyelitis or systemic lupus erythematosus. The important function of TLRs was confirmed in some chronic inflammatory disorders, such as: asthma, rheumatoid arthritis and

inflammatory joint disease. TLRs are also involved in the regulation of pro-inflammatory cytokines production. The malfunction may lead to tissue damage. The best example for this is sepsis, which is the result of uncontrolled TLR4 signaling. Sepsis and the sequential multiple organ failure/disfunction syndrome (MOF/MODS) followed by septic shock are the most common cases of death in the intensive care units (Stone 1994). Septic shock is comparable with the effect that is initiated with the LPS released by the microorganisms during the infection. The production of the inflammatory factors can even result in death. As SIGIRR can function as a negative regulatory factor, especially in the case of TLR4 it can be a step in providing the therapeutic approach against sepsis. The function of SIGIRR in controlling the other diseases in which the TLR/IL-1R family may contribute has not been shown yet. Targeting IL-1 and other cytokines with single cytokine- or cytokine receptor antagonists has already become part of therapeutic regimens of inflammatory diseases such as rheumatoid arthritis in humans (Dinarello 2005, Furst 2005). The concept of targeting signaling molecules of the TLR/IL-1R superfamily may provide a related but less restricted strategy (Ulevitch 2004).

Thus, a better understanding of the factors that regulate immune activation, including orphan receptor SIGIRR, may eventually expand the armament of anti-inflammatory drugs that allow appropriate interventions where necessary.

## 1.5. AIM OF THE RESEARCH PROJECT

This study was motivated by two observations: first, previous observation that TLR4 on intrinsic renal cells as well as bone marrow-derived cells contributes to innate immunity in infective pyelonephritis (Patole 2005), and second, SIGIRR, a negative regulator of TLR/IL-1R signaling, was reported to be expressed at high levels in the kidney (Polentarutti 2003, Wald 2003). We hypothesized that SIGIRR would control inappropriate TLR signaling in the kidney, and if so, that SIGIRR on renal myeloid cells and non-immune intrinsic renal cells would contribute to this phenomenon. To reveal the function of this protein in the kidney we characterized the SIGIRR expression pattern and performed structural and functional studies on the regulation and the expression of this molecule. As over-expression of SIGIRR in cell lines can lead to inhibition of immune responses, this work investigates the possibility of using SIGIRR as anti-inflammatory factor. However, the identification of a ligand or a soluble functional form of the protein would be in this case necessary. This study also questioned the effect of SIGIRR on the development of the autoimmune disease, systemic lupus erythematosus (SLE). The role of TLRs in autoimmunity is not yet well defined. Practically, the effects of SIGIRR on the development of autoimmune disease are to be analysed in an in-vivo model.

To address these issues, several *Sigirr*-deficient murine cell populations were used. The phenotypic characterization of the mouse lines and functional characterisation of cell types were used to elucidate the role of SIGIRR in the kidney.

# 2. MATERIAL AND METHODS

## 2.1 MOLECULAR BIOLOGY METHODS

### 2.1.1. Cloning of human and murine SIGIRR

The polymerase chain reaction (PCR) was used in order to amplify DNA from genomic DNA or cDNA. DNA encoding N-terminal FLAG tagged murine SIGIRR was amplified by PCR from C57BL/6 mice cDNA from kidney using the following primers:

Fw.-5′GCCACCATGGACTACAAAGACGATGACGATAAAGGAGCAGGTGTCTGT3′

Rv-3′CTCCTACTACACATCCTATAG5′

The PCR product was digested with Hind III and EcoRV and ligated into pCR3 vector (Invitrogen, Karlsruhe, Germany).

The following reagents were added for a PCR reaction:

1 µl cDNA

2.5 µl 10x HotStar PCR buffer (Qiagen GmbH, Hilden, Germany)

0.5 µl 25 mM dNTP mix

0.25 µl Fw. primer (100 pM)

0.25 µl Rv. primer (100 pM)

0.25 µl HotStar polymerase (Qiagen GmbH, Hilden, Germany)

5 µl Solution Q (Qiagen GmbH, Hilden, Germany)

$H_2O$ to a total volume of 25 µl

Samples were amplified in a thermocycler (Biometra Uno2) with the following conditions: The programs used had a 15-min incubation step at 94°C before starting the cycle. The next step was for 1 min at 94°C, annealing was performed at 61°C, below the melting point of the primers for 75 sec. The next step in the cycle was incubation at 72°C for 2 min. The cycle was repeated 30 times and the program finished with 10 min incubation at 72°C. The samples were used directly for electrophoresis.

DNA encoding human SIGIRR was amplified by PCR from cDNA from human kidney using the following primers:

Fw. - 5´GATCCGCCACCATGCCAGGTGTCTGT3´

Rv. - 3´TTCCTACTATACATCCTATAG5′

The PCR product was digested with BamH1 and EcoRV and ligated into pCR3 vector (Invitrogen, Karlsruhe, Germany). Samples were amplified in a thermocycler (Biometra Uno2) with the following conditions: The programs used had a 15-min incubation step at 94°C before starting the cycle. The next step was for 1 min at 94°C, annealing was performed at 42°C below the melting point of the primers for 60 sec. The next step in the cycle was incubation at 72°C for 2 min. The cycle was repeated 30 times and the program finished with 10 min incubation at 72°C. The samples were used directly for electrophoresis.

**2.1.2. Electrophoresis of DNA**

The DNA sample to be analyzed was mixed with one tenth volume of DNA loading buffer (600 µl 50% glycerine; 20 µl 50 x TAE; 380 µl water; a few crystals of bromophenol blue). The samples were separated in agarose gels in 1 x TAE buffer (40 mM Tris; 0.02 mM acetic acid; 1 mM EDTA; pH 8.0). The concentration of agarose varied depending on the samples to be run: 0.5% for digested genomic DNA to 2% for small fragments (250 bp or less). The agarose gel was prepared by boiling 1g of agarose in 100 ml 1 x TBE, add 4 µl ethidium bromide (10 mg/ml, Sigma-Aldrich, Taufkirchen, Germany). The fragments are separated by electrophoresis according to size and can be visualized in UV light due to the presence of ethidium bromide between the DNA strands. Ladder Mix (Invitrogen, Karlsruhe, Germany) was used as size standard. After visualizing the DNA in an agarose gel, the band to be purified was excised from the gel and transferred into a 1.5 ml microcentrifuge tube. The amplified fragments of DNA were purified from agarose-gel with the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany).

### 2.1.3. Ligation of DNA fragments

The cDNA fragments, which were amplified in PCR reaction were digested with desired restriction enzymes and ligated into pCR3 vector (digested with the same enzymes). T4 ligase (Invitrogen, Karlsruhe, Germany) was used for ligation. 6µl of PCR product and 2 µl of vector DNA were incubated with 1 µl ligase, 1 µl 10 x T4 ligase buffer (Invitrogen, Karlsruhe, Germany). Both ligations were incubated 48 hours at 16°C. After ligation the mixtures were used directly for electrotransformation of DH5a cells and streaked out on LB plates complimented with ampicillin (100 µg/ml). Single colonies were used for inoculation of liquid medium and after overnight incubation used for plasmid isolation.

### 2.1.4. Generation of competent cells for electro-transformation

A fresh E. coli (DH5α) culture was prepared by inoculating 10 ml LB medium (1% Bacto-Tryptone, 0.5% Bacto-Yeast extract, 0.5% NaCl, pH 7.5) with a single colony and incubating overnight. 500 ml LB medium were inoculated with 5 ml (1/100) of the overnight culture and grown to a density of $OD_{600}$ 0.5. The cell suspension was chilled on ice for 30 min and centrifuged at 4000 g for 15 min at 4°C. Cells were resuspended in 500 ml ice cold H2O and centrifuged again. The pellet was resuspended in 250 ml ice cold $H_2O$, centrifuged and resuspended in 20 ml of ice cold 10 % glycerol. After another centrifugation the pellet was resuspended in 2 ml of ice cold 10 % glycerol and stored in 40 µl aliquots at – 70°C.

### 2.1.5. Transformation of bacterial cells by electroporation

50 µl of competent cells were thawed on ice, 0.5 µl of ligation mix was added and left on ice. The cell suspension was then placed in a chilled, dry and sterile electroporation cuvette (0.2 cm) in a Gene Pulser (Bio-Rad) and pulsed once at 25 µF, 2.5 kV (Pulse Controller, Bio-Rad: 200 Ω). Cells were then immediately resuspended in 1 ml SOC medium (2% Bacto-Tryptone; 0.5% Bacto-yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose). The cell suspension was incubated at 37°C for 1 h on a shaker in tube and then plated on LB-agar plates containing the selective antibiotics. Plates were incubated over night at 37°C. Each colony from a plate was slightly

touched with a sterile pipette tip and the bacterial cells on the tip were re-suspend in 2 ml LB medium containing antibiotic for selection (Ampicilin). Inoculated colonies were grown over night for the small scale plasmid preparation.

**2.1.6. Small scale plasmid preparation – mini prep**

This quick purification was used for testing which colony of bacteria express the right construct. A 1.5 ml aliquot of an overnight culture of E. coli was centrifuged in 1.5 ml sterile microcentrifuge tubes for 30 sec. The supernatant was discarded. The bacterial pellet was resuspended in 100 µl lyse buffer (25 mM Tris HCl pH 7.5; 10 mM EDTA; 50 mM glucose; RNAse 0.1 mg/ml). Then the 200 µl of alkalizing buffer was added (0.2 M NaOH, 1% SDS) and the tubes were incubated for 5 min. on ice followed by adding 150 µl of 5 M KAc solution. After centrifugation step (14000 rpm, 3 min) the supernatant was transferred into a new microcentrifuge tube and extracted with phenol/chloroform/isoamylalcohol (25:24:1, v/v/v). After 5 min centrifugation at RT, the upper phase was transferred to a new microcentrifuge tube, mixed with 500 µl 100% ethanol, incubated 1 hour at -20°C and centrifuged for 15 min. The pellet was then washed with 70% ethanol, dried and dissolved in 20 µl of water.

**2.1.7. Analysis of DNA with restriction enzymes**

2-4 U of the desired restriction enzymes (New England BioLabs, Frankfurt am Main, Germany) were added for 1 µg DNA in the appropriate restriction buffer. The restriction mix was incubated for 1 or 2 h at appropriate temperatures. The following restriction enzymes or enzyme combinations were used to detect the presence of a vector with the desired insert after ligation and transformation: BamHI, EcoRV, SacI.

**2.1.8. RNA isolation 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. The 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 xg for 30 seconds. The flow-through was discarded and the digestion with DNAse was performed on the columns (Qiagen GmbH, Hilden, Germany DNAse digestion kit). Then the columns were loaded with 700µl of buffer RW1 and centrifuged at 8000 xg 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 xg 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 150 µl 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.

**2.1.9. Isolation of RNA from tissues**

The RNA isolation protocol was suitably modified from Chomczynski's method (Chomczynski P 1987). DNAse digestion was included. 3 ml of solution D containing 8 µl 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 300 µl 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 until a milky white suspension resulted. The

falcon tube was then placed on ice for 15 min and centrifuged at 4000 xg 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 xg 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 and 1 ml solution. The pellet was dissolved in buffer containing DNAse, RNAse inhibitor, 1mM DTT, 0.05 M TrisHCl and 5 mM $MgCl_2$ and incubated for 30 min at 37°C. After the incubation 15 µl of 2 M sodium acetate was added and the phenol/chloroform/isoamyl alcohol extraction was performed. The water phase 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 xg 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 xg 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 10 µl 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 2 µl 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 1.6 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.

**2.1.10. cDNA synthesis and real-time PCR analysis**

The RNA samples isolated according to the procedure detailed above were diluted in DEPC water to a concentration of 1 µg / 20 µl. A master mix was prepared with reagents such as 9 µl of 5 x 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 40 U/µl RNasin (Promega, Mannheim, Germany), 0.5 µl of 15 µg/ml linear acrylamide (Ambion Ltd,

Cambridgeshire, UK), 0.5 µl of Hexanucleotide (Roche, Mannheim, Germany), 1 µl of Superscript (Invitrogen, Karlsruhe, Germany) or dd$H_2O$ in case of the controls. The master mix was made to a volume of 15 µl and added to 1 µg / 20 µl 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 at placed 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 488 nm. 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). 18 µl of the mastermix was pipetted into each well of a 96-well plate and 2 µl 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 PE Biosystems, Weiterstadt, Germany.

**2.1.11. Northern blot**

Total kidney and spleen RNA was isolated and analyzed for the degradation and purity (analytical MOPS gel; OD by 260 and 280 nm). Every 5 µg RNA sample was dried, dissolved in NB-loading solution (15 µl formamide, 5 µl formaldehyde, 3 µl10 x MOPS, 7 µl DEPC-treated water), incubated 10 min in 65°C and separated by electrophoresis on a 1% formaldehyde/MOPS agarose gel (1% agarose, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 6 % formaldehyde) in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA; pH 7.0). To see the progression of the separation dye (loading buffer containing 0.25 % bromophenol blue) was run next to the samples in a separate well. The separation was performed RNAse-free mini gel apparatus; first 10 min at 100 V followed by 2-3 hours at 200 V. The RNA was transferred on the GeneScreen membrane for 24 hours in 10 x SSC buffer (1.5 M NaCl, 0.15 M Na-citrate), baked (1 hour at 80°C between 2 sheets of 3 MM paper) and cross-linked with UV light (50 mJoule in Bio-Rad Gene Linker UV Chamber).

The DNA-probes syntheses were performed using the Prime-it Random Primer Labelling Kit (Stratagene, La Jolla, CA). P32-labeled DNA probes were complementary to 243bp N-terminus part (cut with HindIII and BstXI) or the full length SIGIRR (the plasmid preparation, enzymes restriction and DNA gel-extraction described above). Efficacy of probe synthesis was checked by PEI chromatography. For hybridisation the QuickHyb Hybridisation Solution was used (Stratagene, La Jolla, CA). For pre-hybridization, the membranes were incubated in a hybridization glass tube with 4 ml of hybridization buffer (Stratagene, La Jolla, CA). After pre-hybridization, the radioactive probe was added to the buffer, gently mixed and incubated for 24 hours at 65°C. The membranes were then washed 5 times with 2 x SSC supplemented with 0.1 % SDS (low stringency buffer) at RT. The

membranes were sealed in plastic bags and exposed on a PhosphorScreen of the PhosphorImager after 24 hours.

## 2.2 CELL CULTURE METHODS AND CYTOKINE DETECTION

### 2.2.1. Cell lines

Human epithelial kidney cells (HEK 293) was derived from embryonic kidney cells transformed by human adenovirus type 5 DNA. 293 cells grow plastic adherent. Cell line was maintained under standard culture conditions (in an incubator set at 37 °C supplied with 5.0 % $CO_2$) in Dulbecco's modified Eagle's medium (DMEM, Biochrom KG, Berlin, Germany) supplemented with 5 % heat-inactivated fetal calf serum (FCS) (Serum Supreme, BioWhittaker, Walkersville, MD, USA), penicillin 100 U/ml and streptomycin 100 µg/ml as described (Complete DMEM medium).

### 2.2.2. Calcium Phosphate transfection

In this method plasmid DNA was introduced into monolayers of eukaryotic cells culture via precipitate that adheres to the cell surface. HEK 293 cells were seeded on six-well plates at a density of 3 x $10^5$ cells/well and grown over night to sub-confluence. Cells in every well were transfected with following transfection mix: 225 µl $H_2O$, 25 µl 2.5M $CaCl_2$ mixed with 5 µg plasmid DNA. After incubation for 10 min. at 37 $^oC$ the DNA mix was vortexed with 250 µl HeBs (0.28 M NaCl; 0.05 M HEPES; 1.5 mM $Na_2HPO_4$; pH 7.05). A precipitate containing calcium phosphate and DNA is formed by slowly mixing HEPES containing solution (HeBs) and solution that contains calcium chloride and DNA. After incubation for at 37 $^oC$ 500 µl of mix was added to the cells. The medium (containing 5 % FCS and 25 mM HEPES) was changed 6 hours later. Thirty hours after transfection, the cells were detached, washed (1x) with PBS and used for protein extraction.

### 2.2.3. Reporter gene analysis

Reporter studies were performed by using lipofectamine (Invitrogen, Karlsruhe, Germany) transfection of primary murine tubular epithelial cell line with an NF-κB responsive luciferase

reporter gene (Promega, Mannhein, Germany). Tubular cells were transfected with constructs (pCR3-mSIGIRR and NFκB) with amount of 2.5 µg of each plasmid (5 µg total). For transfection cells were seeded at the density of 1,5 x $10^5$ into 3.5 cm dishes and grown overnight in 37 $^oC$. Then cells were transfected with expression plasmids coding molecules of interest by the lipfectamine method (1 µg DNA + 1 µl lipofectamine; 30 min RT incubation). 24 hours after transfection cells were stimulated with TLR ligands for 24 another hours. Cells were lysed, and luciferase activity was assessed using Reporter lysis buffer and Luciferase Reporter reagent (Promega, Mannhein, Germany).

**2.2.4. Primary cell culture – tubular epithelial cells**

Kidney cell suspensions were prepared by mashing the kidney from one mouse in 250 µl of cold HBSS medium supplemented with 10 mM HEPES, 10% FCS and 1% PS. The suspension was applied onto 30 µm pre-separation filters (Miltenyi Biotec GmbH; Bergisch Gladbach, Germany), centrifuged (300 x g for 10 min), re-suspended in complete DMEM medium (10 % FCS) and incubated at 37 °C for 1 bis 2 hours on the Petri dish so that the monocytes in the cell suspension adhere. The non-adherent cells from the suspensions were than collected, centrifuged and re-suspended in K1 medium (DMEM supplemented with 10 % FCS, 1 % PS, 10 mM HEPES, 1 % ITS (insulin, transferrine, selenium) and hormones/growth factors mix: EGF, PGE-1, T3, ITSS, hydrocortisone). Cells were plated on 12 or 24 well collagen type IV- treated plates. Plates were coated with 6µg/ml collagen IV (Sigma-Aldrich, Taufkirchen, Germany) in PBS overnight in 4 °C. The cells were grown till they were 70-80 % confluent. K1 medium was changed every 3-4 days.

**2.2.5. Primary cell culture – mesangial cells**

Kidney cell suspensions were prepared from the cortex (medulla was removed) by mashing it in 250 µl of cold complete RPMI medium. The suspension was applied onto 150, 103, 63, 50 and 45 µm sieves, rinsed with cold PBS, centrifuged (4000 rpm for 7 min), re-suspended in complete RPMI medium and applied onto 30 µm Pre-separation Filters (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Glomeruli remained on the filter. The filter was swapped upside down and rinsed with PBS containing 1 mg/ml collagenase IV. Glomeruli were incubated in this solution for 15-20 min at

37 °C. The cells were then centrifuged and re-suspended and plated in RPMI medium complemented with 20 % FCS, 1 % PS, 1 % ITS (insulin, transferrine, selenium). Medium was changed every 4-6 days; the first passage was made after 16-20 days.

**2.2.6. Primary cell culture - splenocyte or adherent spleen monocytes**

Spleens were isolated from the mice, placed in a petri-dish containing complete RPMI medium and mashed with the help of forceps, this coarse suspension was then passed through a 30 micron steel wire mesh and collected in a sterile petri-dish. This suspension was then centrifuged at 1600 RCF for 4 min at 4 °C to obtain a pellet. The pellet thus obtained was washed with sterile PBS and the obtained pellet was resuspended in 0.83 % ammonium chloride solution so as to haemolyse the red blood cells. This was followed by a washing steps (2X) as mentioned above, passed through a pre-separation filter to obtain single cell suspension (Miltenyi Biotec GmbH; Bergisch Gladbach, Germany). Finally the cells were centrifuged, supernatant was discarded and the pellet was resuspened in complete RPMI medium and cell counts were done. A 10 x $10^6$ or 5 x $10^6$ of cells was plated in 6 or 12 well plates and incubated at 37 °C for 24 hrs under standard culture conditions. The culture medium was replaced with fresh complete RPMI medium so as to obtain the adherent spleen monocytes, ready to be used for stimulation experiments.

**2.2.7. Primary cell culture - bone marrow-derived dendritic cells**

Bone marrow was isolated from the tibia and femurs from the fore and the hind limbs of the mice. Muscle tissue and the bone caps were removed. A 0.45 x 13 mm needle fitted to a 1 ml syringe, filled with complete RPMI medium, was inserted to one end of the bone so as to flush the bone marrow from the other end and collected in a sterile petri-dish. In this manner, all bones were carefully flushed to obtain bone marrow which was then centrifuged at 1600 RCF for 4 min at 4 °C to obtain a pellet. The pellet thus was washed with sterile PBS and processed for hemolysis and washes as described above. Finally, the cells were centrifuged, resuspened in complete RPMI medium with 50 ng/ml human recombinant Flt3 ligand (Immunotools, Friesoyth, Germany) or 1 ng/ml rmGM-CSF (Immunotools, Friesoyth, Germany) and cell counts were done. Bone marrow isolates were cultured

for 8 -10 days in complete RPMI with 50 ng/ml human recombinant Flt3 ligand or 1 ng/ml rmGM-CSF. Medium was changes every 3 days.

**2.2.8. Primary cell culture – renal APC**

Antigen presenting cells were prepared from kidneys as follows. Kidney cell suspensions were prepared by mashing the kidney from one mouse in 250 µl of cold 10 % FCS-1 % PS-RPMI medium. The suspension was applied onto 30 µm Pre-separation Filters (Miltenyi Biotec GmbH; Bergisch Gladbach, Germany), centrifuged (300 x g for 10 min) and re-suspended in wash buffer (PBS containing 0.5 % BSA and 2 mM EDTA). CD11b+ cells isolation was performed using CD11b magnetic beads and LD MACS separation columns (both Miltenyi Biotec GmbH; Bergisch Gladbach, Germany). 1 x $10^7$ cells were re-suspended in 90 µl wash buffer and 10 µl of magnetic beads were added. The cells were incubated with beads for 20 min in the cold room. The cells were then centrifuged, washed and re-suspended in cold wash buffer (1x$10^8$ cells in 2 ml buffer). The suspension was applied onto LS MACS Separation column, which was previously placed in magnetic field and adjusted with 3 ml wash buffer. The flow through was collected as a CD11b-depleted cell fraction, plated and grown in a condition identical to these of primary tubular epithelial cells. After washing (3 x 3 ml of wash buffer) column was removed from magnetic field and cells were eluted with 5 ml wash buffer, centrifuged and plated (3 x $10^5$ cells/ml) in 10%FCS-1%PS-RPMI medium supplemented with 1 ng/ml rmGM-CSF (ImmunoTools, Friesoyth, Germany) and grow till they were 70-80 % confluent. Medium was changed every 3-4 days.

**2.2.9. Stimulation experiments**

Primary cells (tubular epithelial cells, spleen monocytes, BMDCs, renal APCs or mesangial cells) for the stimulations were prepared from organs of 6 week old mice in the C57BL/6 background and grown in culture according to standard protocols mentioned above. Cells were treated with medium control or IFN-γ 100 U/ml (PeproTech, Rocky Hill, NJ) + TNF-α, 500 U/ml (ImmunoTools, Firesoythe, Germany) or TLR ligands as follows: TLR1/2: Pam3Cys 1µg/ml (Alexis Biochemicals, Grünberg, Germany), TLR3: pI:C 50 µg/ml (Sigma-Aldrich, Taufkirchen, Germany), TLR4: ultra

pure LPS 1 µg/ml (Invivogen, San Diego, CA), TLR9: CpG-DNA 1668 1 µM (TibMolbiol, Berlin, Germany). Other ligands used for the stimulation were: IL-1beta (10ng/ml; ImmunoTools, Friesoyth, Germany) or IL-18 (10ng/ml ImmunoTools, Friesoyth, Germany). Ligands were preincubated with polymyxin B sulphate (Sigma-Aldrich, Taufkirchen, Germany) (50 µg/ml) to neutralize possible LPS contaminations. After various time intervals 6, 12, 18 or 24 hours (indicated in figures) culture supernatants were collected and cytokine levels were determined using a commercial ELISA kits. RNA from cells was prepared for mRNA analysis using the Qiagen RNasy Mini Kit (Qiagen GmbH, Hilden, Germany). For additional experiments 50 mg slices were cut with the scalpel from kidneys of SIGIRR-deficient or wild type C57BL/6 mice, placed in DMEM 10% FCS medium. The kidney slices were stimulated for 24 hours with either medium or 1 µg/ml ultra pure LPS. For the stress-conditions experiments NaCl (600 mM), albumin (10 mg/ml) or the 42°C were used. Supernatants were collected for ELISA. In all stimulation experiments all cell types were unstarved (unless mentioned otherwise).

U1snRNP (which is conserved between species) was purified from HeLa cell nuclear extracts (Bochnig P Eur J Biochem. 1987). The anti-Sm (B/D) antibody clone Y12, mouse IgG3 isotype28 was purified from Y12 hybridoma supernatant, CpG 2216 oligonucleotides were comertialy avalable (MWG Biotech, Munich, Germany). Poly-I:C RNA, poly-U RNA (Amersham Biosciences, Freiburg, Germany), and oligoribonucleotides (Curevac, Tübingen, Germany) were used as indicated.

Bone marrow cells from wild-type and knockout mice were cultured with 20 ng/mL human recombinant Flt3L (R&D Systems, Wiesbaden, Germany) in complete medium for 7 days to generate greater than 90 % CD11c+ DCs with 40 % to 50 % CD11blow/CD86low/B220high PDCs and 40 % to 50 % CD11bhigh/B220low DCs. On day 7, cells were harvested, resuspended in fresh medium, and seeded at 4 x $10^5$ cells/well; 100 µL/well in 96-well plates. RNAs and the isolated U1snRNP were preincubated with DOTAP cationic liposomes (Carl Roth, Karlsruhe, Germany) for 30 min RT. Y12 antibody was incubated with U1snRNP in PBS for 15 minutes on ice plus 5 minutes at 37°C. Stimuli were added in 100 µL volume per well (concentrations indicated) for 24 hours.

### 2.2.10. Cytokine measurements

Cytokine levels in sera or cell culture supernatants were determined using commercial ELISA kits: IL-6, IL-12p40, CCL2 or CXCL2 (OptEiA, BD Pharmingen, San Diego CA or R&D Systems, Wiesbaden, Germany) following the protocol provided by the respective manufacturers. The 96-well plate was first coated with 100µl/ well capture antibody (anti-mouse cytokine) at recommended dilution in 0.2 M Sodium phosphate buffer of specified pH and placed overnight at 4 °C. The wells were then aspirated, washed with >200 µl wash buffer (PBS pH 7 with 0.05 % Tween-20) and the plate was blocked with >200 µl/well assay diluent (PBS pH 7 with 10 % FCS) and incubated at room temperature for 1 hour. This was followed by aspiration, 2 washes as described above, and 100 µl of standard or sample (cell supernatant or the mice serum) was pipette to appropriate well and the plate was incubated for 2 hours at room temperature. The plate was then aspirated, washed five times and 100 µl working detector (biotinylated anti mouse cytokine or detection antibody with avidin-horse raddish peroxidase conjugate) was added to each well and incubated at room temperature for 1 hour. This was followed by an aspiration and wash step (>5 washes). The TMB substrate solution (BD Biosciences, Hamburg, Germany) was then added to each well at a volume of 100 µl and incubated for 30 minutes. The stop solution (1 M phosphoric or 2N sulphuric acid) was then added to each well, and absorbance was measured at prescribed wave length (nm), using an automatic plate reader.

### 2.2.11. Proliferation assay

Proliferation of cells was assessed using *CellTiter 96 Proliferation Assay* (Promega, Mannheim, Germany). The CellTiter 96 Aqueous One Solution contains a novel tetrazolium salt compound (MTS) and phenazine ethosulfate that serves as an electron coupling reagent. The solution remains stable normally, while the MTS is bioreduced by the NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (such as proliferating cells). The cells isolated according to the procedure described above ($1 \times 10^5$) were incubated in 96-well plates in 100 µl complete RPMI medium and treated with 5 µg/ml CpG-ODN 1668 or 1 µg/ml LPS for a period of 72 hours under standard culture conditions. To each well with different treatments, 20 µl CellTiter 96 Aqueous One

Solution was added and incubated at 37 °C for 4 hours and the optical density (OD) was measured at 492 nm for comparing the cell proliferation.

### 2.2.12. Phagocytosis assay

Isolated intrarenal myeloid cells were cultured till they were 70% confluent in RPMI complete + GM-CSF medium. The cells were incubated with 1 mg/ml FITC-labeled dextran (70000 kD; Sigma-Aldrich, Taufkirchen, Germany) for 90 min. As a control for non-specific dextran attachment, 0.02 % azide was added or cells were cultured at 4°C to stop energy-dependent cellular functions. To determine phagocythic activity, the take up of fluorescence beads was detected by flow cytometry.

## 2.3. PROTEIN ANALYSIS

### 2.3.1. Protein extraction from tissues

Organs from mice were manually dissected, mashed to powder in the liquid nitrogen and homogenized in 1ml lysis buffer (50 mM Tris HCl, pH 7.5; 150 mM NaCl; 100 µM sodium orthovanadate, 0.5 % sodium deoxycholat, 4 % NP-40, 2 % Triton-X-100; 5 mM EDTA; 300 mM sucrose; proteases inhibitor tablets COMPLETE (Roche, Mannhein, Germany). The solution was homogenised with hand homogenizer, left for 30 min in 4$^{o}$C on over-head shaker and centrifuged for 45 min at 30000 x g. The supernatant was tested for the protein concentration (Bradford test, BioRad, München, Germany).

### 2.3.2. Western blot

Extracted proteins were incubated in 2 x Laemmli buffer (Tris 30 mM; Glycerol 5 %; 1% SDS; 0.0025 % bromophenol blue) with beta-mercaptoethanol for 30 minutes at 65°C, resolved by 12 % SDS-PAGE, and transferred to an methanol-activated PVDV Immobilon-P membrane (Millipore, Eschborn, Germany) using the BioRad Semi-Dry Blotting System (BioRad, München, Germany). Electrotransfer was performed for 1 h at 25 V. To verify successful transfer of protein and note the

position of the marker (peqGOLD protein marker; PeqLab, Erlangen, Germany), the membrane was stained for 10 min in Ponceau solution (0.5 % PonceauS in 1 % acetic acid) and destained with $H_2O$. After blocking with 1 % western blocking solution (Roche, Mannhein, Germany) the filter was incubated with a goat polyclonal anti-SIGIRR antibody (1:1000; R&D Systems, Wiesbaden, Germany) over night in 0.5 % Western blocking solution (Roche, Mannhein, Germany). Immune complexes were visualized using a peroxidase-conjugated donkey anti-goat IgG antibody (1:10000, Dianova, Hamburg Germany) for 1 hour in 0.5 % Western blocking solution and processed for detection by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech Europe, Freiburg, Germany). After every antibodie addition, the membrane was washed 5 times with TBS or TBS-Tween.

Before digestion with PNGaseF (New England BioLabs, Frankfurt am Main, Germany) protein isolates were denaturised at 95°C for 10 min in buffer containing 0.5 % SDS and 0.04 % DTT. Digestion was performed for 1h at 37°C in buffer containing 0.05 M sodium phosphate pH 7.5 and 1 % NP-40. Benzyl-N-Acetygalactosamine (Sigma-Aldrich, Taufkirchen, Germany) was used as an inhibitor for O-glycosylation at 4 mM final concentration. The cells with or without inhibitors were grown on 90 mm dish for 48 h. Media containing fresh inhibitor (4 mM) were changed two times during this period.

### 2.3.3. Tissue immunostaining

From kidneys collected as mentioned above, two-micrometer-thick paraffin-embedded sections were cut and processed for immunohistochemical staining performed on paraffin-embedded sections. Upon isolation the tissue pieces were placed in plastic histocassettes and dipped in formalin. The formalin solution was prepared using 500 ml of 40 % formaldehyde in PBS (32.5 g $Na_2HPO_4$ and 20 g $NaH_2PO_4$ in 4.5 L $ddH_2O$ water, pH 7.4). The blocks were then infiltrated and embedded with paraffin and sections were cut in ribbons and mounted on slides. 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).

Staining for SIGIRR was performed on acetone-fixed frozen section using the polyclonal anti-

SIGIRR antibody (1:50). A PE-labeled donkey anti-goat IgG antibody was used for detection (1:10000, Dianova, Hamburg, Germany). FITC-phalloidin (Invitrogen, Karlsruhe, Germany) was used for staining tubular brush border (F actin). Negative controls included incubation with a respective isotype antibody.

### 2.3.4. Immunostaining of cultured cells for SIGIRR

Staining for SIGIRR and F-actin in tubular epithelial cells was performed on 3.7 % formaldehyde-fixed cell monolayer, permeabilized with 0.5 % Triton-X-100. Polyclonal anti-SIGIRR antibody (1:200) and a PE-labelled donkey anti-goat IgG (1:500) were used for detection. Negative controls included incubation with a respective isotype antibody. In the next step, a suitably labelled secondary antibody was used with respective detection system.

### 2.3.5. Immunostaining for nuclear p50

Staining for nuclear p50 in tubular epithelial cells and spleen monocytes was performed on 3.7 % formaldehyde-fixed cell monolayer, permeabilized with 0.5 % Triton-X-100. The cells were pre-stimulated with the TLRs ligands for 1 hour just befor fixation to activate the p50 translocation to the nucleus. A p50 (NLS) antibody (1:100, Santa Cruz, Heidelberg, Germany) was used in order to detect nuclear translocation of NF-κB (Fagerlund 2005). Negative controls included incubation with a respective isotype antibody. In the next step, a suitably labelled secondary antibody was used with respective detection system.

### 2.3.6. Flow cytometry

Flow cytometry of primary spleen monocytes and tubular epithelial cells was performed using the goat polyclonal anti-SIGIRR antibody. For intracellular staining, cells were fixed with 1% paraformaldehyde and permeabilized with permeabilization buffer (PBS, 0,5 % BSA, 0,5 % saponin) at room temperature for 20 min. Primary cells were harvested using methods described above, involving hemolysis, passage through preseparation filter to obtain single cell suspension and wash steps, under cold conditions on ice. All cell types, after appropriate stimulation procedures were

harvested, resuspended in 50-100 µl PBS, and incubated with the respective primary or secondary antibodies (wherever applicable) or isotype controls at prescribed dilutions for 60 minutes each, followed by a washing step, resuspended in PBS for FACS analysis. A polyclonal PE-labelled donkey anti-goat antibody was used for detection (1:1000, Dianova, Hamburg, Germany). A goat IgG (BD Pharmingen) was used as isotype control. FACS analysis was conducted using a FACScalibur machine and CellQuest software (BD Pharmingen). The renal APCs surface staining was performed using PE-labeled mouse anti-CD11c (BD Pharmingen, Hamburg, Germany).

## 2.4. IN VIVO EXPERIMENTS AND STATISTIC

### 2.4.1. Animal studies

*SIGIRR*-deficient mice were generated by homologous recombination of the *SIGIRR* gene by the group of prof. A. Mantovani as previously described (Garlanda 2003), genotyped, and backcrossed to the C57BL/6 strain (Charles River Laboratories, Sulzfeld Germany) to the generation F6. C57BL/6 *lpr/lpr* or MRL *lpr/lpr* mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and Jackson Laboratories (Manheim Germany) respectively and backcrossed together with *SIGIRR-/-* strain to generate the C57BL/6 *lpr/lpr SIGIRR-/-* or MRL *lpr/lpr SIGIRR-/-* mice. Genotyping was performed by PCR with appropriate primers. Mice were housed in groups of 5 mice in filter top cages with a 12 hour dark/light cycle and unlimited access to food and water. Cages, nest lets, food and water were sterilized by autoclaving before use. All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities.

Tubular epithelial kidney cells, mesangial renal cells, spleen monocytes, bone marrow derived dendritic cells (BMDCs) or renal dendritic cells were isolated from the C57BL/6, SIGIRR-/- or the lpr/lpr-SIGIRR knock out mice. All mice were sacrificed by cervical dislocation. The whole spleen tissue was removed and processed for splenocytes isolation and culture as described below. For obtaining the BMDCs, whole bones from the femur from the fore and hind limbs were separated and processed as described below. Kidneys were removed for the preparation of tubular epithelial and

mesangial cells.

### 2.4.2. Serum IgGs and urine protein measurement

Blood and urine samples were collected from each animal every 30 days. The urine protein ratio was determined by the albumin ELISA (Bethyl Labs, Montgomery, TX, USA). Anti-dsDNA antibodies levels in serum samples were determined by ELISA: NUNC maxisorp ELISA plates were coated with poly-L-lysine (Trevigen, Gaithersburg, MD, USA) and mouse embryonic stem cell dsDNA. After incubation with mouse serum dsDNA-specific IgG, IgG1, IgG2a, IgG2b, IgG3 were detected by ELISA (Bethyl Labs, Montgomery, TX, USA). All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities.

### 2.4.3. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Comparison between groups was performed using Student's *t*-test. A value of $p < 0.05$ indicated statistical significance. Error bars represent standard error of the mean (SEM).

# 3. RESULTS

## 3.1. EXPRESSION OF SIGIRR

### 3.1.1. *Sigirr* expression is strain- and gender- dependent

Genetic variation between mouse strains has recently been characterized in detail using single nucleotide polymorphisms (Wade 2002). Differences in genetic background between mouse strains affect gene expression, which explains phenotypic differences. Genomic variability could be correlated with high levels of single nucleotide polymorphisms (SNPs) occurring between mouse strains. Before we chose the right background for backcrossing the *Sigirr*-deficient mice we investigated the level of *Sigirr* expression in different mouse strains. *Sigirr* expression levels in renal tissue from three different inbred mouse strains (C57BL/6, BALB/c and C3H/HeNCrl) were determined. Total RNA from three to six individuals per strain was isolated, 1 µg total RNA was reversed transcribed and investigated for *Sigirr* expression by the quantitative real time PCR. Significantly lower expression for *Sigirr* was shown in BALB/c and C3H/HeNCrl compared to C57BL/6 strain. Moreover *Sigirr* might be a gene which is sex-dependently regulated because male express significantly more SIGIRR than the female of the same strain and age, but interestingly only in C57BL/6 mice (Figure 5).

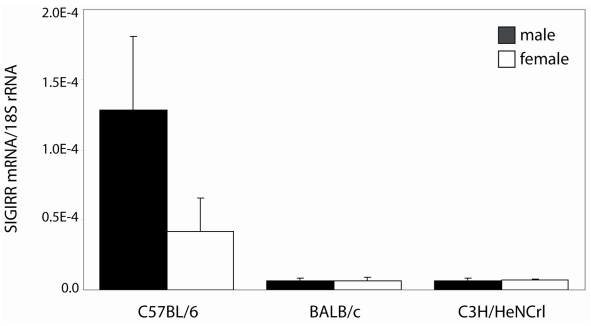


*Figure 5.* ***Sigirr* expression in C57BL/6, BALB/c and C3H/HeNCrl mice**: mRNA was extracted from kidneys of 6 weeks old mice with different genetic backgrounds (C57BL/6, BALB/c and C3HHeNCrl) and different gender as indicated (n=3-6). *Sigirr* mRNA expression levels were determined by real-time RT-PCR and expressed as mean of the ratio SIGIRR /18s-rRNA ± SEM.

For further experiments we chose 6 weeks old males with the C57BL/6 background hoping to see the most striking differences in phenotype of *SIGIRR*-deficient and wild type mice.

### 3.1.2. *Sigirr* expression in solid organs

*Sigirr* mRNA level was analysed by real-time RT-PCR in solid organs of 6 week old C57BL/6 mice. RT-PCR screening with mouse *Sigirr* -specific primers on a variety of solid organs revealed its wide expression. High levels of *Sigirr* mRNA were found in kidneys, i.e. 3-fold compared to respective *Sigirr* levels in spleen (Figure 6, left). By contrast, brain, heart, lung, liver, small intestine, colon, skin, and muscle expressed *Sigirr* mRNA at lower levels as in spleen. The prominent expression of SIGIRR in kidneys of 6 week old mice was confirmed on the protein level by Western blot (Figure 6, right).

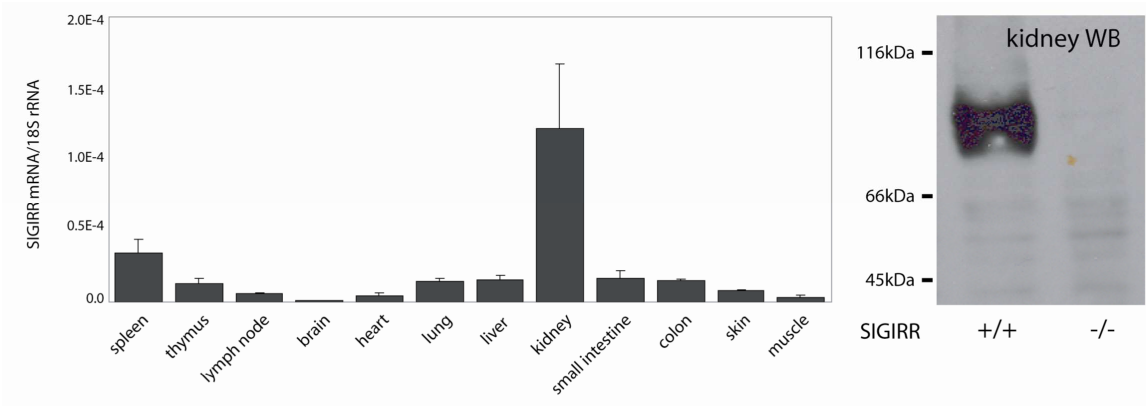


*Figure 6.* ***Sigirr* expression in C57BL/6 mice:** (left): mRNA was extracted from organs of C57BL/6 mice of 6 weeks age (n=6). *Sigirr* mRNA expression levels were determined by real-time RT-PCR and expressed as mean of the ratio *Sigirr* /18s-rRNA ± SEM; (right): SIGIRR protein expression was determined by Western blot analysis. Proteins were prepared from kidneys of 6 week old C57BL/6 wild-type mice or *Sigirr* -deficient mice as indicated.

Because TLR expression has been reported to be age-dependent (Renshaw 2002), *Sigirr* mRNA levels were analysed in C57BL/6 mice of 10 days, 6 weeks or 1 year of age. In most organs *Sigirr* mRNA levels declined from young to old age (Figure 7). Interestingly, *Sigirr* mRNA levels in 6 week old C57BL/6 mice were 7-8-fold higher as compared to 10 day or 1 year old mice. These data show that *Sigirr* is expressed at high levels in kidneys of adolescent C57BL/6 mice.

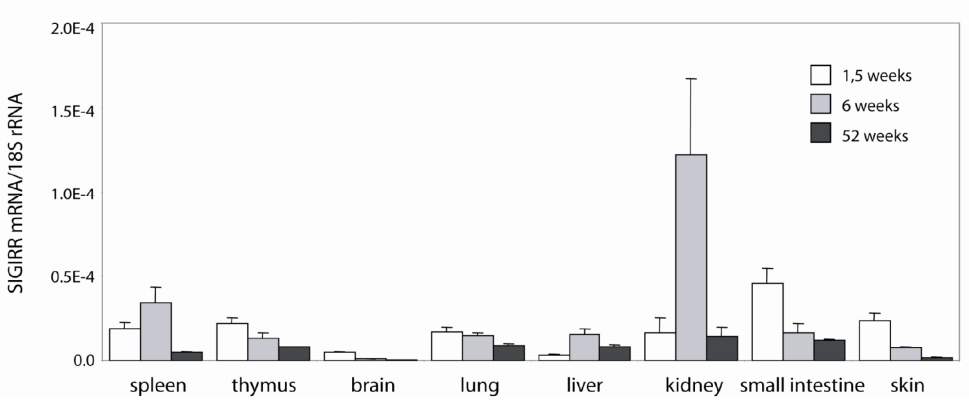


*Figure 7.* ***Sigirr* expression in C57BL/6 mice**: mRNA was extracted from organs of C57BL/6 mice of different age (1,5 weeks; 6 weeks; 52 weeks) as indicated (n=3-6). *Sigirr* mRNA expression levels were determined by real-time RT-PCR and expressed as mean of the ratio SIGIRR /18s-rRNA ± SEM.

### 3.1.3. *Sigirr* is expressed at high levels in intrarenal myeloid cells and renal tubular epithelial cells

Next the source of renal *Sigirr* expression was determined. Kidneys of 6 week old C57BL/6 mice were carefully dissected into cortex and medulla and real-time RT-PCR was performed for *Sigirr* mRNA. Cortex and medulla from kidneys of 6 week old C57BL/6 mice expressed equal levels of *Sigirr* mRNA, suggesting a tubular or vascular origin rather than glomerular cells, which locate to the renal cortex (Figure 8, left).

In order to determine the cellular origin of renal *Sigirr* expression, tubular epithelial cells, mesangial cells and intrarenal myeloid cells were isolated from 6 week old C57BL/6 mice.

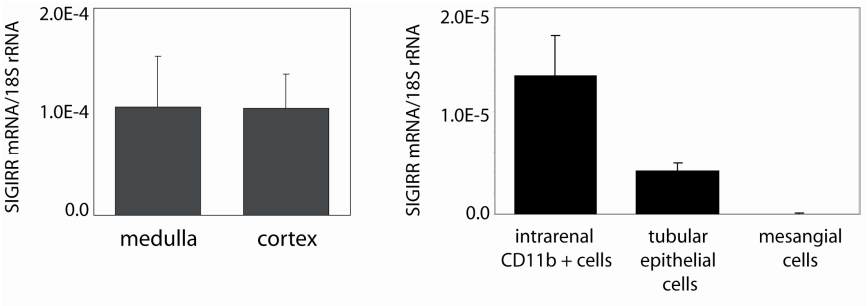


*Figure 8.* ***Sigirr*** **expression in renal cells:** (left): Renal cortex and medulla were dissected from 6 week old C57BL/6 mice (n = 5) and RNA was extracted. *Sigirr* mRNA levels were determined by real-time RT-PCR and expressed as mean of the ratio *Sigirr* /18s-rRNA ± SEM (right): Primary cells were isolated from of 6 week old C57BL/6 mice as described in methods. *Sigirr* mRNA levels were determined as before by real-time RT-PCR and expressed as mean of the ratio Tir8/Sigirr/18s-rRNA ± SEM. N.d. = not detected.

In fact, unlike primary mesangial cells tubular epithelial cells and resident CD11b/F4/80-positive renal myeloid cells both expressed SIGIRR mRNA (Figure 8, right). The latter cells localize to the interstitium of the renal cortex and medulla of mice (Figure 9). These data indicate that the profound renal SIGIRR expression originates from tubular epithelial cells and intrarenal immune cells, i.e. resident antigen-presenting cells.

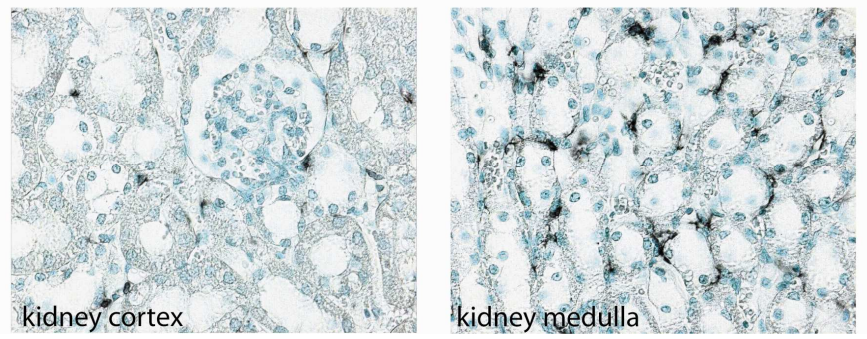


*Figure 9.* **Immunohistochemistry of F4/80-positive antigen presenting cells (APC) in the kidney:** Kidney slides were obtained from 6 weeks old C57BL/6 wild type mice. The slides were stained with anti-F4/80 antibodies for intrarenal antigen-presenting cells (black) in kidney cortex (left) and medulla (right); original magnification x200.

The transmembrane molecule SIGIRR has been reported to suppress LPS or IL-1 signaling in Jurkat cells by interacting with the intracellular domain of TLR4 and both extracellular Ig domain and the intracellular TIR domain of IL-1R (Qin 2005). Thus, SIGIRR should localize to outer membranes of these cells.

Flow cytometry using a polyclonal anti-mSIGIRR antibody revealed surface expression of SIGIRR protein on primary tubular epithelial cells prepared from wild-type mice (Figure 10, left), but not from SIGIRR-deficient mice (Figure 10, right).

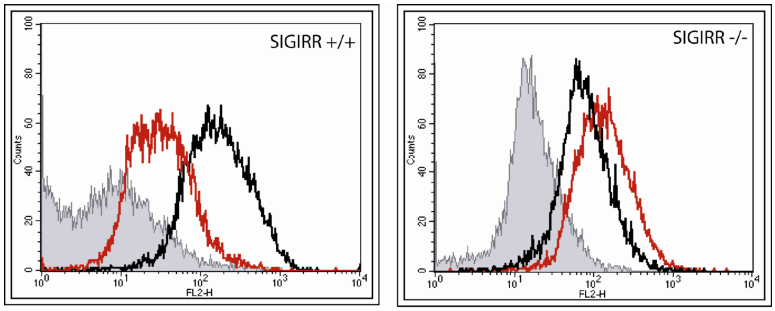


*Figure 10.* **SIGIRR expression in tubular epithelial cells:** left and right: Flow cytometry for SIGIRR was performed using primary tubular epithelial cells prepared from wild-type mice (left) and *Sigirr* -deficient mice (right) as indicated. Surface expression of SIGIRR (black line) is indicated by a fluorescence shift compared to the isotype control antibody (red line). Grey area represents the secondary antibodies control.

The cellular distribution of SIGIRR protein was confirmed by SIGIRR immunostaining on cultured primary tubular epithelial cells (Figures 11A and 11B) and kidney sections prepared from 6 week old C57BL/6 mice (Figure 11C and 11D). Positive staining signals were detected at basolateral and luminal membranes of proximal and distal tubular epithelial cells. In proximal tubular epithelial cells the colocalisation with the luminal brush border (costainined with FITC-phalloidin) was particularly apparent (Figure 11C). Glomeruli stained negative for SIGIRR (not shown). Negative controls included staining cells or renal sections from *Sigirr*-deficient mice (Figures 11B and 11D), omitting the primary antibody (not shown) or preabsorbing the primary antibody with murine SIGIRR-overexpressing HEK293 cells (not shown). Thus, SIGIRR is expressed on basolateral and luminal

membranes of proximal and distal tubular epithelial cells in the mouse kidney. In order to assess the subcellular expression of SIGIRR protein the *Sigirr*-deficient mice were used as a control for the specificity of the detection methods applied. The lack of SIGIRR protein in kidneys of *Sigirr*-deficient mice was confirmed by Western blot using a polyclonal anti-mSIGIRR antibody (Figure 6, right).

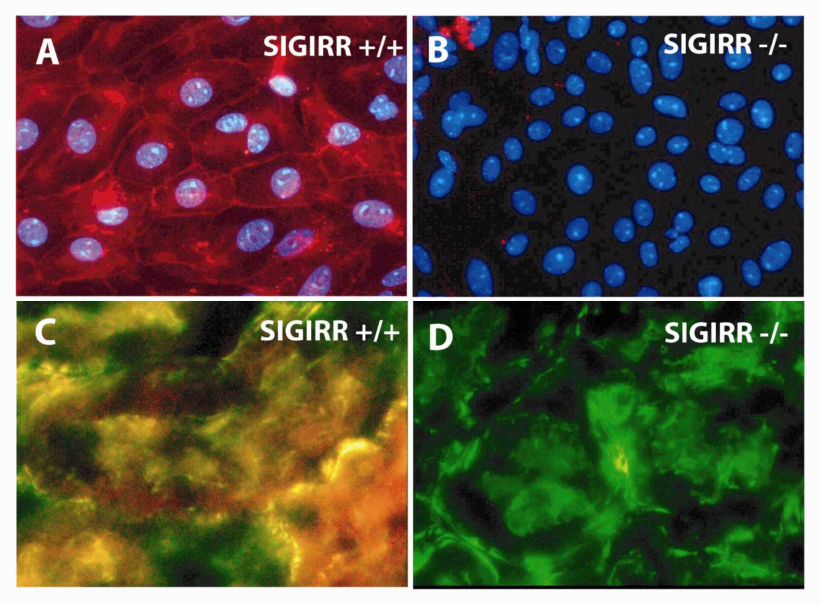


*Figure 11.* **SIGIRR expression in tubular epithelial cells:** A and B: SIGIRR immunostaining with PE-labelled secondary antibody confirms SIGIRR expression on the cellular surface in confluent growing primary tubular epithelial cells prepared from wild-type mice (A). Tubular epithelial cells from *SIGIRR*-deficient mice lack respective positive signal (B). DAPI staining of cell nuclei is seen in blue (original magnification x 400). C and D: Renal sections were prepared from 6 week old C57BL/6 wild-type mice and *SIGIRR*-deficient mice and stained for SIGIRR with a PE-labeled secondary antibody. FITC-phalloidin stains brush border in proximal tubular epithelial cells and cell-cell contacts in green. A yellow signal is obtained at basolateral membranes and brush border as a result of colocalization of red SIGIRR staining and green phalloidin staining (C). The SIGIRR signal is absent when renal sections taken from *SIGIRR*-deficient mice were stained accordingly (D), original magnification x 400.

## 3.2. REGULATORY FUNCTION OF SIGIRR

### 3.2.1. Cloning of SIGIRR

Accession numbers NM_021805 (*homo sapiens Sigirr*) and NM_023059 (*mus musculus Sigirr*) from the CoreNucleotide PubMed library were used to design the primers in order to isolate full-length *Sigirr* cDNA from mouse kidney cDNA and human kidney cDNA (for primer sequences see material and methods). The sequences of the human clones contained an open reading frame of 1233 bp which predicted a protein of 410 aa. The mouse clones contained an open reading frame of 1230 bp coding a 409aa protein. The full length SIGIRR contains a short extracellular region (118aa) that corresponds to a single Ig domain. The C-terminal region of SIGIRR, intracellular TIR domain is longer than the typical TIR domain of TLR/IL-1R family members. Also conserved amino acids in the TIR domain, which were shown to be essential for the signaling are changed in SIGIRR compared with others TLR/IL-1R family members.

In order to obtain a full length *Sigirr* cDNA, the PCR amplified products were cut with the restriction enzymes, directly ligated into pCR3 vector (Figure 12) and placed into competent bacteria by the transformation. The quality of the constructs was verified by the restriction enzymes and sequencing. Generated mouse construct contained additionally the FLAG-tag on the N-terminus. The constructs were used later in the transfection experiments, northern blot analysis as well as some other not mentioned in this work side experiments.

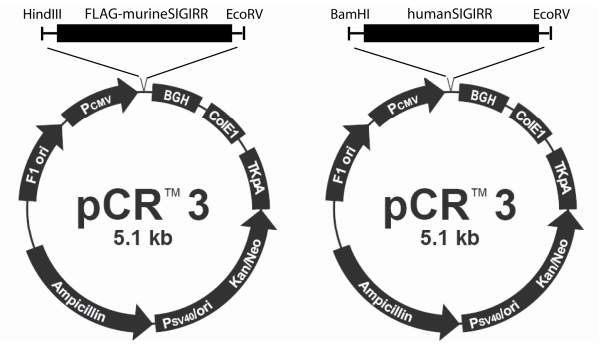


*Figure 12.* **Schematic presentation of the *Sigirr* constructs used in experiments:** Both murine and human *Sigirr* were cloned into pCR3 vector. Murine *Sigirr* was additionally enriched in the FLAG sequence.

### 3.2.2. SIGIRR inhibits TLR-ligands mediated NF-κB activation

In order to examine the potential function of SIGIRR protein in the signalling pathway starting from toll like receptors, the construct containing the open reading frame of murine SIGIRR was overexpressed in murine tubular epithelial cell line. TLR ligands, i.e. Pam3Cys (TLR1/2), poly I:C RNA (TLR3), LPS (TLR4), and CpG-DNA (TLR9) caused 24 h after stimulation increase in NF-κB activation in tubular epithelial cell line. This effect was decreased by transfection with the plasmid encoding SIGIRR (Figure 13). Thus, overexpression of SIGIRR reduced TLR mediated NF-κB reporter activity, indicating an inhibitory function of SIGIRR in TLRs dependent NF-κB actibation. The same experiment was performed in 293HEK cell line. However only very weak inhibition was observed in the 293HEK cells (using the human *Sigirr* construct), which express TLRs only on the basal level suggesting that the effect was TLRs dependent (data not shown).

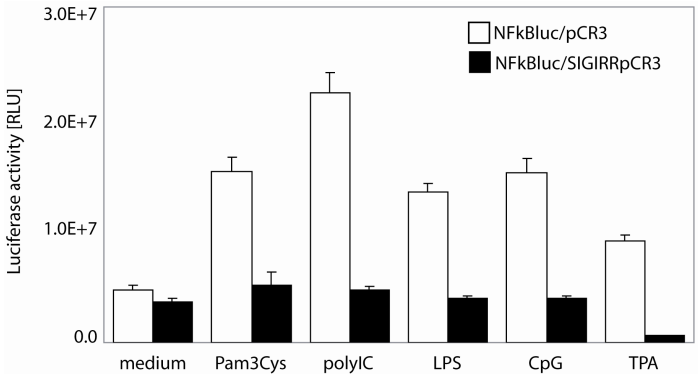


*Figure 13.* **Expression of SIGIRR inhibits the activation of NF-κB:** tubular epithelial murine cell line was co-tranfected with 1µg expression plasmid encoding for murine SIGIRR along with the NFκB reporter plasmid driving luciferase expression. Luciferase activity was messured as described in materials and methods. TPA was used as a positive control. Bars represent mean values from two independent experiments ± SEM.

### 3.2.3. SIGIRR is a negative regulator of TLR/IL-1R signaling in immune but not in tubular epithelial cells

The strong expression of SIGIRR in tubular epithelial cells suggests an inhibitory effect on LPS-induced gene expression, as reported for SIGIRR in splenocytes (Wald 2003). In order to address this

question, primary tubular epithelial cells and spleen monocytes were isolated either from 6 week old wild-type mice or from age-matched mice with various deletions of genes involved in TLR signaling. *CXCL2* mRNA expression was determined by real-time RT-PCR as a readout for LPS-induced chemokine expression. 24 h of stimulation with 1 µg/ml LPS increased *CXCL2* mRNA expression in monocytes and tubular epithelial cells (Figure 14). This effect was mediated through TLR4 as tubular epithelial cells prepared from *Tlr4*-deficient mice did not induce *CXCL2* mRNA in response to LPS. *MyD88*-deficient monocytes showed a reduced response consistent with a contribution of MyD88 on TLR4 signaling which involves additional adaptor molecules. In TLR3- or *Tlr9*-deficient monocytes and tubular epithelial cells LPS-induced CXCL2 expression was not affected. By contrast, *Sigirr*-deficient monocytes mice showed an enhanced induction of LPS-induced *CXCL2* mRNA as compared to wild-type monocytes. Interestingly, in tubular epithelial cells lack of SIGIRR had no effect of LPS-induced *CXCL2* mRNA levels, suggesting that SIGIRR does not inhibit LPS-induced *CXCL2* expression in tubular epithelial cells.

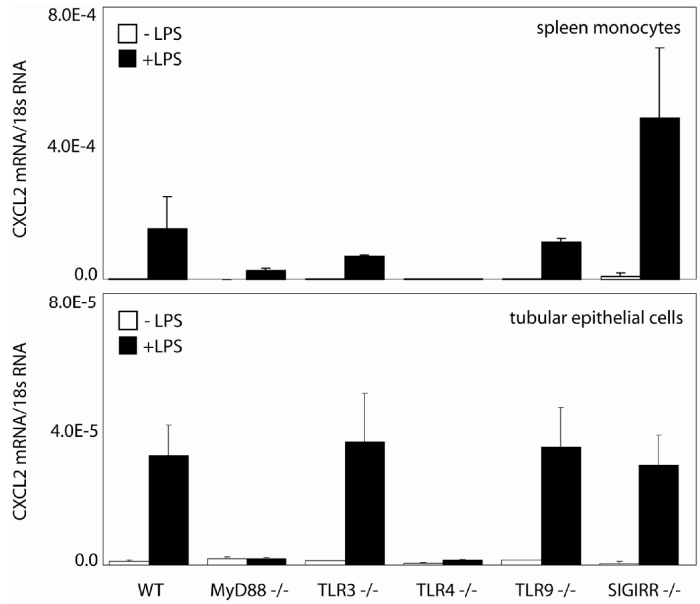


*Figure 14.* **SIGIRR and cell type-specific responses to LPS.** Monocytes (A) and tubular epithelial cells (B) were prepared from 6 week old mice of different strains with C57BL/6 background as indicated (*wild type, MyD88-/-, Tlr3-/-, Tlr4-/-, Tlr9-/- and Sigirr-/-*). Cells were stimulated either with medium or *E.coli* LPS (1 µg/ml) for 24 h. CXCL2 production was determined by real-time RT-PCR and expressed as mean of the ratio CXCL2/18s-rRNA ± SEM. Data represent means ± SEM from 3 independent experiments.

Next it was examined whether the lack of SIGIRR-mediated TLR inhibition in tubular epithelial cells is restricted to TLR4 signaling. Thus, monocytes and tubular epithelial cells were prepared from *Sigirr*-deficient and wild-type mice as above and incubated for 24 h with ligands for respective TLRs: Pam3Cys (ligand for TLR1/2), poly I:C RNA (ligand for TLR3), LPS (ligand for TLR4), and CpG-DNA (ligand for TLR9). CCL2 production was measured by ELISA as another marker for TLR-induced chemokine secretion. Compared to wild-type monocytes *Sigirr*-deficient monocytes produced increased amounts of CCL2 when exposed to TLR ligands (Figure 15). This suggests the inhibitory function of SIGIRR in these cells. Surprisingly this effect was not restricted only to the LPS signalling but to the all used TLR ligands. This effect was not observed in tubular epithelial cells (Figure 15). No response to CpG-DNA was observed in tubular epithelial cells due absence of TLR9 on these cells. These data show that signaling through TLR1/2, -3, and -4 is independent of SIGIRR in tubular epithelial cells, but not in monocytes.

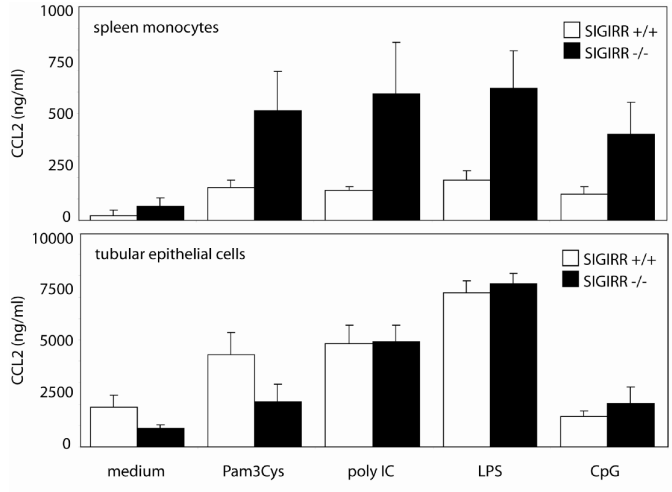


*Figure 15.* **SIGIRR and TLR signaling**: Monocytes and tubular epithelial cells were prepared from 6 week old *Sigirr*-deficient or wild-type mice as indicated. Cells were stimulated either with medium, Pam3Cys, pI:C RNA, LPS or CpG-DNA for 24 h. CCCL2 production was determined in supernatants by ELISA. Data represent means ± SEM from 3 independent experiments.

LPS-induced chemokine expression is mediated through NF-κB. Because transfection of primary spleen monocytes and tubular cells with a NF-κB reporter gene contruct was limited by low cell survival we used a p50 (NLS) antibody to determine nuclear translocation of NF-κB as a marker of NF-κB activation. CD11b positive monocytes and tubular epithelial cells were stained after 1 h of stimulation with either medium or LPS (Figure 16). The data obtained were consistent with that of LPS-induced CCL2 production, which excludes a role for SIGIRR on LPS-induced NF-κB activation in tubular epithelial cells. These data indicate that SIGIRR has cell-type specific functions, i.e. an inhibitory effect on LPS-induced TLR4 signaling in monocytes, which is absent in tubular epithelial cells. LPS-induced TLR signaling is independent of SIGIRR in tubular epithelial cells.

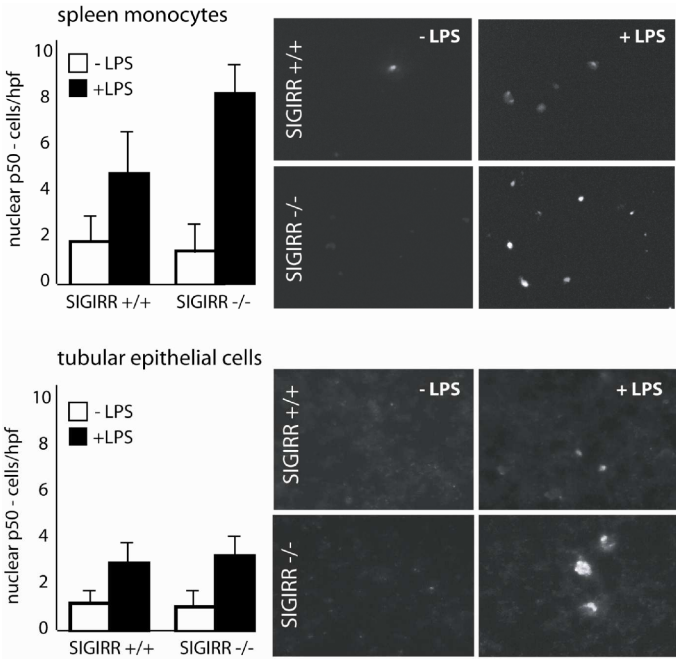


*Figure 16.* **SIGIRR and cell type-specific responses to LPS.** Monocytes and tubular epithelial cells were prepared from 6 week old *Sigirr*-deficient or wild-type mice as indicated. A: After 1 h of stimulation with LPS (1 µg/ml) the cells were stained with a p50 antibody for detection of NFκB activation as described in methods. Data represent means ± SEM of positive nuclei per high power field (hpf).

The same cell type-specific function of SIGIRR was found for signaling via receptors of the IL-1R subfamily, e.g. the IL-18 receptor (Qin 2005). Lack of SIGIRR did not affect IL-18-induced CCL2 production in tubular epithelial cells, while CCL2 production was enhanced in *Sigirr*-deficient monocytes (Figure 17, right and left respectively). These data show that SIGIRR does not affect signaling through TLR/IL-1Rs in tubular epithelial cells.

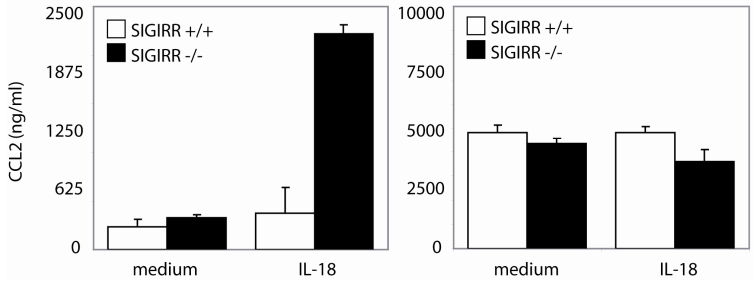


*Figure 17.* **SIGIRR and IL-receptors signaling**: Monocytes (left) and tubular epithelial cells (right) were prepared from 6 week old *Sigirr*-deficient or wild-type mice as indicated. Cells were stimulated either with medium or IL-18 for 24 h. CCL2 production was determined in supernatants by ELISA. Data represent means ± SEM from 3 independent experiments.

In the kidney the renal tubules are surrounded by a dense network of dendritic cells (Soos 2006). Resident intrarenal myloid cells and tubular epithelial cells, contribute to renal TLR4 signaling in infective pyelonephritis with uropathogenic *E. coli* UPEC (Patole 2006). Hence, it was hypothesized that lack of SIGIRR would enhance LPS-induced renal chemokine release as a result of uncoupling TLR4 signaling in both cell types. In fact, kidney slices from *Sigirr*-deficient mice produced much more CCL2 and CXCL2 upon exposure to 1 μg/ml UPEC LPS for 24 h (Figure 18, upper panel). However, when primary tubular epithelial cells or CD11b positive renal myeloid cells were exposed to LPS, lack of SIGIRR was found to enhance LPS-induced CCL2 and CXCL2 production only in the myeloid cells but not in the tubular epithelial cells (Figure 18, middle and lower panel). These data suggest that the suppressive effect of SIGIRR on renal TLR4 signaling relates to SIGIRR in intrarenal myeloid cells and is independent of SIGIRR in tubular epithelial cells.

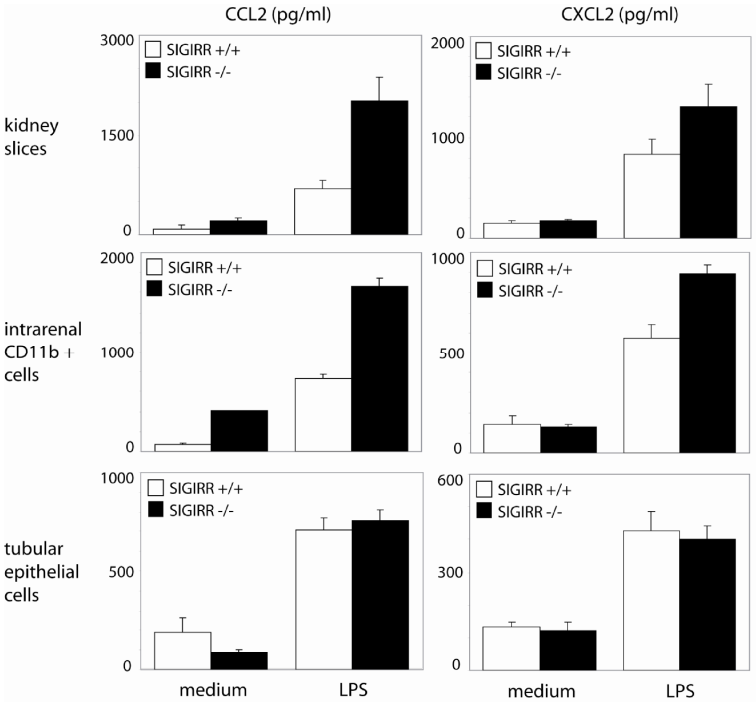


*Figure 18.* **SIGIRR and LPS-induced CCL2 and CXCL2 production in mouse kidneys:** (upper panel): Kidney slices, freshly prepared from wild-type and *Sigirr*-deficient mice, were placed in medium and exposed to 1 μg/ml ultrapure LPS. After 24 h supernatants were harvested and CCL2 (left) and CXCL2 production (right) were measured by ELISA. Data represent means ± SEM from 3 independent experiments. Intrarenal myeloid cells (middle) were prepared from 6 week old wild-type and *Sigirr*-deficient mice by CD11b-magnetic bead isolation and cultured with GM-CSF as mentioned in methods. Tubular epithelial cells (lower panel) were prepared as before. The cells were incubated with either 1 μg/ml ultrapure LPS in medium or medium alone for 24 h. Data represent means ± SEM from 3 independent experiments.

### 3.2.4. SIGIRR is a suppressor of TLR mRNA expression in monocytes but not in tubular epithelial cells

Next it was examined whether the cell type specific effects of SIGIRR on TLR signaling relate to a modulatory effect on *Tlr* expression. Primary spleen monocytes and tubular epithelial cells were kept under normal culture conditions and *Tlr1-9*, and *-11* mRNA expression levels were determined by real-time RT-PCR. Wild-type monocytes expressed nearly all TLRs (Figure 19). *Sigirr*-deficient monocytes expressed much higher levels of *Tlr1* (13-fold), *Tlr5* (23-fold), *Tlr6* (7-fold), *Tlr7* (8-fold), *Tlr9* (12-fold), and *Tlr11* (23-fold) as compared to spleen monocytes isolated from wild-type mice. Lack of SIGIRR did not affect mRNA expression of *Tlr2, -3, -4,* and *-8* in spleen monocytes. By contrast, wild-type tubular epithelial cells, which expressed *Tlr1-4* and *-11*, revealed mRNA

expression levels similar to *Sigirr*-deficient tubular epithelial cells (Figure 19). Apparently, SIGIRR is a suppressor of *Tlr1, -5, - 6, -7, -9,* and *-11* mRNA expression in spleen monocytes, but has no effect on the TLRs expressed by tubular epithelial cells.

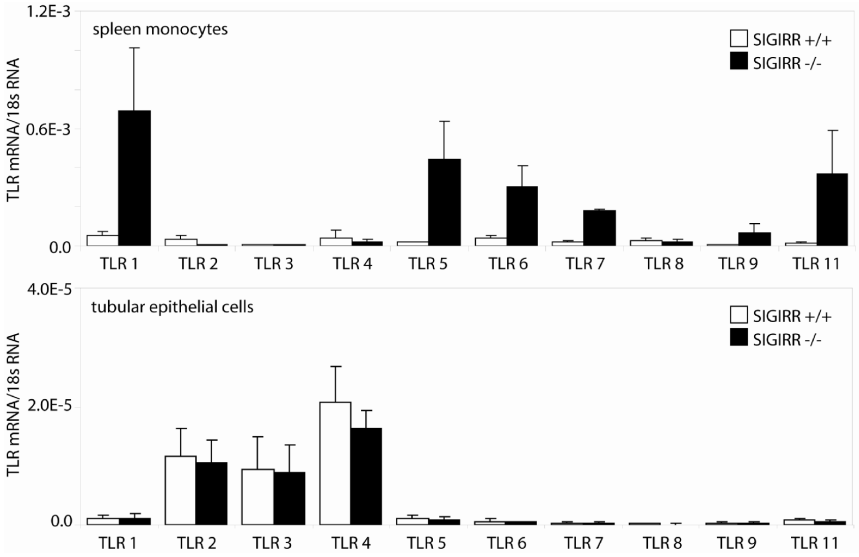


*Figure 19.* **SIGIRR modulates *Tlr* mRNA expression :** Monocytes and tubular epithelial cells were prepared from 6 week old *Sigirr*-deficient or wild-type mice as indicated. *Tlr1-9* and *-11* mRNA expression levels were determined by real-time RT-PCR and expressed as mean of the ratio TLR/18s-rRNA ± SEM.

### 3.2.5. SIGIRR is a suppressor of *Tlr* mRNA expression in dendritic cells

Most renal DCs positive for the CD11c marker posses also CD11b and F4/80 markers indicating that the latter are not only specific for the macrophages in the kidney (Kruger 2004, Soos 2006). Others express macrophage markers (e.g., F4/80 and CD11b) instead the typical for DCs CD11c, and were despite of this fact classified as dendritic cells because of the CX3CR1 expression on the surface (Soos 2006). By contrast the DCs residing in lymphoid tissues such as the spleen show the minor expression of F4/80 macrophage marker (Kruger 2004). CD8a and B220 markers which are typical for lymphoid and plasmacytoid DCs, respectively are not expressed on renal DCs. Like others tissue-resident immature DCs at steady state, also renal DCs show high level of major histocompatibility complex (MHC) class II expression, but low CD80, CD86, and CD40 expression (Soos 2006). Thus,

renal dendritic cells are not a homogeneous cell population and they differ from the DCs found in the lymphoid tissues, but also differs from the DCs found in mucosal tissues.

Because the expression of SIGIRR in renal CD11b positive cells is significant and there are striking differences in SIGIRR glycosylation in these cell types we decided to characterise better the myeloid cells in the kidney. The number of tubular cells exceeds the number of myeloid cells in the healthy kidney and many reports support a functional role of the intrarenal network of myeloid cells in the healthy kidney (Kruger 2004; Soos 2006). We isolated the intrarenal CD11b positive cells from both wild type and *Sigirr*-deficient mice. We have performed flow cytometry for CD11c on the prepared this intrarenal resident cell population and found that 45 % of the CD11b positive cells were also positive for CD11c (data not shown). We decided to investigate this mixed cell population of intrarenal myeloid cells.

Next it was examined whether the SIGIRR has an effect on TLR expression in antigen presenting cells. Primary bone marrow derived dendritic cells or intrarenal myeloid cells were cultured with FLT3 or GM-CSF and *Tlr1-9*, and *-11* mRNA expression levels were determined by real-time RT-PCR (Figure 20). Wild-type dendritic/myeloid cells expressed nearly all *Tlrs*. *Sigirr*-deficient bone marrow dendritic cells cultured with GM-CSF expressed higher levels of *Tlr1* (8-fold), *Tlr2* (13-fold), *Tlr3* (7-fold), *Tlr4* (4-fold), *Tlr5* (21-fold), *Tlr6* (3-fold), *Tlr7* (5-fold), *Tlr8* (5-fold), *Tlr9* (10-fold) and *Tlr11* (20-fold) as compared to bone marrow dendritic cells cultured with GM-CSF, which were isolated from wild-type mice. Similar folds increase were observed by the bone marrow derived dendritic cells cultured with FLT3L (Figure 20, middle), however the *Tlr* expression was significantly lower than in the case of the cells cultured with GM-CSF.

Lack of SIGIRR did not affect that strongly the *Tlr* mRNA expression in the myeloid cells isolated from the kidneys. The intrarenal myeloid cells expressed even strongly the tested *Tlrs*. However the differences between *Sigirr*-deficient and wild type cells are not that visible like by the bone marrow derived cells. *Sigirr*-deficient intrarenal myeloid cells expressed only slightly higher levels of *Tlr1* (3-fold), *Tlr2* (2-fold), *Tlr3* (1,5-fold), *Tlr4* (2-fold), *Tlr5* (4-fold), *Tlr6* (4-fold), *Tlr7* (3-fold), *Tlr8* (2-fold), *Tlr9* (3-fold) and *Tlr11* (2-fold) as compared to intrarenal myeloid cells, which were isolated from wild-type mice. Apparently, SIGIRR is a suppressor of *Tlrs* mRNA expression in antigen

presenting cells, but it is dependent on the stage of the development of these cells and also from the local environment.

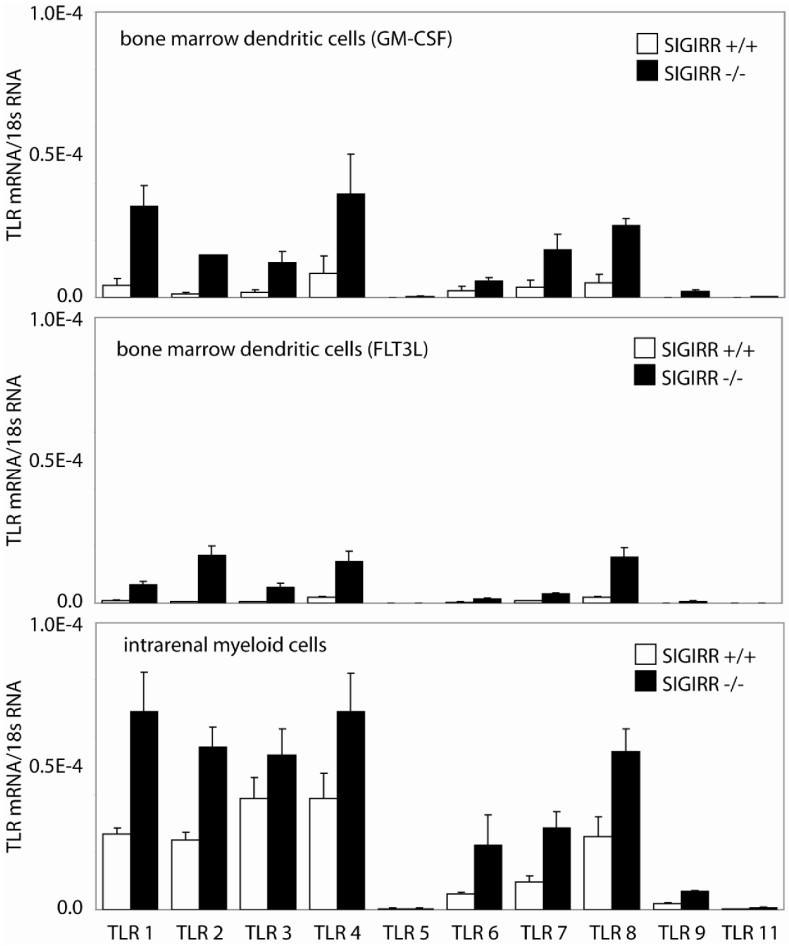


*Figure 20.* **SIGIRR modulates *Tlr* mRNA expression.** Bone marrow dendritic cells stimulated with Flt3L (upper panel) bone marrow dendritic cells stimulated with GM-CSF (middle) or intrarenal myeloid cells (lower panel) were prepared from 6 week old *Sigirr*-deficient or wild-type mice as indicated. *Tlr 1-9* and *-11* mRNA expression levels were determined by real-time RT-PCR and expressed as mean of the ratio TLR/18s-rRNA ± SEM.

### 3.2.6. SIGIRR is a suppressor of TLR signaling in dendritic cells

Next, we questioned whether cells respond to TLR ligands as predicted from their respective TLR expression profile. It was examined whether the lack of SIGIRR-mediated TLR inhibition in antigen presenting cells affecting the TLRs signaling. Thus, bone marrow derived or intrarenal antigen presenting cells were prepared from *Sigirr*-deficient and wild-type mice as above and incubated with ligands for respective TLRs: Pam3Cys (TLR1/2), poly I:C RNA (TLR3), LPS (TLR4), imiquimod (TLR7) and CpG-DNA (TLR9). Bone marrow dendritic cells (cultured with GM-CSF or Flt3L) or the intrarenal myeloid cells from wild-type mice produced IL-6 and IL-12 in response to TLR ligands (Figure 21). However, the ability of *Sigirr-/-* cells to produce these inflammatory cytokines in response to the same ligands was signicantly increased in case of all three tested cell types. Moreover, the production of IL-6 and IL12 was higher in case of bone marrow dendritic cells cultured with GM-CSF and the intrarenal myeloid cells than in case of bone marrow dendritic cells cultured with Flt3L. These differences may be due to the different expression levels of the TLRs.

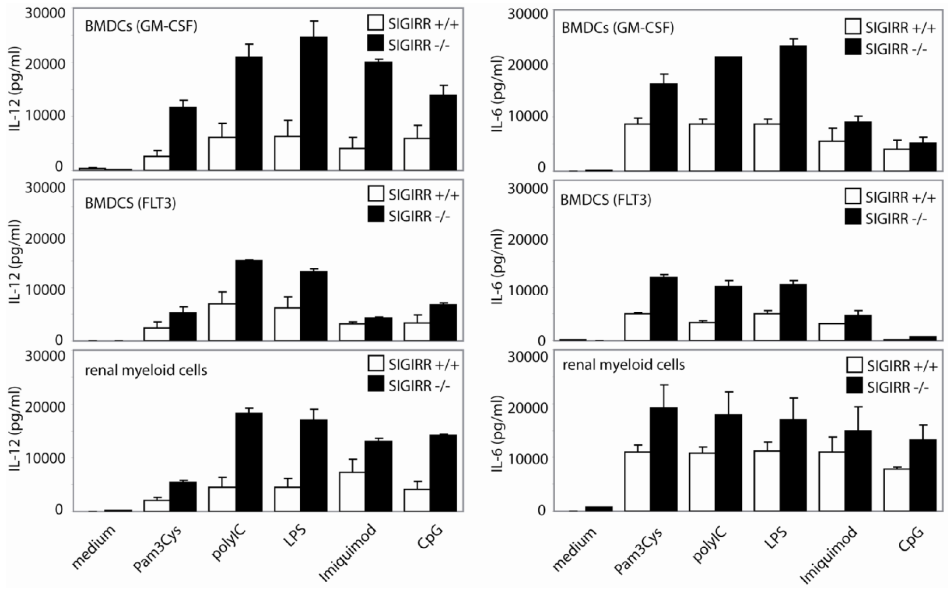


*Figure 21.* **SIGIRR modulates TLR-induced IL12 and IL-6 production.** Bone marrow dendritic cells stimulated with GM-CSF (upper panel) bone marrow dendritic cells stimulated with Flt3L (middle) or intrarenal myeloid cells (lower panel) were prepared from 6 week old *Sigirr*-deficient or wild-type mice as indicated. Cells were stimulated either with medium, Pam3Cys, pI:C RNA, LPS, imiquimod or CpG-DNA for 24 h. IL-12 (left) or IL-6 (right) production was determined in supernatants by ELISA. Data represent means ± SEM from 3 independent experiments.

Also CCL2 (MCP-1) production was measured by ELISA as another marker for TLR-induced chemokine secretion (here the tubular epithelial cells included). Compared to wild-type bone marrow dendritic cells cultured with GM-CSF and intrarenal myeloid cells *Sigirr*-deficient suitable cell populations produced increased amounts of CCL2/MCP1 when exposed to additional TLR ligands, i.e. Pam3Cys (TLR1/2), poly I:C RNA (TLR3), LPS (TLR4), imiquimod (TLR7) and CpG-DNA (TLR9). Only very small differences were observed in the production of CCL2 between wild-type and *Sigirr-/-* bone marrow derived dendritic cells cultured with Flt3L in response to all tested TLR ligands, indicating that development stage of the dendritic cells plays an important role in the signalling of these cells. No effect was observed in tubular epithelial cells, indicating that signaling through TLRs is independent of SIGIRR in tubular epithelial cells, but not in antigen presenting cells. However the presented data proved that SIGIRR is a strong negative regulator of TLR mediated signalling in DCs (cultured with GM-CSF) and the intrarenal myeloid cells (Figure 22).

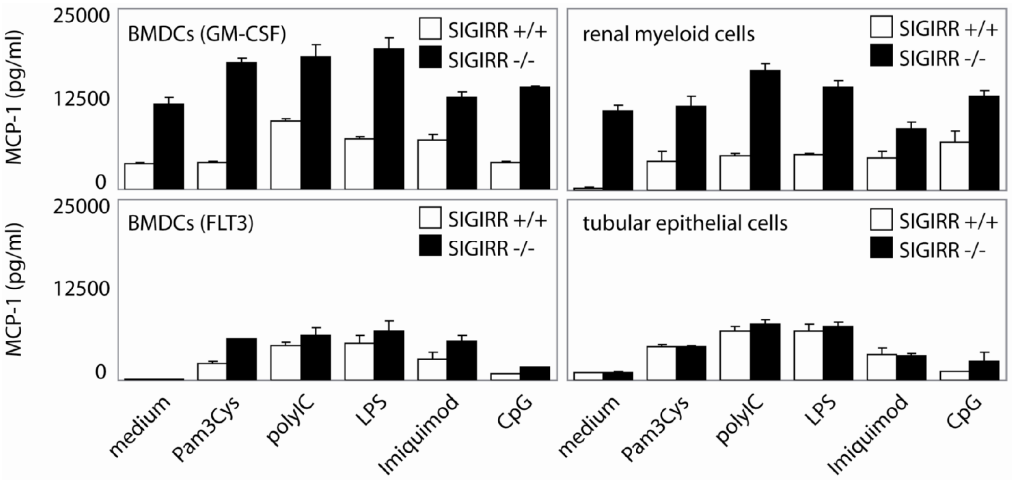


*Figure 22.* **SIGIRR modulates TLR-induced MCP1/CCL2 production.** Bone marrow dendritic cells stimulated with GM-CSF, intrarenal myeloid cells, bone marrow dendritic cells stimulated with Flt3L or tubular epithelial cells were prepared from 6 week old *Sigirr*-deficient or wild-type mice as indicated. Cells were stimulated either with medium, Pam3Cys, pI:C RNA, LPS, imiquimod or CpG-DNA for 24 h. CCL2/MCP-1 production was determined in supernatants by ELISA. Data represent means ± SEM from 3 independent experiments.

## 3.3. REGULATION OF SIGIRR EXPRESSION

### 3.3.1. The regulation of *Sigirr* expression is cell type-specific

It was determined whether *Sigirr* expression is regulated by the presence of TLRs by assessing *Sigirr* mRNA levels in monocytes and tubular epithelial cells isolated from 6 week old mice deficient for either *MyD88* or *Tlrs* that are expressed by both cell types, i.e. TLR2, -3, and -4. In *Tlr4*-deficient monocytes *Sigirr* mRNA levels were found to be increased 5-fold as compared to monocytes prepared from wild-type mice (Figure 23). By contrast, lack of TLR2, -3, or MyD88 had no major effect on *Sigirr* mRNA expression in monocytes. In tubular epithelial cells, lack of TLR4 had a similar effect on *Sigirr* mRNA levels (Figure 23). However, *Sigirr* mRNA expression was reduced in tubular epithelial cells prepared from mice lacking TLR2 or -3. As in monocytes lack of MyD88 did not affect *Sigirr* mRNA expression. While TLR2 and -3 seem to have slightly different impact on *Sigirr* mRNA expression in monocytes and tubular cells, the presence of TLR4 downmodulates *Sigirr* mRNA in both cell types.

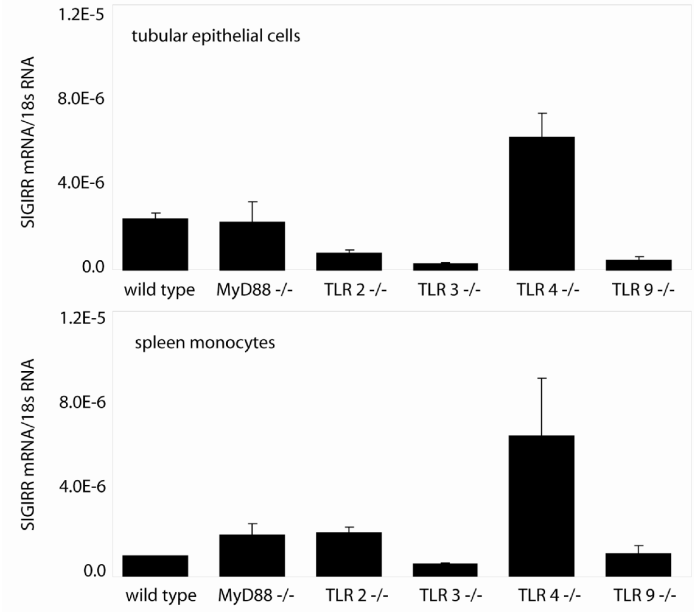


*Figure 23.* **Regulation of *Sigirr* by TLR:** Spleen monocytes and tubular epithelial cells were prepared from 6 week old mice of different strains as indicated. *Sigirr* mRNA expression levels were determined by real-time RT-PCR and expressed as mean of the ratio TLR/18s-rRNA ± SEM.

As *Tlr4* deficiency was associated with increased *Sigirr* mRNA expression in monocytes and tubular epithelial cells, LPS stimulation should suppress *Sigirr* mRNA levels. To test this hypothesis the monocytes and tubular epithelial cells were stimulated with LPS or a combination of IFNγ and TNFα. In fact, both LPS and IFNγ/TNFα reduced *Sigirr* mRNA levels in tubular epithelial cells (Figure 24). By contrast, LPS and IFNγ/TNFα both increased *Sigirr* expression in monocytes in a dose dependent manner with highest mRNA levels after 18 h (Figure 24). These data show that *Sigirr* is regulated differently in tubular epithelial cells and monocytes by LPS as well as by IFNγ and TNF.

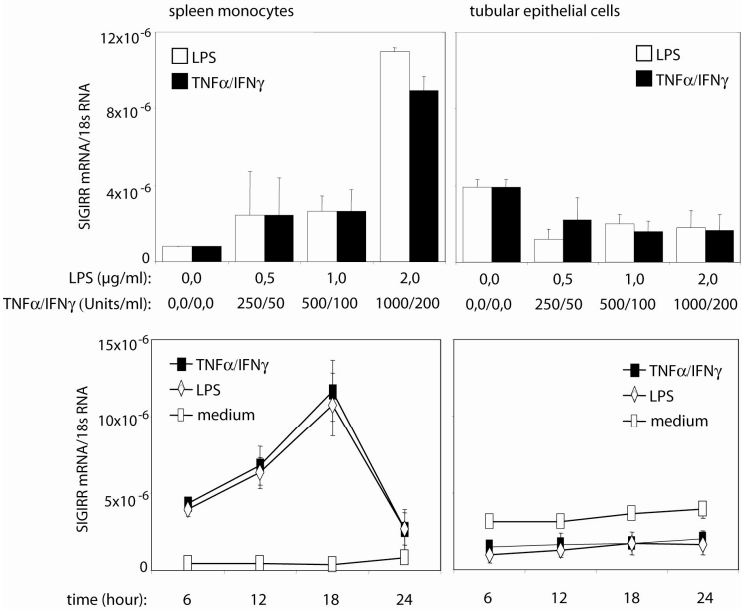


*Figure 24.* **Regulation of *Sigirr* by IFNγ and TNFα:** Monocytes (left) and tubular epithelial cells (right) were prepared from 6 week old C57BL/6 mice; (upper panel): cells were stimulated with either medium, LPS or IFNγ + TNF (different concentrations as indicated). *Sigirr* mRNA expression levels were determined after 18 h by real-time RT-PCR and expressed as mean of the ratio SIGIRR/18s-rRNA ± SEM. (lower panel): cells were stimulated with either medium, LPS (1 µg/ml) or IFNγ (100 U/ml) + TNF (500 U/ml) *Sigirr* mRNA expression levels were determined after 1, 6, 12, 18 or 24 h by real-time RT-PCR and expressed as mean of the ratio SIGIRR/18s-rRNA ± SEM.

### 3.3.2. Immune cells and renal tubular epithelial cells both express full length *Sigirr*

Alternative splicing is common in members of the TLR/IL-1R family (Wells 2006). To test whether kidney and spleen cells express different splice variants of the *Sigirr* gene, the real-time RT-PCR using primers specific for the intracellular or extracellular domain of *Sigirr* was performed (Figure 25). Spleen monocytes and tubular epithelial cells expressed comparable levels of both the intracellular and extracellular domain of *Sigirr* (Figure 25).

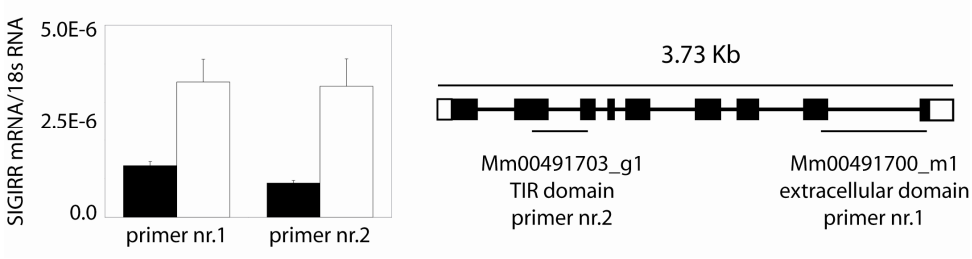


*Figure 25.* ***Sigirr*** **transcripts:** Tubular epithelial cells and spleen monocytes were prepared from 6 weeks old *Sigirr*-deficient or wild-type mice. An expression level of *Sigirr* mRNA was determined by using primers specific for either the extracellular (primer 1) or the intracellular domain (primer 2) of *Sigirr*. The respective mRNA expression levels using primer 1 and primer 2 were determined by real-time RT-PCR using cDNA prepared from monocytes (black) and tubular epithelial cells (white) and expressed as mean of the ratio to 18s-rRNA ± SEM.

Only one splice variant of murine *Sigirr* mRNA was characterized using the Genomatix library. This was confirmed by Northern Blot analysis. Northern blot analysis of mouse *Sigirr* showed that it is expressed in both kidney and spleen. We found hybridization to RNA from kidney and spleen. Total RNA was extracted from kidneys and spleens from wild type and *Sigirr*-deficient mice. The RNA was hybridized with a $P^{32}$ –labeled full length (data not shown) or extracellular part of *Sigirr* (Figure 26) DNA probe. A single *Sigirr* mRNA transcript of 3.5 kB was detected in both kidney and spleen and no splice variants were detected (Figure 26).

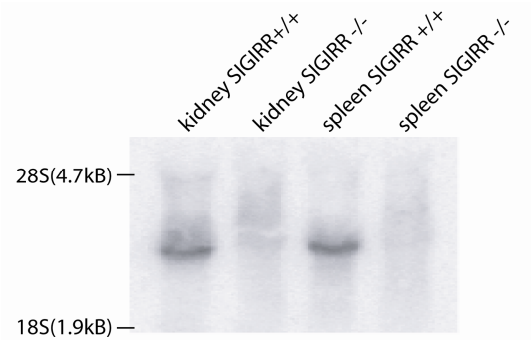


*Figure 26.* ***Sigirr*** **transcripts:** Northern blot analysis was performed on RNA isolates (10 µg per lane) from spleens and kidneys of wild-type (WT) and *Sigirr*-deficient mice (KO) as described in methods. Note a single *Sigirr* transcript of 3.5 kB in spleen and kidney of wild-type mice. The quality of RNA used in the experiment was investigated by the agarose RNA gel (28S and 18S) and by the RNA concentration measurements (OD 260/280).

## 3.4. POSTTRANSLATIONAL MODIFICATION OF SIGIRR

### 3.4.1. SIGIRR glycosylation is cell type specific

When tubular epithelial cells and monocytes both express full length SIGIRR the cell type-specific effects on TLR signaling may relate to posttranslational modifications. Like the other IL-1R family members, SIGIRR is highly glycosylated. There are five putative sites for N-glycosylation (N–X–S/T, where X is any amino acid except proline) within the extracellular region mouse SIGIRR. The more complexed O-linked glycosilation is not easy to predict in this case but SIGIRR have several threonine and several serines which might be O-glycosilated. The molecular weight of the native mouse SIGIRR differs from the amino acids sequence-calculated form of SIGIRR suggesting extensive glycosylation , which is consistent with the previous speculations (own data, Thomassen 1999).

Thus, we tested whether SIGIRR is differentially glycosylated in the intrarenal immune cells and tubular epithelial cells. PNGaseF was used as a tool which can digest N-linked sugar components, but not N-glycans with fucose linked α-1.3 to the Asn-bound N-acetylglucosamine, O-linked oligosaccharides or glycosylphosphatidylinositol (GPI) lipid anchors from glycoproteins, and hence,

allows the detection of glycosylation variants.

In fact, Western blot of total kidney protein isolates revealed two SIGIRR–specific bands of 75 and 90 kDa (Figure 27, middle). Next we prepared renal cell suspensions from isolated CD11b positive myeloid cells and CD11b negative renal cells of non-myeloid origin. The renal myeloid cells expressed the larger 90 kDa form of SIGIRR while the non-myeloid renal cells predominantely expressed the smaller form of 75 kDa (Figure 27, middle). Protein size depends on protein glycosylation. We tested the glycosylation of SIGIRR in the two types of renal cells by digesting protein isolates from renal myeloid and non-myeloid cells with PNGaseF. This glycosidase reduced the molecular mass of SIGIRR in both CD11b positive and negative renal cells exactly in the same pattern like in case of kidney and spleen (Figure 27, left). CD11b positive and negative renal cells both carry SIGIRR glycosylated at identical sites, digested by PNGaseF to a 60 kDa protein. The CD11b negative (non-immune) renal cells, however, contained additional SIGIRR glycosylation variants that are digested by PNGaseF to two other variants with a size of approximately 45kDa and 50 kDa.

When the protein exctracts were digested with endo-O-glycanase, which removes the distal Gal-beta-(1,3)-GalNAc no significant differences in non-digested and digested SIGIRR size were detected in both renal tubular epithelial and renal CD11b+ cells. Since the digestion with this enzyme results often in only very small shifts on a gel we concluded that SIGIRR size is too big and the detected signal surface is too wide to observe significant shift on a gel after digestion of the protein. It might be that SIGIRR lack the Gal-beta-(1.3)GalNAc links in both cell types (data not shown).

Although protein O-glycosylation is a major posttranslational modification, it is poorly understood compared with N-glycosylation. Jacalin (lectin from the jackfruit *Artocarpus integrifolia*) is an important tool in functional analysis of mucin-type glycoproteins and glycopeptides. Mucin-type O-glycosyaltion starts with the attachment of alpha N-acetylgalactosamine (α-GalNAc) to a Ser/Thr residue and then proceeds through the transfer of various sugars. To test if the SIGIRR in both tubular epithelial and renal CD11b+ cells is O-glycosylated the immunostaining with HRP-conjugated jacalin was performed. Jacalin bound rather unspecific to the membrane. It was

impossible to distinguish the SIGIRR specific bands, even when the protein-extracts from *SIGIRR*-deficient mice were separated on the same gel (data not shown). Jacalin-based specification of SIGIRR glycosylation may not be readily feasilble with whole-organ/cell protein-extracts. Immunostaining with the monoclonal O-linked N-acetyloglucosamine antibody CTD110.6 gave really weak signal, and there were several proteins having similar size like SIGIRR (which appear on the gels of *SIGIRR*-deficient cells and which are heavily glycosylated; data not shown).

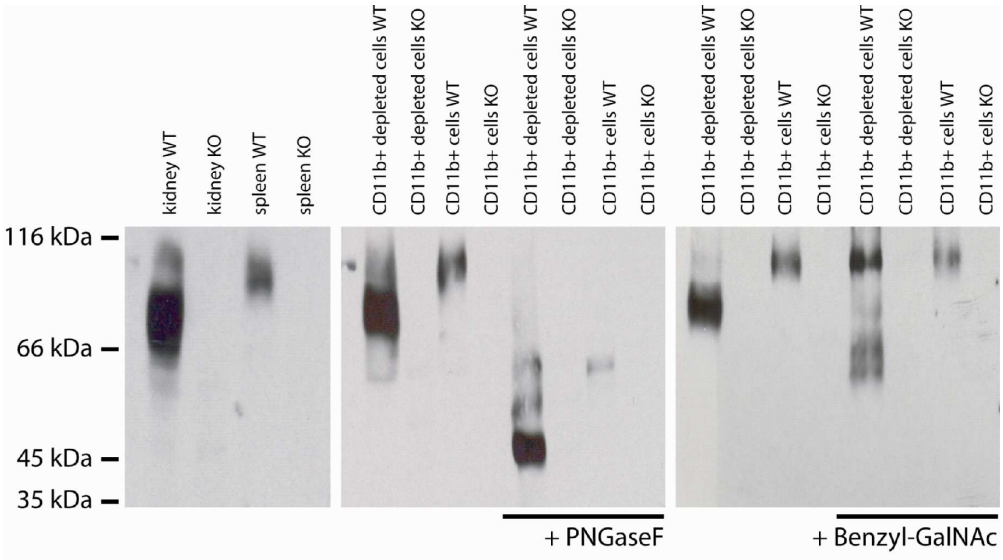


*Figure 27*. **SIGIRR glycosylation:** Western blot was performed on protein isolates derived from total kidney or from renal cell suspensions seperated by magnetic beads isolation for CD11b positive and negative cells, i.e. renal myeloid cells and mostly tubular epithelial cells. Note that the smaller but predominant of the two SIGIRR -specific bands seen in total kidney isolates derives exclusively from CD11b negative renal cells. The specificity of the bands is indicated by their absence in isolates from *Sigirr*-deficient mice. PNGase digests the SIGIRR glycoforms from bot cell types to smaller but still different proteins indicative of cell-type specific N-glycosylation of SIGIRR (middle). Inhibiting O-glycosylation with benzyl-GalNAc reduces the size of the smaller glycoform of SIGIRR in renal CD11b- cells but not the larger form of SIGIRR present in both cell types (right).

To further investigate the O-glycosylation of SIGIRR in renal immune and non-immune cells, benzyl-N-acetylgalactosamine (benzyl-GalNAc), an inhibitor of O-glycosylation, was used during cells culturing. Benzyl-GalNAc treatment did not change the size of the larger glycoform of SIGIRR in renal myeloid and non-myeloid cells (Figure 27, right); hence, the larger SIGIRR glycoform is not

O-glycosylated. By contrast, exposing renal non-immune cells to benzyl-GalNAc reduced the size of the smaller predominant SIGIRR glycoform to about 60 kDa, suggestive of O-glycosylation of this protein (Figure 27, right). These data show that the cell type-specific functions of SIGIRR in the kidney do not relate to different splicing but are associated with different glycosylation variants.

The inhibition of O-glycosylation by benzyl-N-acetylgalactosamine we noted a significant reduction of the SIGIRR size in protein extracts from renal tubular epithelial cells but not from myeloid renal cells, like already mentioned above. This would indicate significant differences in SIGIRR O-glycosylation in these cell types.

## 3.5. OTHER FUNCTIONS OF SIGIRR

### 3.5.1. SIGIRR influences the maturation of the intrarenal myeloid cells

Since we observed such striking differences in the signalling of the *Sigirr* deficient and wild type antigen presenting cells as well as the differences between these cells and tubular epithelial cells we decided to investigate the other features of both cell populations. Mature DCs unlike the macrophages are efficient T cell activators and posses little phagocytic and bactericidal activity. However the maturity of renal DC is rather low (Kruger 2004) compared with DCs from lymphoid organs (Merad 2002). Higher phagocytic activity and less efficient T cell activation of renal DCs may be an evidence for some macrophage functionality (Kruger 2004), and that under infectious circumstances renal CD11c+ cells may take over the macrophage effector functions (Serbina 2003).

Intrarenal myeloid cells from *Sigirr*+/+ and *Sigirr*-/- mice were compared for their ability to take up FITC–dextran. LC, immature DC and macrophages all showed high levels of endocytoxic activity as compared to negative control cells by which the take up experiment was performed at 4$^{o}$C in the presence of natriumacid (data not shown). The FITC–dextran take up by wild type myeloid cells showed the one clear peak. In contrast, *Sigirr*-deficient cells showed the two populations of the cells which were able to internalise the dextran (Figure 28).

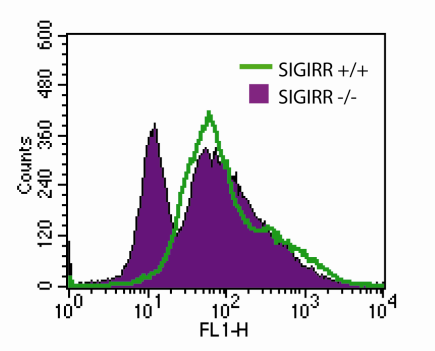


*Figure 28.* **Comparison of FITC-dextran take up by the intrarenal myeloid cells:** SIGIRR deficient and wild type intrarenal CD11b+ myeloid cells were prepared like described in material and methods and compared in their ability to take up the FITC-labeled dextran. After 90 min. incubation with the FITC labelled dextran the cells were collected, washed and analysed by the flow cytometry.

The immature dendritic cells found under surface epithelia and in the solid organs (including kidney) are not very potent stimulators of the T cells (Guermonprez 2002, Steinman 1995). The immature phenotype is characterised by the low level of costimulatory molecules and a high ability to take up the antigens (Cella 1997, Mellman 2001). Mature, already active dendritic cells rapidly loose the ability to take up the antigens. In the case of *Sigirr*-deficient cells we observe two populations of cells. We conclude that the first peak represent the mature dendritic cells which shows lower take up of the antigen but are able to produce more proinflammatory cytokines and may activate better the T cells. The second peak observed also by the wild type cells represents the immature dendritic cells where antigen take up is really potent.

### 3.5.2. SIGIRR does not influence the proliferation of tubular epithelial and antigen-presenting cells

Since cell growth is a result of interplay between a variety of cellular processes involving rearrangements of the cytoskeleton, growth rate of *Sigirr* knock-out cells was determined and compared with that of wild-type tubular epithelial cells. The cell proliferation was investigated by *CellTiter 96* proliferation assay in complete medium at 37°C after the stimulation with the TLR

ligands. Incubation of the tubular epithelial renal cells with TLR ligands, i.e. Pam3Cys (TLR1/2), poly I:C RNA (TLR3), LPS (TLR4), and CpG-DNA (TLR9) resulted in dose dependent decrease of cell proliferation. Under all conditions, no significant differences between *Sigirr*-/- and *Sigirr*+/+ cells were observed (Figure 29). Thus, SIGIRR has no influence on proliferation of renal tubular epithelial cells.

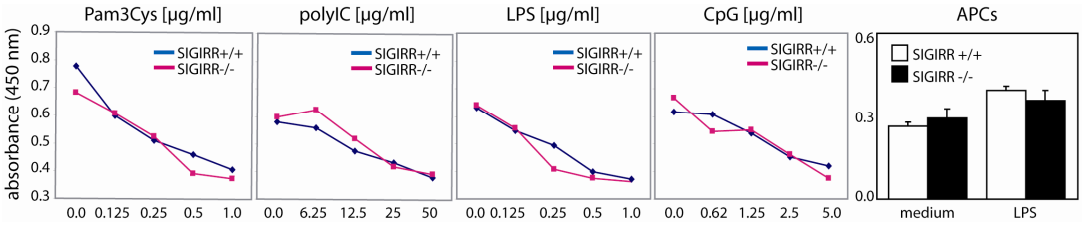


*Figure 29.* **Renal tubular epithelial cells proliferation:** (curves): Renal tubular epithelial cells were isolated from wild type or *Sigirr*-deficient mice and were cultured with different TLR ligands: Pam3Cys, polyIC, LPS, CpG or complete medium for 72 h. (Bars): Bone marrow dendritic cells (APCs) were isolated from wild type or *Sigirr*-deficient mice and incubated with LPS or with medium for 72 h. Cells proliferation was assessed by *CellTiter 96* assay.

The proliferation of antigen presenting cells was also not affected by the SIGIRR. By contrast the antigen presenting cells multiply better then the tubular cells after the stimulation (was shown only for LPS).

### 3.5.3. The function of SIGIRR during stress conditions

Some proteins that are not essential under optimal culturing conditions might play a role under stress conditions. Since SIGIRR is expressed in the renal tubular epithelial cells it is possible that it is responsible for the survival in the osmotic stress conditions. Synthesis of stress proteins and accumulation of compatible osmolytes are responsible for recovery of cells in response to exposure of cells to high osmolarities (Kwon and Handler, 1995). Kidney cells should be able to quickly respond to changes in cell volume and survive the osmotic stress. For this reason, growth of *Sigirr*-deficient cells was determined under different osmotic conditions and compared with that of the wild-type cells. The production of the inflammatory cytokines by the wild type and *Sigirr* knock-out cells

in the presence of increased osmolarity was determined. Growth complete medium was supplemented with NaCl and cultures were grown in this medium under optimal conditions. In the presence of NaCl the cells increased the production of cytokines. There were no significant differences in the *CXCL2* expression between wild type and *Sigirr* knock-out tubular epithelial cells (Figure 30, upper panel). These results suggest that the SIGIRR does not affect the cell metabolism under conditions of increased osmolarity. Similar results were obtained after incubating the cells with different concentration of glucose, urea or raffinose (data not shown).

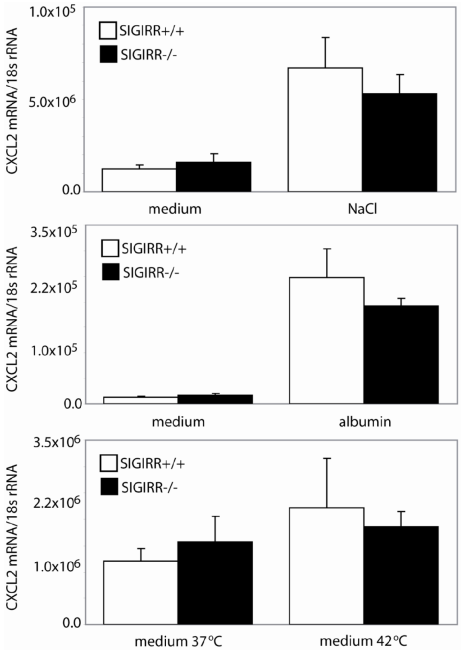


*Figure 30.* **The function of SIGIRR during stress conditions**: Tubular epithelial cells were prepared from 6 week old *Sigirr*-deficient or wild-type mice as indicated. Cells were cultured under osmotic stress conditions for 24 h (upper panel); stimulated with albumin or medium for 24 h (middle); cultured under temperature stress conditions for 24 h (lower panel). *CXCL2* mRNA expression levels were determined by real-time RT-PCR and expressed as mean of the ratio CXCL2/18s-rRNA ± SEM.

Albumin, a major blood protein, is retained in the blood because it does not cross the glomerular filter into the kidney. The fraction that is filtered is reabsorbed by proximal tubule cells, which efficiently removes albumin from the filtrate. In many renal diseases, injury to the glomerulus breaks down the barrier function of the glomerulus, leading to excess filtration of albumin, which is a well known marker for renal disease with a direct correlation between albuminuria and the progression of chronic kidney disease to end-stage renal disease. Exposure to high concentrations of albumin leads to tubular interstitial disease, possibly by inducing apoptosis. The renal tubular epithelial cells were cultured in the presence of the albumin and *CXCL2* was again used as a read out of inflammatory responses. The level of *CXCL2* expression was equal in both wild type and *Sigirr*-deficient tubular epithelial cells exposed to albumin (Figure 30, middle).

Wild-type cells and *Sigirr* knock-out renal tubular epithelial cells were also grown under conditions of high-temperature stress. During the first hour of exposing the cells to heat stress, the cells usually produce heat shock proteins. However, longer heat shock conditions leads to the shift to the normal pattern of protein synthesis. To test if the SIGIRR is responsible for the stabilization of the cells exposed to the temperature stress the tubular epithelial cells which normally grow at 37ºC, were maintained at a temperature of 42ºC (constantly or for the short 1-hour-long period of time). The effect of continuous heat stress on cell growth was analyzed as were the modifications occurring with protein synthesis, cell growth and cell apoptosis during both long- and short-term heat stresses (Figure 30; data not shown).

The reaction on all tested stress conditions seems to be SIGIRR independent. Investigation of the tubular epithelial cells apoptosis and necrosis during same stress conditions revealed similar results (data not shown).

### 3.5.4. SIGIRR is not a cell-cell contact molecule:

Next we investigated a possible role of SIGIRR as a cell adhesion or contact molecule. Cell Adhesion Molecules (CAMs) are proteins located on the cell surface responsible for the binding with other cells or with the extracellular matrix (ECM) during cell adhesion. These proteins are like SIGIRR, transmembrane receptors. Many of the cell adhesions molecules are highly glycosylated. Some of them, like immunoglobulin-superfamily CAMs (NCAMs Neural Cell Adhesion Molecules, Intercellular adhesion molecules - ICAMs, VCAM-1 Vascular Cell Adhesion Molecule, PECAM-1 Platelet-endothelial Cell Adhesion Molecule, L1 and CHL1) contain also the immunoglobulin domains.

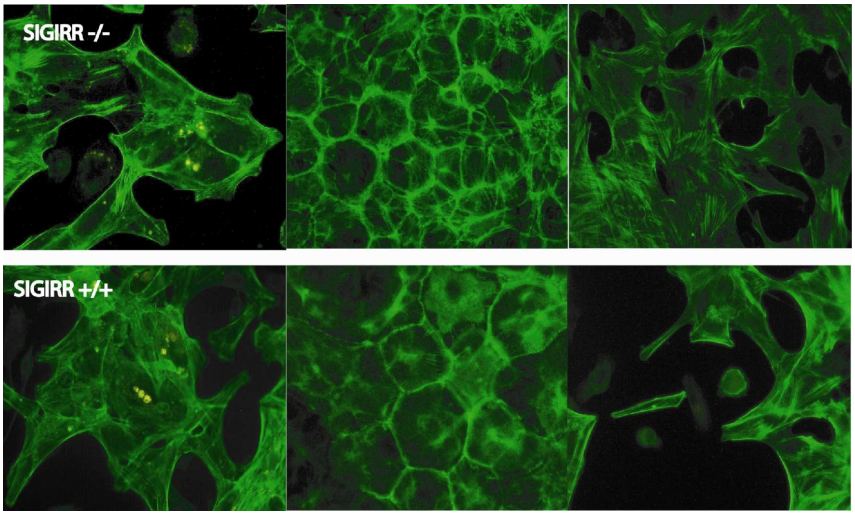


*Figure 31*. **Staining of microfilaments in tubular epithelial cells.** Tubular epithelial cells were prepared from 6 week-old *Sigirr*-deficient or wild-type mice as indicated. Cells were cultured under normal conditions on the glass plates coated with collagen IV. Staining of microfilaments was performed with FITC-phalloidin. Every result consists of three photos representing the typical forms of tubular epithelial cells found in the culture.

Cell adhesion proteins hold together the components of solid tissues and are important for the function of migratory cells like white blood cells. Regulation of cell adhesion proteins is important during embryonic development for the process of morphogenesis. Cell adhesion proteins are also important for interactions that allow viruses and bacteria to enter or damage the cells. The cellular structures such as the cell cytoskeleton are together with the cell membrane responsible for the structural integrity of the cell. Since no differences between *Sigirr*-/- and *Sigirr*+/+ renal tubular

epithelial cells were observed as it comes to cell proliferation and cell survival, the structure of the cytoskeleton and the cell shape were analyzed. The cells were grown on the collagen or on matrigel in the complete medium. After 10 days the cells with the 70% of confluence were stained with phalloidin to stain the cytoskeleton. It is often impossible to distinguish any interior detail with conventional microscopy so the stained cells were also carefully studied under the fluorescent or the confocal microscope. No differences in the structure between *Sigirr*+/+ and *Sigirr*-/- tubular epithelial cells were observed (Figure 31). Both cell populations formed identical cell formation and the contacts between the cells were not affected.

## 3.6. THE ROLE OF SIGIRR IN SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

### 3.6.1. Characterization of C57BL/6 *lpr/lpr* mice

Although TLRs are crucial in the activation of the antimicrobial immune response they can also recognize self-antigens released from stressed or damaged host cells, and such self-recognition may lead to development of autoimmune disease (Anders 2007, Viorritto 2007). Because SIGIRR seems to be a negative regulator for many TLRs it may have a function also in development of autoimmunity (Wald 2004, Garlanda 2003). SLE is a chronic autoimmune condition with a wide spectrum of clinical manifestations, characterized by the production of auto-antibodies to components of the cell nucleus. Lupus nephritis, a form of immune-complex glomerulonephritis is one of the disease manifestations of SLE.

To investigate the role of SIGIRR in SLE we used a C57BL/6 *lpr/lpr* mouse model. It has been demonstrated that the *lpr* gene codes for a mutant Fas/CD95/Apo-1, that leads to abnormal transcription and greatly reduced expression of the Fas/CD95/Apo-1 receptor, which is expressed on variety of hematopoietic cells (T cells, B cells, monocytes, and granulocytes). It is responsible for regulation of apoptosis of these cells during the immune response. The *lpr* mice suffer from anomalies of the B-cell compartment, concerning both early and late differentiation stages. C57BL/6 *lpr/lpr* mice are a model for human lupus nephritis because renal disease in these mice develops

secondary to dsDNA autoantibody production and renal immune complex deposition. However, these changes appear relatively late, not like in case of other mice disease model MRL *lpr/lpr* mice where, in female the serum levels of double-stranded DNA (dsDNA) antibodies progressively increased from 8 weeks of age. Increasing levels of serum dsDNA autoantibodies are associated with immune complex deposits in glomeruli. In C57BL/6 *lpr/lpr* mice abnormalities of immune system develop around 24$^{th}$ week of age. Homozygotes mice for the *lpr* gene develop an age-dependent lupus-like autoimmune disease and a severe lymphadenopathy and splenomegaly.

Mice were breed under sterile conditions (see material and methods) and the visible symptoms of the disease were carefully investigated. The first notes about the increased lymph nodes in C57BL/6 *lpr/lpr* mice were made after 16 weeks of their life, however these first changes appeared so early only in around 20 % of C57BL/6 *lpr/lpr* mice. Also the typical visual features like the butterfly rashes were observed first after 32 weeks (Figure 32). After 32 and later after 48 weeks the lymph nodes (weight 1.1 g ± 0.3) of the C57BL/6 *lpr/lpr* mice were significantly increased. Also the spleens were around 2-3 times increased as compared to the 16 weeks old C57BL/6 *lpr/lpr* mice. The average weight of spleens from the 48 weeks old female C57BL/6 *lpr/lpr* mice was approximately 0.23 g ± 0.4, whereas weight of spleens from the 16 weeks old female from the same background was 0.11 g ± 0.2. At the 48$^{th}$ week of life mice lost around 30% of the hair (mostly the front side, figure 32), however despite of all these symptoms they were still active.

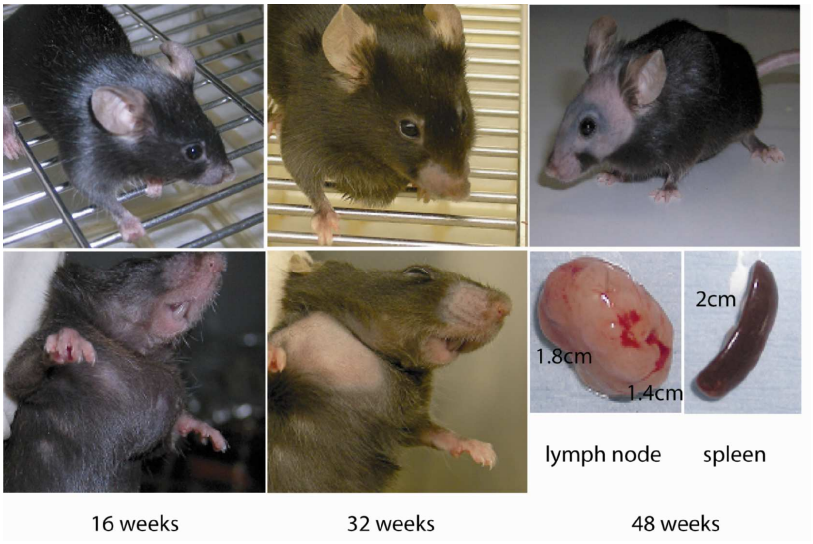


*Figure 32*. **C57BL/6 *lpr/lpr* mice morphology:** mice were kept under the sterile conditions. The documentation was made every month. First visible syndromes of the SLE were detectable after 16 weeks (only small changes in around 20% of the mice). Later (32, 48 weeks) mice develop lymphadenopaty and severe skin lesions.

The antibodies and levels of proinflammatory cytokine in serum as well as the albumin level in the urine were monitored every 30 days.

Serum levels of IL-12 in C57BL/6 *lpr/lpr* mice were determined by ELISA. We observed that C57BL/6 *lpr/lpr* mice did not increase dsDNA-specific antibodies production over a period of 360 days. By contrast, the age-dependent increase in the proinflammatory cytokine IL-12 level was observed (Figure 33). Also the level of IgG in the serum did not increase (data not shown). Importantly, in spite of lack of increased level of autoantibodies these mice developed clinical features of systemic inflammatory disease.

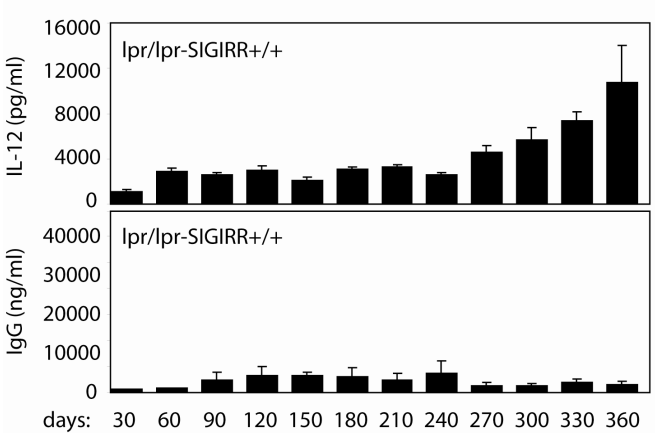


*Figure 33.* **Serum and proinflammatory cytokines analysis from C57BL/6 *lpr/lpr* mice:** Mice were bled every month and the serum samples were analysed for IL-12 or anti dsDNA IgGs. . Data represent means ± SEM from 6 independent mouse sera.

To determine whether in the C57BL/6 background the *lpr/l*pr mutation influences the progression of renal disease, we quantified protein excretion in the urine that was collected over one year, every 30 days. At 1 to 4 months of age, the protein levels in C57BL/6 *lpr/lpr* mice were low, but as the mice aged, proteinuria, as measured by albumin ELISA in the urine, began to increase. By 5 month of age all animals had slightly increased urinary protein concentrations of 5 to 7 µg/ml (Figure 34). The albumin values measured by ELISA are however too low to speculate about kidney damage. This fact can support the already published observation that lupus nephritis is a very complex disease and it does not develop in every genetic background in mice.

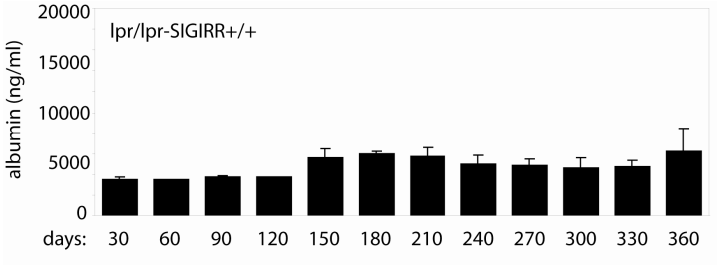


*Figure 34.* **Proteinuria in C57BL/6 *lpr/lpr* mice:** Urine was collected every month from C57BL/6 *lpr/lpr* mice (n = 6). Urinary protein, albumin levels were measured by ELISA. Data represent means ± SEM from 6 independent mouse urine samples.

### 3.6.2. SIGIRR affects immune complex-dependent DCs activation

Infections are known to induce disease flares in individuals with autoimmune pre-disposition. Hence a role for SIGIRR, leading to aggravation of lupus nephrits was hypothesized.

Viral dsRNA, bacterial DNA and components of bacterial cell wall are known to activate DCs to secrete cytokines which are associated with the disease activity in SLE. Because SIGIRR is expressed by murine plasmacytoid and myeloid DCs, we hypothesized that the lack of this receptor could influence the function of DC subsets in autoimmune disease. In order to determine the pathogenic role of SIGIRR in lupus, C57BL/6 *lpr/lpr* mice were crossed with C57BL/6 *SIGIRR-/-* mice. The mice in the C57BL/6 genetic background were used for these experiments. Because a major functional consequence of pDC activation by TLRs is the secretion of proinflammatory cytokines, we determined their concentration in vitro as well as in vivo, in serum of C57BL/6 *lpr/lpr* or C57BL/6 *lpr/lpr SIGIRR-/-* mice.

DNA-containing immune complexes (ICs) have been shown to activate TLR9, the receptor for bacterial CpG DNA, in B lymphocytes and DCs (Leadbetter 2002, Boule 2004, Means 2005). Also ICs containing small nuclear ribonucleoproteins snRNP, a second major autoantigen in SLE, stimulate dendritic cells, leading to the production of proinflammatory cytokines. U1snRNP complexes (U1snRNA within U1snRNP) recognition is dependent mostly but not exclusively on TLR7 but independent of TLR3 (Savarese 2006).

We used snRNPs (specifically U1snRNPs) complex to investigate the role of SIGIRR in the DCs activation during SLE. SnRNP are the major SLE autoantigens in addition to dsDNA. Bone marrow dendritic cells were isolated from C57BL/6 *lpr/lpr* or C57BL/6 *lpr/lpr Sigirr-/-* mice; incubated with different TLR ligands or medium for 24 h. DCs were incubated with purified U1snRNP complexed with cationic liposomes or with control stimuli poly-U ssRNA/DOTAP (as TLR7 ligand), poly-I:C dsRNA/DOTAP (as ligand for TLR3 and other dsRNA recognition receptors), LPS (as TLR4 ligand) and CpG 2216 oligonucleotide (as TLR9 ligand). Bone marrow dendritic cells showed an increase in IL-12p40 and IL-6 release after exposure to all tested stimuli, namely LPS, CpG, pI:C RNA, polyU RNA, imiquimod, Y12-U1snRNP and U1snRNP complex. These data indicate that all stimuli used

induce the production of proinflammatory mediators, suggesting that infections that can lead to immune activation and cytokine release may associate with disease flares during ongoing lupus. Surprisingly, in case of stimulation with LPS, CpG, pI:C RNA, polyU RNA and imiquimod no differences in cytokines production between dendritic cells from C57BL/6 *lpr/lpr Sigirr*+/+ and C57BL/6 *lpr/lpr Sigirr-/-* mice were observed (Figure 35 and 36). SIGIRR seems to have no effect on the IL-6 and IL-12 production after stimulation with mentioned ligands in the C57BL/6 *lpr/lpr* mice. In dendritic cells from mice of this background the function of SIGIRR as a negative regulator of TLR signaling is questionable from our observations. The lpr mutation seems to have an effect similar to SIGIRR in DCs. The production of proinflammatory cytokines is increased (compare with the Figure 21, Flt3L-DCs). However SIGIRR does not have the expected, additive effect on cytokine production (Figure 35 and 36).

It is known that CpG DNA can aggravate autoimmune tissue injury locally by activation of tissue macrophages (Tsunoda 1999); that genomic DNA released by dying cells can induce APC maturation (Ishii 2004) and inhibitors of DNA methylation can induce SLE in humans (Richardson 2003). Similar reports were delivered about the viral RNA. SIGIRR does not affect production of cytokines in *lpr/lpr* mice after stimulation with CpG, polyU, polyIC or imiquimod. *Sigirr*-deficient BMDC cells responded to the polyIC/polyU, LPS, imiquimod and CpG, the ligands for TLR3, TLR4, TLR7 and TLR9 respectively to the same extent as *Sigirr*-sufficient cells.

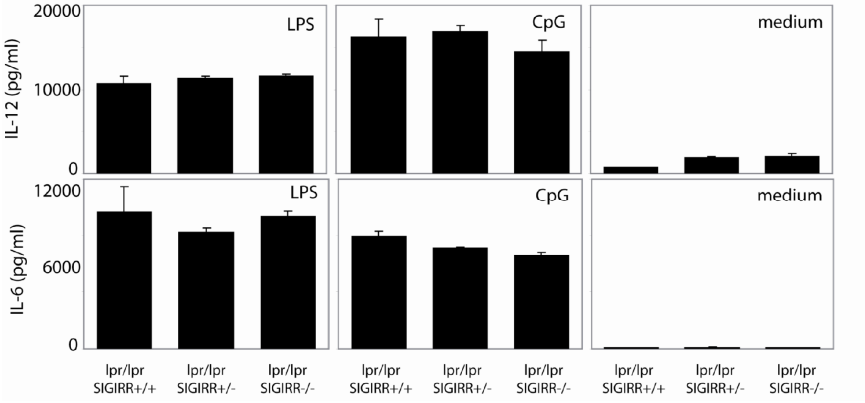


*Figure 35.* **Influence of *Sigirr* deficiency on DC activation of by TLR4 and TLR9 ligands:** DCs generated from C57BL/6 *lpr/lpr*, C57BL/6 *lpr/lpr Sigirr+/–* and C57BL/6 *lpr/lpr Sigirr–/–* mice were incubated with the indicated stimuli (ultra pure LPS 1 µg/ml; CpG 2216 0.1 µM or medium alone) for 24 h. (upper panel) IL-12 and (lower panel) IL-6 were measured in the supernatants by ELISA. Mean values and standard deviations are shown (n = 3).

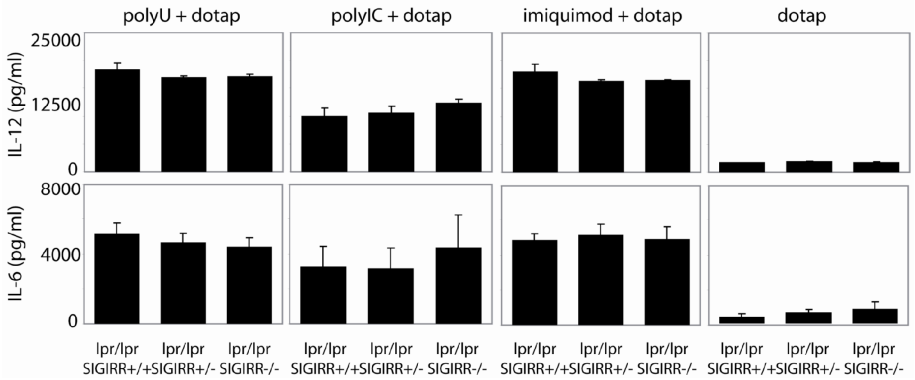


*Figure 36.* **Influence of *Sigirr* deficiency on DC activation of by TLR3 and TLR7 ligands:** DCs generated from C57BL/6 *lpr/lpr*, C57BL/6 *lpr/lpr Sigirr+/–* and C57BL/6 *lpr/lpr Sigirr–/–* mice were incubated with the indicated stimuli (10 µg/mL poly-U with 12.5 µg/mL DOTAP; 20 µg/mL poly IC with 12.5 µg/mL DOTAP; 10 µg/mL imiquimod with 12.5 µg/mL DOTAP or DOTAP alone) for 24 h. (upper panel) IL-12 and (lower panel) IL-6 were measured in the supernatants by ELISA. Mean values and standard deviations are shown (n = 3).

BMDCs produced cytokines in response to ICs namely Y12-antibody-U1snRNP or U1snRNP, whereas the Y12 antibodies or U1 alone consistently elicited a weak but detectable response, similar to the medium control. This response depended most likely on IC formation, as Y12-U1snRNP and U1snRNP show some differences in the ability to stimulate the cytokines release. As one can see on both figures, also the single allele deletion (*Sigirr*+/-) is able to affect the IL-6 and IL-12 production.

The role of SIGIRR for recognition of U1snRNP was investigated by comparing cytokine production of DCs derived from bone marrow cells of C57BL/6 *lpr/lpr*, C57BL/6 *lpr/lpr Sigirr*+/– or C57BL/6 *lpr/lpr Sigirr*–/– mice in response to U1snRNP/DOTAP and U1snRNP/Y12 antibody IC.

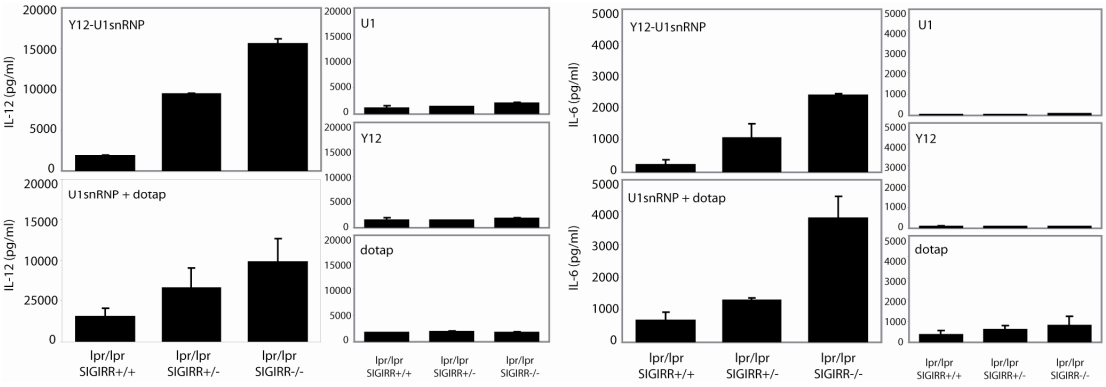


*Figure 37.* **Influence of *Sigirr*-deficiency on DC activation of by U1snRNP:** DCs generated from C57BL/6 *lpr/lpr*, C57BL/6 *lpr/lpr Sigirr*+/– and C57BL/6 *lpr/lpr Sigirr*–/– mice were incubated with the indicated stimuli (10 µg/mL U1snRNP with 12.5 µg/mL DOTAP or 20 µg/mL U1snRNP with 50 µg/mL Y12-antibody) for 24 h. Columns 2 and 4 represent the negative controls (U1, Y12 or dotap). (left) IL-12 (right) IL-6 cytokine levels were measured in the supernatants by ELISA. Mean values and standard deviations are shown (n = 3).

These data suggest that TLR2, 3, 4, 7 and 9 agonists induce the production of IL-12p40 and IL-6 in both dendritic cells isolated from C57BL/6 *lpr/lpr* or C57BL/6 *lpr/lpr Sigirr*-deficient mice and that SIGIRR affect only signalling initiated by Y12-U1snRNP and U1snRNP.

We showed that U1snRNP complexed with cationic lipid or anti-Sm autoantibody induced inflammatory cytokine responses in murine bone marrow derived DCs. IL-12 and IL-6 induction by U1snRNP required an intracellular delivery because U1snRNP alone had very low

immunostimulatory activity (data not shown). The complexation with cationic liposomes could be replaced by the formation of ICs with purified anti-Sm monoclonal antibody Y12, which are internalized by DCs through Fc receptors. Cytokine responses were observed after stimulation with Y12-U1snRNP complex but not with Y12 antibody alone (Figure 37). U1snRNP was only active when it was internalized into the endosomal compartment, as has been shown for human SLE ICs containing DNA (Bave 2003, Means 2005). The *lpr/lpr* dendritic cells which were deficient for *Sigirr* showed an enhanced induction of IC-induced proinflammatory cytokines levels compared to *lpr/lpr* dendritic cells. The cells heterogeneous for *Sigirr* showed only minor but still significant effect (Figure 37). Thus, SIGIRR has a negative effect on the IC-induced cytokine production in murine dendritic cells.

### 3.6.3. SIGIRR negatively regulates serum cytokines and serum anti dsDNA IgGs in C57BL/6 *lpr/lpr* mice

Thus, having demonstrated the effect of SIGIRR on IL-6 and IL-12p40 secretion in antigen-presenting cell subsets that were isolated from C57BL/6 *lpr/lpr* or C57BL/6 *lpr/lpr Sigirr-/-* mice in vitro, we next studied anti dsDNA IgGs serum level changes in C57BL/6 *lpr/lpr Sigirr+/+* or C57BL/6 *lpr/lpr Sigirr-/-* mice. Serum was collected from both autoimmune C57BL/6 *lpr/lpr* mice and C57BL/6 *lpr/lpr* mice deficient in the expression of *Sigirr* and the IgG level was determined by ELISA. Sera from all the C57BL/6 *lpr/lpr* or C57BL/6 *lpr/lpr Sigirr*-defficient offspring contained anti-dsDNA antibodies and IL-12. However the C57BL/6 *lpr/lpr Sigirr-/-* mice had significantly increased serum levels of both IL-12p40 and antibodies as compared with C57BL/6 *lpr/lpr Sigirr +/+* mice (Figure 38). It is therefore likely that SIGIRR is involved in both the in vivo production of auto-antibodies, as well as the subsequent IC-stimulation of B cells.

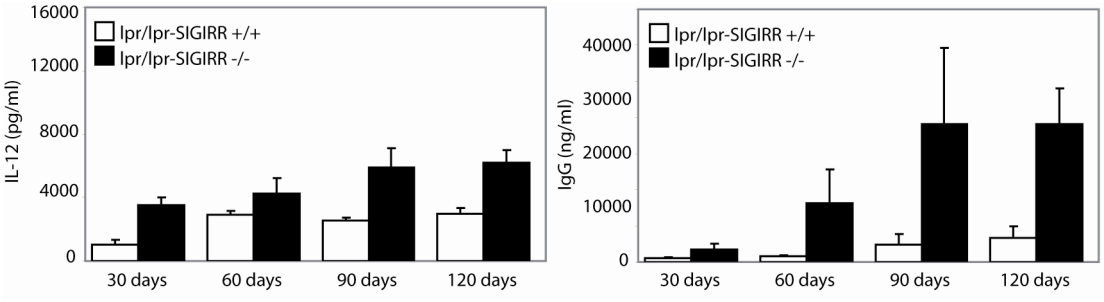


*Figure 38.* **IL-12 and IgG serum levels of C57BL/6 *lpr/lpr Sigirr -/-* mice:** Serum IL-12p40 and dsDNA IgG autoantibody levels in C57BL/6 *lpr/lpr* (n=6) and C57BL/6 *lpr/lpr Sigirr-/-* (n=6) mice were determined by ELISA. Serum was obtained every 30 days. Data represents means ± SEM.

From the above results, one would predict that lack of SIGIRR would be associated with more severe autoimmune tissue injury in C57BL/6 *lpr/lpr* mice. *In vivo*, C57BL/6 *lpr/lpr Sigirr-/-* mice have increased serum IL-12p40 as well as anti DNA IgGs levels. Lack of SIGIRR aggravates SLE in C57BL/6 *lpr/lpr* mice. C57BL/6 *lpr/lpr Sigirr-/-* sera had increased concentrations of every IgG isotype, but the most prominent increases were in IgG1, IgG2b and IgG3 (Figure 39). The isotypes affected by the absence of SIGIRR are the immunoglobulin isotypes often associated with inflammation and autoimmunity (Nimmerjahn and Ravetch, 2005). IgG3 has been reported to play a central role in the development of nephritis in MRL/Mp *lpr/lpr* mice (Takahashi et al., 1991). Taken together, these data indicate that the genetic absence of SIGIRR led to increased disease activity and global immune activation in C57BL/6 *lpr/lpr Sigirr-/-* mice.

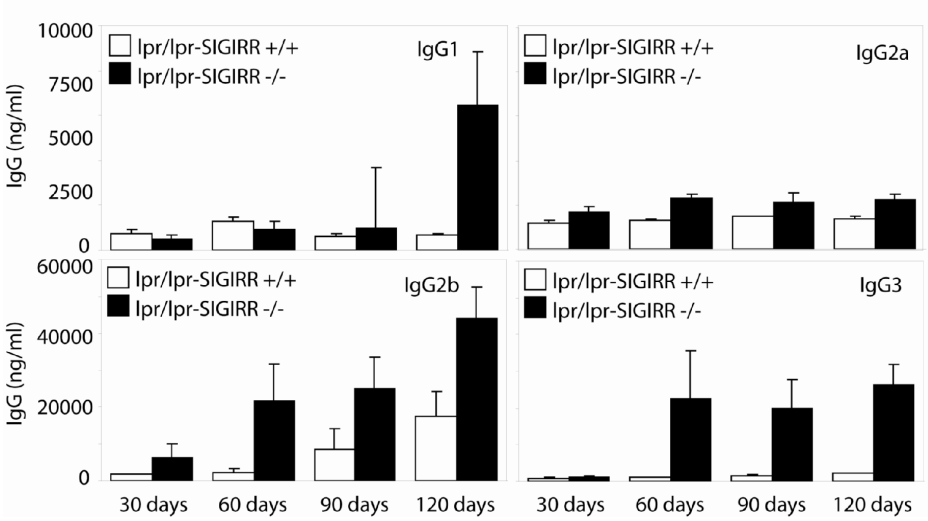


*Figure 39.* **IgGs isotypes analysis of C57BL/6 *lpr/lpr Sigirr-/-* mice:** Serum anti dsDNA autoantibody isotypes IgG1, IgG2a, IgG2b, IgG3 levels in C57BL/6 *lpr/lpr* (n=6) and C57BL/6 *lpr/lpr Sigirr-/-* (n=6) mice were determined by ELISA. Serum was obtained every 30 days. Data represents means ± SEM.

### 3.6.4. Further abnormalities in C57BL/6 *lpr/lpr Sigirr-/-* mice

Aggravation of renal disease is usually demonstrated by an increase in proteinuria. We did not observed increased urine albumin concentration in C57BL/6 *lpr/lpr Sigirr*-deficient mice as compared with the C57BL/6 *lpr/lpr* mice up to the age of 18 weeks. By 18 wk of age in the control mice as well as in the *lpr/lpr Sigirr-/-* mice, all investigated animals had urinary protein concentrations of around 4 µg/ml (Figure 40). These results were confirmed by ELISA analysis of urinary albumin. Thus, both tested group of mice did not developed proteinuria at age of 120 days. It is however not clear if this trend is going to be stable or if the C57BL/6 *lpr/lpr Sigirr*-deficient mice do develop the proteinurie later. Further experiments are necessary to answer this question.

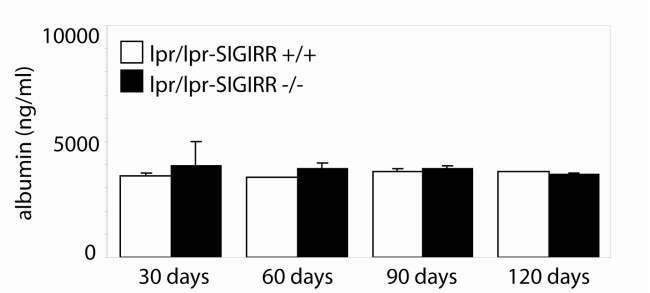


*Figure 40.* **Proteinuria in C57BL/6 *lpr/lpr Sigirr-/-* mice:** Urine from C57BL/6 *lpr/lpr* (n = 6) and C57BL/6 *lpr/lpr Sigirr-/-* mice (n = 6) was collected every month. Urinary protein levels were measured by albumin ELISA. Data represent means ± SEM from 6 independent mouse urine samples.

C57BL/6 *lpr/lpr Sigirr-/-* mice had normal appearance, growth, size and fertility. However they showed increased and progressive skin lesions, lymphadenopathy and mortality.

Having observed the clear effects of *Sigirr* deficiency on autoantibody production and DC activation, we then determined whether the absence of this receptor had a significant impact on the manifestation of clinical autoimmune disease. We found that C57BL/6 *lpr/lpr Sigirr*-deficient mice had a significant increase in the incidence and severity of autoimmune skin disease compared to wild-type littermates. Concordant with this were increased lymphadenopathy and splenomegaly in C57BL/6 *lpr/lpr Sigirr*-deficient mice (Figure 41). Increased spleen and lymph node weight in C57BL/6 *lpr/lpr Sigirr*-deficient mice is due to accumulation of lymphocytes in these organs. However, we do not have enough data to perform good statistical analysis of these phenomena yet. The investigation of the new cohort is needed to answer precisely all the interesting problems.

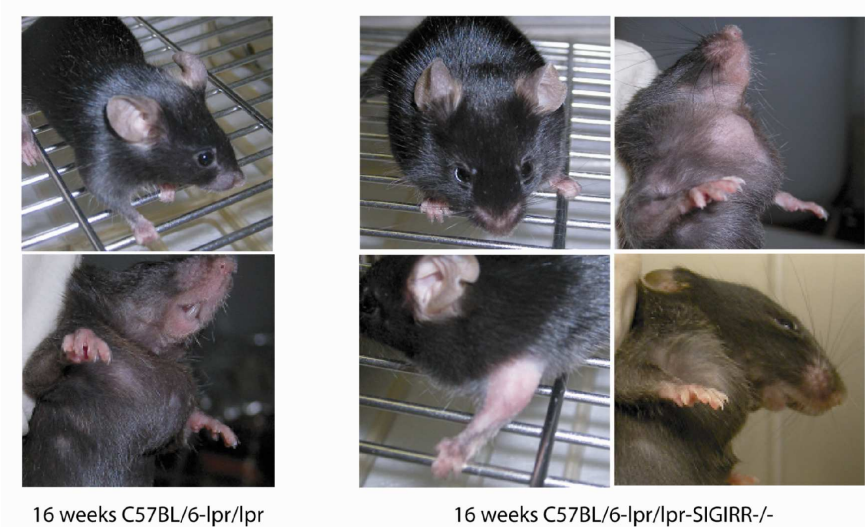


*Figure 41*. **C57BL/6 *lpr/lpr Sigirr-/-* mice morphology:** mice were kept under the sterile conditions. The documentation was made every month. First visible syndromes of the SLE were detectable after 12 weeks. In the age of 16 weeks mice have already severe lymphadenopathy and they start loosing the hair.

The typical visible abnormalities associated with the disease appear first after 16 - 24 weeks in C57BL/6 *lpr/lpr* mice, whereas C57BL/6 *lpr/lpr Sigirr*-deficient mice develop the skin lesions and lymphomegaly after 12 weeks of life (Figure 41). In addition, there was very little skin disease in C57BL/6 *lpr/lpr* mice at 16 weeks of age, whereas skin disease was evident in C57BL/6 *lpr/lpr Sigirr*-deficient mice (observed however only in around 20% of investigated mice). The development of these changes seems to be much faster in the C57BL/6 *lpr/lpr Sigirr*-deficient mice.

Although we observed an increase in nearly every marker of disease severity in autoimmune C57BL/6 *lpr/lpr Sigirr*-deficient mice, it remained unclear whether these disease markers truly affected mortality. We therefore allowed C57BL/6 *lpr/lpr* and C57BL/6 *lpr/lpr Sigirr*-deficient mice littermates to develop spontaneous disease, and we monitored them without intervention until the time of death. We found that C57BL/6 *lpr/ Sigirr*-deficient mice had accelerated mortality relative to *lpr/lpr* controls (Figure 42). All 20 mice in C57BL/6 *lpr/lpr* group survived up to 12 weeks of age

and only 15 mice in C57BL/6 *lpr/lpr Sigirr*-deficient group survived to 12 weeks of age; a formal analysis in a new cohort is ongoing.

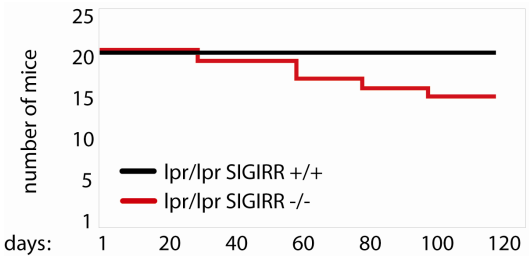


*Figure 42.* **Kaplan-Meier curves:** Survival of C57BL/6 *lpr/lpr* and C57BL/6 *lpr/lpr Sigirr-/-* mice was compared. All studied mice were kept sterile. *Lpr/lpr Sigirr*-deficient mice showed diminished survival than the control mice group.

We showed that C57BL/6 *lpr/lpr* mice lacking SIGIRR developed more severe clinical disease and show that it leads to early mortality.

Our experiments with C57BL/6 *lpr/lpr* mice followed the observation which we made with the *Sigirr* deficiency in the MRL *lpr/lpr* background. We were able to generate only the MRL *lpr/lpr Sigirr+/-* mice. They initially appeared healthy, and daily observation did not reveal any behavioral abnormalities. However, after reaching 10 weeks of age, there was a marked increase in the mortality rate of MRL *lpr/lpr Sigirr+/-* mice. We were not able to generate the *SIGIRR*-deficient *lpr/lpr* mice in the MRL background. Thus, the heterozygote of *Sigirr* was sufficient for the 100 % mortality in the MRL *lpr/lpr* background. This fact is clear evidence for the function of SIGIRR in the development of the autoimmune disease.

# 4. DISCUSSION

## 4.1. FUNCTION OF SIGIRR IN THE KIDNEY

SIGIRR is an orphan receptor of the IL-1R/Toll-like receptor family with regulatory functions on TLR signalling and is expressed at high levels in the human and murine kidney. The TLR/IL-1R superfamily has a central role for initiating innate antimicrobial immunity and, hence, may contribute to renal pathology in infective pyelonephritis (Akira 2006, Anders 2004). Under normal conditions, the kidney maintains a sterile environment, but renal tubular epithelial cells express TLR1-4 and -11 and can produce proinflammatory cytokines and chemokines in response to respective TLR ligands (Tsuboi 2002, Zhang 2004, Hung 2006, Yang 2006). Moreover, TLR4 and TLR11 mediate renal chemokine production and subsequent neutrophil recruitment during ascending urinary tract infection with uropathogenic *Escherichia coli* (Zhang 2004, Patole 2005). Furthermore, renal dendritic cells contribute to innate immunity in the kidney as they are activated via TLRs either by pathogens directly or by endogenous molecules such as Tamm-Horsfall protein (Saemann 2005). We hypothesized that SIGIRR may control inappropriate TLR signaling in the kidney, and if so, that SIGIRR on renal myeloid cells and non-immune intrinsic renal cells would contribute to this phenomenon. This study confirmed only the first part of this hypothesis, but data presented here do not support the latter. SIGIRR has cell type-specific functions and is regulated differently in tubular epithelial cells.

SIGIRR mRNA was previously shown to be expressed in most murine and human organs with a preference to epithelial tissues, i.e. kidney, lung, gut, and cornea (Polentarutti 2003, Wald 2003, Garlanda 2004, and Huang 2006). The data presented here show that SIGIRR mRNA and protein are expressed at high levels in kidneys of 6 week old mice. Interestingly, renal SIGIRR mRNA levels were much lower in newborn mice and also decline with aging. This represents a new finding, as high renal SIGIRR levels reported in previous studies referred to the analysis of 8-10 week old mice

(Polentarutti 2003). An age-dependent expression of SIGIRR was also found for other solid organs, i. e. spleen, liver, lung, and skin. The reason for age dependent SIGIRR expression remains elusive, but a decline in TLR expression and function with aging has also been reported (Renshaw 2002). Reduced expression and function of TLRs with aging thus impacts both the quality and magnitude of host innate and adaptive immune responses to bacterial and fungal infections by the altered inflammatory and priming environment. It is possible that SIGIRR as a putative regulator of TLRs signaling is being down regulated together with the TLR receptors.

In these studies we showed that *Sigirr* expression is also strain dependent and sex dependent. C57BL/6 male mice expressed SIGIRR most. The differences in *Sigirr* expression in C3H/HeNCrl, BALB/c and C57BL/6 mice may be also due to the differences in the TLR expression profile (data not shown; Liu 2002). It is not surprising that we observed striking differences in the *Sigirr* expression level between different mouse strains and different gender. Difference in gene expression may involve many factors. Gender differences clearly affect the immune system, and female are more likely than male (also by humans) to develop autoimmune diseases like diabetes, lupus, and rheumatoid arthritis.

SIGIRR protein localized to tubular epithelial cells on immunostaining which is consistent with the recent description of *Sigirr* mRNA in these cells evidenced by *in situ* hybridization (Polentarutti 2003), by quantitative real-time RT-PCR, and flow cytometry from tubular epithelial cells prepared from mouse kidneys. Both flow cytometry and cellular SIGIRR staining localized SIGIRR protein to the outer cell membrane, but not in the cytoplasm of tubular epithelial cells. Immunostaining of the renal slices did not reveal significant SIGIRR expression in other intrinsic renal cell types, including vascular endothelial cells, mesangial cells or glomerular visceral epithelial cells, i.e. podocytes. Thus, renal SIGIRR expression originates from tubular epithelial cells. Futhermore SIGIRR staining was found in all segments of the nephron which harbour tubular epithelial cells of different phenotypes and functions. In fact, comparable *Sigirr* mRNA expression levels were found in renal cortex and medulla. We conclude that adult mice express high levels of SIGIRR in the kidney and that the SIGIRR expression originates from tubular epithelial cells. However, our later observations revealed that SIGIRR is also strongly expressed in the intrarenal myeloid cells, which form a kind of network

in the kidney. The results presented here show that SIGIRR has a unique pattern of expression that includes tubular epithelial cells, monocytes and intrarenal myeloid cells but not mesangial cells. The finding that intrarenal myeloid cells have such high *Sigirr* transcript level was unexpected. The expression of SIGIRR in antigen presenting cells is consistent with the view that this molecule has a regulatory role in kidney. SIGIRR was shown in these studies as a negative regulator of LPS signalling in the heterogeneous kidney cell suspension/kidney slices.

This study clearly demonstrates that SIGIRR suppresses renal CCL2 production upon exposure to LPS. This is consistent with the exacerbation of various other inflammatory disease models that have been induced in *Sigirr*-deficient mice, e.g. endotoxic shock (Wald 2003), dextran-induced colitis (Garlanda 2004), and *Pseusomonas aeruginosa* keratitis (Huang 2006). Recognition of LPS in the kidney may involve various cell types which can broadly be classified into renal immune cells and non-immune cells. Intrarenal immune cells are mainly resident antigen-presenting cells which originate from the bone marrow and that form a dense network in the interstitial space that grid the tubular compartment in the healthy kidney (Kruger 2004, Soos 2006). Consistent with the previous description of SIGIRR`s function in dendritic cells and monocytes (Polentarutti 2003; Wald 2003) we found that SIGIRR suppresses TLR 2, -3, -4, -7 and -9 signaling in intrarenal antigen presenting cells as shown by production of proinflammatory cytokines. The inhibitory effect of SIGIRR on TLR signaling is mediated by its intracellular TIR domain, which does not retain two amino acids (Ser447 and Tyr536) in the highly conserved TIR domain (Thomassen 1999, Qin 2005).

We tested different immune cells and their response to TLR ligands. We conclude that SIGIRR is able to inhibit the TLR -2, -3, -4 and -9 signaling in monocytes (CCL2 production; CXCL2 data not shown). Similar results we obtained during testing the different kind of bone marrow dendritic cells (cultured with GM-CSF or FLT3L) and their responses to LPS and other TLR ligands.

Data presented here confirm the inhibitory effect of SIGIRR on TLR signaling in immune/antigen presenting cells because ligands for TLR1/2, -3, -4, -7 and -9 induced much higher levels of

proinflammatory cytokines production (IL-6, IL-12 or CCL2) by *Sigirr*-deficient antigen presenting cells as compared to cells prepared from wild-type mice. However, the response of FLT3L-DCs was weaker than in the case of GM-CSF-DCs or intrarenal myeloid cells. Furthermore in case of FLT3L-DCs we did not observed much difference between *Sigirr* deficient and wild type cells in CCL2 production. Moreover, the CCL2 production of GM-CSF-DCs and intrarenal myeloid cells showed striking differences between *Sigirr-/-* and *Sigirr+/+* genotypes so that the stimulation with the ligands did not really affected the level of produced CCL2. Thus SIGIRR down-regulate the IL-6 and IL-12 production after stimulation with TLR ligands in immune cells. Moreover, the lack of SIGIRR results in the constant inflammatory state caused by the CCL2 production. It was already reported that a balance of IL-12 and MCP-1/CCL2 produced by DC is crucial in determining the fate of immune responses. MCP1 tends to develope Th2 responses, whereas IL-12 tends to induce Th1 responses. Whether the *Sigirr*-deficient mice have constantly elevated Th2 responses remains to be elucidated. This modulatory effect on TLR signaling was not observed in primary tubular epithelial cells. In fact, the ligands for TLR1/2, -3, and -4 induced similar amounts of CCL2 in *Sigirr*-deficient or wild-type tubular epithelial cells. As tubular epithelial cells do not express TLR9, CpG-DNA did not induce CCL2 production (Tsuboi 2002).

In addition, TLR signaling may be regulated through TLR expression (Liew 2005), which is likely to be SIGIRR-dependent. We demonstrated that the expression levels of mRNAs for almost all TLRs were higher in bone marrow derived DC, spleen monocytes and renal myeloid cells from *Sigirr-/-* mice than in those from C57BL/6 wild type mice. However, the differences between *Tlr 2, 3* and *4* mRNA levels were not significant in case of monocytes, which expressed low level of these receptors. In fact, *Sigirr*-deficient spleen monocytes expressed increased levels of *Tlr1, -5, -6, -7, -9,* and *-11* which may contribute to the enhanced TLR signaling observed in these cells. However, ligation of TLR2, -3, and -4 did also show increased CCL2 production in *Sigirr*-deficient monocytes despite identical *Tlr* expression levels as compared to wild-type monocytes. The bone marrow derived DCs cultured with FLT3L expressed lower *Tlrs* mRNA levels than the same cells cultured with GM-CSF or the intrarenal myeloid cells. This can be explained by the lower maturation stage of these cells. Intrarenal myeloid cells expressed very high mRNA levels of all *Tlrs* (except *Tlr5* and

*Tlr11*). Like already mentioned above, we observed even more elevated *Tlrs* mRNA levels in the *Sigirr* knock-out mice. Thus SIGIRR is a negative regulator of TLRs expression in the immune cells. SIGIRR suppresses expression of a subset of TLRs in immune cells and this suppression is cell type-specific because it was not found in renal tubular epithelial cells. Thus, in the kidney the regulatory role of SIGIRR on TLR signaling should be restricted to immune/antigen presenting cells. Thus SIGIRR regulates negatively the TLR signalling exclusively in immune/antigen presenting cells but not in renal tubular epithelial cells (non-immune cells).

In previous studies Wald, *et al*. reported a similar role for SIGIRR in renal cell suspensions but in their study kidney cell suspensions were prepared from *Sigirr*-deficient mice in a mixed genetic background (Wald 2003). Exposure to LPS or CpG-DNA increased NF-κB activation as compared to cells prepared from wild-type mice (Wald 2003). Also *in vivo* systemic endotoxin challenge was carried out in both recently reported strains of *Sigirr*-deficient mice (Wald 2003, Garlanda 2004). While Wald et al. observed a reduced threshold to lethal endotoxin challenge, Garlanda, et al could not detect a difference in LPS-induced mortality between *Sigirr*-deficient and wild-type mice (Garlanda 2004). However, both studies used *Sigirr*-deficient mice in a mixed genetic background and littermates as wild-type controls. In the present study we applied more stringent preparation techniques for primary tubular cells, as we found that kidney cell suspensions prepared following the protocol reported by Wald, *et al*. are commonly contaminated by myeloid cells, compromising conclusions on the role of SIGIRR in tubular epithelial cells. By using the protocol reported by Tsuboi, *et al*. the primary tubular epithelial cell population was >95% (Tsuboi 2002). Furthermore, we have now backcrossed *Sigirr* mutants for 6 generations into the C57BL/6 background and prepared cells used in the experiments above from these mice with a predicted >95% identical background as compared to wild-type controls (Sigmund 2000). In fact, by using a pure tubular epithelial cell population no impact of SIGIRR on TLR signaling could be detected. SIGIRR do not influence the TLRs expression level as well as signalling of the TLRs, which suggest no TLR regulatory function in tubular epithelial cell populations. Obviously, intrarenal antigen-presenting cells and tubular epithelial cells contribute to renal TLR4 signaling. Although SIGIRR is expressed

by both cell types, SIGIRR suppresses TLR signaling only in intrarenal antigen-presenting cells and not in tubular epithelial cells. While reducing TLR signaling in renal antigen-presenting cells may avoid inappropriate immunity-related tissue injury, an unrestricted activation of tubular cells may support the induction of necessary epithelial stress response elements to cope with microbial infection.

During differentiation, DC up-regulate the expression of MHC class I and class II and costimulatory molecules and thus increase their efficiency as APC (Banchereau 2000, Langenkamp 2000, Liu 2001, Mellman 2001, Reis e Sousa 2001). We found that DCs from *Sigirr-/-* show weaker take up of antigen, which suggests that the *Sigirr-/-* DCs maturate faster and are rather better at cytokine production as at the antigen take up. This would explain the increased cytokine production of *Sigirr-/-* DCs after stimulation with TLRs ligands. TLR signalling is important for DC maturation, characterized by cytokine production, up-regulation of costimulatory molecules, and an increased ability to activate T cells (Kaisho 2001). Thus DCs from *Sigirr-/-* C57BL/6 mice are more mature than those from wild type C57BL/6 mice. Taken together, these results suggest that differences in *Tlr* gene expression levels in DC may reflect differences in the composition of DC subsets at different maturation stages in *Sigirr*-deficient and wild type C57BL/6 mice. However the expression levels of TLRs are not known at the protein level. On the other hand reactivities of DC to microbial molecules in *Sigirr* knock-out and wild type C57BL/6 mice may not be explained by differences in TLR expression levels alone.

Interestingly, SIGIRR is differentially regulated in antigen presenting and renal tubular epithelial cells. LPS challenge suppressed *Sigirr* mRNA production up to 24 hours in tubular epithelial cells which is consistent with downmodulation of renal *Sigirr* mRNA after intravenous injection of 1 μg LPS in mice (Polentarutti 2003, Wald 2003). By contrast, both LPS and TNF/IFN-γ have opposite effects on *Sigirr* mRNA expression in spleen monocytes, a finding consistent with the recent observation of Tir8/Sigirr expression in monocytes of patients with sepsis (Adib-Conquy 2006). The

same cell type-specific response was observed when monocytes and tubular epithelial cells were stimulated with TNF/IFN-γ in a dose dependent manner. Thus, proinflammatory stimuli, i.e. LPS and TNF/IFN-γ, have opposite effects on *Sigirr* mRNA expression in spleen monocytes and tubular epithelial cells. Based on our finding that SIGIRR regulates TLR expression in monocytes we questioned whether TLRs regulate SIGIRR expression. In fact, *Sigirr* mRNA levels were elevated in spleen monocytes and tubular epithelial cells prepared from *Tlr4*-deficient mice of the same genetic background. This indicates that TLR4 signaling suppresses SIGIRR expression in both antigen presenting and tubular epithelial cell types. Lack of *Tlr2* and *Tlr3* suppressed *Sigirr* mRNA in tubular epithelial cells but not in spleen monocytes. Obviously, TLRs selectively regulate *Sigirr* expression in a cell type-specific manner. Appearantly, SIGIRR expression is regulated by multiple factors, i.e. proinflammatory factors, TLR signaling, and age.

Why is SIGIRR expressed in the tubular epithelial cells? Is there a mechanism that is able to switch off SIGIRR function in these cells? What are the factors that control the cell type-specific functions of SIGIRR? Alternative splicing is common for the members of the IL-1R/TLR superfamily and their signaling molecules (Schnare 2001, Wells 2006, Bergers 1994, Rossler 1995). For example, the *T1/ST2* gene encodes two splice variants (Lohning 1998). The transmembrane ST2L that has inhibitory effects on TLR signaling similar to SIGIRR and the soluble ST2, a truncated ST2 protein lacking the intracellular TIR domain is required for signaling (Lohning 1998). Because ST2L is selectively expressed by immune cells and soluble ST2 selectively by non-immune cells (Rossler 1993), we hypothesized that the same could be true for SIGIRR. Therefore, we intended to test whether tubular cells express a splice variant of *Sigirr* that lacks the intracellular TIR domain. By using PCR primers for the extracellular and intracellular domain as well as Northern blotting we essentially excluded alternative splicing of *Sigirr*. By amino acid alignment and data base search, *Sigirr* was not found to encode any of potential functional domains, such as EGF-like repeats and a MAM domain, as well as an RGD integrin binding motif. These structures are commonly found in secreted proteins. By Western blotting and immunocytostaining, we found that the SIGIRR protein was localized on the cell surface, but not in the culture medium. Thus SIGIRR has no soluble form.

Posttranslational modifications such as glycosylation can also alter the function of immune mediators (Daniels 2002). Furthermore, N-glycans have been shown to play a general role in protein folding and protein sorting in biosynthetic traffic of polarized renal tubular epithelial cells (Scheiffele 1995). SIGIRR`s five putative glycosylation sites and its predicted and actual molecular weight indicate extensive glycosylation (Thomassen 1999). We confirmed glycosylation of SIGIRR by PNGaseF digestion or inhibition of O-glycosylation and Western blotting. Interestingly, digestion with PNGaseF leads to production of 2 additional glycoforms of SIGIRR in the kidney. These results suggest that this additional SIGIRR glycoform which is expressed in some renal cells contains N-glycans (not all N-glycans can be cleaved by the PNGaseF) or O-glycans which cannot be cleaved by PNGaseF. The smaller form of SIGIRR found in renal CD11b negative (i.e. mostly tubular epithelial) cells is O-glycosylated as shown by benzyl-GalNAc inhibition, but myeloid cell SIGIRR is not. By contrast, both the myeloid and non-myeloid cell SIGIRR forms were markedly reduced in size after digestion with PNGaseF, which is suggestive of extensive N-glycosylation. Thus, SIGIRR in renal tubular epithelial cells is N- and O- glycosylated, whereas SIGIRR in intrarenal CD11b positive myeloid cells lacks O-glycosylation which can be inhibited by benzyl-GalNAc. The oligosaccharide structures of many glycoproteins play an important role in the folding of proteins and biological activities (Rademacher 1988). Since the targeting and activity of many glycoproteins can be affected by their glycosylation it would be of great interest to investigate the glycosylation of SIGIRR more detailed. Maybe changes in particular sugar residues would affect the function of the SIGIRR in tubular epithelial cells and immune cells and help understand the differences in signalling in these both cell types. Additional work is needed to address this question.

In this report we demonstrate that SIGIRR is not only an extensively N glycosylated, but also O-glycosylated membrane glycoprotein. SIGIRR has not been described before to carry O-glycans. There is also number of studies that suggest a link between O-glycosylation and apoptosis (Yin 2003, Ren 2004, Zachara 2004). But we did not observe any correlation between the presence of SIGIRR and susceptibility to apoptosis or to stress.

SIGIRR is characterized by the presence of an Ig-like domain, and this fact may suggest that it mediates interactions with other Ig-like adhesion molecules. Because of the fact that SIGIRR is highly glycosylated (like many others adhesions molecules) SIGIRR may play a role in establishing the contacts between the cells and stabilizing the integrity of tubular epithelial cell populations in the in vitro culture. From microscopy and proliferation observations we conclude that SIGIRR does not play a role in these processes, but to answer this question precisely additional work on this field would be needed.

In the present study, analysis of the response of *Sigirr*-deficient cells to a variety of stresses demonstrated that heat shock, hyperosmotic shock, and increased albumin concentrations are not the factors which are affected by the presence/absence of SIGIRR. All three stress sorts are physiologically important stress factors in mammalian kidney and they can affect cellular protein functions and alter biosynthetic processes.

Both kidney epithelial cells and kidney myeloid cells play an important role in the immunity of the urinary tract and both are using pattern recognition receptors (PRR) to initiate the response to microbial organisms or injury. Renal tubular cells are also capable to present the antigens, which make them perfect initiators and regulators of immune system in the kidney (Kelley 1993). They respond to local infection by the release of variety of cytokines and chemokines that affect the cellular component of the innate immune response. The maturation and recruitment of renal APCs, therefore might depend on the tubular cells.

## 4.2. FUNCTION OF SIGIRR IN SYSTEMIC AUTOIMMUNITY

SLE is an autoimmune disease characterized by spontaneous lymphoproliferation, expansion of autoreactive B and T cells, and production of polyclonal autoantibodies against numerous nuclear antigens (Kotzin 1996, Lipsky 2001). Disease-related autoantibodies in SLE focus on dsDNA and chromatin and RNA-containing Ags such as Smith Ag and RNP or ribosomal components (Egner, 2000; Muro, 2005). Anti-dsDNA antibodies are thought to play a crucial role in the pathogenesis of lupus nephritis and are responsible for the tissue damage (Hahn 1998, Winfield 1977). They are found in 70 % of patients with SLE. In many patients with SLE, increased renal disease activity is associated with rising titres of anti-DNA antibodies (Bootsma 1995). RNA and RNA/protein macromolecules, such as Sm/RNP, constitute a second major category of autoantigen frequently targeted in systemic autoimmune diseases such as SLE. Around 30 % of SLE patients have circulating anti–Smith (Sm) antibodies recognizing the 7 Sm proteins (B, D1, D2, D3, E, F, G), which are common to all small nuclear ribonucleoproteins (snRNPs) and which associate with U snRNA (U1, 2, 4, 5). In addition, 40 % of SLE patients have anti-RNP antibodies, which specifically bind to the proteins within U1snRNPs (Migliorini 2005). The role for TLRs in stimulating dendritic cells (DCs) in autoimmune disease was suggested by the finding that DCs secrete inflammatory cytokines via a TLR9- or TLR7-dependent mechanism upon stimulation with nucleic acid-containing immune complexes (Boule 2004; Means 2005; Savarese 2006).

Defects in apoptosis, clearance of immune complexes (ICs), or regulatory cells function may lead to uncontrolled activity of self-reactive T- and B-lymphocytes and to the production of tissue-damaging autoantibodies and ICs. Autoantibody production, immune complex deposition and local inflammatory cytokine and chemokine production by infiltrating immune cells as well as intrinsic renal cells cause renal tissue injury in SLE. It has been demonstrated that ICs containing nucleic acids can directly activate murine B lymphocytes and DCs, which is contributing to the development of SLE (Bave 2000, Lovgren 2004, Leadbetter 2002).

In these studies we show that in C57BL/6 *lpr/lpr* mice, lymphoproliferation and dsDNA autoantibody production progress with age, but the development of the SLE in the C57BL/6 background is very slow. We showed that C57BL/6 *lpr/lpr* mice do not develop proteinuria and kidney damage, which is consistent with previous studies (Nagata 1995). An intact FasL/Fas system is required to limit certain inflammatory responses, and systemic inflammation, including renal inflammation in C57BL/6 *lpr/lpr* mice (Fleck 1998). The best-studied strains of mice that spontaneously develop a lupus-like pathology are the New Zealand Black/New Zealand White hybrid strain (NZB/WF1); the MRL/Mp *lpr/lpr* strain, which carries the *lpr* mutation of the FAS receptor gene; and the BXSB strain, which carries the Y chromosome autoimmune accelerator (*Yaa*) gene (Theofilopoulos and Dixon 1985). Extensive genetic mapping studies in all three strains have identified multiple strain specific intervals associated with disease susceptibility. It is interesting that the progression of SLE in C57BL/6 *lpr/lpr* mice, showed in this study is independent of DNA autoantibody production and proteinuria which may relate to the specific genetic background.

In our studies we used the C57BL/6 background to introduce the additional *Sigirr* mutation to the already preexisting *lpr/lpr* mutation. We were forced to use this genetic background because we were not able to generate double *lpr/lpr Sigirr-/-* mutation in MRL mice, which according to our previous data, would be more suitable for the lupus nephritis investigation. However, backcrossing to MRL background appeared to be very problematic because of the high mortality rate of these mice. We concluded that SIGIRR plays an important role in the development of SLE, but were unable to show this by histology and serum analysis. The *lpr/lpr Sigirr-/-* phenotype was investigated in C57BL/6 background, which appeared to be less severe in development of autoimmunity.

It is known that viral or bacterial infections can aggravate disease activity in pre-existing SLE, but the role of SIGIRR in this context is hypothetical. Nucleic acids have immunomodulatory functions as they are recognized by TLRs. TLR3 recognizes double-stranded RNA, TLR7 and TLR8 recognize single-stranded RNA, CpG-DNA is a ligand for TLR9, and all of these TLRs are expressed in the nephritic kidney. These nucleic acid-specific TLRs are localized within an intracellular compartment (Barton 2006). Chloroquine and other inhibitors of endosomal acidification prevent signaling through

TLR3, TLR7/8, and TLR9, which argues for endosomal maturation as a critical step in this process (Diebold 2004). TLR3 or TLR7 as well as TLR9 signaling can trigger the exacerbation of established immune complex disease in MRL lpr mice (Patole 2007, Anders 2004, Pawar 2006). Viral dsRNA can aggravate lupus nephritis locally through TLR3 on renal macrophages, dendritic cells, and glomerular mesangial cells (Patole 2005). Small nuclear RNA does activate B cells and dendritic cells via TLR7 (Savarese 2006). Bacterial CpG-DNA increases production of dsDNA autoantibodies and glomerular IgG deposits (Anders 2003). However all mentioned TLRs use different signalling pathways. For example, dsRNA-induced disease activity is independent of B cell activation and humoral anti-chromatin immunity in experimental SLE and therefore differs from effects of circulating TLR7 ligands and bacterial CpG-DNA (Patole 2007). Moreover, only CpG-DNA, but not polyI:C or imiquimod induce lupus nephritis in young MRL *lpr/lpr* mice, most likely due to its potential to activate B cells to produce autoantibodies and to secrete much higher levels of proinflammatory cytokines in immune cells as seen with TLR3 or TLR7 ligands (Pawar 2006). Interfering with nucleic acid-specific TLRs may offer a new understanding of the pathogenesis of a number of kidney diseases and potentially new targets for therapeutic intervention (Lenert 2005).
Here we hypothetized that SIGIRR, which is involved in processes of negative regulation of nucleic acid-specific TLRs may play a role in regulation of autoimmune tissue injury. In this study we used the model of spontaneous lupus-like immune disease in C57BL/6 *lpr/lpr* mice to study the effects of SIGIRR on SLE development. For the first time, this study provides evidence for SIGIRR, a negative regulator of TLRs to regulate proinflammatory cytokine and chemokine production in response to immune complexes.

Recently, independent research groups provide evidence that blocking of some TLRs may be a crucial approach in developing the therapeutic strategy against SLE. TLR7 overexpression is associated with antinuclear autoantibody production and lupus-like disease in mice (Pisitkun 2006, Subramanian 2006). Contrarily, *Tlr7*-deficient MRL *lpr/lpr* mice show less lymphoproliferation, less activation of plasmacytoid dendritic cells, and less autoimmune lung and kidney injury (Christensen 2006). Moreover, TLR7 blockade with synthetic oligodeoxynucleotides with immunoregulatory

sequences (IRS 661) substantially reduce autoimmune tissue lung and kidney injury (Pawar 2007), which is consistent with the phenotype of *Tlr7*-deficient MRL *lpr/lpr* mice (Christensen 2006). IRS 661 significantly reduced the number of CD4/CD8 double negative T cells in spleen (Pawar 2007), a population which continuously expands in MRL *lpr/lpr*, because of the inability to delete autoreactive T cells via the interaction of Fas with the Fas-ligand in these mice (Cohen 1991). TLR7 is required to generate anti-Sm RNP IgG (Christensen 2006) and serum levels of anti-Sm RNP IgG were also reduced with injection of IRS 661 (Pawar 2007). Furthermore, IRS 661 reduces the serum levels of anti-dsDNA IgG2a and IgG2b as well as glomerular deposits of IgG2a and complement factor C3c. The IRS 661 reduces also the production of CCL2 and CCL5 in kidney and macrophage and lymphocyte infiltrates in kidney MRL *lpr/lpr* mice (Pawar 2007). The contribution of TLR9 to the pathogenesis of lupus must involve different mechanisms; lack of TLR9 is associated with less chromatin-specific autoantibodies and with a higher activation state of plasmacytoid dendritic cells (Christensen 2006). However, injections with TLR7 plus TLR-9-antagonistic oligodeoxynucleotides had similar protective effects on kidney and lung disease in MRL *lpr/lpr* mice (Pawar 2007), that is comparable to what has been observed with oligonucleotide antagonists specific for TLR9 only in the same lupus model (Patole 2005) or in NZB/NZW mice (Dong 2005).

In our studies we observed that the lack of negative regulator of TLRs, SIGIRR is able to increase the anti-dsDNA antibody production as well as the production of proinflammatory cytokines such as IL-6 and IL-12. C57BL/6 *lpr/lpr Sigirr-/-* mice have elevated anti-dsDNA IgGs levels compared to *lpr/lpr* controls. SIGIRR seems to have mostly an influence on IgG1, IgG2b and IgG3 levels. We did not observe striking differences in case of IgG2a subtype. This suggests that SIGIRR has a protective role in autoimmunity. However, the mechanism of SIGIRR function may differ from this observed in case of TLR7 or TLR9 blockade with the antagonists. SIGIRR is most probably blocking the signaling of TLRs by the interaction with their TIR domain, not by affecting the ligand binding. Moreover, SIGIRR is probably able to inhibit at the same time more than only one signaling pathway by interacting with different TLRs at the same time as well as with the adaptor molecules. The

function of SIGIRR in blocking the development of SLE may be more complex and may involve a larger part of the innate immunity.

Aggravation of SLE in C57BL/6 *lpr/lpr* mice is supported by the activation of immune cells and production of proinflammatory cytokines. There is much evidence supporting the theory that DCs play a central role in infection-associated development of autoimmunity. For example, these cells constitutively express most TLRs in mice and humans (Hornung 2002, Muzio 2000). Ex vivo activation of DCs and transfer of such cells into mice that are prone to autoimmune myocarditis was sufficient to initiate overt myocarditis (Eriksson 2003); and aggravation of lupus nephritis can be triggered by injections of recombinant IL-12 in MRL *lpr/lpr* mice (Huang 1996). Moreover, the stimulation of TLR4 and TLR9 on DCs blocks the suppressor activity of CD4+CD25+ regulatory T cells via the secretion of IL-6 (Pasare 2003). The role of regulatory T cells in SLE remains to be elucidated. However, it is already known that their number is reduced in peripheral blood of SLE patients with active disease (Crispin 2003, Liu 2004). SIGIRR inhibitory function therefore may modulate adaptive immunity in SLE as well.

Bone marrow–derived dendritic cells produce proinflammatory cytokines, chemokines, and type I interferons upon stimulation with the TLR ligands or immune complexes. However, only stimulation with the immune complexes increases the proinflammatory cytokine production in C57BL/6 *lpr/lpr Sigirr-/-* mice. When exposed to LPS, CpG, imiquimod, pI:C RNA or polyU RNA, dendritic cells cells expressed large amounts of IL-6 and IL-12, proinflammatory mediators that are known to be involved in progression of SLE. However, no significant differences between *Sigirr+/+* and *Sigirr-/-* cells were detected in the investigated C57BL/6 *lpr/lpr* background. Moreover, in this genetic background *Sigirr* deficiency does not seem to affect the production of cytokines by dendritic cells. We did not expect this rather surprising phenomenon. The *lpr* mutation however, is abele to change phenotype of *Sigirr-/-* cells that we observed in in vitro experiments with immune cells. The production of cytokines by the dendritic cells isolated from *Sigirr+/+ lpr/lpr* or *Sigirr-/- lpr/lpr* mice was comparable.

Recently, many studies focused on TLR7 and proposed this receptor as a novel and potential therapeutic target in systemic lupus erythematosus. *Tlr7*-deficient and wild-type mice revealed that TLR7 contributes to the production of antibodies against the Smith antigen of Sm-RNP RNA (Christensen 2006). This is interesting, because the lupus autoantigen U1snRNP RNA was identified as an endogenous ligand for TLR7 (Savarese 2006, Vollmer 2005). Furthermore, 564 immunoglobulin transgenic mice produce large amounts of anti-RNA, -DNA, and -nucleosome antibodies of the IgG2a and IgG2b isotype that cause nephritis, a phenomenon which was abrogated in *Tlr7*-deficient mice (Berland 2006)

Based on the present evidence of a proinflammatory role of TLR7 for lupus we hypothesized that inhibition of TLR7 would have beneficial effects on experimental lupus. This study shows that SIGIRR plays a role in regulation of proinflammatory cytokine production by murine dendritic cells in response to immune complexes like U1snRNP autoantigen. U1snRNP immune complexes can stimulate pDC for type I IFN and IL-6 production via a TLR7-dependent pathway (Savarese 2006). U1snRNP can directly stimulate pDC for IFN-α production in a TLR7-dependent manner and stimulate monocytes for TNF-α production via a TLR8-dependent pathway (Vollmer 2005). SIGIRR is able to inhibit the production of cytokines after stimulation with the U1snRNP immune complex. SIGIRR exclusively regulates the mechanism of immunostimulation by nuclear self-antigens, shown here using the U1snRNP autoantigen. The mechanisms and the exact TLRs involved in this process are however not known. Immune complexes require to be delivered to the cytoplasm and this suggests that the endosomal TLRs (TLR3, -7 and -9) are involved in the inflammation process. One may assume that immune complex–induced maturation of DCs would enhance humoral immunity against chromatin, an important autoantigen in SLE. In fact, the data from this study clearly show that in C57BL/6 *lpr/lpr* mice, the deficiency of SIGIRR provides a signal for B cell activation, consistent with the finding that serum DNA autoantibody levels were elevated in *Sigirr*-deficient C57BL/6 *lpr/lpr* mice. Thus, lack of SIGIRR may affect global B cell activation by increased production of cytokines as a response to endogenous stimuli. Thus, the autoantibody production in vivo may well reflect direct effects of the IC on B cells, as well as indirect effects mediated by IC activation of DCs and subsequent induction of cytokine production or priming of helper T cells. Additional studies will

be necessary to evaluate the relative importance of DC and B cell intrinsic events. Overall, the presented data establish a role for SIGIRR in SLE. Our data suggest that TLR regulation plays an important role in the pathogenesis of lupus. The findings from this study may help to identify novel targets for the treatment of lupus. Pharmacologic agents interfering with the internalization of IC-containing nuclear autoantigens, endosomal maturation, and binding of endogenous ligands to the endosomally localized TLRs or targeting the regulatory molecules such as SIGIRR may help to forward immunotherapies for the treatment of SLE. We conclude that SIGIRR is one of the mechanisms that may protect the host from the exacerbation of pre-existing SLE. Thus, the roles of TLRs and their regulators for the evolution of specific autoantibodies may even be more complex and requires a detailed analysis of immune cell subsets.

In summary our data suggest that SIGIRR is involved in inhibition of TLR7 which, together with previous data, may lead to reduction of autoantibody production and prevents autoimmune tissue injury in experimental lupus. These data support the concept that TLRs signalling contributes to the pathogenesis of autoantibody production and autoimmune tissue injury in SLE and propose TLR negative regulation of TLRs as a novel therapeutic concept for lupus.

In summary, resident renal antigen-presenting cells contribute to TLR-mediated antimicrobial immunity in the kidney and this function is controlled by SIGIRR, an orphan receptor of the Il-1R family. Thereby, SIGIRR inhibits an inappropriate innate immune response in the kidney. However, SIGIRR does neither inhibit TLR signaling nor reduce TLR expression in tubular epithelial cells supporting their role as sensors of microbial infection in the kidney. Furthermore, SIGIRR is differentially regulated in immune/antigen presenting cells and tubular epithelial cells. Our data further support the idea that posttranslational modifications rather than alternative splicing account for the cell type-specific functions of SIGIRR. Ligands for SIGIRR remain unknown, but it has been shown that SIGIRR cannot bind to any known ligand of the Toll/IL-1R (TIR) superfamily such as LPS or IL-1, subsequently activating NF-κB signaling (Thomassen 1999).

Our data support the hypothesis that SIGIRR deficiency hyper-activates immune cells, and thus contributes to the pathogenesis of lupus. Physilogically, SIGIRR supresses the activity of SLE disease. Vice versa , lack of function (LOF) mutation of SIGIRR may predispose to SLE.

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## *List of publications:*

**Lech M**, Garlanda C, Mantovani A, Kirschning CJ, Schlondorff D, Anders HJ. Different roles of TiR8/Sigirr on toll-like receptor signaling in intrarenal antigen-presenting cells and tubular epithelial cells. Kidney Int. 2007 May 2; [Epub ahead of print]

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