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Bc. Zdeňka Hanusová

Fyziologická úloha proteinu SIGIRR v časném embryonálním vývoji

Physiological role of SIGIRR in early embryonic development

Diplomová práce

Školitel: RNDr. Dominik Filipp, CSc.

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Abstract

IL-1 receptor/Toll-like receptor (IL-1R/TLR) supefamily represents a group of proteins that share highly conserved TIR domain in their cytoplasmic region. Signal transduction mediated by TIR-containing proteins involves the activation of NF-κB transcription factor and thus the members of this superfamily play a key role in many physiological responses related to innate immune defense and inflammation. SIGIRR (single immunoglobulin IL1R-related molecule) is a recently discovered member of the IL-1R family, however it differs from the other group members by its unique structural features. SIGIRRhas been so far considered to be an 'orphan' receptor as no SIGIRR ligand has been identified yet. Moreover, SIGIRR itself is not capable to induce the NF-κB activation. Instead, SIGIRR is supposed to act as a negative regulator for IL-1Rs/TLRs mediated inflammation. Its inhibitory function has been implemented in several signalling pathways in various cell types and tissues including the kidney, the digestive tract and the lung. Recent reports also suggest that SIGIRR could play a role in early embryonic development. The main aim of this thesis is to characterize the mechanism how SIGIRR negative regulatory function in IL-1R/TLR signalling pathway is delivered. Here we describe the establishment of transduced cell lines suitable for biochemical and microscopic analyses of SIGIRR in hematopoietic cells. We also demonstrate that the difference in membrane distribution of SIGIRR in hematopoietic versus non-hematopoietic origin could at least partly explain our observation that SIGIRR does not function as a negative regulator of triggered TLR signalling, in at least some hematopoietic cells. By employment of BWZ.36 reporter cell line we established a model suitable for screening of SIGIRR binding molecules allowing to identify putative SIGIRR interacting ligands. Finally we show that SIGIRR is expressed in early stages of murine development in both $CD11b^{+}TLR2^{+}$ embryonic phagocytes and non-immune cells where its presence affects the expression level of other related TIR domain-containing proteins. These results are first to describe the potential dependency of SIGIRR function on its membrane distribution and provide a new insight into possible mechanisms of SIGIRR function, suggesting that SIGIRR can curb the triggering of TLR signalling in various cell types and tissues, including embryo.

Key words: SIGIRR, IL-1 receptor family, Toll-like receptors, TIR-containing adaptors, negative regulation, embryonic development.

Abstrakt

Receptory skupiny IL-1 a Toll-like receptory (IL-1R/TLR) jsou proteiny, které ve své cytoplasmatické části sdílejí vysoce konzervovanou Toll/IL-1R (TIR) doménu. Signalizace zprostředkovaná těmito proteiny zahrnuje aktivaci transkripčního faktoru NF-κB a členové této skupiny se tedy výrazně podílejí na řadě reakcí, jež jsou v těle součástí vrozené imunity a zánětlivého procesu. Protein SIGIRR (single immunoglobulin IL1R-related molecule) je nedávno objeveným členem rodiny IL-1 receptorů, který se od ostatních členů skupiny liší svojí unikátní proteinovou strukturou; prozatím také nebyl identifikován žádný jeho ligand. SIGIRR sám není schopen zprostředkovat aktivaci NF-κB a jeho hlavní funkce zřejmě spočívá v negativní regulaci zánětlivých reakcí vyvolaných ostatními receptory skupiny IL-1R/TLR. Jeho inhibiční účinky již byly popsány pro několika signálních drah probíhajících v různých typech buněk a tkání, především pak v ledvinách, zažívacím traktu a plicích. Nedávná zjištění také naznačují, že protein SIGIRR by mohl hrát důležitou roli v časném embryonálním vývoji. Hlavním cílem této práce je přispět k pochopení mechanismů, kterými je zprostředkována funkce proteinu SIGIRR jako negativního regulátoru IL-1R/TLR signalizace. Pro účely biochemických a mikroskopických analýz proteinu SIGIRR v hematopoietických buňkách jsme vytvořili modelové buněčné linie. Ukázali jsme, protein SIGIRR vykazuje odlišnosti v distribuci a aktivitě v buňkách různého původu a také jsme prokázali, že SIGIRR nepůsobí jako negativní regulátor TLR signalizace v různých hematopoietických buňkách. S využitím reportérové buněčné linie BWZ.36 jsme vytvořili model vhodný pro testování možných molekul vázajících SIGIRR a umožňující tak identifikaci a další studium jeho interakčních partnerů. Stanovili jsme, že protein SIGIRR je exprimován v raných fázích myšího embryonálního vývoje, a to jak $CD11b^{+}TLR2^{+}$ embryonálními fagocyty, tak neimunitními buňkami. Ukázali jsme, že vybrané proteiny obsahující TIR doménu vykazují odlišnou kinetiku exprese v průběhu rané embryogeneze při srovnání SIGIRR-deficientních a wild-type myší. Předkládané výsledky jako první popisují možnou závislost aktivity proteinu SIGIRR na jeho membránové distribuci. Práce také poskytuje nový pohled na možné mechanismy, jimiž je funkce proteinu SIGIRR zprostředkována, naznačující, že SIGIRR by mohl bránit nežádoucímu spuštění TLR signalizace v různých buněčných typech a tkáních, včetně vyvíjejícího se embrya.

Klíčová slova: SIGIRR, rodina receptoru pro IL-1, receptory skupiny Toll, adaptory s TIR doménou, negativní regulace, embryonální vývoj.

Contents

List of abbreviations

2-ME	2-mercaptoethanol
Ab	antibody
ANOVA	analysis of variance
APC	antigen-presenting cell
BFB	bromophenol blue
BSA	bovine serum albumin
CasC3	cancer susceptibility candidate 3
CD	cluster of differentiation
cDNA	complementary DNA
CPRG	chlorophenolred-ß-D-galactoside
DAB	3,3´-diaminodbenzidine
DAMP	danger-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DNA	deoxyribonucleic acid
E	day of embryonic development
EDTA	ethylenediaminetetraacetic acid
EEC.B	early embryonic cell line B
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
EYFP	enhanced yellow fluorescent protein
FCS	fetal calf serum
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
HA	hemagglutinin
HAEC	human airway epithelial cell line
HBSS	Hank's balanced salt solution
HEK293T	human embryonic kidney 293T cell line
HRP	horseradish peroxidase
Hsp	heat shock protein
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-1R	interleukin-1 receptor
IMDM	Iscove's modified Dulbecco's medium
IRAK	interleukin-1 receptor-associated kinase
IRES	internal ribosome entry site

LM	laurylmaltoside
LPS	lipopolysaccharide
LRR	leucin-rich repeate
MAL	MyD88 adaptor-like
MEM	minimum essential medium
MSCV	murine stem cell virus
MyD88	myeloid differentiation primary-response gene 88
NK	natural killer
NF-κB	nuclear factor-kappa B
NLR	Nod-like receptor
OD	optical density
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptor
qRT-PCR	quantitative real-time polymerase chain reaction
RLR	RIG-I-like receptors
RNA	ribonucleic acid
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SARM	sterile α and HEAT/armadillo motif protein
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIGIRR	single Ig IL-1R-related molecule
S.O.C.	super optimal broth with catabolite repression medium
TBE	tris/borate/EDTA buffer
TBS-T	tris-buffered saline Tween 20
TIGIRR	triple Ig IL-1R-related molecule
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-alpha
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFN-β
WB	Western blot
WT	wild-type

1 Introduction

Immune system represents the capability of organism to recognize and resist harmful exogenous pathogens and also dangerous structures of endogenous origin. To ensure proper operation of these processes, immunity involves two basic components – innate (antigen-nonspecific) and adaptive (antigen-specific) immune responses. Both mechanisms exist side-by-side. Innate immunity represens the first line of defense and provide immediate reaction against infection. On the other hand, although adaptive immunity is characterized by slow starting, it could confer specific long-lasting and protective immunity to the host.

One of the most powerful immune tools is represented by a set of highly-conserved group of Interleukin-1 receptor (IL1R)-like and Toll-like receptor (TLR) proteins, that play key roles in many physiological reactions related to innate immunity and inflammation (O'Neill, 2008). These proteins share common cytoplasmic region called Toll-IL1R (TIR) domain, which is involved in the activation of NF-κB transcription factor. The activation of signalling cascade has to be tightly regulated in order to prevent trigerring of potentially devasting local and systemic inflammatory reactions. Several distinct molecules had been identified to act as negative regulators for IL-1R/TLR system, such as IL-1R antagonist (Muzio et al., 1995), transmembrane regulator ST2 (Brint et al., 2004) or intracellular proteins IRAK-M (Kobayashi et al., 2002) and MyD88s (Janssens et al., 2002). Recent evidence suggests that protein SIGIRR (single Ig IL-1R-related molecule, also known as TIR8) could also be a part of this complicated regulatory network. This transmembrane protein posseses unique structural features that distinguishes it from other members of ILR family and mediates suggested negative effects of SIGIRR to IL-1R/TLR signalling. Up till now it has been shown that SIGIRR is expressed in many distinct cell types in various tissues. The results of previous experiments performed in our lab also point to a relatively high level of SIGIRR expression in early embryonic stages of murine development (E6.5 – E8.5) (Vavrochová, 2010) .

The main aim of this thesis is to contribute to understanding of the role of SIGIRR as a negative regulator of IL-1R/TLR signalling pathway. We will describe the development of various SIGIRR constructs and establishment of transduced cell lines suitable for biochemical analyses of SIGIRR. We will also show the difference in the distribution and activity of SIGIRR between cells of hematopoietic origin and non-hematopoietic cells and thus provide a new insight into possible mechanisms of SIGIRR function. A newly established model based on

BWZ.36 reporter cell line which is suitable for screening and study of possible SIGIRR interacting partners will be presented in the following part of this thesis. Finally, we will focus on the role of SIGIRR at embryonic level. We will show the expression profile of SIGIRR in embryonic phagocytes and non-immune cells and also describe the distinct kinetics of expression for selected TIR domain-containing proteins in the wild-type and SIGIRR$^{-/-}$ embryos, suggesting that SIGIRR could represent a molecular brake preventing the IL-1R/TLR signalling from potentially harmful trigerring in early stages of embryonic development.

2 Literature review

This section of the thesis presents the brief overview of IL-1R/TLR signalling. We will describe particular members of this protein family and various mechanisms of signal transduction triggered by this type of receptors, with special emphasis on related regulatory processes. Further we will sumarize the current knowledge about SIGIRR, recently discovered member of IL-1R family with inhibitory potential, and describe its structure, expression and function. We will also elaborate on our preliminary results concerning the early embronic cells where a relatively high expression level of SIGIRR were detected. This led us to propose that SIGIRR could represent a potent negative regulator of inflammatory processes in early stages of murine embryonic development.

2.1 Innate immune responses: the first line of defence

The immune system of higher vertebrates is traditionally divided into two components, innate and adaptive immunity. The innate immunity relies on a set of ready-to-go mechanisms which can mediate the recognition of potentially danger signals and subsequent triggering of an appropriate response. The adaptive immune system is on the other hand characterized by the ability to provide antigen-specific responses and immunological memory. Key players in these processes are antigen receptors expressed on T and B lymphocytes. These cell surface structures are randomly generated by a unique process of gene rearrangement that ensures their practically unlimited diversity. Both parts actively cooperate and regulate each other. Moreover, innate immune system plays an essential role in the activation of the adaptive immune responses (Medzhitov and Janeway, 1998).

Innate immune mechanisms are constantly present in the host, meaning that they are ready to respond to pathogen invasion or tissue damage at any given time and do not require an induction period for their proper function. Several important components of innate immunity are typically distinguished and include anatomical barriers, normal intact commensal flora, antibacterial molecules and also inflammatory and phagocytic responses. We will focus here on the last mentioned processes as SIGIRR is predominantly involved in this type of immune action.

2.1.1 Phagocytosis and pathogen recognition, basic processes in innate immunity

Phagocytosis is a term describing the process when solid particles are engulfed by cell membranes. This process is on molecular level relatively conserved and its evolutionary invention could be tracked even before the development of multicellularity (Stuart and Ezekowitz, 2008). Phagocytosis was discovered in 1882 by Russian embryologist Elie Metchnikoff who first to describe this phagocytic function as 'eating to defend' and propose that phagocytosis represents a cell-mediated immune response (Tauber, 2003). Based on their morphology and biochemical features, so-called professional phagocytes involved in immune processes could be divided into two groups, polymorphonuclear phagocytes represented by 3 types of granulocytes (neutrophilic, basophilic and eosinophilic) and also mononuclear phagocytic cells. This category of cells comprises monocytes, tissue macrophages and also dendritic cells, which represent the main type of professional APCs (antigen-presenting cells) involved in the stimulation of T cells. Dendritic cells thus serve as mediators between innate and adaptive branch of immunity (Banchereau and Steinman, 1998). However, the detailed categorization of phagocytic cells and their subtypes has still not been established and remains the object of interest.

Many mechanisms of innate immune responses, such as triggering the inflammation, are dependent on the recognition of evolutionarily conserved structures, designated as PAMPs (pathogen-associated molecular patterns). Limited number of so called pattern recognition receptors (PRRs) serves these purposes. Several classes of PRRs, such as Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) have been discovered and characterized so far. Together PRRs represent a potent group of germ-line encoded receptors with ability to mediate the host defense against various types of pathogens, both extracellular and intracellular, through recognition of distinct classes of molecules including proteins, lipids, carbohydrates and nucleic acids (Figure 2.1). Several comprehensive and excellent reviews have recently been published on this topic (Kawai and Akira, 2010; Medzhitov, 2007; Takeuchi and Akira, 2010).

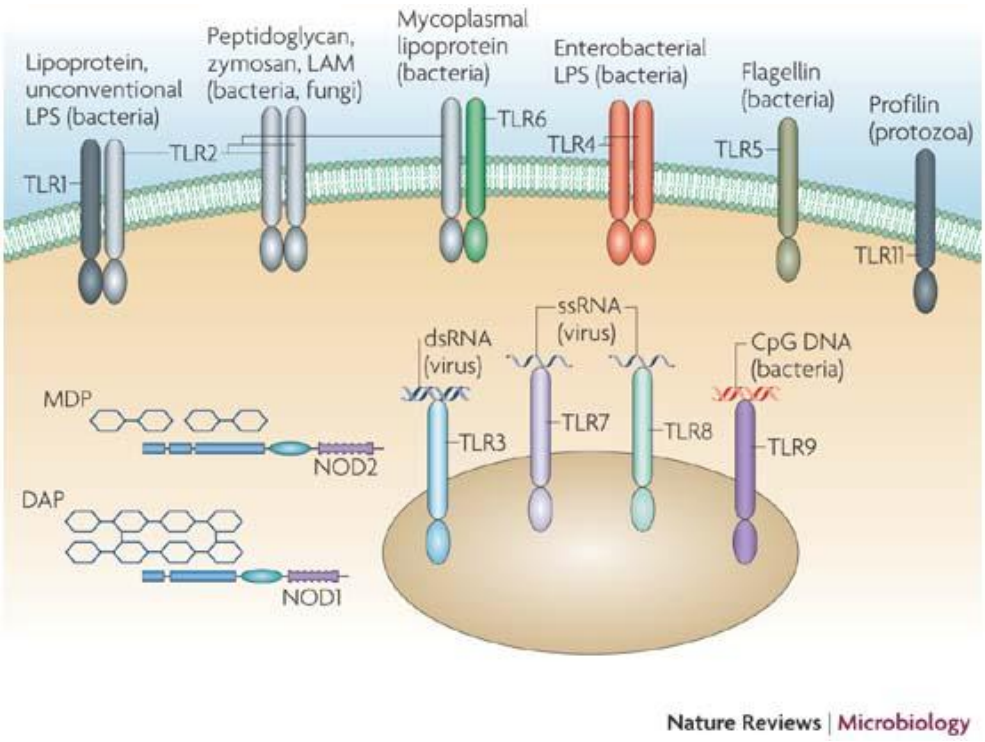

Figure 2.1 Illustration of various selected pattern recognition receptors and their exogenous ligands. DAP, diaminopimelic acid; ds, double-stranded; MDP, muramyl dipeptide; LPS, lipopolysaccharide; LAM, lipoarabinomannan; ss, single-stranded. Taken from (Kaufmann, 2007).

PAMPs are besides other things characterized by being invariant among various microorganisms of a given class, but are never present in host cells. Thus, they could be used for distinguishing the 'self' from 'non-self'. However, several PRRs are also able to recognize endogenous ligands, which could serve as 'danger' signals, also called DAMPs (danger-associated molecular patterns). These factors, produced by necrotic cells as a consequence of infection, inflammation or other type of cellular stress, could mediate the triggering of PRR signalling and thus protect the host from undesirable tissue damage. Discovery of such molecules supports the so called 'danger theory', proposed by Polly Matzinger. Based on this hypothesis the triggering of immune responses is initiated upon the recognition of endogenous danger signal originated from infected or somehow injured cells rather than by recognizing the 'non-self' structures (Matzinger, 2002). So far, this interesting model has been scrutinized in the context of TLR signalling (Asea et al., 2002; Park et al., 2004; Vabulas et al., 2002).

2.2 The IL-1R/TLR superfamily

The interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily was at first defined at 1998 (O'Neill and Greene, 1998). All members of this group share one common feature, the presence of Toll/IL-1 receptor (TIR) domain in their cytoplasmic region. This domain is required for the initiation of signalling pathways mediated by IL-1R/TLR proteins, as it is responsible for the interaction and recruitment of various adaptor molecules involved in signal transduction.

The journey leading to classification of IL-1R/TLR proteins as a separate superfamily began in the early 1980s with the discovery of IL-1, a potent mediator capable to affect almost every cell type and tissue in the body (Dinarello, 2010). Later, the receptor for IL-1, containing 3 extracellular Ig domains (IL-1R1), was described (Sims et al., 1988). However, at this time, the intracellular part of IL-1R1 did not show a homology with any other known protein. This situation changed dramatically in 1991 when it was found that IL-1R1 and the *Drosophila melanogaster* protein Toll (dToll) have common intracellular domain (Gay and Keith, 1991), later designated as 'TIR domain'. This observation was surprising, because not only that the extracellular region of Toll was distinct from IL-1R1 and composed of leucine-rich repeats (LRRs), but Toll also did not participate in the inflammatory responses and its role was in the determination of dorso-ventral embryonic polarity (Anderson et al., 1985; Hashimoto et al., 1988). Later, dToll was shown to mediate the resistance against fungal infections (Lemaitre et al., 1996). These discoveries were further followed by the identification of human Toll-like receptor with capability to activate the transcription factor NF-κB (Medzhitov et al., 1997), and demonstration of its function in innate immune responses to lipopolysaccharid (LPS) (Poltorak et al., 1998). Since then, an intensive study of the IL-1R1/TLR superfamily led to discovery of many other TIR-containing proteins whose fuction and regulation is still under investigation.

2.2.1 Structural characteristics of the IL-1R/TLR family members

Proteins of the IL-1R/TLR superfamily are defined by the presence of cytoplasmic TIR domain. Thus, IL-1Rs are similar to TLRs in the intracellular region, but their extracellular parts differ markedly. The extracellular N-terminal domain of TLRs contains approximately 16 - 28 tandem copies of leucine-rich repeats (LRR), whereas the extracellular part of IL-1Rs comprises 3 immunoglobulin (Ig) domains. The only exception here is represented by SIGIRR,

which possesses only one extracellular Ig domain. Another group of TIR-containing proteins is represented by adaptor molecules such as MyD88, TRIF or SARM (Figure 2.2). These intracellulary localized proteins are involved in signal trasduction upon activation of receptors from IL-1R/TLR superfamily. Although IL-1Rs and TLRs bind different ligands, many components involved in signal transduction are common to both the TLR and IL-1R pathways because of their shared TIR domains (Medzhitov, 2001).

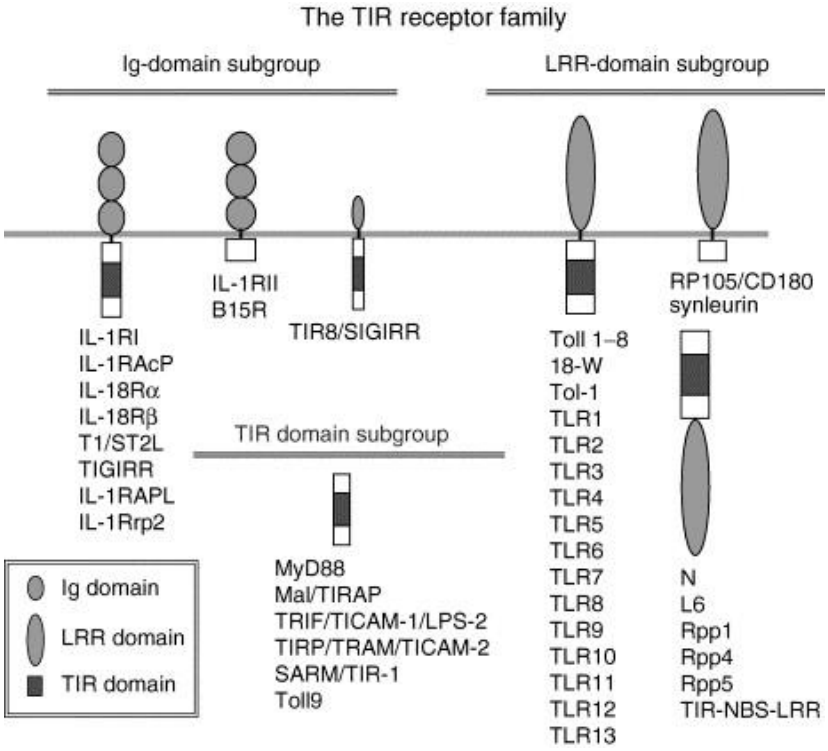

Figure 2.2 Members of the IL-1R/TLR superfamily. These proteins share common TIR domain in their intracellular region and thus this group is sometimes reffered to as the 'TIR receptor family'. In contrast, extracellular regions of IL-1Rs and TLRs are structurally unrelated and are composed of Ig domains (IL-1Rs) or leucin-rich repeat (LRR) motifs. Intracellular TIR-containing adaptor proteins mediate the signal transduction after IL-1Rs or TLRs activation. Taken from (Boraschi and Tagliabue, 2006).

2.2.2 Members of the IL-1R subfamily

IL-1 receptor family represents a group of closely related proteins; many of their encoding genes are clustered in a short region of human chromosome 2. So far, 10 members of the IL-1R family has been identified in mice and humans (Table 2.1). This group comprises six receptors (IL-1R1, IL-18Rα, ST2, TIGIRR-1, TIGIRR-2, and IL-1Rrp2), two coreceptors (IL-1RAcP and IL-18Rβ), an inhibitory orphan receptor (SIGIRR) and also a decoy receptor (IL-1RII)

(Casanova et al., 2011; Dinarello, 2009). Although IL-1RII lacks the TIR domain, it is still considered to be a part of this family due to its high similarity to the IL-1RI. Considering this group of proteins, the only members without proposed function are TIGIRR-1 and TIGIRR-2. Interestingly, TIGIRR-2 mutation has been found to associate with X-linked retardation in humans (Carrie et al., 1999). The fundamental knowledge about function of the other proteins from this family will be described below.

Name	Ligand	Expression
IL-1RI (membrane and soluble form)	Initiates and amplifies the immune and inflammatory response upon binding the agonist ligands IL-1α and IL-1β; inhibited upon binding the antagonist ligand IL-1Ra; the soluble form binds IL-1F10	Expressed by all cells responsive to IL-1, predominant type of IL-1R on T cells, fibroblasts, epithelial and endothelial cells
IL-1RII (membrane and soluble form)	Binds IL-1β and less efficiently IL-1α and IL-1Ra; decoy receptor, unable to initiate signal transduction. Both membrane and soluble form have inhibitory activity	Expressed by many cell types, particularly abundant on B cells, mononuclear phagocytes, polymorphonuclear leukocytes, and bone marrow
IL-1RAcP (membrane and soluble form)	Coreceptor for IL-1RI responsible for signaling after binding IL-1α or IL-1β; can form inactive complexes with IL-1RII bound to IL-1; coreceptor for IL-1Rrp2 in activation by IL-1F6, IL-1F8, and IL-1F9	Expressed by all cells responsive to IL-1
IL-18Rα (IL-1Rrp1)	Binds IL-18 and IL-1F7	Mononuclear phagocytes, neutrophils, Th1 cells, NK cells, endothelial cells, smooth muscle cells
IL-18Rβ (AcPL)	Coreceptor for IL-18Rα responsible for signaling after binding of IL-18	Mononuclear phagocytes, neutrophils, Th1 cells, NK cells, endothelial cells, smooth muscle cells
T1/ST2 (membrane and soluble form)	Orphan receptor; negative regulator of IL-1R, TLR2, and TLR4	Th2 cells, mast cells, fibroblasts
TIGIRR (TIGIRR-1)	Orphan receptor	Skin; less in liver, placenta, fetal brain
IL-1RAPL (TIGIRR-2)	Orphan receptor; Ca^{2+}-dependent inhibition of exocytosis; involved in X-linked mental retardation	Heart, brain, ovary, skin; less in tonsils, fetal liver, prostate, testis, small intestine, placenta, colon
IL-1Rrp2	Binds the agonists IL-1F6, IL-1F8, and IL-1F9, as well as IL-1F5 (antagonist of IL-1F9)	Lung, epididymis, lower levels in testis, and cerebral cortex (nonneuronal)
TIR8 (SIGIRR)	Orphan receptor; negative regulator of TLR/IL-1R signaling	Ubiquitous; highly expressed in intestinal epithelial cells

Table 2.1 Members of the IL-1R family. Taken from (Boraschi and Tagliabue, 2006).

The ligands for IL-1R family are represented by a group of cytokines designated as IL-1 family (Figure 2.3). Well-known are molecules IL-1, IL-18 and IL-33, but the role of novel members of the this family in signalling mediated by IL-1Rs is less well defined. However, IL-1F6, -F8 and -F9 are recognized by IL-1Rrp2 (also known as IL-1RL2) with IL-1Racp coreptor and activate NF-κB and MAP kinases, similarly as IL-1, IL-18 and IL-33 do (Towne et al., 2004). IL-1F5 on the other hand serves as an antagonist for these cytokines (Blumberg et al., 2007).

IL-1R1 together with accesory protein IL-1Racp forms a receptor complex for IL-1, both α and β. The response is initiated by binding of ligand to IL-1R1, subsequent recruitment of the IL-1Racp (also IL-1RAP) and induction of further signalling (Greenfeder et al., 1995).

This patway could be blocked by IL-1Ra (IL-1R antagonist) (Eisenberg et al., 1991). This cytokine not only prevents the binding of IL-1 to IL-1R1, but also does not allow the recruitment of IL-1Racp. IL-1Ra expression is induced by various inflammatory stimuli in many tissues. Another regulator for IL-1R1 signalling is IL-1RII. This protein could bind IL-1, but is has only a short cytoplasmic region which is unable to mediate the signalling; thus, IL-1RII acts as a decoy receptor (Colotta et al., 1993). IL-18 is recognized by IL-18R formed by two subunits.

IL-33, recently discovered cytokine from IL-1 family, has been shown to serve as a ligand for ST2 (also called IL-33Rα) (Schmitz et al., 2005). Upon IL-33 recognition, ST2 recruits the coreceptor molecule IL-1Racp to initiate the activation of MAPK signalling pathway (Brint et al., 2002). It was found that ST2 is highly expressed by Th2 lymfocytes and probably plays a role in Th2 activation (Lohning et al., 1998). Beside this, ST2 possesses a regulatory function and is involved in suppression of IL-1R and several TLR mediated signalling pathways (Brint et al., 2004). Moreover, soluble form of ST2 generated by alternative mRNA splicing could bind to IL-33 and inhibits its activity (Hayakawa et al., 2007; Palmer et al., 2008). SIGIRR is another protein from IL-1R family, which acts as a negative regulator for IL-1R/TLRs signalling. Details about this protein, its structure, expression and function will be described in Chapter 2.3.

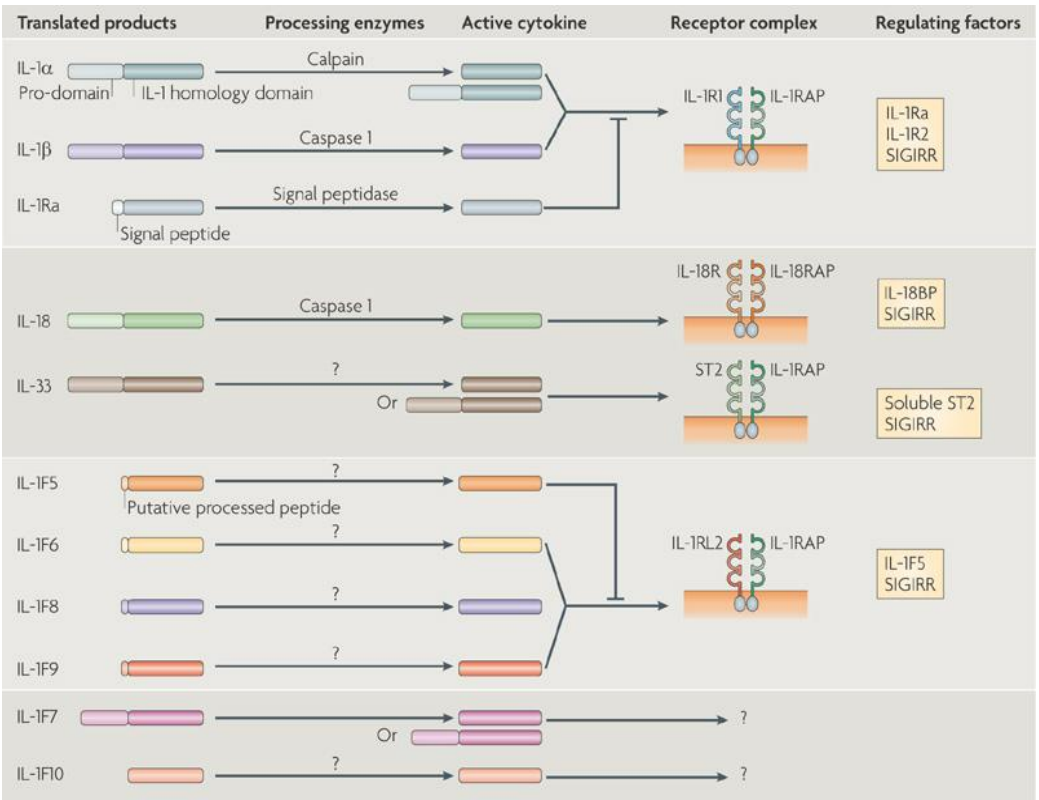

Figure 2.3 Interleukin-1 family members, their receptors and regulation. AP, accessory protein; IL-18BP, IL-18 binding protein; R, receptor; Ra, receptor antagonist; RL, receptor-like. Taken from (Sims and Smith, 2010).

2.2.3 Toll-like receptors and their ligands

Toll-like receptors are expressed mainly by innate immune cells represented by macrophages, dendritic cells, neutrophils or NK cells. However, their expression in other cell types such as lymphocytes, endothelial cells and fibroblasts has been also well described (Chang, 2010). Thus, TLRs are considered to be very important receptors triggering the innate immune responses. Humans express ten TLRs (TLR1 to TLR10), whereas twelve TLRs (TLR1 to TLR9, TLR11 to TLR13) were found in mice.

Each TLR could recognize distinct PAPMs derived from viruses, bacteria, mycobacteria, fungi, or parasites. However, it seems that TLRs could also detect endogenous ligands serving as 'danger' signals (Chapter 2.1.1). So far, ligands have been identified for all TLRs except for human TLR10 and mouse TLR12. Very recently it was shown that bacterial RNA represents a ligand for TLR13 (Hidmark et al., 2012). Basic overview of TLRs and their ligands is shown in Table 2.2.

TLRs are localized in the distinct cellular compartments and this distribution reflects in which of the compartments their ligands are found. TLR1, TLR2, TLR4, TLR5 and TLR6 and are expressed on the cell surface where they could detect molecular components exposed on the surface of pathogens (Hayashi et al., 2001; Poltorak et al., 1998; Takeuchi et al., 2001; Takeuchi et al., 2002; Yang et al., 1998; Zhang et al., 2004). By contrast, TLR3, TLR7, TLR8 and TLR9 are expressed within intracellular vesicles, which enable these TLRs to recognize viral and bacterial nucleic acids (Alexopoulou et al., 2001; Diebold et al., 2004; Heil et al., 2004; Hemmi et al., 2000). Recently it was shown that TLR11 and TLR13 are also localized intracellulary (Pifer et al., 2011; Shi et al., 2011).

The exact mechanism of how TLRs could recognize their antigen has not been fully determined yet. It seems that some TLRs could bind their ligands independently, such as TLR3 and TLR9 (Kleinman et al., 2008; Park et al., 2008), whereas other need coreceptors (Lee et al., 2012). LPS-recognition mediated by TLR4 is supposed to be dependent on accesory proteins LBP (LPS-binding protein), CD14 and MD2 (Palsson-McDermott and O'Neill, 2004), however, the precise mechanism of this process has not been fully elucidated yet. It has been shown that TLRs could also form heterodimers (such as TLR1/2) and homodimers (such as TLR3/3) after association with their ligands; the crystal structure of these complexes has been described recently (Jin et al., 2007; Park et al., 2009).

TLR	Location of TLR	PAMPs recognized by TLR	Co-receptor (s)	Signaling adaptor	Transcription factor(s)	Effector cytokines induced
TLR1/2	Plasma membrane (cell surface)	Triacyl lipopeptides (Bacteria and Mycobacteria)	Hetrodimer of TLR1/2 forms a functional receptor	TIRAP, MyD88	NFκB	Inflammatory cytokines (TNF-α, IL-6 etc.)
TLR2	Plasma membrane (cell surface)	Peptidoglycan (Gram-positive bacteria), LAM (Mycobacteria), Hemagglutinin (Measles virus), phospholipomannan (*Candida*), Glycosylphosphophatidyl inositol mucin (*Trypanosoma*)	CD36, RP105	TIRAP, MyD88	NFκB	Inflammatory cytokines (TNF-α, IL-6 etc.)
TLR3	Endosome	ssRNA virus (WNV), dsRNA virus(Reovirus), RSV, MCMV		TRIF	NFκB, IRF3,7	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs
TLR4	Plasma membrane (cell surface)	LPS (Gram-negative bacteria), Mannan (*Candida*), Glycoinositolphospholipids (*Trypanosoma*), Envelope proteins (RSV and MMTV)	MD2, CD14, LBP, RP105	TIRAP, MyD88, TRAM and TRIF	NFκB, IRF3,7	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs
TLR5	Plasma membrane (cell surface)	Flagellin (Flagellated bacteria)		MyD88	NFκB	Inflammatory cytokines (TNF-α, IL-6 etc.)
TLR6/2	Plasma membrane (cell surface)	Diacyl lipopeptides (Mycoplasma), LTA (Streptococcus), Zymosan (*Saccharomyces*)	Hetrodimer of TLR6/2 or dectin-1 forms a functional receptor	TIRAP, MyD88	NFκB	Inflammatory cytokines (TNF-α, IL-6 etc.)
TLR7	Endosome	ssRNA viruses (VSV, Influenza virus)		MyD88	NFκB, IRF7	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs
TLR8[#]	Endosome	ssRNA from RNA virus		MyD88	NFκB, IRF7	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs
TLR9	Endosome	dsDNA viruses (HSV, MCMV), CpG motifs from bacteria and viruses, Hemozoin (Plasmodium)		MyD88	NFκB, IRF7	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs
TLR11[*]	Plasma membrane (cell surface)	Uropathogenic bacteria, profillin-like molecule (*Toxoplasma gndii*)		MyD88	NFκB	Inflammatory cytokines (TNF-α, IL-6 etc.)

LAM, Lipoarabinomannan; WNV, West Nile virus; RSV, Respiratory syncytial virus; MCMV, Murine cytomegalovirus; MMTV, Mouse mammary tumor virus; LTA, Lipoteichoic acid; VSV, Vesicular stomatitis virus; HSV, Herpes simplex virus and CpG, Cytidine-phosphate-guanosine.

[#] Human TLR8.

[*] Expressed in mouse.

Table 2.2 Overview of Toll-like receptors, their localization and ligands. Notably, although TLR11 is stated here as a plasma membrane protein, recently is was shown that it is localized intracellulary. See the main text for details. Taken from (Kumar et al., 2009).

2.2.4 IL-1Rs/TLRs mediated signalling pathways and their regulation

Although receptors from IL-1R and TLR family posses different extracellular domains and thus recognize different ligands, they share common intracellular components involved in signal transduction. Upon activation by their ligands, IL-1R1, IL-18R, ST2 and TLRs recruit the TIR-containing cytosolic adaptors which then initiate the downstream signaling pathway leading to the activation of transcription factors such as NF-κB, IRFs and AP-1. It has been demonstrated that the interaction between TIR domains of receptors and adaptors is necessary for this process. Several interesting reviews have been dedicated to this area (Jenkins and Mansell, 2010; Kumar et al., 2009; Takeda and Akira, 2004). Here, we will briefly summarize the basic processes of IL-1R/TLR signalling, as illustrated also in Figure 2.4.

IL-1R1, IL-18R, and ST2 have all been shown to signal via MyD88 (myeloid differentiation primary-response gene 88) (O'Neill, 2000). Once activated with its ligand, MyD88 is recruited to the receptor through its TIR domain. This interaction allows the recruitment of another proteins involved in signal transduction, IRAKs (interleukin-1 receptor-associated kinase) 1, 2 and 4. IRAK4 is the first one which becomes activated and mediates the phosphorylation of IRAK1. As a consequence, TRAF6 (TNF receptor associated factor 6) is recruited to the signalling complex. TRAF6 is an E3 ubiquitin ligase and together with proteins UBC13 and UEV1A catalyzes the activation of a downstream kinase TAK1 (TGF-β-activated kinase 1). This kinase then mediates the activation of IKK (inhibitor of NF-κB kinase) complex which catalyzes the subsequent degradation of IκB (inhibitor of NF-κB). As a consequence, the transcription factor NF-κB (also known as p50/p65 heterodimer) is free to translocate from the cytosol to the nucleus. JNK/p38 MAP kinases could also be activated by TAK1 and mediate the activation of another transcription factor, AP-1. Both NF-κB and AP-1 could induce the expression of target pro-inflammatory genes such as TNF-α, IL-1β, IL-6 and IL-12.

The signalling pathway decribed above is used also by all members from the TLR group, except TLR3. The MyD88-independent, but TRIF (TIR-domain-containing adaptor protein inducing IFN-β)-dependent pathway is employed after activation of the TLR3 signalling. In this pathway, TRIF interacts with TRAF3 which then form a complex with TBK1 (TANK-binding protein 1) and noncanonical IKKs. This results in the dimerization and activation of IRFs (interferon regulatory factor) 3 and 7, which then translocate into the nucleus and activate the transcription of type I IFN and IFN-inducible genes. TRIF could also

mediate the activation of NF-κB and MAP kinases by interaction with TRAF6. TLR4 is the second receptor that could also mediate the signallization by TRIF-dependent pathway.

Beside MyD88 and TRIF, other TIR-containing molecules have been identified to mediate the signal transduction via TLRs. TIRAP (TIR domain-containing adaptor protein, also known as MAL, MyD88-adaptor-like protein) is involved in the MyD88-dependent signalling triggered by TLR2 and TLR4 (Fitzgerald et al., 2001), whereas TRAM (TRIF-related adaptor molecule) is required for activation of the TRIF-dependent patway by TLR4 (Fitzgerald et al., 2003). The least well characterized of these adaptors is SARM (sterile α- and armadillo-motif-containing protein). It was found that SARM might be a negative regulator of TRIF-dependent signalling (Carty et al., 2006). However, this was shown for human, but not for murine SARM (Kim et al., 2007), and thus the function of this protein has to be further investigated.

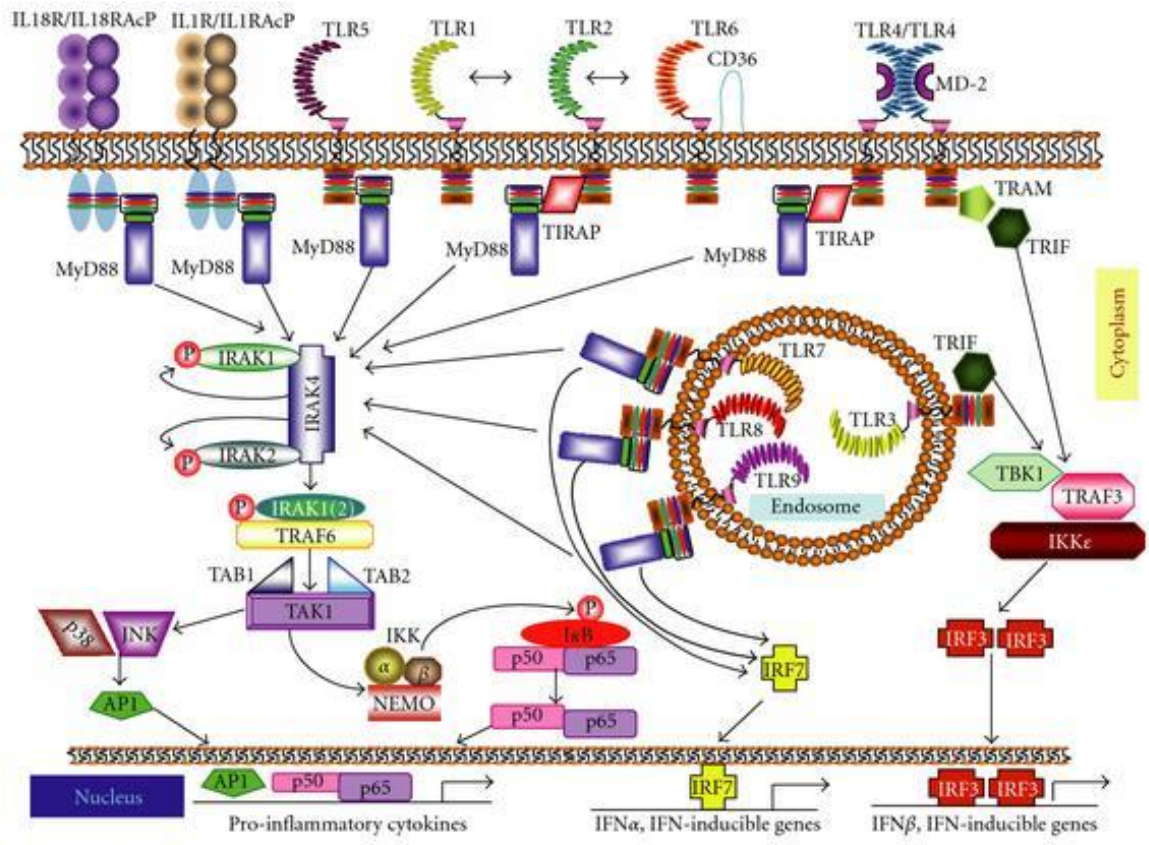

Figure 2.4 TLR/IL-1R signalling pathways. Both IL-1R-like and Toll-like receptors trigger their signalling via TIR-containing adaptor proteins. After the activation of IL-1R1, IL-18R, ST2 and TLRs (except TLR3), MyD88 associates with the receptor and mediate the further recruitment of IRAKs, TRAF6, and the TAK1 complex, leading to the activation of NF-κB and AP-1 transcription factors. TLR3 and TLR4 could signal via TRIF-dependent pathway, leading to activation of transcription factors IRF3 and IRF7. See the main text for details. Taken from (Loiarro et al., 2010).

IL-1R/TLR signalling pathways has to be tightly regulated because their inappropriate activation and triggering of uncontrolled inflammation processes could cause severe tissue damages and can contribute to the pathogenesis of many human diseases. So far, many molecules and disctinct mechanisms that could supress the IL-1R/TLR mediated signalling at multiple levels have been identified. As the negative regulation of immune responses mediated by members of the IL-1R family has been briefly described in Chapter 2.2.2, this part will be devoted mainly to the negative regulation of TLRs.

Various mechanisms could be employed in order to regulate the TLR-mediated immune response. One of the most important way is represented by reducing the TLRs levels. This could be mediated by inhibition of TLR expression by anti-inflammatory cytokines, especially TGF-β (transforming growth factor β) (McCartney-Francis et al., 2004). Another way is represented by degradation of TLRs through ubiquitylation. Several ubiquitin ligases were identified to posses a regulatory function for TLR signalling, such as Triad3A (Chuang and Ulevitch, 2004) or SOCS-1 (Suppressor of cytokine signalling-1) (Mansell et al., 2006). Recent reports show that also de-ubiquitinating enzymes are involved in controlling of TLR signalling (Boone et al., 2004).

Biological activities of TLRs are further under the control of a broad range of distinct types of molecules, including microRNAs (Sheedy et al., 2010; Taganov et al., 2006). One of the many TLRs regulators are soluble decoy receptors. So far, sTLR2 and sTLR4 were described (Iwami et al., 2000; LeBouder et al., 2003). Another potent regulator of TLR signalling is MyD88s, a spliced form of MyD88, which could prevent the IRAK4 recruitment upon activation of the signalling (Janssens et al., 2002). A negative regulation of signal transduction is exerted also by IRAKM, which could inhibit the dissociation of IRAK1 from MyD88 and thus block the formation of IRAK1/TRAF6 complex (Kobayashi et al., 2002). Other proteins involved in regulation of TRL-mediated pathways could be represented by TRAF4 (Takeshita et al., 2005), ATF3 (Activating transcription factor 3) (Gilchrist et al., 2006; Whitmore et al., 2007), TIPE2 (Sun et al., 2008), SHP1 and 2 (Src homology 2 domain-containing protein tyrosine phosphatase (An et al., 2008; An et al., 2006) . More examples and additional information could be found in Table 2.3.

An important role in TLRs regulation play transmembrane protein regulators from IL-1R family, ST2 and SIGIRR. The function of ST2 was outlined in Chapter 2.2.2, SIGIRR will be discussed in Chapter 2.3.

Regulator	Expression and induction	Affected TLR	Possible mechanism	References
sTLR2	Constitutively expressed in breast milk and plasma	TLR2	TLR2 antagonist	52
sTLR4	ND	TLR4	Blocks interaction of TLR4 and MD2	51
MyD88s	LPS-induced expression, mainly in spleen	TLR4	MyD88 antagonist	55,56
IRAKM	LPS-induced expression by monocytes	TLR4,9	Inhibits phosphorylation IRAK1	60
SOCS1	LPS- and CpG-induced expression by macrophages	TLR4,9	Suppresses IRAK	65,66
NOD2	ND	TLR2	Suppresses NF-κB	73
PI3K	Constitutively expressed by most cells	TLR2,4,9	Inhibits p38, JNK and NF-κB function	80
TOLLIP	Constitutively expressed in most tissues	TLR2,4	Autophosphorylates IRAK1	81–83
A20	LPS-induced expression by macrophages	TLR2,3,4,9	De-ubiquitylates TRAF6	89
ST2L	LPS-induced expression by macrophages	TLR2,4,9	Sequesters MyD88 and MAL	100
SIGIRR	Mainly expressed by epithelial cells and immature dendritic cells but downregulated by activation	TLR4,9	Interacts with TRAF6 and IRAK	110,111
TRAILR	Constitutively expressed by most cells	TLR2,3,4	Stabilizes IκBα	114
TRIAD3A	Constitutively expressed by most cells and tissues	TLR4,9	Ubiquitylates TLRs	116

IRAK, interleukin-1 receptor-associated kinase; LPS, lipopolysaccharide; ND, not determined; NF-κB, nuclear factor-κB; NOD2, nucleotide-binding oligomerization domain protein 2; PI3K, phosphatidylinositol 3-kinase; SIGIRR, single immunoglobulin interleukin-1-related receptor; SOCS1, suppressor of cytokine signalling 1; sTLR, soluble decoy TLR; TOLLIP, Toll-interacting protein; TRAF, tumour-necrosis factor-receptor-associated factor; TRAILR, tumour-necrosis factor-related apoptosis-inducing ligand receptor.

Table 2.3 Overview of TLRs negative regulators. Taken from (Liew et al., 2005).

2.2.5 Role of lipid rafts in regulation of TLR signalling

Lipid rafts are small, heterogeneous, sphingolipid- and sterol-rich membrane-microdomains that are thought to organize agonist-induced interactions among signalling proteins (Simons and Ikonen, 1997). They have been demonstrated to play an important regulatory role in multiple cellular processes such as membrane sorting or signal transduction. From a biochemical point of view, lipid rafts appear as detergent-resistant membranes (DRMs), detergent-insoluble glycolipid-enriched complexes (DIGs) or glycosphingolipidenriched membranes (GEMs) (Horejsi et al., 1998).

Although the best established role for lipid rafts as signalling platforms have been demonstrated in T and B cell receptor activation (Cheng et al., 2001; Janes et al., 2000),

recent reports indicate that also LPS induced interactions between TLR4 and other signalling proteins are mediated by lipid rafts.

The first report related to TLR4 redistribution to lipid rafts upon LPS stimulation was provided by (Triantafilou et al., 2002). They described that several molecules involved in the bacterial recognition, such as CD14, Hsp70 or Hsp90 were constitutively found in lipid rafts, whereas other molecules such as TLR4, GDF5 and CXCR4 were recruited to lipid rafts following the LPS stimulation. Together, upon LPS stimulation these molecules form a complex within the lipid rafts. It has been also shown that TLR4 highly co-localized with caveolin-1 (cav-1) in caveolae membrane fraction in peritoneal macrophages (Nakahira et al., 2006) and also in human aortic endothelial cells (Walton et al., 2006). Recent evidence suggests that cav-1 could interact with TLR4 and inhibit the LPS-induced pro-inflammatory cytokine (TNF-α, IL-6) production in murine macrophages (Wang et al., 2009). Raft levels of TLR4 and the cell responsiveness to TLR2, TLR4, TLR7 and TLR9 ligands are probably directly associated with raft cholesterol levels (Yvan-Charvet et al., 2008; Zhu et al., 2008; Zhu et al., 2010). Moreover, hypercholesterolemia increased macrophage raft cholesterol in mice and humans *in vivo* and also increased cell responsiveness to LPS (Madenspacher et al., 2010; Oiknine and Aviram, 1992). On the other hand, it has been previously described that in a different context, increased cholesterol level can also be associated with reduced macrophage inflammatory function (Haga et al., 1989; Hamilton et al., 1990). According to these findings, lipid trafficking pathways in macrophages are now supposed to be critical determinants for their TLR signalling.

2.3 SIGIRR, an important regulator of IL-1R/TLRs based immune responses

Single-Ig-Il-1R related molecule (SIGIRR) is a structurally distinct member of the IL-1R family. The first report which demonstrated its unique stuctural characteristics preventing it from triggering the NF-κB signalling was published in 1999 (Thomassen et al., 1999). Moreover, SIGIRR is considered to posses an inhibitory function which can regulate IL-1Rs/TLRs mediated inflammation. So far, this has been reported for several signalling pathways in distinct cell types and tissues such as digestive tract, kidney and lung. The fundamental overview of these findings is described below.

2.3.1 Structure of SIGIRR gene and protein

Human SIGIRR gene comprises of 10 exons and is localised on chromosome 11, whereas murine SIGIRR gene is located into chromosome 7 and organized in 9 exons (Thomassen et al., 1999). Thus, SIGIRR gene is not a part of a cluster containing other IL-1R family members. The cloning of full-length SIGIRR cDNA revealed that human SIGIRR protein consists of 410 amino acids. Mouse SIGIRR cDNA encodes for a 409 amino acid long protein.

Structural characteristic of SIGIRR protein is unique among all members of IL-1R family (Thomassen et al., 1999) and shows some interesting features. Several parts could be distinguished within the SIGIRR protein – an extracellular Ig domain, a transmembrane domain, intracellular TIR domain and a long cytolaplasmic tail (Figure 2.5). Unlike most other members of this family, SIGIRR possesses the only extracellular Ig domain, which probably does not support ligand binding. Interestingly, its highly-conserved intracellular TIR domain has two amino acids substitutions (Ser447 and Tyr546, replaced by Cys222 and Leu305), which probably prevents SIGIRR to participate in NF-κB activation durung signal transduction. Moreover SIGIRR contains a unique C-terminal 95 amino acid sequence. This domain is probably responsible for SIGIRR-mediated negative regulation of signalling triggered via Toll-like and IL-1 receptor complexes in many cell types and tissues.

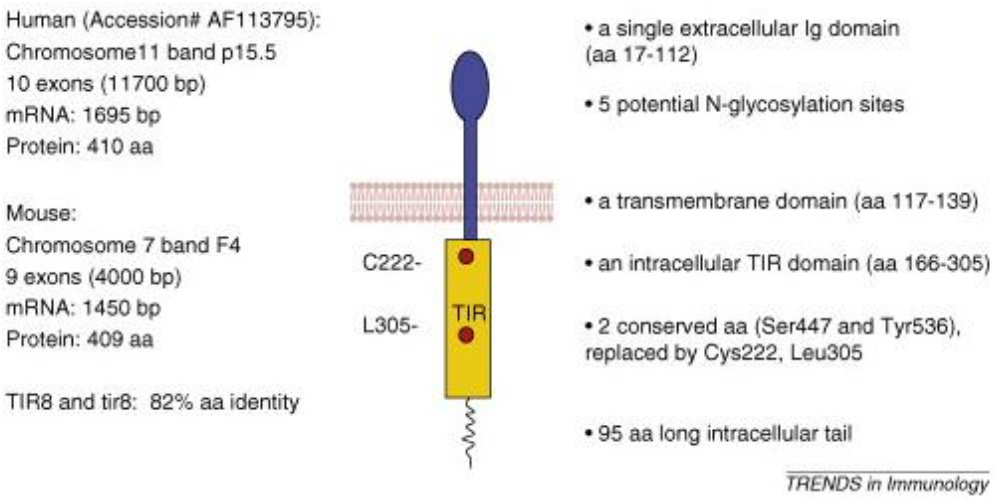

Figure 2.5 Organization of SIGIRR gene and protein. SIGIRR protein possesses some unique structural characteristics – it has only one extracellular Ig domain, two amino acid substritution in its TIR domain and also has a unique cytoplasmic tail probably with inhibitory function. Taken from (Garlanda et al., 2009).

SIGIRR is supposed to be heavily glycosylated; its sequence contains 5 possible extracellular N-glycosylation sites and results from Western blot analysis confirmed this assumption (Lech et al., 2007; Thomassen et al., 1999).

2.3.2 Expression profile of SIGIRR

Screening analyses confirmed that SIGIRR is expressed in various animals. Moreover, it has been shown that the basic pattern of SIGIRR expression among various tissues does not differ between species and also that the cDNA obtained from distinct species show high homology between each other (Riva et al., 2009). These findings suggest that SIGIRR represents a relatively highly conserved protein not only in the meaning of sequence homology, but also regarding the expression profile.

SIGIRR is expressed in a broad range of tissues (Polentarutti et al., 2003; Thomassen et al., 1999; Wald et al., 2003). As illustrated in Figure 2.6, the highest levels of SIGIRR expression could be detected within the kidney, the digestive tract, the lung, the liver and also in lymphoid organs. Considering the kidney, SIGIRR is expressed in renal tubular epithelial cells and renal antigen-presenting cells (Lech et al., 2007). Epithelial cells also produce SIGIRR in digestive tract (Xiao et al., 2007). The brain, where SIGIRR expression was discovered, represents another important source of SIGIRR (not included in Figure 2.6) (Costelloe et al., 2008; Polentarutti et al., 2003). here, SIGIRR is expresed in neurons, microglia and astrocytes (Andre et al., 2005) where it modulates the inflammatory responses (Costello et al., 2011; Watson et al., 2010).

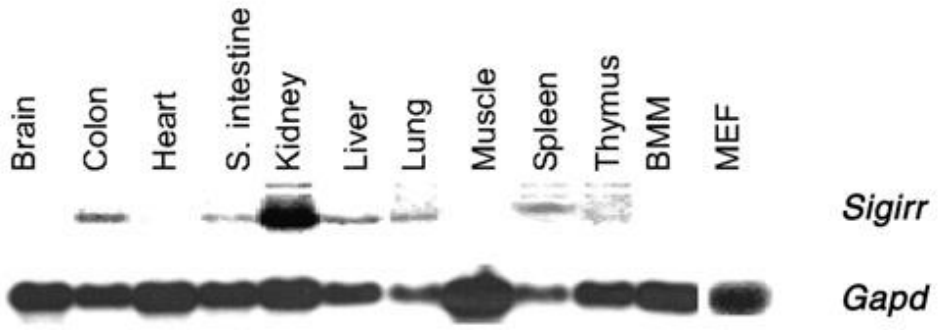

Figure 2.6 SIGIRR expression among distinct tissues. Using Nothern blot analysis, SIGIRR has been identified to be expressed in various murine tissues, specifically reaching its highest level in the kidney. Taken from (Wald et al., 2003).

Considering various types of immune cells, SIGIRR is expressed by NK cells, B cells, monocytes and immature dendritic cells (Garlanda et al., 2004; Polentarutti et al., 2003). Although macrophages were originally described as SIGIRR-negative (Wald et al., 2003), recent evidence suggests that SIGIRR is expressed not only in adult murine peritoneal macrophages (Vavrochová, 2010), but also in human monocyte-derived macrophages (Drexler et al., 2010a). However, the expression levels of SIGIRR in human primary cells substantially decreases during their differentiation (Drexler et al., 2010a). Moreover, a specific control mechanism for DC activation dependent on interaction of SIGIRR with MyD88 has recently been proposed (Drexler et al., 2010b).

The expression of SIGIRR can be down-regulated by LPS (Huang et al., 2006; Polentarutti et al., 2003). On the other hand, SIGIRR production is increased in intestinal cells during stress, infection or inflammation (Gopal et al., 2008; Kadota et al., 2010) and also in monocytes and Peyer's patch dendritic cells during sepsis (Adib-Conquy et al., 2006; Davies et al., 2010). Reduced expression of SIGIRR has been observed in patients with psoriatic arthritis (Batliwalla et al., 2005), Crohn's disease (Naito et al., 2006) and asymptomatic bacteriuria (Ragnarsdottir et al., 2007), suggesting a possible immunosuppressive role of SIGIRR.

2.3.3 SIGIRR function and regulation

SIGIRR protein shows a unique structural organization, which mediates its regulatory function on various immune processes. The main role of SIGIRR is considered to be a negative regulator of IL-1R/TLR mediated signalling pathways.

To date, it has been described that SIGIRR inhibits IL-1R1 and TLR4 induced NF-κB activation in various cell line models, such as HEK293T, HeLa, HepG2, Jurkat, A549 or HAEC cells (Adib-Conquy et al., 2006; Huang et al., 2006; Polentarutti et al., 2003; Qin et al., 2005; Tian et al., 2010; Wald et al., 2003; Zhang et al., 2011). NF-κB reporter system was also employed in order to examine the role of various parts of SIGIRR molecules to its inhibitory properties. It has been shown that truncated SIGIRR specifically lost their ability to negatively regulate the activation of NF-κB transcription factor upon triggering of IL-1R/TLR signalling (Polentarutti et al., 2003; Qin et al., 2005). These experiments also provided an insight into possible mechanisms of SIGIRR mediated regulation of immune responses. As illustrated in Figure 2.7, obtained results suggest that the extracellular Ig domain is involved in inhibition of IL-1R1 signalling by interfering with the heterodimerization of IL-1R and IL-1R accessory

protein (IL-1RAcp) (Qin et al., 2005), whereas the intracellular part of SIGIRR interferes with both IL-1 and TLR4 signalling (Polentarutti et al., 2003; Qin et al., 2005). The recruitment of various signalling components to receptors is probably prevented by interaction of SIGIRR and other proteins through their TIR domains. This statement is supported also by findings that SIGIRR is able to interact not only with receptors such as IL-R1 and TLR4, but also with adaptor protein TRAF6, IRAK1 and MyD88 (Drexler et al., 2010b; Qin et al., 2005; Wald et al., 2003). However, the exact way of how SIGIRR regulatory effects are mediated remains to be further investigated. Recently, the interaction between SIGIRR and protein PALM3 (paralemmin-3) was described (Chen et al., 2011), but the specific role of this connection has to be elucidated.

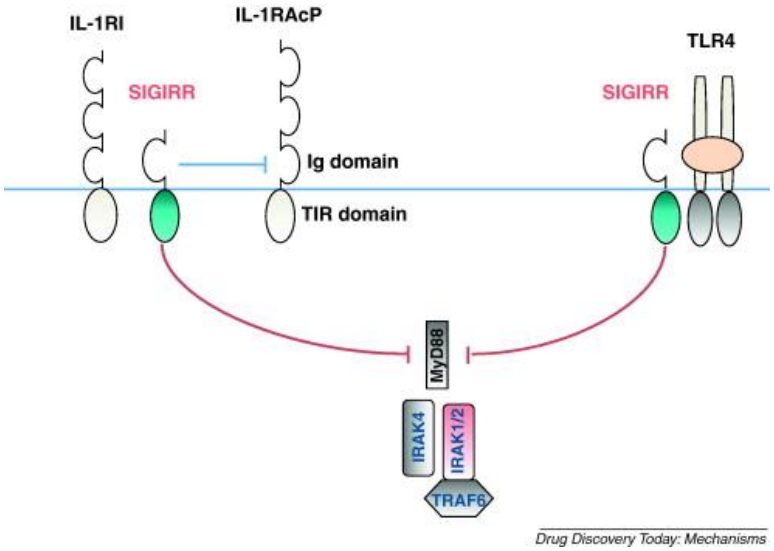

Figure 2.7 SIGIRR negatively regulates IL-1R and TLR signaling through distinct mechanisms. SIGIRR inhibits both IL-1R and TLR4 signaling by attenuating the recruitment of MyD88 to the TIR domain of the receptor. In addition, SIGIRR also prevents the heterodimerization of IL-1R with its accessory protein. Taken from (Zhao et al., 2011) .

SIGIRR also regulates signalling mediated through other receptors of IL-1R/TLR family, although these effects have not been examined in such details as in the case of signalling by IL-1R1 and TLR4. SIGIRR could negatively regulate the signalling triggered by IL-18 (Adib-Conquy et al., 2006) and also IL-33/ST2 mediated signalling through interaction of SIGIRR with ST2 (Bulek et al., 2009). Concerning the TLRs, it has been reported that SIGIRR exerts its negative function in the regulation of TLR3 response in kidney monocytes (Lech et al., 2007) and evidence points also to its involvement in the inhibition of TLR5, TLR7 and TLR9 mediated

signalling (Garlanda et al., 2004; Lech et al., 2008; Zhang et al., 2011). A computational approach was also employed to study the influence of SIGIRR on TLR signalling and the model of SIGIRR interaction with TLR4 and TLR7 have been developed as illustrated in Figure 2.8 (Gong et al., 2010). However, distinct cell types differ in their ability to employ SIGIRR into the network of regulatory processes. The reason for this discrepancy is not clear and has to be further investigated.

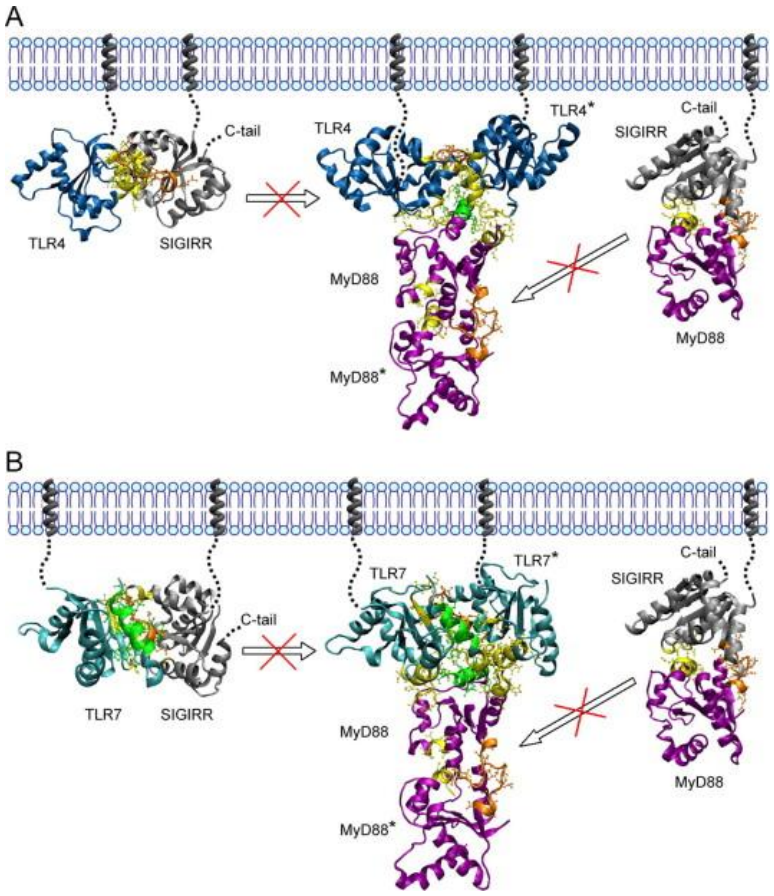

Figure 2.8 Models if SIGIRR inhibiting MyD88-dependent TLR4 (A) and TLR7 (B) signalling. Taken from (Gong et al., 2010).

SIGIRR possesses only one extracellular Ig domain, which is likely not enough to ensure ligand-specific binding. Thus, SIGIRR is sometimes classified as an 'orphan' receptor and so far no structure has been identified as *bona-fide* SIGIRR ligand. Nevertheless, the recent evidence suggests that IL-1F5 (also called IL-36 receptor antagonist and designated as IL-36RA or IL-36RN) could mediate the anti-inflammatory processes in glial cells through

induction of IL-4 production, which is dependent on the interaction of IL-1F5 with SIGIRR (Costelloe et al., 2008). However, it remains to be further elucidated, whether this novel member of IL-1 family indeed binds to SIGIRR or not.

2.3.4 Role of SIGIRR in regulation of distinct immune processes

In vivo, SIGIRR represents an important signalling element participating in many physiological responses. Recent studies were focused on to reveal the role of SIGIRR in inflammatory processes taking place in tissues with high levels of SIGIRR expression, e.g. in the digestive tract, the kidney and the lung. Recently reported evidence suggests that SIGIRR is also involved in the process of T-cell polarization and supresses the Th17 cell differentiation (Gulen et al., 2010). To help the better understanding of the SIGIRR function *in vivo*, two SIGIRR$^{-/-}$ mouse models have been established (Garlanda et al., 2004; Wald et al., 2003). Probably due to the different genetic background these mice show differences in the immune responses. Garlanda and coworkers reported that SIGIRR$^{-/-}$ mice display normal inflammatory reactions at sites other than the gastrointestinal tract, including normal inflammation in response to LPS (Garlanda et al., 2004), whereas others revealed that SIGIRR-deficient mice showed increased susceptibility to systemic administration of LPS (Wald et al., 2003).

The first area of interest in the field of SIGIRR *in vivo* studies is represented by intestinal inflammation. Treatment of SIGIRR$^{-/-}$ mice with DSS (dextran sodium sulphate) led to increased severity of colitis in these mice compared to wild-type controls (Garlanda et al., 2004; Xiao et al., 2007). This phenotype was associated with increased weight loss, tissue damage, leukocyte infiltration, pro-inflammatory cytokine production and also mortality. SIGIRR$^{-/-}$ mice also show increased susceptibility to drugs capable to induce colitis-associated cancer (CAC), a colorectal disease that arises in patients suffering from chronic inflammatory bowel disease (IBD). Mice subjected to this treatment displayed increased rate of cell proliferation (Figure 2.9), local inflammatory processes and production of inflammatory cytokines (Garlanda et al., 2007b). The increased colonic tumorogenesis, dependent on the presence of comensal bacteria in the gastrointestinal tract, was also described for SIGIRR$^{-/-}$ mice (Xiao et al., 2010).

Whereas it has been reported that SIGIRR is not expressed by human leukemic B lymphocytes (Muzio et al., 2009), SIGIRR$^{-/-}$ mice displayed accelerated expansion of monoclonal B cells in mouse model of chronic lymphotic leukemia (Bertilaccio et al., 2011).

Together, the observations described above support the importance of chronic inflammation in malignancy and several reviews are devoted to this interesting phenomenon (Hanahan and Weinberg, 2011; Mantovani et al., 2008).

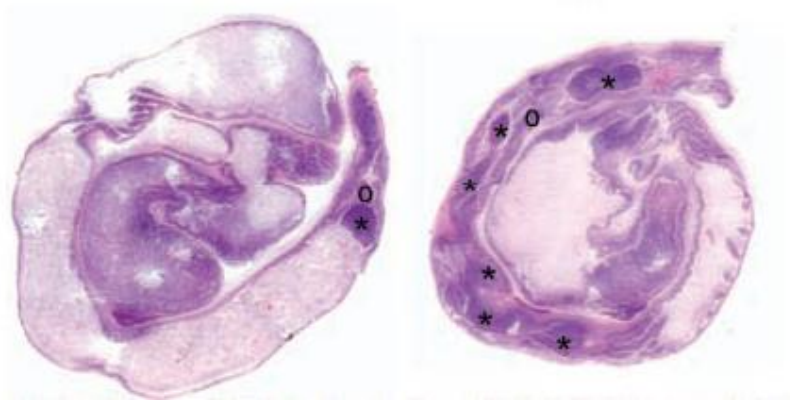

Figure 2.9 Proliferating lessions in rectal lumen of Sigirr$^{+/+}$ (A) and Sigirr$^{-/-}$ (B) mice. * marks the high-grade adenoma, o marks the low-grade adenoma. Taken from (Garlanda et al., 2007b).

The role of SIGIRR in various infections has also been investigated. In a model of keratitis induced by *Pseudomonas aeruginosa*, it has been described that SIGIRR in coordination with ST2 promotes the host resistance to infection with this bacteria by down-regulation of TLR signalling and consequent production of pro-inflammatory cytokines (Huang et al., 2007; Huang et al., 2006). SIGIRR also plays a role in dampening the inflammation and tissue damage in tuberculosis and fungal infection. SIGIRR$^{-/-}$ mice showed exaggerated susceptibility to infection with *Mycobacterium tuberculosis*, together with increased serum level of TNF-α and IL-1β (Garlanda et al., 2007a). Similarly, the inflammatory processes and susceptibility to infection with *Candida albicans* or *Aspergillus fumigatus* were higher in SIGIRR$^{-/-}$ mice (Bozza et al., 2008). This phenomenon was also linked to the activation of the Th17 pathway and reduced Treg cell activation.

SIGIRR is also involved in the regulation of autoimmune processes. Altough the lack of SIGIRR itself does not induce the autoimmunity, it has been discovered that SIGIRR deficiency is associated with autoimmune injuries in B6$^{lpr/lpr}$ mice (Lech et al., 2008). These mice developed a set of severe autoimmune diseases, including lupus nephritis within first 6 months of life and also displayed enhanced activation of dendritic cells and B lymphocytes, which led to an increased expression of inflammatory and anti-apoptotic molecules and production of autoantibodies against multiple nuclear lupus autoantigens. These evidence suggest that the presence of SIGIRR could probably prevent murine lupus, either spotaneous

(Lech et al., 2008) or drug-induced (Lech et al., 2010). SIGIRR deficiency is associated with increased inflammatory responses during murine kidney allotransplantation (Noris et al., 2009). Considering the kidney, postischemic acute renal failure is supposed to be prevented by SIGIRR (Lech et al., 2009), whereas postobstructive renal interstitial fibrosis is not (Skuginna et al., 2011). Finally, SIGIRR$^{-/-}$ mice displayed increased pathological changes compared to wild-type controls when a mouse model of rheumatoid arthritis was employed (Drexler et al., 2010a). Together, these reports demonstrate that SIGIRR is a very important molecule critically involved in the regulation of a complex inflammatory signalling network.

2.3.5 Expression of SIGIRR in early stages of murine development

It has been already described above (Chapter 2.3.2) that SIGIRR expression is detectable in various cell types and tissues in adults. The interesting finding recently came out also about SIGIRR expression during embryonic development (Vavrochová, 2010). Results obtained from experiments performed in our lab showed that SIGIRR is highly expressed in very early stages of murine embryonic development (E6.5 – E8.5), then its expression decreases. Whole-mount immunohistochemistry of the E7.5 embryo confirmed that SIGIRR is expressed across all embryonic tissues in this phase of development (Figure 2.10). These findings suggest that SIGIRR can mediate a negative regulation of IL-1R/TLR signalling pathway in embryonic cells and thus could be involved in processes of preventing and dampening the inflammation and tissue damage in this period of development and playing a crutial role in the maintenance of embryonic homeostasis.

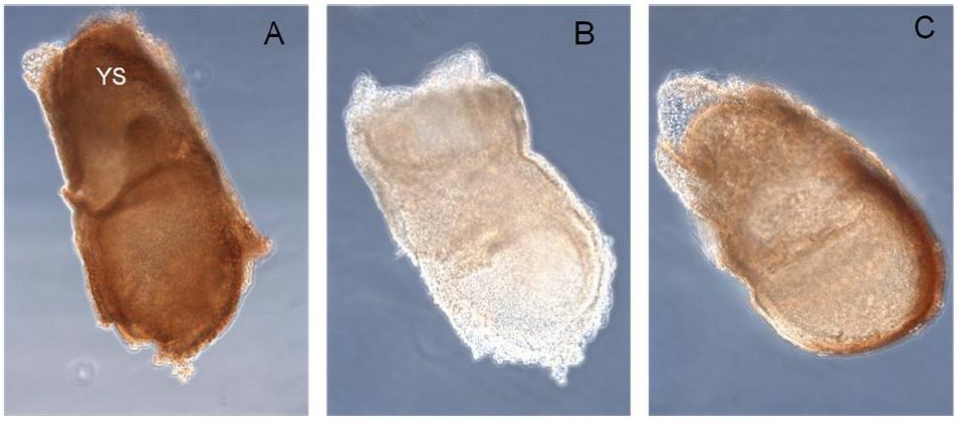

Figure 2.10 SIGIRR is expressed in murine E7.5 embryo. Whole-mount-immunohistochemistry of murine E7.5 embryo revealed that SIGIRR is ubiquitously expressed in this stage of embryonic development (A). Embryos stained with anti-Brachyury antibody (C) served as a positive, whereas and staining with HRP-conjugated secondary antibody (B) only as a negative control. YS – yolk sac. Taken from Vavrochová, 2010.

3 Materials and methods

3.1 Materials

3.1.1 Mice

Three mice strains were used: wild-type ICR (CD1) and C57Bl/6J mice and knock-out SIGIRR$^{-/-}$/TIR8$^{-/-}$ mice (a gift from prof. A. Mantovani, Istituto Clinico Humanitas, *Milan, Italy*) (Garlanda et al., 2004). All mice were housed in the pathogen-free animal facility at the Institute of Molecular Genetics (*Prague, Czech Republic*).

3.1.2 Cell lines and cell culture maintenance

SYF and HEK293T cells and their transfectants were cultured with IMDM (*Gibco*) supplemented with 10 % FCS (*Gibco*) and antibiotics (100 U/ml of penicillin and 10 μg/ml of streptomycin) (*Sigma-Aldrich*). Cell lines J774.2, RAW264.7, BWZ.36, DC2.4 and their transfectants were maintained in RPMI 1640 (*Sigma-Aldrich*) supplemented with FCS and antibiotics as described above. Murine pre-B cell line 70Z/3 (also its transfectants) was cultured with RPMI 1640 Medium - GlutaMAX™-I (*Gibco*) containing 10 % FCS, 0.05 mM 2-ME and antibiotics as described above.

3.1.3 Bacterial strain

TOP10 Chemically Competent *Escherichia coli* strain (*Invitrogen*) was used in all experiments comprising bacterial transformation.

3.1.4 Vectors

Full-length SIGIRR cDNA was at first cloned into pEYFP-N3 plasmid (Figure 3.1), which encodes a yellow-green variant of GFP and allows to express genes as fusions to the N terminus of EYFP, and also into pIRES2-EGFP plasmid (*Clontech*) (Figure 3.1) – this vector contains the sequence for IRES followed by the sequence for EGFP, which allows the co-expression of selected gene and this fluorescent tag. For follow-up experiments, retroviral plasmids were used – the construct for fusion SIGIRR-EYFP protein was cloned into the retroviral vector pLXSN (*Clontech*) (Figure 3.2) and the construct for chimeric SIGIRR-CD8α-CD3ζ protein was inserted into the pMC83IG plasmid (the type I transmembrane retroviral CD3ζ-fusion cassette vector, derived from pMCIG vector) (Mesci and Carlyle, 2007).

Other SIGIRR constructs were inserted into the MSCV-based retroviral vector pMigR1 (Pear et al., 1998) (Figure 3.2). For retroviral triple transfection (Chapter 3.2.10), plasmids encoding *Env* and *Gag/Pol* genes were used.

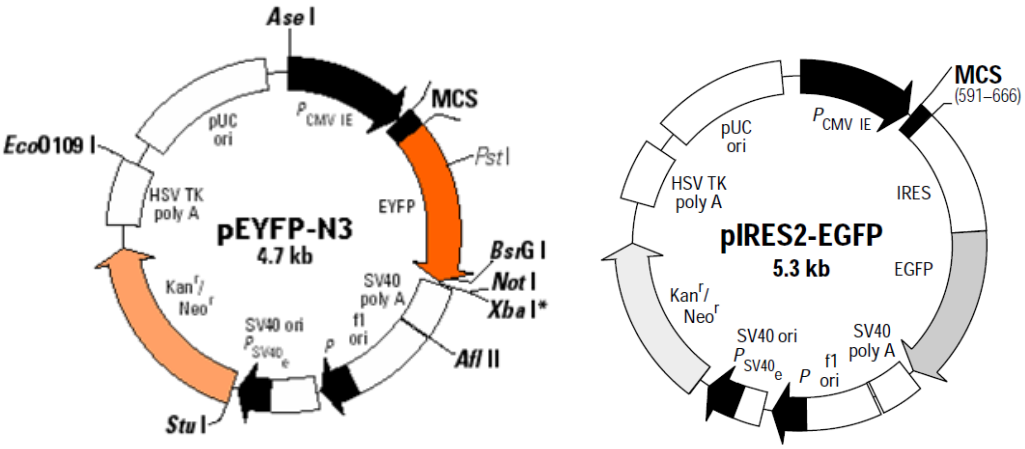

Figure 3.1 Restriction maps for vectors pEYFP-N3 (left; taken and adjusted from http://www.liv.ac.uk/physiology/ncs/catalogue/Cloning/pEYFP-N1-Map.htm) and pIRES2-EGFP (right; taken from http://www.dmlim.net/vectors/pIRES2-EGFP/pIRES2-EGFP-map.pdf).

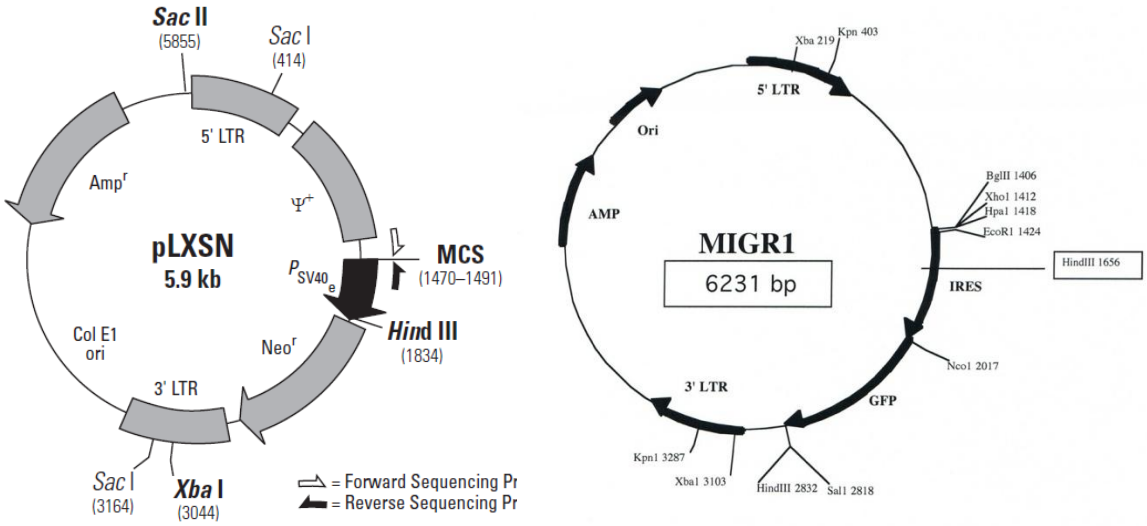

Figure 3.2 Restriction maps for vectors pLXSN (left; taken from http://www.clontech.com) and pMigR1 (right; taken from http://addgene.org/static/data/63/41/ba263e96-af63-11e0-90fe-003048dd6500.jpeg).

3.1.5 Antibodies

All used antibodies are listed below in the Table 3.1.

Primary antibodies			
Name	**Origin**	**Polyclonal/Conjugate**	**Producer**
Anti-Brachyury	Goat	Polyclonal	Santa Cruz Biotechnology
Anti-CD11b	Rat	PE	eBioscience
Anti-GFP	Rabbit	Polyclonal	Abnova
Anti-HA	Rabbit	Polyclonal	Sigma-Aldrich
Anti-SIGIRR	Goat	Polyclonal	R&D Systems
Anti-TLR2	Rat	Biotin	eBioscience
Secondary antibodies			
Name	**Origin**	**Polyclonal/Conjugate**	**Producer**
Anti-Goat IgG (H+L)	Donkey	Alexa555	Invitrogen
Anti-Goat IgG (H+L)	Rabbit	HRP	Invitrogen
Anti-Mouse IgG (H+L)	Goat	Alexa555	Invitrogen
Anti-Rabbit IgG (H+L)	Goat	Alexa555	Invitrogen
Anti-Rabbit IgG (H+L)	Goat	HRP	Bio-Rad Laboratories
Streptavidin conjugates			
Name		**Polyclonal/Conjugate**	**Producer**
Streptavidin		FITC	eBioscience
FC block			
Name	**Origin**	**Polyclonal/Conjugate**	**Producer**
Anti-CD16/32	Rat	Purified	BD Pharmingen

Table 3.1 List of used antibodies.

3.1.6 Primers

Primers were designed by Roche Universal Probe Library[1] and the Primer3 program[2]. All used primers are listed in the Table 3.2.

Gene	Forward primer (5´→ 3´)	Reverse primer (5´→ 3´)
CasC3	TTCGAGGTGTGCCTAACCA	GCTTAGCTCGACCACTCTGG
IL1R1	TTGACATAGTGCTTTGGTACAGG	TCGTATGTCTTTCCATCTGAAGC
IL-1β	TGTAATGAAAGACGGCACACC	TCTTCTTTGGGTATTGCTTGG
IL-6	TGATGGATGCTACCAAACTGG	TTCATGTACTCCAGGTAGCTATGG
IRAK	GGATCAGCTCCACCTTCAGA	CCCAGAAGAATGTCCAGTCG
MyD88	TGGCCTTGTTAGACCGTGA	AAGTATTTCTGGCAGTCCTCCTC
SIGIRR	GAAGAGCCATGGCAGGTG	GCTGTGCAATTCAAAGCAAC
ST2	GACCTGTTACCTGGGCAAGAT	GGCCAGAACAAACACCTGTC
TLR3	ATACAGGGATTGCACCCATA	CCCCCAAAGGAGTACATTAGA
TLR4	GGACTCTGATCATGGCACTG	CTGATCCATGCATTGGTAGGT
TLR5	ACGCACCACACTTCAGCA	AGCCTCGGAAAAAGGCTATC
TLR7	TGCCTTCTCTCTGTCTCAGAGGACT	TCCTGAGAAGGGAGCCAAGGAC
TLR9	CGCCCAGTTTGTCAGAGGGAGC	GGTGCAGAGTCCTTCGACGGAG
TNF-α	TCTTCTCATTCCTGCTTGTGG	GGTCTGGGCCATAGAACTGA
TRAF6	TTGCACATTCAGTGTTTTTGG	TGCAAGTGTCGTGCCAAG
TRIF	ACCAGGGACCGGGAGATCTACCA	CAAAGATGCTGGAGGGCGGCA

Table 3.2 List of murine primer pairs used for qRT-PCR analyses.

[1] ProbeFinder version 2.45; http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp.
[2] Version 0.4.0; http://frodo.wi.mit.edu/primer3/.

3.2 Methods

3.2.1 Isolation and stimulation of embryos and peritoneal macrophages

Mice were set up together for mating in the evening and the presence of vaginal plugs was checked next morning. Embryos were then collected from timed matings at embryonic days 7.5, 8.5 and 9.5 – pregnant mice were sacrified by cervical dislocation and embryos were dissected out of the decidua. Peritoneal magrophages were isolated by injection of cold PBS into the peritoneal cavity and collection of the cell suspension containing fluid.

For LPS stimulation purposes peritoneal macrophages or embryonic cells were placed into RPMI-1640 (*Sigma-Aldrich*) supplemented with 10 % FCS and antibiotics (100 U/ml of penicillin and 10 µg/ml of streptomycin). Samples were cultured for 24 hours at 37 °C in the presence or absence of LPS (500 ng/ml) derived from *Salmonella typhimurium* (*Difco*) and then subjected to RNA isolation and qRT-PCR analysis (Chapter 3.2.2, 3.2.3).

3.2.2 RNA isolation and reverse transcription

RNA isolation from cultured and sorted cells and whole embryos collected at embryonic days 7.5 – 8.5 was performed with Rneasy® Plus Micro Kit (*Qiagen*). RNA from whole embryos collected at developmental stage E9.5 was isolated by NucleoSpin® RNAII kit (*Machery-Nagel*). Work procedure was performed according to manufacturer´s instructions. RNA purity and concentration was then determined spectrophotometricly using NanoDrop 1000 instrument (*Thermo Scientific*).

For reverse transcription of isolated RNA into cDNA, RevertAid™ Premium Reverse Transcriptase, RiboLock™ RNase Inhibitor, Random Hexamer Primer and dNTPmix (*Thermo Scientific*) were used. The reaction was performed using XP cycler (*Bioer Technology*) according to the manufacturer's protocol.

3.2.3 Quantitative real-time PCR (qRT-PCR)

cDNAs obtained in reverse transcription were subjected to qRT-PCR analyses to measure the expression level of selected genes. All experiments were performed on the LC480 machine (*Roche*) with the reaction mixture composed of SYBR Green I Master Mix (*Roche*), primer mix (forward and reverse, each 8 µM) and appropriate cDNA. The evaluation of gene expression level was based on the relative quantification model (Pfaffl, 2001) with normalization to referential endogenous housekeeping gene CasC3.

3.2.4 Flow cytometry and cell sorting

To prepare a single cell suspension, embryos were treated with dispase (*Invitrogen*) for 10 minutes at 37 °C. Cultured cells or single cell suspensions were then washed with HBSS without Ca^{2+} and Mg^{2+} supplemented with 1 % FCS and incubated with anti-CD16/32 antibody (*BD Pharmingen*) for 15 minutes at 4 °C to block the Fc receptors. After this step, cells were stained with antibodies in appropriate combinations and washed with HBSS without Ca^{2+} and Mg^{2+} supplemented with 1 % FCS prior FACS analysis. For determination of the transfection and the retroviral spinning-mediated infection efficiency (Chapter 3.2.9, 3.2.10) cells were examined for the GFP fluorescence. All FACS measurements were performed using LSRII instrument (*BD Biosciences*) in the presence of Hoechst 33258 dye to exclude dead cells. Cell sorting experiments were performed by Zdeněk Cimburek using Influx cell sorter (*BD Biosciences*). Collected data were analysed using FlowJo statistic software.

3.2.5 DNA cloning

The DNA cloning procedure was divided into several parts: (1) generation of DNA fragments by PCR, (2) ligation of these fragments into the cloning vector, (3) multiplication of the vector DNA in bacteria and its isolation, (4) sequencing of selected plasmid clones, (5) digestion of the non-mutated plasmid, (6) insertion of the appropriate coding sequence into the target vector, (7) sequencing of selected vector clones, (8) cell line transfection using non-mutated vector clone. Full-length or C-terminal modified SIGIRR DNA was generated by PCR using set of primers listed in Table 3.3.

Construct / Vector	Forward primer (5´→ 3´)	Reverse primer (5´→ 3´)
SIGIRR / pEYFP-N3	CTCGAGGCCATGGCAGGTGTCTGTGACATG	GAATTCCCCACATCATCCTCAGACAC
SIGIRR / pIRES2-EGFP	CTCGAGGCCATGGCAGGTGTCTGTGACATG	GAATTCCTACACATCATCCTCAGACACGAG
SIGIRR-extra / pMC83IG	CTCGAGGCCATGGCAGGTGTCTGTGACATG	GCGGCCGCATGGCCAGCAGGGCCAGCT
SIGIRR-EYFP / pLXSN	CTCGAGGCCATGGCAGGTGTCTGTGACATG	CTCGAGTTACTTGTACAGCTCGTCCAT
SIGIRR-HA / pMigR1	GAATTCGCCATGGCAGGTGTCTGTGACATG	GAATTCCTAAGCGTAATCTGGGACGTCGTATGGGTAGCCGCCGCCGCCCACATCATCCTCAGACAC

Table 3.3 List of primer pairs used for SIGIRR DNA cloning. Restiction sites are underlined. CTCGAG = XhoI, GAATTC = EcoRI, GCGGCCGC = NotI.

The PCR reaction was performed with High Fidelity PCR Enzyme Mix (*Fermentas*) using XP cycler (*Bioer Technology*). DNA fragments were cloned into the pJet1.2/blunt cloning vector (*Fermentas*) according to manufacturer's instructions. The ligation mixture was used for bacterial transformation (Chapter 3.2.6) and single transformed colonies were then subjected to plasmid isolation (Chapter 3.2.7) and sequencing. Sequencing was performed by The laboratory of DNA sequening (*Faculty of Science, Charles University in Prague*) using Genetics Analyzer (*Applied Biosystems*). Appropriate clones were then digested with suitable restriction enzymes and examined by agarose gel electrophoresis (Chapter 3.2.8). DNA fragments isolated from gel using Zymoclean™ Gel DNA Recovery Kit (*Zymo Research*) were then inserted into target vectors with T4 DNA ligase (*Fermentas*) to produce recombinant SIGIRR protein. Plasmids were multiplied in bacteria and sequenced as described above. Appropriate cell lines were then transfected (Chapter 3.2.9, 3.2.10) with non-mutated vector clones.

3.2.6 Bacterial transformation and plasmid isolation

TOP10 Chemically Competent bacteria (50 µl aliquot, *Invitrogen*) were incubated with 1 µg of plasmid DNA for 20 minutes on ice and then subjected to the heat shock (42 °C, 1 minute). After chilling down on ice for 2 minutes, 200 µl of S.O.C. Media (*Invitrogen*) was added and then the bacteria were shaken at 37 °C incubator. After 45 minutes, bacteria were plated out onto agar plates supplemented with appropriate antibiotics and incubated overnight at 37 °C to allow the growth of bacterial colonies.

3.2.7 Plasmid isolation

Single transformed bacterial colonies were used to generation of bacterial cultures by incubating overnight at 37 °C to allow the multiplication of bacteria. Plasmids were isolated either by ZR Plasmid Miniprep™ kit (for low scale bacterial cultures, *Zymo Research*) or by NucleoBond Xtra Midi Plus kit (for medium scale amounts, *Machery-Nagel*) according to manufacturer's instructions. The purity and concentration of plasmid DNA was then measured spectrophotometricly using NanoDrop 1000 instrument (*Nanodrop Technologies*). The size and integrity of plasmid DNA was verified by the restriction reaction with appropriate DNA endonucleases, when the reaction mixture was incubated at 37 °C for 1 hour and analysed by agarose gel electrophoresis as described below (Chapter 3.2.8).

3.2.8 Agarose gel electrophoresis

DNA containing samples were mixed with the BFB gel loading dye and loaded onto the 0.8% agarose gel supplemented with the ethidium bromide (1 μg/ml). GeneRuler™ DNA Ladder Mix (*Thermo Scientific*) was used as the standard molecular weight marker. Gels were run in the TBE buffer and DNA fragments were separated by applying a constant voltage. Finally, gels were illuminated with the UV radiation and their images were taken.

3.2.9 Transient cell line transfection

HEK293T cells were transfected using Lipofectamine™ LTX and PLUS™ Reagent (*Invitrogen*) according to manufacturer's instructions. Briefly, 0,4 × 10^6 HEK293T cells were cultured overnight in a 6-well plate; Opti-MEM® I Reduced Serum Medium (*Gibco*) supplemented with 10 % FCS was used as a culture medium. Transfection complexes were prepared as follows: 3 μg of plasmid DNA was diluted in 500 μl of Opti-MEM® I Reduced Serum Medium and 3 μl of PLUS™ reagent was added. The mixture was then incubated at room temperature. After 5 minutes, 6 μl of Lipofectamine™ LTX was added to the diluted DNA and the mixture was incubated for another 30 minutes at room temperature to allow formation of DNA-lipid complexes. The mixture was then added dropwise to the appropriate well containing HEK293T cells. Cells were incubated at 37 °C CO_2 incubator for 24 hours prior further processing; medium was changed to IMDM, 10 % FCS, after 6 hours.

For immunofluorescence labelling purposes (Chapter 3.2.13), 8 × 10^4 HEK293T cells were cultured on glass coverslips placed in a 24-well plate. Transfection was performed with the mixture containing 0.5 μg of plasmid DNA diluted in 100 μl of Opti-MEM® I Reduced Serum Medium, 0.5 μl of PLUS™ reagent and 1 μl of Lipofectamine™ LTX under the same conditions as described above.

3.2.10 Retroviral cell line infection

At first, retroviral supernatants were generated by triple transfection of HEK293T cells using Lipofectamine™ LTX and PLUS™ Reagent as described above, only with a small modification: for preparation of DNA-lipid complexes, 1 μg of plasmid DNA together with 1 μg of *Env* and 1 μg of *Gag/Pol* encoding vectors was used. In paralell with transfection procedure, 0,3 × 10^6 J774.2, 70Z/3 or BWZ.36 cells were cutured in a 6-well plate overnight. 24 hours post transfection, virus supernatant from HEK293T cells was harvested,

supplemented with polybrene (*Sigma-Aldrich*) to a final concentration of 8 µg/ml and used for spinning-mediated infection of target cells at 1800 RPM for 45 minutes at room temperature. This infection process was repeated 24 hours later with a fresh supernatant, and stable transfectants were then sorted our by FACS at day 2 following infection.

3.2.11 SDS-PAGE and Western blot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) samples were prepared using SDS sample buffer, loaded onto polyacrylamide gels (8 % to 12 %) and run in SDS running buffer (25 mM Tris, 192 mM glycin, 0.1 % (w/v) SDS). PageRuler* Prestained Protein Ladder (*Thermo Scientific*) was used as a standart molecular weight marker. Resolved proteins were transfered to Immobilon-FL 0.45 µm PVDF membrane (*Millipore*) by blotting in transfer buffer (20 mM Tris, 156 mM Glycine, 20 % MetOH). Membranes were washed in TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05 % Tween) and blocked for 1 hour in 5 % non-fat milk diluted in TBS-T. Blots were incubated for 1.5 hour with primary antibodies, washed 5 times in TBS-T and then stained with appropriate secondary antibodies conjugated to horseradish peroxidase (HRP). After 5 washes in TBS-T membranes were developed by incubation with the ECL substrate for 1 minute and the chemiluminiscent signal were visualised by exposure of membranes to X-ray films.

3.2.12 Gel filtration chromatography

This experimental procedure is based on previously described protocol (Cinek and Horejsi, 1992) and was used with minor modifications. Briefly, small columns (5 ml pipette tips closed with a piece of glass wool) were filled with 2 ml of Sepharose 4B beads (*Sigma-Aldrich*) and then equilibrated with two volumes of the lysis buffer at 4 °C. TKM buffer (50 mM Tris-HCl pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA pH 8.0,) supplemented with 0.5 % Brij® 58 (*Sigma-Aldrich*) or 1 % laurylmaltoside (LM, n-Dodecyl-β-D-maltoside) (*Calbiochem*) was used for this purpose. Cells were lysed in 200 µl of TKM- Brij® 58 or TKM-LM buffer supplemented with complete protease inhibitor cocktail (*Roche*) for 30 minutes at 4 °C and then centrifuged (3000 RPM, 4 °C). Supernatant was loaded onto the columns at 4 °C and 12 fraction were then collected by elution with the lysis buffer (200 µl/5 minutes per one fraction). Collected fractions were then used for the preparation of SDS-PAGE samples and subjected to electrophoresis and Western blot analysis.

3.2.13 Immunofluorescence labelling of cultured cells

To visualize the localization and distribution of proteins, cells were subjected to immunofluorescence labelling. In case of using J774.2 cell line, 6×10^4 cells were cultured on glass coverslips placed in a 24-well plate. HEK293T cells were prepared as described above (Chapter 3.2.9). After 24 hours, cells were washed with PBS and fixed for 15 minutes in 4 % PFA. Then they were washed, incubated with 15 mM NH_4Cl in PBS for 5 minutes and rinsed with PBS. The blocking step was performed using PBS supplemented with 0.3 % Triton X-100 (PBT) and 5 % FCS. After 1 hour, cells were washed with PBT and incubated with primary antibody diluted in PBT for 1.5 hour. Samples were washed 6 times with PBT and then incubated with secondary antibody conjugated with appropriate fluorophore for 45 minutes. After 6 washes with PBT, the glass coverslips were mounted using Vectashield Mounting Medium with DAPI (*Vector*) to visualise also the cell nucleus. Samples were analyzed by confocal microscopy using Leica DMI6000 confocal microscope with TCS SP5 AOBS Tandem and HCX PL APO 40x/1,25-0,75 Oil CS UV objective. Images were then processed with LAS AF Lite software (*Leica Microsystems*).

3.2.14 Whole-mount embryo immunohistochemistry

Embryos collected at the developmental stage E7.5 were fixed overnight with 4 % PFA at 4 °C. Samples were then washed six times in PBS at room temperature and incubated with solutions as follows: 15 mM NH_4Cl in PBS (30 minutes, room temperature), 100 mM glycine in PBS (30 minutes, room temperature) and 1 % H_2O_2 in PBS (3 hours, room temperature). The blocking step was performed with incubation of embryos in PBSMT (2 % non-fat milk, 0.5 % Triton X-100 (v/v) in PBS) for 2×1 hour. Embryos were then stained with primary antibody diluted in PBSMT overnight at 4 °C and next day washed 5 times (1 hour each) with PBSMT (2 times at 4 °C, 3 times at room temperature). The same scheme was applied to staining with secondary antibody conjugated to HRP. After the last wash, embryos were rinsed with PBTX (0.2 % (v/v) BSA, 0.5 % Triton X-100 (v/v) in PBS), washed with this solution for 20 minutes and incubated in dark with DAB substrate at room temperature. After 30 minutes, H_2O_2 was added to a final concentration of 0.03 % and samples were rocked for 5 minutes. Embryos were then washed 3 times in PBTX, post-fixed in 4 % PFA and images of them were taken by SZX9 microscope (*Olympus*).

3.2.15 Stimulation of surface IgM expression in 70Z/3 cells

For induction of IgM expression in 70Z/3 derived cell lines, cells were placed into 96-well plate (8×10^4 cells per well) and incubated at 37 °C with various concentration of LPS (*Difco*) for 5 to 20 hours. Cells were stained with the Anti-Mouse-Alexa555 conjugated antibody (*Invitrogen*). The surface expression of IgM was examined by FACS (Chapter 3.2.4).

3.2.16 BWZ assay

For stimulation of BWZ cells with antibodies, 96-well plates were pre-coated with antibodies diluted in PBS for 4 hours at 37 °C. Plates were washed with PBS and wild-type or transfected cells were added to appropriate wells (5×10^4 cells per well). After overnight incubation at 37 °C and washing of plates with PBS, cells were lysed in Z-buffer (100 mM 2-ME, 9 mM $MgCl_2$, 0.125% NP-40, 0.15 mM CPGR in PBS) at 37 °C. After 4 hours, 1 M glycine in PBS was added to each well and plates were read at 595 nm by Multiscan EX (*Thermo Scientific*). For stimulation assays with soluble reagents, BWZ cells were incubated overnight at 37 °C with PMA (10 ng/ml) and ionomycin (0,5 µM) or various concentrations of IL-1β (*Immunotools*) and developed as described above.

3.2.17 ELISA

The wild-type or transfected J774.2 cells were incubated for 24 hours in the presence or absence of LPS (100 ng/ml); for these stimulation purposes, 3 different types of LPS (*Difco, Invivogen, Sigma-Aldrich*) were used. Supernatants were harvested from all samples and used for quantitative examination of the TFN-α level by ELISA (enzyme-linked immunosorbent assay). The experiment was performed using Mouse TNF alpha ELISA Ready-SET-Go!® (*eBioscience*) according to manufacturer's instructions and plates were analysed at 450 nm by Multiscan EX (*Thermo Scientific*).

3.2.18 Statistical analysis

The data from stimulation experiments are presented as means plus SD unless stated otherwise. The significance between groups was determined either by a Student's *t*-test or a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls multiple comparisons test to determine which data sets were significantly different from each other. Statistical tests were performed using Prism 6 and InStat 3.10 software (*GraphPad*).

4 Results

4.1 Aim of the thesis

Protein SIGIRR has recently been identified as a negative regulator that influences NF-kB signalling through cytokine receptors as well as Toll-like receptors. Its expression has been detected in many distinct cell types and tissues and previous results obtained in our lab show, that SIGIRR is also expressed in early stages in embryonic development.

The main aim of this thesis is to contribute to the elucidation of the mechanism underpinning the negative regulatory role of SIGIRR in IL-1R/TLR signalling pathway. To achieve this goal we set out these following tasks:

I. To develop various SIGIRR constructs and establish transduced cell lines suitable for biochemical and microscopic analyses of SIGIRR funtion in hematopoietic cell lines.
II. To test the regulatory role of SIGIRR in cells of hematopoietic origin and thus confirm or rule out its negative regulatory function in these cells and provide a new insight into possible mechanisms of SIGIRR function.
III. To establish a new screening model based on BWZ.36 reporter cell system suitable for binding and subsequent identification of possible SIGIRR ligands.
IV. To assess the expression and the role of SIGIRR during early embryonic development using SIGIRR deficient mouse strain.

4.2 Establishment of SIGIRR-overexpressing cell lines

Protein SIGIRR is supposed to negatively influence signalling triggered via receptors from IL-1R1/TLR family, thus, at first we wanted to investigate SIGIRR inhibitory function in various cell lines. To performe this we designed a set of constructs in order to create various cell lines transfectants suitable for biochemical and microscopic analyses of SIGIRR.

As the pilot experiment for this part of our study, the full-length SIGIRR DNA was amplified by PCR using specific primers (Table 3.3) and murine E7.5 cDNA as a template. Following the protocol described above (Chapter 3.2.5) the SIGIRR coding sequence was ligated into the pEYFP-N3 vector (Figure 3.1) and HEK293T cells were transfected with this plasmid using lipofection (Chapter 3.2.9). Transfected cells were designated as HEK293T/SIGIRR-EYFP cells; as a control, HEK293T cells transiently transfected with empy pEYFP-N3 plasmid (HEK293T/EYFP) were used.

DNA from pSIGIRR-EYFP-N3 plasmid was used as a template for amplification of another SIGIRR contruct (Figure 4.1) which was cloned into the pIRES2-EGFP vector (Figure 3.1). This plasmid was lipofected into HEK293T cells (designated as HEK293T/SIGIRR). Cells transfected with empty pIRES2-EGFP vector (HEK293T-EGFP) were used as a control.

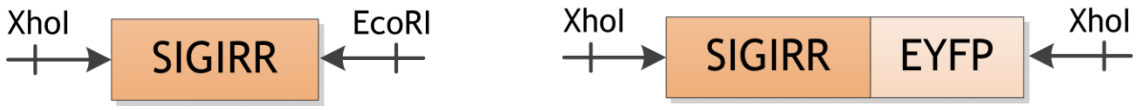

Figure 4.1 Scheme of full-legth SIGIRR construct cloned into the pIRES2-EGFP vector (left) and into the retroviral pLXSN vector (right).

4.2.1 Establishing SIGIRR stable infectants using retroviral infection system

As the stability of transiently transfected cells was not satisfiying and cells usually lost their ability to express inserted protein in a few days post transfection, we decided to develop several stable transformed systems using retroviral infection (Chapter 3.2.10). Three different constructs were designed to serve various purposes.

To create a stable system expressing SIGIRR with a fluorescent tag, which would be suitable for microscopical analyses, a retroviral vector pLXSN (Figure 3.2) was employed. SIGIRR-EYFP coding sequence was amplified by PCR from pSIGIRR-EYFP-N3 plasmid DNA, which served as a PCR template also for the other constructs described below. SIGIRR-EYFP construct (Figure 4.1) was then ligated into the target vector following the previously described protocol (Chapter 3.2.5) and used for retroviral infection of murine macrophage-like cell line, J774.2. Transduced cells after sort of EYFP-positive cells were designated as J774.2/SIGIRR-EYFP.

Bicistronic pMigR1 vector (Figure 3.2) was used as a vehicle for SIGIRR protein modified with hemagglutinin (HA) tag at the C terminus (Figure 4.2). Introduction of this plasmid into target cells allows the identification of transduced cells based on the expression of free EGFP. Murine pre-B cell line 70Z/3 and also J774.2 cells were used for retroviral infection with this vector. EGFP-positive cells were sorted out and designated as 70Z/3.SIGIRR-HA or J774.2/SIGIRR-HA cells. Stably transduced 70Z/3.MigR1 and J774.2/MigR1 cell lines were also generated and used as controls.

For developing a model suitable for screening of putative SIGIRR-interacting ligands, BWZ.36 reporter system was used. The BWZ murine thymoma cell line contains a transgenic LacZ gene under the control of NFAT promotor element (Sanderson and Shastri, 1994). These cells were transduced with chimeric protein consisting of SIGIRR extracellular domain and CD3ζ signalling domain (Figure 4.3); pMC83IG vector was used for this purpose. Cross-linking of the fusion receptor triggers the expression of β-galactosidase in BWZ cells, which could be used either for identification of possible ligands for surface molecules or screening of monoclonal antibodies against specific cell surface antigens (Mesci and Carlyle, 2007). Cells transduced with chimeric protein were designated as BWZ/SIGIRR, control cells transduced with empty vector as BWZ/MigR1.

Figure 4.2 Scheme of the construct representing SIGIRR protein modified with HA tag.

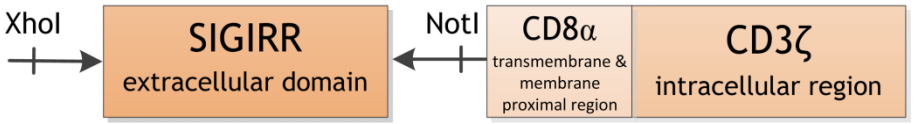

Figure 4.3 Scheme of chimeric SIGIRR-CD8α-CD3ζ protein for transduction of BWZ cells.

During our experimental work we were at first simple transfering the virus-containing supernatant from packaging cells to target cells, but this way of infection had very low efficency. By employment of the retroviral spinning-mediated infection (spin infection) we were able to achieve 10-fold increase in the efficiency of infection (up to 80 %; data not shown). Thus, using transient transfection and stable transduction we established a set of lines overexpressing SIGIRR for follow-up analyses.

4.3 Studies focused on SIGIRR as a TLR signalling negative regulator

According to the results of many experiments presented in the literature, SIGIRR is supposed to inhibit the NF-κB signalling pathways triggered by several IL-1 and TLR family receptors in many cell types. To further broaden the range of our knowledge regarding this area, we decided to investigate the inhibiting potencial of SIGIRR in several ways using different cell lines.

4.3.1 SIGIRR does not inhibit the signalling in 70Z/3 cells upon LPS stimulation

70Z/3 cell line represents a powerful tool to study the LPS responsiveness under various conditions. These cells are derived from murine B lymphoma and constitutively express μ heavy chains which remain intracellular (Paige et al., 1978). Treatment of 70Z/3 cells with LPS or some cytokines such as IFN-γ triggers the synthesis of κ light chains and thus causes the surface IgM expression in these cells (Briskin et al., 1988; Paige et al., 1978), which could be easily measured by FACS. Here we decided to overexpress SIGIRR in 70Z/3 cells and perform a set of stimulation experiments in order to examine the possible role of SIGIRR as an inhibitor of TLR signalling in 70Z/3 cells.

At first we tried to determine the dose-dependent stimulation profile of 70Z/3 cells. Parental or transduced 70Z/3 cells were subjected to treatment with various LPS doses for 16 hours and the presence of surface IgM was measured by FACS (Chapter 3.2.15). Our results show that even low levels of LPS (0.1 μg/ml) could induce the expression of surface IgM in 70Z/3 cells and the maximum response could be obtained by using LPS concentrations starting at 1 μg/ml (Figure 4.4). We also revealed that there are no dramatic differences in surface IgM expression between parental and transduced cell lines. However very surprising was the finding that the subset of IgM^+ cells was in all cases slightly higher in SIGIRR expressing population compared to negative controls. This outcome is in the opposite of what we expected, considering that SIGIRR probably serves as a negative regulator of LPS-triggered signalling and thus its presence should significantly decrease the LPS response of 70Z/3 cells.

We also compared the response of 70Z/3 cells incubated with LPS for different time periods from 5 to 20 hours. According to our findings, IgM starts to clearly appear on the cell surface after 10 hours of the LPS stimulation (Figure 4.4). The overall stimulation profile was again very similar for all tested cell lines, which could be verified by FACS data (Figure 4.5).

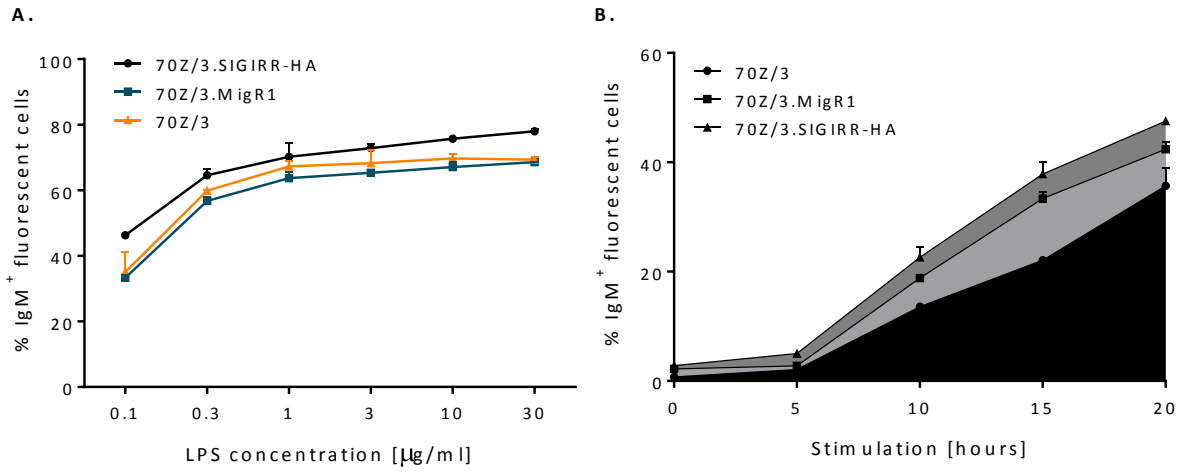

Figure 4.4 The stimulation profile of 70Z/3 cells and their transductants. No dramatic diferrence in surface IgM expression between cell lines could be observed upon incubation of cells with various doses of LPS for 16 hours (A) or upon treatment of cells with 100 ng LPS/ml for different time periods (B).

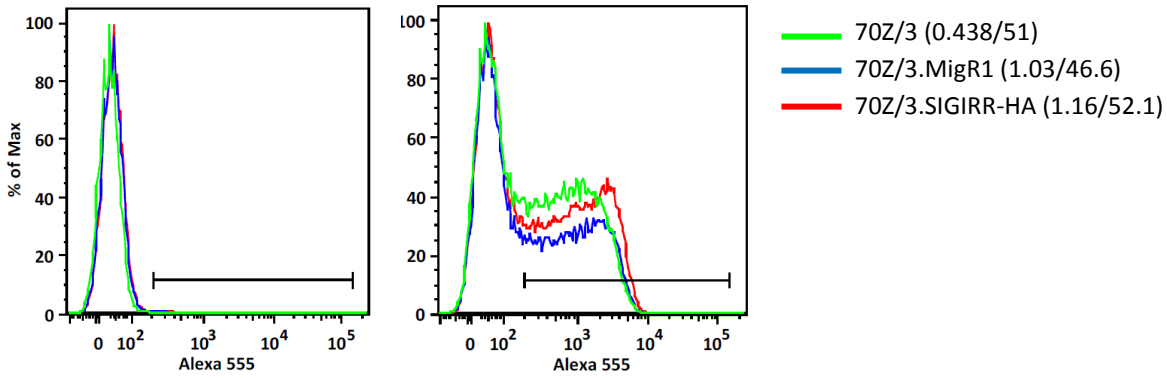

Figure 4.5 FACS analysis of IgM$^+$ cells in 70Z/3 populations. Cells were stimulated with 100 ng LPS/ml for 16 hours (right) or left untreated (left). Cell were stained with anti-mouse IgG-Alexa555 conjugated antibody. Histogram curves obtained by FACS analysis show the similar trend for all tested cell lines. The black marker defines IgM$^+$ population; numbers in legend denote the % of IgM$^+$ cells in non-treated/treated subset. Representative graphs are selected from several independent experiments.

In sum, these results suggest that TLR signalling in 70Z/3 cells is not influence by the presence of SIGIRR. This phenomenon could be caused by disrupted spatial structure of SIGIRR. Another possible explanation could be that overall signalling conditions in this cell line doues not to involve SIGIRR correctly in their NF-κB signalling pathways and thus cells overexpressing SIGIRR show no significant difference in surface IgM expression upon LPS stimulation.

4.3.2 Analysis of TNF-α production in J774.2 transductants

To test and compare the SIGIRR-dependent LPS responsiveness also in a different cell line then 70Z/3, we measured the TNF-α expression levels by ELISA also in murine macrophage-like cell line J774.2, infected or not with MigR1, MigR1-SIGIRR-HA (SIGIRR-HA) and pLXSN-SIGIRR-EYFP (SIGIRR-EYFP). To make sure that different types of LPS will not affect the outcome of signaling, cells were stimulated with LPS derived from two different bacterial species: *Escherichia coli* available from two different commercial sources, *Invivogen* (designated as LPS1) and *Sigma-Aldrich* (further reported as LPS2), and *Salmonella typhimurium* (available from *Difco*) (designated as LPS3). TNF-α containing supernatans were then processed as described above (Chapter 3.2.17).

We found out that all cell lines infectants responded to all three batches of LPS with significantly increased levels of TNF-α (Figure 4.6). The only exception in comparison with other infectants were supernatants derived from the empty vector control J774.2/MigR1, whether stimulted or not, that exhibited unusually low levels of TNF-α independently of the batch of LPS used (Fig.4.6 B). To normalize these results with those obtained from other infectants and noninfected parental cell line, we calculated the index of stimulation, by dividing the levels of TNF-α production after stimultion to that measured in an unstimulted cell culture.

The results are quite striking and not consistent with the inhibitory role of SIGIRR in TLR signaling. Specifically, and as illlustrated in Figure 4.7, SIGIRR-overexpressing infectants do not show the expected trend to inhibit the LPS-triggered signalling leading to decreased levels of TNF-α. Beside this, J774.2/SIGIRR-HA cell line produced approximately 2-times more TNF-α compared to the parental J774.2 cell line treated with either batch of LPS. Thus, we concluded that SIGIRR, in case of TLR4 signalling in macrophage cell line J774.2, failed to act as a its negative regulator. To verify whether this is a more general phenomenon in macrophage-related cells, we extended our study to primary cells.

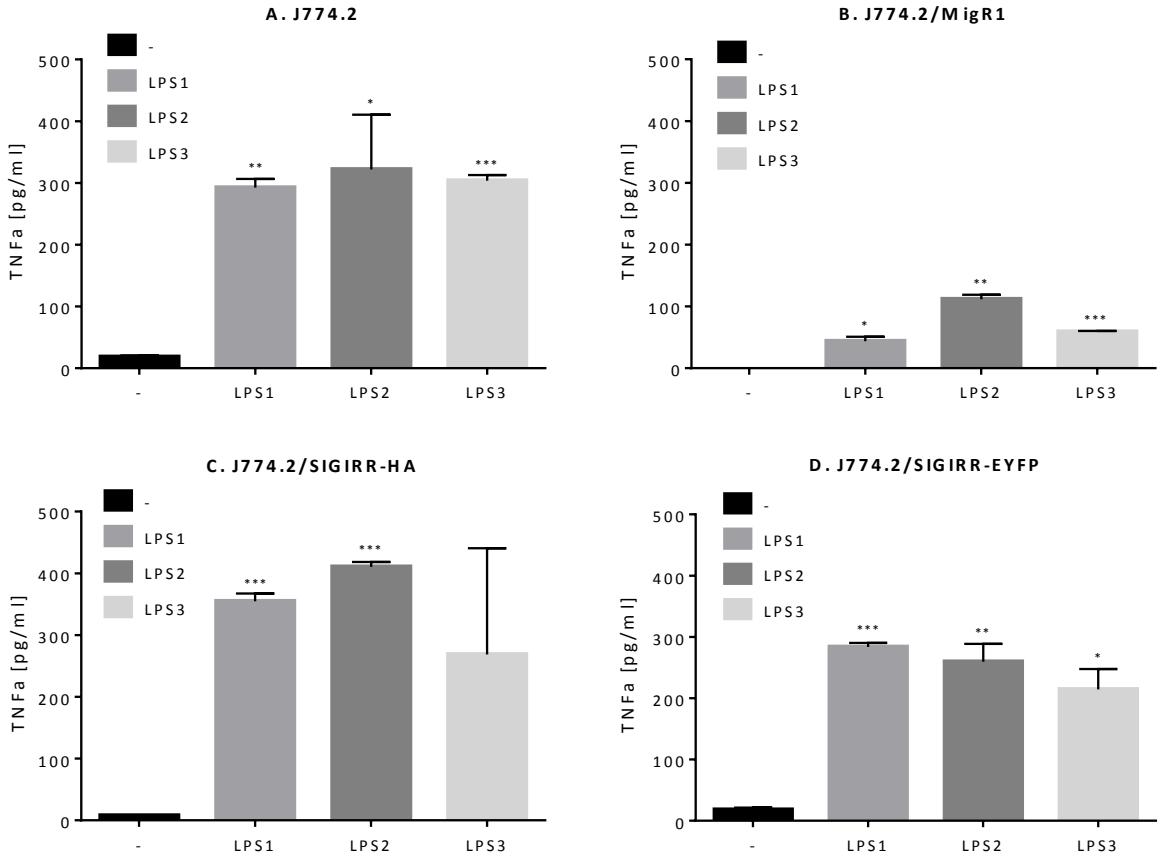

Figure 4.6 Stimulation of J774.2 cell lines with LPS. All cell lines show significant increase in TNF-α production upon stimulation with several types of LPS measured by ELISA (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$), but SIGIRR-overexpressing cells do not significantly differ in TNF-α levels compared to parental J774.2 cells. Data are presented as mean plus SD, n=2.

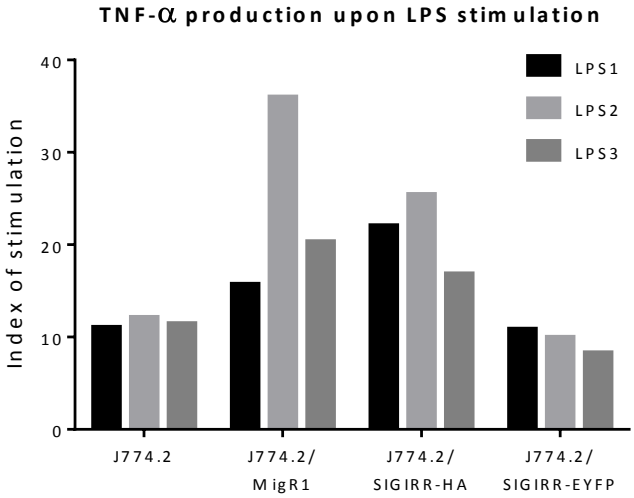

Figure 4.7 Overexpression of SIGIRR in J774.2 cells does not decrease the TNF-α production upon LPS treatment. On contrary, the calculated fold change in TNF-α expression between non-stimulated cells and cells stimulated with LPS is approximately 2-times higher for J774.2/SIGIRR-HA cells compared to parental J774.2 cells. SIGIRR-EYFP showed comparable index of stimulation as the parental cell line.

4.3.3 Stimulation of peritoneal macrophages from SIGIRR$^{-/-}$ mice with LPS

To assess the role of SIGIRR in primary cells functionaly related to immune system, we investigated the effect of SIGIRR on TLR4 signalling macrophages. The peritoneal macrophages obtained from the wild-type and SIGIRR$^{-/-}$ mice on C57Bl/6 background (further as B6) were stimulated or not with LPS as described above (Chapter 3.2.1). The expression of several important proinflammatory cytokines, specifically TNF-α, IL-1β and IL-6, were assessed by isolating RNA from cell cultures and subjecting them to qRT-PCR analysis.

Our results show that the cytokine production is not strongly affected by SIGIRR absence, nevertheless the detected basal levels of IL-1β and IL-6 are approximately 5 and 3 times higher in SIGIRR$^{-/-}$ cells, respectively, compared to the wild-type control. This difference in the expression levels of these cytokines is preserved also in these cells when stimulated with LPS (Figure 4.8). The same trend for increased levels of cytokine in SIGIRR$^{-/-}$ cells compared to the wild type cells was observed for the expression of TNF-α in unstimulated samples. This finding suggests that rather than being a *bona fide* negative regulator of activated TLR signalling, i.e. affecting the outcome of signalling, SIGIRR rather functions to control the basal level of expression of proinflammatory cytokines through so far uncharacterized, TLR-ligand independent mechanism. This is certainly the area of interest which should be further explored to understand the molecular basis for its role in setting the homeostatic background levels of immune cytokines.

Thus, our results demonstrate that SIGIRR is involved in the regulation of cytokine homeostasis during a normal physiology, especialy when it comes to basal cytokine production in macrophages. This sharply contrast with its previously documented negative regulátory role in TLR signalling in non-hematopoietic cells, such as epithelial cells, neurons as wel as glial cells in CNS. So, it seems that SIGIRR function is cell content-depend. To understand these differences, we perform battery of tests to reveal potential discrepancies in the expression, localization and membrane distribution of SIGIRR in non-hematopoietic cells and macrophages.

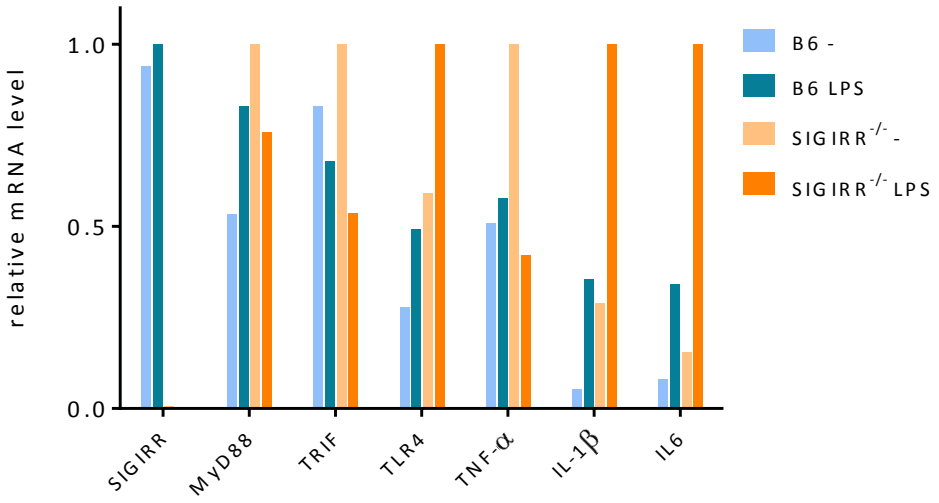

Figure 4.8 The presence or absence of SIGIRR in peritoneal macrophages does not change the trend in their cytokine production upon LPS stimulation, but probably could influence their common expression levels. The expression of selected genes was normalized to CasC3 mRNA levels. Standard deviations are not indicated as the measurment was performed on a single sample and also to its highest value, which was for the better visualisation adjusted to a reference value „1".

4.4 Western blot analysis of SIGIRR transfectants and transductants

Because of our findings that SIGIRR does not act a negative regulator of TLR-mediated signalling in case of distinct hematopoietic cells (Chapter 4.3) we wanted to examine the model cell lines in more details. Therefore we decided to check whether SIGIRR is really overexpressed by these cells using Western blot analysis.

At first we wanted to know, whether the transient transfection of HEK293T with plasmid containing fusion SIGIRR-EYFP construct was efficient. Therefore we tested this cell line by Western blot analysis using anti-SIGIRR antibody and also anti-GFP antibody. We were able to clearly detect the band representing SIGIRR-EYFP protein with the molecular weight approximately 150 kDa (Figure 4.9).

We also examined the expression of the fusion SIGIRR-EYFP protein in stable transduced J774.2/SIGIRR-EYFP cells and according to our expectation we detected the band with same molecular weight as in case of HEK293T/SIGIRR-EYFP cells (Figure 4.10), suggesting that the transduction was successful.To test the expression of HA-tagged SIGIRR in J774.2 and 70Z/3 cells we analysed their lysates by Western blot using anti-SIGIRR and anti-HA antibody (Figure 4.10). At both cases we were able to detect the band with molecular weight approximately 100 kDa. This band represents the expression of SIGIRR-HA protein in stable transduced cells.

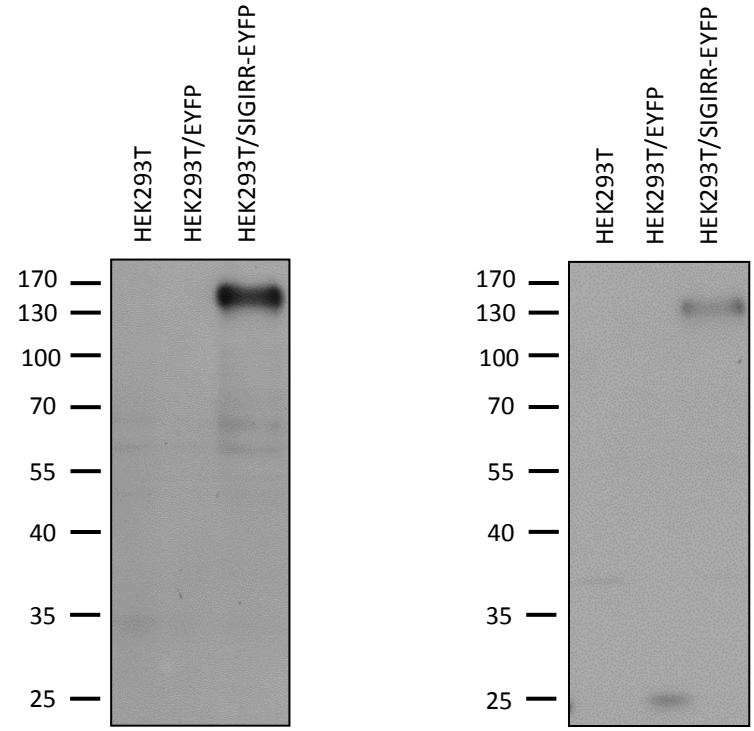

Figure 4.9 Western blot analysis of HEK293T/SIGIRR-EYFP cell line. Western blot was stained with anti-SIGIRR (left) or anti-GFP (right) antibody.

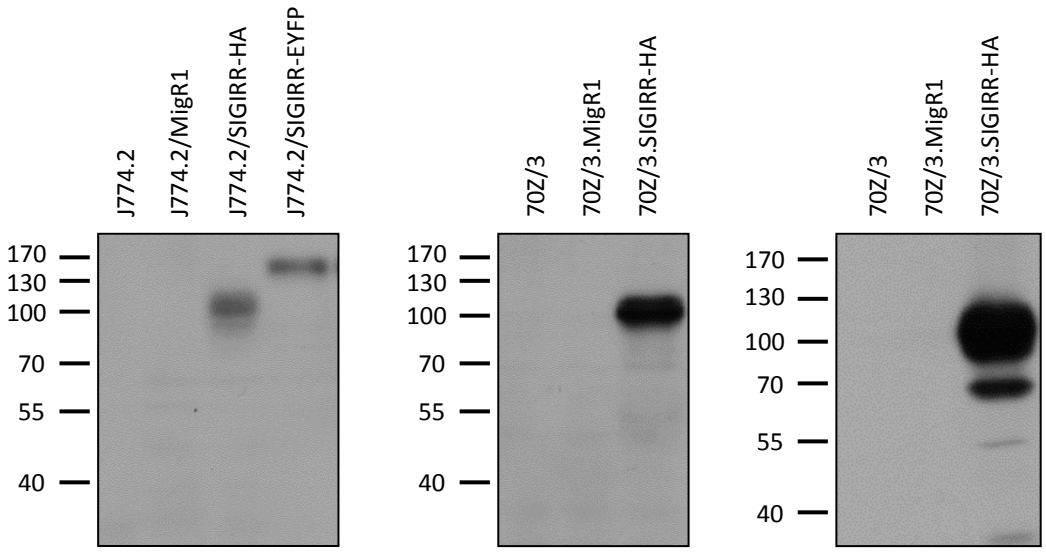

Figure 4.10 Western blot analysis of J774.2 and 70Z/3 transduced cell lines. Western blot was stained with anti-SIGIRR (left and middle panel) or anti-HA (right panel) antibody.

According to the information available in literature, predicted molecular weight of SIGIRR is approximately 40 – 90 kDa. Surprisingly, the molecular weight of proteins expressed in our cell lines was higher than expected. To exclude the possibility that SIGIRR could form molecular complexes containing identical subunits, we treated cell lysates with SDS sample buffer supplemented with 8 M urea when preparing WB samples. The presence of urea in buffer should lead to complete reduction and solubilisation of proteins and is used especially for membrane and nuclear non-histon proteins. To avoid the aggregation of protein caused by high temperatures we also tried not to boil the WB samples before electrophoresis. According to our results (data not shown), none of these conditions or their combination had an influence on the protein molecular weight. Therefore we decided to create a full-length SIGIRR construct with no modifications, thus representing the physiological variant of this protein. This construct was introduced into HEK293T cells. Western blot analysis showed that the molecular weight of overexpressed SIGIRR protein was approximately 60 kDa (Figure 4.11). Why SIGIRR shows such a heterogenity in molecular forms could be explained by their different glycosylation, as SIGIRR is supposed to be heavily glycosylated. Another explanation could be that in some cases SIGIRR forms dimers which are not sensitive to standard ways of reduction. Thus, we showed that the unability of SIGIRR to inhibit the TLR signalling was not caused by the insufficient expression of SIGIRR. Our findings also suggest that different variants of SIGIRR protein are results of distinct processing pathways in various cells that create the diversity in molecular forms found in given cell lines.

4.5 SIGIRR is a membrane-localized protein

As we confirmed that our model cell lines are overexpressing SIGIRR, we wanted to further investigate the possible explanation of why SIGIRR did not act a negative regulator for signalling triggered via TLRs in transduced cells. SIGIRR is predicted to be a transmembrane protein and localize within cell membranes, so we decided subjected various transfected or tranduced cells lines established as decribed above (Chapter 4.2) to confocal microscopy to in order to test the localization of SIGIRR in these cell lines.

At first we examined the localization of fusion SIGIRR-EYFP protein transiently transfected into HEK293T cells. Fluorescent signals corresponding with this protein were clearly detectable at the plasma and also inner cell membranes (Figure 4.11). In contrast with

this, the EYFP fluorescence detected in control HEK293T cells transfected with empty plasmid was difusely localized through the cell. Thus, both cell lines could be easily distinguished.

The cellular localization of SIGIRR was further analyzed by immunofluorescence labelling of HEK293T/SIGIRR EYFP cells with anti-SIGIRR antibody as the primary antibody. Positive fluorescent signals were observed only in cells expressing fusion SIGIRR-EYFP protein, but not in non-transfected cells (Figure 4.12) or cells transfected with empty vector (data not shown). Both EYFP (corresponding to SIGIRR) and Alexa555 (corresponding to anti-SIGIRR antibody) fluorescent signals were co-localized.

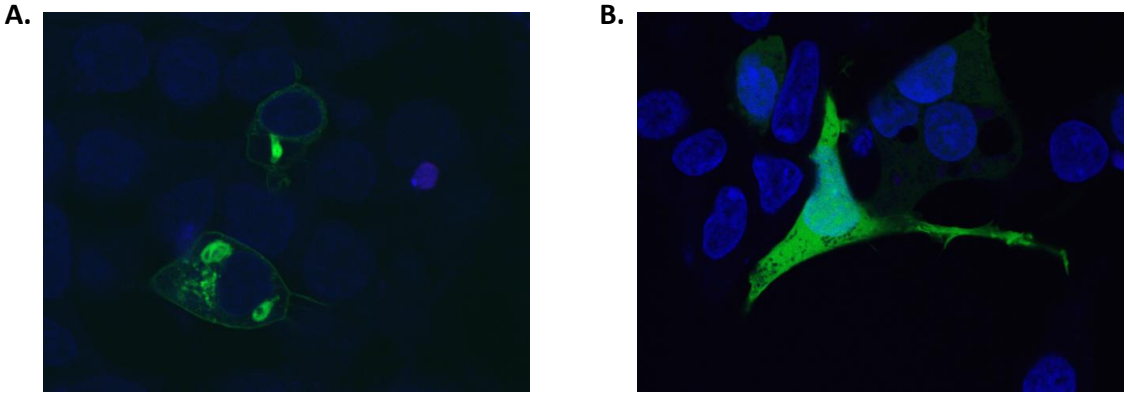

Figure 4.11 The EYFP fluorescence detected in HEK293T/SIGIRR-EYFP cells (A) or HEK293T/EYFP (B) cells. Fusion SIGIRR-EYFP protein is membrane localized, whereas the free EYFP is distributed diffusely in the cell. All cells were co-stained with DAPI (blue). Images were taken with Leica SP5 confocal microscope with total magnification 400x.

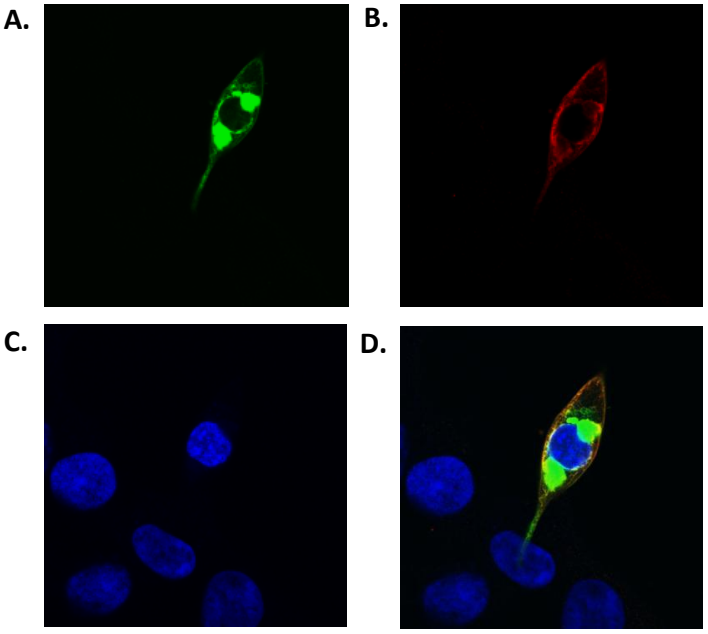

Figure 4.12 Staining of HEK293T/SIGIRR-EYFP cells with anti-SIGIRR antibody. Pictures are illustrating the fluorescent signals for EYFP (A), Alexa555 (B) and DAPI (C). EYFP and Alexa555 signals are co-localized (D). Images were taken with Leica SP5 confocal microscope with total magnification 400x.

We also analysed J774.2/SIGIRR-EYFP cells and their fluorescence. The EYFP fluorescence appered to correspond mainly with the plasma membrane of transduced cells, whilst the fluorescent signals of free GFP expressed by control J774.2/MigR1 cells were again difused through the whole cell (Figure 4.13).

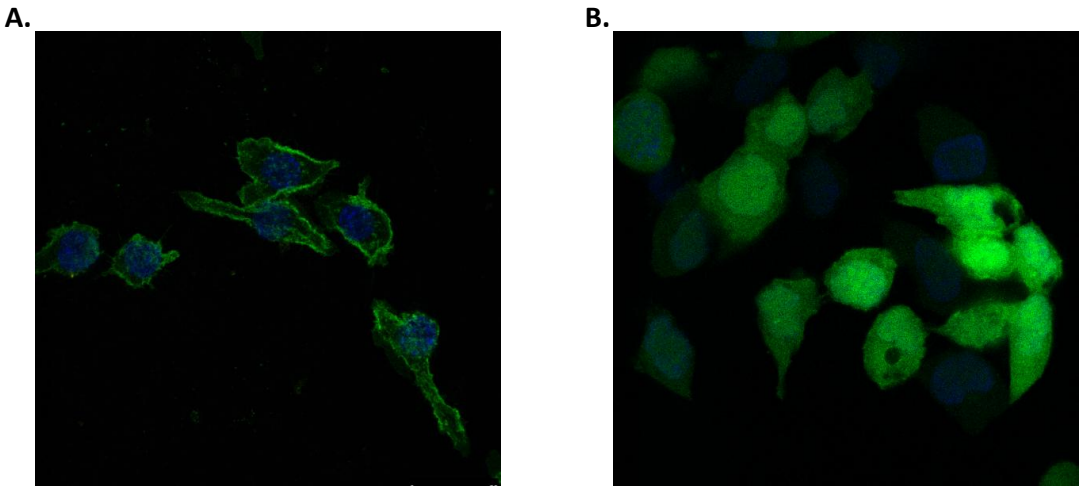

Figure 4.13 Fluorescence of J774.2 transductants. Fusion SIGIRR-EYFP protein in J774.2/SIGIRR-EYFP cells is distributed mainly throuht the plasma membrane (A), expression of free GFP in control J774.2/MigR1 cells is difused (B). All cells were co-stained with DAPI (blue). Images were taken with Leica SP5 confocal microscope with total magnification 400x.

Finally we performed the immunolabelling of J774.2 transductants with anti-SIGIRR antibody as described above. The fusion SIGIRR-EYFP and also HA-modified SIGIRR protein was recognized with the anti-SIGIRR antibody (Figure 4.14). EYFP and Alexa555 signals were co-localized in case of cells expressing fusion protein, whereas the fluorescent signals detected for J774.2/SIGIRR-HA cells were different. Difusely localized GFP signal corresponds to the expression of free GFP as a marker of transduction, membrane-associated Alexa555 flurescence is observed when labelling the SIGIRR protein. As it could be observed, distribution of Alexa555 fluorescence is similar for both SIGIRR-EYFP or SIGIRR-HA protein, which suggests that the presence of tag expression does not affect the cellular localization of SIGIRR protein in J774.2 cells.

Our microscopical analyses confirmed that SIGIRR expressed by model cell lines localizes into the membrane compartment, meaning that SIGIRR function as a negative regulator for TLR signalling was not influenced by its defective localization within membranes of these transduced cells.

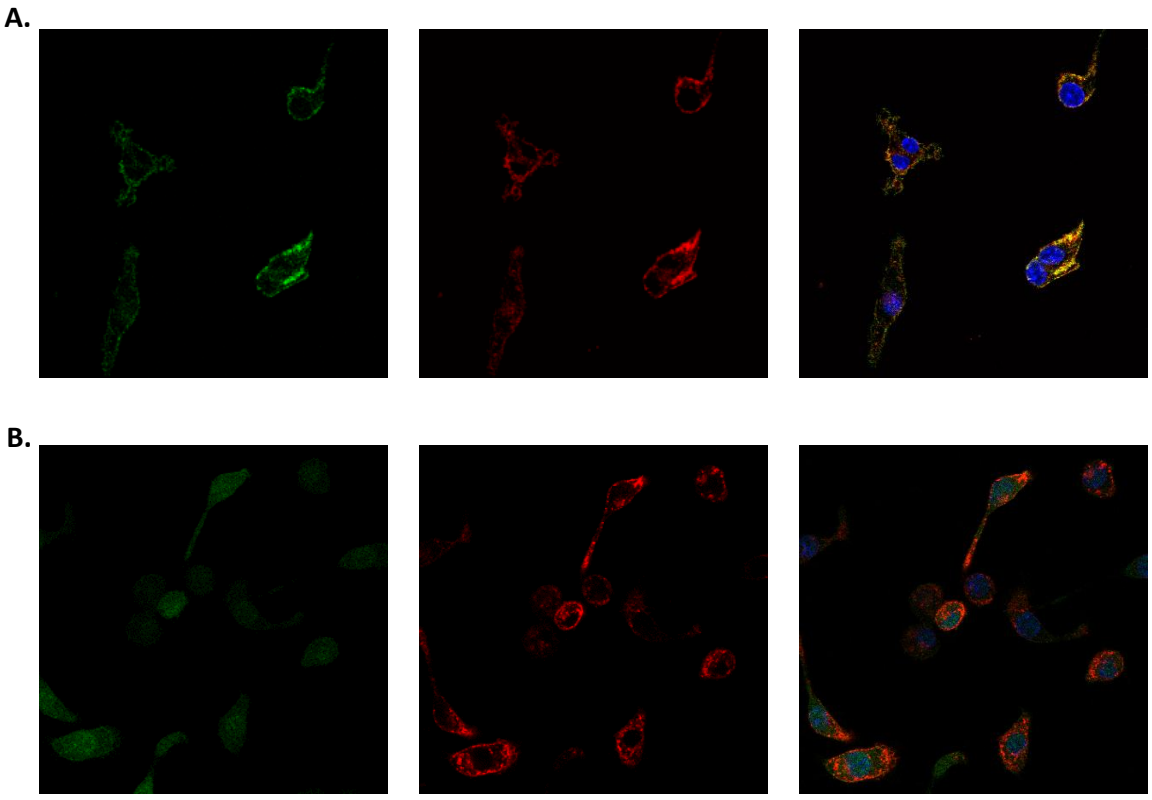

Figure 4.14 Immunolabelling of J77.2 transductants. Fusion SIGIRR-EYFP protein in J774.2/SIGIRR-EYFP cells (A) and SIGIRR-HA protein in J774.2/SIGIRR-HA cells (B) share the common distribution pattern (green – EYFP/GFP fluorescence; red – Alexa555 fluorescence; blue – DAPI staining). Images were taken with Leica SP5 confocal microscope with total magnification 400x.

4.6 SIGIRR could be found in at least three distinct molecular forms

During our Western blot analyses of SIGIRR-expressing cell types we discovered that several molecular forms of SIGIRR could be detected, each with different molecular weight. This finding led us to idea to explore them in more details, as this heterogenity could play a role in mediating of SIGIRR function and thus be at least partially responsible for our observations that SIGIRR did not modulate the TLR signalling in our model cell lines.

At first we examined the presence of SIGIRR not only in transduced cell lines, but also in samples obtained from another cell types (Figure 4.15). Especially we were interested in the cell line designated as EEC.B, which was derived in our laboratory from E7.5 ckit$^+$ TLR2$^+$ embryonic cells (Balounová, unpublished data). We found out that this particular cell line expresses endogenous SIGIRR with molecular weight approximately 140 kDa and thus this represents the first molecular variant of SIGIRR (designated as SIGIRR-140). The second molecular form with molecular weight approximately 100 kDa was detected in J774.2 cells

tranduced with SIGIRR protein modified with HA tag and also in lysate from mouse kidney cells (SIGIRR-100). The last molecular form was represented by full-lenght SIGIRR expressed by transfected HEK293T cells (SIGIRR-60).

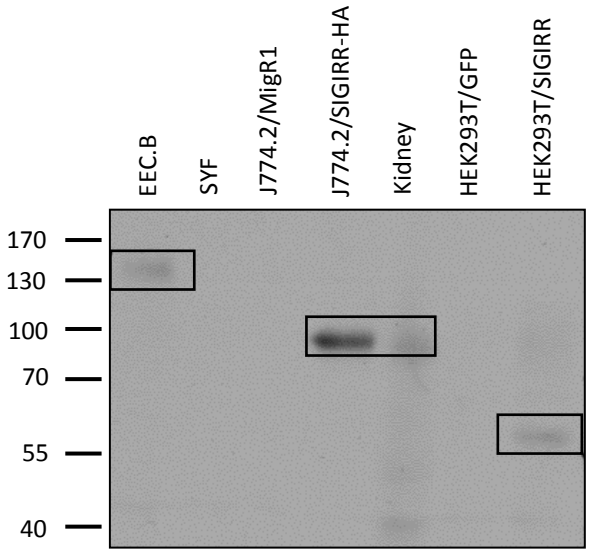

Figure 4.15 SIGIRR could be found in at least three molecular forms with distinct molecular weights. Western blot was stained with anti-SIGIRR antibody; three different SIGIRR forms (labeled by black borders) could be detected.

To further investigate the differences between distinct forms of SIGIRR we decided to characterize a size-dependent membrane distribution of SIGIRR molecular forms using gel filtration chromatography. This approach alows to simply fractionate cell lysates according to the size of molecular complexes present across membranes and their three-dimensional structure. For this purposes we performed gel filtration of EEC.B, J774.2/SIGIRR-HA and HEK293T/SIGIRR cells (Figure 4.16). Our results from gel filtration using Brij58 detergent show us that two of three distinct SIGIRR molecular forms are mainly enriched in high molecular weight fractions (4 + 5), whereas the smallest form is associated preferably with fractions representing smaller associated complexes. Because various lipid raft markers typically co-fractionate with high molecular weight fractions, our findings could suggest that SIGIRR-100 and SIGIRR-140 are in resting cells at least partially located into lipid rafts. This hypothesis is also supported by our observations from experiments when Brij58 was replaced with laurylmaltoside (LM). Presence of this detergent disrupts protein-lipid, but preserves protein-protein interactions and thus causes lipid rafts desintegration. When using LM, all froms of SIGIRR co-fractionated mainly with low molecular weight size complexes.

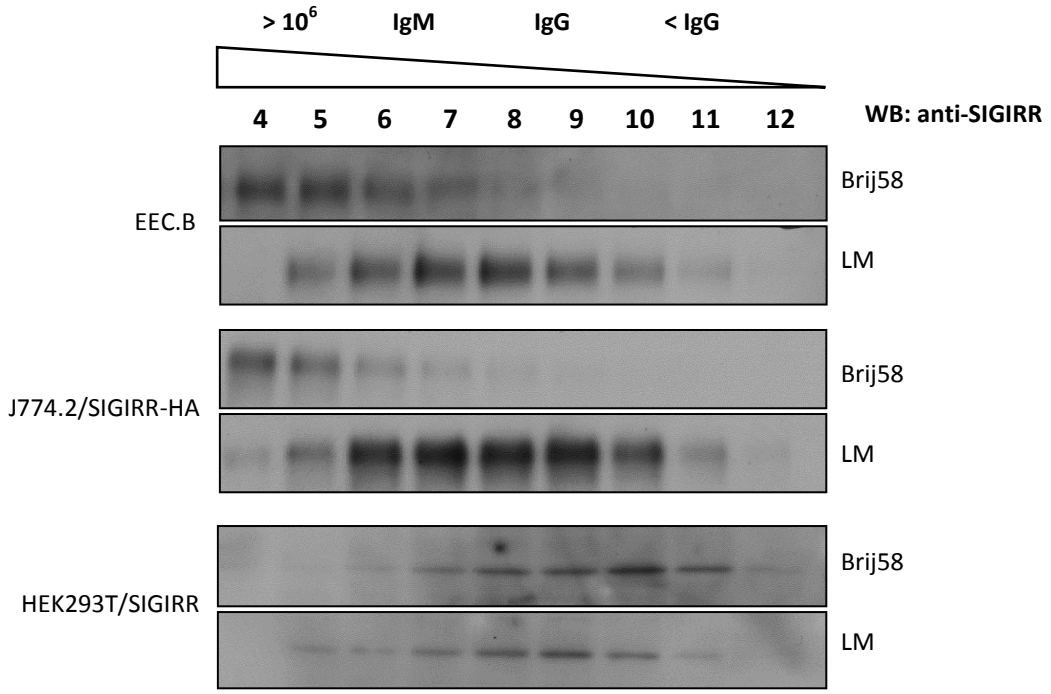

Figure 4.16 Fractionation of cells lysed in TKM buffer supplemented with 0,5 % Brij58 or 1 %LM using Sepharose 4B gel filtration chromatography. Fractions contraining SIGIRR were examined by Western blot using anti-SIGIRR antibody. Approximate molecular weight size marker is shown on the top.

Thus, we provided an evidence that SIGIRR could be found in at least three molecular forms, which differ not only in the meaning of their molecular weight, but also in their membrane distribution. To our best knowledge this finding represents the first report on membrane distribution of SIGIRR and it will be very interesting to furhter investigate the specific profile of possible SIGIRR localization into lipid rafts.

According to our observations, non-hematopoietic cells show the expression of SIGIRR form with lower molecular weight, located into smaller molecular complexes. On the other hand, distint cells of hematopoietic origin express this protein in molecular forms possibly located into lipid rafts. Signalling via TLRs in our model hematopoietic cell lines was probably not affected by SIGIRR because they possess this particular type of SIGIRR variant. Thus, our results suggest that the membrane distribution of SIGIRR is probably dependent on the cell type and could influence the overall SIGIRR ability to negatively regulate signalling pathways triggered via IL-1R/TLRs.

4.7 BWZ model is suitable for screening of possible SIGIRR ligands

SIGIRR is at present considered to be a so called orphan receptor, which means that o ligand no *bona-fine* ligand has been so far identified to interact with SIGIRR. However, recent experiments have suggested that SIGIRR could interact with one of the IL-1 family member, IL-1F5. According to these findings we decided to prepare a model suitable for screening of possible SIGIRR interaction partners. To arrange this we employed BWZ.36 reporter cell line whose stimulation leads to the measurable expression of β-galactosidase. These cells were transduced either with empty plasmid (BWZ/MigR1) or with vector encoding chimeric SIGIRR-CD8α-CD3ζ protein (BWZ/SIGIRR). Cells were then subjected to the set of stimulation experiments to identify whether the model is working properly and could be used for screening analyses (Chapter 3.2.16).

At first we looked at cells overall-signalling capability. Wild-type BWZ cells and their transductants were subjected to stimulation with PMA and ionomycin to reveal whether the ability of cells to express the β-galactosidase upon enhancement was not broken during the transfection. The result showed us that stimulation of either original or transfected cells with these common activators led to the significantly increased levels of β-galactosidase expression compared to non-stimulated state (Figure 4.17).

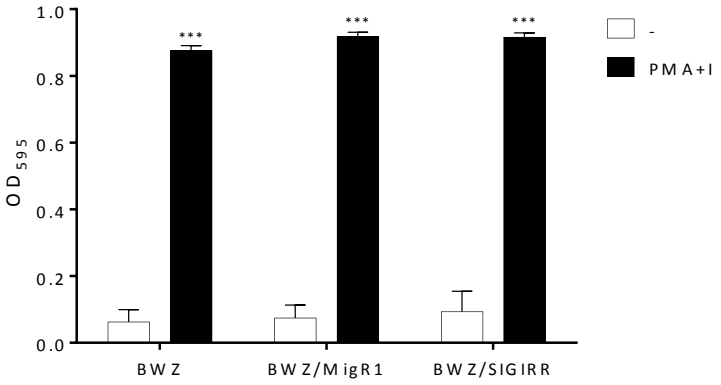

Figure 4.17 Wild-type BWZ cells and their transfectants are stimulated with PMA and ionomycin. Cells were left unstimulated or stimulated overnight with PMA (10 ng/ml) and ionomycin (0.5 μM). Treatment with PMA and ionomycin (PMA+I) enhanced the β-galactosidase expression in all cell lines compared to non-stimulated controls with high significance (***p < 0.001). Data are presented as mean plus SD, n=3.

Then we were interested whether the spatial structure of SIGIRR extracellular domain was disrupted or not because of the joining with different intracellular domain. To test this we stimulated wild-type BWZ cells and transfectants with anti-SIGIRR antibody recognizing the extracellular part of SIGIRR molecule. BWZ/SIGIRR, but not other infectants, expressed the β-galactosidase at significantly higher level compared to non-stimulated controls (Figure 4.18 A). To exclude the possibility of false-positive results which could be caused by changes in the spatial structure of SIGIRR extracellular domain we also stimulated all three types of cell populations with various concentrations of IL-1β. This cytokine should not interact with SIGIRR and this was also confirmed by results of our experiments. Even high levels of IL-1β did not activated the β-galactosidase expression in BWZ cell lines (Figure 4.18 B).

Our results demonstrated that the BWZ/SIGIRR cell line is able to specifically recognize and signal SIGIRR-interacting molecules, Thus, we established a model system suitable for identification and further study of possible SIGIRR ligands.

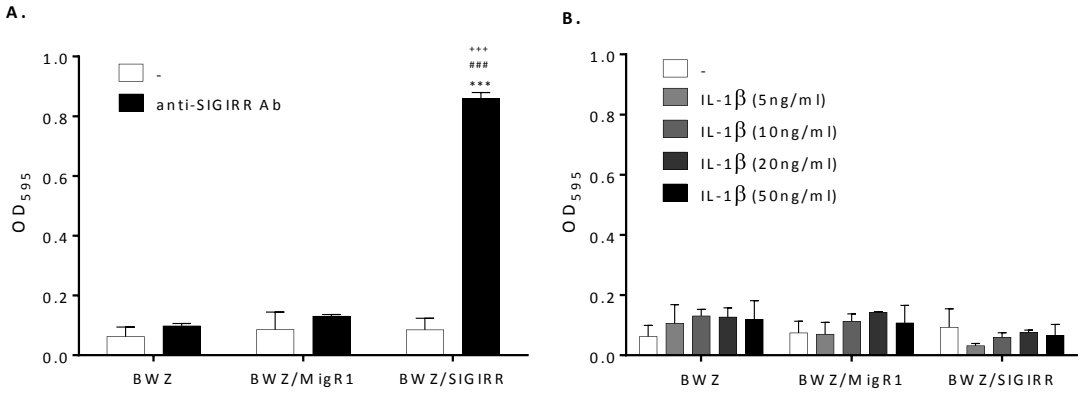

Figure 4.18 A. Incubation with anti-SIGIRR antibody enhances the production of β-galactosidase in BWZ/SIGIRR cells. Highly significant results were obtained when comparing treated and non-treated BWZ/SIGIRR cells (***p < 0.001), treated BWZ/SIGIRR and BWZ/MigR1 cells (###p < 0.001) and treated BWZ/SIGIRR and BWZ (+++p < 0.001) cells. **B. Treatment with IL-1β did not stimulate the production of β-galactosidase in wild-type or transduced BWZ cells compared to non-treated controls.** Data are presented as mean plus SD, n=3.

4.8 Analysis of SIGIRR function during embryonic development

Recently obtained results from experiments performed in our lab showed that SIGIRR is expressed in unusually high levels during early stages (E6.5 – E8.5) of murine embryonic development (Vavrochová, 2010). According to this finding we decided to further evaluate the role of SIGIRR in early embryogenesis using previously established SIGIRR$^{-/-}$ mouse model.

First we asked what is the cellular source of SIGIRR expression in early embryogenesis. Knowing that E7.5 CD11b^{+} TLR2^{+} phagocytes express the whole spectrum of TLRs and their adaptor proteins, we decided to compare the level of SIGIRR expression in E7.5 – E9.5 CD11b^{+} TLR2^{+} phagocytes as compared to CD11b^{-} TLR2^{-} nonimmune cells.

Cells obtained from wildtype ICR embryos (E7.5 – E9.5) were sorted our for CD11b^{+} TLR2^{+} population representing embryonic phagocytes. Double negative (CD11b^{-} TLR2) non-hematopoietic cells were also collected. Subsequent qRT-PCR analysis revealed that starting embryonic day 8.5 SIGIRR is expressed in higher levels by phagocytic cells (Figure 4.19 A). This correlates with our previously described finding that these cells are also main producers of various TLRs during this phase of embryonic development. Interestingly, at embryonic day 7.5 when the overall expression of SIGIRR is supposed to reach its highest level (Figure 4.19 B), SIGIRR is extensively produced by both phagocytic and non-immune cells. This finding suggests that during early stages of murine development SIGIRR could play a general role and might serve as a global inhibitor of inflammation, maintaining embryonic homeostasis.

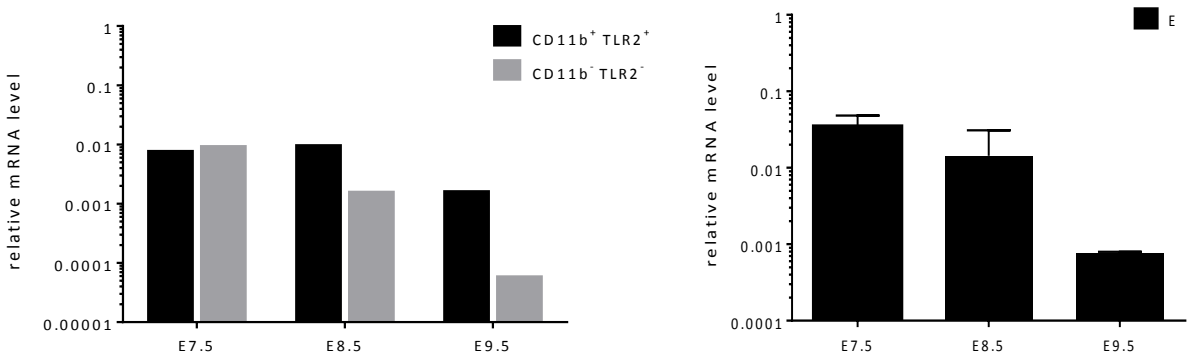

Figure 4.19 qRT-PCR analysis of SIGIRR expression during early embryogenesis. RNA from sorted cells was reversely transcribed into cDNA and subjected to qRT-PCR (A). The expression of SIGIRR was normalized to CasC3 mRNA level; standard deviations are not indicated as the measurment was performed on a single sample. Expression profile of SIGIRR in whole embryos (E) is presented here for comparison (B).

4.8.1 SIGIRR expression in E7.5 embryos from C57Bl/6 mice

To confirm our previous results from whole-mount immunohistochemistry performed on ICR E7.5 embryos (Chapter 2.3.5) we repeated this experiment using E7.5 embryos collected from C57Bl/6 mice (here referred as B6 mice). As the SIGIRR$^{-/-}$ strain is on C57Bl/6 background, we were interested whether the B6 wild type embryos also show the same pattern of SIGIRR expression as embryos from ICR mice. The procedure was performed according to protocol described above (Chapter 3.2.14). Embryos were stained with a different type of anti-SIGIRR antibody compared to the original experiment in order to increase a chance to reveal possible false positive result. Embryos stained with antibody against Brachyury, a protein expressed in this stage by cells forming the primitive streak, served as a positive control (Figure 4.20 B). As a negative control, embryos stained with the secondary antibody only were used (Figure 4.20 C). Our results confirmed the previously described findings about spatial expression profile of SIGIRR, which is almost evenly distributed through the whole embryo in day 7.5 of murine embryogenesis (Figure 4.20 A).

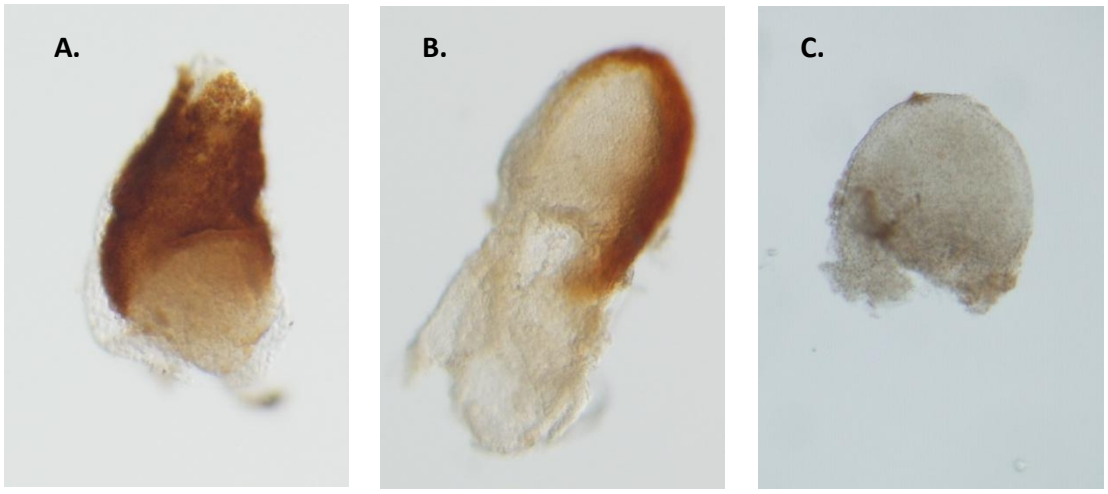

Figure 4.20 Whole-mount embryo immunohistochemistry. E7.5 embryos collected from B6 mice were strained with anti-SIGIRR Ab (A), anti-Brachyury Ab (B) or secondary HRP-conjugated Ab only (C) and the signal was developed using DAB as a substrate. Images were taken with Olympus SZX9 microscope with total magnification 57x.

4.8.2 Absence of SIGIRR does not affect the SIGIRR$^{-/-}$ mice breeding performance

As we discovered, SIGIRR is highly expressed during murine embryogenesis, which suggests that this protein could have an important role in this process. To investigate whether SIGIRR$^{-/-}$ mice thus do not suffer from breeding difficulties we looked at the breeding performance of this strain and also of some other strains.

As illustrated in Figure 4.21, collected data do not indicate that breeding of TIR8 strain is somehow impaired in the meaning of number of pups per litter. However, it has to be considered that mice are kept in SPF facility and thus protected from the exposure to environmental antigens what in turn can mask the effect of SIGIRR deficiency. Nevertheless, more detailed analysis has to be performed in order to make any conclusion about the breeding efficiency of SIGIRR$^{-/-}$ strain when compared to other strains.

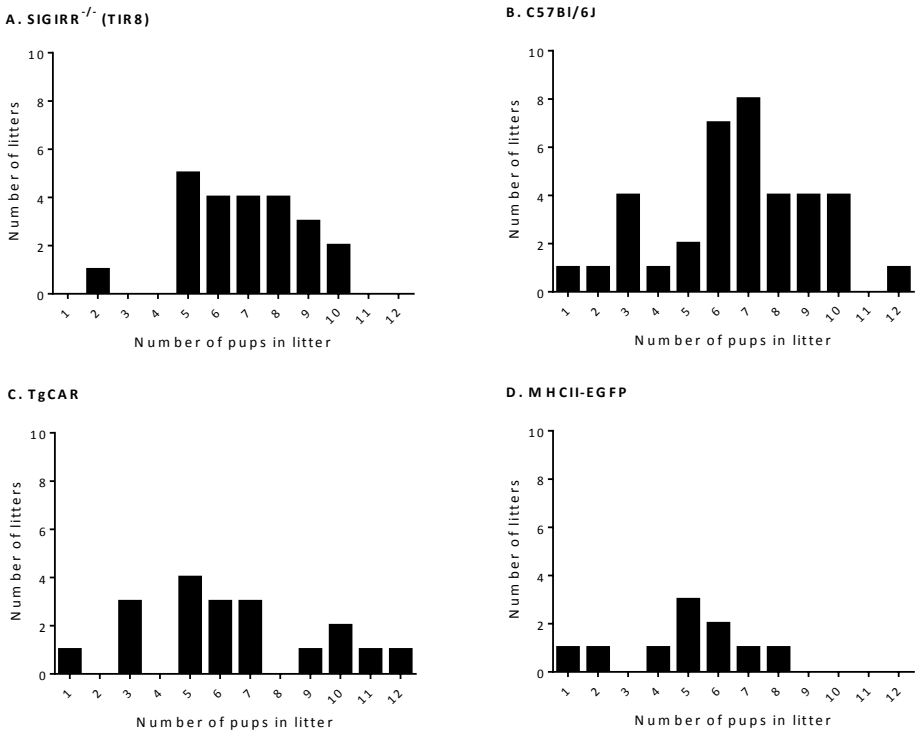

Figure 4.21 Illustration of breeding performance of various mice strains. TIR8 (SIGIRR$^{-/-}$) n=23; C57Bl/6J n=37; TgCAR n=19; MHCII-EGFP n=10.

4.8.3 Expression profile of innate immune molecules in SIGIRR$^{-/-}$ embryos

Using qRT-PCR approach we determined the expression levels of selected genes which are involved in signalling pathways negatively regulated by SIGIRR. For this purposes RNAs isolated from the wild-type and SIGIRR$^{-/-}$ embryos at embryonic stages E7.5 – E9.5 were used. Our results revealed that both receptors (IL-1R1 and TLRs) and adaptor molecules, that are part of signalling pathway suggested to be regulated by SIGIRR, are predominantly expressed at significantly lower levels in E7.5 and E9.5 SIGIRR$^{-/-}$ embryos (Figure 4.22). This interesting and for the first time described phenomenon allows us to speculate that in the wild-type embryos SIGIRR could act as a brake, preventing too easy and unwanted triggering of IL-1R1/TLRs signalling. It seems that to ensure a proper function of appropriate signalling molecules they are expressed at higher levels here in order to overcome the barrier caused by the presence of SIGIRR. On the other hand, in SIGIRR$^{-/-}$ embryos no such block exists and thus the expression of signalling molecules here is lower and probably on the basal level sufficient for proper signal transduction.

Surprisingly, this phenomenon has not been observed in case of IL-1R1 expression in E7.5 embryos, where practically no difference was detected between wild-type and SIGIRR$^{-/-}$ mice. This could suggest that in this stage of development, murine embryos are more sensitive to triggering of TLR signalling and thus expression levels of IL-1R1 do not need to be buffered by SIGIRR presence.

When comparing the expression of signalling molecules at stage E8.5, several genes show similar trend as described above, whereas MyD88, TLR3, TLR4 and TLR5 (this one also at stage 9.5) have opposite expression profile. Whether is this caused by global changes in signalling capacity of embryonic cells or by some other reason remains to be elucidated.

In summary, we described the expression kinetics for selected TIR-containing protein during early embryogenesis in wild-type and SIGIRR$^{-/-}$ mice. Our results show that expression profiles of these molecules differ for wild-type and SIGIRR$^{-/-}$ embryos and their expression is predominantly lower in the absence of SIGIRR. This could suggest that SIGIRR act as a blocator preventing the inappropriate signalling triggered via IL-1Rs/TLRs in early stages of embryonic development.

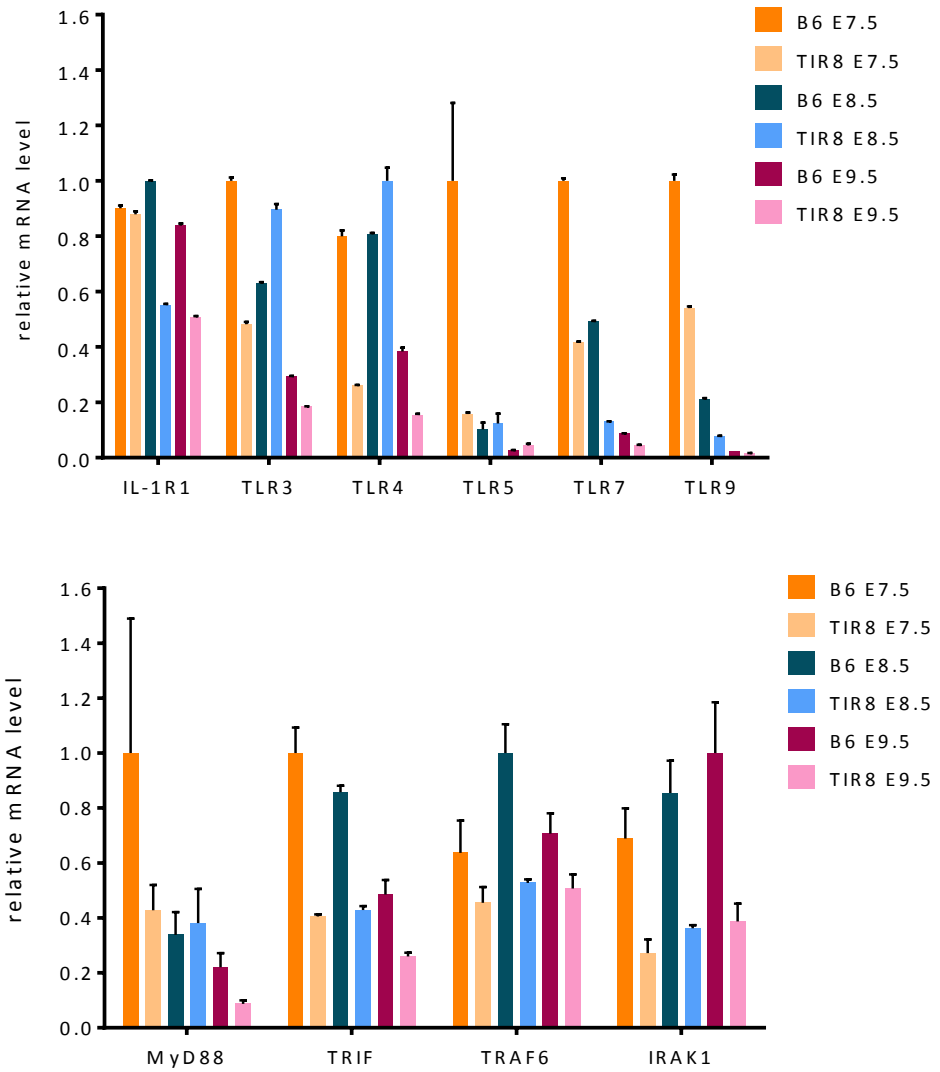

Figure 4.22 Expression kinetics of receptor and adaptor molecules involved in signalling pathways regulated by SIGIRR. Total RNA was isolated from B6 or SIGIRR$^{-/-}$ embryos in different stages of development and subjected to qRT-PCR. Expression level for each gene was normalized to CasC3 mRNA levels and also to its highest value, which was for the better visualisation adjusted to a reference value „1".

4.8.4 LPS stimulation of SIGIRR$^{-/-}$ embryonic cells

To evaluate the possible role of SIGIRR as a negative regulator of immune responses during murine development we tried to stimulate embryonic cells with LPS. The wild-type and SIGIRR$^{-/-}$ embryos from stages E7.5 and E8.5 were isolated and single cell suspensions were prepared. Cells were then incubated with or without LPS as described in Chapter 3.2.1 and subjected to qRT-PCR analysis.

In general, our results do not show strong differences between expression profiles of selected cytokines in the wild-type and SIGIRR$^{-/-}$ embryonic cells. However, detected levels of IL-1β are considerably higher when comparing E7.5 cells in the presence or absence of SIGIRR. This finding supports our previously stated hypothesis about SIGIRR functioning as a brake for signal transduction. This trend is not observed in E8.5 embryonic cells, which could be a consequence of finding that SIGIRR expression peaks at embryonic day 7.5 and then decreases. Thus, from stage E8.5 SIGIRR may not be as influential for the control of innate immune signalling. However, further experiments have to be performed in order to identify the precise and specific role of SIGIRR in maintaining embryonic homeostasis.

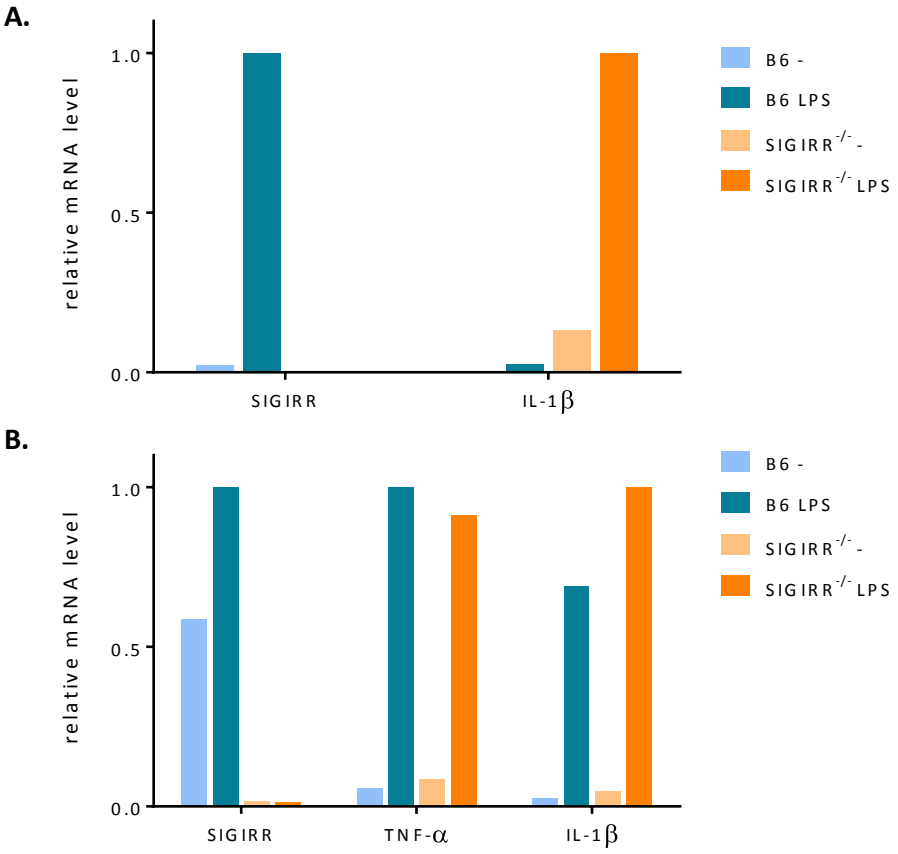

Figure 4.23 Stimulation of embryonic cells with LPS. B6 or SIGIRR$^{-/-}$ embryonic cells from E7.5 (A) or E8.5 (B) embryos were subjected to LPS stimulation and subsequent RNA isolation and qRT-PCR analysis. The expression of selected genes was normalized to CasC3 mRNA level and also to its highest value, which was for the; standard deviations are not indicated as the measurment was performed on a single sample. TNF-α expression is not stated for E7.5 embryonic cells because the product was undetectable at this assay.

5 Discussion

Immune responses are mediated through many distinct mechanisms and number of various types of molecules are involved in the complex network of immune processes. Very important group of proteins is represented by the superfamily of IL-1R/TLR molecules that participate in many physiological reactions related to innate immune and inflammatory responses (O'Neill, 2008). These proteins share highly-conserved TIR domain in their intracellular region capable to induce signallization leading to the activation of transcription factors NF-κB, AP-1 and IRFs. Together, members from IL-1R/TLR superfamily are able to mediate the immune response to a broad range of various inflammation stimuli.

The activation and progress of this powerful signalling network is strictly regulated to preclude the trigerring of inappropriate and potentially harmful inflammatory reactions. Several molecules exerting a supressor function on the IL-1R/TLR mediated signalling has been identified so far. SIGIRR, a structurally distinct member of the IL-1R family, is one of them (Thomassen et al., 1999). SIGIRR itself could not mediate the regulation of downstream transcription factors, but accumulated data suggest that its main role is to negatively regulate the signalling triggered by IL-1R/TLR group (Adib-Conquy et al., 2006; Bulek et al., 2009; Garlanda et al., 2004; Lech et al., 2007; Lech et al., 2008; Zhang et al., 2011). While such inhibitory function of SIGIRR has been implemented in various signalling pathways in distinct cell types, the best such evidence has been provided for the inhibition of IL-1R1- and TLR4-mediated signalling (Adib-Conquy et al., 2006; Huang et al., 2006; Polentarutti et al., 2003; Qin et al., 2005; Tian et al., 2010; Wald et al., 2003; Zhang et al., 2011). Despite the on-going research, the precise mechanism which mediates the SIGIRR inhibitory function is still unknown. SIGIRR is expressed in many cell types and tissues in adults (Polentarutti et al., 2003; Wald et al., 2003), but recent data also document an unusually high level of SIGIRR expression during early stages of embryonic development (E6.5 – E8.5) (Vavrochová, 2010). That suggests that SIGIRR could serve as a global inhibitor of inflammation not only in adulthood, but also during embryogenesis.

The main aim of this thesis was to elucidate the mechanisms underpinning the negative regulatory role of SIGIRR in IL-1R/TLR signalling, with a special emphasis on the characterization of SIGIRR function during embryonic development. Because of adversities associated with experiments performed on murine embryos, such as the requirement for

considerable amount of time-pregnant females, high degree of difficulty to isolate and manipulate young embryos, technically difficult and time consuming experimental settings as well as significant limitation of cellular material obtained, we at first decided to use and create the set of various cell lines suitable for primary biochemical and microscopical analyses of SIGIRR. We established stably transduced pre-B 70Z/3 and macrophage-like J774.2 cell lines overexpressing SIGIRR (70Z/3.SIGIRR-HA, J774.2/SIGIRR-HA, J774.2/SIGIRR-EYFP) as models to study SIGIRR function in murine hematopoietic cells (Chapter 4.1).

Stimulation of 70Z/3 cells with LPS did not show any significant difference in the term of activation status of 70Z/3.SIGIRR-HA cells compared to their negative controls (Figure 4.4, Figure 4.5). Since LPS-mediated surface expression of IgM in these cells requires the activation of NF-κB and the ectopically expressed SIGIRR failed to influenced LPS signalling in these cells, these data represented the first hint that SIGIRR might not necesserily function as a negative regulator of LPS signalling pathway. To further explore this notion, we also performed experiment with parental or SIGIRR-transduced macrophage-like cell line J774.2 and the levels of secreted TNF-α were measured in supernatants from LPS treated and non-treated samples. Contrary to recently publised reports, data showed that the presence of HA-tagged SIGIRR in J774.2 cells resulted not in downregulation, but in approximately 2-fold increase in TNF-α production in these cells compared to parental cells. Another J774.2 infectants expressing EYFP-tagged SIGIRR- did not show any difference (Figure 4.7). This dicrepancy between infectants could be caused by the presence of relatively large EYFP tag on SIGIRR C-terminus compared to a short HA tag, likely preventing SIGIRR-mediated regulation of TNF-α expression. Nevertheless, these experiments suggested that at least in immune cells of hematopoietic origin, SIGIRR is not involved in the negative regulation of IL1R/TLR signaling.

Interestingly, examining the expression levels of pro-inflammatory cytokines in primary macrophages isolated from wild-type and SIGIRR$^{-/-}$ mice on C57Bl/6J background, we found that detected basal levels of IL-1β, IL-6 and TNF-α are higher in the absence of SIGIRR (figure 4.8). This suggests that SIGIRR could represent a molecular brake negatively regulating the basal level of proinflammatory cytokine expression. This is so far undescribed and largely unexplored mechanism of SIGIRR function. Thus, our results failed to confirm that SIGIRR is involved in the negative regulation of activated TLR4 signalling, as documented for non-hematopoietic cells, and suggests that at least in macrophages it can act independently of TLR

signaling by setting the baseline of cytokine expression. While the molecular mechanism is not obvious, several possible reasons could serve as explanations of this observed effect.

We explore several avenues to explain this unexpected finding. We were looking for an evidence which should reveal physiologically-relevant difference in the „behavior“ of SIGIRR expressed in the hematopoietic (macrophages, pre-B cells) versus non-hematopoietic cells (epithelial cells, HEK cell line) and thus would provide a clue causing this interesting phenomenon. Initially, Western blot analysis of transduced cell confirmed that SIGIRR is overexpressed comparably in these two type of cells. Previous analyses showed that SIGIRR is a transmembrane protein (Zhang et al., 2011), so we were also interested in whether transduced cell line do not express defective version of SIGIRR, which is not be able to localize on cell membranes. Microscopical analysis of SIGIRR infectants confirmed that SIGIRR is present in the membrane compartment of both hematopoietic and non-hematopoietic cells, meaning that the discrepancy in SIGIRR function was not influenced by its defective localization within membranes (Figure 4.11 – 4.14).

Interestingly, we were able to detect that SIGIRR is present in various molecular forms in distinct cell lines (Figure 4.9 and 4.10). As it has been shown previously, SIGIRR could be glycosylated to various level and complexity (Lech et al., 2007) and thus, we speculated, that observed cell type-dependent functional differences of SIGIRR are likely due to their glycosylation pattern and distinct processing pathways specific for a given cell line and cell type. Indeed, and as revealed by a comparative Western blot analysis (figure 4.15), the molecular weight of glycosylated SIGIRR expressed in some macrophage cell line (~100-130kDA) and non-hematopoietic epithelial cells (~55-60kDA), are clearly distinct. That suggests that the pattern of glycosylation could, at least partly, account for functional differences of SIGIRR in cell-type specific manner.

We also explored the membrane distibution of various SIGIRR molecular forms. Using gel filtration we were able to fractionate lysates from cells expressing various form of SIGIRR according to the size of molecular complexes present across membranes and their three-dimensional structure. Our analysis revealed, that SIGIRR variants expressed in hematopoietic cells is preferentially present in the heavy-molecular weight fractions, which suggests that SIGIRR could be localized into lipid rafts (Figure 4.16). On the other hand, molecular form of SIGIRR expressed by epithelial HEK293T largely partitioned to membrane complexes with lower molecular weight. According to our best knowledge, these results are first to

characterize the membrane distibution of SIGIRR. Our finding also suggest that the membrane distribution of SIGIRR is probably dependent on the cell type and could influence the overall SIGIRR ability to negatively regulate signalling pathways triggered via IL-1R/TLRs.

So far, no ligand has been identified for SIGIRR. Nevertheless, recent findings suggest that SIGIRR could interact with IL-1F5, a member of IL-1 cytokine family. Together, these two molecules are supposed to regulate the inflammatory responses in the brain (Costelloe et al., 2008). As we are interested in searching for such SIGIRR inteacting partners, the next part of this thesis was devoted to the eshablishment of a model system suitable for identification and further study of possible SIGIRR binding partners. We employed the BWZ.36 reporter cell line, which contains a transgenic LacZ gene under the control of NFAT promotor element (Sanderson and Shastri, 1994). These cells were transduced with chimeric SIGIRR protein, which possesses CD3ζ intracellular signalling domain. When a putative ligand binds to the extracellular part of a protein of interest, CD3ζ mediates the activation of a downstream signalling cascade leading to the measurable expression of β-galactosidase. BWZ/SIGIRR infectants were subjected to a battery of test, which proved their ability to serve as a model suitable for identification the SIGIRR interacting partners (Figure 4.17 and 4.18). Thus, we established a reporter cell line suitable to identify putative SIGIRR ligands. This system will be further explored in SIGIRR research ongoing in our laboratory.

Results of experiments performed in our lab suggest that SIGIRR is highly expressed during murine early embryogenesis, especially in the stage E7.5 of the development (Vavrochová, 2010). According to these findings we proposed a hypothesis that SIGIRR could be involved in the maintenance of embryonic homeostasis. Thus, we designed a set of experiments in order to contribute to the understanding of this possible SIGIRR function. We found out that SIGIRR is extensively produced by both $CD11b^+$ $TLR2^+$ phagocytic and $CD11b^-$ $TLR2^-$ non-immune embryonic cells in E7.5 embryos, whereas at stages E8.5 and E9.5 of the development SIGIRR was predominantly expressed in $CD11b^+$ $TLR2^+$ phagocytic immune cells (Figure 4.19). Embryonic phagocytes are supposed to be the main embryonic source of TLRs and their adaptors (Vavrochová, 2010). However, at E7.5, both $CD11b^+TLR2^+$ and $CD11b^-TLR2^-$ cells expressed very low levels of these immune molecules. Thus, the high level of SIGIRR and minute amounts of TLRs and their adaptors expressed at E7.5 stronly argues, in addition to TLR-dependent, also for TLR-independent role of SIGIRR in early stages of murine embryonic development. One possible explanation could be that SIGIRR is involved in regulation of

inflammatory processes in developing embryos through its activation via IL-1F5. However, according our results, IL1-F5 is not expressed during embryogenesis (data not shown) and thus another mechanism of SIGIRR function has to be explored.

In following experiments we examined the SIGIRR$^{-/-}$ mouse model in more details. As previously documented, SIGIRR$^{-/-}$ mice showed increased susceptibility to various infections (Bozza et al., 2008; Garlanda et al., 2007a; Huang et al., 2006) and inflammation stimuli (Garlanda et al., 2004; Garlanda et al., 2007b; Xiao et al., 2007; Xiao et al., 2010), which indicates, that it is a „sentinel sensor" regulating the threshold for triggering the inflammatory processes. However, we did not observe that the breeding performance of this strain is somehow impaired (Figure 4.21). We also described the expression kinetics for selected TIR-containing protein during early embryogenesis in the wild-type and SIGIRR$^{-/-}$ mice. Our findings showed that the expression of these molecules differs markedly between the wild-type and SIGIRR$^{-/-}$ embryos and their expression levels are predominantly lower in the absence of SIGIRR (Figure 4.22). This could suggest that SIGIRR act as a molecular blocker preventing the inappropriate signalling triggered via IL-1Rs/TLRs in early stages of embryonic development. Results from experiment, whereby E7.5 and E8.5 wild-type of SIGIRR$^{-/-}$ embryonic cells were stimulated with LPS, showed that levels of TNF-α and IL-1β expression are higher for SIGIRR-negative cells (Figure 4.23). This correlates with our results from analogous experiment with peritoneal macrophages (Figure 4.8).

Thus, several interesting findings concerning SIGIRR protein were revealed during our experimental work which further contributed to the elucidation of its physiological role. In the near future, we would like to focus our effort on more detail characterization of distinct SIGIRR molecular forms and provide more comprehensive insight into their membrane distribution, i.e. whether its lipid rafts residency status changes upon activation of cells with TLR or IL-1 receptor ligands. We also intend to carry out a set of screening assays for possible SIGIRR ligands using BWZ/SIGIRR cell line which establishment was described in this thesis. Moreover, further analyses dedicated to elucidating the specific role of SIGIRR in embryos, such as additional stimulation experiments using SIGIRR$^{-/-}$ murine model, microarray comparison of embryonic SIGIRR^{+} and SIGIRR^{-} cells or experiments devoted to the characterization of newly established EEC.B cell line are planned to be performed. The detailed description of mechanisms how SIGIRR could mediate the regulation of signalling pathways triggered by receptors from IL-1R/TLR superfamily will advance our understand of

the whole network of tightly regulated, context-specific innate immune responses and in a long run might even improve the treatment of some diseases which are regulated by the same signaling network.

6 Conclusions

Our experiments demonstrated that SIGIRR does not inhibit the TLR4-mediated signalling in model hematopoietic cell lines and primary macrophages and highlights a possible importance of SIGIRR glycosylation, membrane distribution and its possible localization into lipid rafts for its function. We described the establishment of reporter BWZ/SIGIRR cell line which allows to identify and study the putative SIGIRR binding molecules. In addition, we showed that SIGIRR is expressed in early embryonic cells, both phagocytic $CD11b^{+}$ $TLR2^{+}$ cells and cells of non-hematopoietic origin. In this context we demonstrated that the expression profile of selected TIR-containing receptors and adaptors during early embryogenesis differs between the wild-type and $SIGIRR^{-/-}$ mice. Obtained results suggest that in macrophages, SIGIRR can downregulate the basal level of expression of pro-inflammatory cytokines in TLR-activation independent manner and thus prevent the inappropriately exacerbated cytokine responses mediated by receptors from IL-1R/TLR family. This is likely a more general phenomenon underpinning the negative regulatory role of SIGIRR in distinct cell types, including those present in developing embryos, where it also modulates the expression levels of other IL-1R/TLR family members and related signalling molecules. Further analyses towards revealing the precise mechanism of SIGIRR-mediated cell-type-dependent regulatory process are warranted.

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