CHARLES UNIVERSITY IN PRAGUE

FACULTY OF SCIENCE

DEPARTMENT OF CELL BIOLOGY

DIPLOMA THESIS

Expression and function of molecules of innate immune system in embryonic phagocytes

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Prague 2010

Affirmation

I hereby declare that I have written this thesis independently under supervision of RNDr. Dominik Filipp, Csc., with the use of listed literature.

Příbram 18. 8. 2010 Tereza Vavrochová

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Acknowledgments

I kindly thank my supervisor RNDr. Dominik Filipp, CSc., for his obliging attitude, valuable advice and criticism while I was writing this thesis. I also thank him for creating a very nice working environment and, last but not least, for understanding and help in solving personal problems.

My big thanks belong to all my colleagues, especially Jana Oujezdská, M.Sc., Aleš Neuwirth, M.Sc., Ondřej Ballek, M.Sc., and Adéla Broučková, M.Sc. who helped me during my experiments and whose patience and friendly support I regarded so much. I would also like to thank Zdeněk Cimburek for his FACS analysis performance, and my parents who supported me financially during my studies.

My special thanks belong to all my friends, especially to Martina Benešová, Viktorie Tothová and Petra Blovská for their help in critical periods of my life and for the wonderful days spent in Prague whether at school or not.

Last but not least I would like to thank my boyfriend Jiří Simonides for his love, care and appreciation, as well as graphic design, proofreading and assistance in translating this work.

Abstract

Phagocytes of an early embryo represent a mixture of myeloid lineages that differ from adult macrophages phenotypically, biochemically and by their origin. Recent studies suggested that there are at least three waves of macrophages populating the early embryo: a maternally-derived one and two waves of extraembryonic, YS-derived origin. In addition, the occurence of early embryonic phagocytes of undetermined origin in developing anterior head mesoderm in evolutionary distinct species is well documented. This origin-related heterogeneity among early embryonic phagocyte subpopulations coupled with the lack of specific markers makes it difficult to distinguish them phenotypically and study their potentially distinct physiological roles in early development.

The aim of this study is to identify and characterize a set of novel markers suitable for identification of embryonic phagocytes. Here, using qRT-PCR approach, we have established the kinetics of expression of Toll like receptors (TLRs) and their TIR-domain containing adaptors during early embryogenesis (E7.5-E12.5) and demonstrate that their major cellular source are indeed phagocytes. Using whole-mount embryo immunohistochemistry we also show that negative regulator of TLR signaling Sigirr is expressed during very early stages of mouse development. Approximately 0.7-1% of cells in E10.5 embryo are of macrophage phenotype characterized by surface coexpression of TLR2, TLR4, CD45, CD14, CD11b and F4/80 antigens. Using reciprocal matings between the wild type and transgenic mice ubiquitously expressing EGFP, in combination with TLR2 staining, we provide evidence that the early occuring, maternally-derived phagocytes are replaced by those of embryonic origin. The microarray analysis of $CD11b^{+}$ $TLR2^{+}$ cells isolated from E10.5 embryos has revealed upregulated expression of a set of genes which could be used for phenotypic characterization of these cells. These results are first to describe the regulated expression of TLRs and other immune-related molecules during mammalian embryogenesis and demonstrate the potential of TLRs to serve as markers for early embryonic phagocytes.

Key words

Embryonic phagocytes, Toll-like receptors, TIR-domain adaptors, phagocyte's markers.

Abstrakt (CZ)

Embryonální fagocyty představují směs myeloidních buněk, které se liší od linie dospělých makrofágů svým původem, fenotypem a biochemicky. Ukazuje se, že embryo je postupně osidlováno nejméně třemi populacemi makrofágů: první z nich pochází od matky a následující dvě z extraembryonálního žloutkového váčku. Prokázán je také výskyt raných embryonálních fagocytů u evolučně rozdílných druhů v hlavovém mezodermu, jejichž původ není doposud objasněn. Různorodost embryonálních fagocytů ve spojení s nedostatkem jejich specifických markerů neumožnuje rozlišovat tyto buňky na základě fenotypu a znesnadňuje studium jejich potenciálně rozdílných fyziologických funkcí v embryonálním vývoji. Cílem této práce je identifikovat nové markery vhodné pro fenotypovou charakterizaci embryonálních fagocytů. Pomocí analýzy qRT-PCR jsme stanovili kinetiku exprese receptorů skupiny Toll (TLRs) a jejich adaptérových proteinů obsahujících TIR doménu v průběhu rané embryogeneze (E7,5-E12,5) a prokázali jsme, že jsou produkovány fagocytickými buňkami. Pomocí imunohistochemie na celém embryu jsme taktéž prokázali, že Sigirr, negativní regulátor TLR signalizace, je rovnoměrně exprimován v embryonálních tkáních během raného vývoje. Přibližně 0,7-1% buněk v embryu starém 10,5 dne tvoří makrofágy charakterizované povrchovou expresí TLR2, TLR4, CD45, CD14, CD11b a F4/80. Pomocí recipročního křížení mezi divokým typem myší a transgenními myšmi, které exprimují EGFP, v kombinaci s barvením pomocí protilátky specifické pro TLR2, jsme prokázali, že rané embryonální fagocyty pocházející od matky jsou postupně nahrazovány fagocyty embryonálního původu. Mikročipová analýza $CD11b^{+}$ $TLR2^{+}$ buněk izolovaných z 10,5 dne starého embrya odhalila soubor genů, které mohou být využity k fenotypové charakterizaci těchto buněk. Předkládané výsledky poprvé popisují specifickou expresi TLRs a dalších molekul souvisejících s imunitou, potenciálně využitelných jako markery embryonálních fagocytů.

Klíčová slova

Embryonální fagocyty, receptory skupiny Toll, adaptéry obsahující doménu TIR, fagocytické markery.

Contents

List of abbreviations

AGM	aorta-gonad-mesonefros
APC	allophycocyanin-conjugated
AP-1	activator protein 1
Casc3	cancer susceptibility candidate 3
CD	cluster of differentiation
cDNA	complementary DNA
c-fms	colony-stimulating factor 1 receptor
CR	complement receptor
CSF-1	colony-stimulating factor 1
CSFR-1	colony-stimulating factor 1 receptor gene
ct	treshold cycle
DC	dendritic cell
DN	$CD11b^-$ $TLR2^-$ cells
DNA	deoxyribonucleic acid
DP	$CD11b^+$ $TLR2^+$ cells
dsDNA	double stranded DNA
DsRed	red fluorescence protein
E	day of embryonic development
EA	erythrocyte antibody
ECFP	enhanced cyan fluorescent protein
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGF-TM7	epidermal growth factor-seven transmembrane receptors
EMR1	EGF-module-containing mucin-like hormon receptor 1
ER	endoplasmatic reticulum
E_{ref}	real-time PCR efficiency of a reference gene transcript
E_{target}	real-time PCR efficiency of target gene transcript
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HPP-CFC	high proliferative potential colony-forming cells

HRP	horseradish peroxidase
HSC	haematopoietic stem cell
HSC/RUs	haematopoietic stem cells/long term repopulating units
IFN	interferon
Ig	immunoglobulin
IKK	IkappaB kinase complex
IL	interleukine
IL-RAcP	IL-1 receptor accessory protein
IL-1R	interleukin 1 receptor
IRAK	IL-1R-associated kinase
IRF	interferon regulatory factor
LAL-assay	limulus amoebocyte lysate assay
LB	lysogeny broth
LBP	LPS binding protein
LPS	lipopolysaccharide
LRR	leucine-rich repeat
M1	classically activated macrophages
M2	alternatively activated macrophages
Mac-1	macrophage-1 antigen
Mal	MyD88 adaptor-like protein
MAP	mitogen-activated protein
MARCO	macrophage receptor with collagenous structure
MD-2	myeloid differentiation protein 2
MHC	major histocompatibility complex
Mitf	microphthalmia transcription factor
MPS	mononuclear phagocyte system
MR	mannose receptor
mRNA	messenger RNA
MyD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor-kappa B
NK	natural killer
O/N	over night
PAMP	pathogen- associated molecular pattern
PAS	paraaortic splanchnopleura

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
Pecy7	phycoerythrin conjugated with cyanine dye Cy7
PerCP	peridinin-chlorophyll protein
PFA	paraformaldehyde
pM	peritoneal macrophage
PRR	pathogen recognition receptor
psc	post coitum
qRT-PCR	quantitative real-time polymerase chain reaction
RES	reticuloendothelial system
RIN	RNA integrity number
RIP	receptor interacting protein
RNA	ribonucleic acid
RNAi	RNA interference
RT	room temperature
SARM	sterile alpha and armadillo-motif-containing protein
sh	short hairpin
Sigirr	single immunoglobulin IL1-R-related molecule
siRNA	small interfering RNA
S.O.C.	super optimal broth with catabolite repression
SR	scavenger receptor
TAK	TGF-beta activated kinase
TAMs	tumor-associated macrophages
TBK1	TANK-binding kinase 1
tg	transgene
TGF	transforming growth factor
T_h	helper T- cell
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor
TRAM	Trif-related adaptor molecule

TRIF	TIR-domain-containing adaptor inducing interferon-beta
Ubc13	ubiquitin-conjugating enzyme 13
Uev1A	ubiquitin-conjugating enzyme E2 variant 1 isoform A
WT	wild type
YS	yolk sac

1. Introduction

Immune system represents host defenses directed towards distinguishing and eliminating foreign, pathogenic or dangerous structures of exogenous or endogenous origin. Immune mechanisms are divided in two main classes which are functionally linked to generate a robust immune responses: innate (antigen-nonspecific) and adaptive (antigen-specific). A seminal discovery of mammalian Toll-like receptors (TLRs) (1) functioning as Pattern Recognition Receptors (PRRs) led to an enormous expansion of innate immunity research (2-4).

The existence of PRRs as a major effector class of innate immune receptors was predicted by Charlie Janeway in 1989 when he formulated the theory of immune recognition of *infectious-non-self* (5). He suggested that PRRs recognize highly conserved microbial structures, so called Pathogen Associated Molecular Patterns (PAMPs), which are shared by large groups of pathogens and in many cases are essential for microbial survival. The fact that PAMPs are absent from the host leads to a perfect ability of PRRs to distinguish self from non-self structures. Interestingly, it seems that some PRRs can be stimulated by endogenous ligands released during the stress or necrotic tissue damage. Moreover, several receptors capable of interaction with both endogenous and exogenous ligands have been already characterized, for instance CR3, CD14, SR- A I/II, MARCO, MR C-lectin domains, CD36 and well studied group of TLRs (6).

TLRs are of great clinical importance, as many inflammatory processes, both infectious and sterile, seems to depend on TLR signaling (7). While expected role of TLRs in distinguishing self-ligands has not been conclusively proven, their involvement in CD36-mediated sterile inflammation was recently demonstrated (8). Up till now, the TLRs have been studied in adult phagocytes only, but the recent experiments conducted in our laboratory revealed that TLRs are also expressed in early stages of embryonic development (manuscript in preparation). These data suggest that recognition of endogenous ligands by TLRs in this pathogen-free environment may critically influence the embryonic homeostasis and developmental tissue remodeling. Revealing the nature of endogenous ligands could represent a critical step towards finding a cure for diseases caused by a chronic sterile inflammatory process (7).

The aim of the first part of this thesis is to summarize the current literature knowledge about embryonic phagocytes, their development, origin and function as well

as to give an overview surrounding the physiology, structure, ligands and signaling of TLRs. However, the main goal is to present our current data demonstrating the physiological overlap between these two areas of research. Specifically, we will show the kinetics of expression of TLRs and other TIR-domain containing immune molecules during early embryogenesis and identify embryonic phagocytes as their cellular source. We will also show that reciprocal mating between the wild type and transgenic mice ubiquitously expressing EGFP, in combination with TLR2 antibody staining, is a suitable approach to study the kinetics of appearance of embryonic and maternal phagocytes. Using microarray analysis we also revealed upregulated expression of several genes in embryonic phagocytes in comparison to embryonic non-phagocytic cells and peritoneal macrophages that could be potentially used for phenotypic distinction among embryonic phagocyte subpopulation. Finally, we describe the methodological approach to study the role of newly identified genes specifically expressed in embryonic phagocytes using siRNA knockdown system in whole embryos.

2. Literature Review

2.1. Elie Metchnikoff: The father of cellular innate immunity

Phagocytosis, the engulfment of solid particles by cell membrane to form an internal phagosom or "food vacuole", is evolutionary highly conserved process that evolved before the appearance of multicellularity (9). The term "phagocyte" derived from the Greek *phagein* (to eat) and *kytos* (cell) was proposed by Russian embryologist Elie Metchnikoff (1845-1916), who was the first to discover phagocytosis as a cell-mediated immune response to foreign particles (10).

Metchnikoff started his career with investigating the microscopic structure and embryology of simple marine organism. As early as 1866, he made his first observation of intracellular engulfment of nutrients by cells in a member of the *Turbelaria*, simple invertebrates without a fully developed intestine (11). However, it took him next 16 years, during which he studied a great diversity of organisms, to perform the celebrated experiment with starfish larvae, providing the very first evidence for a dual role of phagocytosis. Consistent with Darwinian theory of

evolution, he immediately foresaw that in primitive organisms such as amoebae, phagocytosis is associated with nutritive function, but in more complex animals it plays an important role in tissue remodeling, wound healing, inflammation and host defence against pathogens (10). Metchnikoff observed two types of phagocytes: microphages (nowadays called granulocytes) involved in defence against acute bacterial infection and macrophages involved in defence against chronic bacterial infection (11).

In spite of Metchnikoff´s research, the last decades of the 19th century were dominated by research physicians such as Emil von Behring, the discoverer of diphtheria antitoxin and Paul Ehrlich, who were focused on soluble factors in the blood such as complement and antibody to account for immune destruction (12). Metchnikoff's view that phagocytes are the main if not the only line of defence against microorganisms led into a conflict with humoralists who opposed him on the basis of specific mechanism (13). Metchnikoff examined this mechanism exemplified in the process of enhanced phagocytosis of bacteria *in vivo* after vaccination of different animals or *in vitro* after mixing with immune serum, but he fail to explain it (14).

The role of antibody and complement remained controversial until this conundrum was resolved by Almroth Wright in 1903 when he defined opsonins as a functional bridge between phagocytosis and humoral factors (13). In 1908 Elie Metchnikoff and Paul Ehrlich shared the Nobel Prize for their discoveries of phagocytes-mediated cellular immunity and antibody-mediated humoral immunity, respectively (15).

2.2. Phagocyte cells

Elie Metchnikoff´s distinction between microphages and macrophages was the first step in dissecting the phagocyte heterogeneity and lineage differentiation. Nowadays, based on their morphology, receptor repertoire and enzymatic features, we recognize two main types of "professional" phagocytic cells: the polymorphonuclear phagocytes represented by neutrophilic, eosinophilic and basophilic granulocytes and mononuclear phagocytes represented by monocytes, and tissue macrophages. The special type of mononuclear phagocytic cells are dendritic cells, which play a key role

in processing and presentation of antigens in the context of MHC molecules and thus link innate and adaptive immune responses (16).

2.2.1. Historical classification of mononuclear phagocytes

Metchnikoff recognized that mononuclear phagocytes residing in various organs and tissue macrophages are closely related and for this reason he introduced the term "macrophage system" (17). In 1924, the macrophage system was elaborated by Aschoff into the Reticuloendothelial System (RES) (18). RES system included not only monocytes and macrophages, but also both phagocytic reticuloendothelial cells lining blood and lymph sinuses and reticular cells of lymphoid organs. Histyocytes and splenocytes were also included into this system. Despite the fact that Aschoff's reticuloendothelial system was criticised and alternative terms and definitions were proposed, e. g. "reticulohistiocyte system" (17), it was well accepted. However, gradual accumulation of data resulted in the need to revise the classification of phagocytic mononuclear cells. Accordingly, in 1969 the committee of The conference on mononuclear phagocytes held in Leiden, proposed the term "Mononuclear Phagocyte System" (MPS) (17). Thus, the RES system which classifies the phagocytic cells by their function was effectively replaced by MPS emphasizing rather their origin and developmental pattern (see chapter 2.2.2).

However, since the beginning of 20th century, Dantschakoff showed that macrophages exist in the yolk sac (YS) during early ontogeny, long before the initiation of bone marrow haematopoiesis (18). Then, sixty years later, Moore and Metcalf determined that macrophages were present during YS haematopoiesis (19) and in 1972 Cline and Moore extended the MPS model by demonstrating that the YS is the site of origin of macrophage precursors, that differentiate in the fetal liver and bone marrow through the monocytic pathway (20). However, as described in following section, continuous influx of new data required further modifications of this system.

2.2.2. The mononuclear phagocyte system

Mononuclear phagocyte system comprises circulating blood monocytes, resident tissue macrophages and their precursor cells in bone marrow – promonocytes and monoblasts (17). MPS unifies these group of phagocytic cells based on their origin, kinetics of development, similarities in the morphology and function. As mentioned above, it was conceptually extended by the emergence of yolk sac macrophages originally thought to undergo their development through a monocytic stage (20). Also, MPS originally proposed that the number of cell divisions occuring in the stage of precursor cells and the local proliferation of mature macrophages contributes very little to the maintenance of resident tissue macrophage populations. The basic features of the MPS are summarized in figure 2.1.

Nowadays, there are several factual reasons to revise the original concept of MPS. The first one is the local proliferation of tissue macrophages and their self-renewal. Recent studies indicate that although monocytes can be precursors for replenishment of tissue-resident macrophage population, the local proliferation of tissue macrophages in adults is significant and has an important role in the renewal and maintenance of these cells (21). Secondly, the ability of haematopoietic stem cells to serve as precursors not only of MPS but also of other cell type including neurons, hepatocytes and renal epithelial cells has been demonstrated (22-26). Furthermore, it has been shown that mature blood monocytes and inflammatory macrophages can transform into vascular elements (23, 27-32). In addition, based on the expression profile in transgenic mice of EGFP construct under CSF-1 receptor promoter, the morphological, phenotypic and functional differences between mononuclear phagocytes and dendritic cells have been recently challenged (33-35). Importantly, macrophages that appear early in the embryo are distinct from those of adults as they do not follow the monocyte pathway (36, 37). Because embryonic phagocytes represent the main subject of my thesis, I will describe their development in more details in the following section (section 2.3).

A major advantage in characterization of MPS provided surface protein markers defined by monoclonal antibodies. One of the most studied antibodies was F4/80 which recognize a 160 kDa cell surface glycoprotein that is a member of the EGF-TM7 family of proteins which shares 68% overall amino acid identity with

human EGF module containing mucin like hormone receptor 1 (EMR1) (38). Although the F4/80 antigen is present on a wide range of mature tissue macrophages, the expression is difficult to detect in lung macrophages and macrophages of lymphoid organs (39) as well as in the dermis (40). Therefore, Hume and colleagues began to study an alternative surface marker, receptor for macrophage colony-stimulating factor (CSFR-1), which is expressed on macrophages and trophoblast cell lineages. The CSFR-1 encoded by *c-fms* proto-oncogene is a tyrosine-kinase receptor binding the macrophage colony-stimulating factor (CSF-1), the lineage-specific growth factor playing a key role in macrophage proliferation, differentiation and survival (33, 41). In 2003, they constructed the *c-fms*-EGFP transgene reporter as a model for studying the mononuclear phagocyte development and function (35).

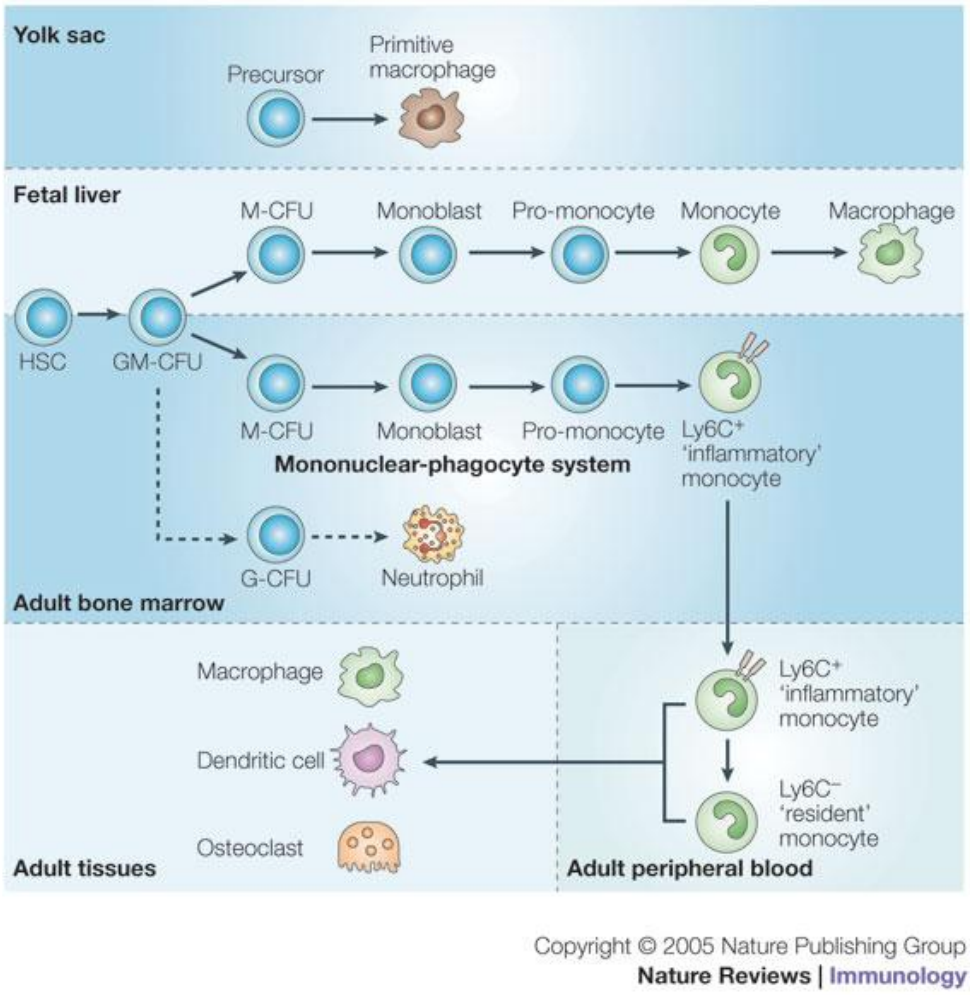


Figure 2.1. Recent model of the mononuclear phagocyte system (Taken from 21). The macrophage-like cells from the yolk sac are distinct from adult macrophages originating in the fetal liver and later in bone marrow (for further details, see chapter 2.3).

2.3. Embryonic phagocytes

This chapter provides an overview of the current knowledge on this unique embryonic cell lineage. To describe their origin and the main developmental paths in more detail, it is important to put these cells into the context of general embryonic haematopoiesis.

2.3.1. Embryonic haematopoiesis

Haematopoiesis is a genetically controlled process where all cellular components of the blood are derived from multipotent and selfrenewing precursors – haematopoietic stem cells (HSCs). Further development of lymphoid and myeloid progenitors from HSCs is tightly regulated by the production of specific growth and differentiation factors in the context of appropriate bone-marrow microenvironment. Lymphoid progenitors then differentiate into NK-, B- or T- cells, whereas myeloid progenitors give rise to erythrocytes, megakaryocytes, mast cells, eosinophils, neutrophils and macrophages (42).

During development of the mammalian embryo, HSCs and their progeny are found at several sites: the yolk sac (YS) (19), paraaortic splanchnopleura (PAS)/aorta-gonad-mesonephros (AGM) region (43-47), liver (48, 49), spleen and thymus (50). In the mouse embryo, liver is considered to be the principal site for haematopoietic expansion and differentiation during fetal life. However, neither liver nor spleen and thymus, which are structurally formed during relatively late stages of gestation, can generate haematopoietic cells *de novo.* Rather, these organs are colonized by HSCs and their progenitor cells generated at earlier stages of embryonic development at haematopoetic sites such as YS and AGM (51).

The extraembryonic YS is the site where the earliest haematopoietic activity is observed. Blood islands are detected in this structure on 7^{th} day of gestation (52) and haematopoiesis at this site progresses through day 13 of the mouse development, when this structure begins to degenerate (51). Mechanism of blood island formation is depicted in figure 2.2. HSCs, derived from the YS prior to establishment of embryonic circulation (E8.5), were originally considered to be the primordial embryonic pluripotent precursors migrating in later stages of development to the fetal liver and

bone marrow (19, 49). However, it turns out, that these cells are incapable to differentiate into lymphoid lineages *in vitro* and they don't possess the long-term reconstitution potential (43, 45). This implies that the YS represents a site of so called primitive haematopoesis where erythrocytes and macrophages of primitive phenotype are produced (53).

However, experiments analysing an emergence of haematopoietic progenitors in mouse embryos came with suprising findings: while the production of primitive erythroid progenitors terminates shortly, definitive erythroid progenitors distinguished by the expression of adult-restricted hemoglobin gene, as well as myeloid and megakaryocytic cells appears in the yolk sac at E8.25, prior to the onset of circulation (54, 55). This concept of the YS-derived first wave of definitive haematopoietic activity is supported by evidence that cells with high proliferative potential colony-forming cells (HPP-CFCs) appear in the YS at early somite pair stage (E8.25) (56). However, the nature of cellular and molecular mechanisms regulating and coordinating the onset of primitive and the first wave of definitive haematopoeisis in the YS structure remains enigmatic.

In addition, it has been shown that at later stages the YS becomes competent to generate and/or expand definitive haematopoietic stem cells/long term repopulating units (HSC/RUs) of YS and/or AGM origin (see below), able to colonize the developing liver with cells migrating directly from AGM (figure 2.3) (57).

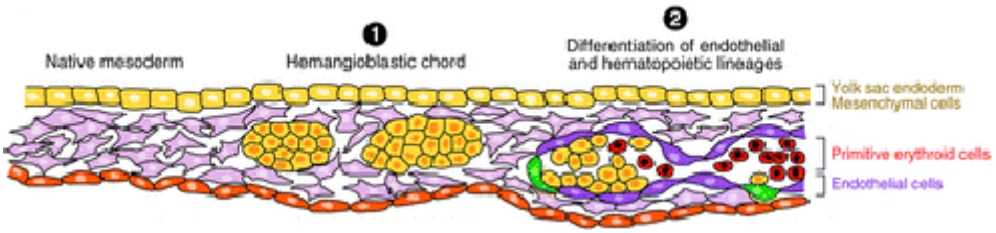


Figure 2.2. Formation of the yolk sac blood islands (Taken from 58). Aggregates of mesodermal cells appear at E7 in the mouse embryo forming the extraembryonic blood islands. Endothelial cells differentiate at the edges of aggregates, while the inner part of aggregates comprise erythrocytes which are called "primitive" because they never reach the final enucleated stage *in situ*.

The second wave of HSCs production is occuring in the intraembryonic paraaortic compartment, called Aorta-Gonad-Mesonephros (AGM), the site where the aorta, gonads and mesonephros begin to develop (45). However, there is no

differentiation of haematopoietic cells in the region of AGM. It is therefore thought, that these intraembryonic cells are released into the circulation to first colonize fetal liver at around E10 and later the spleen and bone marrow, where they further expand and differentiate (59). These marrow-engrafting stem cells give rise to lymphoid and definitive erythromyeloid progenitors which are necessary for sustained generation of blood cells during the life of an individual.

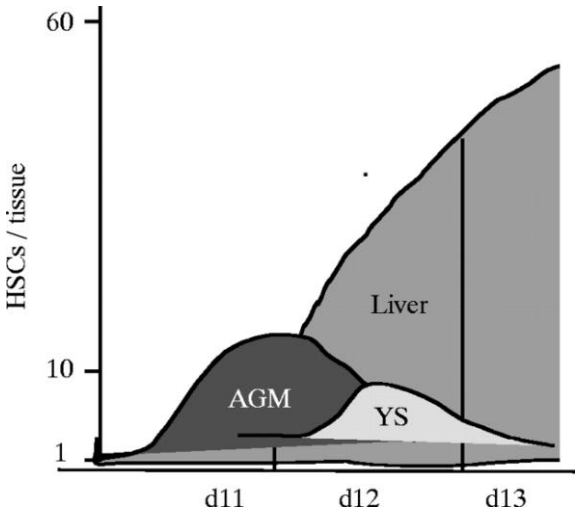


Figure 2.3. Schematic representation of fetal liver colonization by HSC/RUs (Taken from 57). Both AGM and YS HSCs contribute to adult haematopoiesis in two subsequent waves that peaks on E10-11 and E12, respectively. The number of HSC/RUs in the liver are measured *in vivo*, whereas the number of HSC/RUs in AGM and YS indicates its amount generated *in vitro*.

Furthermore, there are studies suggesting that the fetal placenta functions as yet another haematopoietic with an important role in HSCs development. Mouse placenta develops by fusion of the allantois to the ectoplacental cone and HSC activity is observed here in parallel with AGM region (60). Although the first evidence of haematopoietic activity of placenta was documented in 60's of 20th century (61, 62), only recently published data from experiments on an avian model revived the interest in this topic (63, 64). Alvarez-Silva with his colleagues were the first to demonstrate that before the liver colonization, the murine placenta contains a relatively large number of multi-potential clonogenic progenitors (65). Their follow-up *in vivo* studies showed the presence of pluripotent HSCs in a placenta with the capacity of self renewal and able to repopulate the entire haematopoietic system of adult hosts that were exposed to radiation (66, 67). A revised model of early haematopoiesis highlighting embryonic tissues and paths of colonization by progenitor cells is depicted in figure 2.4.

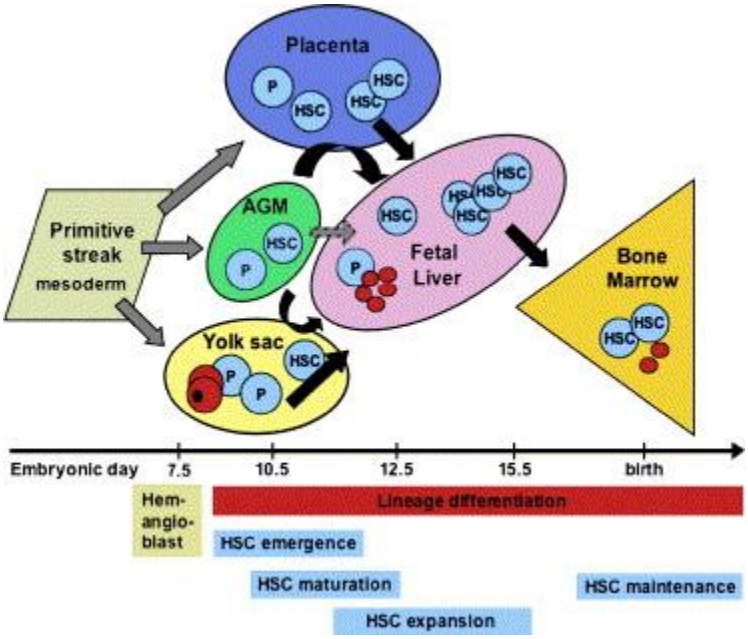


Figure 2.4. The sequence of haematopoietic colonization events in mouse development (Taken from 60). The revised model of early haematopoiesis proposes several pre-liver haematopoietic anatomical sites participating in fetal HSC development: the primitive streak, which is a source of mesoderm that give rise to the hemangioblast (common precursor for haematopoietic and endothelial cells), extraembryonic yolk sac (YS), intraembryonic AGM region and placenta, a putative novel site of HSCs production and supportive niche for their maturation/expansion. While it has been shown that progenitor cells originally derived from the yolk sac are unable to generate lymphoid progenitors, AGM could give rise to all haematopoietic lineages (43). However, after the blood connection between the YS and embryo is established at E8.5, the YS then can produce *in vitro* both myeloid and lymphoid lineage, due to being colonized by the AGM derived progenitor cells (43). While the migration of progenitor cells from the YS- and AGM into the fetal liver at day 9.5-10 and 10-10.5, respectively, has been well documented (45, 68), it was thought that only AGM-derived progenitor cells contribute to the definitive haematopoiesis (45). Recent findings revealed that also YS (69) and placenta (60) contribute to adult haematopoiesis (see the text for details). Arrows indicates possible sequential colonization of haematopoietic sites by progenitors and HSCs: the gray arrows indicate routes of HSCs through embryonic tissues before the establishment of circulation; black arrows represent HSCs migration through major vascular connection between the organs; arrow with a dotted line represent hypothesized extravascular migration between the AGM and fetal liver.

2.3.2. Origin of phagocytes in developing embryo

According to a general scheme of mammalian embryonic haematopoiesis, the yolk sac and fetal liver are the main sites for phagocyte development. Although the existence of macrophages in the yolk sac has been reported as early as 1908 (18), more complex analysis – describing development and differentiation of these cells in the mouse embryo, were conducted by Naito et al. in the last decade of the 20th century (36, 37). Using the surface marker F4/80, characteristic morphological features and ultrastructural peroxidase cytochemistry, these authors defined two different lineages of macrophages termed as "primitive" and "monocyte-derived". Primitive macrophages are detected in blood islands of the yolk sac at around day 9 of gestation (37). These round cells contain a large euchromatic nucleus, they lack peroxidase activity, are positive for F4/80 (36, 37) and negative for the presence of S100 proteins, S100A8 and S100A9 (70). Differentiation of these cells into fetal macrophages, characterized by clearly distinguishable intracellular organelles and extended filopodia begins on day 10 (36, 37). Afterwards, they spread into a mesenchymal layer and later to the extra-embryonic coelom where they mature. Finally, actively proliferating fetal macrophages migrate via blood vessels and colonize various embryonic tissues (37).

Promonocytes and monocytes are detected as a minor population in the blood islands of the YS only one day after development of fetal macrophages, on day 11 (71). Compared with the primitive or fetal macrophages, promonocytes/monocytes have peroxidase activity, never express mature macrophage marker F4/80, lack erythrocyte antibody (EA)-rosette formation and immune phagocytosis (table 2.1). Observed differences in these basic phenotypic markers led Naito and his colleagues to conclude that primitive/fetal macrophages represents a separate lineage originating from haematopoietic cell in YS, prior to the appearance of monocytes (figure 2.5).

Table 2.1. Ultrastructural, cytochemical, immunoelectron microscopic and functional features of primitive/fetal macrophages and yolk sac promonocytes/monocytes (Taken from 37).

	Primitive macrophage	Fetal macrophage	Yolk sac promonocyte	Yolk sac monocyte
Nucleus	Round, indented	Indented	Indented	Reniform
Nucleolus	Large	Medium-sized	Large	Medium-sized
Chromatin	Euchromatic	Heterochromatic	Heterochromatic	Heterochromatic
N/C ratio	≧ 1	< 1	< 1	< 1
rER	Few	+	+ +	+ +
Golgi	Poor	+	+ +	+
Lysosome	−	+	+	+
Phagosome	−	+	−	−
Pinocytic vesicle	Few	+	+	+
Polyribosome	+ +	+	+	+
Filopodia	+	+	−	−
Microvilli	+	+	+	+
Pseudopodia	−	+	−	−
Peroxidase				
Nuclear envelope	−	−	+	−
rER	−	−	+	−
Golgi	−	−	+	−
Granule	−	−	+	+
F4/80	+	+	−	−
EA rosette formation	+	+	−	−
Immune phagocytosis	+	+	−	−
Latex phgocytosis	Slight	+	−	−

N/C ratio, nucleocytoplasmic ratio; rER, rough endoplasmic reticula; −, absent; +, present; + +, abundant.

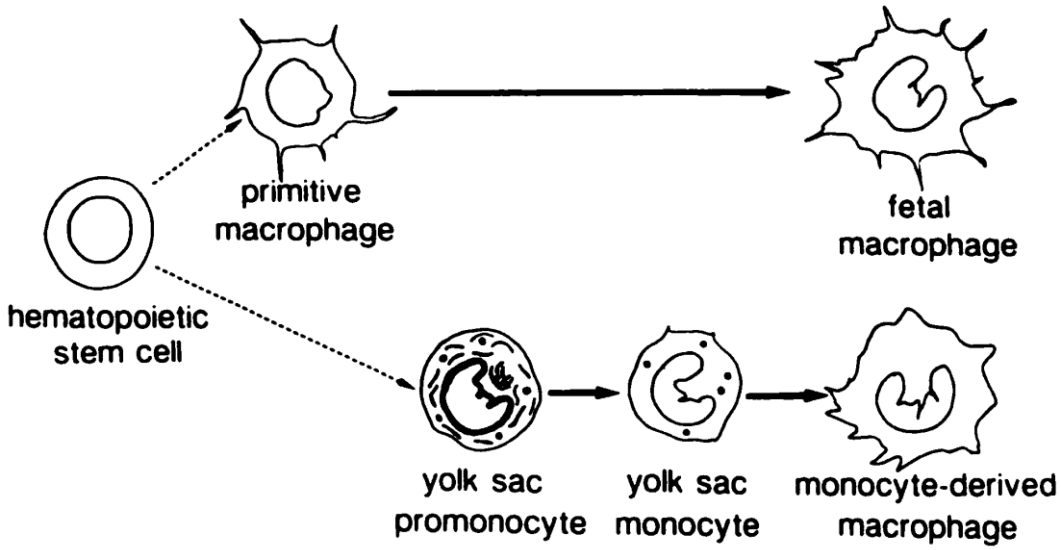


Figure 2.5. A postulated schematic diagram of the macrophage development in the mouse yolk sac haematopoiesis (Taken from 37).

The developmental model of two separated and independent macrophages lineages (primitive/fetal and monocytic/adult) found its support in studies involving phylogenetically distinct classes of animal embryos such as from drosophila, zebrafish, frog, avian and rodent. As a general rule, embryonic phagocytes were found in the anterior head mesoderm prior to the appearance of yolk sac phagocytes and establishment of circulation (72-77). Because of evolutionary conserved location of appearance of embryonic phagocytes, the head mesenchyme is also considered as a potential site of their origin (18). While both head mesoderm- and yolk sac-residing phagocytes remain extravascular and are able to proliferate, the relationship between these two populations has not been firmly established. Specifically, while a contribution of the YS to the pool of early avian macrophages found in head structures was suggested, migration routes as well as the nature of their immediate YS-derived precursor(s) are unknown (75, 78). Moreover, whether these embryonic phagocytes persist in the adulthood and form a self-renewable source of tissue macrophages, remains also uncertain too.

2.3.2.1. New insight into origin of phagocytes in developing embryo

In 2005, Bertrand et al. characterized the existence of three different embryonic YS-derived macrophage populations through their phenotypic and differentiation potential analysis in CX_3CR1^{GFP} transgenic embryos (E7.5-E10.5) (69). CX_3CR1 is a fractalkine receptor expressed by monocytes, tissue macrophages and dendritic cells (79), and its targeted replacement by EGFP allows reliable detection of myeloid lineages.

The first wave of macrophages detected in embryos obtained from the cross between CX_3CR1^{GFP} male and wild-type female, was considered as maternally derived, as these cells were negative for EGFP. These mature macrophages (expressing Mac-1, F4/80, CD45) are detected in the YS mesoderm and adjacent structures on day 7.5 to 9.5 and are considered to be completely replaced by macrophage populations appearing later in development. Maternally-derived macrophages are thought to play a scavenger role before the establishment of embryo´s own phagocyte population.

The second wave of macrophages occurs slightly later (E8.5) and seems to originate from monopotent precursors in the YS displaying $CD45^-$/c-Kit^+ phenotype which differentiate into CX_3CR1^+ $F4/80^+$ macrophages. Authors suggest that these two populations correspond to those which Naito with colleagues called as "primitive macrophages". They considered $F4/80^+$ cells as maternally-derived, and proliferating macrophages as those derived from macrophage restricted precursors.

Finally, the third wave appears in the YS shortly after the second one and its precursors display erythromyeloid potential, what is the only important difference of the two. Both second and third wave of YS-derived precursors differentiate to adult macrophage precursors and thus seem to contribute to the process of definitive haematopoiesis. However, whether the YS-derived macrophages also contribute to a pool of resident macrophages, remains to be evaluated. These new findings concerning the origin of embryonic phagocytes are summarized in the figure 2.6.

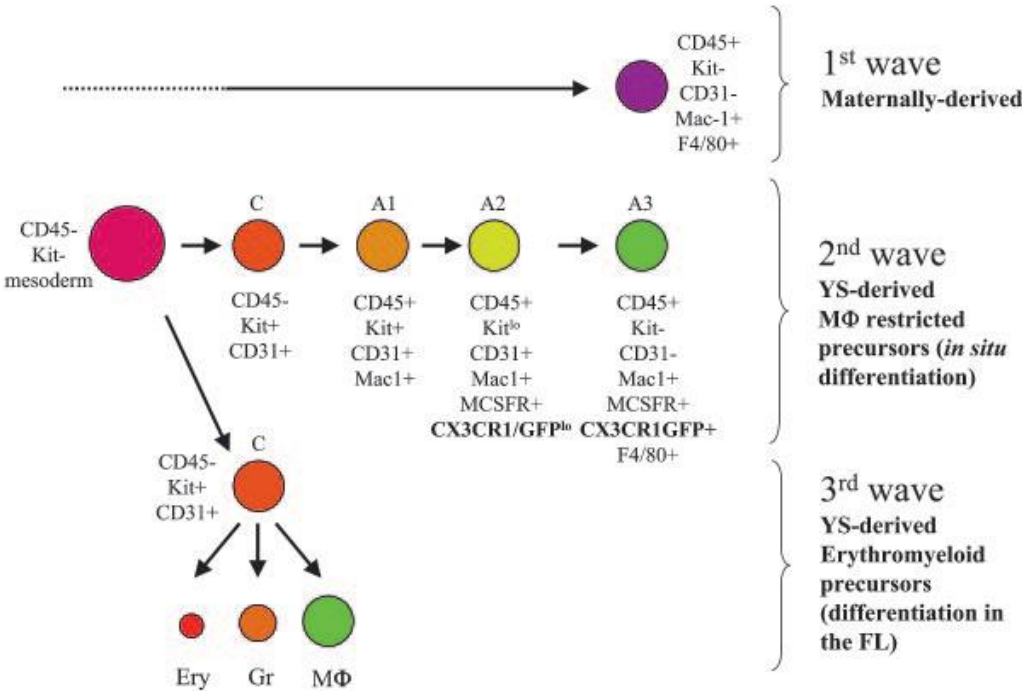


Figure 2.6. Three waves of YS macrophage populations characterized in transgenic CX_3CR1^{GFP} embryos by their phenotype, origin, differentiation potential and lineage relationships (Taken from 69).

2.3.3. Function of embryonic macrophages

The similarity in the spatio-temporal kinetics of appearance of early embryonic macrophages in various species across animal kingdom attests to their important and evolutionary conserved role during embryogenesis and development. There's a general consensus that the main function of embryonic phagocytes is to eliminate apoptotic, effete and senile cells. Their essential role in tissue remodelling is also well documented (18). This fits with the Metchnikoff's original idea positioning phagocytes as the cells central for embryonic homeostasis. This function is delivered via the surveillance of embryonic development through the process of sterile inflammation (13). It is important to emphasize, that the involvement of macrophages in the maintenance of embryonic homeostasis is independent of their role in immune responses (80). This model also supports the notion that the presence of phagocytes in evolutionary lower organisms that lack elaborate immune system observed in vertebrates and mammals is crucial for early stages of ontogenesis. The very same notion applies for vertebrate species at early developmental stages when the immune system is not fully developed.

Another biologically important attribute of macrophages is their secretory plasticity. Macrophages are well known to produce and secrete a multitude of immune, neuroendocrine, reactive oxygen/nitrate mediators and growth factors that can regulate, and affect the proliferation, differentiation and other physiological functions of adjacent as well as distant cells. In accordance with the site and time of their appearance during development, macrophages are able to secrete cytokines supporting haematopoiesis such as erythropoietin (81), IFNα (82), TGFβ (83) and trombospondine (84), cytokines important for vascularization of embryonic tissues (73) and components of basement membranes like proteoglycans, laminin, tiggrin, glutactin and collagen (85, 86). In addition, microchip analyses utilizing RNA derived from macrophages activated with well-defined stimuli showed that a plethora of other humoral factor can be synthesize and secreted into surrounding environment (87).

At present time, based on functional considerations, three distinct populations of macrophages are recognized: classically activated (participating in host defence, originally designated M1), wound-healing (M2) and regulatory macrophages (80). Each of these populations secretes distinct set of cytokines that underpin their physiological functions. However, at present, we have only a very limited amount of

data pertinent to their appearance and secretion profiles, synergistic effect of cytokine co-production as well as the nature of stimuli and signaling pathways controlling their production in time and environment-dependent fashion in early E7.5-11.5 embryo (80, 87).

Interestingly, and in this context, a recent microarray analysis of $EGFP^+$ macrophages isolated from embryonic tissues revealed significant similarities with tumor-associated macrophages (TAMs) (87). Both embryonic macrophages (E15.5) and TAMs express number of M2-associated genes which are involved in wound-healing and angiogenesis, suppress inflammation and/or support the scavenger role (21). Because of the likehood that TAMs and embryonic macrophages share trophic mechanism, the authors of this study suggested that understanding the role of macrophages in embryonic development can be employed to therapeutic applications in malignancy (87).

In addition, experiments conducted on zebrafish embryos showed that early embryonic macrophages can eradicate not only apoptotic cells but also both the gram-negative and gram-positive bacteria before the appearance of any lymphocytes and thus can protect the embryos against infection (73).

2.3.4. Visualizing of embryonic macrophages

Detection and distinction of macrophage subpopulations in early stages of development is based on their morphology, enzymology and surface markers (table 2.1 and table 2.2). Among the best established markers for phenotypic characterization of mouse YS phagocytes is the mRNA or protein product of the CSF-1 receptor gene *Csf1r (c-fms)* (76), Mac-1 integrin (CD11b) (88), F4/80 (89), mannose receptor (90) and microphthalmia transcription factor *(mitf)* gene product (91). Two chemotactic factors, S100A8 (MRP-8) and S100A9 (MRP-14) are markers for the onset of definitive myelopoiesis in the liver so they distinguish definitive embryonic macrophages from YS-derived macrophages where these genes are never expressed (91-93).

Table 2.2. Macrophage markers used for describing of embryonic phagocytes in various species (Taken from 70).

	Marker	Drosophila	Fish	Xenopus	Birds	Rodents	Human
Receptors	RMI					+	
	F4/80					+	
	Mac-1					+	
	Mannose receptor					+	
	c-fms					+	
	Scavenger receptor	+					
	crq receptor	+					
	SR-CI					+	
	ABC transporter					+	
	DEP-1					+	+
	Lectin binding						+
	CD68						
Enzymes	Lysozyme				+	+	+
	PU.1					+	
Transcription factors	Mitf					+	
	glide/gcm	+					
	L-plastin		+				
Other	Protein-X	+					
	MDP-1	+					
	WLC 15		+				
	XL-1			+			

In recent years, an advanced approach for detection of embryonic macrophages by creating a transgene mice with fluorescent protein expressed under lineage-specific promoters, has been used. In this way, transgenic lineage labels allow not only to observe macrophages in live organism (figures 2.7. and 2.8) but also to discern embryonic lineage of macrophages from maternally-derived macrophages in embryos obtained from crossing transgene males with wild type (WT) females. Table 2.3 summarizes selected transgenic markers of embryonic phagocytes in various species.

Table 2.3. Transgenic markers using for visualization of embryonic phagocytes (Taken from 94).

	Promoter/gene	Marker	Expression specificity
Fruitfly Drosophila *melanogaster*	Collagen IV (Cg-lacZ, Cg-Gal4)	lacZ, nEGFP[a]	Phagocytosing plasmatocytes embryonic/larval hemocytes,
	Hemolectin (hml-Gal4)	nEGFP	Embryonic/larval hemocytes
	Glial cell missing(gcm) (gcm-lacZ, gcm-Gal4)	lacZ, nEGFP	Early plasmatocytes, some glial cells
	Hemese (hemese-Gal4)	nEGFP	Embryonic, including early, plasmatocytes
Zebrafish Danio rerio	pu.1(spi1) (spi1-EGFP)	EGFP	Embryonic phagocytes, myeloid lineage (incl. granulocytes)
	Lysozyme C (lysC-EGFP,DsRed2)	EGFP, DsRed2	Embryonic phagocytes, myeloid lineage (incl. granulocytes)
Clawed frog *Xenopus laevis*	Lurp1 (Lurp1-EGFP,DsRed1)	EGFP, DsRed1	Embryonic phagocytes, some myeloid cells in adult
Mouse Mus *musculus*	PU.1 (PU.1$^{EGFP/1}$)	EGFP	Embryonic expression not described[b], myeloid, B lymphocytes
	CX$_3$CR1 (CX$_3$CR1$^{EGFP/1}$)	EGFP	In embryonic macrophages from 9.5 dpc, subset of monocytes macrophages in adult animals
	Csf1r(c-fms) (c-fms-EGFP)	EGFP	Trophoblast cells, embryonic phagocytes from 9.5 dpc, myeloid, B cells in adult
	Modified Csf1r(c-fms) (Csf1rDP$_{tro}$ - Gal4/UAS-ECFP)	ECFP	Embryonic phagocytes (precursors) from 8.5 dpc, subset of macrophages in adult

[a]Nuclear-localized EGFP, expressed from the UAS-NLS-EGFP, a Gal4 responder line.
[b]While embryonic expression of this reporter has not been reported, known expression pattern of the *PU.1* gene suggests that the reporter allele is likely to be functional during embryogenesis in definitive embryonic macrophages.

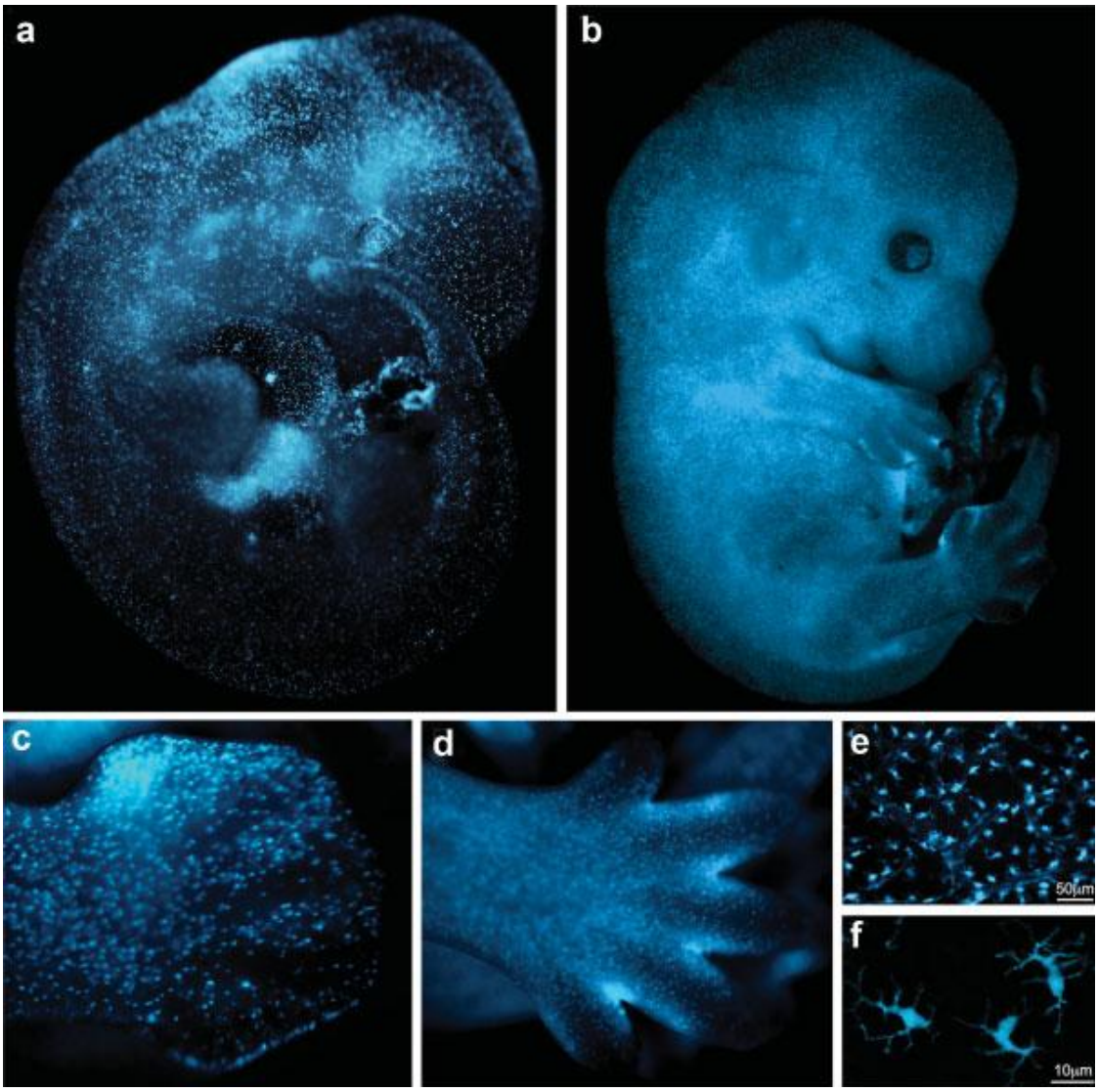


Figure 2.7. Embryonic macrophages visualized using *MacBlue* transgenic mice (*Csf1rΔP*$_{tro}$-Gal4/UAS-ECFP) (Taken from 94). (a) Embryonic macrophages are dispersed throughout E11,5 embryo with highest levels of ECFP in the fetal liver and anterior part of the embryo. **(b)** Embryonic macrophages in E12.5 embryo. Supposedly, the higher level of macrophages is caused by increasing number of definitive macrophages differentiating in the fetal liver. **(c-d)** Hindlimb of an E11.75 and E13.5 embryo, respectively. ECFP$^+$ cells were observed especially in areas of increased cell death, such as anterior and interdigital region of developing footplate. **(e)** Macrophages in the yolk sac of E14.5 embryo. They are evenly deployed and many of them are juxtaposed to the blood vessels. **(f)** Confocal imaging-based 3D reconstruction of ECFP$^+$ Langerhans cells of the skin.

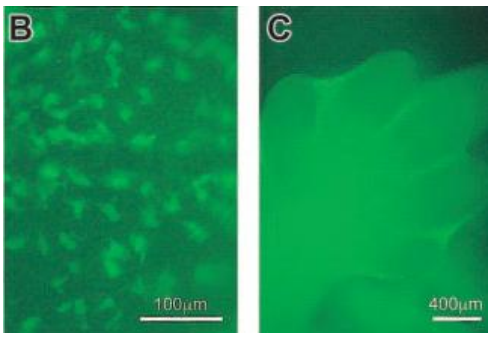


Figure 2.8. Visualizing of embryonic cells using *MacGreen* transgenic mice (c-*fms*-EGFP) (Taken from 35). (b) $EGFP^+$ cells in the yolk sac of E9.5 embryo. **(c)** Hindlimb of an E12 embryo. $EGFP^+$ cells are concentrated in interdigital necrotic zones.

Because detection of the surface-specific markers is an efficient approach to reliably obtain distinct and pure macrophage populations by using FACS sorting technique, there´s a necessity to identify additional set of surface markers expressed exclusively on embryonic phagocytes. In the best case scenario, these newly identified markers would be suitable for phenotypic distinction among embryonic phagocyte subpopulations. In this context, our recent data provide the very first evidence that TLRs are expressed at early stages of embryonic development (figure 2.9). This finding is seemingly surprising as TLRs are prototypic innate immune receptors recognizing predominantly microbial invariant structures. However, their expression during embryonic development in a microbe-free environment points to a possibility of TLR-mediated recognition of putative endogenous ligands. This would be a paradigm changing shift implicating TLR signaling in embryonic homeostatic regulatory mechanisms. This highly intriguing possibility led us to initiate an investigation on physiological role of TLR in embryonic homeostasis. Thus, the following chapter will briefly consider these molecules in general terms.

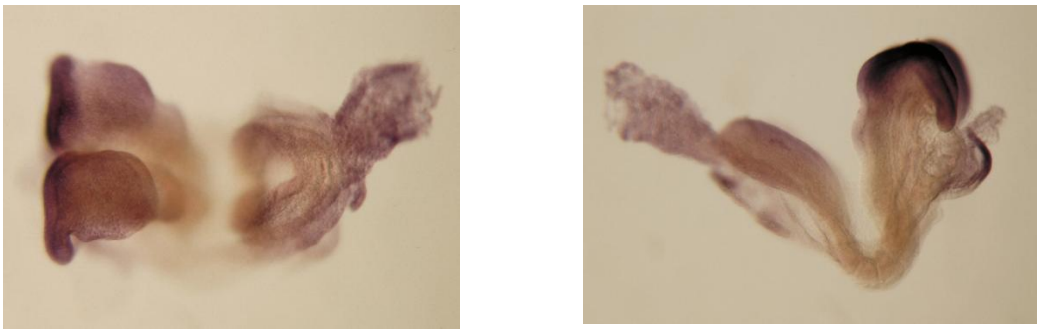


Figure 2.9. Localization of TLR4 mRNA in E8.5 embryo (Filipp et al., unpublished). TLR4 mRNA was localized in the embryo using whole mount in situ RNA hybridization. Expression of TLR4 is localized predominantly to developing head structures of embryo.

2.4. Mammalian Toll like receptors

Toll like receptors are considered as important receptors triggering innate immune responses. Upon their interaction with ligands derived from conserved microbial structures, several distinct signaling pathways are initiated resulting in global and robust changes in the gene expression. This, in turn, leads to effective innate immune responses and regulated development of acquired, antigen-specific immunity. In addition, there is a growing body of evidence that TLRs can also sense the presence of endogenous ligands induced by stress, necrosis, aseptic injury and irritation implicating their involvement in sterile physiological inflammation. The fundamental features of TLR physiology are outlined below.

2.4.1. Search for mammalian TLRs

Critical involvement of transmembrane protein Toll in the process defining dorso-ventral polarity in developing drosophila embryo (95, 96) and its participation in mediating innate immune responses to fungal infection in the same organismus were important prerequisite for its discovery in mammals (97).

The very first report describing the human Toll-like receptor gene was by Nomura and colleagues in 1994 (98) and genetically mapped by Testa's group in 1996 (99). One year latter, Janeway's team provided the very first functional analysis demonstrating that a constitutively active form of human TLR4 (at that time the only described human TLR gene) induces the activation of NF-κB, resulting in the expression of genes required for initiation of adaptive immune responses (1). This seminal discovery was quickly complemented by an excellent study by Bruce A. Beutler and colleagues demonstrating the principal function of human TLR4 gene in innate immune responses to lipopolysaccharide (LPS) (100). Notably, they showed that mice carrying a specific mutation in TLR4 gene are refractory to LPS stimulation and don't die of septic shock. This identified TLR4 as a key signal-transducing receptor for LPS and strongly suggested that other TLRs might also recognize other immunologically-relevant microbial structures. Since then, an intense search has so far identified 10 human and 12 murine TLRs. TLR1-TLR9 are expressed in both mice and

humans, whereas TLR10 is expressed only in humans. In contrast, mice express also TLR11, TLR12 and TLR13 which lack functional homologs in humans.

2.4.2. Functional domains of TLRs

The TLRs are type I integral membrane glycoproteins with molecular weights ranging from 90 to 115 kDa. As their intracellular portion share a significant homology with the cytoplasmatic region of IL-1 receptor (IL-1R) we classify them as Toll/IL-1 Receptor (TIR)-domain containing proteins. (1, 101) In contrast, the extracellular domains of both types of receptors differ markedly; the IL-1R contains three immunoglobulin-like domains, whereas TLRs consist of 19-25 tandem copies of leucine-rich repeats (LRR) (figure 2.10).

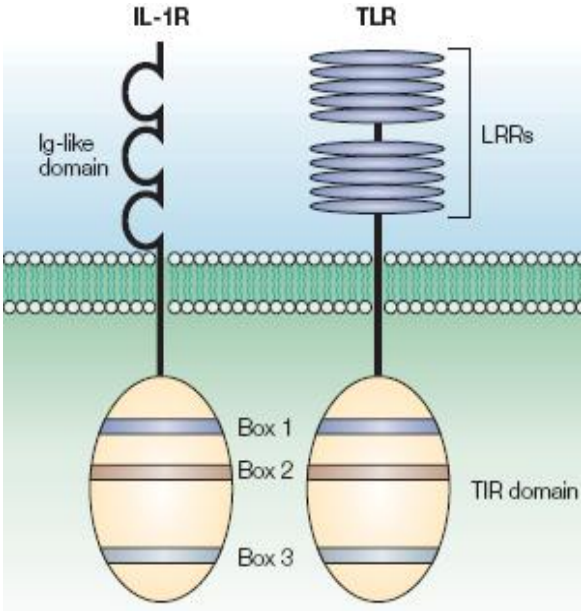


Figure 2.10. TLR structure (Taken from 11). TLRs and IL-1R share a highly conserved cytoplasmatic domain termed as the Toll/ IL- 1R (TIR) domain, which consists of three homologous segments (designated as boxes 1, 2, 3). In contrast, TLR and IL-1R extracellular regions are structurally unrelated and are composed of leucine-rich repeat (LRR) motifs and three immunoglobulin (Ig)-like domains, respectively.

2.4.3. Expression of TLRs, their subcellular localization and ligands

TLRs are predominantly expressed by innate immune cells like macrophages, neutrophils, dendritic cells, mast cells and NK cells. In addition, their expression in other cell types such as lymphocytes, endothelial cells, skin keratinocytes, fibroblasts and cancer cells has been also well documented (102).

There are two main subcellular localizations of TLRs. Whereas TLR1, 2, 4, 5, 6, 10 and 11 are all expressed on the plasma membrane, TLR3, 7, 8 and 9 are found in intracellular compartments such as endosomes (103). This apparent dichotomy in their localization is dictated by the requirement to recognize particular PAMP of infectious agents. Specifically, those TLRs expressed on the plasma membrane usually recognize bacterial structures exposed on the surface of microbes (100, 104-107), while intracellularly positioned TLRs sense viral and bacterial nucleic acids (108-111). This is strategically important as the viral and intracellular pathogen`s bacterial life cycle is largely restricted to this intracellular compartment in general, and to endosomes in particular (112). The list of ligands and their origin are listed in table 2.4. As the recognition of some ligands of endogenous nature (also listed in this table) by TLRs is still a subject of serious scientific discussions, I will discuss this contentious issue in more details in a separate chapter (see 2.4.5).

Important is also the question about the mode of TLR ligand recognition. While there is a strong evidence that at least some TLRs recognize their ligands directly and independently, some of them need co-receptors for efficient ligand binding and signal transduction (3). In particular, it has been demonstrated that dimerised TLR3 and TLR9 are able to bind their respective nucleic acid ligands directly via C-terminal part of their extracellular leucine-rich repeat domains (113, 114). On the other hand, TLR4-mediated LPS signaling requires at least three accessory proteins: (i) acute phase protein LBP (LPS-binding protein) (115) which forms a high-affinity complex with the lipid A moiety of LPS, (ii) CD14, a glycosylphosphatidylinositol-anchored protein preferentially expressed on the surface of myeloid cells (116-118) which appears to present and load LPS on TLR4 and (iii) MD-2, a secreted glycoprotein which acts as en extracellular adaptor protein of TLR4 and enhances LPS responsiveness (119, 120). Whereas TLR4 expressing cells that lack MD-2 are refractory to LPS challenge (121, 122), CD14 knockout mice showed notable decreased, yet significant responses to LPS

suggesting that CD14-independent LPS recognition mechanism by TLR4 still exists (123, 124). However, whether TLR4 also binds LPS directly or not has not been solved to satisfaction as several studies provided opposing results on this issue (125-128).

Table 2.4. TLRs and their ligands (Taken from 2).

Table 1 | **Toll-like receptors and their ligands**

Receptor	Ligand	Origin of ligand
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria
	Soluble factors	*Neisseria meningitidis*
TLR2	Lipoprotein/lipopeptides	Various pathogens
	Peptidoglycan	Gram-positive bacteria
	Lipoteichoic acid	Gram-positive bacteria
	Lipoarabinomannan	Mycobacteria
	Phenol-soluble modulin	*Staphylococcus epidermidis*
	Glycoinositolphospholipids	*Trypanosoma cruzi*
	Glycolipids	*Treponema maltophilum*
	Porins	*Neisseria*
	Atypical lipopolysaccharide	*Leptospira interrogans*
	Atypical lipopolysaccharide	*Porphyromonas gingivalis*
	Zymosan	Fungi
	Heat-shock protein 70*	Host
TLR3	Double-stranded RNA	Viruses
TLR4	Lipopolysaccharide	Gram-negative bacteria
	Taxol	Plants
	Fusion protein	Respiratory syncytial virus
	Envelope protein	Mouse mammary-tumour virus
	Heat-shock protein 60*	*Chlamydia pneumoniae*
	Heat-shock protein 70*	Host
	Type III repeat extra domain A of fibronectin*	Host
	Oligosaccharides of hyaluronic acid*	Host
	Polysaccharide fragments of heparan sulphate*	Host
	Fibrinogen*	Host
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides	*Mycoplasma*
	Lipoteichoic acid	Gram-positive bacteria
	Zymosan	Fungi
TLR7	Imidazoquinoline	Synthetic compounds
	Loxoribine	Synthetic compounds
	Bropirimine	Synthetic compounds
	Single-stranded RNA	Viruses
TLR8	Imidazoquinoline	Synthetic compounds
	Single-stranded RNA	Viruses
TLR9	CpG-containing DNA	Bacteria and viruses
TLR10	N.D.	N.D.
TLR11	N.D.	Uropathogenic bacteria

*Possibility of contamination with microbial components; N.D., not determined

2.4.4. TLR signaling components and pathways

TLR signaling pathway is relatively well understood and several excellent reviews have been dedicated to this issue (2-4, 103). Upon ligand binding, TLRs dimerise and recruite proximal TIR-domain containing adaptor proteins initiating downstream signaling leading to the activation of transcription factors such as NFκB, IRFs and AP-1. So far, five TLR adaptor proteins have been discovered: MyD88 (myeloid differentiation primary-response gene 88), Mal (MyD88-adaptor-like protein)/TIRAP (TIR domain-containing adaptor protein), TRIF (TIR-domain-containing adaptor protein inducing IFN-β), and TRAM (TRIF-related adaptor molecule). Lastly, the adaptor protein SARM (sterile α- and armadillo-motif-containing protein) seems to have multiple roles and functions which are species-dependent and differ in variety of conditions (129). As the details of the TLR signaling pathways are beyond the scope of this thesis, schematic cartoon and concise summary of TLR pathways are depicted in figure 2.11 and accompanying figure legend.

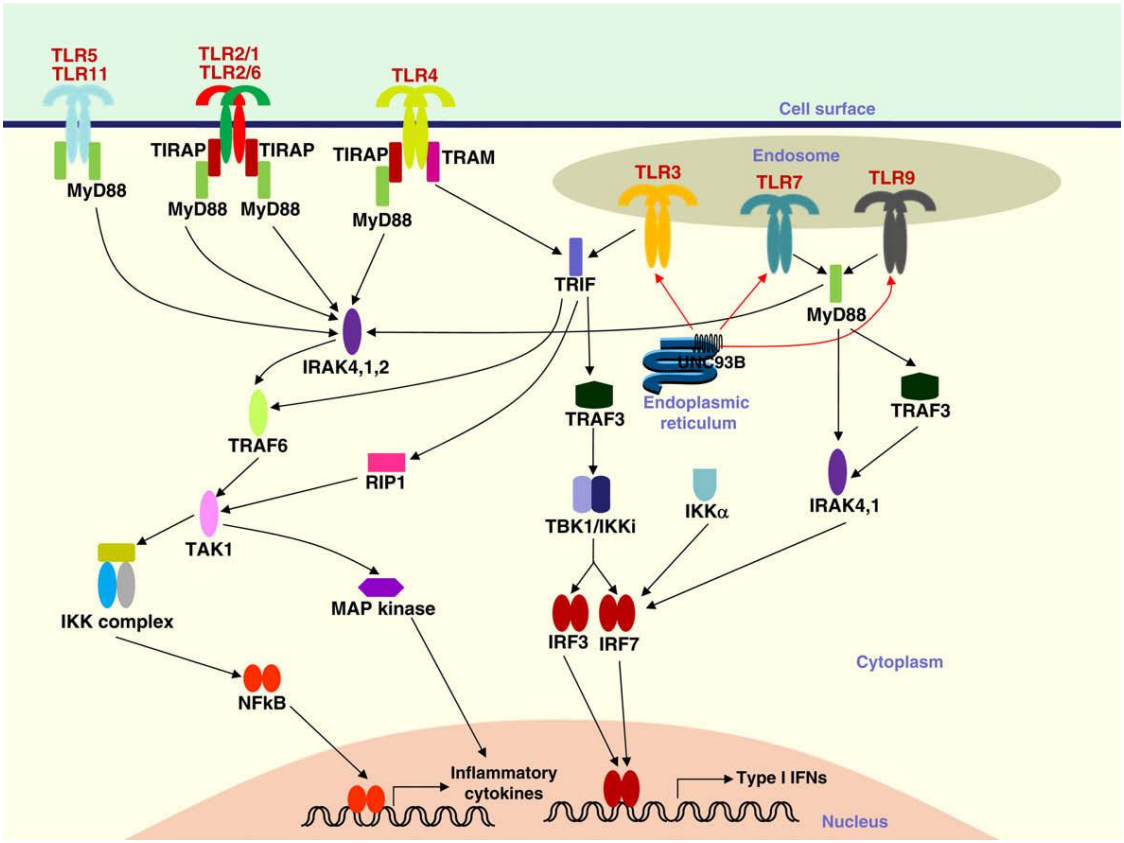


Figure 2.11. Signaling pathway of TLRs (Taken from 3). The engagement of all TLRs, except TLR3, with their respective ligands recruits MyD88, IRAKs and TRAF6. In turn, TRAF6 forms a complex with Ubc13 and Uev1A and via lysine 63-polyubiquitinylation activates the TAK1 pathway. This leads to activation of NFκB and AP-1 transcription factors through IKK complex and MAP kinases, respectively. NFκB and AP-1 translocate into the nucleus and induce the expression of target genes such as TNFα, IL6, IL1β and IL12. In addition to MyD88 pathway, TIRAP adaptor protein is involved in the signaling through TLR1/2, TLR2/6 and TLR4. TLR3 is the only TLR that uses the TRIF-pathway exclusively. TLR4 can also signal via MyD88-independent signaling pathway by recruitment of TRIF. However it requires the additional linker adaptor TRAM which is not required for endosom-residing TLR3. It is suggested that TLR4 activation by ligand binding at the plasma membrane triggers the MyD88-dependent pathway, then it gets internalize and activates TRIF pathway in the second step. This is in accordance with hypothesis that endosomal TLRs rather activate IRF transcription factors whereas plasma membrane TLRs activate NFκB pathway. TLR3, TLR7/8 and TLR9 are transported to the endosomes via ER-resident membrane protein UNC93B. In the TRIF-dependent pathway, TRIF interacts with TRAF3 which forms a complex with TBK1 and IKKi and leads to activation of IRF3 and IRF7. Activated IRFs dimerise and translocate to the nucleus where they induce expression of type I IFN and IFN-inducible genes. Additionally, TRIF can also interacts with TRAF6 and RIP1 resulting in NFκB and MAP kinases activation. In plasmatocytoid dendritic cells expressing TLR7/8 and TLR9, both NFκB and MAP kinase signaling pathways as well as type I IFN pathway can be induced.

2.4.4.1. Negative regulation of TLR signaling

As an uncontrolled TLR signaling leads to deleterious and inappropriate inflammatory responses, it is tightly regulated by elements of negative regulatory feedback loop mechanisms (table 2.5). Among them, an important role play the group of transmembrane protein regulators (figure 2.12), especially ST2 and Single Immunoglobulin Interleukin-1-Related Receptor (SIGIRR), both containing an intracellular TIR domain.

The ST2 gene (also known as T1, Fit-1 or DER4) was originally identified in murine fibroblasts as a late response gene strongly induced by the Ha-*ras* and v-*mos* oncogenes and by the serum (130, 131). Alternative splicing of ST2 gene results in two mRNA species: an abundant secreted glycoprotein (sST2) and a rare longer transmembrane form (ST2L) (132-134). ST2L has been shown to be expressed on mast cells (135) and Th2 cells but not Th1 cells (136-138). Recently it was found, that upon IL-33 stimulation, ST2 forms a complex with IL-RAcP, which is involved in Th2-mediated immune responses (139). Although ST2L is able to activate MAPK pathway, it does not activate NF-κB (140). ST2L suppresses IL-1R, TLR2, TLR4 and TLR 9 but not TLR3 signaling by sequestration of MyD88 and Mal through its TIR domain (141). Furthermore, ST2L has a key function in endotoxin tolerance (141).

The Sigirr gene (also known as TIR8) is an orphan receptor which is highly conserved among species and throughout the evolution (142). Its single extracellular immunoglobulin domain does not support ligand binding and its intracellular TIR domain cannot activate NF-κB because it lacks two essential amino residues (Ser447 and Tyr536) (143). Importantly, Sigirr differs from other TLR/IL-1 superfamily members by presence of unique 95 amino acids long tail with inhibitory properties (143). Sigirr is ubiquitously expressed at higher levels in the kidney, liver, lung, lymphoid organs and the gut where the expression is particularly high in epithelial cells (144, 145). It is expressed by NK cells, B cells, monocytes and immature dendritic cells with reduced expression after maturation (144, 146, 147). Consistent with high expression of Sigirr in the gut it has been shown that it is involved in regulating inflammation in the gastrointestinal tract (146, 148) and it also contributes to the control of autoimmunity by inhibition of DCs activation upon exposure to lupus immune complexes (149). Moreover, it has been shown that Sigirr is a key molecule involved in dampening inflammation and tissue damage in both tuberculosis and

fungal infections (150, 151). It has been shown that Sigirr negatively regulates TLR4, TLR7 and TLR9 signaling (146, 147, 149, 152, 153). Interaction with other members of TLR family has not been established so far.

Table 2.5. Negative regulators of TLRs (Taken from 154).

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

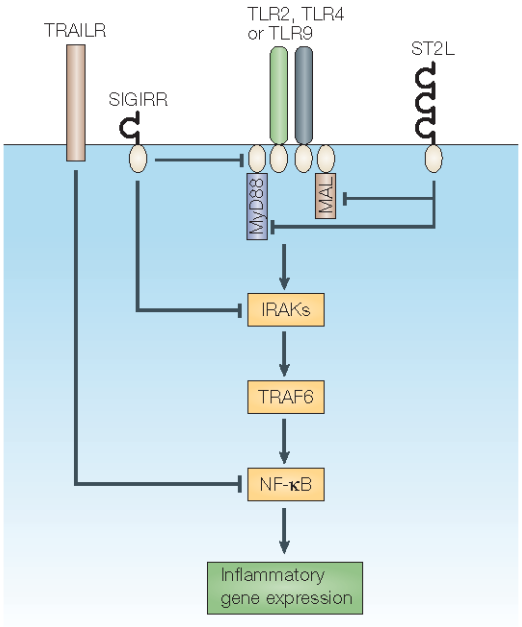


Figure 2.12. Transmembrane regulators of TLR signaling (Taken from 154).
ST2L blocks TLR signaling by sequestering TIR domains of MAL and Myd88 and thus suppressing NF-κB activation induced by TLR2, TLR4 and TLR9. Sigirr downregulates TLR signaling by its interaction with IRAK and TRAF6 via its TIR domain but the precise mechanism of action is unclear. Both ST2L and Sigirr can affect only MyD88-dependent TLR signaling pathways. By contrast, TRAILR can also suppress MyD88-independent TLR pathway because it acts later in TLR signaling by stabilizing the inhibitor of NF-κB and thus prevents it from degradation.

2.4.5. Danger theory and endogenous TLR ligands

The decisive role of TLRs in innate and adaptive responses to pathogen-mediated infection has been conclusively proven. In addition, contribution of TLRs to autoimmunity and/or sterile inflammation has been under close scrutiny (7, 155-157). In this regard, it is important to demonstrate that these receptors mediate responses to endogenous stimuli. So far several candidates for endogenous ligands have been suggested, most of them implicated in TLR2 and TLR4 signaling (158-168). Discovery of these endogenous ligands for TLRs as potent activators of innate immune system fits well with the "danger theory" proposed by Polly Matzinger (169). It suggests that the immune system evolved to recognize endogenous danger signals released from injured and stressed cells, rather than "nonself" signals. If correct, many autoimmune diseases could find the cure by blocking TLR signaling (7).

2.4.5.1. Problems surrounding the identification of true endogenous TLR ligands

There are more than 20 reports documenting the stimulatory potential of distinct putative endogenous TLR ligands (170). However, it has been shown that in

many cases, their cytokine-inducing effects may be due to contamination by LPS and bacterial lipoproteins (155-157, 171), as these ligands were mainly produced in prokaryotic expression systems. In addition, the possible effect of bacterial contamination in these recombinant samples cannot be excluded due to methods employed for assessment of this contamination. For example, it was shown that LAL-assay, a method routinely used for determination of contaminating levels of free LPS, is unable to detect any other bacterial contaminants, like lipopeptides (172). Likewise, this assay precludes detection of proteins able to sequester LPS, for example LBP and bactericidal/permeability inducing protein. Thus, in their presence, LPS can be "shielded" from detection by LAL-assay and LPS became virtually undetectable (173). In consideration of this notion, alternative techniques for the preparation of TLR ligands utilizing eukaryotic recombinant expression systems have been used. However, it has been reported that recombinant proteins derived from eukaryotic system also contained bacterial products due to mycoplasma contamination and/or the usage of tag-cleaving enzymes originally produced in bacterial systems (170, 174). Thus, due to the lack of rigorous control methodologies able to establish if the reported putative host ligands of TLRs are of endogenous or bacterial nature, immunostimulatory activities of endogenous TLR ligands should be taken with a reasonable caution.

On the other hand, data obtained in our laboratory (see the result section) demonstrated the expression of TLRs on phagocytes in early stages of embryonic development. As this development takes place in a sterile environment, this finding suggests a physiological importance of TLRs endogenous ligands. Consequences could be far reaching. Specifically, that TLRs might indeed recognize both exogenous and endogenous structures what would significantly extend and modify our current paradigm concerning TLRs as receptors functioning to recognize microbial metabolites exclusively. Whether the signaling pathways engaged upon recognition of endogenous and microbial ligands are identical or exhibit significant differences, remain to be established. However, recently described involvement of TLR4/TLR6 dimers in CD36-mediated signaling to endogenous ligands (8) as well as our own finding that Sigirr is ubiquitously expressed in very early stages of embryonic development (section 4.3.3), further supports the idea about the distinct role of TLR signaling in the maintenance of homeostasis under sterile conditions as well as distinct and specific signaling pathways used for this purpose.

3. Materials and methods

3.1. Materials

3.1.1. Mice

Mouse strains ICR (CD1) and B6CF2XB6 were used. Mice were housed in the pathogen-free animal facility at Institute of Molecular Genetics *(Prague, Czech Republic).*

3.1.2. Bacterial strain

TOP 10 bacterial strain *Escherichia coli (Invitrogen)*

3.1.3. Vector

RNAi-Ready pSIREN-DNR-DsRed-Express Donor Vector *(Clontech)*

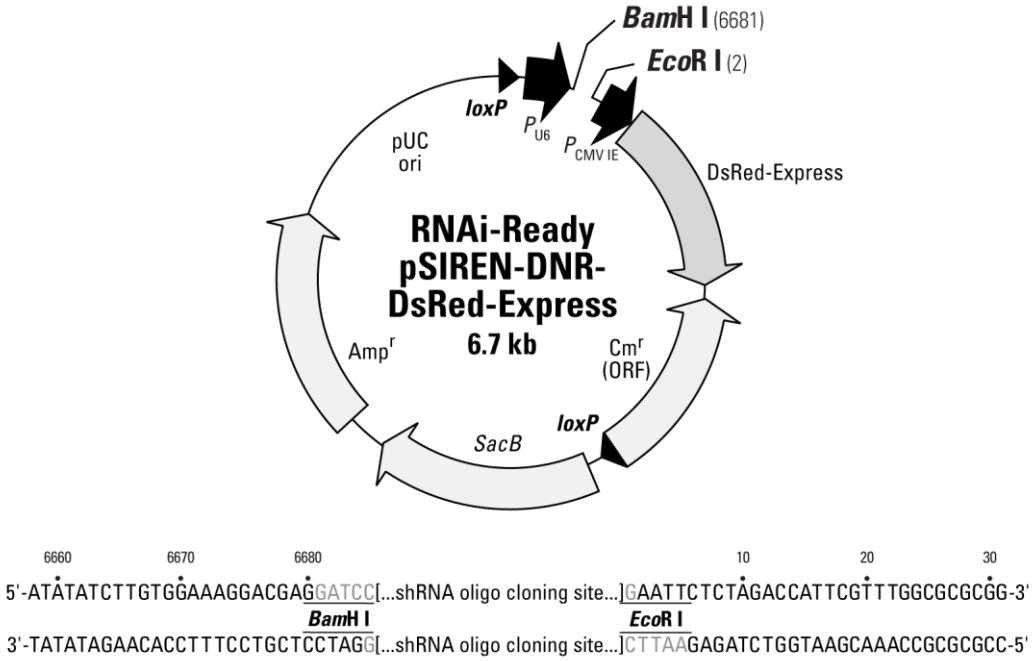


Figure 3.1. Restriction map and Cloning Site of the RNAi-Ready pSIREN-DNR-DsRed-Express Donor Vector (Taken from www.clontech.com).

3.1.4. Antibodies

All antibodies used are listed in table 3.1.

Table 3.1. Primary and secondary antibodies and their specifications

Name of Antibodies	Source of Ab origin	Conjugate/purified	Source of Ab
Fc blocking			
Anti-CD16/32	Rat	Purified	BD Pharmingen
Primary Antibodies			
Anti-SIGIRR	Goat	Polyclonal	Santa Cruz Biotechnology
Anti-Brachyury	Goat	Polyclonal	Santa Cruz Biotechnology
Anti-CD11b	Rat	PE	eBioscience
Anti-CD11b	Rat	FITC	eBioscience
Anti-TLR2	Rat	Biotin	eBioscience
Anti-TLR2	Rat	Alexa647	eBioscience
Anti-TLR4	Rat	PE	eBioscience
Anti-CD45	Rat	PerCP	BD Pharmingen
Anti-F4/80	Rat	PE-Cy7	eBioscience
Anti-CD14	Rat	APC	eBioscience
Secondary Antibodies-IHC			
Anti-Goat IgG (H+L)	Rabbit	HRP	Invitrogen
Streptavidin conjugates			
Streptavidin		FITC	eBioscience
Streptavidin		APC	eBioscience

3.1.5. Commercial Kits

RNeasy Plus Micro Kit *(Qiagen)*

NucleoSpin RNA II *(Macherey Nagel)*

SuperScript™ III Reverse Transriptase *(Invitrogen)*

ZR Plasmid Miniprep™-Classic *(Zymo Research)*

Jetstar Plasmid Purification Midi Kit *(Genomed)*

LightCycler® 480 SYBR Green I Master *(Roche)*

Mouse_WG6_v2 Expression BeadChip Kit *(Illumina)*

3.1.6. Primers

Primers were designed using the Primer3 program (version 0.4.0; http://frodo.wi.mit.edu/primer3/) and Roche Universal Probe Library (ProbeFinder version 2.45; http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp) (table 3.2).

Table 3.2. List of used primers

Gene	Forward primer (5′→ 3′)	Reverse primer (5′→ 3′)
Casc3	TTC-GAG-GTG-TGC-CTA-ACC-A	GCT-TAG-CTC-GAC-CAC-TCT-GG
TLR1	CAA-TGT-GGA-AAC-AAC-GTG-GA	TGT-AAC-TTT-GGG-GGA-AGC-TG
TLR2	GTT-CAT-CTC-TGG-AGC-ATC-CG	ACT-CCT-GAG-CAG-AAC-AGC-GT
TLR3	CAC-AGG-CTG-AGC-AGT-TTG-AA	TTT-CGG-CTT-CTT-TTG-ATG-CT
TLR4	GGA-CTC-TGA-TCA-TGG-CAC-TG	CTG-ATC-CAT-GCA-TTG-GTA-GGT
TLR5	ACG-CAC-CAC-ACT-TCA-GCA	AGC-CTC-GGA-AAA-AGG-CTA-TC
TLR6	TTC-CCA-ATA-CCA-CCG-TTC-TC	CTA-TGT-GCT-GGA-GGG-TCA-CA
TLR7	AAT-CCA-CAG-GCT-CAC-CCA-TA	CAG-GTA-CCA-AGG-GAT-GTC-CT
TLR8	CAA-ACG-TTT-TAC-CTT-CCT-TTG-TC	AAT-CAC-AAG-GGA-GTT-GTG-CC
TLR9	CTC-GGA-ACA-ACC-TGG-TGA-CT	ACT-GGA-GGC-GTG-AGA-GAT-TG
TLR11	ATG-GGG-CTT-TAT-CCC-TTT-TG	GATGTTATTGCCACTCAACCA
TLR12	TTT-CAA-GCA-CTG-GCC-TAA-CC	GAA-GCC-TAG-GCA-TGG-CAG-T
TLR13	ATG-AAT-GGG-AGC-AGC-TTT-GT	CGG-CAG-AGA-AAA-TCC-TAC-TAA
MyD88	TGG-CCT-TGT-TAG-ACC-GTG-A	AAG-TAT-TTC-TGG-CAG-TCC-TCC-TC
TRIF	GTG-TGA-CTA-GAA-GAG-AAA-GAG	CCC-CAA-GGA-AGA-GAC-ACA
TRAM	CTG-CTT-GCG-TGG-TTT-TGT-GGA-G	CCG-GGG-AAG-AGG-GCT-GTT
SARM	GCT-TGC-TGG-AGC-AGA-TCC-T	TCA-CGC-CTA-GAC-CGA-TGC
MAL	TCA-CCC-CCG-AGT-CAC-AGT-TCA-C	AGG-CCG-GAC-AGC-AGG-GGT-ATG
SIGIRR	GAC-ATG-GCC-CCT-AAT-TTC-CT	GAC-CCA-GAA-GTC-CTC-ATG-GA
Mrc1	GGA-CGA-GCA-GGT-GCA-GTT	CAA-CAC-ATC-CCG-CCT-TTC
Sta1	GAT-CTT-CAG-CCG-CCT-ATT-GA	GAT-GGG-ACG-CAT-CCT-GAA
Cx3CR1	CAA-AAT-TCT-CTA-GAT-CCA-GTT-CAG-G	AAG-TTC-CCT-TCC-CAT-CTG-CT
Asb2	TCT-CTT-TGT-TGC-CCA-GAC-CT	TTC-CAG-AAG-AGG-GAC-TCA-GC
Msr2	GCC-TTT-GAT-TGT-GGA-CAT-GA	ATC-TTC-AGA-AAG-TGC-TGG-GTA-A
Slc40A1	TGT-TGT-TGT-GGC-AGG-AGA-AA	AGC-TGG-TCA-ATC-CTT-CTA-ATG-G
Gpr86	GTG-GGT-TGA-GCT-AGT-AAC-TGC-C	GTG-GTG-TTG-ATT-GTC-CCG-A
Eg245190	ACT-GTT-GGA-CCG-GCC-TCT	TAT-CCC-TTC-CCC-GCT-CTC
Gpr84	TGT-GAA-AAC-TGG-GAA-CCT-CA	GCC-CAA-CAC-AGA-CTC-ATG-G
Aif1	GGA-TTT-GCA-GGG-AGG-AAA-AG	TGG-GAT-CAT-CGA-GGA-ATT-G
MPO	GGA-AGG-AGA-CCT-AGA-GGT-TGG	TAG-CAC-AGG-AAG-GCC-AAT-G
Gas6	GGG-GAA-TGG-ATT-TGC-TAC-CT	TTA-ACT-TCC-CAG-GTG-GTT-TCC
Ccl4	GCC-CTC-TCT-CTC-CTC-TTG-CT	GGA-GGG-TCA-GAG-CCC-ATT
Ccl3	CAA-GTC-TTC-TCA-GCG-CCA-TA	GGA-ATC-TTC-CGG-CTG-TAG-G
CD79b	CAC-ACT-GGT-GCT-GTC-TTC-CA	CAC-TGC-TTG-TCA-TTG-CTG-GT
Cor1a	CTA-CTT-GGG-AGG-GGT-CAC-G	TTT-GCT-GGA-GCG-AAC-CAC

3.1.7. Enzymes

BamHI *(Fermentas)*
EcoRI (*New England Biolabs)*
Dispase *(Invitrogen)*

3.2. Methods

3.2.1. Embryos/peritoneal macrophages isolation

The mice were set up for mating in the evening. The next morning, when the presence of vaginal plug was confirmed, that day was designated E0.5. Immediately after time-pregnant females were sacrificed by cervical dislocation, embryos were delivered from the uterus and then dissected out of the decidua with the yolk sac and the amnion pulled away from the embryo.

Peritoneal macrophages were isolated by lavage of peritoneum with cold PBS.

3.2.2. RNA isolation

For RNA isolation from both the whole 7.5-8.5 day old embryos and sorted cells, the commercial kit RNeasy Plus Micro Kit was used. For RNA isolation from the whole 9.5-12.5 day old embryos and the peritoneal macrophages, NucleoSpin RNA II was used. Work procedure was performed in accordance with manufacturer´s instructions.

3.2.3. Determination of RNA purity and concentration

RNA concentration was determined spectrophotometricly at wavelenght of 260 nm using NanoDrop 1000 *(Thermo Scientific).* An A260 reading of 1.0 is equivalent to about 40 μg/ml of RNA. RNA purity was verified by ratio specification of A_{260}/A_{280} absorbance which varied between 1.8 and 2.0. The quality of RNA which was used for

microarray analysis was assessed electrophoreticly using BioAnalyser 2100 *(Agilent)* (performed by the staff of IMG Genetics and Bioinformatics core facility). Only RNA samples with RNA integrity number (RIN) higher than 7 were used for further analysis.

3.2.4. Reverse transcription

Isolated RNAs were reverse transcribed into cDNA using SuperScript™ III Reverse Transcriptase. For one 20µl reaction, 100ng or 500ng of RNA isolated from whole embryos or 100ng of RNA isolated from sorted embryonic cells and adult peritoneal macrophages were used. The reaction was performed according to manufacturer's instructions.

3.2.5. Quantitative real-time PCR (qRT-PCR)

qRT-PCR method measures the level of fluorescence which increase with raising amount of amplified DNA during each PCR cycle. For a direct measurements of DNA amplification in PCR reaction, fluorescent dyes able to intercalate into dsDNA are used, such as Sybr green. The treshold cycle value (Ct) corresponds to the cycle in which the measured fluorescence level is higher than the fluorescence of the background. The more RNA templates present at the start of amplifying reaction, the lower Ct is obtained.

PCR experiments using hot start polymerase with Sybr green dye were performed on the LC480 machine *(Roche)*. cDNA (20µl) was firstly diluted by adding PCR-grade water to final volume of 100µl. Every reaction mixture was prepared in a well in 384 well-plate with the following composition:

2,5 µl	LightCycler® 480 SYBR Green I Master
0,5 µl	primer mix (each 8µM)
2 µl	cDNA

Following program was used for amplification reaction:

DNA polymerase activation:	95°C	7min	
Denaturation:	95°C	10s	
Annealing:	60°C	20s	45x
Elongation:	72°C	20s	
Acquiring:	78°C	1s	
Meassuring of melting temperature:	95°C	15s	
	65°C	1min	
continuous to	97°C	(0,06°C/s)	
	37°C	1min	

The quantification of gene expression was calculated as follows: the relative expression ratio = $(E_{target})^{Cp\ target\ (control\text{-}sample)}/\ (E_{ref})^{Cp\ ref\ (control\text{-}sample)}$ (175) was employed to calculate expression of target genes. The expression of target genes was normalized to housekeeping gene Casc3. In negative control samples, the cDNA template was replaced by H_2O. Unless stated otherwise, three different and independent samples, each analysed in duplicates or triplicates, were used for an assessment of each single gene expression.

3.2.6. Flow cytometry (FACS) and cell sorting

Single cell suspension from embryos was prepared by dispase treatment (at the final concentration 500mg/ml) for 10 min at 37°C. Cells were then washed in 1% FCS *(Invitrogen)* supplemented PBS, filtered through 40µm cell strainer *(BD Biosciences)*, pelleted by centrifugation and washed three times with 1% FCS in PBS. To block the Fc receptors, cells were incubated with anti-CD16/32 antibody for 10 min at 4°C. Cells were stained with directly conjugated antibodies or sequentially, with primary and then secondary antibodies in appropriate combinations. Cells were washed twice and analysed using LSRII in the presence of Hoechst 33528 dye *(Roche)* to exclude dead cells. For sorting purposes FACSVantage SE equipment was used. Cell sortings were performed by Zdeněk Cimburek.

3.2.7. Immunohistochemistry of whole-mount embryo

The procedure was performed as previously described (176) with minor modifications. Briefly, embryos (E7.5) were fixed O/N in 3.8% PFA *(Polysciences, Inc.*) in PBS (pH 7.2) at 4°C. Then they were washed 6x in PBS at RT for 5 min, following successive incubations in 15mM NH_4Cl in PBS at RT for 30 min, 100mM glycine in PBS incubation for 30 min and 1% H_2O_2 in PBS at RT for 3 hours. Then the blocking steps and the staining procedure were performed according to the original protocol. Embryos were then inspected and their images taken by Leica DM6000 B microscope.

3.2.8. Microarray analysis

mRNAs from both $TLR2^+$ $CD11b^+$ double positive (DP), $TLR2^-$ $CD11b^-$ double negative (DN) sorted cells derived from 10.5 old day embryo (E10.5) and $TLR2^+$ $CD11b^+$ DP peritoneal macrophages were isolated and the RIN number was determined (sections 3.2.2 and 3.2.3). The gene expression was analyzed using Mouse-WG6-v2-Expression BeadChip Kit (performed by the staff of IMG Genetics and Bioinformatics core facility). Microarray data were analyzed using the Partek and David softwares.

3.2.9. Bacteria plasmid transformation

50µl aliquote of TOP 10 chemocompetent bacteria were incubated with 1µg of plasmid DNA for 20 min on ice and then heatshocked at 42°C for 1 min. After chilling down on ice for 2 min, 200µl of S.O.C. medium *(Invitrogen)* was added and the bacteria were shaked at 37°C for 1 hour. Bacteria were then plated out on agar plates supplemented with ampicilin and incubated O/N in 37°C.

3.2.10. Plasmid isolation

Single transformed bacterial colonies were picked up from agar plates and transferred to LB medium *(Gibco)* supplemented with 100μg/ml of ampicilin and shaked at 37°C O/N. Plasmids were isolated either by ZR Plasmid Miniprep™-Classic kit designed for small bacterial cultures (up to 3ml) or by Jetstar Plasmid Purification Midi Kit for medium scale amounts (50 ml) according to manufacturer's instructions. For the purpose of injection into mice, plasmid DNA was finally dissolved in Ringer´s buffer (8,6g/l NaCl; 0,3g/l KCl; 0,25g/l $CaCl_2$; injection H_2O). Integrity of plasmid DNA and its size was verified by restriction reaction with BamHI and EcoRI endonucleases (4U/μg DNA) at 37°C for 45 min and analysed by gel electrophoresis (data not shown).

3.2.11. Knockdown of gene expression in embryos: pilot experiments

To test a recently developed approach for knocking-down the expression of specific genes during early ontogenesis (177), we used the red fluorescent gene (DsRed) expressing vector to monitor its distribution in early E10.5 embryos upon its administration into pregnant females. Specifically, 10-50μg of pSIREN-DNR-DsRed-Express Vector dissolved in 250μl of Ringer´s buffer was injected into tail vein of time-pregnant mice at 7 days post coitum (psc). Three days later, mice were sacrificed by cervical dislocation, embryos were delivered from the uterus and plasmid delivery was assessed by both fluorescence microscopy and FACS analysis. If efficient, this approach should result in ubiquitous expression of DsRed in the embryo as illustrated in the figure 3.2.

Figure 3.2. Assessment of an ubiquitous DsRed expression in embryos (Taken from 177). Two embryos (E10.5) from pSIREN-DNR-DsRed-Express Vector injected mother display different levels of DsRed intensity according to delivered plasmid efficiency.

4. Results

4.1. Expression of TLRs in mouse embryo

So far, the mode of expression and function of TLRs have been studied extensively in adult phagocytes only. However, our recent study revealed that TLR4 is expressed in early stages of embryonic development (section 2.3.4). These data strongly suggest that TLR signaling could be involved in the maintenance of homeostasis in developing embryos.

4.1.1. Expression profile of TLRs and TLR adaptor proteins

Using qRT-PCR approach, we first determined expression levels of all TLRs and their adaptor proteins in early stages of mouse development (E7.5-E12.5) (figure 4.1). Because of technical reasons, TLR8 is not involved in the analysis. On the basis of their kinetics of expression, we divided TLRs into two groups. The first one, including TLR1 and TLR 6-13, is characterized by gradual increase of expression with the maximum increment between day 9.5 and 10.5 psc. The second group, including TLR 2-5, exhibits a bimodal profile of expression with the lowest value at around day 9.5 (figure 4.1 A). The gene expression of TLR adaptor proteins follows a different kinetics profile: their expression reaches its maximum in very early stages of development (E7.5-8.5 psc), then decreases significantly to its minimum around E9.5-10.5 psc and finally it slightly levels up. TRAM represents a somewhat diverted version from this profile with more symmetrical bimodal distribution in the very early (E7.5-8.5) and later (E11.5-12.5) stages of development (figure 4.1 B). The only exception is SARM with gradual increase in its expression, with the maximum at E12.5. It is of note that expression levels among different TLRs and their adaptors, highlighted in figure 1, was not possible to compare as the mathematical analysis of their expression, measured by qRT-PCR, precluded the effect of primer efficiency factor.

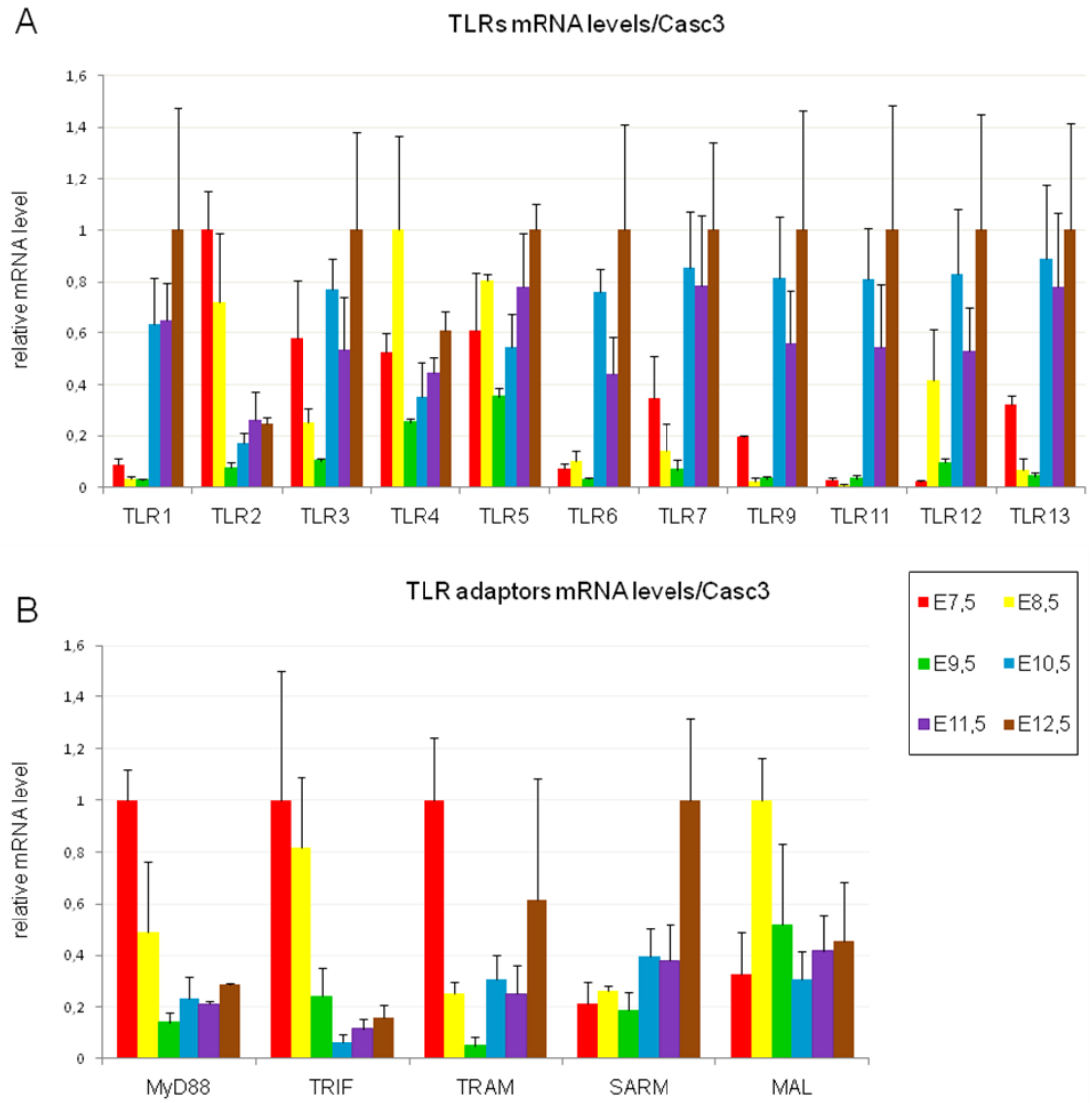


Figure 4.1. TLRs and TLR adaptors are expressed in early stages of mice development. Total RNAs from whole embryos in different stages of mice development were reverse transcribed using SuperScript™ III Reverse Transcriptase. qRT-PCR was performed using LightCycler® 480 SYBR Green I Master and the expression of TLRs (A) and TLR adaptor proteins (B) was normalized to Casc3 mRNA levels. For the purpose of better visualization, all values of expression profile for each gene tested were normalized to its highest value which was adjusted to a reference value "1". Embryonic day-related color code placed in (B) applies to (A) as well.

4.1.2. Surface expression of TLRs and other markers of embryonic phagocytes is restricted to CD11b$^+$ cells

To confirm the expression of selected TLRs on a protein level and to identify in which type of cells they are expressed, we performed FACS analysis of 10.5 day old embryo. As illustrated in figure 4.2 A, approximately 1% of all embryonic cells are positive for CD11b. Nearly all CD11b positive embryonic phagocytes are also positive for CD14 (figure 4.2 A). As mentioned in section 2.3.3, CD11b is a prominent surface marker of embryonic phagocytes (88). In adult mice is CD11b expressed on the surface of many leukocytes involved in the innate immune system, including monocytes, macrophages, granulocytes and NK-cells (178). Expression of CD14 (described in the section 2.4.3) on the embryonic phagocytes has not been previously demonstrated. When gated on this double-positive cell population (red square in figure 4.2 A), it is also positive for both haematopoietic marker CD45 and macrophage marker F4/80. As illustrated in the figure 4.2 B, these cells (red square) are ~99% positive for both TLR2 and TLR4 (figure 4.2 C).

These results are first to characterize the surface expression of TLRs and CD14 in early stages of mammalian embryogenesis and suggest their use as specific phenotypic markers of embryonic phagocytes.

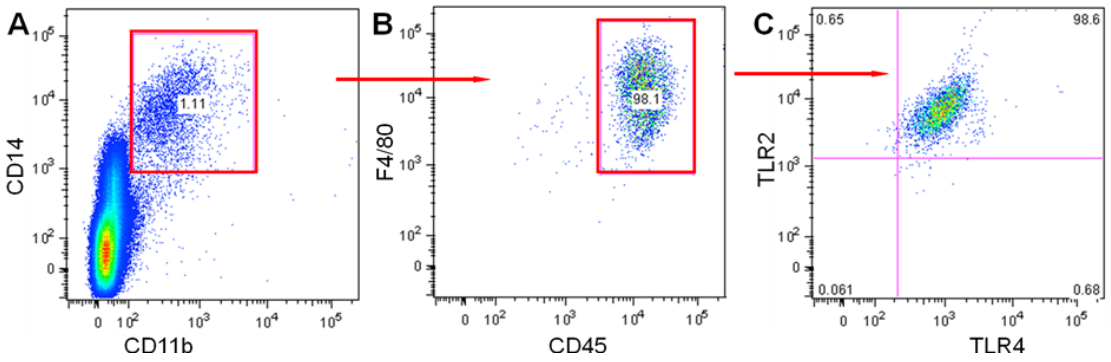


Figure 4.2. Expression of TLRs and other embryonic surface markers on CD11b$^+$ phagocytes (E10.5). Single suspension was stained with indicated antibodies. FACS analysis was performed on LSRII. See the main text for details.

4.1.3. Expression of TLRs and TLR adaptors on CD11b$^+$ TLR2$^+$ sorted cells

In the next step we asked the question whether all TLRs and their adaptors are expressed on the CD11b$^+$ TLR2$^+$ double-positive (DP) phagocytic cells and whether their expression is restricted to these cells. DP and CD11b$^-$ TLR2$^-$ double-negative (DN) cells were sorted out from 10.5 days embryo (figure 4.3 A) and mRNA isolated from these cells was subjected to qRT-PCR (figure 4.3 B). Results showed a complex expression pattern of these molecules in embryonic cells. The vast majority of TLRs are predominantly expressed on CD11b$^+$ TLR2$^+$ embryonic phagocytes. Notably, the expression of TLR1, 2, 4, 6, 7, 9 and 13 is approximately 50-100 times higher in DP than in DN E10.5 cells. 10-fold difference in expression levels was revealed for other two receptors TLR3 and TLR5. The expression of TLR12 seems to be roughly equal in DP and DN embryonic cells. TLR11 represents the only receptor with relatively higher expression in DN over DP embryonic cells. Similarly, while the adaptor proteins TRAM, MyD88 and MAL display approximately 100, 50 and 4 fold higher expression in DP cells, respectively, the expression of TRIF in DP and DN cells seems to be equal. On contrary, the expression of adaptor protein SARM, which has been lately associated with a non-immune function in neuronal cells (179, 180), is about 20 times higher in DN than in DP embryonic cells.

Thus, the expression of TLRs and their adaptors is not exclusive to CD11b$^+$ TLR2$^+$ embryonic phagocytic cells. Rather, they are also expressed, even though at much lower levels, in non-phagocytic cells. Whether these levels reflect a ubiquitous low level of expression in non-phagocytic cells or rather their relatively higher expression in a certain subpopulation of DN embryonic cells, remain to be determined. However, as demonstrated in the figure 4.3, it seems that 50-100 fold difference in TLR gene expression is sufficient to distinguish TLR-positive phagocytes from TLR/CD11b-negative non-phagocytic cells.

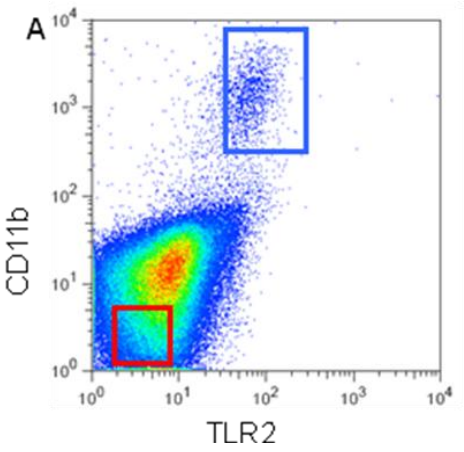
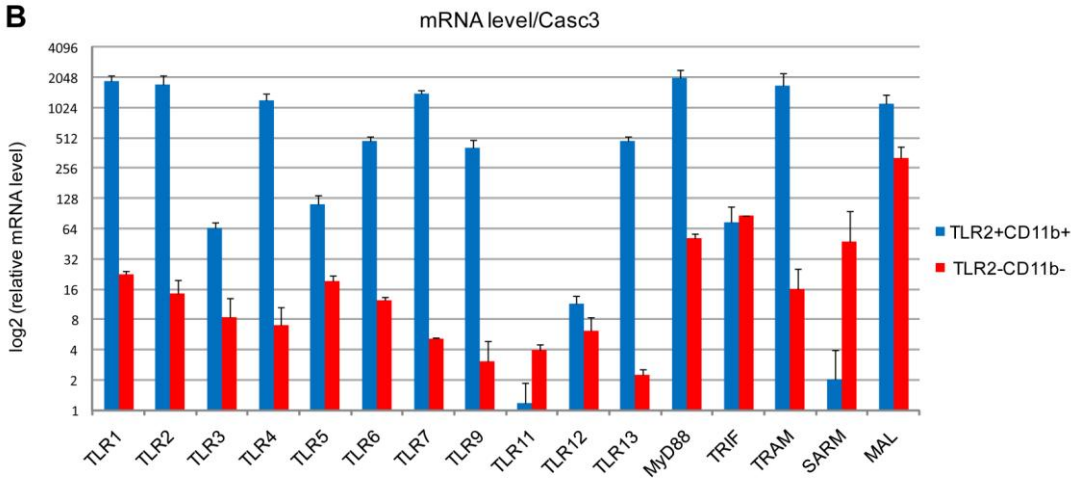


Figure 4.3. Expression levels of TLRs and TLR adaptors in E10,5 murine embryonic phagocytes. A. $CD11b^+$ $TLR2^+$ cells (blue square) and $CD11b^-$ $TLR2^-$ (red square) were sorted out from a single cell suspension of a whole embryo using FACS sorter. **B**. mRNA expression was assessed using qRT-PCR and normalized to Casc3 mRNA levels.



4.2. Distinction between embryo- and maternally-derived phagocytes

To investigate whether TLR positive phagocytes present in embryos are indeed of embryonic origin or represent adult myeloid cells derived maternally, we crossed wild-type (WT) ICR female mice with transgenic B6CF2XB6 male mice expressing EGFP under β-actin promotor (tg-EGFP) and their E10.5 embryos were analysed. Cells of embryonic origin should be $EGFP^+$ (as all embryonic cells carry the EGFP allele from a male parent), whereas maternally-derived cells (migrating from $EGFP^-$ female parent) are EGFP negative. However, as illustrated in figure 4.4 (left panel), our experiments showed that $EGFP^+$ cells comprise only about 50-75% of total embryonic cells. This number has not increased even when both parents used for mating were transgenic for EGFP (data not shown). Thus, due to developmental and/or epigenetic

regulation of the transgene expression we were not able to distinguish between the embryo-derived cells that loss or never retained EGFP expression and those migrated to embryo from EGFP negative mother. Inspite of this obstacle, when we gated on live cells, it became obvious that the vast majority of TLR2^{+} phagocytes, also positive for F4/80 and CD45 are of embryonic origin, as they are EGPF positive (figure 4.4, middle and right panel).

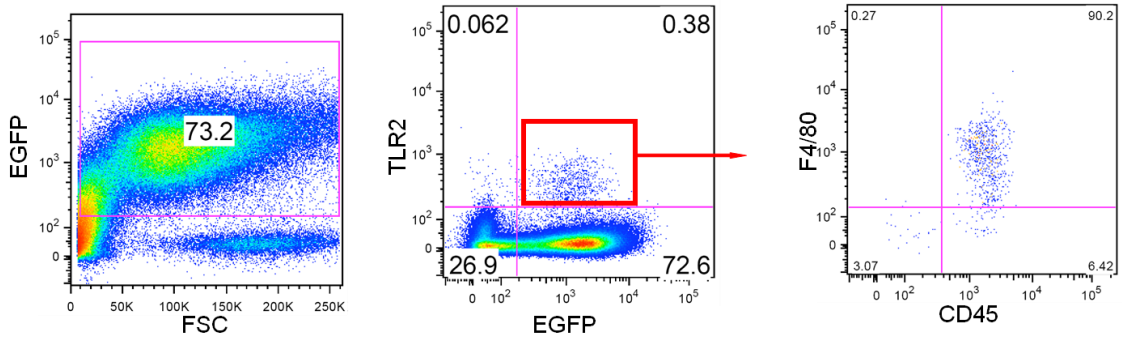


Figure 4.4. The majority of embryo-derived phagocytes are of embryonic origin. To distinguish maternal and embryo-derived phagocytes, WT female mice were crossed with males expressing EGFP under β-actin promoter. Suspension of cells from E10.5 embryos was prepared using dispase treatment. Cells were stained with anti-TLR2-Alexa 647, anti CD45-PerCP and anti-F4/80-PECy7 and the fluorescence was measured by LSRII FACS. Embryonic cells express EGFP while maternal cells invading the embryo don't.

To obtain an insight into the kinetics of appearance of embryonic-derived TLR2-positive phagocytes in early embryos, we performed FACS analysis of EGFP^{+} embryos from mating between the WT females and tg-EGFP males at different stages of development (figure 4.5). Results obtained demonstrate that embryonic TLR2^{+} cells appear in the embryo at around day 8.5-9.5 and their frequency then continually increases from approximately <0.1% (E8.5) to ~2% in E13.5. On the other hand, while the maternally derived EGFP^{-} TLR2^{+} population is much less abundant compare to EGFP^{+} TLR2^{+}, we cannot exclude the possibility that at least some portion of it comes from embryonic-derived EGFP^{+} TLR2^{+} cells that lost or never retain the EGFP expression.

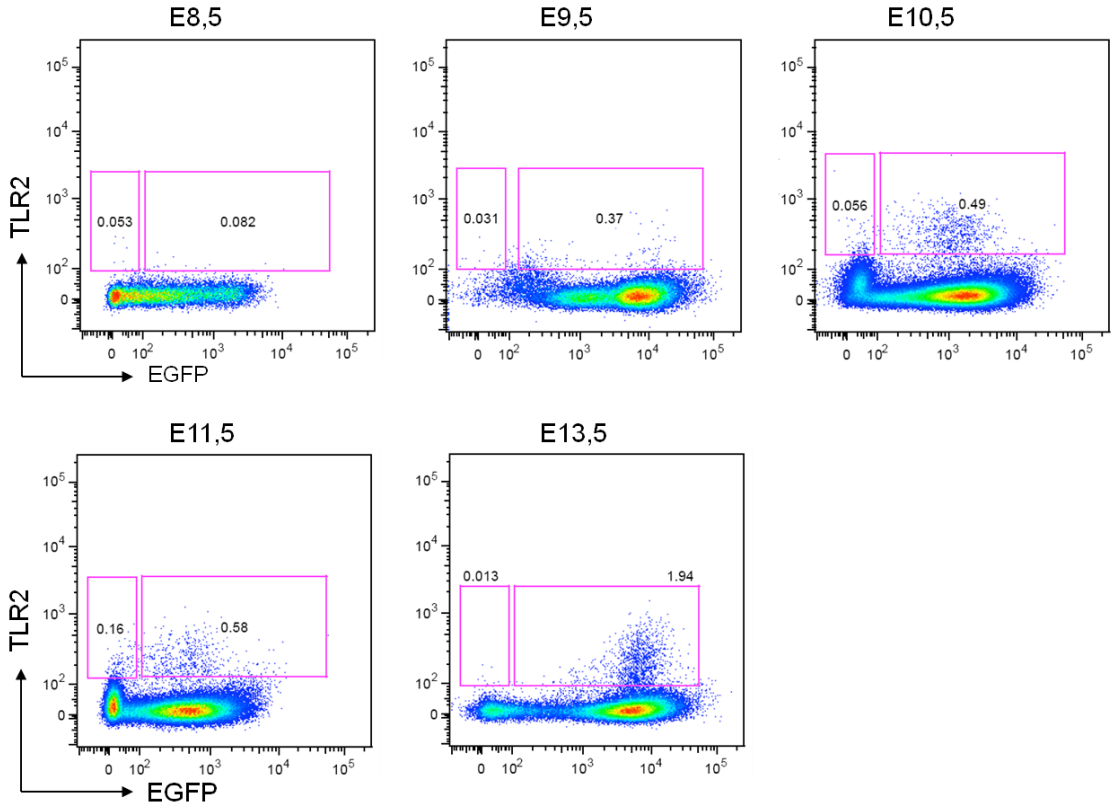


Figure 4.5. Embryonic TLR2$^+$ cells start to populate the embryo at around E9.5. Wt female mice were crossed with transgenic males expressing EGPF under β-actin promoter. Heterozygous EGFP$^+$ embryos at different stages of development were collected and stained with anti-TLR2 antibody. Fluorescence was measured by LSRII FACS. EGFP positive cells were considered to be embryonic. The proportion of EGFP$^+$ TLR2$^+$ (embryonic-derived) and EGFP$^-$ TLR2$^+$ cells is indicated in the purple rectangle in the middle and left part of each dot plot, respectively.

A series of complementary experiments with matings between tg-EGFP females and WT male that mapped the kinetics of appearance of maternally-derived, adult EGFP-positive phagocytes in early embryos, were performed by my colleague in the lab, Jana Oujezdská. I will briefly comment on these results in the discussion.

4.3. Negative regulators of TLRs are expressed in early stages of mice development

4.3.1. Expression profile of ST2 and Sigirr

Our analysis of expression of innate immune molecules in early embryos also included other genes containing TIR signaling domain. Specifically, we determined the expression profile of two transmembrane receptors ST2 and Sigirr, negative regulators of TLRs signaling (section 2.4.4.1). RNAs from whole embryos derived from time-pregnant females, were reverse transcribed and expression levels of ST2 and Sigirr were analyzed using qRT-PCR. Surprisingly, while the kinetics of expression of ST2 gradually increases from very low levels at day 7.5-9.5 up to 20-fold in day 12.5 (figure 4.6 A), mRNA level of Sigirr exhibited completely opposite trend reaching its maximum at day 7.5 and staying on its minimum between day 9.5-12.5 of embryonic development (figure 4.6 B). Different expression profiles of ST2 and Sigirr provided thus further evidence that these molecules fulfill distinct and very specific functions during early embryogenesis.

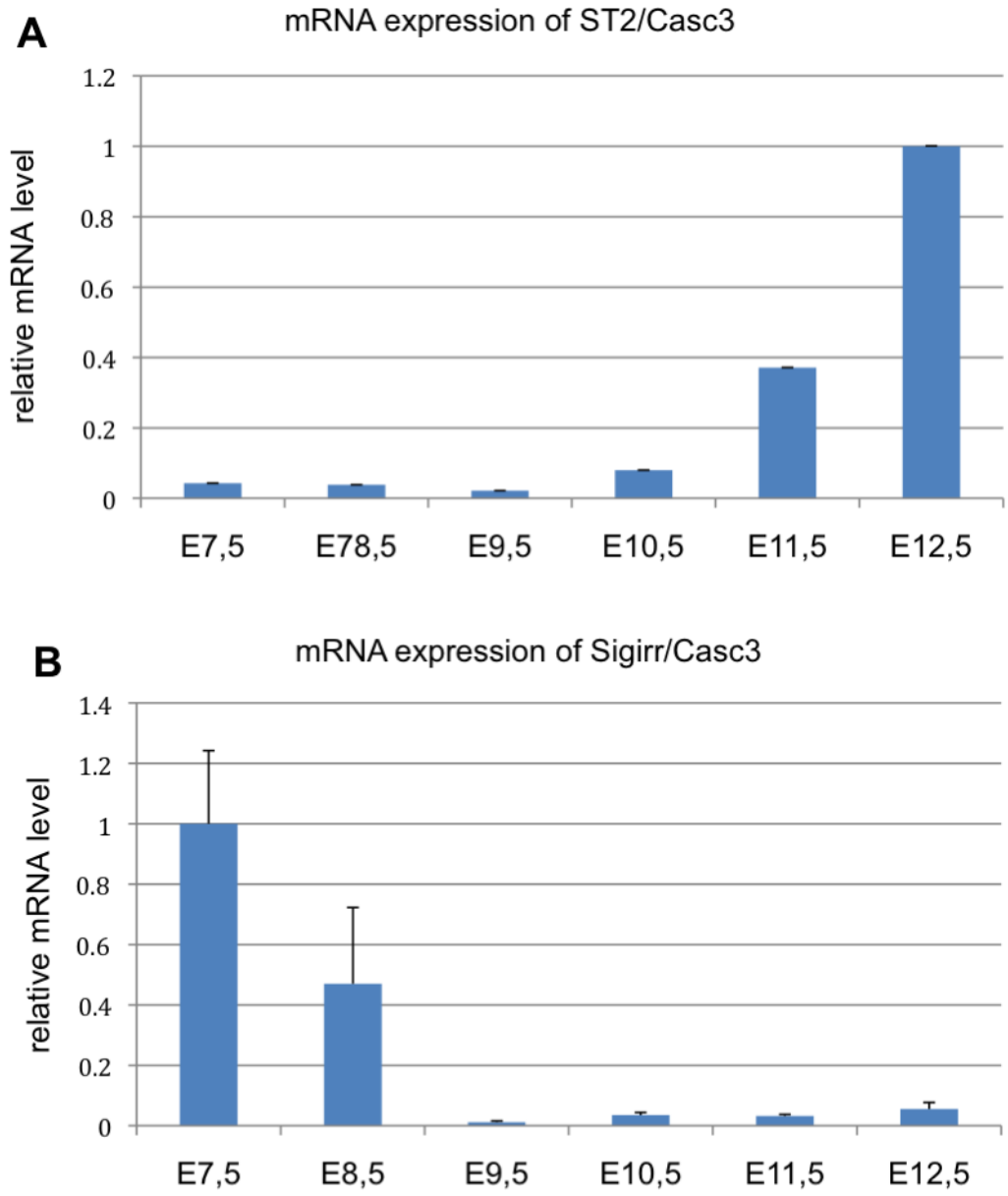


Figure 4.6. Distinct kinetics of gene expression of ST2 and Sigirr, two negative transmembrane regulators of TLR signaling during early stages of mice development. Expression levels of transmembrane form of ST2 (A) and Sigirr (B) were assessed using the total mRNAs isolated from whole embryos at indicated day of embryonic development followed by qRT-PCR. Expression levels were normalized to Casc3 mRNA.

4.3.2. Sigirr is expressed in CD11b^{+} TLR2^{+} cells

Above described results showed that Sigirr is highly expressed in early stages of development (E7.5-8.5) and then its expression temporarily ceases (E9.5-12.5). This prompted us to assess its cellular source in developing embryos. Towards this end, we first looked at Sigirr expression in sorted CD11b^{+} TLR2^{+} embryonic phagocytes as compared to CD11b^{-} TLR2^{-} double-negative embryonic cells derived from E10.5 embryos, e.g. when Sigirr expression is on its minimum. Interestingly, qRT-PCR analysis revealed its relatively high expression in double-positive cells (DP) compared to a low expression in double-negative (DN) sorted cells (figure 4.7).

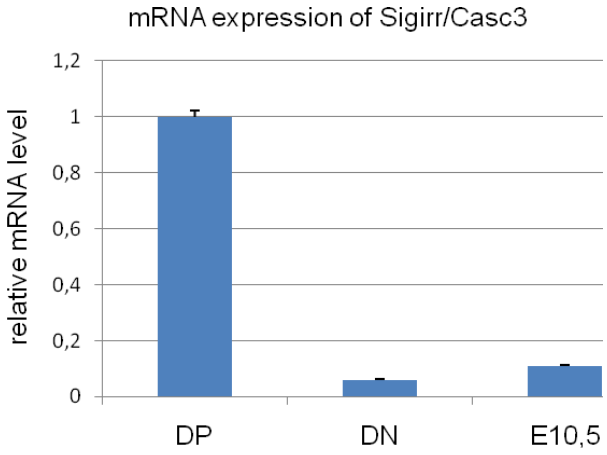


Figure 4.7. Sigirr is expressed in E10.5 embryonic phagocytes. Sigirr mRNA expression in CD11b^{+} TLR2^{+} cells (DP), CD11b^{-} TLR2^{-} (DN) and a whole embryo (E10.5) was assessed using qRT-PCR and normalized to Casc3 mRNA levels.

A very low frequency of embryonic phagocytes in E7.5 and 8.5 embryos disallowed us to sort CD11b/TLR2 double positive and negative cells and thus identify the main source of Sigirr during these stages of embryonic development using qRT-PCR. To circumvent this limitation, we used an alternative approach described in the next section.

4.3.3. Sigirr whole-mount immunohistochemistry of E7.5 embryo

The whole-mount immunohistochemistry was performed on 7.5 day old embryos as described in the Methods section (3.2.7) The staining with two goat antibodies, one directed against brachyury gene product, used here as a positive control, and the other specific for Sigirr, was followed by HRP-conjugated rabbit anti-goat IgG. Brachyury is a protein encoded by the *T* gene, which is required for normal mesoderm development and definition of body axis. In mice, it is expressed in the inner cell mass of blastocyst and later, by the cells forming the primitive streak of embryo (figure 4.8 C). As illustrated in the figure 4.8 A, Sigirr expression on a protein level is readily detectable throughout the entire mouse embryo including its extraembryonic parts such as the yolk sac (YS). To our best knowledge this is the very first report on spatio-temporal expression profile of Sigirr in early embryo. While its role in embryonic development remains enigmatic, our expression data are indicative of its important physiological role as the global negative regulator of inflammation during early stages of embryogenesis.

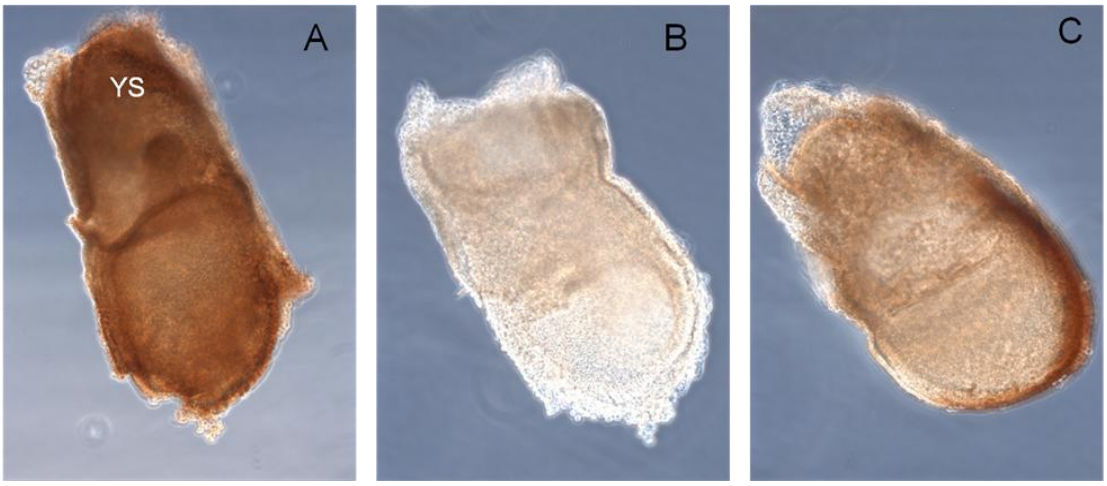


Figure 4.8. Immunohistochemistry of whole E7.5. (A) anti-Sigirr, (B) negative control, (C) anti-Brachyury. Leica DM6000B, magnification 10x.

4.4. Microarray analysis of CD11b$^+$ TLR2$^+$ E10.5 embryonic cells

Number of suitable markers available to study and isolate CD11b$^+$ TLR2$^+$ embryonic phagocytes is limited. To identify novel useful determinants of these cells we compared their expression profile with the profile of adult peritoneal macrophages and CD11b$^-$ TLR2$^-$ embryonic cells.

4.4.1. Comparative microarray analysis of CD11b$^+$ TLR2$^+$ E10.5 embryonic cells and CD11b$^+$ TLR2$^+$ peritoneal macrophages

In the first step, to identify genes expressed in myeloid cells that are preferentially expressed during early stages of development, we compared expression profiles of CD11b$^+$ TLR2$^+$ double-positive (DP) cells sorted from 10.5 day old embryos and CD11b$^+$ TLR2$^+$ adult peritoneal macrophages (pM). The microarray analysis was performed using the Mouse-WG6-v2 Expression BeadChip Kit (Illumina). Analysis of data initially identified more than 100 genes with significantly upregulated expression (≥8-fold) in embryonic macrophages. Gene ontology analysis showed that these genes participate in a wide spectrum of biological processes, mostly in the metabolism, cell cycle, surface receptor signaling and signal transduction and cytoskeletal organization (figure 4.9).

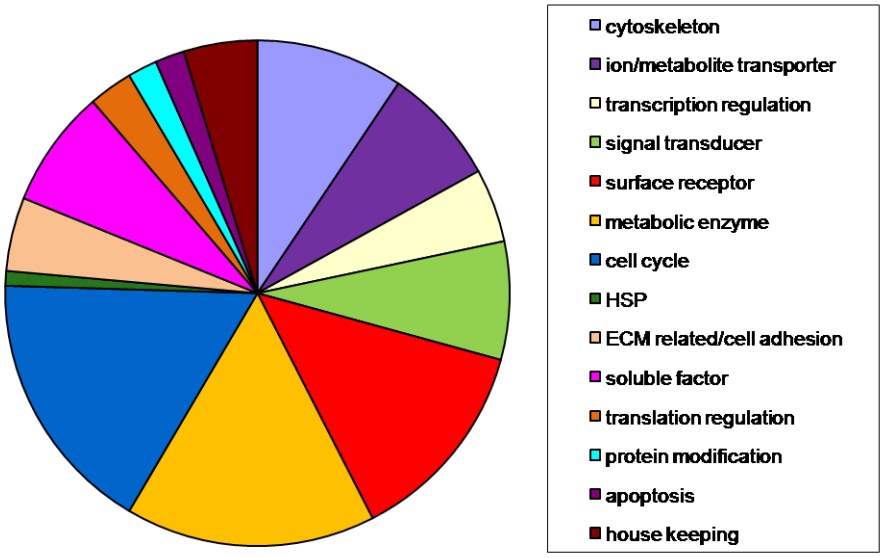


Figure 4.9. Biological process ontology of genes highly upregulated in CD11b$^+$ TLR2$^+$ E10.5 embryonic cells (DP) as compared to CD11b$^+$ TLR2$^+$ peritoneal macrophages (pM) . Microarray analysis identified 107 transcripts with significantly enhanced expression in the embryonic cells (unadjusted p-value ≤0.03, fold change ≥ 8). The functional assignment of selected genes were analysed using the software David and SwissProt database available on EXPASy Proteomic Server.

4.4.2. Comparative microarray analysis of embryonic E10.5 CD11b$^+$ TLR2$^+$ and CD11b$^-$ TLR2$^-$ cells

The above described microarray analysis identified genes highly enriched in CD11b$^+$ TLR2$^+$ embryonic phagocytes compared to adult peritoneal macrophages (pM). However, it precluded us to reveal those genes that are highly expressed in CD11b$^+$ TLR2$^+$ double-positive (DP) embryonic phagocytes and not in the remaining cells of developing embryo. For example, if a particular gene is expressed in both DP embryonic phagocytes and pM, but not in CD11b$^-$ TLR2$^-$ double negative (DN) embryonic cells, it would escape the previous screen. To rectified this deficiency, we performed an additional microarray analysis comparing expression profile of DP with DN embryonic cells isolated from E10.5 embryo. The analysis of data was performed as described for the previous microarray (section 4.4.1, figure 4.9). In total, 218 genes with at least eight-fold difference in expression level were found significantly upregulated in embryonic DP cells as compared to DN cells. Their biological process ontology analysis is presented in the figure 4.10. Interestingly, there is a significant increase in the proportion and the total number of signal transducing molecules

revealed by this microarray as compared to the previous one. This is not surprising, as DP embryonic phagocytes and DN embryonic cells represent developmentally and functionally different cell types and should utilize distinct set of signaling modules while DP embryonic phagocytes and adult peritoneal macrophages both belong to myeloid lineages which related signaling pathways.

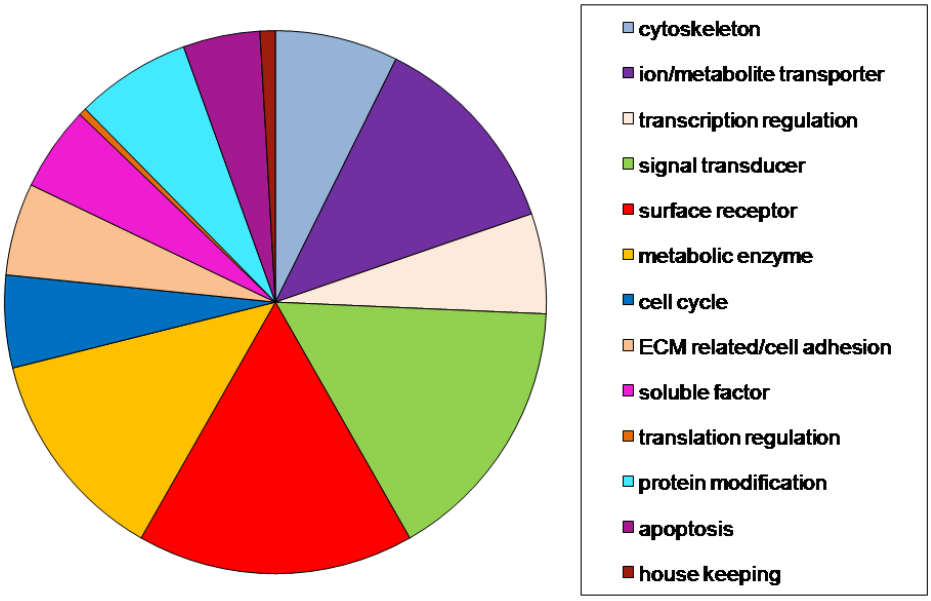


Figure 4.10. Microarray analysis of CD11b$^+$ TLR2$^+$ and CD11b$^-$ TLR2$^-$ E10.5 cells. Based on CD11b and TLR2 antibody staining profile, E10.5 embryonic cells were sorted and the mRNA profiles of double-positive and double-negative cells were compared using Mouse-WG6-v2 Expression BeadChip Kit. 218 transcripts were considered to be significantly enriched in embryonic CD11b$^+$ TLR2$^+$ cells according to selected criteria (Partek software, unadjusted p-value $\leq$ 0.03; fold change $\geq$ 8). The functional assignment of selected genes were analysed using the software David and SwissProt database available on EXPASy Proteomic Server.

Our main aim was to find suitable markers of embryonic phagocytes that would allow us to discriminate them from embryonic DN cells and potentially also from adult macrophages. A simple comparison of the two microarray datasets of genes has revealed that there are 21 highly upregulated genes in embryonic CD11b$^+$ TLR2$^+$ cells which exhibit significantly lower expression in both CD11b$^+$ TLR2$^+$ peritoneal macrophages and CD11b$^-$ TLR2$^-$ embryonic cells (figure 4.11 and table 4.1).

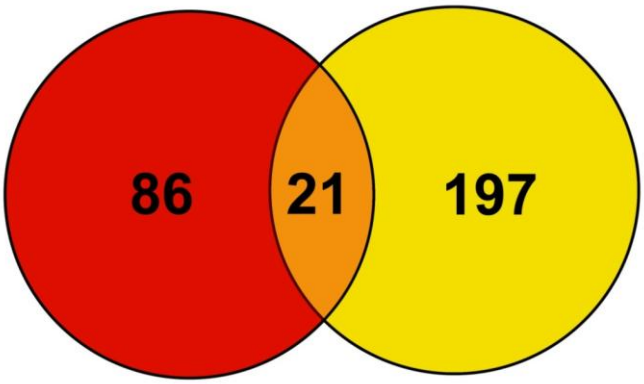


Figure 4.11. Comparative analysis of microarray data revealed 21 genes with high expression in CD11b$^+$ TLR2$^+$ double positive (DP) E10.5 cells. DP E10.5 cells were sorted and their mRNA profiles were compared to mRNA profiles of CD11b$^+$ TLR2$^+$ peritoneal macrophages (pM) or embryonic CD11b$^-$ TLR2$^-$ double-negative (DN) cells. 107 and 218 transcripts were significantly enriched in embryonic DP cells as compared to pM (red circle) and DN (yellow circle), respectively. In total, 21 genes upregulated in DP cells were significantly downregulated in both pM and DN (see the list of genes in the table 4.1).

Table 4.1. Genes specifically enriched in CD11b$^+$ TLR2$^+$ E10.5 cells. Gene annotation was performed using NCBI Gene Entrez database (http://d360prx.biomed.cas.cz:2259/gene) and the David software.

gene	official full name	surface	function/characteristization
Aif1	Allograft inflammatory factor 1	no	organelle, cytoskeleton organization and biogenesis, role in M∅ activation and function
Asb2	Ankyrin repeat and SOCS box protein 2	no	protein modification, protein ubiquitination
Ccl3	C-C motif chemokine 3	secreted	small chemokine, cytokine-cytokine receptor interaction, Toll-like receptor signaling pathway
Ccl4	C-C motif chemokine 4, MIP-1beta	secreted	chemotaxis, cytokine-cytokine receptor interaction, Toll-like receptor signaling pathway
CD79b	B-cell antigen receptor complex-associated protein beta-chain	yes	cell surface receptor linked B signaling pathway
COR1a	Coronin 1a	no	actin binding protein
Cx3CR1	Chemokine (C-X3-C) receptor 1, Fractalkine receptor	yes	G protein coupled receptor activity, CX3C Fractalkine chemokine receptor
Eg245190	Putative uncharacterized protein Fragment	no	leucin rich domain
GAS-6	Growth arrest-specific protein 6 Precursor	yes	cell morphogenesis, regulation of cell growth, regulation of cell size
Gpr84	G protein-coupled receptor 84	yes	Rhodopsin-like GPCR superfamily, G-protein coupled receptor activity, signal transducer activity

Gpr86	P2Y purinoceptor 13 (P2Y13),G-protein coupled receptor 86	yes	Rhodopsin-like GPCR superfamily, UDP-glucose receptor, P2Y13 purinoceptor
IGH-6*	Immunoglobulin heavy chain C gene segment	yes	antigen binding, signal transducer activity, receptor activity
MPO	Myeloperoxidase	no	heme protein synthesized during myeloid differentiation that constitutes the major component of neutrophil azurophilic granules, produces hypohalous acids central to the microbicidal activity of neutrophils
MRC1	Macrophage mannose receptor 1	yes	mediates the endocytosis of glycoproteins by macrophages, binds both sulfated and non-sulfated polysaccharide chains, phagocytic receptor for bacteria, fungi and other pathogens
Msr2	Fc receptor-like S	yes	scavenger receptor
Ptgds2*	Glutathione-requiring prostaglandin D synthase	no	organic acid metabolic process, lipid metabolic process, fatty acid metabolic process
SlcO2B1	Solute carrier organic anion transporter family member 2B1,Slc21a9	yes	organic anion transporter polypeptide OATP, mediates the Na+-independent transport of organic anions such as taurocholate, the prostaglandins PGD2, PGE1, PGE2, leukotriene C4, thromboxane B2 and iloprost
Slc40A1*	Ferroportin-1	yes	iron ion binding, transition metal ion transport
Slc46A3*	Solute carrier family 46 member 3	yes	transmembrane transport
STA1	Stabilin-1	yes	scavenger receptor for acetylated low density lipoprotein, binds to both Gram-positive and Gram-negative bacteria and may play a role in defense against bacterial infection, blood vessel development, receptor-mediated endocytosis, scavenger receptor activity
TnnI2*	Troponin I	no	striated muscle thin filament, actin cytoskeleton

*So far, these genes have not been analysed by qRT-PCR (see the text for details).

Using the NCBI Gene Entrez database and the software David, we have annotated all 21 genes based on available information relevant to each particular gene specific ontology (function, localization and biological process) (table 4.1). To validate the expression data obtained from mRNA microarrays, we have so far performed qRT-PCR analysis on 16 out of 21 of these genes. Specifically, we have compared the expression level of each particular gene in DP and DN E10.5 cells (data not shown). In total, 11 genes were confirmed to display an enhanced expression in embryonic $CD11b^{+}$ $TLR2^{+}$ but not in embryonic $CD11b^{-}$ $TLR2^{-}$ and were chosen for next analysis.

To get a more complex insight into the expression pattern of these 11 genes, we simultaneously measured their relative expression levels in sorted embryonic DP and DN cells and also in the population of the adult peritoneal macropages (pM). As

illustrated in figure 4.12, our analysis confirmed significant, 50 up to more than 1000-fold differences in gene expression levels between DP and DN E10.5 cells. Based on the scale of this difference, selected genes could be split into two groups. The first group involves CX3CR1, Mrc1, Grp86, Grp84, Aif1, Ccl4 and Msr2, genes with 500-1000 fold difference in expression while this difference in the remaining gene are somewhere between 50-100 fold. Differences in the expression were less pronounced between DP E10.5 cells and pM, however, with a few exceptions, they also exhibited at least 8-fold enrichment in the DP cells. Only in case of two out of 11 selected genes, COR1a and Msr2, the 8-fold difference in expression between DP embryonic cells and pM (default threshold set up in microarray data analyses) was not confirmed.

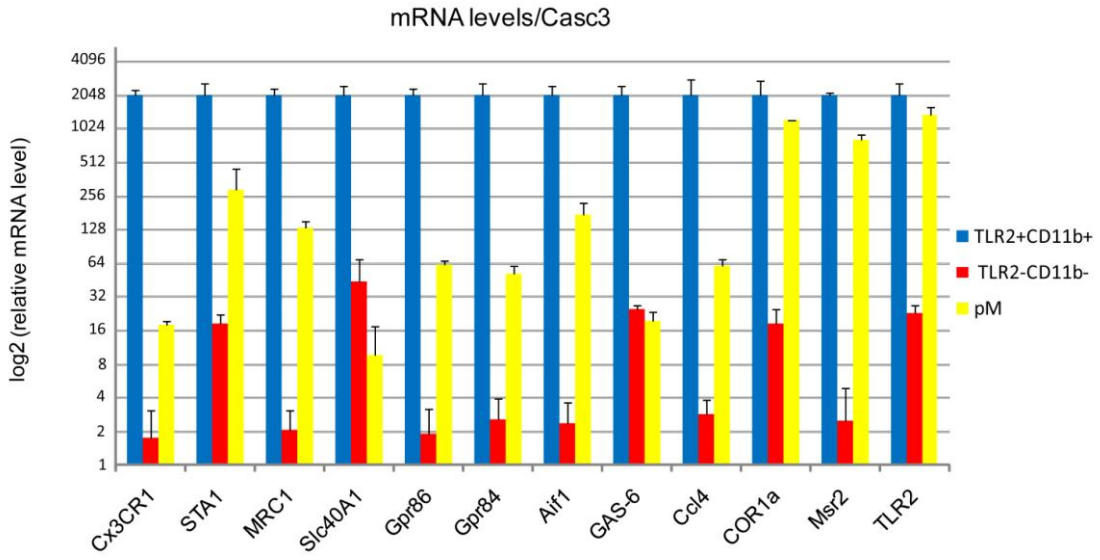


Figure 4.12. Potential markers of embryonic phagocytes. $CD11b^{+}TLR2^{+}$ (DP) cells and $CD11b^{-}TLR2^{-}$ (DN) were sorted from whole embryo (E10.5) using FACS sorter. mRNA expression of selected genes was assessed on DP- and DN cells as well as on peritoneal macrophages (pM) using qRT-PCR. Expression levels were normalized to Casc3 mRNA levels. TLR2 served as the positive control.

However, it is important to emphasize that regardless of their level of expression in pM, the solely criterium for each of these genes to become a useful marker of embryonic phagocytes is its highly enriched level of expression in $CD11b^{+}$ $TLR2^{+}$ embryonic phagocytes as compared to $CD11b^{-}$ $TLR2^{-}$ embryonic cells.

To assess the potential of 11 selected genes to serve not only as generic markers of embryonic phagocytes but also as specific biomarkers of different embryonic phagocyte lineages, we have established the kinetics of their mRNA expression within the period of early embryogenesis. The rationale behind this analysis is that as the maternally- and the two YS-derived macrophage lineages appear in the embryo sequentially, one after the other between day 7.5 to 9.5/10.5 (see section 2.3.2.1 for details), divergent kinetics of gene expression among selected genes would indicate their bias for lineage-specific expression. The expression profile of Sigirr served as the positive control. As presented in the figure 4.13, the general trend of Sigirr expression (high in E7.5-8.5, and low in E9.5-12.5) corresponded to its previously established profile (section 4.3.1, figure 4.6 B).

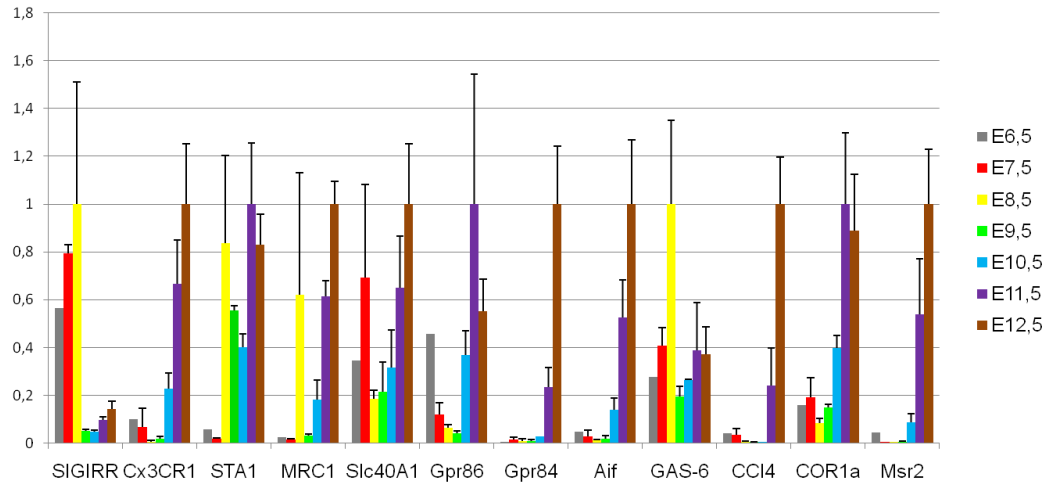


Figure 4.13. The kinetics of expression of selected potential markers of embryonic macrophages. mRNAs isolated from embryos at different stages of mice development were reverse transcribed using SuperScript™ III Reverse Transcriptase. qRT-PCR was performed using LightCycler® 480 SYBR Green I Master and expression of selected genes was normalized to Casc3 mRNA levels. Sigirr served as the positive control. A standard deviation of E6.5 values is not indicated because the measurement was performed on a single sample (see the text for details).

Figure 4.13 illustrates distinct expression profiles among genes tested. Five out of 11 genes: Sta1, Mrc1, GAS-6, Slc40A1, and to a certain extend also COR1a exhibited a bimodal distribution of gene expression with the first peak reaching maximum on day 7.5-8.5, then falling on day 9.5-10.5 and then raising again. It is surprising that similar to Sigirr gene, Slc40A1, Grp86, GAS-6 and partially also COR1a genes showed a relatively high expression already in 6.5 day old embryo. The remaining seven genes showed a time-dependent, gradual enhancement of gene expression with a maximum increase between 10.5 and 11.5 day of development. Thus, differential kinetics of expression of 11 selected genes in early stages of ontogenesis suggests that they could be used as lineage-specific markers of embryonic phagocytes. It is of note that gene expression analysis (figure 4.13) conducted on the sample derived from 6.5 day old embryos was performed in a single measurement for each gene due to an insufficient amount of isolated RNA (counting in total about 50 embryos from a cohort of several time-pregnant female mice).

4.5. Experiments in progress

4.5.1. *In vivo* gene silencing in embryo

To investigate the function of selected genes in development, in the maintenance of early embryonic homeostasis and physiology of phagocytes, we conducted a series of experiments to downregulate their expression in embryos using short hairpin (sh) RNAs (177). For this purpose we used a recently described protocol based on the use of pSIREN-DNR-DsRed-Express plasmid *(Clontech)* (section 3.2.11). As described in several papers devoted to this approach, plasmid carrying shRNA specific for a gene under investigation is injected into time-pregnant mice and through the circulation is delivered to developing embryos where the encoded shRNA is ubiquitously expressed causing silencing in gene-specific manner (177, 181). Advantage of this system is that intensity of fluorescent signal (DsRed protein) usually correlates with the severity of transcript knock-down. Our preliminary data are not as positive as expected because the microscopic examination of embryos derived from plasmid or PBS injected pregnant females revealed no difference in their fluorescent intensity. However, as illustrated in the figure 4.14, when a single cell suspension was

prepared from these embryos, a small population of red-positive embryonic cells from plasmid injected, but not PBS injected mice, was observed upon FACS examination. Thus, further optimization of this protocol is needed for successful application of this method.

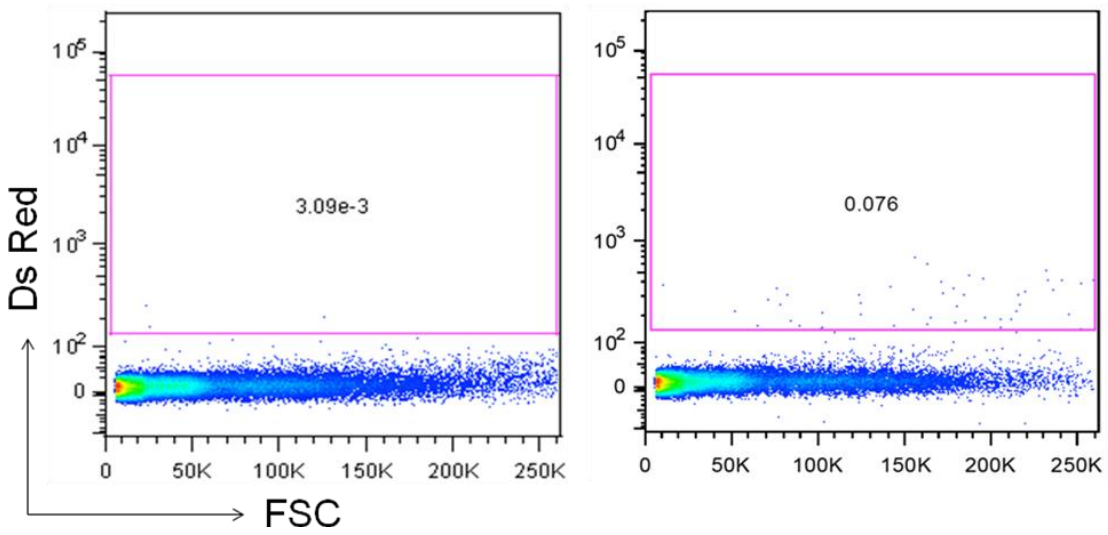


Figure 4.14. Expression of DsRed fluorescent protein in cells derived from E10.5 embryos developing in time-pregnant females injected with PBS (left) or DsRed expressing plasmid (right). Fluorescent intensity was measured using LSRII equipment.

4.5.2. Embryo whole-mount in situ hybridization of TLRs

Currently, we are mapping the spatial expression profile of some of TLRs in the embryo. The rationale for this type of experiment stems from TLRs differential kinetics of expression described in this work. In this context, it would be very interesting to determine which TLRs are expressed in the head region of developing embryos. In addition, remarkable differences in spatio-temporal kinetics of appearance of TIR-domain containing proteins such as TLR4 and Sigirr warrant further investigation in this line of research.

4.5.3. Embryo whole-mount immunohistochemistry

At present, we are also testing whether antibodies commercially available to some of those 11 selected genes could be used as lineage-specific markers of embryonic phagocytes.

5. Discussion

Phagocytes are considered to be among the oldest cell types in animal kingdom (182) and were studied by Elie Metchnikoff more than a century ago. One of the main goals of phagocyte research conducted throughout the last three decades was to clarify the origin of well-known adult phagocytes in embryo. Accumulated data indicated that phagocytes which appear in early mice development (E7.5-E12.5) represent a unique embryonic macrophage lineage that differs from adult macrophages phenotypically, biochemically and by their origin. However, the latest studies suggested that there are at least three waves of macrophages populating an early embryo: a maternally-derived one and two waves of extraembryonic, YS-derived phagocytes. Moreover, the origin of embryonic phagocytes appearing in an anterior head mesoderm before the establishment of any circulation remains obscure. While both head mesoderm- and yolk sac-residing phagocytes are able to proliferate, the relationship between theses populations has not yet been established.

The main goal of our investigation presented in this thesis was to identify a set of surface markers expressed on embryonic phagocytes suitable for phenotypic distinction among distinct lineages of embryonic phagocyte populations. On the basis of our previous unpublished data showing expression of TLR4 in early stage of mouse development (figure 2.9), we characterized the expression of a set of TLRs and other TIR domain-containing signaling molecules on mRNA and protein levels during early embryogenesis, between E7.5 and E12.5 (figure 4.1-4.8). As illustrated in figure 4.1, all TLRs tested (except TLR8 which was excluded due to technical problems) and their adaptor proteins are expressed in early stages of embryonic development.

We observed two distinct types of kinetics profiles of TLRs and other TIR domain-containing molecules: either a bimodal distribution or a gradually increasing gene expression over time. Interestingly, a common feature of genes associated with

these types of kinetics profile is a pronounced, very low expression on 9.5 day of development. This observation correlates well with our own data derived from matings between wild-type male and transgenic B6CF2XB6 female mice expressing EGFP under β-actin promoter, which showed that $TLR2^+$ macrophages present during the very early stages of mouse development (E7.5) are maternally-derived and this population rapidly disappears (Oujezdská et al., unpublished). Thus, we explain the rapid decrease of expression of TLRs and other TIR domain-containing molecules at day 9.5 as a direct consequence of disappearance of the transient population of extraembryonic maternally-derived macrophages. On contrary, and as demonstrated in this work (section 4.2 and figure 4.5), the embryo-derived $TLR2^+$ macrophages begin to appear in the embryo around day 9.5, a day later than the second wave of committed macrophage precursors described by Bertrand et al. (69). We believe that these two populations of cells are identical by their ontogenetic origin, and a discrepancy between 8.5 and 9.5 day of their appearance is caused by the fact that Bertrand at al. detected these cells in the YS, whereas the population of cells prepared for our analysis was derived directly from embryonic tissues. Thus, as phagocyte population is the major cellular source of TLRs and TIR domain-containing molecules, their lowest expression levels on day 9.5 results from the culmination of disappearance of maternally-derived phagocytes on one hand and initial low levels of just appearing, embryo-derived $TLR2^+$ phagocytes, on the other hand.

To conclude that TLRs and their adaptor proteins are present predominantly on the population of phagocytic cells, we analysed their expression on embryonic phagocytes positively sorted for phagocytic marker CD11b and TLR2 (figure 4.3) as well as on non-phagocytic cells. While the most of TLRs and their adaptors are indeed highly enriched in DP embryonic cells, these cells are certainly not their exclusive source. Surprisingly, non-phagocytic cells also express these molecules, albeit, in general, at lower levels. However, because embryonic non-phagocytic cells represent up to 99% of the total population of embryo, their contribution to a total pool of these receptors and adaptors is quite significant. That is especially true for TLR 3, 5, 11 and 12 and adaptor proteins MAL, TRIF and SARM. Whether the low expression levels of TLRs and their adaptor proteins is characteristic for all non-phagocytic cells or it is cell-type restricted, remains to be elucidated.

Because of the striking fact that TLRs, recognizing molecular structures specific for microbial pathogens, are expressed in early embryo which develops in

pathogen free environment, it suggests that TLR signaling is involved in the regulation of embryonic homeostasis and recognition of endogenous ligands leading to non-infectious, sterile inflammatory and immune responses. However, due to potential contamination by LPS of molecules derived from potential endogenous ligands used in several studies, the direct involvement of TLRs in recognition of endogenous ligands has never been conclusively proven. Resolving this issue is of immense importance because it appeared that many diseases like rheumatoid arthritis, ankylosing spondylitis, psoriasis, Crohn´s disease or systemic lupus erythematosus are caused by sterile inflammation and their treatment with inhibitors of TLR signaling would potentially provide a cure (7). This notion is gaining a momentum, as it has been recently shown that TLR4 and TLR6 heterodimer is involved in CD36-mediated signaling to endogenous ligands (8).

On the other hand, however, it can´t be excluded that TLRs expressed in embryos play a microbe-recognizing role, the same role as in adults. When an infection occurs by transplacental transmition, pathogens can be recognized by TLRs which trigger signaling pathways leading to the expression of molecules which support the local inflammation and thus curbing the infection. This scenario found its support in experiments conducted on the zebrafish embryo where the ability of early embryonic macrophages to engulf both gram-negative and gram positive bacteria and thus protect the embryo against infection, before the appearance of any lymphocytes, has been demonstrated (73). Our own experiments conducted on murine embryonic macrophages also demonstrated their ability to phagocytose opsonized bacteria (Fišerová, unpublished results).

Moreover, in this work we report an interesting finding that the transmembrane negative regulator of TLR signaling, Sigirr, is highly expressed in very early stages (E6.5 to 8.5) of mouse development and then its expression rapidly decreases (figure 4.6). The immunohistochemistry showed that Sigirr is evenly distributed throughout the E7.5 embryo (figure 4.8). When we tested its expression on sorted cells derived from E10.5 embryo, e.g. when its expression is downregulated, it turned out that Sigirr is expressed at higher levels in $CD11b^{+}$ $TLR2^{+}$ phagocytes than in $CD11b^{-}$ $TLR2^{-}$ non-phagocytic cells (figure 4.7). We found that Sigirr-positive $CD11b^{+}$ $TLR2^{+}$ embryonic phagocytes also express mature macrophage marker F4/80 (figure 4.2 and 4.7) and that F4/80-positive adult peritoneal macrophages express Sigirr as well (data not shown). These findings contrast with the information available from literature sources which

reference the adult macrophage population as Sigirr-negative (154). The source and reason for this discrepancy are at present unknown.

In adults, Sigirr is highly expressed on epithelial cells and immature dendritic cells where it is able to negatively regulate TLR4, TLR7 and TLR9 signaling. This notion is supported by experiments where Sigirr deficient mice showed increased susceptibility to tuberculosis or fungal infections in comparison to wild type mice (150, 151). Its unusually high expression during very early stages of embryogenesis suggests that it can function as a global negative inhibitor of inflammatory responses in this period of development. Sigirr may be involved in dampening of inflammation and tissue damage and thus prevent the embryo from inappropriate activation of inflammatory responses to endogenous and exogenous stimuli and thus in turn contribute to the maintenance of embryonic homeostasis. Relatively high expression of TLR adaptor proteins MAL and TRIF in embryonic non-phagocytic cells (figure 4.3) make them suitable candidates for playing role in Sigirr-mediated TLR-independent signaling. Alternatively, Sigirr can itself negatively affect TLR signaling pathways in $CD11b^{+}$ $TLR2^{+}$ embryonic phagocytes. However, the precise role of Sigirr in very early stages of embryonic development as well as its functional relationship to TLR signaling in these early stages of ontogenesis remains to be determined.

In addition to occurence of TLRs on embryonic phagocytes, the microarray analysis of $CD11b^{+}$ $TLR2^{+}$ cells isolated from the E10.5 embryos has revealed significantly upregulated expression of several novel genes in comparison to their expression in both mouse peritoneal macrophages and $CD11b^{-}$ $TLR2^{-}$ cells (table 4.1) These genes have a potential to serve as novel markers for detection and isolation embryonic phagocytes. We believe, that the characterization of these molecules will further advance our understanding of the function of innate immune molecules during embryonic development and will provide a valuable insight into functional properties of embryonic phagocyte subpopulations. Already demonstrated function of embryonic phagocytes is the elimination of apoptotic cells, but their ability to secrete a vast array of immune, neuroendocrine, reactive oxygen/nitrate mediators and growth factors that can modify and alter not only their own physiology but also the function of other cells suggests their important, yet undiscovered roles in embryonic development. Recent experiments conducted in our lab shed a new light on the ability of embryonic cells to produce variety of cytokines in response to TLR stimuli (Oujezdská, unpublished

results). Dissecting the signaling pathways and specific functions of these cytokines in early embryonic development will be one of the main themes of our future work.

6. Conclusions

- These results are first to characterize the temporal and spatial expression of TLRs and their adaptor proteins on early embryonic phagocytes. It also demonstrates expression of CD14 on these cells.
- Negative regulator of TLR signaling Sigirr is expressed in early stages of the mouse development and is evenly distributed throughout embryo.
- Embryonic $TLR2^+$ phagocytic cells appear in the embryo at around day 9.5 and they replace the maternally-derived macrophage population continually. At E10.5, the most of phagocytes are of embryonic origin; these cells are of $TLR2^+F4/80^+CD45^+$ phenotype.
- According to mRNA microarray and qRT-PCR data, there are at least 11 putative specific embryonic phagocyte markers which could allow discrimination of embryonic phagocytes from the maternal ones.

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