BACHELOR THESIS

Embryonal phagocytes: their identification, origin and functions

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Affirmation

I hereby declare that I have written this thesis independently, with the use of listed literature.

Příbram 10. 8. 2008

Tereza Vavrochová
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Abstract

Embryonal phagocytes represent a unique cell lineage which differ from those of adult macrophages phenotypically, biochemically and by their origin as they do not follow the monocytic pathway. In spite of the fact that this population is evolutionary very conserved from insects to mammals, there is still a paucity of information about their physiology and function. Critical is the lack of surface markers which would allow their reliable isolation with subsequent manipulation and microscopic visualization. Recent finding in our laboratory that Toll-like receptors (TLRs) and the macrophage differentiation marker CD14 are expressed in early stages of mouse development could represent a critical step towards overcoming this issue. The first part of this thesis summarises the current knowledge concerning the embryonal phagocytes, their origin, function a identification. The following part is focused on TLRs, their ligands, signaling and involvement of TLRs in defense responses of individuals to environmental pathogens. In the experimental section we briefly report the gene and protein expression analyses of innate immune molecules in the mouse 10.5 day old embryo. Specifically, qRT-PCR analyses detected relatively high expression levels of TLR2 and TLR4. FACS analysis of total single cell suspension showed that about 1.0% of cells were positive for the phagocytic surface marker CD11b. 75% of these cells exclusively were also positive for CD14, TLR2 and TLR4. These results are first to characterize the expression of TLRs in early stages of mammalian embryogenesis and suggest the existence of TLRs endogenous ligands.

Key words

Embryonal phagocytes, hematopoiesis, macrophages, innate immunity, Toll-like receptors, endogenous ligands, sterile inflammation.
Abstrakt

Embryonální fagocyty představují ontogeneticky samostatnou vývojovou buněčnou linii, která se liší od linie dospělých makrofágů fenotypově, biochemicky a původem, neboť neprochází stádiem monocytárního vývoje. Množství informací o fyziologii a funkci této populace buněk je stále omezené navzdory tomu, že je evolučně značně konzervovaná od hmyzu až k savcům. Jedním z hlavních problémů je zejména neznalost vhodných povrchových markerů, které by umožnily spolehlivou izolaci, manipulaci a mikroskopické pozorování těchto buněk. Výsledky našich pokusů ukázaly, že receptory skupiny Toll (TLRs), jakož i makrofágový diferenciační marker CD14, jsou exprimovány v raných stádiích vývoje myší. Tato zjištění by mohla představovat zásadní pokrok v řešení problému nedostatku povrchových markerů. První část této bakalářské práce se věnuje aktuální znalosti o embryonálních fagocytech, jejich původu, funkci a identifikaci. Následující část je zaměřena na TLRs, jejich ligandy, signalizaci a účast TLRs na obranných odpovědích jedince vůči vnějším patogenům. V praktické části stručně shneme analýzy genové a proteinové exprese molekul vrozeného imunitního systému v myším embryu starém 10.5 dne. Konkrétně qRT PCR analýzy prokázaly relativně vysokou expresi TLR2 a TLR4. Průtoková cytometrická analýza vykonaná na celkové buněčné suspenzi ukázala, že přibližně 1.0% buněk bylo pozitivní na povrchový fagocytearní marker CD11b. 75% těchto buněk bylo také pozitivní na CD14, TLR2 a TLR4. Tyto výsledky jako první popisují expresi TLRs v raných stádiích embryogeneze savců a poukazují na možnost existence jejich endogenních ligandů.

Klíčová slova

Embryonální fagocyty, krvetvorba, makrofágy, vrozená imunita, receptory skupiny Toll, endogenní ligandy, sterilní zánět.
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List of abbreviations

AGM  aorta- gonad- mesonefros
APC  antigen- presenting cell
APC-conjugated  allophycocyanin-conjugated
API  activator protein 1
CD  cluster of differentiation
cDNA  complementary DNA
C-FMS  colony-stimulating factor 1 receptor
CP  crossing point
CR  complement receptor
DC  dendritic cell
DNA  deoxyribonucleic acid
dsRNA  double-stranded ribonucleic acid
E  day of embryonic development
EA  erythrocyte antibody
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
FBS  fetal bovine serum
FITC  fluorescein isothiocyanate
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
HSC  hematopoietic stem cell
Hsp  heat-shock protein
IFN  interferon
Ig  immunoglobulin
IKK  IkappaB kinase complex
IL  interleukin
IL-1R  interleukin 1 receptor
IRAK  IL-1R-associated kinase
IRF  interferon regulatory factor
LBP  LPS binding protein
LPS  lipopolysaccharide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRR</td>
<td>leucine-rich repeat</td>
</tr>
<tr>
<td>LTA</td>
<td>lipoteichoic acid</td>
</tr>
<tr>
<td>Mac-1</td>
<td>macrophage-1 antigen</td>
</tr>
<tr>
<td>Mal</td>
<td>MyD88 adaptor-like protein</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen-activated protein</td>
</tr>
<tr>
<td>MARCO</td>
<td>macrophage receptor with collagenous structure</td>
</tr>
<tr>
<td>MD-2</td>
<td>myeloid differentiation protein 2</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MPS</td>
<td>mononuclear phagocyte system</td>
</tr>
<tr>
<td>MR</td>
<td>mannose receptor</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>OPN</td>
<td>osteopontin</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PRR</td>
<td>pathogen recognition receptor</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RES</td>
<td>reticuloendothelial system</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SARM</td>
<td>SAM and ARM-containing protein</td>
</tr>
<tr>
<td>SR</td>
<td>scavenger receptor</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single-stranded ribonucleic acid</td>
</tr>
<tr>
<td>TAB</td>
<td>TAK binding protein</td>
</tr>
<tr>
<td>TAK</td>
<td>TGF-beta activated kinase</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>T\textsubscript{H}</td>
<td>helper T-cell</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR domain-containing adaptor protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>Trif-related adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adaptor inducing interferon-beta</td>
</tr>
<tr>
<td>Ubc13</td>
<td>ubiquitin-conjugating enzyme 13</td>
</tr>
<tr>
<td>Uev1A</td>
<td>ubiquitin-conjugating enzyme E2 variant 1 isoform A</td>
</tr>
<tr>
<td>YS</td>
<td>yolk sac</td>
</tr>
</tbody>
</table>
1. Introduction

Phagocytosis, the engulfment of solid particles by cell membrane to form an internal phagosome or "food vacuole", is highly conserved process performed by unicellular organism and many cell types found in metazoans (1). The term "phagocyte" derived from the greek phagein (to eat) and kytos (cell) was proposed by Russian embryologist Ilya Metchnikoff (1845-1916), who discovered the phenomenon of phagocytosis (2). His seminal work with starfish larvae provided the very first evidence for a dual role of phagocytosis: in primitive organisms such as amoebae it 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 (2).

Metchnikoff fiercely defended his view that phagocytes were the main if not the only line of defence against microorganism, which led into a conflict with humoralists, who were focused on soluble factors in the blood (i.e. complement and antibody) to account for immune destruction (3). This fight was eventually resolved by positioning opsonins as a bridge between cellularists and humoralists by Almroth Wright in 1903 (3). Thus in 1908 Ilya Metchnikoff shared the Nobel Prize in Physiology or Medicine with Paul Ehrlich; this prize was awarded as a part of compromise to Metchnikoff for his discovery of the major types and functions of phagocytes as the basis of cellular immunity and to Ehrlich for his studies on antibodies and humoral immunity (4).

For recognition of conserved microbial components there are various kinds of receptors on phagocytes, called Pathogen Recognition Receptors (PRR) (5). Moreover, it seems that some of PRRs can be stimulated by endogenous ligands, i.e. molecules which are being released during stress or necrotic tissue damage. A few of these 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 Toll-Like Receptors (TLRs) (6).

TLRs are of great importance, as many inflammatory processes, both infectious and sterile, seems to depend on TLR signaling (7). In this regard, however, an expected role of TLRs in distinguishing self-ligand leading to sterile inflammation has not been conclusively proven. Up till now, the TLRs have been studied in adult phagocytes only, but the recent experiments conducted in our laboratory revealed that TLRs and CD14
are expressed in early stages of embryonal development (unpublished). These data suggest that recognition of endogenous ligands by TLR in this pathogen-free environment may critically influence the embryonal 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 main goal of my thesis is to describe origin, function and identification of embryonal phagocytes. I will also give an overview on TLRs and briefly show and discuss expression of TLR2, TLR4 and CD14 on this unique embryonal cell population.

Before we consider the main features of embryonic phagocytes and place them in the immune system as a whole, let's briefly consider the immunity in general terms.

2. Immune System

Immune system is an aggregate of mechanisms ensuring the integrity of an organism by distinguishing and eliminating foreign or potentially dangerous own structures. Immune mechanisms can be divided in two basic classes: innate (antigen nonspecific) and adaptive (antigen specific).

2.1. Innate Immunity

In comparison with adaptive immune system, the innate immune system is evolutionarily older and it exist in both vertebrates and invertebrates as well as in plants. The innate immunity mechanism consists of two arms: humoral and cellular components. Humoral components are represented by complement system, interferons, lectins, defensins and other serum proteins. The complement consist of serum and membrane proteins which interact among themselves and with other immune molecules as well. Their sequential interactions on the surface of pathogens lead to pathogen lysis and opsonization. Some complement parts are also effective chemotactic elements.

Cellular component of innate immunity are mast cells, basophils and phagocytes represented mostly by neutrophilic and eosinophilic granulocytes, monocytes,
macrophages and dendritic cells. A signature characteristic of these cells is their ability to mount a non-clonal and nonspecific responses (8). These responses are ever present due to the engagement of germ-line encoded PRR (9). The existence of PRR was predicted by Charlie Janeway in 1989 (5) when he formulated the theory of immune recognition of infectious-non-self. He suggested that PRRs have been evolving under very strong pressure of selection generated by pathogens and therefore they are not specific to a particular pathogen. Rather, they recognize highly conserved microbial structures, so called Pathogen Associated Molecular Patterns (PAMPs), which are in many cases essential for microbial survival. The fact that PAMPs are never present in the host results in a perfect ability of all PRRs to distinguish self from non-self. Presence of these mechanisms is not contingent on infection, they are always present in an organism and thus their response reaction to a microbial challenge is very fast (in minutes).

For many years the innate immunity has been regarded to be of inferior importance when compared to acquired immunity. The discovery of mammalian TLRs at the end of 20th century (10) as a major class of PRRs and characterization of their microbial ligands (11) led to an enormous expansion of innate immunity research. Pathogen recognition by TLRs leads to two important outcomes: (i) activation of signaling pathway inducing antimicrobial genes and inflammatory cytokines expression and (ii) activation of innate immunity as a critical step for development of antigen-specific adaptive immune responses executed by T- and B- cells as well as NK-T cells (9).

Dendritic cells (DCs) play a key role in linking innate and adaptive immune responses (12). There are several types of DCs in human and mice each expressing a specific set of TLRs. Binding of TLR ligand to TLR on immature DC induces the process of their maturation. Maturating DC migrate from tissues to lymphatic nodes and other secondary lymphatic organs and differentiate into activated Antigen-Presenting Cells (APCs) which effectively activate antigen-specific T-lymphocytes. This is caused particularly by high expression of MHC, co-stimulatory molecules (CD80 and CD86) and adhesive molecules. Mature DCs also secrete a wide variety of cytokines, particularly interleukin (IL)-12 which induce T helper (T\bf{H}) cells differentiation to T\bf{H}1 effector cells (Figure 1) (13). Furthermore there are γδ T- cells and NK- T cells, which exhibit some characteristic features typical for both innate and
adaptive system, thus they likely represent a cellular link between innate and adaptive immunity.

Figure 1. Role of dendritic cells in innate and adaptive immunity (Taken from 14). Toll-like receptors (TLRs) expressed on antigen-presenting cells (APCs) such as dendritic cells recognize pathogen-derived components and induce upregulation of co-stimulatory molecules (CD80, CD86) and also inflammatory cytokines such as interleukin (IL)-12. After pathogen engulfment the dendritic cells present pathogen-derived peptide antigens to naive T-cells. Thus, phagocytosis mediated antigen presentation together with TLR-mediated expression of co-stimulatory molecules and inflammatory cytokines lead to the development of antigen-specific adaptive immunity, especially differentiation of T-cells into T helper (Th)1 effector cells.

2.2. Adaptive Immunity

Adaptive part of immune system based on T- and B- lymphocytes was developed at about 400 millions years ago and is known only in vertebrates where both innate and adaptive mechanisms are indispensable and they cooperate with each other.
As the description of adaptive immune system and it’s functions is beyond the scope of this thesis, the following table only briefly summarizes the main differences between Innate and Adaptive immune systems.

Table 1. Innate and adaptive immunity (Taken from 15)

<table>
<thead>
<tr>
<th>Property</th>
<th>Innate immune system</th>
<th>Adaptive immune system</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Receptors</strong></td>
<td>Fixed in genome</td>
<td>Encoded in gene segments</td>
</tr>
<tr>
<td></td>
<td>Rearrangement is not necessary</td>
<td>Rearrangement necessary</td>
</tr>
<tr>
<td><strong>Distribution</strong></td>
<td>Non-clonal</td>
<td>Clonal</td>
</tr>
<tr>
<td></td>
<td>All cells of a class identical</td>
<td>All cells of a class distinct</td>
</tr>
<tr>
<td><strong>Recognition</strong></td>
<td>Conserved molecular patterns (LPS, LTA, mannans, glycans)</td>
<td>Details of molecular structure (proteins, peptides, carbohydrates)</td>
</tr>
<tr>
<td><strong>Self-Nonself discrimination</strong></td>
<td>Perfect: selected over evolutionary time</td>
<td>Imperfect: selected in individual somatic cells</td>
</tr>
<tr>
<td><strong>Action time</strong></td>
<td>Immediate activation of effectors</td>
<td>Delayed activation of effectors (3-5 days)</td>
</tr>
<tr>
<td><strong>Response</strong></td>
<td>Co-stimulatory molecules</td>
<td>Clonal expansion or anergy</td>
</tr>
<tr>
<td></td>
<td>Cytokines (IL-1, IL-6)</td>
<td>IL-2</td>
</tr>
<tr>
<td></td>
<td>Chemokines (IL-8)</td>
<td>Effector cytokines: (IL-4, IFN)</td>
</tr>
</tbody>
</table>

3. Early hematopoiesis

All cellular components of the blood are derived from a self-renewing, multipotent precursors- hematopoietic stem cells (HSCs), in a process known as haematopoiesis. HSCs develop into lymphoid and myeloid progenitors. Lymphoid progenitors differentiate into NK-, B- or T- cells, whereas myeloid progenitors
differentiate into erythrocytes, megakaryocytes, mast cells, eosinophils, neutrophils and macrophages.

During development, HSCs and their progeny are found at different sites. In the mouse, the earliest hematopoietic activity can be seen on 7th day of gestation in blood islands of the yolk sac (YS) (16). It had been assumed for a long time that the YS is the only pre-liver embryonic haematopoietic site where HSCs originate and afterwards they migrate to the fetal liver and later to the bone marrow (17, 18). However, this view has been challenged by the observation demonstrating that hematopoietic precursor cells, originating from the YS before the blood connection between the embryo and the YS is established (E8.5), have limited differentiation potential in vitro and also are incapable of long-term reconstitution of adult animals (19, 20). So, as a consequence, the YS is considered to be a site of primitive hematopoiesis, where only primitive erythrocytes and some primitive macrophages originate (21).

According to the above, most stem cells that will be found in the adult bone marrow and in circulation are derived from cells that appear slightly later (E10) and in different location. This second wave of HSC production occurs in the intraembryonic paraaortic compartment, called Aorta-Gonad-Mesonephros (AGM), the site where the aorta, gonads and mesonephros begin to develop (20). Nowadays it is thought, that these intraembryonic cells first colonize fetal liver at around E10 and later the spleen and bone marrow. AGM-derived progenitor cells are therefore responsible for definitive haematopoiesis in all haematopoietic organs later in the development (22). Also it has been shown that an increased in differentiation potential of yolk sac cells after the establishment of circulation is probably due to the colonization of yolk sac by intraembryonic AGM cells (19). A model of early hematopoiesis highlighting embryonic tissues where general and alternative paths of colonization by progenitor cells occur is depicted in figure 2.
Figure 2. Sequence of hematopoietic colonization events in the mouse development (Taken from 23). The general model of early haematopoiesis proposes that there are two pre-liver haematopoietic sites where the founder cells for the blood system are produced: extraembryonic yolk sac (YS) and intraembryonic AGM region. The first differentiated primitive erythrocytes and progenitors of primitive-type of macrophages arise in the yolk sac around day 7 of the mouse development (16). In contrast, undifferentiated, multipotent progenitors are detected in AGM beginning at day 7.5 onwards (24) but are able to differentiate only after repopulating the fetal liver at day 10 and later bone marrow (25). 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 (19). However, after the blood connection between the YS and embryo becomes established at E8.5, the YS then can produce in vitro both myeloid and lymphoid lineage, due to to being colonized by the AGM derived progenitor cells (19). Despite the fact that 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 documented (20, 26), only AGM derived progenitor cells contribute to the definitive hematopoiesis (20). Arrows indicates the sequential colonization of hematopoietic sites by progenitor cells starting from the YS and AGM: full arrows represent a general model (22); broken arrows indicate suggested path of colonization based on in vitro experiments (17, 19); arrows with the question mark represent an alternative path of fetal spleen and bone marrow repopulation based on in vivo transplantation experiments (20, 27).
3. 1 Embryonal phagocytes

3. 1. 1. Historical classification of phagocytes

All phagocytic cells were originally classified together in 1924 by Aschoff as a part of Reticuloendothelial System (RES) (28). Besides monocytes and macrophages, the reticuloendothelial system includes both phagocytic endotelial cells lining blood sinuses and also reticular cells of lymphoid organs, whose ability to take up the vital dye was thought by him to result in active phagocytosis. In 1972 Ralph van Furth and Cohn (29) proposed the term „Mononuclear Phagocyte System“ (MPS), which classifies the cells by their origin, not by their function as RES. This lead to RES be replaced by MPS. Mononuclear phagocyte system has been defined as a family of cells comprising circulating blood monocytes, resident tissue macrophages and their precursor cells in bone marrow (promonocytes, monoblasts) (29). Later, Moore and colleagues extended this model by demonstrating that the YS is the site of origin of macrophage precursors (17), which differentiate in the fetal liver and bone marrow through the monocytic pathway (30). However, this concept of the MPS has been challenged by an evidence that macrophages found in an embryo differ from those of adults phenotypically, biochemically and, importantly, developmentally, as they do not follow the monocyte pathway (31, 32).

3. 1. 2. Origin of phagocytes in developing embryo

In accordance with a general path of early hematopoiesis described above, phagocytes in mammalian embryo develop in two sites, yolk sac and fetal liver. While the appearance of adult hepatic monocytes/macrohages coincides with the onset of definitive hematopoiesis in the liver at day 11, embryonic phagocytes are detected in blood islands of the yolk sac at around day 9 of gestation (32). These primitive macrophages are round, have a large euchromatic nucleus, poorly developed microvilli and they never showed peroxidase activity (31, 32). On day 10 they differentiate into fetal macrophages with more evolved intracellular organelles and extended filopodia (31, 32). Fetal macrophages seem to leave the blood islands, migrate into mesenchymal
layer and then to the extra-embryonic coelom where they mature. Subsequently they move via blood vessels, actively proliferate and colonize embryonic tissues (32).

Promonocytes and monocytes appear in the blood islands of the YS on day 11 (one day after development of fetal macrophages) but they form a minor community here. The differentiation of monocytic cells is thought to be completed by the middle stage of hepatic haematopoiesis in the fetal mouse liver (33). 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). These events imply that primitive/fetal macrophages constitute a separate lineage arising straight from hematopoietic cell in YS before the appearance of monocytes (Figure 3).

Moreover, in contrast to the fetal macrophages, the monocytes, as well as monocyte-derived macrophages, do not possess proliferative capacity under normal steady state conditions (29, 34) which also implies that embryonic macrophages are an independent, self-sustaining cell population.

![Diagram](https://example.com/diagram.png)

Figure 3. A postulated schematic diagram of the macrophage development in the mouse yolk sac hematopoiesis (Taken from 32)
<table>
<thead>
<tr>
<th></th>
<th>Primitive macrophage</th>
<th>Fetal macrophage</th>
<th>Yolk sac promonocyte</th>
<th>Yolk sac monocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus</td>
<td>Round, indented</td>
<td>Indented</td>
<td>Indented</td>
<td>Reniform</td>
</tr>
<tr>
<td>Nucleolus</td>
<td>Large Euchromatic</td>
<td>Medium-sized</td>
<td>Large Heterochromatic</td>
<td>Medium-sized</td>
</tr>
<tr>
<td>Chromatin</td>
<td>≥ 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>NC ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rER</td>
<td>Few</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Golgi</td>
<td>Poor</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lysosome</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phagosome</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pinocytic vesicle</td>
<td>Few</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Polysomes</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Filopodia</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Microvilli</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pseudopodia</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Peroxidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear envelope</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>rER</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Golgi</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Granule</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F4/80</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EA rosette formation</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Immune phagocytosis</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Latex phagocytosis</td>
<td>Slight</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

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

Table 2. Ultrastructural, cytochemical, immunoelectron microscopic, functional features of primitive/ fetal macrophages and yolk sac promonocytes/ monocytes (Taken from 32)

This theory of two separated macrophages lineages (primitive/ fetal and monocytic/ adult) is additionally supported by several studies involving drosophila, zebrafish, frog, avian and rodent embryos that documented the occurrence of phagocytes in anterior head mesoderm well before the establishment of any circulation and the appearance of yolk sac phagocytes (35-40). Although using the YS- chimeras (a chick embryo grafted on a quail YS and vice versa) it has been shown that some of the early avian macrophages come from the YS (38, 41), their migration path was not established and additional presence of local stem cells in the head never rejected. Because of the fact that the presence of embryonal phagocytes in the head mesenchyme is evolutionary conserved from drosophila to mammals, this area is also considered to be a potential site of their origin (28). While both head mesoderm- and yolk sac-residing phagocytes remain extravascular and are able to proliferate, the relationship between these two populations has not yet been established. Another unresolved question is whether these embryonal phagocytes are retained in the adult and therefore forming a source of tissue macrophages with a capacity for self-renewal.
3. 1. 3. Function of embryonal phagocytes

The conservation of early embryonic macrophages throughout evolution suggests their important role during embryogenesis and development. It seems that their main function is elimination of cells that have undergone autonomous programmed cell death and thus tissue remodelling is assumed to be their primordial task (28). This proposed role is in accordance with Metchnikoffs original thesis suggesting that the primary function of phagocytes was the surveillance of embryonic development through the process of sterile inflammation responsible for removal of destroyed, effete and senile cells. This would explain the existence of phagocytes in organisms without extensive immune system or the existence of phagocytes in organisms in the very early stages of ontogenesis when the immune system is not fully developed.

Moreover, the ability of macrophages to secret a vast array of immune, neuroendocrine, reactive oxygen/nitrate mediators and growth factors that can control, modulate or affect the physiology and function of other cells suggest their additional functions. Specifically, it has been documented that embryonic macrophages can produce erythropoietin (42), IFNα (43), TGFβ (44) and trombospodine (45), cytokines supporting vascularization of embryonic tissues (36) and components of basement membranes like proteoglycans, laminin, tiggrin, glutactin and collagen (46, 47). However, there is a significant lack of information about stimuli and signaling pathways leading to the production of these mediators.

In addition, experiments conducted on the zebrafish embryo 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 (36).

3. 1. 4. Identification of embryonal phagocytes

Presence of macrophages in early stages of development is based on their morphology, enzymology and surface markers (Table 2 and Table 3). Up till now, the only surface markers used for phenotypic characterization of mouse YS phagocytes were Mac-1 integrin (CD11b/CD18) (48), the receptor for macrophage colony-
stimulating factor (c-fms) (39), F4/80 (49) and mannose receptor (50). Because surface markers are the only way how to reliably obtain a unique and pure population of embryonal phagocytes by using FACS sorting, there’s necessity to identify other receptors expressed exclusively on these cells. Interestingly, data recently obtained in our laboratory provide the very first evidence that TLRs and CD14 are indeed expressed at early stages of embryonic development. The fact that TLRs recognizing predominantly specific molecular patterns characteristic for pathogens are expressed in pathogen free environment during the early stages of embryonic development is striking and suggest a role for TLRs in the recognition of endogenous ligands. Thus, TLR signaling is probably a very important part of embryonic homeostatic regulatory mechanism.

Table 3. Macrophage markers used for describing of embryonic phagocytes in various species
(Taken from 51)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Drosophila</th>
<th>Fish</th>
<th>Xenopus</th>
<th>Birds</th>
<th>Rodents</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMI</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4/80</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mac-1</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannose receptor</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-fms</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scavenger receptor</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>crg receptor</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR-C1</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABC transporter</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEP-1</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lectin binding</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD68</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Enzymes</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lysozyme</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PU.1</td>
<td>+</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Transcription factors</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mstf</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glide/gcm</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-plasmin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein-X</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDP-1</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WLC 15</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL-1</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
4. Toll like receptors

As mentioned in chapter 2, one of the innate immune mechanisms which detects the pathogens employs family of TLRs. When microbial components are recognized by these receptors, signaling pathways are initiated and lead to changes in the gene expression, which control innate responses and the development of antigen specific acquired immunity. In addition, there are reports suggesting that TLRs also recognize endogenous ligands induced during stress, necrosis, aseptic injury and irritation and thus participating in sterile physiological inflammation. The most important aspects of TLR physiology are outlined below.

4.1 Discovery of TLRs

The discovery of the TLR family began with the identification of Drosophila protein Toll required for dorso-ventral axis formation during embryogenesis (52). Following studies have shown that Toll also has an essential role in the fly’s innate immune response to the fungus Aspergillus fumigatus (53). The first announced human Toll-like receptor was described by Nomura et al. in 1994 (54) and mapped by Taguchi et al. in 1996 (55). In 1997, Medzhitov et al. (10) showed that a constitutively active human TLR mutants can induce the activation of NF-κB and pathways leading to expression of certain genes necessary for initiating an adaptive immune response. The function of TLRs was discovered by Bruce A. Beutler and colleagues (56) who showed that mice resistant to LPS contained mutations in TLR4. This identified TLR4 as a key receptor for LPS and strongly suggested that other TLRs might also recognize microbial structures. Since then, a massive search has so far identified 11 human and 13 mouse TLRs orthologs that respond to a variety of agonists (57).
4. 2. Structure of TLRs

The TLRs are type I integral membrane glycoproteins and on the basis of significant homology in cytoplasmatic region we classify them into large superfamily including interleukin-1 receptor (IL-1R) (10, 58). This cytoplasmic portion of TLRs and IL-1Rs is highly conserved and is known as the Toll/IL-1R (TIR) domain (59). Despite this similarity, the extracellular domains of both types of receptors differ markedly; the IL-1Rs contains three immunoglobulin-like domains, whereas TLRs consist of 19-25 tandem copies of leucine-rich repeats (LRR) (Figure 4).

![TLR structure](image)

**Figure 4. TLR structure (Taken from 11).** Toll-like receptors and interleukin receptors (IL-1Rs) have a highly conserved cytoplasmatic domain termed as the Toll/IL-1R (TIR) domain, which is characterised by the presence of three homologous short motifs (boxes 1, 2, 3). By contrast, their extracellular regions are structurally unrelated: IL-1Rs contain three immunoglobulin (Ig)-like domains, whereas TLRs have leucine-rich repeat (LRR) motifs.

4. 3. TLRs, their ligands and localization

Based on subcellular localization, TLRs can be classified into two groups. The first group includes TLR1, 2, 4, 5, 6, 10 and 11 which are all expressed on the cell’s surface (57). These TLRs can sense especially bacterial structures: TLR2 in association with TLR1 or TLR6 discriminates between triacylated (60) and diacylated (61)
lipoproteins, respectively; TLR4 recognizes LPS (56); TLR5 senses flagellin (62); and mouse TLR11 is involved in detection of uropathogenic bacteria (63). The second group includes TLR3, 7, 8 and 9, which are found almost exclusively in intracellular compartments such as endosomes and they sense viral and bacterial nucleic acid in particular (57): TLR3 and TLR7/8 recognize viral dsRNA (64) and ssRNA (65, 66) respectively, TLR9 senses nonmethylated CpG DNA (67).

Importantly, TLRs have been suspected of binding to endogenous molecules as well. For example, besides of variety of exogenous ligands, TLR4 is also triggered by host heat shock proteins (68), some host-derived oligosaccharides (69), fibrinogen (70) and fibronectin (71). Because recognition of endogenous ligands by TLRs is in accordance with our data describing the expression of TLRs on embryonic phagocytes and the identification of these ligands will be subject of my future studies, I will discuss this point in more details in a separate chapter. The main ligands recognized by TLRs are summarized in table 4.

In addition, there are some TLRs which require accessory proteins to recognize their ligands. A good example is recognition of LPS by TLR4. LPS is initially bound to serum protein LBP (LPS-binding protein) and this complex is subsequently identified by CD14, a glycosylphosphatidylinositol-anchored protein preferentially expressed on monocytes, macrophages and neutrophils (72). Another component of LPS receptor complex is MD-2, a molecule which associates with the ectodomain of TLR4 and enhances LPS responsiveness (73). Nevertheless, it’s important to say that there is still controversy whether TLR4 binds its ligand directly or not. Several groups indicate that recognition of LPS by TLR4 involves direct binding, while others have proposed that LPS interaction is aided by CD14 and MD-2 (74-77).
Table 4. TLRs and their ligands (Taken from 11)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Origin of ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Tricarb lipoprotein, soluble factors</td>
<td>Bacteria and mycobacteria, Neisseria meningitides</td>
</tr>
<tr>
<td>TLR2</td>
<td>Lipopolysaccharide, Peptidoglycan, Lipoethic acid, Lipoprotein-lipoprotein, Lipoaminobutyran, Phenol-soluble modulin, Glycolipids, Glycolipids, Porins, Atypical lipopolysaccharide, Atypical lipopolysaccharide</td>
<td>Various pathogens, Gram-positive bacteria, Gram-negative bacteria, Mycobacteria, Staphylococcus epidermidis, Trypanosoma cruzi, Treponema pallidum, Neisseria, Leptospira interrogans, Porphyromonas gingivalis</td>
</tr>
<tr>
<td>TLR3</td>
<td>Double-stranded RNA</td>
<td>Viruses</td>
</tr>
<tr>
<td>TLR4</td>
<td>Lipopolysaccharide, Tezol, Fusion protein, Envelope protein, Heat-shock protein 60, Heat-shock protein 70, Type III repeat extracellular domain A of fibronectin, Oligosaccharides of hyaluronic acid, Poly saccharides fragments of heparan sulphate, Fibrinogen</td>
<td>Gram-negative bacteria, Plants, Respiratory syncytial virus, Mouse mammary-tumour virus, Chlamydia pneumoniae, Host, Host, Host, Host</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td>Bacteria</td>
</tr>
<tr>
<td>TLR6</td>
<td>Diacyl lipopolysaccharide, Lipoethic acid, Zymosan</td>
<td>Mycoplasma, Gram-positive bacteria, Fungi</td>
</tr>
<tr>
<td>TLR7</td>
<td>Imidazolquinoline, Loxoribine, Bropirimine, Single-stranded RNA</td>
<td>Synthetic compounds, Synthetic compounds, Synthetic compounds, Viruses</td>
</tr>
<tr>
<td>TLR8</td>
<td>Imidazolquinoline, Single-stranded RNA</td>
<td>Synthetic compounds, Viruses</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG-containing DNA</td>
<td>Bacteria and viruses</td>
</tr>
<tr>
<td>TLR10</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>TLR11</td>
<td>N.D.</td>
<td>Uropathogenic bacteria</td>
</tr>
</tbody>
</table>

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

4.4. TLR signaling

In a large part, the recognition specificities of the TLRs have been determined and numerous studies described their signaling mechanisms (Figure 5) (reviewed in 11, 14, 57, 78, 79). Essential to TLR transduction pathways are the receptor proximal, TIR domain-containing adaptor proteins: MyD88, Mal (MyD88 adaptor-like)/TIRAP (TIR
domain-containing adaptor protein), Trif (TIR-domain-containing adaptor inducing interferon-beta), TRAM (Trif-related adaptor molecule) and SARM (SAM and ARM-containing protein) (80). MyD88 is a central adaptor protein used by all TLRs with the exception of TLR3. After ligand binding, TLRs dimerize and recruit MyD88 through interaction of their respective TIR domains. MyD88 then associates with IL-1R kinases IRAK1 and IRAK4. IRAKs interact with TRAF6, which subsequently forms a complex with UBC13 and Uev1A to promote the synthesis of lysine 63-linked polyubiquitin chains, leading to activation of TAK1/TAB1/2/3. This aggregate targets both IKK complex and MAP kinase pathway resulting in the activation of NFκB and AP1 transcriptions factors. NFκB and AP1 translocate to the nucleus and induce the expression of proinflammatory cytokines TNFa, IL6, IL1β and IL12.

Additionally, analysis of MyD88-deficient mice showed some unexpected features in signaling downstream of TLR3 and TLR4. Macrophages and DCs derived from MyD88 knockout mice do not produce inflammatory cytokines after stimulations by their ligands, however, activation of NF-κB and MAP kinase signaling pathway through TLR4 is observed with delayed kinetics (81). This finding indicates that stimulation of TLR4 facilitates at least two signal- transduction pathways: The MyD88-dependent pathway involves the early phase of NF-κB and MAP kinases activation which leads to the production of inflammatory cytokines; The MyD88-independent pathway leads to activation of interferon (IFN)-regulatory factor (IRF3) as well as to activation of the late phase of NF-κB and MAP kinases, both of which lead to the production of IFN-β and the expression of IFN- inducible genes (11). Approvingly, the TLR3 mediated pathway also triggers transcription factor IRF3 and therefore induces IFN-β in a MyD88-independent manner. Production of type I IFN and IFN-inducible genes occurs also in plasmacytoid dendritic cells (pDCs) expressing TLR7/8 and TLR9. Transcription factor IRF7 is here directly phosphorylated by MyD88- recruited IRAK 1 and then translocated to the nucleus (82). Therefore, the recruitment of different combinations of adapter proteins to individual TLRs translates into the activation of common and unique pathways. This results in the production of pathogen-tailored combinations of inflammatory cytokines and anti-viral type I interferons (83).
Figure 5. TLRs signaling pathways (Taken from 57). After ligand binding, all TLRs with the exception of TLR3 recruit MyD88, IRAKs and TRAF6 to activate the Ubc13/TAK1 pathway. The TAK1 then activates the IKK complex (inhibitor of nuclear factor-κB (κB)-kinase complex) composed of IKKα, IKKβ, and IKKγ/NEMO, which catalyzes phosphorylation of IκB proteins leading to its ubiquitylation and subsequent degradation. This allows NF-κB to translocate to the nucleus and induce the expression of target genes. TAK1 also phosphorylates the mitogen-activated protein (MAP) kinases, which mediates AP-1 activation. IRF5 binds MyD88-IRAK4-TRAF6 complex and after phosphorylation moves to the nucleus. NF-κB, AP-1 and IRF5 control the expression of inflammatory cytokine genes. TIRAP adapter is involved in the MyD88-dependent signaling through TLR4, TLR1/2 and TLR2/6. TLR4 also recruits TRIF adapter in MyD88 independent pathway which engages TBK1 and IKKi leading to the activation of IRF3. Activated IRF3 dimerize, translocate into nucleus and induce expression of type I IFN and IFN- inducible genes. TRAF 3 forms a complex with TBK1 and IKKi. TRIF interacts with TRAF6 and RIP1, which mediate NF-κB activation. While TRIF-dependent TLR4 signaling requires the presence of TRAM adapter, endosome-residing TLR3 utilizes TRIF exclusively. In pDCs expressing TLR7/8 and TLR9, a signaling complex consisting of MyD88-TRAF6-IRAK4-OPN-i-TRAF3-IKKα-IRAK1-IRF7 is formed. IRF7 is directly phosphorylated by MyD88-recruited IRAK1 and subsequently translocated to the nucleus to induce expression of type I IFN and IFN- inducible genes. IKKα is also capable of phosphorylating IRF7.
4.5. Endogenous ligand of TLRs

It has been shown that TLRs play a crucial role in innate and adaptive immune responses to pathogen infection, however, whether they are also capable of mediating similar responses to endogenous stimuli and thus contribute to autoimmunity and/or to sterile inflammation remains uncertain. As a first step towards answering this question, a few candidates for endogenous ligands that function through TLRs have been identified (68-71, 84-90) and some of them are listed in TABLE 4. Discovery of these host-derived ligands for TLRs as a potent activators of innate immune system and also the adaptive immune system demonstrated by the upregulation of costimulatory molecules in dendritic cells demandes to revise the original „self- nonself“ immunesurveillance hypothesis (91). Therefore Polly Matzinger proposed the „danger“ theory whereby the immune system evolved primarily to recognize danger signals released from injured cells, such as those exposed to pathogens, toxins, mechanical damage and so on, rather than nonself signals (92). Participation of TLRs in sensing endogenous ligands may have very important physiological, immunological and pathological implications (7, 93-95). 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 based on comprehensive „upstream“ blockade of TLR signalling would potentially provide a cure (7).

4.5.1. Endogenous ligands versus contaminants

Recent evidence suggest that the cytokine-inducing effects of putative endogenous ligands may be due to the effects of contaminants such as LPS and bacterial lipoproteins (93-96). Because the recombinant products are produced by Escherichia coli, the final preparations may be contaimned with bacterial components. Similarly, purified preparations also frequently contain LPS (91, 94). Although there were many experiments performed to rule out the possibility of LPS contamination being responsible for erroneous attribution of endogenous molecules as potent TLRs stimuli, many unclarities still remain (91, 94). Nowadays, it is still not conclusively
proven that the reported putative host ligands of TLRs are of endogenous or bacterial nature.

In contrast, several studies clearly documented an involvement of TLRs in sensing of endogenous agents. For example, in 2002 Elizabeth A. Leadbetter et al. discovered that TLR signalling participate in autoimmunity through recognition of endogenous DNA by TLR9 (97). In 2003 Daniel R. Goldstein et al. showed that allografts rejection in mice with targeted disruption of the adaptor MyD88 proceeds slower than in the control mice, what implicates TLRs in this process (98). In addition, TLRs also may recognize TNF α, cytokine which is mainly produced by TLR signaling and is the principal effector cytokine shared by infectious and sterile inflammation (7). Last but not least, there are our data demonstrating the expression of TLRs in early stages of embryonic development and suggesting the existence of TLRs endogenous ligands. These findings indicate that both exogenous and endogenous agents might signal through the same set of evolutionarily conserved proteins what opens the door for challenging the current dogma defining TLRs as the receptors characteristic for microbial structures.

5. Experimental part

In this part, I will briefly describe the results highlighting the expression of TLRs in embryonal mouse development.

5. 1. Main objectives

(i) Quantification of TLR1-8 in 10.5 day old embryo
(ii) Detection of surface expression of TLR2 and TLR4 in 10.5 day old embryo
(iii) Identification of the cell type expressing TLRs
5. 2. Materials and methods

Mice:

CD1 mice were used. The day when a vaginal plug first appeared (midday) was designated E0.5. Immediately after sacrifice by cervical dislocation, embryos from 10.5 day were delivered from the uterus and then dissected out of the decidua with the yolk sac and the amnion pulled away from the embryo.

Real-time RT-PCR (qPCR):

Total RNA from E10.5 embryo isolated with NucleoSpin RNA II kit (Macherey-Nagel; Germany, Düren) with subsequent DNase treatment (Invitrogen, U.S.A., Carlsbad) was reverse transcribed into cDNA using Superscript II (Invitrogen, U.S.A., Carlsbad) and random hexamers (Promega; WI U.S.A., Madison) according to manufacture instructions. The expression of mouse target genes (TLR1-8) was normalized to endogenous housekeeping gene (GAPDH). Primers were designed using the Primer3 program. PCR experiments using hot start polymerase with Sybr green dye (Roche) were performed on the LC480 machine (Roche). Each single experiment (sample) was done in duplicate. Relative quantification model using following formula: ratio = (E_{target})^{Cp}_{target (control-sample)} / (E_{ref})^{Cp}_{ref (control-sample)} (99) was employed to calculate expression of target genes.

Facs analysis:

Single cell suspension from 10.5 day old embryo was prepared by dispase (Invitrogen, USA, Carlsbad) treatment (100 µl per 1,5 ml PBS) 10 min at 37°C. After incubation the cells were washed in 1% Fetal bovine serum (FBS) (Invitrogen, USA, Carlsbad) supplemented PBS, filter through 40 µm BD Falcon cell strainer (BD Biosciences; Belgium, Erembodegem), pelleted by centrifugation and washed three times with 1% FBS in PBS. To block the Fc receptors, cells were incubated with anti-CD16/32 antibody for 10 min at 4°C. Cells were then stained with FITC-conjugated anti-CD11b, APC-conjugated anti-CD14, PE-conjugated anti-TLR4 and biotin-
conjugated anti-mouse TLR2 (all five antibodies mentioned above were from eBioscience; U.S.A., San Diego) followed by staining with the streptavidin-Qdot conjugate (Invitrogen, USA, Carlsbad) for 30 min. Cells were washed twice and analysed using LSII in the presence of Hoechst 33528 dye (Roche) to exclude dead cells. Analyses were performed by Zdeněk Cimburek.

5.3. Results

5.3.1. TLR 1-8 are expressed in 10.5 day embryo.

As illustrated in figure 6, the expression of all TLRs tested in this experiment was detected and quantified. TLR2 and TLR4 are the most abundantly expressed receptors of this family, while TLR6 and TLR7 are expressed at lower level. TLR1, 3, 5 and 8 displayed intermediate levels. Difference between TLR2 and TLR7 as the most and the least expressed TLRs, respectively, is approximately 6-fold. Bar graph in figure 6 represents the result of two independent experiments.

![TLRs mRNA level/GAPDH](image)

Figure 6. Quantification of expression levels of TLRs in 10.5 day embryo

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5.3.2. Expression of TLRs and CD14 is restricted to CD11b+ embryonal phagocytes.

CD11b also known as Mac-1 is a surface-specific marker of embryonal phagocytes (48). In adult mice it is expressed on the surface of many leukocytes involved in the innate immune system, including monocytes, macrophages, granulocytes and NK-cells (100). CD14 is a membrane-associated glycosylphosphatidylinositol-linked protein expressed predominantly on adult monocytes and macrophages, weakly on neutrophils (72). Its expression on the embryonal phagocytes has not been previously demonstrated. As illustrated in figure 7A, approximately 1% of all embryonal cells staines positive for CD11b (Figure 7A). About 75% of all CD11b positive embryonal phagocytes are also positive for CD14 (Figure 7A). When gated on this CD14 and CD11b double-positive phagocytic cell population (red square in figure 7A), they are 99,7% and ~70% positive for TLR2 and TLR4 markers, respectively (Figure 7B). Thus, TLR2 and TLR4 are expressed on the surface of a vast majority of embryonal phagocytes of E10.5 embryo. While figure 7A and B show a concomitant expression of CD11b, CD14, TLR2 and TLR4, histograms in figure C and D illustrate the single staining profile of TLR2 and TLR4 markers on CD11b/CD14 positive embryonal phagocytes, respectively.

These results are first to characterize the expression of TLRs and CD14 on embryonal phagocytes in early stages of mammalian embryogenesis and suggest the existence of TLRs endogenous ligands.
Figure 7. Expression of CD14, TLR2 and TLR4 on the surface of CD11b+ embryonal phagocytes (E10.5). Single cell suspension was stained with indicated antibodies and FACS analysis was performed on LSRII. See text for details.
5. 4. Outlines of author’s future experimental work

My experimental project for diploma thesis will be focused on following goals: (i) quantification of expression profile of all mouse TLRs on 5.5-12.5 day-old embryos and their embryonal phagocytes using qRT-PCR technique; (ii) Detection of surface expression of the most abundantly expressed TLRs using FACS analysis and Western blotting techniques. I will also attempt to use surface expression of TLRs to physically localize embryonal phagocytes in early embryos; (iii) identification of novel innate immune receptors and molecules expressed in embryonic phagocytes by a microchip analysis; (iv) isolation and characterization of endogenous ligands for selected TLRs by using recently described method based on the generation and screening of monoclonal antibodies specific for cell surface antigens.

We will be focused on utilizing the primary mouse embryonic cells as a source of phagocytes. Methodological approaches will include FACS analysis, Western blotting, standard DNA and RNA recombinant technology, fluorescent microscopy and live video imaging, cDNA microarray analyses and transgenesis, enabling genetic and biochemical characterization of innate immune molecules involved in embryonic homeostasis and sterile inflammation.

6. Conclusions

Phagocytes are considered to be among the evolutionary oldest cell types (101) and were studied by Elie Metchnikoff more than a century ago. One of the main goals throughout the last three decades was to clarify the origin of well-known adult phagocytes in embryo. Observations have shown that the ontogeny of phagocytes during early stages of embryonic development differs from that occurring during adult development and that the embryonic phagocytes bypass the monocytic pathway. However, the origin of embryonal phagocytes still remains obscure because of establishment of circulation between YS and embryo on day E8.5. At this time the cells from AGM which are probably the site of origin of monocytic/adult macrophages can colonize YS and vice versa. However, due to the lack of surface markers that would
distinguish YS-derived phagocytes (until now characterized only by surface expression of Mac-1 integrin, c-fms, F4/80 and mannose receptor) from those of AGM origin, an independent ontogenetic origin of these two phagocytic lineages can't be ascertained. Moreover, the origin of embryonic phagocytes which appear in anterior head mesoderm before the establishment of any circulation also remains obscure. While both head mesoderm- and yolk sac- residing phagocytes remain extravascular and are able to proliferate, the relationship between these two populations has not yet been established.

For determination of the origin and differentiation pathways as well as functional importance of embryonal phagocytes is necessary to identify a novel set of surface receptors which would allow their more comprehensive phenotypic characterization. Towards this end, data recently obtained in our laboratory provide the very first evidence that at least some TLRs are expressed at early stages of embryonic development. The fact that TLRs recognizing molecular structures specific for microbial pathogens are expressed in early embryo which develops in pathogen free environment is striking and suggest a role for TLRs in the recognition of endogenous ligands. Although there are several studies indicating the participation of TLRs in recognition of endogenous ligands leading to non-infectious inflammatory and immune responses, their direct involvement has never been conclusively proven because of putative contamination of endogenous molecules by LPS. Resolving this issue is of immense important 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 based on comprehensive 'upstream' blockade of TLR signaling would potentially provide a cure.

Undoubtedly, discovery of TLRs expression on the embryonic phagocytes as well as the future identification of a new set of receptors expressed on these cells will provide a valuable insight into their functional properties. Already demonstrated function of embryonal phagocytes is elimination of apoptotic cells, but their ability to secret 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. We believe that further identification and characterization of signaling molecules expressed in embryonal phagocytes will further advance our understanding of the function of innate immune molecules during embryonal development.
7. References


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