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TIM FAMILY MOLECULES IN HEMATOPOIESIS

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Abstract

Hematopoietic cells, i.e., erythrocytes, platelets and white blood cells, differentiate from hematopoietic stem cells in a process that is similar in vertebrates. Hematopoiesis is regulated by molecules expressed by both the hematopoietic stem and progenitor cells and the surrounding microenvironments. Knowledge of these molecules is important since many of the genes involved in normal hematopoiesis are mutated in leukemia. Furthermore, this information can be utilized in more efficient isolation and expansion of hematopoietic cells *in vitro*. However, these molecules are not yet sufficiently characterized.

Transmembrane immunoglobulin and mucin domain (TIM) genes form a known family of immunoregulators. In mammals, TIM-4 is expressed by antigen presenting cells, while TIM-1, TIM-2 and TIM-3 are expressed by T cells, in which they regulate differentiation of T_H cells. The role of TIM molecules in hematopoiesis has not yet been investigated.

The aim of this thesis work was to identify and analyze novel molecules involved in embryonic hematopoiesis using chicken and mouse as model organisms. This was carried out by generating a cDNA library of hematopoietic stem and progenitor cells from embryonic chicken para-aortic region. Both previously known and novel candidate genes were identified from the library. Among them, we found homologs to *tim* genes. Their expression and role in hematopoiesis was studied further.

TIM-2 expression was shown to be tightly governed during B cell development. It is expressed by common lymphoid progenitors and highly proliferative large-pro and large pre-B cells during both fetal liver and adult bone marrow hematopoiesis.

In mouse, *tim-4* expression was restricted to fetal liver $CD45^+F4/80^+$ cells. Furthermore, two distinct populations were identified: $F4/80^{hi}TIM\text{-}4^{hi}$ and $F4/80^{lo}TIM\text{-}4^{lo}$. The results suggest that the $F4/80^{hi}TIM\text{-}4^{hi}$ cells are yolk sac-derived macrophages and the $F4/80^{lo}TIM\text{-}4^{lo}$ cells myeloid progenitors.

This work shows for the first time that TIM family molecules are expressed during hematopoiesis. TIM-2 and TIM-4 are expressed by specific cell types during hematopoietic cell development, and in the future they may be utilized as markers in isolation of hematopoietic progenitor cells.

Keywords: B cell development, fetal liver, gene expression, hematopoiesis, myeloid progenitor cells, para-aortic region, transmembrane immunoglobulin and mucin domain containing molecule

Syrjänen, Riikka, TIM-molekyylien rooli verisolujen kehityksessä.
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Tiivistelmä

Verisolut eli punasolut, verihiutaleet ja immuunipuolustuksessa tärkeät valkosolut kehittyvät alkion veren kantasoluista prosessissa, joka on kaikissa selkärankaisissa samankaltainen. Veren kanta- ja esisolujen sekä ympäröivän mikroympäristön tuottamat molekyylit säätelevät hematopoieesia eli verisolujen kehitystä. Näiden molekyylien tunteminen on tärkeää, sillä useat normaalia verisolujen kehitystä säätelevät geenit ovat osallisena myös verisyöpien synnyssä. Lisäksi tätä tietoa on mahdollista hyödyntää verisolujen tehokkaammassa eristämisessä ja kasvattamisessa hoitoja varten.

Immuunipuolustuksen solut, kuten syöjäsolut eli makrofagit ja T-solut, ilmentävät TIM-molekyylejä (Transmembrane Immunoglobulin and Mucin). Ne toimivat immunologisen vasteen säätelyssä sekä solusyönnissä, mutta niiden roolia verisolujen kehittymisessä ei ole selvitetty aikaisemmin.

Tässä väitöstutkimuksessa etsittiin uusia hematopoieesiin vaikuttavia geenejä käyttäen malli-eläiminä sekä kanaa että hiirtä. Tutkimuksessa luotiin geenikirjasto kanan alkion para-aortaalisen alueen veren kanta- ja esisoluista. Kirjastosta tunnistettiin useita ennalta tiedettyjä sekä uusia verisolujen kehitykseen vaikuttavia geenejä. Tutkimuksessa analysoitiin tarkemmin kirjastosta löytyneiden TIM-geeniperheen jäsenten ilmentymistä ja roolia verisolujen kehityksessä.

Tutkimuksessa osoitettiin, että TIM-2 proteiinin ilmentymistä säädellään tarkasti B-solujen kehityksen aikana. Lymfosyyttien yhteiset esisolut sekä suuret pro-B- ja pre-B-solut ilmentävät TIM-2 proteiinia B-solukehityksen aikana sekä alkion maksassa että aikuisen luuytimessä.

Hiiren alkiossa *tim-4* geenin ilmentyminen oli rajoittunut maksaan, jossa erottui kaksi erillistä solupopulaatiota: F4/80^{hi}TIM-4^{hi} ja F4/80^{lo}TIM-4^{lo}. Tutkimuksen tulokset viittaavat siihen, että maksan F4/80^{hi}TIM-4^{hi} solut ovat ruskuaispussista lähtöisin olevia syöjäsoluja ja F4/80^{lo}TIM-4^{lo} solut myeloidisen linjan esisoluja.

Tämä tutkimus on ensimmäinen osoitus TIM-molekyylien ilmentymisestä kehittyvissä verisoluissa. Havaitsimme, että TIM-2- ja TIM-4-molekyylejä ekspressoidaan tietyissä soluissa verisolujen erilaistumisen aikana, joten tulevaisuudessa niitä on mahdollista käyttää merkkiproteiineina hematopoieettisten solujen esiasteita eristettäessä.

Asiasanat: alkion maksa, B-solujen kehitys, geeniekspressio, hematopoieesi, myeloidiset esisolut, para-aortaalinen alue, transmembrane immunoglobulin and mucin domain containing -molekyyli

To all my family

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Abbreviations

AGM	aorta-gonads-mesonephros
APC	antigen presenting cell
BCR	B cell receptor
CD	cluster of differentiation
CD45	cluster of differentiation 45 (common leukocyte antigen)
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
c-kit	proto-oncogene c-kit (tyrosine-protein kinase kit/CD117)
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
DC	dendritic cell
DN	double negative cell
DNA	deoxyribonucleic acid
DP	double positive cell
dpc	days post coitum
ED	embryonic day
EEF1A	eukaryotic translation elongation factor 1 alpha
FACS	fluorescence activated cell sorting
Fc	Fragment, crystallizable region
FCγR	Fragment, crystallizable gamma receptor II/III (CD32/16)
FL	fetal liver
Flt3	fms-related tyrosine kinase 3 (CD135)
FZ6	frizzled 6
GAL9	galectin-9
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA-1	globin transcription factor 1
GMP	granulocyte-macrophage progenitor
HMGB1	high mobility group box 1 protein
HPC	hematopoietic progenitor cell
HSC	hematopoietic stem cell
IFN-γ	interferon gamma
Ig	immunoglobulin
IgV	immunoglobulin variable domain
IL	interleukin
IL-7Rα	interleukin seven receptor alpha

Lin^-	lineage marker negative
LMPP	lymphoid primed multi-potent progenitor
LMIR5	Leukocyte mono-immunoglobulin-like receptor 5 (CD300b)
LSK	$Lin^-Sca\text{-}1^+c\text{-}kit^+$
LT-HSC	Long-term reconstituting HSC
Mac-1	Macrophage-1 antigen (integrin αMβ2)
MD1	lymphocyte antigen 86
MEP	megakaryocyte-erythrocyte progenitor
MHC	major histocompatibility complex
MILIBS	metal-ion-binding-site
MPP	multi-potent progenitor
mRNA	messenger RNA
NK	natural killer cell
NKT	natural killer T cell
PA	para-aortic
PAF	para-aortic foci
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGFRB	platelet derived growth factor receptor beta
PS	phosphatidylserine
qPCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
Runx1	Runt-related transcription factor 1
Sca-1	stem cell antigen 1 (Ly-6a)
S.D.	standard deviation
Sema4A	Semaphorin 4A
SSH	suppression subtractive hybridization
TCR	T cell receptor
T_H	T helper cell
TIM	Transmembrane (or T cell) immunoglobulin and mucin domain containing molecule
TLR	toll-like receptor
T_{reg}	regulatory T cell

List of original publications

The thesis is based on the following articles, which are referred to in the text by their Roman numerals (I–III).

I Säynäjäkangas R*, Uchida T & Vainio O (2009) Differential gene expression in $CD45^+$ cells at para-aortic foci stage of chicken hematopoiesis. Scandinavian Journal of Immunology 70: 288–294.

II Syrjänen R, Petrov P, Glumoff V, Fang S, Salven P, Savolainen E-R, Vainio O & Uchida T (2014) TIM-family molecules in embryonic hematopoiesis: fetal liver TIM-4^{lo} cells have myeloid potential. Experimental Hematology 42: 230–240.

III Syrjänen R, Petrov P, Glumoff V, Savolainen E-R, Fang S, Salven P, Vainio O & Uchida T (2014) Differential expression of TIM-2 during B cell development. Manuscript.

*Syrjänen *née* Säynäjäkangas

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1 Introduction

Hematopoiesis, i.e., the formation of blood cells, gives rise to cells required for the oxidation of tissues, blood clotting and the immune responses against pathogens. Hematopoietic stem cells (HSC) arise during embryonic development from the dorsal aorta (1, 2). From there, the HSC migrate into the primary embryonic hematopoietic organs, which are the underlying para-aortic (PA) mesenchyme in chickens and fetal liver in mammals (3, 4). Later in development, hematopoietic stem and progenitor cells (HPC) migrate further into bone marrow, thymus and spleen and into the Bursa of Fabricius in avians, to differentiate and expand.

These hematopoietic organs constitute microenvironments that provide signals required for the differentiation, proliferation and migration of HSC and HPC. In addition, HSC and HPC themselves produce molecules that regulate hematopoiesis. These include cytokines, chemokines, growth factors and transcription factors. However, these molecules have not yet been sufficiently characterized. Diagnostics and treatments of blood diseases, like leukemia, utilize knowledge of these molecules. Increased information on them can improve techniques used for *in vitro* generation and expansion of hematopoietic cells. Many of the HSC and HPC populations can be defined based on their surface antigen expression. Further information on these markers would greatly improve the isolation of HSC and HPC. In addition, many genes involved in the normal hematopoiesis are mutated in leukemia (5). Therefore, knowledge of these molecules helps in understanding the mechanisms of malignant transformation and eventually in development of treatments.

T cell immunoglobulin and mucin domain molecules (TIM) form a family of type I glycoproteins. In mammals, TIM family members are expressed by several types of immune cells including T cells, B cells and antigen presenting cells (APC). TIM molecules are known to function in immunity, e.g., in regulation of T helper (T_H) cell responses (6–8) and in removal of apoptotic bodies through phosphatidylserine (PS) binding (9–12). TIM-3 has been reported to be overexpressed by leukemia stem cells (13), but the role of the TIM molecules in hematopoiesis is unknown.

In order to find and analyze novel molecules involved in embryonic hematopoiesis, a complementary DNA (cDNA) library of HSC and HPC from chicken PA region was constructed. The library provided both previously identified and novel candidate genes. These included homologs to chicken *tim-1*

and *tim-4*. The expression and role of TIM family molecules during hematopoietic cell differentiation in chicken and mouse was further studied in this thesis work.

2 Review of literature

2.1 Hematopoiesis

All blood cells are produced from hematopoietic stem cells in a process called hematopoiesis. There are two types of hematopoiesis, primitive and definitive. Primitive hematopoiesis produces primitive erythrocytes, macrophages and megakaryocytes that are required during embryonic development and growth. Primitive HSC are not capable of long-term production of blood cells, and the majority of their progeny disappear shortly after birth. The role of definitive hematopoiesis is to produce all lineages of blood cells throughout the individual's life span. Definitive HSC can self-renew and their derivatives reside in the bone marrow of an adult individual.

HSC possess two properties. First, they can undergo self-renewal to continuously supply blood cells, and secondly, they are multi-potent, thus capable of producing all the cells of the hematopoietic lineages (1, 14). During hematopoiesis, stem cells gradually lose their self-renewal capacity and multi-potentiality, as they differentiate into certain lineages. This is a step-wise process that is conducted by the microenvironment of the tissues the cells reside in. The hematopoietic microenvironment consists of molecules secreted by the surrounding cells, e.g., stromal cells in the bone marrow and the thymus. These molecules include growth factors, transcription factors, cytokines, chemokines, and micro RNAs (ribonucleic acid). Expression of these molecules is strictly regulated in a temporal and spatial fashion by both genetic and epigenetic mechanisms.

Epigenetic changes, including histone modifications and DNA methylation, are especially important for self-renewal and lineage specification as they allow timed activation of developmental genes. These epigenetic programs can also be passed down through subsequent cell divisions allowing cells to remember their acquired lineage-related gene expression profiles (15).

In has been suggested that histone acetylations maintain loose chromatin structure during early differentiation so that multi-lineage affiliated genetic programs are easily accessed (16). In addition, many genomic regions have been shown to maintain a bivalent structure characterized by both activating and repressing histone modifications (17). These poised regions have been suggested to indicate pre-priming of HSC into certain lineages or to allow developmental

flexibility (18). These epigenetic markers are lost during further histone modifications, which are often associated with loss of self-renewal and multi-potency. For example, silencing *octamer-binding transcription factor* (*oct4*) gene results in loss of pluripotency in stem cells (19, 20).

In addition to histone modifications, timed activation of genes by promoter DNA demethylation has been shown to be important for both human and mouse hematopoiesis (21–24). Genome-wide analysis of promoter methylation has revealed that many genes are initially methylated in HSC and multi-potent progenitors (MPP) and later specifically activated by demethylation (21, 24). These genes include e.g., granulocyte-macrophage lineage-associated *myeloperoxidase* (*Mpo*) and T cell-associated *SRC family kinase* (*Ikt*) (21). Recently, evidence on e.g., RNA-dependent DNA-methylation (25) and direct micro RNA-DNA binding-associated gene silencing (26) has indicated that non-coding RNAs also participate in epigenetic chromatin modifications (27).

In this thesis, hematopoiesis is described mainly as it takes place in the mouse. However, most of the events and mechanisms of hematopoiesis are similar in all vertebrates.

2.1.1 Hematopoietic stem cells

Adult mouse HSC can be recognized by their high surface expression of stem cell antigens c-kit (proto-oncogene c-kit/tyrosine-protein kinase kit/CD117) and Sca-1 (stem cell antigen 1/Ly-6a) and lack of lineage marker expression (Lin) (28). They are therefore designated as LSK cells ($Lin^-c\text{-}kit^+Sca\text{-}1^+$). Lineage markers are defined by an antibody cocktail that recognizes cells from the major hematopoietic lineages including T lymphocytes, B lymphocytes, monocyte/ macrophage, granulocytes, natural killer (NK) cells and erythrocytes. The LSK population is rather heterogeneous, and HSC can be further enriched among them as CD34 and Flk2 (fetal liver kinase 2) negative (29). Later, HSC have also been defined as $CD150^+CD48^-CD244^-$ cells which largely overlap with the $CD34^-Flk2^-$ LSK population (30, 31).

Long-term reconstituting HSC (LT-HSC), identified as $Lin^-c\text{-}kit^+Sca\text{-}1^+CD150^+CD48^-CD34^-Flt3^-$, are the only population capable of long-term hematopoietic reconstitution (Fig. 1) (32–34). When the LT-HSC differentiate, they become short-term reconstituting HSC (ST-HSC, $Lin^-c\text{-}kit^+Sca\text{-}1^+CD150^+CD48^+CD34^{-/+}Flt3^-$) which are incapable of longer-term reconstitution due to their limited self-renewal capability (35). During further differentiation,

short-term reconstituting HSC lose their ability to self-renew and differentiate into multi-potent progenitors (MPP) identified as $Flt3^+$ (fms-related tyrosine kinase 3) LSK (32–34). MPP have still retained multi-lineage capability but can reconstitute only transiently (34, 36).

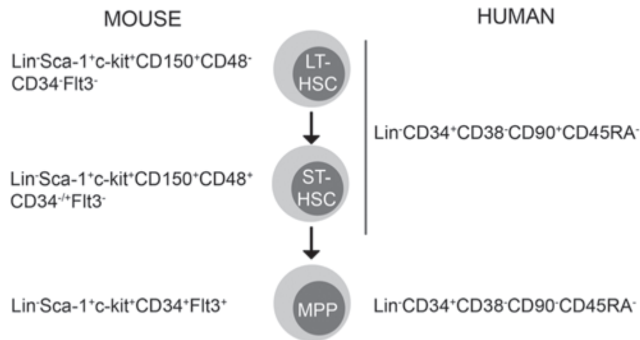


Fig. 1. Markers for long-term and short-term reconstituting hematopoietic stem cells (LT-HSC and ST-HSC, respectively) and multi-potent progenitors (MPP) in mouse and humans.

The same surface markers can generally be used to isolate fetal and adult HSC and HPC although some differences have been identified. These differences are described in later chapters where the embryonic HSC and HPC are discussed further.

2.1.2 Emergence of the hematopoietic stem cells in the embryo

HSC are known to arise in several intra- and extra-embryonic sites including yolk sac, aorta-gonads-mesonephros region (AGM), allantois/placenta, major arteries (umbilical and vitelline arteries) and endocardium of the heart (1, 2, 37–41) (Fig. 2). The processes related to HSC emergence are similar in the different sites although the arising cells can be phenotypically and functionally different and produce distinct types of stem and progenitor cells (e.g., primitive and definitive) (42). The exact role of each site in hematopoiesis still remains under debate (43).

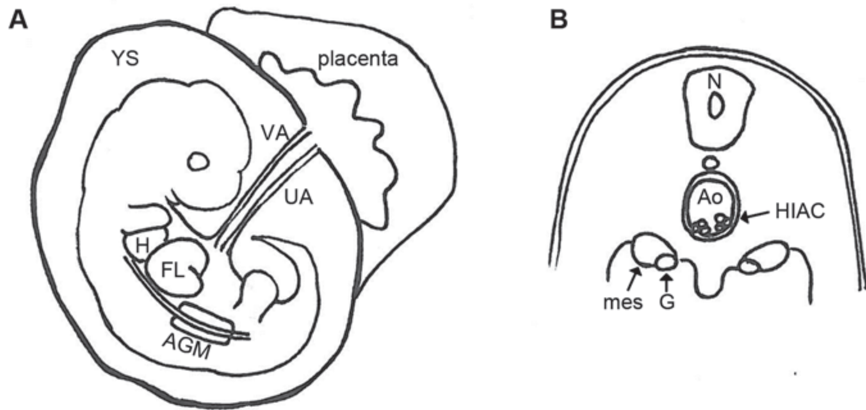


Fig. 2. Hematopoietic sites in 11.5 dpc mouse embryo. A) Longitudinal section and B) cross section. YS, yolk sac; AGM, aorta-gonads-mesonephros region; VA, vitelline artery; UA, umbilical artery; H, heart; FL, fetal liver; N, notochord; Ao, aorta; HIAC, hematopoietic intra-aortic cluster; mes, mesonephros; G, gonad.

Yolk sac

First HSC arise in the extra-embryonic site yolk sac as blood islands at 7.5 dpc (days post coitum) in mouse and at ED (embryonic day) 1 in chicken (1, 37). These first HSC express c-kit, CD34, and CD41 (44). The yolk sac remains the only source of hematopoietic cells until blood circulation starts. It is considered to provide only short-term myeloid reconstitution in the form of primitive erythrocytes, macrophages and megakaryocytes (45). These cells are required for the development and growth of the embryo.

It has been suggested that some yolk sac-derived HSC also contribute to definitive hematopoiesis. For example, progeny of primitive macrophages constitute at least some of the resident tissue macrophages in the adults, including microglia, Kupffer cells and Langerhans cells (46, 47).

Aorta-gonads-mesonephros region

The first evidence for an intra-embryonic source of definitive HSC came from sophisticated chicken-quail chimera experiments by Dieterlen-Lièvre (2). She crafted quail embryos into chicken yolk sacs before or shortly after the establishment of vascularization and then assessed contribution of each species

into the developing hematopoietic system based on the differences on the nuclei. The results showed that cells giving rise to definitive hematopoiesis did not originate from the yolk sac (chicken), as was previously thought, but were of intra-embryonic origin (quail) instead. Later, this intra-embryonic origin of definitive HSC has been confirmed and characterized in avian, amphibians and mammals as the AGM, also known as the para-aortic splanchopleura (48–53). Furthermore, the aortic region, but not the surrounding urogenital compartment of the AGM, has been established as the source for HSC (54).

The HSC arise from the ventral wall of the aorta as hematopoietic intra-aortic clusters at 8.5–11.5 dpc in mouse, from ED 3 in chicken and from 24 days of gestation in humans (3, 53, 55–57) (Fig. 2B). AGM creates only a very limited number of HSC but is the most potent source of multi-lineage reconstituting progenitors (58). The AGM HSC can be identified by their expression of e.g., c-kit, CD34 and CD41 (44). In addition, HSC begin to express the common leukocyte antigen CD45 (cluster of differentiation 45) and the HSC marker CD150 during AGM hematopoiesis (44, 59).

Hematopoietic and endothelial cells originate in close proximity and were therefore suggested already a century ago to arise from a common ancestor called the *hemangioblast* (reviewed in (60, 61)). Aortic cells giving rise to HSC do indeed express markers for both endothelial (e.g., CD31/PECAM-1, endothelial-specific receptor tyrosine kinase and vascular endothelial cadherin) and hematopoietic lineages (e.g., AA4.1/CD93, CD45 and CD41) (62, 63), and knock-out of molecules common to both cell types, e.g. mouse *flk1* (fetal liver kinase 1/vascular endothelial growth factor receptor 2) and zebra fish *cloche* result in impairment of both hematopoietic and endothelial tissues (64, 65). However, there is still no direct evidence showing differentiation of HSC and endothelial cells from a common origin. Instead, an alternative theory where HSC bud from already differentiated endothelial cells of the aorta referred to as *hemogenic endothelium* has been suggested and is supported by several studies (reviewed in (60, 61)). Time-lapse imaging has given the most direct evidence by visualizing the budding of the endothelial cell from the vascular wall into a free-moving hematopoietic cell in real time (66–70). Interestingly, a study by Lancrin *et al.* (71) has connected the two theories by suggesting that hemangioblast first gives rise to hemogenic endothelium which then generates HSC.

Molecular mechanisms behind the HSC emergence are not yet fully understood, but some key signals known to be indispensable for their formation include initiation of the circulation, nitric oxide, Runx1 (Runt-related

transcription factor 1) and Notch1. The blood flow, starting at 8.5 dpc in mouse, causes mechanical pressure in the form of shear stress that activates the aorta to produce nitric oxide, which in turn increases expression of the transcription factor Runx1 in the $CD45^{+}c\text{-}kit^{+}$ cells in the aorta (72). Expression of Runx1 precedes the emergence of HSC in the AGM and has been shown to be indispensable for the intra-aortic cluster, hematopoietic progenitor, and HSC formation (73, 74). During AGM hematopoiesis, Notch1 is important in the endothelial-hematopoietic transition (75, 76).

Other hematopoietic sites

In addition to AGM and yolk sac, also other sites contribute to the HSC pool. The allantois is an extra-embryonic organ involved in gas exchange, excretion and bone formation and in avian, in shell calcium resorption. In mammals, allantois forms part of the placenta. Transplantation studies by Till and McCulloch and Dancis *et al.* were the first ones to establish the placenta as a source of HSC (77–79). These findings were forgotten for decades until Caprioli and colleagues discovered that the allantois in chicken has hematopoietic potential (38, 39). These and later studies have now established the allantois/placenta as an independent site for definitive HSC emergence and amplification (80–82). It has been suggested that the role of placenta and allantois is to provide HSC later in embryonic development, after the AGM hematopoiesis has ceased (82).

Vitelline and umbilical arteries have been shown to generate HSC capable of long-term multi-lineage hematopoietic repopulation (40, 83). The umbilical artery connects the aorta to the placenta and the vitelline artery the aorta to the yolk sac. Their role in hematopoiesis has not yet been much studied, and their contribution to the hematopoiesis is likely to be low (43). The emergence of HSC in the major arteries has been suggested to be initiated by the physical pressure from blood flow in a similar mechanism as in the dorsal aorta.

Also hemogenic endocardium of the heart was recently suggested as a source for transient definitive hematopoietic progenitors by Nakano *et al.* (41). They detected hemogenic activity from a subset of endocardial cells which also expressed key cardiac transcription factors NKx2-5 (NK2 homeobox 5) and Isl1 (insulin gene enhancer protein). Close relationship between hematopoietic and cardiac tissues has been suggested before (84–86) and therefore it is possible that the hematopoietic cells arise from multi-potent cardiovascular progenitors (41).

2.1.3 Differentiation and proliferation of the hematopoietic stem cells takes place in the para-aortic foci and fetal liver

Definitive HSC do not reside for long at their site of origin; they migrate into other sites which provide the microenvironment required for expansion, differentiation and further migration into lineage-specific organs (60). In chicken, HSC start to cluster and ingress into the underlying mesenchyme, giving rise to para-aortic foci (PAF) at ED 4–10 (3). In mammals, the HSC migrate further into the fetal liver (FL), most likely through the bloodstream. In mouse, this happens from 10 dpc until the first two to four days after birth (4, 87). β1-integrin is required for the colonization of FL in mice as HSC deficient for it accumulate in the circulation and do not home into FL (88, 89). Yolk sac-derived HSC and HPC also differentiate in the PAF/FL.

FL HSC can be characterized with several surface markers including AA4.1, CD150 and CD34 (31, 90–92). They can also be identified as c-kit^{+}Sca-1^{+}Lin$^{-/lo}$Mac-1^{+}Thy1.1^{lo} (93). Interestingly, Mac-1 (Macrophage-1 antigen/integrin αMβ2), which is generally known as a marker for macrophages, is only expressed by FL HSC and not by adult bone marrow HSC. FL HSC differ from their adult counterparts also by their superior proliferative capacity.

Expanding the definitive HSC is an important function of the PAF/FL because AGM only gives rise to a limited number of HSC. In the mouse FL, the number of HSC has been shown to increase almost 40-fold between 12 and 16 dpc while the cells still maintain their repopulating capability (94). The numbers of myeloid and B cell progenitors also increase in the FL until birth (95, 96). Interestingly, the number of T cell progenitors in FL is at the highest already at 13 dpc (97). Known factors involved in FL HSC/HPC expansion include c-myb, PU.1, angiopoietin-like factors and Sox17 (98–101).

Recently, early AGM- and yolk sac-derived HSC were shown to have lower reconstituting ability than later HSC and were thus suggested as immature HSC (102). Furthermore, the study suggested that these cells mature in FL by acquiring HSC properties including surface marker expression and improved long-term reconstitution activity, thus highlighting the function of FL as a site for HSC differentiation. Also HPC differentiate in PAF/FL, although most lineages do not reach full maturity there. Both yolk sac- and AGM-derived erythrocytes can differentiate into mature cells within the erythroblast islands of the fetal liver (103). In mammals, also B cells differentiate close to maturity in FL. Later in development, HSC and HPC migrate from PAF/FL into thymus, spleen and in

chicken, also into bursa of Fabricius. HSC and HPC also migrate into bone marrow during late gestation and the early post-natal life (104, 105). In adults, bone marrow functions as the pool of hematopoietic stem and progenitor cells during the whole life span (106). Lineage differentiation taking place in FL and the later hematopoietic sites is described in more detail in chapters 2.1.5 and 2.1.6.

2.1.4 Lineage commitment

Hematopoietic cell differentiation happens as a step-wise process during which the cells lose their self-renewal ability and multi-potentiality. This is usually referred to as lineage commitment or lineage restriction and it describes the developmental potential of each differentiation phase (reviewed in (107, 108)). However, the importance of *in vivo* cell fate, meaning the actual physiological outcome of HPC, should also be taken into account when lineage commitment is discussed (107).

The classical model for hematopoiesis suggests that lymphoid and myeloid lineages separate directly from multi-potent progenitors (Fig. 3A). This model was originally not based on experimental data but on the historical myeloid-lymphoid dichotomy established already more than 100 years ago (109, 110). The model was later supported by the identification of common lymphoid (CLP) and myeloid progenitors (CMP) from mouse bone marrow by Weissman lab (111, 112). They purified mouse bone marrow $Lin^{-}IL\text{-}7R\alpha^{+}c\text{-}kit^{lo}Sca\text{-}1^{lo}$ cells that could give rise to T, B and NK cells but not to cells of the myeloid lineage, and designated them as CLP (112). Next, CMP, which subsequently give rise to megakaryocyte-erythrocyte progenitors (MEP) and granulocyte-macrophage progenitors (GMP), were also characterized (111). These findings were further supported by identification of the corresponding progenitors from mouse FL (113, 114). However, the CLP identified from FL also harbor macrophage potential (114). Indeed, there are also other studies including characterization of progenitor populations based on more surface markers and single-cell clonal analyses which suggest that lineage commitment may not follow the classical route (reviewed in (107, 108, 115, 116)). These reports mainly question the notion of the early segregation of the myeloid and lymphoid lineages and also include several branching points for myeloid lineages. Therefore, several revised models of lineage commitment have been proposed.

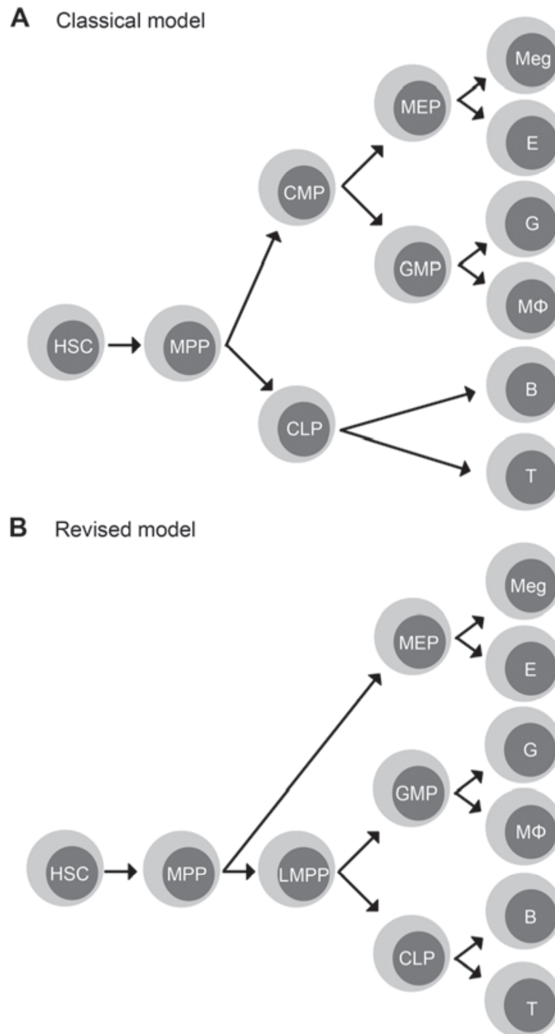


Fig. 3. Models of hematopoietic lineage commitment. A) Classical model B) Revised model. HSC, hematopoietic stem cell; MPP, multi-potent progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-macrophage progenitor; LMPP, lymphoid primed multi-potent progenitor; Meg, megakaryocyte/platelet; E, erythrocyte; G, granulocyte; MΦ, macrophage; B, B cell; T, T cell.

First of all, Adolfsson *et al.* showed that $Flt3^+$ LSK cells, which are generally considered as MPP, have both T and B lymphoid and granulocyte-macrophage

potential but only little or no megakaryocyte-erythrocyte potential, and designated these cells as lymphoid primed multi-potent progenitors (LMPP) (117). Distinct patterns of multi-lineage transcriptional priming also suggest that megakaryocytes and erythrocytes branch out from the HSC separately from other myeloid lineages (118–121). Taken together, these data suggest an alternative model for lineage commitment (115) (Fig. 3B).

On the other hand, results obtained from a clonal assay system by Kawamoto *et al.* (122) which enables analysis of differentiation of a single cell into B, T and myeloid lineages suggest that T and B cells do not develop from a common precursor. Instead, bipotent progenitors giving rise to myeloid and B or myeloid and T cells were detected from FL. Based on this and further analyses (122–125), a myeloid-based model of lineage restriction was suggested (126). Key points in this model are the presence of CMLP (common myelo-lymphoid progenitors), the retention of myeloid potential after segregation into B and T lineages, and the absence of CLP.

The validity of the different models still remains under discussion (Fig. 3). It is also possible that lineage commitment differs between adult and embryonic hematopoiesis (115). For example, embryonic but not adult hematopoiesis fits the myeloid-based model very well (107). Some differences may also result from the fact that the models are based on different experimental systems and focus on different aspects of the lineage commitment (107). However, particular points can be generally agreed on. First of all, lineage restriction is a gradual event and retention of lineage potential is thus not an all-or-nothing issue (127). This means that progenitors retain potential to give rise to other lineages until more mature stage of development (128). Secondly, the progenitors also express multiple lineage-affiliated genes, also referred to as promiscuous gene expression, during their differentiation into certain lineage (129–133). Such robustness in lineage restriction allows a rapid reaction to changing environmental factors, for example by providing quick expansion of myeloid fraction as a response to infections. Lastly, HSC can reach a specific cell fate through more than one type of intermediate progenitors (128).

2.1.5 Myeloid cell differentiation

Myeloid cells function in innate immunity and provide the first non-antigen-specific line of defense against pathogens. They include megakaryocytes/ platelets, granulocytes, monocytes/macrophages and mast cells. Also erythrocytes

are classically thought to belong to myeloid lineage. Dendritic cells (DC) may differentiate from both myeloid and lymphoid progenitors but are described under myeloid differentiation in this thesis. As discussed previously, the first myeloid cells to emerge during development are yolk sac-derived primitive macrophages, erythrocytes and megakaryocytes. Later in development, definitive myeloid progenitors differentiate in the FL and in adult in bone marrow.

Myeloid progenitors can be distinguished both in FL and adult bone marrow among Lin$^-$ckit$^+$Sca1$^-$ cells based on their expression of FCγR (fragment, crystallizable gamma receptor II/III) and CD34 (111, 113). CMP can be identified as Lin$^-$c-kit$^+$Sca-1$^-$CD34$^+$FCγR^{lo}, the MEP as Lin$^-$c-kit$^+$Sca-1$^-$CD34$^-$FCγR^{lo} and the GMP as Lin$^-$c-kit$^+$Sca-1$^-$CD34$^+$FCγR^{hi} (Fig. 4). Later, shared macrophage-dendritic cell progenitors have also been shown to differentiate from CMP (134).

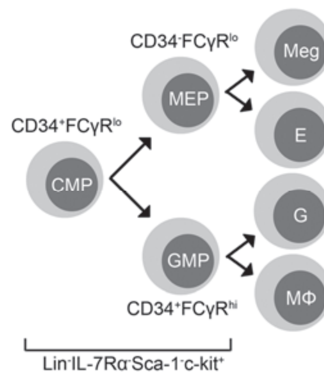


Fig. 4. Markers for myeloid progenitor cell populations in mouse fetal liver and adult bone marrow. CMP, common myeloid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-macrophage progenitor; Meg, megakaryocyte/platelet; E, erythrocyte; G, granulocyte; MΦ, macrophage.

Transcription factor PU.1 is required for myeloid cell development. Interestingly, it can also be used to separate two distinct cell populations: PU.1-expressing cells have macrophage-granulocyte potential while PU.1-deficient cells have erythroid potential (135, 136). Furthermore, PU.1 knock-out mice lack CMP and CLP but have relatively normal numbers of erythrocytes and MEP (137, 138). This suggests that the role of PU.1 in myeloid development is to conduct lineage choice.

The development of mast cells has not yet been well characterized. Mast cell precursors can be found in fetal mouse blood from 15.5 dpc until birth and in very low numbers in adult blood (139, 140). Differentiation both from MPP and from CMP has been suggested as a source for the mast cell precursors (141, 142). Development of other myeloid lineages is better understood and is described below.

Megakaryocyte and erythrocyte development

Megakaryocytes and erythrocytes belong to the same lineage and can arise from MPP and their common progenitor MEP (143) (Fig. 3). Megakaryocyte and erythrocyte progenitors share many of the growth factor receptors and transcription factors. These include erythropoietin and thrombopoietin receptors and GATA-1 (globin transcription factor 1), GATA-2, FOG-1 (friend of GATA-1) and NF-E2 (nuclear factor, erythroid-derived 2) (144–150).

Erythrocytes are the first hematopoietic cells to differentiate during embryonic development, and in adult blood they are the most common cell type. Primitive erythrocytes are often nucleated, larger than definitive erythrocytes, and they express fetal hemoglobins (reviewed in (151)). They support the rapid growth of the embryo by providing oxygen to the tissues. Erythrocyte development begins in the yolk sac blood islands and then moves into FL erythroblast islands, and it depends on Wnt/β-catenin signaling pathway (152–154). Progressive stages of erythrocyte development include erythroid burst-forming unit (BFU-E), erythroid colony forming unit, pro-erythroblast, basophilic erythroblast, polychromatic erythroblast, orthochromatic erythroblast and reticulocyte (reviewed in (151)). These stages can be distinguished by expression of Ter119, CD71, c-kit and CD44 (155, 156).

Megakaryocytes develop from a megakaryocyte colony-forming unit through megakaryoblast and promegakaryocyte stages (reviewed in (157)). During maturation, they grow, become polyploidic through endomitosis and accumulate large amounts of cytoskeletal proteins and a highly tortuous invaginated membrane system. Then platelets are released from mature megakaryocytes through cytoskeletal structures called proplatelets (158, 159). Primitive megakaryocytes are larger and more reticular than their definitive counterparts and they are required for blood clotting during development, and especially during birth (143).

Development of other myeloid lineages

Granulocytes, macrophages and dendritic cells have been suggested to differentiate from GMP or bipotential lympho-myeloid progenitors (Fig. 3). Important growth factors involved in their development include granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) (reviewed in (160)). Also granule components like lactoferrin and neutrophil gelatinase are important in granulocyte and macrophage differentiation. Transcription factor C/EBPα (CCAAT/enhancer-binding protein alpha) is required for production of GMP from CMP, and PU.1 and IRF8 (interferon regulatory factor 8) in granulocyte versus macrophage lineage choice (reviewed in (161)). PU-1 is required for macrophage – DC fate decision from macrophage-dendritic cell progenitors (162).

Granulocytes include neutrophils, eosinophils and basophils. C/EBPε (CCAAT/enhancer binding protein epsilon) and GFI1 (growth factor independent 1) transcription factors are involved in the specification from GMP into granulocytes (reviewed in (161)). The subsequent developmental stage after GMP is myeloblast, which in turn is followed by the promyelocyte stage, where irreversible commitment to granulocyte lineage takes place (reviewed in (163)). The cells also start to produce granules. This continues until the end of the next myelocyte stage. During the subsequent metamyelocyte and band stages, the cells become smaller and the nucleus is condensed until it reaches the segmented structure characteristic of mature granulocytes.

Macrophage development also progresses through distinguishable stages (Fig. 5). These include granulocyte-macrophage colony-forming unit, macrophage colony-forming unit, pro-monocyte and monocyte. During differentiation, the macrophage precursors acquire granules and vacuoles which are characteristic of mature macrophage morphology. They also gradually start to express macrophage lineage-associated surface markers including F4/80, BM8 and Mac-1 (164, 165). Also ER-MP family molecules are expressed differentially during macrophage development: ER-MP12 (CD31/PECAM1) and ER-MP20 (Ly-6C) are expressed by progenitor cell subsets but not by mature macrophages (166, 167) (Fig. 5).

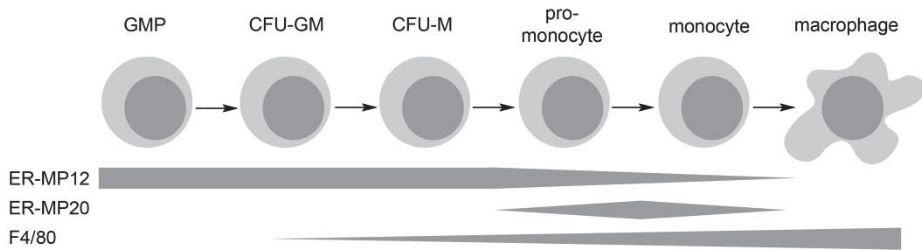


Fig. 5. Schematic representation of the development of macrophages from granulocyte-macrophage progenitors (GMP). CFU-GM, granulocyte-macrophage colony-forming unit; CFU-M, macrophage colony-forming unit.

During development, primitive macrophages are required for clearance of apoptotic cells and tissue remodeling (103, 152, 168). These yolk sac-derived macrophages have recently been suggested to be separable from embryo-derived macrophages by their high expression of the macrophage marker F4/80 (47). Furthermore, these yolk sac-derived macrophages contribute to definitive hematopoiesis in the adults as self-renewable resident tissue macrophages (46, 47). On the other hand, AGM-derived macrophage precursors and their progeny are considered to constitute the adult phagocyte system (47).

DC can arise from CMP and CLP (169, 170)) and Flt3 has been shown to be indispensable for their development from both lineages (171). Myeloid DC are generated from CMP through macrophage-dendritic cell progenitor, common DC progenitor and pre-DC stages in bone marrow (134, 172–174). Finally, the pre-DC acquire mature phenotype and morphology in lymph nodes. Lymphoid DC have been shown to arise from CLP, but also more differentiated early T cell progenitors in thymus can give rise to them (169, 175).

2.1.6 Lymphoid cell differentiation

Lymphocytes include T and B cells, natural killer (NK) and natural killer T (NKT) cells. T and B cells are responsible for the adaptive immune responses: T cells function in cell-mediated immunity whereas B cells function in humoral immunity by producing antibodies. Instead, NK and NKT cells are cytotoxic lymphocytes that function in innate immunity against viral infections and tumors. The function of both T and B cells relies largely upon their capability to recognize a wide variety of specific antigens. They acquire this attribute via receptor gene rearrangements followed by selection of functional but not self-reactive variants.

Based on different models of lineage commitment, lymphocytes have been suggested to differentiate from CLP or myeloid-B and myeloid-T bipotent progenitor cells (Fig. 3). In adult bone marrow, CLP can be identified as Lin^{-}IL-7Rα^{+}c-kit^{lo}Sca-1^{lo} and in FL as IL-7Rα^{+}B220$^{-/lo}$c-kit^{lo}Sca-1^{lo} cells (114, 176). CLP have later been shown to serve mainly as B cell progenitors (177, 178). However, direct progenitors upstream of T and B cell development usually express IL-7Rα (IL-7 receptor alpha) and IL-7 has been shown to be indispensable for lymphoid development (179). IL-7 is not directly involved in lineage commitment but instead regulates the immunoglobulin gene rearrangements, e.g., by maintaining EBF1 (early B-cell factor 1) transcription factor expression.

Also NK and NKT cells differentiate from CLP (176, 180). However, also myeloid progenitors have been suggested to give rise to NK cells, their origin thus still remaining under controversy (181–183).

B cell development

Early embryonic B cell development takes place in the FL in mouse and in the Bursa of Fabricius in chicken starting at 14 dpc and ED 8–14, respectively (184–186). Early B cell development moves from the FL and bursa into the bone marrow shortly after birth. There are also other differences in avian and mammalian B cell development (reviewed in (187)), e.g. in chicken the gene rearrangements include somatic gene conversion (188). Here, B cell development is described as it happens in the mouse using Philadelphia (Hardy) nomenclature (189–191).

Different steps of B cell development are characterized comprehensively based on the rearrangement status of the immunoglobulin (Ig) heavy and light chain genes and the expression of differentiation markers on the cell surface (Fig. 6) (192–194). In the bone marrow, B cell development is a continuous process but in FL, it happens stepwise, and all the stages are not present simultaneously (189, 195). However, B cell developmental stages can be identified with the same surface markers in FL and bone marrow (195).

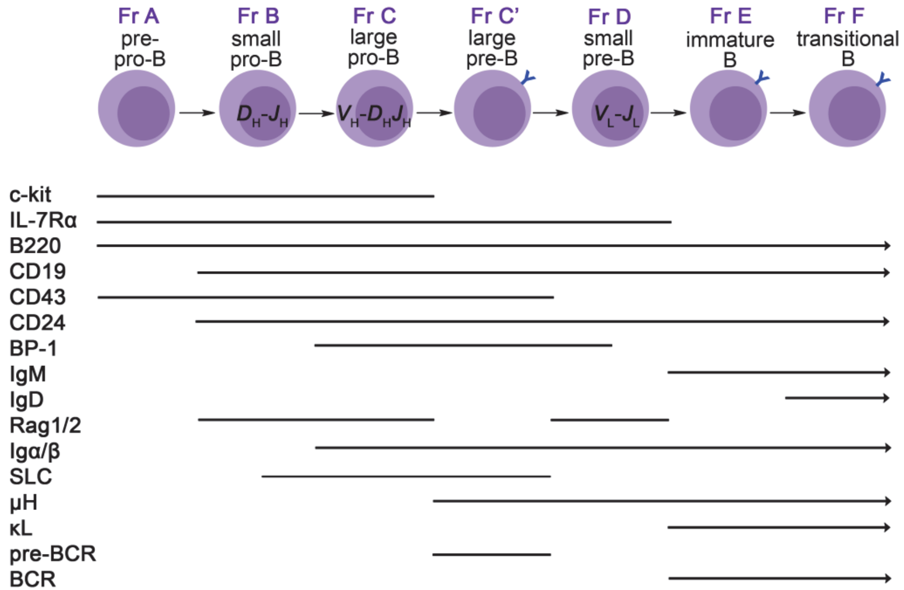


Fig. 6. Diagram showing development of B cells in mouse including immunoglobulin rearrangement events and expression of B lineage genes and surface markers. Fr, Fraction. Dash (-) represents immunoglobulin rearrangement events.

The earliest B cell progenitors, pre-pro-B cells (Fr A, $B220^+CD43^+CD24^-BP\text{-}1^-$) can be recognized by the expression of high molecular weight form of the CD45 (B220) (196). Pre-pro-B cells are heterogeneous and retain some T and NK cell potential (191, 197, 198). However, Hardy *et al.* (195) have identified that AA4.1-expressing cells within the pre-pro-B fraction are the only ones capable of B cell colony formation on *in vitro* assays.

B cell commitment takes place at the subsequent pro-B cell stage (Fr B, $B220^+CD43^+CD24^+BP\text{-}1^-$). It is associated with the upregulation of B-lineage transcription factors Pax5 (paired box 5), EBF1 (early B-cell factor 1), E2A (transcription factor 3) and Ikaros, which repress B lineage inappropriate genes and induce B cell-specific transcripts and eventually CD19 expression on the cell surface (199–202). In addition, recombination-activating genes 1 and 2 (Rag1 and Rag2) are expressed, resulting in the rearrangement of immunoglobulin heavy chain diversity (D_H) and joining (J_H) segments (203–205).

Next, at the large/late pro-B cell stage (Fr C, $B220^+CD43^+CD24^{lo}BP\text{-}1^+$), heavy chain variable region (V_H) is rearranged with the D_HJ_H segment creating a full-length μ heavy chain (206). μ heavy chain then associates with invariant

surrogate light chain, consisting of λ5 and VpreB proteins (207, 208). This complex is expressed on cell surface at the large/early pre-B cell stage (Fr C', $B220^{+}CD43^{+}CD24^{hi}BP\text{-}1^{+}$) and associates with Igα and β chains to form pre-BCR (B cell receptor/membrane bound antibody) complex (207–209). Expression of the pre-BCR on the cell surface at the large pre-B cell stage is an important checkpoint in the B cell development (210). If the IgH has successfully rearranged and provides strong enough signaling, Rag1/2 (recombination activating genes 1 and 2) and surrogate light chain genes are downregulated and the heavy chain undergoes allelic exclusion (206, 211, 212). Cells with functional pre-BCR also undergo a highly proliferate phase referred to as clonal expansion (213, 214).

After the clonal expansion, Rag genes are again upregulated to initiate immunoglobulin light chain rearrangement (V_L to J_L) (204, 214, 215). Cells at this stage are called small/late pre-B cells (Fr D, $B220^{+}CD43^{-}IgM^{-}IgD^{-}$). The completely rearranged Ig heavy and light chains then form the BCR, which is expressed on the cell surface at the immature B cell stage (Fr E, $B220^{+}CD43^{-}IgM^{+}IgD^{-}$) (216–218). This stage is a second important checkpoint in the B cell development because it monitors the IgL rearrangement and also self-reactivity (reviewed in (219)). If the newly-formed BCR provides adequate signaling, B cells are positively selected and allowed to migrate into spleen where further maturation takes place. Autoreactive or poorly signaling B cells can be rescued by receptor editing, are eliminated by apoptosis or enter a state of anergy (220–223).

B cells arising from FL precursors, also known as B1 cells, differ in their phenotype and function from the bone marrow-generated adult type B cells (B2 cells) (224, 225). B1 cells are distinguishable by their surface expression of CD5 and they are maintained through adulthood through self-renewal. They are considered innate-like cells because they express oligoclonal BCR, allowing them to respond rapidly to pathogens, and they can also act as APC (226).

T cell development

Instead of fetal liver and bone marrow, full T cell maturation takes place in a separate organ, the thymus. Despite some minor differences, T cell development in mammals and chicken is remarkably similar. The thymus is seeded continuously during mouse development, starting at 11 dpc from FL and after 16 dpc from bone marrow. During chicken embryonic development, the thymus is seeded in three subsequent waves (227). Progenitors in the first wave at ED 6–8

originate from the PAF and in the following two waves at ED 12–14 and ED 18 to hatching from bone marrow (227, 228).

Depending on the experimental design and the model used, several FL and bone marrow progenitors (MPP, LMPP, CLP) have been reported to have potential to migrate into thymus (176, 177, 229–233). These progenitors enter the thymus at the corticomedullary junction and differentiate into the earliest thymic progenitors characterized as $Lin^{-}CD44^{+}c\text{-}kit^{+}CD25^{-}$ (177). During the subsequent differentiation, cells migrate through the different thymic subcompartments which provide proper timing and type of interaction with the surrounding stromal cell microenvironment. Different stages of the T cell development are well characterized by surface antigen expression of CD24, CD44, CD4, CD8 and rearranged T cell receptor (TCR) chains (Fig. 7).

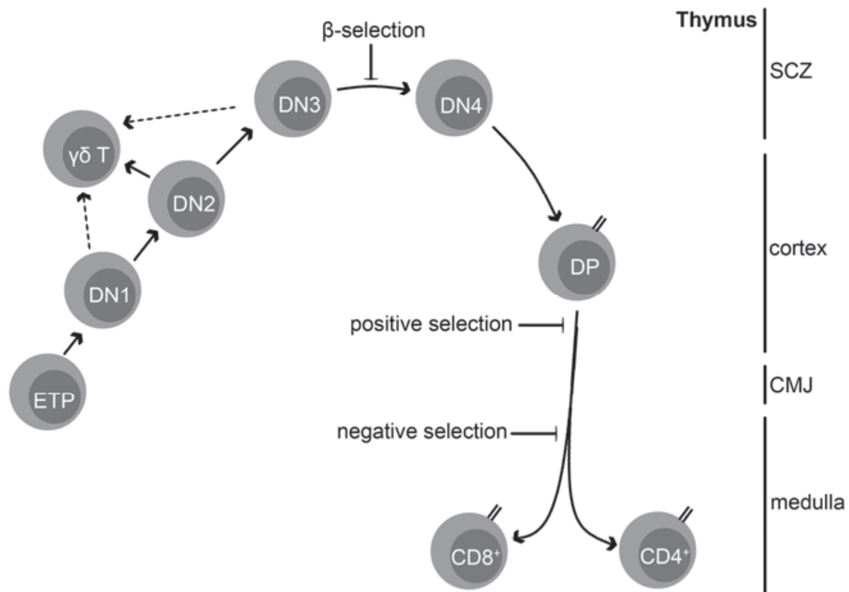


Fig. 7. Schematic representation of T cell development in thymus. ETP, earliest thymic progenitor; DN, double negative; DP, double positive; II, αβ T cell receptor; SCZ, subcapsular zone; CMJ, corticomedullary junction.

Notch family molecules are required for the T cell development. Signaling through Notch gradually induces a T lineage-associated genetic program and inhibits other cell fates (232–236). Notch1 and its ligand Delta-like4 (DL4) are especially important for the T lineage specification at the DN1 (double negative

1) stage defined as $CD4^-CD8^-CD44^+CD25^-$ (235). After proliferation and differentiation in the corticomedullary junction (237), DN1 cells migrate deeper into cortex and differentiate into DN2 thymocytes ($CD4^-CD8^-CD44^+CD25^+$) in which TCRβ, -γ and -δ genes begin to rearrange (238, 239). TCRαβ versus -γδ T cell fate is determined at the DN2 stage based on which gene rearrangement is successful. γδ T cells are prominent during embryonic development and they are thought to represent a more ancient or innate-like T cells (reviewed in (240)). They can recognize a variety of proteins and other types of antigens and are abundant in epithelia. Cells selected for αβ fate continue their differentiation by going through the DN3 stage ($CD4^-CD8^-CD44^-CD25^+$) and β-selection, an important developmental checkpoint, where cells expressing successfully rearranged β-chain as pre-TCR are positively selected for further stages (239). After DN3, cells have undergone irreversible commitment to T cell lineage. In the following DN4 stage identified as $CD4^-CD8^-CD44^-CD25^-$, the cells start to migrate back towards the medulla and upregulate CD4 and CD8, thereby differentiating into DP (double positive, $CD4^+CD8^+$) cells (237). DP cells rearrange the TCRα gene and start expressing the complete TCRαβ. These cells then undergo positive and negative selection. T cells that have recognized major histocompatibility complex (MHC)-self antigen complexes are positively selected and the ones that do not die by neglect (241). When the cells reach the medulla, they undergo negative selection, where cells having too high affinity for MHC are removed by apoptosis to prevent generation of autoreactive T cells (241). Surviving T cells then downregulate either CD4 or CD8 and become single positive αβ T cells that migrate into lymph nodes and periphery to conduct their function.

$CD4^+$ T cells help other immune cells through cell – cell interactions and by producing cytokines and are therefore called helper T cells. Also regulatory T cells (T_{reg}) differentiate from $CD4^+$ T cells. $CD8^+$ cells, on the other hand, produce cytotoxins called perforins and granzymes which kill infected or tumor cells by inducing apoptosis. Therefore they are referred to as cytotoxic or killer T cells.

2.1.7 Hematopoiesis in humans

Human HSC transplantation, which is used for treatment of hematological malignancies, is the first and most commonly utilized form of stem cell therapy. HSC are available from umbilical cord blood, bone marrow, and peripheral blood.

A lot of effort has been dedicated to investigating hematopoiesis in humans (242, 243) with techniques including *in vitro* culture methods, and transplantation of human HSC and HPC into humanized mice (244–246).

Many aspects of hematopoiesis are similar in humans and other vertebrates. These include the physiological events, e.g., emergence of HSC in yolk sac and AGM-region and cell differentiation first in FL and later in bone marrow and thymus (247). The basic principles of HSC multi-potency and self-renewal also apply.

However, there are also several differences. First of all, markers defining HSC and HPC populations are not the same (Table 1). Human HSC and HPC can be found among $CD34^+$ cells, and HSC can be further enriched among them as $Lin^-CD34^+CD38^-CD90^+CD45RA^-$ (Fig. 1) (248–251). On contrary to mouse, human HSC are also $CD150^-$ and $Flt3^+$ (252, 253). Some of the growth factors and cytokines are different, e.g., IL-7 is not adequate to support development of human B cells in culture (254). Also biological differences including body size, genomic diversity and length of lifespan must be considered. These differences highlight the importance of complementary research conducted in parallel in humans and model organisms (255).

Table 1. Markers defining mouse and human hematopoietic stem and progenitor cell populations in adults.

Population	Mouse phenotype	Human phenotype
HSC	$Lin^-c\text{-}kit^+Sca\text{-}1^+CD150^+CD48^-CD34^-Flt3^-$	$Lin^-CD34^+CD38^-CD90^+CD45RA^-$
MPP	$Lin^-Sca\text{-}1^+c\text{-}kit^+CD34^+Flt3^+$	$Lin^-CD34^+CD38^-CD90^-CD45RA^-$
CMP	$Lin^-IL\text{-}7R\alpha^-Sca\text{-}1^-c\text{-}kit^+CD34^+FC\gamma R^{lo}$	$Lin^-CD34^+CD38^+CD45RA^-CD135^+CD10^-CD7^-$
GMP	$Lin^-IL\text{-}7R\alpha^-Sca\text{-}1^-c\text{-}kit^+CD34^+FC\gamma R^{hi}$	$Lin^-CD34^+CD38^+CD45RA^+CD135^+CD10^-CD7^-$
MEP	$Lin^-IL\text{-}7R\alpha^-Sca\text{-}1^-c\text{-}kit^+CD34^-FC\gamma R^{lo}$	$Lin^-CD34^+CD38^+CD45RA^-CD135\text{-}CD10^-CD7^-$
CLP	$Lin^-c\text{-}kit^+Sca\text{-}1^+IL\text{-}7R\alpha^+$	$Lin^-CD34^+CD38^+CD45RA^+CD10^+CD7^-$

2.1.8 Strengths and limitations of different model organisms in developmental hematopoiesis

Several different animal models have been utilized in developmental hematopoiesis. Avians and amphibians have traditionally been used in developmental biology due to the relatively easy availability and large size of the embryos (reviewed in (60)). In addition, possibility for transplantation of embryonic tissues and cells enables cell fate studies. These include the chicken-

quail chimeras providing the first evidence on intra-embryonic origin of definitive HSC (see 2.1.2) (2). However, there are some limitations on the availability of antibodies, *in vitro* culture systems and knock-out animals in avian and amphibian systems. Some of these obstacles are overcomed by using, as a model, zebra fish (*Danio rerio*), which offers transparent rapidly growing embryos (256–258). Markers for zebra fish HSC and HPC are well characterized and transgenic animals, fluorescent reporter systems, lineage-tracing and anti-sense technology are available. However, lack of yolk sac and the evolutionary distance from humans have limited studies on zebra fish.

Today, mouse (*Mus musculus*) is the most commonly used animal model in hematopoiesis because of its close relation to humans, well characterized HSC and HPC, availability of antibodies and *in vitro* systems and easiness to create knock-out and reporter mice. However, mouse embryos are relatively small and timing of the embryonic development is not as straightforward as it is in e.g. avians and zebra fish.

The first part of this thesis work utilizes chicken (*Gallus gallus domesticus*) as a model organism (I). In the later parts (II and III), the analysis was extended to mouse to gain knowledge on TIM family molecules also in mammals and to utilize the better availability of antibodies and *in vitro* culture systems. In chicken, the work focuses on the PAF, which provides the microenvironment for HSC and HPC proliferation and differentiation, and in mouse, on the corresponding FL stage of hematopoiesis.

2.2 TIM molecules

TIM molecules constitute a family of type I glycoproteins. The family consists of three members (TIM-1, TIM-3 and TIM-4) in humans, four (TIM-1-4) in mouse (259) and two (TIM-1 and TIM-4) in chicken (260) (Table 2). In addition, there are four uncharacterized TIMs (TIM-5-8) present in rodents which are suspected to be pseudogenes. However, TIM-8, which is also known as Dppa1 (Developmental pluripotency associated 1), can have functions in cell differentiation (261). Structural and functional studies strongly suggest that human and mouse TIM-1, TIM-3 and TIM-4 are homologs and that mouse TIM-2 is highly homologous to human TIM-1 (259, 262, 263). This thesis work focuses on chicken TIM-1 and TIM-4, and mouse TIM-1, TIM-2, TIM-3 and TIM-4.

Table 2. Expression and function of TIM molecules in the immune system. Expression is stated only in humans, rodents and chicken.

Molecule	Species	Expression	Known functions	References
TIM-1				
	human	activated T cells	Regulation of T cell responses	(6, 264–266)
	rodents	B cells	Unknown	(6, 267)
	chicken	APC	Positive regulation of T cell responses	(268–271)
		kidney epithelial cells	PS binding mediated phagocytosis	(11)
TIM-2				
	rodents	T_{H2} cells	Negative regulation of T_{H2} responses	(272–275)
		B cells	Unknown	(274, 275)
TIM-3				
	human	T_{H1}, T_{H17}, $CD8^+$ T cells, T_{regs}	Regulation of T cell responses	(7, 8, 276–278)
	rodents	monocytes/MΦ, DC, mast cells	Regulation of immune responses	(12, 269, 279, 280)
		APC	PS binding mediated phagocytosis	(12, 281)
TIM-4				
	human	APC	PS binding mediated phagocytosis	(9, 10, 282)
	rodents		Regulation of T cell responses	(283–285)
	chicken			

In chicken, *tim* genes are located on chromosome 13, and in mouse and human in the T cell and Airway Phenotype Regulator (TAPR) region in syntenic chromosomal regions 11B1.1 and 5q33.2, respectively (259). The mouse TAPR region was identified as a site associated with asthma susceptibility, as a separate site from the IL gene cluster located in the same chromosome (259). The TIM genomic region and certain *tim-1* and *tim-3* polymorphisms have been linked with allergic asthma and atopic disease susceptibility in several studies in humans and mouse (259, 286–291).

TIM proteins are composed of an N-terminal IgV-domain (immunoglobulin variable domain), a mucin-like domain, a transmembrane region and a short cytoplasmic tail with putative tyrosine phosphorylation sites in TIM-1, TIM-2 and TIM-3 (259) (Fig. 8). The IgV and mucin domains contain several N- and O-glycosylation sites. Determination of the crystal structures of murine TIMs have revealed that sequence differences in the IgV and mucin domains enable the differential ligand-binding capabilities of the family members (282). However, there is a conserved metal-ion-binding-site (MILIBS) in the IgV domain of TIM-1, TIM-3 and TIM-4 that mediates many of the TIM-ligand interactions.

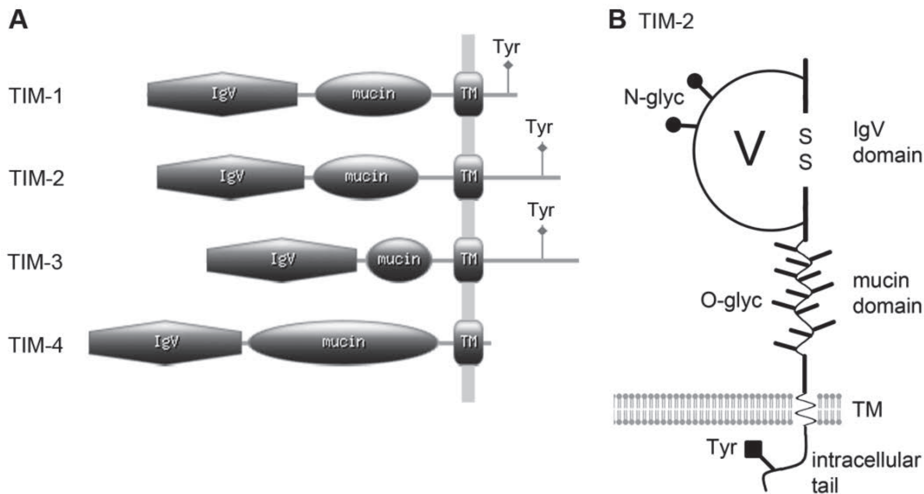


Fig. 8. A) Domain organization of the characterized mouse TIM-family members. B) Schematic structure of mouse TIM-2 protein. Only experimentally confirmed phosphorylation sites are shown (Tyr). Locations of N- and O-glycosylation sites (N-glyc and O-glyc, respectively) are presented as rough indications. IgV, immunoglobulin variable domain; TM, transmembrane domain.

TIMs are known to be expressed by liver and kidney cells and broadly by a variety of immune cells including T cells, B cells and APC (Table 2). They are known to conduct distinct functions both in adaptive and innate immunity, including T_{H1}-T_{H2} regulation (6–8) and removal of apoptotic bodies through PS binding (9–12). Even though TIMs are known to operate in several immunological responses, it has not been clarified whether they also have a role in the development of cells of the immune system.

2.2.1 TIM-1 as a T cell co-stimulatory molecule

TIM-1 was first discovered as hepatitis A virus cellular receptor 1 (HAVCR-1) in human and African green monkey (292, 293) and as kidney injury molecule 1 (KIM-1) in rat (294). In addition to hepatitis A virus (HAV), TIM-1 can bind PS, TIM-4 and itself (9, 11, 284). There is also some evidence that TIM-1 interacts with LMIR5 (Leukocyte mono-immunoglobulin-like receptor 5, CD300b) and IgA, but the true relevance of these interactions is unknown (295, 296).

TIM-1 was the first family member whose role was studied in the immune system as it was found to be expressed on activated but not naïve $CD4^+$ T cells and to function as a T cell co-stimulatory molecule both *in vitro* and *in vivo* (6, 259). Originally it was thought to positively regulate T_{H2} responses which are predominant in parasitic infections and allergy (297). Agonist antibody against TIM-1 promotes T_{H2} proliferation and production of the T_{H2} cytokine IL-4, prevents the development of tolerance and increases airway inflammation (6). Similar effects can also be seen as a result of TIM-4-TIM-1 interaction (284). Also NKT cells may promote T_{H2} responses via TIM-1: they constitutively express TIM-1 and TIM-1 co-stimulation on them enhances IL-4 production and inhibits IFN-γ (interferon gamma) production (270, 271).

Interestingly, TIM-1 ligation has also been shown to have opposing effects, including inhibition of T_{H2} cell responses, promotion of T_{H1} and T_{H17} commitment and diminished inflammation (264, 265, 298). A particular TIM-1 antibody can also impair immunosuppressive effects of T_{reg} cells (298). The differential T cell responses are suggested to be caused by separate ligand-binding sites (e.g. IgV and mucin domains) and different ligand binding avidities, which may result in differences in downstream signaling (264, 265). To date, all other known T cell co-stimulatory molecules (e.g., CD28, inducible T cell co-stimulator, cytotoxic T-lymphocyte-associated protein 4 and programmed cell death 1) can be categorized as positive or negative co-stimulators, and TIM-1 could therefore be the first known molecule to both activate and inhibit T cell responses (264).

Downstream signaling caused by TIM-1 expression on T cells has been studied. TIM-1 expression increases the activity of NFAT (nuclear factor of activated T cells) and AP-1 (activator protein 1) transcription factors (266). This activation depends on phosphorylation of tyrosine 276 (Y276) in the cytoplasmic tail of TIM-1, possibly by Fyn kinase (299). In another study, TIM-1 expressed in Jurkat T cells was shown to co-localize with CD3 and to be recruited to the TCR signaling complex, which results in phosphorylation of TIM-1, Zap70 (ζ-associated protein of 70 kDa) and Itk (IL-2 induced T cell kinase) (300). The MAPK/ERK (Mitogen-activated protein kinase/extracellular signal regulated kinase) pathway has also been suggested as a down-stream signaling target of TIM-1 ligation (301).

TIM-1 deficient mice have been analyzed in order to gain further insight on the role of TIM-1 in T cell responses. In general, TIM-1 knock-out mice are healthy and show only modest phenotypical changes (267, 302, 303). Wong *et al.* (267) did not detect any changes in T_{H2} responses whereas Curtiss *et al.* (303)

found slight increases in production of T_{H2} and T_{H17} cytokines. Mice where the mucin domain of the TIM-1 molecule was deleted developed autoimmunity, abnormal double-negative T cells, and autoantibodies after aging, possibly because of defective B regulatory cells (302). Indeed, the studies on knock-out mice have highlighted the importance of TIM-1 expression on B cells instead of T cells. TIM-1 expression was detected in splenic B cells and particularly in germinal center B cells after IgM induction through BCR-signaling (6, 265, 267). However, B cell responses and germinal center B cell differentiation were not affected in TIM-1 knock-out mice (267).

TIM-1 is also expressed constitutively by innate immune cells including dendritic cells and mast cells (268, 269). TIM-1 expressed by DCs promotes effector T cell responses and inhibits regulatory T cell responses via upregulation of costimulatory molecules and proinflammatory cytokines (268). Interfering with TIM-1-TIM-4 interaction can enhance T_{H2} activation by mast cells.

PS is expressed on the surfaces of apoptotic cells as a recognition marker for phagocytic cells (304). TIM-1 can bind PS and mediate the uptake of apoptotic cell (9, 11). This has been studied especially in kidney injury, where TIM-1-expressing kidney epithelial cells can transform into phagocytes and engulf injured cells through PS binding (11). The TIM-1^{+} epithelial cells may also promote tissue dedifferentiation and proliferation and attenuate inflammation (11, 305). Because many studies have shown that TIM-1 is upregulated in injured kidney, it is nowadays used as a diagnostic marker protein for acute kidney injury (306–308).

2.2.2 TIM-2 as a negative regulator of T_{H2} responses

The TIM-2 molecule exists only in murines and it is therefore the least known family member (Table 2). However, mouse TIM-1 and TIM-2 genes are almost equally homologous to human TIM-1 (41% and 36% respectively) and have likely arisen as a duplication of the TIM-1 locus (259). Therefore it has been suggested that the murine TIM-2 may have overlapping functions with human TIM-1 (263).

Like TIM-1, TIM-2 is expressed by kidney and liver cells and cells of the immune system including T_{H2} and B cells (272, 274, 275). To date, two TIM-2 ligands have been suggested: H-ferritin, which is involved in iron transport and storage, and Semaphorin 4A (Sema4A), which is involved in immunoregulation (274, 309). In addition, TIM-2 has been detected to function ligand-independently

(310). However, this could be a result of TIM-2 homodimerization similarly to TIM-1 (282, 310, 311). In contrast to other family members, TIM-2 is not able to bind PS or other phospholipids because it lacks critical amino acids in the MILIBS (12, 282).

Earliest studies on TIM-2 focused on its role in regulation of T_H responses. TIM-2 is expressed only in low levels by naïve T cells and T_{H1} cells but its expression is upregulated in T_{H2} cells, particularly by late differentiation (272–274) (Fig. 9). Blockade of TIM-2 causes inhibition of T_{H1} and induction of T_{H2} responses and less severe experimental autoimmune encephalomyelitis, a multiple sclerosis disease model in mice (272). In addition, mice lacking TIM-2 show T cell hyperproliferation, overexpression of T_{H2} cytokines and aggravated lung inflammation in airway inflammation model (272, 275). These are thought to be a result of preferential expansion or survival of the T_{H2} cells instead of negative regulation of T_{H1} responses. Kumanogoh *et al.* (309) identified Sema4A as a possible ligand for TIM-2 in the T_{H2} responses and showed that Sema4A – TIM-2 binding induces tyrosine phosphorylation of TIM-2. This suggests that TIM-2 is a receptor capable of transmitting signaling. Furthermore, TIM-2 expression can impair induction of NFAT (nuclear factor of activated T cells) and AP-1 (activator protein 1) dependent transcription, suggesting that it may be a possible signaling route for TIM-2 action (310). Taken together, these studies suggest that TIM-2 is required for the negative regulation of T_{H2} responses and allergic disease.

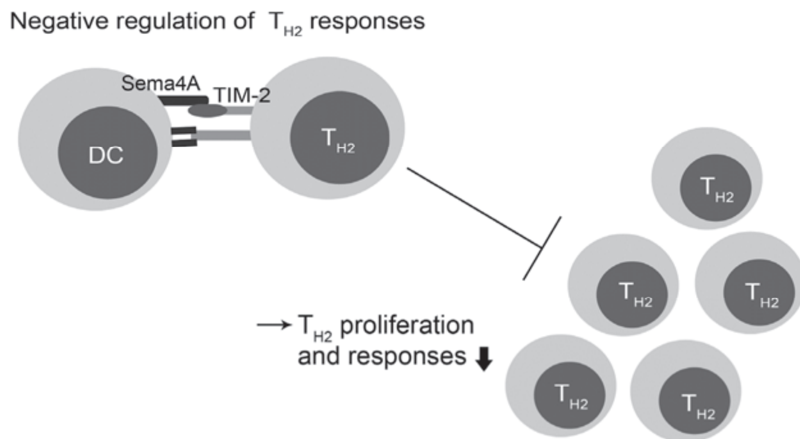


Fig. 9. Schematic representation of the role of TIM-2 in immune response.

TIM-2 can mediate uptake of H-ferritin, but not L-ferritin into cells in a similar fashion as other TIM molecules bind and engulf PS and apoptotic bodies, although through a different ligand binding site (274). The ferritin protein complex consists of 24 subunits of H- and L-ferritin and is generally known for iron binding and transport into cell (312). Interestingly, ferritin is also expressed during inflammation, binds into myeloid and lymphoid cells and regulates proliferation of immune cells (313–316). It inhibits T cell response to mitogens, impairs B cell maturation and can act as an immunosuppressor. Interaction with TIM-2 has been suggested to provide a connection for the immune regulatory functions of the ferritin (317).

Like TIM-1, TIM-2 is expressed by B cells, particularly by germinal center B cells in the spleen (267, 274, 275). However, the function and significance of TIM-2 expression by B cells has not yet been solved. TIM-2 is also expressed in the kidney, especially by renal tubule cells, and in liver bile duct epithelial cells, where it is possibly involved in transporting ferritin into or out of bile (274). Oligodendrocytes express TIM-2 which functions in internalization of H-ferritin required for the production of myelin during differentiation (318).

Even though TIM-2 has not been studied as much as the other family members, it is the only one whose role has been studied in embryonic development. Watanabe *et al.* identified TIM-2 as molecule expressed by 14.5 dpc mouse fetal liver hepatocytes (319). Binding of TIM-2-human Fc fusion protein with TIM-2 inhibited the differentiation of hepatocytes *in vitro* and knock-out of *tim-2* expression with small interfering RNA upregulated the expression of liver differentiation genes. Together, these results suggested that TIM-2 negatively regulates differentiation of hepatocytes. However, $CD45^+$ hematopoietic cells were excluded from these analyses completely, thus leaving open the question of the possible role of TIM-2 in fetal liver hematopoiesis.

2.2.3 TIM-3 as a regulator of adaptive and innate immune responses

TIM-3 is present in humans, rodents and other mammals (0). It is often referred to as hepatitis A virus cellular receptor (HAVCR-2) even though it cannot directly bind hepatitis A virus. TIM-3 is known to conduct diverse effects both in adaptive and innate immunity and it has at least four ligands: PS, galectin-9 (GAL9), high mobility group box 1 protein (HMGB1) and human leukocyte antigen B (HLA-B) -associated transcript 3 (Bat3) (7, 12, 320, 321).

The mucin domain of the TIM-3 is relatively short (Fig. 8) and so far both functional and structural studies have suggested that only its IgV domain is capable of ligand binding (320, 322, 323). On the other hand, the cytoplasmic tail of TIM-3 is longer and more complex than that of other TIMs and it contains several putative tyrosine phosphorylation sites (259). It is known that at least one of the tyrosines, Y265, is phosphorylated with Ikt (interleukin inducible T cell kinase) upon GAL9 binding into mouse T cells (324). NFAT (nuclear factor of activated T cells)/ AP-1 (activator protein 1), ERK/MAPK (mitogen-activated protein kinase/extracellular signal regulated kinase) and NF-κB (nuclear factor κB) signaling routes have been suggested to be involved in TIM-3 signaling both in adaptive and innate immune cells although the specifics have not been determined yet (279, 325–327).

TIM-3 is not expressed in naïve T cells. However, after several rounds of proliferation, TIM-3 expression is upregulated in T_{H1} cells, which mediate immune response against intracellular pathogens such as viruses and bacteria (7). Blocking TIM-3 – ligand interaction with a TIM-3 Ig fusion protein increases production of IFN-γ and hyperproliferation of T_{H1} cells, suggesting TIM-3 is a negative regulator of T_{H1} responses (322). In addition, a study by Zhu *et al.* (7) showed that a T_{H1} cytokine, IFN-γ, secreted by $CD4^+$ and $CD8^+$ T cells upregulated GAL9 expression by e.g. T_{regs}, B cells and mast cells. TIM-3 then binds GAL9, which results in inhibition of T_{H1} responses through increased apoptosis (7, 328). Binding of another TIM-3 ligand, human leukocyte antigen B (HLA-B)-associated transcript 3, can instead rescue T cells from the GAL9-induced apoptosis (321). Several studies have also shown that TIM-3 has a role in T_{H17} differentiation (329–331).

The T cell inhibitory functions of TIM-3 have also been highlighted in tolerance and transplantation (322, 331, 332). Blocking the TIM-3-GAL9 pathway decreases activity of T_{regs} (278, 333, 334) and correspondingly, interfering with TIM-3 with small hairpin RNA increases T_{reg} production (331). TIM-3-deficient mice have impaired tolerance induction and they reject islet allografts in diabetic mice (335). Furthermore, interfering with TIM-3-GAL9 signaling leads to accelerated rejection of cardiac allografts in mice as a result of increased donor-specific alloantibody production, increased T_{H1} and T_{H17} polarization and suppression of T_{regs} (330).

Interestingly, several studies have suggested a thus far unknown ligand for TIM-3 in T cell responses (322, 335, 336). The putative ligand can be detected with TIM-3-Ig fusion protein on naïve effector, regulatory and memory T cells

and DCs. Expression of TIM-3 and its unknown ligand are upregulated during T cell activation, and their interaction is suggested to suppress T cell cytokine production and proliferation (336).

On innate immunity, TIM-3 can conduct both positive and negative regulation. APCs, e.g. mouse $CD11^+$ DC and microglia and human monocytes and DC express TIM-3 constitutively, and TIM-3 signaling in these cells increases inflammation by upregulation of costimulatory receptors and cytokines (12, 279). On the other hand, inhibiting TIM-3 signaling on human monocytes increases cytokine production via Toll-like receptor (TLR) stimulation (280). In addition, innate immune responses are regulated by TIM-3 expressed by T cells by promoting the expansion of $CD11^+$ DC (337). TIM-3 expressed by APC can also mediate phagocytosis through binding of PS, which can result in cross-presentation of antigens to $CD8^+$ T cells (12, 281). Interestingly, binding of PS does not block the binding site of GAL9 because they bind on the opposite sites of the IgV domain. TIM-3 expressing T cells are not capable of phagocytosis (281). However, cross-linking of TIM-3 by apoptotic cells is suggested to affect immune response by inducing cytokine production or by providing pro-apoptotic signals to the T cell (7, 8, 279).

Opposed effects in TIM-3 mediated immunoregulation may be a result of differing downstream signaling (279, 324). However, it is not known how the divergent regulatory effects of TIM-3 are eventually balanced in immune responses. Because TIM-3 is expressed constitutively on APCs and upon activation in T cells, Kane (338) has suggested that TIM-3 increases T cell activation and differentiation during early immune response while helping to limit the response in later stages.

The role of TIM-3 has been studied in several diseases. Multiple sclerosis patients have impaired immunoregulation of autoreactive T cells due to dysregulated TIM-3 pathway (336, 339). In cystic fibrosis, TIM-3 is overexpressed in airways and it promotes abnormal neutrophil influx and eventually prolonged inflammatory response (340). TIM-3 expression has also been detected from leukemic stem cells but not from normal HSC in acute myeloid leukemia patients, suggesting a possible dysregulation of TIM-3 in myelopoiesis (13, 341, 342). TIM-3 overexpressed by tumor-associated DCs can also suppress innate immune responses against nucleic acids (320). This occurs through binding of HMGB1, which is known to be important in activation of innate immunity as a response to nucleic acids via e.g. TLRs (343). Nucleic acids

are normally transported into endosomes by HMGB1, but this is prevented by the interaction with TIM-3, resulting in attenuated tumor immunity (320).

TIM-3 is expressed by exhausted T cells in human cancer and *in vitro* cancer models (344–347). Exhausted T cells fail to proliferate and perform their normal functions like cytotoxicity and cytokine secretion (reviewed in (348)). Notably, TIM-3 is usually co-expressed with another known T cell exhaustion marker PD-1 (programmed cell death-1) and together they mark the most dysfunctional T cells (344, 346, 347). Furthermore, co-blocking TIM-3 and PD-1 restores $CD8^+$ T cell function and limits tumor growth in several types of cancer (349). Similar TIM-3-mediated T cell exhaustion is associated with human immunodeficiency virus and hepatitis C virus infections (277, 350).

2.2.4 TIM-4 in phosphatidylserine -mediated phagocytosis

TIM-4 has been independently cloned as SMUCKLER (spleen, mucin-containing, knock-out of lymphotoxin), which is downregulated in the spleen of lymphotoxin α- and β-deficient mice (351). In addition to mammals, it can be found in chicken (Table 2) (260). TIM-4 is the only TIM family member that does not have any phosphorylation sites in its cytoplasmic tail and therefore it is not likely to be involved in direct inward signaling (352). Instead, its main function appears to be PS-mediated phagocytosis (9, 282). It can also bind TIM-1 and LMIR5 although the biological importance of TIM-4-LMIR5 binding is so far unknown (284, 295).

TIM-4 differs from other TIM molecules also in its expression pattern: it cannot be found in T and B cells, but is instead found in certain APC populations (9, 10, 284, 353). In humans, TIM-4 is expressed by tingible-body macrophages in germinal centers of tonsils and white pulp of spleen (9). In mice, TIM-4 is expressed by resident $F4/80^+$ peritoneal macrophages, splenic $MOMA\text{-}1^+$ marginal zone metallophilic macrophages, $CD11^+$ DCs, and peritoneal B1 cells but not by plasmacytoid DC, for example (9, 10, 281, 283, 284, 351). In fact, TIM-4 appears to be expressed by APC only at certain stages and is replaced by other PS receptors when cells are involved in different immune responses. For example, peritoneal macrophages lose TIM-4 and instead start to express TIM-3 and MFG-E8 (milk fat globule-EGF factor 8 protein) upon thioglycollate-elicition (10, 12).

Several structural and functional studies have confirmed that TIM-4 binds PS (9, 10, 282), which is translocated on the surface of apoptotic cells as a marker for phagocytic cells (reviewed in (304)) (Fig. 10). Clearance of the apoptotic bodies

is important for maintaining tissue homeostasis as failure results in e.g. autoantibody production and inflammation (reviewed in (354)). The conserved MILIBS cleft of the IgV domain is required for the PS binding into TIM-4 (282). The engulfment phase of phagocytosis also requires TIM-4, although there is contradicting data on the exact domain requirement (352, 355). Wong *et al.* showed that the intracellular tail of TIM-4 is required for the ingestation process (355) whereas Park *et al.* demonstrated that the transmembrane domain is indispensable (352). The mechanism for TIM-4-mediated engulfment appears to utilize cytoskeleton and myosin motor proteins but not any of the known phagocytosis signaling pathways (262, 352).

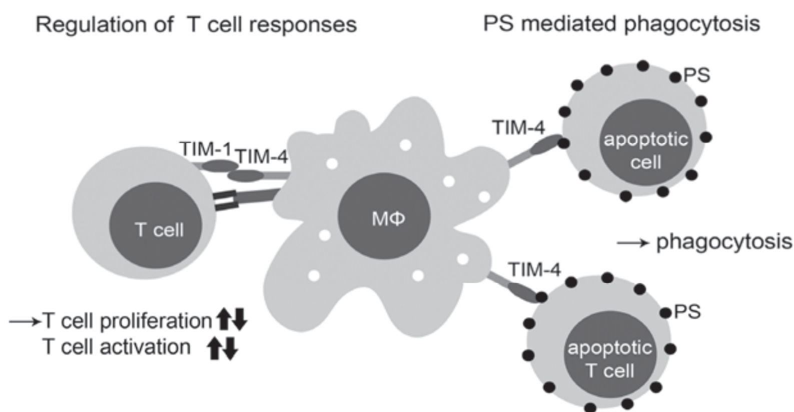


Fig. 10. Schematic representation of the roles of TIM-4 in immune response. TIM-4 mediates phagocytosis of apoptotic cells by APC through binding of PS. It also regulates T cell response both negatively and positively (see below). MΦ, macrophage; PS, phosphatidylserine.

Similarly to TIM-4 expression, requirement of TIM-4 for PS-mediated phagocytosis is restricted only to particular APC populations. Wong *et al.* and Rodriguez-Manzanet *et al.* showed that knock-out of TIM-4 results in incapability to phagocytose in peritoneal macrophages and B1 cells, but not in splenic APCs (353, 355). TIM-4 deficiency also results in an increased number of macrophages in the peritoneum, hyperactivation of lymphocytes and signs of systemic autoimmunity, e.g. production of anti-double stranded DNA.

TIM-4 functions in adaptive immunity by both enhancing and inhibiting T cell differentiation and co-stimulation (283, 284) (Fig. 10). TIM-4 inhibitory

functions have been shown to be a result of reduction in memory T cell numbers due to PS-mediated engulfment of apoptotic antigen-specific T cells (285). Instead, binding of TIM-1 to TIM-4 promotes T cell activation, division and survival by phosphorylation of signaling molecules LAT (linker of activated T cells), Akt (protein kinase B) and ERK1/2 (extracellular signal regulated kinase 1/2) (284, 301). In naïve T cells, TIM-4 can both inhibit and promote activation (283, 284). Since TIM-1 is not expressed by naïve T cells, it was suggested that they express a yet unknown ligand for TIM-4 (283). TIM-4 blockage has also been shown to cause increased induction of T_{reg} from naïve $CD4^+$ T cells and enhanced allograft survival in mouse model of skin transplantation (356). Besides tolerance, TIM-4-mediated regulation of T cell responses may also have clinical significance in autoimmune diseases like rheumatoid arthritis (357).

3 Aims of the study

The objective of this work is to identify and analyze novel molecules involved in hematopoiesis especially at the chicken PAF and corresponding mouse FL stage of development. The specific aims were:

1. To create a cDNA library of chicken para-aortic $CD45^+$ cells in order to identify novel molecules involved in hematopoiesis (I).
2. To study the expression of TIM-1 in chicken and mouse during embryonic development (II).
3. To study the expression of TIM-2 in mouse FL and adult bone marrow hematopoiesis and investigate the hematopoietic potential of TIM-2^+ FL cells (III).
4. To study the expression of TIM-3 in mouse during embryonic development (II).
5. To study the expression of TIM-4 in chicken and mouse hematopoiesis and analyze the hematopoietic potential of mouse TIM-4^+ FL cells (II).

4 Materials and methods

4.1 Experimental animals and tissue collection

Animal care and tissue collection were conducted in strict accordance with the experimental animal ethical committee of the University of Oulu. Embryos from MHC-homozygous chicken strains H.B2, H.B15 and H.B21 with MHC haplotypes B2, B15 and B21 (358), respectively, from the colonies at the Department of Medical Microbiology and Immunology, University of Oulu, were obtained by incubating eggs at 38 °C in a humidified atmosphere.

C57/BL6 mice from the Laboratory Animal Center of the University of Oulu were mated to obtain embryos. The day of vaginal plug observation was considered 0.5 dpc.

All embryonic tissues were isolated microsurgically into PBS (phosphate buffered saline) on ice. Single cell suspension was prepared by pressing the tissues twice through Nylon Net Filters (Millipore, Boston, USA) by using physical pressure with a 1 ml syringe plunger and/or flushing with PBS. Bone marrow cells were isolated from femurs and tibia of mice over the age of 8 weeks by injecting PBS into the bone to flush out the cells.

4.2 Magnetic cell sorting of chicken para-aortic cells

Dead cells were removed by labeling them unspecifically with magnetic MicroBeads conjugated with goat anti-mouse Ig (Miltenyi Biotech, Auburn, USA) and run through an MS column in VarioMACS magnetic cell sorter (Miltenyi Biotech). The unlabeled flow through was collected as live cells which were labeled with a mouse anti-chicken-CD45 antibody (AbD Serotech, Oxford, UK) followed by a secondary goat anti-mouse Ig conjugated with MicroBeads. $CD45^+$ cell fraction was isolated with the VarioMACS with an MS column optimized for positive selection of rare cells. The negative fraction was purified further by passing the cell suspension through a depletion optimized LD column.

4.3 Flow cytometric analysis and cell sorting

4.3.1 Flow cytometric analysis of chicken cells

Magnetically isolated chicken para-aortic $CD45^+$ and $CD45^-$ cells were stained with a FITC-conjugated goat anti-mouse IgG-antibody (AbD Serotech) to estimate the purity of the cell fractions. The dead cells were labeled with 1 µg/ml propidium iodide (Sigma-Aldrich, St. Louis, USA).

Frequency of macrophages was analyzed by staining para-aortic cells with a mouse anti-chicken KUL01 antibody (359) (a kind gift from Professor B. Goddeeris, University of Leuven, The Netherlands) and a goat anti-mouse IgG1-PE secondary antibody and a FITC-conjugated monoclonal CD45 antibody (AbD Serotech).

4.3.2 Flow cytometric analysis and sorting of mouse cells

Before antibody staining of mouse fetal liver cells red blood cells were lysed with 0.14 M NH_4Cl, 0.02 M Tris-HCl solution for 6 min, and the Fc-receptors were blocked by treating the cells with 10% mouse plasma for 35 min at 4 °C.

All used anti-mouse antibodies can be found in Appendices. Corresponding isotype control antibodies were used as negative controls in all assays. Dead cells were excluded by gating based on DAPI NuvBlue™ Fixed Cells Stain or SYTOX® AADvanced™ Dead Cell Stain Kit (Molecular Probes Inc., Eugene, USA). Other possible gating strategies are reported above each FACS (fluorescence activated cell sorting) blot.

Purity of the sorted cell populations was confirmed to be satisfactory with FACS analysis. In addition, frequency of impurities was taken into account in predicting the data.

4.3.3 Data acquisition and cell sorting

FACSAriaII (Becton-Dickinson) was used for cell sorting and FACScan, FACSCalibur, LSR Fortessa or FACSAriaII (Becton-Dickinson) for flow cytometry analyses. Data analysis was performed with BD CellQuest Pro 5.1.1, CyflogicTM (CyFlo Ltd, Turku, Finland) or FlowJo versions 7.6.5 and v.10 (Treestar, Ashland, USA). Representative figures from two to four independent experiments are shown.

4.4 RNA isolation and cDNA synthesis

Total RNA was isolated from the magnetically sorted CD45$^+$ and CD45$^-$ chicken para-aortic cell fractions with an RNeasy® Plus Mini Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. SMART™ PCR cDNA Synthesis Kit (Clontech, Palo Alto, USA) was used to synthesize cDNA as described by the manufacturer.

Total RNA from mouse embryonic tissues was isolated by Trizol reagent (Roche, Basel, Switzerland) with Dnase I (Roche) treatment. Total RNA from sorted cells was isolated with RNeasy® Plus Micro kit (Qiagen, Düsseldorf, Germany). cDNA was synthesized with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, USA) from 1 µg (tissues) or 100 ng (sorted cells) RNA.

4.5 cDNA library

4.5.1 Construction of the cDNA library

Suppression subtractive hybridization (SSH) was performed with cDNA from the ED 7 chicken CD45$^+$ para-aortic cells as a tester and cDNA from the CD45$^-$ para-aortic cells as a driver, using the PCR-Select™ cDNA Subtraction Kit (Clontech) (360) according to the manufacturer's recommendations except for the modifications described below. Preliminary reverse transcriptase PCR experiments indicated that expression of the housekeeping genes *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*) and *actin*, *beta* were 15 to 27 times higher in the CD45$^+$ cell fraction than in the CD45$^-$ cell fraction (data not shown). In order to compensate for this difference, the driver to tester ratio was increased by 3-fold. First hybridization was carried out for 10 h. In the first PCR, samples were incubated at 75 °C for 5 min, at 94 °C for 25 sec followed by 25 cycles of 10 sec at 94 °C, 30 sec at 66 °C, 90 sec at 72 °C. The second PCR was carried out for 11 cycles of 10 sec at 94 °C, 30 sec at 68 °C and 90 sec at 72 °C.

Subtraction efficiency was estimated by PCR using unsubtracted cDNA from CD45$^+$ cells and the subtracted cDNA as templates. PCR was performed according to the PCR-Select™ cDNA Subtraction Kit manual except for the changes described below. The concentration of subtracted template cDNA was 5 times higher than that of the unsubtracted template to detect even trace amounts of the housekeeping gene expression. The PCR was carried out for 15, 20, 25 and

30 cycles. The analyzed genes were *cd45* and the housekeeping genes *gapdh* and *eukaryotic translation elongation factor 1 alpha* (*eef1a*). All PCR primers were purchased from Oligomer (Helsinki, Finland) and their sequences are shown in Appendix Primers.

The cDNA obtained from the SSH was purified with an E.Z.N.A.™ Cycle pure Kit (Omega bio-tek, Doraville, USA) and ligated to pGEM-T Easy plasmid (Promega Corporation, Madison, USA) according to the manufacturer's protocol. The plasmids were transformed into competent JM109 *Escherichia coli* cells (Promega Corporation) following manufacturer's heat-shock protocol. The library was plated on lysogeny broth-ampicillin (100 µg/ml; Sigma Aldrich) agar plates containing 40 µg/ml 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-gal; AppliChem GmbH, Darmstadt, Germany) and 120 µM isopropyl β-D-1-thiogalactopyranoside (IPTG; Fermentas, St Leon-Rot, Germany). White colonies were picked and PCR was performed with M13F and M13R primers (see Appendix Primers) in order to confirm the existence of over 200 base pair long single inserts. Clones containing suitable inserts were grown in 5 mL lysogeny broth media with 100 µg/mL ampicillin at 37 °C over night. The plasmids were isolated with a NucleoSpin® Plasmid Quick pure Kit (Machery-Nagel, Duren, Germany).

4.5.2 Bioinformatic analysis of the cDNA library

Forward and reverse strands were sequenced from cDNA clones by using M13 primers (see Appendix Primers). BigDyeTerminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, USA) and ethanol precipitation purification were used for sample preparation and ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) for sequencing the samples.

cDNA sequences from the differential library were identified by BLAST searches in NCBI database (http://blast.ncbi.nlm.nih.gov/). AmiGO search engine from the Gene Ontology database (http://www.geneontology.org) was used for giving biological process and cellular component gene ontology annotations for the cDNAs (361). The number of clones representing each gene was taken into account. Annotation terms were categorized into groups.

4.6 PCR and quantitative real-time PCR

All PCR and quantitative real-time PCR (qPCR) primers used in the studies can be found in the Appendix Primers and they were purchased from Oligomer.

To confirm differential expression after SSH, the expression of the following chicken genes: *gapdh*, *cd45*, *tim1*, *tim4*, *frizzled 6* (*fz6*), *platelet-derived growth factor receptor beta* (*pdgfrb*), *lymphocyte antigen 86* (*md1*) as well as *tlr2*, *tlr4* and *tlr15* were analyzed using unsubtracted cDNA from $CD45^+$ cells (0.4 ng) and excess amount (3.6 ng) of the unsubtracted cDNA from the $CD45^-$ cells as templates. PCR conditions were: 94 °C for 20 sec followed by 30 cycles of 20 sec at 94 °C, 20 sec at 56 °C and 45 sec at 72 °C. Amplified fragments were analyzed by 1% agarose gel electrophoresis with GeneRuler 1 kb DNA ladder (Fermentas) as the molecular size standard.

For expression analysis of chicken *tim-1* and *tim-4* RNA in embryonic tissues, the same PCR conditions were used with the exception that 58 °C was used as the annealing temperature.

The mouse qPCR primer pairs were designed to span exon-intron boundaries to identify possible genomic DNA contamination. cDNA from mouse tissues or sorted FL cells was used as template in qPCR reactions with Maxima SYBR Green qPCR master mix (Fermentas, Vilnius, Lithuania). All samples, including negative controls, were done in two technical duplicates. The samples were denatured at 95 °C for15 minutes and subjected to 40–45 cycles of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 45 seconds with Rotor-Gene RG-3000 (Corbett research, Sydney, Australia). Specificity of the product was confirmed by determining their size on 1.2% agarose gel and by inspection of melting curves. Results are expressed as a relative expression level calculated as ratio of target genes to housekeeping gene *gapdh*. Mean with standard deviation (S.D.) of at least three independent experiments are shown.

4.7 Whole mount *in situ* hybridization

Plasmids from the cDNA library (see part 4.6) containing segments homologous to chicken *tim-4* and *pu.1* genes were used as templates for transcribing probes with DIG RNA labeling kit (Roche, Basel, Switzerland) to obtain antisense and sense probes. Specificity of probes was confirmed with dot blot against the respective plasmid and tissue samples known to express the gene in question.

Abdominal cavities of the chicken embryos were opened and heart, lungs, liver, gastro-intestinal organs, gonads and mesonephros were removed for efficient probe penetration. Embryos were fixed in 4% paraformaldehyde in PBS with 0.1% Tween 20 for overnight at 4 °C with gentle rolling, dehydrated with a series of methanol/PBS washes and stored at −20 °C in methanol. Fixed embryos were bleached with 6% H_2O_2 for 30 min at room temperature with agitation and rehydrated with methanol/PBS washes. Hybridization was performed according to Henrique *et al.* (362) with the exception that hybridization temperature was 70 °C. Probes were detected with DIG Nucleic acid detection kit (Roche) according to the manufacturer's instructions. NBT/BCIP (nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate) color reaction was observed under a stereo microscope. For making sections, the embryos were postfixed (60% ethanol, 5% acetic acid, 11.4% formaldehyde) at room temperature for 2 h with gentle rolling. Embryos were dehydrated in ethanol series and embedded in paraffin wax. 5-µm sections were cut and observed under Leica DM 2000 microscope with 200x magnification.

4.8 Morphological analysis

Sorted FL cells were cytospinned into slide that was fixed with methanol for 10 minutes and stained with May-Grünwald/Giemsa. Cells were visualized with Leica DM 2000 microscope (magnification x200).

4.9 Phagocytosis assay

Phagocytosis ability of FL cells was studied with Alexa Fluor 488 labeled Zymosan A *Saccharomyces cerevisiae* BioParticles® Fluorescent Particles and Opsonizing Reagent (Molecular probes Inc.) in concentration of 100 BioParticles/cell according to the manufacturer's protocol. FACSAriaII (Becton-Dickinson) and Zeiss LSM780 confocal microscope were used for analysis.

4.10 *In vitro* colony-forming cell assays

Colony-forming cell assays are based on the ability of hematopoietic stem and progenitor cells to proliferate and differentiate into colonies in semi-solid media in response to cytokine stimulation. Myeloid colony forming abilities were analyzed with MethoCult M3434 medium (StemCell Technologies, Grenoble,

France) which contains the following cytokines: stem cell factor (SCF, IL-3, IL-6 and erythropoietin (Epo) which promote the growth of erythrocyte, granulocyte, macrophage and megakaryocyte colonies. Lymphoid colony-forming cell abilities were tested with MethoCult M3630 medium (StemCell Technologies) which contains IL-7 promoting the growth of pre-B cell colonies.

FACS-sorted cells were plated on MethoCult. After incubation in a humidified atmosphere with 5% CO_2 at 37 °C for twelve days for myeloid and seven days for lymphoid cultures, colonies were characterized based on morphology and counted under an inverted microscope. Complete MethoCult culture plate in addition to a few individual colonies of each type were collected into PBS and analyzed for morphology as described in 4.8.

4.11 Statistical analysis

One-way analysis of variance (ANOVA) was used to test if the expression levels or phagocytosis rates were significantly different between different tissues and cell types. Tukey's post hoc test was used for pair-wise comparisons when appropriate. Kruskal-Wallis test was used for nonparametric analyses. The analyses were conducted with IBM SPSS Statistics 19 (IBM, Chicago, IL, USA). The difference was considered to be significant if p value was less than 0.05.

5 Results

5.1 Gene expression profile of ED 7 chicken CD45$^+$ para-aortic cells

Gene expression profile of HSC and HPC was created in order to identify novel genes involved in hematopoiesis. The results are reported in the first article (I).

5.1.1 Construction of the differential CD45$^+$ cDNA library

A differential cDNA library that represents genes expressed only by CD45$^+$ cells from the ED 7 chicken para-aortic region was created with SSH. In SSH, genes expressed by both the driver and tester cells, e.g. housekeeping genes, are subtracted, and the genes expressed only by the target cell population, the tester, are enriched.

Frequency of hematopoietic cells (CD45$^+$ cells) in the original PA region tissue sample was 10%. Magnetic cell sorting was used to enrich the frequency of CD45$^+$ cells to 85% (see I, Fig. 1.). The negative fraction, used as a driver in the SSH, contained 3% of CD45$^+$ cells after purification. This was considered not to affect the efficiency of subtraction.

After the subtraction, its efficiency was confirmed by PCR using subtracted and unsubtracted cDNAs as templates (see I, Fig. 2.). Housekeeping genes *gapdh* and *eef1a* were only weakly visible after 30 cycles using subtracted cDNA as template while unsubtracted cDNA gave clear bands already after fifteen cycles. *cd45* gene was amplified more from the subtracted cDNA indicating enrichment of differentially expressed genes. Successfully subtracted cDNA was ligated into plasmid vector and cloned in *Escherichia coli* to create the cDNA library.

5.1.2 Identification and analysis of the differentially expressed genes

To identify genes from the differential cDNA library, 410 clones were isolated and sequenced. By sequence homology search, 99 distinct known genes and eight functionally unknown genes were found. In addition, 16 clones showed homology to mitochondrial or ribosomal genes. They were considered as housekeeping genes and therefore excluded from further analysis.

The most frequently occurring genes in the library were *defensins beta 7* (54 clones), *beta 1* (21 clones) and *beta 2* (17 clones) in addition to *goose-type lysozyme* (37 clones), *leukocyte cell-derived chemotaxin 2* (30 clones), *angiogenin* (24 clones), *pdgfrb* (12 clones) and *heterochromatin-associated protein MENT* (12 clones). Two clones showed homology to chicken *cd45* gene.

Identified genes were annotated with gene ontology terms according to cellular component and biological processes and further classified into larger groups (Fig. 11). A full list of identified genes with their gene ontology annotations according to cellular component can be found as Appendix III and annotations according to biological processes as Appendix IV.

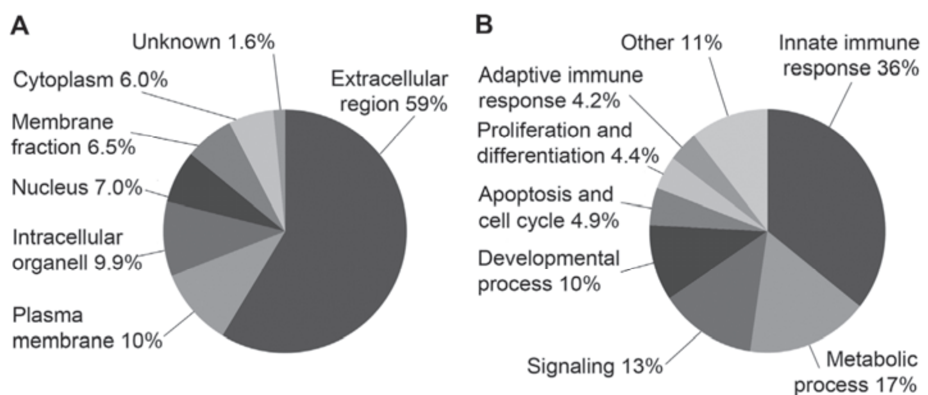


Fig. 11. Classification of differentially expressed genes. Differentially expressed genes identified from the subtractive library were searched for gene ontology terms according to A) cellular component and B) biological process, and then classified into groups.

Based on cellular component, most molecules found in the library are located in the extracellular region (59%, 225 clones, Fig. 11A). Other clones were classified in plasma membrane (10%, 40 clones), intracellular organelles (9.9%, 38 clones), nucleus (7.0%, 27 clones), membrane fraction (plasma membrane excluded, 6.5%, 25 clones) and cytoplasm (6.0%, 23 clones). Six clones (1.6%) did not have any gene ontology for cellular component in the database and are therefore shown as ‘unknown’.

According to biological processes, 36% (137 clones) of the clones were classified in innate immune response, 17% (64 clones) in metabolic process, 13% (51 clones) in signaling, 10% (39 clones) in developmental processes, 4.9% (19

clones) in apoptosis and cell cycle, 4.4% (17 clones) in proliferation and differentiation and 4.2% (16 clones) in adaptive immune response (Fig. 11B and Fig. 3 in article I). Eleven per cent of the genes (41 clones) could not be categorized into any of the groups and were therefore classified in faction 'Other'.

The high amount of innate immunity-related genes in the library was unexpected. Since yolk sac-derived macrophages are a likely source for these clones, the amount of macrophages was analyzed by FACS. Indeed, 50% of the $CD45^{+}$ cells in the PA region were confirmed as macrophages (see I, Fig. 4).

Differential expression of selected interesting development- or immunity-related genes were confirmed further by PCR (Fig. 12). *tim-1*, *tim-4*, *tlr2*, *tlr4*, *tlr15*, *pdgfrb* and *md1* were amplified only or with much higher abundances from $CD45^{+}$ than $CD45^{-}$ cDNA. *fz6* was amplified higher from $CD45^{-}$ than $CD45^{+}$ cDNA, and was considered as a false positive in the cDNA subtraction.

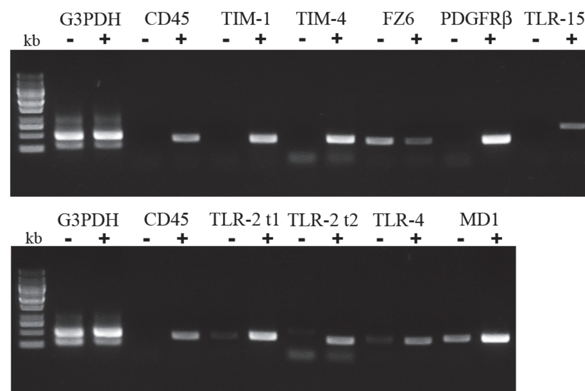


Fig. 12. Differential expression of lymphoid and myeloid genes by chicken $CD45^{+}$ cells. Expression of selected genes identified from the subtractive library was analyzed by PCR. cDNA templates were prepared from $CD45^{-}$ (-) and $CD45^{+}$ (+) cells in ED 7 PA region. Reprinted from article I with permission from John Wiley and Sons.

5.2 Expression of TIM-1 in chicken and mouse embryo

TIM-family molecules detected from the cDNA library (I) are known regulators of immune response but their role in hematopoiesis had not been investigated prior to this study. Therefore they were selected for further analysis on their expression and role in hematopoiesis.

tim-1 expression was further examined from various embryonic chicken tissues by PCR to see if it showed hematopoietic specificity (Fig. 13). *tim-1* messenger RNA (mRNA) was detected from embryonic tissues at ED 4 and ED 7, but not at ED 13. Especially high mRNA expression was seen in the AGM region at ED 4 and in the PA region, liver and mesonephros at ED 7. This data indicates that in addition to ED 7 PA region, *tim-1* is expressed earlier in chicken development in the AGM, which is the site for HSC emergence.

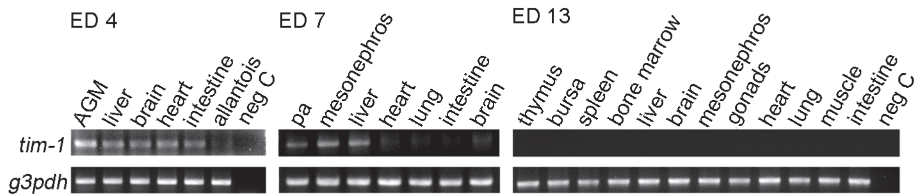


Fig. 13. *tim-1* expression during chicken embryonic development. Gene expression was analyzed from ED 4, 7 and 13 tissues. Representative data from two to four experiments is shown. AGM, aorta-gonads-mesonephros region; ED, embryonic day; neg C, negative control; PA, para-aortic region. Modified from Fig. 1 in article II with permission from Elsevier.

In order to gain knowledge on TIMs in mammals, *tim-1* mRNA expression was also analyzed in mouse (Fig. 14A). Since this work focuses on FL hematopoiesis, tissues from the time of mouse FL colonization (11 dpc) and at the FL hematopoiesis (12.5 dpc) were used. Highest *tim-1* mRNA expression was detected in the lung at 12.5 dpc. Also AGM showed slightly more *tim-1* expression than other tissues at 11 dpc. However, the corresponding PA region showed only very little *tim-1* expression at later 12.5 dpc stage.

Even though *tim-1* mRNA expression was low in FL, we wanted to confirm it on protein level by FACS (Fig. 14B-C). TIM-1 was expressed by 2.0% of 13.5 dpc mouse FL cells, and 47% of these were CD45$^+$, indicating they are hematopoietic. The TIM-1$^+$CD45$^-$ cells are either erythrocytes, which reside in FL in large quantities, or hepatocytes.

It is known that *tim* family members are expressed by macrophages in adult mammals (9, 10, 284) and thus F4/80 antibody specific for macrophages (164) was also used in FACS analysis. The CD45$^+$TIM-1$^+$ population comprised both F4/80$^+$ and F4/80$^-$ cells (38% and 62%, respectively, Fig. 14C).

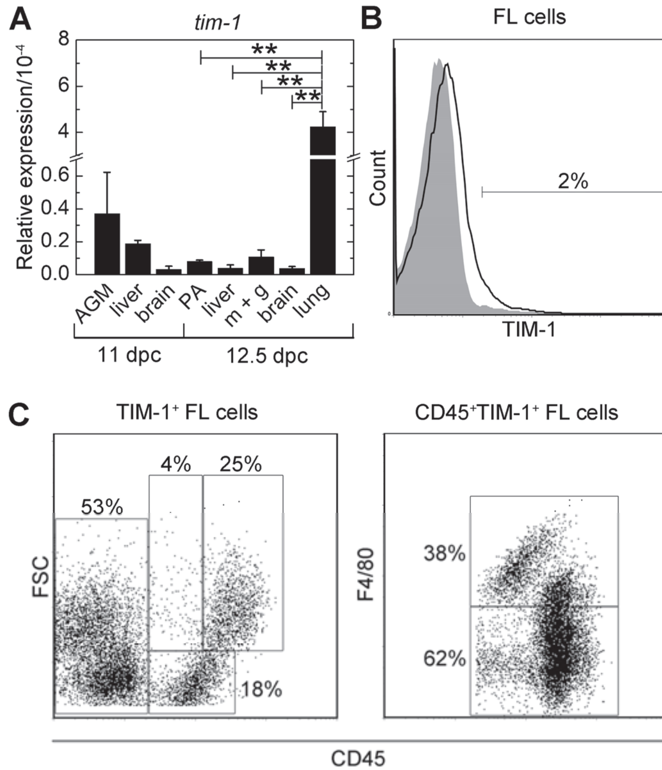


Fig. 14. TIM-1 expression during mouse embryonic development and in 13.5 dpc fetal liver. A) *tim-1* gene expression was analyzed with qPCR. Relative mRNA level normalized to *gapdh* is shown. B) TIM-1 protein expression in 13.5 dpc FL was analyzed with FACS. TIM-1 staining (black line) with control (grey area) and C) TIM-1, CD45 and F4/80 triple stainings are shown. Modified from Fig. 2 and Fig. 4 in article II with permission from Elsevier.

5.3 Expression and role of TIM-2 in mouse embryo

The *tim-2* gene does not exist in chicken so its expression and role in hematopoiesis was analyzed in mouse only.

5.3.1 Expression of tim-2 mRNA in mouse embryonic tissues and fetal liver

Similarly to *tim-1*, qPCR was performed from several mouse embryonic tissues at 11 and 12.5 dpc. *tim-2* mRNA was detected predominantly in FL at both stages (see III, Fig. 1A). The expression was almost four times higher at 12.5 dpc than at 11 dpc. The relative expression level of *tim-2* mRNA was considerably higher than *tim-1*, *tim-3* or *tim-4* expression levels. Since FL is the major hematopoietic site at this stage of mouse development, *tim-2* is assumed to be involved in early hematopoiesis.

We sorted 13.5 dpc FL cells into populations based on CD45 expression and cell size and studied *tim-2* mRNA expression by qPCR to further investigate the cells expressing *tim-2* (see III, Fig. 1B). The expression level of CD45 is known to vary in HSC and HPC at different developmental stages. For example, in yolk sac and AGM, $CD45^{lo}$ cells have the highest hematopoietic potential (363, 364). 13.5 dpc livers were used because they contain substantially more cells than 12.5 dpc liver but still represent the fetal liver stage of hematopoiesis (94, 112). The analysis showed that *tim-2* mRNA is expressed by $CD45^{+}$ cells, especially by $CD45^{hi}$ ($CD45^{high}$) and $CD45^{lo/sm}$ ($CD45^{low/small}$) cells. Since FL $CD45^{+}$ cells contain macrophages, F4/80 antibody specific for them was added into the sort setup. *tim-2* mRNA was detected especially from $CD45^{hi}F4/80^{-}$ population (see III, Fig. 1C) which presumably contains HSC and HPC. The $CD45^{lo/sm}F4/80^{-}$ cells could not be analyzed because of their low number.

5.3.2 Expression of TIM-2 protein in mouse fetal liver

FACS analysis showed that 2.0% of 13.5 dpc FL cells were expressing TIM-2 protein. Half of the $TIM\text{-}2^{+}$ cells were also $CD45^{+}$ (see III, Fig. 2A), suggesting that TIM-2 is expressed by hematopoietic cells. In order to study these cells further, we sorted 13.5 dpc FL cells based on CD45 and TIM-2 expression into $CD45^{-}TIM\text{-}2^{-}$, $CD45^{+}TIM\text{-}2^{-}$, $CD45^{-}TIM\text{-}2^{+}$ and $CD45^{+}TIM\text{-}2^{+}$ populations (see III, Fig. 2A). Cellular morphology suggests that the $CD45^{-}TIM\text{-}2^{-}$ population consists of erythrocytes (see III, Fig. 2B) at several developmental stages. The $CD45^{+}TIM\text{-}2^{-}$ population contains three types of cells: macrophages at different developmental stages with varying numbers of vacuoles, mature and developing granulocytes, e.g., neutrophils, and immature cells with unorganized chromatin and primitive nuclei. The latter are presumably HSC and HPC. Cells in the

$CD45^+TIM\text{-}2^+$ population resemble $CD45^+TIM\text{-}2^-$ population with the exception that they contain less macrophages and developing myeloid cells. $CD45^-TIM\text{-}2^+$ cells appear similar as $CD45^-TIM\text{-}2^-$ cells.

Sca-1 and c-kit expression was detected from both $CD45^+TIM2^+$ (79% and 52%, respectively) and $CD45^+TIM2^-$ populations (38% and 69%, respectively), suggesting that these populations contain stem and progenitor cells (see III, Fig. 2C). $CD45^+TIM2^+$ cells contained twice as many $Sca\text{-}1^+$ cells and evidently less $c\text{-}kit^+$ cells than $CD45^+TIM2^-$ population.

5.3.3 Expression of TIM-2 in mouse fetal liver and bone marrow hematopoietic stem and progenitor cell populations

HSC and HPC populations were analyzed for TIM-2 expression (28, 111, 113, 114, 176). In FL, HSC can be found among LSK cells (28) which contained 5.7% $CD45^+TIM\text{-}2^+$ cells (Table 3).

Table 3. Expression of TIM-2 in 13.5 dpc fetal liver and adult bone marrow hematopoietic stem and progenitor cell populations was analyzed with FACS. See III, Fig. 3, 4 and 5 for FACS blots.

Cell population	Phenotype	TIM-2 positivity (%)
FL		
LSK	$Lin^-Sca\text{-}1^+c\text{-}kit^+$	5.8
CMP	$Lin^-IL\text{-}7R\alpha^-Sca\text{-}1^-c\text{-}kit^+CD34^+FC\gamma R^{lo}$	0.3
GMP	$Lin^-IL\text{-}7R\alpha^-Sca\text{-}1^-c\text{-}kit^+CD34^+FC\gamma R^{hi}$	0.6
MEP	$Lin^-IL\text{-}7R\alpha^-Sca\text{-}1^-c\text{-}kit^+CD34^-FC\gamma R^{lo}$	0.2
CLP	$IL\text{-}7R\alpha^+B220^{-/lo}c\text{-}kit^{lo}Sca\text{-}1^{lo}$	10
Adult bone marrow		
CMP	$Lin^-IL\text{-}7R\alpha^-Sca\text{-}1^-c\text{-}kit^+CD34^+FC\gamma R^{lo}$	0.6
GMP	$Lin^-IL\text{-}7R\alpha^-Sca\text{-}1^-c\text{-}kit^+CD34^+FC\gamma R^{hi}$	0.4
MEP	$Lin^-IL\text{-}7R\alpha^-Sca\text{-}1^-c\text{-}kit^+CD34^-FC\gamma R^{lo}$	0.0
CLP	$Lin^-c\text{-}kit^+Sca\text{-}1^+IL\text{-}7R\alpha^+$	39

Analysis of myeloid progenitor cell populations defined by CD34 and FCγR (111, 113) showed that fetal liver and adult bone marrow CMP, GMP and MEP do not express TIM-2 (Table 3). We also determined TIM-2 expression on CLP defined as $IL\text{-}7R\alpha^+B220^{-/lo}c\text{-}kit^{lo}Sca\text{-}1^{lo}$ in FL (114) and as $Lin^-c\text{-}kit^+Sca\text{-}1^+IL\text{-}7R\alpha^+$ in adult bone marrow (176). Our analysis showed that 10% of FL and 39% of adult bone marrow CLP were $TIM\text{-}2^+$ (Table 3). It is noteworthy that bone marrow

$CD45^+TIM\text{-}2^+$ population contained significantly more IL-7Rα$^+$ cells than $CD45^+TIM\text{-}2^-$ cells (76% and 10%, respectively; see III, Fig. 5B, lower panel). These data imply that TIM-2 is expressed by CLP in mouse FL and adult bone marrow.

5.3.4 Hematopoietic potential of TIM-2$^+$ fetal liver cells

Hematopoietic colony-forming cell potential of the $CD45^+TIM\text{-}2^+$ cells was studied by *in vitro* assays. First, FL cells were sorted as earlier (see 5.3.2 and III, Fig. 2A) and purities of the sorted populations verified with FACS. In the myeloid CFU-assay, $CD45^+TIM\text{-}2^-$ cells generated a total of 125±25 colonies per 1,000 plated cells (Table 4). These included erythroid-, granulocyte-macrophage- and granulocyte-erythroid-megakaryocyte-macrophage-CFU indicating that $CD45^+TIM\text{-}2^-$ cells are multi-potent and thus contain HSC and myeloid progenitors. $CD45^+TIM\text{-}2^+$ FL cells gave rise to only 6.3±8.8 colonies per 1,000 plated cells. However, there are a number of residual $CD45^+TIM\text{-}2^-$ cells, i.e., HSC and HPC, in the $CD45^+TIM\text{-}2^+$ population after FACS sorting. Therefore the colonies generated from $CD45^+TIM\text{-}2^+$ cells presumably arose from cell sorting impurities. No colonies were generated from the $CD45^-TIM\text{-}2^-$ and $CD45^-TIM\text{-}2^+$ cells on the myeloid CFU-assay.

Table 4. Myeloid colony-forming potential of CD45 and TIM-2 defined 13.5 dpc mouse fetal liver cells. Colony formation was analyzed with 1,000 plated cells and is shown as mean (n) and S.D.

Population	CFU-GM n (S.D.)	BFU-E n (S.D.)	CFU-GEMM n (S.D.)	Total n (S.D.)
$CD45^-TIM2^-$	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
$CD45^+TIM2^-$	108 (23)	1.3 (0.4)	16 (2.5)	125 (25)
$CD45^-TIM2^+$	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
$CD45+TIM2^+$	6.3 (8.8)	0.0 (0.0)	0.0 (0.0)	6.3 (8.8)

B lymphoid CFU-assay is not as efficient in generating colonies from FL cells as myeloid CFU-assay and therefore 7,500 cells were plated. Both $CD45^+TIM\text{-}2^+$ and $CD45^+TIM\text{-}2^-$ populations had potential to create pre-B cell colonies as they gave rise to 8.4±3.2 and 7.9±1.4 colonies, respectively (Table 5). As in the myeloid CFU-assay, $CD45^-TIM\text{-}2^-$ and $CD45^-TIM\text{-}2^+$ cells had no hematopoietic potential in the B lymphoid assay. Taken together, these results suggest that

CD45$^+$TIM-2$^+$ FL cells have B lymphoid, but no myeloid hematopoietic colony-forming cell potential.

Table 5. B lymphoid colony-forming potential of CD45 and TIM-2 defined 13.5 dpc mouse fetal liver cells. Colony formation was analyzed with 7,500 plated cells and is shown as mean (n) and S.D.

Population	CFU-B n (S.D.)
CD45$^-$TIM2$^-$	0.0 (0.0)
CD45$^+$TIM2$^-$	7.9 (1.4)
CD45$^-$TIM2$^+$	0.0 (0.0)
CD45$^+$TIM2$^+$	8.4 (3.2)

5.3.5 Expression of TIM-2 during B cell development in fetal liver and adult bone marrow

Since the CD45$^+$TIM-2$^+$ cells showed B lymphoid potential, we assessed TIM-2 expression during different stages of B cell development in FL and adult bone marrow (Table 6). TIM-2 and CD45 expression were analyzed from B cell developmental stages defined by Philadelphia (Hardy) model (189–191). 13.5 dpc FL does not contain the most mature stages of B cell differentiation, the small pre-B, immature B and transitional B (fractions (Fr) D to F) and therefore we also analyzed 18.5 dpc FL. Large pro-B (Fr C) and large pre-B (Fr C') cannot be distinguished clearly in FL and were therefore analyzed together (Fr C – C').

Table 6. Expression of TIM-2 during B cell development was analyzed with FACS. For 13.5 and 18.5 dpc FLs, fractions C and C' were analyzed together. Fractions D – F are not yet present at 13.5 dpc fetal liver and were therefore not analyzed (N/A). See III Fig. 7 for FACS blots.

<table>
<tr><th rowspan="2">Population</th><th rowspan="2">Phenotype</th><th colspan="3">TIM-2 positivity (%)</th></tr>
<tr><th>13.5 dpc FL</th><th>18.5 dpc FL</th><th>adult bone marrow</th></tr>
<tr><td>Fr A pre-pro-B</td><td>B220$^+$CD43$^+$CD24$^-$BP-1$^-$</td><td>0.6</td><td>0.5</td><td>7.9</td></tr>
<tr><td>Fr B small pro-B</td><td>B220$^+$CD43$^+$CD24$^+$BP-1$^-$</td><td>2.9</td><td>0.2</td><td>5.5</td></tr>
<tr><td>Fr C large pro-B</td><td>B220$^+$CD43$^+$CD24^{lo}BP-1$^+$</td><td rowspan="2">34</td><td rowspan="2">14</td><td>92</td></tr>
<tr><td>Fr C' large pre-B</td><td>B220$^+$CD43$^+$CD24^{hi}BP-1$^+$</td><td>74</td></tr>
<tr><td>Fr D small pre-B</td><td>B220$^+$CD43$^-$IgM$^-$IgD$^-$</td><td>N/A</td><td>3.6</td><td>0.1</td></tr>
<tr><td>Fr E immature B</td><td>B220$^+$CD43$^-$IgM$^+$IgD$^-$</td><td>N/A</td><td>14</td><td>0.9</td></tr>
<tr><td>Fr F transitional B</td><td>B220$^+$CD43$^-$IgM$^+$IgD$^+$</td><td>N/A</td><td>52</td><td>6.4</td></tr>
</table>

In 13.5 dpc FL, TIM-2 expression was not observed from pre-pro- (Fr A) and pro-B cells (Fr B), which contained 0.6% and 2.9% TIM-2^+ cells, respectively (Table 6 and III, Fig. 7A). Instead, 34% of the subsequent large pro-B and large pre-B cells (Fr C – C') were TIM-2^+.

In 18.5 dpc FL, TIM-2 expression pattern was similar: it was not expressed by pre-pro- (0.5%) and pro-B cells (0.2%), but 14% of large pro-B and large pre-B cells were TIM-2^+ (Table 6 and III, Fig. 7B). At the subsequent small-pre B cells (Fr D), TIM-2 expression was downregulated as only 3.6% of cells were TIM-2^+. The following immature B cells (Fr E) and transitional B cells (Fr F) showed TIM-2 upregulation (14% and 52%, respectively).

In adult bone marrow, the pre-pro- and small pro-B cells also showed only a low frequency of TIM-2^+ cells (7.9% and 5.5%, respectively), but the expression was dramatically upregulated in large pro-B and large pre-B cells, as 92% and 74% of the cells were TIM-2^+, respectively (Table 6 and III, Fig. 7C). TIM-2 expression is turned completely off at the following small pre-B (0.1%) and immature B cell (0.9%) stages and then slowly upregulated again in the transitional B cell stage where 6.4% of the cells are TIM-2^+. These results demonstrate that TIM-2 expression is tightly governed during B cell development in FL and adult bone marrow where it is specifically expressed by large pro- and large pre-B cells and transitional B cells (Fig. 15).

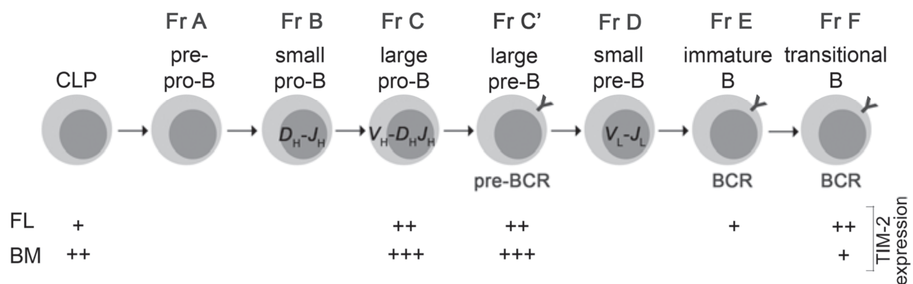


Fig. 15. Expression of TIM-2 during B lymphopoiesis in fetal liver and adult bone marrow. Expression is shown with plus (+) signs. pre-BCR and BCR expression are also indicated. Dash (-) represents immunoglobulin gene rearrangement events. Fr, Fraction.

5.4 Expression of TIM-3 in mouse embryo

The *tim-3* gene does not exist in chicken and therefore its expression was analyzed in mouse only. qPCR showed *tim-3* mRNA expression in all studied tissues; however, it was highest in FL at 12.5 dpc (Fig. 16A).

The expression in FL was confirmed on protein level by FACS (Fig. 16B-C). 1.1% of 13.5 dpc mouse FL cells were stained with TIM-3 antibody. All TIM-3$^+$ cells were also CD45$^+$. In addition, the majority of the CD45$^+$TIM-3$^+$ FL cells were F4/80$^+$ (76%), suggesting that macrophages are a major population expressing TIM-3.

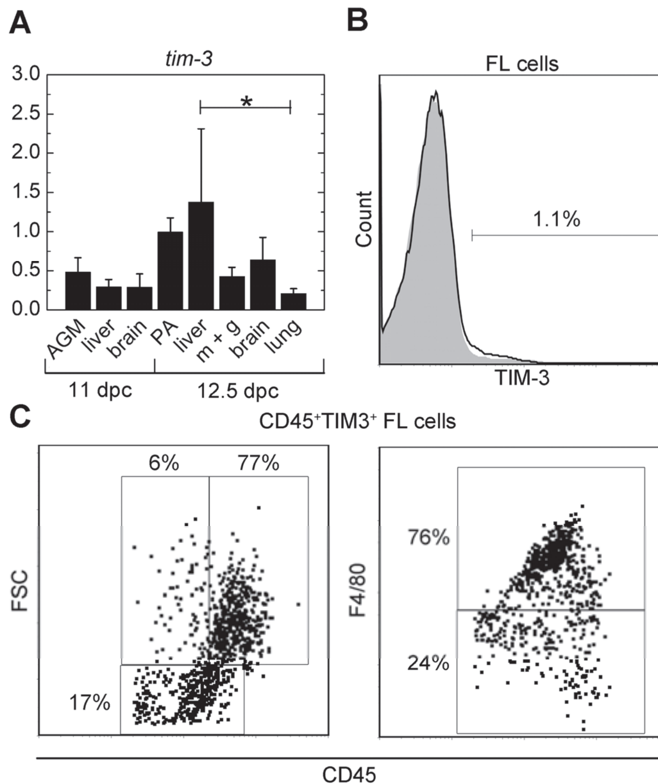


Fig. 16. TIM-3 expression during mouse embryonic development and in 13.5 dpc fetal liver. A) *tim-3* gene expression was analyzed with qPCR. Relative mRNA level normalized to *gapdh* is shown. B) TIM-3 protein expression in 13.5 dpc FL was analyzed with FACS. TIM-3 staining (black line) with control (grey area) and C) TIM-3, CD45 and F4/80 triple stainings. Modified from Fig. 2 and Fig. 4 in article II with permission from Elsevier.

5.5 Expression and role of TIM-4 in chicken and mouse embryo

In the first part of this work, *tim-4* expression was detected in $CD45^+$ cells from the chicken PA region (I). Its expression and role were further studied in chicken and mouse.

5.5.1 Expression of TIM-4 in chicken and mouse embryo

When PCR was conducted on chicken embryonic tissues, *tim-4* mRNA was amplified from all the analyzed tissues at all developmental stages (Fig. 17A). The expression was highest in hematopoietic tissues, para-aortic region (ED 7) and bone marrow (ED 13), as well as in non-hematopoietic tissues mesonephros (ED 13) and liver (ED 7, ED 13).

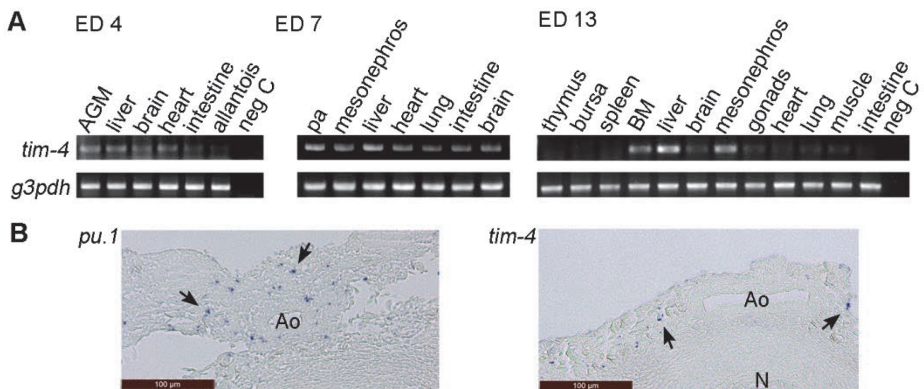


Fig. 17. A) *tim-4* gene expression was analyzed from ED 4, 7 and 13 tissues. Representative data from two to four experiments is shown. Modified from Fig. 1 in article II with permission from Elsevier. B) *In situ* hybridization of chicken ED 7 PA regions with probes against *pu.1* and *tim-4*. Arrows mark signal. AGM, aorta-gonads-mesonephros region; ED, embryonic day; neg C, negative control; PA, para-aortic region; Ao, aorta; N, notochord.

tim-4 mRNA was also visualized by whole mount *in situ* hybridization which showed *tim-4* positive cells distributed in the PA region and surrounding tissues (Fig. 17B). Transcription factor *pu.1*, known to be expressed by hematopoietic cells including HSC, HPC and macrophages (365), was used as a positive control. It was detected similarly in the PA region. Compared to *pu.1*, *tim-4* signal strength was weaker and positive cells were less frequent. *tim-4* is expressed by

macrophages in adults (9, 10, 284, 353), and therefore the detected scattered expression in the chicken embryo might also be from macrophages.

tim-4 mRNA expression was also studied in mouse embryo (Fig. 18A). Some expression was detected in all studied tissues, but it was evidently high in FL, which might suggest a role in hematopoiesis.

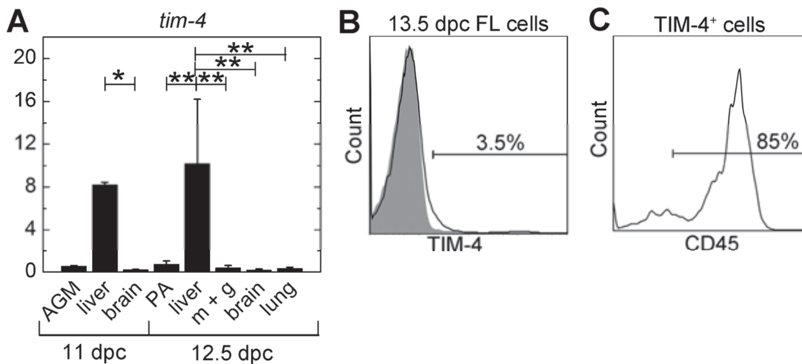


Fig. 18. TIM-4 expression during mouse embryonic development and in 13.5 dpc fetal liver. A) *tim-4* gene expression was analyzed with qPCR. Relative mRNA level normalized to *gapdh* is shown. B) TIM-4 protein expression in 13.5 dpc FL was analyzed with FACS. TIM-4 staining (black line) with control (grey area). C) CD45 expression in TIM-4^{+} fetal liver cells. Modified from Fig. 2 and Fig. 5 in article II with permission from Elsevier.

Because FL was the major tissue where *tim-4* mRNA was observed, the expression was analyzed further in different FL cell populations, similarly to *tim-2* (see 5.3.1). Among the four distinct populations – CD45^{-}, CD45$^{lo/la}$, CD45$^{lo/sm}$ and CD45^{hi} – highest *tim-4* mRNA expression was detected in the CD45$^{lo/sm}$ population (see II, Fig. 3A-B). When F4/80 antibody specific for macrophages (164) was added into the sort set up, *tim-4* mRNA was detected only in CD45^{+}F4/80^{+} cells (see II, Fig. 3C-D). *tim-4* mRNA expression could not be analyzed reliably in CD45$^{lo/sm}$F4/80^{-} cells because of low cell yield since most CD45$^{lo/sm}$ cells were F4/80^{+}.

TIM-4 expression was also confirmed on protein level by flow cytometry (Fig. 18B-C). It was detected on 3.5% of 13.5 dpc mouse FL cells, the majority of which were CD45^{+} (85%). When different CD45 expressing populations were analyzed, TIM-4^{+} cells were seen particularly in CD45$^{lo/sm}$ population (see II, Fig.

5C), which is expected to be the most primitive (363, 364). Furthermore, all TIM-4^+ cells were $F4/80^+$. All these results are in concordance with the analysis of *tim-4* mRNA.

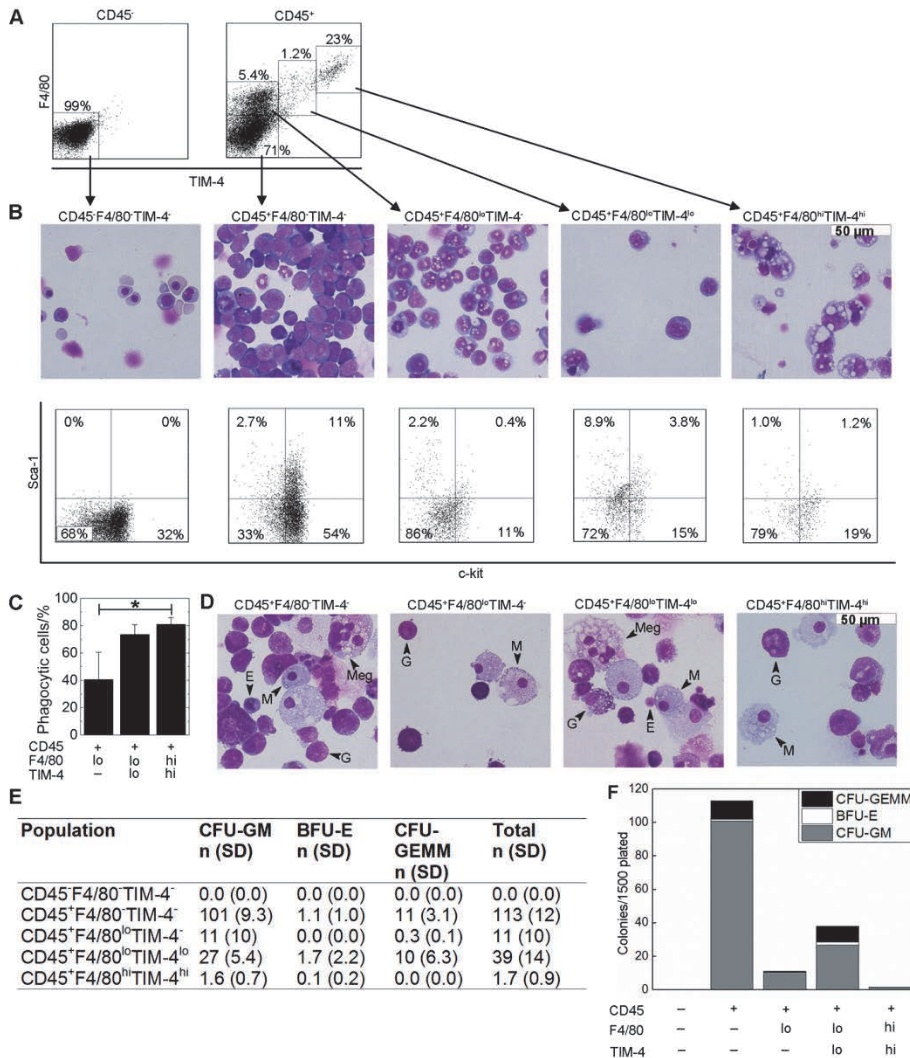
5.5.2 Characterization of TIM-4^+ FL cells

Two TIM-4^+ populations, TIM-4^{lo} and TIM-4^{hi}, were observed (Fig. 19A). TIM-4^{lo} cells were also $F4/80^{lo}$, and TIM-4^{hi} cells $F4/80^{hi}$. These populations were FACS sorted together with $CD45^+F4/80^{lo}TIM4^-$, $CD45^+F4/80^-TIM4^-$ and $CD45^-F4/80^-TIM4^-$ populations and their cellular morphology and stem cell marker expressions were analyzed (Fig. 19B-C).

The $CD45^-F4/80^-TIM4^-$ population contained erythrocytes at several differentiation stages (Fig. 19B, upper panel). The $CD45^+F4/80^-TIM4^-$ population consisted of macrophages, granulocytes and cells with big primitive nuclei with loose chromatin, as is typical of stem and progenitor cells. The $CD45^+F4/80^{lo}TIM4^-$ and $CD45^+F4/80^{lo}TIM4^{lo}$ populations also contained primitive cells and rather undifferentiated macrophages with only few vacuoles and granules. On the other hand, the $CD45^+F4/80^{hi}TIM4^{hi}$ population contained large granular and vacuolar macrophages.

Expression of known markers of stem cells (c-kit and Sca-1) was also studied in the TIM-4, CD45 and F4/80 defined populations (Fig. 19B, lower panel). The $CD45^+F4/80^-TIM4^-$ population contained the highest frequency of c-kit^+Sca-1^+ double positive (11%) and c-kit^+ single positive (54%) cells. Among F4/80 expressing populations, most c-kit^+Sca-1^+ double positive (3.8%) and Sca-1^+ single positive (8.9%) cells were found in the $CD45^+F4/80^{lo}TIM4^{lo}$ population. The $CD45^+F4/80^{hi}$TIM-4^{hi} population contained the highest frequency of c-kit^+ single positive cells (19%), whereas most $CD45^+F4/80^{lo}$TIM-4^- cells were c-kit^- to c-kit^{lo} and Sca-1^-. Taken together, these data indicate that $TIM4^{lo}$ and TIM-4^{hi} populations may contain undifferentiated cells in addition to mature macrophages.

Since the macrophages contained in $F4/80^{lo}TIM4^-$, $F4/80^{lo}TIM4^{lo}$ and $F4/80^{hi}TIM4^{hi}$ populations had distinct morphologies, we studied their capability to phagocytose Zymosan A beads. Interestingly, the TIM-4 expressing fractions did not differ in their ability to phagocytose as demonstrated by FACS analysis (74% and 81%, respectively, Fig. 19C) and further confirmed by microscopy (data not shown). $F4/80^{lo}TIM4^-$ cells were also capable of phagocytosis (41%) but not as efficiently as TIM-4 positive cells.



E

Population	CFU-GM n (SD)	BFU-E n (SD)	CFU-GEMM n (SD)	Total n (SD)
CD45⁻F4/80⁻TIM-4⁻	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
CD45⁺F4/80⁻TIM-4⁻	101 (9.3)	1.1 (1.0)	11 (3.1)	113 (12)
CD45⁺F4/80^lo TIM-4⁻	11 (10)	0.0 (0.0)	0.3 (0.1)	11 (10)
CD45⁺F4/80^lo TIM-4^lo	27 (5.4)	1.7 (2.2)	10 (6.3)	39 (14)
CD45⁺F4/80^hi TIM-4^hi	1.6 (0.7)	0.1 (0.2)	0.0 (0.0)	1.7 (0.9)

Fig. 19. CD45, F4/80 and TIM-4 defined 13.5 dpc mouse fetal liver populations show different hematopoietic potential. (A) FL cells were sorted into five populations based on CD45, F4/80 and TIM-4 expression. (B) The populations were analyzed for cellular morphology by May-Grünwald/Giemsa staining of cytospin samples, and for c-kit and Sca-1 expression by FACS. Representative figures from three independent experiments are shown with mean values. (C) Engulfment of Zymosan beads by FL macrophage populations. Percentage of Zymosan A–Alexa Fluor 488 positive cells is shown as mean and S.D. from three independent experiments. * $p < 0.05$. (D) Hematopoietic differentiation of 1,500 sorted cells was analyzed by CFC-assay. Cells

from all the colonies were pooled together as cytospins and cellular morphologies were visualized with May-Grünwald/Giemsa stain. (E-F) Colony counts are shown as mean and S.D. from three independent experiments with duplicated plates in each. GEMM = granulocyte-erythrocyte-macrophage-megakaryocyte, E = erythrocyte, M = macrophage, G = granulocyte, Meg = Megakaryocyte. Reprinted from article II with permission from Elsevier.

5.5.3 Hematopoietic potential of TIM-4^{+} mouse FL cells

Next, we investigated whether TIM-4-expressing populations have hematopoietic capability. CD45^{+}F4/80^{-}TIM4^{-} cells were used as positive control. They had the highest colony-forming ability (113±12) in the CFU-assay and gave rise to all types of myeloid colonies (Fig. 19D-F). This, together with the morphology and c-kit/Sca-1 expression, indicates that CD45^{+}F4/80^{-}TIM4^{-} cells contain HSC and HPC, as expected.

CD45^{+}F4/80^{lo}TIM-4^{lo} cells had the highest myeloid colony-forming cell activity (39±14 colonies) among the F4/80 expressing populations. They could generate all types of colonies but were biased towards granulocyte-erythroid-megakaryocyte-macrophage-CFU production. The CD45^{+}F4/80^{lo}TIM-4^{-} population generated especially small granulocyte, macrophage and granulocyte-macrophage colonies (11±10), which consisted of less than a hundred cells each. CD45^{+}F4/80^{hi}TIM-4^{hi} cells produced a very low number of colonies (1.7±0.9), which most likely arose from FACS sorting impurities. The CD45^{-}F4/80^{-}TIM-4^{-} population, which contains hepatocytes and erythrocytes, did not produce any colonies even when as many as 20,000 cells were plated.

Next we analyzed whether the difference in hematopoietic potential of TIM4^{lo} and TIM-4^{hi} cells could be explained by distinction in the expression of CD34 and FCγR, known myeloid progenitor markers (111, 113), or ER-MP12 (CD31/PECAM-1) and ER-MP20 (Ly-6C), which are known to be expressed by hematopoietic progenitor cells but not by mature macrophages (166, 167). Enrichment of neither TIM-4^{lo} nor TIM-4^{hi} cells was seen in the CMP, GMP or MEP populations (See II, Fig. 7A) although a very small number of TIM-4^{lo} cells did express MEP (CD34^{-}FCγR^{lo}) and CMP (CD34^{+}FCγR^{lo}) phenotype. TIM-4^{hi} cells were mostly Lin^{-}c-kit^{+}Sca-1^{-} and therefore did not contain significant numbers of cells with myeloid progenitor phenotypes. TIM-4^{lo} cells contained ER-MP12^{hi}20^{-} population (See II, Fig. 7B and Fig. 18) which is known to include undifferentiated hematopoietic cells including macrophage-colony forming cells

(165, 366). Some TIM-4^{lo} cells were also ER-MP12^{-}20^{-} or ER-MP12^{lo}20^{-}, which are known to contain mostly mature cells. TIM-4^{hi} cells contained mostly ER-MP12^{-}20^{-} cells and some ER-MP12^{lo}20^{-} cells. Taken together, these data suggest that TIM-4 is expressed in myeloid progenitor cells from the GMP/MEP stage onwards (Fig. 20).

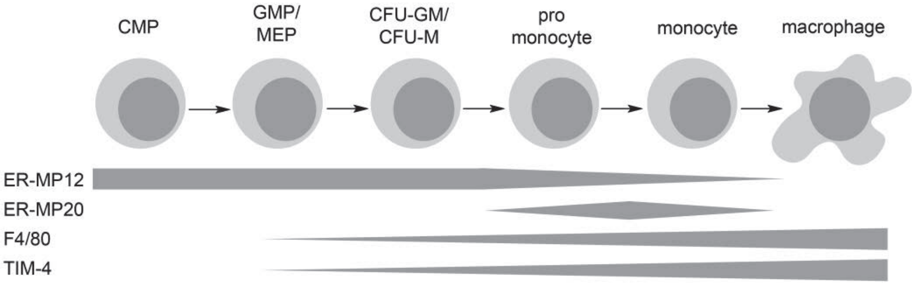


Fig. 20. Schematic representation of TIM-4 and surface marker expression during myeloid cell development in mouse FL. F4/80 and TIM-4 expression are shown based on the results of this study. CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte-erythrocyte progenitor; CFU-GM, granulocyte-macrophage colony forming unit; CFU-M, macrophage colony-forming unit. Reprinted from article II with permission from Elsevier.

6 Discussion

There is an increasing demand for markers to identify hematopoietic stem and progenitor cell populations for diagnostic and therapeutic purposes. Furthermore, knowledge on molecules conducting the differentiation and proliferation of HSC and HPC is required for their *in vitro* expansion. Therefore, we have constructed a subtractive cDNA library of genes differentially expressed by $CD45^+$ cells in the ED 7 PA region of chicken embryos (I). The para-aortic region is an equivalent to human and mouse FL and provides a microenvironment for HSC and HPC proliferation and differentiation prior to their migration into lineage-specific organs (60). Except for erythroid differentiation, little is known about the molecules conducting the differentiation and lineage commitment of HPCs at this stage of development in either mammals or avians (60, 152).

6.1 Gene expression profile of $CD45^+$ PAF cells from chicken (I)

Gene expression profiles have previously been created from normal HSC and HPC in AGM (367), FL (368, 369) and thymus (370) of mouse embryo as well as from adult bone marrow (133, 371, 372). In addition, gene expression has been analyzed in human HSC (368) and malignant hematopoietic cells (373, 374). Most of these studies are based on microarray technology which is optimal for analyzing a large number of transcripts at a time and thus overviewing complex gene regulation. However, microarray technology is not ideal for the detection of rare transcripts and novel regulators. Instead, we used a suppression subtractive hybridization method to analyze differentially expressed genes. Kinetics of subtractive hybridization allows normalization of the library, thus increasing chances to detect rarely expressed transcripts and identification of novel genes involved in the regulation of hematopoiesis.

Annotations by gene ontology according to cellular localization and biological processes were made for the cDNAs identified from the subtractive library. Based on cellular localization, most transcripts were subcomponents of extracellular region or plasma membrane, suggesting involvement in cell-cell interactions and/or signaling. Some genes were classified into the nucleus and are thus likely to be involved in regulation of transcription, e.g. as transcription factors. Based on biological processes, a variety of genes related to signaling, development, proliferation, differentiation and apoptosis were identified and are therefore suggested to be involved in early hematopoiesis. In line with a report on

adult mouse bone marrow LT-HSCs, a large number of metabolism-related transcripts were also found to be differentially transcribed (372).

Surprisingly, innate immunity-related genes such as *defensin* were highly expressed by hematopoietic cells at this stage. Macrophages originating from yolk sac are known to emerge prior to the onset of definitive hematopoiesis and to function in scavenging apoptotic cells during the early embryonic development (168, 375). Therefore, it is possible that yolk sac-derived macrophages are a source of the innate immunity-related genes in our study. The fact that about half of the $CD45^+$ cells in the PA region were macrophages may partly explain the large number of those transcripts.

In addition to yolk sac-derived macrophages, indiscriminate activation of multiple gene programs in HSC and HPC is presumably the reason for the occurrence of the innate immunity-related transcripts. Multiple lineage-affiliated genes are known to be promiscuously expressed by HPCs during lineage commitment (129–132). Akashi *et al.* (133) have also shown that genes related to mature cell functions are expressed by HSC and HPC prior to, and during, lineage commitment. These reports have suggested that at the early stages of hematopoiesis, epigenetic mechanisms maintain the chromatin structure open for multiple lineage programs (16). This allows progenitor cells to be flexible in fate decision and to differentiate readily upon different biological stimuli.

Several genes known to be involved in chicken and mammalian hematopoiesis were reidentified including *pu.1*, *cd45*, *pdgfrb* and *granulocyte-macrophage colony-stimulating factor*, thus supporting the high quality of the cDNA library. Therefore, the library should also reveal novel molecules which are functional in hematopoiesis at PAF stage. For instance, *tim-1* and *tim-4* were found in the library and selected for further analyses.

6.2 Expression of TIM molecules

Even though the role of TIM molecules in the regulation of T_H cell proliferation and PS binding has been studied extensively (7, 264, 272), their role in embryonic hematopoiesis is not yet known. The only indication of the function of TIM family members in hematopoiesis has been the detection of TIM-3 from leukemic stem cells in acute myeloid leukemia patients (13, 341, 342). Interestingly, normal human HSC were not shown to express TIM-3 in these studies.

The identification of *tim-1* and *tim-4* from the $CD45^+$ para-aortic cells elicits a possible involvement of TIM molecules in embryonic hematopoiesis (I). Indeed,

in the articles II and III we show for the first time that TIM family molecules are expressed by hematopoietic tissues and cells during embryonic development and that TIM-2 and TIM-4 expressing mouse FL cells have hematopoietic potential. These findings are summarized in Table 7 and will be discussed below.

Table 7. Major findings from studies II and III on the expression and hematopoietic potential of TIM family molecules in mouse. BM, bone marrow; N/A, not analyzed.

Molecule	Expression	*In vitro* hematopoietic potential	Article
TIM-1	AGM	N/A	II
TIM-2	FL: LSK, CLP, large pro-B and pre-B, transitional B	B lymphoid	III
	BM: CLP, large pro-B and pre-B, transitional B	N/A	III
TIM-3	FL: F4/80^{+} cells	N/A	II
TIM-4	FL: CD45^{+}F4/80^{lo}	myeloid multi-lineage	II
	FL: CD45^{+}F4/80^{hi}	no potential	II

6.2.1 TIM-1 is expressed during HSC emergence (II)

First, we investigated the specificity of tim-1 expression in tissues from chicken embryos at hematopoietically characteristic developmental stages. The expression of *tim-1* was higher in AGM than in other tissues at ED 4. At this stage, HSC are generated by the hemogenic endothelium of the dorsal aorta (376), and therefore it is possible that TIM-1 is involved in HSC emergence. Lower *tim-1* expression in the PA region at ED 7 might be due to HSC losing *tim-1* expression when they start to proliferate and differentiate.

To expand the study to mammals, we analyzed the expression of *tim* genes quantitatively in mouse embryos. Similarly to chicken, *tim-1* expression was clear in 11 dpc mouse AGM, the site of HSC emergence (376), but very low in 12.5 dpc FL, the subsequent hematopoietic site (60). Even though the *tim-1* expression level was rather low, these data, together with the results in chicken, imply that TIM-1 is involved in HSC emergence. This represents an intriguing future topic of investigation.

6.2.2 TIM-2 is expressed by CLP and large pre- and pro-B cells in fetal liver and adult bone marrow (III)

Mouse embryonic and adult kidney and liver cells and mature B and T_{H2} cells are known to express TIM-2 (272, 274, 319). We detected *tim-2* transcription to be

high in FL. Furthermore, we detected TIM-2 mRNA and protein expression in both $CD45^-$ and $CD45^+$ FL cells. Earlier, Watanabe *et al.* (319) have also detected TIM-2 expression in $CD45^-$ FL cells which presumably include erythrocytes and hepatocytes. On the other hand, TIM-2 expression in $CD45^+$ FL cells suggests that TIM-2 is also expressed by HSC and/or HPC. We detected *tim-2* expression particularly in $CD45^{hi}$ cells. The most primitive and multi-potent HSC in yolk sac and AGM have been shown to be $CD45^{lo}$ (363, 364). Furthermore, definitive HSC have been shown to acquire CD45 expression during their maturation and differentiation in FL (44, 59). $CD45^+TIM-2^+$ cells expressed c-kit and Sca-1 and also had primitive morphology, further supporting the idea that they include HSC/HPC.

We showed that a clear fraction of LSK cells from FL were $TIM-2^+$. LSK population is known to be heterogeneous as it contains LT-HSC as well as MPP and CLP (33, 176). Indeed, we detected TIM-2 expression both on FL and bone marrow CLP. Instead, myeloid progenitor cell populations CMP, GMP or MEP from FL and bone marrow did not express TIM-2. In concordance with this, $CD45^+TIM-2^+$ cells showed B-lymphoid, but not myeloid *in vitro* colony-forming cell potential, suggesting they are not multi-potential. Taken together, these data suggest that $CD45^+TIM-2^+$ cells are likely not HSC, MPP nor myeloid progenitors, but lymphoid progenitors.

During B cell development, large pro-B cells undergo V_H to D_HJ_H rearrangement and start to express immunoglobulin μ heavy chain (Figure 4). The μ heavy chain together with the surrogate light chain forms the pre-BCR complex which is then expressed on the cell surface (208, 209, 377). This transition from large pro- to large cycling pre-B cells is an important checkpoint in B cell development and requires adequate signaling through the pre-BCR (210, 378). Cells passing this checkpoint go through clonal expansion in response to IL-7, which is followed by cell cycle arrest, initiation of immunoglobulin light chain rearrangement and further B cell differentiation (214, 215, 379). During the differentiation stages that follow, cell proliferation is relatively low. We observed that TIM-2 is highly expressed in the proliferating large pro- and large pre-B cells, then down-regulated and later expressed again when cells differentiate into transitional B cells. Such drastic changes in TIM-2 expression during B cell development are unlikely to be just a result of promiscuous gene expression known to be common in HSC and HPC (132, 133). In adults, splenic germinal center B cells express TIM-2 (274). Germinal center B cells undergo affinity maturation and class-switch recombination (380–382) and interestingly, are also a

highly proliferative B cell population (383, 384). Given that TIM-2 expression is upregulated in B cells specifically at the expansive stages, it is possible that TIM-2 has a role in ensuring proper proliferation of B cells.

TIM-2 has been reported to be a negative regulatory molecule as TIM-2 deficiency leads to T_{H2} cell hyperexpansion and cytokine overexpression (275). Furthermore, TIM-2 has been suggested to suppress fetal hepatocyte proliferation (319). Therefore, we suggest that the role of TIM-2 in B cell development involves negative regulation of cell proliferation, possibly by arresting expansion and/or differentiation of large pro-B and pre-B cells with unsuccessfully rearranged μ heavy chain or inadequate pre-BCR signaling. Regulation at the pre-B cell stage is important to prevent uncontrolled expansion and genomic instability (218, 385) and is further highlighted by the fact that most childhood acute lymphoblastic leukemia (ALL) cases arise from pre-B cells (378, 386). Similarly, control of expansion is important in germinal center B cells which are the most common source of adult B cell lymphomas (387).

6.2.3 TIM-3 is expressed by macrophages in mouse fetal liver (II)

tim-3 mRNA was widely expressed in the analyzed tissues, while in FL, TIM-3 protein was detected in $CD45^{+}F4/80^{+}$ cells. Macrophages of adult mouse have been reported to express TIM-3 (12) and it is therefore possible that also the TIM-3 expressing cells in the different embryonic tissues are macrophages, which are known to remove dead cells and promote tissue development during embryogenesis (168, 388).

6.2.4 TIM-4^{lo} cells are myeloid progenitors (II)

Chicken *tim-4* mRNA was detected in multiple tissues, especially at ED 4–7. Furthermore, *tim-4* expression was widespread in ED 7 PA region and surrounding tissues. Since TIM-4 is expressed by APCs in the adult immune system (9, 10, 301) in mice and humans, its broad expression in chicken embryo might be due to transcription by yolk sac-derived macrophages.

Interestingly, *tim-4* expression showed a more restricted expression pattern in mouse as it was only detected in FL. However, both TIM-4 mRNA and protein were expressed only by $F4/80^{+}$ cells, suggesting that TIM-4 in the mouse FL is also expressed by macrophages. TIM-4 expression was detected particularly on $CD45^{lo}$ cells, which are more primitive than $CD45^{hi}$ cells in mouse embryonic

AGM region and yolk sac (363, 364). In addition, F4/80^{lo}TIM-4^{lo} cells had more primitive morphology than F4/80^{hi}TIM-4^{hi} cells. Taking these findings together, we propose that the F4/80^{lo}TIM-4^{lo} cells are hematopoietic progenitors whereas F4/80^{hi}TIM-4^{hi} cells are mature macrophages.

Next we compared the expression of stem cell markers and myeloid colony forming cell potential of TIM-4^{hi} and TIM-4^{lo} cells. The F4/80^{hi}TIM-4^{hi} cells did not give rise to a substantial number of colonies in CFU assay. In addition, the F4/80^{hi}TIM-4^{hi} cells had the morphology of macrophages and they were capable of phagocytosis, suggesting that they are differentiated macrophages (47). Furthermore, F4/80^{hi} macrophages in mouse FL have previously been shown to originate from yolk sac (47). Yolk sac-derived macrophages are known to differ from definitive hematopoietic cells by their phenotype (389, 390), which may explain the detected high c-kit expression in the F4/80^{hi}TIM-4^{hi} cells. Yolk sac-derived F4/80^{hi} macrophages are also able to self-renew and to persist as tissue macrophages even after birth (47), and c-kit signaling is known to promote the survival of hematopoietic cells (391, 392), e.g., mast cells (393). F4/80^{hi}TIM-4^{hi} macrophages may utilize a similar mechanism.

Based on morphology, TIM-4^{lo} cells include both immature macrophages and undifferentiated cells, which are possibly myeloid progenitors. The c-kit/Sca-1 expression of the F4/80^{lo}TIM-4^{lo} cells and the results of *in vitro* differentiation assay support this notion. The F4/80^{lo}TIM-4^{lo} population gave rise to multi-potential GEMM-colonies suggesting that TIM-4 may be expressed already at multi-potent progenitor or CMP stage. However, the TIM-4^{lo} population contained only few CMP as defined by CD34^{+}FCγR^{lo} (111, 113). ER-MP12 and ER-MP20 are known to be expressed by hematopoietic progenitor cells but not by mature macrophages (166, 167). The TIM-4^{lo} cells contained a substantial ER-MP12^{hi}20^{-} population which is presumed to contain undifferentiated cells, including ones giving rise to macrophage-CFU (366, 394). Taken together, these data suggest that TIM-4^{lo} cells in mouse FL are myeloid progenitors and that in the future, TIM-4 could be utilized as a marker in myeloid progenitor cell isolation or characterization.

6.3 Remaining questions and future prospects

6.3.1 Gene expression profiling of hematopoietic stem and progenitor cells

The suppression subtractive hybridization method assumes that housekeeping gene expression levels of the used tester and driver populations are equal (360). However, we detected that housekeeping gene expression was significantly higher in the tester cells ($CD45^+$). This high expression is likely a result of the high metabolism of HSC and HPC (372) and could result in insufficient subtraction of the housekeeping genes and other transcripts shared by the driver and tester cells. To avoid this, we adjusted the driver ($CD45^-$) cDNA amount accordingly. As a result, the subtraction was highly stringent and the library became highly specific for the $CD45^+$ population.

The para-aortic $CD45^+$ cell fraction used in this study was considered to contain HSCs, early HPCs and lymphoid and myeloid progenitors but not erythrocytes. However, we observed that half of the $CD45^+$ cells in the PA region were macrophages. Based on this observation, it appears that using $CD45^+KUL01^-$ cells, instead of $CD45^+$ cells, as a target population would have provided a library more enriched with transcripts from actual HSC and HPC. However, the constructed cDNA library consists of transcripts expressed by a heterogeneous group of hematopoietic stem, progenitor and mature cells. Regulation of hematopoiesis is known to be very dynamic between different developmental stages (395). Therefore, comparison of expression profiles at consecutive developmental stages would give information on regulatory programs activated at specific developmental windows.

Although this thesis focused on the analysis of TIM family molecules, the differential cDNA library provides also other genes possibly involved in the regulation of hematopoiesis. Such genes include *tlr2*, *tlr4* and chicken-specific *tlr15*, which are known as pattern recognition receptors functioning in innate immunity (396). The first evidence suggesting a role for TLRs in hematopoietic development was provided by Nagai *et al.* (397). They reported that *in vitro* stimulation of mouse TLR-2 and TLR-4 expressed by HSCs and early HPCs with synthetic ligand or lipopolysaccharide resulted in enhanced myeloid differentiation in a Myd88 (myeloid differentiation primary response gene 88)-dependent manner. In addition, the fate of common lymphoid progenitors was biased toward dendritic cells. Since then, similar observations have been reported

with human bone marrow $CD34^+$ cells and mouse Lin^- HSC and HPC (398, 399). Collectively, these findings have demonstrated a possible role for TLRs in vertebrate hematopoiesis as regulators of myeloid differentiation. It is also probable that TLRs are expressed as regulators of myeloid differentiation by PAF/FL HPCs at the initial stage of lineage decision.

Recent advances in the field of genomics and proteomics enable more through-out and high-resolution analyses than conducted in this thesis work. Such studies would also provide important insight on chicken PAF and mammalian FL hematopoiesis. For example, using ChipSeq (chromatin immunoprecipitation sequencing) profiling together with deep sequencing allows detection of DNA binding targets including transcription factors and micro RNA (400, 401). Further insight into regulation on gene expression can also be acquired by combining transcriptional data with epigenetic profiles. In the field of hematology, these methods have been used for example in distinguishing human acute lymphoblastic leukemia from other types of leukemia and healthy samples based on DNA methylation profiles (374, 402–404).

6.3.2 TIM family molecules

The results presented in this thesis provide a basis for further studies on the role of TIM family molecules in hematopoiesis. In particular, the results suggest that TIM-2 and TIM-4 have roles in lymphoid and myeloid development, respectively. Since the data provided in the articles II and III are mainly descriptive, further functional studies are required to fully understand the role and importance of TIM molecules in hematopoiesis.

Since TIM-1, TIM-3 and TIM-4 are homologous between human and mouse, and mouse TIM-2 is highly homologous to human TIM-1 ((259, 262, 263)), the results from this thesis also give direction for further investigations on the role of TIM molecules in human hematopoiesis. Since we show that TIM-2 is expressed in B cell proliferation, its homolog TIM-1 may have a similar expression pattern in humans. Furthermore, TIM-1 expression should be analyzed in human B cell lymphoma lines since our results suggest a possible negative regulatory role for TIM-2 in B cell proliferation. It would also be interesting to analyze possible expression and role of TIM-4 in human myelopoiesis, e.g. on bone marrow HPC.

Earlier studies on TIM-deficient mice have not shown any apparent abnormalities in the numbers and frequencies of immune cells in adult mice (272, 275, 302, 303, 335, 353, 355). These studies did not investigate the development

of hematopoietic cells during embryonic or adult hematopoiesis and possible defects could therefore not be detected. On the other hand, TIM molecules may not be indispensable for hematopoiesis, or they may have functional redundancies with other molecules. Nevertheless, including analyses of knock-out mice in articles II and III would have provided hints for the function of TIM molecules. In addition, since we used only *in vitro* colony-forming cell assays, it would be beneficial to address the lineage potential of TIM-2- and TIM-4-expressing FL cell populations by *in vivo* transplantation studies.

Since TIM-2 has been reported to be expressed by activated T_{H2} cells in adult mice (272, 274) and we detected it on CLP, it would be interesting to analyze further its expression and role during T cell differentiation. On the other hand, the classical CLP from bone marrow have been reported to create mostly B, but not T cells when injected into sublethally irradiated mice and have therefore been suggested to primarily contain progenitors of B cell lineage (176–178). Furthermore, thymus-seeding cells have been suggested to be a subpopulation of MPP, upstream of CLP (405, 406).

A possible mechanism for TIM-2 function in B cell development is through the binding of Sema4A. Sema4A is known to be expressed by, e.g., dendritic cells, T_{H1} cells and resting B cells, and it can increase activation and differentiation of T cells (309, 407). However, this interaction has not been observed in further studies (272, 274, 408) and its biological importance is therefore hard to estimate. Another potential role for TIM-2 in B cell development could be the uptake of H-ferritin into cells. This function of TIM-2 was originally reported in T and bile duct cells (274, 318) and later in oligodendrocytes and A20 B cell line (318, 409). Ferritin protein complexes function in transport and intracellular storage of iron, which is required in adequate but not excessive amounts for normal cell differentiation and growth (410). For example, H-ferritin overexpression is associated with development of carcinomas, including radiation-induced leukemia/lymphoma in mice (411). On the other hand, ferritin inhibits proliferation of granulocyte-macrophage lineage, erythrocytes, B cells and T cells (313, 412–416), and this effect has been suggested to be mediated by TIM-2 (317, 409). However, in another study, *in vitro* proliferation of B cells and the differentiation of plasma cells were not affected by iron (417). Instead, this study reported that iron suppressed B cell class-switch recombination by inhibiting the function of AID (activation-induced cytidine deaminase). Nevertheless, interaction of H-ferritin and TIM-2 during early B cell development should be investigated further.

We could not detect significant TIM-4 expression on CMP, GMP and MEP even though the results of the colony-forming assay suggested otherwise. Instead, ER-MP12^{hi}20^{-} cells were detected in TIM-4^{lo} populations. ER-MP12 and -20 markers give a general idea of the TIM-4 expression during myelopoiesis, but the exact development stage of the TIM-4^{lo} cells cannot be predicted. Unfortunately, there is not yet adequate knowledge on markers during myeloid and macrophage development to improve the analysis. However, based on article II, it appears that TIM-4 could be one such marker. Analyzing its role and expression further during bone marrow myelopoiesis would also be beneficial.

It will also be intriguing to find out the possible functional role of TIM-4 in myeloid cell differentiation. Since TIM-4 does not have any signaling motifs in its cytoplasmic tail (352), it is likely that its function is mediated by a ligand or co-receptor. So far, three TIM-4 ligands, PS, TIM-1 and LMIR-5, have been identified (9, 282, 284, 295). In addition, an unidentified ligand for TIM-4 has been suggested to be expressed by naïve T cells (283). Indeed, TIM-4 has been shown to enhance T cell division and survival through binding of TIM-1 (284, 301). Discovering the possible mechanism will, however, require further studies.

7 Conclusions

The objective of this thesis was to identify and analyze novel molecules involved in chicken and mouse hematopoiesis. The main findings of each publication are summarized below.

I A subtractive cDNA library was constructed and genes expressed differentially by $CD45^+$ cells from the ED 7 chicken PA region were identified. Annotation of the identified genes revealed a variety of biological processes, in line with the idea of promiscuous expression of multiple lineage genes in HSC and HPC. Several candidate genes, which may have roles in early hematopoiesis, were provided in the study.

II We showed for the first time that TIM molecules are expressed in hematopoietic organs during embryonic development. Furthermore, *tim-1* gene expression was detected in chicken and mouse embryos in the AGM region at the time of HSC emergence while *tim-3* mRNA was widely expressed in different tissues. Instead, *tim-4* expression was restricted to FL and two distinct populations were identified: $F4/80^{hi}TIM\text{-}4^{hi}$ and $F4/80^{lo}TIM\text{-}4^{lo}$. We show that the $F4/80^{hi}TIM\text{-}4^{hi}$ cells are likely yolk sac-derived macrophages and the $F4/80^{lo}TIM\text{-}4^{lo}$ cells myeloid progenitors.

III We detected TIM-2 expression to be strictly regulated during B cell development. It is expressed by CLP and large-pro and large pre-B cells and transitional B cells both in FL and adult bone marrow. TIM-2 is known to negatively regulate proliferation of T_{H2} cells and hepatocytes, and we therefore discuss its possible role in the regulation of B cell development.

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Appendix I

Table 8. Antibodies used in the flow cytometry experiments.

Name	Clone	Labels	Source
Anti-chicken antibodies			
CD45	LT40	FITC	AbD Serotech
CD45	LT40	unlabeled	AbD Serotech
IgG secondary antibody		FITC	AbD Serotech
KUL01	KUL01	unlabeled	(359)
IgG1 secondary antibody		PE	AbD Serotech
Anti-mouse antibodies			
CD16/32 (FCγRIII/II)	93	PerCP-Cy5.5, APC	Biolegend
CD24 (HAS)	M1/69	PerCP-Cy5.5	Biolegend
CD31 (ER-MP12)	390	PE-Cy7	Biolegend
CD34	RAM32	FITC	Becton Dickinson
CD43	S11	APC	Biolegend
CD45	I3/2.3	FITC, APC-Cy7	Southern Biotech
CD45R (B220)	RA3-6B2	PE, APC-Cy7	Biolegend
CD117 (c-kit)	2B8	PerCP, APC-Cy7	Biolegend
CD127 (IL-7Rα)	A7R34	FITC, Brilliant Violet 421	eBioscience
F4/80	Cl:A3-1	FITC, APC	AbD Serotech
IgD	11-26c.2a	FITC	Biolegend
IgM	RMM-1	PerCP-Cy5.5	Biolegend
Ly-6A/E (Sca-1)	E13-161.7	PE, PE-Cy7, APC	Biolegend
Ly-6C (ER-MP20)	HK1.4	Alexa Fluor 700	Biolegend
Ly-51 (BP-1)	6C3	FITC	Biolegend
TIM-1	RMT1-4	PE	Biolegend
TIM-2	F37-2C4	PE, Alexa Fluor 647	Biolegend
TIM-3	RMT3-23	PE	Biolegend
TIM-4	F31-5G3	PE	Biolegend
Lineage marker cocktail (Lin: CD3, Ly-6G/Ly-6C, B220, TER-119, CD11b)	17A2, RB6-8C5, RA3-6B2, Ter119, m1/70	Pacific Blue	Biolegend

Appendix II

Table 9. PCR and qPCR primers used in the study.

Primer name	Primer sequence
Chicken primers	
chCD45_F	TGGCTCTGTGTCTGTAAGAGCA
chCD45_R	TCAGGGCCCTCGAGTTATATCT
chGAPDH_F	ATGCCATCACAGCCACACAGAA
chGAPDH_R	TTGGATGCCATGTGGACCATCA
chEEF1a_F	AGGCTGATTGTGCTGTCCTGAT
chEEF1a_R	ACGCAGAGGTTTGTCAGTTGGA
chFZ6_F	AGCCGAGGAAGGTGAAAACAGT
chFZ6_R	CCGCATCAGTTTTACGCATGGA
chMD1_F	TGCATCAATGCCAGCACAGAGT
chMD1_R	AACAGTGGCGCGATCTTCGTTA
chPDGF-Rβ_F	ACGAGTCCTGAAGTGGGAACTG
chPDGF-Rβ_R	TGTGGAAGAGCACTTCTCACCT
chTIM-1_F	ACCCAGAAGTAACCTCAAGCCT
chTIM-1_R	ACACACTTAGCTGCAGAGCAGA
chTIM-4_F	TGGCGGTGCTCGAAGTATTTGA
chTIM-4_R	TGCCATTGCACTTGGAGTGT
chTLR-2 t1_F	AGCTGGATTTCTCGCACTTTCG
chTLR-2 t1_R	CGCTTATGCTCCAGCAACACAA
chTLR-2 t2_F	TGGGAAGTGGATTGTGGACAAC
chTLR-2 t2_R	TCCAGTAGAGGATGGCTACAGT
ch_TLR-4_F	GGCTTCGAGCAAAAGTTTGGGA
ch_TLR-4_R	TCTCAAAGGAGTTGCCTGCCAT
ch_TLR-15_F	AAGTGTCCAACTGCTCCATCGT
ch_TLR-15_R	AGTGATGGCGTTGTCGCTAATG
Mouse primers	
mGAPDH_315F	GGTGCTGAGTATGTCGTGGA
mGAPDH_455R	GTGGTTCACACCCATCACAA
mTBP_653F	CTGGAATTGTACCGCAGCTT
mTBP_783R	ATGATGACTGCAGCAAATCG
mTIM1_651F	CTGGAGTAATCACACTGAAGCAA
mTIM1_724R	TGCCAACATAGAAGCCCTTAGTA
mTIM2_310F	GGTGGTCCCTATTGCTGTGT
mTIM2_407R	GTTGGTGGACTCGTGGAAAT
mTIM3_614F	CCCTGGCACTTATCATTGGT
mTIM3_710R	GGCAAGTTGGCCAGTGTAAT
mTIM4_584F	CACCTGGCTCCTTCTCACAA (284)
mTIM4_761R	GTCGTCAGCTGTGAAGTGGA
M13 plasmid primers	
M13_F	CGCCAGGGTTTTCCCAGTCACGAC
M13_R	TCACACAGGAAACAGCTATGA

Appendix III

Table 10. List of differentially expressed genes identified from the subtractive library. Genes are presented in groups by gene ontology annotations according to cellular localization.

Accession no	Gene name	No of clones
Extracellular region		*225*
NM_001001194	Defensin beta 7	54
X61002	Goose-type lysozyme	37
NM_205478	Leukocyte cell-derived chemotaxin 2	30
NM_001007942	Angiogenin 2	24
NM_204650	Defensin beta 1	21
NM_204992	Defensin beta 2	17
NM_001004399	Lymphocyte antigen 86 (md1)	9
NM_001031609	Selenoprotein P precursor	7
XM_426613	Complement component factor h	6
AJ278103	Coagulation factor XIIIA	3
AY621308	Defensin beta 6	3
XM_001233306	Kazal-type serine proteinase inhibitor	3
AY621306	Defensin beta 4	2
NM_204811	Prosaposin	2
XM_415013	Cystatin F	1
XM_418301	Lymphocyte antigen 96 (md2)	1
XM_415257	Macrophage stimulating 1	1
AY817057	Myeloid antimicrobial peptide 27	1
NM_204969	Platelet-activating factor acetylhydrolase	1
XM_419377	Secretogranin I precursor	1
NM_001030617	T-cell immunoglobulin and mucin domain containing 1	1
Plasma membrane		*40*
XM_001233829	Platelet-derived growth factor receptor beta	12
XM_417701	Lysosomal associated multispanning membrane protein 5	6
AB046119	Toll-like receptor 2	4
XM_001236952	Immunoglobulin-like receptor CHIR-B2	3
AY549497	Annexin A1	2
NM_204417	Protein tyrosine phosphatase, receptor type C (CD45)	2
AF115332	Adenosine A3 receptor	1
NM_001079484	Cell surface glycoprotein CD200 receptor	1
EF524205	Chemokine (C-C motif) receptor 5	1
L21719	C-mer proto-oncogene tyrosine kinase	1
XM_416862	Granulocyte-macrophage colony-stimulating factor receptor subunit alpha	1
X65292	High density lipoprotein binding protein	1

Accession no	Gene name	No of clones
XM_001235105	Macrophage mannose receptor precursor (CD206)	1
XM_001234121	Neural cell-adhesion molecule	1
NM_001006149	T-cell immunoglobulin and mucin domain containing 4	1
NM_001030693	Toll-like receptor 4	1
DQ267901	Toll-like receptor 15	1
Intracellular organell		*38*
NM_205344	Heme oxygenase 1	11
S49650	Cathepsin D	4
XM_416070	Glucosamine (n-acetyl)-6-sulfatase precursor	4
AJ719417	Adaptor related protein complex 1, mu 1 subunit	2
X03509	Creatine kinase, brain	2
XM_421328	Legumain	2
NM_001006453	N-acylsphingosine aminohydrolase 1	2
XM_416756	1-acyl-sn-glycerol-3-phosphate acyltransferase gamma	1
NM_001007948	ARP1 actin-related protein homolog A, centractin alpha	1
XM_001231327	Coactosin-like 1	1
J02912	Destrin	1
XR_026868	Importin 7	1
XM_421661	Lipase A, lysosomal acid, cholesterol esterase (Wolman disease)	1
XM_420095	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide	1
XM_419802	SEC63 homolog	1
AJ416889	Sulfotransferase 1C	1
M23082	Tropomyosin	1
NM_001012917	WAS/WASL interacting protein, family member 1	1
Nucleus		*27*
AF053401	Heterochromatin-associated protein MENT	12
NM_205023	Hematopoietic transcription factor pu.1 (spi-1)	5
NM_205208	Cysteine and glycine-rich protein 2	2
XM_001237031	Ras-related C3 botulinium toxin substrate 2	2
XM_416493	Cystatin A	1
NM_001030848	Eukaryotic translation initiation factor 6	1
AY675346	Exosome component 9	1
XM_001234311	Thymosin beta 4	1
D28600	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B	1
XM_422151	Zinc finger homeobox protein 1b (SMADIP1)	1
Membrane fraction (plasma membrane excluded)		*25*
X61200	S100 calcium binding protein A9 (MRP-126)	4
NM_001030339	CD81	3
AY283064	Frizzled-6	2
X71786	Integrin, beta 2 (CD18)	2
NM_205181	Stathmin-like 2	2

Accession no	Gene name	No of clones
NM_001004766	6-alpha-L-fucosyltransferase	1
XM_425997	Chimerin 2	1
NM_001100286	Cytochrome B-245 heavy chain	1
XM_416309	Cytoskeleton-associated protein 4	1
XM_418545	Dipeptidyl aminopeptidase-like 6	1
XM_416477	Mannose-6-phosphate receptor	1
XM_418351	Metadherin	1
NM_206983	Phospholipase A receptor 180 kDa	1
NM_204302	Presenilin 2	1
NM_001145227	Solute carrier family 23 (nucleobase transporters), member 2	1
XM_417242	Solute carrier organic anion transporting polypeptide 2b1	1
XM_419915	Transmembrane protein 14A	1
Cytoplasm		*23*
NM_001008440	Lymphocyte cytosolic protein 1	6
L15386	Glutathione S-transferase	3
NM_204342	Lymphocyte specific 1	3
DQ272298	Deoxyribonuclease II precursor	2
DQ212711	Protein kinase, 5' AMP-activated, gamma 2 non-catalytic subunit	2
U25026	Cell division cycle 37	1
NM_205423	Cytosolic phospholipase A2	1
XM_421965	Isocitrate dehydrogenase 1 (NADP+), soluble	1
XM_001232660	Myosin, heavy polypeptide 9, non-muscle	1
NM_001039272	Neutrophil cytosolic factor 4	1
X02009	Ovotransferrin	1
NM_001079717	Peptidase D	1
Unknown GO annotation		*6*
NM_001031365	Cell cycle associated protein 1 (CAPRIN1)	2
XR_027079	CD180	2
XM_416367	Gonad expressed transcript	1
NM_001008448	Oxysterol binding protein-like 2	1

Appendix IV

Table 11. List of differentially expressed genes identified from the subtractive library. Genes are presented in groups by gene ontology annotations according to biological processes.

Accession no	Gene name	No of clones
Innate immune response		*137*
NM_001001194	Defensin beta 7	54
NM_205478	Leukocyte cell-derived chemotaxin 2	30
NM_204650	Defensin beta 1	21
NM_204992	Defensin beta 2	17
XM_426613	Complement component factor h	6
AY621308	Defensin beta 6	3
AY621306	Defensin beta 4	2
XM_001231327	Coactosin-like 1	1
NM_001100286	Cytochrome B-245 heavy chain	1
AY817057	Myeloid antimicrobial peptide 27	1
NM_001039272	Neutrophil cytosolic factor 4	1
Metabolic process		*64*
X61002	Goose-type lysozyme	37
S49650	Cathepsin D	4
XM_416070	Glucosamine (n-acetyl)-6-sulfatase precursor	4
L15386	Glutathione S-transferase	3
DQ272298	Deoxyribonuclease II precursor	2
NM_204811	Prosaposin	2
DQ212711	Protein kinase, 5' AMP-activated, gamma 2 non-catalytic subunit	2
XM_416756	1-acyl-sn-glycerol-3-phosphate acyltransferase gamma	1
XM_418545	Dipeptidyl aminopeptidase-like 6	1
AY675346	Exosome component 9	1
X65292	High density lipoprotein binding protein	1
XM_421965	Isocitrate dehydrogenase 1 (NADP+), soluble	1
NM_001008448	Oxysterol binding protein-like 2	1
NM_001079717	Peptidase D	1
NM_204969	Platelet-activating factor acetylhydrolase	1
NM_001145227	Solute carrier family 23 (nucleobase transporters), member 2	1
AJ416889	Sulfotransferase 1C	1
Signaling		*51*
XM_001233829	Platelet-derived growth factor receptor beta	12
NM_205344	Heme oxygenase 1	11
NM_001004399	Lymphocyte antigen 86 (md1)	9
AB046119	Toll-like receptor 2	4
XM_001236952	Immunoglobulin-like receptor CHIR-B2	3

Accession no	Gene name	No of clones
X71786	Integrin, beta 2 (CD18)	2
NM_205181	Stathmin-like 2	2
NM_001004766	6-alpha-L-fucosyltransferase	1
AF115332	Adenosine A3 receptor	1
EF524205	Chemokine (C-C motif) receptor 5	1
L21719	C-mer proto-oncogene tyrosine kinase	1
XM_418301	Lymphocyte antigen 96 (md2)	1
XM_001234121	Neural cell-adhesion molecule	1
NM_001030693	Toll-like receptor 4	1
DQ267901	Toll-like receptor 15	1
Developmental process		*39*
NM_001007942	Angiogenin 2	24
X03509	Creatine kinase, brain	2
AY283064	Frizzled-6	2
XM_421328	Legumain	2
NM_001006453	N-acylsphingosine aminohydrolase 1	2
XM_425997	Chimerin 2	1
XM_421661	Lipase A, lysosomal acid, cholesterol esterase (Wolman disease)	1
XM_415257	Macrophage stimulating 1	1
XM_001232660	Myosin, heavy polypeptide 9, non muscle	1
XM_001234311	Thymosin beta 4	1
D28600	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B	1
XM_422151	Zinc finger homeobox protein 1b (SMADIP1)	1
Apoptosis and cell cycle		*19*
AF053401	Heterochromatin-associated protein MENT	12
NM_204342	Lymphocyte specific 1	3
AY549497	Annexin A1	2
U25026	Cell division cycle 37	1
J02912	Destrin	1
Proliferation and differentiation		*17*
NM_205023	Hematopoietic transcription factor pu.1 (spi-1)	5
NM_001030339	CD81	3
XR_027079	CD180	2
NM_205208	Cysteine and glycine-rich protein 2	2
XM_001237031	Ras-related C3 botulinium toxin substrate 2	2
XM_416493	Cystatin A	1
NM_205423	Cytosolic phospholipase A2	1
NM_204302	Presenilin 2	1
Adaptive immune response		*16*
NM_001008440	Lymphocyte cytosolic protein 1	6
X61200	S100 calcium binding protein A9 (MRP-126)	4
NM_204417	Protein tyrosine phosphatase, receptor type C (CD45)	2

Accession no	Gene name	No of clones
XM_415013	Cystatin F	1
NM_001030617	T-cell immunoglobulin and mucin domain containing 1	1
NM_001006149	T-cell immunoglobulin and mucin domain containing 4	1
NM_001012917	WAS/WASL interacting protein, family member 1	1
Other		*41*
NM_001031609	Selenoprotein P precursor	7
XM_417701	Lysosomal associated multispanning membrane protein 5	6
AJ278103	Coagulation factor XIIIA	3
XM_001233306	Kazal-type serine proteinase inhibitor	3
AJ719417	Adaptor related protein complex 1, mu 1 subunit	2
NM_001031365	Cell cycle associated protein 1 (CAPRIN1)	2
NM_001007948	ARP1 actin-related protein homolog A, centractin alpha	1
NM_001079484	Cell surface glycoprotein CD200 receptor	1
XM_416309	Cytoskeleton-associated protein 4	1
NM_001030848	Eukaryotic translation initiation factor 6	1
XM_416367	Gonad expressed transcript	1
XM_416862	Granulocyte-macrophage colony-stimulating factor receptor subunit alpha precursor	1
XR_026868	Importin 7	1
XM_001235105	Macrophage mannose receptor precursor (CD206)	1
XM_416477	Mannose-6-phosphate receptor	1
XM_418351	Metadherin	1
X02009	Ovotransferrin	1
NM_206983	Phospholipase A receptor 180 kDa	1
XM_420095	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide	1
XM_419802	SEC63 homolog	1
XM_419377	Secretogranin I precursor	1
XM_417242	Solute carrier organic anion transporting polypeptide 2b1	1
XM_419915	Transmembrane protein 14A	1
M23082	Tropomyosin	1

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